Development and Evaluation of a Generic HPLC-Tandem MS Screening Method for the Detection of Potential Biomarkers for Reactive Intermediates

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von
Karoline Simon, geb. Scholz
aus Bad Königshofen

Würzburg 2006

Eingereicht am:.....

bei der Fakultät für Chemie und Pharmazie

Gutachter:.....

Gutachter:.....

der Dissertation

Prüfer:.....

Prüfer:.....

Prüfer:.....

des Öffentlichen Promotionskolloquiums

Tag des Öffentlichen Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

To my dear family

TABLE OF CONTENTS

ABBREVIATIONS

$b_{0.5}$	full width at half of peak maximum
CAD	collision gas for collisional activated dissociation
CE	collision energy
CID	collision induced dissociation
CNL	constant neutral loss
cps	counts per second
CUR	curtain gas
CXP	collision cell exit potential
DNA	desoxyribonucleic acid
DP	declustering potential
ELISA	enzyme-linked immunosorbent assay
EP	entrance potential
EPI	enhanced product ion
ESI	electrospray ionization
GAS 1	nebulizer gas
GAS 2	turbo gas
GC-MS	gas chromatography coupled to mass spectrometer
GST	glutathionyl-S-transferase
HPLC-MS/MS	high performance liquid chromatography coupled to tandem mass spectrometer
HPLC-UV/Vis	high performance liquid chromatography coupled to ultraviolet-visible spectroscopy
IDA	information dependent association
IS	ion spray voltage
K_m	Michaelis-Menten-constant
LIT	linear ion trap

LOD	limit of detection
LOQ	limit of quantitation
MPO	myeloperoxidase
MRM	multiple reaction monitoring
MS	mass spectrometer
MS/MS	tandem mass spectrometer
NADPH	nicotinamide adenine dinucleotide phosphate
NSAID	non-steroidal anti-inflammatory drug
S_s	peak symmetry: $b_{0.05}/2A$ with $b_{0.05}$: full width at 1/20 of peak maximum and A: distance between the ascending curve at 1/20 of the peak maximum and the line vertical through the peak maximum
S/N	signal to noise
PrIS	precursor ion scan
PMA	phorbol-12-myristate-13-acetate
ROS	reactive oxygen species
SIM	single ion monitoring
SRM	single reaction monitoring
thMRM	theoretical multiple reaction monitoring
UDP-GA	uridine-5'-diphosphate-glucuronic acid
UGT	UDP-glucuronyltransferase

1 INTRODUCTION AND BACKGROUND

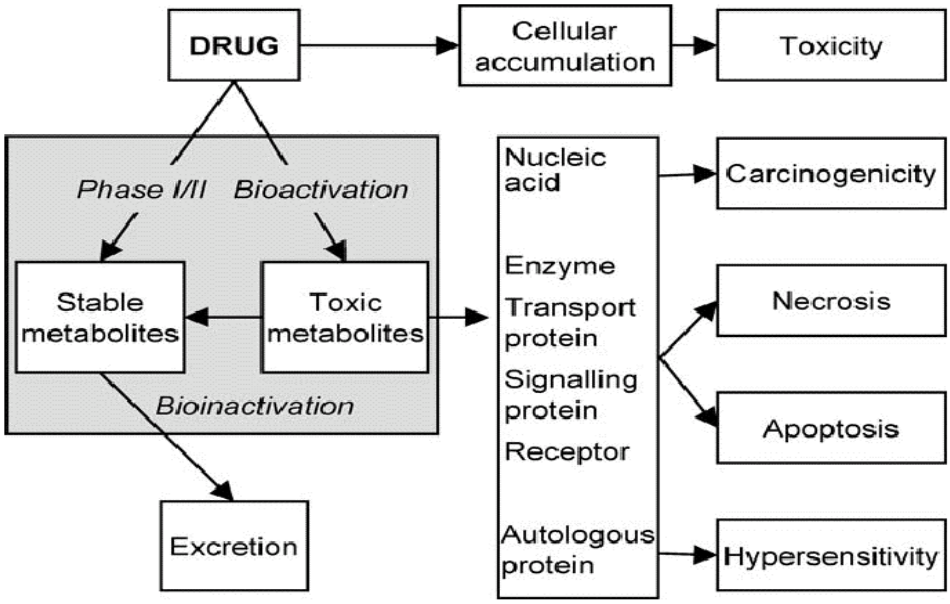
1.1 Significance of Reactive Intermediates

"All things are poison and nothing (is) without poison; only the dose makes that a thing is no poison." This statement by Paracelsus is often cited and still valid. However, although most toxic effects occur dose-dependently, some adverse toxic reactions cannot be related to dose. Most of the time, such adverse effects can be explained by the pharmacodynamic or pharmacokinetic characteristics of a compound. In the last decades, however, some compounds, particularly clinically used drugs and industrial chemicals, have been described to cause rare toxicities in a small group of individuals. This observation is termed idiosyncratic toxicity and is characterized by low incidence (< 1 : 5,000) with interindividual susceptibility, sometimes delayed occurrence and reactive metabolite formation without apparent correlation to pharmacokinetics or pharmacodynamics of a drug [1]. Interindividual susceptibility towards toxic effects of specific compounds may result from various factors including genetic predisposition, inflammation, oxidative stress, specific diseases or co-medication. Co-medication with a cytochrome P450 3A4 (CYP3A4) inducer, for instance, may increase the concentration of a reactive metabolite of the compound of interest that is formed by this isoenzyme. Alternatively, induction of oxidative stress may decrease the cellular concentrations of glutathione (GSH) that are required for trapping reactive intermediates. Reactive intermediates are not only related to idiosyncratic toxicity but are also described for causing dose-dependent hepatotoxicities and other damages (Scheme 1).

In 1994, over 100,000 cases of deaths had been associated with such adverse drug reactions in the U.S. [2]. These toxic side effects have often led to black box warning or withdrawal of drugs from the market, e.g. clozapine, bromfenac, or troglitazone [3]. From 1975 to 1999, 2.9% of drugs have been withdrawn from the market, from which 26.2% were associated to hepatic, 10.5% to hematological, and 6.3% to dermatological effects with severe consequences for patients‘ lifes [4, 5]. Since the development of a successful drug costs about 800 million dollars and takes about 12 to 15 years of investigations, such a withdrawal from the market means loss of time and resources during drug development and severe damage of patients lifes. To avoid such incidences, the industry is strongly interested in early detection of such

reactive intermediate forming drugs during drug discovery and development processes.

The common reason for toxicity of reactive intermediates and metabolites is their electrophilic character and high affinity towards tissue nucleophiles like sulfhydryl and amino groups. The electrophilic acitivity of a compound is characterized by the principle of hard and soft acids and bases. Hard electrophiles show great affinity towards hard nucleophiles, soft electrophiles covalently bind to soft nucleophiles [6]. Reactive intermediates include α-β-unsaturated aldehydes, iminoquinones, halogenated hydrocarbons, heterocyclic amines, and diolepoxides that may be generated by metabolic processes. Besides clinically used drugs, various other compounds are potential substrates for bioactivation. Environmental agents as polycyclic aromatic hydrocarbons and constituents of cigarette smoke such as acrylamide, 1,3-butadiene, ethylenoxide, and benzene are some examples of compounds that are known to generate reactive metabolites and can cause severe injuries [7-10]. Numerous industrial compounds such as bisphenol A, styrene, toluene, vinylchloride, trichlorethene, and perchlorethene are reactive by themselves or after biotransformation [11]. Additionally, dietary isothiocyanate derivatives in mustard or sulfurophane in broccoli, safrole in pepper, and also mycotoxins can be included in this group of potentially reactive compounds [12-14].



Scheme 1: Overview of metabolic activation of xenobiotics and possible toxic effects published by Park et al. [15].

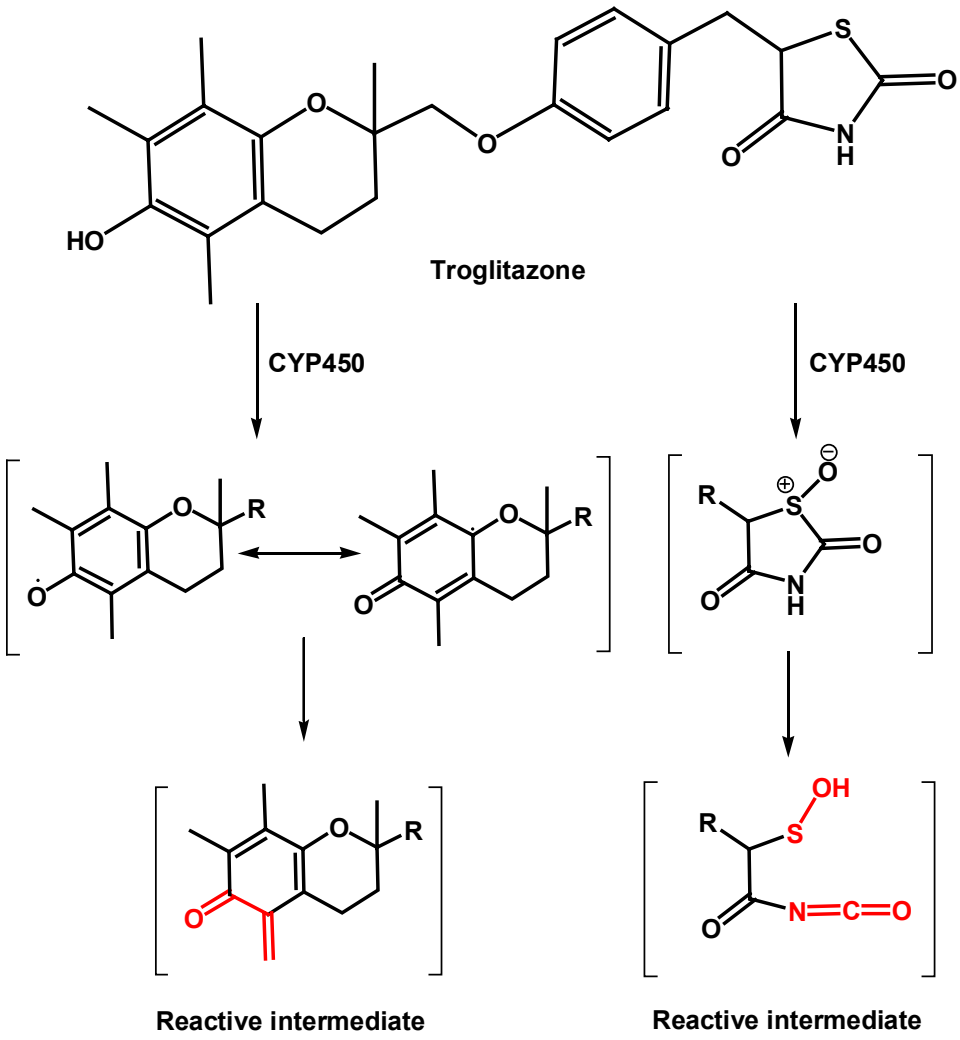
1.2 Formation of Reactive Intermediates

Adverse drug reactions are often attributed to covalent binding of electrophiles to cellular macromolecules leading to direct tissue injuries. Electrophiles are predominantly formed after biotransformation. In general, metabolism of lipophilic compounds consists of two biotransformation steps, i.e. phase I and phase II metabolism to functionalize the compound (increased water-solubility) and to eliminate the effective xenobiotic from the organism respectively. The metabolic conversion of lipophilic compounds into water-soluble derivatives is the main intention to excrete a compound. Metabolism mainly occurs in the liver and intestine and generally includes a) insertion of polar groups, predominantly by CYP450 isoenzymes which oxidize, reduce, hydrolyze, or decarboxylate the compound (phase I metabolism) and b) conjugation with endogenous substrates such as glutathione or activated glucuronic acid to detoxify or inactivate the compound (phase II metabolism). However, these processes may also lead to bioactivation of a compound, i.e. formation of electrophilic metabolites, as described for several drugs such as acetaminophen, troglitazone, and diclofenac.

The analgesic drug acetaminophen (see chemical structure in chapter 4.1.4.1.1) is oxidized to *N*-acetyl-p-benzoquinone imine (NAPQI) by various CYP450 isoenzymes,

including CYP2E1. NAPQI is highly reactive for conjugation with nucleophiles, e.g. proteins and other macromolecules. At clinically used doses, acetaminophen is mainly metabolized by glucuronidation or sulfation and NAPQI (< 5% of administered dose of acetaminophen) is rapidly detoxified by conjugation with glutathione (GSH) and excreted as the corresponding mercapturic acid in urine. However, at high doses (> 10 g in humans), sulfation is saturated and GSH is depleted by as much as 90% [16]. Thus, accompanied with a lack of detoxifying agents, NAPQI is formed in high concentrations and can covalently bind to liver proteins and other macromolecules leading to severe liver damage [16]. Primary cellular targets have been postulated to be mainly mitochondrial proteins, resulting in a loss of energy production and cellular ion control with an ongoing loss in calcium homeostase [17].

The insulin sensitizing PPARγ (peroxisome proliferator-activated receptor gamma) agonist troglitazone (see chemical structure in chapter 4.1.4.1.3) was withdrawn from the U.S. market in 2000 after several cases of hepatotoxicity (in 1.9% of patients) [18, 19]. Troglitazone is exemplary for idiosyncratic toxicity. The exact mechanism of troglitazone-mediated liver failure remains unknown, although formation of reactive metabolites is discussed as a contributing factor [20]. Various potentially reactive metabolites have been described and point to the thiazolidine ring and the chromane moiety as the sites of metabolic activation [21]. Troglitazone undergoes a) opening of the thiazolidine ring and *S*-oxidation which can be converted to an electrophilic α-keto isocyanate derivative at two different sites and b) one electron oxidation to form a phenoxy radical that is further metabolized to quinone methide that may also serve as electrophile [20] (Scheme 2). The latter cannot be formed by rosiglitazone or pioglitazone, two drugs which are structurally related to troglitazone but which are not associated with incidences of hepatotoxicity (for chemical structures see page 116). This issue may be due to the lack of the chromane moiety in these drugs, compared to troglitazone.

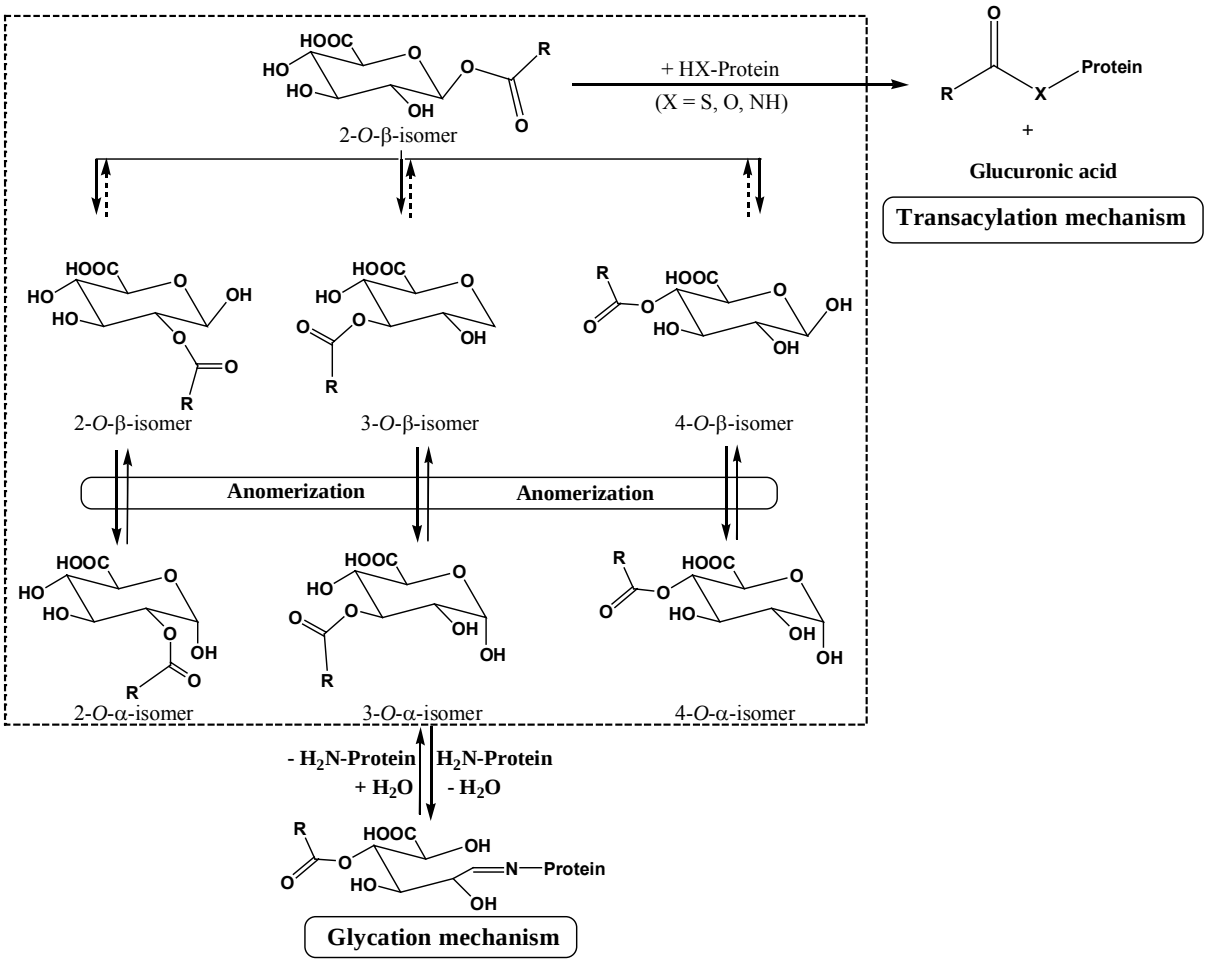


Scheme 2: Proposed scheme for the bioactivation of troglitazone upon CYP450-mediated oxidation of the chromane moiety and the thiazolidindione ring to reactive intermediates. The reactive substructures are marked in red.

The non-steoidal anti-inflammatory drug (NSAID) diclofenac (see chemical structure in chapter 4.1.4.1.2) causes severe liver damage in one to two cases per million prescriptions [22]. This hepatotoxic effect may be explained by the biotransformation pathways of diclofenac. Besides other metabolic processes, diclofenac is metabolized by CYP2C9 to 4´-hydroxydiclofenac and by CYP3A4 to 5-hydroxydiclofenac. Particularly, 5-hydroxydiclofenac is very unstable and may be oxidized to an iminoquinone. This intermediate is electrophilic and can be readily conjugated to macromolecules such as proteins. It is thought to be responsible for inhibition of CYP3A4 by binding to the active site of the isoenzyme immediately after formation [23]. 4´-Hydroxydiclofenac may also act as precursor compound for the generation of a protein-reactive iminoquinone, but is described as less susceptible to

oxidation and protein adduction than its regioisomer 5-hydroxydiclofenac [23]. However, these electrophiles seem to have little influence on the occurrence of diclofenac-mediated hepatotoxicity rather than the alternative biotransformation of diclofenac by direct glucuronidation of its carboxylic group to a protein-reactive acyl glucuronide. Carboxylic acids display an additional group for potential bioactivation by phase II metabolism. Carboxylic acids, as present in diclofenac or other non-steroidal anti-inflammatory drugs, can be conjugated with activated glucuronic acids (UDP-glucuronic acid) to form acyl glucuronides, catalyzed by the enzyme UDP-glucuronyltransferase (UGT). This membrane-bound enzyme class catalyzes the transfer of UDP-glucuronic acid to xenobiotics containing nucleophilic substructures such as alcohols, thiols, amines, hydroxylamines, and carboxylic acids. The main part of glucuronidation reactions are detoxifying, except for formation of acyl glucuronides. Compared to highly reactive electrophiles generated by phase I metabolism which mostly bind to macromolecules at the site of formation, acyl glucuronides can leave the site of origin and may cause damage in target tissues that are not directly related to the formation of such metabolites [24]. Thus, correlation of specific toxicity with generation of such reactive metabolites is difficult. Acyl glucuronides, however, are susceptible to nucleophilic substitution or hydrolysis. Glucuronidation of carboxylic acids initially provides the labile 1-O-β-acyl glucuronide that may, besides hydrolysis to the aglycone, undergo different reactions which are a) direct transacylation with nucleophilic heteroatoms of amino acids or b) acyl migration via an ortho-acid ester intermediate forming the relatively stable regioisomers 2-, 3-, and 4-O-β-glucuronide, and their anomerized α-isomers. The latter may be bound to proteins after ring fission via Schiff base formation (Scheme 3) [24], analogous to non-enzymatic glycation of albumin by glucose and other sugars [25].

Another possible activation pathway of carboxylic acids, as present in diclofenac, is the formation of Acyl-CoA thioesters as described for NSAIDs. These intermediates are even more reactive to proteins *in vitro* than the corresponding acyl glucuronides [26]. Thioesters show greater reactivity towards amino groups than carboxylic esters (stabilities: thioesters < esters < amides) [24]. Primary amino groups are the preferred sites for electrophilic substitution of acyl glucuronides. Acyl glucuronides are potentially reactive for conjugation to proteins and other macromolecules and should be considered in toxicity studies in drug development processes.



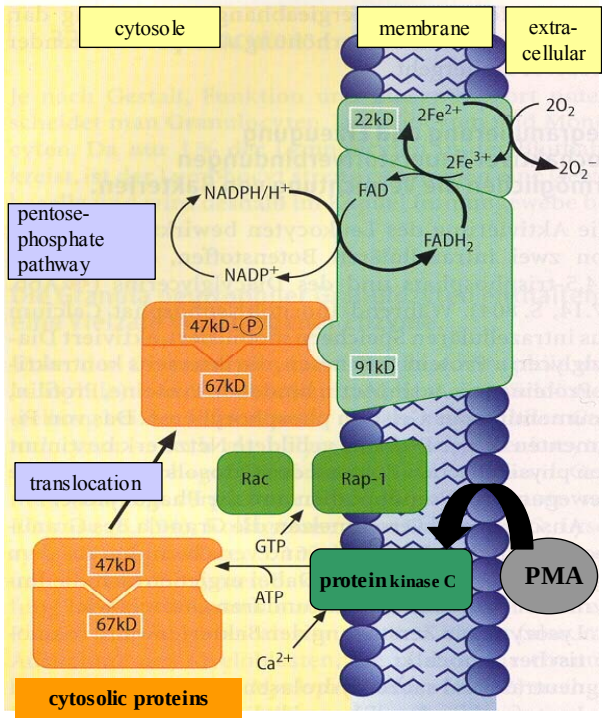
Scheme 3: Possible reaction mechanisms of acyl glucuronides including acyl migration to more stable regioisomers and anomerization (i.e. conversion from β-isomers to α-isomers and back) together with transacylation (with loss of glucuronide) and glycation mechanisms (glycation of the protein with the ring opened acyl glucuronide (modified from Bailey et al. [27]).

Reactive metabolites may also be formed by other phase II metabolism pathways. Bioactivation of xenobiotics may, for instance, occur by GSH conjugation. Some xenobiotics such as haloalkanes and -alkenes are susceptible to β-lyase, an enzyme present in the kidneys. Enzymatic degradation of GSH adducts yields cysteine conjugates, which are substrates for β-lyase [28]. β-Lyase cleaves the carbon sulfur bond leading to thiols and, sometimes, to protein-reactive thioketenes [29]. Dihaloalkanes may even generate highly reactive episulfonium ions after GSH conjugation and enzymatic degradation (Scheme 5). Sulfation of *N*-hydroxy arylamines may generate a reactive nitrenium ion which is genotoxic due to its affinity to DNA [30].

However, toxicological responses may be also immune-mediated. Such toxicities include drug hypersensitivities like agranulocytosis, skin rash, and epidermal necrolysis. Basically, two different hypothesis for immune-related toxicities are discussed, which are a) formation of reactive intermediates which bind to cellular macromolecules to form haptens and induce immune response and b) occurrence of cell damages or other “danger signals” that activate the immune system [31].

In general, the immune system consists of the unspecific cellular immune system with macrophages, granulocytes, and natural killer cells and the specific immune system, including primarily lymphocytes. A major function of the unspecific immune response is the phagocytation and destruction of the infectious agents and other foreign compounds that may be harmful to the organism. Neutrophils, for instance, are essential for cellular immune response and destroy such agents by release of aggressive chemicals. Such substrates, mostly of oxidative origin (reactive oxygen species (ROS)), are generated by the “respiratory burst” after stimulation. Neutrophils, generally for phagocytosis of bacterial and viral agents, possess primary and secondary granules with different functions and enzymes that catalyze formation of ROS. They can be stimulated by a variety of substances, such as lipopolysaccharide, a component present in bacterial cell walls or the tumor promoting agent phorbol-12-myristate-13-acetate (PMA). The initial step of the “respiratory burst” is the activation of the multicomponent enzyme NADPH oxidase. In resting neutrophils, the five main components of this enzyme are divided between the cytosol and the membranes of secretory vesicles and specific granules (secondary granules). The two membrane-bound components build a heterodimeric flavohemoprotein known as cytochrome b_{558}. Stimulation of neutrophils results in the formation of two intracellular messengers, inositol-1,4,5-triphosphate and diacylglycerine, and subsequently in mobilization of intracellular calcium from vesicles with activation of protein kinase C. This enzyme initiates phosphorylation of the cytosolic components. Upon phosphorylation, the entire cytosolic complex translocates to the membrane, where it joins with cytochrome b_{558} to form a fully functional oxidase (Scheme 4). The synthetic stimulant PMA, used in the present study for activation of neutrophils, directly activates protein kinase C. The active NADPH oxidase catalyzes the one electron reduction of oxygen to superoxide anion (O_2^-), using NADPH as electron donor. Subsequent reactions lead to formation of

H_2O_2, $OH^{\cdot}$, and eventually, catalyzed by myeloperoxidase (MPO) to hypochloric acid (HOCl) (Equations 1 – 4).



$$NADPH + 2\,O_2 \xrightarrow{\text{NADPH oxidase}} NADP^+ + H^+ + 2\,O_2^- \quad (1)$$

$$2\,O_2^- + 2\,H^+ \xrightarrow{\text{superoxiddismutase}} H_2O_2 + O_2 \quad (2)$$

$$O_2^- + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + O_2 \quad (3)$$

$$H_2O_2 + Cl^- \xrightarrow{\text{myeloperoxidase}} H_2O + OCl^- \quad (4)$$

Scheme 4: Mechanism of activation of NADPH oxidase with subsequent formation of ROS and target enzyme of the respiratory burst stimulant PMA, according to [32] (modified). Formation of reactive oxygen species during the respiratory burst are shown in equations 1 – 4. The catalyzing enzymes are included.

Such ROS are extremely reactive and may convert drugs and other xenobiotics to reactive intermediates. Although of minor importance for the general metabolism of a drug, such protein-reactive intermediates may also contribute to e.g. immune-mediated agranulocytosis. Agranulocytosis is defined as a decrease in number of circulating neutrophils to < 0.5 x 10^9/l blood (physiologically 4.5 x 10^9/l) and is often associated with sometimes antipsychotics or antiepileptic drugs, and NSAIDs.

The atypical neuroleptic drug clozapine, for instance, is described to occasionally cause agranulocytosis (0.8% in the first year of treatment) [33]. However, this drug is still in use for patients with treatment-resistent schizophrenia. Based on the severe side effects, patients require close monitoring for a contingent decrease in neutrophil number. The mechanism of toxicity has yet to be elucidated but may be due to toxic metabolites formed from clozapine. The reactive and potentially toxic metabolite of clozapine is the nitrenium ion, that can be generated by cytochrome P450 (CYP450) isoenzymes in liver (mainly by CYP1A2) and in neutrophils [34, 35].

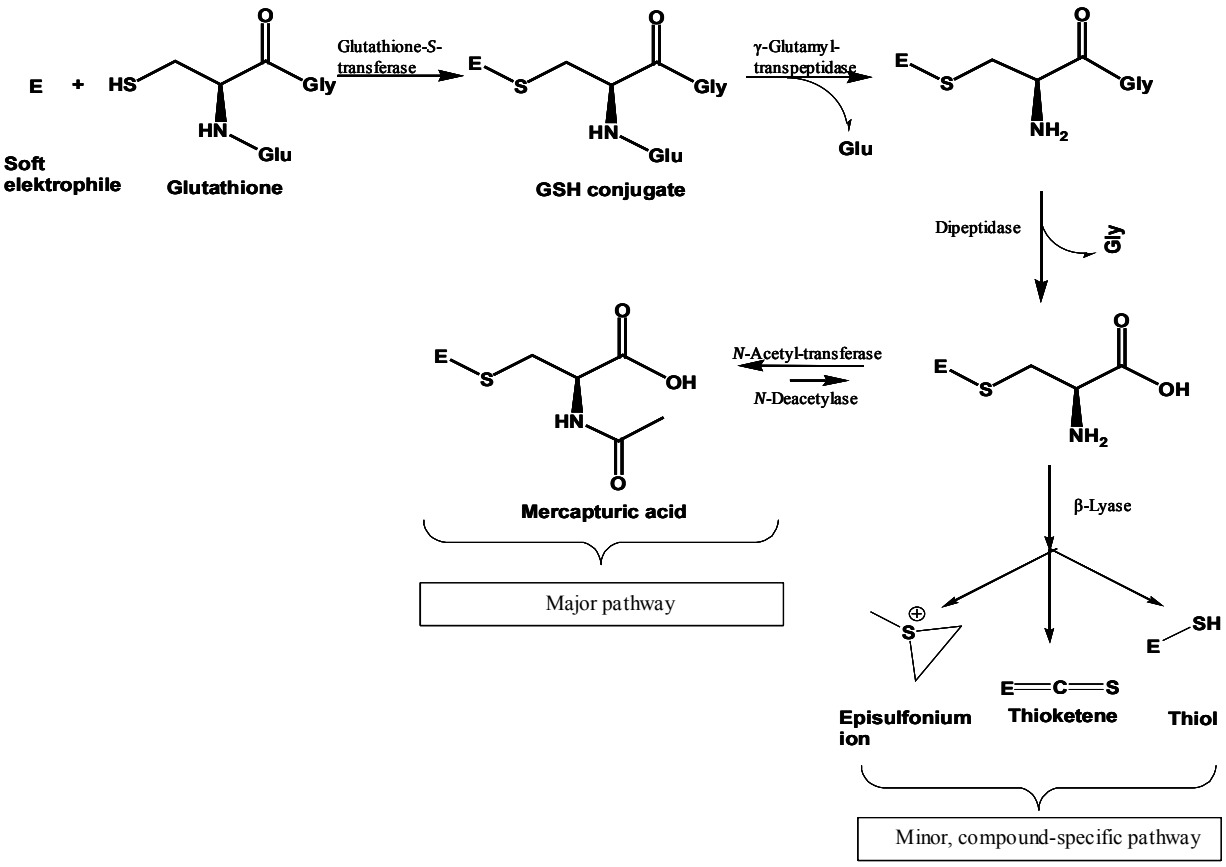
Carbamazepine is one of the most common drugs used against epilepsy. However, it has been associated with idosyncratic toxicities involving cutanous, hematological, immunological, renal, and hepatic disorders [36, 37]. These adverse reactions are thought to result from reactive metabolites formed by different CYP450 isoenzymes. One major metabolite of carbamazepine is 2-hydroxycarbamazepine which is further metabolized to an iminoquinone by CYP450-dependent oxidative mechanisms [38]. This reactive intermediate can undergo GSH conjugation or NADPH-dependent reduction to 2-hydroxyiminostilbene [38]. 2-Hydroxyiminostilbene, however, may be oxidized to an iminoquinone derivative again, e.g. by HOCl released by activated neutrophils [39].

The different adverse toxic effects caused by acetaminophen and troglitazone (liver damage) versus clozapine and carbamazepine (immune-mediated toxicity) may give the assumption for tissue-specific reactive intermediate formation and corresponding tissue-specific reactions. Due to the aggressive oxygen species present in this cell type, reactions may occur that differ from those in hepatocytes.

1.3 Detoxification

Conjugation of phase I-mediated reactive intermediates with the thiol group of the cysteine residue of GSH is one of the most important detoxifying reactions *in vivo* and protects macromolecules from electrophilic attack [40]. GSH-conjugates are formed either non-enzymatically or enzymatically by glutathione-*S*-transferases (GSTs). This enzyme group presents a multigene family of isoenzymes with broad specificity. Mammalian cells contain mainly cytosolic and some membrane-bound GSTs which are divided in six subfamilies, the α, μ, π, θ, ω, and ζ class [41]. Polymorphism are described for some isoenzymes, e.g. GST M1 (μ class) and GST

T1 (θ class) [42]. These isoenzymes are related to adverse reactions such as increased incidence of cigarette-mediated cancer for GST M1 depleted genotypes. GSTs catalyze or promote reactions of GSH with electrophiles. After conjugation, GSH adducts are mainly catabolized to cysteine-conjugates by γ-glutamyl-transpeptidase and cysteinyl-glycine dipeptidase that are present in liver and kidneys. After acetylation of the cysteine moiety via cysteine-*S*-conjugate *N*-acetyltransferase (not to be mixed with the polymorphic *N*-acetyltransferase), the corresponding mercapturic acids are formed which are predominantly excreted with urine [29] (Scheme 5). Some mercapturic acids are further oxidized to corresponding sulfoxides as recently shown for the mercapturic acid of fluoromethyl-2,2-difluoro-1-(trifluoromethyl)vinyl ether *in vitro* or for 15-A(2t)-isoprostane *in vivo* [43, 44]. Therefore, formation of sulfoxides should be taken into account when drug toxicity is determined *in vivo*.



Scheme 5: Possible metabolic pathways of electrophilic xenobiotics (E = soft electrophiles) to mercapturic acids and other, partially protein-reactive glutathione-mediated metabolites (thioketene, episulfonium ions).

1.4 Methods for the Generation and Detection of Reactive Intermediates

1.4.1 *In vitro* Methods

Reactive intermediates show high reactivity towards macromolecules under physiological conditions resulting in very short half-lives. Thus, detection of such electrophiles during drug development processes is necessary. The pharmaceutical industry uses several *in vitro* assays to generate and trap such reactive intermediates and obtain information about the chemical reactivity of a newly discovered compound.

Formation of reactive intermediates is generally performed in liver microsomal incubations in the presence or absence of NADPH to examine possible influence of reductive agents on the occurrence of electrophiles. The liver contains most of the metabolic active CYP450 isoenzymes. In hepatocytes, these enzymes are mostly bound to the smooth endoplasmatic reticulum, which is used to prepare microsomes for incubation studies on phase I metabolism. Since the occurence of reactive intermediates is often a direct result of phase I metabolism, microsomal incubation displays the best method for the generation of such electrophiles. Phase II substrates, which may capture electrophiles and prevent them from detection, are not present in microsomes. However, they may be added to a microsomal incubation solution as performed for generation of acyl glucuronides in this study.

To obtain reactive intermediates that potentially cause immune-mediated toxicities, investigators incubate compounds with activated neutrophils, myeloperoxidase/H_2O_2 systems, hypochloric acid or horseradish peroxidase [45-47]. These assays imitate the highly reactive milieu formed by reactive oxygen species of immune cells.

Covalent protein binding of reactive intermediates is often determined with radiolabeled compounds [48]. Binding of radiolabeled compounds to proteins may result in overestimation of the toxic potential of a compound due to detection of all protein adducts, although some may not cause toxic effects *in vivo*. In addition, synthesis of radiolabeled substances costs time and money.

The favoured trapping agent for reactive intermediates is GSH. Protein-reactive metabolites are mainly trapped with GSH, demonstrating the natural reaction pathway of most electrophiles [49]. Alternatively, the GSH degradation product *N*-acetyl-L-cysteine may be used for trapping reactive intermediates. Both substrates, *N*-acetyl-L-cysteine and GSH, use the sulfhydryl group of cysteine as nucleophile. Conjugation of reactive intermediates with *N*-acetyl-L-cysteine reflects the real chemical affinity towards electrophiles due to the lack of reactions catalyzed by GST. Moreover, since most mercapturic acids are excreted with urine, detection of mercapturic acids as biomarkers for reactive intermediate formation displays a non-invasive method for *in vivo* data acquisition.

Other electrophiles such as acyl glucuronides are classified as hard electrophiles and preferentially bind to *N*-containing nucleophiles like L-lysine or tyrosine to form stable amides [24]. L-Lysine adducts are described for several endogenous and exogenous substances as malondialdehyde [50], furan [51], acroleine [52], or diclofenac [53] *in vitro* and *in vivo*. L-Lysine is an essential amino acid of natural origin with a terminal primary amino group present in many enzymes and proteins and a possible target for covalent binding [54, 55]. Although glycation is discussed as the major reaction pathway of acyl glucuronides, the reactivity and susceptibility of 1-O-β-glucuronide towards proteins and other macromolecules should be considered in toxicity studies.

1.4.2 *In vivo* Methods

Detectable end products of reactive intermediates are DNA adducts, protein adducts, GSH adducts, or mercapturic acids. The availability of analyte containing matrix is therefore limited to blood, urine, or feces in living animals and tissue post mortem. DNA-adducts can be stable and detectable for a prolonged period of time, but are often repaired and removed by depurination. Although DNA adducts are excreted as nucleic acid adducts in urine, the high variability and background excretion may cause non-reproducible results. However, the main disadvantage of DNA and protein adducts is the invasive sampling of blood and the low amount of material available for analysis. Moreover, the distribution of some drugs and compounds in different tissues and body fluids requires great amounts of these matrices to obtain detectable amounts of analyte. GSH adducts are excreted biliary making collection complex.

Thus, collection of these body fluids or tissues is intricate and generally limited to animal studies. Mercapturic acids, however, display a valuable option to simply, rapidly, and non-invasively reconstruct the bioactivation of a compound in urine. Additionally, *in vitro* assays with *N*-acetyl-L-cysteine as trapping agent may provide ideas on the biotransformation pathway and possible generation of reactive metabolites. Moreover, mercapturic acids show an identical substructure that may be useful in the development of a method for the general detection of mercapturic acids to obtain also unknown mercapturic acids of a compound.

Phase II metabolites that are bioactivated to acyl glucuronides are either conjugated with macromolecules, or, are stabilized by acyl migration to form regioisomers that may also bind to macromolecules. Acyl glucuronides were mainly detected by usage of β-glucuronidase after stabilization and extraction from biological fluids or direct detection of protein adducts, partly degraded to peptides by various proteases [50, 56]. Alternatively, based on the affinity of acyl glucuronides to primary amines they were mostly detected as L-lysine adducts in urine [57-59]. Thus, development of a method for the detection of L-lysine adducts may be an useful option for the detection of such reactive phase II metabolites.

1.4.3 Analytical Methods

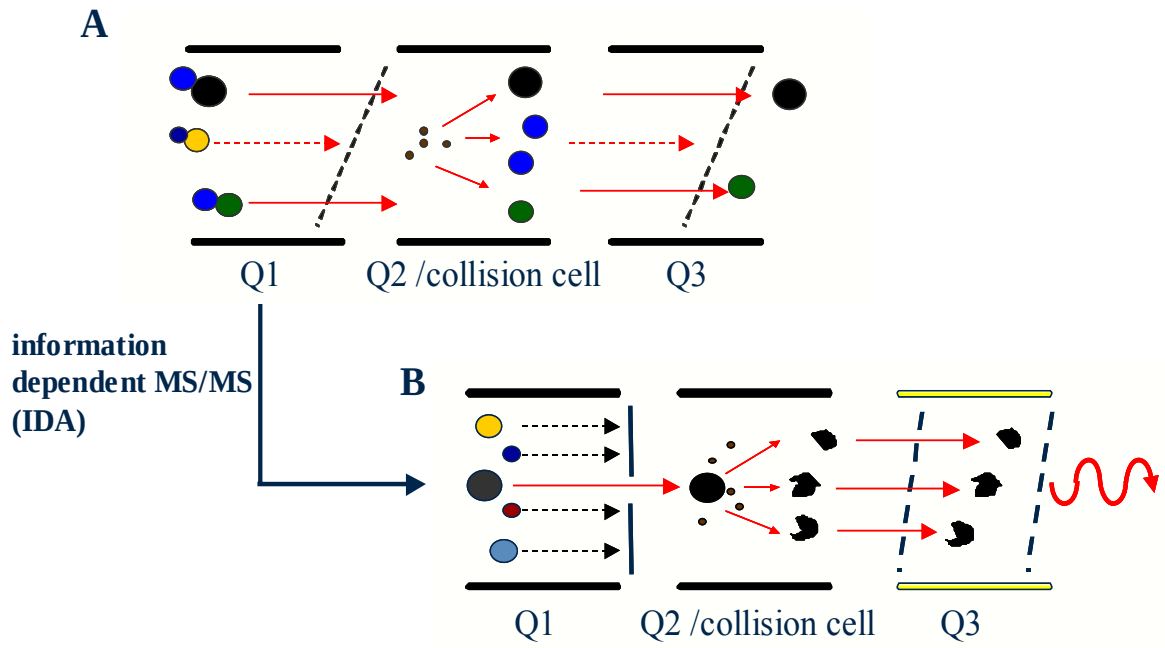
Detection and quantitation of unknown analytes is an important challenge to analytical methods, particularly when analytes are present in low concentrations in complex matrices such as biological fluids. Therefore, highly sensitive analytical methods have to be developed to screen blood or urine samples for xenobiotics and their metabolites.

Metabolites such as mercapturic acids and acyl glucuronides have been detected by various analytical instruments mainly based on HPLC coupled with fluorescence or UV detection instruments, and mass spectrometry (MS). In addition, mercapturic acids were detected in matrix after derivatization using GC/MS [50, 60]. Enzyme-linked immunosorbent assays (ELISA) for the detection of specific mercapturic acids have also been described previously, but with less accuracy compared to HPLC-tandem MS (MS/MS) methods [61, 62]. Over the recent years, HPLC-MS/MS has extensively been applied for the detection and quantitation of specific phase II

metabolites in urine [63, 64]. These hydrophilic analytes are well ionized by electrospray. In electrospray sources, ionization of the analytes occurs by high voltage. A nebulizer gas stabilizes the spray. After ionization, droplets with charged analytes embedded in HPLC solvent are formed. These droplets are evaporated by high in-source temperature and turbogas until coulomb explosion (rejection of identically charged ions within a droplet is greater than the surface tension of the droplet) occurs. The charged “quasi” molecular ions (precursor ions) are introduced into the mass spectrometer after they passed a curtain gas to preserve contaminants and solvent drops from entering the MS.

Modern mass spectrometers offer several scan techniques like constant neutral loss or precursor ion scan to generate screening strategies for improved specificity and sensitivity of a metabolite group in complex matrices. Generally, tandem mass spectrometers consist of three quadrupoles, i.e. Q1, Q2, and Q3. The quadrupoles Q1 and Q3 filter ions, whereas Q2 serves as collision cell for fragment ion formation. In Q2, the charged “quasi” molecular ion that was previously filtered by Q1, collides with gas molecules (i.e. nitrogen) and is dissociated to specific product ions. These are either scanned (product ion scan) or one or more specific fragment ions are filtered by Q3 (multiple reaction monitoring (MRM)). Novel hybrid instruments such as quadrupole linear ion trap MS (QTRAP), use Q3 as a linear ion trap (LIT) in which fragment ions can be collected for improved product ion mass spectra (enhanced product ion (EPI) scan). Q1 and Q3 with collision induced dissociation (CID) in the collision cell can also filter out and scan masses with a specific mass difference, i.e. a constant neutral loss (CNL) of the precursor ion that occurred in the collision cell. When CID is used and Q3 is set to filter a specific fragment ion, Q1 can scan for those precursor ions that are fragmented to the defined target product ion (precursor ion scan (PrIS). The new generation of mass spectrometers offers the possibility to combine specific survey scans, such as CNL scan or PrIS, with enhanced product ion scans to acquire a MS/MS mass spectrum of the desired signal within a single run. This is performed by the so called information dependent acquisition (IDA) feature (Scheme 6). In addition to the specificity of the survey scans, this mass spectrum, obtained with a linear ion trap, provides spectral data to characterize the structure of unknowns or to confirm known analytes.

The methods, developed so far for the detection of phase II metabolites, are mostly compound specific and do not permit screening for unknown metabolites [65-68]. Therefore, a general method for the detection of a specific group of metabolites would provide also signals of yet unknown derivatives. For such a procedure, a metabolite group with identical substructures has to be selected. This selection criterion may be a specific product ion such as *m/z* 113 in glucuronides [69] or a specific neutral loss such as 162 Da involving a glucose substructure as present in glycosides [70] or the neutral loss of 129 Da of glutathione metabolites in positive ion mode [71]. Mercapturic acids may also show an identical substructure that may be used for the development of a general HPLC-MS/MS method. Additionally, they can be rapidly obtained by simple urine collection and show sufficient stability. Acyl L-lysine adducts may also provide a metabolite group with common structural properties that can be used for the development of a generic HPLC-MS/MS method for the detection of reactive acyl glucuronides.



Scheme 6: Scheme of the scanning series of an IDA method, combining a constant neutral loss survey scan (A) with an enhanced product ion (EPI) scan, using a linear ion trap (B). Q1, Q2, and Q3 describe the quadrupoles for ion transport and filtering, wheras Q2 serves as collision cell (nitrogen as collision gas (●)) in both scan modes. In (B), Q3 is used as a linear ion trap to collect fragment ions generated by Q2.

1.5 Applicability of a HPLC-MS/MS Screening Method for the Detection of Mercapturic Acids and L-Lysine Conjugates in Drug Development

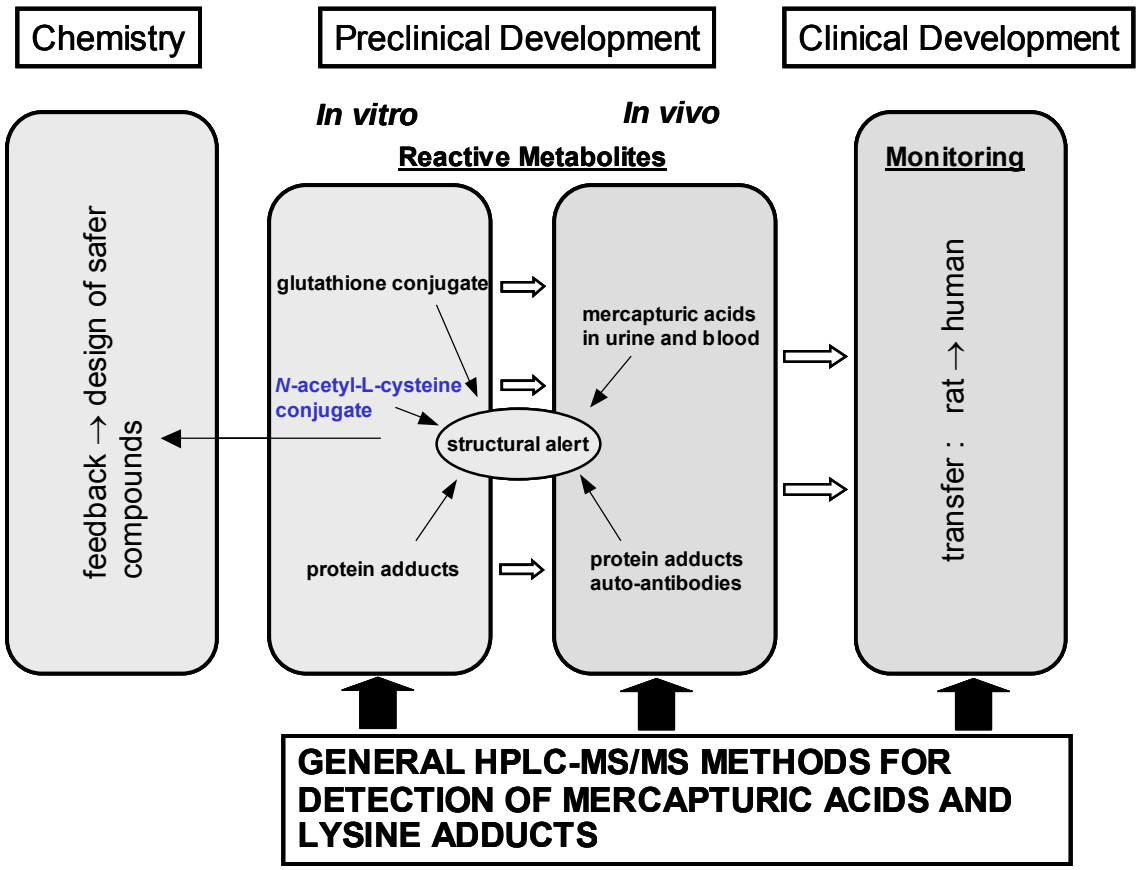
Prior to approval of a drug by regulatory authorities, a compound has to pass several tests on toxicity together with other pharmacodynamic and pharmacological studies (Scheme 7). If a compound shows toxic effects *in vitro* and, later on, *in vivo* during drug development processes, this drug candidate must be considered as not applicable to humans. Thus, already in drug development processes, risk and costs can be limited by early detection of compounds that show such reactivity. Also environmental and chemical compounds, such as vehicle emissions, acrylamide, or 1,3-butadiene partially show biotransformation to reactive metabolites *in vivo* and display a second group of potentially reactive xenobiotics [72-74].

Nucleophilic substitution of electrophiles with *N*-acetyl-L-cysteine or L-lysine may display a useful opportunity to trap reactive intermediates of a compound of interest. Despite simple generation and detection of such adducts *in vitro* under defined conditions and with high concentration, these metabolites have to be detected also in *in vivo* samples containing much less amounts of such metabolites.

GSH adducts provide information about reactivity and potential toxicity of a compound. However, *in vivo*, generation of mercapturic acids in urine and blood are determined. Trapping reactive intermediates with *N*-acetyl-L-cysteine already *in vitro* may display an opportunity to obtain metabolites of reactive intermediates that may be transferred directly to animal studies and, later on, to humans. Mercapturic acids are previously described as metabolites of reactive intermediate forming compounds. They can be directly correlated to exposure *in vivo* and are simple and rapid to obtain. In addition, standards for metabolite identification may be rapidly generated and simplify metabolite assignment in urine. Thus, mercapturic acids fulfill most of the claims on a biomarker for detection of reactive intermediates [75]. Several publications demonstrate the applicability of HPLC-MS/MS instruments to detect mercapturic acids as biomarkers for certain, potentially reactive compounds such as the mercapturic acids of lipid peroxidation products, contaminants in cigarette smoke, or of industrially used compounds [68, 76, 77]. A general method for the detection of mercapturic acids would provide the possible toxic potential of a compound without time consuming method development and optimization for specific metabolites. The

same method may be applied to *in vitro* and *in vivo* samples with high throughput. In addition, unknown mercapturic acids of reactive intermediates which cannot be predicted by the chemical structure of a compound may be obtained as well. If *in vitro* data suggested a structural alert of a compound, early testing of similar drugs with different substructures might be useful. Combining *in vitro* assays of different tissue and cell types may enhance the possibility of anticipating potentially serious adverse drug reactions. However, particularly the occurrence of idiosyncratic toxicities that are not observed in animal studies or in *in vitro* tests present a problem in drug development and risk assessment of a new compound. Interindividual susceptibility and the genetically diverse human patient populations will always limit the ability to predict such toxic effects. Thus, reactive intermediate forming compounds have to be generally avoided from the beginning of drug design and should be closely monitored during drug development.

