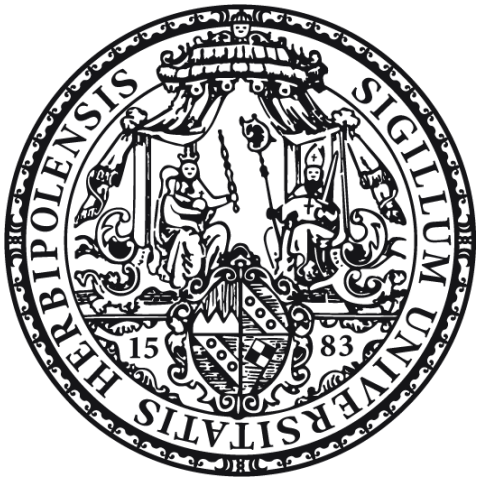
Improving the quality analysis of monographed drugs - dapsone, baclofen, acarbose and other selected APIs

Dissertation zur Erlangung des naturwissenschaftlichen
Doktorgrades an der Fakultät für Chemie und Pharmazie
der Julius-Maximilians-Universität Würzburg



vorgelegt von

Adrian Dieter Leistner

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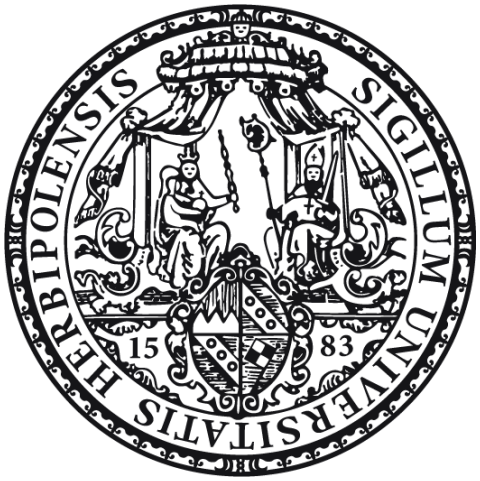

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Eingereicht bei der Fakultät für Chemie und Pharmazie am

_____

Gutachter der schriftlichen Arbeit

1. Gutachter: _____

2. Gutachter: _____

Prüfer des öffentlichen Promotionskolloquiums

1. Prüfer: _____

2. Prüfer: _____

3. Prüfer: _____

Datum des öffentlichen Promotionskolloquiums

_____

Doktorurkunde ausgehändigt am

_____

„Jedes Naturgesetz, das sich dem Beobachter offenbart, läßt auf ein höheres, noch unerkanntes schließen.“

Alexander von Humboldt

~ Meiner Familie ~

DANKSAGUNG

Die hier vorliegende Arbeit entstand auf Anregung und unter Anleitung von

Frau Prof. Dr. Ulrike Holzgrabe

am Lehrstuhl für Pharmazeutische und Medizinische Chemie
des Instituts für Pharmazie und Lebensmittelchemie
der Julius-Maximilians-Universität Würzburg.

Ich danke ihr für die freundliche Aufnahme in ihren Arbeitskreis, die interessanten und abwechslungsreichen Projekte, das stets in mich gesetzte Vertrauen sowie die Unterstützung und Hilfe in jeder Phase meiner Promotion.

Des Weiteren möchte ich mich bei der Hanns-Seidel-Stiftung für die Förderung dieser Arbeit in Form eines Begabtenstipendiums aus den Mitteln des Bundesministeriums für Bildung und Forschung (BMBF) bedanken.

Weiterer Dank gilt allen Kolleginnen und Kollegen - ehemaligen sowie aktuellen - des AK Holzgrabe, die diese Zeit so angenehm gestaltet haben:

Alex, Anja, Antonio, Christiane, Christine, Cristian, Curd, Daniela, Emilie, Flo, Jens, Jonas U., Jonas W., Joshi, Klaus, Laura, Liling, Lina, Lu, Lukas, Markus, Mohamed, Nelson, Niclas, Nicolas, Patrick, Paul, Rasmus, Ruben, Sebastian S., Sylvia, Theresa L., sowie auch Martina und Frau Möhler;
Eva-Maria, Renate und Silke; Christoph, Karl und Matthias.

Ich danke Prof. Dr. Oliver Scherf-Clavel für seine Hilfe bei der Diskussion analytischer Fragestellungen.

Weiterer Dank gilt den Kooperationspartnern von Thermo Fisher Scientific und Arevipharma für die stets unkomplizierte und produktive Zusammenarbeit:

Jon Bardsley, Serdar Bilgesoy, Dr. Benjamin Eggart, Dr. Susanne Fabel, Dr. Paul Gamache, Sylvia Grosse, Dr. Tibor Müllner, Werner Schneider und Dr. Frank Steiner

Dr. Dirk Jung und Dr. Stephan Haerling

“Hvala” an die serbischen Kolleginnen Ana, Bojana, Jovana und Nevena für die erfolgreiche Kooperation.

Danke an Jens, Jonas U., Jonas W., Rasmus, Sebastian S. und Sebastian Z. für die gemeinsame Betreuung des 8. Semesters.

Ein besonderer Dank gebührt meiner Familie und meinen Freunden, die mir stets Rückhalt gegeben haben.

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List of abbreviations

API	active pharmaceutical ingredient
APS	aminopropyl silyl
C18	octadecyl silyl
CAD	charged aerosol detector
DAD	Diode Array Detector
DoE	design of experiments
EDQM	European Directorate for the Quality of Medicines & HealthCare
ELSD	Evaporative Light Scattering Detector
EMA	European Medicines Agency
EU	European Union
EFPIA	European Federation of Pharmaceutical Industries and Associations
FDA	U.S. Food and Drug Administration
GC	gas chromatography
HILIC	hydrophilic interaction chromatography
HPLC	high performance liquid chromatography
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
Imp.	Impurity
IPC	ion pairing chromatography
JP	Japanese Pharmacopoeia
LoD	limit of detection

LoQ	limit of quantification
MS	mass spectrometry
PDE	permitted daily exposure
PGC	Porous graphite carbon
PGI	potential genotoxic impurities
Ph. Eur.	European Pharmacopoeia
Ph. Int.	International Pharmacopoeia
PFP	pentafluorophenyl
PFV	power function value
PMI	potential mutagenic impurities
QSAR	quantitative structure–activity relationship
RP	reversed phase
RSD	relative standard deviation
SMD	Sauter mean diameter
TFA	trifluoroacetic acid
TLC	thin-layer chromatography
TTC	Threshold of Toxicological Concern
USP	United States Pharmacopeia
UV	ultraviolet
UV/VIS	ultraviolet - visible

1. Introduction

The quality of a pharmaceutical product is directly dependent on the purity of its active pharmaceutical ingredients (API). In order to guarantee the highest possible standards, purity control is a highly regulated field of activity. There are several quality guidelines issued by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), whose members include regulatory agencies such as the European Commission or the U.S. Food and Drug Administration (FDA) and industry associations such as European Federation of Pharmaceutical Industries and Associations (EFPIA). They aim to promote public health through international harmonization [1]. Further, pharmacopoeias such as the European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopeia (USP) describe methods for the analysis of APIs and excipients. They publish general and specific monographs to ensure adequate quality of the resulting drugs [2, 3].

1.1. Elaboration of a monograph

A few years before patent expiry, the monograph development process starts [3, 4]. For example, APIs used in drugs that are approved by EU member states can begin a “P4 elaboration” of a new monograph [4-6]. Therefore, a lot of data (analytical procedures, analytical specifications, impurities, degradation products and results of the stability tests on the active substance) and samples (API and impurities) must be sent to the EDQM and a draft monograph will be prepared in compliance with the *Technical Guide for the elaboration of monographs* by the corresponding expert group at the EDQM and then published in *Pharmeuropa* [5, 6]. Regulatory authorities and international pharmaceutical industry can comment on the published monograph, which will be considered by the expert group. The final monograph will be added to the next edition or supplement of the Ph. Eur. after acceptance by the commission.

Any existing monograph and all general chapters can be revised, if necessary. In case of a changed manufacturing process of the tested substance or in case of newly emerged scientific knowledge, the methods should be adapted or reviewed thoroughly. Often monographs of substances that are very similar are adapted as part of an harmonization process of the monographs [4]. Furthermore, an international harmonization with the USP and JP is pursued.

1.2. Related substances in pharmacopoeia and their determination

The purity of an API can be monitored and controlled by a series of tests, that are included in the respective Ph. Eur. monographs. For example, residual solvents are controlled in addition to organic and inorganic impurities [7]. Most monographs include a transparency statement, which provides the impurities known to be detectable by the monograph's described tests. Whether an impurity should be classified as to be specified or not, also depends on the daily dose of the drug substance. Therefore, the ICH Q3A (R2) guideline and analogously the general monograph on *Substances for pharmaceutical use (2034*) propose thresholds considering the maximum daily dose (see Table 1). Additionally, toxicology related limits have to be respected [2, 8]. Not each of the impurities listed in the transparency list is also limited individually. Some of the compounds are only listed for supplementary information but are nevertheless ultimately limited by the sum of impurities parameter if they exceed the disregard limit.

The respective method of the section “Related substances” enables to quantify the specified impurities, which have acceptance criteria, and other detectable impurities, which might be present. For the latter, the section related substances of the general monograph on *Substances for pharmaceutical use (2034*) must be consulted to determine whether this impurity must be classified as to be identified or qualified according to the thresholds [2]. Of note, peaks below the reporting threshold are not considered for the analysis.

Table 1: Thresholds for impurities according to ICH guideline Q3A (R2) and the general monograph on Substances for pharmaceutical use (2034) [2, 8]

Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
≤ 2 g	0.05%	0.10% or 1.0 mg*	0.15% or 1.0 mg*
> 2 g	0.03%	0.05%	0.05%

* whichever is lower

For the specified impurities, the monograph lists respective limits that may not be exceeded. All other detectable impurities are limited by a value for the respective unknown impurities or by the sum of impurities value if the impurities exceed the disregard limit of the monograph. A shortcoming of this approach is that theoretically many impurities can be contained under the disregard limit, but in the result of the Related substances' test, they are not reported. Therefore, almost all methods of a monograph are necessary to adequately characterize the quality of an API.

The test for related substances is performed mostly chromatographically since the compounds must necessarily be separated. In the simplest case, this is done by thin-layer chromatography (TLC), test solution and corresponding dilutions (e.g., 1:100 or 1:500) are being added to the TLC plate. For quantification, the non-diluted test solution must not contain any additional spot that is more intense than the main spot of the diluted solution. One example thereof was the test for *Related substances* of dapsone [9], which was later replaced by a HPLC-UV method that has resulted from this work. For the impurity analysis of dapsone, the Ph. Eur. used several dilutions of test and reference solutions, which were separately applied to the plate. After development and air drying, the plate was sprayed with a derivatization agent and examined in daylight. No spot other than the main spot was allowed to be more intense than the reference solution (1:100) and no two spots were allowed to be more intense than the diluted reference solution (1:500). However, this method did not allow a more accurate assessment of purity but could only check whether very rough limits were met or not. Additionally, TLC methods are usually not very sensitive.

Therefore, these simple TLC methods are gradually replaced by modern HPLC methods during monograph revision. As mentioned above since Ph. Eur. 10.6. the test for related substances of dapsone has been replaced by a HPLC-UV method. It now specifies three impurities and limits other impurities to less than 0.10% each. Thus, the impurity profile can now be better studied and the new limits for impurities imply a purer API [10].

1.3. Techniques for the control of impurities

Different affinity of analytes to two immiscible phases enables chromatographic separation. Herein, the analytes are distributed between the stationary and the mobile phase and hence are separated due to different travel speeds.

The mobile phase may be a liquid, gas, or supercritical fluid and is passed through a chromatographic column, which contains a solid or liquid stationary phase immobilized on a solid or gel. In the chromatographic process, analytes are entrained along by the mobile phase and retained at the stationary phase by various mechanisms of interaction, such as adsorption, ionic interaction, mass distribution, size exclusion or stereochemical interaction. TLC, gas chromatography (GC) and high-performance liquid chromatography (HPLC), which is the gold standard in the analysis of APIs, drugs, and their impurities, are predominantly used for pharmaceutical purity analysis [11].

An HPLC instrument basically consists of a pumping system, an injector, a chromatographic column, a detector, and a data acquisition system. The mobile phase is pumped from the storage flask with a constant rate through the instrument [12].

As a stationary phase, a RP column is often used. A typical mobile phase for RP chromatography consists of a mixture of water and organic modifiers, e.g., acetonitrile, methanol, tetrahydrofuran or acetone. Typically the aqueous solution usually consists of buffering agents like phosphate, acetate or formate buffers, which are used to adopt a defined pH value [12, 13]. Its selection also depends on the type of detector used for the analysis. For example, volatile additives and buffering agents (like formic or acetic acid and their ammonium salts) must be used with aerosol-based detectors, like the charged aerosol detector (CAD) [14-16] or MS detectors [17-19]. Furthermore, ion pairing agents can be added to the mobile phases to perform ion pairing chromatography (IPC) for anions or cations on a common RP stationary phase. Therefore, e.g., alkylammonium and alkylsulfonate salts, are used, respectively [20].

For the chromatographic separation, various types of column materials are available. The column selection highly depends on the physicochemical properties of the analytes. Typically, derivatized reversed phase silica gel phases (e.g., C8, C18, phenyl, or PFP) are utilized for the drug analysis. For more challenging separation problems, porous graphite, mixed-mode or HILIC phases are applied [21-24]. Specially modified stationary phases, e.g., amylose or cellulose derivatives, are employed for chiral separations [25-27].

In general, smaller column particles (as utilized in UHPLC) lead to better efficiency than larger ones, but generate a higher backpressure [28], which can be a problem for older HPLC systems, that mostly can only operate up to 400 bar. E.g. *Yang* showed, that the switch from 4.0 µm particles to 1.7 µm lead to an increase of the plate number

from 2000 to 7500, which indicates better separation efficiency [29]. The influence of the linear flowrate u_x on the resulting backpressure P is expressed in Eq. 1 as a modification of *Darcy's law* [30, 31], which nicely describes the influence of the particle diameter d_p of the packing on the backpressure. The factor f depends on particle packing and shape of the column material, η is the viscosity of the solvent, r is the column radius and L is the column length.

$$P = f \frac{u_x \eta L}{\pi r^2 {d_p}^2} \qquad \text{Eq. (I)}$$

Today, a higher backpressure is not a problem anymore, when the separation is performed by means of a modern UHPLC systems, that can operate at pressures up to 1000 bar [32]. Moreover, it can be beneficial for reasons of solvent and time savings, to use smaller particles in combination with a narrower and shorter column but equivalent separation efficiency [33].

As described before, porous graphite carbon (PGC) is a column material with unique mechanism of retention and therefore it might be an alternative for complex separations. On the one hand PGC shows retention comparable to a common octadecyl silyl (C18) stationary phase. In addition, however, it also provides retention for polar analytes, which is achieved by a polar retention effect of the graphite surface. Furthermore, it can be used in quite harsh conditions like high temperatures, very acidic and basic pH values [34-38].

"Hydrophilic Interaction Chromatography" (HILIC) can be an alternative for the analysis of polar compounds, as they are mostly too polar to be retained by RP columns. Small amounts of water in the mobile phase form a layer of aqueous phase on the surface of the stationary phase, which is able to interact with the hydrophilic regions of the polar analytes and thus support separation. HILIC can be useful for the separation of small molecules, like amino acids [34-36] or of polar sugars [21, 37, 38].

An ideal detector has to be sensitive to very low concentrations, independent of the type of analyte, linear over a wide range of concentration, independent of the solvent and temperature used, and prevents peak broadening.

Of course, such an ideal detector does not exist, and compromises must always be made, even though today's detectors often come close to an ideal detector.

1.3.1. UV detector

The ultraviolet (UV) and ultraviolet-visible (UV/VIS) detectors are the most common HPLC detector types since most analytes absorb light in the UV or visible spectrum. According to the *Beer-Lambert law*, the absorbance of the analyte and its concentration form a linear relationship, which allows simple evaluation of the analysis [13, 39, 40].

There are two general types of detectors - simple photocells or photodiode arrays. In contrast to the simple photocell detector, the Diode Array Detector (DAD), allows to record whole UV/VIS spectra in the previously defined range and thus can check peak purity within analysis and allows the simultaneous record of more than one single wavelength [41].

One major advantage of the UV detector is, that it is simple to use, even with gradient elution. However, it can only be applied with a non-absorbing mobile phase. Therefore, the cutoff wavelengths (a wavelength below the solvent absorbs too strong) of the solvents used must be considered, which are displayed in Table 2.

Next, the UV detector is a non-destructive detector and in the case of preparative HPLC analysis, the respective compounds can be gathered in a fraction collector and thus can be further used for other experiments.

Table 2: Typical solvent used for HPLC and their respective UV cutoff [13]

Solvent	**UV cutoff (nm)**
Acetone	330
Acetonitrile	190
Ethanol	210
Isopropanol	205
Methanol	205
Tetrahydrofuran	212
Water	190

However, there are also some drawbacks associated with the use of the UV detector. E.g., changes of the temperature within the chromatographic system influences the refractive index of the eluent and thus changes the absorption and thereby causes baseline drift [42]. Also, peak broadening due to temperature-dependent diffusion

processes cannot be excluded. Modern instrumentations have reduced this issue by holding the temperature in the detector constant.

Next, the sensitivity of the detector, especially at low wavelengths is dependent on the UV lamps used. Furthermore, the detector should not be used for the determination of very large and very small analyte amounts at the same time. In a standard flow cell small amounts of analytes are not sufficiently recorded, as their signal is broadened. In this case, special flow cells of smaller volume need to be used. The other way round, for higher amounts of analyte, the detector would reach its detection limit and the amount is not recorded correctly. Therefore, the operator must choose the correct flow cell setting for the respective analytical task.

Probably the biggest shortcoming of the UV detector is that analytes without a chromophore cannot simply be detected. To overcome this problem, universal detectors such as the charged aerosol detector (CAD) can be utilized either as single detector or as a hyphenated detector setting behind the UV detector.

1.3.1. Charged aerosol detector

Among the universal detectors, the Evaporative Light Scattering Detector (ELSD), which was commercially introduced in the early 1980s [43], is now routinely used for the assay of quality influencing compounds in several herbal drugs [44-49], for the assay of phospholipids [50, 51], for the composition of triglycerides in sesame oil [52], or for limit tests for substances lacking a chromophore [53, 54]. However, the use within the related substances test of APIs seems to be not the best choice, since the detector can generate spike peaks due to the large amount of the main signal of the API, when concentrated solutions - as needed within the related substances tests - were injected [55]. The Ph. Eur. monographs for related substances using an ELSD, advice to use a valve to cut the main peak out and thereby to save the detector [56, 57].

This problem was solved by the charged aerosol detector (CAD), which was already patented in 2001. However, the first generation of the CAD commercially available was introduced in 2005 [14].

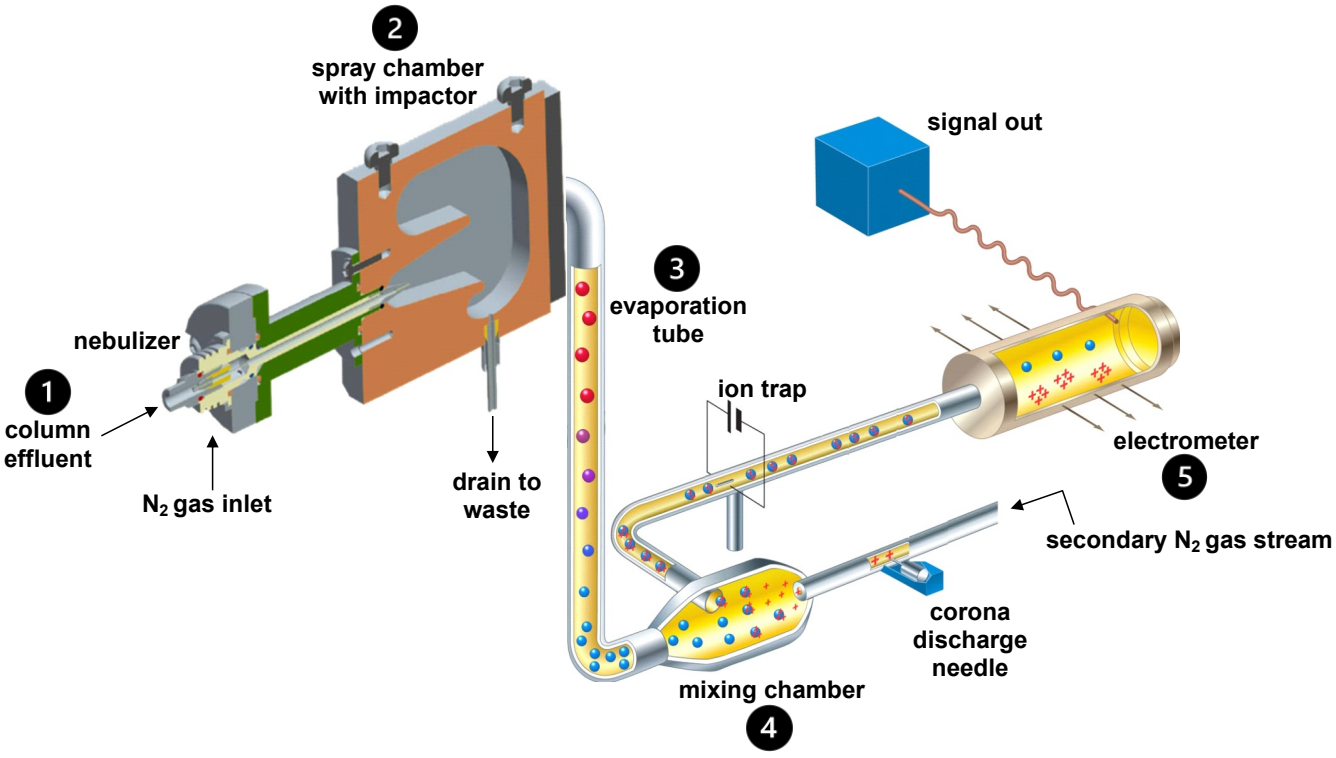

Figure 1: Schematic operating principle of the CAD
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Its detection principle (see Figure 1) is based on the measurement of the charge from previously dried and charged aerosol particles: The HPLC eluate (1), entering the

CAD, is nebulized by means of nitrogen gas. The resulting droplets are not of the same size. The ones, that are too big are drained to waste (2), while the smaller ones are transferred by a nitrogen stream to an evaporation tube (3), where the incoming aerosol is dried. Next, in the corona charging chamber, positive charge is transferred from nitrogen to the dried particles (4). After passing an ion trap for small ions, a collector captures the charged particles, and an electrometer (5) measures the positive charge reaching the collector.

For those nebulization processes, the droplet size, more precisely the Sauter mean diameter (SMD) can be predicted by *the Nukiyama-Tanasawa* equation [14, 58],

$$SMD = \frac{585 \times \sigma^{0.5}}{(v_g - v_l) \times \rho^{0.5}} + 597 \left(\frac{\eta}{(\sigma \times \rho)^{0.5}}\right)^{0.45} \times \left(1000 \frac{Q_l}{Q_g}\right)^{1.5} \qquad \text{Eq. (II)}$$

where σ is the surface tension of the eluent, v_g and v_l are the axial speeds of the gas or the liquid, respectively. ρ describes the density of the eluent, while η is its dynamic viscosity. Q_l and Q_g represent the flow rate of the eluent and of the gas.
Due to the design of the detector, droplets of the primary aerosol that are too large are removed by impact to the wall of the spray chamber, and with them the analytes they contain. Consequently, eluents i.e., organic solvate that form smaller droplets (e.g., those with low viscosity or low surface tension) allow a larger fraction of the analyte to enter the evaporation tube and thus producing a higher signal. The CAD signal itself depends on the mean charge per dried particle, which in turn depends on the diameter d of the dried aerosol particles [14, 59, 60]. This can be described by Eq. (III) within a relationship to the aerosol particle diameter D:

$$d = D \times \left(\frac{C_s}{\rho_s}\right)^{\frac{1}{3}} \qquad \text{Eq. (III)}$$

A nonlinear relationship between d and the solute mass concentration C_s is indicated by the exponent of the equation. The solute density ρ_s also influences the diameter of the dried particles. To compensate this nonlinear response, the analyst can theoretically multiply the obtained peak areas by the cube root of the respective density [61]. However, in practice the analytes of the related substances tests are of comparable physicochemical properties and thus this compensation is not needed.

The obtained dried particles are charged by encountering a gas stream, which in turn has been positively charged by a corona discharge [62]. Later, they are led to a collector, which converts the charge into an output signal. The response of the analytes is approximately mass flow dependent. Consequently, an equal signal can be generated by the same mass of all nonvolatile analytes. Thus, it is a sensitive and almost universal detector [63].

The CAD, like all aerosol-based detectors, generates a non-linear response that can be described by a power law function:

$$A = a\left(m_{inj}\right)^{b} \qquad \text{Eq. (IV)}$$

Thereby, the relationship between the peak area response A and the analyte mass injected m_{inj} is described. The factors a and b depend on the experimental conditions. Only for $b = 1.0$ the response is linear [60, 64]. However, for most analytes, the CAD signal is not linear over the entire working range of the detector, but it may be quasi-linear within a small range of two orders of magnitude [65]. A double logarithmic transformation of the calibration curve can help to linearize the CAD signal over a wider range [64]. For linearization, the power function value (PFV) can alternatively be applied after optimization for the respective compound [66]. Modern generations of CAD also allow the temperature of the evaporation tube to be controlled, providing a tool for method optimization to achieve better performance over a wider range [67]. Under Ph. Eur. today, the CAD has to be used for the related substances tests of topiramate and vigabatrin as well as for the contrast agent gadobutrol [68-70].

Taken together, the usage of the CAD can be beneficial for compounds lacking a chromophore. However, in most cases UV detection is superior since most analytes have a chromophore and then the UV detector performs to its full sensitivity strengths. The hyphenation of UV detectors and charged aerosol detectors allows sensitive detection of analytes carrying a chromophore, while at the same time the analytes without a chromophore are not missed. The combined setup can thus be an optimal detector assembly [71].

1.4. More impurities than specified within the pharmacopoeia

As stated in previous sections of this work not all impurities of an API are listed in the transparency list of the pharmacopoeia. Several of them are simply unknown. For the development of new drugs, the impurity profile of the drug is kind of a black box. This is a real problem for the pharmaceutical industry, as it has to prove the efficacy, safety and appropriate quality of the drugs for its patients [72]. Consequently, pharmaceutical manufacturer must know everything about their products. As part of the approval process of a drug, companies must therefore submit evidence of their drug's quality. This also includes the by-products that arise. But how is that done?

One option for that is a deep evaluation of the whole manufacturing process itself, including all potential side reactions and impurities of reagents and solvents. Therefore, reaction matrices can be built up for each step of the synthesis. These matrices have to be evaluated, whether the reaction probably takes place or not [73]. After that, it is necessary to check which of the potential impurities are present in the product and then quantify them in a targeted approach.

Another option is, that scientists find additional impurities by chance, that were not under observation. E.g., within the impurity profiling of dapsone one additional impurity was found and identified [74]. Those findings must be explained, added to the reaction matrix and further evaluated.

Last but not least, solvents are often neglected in their importance; they may either undergo reactions or contribute to contaminated products through their impurities [75]. Taken together, it is not so easy to find impurities unless you are specifically looking for them. Structurally related substances can be easier detected than those whose chemical structure is not related to the API. Therefore, untargeted approaches should be applied in addition, to supplement impurities that are not under observation [76, 77]. It goes without saying that only those analytes can be found for which the respective detector is suitable. A setup with different complementary detectors would therefore be quite useful for the analytics of the development phase of a drug [15].

1.5. References

[1] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, Overview of ICH. https://admin.ich.org/sites/default/files/2021-06/OverviewOfICH_2021_0614.pdf, 2021, (accessed 12 July 2022).

[2] European Pharmacopoeia 10th ed., Substances for pharmaceutical use (2034), Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2020.

[3] The United States Pharmacopeial Convention, An Overview of USP Monographs. https://www.usp.org/sites/default/files/usp/document/about/public-policy/monograph-basics.pdf (accessed 12 July 2022).

[4] European Directorate for the Quality of Medicines & HealthCare, Elaboration & Revision. https://www.edqm.eu/en/elaboration-revision (accessed 12 July 2022).

[5] European Directorate for the Quality of Medicines & HealthCare, Procedure 4 (P4). https://www.edqm.eu/documents/52006/0/P4procedureAllyouNeedtoKnow2.pdf/85d459b5-fadb-8c98-acb3-b3a22a8c7349?t=1640092701402 (accessed 12 July 2022).

[6] European Directorate for the Quality of Medicines & HealthCare, Technical guide for the elaboration of monographs, https://www.edqm.eu/documents/52006/66555/02-technical-guide-elaboration-monographs-8th-edition-2022.pdf/46b07f46-779d-687d-1455-74d8a9a515ee?t=1656321894803, 2022 (accessed 12 July 2022).

[7] European Pharmacopoeia 10th ed., 5.10. Control of impurities in substances for pharmaceutical use, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2020.

[8] ICH Harmonised Tripartite Guideline, Impurities in new drug substances Q3A (R2), Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland, 2006.

[9] European Pharmacopoeia 9th ed., dapsone monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2017.

[10] European Pharmacopoeia 10.6, dapsone monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[11] European Pharmacopoeia 10th ed., 2.2.46. Chromatographic separation techniques, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2020.

[12] European Pharmacopoeia 10th ed., 2.2.29. Liquid chromatography, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2020.

[13] L.R. Snyder, J.J. Kirkland, J.W. Dolan, Introduction to modern liquid chromatography, third edition, John Wiley & Sons, 2011.

[14] P.H. Gamache, Charged aerosol detection for liquid chromatography and related separation techniques, John Wiley & Sons, 2017.

[15] T. Muellner, Acworth, I., Gamache, P., Getting the most out of your charged aerosol detector-Factors influencing charged aerosol detector performance, https://assets.thermofisher.com/TFS-Assets/CMD/brochures/tg-73914-hplc-cad-factors-influencing-performance-tg73914-en.pdf, 2021, (acessed 16 september 2022).

[16] M. Plante, F. Kusinitz, I. Acworth, Effect of Mobile Phase Quality on Analytical Performance of Corona Charged Aerosol Detectors, https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/TN-159-LC-CAD-Mobile-Phase-TN71390-EN.pdf, 2016, (acessed 16 september 2022).

[17] C.-K. Lim, G. Lord, Current developments in LC-MS for pharmaceutical analysis, Biol. Pharm. Bull., 25(5) (2002) 547-557.

[18] X. Subirats, E. Bosch, M. Rosés, Buffer considerations for LC and LC–MS, LC GC N. Am., 27(11) (2009) 1000–1004.

[19] J. Dolan, A guide to HPLC and LC-MS buffer selection, ACE (2009), https://www.hplc.eu/Downloads/ACE_Guide_BufferSelection.pdf,
(acessed 04 september 2022).

[20] T. Cecchi, Ion-pair chromatography and related techniques, CRC Press, 2009.

[21] B. Dejaegher, Y. Vander Heyden, HILIC methods in pharmaceutical analysis, J. Sep. Sci., 33(6-7) (2010) 698-715.

[22] K. Zhang, X. Liu, Reprint of “Mixed-mode chromatography in pharmaceutical and biopharmaceutical applications”, J. Pharm. Biomed. Anal., 130 (2016) 19-34.

[23] T. Hanai, Separation of polar compounds using carbon columns, J. Chromatogr. A, 989(2) (2003) 183-196.

[24] S. Ahuja, M. Dong, Handbook of pharmaceutical analysis by HPLC, Elsevier, 2005.

[25] I. Ali, V.D. Gaitonde, H.Y. Aboul-Enein, A. Hussain, Chiral separation of β-adrenergic blockers on CelluCoat column by HPLC, Talanta, 78(2) (2009) 458-463.

[26] S. Grybinik, Z. Bosakova, An overview of chiral separations of pharmaceutically active substances by HPLC (2018–2020), Monatsh. Chem., 152(9) (2021) 1033-1043.

[27] Y. Okamoto, T. Ikai, Chiral HPLC for efficient resolution of enantiomers, Chem. Soc. Rev., 37(12) (2008) 2593-2608.

[28] J.E. MacNair, K.D. Patel, J.W. Jorgenson, Ultrahigh-pressure reversed-phase capillary liquid chromatography: isocratic and gradient elution using columns packed with 1.0-μm particles, J Anal Chem, 71(3) (1999) 700-708.

[29] Y. Yang, C.C. Hodges, Assay transfer from HPLC to UPLC for Higher Analysis Throughput, LC GC Chromatography, (2005) 31-35, https://cdn.sanity.io/files/0vv8moc6/chroma/8f7d046b34df8841f2af4b5a8deb63b60c3de690.pdf/article-164650.pdf (acessed 20 august 2022).

[30] D.C. Harris, Quantitative Chemical Analysis, 8 ed., W.H. Freeman, New York, NY, 2010.

[31] T. Farkas, G. Zhong, G. Guiochon, Validity of Darcy's law at low flow-rates in liquid chromatography, J. Chromatogr. A, 849(1) (1999) 35-43.

[32] S. Fekete, J. Schappler, J.-L. Veuthey, D. Guillarme, Current and future trends in UHPLC, Trends Anal. Chem., 63 (2014) 2-13.

[33] Agilent Technologies Inc., UHPLC and HPLC Method Optimization: Secrets You Can Put In Use Today., 2017, https://www.agilent.com/cs/library/slidepresentation/public/UHPLC_and_HPLC_Method_Optimization07112017.pdf, (accessed 17 September 2022).

[34] L. Pereira, Porous graphitic carbon as a stationary phase in HPLC: theory and applications, J. Liq. Chromatogr. Relat. Technol., 31(11-12) (2008) 1687-1731.

[35] Y. Daali, S. Cherkaoui, X. Cahours, E. Varesio, J.L. Veuthey, High performance liquid chromatography of acarbose and its metabolite on porous graphitic carbon column, J. Sep. Sci., 25(5-6) (2002) 280-284.

[36] X. Ji, G. Hu, Q. Zhang, F. Wang, C. Liu, Simultaneous analysis of heparosan oligosaccharides by isocratic liquid chromatography with charged aerosol detection/mass spectrometry, Carbohydr Polym., 152 (2016) 337-342.

[37] T.E. Bapiro, F.M. Richards, D.I. Jodrell, Understanding the Complexity of Porous Graphitic Carbon (PGC) Chromatography: Modulation of Mobile-Stationary Phase Interactions Overcomes Loss of Retention and Reduces Variability, J. Anal. Chem., 88(12) (2016) 6190-6194.

[38] C. West, C. Elfakir, M. Lafosse, Porous graphitic carbon: A versatile stationary phase for liquid chromatography, J. Chromatogr. A, 1217(19) (2010) 3201-3216.

[39] R. Pawellek, U. Holzgrabe, Performance of ion pairing chromatography and hydrophilic interaction liquid chromatography coupled to charged aerosol detection for the analysis of underivatized amino acids, J. Chromatogr. A, 1659 (2021) 462613.

[40] H.C. Prinsen, B. Schiebergen-Bronkhorst, M. Roeleveld, J. Jans, M. de Sain-van der Velden, G. Visser, P. Van Hasselt, N. Verhoeven-Duif, Rapid quantification of underivatized amino acids in plasma by hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass-spectrometry, J. Inherit. Metab. Dis., 39(5) (2016) 651-660.

[41] A. Socia, J.P. Foley, Direct determination of amino acids by hydrophilic interaction liquid chromatography with charged aerosol detection, J. Chromatogr. A, 1446 (2016) 41-49.

[42] K. Schilling, J. Krmar, N. Maljurić, R. Pawellek, A. Protić, U. Holzgrabe, Quantitative structure-property relationship modeling of polar analytes lacking UV chromophores to charged aerosol detector response, Anal. Bioanal. Chem., 411(13) (2019) 2945-2959.

[43] B. Buszewski, S. Noga, Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique, Anal. Bioanal. Chem., 402(1) (2012) 231-247.

[44] D.F. Swinehart, The Beer-Lambert Law, J. Chem. Educ., 39(7) (1962) 333.

[45] M. Swartz, HPLC DETECTORS: A BRIEF REVIEW, J. Liq. Chromatogr. Relat. Technol., 33(9-12) (2010) 1130-1150.

[46] M.W. Dong, J. Wysocki, Ultraviolet detectors: perspectives, principles, and practices, LC GC N. Am., 37(10) (2019) 750-759.

[47] G. Openhaim, E. Grushka, Temperature-dependent refractive index issues using a UV–visible detector in high-performance liquid chromatography, J. Chromatogr. A, 942(1) (2002) 63-71.

[48] L.-E. Magnusson, D.S. Risley, J.A. Koropchak, Aerosol-based detectors for liquid chromatography, J. Chromatogr. A, 1421 (2015) 68-81.

