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Chapter 4

Analytical Methods for Quantification of Drug Metabolites in Biological Samples

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1. Introduction

The study of the metabolic fate of drugs is an essential and important part of the drug development process, research of drug metabolism pathways, drug-drug interactions, drug-herb interactions, influence of genetic polymorphisms and other factors that influence the phase I and/or II metabolism of a drug. Different *in vitro* methods, from subcelullar to organ range, and *in vivo* studies are applied for the clarification of drug metabolism. The analysis of metabolites in complex biological matrices is a challenging task therefore several analytical methods for qualification and quantification of drug metabolites are used. Liquid chromatography coupled with mass spectrometry (LC-MS) has become the most powerful analytical tool for screening and identification of drug metabolites in biological matrices. However, adequate sample preparation is a key prerequisite aspect of successful quantitative and qualitative bioanalysis. Different approaches for metabolite quantification in biological samples from direct quantification, indirect quantification through parent drug after metabolite hydrolysis to quantification supported by using response factors between drug and their metabolites are often used. The most frequently used method for quantification is liquid chromatography coupled to different detectors such as mass spectrometer or UV detector. The LC-MS/MS methods are considered as most appropriate for determination of drugs and their metabolites and are also best suited for high throughput analysis. However, in LC-MS/MS assays, matrix effect and selection of suitable internal standards should be adequately addressed.

2. Background of drug metabolism

The study of the metabolic fate of drugs is an essential and important part of the drug development process. During drug evaluation the research of drug metabolism is of high importance especially when metabolites are pharmacologically active or toxic or when a

drug is extensively metabolized [1]. Interindividual differences in drug metabolism also lead to the research of factors that affect drug metabolism [2, 3]. Moreover, a metabolism of toxic substances is also frequently investigated [4].

In early discovery, drug metabolism input provides a basis for choosing chemical structures and lead compounds with desirable drug metabolism and pharmacokinetic (DMPK) or safety profiles [5, 6]. It is the fact that the shift of the rate of drug attrition from 40% in 1990 to 10% in 2000 was due to increased efforts in applying DMPK principles for drug development. Beside traditional drug metabolism research that focuses on absorption, distribution, metabolism and excretion *in vitro* and *in vivo* studies, the knowledge about pharmacogenetics, pharmacogenomics and transporters brought many advances in drug metabolism research [5]. For the feasibility to successfully monitor the drug metabolism, suitable bioanalytical methods have to be developed and validated. Studies of metabolic fate of drugs in living systems may be divided into three areas: 1) elucidation of biotransformation pathways, 2) determination of pharmacokinetics of the parent drug and/or its primary metabolites and 3) identification of chemically-reactive metabolites that are important in drug-induced toxicity [7].

Metabolism is a process of biotransformation when drugs are transformed into a different chemical form by enzymatic reactions. Mainly, metabolism increases drug hydrophilicity and decreases the toxicity and activity of most drugs. On the other hand, the biotransformation reactions could lead to bioactivation of drugs in which case the metabolite is more toxic and/or more active than the parent drug (reactive metabolite formation) [8]. The mechanism of bioactivation of drugs may be classified into following categories: biotransformation to stable but toxic metabolites, biotransformation to electrophiles, biotransformation to free radicals and formation of reactive oxygen metabolites. Additionally, bioactivations are also the transformations of a prodrug, promoiety or bioprecursor prodrug to a more effective metabolite [9]. Prodrug approach is commonly used in order to overcome the poor bioavailability of the active form of the drug. In case when prodrug consists of two pharmacologically active drugs that are coupled together in a single molecule it is called promoiety. Another type of prodrug is a bioprecursor drug which does not contain a carrier or promoiety, but results from a molecular modification of the active agent itself [9].

There are several factors influencing drug metabolism such as genetic, physiologic, pharmacodynamic and environmental factors. CYP2D6, CYP2C19, CYP2C9, CYP3A4, CYP3A5 are enzymes that are responsible for metabolism of many marketed drugs and are also highly polymorphic [10]. Many non-cytochrome P450 drug metabolizing enzymes also play important role in the metabolism of a variety of drugs. Among them polymorphisms of thiopurine methyltransferase (TPMT), butyrylcholinesterase, N-acetyltransferase (NAT) and UDP-glucuronosyltransferase (UGT) influence the metabolism of drugs [11]. Different physiological factors such as age, sex, disease state, pregnancy, exercise, circadian rhythm and starvation lead to the impaired metabolism among subjects and should be taken into consideration when evaluating the drug metabolism. Dose, frequency, route of

administration, tissue distribution and protein binding of the drug affect its metabolism. Moreover, environmental factors such as environmental chemicals, co-administered drugs, tobacco, smoking, alcohol drinking and dietary constituents may change not only the kinetics of enzyme reaction but also the whole pattern of metabolism, thereby altering the bioavailability, pharmacokinetics, pharmacologic activity or the toxicity of the drug [10, 11].

3. Drug metabolic pathways

Drugs are metabolized by different reactions that are classified into two groups: phase I and phase II. Phase I reactions include oxidation, reduction and hydrolysis. The function of phase I reactions is to introduce a new functional group within a molecule, to modify an existing functional group or to expose a functional group that is a substrate for phase II reactions. Phase I reactions are responsible for enhancement of drugs' hydrophilicity and consequently facilitate the excretion. Phase II reactions represent conjugating reactions and mainly further increase the hydrophilicity and facilitate the excretion of metabolites from the body [10]. Enzymes that catalyze phase I reactions include microsomal monooxygenases (cytochrome P450, flavin-dependent monooxygenase) and peroxidases, cytosolic and mitochondrial oxidases, reductases and hydrolytic enzymes. Cytochrome P450 enzymes may catalyze aliphatic hydroxylation, N-, O-, S-dealkylation, oxidative dehalogenation, epoxidation [6]. The participation (%) of hepatic CYP450 isoforms in the metabolism of clinically important drugs is as follows: 3A4/5 (36%), 1A1 (3%), 1A2 (8%), 2B6 (3%), 2C8/9 (17%), 2C18/19 (8%), 2D6 (21%), 2E1 (4%) [10]. Flavin-dependent monooxygenase, a flavoprotein, is a microsomal monooxygenase that is not dependent on cytochrome P450. It is capable of oxidizing nucleophilic nitrogen and sulfur atoms [6, 10]. Other typical phase I oxidation enzymes are monoamineoxidase (MAO), diamineoxidase (DAO), cyclooxygenase (COX), alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), molybdenum hydroxylase (include aldehyde oxidase, xanthine oxidase and xanthine dehydrogenase). In addition to promoting oxidative metabolism, cytochrome P450 enzymes may also catalyze reductive biotransformation reactions for the reduction of azo and nitro compounds to primary amines [10, 12]. Hydrolytic enzymes that consist of non-specific esterases and amidases are also a member of phase I enzymes of metabolism [6, 10].

Phase I reactions may be followed by phase II reactions; however preceding phase I reactions are not a prerequisite. Phase II enzymes are highly capable of polarizing lipophilic drugs through conjugation with a polar substrate that facilitates excretion [13]. Contrary to phase I reactions, phase II reactions demand energy to drive the reaction. Energy is usually consumed to generate a cofactor or an activated intermediate then utilized as co-substrate [6]. Phase II reactions are catalysed by UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), N-acetyltransferases (NAT), glutathione-S-transferases (GST) and methyltranserases [6, 10, 13]. Of the conjugating reactions glucuronidation, which catalyzes the transfer of glucuronic acid to aliphatic and aromatic compounds, is the most important. UGTs are able to form O-, N- and S-

glucuronides and require uridine diphosphate glucuronic acid for glucuronide formation [6, 10]. SULT is the enzyme responsible for the formation of sulfate esters in the presence of co-substrate 3′-phospohoadenosine-5′-phosphosulfate (PAPS). Aromatic amines, hydrazines, sulfonamides and certain aliphatic amines are biotransformed to amides in a reaction catalyzed by N-acetyltransferase and utilize acetyl coencyme A as cofactor [6]. Another important conjugating reaction is a conjugation with glutathione which is present in many cells at high concentrations. Glutathione conjugation captures reactive electrophiles and transforms them to stable, often non-toxic tioethers [6]. Methylation is a process that results in a formation of O-, N- and S-methylated products by the transfer of methyl group from methionine [10].

4. Models for evaluation of drug metabolism

In this chapter different *in vitro* and *in vivo* models for the evaluation of drug metabolism are presented. Advantages and disadvantages of subcellular fractions (microsomes, recombinant enzymes, cytosolic liver fractions, liver S9 fraction), cellular fractions (isolated hepatocytes, immortal cell lines, liver slices, perfused liver), *in vivo* animal and human studies will be presented.

Biotransformation occurs in liver, intestine, kidney, lungs, brain, nasal epithelium and skin. Since liver is the most important organ for drug metabolism [14, 15] the liver-based *in vitro* technologies for evaluation of drug metabolism are presented below. *In vitro* models that range from whole cell system to enzyme preparations are now increasingly applied for quantitative and qualitative assessment in preclinical drug development, post-approval routine checks, identification of metabolic determinant factors and prediction of drug-drug, herb-drug and food-drug interactions [15].

4.1. Recombinant human CYP and UGT enzymes (supersomes, baculosomes)

Recombinant human CYP and UGT enzymes have proven to be a useful tool in *in vitro* biotransformation studies. This *in vitro* model, referred to also as supersomes or baculosomes, is produced by transfection of insect cells with cDNA for human CYP and UGT by baculo virus, namely insect cells lack endogenous CYP and UGT activity. The advantage of this system is that enzyme activity of one single CYP or UGT isoform is expressed and therefore the assessment of individual metabolic enzyme and its contribution to the metabolic pathway could be performed. Additionaly, this *in vitro* system could be used also for the evaluation of drug-drug interactions. Moreover, due to availability of supersomes with different CYP and UGT genotypes, the influence of different polymorphisms on drug biotransformation could be estimated. Currently, all common human CYPs and UGTs co-expressed with NADPH-cytochrome P450 reductase are commercially available. The disadvantage of this *in vitro* model is the latency of glucuronidation because the active site of UGT is shielded behind a hydrophobic barrier. To resolve this problem a pore-forming agents such as alamethicin are used [14-18].

When performing the experiment with supersomes, the experiment with control nontransfected supersomes should be conducted. A NADPH regenerating system (NRS), which consists of β-NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase, or NADPH is required in the incubation for the evaluation of CYP activity and uridine diphospoglucuronic acid (UDPGA) has to be added as a cofactor when evaluating UGT enzyme activity [14-16].

4.2. Human liver microsomes (HLM)

HLM are vesicles of hepatocyte endoplasmic reticulum obtained by differential centrifugation of liver preparations (homogenates) from fresh human liver, liver slices, liver cell lines and primary hepatocytes. This subcellular fraction is a rich source of following enzymes: cytochrome P450s, flavin-monooxigenase (FMO), carboxyl esterases, epoxyde hydrolase and UGTs. Therefore, HLM are most frequently utilized *in vitro* model in drug metabolic profiling and drug interaction studies. Moreover, the influence of specific isoenzymes is studied using liver microsomes in the presence of specific inhibitors. There are interindividual variations in the activity of human liver microsomes; therefore they can be utilized also to study interindividual variability. In case of general estimation of drug metabolism, pooled microsomes from a large bank of individual liver tissues can be used to overcome the influence of interindividual variability. Microsomes from other human organs (intestine, kidney, lung) [19] are also available and are utilized to evaluate extrahepatic metabolism. Additionally, gender-specific microsomes are available for the estimation of gender-based discrepancies in drug biotransformation. In drug discovery process HLM are used for metabolite identification, evaluation of interspecies differences in drug metabolism, prediction of *in vivo* clearance, reaction phenotyping and metabolic pathway identification [14-18, 20].

