# Development of simple and cost-effective High Performance Liquid Chromatography methods for quality control of essential beta-lactam antibiotics in low- and middle-income countries

## DISSERTATION

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# `List of Abbreviations

| | |
|---|---|
| 6-APA | 6-amino penicillanic acid |
| 7-ACA | 7-aminocephalosporanic acid |
| 7-ADCA | 7-aminodeacetoxycephalosporanic acid |
| ADR | Adverse Drug Reactions |
| AMP | Ampicillin |
| API | Active Pharmaceutical Ingredient |
| BBB | Blood brain barrier |
| BE | Bioequivalence |
| BFArM | Federal Institute for Drugs and Medical Devices (Germany) |
| BP | British Pharmacopeia |
| CFL | Cefalexin |
| CFT | Cefotaxime |
| CFZ | Cefazolin |
| CFZ | Cefazolin |
| cGMP | current Good Manufacturing Practices |
| CLS | Cilastatin |
| CLV | Clavulanic acid |
| CLX | Cloxacillin |
| CRF | Central Research Fund |
| CRS | Certified Reference Standard |
| CTX | Cetriaxone |
| CTX | Ceftriaxone |
| DEG | Diethylene glycol |
| DML | Drug Manufacturing License |

| | |
|---|---|
| DNA | Deoxyribonucleic acid |
| DRAP | Drug Regulatory Authority of Pakistan |
| DTL | Drug Testing Laboratory |
| EMA | European Medicine Agency |
| EML | Essential Medicine List |
| FDA | United States Food and Drug Administration |
| FPP | Finished Pharmaceutical Products |
| FY | Fiscal year |
| GDP | Gross Domestic Product |
| GMP | Good Manufacturing Practices |
| GMP | Good Manufacturing Practices |
| HPC | Hydroxy propyl cellulose |
| HPLC | High Performance Liquid Chromatography |
| ICH | International Conference on Harmonization of Technical Requirements |
| IMI | Imipenem |
| IMPACT | International Medicinal Product Anti-counterfeiting Taskforce |
| IR | Infrared |
| ISO | International Organization for Standardization |
| LB | Live births |
| LMIC | Low- and Middle- Income Countries |
| MEDS | Mission for Essential Drugs and Supplies |
| MHRA | Medicines and Healthcare products Regulatory Agency of the UK |
| MIC | Minimum Inhibitory Concentration |
| MMTD | methylthiadiazole thiol |
| MRP | Meropenem |
| NMT | Not more than |

| | |
|---|---|
| NRA | National Regulatory Authority |
| PDCU | Provincial Quality Control Unit of Punjab |
| PenG | Penicillin G |
| PenGb | Benzathine Penicillin |
| PenGp | Procaine benzyl penicillin |
| PenV | Penicillin V |
| Ph.Eur. | European Pharmacopoeia |
| Ph.Int. | International Pharmacopoeia |
| PPR | Piperacillin |
| PVP | Polyvinylpropylene |
| qNMR | Nuclear Magnetic Resonance |
| RP-HPLC | Reverse Phase Liquid Chromatography |
| RUD | Rational Use of Drugs |
| SF | Substandard and Falsified Medicinal Products |
| SS | System suitability |
| SSFCs | substandard/spurious/falsely-labelled/falsified and counterfeit medicines |
| TB | Tuberculosis |
| TDI | Total Daily Intake |
| TFDA | Tanzanian Food and Drug Authority |
| TLC | Thin Layer Chromatography |
| TZB | Tazobactam |
| USD | United States Dollar |
| USP | United States Pharmacopoeia |
| UV | Ultraviolet |
| WHO | World Health Organization |

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# INTRODUCTION

## CHAPTER ONE

## 1.1. Quality of antibiotics

### 1.1.1. Background

Quality issues in pharmaceuticals had been the major point of concern since the past two decades (1-5), especially with reference to developing countries (3, 6, 7). Old and extensively used molecules including antibiotics are more prone to be counterfeited (8). Antibiotics were found to be 8-10 times more commonly reported for quality issues than any other class of medicines (8). According to International Medicinal Product Anti-counterfeiting Taskforce (IMPACT) of World Health Organization (WHO), one average figure cannot be used for prevalence of counterfeit medicines, as the situation varies markedly across various economic and geographical regions of the world (9). These figures range from 1% for developed countries, to 10-30% for various parts of developing world markets (9). Anti-malarial and antibiotics are the therapeutic classes that are mostly investigated and reported for quality issues (1, 3, 10). Beta-lactams are the major class of antimicrobials, constituting about 65% of the global antibiotic market (11), and 50% of the total reported cases of counterfeit antibiotics (12). Oral dosage forms are the most counterfeited formulations for antibiotics (12), therefore most of the studies are based upon the investigation of  solid oral dosage forms (3).

Extensive literature on quality issues of antimicrobials is available for the last two decades (8, 13, 14). In 1991-92, thirteen essential drugs were tested for stability after storage in rural settings of Zimbabwe and 2 out of 10 samples of ampicillin injection failed the assay test in the initial pre-treatment testing with 87-91% content (15). A study conducted in 1997, for the samples of chloroquine and antibacterial drugs including amoxicillin, ampiclox, co-trimoxazole and tetracycline from Nigeria and Thailand reported 36.5% substandard medicines with either no active ingredient, low or higher amount of active pharmaceutical ingredient (API) (7). The field testing study carried out in 2009 on essential antimicrobials, conducted in the two cities of India showed a prevalence of 5% and 12% in Chennai and New Delhi, respectively (16). The 2014 survey on antimalarials and amoxicillin in Papua New Guinea showed that 48.3% of medicine sampled at all levels of health system exhibited poor quality when tested using High Performance Liquid Chromatography (HPLC) methods (17). Another study for amodiaquine and amoxicillin conducted in Papua New Guinea in the same period reported that none of the 14 samples complied with the set of quality

control tests including weight variation, content uniformity, Thin Layer Chromatography (TLC) and dissolution tests. A 2014 survey showed, 19% of 854 samples failing either visual and physical inspection, or dissolution or TLC test for anti-tuberculosis drugs sampled from Almaty, Interdistrict Tuberculosis (TB) dispensary in Kazakhstan (18). In 2014, WHO circulated drug alerts on falsified bulk packages of amoxicillin, quinine and sulfadoxime-pyrimethamine found in public sector supplies of Niger (19). According to statistics from WHO Global Rapid Alert System (July 2013 till August 2016), anti-infectives for systemic use and anti-parasitic agents constitute the two major classes of substandard/spurious/falsely-labelled/falsified and counterfeit medicines (SSFCs) (20).

The literature on substandard and falsified medicines is primarily concentrated on the antimalarial medicines with the studies conducted mostly in sub-Saharan Africa and in some parts of southeast Asia. A review of studies on antimalarial agents discusses 7 surveys from southeast Asia (Cambodia, Laos, Thailand, Mayanmar, India, China and Vietnam) and 21 surveys from sub-Saharan Africa (Uganda, Cameroon, Kenya, Democratic Republic of Congo, Burkina Faso, Madagascar, Senegal, Ghana, Nigeria, Tanzania, Ethiopia, Gabon, Ghana, Mali, Mozambique, Sudan, Zimbabwe, Burundi, Angola, Rawanda and Chad) carried out within 1999-2011(21). The total samples tested in the surveys were 1437 and 2634 for southeast Asia and sub-Saharan Africa, respectively, and showed 35% of samples failing chemical analysis in both groups. 36% of samples from southeast Asia were found falsified and 46% failed packaging analysis whereas 20% samples from three studies for sub-Saharan Africa were found falsified.

Some recent studies show lower prevalence of poor-quality medicines in certain regions and settings. The 2016 Tanzanian survey based on risk based model using Minilab® and HPLC techniques showed an overall failure rate of 7.4% for essential antimicrobials and other medicines, the failures were largely due to one temperature sensitive product (22). Constant strategic improvements in the surveillance and testing capacities of the Tanzanian Food and Drug Authority (TFDA), resulted in the curtailing the problem of high prevalence of poor quality antimalarials (23). A TFDA surveillance study during 2012-2015 using 2-tier testing strategy with Minilab® and compendial methods for antimalarial samples showed a failure rate of 0.2% and 2.1% using Minilab® disintegration and TLC tests, respectively, whereas all of the 5%

(7/147) samples failing the confirmatory tests were found to be locally manufactured products, an observation also documented for disintegration test of paracetamol samples by the same researchers (24). In a 2016 study conducted in Malawi, Minilab® TLC method identified 2 substandard and 1 falsified sample containing paracetamol and cotrimoxazole instead of sulfadoxime/pyrimethamine from the 28 samples collected from 4 districts. Majority of these samples were generic products from a manufacturer not registered with National Regulatory Authority (NRA) of Malawi (25). In 2017, two studies by Lutz Heide et al., studied quality of medicines using Minilab® and compendial methods for samples from faith-based drug supply organizations from Southern Malawi, and from Asia and Africa. In the first study, 155 samples comprising of 6 antimalarials and 6 antibiotics were sampled from 31 facilities including public and faith-based organization as well as private facilities of Southern Malawi. 7 out of 155 samples were found substandard, with 6 out of specification samples sourcing from private facilities supplies and 1 sample was found falsified (26). In the second study, 85 different therapeutic categories were tested and 21 out of 869 (2.4%) samples were found SF with 12 containing wrong APIs and the study showed highest failure rate for antimalarial agents (9.5%). The failure rates were 7.1%, 2.7% and 1.1% for Cameroon, Democratic Republic of Congo, and Niger, respectively (27).

Though the problem of poor-quality medicines is concentrated mainly in the developing countries and has been extensively studied in certain specific geographical regions, it is not restricted to a one region or economic market. Recently, a case of falsified Hepatitis B medicine has been reported in Japan (28, 29). In 2007, a case of unfractionated heparin contaminated with oversulphated chondriatin sulfate claimed more than 100 lives in USA (30). More stringent controls on the quality of medicines are under taken globally, including implementation of "Track and Trace" system. India, China and Thailand are the most common suppliers of the poor-quality medicine in the global markets (20). Indian pharmaceutical Industry is facing lot of warnings and withdrawal of United States Food and Drug Administration (FDA) approvals since the closure of Ranbaxy Laboratories plants in May 2013 (31, 32).

A very narrow view of the global situation of the poor-quality medicine can be made through available data as only 9.4% of total safety reports sent to Uppsala Monitoring

Centre are from low- and middle-income countries (LMICs) (20). It is advocated that more data should be made public from the regulatory and pharmaceutical industries (20). The data from developed countries facilitate understanding of the problem in well-controlled drug markets (33, 34). However, more efforts need to be made by the developing countries to device effective regulatory strategies and increase transparency as shown by TFDA (23).

Absence of a proper post-marketing surveillance system and pharmacovigilance contributes to aggravation of the problems related to the quality of medicines. Encouraged reporting and early detection leads to gradual improvements in the health system. The reflexes for reporting, managing product recalls and timely action on the quality failures identified during post marketing surveillance is missing in the countries with poor regulatory systems (35). In addition to an older document on combating counterfeit drugs (1999), WHO has recently introduced guidelines on testing of "suspect" falsified medicines for early identification, documentation and initiating appropriate response (36, 37). The two landmark documents published in November 2017 by WHO (38, 39) provide the most comprehensive and latest evidence on the socioeconomic impact, the current data as well as recommendations on the problem of poor-quality medicines.

### 1.1.2. The updated definition:

On the 29th May 2017 the WHO gave a "new" definition of substandard and falsified (SF) medical products, detailed as follows whereas the term counterfeit is withdrawn from this context (40):

"*Substandard medicines*, also called "out of specification", these are authorized medical products that fail to meet either their quality standards or their specifications, or both.

*Falsified medicines* are the medical products that deliberately/fraudulently misrepresent their identity, composition, or source. Any consideration related to intellectual property rights does not fall within this definition. "Identity" shall refer to the name, labelling or packaging or to documents that support the authenticity of an authorized medical product. "Composition" shall refer to any ingredient or component of the medical product in accordance with applicable specifications authorized/recognized by National Registration and Regulatory authority. "Source"

shall refer to the identification, including name and address, of the marketing authorization holder, manufacturer, importer, distributor, or retailer, as applicable.

Medical products should not be considered as falsified solely on the ground that they are unauthorized for marketing in a given country"

### 1.1.3. Impact of quality failure of antibiotics on healthcare system

Ensuring quality of antimicrobial agents is an important concern worldwide, as the failure to deliver the intended quality can lead to serious implications on health care system (8, 12, 13, 41). Quality failure of antibiotic can be direct result of the poor manufacturing standards or due to inappropriate storage and handling of medicine (7, 20). The most common quality issue is having less than the claimed content of active ingredient, followed by absence of active pharmaceutical ingredient (API), excessive amounts of API and presence of impurities and contaminants (3).

Poor quality is also closely related to the improper storage of pharmaceuticals (Figure 1.1). Certain antimicrobials like artesunate and co-amoxiclav are particularly sensitive to humidity and temperature variations (6). Refrigeration and cold room facilities as well as ensuring uninterrupted power supplies for these temperature sensitive stocks is challenging for resource-limited settings. Furthermore, sale of medicines as loose tablets and capsules can degrade the light and moisture sensitive medicines. This practice is predominant in physician operated dispensaries as well as public sector dispensaries due to cost savings.

The effects of poor-quality of antibiotics on health care can be categorized as the direct effects and indirect (subsequent) effects. The direct effects include therapeutic failure, non-compliance, adverse drug reactions, failures of 5Rs (right drug, right dose, right frequency, right route, right handling) and antibiotic resistance. Each of these direct effects can lead to one another resulting in a domino effect. Indirect effects include failure of antibiotic stewardship programs and high treatment costs (Figure 1.1).

### 1.1.3.1.     Therapeutic Failure

Less than the specified amount of active ingredient (under dose) or adulteration of the drug with cheaper API or inactive material lead to therapeutic failure. Wrong API or inactive materials are reported in many falsified products (25). Low API content can adversely affect the therapeutic outcome of narrow therapeutic index anti-

infectives leading to increased morbidity and mortality, one of the impacts of substandard medicines (42, 43) .

## 1.1.3.2. Non-Compliance

Distrust of the patient on quality of medicine leads to compliance issue. Patients may disrupt the antibiotic treatment before the recommended period or may not comply with the proper use of medicine needed to obtain the required therapeutic effect, subsequently contributing to the increase in antimicrobial resistance. Contaminants and impurities in the substandard product can cause adverse drug effects leading to patient non-compliance. Poor quality medicine has also been identified as a reason for lack of motivation of people in accessing the public sector facilities in poor health settings (44).

## 1.1.3.3. Adverse drug reactions

### 1.1.3.3.1. High API content

Substandard medicine with higher than the specified limits of API increases the risk of adverse drug reactions (ADR) and so inadvertently effects the patient compliance incurring additional treatment costs. Presence of higher than the acceptable content of API has been reported in substandard antimalarial and antibacterial agents reported in Pakistan, Nigeria and Thailand in different studies (7, 45).

### 1.1.3.3.2. Pharmaceutical impurities-adverse reactions and toxicological effects

The control of pharmaceutical impurities is of crucial importance for ensuring safety of pharmaceuticals (46). The term impurities refer to any substance in the pharmaceutical preparation that is not a part of active pharmaceutical ingredient/s (API) or excipients. Their type and amount are controlled by the limits provided in pharmacopoeia monographs. Non-compliance to these quality specifications renders the pharmaceutical product as substandard. Qualification of the impurities to assess their biological safety and toxicity potential is carried out in the drug development phase and their quantity is limited by the stringent guidelines (47-49) .

Contamination of pharmaceuticals by erroneous material, adulterant, presence of a higher content of known or unknown impurities make the product substandard and unsuitable for clinical use. Inadvertent contamination of medicine by any erroneous material is a serious problem seen time to time as result of non-compliance to Good

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Manufacturing Practices (GMP). Serious toxicological reactions can emerge demanding additional medical care and thereby increasing health care cost (2, 50). Contamination of pyrimethamine in cardiovascular medicine in Pakistan and contaminated heparin in the USA are two prominent examples (35, 51). Pharmaceutical impurities are mainly the starting material, intermediates, by-products, and degradation products of the active ingredient. Controlling the impurities level by using more purified API is a successful strategy in minimizing serious adverse reactions as seen in case of vancomycin (52). Identification of new impurities e.g., in case of trimethoprim led to changes in the analytical method for the test of Related Substance (53, 54). Limit test for levomethorphan in dextromethorphan finished pharmaceutical products (FPPs) has been introduced in the recent edition of International Pharmacopoeia (Ph.Int.) to ensure prevention of safety issues as reported in 2012 (55). In 2012, similar incidence occurred twice in Pakistan (60 deaths) and in Paraguay (11 children intoxicated and 1 death) with consumption of API from Indian source having the high levels of toxic enantiomer, levomethorphan in dextromethorphan batches (56, 57).

Change in the origin and method of synthesis are not permitted as it can give rise to new impurities and without proper identification and control of these impurities serious consequences regarding patient safety might emerge. Investigation of heparin case using quantitative Nuclear Magnetic Resonance (qNMR) spectroscopy revealed new impurities from a Chinese sourced product (50, 58).

Mortality and morbidity incidences followed by the consumption of contaminated medicines are reported with ambiguous supply chain, overlooked quality failures in imported products and raw materials, and GMP non-compliances. Let alone, the cases of diethylene glycol poisoning has emerged globally at various times and at various places (59).Diethylene glycol is an antifreeze agent that causes acute renal failure on intake. It has been fraudulently used as adulterant in place of glycerine, in pharmaceuticals and hygiene related products. These cases were reported in 1995 in Bangladesh (60), in 1997 in Haiti (61), and ten years later in 2006 in Panama with Chinese source (100 children died) (62).

Allergenicity is one of the major toxic properties of impurities in penicillins. Penicillins and their synthesis impurity 6-aminopenicillanic acid (6-APA) convert to pencilloyl

moiety by ring cleavage. These small molecules form haptens by reacting with the proteins from fermentation medium and cause penicillin urticarial reaction (63, 64). Other degradants like penicilloic acid, penaldic acid and penicillamine are minor antigenic determinants (63, 64). The high molecular weight oligomeric degradation product of penicillins and cephalosporins are also investigated for their antigenic potential (63, 65). Cross allergenicity between penicillin and cephalosporins is up to 5-6%. Non-penicillin beta-lactams also possess similar allergenic potential. The FDA guidelines for good manufacturing practices (GMP) require the production of beta-lactam antibiotics in separate facilities than other pharmaceuticals to avoid cross contamination (64).

### 1.1.3.4.    Antimicrobial resistance

### 1.1.3.4.1.    Effect of API content on antimicrobial resistance

Substandard antimicrobials containing lower than the specified content of API or poor dissolution and disintegration profile fail to deliver the dose required to attain the plasma levels above minimum inhibitory concentration (MIC). Use of substandard medicines presents ideal condition for the selection of resistant organisms, therefore "assurance of uninterrupted access to essential medicines of assured quality" is accounted as an  integral part of WHO policy package to combat antimicrobial resistance (66). Treatment failure due to suboptimal dose of antibiotics produces long term consequences on health system including antimicrobial resistance (20, 67, 68). Low content of API in the antimalarials is documented as the cause of the development of antimalarial resistance in Africa (69). The high incidence of typhoid resistance in Burma has been associated with the prevalence of low quality antimicrobials (67). A similar situation is confronted in south of Pakistan where multidrug resistance typhoid is seen on rise, in particular with the treatment of choice ceftriaxone (70). Though most of the antibiotics possess a wider therapeutic window but the degree of quality issues, their high prevalence and poor handling can play evident role in the therapeutic failure and ultimately leading to antimicrobial resistance. The role of substandard medication on noncompliance leading to high microbial resistance development is discussed earlier (see 1.1.2.2).

## 1.1.3.4.2. Potential role of pharmaceutical impurities in antimicrobial resistance development

High structural similarities between the parent compound and their impurities as well as low antibacterial activity increase the possibility of the interaction of these impurities with the microbiological system over the long period of exposure and facilitate resistance development. Safe disposal of antimicrobials and their deactivation prior to disposal is strongly advocated to prevent development of microbial resistance (71, 72). Pharmaceutical impurities of commonly used antibiotics in human and veterinary medicine are studied for their possible effect on resistant development (73, 74).

Loss of antimicrobial activity might occur when antibiotics are subjected to certain pharmaceutical processes like radio sterilization (75). Caution needs to be placed in selecting such procedures to ensure that the antibacterial activity of the active pharmaceutical ingredient is not compromised.

## 1.1.3.5. Failure of 5 R's

Poor packaging and inadequate labelling of medicines is commonly observed in countries with weak regulatory settings (21). A study by TFDA reports 9.1% of the samples of antimalarials to have poor packaging information (24). According to WHO, 5% of the counterfeit drugs have packaging errors (30). This leads to improper drug administration and drug misadventures. For the medicine to be effective it is important that 5R's of right route, right dose, right frequency, and right handling are followed (Figure 1.1). These 5R's are derived from proper medication administration in nursing (76).

Most of the paediatric oral formulations of antibiotic and parenteral preparations need reconstitution prior to administration. The choice of solvent and diluents used for reconstitution and the diluents for preparation of intravenous infusions must be according to the manufacturer's directions. Adequate information need to be provided to the users by manufacturer in this regard, including the time of disposal after the reconstitution (77). In resource limited settings, insufficient or poor labelling, poor personnel training, lack of access to unbiased drug information and insufficient supplies for proper and safe administration of antibiotics lead to mishandling and degradation of the drug prior to administration to the patient.

## 1.1.3.6. Effect on rational use of antibiotics/antibiotic stewardship programs

Antibiotic stewardship programs are a crucial tool for controlling the spread of antibiotic resistance and multiple drug resistance conditions. However, it becomes increasingly difficult for practitioners to adhere to the guidelines for rational prescribing and follow the recommendations of antibiotic stewardship programs when the treatment outcomes are doubtful or compromised because of poor quality of antibiotics. Deviations from the recommended choice of antibiotics are made. Frequent and faster switching to the high cost antibiotics results in sabotaging the entire system of the rational antibiotic usage (Figure 1.1).

## 1.1.3.7. Cost implication of poor quality of antimicrobials

The ultimate consequence of bad quality medicines and in-particular antibiotics is the over-all increase the health systems cost and increase in disease burden. A substandard drug can be both over and under dose, resulting in therapeutic failure or an undue adverse reaction. In both cases the outcome is discontinuation of drug therapy or switching over to the expensive antibiotics (67). Absence of API and low API content results in the treatment failure and leads to patient's distrust on health system, non-compliance, morbidity and mortality, and an ultimately increase in treatment cost.

One direct cost impact of poor-quality of medicine is the shifting of the preference of the prescriber, patient and procurement agencies towards the costly innovator and branded medicines particularly from the transnational companies. A colossal difference exists in prices between the innovator and generic medicines and similarly between products from the local market and the imported transnational products. Inability to afford the antimicrobial agents by the public health agency can result in the outbreak of epidemic due to the spread of infectious disease, escalating the problem beyond the capacity of the health system (45). Patients in remote and rural settings take partial therapy and even single dose of antibiotics because of cost constraints. The prevalence of poor-quality medicines also differ in urban and rural settings (17).

Generic medicine is an effective tool for ensuring access of essential medicines to public and avoiding such crisis. However, poor-quality of medicines and the

medicines with doubtful quality status led to the withdrawal of generic medicines policy in countries like Pakistan (78).

Generic medicine advocacy weakens in poor regulatory settings (20). Donor agencies are seen to rely more on the innovator or branded medicine from transnational companies thus causing undue increase the cost (60). However, with the use of prequalification procedures, locally produced medicines proved to be equally effective as the imported brands of anti-tuberculosis medicines as reported in a study from Pakistan (79).

Bioequivalence studies were introduced in 1984 in USA as a requirement to prove generic equivalence. Intravenous medicaments and bio-waivered classes of medicines are technically exempted from the bioequivalence. Mandatory bioequivalence for product registration is yet to be effectively implemented in many parts of the world like India and Pakistan. The two countries have recently initiated regulation in this regard.

In a comprehensive review on the studies conducted for comparison of brand and innovator antimicrobials, only two out of twenty-two studies using microbial and pharmaceutical assay techniques gave evidence of brand/innovator and generic antimicrobial equivalence. The rest proved the dominance of the brand and innovator medicine. In contrast, only four out of thirty-four bioequivalence studies conducted on different antimicrobial agents showed that the generic medicines (comparator) was significantly inferior to the innovator brand, rest proved otherwise (14).
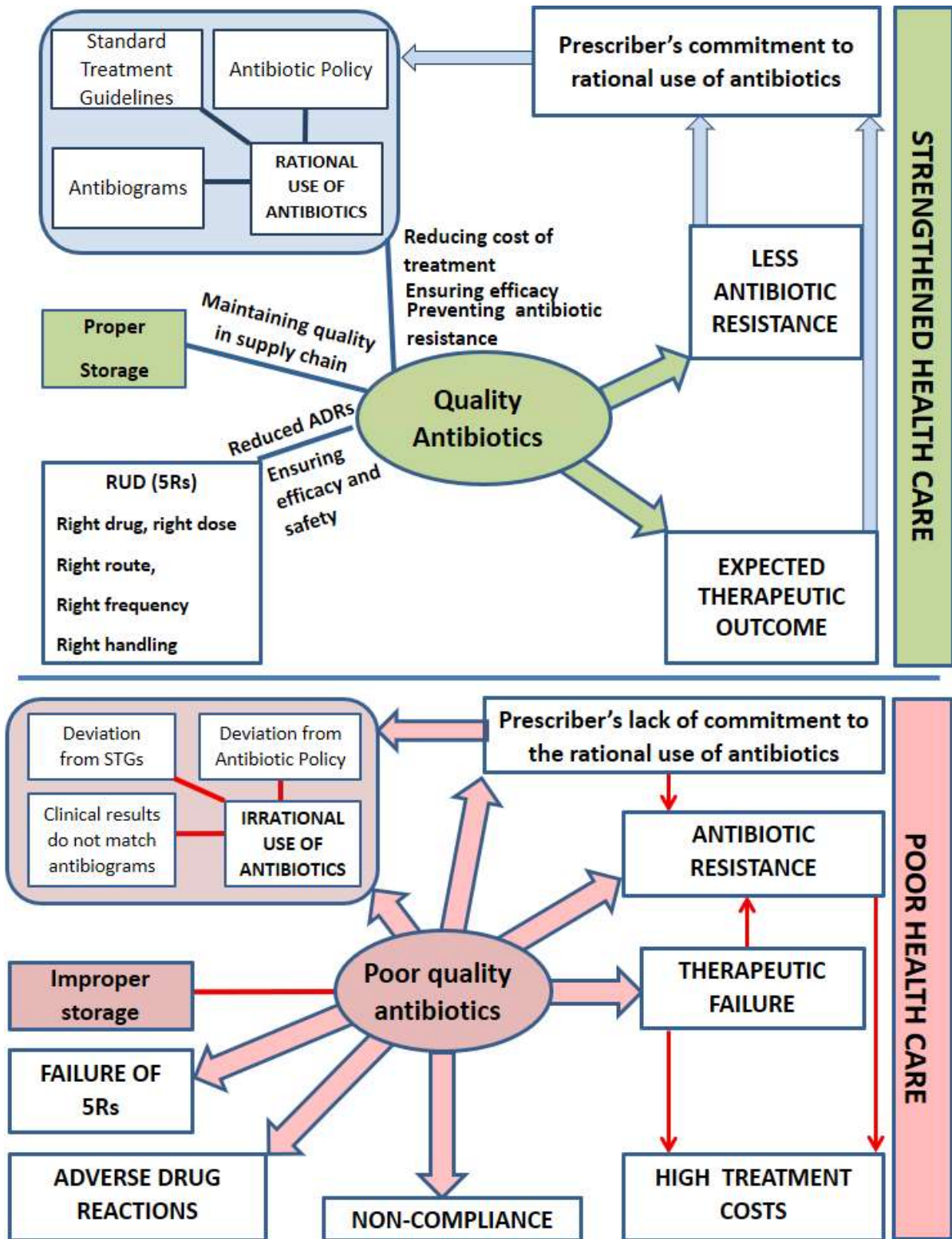
Figure 1.1. Impact of quality failure of antibiotics on health system

## 1.1.4. Quality assessment methods for antibiotics:

Antibiotics belong to the class of pharmaceuticals that are originated from fermentation or semisynthetic source. Assay and test for related substances are the key analytical tests carried out on the antimicrobials along with other general and dosage form specific tests like dissolution, disintegration, sterility, and pyrogen testing. The nature of pharmaceutical impurities is closely linked to the procedures adopted for synthesis of API. Changes in synthesis method lead to production of different impurities and requires improvement in the impurity test method (53). More stringent limits on the presence of impurities in pharmaceuticals especially for fermentation and semi synthetic products are being introduced (80).

Identification of the known and unknown impurities in antibiotics and limiting their concentration in the final product is therefore an integral part of the assurance of quality and efficacy of antibiotics and is discussed in detail in the later section of this chapter. After identification, impurities are investigated for their potential toxicities and biological effects though a qualification process (47, 48). Biological evaluation of the impurities is carried out for the known impurities for their possible toxicological effects as part of the product registration requirements laid by International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines (47-49). Antimicrobial resistance as well as mutagenic and genotoxic potential of impurities of antibiotics are a huge concern owing to the widespread use of antibiotics (81).

### 1.1.4.1. Techniques for assay and impurity profiling of antibiotics:

HPLC is used as a gold standard for the assay and impurity profiling methods intended for routine regulatory and quality control procedures. Due to the polar nature of antibiotics, reverse phase liquid chromatography (RP-HPLC) with predominantly gradient elution has widely been used for the impurity profiling of antibiotics. Very few monographs of antibiotics e.g., cephradine capsules monograph by British Pharmacopoeia (BP) 2013 include TLC for impurity profiling. Biological assays had also been part of the pharmacopoeia for the antimicrobials and employed use of standard reference microorganisms for the determination of antimicrobial efficacy of the antibiotics. Dilution method involving optical density measurement and disc plate methods using diameter of zone of inhibition are used to carry out a bio-assay for

certain antibiotics. Bio-assay is also used for the potency determination of standards of antibiotics. Potentiometric titration is also used for assay determinations of certain antibiotics and was more popular two decades earlier. Out of the 19 molecules included for study in this document only two molecules (cilastatin and procaine benzylpenicillin) have assay methods based upon wet chemistry in the recent editions of pharmacopoeia (82, 83).

Antibiotics are also part of the Minilab® Field testing kit that includes colorimetric and thin layer chromatographic tests (84). Colorimetric tests were initially used for field testing by WHO for checking the identity of medicine in field and remote areas. Colorimetric tests and TLC are still used as identification tests in the pharmacopoeia.

## 1.1.4.2.      General concepts of quality of pharmaceuticals

In practice, maintenance of quality is the combination of compliance to current Good Manufacturing Practices (cGMP) and the detailed set of analytical tests specified by the pharmacopoeia. The former being regulated through the licensing procedures of the national regulatory authorities of the countries. It includes approval of manufacturing premises, evaluation of product dossiers, field inspections for cGMP compliance to obtain and maintain the licensing status. The other quality segment comprises of analytical testing of raw materials (API and excipients) and FPPs and its record maintained by the manufacturing facility.

From analytical perspective, the quality of APIs is mainly regulated by specifications provided in pharmacopoeia monographs. These include assay and impurity tests as the key analytical procedures among many others that ensure quality of the raw materials and the FPPs.  European Pharmacopoeia (Ph. Eur.), United States Pharmacopoeia (USP), British Pharmacopoeia (BP) and the International Pharmacopoeia (Ph. Int.) are the major pharmacopoeia used globally. The latter one is published by World Health Organization (WHO) and has its unique role in offering simple, reliable and cost-effective analytical methods for better compliance by the countries with resource-limited settings.

ICH guidelines provide comprehensive documents for the new pharmaceuticals emerging in the market and works as a collaborative document prepared by expert agencies from European Union, United States of America, and Japan (Tripartite) (47-49) .

### 1.1.4.3. Pharmaceutical impurities

Pharmaceutical impurities include organic impurities (also generally termed as related substances), inorganic impurities and residual solvents.

The term related substances technically refer to the chemical structures closely related to API and may share similar biological activity. Related substances cover a list of impurities including starting material, intermediates, degradation products and process related impurities (85).

### 1.1.4.4. Classification of organic impurities:

### 1.1.4.4.1. Synthesis impurities:

Synthesis impurities include starting material (e.g., 6-amino penicillanic acid for penicillins and 7-aminpcephalosporanic acid for cephalosporins), and the substances formed during the synthesis including intermediates (e.g., deacyl ceftriaxone in ceftriaxone) they are produced as a product of the main reactions of the synthesis process and are mostly isolated and characterized, final intermediates (last compound in the synthesis chain before conversion into the final product), transformation products (products that may be produced in the reaction), by-products theoretical or potential impurities). Pharmacopoeia provide the limits for the content of concomitant substances in certain APIs (e.g., cephalexin in cephradine, 4-hydroxyphenoxymethylpenicillin in phenoxymethyl penicillin) (86, 87).

### 1.1.4.5. Process impurities:

These impurities were not the part of the initial raw materials but are generated as result of the processes involved in drug formulation. Various processes like radio-sterilization, coating, granulation etc. can contribute to the formation of these impurities.

Signal impurities: The term signal impurity is used for the process and degradation impurities to provide key information about the processes involved in the drug formulation, they require specific identification and quantification tests.

### 1.1.4.6. Degradation products:

Decomposition of the API due to various physico-chemical factors result in generation of the degradation products or degradants.

## 1.1.4.7.    Interaction impurities:

The impurities produced by chemical or physical interaction between the different components of the drug product (API, excipient, container and closure). Excipients are pharmacologically inert substances that are used to develop the API into a dosage form. However, based upon the chemical nature of the API and excipients, interactions can occur generating additional impurities and may inadvertently effect the quality of the product (88). Acetylation products are formed by aspirin when formulated with codeine or sulfadiazine.  Ester and amide formation was observed with the use of citric acid in the formulation of 5-aminosalicylic acid (89). Benzocaine interacts with polyvinyl acetate phthalate to form an interaction impurity.

Primary and secondary amines can interact with reducing sugars forming amines or imines, respectively by Milliard reaction (e.g., gabapentin and pregabalin with lactose) (88). Excipients containing double bonds like sodium stearyl fumarate and sorbitan monooleate can react with primary amines (e.g. fluvoxamine maleate) like a Michael addition (90). The impurities in excipients are important when the excipient-to-API-ratio is high. Certain excipients contain characteristic impurities carboxylic acids in microcrystalline cellulose and formaldehyde in talc (90). Polymeric ethers, polyvinylpropylene based excipients and hydroxypropylcellulose generate peroxides on degradation. Some of these substances can be reactive species and may form interaction impurities with API and other components of the formulation.

Apart from these any foreign or extraneous substance that is added to the product deliberately or accidently are also categorized under the term impurities. The pharmaceutical product is meant to be free from such contaminants.

Stereochemistry of the compounds plays a crucial role in the activity of the medicines. Change in stereoisomerism can result in loss of activity (L-isomers of amoxicillin and ampicillin, *E*-isomers of cephalosporins) or production of highly toxic isomers (levomethorphan in dextromethorphan) (56, 57, 63). However, the identification and quantification of these isomers is a challenging aspect and may need sophisticated techniques like chiral chromatography methods. These isomers are included in the tests for related substances. Optical rotation is also done for determination of the dextro- and levo-rotatory forms of the biologically active substances. Forced degradation is an important tool in the investigation of potential impurities of medicinal

agents (91).

The investigation of impurities in FPP can be stemmed to the set of impurities that were contributed by the active pharmaceutical agent and the raw material or produced or added during the processing of formulation into finished product or produced during handling and storage.

### 1.1.4.8. Limits of pharmaceutical impurities

Pharmacopoeia monograph define a *disregard limit* above which all present impurities are summed to give the amount of *total impurities*. The limits of total and individual impurities are also defined. Impurities with the known chemical structure are termed identified impurities whereas the impurities for which only limited information like retention time is known are termed unidentified impurities. Specified impurities are the impurities with known structure. The list of impurities given by the pharmacopoeia as the actual or organic impurities of an API are termed known impurities and provision of comprehensive information about the known impurities is part the new drug and investigational drug registration process regulated under the ICH guidelines Q3A (R2) for drug substances and Q3B (R2) for drug products (47, 48). Limits of pharmaceutical impurities are calculated on the basis of the total daily dose of API. The threshold limit for reporting, identification (structural elucidation) and qualification (biological safety) by Ph.Eur., European Medicines Agency (EMA) and the ICH guidelines are tabulated in Table 1.1 and 1.2 and are mandatory part of the drug registration process (47, 48, 80, 92). The limits given by the ICH guidelines and Ph. Eur. are not applicable to the products of fermentation and semi-synthetic products derived from fermentation.

ICH guideline Q6A provides decision trees for establishing acceptance criterion for a specified impurity and/or a degradation product in a new drug substance whereas algorithm for carrying out safety studies or reducing the level of impurities for confirmation to the identification and qualification thresholds for impurities in drug substances and drug products are provided in ICH guidelines Q3A/B(47, 48, 93).

Introduction

Table 1.1    Thresholds for impurities in semi synthetic antibiotics for human use by guidelines on setting specifications for related substances in antibiotics by European medicines agency (API and FPP) and Ph. Eur. (for API only)

| Max Daily Dose | Reporting threshold- API, FPP (%) | Identification threshold- API, FPP (%) | Qualification threshold- API, FPP (%) |
|---|---|---|---|
| ≤ 2g/day | 0.05, 0.1 | 0.1, 0.2 | 0.15, 0.2 |
| > 2g/day | 0.03, 0.1 | 0.05, 0.2 | 0.05, 0.2 |

Table 1.2    Thresholds for impurities in API and FPP according to ICH Harmonized Tripartite Guidelines (Q3A (R2) and (Q3B (R2)

| Max Daily Dose | Reporting threshold (%) | Identification threshold (%) | Qualification threshold (%) |
|---|---|---|---|
| *API* | | | |
| ≤ 2 g/day | 0.05, | 0.1, 0.2 | 0.15, 0.2 |
| > 2 g/day | 0.03, | 0.05, 0.1 | 0.05, 0.15 |
| *FPP* | | | |
| ≤ 1 g/day | 0.1 | | |
| > 1 g/day | 0.05 | | |
| < 10 mg | | 1.0% or 5 µg TDI | 1.0% or 5 µg TDI |
| 10 mg-100 mg | | 0.5% or 20 µg TDI | 0.5% or 200 µg TDI |
| > 10 mg-2 g | | 0.2% or 2mg TDI | 0.2% or 3mg TDI |
| > 2 g | | 0.10% | 0.15% |

## 1.1.4.9.      Identification and qualification of impurities:

The identification of impurities implies the structural elucidation of the impurities. Presence of impurities in trace amounts and their instability frequently pose a challenge in identification. Matching of retention time and spiking with known standards are the simple approaches for confirmation of identity of the new impurities. Use of PDA detector helps in ensuring peak purity. Forced degradation augments the amount of impurity before isolation using preparative TLC or HPLC. The isolated impurity can be analysed by means of mass, Infrared, UV, NMR spectrum for structure elucidation. In case of failure of separation, synthesis or X-ray diffraction studies are done. Modern structural identification tools include mass spectrum, NMR or IR based hyphenated techniques (85, 94).

Qualified impurities are the impurities actually found in the pharmaceuticals and proven safe by the biological screening processes. ICH M7 guidelines for assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals are employed in order to limit potential carcinogenic risk (49). All actual and potential impurities with known structure are evaluated for DNA reactivity or mutagenic potential using databases and literature search. Structural alerts are identified by *in silico* assessment and then a bacterial mutation assay e.g., Ames test is performed. Impurities are classified from Class 1-5 on basis of their mutagenic and carcinogenic potential. An acceptable intake limit of 1.5 and 20 µg/day are employed for class 2 and 3 impurities in the drug substance used for chronic administration and for anti-infectives for less than 12 months use, respectively (46). Class 3 and 4 are the impurities without structural alerts or with structural alerts but proven non-mutagenicity, and are controlled using the algorithm provided in ICH Q3A/B guidelines (47, 48). An impurity is considered qualified when it has been tested in preclinical or clinical studies at the level equal or higher than that found in the marketed product or on presence of the safety data. Actual testing for gene mutation (Ames test), or determination of chromosomal aberration (mouse lymphoma assay) and a general toxicity test (rat study) need to be performed, in case of absence of such data (46).

## 1.2. Essential beta-lactam antibiotics and their impurities

### 1.2.1.     Background

Beta-lactam antibiotics are the one of the oldest and most popularly used class of antibiotics (95) constituting of about 65% of the total global antibiotic market (11). Since the discovery of penicillin in 1929 by Sir Alexander Fleming, dozens of new molecules have entered the pharmaceuticals market as the semi-synthetic derivatives of penicillins and their closely related group of cephalosporins. The microbiological origins for penicillins, cephalosporins, carbapenems, and clavulanic acid include *Penicillium notatum* and *P. chrysogeneum*, *Cepahlosporium spp.,* *Streptomyces cattleya*, and *Streptomyces clavuligerus*, respectively. More than 50 cephalosporins are discovered and many are popularly used clinically. By 2013, 34 beta-lactam molecules were registered for clinical use by FDA (64).The molecules as old as benzyl penicillin still have an unchallenging role for their usefulness in the specific illnesses like syphilis.

The term beta-lactam refers to the, a 4-membered cyclic amide ring that is the essential pharmacophore (63). Beta-lactam group of antibiotics include penicillins, cephalosporins, carbapenems and monobactams (63). These are cell wall synthesis inhibitors and are bactericidal in nature (96). Beta-inhibitors are compounds that are used concomitantly with beta-lactam antibiotics to provide stability against beta-lactamase producing microorganisms (63, 97).

15 out of 29 key ACCESS antibiotics in WHO 20th model Essential Medicines List (WHO-EML) belong to beta-lactam group of antibiotics. ACCESS group includes antibiotics of vital significance in any health systems and is emphasized for assuring availability, affordability and quality by WHO. Two beta-lactamase inhibitors are also included in the same list. Four of these beta-lactams (cefixime, ceftriaxone, cefotaxime and ceftazidime) are shared with the WATCH group. WATCH group of antibiotics include antibiotics with high resistance potential and are recommended as first and second line medicines only in specific and limited number of indications (98). The current section discusses the compendial specifications (82, 83) for the 19 beta-lactam antibiotics and related molecules included in the ACCESS group list (Table 1.3) and the challenges faced for their routine quality control. The structural formula of these essential beta-lactam antibiotics and their known impurities are shown in Figure 1.2-1.26.

# Introduction

The beta-lactam antibiotics vary at their side chains of 6-aminocephalosporanic acid (6-APA) for penicillins and 7-aminocephalosporanic acid (7-ACA) for cephalosporins providing them diversity in their stability, antimicrobial spectrum and pharmacokinetic properties.

Additional to the Ph.Eur. 9.2 (82) list of known impurities, USP 40 (83) includes some more impurities for ampicillin (AMP1-4), ceftriaxone (CTX 1-2), cefotaxime (CFT Imp 1-3), cefazolin (CFZ Imp 1-4), cloxacillin (CLX Imp 1-2) and piperacillin (PPR Imp 1-20), shown in Figure 1.5-1.26. Similarly, CFZ-Imp J and CFZ-Imp K from Ph. Eur. are not enlisted by USP 40. Two new impurities of PenG-Imp G and Imp F (iso-penicillin F and dihydropenicillin F) are recently included in Ph.Eur. Six known impurities are listed in Ph.Eur. 9.2 for piperacillin API, in contrast to only 4 impurities mentioned in the monograph for API and piperacillin injection in USP 40 whereas, the monograph for tazobacatam and piperacillin for injection includes 20 additional impurities of piperacillin. However, some of the known impurities in pharmacopoeia are not meant to be reported.

General impurities of beta-lactam antibiotics include starting materials like 6-amino penicillanic acid (6-APA) for penicillins and 7-aminocephalosporanic acid (7-ACA) for cephalosporins, substances used in the fermentation for side chain substitutions like phenyl glycine (AMP-Imp L, CFL-Imp A), hydroxyl phenyl glycine (AMX-Imp I, cefadroxil), side chain chemical substituents like thiotriazinone/triazine analog (CTX-Imp C) and pyridine (CTZ-Imp F), ring open structures (penicilloic and penilloic acids of aminopenicillins), oligomers and co-oligomers (amino penicillin dimers and trimers), biologically less active or inactive stereoisomers (*E*-isomers of cephalosporins and L-isomers of aminopenicillins) along with various degradation products and synthesis intermediates (99). Various thiazolyl derivatives are used for the acylation of 7-ACA in cephalosporins synthesis, and may be found in the final product e.g., CTX-Imp D and CFZ-Imp E (99).

Table 1.3     Salient features and route of administration of ACCESS beta-lactams.

| | Beta-lactam Antibiotic | Salient features | Route of administration |
|---|---|---|---|
| | PENCILLINS | | |
| 1. | Benzylpenicillin (PenG) | Natural penicillin | Intravenous |
| 2. | Benzathine penicillin (PenGb) | Long acting | Intramuscular |
| 3. | Procaine benzylpenicillin (PenGp) | Long acting | Intramuscular |
| 4. | Phenoxymethylpenicillin (PenV) | Acid resistant | Oral |
| 5. | Amoxicillin (AMX) | Acid resistant, extended spectrum | Intravenous, oral |
| 6. | Ampicillin (AMP) | Acid resistant, extended spectrum | Intravenous, oral |
| 7. | Cloxacillin (CLX) | Acid-resistant, penicillinase stable | Intravenous, oral |
| 8. | Piperacillin (PPR) | Antipseudomonal | Intravenous |
| | CEPHALOSPORINS | | |
| 9. | Cefalexin (CFL) | First generation, surgical prophylaxis | Intravenous, oral. |
| 10. | Cefazolin (CFZ) | First generation-high bone penetration, surgical prophylaxis | Intravenous, oral |

|      | Beta-lactam Antibiotic | Salient features | Route of administration |
|------|------------------------|------------------|-------------------------|
| 11.  | Cefotaxime (CFT)       | Third generation | Intravenous |
| 12.  | Ceftriaxone (CTX)      | Third generation- long acting | Intravenous, Intramuscular |
| 13.  | Ceftazidime (CTZ)      | Third generation- antipseudomonal | Intravenous |
| 14.  | Cefixime (CFX)         | Third generation | Oral |
|      | CARBAPENEMS            |                  |  |
| 15.  | Meropenem (MRP)        | Broad spectrum- crosses BBB | Intravenous |
| 16.  | Imipenem (IMI)*        | Broad spectrum- does not cross BBB | Intravenous |
|      | *Beta-lactamase inhibitors* |             |  |
| 17.  | Clavulanic acid (CLV)  | Used with amoxicillin | Intravenous, oral |
| 18.  | Tazobactam (TZB)       | Used with piperacillin | Intravenous |
|      | *Others*               |                  |  |
| 19.  | Cilastatin (CLS)       | Used with imipenem to increase its half-life | Intravenous |

*alternate to meropenem when used other than CSF infections.*
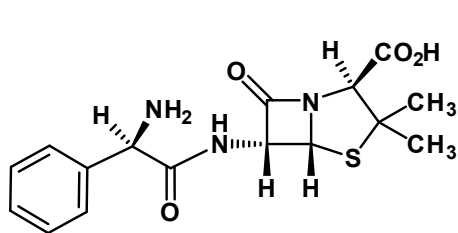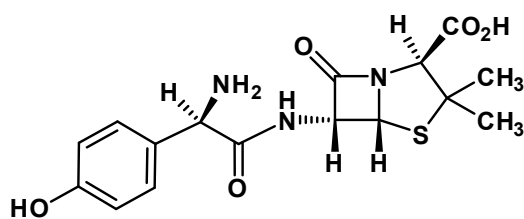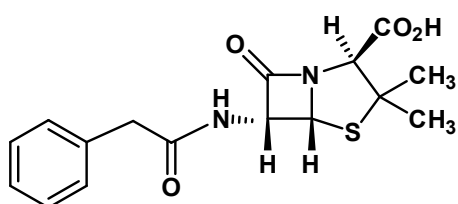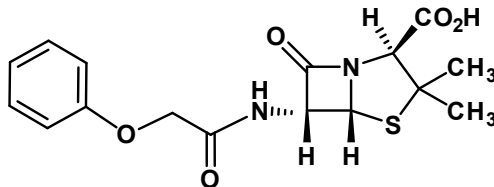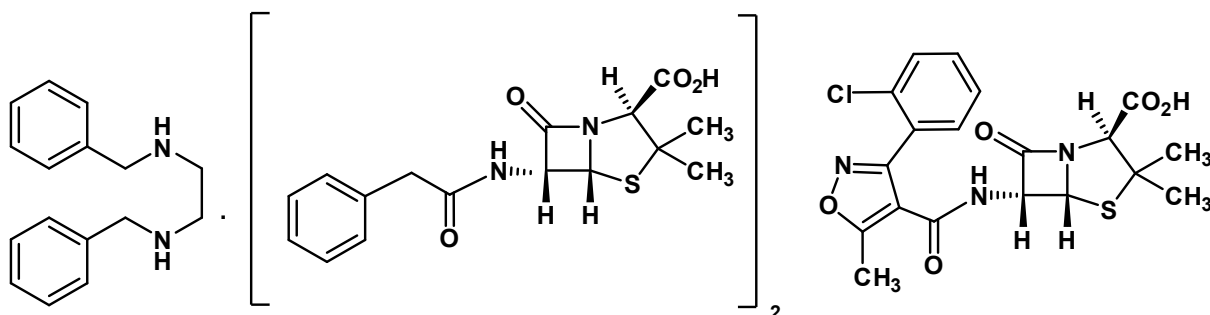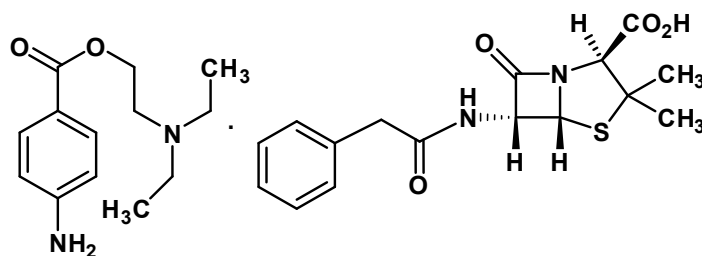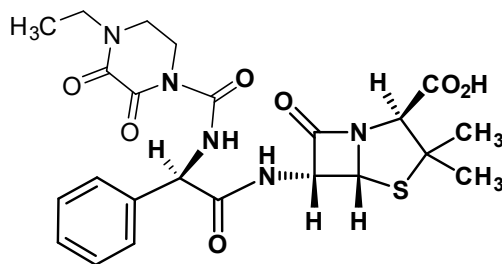
Figure 1.2.   Chemical structures of essential penicillins
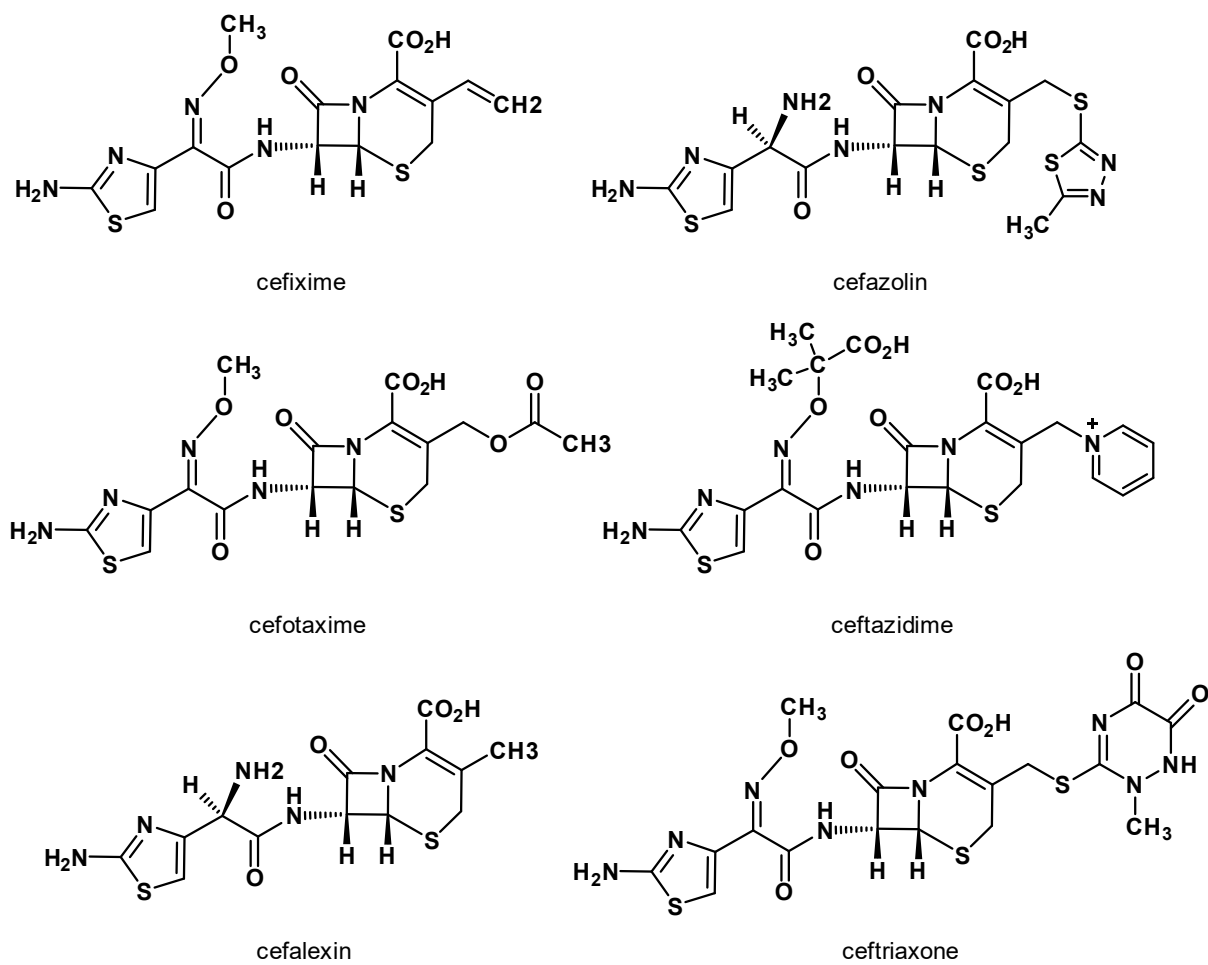
Figure 1.3. Chemical structures of essential cephalosporins
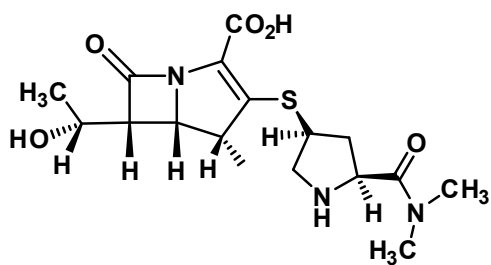
meropenem
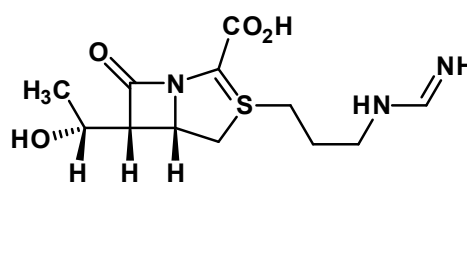
imipenem
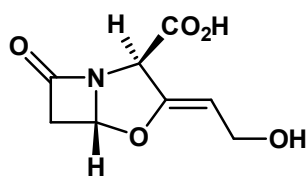
clavulanic acid
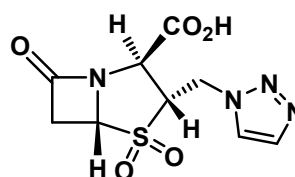
tazobactam
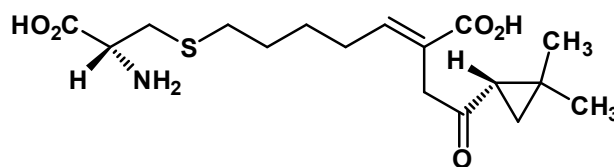
cilastatin

Figure 1.4. Chemical structures of essential carbapenems, beta-lactamase inhibitors and cilastatin

AMX-Imp A/AMP-Imp A (6-APA)

AMX-Imp B/AMP-Imp B (L-isomer)

$R_3$= 2-hydroxyphenylglycine, $R_4$= H; AMX-Imp I

$R_3$= H, $R_4$=phenylglycine; AMP-Imp E

$R_3$=phenylglycine, R4= H  AMP-Imp I

AMX-Imp C/AMP-Imp C
(diketopiperazine)

AMX-Imp F/AMP-Imp E
(phenylpyrazinol)

R2= $CO_2H$, AMX-Imp D/AMP-Imp D
(penicilloic acids)

R2= H, AMX-Imp E/AMP-Imp F
(penilloic acids)

AMX-Imp I/AMP-Imp L

(hydroxyphenylglycine/phenylglycine)

AMP-Imp G  (diphenyldiketopiperazine)

AMX-Imp H/AMP-Imp K
(pivaloylhydroxyphenylglycine/pivaloylphenylglycine)

Figure 1.5.  Chemical structure of impurities of amoxicillin (AMX) and ampicillin (AMP)
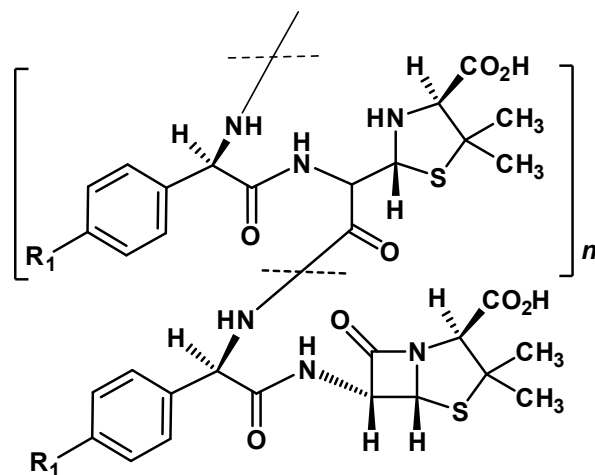
AMX-Imp J/AMP-Imp M
(co-oligomer of penicilloic acid and aminopenicillin)
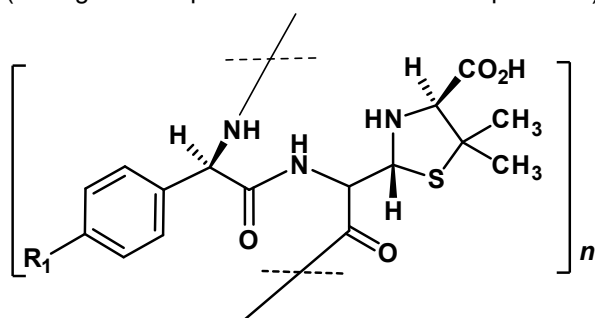
AMX-Imp K/AMP-Imp N (oligomer of penicilloic
acid of aminopenicillin/ open ring oligomer)
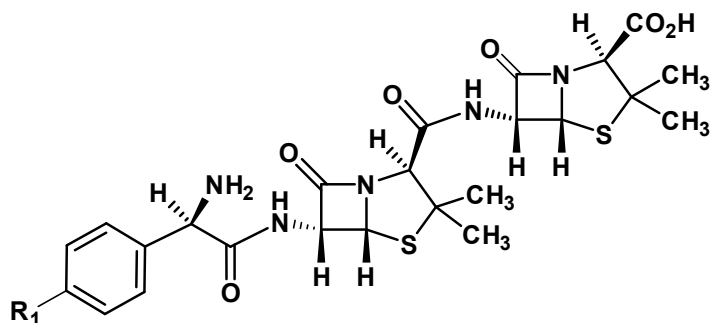
AMX-Imp L /Amp Imp 5 (6-APA amoxicillin amide/penicillanyl ampicillinamide)
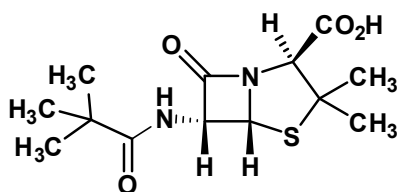
AMP-Imp J (pivaloyl aminopenicillanic acid)

Figure 1.6. Chemical structures of impurities of amoxicillin (AMX) and ampicillin (AMP)
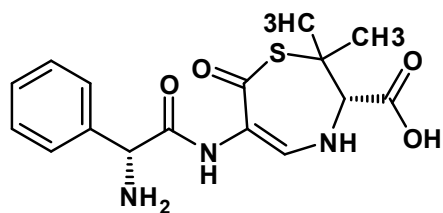
AMP -Imp 1 (ampicllin thiazepine analog)
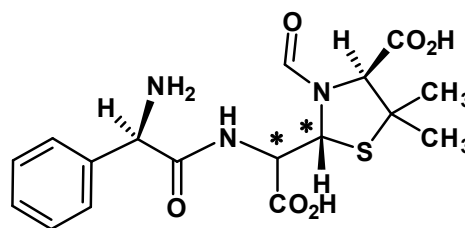
AMP- Imp 2 (*N*-formyl ampicilloic acid)
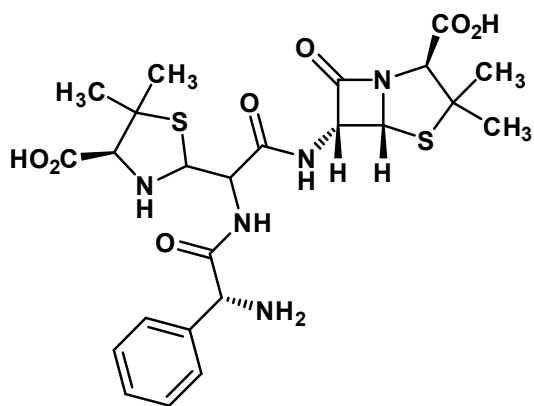
AMP- Imp 3 (ampicilloyl aminopenicillanic acid)
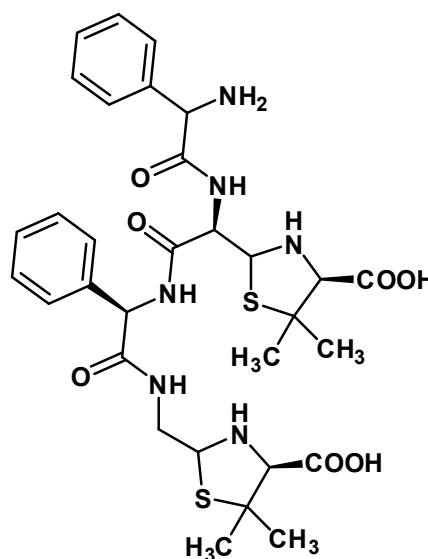
AMP- Imp 4 (ampicillin open ring dimer)
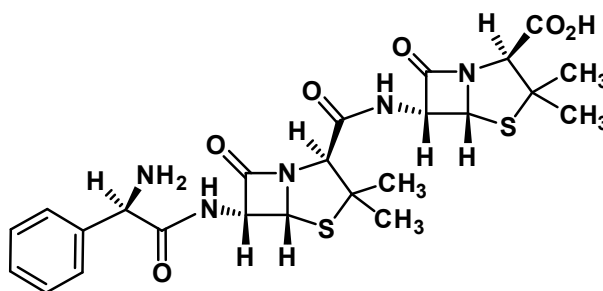
AMP-Imp 5  (penicillanyl ampicillinamide)

Figure 1.7.   Chemical structures of additional impurities of ampicillin in USP 40.