[49] European Pharmacopoeia 10.8, akebia stem monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[50] European Pharmacopoeia 10.8, astragalus mongholicus root monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[51] European Pharmacopoeia 10.8, black cohosh monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[52] European Pharmacopoeia 10.8, coix seed monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[53] European Pharmacopoeia 10.8, isatis root monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[54] European Pharmacopoeia 10.8, platycodon root monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[55] European Pharmacopoeia 10.8, egg phospholipids for injection monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[56] European Pharmacopoeia 10.8, soya phospholipids for injection monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[57] European Pharmacopoeia 10.8, sesame oil, refined monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[58] European Pharmacopoeia 10.8, sucrose monopalmitate monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[59] European Pharmacopoeia 10.8, sucrose stearate monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[60] S. Almeling, U. Holzgrabe, Use of evaporative light scattering detection for the quality control of drug substances: Influence of different liquid chromatographic and evaporative light scattering detector parameters on the appearance of spike peaks, J. Chromatogr. A, 1217(14) (2010) 2163-2170.

[61] European Pharmacopoeia 10.8, hydroxypropylbetadex monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[62] European Pharmacopoeia 10.8, sulfobetadex sodium monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[63] S. Nukiyama, An Experiment on the Atomization of Liquid by means of an Air Stream (1. Report), J. Soc. Mech. Eng., Japan 4 (1938) 128-135.

[64] K.-S. Woo, D.-R. Chen, D.Y.H. Pui, W.E. Wilson, Use of Continuous Measurements of Integral Aerosol Parameters to Estimate Particle Surface Area, Aerosol Sci. Technol., 34(1) (2001) 57-65.

[65] T. Vehovec, A. Obreza, Review of operating principle and applications of the charged aerosol detector, J. Chromatogr. A, 1217(10) (2010) 1549-1556.

[66] S. Matsuyama, Y. Orihara, S. Kinugasa, H. Ohtani, Effects of densities of brominated flame retardants on the detection response for HPLC analysis with a corona-charged aerosol detector, Anal. Sci., 31(2) (2015) 61-65.

[67] A. Medved, F. Dorman, S.L. Kaufman, A. Pöcher, A new corona-based charger for aerosol particles, J. Aerosol Sci., 31 (2000) 616-617.

[68] M. Menz, K. Lovejoy, I. Acworth, P. Gamache, F. Steiner, Charged aerosol detection - factors affecting uniform analyte response, https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/tn-72806-uhplc-charged-aerosol-detection-tn72806-en.pdf, 2021, (acessed 02 September 2022).

[69] P. Gamache, B. Eggart, K. Lovejoy, and I. Acworth, Charged aerosol detection – use of the power function and robust calibration practices to achieve the best quantitative results, https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/tn-73299-lc-cad-calibration-tn73299-en.pdf, 2019,
(acessed 02 September 2022).

[70] J.P. Hutchinson, J. Li, W. Farrell, E. Groeber, R. Szucs, G. Dicinoski, P.R. Haddad, Universal response model for a corona charged aerosol detector, J. Chromatogr. A, 1217(47) (2010) 7418-7427.

[71] R. Pawellek, T. Muellner, P. Gamache, U. Holzgrabe, Power function setting in charged aerosol detection for the linearization of detector response–optimization strategies and their application, J. Chromatogr. A, 1637 (2021) 461844.

[72] K. Schilling, R. Pawellek, K. Lovejoy, T. Muellner, U. Holzgrabe, Influence of charged aerosol detector instrument settings on the ultra-high-performance liquid chromatography analysis of fatty acids in polysorbate 80, J. Chromatogr. A, 1576 (2018) 58-66.

[73] European Pharmacopoeia 10.8, gadobutrol monohydrate monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[74] European Pharmacopoeia 10.8, topiramate monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[75] European Pharmacopoeia 10.8, vigabatrin monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[76] U. Holzgrabe, K. Schilling, R. Pawellek, O. Scherf-Clavel, With united forces against impurities, GIT-Labor-Fachzeitschrift 1-2/2021 (2021) 24-26, https://analyticalscience.wiley.com/do/10.1002/was.000600106 (accessed 02 september 2022).

[77] European Medicines Agency, From laboratory to patient: the journey of a centrally authorised medicine. EMA/103813/2018 Rev. 1, (2018), https://www.ema.europa.eu/en/documents/other/laboratory-patient-journey-centrally-authorised-medicine_en.pdf (acessed 14 july 2022).

[78] A. Leistner, S. Haerling, J.-D. Kreher, I. Becker, D. Jung, U. Holzgrabe, Risk assessment report of potential impurities in cetirizine dihydrochloride, J. Pharm. Biomed. Anal., 189 (2020) 113425.

[79] A. Leistner, U. Holzgrabe, Impurity profiling of Dapsone using gradient HPLC method, J. Pharm. Biomed. Anal., 198 (2021) 113982.

[80] H. Buschmann, U. Holzgrabe, D. Jung, „Unerwartet “verunreinigt, Deutsche Apotheker Zeitung, (2019) 159-20.

[81] J. Wohlfart, E. Jäckel, O. Scherf-Clavel, D. Jung, M. Kinzig, F. Sörgel, U. Holzgrabe, Impurity profiling of bisoprolol fumarate by liquid chromatography-high-resolution mass spectrometry: A combination of targeted and untargeted approaches using a synthesis reaction matrix and general unknown comparative screening, Journal of Chromatography Open, 1 (2021) 100012.

[82] O. Scherf-Clavel, M. Kinzig, A. Besa, A. Schreiber, C. Bidmon, M. Abdel-Tawab, J. Wohlfart, F. Sörgel, U. Holzgrabe, The contamination of valsartan and other sartans, Part 2: Untargeted screening reveals contamination with amides additionally to known nitrosamine impurities, J. Pharm. Biomed. Anal., 172 (2019) 278-284.

2. Aim of the thesis

The analysis of APIs, in particular its purity profiling, is an important field of activity nowadays. Even though many details about long-established APIs are already known, new impurities of APIs might occur.

The aim of this thesis is to further improve the quality analysis of monographed APIs and thus to contribute to the supply of the population with pure active ingredients. For this purpose, various aspects of impurity analysis should be studied in more detail. The thesis can therefore be divided into four main topics:

1. Cetirizine. The drug ought to be chosen as an example for a detailed risk assessment of the manufacturing process of the API. The process of a risk assessment should be highlighted in detail. In this context, it was aimed to examine the impurity profile in more detail using reaction matrices. Subsequently, it will be checked whether the impurities actually found in the API occur in them or further evaluations are necessary.

2. Dapsone. It was aimed to develop and validate an HPLC method that could replace the existing TLC method for related substances. In addition to the method intended for pharmacopeial use, a somewhat simplified method should also be developed. It is being considered to be used in countries with less well-equipped laboratories and still offers an improvement in comparison to the TLC method.

3. Baclofen. It was aimed to develop and validate an improved HPLC method that could replace the existing pharmacopoeial method for related substances. In addition, CAD detection should be used to detect impurities without chromophore, if any are present.

4. Acarbose. Due to the weak chromophore of the API and its impurities, the studies aimed for a method making use of CAD detection. Since beside the detection of the analytes, their separation is challenging, several detection techniques should be evaluated. Alternative methods for the impurity profiling of acarbose were aimed to be developed and validated.

3. Results

3.1. Risk assessment report of potential impurities in cetirizine dihydrochloride

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https://doi.org/10.1016/j.jpba.2020.113425

Abstract

Recently, the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) had reported on “unexpected” impurities in a couple of sartans, ranitidine, and metformin. These events led to a lot of discussion with regard to the risk assessment for the production process itself. Most of these discussions covered the field of nitrosamine impurities only, but that would be too short-sighted. One should expand that scope. It is impossible to synthesize a 100 % pure compound which holds true for all active pharmaceutical ingredients (APIs). Different synthetic routes result in different impurity profiles. Therefore, pharmacopoeias try to consider all possible impurities that can arise from different drug synthesis routes in one a single monograph for impurity assessment. However, API manufacturers cannot simply rely on the impurity profile reported in pharmacopoeias for the production of a high-quality product. They have to implement a whole risk assessment to rate the presence of impurities in the API. Here, a strategy to evaluate and minimize the load of potential risks of impurities during the manufacturing process of the drug substance cetirizine dihydrochloride within the frame of a detailed risk assessment report is demonstrated.

1. Introduction

Recently, the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) had reported on “unexpected” impurities, the nitrosamines, in a couple of sartans [1], ranitidine [2] and metformin [3]. These events led to a lot of discussion with regard to the risk assessment for the production process that not only comprises the starting materials and reactants but also auxiliary materials, solvents,

and possible side products [4]. Most of these discussions covered the field of nitrosamine impurities only, but this would be too short-sighted. Therefore, we present a typical assessment which has to be performed for all manufacturing processes and for all drugs. As a representative drug substance, we have chosen Cetirizine dihydrochloride (Figure 1), later described as cetirizine.

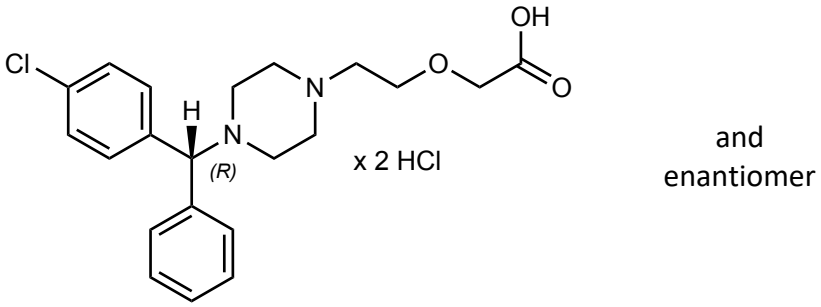


Figure 1: Cetirizine: (RS)-2-((4-((4-chlorophenyl)phenylmethyl)piperazin-1-yl) methoxy)acetic acid dihydrochloride

Cetirizine (ATC R06E07) [5] is a H_1-antihistaminic drug of the second generation. It is commonly used for the treatment of seasonal allergic rhinitis, perennial rhinitis, and chronic idiopathic urticaria [6]. It was patented in 1981 [7, 8] and came into medical use in 1987 under the brand name Zyrtec® [9]. Enantiomeric pure Levocetirizine is marketed as well. Today, cetirizine is a very commonly applied drug. Thus, many generic brands are available on the market without prescription. In 2016, more than 16 million packages of oral antihistaminic drugs were sold in pharmacies [10].

In general, it is impossible to synthesize a 100 % pure compound which holds true for all active pharmaceutical ingredients (API). They are always accompanied by impurities which can be organized in the following classes [11]: organic impurities (process- and drug-related), often called related substances; inorganic impurities; residual solvents [12]; potential mutagenic impurities (PMI) and potential genotoxic impurities (PGI) [13]. In the last 20-30 years, the PGI have drawn the attention of the regulatory authorities.

A typical example is the recently found *N*-nitrosodimethylamine (NDMA) in valsartan which occurred due to a change of the synthetic pathway [14]. Besides the formation of synthesis side products, problems might also arise from solvents due to degradation, solvent recovery, and cross-contamination in the manufacturing processes. Thus, insufficient control of impurities can quickly end up in a big problem. Hence, it is very important to analyze each synthesis step for the possible formation of side-products and to define the purity of all reagents and solvents used. Consequently, the ICH

guideline Q3A(R2) on Impurities in New Drug Substances states that “The applicant should summarize the actual and potential impurities most likely to arise during the synthesis, purification, and storage of the new drug substance. This summary should be based on sound scientific appraisal of the chemical reactions involved in the synthesis, impurities associated with raw materials that could contribute to the impurity profile of the new drug substance, and possible degradation products. This discussion can be limited to those impurities that might reasonably be expected based on knowledge of the chemical reactions and conditions involved. In addition, the applicant should summarize the laboratory studies conducted to detect impurities in the new drug substance.” [11]. Hence, some sort of risk assessment has been published by the regulatory authorities (EMA) for the NDMA occurrence in valsartan [4]. However, this should have already been done by the manufacturer upon changing the synthesis conditions. It is essential, that there are adequate methods to control all various sub-classes of impurities as defined in ICH Q3A or ICH M7.

In this article, a strategy to evaluate and minimize the load of potential risks of impurities during the manufacturing process of the drug substance cetirizine within the frame of a detailed risk assessment report is demonstrated.

As a first step in the control of an API, the European Pharmacopoeia (Ph. Eur.) or the United States Pharmacopoeia (USP) should be consulted for controlled impurities. Next, the respective synthesis pathway has to be evaluated for the formation of potential impurities, residual solvents, or PGIs in order to find out whether the chosen synthesis strategy is in accordance with the one considered for the elaboration of the pharmacopoeial monograph. Basically, the monograph offers a starting point for a risk assessment. In addition, the aim of the assessment is to retrieve impurities that are either process-related, specific or have a higher risk potential (PGI) than the monographed impurities. For cetirizine, the Ph. Eur. [15] limits six specified impurities A - F to be not more than 0.15%, each. Other impurities than A - F are regarded as unspecified impurities and must be below 0.10%. Peaks below a threshold of 0.05% can be disregarded when calculating the total amount of impurities. Of note, PGIs and PMIs are not captured by these methods. They must be controlled by a separate suitable test.

The impurities considered in the Ph. Eur. are summarized in Table 1. Additionally, the origin is given.

Table 1: Impurities listed in the European Pharmacopoeia

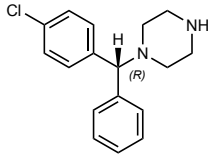
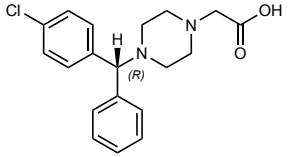
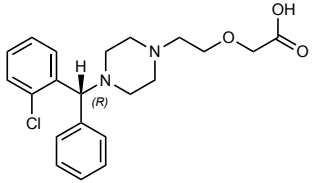
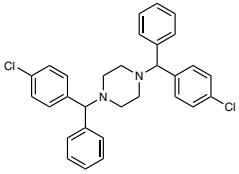
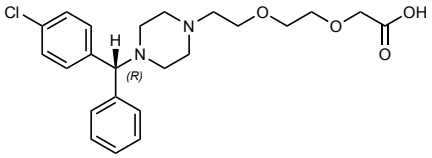
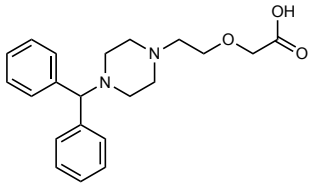
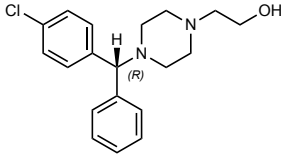
Impurity	Name	Structure		Origin
A	(*RS*)-1-[(4-chlorophenyl)phenylmethyl]piperazine		and enantiomer	Arises during synthesis due to impurity in starting material
B	(*RS*)-2-[4-[(4-chorophenyl)phenylmethyl]piperazin-1-yl]acetic acid		and enantiomer	Arises during synthesis due to impurity in starting material or Oxidation of imp. G
C	(*RS*)-2-[2-[4-[(2-chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]acetic acid		and enantiomer	Impurity in starting material leads to Regio-isomer
D	1,4-bis[(4-chlorophenyl)phenylmethyl]piperazine			Condensation of two molecules of the starting material with piperazine originating as impurity in starting material
E	(*RS*)-2-[2-[2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]ethoxy]acetic acid (Ethoxy cetirizine)		and enantiomer	Impurity in piperazinoethanol (starting material) leads to impurity
F	2-[2-[4-(diphenylmethyl)piperazin-1-yl]ethoxy]acetic acid			Impurity in starting material leads to impurity
G	(*RS*)-2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl]ethan-1-ol		and enantiomer	Intermediate

Table 2: USP monographed cetirizine impurities.

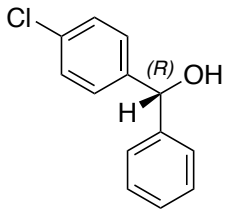
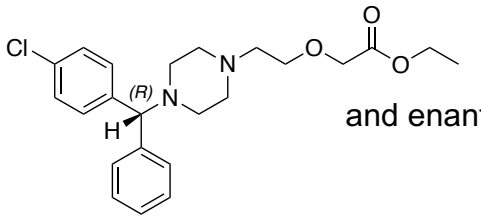
USP Impurity	Name	Structure	Origin
4-Chlorobenzhydrol	(*RS*)-(4-chlorophenyl)(phenyl)methanol	 and enantiomer	Impurity in starting material or water in starting material
Dimer ≙ Ph.Eur. Imp. D	1,4-bis[(4-chlorophenyl)phenylmethyl]piperazine	See Table 1	
2-Chlorocetirizine ≙ Ph.Eur. Imp. C	(*RS*)-2-[2-[4-[(2-chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]acetic acid	See Table 1	
Cetirizine related compound A ≙ Cetirizine ethyl ester	ethyl (*RS*)-2-(2-(4-[(3-chlorophenyl)phenylmethyl]piperazin-1-yl)ethoxy) acetate	 and enantiomer	Insufficient saponification of intermediate
Deschlorocetirizine ≙ Ph.Eur. Imp. F	(2-[2-[4-(diphenylmethyl)piperazin-1-yl]ethoxy]acetic acid	See Table 1	
CBHP ≙ Ph.Eur. Imp. A	(*RS*)-1-[(4-chlorophenyl)phenylmethyl]piperazine	See Table 1	

Table 3: Alternative USP monographed cetirizine impurities.

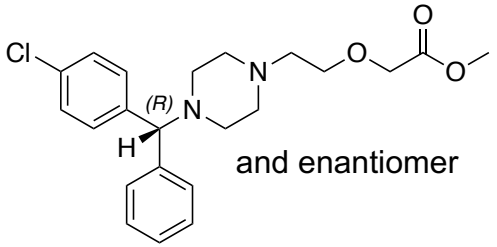
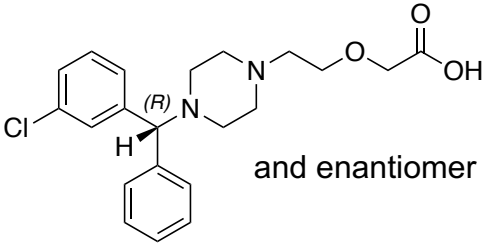
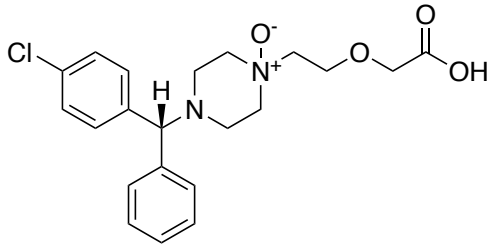
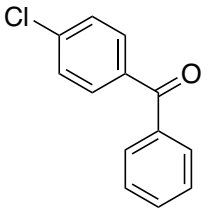
USP Impurity	Name	Structure	Origin
Deschlorocetirizine ≙ Ph.Eur. Imp. F	2-[2-[4-(diphenylmethyl)piperazin-1-yl]ethoxy]acetic acid	See Table 1	
Cetirizine ethanol ≙ Ph.Eur. Imp. G	(*RS*)-2-[4-[(4-chlorophenyl)phenylmethyl]piperazine-1-yl]ethanol	See Table 1	
CBHP ≙ Ph.Eur imp.A	(*RS*)-1-[(4-chlorophenyl)phenylmethyl]piperazine	See Table 1	
2-Chlorocetirizine ≙ Ph.Eur. Imp. C	(*RS*)-2-[2-[4-[(2-chlorophenyl)phenylmethyl)piperazin-1-yl]ethoxy]acetic acid	See Table 1	
Cetirizine methyl ester	(*RS*)-Methyl 2-(2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl] ethoxy)acetate	 and enantiomer	Insufficient saponification of intermediate or esterification due to process-related imp. of solvent in precipitation process
3-Chlorocetirizine	(*RS*)-2-[2-[4-[(4-chlorophenyl)phenylmethyl)piperazin-1-yl]ethoxy]acetic acid	 and enantiomer	Impurity in starting material leads to Regio-isomer
Cetirizine acetic acid ≙ Ph. Eur. Imp B	(*RS*)-2-[4-[(4-chorophenyl)phenylmethyl]piperazin-1-yl]acetic acid	See Table 1	

Table (3): Continued

USP Impurity	Name	Structure	
Cetirizine N-oxide	(*RS*)-2-[2-[4-[(4-chlorophenyl)phenylmethyl)piperazin-1-yl]ethoxy]acetic acid N-oxide	 and enantiomer	
4-Chlorobenzhydrol	(*RS*)-(4-chlorophenyl)(phenyl)methanol	See Table 2	
4-Chloro-benzophenone	(4-chlorophenyl)phenylmethanone		Impurity in staring material or oxidation of starting material
Dimer ≙ Ph.Eur. Imp. D	1,4-bis[(4-chlorophenyl)phenylmethyl]piperazine	See Table 1	

The monograph “Cetirizine dihydrochloride” in USP 42 [16] allows not more than 0.1% of each specified impurity. This limit is valid for unspecified impurities as well. Peaks below 0.02% can be disregarded.

It can be seen from Table 2 that the Ph. Eur. impurities B, G, and E are not included in the USP monograph, indicating that different production processes might have been considered when establishing the monographs. Furthermore, the USP limits the specified impurities to 0.1% while the Ph. Eur. limits these impurities to 0.15%.

If cetirizine ethanol ((2-(4-((4-chlorophenyl)phenylmethyl)piperizin-1-yl)ethanol) or cetirizine acetic acid are present in the test substance, a different impurity profile is expected and an alternative test is recommended to be performed according to the USP, resulting in the impurity profile listed in Table 3.

Of course, this list is not a conclusive enumeration. For example, there are some additional impurities in finished dosage forms [17-20]. However, this publication focuses on impurities that are likely to be present in the API, only.

As already mentioned, the impurities listed in Pharmacopoeias are related to the synthesis pathway. Thus, some exemplary synthesis pathways used in production scales are summarized below.

2. Synthesis of Cetirizine

The synthesis of cetirizine given in the European patent EP 58 146 [8] starts off with the nucleophilic substitution of 4-chlorobenzhydryl chloride (1) with sodium carbonate solution giving the substituted piperazine derivative (3). Next, the carbamate is hydrolyzed with hydrochloric acid to achieve (4) and subsequently the cetirizine methyl ester is formed by nucleophilic substitution using methyl(2-chloroethoxy)acetate. Finally, the ester is saponificated with potassium hydroxide to yield cetirizine (Figure 2).

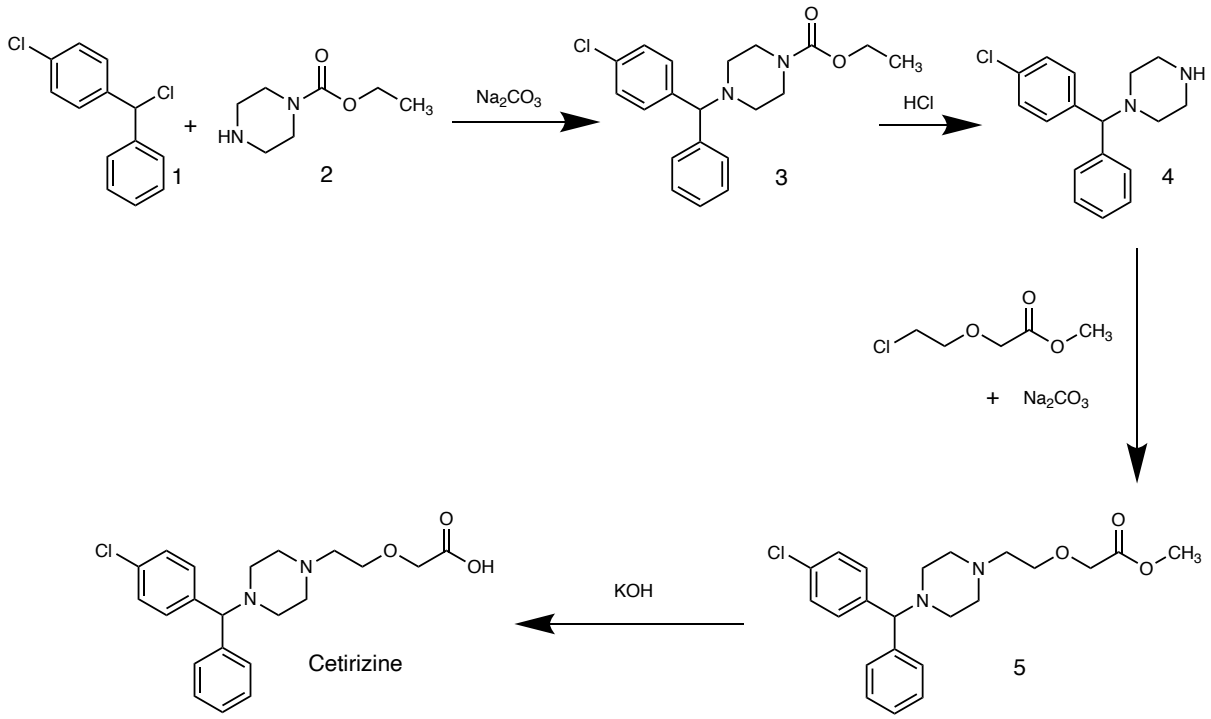


Figure 2: Synthetic pathway according to EP 58 146.

A more recent pathway is described by *Reiter et al*. [21]. It starts one step ahead as can be seen in Figure 3. To form the 4-chlorobenzhydryl chloride, 4-chloro-benzophenone is reduced by sodium borohydride and the obtained alcohol is activated with thionyl chloride to give 4-chlorobenzhydryl chloride (1) followed by a nucleophilic substitution with piperazinoethanol. After washing with water and a further nucleophilic substitution, an amide is formed which is hydrolyzed to achieve the final API.

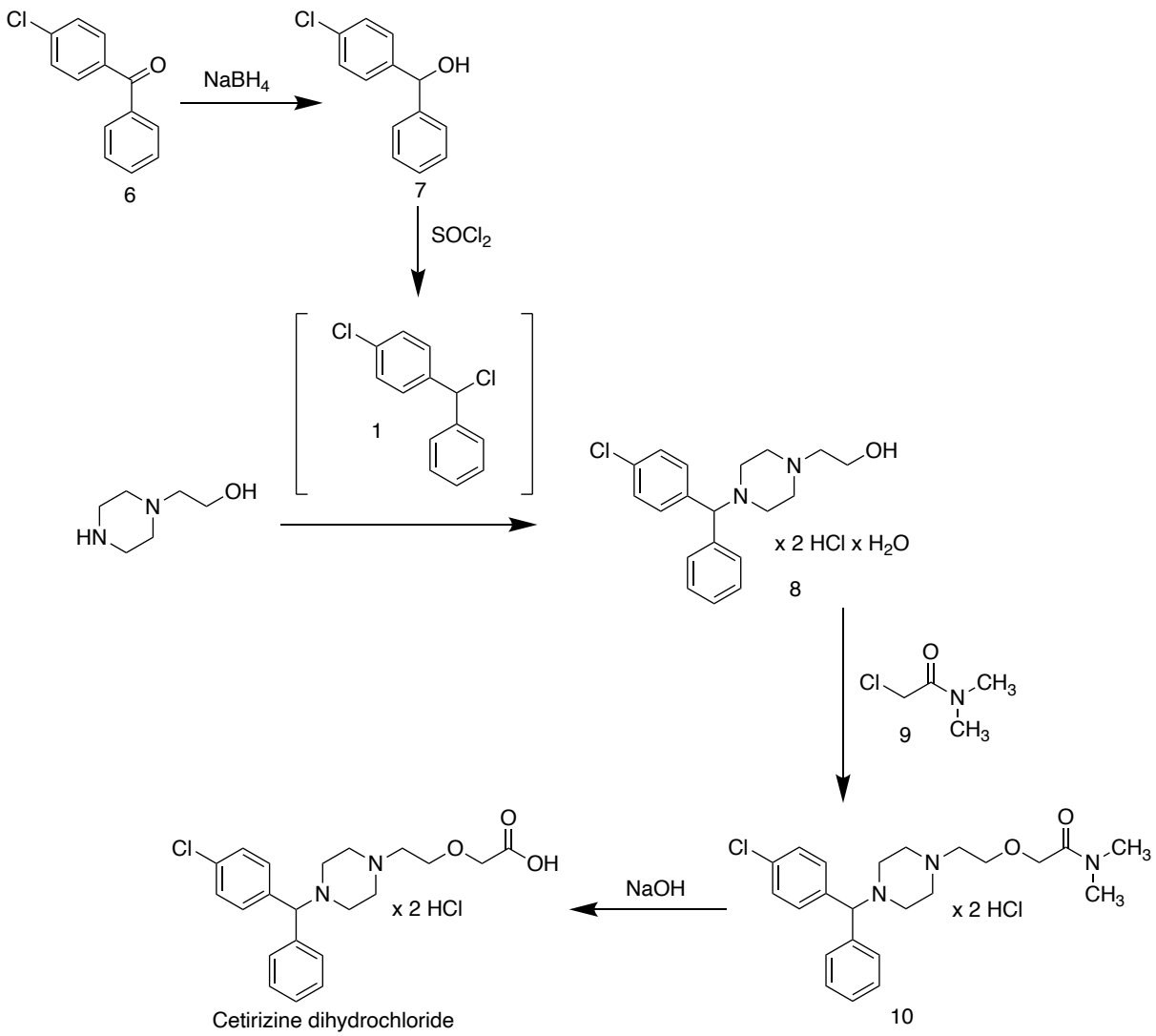


Figure 3: Synthetic pathway according to Reiter et al. [16].

Of note, the reactive compound 2-chloro-*N,N*-dimethylacetamide (9) used for the last nucleophilic substitution is synthesized from 2-chloroacetyl chloride and dimethylamine. Therefore, it is essential to ensure that the starting material does not contain NDMA which might be formed during the synthesis when the inorganic impurity nitrite is present during the manufacturing process. If pure chemicals are used, this risk does not exist. Nevertheless, it must be ensured that nitrite is absolutely absent in processes dealing with dimethylamine.

Different synthetic routes result in different impurity profiles. Therefore, pharmacopoeias try to consider all possible impurities that can arise from different drug synthesis routes in a single monograph for impurity assessment.

However, API manufacturers such as Arevipharma cannot simply rely on the impurity profile reported in pharmacopoeias for the production of a high-quality product (cf. Figure 4). They have to implement a whole risk assessment to rate the presence

of impurities in the respective API. The potential impurities most likely occurring during synthesis, purification, and storage are assessed based on sound scientific assessment of the chemical reactions involved in the synthesis, impurities associated with starting materials, intermediates, reagents, auxiliaries, and catalysts. All possible impurities contribute to the impurity profile in addition to possible degradation products. The risk assessment is necessary even when using previously described synthesis pathways because slight changes of the procedures, e.g. changing the supplier of reagents, as well as slight modifications of the reaction conditions can have a substantial influence on the impurity profile. Organic impurities, residual solvents, and PGIs have to meet the requirements of the ICH guidelines Q3A “Impurities in new drug substances”, Q3C “Residual solvents”, and M7 “Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk” [11-13].

For synthesis of the API, Arevipharma starts by converting 4-chlorobenzhydryl chloride with piperazinoethanol to form the base (8). Next, the carboxylate (11) is formed. The acid is released with an aqueous solution of hydrochloric acid and the dihydrogen chloride is eventually precipitated with hydrogen chloride gas in acetone.

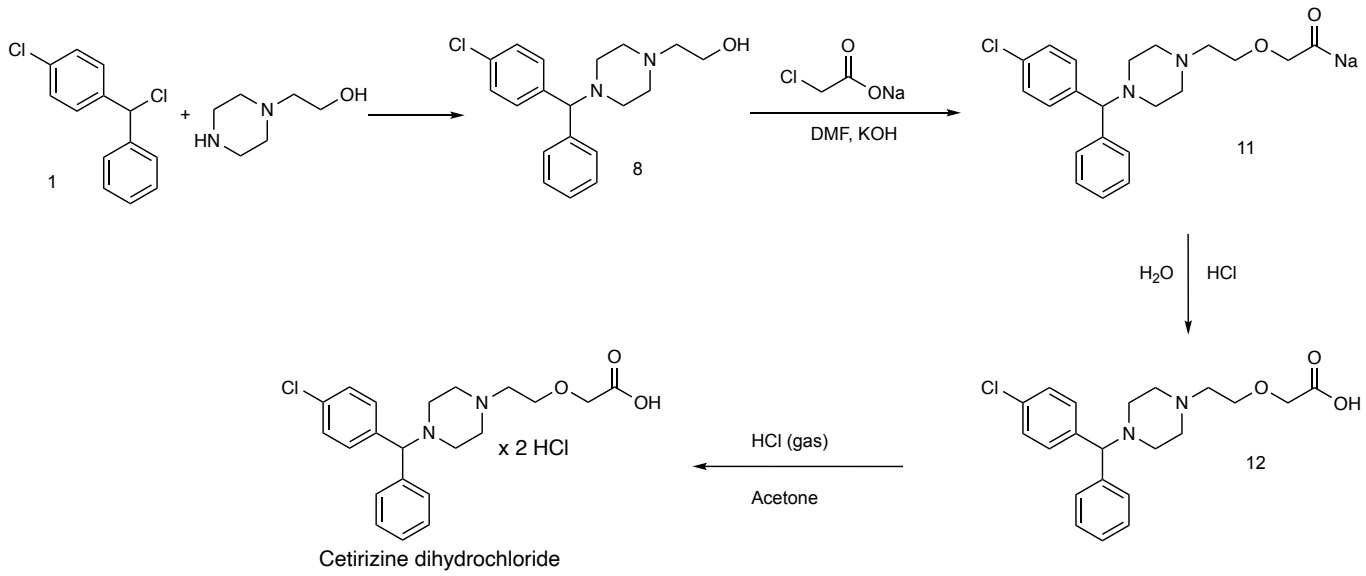


Figure 4: Synthetic pathway assessed by Arevipharma – a process based on WO2004103982 and IN2007MU00826.

3. Arising of impurities

One source of impurities is the starting material used for synthesis of the API itself. Not all starting materials are specified or even listed in the European Pharmacopoeia. For example, this holds true for 2-piperazinoethanol. For dimethylformamide, no impurities are specified but the degradation to dimethylamine and carbon monoxide has to be considered. Only the content of water is limited to 0.1%. Acetone is monographed in the European Pharmacopoeia limiting the impurities methanol and isopropanol to 0.05% (v/v) and benzene to 2 ppm (v/v). Any other impurity might be present at a limit of less than 0.05% (v/v). Water may be present in a maximum content of 3 g/L [15]. Nonetheless, it is allowed to manufacturers to set their own specification limits for their solvents (Table 4). Therefore, the purity of the starting material has to be specified and controlled well by setting narrow in-house limits for potential impurities to achieve a high-quality drug product. A good specification of the starting material is one of the most effective control strategies to reduce the load of impurities.

Table 4: Selection of starting material and solvents including the specification thereof.

Starting material/ Solvent (CAS)	Observed and potential impurities	Content
2-Piperazinoethanol (103-76-4)	piperazine 1-((2-hydroxyethoxy)ethyl)piperazine each unspecified single impurity water	≤0.10% ≤0.10% ≤0.10% ≤0.3%
Acetone, pure (67-64-1)	methanol isopropanol benzene diacetone alcohol water	≤0.1% ≤0.1% ≤0.02% ≤0.1% ≤0.2%
Dimethylformamide (68-12-2)	dimethylamine formamide methanol every other single impurity water	≤0.05% ≤0.05% ≤0.01% ≤0.05% ≤0.05%

Secondly, potential impurities might originate from solvents used in the manufacturing process. Their purity has to be specified and controlled analogously to the starting materials. Third, potential impurities originating from reagents used in the manufacturing process have to be specified as well. Luckily, in the assessed cetirizine

synthesis no organic impurities originating from the reagent materials are expected and thus, this potential source of impurities can be neglected.
Potential impurities originating from the reaction itself are probably the most important source. Therefore, a rigorous impurity and carry-over assessment was developed in order to address the formation and fate of impurities during the whole manufacturing process of cetirizine dihydrochloride. It is necessary to evaluate all sources of potential impurities and their transformations during the process. For this, a two-step approach is applied:

1. - Identification of impurities using a reaction matrix

2. - The risk of carry-over of impurities likely to be present in the active substance and further assessment of their mutagenic potential (risk assessment)

The reaction matrix built contains starting materials as well as reagents, auxiliaries, intermediates, solvents, and their impurities of each step of the process. Additionally, the chemical reactions to potential contaminants were examined under the process conditions. Each reaction and purification step of the whole manufacturing process depends on a detailed evaluation.
A reaction matrix for the following exemplary reaction is given below. The compounds A and B form the products C and D in a reaction using solvent S.

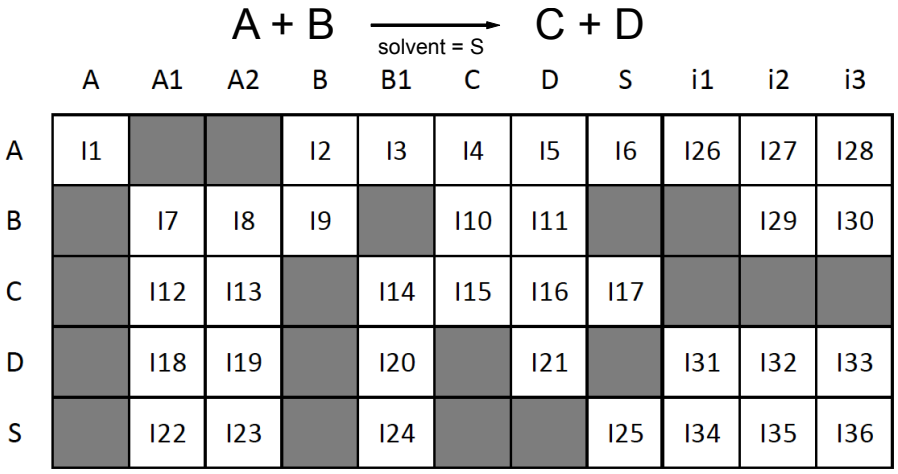


Figure 5: Schematic reaction matrix to evaluate the arise of impurities.

