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Timo Rousu

LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY IN DRUG METABOLISM STUDIES

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Abstract

Drug metabolite profiling and identification studies are nowadays regularly conducted with liquid chromatography (LC) coupled with mass spectrometry (MS) as an analytical tool. The speed, selectivity and sensitivity of modern LC–MS instruments have been significantly increased in recent years. Especially the use of ultra-high-performance LC (UHPLC) in combination with a modern high-resolution MS instrument offers high full scan detection sensitivity, mass accuracy and the detection of both expected and unexpected metabolites in a single LC–MS run. The present study showed that no single LC–MS conditions were suitable for the analysis of a large group of structurally diverse compounds. The testing of optimum conditions for each individual compound led to more high-quality data when chromatographic retention behavior and mass spectrometric ionization efficiency for *in vitro* metabolite profiling were considered. The developed LC–MS methods were applicable for measuring both the disappearance of the parent compound and the formation of metabolites. Tentative metabolite identification was based on the measured accurate mass time-of-flight (TOF) MS data. In the second part, a rapid and sensitive assay was designed and built for the trapping, screening and characterization of reactive metabolites *in vitro*. In total, 78 trapped reactive metabolite conjugates were detected and identified based on accurate mass data using 12 structurally different test compounds. The majority of the detected conjugates were reported for the first time. Amine-containing compounds, that formed methylated and cyanide-trapped products after CYP-mediated reaction steps in human liver microsomal (HLM) incubations, were studied further. The observed methylated cyano conjugates were shown to be experimental artifacts, i.e., metabonates. The study also describes the use of traditional high-performance LC (HPLC) and the more modern UHPLC coupled to time-of-flight, triple quadrupole and hybrid linear ion trap mass spectrometers in drug metabolism studies, and reviews on how to choose the most suitable LC–MS system for metabolite profiling purposes in drug discovery and early drug development.

Keywords: drug metabolism, drugs, in vitro, LC–MS, liquid chromatography, mass spectrometry, reactive metabolites

Rousu, Timo, Nestekromatografia–massaspektrometria lääkeainemetaboliatutkimuksessa
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Luonnontieteellinen tiedekunta, Kemian laitos, PL 3000, 90014 Oulun yliopisto
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Tiivistelmä

Nestekromatografia (LC) yhdistettynä massaspektrometriaan (MS) on nykyaikana yleisesti käytetty analyysimenetelmä lääkeaineiden aineenvaihduntatuotteiden (metaboliittien) havaitsemisessa ja tunnistamisessa. Modernien LC–MS -laitteiden nopeus, selektiivisyys ja herkkyys ovat merkittävästi parantuneet viime vuosina. Käytettäessä ultrakorkean suorituskyvyn nestekromatografia (UHPLC) yhdessä nykyaikaisen korkean massaresoluution MS-laitteen kanssa on mahdollista havaita kaikki sekä odotetut että odottamattomat metaboliitit yhdellä kertaa. Tutkimalla suurta joukkoa rakenteellisesti erilaisia yhdisteitä voitiin todeta, että yksittäiselle yhdisteelle optimoidut mittausolosuhteet johtivat korkealaatuisempaan dataan kuin yleiset ei-optimoidut olosuhteet, kun arvioitiin sekä kromatografista piikin profiilia ja pidättymistä että ionisaatiotehokkuutta. Yksikään yksittäinen analyysiolosuhde ei myöskään soveltunut kaikille yhdisteille. Tutkimuksessa kehitetyillä LC–MS -analyysimenetelmillä tutkittiin sekä kanta-aineen häviämistä että metaboliatuotteiden muodostumista in vitro -menetelmillä. Alustava metaboliatuotteiden tunnistus perustui tarkan massan mittaukseen lentoaikamassaspektrometrillä (TOFMS). Tutkimustyön seuraavassa vaiheessa kehitettiin nopea ja herkkä analyysimenetelmä reaktiivisten metaboliittien pyydystämiseen, havaitsemiseen ja tunnistamiseen ihmisen maksamikrosomivalmisteista in vitro -menetelmin. 12 testiyhdisteelle havaittiin kaikkiaan 78 erilaista reaktiivisen metaboliitin konjugaatiotuotetta, jotka tunnistettiin tarkan massan perusteella. Suurin osa tunnistetuista konjugaatiotuotteista raportoitiin ensimmäistä kertaa. Amiineja sisältäville testiyhdisteille havaittiin muodostuvan sytokromi P450 (CYP) entsyymien katalysoimien reaktioiden välityksellä metyloituneita ja syanidianionilla konjugoituneita metaboliatuotteita. Tarkempien tutkimusten jälkeen näiden todettiin olevan koejärjestelyistä johtuvia artefaktoja, toisin sanoen metabonaatteja, eivätkä todellisia reaktiivisten metaboliittien konjugaatiotuotteita. Tässä tutkimuksessa arvioitiin myös perinteiseen korkean suorituskyvyn nestekromatografiin (HPLC) sekä uudempaan UHPLC-laitteistoon kytkettyjen lentoaika-, kolmoiskvadrupoli- ja hybridimallisten ioniloukkumassaspektrometrien soveltuvuutta aikaisen lääkekehitysvaiheen metaboliatutkimuksiin.

Asiasanat: aineenvaihdunta, aineenvaihduntatuotteet, analyysimenetelmät, in vitro -menetelmä, lääkeaineet, massaspektrometria, nestekromatografia

nihil boni sine labore

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Espoo, April 2012 Timo Rousu

Abbreviations

AA	acetic acid
ACN	acetonitrile
ADR	adverse drug reaction
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APPI	atmospheric pressure photoionization
CYP	cytochrome P450 enzyme
DMSO	dimethyl sulfoxide
ECVAM	European Centre for the Validation of Alternative Methods
EMS	enhanced full mass scan
EPI	enhanced product ion scan
ER	enhanced resolution scan
ESI	electrospray ionization
FA	formic acid
FWHM	full width at half maximum
GC	gas chromatography
GSH	glutathione (reduced form)
GST	glutathione S-transferase
HLH	human liver homogenate
HLM	human liver microsomes
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
IADR	idiosyncratic adverse drug reaction
IDA	information dependent acquisition
IT	ion trap
KCN	potassium cyanide
LC	liquid chromatography
LLE	liquid-liquid extraction
MDF	mass defect filter
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)

NL	neutral loss scanning
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PI	precursor ion scanning
PPT	protein precipitation
Q	quadrupole mass analyzer
QqLIT	hybrid linear ion trap triple quadrupole (mass spectrometer)
QqQ	triple quadrupole (mass spectrometer)
Q-Trap	hybrid linear ion trap triple quadrupole mass spectrometer from Applied Biosystems / MDS Sciex
RLH	rat liver homogenate
RLM	rat liver microsomes
RP	reversed phase
SCA	semicarbazide
S/N	signal-to-noise ratio
SPE	solid phase extraction
SRM	selected reaction monitoring
SULT	sulphotransferase
TOF	time-of-flight (mass spectrometer)
UDPGA	uridine 5'-diphospho-glucuronic acid
UGT	UDP-glucuronosyltransferase
UHPLC	ultra-high-performance liquid chromatography
UPLC	ultra-high-performance liquid chromatograph from Waters

List of original articles

This thesis is based on the following publications, which are referred to in the text by Roman numerals I–V.

I Rousu T, Hokkanen J, Pelkonen O & Tolonen A (2010) Applicability of generic assays based on liquid chromatography–electrospray mass spectrometry to study *in vitro* metabolism of 55 structurally diverse compounds. Frontiers in Drug Metabolism and Transport 1(10): 1–13.

II Pelkonen O, Tolonen A, Rousu T, Tursas L, Turpeinen M, Hokkanen J, Uusitalo J, Bouvier d'Yvoire M & Coecke S (2009) Comparison of Metabolic Stability and Metabolite Identification of 55 ECVAM/ICCVAM Validation Compounds between Human and Rat Liver Homogenates and Microsomes – a preliminary Analysis. Alternativen zu Tierexperimenten 26(3): 214–222.

III Rousu T, Pelkonen O & Tolonen A (2009) Rapid detection and characterization of reactive drug metabolites *in vitro* using several isotope-labeled trapping agents and ultra-performance liquid chromatography/time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry 23(6): 843–855.

IV Rousu T & Tolonen A (2011) Characterization of cyanide-trapped methylated metabonates formed during reactive drug metabolite screening *in vitro*. Rapid Communications in Mass Spectrometry 25(10): 1382–1390.

V Rousu T, Herttuainen J & Tolonen A (2010) Comparison of triple quadrupole, hybrid linear ion trap triple quadrupole, time-of-flight and LTQ-Orbitrap mass spectrometers in drug discovery phase metabolite screening and identification *in vitro* – amitriptyline and verapamil as model compounds. Rapid Communications in Mass Spectrometry 24(7): 939–957.

The present author is the primary author of I, III–V and co-author of II. Most of the experimental analytical work related to II was the work of the present author.

Contents

1 Introduction

The mission of pharmaceutical research companies is to develop safe and effective drugs, and bring them to market for the treatment of diseases in the shortest possible time. The drug discovery phase starts with the identification and validation of the molecular target (e.g. protein, gene or receptor) that is associated with the disease with which the potential drug interacts. High-throughput screening (HTS) is the most common way to test hundreds of thousands of different chemical compounds for their ability to modify the target. A few lead compounds are selected for the assessment of safety, and are tested for their absorption (A), distribution (D), metabolism (M), excretion (E) and toxicological (T) properties. These early development phases of ADMET studies are conducted in test systems (*in vitro*), in living organisms (*in vivo*) and by computational models (*in silico*). The drug development process continues with lead optimization, where numerous structural derivatives or analogues are synthesized and tested for, for example, selectivity to modify the target, tolerability, potential side effects, optimal pharmaceutical formulation and manufacturing process. The preclinical discovery and development phase lasts for about 3–6 years, and is followed by clinical trials (phase I–III) for about 6–7 years. The total time needed for a drug molecule to progress from discovery to an approved drug is about 10–15 years.