PenG-Imp A/ PenV-Imp A

PenG-Imp B/PenGp-Imp E
(phenylacetic acid)

PenV-Imp B
(phenoxyacetic acid)

PenV-Imp A (benzylpenicillin)

R₁= phenyl; PenG-Imp C
(4-hydroxybenzylpenicillin)

R₁= phenoxy; PenV-Imp D
(4-hydroxyphenoxymethylpenicillin)

PenG-Imp E; R₁=phenyl, R₂=CO₂H (penicilloic acids)

PenG-Imp F; R₁=phenyl, R₂=H (penilloic acids)

PenV-Imp E; R₁=phenoxy, R₂=CO₂H (penicilloic acids)

PenV-Imp F; R₁=phenoxy, R₂=H (penilloic acids)

PenG-Imp D = PenGb-Imp D=PenGp-Imp D

PenG-Imp E = PenGb-Imp E=PenGp-Imp B

PenG-Imp F=PenGb-Imp F= PenGp-Imp F

PenG-Imp D (penillic acid)

PenGp-Imp A (4-aminobenzoic acid)

PenG-Imp G (iso-penicillin F)

PenGb-Imp C
(benzylpenicilloic acids benzathide)

PenG-Imp F (dihydropenicillin F)

Figure 1.8.  Chemical structures of known impurities of benzylpenicillin (PenG), benzathine benzylpenicillin (PenGb), procaine benzylpenicillin PenGp) and phenoxymethyl penicillin (PenV)

R₂= CO₂H; Clx-Imp A (penicilloic acids)

R₂= H; Clx-Imp B (penilloic acids)

CLX-Imp D

CLX-Imp C (6-APA)

CLX-Imp E (6-APA cloxacillin amide)

CLX-Imp 1 (tiocloxacillin)

CLX-Imp 2 (cloxacillin penicilloic penicillamide)
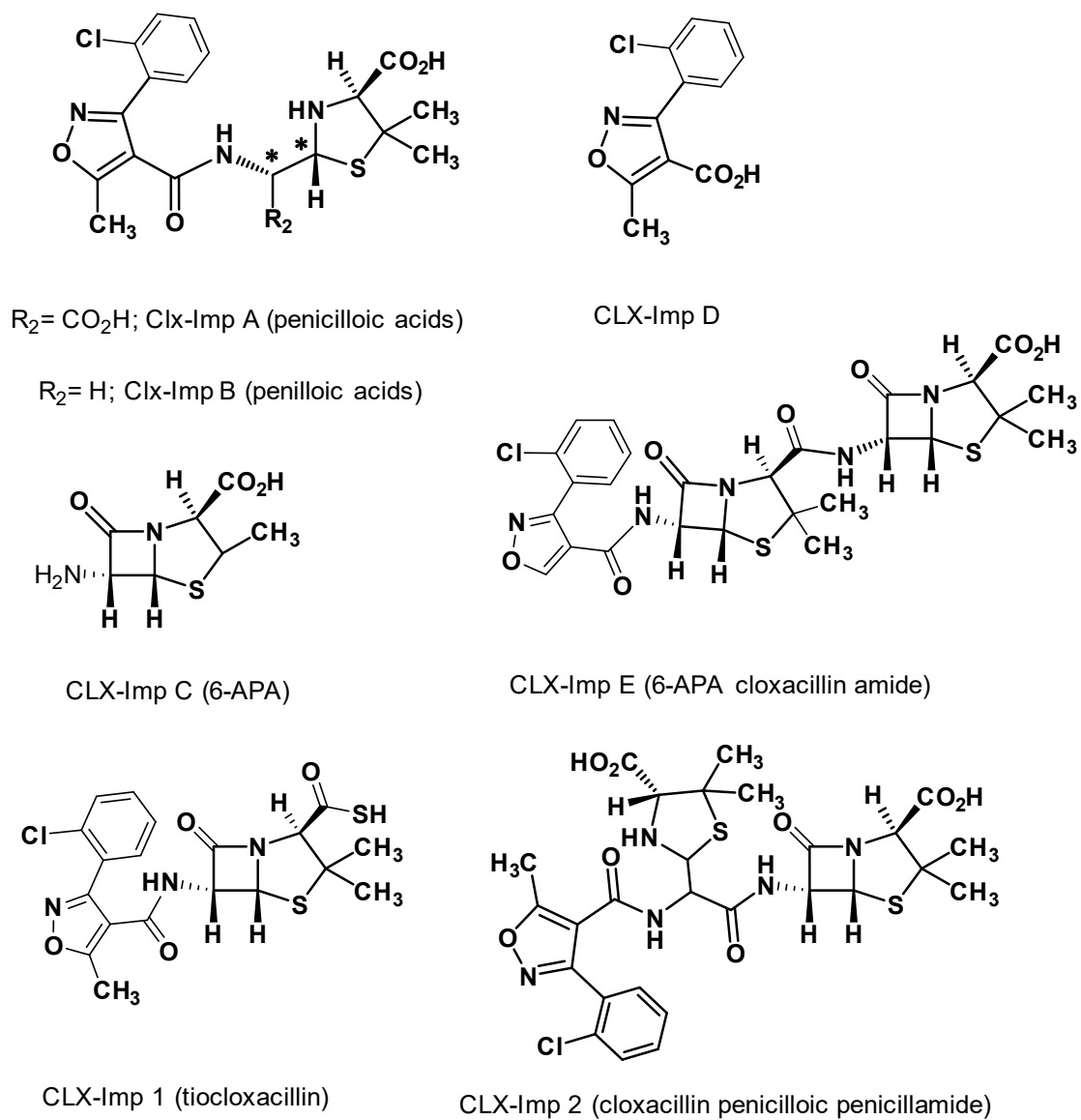
Figure 1.9.   Chemical structures of known impurities of cloxacillin (CLX)

CFR-Imp A/CFL-ImpB (7-ADCA;
7-aminodeacetoxycephalosporanic acid)

CFR-Imp E

CFR-Imp B (D-dihydrophenylglycine)

CFR-Imp F/CLX-Imp D

CFR-Imp C & CFR-Imp D
(isomer 1 & 2)

CFR-Imp G/CFL-Imp E
(7-ADCA pivalamide)

CFL-Imp A (D-phenylglycine)

and epimer at C*

CFL-Imp F(delta-2-cefalexin)

CFL-Imp C (phenylglycylcefalexin)
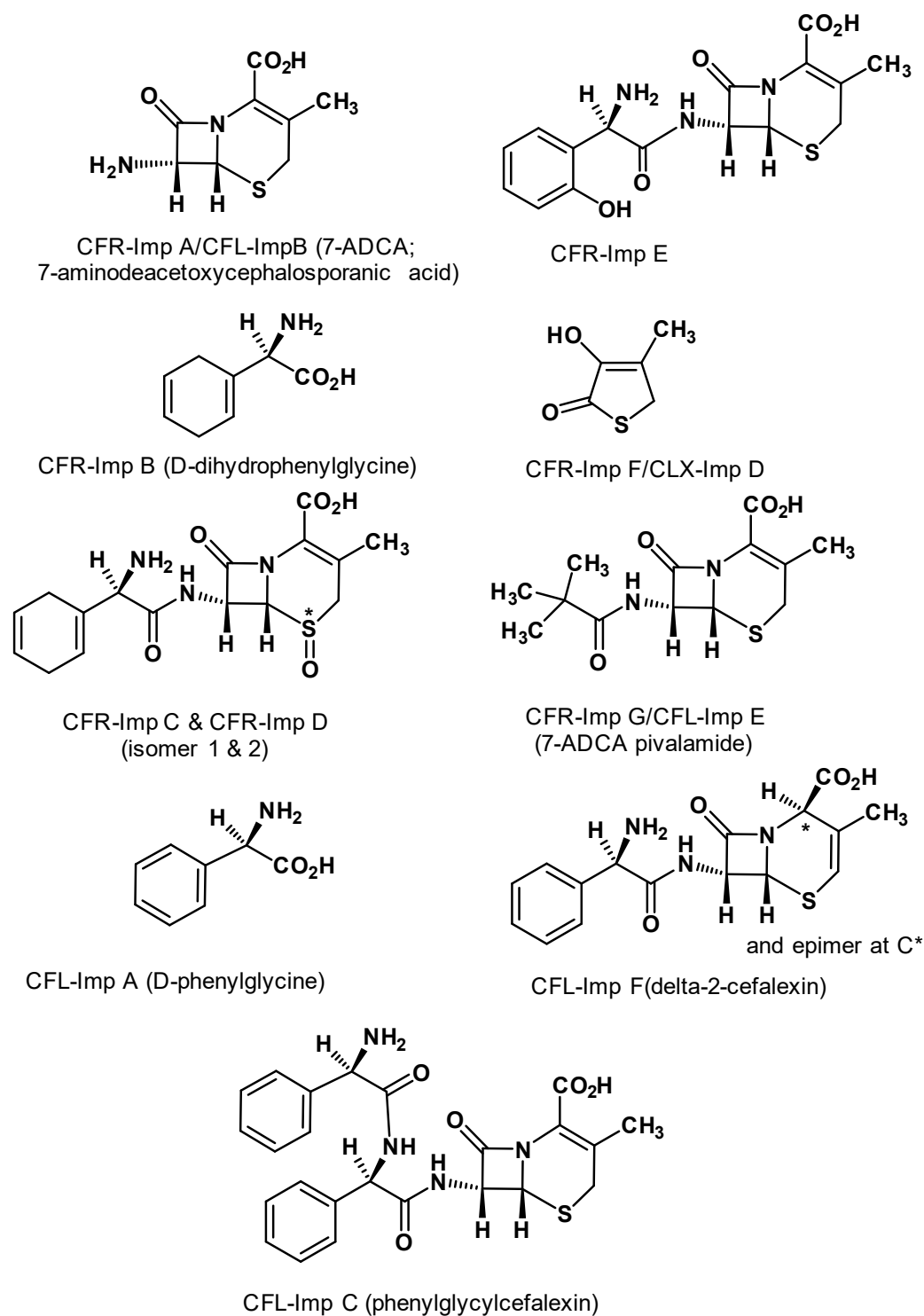
Figure 1.10. Chemical structures of known impurities of cefradine (CFR) and cefalexin (CFL).

CFZ-Imp A

(deacyltated cefazolin)

CFZ-Imp E

(methylthiadiazole thiol)

(MMTD)

CFZ-Imp H (7-ACA)

CFZ-Imp B

(cefazolin pivaloyl)

CFZ-Imp I

(cefazoloic acid)

CFZ-Imp C

(cefazolin 3-methyl analog)

CFZ-Imp J

CFZ-Imp D

(cefazolin acetoxy analog)

CFZ-Imp K

(cefazolinamide)

CFZ-Imp G

(cefazolin lactone)

CFZ-Imp L

(cefazolin epimer)
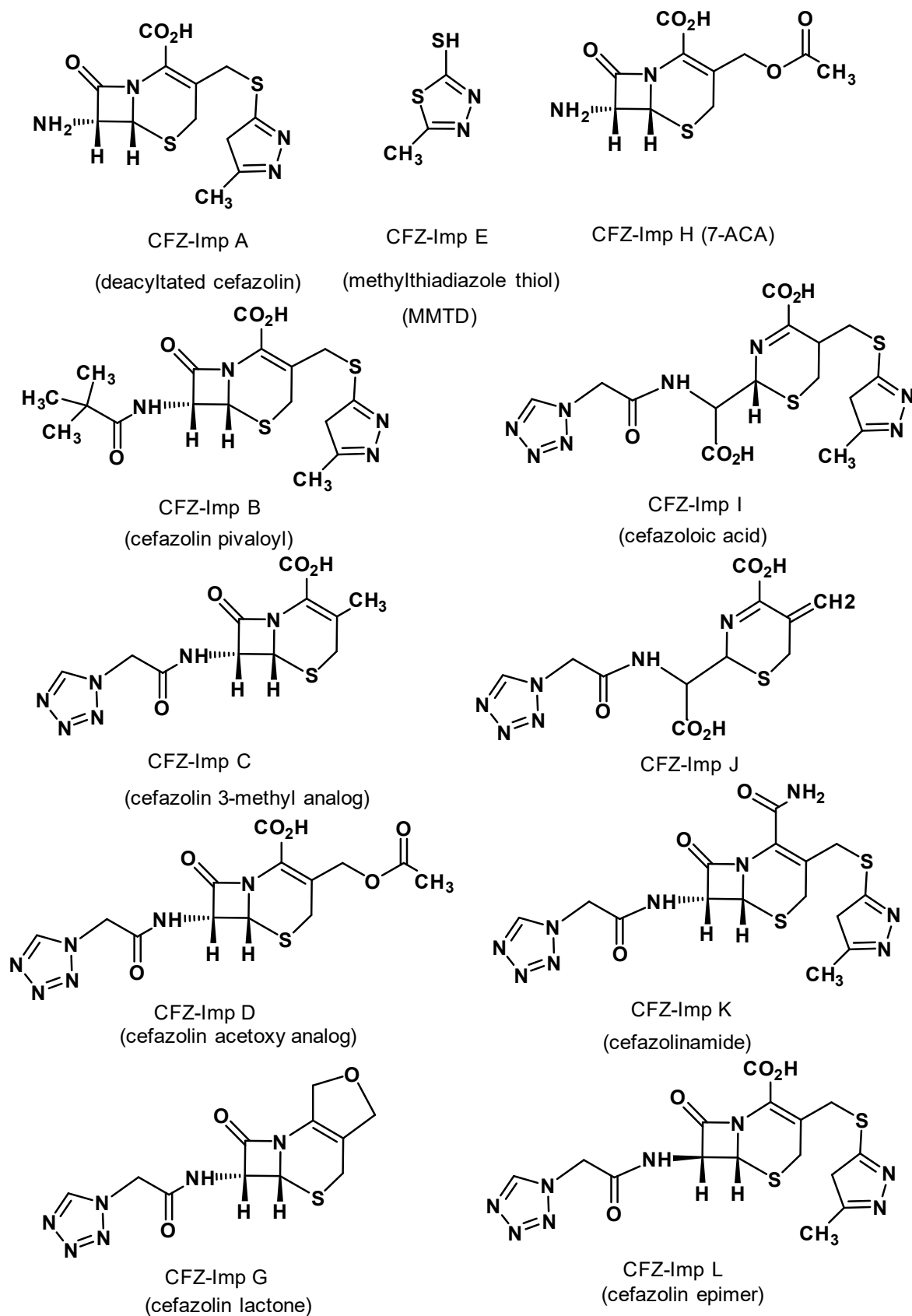
Figure 1.11. Chemical structures of cefazolin (CFZ) impurities

**CFZ-Imp 1**
(2-(1*H*-tetrazol-1-yl)acetic acid)

**CFZ-Imp 3**
(cefazolin 3-hydroxymethyl)

**CFZ-Imp 2**
*N*-(2,2-dihyroxyethyl)-2-(1H-tetrazol-1-yl)
acetamide

**CFZ-Imp 4**
(cefazolin open ring lactone)
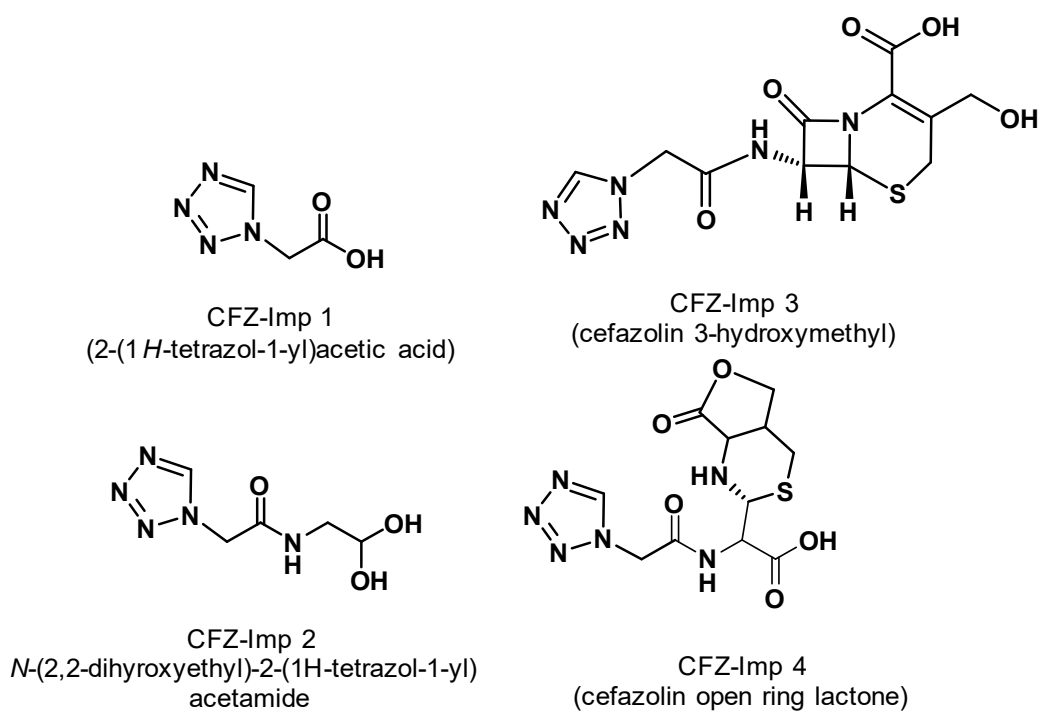
Figure 1.12. Chemical structures of additional cefazolin impurities from USP 40



CFX-Imp A; R=$CO_2H$

CFX-Imp B; R=H

CFX-Imp D

CFX-Imp C (cefixime 7-epimer)

CFX-Imp E; $R_2$=$CH_3$, $R_3$=H

CFX-Imp F; $R_2$=CH=CH, $R_3$=$C_6H_5$
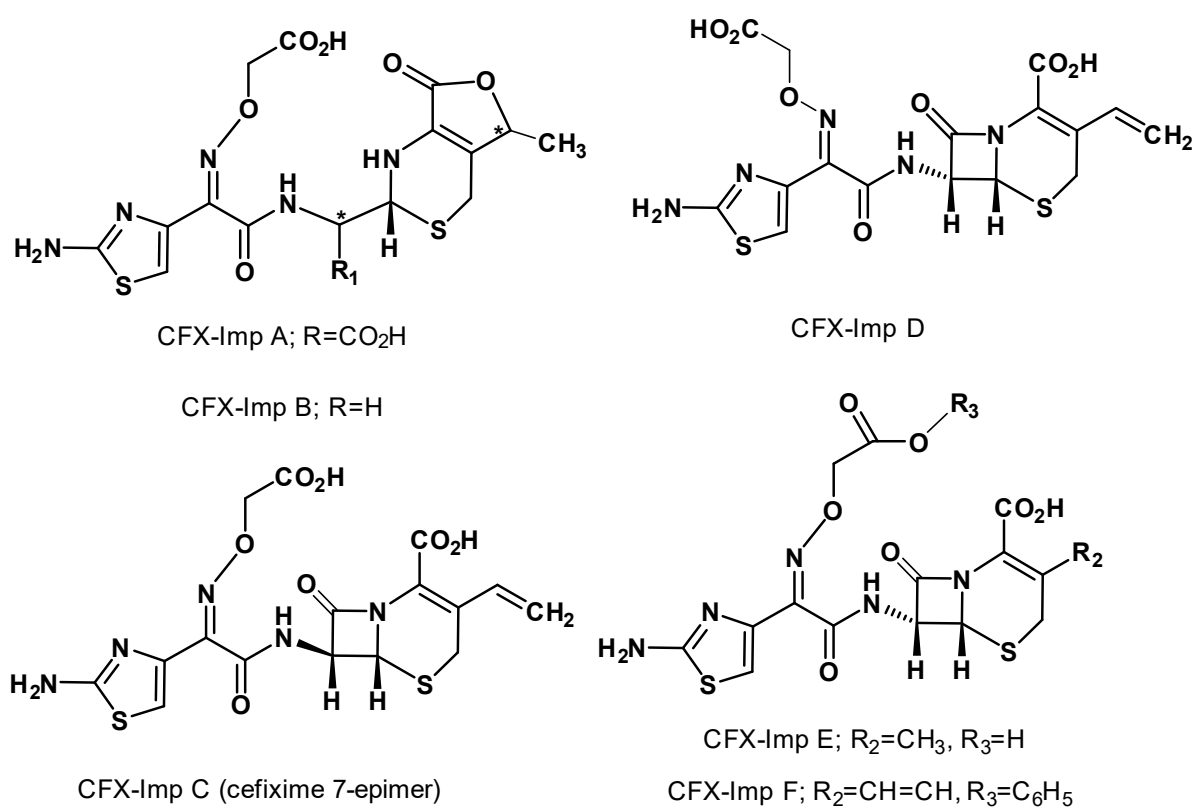
Figure 1.13. Chemical structures of known impurities of cefixime (CFX)

CFT-Imp A $R_1=R_2=$ H
(deacetoxycefotaxime)

CFT-Imp B $R_1=$OH, $R_2=$ H
(deacetylcefotaxime)

CFT-Imp C $R_1=$O-CO-CH$_3$,$R_2=$ CHO
(*N*-formylcefotaxime)

CFT-Imp D
(*E*-isomer)

CFT-Imp E
deacetylcefotaxime lactone

CFT-Imp F
(cefotaxime dimer)

CFT-Imp G
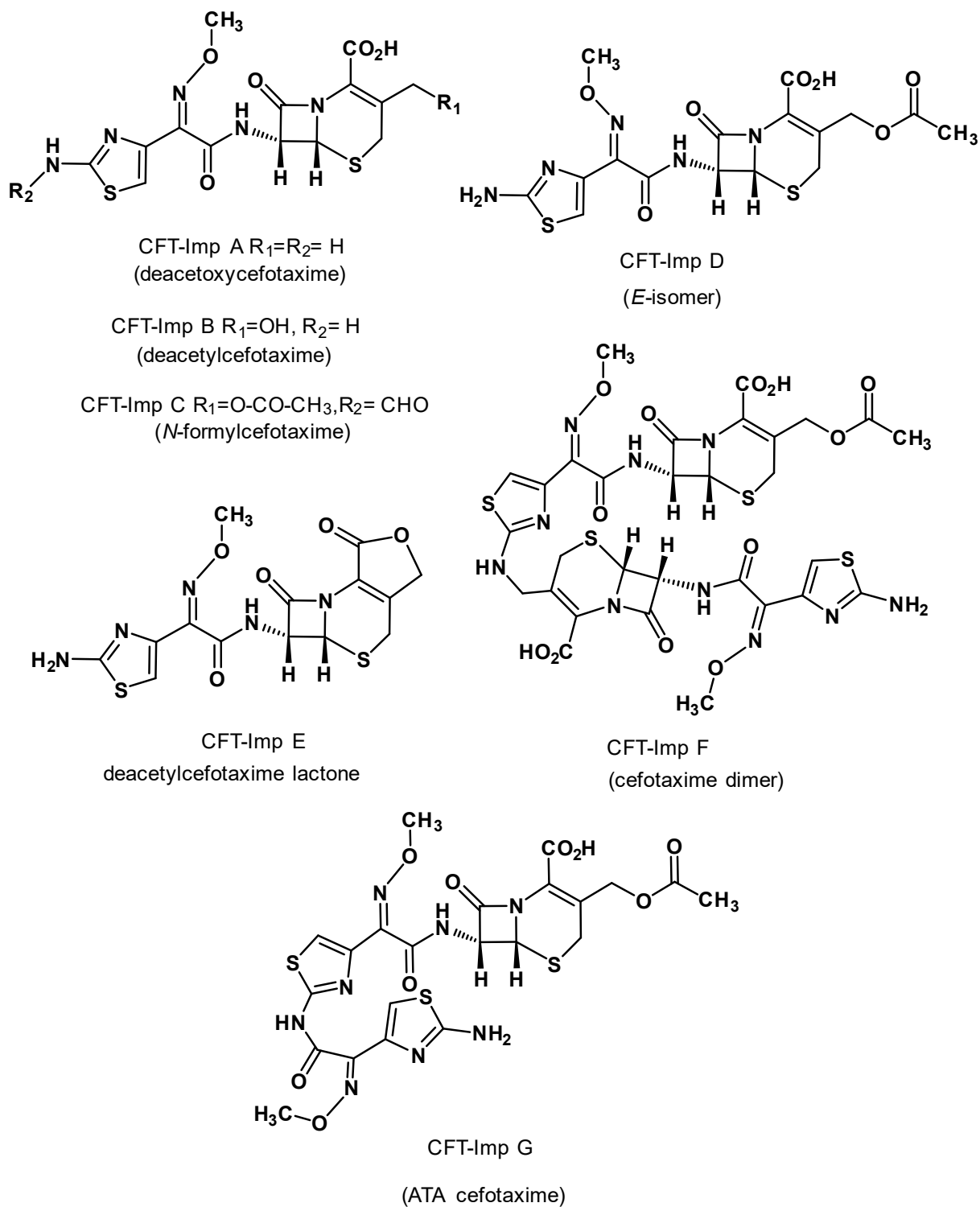
(ATA cefotaxime)

Figure 1.14. Chemical structures of impurities of cefotaxime (CFT)
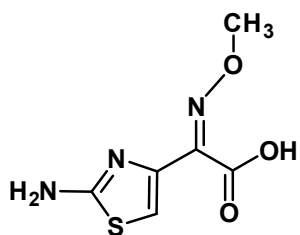
CFT-Imp 1

((Z)-2-(2-Aminothiazol-4-yl)-2-(methoxyimino)acetic   acid)
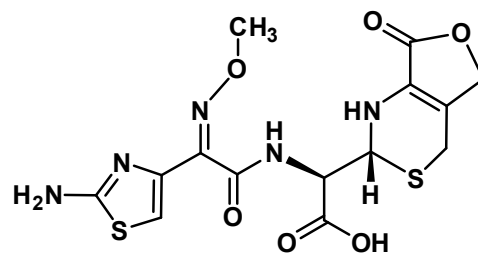
CFT-Imp 2

(cefotaxime open ring lactone)
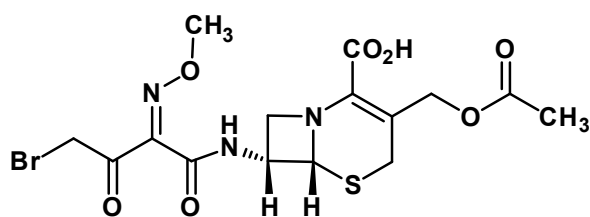
CFT-Imp 3

(bromoacetyl analog)

Figure 1.15. Chemical structures of additional impurities of cefotaxime (CFT) from USP 40.
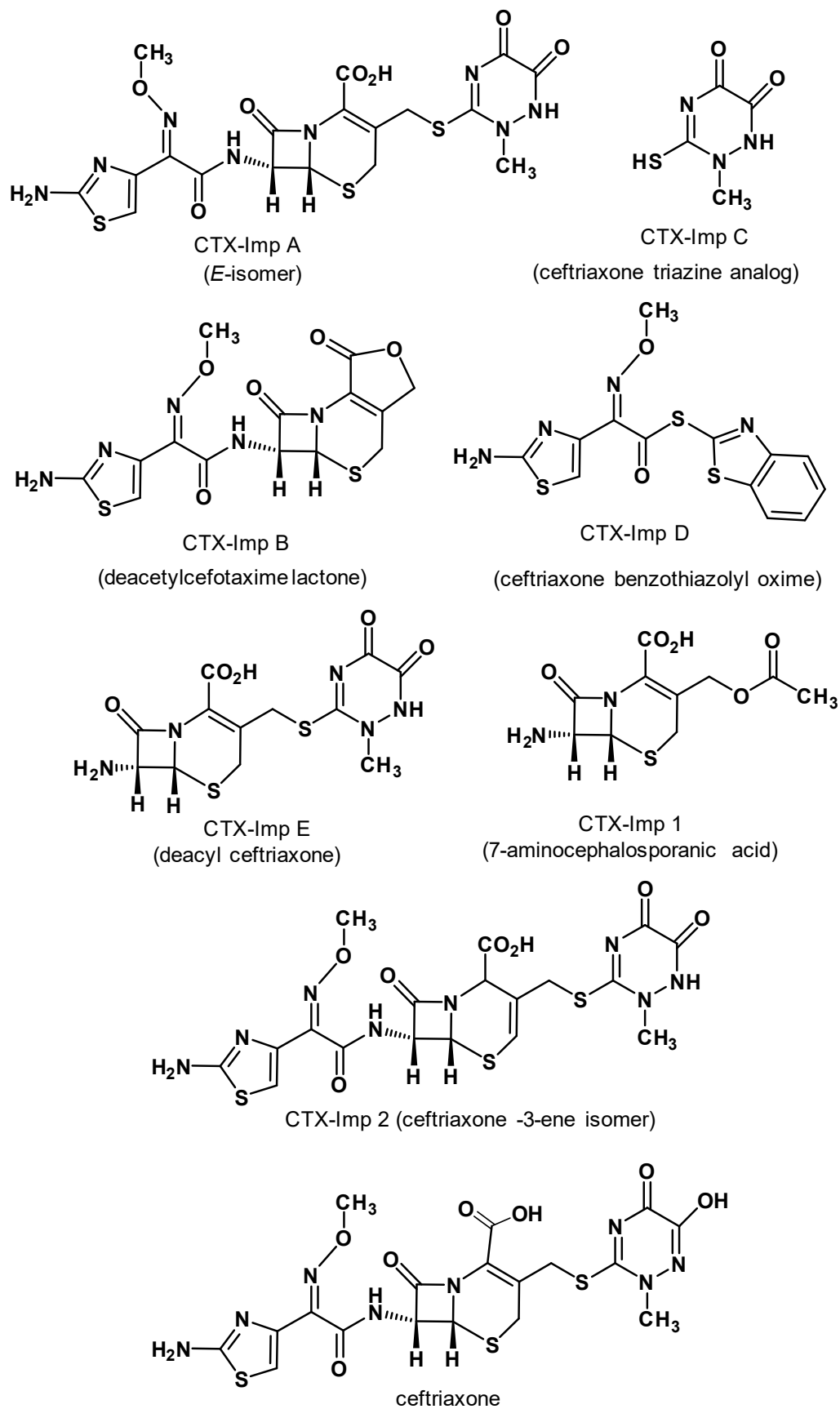
Figure 1.16. Chemical structures of known impurities of ceftriaxone (CTX)
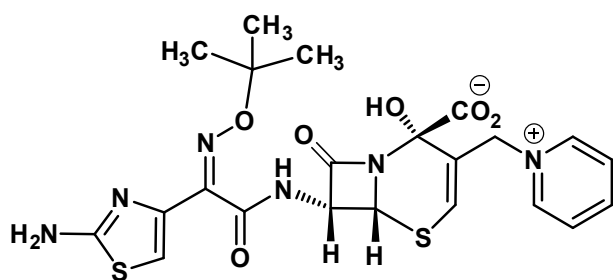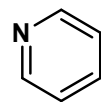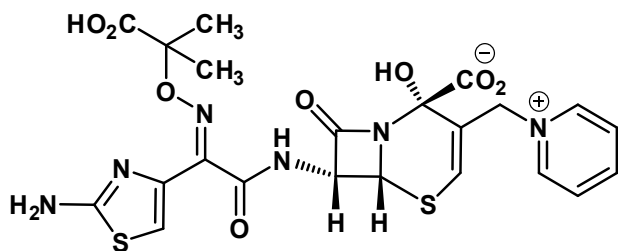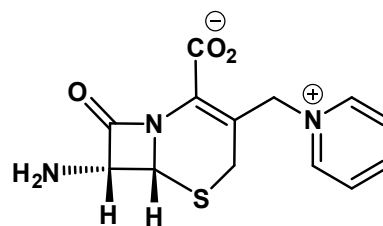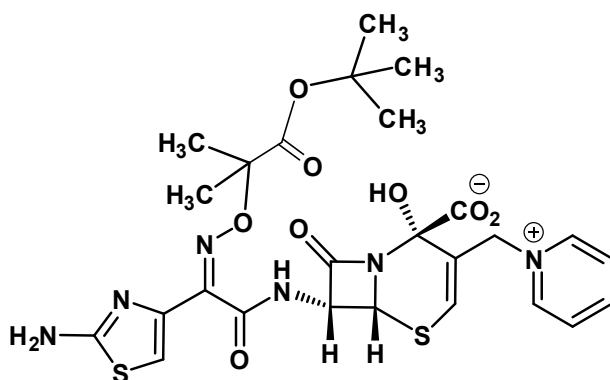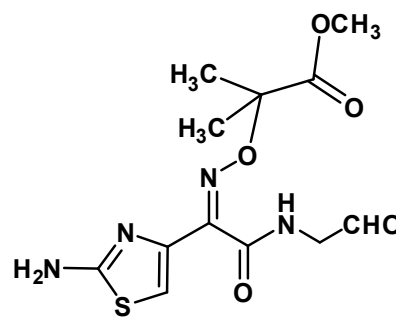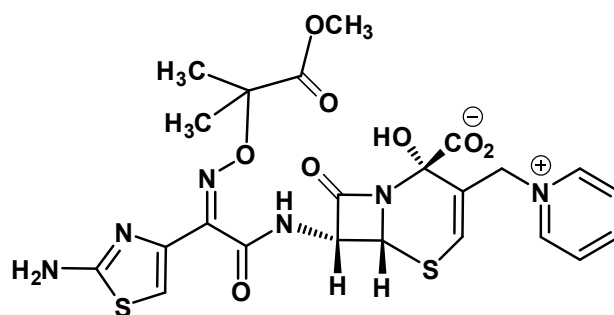
Figure 1.17. Chemical structure of known impurities of ceftazidime (CTZ)
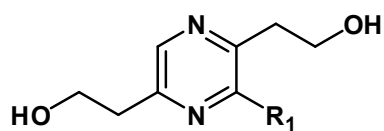
CLV-Imp A; R$_1$=H

CLV-Imp B; R$_1$=CH$_2$-CH$_2$-CO$_2$H

CLV-Imp C; R$_1$=CH$_2$-CH$_3$
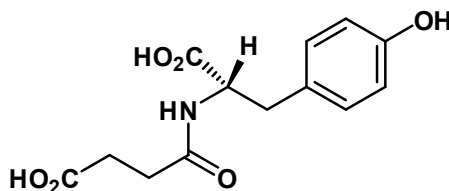
CLV-Imp G
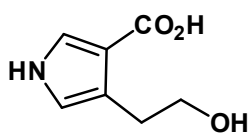
CLV-Imp D
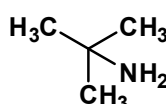
CLV-Imp H
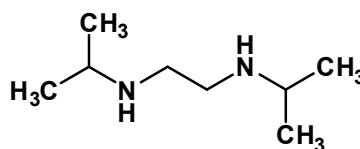
CLV-Imp L
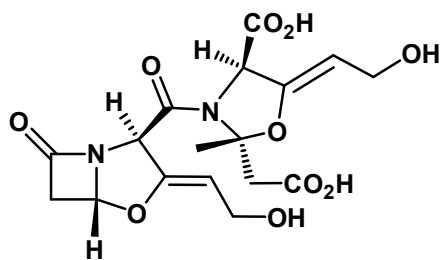
CLV-Imp E
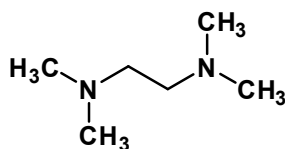
CLV-Imp J
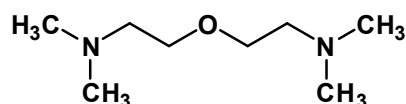
CLV-Imp M
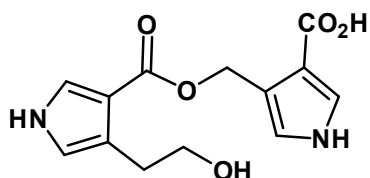
CLV-Imp F
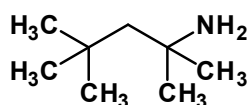
CLV-Imp K

Figure 1.18. Chemical structures of known impurities of clavulanic acid (CLV)
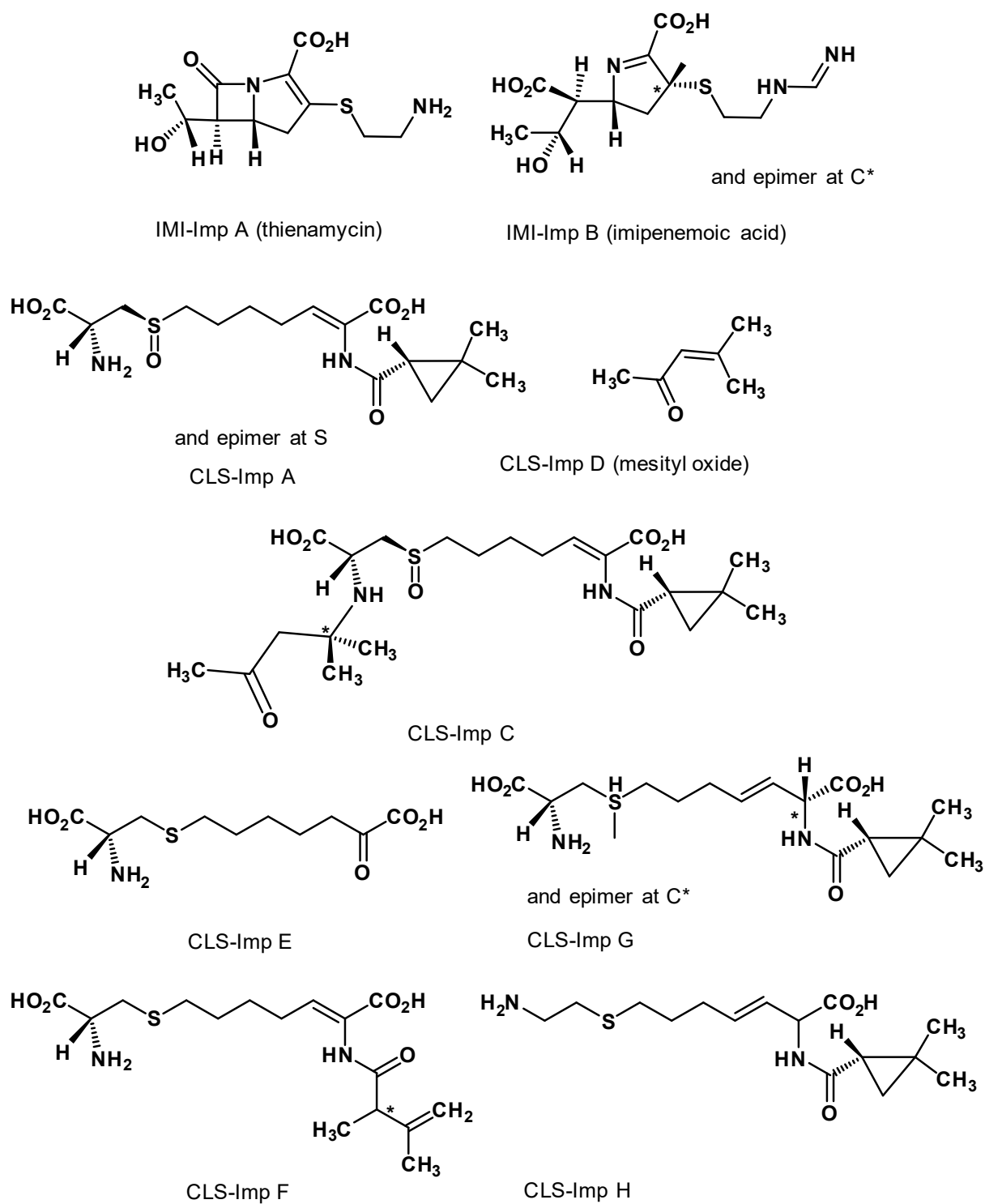
Figure 1.19. Chemical structure of known impurities of imipenem (IMI) and cilastatin (CLS)
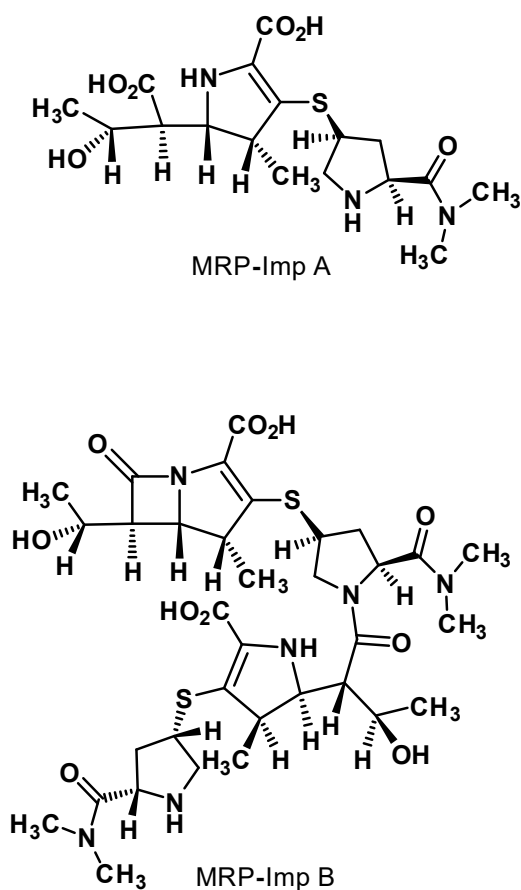
Figure 1.20. Chemical structures of impurities of meropenem (MRP)
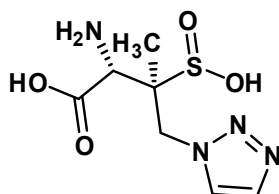


tazobactam related compound A

Figure 1.21. Chemical structure of impurity of tazobactam (TZB)
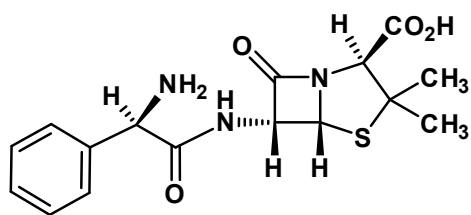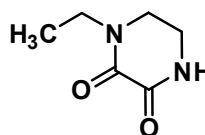
PPR-A (ampicillin)

PPR-E (1-ethylpiperazine-2,3-dione)
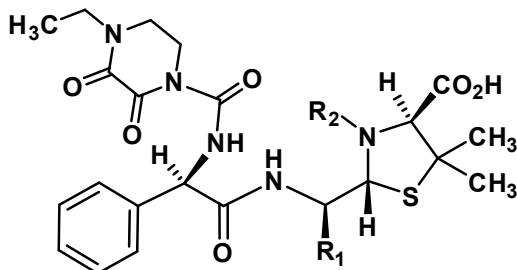
PPR-B, $R_1$=CO$_2$H, $R_2$ = H (piperacillin penicilloic acid)

PPR-C, $R_1$=H, $R_2$ = H (piperacillin penilloic acid)

PPR-B, $R_1$=CO$_2$H, $R_2$ = -COCH$_3$ (acetylated piperacillin penicilloic acid)
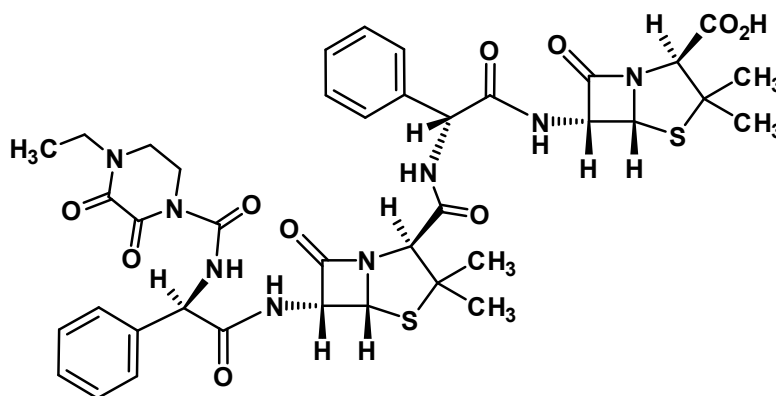
PPR-D (piperacillinylampicillin)

Figure 1.22. Chemical structures of impurities of piperacillin (PPR)

PPR-Imp 1
(formyl penicillamine)

PPR-Imp 2
(piperazinedionecarbonyl D-phenylglycine)

PPR⁻ Imp 3
(L-piperacillin)

PPR-Imp 7
(piperacillin methyl ester)

PPR-Imp 8

(piperacillin dimer ethyl ester)
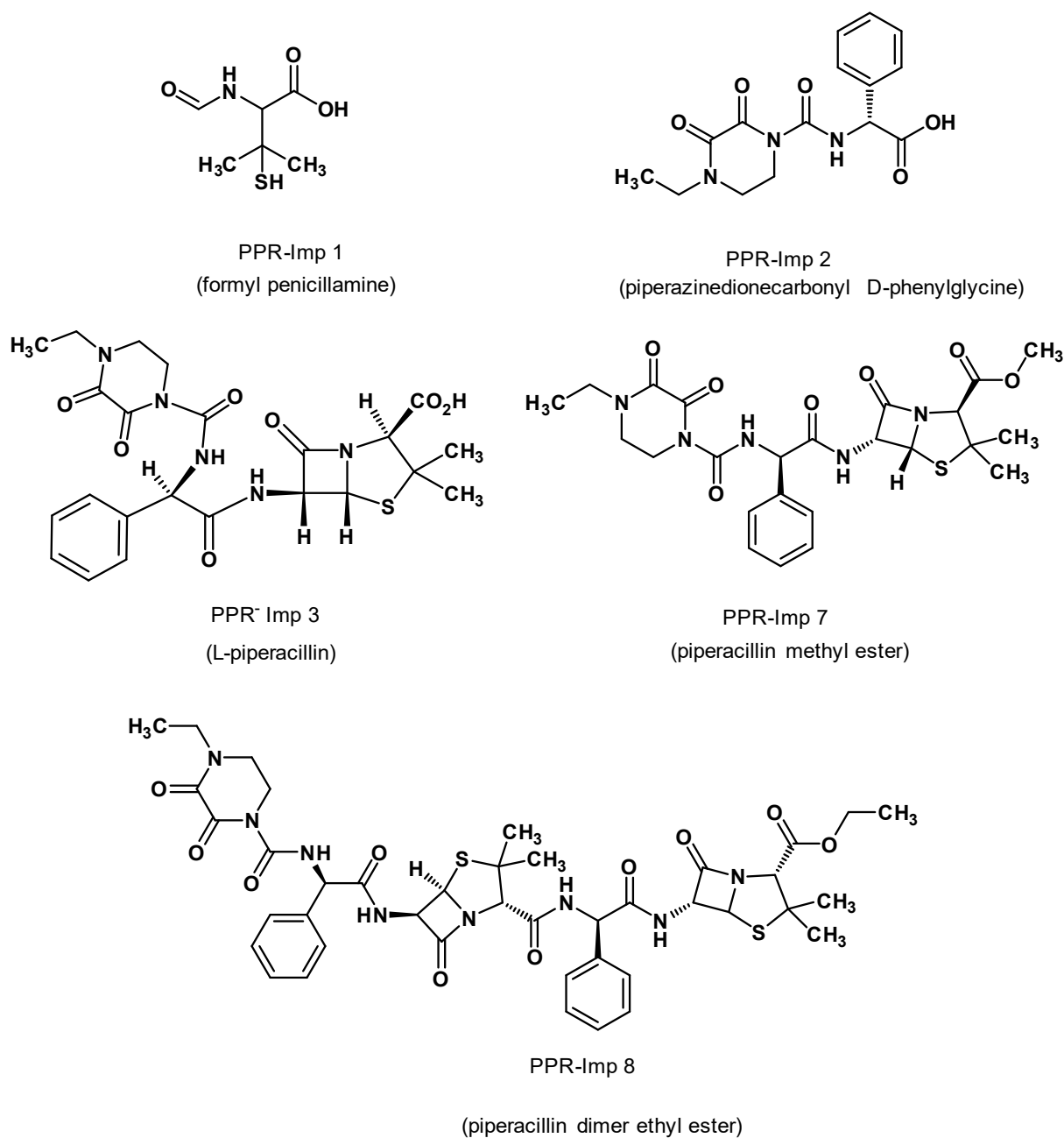
Note: PPR-Imp 4, 5 and 6 are unidentified impurities mentioned in USP 40

Figure 1.23. Chemical structures of impurities of additional impurities of piperacillin (PPR) in USP 40 (Imp 1- Imp 8)

PPR-Imp 9

(piperacillin dimer thiazolamide derivative)

PPR-Imp 10

(piperacillin penicillamide)