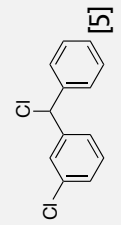
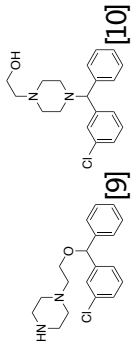
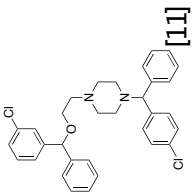
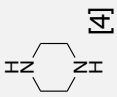
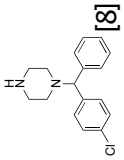
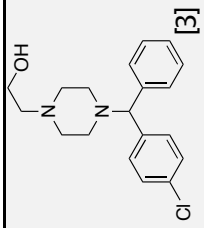
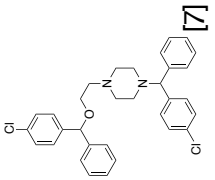
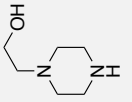
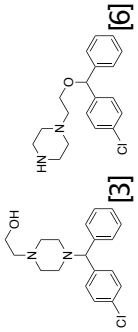
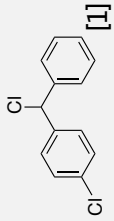
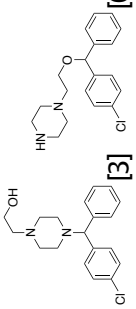
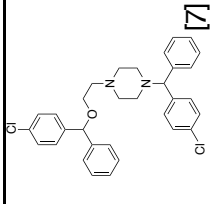
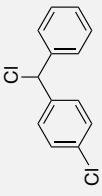
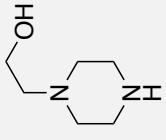
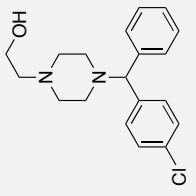
In this matrix, all used starting materials, reagents, auxiliaries, intermediates, and solvents as well as their known and potential (concluded from their manufacturing process) impurities are listed in the first row. To clarify the matrix given in Figure 5,

starting material A, its impurities A1 and A2 as well as B and impurity B1, products C and D, solvent S are put in the first row and all the major compounds given in the reaction scheme above are filled in the first column. If this reaction forms intermediates, they are put in the first row as well (e.g. i1, i2 or i3). Each table element has to be evaluated for potential reaction products in between the corresponding row and column element. The reaction products are continuously numbered in this scheme as impurities I1 to I36.

To explain the procedure in more detail, the first reaction step (Figure 4) is taken as an example (see Table 5): In the first row of the matrix, the starting materials (1) and (2) and the product (3) as well as a selection of the known impurities of the starting material (4) and (5) can be found. As described, the first column is filled with the major compounds (1), (2) and (3). Each table element had been evaluated for a potential reaction. E.g., the starting material (1) reacts with starting material (2) to give the product (3) but in addition it also reacts to the impurity (6). To simplify the scheme a selection is shown only.

It was assumed that it is unlikely that reagents and their impurities are reacting with each other because of their co-existence in the starting material. This assumption applies only, if activating effects (e.g. pH value, catalysts, temperature) are absent and an activation by chemical transformation can be excluded. Nevertheless, these assumptions have to be assessed carefully. At this point of the assessment, the use of reaction matrices can be a powerful tool. Second, it is unlikely that two impurities of starting material or reagents are reacting with each other due to their low concentration. One exception was defined: In case of solvent, impurities can react with each other because they are present in higher quantities. Furthermore, it was assumed that at some point further conversion of impurities into new species is unlikely due to incomplete reaction and decreasing concentration of reactants (exception: reactant = solvent).

Table 5: Selection of starting material and impurities to build a reaction matrix for the first step [numbered according to Arevipharma].

	–		
		–	–
		–	–
		–	–
	–		
			

Creating the final matrix can be compared to a rolling circle (Figure 6): After evaluating the reaction matrix for the first time, the processor has to look at the molecules. If they carry reactive groups, they have to be added to the first row of the matrix. Their interactions with the major compounds have to be considered until no “reactive” impurities are existent.
Next, the chance for the carry-over of potential impurities has to be evaluated. The probability of carry-over is classified in four categories:

1 - Unlikely to be carried over
2 - Likely to be depleted, but potential carryover
3 - Likely to be carried over
4 - PGIs that are likely to be present in the final API

Impurities that are classified as group 2, 3, or 4 substances were transferred to the reaction matrix of the next process step. The decision whether an impurity/compound is carried over into the next step is based on an evaluation of key chemical and physical properties like reactivity, melting/boiling point, solubility, distribution during extraction, and crystallization behavior. This semi-quantitative approach based on theoretical purging factors as described by [22] has been applied to several processes over the last years.
Degradation products, which might impact the shelf-life specifications, can be identified by stability studies and stress tests of the environmental factors pH, light, oxygen, temperature, and humidity. These tests are not part of this assessment – but might be the starting point of a follow-up review considering the systematic review of the degradation profile and degradation pathways.

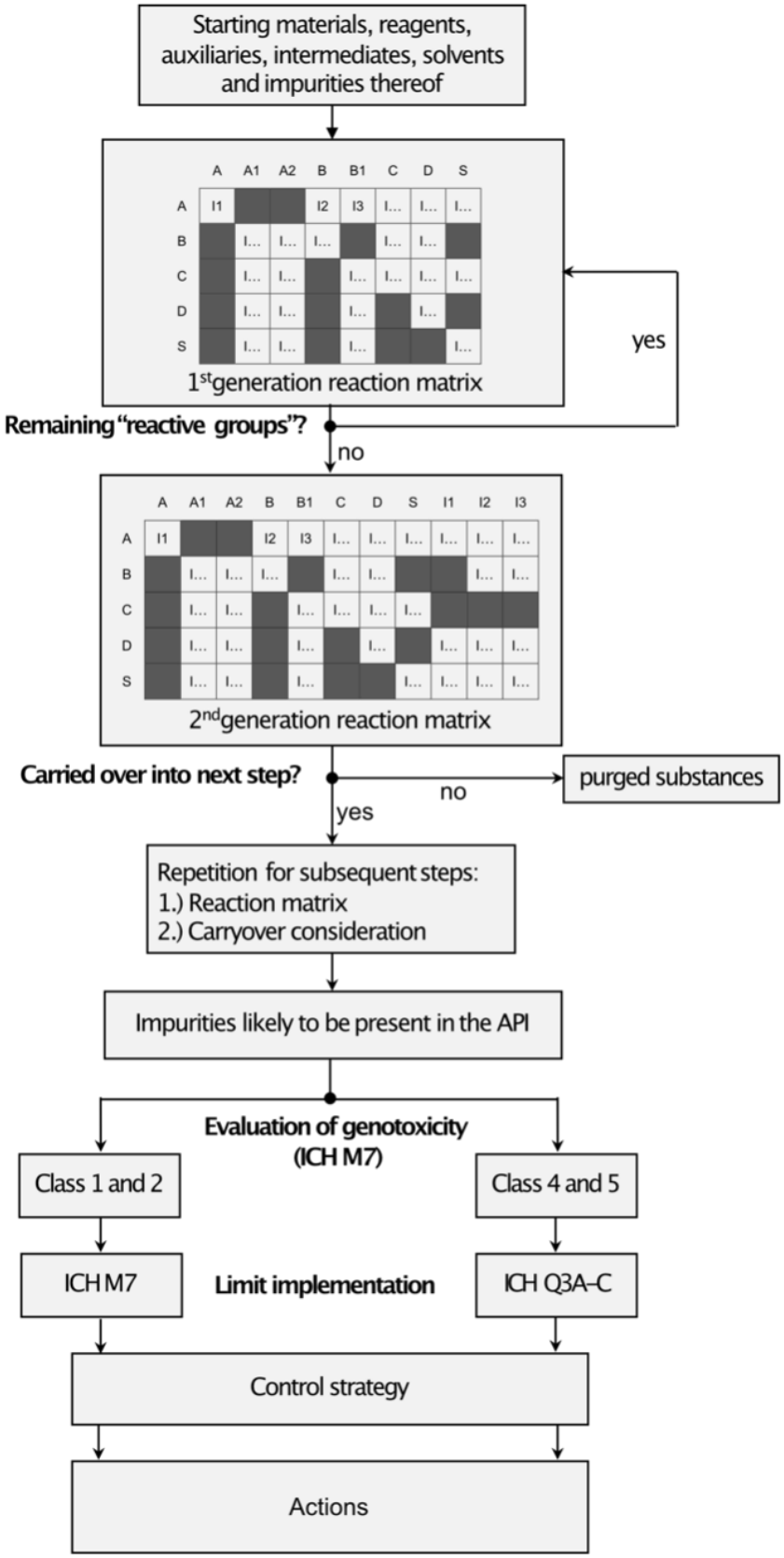


Figure 6: Schematic flow chart how to build up the entire risk assessment.

4. Potential genotoxic impurities

PGIs have attracted the attention of regulatory authorities over the last 20-30 years. The ICH guideline M7 outlines recommendations for assessment and control of mutagenic impurities that reside or are reasonably expected to exist in the final drug substance or product. The “Threshold of Toxicological Concern” (TTC) concept was developed to define an acceptable intake for any unstudied chemical that poses a negligible risk of carcinogenicity or other toxic effects. The methods upon which the TTC is based are generally considered to be very conservative. PGIs are initially identified by their chemical structure being associated with genotoxic activity.

The hazard assessment was done for all impurities that are likely to be present in the API according to the carryover considerations. Therefore, all impurities are categorized in five classes in conformity to ICH M7. For example, Class 1 compounds are defined as mutagenic carcinogens. They must be controlled at or below a specific acceptable limit. At the other end of the list, Class 5 compounds do not carry alerting structural elements/groups and are treated as non-mutagenic impurities. In a nutshell, it is possible to categorize each compound according to its mutagenic hazard into these five classes and decide whether or not it is necessary to define limits due to otherwise existing health risks. In accordance to ICH M7 Guideline Class 4 and Class 5, impurities are treated like non-mutagenic substances and their limits have been defined in accordance to the ICH Q3 A-D guideline.

For sake of completeness it should be noted, that quantitative structure–activity relationship (QSAR) tools also exist. They can be used to support these kinds of assessments as well.

5. Residual solvents

The ICH Q3C guideline provides applicable limits for residual solvents based on the permitted daily exposure (PDE) and categorizes solvents in three classifications in accordance to their toxicity:

“*Class 1 solvents:* *Solvents to be avoided.* Known human carcinogens, strongly suspected human carcinogens, and environmental hazards.

Class 2 solvents: *Solvents to be limited.* Non-genotoxic animal carcinogens or possible causative agents of other irreversible toxicity such as neurotoxicity or teratogenicity. Solvents suspected of other significant but reversible toxicities.

Class 3 solvents: *Solvents with low toxic potential.* Solvents with low toxic potential to the human body; no health-based exposure limit is needed. Class 3 solvents have PDEs of 50 mg or more per day" [12].

Of note, Class 1 solvents that might be present in another solvent (e.g. toluene or acetone containing benzene) are considered as well.

6. Risk of the formation of PGI under synthesis conditions

The manufacturing process includes the precipitation of the hydrochlorides in acetone followed by drying at elevated temperatures. Under these conditions it is possible that mesityl oxide is formed [23] and reacts with hydrogen chloride having emerged from the ammonium salts.

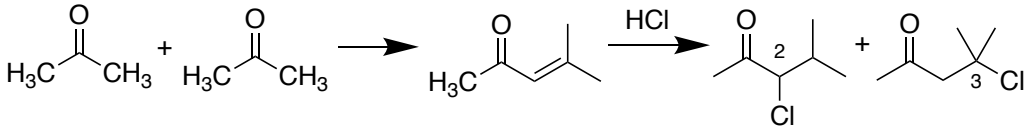


Figure 7: Formation of mesityl oxide and hypothetical addition of HCl generating 2-Cl and 3-Cl substituted compounds.

Two possible regioisomers can be formed during a reaction of hydrochloric acid to mesityl oxide (Figure 7). It is known that adding hydrochloric acid results in the selective formation of the higher substituted halide (Markovnikov's rule). [24] The 2-chloro derivative can only be generated if a tertiary amine is present (Baylis–Hillman like reaction) [24], but the precipitated hydrochlorides are quaternary ammonium salts with essentially no nucleophilicity. Therefore, it is plausible that only the 3-chloro derivative is likely to be present after the drying process. Considering the maximum daily dose of 20 mg cetirizine dihydrochloride per day, the identification threshold is 0.10 % according to ICH Q3A. Lower thresholds can be appropriate if the impurity is unusually toxic, e.g. Class 4-5, ICH M7. Here, the reporting threshold for impurities is set to 0.05%.

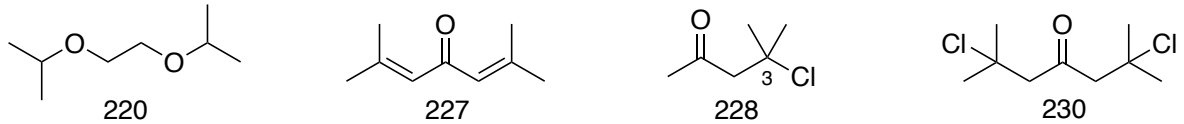


Figure 8: Impurities 220, 227, 228 and 230.

Impurities 220, 227, 228, and 230 (Figure 8) have been classified as Class 3 compounds due to their structural alert as an alkylating agent but with no mutagenicity data available (ICH M7). [13] According to ICH M7, the daily intake of 1.5 µg of a single

mutagenic substance is acceptable for a treatment duration of more than 10 years to lifetime. Of note, for some sub-classes the limit is even higher. Nevertheless, a very conservative way was chosen.

Taking into account the permitted daily exposure (PDE) of 20 mg cetirizine dihydrochloride, a limit of 75 ppm of a single potential genotoxic contaminant (PGI) was calculated ($\frac{1.5\ \mu g}{20\ \mu g} = 75\ ppm$) [9].

As directed according to ICH M7 guideline, no routine testing is necessary when it can be guaranteed that levels of the impurity in the drug substance are less than 30% of the acceptable limit for at least six consecutive pilot scale or three consecutive production scale batches. 30% of the lowest limit of 75 ppm of an individual PGI in cetirizine are calculated to be 22 ppm.

If there are at least three known mutagens with unknown carcinogenic potential (class 2 according to ICH M7) or impurities with alerting structure, unrelated to the structure of the drug substance (class 3 according to ICH M7), the total PGIs have to be limited to a total daily intake of 5 µg/day. Analogue to the criteria above, a limit of 250 ppm of multiple PGIs can be calculated. 30% of the lowest limit of 250 ppm of multiple PGIs in cetirizine dihydrochloride are calculated to be 75 ppm. Hence, if the manufacturer can prove to reliably produce batches containing <22 ppm of individual PGI and <75 ppm of total PGIs, no testing is needed. During the hazard assessment, four impurities were identified to be candidates for genotoxic impurities. They are illustrated in Figure 8.

Taken together, 222 compounds have been identified in this assessment as potential impurities. The carry-over of most of them can be neglected due to their physicochemical properties. Fortunately, only a few of them will be found in the final API product in very low amounts.

7. Control strategy

To identify the components an extensive fate and purge assessment was performed in accordance to [22] based on reactivities, solubilities, volatilities, ionizability, and crystallization behavior. However, the theoretical risk assessment of the presence of impurities and their carry-over to the final API based on predictions made from chemical knowledge is only one component of an impurity risk assessment. The second centerpiece – the implementation of an adequate control strategy – is a crucial

part of an impurity risk assessment, which can be challenging due to the different level impurities have to be controlled at.

Beginning from starting materials, solvents and auxiliaries via intermediates up to the final API adequate specifications are established and justified based on the underlying risk of potential impurity contamination. In this context, impurities that might be present or might be carried-over are controlled – and the process capability for their depletion is continuously monitored.

For impurities that are evaluated to pose a lower risk of carry-over due to process understanding and down-stream process capability for depletion, no routine test according to the established control strategy is performed. In these cases, a study providing evidence for absenteeism is performed in addition to the theoretical assessment.

Related substances that are not covered by routine analytical tests, were subject to HPLC-MS tests of production scale batches of cetirizine dihydrochloride. An HPLC-MS method adopted to the Ph. Eur. HPLC method has been performed. However, sulfuric acid had to be replaced by trifluoroacetic acid due to the conditions needed for LC-MS. The mobile phase used consists of a mixture of seven parts of water pH 1.2 (adjusted with trifluoroacetic acid) and 93 parts of acetonitrile. For separation, a Thermo Hypersil Silica 5µm, 250 x 4.6 mm column was used. The experiments were performed on a Thermo/Dionex Ultimate 3000 HPLC system coupled with a Thermo/Dionex MSQ+ mass spectrometer using ESI positive/negative switching ionization mode covering a mass range of 100 – 1000 m/z.

Using the molar masses, sum formulae have been determined and compared with potential structures. Of course, with this procedure it is not possible to distinguish between regio- and stereoisomers. Identification of the present isomers of the masses detected was achieved by spiking experiments. The identified isomers were qualified as reference standards and detection methods were validated. Validation studies confirmed the suitability of the analytical procedure for testing of the related substances of cetirizine dihydrochloride.

These HPLC-MS investigations - supported by spiking experiments - allowed the identification of seven impurities. Out of these seven compounds (Table 6), six can be identified by the risk assessment. The presence of the remaining one (cetirizine ethyl ester) above the acceptable limit is excluded due to the solvent specifications. In addition, it is controlled in routine testing.

Table 6: Compounds detected, their high-resolution masses, possible sum formulae and structures.

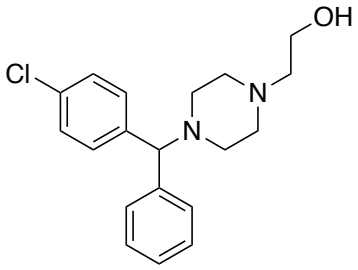
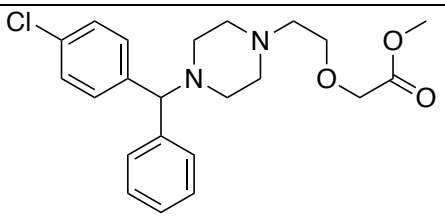
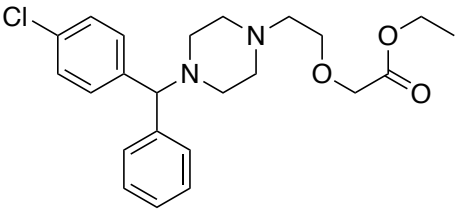
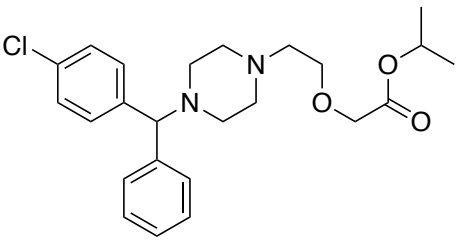
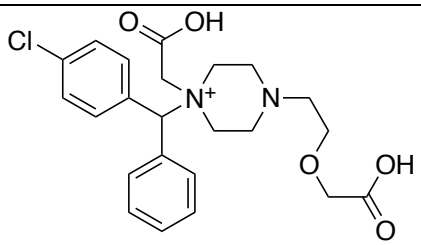
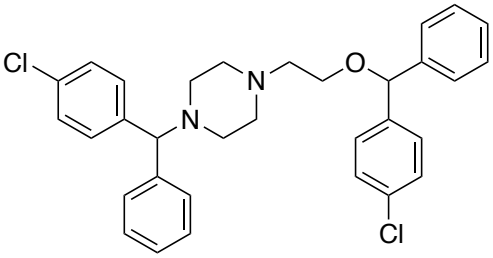
m/z	Calculated sum formula	Possible structure (only one isomer shown)
331.1572	$C_{19}H_{24}ON_2Cl$	
389.1627	$C_{21}H_{26}O_3N_2Cl$	Cetirizine
403.1783	$C_{22}H_{28}O_3N_2Cl$	
417.1939	$C_{23}H_{30}O_3N_2Cl$	
431.2	$C_{24}H_{32}O_3N_2Cl$	
447.1682	$C_{23}H_{28}O_5N_2Cl$	
531.2	$C_{32}H_{32}ON_2Cl_2$	

Figure 9 shows that the method used is not ideal. Especially the quantification of Cetirizine methyl ester might probably not be correct. Its peak is overlaid by the main peak of Cetirizine. Normally, peak overlay is not a problem of MS detection, but here it has to be considered that the MS detector is probably at the limit of saturation and not operating linear anymore due to the high amount of API injected.

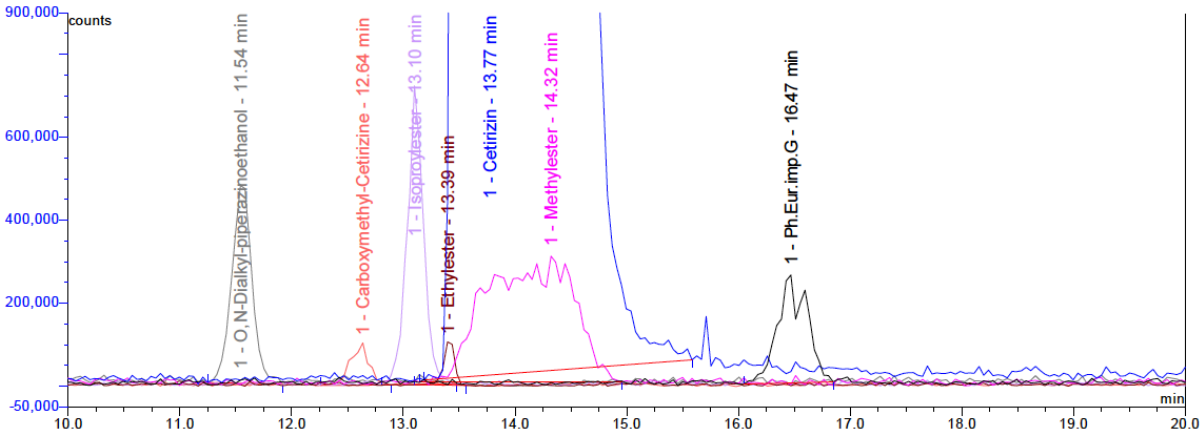


Figure 9: Overlay of extracted ion chromatograms of an exemplary batch.

Besides related substances, the absence of other impurities like PGIs, PMIs and non-related substances that poses a certain risk as assessed, was demonstrated using validated specific methods with sufficient sensitivity (Section 6). The batch analysis data is provided. Hence, these analytical investigations and absence studies support the theoretical assessment and complement the established control strategy.

8. Conclusion

The identification process including considerations of carry-over of potential impurities revealed a number of compounds that are likely to be present in the final API cetirizine dihydrochloride. These candidates were evaluated for their potential genotoxicity (PGI) and have been classified into five groups in accordance to the ICH M7 Guideline.

The majority of the examined compounds are non-alerting structures which are related to the drug substance. Some of these impurities are controlled by starting material specifications and therefore, no additional testing is necessary. A good specification is one of the most effective control strategies to reduce the load of impurities. The presence of impurities, which cannot be controlled by other specifications, was evaluated by mass screening experiments. Seven impurities were identified. For all other compounds of the matrix, absence was proved.

Furthermore, residual solvents were identified and classified in agreement with the ICH guidelines. The batch analysis using validated test methods provided evidence that

- the class 1 solvent benzene was always found to be below the threshold for routine test,
- the class 2 solvents dimethylformamide, methanol, and toluene were always found to be below the threshold for routine test
- the class 3 solvents acetone and 2-propanol were found at very low concentration levels (cf. Figure 4).
- In addition, these solvents are covered by the test for *Loss on drying* and thus in routine analysis, a specific test for these solvents is not required.

A routine analysis has been established for dichloromethane and mesityl oxide. Furthermore, methods for detecting the volatiles ethyleneglycol and ethyleneglycoldimethylether were validated by analysis of ten production scale batches. The content of these volatiles is always below 10% of the limit derived from ICH Q3C. Therefore, a further routine test is not required.

Degradation products can be identified by stability studies and stress tests of the environmental factors pH, light, oxygen, temperature, and humidity. These tests are not part of this assessment – but might be the starting point of a follow-up review considering the systematic review of the degradation profile and degradation pathways. In a next step, impurities arising in finished dosage forms can be evaluated as well. In literature, some additional impurities are shown for drug formulations of cetirizine [17-20]. Maybe it would be possible to build up a similar risk assessment - as it is described for the API - to identify all possible impurities in the finished dosage form as well.

All synthetic pathways creating the API should be evaluated in a risk assessment. Unfortunately, not all changes of the conditions can be considered. Therefore, new findings have to be reported and the assessment must be adopted or repeated when any procedure is modified.

This representatively described typical risk assessment of Cetirizine dihydrochloride has to be performed for all manufacturing processes and for all marketed drugs. By discussing not only the starting materials and reactants but also auxiliary materials, solvents, and possible side products thereof, probably all possible impurities might be identified and a further “unexpected” observation - as described for sartans, ranitidine, and metformin - could be prevented.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] European Medicines Agency, Sartan medicines: companies to review manufacturing processes to avoid presence of nitrosamine impurities, EMA/248364/2019 Rev 1, 2019.

[2] U. S. Food and Drug Administration, Laboratory Tests | Ranitidine, 2019, https://www.fda.gov/drugs/drug-safety-and-availability/laboratory-tests-ranitidine, (acessed 03.11.2019).

[3] European Medicines Agency, EMA Update on Metformin Diabetes Medicines, EMA/660975/2019, 2019.

[4] European Medicines Agency, Information on Nitrosamines for Marketing Authorisation Holders, EMA/189634/2019, 2019.

[5] WHO Colloboration Center for Drug Statistics Methodology, https://www.whocc.no/atc_ddd_index/?code=R06AE07, 2018 (accessed 12.08.2019).

[6] D. Singh-Franco, H.L. Ghin, G.I. Robles, N. Borja-Hart, A. Perez, Levocetirizine for the treatment of allergic rhinitis and chronic idiopathic urticaria in adults and children, Clin Ther, 31 (2009) 1664-1687. https://doi.org/10.1016/j.clinthera.2009.08.015

[7] J. Fischer, C.R. Ganellin, Analogue-based Drug Discovery, Wiley 2006.

[8] E.T. Fischer, Svend Peter, EP 58 146: A Process for the Preparation of 2-2-4-(diphenylmethyl)-1-piperazinyl]ethoxy Acetic Acid Compounds or Salts Thereof, 1981.

[9] GSK, ZYRTEC Datasheet, https://medsafe.govt.nz/profs/Datasheet/z/zyrtecsol.pdf, 2012, (accessed 19.03.2020).

[10] N. Tröbitscher, Heuschnupfen: Verordnungen nehmen zu. https://www.apothekeadhoc.de/nachrichten/detail/markt/antillergika-heuschnupfen-verordnungen-nehmen-zurhinologika-antihistaminika/, 2017, (accessed 20.12.2019).

[11] ICH Hormonised Triparite Guideline, Impurities in new drug substances Q3A (R2), Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland, 2006.

[12] ICH Hormonised Triparite Guideline, Impurities: Guideline for residual solvents Q3C (R7), Current Step, 4 (2018) 1-25.

[13] ICH Hormonised Triparite Guideline, Assessment and Control Of Dna Reactive (Mutagenic) Impurities In Pharmaceuticals To Limit Potential Carcinogenic RISK M7, International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH): Geneva, 2017.

[14] T. Nguyen, A Side Reaction May Have Led to Impurities Found in Valsartan Heart Drugs, 2019.

[15] European Pharmacopoeia 9th edition, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2017.

[16] United States Pharmacopeia 42, US Pharmacopeial Convention, Rockville, MD, USA, 2018.

[17] A. Jaber, H. Al Sherife, M. Al Omari, A. Badwan, Determination of cetirizine dihydrochloride, related impurities and preservatives in oral solution and tablet dosage forms using HPLC, J. Pharm. Biomed. Anal., 36 (2004) 341-350, https://doi.org/10.1016/j.jpba.2004.07.002.

[18] F.S. Javid, A. Shafaat, A. Zarghi, Determination of cetirizine and its impurities in bulk and tablet formulation using a validated capillary zone electrophoretic method, J. Anal. Chem., 69 (2014) 442-447, https://dx.doi.org/10.1134/S1061934814050037.

[19] Z. Zhou, Non-target impurity profiling of marketplace Cetirizine using high-resolution mass spectrometry and multivariate data analysis, Rapid Commun. Mass Spectrom., 30 (2016) 1941-1950. http://dx.doi.org/10.1002/rcm.7675

[20] S. Rudaz, S. Souverain, C. Schelling, M. Deleers, A. Klomp, A. Norris, T. Vu, B. Ariano, J.-L. Veuthey, Development and validation of a heart-cutting liquid chromatography–mass spectrometry method for the determination of process-related substances in cetirizine tablets, Anal. Chim. Acta, 492 (2003) 271-282. https://doi.org/10.1016/S0003-2670(03)00339-8

[21] J. Reiter, P. Trinka, F.L. Bartha, L. Pongó, B. Volk, G. Simig, New Manufacturing Procedure of Cetirizine, Org. Process Res. Dev., 16 (2012) 1279-1282. http://dx.doi.org/10.1021/op300009y

[22] A. Teasdale, Genotoxic impurities: strategies for identification and control, John Wiley & Sons, 2011.

[23] M. Konieczny, G. Sosnovsky, Novel Aspects in the Preparation of Phorone, Zeitschrift für Naturforschung B, 33 (1978) 454-460.

[24] J.Clayden, N.Greeves, S. Warren, Organische Chemie, Springer Spektrum, Berlin; Heidelberg, 2013.

3.2. Dapsone

3.2.1. Impurity profiling of dapsone using gradient HPLC method

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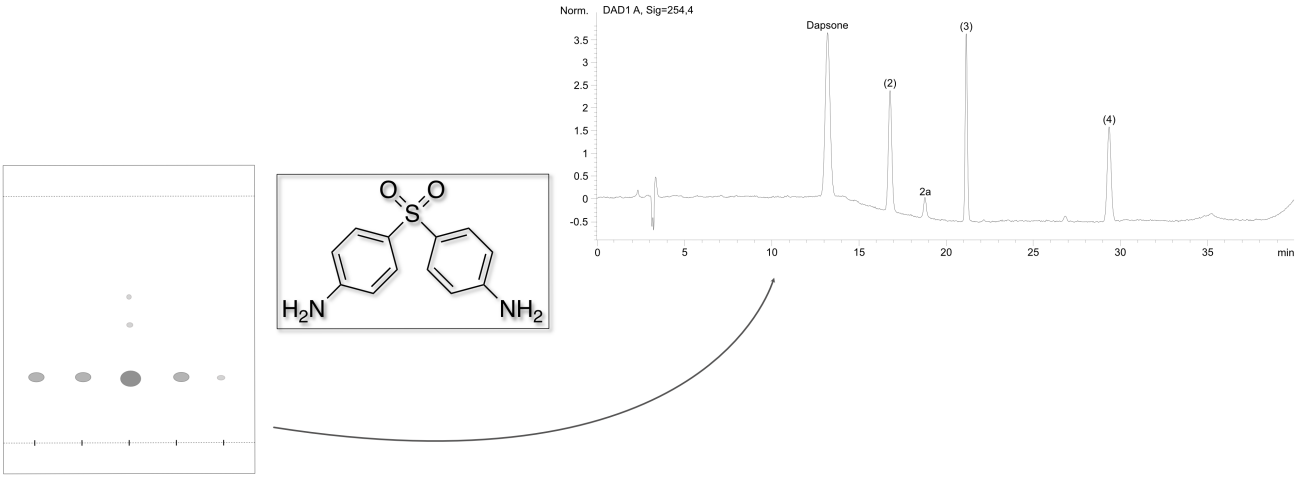
https://doi.org/10.1016/j.jpba.2021.113982

Abstract

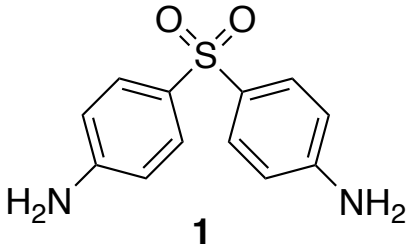
The quality control of active pharmaceutical ingredients (APIs) is a very important aspect for drug products entering the market. However, also for the well-established drugs, there ought to be a state-of-the-art impurity control. Some of the pharmacopoeial tests for related substances still make use of thin layer chromatography, even though selectivity and sensitivity are suboptimal. Here, we report on the development of a new gradient high performance liquid chromatography (HPLC) method for dapsone in order to replace the currently described pharmacopoeial TLC method. The separation of all relevant components was achieved on a C18 stationary phase (Waters XTerra® RP_{18} 5 µm 4.6 × 250 mm) using a water-acetonitrile gradient. A limit of detection (LOD) of 0.02% was registered for all specified impurities. Additionally, within this study an “impurity of an impurity” was identified by means of LC–MS/MS.

Graphical Abstract



1. Introduction

Nowadays, potential new drug substances must pass a detailed registration process before entering the European market [1]. Safety, efficacy, and good quality must be demonstrated for new innovative medicines. However, not all drugs on the market can be categorized as “new” or “innovative”. Some of them are already in use for 100 years. For these well-established drugs, there ought to be a control mechanism for quality and safety in place. The European Pharmacopoeia (Ph. Eur.) aims at doing this. The general monograph 5.10. “Control of impurities in substances for pharmaceutical use” [2] states that “[all monographs] are designed to ensure acceptable quality for users”. Due to the constant increase of knowledge, some of the tests for related substances are no longer state-of-the-art. In the cases of “old” drugs, thin layer chromatography (TLC) tests are sometimes still in use, even though selectivity and sensitivity are not sufficient anymore. Hence, they have to be replaced by HPLC tests [3]. In addition, new production pathways lead to new production-related impurities that must be included in the test for related substances. When considering these constantly changing challenges, it is obvious that periodically and critically reviewing the already established monographs is essential. We chose the long-term used substance dapsone (Figure 1) as a representative drug to exemplarily demonstrate this process.



1

Figure 1: Molecular formula of dapsone

1.1. Pharmaceutical use of dapsone

Mycobacteria, such as *Mycobacterium leprae* [4] or *Mycobacterium tuberculosis*, can cause very severe diseases such as leprosy or tuberculosis. The physicochemical properties of most common antibiotic compounds are too hydrophilic to pass through the outer mycomembrane [5, 6], while dapsone (Figure 1) is lipophilic enough to penetrate to its target, the bacterial folic acid biosynthesis [5, 7]. Additionally, dapsone is also used as a supporting agent against protozoal infections such as malaria [8].

1.2. Synthesis of dapsone

Since most of dapsone's impurities are production-related, the synthesis pathways have to be examined carefully. The first dapsone synthesis has already been described in 1908 by *E. Fromm and J. Wittmann* [9, 10]. Another common synthesis was described by *S. Sugasawa and K. Sakurai* [11].

In modern manufacturing processes, it is sometimes advantageous to apply a continuous process. One example is described in patent CN103819372 [12], making use of 4,4'-dichlorodiphenyl sulfone (9) as starting material. In an Ullmann-type [13-15] coupling reaction, (9) is aminated under high pressure with ammonium hydroxide and copper (I) chloride as catalyst (see Figure 2). According to information on the webpage [16], Atul Ltd, an Indian manufacturer of dapsone, is following this pathway. Here, batches of Atul have been checked by means of the final HPLC methods.

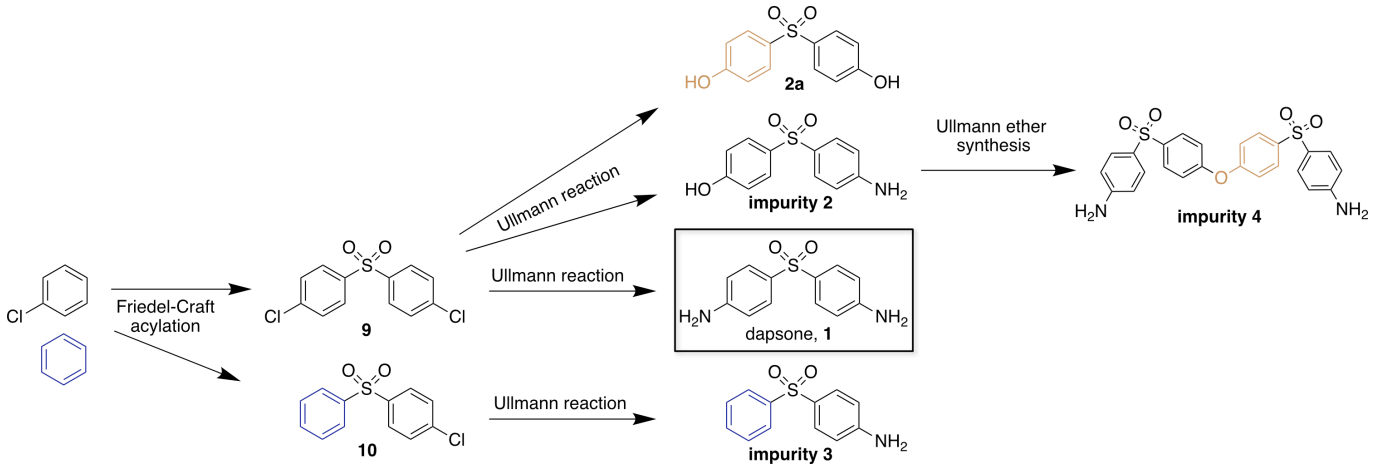


Figure 2: Schematic formation of dapsone and its impurities.