NADPH or NRS is required in the incubation for the estimation of CYP activity. In order to evaluate the UGT activity UDPGA and alamethicin (pore-forming reagent) are required [14-16].

The advantages of HLM are ease of use, low costs, best-characterized *in vitro* model for estimation of drug biotransformation, easy storage, appropriate for studying of interindividual and population-based variation, long term storage, provide qualitative estimations of *in vitro* drug metabolism, convenient tool for high throughput screening of compounds, appropriate for lead compound optimization studies and drug interaction studies. However, some disadvantages of HLM also exist. HLM are not appropriate for quantitative estimation of drug biotransformation because of absence of enzymes like NAT, GST and SULT and cofactors needed. This limits the expected metabolic competition and formation of some *in vivo* present metabolites. Another drawback is a very difficult assessment of the fraction of drug bound to plasma proteins versus to microsomes which is an important factor in the estimation of *in vivo* biotransformation [14-16, 18].

4.3. Cytosolic fraction

Cytosolic fraction is an *in vitro* model that has not been used very often so far. Like HLM, cytosol is produced by differential centrifugation of liver homogenate. Soluble enzymes of phase II such as NAT, GST, SULT, carboxylesterase, soluble epoxide hydrolase, diamine oxidase, xanthine oxidase and alcohol dehydrogenase are expressed in cytosolic fraction, but only first three are expressed at higher concentration. This *in vitro* model requires cofactors like acetyl coA, dithiothreitol and acetyl coA-regenerating system for NAT, PAPS for SULT, glutathione for GST activity [14-16, 18].

The main advantage is the presence of only aforementioned enzymes at higher concentrations than in liver S9 fraction. The biotransformation by NAT, GST or SULT can be studied separately or in combination depending on the cofactors added. The main disadvantage is the absence of UGT and therefore glucuronidation cannot be studied by this model [14-16, 18].

4.4. S9 fractions

S9 fraction contains both microsomal and cytosolic fractions and consequently expresses a wide range of metabolic enzymes – CYP, FMO, carboxylesterases, epoxide hydrolases, UGT, SULT, methyl transferases, acethyltransferases, GST and others. This *in vitro* model could be employed for metabolic, toxicity and mutagenicity studies. Similar to upper mentioned *in vitro* models the addition of cofactors is needed; NADPH or NRS for CYP, UDPGA for UGT, acetyl coA, dithiothreitol and acetyl coA-regenerating system for NAT, PAPS for SULT and glutathione for GST [14-16, 18, 20].

The main advantage over microsomes and cytosolic fraction is a more complete representation of the metabolic profile due to the presence of phase I and phase II enzymes. However, a disadvantage is the overall lower enzyme activity in the S9 fraction compared to microsomes and cytosol, which may leave some metabolites unnoticed [14-16, 18].

4.5. Cell lines

This *in vitro* model is less popular than other described models due to dedifferentiated cellular characteristics and lack of complete expression of all families of metabolic enzymes. The sources of cell lines are primary tumors of liver parenchyma. Currently available cell lines are Hep G2, Hep 3B, SNU-398, SNU-449, SNU-182, SNU-475, BC2, PLC/PRE/5, C3A, SK-Hep-1 and among them Hep G2 cell line is most frequently used for biotransformation studies. The metabolic activity of liver cell lines is generally low compared to freshly isolated human hepatocytes. Metabolic activity of some metabolic enzymes is even not detected. The problem of low activity could be partly overcome by the pretreatment of cell lines by inducers of various metabolic enzymes. But still the induced activity is below the enzymatic activity in freshly isolated human hepatocytes. Liver cell lines require appropriate culture medium, whose composition significantly influences the metabolic activity. The described *in vitro* model is easy to culture and have stable enzyme

concentration. On the other hand, the absence or low expression of most important phase I and phase II drug metabolizing enzymes limits the application of this *in vitro* model. Moreover, metabolites are not easily detected and it is difficult to investigate individual enzymes due to their low expression level [14-16].

4.6. Transgenic cell lines

Transgenic cell line is a cell line that recombinantly expresses human phase I and/or phase II enzymes. All important human CYPs and UGTs have been expressed in this way to overcome the limitations of liver cell lines. Cell lines may be transfected at high efficiency using protoplast fusion. The main advantages are the ease of culturing, high expression of CYP and UGT isoenzymes, possibility to study single enzyme reactions and the influence of one isoenzyme or a combination of a number of isoenzymes. This *in vitro* model can also be used in the study of metabolite structures, pharmacological elucidation and to assess drug-drug interactions. The main drawback is that only one or a few of isoenzymes are expressed, therefore the complete *in vivo* situation cannot be reflected. Moreover, transgenic cell lines are more expensive than other enzyme-based technologies [14-16, 18].

4.7. Hepatocytes

Hepatocytes are well-established, well-characterized and frequently used *in vitro* model in drug biotransformation research. This *in vitro* model could be employed for the evaluation of metabolic stability, metabolite profiling and identification, drug efficacy, hepatic proliferation, hepatotoxicity and drug-drug interactions. Phase I and phase II drug metabolism pathways can be studied by the use of primary hepatocytes and cultured hepatocytes. Like with microsomes interindividual variation can be observed with hepatocytes. This can be overcome by using mixture of hepatocytes from different donors. Cryopreservation of hepatocytes offers many advances in the experimentation, namely activity of most phase I and phase II enzymes is retained.

Primary hepatocytes are obtained by collagenase perfusion of whole liver or a part of liver. This *in vitro* system has strong resemblance of *in vivo* situation due to heterogeneity of enzyme expression in human liver and preservation of drug metabolizing enzymes at *in vivo* levels. Another advantage of primary hepatocytes is the ease of use and high throughput. The important disadvantage is the drop of hepatocytes viability during incubation period (viable 2-4 hrs). Moreover, lack of liver non-hepatocyte cells which may be necessary for cofactor supply, lack of cell polarity, cell-cell and cell-matrix contacts limits the *in vivo* resemblance [14-18, 20].

After isolation, hepatocytes can be cultured in a monolayer in order to prolong the viability to 4 weeks. This characteristic in combination with the prolonged regulatory pathways allows the use of this *in vitro* model in studies of up-regulation or down-regulation of metabolic enzymes. However, cultured hepatocytes gradually lose viability and liver specific function. Many factors influence the morphology and functions of hepatocytes in

culture: medium formulation, extracellular matrix, initial cell suspension and density, drug concentrations. Hepatocytes could also be cultured in a sandwich configuration where hepatocytes are placed between two layers of gelled extracellular matrix. This type of culture retains liver hepatocyte specific functions for a longer period [18, 20].

4.8. Liver slices

Liver slices and hepatocytes are the most physiologically relevant *in vitro* techniques used for quantitative and qualitative measurement of hepatic phase I and phase II metabolism of drugs due to full complement of enzymes and cofactors. High-precision tissue slicers (e.g. Krumideck slicer, Brenden-Vitron slicer) are used for the production of liver slices of uniform thickness (less than 250 μm). The advantage of liver slices over hepatocytes lies in the intact structure of liver tissue containing hepatic and non-hepatic cells, normal spatial arrangement and possibility of morphological studies. The described *in vitro* model allows higher throughput compared to isolated perfused liver. Another advantage is the non-requirement for digestive enzymes and consequently the preservation of intact tissue structure. Moreover, no addition of cofactors is needed for enzyme activity. However, some disadvantages of this model are known: decrease of CYP activity in short time due to impaired diffusion of nutrients and oxygen in the liver slice, damaged cells on the outer sides of the slice, inadequate tissue penetration of the test medium, short viability period (5 days), lack of optimal cryopreservation procedures and a need for expensive equipment [14-16, 18, 20].

4.9. Isolated perfused liver

Isolated perfused liver gives an excellent representation of the *in vivo* situation but it is not used frequently due to practical inconveniences. Normally animal liver tissue on a small scale is used, but never human liver tissue. The additional advantages of this *in vitro* model are also three-dimensional architecture, presence of hepatic and non-hepatic cell types, possibility to collect bile. The important disadvantages of this model are: poor reproducibility, functional integrity limited to 3 hours, difficult handling, poor perfusion of cells by nutrients and oxygen, low throughput and no availability of human liver. This model is useful only in case when bile secretion is the subject of research [14-16].

4.10. Animal and human *in vivo* studies

The identity of metabolites present in any matrix of animal or human provides essential information about the biotransformation pathways involved in the clearance of a drug. When the metabolite profiling of a parent drug is similar qualitatively and quantitatively between animal and human, we can assume that potential clinical risks of parent drug and metabolite have been adequately investigated during nonclinical studies. When a difference arises between *in vitro* and *in vivo* findings, the *in vivo* results should always take precedence over *in vitro* studies [21]. The FDA guidance encourages the identification of differences in

drug metabolism between animals and humans as early as possible during the drug development process in order to find unique human metabolites and major metabolites [1, 21]. FDA defines that metabolites will need to undergo additional safety evaluation when steady-state systemic exposure to metabolite in humans exceeds 10% of parent drug exposure (disproportionate metabolite) [1].

The results of aforementioned *in vitro* studies can be correlated to *in vivo* situation and vice versa. This multidisciplinary approach of translational medicine yields an insight into complex mechanisms of drug disposition. The principle of translational medicine is presented on raloxifene, a selective estrogen receptor modulator, which exhibits quite large and unexplained interindividual variability in pharmacokinetics and pharmacodynamics [2, 3, 19, 22]. The gained knowledge about drug pharmacokinetics and pharmacodynamics insures a safer and more effective treatment strategy in the clinical setting.

5. Qualitative evaluation of metabolites

The known identity of metabolites is the prerequisite for a suitable metabolic assessment of drugs. Liquid chromatography coupled with mass spectrometry has become the most powerful analytical tool for screening and identification of drug metabolites in biological matrices. A short overview of analytical strategies for identification of metabolites will be provided. More information regarding metabolite identification can be found in following review articles [7, 23-27]. The selection of suitable LC-MS instrumentation is needed for qualitative evaluation of metabolites. Moreover, this issue is also important for quantitative evaluation of metabolites as discussed in section 8. Additionally, some examples for metabolite identification using LC-MS/MS will be provided in this section.

5.1. LC-MS instrumentation

5.1.1. Ionization techniques

A LC-MS ion source has the double role of eliminating the solvent from the LC eluent and producing gas-phase ions from the analyte. The application of atmospheric pressure ionization (API) methods has provided a breakthrough for the LC-MS systems and has brought it to the forefront of analytical techniques. Some ion sources such as API operate at atmospheric pressure where others like electron impact (EI) or chemical ionization (CI) operate in vacuum. While soft API interfaces, in particular electrospray, produce molecular ions with minimal fragmentation, high energy sources like EI mostly generate fragment ions. API techniques are most widely used for metabolite detection, identification and quantification [7, 28] due to the ability to operate at atmospheric pressure, good compatibility with reversed phase chromatography and generation of intact molecule ions at very high sensitivity. All three API techniques: electrospay ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) are complementary.