L-Lysine conjugates are described to be formed *in vivo* and, thus, present an opportunity to detect nitrogen-affine electrophiles, such as acyl glucuronides, *in vitro* and *in vivo,* similar to mercapturic acids [57, 78]. However, the applicability of L-lysine adducts as biomarker for assessment of reactivity of a compound in drug development processes has yet to be elucidated and seems to be difficult due to the fact that L-lysine is part of many proteins and substrates.



Scheme 7: Overview of drug discovery and early development processes with possible application of the developed HPLC-MS/MS screening methods for the detection of mercapturic acids and L-lysine adducts.

2 OBJECTIVE

The aim of this work was the development and evaluation of a generic HPLC-tandem MS (MS/MS) screening method to detect potential biomarkers for reactive intermediates *in vitro* and *in vivo*. The method should fulfill specific conditions such as specifity, sensitivity, and robustness combined with simple and rapid sample preparation for the detection of the analytes of interest.

Mercapturic acids were foreseen as potentially promising biomarkers for reactive metabolites. Main focus of this work was to establish a generic method to measure mercapturic acids in different matrices by means of HPLC-MS/MS. Based on common adduct properties of the available standards in positive or negative ion mode, a generic analytical method for the detection of mercapturic acids should be developed and evaluated. All parameters involved in this data acquisition should be optimized to yield highest sensitivity and reproducibility. A number of parameters of the MS/MS instrument affect sensitivity and fragmentation spectrum of the precursor ion and were subject to optimization [79, 80]. Comparison of different scanning types, including CNL, PrIS, and MRM as specific survey scan modes would provide the most sensitive and specific method for the detection of such metabolites. Validation of the methods would show the applicability of the method for quantitation studies. A panel of model compounds with different chemical structures known to covalently bind to proteins and form GSH adducts should be tested *in vitro* to generate and trap reactive intermediates. Based on the toxicological relevance for the model compound, various incubation assays, i.e. rat and human liver microsomes and human neutrophils should be performed to cover different options for generation of reactive intermediates. As a general screening method, structural identification of the metabolites would be of minor importance rather than generation and optimization of detection of such signals. Application of this method to *in vivo* situation would confirm the applicability and sensitivity of the method in urine.

The principle of common adduct properties of one metabolite group should be expanded to reactive intermediates that conjugate with substrates different from GSH. L-Lysine as a nitrogen-containing trapping agent for protein-reactive metabolites such as acyl glucuronides should provide a tool for this investigation. Evaluation of the method *in vitro* and *in vivo* should demonstrate the applicability of this metabolite group for assessment of acyl glucuronide formation.

3 MATERIALS AND METHODS

3.1 Instrumentation

HPLC-tandem MS (MS/MS) experiments were performed with an HPLC system consisting of an Agilent quaternary solvent pump and an Agilent Autosampler Series 1100 (Agilent Technologies, Waldbronn, Germany) coupled to a QTRAP 2000 mass spectrometer (Applied Biosystems, Darmstadt, Germany) with a TURBO-Ionspray source. Alternatively, data were obtained on an API 3000 triple stage mass spectrometer with an electrospray ionization (ESI) source (Applied Biosystems, Darmstadt, Germany). Analyst software 1.4 and 1.4.1 were used for data aquisition. The centrifuges used were a Heraeus Megafuge 1.0R (Hanau, Germany) for cooled operation and an Eppendorf Centrifuge 5415C (Hamburg, Germany). A Speed Vac Plus SC110A from ThermoLife Sciences (Egelsbach, Germany) was used for evaporation of solvents. For aseptic preparation of neutrophils, a Gelaire®BH26 Laminar Airflow (Flow Laboratories, Meckenheim, Germany) was used. Cell counts were performed using a Coulter Z1 (Coulter Electronics, High Wycombe, UK). Purity of cell solutions was estimated using a BD LSR flow cytometer (BD Biosciences, Heidelberg, Germany).

3.2 Chemicals and Reagents

Unless otherwise indicated, all chemicals were purchased from Sigma/Aldrich (Taufkirchen, Germany) and were of the highest purity level available. Buffer salts for neutrophil isolation were obtained from Merck (Darmstadt, Germany). Supplements for culture-media were bought from Hyclone (Logan, Utah, USA). *N*-acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine, *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine, *N*-acetyl-*S*-(4-chlorobenzyl)-L-cysteine, *N*-acetyl-*S*-(2-fluorobenzyl)-L-cysteine, *N*-acetyl-*S*-(4-bromobenzyl)-L-cysteine, *N*-acetyl-*S*-(4-methoxybenzyl)-L-cysteine, *N*-acetyl-*S*-(4-*tert*-butylbenzyl)-L-cysteine, *N*-acetyl-*S*-(1,1-dichloro-2,2-difluoroethyl)-L-cysteine, and *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine were synthesized and characterized as described previously [81, 82]. *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine, *N*-acetyl-*S*-phenyl-L-cysteine, and *N*-acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine were obtained from Toronto Research Chemicals (North York, Canada). *N*-acetyl-*S*-[5-(acetylamino)-2-hydroxyphenyl]-L-cysteine (acetaminophen mercapturic acid, AAP-MA), *N*-acetyl-*S*-

(1,4-dihydroxynonan-3-yl)-L-cysteine (DHN-MA) [67], and *N*-acetyl-*S*-(5-hydroxy-2-pentyltetrahydrofuran-3-yl)-L-cysteine (HNE-MA) were kindly supplied by F. Hoffmann-La Roche (Basel, Switzerland). Rat and human liver microsomes and baculovirus expressed CYP450 isoenzymes were purchased from Gentest (NatuTec, Frankfurt/Main, Germany).

3.3 Syntheses

3.3.1 Syntheses of Sulfoxides of Mercapturic Acid Standards

Syntheses of sulfoxides of the mercapturic acid standards were performed according to procedures described by Werner et al. with some alterations [81]. Mercapturic acid standards (100 µg) were dissolved in glacial acetic acid (100 µl). Hydrogen peroxide (3%, 100 µl) was added to the stirring solution at 0°C and left stirring to react for one hour on ice and three hours at room temperature. At the end of the reaction, solutions were dried using a speedvac and the residues were reconstituted in CH_3OH/H_2O (1:2). Identity was confirmed by determination of the parent mass and assignment of the fragment ions detected in negative and positive ion mode.

3.3.2 Synthesis of Acetaminophen Mercapturic Acid

Chemical Synthesis of *N*-(3,4-Dihydroxyphenyl)-acetamide (3-OH-AAP). The derivative of acetaminophen was synthesized according to the method used by Hinson et al. [83]. 4-Nitrocatechol (500 mg, 3.2 mmol in 5 ml H_2O) was added dropwise to an alkaline sodium dithionite suspension (2.2 g, 12.8 mmol in 7.2 ml of 10% NaOH) under an atmosphere of nitrogen and left to reaction for 35 minutes. Acetylation of the primary amine was accomplished with an excess of acetic anhydride (1071 µl, 11.2 mmol). The suspension was acidified to pH 2 with concentrated HCl (36%) and the product was extracted four times with 10 ml ethylacetate. The organic phase was dried under nitrogen and the residue was cristallized with CH_3OH /CH_2Cl_2 (1:2) to yield pale white cristals. MS/MS analysis of the product obtained ions at *m/z* 166 in negative ion mode and at *m/z* 168 in positive

ion mode, respectively. Fragmentation pattern by HPLC-MS/MS confirmed the identity of the product.

Microsomal Synthesis of *N*-acetyl-*S*-[5-(acetylamino)-2,3-dihydroxyphenyl]-L-cysteine (3-OH-AAP-MA). The reaction medium for the formation of the mercapturic acid of 3-OH-AAP was identical to the microsomal incubation for the generation of mercapturic acids described in chapter 3.4.2.5. *N*-acetyl-L-cysteine was added to the incubation mix after five minutes of preincubation in a shaking water bath at 37°C. Incubation solution without addition of substrate served as control. Characterization of 3-OH-AAP-MA was performed by HPLC-MS/MS using a QTRAP 2000.

3.3.3 Syntheses of L-Lysine and Acetyl-L-lysine Adduct Standards

The commercially available acyl halides benzoyl chloride, 3,5-diethoxybenzoyl chloride, benzoyloxymethylbenzoyl chloride, and 3,4-dimethoxybenzoyl chloride were used for the generation of acyl lysine conjugates. L-Lysine (100 µmol was dissolved in 1 ml of 10mM NaOH). Acyl halide (100 µmol) was added directly to the lysine solution and stirred at room temperature over night. The same procedure was performed with N^{α}-acetyl-L-lysine and N^{ε}-acetyl-L-lysine. Samples were dried using a speedvac and pellets were dissolved in CH_3OH for analysis. Precursor ion and EPI mass spectra in both ion modes were recorded and analyzed to confirm the formation of the respective acyl L-lysine adducts.

3.4 Mercapturic Acids

3.4.1 Generation of HPLC-MS/MS Methods

HPLC Conditions for Method 1 and 2:

Precolumn: C18 ODS (4 x 3 mm, Phenomenex, Aschaffenburg, Germany)

Column: Reprosil Pur C18 AQ 5 µm (150 x 2 mm, Dr. Maisch, Ammerbuch, Germany)

Solvent A: 5 mM ammonium acetate pH 6.8

Solvent B: acetonitrile

Flow rate: 250 µl/min

Gradient: gradient from 5% B to 50% B within 16 minutes, constant at 50% B for 4 minutes, linear increase from 50% B to 100% B within 1 minute, hold at 100% B for 2 minutes, back to starting conditions within 1 minute, and re-equilibration for 6 minutes

HPLC Conditions for Methods 3 to 5:

Precolumn: Reprosil Pur C18 AQ 5 µm (10 x 2 mm, Dr. Maisch, Ammerbuch, Germany)

Column: Reprosil Pur C18 AQ 5 µm (150 x 2 mm, Dr. Maisch, Ammerbuch, Germany)

Solvent A: 0.1% of formic acid

Solvent B: acetonitrile

Flow rate: 250 µl/min

Gradient: linear gradient from 10% B to 90% B within 25 minutes, back to starting condition within 1 minute, and re-equilibration of the column for 10 minutes

3.4.1.1 Generation of the IDA CNL/negative EPI Method (method 1)

Mercapturic acids were detected with a constant neutral loss (CNL) survey scan (129 Da) after negative ionization with an ionspray voltage (IS) of -4,200 V and a source temperature of 400°C. Further characterization of the mercapturic acids detected was performed by a combination of this survey scan with one enhanced product ion (EPI) scan using Q3 as linear ion trap (LIT). Conversion of the scan modes was controlled by the information dependent acquisition (IDA) software of Analyst 1.4. After optimization of the method, instrument settings and compound-dependent parameters for the CNL 129 survey scan were as follows:

Table 1: Instrument settings and compound-dependent parameters for the detection of mercapturic acids with IDA CNL/negative EPI.

CUR [psi]	IS [V]	Temp	GAS 1 [psi]	GAS 2 [psi]	DP [V]	EP [V]	CE [V]	CXP [V]
30	-4,200	400	45	50	-50	-10	-20	-2

Q1 resolution was set unit, Q3 resolution was set low. No settling time was necessary for the adjustment of the compound-dependent parameters between the two experiments CNL 129 Da and EPI scan due to identical parameters such as declustering-, entrance-, collision cell exit potential (DP, EP, CXP), and collision energy (CE). Mass resolution (MR) pause between each mass scan was 5 ms and dwell time was fixed to three seconds for the scan of 251 masses in the suvey scan mode. Nitrogen was used as curtain gas (CUR), nebulizing gas (GAS 1), turbogas (GAS 2), and collision gas (CAD: medium for CNL survey scan and high for EPI scan). The IDA Software allowed a signal for EPI scan with dynamic LIT fill time, when signal intensity in the survey scan exceeded 3,000 cps. This target ion was then ignored for the following 40 seconds. Mass tolerance was set to 0.25 Da.

3.4.1.2 Generation of the MRM Method for Quantitation of Acetaminophen Mercapturic Acid (AAP-MA) in Rat Urine (method 2)

Data acquisition was performed in MRM mode by monitoring the mass transitions of *m/z* 311.1 to *m/z* 182.1 for AAP-MA and *m/z* 328.1 to *m/z* 199.1 for the internal standard *N*-acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine, respectively. A dwell time of 500 ms for each mass transition was used. Instrument settings were copied from method 1. A volume of 5 µl was injected into the HPLC-system.

3.4.1.3 Generation of the IDA CNL/negative EPI Method after Optimization (method 3)

Instrument and parameter settings (Table 1) were transferred from method 1. EPI scan mode with two different collision energies, controlled by IDA software, was applied only when a signal in the survey scan exceeded an intensity of 500 cps. After detection and fragmentation, target ions with the same mass were excluded for the

following 40 seconds. Fragmentation was obtained with collision energies of –20 V and –60 V.

Table 2: MS/MS parameter settings for the characterization of mercapturic acids with EPI after CNL survey scan in negative ion mode.

Experiment (negative ion mode)	**CNL 129**	**EPI**
m/z*	200 - 450	50 - 500
CAD	medium	high
Resolution	Q1: unit Q3: low	Q1: unit
MR pause [ms]	5	5
Scan rate [mass units/s]	3 s dwell time	4,000
Q0 trap	-	no
Settling time [ms]	700	-
LIT fill time [ms]	-	50

*adapted to expected metabolite masses

3.4.1.4 Generation of the IDA thMRM/negative EPI Method (method 4)

Instrument and compound-dependent parameters were identical to the IDA CNL/negative EPI method described in Table 1. However, a MRM scan instead of a CNL scan was used as survey scan with 251 mass transitions based on the characteristic neutral loss of 129 Da. Each mass transition was scanned for 5 ms and MR pause was reduced to 2 ms.

For comparison between IDA thMRM/negative EPI (251 mass transitions with a single dwell time of 5 ms) and non-IDA single MRM scan (25 mass transitions with a single dwell time of 50 ms), instrument and compound-dependent settings were identical to method 3. For analysis of mercapturic acid standards in single MRM scan mode, IDA was not inserted into the method. Mass transitions according to the neutral loss of 129 Da were selected for data acquisition.

3.4.1.5 Generation of the IDA CNL/positive EPI Method (method 5)

In a separate method, the CNL scan was obtained in the negative ion mode, whereas fragmentation was performed in positive ion mode to obtain more effective fragmentation for identification. To set the correct mass for the EPI scan after positive ionization, an enhanced resolution (ER) scan in positive or negative ion mode had to be interposed depending on the quality of the isotopic pattern in the survey scan. To remain with acceptable cycle time, only one EPI scan at CE 20 V was performed and dwell time in the survey scan was reduced to 1.5 s. Positive ionization was obtained with IS 5,500 V and DP 40 V.

Table 3: MS/MS parameter settings for the characterization of mercapturic acids with EPI scan in positive ion mode after CNL survey scan in negative ion mode and an enhanced resolution scan (ER) with either polarity.

Experiment	**CNL 129 (negative ion mode)**	**ER (neg./pos. ion mode)**	**EPI (positive ion mode)**
m/z*	200 - 450	best ion	50 - 500
CAD	medium	high	high
Resolution	Q1: unit Q3: low	Q1: open	Q1: low
MR pause [ms]	5	5	5
Scan rate [mass units/s]	1.5 s dwell time	1,000	4,000
Q0 trap	-	no	no
Settling time [ms]	700	700	200
LIT fill time [ms]	-	50	50

*adapted to expected metabolite masses

3.4.1.6 Method for Identification of the Isobaric Acetaminophen Metabolites (method 6)

Parameter settings such as IS, CUR, DP, and CE of method 5 were used. The gradient (flow rate of 250 µl/min) started with 5% of acetonitrile and increased to 15% of acetonitrile within 20 minutes. The polar solvent consisted of 0.1% of formic acid in water. Data acquisition was performed in the MRM mode by monitoring five mass transitions for the identification of the acetaminophen metabolites in the positive ion mode, namely *m/z* 329.1 to *m/z* 224.1, to 200.1, to 182.1, to 156.1, and to 140.1 (dwell time of 200 ms each). A volume of 10 µl was injected into the HPLC-MS/MS system.

3.4.2 Sample Preparation

3.4.2.1 Isolation and Preparation of Neutrophils from Human Whole Blood

The studies were carried out according to the Declaration of Helsinki after approval by the Regional Committee of the University of Wuerzburg, Germany, prior to written informed consent by the subjects.

Human whole blood (24 ml) was taken from healthy adult volunteers (aged 24 to 29 years) and kept in heparinized tubes at room temperature until isolation (time until isolation < 60 minutes). Isolation was performed according to the method described by Greulich T. [84]. Granulocytes, lymphocytes, and erythrocytes were separated from the other blood constituents by carefully overlaying the blood with 37°C warm Histopaque (density of 1.077 mg/ml) and centrifugation at 535 x g for 30 minutes at room temperature. The two upper phases were discarded and sedimentation of the erythrocytes was achieved by addition of 3% dextrane solution in sterile water (24 ml) and incubation for 15 minutes. The supernatant containing the neutrophils was carefully separated from the sediment and centrifuged at 350 x g to obtain neutrophils as pellet. The supernatant was discarded and the pellet was resuspended in 5 ml of PBS_{def} buffer (PBS_{def} buffer consisted of 2.7 mmol KCl, 141.3 mmol NaCl, 3.2 mmol Na_2HPO_4 x 2 H_2O, and 1.5 mmol KH_2PO4 in 1 l H_2O). Any remaining erythrocytes were denaturated by lysis using hypotone 0.2% NaCl (20 ml). To avoid lysis of the osmotically more stable neutrophils, an equal amount of

hypertone NaCl solution (1.2% NaCl) was added 45 seconds after addition of hypotone NaCl solution to restore isotony. The suspension was centrifuged at 1,737 x g for 10 minutes at 12°C. The supernatant was discarded and the pellet was resuspended in PBS_{def} buffer. This lysis step was repeated 3 to 4 times (centrifugation now at 630 x g for 10 minutes at 12°C) until a pale white pellet was obtained. Isolated neutrophils were resuspended in RPMI 1640 buffer supplemented with 1 mM L-glutamine, 0.5 mM sodium-pyruvate, 5 µM HEPES buffer, penicillin/streptomycine (100 U/ml, i.e. 100 mg/ml), and non-essential amino acids (100x) (5 ml/l buffer) and kept at room temperature until incubation, which was performed within 60 minutes from separation. Cells were counted on a Coulter Z1 (cells with sizes between 7.5 µm and 30 µm). Purity and viability of cells were estimated on a BD LSR flow cytometer by determination of granularity and cell size of the suspended cells. For incubation tests, the purity of the cell suspension was taken into account. All solutions were sterile and working steps were performed under laminar air flow.

3.4.2.2 Sample Preparation of Microsomal Incubation Solutions and Urine Samples

After generation of mercapturic acids *in vitro* or *in vivo*, proteins were precipitated by the addition of acetonitrile (0°C) to the sample (1:2 v/v), followed by centrifugation at 15,000 x g for 20 minutes. The supernatant was removed and the organic phase was evaporated under a stream of nitrogen. Internal standard of *N*-acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine was added to the solution to obtain a final concentration of 2 µg/ml when quantitative analysis of metabolite formation was of interest.

3.4.2.3 Sample Preparation for Determination of LOD with Method 3

For limit of detection (LOD) determination, a stock solution of the mercapturic acid standards described in chapter 3.2 was prepared with a concentration of 2.5 µg/ml CH_3OH/H_2O (1:2 v/v) for each mercapturic acid. A dilution series including a 1:2 dilution with either urine or CH_3OH/H_2O was performed to obtain concentrations of each substance of 0.04, 0.08, 0.16, 0.2, 0.4, 0.8, 1.6, and 4.2 µg/ml. For LOD determination in matrix, urine from an untreated male Wistar rat was used. Each standard dilution was measured three times. LOD level was defined as the

concentration of analyte showing a signal with a CNL of 129 Da and successful recording of at least two out of three MS/MS mass spectra.

3.4.2.4 Sample Preparation for Validation of Method 3 and 4

Individual rat urine samples of the three dose groups of the acetaminophen (AAP) rat study described in chapter 3.4.2.6 were pooled (control, low and high dose pool) and precipitated as described before. Pooled low dose and control dose urine were diluted 1:10, pooled high dose urine 1:100 with water. Calibration was performed with concentrations of 0.3, 1, 3, 10, 30, 100, and 300 µg/ml of acetaminophen mercapturic acid (AAP-MA) in pooled control urine. The amount of urine was the same in all calibration samples. Two control urine samples spiked with AAP-MA standard (1.5 µg/ml and 25 µg/ml respectively) were used for the determination of recovery. Internal standard *N*-acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine was added to reach a final concentration of 2 µg/ml in each sample. All samples were measured ten times in a row in three different blocks (external samples were measured between the interday determinations). For LOD and LOQ determination, two control pool samples were spiked with 0.025 µg/ml and 0.1 µg/ml of AAP-MA standard and measured five times with IDA CNL/negative EPI and IDA thMRM/negative EPI (method 3 and 4) respectively. To demonstrate the potential to perform quantitative measurements with these methods, they were compared with a MRM method scanning for one quantifier (*m/z* 311.1 to *m/z* 182.1) and one qualifier (*m/z* 311.1 to *m/z* 139.1) for AAP-MA and *m/z* 328.1 to *m/z* 199.1 for the internal standard using the corresponding optimized compound-dependent parameters (Table 9).

3.4.2.5 Generation of Mercapturic Acids *in vitro*

Liver Microsomes. Human or rat liver microsomes were incubated with diclofenac, bisphenol A, acetaminophen, bifonazole, clozapine, and carbamazepine (stock solutions identical to incubation with neutrophils) or troglitazone (stock solution of 10 mM in dimethylsulfoxide) at 37°C for 60 minutes and additionally 30 minutes for acetaminophen in a shaking water bath. Protein concentration were 1 mg/ml. Incubation volume was 500 µl and consisted of 0.1 M sodium phosphate buffer (pH 7.4), 1 mM magnesium chloride, 10 mM glucose-6-phosphate, 1 mM NADP, 0.5 U/mL glucose-6-phosphate-dehydrogenase, protein, and substrate (final

concentration of 50 µM (diclofenac and carbamazepine) or 100 µM (all others); final concentration of organic solvent did not exceed 1% in the incubation mixture). *N*-acetyl-L-cysteine was added after five minutes of preincubation to reach a final concentration of 10 mM. Incubations without substrate served as controls. NADP, glucose-6-phosphate, and *N*-acetyl-L-cysteine solutions were freshly prepared.

Baculovirus Expressed Human CYP450 Isoenzymes. Diclofenac stock solution was incubated with recombinant CYP2C9 and CYP3A4 isoenzymes (1 mg/ml protein) in the presence of *N*-acetyl-L-cysteine under identical conditions as described above for the microsomal generation of mercapturic acids. The mercapturic acid of acetaminophen was obtained with human recombinant CYP2E1 under identical conditions.

Neutrophils. Cells were centrifuged and resuspended in HBSS buffer at a concentration of 4 x 10^6 cells/ml. Aliquots of cell suspension (500 µl) were incubated with diclofenac (DCF), bisphenol A (BPA), acetaminophen (AAP), bifonazole, clozapine (10 mM stock solutions either in water (DCF, AAP), C_2H_5OH (bifonazole) CH_3OH (BPA) or dimethylsulfoxide (clozapine)), or carbamazepine (5 mM stock solution in C_2H_5OH), both in the presence and absence of 10 nmol phorbol-12-myristate-13-acetate (PMA). Final concentrations of the substrates were 100 µM (AAP, bifonazole, clozapine, BPA, and DCF) and 50 µM (DCF and carbamazepine), respectively. After 30 minutes of incubation at 37°C in a shaking water bath, *N*-acetyl-L-cysteine at a final concentration of 10 mM was added to each assay and incubation was continued for 60 minutes, then sonicated for five minutes and freezed at -20°C. Prior to analysis, the samples were thawed and centrifuged at 15,000 x g for 20 minutes. Incubations without substrate served as controls. A volume of 50 µl of the supernatant was injected into the HPLC-MS/MS system.

3.4.2.6 Generation of Mercapturic Acids by Rats

Acetaminophen. Nine male Sprague-Dawley rats (8-9 weeks old on delivery) were kept in Macrolon cages (3 animals per cage) on a 12 hours light/dark cycle and allowed free access to standard laboratory chow and tap water (sterilized). Rooms were maintained at a temperature of 22 ± 2°C and humidity of 50-60%. After six days of acclimatization in metabolic cages, the rats were divided into three groups (n = 3/group) and were administered a single oral dose of acetaminophen by gavage

(low dose: 20 mg/kg body weight (b.w.) and high dose: 640 mg/kg b.w.) dissolved in 20% Tween 80-saline solution. The control group received an equal volume of vehicle. Urine samples were collected from -24-0 hours (predose) and from 0-24 hours (postdose) and were stored at -80°C until analysis. Creatinine levels were determined by the Central Laboratory at the University of Wuerzburg.

Diclofenac. Samples from this study were kindly provided by F. Hoffmann-La Roche (Basel, Switzerland). Briefly, six rats (3 groups) were orally administered 10 mg/kg b.w. of diclofenac by gavage. Urine was collected 6 hours (sacrifice of group 1), 18 hours (sacrifice of group 2), 24 hours, and 48 hours (sacrifice of group 3) after administration. Three other rats were administered 20 mg/kg b.w. of diclofenac by gavage and urine was collected after 24 hours.

3.4.2.7 Generation of Mercapturic Acids of Acetaminophen by Humans

The studies were carried out according to the Declaration of Helsinki after approval by the Regional Committee of the University of Wuerzburg, Germany, prior to written informed consent by the subjects.

Human urine samples were obtained from ten healthy volunteers who received the pharmacologically effective dose of 500 mg acetaminophen (Paracetamol ratiopharm®, Ulm, Germany) or a dose of 50 mg. Morning urine was collected before (control urine) and 8 hours after administration to acetaminophen. Proteins were precipitated by the addition of acetonitrile (0°C) to the sample (1:2 v/v), followed by centrifugation at 15,000 x g for 20 minutes. The supernatant was removed and the organic phase was evaporated under a stream of nitrogen. For quantitation of renal elimination of the mercapturic acid of acetaminophen, creatinine levels were determined in the Central Laboratory at the University of Wuerzburg.

3.4.3 Determination of DHN-MA and HNE-MA in Rat Urine

N-acetyl-*S*-(1,4-dihydroxynonan-3-yl)-L-cysteine (DHN-MA) and *N*-acetyl-*S*-(5-hydroxy-2-pentyltetrahydrofuran-3-yl)-L-cysteine (HNE-MA) signals were detected in urine samples of rats before and after administration of acetaminophen (method 1). Peak areas of HNE-MA and DHN-MA were determined with Analyst 1.4. and expressed relative to creatinine. Urine samples were not diluted.

3.4.4 Quantitation of Acetaminophen Mercapturic Acid in Rat Urine

To generate two calibration curves, standard dilutions were prepared in diluted control urine (1:10 in water) with standard AAP-MA at concentrations ranging from 0.5 µg/ml to 100 µg/ml for the quantitation of AAP-MA in low dose urine and 50 µg/ml to 500 µg/ml for high dose urine, due to non-linearity over the whole concentration range. *N*-acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine (2 µg/ml) served as internal standard. For quantitation, urine samples were diluted 1:10 in water. Data acquisition was performed in MRM mode using method 2. Measurements of each sample were performed three times. Quantitation of AAP-MA in rat urine was determined with Analyst 1.4 and expressed relative to creatinine.

3.4.5 Quantitation of Acetaminophen Mercapturic Acid in Human Urine

Standard dilutions were prepared in pooled control urine (1:5 dilution in water) with standard AAP-MA at concentrations ranging from 0.3 to 300 µg/ml and the internal standard *N*-acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine. Data acquisition was performed in MRM mode using MRM conditions described in method 2, but with the optimized chromatographic conditions (0.1% of formic acid and acetonitrile as HPLC solvents). Quantitation of AAP-MA in human urine was determined with Analyst 1.4 and expressed relative to creatinine.

3.5 L-Lysine Adducts

3.5.1 Generation of the IDA CNL/negative EPI Method for the Determination of Acyl Glucuronides

HPLC conditions, instrument-, and parameter settings were identical to method 3 (Table 2). In the survey scan 300 masses were scanned for a constant neutral loss of 176 Da in negative ion mode for 3 seconds. EPI scan (CE –10 V) of the target ions was performed when signal intensity exceeded 1,000 cps.

3.5.2 Development and Generation of the HPLC-MS/MS Method for the Determination of L-Lysine Conjugates (method 7)

100 µl of the stock solutions of seven commercially obtained L-lysine standards N^{ε}-(*tert*-butoxycarbonyl)-L-lysine, N^{ε}-methyl-L-lysine HCl, N^{α}-(benzyloxycarbonyl)-L-lysine, N^{ε}-glutamyl-L-lysine, N^{ε}-(2,4-dinitrophenyl)-L-lysine HCl, saccharopine (N^{ε}-(L-glutar-2-yl)-L-lysine), and biocytine HCl (N^{ε}-biotinyl-L-lysine) with concentrations of 1 mg/ml solvent (standards dissolved in CH_3OH except for saccaropine and N^{ε}-g-glutamyl-L-lysine which were dissolved in water) were mixed to reach a single concentration of 143 µg/ml each.

HPLC Conditions:

Precolumn: Reprosil Pur C18 AQ 5 µm (10 x 2 mm, Dr. Maisch, Ammerbuch, Germany)

Column: Reprosil Pur C18 AQ 5 µm (150 x 2 mm, Dr. Maisch, Ammerbuch, Germany)

Solvent A: 0.1% of formic acid

Solvent B: acetonitrile

Flow rate: 250 µl/min

Gradient: isocratic at 0% B for 2 minutes followed by a linear gradient to 50% B within 18 minutes and to 100% B within another 5 minutes; back to starting conditions within 3 minutes, and re-equilibration for 10 minutes

MS Conditions:

Analysis of L-lysine conjugates was performed with a 3 seconds persistent survey scan for precursor ions (mass range of 251 masses) that showed fragmentation to *m/z* 84 after positive ionization. Masses with the occuring fragment ion at *m/z* 84 were then triggered for an EPI scan in positive ion mode and a collision energy (CE) of 30 V. EPI scan with dynamic LIT fill time was performed only when signals in the survey scan occurred that exceeded 1,000 cps of intensity. Exclusion of former target ions took 40 seconds. Other IDA criteria and EPI settings were identical to method 3.

For determination of L-lysine conjugates, the following instrument and parameter settings were used:

Table 4: Optimzed MS/MS settings for the characterization of L-lysine conjugates after positive ionization.

CUR [psi]	IS [V]	Temp	GAS 1 [psi]	GAS 2 [psi]	DP [V]	EP [V]	CE [V]	CXP [V]
40	5,000	400	60	60	20	10	30	3

3.5.3 Generation of HPLC-MS/MS Method for the Detection of L-Lysine Adducts on an API 3000 (method 8)

Determination of acyl L-lysine adducts of diclofenac and ibuprofen was performed on an API 3000 instrument with a source temperature of 400°C and an ionspray voltage (IS) of 5,000 V. Nitrogen was used for nebulizing, curtain and collision gas (NEB 11, CUR 8, CAD 4). Compound-dependent parameters as DP, EP, CE, and CXP were the same as used for analysis on the QTRAP 2000 instrument. Detection of L-lysine adducts was performed in MRM scan mode. Specific mass transitions were monitored for the determination of acyl L-lysine adducts (diclofenac: *m/z* 424.2 to *m/z* 250.1, *m/z* 129.1, and *m/z* 84.1 amu and *m/z* 440.2 to *m/z* 147.1, *m/z* 130.1, and *m/z* 84.1) and for the internal standard N^{ε}-(2,4-dinitrophenyl)-L-lysine (*m/z* 313.2 to *m/z* 84.1) with dwell times of 100 ms for each mass transition.

3.5.4 Sample Preparation

3.5.4.1 Sample Preparation for the Determination of Sensitivity of L-Lysine Adducts

Dilution of the stock solution mixture were prepared ranging from 1:100 to 1:100,000 in H_2O (1:100, 1:500, 1:1,000; etc.) and measured once. According to the detection of some L-lysine standards close to the LOD, the dilution 1:100 (1.43 µg/ml) was used for analysis. For determination of the sensitivity in matrix human control urine, rat control urine, and control incubation solution, the stock solution mixture was

diluted 1:50 with CH_3OH/H_2O (1:2 v/v) before it was mixed with an equal volume of the according matrix to obtain identical concentrations. A volume of 5 µl were injected for analysis (method 7). Each sample was measured five times.

3.5.4.2 Generation of L-Lysine Adducts in Rat Liver Microsomes

Acyl glucuronides were formed upon incubation of either diclofenac, ibuprofen, bromfenac, or sodium benzoate (10 mM stock solution in water) with rat liver microsomes in the presence of UDP-glucuronic acid (UDP-GA) as described previously with some modifications [85]. Incubation solution consisted of liver microsomes (1 mg protein/ml incubation solution), the glucuronidation activator alamethicine (25 µg/mg protein), 20 mM $MgCl_2$, and UDP-GA (5 mM final concentration) in 50 mM Tris buffer (adjusted to pH 7 with HCl). Liver microsomes were activated with alamethicine on an ice bath 30 minutes before incubation was started. Tris buffer, $MgCl_2$, and substrate was mixed and placed in a shaking water bath (37°C) 10 minutes prior to incubation start. Reaction was initiated by pooling the activated microsomes with the substrate mixture and adding UDP-GA. Incubation without substrate served as control. 30 minutes after incubation at 37°C in a shaking water bath, the samples were vortexed and centrifuged at 15,000 x g for 10 minutes. An aliquot of each sample was removed and 3% of formic acid in acetonitrile was added to stabilize the formed acyl glucuronides (proportion: 1:3.5 incubation solution). L-lysine was added to reach a final concentration of 50 mM. The pH value was adjusted to 8.5 with 10% of ammonia. The samples were vortexed and incubated at 60°C for 60 minutes (using a heating block). Samples were analyzed directly or stored at –20°C until analysis. Prior to analysis, the samples were thawed and centrifuged at 15,000 x g for 10 minutes. Internal standard (N^{ε}-(2,4-dinitrophenyl)-L-lysine HCl) was added to reach a final concentration of 20 ng/ml.

3.5.4.3 Generation of L-Lysine Adducts in Human Urine Samples

Diclofenac urine samples were obtained from five volunteers (four males and one female, aged 19 to 52 years), who were recruited from the Hospital of the University of Wuerzburg (medical surgery). These subjects have been administered Voltaren Resinat® (75 mg diclofenac-Na, Nuernberg, Germany) twice a day over at least two days. Urine was collected 5 hours and 8 hours after dosage, respectively. Creatinine

levels were routinely determined in the Central Laboratory at the University of Wuerzburg

Urine was freshly thawed at room temperature and L-lysine was added to reach a final concentration of 50 mM. The mixture was heated for 60 minutes to 60°C in a heating block. After heating, samples were centrifuged at 15,000 x g for 10 minutes prior to analysis. Internal standard (N^{ε}-(2,4-dinitrophenyl)-L-lysine HCl) was added to the supernatant (final concentration of 20 ng/ml). Urine samples without L-lysine and control urine from individuals not exposed to drugs served as controls.

Statistics:

Data are given as mean ± standard deviation. Comparison between data were performed with ANOVA followed by two sided *t* test. $p < 0.05$ was considered significant.

4 RESULTS

4.1 Mercapturic Acids

4.1.1 Analytical Method Development

Based on the idea that mercapturic acids formed from conjugation of reactive intermediates with glutathione may serve as potential biomarkers for the detection of reactive metabolites, the aim of this study was to establish a general method for the determination of these metabolites. With recent advances in analytical techniques, methods are now available that allow the sensitive and selective detection of these metabolites. HPLC separation coupled with modern MS/MS technique demonstrates a specific and sensitive potential for the detection and characterization of mercapturic acids in matrix without complex sample preparation and derivatization.

4.1.1.1 Ionization and Fragmentation Pattern of Mercapturic Acid Standards and their Sulfoxides obtained with MS/MS Techniques

To establish a HPLC-MS/MS method for the general detection of structurally diverse mercapturic acids and their sulfoxides, specific fragmentation patterns of characteristic substructures were determined. A wide range of mercapturic acids was directly injected into the mass spectrometer and fragmentation experiments in negative and positive ion mode were performed. The most prominent fragment ions of mercapturic acids and their sulfoxides are given in Table 5 -Table 8.

In negative ion mode, all the available standards including the corresponding sulfoxides showed a common constant neutral loss (CNL) of 129 Da, resulting from cleavage of the thioether (Scheme 8). The fragment ions generated by this CNL were the most prominent signals detected. The CNL scan showed improved selectivity compared to Q1 full scan due to the possibility to selectively determine only such signals that showed the specific loss. Thus, sensitivity was increased. With positive CID, a product ion at *m/z* 130 and/or a CNL of 163 Da was observed in the mass spectra of many of the mercapturic acid standards. The product ion at *m/z* 130 was assigned to 2-acetylamino-propionic acid and was observed for mercapturic acid standards and their sulfoxides, whereas *m/z* 163 appeared only for mercapturic acids

and corresponded to the loss of the entire *N*-acetyl-L-cysteine moiety (Scheme 8). However, neither a common fragment ion nor a common CNL occurred in the positive ion mode. Thus, further development of the method was performed with a CNL 129 Da survey scan in negative ion mode.

Table 5: Most prominent fragment ions of mercapturic acid standards detected in the negative ion mode.

Compound	**$[M - H]^-$**	**Fragment ions detected**							
N-Acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine	256	**127**[a]	120	91					
N-Acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine	256	**127**[a]	91						
N-Acetyl-*S*-(4-chlorobenzyl)-L-cysteine	286	**157**[a]	121	111					
N-Acetyl-*S*-(2-fluorobenzyl)-L-cysteine	270	**141**[a]	121	95	75				
N-Acetyl-*S*-(3-hydroxypropyl)-L-cysteine	220	178	**91**[a]	89	84	54			
N-Acetyl-*S*-(4-bromobenzyl)-L-cysteine	330	**201**[a]	121	79					
N-Acetyl-*S*-(4-methoxybenzyl)-L-cysteine	282	**153**[a]	138	137	109	92	84	57	
N-Acetyl-*S*-(4-*tert*-butylbenzyl)-L-cysteine	308	**179**[a]	163	122	84	57			
N-Acetyl-*S*-[5-(acetylamino)-2-hydroxyphenyl]-L-cysteine	311	**182**[a]	139	58					
N-Acetyl-*S*-(1,1-dichloro-2,2-difluoroethyl)-L-cysteine	294	**165**[a]	145						
N-Acetyl-*S*-phenyl-L-cysteine	238	111	**109**[a]						
N-Acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine	328	**199**[a]	169	153	135	120	105	88	46
N-Acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine	292	**163**[a]	84						

[a] **fragment ion that experienced a constant neutral loss of 129 Da**

Table 6: Most prominent fragment ions of the mercapturic acid standards detected in the positive ion mode.

Compound	$[M + H]^+$	Fragment ions detected
N-Acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine	258	216 199 180 170 134 127 83
N-Acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine	258	216 195 170 131 127 92 83 56
N-Acetyl-*S*-(4-chlorobenzyl)-L-cysteine	288	246 200 130[a] 125[b] 89 56
N-Acetyl-*S*-(2-fluorobenzyl)-L-cysteine	272	230 213 184 167 133 130[a] 109[b] 83
N-Acetyl-*S*-(3-hydroxypropyl)-L-cysteine	222	180 163 130[a] 117 91 84 59[b]
N-Acetyl-*S*-(4-bromobenzyl)-L-cysteine	332	273 227 169[b] 130[a] 90 84 59
N-Acetyl-*S*-(4-methoxybenzyl)-L-cysteine	284	121[b] 106 95 91 78
N-Acetyl-*S*-(4-*tert*-butylbenzyl)-L-cysteine	310	147[b] 132 117 105 91
N-Acetyl-*S*-[5-(acetylamino)-2-hydroxyphenyl]-L-cysteine	313	271 225 208 182 166 149 140 96 84
N-Acetyl-*S*-(1,1-dichloro-2,2-difluoroethyl)-L-cysteine	296	228 214 186 168 132 130[a] 116 83 59
N-Acetyl-*S*-phenyl-L-cysteine	240	198 181 135 130[a] 109 91 84 65 56
N-Acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine	330	288 242 199 197 186 151 109 77 58
N-Acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine	294	252 235 206 170 163 119

[a]product ion at *m/z* 130

[b]fragment ion that experienced a constant neutral loss of 163 Da

Table 7: Most prominent fragment ions of the sulfoxides of mercapturic acid standards detected in the negative ion mode.

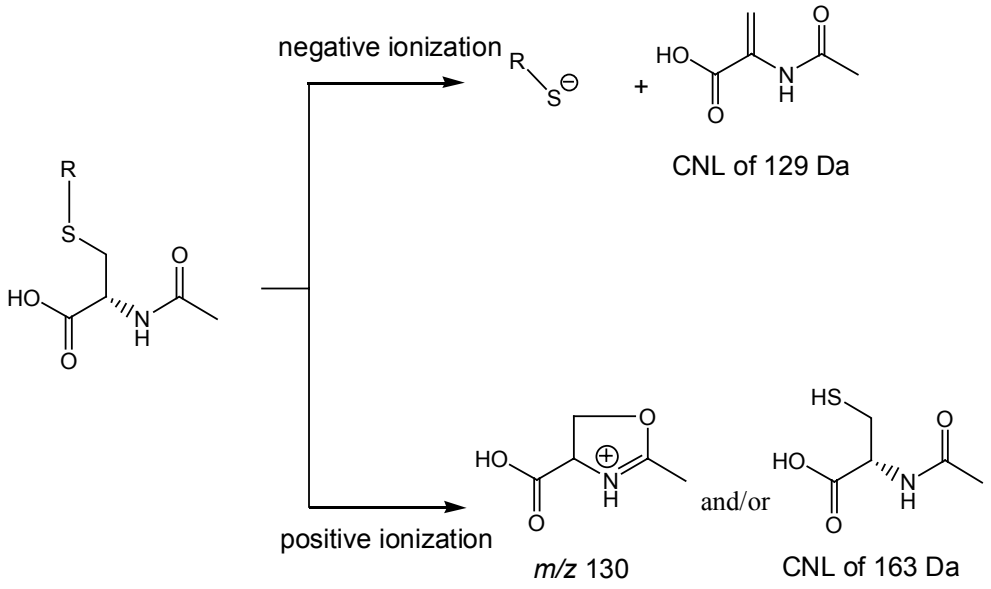
Sulfoxide of the compound	**$[M - H]^-$**	**Fragment ions detected**
N-Acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine	272	**143**[a] 132 120 107
N-Acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine	272	272 236 210 **143**[a] 107 81
N-Acetyl-*S*-(4-chlorobenzyl)-L-cysteine	302	177 **173**[a] 155 115 100 84 74
N-Acetyl-*S*-(2-fluorobenzyl)-L-cysteine	286	177 **157**[a] 137 115 100 84
N-Acetyl-*S*-(3-hydroxypropyl)-L-cysteine	236	178 116 **107**[a] 84 59
N-Acetyl-*S*-(4-bromobenzyl)-L-cysteine	346	**217**[a] 199 177 171 81 79
N-Acetyl-*S*-(4-methoxybenzyl)-L-cysteine	298	177 **169**[a] 136 123 115 100 84
N-Acetyl-*S*-(4-*tert*-butylbenzyl)-L-cysteine	324	**195**[a] 177 115 100 84 74
N-Acetyl-*S*-[5-(acetylamino)-2-hydroxyphenyl]-L-cysteine	327	**198**[a] 180 165 150 138 128
N-Acetyl-*S*-(1,1-dichloro-2,2-difluoroethyl)-L-cysteine	310	**181**[a] 161 132 116 83
N-Acetyl-*S*-phenyl-L-cysteine	254	**125**[a] 97 93 77 65
N-Acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine	306	**177**[a] 132 128 120 112 84 65

[a] **fragment ion that experienced a constant neutral loss of 129 Da**

Table 8: Most prominent fragment ions of the sulfoxides of mercapturic acid standards detected in the positive ion mode.

Sulfoxide of the compound	**$[M + H]^+$**	**Fragment ions detected**							
N-Acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine	274	232	186	150	130 [a]	112			
N-Acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine	274	218	152	130[a]	112	88	84	56	43
N-Acetyl-*S*-(4-chlorobenzyl)-L-cysteine	304	296	125	118	88	74	56		
N-Acetyl-*S*-(2-fluorobenzyl)-L-cysteine	288	270	109	106	102	88	70	57	
N-Acetyl-*S*-(3-hydroxypropyl)-L-cysteine	238	178	163	150	130[a]	114	88	84	
N-Acetyl-*S*-(4-bromobenzyl)-L-cysteine	348	260	171	130[a]	118	106	88	74	
N-Acetyl-*S*-(4-methoxybenzyl)-L-cysteine	300	153	147	121	106				
N-Acetyl-*S*-(4-*tert*-butylbenzyl)-L-cysteine	326	147	132	130[a]	119	117	105	91	
N-Acetyl-*S*-[5-(acetylamino)-2-hydroxyphenyl]-L-cysteine	329	227	200	182	155	140	130[a]	88	84
N-Acetyl-*S*-(1,1-dichloro-2,2-difluoroethyl)-L-cysteine	312	270	230	170	130 [a]	112			
N-Acetyl-*S*-phenyl-L-cysteine	256	214	150	130 [a]	112				
N-Acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine	308	266	220	167	130 [a]	112			

[a] product ion at *m/z* 130



Scheme 8: Common constant neutral loss and frequent fragmentation of mercapturic acid standards and their corresponding sulfoxides (except for CNL 163 Da) after negative or positive ionization. Fragment ion at *m/z* 130 was previously described for disulfides of biothiols by Rubino et al. [86]

4.1.1.2 Optimization of HPLC Conditions

Initial optimal selection of HPLC solvent was performed with single standards. Each mercapturic acid (100 µg/ml) was directly introduced into the mass spectrometer using a syringe pump (flow rate of 50 µl/min). A t-junction enabled the mixture of the standard solution with one of three different mobile phases (0.1% of formic acid (FA), 5 mM ammonium formate pH 3, and 5 mM ammonium acetate pH 6.8, each with 300 µl/min flow rate). Under these conditions, 5 mM ammonium acetate pH 6.8 was found to be the best buffer for the detection of all standards with CNL scan in the negative ion mode due to highest ionization rates, followed by ammonium formate and formic acid.

Chromatographic separation was tested on a Synergi polar RP18 column with a mixture of the standards in a concentration of 5 µg/ml each and with the HPLC solvents 5 mM ammonium acetate pH 6.8 and acetonitrile. The gradient used for method 1 and 2 was developed to obtain good peak shape with an ideal signal to noise ratio. Full baseline separation was considered to be of minor importance since identification was achieved by the different masses of the mercapturic acid standards. However, differences in ionization efficiency between the single mercapturic acid standards were apparent (most unpolar *N*-acetyl-*S*-(4-*tert*-

butylbenzyl)-L-cysteine better than most polar compound *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine) presumably due to the proportion between the organic, more volatile HPLC solvent acetonitrile and the polar HPLC solvent 5 mM ammonium acetate at the moment of elution of the standards.

Due to the instability of the Synergi Polar column at neutral pH (ammonium acetate with pH 6.8), further chromatographic separation was performed on a Reprosil Pur AQ column with broader pH stability (pH 1 to pH 10). Tests with the three HPLC solvents on this column resulted in slightly better peak shapes and, for some mercapturic acids, a higher sensitivity with 0.1% of formic acid than with the ammonium acetate buffer. For instance, 5 µg/ml *N*-acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine with ammonium acetate showed a peak width at half height of $b_{0.5} \leq 0.14$ minutes and a peak symmetry of $S_s = 1.11$ in contrast to 0.1% of formic acid with $b_{0.5} \leq 0.13$ minutes and $S_s = 1.02$. Changing the solvent to 0.1% of formic acid additionally enabled an increase of the organic phase fraction in the starting point and gained sensitivity especially in the beginning of the run, due to the reduced polarity of the mercapturic acids in acidic solvents. Final HPLC optimization was therefore performed using a Reprosil Pur AQ column with 0.1% FA and acetonitrile as mobile phases (Figure 1).

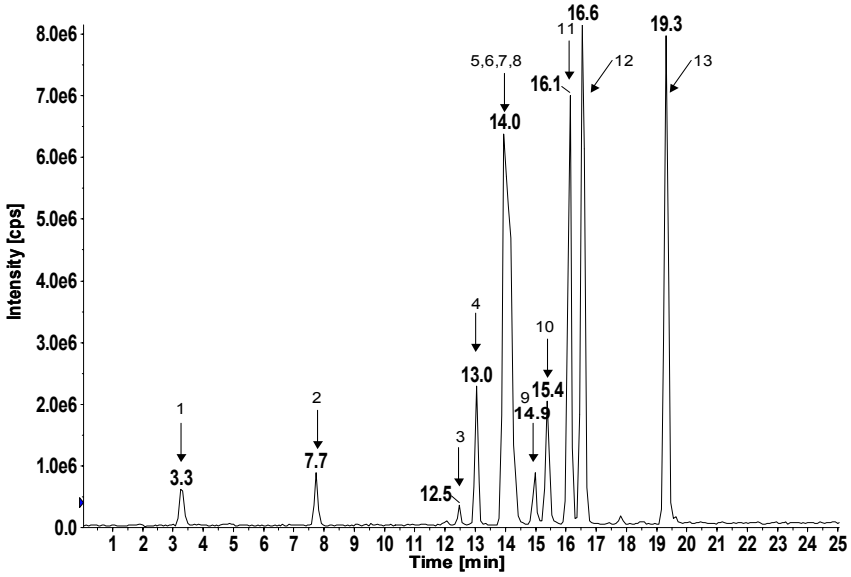


Figure 1: HPLC chromatogram of mercapturic acid standards with 5 µg/ml CH_3OH/H_2O (1:2 v/v) each, obtained with CNL 129 Da survey scan on a Reprosil Pur AQ column with optimized HPLC gradient. Assignment of each peak (no. 1-13) is given in Table 9.