1.3. Pharmacopoeial quality control

The current monograph of dapsone described in the European Pharmacopoeia makes use of a TLC method for the assessment of related substances which consist of development, air drying, and subsequent spray-coating with a solution of 4-dimethylaminocinnamaldehyde in ethanolic hydrochloric acid. Examination under daylight allows for detecting one impurity a limit lower than 1.0%. If there are two impurities present, not more than 0.2% of each is allowed, respectively [2]. Using slightly different methods, the International Pharmacopoeia (Ph. Int.) [17] and the United States Pharmacopeia (USP) [18] also use this TLC methodology for their tests for related substances. Furthermore, the USP applies normal phase chromatography for the assay of dapsone.

As already mentioned, the aim of this study was to develop a state-of-the-art HPLC method for the Ph. Eur. [19]. The amount of the impurities 2, 3, and 4 in the investigated batches was higher than 0.1% each. Thus, they have to be regarded as specified impurities.

All of them are side products of the dapsone synthesis. As can be seen in Figure 2, impurity 3 can be present because the starting material chlorobenzene contains small amounts of benzene, which gave the monochloro compound (10) upon Friedel-Crafts acylation [20], followed by the Ullmann reaction. Impurity 2 and impurity 4 originate from the manufacturing process as a side product and follow-up product, respectively. Instead of the nucleophile ammonia which is required during the Ullmann reaction, water is involved in replacing the chlorine substituents to form impurity 2, which represents a reactant for an Ullmann ether synthesis itself, resulting in impurity 4 [21].

1.4. Analytical methods in literature

To the best of our knowledge, five analytical methods are reported in the literature, i.e. two for the quantification of dapsone in human plasma [22, 23], the assay in tablets [24], the impurity assessments by using microbore column chromatography with fluorometric quantitation of the obtained fractions [25], and a normal phase chromatography method using UV detection [26]. The latter method showed acceptable peak separation but a low sensitivity due to a high noise level. In 2014, an ecofriendly RP-method for the determination of dapsone was presented [27]. However, a sensitive method for quality evaluation is still needed.

2. Experimental

2.1.Chemicals and reagents

Dapsone reference standards and all impurities were obtained from the European Directorate for the Quality of Medicines & HealthCare (EDQM), Strasbourg, France. Acetonitrile and methanol, both HPLC grade (>99.9% purity), were purchased from Sigma-Aldrich Chemie GmbH (Darmstadt, Germany). Ultra–pure water was produced by a water purification system from Merck Millipore (Darmstadt, Germany). All solutions were filtered through a 0.2 µm PTFE filter supplied by VWR international GmbH (Darmstadt, Germany).

2.2. Apparatus

The method development was performed on an Agilent 1100 modular chromatographic system consisting of an online vacuum degasser, a binary pump G1312A, an auto sampler G1313A, a thermostated column compartment G1316A and a diode-array detector G1315B (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Agilent ChemStation® Rev. B03.02 software was used for data processing. The dwell volume of the Agilent system used for method development was 1.307 mL.

High resolution tandem mass spectrometry experiments were performed on a QqTOF system consisting of an Agilent Infinity II chromatographic system (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) coupled to a SCIEX X500R QTOF mass spectrometer (SCIEX, Concord, Ontario, Canada) equipped with a Turbo V™ source (SCIEX, Concord, Ontario, Canada). The mass spectrometric conditions for the QqTOF experiments are listed in Table 1.

Table 1: Mass spectrometric conditions for the QqTOF experiments.

Ionization:	ESI, positive mode
Ionspray voltage:	5500 V
Curtain gas:	20 psi
Ion source gas 1	50 psi
Ion source gas 2	50 psi
Temp.:	575 °C
Collision energy:	25 V

2.3. Chromatographic procedure

As stationary phase, a column with end-capped octadecylsilyl silica gel for chromatography was used as stationary phase (a XTerra® RP18 5 µm 4.6 x 250 mm column; Waters Corporation, Milford, MA, USA). The chromatographic system was operated using gradient elution at ambient temperature and UV detection at 254 nm. Mixtures containing acetonitrile and water (25:75 v/v) - labeled A - and (75:25 v/v) – labeled B - were used: 0-10 min: 0% B; 10-20 min: 0→50% B; 20-35 min: 50% B; 35-36 min: 50→0% B; 36-40 min: 0% B, respectively. The flow rate was set to 1.0 mL/min and the injection volume was 20 µL. The amount of impurities within the batches was measured in sextuple on two different days.

For the high-resolution mass spectrometry experiments, 0.1 % (v/v) of formic acid was added to the mobile phase due to better ionization. The experiments were performed with ESI in positive mode.

2.4. Preparation of sample and stock solutions

For the sample solutions, 40 mg of dapsone were dissolved in 100 mL of a mixture of acetonitrile-water (50:50, v/v). Stock solutions (40 µg/mL) of the respective impurities - in an acetonitrile-water mixture (50:50, v/v) - were prepared for spiking the sample solution. The system suitability solution of an equal concentration level was prepared by spiking 100 µL of the sample solution with 1000 µL of each impurity stock solution and diluting this mix with the acetonitrile-water mixture (50:50, v/v) to 100 mL.

All solutions were stored at ambient temperature.

3. Results and discussion

3.1. HPLC method development

Gradient elution was chosen for the development of the method. For optimization, the parameters temperature, composition of the organic modifier of the mobile phase, flow rate, and column length were varied. The injection volume was set to 20 µL. Dapsone shows UV absorbance maxima at 260 nm and 295 nm [2], which is different for its impurities. Nevertheless, all considered substances show sufficient absorption at 254 nm. Therefore, UV detection was performed at this wavelength.

To keep the system as simple as possible, a standard end-capped octadecylsilyl silica gel for chromatography column was used. The column compartment and the

autosampler were operated at ambient temperature. Flow rate was set to 1.0 mL/min. A binary gradient was applied using water and acetonitrile.

Since not all impurities are soluble under the HPLC starting conditions, a mixture of acetonitrile and water (50:50 v/v) was used to prepare the sample solutions.

Dapsone and its specified impurities 3 and 4 (Figure 2) are structurally very similar and all consist of anilino moieties. However, impurities 2 and 2a have an acidic phenol group which is increasingly deprotonated at pH values higher than 7. This causes co-elution of dapsone and impurity 2 or even a change in their elution order. Slight deviations from the pH value 7 of the mobile phase (± 0.2) are acceptable but already led to a small decrease in resolution. Nevertheless, no buffer adjuvants are necessary to obtain a good peak shape.

Of note, impurity 2 CRS contains the detectable impurity 2a which cannot be found in the representative batches of the drug substance dapsone. Upon spiking experiments with impurity 2 CRS, it was separated from all other peaks. This “impurity of the impurity” was identified by high resolution tandem mass spectrometry (section 3.1.3) as 4,4' sulfonyldiphenol (2a), also known as Bisphenol S and likely to be a second side product of the Ullmann conversion of 9 (Figure 2).

3.1.1. Validation

The method was validated with regard to specificity, linearity, range, precision, accuracy, LOD/LOQ, and robustness according to the guideline Q2(R1) of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [28].

3.1.1.1. Specificity:

A solution containing the substance spiked with the impurities was used in order to assign the peaks. All impurities were very well separated from dapsone and from each other (Figure 3).

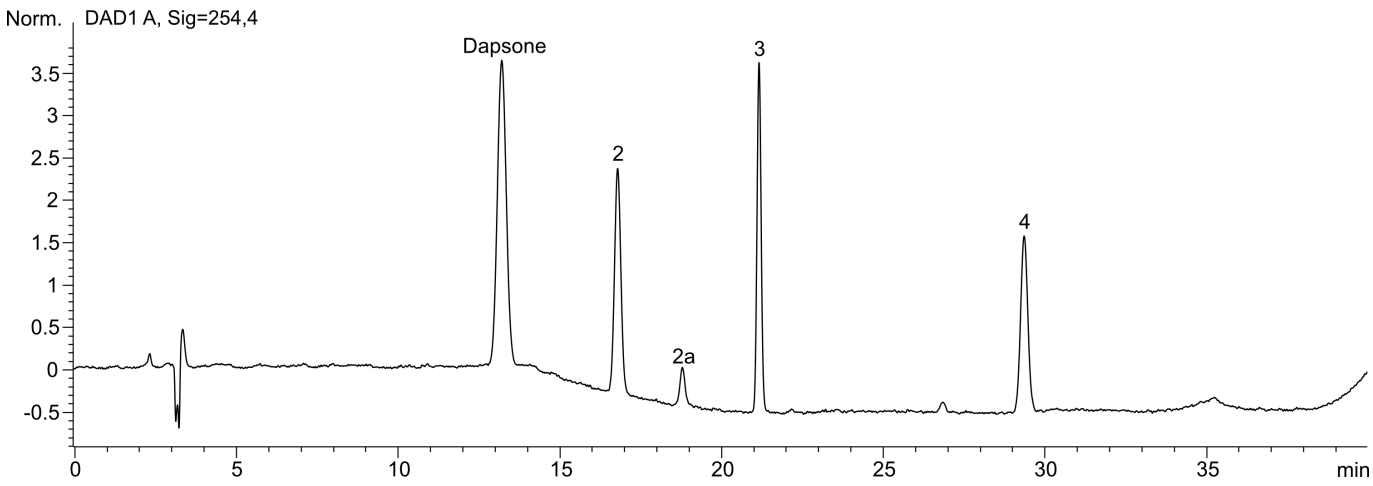


Figure 3: A typical chromatogram obtained with an impurity-spiked dapsone solution. peak labeling: Dapsone, impurity 2, 2a imp. of impurity 2, impurity 3 and impurity 4; column: Waters XTerra®RP18 5 µm 4.6 × 250 mm; The chromatographic system was operated with a flow rate of 1.0 mL/min using gradient elution at ambient temperature and UV-detection at 254 nm. Further chromatographic conditions for this gradient HPLC can be seen in section 2.3.

3.1.1.2. Linearity and range

The linearity and range were determined over a calibration range of 0.05 to 0.40% for all impurities (eight equally distributed concentration levels) relative to a sample concentration of 400 µg/mL. The linearity of dapsone itself was established in a range of 80 - 120% for sake of completeness in order to show that the method is able to determine the content as well.

The data obtained (cf. Figure 4) show a relative standard deviation (RSD) on every level for every impurity below 3.96%. As displayed in Table 2, the coefficient of determination (R2) was higher than 0.9999 for each calibration curve.

Table 2: Parameters of the linear regression of analyte concentration and peak area, response factors obtained from calibration and the relative retention to dapsone for all impurities using gradient method.

Substance	R^2	slope	y-intercept	LOQ*	LOD*	response factor	relative retention
Impurity 2	0.9999	152.67	-0.1102	0.04 %	0.014 %	0.6	1.29
Impurity 3	0.9999	105.17	0.0073	0.04 %	0.014 %	0.4	1.67
Impurity 4	0.9999	129.99	0.0703	0.04 %	0.013 %	0.5	2.26

* in per cent of the sample solutions concentration

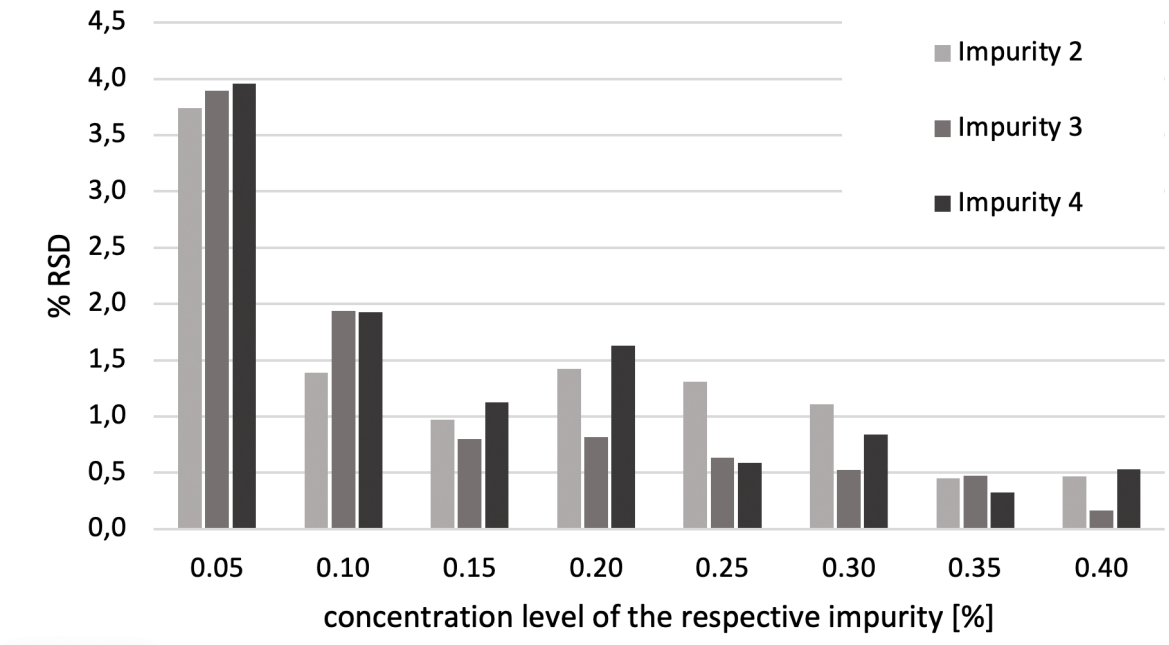


Figure 4: RSD of each calibration level for every impurity obtained from the calibration curves (sextuple injection).

3.1.1.3. Accuracy

Spiked sample solutions were used to assess accuracy. To calculate the correct content of the impurities, it is necessary to determine the response factors. According to the Ph. Eur. 10 (chapter 2.2.46), a response factor has to be considered if it is outside of the range between 0.8 and 1.2. The response factors for all specified dapsone impurities are out of this range and therefore must be used (cf. Table 2). They were determined by preparing separate solutions with known content of dapsone and of the impurities which were subsequently measured. The respective response factor can be obtained with the following equation, while A_i represents the area of the peak due to the impurity, A_s the area of the peak due to dapsone, C_s the concentration of dapsone in milligrams per milliliter and C_i the concentration of the impurity in milligrams per milliliter:

$$response\ factor = \frac{A_i}{A_s} \times \frac{C_s}{C_i}$$

The recovery rate was calculated at the lower end of the calibration curves, at the specification limit and as well on the upper end of the calibration curve and found between 89 and 111% (n = 6; RSD = 0.45–4.79%) on every level.

Repeatability and precision were determined using two real batch samples. All batches contained the three specified impurities. Therefore, no further spiking tests are

necessary to show repeatability and precision. The RSD intra-day was between 0.4 and 1.7% (n = 6) and inter-day determined on two consecutive days between 0.4 and 1.2% (n = 12), indicating a sufficient precision.

To check the stability of the sample solution, storage at room temperature was examined for three days, one week, one month, 90 days, and one year. The sample solution was found to be very stable. Even the solutions stored in absence of light for one year did not show an increase of detectable degradants at a level higher than 0.05%, each.

3.1.1.4. LOD/LOQ

For the treatment of leprosy, a common daily intake of 100 mg of dapsone is necessary [7]. For other indications, the dosage of dapsone varies between 50 and 400 mg per day [29]. Based on this daily intake and with regard to the general chapter of the European Pharmacopoeia “Substances for Pharmaceutical use”, the reporting threshold for impurities is set to 0.05% [2, 30].

The calibration curves were as well used to calculate the LOD and the with regard to ICH guideline Q2(R1) [28] applying the following equations:

$$LOD = \frac{3.3\sigma}{b} \qquad LOQ = \frac{10\sigma}{b}$$

with σ being the standard deviation of the response and b being the slope of the calibration curve, respectively [31]. The data are summarized in Table 2. The LOQs of 0.04% for each specified impurity are sufficient for quality assessment.

3.1.1.5. Robustness

For checking the robustness of the method, chromatographic parameters were varied in the following ranges: temperature of the column compartment ± 5°C, flow rate ± 0.15 mL/min and acetonitrile content ± 1% (v/v). As can be seen from Figure 5, all variations have a small and thus acceptable influence on the separation of all components. However, the acetonitrile content of the mobile phase exhibits the largest influence on robustness. Varying it by 4% (v/v) causes a retention time shift of about 20 min for impurity 4 (data not shown). Thus, it can be concluded that the method is not robust against changes of the acetonitrile portion. Furthermore, its content must be within the stated range of ± 1% (v/v) to achieve acceptable separation.

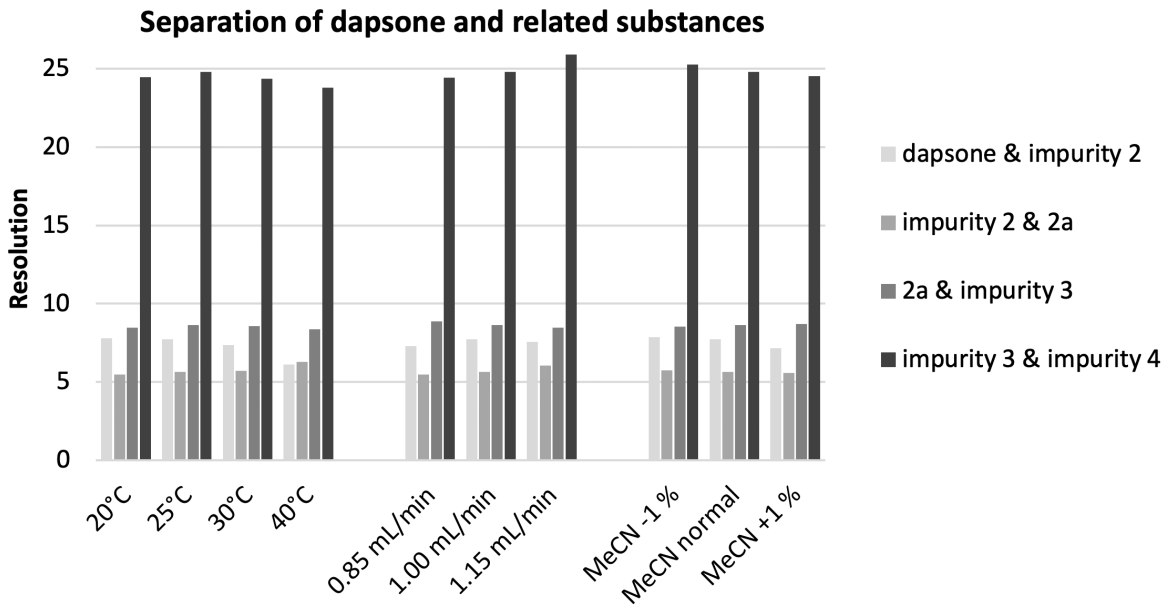


Figure 5: Robustness study for the related substances method of dapsone.

3.1.2. Batch results

Two batches of dapsone have been tested by means of the newly developed method (see Table 3). Both of them contain all impurities at a low level; consequently, all have to be regarded as specified impurities because the content is higher than 0.10% [2, 30].

Table 3: Results of two dapsone batches.

Impurity	Content [%] batch #1	Content [%] batch #2
2	0.17	0.17
3	0.23	0.25
imp. 25.8 min*	0.04	0.04
4	0.26	0.25
Sum of impurities	0.66	0.67

* unknown impurity below reporting threshold

3.1.3. Identification of impurity 2a

The QqTOF experiments showed an exact m/z of 250.056 for the peak of impurity 2, while the peak of impurity 2a delivered an exact m/z of 251.041. The mass difference of about one Dalton and the chromatographic behavior of impurity 2a suggest the suspicion that the amino substituent of impurity 2 is replaced by a hydroxy group.

This assumption could be confirmed by the corresponding fragment spectra (Figure 6). The spectrum of impurity 2a shows fragment ions with the sum formula of $C6H5O3S^+$ (m/z 156.997), only, while the one for impurity 2 shows fragments of the sum formula $C6H6NO2S^+$ (m/z 156.013) and $C6H5O3S^+$ (m/z 156.997), respectively.

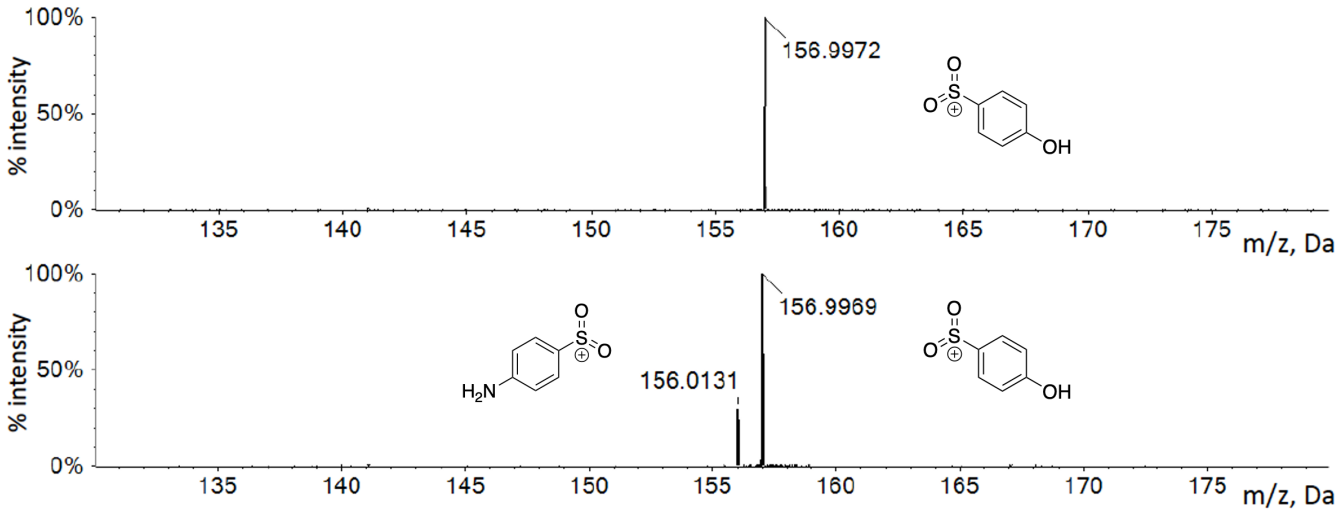


Figure 6: Fragment spectra of impurity 2 (down) and 2a (up) obtained with the QqTOF system; ionization mode: ESI, positive polarity; ionspray voltage: 5500 V; curtain gas: 20 psi; ion source gas 1: 50 psi; ion source gas 2: 50 psi; temp.: 575°C; collision energy: 25 V.

4. Conclusion

The TLC test for related substances of the current monograph of dapsone allows the visual comparison of two concentration levels (0.2 % and 1%), only. The gradient HPLC methods allow an appropriate purity control of dapsone. Compared to the currently described pharmacopoeial TLC test, the LOQ could be reduced to 0.05% which makes the gradient HPLC method appropriate for pharmacopoeial purposes.

CRediT authorship contribution statement

Adrian Leistner: Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing. **Ulrike Holzgrabe:** Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

Thanks are due to the Hanns Seidel Foundation, the German Federal Ministry of Education and Research (BMBF), and the Federal Institute of Drugs and Medical Devices (BfArM) for financial support for the Elaboration of the Dapsone Monograph, as well as Rasmus Walther and Jonas Wohlfart (University of Würzburg) for performing the high-resolution mass spectrometry experiments.

References

[1] European Medicines Agency, From laboratory to patient: the journey of a centrally authorised medicine. EMA/103813/2018 Rev. 1, (2018), https://www.ema.europa.eu/en/documents/other/laboratory-patient-journey-centrally-authorised-medicine_en.pdf, (accessed 08 July 2020).

[2] European Pharmacopoeia 10th edition, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2020.

[3] C. Smith, Disadvantages & Advantages of an HPLC, 2018, https://sciencing.com/disadvantages-advantages-hplc-5911530.html, (accessed 20 February 2020).

[4] S. Sasaki, F. Takeshita, K. Okuda, N. Ishii, Mycobacterium leprae and leprosy: a compendium, Microbiol Immunol., 45 (2001) 729-736, http://doi.org/10.1111/j.1348-0421.2001.tb01308.x.

[5] D. Steinhilber, M. Schubert-Zsilavecz, H.J. Roth, Medizinische Chemie, 2 ed., Deutscher Apotheker Verlag Stuttgart, Stuttgart, 2010.

[6] A.H. Delcour, Outer membrane permeability and antibiotic resistance, Biochim. Biophys. Acta., 1794 (2009) 808-816, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2696358/pdf/nihms114274.pdf, (accessed 25 February 2020).

[7] K. Aktories, U. Förstermann, F.B. Hofmann, K. Starke, Allgemeine und spezielle Pharmakologie und Toxikologie: Begründet von W. Forth, D. Henschler, W. Rummel, Elsevier Health Sciences, 2017.

[8] G. Wozel, J. Barth, Current aspects of modes of action of dapsone, Int. J. Dermatol., 27 (1988) 547-552, https://doi.org/10.1111/j.1365-4362.1988.tb02401.x.

[9] R.S. Vardanyan, V.J. Hruby, 34 - Antimycobacterial Drugs, in: R.S. Vardanyan, V.J. Hruby (Eds.) Synthesis of Essential Drugs, Elsevier, Amsterdam, 2006, pp. 525-534, https://doi.org/10.1016/B978-044452166-8/50034-0.

[10] E. Fromm, J. Wittmann, Derivate des p-Nitrothiophenols, Ber. Dtsch. Chem. Ges., 41 (1908) 2264-2273, https://doi.org/10.1002/cber.190804102131

[11] S. Sugasawa, K. Sakurai, Friedel-Crafts-Reactions with Halogenides Containing Sulfur (I) - A Synthesis of 4, 4'-Diamino-diphenylsulfone, Yakugaku Zasshi, 60 (1940) 22-24, https://doi.org/10.1248/yakushi1881.60.1_22.

[12] L. Chen, H. Wu, F. Mao, J. Wu, Q. Niu, (2014), Process for the preparation of 4,4'-diaminodiphenylsulfone (CN103819372 (A)), Jiangsu Aolunda High-Tech Industry Co., Ltd., https://worldwide.espacenet.com/publicationDetails/biblio?CC=CN&NR=103819372A&KC=A&FT=D&ND=3&date=20140528&DB=&locale=.

[13] D. Ma, Q. Cai, H. Zhang, Mild Method for Ullmann Coupling Reaction of Amines and Aryl Halides, Org. Lett., 5 (2003) 2453-2455, https://doi.org/10.1021/ol0346584.

[14] Y. Zhu, Y. Wei, A simple and efficient copper-catalyzed amination of aryl halides by aqueous ammonia in water, Can. J. Chem., 89 (2011) 645-649, https://doi.org/10.1139/v11-051.

[15] Ullmann Reaction, https://www.organic-chemistry.org/namedreactions/ullmann-reaction.shtm, (accessed 09 April 2020).

[16] Atul Ltd., Sulfones, https://www.atul.co.in/wp-content/uploads/polymer-performance-material-liabrary/Sulfones%20catalogue_Atul%20Ltd.pdf, (accessed 16 October 2020).

[17] World Health Organization, The International Pharmacopoeia, 9th edition [electronic resource], Genva, Switzerland, 2019.

[18] United States Pharmacopeia 43, US Pharmacopeial Convention, Rockville, MD, USA, 2020.

[19] B. Renger, Pharmaceutical analysis in quality control, 2008, https://www.europeanpharmaceuticalreview.com/article/868/pharmaceutical-analysis-in-quality-control/, (accessed 19 February 2020).

[20] Y. Zhou, L. Cheng, J. Luo, Y. Zhang, L. Lai, (2018), Process for preparation of 4,4'-dichlorodiphenyl sulfone (CN108047101 (A)), Jiujiang Zhongxing Medicine & Chemical Co. Ltd., https://worldwide.espacenet.com/searchResults?DB=EPODOC&submitted=true&locale=en_EP&ST=singleline&compact=false&query=CN108047101.

[21] C. Sambiagio, S.P. Marsden, A.J. Blacker, P.C. McGowan, Copper catalysed Ullmann type chemistry: from mechanistic aspects to modern development, Chem. Soc. Rev., 43 (2014) 3525-3550, https://doi.org/10.1039/C3CS60289C.

[22] S. Kwadijk, J.S. Toraño, High-performance liquid chromatographic method with ultraviolet detection for the determination of dapsone and its hydroxylated metabolite in human plasma, Biomed. Chromatogr., 16 (2002) 203-208, https://doi.org/10.1002/bmc.132.

[23] J. Moncrieff, Determination of dapsone in serum and saliva using reversed-phase high-performance liquid chromatography with ultraviolet or electrochemical detection, J. Chromatogr. B Biomed. Sci. Appl., 654 (1994) 103-110, https://doi.org/10.1016/0378-4347(93)E0439-W.

[24] M. Madhu, V. Sreeram, A. Nagendrakumar, T. Reddy, A Newer RP-HPLC Method for the Estimation of Dapsone in Bulk and In Pharmaceutical Formulations, Am. J. PharmTech Res., 4 (2014).

[25] G. Gordon, D. Ghoul, J. Peters, Identification and quantitation of impurities in dapsone preparations, J. Pharm. Sci., 64 (1975) 1205-1207, https://doi.org/10.1002/jps.2600640717.

[26] C. Mannan, G.-J. Krol, B. Kho, High-speed liquid chromatographic analysis of dapsone and related compounds, J. Pharm. Sci., 66 (1977) 1618-1623, https://doi.org/10.1002/jps.2600661129.

[27] L. Beheshti-Maal, L. Navidpour, M. Afshar, An ecofriendly and stability-indicating RP-HPLC method for determination of dapsone: Application to pharmaceutical analysis, Chiang Mai J. Sci, 43 (2016) 620-629.

[28] ICH harmonised triparite Guideline, Validation of analytical procedures: text and methodology Q2 (R1), International conference on harmonization, Geneva, Switzerland, 2005.

[28] J. Zuidema, E.S.M. Hilbers-Modderman, F.W.H.M. Merkus, Clinical Pharmacokinetics of Dapsone, Clin. Pharmacokinet., 11 (1986) 299-315, https://doi.org/10.2165/00003088-198611040-00003.

[30] ICH harmonised triplicate Guideline, Impurities in new drug substances Q3A (R2), Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland, 2006.

[31] M.M. Sanagi, S.L. Ling, Z. Nasir, D. Hermawan, W.A. Wan Ibrahim, A.A. Naim, Comparison of signal-to-noise, blank determination, and linear regression methods for the estimation of detection and quantification limits for volatile organic compounds by gas chromatography, J. AOAC Int., 92 (2009) 1833-1838, https://doi.org/10.1093/jaoac/92.6.1833.

3.2.1. Challenges within the isocratic method development

Probably the simplest type of HPLC analysis is an isocratic one, which is usually quite easy to perform. However, the development of an isocratic HPLC method for dapsone was quite challenging:

The objective of the study was to develop an isocratic HPLC method capable of separating all relevant impurities of dapsone within an acceptable time of 60 min. Furthermore, the aim was to avoid the expensive acetonitrile (substitution by methanol) to make the method suitable for low- and middle-income countries. Based on the pharmacopoeial like gradient method, the same conditions (column, flow rate, temperature and injection volume) were chosen to start the isocratic method development. The influence of the methanol concentration in the mobile phase on the separation was studied from 40% (v/v) to 50% (v/v). The experiments cf. Figure 1 resulted in a co-elution of dapsone and impurity 2 or a too long elution time for impurity 4, respectively.

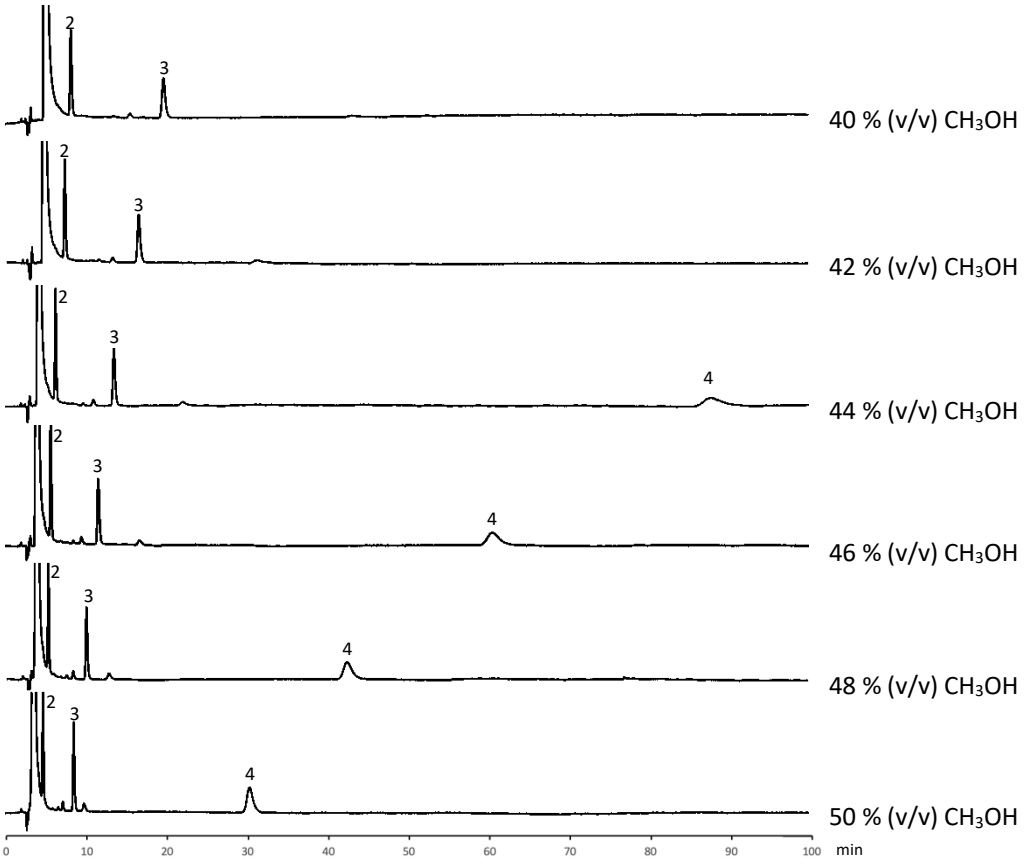


Figure 1: Influence of the methanol concentration in the mobile phase; column temperature 25 °C; flow rate 1.0 mL/min;
column: Knauer Eurospher II 100-5 C18H 250 x 4.6 mm and UV detection;
peak labeling: impurity 2, impurity 3 and impurity 4

Since a shorter column, a higher flow rate and the random variation of the composition of the mobile phase did not result in a satisfactory separation, a design of experiments (DoE) approach using DryLab®4 was applied.

The DryLab®4 DoE modeling software allows to predict chromatographic experiments with only a few entrance experiments by generating a cubic design space. The parameters of gradient steepness (tG), temperature and variations in the composition of the organic mobile phase (tC) were chosen to calculate this ternary model and to build a cube by keeping all other conditions constant. Of note, twelve tests are sufficient to calculate a model containing a million virtual experiments. Due to the coloring the predicted peak separation can be represented (see Figure 2-A*)*. A good separation can be expected by employing a mobile phase consisting of water and organic modifier being 40 % (V/V) of methanol in acetonitrile (cf. Figure 2-B and 2-C).