PPR-Imp 11

(piperacillin dimer)
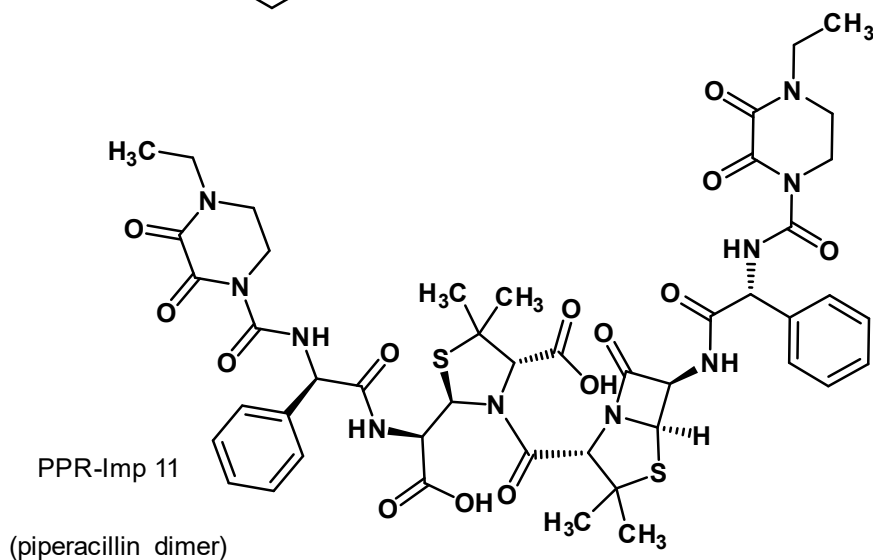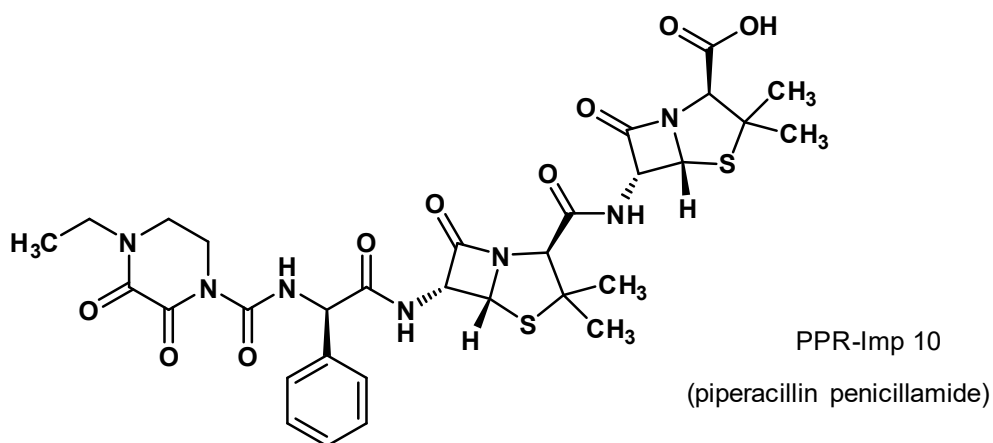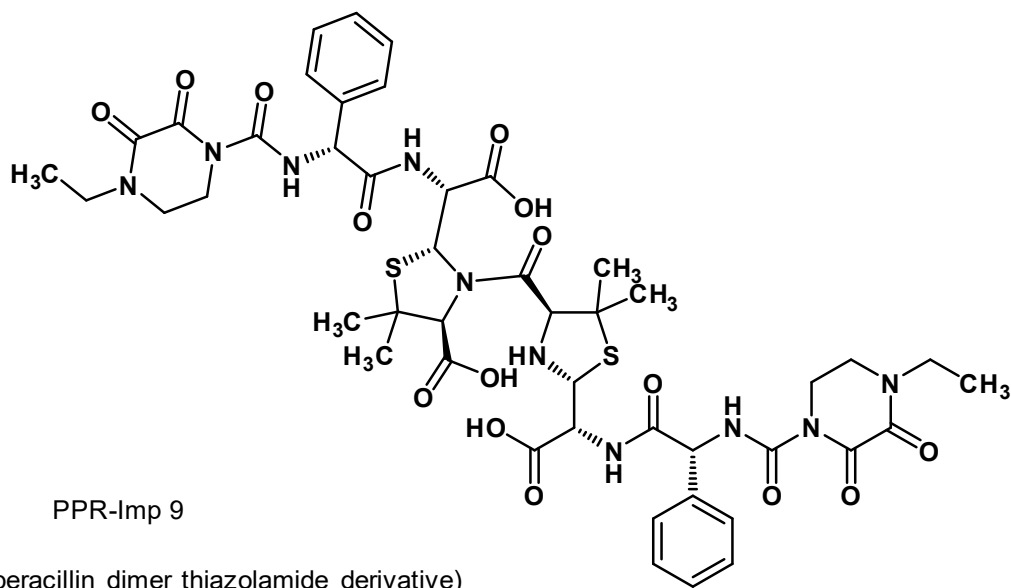
Figure 1.24. Chemical structures of impurities of additional impurities of piperacillin (PPR) in USP 40 (Imp 9- Imp 11)
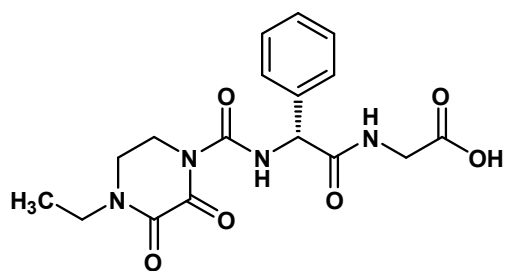
PPR-Imp 12

(piperazinedionecarbonyl  D-phenylglycylglycine)
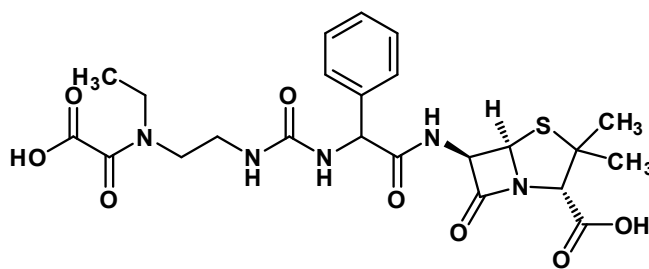
PPR-Imp 15

(piperacillin oxalylamide)
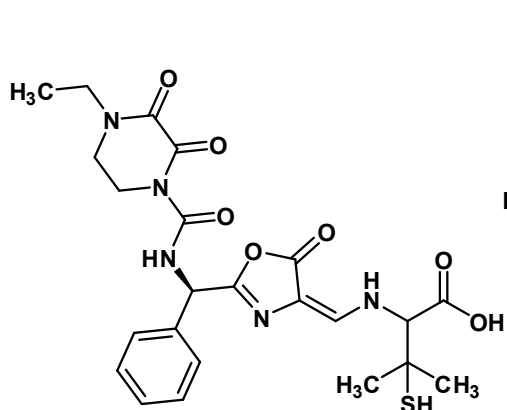
PPR⁻ Imp 13

piperacillinpenicillenic  acid
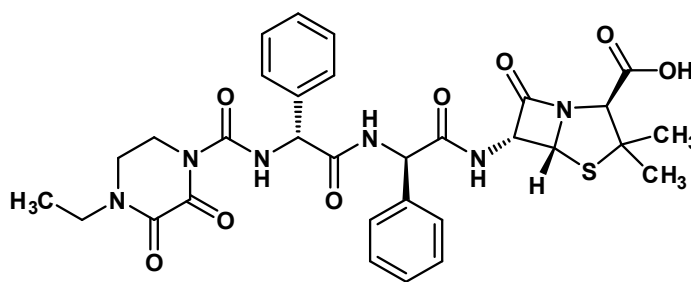
PPR-Imp 16

(piperazinedione-carbonyl  D-phenyl-
gylcylampicillin)
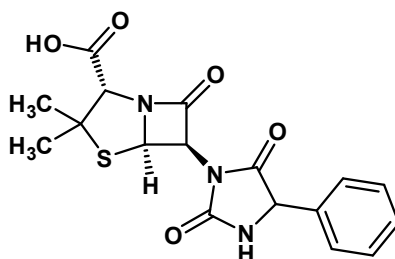
PPR-Imp 14

(ampicillin hydantoin analog)

Figure 1.25. Chemical structures of impurities of additional impurities of piperacillin (PPR) in USP 40 (Imp 12- Imp 14)
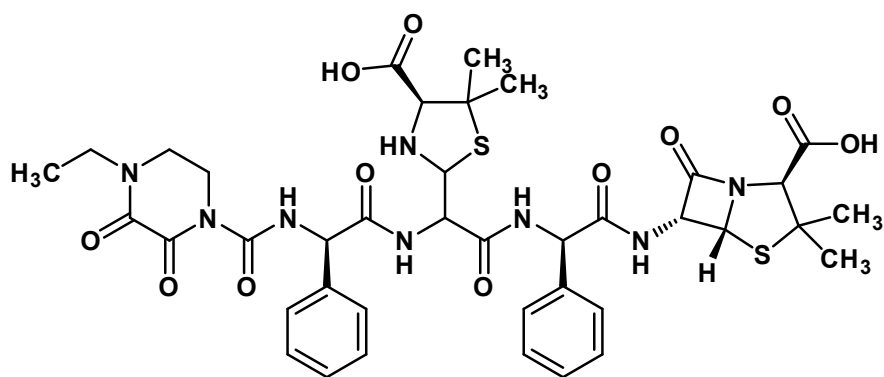
PPR⁻ Imp 17

(open ring piperacillinylampicillin)
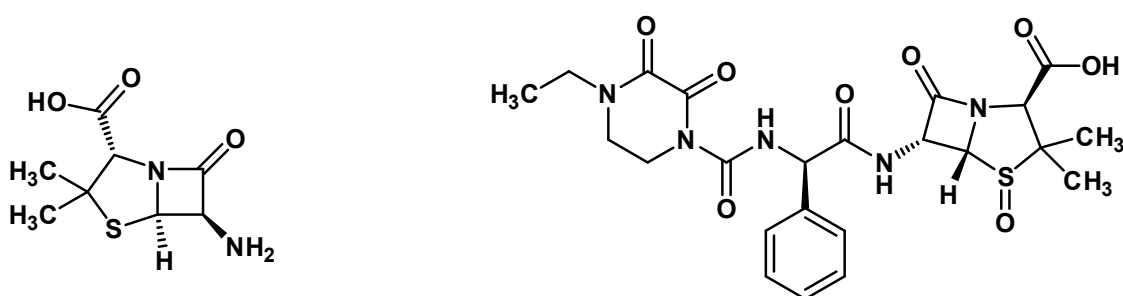


PPR-Imp 18

(6-aminopenicillanic acid)



PPR-Imp 19

9piperacillin sulfoxide)
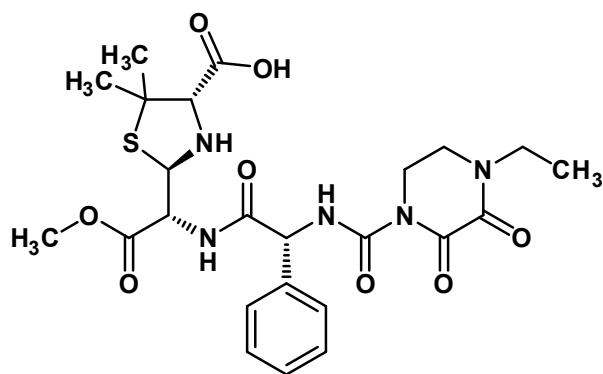


PPR-Imp 20

(piperacillin methyl penicilloatte)

Figure 1.26. Chemical structures of impurities of additional impurities of piperacillin (PPR) in USP 40 (Imp 17- Imp 20)

## 1.2.2. Compendial limits for assay and impurity tests

Comparison of the assay limits for APIs of beta-lactam antibiotics from Ph.Eur. 9.2 and USP 40 (Figure 1.27) shows that similar limits are practiced by both pharmacopoeia for most of the molecules but a significant difference is present for the assay limits of cephalexin, ceftriaxone, and clavulanic acid(82, 83). In case of USP the assay units are sometimes given in terms of potency expressed as µg/mg (which were then converted to mg/mg% for in Figure 1.27) whereas assay limits are described as percentage content by Ph. Eur. and BP.

Table 1.4 -1.6 describe the current limits of impurities of beta-lactam antibiotics for total impurities, unspecified impurities and specified impurities by the Ph. Eur. 9.2 and USP 40. Although the limits described are generally similar in the two pharmacopoeias, some monographs including amoxicillin, ampicillin, ceftriaxone, cefixime, meropenem and clavulanic acid show considerable difference in the limits provided by the two pharmacopoeias.
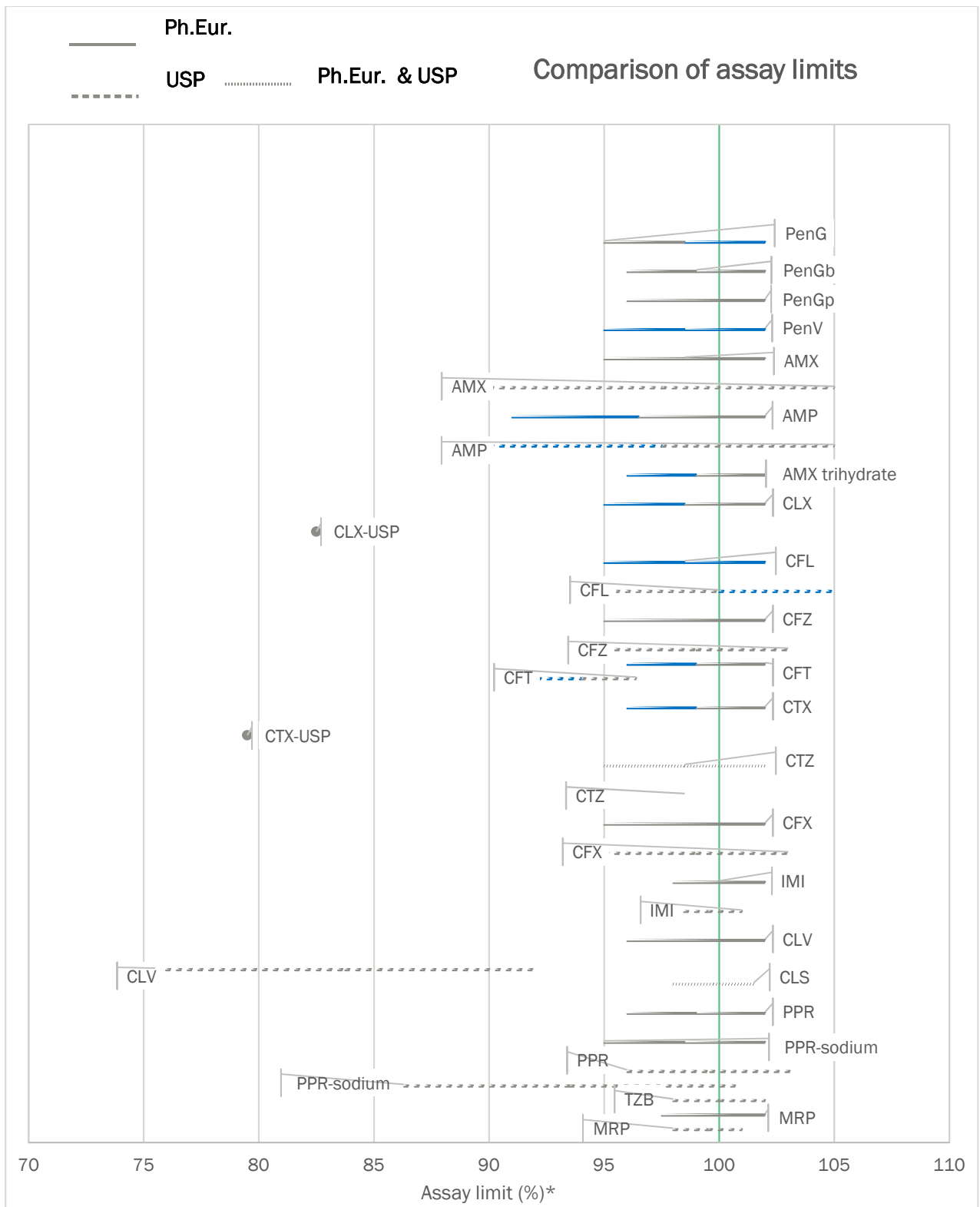
Figure 1.27. Assay limits for essential beta-lactams APIs according to Ph. Eur. 9.2 and USP 40.

Table 1.4     Limits for total, individual and specified impurities of essential beta-lactam antibiotics in API by European Pharmacopoeia 9.2 (82)

| | Beta-lactam Antibiotic | Total impurities (NMT %) | Unspecified individual impurity (NMT %) | Specified impurities (NMT %) |
|---|---|---|---|---|
| 1. | Benzylpenicillin | 3.0 (0.05) | 0.2 | Imp E: 2.0, Imp F: 1.0 (sum of epimers for Imp E, F), Imp B: 0.5, Imp A, C, D, G, H: 0.2 |
| 2. | Benzathine penicillin | - | 0.05 | Imp C: 2.0 |
| 3. | Procaine benzylpenicillin | - | 1.0 | Imp A: 0.024 |
| 4. | Phenoxymethyl-penicillin | 3.0 (0.05) | 0.15 | Imp E, F: 1.0 (sum of epimers), Imp B: 0.2 |
| 5. | Amoxicillin sodium Amoxicillin trihydrate | 9.0 (0.1) | 2.0 1.0 | Imp J: 3.0 |
| 6. | Ampicillin sodium Ampicillin trihydrate | - - | 2.0 1.0 | Imp M: 4.5 - |
| 7. | Cloxacillin | 5.0 (0.05) | 1.0 | - |
| 8. | Piperacillin | | 2.0 | |
| 9. | Cefalexin | 3.0 (0.05) | 1.0 | Imp B: 1.0 |
| 10. | Cefazolin | 3.5 (0.05 | 1.0 | - |
| 11. | Cefotaxime | 3.0 (0.05) | 0.2 | Imp A, B, C, D, E, F: 1.0 |

Table 1.4 *(cont'd)*

|  | **Beta-lactam Antibiotic** | **Total impurities (NMT %)** | **Unspecified individual impurity (NMT %)** | **Specified impurities (NMT %)** |
|---|---|---|---|---|
| 12. | Ceftriaxone | 4.0 (0.1) | 1.0 | - |
| 13. | Ceftazidime | 5.0 (0.05) | 0.1 | Imp A, B, G: 0.2, Imp F: 500 ppm |
| 14. | Cefixime | 4.5(0.1) | 1.5 | - |
| 15. | Meropenem | 1.1 (0.05, **0.03†**) | 0.1, 0.05† | Imp A: 0.5, Imp B: 0.3 |
| 16. | Imipenem | 1.5 (0.05) | 0.1 | Imp A: 1.0, Imp B: 0.3 (for each epimer) |
|  | *Beta-lactamase inhibitors and other adjuvants in therapy* | | | |
| 17. | Clavulanic acid | 2.0 (0.05) | 0.2 | Imp E, G: 1.0 |
| 18. | Cilastatin | 1.0 **(0.03)** | 0.05 | Imp A: 0.5 for each epimer, Imp C: 0.4, Imp E: 0.3, Imp B, F, H: 0.1, Imp G: 0.1 for each epimer |

*† meropenem is produced by fully synthetic method*

NMT= Not more than

Table 1.5    Limits for total, individual and specified impurities of essential beta-lactam antibiotics in API by United States Pharmacopoeia (USP 40) (83)

| | Beta-lactam Antibiotic | Total impurities (NMT %)-disregard limit (%) | Unspecified individual impurity (NMT %) | Specified impurities (NMT %) |
|---|---|---|---|---|
| 1. | Amoxicillin | 5.0 **(0.03)** | 1.0 | Imp A: 0.5, Imp C-E, G, J, L, M: 1.0 |
| 2. | Cloxacillin | 5.0 (0.05) | 1.0 | All known impurities are specified: 1.0 |
| 3. | Piperacillin/PPR sodium -Procedure 1 -Procedure 2 | 3.8 (Sum of Imp A, B, D, E, F) | NA | -Imp A: 0.2, B: 1.0, E: 0.2, F: 0.4. For PPR. Sodium: Imp E is not to be reported, Imp A is not specified, Imp F: 1.0, Imp B: 3.5) Imp D: 2.0 |
| 4. | Cefalexin | 5.0 (NA) | 1.0 | |
| 5. | Cefazolin | 3.5 (NA) | 0.1 | Imp E: 0.5, Imp A-D, G, H, I: 1.0, Imp 1 & 2: 1.0, Imp 3 & 4: 0.5 |
| 6. | Cefotaxime -Procedure 1 -Procedure 2 | 3.0 (0.05) 3.0 (0.05) | 0.2 0.2 | Imp G: 0.2, Imp 1: 0.15, Imp 2: 0.15, Imp 3: 0.15 |
| 7. | Ceftriaxone | 2.5 (0.1) | 0.2 | Imp B: 0.5, Imp D: 0.2, Imp C, E, A: 1.0, Imp 1: 0.5, Imp 2: 0.3 |

Table 1.5 *(cont'd)*

| | Beta-lactam Antibiotic | Total impurities (NMT %)- disregard limit (%) | Unspecified individual impurity (NMT %) | Specified impurities (NMT %) |
|---|---|---|---|---|
| 8. | Cefixime | 2.0 (NA) | 1.0 | |
| 9. | Meropenem | 0.3 | 0.1 | NMT than 0.3 for any sum of any two major impurities |
| | Beta-lactamase inhibitors and other adjuvants | | | |
| 10. | Clavulanic acid -Procedure 1 -Procedure 2 | 1.0 (NA) - | - - | Clavam-2-carboxylate potassium: 0.01 |
| 11. | Cilastatin | Purity NLT 98.5% | 0.5 | |
| 12. | Tazobactam | 0.3 (sum if all impurities except Imp A | 0.1 | Imp A: 1.0 |

NA= not available in monograph

Table 1.6 Limits for total, individual and specified impurities of ampicillin by United States Pharmacopoeia (USP 40)

| | Beta-lactam antibiotic-procedure | Total impurities (NMT %)-disregard limit (%) | Unspecified individual impurity (NMT %) | Specified impurities (NMT %) |
|---|---|---|---|---|
| 1. | Procedure 1 | 3.0 | 0.25 | Imp A:0.5, Imp C1 and C2 (epimers): 0.4, 0.3 respectively, Imp F: 1.0, Imp L: 0.5, Imp N, 0.6, Imp M1 (dimer): 1.0, Imp M2 (trimer): 0.4, AMP-Imp1: 0.3 |
| 2. | Procedure 3 | 5.0 | 0.1 | Imp A-C, E-N: 1.0 including Imp M1 (dimer) and Imp M2 (trimer) and Imp N (ampicillin open ring dimer) |
| 3. | Procedure 4 | 5.0 | 1.0 | Imp A, B and L: 0.5, Imp C, D1 and D2, E, F1 and F2, and Imp M1: 1.0, AMP Imp 3 (ampicilloyl aminopenicillanic acid): 0.5, AMP Imp 5 (penicillanyl ampicillinamide): 1.0. |

## 1.3. Assay and impurity profiling methods and challenges faced by the resource limited settings

### 1.3.1. Background

Quality assurance of pharmaceuticals demands expertise of highly trained personnel, provided with the high technology instruments and the running budget for purchase of chemicals and maintenance of the facility. High sample loads and lack of finances in resource limited settings of LMICs lead to compromises in performing the essential set of experiments.

The incidents of poisoning and deaths due to contamination of cardiovascular medicine in Pakistan and the absence of capacity to detect, investigate and address the issue in time by the public sector regulatory facility showed the gravity of negligence and the wide gap between the analytical and regulatory resources of the developed, developing and under-developed world (35).

This section is based upon the routine quality analysis of these molecules in the industry and by the regulatory laboratory.

### 1.3.2. The ideal assay and impurity profile test method for resource limited settings

Limitations of the resource limited settings studied by analytical scientists advocate the need of development of simple and cost-effective methods using cheap and routine laboratory chemicals for the essential medicines. A concept of intermediary methods practiced by countries like Tanzania successfully ensured high sample analysis rate leading to the control in the prevalence of poor quality and substandard medicine. Enhancing capacity of the regulatory settings by providing these simplified methods of analysis offers a practical approach for combating the menace of substandard and falsified medicines.

A simple and cost-effective HPLC method for beta-lactam antibiotics can be referred to a reverse phase isocratic method that is carried out using routine chemicals and solvents like phosphate buffer and methanol employing routine C18 reverse phase HPLC columns (23).

## 1.3.3. Compendial methods and challenges for resource limited settings.

## 1.3.3.1. Cost intensive and sophisticated chromatographic conditions

Expensive and sophisticated choices in analytical methods can make the routine quality control tests difficult to manage within the limited capacity of the LMICs. The key chromatographic conditions that pose challenge to the resource limited settings are as follows:

- Gradient elution

- Ion pair chromatography

- Use of acetonitrile as organic content of mobile phase

- Use of columns other than C18 stationary phase

- Column particle size lower than 5 μm

- Need of reference standards for system suitability test, and peak identification of impurities

- use of column oven

Use of ion pair reagents entail high cost, permanent adherence to the stationary phase, and problems like low method robustness. Pharmacopoeia guidelines allow variations in the columns and methods settings within certain ranges. Higher than the specified particle size of column packing adversely effects the method resolution and is not recommended. Methods should be robust enough to operate within the slight variation of the ambient temperature due to poor temperature control facilities in resource limited settings. Shorter run time is preferred to allow less use of solvents and energy resources and to increase the capacity to analyse high number of samples. Similarly, methods employing use of column oven add to the energy and logistic expenditure. The recommended range of optimum pH range for column use is based upon room temperature conditions. Continuous use of organic concentration of lower than 10% can result in the phase collapse of reverse phase columns. Isocratic elution do not require extra time for re-equilibration as needed for gradient elution methods and are also easier to use even when slight changes in chromatographic settings (e.g., column specifications and flow rate) are needed (100).

Hence, reverse phase C18 column with a particle size of 5 μm and methanol as organic component in place of acetonitrile provide an efficient model for methods

suited to the resource limited settings (23). The *in-situ* preparation of impurities from the cheap and accessible chemicals can be an alternate to buying expensive impurity standards.

### 1.3.3.2. Multiple methods needed for impurity profiling of an API

Impurities of an API are mostly very similar in the physico-chemical characteristics of the API. However, some impurities can possess an altogether different behavior, differing significantly in the solubility, size or stability characteristics and thus requiring use of two or more methods to ensure complete impurity profiling of the API e.g., ampicillin, piperacillin and ceftazidime (Table 1.7)

### 1.3.4. Current compendial methods for assay and impurity profiling of beta-lactam antibiotics

Assay and impurity profiling constitute the two crucial analytical tests carried out on API and FPPs. According to WHO member countries must ensure the provision of quality assured essential medicine to its people. Beta-lactam antibiotics included in the ACCESS/ WATCH group of 20th model WHO essential medicines list, were selected for collecting available data on compendial methods. The requirements for assay and impurity testing in accordance with the latest editions of European Pharmacopoeia (Ph. Eur. 9.0) and United States Pharmacopoeia (USP 40) was tabulated and the chromatographic settings posing challenges for the resource limited settings (see section 1.3.2.2 for criteria) were written in bold (Table 1.7).

Pharmacopoeia offer limited flexibility for variation in chromatographic settings from the specifications in the monograph. However, certain parameters like particle size diameter are very restrictive. Only the column of particle size equal or lower than the recommended dimensions can be used due to its crucial effect on the resolution. Changes in the chromatographic settings for gradient method is much more difficult than isocratic because of the chances of possible shifts in peaks and wrong peak assignment (100, 101).

### 1.3.5. Revised guidelines for the impurities in antibiotics, method sensitivity and reporting level for impurities:

According to the recent guidelines the reporting limit applicable to antibiotic for human use with daily dose ≥ 2 g is 0.03% (Table 1.1 & 1.2). However, only the monographs for amoxicillin, meropenem and cilastatin include disregard limit as low as 0.03% (see

bold figures in Table 1.4 and 1.5). Hence, there is a need for revision of compendial methods to ensure that the threshold for reporting of 0.03% is achieved.

Table 1.7    Current HPLC methods for assay and test for related substances of essential beta-lactam antibiotics from European Pharmacopoeia (Ph. Eur. 9.2) and United Stated Pharmacopoeia (USP 40)

|  | **Beta-lactam antibiotic** <br> *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | **Method details** <br> *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
|---|---|---|
| M1. | Benzylpenicillin potassium <br> Benzylpenicillin sodium <br> *Ph. Eur. 9.2 (04/2017)- related substances* | *End capped C18 column (150 X 4.6 mm, 3 µm)* <br> **Gradient elution** <br> Mobile phase A-phosphate buffer pH 3.4: methanol: water (10:30:60 v/v/v), <br> Mobile phase B-phosphate buffer pH 3.4: methanol: water (10:55:35 v/v/v), flow rate: **1.5 mL/min**, 225 nm, **50 °C**- 30 min. <br> Gradient (A/B, v/v):  0-7 min (70/30), 7-17 min (70/0), 17-22 min (0/100) <br> *Requirements for SS solution: CRS containing Imp A-H, SS include Imp F, C and D* |
| M2. | Benzylpenicillin potassium <br> Benzylpenicillin sodium <br> *Ph. Eur. 9.2 (04/2017)- assay* | -same as M1- <br> Isocratic elution <br> Initial isocratic conditions with flow rate to **1.2 mL/min**, 10 min |
| M3. | Benzylpenicillin potassium <br> Benzylpenicillin sodium <br> USP 40-*assay* | *C18 column (100 X 4.6 mm, 5 µm)* <br> Isocratic elution <br> Mobile phase-0.01 M phosphate buffer: methanol (60:40), v/v), flow rate: 1.0 mL/min, 220 nm, ambient. <br> *Requirements for SS solution: 2-pheylacetamide* |
| M4. | Benzathine penicillin <br> *Ph. Eur. 9.2 (01/2008)- related substances* | *End capped C18 column (250 X 4.0 mm, 5 µm)* <br> Isocratic elution <br> Mobile phase A-phosphate buffer (34 g/L), pH 3.5: methanol: water (10:30:60 v/v/v), |

| | Beta-lactam antibiotic | Method details *(Cont'd)* |
|---|---|---|
| | *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
| | | Mobile phase B-phosphate buffer (34 g/L), pH 3.5: methanol: <br><br> water (10:60:30 v/v/v), <br><br> Gradient (A/B, v/v): 0-7 min (75/25), 10-20 min (to 0/100), 20-55 min (0/100), 55-70 min (to 75/25) re-equilibration**,** flow rate: 1.0 mL/min, 220 nm, **40 °C**-32 min. <br><br> *Requirements for SS solution: Imp C in test/reference solution of PenGb* |
| M5. | Benzathine penicillin <br><br> *Ph. Eur. 9.2 (01/2008)-assay* | *End capped C18 column (250 X 4.0 mm, 5 µm)* <br><br> Isocratic elution <br><br> Mobile phase-phosphate buffer (34 g/L), pH 3.5: methanol: water (10:35:55 v/v/v), flow rate: 1.0 mL/min, 220 nm, **40 °C**- 32 min. <br><br> *Requirements for SS solution: Imp C in test/reference solution of PenGb* |
| M6. | Benzathine penicillin <br><br> *USP 40-assay* | *C18 column (300 X 4.0 mm, 5 µm)* <br><br> isocratic elution <br><br> Mobile phase A: 0.05 M phosphate buffer, pH 6.0: **acetonitrile** (4:1 v/v), flow rate: **2.0 mL/min**, 225 nm. <br><br> *Requirements for SS solution: PenV-potassium* |
| M7. | Procaine benzylpenicillin <br><br> *Ph. Eur. 9.2 (01/2008)-related substances* <br><br><br> *Ph. Eur. 8.0 (01/2008)-assay* | *C18 column (250 X 4.6 mm, 5 µm)* <br><br> Isocratic elution <br><br> Mobile phase-acetonitrile: water: phosphate buffer: **tetrabutylammonium hydroxide** pH 7.0 (25:25:50, v/v), flow rate: **1.75 mL/min**, 225 nm. <br><br> *Requirements for SS solution: 4-aminobenzoic acid* <br><br> -as above- separate sample solution for assay |
| | Procaine benzylpenicillin <br> USP 40-*assay\** | *Iodometric Assay* |

| | Beta-lactam antibiotic | Method details *(Cont'd)* |
|---|---|---|
| | *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
| M8. | *Content of PenG and procaine* | *C18 column (300 X 3.9 mm, 10 µm)*<br><br>Isocratic elution<br><br>Mobile phase-acetonitrile: water: phosphate buffer: **tetrabutylammonium hydroxide,** pH 7.0 (25:25:50, v/v/v), flow rate: 1.0 mL/min, 235 nm.<br><br>*Requirements for SS solution: PenV-potassium* |
| M9. | Phenoxymethylpenicillin, Phenoxymethylpenicillin potassium<br><br>*Ph. Eur. 9.2 (04/2017)- related substances*<br><br><br><br><br><br><br><br><br><br><br><br>*Ph. Eur. 9.2 (04/2017)- assay* | *End capped C18 column (150 X 4.6 mm, 3 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A-phosphate buffer pH 3.4: methanol: water (10:30:60 v/v/v),<br><br>Mobile phase B-Phosphate buffer pH 3.4: methanol: water (5:60:35 v/v/v), flow rate: **1.5 mL/ min**, 254 nm, **50 °C**- 32 min<br><br>Gradient (A/B, v/v): 0-2 min (85/15), 2-5 min (to 70/30), 5-17 min (70/30), 17-22 min (to 0/100), 22-32-reequilbriation<br><br>*Requirements for SS solution: epimers of Imp F in PenV system suitability CRS containing (Imp B, D, E and F)*<br><br>-as above- with separate sample solutions |
| M10. | Phenoxymethylpenicillin potassium<br><br>USP 40–assay of PenV and limit of PenV-Imp D | *C18 column (300 X 4.0 mm)*<br><br>isocratic elution<br><br>Mobile phase-**acetonitrile**: glacial acetic acid: water (350: 5.75: 650 v/v/v), flow rate: 1.0 mL/min, 254 nm.<br><br>*Requirements for SS solution: PenG-potassium* |
| M11. | Phenoxymethylpenicillin potassium<br><br>USP 40–limit of phenoxyacetic acid | *C18 column (250 X 4.0 mm, 5 µm)*<br><br>isocratic elution<br><br>Mobile phase-**acetonitrile**: glacial acetic acid: water (35: 1: 65 v/v/v), flow rate: 1.0 mL/min, 254 nm.<br><br>*Requirements for SS solution: PenG-potassium* |

| | Beta-lactam antibiotic<br><br>*Pharmacopoeia Ed. (revision date)- Assay/or related substances* | Method details *(Cont'd)*<br><br>*Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
|---|---|---|
| M12. | Amoxicillin<br><br>*Ph. Eur. 8.0 (01/2013)- related substances*<br><br><br><br><br><br><br><br>*Ph. Eur. 8.0 (01/2013)- assay* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A-phosphate buffer pH 5.0: **acetonitrile** (99:1, v/v),<br><br>Mobile phase B-phosphate buffer pH 5.0: **acetonitrile** (80:20, v/v)<br><br>Gradient (A/B, v/v): 0-t$_R$ min (92/8), tR- (tR+ 25) (to 0/100), tR- (tR+ 25) + (tR+ 40) (0/100), (tR+ 40)-(tR+ 55) (to 92/8), re equilibration, flow rate: 1.0 mL/min, 254 nm, **55 min.**<br><br>*Requirements for SS solution: cefadroxil*<br><br>-initial isocratic conditions as above- with separate sample solution |
| M13. | Amoxicillin<br><br>*USP 40-related substances* | *C18 column (100 X 4.6 mm, 5 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A-phosphate buffer pH 5.0<br><br>Mobile phase B-methanol<br><br>Gradient (A/B, v/v): 0-10 min (97/3), 10-22 min (to 75/25), 22-26 min (to 97/3), re-equilibration, flow rate**: 1.5 mL/min**, 210 nm, **40 °C, 30 min.**<br><br>**Temperature of auto-sampler: 4 °C**<br><br>*Requirements for SS solution: AMX-Imp A and AMX-Imp D* |
| M14. | Amoxicillin<br><br>*USP 40-assay* | *C18 column (250 X 4.0 mm, 5 µm)*<br><br>Isocratic elution<br><br>Mobile phase-phosphate buffer pH 5.0: acetonitrile (24:1, v/v), flow rate: **1.5 mL/min**, 230 nm. |
| M15. | Ampicillin sodium<br><br>*Ph. Eur. 9.0 (01/2017)- related substances*<br><br>Ampicillin trihydrate<br><br>*Ph. Eur. 9.0 (01/2008)- related substances* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A: dilute acetic acid (0.5 ml) + 0.2 M potassium dihydrogen phosphate (50 mL) + acetonitrile (50 mL + water up to 1000 mL |

| | Beta-lactam antibiotic *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | Method details *(Cont'd)* *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
|---|---|---|
| | *Ph. Eur. 9.0 (01/2017)- assay* Ampicillin trihydrate<br><br>*Ph. Eur. 9.0 (01/2008)- assay* | Mobile phase B: dilute acetic acid (0.5 mL) + 0.2 M potassium dihydrogen phosphate (50 mL) + **acetonitrile** (400 mL) + water up to 1000 mL<br><br>Gradient (A/B): 0-$t_R$ min (85/15, $t_R$- ($t_R$+ 30) (to 0/100), $t_R$- ($t_R$+ 30) + ($t_R$+ 45) (0/100), ($t_R$+ 45)- ($t_R$+ 60) (85/15), flow rate: 1.0 mL/min, 254 nm, 60 min<br><br>-as above using initial isocratic conditions |
| M16. | Ampicillin sodium/Ampicillin trihydrate<br><br>*USP 40-assay and related substances* | *C18 column (150 X 4.6 mm, 5 μm)*<br><br>**Gradient elution**<br><br>Phosphate buffer: 6.54 g/L of potassium dihydrogen phosphate + 0.34 g/L of dipotassium hydrogen phosphate, pH 5.5<br><br>Mobile phase A-**acetonitrile**: phosphate buffer (2:23, v/v)<br><br>Mobile phase B-**acetonitrile**: solution A (3:7, v/v)<br><br>Gradient (A/B, v/v): 0-6 min (100/0), 6-15 min (to 0/100), 15-16 min (0/100), 16-18 min (100/0), 18-20 min (100/0), flow rate: **1.5 mL/min**, 230 nm. |
| M17. | Ampicillin sodium/Ampicillin trihydrate<br><br>*USP 40-related substances (procedure 3-when the sample is expected to contain phenyl pyrazinol, pivaloyl phenyl glycine, pivaloyl amino penicillanic acid, diphenyldiketopiperazine and open ring dimer)* | *C8 column (150 X 4.6 mm, 5 μm)*<br><br>**Gradient elution**<br><br>Mobile phase A-6.54 g/L of potassium dihydrogen phosphate + 0.34 g/L of dipotassium hydrogen phosphate, pH 5.5<br><br>Mobile phase B-**acetonitrile**: solution A (2:23, v/v)<br><br>Gradient (A/B, v/v): 0-20 min (98/2), 20-40 min (to 90/10), 40-50 min (to 80/20), 50-55 min (75/25), 55-60 min (75/25), 60-62 min (to 98/2), flow rate: **1.5 mL/ min,** 220 nm, **40°C**<br><br>**Autosampler temperature: 4°C** |

| | Beta-lactam antibiotic | Method details *(Cont'd)* |
|---|---|---|
| | *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
| M18. | Ampicillin sodium/Ampicillin trihydrate<br><br>*USP 40- related substances (procedure 4- when the sample is expected to contain ampilloyl aminopenicillanic acid and penicillanyl ampicillinamide)* | *C18 column (150 X 4.0 mm, 3 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A-3.4 g/L of disodium hydrogen phosphate dodecahydrate + 1.4 g/L of potassium dihydrogen phosphate, pH 5.5<br><br>Mobile phase B-**acetonitrile**<br><br>Gradient (A/B, v/v): 0 min (99/1), 0-1.5 min (to 95/5), 1.5-6.5 min (to 90/10), 6.5-7.5 min (to 89/11), 7.5-13.5 min (to 84/16), 13.5-16.5 min (to 75/25), 16.5-18 min (to 60/40), 18-25 min (99/1), re-equilibration, 35 min, flow rate: **1.3 mL/ min**, 220 nm, **40 °C.**<br><br>**Autosampler temperature: 4 °C** |
| M19. | Cloxacillin sodium<br><br>*Ph. Eur. 8.0 (01/2013)- related substances*<br><br><br>*Ph. Eur. 8.0 (01/2013)- assay* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>Isocratic elution<br><br>Mobile phase A-phosphate buffer pH 5.0: acetonitrile (75:25, v/v), Flow rate: 1.0 mL/ min, 225 nm, ambient.<br><br>-as above- using separate sample solution |
| M20. | Cloxacillin sodium<br><br>*USP 40-related substances* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A: 1.18 g/L **sodium hexanesulfonate** monohydrate and 0.8mL/L of ammonium hydroxide. pH 2.9-3.1<br><br>Mobile phase B:  **acetonitrile**<br><br>Gradient (A/B, v/v): 0 min (80/20), 0 to 30 min (to 35/65), re-equilibration, flow rate**: 1.5 mL/min**, 225 nm, **40 °C**. Temperature of auto-sampler **4 °C.** |
| M21. | Cloxacillin sodium<br><br>*USP 40-assay* | -as in impurity method (M020)- with following modification in gradient<br><br>Gradient (A/B, v/v): 0-2 min (45/55), 2-2.5 min (to 35/65), 2.5-5 min (35/65), re-equilibration. Temperature of auto-sampler **4 °C.** |

| | Beta-lactam antibiotic | Method details *(Cont'd)* |
|---|---|---|
| | *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
| M22. | Piperacillin<br><br>*Ph. Eur. 9.2 (01/2017)- related substances* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A-water: 31.2 g/L of sodium dihydrogen phosphate: 80 g/L **tetrabutylammonium hydroxide**, pH 5.5: **acetonitrile** (576:200:24:200) |
| | *Ph. Eur. 9.2 (01/2017)- Assay* | Mobile phase B-water: 31.2 g/L of sodium dihydrogen phosphate: 80 g/L **tetrabutylammonium hydroxide**, pH 5.5: **acetonitrile** (126:200:24:650)<br><br>Gradient (A/B, v/v): $0-t_R$ min (88/12), tR- (tR+ 30) (to 0/100), (tR+ 30)- (tR+ 45) (to 88/12) re equilibration, flow rate**: 1.5 mL/min**, 225 nm, **40 °C**.<br><br>*Requirements for SS solution: ampicillin*<br><br>-as above- using initial composition |
| M23. | Piperacillin/PPR sodium<br><br>USP 40-Assay<br><br><br><br>*Related substances Imp B, F, A and E* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>Isocratic elution<br><br>Mobile phase-methanol: water: 0.2 M phosphate dihydrogen phosphate: 0.4 M **tetrabutylammonium hydroxide** (450:447:100:3 v/v/v/v), flow rate: 1.0 mL/min, 220 nm.<br><br>*Requirements for SS solution: ampicillin*<br><br>-as above- using separate standard solutions |
| M24. | Piperacillin<br><br>USP 40-*related substances Imp D and total impurities* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>Isocratic elution<br><br>Mobile phase-methanol: water: 0.2M phosphate dihydrogen phosphate: 0.4 M **tetrabutylammonium hydroxide** (615:282:100:3 v/v/v/v), flow rate: 1.0 mL/min, 220 nm.<br><br>*Requirements for SS solution: ampicillin* |

| | Beta-lactam antibiotic | Method details *(Cont'd)* |
|---|---|---|
| | *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
| M25. | Cephalexin monohydrate<br><br>*Ph. Eur. 8.0 (04/2008)-related substances* | ***Spherical*** *C18 column (100 X 4.6 mm, 5 μm)*<br><br>**Gradient elution**<br><br>Mobile phase A-Phosphate buffer pH 5.0,<br><br>Mobile phase B-methanol,<br><br>Gradient (A/B, v/v): 0-1 min (98/2), 1-20 (to 70/30), re-equilibration, flow rate: **1.5 mL/min**, 220 nm. |
| M26. | Cephalexin monohydrate<br><br>*Ph. Eur. 8.0 (04/2008)-assay* | *C18 column (250 X 4.6 mm, 5 μm)*<br><br>Isocratic elution<br><br>Mobile phase-methanol: acetonitrile: phosphate buffer: water (2:5:10:83 v/v/v/v), flow rate: **1.5 mL/min**, 254 nm. |
| | **Beta-lactam antibiotic**<br><br>*Pharmacopoeia Ed. (revision date)- Assay/or related substances* | **Method details *(Cont'd)***<br><br>*Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
| M27. | Cephalexin monohydrate<br><br>and cephalexin HCl<br><br>*USP 40-related substances* | *C18 column (250 X 4.6 mm, 5 μm)*<br><br>**Gradient elution**<br><br>Mobile phase A- 1 g/L **sodium 1-pentasulfonate** with 15 mL of trimethylamine, pH 2.5<br><br>Mobile phase B-1 g/L **sodium 1-pentasulfonate** with 15 mL of trimethylamine and 300 mL of water, pH 2.5 and added 350 ml each of **acetonitrile** and methanol<br><br>Gradient (A/B, v/v): 0-1 (100/0), 1-33.3 (to 0/100), 33.3-34.3 (0/100), re-equilibration, 40 min, flow rate: 1.0 mL/min, 254 nm. |
| M28. | Cephalexin monohydrate<br><br>and cephalexin HCl<br><br>*USP 40- assay* | *C18 column (250 X 4.6 mm, 5 μm)*<br><br>Isocratic elution<br><br>Mobile phase A-0.985 g/L **sodium 1-pentasulfonate** in a mixture of acetonitrile: methanol; trimethylamine: water (20:10:3:170), adjusted to pH 3.0, flow rate: 1.0 mL/ min, 254 nm. |

| | Beta-lactam antibiotic | Method details *(Cont'd)* |
|---|---|---|
| | *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
| M29. | Cefazolin *Ph. Eur. 8.0 (04/2014)- related substances* | *C18 column (125 X 4.0 mm, 3 µm)* **Gradient elution** Mobile phase A-14.54 g/L disodium hydrogen phosphate dodecahydrate and 3.53 g/L of potassium dihydrogen phosphate Mobile phase B-**acetonitrile** Gradient (A/B, v/v): 0-2 min (98/2), 2-4 min (to 85/15), 4-10 min (to 60/40), 10-11.5 min (35/65), 12-15 min (to 98/2), 15-21 min (98/2), 21 min, flow rate: **1.2 mL/min**, 254 nm, **45 °C** |
| M30. | Cefazolin *Ph. Eur. 8.0 (04/2014)- assay* | *C18 column (250 X 4.6 mm, 5 µm)* Isocratic elution Mobile phase A: 10 volumes of **acetonitrile** + 90 volumes of solution (2.77 g/L disodium hydrogen phosphate dodecahydrate +1.86 g/L citric acid) Flow rate: 1.0 mL/ min, 270 nm, ambient |
| M31. | Cefazolin *USP 40-related substances* | *C18 column (250 X 4.6 mm, 5 µm)* Solution 1-6.8 g/L potassium dihydrogen phosphate in water Mobile phase A-6.8 g/L potassium dihydrogen phosphate in water, pH 6.8 using 10% sodium hydroxide. **Gradient elution** Mobile phase B-**acetonitrile** + solution 1 (1:1, v/v) Gradient (A/B):  0-7 min (98/2), 7-15 min (to 85/15), 15-30 min (to 80/20), 30-35 min (80/20), 35-40 min (to 50/50), 45-50 min (50/50), 50-55 min (98/2), 55-65 min (98/2), 65 min, flow rate: **1.5 mL/min**, 210 and 254 nm, **30 °C.** |
| M32. | Cefazolin *USP 40-assay* | *C18 column (300 X 3.9 mm, 10 µm)* Isocratic elution Buffer A-0.9 g/L anhydrous dibasic sodium phosphate + 1.3 g/L citric acid monohydrate in water |

| | Beta-lactam antibiotic<br><br>*Pharmacopoeia Ed. (revision date)- Assay/or related substances* | Method details *(Cont'd)*<br><br>*Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
|---|---|---|
| | | Buffer B-5.7 g/L anhydrous dibasic sodium phosphate + 3.6 g/L potassium dihydrogen phosphate in water<br><br>Mobile phase-**acetonitrile**: buffer A (10:90, v/v)<br><br>Flow rate: **2.0 mL/min**, 254 nm. |
| M33. | Cefotaxime sodium<br><br>*Ph. Eur. 8.0 (01/2008)- assay and related substances* | *C18 column (150 X 3.9 mm, 5 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A-7.1 g/L disodium hydrogen phosphate dodecahydrate, pH 6.25<br><br>Mobile phase B-methanol<br><br>Gradient (A/B, v/v)): 0-7 min (86/14), 7-9 min (to 82/18), 9-16 min (82/18), 16-45 min (to 60/40), 45-50 min (to 60/40), 50-55 min (to 86/14), 55-60 min (86/14), 55 min, flow rate: 1.0 mL/min, 235 nm, **30 °C.** |
| M34. | *Cefotaxime*<br><br>*USP 40- assay and related substances*<br><br>Also for Cefotaxime injection<br><br>*BP 2012-related substances* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>Isocratic elution<br><br>Solution A-3.5 g/L of potassium dihydrogen phosphate + 11.6 g/L disodium hydrogen phosphate dodecahydrate, pH 7.0<br><br>Solution B-methanol 375 ml<br><br>Mobile Phase-solution A (1L) + solution B (375ml), flow rate-1.0 mL/ min, 235 nm, 48 min<br><br>(USP method uses a premixed solution for the equilibration and initial isocratic phase as mobile phase A). |
| M35. | Ceftriaxone sodium<br><br>*Ph. Eur. 8.0 (01/2008)- assay and related substances*<br><br>*USP 40-assay and related substances* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>Isocratic elution<br><br>Buffer 7.0-0.067 M potassium dihydrogen phosphate, pH 7.0 prepared from solution 1 and 2 (38.9 mL + 61.1 mL respectively)<br><br>Solution 1-0.908g potassium dihydrogen phosphate in 100 mL |

| | Beta-lactam antibiotic *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | Method details *(Cont'd)* *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
|---|---|---|
| | Note: Ceftriaxone Injection assay method is slight alteration of this method | Solution 2-2.38 g disodium hydrogen phosphate in 100 mL<br><br>Buffer 5.0-2% w/v citric acid, pH 5.0<br><br>Solution A-water: buffer 7.0: buffer 5.0: **acetonitrile** (440: 55:5:500, v/v/v)/v)<br><br>Mobile phase-2g each of **tetradecylammnoium bromide** and **tetraheptylammonium** bromide are dissolved in solution A (1L)<br><br>Flow rate: 1.5 mL/ min, 254 nm, ambient, 20-30 min |
| | Ceftriaxone injection *USP 40-assay* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>Isocratic elution<br><br>Buffer 7.0- 0.067 M potassium dihydrogen phosphate, pH 7.0 prepared from solution 1 and 2 (38.9 mL + 61.1 mL respectively)<br><br>Solution 1- 0.908 g potassium dihydrogen phosphate in 100 mL<br><br>Solution 2-2.38 g disodium hydrogen phosphate in 100 mL<br><br>Buffer 5.0-2% w/v citric acid, pH 5.0<br><br>Solution A- water: buffer 7.0: buffer 5.0: **acetonitrile** (440: 55:5:500, v/v/v)/v)<br><br>Mobile phase-3.2 g **tetraheptylammonium bromide** are dissolved in solution A (1L), flow rate: **1.2 mL/ min**, 270 nm, 20-30 min. |
| M36. | Ceftazidime pentahydate *Ph. Eur. 8.0 (01/2013)- related substances* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A-3.6 g/L disodium hydrogen phosphate dodecahydrate + 1.4 g/L of potassium dihydrogen phosphate, pH 3.4<br><br>Mobile phase B-**acetonitrile** |

| | Beta-lactam antibiotic | Method details *(Cont'd)* |
|---|---|---|
| | *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
| | | Gradient (A/B, v/v)): 0 min (96/4), 0-4 min (to 89/11), 4-5 min (89/11), 5-8 min (to 84/16), 8-11 min (to 80/20), 11-15 min (to 50/50), 15-18 min (to 20/80), 18-22 min (20/80), re-equilibration, 30 min, flow rate: **1.3 mL/ min**, 254 nm, **40 °C** |
| M37. | Ceftazidime pentahydrate<br><br>*Ph. Eur. 8.0 (01/2013)- Impurity F* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>Isocratic elution<br><br>Mobile phase-ammonium dihydrogen phosphate buffer (28.8 g/L), pH 7.0: acetonitrile: water (8:24:68, v/v/v), 10 min, flow rate: 1.0 mL/ min, 255 nm |
| M38. | Ceftazidime pentahydrate<br><br>*Ph. Eur. 8.0 (01/2013)- assay* | ***C6 (hexasilyl silica gel) column** (150 X 4.6 mm, 5 µm)*<br><br>Isocratic elution<br><br>Mobile phase: disodium hydrogen phosphate (4.3 g/L) + 2.7 g of potassium dihydrogen phosphate in 980 ml of water + 20 ml of **acetonitrile**, 6 min<br><br>Flow rate: **2.0 mL/min**, 245 nm |
| M39. | Ceftazidime pentahydrate<br>*USP 40-assay* | *C18 column (150 X 4.6 mm, 5 µm)*<br><br>Isocratic elution<br><br>Buffer 7-anhydrous disodium hydrogen phosphate 4.3 g+ 2.7 g of potassium dihydrogen phosphate in 100 mL of water<br><br>Mobile phase-20 mL **acetonitrile** + 100 mL of buffer 7 diluted to 1000 mL with water, flow rate: **2.0 mL/min**, 254 nm |
| | Ceftazidime for injection<br><br>*USP 40-Limit for Impurity F* | *C18 column (250 X 4.6 mm, 5 µm)*<br><br>Isocratic elution<br><br>Mobile phase A- 0.25 M ammonium dihydrogen phosphate buffer (100 mL) + **acetonitrile** (300 mL) + water (to make 1000 mL), flow rate: **1.6 mL/ min**, 254 nm |

| | Beta-lactam antibiotic | Method details *(Cont'd)* |
|---|---|---|
| | *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
| | For ceftazidime injection with arginine<br><br>*USP40- content of arginine* | *Saturator pre-column: L27, porous silica with particle size 30-50 µm (500 X 4.6 mm)*<br><br>*Analytical column: L20, dihydroxypropane groups chemically bonded to porous silica (250 X 4.0 mm)*<br><br>Isocratic elution<br><br>Mobile phase-1.15 g/L of ammonium dihydrogen phosphate, pH 2.0: acetonitrile (25:75, v/v), flow rate: 1.0 mL/min, 206 nm |
| M40. | Cefixime<br><br>*Ph. Eur. 8.0 (01/2008)- assay and related substances* | *C18 column (125 X 4.0 mm, 5 µm)*<br><br>Isocratic elution<br><br>Solution A-8.2 g/L of **tetrabutylammonium hydroxide, pH 6.5**<br><br>Mobile phase-acetonitrile: solution A (25:75, v/v)<br><br>Flow rate: 1.0 mL/min, 254 nm, **40°C** |
| M41. | Cefixime<br><br>*USP 40-assay and related substances* | *C18 column (125 X 4.6 mm, **4 µm**)*<br><br>Isocratic elution<br><br>Solution A-25ml of 0.4 M (103.8 g/L) of **tetrabutylammonium hydroxide, pH 6.5** (0.01M= 2.6 g/L)<br><br>Mobile phase-**acetonitrile**: solution A (25:75, v/v)<br><br>Flow rate: adjustable to achieve RT of cefixime= 10min, 254 nm, **40 °C** |
| M42. | Imipenem monohydrate<br><br>*Ph. Eur. 9.0 (04/2013)- assay and related substances* | *End-capped C18 column (150 X 4.6 mm, **3 µm**)*<br><br>**Gradient elution**<br><br>Solution A-0.32 g/L anhydrous sodium dihydrogen phosphate + 1.04 g anhydrous disodium hydrogen phosphate, pH 7.3<br><br>Mobile phase A-**acetonitrile**: solution A (0.7:99.3, v/v)<br><br>Mobile phase B-**acetonitrile**: solution A (25:75, v/v) |

| | Beta-lactam antibiotic<br><br>*Pharmacopoeia Ed. (revision date)- Assay/or related substances* | Method details *(Cont'd)*<br><br>*Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
|---|---|---|
| | | Gradient (A/B, v/v)): 0-9 min (100/0), 9-24 min (to 68/32), 24-24.5 min (to 50/50), 24.5-29 min (50/50), re-equilibration, 40 min, flow rate: 1.0 ml/min, 210 nm, **30 °C** |
| M43. | Imipenem monohydrate<br><br>*USP 40-assay* | *C18 column (300 X 4.6 mm)*<br><br>Isocratic elution<br><br>**Mobile phase-2.0g/L of sodium 1-hexanesulfonate in phosphate buffer, pH 6.8**<br><br>Flow rate: **2.0 ml/min**, 254 nm, **50 °C** |
| M44. | Potassium clavulanate<br><br>*Ph. Eur. 7.0 (07/2010)-related substances* | *C18 column (100 X 4.6 mm, 3 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A-0.05 M (7.8 g/L) sodium dihydrogen phosphate, pH 4.0<br><br>Mobile phase B-methanol: mobile phase A (1:1, v/v)<br><br>Gradient (A/B, v/v)): 0-4 min (100/0), 4-15 min (to 50/50), 15-18 min (50/50), re-equilibration, 28 min<br><br>Flow rate: 1.0 ml/ min, 230 nm, **40 °C** |
| M45. | Potassium clavulanate<br><br>*Ph. Eur. 7.0 (07/2010)-assay* | *C18 column (300 X 4.6 mm, 10 µm)*<br><br>Isocratic elution<br><br>Mobile phase-15 g/L sodium dihydrogen phosphate, pH 4.0: methanol (95:5, v/v)<br><br>Flow rate: 1.0 ml/ min, 230 nm, ambient |
| M46. | Potassium clavulanate<br><br>*USP 40- related substances* | *Same as M044 with changed underlined below*<br><br>*C18 column (100 X 4.6 mm, 5 µm)*<br><br>**Gradient elution**<br><br>Mobile phase A-0.05 M sodium dihydrogen phosphate, pH 4.0<br><br>Mobile phase B-methanol: mobile phase A (1:1, v/v)<br><br>Gradient (A/B, v/v)): 0-4 min (100/0), 4-15 min (to 50/50), 15-18 min (50/50), 18-24 min (100/0), re- |

| | Beta-lactam antibiotic | Method details *(Cont'd)* |
|---|---|---|
| | *Pharmacopoeia Ed. (revision date)- Assay/or related substances* | *Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
| | | equilibration time 15 min, 39 min<br><br>Flow rate: 1.0 ml/ min, 230 nm, **40 °C** |
| M47. | Potassium clavulanate<br><br>*USP 40- related substances (for limit of clavam -2 carboxylate potassium)* | *C18 column (300 X 4.0 mm, 3-10 **μm**)*<br><br>**Isocratic elution**<br><br>Mobile phase-0.1 M sodium dihydrogen phosphate, pH 4.0<br><br>Flow rate: 0.5 ml/ min, 210 nm, ambient |
| M48. | Potassium clavulanate<br><br>*USP 40- assay* | *C18 column (300 X 4.0 mm, 3-10 **μm**)*<br><br>**Isocratic elution**<br><br>Solution A-7.8 g/L sodium dihydrogen phosphate, pH 4.4<br><br>Mobile phase: methanol: solution A (1:19, v/v)<br><br>Flow rate: **2.0 ml/ min**, 220 nm, ambient |
| M49. | Cilastatin<br><br>*Ph. Eur. 9.0 (01/2017)- related substances* | *End-capped C18 column compatible with **100% aqueous mobile** phase (250 X 4.6 mm, 5 μm)*<br><br>**Gradient elution**<br><br>Mobile phase A-phosphate buffer, pH 3.25<br><br>Mobile phase B-**acetonitrile**: mobile phase A (1:1, v/v)<br><br>Gradient (A/B, v/v)): 0-3 min (100/0), 3-28 min (to 90/10), 28-38 min (90/10), 39-63 min (to 50/50), 63-78 min (to 30/70), 78- 88 min (30/70), re-equilibration, 92 min, flow rate: **2.0 ml/ min**, 210 nm, **50 °C** |
| | Cilastatin -*Ph. Eur. 9.0 (01/2017) and USP 40 - assay* | Potentiometric titration |
| M50. | Cilastatin<br><br>*USP 40-related substances* | *End-capped C18 column compatible with **100% aqueous mobile** phase (250 X 4.6 mm, 5 μm)*<br><br>**Gradient elution**<br><br>Mobile phase A-phosphoric acid (1:1000, v/v): **acetonitrile** (70:30, v/v) |

|  | **Beta-lactam antibiotic** <br><br>*Pharmacopoeia Ed. (revision date)- Assay/or related substances* | **Method details** *(Cont'd)* <br><br>*Column type (length x internal diameter, particle size),* elution method, mobile phase, flow rate, detection wavelength, column temperature, run time. |
|---|---|---|
|  |  | Mobile phase B-phosphoric acid (1:1000, v/v) <br><br> Gradient (A/B, v/v)): 0 min (15/85), 0-30 min (to 100/0), re-equilibration, 40 min, flow rate**: 2.0 ml/ min**, 210 nm, **50 °C** |
| M51. | Tazobactam <br><br>*USP 40- assay* <br><br><br><br><br><br><br><br><br><br><br> *USP 40- related substances* | *C18 column (250 X 460 mm, 5 µm)* <br><br> **Isocratic elution** <br><br> Mobile Phase-1000 ml of 1.32 g/L ammonium phosphate, pH 2.5 + 30 ml **acetonitrile**, flow rate: **1.5 ml/ min**, 210 nm, ambient <br><br> Temperature of sample solutions and blank: 3 **°C** or inject the solutions immediately after preparation <br><br> **Autosampler at 3 °C** <br><br> Same as above with blank and separate sample solution |
| M52. | Meropenem <br><br>*Ph. Eur. 9.0 (01/2017)- related substances* <br><br>*USP 40- chromatographic purity* <br><br><br><br><br> *Ph.Eur. 9.0-Assay* | *C18 column (250 X 460 mm, 5 µm)* <br><br> *Base-deactivated end-capped octadecylsilyl silica gel for chromatography* <br><br> **Isocratic elution** <br><br> Solution A: 0.1% v/v triethylamine in water, pH 5.0 <br><br> Mobile phase: acetonitrile: solution A (7:100), flow rate: **1.6 ml/ min**, 220 nm, **40 °C** <br><br> Same as above, with separate sample |
| M53. | Meropenem <br><br>*USP 40-assay* | *C18 column (250 X 460 mm, 5 µm)* <br><br> **Isocratic elution** <br><br> Solution A: 0.1% v/v triethylamine in water, pH 5.0 <br><br> Mobile phase: <u>methanol</u>: solution A (1:5), flow rate: **1.5 ml/min**, 300 nm, ambient |

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# 2

# AIMS AND OBJECTIVES

**CHAPTER TWO**

## 2.1. Aim:

The study is aimed at the investigation of quality of API and FPPs of beta-lactam antibiotics and development of their simple and efficient quality control methods.