Most drugs are eliminated from the human body by enzymatic biotransformation reactions catalyzed by cytochrome P450 enzymes (oxidative phase I metabolism) and different transferase enzymes (conjugative phase II metabolism). Metabolism and metabolic reactions determine to a great extent the pharmacokinetic properties of drugs, and are often behind bioavailability problems. [1] Although metabolism is in general a beneficial process, it can result in either reversible or irreversible adverse drug reactions (ADRs). [2] Especially reactive metabolites are believed to be behind unexpected drug-induced toxicity and other adverse reactions. [3]

Metabolite profiling and identification studies in drug discovery and early development are nowadays regularly conducted from *in vitro* preparations with liquid chromatography (LC) coupled with mass spectrometry (MS). [4, 5] The speed, selectivity and sensitivity of the LC–MS instruments available for drug metabolism studies have been significantly increased in recent years, enabling higher throughput and faster turnaround time. [6] Ultra-high-performance LC (UHPLC) systems provide good chromatographic resolution in the separation of

drugs and metabolites from each other and from interfering matrix components. [7, 8] Metabolite profiling in early phases of drug development are often conducted using very fast generic LC–MS methods to obtain high throughput, but the optimization of chromatographic performance and mass spectrometric ionization efficiency very often improves the quality of the measured data. [6] Low-resolution triple quadrupole (QqQ) mass spectrometers operated in either neutral loss (NL) or precursor ion (PI) scanning modes are traditionally used in metabolite screening, but they rely on similar fragmentation behavior as the parent drug, and unexpected metabolites are thus easily missed. [9] High-resolution MS instruments offer high full scan detection sensitivity, mass accuracy and the detection of both expected and unexpected metabolites in a single LC–MS run, thus the identification of biotransformations, and their approximate sites as well, is both cost- and time-effective. [6] Modern LC–MS instruments are also capable of measuring both qualitative and quantitative data simultaneously. [10]

The present study describes the use of high-performance LC (HPLC) and UHPLC coupled to time-of-flight (TOF), QqQ and hybrid linear ion trap mass spectrometers in drug metabolism studies, and reviews on how to choose the most suitable LC–MS system for metabolite profiling purposes in drug discovery and early drug development. LC–MS methods were developed and applied to monitor both substrate depletion and metabolite formation for a large number of structurally diverse compounds, and generic LC–MS based assays were evaluated considering chromatographic retention behavior and mass spectrometric ionization efficiency for *in vitro* metabolite profiling. The trapping, screening and characterization of reactive metabolites *in vitro* are also described.

2 Review of literature

2.1 Drug metabolism

Chemicals that enter the human body, intentionally or unintentionally, are cleared mainly by one of three mechanisms: metabolism, renal or bile excretion. [11–13] The relative proportions of major clearance routes are illustrated in Figure 1. Metabolism is the primary route with a share of about 70%, followed by renal clearance with a about 25% share and biliary excretion with the lowest share, although being the major route of clearance for some compounds, e.g., pravastatin. [14, 15] Most drugs are eliminated from the human body by oxidative (phase I) and conjugative (phase II) metabolism. [12, 16] In a metabolic reaction the drug compound is subjected to chemical alteration or degradation. The metabolite of a drug is often more hydrophilic than the drug itself, thus metabolism usually aims at the chemical modification of hydrophobic drug molecules into more easily excreted species. [17] The metabolite can also be active with the desired therapeutic effect instead of the parent drug that is thus administered as a pro-drug. [16] The activation of a drug by metabolism can also result in a toxic product. [2]

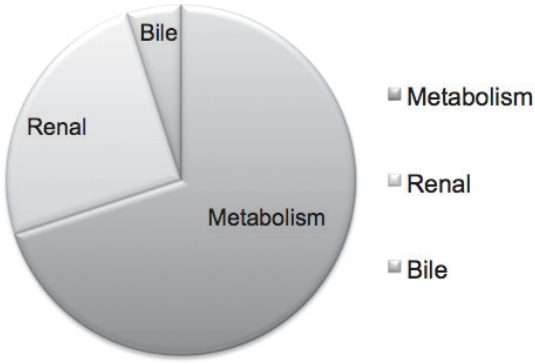

Fig. 1. Relative proportions of major clearance mechanisms of drugs from the human body. Modified from [13].

2.1.1 Oxidative phase I metabolism

The human liver has a high concentration of cytochrome P450 (CYP) enzymes [16], and is the first intestinal organ that a drug molecule passes through after

absorption from the gut. Since CYPs are the principal enzymes that catalyze oxidative drug metabolism [18], the *in vitro* studies of drug metabolism with different liver preparations are of great importance and widely conducted.

CYP enzymes belong to a superfamily of hemoproteins. The main function of CYPs is to catalyze the oxidation of its substrate. [19] CYPs that are involved, and thus have the greatest influence on drug metabolism in humans, belong to CYP1, CYP2 and CYP3 families. [18, 19] The relative proportions of these three CYP families are illustrated in Figure 2. CYP1A2 is the most important member of the CYP1 enzyme family in human hepatic metabolism, while the most important enzymes in the CYP2 family are 2A6, 2B6, 2C8, 2C9, 2C19, 2D6 and 2E1. [20] CYP3A4 is the most important member of the CYP3 family and this single enzyme metabolizes the largest proportion of drugs that are currently available. [13, 19]

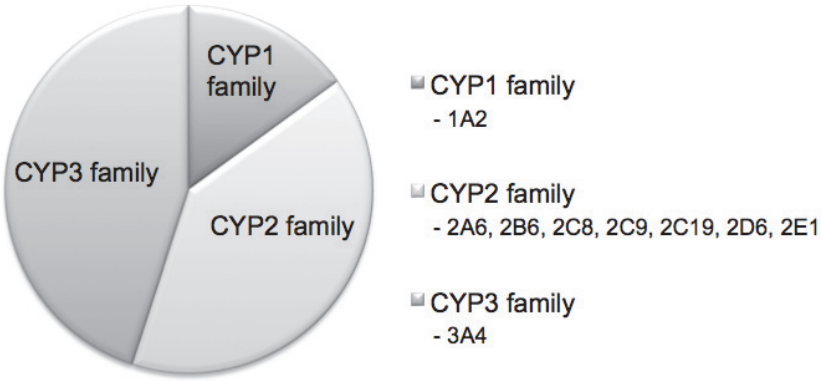

Fig. 2. Relative proportions of the three most important CYP families, and the most important enzymes within these families, responsible for the hepatic metabolism of most drugs in humans. Modified from [13].

Important phase I biotransformations that CYP enzymes catalyze include hydroxylation of C–H bonds, N/S-oxidation, deamination, dehalogenation and N/S/O-dealkylation. A desaturation reaction proceeds via oxidation of C/N/S and subsequent generation of a dehydrogenation product. [16] Although oxidative ester cleavage by CYP enzymes is possible, the vast majority of non-CYP-mediated phase I metabolic reactions are ester or amide hydrolysis by esterases or amidases, respectively. [13, 16] CYP-mediated metabolism of a drug can be catalyzed by only one or multiple different CYP enzymes. [19]

The metabolic fate of drugs in humans varies between individuals because the expression of CYP enzymes has numerous variations. Inter-individual variations rise from e.g. genetic factors of the population (Caucasian, Asian, Afro-American, etc.) and environmental factors like diet, alcohol consumption, cigarette smoking and medication. [21–23] Therefore, e.g. other co-administered drugs can result in the induction or inhibition of the metabolizing CYP enzymes, thus the intended plasma level can be increased or decreased drastically beyond desired concentration leading to failure in dosing and treatment, or even toxicity causing severe damage.

2.1.2 Conjugative phase II metabolism

Phase II metabolism of drugs is often referred to using the more distinctive name, conjugative metabolism. In a conjugation reaction a molecule is conjugated with a polar group by an enzyme (transferase), after that the molecule is usually easily excreted. Conjugation typically results in inactivation of the drug or metabolite. [24] Phase II metabolites can be either direct conjugations to the parent drug or they can be preceded by oxidative phase I metabolism. Heteroatoms are often the sites on drugs where many conjugation reactions occur, of which N/O/S-conjugates are the most common. [25, 26]

The most common conjugation reaction is the addition of glucuronic acid by UDP-glucuronosyltransferases (UGTs), i.e. glucuronidation [26–29], which is an endogenous reaction. Other highly important endogenous conjugative enzymes include glutathione S-transferases (GSTs) and sulphotransferases (SULTs) that produce glutathione (GSH) and sulfate conjugates, respectively. [25, 30] Acetylation and methylation, as well as different amino acid (e.g. glycine, cysteine, taurine) conjugations, are also important metabolic routes of drugs. [31–33]

2.1.3 Reactive metabolites and trapping agents

Different chemically reactive species, e.g., quinones, quinoneimines, epoxides and aldehydes can be formed from drug molecules via bioactivation. [34] Bioactivation products are usually referred to as reactive metabolites, a categorization that is also frequently assigned to free radicals. A common chemical property of reactive metabolites is that most of them are electrophiles, and thus reactive with nucleophiles. Both electrophiles and nucleophiles are often

categorized as either “soft” or “hard” depending on the electron density of the species. [35]

Bioactivation, and thus reactive metabolite formation, is one context of drug-induced toxicity. [2] Reactive drug metabolites can form covalent adducts with cell proteins or DNA, and this binding to proteins may lead to cell damage. [36–38] An established example of bioactivation is illustrated in Figure 3, in which acetaminophen (a common pain killer and fever reducer) forms a reactive quinoneimine metabolite via CYP-mediated N-oxidation and subsequent rearrangement to hepatotoxic “NAPQI”. [39, 40] The formed “NAPQI” is a “soft” electrophile and is thereafter detoxified by a glutathione conjugation, which is a “soft” nucleophile.

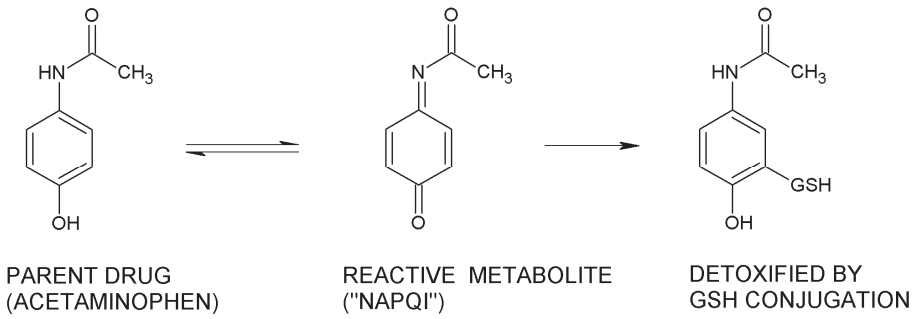

Fig. 3. Scheme of bioactivation of a drug molecule (acetaminophen) to a reactive quinoneimine metabolite (“NAPQI”) that is subsequently detoxified by GSH conjugation.