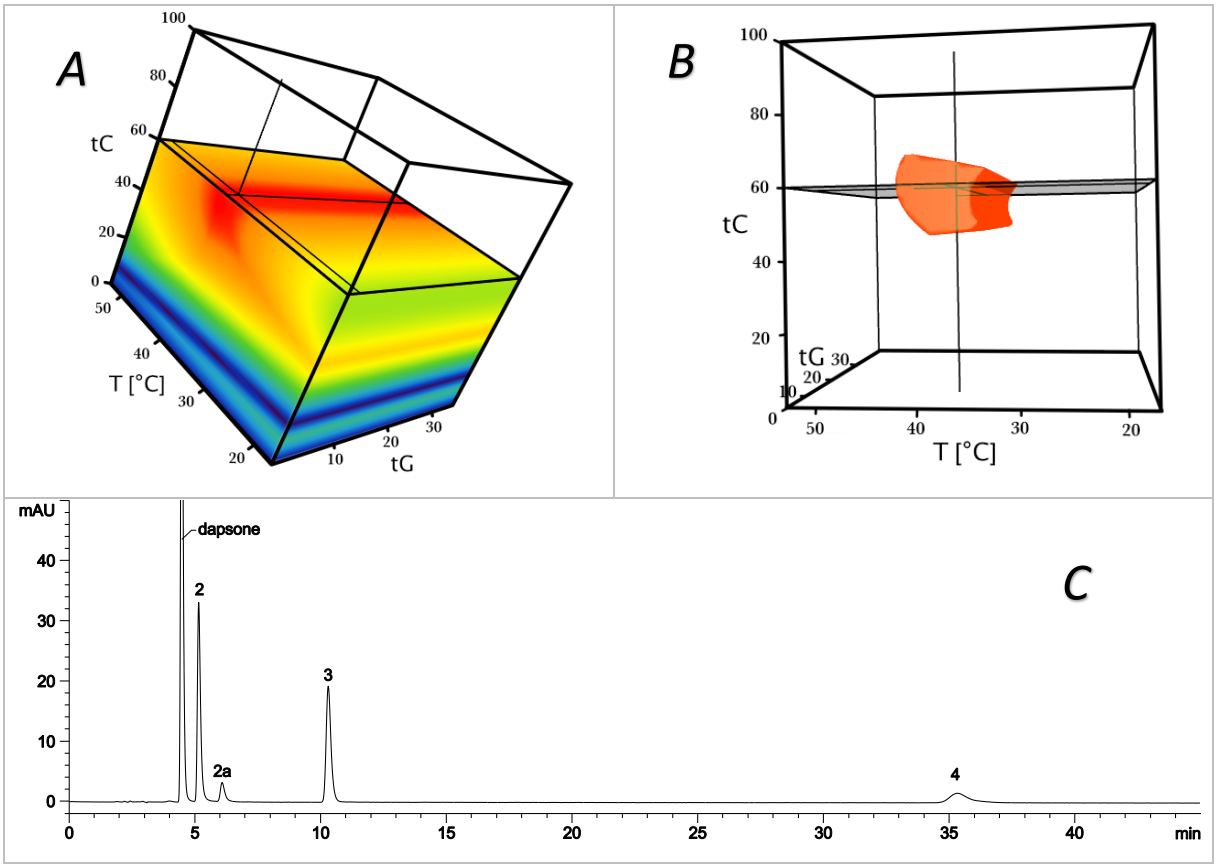


Figure 2: A – calculated representative DryLab®-cube; B – design space with best resolution; C- Chromatogram of a dapsone solution spiked with its impurities at the DoE predicted optimum conditions (blue color means poor separation of the peaks and red color good peak separation)

However, this improvement compared to pure acetonitrile or methanol is not sufficient. Moreover, as stated before, the usage of the expensive acetonitrile is not suitable for

low- and middle-income countries. Moreover, the predicted optimum composition of 45% (v/v) methanol is suitable for spiked batches at a low concentration level only. Using this method with real batches, again results in a co-elution of dapsone and impurity 2.

To overcome the resolution problem, even the usage of normal phase chromatography based on [1] was performed. This approach resulted as expected in an inversion of the elution order. Also, the critical peak pair was separated well (data not shown) and the solvents needed for this normal phase chromatography were only available in an insufficient quality. The limit of detection (LOD) was too high due to a very high noise, which makes this method inappropriate for the intended use.

Since baseline separation of all components of a real batch is isocratically impossible, Dapsone, impurity 2 and impurity 3 were quantified in one run (method A) and impurity 4 in a second run (method B) using a slightly different mobile phase (Figure 3). With this approach of a “split run” method, the total time of analysis for one sample can be reduced to less than one hour, and the LODs/LOQs, especially for impurity 4, are much lower compared to those obtained with a long single isocratic run. A standard *end-capped octadecylsilyl silica gel for chromatography column* was used to perform the isocratic experiments using a mobile phase composed of methanol : water (38:62, V/V) and methanol : water (65:35, V/V), respectively. The experiments were performed at ambient temperature, a flow rate of 1.0 mL/min, and an injection volume of 20 µL.

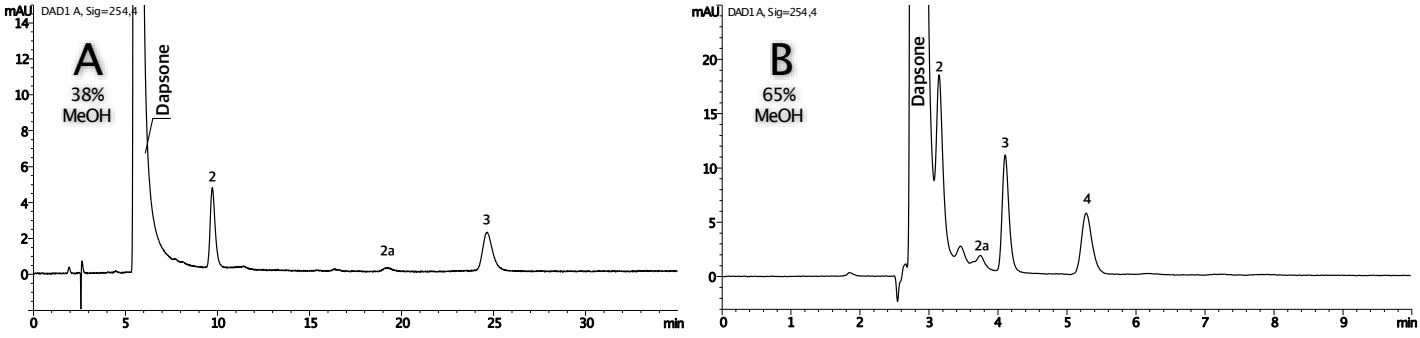


Figure 3: Typical chromatograms obtained with spiked sample solution of dapsone. Dapsone, impurity 2, 2a imp. of impurity 2, impurity 3 and impurity 4

[1] C. Mannan, G.-J. Krol, B. Kho, High-speed liquid chromatographic analysis of dapsone and related compounds, Journal of pharmaceutical sciences, 66 (1977) 1618-1623.

3.3. Impurity Profiling of Baclofen Using Gradient HPLC–UV Method

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Chromatographia 2021, 84.10, 927-935.

https://doi.org/10.1007/s10337-021-04079-y

Abstract

The $GABA_B$ receptor agonist baclofen is a medication commonly used for the treatment of muscle spasticity. It is an amino acid and related to the neurotransmitter GABA. In this study, we developed a new, gradient high-performance liquid chromatography (HPLC) method for the impurity assessment of baclofen, which is appropriate for pharmacopoeial purposes. Since the impurities related to the synthesis pathway are acids, zwitterionic, or neutral, the method development is challenging. However, the separation of all components was achieved on a C18 stationary phase using a water–acetonitrile–trifluoroacetic acid gradient. A limit of detection (LOD) of at least 0.02% was registered for all specified impurities. Additionally, CAD detection was performed to detect potential impurities lacking off a chromophore. The baclofen batches analyzed are far more pure than expected. All impurities were found below the specification limit, and thus, they can be regarded as unspecified. Moreover, the required runtime could be significantly reduced compared to the current USP or Ph. Eur. method.

1. Introduction

Baclofen (Figure 1) is widely used for the treatment of muscle spasticity often occurring with multiples sclerosis, degenerative traumatic spinal cord diseases, and paraplegic syndromes [1]. Additionally, baclofen is today off-label used to treat alcoholism [2]. Due to its structural similarity to the neurotransmitter γ- aminobutyric acid (GABA), baclofen is an agonist at the $GABA_B$ receptor in the spinal cord acting as muscle relaxant [3]. Commonly, 5-120 mg of the drug are administered orally per day [4], mostly in three-

to-four equal dosages [5]. In addition, intrathecal infusions are on the market that are directly delivered to the cerebrospinal fluid space.

Since most of the impurities of an API are production-related, the synthesis pathways [3, 6-8] have to be studied carefully, to identify potential impurities. The pathways for formation of potential impurities, named by some manufacturers, are displayed in Figure 1.

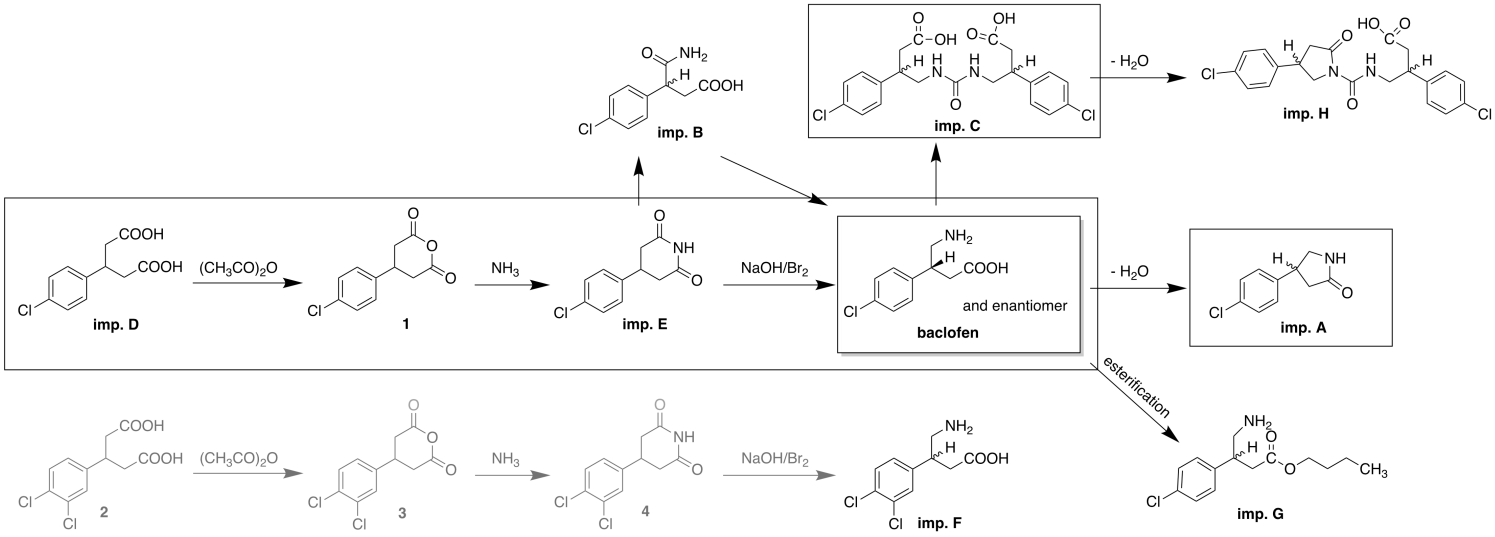

Figure 1: Exemplary synthetic pathway for the formation of baclofen and its impurities

The synthesis pathway (see the large box in Figure 1) described by Keberle's group starts off with 3-(4-chlorophenyl)pentanedioic acid, being impurity D, followed by the formation of the anhydride (1), which is converted to the imide using ammonia. The latter imide, being impurity E, is hydrolyzed to either baclofen or to the amide-acid side product impurity B. Impurity F occurs in case a wrong starting product was used, being the 3-(3,4-dichlorophenyl)pentanedioic acid (2), which was also converted to the respective glutarimide (4). Impurity A is an intramolecular cyclisation product and preferentially formed under dry and warm conditions [9]. Analogously, impurity H is formed out of impurity C, which is a reaction product of two molecules of baclofen with carbonic acid. Of note, baclofen and the impurities A, B, F and G are each racemic compounds. In contrast, the impurities C and H have two stereocenters, resulting in three or four stereoisomers, respectively. Therefore, impurity C represents the diastereomeric mixture of a pair of enantiomers and of the meso compound, while impurity H is the diastereomeric mixture of two pairs of enantiomers.

The related substance test of the current monograph of baclofen, described in The Japanese Pharmacopoeia (JP XVII), makes use of isocratic reversed phase

chromatography with UV detection at 268 nm applying a simple C18 column (10 µm; 250 x 4.0 mm) and a mixture of methanol and diluted acetic acid as mobile phase [10]. However, the monograph does not specify any impurities, but limits a single impurity at a level of 1.0% and the total sum of impurities at a level of 1.5%, respectively. These limits are determined by evaluating the peak heights of peaks other than baclofen in the test solution in comparison to accordingly diluted baclofen solutions. The European Pharmacopoeia (Ph. Eur.) 10.0th ed. specifies and limits impurity A at a level of 1.0% and a total of 2% using an isocratic ion pair reversed phase method [11]. Using the suitable LiChrospher® 100 RP-18e (5µm) 250 x 4.0 mm column, baclofen is eluted after 4 min (cf. Figure S-1). According to the pharmacopoeial related substances test, a total run time of 20 min being the 5-fold of the retention time of baclofen is sufficient. In contrast, the United States Pharmacopeia (USP) 43 applies a gradient method by means of sodium pentanesulfonate as ion-pairing reagent and a mixture of acetonitrile and methanol using a column whose particle size was decreased to be 5 µm. Again, impurity A is limited to 1.0% and other impurities may be present at levels below 0.10%, while in sum a level of 2.0% impurity may not be exceeded [12].
Beside the different methods of the Pharmacopoeias, several methods for the content determination of baclofen are reported in the literature [13-15]. However, methods for impurity profiling of baclofen are quite rare. For the quantitation of baclofen and its impurities in injection formulations, an UPLC method was reported by Ga et al. being able to separate and quantify baclofen, imp. A and imp. B [16]. Elagawany et al. described a TLC method for the determination of at least two baclofen impurities [17], which can be considered as outdated, because of the typically low sensitivity of TLC.

Since the manufacturers announced additional impurities, the related substances test needs a revision. This newly developed method is a candidate to replace the current related substances test. Due to the daily dose of 30 to 75 mg, unspecified impurities should not exceed 0.1% [5, 18]. For the development of a new pharmacopoeial method, the impurities A and C, displayed in the small boxes of Figure 1, should be regarded as specified because they occur potentially at levels higher than 0.1%. Moreover, it can be assumed that the other impurities, being also related substances, will be detected as unspecified impurities by means of this newly developed method. Since only impurity A and C are available, a CAD detection was performed in order to find additional impurities without a chromophore.

Experimental

Chemicals and reagents

Baclofen reference standards and the impurities A and C were obtained from the European Directorate for the Quality of Medicines & HealthCare (EDQM), Strasbourg, France. Trifluoroacetic acid (TFA) (>99% purity), acetonitrile and methanol, HPLC grade (>99.9% purity), sodium pentanesulfonate (>99.0% purity), sodium hexanesulfonate (>98% purity), phosphoric acid and acetic acid, analytical grade (>99% purity) were purchased from Sigma-Aldrich Chemie GmbH, (Darmstadt, Germany). Potassium dihydrogenphosphate for HPLC was purchased from VWR international GmbH (Darmstadt, Germany). Ultra-pure water was freshly produced by a water purification system from Merck Millipore (Darmstadt, Germany). All solutions were filtered through a 0.2 µm PTFE filter (VWR international GmbH, Darmstadt, Germany).

Apparatus

HPLC measurements were performed on an Agilent 1100 modular chromatographic system consisting of an online vacuum degasser, a binary pump G1312A, an auto sampler G1313A, a thermostated column compartment G1316A and a diode-array detector G1315B (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Agilent ChemStation® Rev. B03.02 software was used for data processing. The dwell volume of the Agilent system used for method development was 1.307 mL. UV detection was performed at 225 nm, except where otherwise stated.

For the experiments using CAD detection, the ESA Corona® charged aerosol detector (ThermoFisher, Courtaboeuf, France) was linked with the HPLC system by a 0.25 mm internal diameter PEEK capillary and a 0.22 µm stainless-steel inlet-frit. An ESA Nitrogen Generator (ThermoFisher, Courtaboeuf, France) was used to produce highly pure nitrogen (99.9%). The gas inlet pressure was set to 35.0 psi. Filter was set to “none” and the electric current range to 100 pA.

To check the volatility of the compounds, experiments with different evaporation temperature settings (30°C, 35°C, 40°C and 45°C) and power function value setting 1.0 were performed on a Thermo Scientific Vanquish™ Flex modular chromatographic system (Thermo Fisher Scientific, Germering, Germany) consisting of a binary pump with online degasser, a thermostatted split sampler (8°C), a thermostatted column compartment with passive pre-heater (both maintained at 35°C), and a variable

wavelength detector in-line with a Vanquish™ Horizon CAD. The CAD was supplied with nitrogen gas from an ESA nitrogen generator (Thermo Fisher Scientific) connected to the in-house compressed air system. The HPLC instrument was controlled, and runs were processed using the Chromeleon® Data System Version 7.2.6 software program (Thermo Fisher Scientific).

Chromatographic procedure

For the development of the new method, an end-capped octadecylsilyl silica gel for chromatography column Luna® 3µm C18(2) (100Å 150x4.6 mm, Phenomenex Ltd., Aschaffenburg, Germany) was used as stationary phase. The system was operated using gradient elution with a column oven temperature of 35 °C and UV detection at 225nm. For the examination, mixtures containing acetonitrile, water, and TFA (20:79.95:0.05, v/v/v), mobile phase A - and (80:19.95:0.05, v/v/v), mobile phase B - were used: 0-2 min: 5% B; 2-3 min: 5-25% B; 3-4 min: 25-35% B; 4-13 min: 35% B; 13-14 min: 35-5% B; 14-16 min: 5% B, respectively. The flow rate was set to 1.0 mL/min and the injection volume was 20 µL.

Preparation of solutions

Sample solution

45 mg of baclofen were accurately weighed and dissolved in 100 mL of mobile phase A.

Stock solutions

Stock solutions (45 µg/mL) of the respective impurities in mobile phase A, were prepared for spiking the sample solution. Of note, impurity C was not available as the free acid, but as the dicyclohexylamine salt of impurity C, only. Thus, a corrected mass (factor 1.8) was weighted and dissolved in order to obtain a stock solution of the required concentration.

System suitability solution

A system suitability solution of equal concentration level (4.5 µg/mL) was prepared by spiking 100 µL of the sample solution with 1000 µL of each impurity stock solution and diluting this mix with mobile phase A to 10.0 mL.

All solutions were stored at 5 °C.

Results and discussion

HPLC method development

First, the current USP and Ph. Eur. method were applied to check, whether they are able to separate and detect the late eluting impurity C, using the system suitability solution and the two batches available. The respective chromatograms of the system suitability solution are displayed in Figure S-1, and the batch results are listed in Table 1. The Ph. Eur. method is unable to detect imp. C within runtime of 20 min and at a sufficient LOQ, because the retention time was eightfold of the retention time of baclofen instead of fivefold as reported in the Ph. Eur. The LOQ of 0.69% is not sufficient to detect impurity C at a low level and thus this method needs further improvement. Using the USP method, impurity C can be evaluated, but again at a very long retention time at the end of the method, running into the risk of missing the peak if columns with a different carbon load are used. Additionally, the overall runtime of about 30 min for each of both pharmacopoeial methods is rather long.

Table 1: Results of two baclofen batches

Impurity	Content [%] batch #1			Content [%] batch #2		
	New method	Ph. Eur.	USP	New method	Ph. Eur.	USP
Imp. at 7.3 min	0.05	n.a.	n.a.	---	n.a.	n.a.
Imp. A	<0.05	0.08	0.09	<0.05	<0.05	<0.05
Imp. C	<0.05	n.a.	<0.05	0.06	<0.05	0.09
Sum of impurities	0.05	0.08	0.09	0.06	0	0.09

Baclofen is an amino acid of zwitterionic character and would, thus, elute very early on a reversed phase column without using ion pairing reagents, while impurity A is neutral and impurity C is charged depending on the pH. In particular, these different physico-chemical properties of the analytes are responsible for the need of gradient elution and ion-pairing reagents when a reversed-phase-column is planned to use. Therefore, a standard end-capped octadecylsilyl silica gel for chromatography column was used to keep the system as simple as possible.

Because usage of longer chain ion pairing reagents with low detection wavelength often causes unacceptable background noise, TFA was applied instead, which is additionally not critical at a detection wavelength of >220 nm [19, 20]. In addition, at low concentration levels, pH values of about 2, can easily be achieved. The column

compartment and the autosampler was operated at 35 °C, while the flow rate was set to 1.0 mL/min.

The UV absorption maximum of baclofen (220 nm) and its specified impurities A and C are quite similar. Since the baseline noise is borderline at this wavelength, the experiments of the optimized method were performed at 225 nm, as in the USP method.

Upon method development, the injection volume was optimized. Since the injection of 10 µL of the sample solution did not achieve a sufficient LOQ, the mass on column had to be increased. Since the injection of a higher concentrated solution was not possible for solubility reason, 20 µL were injected, which resulted in a satisfactory LOQ of at least 0.05%.

Analogously, the chromatographic conditions, such as the temperature of the column compartment, the flow rate and the TFA content were optimized. Its influence on the separation can be seen in Figure 2. Beside the TFA concentration, the variations have a small influence on the separation only. The latter shows the best separation used at a concentration level of 0.05%.

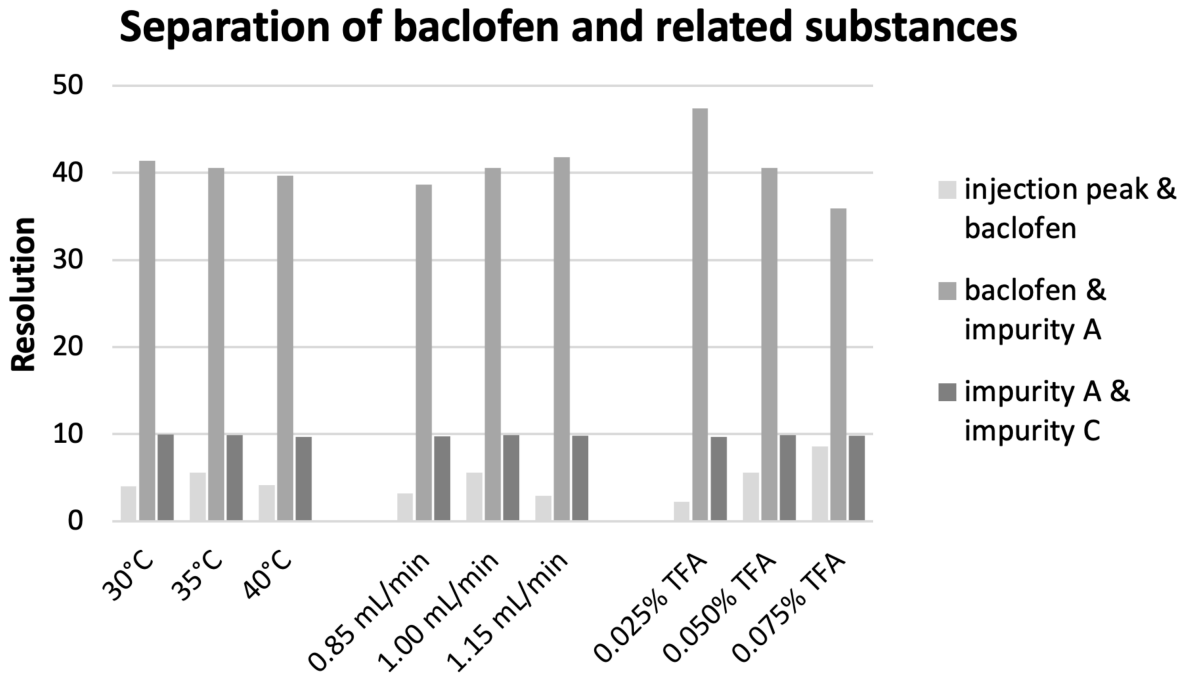

Figure 2: Robustness study for the related substances method of baclofen

As already stated, baclofen and its related substances are of different physio-chemical properties, and thus, gradient elution is necessary to guarantee elution within acceptable time. The starting conditions were selected for dissolution of baclofen and

its impurities and additionally to avoid co-elution of the injection peak and baclofen. A gradient was chosen, to achieve sufficient resolution between potential impurities eluting between baclofen and imp. A and a total run time of less than 16 min.

CAD detection is especially utilized for substances lacking off a chromophore or those with a weak one, only. Since the CAD response is independent of physiochemical properties, it is suitable to look for known and unknown impurities. As at low levels, impurity C is not easy to quantify by means of UV detection correctly (shallow peak height and baseline noise), CAD detection experiments were performed. This was possible because the final HPLC method is suitable for CAD due to the volatility of the mobile phase. In Figure 3 the hyphenated detection of UV and CAD is shown for the system suitability solution. Interestingly, the solution of batch #1 did not show any other impurity (see Figure 4), which are expected to elute after baclofen due to their structure, while the solution of batch #2 contained an unspecified impurity eluting close to impurity A. Chromatograms of both batches and the system suitability solution are displayed in the Supplementary Information (Figure S 2).

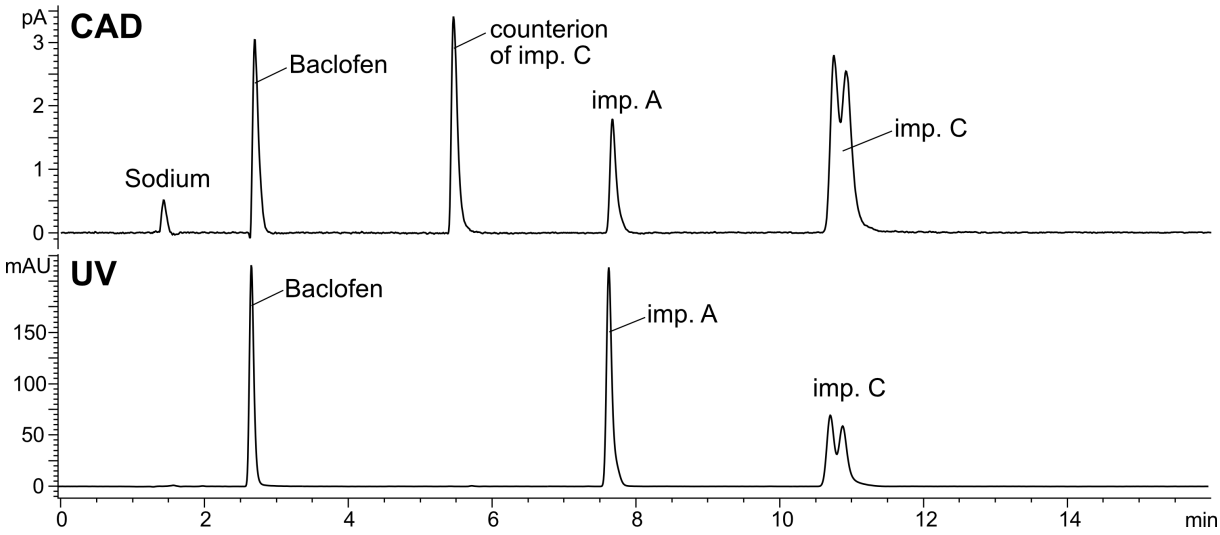

Figure 3: A typical chromatogram obtained with the system suitability solution. peak labeling: baclofen, impurity A and impurity C; column: Phenomenex Luna® 3µm C18(2) 100Å 150x4.6 mm; The chromatographic system was operated with a flow rate of 1.0 mL/min using gradient elution at 35°C, UV (225 nm) and CAD detection. Further chromatographic conditions for this gradient HPLC can be seen in section 2.3

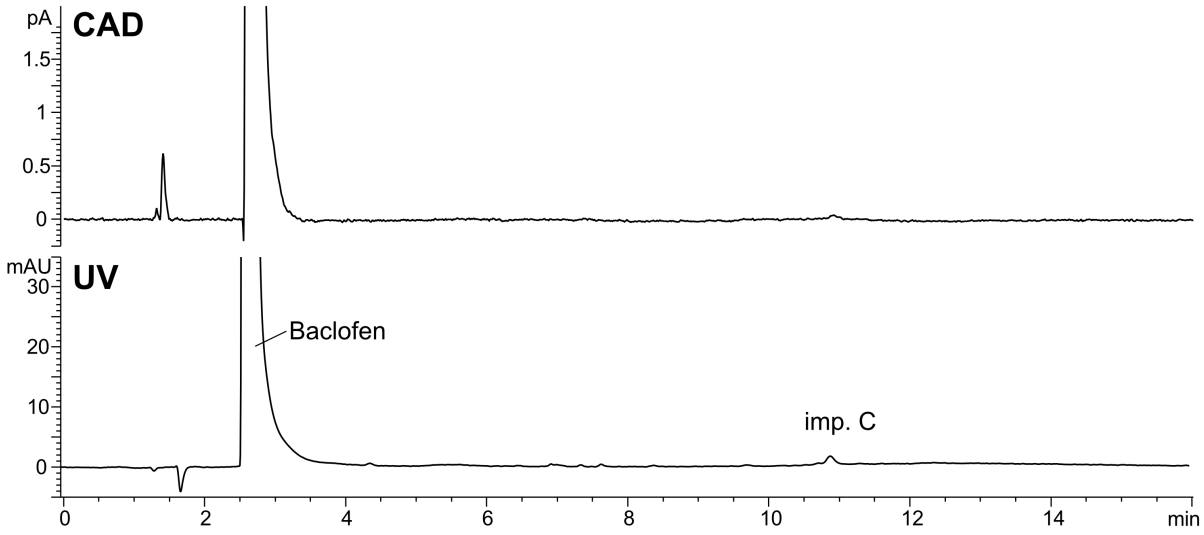

Figure 4: Chromatogram obtained with a sample solution of batch #1 by means of UV and CAD detection. Chromatographic conditions for this gradient HPLC can be seen in section 2.3

Of note, the comparison of UV and CAD detection revealed a substantial difference in peak height, which is due to the semi-volatility of impurity A within CAD detection (cf. Figure 5 and Figure S-3). Moreover, the counterion of impurity C being dicyclohexylamine is detected, but no additional impurity. Since a UV detection is the mostly used in quality assessment, the validation is performed using UV detection.

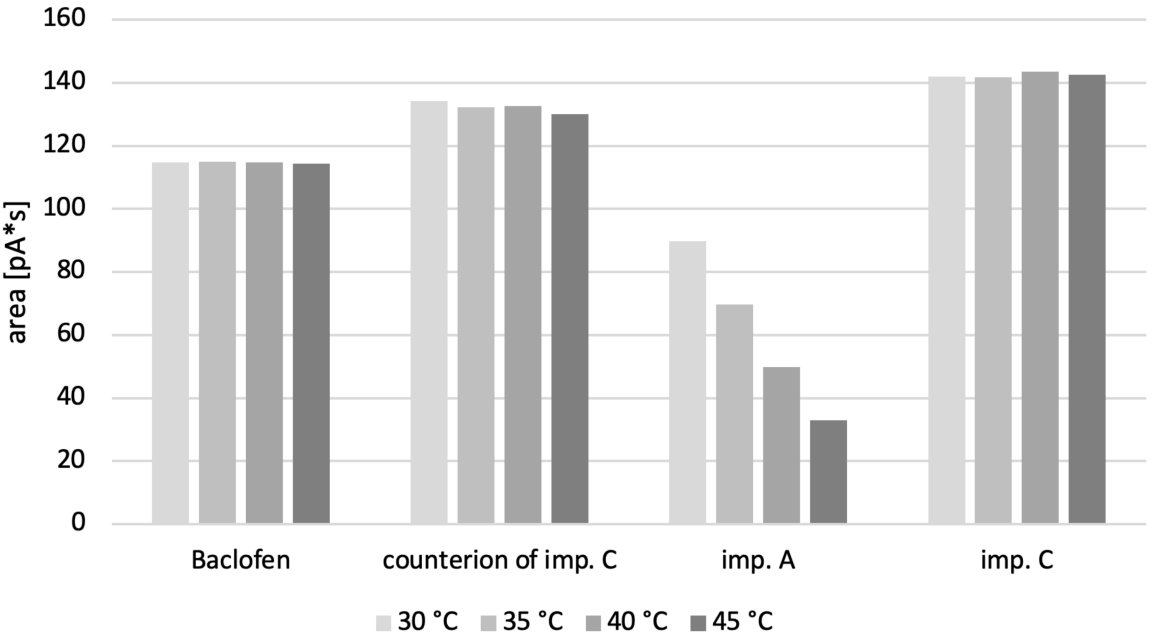

Figure 5: Influence of the evaporation temperature on the peak area of the respective CAD signals

Validation

The method was validated with regard to specificity, linearity, range, precision, accuracy, LOD/LOQ, and robustness according to the guideline Q2(R1) of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [21].

Specificity: The system suitability solution containing equal amounts (4.5 µg/mL) of baclofen and its impurities A and C was used to assign the peaks. All impurities were baseline separated from baclofen and from each other (Figure 3). Additionally, it was even possible to achieve a slight separation of the diastereomeric mixture of impurity C.

Linearity and range: To determine linearity and range, a calibration range of 0.05 to 0.40% for both impurities (eight concentration levels equally distributed) was established (see Figure S 3).

As displayed in Table 2, the coefficient of determination (R^2) was higher than 0.9995 for each calibration curve, and a relative standard deviation (RSD) on every level for both impurities below 4.76% was obtained (Supplementary Information Figure S-4).

Table 2: Parameters of the linear regression of analyte concentration and peak area, response factors obtained from calibration and the relative retention to baclofen for all impurities

Substance	R^2	slope	y-intercept	LOQ*	LOD*	response factor	relative retention
Impurity A	0.9995	243.00	-0.7981	0.04 %	0.014 %	1.2	2.83
Impurity C	0.9995	206.58	-1.7173	0.05 %	0.016 %	1.0	3.99

* in per cent of the sample solutions concentration

Accuracy: For the assessment of accuracy, spiked sample solutions were used. According to the Ph. Eur. 10 (chapter 2.2.46.) [11], a response factor has to be applied if it is out of the range 0.8 - 1.2, for the correct calculation of the content of the impurities. The respective response factor can be obtained with the following equation, while A_i represents the area of the peak due to the impurity, A_s the area of the peak due to baclofen, C_s the concentration of baclofen in milligrams per milliliter and C_i the concentration of the impurity in milligrams per milliliter:

$$response\ factor = \frac{A_i}{A_s} \times \frac{C_s}{C_i}$$

All response factors (Table 2) are within this range and no further correction is necessary. Recovery rates (cf. Figure S-5) were calculated at the lower end of the calibration curves, at the specification limit for imp. C (0.15%) as well as on the upper end of the calibration curve and found between 96 and 111% (n = 6; RSD = 0.44–4.46%) on every level.

All batches contained at least one of the specified impurities at a very low level. For the assessment of *repeatability and precision*, two real batch samples, spiked with 0.25% of each impurity were used. The RSD intra-day was between 0.76 and 0.85% (n = 6) and inter-day determined on two consecutive days between 0.75 and 1.16% (n = 12), indicating a sufficient precision.

The formation rate of the lactam impurity A out of baclofen in aqueous solution is pH-dependent [9]. For solutions of low pH values as applied in the mobile phase, this process is supposed to be very slow. Nevertheless, sample solutions were stored at room temperature for one month to check its stability. Beside the approximate doubling of the content of imp. A., an increase of an additional unknown decomposition product at a very low level was observed. Therefore, all solutions were stored at 5 °C to ensure sufficient stability. Forced degradation studies reported in the literature, confirm this observation. Ahuja reported a slow formation of impurity A, while dos Santos showed high stability of baclofen under forced degradation conditions [9, 22].

LOD/LOQ: Since baclofen is used in a daily dosage range of 5-120 mg [4], a reporting threshold for impurities is defined to be 0.05% according to chapter “Substances for Pharmaceutical use” of the European Pharmacopoeia [11, 18]. LODs and LOQs are calculated by means of the calibrations curves and with regard to ICH guideline Q2(R1) [21] using the following equations.

$$LOD = \frac{3.3\sigma}{b} \qquad LOQ = \frac{10\sigma}{b}$$

with σ being the standard deviation of the response and b being the slope of the calibration curve, respectively [23]. As can be seen from Table 2, the LOQs meet the requirement of 0.05 % for quality assessment.

Robustness: To check the robustness of the method, relevant measuring parameters were varied in the following ranges: temperature of the column compartment ± 5 °C, flow rate ± 0.15 mL/min and TFA content ± 0.025% (v/v). As can be deduced from Figure 2, most variations have a small influence on the separation of all components. Only the TFA content is critical.

System suitability: To ensure system suitability, the pharmacopoeias make use of critical separation pair. However, a real critical peak pair does not exist for this method. Since it was one goal, to have enough time space for potential impurities eluting between the main peak and imp. A, the most the most critical separation within this application is due to the injection peak and baclofen.

Batch results

This new method was used to test two batches of baclofen (see Table 1). Both batches are very pure. Batch #2 contains impurity A and an unspecified impurity at a very low level, while impurity C was below detection limit. As can be seen from Figure S-2, the unspecified impurity is detected by UV and CAD, while imp. A is present at a UV-detectable level, only. The other batch contained a low amount of impurity C, while impurity A is not detected at a level to be reported. Of note, this batch solution contains one of the diastereomers of impurity C, only. According to the rules of the Ph. Eur., only impurity C has to be regarded as specified.

A mass balance approach carried out confirms, that 99.7% and 99.8% of the sample mass can be attributed to baclofen and its specified impurities, respectively.