## 2.2. Objectives:

The study is designed for attaining the following objectives:

1) evaluation of API and FPP samples of beta-lactams from Pakistan for assay, impurity profile and investigation of unknown impurities;

2) preparation, isolation and synthesis of impurities of beta-lactam antibiotics;

3) development of simple and cost-effective RP-HPLC based assay and impurity profiling methods for essential beta-lactams.

# RESULTS

## CHAPTER THREE

# 3.1 Method development and validation for assay and impurity profiling of ceftriaxone using reverse phase liquid chromatography

**Abstract**

Challenges in pharmaceutical analysis regarding essential antibiotics call for provision of sensitive, yet simple, economical, and reproducible methods for use across the diverse global regulatory settings. A set of two RP-HPLC methods (A and B) was developed for assay and impurity profiling of ceftriaxone as an alternate to the complex and cost-intensive ion pair chromatographic method used by the European Pharmacopoeia (Ph.Eur.) and the United States Pharmacopoeia (USP). In method A, isocratic elution on Zorbax Eclipse Plus C18 column (150 X 4.6 mm, 5 µm) was carried out with 0.02 M potassium dihydrogen phosphate buffer (pH 5.0)-methanol (83:17, v/v) at 0.8 mL/min using a detection wavelength of $\lambda$ = 254 nm for the assay and impurity profiling of ceftriaxone. This method quantifies five known impurities of ceftriaxone as well as a group of hitherto unknown impurities, of which one was identified as deacetylcefotaxime. Simple *in-situ* preparation of three impurities (two known and one unknown) was performed. Relative retention times and correction factors were determined for identification and quantification of individual impurities. Because impurity D of ceftriaxone could not be eluted using method A, method B was developed using isocratic elution on a Knauer C18A column (150 X 4.6 mm, 5 µm) with 0.1% phosphoric acid (v/v)-acetonitrile (65:35, v/v) at 0.8 mL/min with a detection wavelength of $\lambda$ = 240 nm. The two methods were validated for specificity, linearity, accuracy, precision, and robustness in accordance with the ICH guidelines. Limits of detection and quantification were determined for the six known impurities of ceftriaxone and were found to be lower than the current reporting limits (0.1% of the nominal test solution concentration) used by the Ph.Eur. and USP, except for impurity A.

**Key words:** ceftriaxone, quality control, impurity profiling, high performance liquid chromatography, beta lactam antibiotics, unknown impurities.

## 1. Introduction

Ceftriaxone is a long acting, parenteral, third generation cephalosporin antibiotic (1-3). In 2011, the World Health Organization (WHO) included it in the 30 priority medicines for mothers and children for its use in children under five years of age for treatment of pneumonia and neonatal sepsis (4). Ceftriaxone is also used as the drug of choice for the treatment of meningococcal meningitis in "extended meningitis belt" of sub-Saharan Africa (5). This clinically crucial antibiotic faces fast development of antibiotic resistance and is included in the WATCH group of the 20th Essential Medicines list (EML) by WHO (6).

According to European Medicines Agency (EMA) guidelines for setting specifications for the impurities in antibiotics reporting, and identification/qualification thresholds of impurities found in semi-synthetic antibiotic for human use (max. daily dose ≥ 2g) are 0.03% and 0.05%, respectively (7). The limits for reporting and identification/ qualification of impurities in finished products are 0.1%, and 0.2%, respectively (7). European Pharmacopoeia (Ph.Eur. 9.2) and United States Pharmacopoeia (USP 40) monographs for ceftriaxone disodium state a disregard limit of 0.1% for reporting of impurities (8, 9).

RP-HPLC assay methods for ceftriaxone alone and in fixed dose dosage forms are reported in the recent literature (10-12). One of the study on quality parameters of generic ceftriaxone also employed modification of compendial method to quantify "unknown" impurities (13). The current pharmacopoeial method for assay and impurity profiling of ceftriaxone is an ion pair chromatography method using 0.1% w/v each of tetraheptylammonium bromide, and tetradecylammonium bromide as ion pair reagents, 5.5% v/v of 0.067 M phosphate buffer (pH 7.0) and 0.5% v/v of 0.01 M citric acid buffers (pH 5.0), along with 50% v/v acetonitrile as an organic solvent (8, 9, 14). It is a complex and cost-intensive method using a flow rate of 1.5 mL/min for the mobile phase and also involving the use of an expensive impurity reference standard (impurity A) for system suitability test.
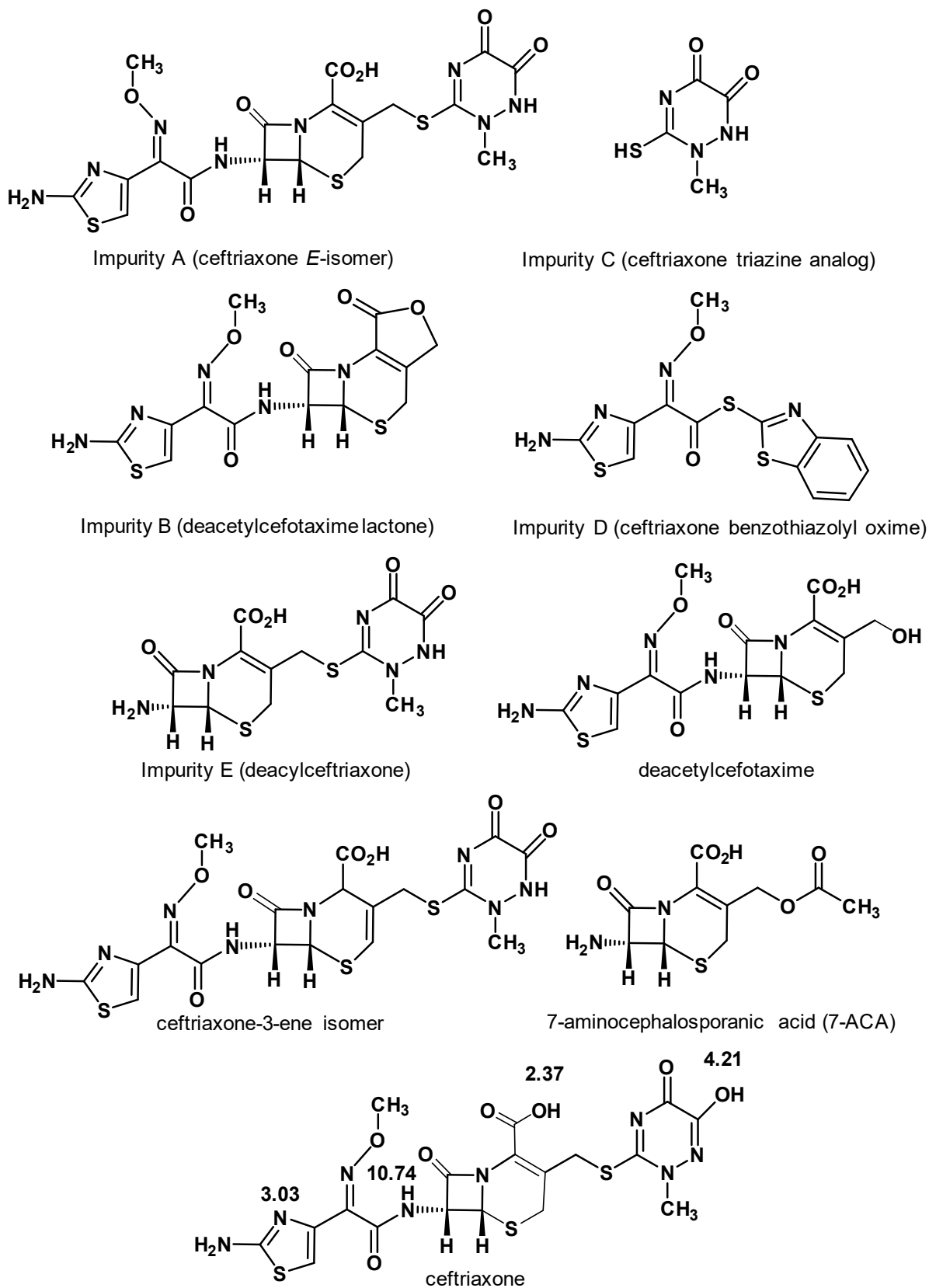
Figure 1.    Chemical structure of ceftriaxone with its acidity constants and its impurities.

Ceftriaxone is produced commercially by various semi-synthetic methods (15-19). Ph.Eur. 9.2 and USP 40 enlist 5 known impurities of ceftriaxone (impurity A-E) with the addition of 7-aminocephalosporanic acid (7-ACA) and ceftriaxone 3-ene isomer in the list of known impurities by USP (Figure 1) (8, 9). The known impurities of ceftriaxone consist of starting materials namely, 7-ACA, ceftriaxone benzothiazolyl oxime ether (impurity D) and thiotriazinone (impurity C) along with reaction intermediate deacylceftriaxone (impurity E) and the degradation products (impurity B and impurity A) (15). Impurity B of ceftriaxone (deacetylcefotaxime lactone) is also a known impurity of cefotaxime (20). Cefotaxime and ceftriaxone differ at C3 substitutions and are expected to have a number of common impurities.

This study was designed to offer routine quality control procedure for use in resource limited settings of middle and low-income countries and therefore, it was aimed to develop simple, sensitive and cost-effective, assay and impurity profiling method for ceftriaxone bulk drug, based on isocratic RP-HPLC using C18 column, and avoiding use of expensive reagents. Most of the compendial assay and impurity test methods for the essential cephalosporins listed in 20th Essential Medicines List (EML) by WHO have one or more of the limiting factors including gradient elution, ion pair chromatography, additional impurity standards needed for system suitability tests, or need of a column other than the C18 column, that makes them difficult for use as routine quality control tests in resource limited settings of low- and middle-income countries. Lack of sophisticated facilities, less number of skilled personnel, inadequate budgets, huge number of samples with the growing problem of substandard and falsified medicines are identified as the few among many other barriers to post-marketing surveillance in developing countries (21, 22). International Pharmacopoeia (Ph.Int.) mandates provision of "less technically demanding alternatives", with consideration on cost of analysis and aims to cover the major public health needs of its member states so that the surveillance and regulatory roles of public institutions remain effective world-wide (23). However, the procedures included in Ph. Int. are mostly adopted from the Ph. Eur. or USP and unable to address the resource issues faced by the developing countries (24). Success stories in strategies used for combating the problem of poor quality medicine reinforce the need of availability of more number of *accessible quality control methods* for use by the resource limited settings that can work as intermediary testing methods (22). Monographs for assay

and impurity profile of ceftriaxone active pharmaceutical ingredient (API) and finished pharmaceutical products (FPPs) are not yet included in the Ph. Int. (25).

The aim of this study is to develop a cheap, robust C18 based RP-HPLC method without use of ion-pair reagents and acetonitrile, and by using phosphate buffer and methanol as the first choice for mobile phase composition. The aim was also to meet the challenge of development of quality control method offering more stringent control of impurities in antibiotics. Simple *in-situ* preparation methods of impurities were included to facilitate peak identification and additional costs of impurity reference standards.

## 2.      Experimental

### 2.1.   Chemicals and reagents

Certified reference standard for ceftriaxone was procured from *Sigma Aldrich* (Laramie, USA). Certified reference standard of impurity A of ceftriaxone (ceftriaxone *E*-isomer) was purchased from the *European Directorate for the Quality of Medicines and Healthcare* (EDQM, Strasbourg, France). Impurity D (ceftriaxone benzothiazolyl oxime) and 7-aminocephalosporanic acid (7-ACA) were purchased from *TCI Europe* (Zwijndrecht, Belgium), impurity C (ceftriaxone triazine analog; thiotriazinone) and impurity E (deacylceftriaxone) from *Abcr GmbH* (Karlsruhe, Germany), and *Carbosynth UK* (Berkshire, United Kingdom), respectively. Impurity B (deacetylcefotaxime lactone) was prepared inhouse using cefotaxime powder for injection procured from *Fresenius Kabi Deutschland GmbH* (Hamburg, Germany) according to the method described in Ph. Eur. 9.2 under monograph for cefotaxime sodium (26).

HPLC grade acetonitrile and methanol were purchased from *VWR* (Fontenay-sous-Bois, France) and *Merck KGaA* (Darmstadt, Germany), respectively. Analytical grade potassium dihydrogen phosphate was obtained from *VWR* (Leuven, Belgium). Phosphoric acid of HPLC grade was purchased from *Sigma Aldrich* (Steinheim, Germany). Other miscellaneous chemicals including potassium hydroxide, sodium hydroxide, hydrochloric acid, acetic acid, ammonium formate etc. were of analytical grade. Water for chromatography was prepared inhouse using a Milli-Q® laboratory water system from Merck Millipore (Darmstadt, Germany).

## 2.2. High performance liquid chromatography instruments

Two Agilent 1100/1200 series HPLC systems (Agilent Waldbronn, Germany) were used for the method development and validation. The systems were equipped with on-line degasser, injector with auto-sampler, a variable wavelength detector or the diode array detector. An external column oven by Beckman Coulter GmbH (Krefeld, Germany) was used. Chemstation for LC 3D systems Rev. B.03.02 from Agilent Technologies (USA) was used for the data acquisition, analysis, and reporting.

Stationary phases including reverse phase C18 columns of various brands were used for the method development; column A: Zorbax Eclipse Plus C18 (150 X 4.6 mm, 5 µm) from Agilent technologies (USA), column B: Eurospher II C18A (150 X 4.6 mm, 5 µm), column C: Eurospher II C18H (125 X 4.6 mm, 5 µm) from *Knauer GmbH* (Berlin, Germany), and column D: XTerra RP$_{18}$ (250 X 4.6 mm, 5 µm) from *Waters* (Milford, MA, USA).

## 2.3. Preparation of buffers, mobile phase and solvents

*0.02 M phosphate buffer, pH 5.0 for mobile phase of method A:* Potassium dihydrogen phosphate (0.02 M) was prepared by dissolving 2.72 g in 1000 mL water and pH was adjusted to 5.0 with 1 M potassium hydroxide.

*0.02 M phosphate buffer, pH 7.0 as solvent for method A:* Potassium dihydrogen phosphate (0.02 M) was prepared by dissolving 2.72 g in 1000 mL water and adjusting the pH to 7.0 with 1 M potassium hydroxide.

*0.1% phosphoric acid as aqueous component of mobile phase for method B:* 1 mL of ortho-phosphoric acid (85%, v/v) was diluted to 1000 mL water.

## 2.4. Preparation of sample and standard solutions (Method A)

*Ceftriaxone:* The solutions for linearity and accuracy experiments were prepared by dissolving 8.0 mg of ceftriaxone in 10.0 mL of phosphate buffer 7.0 (stock solution of ceftriaxone; 0.8 mg/mL) and diluting appropriately. Standard solutions for calibration curve were prepared in the range of 0.24-0.72 mg/mL (five points: 0.24, 0.36, 0.48, 0.60, and 0.72 mg/mL) corresponding to 40%, 60%, 80%, 100% and 120% of the test solution A concentration (0.6 mg/mL). Low level calibration curve in the range of 0.2-

12.2 µg/mL (eight points: 0.2, 0.3, 0.6, 2.6, 5.0, 7.4, 9.8, and 12.2 µg/mL) was prepared corresponding to 0.03-2.03% of test solution A for determination of correction factors of impurities.

*Impurity A, B, C, E and 7-ACA:* Stock solutions (0.2 mg/mL) of impurity C, 7ACA and impurity E were prepared by dissolving 10.0 mg of each in 50.0 mL of phosphate buffer 7.0, while 1.0 mg of each of impurity A and impurity B were dissolved separately in 10.0 mL of solvent A to prepare their impurity stock solutions (0.1 mg/mL). All stock solutions were diluted to working solutions of 0.02 mg/mL and 0.002 mg/mL for each impurity. Standard calibration solutions were prepared separately for impurity E, impurity C and 7-ACA, (range: 0.2-12.2 mg/mL, eight points: 0.2, 0.3, 0.6, 2.6, 5.0, 7.4, 9.8, and 12.2 µg/mL), for impurity B (range: 0.3-12.2 mg/mL, seven points: 0.3, 0.6, 2.6, 5.0, 7.4, 9.8, and 12.2 µg/mL) and for impurity A (range: 2.6-12.2 mg/mL, five points: 2.6, 5.0, 7.4, 9.8, and 12.2 µg/mL). Samples for accuracy experiments were prepared by spiking solution of ceftriaxone (0.1 mg/mL) prepared from stock solution of ceftriaxone, with stock solution of respective impurities to produce different impurity concentration levels. Levels used for impurity E and C being 0.03, 0.05, 0.1, 0.5, 1.0, 1.5, and 2.0%, for 7-ACA as 0.03, 1.0, and 2.0%, for impurity B being 0.05, 0.1, 1.0, and 2.0% and for impurity A as 1.0 and 2.0% of test solution A.  An unspiked solution of ceftriaxone (0.1 mg/mL) was used as a control during accuracy experiments.

## 2.5.    Preparation of sample and standard solutions (Method B)

*Ceftriaxone:* 10.0 mg of ceftriaxone was dissolved in 10.0 mL of 50%, v/v acetonitrile (ACN (50%)) to prepare the stock solution (1 mg/mL) which was then diluted to a working solution of 0.05 mg/mL for preparation of standard and sample solutions.

*Impurity D:* 10.0 mg of impurity D of ceftriaxone was dissolved in 50.0 mL of ACN (50%). This stock solution (0.2 mg/mL) was then diluted to a working solution of (0.05 mg/mL) for preparation of standard and sample solutions.

Standard solutions of ceftriaxone and impurity D were prepared separately for constructing their calibration curves (Range: 0.3-24.3 mg/mL, five points: 0.3, 0.9, 2.7, 8.1, and 24.3 µg/mL) corresponding to 0.03%-2.43% of test solution B, using their respective working solutions.

Sample solutions for accuracy experiments were prepared by spiking a solution of ceftriaxone (0.1 mg/mL) with a known amount using working solution of impurity D. Spiking was done at seven levels being 0.03%, 0.05%, 0.1%, 0.5%, 1.0%, 1.5%, and 2.0% of test solution B. An unspiked solution of ceftriaxone (0.1 mg/mL) was used as a control during accuracy experiments.

## 2.6.  Preparation of impurities of ceftriaxone

*Preparation of deacetylcefotaxime as a system suitability solution:* The deacetylcefotaxime solution was prepared by using the exposure of ceftriaxone solution (1 mg/mL) in 0.02 M phosphate buffer, pH 5.0 to $\lambda$ = 254 nm for 48 h.

*Deacetylcefotaxime lactone (Impurity B):* Impurity B of ceftriaxone was prepared using the acidic hydrolysis of cefotaxime at 40°C for 2 h in accordance with the method described in monograph for cefotaxime in Ph.Eur. 9.2 (20). The final solution was lyophilized and re-dissolved in 25 ml of water for purification by extraction using 25 mL of ethyl acetate in three steps. The ethyl acetate extract was then dried under vacuum and identity of the impurity was confirmed by LC/MS (m/z = 395.90).

*Identification solution for impurity A-using daylight exposure:* The test solution of ceftriaxone (0.6 mg/mL) in 0.02 M phosphate buffer, pH 7.0 was exposed to daylight for 24 h.

## 2.7.  Solutions for robustness studies

For method A, 1) the system suitability solution and 2) ceftriaxone (0.6 mg/mL) spiked with impurity E, impurity C, 7-ACA, impurity B and impurity A in concentration of 2% of test solution A were used for robustness studies, whereas for method B included 1) solution of ceftriaxone and impurity D both in the concentration of 2% of the test solution B prepared in ACN (50%), and 2) a stock solution of impurity D which was prepared in methanol (0.2 mg/mL) and diluted with ceftriaxone working solution and ACN (50%) to produce concentration of 1% of test solution B for both ceftriaxone and impurity D.

*2.7.1. Standard solutions for calibration curve in the range of limits of detection (LOD):*

For method A, six standard solutions for a low-level calibration curve in the range of the limit of quantification were prepared. For impurities A, C, E and 7-ACA the concentration range of 0.05-0.3 µg/mL (0.05, 0.1, 0.15, 0.2, 0.25, 0.3 µg/mL) was used. For impurity B dilutions in the range of 0.4-1.4 µg/mL (0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 µg/mL), and for impurity A in the range of 1.3-12.2 µg/mL (1.3, 2.6, 5.0, 7.4, 9.8, and 12.2 µg/mL) were prepared. For method B, a standard curve for LOQ and LOD determination was prepared in the range of 0.25-2.75 µg/mL (0.25, 0.75, 1.25, 1.75, 2.25, and 2.75 µg/mL).

All solutions containing ceftriaxone were stored protected from light and brown colored glassware was used for preparing and storing ceftriaxone solutions. All the analytes were first dissolved using ultra sonication for 10 min with a portion of solvent, followed by dilution to final volume. All the standard and sample solutions were prepared separately for each impurity and the solutions were prepared fresh or used after thawing from storage at -20°C.

## 3.    Results and discussion:

### 3.1.    *Target LOQ and limits for reporting of impurities of ceftriaxone*

The LOQ of proposed method was targeted to achieve low reporting threshold levels for impurities (0.03% of the test solution) in accordance with the EMA guidelines for setting specification for impurities in antibiotics (7), based upon the maximum daily dose of ceftriaxone (max. daily dose > 2g). The threshold levels for impurities, in accordance with various reference documents are shown in Appendix I, Table A1 (8, 9). Currently only a few monographs for APIs of essential beta lactam antibiotics (6) in Ph.Eur. and USP include the disregard limit as low as 0.03%, including the monograph for amoxicillin (27), meropenem (28) and cilastatin (29).

The target of method sensitivity of 0.03% was met partially, as the LOQ of impurity C, impurity E, 7-ACA and impurity D were found ≤ 0.03% whereas the LOQ of impurity B was equal to 0.05%. Method A was found less sensitive for impurity A as LOQ was found to be 0.43% of the test solution A (Table 4).

*3.2. Identification of an unknown peak group and confirmation of impurity of ceftriaxone.*

The method has an advantage of separating a peak group of three unknown impurities (Figure 2a). The solutions of ceftriaxone reference standard as well as the finished products prepared in phosphate buffer, pH 5.0, show presence of peak group with retention time (RT) of 4.1-5.3 min (rel.RT=0.6-0.8), which is resolved from the principal peak with a resolution of ≥ 2.0 (Figure 2a).

Increase in area of these peaks is observed on overnight storage. Moreover, on exposure of this solution at $\lambda$ = 254 nm for 48 h reduced ceftriaxone to 50% of its initial peak area and increase the second peak of the unknown peak group. This second peak was identified as deacetylcefotaxime (Figure 1) being a known impurity of cefotaxime. It appears at 4.9 min in method A (rel.RT= 0.7) and was identified by matching the retention time of deacetylcefotaxime produced using cefotaxime by method stated in USP (30, 31) and a reported method (32). Production of deacetylcefotaxime by the using UV exposure of ceftriaxone solution in phosphate buffer, pH 5.0 and by treating its solution in water at 70 ℃ for 1 h are the new observations reported for the first time in this study. The earlier report of production of this impurity from ceftriaxone used acid-based degradation (33). The current study documents the presence of deacetylcefotaxime in ceftriaxone CRS for the first time (Figure 3).
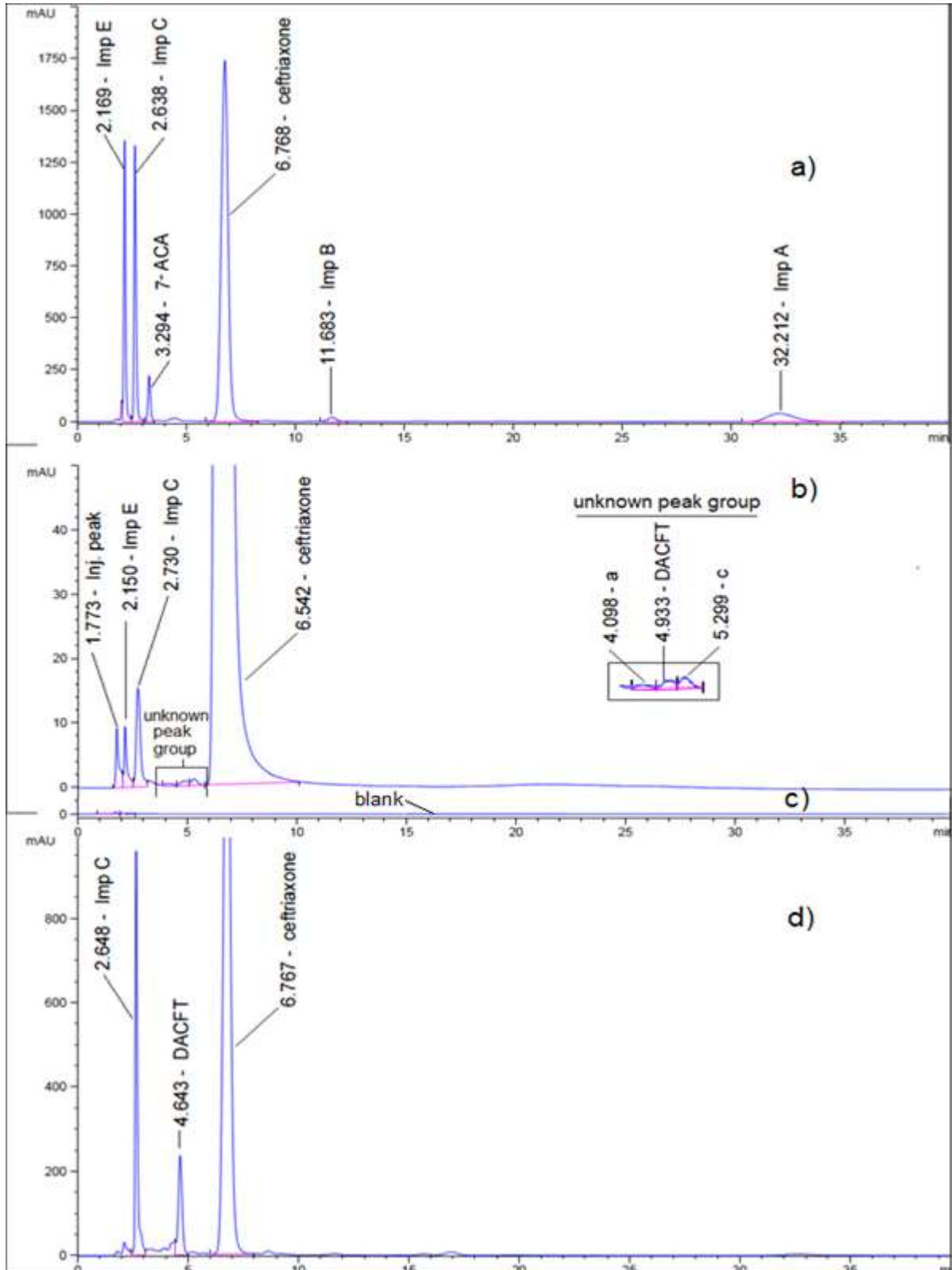
Figure 2.    Method A sample chromatograms; a) ceftriaxone (0.6 mg/mL) with Imp E, Imp C, Imp A (0.1 mg/mL), 7-ACA, and Imp B (0.05 mg/mL), b) ceftriaxone standard 0.6 mg/mL with unknown peak group, c) blank and d) deacetylcefotaxime (DACFT) solution spiked with ceftriaxone standard (0.6mg/mL).

3.2.1.    Simple *in situ* preparation methods for impurities of ceftriaxone

Preparation of impurity B, impurity A and deacetylcefotaxime were carried out in this study using ceftriaxone as starting material to aid system suitability testing and identification of peaks.  Deacetylcefotaxime (Figure 1) is for the first time, identified in this study to be present in ceftriaxone CRS (Figure 2a). This impurity is produced by exposing the solution of ceftriaxone in 0.02 M phosphate buffer (pH 5.0) to UV ʎ = 254 nm for 24-48 h (Figure 2a), or on overnight storage. Alternatively, heating cefotaxime (32) or ceftriaxone solution in water at 70 ℃ for 1 h, or treatment of solutions of cefotaxime with sodium carbonate  (30, 31) or with acetic acid (33) (Table 1).

Impurity B is prepared by acid hydrolysis of cefotaxime at 40 ℃ for 2 h, as described under tests for related substances in Ph.Eur. 9.2, Monograph for of cefotaxime (20). The method was tested in this study by using ceftriaxone as a starting material, and it produced the same results.

Impurity A is the *E*-isomer of ceftriaxone and elutes as the last peak in the developed method. It was produced by daylight exposure of standard solution of ceftriaxone for 24 h and was confirmed by spiking with impurity A standard (Figure 3). Impurity A eluted at rel.RT of 4.8-4.9, 4.4, 5.3 on column A, B, C and D, respectively, using method A. These rel.RT values are with respect to the retention time of ceftriaxone on column A, B, C, and D, respectively. In general, *E*-isomers of cephalosporins are produced under acidic conditions or upon heating (34), and are less active and more unstable than the Z-isomer (1). USP have recently included light protection for the standard and test solutions for assay and impurity of ceftriaxone in its monographs (8, 14). Storage recommendation by the compendial monographs for the bulk drug and finished products of ceftriaxone also include light protection (9, 14)
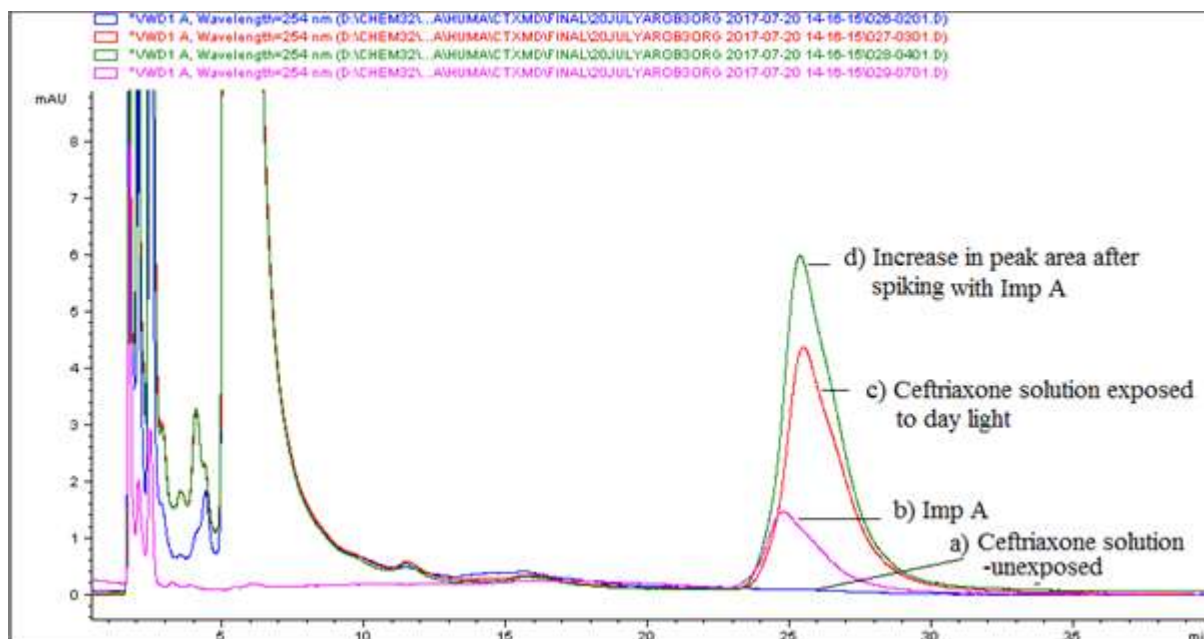
Figure 3.  Impurity A produced in solution of ceftriaxone on exposure to daylight. a) ceftriaxone solution stored in brown glass vials, b) solution of impurity A CRS, c) ceftriaxone solution exposed to day light for 24 h, d) increase in peak area after spiking ceftriaxone solution exposed to daylight with impurity A CRS.

3.2.2. Degradation products of ceftriaxone in various conditions

Degradation products of cefotaxime were studied in various conditions and solvents in a recent investigation (32) including production of deacetylcefotaxime and cefpodoxime on heating at 70°C for 1 h using water and 50% methanol (v/v) as solvents, respectively. Due to the close structural similarity of ceftriaxone and cefotaxime a similar set of experiments has been employed using ceftriaxone in this study to gain more information on the degradation profile, impurity preparation methods and the choice of solvent for ceftriaxone. Table 1 summarizes results of different experiments done in the current study and from literature that produced degradation products of ceftriaxone and cefotaxime. These experiments reinforce previous studies (32, 33) that the choice of solvent and the handling conditions are crucial to the stability of cephalosporins and need to be studied during method development.

Table 1        Similarity in degradation products produced by ceftriaxone and cefotaxime at various conditions

| Treatment | Impurity(s) produced | |
|---|---|---|
| | **with cefotaxime** | **with ceftriaxone** |
| Acid hydrolysis at 40°C for 2 h | deacetylcefotaxime (32) | deacetylcefotaxime* |
| Solution in 50% methanol at 70°C for 1h | Cefpodoxime  (32) | Various unknown peaks appeared* |
| Heating aqueous solution at 70°C for 1h | deacetylcefotaxime (32) | Impurity C and deacetylcefotaxime* |
| Day light exposure | | Impurity A* |
| Exposure of solution in phosphate buffer, pH 5.0 to 254 nm for 24-48 h | | Impurity C and deacetylcefotaxime * |
| Treatment with sodium bicarbonate | deacetylcefotaxime (30, 31) | |
| Treatment with acetic acid | | deacetylcefotaxime, impurity C and reversible spiro isomer (33) |
| In phosphate buffer, pH 5.0 | | Impurity C, peak group with set of three peaks including deacetylcefotaxime * |

*performed in current study.*

### 3.3.    Method development and optimization

### 3.3.1.    Overview of methods

The details of chromatographic settings of the two developed methods are provided in the Table 2. The chromatographic attributes of the analytes separated using method A, needed for peak identification and quantification are shown in Table 3. Figure 2a-d shows the successful separation of ceftriaxone and its known and unknown impurities using method A settings.

3.3.2. *pH-optimization for mobile phase buffer used in method A*

Ceftriaxone possesses acidic groups with $pK_1$ (COOH) = 2.37, $pK_2$ (amide) = 10.74, and $pK_3$ (hydroxyl triazinone group) = 4.21 along with a basic group with $pK_4$ (thiozolamine) = 3.03 (35). pH 5.0 offers deprotonation of the carboxylic and hydroxyl triazinone group with protonated amide as shown in Figure 1.

An isocratic elution on column A using 0.02 M dihydrogen potassium phosphate buffer (pH 5.0) with 17% methanol (v/v) as mobile phase was chosen as initial settings for method A, using ceftriaxone solution (0.6 mg/mL) with impurity E, impurity C, impurity A (0.1 mg/mL each) and impurity B and 7-ACA (0.05 mg/mL each); ceftriaxone solution (0.6 mg/mL) and solution of deacetylcefotaxime spiked with ceftriaxone standard solution (0.6 mg/mL) (Figure 2).

First, pH range was optimized by variation between pH 4.5-7.5. Deacetylcefotaxime and ceftriaxone peaks were identified as a critical peak pair whereas, retention time of impurity A remained consistent at pH above 5.5 but increased to 57 min at pH 4.5. Fine tuning was done within the narrow optimal pH of 4.9-5.3 showing maximum resolution for the critical peak pair at pH 5.0 (Appendix I, Figure A1). The minimum resolution of Rs = 1.6 was observed between the unknown peak c (the last peak in the unknown peak group) and ceftriaxone at pH 5.2 showing the adequate robustness of the method on slight change in pH.

The optimized method at pH 5.0 provided elution of ceftriaxone at the retention time of 6-7 min with Rs of ≥ 2 between all known impurities as well as between ceftriaxone and its known impurities (Figure 2a and Appendix I, Figure A1). The final chromatographic settings for method A include isocratic elution of 0.02 M potassium dihydrogen phosphate, pH 5.0: methanol (83:17%, v/v) at 0.8 mL/min with a detection wavelength $\lambda$ = 254 nm on column A at ambient temperature, with an injection volume of 20 µL.

In the developed method, impurity A elutes as a last signal at 29-33 min and is well resolved from principal and the other impurity peaks but with less sensitivity (LOQ = 0.43%). It was noted that, column D elutes impurity A at 23 min with sharper peak and three times higher peak height, providing better signal-to-noise ratio. However, column D was unable to provide base line separation for 7-ACA and impurity C (Rs = 0.8).

Table 2        Chromatographic settings of the developed methods

|  | *Method A* | *Method B* |
|---|---|---|
| *Stationary Phase* | Octadecylsilanyl (C18) | Octadecylsilanyl (C18) |
| *Column dimensions* | 150 mm X 4.6 mm, 5 µm particle size | 150 mm X 4.6 mm, 5 µm particle size |
| *Brand* | Agilent Eclipse Plus | Knauer C18A |
| *Elution* | Isocratic | Isocratic |
| *Mobile phase* | 0.02 M potassium dihydrogen phosphate, pH 5.0: methanol (83:17%, v/v) | 0.1% phosphoric acid: acetonitrile (65:35%, v/v) |
| *Detection wavelength* | 254 nm | 240 nm |
| *Flow rate* | 0.8 mL/min | 0.8 mL/min |
| *Injection volume* | 20 µL | 20 µL |
| *Run time (min)* | 40 min | 25 min |
| *Solvent* | 0.02 M potassium dihydrogen phosphate, pH 7.0 | acetonitrile 50%, v/v |
| *System suitability test* | Rs ≥ 2.0 using the system suitability test solution |  |
| *Temperature* | ambient | ambient |
| *Concentration of test solution* | test solution A (0.6 mg/mL) | test solution B (1.0 mg/mL) |

Table 3        Retention time (RT), relative retention time (RRT) and correction factor (CF) for impurities using method A

| *Impurity* | *RT (min)* | *RRT* | *k' (capacity factor)* | *CF (if needed)* |
|---|---|---|---|---|
| *Impurity E* | 2.1 | 0.3 | 1.0 | Not required |
| *Impurity C* | 2.6 | 0.4 | 1.2 | 0.7 |
| *7-ACA* | 3.3 | 0.5 | 1.6 | 1.5 |
| *Unknown peak group* | 4.1-5.3 | 0.6-0.8 |  |  |
| *Deacetylcefotaxime* | 4.8 | 0.7 | 2.3 | -- |
| *Ceftriaxone* | 6.6 | 1.0 | 3.1 | -- |
| *Impurity B* | 11.8 | 1.8 | 5.6 | 1.4 |
| *Impurity A* | 27.6-32.6 | 4.8-4.9 | 13.0-15.5 | 1.4 |

### 3.3.3. Impurity D of ceftriaxone and need of separate method

Impurity D, 2 mercaptobenzothiazolyl(Z)-2-(2-aminothiazol-4-yl is the acylating agent used in synthesis of ceftriaxone and other cephalosporins for conversion with 7-ACA (18, 19). During the synthesis of ceftriaxone, the toxic by-product 2-mercaptobenzothiazole is produced which is difficult to remove from the final product (16, 17, 36, 37). Impurity D of ceftriaxone was found unstable in methanol. LC/MS investigation revealed a transesterification giving 2-mercaptobenzothiazole and *Z*- and *E*- isomers of (2*Z*)-methyl 2-(2-amino-1,3-thia-zol-4-yl)-2-(methoxy-imino)ethanoate (m/z=215.9), which  was already reported (38) (Figure 4). In acetonitrile impurity D is stable.



Impurity D
(ceftriaxone benzothiazolyl oxime ether)
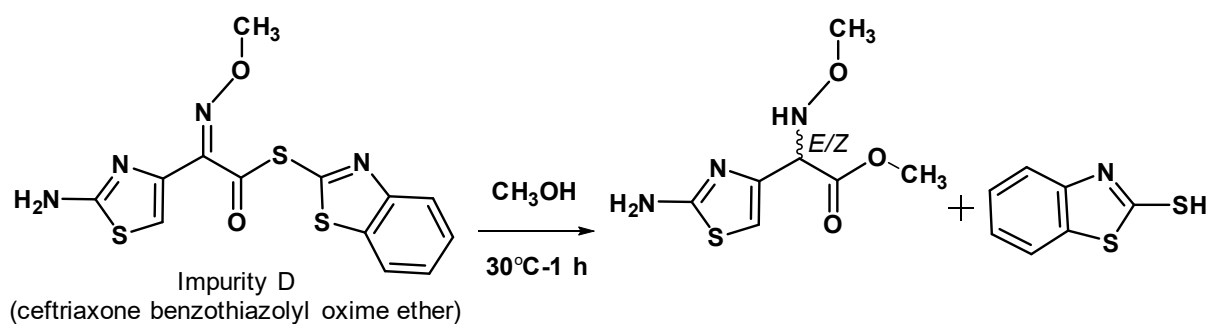
Figure 4.    Scheme of reaction of impurity D with methanol (38)

The significant difference in the solubility and lipophilicity of impurity D (predicted Log P = 3.28) (39) from other analytes of interests did not allow elution of impurity D using method A therefore method B was developed. Since impurity D is stable in acetonitrile, a mixture of 0.1% phosphoric acid (v/v) with 35% acetonitrile (v/v) was found as a suitable composition and was used at a flow rate of 0.8 mL/min on column B with the detection wavelength of $\lambda$ = 240 nm (Table 2). The separation of impurity D, ceftriaxone and the degradants of impurity D in methanol was achieved using method B with elution of ceftriaxone at 2.2 min, 2-mercaptobenzothiazole at 9.7 min (RRT = 4.4) and impurity D at 20.6 min (RRT = 9.4) (Figure 5).
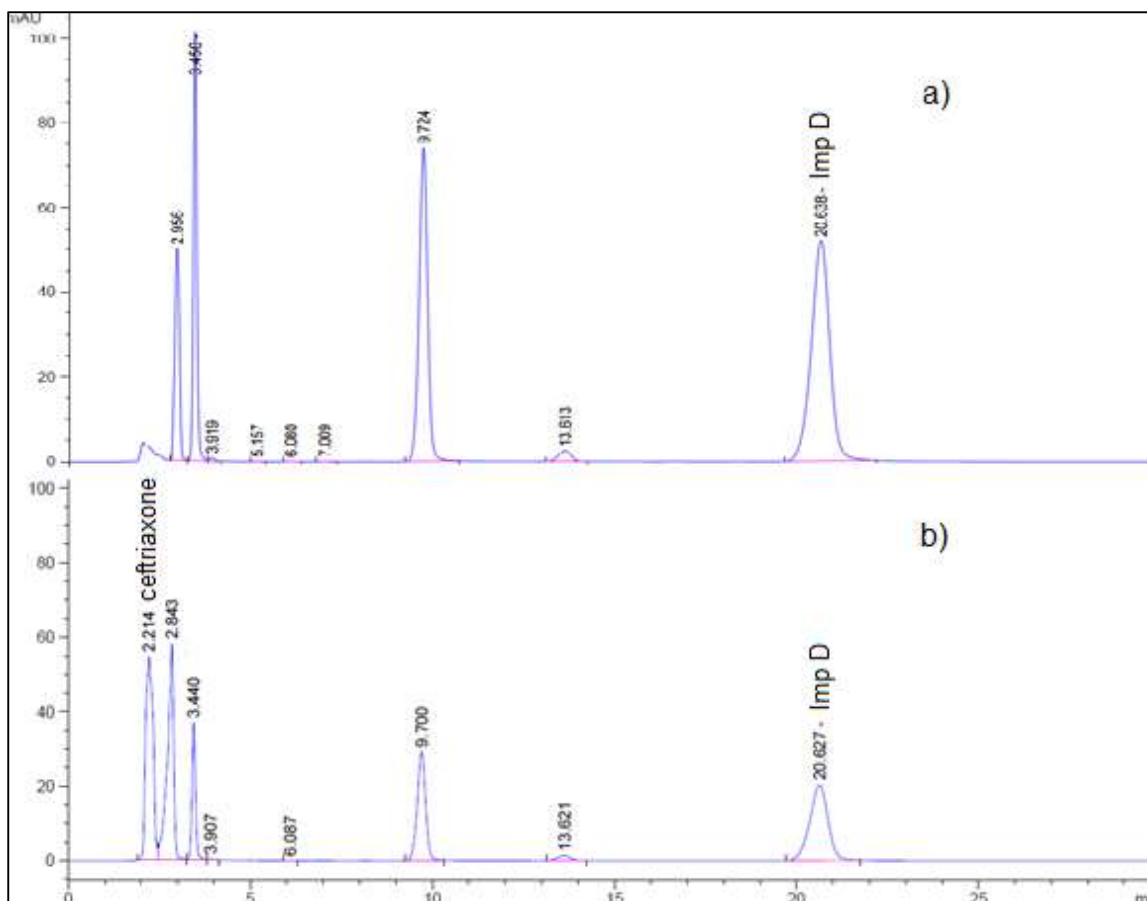
Figure 5.    a) Impurity D degradation in methanol (0.05 g/mL), showing 3 main peaks for degradants at 2.95, 3.45, 9.72 min and impurity D peak at 20.63 min. b) ceftriaxone and degraded impurity D (0.01 mg/mL of each) showing resolution of ceftriaxone and the first peak of impurity D degradant (Rs =1.8).

*3.4.    Stability and solvent selection*

Because of the stability issues observed for ceftriaxone (32, 33) and impurity D (38), the suitable solvent were found to be 0.02 M dihydrogen phosphate buffer (pH 7.0) and acetonitrile 50%, v/v, for method A and B, respectively. The current pharmacopoeial methods also use phosphate buffer, pH 7.0 as a major buffer component with the 50% acetonitrile as the solvent (8, 9, 14, 40).

3.5.    **Method validation-assay of ceftriaxone using method A**

Validation of developed analytical methods were conducted in accordance with the International Conference on Harmonization (ICH) (Q2(R1) (41) .

### 3.5.1. Specificity

The method was found to be specific for the quantification of ceftriaxone and its impurities with a resolution of Rs ≥ 2.0 studied using the spiked sample (ceftriaxone = 0.6 mg/mL, impurity E, C, A= 0.1 mg/mL, 7-ACA and impurity B = 0.05 mg/mL) (Figure 2 and Appendix I, Figure A1). The peaks of all the spiked analytes were found to be pure on analysis using diode array detector.

### 3.5.2. Linearity:

A five-point calibration curve was constructed in the range of 0.24-0.72 mg/mL to calculate coefficient of regression ($R^2$ value). The regression equation for ceftriaxone was y=64709x + 72.09 with $R^2$ value of 1.0000 (Table 5). Of note, USP includes only the lower limit of 795 µg/mg, as acceptable limit of content in ceftriaxone bulk drug (Appendix I, Table A1).

### 3.5.3. Accuracy

Recovery of ceftriaxone solution for 80%, 100% and 120% samples was within 98.6-101.3%, for all the three days. The inter-day accuracy was found to be 100.0% (n = 3, RSD ≤ 1.3) for each level.

### 3.5.4. Precision

Reproducibility of the method was determined by analysing the data from multiple injections (n = 6) of test solution of ceftriaxone (0.60 mg/mL). The intra-day relative standard deviation (RSD) for retention time, peak area and peak height was within 0.1-1.3% showing adequate reproducibility of the method.

### 3.5.5. Stability

Recovery of standard solution corresponding to test solution concentration (0.6 mg/mL) analysed after 24 h of storage at room temperature in brown glass vials was found to be 100.8%. A variation of 2.13% in 24 h is documented for ceftriaxone using compendial method (42) and prompt use of standard and sample solutions is advised in one (40) of the three monographs for ceftriaxone by USP (8, 14).

## 3.6. Method validation-test for related substances of ceftriaxone using method A

Validation of method A was carried out for quantification of impurity E, impurity C, 7-ACA, impurity B and impurity D. Accuracy, precision and linearity experiments were repeated for all the impurities for three days, except for impurity A for which the data for only one day could be obtained.

### 3.6.1. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ values were determined for the impurity E, impurity C, 7ACA and impurity B and impurity A by constructing calibration curve in the concentration range close to the limit of quantitation of each impurity. The signal-to-noise ratio (S/N) obtained for each impurity of ceftriaxone at or close to (shown in bold) the calculated LOD and LOQ values are given in Table 4.

Table 4        The observed S/N ratio of impurities of ceftriaxone at LOD and LOQ

| | Limits of detection (LOD) | | | Limits of quantification (LOQ) | | |
|---|---|---|---|---|---|---|
| | µg/mL | as % of TS | S/N | µg/mL | as % of TS | S/N |
| Impurity E | **0.04** | 0.007 | 24 | 0.1 | 0.01 | 39 |
| Impurity C | **0.01** | 0.002 | 6.6 | **0.05** | 0.01 | 33 |
| 7ACA | **0.03** | 0.005 | 8 | 0.1 | 0.02 | 15 |
| Impurity B | **0.2** | 0.033 | 6.2 | **0.3** | 0.05 | 15 |
| Impurity A | 1.3 | 0.217 | 7.7 | 2.6 | 0.43 | 13 |
| Impurity D | 0.03 | 0.003 | 3.3 | 0.1 | 0.01 | 14 |

*TS= concentration of API in test solution (for method A=0.6 mg/mL, for method B= 1.0 mg/mL)*

The limits of quantification were lower than the disregard limit used by the pharmacopoeia (0.1% of the test solution) in monographs for ceftriaxone API and FPP for all the impurities except impurity A. The LOQ value determined for impurity E, impurity C and 7-ACA was ≤ 0.03% and for impurity B it was 0.05%. The LOQ for impurity A was found to be 4 times higher than the reporting level of 0.1%.

### 3.6.2. *Linearity*

Method A has proven linearity of $R^2 \geq 0.9990$ for the six analytes within the concentration range given in Table 5. Impurity C has been reported in upto 1% concentration in the finished product samples of ceftriaxone (43). Three individual sets of standard solutions were injected to get the linearity data. The correction factors for impurities of ceftriaxone are shown in Table 3.

Table 5        Linearity data for ceftriaxone and its impurities on method A

| Impurity | Range | Range as % of test solution A | Regression equation | $R^2$ |
|---|---|---|---|---|
| Impurity C | 0.2-12.2 µg/mL | 0.03-2.03% | $y=82291x-0.304$ | 1.0000 |
| Impurity E | 0.2-12.2 µg/mL | 0.03-2.03% | $y=69032x-2.544$ | 0.9999 |
| 7-ACA | 0.2-12.2 µg/mL | 0.03-2.03% | $y=39735x-0.024$ | 0.9998 |
| Impurity B | 0.3-12.2 µg/mL | 0.05-2.03% | $y=43078x+1.413$ | 0.9998 |
| Impurity A | 2.6-12.2 µg/mL | 0.43-2.03% | $y=44461x-25.955$ | 0.9997 |
| Ceftriaxone | 0.2-12.2 µg/mL | 0.03-2.03% | $y=60917x-5.732$ | 0.9996 |
| Ceftriaxone | 0.26-0.72 mg/mL | | $y=64709x+72.09$ | 1.0000 |

### 3.6.3. *Accuracy*

The method was found of adequate accuracy with the calculated recovery ranging from 75.2.0-110.6% for the impurities analysed at the tested levels shown in Table 5. The mean peak area of either triplicate or sextuple injections was used for recovery calculation. The inter-day RSD ranged from 0.7-7.6% with the exception for impurity B (Table 5)

### 3.6.4. *Precision*

Intra- and inter-day precision was determined by calculating RSD with respect to peak area and retention time for the spiked samples at the lowest (0.03%), intermediate (1.0%) and highest (2.0%) levels by injecting in sextuples on three days (levels included for precision study are shown in bold in Table 6). Intra-day RSD for retention time and peak area was $\leq 2.0\%$ (n = 6) for all the samples, with the exception of peak area repeatability for 0.03% level of impurity C and 7-ACA which were in the range of

4.2-13.5% and 1.8-3.4%, respectively. Inter-day RSD with respect to retention time and peak area was within 0.75-6.1% (n=3).