As early as possible detection and identification of the formation of reactive metabolites is advised in the early stages of the drug development process. [7, 34, 41, 42] The later the drug development process fails, the more money and resources are wasted. The failure by toxicity issues can originate from either predictable, usually dose-dependent, or unpredictable idiosyncratic adverse drug reactions (IADRs). [43] Also, approved drugs have been withdrawn from the market because of these IADRs, that are partially caused by reactive metabolite-induced toxicity. [44]

Various trapping agents have been used to trap reactive metabolites from *in vitro* incubations. The previously described GSH is a naturally occurring nucleophile in the human body, and is the most widely used trapping agent in reactive metabolite screening assays. [45] GSH is a “soft” nucleophile that is

reactive with “soft” electrophiles such as quinoneimines, nitrenium ions, arene oxides, quinones and imine methides. [41] Cyanide anion is a “hard” nucleophile and an effective trapping agent for iminium ions that can be formed from amine-containing drugs. [46] Semicarbazide (SCA) is often used in reactive metabolite screening assays due to its known capability to trap aldehydes. [47, 48] Methoxylamine has also been used to trap aldehydes. [49, 50] The specificity of the reactive metabolite trapping assays with MS detection is increased by the use of mixtures of stable isotope-labeled and non-labeled versions of the trapping agents, as the real positives have an easily distinguishable molecular ion doublet in mass spectra. [51–53] Numerous customized trapping agents have been developed and applied to reactive metabolite screening assays including N-acetylcysteine [54], derivatives of GSH [55–58] and peptides. [59, 60] Some of the recently introduced trapping agents have been modified to be effective in simultaneously trapping both “soft” and “hard” electrophiles. [61]

Several studies, especially in the past few years, have been conducted to characterize reactive metabolites *in vitro*. The usual outcome and evidence of reactive metabolite formation has been based on LC–MS detection of conjugates of reactive species with nucleophilic trapping agents or irreversible covalent binding to proteins utilizing radiolabelled compounds. [62, 63] The sole detection of trapped reactive metabolites from *in vitro* assays cannot correlate to covalent binding to proteins *in vivo*. [5, 43, 62, 64] Valuable information about the chemical and metabolic stability of the compound is obtained in *in vitro* reactive metabolite screening assays, although undisputable evidence of possible ADRs is not achieved.

2.1.4 In vitro enzyme sources

Different *in vitro* models are universally used in drug metabolism studies. The majority of metabolite screening studies are conducted with liver microsomes. Liver microsomes contain enzymes that provide information about CYP-mediated phase I metabolism and UGT-mediated phase II metabolism, but appropriate cofactors need to be added. [1, 17, 65] Liver homogenate and the subcellular S9 fraction are also widely used in metabolism studies providing additional information about other phase II metabolism, e.g., sulpho conjugations, but appropriate cofactors are also needed. [1, 65] Recombinant CYPs can be used to substantiate if the metabolites are formed via a specific CYP isoform. [66] Hepatocytes, and liver slices as well, are encouraging in metabolism studies, as

they provide the full complement of drug metabolizing enzymes and cofactors, and thus offer the most comprehensive metabolism data. [65, 67, 68] Also immortalized cell lines, such as HepaRG, [69] have been used in metabolism studies.

2.2 Liquid chromatography–mass spectrometry (LC–MS) in drug metabolite profiling and identification

Metabolite profiling studies in humans, and other toxicity species as well, are encouraged in early phases of drug discovery and development. [70–76] Separation of analytes from each other and from the matrix components in drug metabolite profiling studies is regularly conducted with LC and rarely with gas chromatography (GC). [77, 78] The strengths of GC lie in the analysis of small, easily volatile and non-polar compounds and is routinely used in, e.g., forensic science in screening of drugs of abuse. [77, 79] On the contrary to GC, derivatization of the analyte is rarely needed when using LC. [80, 81] The identification of biotransformations and their approximate sites is rapid and time-effective when analysis is conducted with LC–MS. Thus speed, selectivity and sensitivity are the main reasons why LC–MS is the most widely adapted analytical tool for studying drug metabolism at the present time. [6, 70, 82]

2.2.1 Sample preparation

Drugs and drug metabolites are often analyzed from *in vitro* preparations or samples of biological origin such as plasma, tissues, urine or bile. [83] Proper sample pretreatment is of utmost importance prior to LC–MS analysis because biological matrices are very complex and contain various endogenous substances. [84–86] Moreover, tissue samples often need to be homogenized before further sample preparation steps, such as extraction. Traditional sample preparation techniques in drug metabolism studies with LC–MS analysis include liquid-liquid extraction (LLE) [87, 88], protein precipitation (PPT) [84], solid phase extraction (SPE) [86, 89] and sometimes even plain dilution, i.e., the “dilute-and-shoot” method. [90]

Sample preparation aims at cleaner samples with less undesired and unpredicted matrix effects and yielding better detection sensitivity with good reproducibility. [86, 91] Non-specific sample preparation techniques are favored in screening type analysis in order to avoid false negatives by losing possible

metabolites upon pretreatment. Protein precipitation with an equal, or two- to four-fold, volume of acetonitrile or methanol is very simple and straightforward procedure and often used in drug metabolism studies. Termination of *in vitro*, e.g. microsomal, incubation reactions with an equal volume of ACN is an analogous treatment to PPT. Supernatants from centrifuged protein-precipitated samples are usually ready to be injected into an LC–MS system as such. A more specific sample preparation, e.g. SPE or LLE, is often used when the analysis is targeted to known compounds and to reduce matrix effects. [83, 92] Very high selectivity is obtained with ion exchange SPE. [93, 94] The sample is usually extracted in LLE or eluted in SPE with an excess of easily volatile organic solvent to ensure complete elution and good recovery, and thus dilutes to some extent. Evaporation of the resulting sample to dryness and subsequent reconstitution into a small volume of LC–MS injection solvent is often conducted to concentrate the sample. This kind of treatment is however limited to stable compounds, as the evaporation step usually involves some heating and also the concentration of the sample may raise solubility issues. [95, 96]

In addition to sample cleanup and concentration, the aim of sample preparation is the efficient removal of interfering matrix components. The co-elution of endogenous plasma phospholipids or other matrix components can cause severe matrix effects by ion suppression or ion enhancement. [84, 86, 97, 98] The matrix effects on the MS response of progesterone analyzed from human plasma are illustrated in Figure 4. [99] The pretreatment of plasma was performed with HybridSPE that has been shown to be effective in the removal of plasma phospholipids, and thus in a reduction in matrix effects [100], and with traditional SPE with a macroporous copolymer sorbent. The top two multiple reaction monitoring (MRM) chromatograms are from post column infusion of progesterone after blank plasma injections, and the representative peak of progesterone is in the bottom MRM chromatogram (c). The baseline of the progesterone MS signal is relatively stable when HybridSPE was used (a), but severe ion suppression is observed with traditional SPE (b), thus even a slight ~0.04 min shift in the retention time of the analyte may increase or decrease the MS response of progesterone drastically (~40%).

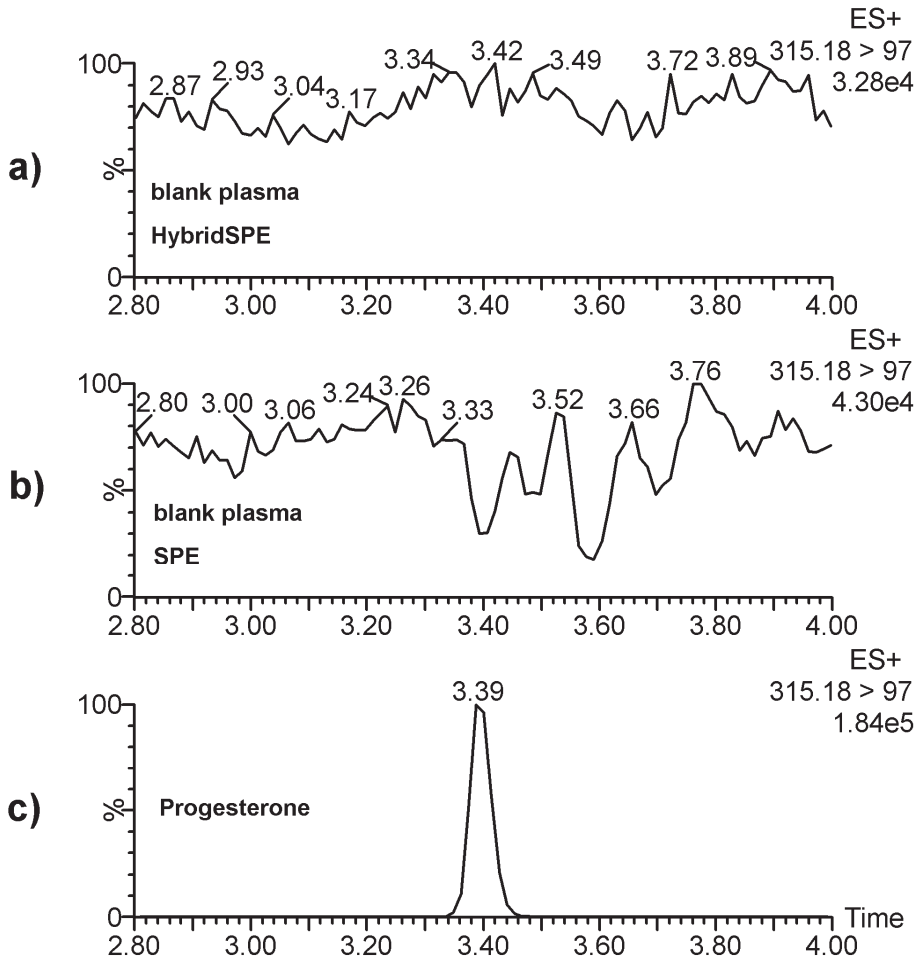

Fig. 4. Post column infusion of progesterone after injections of blank plasma with sample preparation by (a) HybridSPE (a relatively stable baseline of analyte MS signal) or (b) SPE (severe ion suppression at the retention time of progesterone). Peak at retention time of 3.39 minutes is a representative chromatographic peak of progesterone (c). Reproduced from [99] with permission of Future Science Ltd.

2.2.2 Chromatographic separation

The main descriptors in LC separation are selectivity, efficiency and resolution. [101] Selectivity describes the ability of the LC system to discriminate different analytes from each other, and is determined as the ratio of retention factors. Selectivity is dependent on the interactions of the analytes with the stationary phase in the column. Also, the LC eluent type and composition may influence, e.g. the analyte ionization, thus affecting the selectivity of the separation. [102] The most convenient way to make a drastic change in the selectivity is to change the column that has a different stationary phase. Efficiency is the measure of the degree of peak dispersion, and is a property of the column. Efficiency is expressed in the number of theoretical plates and is calculated from retention time and peak width. Efficiency is proportional to the column length, thus the longer the column, the higher the efficiency. Resolution encompasses selectivity and efficiency. Resolution describes the ability of the column to resolve analytes to separate peaks. The resolution of the separation can be improved by increasing either selectivity or efficiency. The effect of selectivity and efficiency on the separation of two analytes from each other, thus to obtain adequate chromatographic resolution, is illustrated in Figure 5, where broad peaks demonstrate poor efficiency and overlapping peaks poor selectivity.