Conclusion

Currently, the major pharmacopoeia are not harmonized with regard to the related substances test of baclofen. Moreover, some API manufacturers gave additional impurities without providing material. For one batch neither the CAD nor the UV detection of the new method developed showed any other impurity beside A and C, which can be detected by means of the new method at a LOD of at least 0.02%. The other batch contained an unknown impurity at a level of 0.05%. Taken together, baclofen is far more pure than described in all international pharmacopoeias; all impurities can be regarded as unspecified, because all of them were found to be below the disregard limit. To document the superiority of this newly developed method, further investigations could be made. The expected specificity, for the currently not available potential impurities shown in the reaction scheme, could be demonstrated and the purity of further batches (e.g., of the same and of other manufacturers) could be analyzed to confirm baclofen as a high purity product.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s10337-021-04079-y.

Acknowledgements

Thanks are due to the Hanns Seidel Foundation and the German Federal Ministry of Education and Research (BMBF) for financial support.

Author contributions

Conceptualization: AL and UH; methodology: AL and UH; formal analysis and investigation: AL and UH; writing-original draft preparation: AL; writing-review and editing: UH; supervision: UH.

Funding

Open Access funding enabled and organized by Projekt DEAL. Adrian Leistner received funding from the Hanns Seidel Foundation and the German Federal Ministry of Education and Research (BMBF).

Data Availability

Not applicable.

Code Availability

Not applicable.

Declarations

Conflict of Interest

Not applicable.

References

[1] D. Steinhilber, M. Schubert-Zsilavecz, H.J. Roth, Medizinische Chemie, 2 ed., Deutscher Apotheker Verlag Stuttgart, Stuttgart, 2010.

[2] R. de Beaurepaire et al., The Use of Baclofen as a Treatment for Alcohol Use Disorder: A Clinical Practice Perspective, Front Psychiatry, 9 (2019), https://doi.org/10.3389/fpsyt.2018.00708R.

[3] C.N. Kent, C. Park, C.W. Lindsley, Classics in chemical neuroscience: Baclofen, ACS Chem Neurosci, 11 (2020) 1740-1755, https://doi.org/10.1021/acschemneuro.0c00254.

[4] G. Geisslinger, S. Menzel, T. Gudermann, B. Hinz, P. Ruth, E. Mutschler, Mutschler Arzneimittelwirkungen : Pharmakologie – Klinische Pharmakologie – Toxikologie, Hirzel Verlag, 2019.

[5] Novartis, Lioresal® 5 - Gebrauchsinformation, 2019, https://www.novartis.de/system/files/product-info/051981_GI_Lioresal_3.pdf, (accessed 05 February 2021).

[6] R.S. Vardanyan, V.J. Hruby, 15. Muscle Relaxants, in: R.S. Vardanyan, V.J. Hruby (Eds.) Synthesis of Essential Drugs, Elsevier, Amsterdam, 2006, pp. 209-218, https://doi.org/10.1016/B978-044452166-8/50015-7.

[7] H. Keberle, J.W. Faigle, M. Wilhelm, Gamma-amino-beta-(para-halophenyl)-butyric acids and their esters, U.S. Patent 3,471,548, 1964.

[8] A. Jagtap, M. Gharpure, N. Shinde, N. Patil, C. Raut, D. Krishnamurthy, A process for the preparation of baclofen, Piramal Enterprises Limited, Patent WO2017009753, https://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=2017009753A1&KC=A1&FT=D, 2017, (accessed 10 January 2021).

[9] S. Ahuja, K. Florey (Ed.), Analytical Profiles of Drug Substances, vol. 14, Academic Press, London (1985) 527-548, https://doi.org/10.1016/S0099-5428(08)60590-0.

[10] Ministry of Health, Labour and Welfare, The Japanese Pharmacopoeia, 17th ed. (JP XVII), Tokyo, 2016, https://www.mhlw.go.jp/file/06-Seisakujouhou-11120000-Iyakushokuhinkyoku/JP17_REV_1.pdf, (accessed 20 November 2020).

[11] European Pharmacopoeia 10th edition, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2020.

[12] United States Pharmacopeia 43, US Pharmacopeial Convention, Rockville, MD, USA, 2020.

[13] O.A. Dukova, E.A. Krasnov, A.A. Efremov, Development of an HPLC Method for Determining Baclofen, Pharm Chem J, 48 (2015) 687-689, https://doi.org/10.1007/s11094-015-1172-5.

[14] A.M. Rustum, Simple and rapid reversed-phase high-performance liquid chromatographic determination of baclofen in human plasma with ultraviolet detection: Application to a pharmacokinetic study, J Chromatogr B Biomed Appl, 487 (1989) 107-115, https://doi.org/10.1016/S0378-4347(00)83012-1.

[15] L. Labat, A. Goncalves, A.R. Marques, B. Duretz, B. Granger, X. Declèves, Liquid chromatography high resolution mass spectrometry for the determination of baclofen and its metabolites in plasma: Application to therapeutic drug monitoring, Biomed Chromatogr, 31 (2017) e3936, https://doi.org/10.1002/bmc.3936.

[16] M. Elagawany, N.F. Farid, B. Elgendy, E.H. Abdelmomen, N.S. Abdelwahab, Baclofen impurities: Facile synthesis and novel environmentally benign chromatographic method for their simultaneous determination in baclofen, Biomed Chromatogr, 33 (2019) e4579, https://doi.org/10.1002/bmc.3936.

[17] Venkata Narasimha Rao Ga, Ultra Performance Liquid Chromatographic Seperation And Quantification Of Baclofen And And Its Potential Impurities In A Injection Formulation, IOSR-JPBS, 13.4 (2018): 29-38., https://doi.org/10.9790/3008-1304022938.

[18] ICH harmonised triparite Guideline, Impurities in new drug substances Q3A (R2), Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland, 2006.

[19] R. Gloor, E.L. Johnson, Practical Aspects off Reverse Phase Ion Pair Chromatography, J Chromatogr Sci, 15 (1977) 413-423, https://doi.org/10.1093/chromsci/15.9.413.

[20] B. Cai, J. Li, Evaluation of trifluoroacetic acid as an ion-pair reagent in the separation of small ionizable molecules by reversed-phase liquid chromatography, Anal Chim Acta, 399 (1999) 249-258, https://doi.org/10.1016/S0003-2670(99)00461-4.

[21] ICH harmonised triparite Guideline, Validation of analytical procedures: text and methodology Q2 (R1), International conference on harmonization, Geneva, Switzerland, 2005.

[22] J. dos Santos, P. Rosa, A.I.H. Adams, Validation of a simple reversed phase-HPLC method for the determination of baclofen in tablets, Drug Anal Res, 2 (2018) 37-43, https://doi.org/10.22456/2527-2616.87929.

[23] M.M. Sanagi, S.L. Ling, Z. Nasir, D. Hermawan, W.A. Wan Ibrahim, A.A. Naim, Comparison of signal-to-noise, blank determination, and linear regression methods for the estimation of detection and quantification limits for volatile organic compounds by gas chromatography, J AOAC Int., 92 (2009) 1833-1838, https://doi.org/10.1093/jaoac/92.6.1833.

Supplementary Information

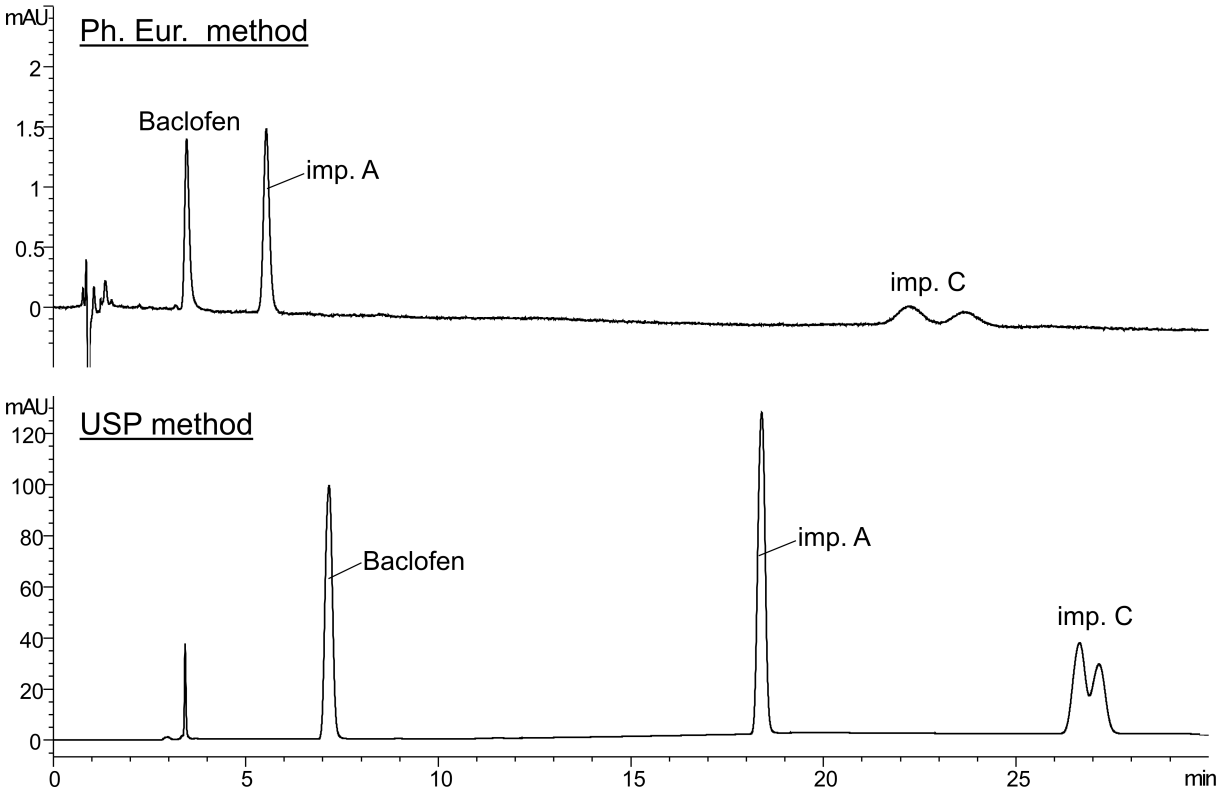

Figure S-1: Chromatograms of the system suitability solution obtained by means of USP and Ph. Eur. related substances test.

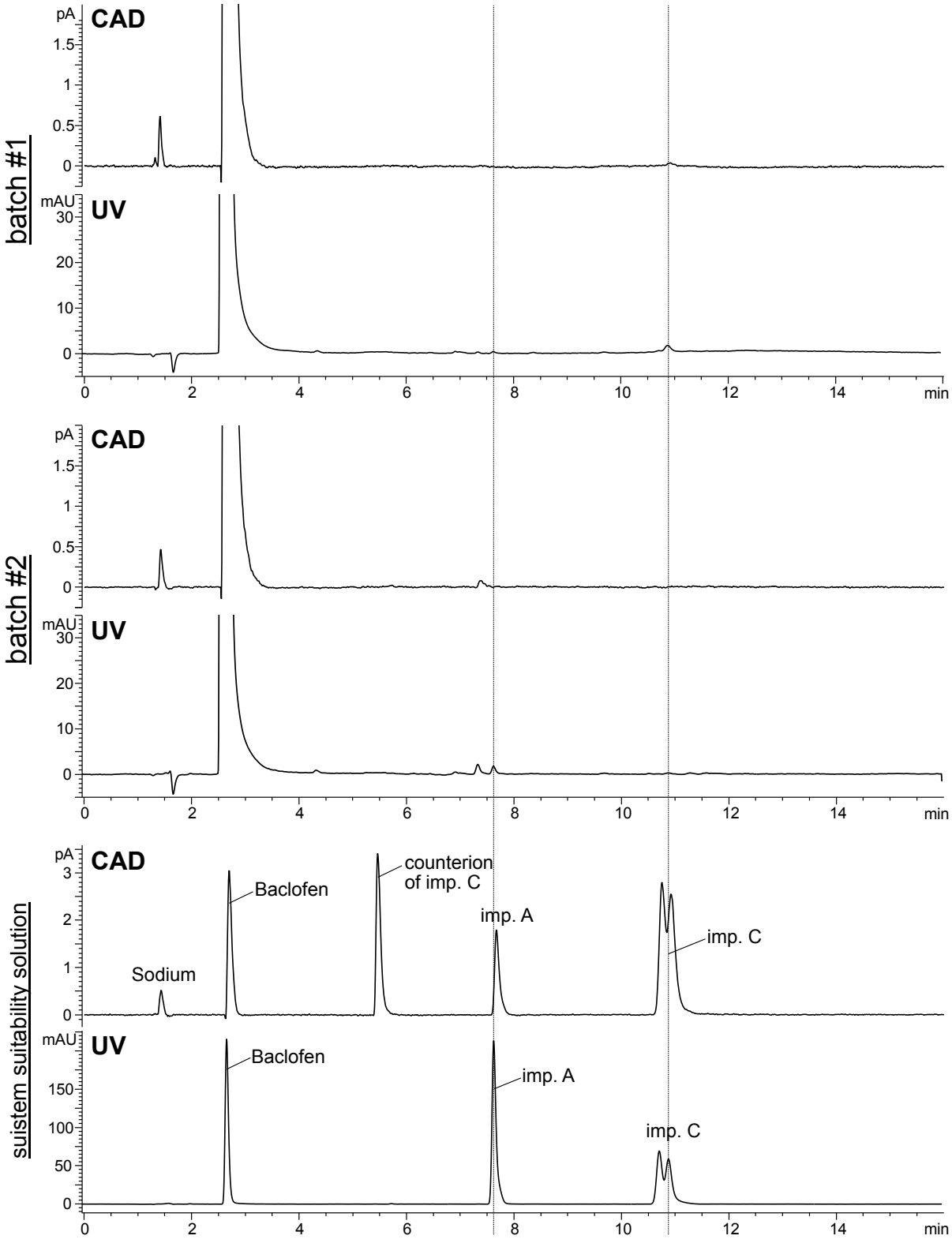

Figure S-2: Chromatograms of batch #1, batch #2 and the system suitability solution obtained by means of hyphenated UV and CAD detection technique. Chromatographic conditions for this gradient HPLC can be seen in section 2.3

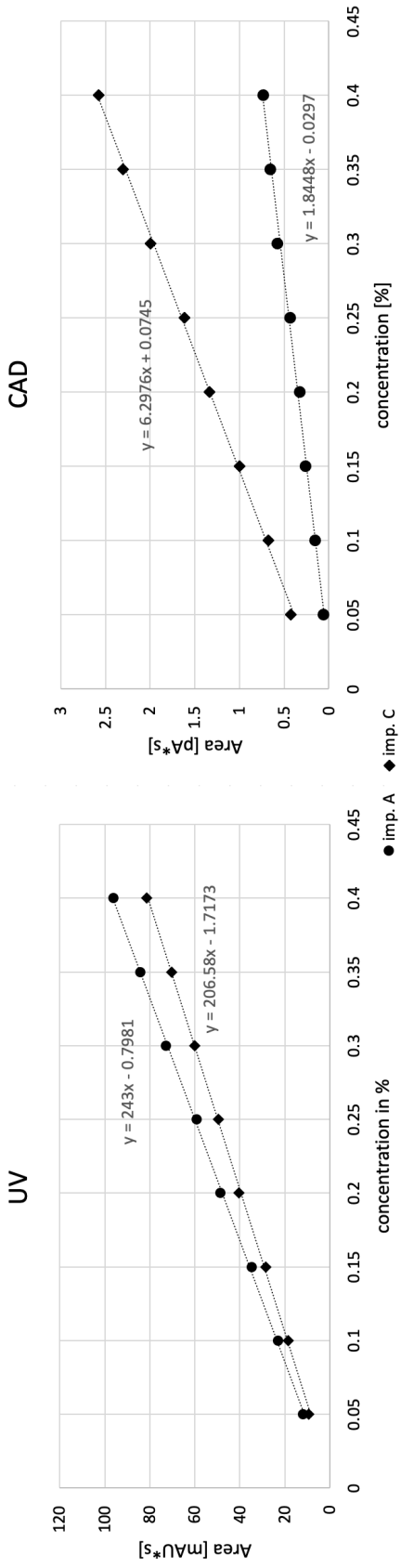

Figure S-3: Calibration curves of UV detection of the impurities A and C within the range of 0.05 to 0.40%. The calibration curves obtained from the hyphenated CAD detection technique, are included for sake of completeness.

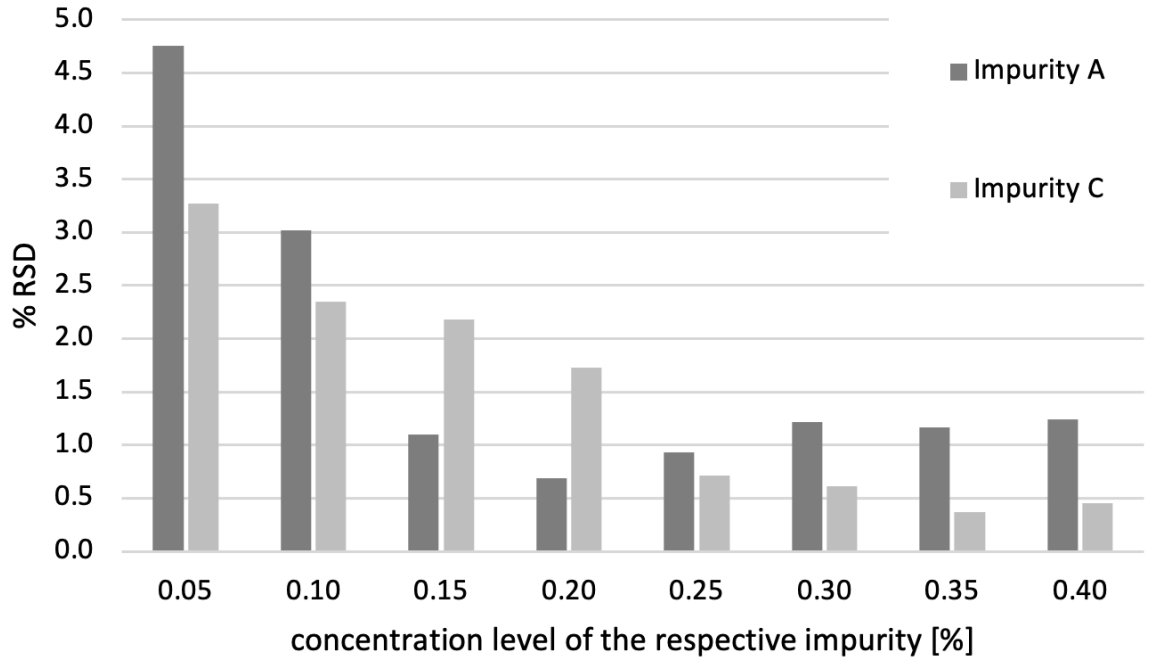

Figure S-4: RSD of each calibration level for every impurity obtained from the calibration curves (sextuple injection)

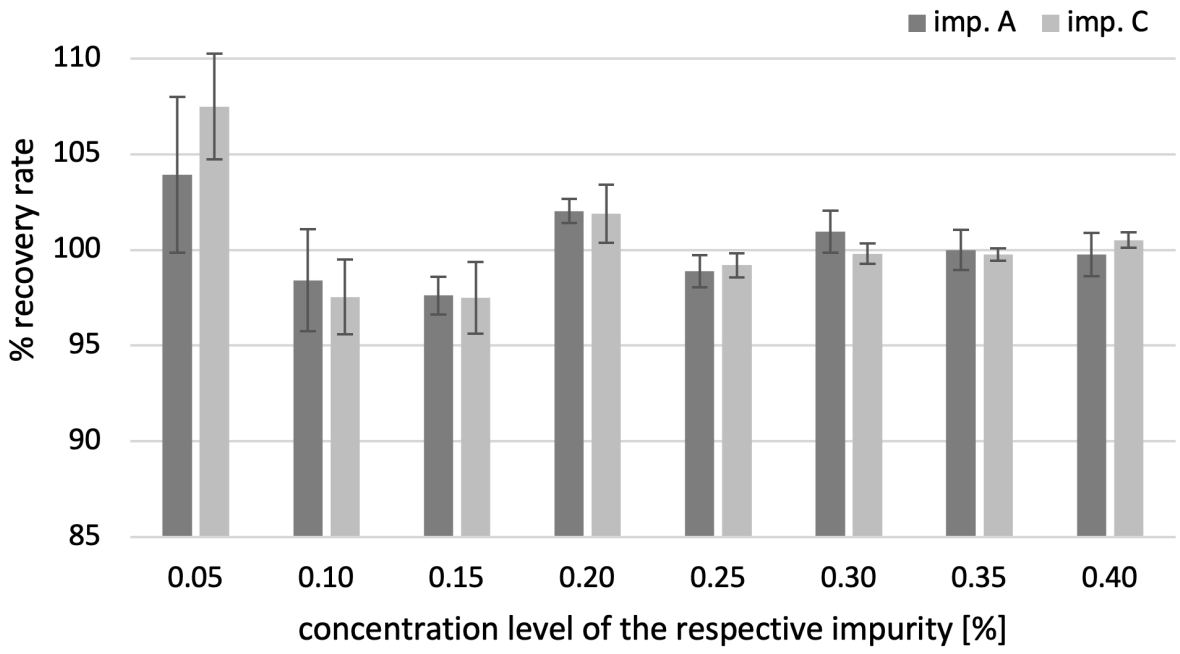

Figure S-5: Mean recovery rates and relative standard error of imp. A and imp. C obtained at UV detection

3.4. Alternative methods to assess the impurity profile of a monographed API using acarbose as an example

Adrian Leistner, Ulrike Holzgrabe

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https://doi.org/10.1016/j.jpba.2022.115063

Abstract

Even though the impurity analysis of the Ph. Eur. is considered well-studied, its methodology should be reviewed periodically to ensure that it is working properly, and all impurities are captured by the section „Related substances" of the monograph. Within this study, the biotechnological produced antidiabetic drug acarbose was chosen to demonstrate some of the advantages as well as the shortcomings arising from the current related substances test of acarbose. Due to its weak chromophore, acarbose is studied by UV detection at 210 nm after being separated on aminopropyl-silyl stationary phases. Thus, the use of alternative detection techniques, such as charge aerosol detection (CAD) and a volatile mobile phase can be beneficial here. Since a simple method transfer to a mobile phase usable with the CAD was not possible, more stable stationary phases were tested. For the rapid determination of the sum of impurities, a method was developed using a pentafluorophenyl column and a mobile phase of 0.1% TFA in water. Maltose and maltotriose were further identified as additional impurities of the API. Furthermore, a method was developed and validated by means of an Amide-HILIC phase, that adequately separated acarbose and all of its impurities. However, the sensitivity of this method needs to be further improved. Additionally, a method was also developed and validated, taking into account the temperature stability of graphite columns. Thus, the separation was carried out at temperatures of about 90 °C to solve the problem of anomerization of acarbose and some of its impurities. In comparison with the other developed methods, the elution order was changed and has to be confirmed, but all components were separated and detected at a sufficient LOQ of 0.10%.

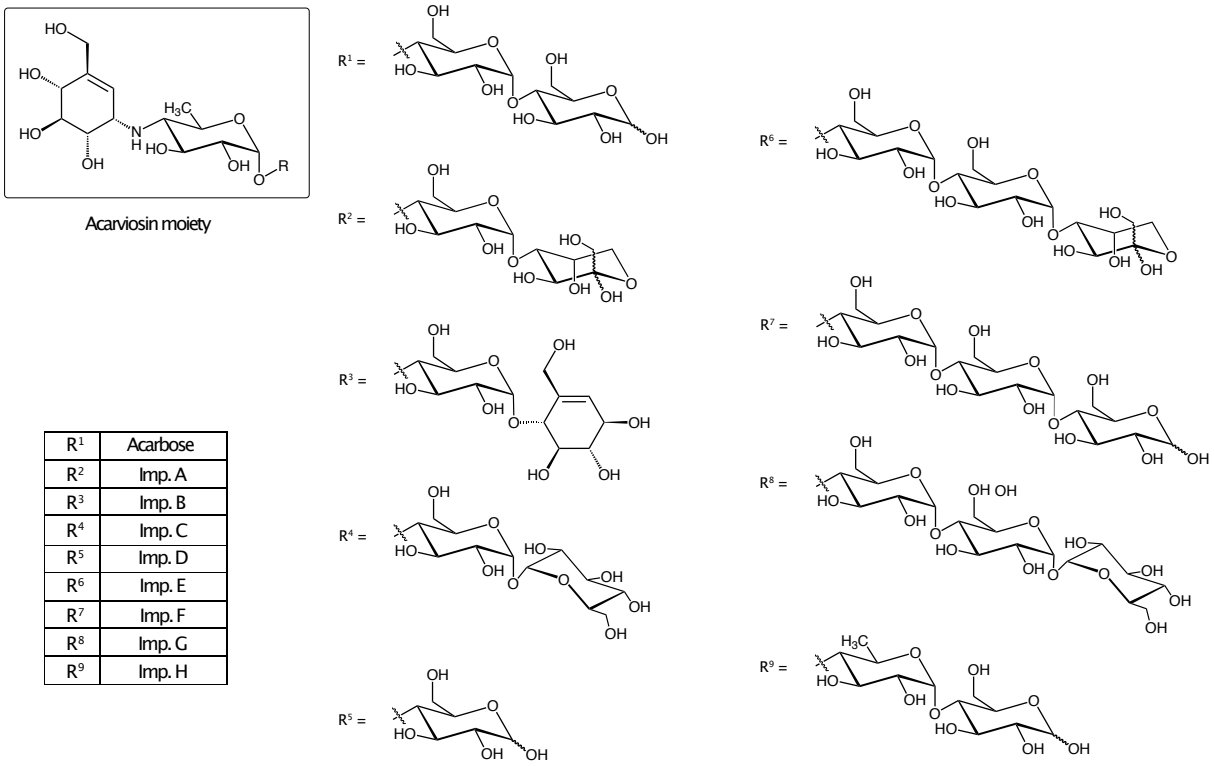
1. Introduction

The impurity profile of active pharmaceutical ingredients (API) monographed in Ph. Eur. can be considered well studied, as it is based on the information provided by the manufacturer of the approved drug products. However, the monographs should be reviewed on a regular basis to ensure that they are still up to date. For example, additional impurities might have been observed, or some of the impurities included simply cannot sufficiently be detected by the current method. Therefore, acarbose was selected as a representative drug to demonstrate some of the advantages as well as the shortcomings arising from the current test for related substances, which has not been changed since its introduction into Ph. Eur. in 2005 [1]. In addition, we present alternative methods as tests for related substances that can also be used to additionally detect those impurities that lack a chromophore.

Acarbose is categorized as an α-glucosidase inhibitor. It inhibits very effective for instance the glucoamylase in the intestine resulting in a delayed release of glucose and other monosaccharides from complex carbohydrates within digestion and is therefore used to treat diabetes mellitus type 2 [2, 3]. Commonly, acarbose is taken three times a day with meals in a total daily dosage of 150 to 300 mg [4].

The structure of the drug acarbose (see Figure 1) imitates the substrates (e.g. starch or sucrose) of several alpha-glucosidases, which are needed for the break-down of complex carbohydrates to monosaccharide units, and consists of an aglycon cyclitol and of an amino sugar forming together the acarviosin part (see left upper corner of Figure 1), which is coupled to two D-glucose units [2]. Due to its physicochemical properties, acarbose is absorbed only at a very small amount. However, absorption is not necessary as its target is located in the gut.

The API is commonly produced by a fermentation process by means of the bacterium *Actinoplanes utahensis* or other strains of *Actinoplanes sp.* [5]. This biotechnological process explains the impurity profile of acarbose. The impurities listed in the transparency list of the Ph. Eur. monograph for acarbose (see Figure 1) differ only in the sugar profile, while the acarviosin part of the molecule is constant [1]. Impurity D consists of only one glucose unit, while beside acarbose, the impurities A, B, C and H have 2 sugar units coupled to the acarviosin part. Compared to acarbose, the impurities E, F and G have one additional sugar unit, thus forming a molecule of five rings.



R^1	Acarbose
R^2	Imp. A
R^3	Imp. B
R^4	Imp. C
R^5	Imp. D
R^6	Imp. E
R^7	Imp. F
R^8	Imp. G
R^9	Imp. H

Figure 1: Impurity profile of acarbose with respect to the Ph. Eur. monograph [1].

Due to the weak chromophore of all these compounds, up to now, the Ph. Eur. evaluates the related substances of acarbose by means of low wavelength detection at 210 nm [1]. For substances lacking of a chromophore or those with a weak one, the Charged Aerosol Detector (CAD) is often an alternative as it is a universal detection technique [6].

Phosphate buffers, that are currently used within the Ph. Eur. method, are not volatile and hence not suitable to be used with the CAD. Therefore, it was mandatory to replace them by volatile additives to use the CAD.

Several analytical methods have been reported on the characterization of acarbose. *Montazeri et al.* could confirm that the USP method of the API acarbose (analogue to the Ph. Eur. monograph) is suitable for the determination in solid dosage forms as well [7, 8]. Now, the later implemented method for tablets [9] is analogue to the USP API monograph but with a less concentrated test solution. *Cherkaoui et al.* compared a CE-UV method with a HPLC-ELSD method using a mixture of methanol and dichloromethane as mobile phase on an amino column [10]. *Daali et al.* reported on the determination of acarbose and its metabolite by means of a porous graphitic carbon

column and different detection techniques, while fluorescence detection after derivatization with 2-aminobenzamide was found to be most sensitive [11]. *Novak et al.* could identify some impurities of acarbose by HPLC-MS and HPLC-NMR [12]. *Pitsch and Weghuber* investigated the separation and quantitation of carbohydrates (including acarbose) in beverages by means of an amide HILIC column [13]. In a review, *Wang et al.* summarized some further analytical methods for the determination of acarbose [14].

2. Experimental

2.1. Chemicals and reagents

Certified Reference Standards (two different batches of acarbose CRS and the CRS for peak identification (an impurity mix of acarbose with the impurities A, B, C, D, E, F and G)) were obtained from the European Directorate for the Quality of Medicines & HealthCare (EDQM), Strasbourg, France. An acarbose API sample (> 95% purity) was purchased from Acros Organics B.V.B.A. (Thermo Fisher Scientific, Waltham, MA, USA).

Trifluoroacetic acid (TFA) (> 99% purity), HPLC grade acetonitrile (> 99.9% purity), maltose (> 99% purity), maltotriose (> 90% purity), phosphoric acid, formic acid and acetic acid were all of analytical grade (> 99% purity) and purchased from Sigma-Aldrich Chemie GmbH, (Darmstadt, Germany). Potassium dihydrogen phosphate and disodium hydrogen phosphate dihydrate for HPLC was purchased from VWR international GmbH (Darmstadt, Germany). Ultra-pure water was freshly produced by a water purification system from Merck Millipore (Darmstadt, Germany).

2.2. Apparatus

The HPLC measurements of the Ph. Eur. related substances' test of Acarbose were performed on an Agilent 1100 modular chromatographic system consisting of an online vacuum degasser G1322A, a quaternary pump G1311A, an auto sampler G1329A, a thermostatted column compartment G1316A, and a variable wavelength detector G1314B (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). UV detection was performed at 210 nm. Agilent OpenLab CDS Rev. C.01.10 [201] software was used for data processing.

All other HPLC experiments were performed on a Vanquish™ Flex modular chromatographic system (Thermo Fisher Scientific, Germering, Germany) consisting of a dual pump F (two independent ternary solvent blending flow streams in one housing) with an online vacuum degasser, a thermostatted split sampler, a thermostatted column compartment with an active pre-heater, and a diode array detector in-line with a Vanquish™ Horizon CAD. The CAD was supplied with nitrogen gas from a Corona Nitrogen 1010 nitrogen generator (Peak Scientific Instruments, Inchinnan, United Kingdom) connected to the in-house compressed air system. The instrument was controlled, and runs were processed using the Chromeleon™ 7.3 Chromatography Data System (Thermo Fisher Scientific). For all CAD experiments,

the evaporation temperature was set at 50 °C, while a power function value of 1.0, a data acquisition rate of 10 Hz, and a filter constant of 5.0 s were used unless properly noted.

2.3. Preparation of solutions

For the preparation of the test solution for the Ph. Eur. method [1], 0.200 g of Acarbose were accurately weighed and dissolved to a volume of 10.0 mL with deionized water. This solution was further diluted with deionized water (1.0 mL to 100.0 mL) to obtain reference solution (a). Additionally, a vial of Acarbose for peak identification (containing Acarbose and its impurities A, B, C, D, E, F and G) was dissolved in 1.0 mL of deionized water to obtain reference solution (b). To test the LOQ of the methods, a solution containing 0.05% API was prepared by diluting reference solution (a). All solutions were stored at 8 °C.

2.4. Chromatographic procedures

2.4.1. Ph. Eur. method

For the performance of the current Ph. Eur. test for related substances of acarbose, an aminopropyl-silyl (APS) column e.g., the used Hypersil™ APS–2 (250 x 4 mm i.d., 5 µm particle size, Thermo Fisher Scientific) is necessary. The experimental conditions require to operate isocratic elution with a flow rate of 2.0 mL/min, an injection volume of 10 µL, and a column compartment temperature of 35 °C. A mixture of 750 mL acetonitrile and 250 mL of an aqueous phosphate solution (0.6 g/L potassium dihydrogen phosphate and 0.35 g/L disodium hydrogen phosphate dihydrate) was used as mobile phase. The separation of acarbose and its impurities A, B, C, D, E, F and G was achieved within 50 min and UV detection at 210 nm was applied.

2.4.2. Method with aminopropyl-silyl column and CAD detection

Analogously to the Ph. Eur. method, experiments with CAD detection and replacement of the phosphate buffer by ammonium acetate (10 mM) were performed on the Hypersil™ APS–2 column (250 x 4 mm i.d., 5 µm particle size, Thermo Fisher Scientific).

Comparatively, experiments on a shorter column with the same column chemistry but with smaller particle size (Hypersil Gold™ Amino, 150 x 4.6 mm i.d., 3 µm particle size, Thermo Fisher Scientific) were performed. Therefore, the flow rate was set to 1.5 mL/min and the mobile phase composition had to be adjusted by increasing the

organic content to 85% acetonitrile and using 25 mM ammonium acetate as aqueous component.

2.4.3. Method with Sugar-D column

A COSMOSIL Sugar-D column (250 x 4.6 mm, 5 µm particle size, Nacalai tesque Inc.) with a polyamine-based stationary phase was used for the method. Therefore, isocratic elution (2.0 mL/min) of either a mix of 82% acetonitrile and water or 82% acetonitrile and aqueous solution of 25 mM ammonium acetate was used. 5 µL were injected and the column oven temperature was set to 35 °C. UV detection at 210 nm was compared with the CAD signal.

2.4.4. Method with pentafluorophenyl propyl column

Experiments were performed on an Accucore™ PFP column (100 x 4.6 mm i.d., 2.6 µm particle size, Thermo Fisher Scientific). The final method comprises isocratic elution (1.0 mL/min) of 100 mM ammonium formate at a column oven temperature of 5°C and CAD detection.

2.4.5. Method with Amid-HILIC column

For the separation of acarbose and its impurities A, B, C, D, E, F and G, an Accucore™ 150 Amide HILIC column (100 x 2.1 mm i.d., 2.6 µm particle size, Thermo Fisher Scientific) was used. Within method development, isocratic runs and gradient experiments were performed. The type of buffer, its concentration and the pH were studied as parameters affecting the separation. Additionally, the influence of the method performance was investigated on two different column dimensions. The final method comprised CAD detection after gradient elution with a flow rate of 0.6 mL/min, column oven temperature setting of 45 °C and an injection volume of 2.5 µL. Mobile phase A was 50 mM ammonium acetate pH 5.8 in water, while mobile phase B was acetonitrile. For the gradient, an initial isocratic step for 35 min with 87.0% of mobile phase B was performed. For the next 10 min, a linear gradient to 84.0% of mobile phase B, followed by a further isocratic step for 35 min with 84.0% of mobile phase B was set. This was followed by a re-equilibration step with 87.0% of mobile phase B for 20 min.

2.4.6. Method with Hypercarb column

For the method development on the Hypercarb™ column (150 x 4.6 mm i.d., 3µm particle size, Thermo Fisher Scientific), the influence on the separation of all relevant

components using no buffer, ammonium formate (50 and 100 mM), formic acid (0.1%) or trifluoroacetic acid (0.1%) was examined. The column oven temperature was varied between 10 °C and 90 °C and the injection volumes of 2.5 and 5.0 µL were compared. Both, experiments with isocratic and gradient elution were performed. For the final CAD method, a gradient using aqueous solution of trifluoroacetic acid (0.1%) as mobile phase A and acetonitrile with 0.1% of trifluoroacetic acid as mobile phase B, was performed. Therefore, the column oven temperature was adjusted to 90 °C, the flow rate was set to 1.0 mL/min and the injection volume to 2.5 µL. An initial isocratic step for 2 min with 8.0% mobile phase B was followed by a linear increase to 13.0% mobile phase B over 24 min and a further isocratic step with 13.0% mobile phase B for 5 min. Within the next 2 min, re-equilibration was performed and prolonged by 5 min post time with 8.0% mobile phase B.