- **Electrospay ionization** is by far the preferred method for metabolite identification and quantification. It is the most universal technique for introducing the molecules into the gas phase and it is most gentle and therefore likely to yield an intact molecular ions. ESI is ideally suited for polar, ionic and thermally labile compounds such as drug metabolites; in particular glucuronides and others phase II metabolites. This technique requires ionization of analytes within solution prior to introduction into ion source and thus works best for fairy basic or acidic compounds. Depending on the voltage polarity, nebulised droplets trapping the ionized analyte will be positively or negatively charged. The reduction in size caused by solvent evaporation accounts for the increase in charge density in the droplet leading to its explosion when repulsive forces between charges exceed the cohesive forces of the droplet. This process occurs repeatedly until gas phase ions are produced [29]. Ions in solution are emitted into gas phase without application of heat making ESI suitable for analysis of thermo labile compounds. Many parameters, such as analyte and solution characteristics: pKa, analyte concentration, other electrolytes in solution, dielectric constant of the solvent, affect the ion formation process [7]. The effects of several mobile phase additives on the ionization efficiency have been reviewed [30] and will be discussed later (section 8). Depending on the chemical structure of an analyte, multiple-charged molecular ions can be formed, which is optimal for the analysis of biological macromolecules (e.g. proteins). Despite the numerous benefits of ESI, it suffers from a shortcoming in that it is susceptible to ion suppression effects from high concentrations of buffer, salts and other endogenous compounds in matrix solutions [23].
- **Atmospheric pressure chemical ionization** is more suited for less polar compounds. Certain classes of compound such as heavily halogenated analoges and highly aromatic compounds will run readily on APCI while giving no or a weak response on ESI [7]. APCI like ESI produces ions based on the API strategy, but thought a completely different process. Here, the liquid eluent is sprayed into heated chamber [450-550°C) where the high temperature of a nebulizer gas flow causes the immediate evaporation of the solvent and the analyte. In addition to volatility at the applied temperature, thermal stability of the analyte is also a prerequisite for the successful application of APCI (e.g. glucuronides may break down and appear in the form of protonated aglycone [31]. Ionization of analytes takes place in gas phase where due to high flux of electrons from corona discharge needle, solvent molecules initially react with electrons and form ions that produce protonated solvent ions through secondary reactions. These protonated solvent ions then transfer a proton to form protonated analytes. For efficient ionization, the employed mobile phase should be volatile and also amenable to gas phase acid-base reactions. APCI technique is less prone to ion suppression and provides a wider dynamic detection range than ESI due to ionization that occurs mainly in gas phase. Also, typically higher flow rate is used with APCI [1-2 mL/min) then that in conventional ESI (0.1-0.5 mL/min) [23].

- **Atmospheric pressure photoionization** is relatively new ionization method. This technique can be used for ionization of analytes that are not easily ionizable by ESI and APCI. APPI has similar application range as APCI but slightly extended toward nonpolar compounds [32]. The APPI ion source is very similar to APCI source, except the APCI corona discharge needle is replaced by photoionzation lamp. Depending on the analyte proton affinity relative to the composition of the mobile phase, either a radical molecular ion (typically for nonpolar compounds) or a protonated molecular ion (typically for polar compound) is obtained. APPI has a potencial in the analysis of drug metabolites but more research is needed to fully understand the important parameters and factors that affect the ionization efficiency [33].

5.1.2. Mass analyzers

The function of mass analyzer is the separation of ions formed in ionization source according to their different mass-to-charge (m/z) ratios. The quality of mass separation is characterized by the degree to which close m/z values can be separated in the mass analyzer. Mass analyzers are classified regarding resolution into low and high resolution instruments. The later ones are associated with another important parameter, mass accuracy, which allows determination of elemental formula of particular analyte. The selection of suitable analyzer is driven by the purpose of the analysis and the instrument performance but also depends on the instrument availability and cost effectiveness.

- **Triple quadrupole instruments (QQQ)** are the most common mass spectrometers in analytical laboratories, having most often been acquired for their evident strengths in high sensitivity quantitative analysis of known analytes. These instruments have been often applied also for metabolite identification due to wide availability and excellent tandem mass (MS/MS) properties. In QQQ, the first quadrupole filters ions of interest, the second quadrupole also called collision cell fragments these ions and further the fragment ions are filtered by third quadrupole before reaching the mass detector. Such QQQ configuration allows performing different scans such as full scan, product ion scan, precursor ion scan (PI), constant neutral loss scan (CNL), single ion monitoring (SIM) and selected reaction monitoring (SRM) or multiple reaction monitoring (MRM). PI and CNL are particularly useful in metabolite identification since both scanning modes do not require previous knowledge about the molecular weight of metabolites. High sensitivity for quantitative purposes is retained only when working in MRM mode, however, the detection sensitivity decreases dramatically when wide mass range is analyzed in a scanning mode. This is one of the major disadvantages of using QQQ for the screening of drug metabolites.
- **Ion trap instruments (IT)** are like QQQ relatively inexpensive and compatible with wide range of ionization interfaces. These analyzers utilize ion trap chamber where ions are trapped and then selectively ejected from the chamber. Additionally, the resonance excitation applied in the trap provides efficient dissociation of the precursor ions to product ions. IT provides more sensitivity for structural elucidation than QQQ due to

its better sensitivity in full scan mode and efficient dissociation of the precursor ions which allows multiple stages mass spectrometry (MS^n). Recently, to address classical ion traps (called also 3D IT) shortcomings of insufficient ion storage efficiency, capacity and deterioration of the mass spectrum and dynamic response range, linear IT has been developed [25]. The detection sensitivity in linear IT is at least two orders of magnitude higher than that in 3D IT. Because of these advantages, linear IT will probably in near future totally replace old 3D IT [23].

- **Triple quadrupole-linear ion traps (QTrap)** combine sensitive QQQ technology with high capacity of linear IT incorporating high trapping efficiencies. In this instrument, the last quadrupole of QQQ is replaced with a linear ion trap, which operates as a mass resolving quadrupole or a linear ion trap. This provides clearly increased metabolite screening capabilities compared to traditional IT or QQQ. QTrap enables high sensitivity, wide range mass scanning and MS^n together with QQQ capabilities, such as PI, CNL and very high sensitive MRM data acquisition.
- **Time of flight (TOF)** analyzers are the most suitable high resolution mass spectrometers for fast and cost-efficient metabolite identification. TOF are relatively simple and capable of recording all formed ions on a microsecond time scale offering high sensitivity detection. Ions are accelerated from the ion interface to a fixed kinetic energy and then pass through a field-free tube to the detector. The time needed for ion to reach the detector is proportional to its m/z ratio. TOF strength lies in its very high detection sensitivity when acquiring wide range data, enabling the simultaneous detection of data for all metabolites of interest in one run. High mass resolution and mass accuracy (< 3-5ppm) enable reliable and accurate identification of metabolites by determination of elemental formula of a metabolite. Additionally, the very high acquisition speed makes them ideal for fast chromatography [24].
- **Triple quadrupole-time of flight (Q-TOF)** instruments combine first mass filter and collision cell of QQQ with TOF as the second mass analyzer. These instruments can operate as true tandem MS while providing accurate mass of the product ions. Most modern Q-TOFs have good linear response and are therefore also suitable for quantitative purposes. However, TOF instruments have not the ability to perform positive/negative switching in one run [24].
- **Orbitrap** is another high resolution analyzer which is a hybrid composed of a linear IT and Fourier transform mass spectrometer. It is an effective alternative to the TOF instruments used for metabolite profiling. Orbitrap is capable of high sensitivity screening over wide mass range, MS^n and tandem mass spectrometry with accurate mass data for both parent and fragment ion. However, it is not suitable for fast chromatography because it suffers from a slow data acquisition [24].
- **Fourier transform-ion cyclotron resonance (FT-ICR)** is the third high resolution mass analyzer. The high sensitivity, accurate mass measurements, high mass resolution and MS/MS capabilities of FT-ICR make it attractive for structural determination of ions. However, the combined requirement of ultra-high vacuum system, superconducting magnets as well as sophisticated data system place the cost of these instruments beyond the means of most laboratories involved in drug metabolism studies [7].

5.2. Strategies for metabolite identification

MS methodology is the most suitable approach for metabolite identification as commonly low concentrations of drug metabolites are present in complex biological matrices. Appropriate LC-MS instrumentation is clearly critical to both, detection and structural elucidation, although alternative non-MS approaches may also be important in cases when MS data alone are not sufficient. Tandem mass spectrometry instruments are beside their key role for metabolite quantification also well suited for qualitative purposes. Tandem mass spectrometry experiments, which allow different scan mode possibilities, are by far most informational techniques for structural characterization of metabolites [23]. But these experiments require a set of injections to perform full scan and other scan analyses to identify metabolites of interest. The drive to more versatile and powerful instruments which can perform intelligent data dependent experiments has led to newer mass analyzers, such as high resolution Q-TOF instruments, which now dominate the metabolite identification field.

5.2.1. Full scan

The non-selective nature of full mass scan acquisition enables detection of practically all ionizable metabolites and giving most complete information in terms of metabolite molecular mass. However, two major disadvantages arise by this approach. Firstly, detection sensitivity using QQQ decreases dramatically when wide mass range is scanned. This obstacle can be overcome by using IT analyzers as its full scan is much more sensitive or even better by using TOF instruments which additionally enable accurate mass determination [23]. In case when only QQQ is available, a practical approach may be applied to improve sensitivity; the whole mass range should be divided to narrow scanning ranges by performing multiple analyses of the same sample. Secondly, other non-metabolite matrix compound may interfere with obtained MS data. A common procedure for metabolite detection involves analysis of test and control samples what then allows subtraction of control sample data. This approach is less successful when complex biological samples, such as plasma and urine, are examined. Expected metabolites in studied samples may be predicted based on biotransformation pathways of parent drugs what enables focused search of these compounds. The most common changes in mass caused by biotransformation are shown in Table 1.

5.2.2. Precursor ion and constant neutral loss scan

PI and CNL are more specific approaches for identification of unknown metabolites. This scan mode is only possible for tandem mass spectrometers and therefore suffers at sensitivity like other QQQ scanning acquisitions. In PI scan mode, the second quadrupole mass filter is set to pass only the selected product ions, while the first quadrupole mass filter scans a range of m/z values. In CNL scan mode, both quadrupoles are scanning m/z values while the m/z difference between the quadrupoles is kept constant. Several phase II metabolites at fragmentation lose a distinct neutral group that can be used for specific

identification of these conjugates. Glucuronides, sulfates and glutathione conjugates are often detected by CNL of m/z 176, 80 and 129, respectively. Typical PI for some drug conjugates in negative ionization mode like aliphatic sulfates, sulfonates and phosphates are m/z 97, 81 and 79, respectively [28]. Although PI and CNL provide high selectivity for identification of metabolites, the methods are based on predicted fragmentation behavior of metabolites what depends to some extent also on abilities of the analyst. Therefore, metabolites with unexpected fragmentation can be missed. Nevertheless, in combination with full scan data, PI and CNL is a powerful tool for metabolite identification.

Biotransformation	Change in molecular formula	Change in mass (Da)
Dehydration	- H_2O	-18
Demethylation	- CH_2	-14
Dehydrogenation	- H_2	-2
Hydrogenation	+ H_2	+2
Methylation	+ CH_2	+14
Hydroxylation	+ O	+16
Epoxidation	+ O	+16
S/N-oxidation	+ O	+16
Hydration	+ H_2O	+18
Dihydroxylation	+ O_2	+32
Acetylation	+C_2H_2O	+42
Sulfation	+SO_3	+80
Glucuronidation	+$C_6H_8O_6$	+176
Glutathione conjugation	+$C_{10}H_{15}O_6N_3S$	+305

Table 1. The nominal mass changes in biotransformation of drugs by common metabolic reactions [28, 34]

5.2.3. Product ion scan

Product ion scan is used for structural characterization of the detected metabolites. In product ion mode, a precursor ion (metabolite) is selected in first quadrupole, fragmented in collision cell and the product ions are then scanned in second quadrupole. Structural information is obtained by interpretation of the fragmentation patterns for both metabolite and parent drug. Complete structural characterization of metabolites may be hindered by the absence of useful product ions in tandem mass spectrometry. To obtain more specific structural data, the use of multistage (MS^n) scan by using ion trap instruments can be provided. The selected product ion can be selectively isolated and further fragmented, narrowing the potential sites of modification and providing a more complete assessment of the metabolite structure.