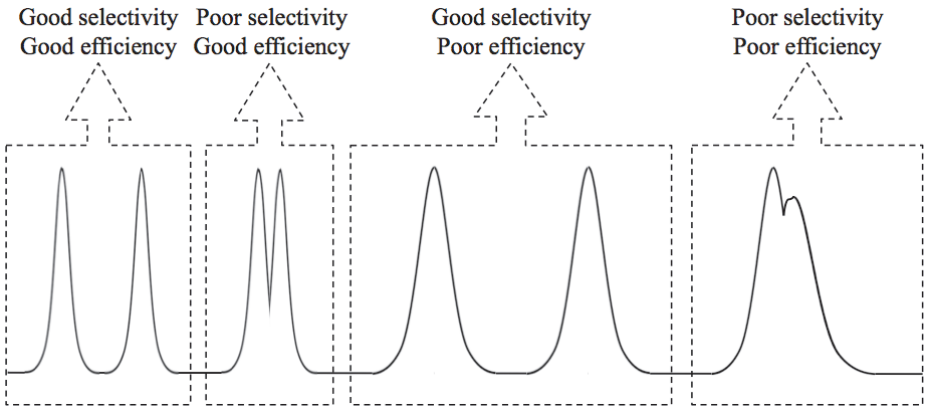

Fig. 5. The complete separation of two chromatographic peaks from each other is achieved with good selectivity. It is equally important to obtain narrow and symmetrical peaks, thus to achieve good efficiency in the separation.

2.2.3 LC system

The basic components in the LC system are solvent reservoirs, high-pressure pump, degasser, injector, column and detector. In the beginning of the 21st century high-performance liquid chromatography (HPLC) has commonly been combined with 50–150-millimeter-long narrow bore columns (inner diameter ~2 mm) packed with 3–5 μm particles in drug metabolism studies. [103] With modern ultra-high-performance LC (UHPLC) systems the chromatographic resolution is increased by the use of smaller particle size (<2 μm) columns. [104–109] This however increases the back pressure of the LC system to even over 10 000 psi, and is thus not applicable with traditional HPLC pumps that are capable of handling about 4 000–6 000 psi back pressures. This disadvantage is partially overcome by the use of novel columns packed with fused-core particles. [110–113]

A gradient elution of a few minutes with a reversed-phase (RP) UHPLC-type analytical column is a commonly used approach in early phase drug metabolism studies, providing fast analysis and adequate separation. [7] The stationary phase in a RP column is carbon-based, non-polar, hydrophobic and usually C18-type, and efficient in the separation of polar compounds. [114, 115] Aqueous conditions and the right pH in the mobile phase provide sufficient retention for polar compounds, and the increase in the proportion of the organic solvent (usually methanol or acetonitrile) increases the solvent strength and the elution of the retained non-polar compounds. The pH governs whether the compound is in ionic or neutral state, and thus also affects the retention of the compound. [114] The pH of the mobile phase in LC–MS applications is controlled and adjusted with miscible and volatile additives, which are compatible with MS, such as acetic acid, ammonium acetate or ammonium hydroxide.

2.2.4 Ionization and mass analyzers

The majority of drug metabolism studies with MS detection utilize atmospheric pressure ionization (API) sources. Electrospray ionization (ESI) is in general the most widely exploited technique, while atmospheric pressure chemical ionization (APCI) and sometimes atmospheric pressure photoionization (APPI) are used to ionize less polar neutral compounds, e.g. steroids. [116-118] In an ESI source the LC flow is directed through a heated high voltage (±1–5 kV) capillary. In the positive ion mode of ESI, the positive charge on the capillary drives negatively charged ions to the capillary wall, and results in a spray of positively charged ions

from the tip of the capillary needle. Vaporization of the excess solvent is aided by a flow of heated nitrogen gas parallel to the LC flow, and the gas phase ions are directed to the MS. [119–124]

Various different quadrupole (Q), TOF and ion trap (IT) mass analyzers have been used to study drugs and their metabolites. The Q mass analyzer can be used to monitor a selected *m/z* value or to scan across a *m/z* range. Triple quadrupole (QqQ) is the most widely spread configuration of quadrupole instruments, and is usually operated at mass unit resolution in selected reaction monitoring (SRM), MRM, product ion scanning, neutral loss scanning (NL) or precursor ion scanning (PI) mode. [125] The SRM/MRM mode of QqQ is usually the operating mode of choice in quantitative bioanalysis, whereas NL and PI scanning modes are used in the screening of metabolites. [9, 126] The NL and PI scanning rely on similar fragmentation behavior as the parent drug and unexpected metabolites are easily missed. In the TOF mass analyzer ions are accelerated and separated in mass according to their flight time. [125] TOF and especially modern hybrid quadrupole TOF (Q-TOF) instruments are capable of very high mass resolution (even over 40 000) with very good mass accuracy, and offer high full scan detection sensitivity, and are thus ideal for metabolite screening, and applicable for quantitative bioanalysis as well. [10] Ion mobility spectrometry (IMS), especially when coupled with TOF, has demonstrated potential applicability in metabolite profiling studies. [127, 128] Even higher mass accuracy than with TOF, is obtained with Orbitrap and Fourier-transform ion cyclotron resonance (FTICR) instruments, but they suffer from a relatively slow data acquisition rate and thus have limitations in very fast LC–MS applications. [125, 129, 130] The conventional quadrupole ion trap and the modern linear ion trap (LIT) are capable of multi-stage tandem mass spectrometry (MS^n) where the cycles of trapping and fragmentation are repeated. [131, 132] The LIT has been fused to different hybrid MS instruments, e.g. the high-resolution LTQ-Orbitrap [129] and the hybrid linear ion trap triple quadrupole (Q-Trap). [131] In the LTQ-Orbitrap very high accurate mass measurements are combined with the high trapping capacity and MS^n function of the ion trap. The ion trap in the Q-Trap is also capable of MS^n as well as high sensitivity scanning. The Q-Trap is usually operated in SRM triggered enhanced product ion MS/MS scanning in metabolite screening. [133]

2.2.5 Data processing

A huge amount of data can be obtained from a single LC–MS run, especially if acquired with a modern high-resolution MS instrument. Different software has been developed for both pre-acquisition predictions and post-acquisition data mining and processing purposes. [134–144] Software that are designed for metabolite screening, such as MetaboLynx and MetWorks, compare and contrast extracted ion chromatograms based on predicted biotransformations from each sample with the control sample. [145] The measured accurate mass data can be processed by a mass defect filter (MDF), that removes biological background ions based on the decimal component of their *m/z* value. [134, 136, 138] A generic dealkylation tool can be used in data processing to identify bonds that have the potential to be cleaved by metabolic reactions. [142] Background subtraction software in combination with a noise reduction algorithm efficiently reduces interfering matrix ion signals in ion chromatograms, and requires no prior knowledge of structures or fragmentation pathways of metabolites. [144, 146–148] An accurate mass-based isotope pattern filtering algorithm can be applied to remove matrix ions, and is applicable for the processing of data measured from stable isotope-labeled compounds or compounds containing natural isotopes, e.g. chlorine. [144, 149]

3 Aims of the study

The goal of this thesis was to develop new LC–MS-based analytical methods to study drug metabolism. Recent developments, especially in MS instruments, are behind the reasons why LC–MS has been adopted as the golden standard in drug metabolism studies. However, there are several types of mass spectrometers available that are used for drug metabolism studies, each with their specific strengths and drawbacks. Besides producing scientifically relevant and novel analytical methods for drug metabolite profiling purposes, some key issues of LC–MS in drug metabolism studies were discussed. The specific aims were:

- To assess if a generic LC–MS method is applicable in comparison to the compound specific method development for metabolite profiling purposes in drug discovery and early drug development. [I]
- To develop and apply LC–MS methods to monitor both substrate depletion and metabolite formation for a large number of structurally diverse compounds. And to characterize similarities and differences between human and rat liver homogenates and microsomes as enzyme sources in *in vitro* metabolite profiling. [II]
- To develop a generic incubation setup and an analytical LC–MS method for the trapping, screening and characterization of reactive metabolites *in vitro*. [III]
- To characterize cyanide-trapped methylated metabolites formed during reactive metabolite screening assay *in vitro*. [IV]
- To assess how to choose the most suitable LC–MS system for metabolite profiling purposes in drug discovery and early drug development. [V]

4 Experimental

The main features of the experimental aspects of this study are described in this section, while more detailed information can be found in the original Articles I–V.

4.1 *In vitro* incubations and sample preparation

The basic incubation mixture consisted of 0.5 mg of microsomal protein *per* ml, substrate in dimethyl sulfoxide (DMSO) and 1 mM nicotinamide adenine dinucleotide phosphate (NADPH). [I–V] The concentration of uridine 5'-diphospho-glucuronic acid (UDPGA) was 1 mM. [I, II, V] For homogenate incubations in I and II also 3'-phosphoadenosine 5'-phosphosulfate (PAPS) and GSH with 0.5 mM and 1 mM concentrations were used as cofactors, respectively. In III and IV the concentration of the trapping agent used was 1 mM, and was a 1:1 mixture of non-labeled and a stable isotope-labeled version. The incubation conditions in terms of species, liver preparation, cofactors, initial substrate concentration and incubation time, as well as the used laboratory chemicals and reagents used in the studies, are described in the original Articles.

In all Articles control incubation without the study compound was performed for each liver preparation used. Also incubations with liver preparation, buffer solution and study compound were conducted to estimate the free fraction of the study compound in the incubation. [I, II]

Each reaction mixture was pre-incubated for 2 minutes in +37 °C in a shaking incubator block. The enzymatic reactions were started by the addition of cofactors and were terminated by the addition of either an equal [III–V] or a two-fold [I, II] volume of ice-cold acetonitrile. Samples were subsequently cooled in an ice bath and the tubes were stored at –18 °C until analysis. Upon analysis the liver microsomal and homogenate incubation samples were thawed at room temperature, shaken and centrifuged for 10 min at 16 100 × *g* and pipetted to Total Recovery [I, II] or Maximum Recovery [III–V] vials.

4.2 LC–MS instruments

The LC–MS instruments used in I–V are listed in Table 1. With Waters Alliance HPLC several different reversed phase columns and mobile phase eluents were used for the analysis of the 55 validation study compounds using both the positive and the negative ion mode of electrospray ionization. [I, II] In III–V the

chromatographic separation was carried out by reversed phase chromatography and gradient elution. With Waters Acquity UPLC chromatograph a Waters BEH Shield RP18 (2.1×50 mm, 1.7 μm) column was used together with an on-line 0.2 μm filter [III–V], while with Thermo Accela HPLC the analytical column was Thermo Hypersil Gold C18 (2.1×50 mm, 3.0 μm). [V] The eluents were 0.1% glacial acetic acid in water and gradient grade acetonitrile with flow rates of 0.2–0.5 ml/min. [III–V] An positive mode of electrospray ionization was used in III–V with all mass spectrometers except for LC–MS/MS experiments using the Quattro premier triple quadrupole mass spectrometer [III] with which both positive and negative ion modes of electrospray ionizations were used.

Table 1. LC–MS instruments in the studies [I–V].