Table 6      Inter-day accuracy by recovery calculation for the impurities of ceftriaxone on method A

| Level- % of TS | Spiked amount (mg/mL) | Inter-day accuracy (Mean recovery (%) ± SD, n = 3) | | | | |
|---|---|---|---|---|---|---|
| | | Impurity C | Impurity E | 7-ACA | Impurity B | Impurity A* MR (%)-D1 |
| 0.03 | 0.0002 | **105.2 ± 3.1** | **109.1 ± 2.0** | **94.5 ± 6.7** | | |
| 0.05 | 0.0003 | 96.6 ± 7.6 | 106.7 ± 5.2 | | 87.0 ± 22.5 | |
| 0.10 | 0.0006 | 95.4 ± 4.4 | 98.6 ± 4.2 | | 88.0 ± 6.5 | |
| 0.50 | 0.0030 | 102.5 ± 1.5 | 93.6 ± 3.2 | | | |
| 1.00 | 0.0060 | **102.0 ± 0.7** | **98.1 ± 1.9** | **97.2 ± 2.7** | 97.6 ± 4.4 | 100.4 |
| 1.50 | 0.0090 | 101.4 ± 1.9 | 92.7 ± 3.5 | | | |
| 2.00 | 0.0120 | **102.7 ± 2.9** | **94.6 ± 3.4** | **93.0 ± 1.8** | **93.7 ± 2.2** | **102.2** |
| Min. recovery observed (level) | | 91.0% (0.1%) | 89.0% (1.5%) | 88.0% (0.03%) | 75.2 (0.05%) | |
| Max. recovery observed (level) | | 108.5% (0.03%) | 110.6% (0.03%) | 100.7% (0.03%) | 108.6 (0.05%) | |

*Data from day 1 (recovery from mean peak area.)*

### 3.6.5. Stability

The percentage change in peak area with reference to initial peak area for standard solution after 24 h was found to be 2.9% and 0.2% for impurity C, 2.4% and 2.8% for impurity E at room temperature (RT) and in refrigerator (RF), respectively. For sample solutions, the change in peak area was found to be 3.2% and 0.8% for impurity C, and 0.5% and 2.4% for impurity E after 24 h on RT and RF storage, respectively. At room temperature 7-ACA standard showed fast decline of 2.8%, 9.4% and 15.0% in peak area after 4, 12 and 24 h, respectively. For sample solution, the change in peak area was 4.35% and 3.0% after 4 h of storage at RT and in RF, respectively. Impurity B standard solution showed 10.0% decrease in peak area after 5 h of RT storage.

### 3.6.6. Robustness

Slight deliberate changes in the final chromatographic settings of method A (Table 2) were performed including temperature (25 °C, 30 °C, and 35 °C), concentration of organic content of mobile phase (± 2%), and flow rate (± 0.2 mL/min). Resolution between all the key analytes was Rs ≥ 1.5 in all the robustness experiments.

Method was reproducible on column B, C and D. with slight difference in the results. Column D produced sharper peaks and good peak bands, with impurity A eluting at 23 min, and peak height three times in comparison to other columns. However, the order of elution of 7-ACA and impurity C was reversed, and their baseline separation was not achieved on this column (Rs = 0.8). On column C (hydrophobic bonding), a longer retention time of 40 min was observed for impurity A. Tailing factor was highest (USP tailing factor = 1.5) on column B (hydrophillic bonding), and baseline resolution of impurity E and 7-ACA was also not achieved (Rs = 0.9).

### 3.7.  Method validation-quantification of Impurity D in ceftriaxone

### 3.7.1. Limits of detection (LOD) and quantification (LOQ)

The LOD and LOQ of impurity was determined on method B in accordance with the methodology described in ICH guidelines and a LOD of 0.03 µg/mL (0.003% of test solution) and LOQ of 0.1 µg/mL (0.01% of test solution) was obtained which produced signal-to-noise ratio of 3.3 and 14, respectively.

### 3.7.2. Linearity

Linearity was determined by constructing a calibration curve using triplicate injections of three individual sets of standard solutions prepared in the range of 0.3-24.3 µg/mL (0.3, 0.9, 2.7, 8.1, 24.3 µg/mL).  The regression equation obtained for impurity D was $y=66094x + 1.3032$ with $R^2$ value of 0.9999 showing good linearity.

### 3.7.3. Accuracy

Recovery for spiked samples was found to be in the range of 82.1%-112.2% with the inter-day RSD ranging from 3.8-7.5% for the seven levels (0.03, 0.05, 0.1, 0.5, 1.0, 1.5 and 2.0%).

### 3.7.4. Precision

The inter-day (intermediate) and intra-day precision was determined by calculating RSD for six replicate injections of sample spiked at highest level (2%). The inter-day RSD was found to be 1.3% and 13.0% for retention time and peak area, respectively. The intra-day RSD (data from three individual solutions prepared on separate days) for retention time and peak area was ≤ 1% and ≤ 1.5%, respectively.

### 3.7.5. Stability

The sample solution of impurity D (2%) was found highly instable with the percentage change of 2.5% from the initial peak area on reinjection after 4 h of room temperature storage.

### 3.7.6. Robustness

Slight deliberate changes in chromatographic settings of method B were made to carry out robustness experiments including variation of temperature (25 °C, 30 °C, 35 °C, and 40 °C), concentration of organic content of mobile phase (± 2%), and flow rate (± 0.2 mL/min). Ceftriaxone, 2-mercaptobenzothiazole, impurity D remained well resolved from each other, and also from any unknown peak (Rs ≥ 2.0) in the solution of ceftriaxone and impurity D (0.02 mg/mL of each analyte) prepared in ACN (50%). The resolution of Rs ≥ 1.0 was obtained between ceftriaxone and any degradant peak from solution of degraded impurity D and ceftriaxone (0.01 mg/mL of each analyte).

### 3.8. Cost and simplicity

The compendial method for assay and impurity profiling of ceftriaxone API costs 4.5 times the combined total cost of method A and B. The developed methods offer simplicity in operation by using isocratic elution, simple mobile phases, lower cost and convenience by requiring only the routine laboratory chemicals, without the need of expensive impurity standards and ion pair reagents. The flow rate of the mobile phase used in developed methods is 0.8 mL/min thereby reducing the solvent consumption.

The RP-HPLC based simple, reproducible methods using methanol and phosphate buffer has been reported in literature for providing a reliable alternate to the expensive ion pair methods (24). Simple and cost-effective methods based on phosphate buffer

and methanol for antimalarial agents reported in literature are in use for routine quality control analysis (22, 24, 44).

## 4. Limitations

In this study, stability issue is encountered for 7-ACA, impurity B and impurity D and a better stability is expected on experimentation with temperature control. The method sensitivity for sensitivity of impurity A can be overcome by using a column offering earlier elution with better signal strength or increasing the injection volume.

## 5. Conclusion

The developed set of methods uses simple isocratic settings, with routine laboratory reagents and offers comparable or in some ways more information on the impurity profile of ceftriaxone in comparison with the expensive ion pair method. The identification of an unknown peak group and a new common impurity between cefotaxime and ceftriaxone suggests further investigations of potential and actual impurities of these molecules. The identification of the toxic degradation product of impurity D and the synthetic impurity of ceftriaxone can be made by using method B. The new methods are cost-effective for routine laboratory analysis and regulatory use and large number of samples can be analysed in limited budget offering them as preferable choice for post marketing surveillance studies. In times of shortages of acetonitrile as seen in past, method A offers assay and partial impurity profiling, thereby allowing the routine quality control of this essential antibiotic go uninterrupted. The two methods (A and B) can be effectively used for assay and impurity profiling of the ceftriaxone bulk drug and further investigation can be made for application of the method for use in quality control of single component FPP or fixed dose combinations of ceftriaxone.

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## 3.2 Development of simple isocratic reverse phase liquid chromatography method for impurity profiling of amoxicillin and clavulanic acid fixed dose combination in solid dosage form

**ABSTRACT**

Coamoxiclav is an essential medicine and a popular fixed dose combination of extended spectrum beta-lactam antibiotic, amoxicillin with beta-lactamase inhibitor clavulanic acid. The combination is used widely for clinical indications in both ambulatory and inpatient care settings. The active pharmaceutical ingredients (APIs) are products of fermentation and semisynthetic origin, and possess a large number of known impurities. A simple, precise and accurate RP-HPLC analytical method using the isocratic elution was developed and validated for quantification of the impurities of amoxicillin in coamoxiclav tablets. Separation was achieved for the two active pharmaceutical ingredients and six impurities of amoxicillin along with a set of eight unknown components, using a standard C18 column (250 X 4.6mm, 5 µm particle size) with 0.02 M potassium dihydrogen phosphate buffer (pH 2.2)-methanol (88:12, v/v), at a flow rate of 1.5 mL/min, in a run time of 65 minutes using a detection wavelength of $\lambda$ = 210 nm. The method was validated using four impurities for specificity, linearity, accuracy, limit of detection and limit of quantification of impurities, and stability of sample and standard solutions. Correction factor and the relative retention time for impurities are provided along with methods for preparation of important impurities, to allow identification and quantification of impurities in absence of impurity reference standards. In contrast to the compendial method for impurity profiling of coamoxiclav tablet which employs acetonitrile as mobile phase component and gradient elution, this method requires cheaper chemicals and simple chromatographic settings and hence is cost effective and practical for use in resource-limited settings.

**Key words:** impurity profile, beta-lactam antibiotics, quality control, RP-HPLC, isocratic elution, fixed dosage forms.

## 1. Introduction

Coamoxiclav, launched in 1981, is a fixed dose combination of the extended spectrum beta-lactam antibiotic, amoxicillin with beta-lactamase inhibitor clavulanic acid (1). It is used widely in the out-patient and in-patient health care settings across the globe (2-5). In 1972, discovery of clavulanic acid provided a promising solution against staphylococcal resistance to penicillins, by acting as a suicide substrate through irreversible inhibition of most beta-lactamases (6, 7). The combination is the first choice of therapy for severe community and hospital acquired pneumonia, sinusitis, exacerbation of COPD, and lower urinary tract infection along with other highly prevalent indications, and is part of the CORE group of antibiotics listed in the 20th essential medicines list (EML) by World Health Organization (WHO) (8). Increasing prevalence of methicillin resistant strains of *Staphylococcus aureus* (MRSA) is a continuing threat to the availability of effective treatment options (9).

The ratio of clavulanic acid to amoxicillin used in oral solid dosage form are 1:2, 1:4, and 1:7 (extended release tablets), with the amount of clavulanic acid being 125 mg in each tablet. The fixed combinations of amoxicillin/clavulanic acid in included in the EML are 125/31.35 mg and 250/62.5 mg for syrup, 500/125 for tablets, and 500/100 and 1000/200 mg for powder for injection.

Amoxicillin is produced semi-synthetically by using 6-aminopenicillanic acid as a starting material whereas clavulanic acid is the fermentation product obtained from *Streptomyces clavuligerus* (10). Both the APIs possess number of known impurities which are mainly their degradation products (Figure 1). Clavulanic acid is a temperature and humidity sensitive substance (10) determining its shelf-life. The injectable formulation of coamoxiclav is recommended for cold (2-8 °C) storage whereas the oral solid dosage forms are now preferably packaged using unit dose blister packing with double aluminum foil. Inadequate storage and poor packaging quality can result in degradation of the API which can lead to therapeutic failure, resistance development and other serious implications to health care system (11).

Amoxicillin is used as trihydrate and sodium salt in oral and parenteral formulations, respectively whereas clavulanic acid is used as a potassium salt. Impurity D constitutes the most common impurity found in the amoxicillin trihydrate followed by impurity C, whereas amoxicillin sodium also contains the dimeric impurity (impurity J) (12). The

chemical structures of amoxicillin, clavulanic acid and their known impurities are shown in Chapter 1-Figure 1.3-1.26.

The provision of quality antibiotics is essential components of the WHO policy package to combat antimicrobial resistance (13). Enabling simpler and cost-effective methods for routine quality control tests of pharmaceuticals improve the regulatory capacity of the national drug testing and surveillance systems (14). In contrast to assay methods, the compendial methods for impurity profiling are mostly based upon sophisticated gradient elution system. The gradient elution RP-HPLC based method for amoxicillin impurity profiling by European pharmacopoeia (Ph.Eur.) is used for impurity profiling of coamoxiclav tablets by British Pharmacopoeia (BP) (15-17). This method employs 0.05 M phosphate buffer, pH 5.0 and acetonitrile as mobile phase components and involves gradient elution at 1.0 mL/min at detection wavelength of $\lambda$ = 254 nm (16, 17). The initial isocratic settings of this method are used for the assay of amoxicillin active pharmaceutical ingredient (API) and finished pharmaceutical product (FPP) (15-17).

The impurity profiling method for amoxicillin API in USP is based upon gradient elution with 0.02 M phosphate buffer. pH 5.0 and methanol, involving elution at 1.5 mL/min at a detection wavelength of $\lambda$ = 210 nm, and requires two impurity standards for the system suitability test (18). In USP, the test for related substances in FPPs of coamoxiclav is only provided for extended release tablets, which is a gradient elution RP-HPLC method employing a short C18 column on phosphate buffer (pH 4.2) and methanol as mobile phase at a flow rate of 1.5 mL/min using the detection wavelength of $\lambda$ = 229 nm (19). For assay of coamoxiclav tablets, BP and USP include an isocratic RP-HPLC method based upon 0.78% w/v sodium dihydrogen phosphate monohydrate buffer (pH 4.4)-methanol (95:5, v/v) at 2 mL/min using a standard C18 column and detection wavelength of $\lambda$ = 220 nm (15, 20). The complete impurity profiling of clavulanic acid requires the use of both HPLC and gas chromatography methods, due to the presence of some low molecular weight and volatile impurities.

The maximum daily dose of clavulanic acid is 375 mg whereas amoxicillin is used in the daily doses of $\geq$ 2 g. Hence according to recent guidelines the reporting limit for the impurities of amoxicillin in FPP is 0.1% (21). The BP 2013 monograph method for related substances in coamoxiclav tablets limit impurity J of amoxicillin to $\leq$ 2% and any other unspecified peak to $\leq$ 1% (15). The USP monograph for extended release coamoxiclav

tablets limits impurity D, impurity C, impurity J and any unspecified impurity to 2.5%, 2.5%, 4.5%, and 0.5%, respectively (22).

The RP-HPLC based impurity profiling methods reported for amoxicillin as API or FPPs, included the methods offering separation of 2-4 impurities using isocratic (23) or gradient elution (24). Three gradient elution methods were reported for complete impurity profiling of amoxicillin (18, 25, 26).

Hoogmartens et al., compared five isocratic methods on various C18 columns (16, 17, 25). Four methods included different phosphate buffer with of 4-7%, v/v of organic content (acetonitrile or methanol) in the pH range of 4.5- 5.0 (25). The fifth method was based on 1.25% acetate buffer-methanol (80:20, v/v) and showed elution of all the analytes within the capacity factor of $k'$ ≤ 13 (23, 25). Lucentini et al. have developed a RP-LC/UV/MS gradient elution  elution method with 0.1%, v/v trifluoroacetic acid (pH 2.1), and acetonitrile as mobile phase components on C18 column separating 13 available impurities of amoxicillin (26).

Use of costly solvents like acetonitrile, need of additional reference standards, specialized columns and the gradient elution setup are impediment in carrying out the critical quality control test on routine basis for the resource limited settings of low- and middle income countries (27).

The aim of this study is to develop a simple and cost-effective RP-HPLC method for impurity profiling of the clavulanic acid and amoxicillin fixed dose combination for solid dosage form included in the essential medicines list (125/500 mg). The new method is aimed to be based on isocratic elution using phosphate buffer and methanol as mobile phase components thereby, avoiding use of acetonitrile, due to its high cost. *In situ* preparation of impurities common in the marketed samples (impurity D, C and J) will be investigated for peak identification. The impurity profiling for impurities of clavulanic acid is not kept in the scope of this method.

## 2.    Experimental

### 2.1.   *Chemical and reagents*

Amoxicillin trihydrate, its impurity A (6-aminopencillanic acid) and impurity I (4-hydroxyphenylglycine) were bought from Sigma Aldrich (Laramie, USA). Lithium

clavulanate was procured from European Directorate for the Quality of Medicines and HealthCare (EDQM) (Strasbourg, France). The remaining impurities of amoxicillin; impurity D (as epimeric mixture of impurity Da and Db; epimers of amoxicilloic acids), impurity F (2-hydroxy-3-(4-hydroxy)-phenylpyrazine), impurity C (diketopiperazine of amoxicillin), were synthesized using methods reported in literature (28-30). Impurity J (amoxicillin dimer) was prepared by using modification of the method reported for preparation of impurity C (28).

HPLC grade methanol, analytical grade potassium dihydrogen phosphate, and orthophosphoric acid were purchased from Merck KGaA (Darmstadt, Germany), Grüssung GmbH (Filsum, Germany), and Sigma-Aldrich Chemie GmbH (Steinheim, Germany), respectively. Purified water was produced by using Milli-Q® laboratory water system from Merck Millipore (Darmstadt, Germany).

The generic fixed dose combination *Amoxiclav 500/125* (Batch No. DB1943, 1A Pharma, Oberhachig, Germany) was used to prepare tablet sample solutions in the validation experiments. Amoxicillin trihydrate vetranal purchased from TCI (Germany) was used for the preparation of impurities.

## 2.2.   Apparatus

Two Agilent 1100/1200 series HPLC systems from Agilent Technologies (Waldbronn, Germany) equipped with online degasser, a binary pump, an autosampler and a variable wavelength detector or a diode array detector were used for method development and validation. An external column oven from Beckman Coulter GmbH (Krefeld, Germany) was also used. The HPLC system was connected to Chemstation® for LC 3D systems Rev. B.03.02 software from Agilent technologies (Waldbronn, Germany) for recording and integration of the chromatograms.

Apart from these the pH meter from Metrohm (Filderstadt, Germany), ultrasonic bath from Brandelin electronic GmbH & Co. KG, (Berlin, Germany), centrifuge from Eppendorf Zentrifugen GmbH (Leipzig, Germany) and analytical balance from Mettler Toledo (Gießen, Germany) were also used.

The standard C18 columns (250 X 4.6 mm, 5 µm particle size) used during method development included column A: Spherisorb® ODS2, from Waters Corporation (Milford,

MA, USA), and Column B: Microsorb® MV from Agilent Technologies (Santa Clara, CA, USA).

## 2.3.   Chromatographic settings:

The analytical method was validated using the standard C18 column, Spherisorb ODS2, (250 X 4.6 mm, 5 µm particle size) as a stationary phase. Isocratic elution was performed with mobile phase consisting of 0.02 M potassium dihydrogen phosphate buffer (pH 2.2)-methanol (88:12, v/v), at a flow rate of 1.5 mL/min with the detection wavelength of 210 nm. An aliquot of 10 µL of sample solutions were injected into the chromatographic system. The column temperature was ambient.

## 2.4.   Preparation of buffer solution for mobile phase:

2.72 g of potassium dihydrogen phosphate was dissolved in 200 ml of water and filtered through 0.45 µm filter. The solution was diluted to 1000.0 mL using water and pH was adjusted to 2.2 using orthophosphoric acid. Mobile phase was sonicated for 20 min before use.

## 2.5.   Preparation of stock, standard and sample solutions for impurities and amoxicillin

*Stock and standard solutions of impurities of amoxicillin:* 2.5 mg of impurity C was dissolved in 20 ml of 15% v/v methanol and the volume was diluted to 50.0 ml. 5.0 mg of each of impurity A and impurity I were dissolved in 5 ml of water together and diluted to a final volume of 50.0 ml with water. 1.0 mg of impurity D was dissolved in 1 ml of water and then diluted to a final volume of 10.0 ml with water. 2.5 mg of amoxicillin trihydrate was dissolved in 50.0 ml of water.

Standard solutions were prepared with regular increments to cover the range of LOQ-2% (30 µg/mL) of the concentration of amoxicillin in test solution. Standard solutions were prepared for amoxicillin and impurity C (range: 0.8-30.8 µg/mL, six points: 0.8, 1.7, 3.4, 7.1, 14.8, 30.8 µg/mL), for impurity A and I (range: 0.2-30.3 µg/mL, six points: 0.2, 0.5, 1.5, 4.1, 11.1, 30.2 µg/mL), and for impurity D (range: 0.6-30.9 µg/mL, six points: 0.6, 1.3, 2.9, 6.4, 14.1, 30.9 µg/mL) by diluting their respective stock solutions with water.

For preparing stock solutions the analytes were added to a small quantity of solvent and sonicated for 15 min. The volume was then made up to the required level using water

and sonicated again for 5 min to prepare the final stock solution. Fresh stock solutions were used each day.

*Preparation of tablet matrix and sample solutions for impurities:* Powdered coamoxiclav tablet (125/500mg) equivalent to 75.0 mg of amoxicillin was weighed, suspended in 30.0 ml of water and sonicated for 15 min followed by making up the volume to 50.0 ml with water. The final solution was mixed and sonicated for 5 minutes before centrifugation at 44,000 rpm for 15 min. The supernatant (1.5 mg/mL) was used as a tablet matrix. Five levels of the spiked samples of impurities including the LOQ, 0.5% (0.0075 mg/mL), 1.0% (0.0150 mg/mL), 1.5% (0.0225 mg/mL) and 2.0% (0.030 mg/mL) of the test solution concentration were prepared by spiking the tablet matrix with the known amount of impurities using their respective stock solutions. Un-spiked matrix solution was analyzed intermittently during the experiments as a blank.

2.6.    Preparation of impurities of amoxicillin:

Impurity D (amoxicillin penicilloic acid as a mixture of diastereomers), impurity F (3-(4-Hydroxyphenyl) pyrazine-2-ol), were prepared for use in validation experiments by methods reported in literature and their identity was confirmed by mass spectrum using LC/MS analysis (29, 30)

Impurity C (diketopiperazine derivative of amoxicillin) and impurity J (amoxicillin dimer) were prepared by following reported method for preparation of piperazinedione derivative of ampicillin (28):

1.0 g of amoxicillin trihydrate (2 capsules of 500 mg strength) was suspended in 20 ml of 10% w/v glucose solution and pH was repeatedly adjusted for 5 h to 9.3 using 2 M sodium hydroxide and then left overnight. Reaction completion was confirmed by TLC on silica gel glass plates by using ethylacetate: acetic acid: water (3:1:1, v/v/v) as mobile phase. The solution was cooled on ice bath and pH was lowered to 2.0 using 5 M HCl. The white precipitate was collected and washed with small amount of water and left to air dry. The dried powder was recrystallized using 80% ethanol. HPLC analysis with developed method showed two products which on LC/MS analysis proved to be impurity C and J with m/z of 366.5 and 731.3, respectively (HPLC purity 98% impurity C and 2% impurity J). A second batch of impurity was prepared by dissolving 2.0 g in 10 ml of 10% w/v glucose solution and following the same method. The percentage content of impurity

C and J in this batch was 66% and 34%, respectively. This batch was used for impurity J spiking.

## 3. Results and discussion

### 3.1. Method development

The method development was based upon the reported methods employing pH value 2-3 (26, 30) for resolution of maximum number of impurities and using isocratic settings (25). The low pH of 2.1 was selected to avoid deprotonation of carboxylic group (pKa = 2.4) of amoxicillin (31), as reported by Lucentini et al. (26). Detection wavelength of 210 nm was selected based on the signal strength of analytes.

pH variation within the range of 2.1-2.4 using a spiking solution of amoxicillin (1.5 mg/ml), clavulanic acid (0.5 mg/ml) with 0.03 mg/ml of impurity I, A, D and C, and 0.01 mg/ml of impurity F and J on the method settings of 0.02 M potassium dihydrogen phosphate buffer (pH 2.1)-methanol (89:11, v/v) and a flow rate of 1.6 mL/min showed that maximum resolution was achieved at pH 2.2 for the 8 known components as well as the 8 unknown peaks (peak 1 and 5 from clavulanic acid and peak 2-4, 6-8 from amoxicillin standard). The settings were improved to 12% organic content and 1.5 mL/min flow rate with Rs value of 1.3 for peak 2 and amoxicillin peak (Figure 1). A corresponding sample chromatogram using marketed coamoxiclav tablets (1.5 mg/mL of amoxicillin) is also shown in Figure 2. The elution profile of amoxicillin, clavulanic acid and the impurities of amoxicillin along with their correction factors on the developed method are shown in Table 1.

The new method is simple and cost effective with the run time approximately the same as the compendial method (15). Moreover, the method is conveniently transferrable to other standard columns (column B). Low buffer concentration and higher solubility of phosphate buffer in methanol reduces the chances of precipitation which is a problem observed with acetonitrile. Hence, the developed method is an improvement of the previously reported methods and provides a cheap and simple solution for the resource limited settings to perform the routine quality control tests for this essential medicine.
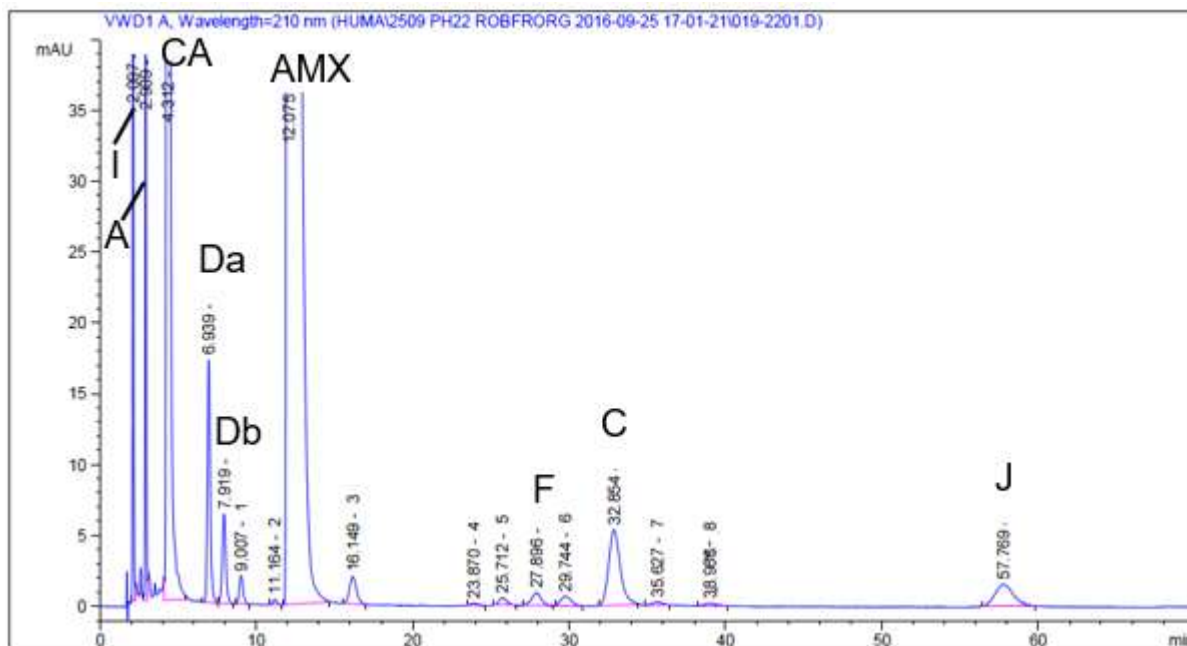
Figure 1.    Sample chromatogram for impurity profiling of amoxicillin with clavulanic
Figure 2.    acid.
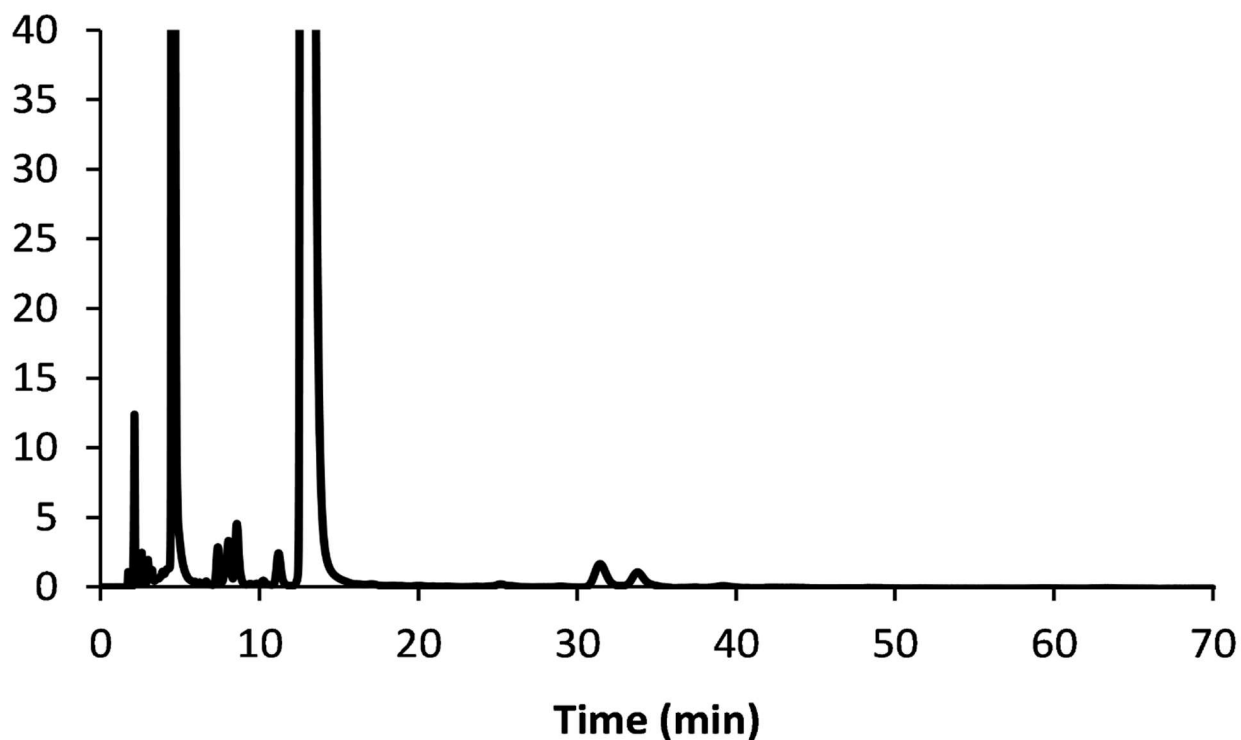


Figure 3.    Sample chromatogram for impurity profiling of coamoxiclav tablet

Table 1.     . Elution profile of amoxicillin, clavulanic acid and impurities of amoxicillin on developed method

| Analyte | RT (min) | rel.RT | Correction factor |
|---|---|---|---|
| Impurity I | 2.1 | 0.17 | Not needed |
| Impurity A | 2.9 | 0.23 | 1.9 |
| *Clavulanic acid* | 4.3 | 0.36 | - |
| Impurity Da | 6.9 | 0.57 | 1.9 |
| Impurity Db | 7.9 | 0.65 | 1.9 |
| *Amoxicillin* | 12.1 | 1.00 | - |
| Impurity F | 27.9 | 2.31 | |
| Impurity C | 32.9 | 2.72 | Not needed |
| Impurity J | 57.8 | 4.78 | |

## 3.2. Method Validation

Method for impurity profiling for coamoxiclav tablets was validated for impurity A, I, D and C using in accordance with the International Conference of Harmonization guidelines ICH Q2 (R1)(32).

### 3.2.1. Specificity:

The method specificity was shown by resolution of Rs ≥ 1.3 for the known and unknown analytes in the spiked sample with the exception of a minor unknown peak (RT = 5.0 min) from amoxicillin standard coeluting with clavulanic acid peak (4.3 min).

### 3.2.2. Limit of detection and limit of quantification (LOD and LOQ)

LOD and LOQ were determined by constructing the calibration curve in the range of limit quantification. The LOD of impurity A, I, D and C were in the range of 0.04 -0.3 µg/mL (0.003-0.02%) whereas the LOQ ranged from 0.1-0.8 µg/mL (0.01-0.05%) (Table 1). The developed method is more sensitive than the required threshold limits of 0.1% and so is applicable for analysis of coamoxiclav FPP.

### 3.2.2.1. Linearity and range:

The method proves to be linear for the concentration range covering LOQ to maximum level 0.03 mg/mL (2% of amoxicillin concentration in test solution). The regression coefficients for all the impurities were above 0.999 (Table 2).

Table 2.        : Limit of detection (LOD) and Limit of quantification (LOQ)

| | Limit of detection | | Limit of quantification | | |
|---|---|---|---|---|---|
| | mg/mL | % of TS | mg/mL | % of TS | S/N |
| Impurity I | 0.0001 | 0.01 | 0.0002 | 0.01 | 28 |
| Impurity A | 0.00004 | 0.003 | 0.0002 | 0.01 | 18 |
| Impurity D | 0.0003 | 0.02 | 0.0006 | 0.04 | 13 |
| Impurity C | 0.0002 | 0.01 | 0.0008 | 0.05 | 12 |

*TS= concentration of amoxicillin in test solution (1.5 mg/mL)*

Table 3.        Linearity data of impurities and amoxicillin.

| | Range (mg/mL, (% of TS)) | | Regression equation | $R^2$ |
|---|---|---|---|---|
| | Min. | Max. | | |
| Amoxicillin | 0.0008 (0.05%) | 0.03 (2%) | y= 12874x - 0.8754 | 1.0000 |
| Impurity I | 0.0002 (0.01%) | 0.03 (2%) | y= 11103x - 0.0314 | 0.9998 |
| Impurity A | 0.0002 (0.01%) | 0.03 (2%) | y= 6864x + 0.3422 | 0.9999 |
| Impurity D | 0.0006 (0.04%) | 0.03 (2%) | y= 6841x - 0.8082 | 0.9999 |
| Impurity C | 0.0008 (0.05%) | 0.03 (2%) | y= 15869x - 1.4151 | 0.9997 |

*3.2.2.2.      Accuracy and precision:*

Spiked samples at LOQ and 0.5%, 1%, 1.5% and 2.0 % level were injected in sextuples and the mean peak area was used for calculation of recovery. The percentage recovery for all the five levels was within 82.0-118.4%, except for once being 73.8% at LOQ level for impurity A. The inter day accuracy for each level was within 80.9-112.5%, with RSD of 1.2-12.1% showing good recovery and reproducibility (Table No. 4).

The intraday precision (RSD) for the retention time and peak area determined from three set of solutions was 0.0-1.0% and 0.2-11.7%, respectively for all the levels.

Table 4.        Intra- and Inter-day accuracy and precision of impurities

| Concentration | | Recovery (%) | | | Inter day accuracy and precision | | |
|---|---|---|---|---|---|---|---|
| %age of TS | amount added (mg/mL) | Day1 | Day 2 | Day 3 | Mean Recovery (%) | SD | RSD (%) |
| **Impurity I** | | | | | | | |
| LOQ 0.01% | 0.0002 | 95.2 | 118.4 | 106.0 | 106.5 | 9.5 | 8.9 |
| 0.50% | 0.008 | 103.9 | 103.8 | 99.4 | 102.3 | 2.1 | 2.0 |
| 1.00% | 0.015 | 104.1 | 105.4 | 100.8 | 103.4 | 1.9 | 1.9 |
| 1.50% | 0.023 | 105.5 | 106.2 | 102.2 | 104.6 | 1.8 | 1.7 |
| 2.00% | 0.030 | 107.2 | 108.1 | 102.9 | 106.1 | 2.3 | 2.1 |
| **Impurity A** | | | | | | | |
| LOQ 0.01% | 0.0002 | 86.9 | 82.0 | 73.8 | 80.9 | 5.4 | 6.7 |
| 0.48% | 0.007 | 97.5 | 98.7 | 100.9 | 99.0 | 1.4 | 1.4 |
| 0.96% | 0.014 | 101.2 | 100.7 | 103.7 | 101.9 | 1.3 | 1.3 |
| 1.45% | 0.022 | 97.6 | 98.6 | 102.9 | 99.7 | 2.3 | 2.3 |
| 1.93% | 0.029 | 96.5 | 96.7 | 99.1 | 97.4 | 1.2 | 1.2 |
| **Impurity D** | | | | | | | |
| LOQ 0.04% | 0.0006 | 118.4 | 87.8 | 103.1 | 103.1 | 12.5 | 12.1 |
| 0.5% | 0.008 | 97.4 | 84.9 | 93.8 | 92.0 | 5.3 | 5.7 |
| 1.0% | 0.015 | 103.7 | 98.4 | 90.7 | 97.6 | 5.4 | 5.5 |
| 1.5% | 0.023 | 101.6 | 86.1 | 89.3 | 92.3 | 6.7 | 7.2 |
| 2.0% | 0.030 | 97.3 | 81.6 | 88.3 | 89.1 | 6.4 | 7.2 |
| **Impurity C** | | | | | | | |
| LOQ 0.05% | 0.0007 | 117.5 | 114.9 | 105.2 | 112.5 | 5.3 | 4.7 |
| 0.47% | 0.007 | 105.2 | 103.1 | 98.2 | 102.2 | 2.9 | 2.9 |
| 0.92% | 0.014 | 102.2 | 104.3 | 96.9 | 101.1 | 3.1 | 3.1 |
| 1.40% | 0.021 | 99.4 | 103.6 | 95.3 | 99.4 | 3.4 | 3.4 |
| 1.92% | 0.028 | 99.6 | 103.6 | 93.5 | 98.9 | 4.2 | 4.2 |

### 3.2.2.3.        Stability:

The standard and sample solutions of impurities were analyzed after 24 h on storage at room temperature. The percentage change in peak area with reference to initial peak

area was calculated. The standard solutions were found more stable than the sample solution for impurity A, D, and C with the percentage change in peak area 0.02-0.5% whereas the values for sample solutions ranged from 0.3-2.0%. For impurity I the percentage change in peak area for standard and sample where 2.4% and 2.7%, respectively (Table 5).

Table 5.        Stability data for standard and sample solutions.

|  | % of TS | Conc. (mg/mL) | % change in peak area in 24 h |
|---|---|---|---|
| Impurity I *(std.)* | 0.7% | 0.011 | 2.4 |
| Impurity I *(sample)* | 1.0% | 0.015 | 2.7 |
| Impurity A *(std.)* | 0.7% | 0.011 | 0.1 |
| Impurity A *(sample)* | 1.0% | 0.015 | 2.0 |
| Impurity D (std.) | 1.0% | 0.014 | 0.5 |
| Impurity D (sample) | 1.0% | 0.015 | 4.7 |
| Impurity C (std.) | 1.2% | 0.019 | 0.02 |
| Impurity C (sample) | 0.9% | 0.014 | 0.32 |

## 4. Conclusion

The new method is simple and cost effective and can be used for impurity profiling of coamoxiclav tablets in resource limited settings. No additional standards, expensive solvents or sophisticated settings like temperature control of column or autosampler is needed. The method is sensitive enough to quantify impurities as low as 0.05% and hence can also be used for the test of related substances for amoxicillin API, as the current threshold for reporting limit for amoxicillin API used by BP and Ph.Eur. is 0.1%. Further investigation can be made for application of the method to other FPPs of amoxicillin as single component and as fixed dose combinations with clavulanic acid. Peak identification can be done with the help of available in-situ methods for preparation of impurities.

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## 3.3 Amoxicillin, coamoxiclav, ceftriaxone and cefotaxime-Synthesis, isolation and identification of impurities of beta-lactam antibiotics

**Abstract**

Pharmaceutical impurities (PI) of beta-lactam antibiotics mainly include starting materials, reaction intermediates, by-products, interaction and degradation products. Compendial methods of impurity profiling require certified impurity standards or *in situ* methods for preparation of peak identification solutions using forced degradation of the API. High cost of impurity standards is an impediment to routine impurity profiling by medicines quality control laboratories of resource limited settings. Simple and cost-effective methods pf preparation of impurities that do not require extensive purification and are not complex in their composition are hence needed.

Impurities of amino-penicillins and third generation cephalosporins are prepared in this study using forced degradation methods. Apart from the use of reported methods new and simpler methods were developed to replace tedious procedures for impurity preparation. Penicilloic acids of amoxicillin, 3-(4-hydroxyphenyl)-pyrazine-2-ol, 2-mercaptobenzothiazole, deacetylcefotaxime lactone were prepared using the reported methods whereas diketopiperazine derivative of amoxicillin, and amoxicillin dimer, were prepared by modification of the method for ampicillin impurities. New methods of preparation were established for deacetyl cefotaxime, deacetyl ceftriaxone and *E*-isomer of ceftriaxone.

Evaluation of the impurities in finished products of beta-lactam antibiotics carried out involving isolation and identification procedures. A group of unknown peaks was observed from grossly deteriorated samples of coamoxiclav and investigated for the possible source and identity.

The results of the study provide critical information for the analyst and researchers in resource limited settings to prepare the pharmaceutical impurities of beta-lactam antibiotics in simple and efficient manner as well as to understand the stability of the parent compounds in different conditions and solvents.

**Key words:** beta-lactam antibiotics, *in-situ* preparation of impurities, coamoxiclav, ceftriaxone, pharmaceutical impurities, impurity profiling.

## 1.     Introduction

Pharmaceutical impurities of beta-lactam antibiotics mainly include starting materials, reaction intermediates, by-products, interaction and degradation products (1, 2). *In situ* methods using forced degradation of the API provide peak identification solutions (3-6) for routine impurity profiling as well as peak assignment of actual and potential impurities during the drug development process (7). Incase impurities constitute the critical peak sets in analytical methods; the presence of impurities is crucial to carry out a valid test (8-10). In general, impurity standards are expensive and are not affordable by the laboratories in low- and middle-income countries for routine quality evaluation procedures.  Instead relative retention time is used for peak identification. Identification of the impurity of peaks require sophisticated techniques including liquid chromatography hyphenated with mass spectrometry (LC-MS, LC-MS/MS), and Nuclear Magnetic Resonance spectroscopy (NMR). In accordance with the International Council for Harmonization (ICH) and European Medicines Agency (EMA) guidelines for setting specifications for impurities in antibiotics the manufacturer must quantify, identify and qualify impurities exceeding the respective threshold limits (11-14). The direct use of forced degradation solutions for peak identification are of limited value in the presence of complex multicomponent peaks. Purification may become inevitable and may involve extensive and sophisticated techniques like column chromatography and preparative HPLC (pHPLC). Methods requiring extensive purification procedures are of less practical value for use in laboratories in developing countries.

Hence, the need of additional impurity standards in analytical testing is an impediment for establishing the test of related substances as part of the routine quality evaluation for pharmaceuticals in developing countries. The chapter aims at the exploration of the available *in-situ* methods for impurities of beta-lactam antibiotics and to develop more synthesis methods, to cover maximum number of known impurities of each essential beta-lactam under study.

The chemical structure of the beta-lactam antibiotics and their impurities are illustrated in chapter one.

**1.1. Objectives**

a.  Investigation of *in-situ* methods of preparation of impurities, using forced degradation and simple laboratory methods

b.  Isolation of impurities from finished products and their identification

c.  Stability of beta-lactams in different laboratory solvents and identification of their degradation products

**2. Experimental**

**2.1. Materials and equipment**

Agilent 1100/1200 series HPLC systems (Agilent Waldbronn, Germany) equipped with on-line degasser, injector with auto-sampler, a variable wavelength detector or the diode array detector were used. An external column oven by Beckman Coulter GmbH (Krefeld, Germany) was used when needed. Chemstation for LC 3D systems Rev. B.03.02 from Agilent Technologies (USA) was used for the data acquisition, analysis, and reporting.

Stationary phases including reverse phase C18 columns Spherisorb® ODS2, from Waters Corporation (Milford, MA, USA), was used for amoxicillin and its impurities. Zorbax Eclipse Plus C18 (150 X 4.6 mm, 5 µm) from Agilent technologies (USA), and Eurospher II C18A (150 X 4.6 mm, 5 µm) were used for impurities of ceftriaxone, and Eurosphere C18H (250 X 4.6 mm, 5 µm) by Knauer GmbH (Berlin, Germany) for co-amoxiclav tablets

Other instruments used for NMR, pHPLC and LCMS included BRUKER Avance III 400 MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany); Agilent 1100 preparative HPLC instrument (Waldbronn, Germany) carrying fraction collector and multiple wavelength detector fitted with C18 column Nucleodur Sphinx, 125 mm X 10 mm, 5 µm particle size from Macherey-Nagel (Gutenberg, France); and Shimadzu LCMS-2020 instrument (Hilden, Germany), with ZORBAX SB-CN, 4.6 x 50 mm, 3.5 µm column from Agilant Technologies (Santa Clara CA, USA), respectively. Other materials included silica gel 60 from Merck for column chromatography. ESI mass spectral data were also acquired on a Shimadzu LCMS-2020 instrument (Hilden, Germany) for samples of ceftriaxone impurities.

## 2.2. Chemicals and materials

Amoxicillin trihydrate, its impurity A (6-aminopencillanic acid) and impurity I (4-hydroxyphenylglycine) were bought from Sigma Aldrich (Laramie, USA). Cefotaxime powder for injection was procured from *Fresenius Kabi Deutschland GmbH* (Hamburg, Germany). Certified reference standard for ceftriaxone was procured from *Sigma Aldrich* (Laramie, USA). Certified reference standard of impurity A of cefotaxime whereas was purchased from the *European Directorate for the Quality of Medicines and Healthcare* (EDQM, Strasbourg, France). ). Impurity D (ceftriaxone benzothiazolyl oxime) and 7-aminocephalosporanic acid (7-ACA) were purchased from *TCI Europe* (Zwijndrecht, Belgium), impurity C (ceftriaxone triazine analog; thiotriazinone) and impurity E (deacylceftriaxone) from *Abcr GmbH* (Karlsruhe, Germany), and *Carbosynth UK* (Berkshire, United Kingdom), respectively

Analytical grades of were purchased from Merck KGaA (Darmstadt, Germany) whereas sodium dihydrogen phosphate monohydrate, orthophosphoric acid were procured from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). HPLC grade acetonitrile and were purchased from VWR (Fontenay-sous-Bois, France) and HPLC grade methanol potassium dihydrogen phosphate, dihydrogen potassium phosphate were procured from Merck KGaA (Darmstadt, Germany). Purified water for preparation of buffer and sample solutions was produced using Milli-Q® laboratory water system from Merck Millipore (Darmstadt, Germany). Other miscellaneous chemicals include sodium bi carbonate, potassium hydroxide, sodium hydroxide and acetic acid used were also of analytical grade.

## 2.3. General methods

The LC-MS method included flow rate of 0.4 mL/min; with ZORBAX SB-CN (4.6 x 50 mm, 3.5 µm). Gradient elution was performed with water and acetonitrile each containing 0.1% formic acid. After 5 min of an isocratic run with 5% acetonitrile, the concentration of acetonitrile was increased to 90% over 5 min and maintained for further 5 min before re-equilibrating to initial 5%. Mass analysis employed electrospray ionization at 350 °C within a variable mass-to-charge (m/z) range of 100-1500 Da.

[1]H spectra scans were measured on a BRUKER Avance III 400 MHz spectrometer using suitable solvent deuterium dioxide-d2 or methanol-d4 or dimethyl sulfoxide-d6 for obtaining.

Preparative HPLC for isolation of impurities of amoxicillin was carried out for purification of impurity F, L and G. Isolation of impurities from finished products of amoxicillin and coamoxiclav using gradient elution of 4-85% v/v of methanol-water over 40 min after an initial isocratic phase of 10 min at 4% v/v methanol-water using 9the flow rate of 3 ml/min.

HPLC methods used for identification of impurities included compendial methods for impurity profiling of amoxicillin, coamoxiclav and ceftriaxone according to Ph.Eur 9.8 (15) and BP 2013 (16) or the developed methods (chapter 2-3).

## 2.4.    Methods for preparation of impurities

### 2.4.1.  Penicilloic acids of amoxicillin (Impurity D of amoxicillin)

### ImpSyn.01. according to Munro et al. (17)

Hydrolysis of a 10 % w/v solution (250 mg in 2.5 ml of water) of amoxicillin trihydrate was carried out using 10 M NaOH at pH 12 for 1.5 to 3 h. Reaction completion was checked by carrying out TLC on silica gel using mobile phase of 1-butanol-ethanol-water (2:1:1) and detecting by exposure to iodine vapours. An Rf value of 0.25 was obtained for amoxicilloate. The solution was diluted with equal volume of water and the pH was adjusted to 7.0 using 5 M HCl.  The penicilloic acids were crystallized by slow addition of 5 ml of 2-propanol with rapid stirring producing a white precipitate on storage at 4 °C. The collected crystals were washed using cold 2-propanol-water (2:1) and dried *in vacuo* over phosphorus pentoxide yielding  65 mg (26%) of 98% ( (HPLC purity) pure amoxicillin penicilloic acid  (17). The mass of *m/z* of 383.5 and ^{1}H NMR spectrum of the prepared impurity were in accordance with those reported in the literature (17).

### ImpSyn.02. according to De Pourcq et al. (18)

Penicilloic acids of amoxicillin were prepared by dissolving 100.0 mg of amoxicillin trihydrate in 0.1 N potassium hydroxide and allowing it to stand for 10 h (18, 19). The solution was neutralized to pH 7.0 using 0.2 M potassium dihydrogen phosphate buffer

and diluting with 50.0 ml of water. A mixture of diastereomers of amoxicillin in the ratio of 45:55 was obtained which changed over time (18).

## 2.4.2. Amoxicillin piperazine-dione and amoxicillin dimer (Impurity C and Impurity J of amoxicillin)

### ImpSyn.03. modification of method from Bundgaard & Larson (20)

1.0 g of amoxicillin trihydrate (2 capsules of 500 mg strength) was suspended in 20 ml of 10% w/v glucose solution and pH was adjusted to 9.3 using 2 M sodium hydroxide solution which was maintained for 7 h and then left to stand overnight. Reaction completion was confirmed by TLC (silica gel; ethylacetate-acetic acid-water (3:1:1, v/v/v)). The spots for amoxicillin and impurity C appeared at the Rf values of 0.47 and 0.89, respectively. The solution was cooled on an ice bath and pH was lowered to 3.0 using 5 M HCl. Pale yellow precipitates were collected and washed with small amount of water and left to air dry. The dried powder was recrystallized using 80% ethanol, yielding 65 mg (6.5%) of off-white powder. HPLC analysis of the powder using developed method showed two products, which on LC/MS analysis proved to be impurity C and J with m/z of 366.5 and 731.3, respectively (HPLC purity 98% impurity C and 2% impurity J)

### ImpSyn.04.: modification of method from Bundgaard & Larson (20)

Impurity J of amoxicillin was prepared by dissolving 2.0 g in 20 ml of 10% w/v glucose solution and following method ImpSyn.03 for impurity C of amoxicillin. White precipitates were obtained with the yield of 90 mg (45%). The percentage purity of impurity C and J in this batch was 66% and 34%, respectively. Two products were observed on LC/MS analysis with m/z of 366.5 and 731.3, corresponding to impurity C and J, respectively (Figure 1).

### ImpSyn.05. according to Ph.Eur. 9.8(3)

200.0 mg of amoxicillin trihydrate in 1 ml of water (20% w/v) treated with 2 M sodium hydroxide to raise the pH to 8.4, was maintained for 4h and 20 h at room temperature, in two batches. Each portion was diluted to100 folds with 0.02 M phosphate buffer, pH 5. The results showed a multi component mixture with impurity C and impurity J in the proportion of 15% and 1% after 4 h (Figure 2a) and 27% and 3% after 20 h (Figure 2b), respectively. An additional peak (2%) eluted after impurity J which might be of amoxicillin trimer was also seen in the solution kept for 20 h. The method is stated by

Ph.Eur. for preparation of solution for peak identification for impurity C and impurity J (21).

### 2.4.3. 3-(4-Hydroxyphenyl) pyrazine-2-ol (impurity F of amoxicillin)
### ImpSyn.06.  according to Phangal and Singh (22)

Amoxicillin trihydrate (1 g) were suspended in 50 ml of water and acidified to pH 3.1 using 5 M HCl. The solution was heated at 75 °C for 6 h and then cooled at room temperature. The resulting mixture was extracted 3 times with chloroform (12.5 ml). The combined organic layers were washed over $Na_2SO_4$ and concentrated under reduced pressure to give a crude product that was fractionated by silica gel chromatography using ethylacetate-hexane-methanol (10:2:0.5 v/v/v)  (22). A clear band of yellow substance was observed which was separated, evaporated to dryness yielding 15 mg (2%) of impurity F of amoxicillin. The product was found to be 97% pure (HPLC purity) and was further confirmed by producing an m/z of 188.9 on LCMS analysis. The ^{1}H NMR spectra was found in agreement with the previously reported in literature (22).

### ImpSyn.07.  : modification of ImoSyn.06 followed by pHPLC

Solution of amoxicillin (200 mg) in water 1 mL was acidified with 1-2 drops of 5 M HCl and heated at 60 °C for 1 h. A three-component mixture was observed on HPLC analysis with XFA7 (33.1%) at 10.6 min, XFA8 (26.9%) at 15.9 min and XF9 (23.2%) at 19.8 min. Pure fractions obtained using pHPLC showed the m/z of 557.1, 563.1 and 188.9 m/z on LCMS analysis, which could be assigned to impurity G, impurity L and impurity F of amoxicillin, respectively.

### 2.4.4. Deacetyl cefotaxime lactone (Impurity B of ceftriaxone/Impurity E of cefotaxime)(23)
### ImpSyn.08. according to Ph.Eur. 9.8 followed by purification:

Deacetyl cefotaxime lactone was prepared using the acidic hydrolysis by addition of 50 ml of dilute HCl (1:5 v/v dilution) to solution of cefotaxime (200 mg) in 200 ml of phosphate buffer, pH 6.25 with 14% methanol at 40°C for 2 h. The solution obtained was diluted with 250 ml of phosphate buffer pH 6.6 along with 50 ml of 8.5% v/v NaOH. This solution was lyophilized and redissolved in 25 mL of phosphate buffer, pH 6.25 for purification by extraction with ethyl acetate (25 mL x3). The ethyl acetate extract was washed over anhydrous sodium sulphate and evaporated to dryness yielding

deacetyl cefotaxime lactone (yield of 83%, HPLC purity 96%). LC/MS analysis was carried out for confirmation of deacetyl cefotaxime lactone (m/z= 395.9).

**ImpSyn.09. Modification of ImpSyn.08 by ceftriaxone instead of cefotaxime as the reactant**

The reaction stated in method ImpSyn.08 was repeated using ceftriaxone as a reactant in place of cefotaxime and HPLC analysis of the diluted reaction mixture showed the formation of deacetyl cefotaxime lactone.

### 2.4.5. Deacetylcefotaxime

**ImpSyn.010. newly identified method**

The deacetylcefotaxime solution was prepared by exposure of a solution of ceftriaxone (100mg) dissolved in 0.02 M phosphate buffer pH 5.0 (100ml), to $\Lambda$=254 nm for 48 h. The solution was analysed using the developed HPLC method showing mainly a three-component mixture including ceftriaxone, the thiotriazinone (impurity C) and a third product which was later identified to be deacetylcefotaxime.

### 2.4.6. E-isomer of ceftriaxone (impurity A)

**ImpSyn.011. newly identified method**

The standard solution of ceftriaxone (10 mg) in 10 ml of 0.02 M phosphate buffer, pH 7.0 was exposed to daylight for 24 h and the obtained showed appearance of a new peak, the identity of which was confirmed by spiking with impurity A standard.

### 2.4.7. Deacyl ceftriaxone (impurity E of ceftriaxone)-

**ImpSyn.012. New method modified from synthesis method of ceftriaxone(24)**

Thiotriazinone (160 mg) and 7-aminocephalosporanic acid (272 mg) were dissolved in water (10 ml) by adjusting the pH to 6.6 using 2 M NaOH solution. The reaction mixture was stirred in oxygen free atmosphere for 18 h at 55-60 °C. The mixture was cooled to room temperature and the pH was lowered to 3.4 using dilute 5 M HCl. Reddish brown to buff color powder was collected on filtration and dried under vacuum as 93% pure (HPLC purity) substance (Figure 3). LCMS analysis showed a significant molecular ion peak at m/z 371.80 (Figure 4).

### 2.4.8. 2-mercaptobenzothiazole (25)

**ImpSyn.013. previously reported by Sharif et al. (25)**

A 1 mg/ml solution was prepared by dissolving 10 mg of impurity D of ceftriaxone in 10 ml of methanol and heating at 30 °C for 1 h. The solution was diluted with 50% v/v acetonitrile in water and was found to be composed of the parent compound, *Z*- and

*E*- isomers of 2-mercaptobenzothiazole and (2*Z*)-methyl 2-(2-amino-1,3-thia-zol-4-yl)-2-(methoxy-imino)ethanoate and their identity was confirmed using LCMS analysis.

## 2.5. Isolation of impurities from finished products and their identification

### 2.5.1. Amoxicillin

7 different batches (1P1, 1P2, 1P4, 2P5, 2P6 and 2P7) of expired and non-expired amoxicillin capsules from Pakistan were analysed for the test of related substances according to the United States Pharmacopoeia (USP) 36. 1P4 contained the highest amount of individual impurities and was extracted with 50% methanol in water for isolation of impurities by pHPLC. Peaks appearing between 17-40 min were collected for identification by using HPLC and LCMS.

### 2.5.2. Coamoxiclav tablets

The impurity profiles were carried out using BP 2013 monograph for the samples of coamoxiclav tablets from Pakistan (Chapter 4). Three samples (CAM 1P2, 1P3 and 2P5) had shown unusual brown appearance of tablet contents and very low assay result for potassium clavulanate content. The comparison of the chromatograms of the samples was carried out on the basis of HPLC analysis.

## 2.6. Effect of solvents under stress conditions

### 2.6.1. Impurities of cefotaxime and ceftriaxone

Solutions of ceftriaxone (1 mg/ml) and cefotaxime (1 mg/ml) were dissolved in water and 50% v/v methanol, separately and a portion of each solution was subjected to 70° C for 1 h. Solutions obtained were adjusted to initial volume and analyzed on the developed method for ceftriaxone.

Separate solutions of ceftriaxone (1 mg/ml) were made in different solvents including water, 0.02 M phosphate buffer 5.0, 0.02 M phosphate buffer 7.0 and 0.02 M ammonium acetate buffer, pH 5.0 and subjected to UV exposure $\lambda$= 254 nm and daylight for 24 h. Pre and post treatment chromatograms were obtained on the developed method. The chromatograms were compared for the appearance and concentration of peaks in each solution.