5.2.4. Multiple reaction monitoring

Although the use of PI and CNL data acquisition improves the selectivity of metabolite detection when comparing with full scan acquisition, all three approaches have reduced sensitivity. For this reason, specific MRM screening may serve as alternative approach for metabolite detection. MRM is the most appropriate acquisition method for quantification of analytes. In this mode, the first quadrupole is set to pass only the selected precursor ion that is fragmented in collision cell and usually the most abundant fragment (product ion) is then filtered in a second quadrupole. Monitoring of specific transition for each analyte yields a superior signal-to-noise ratio with significantly higher selectivity. Utilizing metabolism prediction and knowledge of the tandem mass fragmentation of the parent drug, the approach gives a significant increase in sensitivity and enables a wide range of potential MRM transitions to be targeted. Although the possibility to overlook metabolites remains the targeting MRM is a powerful alternative for metabolite detection when sensitivity is an issue. Single ion monitoring is is another option to overcome low sensitivity of QQQ screening techniques. SIM is less specific and sensitive acquisition compared to MRM but may provide advantages when the potential metabolite fragmentation pattern cannot be predicted correctly. In this case a multiple SIM transitions of the predicted metabolites are performed, which are set accordingly to the expected nominal mass changes regarding to parent drug (Table 1).

5.2.5. High resolution mass spectrometry

The most widespread analyzer providing high mass accuracy (TOF, Orbitrap, FT-ICR) used in metabolite identification is TOF instrument. The specificity in the detection of metabolites with high resolution is significantly higher than that with unit resolution QQQ or IT instruments where the ion chromatograms can be recorded using a 0.1 mass unit window. The high selectivity provides also better sensitivity for the detection of metabolites. It was reported that detection limits for several drugs were 5-25 times better with accurate mass TOF, than with nominal mass TOF (same unit level than at QQQ) [28]. Accurate mass measurements enable to determine the elemental formula of metabolites. Moreover, exact mass shift enables the establishment of the change in molecular formula of the parent drug. For example, metabolites formed by hydroxylation and dehydrogenation (at same time) are, in this way separated from those formed via methylation, in spite that both reaction increase the molecular weight by 14 (Table 1) [24]. The benefit of reliable accurate mass measurements for structural elucidation of unknown metabolites is therefore extremely high. However, metabolites with the same exact mass cannot be distinguished by analyzers. In this case other approaches are needed. Ion mobility time-of-flight mass spectrometry (IM-MS), which separates ions on the basis of their m/z ratios as well as their interactions with a buffer gas, is very convenient. The main advantage of IM-MS is the potential for separation of metabolite isomers without chromatographic separation which makes it a powerful analytical tool for investigation of complex samples [35].

Q-TOFs are the key high resolution instruments for drug metabolism research. Q-TOF instruments provide sufficient mass resolution (up to 40,000) and accurate mass measurements (below 1 ppm). In addition, they can operate at relatively high scanning rates, which are considered as the main drawback of most of the Orbitrap based instruments. On the other hand, Orbitrap analyzers provide a resolving power of up to 100,000 with mass accuracy below 1 ppm. FT-ICR analyzers provide ultrahigh mass resolving power greater than 200,000 but high purchasing and maintenance cost are beyond financial capabilities of most routine laboratories [27].

5.2.6. Other approaches

Other approaches can be applied to provide specific structural information in cases when MS data are not sufficient to determine metabolite structure. Hydrogen/deuterium exchange LC-MS allows studying mechanisms of MS fragment ion formation and metabolic pathways of drugs, as well as differentiate the structures of isomeric metabolites [7]. Metabolites can be isolated and purified from the incubations, followed by structural analysis by NMR. Alternatively, LC-NMR analysis can be performed on biological samples with minimal sample processing but certain limitations occur with this technique, such as lower sensitivity compared with LC-MS and the requirements of relatively expensive deuterated buffers in mobile phase. More recently, LC-NMR has been coupled with MS which enables simultaneous metabolite structure elucidation [25]. Tentatively identified structure of metabolites may also be synthesized and LC-MS data for these compounds are compared with data from the actual metabolites.

5.3. Examples of metabolite identification

Tandem mass spectrometry is well suited for identification of phase II metabolites [36]. As example for this approach, the elucidation of three raloxifene glucuronides in urine as well as their identity confirmation after bioproduction by using QQQ is provided [37]. Chromatograms of each bioproduced glucuronide standard obtained in ESI positive full scan mode gave only one chromatographic peak where MS spectra of each peak showed strong molecular ions at m/z 650, 650 and 826 for two raloxifene monoglucuronides and diglucuronide, respectively. Nominal mass shift of 176, 176 and 2 x 176 Da compared to parent drug (m/z 474) is characteristic for the structure of monoglucuronide and diglucuronide metabolites (Table 1). Product ion scan showed the same mass spectra for both predicted monoglucuronides: fragmentation of the parent ion m/z 650 to 474 and 112. Product ion spectra confirmed also diglucuronide structure by two subsequent m/z 176 neutral losses from the parent molecular ion (m/z 826), giving fragments of monoglucuronide (m/z 650) and of parent raloxifene (m/z 474) as well as additional m/z 112 fragment of raloxifene (N-ethyl-piperidine). Additionally, constant neutral loss scan (m/z 176) and precursor ion scans (m/z 112 and 474) in urine sample have been performed. The analysis in all three cases gave three distinct peaks in chromatograms at retention times for the diglucuronide, and both monoglucuronides (data not shown, but same retention times as in Figure 1) confirming again the structure of metabolites.

Another important point had to be considered, good chromatography was needed in order to separate both monoglucuronides since they cannot be distinguished based on MS. Representative LC-MS/MS chromatogram, using MRM acquisitions for quantitative purposes of raloxifene and its three metabolites in urine sample is shown in Figure 1.

Identification of bisphenol A glucuronide and deuterated bisphenol A glucuronide in microsomal incubations [4] is another example. Twin peaks of metabolites with known mass difference [14] (Da in this case) are helpful for studying fragmentation paths. Product ion scan in ESI negative ionization mode for bisphenol A glucuronide (m/z 403) showed fragments m/z 227 (bisphenol A), 212 (bisphenol A fragment - loss of CH_3) and 113. The molecular ion of deuterated bisphenol A glucuronide fragments from m/z 417 to m/z 241 (deuterated bisphenol A), 223 (fragment - additional loss of CD_3) and 113. Fragment m/z 113, which is present in both cases represent a glucuronic acid fragment in negative ionization with subsequent loss of H_2O and CO_2.

In case of reactive metabolite studies there are typical approaches to identify glutathione conjugates: increased mass shift 305 Da according to the parent, constant neutral loss of pyroglutamic acid (m/z 129) in the positive ionization mode and/or precursor ion of m/z 272 in the negative ionization mode [34, 38]. Recently, an *in vitro* bioactivation study using these identification approaches has confirmed that bazedoxifene does not show the formation of glutathione conjugates compared to raloxifene what offers an improved safety profile of this third generation drug relative to other available SERMs [39].

The glucuronide metabolites may be also simply verified by using ß-glucuronidase which provides the conversion of the glucuronide to its aglycone (see next section). If the conversion is complete, this approach is valid for determination of the metabolite stock solution concentrations when small amounts of glucuronide standards are obtained or available [37].

However, for more demanding application QQQ is usually not satisfactory. Identification of phase I and phase II metabolites of two antineoplastic agents was demonstrated by use of Q-TOF [40]. In this study, 32 metabolites for dimefluron and 28 metabolites for benfluron were detected in the rat urine within 25 min chromatographic run. The identification of individual biotransformation was performed using high mass accuracy measurements for both full scan and tandem mass spectra by extracted ion chromatograms for expected masses of metabolites together with the information about characteristic neutral loss. Another study compared QQQ, linear IT (QTrap), TOF and Orbitrap instruments for identification of microsomal metabolites of verapamil and amitriptyline [41]. Only TOF found all 28 amitriptyline and 69 verapamil metabolites; both expected and unexpected. The TOF offered sensitivity and high mass resolution and also lowest overall time consumption together with the Orbitrap. Orbitrap also showed good mass resolution but was less sensitive, resulting in some metabolites not being observed. Approaches with QQQ and Q-Trap provided the highest amount of fragment ion data for structural elucidation, but being unable to produce very high important accurate mass data, they suffered from many false negatives and especially with the QQQ from very high overall time consumption.

6. Approaches for metabolite quantification

The demand for analyses of low-level drugs in complex biological samples has increased significantly in last years. New pharmaceuticals have typically high potency, so small doses are given and therefore the detection limits of these drugs and their metabolites are of great importance. Selective and sensitive analytical methods for the quantitative evaluation of these analytes are critical for the successful conduction of pharmacological studies. Metabolite quantification is always required when the metabolite is toxic or pharmacologically active or when the concentration of metabolite reaches or exceeds the parent drug concentration in plasma. Different approaches for metabolite determination in biological samples have been used which can be generally divided to direct quantification, indirect quantification through parent drug after metabolite hydrolysis or quantification supported by using response factors between drug and their metabolites. The key role in the selection of the particular approach is driven by the availability of suitable authentic standards. Hence some examples of metabolites production will be also shown here.

6.1. Direct quantification

Direct quantification is the most appropriate approach for metabolite determination in biological matrices but two major points need to be considered. Firstly, in general metabolites are much more hydrophilic than parent drug, especially glucuronides [34]. That fact has represented a hindrance for direct metabolite determination because chromatographic separation between these polar analytes and interfering matrix components could not be achieved in many cases. However, this problem has been overcome by advent of powerful liquid chromatography-tandem mass spectrometry instruments which allow direct quantification of these metabolites [42]. LC-MS/MS nowadays play predominant role in bioassays for pharmacokinetic and metabolism studies due to its inherent specificity, sensitivity and speed. Secondly, appropriate authentic standards are needed for reliable and accurate quantification in biological samples. Proper validation of analytical methods includes preparation of calibration and control samples in given biological matrices using suitable reference standards. Authentic metabolite standards are often not commercially available, particularly in the case of new drugs or drugs of abuse. Moreover, available metabolites may be very expensive and therefore not accessible for every research group, especially not in academic sphere. Furthermore, stable isotope labeled standards of metabolites, which are most convenient internal standards for LC-MS/MS analyses, are even less available and/or more expensive than unlabeled metabolites.

In such situation question may arise why not quantitate metabolites concentration based on parent drug calibration curve as this standard are freely accessible. Modified structure of metabolites may change the response to quite diverse extent among various liquid chromatography detection systems. Mass spectrometry using atmospheric pressure ionization sources is very prone to this issue as the intensity of the MS signal strongly

depends on the analyte even at small structural changes. It has been reported that the response in ESI-MS differed by factor 25 for two oxidative isomeric metabolites with same chemical formula [43] or that no signal in contrary to parent drug has been observed for metabolite in positive ionization ESI. Detection of metabolite was in this case possible only in negative ionization mode [26]. However, with the commonly and easily used UV detection, the metabolites have often the same chromophore as the parent drug (but not always [40], hence giving similar response. But the main limitation of this technique in pharmacokinetic studies lies in not sufficient sensitivity and also in lower selectivity as some compounds does not have UV absorption at a wavelength to distinct it from the background. In contrast to UV, fluorescence and electrochemical detection can be very selective and sensitive. For electrochemical detection the response may also be very dependent on structure, especially for phase I metabolites which usually possess changed oxido-reductive properties compared to parent drug [34]. Beside that both detector systems are very specific what makes them of limited applicability.