Article	Liquid chromatograph	Mass spectrometer	Controller software
I, II	Waters Alliance 2695 HPLC	Micromass LCT	MassLynx 4.0
I, II	Waters Alliance 2695 HPLC	Micromass Quattro micro	MassLynx 4.0
III–V	Waters Acquity UPLC	Waters LCT Premier XE	MassLynx 4.1
III–V	Waters Acquity UPLC	Waters Quattro Premier	MassLynx 4.1
V	Waters Acquity UPLC	AB/MDS Sciex 4000 Q-Trap	Analyst 1.4.2
V	Thermo Accela HPLC	Thermo Scientific LTQ-Orbitrap	Xcalibur 2.0.7

5 Results and discussion

The main results and discussions of I–V are summarized in this section. In the first part of the study the applicability of generic LC–MS methods in comparison to compound-specific method development was studied and applied to drug metabolism studies. [I, II] In the second part LC–MS methods were developed and applied for the screening and characterization of reactive drug metabolites. [III, IV] Finally, different LC–MS instruments for qualitative analysis were compared and evaluated. [V]

5.1 LC–MS in screening of stable drug metabolites [I, II]

5.1.1 Applicability of generic screening method [I]

Chromatographic retention behavior and mass spectrometric ionization efficiency were the two most important factors considered when LC–MS methods for metabolite profiling purposes were developed. [I, II] The applicability of generic LC–MS methods for metabolite profiling in drug discovery and early drug development was evaluated using six different mobile phase pH conditions and acquiring the data with both positive and negative ion modes of electrospray ionization. A generic gradient elution profile with 2–2–60–90–90% acetonitrile in 0–1–3–5–6 minutes was applied. Performances, assessing both ionization efficiency and detection sensitivity of the LC–MS methods for the study compounds, are summarized in Table 2. The large group (55) of study compounds consisted of compounds classified as acid (10), amphoteric (3), base (25) or neutral (17). The study compounds and their classification, as well as calculated pK_a values, are presented in Table 3. The calculated pK_a values were used as a guideline for the classification, which were verified with experimental pK_a values from literature when applicable. The vast majority of the compounds were drugs, and they were selected from the set of validation study compounds provided by ECVAM. The ECVAM list was not based on chemical classes (acids, bases, neutrals) but rather on different toxicities of the compounds. Four of the originally selected compounds were changed because of analytical difficulties, mostly due to being small polyhalogenated compounds with very weak ionization properties with ESI and poor suitability for HPLC analysis. Also very small and

volatile compounds requiring special incubation and analytical conditions were omitted.

Table 2. Performances of the LC–MS methods with variable mobile phase pH conditions to the analysis of 55 study compounds when monitoring both substrate disappearance and metabolite formation using either positive or negative ion mode of electrospray ionization. Modified from [I].

LC–MS properties	pH 2.8		pH 3.2		pH 4.4		pH 6.0		pH 6.7		pH 9.8	
	ESI+	ESI−	ESI+	ESI−	ESI+	ESI−	ESI+	ESI−	ESI+	ESI−	ESI+	ESI−
++	25	5	25	13	32	17	19	16	11	14	19	12
+	8	8	8	4	7	3	4	0	4	2	5	4
(++)	4	1	4	0	3	0	24	1	18	0	17	2
-	18	41	18	38	13	35	8	38	22	39	14	37
Compounds with ++	29		35		41		27		18		25	

++, Good ionization efficiency and detection sensitivity to monitor both substrate disappearance and metabolite formation, good chromatographic peak shape and retention behavior.

+, Good enough ionization efficiency and detection sensitivity to monitor substrate disappearance but not metabolite formation, good chromatographic peak shape and retention behavior.

(++), Good ionization efficiency and detection sensitivity to monitor both substrate disappearance and metabolite formation, but either poor chromatographic peak shape or inadequate retention behavior.

-, Poor ionization efficiency and detection sensitivity to monitor even substrate disappearance.

Good ionization efficiency and detection sensitivity (marked "++" in Table 2) was clearly dependent on the pH of the mobile phase, and the best results were obtained at pH 4.4. At this pH, in total 41 out of the 55 study compounds showed adequate LC–MS performance for monitoring both substrate disappearance and metabolite formation, which was defined as a peak with a signal-to-noise ratio (S/N) exceeding 10 (from a sample corresponding to 1% concentration of the initial incubation concentration of 25 μM). Sufficient ionization efficiency and detection sensitivity to monitor only substrate disappearance (marked "+" in Table 2) was defined as detected peak (S/N >3) from 4% concentration of the actual initial incubation concentration. Apparent chromatographic problems (marked "(++)" in Table 2), i.e. peak tailing/fronting or insufficient retention, were evident at higher pH conditions. Poor peak shape was specified as asymmetric factor A/B <0.4 (tailing peak) or A/B >2 (fronting peak), where A equals peak width before the peak top and B equals peak width after the peak top (both measured from 5% peak height). The criterion for sufficient retention behavior was set so that normalized retention time (retention time minus dead time) should be at least ten times the peak width (measured from 5% peak height).

Table 3. The classification of the 55 study compounds in I and II, and their calculated pK_a values.

Compound	Class	Acid pK_a*	Base pK_a**	Compound	Class	Acid pK_a*	Base pK_a**
Amitriptyline	Base	-	9.0	Acetaminophen	Acid	9.5	–0.5
Amphetamine	Base	-	9.8	Chloramphenicol	Acid	14	–3.1
Antipyrine	Base	-	1.6	Cycloheximide	Acid	16	-
Atropine	Base	14	8.9	Diphenylhydantoin	Acid	7.1	-
Caffeine	Base	-	3.5	Gibberellic acid	Acid	15	-
Carbamazepine	Base	-	–3.5	Ibuprofen	Acid	4.1	-
Dextropropoxyphene	Base	-	9.2	Pentobarbital	Acid	8.0	-
Diazepam	Base	-	4.8	Phenobarbital	Acid	7.6	-
Diphenhydramine	Base	-	9.4	Propylparaben	Acid	8.9	-
Disopyramide	Base	-	9.7	Warfarin	Acid	18.9	-
Haloperidol	Base	-	8.2	Abamectin	Neutral	14	–4.4
Isoniazid	Base	12	4.1	Aflatoxin b1	Neutral	-	-
Maprotiline	Base	-	10	Busulfan	Neutral	-	-
Methadone	Base	-	9.1	Carbaryl	Neutral	-	5.5
Methylphenidate	Base	-	9.2	Chlorpyrifos	Neutral	-	–4.6
Nicotine	Base	-	8.0	Colchicine	Neutral	15	–3.2
Orphenadrine	Base	-	9.4	Dibutyl phthalate	Neutral	-	-
Physostigmine	Base	14	8.5	Diethyl phthalate	Neutral	-	-
Procainamide	Base	-	9.2	Digoxin	Neutral	17	–4.5
Propranolol	Base	-	10	Diuron	Neutral	15	–2.1
Quinidine	Base	14	11	Fenpropathrin	Neutral	11	-
Strychnine	Base	-	7.9	Fenvalerate	Neutral	11	-
Theophylline	Base	11	2.4	Fipronil	Neutral	-	–2.9
Thioridazine	Base	-	9.6	Malathion	Neutral	-	
Verapamil	Base	-	8.3	Metalaxyl	Neutral	-	–1.3
Aminoglutethimide	Amphoteric	11	4.3	Parathion	Neutral	-	-
Aminopterin	Amphoteric	4.7	7.1	Rotenone	Neutral	-	-
Nalidixic acid	Amphoteric	6.1	3.2				

* Calculated strongest acidic pK_a value, ** Calculated strongest basic pK_a value.

None of the tested mobile phase pH conditions were able to provide adequate analytical conditions, even to monitor substrate disappearance, for all of the compounds. Warfarin (acid) and diuron (neutral) had equal and very good LC–MS properties regardless of ionization polarity or pH. Among these two also carbamazepine (base), diazepam (base), aflatoxin b1 (neutral), colchicine (neutral), metalaxyl (neutral) and rotenone (neutral) had very good LC–MS properties using the positive ion mode of ESI, and chloramphenicol (acid),

digoxin (neutral) and fipronil (neutral) using the negative ion mode of ESI in all pH conditions. Isoniazid (base), fenpropathrin (neutral) and parathion (neutral) were detected only at pH 6.0 in the positive ion mode. Generally the negative ion mode was more applicable to acidic compounds in all pH conditions, while basic compounds had better ionization efficiency with the positive ion mode of ESI. The positive ion mode and pH 6.0 were generally the most suitable for the analysis of neutral compounds. Positive ion mode ESI generally covered more compounds at almost every pH, but the selection of study compounds (bases 25/55, acids 10/55) may affect the results to some extent. The column selection for the generic screening method may have an effect on the results. Waters XBridge Shield RP18 was selected based on good experience with the stationary phase and that it was able to cover the whole pH range used in the study. Also the relatively high concentration of organic solvent in the injection solvent (50% acetonitrile) may affect the peak shapes of early eluting compounds in all pH conditions.

The effect of pH on chromatographic peak shape and retention behavior is illustrated in Figure 6. The extracted ion chromatograms of nalidixic acid show very good peak shapes and retention when formic acid (pH 2.8), acetic acid (pH 3.2) or a mixture of acetic acid and ammonium acetate (pH 4.4) are used in the mobile phase (traces a,b and c, respectively), but poor peak shape when ammonium formate (pH 6.0) and ammonium acetate (pH 6.7) are used (traces d and e), and poor peak shape and decreased retention when ammonia (pH 9.8) is used in the mobile phase (trace f).

By the analysis of a large group of study compounds with structural differences, some cautious generalizations could be made based on the obtained results.

- No single or even two different conditions were suitable for covering the whole set of compounds, stressing that the testing of optimum conditions for each individual compound clearly leads to more high-quality data.
- Slightly, but not excessively acidic pH conditions would be optimum for generic LC–ESI–MS analysis of a large set of compounds.
- Poor chromatographic performance, rather than poor ionization efficiency, may often be the limiting factor in analysis of basic compounds. Although recent advances in liquid chromatographic techniques utilizing sub-2 μm particles and better column chemistries to some extent improve the problem

with poor peak shapes, thus leading to a situation where generic chromatography works increasingly better.