Impurity D of ceftriaxone was dissolved in pure methanol and acetonitrile, separately, to produce 1 mg/mL solutions. These solutions were then diluted with 50% v/v dilution

of their respective solvents in water, respectively was subjected to HPLC and LCMS analysis.

## 3. Results and Discussion

*Penicilloic acids of amoxicillin:* Methods for producing penicilloic acids of amoxicillin (AMX-Imp D) involve alkaline degradation (17, 19, 26) and are obtained as a product of crystallization (17), or as lyophilized products (26) or as solution for spiking and peak identification during HPLC method development (19). Crystalized product was obtained using Imp Syn.01 and a solution with racemic mixture of amoxicilloate diastereomers (Da and Db) was obtained using Imp.Syn.02 methods, respectively. The ratio of the two diastereomers changed on storage in aqueous medium and hence the cumulative area was used to calculate percentage purity.

*Diketopiperazine derivative (AMX-Imp C) and amoxicillin dimer (AMX-Imp J):* These impurities were produced in this study using alkaline hydrolysis of amoxicillin dissolved in glucose solution using a reported method for preparation of diketopiperazine derivative of ampicillin (20). A two-component mixture containing AMX-Imp C and AMX-Imp J was obtained with their ratios dependent on the concentration of amoxicillin used in the parent solution. A concentrated solution with 20% w/v amoxicillin produced 66% AMX-Imp C and 33% AMX-Imp J whereas 10% w/v of amoxicillin produced AMX Imp C (93%) as major component, containing only 7% of AMX Imp J (Figure 1a). Two prominent ionic species with m/z of 366.5 and 731.3 were confirmed in LCMS of both solutions. (Figure 1b)

The European Pharmacopoeia (Ph.Eur.) describes a *in situ* preparation of peak identification solution by alkaline hydrolysis of amoxicillin solution in water for Imp C and Imp J of amoxicillin (3). However, this method yields a multi-component solution making it relatively difficult to clearly identify the two impurity peaks using the given relative retention times. The compendial method for in situ preparation of impurity C and J was performed given the reaction time of 4h and 20h showing the two impurities along with other unknown peaks (Figure 2a-b).

Roet et al. (27) and later Zhu et al. have used an extensive time consuming method employing purification with size exclusion chromatography and ion exchange chromatography for preparation of these impurities for amino penicillins (28) using the reaction mixture described by Ph.Eur (3). The method used in the current study is

more simple, reproducible and cost-effective and is used for the first time for preparation of amoxicillin dimer and hence is offered as a better method to the one in use by Ph.Eur.
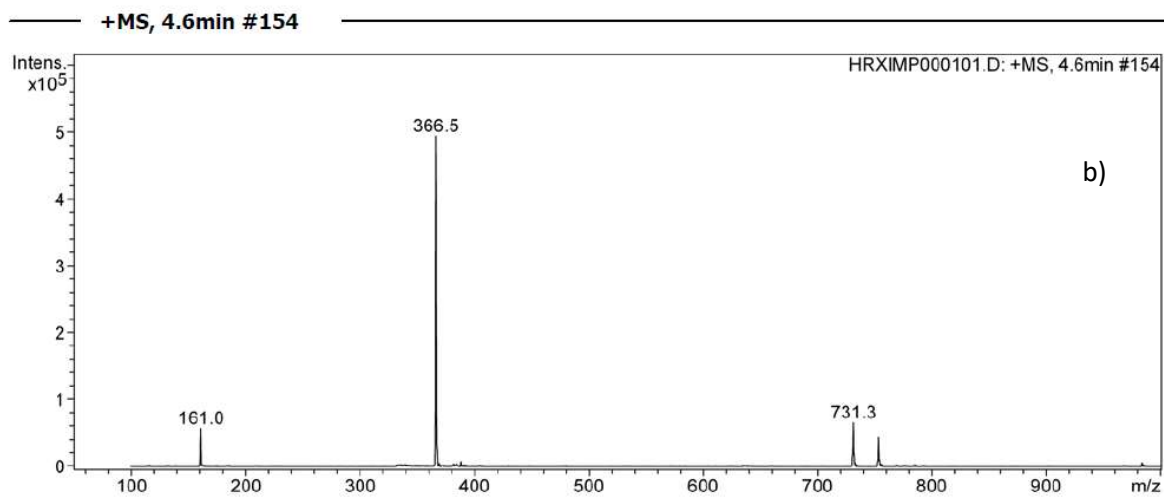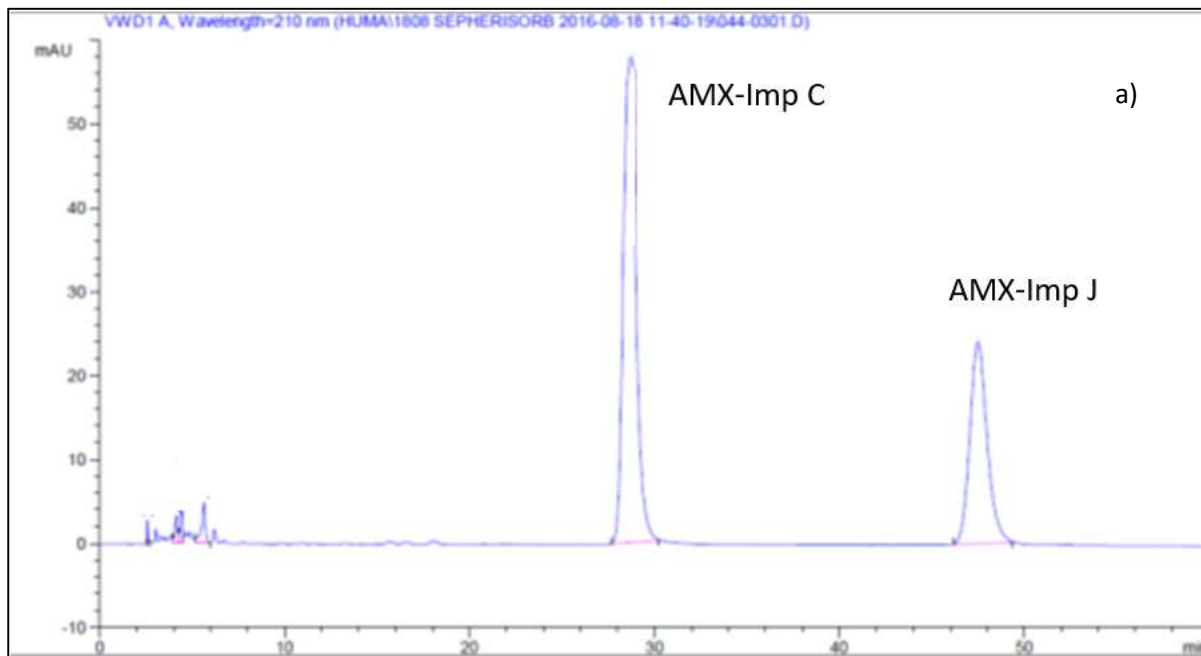


Figure 1. Impurity C and Impurity J of amoxicillin prepared by the new method analyzed on a) HPLC and b) LCMS m/z of 366.5 and 731.3
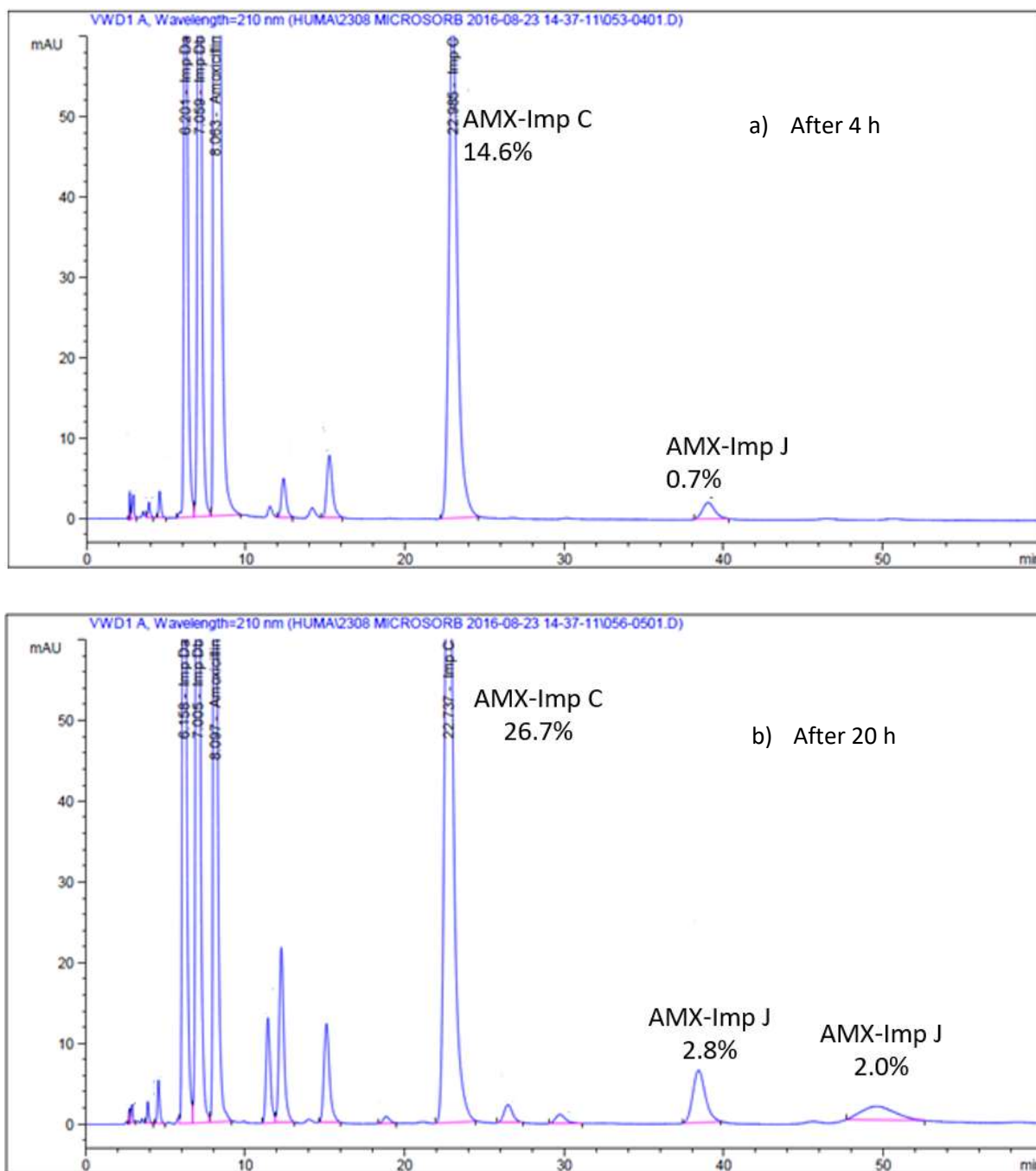
Figure 2 Peak identification solution prepared by using method from European Pharmacopoeia after a) 4 h and b) 20 h of alkaline hydrolysis analyzed using C18 column Microsorb Varian MV (250 mm x 4.6 mm, 5 μm particle size) at 0.02 M potassium dihydrogen phosphate buffer, pH 2.1-methanol (83:17% v/v) and 1 mL/min flow rate.

*Impurity F of amoxicillin (3-(4-hydroxyphenyl)pyrazine-2-ol:* It was prepared by the method reported in literature (22) but a very low yield was obtained of the pure compound. The reaction mixture showed a multi-component mixture by HPLC analysis

with a very small proportion of impurity F. A simpler method of producing impurity F in simple laboratory settings was devised using forced degradation of amoxicillin. 1 ml of aqueous 20% w/v amoxicillin suspension in water was heated at 60 °C for 1 h after acidification with few drops of 5 M HCl, separately. The latter reaction showed three component mixture on HPLC analysis which were found to be impurity F, impurity G and impurity L by LCMS in the ratio of 30-35% each hence providing a simplified method for preparation of peak identification solution for three impurities. The solution could be used without further purification by simple neutralization or dilution with 0.02 M phosphate buffer 5.0.

Impurities of ampicillin and amoxicillin are very similar in chemical structure and can be prepared by the same scheme of experiments (17, 20). Dimer of ampicillin by using *in situ* impurity preparation method  from Ph.Eur. (29) also yield a multi-component mixture from which accurate identification of the dimer peak is difficult.

Diketopiperazines are included in compendial monographs as major specified impurities of sodium salts of amino penicillins (21) along with the dimeric impurities, which produce serious allergic reactions (30).

*Impurities of ceftriaxone and cefotaxime*

*Deacetyl cefotaxime:*        A new method of preparation for deacetyl cefotaxime was introduced in this study using exposure of ceftriaxone solution to ultraviolet radiations. Deacetyl cefotaxime is a known impurity of cefotaxime and is reported to be produced by forced degradation of cefotaxime using sodium carbonate (6), and also by heating at 70° C in water (31). However, the current study is the first to investigate the degradation of ceftriaxone for formation of deacetyl cefotaxime. The only earlier report for production of deacetyl cefotaxime from ceftriaxone is on using acetic acid buffer, pH 4 as a solvent (32).

The results mentioned in chapter 2 of this document provide the first report of identification of deacetyl cefotaxime in the fresh solution of ceftriaxone CRS. Using the 0.02 M phosphate buffer 5.0 as solvent increases the concentration of deacetyl cefotaxime over 24 h and significant increase is seen on exposure to UV $\lambda$= 254 nm for 48 h. This effect was not observed when ammonium acetate, pH 5.0 was used as a solvent. Tian et al. (32) has shown that reversible isomer of ceftriaxone was produced in acetate and formate buffers with deacetyl cefotaxime as a one of the

product. They observed that these products were not formed on using phosphate buffers and solutions containing chloride ions. Their study does not discuss deacetyl cefotaxime concentration and has only shown the structure with the m/z data.

*.E-isomer of ceftriaxone (CTX-Imp A):* Solutions of ceftriaxone at pH 7.0 exposed to daylight showed appearance of a new peak which was identified as *E*-isomer of ceftriaxone (CTX-Imp A) through spiking. The current study is the first report of production of this impurity. CTX-Imp A is an inactive diastereomer of ceftriaxone and is produced in a concentration of 2% w/v in the current experiments by storage of solution in daylight. Further investigation need to be carried out to observe similar degradation pattern in cefotaxime and other closely related molecules.

The production of *E*-isomer might be the possible explanation of the changes in USP 40 monographs for ceftriaxone instructing the product solutions to be protected from light. However, the instruction for the same are not available in clinical settings. The effect of light and temperature collectively on the different intravenous admixtures of ceftriaxone is important for study with respect to low- and middle- income countries where temperature control in hospitals is not available throughout the country. *E-isomer* is an inactive isomer of ceftriaxone and hence may be a reason for the failed efficacy of the medicine in poor- resourced settings.

*Deacetyl ceftriaxone (Impurity E of Ceftriaxone): It* was prepared by the method scheme describe in a patent related to synthesis of thiazolyl compounds. The reactants were changed to 7-aminocephalosporanic acid and thiotriazinone and the reaction condition included pH 6.6 at 50-60°C for 12 hrs. The product obtained showed the HPLC profile (96.4% purity) (Figure 3) and mass spectrum (m/z = 371.3) as reported by the commercial vendor of impurity of ceftriaxone (Figure 4)

*2-Mercaptobenzothiazole:* Impurity D of ceftriaxone remained stable in acetonitrile solution but degraded to two separate entities on dissolving in methanol shown by the LCMS analysis of both solutions. This instability with methanol is also reported earlier in literature (25) where the solution of impurity D of ceftriaxone is dissolved in methanol and heated at 30 °C for 1 h to produce the degradation products. One of the degradation products 2-mercaptobenzathiazole is a reported carcinogen and is also a synthesis impurity of ceftriaxone in methods involving impurity D as reactant. This simple *in situ* preparation of 2-mercaptobenzothiazole can be used for spiking and

identification of impurity peak in the regular chromatographic analysis. The parent solution can be left to crystallize to yield yellow crystals of (2Z)-methyl 2-(2-amino-1,3-thia-zol-4-yl)-2-(methoxy-imino)ethanoate (25) leaving 2-mercaptobenzathiazole in the mother liquor.
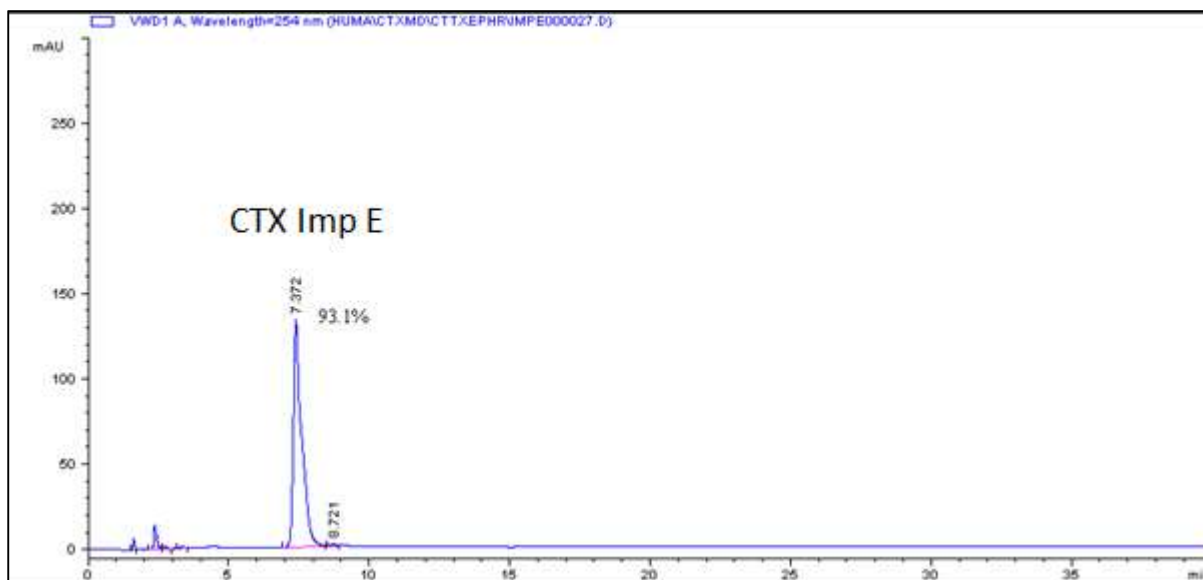


Figure 3: Ceftriaxone impurity E (CTX Imp E) analysed using compendial method for ceftriaxone analysis
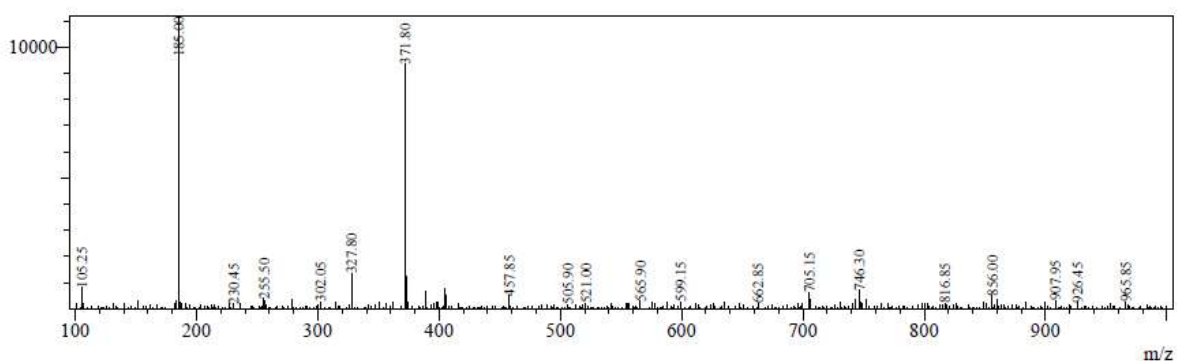


Figure 4: LCMS analysis of the impurity E of ceftriaxone

*Isolation of impurities from finished dosage form of amoxicillin capsules:* Three peak groups were seen for fraction 91, 109 and 118 at 17-18, 21-22, and 23-24 min on analytical column using a methanol water gradient of 4 to 85% methanol over 40 min. Comparison of the LCMS chromatograms of these fractions showed 731.5 m/z (AMX

Imp J), 581.5 m/z (AMX-Imp M), 366.5 m/z (AMX-Imp C) along with entities of 320, 280, 318 and 613.4 m/z (Table 6).

Table 6. LC-MS results of pHPLC fractions from amoxicillin capsule (AMX1P4).

| Fraction No. | Peak group & RT (min)* | | | Mass (m/z) observed against elution time (min) in LC-MS analysis | | | | |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | 8 | 10.6 | 12 | 12.6 | 13 |
| | **17-18** | **21-22** | **23-24** | | | | | |
| 91 | | | present | 366.3 549.0 | | 336.6 | **318 613.4** | 425.6 368.9 **581.5** |
| 109 | Present | Present | present | 366.4 | **731.5** | 320 280 | **318 613.4** | |
| 118 | Present | Present | | 366.5 | **731.5** | 320 280 | | |
| Possible impurities | | | | **C** | **J** | | | **M** |

*Unknown peaks in the impurity profile of coamoxiclav tablets*

Unusual solubility and impurity profile was observed for one of the coamoxiclav tablets sample (CAM1P3) showing the presence of an additional impurity at RRT 6.0 (4.4 %), along with higher concentration of impurity at RRT 3.6 (2.6%) and an additional peak group between RRT 3.6 and 3.8 (Figure 5). However, the concentration of specified impurity (amoxicillin dimer) in CAM1P3 was lower than other samples.
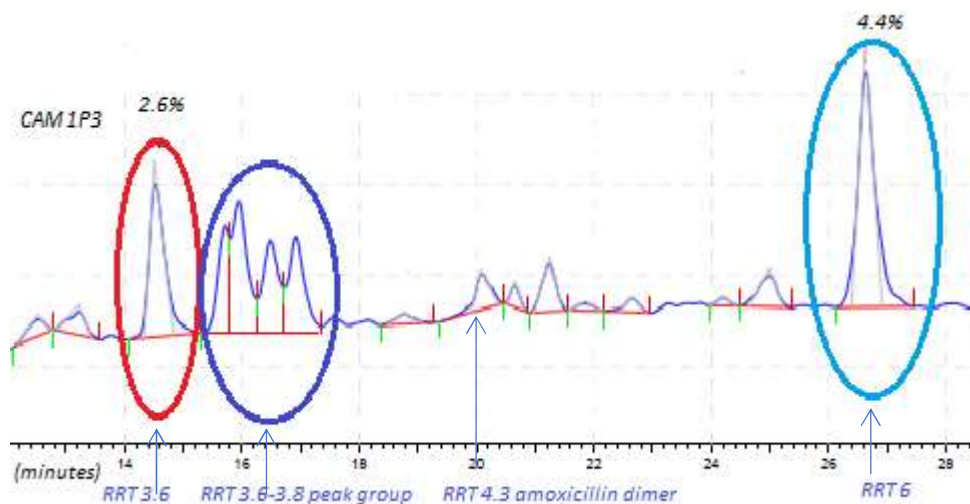


Figure 5. Impurity profile of CAM 1P3 showing unusual peaks

The compendial method for coamoxiclav tablets by BP 2013, employs the method for impurity profiling of amoxicillin states by Ph. Eur 9.0 and do not provide any information on the clavulanic acid impurities. Resolution and detection of clavulanic acid impurities need HPLC-UV and gas chromatography (GC) methods as lot of low molecular

impurities are reported. To investigate the origin of these peaks separate samples of clavulanic acid and amoxicillin and their mixture was analyzed as fresh and after 24 h solutions, still the results did not provide any conclusive information on the unknown peaks.

## 4. Conclusion

Forced degradation and investigation of the choice of solvents in different stress condition is an effective tool for identification of methods for the preparation of impurities of beta-lactam antibiotics. The information gained in the current study was important for making decisions in the method development process, in particular the selection of solvent and pH selection and stability of samples. UV exposure and elevated temperatures are the conditions promoting the impurity production, and the pH of solvent is of crucial importance in this process.

Impurity analysis of finished products with an unusual assay should be investigated for the impurity profiling in detail. Molecules with closely related structures like ceftriaxone and cefotaxime as well as amoxicillin and ampicillin shared the same methods for impurities preparation. Hence in case unusual impurities are produced in one molecule, the investigation must be replicated for the other similar molecules exclude the possibility of the preparation of the impurities. The information accessed in these experiments are not only useful for the analytical testing and method development but are also crucial for the safe and efficacious use of the antimicrobials in the clinical settings.

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# 3.4 Assay and impurity profiling of finished pharmaceutical products of essential beta-lactam antibiotics from Pakistan and other sources using compendial methods.

**Abstract**

The global commitment of access to essential medicines, demands not only the assurance of availability and affordability but also of the quality of the medicines delivered to the public. Use of substandard anti-infective agents is associated with major implications on health care system, notably the emergence of resistance strains. However, there is a serious dearth of data for Pakistan on the subject, and the current study is a trial project to assess the quality of essential beta-lactam antibiotics sampled from the various points in supply chain from different cities of Pakistan. Few samples were also included from Democratic Republic of Congo (DRC) and other countries. Analysis was carried out using the assay and impurity profiling methods from the compendial monographs.

57.1% (12/21) of the total beta-lactam samples from Pakistan and DRC (44.4% (4/9) amoxicillin (500 mg) capsules/tablets, 50.0% (3/6) coamoxiclav (500/125 mg) tablets, 83.3% (5/6) ceftriaxone injection (1 g) analysed in the study were found substandard with low API content. 85.7% (18/21) of these samples were from Pakistan. Ceftriaxone injections from Pakistan and DRC showed API content within the range of (78.6-90.7%) which is lower than the acceptable limits by British Pharmacopoeia. Amoxicillin and ceftriaxone FPP samples complied with the impurity tests.  All the samples were analysed within the shelf life period, with the exception of coamoxiclav tablets (shelf life: -10 to -18 months). Three samples of coamoxiclav from Pakistan from local manufacturers had unusual appearance of tablet content and showed high degradation of clavulanic acid with individual impurities exceeding the threshold of 1%. A systematic increase in the impurity content was observed after shelf life in coamoxiclav samples which was not observed in the single component amoxicillin tablets. Packaging played significant role in product stability of coamoxiclav tablets. Based on the assay of amoxicillin and ceftriaxone samples, the failure rate calculated

for the total number of samples, samples from Pakistan and from DRC was 60.0% (9/15), 50.0% (6/12), and 100.0% (3/3), respectively. Hence, essential beta-lactam antibiotics are prospective candidate for post marketing surveillance studies to gain prevalence data on quality of medicines in Pakistan and develop effective strategies to combat this problem.

**Key words:**

Beta-lactam antibiotics, quality control, amoxicillin, coamoxiclav, ceftriaxone, substandard/falsified medicines, poor-quality, oral solid dosage forms, Pakistan, DRC.

## 1.      Introduction

Poor-quality of medicines is a challenge particularly faced by the low- and middle-income countries (1, 2), causing cost and long-term implications on health care system like antimicrobial resistance on using poor quality antimicrobials (3). Post marketing surveillance is an essential part of the regulatory control that helps identify and rectify quality issues in pharmaceutical supply chain (4). This information is often not available for developing countries as most of the data lies with pharmaceutical industries or regulatory agencies and is not shared publicly. Conflict of interests exists in disclosing quality failures by both the bodies (5). Studies on quality of anti-malarial agents has helped in identifying the magnitude and the complexity of the problem in various parts of African continent, and provided information on designing effective strategies to combat the menace of substandard and falsified medicine (6). However, these studies need to be expanded to the other classes of essential medicines and in the other low- and middle-countries, to identify the depth of issue and built effective strategies. Limited data is available for Pakistan in this regard, mainly consisting of the case reports of high number of fatalities associated with poor quality of medicines (7-9).

Beta-lactam antibiotic are one of the oldest and most widely used antibiotics constituting more than 50% of the antibiotics listed in the essential medicines list by World Health Organization (10).  These agents are widely used in the clinical care for treatment against minor infections to critical life-threatening conditions. Antibiotics are among the most reported class of pharmaceuticals with quality issues and oral solid dosage forms of beta-lactam antibiotics are among the most counterfeited dosage forms reported for antimicrobials (2, 3, 11). Poor regulatory controls in developing countries result in high number of substandard medicine being present in the pharmaceutical supply chain, majority of it being the popularly used generic medicine (11).

The purpose of this study is to analyse finished products samples collected from Pakistan and other similar sources for assay and test for related substances in accordance with the methods described in British Pharmacopoeia 2013 (BP 2013)(12), European Pharmacopoeia, Ed. 8.0 (Ph.Eur. 8.0)(13) and United States

Pharmacopoeia Ed. 38 (USP 38)(14). Few key objectives were outlined for this study, including:

a.      Evaluation of quality of collected samples of finished pharmaceutical products using compendial methods for assay and test for related substances.

b.      Investigation for presence of unknown impurities in the samples and identification of key impurities found in the expired products.

## 2.      Experimental

2.1.    Standards:

Amoxicillin trihydrate certified reference standard (CRS), potassium clavulanate CRS and ceftriaxone CRS and 6-aminopenicillanic acid (6-APA) were procured from Sigma Aldrich (Laramie, USA).

2.2.    Finished product samples: (Appendix III, Table A1-3)

2.3.     Other materials:

Analytical grades of sodium dihydrogen phosphate monohydrate, tetradecylammonium bromide and tetraheptylammonium bromide, sodium di-hydrogen phosphate dodecahydrate, citric acid, orthophosphoric acid were procured from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). HPLC grade acetonitrile and methanol were purchased from VWR (Fontenay-sous-Bois, France) and Merck KGaA (Darmstadt, Germany), respectively. Purified water for preparation of buffer and sample solutions was produced using Milli-Q® laboratory water system from Merck Millipore (Darmstadt, Germany).

2.4.    Instruments and related apparatus:

2.4.1. HPLC system:

Two HPLC systems 1100/1200 series from Agilent Technologies (Waldbronn, Germany) and another HPLC, System Gold from Beckman Coulter GmbH (Krefeld, Germany) were used for the assay and impurity profiling of the finished dosage form. The systems were equipped with injector with auto-sampler, binary pump and a

variable wavelength detector. Agilent systems also included online degasser. An external column oven from Beckmann Coulter GmbH (Krefeld, Germany) was used when required. The softwares for recording and integration of chromatograms were Chemstation for LC 3D system Rev. B.03.02 from Agilent Technologies (Waldbronn, Germany) and 32 Karat software by SCIEX (MA, USA) for the HPLC systems from Agilent Technologies and Beckmann Coulter GmbH, respectively.

## 2.4.2. Stationary phase:

Assay and impurity tests for amoxicillin capsule and coamoxiclav tablet were carried out on C18 columns, 250 x 4.6 mm with 5 μm particle size columns including column A: Hypersil Gold by Thermo Scientific (Darmstadt, Germany), column B: Eurosphere C18H by Knauer GmbH (Berlin, Germany) and column C: Synergy Fusion RP (250 x 4.6 mm with 5 μm particle size ) by Phenomenex (Torrance, CA, USA), whereas assay of ceftriaxone injections was carried out on column D: Hypersil ODS by Thermo Scientific (Darmstadt, Germany).

## 2.4.3. Other instruments

Apart from these the pH meter from Metrohm (Filderstadt, Germany), ultrasonic bath from Brandelin electronic GmbH & Co. KG, (Berlin, Germany), centrifuge from Eppendorf Zentrifugen GmbH (Leipzig, Germany) and analytical balance from Mettler Toledo (Gießen, Germany) were used.


## 2.5. Preparation of mobile phases and solvent:

_Amoxicillin capsule-assay and test for related substances (BP 2013/Ph.Eur.8.0) and for coamoxiclav test for related substances (BP 2013)(15-17):_

_0.05 M potassium dihydrogen phosphate, pH 5.0:_ 6.805 g of potassium dihydrogen phosphate was dissolved in 200 mL of water and filtered. The volume was made up to 500.0 mL and pH was adjusted to 5.0 ± 0.02 using 2 M sodium hydroxide solution (8.5 g in 100 mL). 250.0 mL of the solution was diluted to 1000.0 mL with water and mixed.

*Mobile phase A/solvent for sample preparation:* 10.0 mL of acetonitrile was added to 990.0 mL of 0.05 M potassium dihydrogen phosphate, pH 5.0 and mixed.

*Mobile phase B:* 200.0 mL of acetonitrile was added to 800.0 mL of 0.05 M potassium dihydrogen phosphate, pH 5.0 and mixed.

## Coamoxiclav tablet-assay using BP 2013/ USP 38 (15, 18):

Mobile phase A: 78.0 g of orthophosphate monohydrate was dissolved in 200 mL of water and filtered. The volume was made up to 1000.0 mL and the pH of solution was adjusted to 4.4 ± 0.02 using orthophosphoric acid and mixed.

*Mobile Phase B:* Methanol.

Water was used as a solvent for sample preparation

## Ceftriaxone-assay and test for related substances using Ph.Eur. 8.0/BP 2013/ USP 38(19-21):

*0.067 M phosphate buffer:* Solution A and B were prepared by dissolving 0.908 g of $KH_2PO_4$ and 2.38 g of disodium hydrogen phosphate dodecahydrate, each in 100.0 mL of water, respectively. A mixture of solution A (38.9 ml) and solution B (61.1 mL) was adjusted to pH 7.0 using 1 M KOH or 20% orthophosphoric acid and filtered.

*Preparation of citric acid buffer:* 2.017 g of citric acid was dissolved in 80 mL of water and pH was adjusted to 5.0 using 10 M sodium hydroxide solution. The solution was diluted to 100.0 mL with water, mixed and filtered.

*Mobile phase/solvent for sample preparation:* 2 g each of tetradecylammonium bromide and tetraheptylammonium bromide were dissolved in 300 mL of a solvent mixture which was prepared using 55 mL of 0.067 M phosphate buffer, pH 7.0, and 5.0 mL of citric acid buffer, pH 5.0, were diluted with 440 mL of water and 500 mL of acetonitrile. The solution was filtered and then remaining volume of solvent mixture was added and mixed.

2.6.    Preparation of standard, sample and system suitability solutions:

*Amoxicillin capsule-assay and test for related substances (BP 2013/Ph.Eur.8.0) and for coamoxiclav test for related substances (BP 2013) (15-17):*

Samples solution of tablets and capsules corresponding to 0.6 mg/mL and 1.5 mg/mL of amoxicillin were prepared in mobile phase A with the help of sonification for assay and impurity test, respectively using the label claim. 1% (v/v) solution was prepared by diluting 1.0 mL of 1.5 mg/mL solution to 100 mL using mobile phase A.

Standard solution of amoxicillin was prepared by dissolving 6.0 mg of amoxicillin in 10.0 mL of mobile phase A using 15 minutes of sonication and shaking.

*Coamoxiclav-assay (BP 2013/USP 38) (15, 18):* Tablet content equivalent to 25 mg of amoxicillin was dissolved in water using 15 minutes of sonication and occasional shaking, the volume was then made up to 50.0 ml with water, centrifuged at 44,000 rpm for 10 min and filtered.

Standard solution was prepared by dissolving 5.0 mg of amoxicillin trihydrate and 2.0 mg of potassium clavulanate in 10.0 mL of water with the help of sonication for 15 min. This standard solution was also used for system suitability test.

*Ceftriaxone-assay and test for related substances using Ph.Eur. 8.0/BP 2013/ USP 38(19-21):* 15.0 mg of ceftriaxone CRS and ceftriaxone powder for injection were dissolved in 25 mL of mobile phase using sonication for 5 min and making up the volume to 50.0 mL for preparation of the standard and sample solutions, respectively.

*2.7.*    Chromatographic settings:

*Amoxicillin capsule-assay and test for related substances (BP 2013/Ph.Eur.8.0) and for coamoxiclav test for related substances (BP 2013) (15-17):* The method was performed on column A (AMX series 1), B (AMX assay-1) and C (amoxicillin assay-2, AMX series 2 and coamoxiclav impurity tests), at a flow rate of 1 mL/min using ambient column temperature. Detection wavelength was 254 nm. 50 µL of sample solutions were injected with isocratic elution using 92% mobile phase A, until the principal peak was eluted. Gradient elution was carried out by increasing the concentration of mobile

phase B to 100% over 25 minutes which was maintained for 15 minutes before returning to the initial isocratic elution and maintained for re-equilibration for 15 min.

*Coamoxiclav tablet-assay using BP 2013/ USP 39 (15, 18):* Isocratic elution using reverse phase HPLC principle was carried using column C, at ambient temperature. 5 volumes of 0.78% w/v sodium dihydrogen orthophosphate monohydrate at pH 4.4 was mixed with 95 volumes of methanol. Flow rate of 2 mL/min was used and detection was performed using 220 nm. The injection volume was 20 µL.

*Ceftriaxone-assay and test for related substances using BP 2013/ USP 38/*Ph.Eur. 8.0 *(19-21)*: Reverse phase HPLC method using ion pair reagent for assay and test for related substances of ceftriaxone. According to this method isocratic elution was carried out on column D at 25 °C at a flow rate of 1.5 mL/min. Detection wavelength was set at 254 nm and injection volume at 20 µL.

## 3.    Results

3.1.1.  Samples collection and sources.

Samples from Pakistan were bought (or received for free in case of public sector supply), disguised as a patient or patient's caregiver. Three samples for ceftriaxone injection were received from a tender supply of two tertiary care hospitals of Lahore with the help of a regulatory pharmacist. Samples were collected from three cities including only the registered drug sale premises (Pharmacy, medical store, wholesaler/distributor and a public-sector tertiary care hospital (TCH) dispensary).

21 different brands of beta-lactam antibiotics FPPs (9 amoxicillin capsules and tablets (500 mg), 6 coamoxiclav tablets (500/125 mg), 3 ceftriaxone injections (1 g) from Pakistan, 3 ceftriaxone injections from DRC were included in the study. Four additional samples included a sample of coamoxiclav tablet (500/125 mg) from Germany, and 3 samples of coamoxiclav tablet (875/125 mg) tablets one each from Pakistan, Syria and Kuwait. For Pakistan the sampling cities included Lahore, Gujranwala and Karachi and the area of sampling included urban, suburban localities and from the cluster of retail outlets outside tertiary care hospitals (o/TCH). Samples from DRC were provided by a pharmacist colleague (Appendix III).

The manufacturers of 19 FPP samples from Pakistan included 6 local manufacturers (LC1-6) and 4 multinational companies (MNC1-4) (Appendix III, Table A1-3). The ceftriaxone injection from DRC included two of Chinese origin (MNC6) and one by a Danish distributor (MNC7).

### 3.1.2. Assay of finished products

Results of assay of 25 batches of beta-lactam antibiotics are shown in Table 2, 3, and 4 for amoxicillin capsules/tablets, coamoxiclav tablets and ceftriaxone injections, respectively.

Assay of amoxicillin capsules/tablets (AMX 1P1-3P9) was carried out in two series (Assay 1 and Assay 2), with a gap of 23 months for the seven samples, showing 2.0-10.6% decrease in the assay content from the initial assay value (Table 2). 44.4% (4/9) of the amoxicillin samples failed the assay (shelf life: 1 to 22 months). Difference in assay content was observed for the same brand collected from different locations and type of facilities. One of the three samples manufactured by MNC2 collected from surrounding of a tertiary care hospital in Karachi failed the assay (88.3%, shelf life: 14 months). Similarly, one of the two samples form MNC1 collected from a medical store from sub-urban area of Lahore showed an assay content of 89.5% (shelf life: 9 months) (Table 2). The failure rate for assay of amoxicillin samples from MNCs was 2/6, and for local products 2/3.

Coamoxiclav tablets (500/125 mg) from Pakistan (CAM 1P1-2P6) showed acceptable assay values for amoxicillin except in case of sample CAM 1P3 from LC5 which showed amoxicillin content of 81.8%. The shelf life of amoxicillin samples was -10 to -18 months (expired) at the time of analysis. The results for clavulanic acid assay (CAM-CA) were significantly low for three samples from Pakistan (CAM1P2, 1P3 and 2P5). CAM 1P3 manufactured by LC5 showed only 1% of the label claim of clavulanic acid and was packed without the desiccant in the bottle, whereas CAM 1P2 and CAM 2P5 manufactured by LC4 contained 88.0% and 52.5%, respectively, and were packed as aluminium blister strips instead of glass bottles (Appendix III, Table A4).

Results-Finished pharmaceutical products

Table 1.        Limits for assay (%) of the finished pharmaceutical products.

| Finished Pharmaceutical Product | Assay Limits (%) | |
| --- | --- | --- |
| | BP 2013 | USP 38 |
| Amoxicillin capsule | 92.5-110.0 | -- |
| *Coamoxiclav tablets*<br>Amoxicillin<br>Clavulanic acid | 90.0-105.0<br>90.0-105.0 | 90.0-120.0<br>90.0-120.0 |
| Ceftriaxone injection | 92.0-108.0 | 90.0-115.0 |

Table 2.        Assay of amoxicillin in FPP and API (%).

| Sample ID | Manufacturer | Sampling location-facility type, city | Assay (%), (remaining shelf life in months) | |
| --- | --- | --- | --- | --- |
| | | | Assay 1 | Assay 2 |
| AMX1P1 | MNC1 | o/THC Pharmacy, Lahore | 95.3 (1) | **86.9 (-23)** |
| AMX1P2 | MNC1 | Medical Store, Lahore | **89.5 (9)** | **83.8 (-15)** |
| AMX1P3 | LC1 | Pharmacy, Lahore | 94.2 (10) | **83.6 (-14)** |
| AMX1P4 | MNC2 | Pharmacy, Lahore | 100.4 (9) | 95.4 (-15) |
| AMX2P5 | LC2 | TCH, Lahore | **90.0 (20)** | **88.0 (3)** |
| AMX2P6 | LC3 | Distributor Gujranwala | **88.2 (22)** | **85.8 (5)** |
| AMX2P7 | MNC2 | Pharmacy Gujranwala | 96.6 (10) | **90.5 (-14)** |
| AMX3P8 | MNC2 | o/THC Karachi | | **88.3 (14)** |
| AMX3P9 | MNC3 | o/THC Karachi | | 98.4 (11) |

*THC (Tertiary care hospital), o/THC (outside THC), Bold figures: out of assay limits (92.5-110.0%).*

The three brands with a low content of amoxicillin and/or clavulanic acid showed unusual brown to blackish brown appearance of the tablet content and produced clear sediments on suspending in water (Appendix III, Table A4). Among the sample from Germany and three samples of extended release tablets from Pakistan, Syria and Kuwait, one sample from Syria showed a higher content of amoxicillin (106.3%) (Table 3) but was in acceptable limits in accordance with USP 38 (Table 1). The assay values

for ceftriaxone injection ranged from 78.6 -83.2% for samples from DRC and 89.7-90.7% for samples from Pakistan (Table 4). Out of the six samples under study, none complied with the assay limits by BP 2013 and only one sample from Pakistan fell within the acceptable limit given by the USP 39 (Figure 1 and Table 3)

Table 3.        Assay of coamoxiclav tablets (%)

| Sample ID | Manufacturer | Sampling location-facility type, city | Assay (%) BP limit = 90.0-105.0% | | Shelf life |
|---|---|---|---|---|---|
| | | | Amoxicillin | K-clavulanate | (months) |
| CAM1P1 | MNC4 | o/THC Pharmacy, Lahore | 99.2 | 92.2 | -12 |
| CAM1P2 | LC2 | Medical Store, Lahore | 95.9 | **88.0** | -12 |
| CAM1P3 | LC1 | Pharmacy, Lahore | **81.8** | **1.0** | -18 |
| CAM1P4 | MNC1 | Pharmacy, Lahore | 95.0 | 101.0 | -15 |
| CAM2P5 | LC2 | TCH, Lahore | 91.3 | **52.5** | -12 |
| CAM2P6 | MNC1 | Distributor Gujranwala | 93.9 | 103.2 | -10 |
| CAM2P7* | MNC1 | Pharmacy Lahore | 97.4 | 101.3 | -14 |
| CAM 1D1 | DLC1 | Germany | 103.3 | 100.2 | -3 |
| CAM 1S1* | SLC1 | Syria | **106.3**** | 91.8 | -6 |
| CAM1K1* | KLC1 | Kuwait | 99.2 | 103.9 | -15 |

*extended release tablets (AMX/CA= 875/125 mg) ** within assay limits of USP 38

Bold figures showing out of specification for assay limits of BP 2013

Table 4.        Assay of ceftriaxone injection (%)

| Sample ID | Sampling location-facility type, city | Assay (%) ± S.D. |
|---|---|---|
| CTX- 1C1 | - | 78.6 ± 0.2 |
| CTX- 1C2 | - | 80.6 ± 0.1 |
| CTX- 1C3 | - | 83.0 ± 0.1 |
| CTX- 1P1 | TCH, Lahore | 89.3 ± 0.2 |
| CTX- 1P2 | TCH, Lahore | 88.7 ± 0.3 |
| CTX- 1P3 | TCH, Lahore | 90.7 ± 0.0 |

3.1.3.  Total impurities:

Impurity profile of twenty-five finished products were carried out using the methods for tests for related substances stated in pharmacopoeia and the summary of results for 21 products from Pakistan and DRC are shown in the Figure 1 as concentration of total impurities (%) against the age of the product (remaining shelf life in months with minus (-) representing expired product). The limits for impurities in finished products are given in Table 5.

Only two samples of amoxicillin AMX 1P1 and AMX 2P4 showed impurity at a concentration close to 1.0% (Figure 3a) on analysis done within the shelf life of the products (AMX series 1). The reanalysis after 30 months (AMX series 2) showed no significant rise in the total impurity content (Figure 3b) but an increase in number of peaks after the rel.RT of 4.5. Only one new sample, AMX 3P8 (shelf life = 13 months) showed the presence of an impurity at rel.RT of 4.4 at upto 1.13% (Figure 3b) and it had also shown low assay content (Table 2). Comparison of the impurity profiles of coamoxiclav FPPs shows that the coamoxiclav fixed dose combinations from Pakistan (CAM 1P1-4, CAM 2P5-7) has a significantly higher number of impurities in comparison to the coamoxiclav sample from Germany, Syria and Kuwait (CAM 1D1, CAM 1S1, CAM 1K1) (Table 6 and Figure 4c). The total impurities in coamoxiclav samples (legend: CAM-PK) from Pakistan also increased in concentration as the product aged, which was not observed for expired amoxicillin FPPs from Pakistan (legend: AMX series 2) (Figure 1). Impurity profile of amoxicillin FPPs (series 2) and

coamoxiclav FPPs are shown in Figure 4a-c. The concentration of amoxicillin dimer was within the specified limits but was close to the upper limit for most of the samples except the sample from Germany (CAM 1D1) and Syria (CAM 1S1) (Table 6).

Limited information was available for identification of known impurities of amoxicillin using the method from BP 2013/ Ph.Eur. 8.0. Information on the rel. RT of impurities was acquired from Ph.Eur. 8.0 (22) and literature (23) as well as from the experimental data generated during the synthesis of impurities (Chapter 5). Figure 2 shows the impurity profile of 10 brands of amoxicillin FPPs with the labelled known impurities. Three known impurities were found in the analyzed samples of ceftriaxone injection using the rel. RT data provided in the monograph (21) (Table 7). Limit for total impurity of ceftriaxone is revised from 2.5% in USP 38 (21) to 5.0% in USP 40 (24).

Table 5.        Limits for total impurities (%) in FPPs.

| | Total impurities | | Any unspecified impurity | | Specified impurities | |
|---|---|---|---|---|---|---|
| | BP 2013 | USP 38 | BP 2013 | USP 38 | BP 2013 | USP 38 |
| Amoxicillin capsule | NA | | ≤ 1% | | NA | |
| Coamoxiclav tablets | NA | NA | ≤ 1% | NA | ≤ 2% amoxicillin dimer | NA |
| Ceftriaxone injection | ≤ 5% | ≤ 2.5% Revised in USP 40 to ≤ 5% | ≤ 1% | ≤ 0.2% | None | ≤ 0.5 impurity B, ≤ 1.0 impurity E, C, A ≤ 0.2 impurity D |

NA= information not specified in the monograph
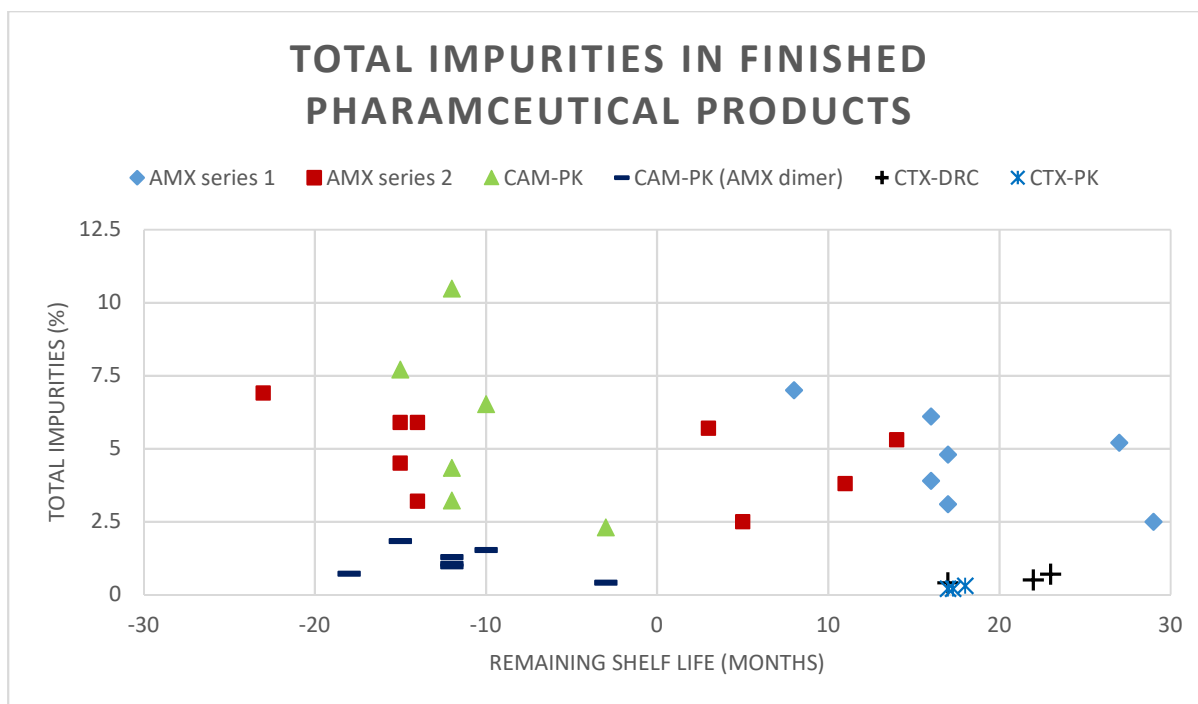
Figure 1.    Concentration of total impurities (%) found in FPPs

Table 6.    Impurity analysis of co-amoxiclav tablets

| Sample Code | Shelf life (months) | Total Impurities* (%) | AMX dimer (%) ≤ 2% | No. of impurities > 1% |
|---|---|---|---|---|
| CAM1P1 | -12 | 3.2 | 1.28 | None |
| CAM1P2 | -12 | 4.3 | 0.96 | None |
| CAM1P3 | -18 | **26.5** | 0.71 | 7(Figure 4c) |
| CAM1P4 | -15 | **7.7** | 1.82 | None |
| CAM2P5 | -12 | **10.5** | 1.05 | 2 (1.2%, 1.3%) |
| CAM2P6 | -10 | **6.5** | 1.52 | 1 (1.3%) |
| CAM2P7* | -14 | **5.3** | 1.60 | None |
| CAM 1D1 | -3 | 2.3 | 0.40 | 1 (1.1%) |
| CAM 1S1* | -6 | 1.7 | 0.65 | None |
| CAM1K1* | -15 | 2.6 | 0.83 | None |

*extended release tablets, ** disregard limit 0.1%, Bold figures show total impurity ≥ 5.0%

Table 7.    Concentration of individual impurities (%) and total impurities (%) in ceftriaxone (CTX) injections from Democratic Republic of Congo (C) and Pakistan (P) in accordance to the limits given by USP 39.

| Impurity Name | Deacetyl cefotaxime lactone | Impurity C | E-isomer of Cefotaxime | Total impurity |
|---|---|---|---|---|
| RRT | 0.2 | 0.48* | 1.4 | |
| **Limit by USP 38 (%)\*\*** | **≤ 0.5** | **≤ 1.0** | **≤ 1.0** | **≤ 2.5\*\*\*** |
| CTX1C1 | - | 0.4 | - | 0.4 |
| CTX1C2 | 0.1 | 0.5 | 0.1 | 0.7 |
| CTX1C3 | - | 0.4 | 0.1 | 0.5 |
| CTX1P1 | - | 0.2 | - | 0.2 |
| CTX1P2 | - | 0.2 | - | 0.2 |
| CTX1P3 | - | 0.2 | 0.1 | 0.3 |

\* Rel.RT assigned using impurity standard, \*\*\* limits from USP 40 ≤ 5.0

\*\*Limit for all individual impurities by British Pharmacopoeia 2013 is ≤ 1%

### 3.1.4. Known and unknown impurities found in the samples
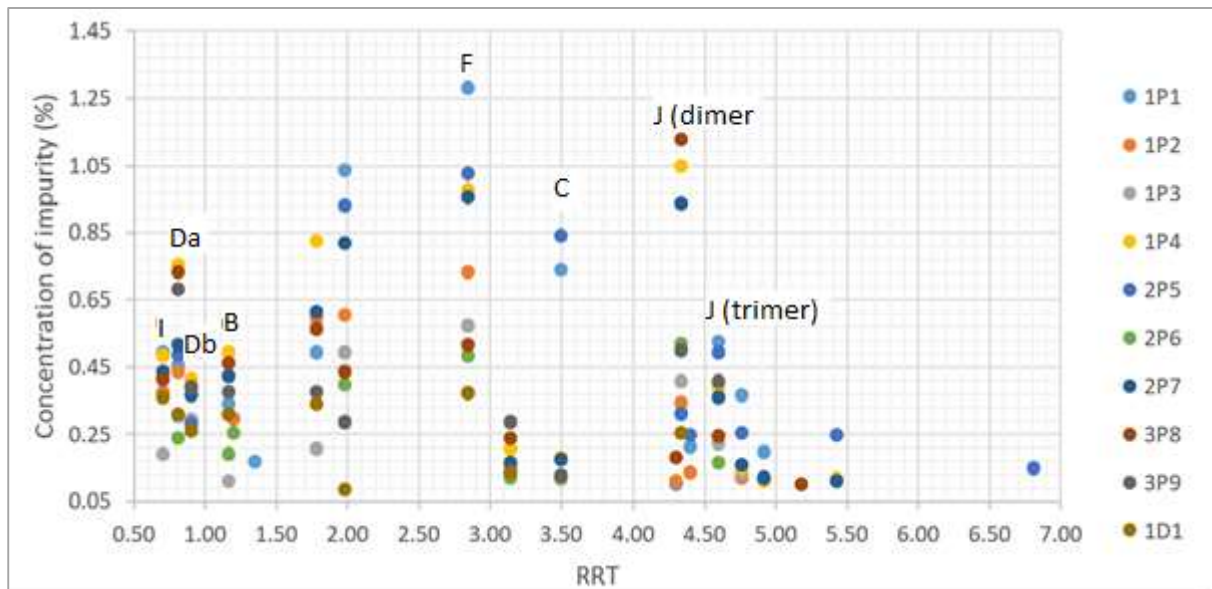


Figure 2.    Impurity profile of 10 brands of amoxicillin capsule/tablets. Known impurities are labelled using alphabetic codes.