Direct quantification can also be performed without suitable standards. For that purpose detectors need to give an equimolar response for all compounds of interest. Additionally, such detectors should be highly sensitive with wide dynamic range, robust and easy to use, compatible with reverse-phase gradient elution and not prone to matrix interferences, namely give a response independent of compound [44]. Although there are sophisticated detectors available, few are used routinely for metabolite quantification. Beside radioactivity detector (RAD) which also require suitable standards (radiolabeled compounds) other compound response independent detectors has been recently discussed elsewhere [25, 44-46]. Such approach has become even more important for metabolite evaluation in the light of recently introduced FDA guidelines on metabolites in safety testing, which recommends that all metabolites greater than 10 percent of parent drug should be examined [1]. Some further examples of metabolite quantification using accelerator MS [47], inductively coupled plasma MS [43], chemiluminescene nitrogen detector [48], quantitative NMR [49] and evaporative light-scattering detector [50] are given.

6.2. Quantification using response factor

Prerequisite to make this approach successful is the chromatographic separation of drug and all metabolites. Quantification is based on using LC-MS/MS in combination with detector that gives an equimolar response independent of the compound, usually with RAD. Response ratio of the metabolite to parent drug on RAD is then correlated to response ratio on LC-MS/MS. Low amounts of metabolites and parent drug in samples are measured by sufficiently sensitive LC-MS/MS, where the analysis of higher amounts allows detection on RAD and due to response factor enables calculating of metabolite concentration. The best way to perform analyses is to combine RAD with MS after liquid chromatography with splitting flow in order to obtain peaks of the metabolites and parent at the same retention times on both detectors [51]. RAD is convenient for such analyses because of the large dynamic range but its use is limited by the availability of radiolabeled standards. However,

the most straightforward detection technique generally found with LC-MS/MS is UV detection. Metabolites can be (semi)quantified using UV response ratio in cases when the parent drug chromophore offers sufficient selectivity, is not altered by metabolism and the metabolites are well separated from other drug related entities and endogenous compounds [46].

This approach may be also reasonable to quantify metabolites in case of limited amounts of authentic standards. After determination of the response factors, metabolites could be then quantified based on calibration curve of parent drug [19]. A constant response factor is absolutely essential and therefore in such cases response factors should be periodically verified. Using the same instrument and without major instrument breakdowns, the response factor seems to be very stable over long periods [52].

6.3. Indirect quantification

Refer to evaluation of glucuronides and other phase II metabolites. These metabolites are determined by cleavage of conjugates to yield the parent drug, which is subsequently detected. This indirect approach has several limitations, including the risk of incomplete hydrolysis, moderate repeatability and time consuming sample preparation [42]. Another drawback is non-selectivity of this procedure toward study of particular metabolite of interest when distinct drug metabolite conjugates are present in sample, like in case of morphine which is transformed to two isomeric metabolites. Morphine-3-glucuronide is an inactive metabolite but morphine-6-glucuronide possesses even greater pharmacological activity than the parent drug [53]. In such cases this approach is not suitable in pharmacokinetic studies as the overall drug concentration including more metabolites is determined in examined biological fluid. However, in the field of toxicology, doping control or drugs of abuse this information may be even more valuable [54, 55]. Nevertheless, direct quantification of metabolites and their indirect quantification via parent drug after metabolite hydrolysis may give comparable results like in case of buprenorphine metabolites [56].

Cleavage of conjugates can be performed by fast chemical hydrolysis or by gentle but time consuming enzyme hydrolysis. Deconjugation by ß-glucuronidase is the predominantly used approach. Different types of enzymes are commercially available but the most frequently used are ß-glucuronidases from *E. coli* or *Helix pomatia*, sometimes combined with arylsulfatase. ß-glucuronidase from *Helix pomatia* provides the advantage of the cleavage of glucuronide and sulfatate conjugates at same time what is important in the field of toxicology [6]. However, the glucuronidase activity is not as high as at *E. coli.* In order to achieve a successful enzyme hydrolysis it is crucial to pay attention on several factors, such as temperature, pH, enzyme origin, enzyme concentration and incubation time [57]. However, cleavage with ß-glucuronidase is not always preferential as for acyl glucuronides (ester conjugates) where alkaline hydrolysis is more suitable [55]. Acid hydrolysis may also be sometimes the better possibility for other glucuronide types [58]. N-glucuronides (primary, secondary and N-hydroxylated amines) are hydrolyzed under mild acidic

conditions but quartenary ammonium glucuronides under basic conditions [25]. Additionally, enzymatic hydrolysis of acyl glucuronides may be hindered due to acyl migration what leads to ß-glucuronidase resistant derivates [59]. Nevertheless, if the ß-glucuronidase treatment is successful for the metabolite of interest, this procedure should be the method of choice.

Another aspect for quantification using this approach has been shown recently [60]. Different benzodiazepines were determined via their metabolites by using acid hydrolysis of urine samples. The parent drug and all metabolites, conjugated as well as non-conjugated (I phase metabolites), were converted to corresponding benzophenone under studied conditions. Such approach reduces the specificity but at same time the overall sensitivity of the method increases, which makes such method suitable for drug abuse monitoring.

6.4. Metabolite production

Alternative approach for direct quantification is to obtain authentic metabolite standards. The chemical synthesis is mainly suitable for achieving phase I metabolites, like O-demetylation, N-demetylation, N-oxidation, carbonyl reduction and other. However, synthesis of the phase II metabolites can be cumbersome and stereochemically demanding and hence go beyond possibilities of most laboratories [34]. Versatile alternative to chemical synthesis is enzyme-assisted *in vitro* production of these metabolites using liver homogenates, liver microscale cultures, cell culture lines or microbial systems where each of these methods has its specific drawbacks [61, 62]. Raloxifene, which is metabolized to two distinct monoglucuronides and one diglucuronide, is an illustrative example for in-house production of authentic standards. Glucuronide yield by chemical synthesis was very low and not sufficient enough to characterize those metabolites. On contrary, the biosynthesis with recombinant human UGT enzymes turned out to be successful in converting parent drug to both monoglucuronides [63]. In last attempt the bioproduction of all three metabolites could be accomplished by using the microorganism *Streptomyces sp* [37]. For more detail about raloxifene *in vitro* metabolism refer to [19]. Availability of both metabolite standards – unlabeled and stable isotope-labeled internal standards is even more important for reliable quantification using LC-MS/MS. Stable isotope labeled metabolites can be obtained by microsomal incubation of labeled drug, of course if it is available and not too expensive [4]. The alternative approach is to use a labeled UDP-glucuronic acid as cofactor in bioproduction of metabolites [62].

Moreover, metabolites can be isolated from urine after oral administration and after purification and characterization they can be used as standards. Bisphenol A glucuronide and its deuterated glucuronide were isolated from rat urine [64]. Recently published work dealing with microsomal bioproduction of the same metabolites [4] revealed some drawbacks of the isolation approach. Beside ethical considerations, the yield of both standards was much lower from animal samples (microgram scale) than microsomal incubates (milligram scale). Additionally, urine as matrix requires also more extensive purification procedure in order to obtain highly pure standards. However, in cases where

metabolites cannot be produced by proposed *in vitro* models the *in vivo* biological samples are then the only media for isolation of those metabolites.

7. Sample preparation

Adequate sample preparation is a key aspect of quantitative bioanalysis and it is usually the most time consuming part of analyses. Interfering matrix compounds, such as proteins, lipids, salts, other endogenous and background compounds, should be removed in sample pretreatment, not only to avoid column clogging and instrument soiling, but also to improve the sensitivity, selectivity and reliability of analyses. Selection of an appropriate preparation procedure depends upon metabolite characteristics, their expected concentrations, the sample size and matrix, and the availability of analytical techniques for analyte quantification. Insufficiently treated samples may cause interfering peaks when using spectroscopic detection techniques such as UV-absorbance or fluorescence. However, analyses by LC-MS/MS are less prone to sample matrix and therefore usually require less pretentious sample clean up. Commonly and widely applied sample preparation techniques include protein precipitation (PP), liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Manual operations associated with sample treatment may be very labor intensive and time consuming and that could be avoided with automation in 96-well plate format or direct sample injection followed by on-line extraction methods.

7.1. Protein precipitation

Protein precipitation (PP) is simple and straightforward method widely used in bioanalysis of plasma samples. It is accomplished by using organic solvent (typically acetonitrile or methanol) or an acid (typically perchloric or trichloroacetic acid). It is followed by centrifugation to separate proteins from liquid supernatant and additionally, supernatant is sometimes diluted with chromatographically compatible solvent (e.g. mobile phase eluent). Supernatant can be directly injected or pre-concentrated after evaporation and reconstitution. Although only proteins are removed, other endogenous compounds remain which can still cause interferences such as matrix effect in mass spectrometry analyses. PP offers a generic and fast sample preparation technique that can be easily automated. The method has been also extended to quantification of drugs and metabolites from whole blood [65]. The same sample preparation technique in not suitable only for plasma but can be transferred to other biological samples such as urine. Moreover, the absence of proteins in these matrices allows direct injection without sample pretreatment. Nevertheless, it is advisable to dilute and filter or centrifuge the samples to reduce matrix effect and to remove eventually present particles [42]. Many examples for metabolite determination using PP, mainly in serum and urine, can be found in recently published review articles [66-68]. PP is also the most convenient method for less complex biological matrices in pharmacokinetic studies, such as hepatocytes [69] or microsomes [4]. In this case protein precipitation by ice-cold methanol (triple volume) at the same time terminates the incubation reaction and introduces internal standard to the final sample.

7.2. Liquid-liquid extraction

To obtain a sensitive analysis for a complex biological media (plasma, urine) liquid-liquid extraction (LLE) or solid phase extraction (SPE) are often required instead of PP. LLE sometimes gives better sample clean up showing less matrix effect in comparison with SPE [70]. Additionally, LLE is in general simpler and may be applicable to almost all laboratories using large variety of available solvents. LLE is also less expensive and flexible as several samples may be prepared in parallels. On the other hand emulsion formation, mutual solubility of analytes in both phases or large volumes of flammable and/or toxic solvents should be considered. In recently published comprehensive overview of methods for measurement of antidepressants and their metabolites in biofluids, many examples of extraction including LLE conditions can be found [67]. Offline methodologies are often very tedious and time consuming, and the risk of sample loss and/or contamination is high. Lack of automation possibilities is therefore another important LLE drawback. However, several research groups have developed different approaches to solve mixing and phase separation problems typically seen in a 96-well plate LLE method [71]. A semi automated LLE procedure using 96-well plates was reported [72].

7.3. Solid-phase extraction

SPE has become very popular and is nowadays considered as a basic technique in many laboratories for sample preparation of drugs and their metabolites from biological matrices. SPE offers several advantages over LLE, including higher recoveries, no problems with emulsions, less solvent consumption and a smaller sample volume requirement. Moreover, automation of sample treatment with high speed and feasibility for treatment of numerous samples at one time is possible. However, a drawback often associated with SPE is their high dead volume, which can lead to loss of sample and may cause dilution of applied samples. SPE column lot production variability or column blockage due to sample viscosity or precipitation may also occur. Columns can be supplied as individual units for manual use and also in 96-well plate format for use with robotic sample processors. The column dead volume has been overcome with a novel 96-well SPE plate that was designed to minimize elution volume (< 25μL). The evaporation and reconstitution step that is usually required in SPE is avoided due the concentration ability of the sorbent [71].