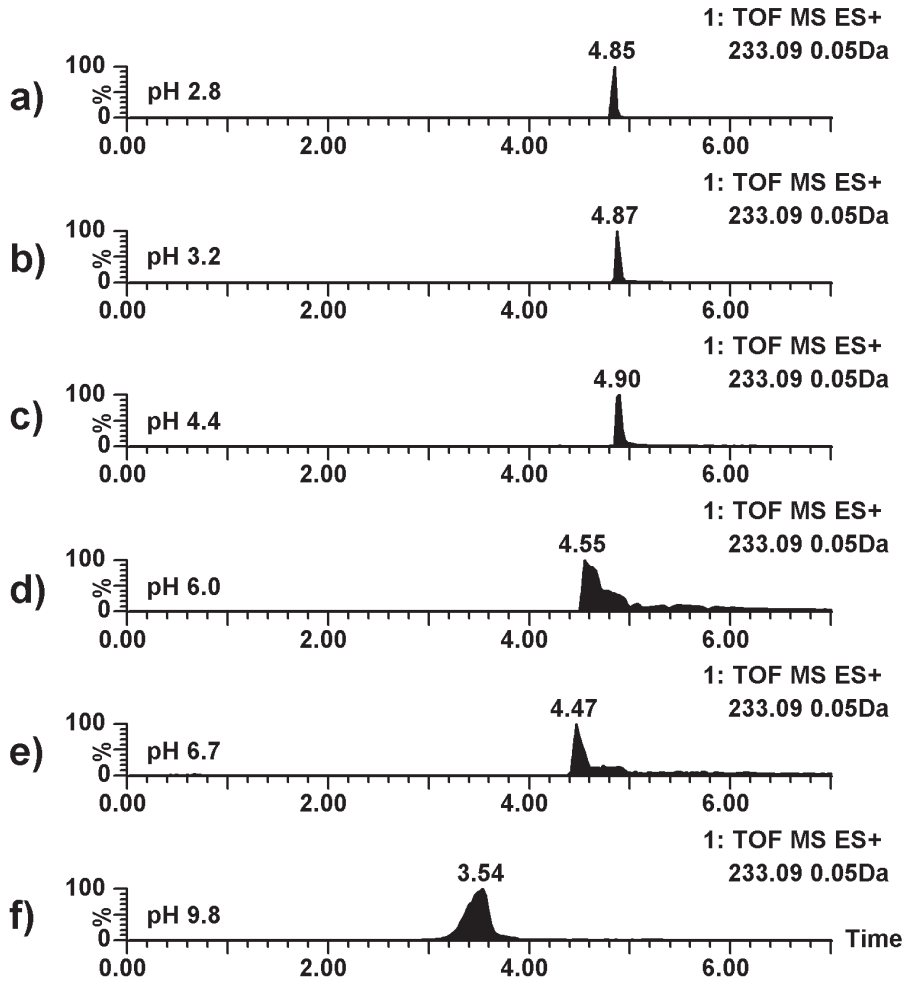

Fig. 6. Positive ion mode extracted ion chromatograms for nalidixic acid at various mobile phase pH-conditions, a) 0.1% formic acid (pH 2.8); b) 0.1% acetic acid (pH 3.2); c) 0.05% acetic acid with 5 mM ammonium acetate (pH 4.4); d) 2 mM ammonium formate (pH 6.0); e) 2 mM ammonium acetate (pH 6.7); f) 10 mM ammonia (pH 9.8). Modified from [I].

5.1.2 Metabolism studies [II]

The generic LC–MS method for metabolite profiling of 55 compounds was evaluated in I, and the data was used as a basis when LC–MS methods were developed and applied to metabolism studies for these same 55 structurally diverse compounds. [I, II] The same validation study compounds as in I, provided by ECVAM, were studied for substrate depletion and stable metabolite formation *in vitro*, using human and rat liver homogenates (HLH and RLH, respectively) and human and rat liver microsomes (HLM and RLM, respectively) to assess similarities and differences between species and liver preparations as enzyme sources. Four of the originally selected compounds were changed because of analytical difficulties, mostly due to being small polyhalogenated compounds with very weak ionization properties with ESI and poor suitability for HPLC analysis. LC–TOFMS method was developed for 52 of the 55 study compounds and LC–MS/MS method for the remaining three. The main categorical results of II are summarized in Table 4.

Table 4. Similarities and differences in the presence or absence of major and minor metabolites between human and rat liver homogenates and microsomes. Modified from [II].

Similarities and differences	Comparison between enzyme source		Comparison between species	
	HLH vs HLM	RLH vs RLM	HLH vs RLH	HLM vs RLM
No metabolites	9*	9*	9*	9*
Metabolite(s) in one, but not in the other	4	3	5	6
Only one metabolite	7**	7**	7**	7**
Major metabolite(s) the same	21	18	14	14
Major metabolite(s) different	10	15	20	18
Minor metabolite(s) the same	11	7	5	2
Minor metabolite(s) different	18	22	28	27

* No metabolites were detected in HLH, HLM, RLH or RLM.

** Same metabolite was detected in HLH, HLM, RLH and RLM.

No metabolites were detected for nine of the compounds (aminopterin, amphetamine, antipyrine, busulfan, digoxin, gibberellic acid, isoniazid,

phenobarbital and theophylline) in any of the sample types; HLH, HLM, RLH or RLM. Metabolites of some compounds were detected only in homogenate but not in microsomes, or vice versa. Metabolites of acetaminophen, aminoglutethimide, caffeine and cycloheximide were detected in HLH but not in HLM. Metabolites of acetaminophen and nicotine were detected in RLH but not in RLM, while metabolites of aminoglutethimide were detected in RLM but not in RLH. In comparison of the species using homogenate, metabolites of aminoglutethimide, caffeine and cycloheximide were detected in HLH but not in RLH, while metabolites of chloramphenicol and pentobarbital were detected in RLH but not in HLH. In comparison, between the species using microsomes, metabolites of cycloheximide and nicotine were detected in HLM but not in RLM, while metabolites of aminoglutethimide, chloramphenicol, pentobarbital and procainamide were detected in RLM but not in HLM. Only one metabolite was detected for carbamazepine, diethyl phthalate, diphenylhydantoin, fipronil, ibuprofen, methadone and parathion, and the same metabolite was detected in all of the sample types HLH, HLM, RLH and RLM for each of these seven compounds.

There were mainly quantitative differences between the detected metabolites, but also species-dependent qualitative differences in metabolite profiles were relatively common; 20 compounds displayed a difference in major metabolites in human and rat homogenates and 18 compounds in human and rat microsomes. For about a half of the compounds some minor metabolites were different between the species. Fourteen compounds (e.g. dibutyl phthalate, methadone and verapamil) had similar major metabolites in both human and rat homogenates, as was observed also in human and rat microsomes. Minor metabolites were observed to be the same between species in homogenates for only five compounds (acetaminophen, carbaryl, diethyl phthalate, dibutyl phthalate and parathion) and in microsomes for only two compounds (carbamazepine and diethyl phthalate). However, dibutyl phthalate was the only compound of these, for which more than one single minor metabolite was detected. In general the differences were greater between the two species with the same enzyme source than were observed in the comparisons between enzyme sources in either human or rat.

An example of the obtained metabolism data in II is presented in Table 5. The observed major (M) and minor (+) metabolites of amitriptyline in both species and liver preparations are marked accordingly. Both major and minor metabolites were the same when comparing HLH and HLM, thus amitriptyline was

categorized to “major metabolites the same” and to “minor metabolites the same” in HLH vs HLM comparison in Table 4. Dissimilar metabolite profiles were obtained in comparison between RLH and RLM. Qualitative differences between species were also evident in both homogenates and microsomes.

Table 5. Observed amitriptyline metabolites in human and rat liver homogenates and microsomes in II (M = major metabolite, + = minor metabolite, - = not detected).

Amitriptyline metabolites	HLH	HLM	RLH	RLM
M1	M	M	M	M
M2	+	+	+	-
M3	+	+	M	M
M4	+	+	+	M
M5	-	-	M	M
M6	-	-	+	+
M7	-	-	M	+
M8	-	-	+	+
M9	-	-	-	+
M10	-	-	-	M

By the analysis of a large group of study compounds with structural differences, some cautious conclusions could be made based on the obtained results.

- Only 4 out of the 55 preselected ECVAM validation study chemicals had to be changed because of analytical problems. All of them were highly halogenated compounds with poor detection with LC–MS. Thus LC–MS based analytical methods were efficient in the primary *in vitro* phase of studying organic chemicals for measuring both the disappearance of the parent compound and the formation, and tentative identification, of metabolites.
- A relatively simple and not too costly experimental setup was used to study the disappearance of the parent compound and the formation, and tentative identification, of metabolites. A single initial substrate concentration (25 μM) was used, which proved to be too low for some compounds (e.g. caffeine) that are low-affinity substrates for its metabolizing enzymes, to produce detectable metabolites with the developed LC–MS method. [150] Remarkably lower substrate concentrations (e.g. 10, 1 and/or 0.1 μM) are however used in metabolic stability studies to avoid saturation of metabolizing enzymes and the underestimation of metabolic disappearance.

[5, 151] Thus the use of a wide range of substrate concentrations would be preferable if both metabolic stability and metabolite formation are studied.

- A preliminary comparison of microsomes and homogenates indicated that differences were not large for most of the compounds, and that for most compounds microsomes were well-suited for both stability and metabolism screening. However, it has to be recognized that microsomes as an enzyme source would not produce most phase II metabolites (e.g. acetaminophen-sulphate). [1]
- A tentative categorical analysis indicated that differences between human and rat preparations were rather modest for most of the compounds. Differences were usually quantitative. Qualitative differences in metabolite profiles were relatively common, about a third of compounds displayed a difference in major metabolite(s) and in about a half of the compounds some minor metabolites were different.

5.2 LC–MS in screening of reactive drug metabolites [III, IV]

5.2.1 Development of screening method [III]

Reactive drug metabolites are difficult to analyze directly by LC–MS methods due to their short lifespan and high reactivity. Reactive metabolites are often electrophiles and thus easily reactive with nucleophiles like glutathione, which is an endogenous scavenger of reactive metabolites in the human body. [35, 41] Traditionally the detection of GSH conjugates is conducted with LC–MS/MS by neutral loss scanning. [63] When coupled with precursor ion scanning the specificity of the method is increased, but it still suffers from the relatively poor sensitivity of the quadrupole mass analyzer when used in scanning mode. [152] GSH is also a quite ineffective trapping agent for "hard" electrophiles, e.g. imines and aldehydes, and is thus not a comprehensive trapping agent for reactive metabolite screening assays *in vitro*.

In study III a rapid and effective screening method was developed for the trapping of reactive metabolites formed in *in vitro* HLM incubations. Twelve structurally different test compounds known to form different kinds of reactive species were studied (e.g. acetaminophen that forms a quinoneimine, clozapine that forms a nitrenium ion, nicotine that forms an iminium ion and pulegone that forms a furan epoxide). [39, 40, 46, 51, 153] Three different trapping agents, with

distinctive trapping capabilities, were used to trap different kinds of reactive species. GSH was used to trap “soft” electrophiles and KCN to trap “hard” electrophiles, thus complementing each other. [41, 63] SCA was used for its known capability to trap aldehydes. [47, 48] Generic conditions were used for chromatographic separation with a highly efficient UPLC system together with very high detection sensitivity of the TOF mass spectrometer. The specificity was further increased by the use of stable isotope-labeled trapping agents, thus the real positives had an easily distinguishable molecular ion doublet in mass spectra. [51–53] The high mass accuracy of TOFMS data provided unambiguous identification of changes in molecular formulae by the formations of reactive metabolite conjugates. Information about other stable phase I metabolism was also provided within the same data. The performance of the developed TOFMS method was compared to the traditional MS/MS method (both NL and PI scanning) using two test compounds for each conjugate type. With QqQMS operated in the positive ion mode the neutral loss scanning of 27/29 Da and 75/78 Da were used for cyanide and semicarbazide conjugates, respectively. Higher detection sensitivity of the TOFMS approach resulted in a higher number of detected SCA conjugates than was observed with the QqQMS, e.g., three SCA conjugates were detected for menthofuran with TOFMS, and only one with QqQMS. Cyanide conjugates were of low abundance in TOFMS data and were not detected at all using QqQMS. Differences in the acquired data using either TOFMS or QqQMS are illustrated in Figure 7 with chromatograms of ethinyl estradiol GSH conjugates. The two top traces (a and b) are negative ion mode PI chromatograms from QqQMS revealing the presence of two conjugates with a distinctive molecular ion doublet from the use of isotope-labeled GSH. The third trace from the top (c) is the MS/MS chromatogram of NL of 129 Da in positive ion mode that shows only one detected conjugate, and the lowest detection sensitivity of the used approaches. Traces (d–j) are extracted ion chromatograms of the ten detected GSH conjugates from the TOFMS data, thus showing superior detection sensitivity of the TOFMS in comparison to the QqQMS.