3. Results and discussion

3.1. Challenges of the impurity analysis of acarbose

As can be seen from Figure 1, all related substances of acarbose are quite similar and differ in the sugar part of the respective molecule only. Some of them are even only isomers of the sugar moiety with the same molecular weight (e.g., M_r = 645: acarbose, imp. A, and imp. C; M_r = 807: imp. E, imp. F, and imp. G). The similarity of the compounds makes their separation and analysis challenging. Moreover, they are not equipped with a strong chromophore. Rather, the one or two double bonds of the molecules under investigation represent a weak chromophore. Moreover, the current Ph. Eur. method is critical for detecting those substances having no chromophore, such as sugar fragments. Therefore, it is better to rely on a universal detection, such as the charged aerosol detection. However, for CAD the mobile phase additives used must be volatile. Consequently, the non-volatile phosphate buffers must be replaced by volatile mobile phase additives, e.g., formic acid, ammonium formate, acetic acid, ammonium acetate, or trifluoroacetic acid.

Of note, for APS columns used by the Ph. Eur. monograph, it is known that the column materials tend to degrade upon usage, which can be easily monitored by the CAD by increasing background current, resulting in an increased noise level and a low LOQ. Therefore, the column chemistry significantly changes over time and the lifetime of this type of column is limited.

Furthermore, most of the analytes show anomerization of the terminal sugar moiety. Consequently, for one impurity, several isomeric forms exist (alpha, beta and open-chain form) and therefore, the potential number of analytes is much higher than initially expected. Even on column this anomerization process takes place and influence the peak shapes significantly. The speed of anomerization can be influenced either by temperature or pH of the mobile phase.

3.2. Advantages of the CAD

Due to its physiochemical properties, (e.g. mass of more than 250 Da [15]), acarbose and its specified impurities can be regarded as non-volatile analytes and their respective CAD signals are practically independent of the evaporation temperature. An increase of the latter can reduce the semi-volatile impurities of the mobile phase and consequently reduce the noise [16]. Best performance criteria were found with an evaporation temperature of 50 °C, which was used for all experiments. The power function value was kept at the default value of 1.0, while the data collection rate was fixed to 10 Hz and a filter constant of 5.0 s was used for all experiments to obtain more comparable results.

The major advantage of the CAD detection of acarbose and its impurities is, that it does not need a correction factor, since the analytes are not volatile, and the detector is mass dependent. Additionally, gradient dependent effects on the signal are minimal within both validated methods and can therefore be neglected.

3.3. Procedure of the method transfer

Initially, the current Ph. Eur. related substances test was performed for reference purposes and a simple method transfer was applied to make the method suitable for CAD detection. Since this was not possible without creating new problems, the column chemistry had to be changed. Finally, two alternative methods with CAD detection were developed successfully.

3.3.1. Comparative measurements by means of the Ph. Eur. method

The experiments following the Ph. Eur. method are intended to be a benchmark for the CAD methods [1]. An exemplary chromatogram of reference solution (b) containing acarbose and its impurities is displayed in Figure 2.

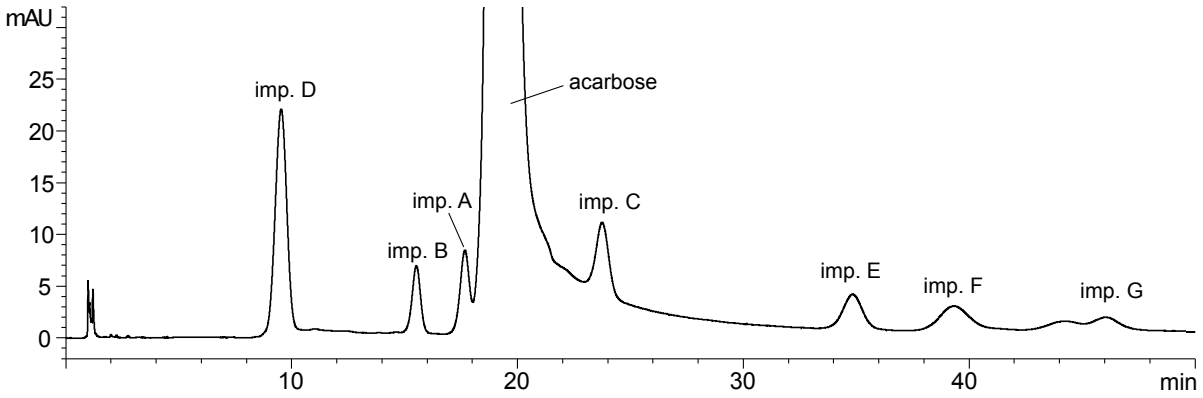


Figure 2: Chromatogram obtained with acarbose reference solution (b) by means of the current Ph. Eur. related substances test for acarbose with UV detection at 210 nm. Column: Hypersil™ APS–2 (250 x 4 mm i.d., 5 µm particle size, Thermo Fisher Scientific); The chromatographic system was operated with a flow rate of 2.0 mL/min using isocratic elution at 35 °C. Further chromatographic conditions for this method can be seen in section 2.4.1.

The method defines two system suitability criteria: The chromatogram obtained from reference solution (b) must be similar to the one supplied by the CRS leaflet (see [17]). Second, the peak-to-valley ratio between impurity A and acarbose should be at least 1.2. The chromatogram obtained for reference solution (b) is very similar to the reference chromatogram of the CRS (see [17]) and the peak-to-valley ratio is 4.4. Hence, the system suitability criteria had been met. Nevertheless, the acarbose peak shows tailing and a small shoulder, as it is also found in the reference chromatogram [17].

3.3.2. Transfer to CAD detection

Since the charged aerosol detection needs a volatile mobile phase, the phosphate buffer had to be replaced by a volatile buffer. The pH of the aqueous phosphate solution used in the Ph. Eur. method is 6.9. Thus, a 10 mM solution of ammonium acetate was used for the replacement. However, the acetate portion increases the UV cut off value and thus increases the UV-dependent noise. Nevertheless, it was still possible to detect acarbose and some impurities at λ = 210 nm but the system suitability criteria of the Ph. Eur. method are not fulfilled as imp. A and acarbose are not separated anymore (see SI-1). Even worse, it was not possible to detect the components at a sufficient LOQ due to an extreme high noise level. In general, the higher the background current, the worse the noise of an elution [16]. Here, background currents of more than 75 pA were observed, while for the mobile phase without a column, values of around 2 pA had been achieved. Same holds true for a

mobile phase without buffer additives. Consequently, these inappropriate noise levels was likely caused by column bleeding, which is also reported in the literature [18, 19]. The amino functionalities of the APS columns generate a locally high pH, which is favorable for their self-destruction. This process can easily be monitored with the CAD by the background current. Attempts to overcome this problem by using a shorter column or adapted solvents were not successful. It can be concluded that silica-based APS phases are not suitable for CAD detection due to the high background current and that polymer-bonded phases described in the literature could be used as an alternative [20, 21].

3.3.3. Column selection

As consequence of the stability issue of the APS stationary phase, a more stable stationary phase had to be used for the acarbose analysis.

The COSMOSIL Sugar-D column has secondary and tertiary amine functions bonded to a high purity spherical silica. A technical note describes the analysis of acarbose [22], using an isocratic elution (2.5 ml/min) of a mixture of acetonitrile and phosphate buffer (82:18) and UV detection at 210 nm. Again, for use with CAD, the phosphate in the mobile phase was replaced with a solution of 25 mM ammonium acetate. Furthermore, the flow rate had to be adjusted to the maximum usable flow rate for the CAD - 2.0 mL/min.

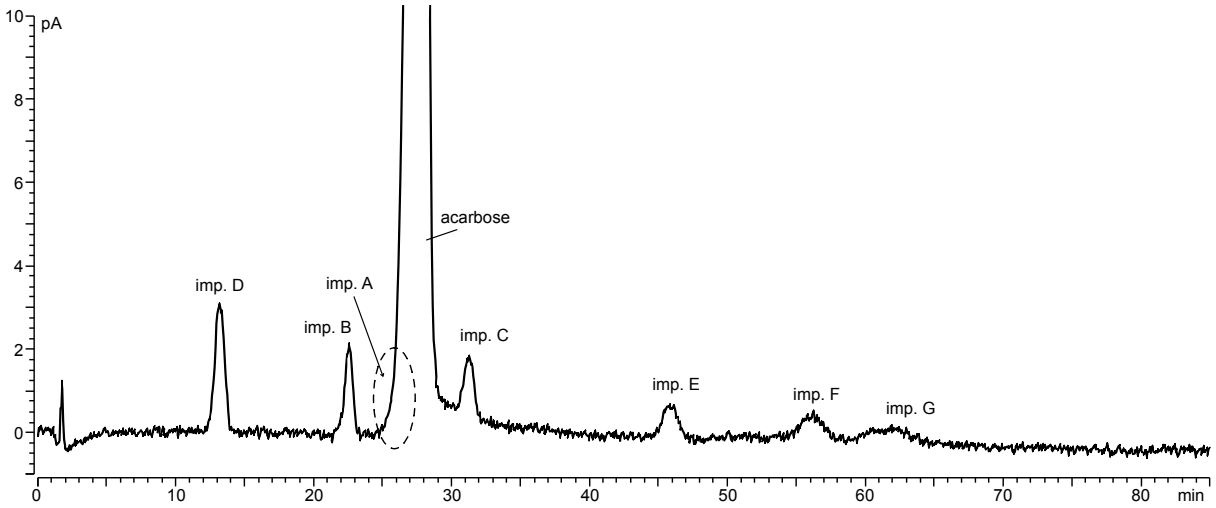


Figure 3: Impurity analysis of acarbose reference solution (b) using Sugar-D column (250 x 4.6 mm, 5 µm particle size) with isocratic elution of a mixture of acetonitrile and water (82:18) at a flow rate of 2.0 mL/min and CAD detection

Since the resulting background current (about 50 pA) was too high for acceptable results, the buffer was replaced by water. An injection of 5 µL reference solution (b)

resulted in a chromatogram with evaluable peaks. However, beside the background current of 10 pA, the critical peak pair of acarbose and imp. A was not sufficiently separated (see Figure 3).

Next, a pentafluorophenyl propyl (PFP) column was tested because of the multiple mechanisms of interaction; hydrophobic, dipole-dipole, aromatic and π-π interactions and hydrogen bonding interactions are appropriate for the separation of polar isomers such as acarbose and its impurities [23]. It was necessary to use the column with the smallest possible amount of organic modifier to ensure retention.

Rapid elution within 10 min was achieved with 1% acetonitrile in aqueous solution of 0.1% TFA as eluent. Acarbose was separated from the impurity peaks (see SI-2), but the impurities were not adequately separated from each other.

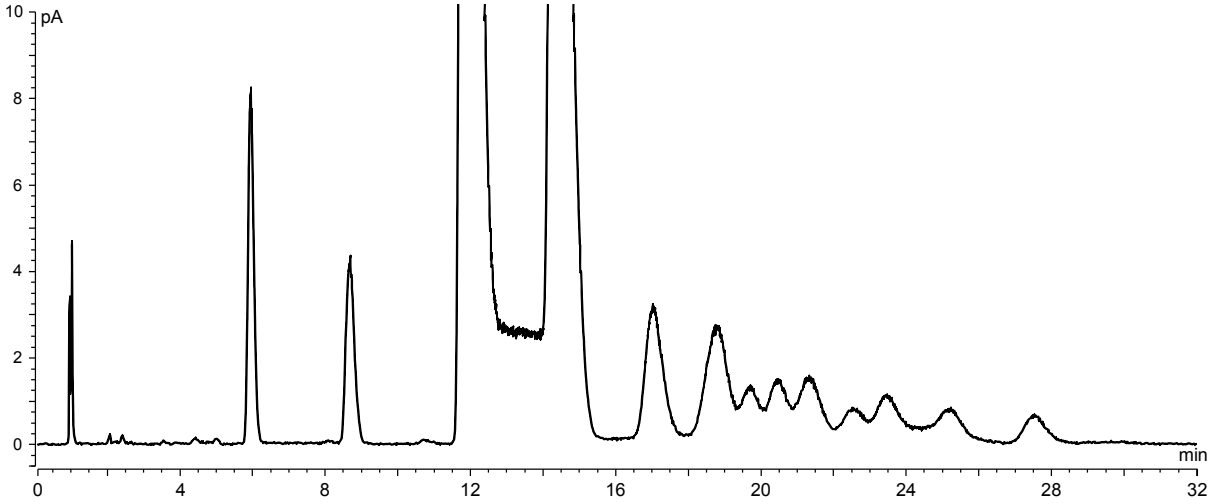


Figure 4: Impurity analysis of acarbose reference solution (b) using Accucore™ PFP column (100 x 4.6 mm i.d., 2.6 µm particle size) with isocratic elution of 100 mM ammonium formate and CAD detection (10 Hz, filter 1.0 s)

Using 100 mM ammonium formate as eluent, the background current was reduced to 0.5 pA, and resulted in a significant reduction in noise without loss of resolution. At the low temperature of 5 °C used for this method, double peaks with no baseline separation appear for some substances in the analysis of acarbose (see Figure 4), indicating an epimerization. At higher temperatures of 70 °C, the rate of epimerization (see section 3.1.) is faster than chromatographic separation and the substances elute as a single, broad peak. Overall, it can be concluded that the method is suitable for the rapid determination of the sum of acarbose impurities, but not for the content evaluation of each single impurity. In addition to the relatively short analysis time, the method is quite sensitive. Injection of a dilute acarbose solution (0.05% based on the test solution)

resulted in two peaks that appeared to be separate and were due to the respective acarbose epimers (see SI-3). In practice, however, the described epimerization reaction takes place, but is hidden in the background noise and cannot be observed in the chromatogram. This method is nevertheless sensitive for the determination of impurities at low levels. In one batch, additional early eluting peaks were observed in the chromatogram. It can be assumed that these are hydrophilic by-products or residues of the fermentation process. By spiking with maltose and maltotriose solutions, two of these unknown peaks (at very low concentrations) could be identified.

Amide HILIC phases, such as Accucore™ 150 Amide HILIC column (Thermo Fisher Scientific), have been proved to be appropriate for the separation of sugars in beverages [13] using a gradient elution (80% to 60% acetonitrile with 10 mM ammonium acetate pH 8.25).
Even though it is advantageous to use the starting mixture of the gradient, an aqueous solution was applied due to solubility problems.
Within the method development, the performance of two different column dimensions of the Amide-HILIC column were tested. The model with 150 x 4.6 mm, 2.6 µm particle size, was compared to the column with 100 x 2.1 mm, 2.6 µm particle size. The latter showed better performance with the CAD as less column material bleeds out and enters the detector. Due to the mass-dependency of the detector, it is better to use columns with reduced inner diameter, to obtain the lowest background current possible, which goes directly with a reduction of noise. The injection of a diluted solution of acarbose (1.0% referred to the test solution) resulted in a small, but identifiable peak for acarbose. However, the signal of a further diluted sensitivity solution (0.05%) could not be identified as a peak. Thus, this method is not shine of best sensitivity (see section 3.3.4.).

For epimerization reasons, elution of a mobile phase of a basic pH and high temperature would be suitable but would destroy the silica-based column. As a compromise, an oven temperature of 45 °C and ammonium acetate buffer pH 5.8 were chosen. As can be seen in Figure 5, acarbose and its impurities A, B, C, D, E, F and G are all baseline separated. However, the runtime of this method is longer than the Ph. Eur. method and needs extra time for re-equilibration. The validation of this method is displayed in section 3.3.4.

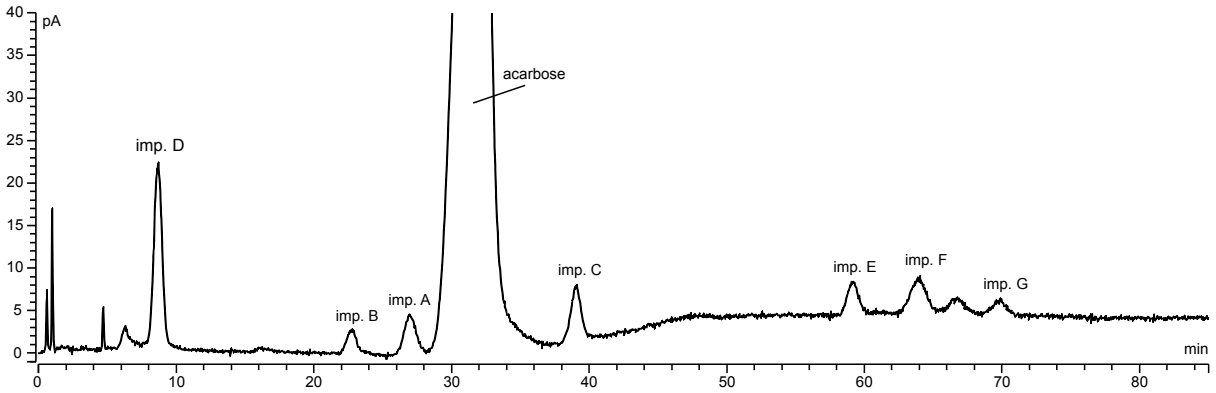


Figure 5: A typical chromatogram obtained with acarbose reference solution (b) solution. column: Accucore™ 150 Amide HILIC 100 x 2.1 mm, 2.6 µm particle size (Thermo Fisher Scientific); The chromatographic system was operated with a flow rate of 0.6 mL/min using gradient elution at 45 °C and CAD-detection. Further chromatographic conditions for this gradient HPLC can be seen in section 2.4.5.

Graphite columns, such as Hypercarb columns, are also suitable for the analysis of polar compounds, like sugars [24-26]. A polar retention effect occurs due to the high affinity of the polar analytes to the graphite surface [24, 27, 28]. Additionally, the column can be used with extreme conditions such as high acidity, high alkalinity, and high temperatures, because there is no material detaching as the stationary phase is just carbon.

As isocratic approaches did not result in a separation or led to very broad peaks without a sufficient sensitivity, a gradient elution was performed (see Figure 6).

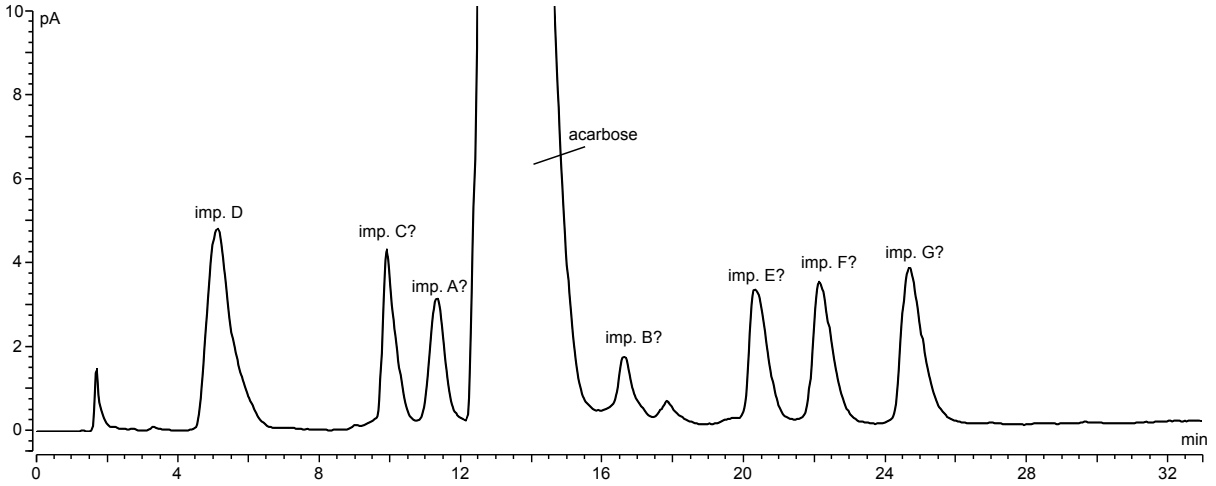


Figure 6: Proposed elution order for the Impurity analysis of acarbose reference solution (b) using Hypercarb™ graphite column (150 x 4.6 mm i.d., 3µm particle size) with gradient elution (mobile phase A: 0.1% TFA in water; mobile phase B: 0.1% TFA in acetonitrile) and CAD detection (10 Hz, filter 1.0 s). Gradient: 0-2 min 8% mobile phase B; 2-26 min 8-13% mobile phase B; 26-31 min 13% mobile phase B; 31-33 min 13-8% mobile phase B.

Since an acidic pH turned out to be beneficial for good separation on the Hypercarb column, in experiments with varying mobile phase composition, the chromatography was performed with 0.1% TFA in the mobile phase (gradient with acetonitrile/water and 0.1% TFA), which resulted in good separation of acarbose, and all impurities within 28 min (see Figure 6). In these initial experiments, it was observed that the stronger the ionic strength of the mobile phase used, the stronger was the retention of the components on the column. For example, retention times of 33 min were observed for Imp. D using 50 mM ammonium formate as mobile phase additive (data not shown), whereas for the final TFA method Imp. D had a retention time of only 5 min in comparison.

Again, double peaks and insufficient separation occurred in experiments performed at low temperatures of 10 °C, but this problem could be solved by using a higher column oven temperature of 90 °C, which is possible with graphite columns. However, the peaks are still quite broad and in case of samples with a high mass on column, acarbose and impurity B would co-elute, hence the injection volume was set to 2.5 µL. This is still sufficient to detect a diluted solution of acarbose (0.05% referred to the test solution) sufficiently (see SI 4).

Comparison of the responses of all methods (see SI-6) indicates a changed elution order for the Hypercarb column. However, the order could not be confirmed, as the single impurities were not available for spiking experiments and a correspond mass detection cannot help due to the same masses of a couple of impurities. The batch results obtained by the measurement of the two CRS of acarbose and of the acarbose delivered by Acros with this method are listed in SI-5.

3.3.4. Validation of Amide-HILIC and Hypercarb method

With regard to ICH Q2(R1) guideline, specificity, repeatability, limit of quantification (LOQ), linearity, range, accuracy and robustness of both Amide-HILIC, and Hypercarb method were investigated [29].

For the assessment of specificity, a solution containing acarbose and its impurities A, B, C, D, E, F and G (ref. solution (b)) was used. Both methods were able to separate all compounds from each other. However, only a suggestion of the peak assignment could be made, since only an impurity mix, and no individual substances were available for final verification.

Repeatability was demonstrated at three different concentration levels (0.20%, 1.00%, and 1.50%) with 6 replications each. The relative standard deviations (%RSD) for the Hypercarb method were in the range of 0.35% to 1.28% indicating a precise method. Indeed, for the Amide- HILIC method, the RSDs (1.01% to 5.35%) are less good.

The limits of quantification (LOQ) were evaluated by visual inspection of the signal-to-noise ratio. To achieve acceptable sensitivity for the Amid-HILIC method, a solution of 0.20% acarbose must be injected. Notwithstanding other challenges, such as baseline separation of all components, injecting a higher concentration or larger amount can lead to overcome the sensitivity problem. For the Hypercarb method, a signal-to-noise ratio of 10:1 for the LOQ - acceptable according to the ICH guideline - was met for a less concentrated solution of 0.10% of acarbose, indicating the good sensitivity of the method.

Linearity and range: A calibration curve including at least five concentration levels was established covering a range from 0.20% to 1.60% ($y = 5.9842x - 0.5620$; $R^2 = 0.9979$) for the Amid-HILIC method and within a range from 0.10% to 1.60% for the Hypercarb method ($y = 1.2466x + 0.0290$; $R^2 = 0.9989$).

Accuracy for the Hypercarb method was determined at three different levels (0.20%, 1.00%, and 1.50%) with mean recoveries ranging from 99.2% to 102.4% (n = 3, %RSD 0.81–1.93%). However, the values of the Amide HILIC method (mean recoveries 97.4% to 107.9% (n = 3, %RSD 1.43–5.06%)) were worse due to the lower precision at the lower level.

For robustness testing of the Amid-HILIC method, the HPLC parameters were varied as follows: column temperature (± 5.0°C), mobile phase flow rate (± 0.1 mL/min), % mobile phase B over the entire gradient range of the method (± 0.5%). These variations already showed that this method is not particularly robust. For example, not all components are eluted within the runtime of the method, if the flow rate is lowered, the acetonitrile content is increased, or the temperature is lowered. For the respective opposite directions, this problem does not apply, and the components are separated accordingly.

For the Hypercarb method, range of the temperature (± 5.0°C), flow rate (± 0.05 mL/min), % acetonitrile over the whole gradient (± 0.5%) and TFA concentration (± 0.05%) were varied. With increased flow rate as well as decreased TFA concentration, peak 3 and acarbose were no longer baseline separated, but their

peak-to-valley ratios of 1.4 and 1.8, respectively, are nevertheless sufficient and the method can be regarded as robust.

3.4. Comparison of the obtained batch results

The batch results of the three different acarbose APIs obtained by the UV Ph. Eur. method were compared to the respective results of the CAD methods on Hypercarb and Amide-HILIC columns. An overview of the results can be seen in Table 1,2 and SI-5.

Table 1: batch results of three different acarbose API by means of the Ph. Eur. test for related substances.

Imp. limits	Acarbose CRS 3	Acarbose CRS 4	Acros API Acarbose
Imp. A ≤ 0.6%	0.20	0.24	0.60
Imp. B ≤ 0.5%	0.16	0.16	0.16
Imp. C ≤ 1.5%	1.07	1.09	0.24
Imp. D ≤ 1.0%	0.35	0.35	0.22
Imp. E ≤ 0.2%	b.r.t.	b.r.t.	b.r.t.
Imp. F ≤ 0.3%	b.r.t	b.r.t.	b.r.t.
Imp. G ≤ 0.3%	b.r.t	b.r.t.	b.r.t.
Unknown imp.			several b.r.t.
Sum of imp.	1.76	1.84	1.22

* b.r.t. = below reporting threshold (0.1%)

For the UV detection of the Ph. Eur., correction factors have to be applied. Therefore, the obtained peak area had to be multiplied by respective correction factors: imp. B = 0.63; imp. D = 0.75; imp. E, F, G = 1.25. For all other components no correction factor is necessary. Peaks below 0.1% (referred to the concentration of the API) can be disregarded. According to the monograph, imp. C is limited to 1.5% (referred to the concentration of the API). The further limits are: imp. D ≤ 1.0%; imp A ≤ 0.6%; imp. B ≤ 0.5%; imp. F and G each ≤ 0.3%; imp. E ≤ 0.2%; all other respective impurities ≤ 0.2%. In sum 3.0% of impurities may not be exceeded. All three batches comply with these limits. However, for the API purchased from Acros, several additional impurities were found below the disregard limit. Of these, maltose and maltotriose were identified as impurities, albeit at a low level.

Due to the described mass-flow dependency of the CAD and the non-volatility of the analytes, no correction factors are necessary with the CAD. The results obtained are in accordance with those of the Ph. Eur. method; however, they provide slightly higher values overall for the sum of the observed impurities. The limits could therefore be exceeded with the CAD method although the active substance complies with the requirements according to Ph. Eur. method. Thus, this ensures that only the purest possible API is used.

Table 2: batch results of three different acarbose API by means of the Amid HILIC method.

Imp. limits	Acarbose CRS 3	Acarbose CRS 4	Acros API Acarbose
Imp. A ≤ 0.6%	0.20	< 0.20	0.39
Imp. B ≤ 0.5%	0.23	0.24	0.27
Imp. C ≤ 1.5%	1.07	1.09	0.24
Imp. D ≤ 1.0%	0.62	0.52	0.43
Imp. E ≤ 0.2%	< 0.20	< 0.20	0.51
Imp. F ≤ 0.3%	b.r.t	b.r.t	b.r.t
Imp. G ≤ 0.3%	b.r.t	< 0.20	0.21
Unknown imp.	b.r.t	b.r.t	0.28 + several b.r.t
Sum of imp.	2.23	2.21	2.33

* b.r.t. = below reporting threshold (0.1%)

The peak assignment could not be confirmed using the respective UV spectra, since they are practically identical for all components. Therefore, a proposal for the elution order for the method on Hypercarb column was made based on the Ph. Eur. and Amide-HILIC measurements: imp. D - imp. C - imp. A – acarbose - imp. B - unknown imp. - imp. E - imp. F - imp. G (see Figure 6 and SI-6). The obtained results of the measured batches (cf. SI-5) are in a comparable order of magnitude as those of the other methods (cf. Table 1 and Table 2).

4. Conclusions

The analysis of the related substances of acarbose is challenging. Since the analytes have only a weak chromophore, CAD detection is an alternative to the currently used low wavelength UV detection. The current Ph. Eur. method for related substances is not optimal, since the stationary APS-phase degrades during the analysis, which can be nicely monitored by the CAD resulting in a high background current. Thus, an easy method transfer to the CAD including the exchange of phosphate by an volatile buffer is practically impossible. Furthermore, since the introduction of the acarbose monograph, a lot of new columns with interesting column chemistry have appeared on the market, from which the acarbose test can profit. Hence, it was possible to develop and validate two alternative CAD methods for the determination of the related substances.

The PFP method is a tool for rapid determination of the sum of impurities of acarbose although not all components are separated. However, it was possible to detect further unknown impurities of an API, which could be partly identified as maltose or maltotriose.

Both, the Hypercarb and the Amide HILIC method are suitable for the replacement of the current Ph. Eur. method. However, the for the Hypercarb method, peak identification must be confirmed.

CRediT authorship contribution statement

Adrian Leistner: Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Visualization **Ulrike Holzgrabe:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

We would like to thank Thermo Fisher Scientific (Germering, Germany) for supplying the Vanquish™ instrumentation and the Thermo Fisher columns. Thanks are due to the Hanns Seidel Foundation and the German Federal Ministry of Education and Research (BMBF) for financial support.

Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.115063.

References

[1] European Pharmacopoeia, 10.0 ed., Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2020.

[2] D. Steinhilber, M. Schubert-Zsilavecz, H.J. Roth, Medizinische Chemie, 2 ed., Deutscher Apotheker Verlag Stuttgart, Stuttgart, 2010.

[3] A.E. Martin, P.A. Montgomery, Acarbose: An α-glucosidase inhibitor, AJHP, 53 (1996) 2277-2290.

[4] Bayer Inc., Product monograph Glucobay, (accessed: 06 April 2022) https://www.bayer.com/sites/default/files/2020-11/glucobay-pm-en.pdf, 2014.

[5] U.F. Wehmeier, W. Piepersberg, Biotechnology and molecular biology of the alpha-glucosidase inhibitor acarbose, Appl. Microbiol. Biotechnol., 63 (2004) 613-625.

[6] K. Schilling, U. Holzgrabe, Recent applications of the Charged Aerosol Detector for liquid chromatography in drug quality control, J. Chromatogr. A, 1619 (2020) 460911.

[7] M. Azam Sadat, A. Mohammadi, N. Adib, A. Naeemy, Development and Validation of a Stability-Indicating HPLC Method for the Determination of Acarbose in Pharmaceutical Dosage Forms, J. Anal. Chem., 73 (2018) 910-916.

[8] United States Pharmacopeia, USP Monographs, Acarbose, USP-NF. Rockville, MD: United States Pharmacopeia, 2022.

[9] United States Pharmacopeia, USP Monographs, Acarbose Tablets, USP-NF. Rockville, MD: United States Pharmacopeia, 2022.

[10] S. Cherkaoui, Y. Daali, P. Christen, J.L. Veuthey, Development and validation of liquid chromatography and capillary electrophoresis methods for acarbose determination in pharmaceutical tablets, J. Pharm. Biomed, 18 (1998) 729-735.

[11] Y. Daali, S. Cherkaoui, X. Cahours, E. Varesio, J.L. Veuthey, High performance liquid chromatography of acarbose and its metabolite on porous graphitic carbon column, J. Sep. Sci., 25 (2002) 280-284.

[12] P. Novak, M. Cindrić, P. Tepes, S. Dragojević, M. Ilijas, K. Mihaljević, Identification of impurities in acarbose by using an integrated liquid chromatography-nuclear magnetic resonance and liquid chromatography-mass spectrometry approach, J. Sep. Sci., 28 (2005) 1442-1447.

[13] J. Pitsch, J. Weghuber, Hydrophilic Interaction Chromatography Coupled with Charged Aerosol Detection for Simultaneous Quantitation of Carbohydrates, Polyols and Ions in Food and Beverages, Molecules, 24 (2019).

[14] Y.-J. Wang, Y.-G. Zheng, Y.-P. Xue, Y.-S. Wang, Y.-C. Shen, Analysis and Determination of Anti-diabetes Drug Acarbose and its Structural Analogs, Curr. Pharm. Anal., 7 (2011) 12-20.

[15] A.W. Squibb, M.R. Taylor, B.L. Parnas, G. Williams, R. Girdler, P. Waghorn, A.G. Wright, F.S. Pullen, Application of parallel gradient high performance liquid chromatography with ultra-violet, evaporative light scattering and electrospray mass spectrometric detection for the quantitative quality control of the compound file to support pharmaceutical discovery, J. Chromatogr. A, 1189 (2008) 101-108.

[16] T. Muellner, Acworth, I., Gamache, P., Getting the most out of your charged aerosol detector-Factors influencing charged aerosol detector performance, (accessed: 06 April 2022) https://assets.thermofisher.com/TFS-Assets/CMD/brochures/tg-73914-hplc-cad-factors-influencing-performance-tg73914-en.pdf, 2021.

[17] EDQM, Information leaflet: Ph. Eur. Reference Standard Acarbose for peak identification CRS batch 4, (accessed: 24 May 2022) https://crs.edqm.eu/db/4DCGI/db/4DCGI/leaflet?leaflet=Y0000427_4, 2016.

[18] B. Porsch, J. Kratka, Chromatographic stability of silica-based aminopropyl-bonded stationary phases, J. Chromatogr. A, 543 (1991) 1-7.

[19] B. Porsch, High-performance liquid chromatography of sugar mixtures containing xylose and arabinose on primary amino-bonded phases, J. Chromatogr. A, 253 (1982) 49-54.

[20] J.R. Benson, D.J. Woo, Polymeric Columns for Liquid Chromatography, J. Chromatogr. Sci., 22 (1984) 386-399.

[21] Shodex, Shodex prospect: Polymer-based Hydrophilic Interaction Chromatography (HILIC) Columns (HILICpak), (accessed: 24 May 2022) https://jimdo-storage.global.ssl.fastly.net/file/95a2cefe-205b-456c-8202-17040b73b836/Shodex_HILIC_HILICpak_Columns.pdf.

[22] Technical Note: „Acarbose Analysis by COSMOSIL Sugar-D“, NACALAI TESQUE, INC., (accessed: 07 April 2022) https://www.nacalai.co.jp/global/download/pdf/Acarbose_Application.pdf.

[23] Phenomenex, Brochure: explore Luna PFP(2), (accessed: 06 April 2022) http://phx.phenomenex.com/lib/5732_I.pdf.

[24] K. Koizumi, High-performance liquid chromatographic separation of carbohydrates on graphitized carbon columns, J. Chromatogr. A, 720 (1996) 119-126.

[25] O. Csernák, B. Rácz, Á. Alberti, S. Béni, Quantitative analysis of 3'- and 6'-sialyllactose in human milk samples by HPLC-MS/MS: A validated method for the comparison of two consecutive lactation periods in the same woman, J. Pharm. Biomed., 184 (2020) 113184.

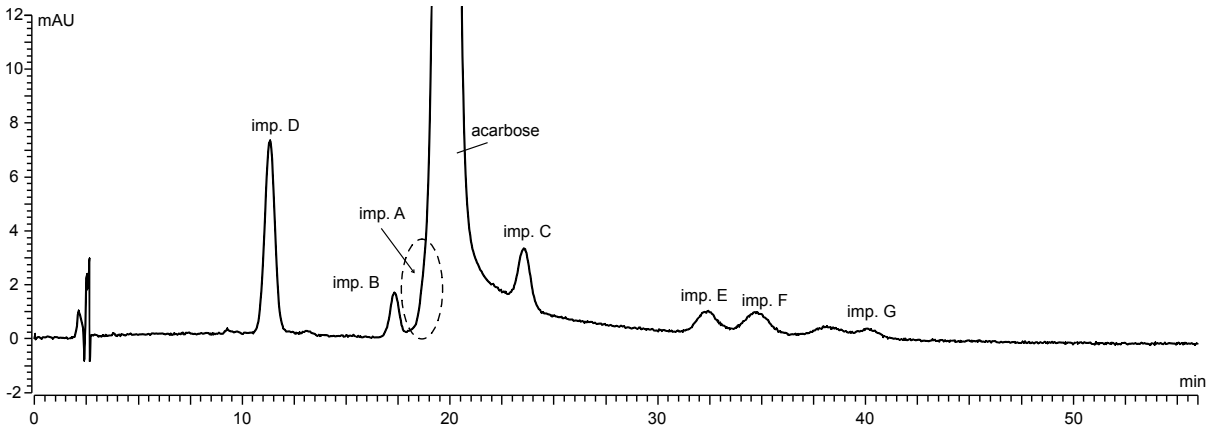
[26] X. Ji, G. Hu, Q. Zhang, F. Wang, C. Liu, Simultaneous analysis of heparosan oligosaccharides by isocratic liquid chromatography with charged aerosol detection/mass spectrometry, Carbohydr. Polym., 152 (2016) 337-342.

[27] L. Pereira, Porous graphitic carbon as a stationary phase in HPLC: theory and applications, J. Liq. Chromatogr. Relat. Technol., 31 (2008) 1687-1731.