SPE is based on chromatographic separation such as liquid chromatography. Wide variety of cartridge types and solvents make SPE procedure suitable for many polar or nonpolar analytes. The extraction procedure can be a generic protocol or can be optimized if better sample clean up is desired. Beside classical reverse phase (e.g. C8 or C18) also polymer reverse phase (e.g. divinylbenzene, N-vinylbenzene), polymer ion-exchange (e.g. weak or strong anion/cation-exchange) or mixed mode ion-exchange sorbents are available. Polymeric reverse phase materials possess both hydrophilic and lipophilic properties and are capable of capturing polar analytes such as drug

metabolites [66]. Another advantage regarding silica based phase is ease of use, since there is no need to keep those phases moistened to maintain interaction. Mix mode ion-exchange and ion-exchange sorbents are even more convenient since strongly retained ionic metabolites allow rigorous washing of cartridge (e.g. 100% methanol) achieving cleaner sample with less matrix interferences [73].

There are now commercially available protein precipitation devices in plate format that allow PP within the plate whilst also removing phospholipids (HybridSPE™ and Waters Ostro™). This novel semi automated sample clean up procedure includes combination of PP and SPE. Proteins in sample are firstly precipitated with organic solvent, then transferred to SPE and directly injected into the analytical instrument. Method is simple, fast and almost free from phospholipids [74]. This sample preparation approach has been successfully applied in metabolism studies of various drugs [75].

7.4. On-line SPE

The on-line SPE offers speed, high sensitivity by the pre-concentrating factor, and low extraction cost per sample, but typically require the use of program switch valves and column re-configurations [71]. Biological samples can be directly injected into liquid chromatographic system without any sample preparations except for aliquoting samples, adding the internal standard and sometimes sample diluting and/or centrifugation. On-line SPE is considered as another dilute and injection approach like protein precipitation, however, it provides cleaner extract with reduced chance for matrix effect. Commonly used columns for on-line SPE are packed with large particles (typically > 20μm) of stationary material, such as polymeric and silica based, which work based on reversed phase, ion-exchange or mixed mode of separation. The combination of large particle size in these narrow bore columns (typically 50x1 mm) and fast flow (typically 3-5 mL/min), called also as turbulent flow chromatography, promotes the rapid removal of proteins with simultaneous retention of the small-molecular analytes of interest. After flushing all the proteins to waste, the direction of the flow is switched; the analytes are back-flushed onto the analytical column for chromatographic separation and detection. Fully integrated home-built or commercial systems enable eluting analytes from the extraction column onto analytical column in narrow bands. That allows multiple injections onto analytical column prior to elution into the instrument detector resulting in better sensitivity [76]. Most on-line SPE approaches use column-switching to couple with the analytical column as well as additional HPLC pump. Various instrument setups and column dimensions can be configured for the fast analysis of drugs and their metabolites in biological matrix at the ng/mL levels or lower [71].

Typically, on-line SPE columns can withstand few hundred injections of diluted plasma or urine samples what depends on the injection volume and sample matrix [77]. Beside mentioned SPE sorbents for turbulent flow chromatography, restricted access materials (RAM), monolithic materials and disposable SPE cartridges are available. The working principle of RAM phases is to isolate macromolecules from the target small molecules in

biological samples based upon their particle sizes and also due the chromatographic interaction. The proteins, that are unable to penetrate into the hydrophobic pores and the hydrophilic outer layer of particles, are first eluted to waste, the smaller molecules penetrate into pores and are additionally retained through the hydrophobic forces [78]. RAM columns may be used either in single column mode, being extraction (SPE) and analytical column at same time, or extraction column in combination with second analytical column. Single column mode approach shows simplicity but is limited due to chromatographic separation power [79]. Monolith phases as extraction sorbents for sample treatment looks promising and has been reviewed recently [77]. Monolith columns may be very convenient as single column mode for high throughput method in LC-MS/MS analysis [80].

8. Analytical methods for metabolite quantification

LC-MS/MS has become the predominant bioanalysis method for pharmacokinetic and metabolism studies due to its inherent specificity, sensitivity and speed. A literature survey of analytical methods for metabolite determination in biological samples undoubtedly confirms that fact. However, HPLC coupled with other detector systems or other separation techniques is often used. As an example, analytical methods for determination of antidepressants and their metabolites [67] are shown. HPLC coupled to different detectors (73%), among them the most popular being mass spectrometry (35%) and UV detection (24%), is the most frequently used analytical method. Applications of electrophoretic and gas chromatography methods for analysis of antidepressants and their metabolites in biofluids have seldom been published in literature (13 and 9%, respectively). Since the data were collected in time frame 2000-2010 [67], the frequency of LC-MS/MS methods is believed to be growing and is nowadays significantly higher because mass spectrometers are lately more accessible. In this section the most frequently used separation techniques as well as detectors will be overviewed with emphasis on LC-MS/MS.

8.1. Liquid chromatography

Good chromatographic separation is prerequisite for reliable and accurate quantification of metabolites in the biological samples. Baseline resolution must be achieved when liquid chromatography is coupled to non-MS detector. Although extensive chromatographic separation using LC-MS/MS is often not necessary, for certain cases, adequate resolution between drugs and various metabolites is required to avoid mass spectrometric interferences. Different metabolites may share the same MRM transition, such as hydroxylate metabolites [81] or glucuronides [37]. An example is shown in Figure 1. Additionally, unstable metabolites, such as N-oxides or glucuronides may be converted to parent drug by in-source dissociation or thermal degradation [79] or in collision cell (ion channel cross-talk). Interferences with endogenous compounds should also be avoided as matrix effect may appear (see 9.1.).

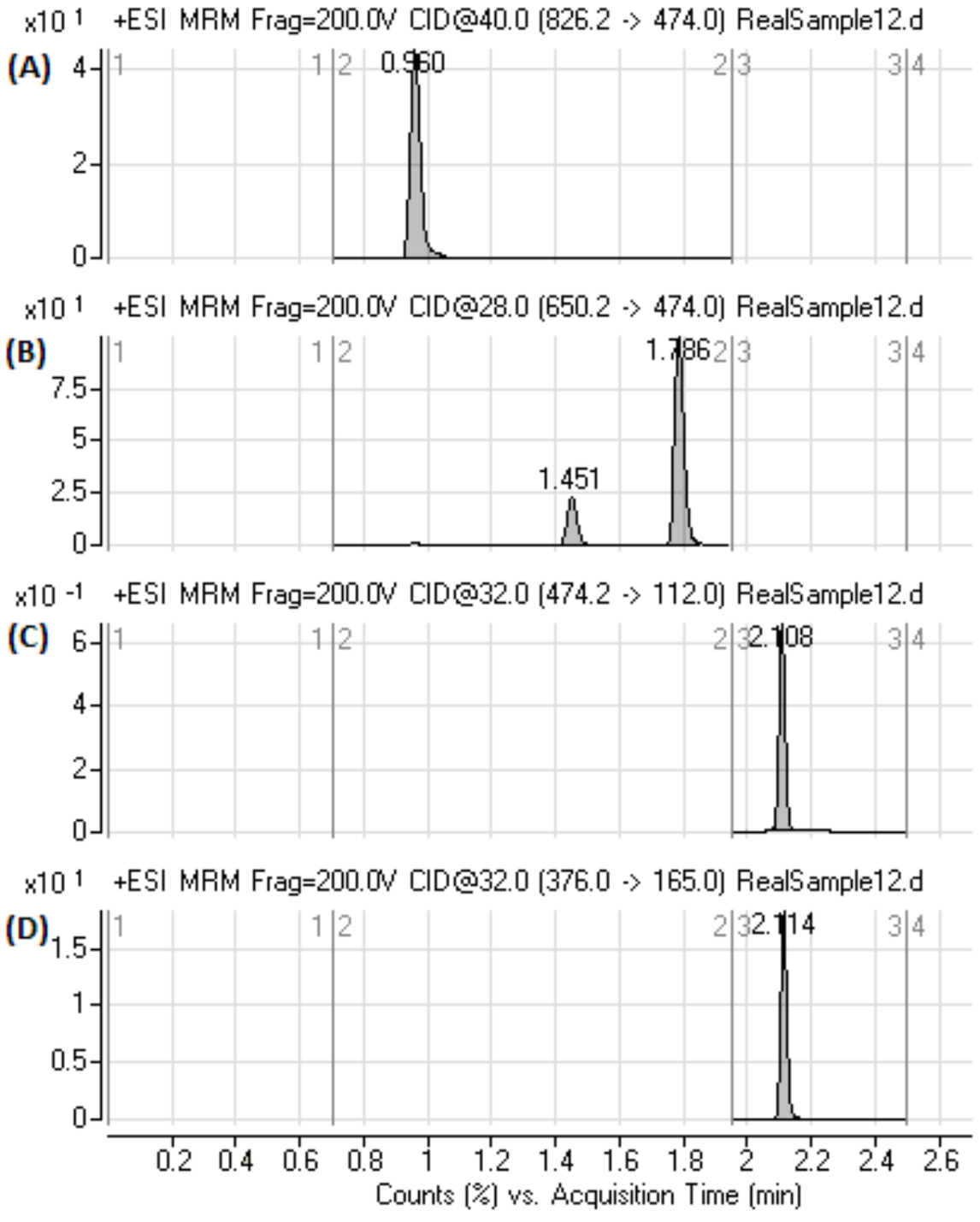

Figure 1. LC-MS/MS chromatogram of urine sample from a patient receiving raloxifene. MRM transitions represent (A) raloxifene diglucuronide, (B) two raloxifene monoglucuronides (C) parent raloxifene, (D) haloperidol as internal standard. For analysis conditions refer to [37].

8.1.1. Reversed phase chromatography

Reversed phase chromatography is most widely used technique in analysis of drugs and their metabolites due to its extensive application to most small molecules which are separated by their degree of hydrophobic interaction with the stationary phase. In most cases, metabolic changes lead to an increased polarity of the metabolite (strong shift for glucuronides and other phase II metabolites as also demonstrated in Figure 1) and therefore decreased retention on this stationary phase in relation to the parent drug [34]. The use of gradient elution is usually required to perform analysis of parent drug and polar metabolites. Common HPLC methods typically use a combination of water and either methanol or acetonitrile containing nonvolatile buffers, such as phosphate buffer and other inorganic additives as mobile phase. However, these nonvolatile additives cannot be recommended for LC-MS/MS because of possible MS contamination and also strong ion suppression effect. Volatile additives are used instead, such as formic or acetic acid (0.1% or lower) or ammonium acetate/formiate (2-10 mM) as salts. In order to maintain consistent chromatographic conditions, the pH of the mobile phase should be two units above or below pKa. C18 column is most commonly used. In some cases for polar metabolites short-chain bonded phases, such as C8, phenyl or cyano are more appropriate. Another effective way to resolve the retention issue is to add ion-paring reagent into mobile phase. The formed neutral ion pars increase retention and also improve peak shape. Among different ion-paring reagents trifluoroacetic acid and other perfluorated acids for basic analytes and for instance nucleoside phosphates for acidic analytes are appropriate for LC-MS/MS analyses [79]. These additives, especially trifluoroacetic acid, should be used at low concentrations because they cause ion suppression.

8.1.2. Hydrophilic interaction chromatography (HILIC)

HILIC using low aqueous/high organic mobile phase is emerging as a valuable supplement to the reversed phase chromatography for the retention of polar analytes [82]. An appropriate amount of water (usually 5-15%) in the mobile phase is suggested for maintaining a stagnant enriched water layer on the surface of the polar stationary phase where the analytes partite. HILIC separates compounds by eluting with strong organic mobile phase against a hydrophilic stationary phase where elution is driven by increasing the water content in the mobile phase [83]. Although some column companies are marketing columns specific for HILIC, most columns used with normal phases, such as pure silica or cyano columns, can operate in HILIC conditions. The highly volatile organic mobile phases, such as methanol and acetonitrile provide low column backpressure and also increased ionization efficiency for MS detection. It has been reported that the ionization responses for basic and acidic polar compounds were enhanced by 5-8 fold in the positive ionization mode and up to 20-fold in the negative ionization mode by the HILIC LC-MS/MS methods as compared to the reversed phase LC-MS/MS method [84]. Low back-pressure allows higher flow rates and may be used for shortening run times, up to several times [85]. Another advantage of HILIC is the possibility to inject higher volumes of organic solvent

onto the column without impairing peak shapes. Therefore, evaporation and reconstitution step of organic extracts after extraction procedure could be omitted making improvement in sample preparation automation and throughput [86].