4.1.1.3 Optimization of Source and Compound-Dependent Parameters

Source parameters such as ion spray voltage (IS), temperature (Temp), and source gases (CUR, Gas 1, Gas 2) were optimized according to the procedure for HPLC optimization with a t-junction. Source parameters depended on flow rate and solvents used for chromatographic separation. A manual change of every single parameter for the achievement of highest sensitivity of the mercapturic acid standards lead to the following optimized settings: IS: –4,200 V, Temp: 400°C, CUR: 30 psi, Gas 1 (nebulizer gas): 45 psi, and Gas 2 (drying gas): 50 psi. The compound-dependent parameters (CDP), i.e. declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP), relevant for the generation of mass spectra were automatically optimized for each standard with “Quantitative Optimization” Software from Applied Biosystems by direct infusion into the MS. DP and CE for the generation of the CNL of 129 Da of the available mercapturic standards varied from –21 to –76 V and from –8 to –28 V, respectively (Table 9). To determine a common DP and CE for highest average sensitivities for all standards, DP and CE were set from –10 to –30 V for CE and –30, –50, and –70 V for DP, respectively. With a DP of –50 V , EP of –10 V, CE of –20 V and CXP of –2 V all standards were detected with good sensitivity.

Table 9: Automated optimized MS/MS parameters of each standard for the generation of the constant neutral loss of 129 Da in the negative ion mode. Results were obtained with the "Quantitative Optimization" feature of Analyst Software.

Compound-dependent parameters	**EP [V]**	**DP [V]**	**CE* [V]**	**CXP [V]**
N-Acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine (3)	-9.5	-31	-12	0
N-Acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine (5)	-11.5	-51	-20	0
N-Acetyl-*S*-(4-chlorobenzyl)-L-cysteine (11)	-5	-56	-16	-2
N-Acetyl-*S*-(2-fluorobenzyl)-L-cysteine (7)	-10	-41	-20	-2
N-Acetyl-*S*-(3-hydroxypropyl)-L-cysteine (1)	-9	-71	-20	0
N-Acetyl-*S*-(4-bromobenzyl)-L-cysteine (12)	-12	-51	-24	-2
N-Acetyl-*S*-(4-methoxybenzyl)-L-cysteine (6)	-4.5	-76	-22	-2
N-Acetyl-*S*-(4-*tert*-butylbenzyl)-L-cysteine (13)	-11	-76	-26	-2
N-Acetyl-*S*-[5-(acetylamino)-2-hydroxyphenyl]-L-cysteine (2)	-5	-56	-20	-4
N-Acetyl-*S*-(1,1-Dichloro-2,2-difluoroethyl)-L-cysteine (8)	-8.5	-61	-16	-4
N-Acetyl-*S*-phenyl-L-cysteine (4)	-9	-71	-22	0
N-Acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine (10)	-4	-26	-16	-4
N-Acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine (9)	-3	-51	-8	-2

*collision energy (CE) according to CNL of 129 Da

4.1.1.4 Development of the IDA CNL/negative EPI Method (method 3)

For improved sensitivity, the CNL scan mode was used as a selective survey scan and combined with an enhanced product ion (EPI) scan for improved mass spectra using the linear ion trap. This combination was triggered by the Analyst Software feature called information dependent acquisition (IDA) and presented the opportunity to obtain fragmentation of only those signals that fulfill the requirements set by the

user in the survey scan. Using IDA provided maximal information in one run and saved time and sample volume. Although the CNL of 129 Da was the most prominent signal after fragmentation and very easily accomplished, collision energy at –20 V was not effective enough to obtain mass spectra with sufficient fragmentation for unequivocal characterization of the signals detected. Therefore, a second EPI scan with a higher collision energy of –60 V was included into the IDA method to receive more information about the chemical structure. With this method cycle time was increased resulting in less data points per peak. However, peak shapes were still acceptable. The addition of a second EPI scan with a higher collision energy provided more fragment ions particularly in the lower mass range.

For parameter optimization of the IDA method, pretests were performed with acetaminophen mercapturic acid (AAP-MA) standard (5 µg/ml) as a reference compound for mercapturic acids in blank rat and human urine. Each variation in the IDA CNL method was measured three to five times and average, absolute standard deviation, precision, and standard error were calculated. Settings with best results were then used for further analysis and for validation.

Intensity Threshold for the Generation of Mass Spectra. Signal intensity that had to be achieved in the survey scan to force the IDA method to switch to the EPI mode influenced the quality of the MS/MS mass spectra. Generally, when threshold for EPI scanning is set too high, it may result in lack of MS/MS mass spectra of low concentrated analytes and, if it is given a low value, it may result in too much “noisy” information. A disadvantage is also the ability to only set one threshold, whereas the different mass traces mostly provide different background levels. When this IDA criteria was changed from 500 cps to 3,000 cps, background signals were not fragmented any more, but mass spectra of mercapturic acids with very low signal intensities were not performed. Thus, threshold of the signals in the survey scan that are further submitted to the collision cell was set to 500 cps.

Change of Collision Gas (CAD). To avoid permanent changes of the CAD gas setting when switching between CNL survey scan (CAD gas default: medium) and EPI scan (CAD gas default: high), CAD gas was also set high in the CNL mode. Results were compared to default CAD gas setting. When CAD gas was set to medium and no time to settle was defined between the switch of the different settings, absolute standard deviation (s.d.) of 0.37, a precision (prec.) of 13.8%, and

a standard error (S.E.) of 0.16 was obtained, whereas a high CAD gas setting increased absolute standard deviation, precision and standard error nearly to a factor of two (Table 10). Thus, CAD gas remained medium in the survey scan.

Settling Time for the CNL scan. To obtain better reproducibility for the CNL scan, particularly because of the different CAD gas settings of the two scan modes, settling time was set to 0 ms (set. 0), 200 ms (set. 200), or 700 ms (set. 700), prior to the CNL scan mode. Calculation of absolute standard deviation, precision, and standard error resulted in better reproducibility when a defined settling time of 700 ms (s.d.: 0.11, prec: 8.3%, and S.E.: 0.05) was set, rather than 200 ms (s.d.: 0.17, prec: 9.6%, and S.E.: 0.05) or no settling time, despite a loss in sensitity (Table 10). Further measurements were therefore performed with a settling time of 700 ms.

Linear Ion Trap Fill Time. The quality for good MS/MS mass spectra also depends on the time the linear ion trap (LIT) can collect fragment ions formed in the collision cell. However, the LIT fill time is limited by the increase of cycle time and potential overfill of the trap. Again, a compromise had to be found between sensitivity and cycle time. A number of different settings are generally possible. Dynamic LIT fill time (DFT) is a means by which the ion trap fill time is adjusted to the ion flux through the mass spectrometer, i.e. short fill time when ion flux is great and longer fill time when ion flux is low, to avoid overfill of the linear ion trap. When DFT is activated, a short scan will adjust the optimal fill time for every precursor ion based on the ion flux before each scan mode. Activation of the “parent area” means that one LIT fill time is defined after a short DFT scan before an entire IDA scan. A manually defined LIT fill time may improve reproducibility due to a reduction of variation in one IDA scan.

The Q0 trapping feature is another option for improved sensitivity and reproducibility. Here, those precursor ions are trapped in Q0 that were previously picked for EPI analysis. Thus, the following four options were tested: a) dynamic LIT fill time with individually fixed LIT fill time for each scan mode (DFT no parent area), b) dynamic LIT fill time with using the time formerly set for the parent ion (DFT with parent area), c) fixed LIT fill time for 50 ms without, and d) with Q0 trapping. Best results for reproducibility were obtained with fixed LIT fill time for 50 ms without Q0 trapping, but sensitivity was slightly better with Q0 trapping (Table 10). Nevertheless, further studies were performed without Q0 trapping due to better reproducibility and improved peak resolution. Optimization of sensitivity could be obtained best with

instrument parameter settings and HPLC solvents rather than the above named parameters. Therefore, such parameters were primarily optimized for improvement of reproducibility.

Table 10: Peak intensity with absolute standard deviation (s.d.), precision, and standard error (S.E.) of AAP-MA after various modifications of the instrument parameter settings using the IDA CNL/negative EPI method. Tests were either performed in human or rat urine samples and analyzed three or five times.

Settings[+]	**n**	**Relative peak areas**[†] **mean ± s.d..**	**Precision [%]**	**S.E.**	**Matrix**
CAD med (set. 0 ms)	5	2.66 ± 0.4	13.8	0.16	Human urine*
CAD high (set. 0 ms)	5	2.45 ± 0.7	28.1	0.31	Human urine*
Set. 200 ms	5	1.74 ± 0.2	9.6	0.07	Human urine*
Set. 700 ms	5	1.37 ± 0.1	8.3	0.05	Human urine*
DFT no parent area	3	0.46 ± 0.06	13.0	0.03	Rat urine
DFT with parent area	3	0.48 ± 0.07	13.9	0.04	Rat urine
Fixed LIT 50 ms	3	0.46 ± 0.02	4.4	0.01	Rat urine
Fixed LIT 50 ms with Q0 trapping	3	0.53 ± 0.06	12.1	0.04	Rat urine

[†]peak area analyte/peak area internal standard

*human urine: at the retention time of AAP-MA, another peak coeluted with identical mass and fragmentation. Thus, peak areas were summed up.

[+]settings that provided best reproducibility were used for the following methods (**bold**)

4.1.1.5 Generation of the IDA thMRM/negative EPI Method (method 4)

Alternatively to the CNL scan mode, an MRM scan mode with calculated mass transitions based on the CNL of 129 Da could be used as survey scan. In this case, 251 exactly defined mass transitions (e.g. *m/z* 221.1 to 92.1, *m/z* 222.1 to 93.1, etc.) were used. To decrease cycle time and obtain optimal sensitivity, the influence of

dwell time on the S/N ratios of the standards was analyzed. A standard mixture was measured three times with different dwell times of 5 ms, 10 ms, and 15 ms respectively for each mass transition. A dwell time of 15 ms showed equal (*N*-acetyl-*S*-(2-fluorobenzyl)-L-cysteine) to up to two times higher S/N ratios (*N*-acetyl-*S*-(4-bromobenzyl)-L-cysteine) than scanning the masses with a dwell time of 5 ms. However, cycle time had to be kept low to obtain enough detection points for the generation of a peak. Due to the high amount of mass transitions scanned during a cycle unit, a dwell time of 5 ms was set and MR pause was shortened to 2 ms resulting in good sensitivity and reproducibility. Other settings were identical to the IDA CNL/negative EPI method (method 3).

4.1.1.6 Generation of the IDA CNL/positive EPI Method (method 5)

Fragmentation of mercapturic acids in positive ion mode provided more product ions for characterization of the signal detected than with negative ion mode. Thus, combining the selective CNL 129 Da with EPI scans in positive ion mode would presumably provide more structural information about the metabolite of interest than EPI scan in negative ion mode. To switch ionization polarities within one run an enhanced resolution (ER) scan necessary for charge state and accurate mass determination had to be inserted prior to the EPI scan. The ER scan picks the most intensive centroid mass from the survey scan for the performance of an EPI scan with switched polarity, i.e. in positive ion mode. The ER scan could be performed either in negative or positive ion mode. Fragmentation was reduced to one EPI experiment due to cycle time that would have exceeded 5 seconds. However, the IDA software sometimes chose the wrong centroid mass for EPI scanning. Due to bad reproducibility in the selection of the right mass in the ER scan mode, this feature was not used for general detection of unknown mercapturic acids.

4.1.2 Comparison of Sensitivity of the Methods

HPLC and MS/MS conditions optimized for IDA CNL/negative EPI method and IDA thMRM/negative EPI method provided LOD levels ranging from 0.3 pmol for *N*-acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine, *N*-acetyl-*S*-(4-bromobenzyl)-L-cysteine, *N*-acetyl-*S*-(4-*tert*-butylbenzyl)-L-cysteine, and *N*-acetyl-*S*-(4-chlorobenzyl)-L-cysteine to

15.5 pmol for *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine on column in CH_3OH/H_2O (Table 11). The LOD levels in urine samples were in the same range for both methods for 10 of 13 standards. For *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine and *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine, LOD in urine could not be determined due to the presence of unknown metabolites with identical mass and identical retention time. *N*-acetyl-*S*-phenyl-L-cysteine showed a LOD level 5-fold higher in urine compared to pure solvent, indicating ion suppression in matrix. Under identical conditions, acetaminophen mercapturic acid showed a five times higher sensitivity in urine with IDA CNL than with IDA thMRM. This feature could not be explained. Overall, the IDA thMRM method was equal to the IDA CNL method for all standards.

Table 11: LOD levels [pmol on column] of standard mercapturic acids spiked to rat urine and to CH_3OH/H_2O (1:2 v/v), respectively. Detection of the signals were obtained by IDA CNL/negative EPI (method 3) and IDA thMRM/negative EPI (method 4). LOD was defined as the concentration of analyte yielding the recording of at least two out of three EPI scans and giving a signal (S/N ratio > 3) with a CNL of 129 Da.

Compound	IDA thMRM		IDA CNL	
	Urine pmol o.c.	CH_3OH/H_2O pmol o.c.	Urine pmol o.c.	CH_3OH/H_2O pmol o.c.
N-Acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine	15.5	15.5	15.5	15.5
N-Acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine	0.8	0.8	0.8	0.8
N-Acetyl-*S*-(4-chlorobenzyl)-L-cysteine	0.3	0.3	0.3	0.3
N-Acetyl-*S*-(2-fluorobenzyl)-L-cysteine	0.4	0.4	0.4	0.4
N-Acetyl-*S*-(3-hydroxypropyl)-L-cysteine	n.d.	2.3	n.d.	2.3
N-Acetyl-*S*-(4-bromobenzyl)-L-cysteine	0.6	0.6	0.6	0.3
N-Acetyl-*S*-(4-methoxybenzyl)-L-cysteine	0.7	0.4	0.7	0.4
N-Acetyl-*S*-(4-*tert*-butylbenzyl)-L-cysteine	0.3	0.3	0.3	0.3
N-Acetyl-*S*-[5-(acetylamino)-2-hydroxyphenyl]-L-cysteine	3.2	3.2	0.6	3.2
N-Acetyl-*S*-(1,1-Dichloro-2,2-difluoroethyl)-L-cysteine	3.4	1.7	6.8	3.4
N-Acetyl-*S*-phenyl-L-cysteine	2.1	0.4	2.1	0.4
N-Acetyl-*S*-(2,4-dinitrophenyl)-L-cysteine	0.6	0.6	0.6	0.3
N-Acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine	n.d.	6.8	n.d.	6.8

n.d. not determined

The CNL methods were compared with the corresponding MRM methods to evaluate each method for specifity and sensitivity with and without insertion of the IDA feature. A mass range from *m/z* 200 to *m/z* 450 was set for CNL and thMRM. CNL was compared with IDA CNL, MRM for 251 mass transitions (thMRM 251) with IDA thMRM for the same 251 mass transitions, and with MRM for 25 mass transitions (thMRM 25) respectively. S/N ratios were slightly, but not significantly higher in the single survey scans with CNL and thMRM compared to the methods using IDA. S/N ratios of thMRM 25 compared to S/N ratios of thMRM 251 were significantly higher (an average of factor 5) (Figure 2). S/N ratios of thMRM 251 compared to CNL was not significantly higher. A comparison of CNL with a mass range of only 25 Da i.e. from *m/z* 200 to *m/z* 225 with thMRM 25 was not performed since the molecular masses of the standards ranged from *m/z* 221 to *m/z* 332.

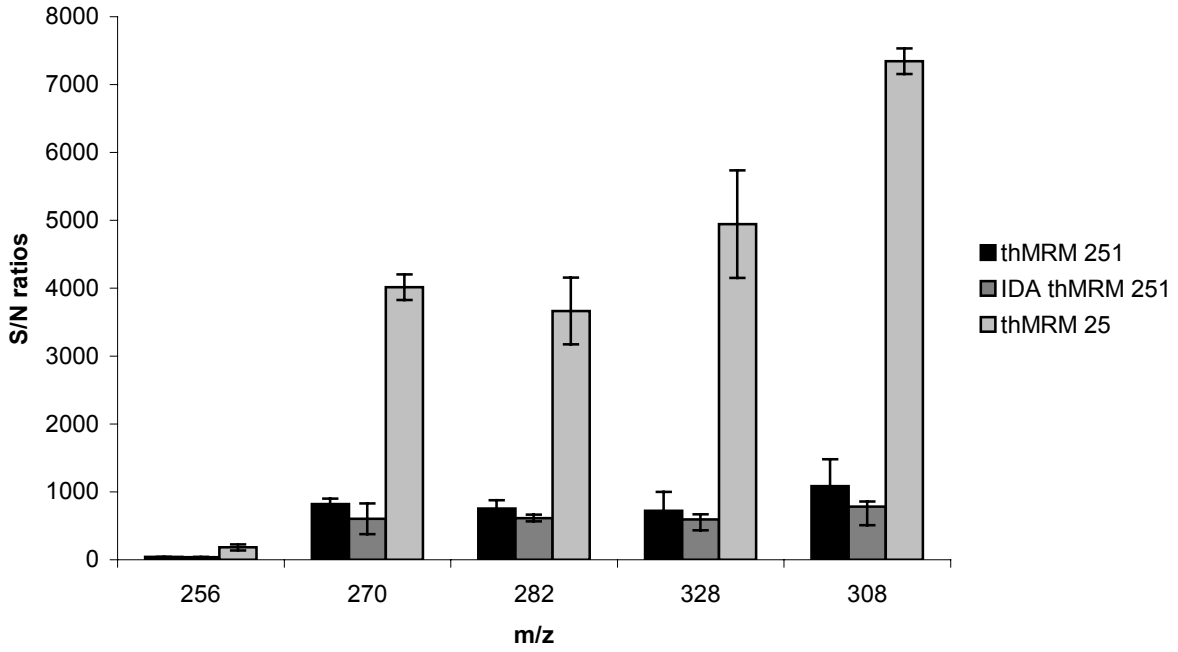


Figure 2: Comparison of average S/N ratios and absolute standard deviation of various mercapturic acid standards determined with MRM scan mode with 25 mass transitions (thMRM 25), 251 mass transitions with IDA (IDA thMRM 251) or without IDA (thMRM 251).

4.1.3 Validation of the Methods

Optimized IDA CNL/negative EPI and IDA thMRM/negative EPI methods were validated with the mercapturic acid standard acetaminophen mercapturic acid (AAP-MA) in blank urine. At a concentration of 0.025 µg/ml of AAP-MA, LOD was reached with a S/N ratio of 3 and LOQ (S/N = 9) was obtained at a concentration of 0.1 µg/ml. Due to validation of the analytical method only, each method and sample was measured ten times in a row in three different blocks. To test IDA methods 3 and 4 as potential quantitation methods, they were compared to the conventional quantitation method, i.e. using MRM scan mode with two mass transitions, the mass transition from *m/z* 311.1 to *m/z* 139.1 (qualifier) and the mass transition from *m/z* 311.1 to *m/z* 182.1 (quantifier) together with the internal standard (*m/z* 328.1 to *m/z* 199.1) with the corresponding optimized compound-dependent parameters. LOD and LOQ were identical for both IDA methods as well as the dynamic range from 0.3 to 100 µg/ml. Accuracy of IDA thMRM ranged from 93% to 118% and is therefore within the accuracy tolerance for a quantitation method (80% to 120% tolerance). For IDA CNL, accuracy was out of the tolerable range (81% to 125%). The regression factor exceeded the value of 0.99 in all methods tested. Precision and standard error of the IDA methods were 3 to 5 times higher than the conventional MRM method in both samples of high dose and low dose spiked urine. Interday validation showed the same results (Table 12). Due to accuracy and precision, the two methods are not optimal for quantitation. Precision should not exceed a value of 3% for analytical methods. However they can be used for qualitative determinations and semi-quantitative predictions.

Table 12: Validation of the IDA CNL/negative EPI method and the IDA thMRM/negative EPI method with acetaminophen mercapturic acid in rat urine and comparison with results of a conventional MRM method.

INTRADAY	n = 10 **IDA thMRM**	n = 10 **IDA CNL**	n = 10 **MRM**
LOD (S/N 3) [µg/ml]	0.025	0.025	n.d
LOQ (S/N 9) [µg/ml]	0.1	0.1	n.d
Dynamic range	0.3 - 100 (quadratic)	0.3 - 100 (quadratic)	0.3 - 30 (linear)
Accuracy [%]	93 - 118	81 - 125	84 - 110
Regression factor	0.9975	0.9967	0.9927
Precision [%] (1.5 µg/ml)	6	6	2
Standard error (1.5 µg/ml)	0.03	0.03	0.01
Precision [%] (25 µg/ml)	11	8	2
Standard error (25 µg/ml)	0.97	0.64	0.15
INTERDAY	n = 3 **IDA thMRM**	n = 3 **IDA CNL**	n = 2 **MRM**
Precision [%] (1.5 µg/ml)	8	10	2
Standard error (1.5 µg/ml)	0.04	0.05	0.01
Precision [%] (25 µg/ml)	10	9	1
Standard error (25 µg/ml)	0.86	0.70	0.09

Both IDA methods are equally sensitive and comparable in reproducibility and robustness. The IDA CNL/negative EPI method was selected for further studies on the generic detection of mercapturic acids generated *in vitro* and *in vivo*. Mass ranges for metabolites of interest could be easily and rapidly changed. The amount of

data obtained was much less than for in the IDA thMRM/negative EPI method and reduced time for data analysis.

4.1.4 Evaluation of the IDA CNL/negative EPI Method for the Detection of Mercapturic Acids

The selected information dependent acquisition method using the constant neutral loss of 129 Da combined with two enhanced product ion scans (IDA CNL/negative EPI) for the general detection and characterization of mercapturic acids was evaluated by generation of mercapturic acids of various compounds known to form reactive intermediates. Acetaminophen, diclofenac, troglitazone, clozapine, bifonazole, carbamazepine, and bisphenol A were chosen as model substrates to test the suitability of the analytical approach for the detection of mercapturic acid formation of xenobiotics in different matrices, including rat and human liver microsomes, baculovirus expressed human CYP450 isoenzymes, and isolated human neutrophils. Differences in mercapturic acid formation detected in these dissimilar assays were evaluated. Detection of mercapturic acids of acetaminophen and diclofenac was also performed in rat urine to evaluate the method for urine samples. Acetaminophen mercapturic acid was detected and quantified in human urine. Novel signals not assigned to known structures were further evaluated and characterized according to identical reference compounds or to their MS/MS mass spectra.

Prior to incubation tests with human neutrophils, these cells had to be isolated from human whole blood (chapter 3.4.2.1) and purity had to be determined on a flow cytometer by FACScan (FACS: Fluorescence activated cell sorting). By measuring forward light scatter (FSC) and side scatter (SSC), size and granularity of the cells could be determined.

When isolated neutrophils were analyzed by flow cytometry, three different cell populations were identified in the resulting dot- and density-plots (Figure 3). The largest population consisted of pure neutrophils due to their great granularity and size. The remaining populations were lymphocytes (similar size with low granularity) [10] and presumably cell fragments (small size with low granularity). By encircling each population, percentage of isolated neutrophils could be calculated. Purities of

about 90% of neutrophils were achieved. Contamination by lymphocytes never exceeded 5%.

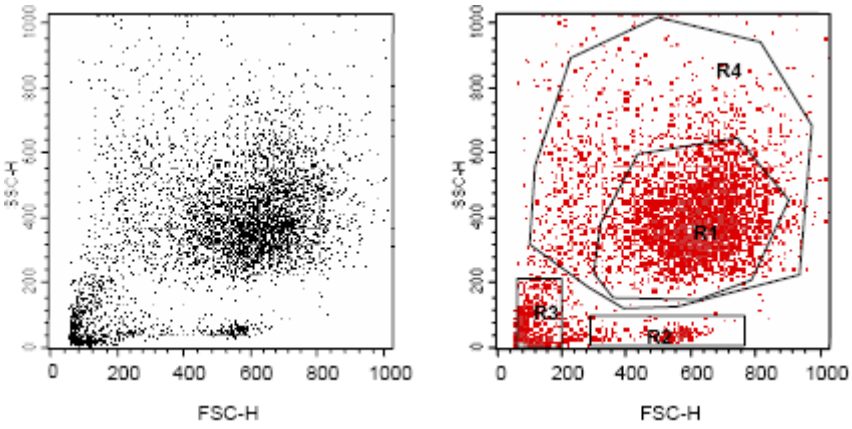
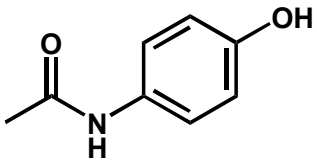


Figure 3: Dot-plot (left) and density-plot (right) of the isolated human neutrophils (5,000 events counted). (**Gate R1:** population of neutrophils with similar granularity and size; **Gate R4:** population of neutrophils; **Gate R2:** lymphocytes; **Gate R3:** cell fragments)

4.1.4.1 Generation and Detection of Mercapturic Acids *in vitro*

4.1.4.1.1 Acetaminophen

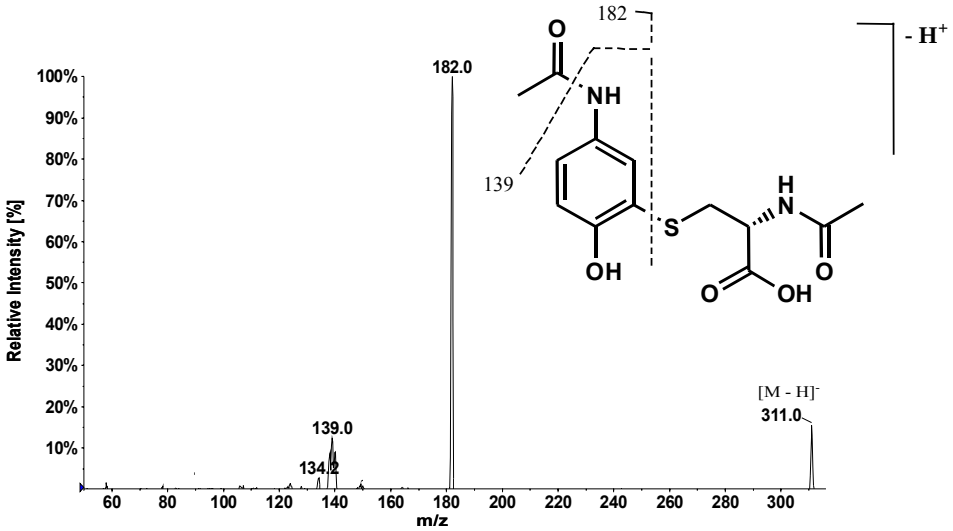


Chemical structure of acetaminophen

Upon incubation of acetaminophen with rat and human liver microsomes in the presence of *N*-acetyl-L-cysteine, one signal occurred with a precursor ion at *m/z* 311. This compound was assigned to acetaminophen mercapturic acid (AAP-MA) according to chromatographic and mass spectrometric properties in positive and negative ion mode that were identical to the chemically synthesized standard (Figure 4). The MS/MS mass spectrum in negative ion mode showed prominent fragment ions at *m/z* 182, *m/z* 139, and *m/z* 134. The fragment ion at *m/z* 182 is formed after cleavage of the sulfur-carbon bond (loss of 129 Da). The product ion at *m/z* 134 originates from an additional loss of water and H_2S, while the fragment ion at *m/z* 139 could be assigned to the cleavage of the acetamide moiety. Analysis of incubation

solution of acetaminophen with recombinant CYP2E1 in the presence of *N*-acetyl-L-cysteine demonstrated the contribution of this isoenzyme to the formation of NAPQI and therefore the conjugation with *N*-acetyl-L-cysteine to form acetaminophen mercapturic acid. In incubations of acetaminophen with activated neutrophils, a small signal with *m/z* 311 (S/N 10) and identical retention time to an authentic standard was detected after addition of *N*-acetyl-L-cysteine, demonstrating the formation of this metabolite in activated neutrophils. The signal did not occur in neutrophils that have not been activated with phorbol-12-myristate-13-acetate (PMA) before. Incubation with human liver microsomes additionally revealed a second signal with *m/z* 327 at a retention time of 6.1 minutes. Further characterization of the metabolite demonstrated formation of 3-hydroxyacetaminophen in human liver microsomal incubations (chapter 4.1.5).

A)



B)

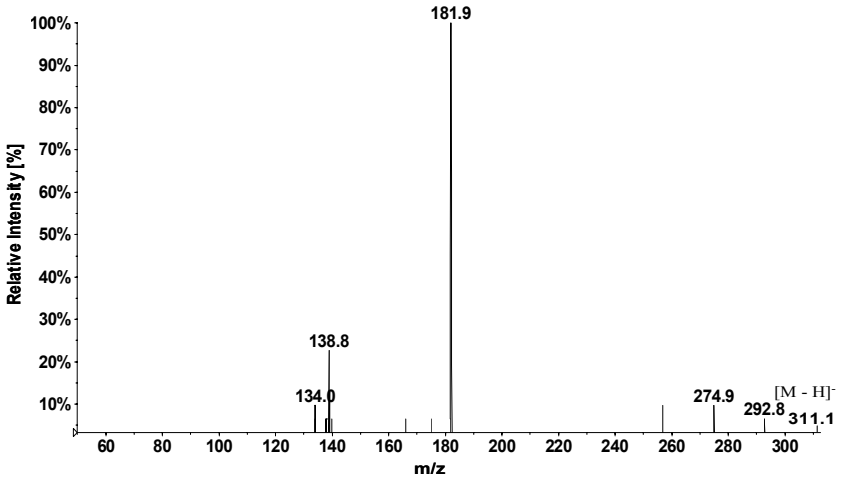
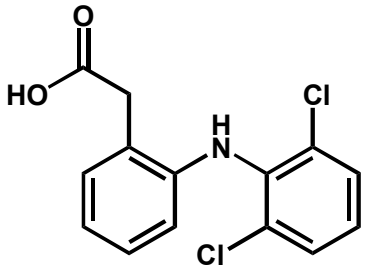


Figure 4: MS/MS mass spectrum of the authentic standard AAP-MA obtained with Quantitation Optimization Software and the proposed fragmentation (A). MS/MS mass spectrum of the signal from rat liver microsomal incubation with acetaminophen in the presence of *N*-acetyl-L-cysteine obtained with IDA CNL/negative EPI with two collision energies, summed in pane B.

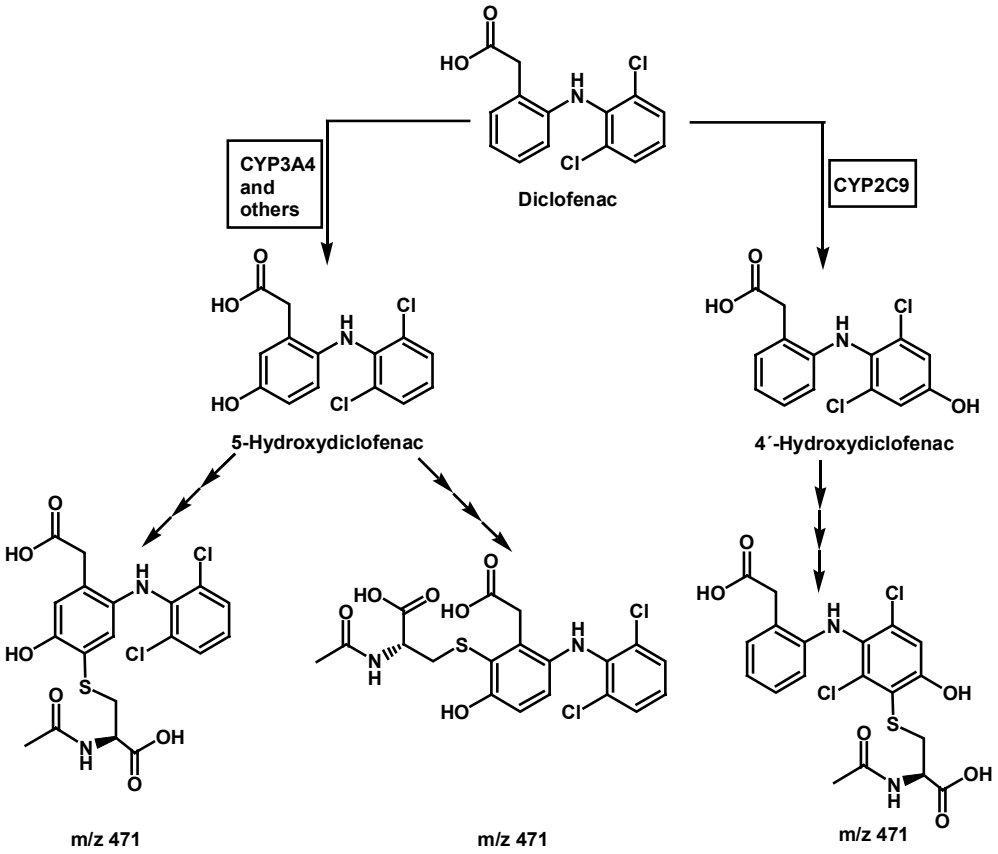
4.1.4.1.2 Diclofenac



Chemical structure of diclofenac

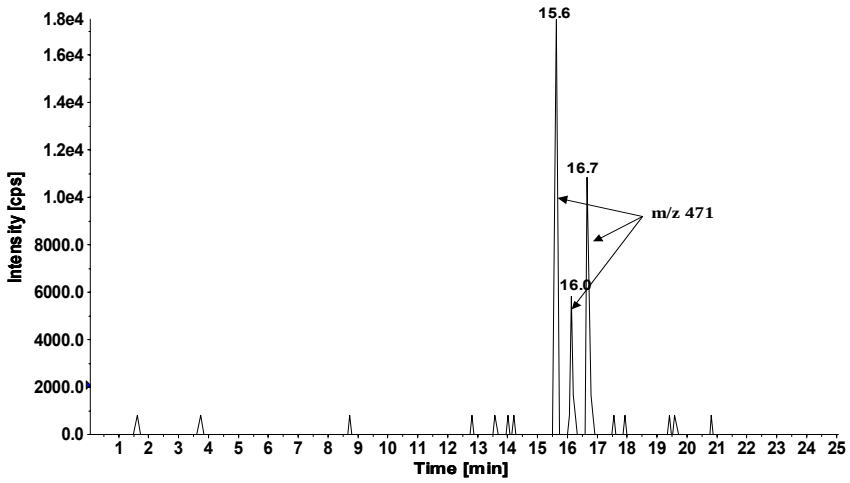
In incubations of diclofenac with hepatic microsomes from rats in the presence of *N*-acetyl-L-cysteine, three metabolites with *m/z* 471 were detected in negative ion mode (Figure 5). After acquisition of product ion spectra using the linear ion trap, the peaks with *m/z* 471 were identified as mercapturic acids of hydroxydiclofenac. Fragmentation of these precursor ions resulted in the formation of predominant product ions at *m/z* 162 and *m/z* 342. The fragment ion at *m/z* 162 was assigned to the *N*-acetyl-L-cysteine moiety, whereas the fragment ion at *m/z* 342 resulted from the neutral loss of 129 Da. The ion at *m/z* 298 was formed by the combined loss of 129 Da and of 44 Da (CO_2). The specific product ion at *m/z* 262 is generated by an additional loss of HCl. In addition, the chlorine isotopic pattern characteristic for the presence of two chlorine atoms in the molecule was used to confirm the presence of

diclofenac metabolites. The mass spectrum of this metabolite after positive ionization has previously been described by Poon et al. and was similar to the mass spectrum obtained in this work [87]. Additionally, diclofenac was incubated with human liver microsomes in the presence of *N*-acetyl-L-cysteine. In this assay, one signal with a precursor ion at *m/z* 471 was detected (t_R = 16.1 minutes), whereas two signals with precursor ions at *m/z* 471 (t_R = 15.7 and 16.7 minutes) occurred in incubation solutions with activated neutrophils. For assignment of the three isobaric mercapturic acids, diclofenac was incubated with the diclofenac metabolizing isoenzymes CYP2C9 and CYP3A4. Diclofenac is metabolized by CYP2C9 to form 4´-hydroxydiclofenac and by CYP3A4 for 5-hydroxylation of diclofenac (Scheme 9) [88, 89]. Thus, according to *N*-acetyl-L-cysteine conjugates formed in the isoenzymatic incubations, the mercapturic acids of 5-hydroxylated diclofenac and 4´-hydroxylated diclofenac was assigned to the signals obtained in rat and human liver microsomal incubations. In incubations of diclofenac with CYP3A4 in the presence of *N*-acetyl-L-cysteine two signals (t_R = 15.7 and 16.7 minutes) were detected and, thus, could be assigned to the mercapturic acids of 5-hydroxydiclofenac. The peak (t_R = 16.2 minutes) observed in incubation with CYP2C9 was assigned to the mercapturic acid of the 4´-hydroxylated diclofenac (Figure 6). One signal with a mass transition from *m/z* 471 to *m/z* 342 (t_R = 16.4 minutes) was observed in incubation solutions and in control samples without substrate. In conclusion, diclofenac was metabolized to the mercapturic acid of 4´-hydroxydiclofenac in human liver microsomal incubation, whereas activated human neutrophils provided the reactive intermediate of 5-hydroxydiclofenac. Incubations with rat liver microsomes revealed the formation of both hydroxydiclofenac metabolites.



Scheme 9: Oxidative metabolic pathways of diclofenac catalyzed by CYP450 isoenzymes, leading to the formation of three isobaric mercapturic acids.

A)



B)

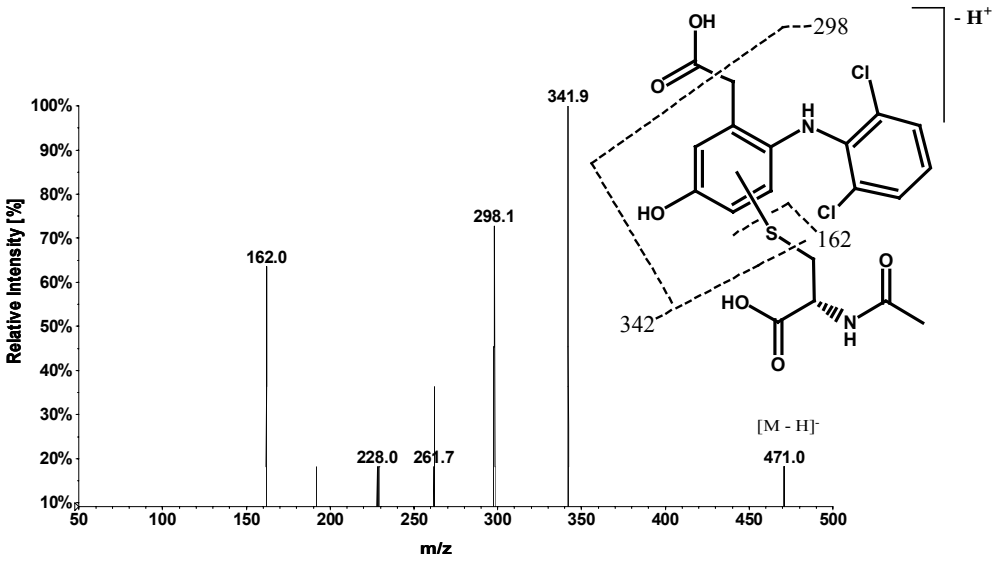
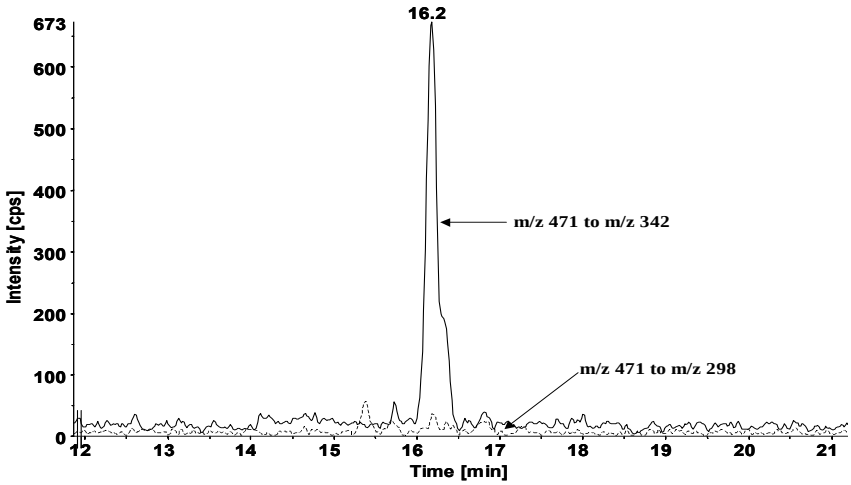


Figure 5: CNL chromatogram (A) and MS/MS mass spectrum of the precursor ion at *m/z* 471 (t_R = 15.6 minutes) (B) of rat liver microsomal incubation with diclofenac in the presence of *N*-acetyl-L-cysteine, obtained with method 3. EPI scan of the peak with t_R = 16.0 minutes was not performed. Mass spectrum of the peak with t_R = 16.7 minutes was identical to the fragmentation pattern shown.

A)



B)

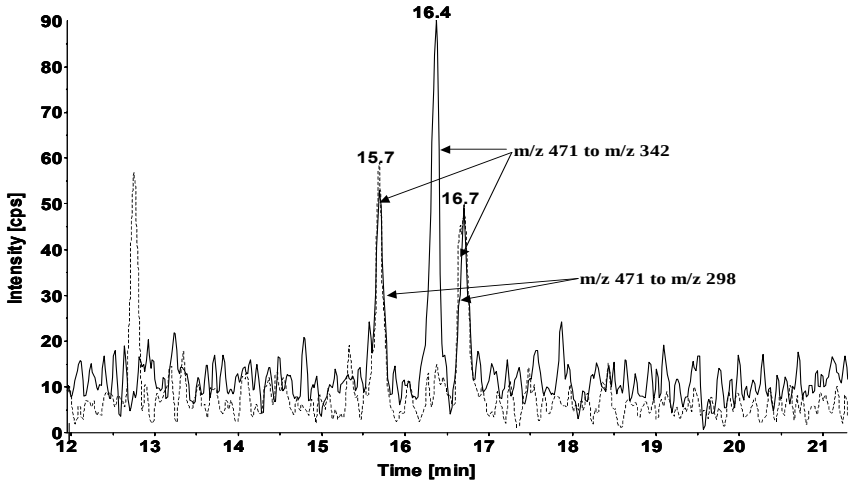
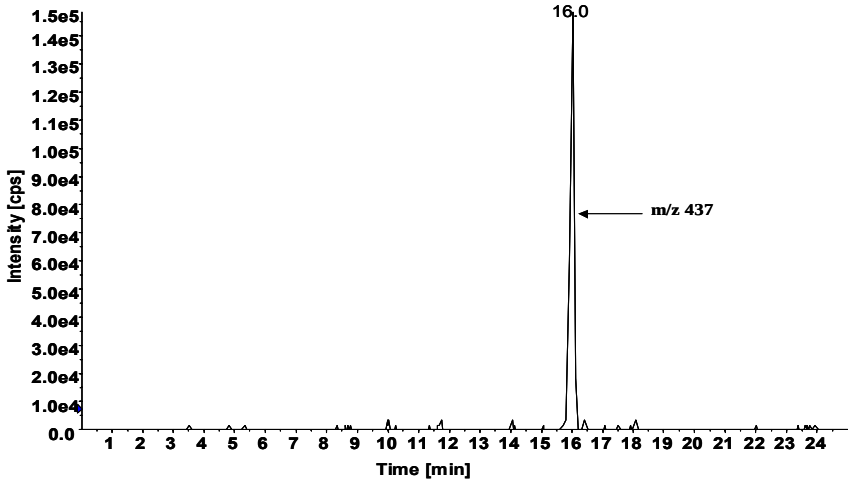


Figure 6: MRM chromatogram of diclofenac incubation with CYP2C9 (A) and CYP3A4 (B) with mass transitions from *m/z* 471 to *m/z* 342 (--) and from *m/z* 471 to *m/z* 298 (---). A signal with mass transition from *m/z* 471 to *m/z* 342 (t_R = 16.4 minutes) occurred in control incubation solution without substrate.

An additional mercapturic acid of a diclofenac metabolite with *m/z* 437 was detected in rat and human liver microsomal incubations of diclofenac in the presence of *N*-acetyl-L-cysteine. The MS/MS mass spectrum in positive ion mode suggested the chemical structure of *N*-acetyl-*S*-{2-[2´-(carboxymethyl)phenylamino]-3-chloro-5-hydroxyphenyl}-L-cysteine (deschlorodiclofenac-MA) as described by Yu et al. [90] for the corresponding GSH adduct of monoclofenac in human liver microsomes. Fragmentation of this mercapturic acid was similar to the fragmentation described by Yu et al. for the glutathione adduct. Typical fragment ions observed were formed by the loss of water (*m/z* 421), an additional loss of HCOOH (*m/z* 375), or loss of HCl

(*m/z* 403) (Figure 7). Additionally, the enhanced resolution (ER) scan in positive ion mode showed the typical chlorine isotopic pattern for one chlorine atom in the molecule (100% to 32%). This metabolite was not observed in incubations with activated neutrophils.

A)



B)

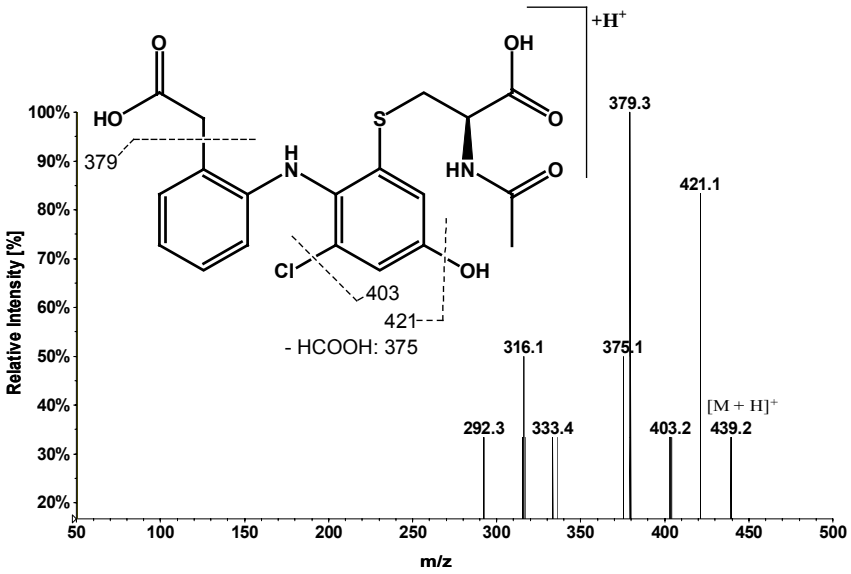


Figure 7: HPLC chromatogram of the precursor ion at *m/z* 437 assigned to *N*-acetyl-*S*-{2-[2´-(carboxymethyl)phenylamino]-3-chloro-5-hydroxyphenyl}-L-cysteine generated in rat liver microsomes (A) and its mass spectrum obtained after positive ionization (B). Data acquisition was performed with CNL survey scan in negative ion mode and EPI scan in positive ion mode (method 5).

A signal with *m/z* 459 was detected only in human liver microsomal incubation of diclofenac in the presence of *N*-acetyl-L-cysteine. The signal showed the chlorine isotopic pattern typical for two chlorine atoms in a molecule (100% to 64%) (Figure 8). The isotopic pattern, the characteristic constant neutral loss for mercapturic acids

and the lack of this signal in control samples suggested a metabolite of diclofenac. With negative ion mode, fragment ions occurred that were assigned to diclofenac metabolite, i.e. *m/z* 330 as the CNL of 129 Da, *m/z* 286 indicating loss of CO_2, and *m/z* 227 which may correspond to the fragment of 229 observed after CID of the mercapturic acid of hydroxydiclofenac. This metabolite was not characterized further.

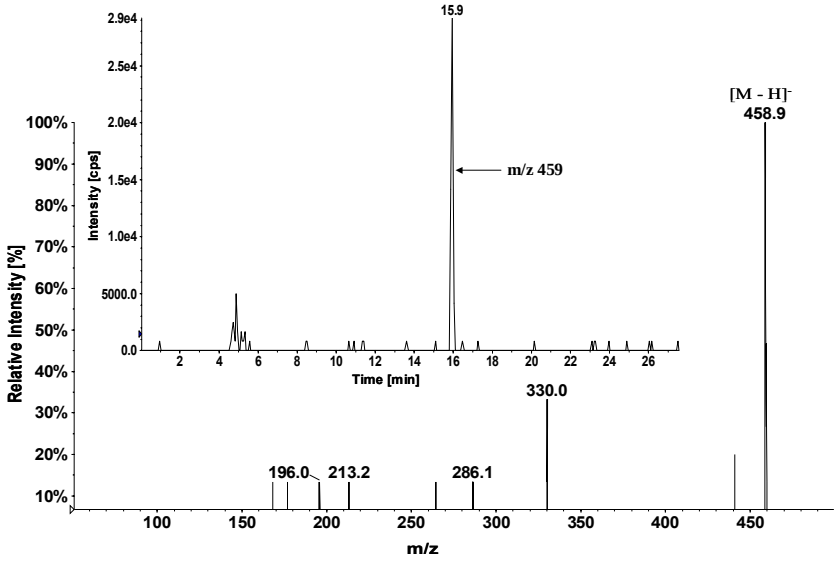
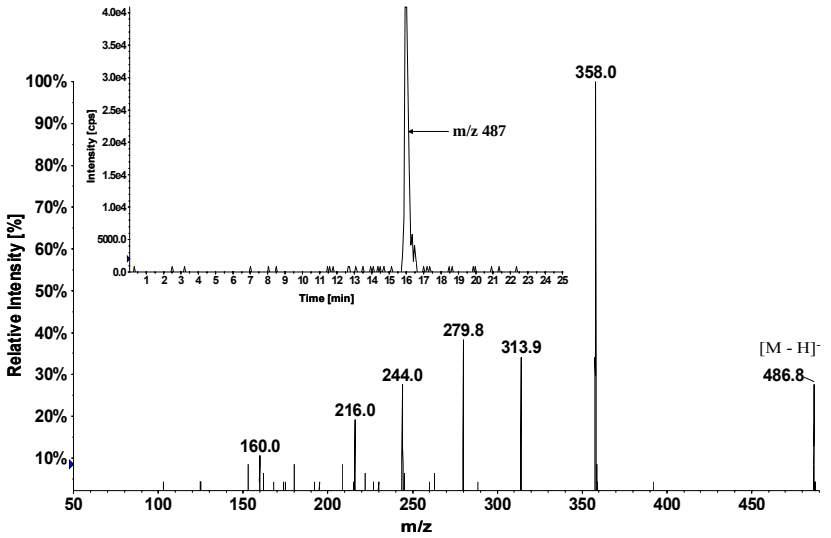


Figure 8: MS/MS mass spectrum and CNL chromatogram of a signal with precursor ion at *m/z* 459 of an incubation of diclofenac with human liver microsomes in the presence of *N*-acetyl-L-cysteine, obtained with method 3.