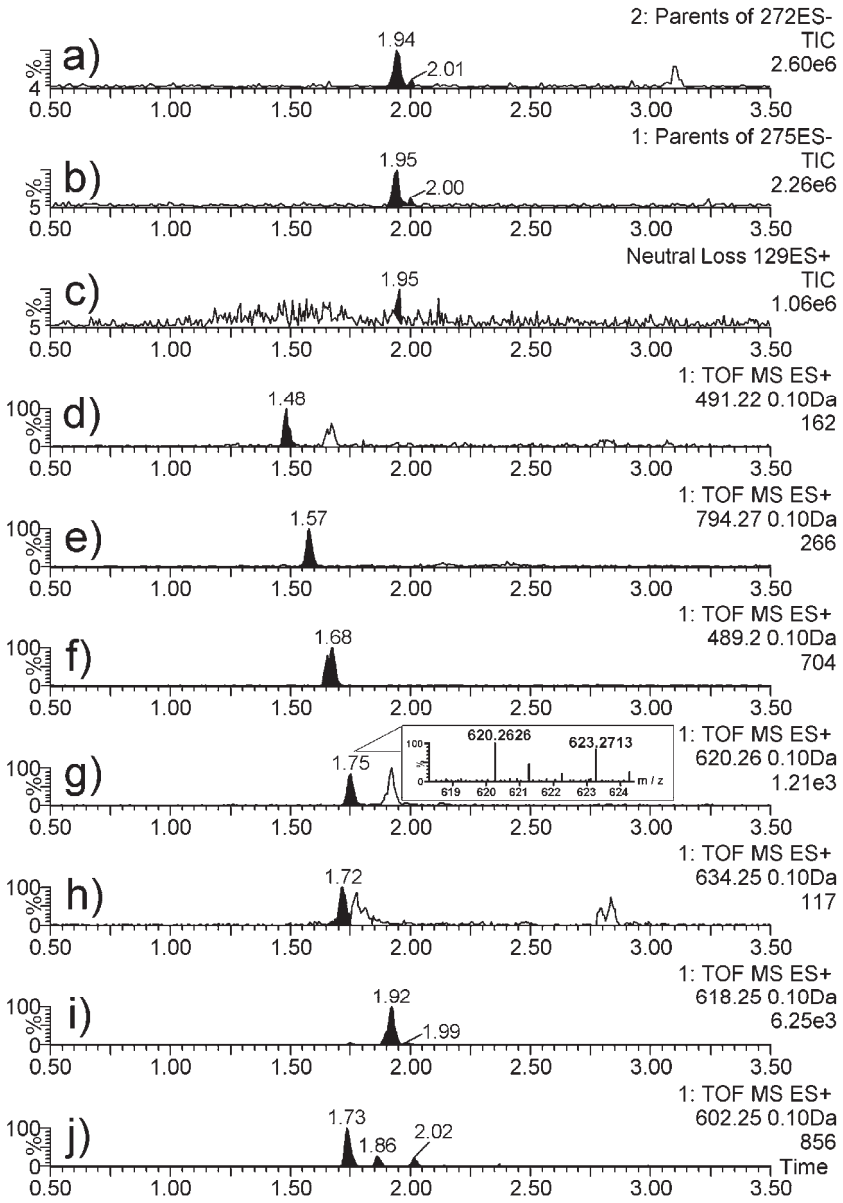

Fig. 7. UPLC–PI–MS/MS chromatograms (a, b), UPLC–NL–MS/MS chromatogram (c), and UPLC–TOFMS ion chromatograms (d–j) for the detected GSH conjugates of ethinyl estradiol. TOFMS spectrum for the molecular ion of the most intense conjugate is shown in the insert. Reproduced from [III] with permission of John Wiley and Sons.

The numbers of detected and tentatively identified reactive metabolite conjugates are listed in Table 6. GSH conjugates were detected for nine compounds, CN conjugates for eight and SCA conjugates for three. All three types of conjugates were detected for menthofuran and ticlopidine. The majority of all trapped reactive species were trapped by GSH, thus being the most applicable trapping agent for this set of compounds. Only cyanide-trapped conjugates were detected for ciprofloxacin, ketoconazole and nicotine, thus suggesting the importance of also using cyanide in addition to GSH as a trapping agent in screening assays. Thirty-four out of the 48 detected GSH conjugates, 14 out of the 25 detected cyanide conjugates and all five detected SCA conjugates were reported, to our knowledge, for the first time in III.

Table 6. The numbers of detected reactive metabolite conjugates with different trapping agents [III].

Test compound	GSH conjugates	CN conjugates	SCA conjugates
Ciprofloxacin	-	5	-
Clozapine	4	6	-
Ketoconazole	-	2	-
Menthofuran	7	1	3
Pulegone	10	-	1
Ticlopidine	6	4	1
1-chloro-2,4-dinitrobenzene	5	-	-
Imipramine	2	3	-
Ethinyl estradiol	10	3	-
Nicotine	-	1	-
Diclofenac	2	-	-
Acetaminophen	2	-	-

5.2.2 Methylated metabonates [IV]

Ciprofloxacin metabolites formed via multiple methylation reactions in a human liver microsomal assay for reactive metabolite trapping with KCN were reported in III. The methylation reaction should not occur metabolically in liver microsomes *in vitro*, thus the origin of these reactions was unclear. A further study was conducted to take a more profound look at the molecular structures of these products by LC–TOFMS and LC–MS/MS.

Amine-containing drugs ciprofloxacin, propranolol and amlodipine were used as test compounds. All of the test compounds formed methylated and cyanide-

trapped products after CYP-mediated reaction steps in incubations with HLM, NADPH and KCN. The methylations most probably originated from a Mannich reaction with formaldehyde although the origin of the formaldehyde involved in the reactions remains unclear. [154] Analogous non-methylated forms of the methylated cyano conjugates were not observed, thus the methylation reaction preceding the cyanide-trapping step was suggested. As the methylation reaction had a role in the formation of the actual reactive and further cyanide-trapped products, the observed methylated cyano conjugates were experimental artifacts, i.e. metabonates, instead of trapped reactive metabolites. [154] This phenomenon should be taken into account when interpreting results from reactive metabolite screening assays with cyanide trapping, as some assays, e.g. isotope-labeled KCN trapping coupled with liquid scintillation detection, cannot distinguish between real cyanide-trapped reactive metabolites and cyanide-trapped metabonates. [155] This in turn stresses that qualitative analysis of the results from reactive metabolite screening assays with cyanide trapping is of great importance to avoiding false conclusions.

The proposed structures of the main metabonates tentatively identified by accurate mass and fragment ion data for the three study compounds in IV are illustrated in Figure 8. It was suggested that reaction pathways to these metabonates each include a methylation step to form an iminium ion out of an imine originating from CYP-mediated reactions of primary or secondary amines in the compounds.

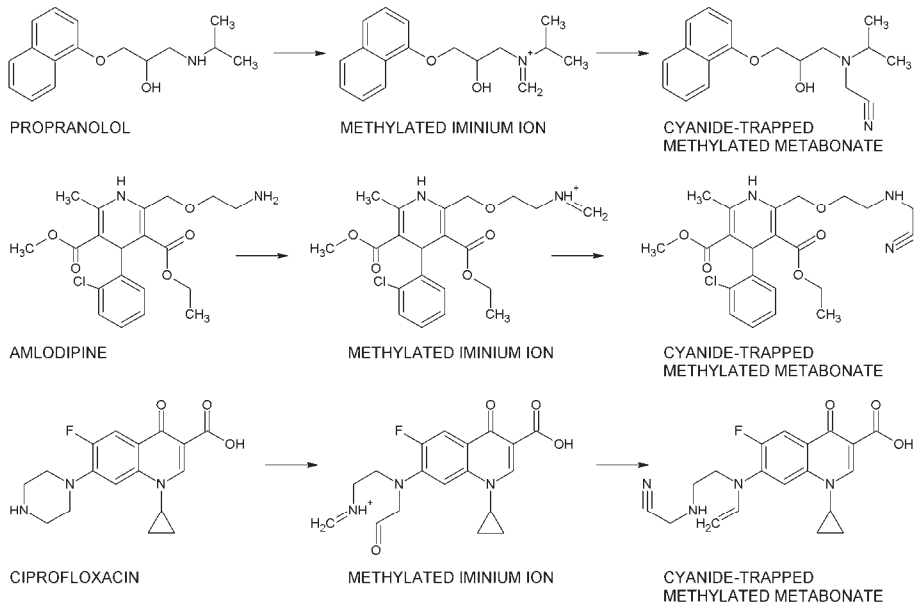

Fig. 8. Proposed structures of methylated intermediate iminium ions and observed and tentatively identified main metabonates from propranolol, amlodipine and ciprofloxacin incubations [IV].

5.3 Comparison of LC–MS instruments for qualitative analysis [V]

Metabolite screening properties of various mass spectrometers widely used in analytical laboratories were compared. A rapid and time-effective "one lab visit only" approach was used, as generally favored in early phases of drug development. [6] Two model compounds, amitriptyline and verapamil which both are known to undergo extensive oxidative metabolism, were studied in human liver microsomal incubations *in vitro* using TOF, QqQ, QqLIT and LTQ-Orbitrap mass spectrometers. The numbers of detected and tentatively identified metabolites with the used MS instruments are shown in Table 7. The main metabolites were detected with all MS approaches, but TOF was the only instrument capable of detecting all of the total observed metabolites showing thus superior performance in comparison to other tested instruments in terms of number of metabolite hits. The test setup with four different LC–MS systems

showed apparent strengths and weaknesses of each instrument type for metabolite profiling purposes. The main aspects are summarized in Table 8.

Table 7. The numbers of amitriptyline and verapamil metabolites detected in HLM incubations with different MS instruments [V].

Compound	Total	TOF	QqQ	QqLIT	LTQ-Orbitrap
Amitriptyline	28	28	10	11	13
Verapamil	69	69	24	21	-*

* No data for verapamil was acquired using LTQ-Orbitrap.

Table 8. The comparison of strengths and weaknesses of different MS approaches in early phase metabolite profiling [V].