8.1.3. Chromatographic approaches for polar metabolites

Metabolites, in particular glucuronides, have typically higher polarity than their parent drugs (Figures 1 and 2). This is the reason that classical reversed phase chromatography (e.g. C18) is sometimes not sufficient enough to maintain appropriate chromatographic retention of these analytes. In such cases already mentioned approaches like short-chain bonded reversed phases and ion-paring reagents (8.1.1.) or HILIC (8.1.2.) may be used. Additionally, mixed-mode columns with an embedded ion-paring group in the reversed phase stationary phase provide the capability for both ion-exchange and hydrophobic interactions in the mobile phase to retain ionizable polar analytes. The mixed-mode column allows retaining hydrophobic analytes by the reversed phase mechanism and hydrophilic analytes by the ion exchange mechanism at higher organic content in the mobile phase [87]. Normal phase chromatography may also be used for retention of polar analytes but due to limited amount of water allowed in the mobile phase, normal phase chromatography interfaced with MS requires complex pretreatment steps for biological samples and therefore has much fewer applications than reversed phase LC-MS/MS [88].

The use of special packing material known as porous graphic carbon (PGC) is another alternative to achieve retention and separation of polar analytes. PGC chromatography commonly employs water, acetonitrile and methanol as the mobile phase but provides markedly greater retention and selectivity for polar analytes than reversed phase columns. For analyte elution PGC normally requires larger organic content in the mobile phase than reversed phase chromatography what consequently results in favorable sensitivity with MS detection [79, 83, 89].

Derivatization of polar analytes results in the reduction of polarity and is therefore another possibility to enhance the chromatographic retention. But this approach is disadvantaged as it is not going toward high throughput, especially in case when the primary purpose of the derivatization is not the detection or stability improvement of the analyte.

8.2. Strategies for high-throughput improvement in liquid chromatography

Current trend in pharmaceutical analysis is the reduction of the analysis time and the increase in sample throughput without sacrificing the separation selectivity. High-throughput bioanalytical assays are typically based on LC-MS/MS but may also be successfully extended to classical HPLC analyses. Approaches to achieve faster analyses include sample preparation (on-line automation or offline semi automation, section 7) and fast liquid chromatography. The later may be in general improved by three approaches: smaller particle size, shorter columns and higher mobile phase flow rates.

8.2.1. Ultra-high performance liquid chromatography (UHPLC)

Reducing the particle diameter from 5.0 μm to 1.7 μm will, in principle, result in a 3-fold increase of efficiency, 1.7-fold increase in resolution, a 1.7-fold in sensitivity, and 3-fold increase in speed [79]. For fast analyses using sub-2μm particle column dimensions are typically 50x2 mm. An additional benefit of UHPLC is the low consumption of mobile phase, where it saves at least 80% compared to HPLC [90]. The high back-pressure resulting in decreased particle size need appropriately designed chromatographic system that would withstand such high pressure (instruments nowadays up to 1200 bars) and also provide at least possible extra column effects. To prevent clogging, manufacturers of UHPLC recommend filtration of both samples and solvents through 0.2 μm filter. Advantages as enhanced separation efficiency, short analysis time and high detection sensitivity make UHPLC coupled with MS/MS an even more powerful analytical support in pharmacokinetic studies [4].

8.2.2. Core-shell column

An emerging alternative to porous particles are porous layer beads, known as core-shell or fused-core particles. The high separation efficiency of core-shell particles is a result of a faster analyte mass transfer from the mobile phase to outer porous layer of the particle. The improved dynamics of analyte movement through these columns result in higher effective peak capacities and separation efficiencies comparable to those fully porous sub-2μm but with advantage of lower back-pressure [91]. This technology is comparable to UHPLC in terms of chromatographic performance but demands neither expensive UHPLC instrumentation nor new laboratory protocols [88]. Commonly available columns, such as Ascentis, Poroshell and Kinetex, use different stationary phases and particle sizes (e.g. Kinetex 1.7 and 2.6 μm) and are widely used with classical HPLC instruments, also in our laboratories. Core-shell columns in combination with UHPLC-MS/MS exhibit excellent performance, as demonstrated in quantification of raloxifene and its three glucuronides [37].

8.2.3. Monolithic chromatography

The use of single rod monolith column is an alternative approach to the chromatographic columns packed with fine particles. The high permeability allows the use of higher flow rates and therefore shorter chromatographic runs, as demonstrated for the separation of bupropion metabolites in 23 seconds or for methylphenidate and its metabolite in 15 seconds [71].

High flow rates may require flow splitting before entering MS. An attractive approach using monolith separation is to combine it with high flow on-line extraction, which allows fast extraction and separation of samples [77]. Current limitations in the application of these columns are the small pH range [2-8], poor temperature resistance, limited column dimensions and stationary phases (C8 and C18) as well as higher costs due to higher mobile phase consumption.

8.3. Other separation techniques

Gas chromatography with mass spectrometry (GC-MS) is most useful for the analysis of trace amounts of organically extractable, non-polar, volatile compounds and highly volatile compounds that may undergo headspace analysis. The GC-MS analysis of polar compounds, such as metabolites, from biological matrices requires analyte extraction into a volatile organic solvent either directly or after chemical derivatization, which typically enhances the volatility of previously non-volatile organic compounds [25]. Most analytes need extensive time-consuming sample preparation including derivatization to become stable, volatile and amenable to the ionization technique. This drawback in throughput necessitated the direction of GC-MS to LC-MS. LC-MS has an advantage over GC-MS method in drug metabolism studies, particularly for low dosed and large drugs, and of course for the analysis of phase II metabolites. However, GC-MS may also have advantages, especially in clinical and forensic toxicology or doping control [54]. GC-MS has been frequently applied for quantification of glucuronides in biological samples but only after treatment with ß-glucuronidase in order to obtain parent drug before analysis [92]. The GC-MS technique is receiving wider acceptance in various classes of antidepressant agents, representing 6% of overall analytical methods for determination of antidepressants and their metabolites [67].

Capillary electrophoresis (CE) is another separation method for quantification of metabolites. This method offers very high resolution capability, high efficiency and short time of analysis. Moreover, CE in many instances can have distinct advantages over HPLC in terms of simplicity, rapid method development, solvent saving and minimal sample requirement [10-30 nL injected) making this technique very interesting for rapid and practical analyses in the biomedical field. However, the main disadvantage is low sensitivity. For this reason, application of CE for analysis of antidepressants and their metabolites is not so widely reported [67]. Applicability of CE using UV-absorbance or mass spectrometry detection was reported for determination of tamoxifene and its phase I metabolites [68].

8.4. Mass spectrometry

Currently, the QQQ using single or multiple reaction monitoring is most often used for quantitative analysis of small molecules in the pharmaceutical industry. QQQ or single stage MS, operating in SIM, is not anymore recommended for reliable bioanalytical quantification, because it suffers from insufficient selectivity in comparison with MRM. SIM can provide the selected ion at certain m/z value, but the matrix or impurity interferences may occur at the same m/z value. Beside lower selectivity, SIM shows also much lower sensitivity in comparison to MRM due to much higher background noise. However, in some specific cases of good chromatographic resolution and the absence of matrix interferences, SIM may be considered as an alternative quantification method. Occasionally, due to the nature of dissociation pathways, resulting in low molecular weight product ions, radial ejection preceding dissociation and/or charge stripping, reliable precursor → product ion

transitions cannot be established. Alternatively, a precursor → precursor scan for reducing noise can be employed [70].

Also other analyzers, such as ion traps and TOF, have been widely and increasly used for metabolite quantification. Especially hybrid instruments which combine a QQQ (Q1 and colission cell) and ion trap or TOF (Qtrap, Q-TOF). These instruments can operate as true tandem mass spectrometry and are usually applied for this purposes. Q-TOF can also operate as TOF and thus provide accurate mass measurments. Additonaly, high resolution of TOF instruments allows the resolution of chromatographic peak from background interferences achieveing better sensitivity. However, it does apear that QQQ using MRM remain about three to five times more sensitive than TOF [93].

The selection of an appropriate ionization technique depends on the analyte characteristics, such as the structure, polarity or molecular weight. In LC-MS/MS analysis three atmospheric pressure ionization techniques cover the whole range of compound polarities and molecular weight: ESI, APCI and APPI. Moreover, the polarity mode can be chosen according to the acidic, neutral or basic properties of the analytes. If the right choice of ionization technique and the polarity mode is not so obvious, all available possibilities should be considered in order to obtain the best response for tested analytes. The softest ionization technique, ESI, is the method of choice for polar and ionic compounds. The advantage of soft ionization is in providing reliable information about molecular weight of the phase II metabolites in comparison to other ionization techniques [42]. For parent drug and phase I metabolites with a lower polarity, APCI and APPI may provide better ionization efficiency and sensitivity [34]. APPI has a similar application range as APCI, but slightly extended toward nonpolar compounds [32]. ESI is generally more susceptible to matrix ionization suppression than APCI [94]. In case of neutral steroids or other poorly ionizable analytes, derivatization can be employed in order to increase detection sensitivity, but additionally the chromatographic retention enhancement of such derivatizated analytes may therefore provide less matrix effect. On the other hand, the disadvantage of derivatization lies in an additional time consuming step for sample preparation [30]. The adjustment of the mobile phase for improving analyte response is much easier compared to derivatization. The effect of mobile phase on ESI efficiency is not well understood and hence the behavior of an analyte in different mobile phase conditions cannot be routinely predicted. Various factors can affect the ionization of analytes in ESI, such as pH, mobile phase additives, flow rate, solvent composition and concentration of electrolytes. It is recommended to evaluate the use of additives (e.g. formic acid, ammonium acetate) and organic modifiers in mobile phase to maximize the ionization efficiency of the analyte, which is highly dependent on its chemical structure. Acidic conditions often promote positive mode ionization of basic compounds and conditions, which are slightly below neutral, neutral or basic, promote negative ionization of acidic compounds.

A dramatic difference in the ESI response can be found even when acetonitrile is replaced by methanol in mobile phase. It was reported that an analyte gave only weak ESI response

in the positive ionization mode in mobile phase containing acetonitrile with formic acid and/or ammonium acetate. But replacement of acetonitrile with methanol in mobile phase gave approximately 25-fold higher response. On the other hand, for the same analyte, mobile phases containing acetonitrile or methanol gave about the same response in negative ionization [79]. Another interesting example is analysis of bisphenol A and its metabolite in biological samples. In order to gain the highest possible sensitivity for bisphenol A and bisphenol A glucuronide, LC-MS/MS conditions were optimized. ESI ionization source operating in negative ionization mode was selected for further optimization of mobile phase. It was found that substitution of acetonitrile/water with methanol/water as mobile phase increased response of parent by approximately two-fold but at the same time decreased response of its metabolite by approximately three-fold. Acetonitrile was selected as organic modifier because metabolite quantification is the main concern of metabolism studies. Additionally, sufficiently high sensitivity is needed for metabolite determination as low concentrations are expected in such studies [4].

8.5. Other detection techniques

UV, fluorescence or electrochemical detectors are usually coupled with liquid chromatograph for determination of drugs and their metabolites. Total analysis time of these methods is often long because baseline chromatographic separation is required for quantification purposes. In terms of reproducibility and robustness, UV and fluorescence detection have an advantage over mass spectrometry. However, methods are less sensitive and specific what requires extensive and time-consuming sample preparation compared to mass spectrometry.