Two additional signals with *m/z* 441 and *m/z* 487 occurred, when diclofenac was incubated with activated human neutrophils. Retention time and MS/MS mass spectra in positive and negative ion mode were identical to the signal observed in rat urine samples (chapter 4.1.4.2.2). The signal was assigned to the metabolite formed by decarboxylation of 5-hydroxydiclofenac and peroxidase-mediated activation as described previously by Miyamoto et al. [91]. The precursor ion at *m/z* 487 suggested formation of either a mercapturic acid of dihydroxylated diclofenac or the sulfoxide of the mercapturic acid of one of the hydroxydiclofenac metabolites (Figure 9). However, unequivocal identification of this metabolite could not be performed due to the lack of available standards.

A)



B)

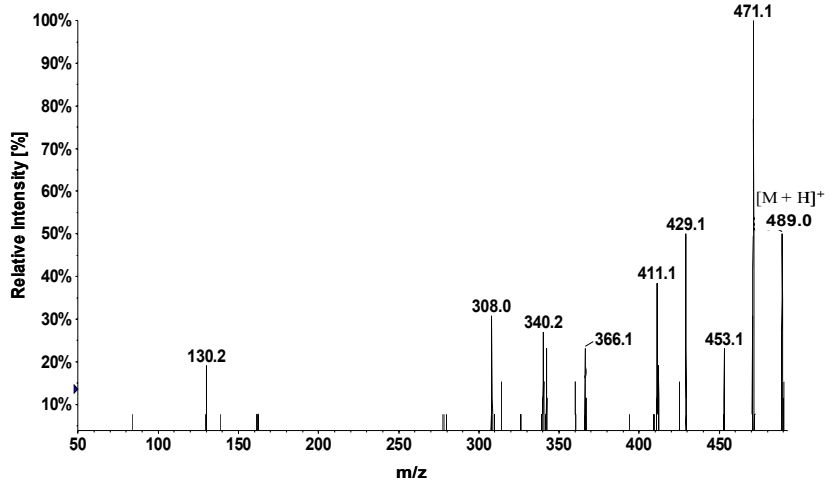
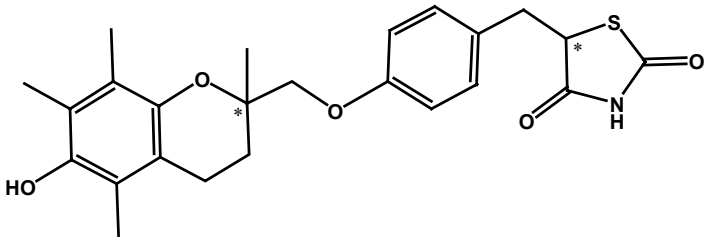


Figure 9: MS/MS mass spectra and CNL chromatogram of the precursor ion at *m/z* 487 in negative ion mode (A) and in positive ion mode (B). The signal was obtained in incubations of diclofenac with activated neutrophils in the presence of *N*-acetyl-L-cysteine with method 3. Mass spectra were obtained with EPI scan at two different collision energies, summed in a single pane.

4.1.4.1.3 Troglitazone



Chemical structure of troglitazone

Upon incubations of troglitazone with rat and human liver microsomes in the presence of *N*-acetyl-L-cysteine, predominant signals with *m/z* 601 and *m/z* 635 were detected. The product ion spectra of these signals were recorded (Figure 10). Two mercapturic acids of troglitazone-derived reactive intermediates were characterized. MS/MS mass spectra of the signals revealed common fragment properties accounting for the constant neutral loss of 129 Da typical for mercapturic acids and an additional loss of H_2S. In contrast to the signal with *m/z* 635, a neutral loss of 43 Da indicated scission of the thiazolidinedione ring of the metabolite with *m/z* 601. The molecular weights and fragmentation patterns of the two *N*-acetyl-L-cysteine adducts in positive and negative ion mode suggested oxidative cleavage of the thiazolidinedione ring (M_r = 636) and oxidation of the substituted chromane moiety to a reactive quinone methide (M_r = 602) yielding electrophiles that were trapped with *N*-acetyl-L-cysteine. In addition, another troglitazone-derived metabolite was revealed using the developed generic screening method. The metabolite with *m/z* 605 showed common constant neutral losses and fragment ions identical to the two mercapturic acids described (Figure 11). The suggested mercapturic acid of troglitazone was related to a GSH conjugate previously described by Alvarez-Sánchez et al. [92]. Identical fragmentation properties of the second signal (t_R = 16.3 minutes) suggested a stereoisomer. Additional signals with *m/z* 310 (t_R = 14.3 minutes), *m/z* 312 (t_R = 3.2 minutes), *m/z* 326 (t_R = 20.3 minutes, S/N close to LOD), *m/z* 328 (t_R = 11.8 minutes) were detected, indicating formation of a panel of different reactive intermediates in the lower mass range. These metabolites may result from metabolic degradation of troglitazone, e.g. to quinones.

A)

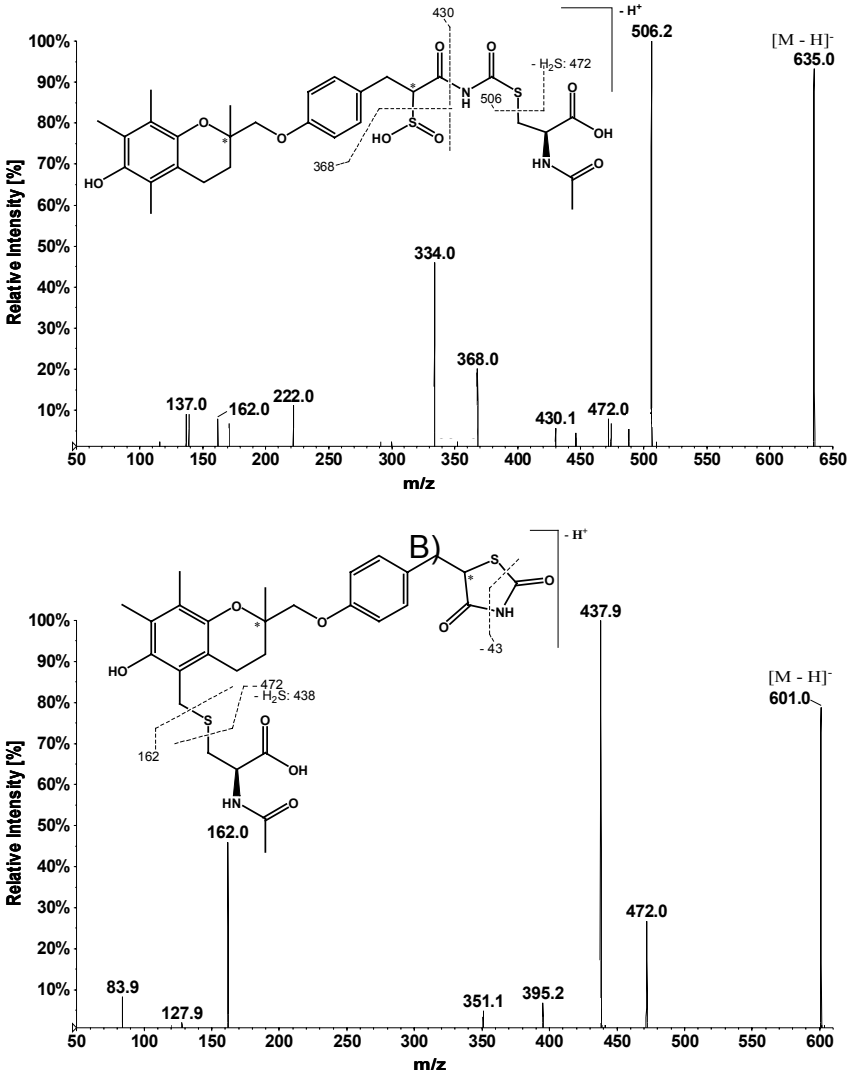
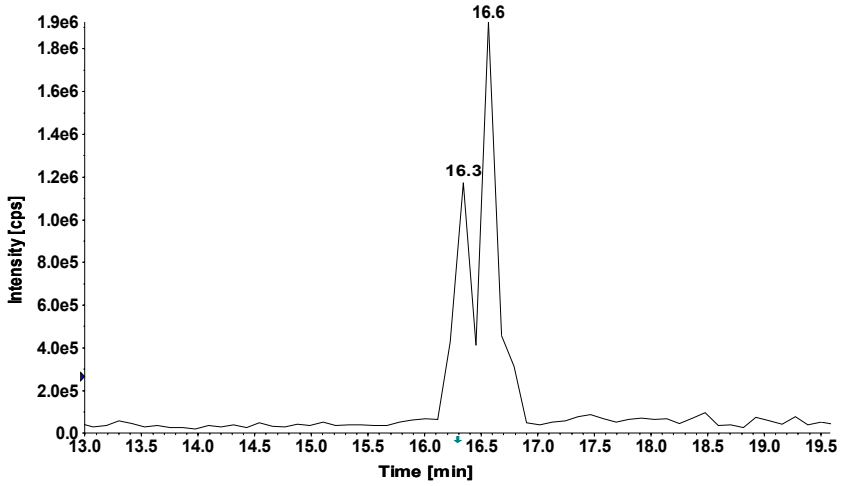


Figure 10**:** MS/MS mass spectra of the signals with *m/z* 635 (t_R = 15.2 minutes) (A) and *m/z* 601 (t_R = 20.0 minutes) (B) detected after incubation of troglitazone with rat liver microsomes in the presence of *N*-acetyl-L-cysteine. Mass spectra were obtained in EPI scan mode after negative ionization. Structures with the proposed fragment ions are included.

A)



B)

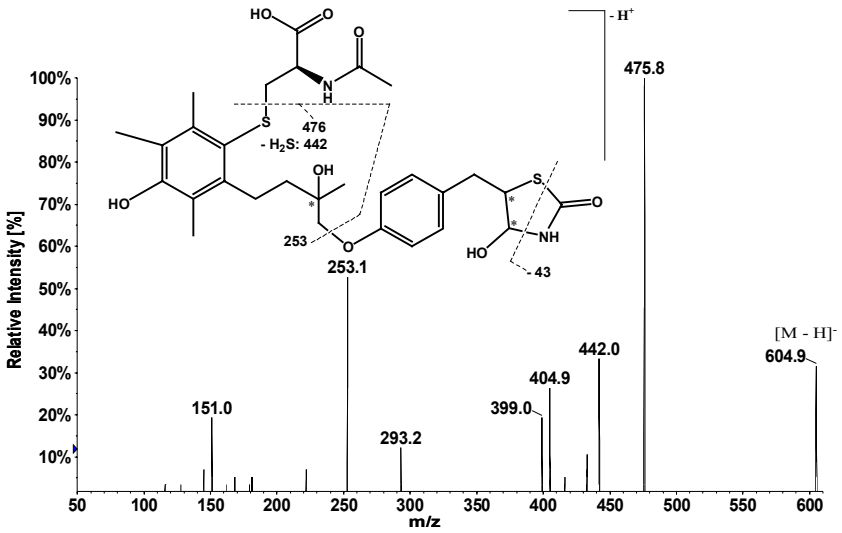
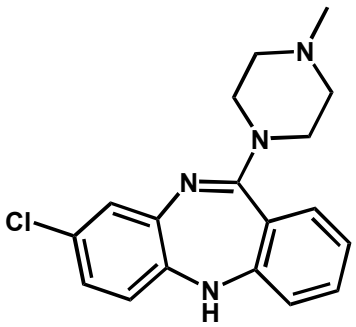


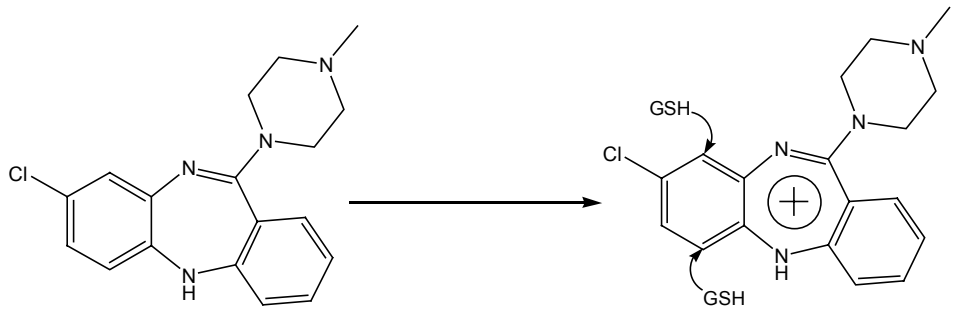
Figure 11: HPLC chromatogram of the metabolites with *m/z* 605 (A) and the corresponding MS/MS mass spectrum of the signal (t_R = 16.6 minutes) obtained in negative ion mode (B). The signals were detected in rat liver microsomal incubation with troglitazone in the presence of *N*-acetyl-L-cysteine. Fragmentation of the second peak (t_R = 16.3 minutes) was identical to B, suggesting a stereoisomer.

4.1.4.1.4 Clozapine



Chemical structure of clozapine

Two metabolites with *m/z* 486 in negative ion mode (t_R = 11.4 minutes and 11.6 minutes) were detected which did not occur in control incubations without substrate. Predominant product ions at *m/z* 357 (characteristic CNL of 129 Da of mercapturic acids), at m/z 259 (additional loss of the *N*-methylpiperazine moiety), and at m/z 223 (additional loss of HCl) were characteristic for the presence of a mercapturic acid adduct of clozapine. Fragmentation patterns after positive ionization were similar to the mass spectrum of the corresponding GSH adducts published by Maggs et al. [93]. According to the MS/MS mass spectra, peaks were assigned to *N*-acetyl-L-cysteine conjugates of clozapine in C6 and C9 position of the 5H-dibenz[b,f]azepine structure (*m/z* 486) previously described by Zhou et al. [94]. Incubations with activated and non-activated neutrophils provided the same mercapturic acids of the clozapine nitrenium ion.

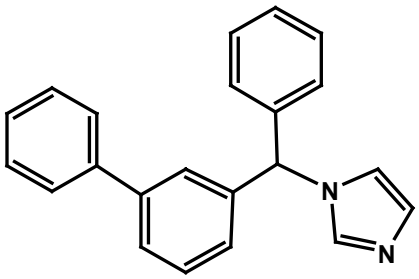


Scheme 10: Proposed bioactivation of clozapine to nitrenium ion and electrophilic sites of the reactive intermediate for conjugation with glutathione.

A metabolite with *m/z* 472 (t_R = 11.1 minutes) occurred only in incubations of clozapine with human liver microsomes. This *N*-acetyl-L-cysteine adduct was not further characterized but may originate from demethylated clozapine due to common

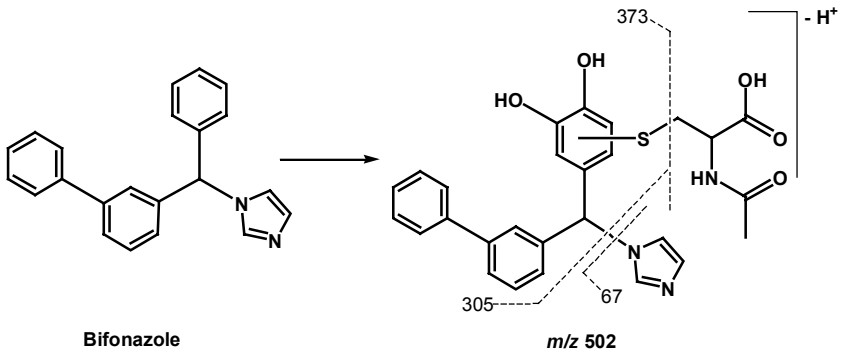
fragmentation properties such as constant neutral loss of 129 Da and additional loss of the piperazine ring to yield a product ion at *m/z* 259.

4.1.4.1.5 Bifonazole



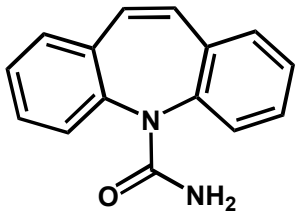
Chemical structure of bifonazole

Upon incubations of bifonazole with human liver microsomes, a mercapturic acid with a precursor ion at *m/z* 502 (t_R = 12.0 minutes) was formed. Fragmentation of this signal in positive and negative ion mode suggested hydroxylation on an aromatic ring at two sites and oxidation to a quinone that was trapped by *N*-acetyl-L-cysteine. Fragmentation after negative ionization provided fragment ions at *m/z* 373, *m/z* 305, and *m/z* 67. Cleavage of the sulfur carbon bond with a CNL of 129 Da as described for all mercapturic acids resulted in the detection of a fragment ion at *m/z* 373. Further cleavage of the imidazole ring formed a fragment ion at *m/z* 305. This ring subsequently provided a product ion at *m/z* 67 (Scheme 11) [95]. In positive ion mode, most prominent fragment ions were detected at *m/z* 436, *m/z* 307, *m/z* 287, and at *m/z* 274. Again, imidazole was dissociated from the remaining molecule to form a product ion at *m/z* 436. Due to this direct loss from the precursor ion at *m/z* 504, *N*-acetyl-L-cysteine conjugation and hydroxylation at the imidazole moiety was excluded. The product ion at *m/z* 307 was formed by cleavage of the sulfur carbon bond. A loss of the entire *N*-acetyl-L-cysteine moiety resulted in detection of a fragment ion at *m/z* 274 (from *m/z* 436). Additionally, mercapturic acids with precursor ions indicative for mono- and dihydroxylated bifonazole were detected in liver microsomal incubations. MS/MS mass spectra showed fragmentation patterns similar to the parent compound bifonazole. Bifonazole did not form any detectable signal with *m/z* 502 in incubation with activated human neutrophils.



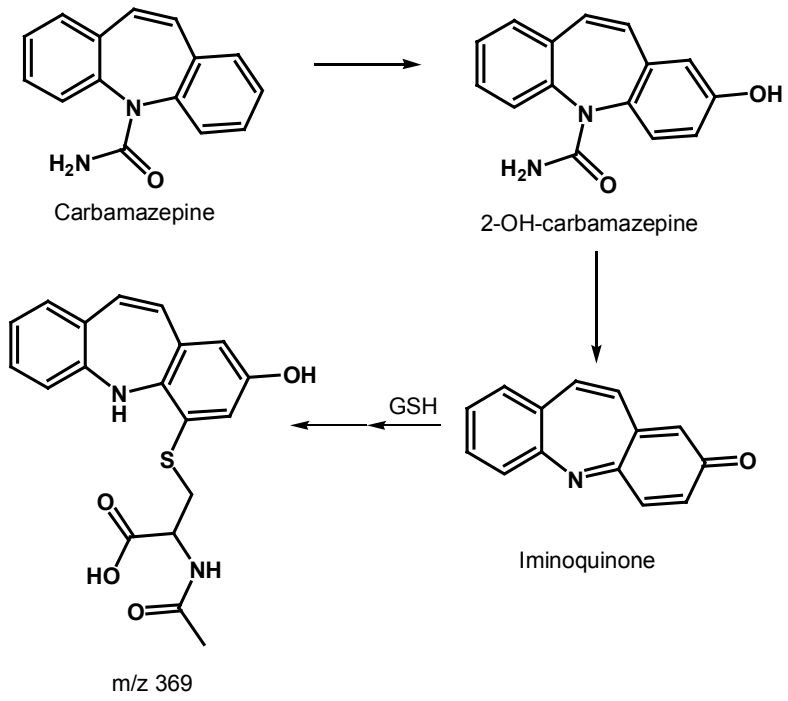
Scheme 11: Proposed metabolism of bifonazole, trapped with *N*-acetyl-L-cysteine with assignment of the fragment ions detected in negative ion mode by HPLC-MS/MS.

4.1.4.1.6 Carbamazepine



Chemical structure of carbamazepine

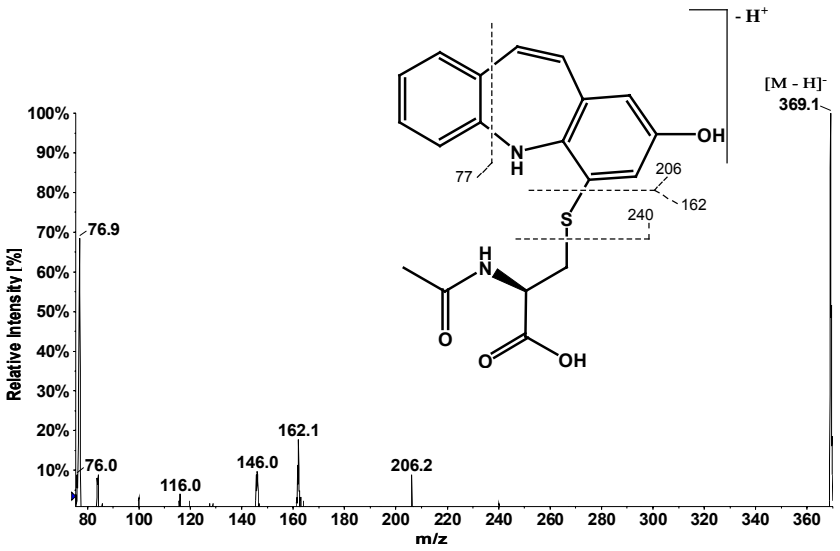
A mercapturic acid of carbamazepine with *m/z* 369 (t_R = 9.3 minutes) was formed in incubations with human liver microsomes and activated human neutrophils. Fragmentation of the metabolite with *m/z* 369 could be obtained in negative and positive ion modes. According to the product ions formed during CID of the precursor ion, a proposal for the fragmentation pattern of the metabolite is given in Figure 12. This mercapturic acid was presumably formed by conjugation of *N*-acetyl-L-cysteine with the reactive iminoquinone after hydroxylation at C2 and hydrolysis as described by Ju et al. [39] for GSH conjugation (Scheme 12).



Scheme 12: Proposed bioactivation of carbamazepine to iminoquinone and conjugation with *N*-acetyl-L-cysteine. The conjugate with m/z 369 was detected with the developed HPLC-MS/MS screening method.

An additional reactive intermediate of carbamazepine that was trapped with *N*-acetyl-L-cysteine provided a precursor ion at *m/z* 351 (t_R = 9.6 minutes) after negative ionization. Fragmentation patterns of this novel metabolite after positive and negative ionization are shown in Figure 13. The common fragmentation behaviour of this metabolite such as loss of $H_2C=C=O$ (- 42 Da), loss of the *N*-acetyl-L-cysteine moiety (- 163 Da) and a common product ion at *m/z* 116 after positive ionization suggests the formation of a carbamazepine-derived mercapturic acid.

A)



B)

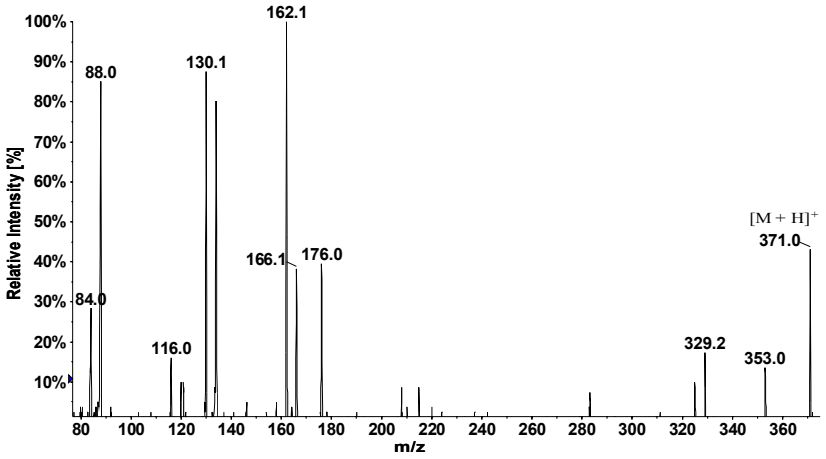
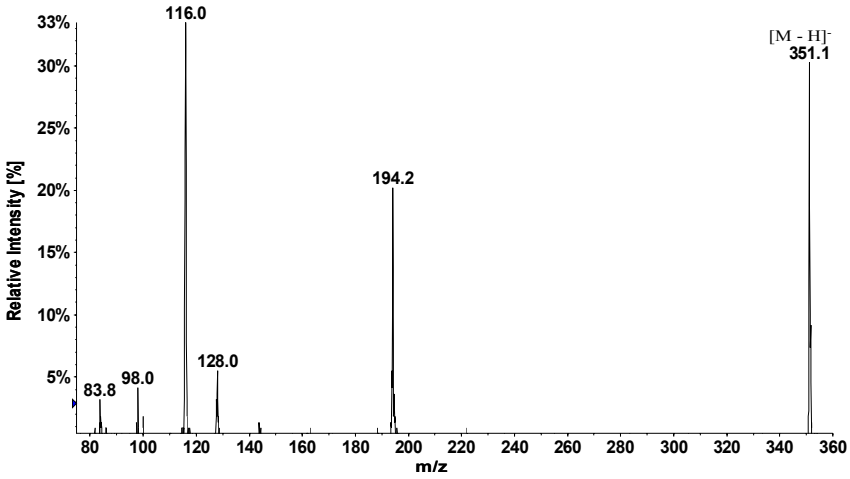


Figure 12: MS/MS mass spectra of the mercapturic acid of a carbamazepine derivative formed in incubation with activated neutrophils. Mass spectra were obtained in negative ion mode (*m/z* 369) (A) and in positive ion mode (*m/z* 371) (B).

A)



B)

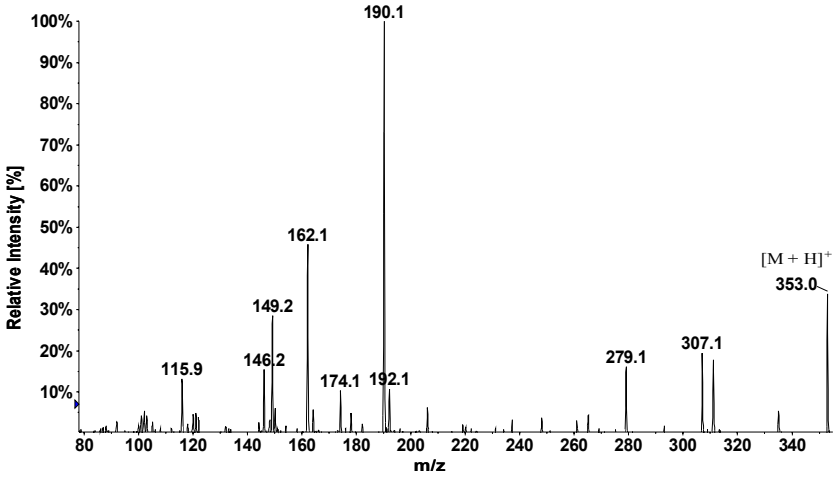
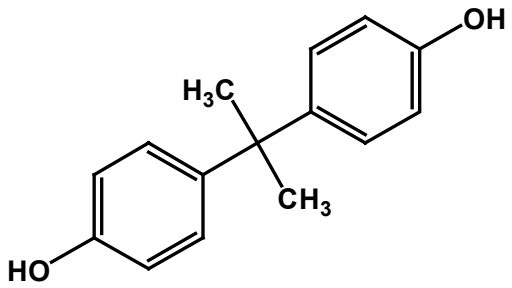


Figure 13: MS/MS mass spectra of an unknown mercapturic acid of carbamazepine formed in incubation with activated neutrophils (M_r = 352 Da). Mass spectra were obtained in negative ion mode (A) and in positive ion mode (B).

4.1.4.1.7 Bisphenol A



Chemical structure of bisphenol A

Incubation of bisphenol A with human liver microsomes and activated human neutrophils provided signals with precursor ions at *m/z* 254 and *m/z* 388 (t_R = 10.5 minutes and 15.8 minutes). Two signals with ions at *m/z* 404 eluted after 14.5 minutes and 15.4 minutes from the column. The metabolite with *m/z* 388 also occurred in non-activated neutrophils. MS/MS mass spectra of these metabolites were obtained in positive and negative ion mode. These metabolites were assigned to a mercapturic acid directly conjugated with bisphenol A (*m/z* 388), two regioisomeric mercapturic acids of the biotransformation product formed by hydroxylation of bisphenol A (*m/z* 404) and cleavage of phenol that was also conjugated with *N*-acetyl-L-cysteine (*m/z* 254*)*. Assignments of the fragment ions after negative ionization are given in Figure 14.

A)

B)

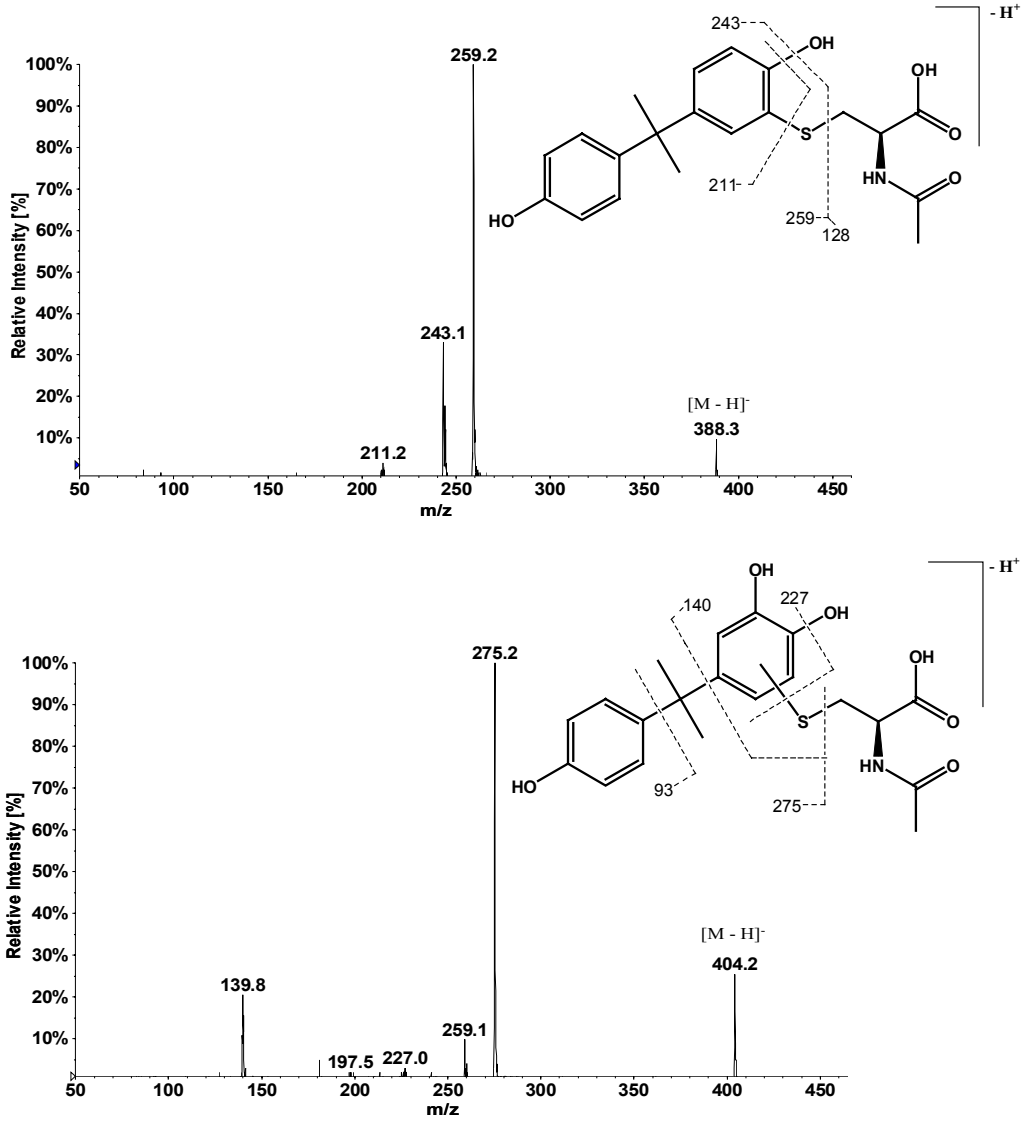


Figure 14: MS/MS mass spectra of the signals with *m/z* 388 (t_R = 15.8 minutes) (A) and *m/z* 404 (t_R = 14.5 minutes) (B) detected after incubation of bisphenol A with human liver microsomes in the presence of *N*-acetyl-L-cysteine. Mass spectra were obtained in EPI scan mode after negative ionization. Assignment of fragmentation structures is included.

4.1.4.2 Detection of Mercapturic Acids *in vivo*

4.1.4.2.1 Acetaminophen

Acetaminophen was administered to rats at two different doses (20 mg/kg and 640 mg/kg b.w.). A third group was administered vehicle only and served as control group. Parameters for liver or renal toxicity, such as creatinine and the transaminase levels ASAT (aspartate aminotransferase) and ALAT (alanine aminotransferase) were routinely determined in the central laboratory of Wuerzburg according to

standard procedures. Urinary creatinine showed no significant change, whereas ALAT and ASAT increased in rats administered a high dose of acetaminophen (Table 13), indicating liver damage, particularly for one rat that showed marked increase in ASAT (factor of 43) and ALAT (factor of 37) compared to the other two high dose rats. Body weight of the three dose groups did not change significantly. However, an average loss in body weight of 9% did occur in the high dose group 24 hours after administration of acetaminophen.

Table 13: Acetaminophen induced effects on body weight, urinary creatinine, and serum transaminase levels (mean ± standard deviation)

	Control dose	**Low dose**	**High dose**
Body weight (predose) [g]	266.2 ± 8.9	269.5 ± 7.0	261.2 ± 14.1
Body weight (postdose) [g]	266.1 ± 5.1	266.2 ± 17.4	237.4 ± 6.9
Creatinine (U0) [mg]$^{+}$	8.6 ± 1.9	10.4 ± 1.3	9.8 ± 0.8
Creatinine (U1) [mg]$^{+}$	9.6 ± 0.5	9.0 ± 0.3	7.9 ± 1.0
ASAT [u/l]†	78.5 ± 0.7	82.7 ± 9.3	273.1 ± 36.1*
ALAT [u/l] †	71.1 ± 10.2	77.3 ± 3.11	169.4 ± 2.2*

$^{+}$ mg creatinine in total volume of urine

†ASAT and ALAT levels in serum after acetaminophen treatment

*mean ± standard deviation of two rats. One rat showed severe liver damage with ASAT of 11,900 u/l and ALAT of 9,380 u/l

Acetaminophen mercapturic acid (AAP-MA), was present in low and high dose urine samples, but not in controls. The extent of formation of AAP-MA was dose-dependent. Identification of the metabolite was demonstrated by identical retention time and mass spectra in positive and negative ion mode compared to the authentic AAP-MA.

Dose-dependent formation of a second metabolite of acetaminophen with *m/z* 327 (t_R = 4.5 minutes) was observed in rat urine samples.

Quantitation of the mercapturic acid was performed by multiple reaction monitoring (MRM) analysis with method 2. A proportion of 2.8 ± 0.7% (low dose group) and

8.1 ± 1.4% (high dose group) of the administered dose of acetaminophen were eliminated as AAP-MA.

In human urine, AAP-MA was evident as a prominent peak after both 50 mg and 500 mg of oral administration of acetaminophen. Identity of the metabolite was confirmed according to retention time and MS/MS mass spectra in positive and negative ion mode. Quantitation of the metabolite showed a renal elimination of AAP-MA of 2.1 ± 0.9% of the administered dose of 500 mg of acetaminophen within eight hours. The same signal was observed in some human control samples. This signal was identified as AAP-MA by separate product ion scans in positive and negative ion mode and comparison with the reference compound.

A second signal with *m/z* 327 was dose-dependently observed in human urine samples, suggesting the formation of either the mercapturic acid of 3-hydroxyacetaminophen (3-OH-AAP-MA) or the sulfoxide of acetaminophen mercapturic acid (AAP-MA-SO).

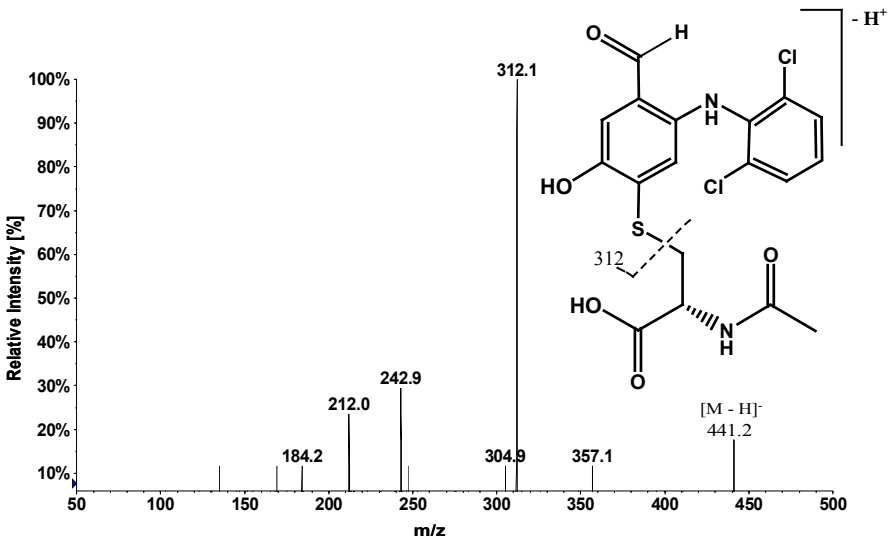
4.1.4.2.2 Diclofenac

Three mercapturic acids of hydroxylated diclofenac were detected in urine samples of rats administered 20 mg/kg b.w. of diclofenac using CNL survey scan in negative ion mode. The signal assigned to the mercapturic acid of 4´-hydroxydiclofenac showed intensities close to the LOD. In combination with an EPI scan in positive ion mode using method 5, two of these metabolites could be identified as the mercapturic acids of 5-hydroxydiclofenac according to retention time, isotopic pattern, and mass spectra identical to the previously obtained metabolites in rat liver microsomal incubations. The mercapturic acids of 5-hydroxydiclofenac were detected in rat urine samples of rats administered 10 mg/kg b.w. of diclofenac whereas the signal with t_R = 15.6 minutes was predominant. The occurrence of this metabolite was time-dependent. According to the S/N ratio and peak areas, most of the metabolized diclofenac was eliminated between 6 and 18 hours after administration of diclofenac. Formation of the mercapturic acid of 4´-hydroxydiclofenac could not be shown at this dose.

A signal with *m/z* 441 was observed in rat urine samples and could be assigned to the mercapturic acid identical to the metabolite detected in incubations of diclofenac

with activated neutrophils. Both showed identical retention times and MS/MS mass spectra in both positive and negative ion mode (Figure 15).

A)



B)

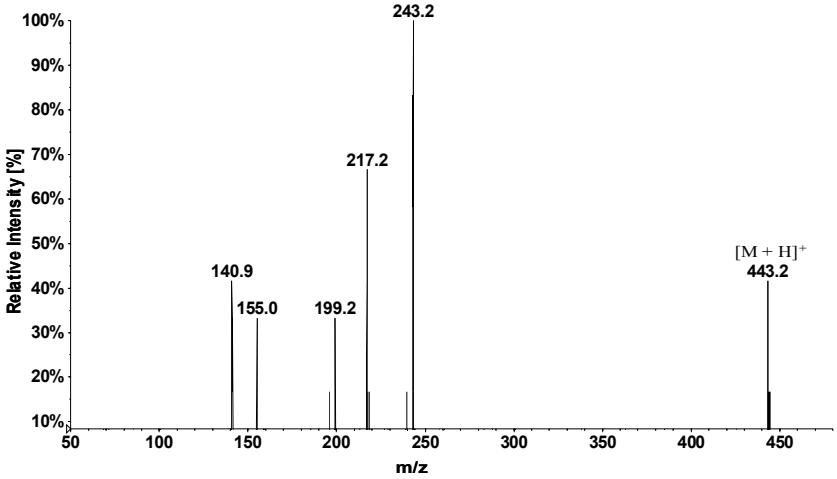


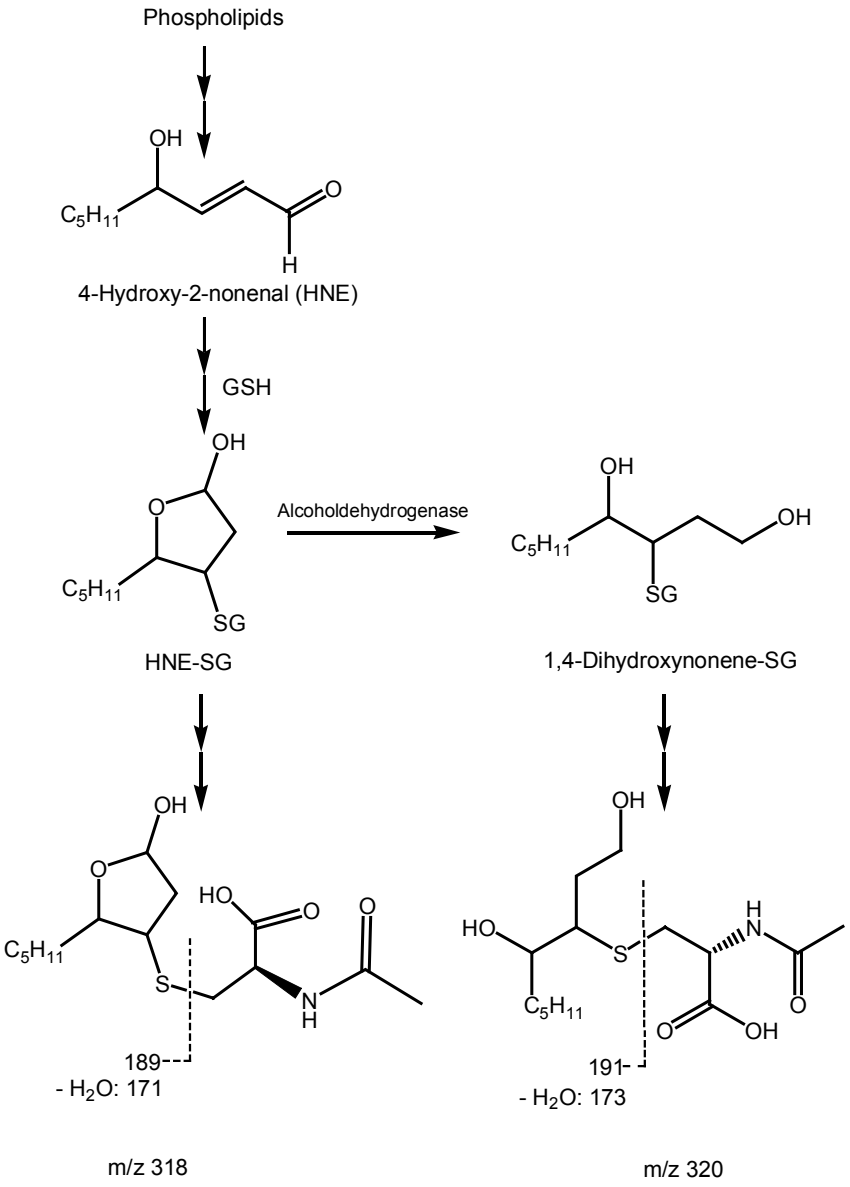
Figure 15: MS/MS mass spectra of *m/z* 441 in negative ion mode (A) and *m/z* 443 in positive ion mode (B) detected in rat urine sample 24 h after administration of 10 mg diclofenac/kg b.w.

In contrast to *in vitro* tests of diclofenac, none of the other signals were detectable in rat urine samples *(m/z* 437, *m/z* 459, nor *m/z* 487).

4.1.4.2.3 Endogenously formed Mercapturic Acids

In urine samples screened for mercapturic acids and sulfoxides, about 40 - 50 signals showed a CNL of 129 Da. Among these, a metabolite with a precursor ion at *m/z* 320 occurred in all rat and human urine samples and could be identified as *N*-acetyl-*S*-(1,4-dihydroxynonan-3-yl)-L-cysteine (DHN-MA), a metabolite of the lipid peroxidation product 4-hydroxy-2-nonene (HNE) (Scheme 13) [67]. The mass spectrum of the metabolite showed fragment ions at *m/z* 191, *m/z* 173, and *m/z* 143 after negative ionization. The fragment ion at *m/z* 191 resulted from the CNL of 129 Da, typical for mercapturic acids, the ion at *m/z* 173 was formed by loss of water, and the third fragment ion at *m/z* 143 described the product ion after a loss of C_2H_6 from the carbon chain of DHN. Identity of DHN-MA was confirmed by fragmentation and retention time identical to DHN-MA standard.

Another metabolite of the lipid peroxidation product was detected in rat and human urine as well as in incubation solutions and was identified as *N*-acetyl-*S*-(5-hydroxy-2-pentyltetrahydrofuran-3-yl)-L-cysteine (HNE-MA) according to mass spectra and retention time identical to the synthesized standard (Scheme 13). The metabolite showed fragmentation to a product ion at *m/z* 189, indicating the common neutral loss of 129 Da. Fragment ions at *m/z* 171 and *m/z* 143 were detected according to fragmentation pattern identical to DHN-MA. The product ion at *m/z* 162 resulted from cleavage of the *N*-acetyl-L-cysteine moiety.

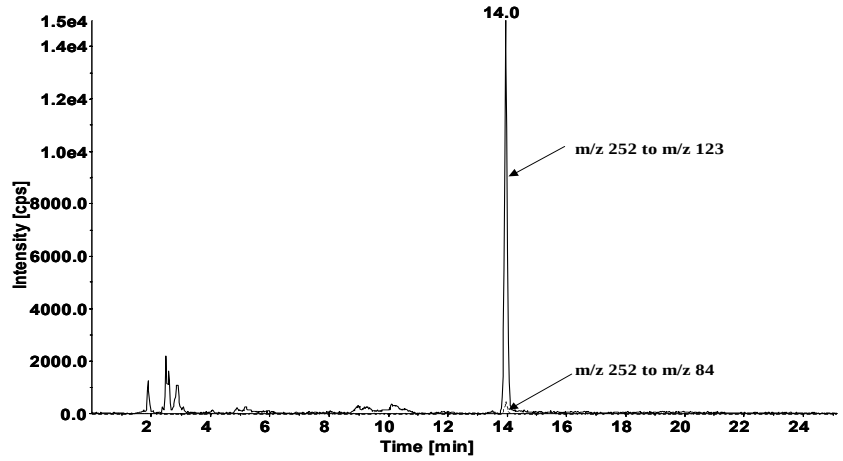


Scheme 13: Proposed metabolic formation of HNE-MA and DHN-MA with assignments of the main fragment ions detected after negative CID.

A third signal could be identified as *N*-acetyl-*S*-benzyl-L-cysteine. It was characterized by its MS/MS mass spectra and comparison to a standard. The peak was observed in 29 of 45 human urine samples tested and showed a precursor ion at *m/z* 252 in negative ion mode (t_R = 13.9 minutes). EPI scan in negative ion mode showed characteristic fragment ions at *m/z* 123, *m/z* 128, and *m/z* 84. Additionally, human urine samples were tested for the presence of this mercapturic acid with a MRM method scanning for two characteristic mass transitions (*m/z* 252 to *m/z* 123 and *m/z* 252 to *m/z* 84) in negative ion mode. The metabolite in human urine showed identical retention time and the two mass transitions in identical relation to the

standard (*m/z* 123 and *m/z* 84: relation of occurrence 15:1), confirming the identity of *N*-acetyl-*S*-benzyl-L-cysteine in human urine (Figure 16). In rat urine, this metabolite was not observed.

A)



B)

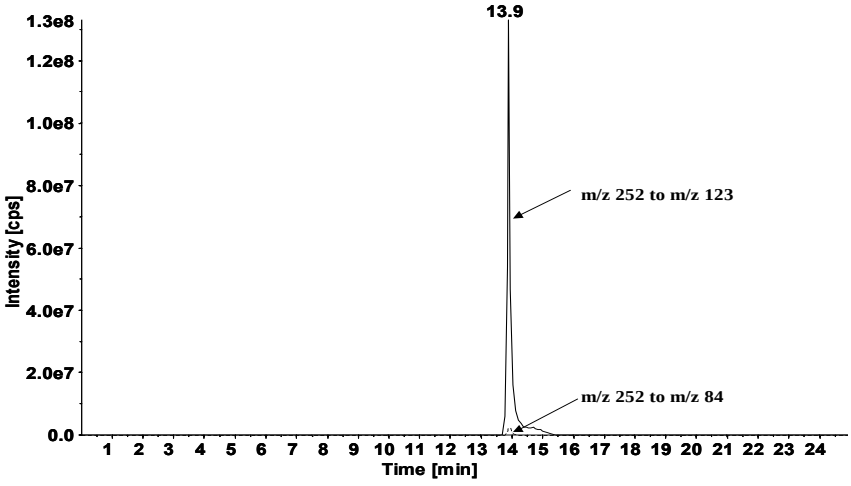


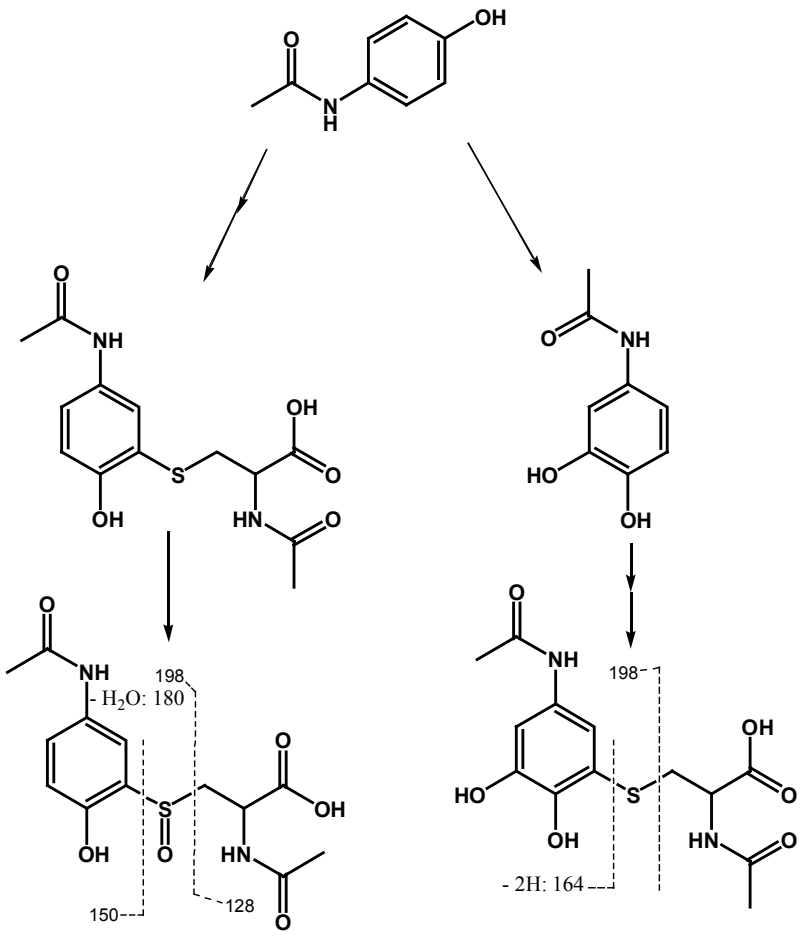
Figure 16: MRM chromatogram of a human urine sample (A) and of *N*-acetyl-*S*-benzyl-L-cysteine standard (10 µg/ml CH_3OH/H_2O 1:2) (B) with two mass transitions from *m/z* 252 to *m/z* 123 (—) and to *m/z* 84 (---) in negative ion mode.