Property	TOF	QqQ	QqLIT	LTQ-Orbitrap
Acquisition	Full scan with 2 parallel functions (1 for molecular ions & 1 for fragmentation)	Precursor ion & neutral loss scans	Predicted SRM-ER-EPI & full scan EMS-ER-EPI	Full scan with data-dependent MS/MS for 2 most abundant ions
Detection sensitivity (with the used approach)	High	Low	Moderate	Moderate
Mass resolution	High (12 000 FWHM)	Low	Low	High (15 000 FWHM), low for MS/MS
Cycle time	0.2 sec	0.3 sec	1.19 & 1.71 sec (amitriptyline), 1.44 & 1.80 sec (verapamil)	~ 1 sec
Quantity of fragment ion data	Moderate	High (but with many acquisitions)	High	Moderate (but mainly from matrix constituents)
Quality of fragment ion data	High (accurate masses obtained)	Low (only low resolution data)	Moderate (good for elucidation but low resolution)	Moderate (as only low resolution data was obtained)
Pre-acquisition workload	Low (no prediction needed)	High (setup with parent compound assuming similar fragmentation of metabolites)	High (manual prediction of SRMs). Moderate if automated prediction of SRMs is possible	Low (no prediction needed)
Post-acquisition workload	Low (software aided data mining, high without software)	High (manual data mining)	Moderate (manual data mining)	Low (software aided data mining, high without software)

The TOF data was acquired using two parallel acquisition functions, with one function for molecular ions using low aperture voltage and one function for fragment ions using high aperture voltage, which is somewhat analogous to the MS^E approach with Q-TOF. [6, 156, 157] With QqQ the data was acquired using precursor ion (PI) and neutral loss (NL) scan functions. [126, 158] To optimize both detection sensitivity and data acquisition speed, all PI and NL experiments were run individually, i.e. in total 21 LC–MS runs per sample for amitriptyline and nine LC–MS runs per sample for verapamil. With QqLIT mass spectrometer, approaches using both predicted single reaction monitoring (pSRM) and full scan (EMS) as a survey scan were used. [133, 159] In either case (pSRM or EMS) the survey scan was linked to enhanced resolution (ER) and enhanced product ion (EPI) scans to produce fragment ion data by information dependent acquisition (IDA). High-resolution (15 000 FWHM) full scan was used as a survey scan with LTQ-Orbitrap experiments with parallel data-dependent acquisition of low-resolution MS/MS data for the two most intense ions in each survey scan. [130]

The TOF instrument showed superior performance in detection sensitivity in comparison to other instruments. The detection sensitivities of the scanning functions of the QqQ instrument were low, but the use of MRM mode data acquisition, although applicable only for known or predicted metabolites, would increase the sensitivity drastically. The sensitivities of the ion trap instruments were considered as moderate with the used approaches. With full scan analysis the LTQ-Orbitrap was able to detect less than half of the metabolites that were found with TOF. The same PI and NL scanning functions that were used with QqQ could have been acquired using the QqLIT instrument, which would most probably have resulted in the detection of the same metabolites that were detected with QqQ. A higher number of PI/NL reactions with QqQ, than fragmentation reactions for the prediction of SRM reactions with QqLIT, were used. With this in mind, the detection sensitivity of the QqLIT was considered to be higher than that of the plain triple quadrupole instrument regardless of fewer metabolite hits obtained with QqQ.

With TOF the total cycle time of 200 milliseconds was superior to other instruments tested, and the high data acquisition rate was very compatible with fast UHPLC chromatography. A total cycle time of around one second was chosen for LTQ-Orbitrap measurements, at the expense of obtained MS/MS data. The low data acquisition rate of the LTQ-Orbitrap instrument required the use of longer LC–MS run times with poorer chromatographic resolution, because longer cycle time, required for higher survey scan resolution or very high-resolution

(>15 000) MS/MS, resulted in missing metabolites due to inadequately defined chromatographic peak shapes. The data acquisition rates of the QqLIT instrument in the used experiments were low and resulted in the longest cycle times of the tested mass spectrometers.

In early phases of drug discovery and development where high throughput is often favored and fast turnaround time is crucial, the strength of the TOF approach was emphasized with the obtained accurate mass fragment ion data, even though the amount of fragment ion data was in many cases lower than those from QqQ and QqLIT experiments. The structural elucidation of biotransformations, and their sites as well, could often be confirmed based on the obtained accurate mass fragment ion data. Although, providing only low-resolution data, the triple quadrupole instruments provided a high amount of fragment ion data that was beneficial and ultimately essential for structural elucidation of biotransformation sites in many cases. Fragment ion data was not obtained for minor metabolites with low abundance with the LTQ-Orbitrap, because the MS/MS data for these metabolites was produced mainly by ions from matrix constituents within the survey scan at clearly higher abundances. This apparent disadvantage could have been overcome by preselecting expected metabolites based on their *m/z* values for data-dependent MS/MS when present, even if ions with higher abundance are detected. In later phases of drug discovery, where more accurate structural elucidation has an increased value and the time used per sample is less critical, the strengths of the LTQ-Orbitrap are more evident. Thus, several high-resolution MS/MS experiments per sample or even per metabolite, in contrast to the early screening phase, are justified.

The overall workload both prior and post acquisition was assessed with the need for prediction of metabolites, setup using parent compound, total acquisition time and data processing. The workloads prior to acquisitions of data were lowest with TOF and LTQ-Orbitrap as no predictions were needed, on the contrary to QqQ and QqLIT instruments, where the predictions and setup prior to acquisitions were laborious. This is further emphasized with large sample series, where low-resolution instruments require individual setup for each compound, but the same setup is possibly applicable for all samples using high-resolution instruments. Software-aided data mining was essential with high-resolution instruments, as a very high amount of data (including expected as well as unexpected metabolites) was obtained from a single run. The workload of the mining of metabolites from this data was relatively low when appropriate software was used, although the results still had to be subjected to manual expert

evaluation and confirmation. With low-resolution instruments the interpretation and evaluation of data was performed manually. Thus, the manual mining of QqQ data was very laborious, and the workload of QqLIT lies somewhere in between that of TOF and QqQ experiments.

6 Summary and conclusions

LC–MS methods were developed and applied to drug metabolism studies. Different MS instruments were coupled with either traditional HPLC or modern UHPLC system to obtain good analytical performance.

This study showed that no single LC–MS conditions were suitable for the analysis of a large group of structurally diverse compounds. [I] Optimization of LC–MS conditions for each individual compound clearly leads to more high-quality data. If generic LC–ESI–MS analysis is intended, slightly acidic pH conditions with fast liquid chromatography utilizing sub-2 μm particles is recommended.

The developed LC–MS methods were applicable for measuring both the disappearance of the parent compound and the formation of metabolites, and therefore was shown to be efficient in the primary *in vitro* phase of drug metabolism studies. [II] LC–TOFMS methods were applied for a large group of study compounds and more specific LC–MS/MS methods were applied for selected compounds when needed. Tentative metabolite identification was based on the measured accurate mass high-resolution TOFMS data. A preliminary comparison of microsomes and homogenates indicated that differences were not large for most of the compounds, and that for most compounds microsomes were well-suited for both stability and metabolism screening. However, it has to be recognized that microsomes as an enzyme source would not produce most phase II metabolites. A tentative categorical analysis indicated that differences between human and rat preparations were rather modest, and usually quantitative, for most of the compounds.

Also in this study, an assay was built up for screening reactive drug metabolites utilizing stable isotope-labeled glutathione, potassium cyanide and semicarbazide as trapping agents and UPLC–TOFMS as an analytical tool. [III] The high mass accuracy of TOFMS data provided unambiguous identification of changes in molecular formulae by the formations of reactive metabolite conjugates. Simultaneous detection of all trapped conjugates and stable phase I metabolites, also from the same data, made the developed screening method very effective. Additionally the ability to use the method with different compounds with the same instrument setup increased throughput. Low initial substrate concentrations could be used with the assay, because the TOFMS provided high detection sensitivity. A large number of trapped metabolite conjugates were detected, and most of them were previously unreported.

Three amine-containing drugs, ciprofloxacin, propranolol and amlodipine, were used to characterize cyanide-trapped methylated metabonates, to study the methylation in more detail. [IV] All of the test compounds formed methylated and cyanide-trapped products after CYP-mediated reaction steps in *in vitro* HLM incubations. As the methylation reaction had a role in the formation of the actual reactive, and further cyanide-trapped products, the observed methylated cyano conjugates were experimental artifacts, i.e. metabonates, instead of trapped reactive metabolites. This phenomenon should be taken into account when interpreting results from reactive metabolite screening assays with cyanide trapping. This in turn stresses that qualitative analysis of the results from reactive metabolite screening assays with cyanide trapping is of great importance to avoiding false conclusions.

Four types of mass spectrometers (TOF, QqQ, LTQ-Orbitrap and QqLIT) were coupled with LC and compared for metabolite screening properties, considering both the ability to detect the metabolites and provide structural information, as well as the issues related to time consumption in laboratory and thereafter in data processing. [V] *In vitro* HLM incubations with amitriptyline and verapamil were used as test samples. TOFMS was shown to be the most suitable of the tested instruments for elucidating as comprehensive a metabolite profile as possible with the lowest overall time consumption.

Some further studies could be performed to obtain even more detailed and high-quality data. The test compound set used in I and II could be analyzed with one or two lower initial substrate concentrations in order to avoid enzyme saturation, and thus more liable results of the disappearance of the compounds. If the study was repeated with a more modern LC–MS instrument, the overall quality and quantity of the obtained data would be improved, thus giving more detailed information about the metabolism. The application of additional enzyme sources, e.g. hepatocytes, would provide even more comprehensive information about the metabolism. The separation efficiency of the 55 study compounds in I and II could be improved by using modern fast liquid chromatography (UHPLC) instead of the traditional HPLC that was used with >3 μm particle size columns. Faster methods with better chromatographic peak shapes could most probably be obtained with UHPLC.

Reactive metabolite screening with other enzyme sources would provide more detailed information about the importance of the formed reactive species, and its significance when correlated to an *in vivo* situation. Also the use of novel trapping agents that are able to trap different kinds of reactive species by

themselves would be a good starting point for further studies. The use of additional enzyme sources could also be applied to study the cyanide-trapped metabonates. More comprehensive studies to characterize the origin, importance and function of formaldehyde and further formation of methylated reaction products would be interesting.

By the use of other modern high-resolution MS instruments, e.g. Q-TOFMS, the quality of MS/MS data in V would be drastically improved. This in turn would lead to more detailed metabolite identification without the need for additional experiments as every ion is fragmented and accurate mass is obtained for each fragment ion. The modern Q-TOFMS instruments are also capable of a mass resolution over 40 000 without limitations on data acquisition rate, and thus very rapid LC–MS methods could be developed and applied.

As a result of this study, new analytical methods to study drug metabolism and new information about stable and reactive metabolites were obtained. The detection and characterization of reactive metabolites is remarkably faster and more efficient with the TOFMS approach developed in this study in comparison to traditional scanning methods with the QqQMS.

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