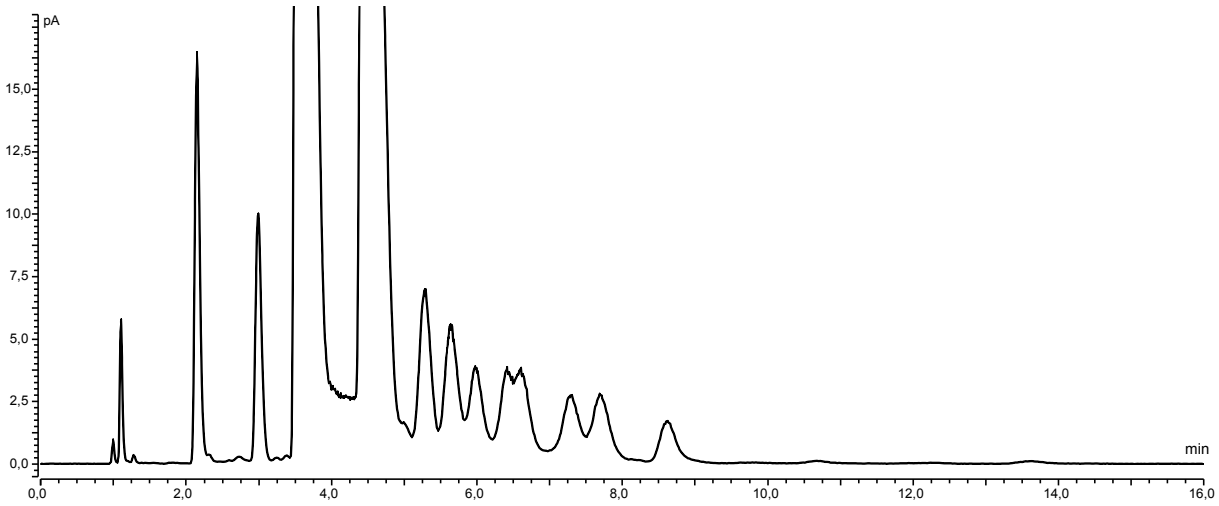
[28] T.E. Bapiro, F.M. Richards, D.I. Jodrell, Understanding the Complexity of Porous Graphitic Carbon (PGC) Chromatography: Modulation of Mobile-Stationary Phase Interactions Overcomes Loss of Retention and Reduces Variability, Anal. Chem., 88 (2016) 6190-6194

[29] ICH harmonised triparite Guideline, Validation of analytical procedures: text and methodology Q2 (R1), International conference on harmonization, Geneva, Switzerland, 2005.

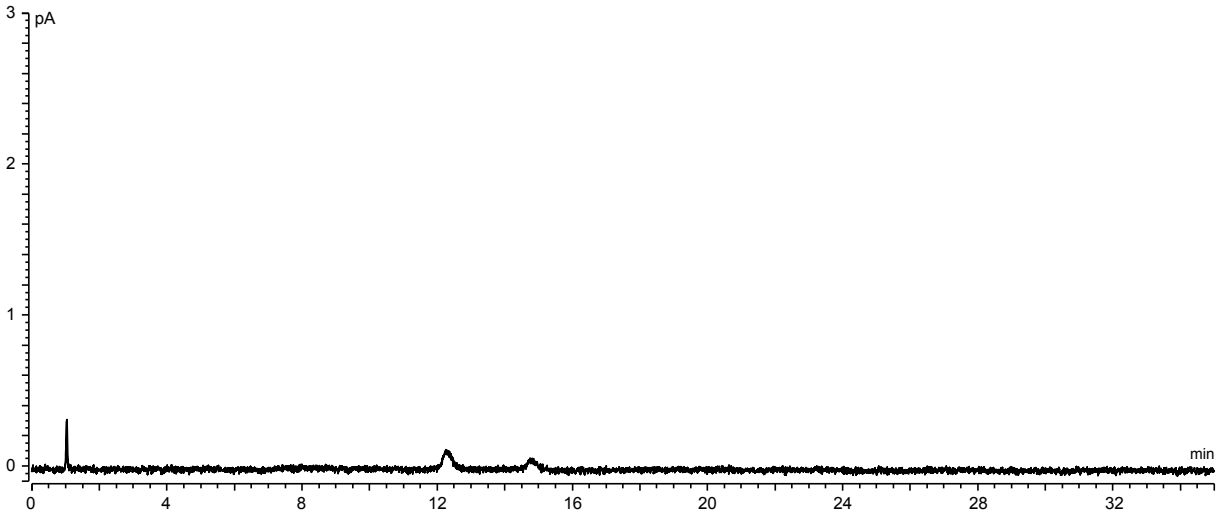
Supplementary Information



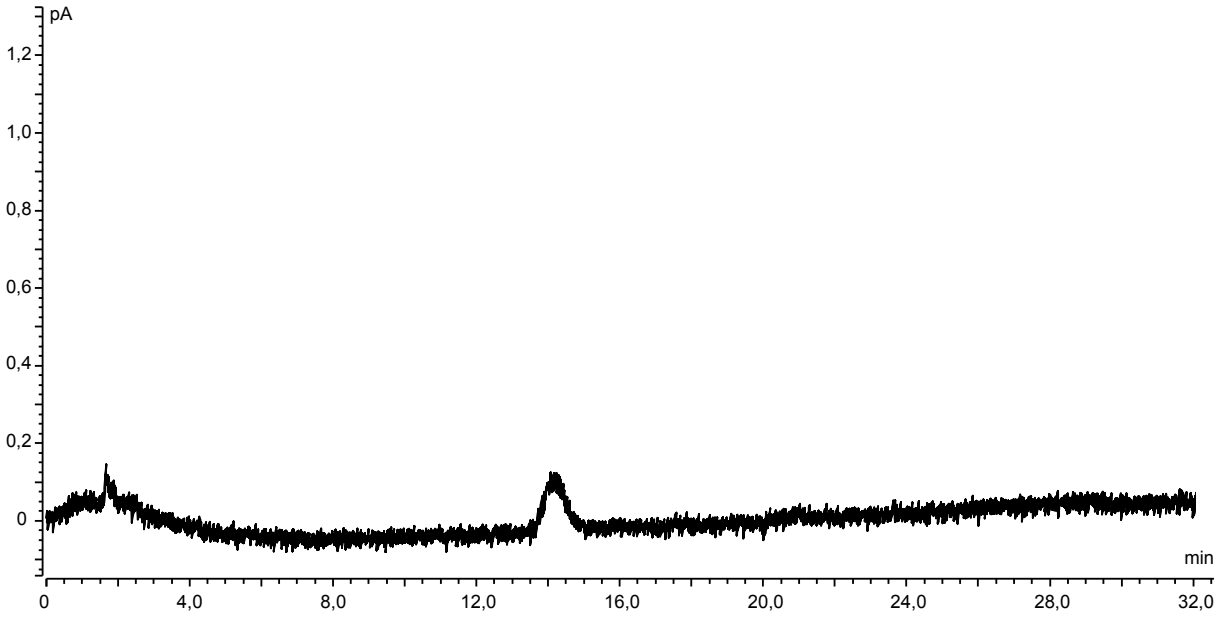
SI - 1: Impurity analysis of reference solution (b) by isocratic elution of 10 mM ammonium acetate (2 mL/min) and UV detection at 210 nm.



SI - 2: Impurity analysis of acarbose reference solution (b) using Accucore™ PFP column (100 x 4.6 mm i.d., 2.6 µm particle size) with isocratic elution of aqueous solution of 0.1% TFA and CAD detection (10 Hz, filter 1.0 s)



SI - 3: Sensitivity analysis of acarbose sensitivity solution (0.05%) using Accucore™ PFP column (100 x 4.6 mm i.d., 2.6 µm particle size) with isocratic elution of aqueous solution of 100 mM ammonium formate and CAD detection (10 Hz, filter 1.0 s)



SI - 4: Sensitivity analysis of acarbose sensitivity solution (0.05%) using Hypercarb™ column (150 x 4.6 mm i.d., 3µm particle size) with gradient elution (mobile phase A: 0.1% TFA in water; mobile phase B: 0.1% TFA in acetonitrile) and CAD detection (10 Hz, filter 1.0 s). Gradient: 0-2 min 8% mobile phase B; 2-26 min 8-13% mobile phase B; 26-31 min 13% mobile phase B; 31-33 min 13-8% mobile phase B.

SI - 5: batch results of three different acarbose API and the results of the injected system suitability solution by means of the Hypercarb method.

Imp. limits	Acarbose CRS 3	Acarbose CRS 4	Acros API Acarbose
Imp. A? ≤ 0.6%	0.18	0.17	0.57
Imp. B? ≤ 0.5%	0.11	0.11	b.r.t.
Imp. C? ≤ 1.5%	1.18	1.17	0.36
Imp. D ≤ 1.0%	0.38	0.39	0.25
Imp. E? ≤ 0.2%	b.r.t.	b.r.t.	b.r.t.
Imp. F? ≤ 0.3%	b.r.t.	b.r.t.	0.42
Imp. G? ≤ 0.3%	0.10	0.10	b.r.t.
Unknown imp.			several b.r.t.
Sum of imp.	1.95	1.94	1.60

* b.r.t. = below reporting threshold (0.1%)

SI - 6: results of the injected system suitability solution by means of the Ph. Eur., Amid-HILIC, and Hypercarb method.

Imp.	Ph. Eur.	Amid-HILIC	Hypercarb
Imp. A?	1.25	1.24	1.24
Imp. B?	0.56	0.78	0.50
Imp. C?	1.4	1.40	1.42
Imp. D	3.02	3.93	3.12
Imp. E?	1.34	1.36	1.49
Imp. F?	1.51	1.19	1.73
Imp. G?	0.72	1.85	2.10
Unknown imp.	0.41	0.92	0.21
Sum of imp.	10.21	12.67	11.81

4. Final Discussion

All projects of this theses aimed to develop tools for an improved impurity profiling of drugs and consequently lead to purer APIs.

In general, it should be pointed out, that the sources of impurities in APIs and medicinal products can be manifold. They can arise, for example, during the synthesis of the APIs through side reactions of the reactants, reagents, catalysts or solvent residues used. In addition, they can also be caused by impurities in the materials used and their reactions with other ingredients [1-6]. For drug products it is even more difficult to establish the whole impurity profile: To the aforementioned API related impurities, those, which are due to side reactions with the excipients or their impurities, must be also considered [7-9].

Even if the impurity profile of the established active ingredients is well-known, it still happens that primarily unexpected impurities occur in the APIs. One example for this is the presence of nitrosamines in sartans, which has been extensively reported in recent years [10-13].

4.1. Risk assessment report

To prevent the occurrence of impurities, which are not completely unexpected due to the synthesis conditions, we have developed an exemplary tool that significantly reduces the occurrence of unexpected impurities, when it was used within synthesis stage. Therefore, we selected cetirizine as model compound to demonstrate the advantages of this tool [14]. From the risk analysis presented in this thesis for the compound cetirizine, it is obvious how many potential impurities can occur during synthesis. In parallel, this tool makes the impurity profiling easier: Targeted analysis can now be performed. So fewer compounds are likely to be overlooked that are actually present. Therefore, such risk analyses should be performed for each drug synthesis and their correctness subsequently verified. With this knowledge of potential impurity profiles, the process control of the synthesis can be adjusted, if necessary, to produce active ingredients that are as pure as possible.

However, a certain residual risk of overlooking impurities still remains. Therefore, a non-targeted approach, as exemplarily described by *Wohlfart et al.*, should be performed in addition [15]. Nonetheless, the basic results of the risk assessment report can be used as a template for other APIs in future.

4.2. New pharmacopoeial methods

For the API dapsone, in the Ph. Eur. only an outdated TLC method (LOQ 0.2%) for purity testing existed [16]. Therefore, a modern LC-UV method was developed for the impurity analysis of dapsone. This new method allows the sensitive (LOQ 0.05%) determination of the impurities and hence was implemented to Ph. Eur. 10.6 [17, 18]. As dapsone is not only used against leprosy, but also used as a supporting agent against malaria, it is often used in countries with less well-equipped control laboratories. Therefore, in addition to the new Ph. Eur. method, a more simplified, isocratic HPLC method was developed. As described in this work, DoE approaches showed that the separation in an acceptable amount of time of all compounds is impossible and thus the sample must be analyzed in two isocratic runs performed with solvents of different elution strength. Both, the new Ph. Eur. method and the simplified ones yield batch results of the same order of magnitude. In addition, the new, stricter pharmacopoeial limits for impurities guarantee that the purest possible API are placed on the market.

Pharmacopoeial methods, on the one hand, can be replaced by newer methods, as exemplified for dapsone. On the other hand, a harmonization process of the major pharmacopoeias (Ph. Eur., USP and JP) is also taking place [19]. Thus, in meetings of Pharmacopoeia Commission it is also discussed, if new methods for the replacement of outdated methodologies are significantly better than those of the other pharmacopoeia. This was the case, for example, for the LC-UV method for baclofen, which was presented here [20]. Although the latter is faster than the USP method, within pharmacopoeial harmonization, the USP method was chosen to be the new Ph. Eur. method for the related substances test of baclofen.

In addition, we used the universal charged aerosol detector to check whether impurities without or with only a weak chromophore are present in baclofen batches. However, beside the counterion of impurity C, no additional impurity could be monitored with the CAD. This might be attributed to the fact that the batches measured were very pure, which of course has a positive effect on drug quality.

4.3. CAD in impurity profiling

Acarbose was chosen as project API, as this compound has only a weak chromophore and some of its impurities might probably not be detected with the pharmacopoeial tests. Therefore, alternative methods for the impurity profiling of acarbose with the CAD as universal detector were developed [21]. However, this was demanding: On the one hand, the separation of known impurities was not easy. On the other hand, also their detection – due to a weak chromophore - was challenging. Since the analytes differed only in the sugar chain, the stationary phase used needs special selectivity [22]. The stationary phase currently used in the pharmacopoeia has the problem that it is not stable and degrades itself over time [23, 24]. This can be observed by means of the CAD, more precisely by an elevated background current. The best results for adequate separation and detection by CAD of acarbose and its impurities could be obtained on an amide-HILIC phase and on a PGC phase. In case of the latter stationary phase, quite harsh conditions with column oven temperatures of 90 °C were necessary to obtain good separation.

Overall, the acarbose study also demonstrated that the APIs contained small amounts of constituents without a chromophore, two of which were identified as the sugar fragments maltose and maltotriose.

Taken together, the impurity profile stated in the pharmacopoeial transparency lists is mostly not conclusive. In order to further improve the quality of monographed APIs, one approach should be to look more closely at the synthesis of the compound in question. Risk analyses can be a suitable tool to get a first impression of the impurity profile to be expected. Furthermore, outdated pharmacopoeial methods should be rapidly replaced by more modern and more sensitive ones. At the same time, the permitted impurity limits reviewed in the revision are to be set as low as possible, based on available batch data, in order to make it mandatory for pharmaceutical manufacturers to produce APIs that are as pure as possible. In these improvement processes, modern stationary phases and modern universal detectors should also be taken into account.

However, one should not think exclusively of high-tech laboratories in the whole development. Methods for appropriate quality analysis should also be developed for rather less well-equipped control laboratories. In this way, the quality of the hopefully very purely produced APIs can also be tested everywhere in the world with a suitable method.

4.4. References

[1] ICH Harmonised Tripartite Guideline, Impurities in new drug substances Q3A (R2), Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland, 2006.

[2] ICH Harmonised Tripartite Guideline, Assessment and Control of DNA reactive (mutagenic) Impurities in Pharmaceuticals to limit potential carcinogenic risk M7(R1), International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, 2017.

[3] K. Zhang, J.D. Pellett, A.S. Narang, Y.J. Wang, Y.T. Zhang, Reactive impurities in large and small molecule pharmaceutical excipients–A review, Trends Anal. Chem., 101 (2018) 34-42.

[4] European Medicines Agency, ICH guideline Q3D (R2) on elemental impurities, European Medicines Agency, Amsterdam, 2022.

[5] European Medicines Agency, ICH guideline Q3C (R8) on impurities: guideline for residual solvents, European Medicines Agency, Amsterdam, 2021

[6] S. Ahuja, Assuring quality of drugs by monitoring impurities, Adv. Drug Deliv. Rev., 59(1) (2007) 3-11.

[7] European Medicines Agency, ICH Q3B (R2) Impurities in new drug products, EMEA, London, 2006.

[8] H. Buschmann, U. Holzgrabe, D. Jung, „Unerwartet “verunreinigt, Deutsche Apotheker Zeitung, (2019) 159-20.

[9] R. Holm, D.P. Elder, Analytical advances in pharmaceutical impurity profiling, Eur. J. Pharm. Sci., 87 (2016) 118-135.

[10] N.A. Charoo, A.A. Ali, S.K. Buha, Z. Rahman, Lesson Learnt from Recall of Valsartan and Other Angiotensin II Receptor Blocker Drugs Containing NDMA and NDEA Impurities, AAPS PharmSciTech, 20(5) (2019) 166.

[11] H. Buschmann, U. Holzgrabe, NDMA in Valsartan, DAZ 2018, Nr. 29, S.22, https://www.deutsche-apotheker-zeitung.de/daz-az/2018/daz-29-2018/ndma-in-valsartan, 2018, (accessed 10.08.2022).

[12] European Medicines Agency, Sartan medicines: companies to review manufacturing processes to avoid presence of nitrosamine impurities, EMA/248364/2019 Rev 1, 2019.

[13] T. Nguyen, A side reaction may have led to impurities found in valsartan heart drugs. https://cen.acs.org/pharmaceuticals/process-chemistry/side-reaction-led-impurities-found/97/web/2019/02, 2019 (accessed 25.05.2020).

[14] A. Leistner, S. Haerling, J.-D. Kreher, I. Becker, D. Jung, U. Holzgrabe, Risk assessment report of potential impurities in cetirizine dihydrochloride, J. Pharm. Biomed. Anal., 189 (2020) 113425.

[15] J. Wohlfart, E. Jäckel, O. Scherf-Clavel, D. Jung, M. Kinzig, F. Sörgel, U. Holzgrabe, Impurity profiling of bisoprolol fumarate by liquid chromatography-high-resolution mass spectrometry: A combination of targeted and untargeted approaches using a synthesis reaction matrix and general unknown comparative screening, Journal of Chromatography Open, 1 (2021) 100012.

[16] European Pharmacopoeia 9th ed., dapsone monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2017.

[17] European Pharmacopoeia 10.6, dapsone monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[18] A. Leistner, U. Holzgrabe, Impurity profiling of Dapsone using gradient HPLC method, J. Pharm. Biomed. Anal., (2021) 113982.

[19] European Pharmacopoeia 10.8, 5.8. Pharmacopoeial Harmonisation monograph, Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2022.

[20] A. Leistner, U. Holzgrabe, Impurity Profiling of Baclofen Using Gradient HPLC–UV Method, Chromatographia, 84(10) (2021) 927-935.

[21] A. Leistner, U. Holzgrabe, Alternative methods to assess the impurity profile of a monographed API using acarbose as an example, J. Pharm. Biomed. Anal., 221 (2022) 115063.

[22] European Pharmacopoeia, 10.0 ed., Council of Europe: European Department for the Quality of Medicines, Strasbourg, France, 2020.

[23] B. Porsch, High-performance liquid chromatography of sugar mixtures containing xylose and arabinose on primary amino-bonded phases, J. Chromatogr. A, 253 (1982) 49-54.

[24] B. Porsch, J. Kratka, Chromatographic stability of silica-based aminopropyl-bonded stationary phases, J. Chromatogr. A, 543 (1991) 1-7.

5. Summary

All presented studies aimed on the improvement of the quality analysis of already monographed drugs. Thereby different LC methods were applied and coupled to i.e., the UV/VIS detector, the CAD or a hyphenation of these detectors, respectively. The choice of the chromatographic system including the detector was largely dependent on the physicochemical properties of the respective analytes.

With the risk-assessment report on the API cetirizine we presented an exemplary tool, that can help to minimize the risk of the occurrence of unexpected impurities. An in-deep analysis of each step within synthesis pathway by means of reaction matrices of all compounds was performed. It is essential to understand the complete impurity profile of all reactants, solvents, and catalysts and to include them in the matrix. Finally, the API of this synthesis was checked if all impurities are identified by this tool. Of note, a shortcoming of such a targeted approach is that impurities can still occur, but they are not captured. This disadvantage can be partially compensated by non-targeted approaches if they are performed in parallel with the other studies that represent most of the impurities. However, this work also shows that even in a supposedly simple synthesis, potentially hundreds of by-products can be formed. For each of them, it must be decided individually whether their formation is probable or how their quantity can be minimized in order to obtain APIs, that are as pure as possible.

In the dapsone project it was aimed to replace the existing old Ph. Eur. TLC method with a modern RP-HPLC method. This was successful and since Ph. Eur. 10.6, the method developed in this work, became a valid monograph. Within the revision process of the monograph, the individual limits for impurities were tightened. However, this new method needs HPLC instrumentation, suitable to perform gradients. As this is not always available in all control laboratories, we also developed an alternative, more simple method using two different isocratic runs for the impurity analysis. The obtained batch results of both, the new pharmacopoeial method and the more simple one, were in a comparable order of magnitude. Furthermore, within the method development stage of the Ph. Eur. method, we could identify one unknown impurity of the impurity reference by high-resolution MS/MS analysis.

Also, in the baclofen project it was aimed to replace the existing Ph. Eur. method with the introduction of an additional impurity to be quantified. A corresponding method was developed and validated. However, due to the harmonization process of the pharmacopoeias, it is currently not used. In addition, we tried to find further, non-

chromophoric impurities by means of the CAD. However, except for one counterion of an impurity, no further impurities were found. Also, the aforementioned new impurity could not be detected above the reporting threshold in the batches analyzed. As the only individually specified impurity A is also present at a low level, it can be concluded that the examined batches of baclofen are very pure.

The use of universal detectors, such as the CAD can be particularly interesting for compounds with no chromophore or those with only a weak chromophore. Therefore, we decided to take a closer look at the impurity profile of acarbose. Currently, acarbose and its impurities are being studied by low wavelength UV detection at 210 nm. Therefore, the question arose whether there are no other impurities in the API that do not show absorption at this wavelength. CAD, which offers consistent detection properties for all non-volatile compounds, is ideally suited for this purpose. However, it was not so easy to use the CAD together with the UV detector, for example, as a hyphenated detection technique, because the Ph. Eur. method uses phosphate buffers. However, this is non-volatile and therefore inappropriate for the CAD. Therefore, an attempt was made to replace the buffer with a volatile one. However, since this did not lead to satisfactory results and rather the self-degradation process of the stationary phase used could be observed by means of the CAD, it was decided to switch to alternative stationary phases. A column screening also revealed further difficulties with acarbose and its impurities: they show an epimerization reaction at the end of the sugar chain. However, since one wanted to have uniform peaks in the corresponding chromatograms, one had to accelerate this reaction significantly to obtain only one peak for each component. This was best achieved by using two stationary phases: PGC and Amide-HILIC. Impurity-profiling methods could be developed on each of the two phases. In addition, as expected, new impurities could be detected, albeit at a low level. Two of them could even be identified by spiking experiments as the sugar fragments maltose and maltotriose.

Taken together, it can be concluded, that this work has contributed significantly to the improvement of the quality analysis of monographed drugs. In addition to the presented general tool for the identification of potential impurities, one of the methods developed, had already been implemented to the Ph. Eur. In an effort to improve the CAD's universal detection capabilities, additional methods have also been developed. Further, new improved methods for the impurity profiling are ready to use.

6. Zusammenfassung

Alle vorgestellten Arbeiten zielten auf die Verbesserung der Qualitätsanalyse von im Europäischen Arzneibuch monographierten Wirkstoffen ab. Dabei wurden verschiedene flüssig-chromatographische Methoden angewandt und z.B. mit dem UV/VIS-Detektor, dem CAD oder einer Kombination dieser Detektoren gekoppelt. Die Wahl des chromatographischen Systems einschließlich des Detektors war weitgehend von den physikochemischen Eigenschaften der jeweiligen Analyten abhängig.

Mit dem Bericht zur Risikoanalyse des Wirkstoffs Cetirizin haben wir ein beispielhaftes Instrument vorgestellt, das helfen kann, das Risiko des Auftretens unerwarteter Verunreinigungen zu minimieren. Es wurde eine eingehende Analyse der einzelnen Syntheseschritte anhand der Reaktionsmatrizen aller Verbindungen durchgeführt. Dabei ist wichtig, das vollständige Verunreinigungsprofil aller Reaktanten, Lösungsmittel und Katalysatoren zu verstehen und in die Matrix mit aufzunehmen. Schließlich wurde produzierte Arzneistoff-Chargen überprüft, ob alle Verunreinigungen mit diesem Werkzeug identifiziert werden konnten. Es soll aber nicht unerwähnt bleiben, dass ein Nachteil eines solch gezielten Ansatzes darin besteht, dass Verunreinigungen im Wirkstoff dennoch vorkommen können, sie aber nicht durch das Tool erfasst werden. Dieser Nachteil kann z.B. durch nicht-zielgerichtete Ansätze kompensiert werden, wenn sie parallel zu den anderen Untersuchungen durchgeführt werden, die bereits den Großteil der Verunreinigungen abbilden können. Diese Arbeit zeigt aber auch, dass selbst bei einer vermeintlich einfachen Synthese potenziell Hunderte von Nebenprodukten gebildet werden können. Für jedes von ihnen muss individuell entschieden werden, ob ihre Bildung wahrscheinlich ist und wie ihre Menge im Endprodukt minimiert werden kann, um möglichst reine Wirkstoffe zu erhalten.

Im Dapsonprojekt wurde versucht, die bestehende dünnschichtchromatographische Ph. Eur.-Methode durch eine moderne HPLC-Methode zu ersetzen. Dies war erfolgreich und seit Ph. Eur. 10.6 wurde die in dieser Arbeit entwickelte Methode zu einer gültigen Monographie. Im Rahmen der Überarbeitung der Monographie sind auch die einzelnen Grenzwerte für Verunreinigungen verschärft worden. Die neue Methode erfordert jedoch ein HPLC-Gerät, mit dem man eine Gradientenelution durchführen kann. Da dies aber nicht immer in allen Kontrolllabors verfügbar ist, haben wir ein alternatives, einfacheres Prozedere entwickelt, bei dem zwei verschiedene isokratische Methoden für die Verunreinigungsanalyse verwendet werden. Die Batch-Ergebnisse der neuen und der einfacheren Methode lagen dabei in einer

vergleichbaren Größenordnung wie die Ph. Eur.-Methode. Darüber hinaus konnten wir eine unbekannte Verunreinigung der Verunreinigungsreferenz durch hochauflösende Massenspektrometrie identifizieren.

Im Rahmen des Baclofen-Projekts sollte die bestehende Ph. Eur.-Methode ersetzt werden und die Monographie durch eine zusätzlich zu quantifizierende Verunreinigung ergänzt werden. Eine entsprechende Methode wurde entwickelt und validiert. Aufgrund des Harmonisierungsprozesses der Pharmakopöen wird sie jedoch derzeit nicht verwendet. Darüber hinaus haben wir versucht, mit Hilfe des CAD weitere, nicht-chromophore Verunreinigungen zu finden. Bis auf ein Gegenion einer Verunreinigung wurden jedoch keine weiteren Verunreinigungen gefunden. Auch die oben erwähnte neue Verunreinigung konnte in den untersuchten Chargen nicht oberhalb des Berichtsgrenzwert nachgewiesen werden. Da auch die sonst einzig individuell zu spezifizierende Verunreinigung A nur in geringem Maße vorhanden ist, kann der Schluss gezogen werden, dass die untersuchten Baclofen-Chargen sehr rein sind.

Der Einsatz von universellen Detektoren wie dem CAD kann besonders für Verbindungen ohne Chromophor oder solche mit nur schwachem Chromophor interessant sein. Daher wurde das Verunreinigungsprofil von Acarbose genauer untersucht. Derzeit werden Acarbose und ihre Verunreinigungen mit Hilfe der UV-Detektion bei 210 nm untersucht. Daher stellte sich die Frage, ob es nicht noch anderen Verunreinigungen im Wirkstoff gibt, die keine Absorption bei dieser Wellenlänge zeigen. Die Detektion mittels CAD, der für alle nichtflüchtigen Verbindungen gleichbleibende Werte liefert, ist für diesen Zweck gut geeignet. Allerdings war es nicht so einfach, den CAD zusammen mit dem UV-Detektor z. B. als gekoppeltes Detektionsverfahren zu verwenden, da die Ph. Eur.-Methode Phosphatpuffer verwendet. Dieser ist jedoch nicht flüchtig und daher für CAD ungeeignet. Es wurde daher versucht, den Puffer durch einen flüchtigen Puffer zu ersetzen. Da dies jedoch nicht zu befriedigenden Ergebnissen führte und vielmehr die Selbstzersetzung der verwendeten stationären Phase mit Hilfe der CAD beobachtet werden konnte, wurde auf alternative stationäre Phasen ausgewichen. Eine Auswahl von verschiedenen Säulen zeigte zudem weitere analytische Schwierigkeiten mit Acarbose und ihren Verunreinigungen: Sie zeigen die Epimerisierungsreaktion am Ende der Zuckeralkohole. Da man für jede Komponente einen Peak in den entsprechenden Chromatogrammen haben wollte, wurde die beschriebene Reaktion durch Temperaturerhöhung beschleunigt. Dies wurde am besten durch die

Verwendung mit folgenden stationären Phasen erreicht: PGC und Amid-HILIC. Auf jeder der beiden Phasen konnten Methoden zur Erfassung der Verunreinigungsprofile entwickelt werden. Darüber hinaus konnten erwartungsgemäß neue Verunreinigungen nachgewiesen werden, wenn auch auf niedrigem Niveau. Zwei von ihnen konnten durch „Spiking“-Experimente als die Zuckerfragmente Maltose und Maltotriose identifiziert werden.

Zusammenfassend lässt sich sagen, dass diese Arbeit einen wesentlichen Beitrag zur Verbesserung der Qualitätsanalyse von monographierten Arzneimitteln geleistet hat. Zusätzlich zu dem vorgestellten allgemeinen Werkzeug zur Identifizierung potenzieller Verunreinigungen wurde eine der entwickelten Methoden bereits in die Ph. Eur. aufgenommen. In dem Versuch, die universellen Detektionsmöglichkeiten des CAD zu verbessern, wurden auch ergänzende Methoden entwickelt. Außerdem sind neue verbesserte Methoden für die Analyse von Verunreinigungsprofilen einsatzbereit.

7. APPENDIX

7.1. List of Publications

Research papers

- Leistner, A.; Haerling, S.; Kreher, J.-D.; Becker, I.; Jung, D.; Holzgrabe, U. *Risk assessment report of potential impurities in cetirizine dihydrochloride*, J. Pharm. Biomed. Anal., **2020**, 189, 113425.
- Leistner, A.; Holzgrabe, U. *Impurity profiling of Dapsone using gradient HPLC method*, J. Pharm. Biomed. Anal., **2021**, 198, 113982.
- Leistner, A.; Holzgrabe, U. *Impurity Profiling of Baclofen Using Gradient HPLC–UV Method*, Chromatographia, **2021**, 84.10, 927-935.
- Pawellek, R.; Krmar, J.; Leistner, A.; Djajić, N.; Otašević, B.; Protić, A.; Holzgrabe, U. *Charged aerosol detector response modeling for fatty acids based on experimental settings and molecular features: a machine learning approach*, J. Cheminform., **2021**, 13.1, 1-14.
- Leistner, A.; Holzgrabe, U. *Alternative methods to assess the impurity profile of a monographed API using acarbose as an example*, J. Pharm. Biomed. Anal., **2022**, 221, 115063.

Other publications

- Pawellek, R.; Leistner, A.; Holzgrabe, U. *Impurity analysis of L-aspartic acid and glycine by HPLC-UV-CAD*,
  URL: https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/can-73761-lc-uv-cad-impurity-l-aspartic-acid-glycine-can73761-en.pdf
- Pawellek, R.; Leistner, A.; Holzgrabe, *Impurity analysis of gabapentin by HPLC-UV-CAD,*
  URL: https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/can-74011-hplc-uv-cad-impurity-analysis-gabapentin-can74011-en.pdf

- Leistner, A.; Holzgrabe, U. *Topiramate impurity analysis: Method migration from a legacy HPLC system to modern instrumentation,*
  URL: https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/can-000572-hplc-vanquish-charged-aerosol-detector-topiramate-can000572-na-en.pdf

- Leistner, A.; Pawellek, R.; Holzgrabe, U. *Evaluating LC methods for enhanced charged aerosol detector response: a case study using underivatized amino acids,*
  URL: https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/can-000721-hplc-vanquish-flex-cad-bcaa-impurities-can000721-na-en.pdf

7.2. Conference Contributions

- Leistner, A.; Pawellek, R.; Holzgrabe, U. *Fine-tuning/ Optimization of CAD performance for pharmaceutical analysis*, DPhG Annual Meeting 2021, Virtual Meeting.

7.3. Documentation of authorship

In this section, the individual contribution for each author to the publications reprinted in this thesis is specified.

Erklärung zur Autorenschaft

Risk assessment report of potential impurities in cetirizine dihydrochloride

Adrian Leistner, Stephan Haerling, Joerg-Detlef Kreher, Ivonne Becker, Dirk Jung, Ulrike Holzgrabe

Journal of Pharmaceutical and Biomedical Analysis 189 (2020) 113425

Adrian Leistner (AL), Stephan Haerling (SH), Joerg-Detlef Kreher (JDK), Ivonne Becker (IB), Dirk Jung (DJ), Ulrike Holzgrabe (UH)

Autor	**AL**	**SH**	**JDK**	**IB**	**DJ**	**UH**						**∑ in Prozent**
Studiendesign	5	2.5			2.5	5						15
Experimentelle Arbeit			7.5	7.5								15
Datenanalyse und Interpretation	25				2.5	2.5						30
Verfassen der Veröffentlichung	20											20
Korrektur der Veröffentlichung					2.5	15						17.5
Koordination der Veröffentlichung						2.5						2.5
Summe	50	2.5	7.5	7.5	7.5	25						100

Erklärung zur Autorenschaft

Risk assessment report of potential impurities in cetirizine dihydrochloride

Adrian Leistner, Stephan Haerling, Joerg-Detlef Kreher, Ivonne Becker, Dirk Jung, Ulrike Holzgrabe

Journal of Pharmaceutical and Biomedical Analysis 189 (2020) 113425

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Ivonne Becker	Dirk Jung	Ulrike Holzgrabe
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Würzburg, 15.11.2022 _____

Prof. Dr. Ulrike Holzgrabe

Erklärung zur Autorenschaft

Impurity profiling of Dapsone using gradient HPLC method

Adrian Leistner, Ulrike Holzgrabe

Journal of Pharmaceutical and Biomedical Analysis 198 (2021) 113982

Adrian Leistner (AL), Ulrike Holzgrabe (UH)

Autor	AL	UH										∑ in Prozent
Studiendesign	5	5										10
Experimentelle Arbeit	30											30
Datenanalyse und Interpretation	7.5	7.5										15
Verfassen der Veröffentlichung	25											25
Korrektur der Veröffentlichung		15										15
Koordination der Veröffentlichung		5										5
Summe	67.5	32.5										100

Erklärung zur Autorenschaft

Impurity profiling of Dapsone using gradient HPLC method

Adrian Leistner, Ulrike Holzgrabe

Journal of Pharmaceutical and Biomedical Analysis 198 (2021) 113982

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Würzburg, 15.11.2022 _____

Prof. Dr. Ulrike Holzgrabe

Erklärung zur Autorenschaft

Impurity Profiling of Baclofen Using Gradient HPLC–UV Method

Adrian Leistner, Ulrike Holzgrabe

Chromatographia 84.10 (2021) 927-935

Adrian Leistner (AL), Ulrike Holzgrabe (UH)

Autor	AL	UH										∑ in Prozent
Studiendesign	5	5										10
Experimentelle Arbeit	30											30
Datenanalyse und Interpretation	7.5	7.5										15
Verfassen der Veröffentlichung	25											25
Korrektur der Veröffentlichung		15										15
Koordination der Veröffentlichung		5										5
Summe	67.5	32.5										100

Erklärung zur Autorenschaft

Impurity Profiling of Baclofen Using Gradient HPLC–UV Method

Adrian Leistner, Ulrike Holzgrabe

Chromatographia 84.10 (2021) 927-935

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Erklärung zur Autorenschaft

Alternative methods to assess the impurity profile of a monographed API using acarbose as an example

Adrian Leistner, Ulrike Holzgrabe

Journal of Pharmaceutical and Biomedical Analysis 221 (2022) 115063

Adrian Leistner (AL), Ulrike Holzgrabe (UH)

Autor	AL	UH										∑ in Prozent
Studiendesign	5	5										10
Experimentelle Arbeit	35											35
Datenanalyse und Interpretation	5	5										10
Verfassen der Veröffentlichung	25											25
Korrektur der Veröffentlichung		15										15
Koordination der Veröffentlichung		5										5
Summe	70	30										100

Erklärung zur Autorenschaft

Alternative methods to assess the impurity profile of a monographed API using acarbose as an example

Adrian Leistner, Ulrike Holzgrabe

Journal of Pharmaceutical and Biomedical Analysis 221 (2022) 115063

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