Table 8.    Identification of impurities in analysed samples using rel.RT reported in literature and experimental data

| Impurity code | rel.RT  (22) | rel.RT (23) | rel.RT of impurity peaks in analysed samples |
|---|---|---|---|
| Impurity I | | 0.41 | 0.70 |
| Impurity Da | | 0.55 | 0.81* |
| Impurity Db | | 0.72 | 0.91* |
| Impurity A | | 0.79 | |
| Impurity B | | 0.86 | 1.17 |
| Impurity E1 | | 2.72 | 1.79 |
| Impurity G | | 2.51 | 1.99 |
| Impurity F | | 3.71 | 2.85* |
| | | H(3.47) | 3.14 |
| Impurity C | 3.4 | C1(3.06) C2(2.96) | 3.50 |
| Impurity J (n=1) | 4.1 | 3.92 | 4.3, 4.35. 4.4 |
| Impurity J (n=2) | 4.5 | 4.37 | 4.60 |
| | | | 4.76 |
| | | | 4.92 |
| | | | 5.43 |
| | | | 6.80 |

*From experimental data



a) Impurity profile of amoxicillin capsules -initial data (series 1)
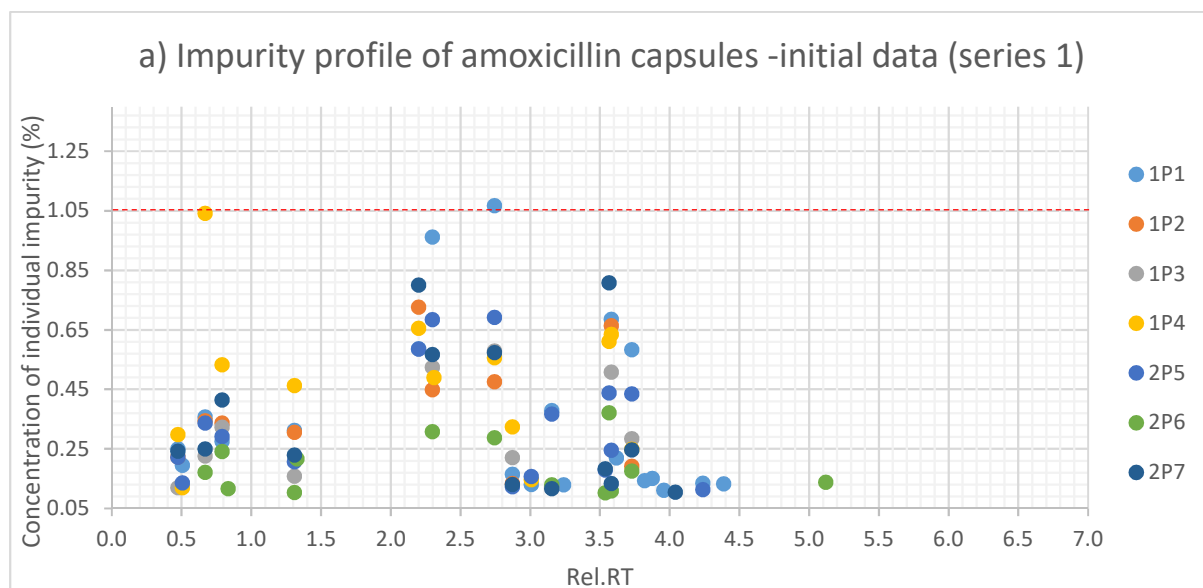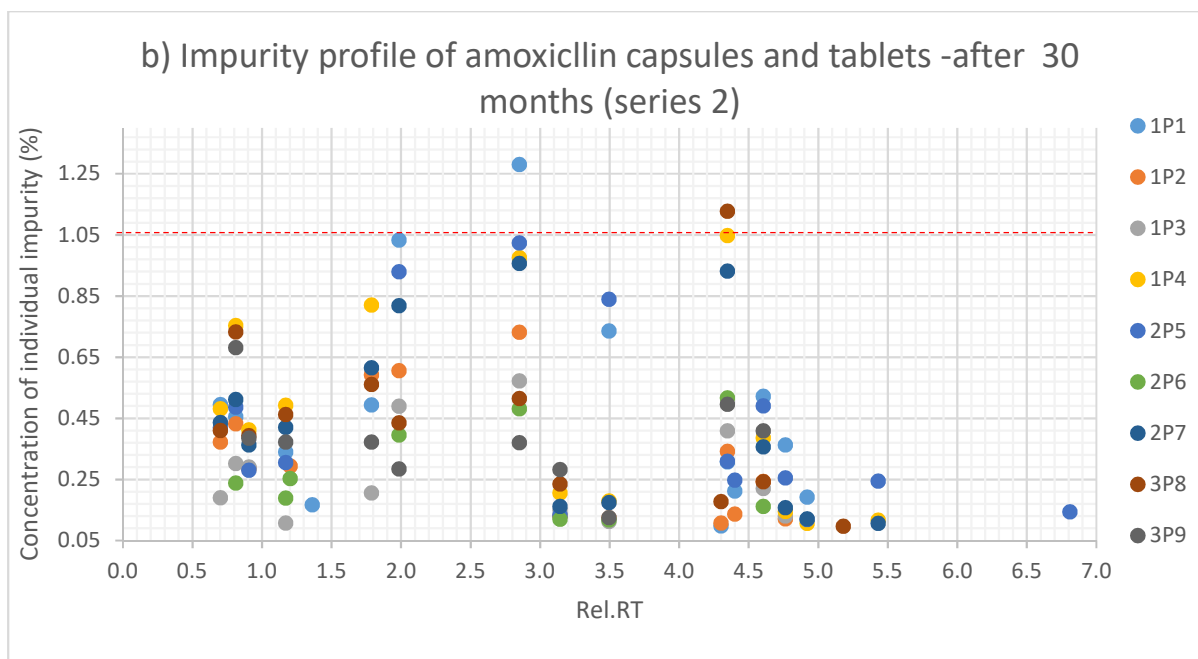
Figure 3.     Impurity of amoxicillin capsules and tablets from Pakistan a) initial b) repeat analysis after 30 months

## 4. Discussion

None of the samples tested in the study, showed absence of API or very low API content, except for the case of clavulanic acid. Products were found substandard with API content below the specified limits of pharmacopoeia. Among the unexpired samples, the lowest content found was 78.4% for ceftriaxone injection from DRC and 88.3% of amoxicillin from a local manufacturer in Pakistan.  The samples with low API content were from local manufacturers from retail or public-sector supplies or from multinational companies from sources like medical stores or retail outlets outside the large tertiary care hospitals.

Difference in the assay of samples of the same brand collected from varying region suggest the presence of genuine and the possible counterfeit products in the market, a problem not uncommon in the poor regulatory settings. Locally manufactured products and public sector tender supplies are commonly questioned with respect to quality. The areas around the public-sector tertiary care hospitals often present a huge private market of pharmacies and medical stores. One of the three samples of amoxicillin capsules from MNC2 that failed to show the adequate API content for amoxicillin was sampled from one such areas of Karachi. Pharmacy and medical store

are the two versions of legal drug sale outlets found in the country, the former being



a) Impurity profile of amoxicllin capsules and tablets (series 2)
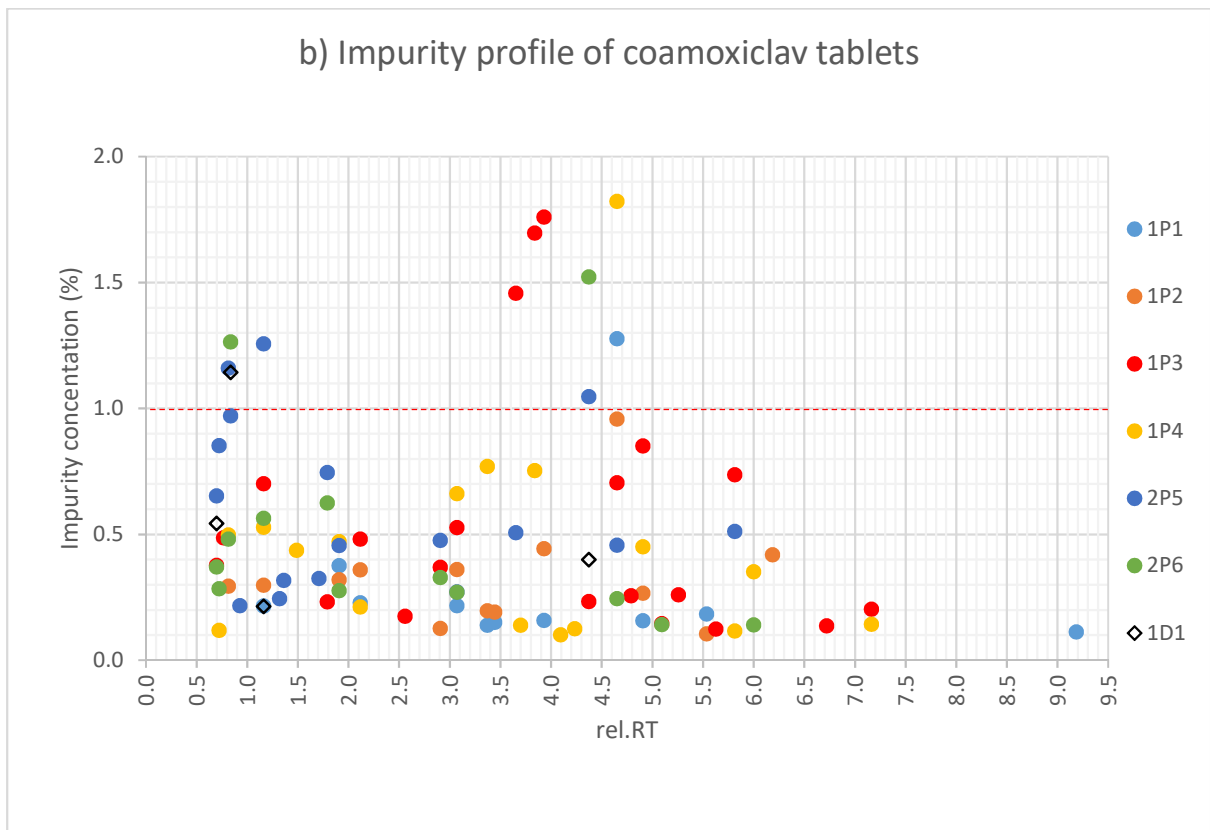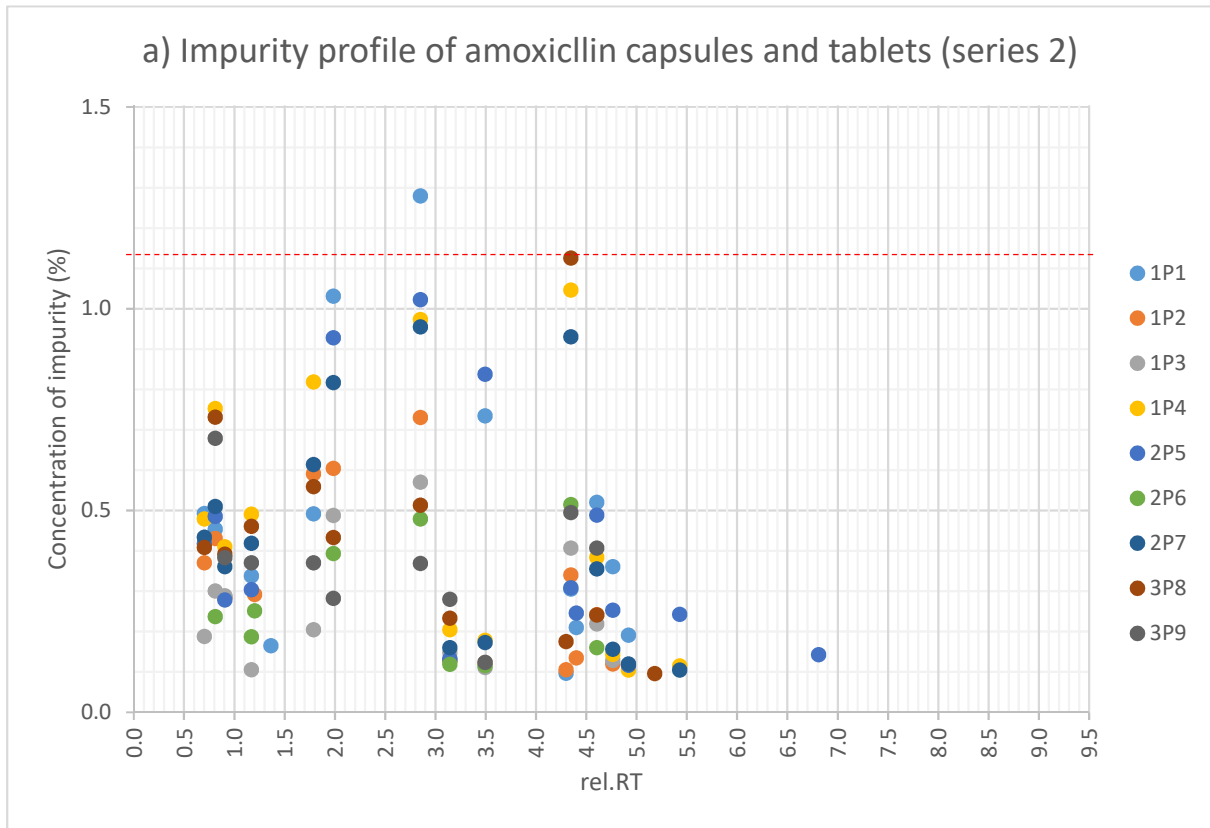


b) Impurity profile of coamoxiclav tablets
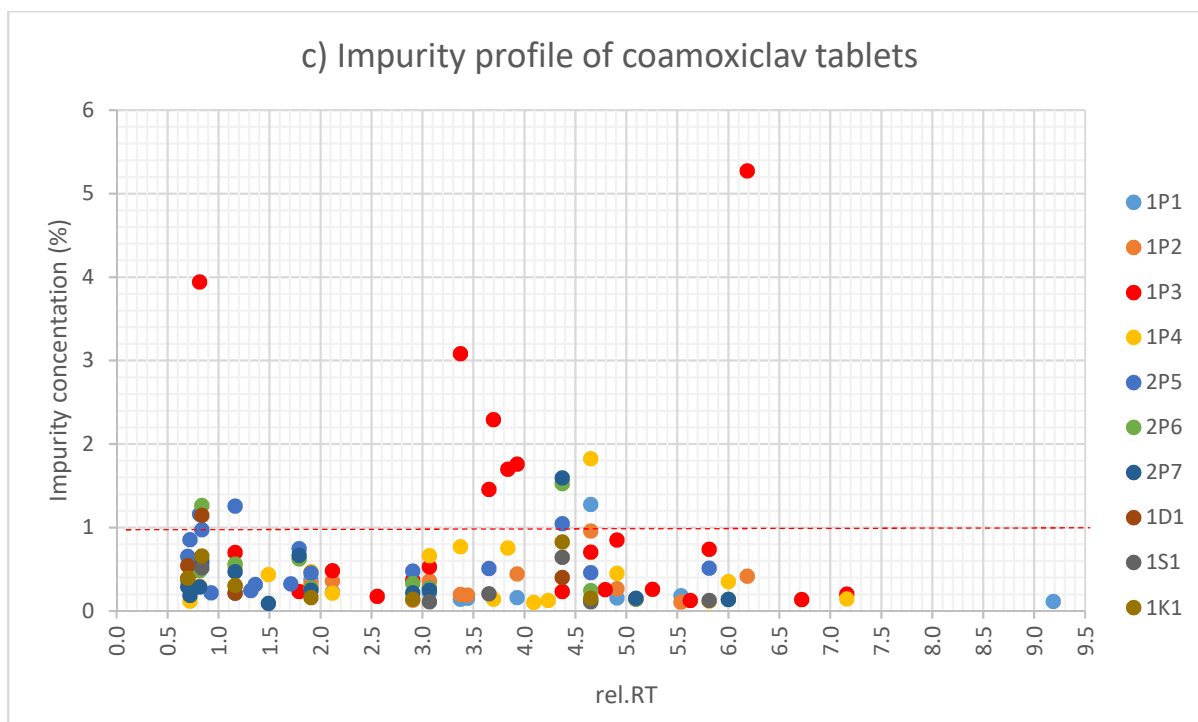
Figure 4. Impurity profile of (a) amoxicillin capsules/tablets, shelf life: 5 to -23 months and (b) coamoxiclav tablets (500/125 mg) from Pakistan and Germany (y-axis: 0.0-2.0%) (c) coamoxiclav tablets (all tested samples), shelf life: -3 to -18 months.

under the license of a pharmacist but not necessarily have the qualified personnel available at the premises. The pharmaceutical regulation in Pakistan is long been criticized for incapability to address the problem of poor-quality medicines and very little information on the quality issues of medicines are available.

A comprehensive study on 96 samples of ceftriaxone injections from 33 manufacturers in Pakistan showed the number of samples with low and high API content as 9.4% (9/96) and 8.3% (8/96), respectively, in accordance with the USP limit of 90-115%. The study also reported failing of all the samples for assay from one of the manufacturers. Among the 9 under dosed samples, 7 were in the range of 75-90% which is in conformity with the results of the current study. The study comments that the quality failures reflect the non-compliance to cGMP with samples also showing low fill volumes (25).

Investigation of 34 generic ceftriaxone injections from 12 countries showed a content within the range of 96.4-100.1%. The total impurities were found to be from 0.22-1.18%, with impurity C of ceftriaxone in the range of 0.18-0.94%. Failure of sterility

test and clarity of the solution were also among the many contraventions reported in that study (26). The current investigation of samples from Pakistan and DRC showed total impurity in the range of 0.2-0.7% with the concentration of impurity C from 0.2-0.5% which is similar to that reported in literature.

The recent report on counterfeit medicines in Peru, an upper middle-income country with a deregulated pharmaceutical sales system and high number of manufacturing facilities, shows that 20% cases for counterfeit medicines reported to General Directorate of Medicines, Supplies and Drugs (DIGEMID), Peru during 1997-2014 included anti-infectives/antibacterial forming the second major therapeutic group after analgesics for counterfeit medicines (27). No such data or open access data base is available for Pakistan and very few studies have been published in this regard.

Extensively used molecules are most commonly encountered with the quality issues and are also preferred choices by the counterfeiters (28). Quality issues for beta-lactam antibiotics have been reported in past in several surveillance studies for substandard and falsified medicines (29-31). A review on counterfeit antibiotics reported globally, documents 30, 4 and 4 case reports for amoxicillin, coamoxiclav, and ceftriaxone, respectively (28) including reports from Pakistan and DRC.

Public sector pharmaceutical purchase system is an area that need to be investigated for the assurance of quality supplies. A survey of health facility supply chain in Papua New Guinea reports 1 out of 47 samples of amoxicillin tablets and capsules to be below the specified limits for assay by BP (92.5-110.0%) (29). In 2014, WHO has circulated alert on the falsified amoxicillin found in the public sector supplies of Niger (32). In adequate antibacterial activity was reported for quinolones in studies involving brand comparisons from Pakistan (33, 34). The WHO prequalification system has evolved the standards of public sector purchases by defining a quality checklist for the essential prerequisites for the facilities to handle such large orders (35). In most instances, the public- sector supplies are demanded in a limited time, which constraints the GMP requirements and compromises the quality of products. Poor quality of medicine in public sector supply systems leads to patient distrust on the health care system (36).The inadequacy of the regulatory system to carry out efficient and complete quality check for pharmaceuticals is another challenge that the resource limited settings in low- and middle-income countries face.  Efforts are made by the

strategies to strengthen the capacity of post marketing surveillance by public sector laboratories resulting in the improvement in the quality of medicine in supply chain (6). In the current study, 5 out of 19 beta-lactam samples from Pakistan (AMP2P5, CAM 2P5 and CTX1P1-3) were from tertiary care public sector supply and all of the samples failed the assay for the acceptable limits given by BP 2013 (Appendix I, Table A1).

The results of coamoxiclav tablets showed extremely low content of clavulanic acid in one of the samples which was marketed without a dessicant pouch. Clavulanic acid is a moisture sensitive and good quality packaging is important for ensuring stability of coamoxiclav tablets (37). The other two products showing low clavulanic acid content were packed in aluminium (Alu-Alu) blister packing showed the amoxicillin content within the specified limits. The other samples from Pakistan were supplied in screw capped amber colored bottles with a dessicant pouch whereas the foreign samples were packaged using Alu-Alu blister packing or PVC-Alu blister packed further in an aluminium pouch (CAM 1K1) (Appendix IV). Three extended release tablets were also tested using the same method and were found within the quality specifications except one sample from Syria had a higher content for amoxicillin. The sample of extended release tablet from Pakistan had higher total impurity content than the other two products (Table 6).

The overall results for quality of beta-lactam antibiotics in Pakistan reflected by the study supports the concerns shown by the policy experts on the inadequacy of the regulatory and market surveillance system for medicines in Pakistan needs restructuring, updating of the infrastructure, training of skilled personnel and strategic policies to improve the current situation (38, 39). The variation in the product quality of same brands samples from different locations reflects poorly regulated pharmaceutical markets like in private pharmacies outside the tertiary care hospitals, suburban areas. Other possible high-risk area are the public sector tender supplies.

## 5.    Limitations

The samples collected in this study were limited in size consisting of single pack or a blister and were not sufficient to perform a proper quality control test. Content from a single tablet or capsule was used in place of taking average content from recommended size of 20 units. Hence, the study results are only limited to represent

the estimated picture for the quality of samples and the term pass or fail is not applicable to it in real sense. The samples were also limited to urban and suburban areas of large cities and the situation can be worse in the rural and remote settings of the country. Hence, a more systematic study including sampling from public and private sector supplies located in urban, sub urban and remote areas sourced from different facility levels and regions of the country need to be initiated for a reliable analysis of the situation.

## 6.    Conclusion

Results of the study provide a glimpse of the situation of quality of beta-lactam antibiotics in Pakistan. The quality of medicines from local manufacturers, tender supplies and remote and peripheral settings need to be particularly evaluated. Though the quality failures observed in the study did not include complete absence of API or huge amounts of unknown impurities, but still a high number of failures and borderline results show an alarming situation. The country must adopt comprehensive and practical strategy for post marketing surveillance to act as a deterrent to the high prevalence of substandard drugs in the market.

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## 3.5   A case study of Pakistan and recommendations for fighting poor-quality medicines

### Abstract

Substandard and falsified medicines have been identified as a huge public health problem in Pakistan and gained national as well as international attention after several incidences of poor-quality medicines claiming lives of hundreds of people in 2011-12. The incidences were a driving force to reform the regulatory structures of the country and prompted the formation of the autonomous "Drug Regulatory Authority of Pakistan". Despite the country possesses a huge pharmaceutical industry that – apart from catering the national pharmaceutical needs – exports to international markets of Middle Eastern and African countries, there is a severe dearth of published literature and scientific evidence for the country regarding medicine quality. This review involves a documentation of the important features of the regulatory framework in Pakistan, its pharmaceutical industry, as well as a compilation and analysis of scientific publications, reports, and other published evidence that can be helpful in documenting the base-line situation of the country. It also documents the progress after the "Fake drug crisis" in 2011, as the country moved towards aggressive reforms by implementing major infrastructural changes towards a rigorous regulatory system. A brief review of the accessible technologies and strategies used in the recent past at global level, especially in the developing countries, are also discussed and recommendations are devised for the country to combat the fight against poor-quality medicine.

**Key words:** Pakistan, drug regulatory athourity, pharmaceutical regulation, substandard, falsified, poor-quality, prevelance

# 1. The case study of Pakistan

Pakistan is among the nations that have recently adopted the concept of an autonomous Drug Regulatory Authority (1, 2) and the country is yet to develop and implement a comprehensive national pharmacovigilance system (1-4). Documentation of the current situation and identification of opportunities in terms of available and tested technologies as well as upcoming regulatory field tools can offer a stepping stone for reforming Pakistan's regulatory systems and deliver safe and efficacious medicines.

## 1.1 The country

Pakistan is a lower-middle income country (5) situated in the west of the Indian Subcontinent and shares its borders with China in north, Afghanistan in west, and Iran in (6) southwest. It is the sixth largest population of the world with over 207 million inhabitants (7) of which 43.4% are below 15 years, and 3.5% are above 65 years of age (8). In the year 2015-16, the per capita public expenditure on health was 45 USD, whereby the World Health Organization (WHO) benchmark is 86 USD (9, 10). Pakistan has not achieved the WHO benchmark of spending 6% of the Gross Domestic Product (GDP) on health within the last ten years (9). The value of 3.1% was recorded for the total health expenditure as percentage of GDP in fiscal year (FY) 2015-16 (10), whereas the highest public sector spending on health was 0.91% of GDP observed in the FY 2016-17 (11). According to National Health Accounts (2015-16), 62.95% of total health expenditure is contributed by out-of-pocket expense whereas the provincial health departments and district government cover only 16.13% and 4.56% of total health expenditure, respectively (10). The infant, neonatal, and under-5 mortality rates per 1000 live births (LB) for 2015 were recorded as 64.6, 46.3, and 79.5, respectively, whereas maternal mortality was 178 per 100,000(12) . The number of deaths due to infectious diseases and respiratory infections constitute a major portion of the total mortality. The current health system and its regulation have long been criticized for the lack of infrastructure, incompetence, and organizational weaknesses (4, 13-15). Absence of pharmacists from the health care system and the lack of sufficient regulatory controls have resulted in the mishandling, misuse, and overuse of pharmaceuticals including antibiotics in the country (15).

## 1.2 Pharmaceutical sector of Pakistan

The country holds a pharmaceutical market of $3.1 million, with systematic anti-

infectives followed by drugs used for gastrointestinal and metabolism disorders representing the major categories of sold pharmaceuticals (10). The larger share (about 60%) of sales goes to domestically produced pharmaceuticals with 95% of the Active Pharmaceutical Ingredients (APIs) being imported (16). So far, the Drug Regulatory Authority of Pakistan (DRAP) has issued more than 850 drug manufacturing licenses (DML) (according to the last report issued from DRAP), but the figure for licenses with active status is around 650 (16). These include licenses covering formulation, basic and semi basic manufacturing (raw material manufacturing), and repacking. However, the list of importers of finished pharmaceutical products including mainly biologicals, vaccines, anticancers, newly approved medicines, contrast medias, etc., exceed the number of pharmaceutical manufacturers. Out of the total licensed manufacturing units in Pakistan, none is approved by the United States Food and Drug Administration (FDA) which is in strong contrast to India and Bangladesh (17). Only one pharmaceutical company holds a European Union Good Manufacturing Practice (GMP) certification and accreditation issued by the Medicines and Healthcare products Regulatory Agency of the UK (MHRA) (18). In early 2018, moxifloxacin tablets produced by Getz Pharma Pvt, Ltd. achieved the status of a "first-ever WHO prequalified pharmaceutical product from Pakistan" (19). Pharmaceuticals manufactured in Pakistan are exported to other countries, mainly to Jordan (80%), Africa, and the Middle East (20). A Central Research Fund (CRF) is operated under the "Drug (Licensing, registration, and advertising) rules" from 1976 (21), according to which 1% of the gross profit of pharmaceutical companies before tax deduction is deposited to the government for supporting research of public and national interests (16, 22). However, since the start of an actual approval of projects under this fund in 2001, only a meagre amount has been consumed, catering to only a handful of projects showing underutilization of funds, lack of planning, and poor execution of policies (23).

## 1.3    Regulatory infrastructure for pharmaceuticals

In Pakistan medicines licensing, manufacturing, registration, pricing, imports, and exports are dealt by the federal government, whereas distribution and sales are regulated by the provincial governments (3). This decentralized regulatory control for drug sales is regarded as a structural weakness of the system by international experts and the situation worsened on devolution of powers from the federal government to

the provinces under 18<sup>th</sup> amendment of the constitution of Pakistan (14). In 2012, the country faced a "Fake Drug Crisis" due to deaths caused by contaminated medicines dispensed from a public-sector facility.

The incidence triggered the establishment of the Drug Regulatory Authority of Pakistan (DRAP) along with the enforcement of the DRAP act (1). DRAP functions as an autonomous body under the Ministry of National Health Services (9). The new organizational structure of DRAP consists of eight technical and five supportive divisions. The division of quality assurance has five field offices supported by federal drug inspectors, assistant drug controllers, and an appellate board. The other seven technical divisions include registration, medical devices, biological drugs, controlled drugs, pharmacy services, health & Over-the-counter, costing, and pricing (24) The pharmacy services division covers pharmacovigilance, clinical trials, regulation of contract research organizations, and research. However, among the stringent drug regulatory systems, the German regulatory agency, Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte, BfArM) , covers drug licensing, pharmacovigilance, medical devices, the federal opium agency, and research under a complex system split into 12 main divisions with 49 sections staffed by around 1000 employees. A special commission works for the licensing (i.e., market authorization) of paediatric medicines. Research comprises of a dedicated division covering pharmacogenomics, pharmacoeidemiology, biostatistics and specialized pharmacology, and experimental neuropsychopharmacology. Moreover, a separate sections work for the falsified medicines and simplified methods, medicine shortages as well as parallel imports. In BfArM, medicines are allotted to the regulatory sections in accordance with their pharmacological category whereas in DRAP only the specialized therapeutic and pharmaceutical groups like anticancers and biologicals are dealt by specialized sections.

By the year 2018, twelve Drug Testing Laboratories (DTLs) are functional in Pakistan with two Central Drug Testing Laboratories (Central DTL in Karachi, and the National Control Laboratory for Biologicals in Islamabad) (2), an appellate laboratory (National Institute of Health, Islamabad), five DTLs in the province of Punjab, and one in each of the other provinces (Sindh, Balochistan, and Khyber Pakhtunkhwah (KPK)). One DTL is located in Azad Jammu and Kashmir. A Federal Drug Surveillance Laboratory, Islamabad, is also in its developmental phase.

Three public sector DTLs (Faisalabad, Lahore and Multan) are ISO 17025 certified whereas none is prequalified by the WHO. One private laboratory in Pakistan gained WHO prequalification in 2014, followed by a voluntary withdrawal after two years (25). Among the other drug testing facilities in the country are the Punjab Drug Testing Laboratory and Research Centre (ISO 17025 certified and accredited by the Government of Punjab) and the Punjab Forensic Sciences Agency.

## 1.4    Identification of medicines quality as a prevailing health sector crisis

Poor quality and shortages of medicines were identified as a major challenge during the rescue and rehabilitation work carried out in the country in the past two decades (26, 27). In 2012, the case of contaminated cardiovascular drugs claimed more than 230 lives ("The Fake Drug Crisis") and became the driving force for the initiation of major reforms regarding infrastructure and regulations of pharmaceuticals in Pakistan (1). A few months later, the country faced another major case of medicine quality failure causing death of hundreds of people from ingestion of a contaminated cough syrup (28).

## 1.5    Interventions for delivery of quality medicines

Pakistan has been fighting the menace of poor quality medicines since a long time. Heavy fines and punishments enacted in the early 90's provided some control and are still used as a deterrent. In 1975, the generic policy of the 1972 Drug Act of Pakistan was withdrawn, as a consequence of suspending 38 pharmaceutical companies for producing substandard medicines (20). In 2005, along with post earthquake rescue operations, WHO established a network being a drug distribution system with early detection and rectification of quality and supply failures in the affected areas (27). The WHO prequalification program is adopted (29) and is accepted countrywide by a number of organizations improving the delivery of quality medicines. In a local study, no significant benefit was found in terms of time taken for smear conversion for the 15-20% more expensive internationally quality assured medicines when compared with locally produced multiple drug resistant tuberculosis medicines purchased through medicines prequalification program (30).

DRAP was enacted soon after the incidence of the 2012 "fake drug crisis", and the multiple health emergencies faced by the country in the form of disease outbreaks (1). The inadequacy of the system to identify the presence of an erroneous substance by

the testing laboratories was evident from the high fatality incidences of contaminated pyrimethamine, in cardiovascular drugs, and substandard dextromethorphan containing high amounts of the toxic levorotatory form (31, 32). DRAP started with clear emphasis on recruiting and developing highly skilled regulatory personnel, modernizing the systems, establishing mechanisms for pharmacovigilance, and upgrading equipment, human resources, and operational systems of the drug testing laboratories (3, 9, 20). However, budgetary allocations are the main hindrances faced by DRAP (20). Health sector reforms are enacted and reinforcement of infrastructure and human resource development as well as external linkages is established to increase the credibility and efficiency of the newly formed DRAP (3, 20, 33). A list of 60,000 registered medicines has recently been made accessible on the DRAP website and the organization aims for adopting a 2D bar coding system as a measure to combat falsified medicines (34).

Other notable interventions include the establishment of a Provincial Quality Control Unit (PDCU) of Punjab. In 2017, PDCU has initiated the dissemination of information on the failed samples to public and health professionals through its web portal (35) and a monthly newsletter (36). 445 Drug Safety Alerts (DSA) were issued by PDCU from 30 August 2017 until 1 October 2018 (13 months) including 313 quality failure reports and 298 reports for medicines for human use which comprised of products declared as substandard, misbranded, adulterated, and spurious. The rest of the quality failures included 21 substandard and misbranded surgical products, three veterinary pharmaceuticals, and seven herbal medicines. Six out of seven samples of herbal medicines contained undeclared sildenafil citrate. One safety alert for Sancos Syrup (Pfizer) was circulated for complete withdrawal of the finished product after instructions from DRAP (35). The product was found to have issues with stability data causing a reduced shelf life (37). Of note, the quality failure reports issued by PDCU included a large number of anti-infective agents predominantly essential beta-lactam antibiotics. Important and alarming examples include coamoxiclav tablets (low content of clavulanic acid and failure of dissolution testing), amoxicillin suspension, ceftriaxone injection, cefixime capsules, and meropenem injection (low API content). Most of the products analyzed by DTLs were sampled from a public-sector tendor supply.

Global safety alerts for communication on recently reported adverse drug reactions, therapeutic goods related problems communicated by the pharmaceutical manufacturers, and information for cancellation of licenses by DRAP constituted 92

DSAs. The remaining alerts included 7 product recalls including a recent product recall for valsartan containing products issued after a global alert for withdrawal of products containing the carcinogenic impurity nitroso-dimethylamine. Data of 3 DSAs was not accessible though the online version of newsletters. However, information is also shared publicly through an official facebook page administrated by PDCU.
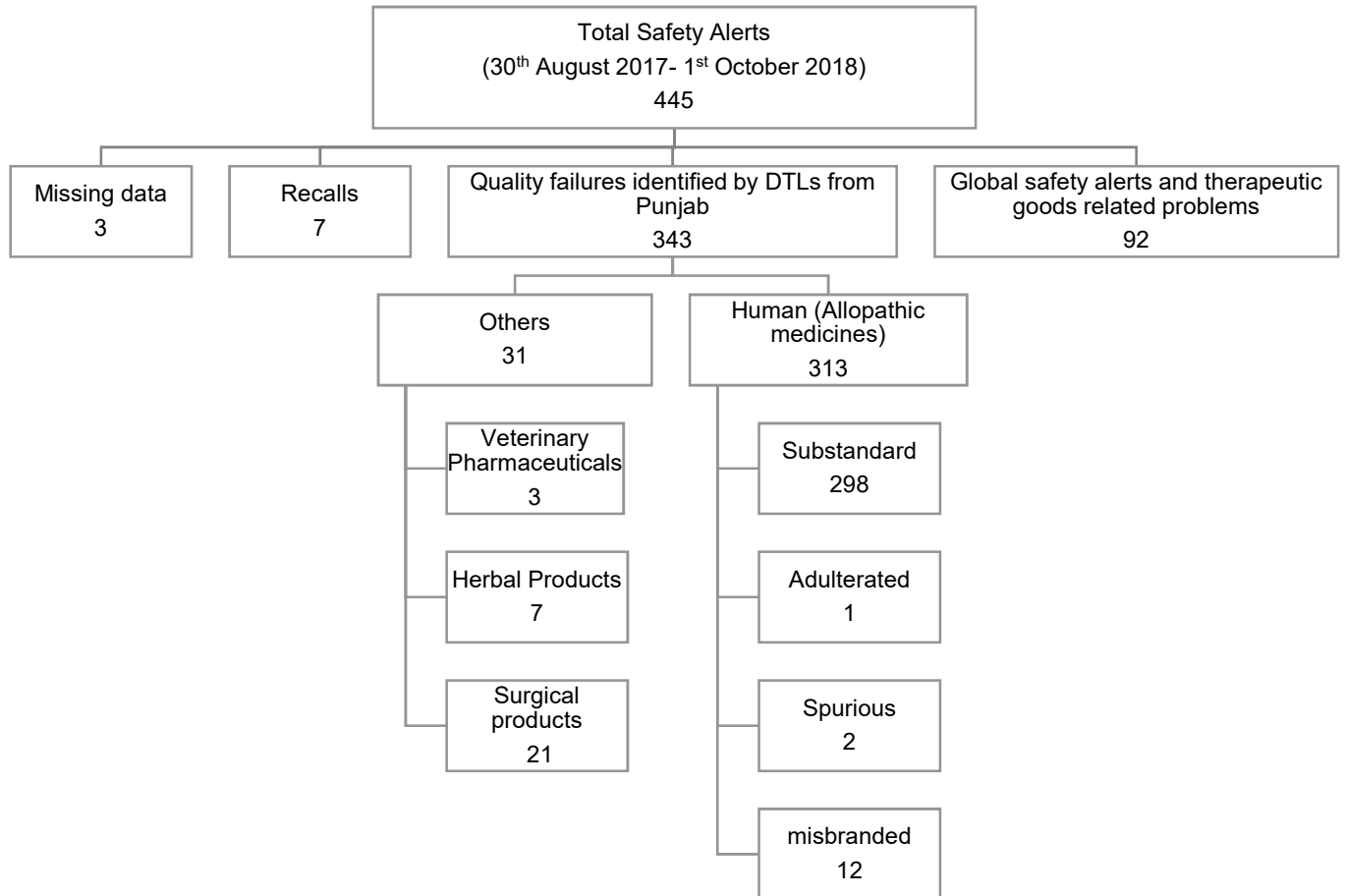


*The terms substandard, misbranded, spurious, and adulterated medicines are according to the definitions given by The Drug Act, 1976 of Pakistan (38)

**Figure 1. Drug safety alerts issued by Punjab Quality Control Unit in 2017 and 2018 (36)**

"Adulterated medicines" also refer to medicines found to be contaminated with dirt or foreign matter (38).

Public accessibility of drug testing data is advocated to promote transparency and to display the status of availability of quality medicines in the country (32). Regarding statistics on quality evaluation and dissemination of safety alerts, the progress of PDCU is exemplary as there are no earlier instances of public sharing of such information in Pakistan (39). However, these figures only represent limited statistics shared publicly by one province. Moreover, all these reports are based upon limited

quality evaluation including physical tests, assay, disintegration, and dissolution tests carried out at the DTLs located in the province of Punjab. Complete compendial testing including impurity tests is not yet covered under the current infrastructure of public drug testing laboratories in Pakistan. Of note, analysis of the national data of recalls by stringent regulatory systems (the UK and Canada) show that contamination (referred to out-of-limit content for impurities and the presence of microbial contamination), stability and packaging defects are the most frequently reported quality failures (40, 41). In view of these figures from well regulated and resourced regulatory facilities, it can be presumed that the countries that omit impurity testing from the routine quality tests may have a higher rate of quality failure than what is reported. This practice weakens the regulatory control, as both industry and regulatory agencies neglect the conduct of impurity profiling placing the population at risk of possible safety issues. The two major cases from Pakistan on quality of medicines are also the result of such neglect. Resource limitation and skilled human resources are the major reasons for this unjustified deliberate omission, followed by other reasons including lack of sensitivity and realization of importance of complete compendial testing.

## 1.6 Literature survey of counterfeit medicines and related studies in Pakistan

The most quoted figure for prevalence of poor quality medicines in Pakistan is 40-50% (42). However, this figure has been criticized for lacking objective data in another publication on drug quality from Pakistan (43). Limited information is available regarding the failure rates documented by public sector DTLs of Pakistan. Out of 9089 samples from the public sector hospitals which were received within three months in 2017, DTL Lahore has reported 3.3% (301 out of 9089) as out-of-specification products (44). A 2010 report states that 2% of approximately 60,000 samples tested in a period of two years at the public sector DTLs failed to comply quality specifications (39).

### 1.6.1 Published information on medicine quality

The case reports, studies, and publications related to counterfeit medicines and related issues reported until December 2017 were compiled and summarised as part of this project to understand the magnitude and severity of the problem of substandard, falsified, and counterfeit medicines in Pakistan. The major sources of information in this regard were scientific publications in peer reviewed journals, research reports,

notifications, and alerts issued by the WHO and other agencies. As media and journalism are the main sources of information for reporting quality issues of medicines, three such reports are also included in the summary (Table 1.1). Drug safety alerts by PDCU were discussed separately in the previous section (see 6.1.5) and are not included in this summary. The published information can be categorized into five classes (cf. Table 1).

## Table 1.1    Summary of published data on the situation regarding poor quality of medicines in Pakistan

| | Reference |
|---|---|
| **A.   International media reports** | |
| 1.   Media Reports on Medicine Quality: Focusing on USAID-assisted countries. 2003-2011 [16 counterfeit cases] | (45) |
| 2.   Stopping fake drugs from Pakistan is too late for victims-2012 [counterfeit drug trafficking cases ] | (46) |
| 3.   Inside deadly Pakistan counterfeit drug trade- 2015 [capacity of regulation and provision of quality medicines] | (47) |
| **B.   Case reports and drug alerts** | |
| 4.   Contaminated drugs are held responsible for 120 deaths in Pakistan (2012) [high dose of pyrimethamine found in cardiovascular drugs isosorbide dinitrate (Isotab), claiming life of more than 120 people] | (31) |
| 5.   WHO drug alert 125- [contamination of batch J093 of Isotab (isosorbide mononitrate) for precaution against the wider circulation of the batch] | (48) |
| 6.   WHO drug alert 126- Levomethorphan contamination in dextromethorphan cough syrup (2012) [Levomethorphan was found in API supplied by the Kanduskar Laboratories, India] | (49) |
| **C.   Investigative analysis of counterfeit cases** | |
| 7.   Epidemic of Plasmodium falciparum malaria involving substandard antimalarial drugs, Pakistan (2003) [generic antimalarial tablets failed the dissolution test, and had high content of active ingredient]. | (50) |
| 8.   Pakistan's deadly cocktail of substandard drugs (March 2012) [chaotic transition of powers and the cases of contaminated drug ] | (14) |
| 9.   Batch J093: Pathology of negligence (2013) [Judicial report of contaminated cardiovascular drug case with evaluation of the regulatory capacity and recommendations to prevent and handle such incidences in future] | (13) |
| **D.   Case referenced in scientific reviews on SSFCS** | |
| 10.   Drug regulators study global treaty to tackle counterfeit drugs (2004) [40-50%] (42) 11.   How to achieve international action on falsified and substandard medicines (2012) | (42) |

| | |
|---|---|
| [Discusses the 2012 fake drug crisis as a possible medicine falsification case if proven that the faulty batch found was found out of specification in the in-house quality control testing and was deliberately allowed to be distributed to hospital] | (51) |
| 12. Substandard drugs: a potential crisis for public health (2014) | (52) |
| **13.** The essential medicines on universal health coverage (2017) [includes fake drug crisis as the major cases of poor quality medicines in the recent years] | (32) |
| **E. Prevalence studies involving pharmaceutical analysis** | |
| 14. Pharmaceutical quality of ceftriaxone generic drug products compared with Rocephin®. [34 generics including 6 products from Pakistan were evaluated on basis of Roche standards and compendial specifications. Overall, sterility test failed for 4 samples and unknown impurity monitored by Roche was found in 5 samples in concentration range of 0.39-1.26%. 30 samples failed the clarity test by USP and 33 products had higher concentration of thiotriazinone (0.22-0.94%, limit ≤ 0.2%).Tricef® from Ali Gohar (Pakistan) failed the assay and content uniformity test, also showing percentage content of thiotriazinone (0.94%) and unknown impurities (1.26%) [2/6] ] | (53) |
| 15. Ofloxacin; Laboratory evaluation of the antibacterial activity of 34 brands representing 31 manufacturers available in Pakistan (2004) [3/34 did not show required antimicrobial activity] | (54) |
| 16. Quality of ceftriaxone injections reality and resonance. (2008)- [15.6% failure rate for ceftriaxone injection] | (43) |

### 1.6.2 Unpublished research reports regarding quality of medicine in Pakistan

1. Ali (55) reported the following cases of substandard pharmaceuticals sampled from various sources in Pakistan in his PhD thesis:

    a. Out of 27 samples of simvastatin API from 24 different sources including China, Italy, Korea, Jordan, and Germany, two samples of Indian origin failed the assay.

    b. Three API samples of cefotaxime sourced from India, Jordan, and Italy were out of specification with respect to impurity content.

    c. 16% of the API samples showed high level of impurities including cefotaxime, glibenclamide, and enalapril.

    d. In total, 22% of all tested oral dosage forms were out of specification.

    e. 22.7% of the products failed dissolution testing including mefenamic acid and diltiazem tablets.

2. Tariq (56) conducted an analysis of ceftriaxone injections from three price categories (low, medium, and high price) sampled from hospitals and retail

pharmacies in Lahore. On analysing the samples using United States Pharmacopoeia monograph and assessing the antimicrobial activity all the results were found to be within the specified limits and showed antimicrobial sensitivity against the tested pathogens.

3. A M. Phil. thesis by Khan (57) reported the assay testing of cefixime capsules from Lahore with TLC and HPLC in accordance with the British Pharmacopoeia method. Out of 14 samples of cefixime capsules, one was identified for low content both by TLC and HPLC assay methods [1/14].

This brief review of literature clearly shows that there is negligible scientific data on the issue of medicine quality in Pakistan with the published data mainly consisting of case reports. The gravity of the problem can be assessed from the emergence of repeated cases of poor-quality medicines in Pakistan. The analytical studies were mainly research projects (PhD or M. Phil. theses) showing mixed results. Academia holds a huge potential for the conduct of such studies, but appropriate technical and financial support is required to develop quality evidence on the subject. None of the initiatives listed above are funded by any public or private funding agencies and thus lack support from major stakeholders including academics, regulators, and manufacturers.

Various barriers and facilitators can be identified from the limited information gathered regarding SF medicinal products in Pakistan. Lack of technical capacity in the field of modern approaches to quality control of pharmaceuticals to combat SF medical products has been clearly established. The key funding institutions like Higher Education Commission and the Central Research Fund (CRF) of Pakistan do not identify the issue as a priority area for provisioning any research funding. On the other hand, international agencies like the WHO and the USP "promoting the quality of medicines" programme are actively supporting capacity building of regulators and industry personnel on the subject (24). However, involvement and ownership of other stakeholders, especially academia and researchers, is still lacking. Collaboration of different stakeholders is crucial to develop sound evidences that can inform policy in a productive manner. Field studies, the use of diverse technical approaches, and studies on tools to quantify and control the issue of substandard and falsified medicine in the country need to be taken up. International literature describes a rich body of data on the prevalence of poor-quality medicines, but mainly raised in sub-Saharan

Africa where the availability of basic field data had resulted in an increased sensitivity to the issue and has driven resources to device solutions with internal and external initiatives (58).

## 2.    Accessible technologies for quality control of pharmaceuticals for low- and middle-countries

A variety of technologies and approaches has been published for the detection of falsified and substandard medicines in developing countries and resource limited settings (59-62) and the comparison of the technologies on the basis of cost, simplicity and performance are also made (60, 61, 63). New technologies and approaches for the determination of the quality of pharmaceutical supplies have emerged in last two decades. Many of the tools suitable for use in LMICs are completely or partially based upon information technology as reviewed by Rasheed et.al.(64). In addition to the modalities discussed in the above literature another valuable approach is of use of intermediary methods established for improving the proficiency and efficiencies of the medicine surveillance systems of resource limited settings (59). Fast, simple and cost-effective HPLC methods for about 10 antimalarial drugs are developed and vaildated for assay and impurity profiling using this approach (59, 65, 66). These methods aim to provide simplicity, reliability and efficiency in the pharmaceutical testing processes at the regulatory laboratories.

## 3.    Global lessons

Clinical failure and increasing resistance to malaria therapy served as a driving force to combat poor quality of medicines in Africa since 1990's. A regional movement was started with antimalarials to which antituberculous and anti-retro viral drugs were added in a later phase. Africa had been the focus of many technological initiatives regarding fast and efficient detection, field testing, and reporting of poor quality medicines (63) including the German Minilab® Project which is successfully run in the continent and has produced significant impact in the reporting of the substandard and falsified medicines.

Rwanda achieved the lowest incidence of substandard and falsified drugs in Africa. It attributes its success to improved country's supply chain, access to high quality medicines in public sector, and drug surveillance system. Coordinated inspections, verifications, and release procedures by the multidisciplinary teams are employed for

importing medicines. A well networked pharmacovigilance system is established containing more than 2400 trained workers (67).

Nigeria has reduced the prevalence of counterfeit substandard and falsified drugs from 41-80% to about 16% (68) by controlling the quality of medicines before crossing the Nigerian borders (69). They ensure the provision of pre-shipment information by the manufacturers/exporters and depute analysts in the source country like India to ensure testing of medicines prior to import.

Höllein et.al. have discussed the two decades journey of the Tanzanian Food and Drug Authority (TFDA) in bringing down the number of counterfeit cases, with its systematic interventions, scientific approach, planning, and international collaboration. Using an intermediary HPLC method, improved analytical facilities, and capacity to test more samples consistently over a number of years had set a deterrent to the counterfeiters (59) .

The WHO prequalification program helped Kenya to reduce the quality failures of medicines. Manufacturers are warned of serious consequences if their product is found to be substandard. The failure rate of samples has reduced from 35-40% to 3-5% as an impact to the strict surveillance procedures by the Mission for Essential Drugs and Supplies (MEDS). MEDS is a non-governmental organization which also owns a WHO accredited quality control laboratory.

Communication of safety alerts and the establishment of a pharmacovigilance system is crucial to the timely identification of poor-quality medicines in the market. Some simple and practical approaches are used in the developed countries as well. "Health Canada" issues the risk communication under five categories applicable to medicine quality, within these the recalls are futher divided into type I-III according to the severity and urgency of the recall (41) whereas drug alerts issued by MHRA are classed in four categories with class 4 not intended for recall and only needs "caution in use" (40). Including the category of recalls is helpful in triggering the appropriate response from target audience.

In Germany, the mechanism of "Rote-Hand-Brief" ("Red Hand Letter" / "Dear Doctor Letter") is used as a safety alert tool with the symbol of a red hand. It is issued by the pharmaceutical manufacturers through the BfArM and the Paul-Ehrlich-Institute (PEI) to communicate information of safety concerns regarding drugs, medicinal products,

vaccines, and biologicals that require immediate action (60). On observing any medicine related quality issue or adverse drug reaction, the health care team members can report e.g. to the "Arzneimittelkommission der Deutschen Apotheker" (AMK) on prescribed performa through different channels which can result in an issuance of a "Rote-Hand-Brief" by the manufacturer after confirming the case by the BfArM. Registered pharmacists and practitioners have access to the on-line software "Identa®" (70) for verifying physical properties of the medicaments which helps in early identification and reporting of falsified medicines in the market. Regular and randomly selected package checking is performed on pharmacy stocks by a pharmacist as a mandatory procedure to identify obviously visible quality defects .

A set of recommendations for Pakistan is provided below based on the brief overview of the acessible technologies regarding quality control of pharmaceuticals for LMICs and important global lessons.

## 3.1 Recommendations for Pakistan

1. **Capacity building**: objective oriented undergraduate training, specifically addressing the training on quality evaluation and pharmacovigilance.

2. **Improved infrastructure**: Mobilization of public and private sector funding for fulfilling essential infrastructure needs and ensuring resource sharing, collaboration and out sourcing for the missing facilities.

3. Adequate funding and promotion of the **research on quality issues** and regional and international collaboration for capacity building and resource sharing (32). Promotion of research in academic institutions should be achieved through development of focused research centres and declaration of SF medicinal products as one of the priority research areas in public sector research funding (58). Industry-academia partnerships for post-marketting surveillance should be encouraged.

4. **Systematic approach to post-marketting surveillance** and increasing efficiency of regulatory laboratories by using strategic sampling and ensuring transparency and public access to data (32).

5. Development of a **pharmacovigilance system** for reporting and efficient action on suspicious medicines. Implementing and encouraging voluntary recalls of substandard medicines identified on the post marketing surveillance activities.

Awareness of public and health care professionals on safe guarding against poor quality medicines by timely identification and reporting should be carried out.

6. **Adopting simple and efficient ideas** in reporting of quality related issues for medicine like "Rote-Hand-Brief" as practiced in Germany. Also ensuring fast networking and stronger communication between regulators and the pharmacy practitioners.

7. Inclusion and promotion of **rapid and cost-effective field testing techniques** for identification of substandard and falsified medicines (59) as well as **intermediary test methods** (simple and cost effective analytical methods) approach for assay and impurity profiling of the key essential medicines identified for quality failures (59, 66).

8. Promoting use of the **WHO prequalification system** and ensuring safety of the supply chain against infiltration of substandard and falsified medicines (26).

9. **Improvement in national GMP standards** to level close to the global GMP requirements set by WHO (55) and adoption of standard internationally accepted definitions regarding quality failures of medicines.

10. A comprehensive multi-component and multidisciplinary **National Action Plan** should be deviced by involving various stakeholders to combat the issue of poor-quality medicines in Pakistan.

## 4. Conclusion

This review has outlined the country situation for Pakistan and the options it has for embarking on the journey to fight against poor-quality medicines. The case reports and investigations listed for Pakistan are suggestive of the need to strengthening of the regulatory systems for premises and GMP inspections, strengthening of analytical laboratories as well as the capacity building on the overall area of controlling counterfeit and substandard medicine in Pakistan. The figure of 40-50% poor quality drugs in Pakistan cannot be defended by the available literature. It is proposed that systematic objective data need to be developed through well planned funded studies for collecting critical statistics regarding substandard and falsified medicinal products in Pakistan.

## 5.    Conflict of Interest

There is no conflict of interest to be disclosed by authors.

## 6.    Author's Contributions

HR and UH conceived the idea as part of HR's PhD research work. HR drafted the initial manuscript which was further refined by inputs from UH and LH. All the authors have read and agreed with the final manuscript.

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## 3.6 Future information technology tools for fighting substandard and falsified medicines in low- and middle-income countries

Mini review

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### ABSTRACT

Substandard and falsified medicines have emerged as a global public health issue within the last two decades especially in low- and middle-income countries. Serious consequences of this problem include a loss of trust and increased financial costs due to less disease control and more frequent complications during therapy. Of note, antimicrobial resistance is an additional long-term implication of poor-quality antimicrobials. This review covers information technology tools including medicines authentication tools as mobile apps and messaging service, 2D barcoding approaches with drug safety alert systems, web based drug safety alerts, radiofrequency identification tags, databases to support visual inspection, digital aids to enhance the performance of quality evaluation kits, reference libraries for identification of falsified and substandard medicine, and quality evaluation kits based on machine learning for field testing. While being easy to access and simple to use, these initiatives are gaining acceptance in low- and middle-income countries. Implementing 2D barcoding based on end-to-end verification and "Track and Trace" systems has emerged as a step towards global security in the supply chain. A breakthrough in web-based drug safety alert systems and data bases was the establishment of the Global Surveillance and Monitoring System by the World Health Organization in 2013. Future applications

include concepts including "lab on a chip" and "paper analytical devices" and are claimed to be convenient and simple to use as well as affordable. The principles discussed herein are making profound impact in the fight against substandard and falsified medicines, offering cheap and accessible solutions.

**Key words:** Information technology; substandard and falsified medicines; field testing; quality evaluation; mobile Apps; medicine authentication tools; Track and Trace.

## 1. Introduction

Low- and middle-income countries (LMICs) are frequently affected by the pandemic of substandard and falsified (SF) medicines. Three quarters of the samples included in the latest World Health Organization (WHO) review were collected in LMICs and show a percentage failure rate of 9.9-10.9% amounting to a crude worth estimate of 30.5 billion USD per annum (1). In May 2017, the WHO revised the definition of substandard and falsified medical products, whereby the term "counterfeit" has been withdrawn from this context (2). In brief, substandard and falsified medicines are differentiated, the latter referring to unclear identity, composition, or source of the respective product.

Among the various solutions and strategies devised for combating poor-quality medicines (3, 4), a substantial portion is based upon information technology tools for fighting falsified medicines (5). Information technology is defined by Merriam-Webster dictionary as *"the technology involving the development, maintenance, and use of computer systems, software and networks for the processing and distribution of data"* *(6).* A variety of technologies and approaches has been published for the detection of falsified and substandard medicines in LMIC and resource limited settings (3, 4, 7, 8) which have also been compared with regard to costs, simplicity, and performance (3, 7, 9). In general, they are easy to operate and access, exhibit low costs, and are very user-friendly (3). The methods are used for initial identification, quality evaluation, as well as an efficient dissemination of information on SF medicines. The WHO proposed a three-pronged approach of prevention, detection, and response in order to tackle the menace of SF medical products (10). This review substantiates the above mentioned WHO approach and covers information technology tools including Medicines Authentication Tools (MAT) as mobile apps and messaging service, 2D barcoding

linked MAT with drug safety alert systems, web based drug safety alerts, Radio Frequency Identification Tagging (RFID) for product tracking, databases for identification of medicinal products to aid visual inspection, digital aids to enhance performance of quality evaluation kits, reference libraries for identification of falsified and substandard medicines employing various analytical techniques, as well as quality evaluation kits based on machine learning for cheap and convenient field testing.

## 2. Classification of IT tools for SF medical products based on usage

All identified information technology tools were classified into five categories based on their field of application (Table 1). The type of tool (mobile application, database, software, or field testing kit), respective examples, its role and the relevance for the end user are incorporated against each application category (Table 1)

### 2.1. Supply chain tracking systems

These are mainly information technology-based solutions used for examining the intact packages at various points within the supply chain up to the consumer level. The concept of end-to-end verification and "Track and Trace" is characteristic for all these services, forming the fundamental basis of a traceable supply chain surveillance. Such systems gain more and more global attention.