4.1.5 Identification and Characterization of the Isobaric Acetaminophen Metabolites

Both, *N*-acetyl-*S*-[5-(acetylamino)-2,3-dihydroxyphenyl]-L-cysteine (3-OH-AAP-MA) and the sulfoxide of acetaminophen mercapturic acid (AAP-MA-SO) provided identical precursor ions at *m/z* 327 in negative ion mode. To discriminate the two analytes, 3-hydroxyacetaminophen (3-OH-AAP) and AAP-MA-SO were synthesized. Further incubation of 3-OH-AAP with rat liver microsomes in the presence of *N*-acetyl-L-cysteine generated the corresponding mercapturic acid. Mass spectrometric characterization of the incubation solution provided two signals with *m/z* 327 (t_R = 6.0 and 6.7 minutes). The signals did not occur in control incubations. MS/MS mass spectra of the peaks in positive and negative ion mode were recorded. Predominant fragment ions at *m/z* 198 and *m/z* 164 occurred in negative ion mode for 3-OH-AAP-MA, presenting the loss of 129 Da (*m/z* 198) and an additional loss of H_2S (*m/z* 164) (Scheme 14). In positive ion mode, fragment ions at *m/z* 269, 224, 182, and 156 occurred as predominant signals. The fragment ion at *m/z* 269 resulted from loss of $H_2C{=}C{=}O$ and loss of water and *m/z* 224 was formed by an additional loss of HCOOH. The product ion at *m/z* 182 occurred by an additional cleavage of an amide. The fragment ion at *m/z* 156 was assigned to protonated 3-mercapto-5-hydroxy-p-quinone imine.

MS/MS mass spectra of chemically synthesized AAP-MA-SO were obtained in positive and negative ion mode. After positive ionization, AAP-MA-SO showed fragment ions at *m/z* 200, 182, and 140, indicating cleavage of the carbon sulfur bond (*m/z* 200), an additional loss of water (*m/z* 182), and loss of $H_2C{=}C{=}O$ (*m/z* 140). Thus, fragmentation patterns differed between 3-OH-AAP-MA and AAP-MA-SO. According to characteristic fragment ions, identity could be confirmed. For instance, the fragment ion at *m/z* 164 (cleavage of the entire *N*-acetyl-L-cysteine moiety) occurred only in the mass spectrum of 3-OH-AAP-MA but not in that of sulfoxide. For the sulfoxide derivative, though, this fragment ion was not detected due to the oxidized sulfur. Moreover, a product ion at *m/z* 164 had not been determined in all standard sulfoxides tested so far (Table 8). On the other hand, the fragment ion at *m/z* 130 only occurred with the sulfoxide of AAP-MA and was also observed in many of the standard sulfoxides. Based on the different mass spectra, a

selective mass transition (*m/z* 329 to 182) for both compounds and specific mass transitions for each single compound (3-OH-AAP-MA: from *m/z* 329 to *m/z* 224 and to *m/z* 156) (AAP-MA-SO: from *m/z* 329 to *m/z* 200 and to *m/z* 140) were chosen for differentiation. A smoother gradient was used to separate AAP-MA-SO from 3-OH-AAP-MA (see method 6).



AAP-MA-SO (m/z 327) 3-OH-AAP-MA (m/z 327)

Scheme 14: Proposed formation of two isobaric metabolites of acetaminophen (3-OH-AAP-MA and AAP-MA-SO) with assignments of the main fragment ions detected after CID in negative ion mode.

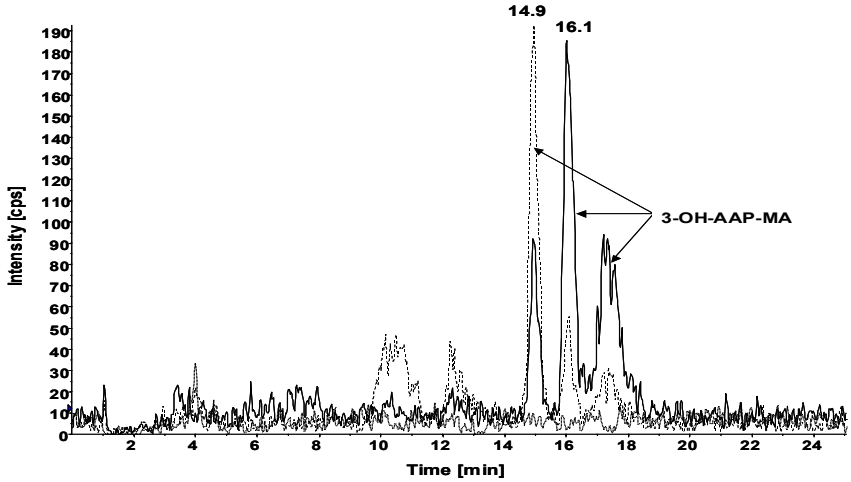
For identification of the signal observed in urine, a human urine sample was spiked with chemically synthesized AAP-MA-SO. The incubation solution of 3-OH-AAP-MA was spiked with AAP-MA-SO. As control, the same human urine sample without spiking and the incubation solution of 3-OH-AAP without addition of AAP-MA-SO was analyzed. Incubation with 3-OH-AAP provided three signals (t_R = 14.8 minutes, 16.0 minutes, and 17.4 minutes) with characteristic mass transitions (from *m/z* 329 to *m/z* 224, to *m/z* 182, and to *m/z* 156). These signals indicated the formation of three

regioisomers of *N*-acetyl-L-cysteine conjugates of 3-OH-AAP. When incubation solution of 3-OH-AAP was spiked with AAP-MA-SO, two additional signals (t_R = 12.4 and 13.3 minutes) with the mass transitions characteristic for AAP-MA-SO (*m/z* 329 to *m/z* 200, *m/z* 182, and *m/z* 140) were detected (Figure 17). In human urine samples without spiking, two signals occurred at retention time and mass transitions identical to AAP-MA-SO. Analysis of human urine showed increasing intensities of the two peaks at t_R = 12.4 and 13.3 minutes when AAP-MA-SO was spiked to the sample. (Figure 18). In summary, the signal with a mass of *m/z* 327 that was detected in human and in rat urine was assigned to the sulfoxide of AAP-MA and its stereoisomer, whereas incubation solution with human liver microsomes formed 3-OH-AAP-MA and did not reveal formation of AAP-MA-SO to detectable amounts.

Table 14: Fragment ions of AAP-MA-SO and 3-OH-AAP-MA obtained in EPI scan mode upon negative and positive CID. Mass spectra were obtained from chemically synthesized AAP-MA-SO and from rat microsomal incubation of 3-OH-AAP fortified with *N*-acetyl-L-cysteine.

Ion mode	**Fragment ions detected**		**Proposed fragmentation**
	AAP-MA-SO [*m/z*]	**3-OH-AAP-MA [*m/z*]**	
negative	327	327	$[M - H]^-$
	198	198	$[M - 129]^-$
	180	-	additional loss of H_2O
	-	164	198 - H_2S
	150	-	198 - S=O
positive	-	329	$[M + H]^+$
	-	269	$[M - H_2C=C=O - H_2O + H]^+$
	-	224	$[269 - HCOOH + H]^+$
	200	-	$[M - 129 + H]^+$
	-	198	$[M - 129 - 2H + H]^+$
	182	-	$[200 - H_2O]^+$
	-	182	$[224 - H_2C=C=O]^+$
	-	164	[*N*-acetyl-L-cysteine + H]$^+$
	-	156	$[198 - H_2C=C=O]^+$
	140	-	$[182 - H_2C=C=O]^+$
	130	-	see scheme 7

A)



B)

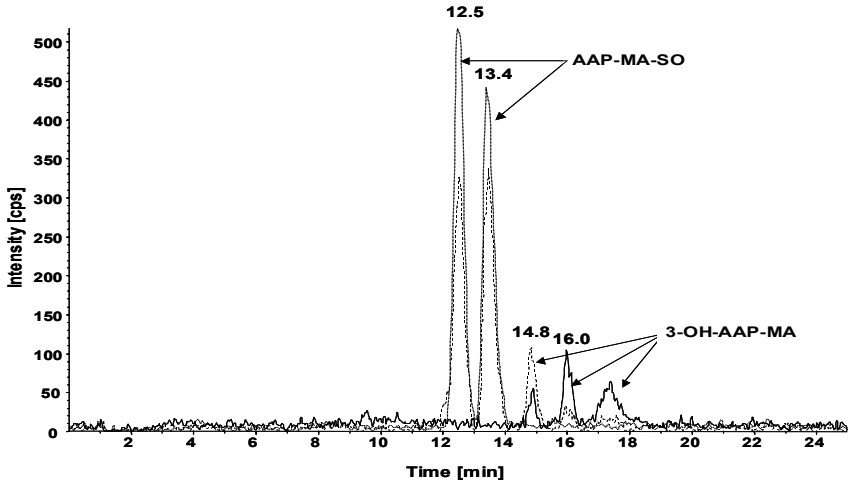
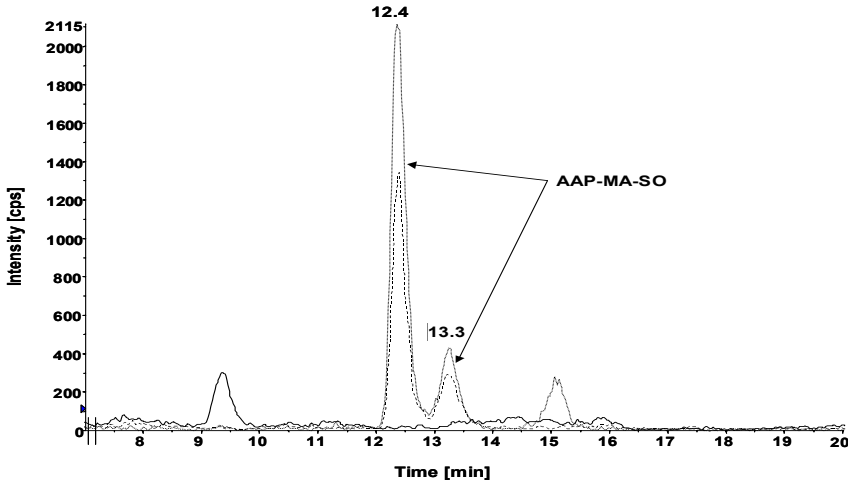


Figure 17: MRM chromatogram of incubation solution of 3-OH-AAP with rat liver microsomes in presence of *N*-acetyl-L-cysteine (A). The same incubation solution was spiked with chemically synthesized AAP-MA-SO (B). MRM chromatogram was obtained with mass transitions from *m/z* 329 to *m/z* 224 (—), *m/z* 182 (---), and *m/z* 140 (···) in positive ion mode.

A)



B)

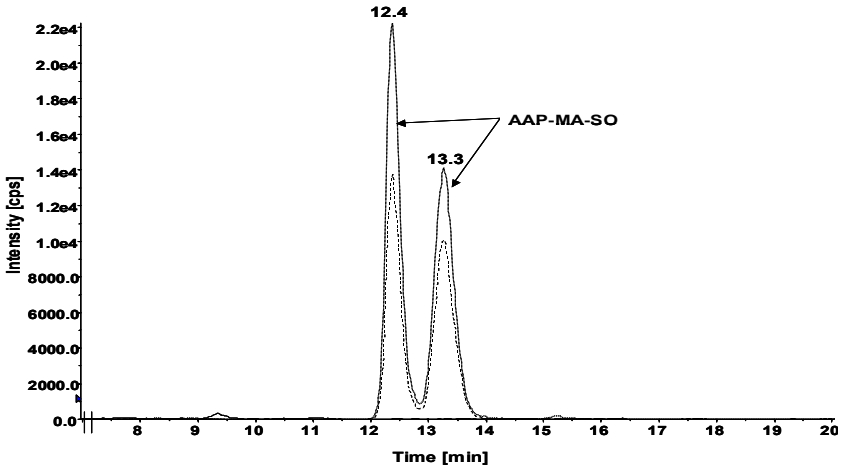


Figure 18: MRM chromatogram of human urine sample of a volunteer administered 500 mg of acetaminophen eight hours before. This sample was either not spiked (A) or spiked (B) with chemically synthesized AAP-MA-SO. MRM chromatogram was obtained with mass transitions from *m/z* 329 to *m/z* 224 (—), *m/z* 182 (---), and *m/z* 140 (···) in positive ion mode.

4.1.6 Dose-dependent Elimination of DHN-MA in Rat Urine after Acetaminophen Administration

A dose-dependent decrease in the renal excretion of DHN-MA and HNE-MA (for chemical structures see Scheme 13) was observed in urine of rats administered acetaminophen. To assess formation of oxidative stress derived mercapturic acids in combination with exposure of GSH depleting compounds such as acetaminophen, peak areas were determined and expressed relative to creatinine. Peak areas of undiluted rat urine samples were determined using method 1. Standard deviation resulted from three different rats in each group. A significant reduction in renal excretion of DHN-MA were determined in urine samples of rats administered 640 mg of acetaminophen. In some samples the S/N ratio of HNE-MA was below LOQ (S/N < 9), so that peak area of this metabolite could not be determined. However, it was evident that excretion of DHN-MA decreased when rats were administered a dose of 640 mg/kg b.w. acetaminophen (Figure 19)

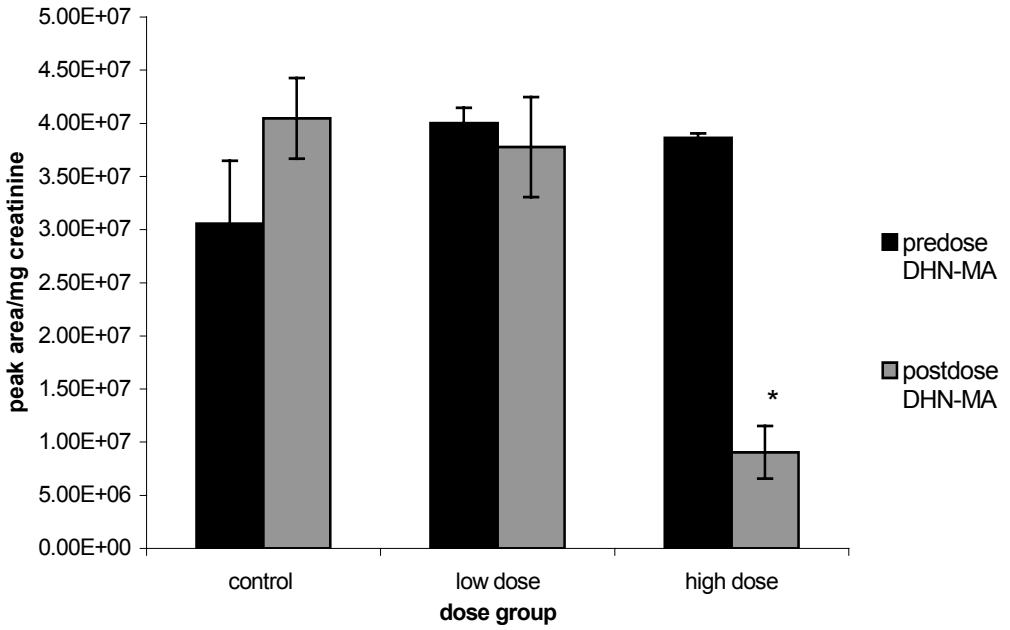
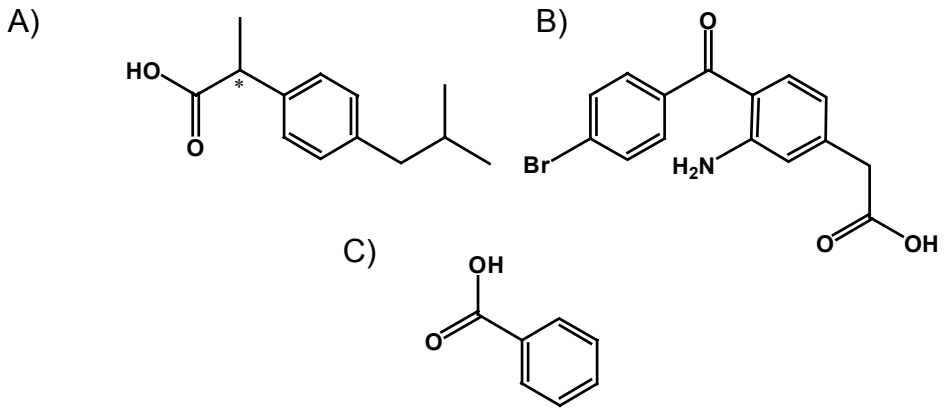


Figure 19: Mean peak areas and standard deviation of detected *N*-acetyl-*S*-(1,4-dihydroxynonan-3-yl)-L-cysteine (DHN-MA) in urine of rats administered acetaminophen in three different doses (n = 3). Peak areas were expressed relative to creatinine. Data acquisition was obtained with method 1. Reduction of DHN-MA excretion in acetaminophen high dose rat urine was significant with $p < 0.05$.

4.2 L-Lysine Adducts

Reactive intermediates formed by biotransformation of various drugs and environmental compounds *in vivo* may not only conjugate with glutathione but are also sufficiently reactive to bind to other nucleophilic endogenous compounds such as amino groups, present in amino acids or nucleic acids. This conjugation may lead to alterations of proteins or DNA and may cause genotoxicity or cytotoxicity, resulting in corresponding damages or influences of the immune system [24, 27]. An exemplary group of such potentially reactive compounds are non-steroidal anti-inflammatory drugs (NSAID), which contain a carboxylic group responsible for their pharmacological effect. This functional group serves as substrate for conjugation with activated glucuronide in phase II metabolism to form acyl glucuronides. Acyl glucuronides are generated by conjugation with activated glucuronic acid (UDP-GA) and catalyzed by glucuronyl transferases (UGTs), which are predominantly present in the lumen of the endoplasmatic reticulum in the liver, kidneys, and gut. Formation of acyl glucuronides may toxify the compound due to the instability of the acyl glucuronides at physiological pH, leading to hydrolysis or positional isomers by acyl migration [24, 96]. Thus, acyl glucuronides can covalently bind to proteins or DNA via transacylation or formation of a schiff base by glycation mechanisms [53]. L-Lysine is one possible target amino acid that may form such conjugates and is used as trapping agent for binding acyl glucuronides in the present study. Glycation of L-lysine has not been considered in the present study due to the lack of such L-lysine adducts as standards. The major focus was on transacylation of various compounds bearing a carboxylic group. To cover this possible side pathway of reactive intermediates, the aim was to develop a sensitive and general method similar to the mercapturic acid method to facilitate detection of L-lysine conjugates *in vitro* and *in vivo*. In the present study, model compounds for generation of protein-reactive acyl glucuronides were diclofenac, ibuprofen, bromfenac, and benzoic acid (chemical structures are given below). These compounds were selected as model compounds for the generation and detection of protein-reactive phase II metabolites.



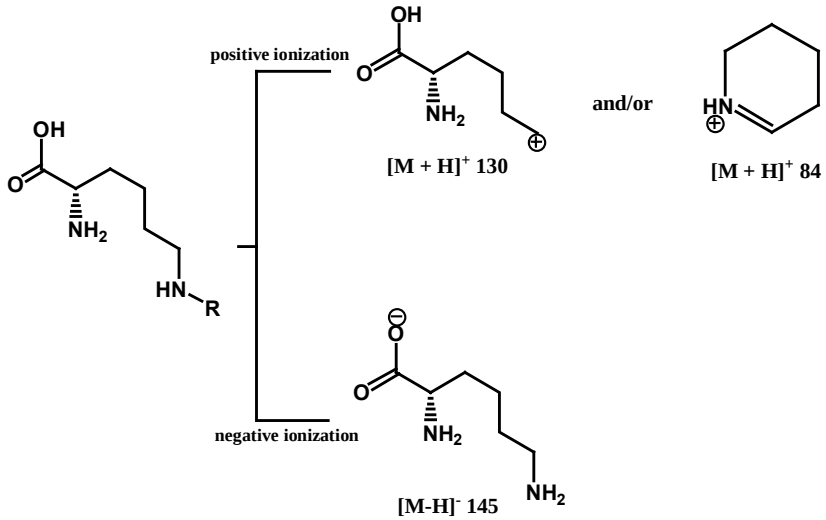
Chemical structures of A) ibuprofen, B) bromfenac, and C) benzoic acid

4.2.1 Analytical Method Development

4.2.1.1 Ionization and Fragmentation Patterns of L-Lysine Standards and Chemically Synthesized L-Lysine Adducts with MS/MS

L-Lysine standards were scanned for possible common neutral losses or product ions. Seven commercially available L-lysine adducts (Table 17) and further L-lysine adducts which were obtained by reaction of acyl halides with L-lysine, N^{α}-acetyl-L-lysine or N^{ε}-acetyl-L-lysine (chapter 3.3.3) were fragmented in negative and positive ion mode. All L-lysine adducts provided a common product ion at *m/z* 84 in the positive ion mode. Additionally, most of the standards showed fragmentation to *m/z* 130 and *m/z* 147, respectively. However, the latter product ions were not common for all of the L-lysine adducts tested. In negative ion mode, a product ion at *m/z* 145 occurred in some of the L-lysine adducts, resulting from cleavage of the L-lysine moiety (Scheme 15). Most prominent fragment ions detected in positive and negative ion mode are given in Table 15 andTable 16.

These product ions occurred also by fragmentation of the *N*-acetyl-L-lysine adduct standards. Except for a single *N*-acetyl-L-lysine adduct (N^{α}-acetyl-ε-(2-benzoyloxymethyl)-phenylcarbonyl-L-lysine), all other reference compounds showed the common product ion at *m/z* 84. Based on this common fragment ion, method development for the detection of L-lysine conjugates was done with precursor ion scan (PrIS) 84. This feature could also be combined with an EPI scan by the IDA software.



Scheme 15: Proposed fragmentation pathway of L-lysine adducts upon CID during mass spectrometric analysis. Fragment ions in positive ion mode were prevíously described by Argirov et al. [97]

Table 15: Fragment ions of L-lysine adducts detected in positive ion mode.

Compounds	$[M + H]^+$	Fragment ions detected								
N^{ε}-(*tert*-Butoxycarbonyl)-L-lysine	247	191	173	147[b]	145	130[c]	128	**84**[a]		
N^{ε}-Methyl-L-lysine	161	144	130[c]	116	98	**84**[a]				
N^{α}-(Benzyloxycarbonyl)-L-lysine	281	237	220	130[c]	128	91	**84**[a]			
N^{ε}-Glutamyl-L-lysine	276	259	213	167	147[b]	130[c]	**84**[a]			
N^{ε}-(2,4-Dinitrophenyl)-L-lysine	313	267	250	216	204	170	158	130[c]	118	**84**[a]
Saccharopine	277	259	213	195	130[c]	**84**[a]				
Biocytine	373	310	227	184	166	130[c]	123	105	97	**84**[a]
N^{α}-Benzoyl-L-lysine	251	233	215	188	122	105	**84**[a]			
N^{ε}-Benzoyl-L-lysine	251	233	215	205	188	105	**84**[a]			
N^{α}-[(3,5-Diethoxyphenyl)acetyl]-L-lysine	339	321	303	276	210	193	165	137	**84**[a]	
N^{ε}-[(3,5-Diethoxyphenyl)acetyl]-L-lysine	339	321	276	193	165	137	**84**[a]			
N^{α}-[(3,4-Dimethoxyphenyl)acetyl]-L-lysine	325	307	289	262	151	147[b]	129	**84**[a]		
N^{ε}-[(3,4-Dimethoxyphenyl)acetyl]-L-lysine	325	307	289	279	262	223	151	147[b]	130[c]	**84**[a]
N^{α}-[(2-Benzoyloxymethyl)-phenylcarbonyl]-L-lysine	385	367	321	263	245	217	188	134	105	**84**[a]
N^{ε}-[(2-Benzoyloxymethyl)-phenylcarbonyl]-L-lysine	385	367	322	263	245	218	188	134	105	**84**[a]

[a] **common fragment ion at *m/z* 84**

[b] precursor ion at *m/z* 147

[c] precursor ion at *m/z* 130

Table 16: Fragment ions of L-lysine adducts detected in negative ion mode.

Compounds	[M - H]⁻	Fragment ions detected
N^{ε}-(*tert*-Butoxycarbonyl)-L-lysine	245	171 145 128 127 93
N^{α}-(Benzyloxycarbonyl)-L-lysine	279	171 152 128 97
N^{ε}-Glutamyl-L-lysine	274	256 213 195 145 128 109
N^{ε}-(2,4-Dinitrophenyl)-L-lysine	311	231 202 188 182 166 162 158 152 145 102
Saccharopine	275	257 240 231 213 196 170 145 128
Biocytine	371	337 328 311 294 282 145
N^{α}-Benzoyl-L-lysine	249	205 181 153 136 127 120 113 97
N^{ε}-Benzoyl-L-lysine	249	205 181 153 136 128 113 120 97
N^{α}-[(3,5-Diethoxyphenyl)acetyl]-L-lysine	337	293 265 248 235 136 108
N^{ε}-[(3,5-Diethoxyphenyl)acetyl]-L-lysine	337	309 281 263 235 136 108
N^{α}-[(3,4-Dimethoxyphenyl)acetyl]-L-lysine	323	249 187 145 121
N^{ε}-[(3,4-Dimethoxyphenyl)acetyl]-L-lysine	323	308 263 145 136 121
N^{α}-[(2-Benzoyloxymethyl)-phenylcarbonyl]-L-lysine	383	261 217 144 132 121 77
N^{ε}-[(2-Benzoyloxymethyl)-phenylcarbonyl]-L-lysine	383	261 243 132 121 77

4.2.1.2 Optimization of Source Parameters and HPLC Conditions

Optimization of the HPLC solvents was performed by automated injection of a master mix of the commercially available L-lysine standards (143 µg/ml each in CH_3OH/H_2O 1:2 v/v) into the MS/MS instrument while the pump provided different solvents with a

flow rate of 250 µl/min. Four different solvents, i.e. 0.1% of formic acid (FA), 5 mM ammonium formate (NH_4FA) pH 3, 5 mM ammonium acetate (NH_4Ac) pH 6, and 5 mM NH_4Ac pH 7 with equal volumes of acetonitrile (50/50 v/v) were used to optimize detection of the L-lysine standards in positive PrIS mode. Best PrIS results were obtained with 0.1% of formic acid as solvent and the instrument and parameter settings specified in chapter 3.5.2. Optimization of chromatography was performed on a Reprosil Pur AQ column with defined MRM mass transitions using *m/z* 84 as product ion and resulted in a chromatogram as shown in Figure 20.

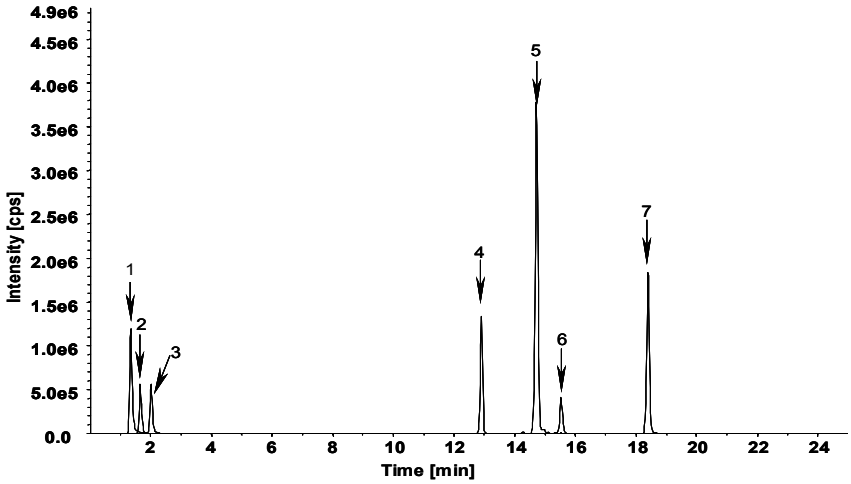


Figure 20: MRM chromatogram of seven L-lysine standards with the optimized HPLC gradient and instrument settings in positive ion mode. Numbers assign the standards given in Table 17.

4.2.1.3 Generation of the IDA PrIS Method

IDA criteria were identical to the optimized parameters for the detection of mercapturic acids. MS/MS spectra obtained with the collision energy and declustering potential as used for the survey scan provided mass spectra sufficient for tentative characterization of a detected signal. Therefore, one EPI scan was inserted into the method. Moreover, cycle time could be reduced to a minimum to obtain optimal peak shapes and maximal intensity. The fragment ion at *m/z* 84 is not very specific, so that for some extracted masses background level were quite high. This enforced an increase of the intensity threshold of the survey scan to 1,000 cps to trigger for EPI scanning. Settling time was not interposed due to identical compound-dependent parameters for both scan modes.

4.2.2 Sensitivities of the L-Lysine Standards in Matrix and Pure Solvent

S/N ratios of each L-lysine standard were determined and are shown in Table 17. Concentrations ranged from 17.5 pmol (biocytin HCl) to 36.5 pmol (N^{ε}-methyl-L-lysine HCl) on column. Although individual LOD levels were not determined, the results give an overview of the sensitivity of the method. In pure solvent, the signal was highest with S/N ratios ranging from 605 (for N^{ε}-*tert*-butoxycarbonyl-L-lysine) to S/N ratio close to LOD (for N^{ε}-glutamyl-L-lysine). When analyzed in human urine, sensitivity decreased by a factor of 1.3 (N^{ε}-glutamyl-L-lysine) to up to 7 (saccharopine) or below LOD. For the extremely polar compound N^{ε}-methyl-L-lysine HCl there was a loss of sensitivity below LOD, due to the elution with the solvent front. Ionization efficiency was low in incubation solution. For comparison of sensitivity, LOD levels of the mercapturic acid standards determined with the IDA CNL method in pure solvent ranged from 0.3 pmol to 15.5 pmol on column.

Table 17: S/N ratios of the L-lysine standards (1.43 µg/ml) in pure solvent and matrix (n = 3).

		H_2O	Human urine	Rat urine	Incubation solution
Compound	$[M + H]^+$	S/N ratio	S/N ratio	S/N ratio	S/N ratio
N^{ε}-Methyl –lysine (1)	161	107 ±19	<LOD	<LOD	<LOD
Saccharopine (2)	277	41 ± 9	6 ± 3	<LOD	4 ± 2
N^{ε}-Glutamyl-L-lysine (3)	276	5 ± 3	3 ± 0	<LOD	<LOD
Biocytine (4)	373	99 ± 28	49 ± 14	41 ± 12	68 ± 15
N^{ε}-(*tert*-Butoxycarbonyl)-L-lysine (5)	247	605 ± 70	144 ± 17	192 ± 59	189 ± 50
N^{α}-(Benzyloxycarbonyl)-L-lysine (6)	281	33 ± 6	25 ± 8	34 ± 8	9 ± 1
N^{ε}-(2,4-Dinitrophenyl)-L-lysine (7)	313	443 ± 61	139 ± 54	169 ± 34	96 ± 18

4.2.3 Generation and Stability of Acyl Glucuronides

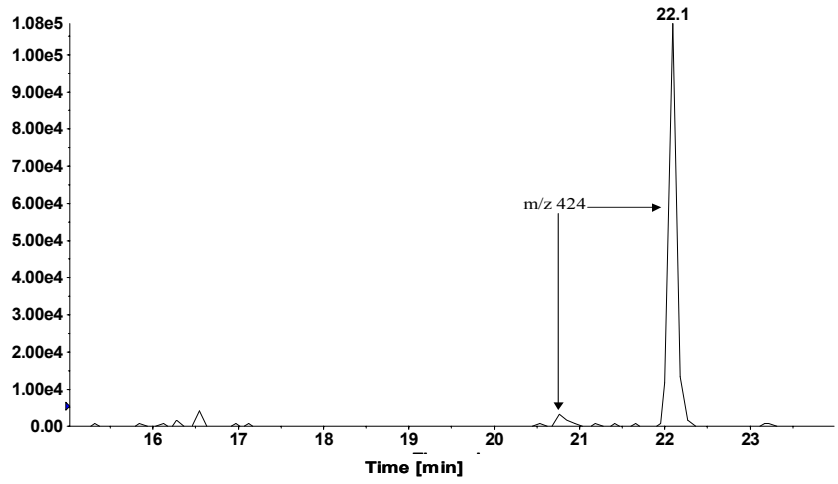
Glucuronidation of carboxylic acid containing compounds was performed by addition of UDP-glucuronic acid (UDP-GA) and alamethicin in the presence of rat liver microsomes. The membrane pore forming peptide alamethicin causes a disruption of the diffusional barrier of the endoplasmatic reticulum membrane to enable the access of substrates and cofactors to the enzyme. Thus, maximal enzyme activity can be achieved [85]. Acyl glucuronides show a decreased stability due to acyl migration to more stable isomers [27]. To test the stability of acyl glucuronides, diclofenac was glucuronated under the previously described conditions. One sample was stabilized by addition of formic acid, the other incubation solution was not stabilized. Both samples were heated to 60°C and the formation and acyl migration of the unstable 1-O-β-acyl glucuronide to its isomers was analyzed. In the sample that was stabilized with formic acid, the 1-O-β-acyl glucuronide was still present after 60 minutes of heating. In the sample that had not been stabilized, 1-O-β-acyl glucuronide vanished below LOD level. After addition of L-lysine, only a small amount of 1-O-β-acyl glucuronide of diclofenac was observed.

4.2.4 Generation and Detection of Acyl L-Lysine Adducts *in vitro*

4.2.4.1 Diclofenac

Incubations of diclofenac in the presence of L-lysine provided a signal with *m/z* 424, indicating transacylation reaction to form the acyl L-lysine adduct. Due to fragmentation pattern of the analyte in positive and negative ion mode, the analyte could be assigned to the L-lysine adduct of diclofenac acyl glucuronide. The fragment ions at *m/z* 278, 250, and 215 are described as characteristic product ions for diclofenac [53, 98, 99]. Product ions at *m/z* 147, 130, and 84 are characteristic for L-lysine fragmentation. Analysis of the sample on an API 3000 mass spectrometer obtained a second peak with identical mass transition for *m/z* 424, indicating covalent binding to the second amino group of L-lysine. This peak was obtained with IDA PrIS method at LOD level. A L-lysine adduct of the corresponding 4´-hydroxydiclofenac was not detected due to the lack of NADPH regenerating system. A further signal with *m/z* 361 and chlorine isotopic pattern were obtained in incubations with diclofenac after addition of L-lysine. This signal was not present in control samples without L-lysine or microsomes.

A)



B)

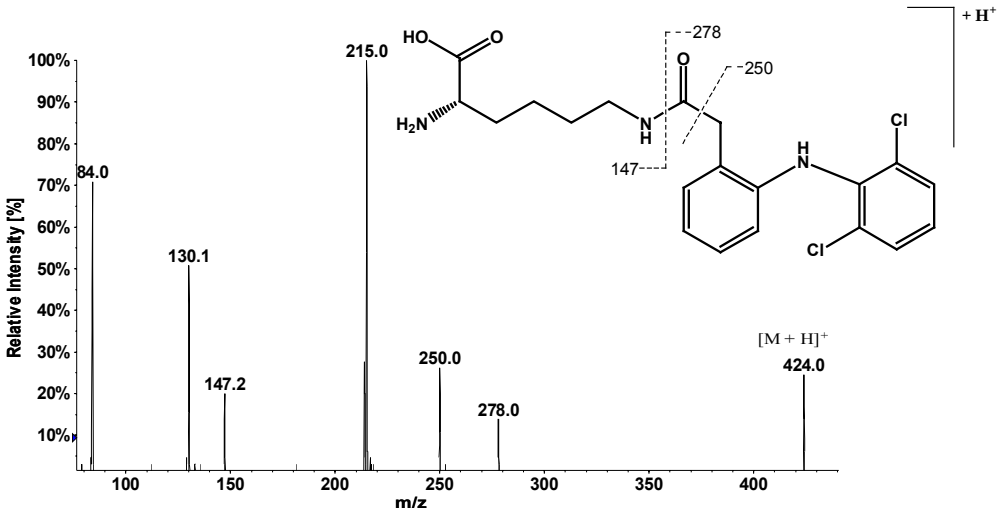


Figure 21: HPLC chromatogram of the precursor ion at *m/z* 424 (A) and MS/MS mass spectrum of the signal (B) obtained in rat liver microsomal incubation with diclofenac after glucuronidation and addition of L-lysine. Data acquisition was performed with IDA PrIS 84. Proposed fragmentation is shown above.

4.2.4.2 Ibuprofen

Ibuprofen formed a metabolite with *m/z* 335 due to the reaction of L-lysine with the acyl glucuronide of ibuprofen by transacylation. Scanning the sample with a single EPI scan, a second peak with similar fragmentation pattern occurred. The fragment ion at *m/z* 84 showed an intensity of only 12%. The low intensity of the fragment ion may be the reason for the lack of the signal when analyzed by IDA PrIS. Analysis on an API 3000 mass spectrometer with characteristic mass transitions obtained three signals with mass transitions from *m/z* 335 to *m/z* 161 (t_R = 22.7 minutes with a S/N of 4 and t_R = 22.9 minutes with a S/N of 12 in comparison to t_R = 24.0 minutes with a S/N 40), indicating formation of N^{α}- and N^{ε}-L-lysine adducts of the stereoisomers of ibuprofen. MS/MS mass spectra could be obtained from two of the three signals. Collision induced dissociation provided characteristic fragment ions e.g. at *m/z* 161 for ibuprofen, as previously described by Ferrer et al. [98], and at *m/z* 147, 130, and 84 for L-lysine after positive ionization and at *m/z* 145 after negative ionization.

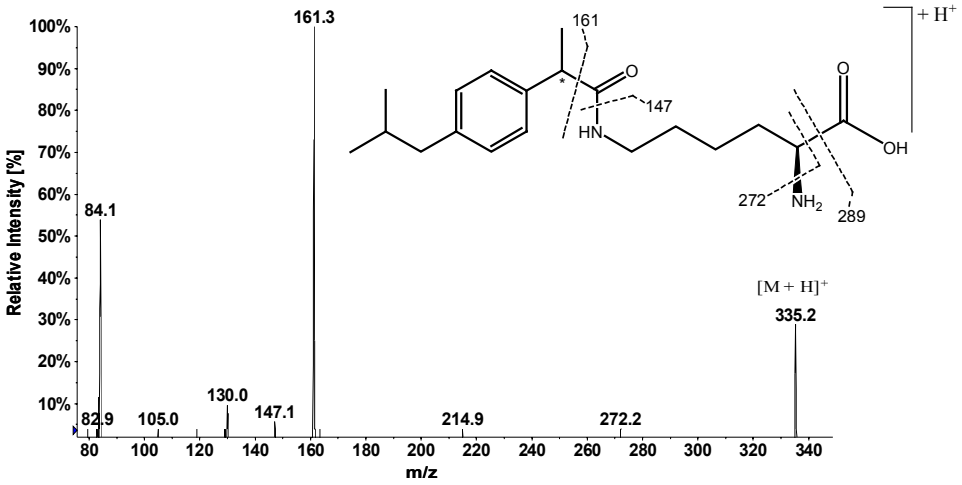
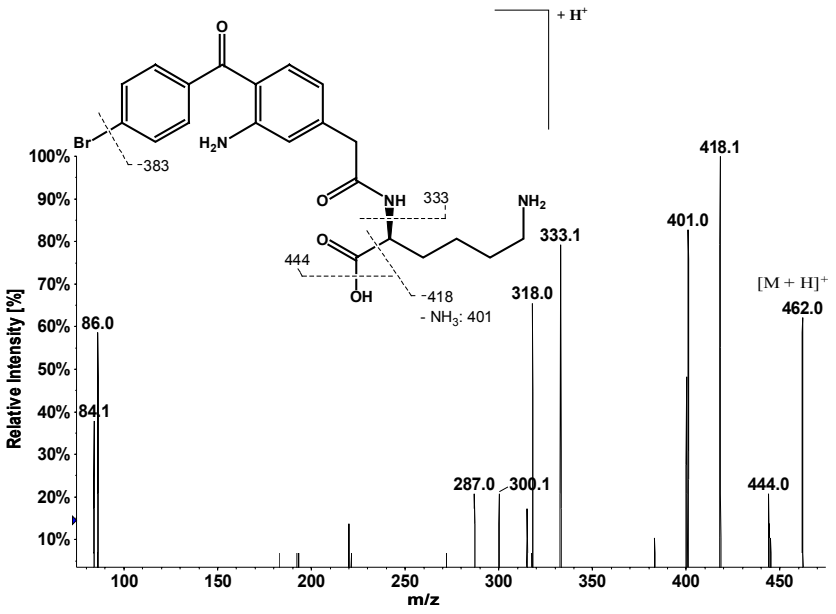


Figure 22: MS/MS mass spectrum of acyl L-lysine adduct of ibuprofen with precursor ion at *m/z* 335, obtained with IDA PrIS method. Proposed fragmentation of the metabolite is shown above.

4.2.4.3 Bromfenac

Two acyl L-lysine conjugates with ions at *m/z* 462 (t_R = 16.5 minutes and 17.5 minutes) were formed by bromfenac incubations, indicating the formation of N^{ε}- and N^{α}- conjugated L-lysine adducts. Characteristic product ions of L-lysine and the bromfenac moiety confirmed the transacylation of the acyl glucuronide of bromfenac with L-lysine. MS/MS mass spectra of both signals are shown in Figure 23. The specific isotopic pattern indicative for the presence of bromine further confirmed the formation of these metabolites.

A)



B)

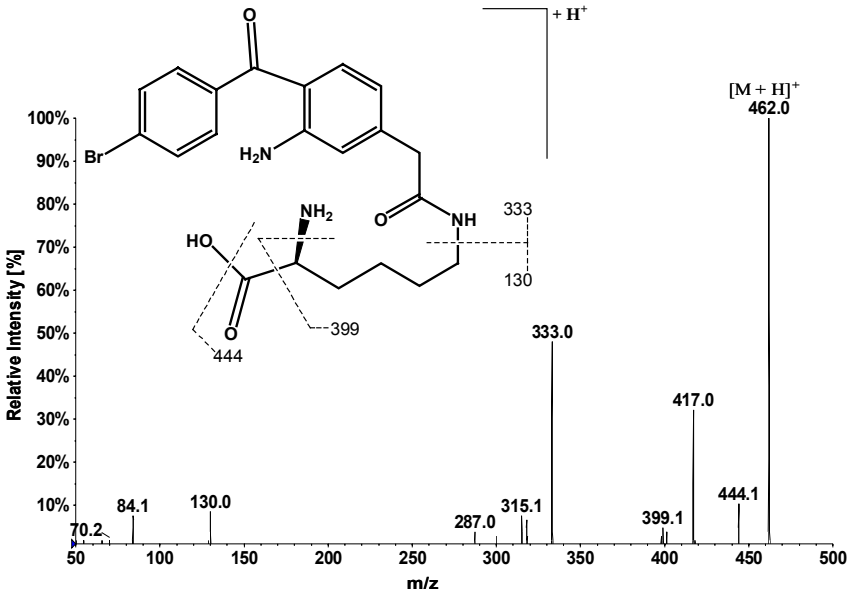


Figure 23: MS/MS mass spectrum of the signal with the precursor ion at *m/z* 462 (t_R = 17.5 minutes) (A) obtained upon rat liver microsomal incubation with bromfenac after glucuronidation and addition of L-lysine. Fragmentation of a second signal (*m/z* 462 and t_R = 16.5 minutes) resulted in similar fragmentation (B). Assignments of the fragment ions are inserted in the figures. Mass spectra were obtained with IDA PrIS.

4.2.4.4 Benzoic acid

Benzoic acid formed two L-lysine adducts at *m/z* 251. Here, standard had been synthesized previously to be able to compare fragmentation and chromatographic behaviour with the metabolite formed *in vitro*. Both standard and incubation solutions provided two peaks with identical mass spectra and retention times (Figure 24). Thus, the two signals were assigned to N^{α}- and N^{ε}-conjugated L-lysine adducts of benzoic acid.

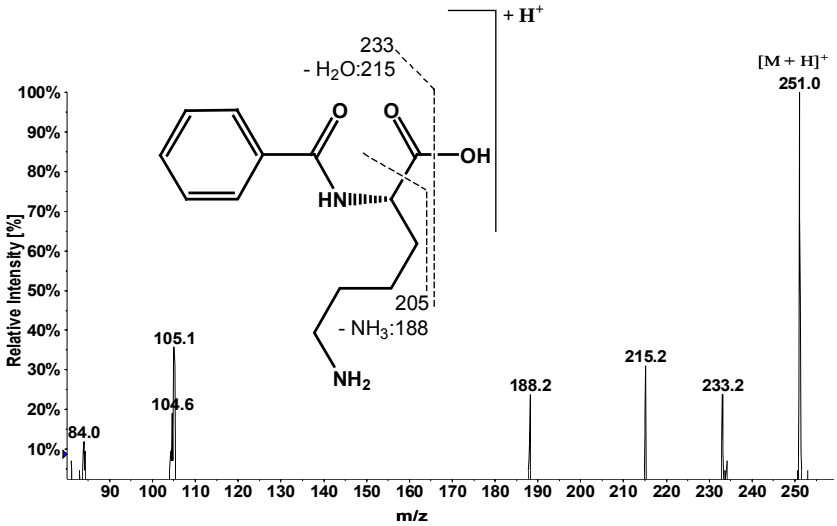


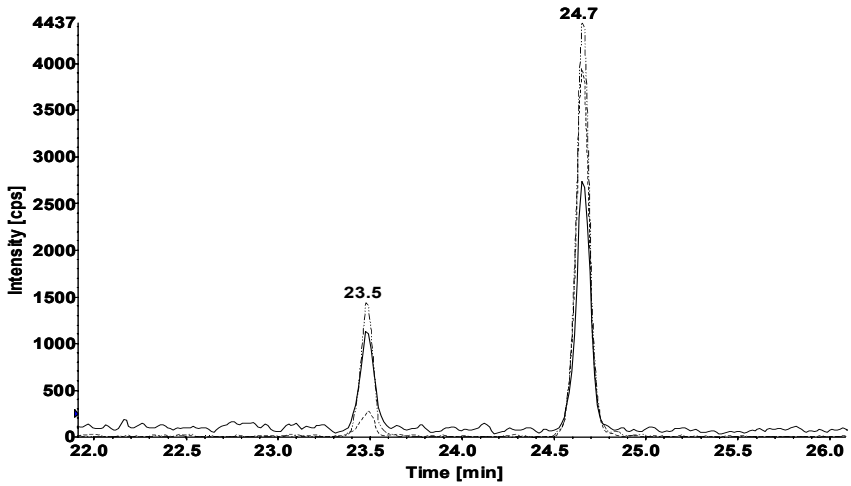
Figure 24: MS/MS mass spectrum of the precursor ion at *m/z* 251 detected in rat liver microsomal incubation with benzoic acid. The second peak provided identical fragmentation with slightly different relations. Reaction of benzoyl chloride with L-lysine provided identical fragmentation and retention time (Table 15).

4.2.5 Detection of L-Lysine Adducts of Diclofenac in Human Urine

A signal with *m/z* 424 was detected in human urine after addition of L-lysine. Fragmentation and chromatographic behaviour was identical to the L-lysine adduct of diclofenac observed in incubations with rat liver microsomes. Scanning the original human urine sample for this L-lysine adduct, this adduct was also present in the sample without L-lysine addition, but with decreased intensity (factor of 3). For further analysis and confirmation of the hypothesis of the presence of L-lysine adducts in human urine without prior addition of L-lysine, the urine samples were concentrated (10 times) and analyzed using method 8 on an API 3000 (Figure 25). Identical retention time and formation of the three product ions in identical relation proved the

presence of a L-lysine adduct of diclofenac in four of five human urine samples five and eight hours after treatment with 75 mg diclofenac-Na.

A)



B)

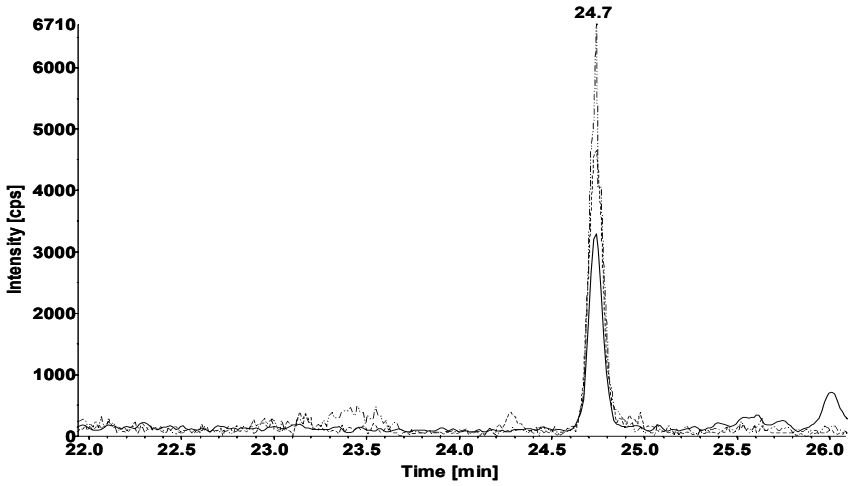


Figure 25: HPLC chromatogram detected in incubation of diclofenac after formation of acyl glucuronide and addition of L-lysine (A) and in human urine of a subject administered diclofenac without addition of L-lysine (B). Human urine sample was ten times concentrated. The chromatogram was obtained in MRM mode with three characteristic mass transitions from *m/z* 424 to *m/z* 250 (—), to *m/z* 129 (-••-), and to *m/z* 84 (---) of the acyl L-lysine adduct of diclofenac in positive ion mode on an API 3000.

5 DISCUSSION

5.1 Mercapturic Acids

5.1.1 Analytical Method Development

The advantage of using the IDA feature of the Applied Biosystems software is the acquisition of a maximum amount of structural information in a single analytical run. In addition, the linear ion trap of the Qtrap instrument offers different MS/MS scan modes to record an enhanced product ion scan of each signal detected by the survey scan. The survey scans include precursor ion scans (PrIS) or constant neutral loss scans (CNL) that are more specific and, thus, more sensitive compared to full scan methods due to reduced data acquisition by detection of only common fragment ions or neutral losses [100]. The intensities of both PrIS and CNL scan modes may be similar when identical scan times are used. However, precursor ion scan may be more sensitive compared to a CNL scan, if a predominant product ion of the analyte is formed and vice versa (i.e. if a predominant neutral loss occurs such as the loss of 162 Da for glycosides [101] or the loss of 129 Da for mercapturic acids as in the present study [102]). To obtain characteristic substructure specific fragmentation pattern of structurally different mercapturic acids, mass spectra were recorded with both polarities in the present work. In negative ion mode, all the available standards showed a CNL of 129 Da. In positive ion mode, a product ion at *m/z* 130 and/or a CNL of 163 Da was observed in the MS/MS mass spectra of many standards. However, a common fragment ion or a CNL did not occur in positive ion mode. These observations are consistent with the reported MS/MS spectra of mercapturic acids derived from styrene, 1,3-butadiene, acroleine, diclofenac, valproic acid, and dopamine, where a CNL of 129 Da occurred in the negative ion mode and a fragment ion at *m/z* 130 was formed in the positive ion mode [77, 87, 103-105].