### 2.1.1. Medicines serialization and verification technology

Medicines are coded using a unique 12-digit serialization and a 2D barcode system with an end-to-end "Track and Trace" process comprising of the following steps (11):

a) Systematic serialization of the products at the manufacturing site. The serialization code uses block chain technology to enable end-to-end decryption (manufacturer to each user in supply chain) offering retrieval of information by an ePedigree-based digital ledger (5). The individual coding applies to both raw materials and finished pharmaceutical products and allows multiple participants to update tracking information, to authenticate, and also to share information using integrated anticounterfeit devices (5).

b) Authentication, i.e. verification of the product authenticity, by scanning the imprinted codes reveals the "authenticity status" and may include additional information, e.g. whether the product is expired, recalled or falsified. The *European Union Falsified Medicines Directive* (EU-FMD) requires this

authentication to be carried out at the point of supply, whereas the *United States Drug Supply Chain Security Act* (DSCSA) demands a verification on each change of ownership during the medicine supply and distribution process, respectively.

c) Decommissioning of the product, if applicable, is required at the final point of supply to the patient in accordance with EU-FMD.

As soon as a product has been scanned for verification, a color-coded pop-up message appears, guiding the user to take appropriate action, e.g. whether a product must be quarantined or can be handed to the patient, respectively. Such warnings and alerts may also be implemented during stocking, dispensing, and supply of medicines, facilitating the identification of suspected or faulty stocks.

The effectiveness of the system is based upon the practice of the end users, i.e. all medicines requiring authentication must be authenticated eventually and a respective action must be taken, accordingly (11). Examples for technology providers are Aegate Holdings Limited, Arvato Systems GmbH, and Solidsoft Reply; they have been assigned by *The European Medicines Verification Organization* for the provision of medication authentication technology within the European Union (11).

"Track and Trace" systems using 2D or data matrix bar coding is gaining world-wide acceptance. Table 2 summarizes the implementation status in different countries. In January 2012, Turkey became the very first country to implement a fully mobile app based verification system (12). SecurPharm, a consortium of the *Federal Union of German Association of Pharmacists* (ABDA) and other professional organizations, launched its pilot project in 2013 involving pharmaceutical manufacturers, wholesalers, and about 400 pharmacies (13-15). The EU-FMD marks 9th February 2019 as deadline for adopting this technology on all prescription medicines throughout all member states (16).

Table 1. Information technology tools for falsified and substandard medicines and medicinal products

| Category / Application | Name | Function | End user |
|---|---|---|---|
| **Supply chain tracking systems** | | | |
| Mobile apps | *mPedigree 2006* | Product authenticity | Up to patient |
| | *Sproxil 2009* | Product authenticity | Up to patient |
| | *Pharmasecure 2007* | Product authenticity + reporting of suspect SF medicine | Up to patient, regulator |
| | *Epothecary* | Product authenticity | Up to patient |
| | *Medsnap (2011)* | Product authenticity | Up to patient |
| Software + digital repository + Mobile App (based on block chain technology) | Aegate Holdings Limited | 2D barcoding and end-to-end verification | Manufacturer and each change of ownership in supply chain up to patient. |
| | Arvato Systems GmbH | | |
| | Solidsoft Reply | | |
| Radio frequency readable tagging of product packs | *RFID* | Product authentication and tracking to unauthorized markets | Manufacturer/regulator |
| Edible Radio frequency readable tagging of unit doses | *Trutag®* | | |
| **Information dissemination** | | | |
| Database | *Global Surveillance for Monitoring System (GSMS)* | WHO global data base for reporting and processing of information on SF medicines | Global regulators-open access |
| Database | *Medication Quality Database (MDQB)* | USP Data base for reporting and processing of information on SF medicines | Global regulators-open access |
| **Quality evaluation** | | | |
| Mobile app | | To improve visual assessment of spots in Minilab® TLC kits | Pharmacy mangers and regulators in field testing |
| Lab on a Chip | Pharmacheck | Field testing kit | |
| Paper analytical devices | Paper analytical devices | Field testing kit | |
| Spectral libraries | Electronic database for reference spectra and fingerprints with or without machine learning | For identification of falsified medicines using Raman, IR, NIR and CD3+ in lab or field set up. | |
| Digital libraries for physical inspection | Identa® | Field testing, regular physical audits | |

Table 2. Implementation status for 2D bar coding of medicines in different countries.

| Region/Country | Directive/Legislation/ Authority | Issue date | Implementation deadline for 2D barcoding |
|---|---|---|---|
| EU and UK | EU Falsified Medicines Directive (EU-FMD)(17) | 2011 | |
| EU | Commission Delegated Regulation (EU) 2016/161(18) | 9th February, 2016 | 9th February, 2019 |
| US | US Drug Supply Chain Security Act (DSCSA) (19) | 27th November, 2013 | serialization: November, 2018 (revised) complete enforcement: late 2023 |
| China | Serialization Mandate (CFDA) (20) | 21st October, 2015 enactment date 1st February, 2016 | |
| Brazil | National Agency for Sanitary Surveillance in Brazil (ANVISA) (21) | 2009 serialization initiation 2013 draft legislation 2014 final proposal | |
| New Zealand | NZ Health information standards (22) | August 2011 | |
| Canada | Joint Technical Statement on Canadian Pharmaceutical Automated Identification and Product Data Requirements | January 2010, revised February 2012 | 1st December, 2017(23) For vaccines (24) Primary level: January 2017 Secondary pack: January 2018 |
| India | Drug authentication and verification application (DAVA) (25) | July 2015 | 1st October, 2015 Revised to March 2016 and 2017 |
| Pakistan | Amendments in Drug (Labelling and Packaging rules), 1986 (Part I-III) system (26) | June, 2015 | June, 2019 |
| Turkey, Argentina, France, South Korea | Already achieved | | |

### 2.1.2. Mobile product authentication

Mobile applications for medication authentication are accessible for large parts of the population, thus directly reaching the patient. Mobile apps and SMS based medicine authentication tools for detecting falsified medicines were pioneered by mPedigree (Nigeria) in 2006 (27), Sproxil (Nigeria) in 2009 (27), İlaç Takip Sistemi (Turkey) in 2010, and Pharmsecure (Nigeria and India) in 2012. These MATs involve the use of a visible or scratchable code which has been printed on the product package by the manufacturer. The patient sends this code to the respective authentication databases using SMS. In reply, a message is received stating whether the stocks inquired are tagged genuine or fake; scannable bar codes are also provided. In Pharmsecure, customers can also capture a photograph of the suspected medicine and send it to the database administrators. Evidence is subsequently transferred to the manufacturer for further investigation. Hence, an early detection of SF medicines is possible, facilitating a rapid response.

The mobile App MyDawa from Ion Kenya, Inc. also uses scratchable codes and can be used for verifying the authenticity of medicines and related medicinal supplies delivered to the Nigerian market. More than 70 international pharmaceutical companies are using the services of Sproxil in Kenya, Ghana, Nigeria, South Africa, and Mali (27). The mPedigree startup was initiated in Nigeria and is now operational in 12 countries across Asia and Africa.

Physicochemical identifiers are a form of authentication token using invisible imprints on a distinct product unit (27). E.g., the Trutag® technology employs edible micro-tagging of tablets using high purity silica which offers low costs and reliable solutions for improved security measures for products and/or medicines prone to falsification and illegal trafficking (28). The product information can be retrieved anywhere using portable TruTag® optical scanners (28).

### 2.1.3. Radio Frequency Identification (RFID)

RFID tags are applicable to products and respective packages using active or passive chips being able to deliver a small set of information, e.g. regarding origin of a particular product (23). Usually, such systems consist of a transponder or a tag affixed to or carried in the product, transmitting various information to the interrogator or the

reader in form of radio waves or wireless signals, respectively. All fields of logistics can benefit from this technique; commonly, passive tags are used which transmit data only when irradiated with a radio signal from an RFID reader in close proximity, thus not requiring any power supply. Modern hardware is designed in a very inconspicuous manner and is mostly constructed as thin labels or small microchips which can be placed inside a product packaging, thus being invisible at first glance but readable from outside.

Although using this technology is recommended by the *U.S. Food and Drug Administration*, the individual costs per unit are quite high when compared to optical coding techniques. However, particularly in the field of monitoring temperature sensitive medicines, active chips are utilized to constantly record the surrounding temperature and thus, to provide a detailed log at any time during and after transport as well as storage.

## 2.2. Databases for information processing and dissemination

The use of drug safety databases and alerts regarding SF medicines has become an essential tool for an efficient and reliable dissemination of information and control of poor-quality medicines in the global medicine supply chains. A remarkable breakthrough in this context is the establishment of the *Global Surveillance and Monitoring System* (GSMS) by the WHO in 2013. Recent landmark reports on SF medicinal products conducted by the WHO presented a literature review of 100 publications with major data contributed from the *Medicines Quality Database* (MQDB) and the GSMS (1).

## 2.3. Quality evaluation in field testing (on spot and in-time results)

Routine quality evaluation procedures require a sophisticated and expensive infrastructure which prove to be a limitation for LMICs [4]. A few novel approaches of developing mobile, convenient, and affordable field kits using machine learning to identify and quantify a medicine based upon its chemical nature (5) or a unique finger print derived from drug interaction analysis are under development (29, 30).

### 2.3.1. Improving performance of GPHF Minilab® through mobile application

The GPHF Minilab® tool kit, established in 1980's by the Global Pharma Health Fund, pioneers the concept of field testing for detection of counterfeit and substandard medicines. It has been operating in 95 countries across the world. The current edition

of the Minilab® manual covers more than 90 active compounds including essential antibacterial and antituberculotic drugs. The kit employs thin layer chromatography with visual evaluation of the respective chromatograms as main analytical technique. Of note, the tests are semiquantitative and thus not suitable for analyzing the content of compounds where the exact dosage is critical, e.g. in the case of antibiotics. However, the limitation of visual evaluation has recently been overcome by introducing a mobile phone application for measuring and comparing the spot intensity (31). In addition, it was shown that during analysis and evaluation, significant deviations from the true content might occur (32, 33).

### 2.3.2. Pharmacheck

The project aims to deliver a "lab on a chip" concept that can give results by color indicators in the field (30, 34). Interaction analysis was performed using *E. coli* to develop fingerprint results for 27 antibiotics. The project is in its developmental stage and aims to provide a fast, low cost, and portable technology that is applicable to the most remote settings. No sample preparation, electric supply, and only minimal training is required (3).

### 2.3.3. Use of spectral libraries for vibrational spectroscopy

Spectroscopic libraries are required in Raman and near infra-red (NIR) spectroscopy as well as in newer technologies like the CD3+ counterfeit device approved by the U.S. Food and Drug Administration or Paper Analytical Devices. These techniques may or may not be linked to machine learning tools for the identification of SF medicines (3, 5, 9), exhibiting low to medium costs and representing valuable tools for detecting SF medicines (3, 5, 9).

### 2.3.4. Use of digital libraries for aiding visual inspection

Visual inspection of the primary and secondary product packages as well as the respective dosage forms holds a key position in immediate identification of falsified medicines. A respective scheme is offered within a WHO guideline (8, 35). In Germany, registered pharmacists and practitioners have access to the online software Identa® for physical identification of medicaments, thus facilitating an early identification and reporting of suspicious medicines. The recall of falsified Pegasys® injection in Germany in 2013, identified during the routine internal audit process, is a prominent example of unravelling falsified medicines using this tool (29, 36).

## 3.   Discussion and conclusion

The constant rise of various types of falsified and substandard medicines demand the incorporation of a fast and effective identification of poor-quality medicines throughout the global supply chain. Efficient and reliable processing of information as well as dissemination of alerts is warranted to minimize the damage caused by the administration of any faulty medicines. Information technology has placed a significant role in providing solutions to both these needs. The majority of currently available applications comprise of medicine authentication tools involving verification of the product packaging. Identification of falsified medicines based upon their active pharmaceutical ingredient (API) content is also anticipated. However, very few applications claim to inform on the amount and purity of the API and innovative approaches are needed to offer accessible solutions in this regard.

Implementing 2D and data matrix barcoding has come up as a unified global strategy. Countries have placed this global intervention program within their respective health system at varying pace. Phase wise realization is seen, with the first step being verifying the serialization and data banking at a local repository (fixed data), proceeding to the second level of enabling data access using a cloud-based repository accessible by multiple users to receive, share, and update information. Equipping pharmacies with the respective soft- and hardware devices including barcode scanners is also a major part of this process. The final stage of the "Track and Trace" process enables a proactive bidirectional system of sending alerts and product warnings that could be linked to electronic prescribing portals to ensure maximum patient safety. However, costs and lack of awareness about the barcoding system had been the major impeding factors in its implementation process. The USA have postponed (37) the process to several deadlines and so is the case with Pakistan, where a large manufacturing industry exists. It is yet to achieve the first milestone of elevating a *Global Trade Identification Number* (GTIN) and serialization to the level of primary packaging by all manufacturers.

India and China are the two main global suppliers of raw materials and finished pharmaceuticals, and their compliance with the implementation of GS1 standards using barcoding holds crucial importance in the success of an end-to-end "Track and Trace" process. The Indian *Drug Authentication and Verification Application* (DAVA) introduced in 2012 was an award-winning project (38). Contrary to that, China has not

yet adopted the global concept of serialization and a separate *China National Drug Code* with serial numbers is issued through its own *Product Identification, Authentication and Tracking System* (PIATS) (21).

Implementation of product serialization and barcoding enables end users, including patients, to identify the authenticity of a product, e.g. through mobile phones equipped with a suitable application for reading the barcodes. This broader accessibility of product identification tools down to the patient level will create more public awareness and a participatory approach for the identification of poor-quality medicines in the supply chain. Increased sensitivity at the consumer level and their engagement in implementing a collective scrutiny solution regarding falsified medicines (5) is particularly important for LMICs where most of the health expenditure is covered by out-of-pocket expense.

"Track and Trace" is a proactive system that updates consumer on the product safety and authenticity at each stage of usage. The collaboration of several systems (e.g., GSMS, Vigibase, and "Track and Trace") will provide a major impact in the delivery of safe and efficacious medicines worldwide. GSMS and Vigibase are two major global data bases, with GSMS targeting only registration of SF medicines, whereas Vigibase is based upon pharmacovigilance. Currently, LMICs are only minor contributors to the global data base on pharmacovigilance. The implementation of GSMS in the WHO m

ember states will increase the sensitization of authorities and masses over drug safety issues.

Certain regions and countries face poor access to medicines due to failure of registration status, unavailability of particular products, high potentials for drug abuse, corruption or pricing issues, all of them representing predisposing factors to illegal medicine trade and diversion (1). Tracking is inevitable to ensure that funded and/or donated supplies reach their intended destinations, of note particularly including conflict and disaster-ridden regions. RFID is the best resource for tracking any unauthorized movement of medicines because of the unique technology design and the possibility to extract and store the respective information in web-based portals or server systems at any time (5). In contrast to 2D barcoding, RFID technologies can be used to identify a product even if it is not in the line-of-sight of the scanner and is detected automatically when in range of the receiver (23). Readers can be portable,

mounted on a post or over head or built in the construction of storage space or buildings, (39) thereby increasing the chances of detection of unauthorized movement of the product or consignment. Moreover, radiowaves can penetrate several layers of packaging and allow batch reading of multiple items at a time (23).

The availability of digital product identification catalogues like "Identa®" for making regular internal audits for stocked medicines invites retail pharmacist to share the role of a regulator in identification of falsified medicines (40). This cost-effective, proactive, and participatory model of fighting against SF medicines practiced in Germany can prove to be effective in LMICs. Availability of such software to health care professionals through mobile applications can make it more user friendly and applicable in remote settings. Training of the field regulatory inspectors on use of such aids can enhance the impact of the intervention. Information technology and digital aids are having a profound impact in the fight against SF medicines and constitute a promising area offering cheap and broadly accessible solutions.

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## 6. Author contributions

HR conceived, designed the review, and wrote first draft of the manuscript; LH and UH refined the draft, and provided intellectual input. All authors contributed to manuscript revision, read, and approved the submitted version.

## 7. Conflict of interest

There is no conflict of interest to disclose.

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# 4

# DISCUSSION AND CONCLUSION

## CHAPTER FOUR

## 4.1. Discussion

The aim of the study performed was to conduct quality evaluation of beta-lactam finished products from Pakistan by carrying out assay and impurity test as well as identify presence of any unknown/new impurities, to prepare and isolate beta-lactam antibiotic impurities and to develop simplified and cost-effective HPLC methods for the quality evaluation (impurity profiling and assay) for their selected molecules.

### 4.1.1. Analysis of FPPs of beta-lactam antibiotics from Pakistan-Fighting the fake's figure

The current study presents a percentage failure rate of 58% (7/12) for amoxicillin and ceftriaxone finished products sampled from Pakistan. The observed ratio of failed samples and the range of API content were 4/9 (range: 88.2-90.0%) and 3/3 (range:89.3-90.7%) for amoxicillin and ceftriaxone FPPs, respectively showing moderate deviation from the compendial specifications. All the three ceftriaxone brands from Democratic Republic of Congo (DRC) failed the assay (range: 78.3-83.6%) with significantly lower assay values (p= 0.04) as compared to the assay of samples from Pakistan. Only three of the expired samples of coamoxiclav analysed in this study, had low content for clavulanic acid (CA) with assay of 1%, 52% and 88%. The sample showing complete deterioration of CA also had a low amoxicillin content (81.8%) and was documented for missing the desiccant pouch in the container during the physical inspection of the product. Few of the coamoxiclav tablets (4/10) were detected with individual impurities higher than 1% and 3 out of10 samples had amoxicillin dimer concentration close to the acceptable limit of 2%.

Prevalence of poor-quality medicines in Pakistan is quoted to be 40-50% (1-3) but this figure is criticized by Obaid for lacking objective data (4) whereas a general sample failure rate of 2% is reported by the public sector Medicines Quality Control Laboratories of Pakistan for the year 2009-10 (5). The results for assay of ceftriaxone injections by Obaid showed a failure rate of 15.6% (according to USP limits: 90.0-115%) with 6 out of 14 substandard samples found in moderate deviation (88<90% and 115<119%) from the compendial limits (4) The lowest assay values for ceftriaxone injection from Pakistan are of 92.6% and 96.4% as reported by Sana et al and Lambert, respectively (6, 7) which are within the acceptable limits.

Recently, a public sector Drug Testing Laboratory in Pakistan has found low clavulanic acid content (76%) in a batch of coamoxiclav from brand reported as substandard in the current study, thus supporting the results of this study (8). The Southeast Asian

and Asia Pacific regions surveillance studies have reported content of amoxicillin capsules in the range of 70-90% and 81-89%, respectively which again is close to the results obtained for amoxicillin in this study (9, 10). Substandard amoxicillin has also been reported in the studies using Minilab Kit and HPLC analysis in Nigeria, Ghana, Thailand and United Kingdom (11, 12). A longitudinal study carried out in 1991-92, concluded that amoxicillin finished product is not vulnerable to harsh storage conditions (13), hence implying that the main reason for substandard product is noncompliance to standards of Good Manufacturing Practices (4). However, low sample size and restricted sources of sampling might be the reason for a higher failure rate reported in the current study (58%), in comparison to the above quoted studies.

A sample of amoxicillin capsule sampled from retail outlets located outside a tertiary care public hospital in Karachi contained 88.3% of claimed API content in contrast to the two samples of the same brand (MNC1) showing 100.4% and 96.6% content that were sampled from Lahore and Gujranwala, respectively. These results pose the chances of the sample from Karachi being falsified and similar suspicion of falsification was also reported in a study for ofloxacin FPPs sampled from various cities of Pakistan (14). Internationally, in 2014, WHO has issued an alert on falsified amoxycillin tablets from Niger (15). The results therefore, reinforce that the essential antimicrobials with high sale volumes are also prone to falsification in poorly regulated markets (16).

It was found that unlike amoxicillin samples, the impurity content increased significantly with the age of coamoxiclav samples (Chapter 4-Figure 4a-c). Protection from humidity and product age was found critical for coamoxiclav formulation. The grossly substandard samples with respect to clavulanic acid were also discolored and produced a clear sediment when attempted to disperse in water. The reason of degradation can be either poor formulation, poor packaging quality or wrong package design, bad storage or combination of these.

The results of this study also second the opinion that the physical inspection should not be overlooked in quality of medicines related surveys(17) as is documented in a recent review on SF medicines with only 2 out of 15 studies having carried out packaging analysis (18).

## 4.1.2. Development of simplified and cost effective HPLC methods for the quality control of beta-lactam antibiotics

Development of simplified and affordable HPLC based quality control methods for beta-lactam antibiotics was found challenging because of many factors discussed below. Most importantly, the wide difference in physicochemical characteristics of parent molecules and various impurities made it challenging to elute them in a simple isocratic system based on mobile phases of phosphate buffer and methanol. Not all of the analytes can be quantified using a single method and additional methods might be employed in such cases for the remaining impurities. Separate methods for a particular impurity is also warranted when multiple methods are used for synthesis of API as different set of impurities are produced by each method. In the current study, it was inevitable to develop a separate method to quantify a known impurity of ceftriaxone that is found in API on using a particular synthesis method. Monographs of ceftazidime and ampicillin by Ph. Eur. and USP also employ multiple methods for complete impurity profiling API (Chapter One-Table 1.8). A set of two methods are used in compendia for complete impurity profiling of clavulanic acid raw material one being reverse phase HPLC and other based upon gas chromatography.

The developed impurity profiling methods were able to achieve the sensitivity of 0.03% for most of the analytes except LOQ of 0.04%, 0.05% and 0.43% for AMC-Imp D, AMX-Imp C, and CTX-Imp A, respectively.

Optimization of pH was found to be the critical step in the method development for impurity profiling of coamoxiclav and ceftriaxone. Moreover, the unstable nature of beta-lactams warranted detailed investigation of solvent to ensure maximum stability and solubility of the targeted analytes.

Beta-lactam molecules possess characteristic amino and carboxylic group as part of their primary structure and hence possess multiple acidity constants. The acidity constant played as an important predictive tool for the solubility owing to the ionic state of the molecule at different pH (19).

In the current study solubility of analytes was found highly dependent on the pH of the solution, 7-aminocephalosporanic acid and deacetyl ceftriaxone were only soluble at pH $\geq$ 6.6. It was also found that ceftriaxone impurity D was unstable in methanolic solutions and the same was supported in literature (20). Stability challenges are also reported by the Liu et al. in the process of method development for cephalosporins

(21) whereas the acetate buffer, pH 4 is reported by Tian et al. to cause formation of reversible isomer ceftriaxone.(22).

High molecular weight polymeric impurities also place a challenge in method development by requiring a longer elution time. The dimeric impurity of amoxicillin (AMX-Imp J) is eluted at 45 min and 58 min in the gradient elution method by Ph.Eur. and the developed isocratic method, respectively. The rel. RT was around 4.5 in both cases.

Moreover, a single run-based assay and impurity method as developed for ceftriaxone in the current study ensures that the less number of samples are needed to be prepared, saving time and consumables needed for multiple chromatographic measurements. Some ion pair chromatography methods used in compendia also provide this added advantage in their methods e.g., cefixime and ceftriaxone (23, 24). However, the current method has an added advantage of being a simple RHPLC method not requiring expensive ion pair reagents.

The need of the additional impurity standards also adds to the cost of the analytical method and is avoided by *in-situ* methods of preparation of impurities in this study. Though beta-lactam antibiotics are old and time-tested molecules, the results of current study and several recent reports (19, 22, 25, 26) emphasize the need of investigation of physicochemical properties of beta-lactam molecules and their impurities. Tange et al. have reported the effect of impurity C of ceftriaxone on formation of its precipitates with calcium (25). This incompatibility is proposed to be the underlying cause of adverse effects of ceftriaxone  and is also studied with respect to brand variations for ceftriaxone injection (26).

## 4.1.3. Development of simplified and cost effective HPLC methods for the quality control of ceftriaxone

New information on the stability and analysis of ceftriaxone were identified during this study which will help in better handling and quality control of the molecule in clinical and laboratory settings. These include: 1) relation between day light exposure and production of impurity A (the inactive *E*-isomer of ceftriaxone), 2) *in-situ* method of preparation of a 'new' impurity, deacetyl cefotaxime, and identified for the first time in the ceftriaxone CRS, 3) a simple, cost effective reverse phase HPLC method based on isocratic elution using phosphate buffer and methanol for assay and impurity profiling of ceftriaxone was developed and validated and 4) a supplementary method

for quantification of impurity D of ceftriaxone and 2-mercaptobenzothiazole the toxic by-product of ceftriaxone synthesis, also an impurity of impurity D.

The analyte 2-mercaptobenzothiazole is of critical importance from safety stand point as the literature reports its difficult removal from the final product i.e., ceftriaxone (27) and is stated to have carcinogenic potential (28). It is to be noted here that the use of impurity D in the synthesis of ceftriaxone is not employed in all methods (29). Of note, the synthesis method employing impurity D as starting material also claims to be producing high yield (30) thus making them potentially a preferable synthesis approach in the markets of API catering developing countries. In July 2018, the API of antihypertensive medicine Valsarten produced by a Chinese source is recalled from the global markets following the identification of a carcinogenic impurity. This case emphasizes the need of post marketing studies to rule out the presence of 2-mercaptobenzothiazole in ceftriaxone sampled from global market.

It is shown in this study that an approximate 2% concentration of impurity A is produced within 24 h by exposure of ceftriaxone solution to daylight. Light protection of ceftriaxone has been recently introduced in the USP 40 for all analytical solutions containing ceftriaxone (31). However, no explanation was available in literature for the reason of change in monograph. Similar instructions for application in clinical settings are yet not found and the product is packaged and marketed in transparent glass bottles instead of amber glass or light protected packing. Hence, the conversion of API to the inactive isomer (CTX-Imp A) should be further investigated in clinical settings at uncontrolled temperature, in varying concentrations, solvent and additives and this information might provide a reason for the loss of efficacy of the injectable in different settings and formulations.

One possible reason for overlooking the formation of E-isomer of ceftriaxone (CTX-Imp A) for a long time can be the close resolution of ceftriaxone API, ceftriaxone-3-ene isomer (rel RT= 1.3) and CTX-Imp A (rel.RT= 1.4) on the compendial method settings, as observed in the sample chromatograms provided by the supplier for the certified reference substance of ceftriaxone (Figure 4.1). During experimentation in our study compendial method provided better resolution between API and its E-isomer than the sample chromatogram suggesting that the resolution is dependent on the type of the column used (Figure 4.2).

The system suitability test in the compendial method is carried out using a dilute solution of ceftriaxone and impurity A (50 µg/mL) with target resolution of ≥ 3.0 and a

tailing factor of API peak ≤ 2. However, the sample chromatogram provided with CRS shows incomplete separation of the impurity peak from the tailed API peak in the test solution concentration (0.3 mg/mL) (Figure 4.1). No information in literature could be found for the ceftriaxone-3-ene isomer mentioned in the list of impurities of ceftriaxone by USP 40 (31, 32).

The current methods provide a clear advantage in this respect by being able to elute *E*-isomer as a last signal in the method. This explains why the issue of light sensitivity and conversion of ceftriaxone to its *E*-isomer triggered by daylight was not caught earlier by the analysts and is identified for the first time in the current study.

In addition to above, wide differences in the threshold limits for the specified impurities for ceftriaxone are identified between the Ph.Eur., BP and USP (Appendix II). International pharmacopoeia by WHO mandates the harmonization of the limits of impurities stated by different pharmacopoeia. Hence, harmonized specifications with respect to impurities of ceftriaxone need to be sought to ensure patient safety.

The literature search has also revealed the use of an additional impurity test method by Roche (7) along with the current compendial RP-ion pair chromatography method for complete impurity profiling (23). This reported additional method (method 2) is a modification of compendial method (23) uses 70% v/v concentration of acetonitrile instead of 50% v/v stated in the compendial method with a flow rate of 1 mL/min in place of 1.5 mL/min. The study reports presence of an unknown impurity by all the generic ceftriaxone products but not by Rocephin, the product of Roche (7). However, no information on the nature of the "unknown impurity" is provided.

A prolonged run on the compendial method was carried out which showed two additional peaks (peak 7 and peak 8 in Figure 4.2) appearing at 20.9 and 6.8 min. Further investigation is needed for identification of these unknown peaks and the unknown impurities reported by Lambert (7).
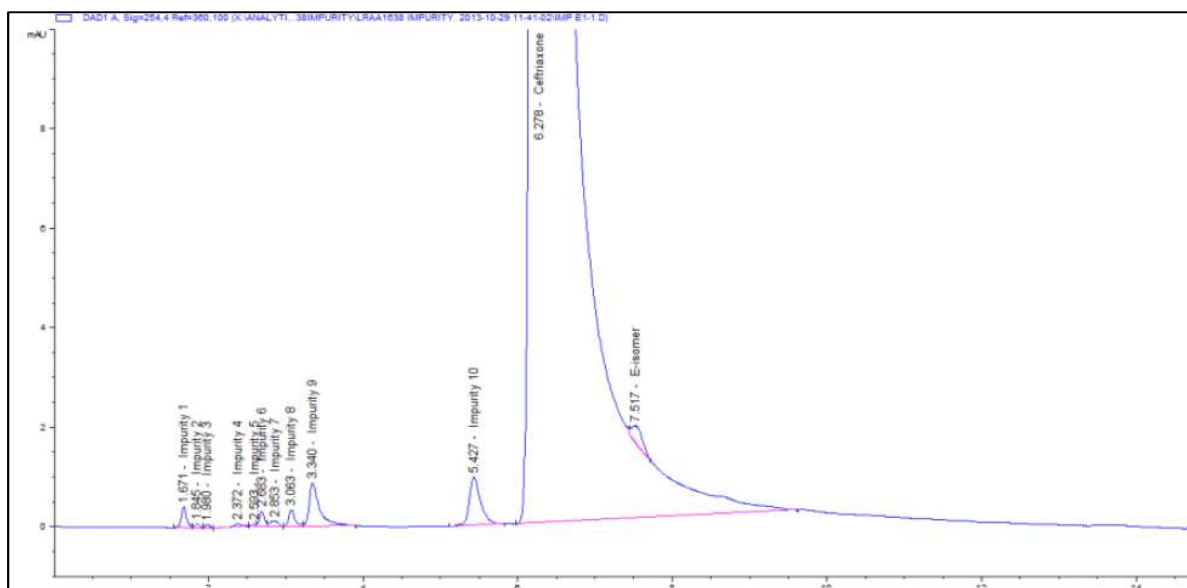
Figure 4.1.   Sample chromatogram supplied by Sigma Aldrich in certificate of analysis of ceftriaxone CRS (Lot: LRAA1638)
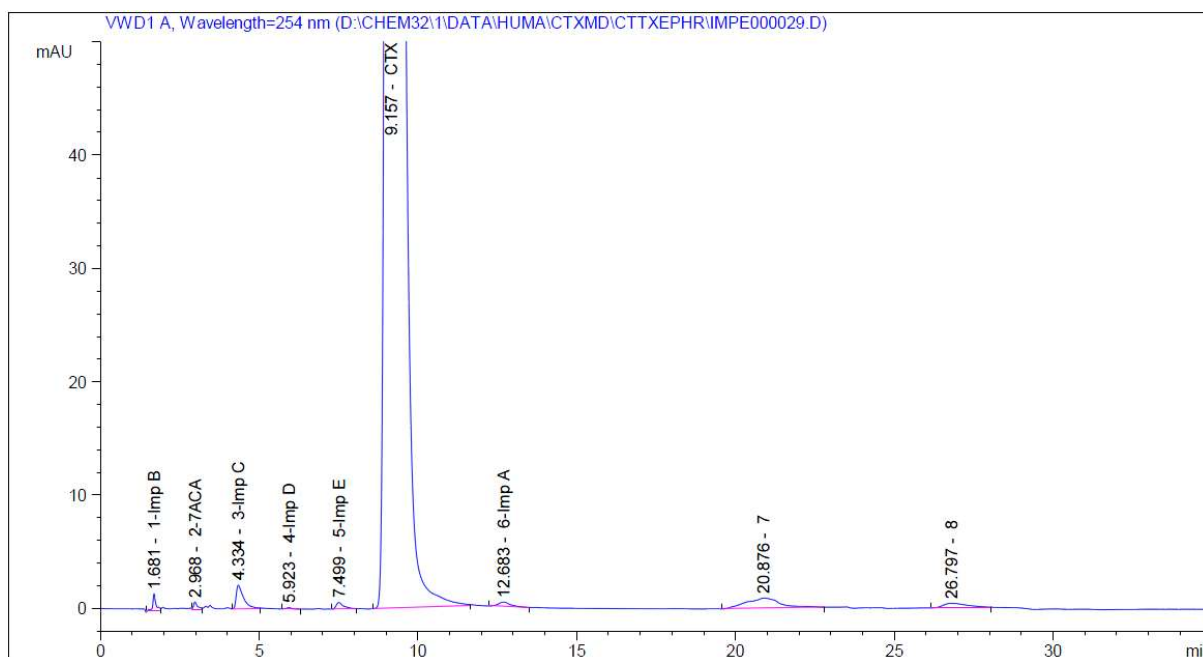


Figure 4.2.   Sample chromatogram of ceftriaxone standard analysed using compendial method (described in chapter 4). Impurity A (*E*-isomer) has rel. RT of 1.4 whereas impurity ceftriaxone 3-ene isomer has an rel. RT of 1.3.

## 4.1.4. Development of simplified and cost effective HPLC methods for the impurity profiling of coamoxiclav tablet

The developed method for impurity profiling successfully resolves the API peaks from the impurities of amoxicillin as well as from the secondary peaks produced by clavulanic acid, amoxicillin peaks CRS with a resolution of ≤1.5. Method was

# Discussion and conclusion

developed according to the laid criterion for simple and cost-effective analysis, discussed earlier and was validated for linearity, precision and accuracy and had shown limit of quantification of ≤ 0.05% for the available impurities of amoxicillin. The previously reported methods for amoxicillin are mostly for carrying out the assay. However, a gradient elution impurity profiling method for amoxicillin is reported using pH 2.1 (33). An isocratic method using methanol- acetic acid 1.25%v/v- (20:80 v/v) as the mobile phase solvent system are studied for partial impurity profiling and method development (34, 35). Analysis of fixed dose combinations of amoxicillin are reported using a validated HPLC method for the quantification of amoxicillin and clavulanic acid in suspensions (36), a few isocratic methods are reported for partial impurity profiling of amoxicillin in finished dosage form (12, 34, 37) resolving the key starting materials used in the synthesis of amoxicillin.

However, no impurity profiling method was reported for the fixed dose combinations of amoxicillin and clavulanic acid. The compendial method used for coamoxiclav tablet employs the gradient elution method for impurity profiling of amoxicillin without any modification. The method is based upon 0.05 M phosphate buffer, pH 5.0 and employs acetonitrile as organic phase. The phosphate buffers are reported to be more prone to precipitation with the use of acetonitrile. Methanol used in the developed method has higher solubility for phosphates. Moreover, the compendial method requires equilibration at a very low organic content (2% v/v) which on long term use can cause change in phase characteristics of the C18 column. An organic concentration of above 5% v/v is advised for safe use on routine C18 columns. Hence, the developed method offers a simple, cheap and efficient analytical solution for quality control of coamoxiclav tablets as the use of acetonitrile as well the use of gradient elution system and high cost ion pair reagents were avoided to ensure that the method is applicable for routine quality control in low- and middle-income countries.

With the availability of impurities of clavulanic acid and remaining known impurities of amoxicillin the unknown peaks already resolved in the developed method can also be identified. The developed method can also be validated for assay and impurity profiling of single ingredient preparations of amoxicillin and other combination dosage forms of coamoxiclav including suspension and extended release tablets.

## 4.1.5. Synthesis and identification of impurities of beta-lactam antibiotics

Preparation of impurities using *in-situ* methods offer cost-saving by avoiding purchase of high cost reference standards for impurities. Forced degradation of APIs has been used in pharmacopoeia for preparation of impurities and this technique is successfully employed in the current study for generating impurities for ceftriaxone and amoxicillin. Study of the similar molecules like cefotaxime and ceftriaxone provided important information and has helped to identify a new impurity in ceftriaxone CRS. Preparation of impurity C and J of amoxicillin was done in this study by using simple hydrolysis and crystallization as described in an analogous method for ampicillin (38). The use of 10% w/v glucose solution as solvent resulted in the complete conversion of the amoxicillin to the set of these two impurities easily crystallizable by lowering pH thus avoiding extensive purification as employed in the previous studies (39). It was observed that changing the concentration of amoxicillin can alter the ratio of Impurity J and C of amoxicillin in the product

Impurity E of ceftriaxone was prepared using a synthesis scheme stated for the preparation of thiazolyl compounds (40). The current study showed that the formation of two known impurities of cefotaxime, deacetyl cefotaxime and decetyl cefotaxime lactone can also be carried out using ceftriaxone in place of cefotaxime, showing their similar degradation pattern (21, 41).

The current study provides the first report on the production of *E*-isomer of ceftriaxone on exposure to daylight which is an important information to be used in the handling of pharmaceutical both in laboratory and clinical settings to ensure its optimum stability.

*In-situ* preparation of impurities is important process in making the impurities available for the analytical and biological evaluation purposes and can reduce the cost tremendously. Moreover, the investigation of the *in-situ* methods reveals important information on the stability and degradation pattern of the molecules which is crucial in understanding of the safety and efficacy of the formulations. Availability of simple *in situ* methods can aid the scientific investigations on impurities of beta-lactam antibiotics, which are otherwise either not available as commercial products or are extremely costly.

## 4.2. Conclusion

The study was able to contribute important scientific information on the essential beta-lactam molecules by provision of new simple and cost effective HPLC based impurity profiling methods for coamoxiclav tablets and ceftriaxone applicable to resource limited settings of LMICs. The study provides novel information on ceftriaxone impurities, its instability in daylight by formation of its *E*-isomer and the possible presence of toxic impurity (2-mercaptobenzothiazole) and the method to quantify. One impurity (deacetyl cefotaxime) was reported from the CRS of ceftriaxone for the first time. Hence, these investigations offer a step forward towards the safe use of this clinically significant molecule.

Forced degradation was found as a promising tool for developing *in-situ* methods for impurity preparation in this study which were used to design new synthesis methods of impurities of amoxicillin and ceftriaxone. The study can be extended to other closely related molecules and to other formulations for understanding the stability and identification of potential "new" impurities as well as to provide further low cost and effective simplified HPLC methods for beta-lactam antibiotics. A partial impurity profiling method for clavulanic acid can also be generated using the method developed for coamoxiclav impurity profiling.

The method for ceftriaxone entails detailed cost evaluation and it is recommended that the cost analysis be included in the analytical methods development for the essential medicines intended for use in low- and middle-income countries. A standardized template can be developed to ensure the homogeneity of the collected data in this regard for its practical application by the decision makers in regulatory sciences.

Hence, beta-lactam antibiotics are vulnerable to quality failures and should be part of the surveillance programs carried out at national level in Pakistan. A 3- or 4-tier strategy based upon risk-based surveillance strategy can provide a more comprehensive, fast and cost-effective information on assessment of the problem in the pharmaceutical supply chain. Physical inspection of the samples and package was of crucial important, for reliable assay and performance test.

The study provides clue that Pakistan faces the problem of poor-quality medicines at different levels of supply chain. However, a conclusive picture can only be drawn using a comprehensive study. Promotion and facilitation of research from the public and private funding sources and awareness of the health professionals and public on safeguarding against the problem is important to curtail the problem of poor-quality

medicines.

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# SUMMARY

## ENGLISH & GERMAN

**Summary (English)**

Assay and impurity profiling of the pharmaceuticals are the key routine quality control methods employed worldwide for which High Performance Liquid Chromatography (HPLC) is the most widely used technique. The ability to carry out these routine laboratory procedures in low- and middle- income countries (LMICs) need the methods to be based upon simple instruments manageable with moderate levels of personnel skill and costs involved.

Simple, convenient, and cost effective reverse phase HPLC methods were developed using phosphate buffer and methanol as mobile phase with C18 column as stationary phase for the impurity profiling and assay of beta lactam antibiotics. Isocratic elution and UV detection was employed in these methods. Impurity profiling method was developed for coamoxiclav tablets and ceftriaxone bulk drug. The method for ceftriaxone included a supplementary method to quantify one of its known impurity (Impurity D of ceftriaxone). This method involved use of acetonitrile where as the two main methods were achieved on the targeted method design, described above. With the exception of impurity A of ceftriaxone, the methods developed can successfully quantify impurities to the concentration as low as ≤0.05%, which is in accordance with the current guidelines for the impurity profiling of antibiotics issued by European Medicines Agency.

As ensuring cost reduction was one of the key objectives of carrying out the method development exercise, *in situ* methods for the preparation of impurities were also identified and some new methods were introduced. The stability of beta lactam antibiotics and the choice of solvent were given due attention during the process of method development revealing information on the presence of new impurities. Deacetyl cefotaxime and 2-mercaptobenzathiazole were identified in this process as new impurities of ceftriaxone currently not listed under known impurities by United States Pharmacopoeia and European Pharmacopoeia. However, deacetyl cefotaxime is a known impurity of cefotaxime whereas the latter molecule is a degradation product of one of the synthesis impurities of ceftriaxone. This substance is reported to be carcinogenic and is resolved using the supplementary method developed for ceftriaxone, hence making its detection and quantification possible. A known inactive impurity of ceftriaxone (Impurity A, E-isomer of ceftriaxone) was` also shown to be

produced by exposure to day light, thus warranting the light protection of the ceftriaxone solution, an information that is of critical importance in the clinical settings.

A series of experimentation was carried out on the finished products of beta lactam antibiotics sampled from Pakistan and few other countries, to identify key quality issues in the samples. Though the limited sample size and convenient sampling did not provide results that could yield a decisive figure for the country status for prevalence of substandard and falsified medical products, but the experiments have clearly indicated that the problems in drug quality do exist and beta lactam antibiotics form a class of high-risk medicine with respect to surveillance for poor-quality medicines. Isolation of unknown impurities was also carried out along with the introduction of new and modified methods for preparation of impurities of beta-lactam antibiotics.

In addition, detailed literature survey was carried out for understanding the complex problem of the poor-quality medicine, impact of poor quality antimicrobials on health care system and the magnitude of the problem at the global level. The country status of Pakistan regarding quality of medicines was recorded based upon the available documentary evidence. The current technologies and strategic options available for low- and middle-income countries in aiding fight for combating poor quality medicines was also laid down to design recommendations for Pakistan. A comprehensive review of the information technology tools used for identification and control of substandard and falsified medicines was also conducted.

## ZUSAMMENFASSUNG

Für die Bestimmung der Reinheit und des Gehaltes von Arzneistoffen wird weltweit maßgeblich die Hochleistungs-Flüssigchromatographie (HPLC) eingesetzt. Damit die entsprechenden Methoden auch in Entwicklungs- und Schwellenländern angewendet werden können, müssen sie zur Verwendung mit sehr einfachen Messgeräten geeignet sein und von auch weniger gut ausgebildetem Personal durchgeführt werden können. Zudem sollten die Kosten möglichst niedrig sein.

Für die Reinheitsanalytik sowie die Bestimmung des Gehaltes von Betalaktam-Antibiotika wurden einfache, praktische und kostengünstige chromatographische Methoden unter Verwendung von Umkehrphasen entwickelt, die als mobile Phase Gemische aus Phosphatpuffern und Methanol sowie als stationäre Phase $C_{18}$-Säulen verwenden. Die Methoden sind isokratisch, die Detektion erfolgt mittels UV/Vis-Spektrometer. Für Coamoxiclav-Tabletten sowie den Arzneistoff Ceftiaxon wurden Methoden zur Reinheitsprüfung entwickelt. Für die Reinheitsprüfung von Ceftriaxon wurde eine zweite Methode benötigt, um eine der bekannten Verunreinigungen (Ceftriaxon-Verunreinigung D) zu quantifizieren. Hierbei musste Acetonitril als Bestandteil der mobilen Phase gewählt werden, wohingegen die beiden Hauptmethoden dem beschriebenen Methodendesign folgten. Außer im Falle der Ceftriaxon-Verunreinigung A können mit den Methoden Verunreinigungen bis zu einem Konzentrationslevel ≤ 0,05 % bestimmt werden, was den Vorgaben aktueller Richtlinien für die Reinheitsanalytik der Europäischen Arzneimittelbehörde entspricht. Da es ein Kernziel während der Methodenentwicklung war, die Kosten für die Analytik möglichst gering zu halten, wurden in situ-Methoden für die Gewinnung von Verunreinigungen entwickelt und einige neue eingeführt. Während des Entwicklungsprozesses wurde besonders auf die Stabilität der Antibiotika und auf die Wahl des Lösungsmittels geachtet, wobei Erkenntnisse über die Anwesenheit neuer Verunreinigungen erlangt werden konnten. Desacetylcefotaxime und 2-Mercaptobenzathiazol wurden als neue Verwandte Substanzen von Ceftriaxon identifiziert und sind derzeit nicht als bekannte Verunreinigen in der United States Pharmacopoeia sowie dem Europäischen Arzneibuch aufgeführt. Desacetylcefotaxime ist eine bekannte Verunreinigung von Cefotaxime, wohingegen das zweite Molekül ein Abbauprodukt einer der Verunreinigungen ist, die während der Synthese von Ceftriaxon entstehen können. Es ist bekannt, dass diese Substanz karzinogen ist, und sie kann mit der erweiterten zweiten Methode für Ceftriaxon erfasst

und quantifiziert werden. Unter Lichteinfluss bildete sich zudem eine weitere bekannte, inaktive Verunreinigung von Ceftriaxon (Verunreinigung A, *E*-Isomeres von Ceftriaxon), weshalb Ceftriaxon-haltige Lösungen vor Licht geschützt aufbewahrt werden sollten. Dieses Wissen ist besonders für den klinischen Bereich relevant.

An einer Reihe von Betalaktam-haltigen Fertigarzneimitteln aus Pakistan und einigen anderen Ländern wurden mehrere Qualitätsuntersuchungen angestellt. Obwohl der geringe Probenumfang und die Methoden der Probensammlung kein eindeutiges Bild darüber zeichnen konnte, ob in einem bestimmten Land Arzneimittelfälschungen besonders häufig auftreten, konnte durch die Versuche dennoch festgestellt werden, dass es Probleme bei der Arzneimittelqualität gibt und dass Betalaktam-Antibiotika eine Hochrisikogruppe darstellen, die besonders gut hinsichtlich des Vorkommens minderwertiger Arzneimittel untersucht werden muss. Zudem wurden unbekannte Verunreinigungen aus den Proben isoliert und neue sowie modifizierte Methoden entwickelt, um Verunreinigungen der untersuchten Substanzen zu gewinnen.

Zudem wurde eine ausführliche Literaturrecherche durchgeführt, um das komplexe Problem minderwertiger Arzneimittel, den Einfluss minderwertiger Antibiotika auf das Gesundheitssystem sowie das globale Ausmaß des Problems zu verstehen. Für Pakistan wurde der *status quo* der Arzneimittelqualität aufgrund vorhandener Dokumentation ermittelt. Außerdem wurde dargelegt, inwiefern die heutigen modernen Technologien und Strategien, die für Entwicklungs- und Schwellenländern zur Verfügung stehen, zur Bekämpfung von qualitativ minderwertigen Arzneimitteln in Pakistan beitragen können. Hierzu wurde eine Übersicht aktueller, moderner digitaler Techniken angefertigt, die für die Aufdeckung und Bekämpfung von minderwertigen und gefälschten Arzneimitteln verwendet werden können.
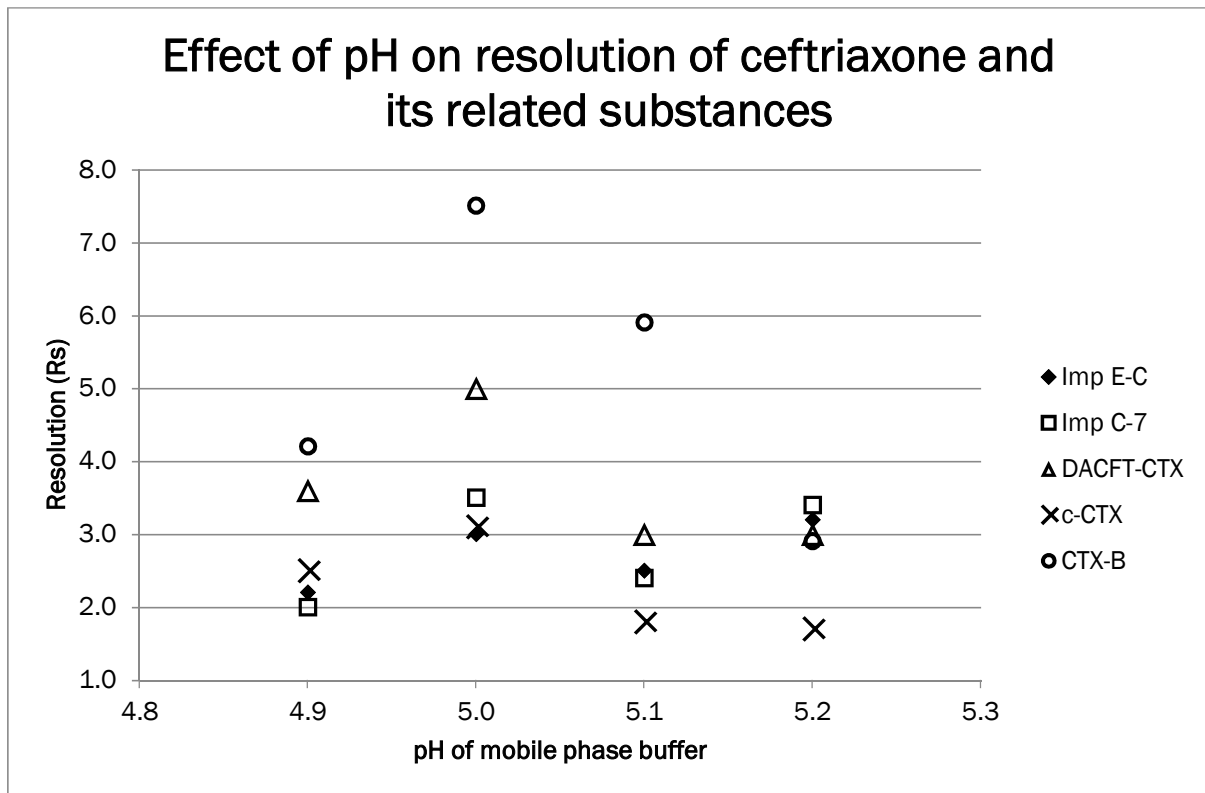
# APPENDICES

**CHAPTER SIX**

S330571

Figure A1      Effect of pH change (4.9-5.3) on resolution ($R_s$) of impurity E-impurity C (Imp E-C), impurity C-7ACA (Imp C-7), deacetylcefotaxime- ceftriaxone (DACFT-CTX), last peak in the unknown peak group-ceftriaxone (c-CTX), ceftriaxone-impurity B (CTX-B).

Appendix I

Table A1      Reporting level, acceptable limits for specified and unspecified impurities and assay limits for ceftriaxone in various reference documents

| Acceptable limits for ceftriaxone as API (for human use)<br>Maximum daily dose of ceftriaxone > 2 g/d | |
|---|---|
| **Reporting level /disregard limit** | **Disregard limit** |
| Ph.Eur. 9.2., -Substance for pharmaceutical use | < 0.03% |
| European Medicines Agency- Guidelines for setting specifications of related impurities in antibiotics-(July 2012) | < 0.03% |
| Ph.Eur. 9.2., Monograph of ceftriaxone sodium | < 0.1% |
| USP 40, Monograph of ceftriaxone sodium | < 0.1 % |
| **Any unspecified impurity** | **Acceptable limit (TL)** |
| Ph.Eur. 9.2., Monograph of ceftriaxone sodium | ≤ 1.0 % |
| USP 40, Monograph of ceftriaxone sodium | ≤ 0.2 % |
| **Specified impurities** | **Acceptable limit (SL)** |
| Ph.Eur. 9.2., Monograph of ceftriaxone sodium | None |
| USP 40, Monograph of ceftriaxone sodium | |
| Impurity E, B, A and 7-ACA | ≤ 0.5 % |
| -Impurity C | ≤ 1.0 % |
| -Impurity D | ≤ 0.2 % |
| -3-ene isomer of ceftriaxone | ≤ 0.3 % |
| **Total impurities** | |
| Ph.Eur. 9.2., Monograph of ceftriaxone sodium | ≤ 4.0 % |
| USP 40, Monograph of ceftriaxone sodium | ≤ 2.5 % |
| **Assay Limits** | |
| Ph.Eur. 9.2., Monograph of ceftriaxone sodium | 96.0-102.0% |
| USP 40, Monograph of ceftriaxone sodium | ≤ 795 µg/mg |

## Appendix II  Supplementary material to Chapter 3.4

Table A 1     Amoxicillin capsules and tablets batch information (coded) sheet

| Sample code | Strength AMX | Brand, manufacturer, Mfg.code | Batch # | Date of expiry |
|---|---|---|---|---|
| AMX1P1 | 500 mg | Karachi, Pakistan (MNC1) | 12E1794 | 05/2014 |
| AMX1P2 | 500 mg | | 13A1906 | 01/2015 |
| AMX1P3 | 500 mg | Karachi, Pakistan (LC1) | C1301 | 02/2015 |
| AMX1P4 | 500 mg | Karachi, Pakistan (MNC2) | GAGAB | 01/2015 |
| AMX2P5 | 500 mg | Karachi, Pakistan (LC2) | 114 | 06/2016 |
| AMX2P6 | 500 mg | Hub, Pakistan (LC3) | 1377 | 08/2016 |
| AMX2P7 | 500 mg | Karachi, Pakistan (MNC2) | CAGBL | 02/2015 |
| AMX3P8 | 500 mg | | EAGCG | 05/2017 |
| AMX3P9 | 500 mg | Karachi, Pakistan (MNC3) | 0936 | 02/2017 |

# Appendix II

Table A2     Coamoxiclav tablets batch information (coded) sheet

| Sample code | Strength AMX | Brand, manufacturer, Mfg.code | Batch # | Date of Mfg. | Date of expiry |
|---|---|---|---|---|---|
| CAM1P1 | 500+125 | Hattar, Pakistan (MNC4) | 3B059 | 02/2013 | 02/2015 |
| CAM1P2 | 500+125 | Karachi, Pakistan (LC4) | C13241 | - | 02/2015 |
| CAM1P3 | 500+125 | Lahore. Pakistan (LC5) | HW628 | 10/2012 | 10/2014 |
| CAM1P4 | 500+125 | Karachi, Pakistan (MNC2) | CAUAJ | 01/2013 | 01/2015 |
| CAM2P5 | 500+125 | Karachi, Pakistan (LC4) | C13301 | - | 04/2015 |
| CAM2P6 | 500+125 | Karachi, Pakistan (MNC2) | CAUCY | 06/2013 | 06/2015 |
| CAM2P7* | 875+125 | Karachi, Pakistan (MNC2) | CASAS | 01/2013 | 01/2015 |
| CAM1D1 | 500+125 | Oberhaching, Germany (DLC1) | DB1943 | | 01/2016 |
| CAM1S1* | 875+125 | Syria (SLC1) | 17390 | 09/2013 | 09/2015 |
| CAM1K1* | 875+125 | Kuwait (KLC1) | 56047 | 12/2011 | 12/2014 |

Table A3  Ceftriaxone injection batch information (coded) sheet

| Sample code | Strength AMX | Brand, manufacturer, Mfg.code | Batch # | Date of Mfg. | Date of expiry. |
|---|---|---|---|---|---|
| CTX1P1 | 1000 mg | Karachi, Pakistan (LC6) | 5048 | 04/2015 | 04/2017 |
| CTX1P2 | 1000 mg | | 5048 | 04/2015 | 04/2017 |
| CTX1P3 | 1000 mg | | 5069 | 05/2015 | 05/2017 |
| CTX1C1 | 1000 mg | Shandong Province, China (MNC5) | C20140105 | 01/2014 | 12/2016 |
| CTX1C2 | 1000 mg | Hebei, China (MNC6) | 148607 | 06/2014 | 06/2017 |
| CTX1C3 | 1000 mg | Denmark. (MNC7) | 143052050 | 06/2014 | 05/2017 |

Table A4 Details of co-amoxiclav tablets used in assay and impurity analysis

| | Strength AMX+CA (mg) | Packaging | Color of tablet content | Sediments in water |
|---|---|---|---|---|
| CAM1P1 | 500+125 | G | off-white | Minor |
| CAM1P2 | 500+125 | A | Brown | Obvious |
| CAM1P3 | 500+125 | G (desiccant missing) | blackish brown | Obvious |
| CAM1P4 | 500+125 | G | off-white | Minor |
| CAM2P5 | 500+125 | A | Brown | Obvious |
| CAM2P6 | 500+125 | G | off-white | Minor |
| CAM2P7* | 875+125 | G | off-white | Minor |
| CAM1D1 | 500+125 | A | off-white | Minor |
| CAM1S1* | 875+125 | A | off-white | Minor |
| CAM1K1* | 875+125 | AA | off-white | Minor |

A= Aluminium foil blister pack

AA=Aluminium foil blister pack in aluminium foil pouch

G=Amber colored screw capped glass bottle with desiccant