To determine the most sensitive survey scan, CNL 129 Da and theoretical mass transitions (thMRM) based on the CNL 129 Da were tested as survey scans for the detection of mercapturic acids. In summary, both methods applied were equally sensitive in all matrices tested. The generic IDA CNL method can be rapidly and easily applied. In contrast, IDA thMRM may be more useful when metabolites with

different substructures and, thus, different specific neutral losses or product ions (e.g. different phase II metabolites of a compound) need to be analyzed in one run. However, then, the method looses its general acceptance. Thus, further data acquisition was performed with IDA CNL. Changing the parameter and mass spectrometer settings such as intensity threshold, collision gas, settling time and linear ion trap fill time improved reproducibility but impaired sensitivity. A compromise was necessary to acquire reproducible data with sufficient sensitivity. Validation of two IDA methods and a conventional MRM method (defined mass transitions with optimized compound-dependent parameters of the analyte of interest) demonstrated that precision and accuracy did not reach the values of the conventional MRM method (chapter 4.1.3). In general, peak areas that are obtained by IDA analysis may show tendencies in the change of concentration of an analyte, but quantitation for pharmacokinetic data acquisition is still recommended to be performed with the most sensitive and reproducible MRM method. Moreover, compared to MRM scanning of only few mass transitions, sensitivity suffered when using the IDA methods due to scanning of over 250 masses in one scanning cycle. In pretests, sensitivity of mercapturic acid standards that were determined with MRM scan mode (13 mass transitions and optimized settings for each compound) was 100 to 4,400 times more sensitive than with the corresponding IDA methods.

Using the IDA methods, LOD levels of the mercapturic acid standards ranged from 0.3 to 15.5 pmol on column depending on matrix and compound. Peak shape and sensitivity of some standards were improved by changing the solvent from initially 5 mM ammonium acetate (pH 6.8) to 0.1% of formic acid. Protonation of the carboxylic groups resulted in reduced polarity and, thus, in delayed elution of the mercapturic acids, so that the initial proportion of the organic phase could be increased to improve the ionization rate of the polar analytes. However, the partial decrease in sensitivity for some mercapturic acid standards (e.g. *N*-acetyl-*S*-(*tert*-butylbenzyl)-L-cysteine) was presumably due to ion suppression caused by the acidic mobile phase of 0.1% of formic acid [106]. The different LOD levels for the standards in different matrices can be explained by the influence of the sample solvents containing buffers and salts on the electrospray ionization of standards eluting close to the solvent front (e.g. *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine). Alternative methods like electrochemical detection, HPLC-UV detection and ELISA have been previously used for the detection of mercapturic acids and provided similar

sensitivities [61, 107]. Previously published methods for the analysis of specific mercapturic acids showed higher sensitivities than the methods developed in this work (Table 18). However, these research groups developed and optimized the analytical methods for defined mercapturic acids. In contrast, the aim of this work was to establish a method for the general screening of mercapturic acids as a tool for detection of reactive intermediates of a wide range of different compounds. In contrast to the published data, the CNL survey scan was combined with EPI scans to obtain information about the chemical structure of the signal detected. Insertion of a second EPI experiment with higher collision energy resulted in enhanced fragmentation and thus improved efficiency of structural elucidation of a signal as shown for 3-OH-AAP-MA and AAP-MA-SO. Due to simultaneous detection and characterization of structurally different mercapturic acids in a single run, a reduction in sensitivity for the single analytes is comprehensible. Moreover, in regard to the generic detection of also yet unknown mercapturic acids, it is more relevant to obtain additional information about the chemical structure of the detected signal than to reach highest sensitivity. Using the developed and validated generic HPLC-MS/MS screening method nearly all of the published mercapturic acids of the compounds tested were detected in different matrices. Additionally, new mercapturic acids were obtained and demonstrate the sensitivity of this method. Moreover, simultaneous detection of corresponding sulfoxides (e.g. in the acetaminophen human study) that have not been described before confirms the strength of this screening method.

Table 18: LOD levels of various mercapturic acids determined with different previously published methods and LOD levels determined in the present study using IDA CNL/negative EPI.

Compound	**Method**	**Matrix**	**Published LOD level [pmol o.c.]**	**LOD levels [pmol o.c.] obtained in this work**	**Reference**
N-Acetyl-*S*-phenyl-L-cysteine	GC-MS/MS (SRM)	urine	0.02	2.1	[108]
N-Acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine	GC-MS (SIM)	urine	0.4	15.5	[109]
N-Acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine,	GC-MS (SIM)	urine	0.4	0.8	[109]
N-Acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine	GC-MS (SIM)	urine	0.001	6.8*	[29]
N-Acetyl-*S*-(3-hydroxypropyl)-L-cysteine	GC-MS/MS (SRM)	urine	0.45	2.3*	[108]
N-Acetyl-*S*-(hydroxy-3-butenyl)-L-cysteine†	LC-MS/MS (MRM)	urine	92	n.d.	[77]
N-Acetyl-*S*-(1,2-dihydroxybutyl)-L-cysteine	LC-MS/MS (MRM)	urine	3.8	n.d.	[77]

*LOD levels determined in CH_3OH/H_2O

†mixture of *N*-acetyl-*S*-(1-hydroxy-3-butenyl)-L-cysteine and *N*-acetyl-*S*-(2-hydroxy-3-butenyl)-L-cysteine

The established IDA methods can be applied to many analytes that provide a CNL or a common product ion for the generation of a specific survey scan. For example, DNA-modifications may be analyzed by this MS/MS technique using the loss of 116 Da (i.e., for etheno-dA or OTA-dG [110, 111]). The loss of 116 Da corresponds to the desoxyribose moiety comparable to the fragmentation described for glycosides by Qu

et al. [101]. The oxidized glucose moiety of glucuronic acids provides a neutral loss of 176 Da and may be used for detection of such phase II metabolites as performed in the present study for the detection of acyl glucuronides. For instance, a method using this loss of 176 Da in a specific MRM mode may be used for the determination of BPA levels in urine and plasma samples. In addition, a product ion at *m/z* 113 is formed from the glucuronide moiety and may be used for the generation of an IDA PrIS method for the general detection of glucuronides [66].

Sample preparation for this method was simple and rapid. Interfering proteins could be precipitated by addition of acetonitrile and separated from the sample by centrifugation. Solid phase extraction (SPE) with two different materials (HLB (hydrophilic-lipophilic balance) and MAX (mixed anion exchange)) was tested to withhold ion suppressing salts, but resulted in either loss of most polar compounds (*N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine with HLB) or loss of the less polar analytes (MAX) during the washing steps. Tests with online sample preparation did not result in an efficient improvement of sensitivity. The injection volume had to be increased to a factor of five to obtain a two times higher detection sensitivity. Due to the small amount of sample available, e.g. in the acetaminophen rat study, online preparation was not performed. However, when analyzing human urine, a much higher sample amount would be available, so that online sample preparation might be useful. Due to the great variance in polarities and chemical behaviour of a metabolite group in urine, loss in recovery of some compounds always has to be accepted when using SPE or online sample preparation as previously shown by Weigel et al. [112].

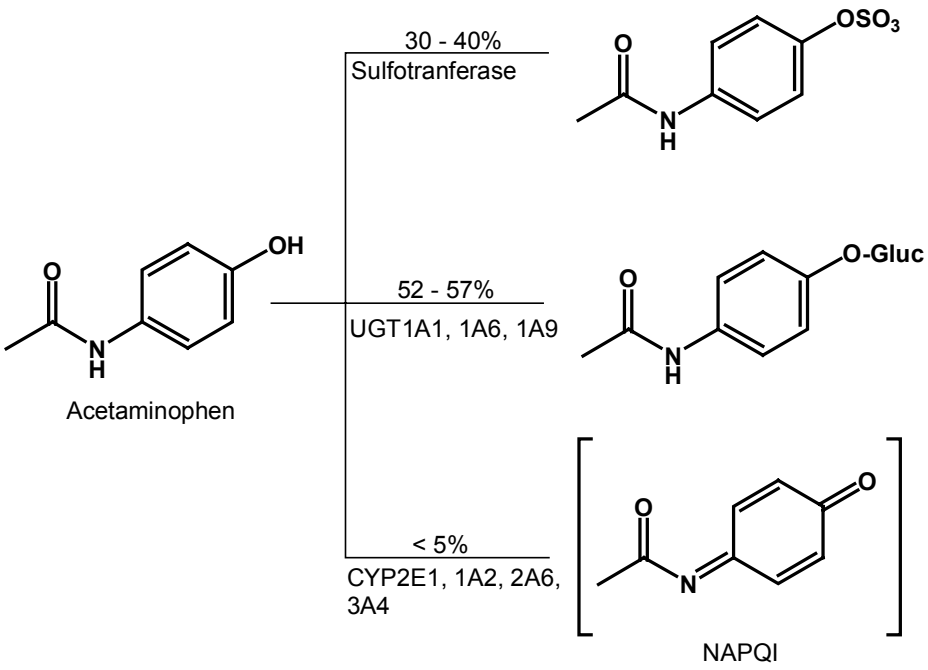
5.1.2 Proof of Concept – Generation of Mercapturic Acids *in vitro*

Testing the drugs acetaminophen, diclofenac, troglitazone, clozapine, bifonazole, carbamazepine, and the industrially used chemical bisphenol A for generation of mercapturic acids, all of them were bioactivated to reactive intermediates and trapped with *N*-acetyl-L-cysteine to form mercapturic acids. Here, well known reactive intermediates were detected which evaluated the developed HPLC-MS/MS method and verified the applicability of the incubation assays to capture potential bioactivation liabilities. In addition, mercapturic acids were detected that have not or just recently been described e.g. for troglitazone [92] or diclofenac [91]. One

metabolite of diclofenac has been previously detected in incubations with activated neutrophils but not *in vivo*.

5.1.2.1 Acetaminophen

Acetaminophen is known to cause severe liver damage when overdosed. The analgesic and antipyretic drug is mainly metabolized by sulfation (30 – 40%) and glucuronidation of the hydroxyl group (52 – 57%) [113]. A minor part, however, is bioactivated by CYP450 isoenzymes, including CYP2E1, CYP1A2, CYP3A4, and CYP2A6 to *N*-acetyl-p-quinone imine (NAPQI) by direct oxidation [16, 114]. At clinically used doses, this metabolite is rapidly detoxified by conjugation to glutathione (GSH) and excreted as its mercapturic acid with urine (Scheme 16). However, at high doses of > 10 g in humans, sulfation of acetaminophen is saturated and GSH is depleted by as much as 90% [16]. Thus, NAPQI is formed in high concentrations with lack of detoxifying agents and covalently bind to liver proteins and other macromolecules leading to severe liver damage [16]. Primary cellular targets have been postulated to be mainly mitochondrial proteins, resulting in loss of energy production and cellular ion control with an ongoing loss in calcium homeostase [17]. Further metabolites of acetaminophen are 3-hydroxyacetaminophen and 3-methoxyacetaminophen, but are of minor importance for the hepatotoxic effect of acetaminophen [115]. Due to the well established research on acetaminophen and its metabolism pathways, this compound was selected for this study to verify and evaluate the HPLC-MS/MS method for *in vitro* and *in vivo* assays.



Scheme 16: Metabolic pathways of acetaminophen in humans after administration of clinically used doses.

Hydroxylation of acetaminophen to 3-hydroxyacetaminophen (3-OH-AAP) and formation of the corresponding mercapturic acid occurred in incubations with human liver microsomes, but not with rat liver microsomes. The corresponding mercapturic acid was detected and indicated species-specific metabolism. The human CYP450 isoenzymes CYP2A6 and CYP2E1 are responsible for the generation of this metabolite as described for the formation of the corresponding GSH adduct with the recombinant isoenzymes. The isoenzyme CYP2E1 is also expressed in rat liver microsomes, in contrast to a lack of expression of CYP2A6 [116]. In incubation tests of acetaminophen with recombinant CYP2E1, no detectable signal for 3-OH-AAP-MA was obtained. Since CYP2A6 is the main isoenzyme for hydroxylation of acetaminophen, 3-hydroxyacetaminophen may only be formed by CYP2E1 to a very low, not detectable amount in incubations with rat liver microsomes [115]. The fact that 3-hydroxyacetaminophen is not as hepatotoxic as NAPQI [117], but conjugates with nucleophilic compounds, demonstrates, that metabolites that are conjugated to *N*-acetyl-L-cysteine or GSH are not unequivocally toxic to the body, but target proteins play also an important role.

NAPQI was formed in every incubation assay tested, including incubations of acetaminophen with activated human neutrophils. In contrast to human liver

microsomes, 3-OH-AAP-MA was not formed in detectable amounts in incubations with activated human neutrophils, demonstrating the tissue-specific and CYP450-mediated formation of this metabolite. The formation of NAPQI in activated neutrophils demonstrates that this cell type may also present a potential target tissue for protein adduct formation by acetaminophen metabolites. However, only very few incidences of agranulocytosis are described for acetaminophen treated patients [118]. Generation of NAPQI in both tissues but with different toxic effects indicates the importance of the target protein to which NAPQI eventually binds [119, 120].

5.1.2.2 Diclofenac

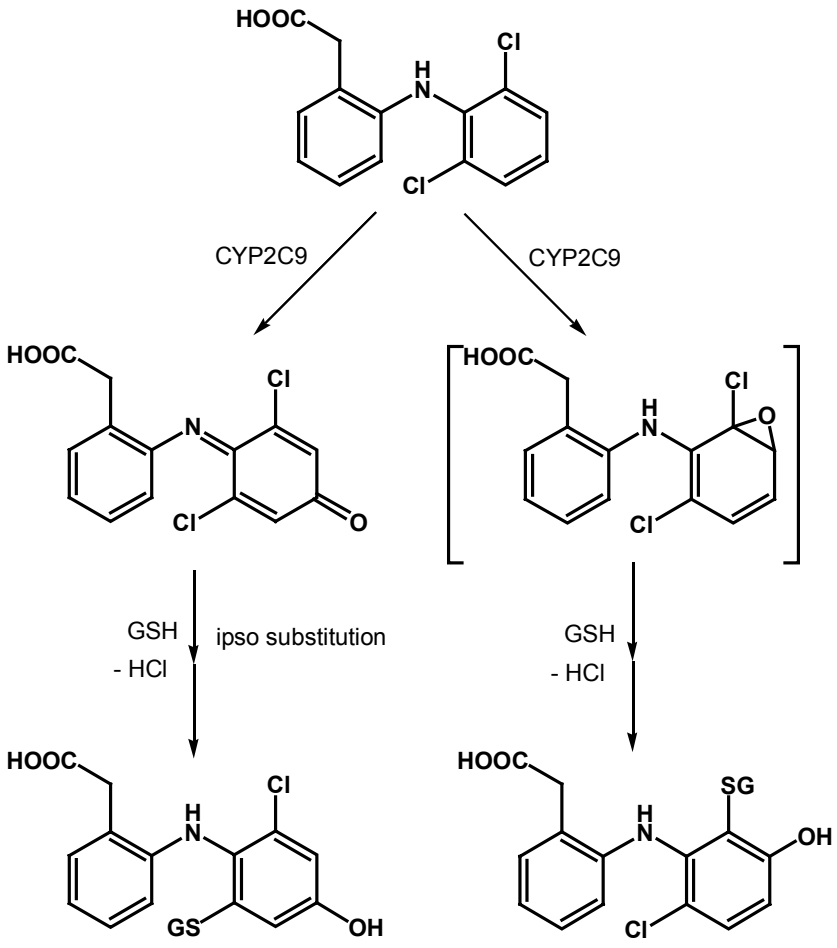
This drug is a non-steroidal anti-inflammatory drug (NSAID) widely used for pain relief. Diclofenac is described to cause rare but severe hepatic injuries, neutropenia, and bone marrow toxicity in patients [121]. The reason for these fatal clinical side effects are still unknown, but are assigned to idiosyncratic toxicity and, thus, may relate to reactive intermediate formation [31]. In liver, bioactivation of diclofenac to reactive metabolites, including C5-hydroxylation and C4´-hydroxylation by the CYP450 isoenzymes CYP3A4 and CYP2C9, respectively, followed by oxidation to protein-reactive quinone imines is described and may attribute to the hepatic necrosis (Scheme 9) [88]. In leucocytes, including neutrophils, CYP450 enzymes play a minor role in activation of xenobiotics. In activated neutrophils, toxification is mainly influenced by hypochloric acid that is generated by the myeloperoxidase (MPO) system of neutrophils [31, 34, 91]. This highly reactive milieu may generate reactive metabolites of diclofenac that are different from hepatic formed metabolites. Thus, neutrophils together with their metabolism processes should be regarded closer to obtain ideas about different reactive intermediate formation of diclofenac in these cells. Although many publications describe the effect of diclofenac on neutrophils, they only examined the reason for the anti-inflammatory effect [122, 123]. However, little is known about the effect neutrophils cause on diclofenac bioactivation. Therefore, diclofenac was incubated with rat and human hepatic microsomes and activated human neutrophils to regard species- and tissue-specific biotransformation of diclofenac.

Three metabolites corresponding to *N*-acetyl-L-cysteine conjugates of 4´-hydroxydiclofenac and 5-hydroxydiclofenac (*N*-acetyl-L-cysteine conjugation at C4

and C6 position of diclofenac) were detected in rat liver microsomal incubation, whereas only 4´-hydroxydiclofenac mercapturic acid was formed in incubation with human liver microsomes. The detection of the three metabolites in rat liver microsomal incubation was consistent with results described by Tang et al. [124]. In human microsomes, Tang et al. described a dose-dependent increase of 5-hydroxylation when diclofenac concentration exceeded 100 µM [88]. This observation suggests that metabolism of diclofenac by CYP2C9 predominantly occurs at lower concentrations up to a saturation of this isoenzyme. Only at higher concentrations (over 100 µM) of diclofenac, the metabolic activation is also mediated by the unselective CYP3A4 which catalyzes hydroxylation of diclofenac at the chemically preferred C5 position. Although CYP3A4 (about 30% of all CYP450 isoenzymes) is the most important isoenzyme involved in 60% of metabolism of xenobiotics, diclofenac is preferentially metabolized by the more specific CYP2C9 (K_m value of 9 µM for 4´-hydroxylation and K_m value of 41 µM for 5-hydroxylation [125]). Thus, the concentrations used in the human liver microsomal incubation study (50 µM and 100 µM) were too low to reach detectable amounts of 5-hydroxydiclofenac mercapturic acids. In rat liver microsomal incubations, the mercapturic acids of 5-hydroxydiclofenac were the predominant metabolites detected. The different formation rates of these diclofenac metabolites indicate species-specific expression and activities of CYP450 isoenzymes. Although 3´-hydroxydiclofenac, 4´,5-dihydroxydiclofenac, and N,5-dihydroxydiclofenac are described as minor metabolites of diclofenac, mercapturic acids of these metabolites have neither been described [125], nor were detected in this study.

In human and rat liver microsomes, another metabolite was detected and assigned to *N*-acetyl-*S*-{2-[2´-(carboxymethyl)phenylamino]-3-chloro-5-hydroxyphenyl}-L-cysteine (deschlorodiclofenac-MA) as recently described by Yu et al. who detected the corresponding GSH adduct in human liver microsomes [90]. In contrast, Yan et al. assigned the metabolite to the regioisomer, i.e. 2´-OH-3´-glutathione-*S*-yl-monoclofenac [126]. Both showed identical mass spectra for the metabolites in positive ion mode by HPLC-MS/MS, whereas Yu et al. additionally confirmed the identity of the metabolite by HPLC/^{1}H-NMR and compared it to a synthetic standard. Yan et al. performed HPLC-MS/MS with isotope labeled GSH for the identification of the metabolite. The mechanism of formation of 4´-OH-2´-glutathione-S-yl-deschlorodiclofenac, as described by Yu et al., is more comprehensible due to

CYP2C9 specific C4´-hydroxylation of diclofenac rather than C2´-substitution of one chlorine atom via an arene oxide intermediate (Scheme 17). Both research groups detected this metabolite only in incubations with human liver microsomes. With the HPLC-MS/MS screening method presented in this study, the metabolite was also detected in rat liver microsomal incubation.



Scheme 17: Proposals for the biotransformation pathways of diclofenac to two different isobaric diclofenac metabolites, published by Yu et al. and Yan et al.

The different characterization of this metabolite demonstrates the disadvantage of HPLC-MS/MS methods for unequivocal identification of metabolites and compounds. Due to their similar structures, regioisomers cannot be differentiated by their mass spectra. Thus, EPI scans of unknown analytes are sufficient for characterization of the detected signal, but do not replace methods like NMR for identification of the analyte of interest. NMR analysis would provide information about the chemical

structure of a metabolite, but relatively high amounts of the isolated compound would be necessary. Thus, the incubation assay had to be scaled up and the metabolite had to be isolated and purified. These processes would cost time and resources. Moreover, ^{13}C-NMR would often be essential for unequivocal characterization of a metabolite. In this work, the developed screening method optimized the characterization of a compound by introducing a second product ion scan with higher collision energy into the method to obtain a maximum of information about the chemical structure of the analyte. Moreover, unequivocal identification is not always necessary to assess, for instance, the chemical reactivity of a compound.

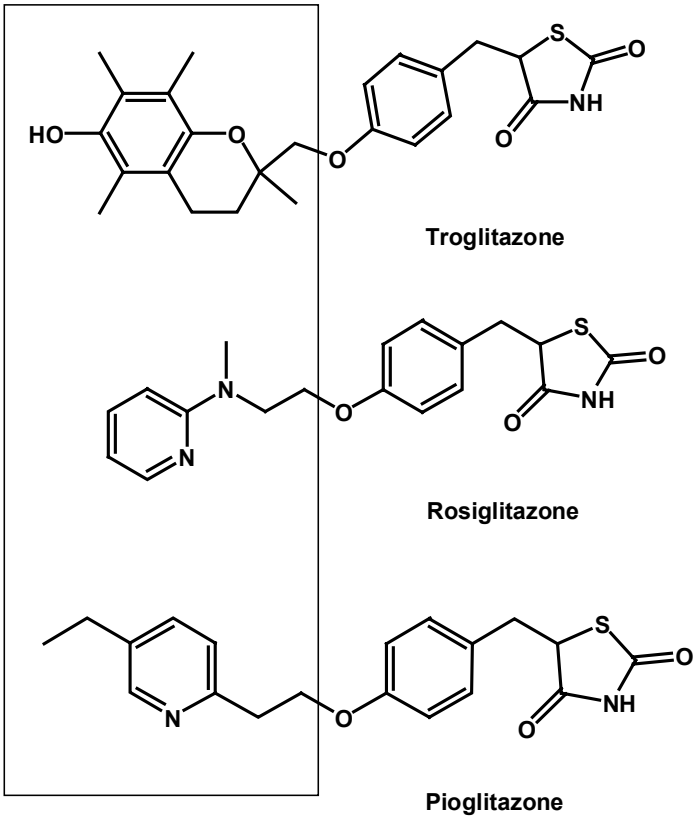
A metabolite of diclofenac with a precursor ion at *m/z* 459 in negative ion mode was detected in human liver microsomes, but not in rat liver microsomal incubations. A metabolite that would correspond to this mercapturic acid has not been described so far and was not further characterized in this work. Nevertheless, the typical chlorine isotopic pattern, the lack of this signal in control samples, and the mass spectrum with fragmentation behaviour similar to diclofenac and its metabolites indicate the presence of a mercapturic acid of a diclofenac metabolite.

The presence of two different metabolites of diclofenac (*m/z* 441 and *m/z* 487) in incubations with activated neutrophils which did not occur in liver microsomal incubation demonstrates that there are tissue-specific differences in the biotransformation of diclofenac. The lack of 4´-hydroxydiclofenac mercapturic acid in activated neutrophils is presumably due to the lack of CYP2C9 and other CYP450 isoenzymes in leucocytes [127-129].

5.1.2.3 Troglitazone

Isolated cases of acute liver toxicity led to the withdrawal of the anti-diabetic drug troglitazone from the market [18]. Formation of reactive metabolites has been suggested as a possible reason for the idiosyncratic hepatotoxicity of troglitazone. Main focus of troglitazone biotransformation is on the potentially reactive chromane moiety, because this structural part of troglitazone is absent in the other clinically used glitazones (pioglitazone and rosiglitazone) that do not cause this toxic effect. Immune-mediated side effects have not been described, so that incubation tests of troglitazone with neutrophils were not performed. Formation of reactive intermediates of troglitazone was tested with rat and human liver microsomes. Since CYP3A4 is

described as the isoenzyme responsible for the bioactivation of troglitazone, a species-specific difference was not expected.



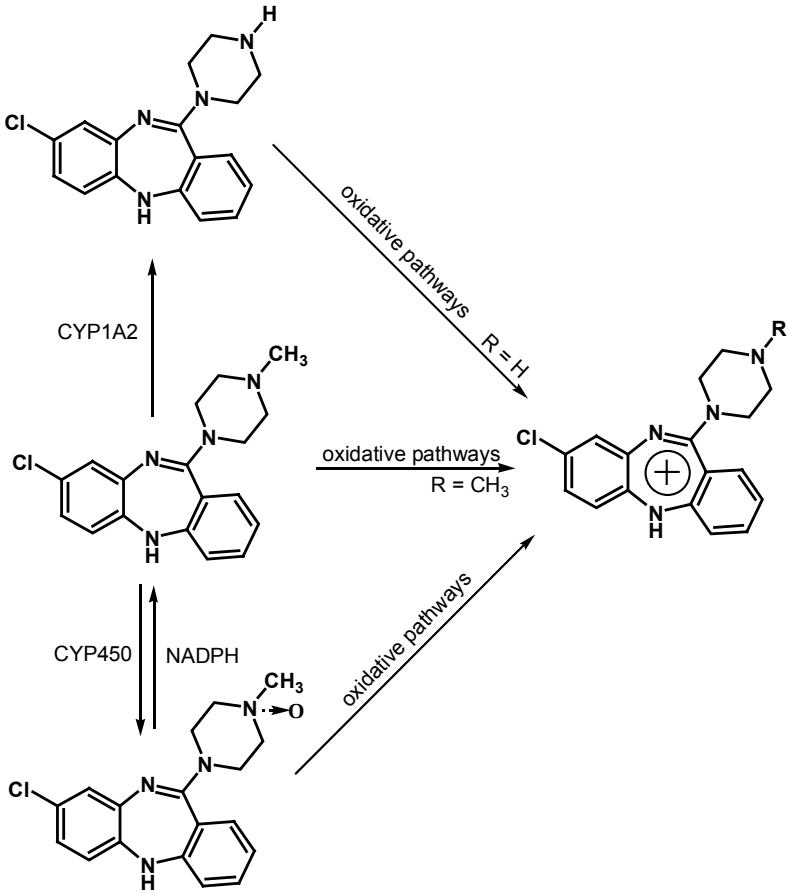
Chemical structures of three different glitazones with differences in structural properties (framed)

Incubation solutions of troglitazone with both hepatic microsomes generated numerous signals, suggesting a great potential for reactive intermediate formation. Predominant *N*-acetyl-L-cysteine adducts with precursor ions at *m/z* 635 and *m/z* 601 in negative ion mode were detected in agreement with the recently described GSH adducts in hepatic microsomes [92]. A new metabolite of troglitazone with a molecular mass of 606 was obtained (Figure 11). According to its mass spectra in positive and negative ion mode, the metabolite was characterized as a mercapturic acid of troglitazone. The corresponding GSH metabolite has been recently described by Alvarez-Sánchez et al. [92]. Detection of mercapturic acids with precursor ions outside the expected mass range (*m/z* 550 to *m/z* 650) demonstrates the importance of scanning a wide mass range to obtain all potentially reactive metabolites.

5.1.2.4 Clozapine

Clozapine is an atypical neuroleptic drug and a model compound for immune-specific idiosyncratic toxicity such as agranulocytosis. The mechanistic processes of drug-induced agranulocytosis are presumably based on the formation of reactive intermediates. Since neutrophils are able to generate reactive intermediates, it seems more likely that these metabolites are responsible for drug-induced agranulocytosis than metabolites formed in the liver. However, since liver necrosis is described as another rare drug-induced side effect of clozapine and bioactivation to reactive metabolites has been observed in neutrophils and liver, incubation tests were performed with both *in vitro* assays.

A signal with a precursor ion at *m/z* 472 was detected only in human liver microsomal incubations, suggesting CYP450-dependent demethylation and activation of clozapine (19 – 27% of clozapine in human liver microsomes is demethylated [35, 130]). The lack of this metabolite in incubations with activated neutrophils may be due to the fact that neutrophils do not or only to a small extent express CYP450 isoenzymes [127, 129]. *N*-acetyl-L-cysteine adducts of a possible *N*-oxidated clozapine were not detected, presumably due to re-reaction to clozapine or formation of the nitrenium ion as described previously (Scheme 18) [130, 131].



Scheme 18:Metabolism of clozapine and bioactivation of the metabolites to the nitrenium ion.

The nitrenium ion itself could be readily trapped with *N*-acetyl-L-cysteine in neutrophils and human liver microsomal incubations (*m/z* 486 in negative ion mode). Surprisingly, this mercapturic acid was also detected in incubations of clozapine with neutrophils that had not been activated before. Clozapine is described to initiate respiratory burst in human neutrophils [132] in the same way as lipopolysaccharide [133]. Based on this fact, clozapine can stimulate neutrophils for oxidative burst on its own and may explain the detection of *N*-acetyl-L-cysteine adducts of clozapine also in non-activated neutrophils. This feature was also observed by Gardner et al. who described protein binding of clozapine without stimulation of neutrophils, unlike other drugs [134].

Although clozapine generated the protein-reactive nitrenium ion in both assays, the drug shows incidence of only 0.03% of liver damage compared to 1% of agranulocytosis [135]. Thus, the question arises why there is such a target-specificity in clozapine toxicity despite the fact that the nitrenium ion is formed in both incubation assays. Gardner et al. tested the formation of reactive intermediates of

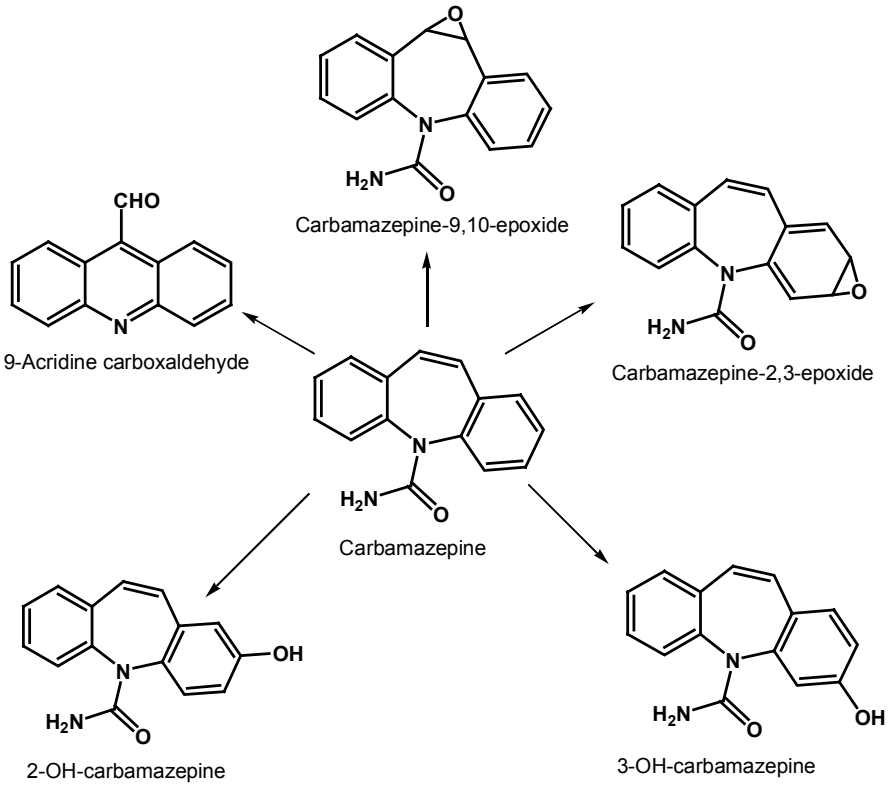
clozapine and olanzapine, a drug with similar chemical structure but no association with induction of agranulocytosis [136]. Gardner et al. obtained similar reactive intermediates for both drugs but detected different protein adducts. These different protein adducts may subsequently result in different effects on protein- and, furtheron, cell function and may explain the clozapine-specific toxicity. Moreover, there may be a difference in the amount of nitrenium ion formed and the presence of nucleophilic trapping agents as GSH to distract the electrophile from binding to macromolecules.

5.1.2.5 Bifonazole

Bifonazole is an antimycotic drug and is commonly topically used in ointments and sprays. Data on metabolism or toxicity studies in mammals are rare. Only direct influence on the activity of CYP450 isoenzymes responsible for the antimycotic effect [137] or its anti-inflammatory activity in granulocytes have been investigated [138]. Due to its chemical structure, hydroxylations of aromates may be initiated by CYP450 enzymes, leading to protein-reactive quinones after oxidation. Thus, bifonazole was included in the incubation tests. Bifonazole was bioactivated to a reactive intermediate and was conjugated with *N*-acetyl-L-cysteine in human liver microsomal incubations but not in incubations with activated neutrophils, indicating a CYP450-dependent metabolism of bifonazole. Moreover, hydroxylation on an aromatic ring of bifonazole, as observed in this assay, is predominantly catalyzed by CYP450 monooxygenases.

5.1.2.6 Carbamazepine

The anti-epileptic drug has been associated with rare adverse reactions such as blood and hepatic disorder. Bioactivation of carbamazepine to iminoquinone, 10,11-epoxide, or 9-acridine carboxaldehyde or other potentially reactive metabolites may contribute to the drug-induced idiosyncratic side effects [35]. To determine possible tissue-specific differences, incubation test were performed with human liver microsomes and human neutrophils.



Scheme 19: Primary main metabolites of carbamazepine, formed by various CYP450 isoenzymes.

The iminoquinone of carbamazepine was generated in incubations with human liver microsomes and activated neutrophils and was trapped with *N*-acetyl-L-cysteine to form a mercapturic acid with a precursor ion at *m/z* 369. Unlike high conversion of carbamazepine to this metabolite in activated neutrophils, the signal of this metabolite was very weak in human liver microsomal incubation (S/N 3), indicating only slight conversion of carbamazepine to iminoquinone with hepatic microsomes. This difference may result from the presence of an NADPH regenerating system in liver microsomal incubation, that presumably reduced the iminoquinone to the more stable 2-hydroxyiminostilbene. Additionally, activated neutrophils with hypochloric acid may generate iminoquinone from iminostilbene more readily than liver microsomes do. Pearce et al. detected the *N*-acetyl-L-cysteine adduct of 2-hydroxyiminostilbene only in the absence of NADPH, indicating the metabolism only under oxidative conditions [38]. Moreover, GST as the reaction accelerating enzyme for GSH conjugation may play an important role in detoxification of reactive metabolites of carbamazepine. Lillibridge et al. described that GST addition to microsomes in the presence of carbamazepine could diminish protein adduct

formation even beyond that seen with GSH alone, indicating an increase in detoxification by GST [139].

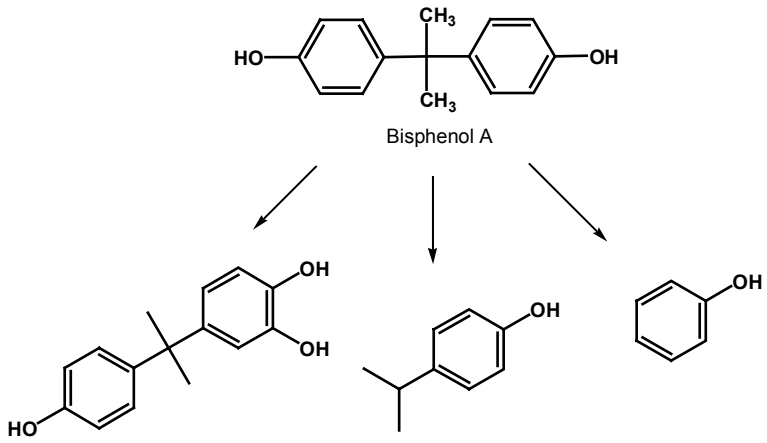
The alternative C3-hydroxylation of carbamazepine does not potentially form a reactive iminoquinone and was not detected as mercapturic acid in this study. Mercapturic acids of the alternative metabolites were not detected either. 9-Acridine carboxaldehyde is electrophilic and has been shown to form protein adducts even in non-activated neutrophils but formed conjugates only with primary amines [140]. This may explain the lack of this metabolite in the assay and, once more, demonstrates the need of complementary assays for the determination of reactive intermediates. In this study *N*-acetyl-L-cysteine adducts of carbamazepine-10,11-epoxide were not formed in detectable amounts, neither with activated neutrophils nor with hepatic microsomes, despite positive results for GSH adduct formation by Bu et al. in human liver microsomal incubations [141]. This difference may be due to variations in GST activities and the protein concentration used.

5.1.2.7 Bisphenol A

Bisphenol A (BPA), an environmental estrogen-like compound, used as a monomer for the production of polycarbonate plastics and as an antioxidant in PVC plastics, can be chemically converted to reactive derivates that bind to DNA and GSH *in vitro* [101, 142, 143]. BPA is a polyphenol and shows substructures similar to a broad spectrum of compounds including flavonoids that are present in many plants and, thus, in food [12]. Although BPA is metabolized extensively to its glucuronide in humans, understanding the metabolism of BPA is important for risk assessment [66]. Moreover, BPA has been described to influence the immune system by affecting cytokines and is discussed as immunotoxic [144]. However, only the direct effects of bisphenol A on various cell types have been examined rather than the effect of bioactivating systems on BPA that may be the initial step for the toxic effects of BPA *in vitro*. Thus, BPA was tested for the generation of reactive intermediates with human liver microsomes and human neutrophils.

Trapping the reactive intermediates of BPA with *N*-acetyl-L-cysteine obtained adducts of phenol, BPA, and its quinone in incubations with human liver microsomes and activated neutrophils. Hydroxylation of BPA to 3-hydroxybisphenol A is described by Jaeg et al. [145]. They also observed cleavage of BPA to phenol and isopropylphenol

in mice liver microsomal incubations and S9 fractions and detected the corresponding GSH adducts (Scheme 20). However, regarding the *in vitro* results of the BPA bioactivation potency and toxicities observed in animal studies, BPA is rapidly deactivated by glucuronidation in humans. A rapid deactivation *in vivo* challenges the relevance of *in vitro* tests for risk assessment. These diverse species-specific effects demonstrate that bioactivation data of a compound in animal studies do not imperatively predict the risks humans are exposed to.



Scheme 20: Metabolites of bisphenol A that were conjugated with GSH and detected in mice liver microsomal incubations [145].

5.1.2.8 Overview and Conclusions of *in vitro* Results

To sum it up, species-specific differences were tested for acetaminophen, diclofenac, and troglitazone. Tissue-specific differences were examined for drugs and chemicals that are associated with immune-mediated toxicities such as acetaminophen, diclofenac, clozapine, and carbamazepine. Bifonazole and bisphenol A were included into the test as compounds with structural properties that potentially form reactive intermediates. A summary of metabolites that were detected in the various *in vitro* assays are given in Table 19.

Table 19: Differences and similarities in formation of mercapturic acids of compounds tested with activated human neutrophils (HN), human liver microsomes (HLM) and rat liver microsomes (RLM). References (Ref.) of the previously published reactive intermediates are listed in the table.

Compound	$[M - H]^-$	Reactive metabolite	HN	HLM	RLM	Ref.
Acetaminophen	311	Quinone imine	+	+	+	[146]
	327	3-OH-AAP	-	+	-	[83]
Diclofenac	437	Deschloro-DCF	-	+	+	[90, 126]
	459	n.d.	-	+	-	novel
	441	Decarboxy-DCF	+	-	-	[91]
	471	5-OH-DCF	+	-	+	[124]
	471	4´-OH-DCF	-	+	+	[124]
	487*	DiOH-DCF/MA-SO	+	-	-	novel
Troglitazone	635	α-Keto-isocyanate		+	+	[20]
	605	Quinone methide		+	+	[92]
	601	Qinone methide		+	+	[20]
	310	n.d.	n.d.	+	+	novel
	312	n.d.		+	+	novel
	326	n.d.		+	+	novel
	328	n.d.		+	+	novel
Clozapine	472*	Demethylated cloz.	-	+	n.d.	[131]
	486	Nitrenium ion	+	+		
Bifonazole	502	Di-OH-bifonazole	-	+	n.d.	novel
Carbamazepine	351	n.d.	+	-	n.d.	novel
	369	2-OH-iminostilbene	+	+		[38]
Bisphenol A	254	Phenol	+	+		
	388	BPA	+	+	n.d.	[145]
	404	OH-BPA	+	+		

*proposals for metabolites are given in the corresponding chapters

In conclusion, the *in vitro* data clearly demonstrate the applicability of the developed and optimized HPLC-MS/MS screening method for the generic detection of mercapturic acids. The generation and detection of reactive intermediates as *N*-acetyl-L-cysteine adducts as shown in this work display complementary methods to the assay with GSH, which is commonly used for the detection of reactive intermediate formation [49]. Reactive intermediates that are detoxified enzymatically by GST-mediated GSH conjugation may lack in these *N*-acetyl-L-cysteine assays. However, this may indicate a GST-catalyzed metabolic pathway of the compound. Detection of tissue-specific mercapturic acids as shown for diclofenac and bifonazole demonstrates that one *in vitro* assay is not sufficient for extensive interpretation of toxicity of a compound, but different tissues and cell types have to be tested prior to further investigations in drug development. Although *in vitro* tests provide a good possibility to search for reactive intermediates they should not be overestimated and do not eliminate the necessity of *in vivo* studies due to the lack of phase II metabolism and detoxification processes *in vitro*. The direct generation of mercapturic acids *in vitro* that presumably occur *in vivo* in urine is of great advantage compared to GSH assays. Urine is a matrix in which mercapturic acids mainly occur and that can be easily and non-invasively collected.

5.1.3 Proof of Concept – Detection of Mercapturic Acids *in vivo*

5.1.3.1 Acetaminophen

Renal excretion of acetaminophen mercapturic acid was determined in humans (2.1 ± 0.9% of the administered dose of acetaminophen) and rats (20 mg/kg b.w.: 2.8 ± 0.7%; 640 mg/kg b.w.: 8.1 ± 1.4%) showing similar results to previously published data [147-149]. Other research groups demonstrated formation of NAPQI corresponding to < 5% of the administered dose of acetaminophen and after overdose an increased formation of 7 to 15% in humans [150, 151]. Interestingly, this metabolite was also detected in unexposed human urine, but not in blank rat urine samples. Acetaminophen is described as a metabolite of various compounds such as aniline and aminophenol [152, 153]. Humans are exposed to those compounds in daily life [154, 155]. Although exposure is low, the sum of all potential compounds forming acetaminophen as a metabolite may yield to detectable amounts of this

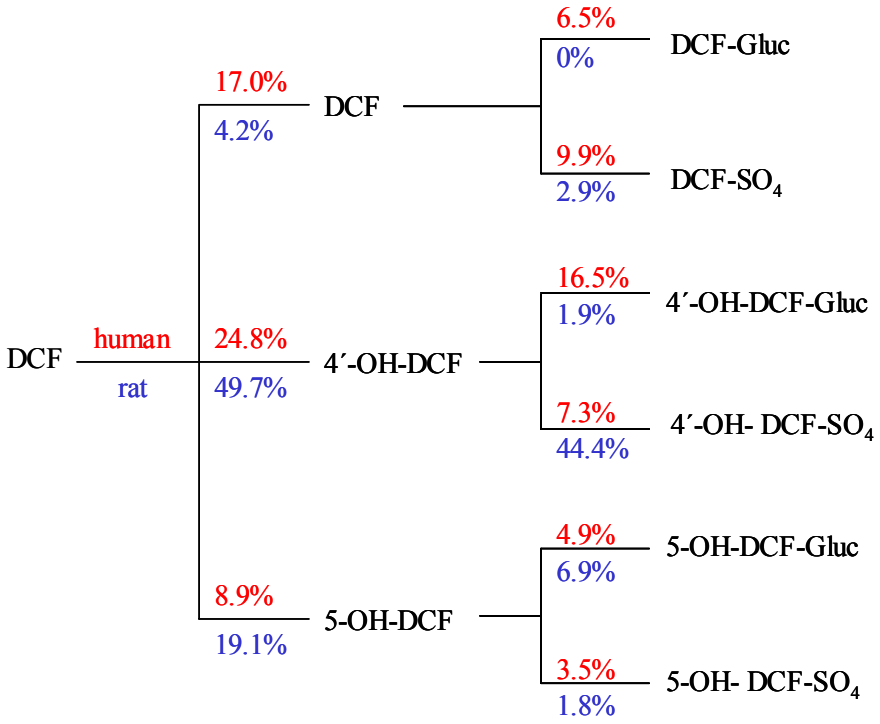
mercapturic acid. The amount of detected acetaminophen mercapturic acid in unexposed humans was up to a factor of 150 times lower than after administration of the clinically used dose, demonstrating quite high exposure rates with such acetaminophen forming compounds. Exposure of humans to these compounds in daily life, including diet or environmental exposure, could explain the lack of this metabolite in rat urine samples. In contrast to humans, rats have defined living and diet conditions. The developed screening method has shown the detection of acetaminophen mercapturic acid in unexposed humans for the first time and demonstrates the sensitivity of this method.

Hydroxylation to 3-hydroxyacetaminophen is described as a further metabolite of acetaminophen and was suggested as mercapturic acid in urine samples of subjects administered this drug [100]. Administration of 3-hydroxyacetaminophen to mice showed depletion of GSH and an increase in release of glutamic-pyruvic transaminase into the blood, but Forte et al. were not able to detect the GSH adduct of 3-hydroxyacetaminophen [117]. In addition, only very small amounts of 3-hydroxyacetaminophen (together with 3-methoxyacetaminophen less than 5% of an administered dose of 500 mg/kg b.w. of acetaminophen) are described to be formed from acetaminophen in mice within 24 hours. Thus, unambiguous identification of this metabolite *in vivo*, though, is still lacking. In the present study, a metabolite with identical mass suggested the presence of *N*-acetyl-*S*-[5-(acetylamino)-2,3-dihydroxyphenyl]-L-cysteine (3-OH-AAP-MA) (Scheme 14). However, sulfoxidation of AAP-MA would result in a metabolite with identical mass. After further characterization, the metabolite was identified as the sulfoxide of acetaminophen mercapturic acid and its stereoisomer by comparison with MS/MS mass spectra of authentic standards in positive and negative ion mode and optimized chromatographic separation of spiked and unspiked urine samples. Different signal intensities of the two stereoisomers suggested stereo-selective oxidation of the sulfur (Figure 18). Up to now, only few sulfoxides have been described, e.g. for fluoromethyl-2,2-difluoro-1-(trifluoromethyl)vinyl ether sulfoxide and *N*-acetyl-L-cysteine sulfoxide of a cyclopentenone isoprostane that also provided a CNL of 129 Da in the negative ion mode and the formation of a product ion at *m/z* 130 in the positive ion mode [43, 156]. Detection of this new acetaminophen metabolite demonstrates that formation of such sulfoxides must be considered when searching for reactive intermediates. Due to the presence of AAP-MA in unexposed human

urine and the detection of the sulfoxide of acetaminophen mercapturic acid after administration of acetaminophen, the actual formation of NAPQI is higher than determined. Only by using a general screening method, as performed here, such metabolites can be detected and may contribute to an improved detection of reactive metabolites.

5.1.3.2 Diclofenac

Diclofenac is used as a non-steroidal anti-inflammatory analgesic drug, with an association to hepatotoxicity and agranulocytosis. Although diclofenac is mainly metabolized by glucuronidation of the carboxylic group (10% to 20% in humans), as much as 50% of the administered dose are hydroxylated to 4´-hydroxydiclofenac by CYP2C9 and excreted as corresponding glucuronide in bile or urine [157]. Other metabolites, including 5-hydroxy-, 3´-hydroxy-, 4´,5-dihydroxydiclofenac and *N*,5-dihydroxydiclofenac, are formed only to a minor extent (< 5% to 10% in humans) [158]. In rats, diclofenac is mainly metabolized by oxidative pathways, forming 4´-OH diclofenac and 5-OH-diclofenac with their glucuronides and sulfates, respectively (about 70%). The metabolites of diclofenac are mainly excreted in urine (Scheme 21) [159].



Scheme 21: Unchanged diclofenac and main metabolites excreted in rat and human urine (% of urinary excretion). Minor metabolites and biliary excretion were not considered [159].

Rats (administered 10 and 20 mg/kg b.w. of diclofenac) excreted two regioisomeric mercapturic acids of 5-hydroxydiclofenac with urine, whereas 4′-hydroxydiclofenac mercapturic acid was detected only in urine samples of rats administered 20 mg of diclofenac/kg b.w. Poon et al. obtained the two mercapturic acids *N*-acetyl-*S*-{3-[2′-(carboxymethyl)phenylamino]-2,4-dichloro-6-hydroxyphenyl}-L-cysteine (4′-hydroxy-diclofenac-MA) and *N*-acetyl-*S*-[4-(carboxymethyl)-5-(2′,6′-dichlorophenyl-amino)-2-hydroxyphenyl]-L-cysteine (5-hydroxydiclofenac-4-mercapturic acid) in rat urine after administration of 50 mg/kg b.w. diclofenac by i.p. application (for chemical structures see Scheme 9) [87]. 4′-Hydroxylation is a CYP2C regioselectively catalyzed reaction. This metabolite is more stable to oxidation than 5-hydroxydiclofenac. 5-Hydroxydiclofenac can be readily oxidized to a quinone imine and further conjugated with GSH [23]. Shen et al. observed protein adducts of 5-hydroxydiclofenac rather than 4′-hydroxydiclofenac. Thus, conjugation of 5-hydroxydiclofenac with *N*-acetyl-L-cysteine seems more reasonable than conjugation of 4′-hydroxydiclofenac and demonstrates the applicability of mercapturic acids as biomarker for reactive intermediate formation.

Another mercapturic acid that was associated with diclofenac was detected in urine samples of rats administered diclofenac. The signal showed a chlorine isotopic pattern in the CNL survey scan. This metabolite was assigned to a benzylic aldehyde derivative of 5-hydroxydiclofenac that has been previously characterized by Miyamoto et al. *in vitro* [91]. Detection of this unexpected metabolite in urine by the developed HPLC-MS/MS method shows the advantage of this generic screening method. The data demonstrate that diclofenac generates a number of yet unknown reactive intermediates that could contribute to the different toxic effects caused by diclofenac.

5.1.3.3 Endogenously Formed Mercapturic Acids

Besides potential covalent binding of reactive intermediates of acetaminophen to cellular proteins, acetaminophen is also known to induce oxidative stress and apoptosis [16]. Incidence of oxidative stress is associated with lipid peroxidation of ω-6-polyunsaturated fatty acids, eventually resulting in formation of 4-hydroxy-2-nonene (HNE) [160]. HNE undergoes various metabolic pathways including formation of 1,4-dihydroxy-2-nonene (DHN). HNE and DHN may be oxidized to reactive aldehydes that may conjugate with GSH. After enzymatic degradation and *N*-acetylation these metabolites are renally excreted as mercapturic acids (DHN-MA and HNE-MA) [161]. HNE-GSH and its degradation products have been proposed as potential biomarkers for oxidative stress based on increased concentrations in liver and plasma after exposure to iron nitrilotriacetate to rats [162]. HNE-MA was detected in all *in vitro* and *in vivo* samples, indicating a certain extent of oxidative stress to cell types without inducers of oxidative stress. By administration of an oxidative stress inducing compound to rats, it is hypothesized that excretion of such oxidative stress markers increases. A semiquantitative statement on the change in renal excretion of such metabolites was tested with acetaminophen treated rats. The fact that DHN-MA excretion was reduced after exposure to a high dose of acetaminophen was in contrast to the expected results that acetaminophen as an inducer of oxidative stress would lead to an increase in formation of these mercapturic acids. The reduced excretion of DHN-MA after high dose treatment of rats suggests a rapid depletion of GSH by increased formation of NAPQI that conjugates with GSH. This conjugation reaction may be competitive to GSH conjugation with HNE and its derivatives. Thus,

the results in the present work advise caution when excretion of HNE-GSH or its degradation products is generally used to predict compound-dependent induction of oxidative stress. This may give false-negative results, particularly, if the administered compound is GSH depleting, for instance by oxidation of GSH or formation of metabolites that bind to GSH. Therefore, other oxidative stress markers such as malondialdehyde or isoprostanes should be considered in such studies [163, 164].

An unknown signal that occurred in human urine samples was assigned to *N*-acetyl-*S*-benzyl-L-cysteine according to mass spectra in positive and negative ion mode and retention time identical to an authentic standard. This signal was not evident in rat urine. *N*-Acetyl-*S*-benzyl-L-cysteine is described as a metabolite of toluene exposure in humans. Toluene is used in certain industrial solvent mixtures by the printing industry. Toluene containing facilities are paints, thinners, glues, and paintbrush. Toluene, besides other volatile organic compounds such as benzene, ethylbenzene, and xylenes (BTEX), also occurs in outdoor and indoor air and is produced by exhaust emissions [165]. It is detected in building and furnishing materials in china [166] and germany [167, 168]. Hippuric acid, *N*-acetyl-*S*-benzyl-L-cysteine, and, to a lower extent, *N*-acetyl-*S*-*p*-toluyl-L-cysteine are the main metabolites of toluene [11]. *N*-acetyl-*S*-benzyl-L-cysteine is formed by hydroxylation to benzylalcohol that undergoes sulfation and is further substituted by GSH to finally be degraded to mercapturic acid [169]. The detection of this metabolite in unexposed subjects is contradictory, though. Angerer et al. detected a background level of 30 µg/l urine of *N*-acetyl-*S*-benzyl-L-cysteine in unexposed humans [169], whereas Inoue et al. did not detect any *N*-acetyl-*S*-benzyl-L-cysteine in unexposed workers (LOD < 0.2 µg/l urine) [170, 171]. Resorption rate of toluene has not been determined in these studies. Allowing for excretion of *N*-acetyl-*S*-benzyl-L-cysteine of unexposed humans of 30 µg/l urine detected by Angerer et al. [169], an average concentration of 0.12 µmol/l. A corresponding concentration of 0.6 pmol on column would result. This value lies within the sensitivity range of the LOD levels (0.3 to 15.5 pmol o.c.) of the standard mercapturic acids determined with the IDA CNL/negative EPI method (Table 11). Thus, detection of *N*-acetyl-*S*-benzyl-L-cysteine with the present screening method is reasonable and, thus, fortifies the data, published by Angerer et al. The minor metabolite of toluene exposure, *N*-acetyl-*S*-*p*-toluyl-L-cysteine, did not occur in detectable amounts.

5.1.4 Applicability of the Developed HPLC-MS/MS Method for the Detection of Mercapturic Acids as Biomarker in Drug Development

Analytical methods to measure mercapturic acids are still considered as rather complicated, expensive and/or slow [75]. This work, however, presents a rapid and sensitive general HPLC-MS/MS method for the screening of mercapturic acids in urine and incubation solutions without complex sample preparation. Within one day, first results can be obtained on a) generation of reactive intermediates of a compound *in vitro* and *in vivo* and b) first structural information about known and unknown mercapturic acids and sulfoxides of this compound. Thus, mercapturic acids can be generated and determined for first ideas on reactivity and possible toxicity of a compound. However, the question remains, if mercapturic acids were suitable biomarkers for toxicity screening in drug development processes. Such a biomarker has to fulfill various requirements such as a) stable with a long half-life, b) specific for also low dose exposures, c) sensitive with a correlated response to increased exposure, d) non-invasively to reduce health risks or discomfort to the subject, e) applicable to *in vitro* and *in vivo,* and f) rapidly and easily prepared and analyzed [75].

Mercapturic acids are generally very stable, although differences may occur depending on the compound moiety *N*-acetyl-L-cysteine is bound to [172]. Specificity and sensitivity, however, may be problematic. Using mercapturic acids as biomarkers for exposure, detection of compound-related mercapturic acids were accomplished by Melikian et al. for *N*-acetyl-*S*-phenyl-L-cysteine and t,t-muconic acid after exposure to benzene [173], or by Imbriani et al. and Sakai et al. for *N*-acetyl-*S*-(methylcarbamoyl)-L-cysteine after exposure to *N,N*-dimethylformamide [174, 175]. All authors detected increased levels of mercapturic acids in urine after exposure to the single compounds. Here, mercapturic acids respond sensitively to exposure of a compound. However, mercapturic acids are not generally suitable as sensitive and specific, as shown for DHN-MA as potential biomarker for oxidative stress with even decreased renal excretion after high dose of acetaminophen in rats. Specificity lacked in the case of the human acetaminophen study, where the same metabolite was also detected in unexposed subjects, presumably due to other compounds that

form this mercapturic acid. Moreover, the complex interindividual differences in metabolism processes involved in the formation of mercapturic acids may influence the excretion of a mercapturic acid. Among these are polymorphisms in activity of metabolizing enzymes (GST M1 [176]; GST T1 [177], 2D6 [65]), induction or inhibition of phase I enzymes like CYP3A4, influenced by many clinically used drugs, and difference in age, gender, lifestyle, or even race [147, 178]. However, this great variance displays the possibility to regard the excretion of all mercapturic acids as a pattern and search for differences in variation of the metabolites. The developed screening method for the detection and characterization of mercapturic acids presents the advantage to scan for all mercapturic acids and their sulfoxides present in urine. Detection of changes of the excreted mercapturic acid pattern may enable predictions on special diseases, genetic polymorphism, or lifestyle and opens a totally new and interesting domain for detection of mercapturic acids [100]. Mercapturic acids can be obtained very easily and non-invasively by collecting urine. Thus, mercapturic acids may serve as potential biomarkers of drug toxicity *in vivo* and *in vitro.*

In conclusion, the developed and validated HPLC-MS/MS method demonstrates a useful tool for the determination and detection of mercapturic acids as biomarkers of drug toxicity. The detection of endogenously formed mercapturic acids (DHN-MA and HNE-MA) as well as mercapturic acids of environmental xenobiotics (benzyl-MA, AAP-MA) shows the sensitivity of the method. Since mercapturic acids and their sulfoxides provide a common neutral loss of 129 Da in negative ion mode, all reactive intermediates that conjugate with *N*-acetyl-L-cysteine can be easily obtained in various matrices. Additionally, first information about the chemical structure can be obtained by the MS/MS mass spectra in the same chromatographic run. The basic principle of this method, i.e. the detection of a metabolite group by their common fragmentation behaviour (here, CNL of 129 Da), is theoretically applicable to other kinds of metabolite groups with a common substructure. Simple incubation assays, as shown in the present work, display a great opportunity to generate reactive intermediates that can be trapped and detected as mercapturic acids. Thus, already in early stages of drug development processes, potentially protein-reactive intermediates of a newly developed compound may be obtained. The drug may be discarded from further investigations or may be modified to a less reactive derivative, if compound-related signals occur during mercapturic acid screening. Unambiguous

identification of such signals is of minor importance rather than the occurrence of the metabolites, which indicate the formation of reactive intermediates and, hence, its potential chemical reactivity. The occurrence of species- and tissue-specific reactive intermediate formation observed with the generic screening method clearly demonstrate that one *in vitro* assay does not cover the broad range of reactive intermediate formation pathways of a compound. Various different *in vitro* systems are necessary to assess the actual risk of a compound and provide information about bioactivation pathways, e.g. CYP450-mediated processes. Additionally, it should be kept in mind that GSH adducts that are enzymatically formed do not occur in the incubation systems with *N*-acetyl-L-cysteine as trapping agent. GST-derived detoxification of reactive intermediates cannot be detected with this method, but this lack of detection may implicate an idea on which GSH adducts are enzymatically formed. Some GSH adducts are degraded chemically or enzymatically (by γ-glutamyl-transpeptidase) to derivatives and escape specific detection for GSH adducts. These metabolites would be detected with the mercapturic acid method [48]. Screening urine samples of rats and humans has also shown the applicability of this analytical method *in vivo* [100].

5.2 Acyl L-Lysine Adducts

In this study, first tests show that acyl L-lysine adducts generate a common product ion at *m/z* 84 in positive ion mode as was previously observed for different lysine adducts [97, 179, 180]. Based on this common fragment ion, a sensitive method was developed for the detection of L-lysine adducts that were previously generated *in vitro*. Mobile phases for highest ionization efficiency and resolution were 0.1% of formic acid in water and acetonitrile as described for a wide range of molecules [181]. Data acquisition was obtained *in vitro* and *in vivo* with specific precursor ion survey scan combined with EPI scan for structural clarification of the detected signal. Four acidic xenobiotics, i.e. diclofenac, ibuprofen, bromfenac, and benzoic acid generated acyl L-lysine adducts after bioactivation of the carboxylic acid by glucuronidation in hepatic microsomes. The presence of other target proteins for acylation and time-dependent degradation to the less reactive regioisomers or hydrolysis of the acyl glucuronide was found to influence acyl L-lysine formation [24]. Kretz-Rommel et al. observed time and dose-dependent covalent binding to hepatocyte protein in

correlation with acyl glucuronide formation by diclofenac, indicating the high affinity of the reactive 1-O-β-acyl glucuronide towards proteins [182]. Such acyl glucuronides may subsequently form adducts with nucleophilic amino acid residues, i.e. L-lysine and tyrosine as *N*-containing nucleophiles and cysteine as *S*-containing amino acid [54, 55, 183]. The data of this study demonstrate that L-lysine is a potential nucleophilic trapping agent for detection of acyl glucuronides and provides an opportunity for the determination of acyl glucuronide formation. Thus, first assessment on reactivity of a compound may be performed. The extent of affinity of acyl glucuronide towards L-lysine depends on the structural chemistry of the compound and sometimes on the stereochemistry of a compound, as described for ketoprofen [53, 54]. Wang et al. ranked the reactivity of carboxylic groups towards amino acids by the degree of α-carbon substitution [53] with acyl glucuronides of acetic acid derivatives as the most reactive metabolite, followed by propionic acid and benzoic acid derivatives.

Scanning urine samples of human subjects after diclofenac dosage demonstrated formation of an acyl L-lysine adduct of diclofenac (chapter 4.2.5). Further investigations will be necessary for unambiguous identification of this metabolite as N^{α}- or N^{ε}-conjugated L-lysine adduct. In this case, characterization will be essential for proving the generation of this metabolite *in vivo* or, due to the presence of L-lysine as free amino acid in urine (250 µmol/g creatinine in adults), a late formation directly in the bladder [184]. Detection of this adduct in human urine demonstrates the applicability of the lysine assay for the detection of acyl glucuronides also *in vivo*.

The developed HPLC-MS/MS method for the detection of L-lysine adducts presents first steps for the establishment of an *in vitro* method for the generation of L-lysine adducts to trap protein-reactive acyl glucuronides together with a general HPLC-MS/MS method for the detection and characterization of such L-lysine adducts. The developed method was based on the principle of a survey scan of a metabolite group with common fragmentation behaviour. The evaluation of this method for *in vitro* and *in vivo* samples demonstrates the common applicability of this principle to a wide range of metabolites. Moreover, the results show that L-lysine is a useful trapping agent for acyl glucuronides *in vitro* and *in vivo.* Thus, reactivity of acyl glucuronides that are discussed to be involved in idiosyncratic toxicity can be elucidated. It is shown that not only glycation mechanisms but also direct transacylation occurs and

that both options have to be considered in studies on drug toxicity. *N*-acetyl-L-cysteine or GSH as alternative trapping agents for acyl glucuronides may be uneffective due to the even higher reactivity of thioesters towards amines [185]. Searching for such thioesters may finally result in underestimation of the risk of the compound.

In conclusion reactive intermediate formation displays an important aspect on reactivity and toxicity of a compound. Early determination of such potentially toxic compounds is essential to save time and resources in drug development processes. L-Lysine adducts as an alternative alert for protein-reactive intermediate formation, particularly for acyl glucuronides, are shown to be useful for detection of *N*-affine electrophiles, even *in vivo*. Although the product ion at *m/z* 84 is not very specific and also occurs as product ion in other compounds such as some mercapturic acids (Table 6), acyl lysine adducts can be detected with this screening method. Using this screening method, an acyl lysine conjugate of diclofenac in human urine was detected for the first time. However, further investigations may establish a more specific fragment ion, for instance, by derivatization of L-lysines [165, 186]. Radiolabeled L-lysine or deuterated L-lysine residues may obtain more specificity. The use of pronase as an unspecific enzyme for the digestion of proteins may provide even more L-lysine adducts and can give ideas on the active site of L-lysine conjugation as well as target proteins [187]. The developed rapid and sensitive HPLC-MS/MS methods for the screening and characterization of mercapturic acids or lysine adducts in various matrices provide the analytical framework for these investigations.

6 SUMMARY

Conjugation of reactive intermediates of drugs with proteins or DNA may result in toxic effects such as hepatotoxicity, agranulocytosis, allergies, tumors, etc. From 1975 to 1999, 2.9% of drugs were withdrawn from the market due to such severe adverse drug reactions [4]. In 1994, more than 100,000 deaths in the USA were related to adverse toxic effects [2]. The development of a successful drug costs about 800 million dollars and takes about 12 to 15 years of research. Therefore, the withdrawal of a drug from the market does not only mean loss of money and resources, but also damages the public image of the company. The damage of patients' lifes is even more important. Thus, formation of chemically reactive intermediates is a widely discussed problem in drug development processes. Early detection of potentially toxic compounds is required for drug discovery and drug development in the pharmaceutical industry. Reactive intermediates that are formed in biotransformation reactions are unstable and bind rapidly to nucleophilic substrates. Conjugation of such electrophilic compounds with glutathione (GSH) is one of the most important detoxifying reactions *in vivo*. Processing of these GSH-conjugates ultimately leads to the formation of renally cleared mercapturic acids, which may also be oxidized to sulfoxides. The affinity of these electrophiles towards nucleophilic substrates such as glutathione is used for determination of reactive intermediate formation *in vitro*. However, reactive intermediates may also be trapped directly with *N*-acetyl-L-cysteine to form mercapturic acids. Thus, mercapturic acids may be generated and detected *in vitro* and non-invasively *in vivo* in urine to assess the reactivity of a compound in early stages of drug development processes. The detection of reactive intermediates as mercapturic acids formed by novel drugs may provide an early assessment of the reactivity of the compound. In addition, generation and detection of reactive intermediates may provide prognoses about possible metabolization and bioactivation pathways of a compound in animals. Therefore, the aim of this work was to develop and evaluate a HPLC-MS/MS screening method for the general detection and characterization of mercapturic acids as biomarkers for reactive intermediate formation *in vitro* and *in vivo*. The objective of this screening method was the simple and rapid detection and characterization of known and unknown mercapturic acids with high throughput and application of the method to several different matrices.

For development of a HPLC-MS/MS screening method, thirteen chemically diverse mercapturic acid standards and their sulfoxides were characterized with regard to common product ions or neutral losses using a mass spectrometer in negative and positive ion mode. The constant neutral loss (CNL) of 129 Da after negative electrospray ionization, resulting from the cleavage between the sulfur and the carbon atom in the *N*-acetyl-L-cysteine moiety, appeared to be common in all compounds. Based on this CNL, a HPLC-MS/MS method was developed and evaluated for the detection of mercapturic acids and sulfoxides in biological matrices. A CNL scan of 129 Da in the negative ion mode was performed using a linear ion trap instrument and was combined with two enhanced product ion (EPI) scans with different collision energies to characterize the detected signals. After optimization of the experimental parameters, the detection limits of the reference substances in rat urine ranged from 0.3 to 15.5 pmol on column (i.e. 20 ng/ml to 800 ng/ml). To obtain higher detection sensitivities of this metabolite group, a similar HPLC-MS/MS screening method was developed and optimized using the multiple reaction monitoring (MRM) scan mode with theoretical mass transitions (thMRM) that corresponded to the CNL of 129 Da. Comparison of the detection limits of the mercapturic acid standards obtained with the two different scan modes (CNL and thMRM) showed no significant difference in sensitivity.

For *in vitro* evaluation of the CNL method, the model compounds acetaminophen, diclofenac, bifonazole, clozapine, troglitazone, carbamazepine, and bisphenol A were screened for formation of reactive intermediates and, hence, detection of the corresponding mercapturic acids. There are basically two types of toxicological effects that are characteristic for adverse drug reactions, i.e. a direct tissue injury, primarily damage of the liver as the main organ of biotransformation and the indirect, immune-mediated effect. Compared to hepatocytes, a more reactive milieu is present in stimulated neutrophils which leads to the formation of reactive intermediates mediated by peroxidase pathways. These bioactivation pathways may differ from reactions that occur in the liver. To determine such differences and to monitor possible species- and tissue-specific toxicities, the model compounds were incubated with stimulated neutrophils and with liver microsomes from rats and humans. The compounds were preincubated in the presence of a NADPH regenerating system and liver microsomes prior to addition of *N*-acetyl-L-cysteine. Neutrophils were purified from human blood and activated by phorbol-12-myristate-13-acetate prior to

incubation. The developed and optimized HPLC-MS/MS screening method was successfully applied to different matrices with a minimum of sample preparation. Using this method, not only known, but also unknown mercapturic acids were detected.

Species-specific differences were observed in incubations of acetaminophen and diclofenac with rat and human hepatic microsomes. For instance, the mercapturic acid of 3-hydroxyacetaminophen was only formed in human liver microsomal incubation, indicating species-specific differences in the biotransformation of acetaminophen. In human liver microsomal incubation of diclofenac, a mercapturic acid with *m/z* 459 was detected that did not occur in incubations with rat liver microsomes. In addition, the metabolite with *m/z* 437 that suggested the formation of *N*-acetyl-*S*-{2-[2´-(carboxymethyl)phenylamino]-3-chloro-5-hydroxyphenyl}-L-cysteine (deschlorodiclofenac-MA) was detected in rat liver microsomal incubation only to a low extent.

Tissue-specific differences in biotransformation of the model compounds in incubations with human neutrophils and human liver microsomes were observed for diclofenac, carbamazepine, clozapine, and bifonazole. In incubations of diclofenac with stimulated neutrophils the metabolite with *m/z* 437 was not obtained. The mercapturic acid at *m/z* 459 and the mercapturic acids of 4´-hydroxydiclofenac (*m/z* 471) related to cytochrome P450 (CYP450) metabolism did not occur either. Interestingly, in contrast to liver, two additional metabolites of diclofenac with precursor ions at *m/z* 441 and *m/z* 487 were detected in incubations with stimulated neutrophils. Based on the mass spectra, the precursor ion at *m/z* 441 presumably originated from a benzylic aldehyde metabolite formed from 5-hydroxydiclofenac after decarboxylation and peroxidase mediated activation by neutrophils. The ion at *m/z* 487 was assigned to a further oxidation of the mercapturic acid of hydroxydiclofenac to the corresponding sulfoxide or to the mercapturic acid of a dihydroxydiclofenac metabolite. Carbamazepine generated the well known protein-reactive quinone imine in both incubation assays. A second mercapturic acid at *m/z* 351 was detected, that has not been described before. Clozapine was bioactivated to the nitrenium ion in both incubation assays. A metabolite at *m/z* 472 was only detected in hepatic microsomal incubation and suggested a CYP450-specific demethylation of clozapine. Microsomal incubation with bifonazole showed a metabolite with *m/z* 502. Based on

its mass spectra, this yet uncharacterized metabolite suggested two hydroxylation reactions and further oxidation to a quinone. Bifonazole did not form any detectable mercapturic acids in incubations with human neutrophils. For bisphenol A and troglitazone, no differences in generation of reactive intermediates between human liver microsomes and activated neutrophils were observed.

The developed HPLC-MS/MS method was also evaluated *in vivo* by analysis of rat and human urine. Drug-related mercapturic acids were detected in urine of rats orally treated with acetaminophen (20 mg/kg and 640 mg/kg b.w.) or diclofenac (10 mg/kg and 20 mg/kg b.w.). Human urine samples were analyzed before and after oral administration of a clinically used dose of 500 mg and 50 mg of acetaminophen, respectively. For acetaminophen, a dose-dependent excretion of the mercapturic acid (*m/z* 311) of the protein-reactive intermediate *N*-acetyl-p-quinone imine (AAP-MA) was detected in rat and human urine. Interestingly, this mercapturic acid occurred also in blank urine samples of human volunteers, indicating other origins of formation, e.g. by exposure to aniline or aminophenol. In addition, a second mercapturic acid with *m/z* 327 occurred dose-dependently in rat and human urine samples after administration of acetaminophen. Further investigations on identification of this metabolite using authentic compounds and comparing their MS/MS mass spectra demonstrated oxidation of AAP-MA to stereoisomeric sulfoxides *in vivo*.

In urine of rats administered diclofenac, *in vivo* formation of the three mercapturic acids of hydroxylated diclofenac was confirmed. In the same samples, the mercapturic acid with *m/z* 441 was detected that has been obtained in incubations with human neutrophils before. The *in vivo* formation of this diclofenac metabolite is described here for the first time.

In addition, three endogenously formed mercapturic acids were detected and identified as *N*-acetyl-*S*-(1,4-dihydroxynonan-3-yl)-L-cysteine (DHN-MA), *N*-acetyl-*S*-(5-hydroxy-2-pentyltetrahydrofuran-3-yl)-L-cysteine (HNE-MA) (*in vivo* and *in vitro*), and *N*-acetyl-*S*-benzyl-L-cysteine (in human urine) by comparing their MS/MS mass spectra with the mass spectra of authentic standards.

In conclusion, the results of the *in vitro* and *in vivo* evaluation demonstrate the advantages of the rapid and generic HPLC-MS/MS screening method for the detection of mercapturic acids, that can be obtained with a minimum of sample

preparation and a high throughput in diverse matrices. The basic principle of this method, i.e. the detection of a metabolite group by their common fragmentation behaviour (here, CNL of 129 Da), is theoretically applicable to other kinds of metabolite groups with a common substructure. In addition, an advantage of the developed HPLC-MS/MS method is the detection of yet unknown mercapturic acids and sulfoxides *in vitro* and *in vivo*. Moreover, by combining the CNL survey scan with EPI scans, characterization of the detected signals without additional measurements is possible. Here, unequivocal identification of those signals is of minor importance rather than the extent of formation of such reactive intermediates. Therefore, this screening method offers the possibility to detect potentially toxic compounds in early stages of drug development processes. However, formation of reactive intermediates does not obligatorily mean that a substance is toxic, but additionally provides information on the biotransformation, besides the potential risk of the compound to generate adverse drug reactions.

Additionally, species- and tissue-specific differences in generation of reactive intermediates were observed with this screening method and, thus, demonstrate the applicability of the method to various matrices. The data obtained also show that, in drug development processes, complementary *in vitro* assays are required for a comprehensive assessment of the reactivity of compounds after bioactivation.

Based on the principle of this method, i.e. common fragmentation of one metabolite group, further methods for the detection of other metabolite groups with common fragmentation behaviour can be developed as has been shown for L-lysine adducts of acyl glucuronides. Acyl glucuronides that are formed after glucuronidation of carboxylic groups are unstable and may conjugate with proteins and amino acids such as L-lysine. L-Lysine with its terminal primary amine represents a potential target of conjugation reactions. Analysis of fragmentation of different L-lysine adducts provided a common product ion at *m/z* 84 in positive ion mode. Based on this common fragment ion, a method was developed for the detection of acyl L-lysine conjugates of various carboxylic acid containing compounds after activation by glucuronidation. Acyl L-lysine adducts were detected in rat liver microsomal incubations with diclofenac, ibuprofen, bromfenac and benzoic acid. An acyl L-lysine adduct was detected in human urine after diclofenac treatment, indicating a possible endogenous conjugation of acyl glucuronides with L-lysine.

7 ZUSAMMENFASSUNG

Konjugation reaktiver Intermediate mit Proteinen oder DNA kann zu toxischen Effekten wie Hepatotoxizität, Neutropenie, Allergien, Tumoren u.a. führen. Zwischen 1975 und 1999 wurden 2.9% der zugelassenen Arzneistoffe wegen Auftretens solcher unerwünschten, toxischen Nebenwirkungen vom Markt genommen [4]. Allein 1994 wurden in den USA über 100.000 Todesfälle aufgrund solcher toxischer Effekte nach Medikamenteneinnnahme beschrieben [2]. Die Entwicklung eines für die Zulassung geeigneten Arzneistoffes dauert durchschnittlich zwischen 12 und 15 Jahre und kostet dem Unternehmen insgesamt ca. 800 Mio $. Wird ein solcher Arzneistoff dann vom Markt genommen, bedeutet das für die Firma nicht nur einen immensen Verlust an Geldern und Resourcen, sondern schädigt auch das Image des Unternehmens. Die Zerstörung der Lebensqualität mit eventueller Todesfolge der Patienten ist dabei besonders tragisch. Daher stellen Substanzen, die reaktive Intermediate bilden können, ein großes Problem in der Arzneistoffentwicklung dar. Aus diesem Grund ist die pharmazeutische Forschungsindustrie daran interessiert, solche potenziell toxischen Substanzen bereits in frühen Phasen der Arzneistoffentwicklung zu erfassen. Elektrophile, reaktive Intermediate sind instabil und reagieren schnell mit nukleophilen Substraten. Die Konjugation reaktiver Intermediate mit Glutathion stellt hierbei einen der Hauptmechanismen der Detoxifizierung im Organismus dar. *In vivo* können enzymatisch geregelte Reaktionen das Glutathionaddukt abbauen und so zur Bildung renal ausscheidbarer Merkaptursäuren führen, die auch zu den entsprechenden Sulfoxiden oxidiert werden können. Die Affinität von reaktiven Intermediaten zu nukleophilen Substraten wie Glutathion wird zur *In-vitro*-Bestimmung reaktiver Metabolite genutzt. Man kann Merkaptursäuren aber auch direkt durch Konjugation mit *N*-Acetyl-L-cystein gewinnen. So können Merkaptursäuren *in vitro* generiert, detektiert und nicht-invasiv auch *in vivo* erfasst werden. Dadurch kann die potenzielle Reaktivität eines Stoffes bereits in der Arzneistoffentwicklung und in der präklinischen Phase abgeschätzt werden.

Ziel dieser Arbeit war es, eine HPLC-MS/MS-Screening-Methode zur generischen Detektion und Charakterisierung von Merkaptursäuren als Biomarker für die Bildung reaktiver Metabolite *in vitro* und *in vivo* zu entwickeln und zu evaluieren. Mit Hilfe der Screeningmethode sollen Merkaptursäuren in verschiedenen Matrices einfach,

schnell und mit einem hohen Probendurchsatz detektiert werden können. Sowohl bekannte und unbekannte Merkaptursäuren reaktiver Substanzen als auch Merkaptursäuren neu entwickelter Substanzen sollen mit dieser Methode erfasst und charakterisiert werden können. Durch Anwendung dieser Methode auf neu entwickelte Arzneistoffe könnten *in vitro* generierte reaktive Intermediate detektiert werden und so frühzeitig Aufschluss über die potenzielle Reaktivität dieses Stoffes geben. Außerdem könnten *in vitro* generierte Merkaptursäuren Vorhersagen über mögliche Metabolisierungs- und Bioaktivierungswege im Tier erleichtern.

Für die Entwicklung der Methode wurden dreizehn chemisch unterschiedliche Merkaptursäurestandards und ihre Sulfoxide in einem Massenspektrometer nach negativer und positiver Ionisierung fragmentiert und ihre Massenspektren auf gemeinsame Fragmentionen oder Verluste von neutralen Molekülfragmenten (Neutralverlust) untersucht. Ein Neutralverlust (constant neutral loss, CNL) von 129 Da nach negativer Ionisierung war charakteristisch für alle Referenzsubstanzen und deren Sulfoxide. Dieser entstand durch Spaltung der Schwefel-Kohlenstoff-Bindung im Merkaptursäureanteil und diente als Basis für die Entwicklung der HPLC-MS/MS-Methode. Dafür wurde ein CNL-Scan auf 129 Da im negativen Ionenmodus durchgeführt. Der CNL-Scan konnte unter Verwendung der vorhandenen Ionenfalle mit zwei Produkt-ionen-Scans (EPI) mit unterschiedlichen Kollisionsenergien kombiniert und für eine Charakterisierung der detektierten Signale verwandt werden. Nach Optimierung der Instrument- und HPLC-Parameter wurden für die einzelnen Referenzsubstanzen Nachweisgrenzen im Bereich von 0.3 bis 15.5 pmol on column (entspricht einem Bereich von 20 ng/ml bis 800 ng/ml) in Rattenurin bestimmt. Um eine höhere Empfindlichkeit bei der Detektion dieser Metabolitenklasse zu erreichen, wurde zusätzlich eine Methode im MRM-Scan-Modus entwickelt und optimiert. Die theoretischen Massenübergänge (thMRM) errechneten sich aus dem CNL von 129 Da. Ein methodischer Vergleich der beiden Scan-Modi (CNL und thMRM) ergab jedoch keinen signifikanten Unterschied in der Nachweisgrenze der Referenzsubstanzen.

Für die *In-vitro*-Evaluierung der CNL-Screening-Methode wurden die Modelsubstanzen Paracetamol, Diclofenac, Troglitazon, Bifonazol, Clozapin, Carbamazepin und Bisphenol A auf die Bildung reaktiver Intermediate hin untersucht. Die toxischen Effekte reaktiver Intermediate können grundsätzlich in zwei Gruppen

aufgeteilt werden. Die erste Gruppe umfasst die direkten Effekte, die sich durch Gewebeschädigung, v.a. der Leber als Hauptmetabolisierungsorgan äußern. Die zweite Gruppe wird durch die indirekten, meist immunabhängigen Effekte charakterisiert. In stimulierten neutrophilen Granulozyten herrscht, im Vergleich zu Hepatozyten, ein reaktiveres Milieu. Dieses Milieu entsteht vor allem durch die Bildung von reaktiven Sauerstoffspezies, die eine Bioaktivierung von Substanzen hervorrufen können. Diese Bioaktivierung kann sich von der Metabolisierung in der Leber unterscheiden. Um solche möglichen Unterschiede zu untersuchen und um eventuell Aufschluß über gewebe- oder speziesspezifische Toxizitäten von Arzneistoffen zu bekommen, wurden die Modellsubstanzen in stimulierten neutrophilen Granulozyten und in Ratten- und Humanlebermikrosomen inkubiert. Die Substanzen wurden vor Zusatz von *N*-Acetyl-L-cystein mit einem NADPH regenerierenden System und Lebermikrosomen vorinkubiert. Die neutrophilen Granulozyten wurden aus humanem Vollblut isoliert und vor der eigentlichen Inkubation mit Phorbol-12-myristat-13-acetat stimuliert. Die entwickelte und optimierte Methode erlaubte die Detektion bekannter und unbekannter Merkaptursäuren in verschiedenen Matrices nach minimaler Probenaufbereitung.

Speziesspezifische Unterschiede in der Bildung von reaktiven Intermediaten zwischen Inkubationen mit Ratten- und Humanlebermikrosomen wurden bei Paracetamol und Diclofenac beobachtet. Die Merkaptursäure des Paracetamolmetaboliten 3-Hydroxyacetaminophen wurde nur in Inkubationen mit humanen Lebermikrosomen detektiert und läßt auf speziesspezifische Unterschiede in der Biotransformation von Paracetamol schließen. In Inkubationen von Diclofenac in humanen Lebermikrosomen wurde ein Metabolit mit *m/z* 459 detektiert, der mit Rattenlebermikrosomen nicht gebildet wurde. Auch der Metabolit mit *m/z* 437, der als *N*-Acetyl-*S*-{2-[2´-(carboxymethyl)phenylamino]-3-chloro-5-hydroxyphenyl}-L-cystein (deschlorodiclofenac-MA) charakterisiert werden konnte, war, im Vergleich zu Inkubationen mit Humanlebermikrosomen, mit geringerer Intensität zu detektieren.

Organspezifische Unterschiede in der Bildung von reaktiven Intermediaten zwischen Inkubationen mit neutrophilen Granulozyten und humanen Lebermikrosomen wurden bei Diclofenac, Carbamazepin, Clozapin und Bifonazol gefunden. Im Vergleich zu Inkubationen mit humanen Lebermikrosomen fehlten in Inkubationen von Diclofenac mit neutrophilen Granulozyten Deschlorodiclofenac-MA (*m/z* 437), die

Merkaptursäure des 4´-Hydroxydiclofenacmetaboliten (*m/z* 471) und der Metabolit mit *m/z* 459. Die Bildung dieser Metabolite ist möglicherweise auf Cytochrom-P450 (CYP450) -spezifische Metabolisierungswege zurückzuführen, die in neutrophilen Granulozyten fehlen. Interessanterweise wurden aber in diesen Proben zwei andere Metabolite mit *m/z* 441 und *m/z* 487 detektiert. Anhand der Massenspektren könnte das Ion mit *m/z* 441 ein Derivat des 5-Hydroxydiclofenac-Metaboliten sein, das nach Decarboxylierung und Peroxidase-abhängiger Bioaktivierung durch neutrophile Granulozyten entsteht. Der andere Metabolit (*m/z* 487) könnte entweder von einem Dihydroxydiclofenacmetaboliten oder von dem Sulfoxid eines der Hydroxydiclofenacmetaboliten stammen. Carbamazepin bildete in beiden Inkubationssystemen das reaktive Iminochinon. Mit stimulierten neutrophilen Granulozyten wurde ein weiterer Metabolit mit *m/z* 351 detektiert, der jedoch nicht näher charakterisiert werden konnte. Das reaktive Nitreniumion des Clozapin wurde sowohl in Inkubationen mit neutrophilen Granulozyten als auch in Inkubationen mit humanen Lebermikrosomen detektiert. Ein Metabolit mit *m/z* 472 konnte nur in Inkubationen mit Lebermikrosomen detektiert werden und weist auf eine CYP450 abhängige Demethylierung von Clozapin hin. In Inkubationen von Bifonazol mit humanen Lebermikrosomen wurde ein Metabolit mit *m/z* 502 detektiert, der nicht in den neutrophilen Granulozytenproben auftrat. Dieser bisher noch nicht charakterisierte Metabolit wurde durch zwei Hydroxylierungsreaktionen an einem der Aromaten und weiterer Oxidation zu einem Chinon gebildet, wie man anhand der Massenspektren nachvollziehen konnte. Bisphenol A und Troglitazon hingegen zeigten keine organabhängigen Unterschiede in der Bildung reaktiver Intermediate.

Die HPLC-MS/MS-Screening-Methode wurde durch Messungen von Ratten- und Humanurinproben auch *in vivo* evaluiert. Arzneistoffbezogene Merkaptursäuren wurden in Urinproben von Ratten gemessen, die über eine Schlundsonde Paracetamol (20 mg/kg und 640 mg/kg K.G.) bzw. Diclofenac (10 mg/kg und 20 mg/kg K.G.) zugeführt bekommen hatten. Humanurin wurde nach Gabe einer therapeutischen Dosis von 500 mg Paracetamol und einer subtherapeutischen Dosis von 50 mg analysiert. Nach Paracetamolgabe konnte die Merkaptursäure des reaktiven *N*-Acetyl-p-benzochinonimins (NAPQI) dosisabhängig im Urin von Ratten und Menschen detektiert werden. Interessanterweise wurde dieser Metabolit auch im Kontrollurin einiger Probanden detektiert. Dies weist auf weitere Bildungsmöglichkeiten dieses Intermediates hin, z.B. durch Exposition gegenüber

Anilin oder Aminophenol. Außerdem wurde ein weiterer Metabolit mit *m/z* 327 dosisabhängig in den Urinproben von Ratte und Mensch detektiert. Durch nähere Untersuchungen zur Identifizierung dieses Metaboliten anhand von Referenzsubstanzen und deren Massenspektren konnte nachgewiesen werden, dass das Merkapturat des NAPQI zu stereoisomeren Sulfoxiden oxidiert wurde. Nach Gabe von Diclofenac an Ratten konnte die Bildung der Merkaptursäuren der hydroxylierten Diclofenacmetabolite *in vivo* nachgewiesen werden. Die bereits erwähnte Merkaptursäure mit m/z 441, die in stimulierten neutrophilen Granulozyten entstand, konnte auch im Rattenurin detektiert werden. Die Bildung dieser Merkaptursäure *in vivo* ist hier zum ersten Mal beschrieben. Mit der HPLC-MS/MS Screening Methode wurden weitere, vom Arzneistoff unabhängige Merkaptursäuren im Urin detektiert. Diese konnten mit Hilfe von Referenzsubstanzen und Massenspektrenvergleich eindeutig als *N*-Acetyl-*S*-(1,4-dihydroxynonan-3-yl)-L-cystein (DHN-MA), *N*-Acetyl-*S*-(5-hydroxy-2-pentyltetrahydrofuran-3-yl)-L-cystein (HNE-MA) (*in vivo* und *in vitro*) und *N*-acetyl-*S*-benzyl-L-cystein (in Humanurin) identifiziert werden.

Schließlich zeigen die Ergebnisse zur *In-vitro*- und *In-vivo*-Evaluierung die Vorteile dieser schnellen und generischen HPLC-MS/MS-Screening-Methode zur Detektion von Merkaptursäuren, die mit minimaler Probenvorbereitung und hohem Probendurchsatz für verschiedene Matrices eingesetzt werden kann. Das Prinzip der Methode ist theoretisch für alle Metabolitengruppen geeignet, die gemeinsame Fragmentierungsmuster zeigen. Mit der speziell für die Detektion von Merkaptursäuren optimierten Screening-Methode können bekannte und unbekannte Merkaptursäuren und ihre Sulfoxide *in vi*tro und *in vivo* erfasst werden. Außerdem ist duch die Verbindung des CNL-Scans mit den EPI-Scans eine Charakterisierung der detektierten Metabolite ohne zusätzliche Messung möglich. Dabei ist die genaue Identität eines detektierten Signals von geringerer Bedeutung als die Bildung solcher Metabolite an sich. Somit können potenziell toxische Substanzen bereits in der frühen Phase der Arzneistoffentwicklung erkannt und eventuell ausgeschlossen werden. Jedoch bedeutet die Bildung reaktiver Intermediate und Detektion von Merkaptursäuren nicht unbedingt, dass eine Substanz toxisch wirken muss, sondern gibt einen ersten Einblick in die Biotransformationswege einer neu entwickelten Substanz und die damit verbundenen potenziellen Gefahren.

Zusätzlich konnten mit dieser Screening-Methode spezies- und gewebespezifische Unterschiede in der Bioaktivierung einer Substanz nachgewiesen und die flexible Einsatzfähigkeit dieser Methode für verschiedene biologische Matrices gezeigt werden. Die unterschiedlichen species- und gewebespezifischen Ergebnisse zeigen zudem, dass komplementäre *In-vitro*-Assays für eine höhere Aussagekraft über die Reaktivität eines Stoffes während der Arzneistoffentwicklung notwendig sind.

Die Screening-Methode, die auf einer gemeinsamen Fragmentierung einer Metabolitengruppe basiert, kann als Grundlage für weitere Methodenentwicklungen zur Detektion anderer Metabolitengruppen mit gemeinsamen Fragmentierungsmuster dienen, wie bei L-Lysin-Addukten von Acylglucuroniden gezeigt werden konnte. Acylglucuronide, die durch Glucuronidierung carbonsäurehaltiger Substanzen entstehen, sind instabil und können Proteine und Aminosäuren binden. L-Lysin mit seinem endständigen primären Amin ist ein potenzielles Substrat für eine solche Konjugation und wurde deshalb zur Erfassung protein-reaktiver Acylglucuronide genutzt. Verschiedene L-Lysin-Addukte ergaben nach Fragmentierung im positiven Ionenmodus ein gemeinsames Produkt-ion mit *m/z* 84. Basierend auf diesem gemeinsamen Fragmention wurde eine Methode entwickelt, die Acyl-L-lysin-Konjugate diverser carbonsäurehaltiger Substanzen nach Aktivierung durch Glucuronidierung erfassen kann. Acyl-L-lysin-Addukte wurden in Inkubationen mit den Modellsubstanzen Diclofenac, Ibuprofen, Bromfenac und Benzoesäure detektiert. Zusätzlich konnte erstmals ein Acyl-L-lysin-Addukt von Diclofenac in Humanurin nachgewiesen werden. Die Detektion dieses Acyl-L-lysin-Adduktes weist auf eine endogene Konjugation des Diclofenac-Acylglucuronides mit L-Lysin hin.

8 REFERENCES

1. Li, A. P. A review of the common properties of drugs with idiosyncratic hepatotoxicity and the "multiple determinant hypothesis" for the manifestation of idiosyncratic drug toxicity. *Chem Biol Interact.* **2002,** *142*, 7-23.
2. Lazarou, J.; Pomeranz, B. H.; Corey, P. N. Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *Jama.* **1998,** *279*, 1200-1205.
3. Walgren, J. L.; Mitchell, M. D.; Thompson, D. C. Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Crit. Rev. Toxicol.* **2005,** *35*, 325-361.
4. Lasser, K. E.; Allen, P. D.; Woolhandler, S. J.; Himmelstein, D. U.; Wolfe, S. M.; Bor, D. H. Timing of new black box warnings and withdrawals for prescription medications. *Jama.* **2002,** *287*, 2215-2220.
5. Amacher, D. E. Reactive intermediates and the pathogenesis of adverse drug reactions: the toxicology perspective. *Curr. Drug Metab.* **2006,** *7*, 219-229.
6. Pearson, R. G.; Songstad, J. Application of the Principle of Hard and Soft Acids and Bases to Organic Chemistry. *J. Am. Chem. Soc.* **1967,** *89*, 1827-1836.
7. Bezabeh, D. Z.; Bamford, H. A.; Schantz, M. M.; Wise, S. A. Determination of nitrated polycyclic aromatic hydrocarbons in diesel particulate-related standard reference materials by using gas chromatography/mass spectrometry with negative ion chemical ionization. *Anal. Bioanal. Chem.* **2003,** *375*, 381-388.
8. Cavalieri, E. L.; Rogan, E. G. Radical cations in aromatic hydrocarbon carcinogenesis. *Free Radic. Res. Commun.* **1990,** *11*, 77-87.
9. Ramesh, A.; Walker, S. A.; Hood, D. B.; Guillen, M. D.; Schneider, K.; Weyand, E. H. Bioavailability and risk assessment of orally ingested polycyclic aromatic hydrocarbons. *Int. J. Toxicol.* **2004,** *23*, 301-333.
10. Smith, C. J.; Perfetti, T. A.; Rumple, M. A.; Rodgman, A.; Doolittle, D. J. "IARC group 2A Carcinogens" reported in cigarette mainstream smoke. *Food Chem. Toxicol.* **2000,** *38*, 371-383.
11. Perbellini, L.; Veronese, N.; Princivalle, A. Mercapturic acids in the biological monitoring of occupational exposure to chemicals. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2002,** *781*, 269-290.
12. Rietjens, I. M.; Boersma, M. G.; van der Woude, H.; Jeurissen, S. M.; Schutte, M. E.; Alink, G. M. Flavonoids and alkenylbenzenes: mechanisms of mutagenic action and carcinogenic risk. *Mutat. Res.* **2005,** *574*, 124-138.
13. Zhang, Y.; Yao, S.; Li, J. Vegetable-derived isothiocyanates: anti-proliferative activity and mechanism of action. *Proc. Nutr. Soc.* **2006,** *65*, 68-75.
14. Scholl, P. F.; Musser, S. M.; Groopman, J. D. Synthesis and characterization of aflatoxin B1 mercapturic acids and their identification in rat urine. *Chem. Res. Toxicol.* **1997,** *10*, 1144-1151.
15. Park, B. K.; Kitteringham, N. R.; Maggs, J. L.; Pirmohamed, M.; Williams, D. P. The role of metabolic activation in drug-induced hepatotoxicity. *Annu. Rev. Pharmacol. Toxicol.* **2005,** *45*, 177-202.

16. James, L. P.; Mayeux, P. R.; Hinson, J. A. Acetaminophen-induced hepatotoxicity. *Drug Metab. Dispos.* **2003,** *31*, 1499-1506.

17. Nelson, S. D. Molecular mechanisms of the hepatotoxicity caused by acetaminophen. *Semin. Liver Dis.* **1990,** *10*, 267-278.

18. Watkins, P. B.; Whitcomb, R. W. Hepatic dysfunction associated with troglitazone. *N. Engl. J. Med.* **1998,** *338*, 916-917.

19. Gale, E. A. Lessons from the glitazones: a story of drug development. *Lancet.* **2001,** *357*, 1870-1875.

20. Kassahun, K.; Pearson, P. G.; Tang, W.; McIntosh, I.; Leung, K.; Elmore, C.; Dean, D.; Wang, R.; Doss, G.; Baillie, T. A. Studies on the metabolism of troglitazone to reactive intermediates in vitro and in vivo. Evidence for novel biotransformation pathways involving quinone methide formation and thiazolidinedione ring scission. *Chem. Res. Toxicol.* **2001,** *14*, 62-70.

21. Smith, M. T. Mechanisms of troglitazone hepatotoxicity. *Chem. Res. Toxicol.* **2003,** *16*, 679-687.

22. Boelsterli, U. A. Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. *Toxicol. Appl. Pharmacol.* **2003,** *192*, 307-322.

23. Shen, S.; Marchick, M. R.; Davis, M. R.; Doss, G. A.; Pohl, L. R. Metabolic activation of diclofenac by human cytochrome P450 3A4: role of 5-hydroxydiclofenac. *Chem. Res. Toxicol.* **1999,** *12*, 214-222.

24. Faed, E. M. Properties of acyl glucuronides: implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metab. Rev.* **1984,** *15*, 1213-1249.

25. Ahmed, N. Advanced glycation endproducts--role in pathology of diabetic complications. *Diabetes Res. Clin. Pract.* **2005,** *67*, 3-21.

26. Li, C.; Benet, L. Z.; Grillo, M. P. Studies on the chemical reactivity of 2-phenylpropionic acid 1-O-acyl glucuronide and S-acyl-CoA thioester metabolites. *Chem. Res. Toxicol.* **2002,** *15*, 1309-1317.

27. Bailey, M. J.; Dickinson, R. G. Acyl glucuronide reactivity in perspective: biological consequences. *Chem. Biol. Interact.* **2003,** *145*, 117-137.

28. Cooper, A. J. Mechanisms of cysteine S-conjugate beta-lyases. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1998,** *72*, 199-238.

29. Voelkel, W.; Friedewald, M.; Lederer, E.; Paehler, A.; Parker, J.; Dekant, W. Biotransformation of perchloroethene: dose-dependent excretion of trichloroacetic acid, dichloroacetic acid, and N-acetyl-S-(trichlorovinyl)-L-cysteine in rats and humans after inhalation. *Toxicol. Appl. Pharmacol.* **1998,** *153*, 20-27.

30. Kadlubar, F. F.; Miller, J. A.; Miller, E. C. Hepatic metabolism of N-hydroxy-N-methyl-4-aminoazobenzene and other N-hydroxy arylamines to reactive sulfuric acid esters. *Cancer Res.* **1976,** *36*, 2350-2359.

31. Uetrecht, J. P. The role of leukocyte-generated reactive metabolites in the pathogenesis of idiosyncratic drug reactions. *Drug Metab. Rev.* **1992,** *24*, 299-366.

32. Petrides P.E. Leukocyten. In: Loeffler G., Petrides P.E:, editors. *Biochemie und Pathobiochemie.* 6th ed. Berlin, Heidelberg: Springer **1998,** p 885-887.

33. Alvir, J. M.; Lieberman, J. A.; Safferman, A. Z.; Schwimmer, J. L.; Schaaf, J. A. Clozapine-induced agranulocytosis. Incidence and risk factors in the United States. *N. Engl. J. Med.* **1993,** *329*, 162-167.

34. Liu, Z. C.; Uetrecht, J. P. Clozapine is oxidized by activated human neutrophils to a reactive nitrenium ion that irreversibly binds to the cells. *J. Pharmacol. Exp. Ther.* **1995,** *275*, 1476-1483.

35. Zhou, S.; Chan, E.; Duan, W.; Huang, M.; Chen, Y. Z. Drug bioactivation, covalent binding to target proteins and toxicity relevance. *Drug Metab. Rev.* **2005,** *37*, 41-213.

36. Shear, N. H. a. S., S.P. Anticonvulsant hypersensivity syndrome. In vitro assessment of risk. *J. Clin. Invest.* **1988,** *82*, 1826-1832.

37. Vittorio, C. C.; Muglia, J. J. Anticonvulsant hypersensivity syndrome. *Arch. Intern. Med.* **1995,** *155*, 2285-2290.

38. Pearce, R. E.; Uetrecht, J. P.; Leeder, J. S. Pathways of carbamazepine bioactivation in vitro: II. The role of human cytochrome P450 enzymes in the formation of 2-hydroxyiminostilbene. *Drug Metab. Dispos.* **2005,** *33*, 1819-1826.

39. Ju, C.; Uetrecht, J. P. Detection of 2-hydroxyiminostilbene in the urine of patients taking carbamazepine and its oxidation to a reactive iminoquinone intermediate. *J. Pharmacol. Exp. Ther.* **1999,** *288*, 51-56.

40. Baars, A. J.; Breimer, D. D. The glutathione S-transferases: their role in detoxification and toxification of xenobiotics. *Ann. Biol. Clin.* **1980,** *38*, 49-56.

41. Nebert, D. W.; Vasiliou, V. Analysis of the glutathione S-transferase (GST) gene family. *Hum. Genomics.* **2004,** *1*, 460-464.

42. Norppa, H. Cytogenetic biomarkers and genetic polymorphisms. *Toxicol. Lett.* **2004,** *149*, 309-334.

43. Milne, G. L.; Gao, L.; Porta, A.; Zanoni, G.; Vidari, G.; Morrow, J. D. Identification of the major urinary metabolite of the highly reactive cyclopentenone isoprostane 15-A(2t)-isoprostane in vivo. *J. Biol. Chem.* **2005,** *280*, 25178-25184.

44. Altuntas, T. G.; Park, S. B.; Kharasch, E. D. Sulfoxidation of cysteine and mercapturic acid conjugates of the sevoflurane degradation product fluoromethyl-2,2-difluoro-1-(trifluoromethyl)vinyl ether (compound A). *Chem. Res. Toxicol.* **2004,** *17*, 435-445.

45. Ju, C.; Uetrecht, J. P. Oxidation of a metabolite of indomethacin (Desmethyldeschlorobenzoylindomethacin) to reactive intermediates by activated neutrophils, hypochlorous acid, and the myeloperoxidase system. *Drug Metab. Dispos.* **1998,** *26*, 676-680.

46. Liu, Z. C.; Uetrecht, J. P. Metabolism of ticlopidine by activated neutrophils: implications for ticlopidine-induced agranulocytosis. *Drug Metab. Dispos.* **2000,** *28*, 726-730.

47. Dalvie, D. K.; O'Connell, T. N. Characterization of novel dihydrothienopyridinium and thienopyridinium metabolites of ticlopidine in vitro: role of peroxidases, cytochromes p450, and monoamine oxidases. *Drug Metab. Dispos.* **2004,** *32*, 49-57.

48. Evans, D. C.; Watt, A. P.; Nicoll-Griffith, D. A.; Baillie, T. A. Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chem. Res. Toxicol.* **2004,** *17*, 3-16.

49. Dieckhaus, C. M.; Fernandez-Metzler, C. L.; King, R.; Krolikowski, P. H.; Baillie, T. A. Negative ion tandem mass spectrometry for the detection of glutathione conjugates. *Chem. Res. Toxicol.* **2005,** *18*, 630-638.

50. Draper, H. H.; Csallany, A. S.; Hadley, M. Urinary aldehydes as indicators of lipid peroxidation in vivo. *Free Radic. Biol. Med.* **2000,** *29*, 1071-1077.

51. Chen, L. J.; Hecht, S. S.; Peterson, L. A. Characterization of amino acid and glutathione adducts of cis-2-butene-1,4-dial, a reactive metabolite of furan. *Chem. Res. Toxicol.* **1997,** *10*, 866-874.

52. Kaminskas, L. M.; Pyke, S. M.; Burcham, P. C. Differences in lysine adduction by acrolein and methyl vinyl ketone: implications for cytotoxicity in cultured hepatocytes. *Chem. Res. Toxicol.* **2005,** *18*, 1627-1633.

53. Wang, J.; Davis, M.; Li, F.; Azam, F.; Scatina, J.; Talaat, R. A novel approach for predicting acyl glucuronide reactivity via Schiff base formation: development of rapidly formed peptide adducts for LC/MS/MS measurements. *Chem. Res. Toxicol.* **2004,** *17*, 1206-1216.

54. Presle, N.; Lapicque, F.; Fournel-Gigleux, S.; Magdalou, J.; Netter, P. Stereoselective irreversible binding of ketoprofen glucuronides to albumin. Characterization of the site and the mechanism. *Drug Metab. Dispos.* **1996,** *24*, 1050-1057.

55. Smith, P. C.; Benet, L. Z.; McDonagh, A. F. Covalent binding of zomepirac glucuronide to proteins: evidence for a Schiff base mechanism. *Drug Metab. Dispos.* **1990,** *18*, 639-644.

56. Bolze, S.; Bromet, N.; Gay-Feutry, C.; Massiere, F.; Boulieu, R.; Hulot, T. Development of an in vitro screening model for the biosynthesis of acyl glucuronide metabolites and the assessment of their reactivity toward human serum albumin. *Drug Metab. Dispos.* **2002,** *30*, 404-413.

57. McGirr, L. G.; Hadley, M.; Draper, H. H. Identification of N alpha-acetyl-epsilon-(2-propenal)lysine as a urinary metabolite of malondialdehyde. *J. Biol. Chem.* **1985,** *260*, 15427-15431.

58. Kalasz, H.; Benko, A.; Szucsp, Z.; Szilagyi, A.; Szarvas, T.; Lengyel, J. HPLC and HPLC-MS analysis of urinary N(epsilon)-monomethyl-lysine. *J. Chromatogr. Sci.* **2005,** *43*, 165-168.

59. Friess, U.; Waldner, M.; Wahl, H. G.; Lehmann, R.; Haring, H. U.; Voelter, W.; Schleicher, E. Liquid chromatography-based determination of urinary free and total N(epsilon)-(carboxymethyl)lysine excretion in normal and diabetic subjects. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2003,** *794*, 273-280.

60. Rooij, B. M. D.; Commandeur, J. N. M.; Vermeulen, N. P. E. Mercapturic acids as biomarkers of exposure to electrophilic chemicals:applications to environmental and industrial chemicals. *Biomarkers.* **1998,** *3*, 239-303.

61. Lohse, C.; Jaeger, L. L.; Staimer, N.; Sanborn, J. R.; Jones, A. D.; Lango, J.; Gee, S. J.; Hammock, B. D. Development of a class-selective enzyme-linked immunosorbent assay for mercapturic acids in human urine. *J. Agric. Food Chem.* **2000,** *48*, 5913-5923.

62. Staatz, C. E.; Taylor, P. J.; Tett, S. E. Comparison of an ELISA and an LC/MS/MS method for measuring tacrolimus concentrations and making dosage decisions in transplant recipients. *Ther. Drug Monit.* **2002,** *24*, 607-615.

63. Li, C.; Grillo, M. P.; Benet, L. Z. In vivo mechanistic studies on the metabolic activation of 2-phenylpropionic acid in rat. *J. Pharmacol. Exp. Ther.* **2003,** *305*, 250-256.

64. Xia, Y. Q.; Miller, J. D.; Bakhtiar, R.; Franklin, R. B.; Liu, D. Q. Use of a quadrupole linear ion trap mass spectrometer in metabolite identification and bioanalysis. *Rapid Commun. Mass Spectrom.* **2003,** *17*, 1137-1145.

65. Lutz, U.; Voelkel, W.; Lutz, R. W.; Lutz, W. K. LC-MS/MS analysis of dextromethorphan metabolism in human saliva and urine to determine CYP2D6 phenotype and individual variability in N-demethylation and glucuronidation. *J. Chromatogr. B Analyt. Technol. Biomed Life Sci.* **2004,** *813*, 217-225.

66. Voelkel, W.; Colnot, T.; Csanady, G. A.; Filser, J. G.; Dekant, W. Metabolism and kinetics of bisphenol a in humans at low doses following oral administration. *Chem. Res. Toxicol.* **2002,** *15*, 1281-1287.

67. Alary, J.; Debrauwer, L.; Fernandez, Y.; Cravedi, J. P.; Rao, D.; Bories, G. 1,4-Dihydroxynonene mercapturic acid, the major end metabolite of exogenous 4-hydroxy-2-nonenal, is a physiological component of rat and human urine. *Chem. Res. Toxicol.* **1998,** *11*, 130-135.

68. Manini, P.; Andreoli, R.; Bergamaschi, E.; De Palma, G.; Mutti, A.; Niessen, W. M. A new method for the analysis of styrene mercapturic acids by liquid chromatography/electrospray tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2000,** *14*, 2055-2060.

69. Weinmann, W.; Schaefer, P.; Thierauf, A.; Schreiber, A.; Wurst, F. M. Confirmatory analysis of ethylglucuronide in urine by liquid-chromatography/electrospray ionization/tandem mass spectrometry according to forensic guidelines. *J. Am. Soc. Mass Spectrom.* **2004,** *15*, 188-193.

70. Zomer, S.; Guillo, C.; Brereton, R. G.; Hanna-Brown, M. Toxicological classification of urine samples using pattern recognition techniques and capillary electrophoresis. *Anal. Bioanal. Chem.* **2004,** *378*, 2008-2020.

71. Haroldsen, P. E.; Reilly, M. H.; Hughes, H.; Gaskell, S. J.; Porter, C. J. Characterization of glutathione conjugates by fast atom bombardment/tandem mass spectrometry. *Biomed. Environ. Mass Spectrom.* **1988,** *15*, 615-621.

72. Straube, E.; Voelkel, W.; Bringmann, G.; Dekant, W. Reaction of nitroso derivatives of dinitropyrenes with sulfhydryl groups of peptides and hemoglobin in vitro and in rats. *Xenobiotica.* **2005,** *35*, 1147-1164.

73. Kellert, M.; Scholz, K.; Wagner, S.; Dekant, W.; Voelkel, W. Quantitation of mercapturic acids from acrylamide and glycidamide in human urine using a column switching tool with two trap columns and electrospray tandem mass spectrometry. *J. Chromatogr. A.* **2006,** *1131*, 58-66.

74. van Sittert, N. J.; Megens, H. J.; Watson, W. P.; Boogaard, P. J. Biomarkers of exposure to 1,3-butadiene as a basis for cancer risk assessment. *Toxicol. Sci.* **2000,** *56*, 189-202.

75. Haufroid, V.; Lison, D. Mercapturic acids revisited as biomarkers of exposure to reactive chemicals in occupational toxicology: a minireview. *Int. Arch. Occup. Environ. Health.* **2005,** *78*, 343-354.

76. Alary, J.; Bravais, F.; Cravedi, J. P.; Debrauwer, L.; Rao, D.; Bories, G. Mercapturic acid conjugates as urinary end metabolites of the lipid peroxidation product 4-hydroxy-2-nonenal in the rat. *Chem. Res. Toxicol.* **1995,** *8*, 34-39.

77. Urban, M.; Gilch, G.; Schepers, G.; van Miert, E.; Scherer, G. Determination of the major mercapturic acids of 1,3-butadiene in human and rat urine using liquid chromatography with tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2003,** *796*, 131-140.

78. Kalasz, H.; Szucs, Z.; Tihanyi, M.; Szilagyi, A.; Lengyel, J. Detection of Nepsilon-monomethyllysine using high-performance liquid chromatography and high-performance liquid chromatography-mass spectrometry. *J. Chromatogr. A.* **2005,** *1079*, 208-212.

79. Moberg, M.; Markides, K. E.; Bylund, D. Multi-parameter investigation of tandem mass spectrometry in a linear ion trap using response surface modelling. *J. Mass Spectrom.* **2005,** *40*, 317-324.

80. Jansen, R.; Lachatre, G.; Marquet, P. LC-MS/MS systematic toxicological analysis: comparison of MS/MS spectra obtained with different instruments and settings. *Clin. Biochem.* **2005,** *38*, 362-372.

81. Werner, M.; Birner, G.; Dekant, W. Sulfoxidation of mercapturic acids derived from tri- and tetrachloroethene by cytochromes P450 3A: a bioactivation reaction in addition to deacetylation and cysteine conjugate beta-lyase mediated cleavage. *Chem. Res. Toxicol.* **1996,** *9*, 41-49.

82. Birner, G.; Bernauer, U.; Werner, M.; Dekant, W. Biotransformation, excretion and nephrotoxicity of haloalkene-derived cysteine S-conjugates. *Arch. Toxicol.* **1997,** *72*, 1-8.

83. Hinson, J. A.; Pohl, L. R.; Monks, T. J.; Gillette, J. R.; Guengerich, F. P. 3-Hydroxyacetaminophen: a microsomal metabolite of acetaminophen. Evidence against an epoxide as the reactive metabolite of acetaminophen. *Drug Metab. Dispos.* **1980,** *8*, 289-294.

84. Greulich, T. Stimulation beta-adrenerger Rezeptoren auf neutrophilen Granulozyten - Effekte auf Adhäsionsmoleküle, Zytokine und intrazelluläre Botenstoffe [dissertation]. Marburg, Germany: Philipps-Universität Marburg.

2004, 165p. Available from archiv.ub.uni-marburg.de/diss/z2005/ 0225/pdf/dtg.pdf

85. Fisher, M. B.; Campanale, K.; Ackermann, B. L.; VandenBranden, M.; Wrighton, S. A. In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. *Drug Metab. Dispos.* **2000,** *28*, 560-566.

86. Rubino, F. M.; Verduci, C.; Giampiccolo, R.; Pulvirenti, S.; Brambilla, G.; Colombi, A. Characterization of the disulfides of bio-thiols by electrospray ionization and triple-quadrupole tandem mass spectrometry. *J. Mass Spectrom.* **2004,** *39*, 1408-1416.

87. Poon, G. K.; Chen, Q.; Teffera, Y.; Ngui, J. S.; Griffin, P. R.; Braun, M. P.; Doss, G. A.; Freeden, C.; Stearns, R. A.; Evans, D. C.; Baillie, T. A.; Tang, W. Bioactivation of diclofenac via benzoquinone imine intermediates-identification of urinary mercapturic acid derivatives in rats and humans. *Drug Metab. Dispos.* **2001,** *29*, 1608-1613.

88. Tang, W.; Stearns, R. A.; Wang, R. W.; Chiu, S. H.; Baillie, T. A. Roles of human hepatic cytochrome P450s 2C9 and 3A4 in the metabolic activation of diclofenac. *Chem. Res. Toxicol.* **1999,** *12*, 192-199.

89. Tang, W. The metabolism of diclofenac--enzymology and toxicology perspectives. *Curr. Drug Metab.* **2003,** *4*, 319-329.

90. Yu, L. J.; Chen, Y.; Deninno, M. P.; O'Connell, T. N.; Hop, C. E. Identification of a novel glutathione adduct of diclofenac, 4'-hydroxy-2'-glutathion-deschloro-diclofenac, upon incubation with human liver microsomes. *Drug Metab. Dispos.* **2005,** *33*, 484-488.

91. Miyamoto, G.; Zahid, N.; Uetrecht, J. P. Oxidation of diclofenac to reactive intermediates by neutrophils, myeloperoxidase, and hypochlorous acid. *Chem. Res. Toxicol.* **1997,** *10*, 414-419.

92. Alvarez-Sánchez, R.; Montavon, F.; Hartung, T.; Paehler, A. Thiazoldinedione bioactivation: A comparison of the bioactivation potentials of troglitazone and pioglitazone using stable isotope-labeled analogues and liquid chromatography tandem mass spectrometry. *Chem. Res. Toxicol.* **2006,** *19*, 1106-1116.

93. Maggs, J. L.; Williams, D.; Pirmohamed, M.; Park, B. K. The metabolic formation of reactive intermediates from clozapine, a drug associated with agranulocytosis in man. *J. Pharmacol. Exp. Ther.* **1995,** *275*, 1463-1475.

94. Zhou, S. F.; Paxton, J. W.; Tingle, M. D.; Kestell, P.; Jameson, M. B.; Thompson, P. I.; Baguley, B. C. Identification and reactivity of the major metabolite (beta-1-glucuronide) of the anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in humans. *Xenobiotica.* **2001,** *31*, 277-293.

95. Troesken, E. R.; Bittner, N.; Voelkel, W. Quantitation of 13 azole fungicides in wine samples by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A.* **2005,** *1083*, 113-119.

96. Boelsterli, U. A. Xenobiotic acyl glucuronides and acyl CoA thioesters as protein-reactive metabolites with the potential to cause idiosyncratic drug reactions. *Curr. Drug Metab.* **2002,** *3*, 439-450.

97. Argirov, O. K.; D., L. N.; J., O. B. Specific MS/MS Fragmentation of Lysine, Arginine, and Ornithine Glycation Products Provides an Opportunity for Their Selective Detection in Protein Acid Hydrolysates and Enzymatic Digests. *Ann. N.Y. Acad. Sci.* **2005,** *1043*, 903.

98. Ferrer, I.; Thurman, E. M. Measuring the mass of an electron by LC/TOF-MS: a study of "twin ions". *Anal. Chem.* **2005,** *77*, 3394-3400.

99. Galmier, M. J.; Bouchon, B.; Madelmont, J. C.; Mercier, F.; Pilotaz, F.; Lartigue, C. Identification of degradation products of diclofenac by electrospray ion trap mass spectrometry. *J. Pharm. Biomed. Anal.* **2005,** *38*, 790-796.

100. Wagner, S.; Scholz, K.; Donegan, M.; Burton, L.; Wingate, J.; Voelkel, W. Metabonomics and biomarker discovery: LC-MS metabolic profiling and constant neutral loss scanning combined with multivariate data analysis for mercapturic acid analysis. *Anal. Chem.* **2006,** *78*, 1296-1305.

101. Qiu, S. X.; Yang, R. Z.; Gross, M. L. Synthesis and liquid chromatography/tandem mass spectrometric characterization of the adducts of bisphenol A o-quinone with glutathione and nucleotide monophosphates. *Chem. Res. Toxicol.* **2004,** *17*, 1038-1046.

102. Scholz, K.; Dekant, W.; Voelkel, W.; Paehler, A. Rapid detection and identification of N-acetyl-L-cysteine thioethers using constant neutral loss and theoretical multiple reaction monitoring combined with enhanced product-ion scans on a linear ion trap mass spectrometer. *J. Am. Soc. Mass Spectrom.* **2005,** *16*, 1976-1984.

103. Sidell, K. R.; Olson, S. J.; Ou, J. J.; Zhang, Y.; Amarnath, V.; Montine, T. J. Cysteine and mercapturate conjugates of oxidized dopamine are in human striatum but only the cysteine conjugate impedes dopamine trafficking in vitro and in vivo. *J. Neurochem.* **2001,** *79*, 510-21.

104. Mascher, D. G.; Mascher, H. J.; Scherer, G.; Schmid, E. R. High-performance liquid chromatographic-tandem mass spectrometric determination of 3-hydroxypropylmercapturic acid in human urine. *J. Chromatogr. B Biomed. Sci. Appl.* **2001,** *750*, 163-169.

105. Gopaul, S. V.; Farrell, K.; Abbott, F. S. Identification and characterization of N-acetylcysteine conjugates of valproic acid in humans and animals. *Drug Metab. Dispos.* **2000,** *28*, 823-832.

106. Schaefer, W. H. a. D., F. jr. Effect of High-Performance Liquid Chromatography Mobile Phase Components on Sensitivity in Negative Atmospheric Pressure Chemical Ionization Liquid Chromatography-Mass Spectrometry. *J. Am. Soc. Mass Spec.* **1996,** *7*, 1059-1069.

107. Toyo'oka, T.; Suzuki, T.; Saito, Y.; Takahashi, A. Electrochemical detection of mercapturic acid derivatives after separation by high-performance liquid chromatography. *J. Chromatogr. A.* **1989,** *475*, 391-399.

108. Stanek, W.; Krenmayr, P.; Scherer, G.; Schmid, E. R. Quantitative determination of N-acetyl(-L-)cysteine derivatives in human urine by tandem mass spectrometry. *Biol. Mass Spectrom.* **1993,** *22*, 133-142.

109. Bloemen, L. J.; Monster, A. C.; Kezic, S.; Commandeur, J. N.; Veulemans, H.; Vermeulen, N. P.; Wilmer, J. W. Study on the cytochrome P-450- and

glutathione-dependent biotransformation of trichloroethylene in humans. *Int. Arch. Occup. Environ. Health.* **2001,** *74*, 102-108.

110. Doerge, D. R.; Churchwell, M. I.; Fang, J. L.; Beland, F. A. Quantification of etheno-DNA adducts using liquid chromatography, on-line sample processing, and electrospray tandem mass spectrometry. *Chem. Res. Toxicol.* **2000,** *13*, 1259-1264.

111. Mally, A.; Zepnik, H.; Wanek, P.; Eder, E.; Dingley, K.; Ihmels, H.; Voelkel, W.; Dekant, W. Ochratoxin A: lack of formation of covalent DNA adducts. *Chem. Res. Toxicol.* **2004,** *17*, 234-242.

112. Weigel, S.; Kallenborn, R.; Huhnerfuss, H. Simultaneous solid-phase extraction of acidic, neutral and basic pharmaceuticals from aqueous samples at ambient (neutral) pH and their determination by gas chromatography-mass spectrometry. *J. Chromatogr. A.* **2004,** *1023*, 183-195.

113. Court, M. H.; Duan, S. X.; von Moltke, L. L.; Greenblatt, D. J.; Patten, C. J.; Miners, J. O.; Mackenzie, P. I. Interindividual variability in acetaminophen glucuronidation by human liver microsomes: identification of relevant acetaminophen UDP-glucuronosyltransferase isoforms. *J. Pharmacol. Exp. Ther.* **2001,** *299*, 998-1006.

114. Hoffmann, K. J.; Axworthy, D. B.; Baillie, T. A. Mechanistic studies on the metabolic activation of acetaminophen in vivo. *Chem. Res. Toxicol.* **1990,** *3*, 204-211.

115. Chen, W.; Koenigs, L. L.; Thompson, S. J.; Peter, R. M.; Rettie, A. E.; Trager, W. F.; Nelson, S. D. Oxidation of acetaminophen to its toxic quinone imine and nontoxic catechol metabolites by baculovirus-expressed and purified human cytochromes P450 2E1 and 2A6. *Chem. Res. Toxicol.* **1998,** *11*, 295-301.

116. Mugford, C. A.; Kedderis, G. L. Sex-dependent metabolism of xenobiotics. *Drug Metab. Rev.* **1998,** *30*, 441-498.

117. Forte, A. J.; Wilson, J. M.; Slattery, J. T.; Nelson, S. D. The formation and toxicity of catechol metabolites of acetaminophen in mice. *Drug Meta. Dispos.* **1984,** *12*, 484-491.

118. Rote Liste ® Service GmbH [Internet]. Frankfurt/Main, Germany:**2006**, Fachinformation; available from www.rote-liste.de

119. Hinson, J. A.; Reid, A. B.; McCullough, S. S.; James, L. P. Acetaminophen-induced hepatotoxicity: role of metabolic activation, reactive oxygen/nitrogen species, and mitochondrial permeability transition. *Drug Metab. Rev.* **2004,** *36*, 805-822.

120. Kaplowitz, N. Idiosyncratic drug hepatotoxicity. *Nat. Rev. Drug Discov.* **2005,** *4*, 489-499.

121. Rote Liste ® Service GmbH [Internet]. Frankfurt/Main, Germany:**2006**, Fachinformation; available from www.rote-liste.de

122. Paino, I. M.; Ximenes, V. F.; Fonseca, L. M.; Kanegae, M. P.; Khalil, N. M.; Brunetti, I. L. Effect of therapeutic plasma concentrations of non-steroidal anti-inflammatory drugs on the production of reactive oxygen species by activated rat neutrophils. *Braz. J. Med. Biol. Res.* **2005,** *38*, 543-551.

123. Gomez-Gaviro, M. V.; Gonzalez-Alvaro, I.; Dominguez-Jimenez, C.; Peschon, J.; Black, R. A.; Sanchez-Madrid, F.; Diaz-Gonzalez, F. Structure-function relationship and role of tumor necrosis factor-alpha-converting enzyme in the down-regulation of L-selectin by non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* **2002,** *277*, 38212-38221.

124. Tang, W.; Stearns, R. A.; Bandiera, S. M.; Zhang, Y.; Raab, C.; Braun, M. P.; Dean, D. C.; Pang, J.; Leung, K. H.; Doss, G. A.; Strauss, J. R.; Kwei, G. Y.; Rushmore, T. H.; Chiu, S. H.; Baillie, T. A. Studies on cytochrome P-450-mediated bioactivation of diclofenac in rats and in human hepatocytes: identification of glutathione conjugated metabolites. *Drug Metab. Dispos.* **1999,** *27*, 365-372.

125. Bort, R.; Mace, K.; Boobis, A.; Gomez-Lechon, M. J.; Pfeifer, A.; Castell, J. Hepatic metabolism of diclofenac: role of human CYP in the minor oxidative pathways. *Biochem. Pharmacol.* **1999,** *58*, 787-796.

126. Yan, Z.; Li, J.; Huebert, N.; Caldwell, G. W.; Du, Y.; Zhong, H. Detection of a novel reactive metabolite of diclofenac: evidence for CYP2C9-mediated bioactivation via arene oxides. *Drug Metab. Dispos.* **2005,** *33*, 706-713.

127. Nagai, F.; Hiyoshi, Y.; Sugimachi, K.; Tamura, H. O. Cytochrome P450 (CYP) expression in human myeloblastic and lymphoid cell lines. *Biol. Pharm. Bull.* **2002,** *25*, 383-385.

128. Starkel, P.; Sempoux, C.; Van Den Berge, V.; Stevens, M.; De Saeger, C.; Desager, J. P.; Horsmans, Y. CYP 3A proteins are expressed in human neutrophils and lymphocytes but are not induced by rifampicin. *Life Sci.* **1999,** *64*, 643-653.

129. Furukawa, M.; Nishimura, M.; Ogino, D.; Chiba, R.; Ikai, I.; Ueda, N.; Naito, S.; Kuribayashi, S.; Moustafa, M. A.; Uchida, T.; Sawada, H.; Kamataki, T.; Funae, Y.; Fukumoto, M. Cytochrome p450 gene expression levels in peripheral blood mononuclear cells in comparison with the liver. *Cancer Sci.* **2004,** *95*, 520-529.

130. Pirmohamed, M.; Williams, D.; Madden, S.; Templeton, E.; Park, B. K. Metabolism and bioactivation of clozapine by human liver in vitro. *J. Pharmacol. Exp. Ther.* **1995,** *272*, 984-990.

131. Williams, D. P.; Pirmohamed, M.; Naisbitt, D. J.; Maggs, J. L.; Park, B. K. Neutrophil cytotoxicity of the chemically reactive metabolite(s) of clozapine: possible role in agranulocytosis. *J. Pharmacol. Exp. Ther.* **1997,** *283*, 1375-1382.

132. Vargas, F.; Rivas, C.; Perdomo, H.; Rivas, A.; Ojeda, L. E.; Velasquez, M.; Correia, H.; Hernandez, A.; Fraile, G. Clozapine prevents apoptosis and enhances receptor-dependent respiratory burst in human neutrophils. *Pharmazie.* **2005,** *60*, 364-368.

133. Sweeney, J. F.; Nguyen, P. K.; Omann, G. M.; Hinshaw, D. B. Lipopolysaccharide protects polymorphonuclear leukocytes from apoptosis via tyrosine phosphorylation-dependent signal transduction pathways. *J. Surg. Res.* **1998,** *74*, 64-70.

134. Gardner, I.; Popovic, M.; Zahid, N.; Uetrecht, J. P. A comparison of the covalent binding of clozapine, procainamide, and vesnarinone to human

neutrophils in vitro and rat tissues in vitro and in vivo. *Chem. Res. Toxicol.* **2005,** *18*, 1384-1394.

135. Kellner, M.; Wiedemann, K.; Krieg, J. C.; Berg, P. A. Toxic hepatitis by clozapine treatment. *Am. J. Psychiatry.* **1993,** *150*, 985-986.

136. Gardner, I.; Leeder, J. S.; Chin, T.; Zahid, N.; Uetrecht, J. P. A comparison of the covalent binding of clozapine and olanzapine to human neutrophils in vitro and in vivo. *Mol. Pharmacol.* **1998,** *53*, 999-1008.

137. Troesken, E. R.; Scholz, K.; Lutz, R. W.; Voelkel, W.; Zarn, J. A.; Lutz, W. K. Comparative assessment of the inhibition of recombinant human CYP19 (aromatase) by azoles used in agriculture and as drugs for humans. *Endocr. Res.* **2004,** *30*, 387-394.

138. Bremm, K. D.; Plenpel, M. Modulation of leukotriene metabolism from human polymorphonuclear granulocytes by bifonazole. *Mycoses.* **1991,** *34*, 41-45.

139. Lillibridge, J. H.; Amore, B. M.; Slattery, J. T.; Kalhorn, T. F.; Nelson, S. D.; Finnell, R. H.; Bennett, G. D. Protein-reactive metabolites of carbamazepine in mouse liver microsomes. *Drug Metab. Dispos.* **1996,** *24*, 509-514.

140. Fuerst, S. M.; Sukhai, P.; McClelland, R. A.; Uetrecht, J. P. Covalent binding of carbamazepine oxidative metabolites to neutrophils. *Drug Metab. Dispos.* **1995,** *23*, 590-594.

141. Bu, H. Z.; Kang, P.; Deese, A. J.; Zhao, P.; Pool, W. F. Human in vitro glutathionyl and protein adducts of carbamazepine-10,11-epoxide, a stable and pharmacologically active metabolite of carbamazepine. *Drug Metab. Dispos.* **2005,** *33*, 1920-1924.

142. Edmonds, J. S.; Nomachi, M.; Terasaki, M.; Morita, M.; Skelton, B. W.; White, A. H. The reaction of bisphenol A 3,4-quinone with DNA. *Biochem. Biophys. Res. Commun.* **2004,** *319*, 556-561.

143. Stopper, H.; Schmitt, E.; Kobras, K. Genotoxicity of phytoestrogens. *Mutat. Res.* **2005,** *574*, 139-155.

144. Sugita-Konishi, Y.; Shimura, S.; Nishikawa, T.; Sunaga, F.; Naito, H.; Suzuki, Y. Effect of Bisphenol A on non-specific immunodefenses against non-pathogenic Escherichia coli. *Toxicol. Lett.* **2003,** *136*, 217-227.

145. Jaeg, J. P.; Perdu, E.; Dolo, L.; Debrauwer, L.; Cravedi, J. P.; Zalko, D. Characterization of new bisphenol a metabolites produced by CD1 mice liver microsomes and S9 fractions. *J. Agric. Food Chem.* **2004,** *52*, 4935-4942.

146. Andrews, R. S.; Bond, C. C.; Burnett, J.; Saunders, A.; Watson, K. Isolation and identification of paracetamol metabolites. *J. Int. Med. Res.* **1976,** *4*, 34-39.

147. Critchley, J. A.; Critchley, L. A.; Anderson, P. J.; Tomlinson, B. Differences in the single-oral-dose pharmacokinetics and urinary excretion of paracetamol and its conjugates between Hong Kong Chinese and Caucasian subjects. *J. Clin. Pharm. Ther.* **2005,** *30*, 179-184.

148. Siegers, C. P.; Loeser, W.; Gieselmann, J.; Oltmanns, D. Biliary and renal excretion of paracetamol in man. *Pharmacology.* **1984,** *29*, 301-303.

149. Chen, C.; Hennig, G. E.; Manautou, J. E. Hepatobiliary excretion of acetaminophen glutathione conjugate and its derivatives in transport-deficient (TR-) hyperbilirubinemic rats. *Drug Metab. Dispos.* **2003,** *31*, 798-804.

150. Prescott, L. F. Paracetamol, alcohol and the liver. *Br. J. Clin. Pharmacol.* **2000,** *49*, 291-301.

151. Bessems, J. G.; Vermeulen, N. P. Paracetamol (acetaminophen)-induced toxicity: molecular and biochemical mechanisms, analogues and protective approaches. *Crit. Rev. Toxicol.* **2001,** *31*, 55-138.

152. Iwersen-Bergmann, S.; Schmoldt, A. Acute intoxication with aniline: detection of acetaminophen as aniline metabolite. *Int. J. Legal Med.* **2000,** *113*, 171-174.

153. Dressler, W. E.; Appelqvist, T. Plasma/blood pharmacokinetics and metabolism after dermal exposure to para-aminophenol or para-phenylenediamine. *Food Chem. Toxicol.* **2006,** *44*, 371-379.

154. Palmiotto, G.; Pieraccini, G.; Moneti, G.; Dolara, P. Determination of the levels of aromatic amines in indoor and outdoor air in Italy. *Chemosphere.* **2001,** *43*, 355-361.

155. Brede, C.; Skjevrak, I. Migration of aniline from polyamide cooking utensils into food simulants. *Food Addit. Contam.* **2004,** *21*, 1115-1124.

156. Sheffels, P.; Schroeder, J. L.; Altuntas, T. G.; Liggitt, H. D.; Kharasch, E. D. Role of cytochrome P4503A in cysteine S-conjugates sulfoxidation and the nephrotoxicity of the sevoflurane degradation product fluoromethyl-2,2-difluoro-1-(trifluoromethyl)vinyl ether (compound A) in rats. *Chem. Res. Toxicol.* **2004,** *17*, 1177-1189.

157. Kumar, S.; Samuel, K.; Subramanian, R.; Braun, M. P.; Stearns, R. A.; Chiu, S. H.; Evans, D. C.; Baillie, T. A. Extrapolation of diclofenac clearance from in vitro microsomal metabolism data: role of acyl glucuronidation and sequential oxidative metabolism of the acyl glucuronide. *J. Pharmacol. Exp. Ther.* **2002,** *303*, 969-978.

158. Bort, R.; Ponsoda, X.; Jover, R.; Gomez-Lechon, M. J.; Castell, J. V. Diclofenac toxicity to hepatocytes: a role for drug metabolism in cell toxicity. *J. Pharmacol. Exp. Ther.* **1999,** *288*, 65-72.

159. Stierlin, H.; Faigle, J. W. Biotransformation of diclofneac sodium (Voltaren) in animlas and in man. II Quantitative determination of the unchanged drug and principal phenolic metabolites in urine and bile. *Xenobiotica.* **1979,** *9*, 611-621.

160. Alary, J.; Debrauwer, L.; Fernandez, Y.; Paris, A.; Cravedi, J. P.; Dolo, L.; Rao, D.; Bories, G. Identification of novel urinary metabolites of the lipid peroxidation product 4-hydroxy-2-nonenal in rats. *Chem. Res. Toxicol.* **1998,** *11*, 1368-1376.

161. Alary, J.; Fernandez, Y.; Debrauwer, L.; Perdu, E.; Gueraud, F. Identification of intermediate pathways of 4-hydroxynonenal metabolism in the rat. *Chem. Res. Toxicol.* **2003,** *16*, 320-327.

162. Voelkel, W.; Alvarez-Sanchez, R.; Weick, I.; Mally, A.; Dekant, W.; Paehler, A. Glutathione conjugates of 4-hydroxy-2(E)-nonenal as biomarkers of hepatic oxidative stress-induced lipid peroxidation in rats. *Free Radic. Biol. Med.* **2005,** *38*, 1526-1536.

163. Kadiiska, M. B.; Gladen, B. C.; Baird, D. D.; Germolec, D.; Graham, L. B.; Parker, C. E.; Nyska, A.; Wachsman, J. T.; Ames, B. N.; Basu, S.; Brot, N.; Fitzgerald, G. A.; Floyd, R. A.; George, M.; Heinecke, J. W.; Hatch, G. E.; Hansley, K.; Lawson, J. A.; Marnett, L. J.; Morrow, J. D.; Murray, D. M.; Plastaras, J.; Robert, L. J. n.; Rokach, J.; Shigenaga, M. K.; Sohal, R. S.; Sun, J.; Tice, R. R.; VanThiel, T. H.; Weilner, D.; Walter, P. B.; Tomer, K. B.; Mason, R. P.; Barett, J. C. Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins and DNA markers of CCl4 poisoning? *Free Radic. Biol. Med.* **2005,** *38*, 698-710.

164. Milne, G. L.; Morrow, J. D. Isoprostanes and related compounds: update 2006. *Antioxid. Redox Signal.* **2006,** *8*, 1379-1384.

165. Soglia, J. R.; Harriman, S. P.; Zhao, S.; Barberia, J.; Cole, M. J.; Boyd, J. G.; Contillo, L. G. The development of a higher throughput reactive intermediate screening assay incorporating micro-bore liquid chromatography-micro-electrospray ionization-tandem mass spectrometry and glutathione ethyl ester as an in vitro conjugating agent. *J. Pharm. Biomed. Anal.* **2004,** *36*, 105-116.

166. Pearce, R. E.; Vakkalagadda, G. R.; Leeder, J. S. Pathways of carbamazepine bioactivation in vitro I. Characterization of human cytochromes P450 responsible for the formation of 2- and 3-hydroxylated metabolites. *Drug Metab. Dispos.* **2002,** *30*, 1170-1179.

167. Schneider, P.; Lorinci, G.; Gebefugi, I. L.; Heinrich, J.; Kettrup, A.; Wichmann, H. E. Vertical and horizontal variability of volatile organic compounds in homes in Eastern Germany. *J. Expo. Anal. Environ. Epidemiol.* **1999,** *9*, 282-292.

168. Topp, R.; Cyrys, J.; Gebefugi, I.; Schnelle-Kreis, J.; Richter, K.; Wichmann, H. E.; Heinrich, J. Indoor and outdoor air concentrations of BTEX and NO2: correlation of repeated measurements. *J. Environ. Monit.* **2004,** *6*, 807-812.

169. Angerer, J.; Schildbach, M.; Kramer, A. S-p-toluylmercapturic acid in the urine of workers exposed to toluene: a new biomarker for toluene exposure. *Arch. Toxicol.* **1998,** *72*, 119-123.

170. Inoue, O.; Kanno, E.; Kasai, K.; Ukai, H.; Okamoto, S.; Ikeda, M. Benzylmercapturic acid is superior to hippuric acid and o-cresol as a urinary marker of occupational exposure to toluene. *Toxicol. Lett.* **2004,** *147*, 177-186.

171. Takahashi, S.; Kagawa, M.; Shiwaku, K.; Matsubara, K. Determination of S-benzyl-N-acetyl-L-cysteine by gas chromatography/mass spectrometry as a new marker of toluene exposure. *J. Anal. Toxicol.* **1994,** *18*, 78-80.

172. van Maanen, M. J.; Doesburg Smits, K.; Damen, J. M.; Heck, A. J.; Beijnen, J. H. Stability of thioTEPA and its metabolites, TEPA, monochloroTEPA and thioTEPA-mercapturate, in plasma and urine. *Int. J. Pharm.* **2000,** *200*, 187-194.

173. Melikian, A. A.; O'Connor, R.; Prahalad, A. K.; Hu, P.; Li, H.; Kagan, M.; Thompson, S. Determination of the urinary benzene metabolites S-phenylmercapturic acid and trans,trans-muconic acid by liquid chromatography-tandem mass spectrometry. *Carcinogenesis.* **1999,** *20*, 719-726.

174. Imbriani, M.; Maestri, L.; Marraccini, P.; Saretto, G.; Alessio, A.; Negri, S.; Ghittori, S. Urinary determination of N-acetyl- S-(N-methylcarbamoyl)cysteine

and N-methylformamide in workers exposed to N, N-dimethylformamide. *Int. Arch. Occup. Environ. Health.* **2002,** *75*, 445-452.

175. Sakai, T.; Kageyama, H.; Araki, T.; Yosida, T.; Kuribayashi, T.; Masuyama, Y. Biological monitoring of workers exposed to N,N-dimethylformamide by determination of the urinary metabolites, N-methylformamide and N-acetyl-S-(N-methylcarbamoyl) cysteine. *Int. Arch. Occup. Environ. Health.* **1995,** *67*, 125-129.

176. Vos, R. M.; van Welie, R. T.; Peters, W. H.; Evelo, C. T.; Boogaards, J. J.; Vermeulen, N. P.; van Bladeren, P. J. Genetic deficiency of human class mu glutathione S-transferase isoenzymes in relation to the urinary excretion of the mercapturic acids of Z- and E-1,3-dichloropropene. *Arch. Toxicol.* **1991,** *65*, 95-99.

177. Haufroid, V.; Jakubowski, M.; Janasik, B.; Ligocka, D.; Buchet, J. P.; Bergamaschi, E.; Manini, P.; Mutti, A.; Ghittori, S.; Arand, M.; Hangen, N.; Oesch, F.; Hirvonen, A.; Lison, D. Interest of genotyping and phenotyping of drug-metabolizing enzymes for the interpretation of biological monitoring of exposure to styrene. *Pharmacogenetics.* **2002,** *12*, 691-702.

178. Parkinson, A.; Mudra, D. R.; Johnson, C.; Dwyer, A.; Carroll, K. M. The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome P450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. *Toxicol. Appl. Pharmacol.* **2004,** *199*, 193-209.

179. Argirov, O. K.; Lin, B.; Ortwerth, B. J. 2-ammonio-6-(3-oxidopyridinium-1-yl)hexanoate (OP-lysine) is a newly identified advanced glycation end product in cataractous and aged human lenses. *J. Biol. Chem.* **2004,** *279*, 6487-6495.

180. Teerlink, T.; Barto, R.; Ten Brink, H. J.; Schalkwijk, C. G. Measurement of Nepsilon-(carboxymethyl)lysine and Nepsilon-(carboxyethyl)lysine in human plasma protein by stable-isotope-dilution tandem mass spectrometry. *Clin. Chem.* **2004,** *50*, 1222-1228.

181. Emmert, J. Mobile Phase Additives for LC-MS. Part 1:Acids - The Most Common Choice..... *Analytix.* **2006,** 8-9.

182. Kretz-Rommel, A.; Boelsterli, U. A. Diclofenac covalent protein binding is dependent on acyl glucuronide formation and is inversely related to P450-mediated acute cell injury in cultured rat hepatocytes. *Toxicol. Appl. Pharmacol.* **1993,** *120*, 155-161.

183. Wells, D. S.; Janssen, F. W.; Ruelius, H. W. Interactions between oxaprozin glucuronide and human serum albumin. *Xenobiotica.* **1987,** *17*, 1437-1449.

184. Dr. Olaf Hagemann [internet]. Dorsten, Germany, **2006** July 06; available from *www.laborlexikon.de/Lexikon/Tabellen/04-Aminosaeuren_im_Urin.htm*.

185. Grillo, M. P.; Hua, F.; Knutson, C. G.; Ware, J. A.; Li, C. Mechanistic studies on the bioactivation of diclofenac: identification of diclofenac-S-acyl-glutathione in vitro in incubations with rat and human hepatocytes. *Chem. Res. Toxicol.* **2003,** *16*, 1410-1417.

186. Cindric, M.; Cepo, T.; Skrlin, A.; Vuletic, M.; Bindila, L. Accelerated on-column lysine derivatization and cysteine methylation by imidazole reaction in a

deuterated environment for enhanced product ion analysis. *Rapid Commun. Mass Spectrom.* **2006,** *20*, 694-702.

187. Scholl, P. F.; McCoy, L.; Kensler, T. W.; Groopman, J. D. Quantitative Analysis and Chronic Dosimetry of the Aflatoxin B(1) Plasma Albumin Adduct Lys-AFB(1) in Rats by Isotope Dilution Mass Spectrometry. *Chem. Res. Toxicol.* **2006,** *19*, 44-49.

9 ACKNOWLEDGMENTS

First of all, I would like to thank PD Dr. W. Voelkel and Prof. Dr. W. Dekant for offering the topic of my PhD thesis as well as their continuing support and particularly for the opportunity to participate in several international congresses and the graduate education program of the German Society for Pharmacology and Toxicology.

I would like to thank Prof. Dr. U. Holzgrabe for supervising my dissertation on behalf of the faculty of chemistry and pharmacy.

My special thanks go to F. Hoffmann-La Roche Ltd, Basel, for the financial support throughout the course of my graduate studies and dissertation and especially to Dr. A. Paehler for his professional and advisory support.

Angela, thank you for your mentorship and discussions, particularly at the end of this dissertation.

Eva Troesken, thank you for so many fruitful discussions, the excellent co-operation, the coffee breaks and the great fun during the Toxkurse. You tried your best to get me back to playing basketball again.

Eva Kopp, thank you for bringing fun and laughter into the lab and for the part you contributed to this work. I will always remember the time, sitting with you and Paul in a boat that does not want to go the direction we do.

Thanks to Andi, Ute, Eva T., Eva K., and Tanja for the wonderful working atmosphere in room 311 and the many motivating and constructive words.

Hannelore, thanks for the many secret meetings on the roof.

Paul and Marco, thank you for solving almost every computer problem.

Nataly, Heike, Ursula, Marco, Max, Silvia, Ursi, Hannelore, Tina, Theresa, Thomas, Marion, Katja, Micha, and Martina, thank you for your support, the great working atmosphere with so many birthday parties and your help.

Martin, thank you for being a good listener and for your encouragement. Sitting in Cassis and finding the right transition from toxicology to marriage was not easy.

Last, but not least, I would like to thank my family for their great support and motivating words. You always believed in me.

10 CURRICULUM VITAE

Karoline Simon, geb. Scholz

3th of July 1976, Schweinfurt, Germany

Dohne 31

45468 Muelheim a.d.Ruhr

Education

07.2003 – present	Graduate study and PhD thesis in toxicology at the Department of Pharmacology and Toxicology, University of Wuerzburg (PD Dr. W. Voelkel) on “Development and Evaluation of a Generic HPLC-MS/MS Screening Method for the Detection of Reactive Intermediate-Related Metabolites *in vitro* and *in vivo*”
07.2003 – present	Participant of the post-graduate education program “Expert in Toxicology” DGPT
05.1998 – 06.2003	University of Wuerzburg, study of pharmacy with six months internship at the Department of Pharmacology and Toxicology, University of Wuerzburg on “Determination of IC_{50} Values of Azole Fungicides Using Fluorescence Spectroscopy”; Degree: Legally Qualified Pharmacist (Approbation)
09.1997 – 04.1998	School of PTA (pharmazeutisch-technische Assistentin), Wuerzburg
09.1995 – 07.1997	Secondary School (Gymnasium Bad Koenigshofen), Degree: Abitur
08.1994 – 05.1995	High School in Freer, Texas, USA, Degree: Graduation
09.1987 – 07.1994	Secondary School (Gymnasium Bad Koenigshofen)
09.1983 – 07.1987	Primary School (Bad Koenigshofen)

Publications

Scholz, K.; Dekant, W.; Voelkel, W.; Paehler, A.
Rapid Detection and Identification of *N*-acetyl-L-cysteine Thioethers Using Constant Neutral Loss and Theoretical Multiple Reaction Monitoring Combined with Enhanced Product Ion Scans on a Linear Ion Trap Mass Spectrometer
J. Am. Soc. Mass Spectrom. 2005, 16, 1976-1984.

Wagner, S.; Scholz, K.; Donegan, M.; Burton, L.; Wingate, J.; Voelkel, W.
Metabonomics and Biomarker Discovery: LC-MS Metabolic Profiling and Constant Neutral Loss Scanning Combined with Multivariate Data Analysis for Mercapturic Acid Analysis
Anal. Chem. 2006, 78, 1296-1305.

Kellert, M.; Scholz, K.; Wagner, S.; Dekant, W.; Voelkel, W.
Quantitation of Mercapturic Acids from Acrylamide and Glycidamide in Human Urine Using a Column Switching Tool with two Trap Columns and Electrospray Tandem Mass Spectrometry
J. Chromatogr. A. 2006, 1131, 58-66

Troesken, E. R.; Scholz, K.; Lutz, R. W.; Voelkel, W.; Zarn, J. A.; Lutz, W. K.
Comparative Assessment of the Inhibition of Recombinant Human CYP19 (Aromatase) by Azoles Used in Agriculture and as Drugs for Humans
Endocr. Res. 2004, 30, 387-394.

Newman, D., Abuladze, N., Scholz, K., Dekant, W., Tsuprun, V., Ryazantsev, S., Bondar, G., Sassani, P., Kurtz, I., Pushkin, A.
Specificity of Aminoacylase III Mediated Deacetylation of Mercapturic Acids
Drug Metab. Dispos., in press.

Posters and Orals at Scientific Meetings

Scholz K., Kopp E. K., Paehler A., Voelkel W.
Detection of Reactive Intermediates as Thioethers Generated by Neutrophils and Microsomes Using a Specific Constant Neutral Loss Scan Combined With Product Ion Scans (Oral)
ASMS 2006, Seattle, USA

Scholz K., Troesken E. R., Manz J., Wienrich B. G., Schoen M. P., Voelkel W.
Resveratrol, Bisphenol A, and Bifonazole form Reactive Metabolites (Poster)
DGPT 2006, Mainz, Germany

Scholz K., Paehler A., Dekant W., Voelkel W.
A Generic LC-MS/MS Method for the Rapid and Sensitive Screening of Bioactivated Drug Metabolites in Rat Urine (Poster)
ISSX 2005, Nice, France

Scholz K., Dekant W., Paehler A., Voelkel W.
Development of a Generic LC-MS/MS Method for the Detection of Mercapturic Acids as Biomarkers of Reactive Metabolite Formation (Poster)
Pfizer Drug Discovery 2004, Kent, UK

Wagner S., Scholz K., Burton L., Voelkel W.
New Tools in Metabonomics: Application of Sensitive MRM Scans Combined with New Scaling Procedures to Detect Reactive Metabolites in Humans (Poster)
ASMS 2006, Seattle, USA

Wagner S., Scholz K., Donegan M., Burton L., Wingate J., Voelkel W.
Metabonomics: LC-MS Based Metabolic Profiling of Mercapturic Acids in Humans (Poster)
DGPT 2006, Mainz, Germany

Voelkel W. and Scholz K.
Highest Sensitivity and Specifity Available by Use of Multiple Reaction Monitoring Mode Combined with Enhanced Product Ion Spectra in Complex Matrix (Poster)
ASMS 2005, San Antonio, USA

Wagner S., Scholz K., Donegan M., Burton L., Wingate J., Voelkel W.
LC-MS Metabolic Profiling of Mercapturic Acids in Humans (Poster)
Advances in Metabolic Profiling 2005, London, UK

Troesken E. R., Scholz K., Lutz R. W., Voelkel W., Zarn J.A., Lutz W.K.
Assessment of the Inhibition of Human Recombinant CYP19 (Aromatase) by Azoles Used in Agriculture and as Drugs for Humans (Poster)
DGPT, 2004, Mainz, Germany

Paehler A.; Haas R.; Scholz K., Voelkel W.
Screening and Characterization of Reactive Drug Metabolites in Vitro and in Vivo by Automated Nanoelectrospray Coupled to Quadrupole Linear Iontrap MS/MS (Poster)
ASMS 2005, San Antonio, USA

Wienrich B. G., Schoen M., Voelkel W., Troesken E.R., Scholz K., Broecker E. B., Schoen M. P.
New Insights into the Molecular Basis of the Antitumoral Activity of Resveratrol (Poster)
33rd Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung 2006, Aachen, Germany