Before the advent of mass spectrometry, UV was the primary detection technique used in pharmacokinetics for quantification of drugs and their metabolites in biological matrices. Although robust, reliable, simple and easy to use, UV detection provides relatively poor sensitivity, especially when the compound of interest has no significant chromophore [44]. However, HPLC coupled with UV detection is still widely applicable for determination of drugs and their metabolites in biological samples [95-97].

In contrast to UV, fluorescence or electrochemical detection can be a very selective and sensitive detection technique. These detectors can extend the sensitivity by 1-3 orders of magnitude if the analyte exhibits, or can be readily derivatized to exhibit, fluorescence or electroactivity [7]. Some drugs such as morphine have good fluorophores which allows its detection without derivatization. For direct determination of morphine and its two glucuronides assays based on liquid chromatography with different detector systems (UV, fluorescence, electrochemical, MS) has been reported. Limits of quantifications for both metabolites were comparable for MS and fluorescence detection but were as expected higher for UV detection [98]. Oxidation vulnerability and native fluorescence properties of most biogenic amines may explain the long history of their quantification by these conventional HPLC detection methods. However, LC-MS/MS methods are rapidly emerging due to its

specificity, sensitivity and high throughput [99]. Electrochemical detection is also very suitable for determination of antioxidants, such as ascorbic acid or glutathione, in biological samples [100].

When analytes do not exhibit fluorescence, electroactivity or have poor UV detection, derivatization can be performed to enhance their detection. In addition, chromatographic retention is enhanced by derivatization what is a very convenient in analysis of polar drug metabolites. Derivatization is an additional step in sample preparation where consideration regarding the stability of derivatizated analyte to solvolysis and thermal degradation need to be addressed. Nevertheless, fluorescence detection is still widely used [67, 68, 101].

9. Aspects of analytical quality

LC-MS/MS is currently considered as the method of choice for quantitative analysis of drugs and their metabolites. The advantages of using this technique in MRM mode due to high sensitivity, selectivity and speed allow developing high throughput methods with little or no sample preparation and minimal chromatographic retention. However, matrix effect may have a significant impact on such LC-MS/MS analyses [94, 102, 103]. Therefore, the evaluation of matrix effect as well as strategies for its elimination or minimization needs to be adequately addressed. Another important parameter for analytical quality is the selection of an appropriate internal standard for the compensation of possible loss of analytes during sample clean up and variations in instrument performance. Other LC-MS/MS issues, such as ion channel cross-talk and carry-over should also not be overlooked. Moreover, metabolite instability may have an influence on the analytical performance and will be additionally addressed here.

9.1. Matrix effect

Matrix effect (ME) is a term that describes any changes in the MS response of analyte that can lead to either a reduced response (ion suppression) or an increased response (ion enhancement) of the LC-MS system. ME is caused by molecules originating from the sample matrix or mobile phase that co-elute with the analyte of interest and therefore interfere with the ionization process in the MS ion source. Several approaches have been proposed to evaluate ME. Among them the post column infusion technique is widely used. Use of this qualitative evaluation technique allows the determination of the matrix effect of endogenous components in blank matrix. During analysis of blank matrix, analyte response is monitored to provide information where in the chromatographic run interferences between the analyte and matrix compounds occur. ME is illustrated as response deviation in the otherwise flat response time trace of the continuously post-column infused analyte [104]. This approach is very useful during method development because it provides information on the retention times where ME has to be expected, which can later be avoided for analyte of interest by optimizing chromatographic conditions.

For quantitative estimation of ME another well recognised approach is more suitable. Matuszewski et al. reported practical approach for the assessment of the absolute and relative ME as a part of validation of bioanalytical LC-MS/MS methods [94]. The difference in response between neat solution sample and post-extraction spiked sample is called the absolute ME, while difference between various lots of post-spiked samples is called the relative ME. As such will an absolute ME primary affect the accuracy and relative ME will affect the precision of the method. The determination of a relative ME is much more important than the determination of absolute ME in the evaluation and validation of bioanalytical method in biofluids [94]. The relative ME caused by interindividual variability in the sample matrix is assessed based on at least 5 lots of different matrices. Relative ME can be expressed as a coefficient of variation at particular concentration level or calculated based on slope lines. For the method to be considered reliable and free from the relative ME, the calculated coefficient of variation of determined slopes in different sources of matrices should not exceed 3-4% [105].

ME is known to be both component and matrix dependent. It was demonstrated that matrix induced ion suppression is especially important for early eluting compounds, such as polar metabolites. Typically, ME more strongly influences lower than higher analyte concentrations [71]. The main source of the commonly observed ME of plasma samples is believed to be endogenous phospholipids and proteins. The lysophospholipids which normally elute earlier in reversed phase chromatography are more likely to cause matrix effects compared to the later eluting phospholipids in spite of the larger concentrations of the latter in plasma [106]. Phospholipids cause ion suppression in both, positive and negative ESI modes and must be removed or resolved chromatographically.

To remove or reduce ME, modification to the sample extraction methodology (SPE or LLE instead of PP) and improved chromatographic separation must be performed. The majority of ME occur in the solvent front of a chromatographic run and if the analytes can be retained to some degree, matrix effects can be minimized. Suitable sample preparation and chromatographic conditions are linked together and form the basis of developing a successful and robust quantitative LC-MS/MS method [102, 103]. Another consideration when dealing with ME is selection of ionization interface. APCI is generally considered to be less prone to ion suppression compared with ESI [94, 105]. However, assay sensitivity and thermal stability of the analyte should be evaluated for eventual APCI application. Reducing the flow rate [20 μl/min or below) directed to ESI source by post column splitting may also reduce or completely remove the ion suppression [107]. Additionally to other approaches UHPLC technology in combination with polymeric mixed-mode SPE and appropriate mobile phase pH may provide significant advantages for reducing ME [73]. Mobile phase additives such as triethylamine and trifluoroacetic acid can also lead to ion suppression. The use of other reagents such as formic or acetic acid, trifluoroacetic acid in conjunction with 10 mM ammonium acetate or addition of 1% propionic acid to the mobile phase may overcome the ME of trifluoroacetic acid. Triethylamine may be replaced with other ion paring reagent such as hexylamine [70].

However, the most efficient way to eliminate the influence of ME on the accuracy and precision of a quantitative analytical method is the use of stable isotope labeled analogs as internal standards [105].

9.2. Selection of internal standard

The selection of a suitable internal standard (IS) is one of the key parameters for establishing a successful LC-MS/MS method. Usually, stable isotope, such as ^{2}H (D, deuterium), ^{13}C, ^{15}N or ^{17}O, labeled standards are most appropriate for compensation of possible loss of analytes during sample clean up and variations in instrument performance (typically caused by matrix effect) since their physicochemical characteristics are practically identical to that of unlabeled analyte. In general, a stable isotope labeled IS is considered to be ideal, since it shows almost same behavior to the analyte of interest in sample preparation, chromatography as well as in ionization process [102]. However, issues like isotopic purity of IS, cross-contamination and cross-talk between MS ion channels, as well as IS stability and isotopic integrity of the label in biological fluid and during sample preparation should be carefully addressed [94].

Mass difference between analyte and IS should be at least 3 Da to avoid signal contribution of the natural isotopes to the signal of IS. Although deuterated IS are most frequently used, several disadvantages need to be considered in some cases, such as different extraction recoveries and retention times between such IS and analyte or exchange of deuterium atoms by hydrogen atoms [108]. For example, differences in retention times for deuterium labeled (d_{16}) and unlabeled bisphenol A compounds is shown in Figure 2, where both, labeled metabolite and parent compound eluted slightly before their unlabeled analogs. Interestingly to mention, deuterium labeled bisphenol A (d_{16}) has all 16 hydrogen atoms substituted by deuterium atoms but is actually d_{14} labeled (mass shift 14 Da in MRM transition, Figure 2) because two deuterium atoms from the functional group (–OD) are easily exchangeable by hydrogen atoms. The observable chromatographic retention time shifts for deuterated analogs depend on the number of deuterated atoms in structure. Deuterated analogs have no retention time shifts up to approximately six deuterium atoms [34] but when more deuterium atoms are included in structure, such as ten, the retention time shift may be significant (up to 1.2 min) [108]. Therefore, other stable isotope labeled IS, such as ^{13}C, are considered as more appropriate [108].

The concentration of IS used for sample preparation is also important and should be approximately at the middle of calibration curve. Optimization of IS concentration is critical to avoid ion suppression by co-eluting analyte leading to standard curve non-linearity [70]. Even if stable labeled isotope IS is used, ME should still be investigated. Namely if ion suppression significantly reduces the signal of both, analyte and IS, the signal to noise ratio may decrease to a point where accuracy and precision may be negatively affected [102].

Problems may occur when more than one compound is determined in the same analytical method. A number of labeled IS identical to number of analyzed compounds would in this case be required [102]. This is not always practically feasible, especially not for stable isotope labeled drug metabolites as their availability is very limited. In such cases, to assure the suitability of the quantification method for determination of drugs and their metabolites, ME evaluation should be carefully addressed [37]. On the other hand, example for simultaneous determination of parent compound and its metabolite using both labeled IS [4] is shown in Figure 2. It is preferred that the labeled IS product ion used for the MRM transition retains the stable isotope moiety as for m/z 403 → 227 (bisphenol A-glucuronide) versus 417 → 241 (deuterated bisphenol A-glucuronide) in Figure 2.

However, labeled ISs are not always available or can be very expensive. As an alternative, structural analogues can be used, with consideration of the structural similarities between the IS and the analyte. To be suitable, the ionization of the analogue must be compared with analyte and should preferably co-elute with the analyte (Figure 1). The selected analog IS should not correspond to any metabolic product of analyte, such as hydroxylated or N-dealkylated metabolites [108]. Nevertheless, in many cases analog ISs or structurally unrelated ISs are not able to compensate the ME [94, 105]. In such situation, other more rigorous approaches to reduce or eliminate ME should be applied (see 9.1.).

9.3. LC-MS pitfalls

Metabolite may produce a molecular ion that is identical to parent ion through in source conversion. Typical metabolites for such conversion are glucuronides, sulfates or N-oxides [79]. N-oxide conversion may also serve for identification purposes as this transformation takes place in APCI source and represent another potential way to differentiate N-oxide from hydroxylated metabolites (both with exact mass) since the later usually do not undergo thermal deoxygenation [23]. Therefore, an inadequate chromatographic separation between parent drug and such metabolites will result in over estimation of parent drug concentration in the presence of these isomeric compounds. The same situation may happen also by ion channel cross-talk in MRM mode, which means that fragment issued from the other scanned transition is still present in collision cell. Metabolites, such as diverse glucuronides, that give the same product ion as parent drug, have to be therefore chromatographically separated. However, for co-eluting compounds, such as analytes and their IS, the absence of cross-talk has to be demonstrated [37].

Carry-over, which is the appearance of an analyte signal in a blank injection subsequently to analysis of high concentration samples, is also a common problem in LC-MS/MS methods. This problem occurs due to retention of analytes by adsorption on active surfaces of the autosamples, solvent lines, extraction columns (e.g. online SPE) or the analytical column. Most carry-over problems can be minimized by an appropriate choice of injector wash solutions and methods, by proper choice of mobile phase and tubings, and by choice of suitable stationary phase and proper variation in the solvent strength [70]. Carry-over becomes prominent after injection of analyte at high concentration and should be assessed

during the validation of the method. Carry-over is usually expressed as percentage of detected analyte in blank sample regarding to its limit of quantification [4] and may significantly lower the sensitivity of the method [109].

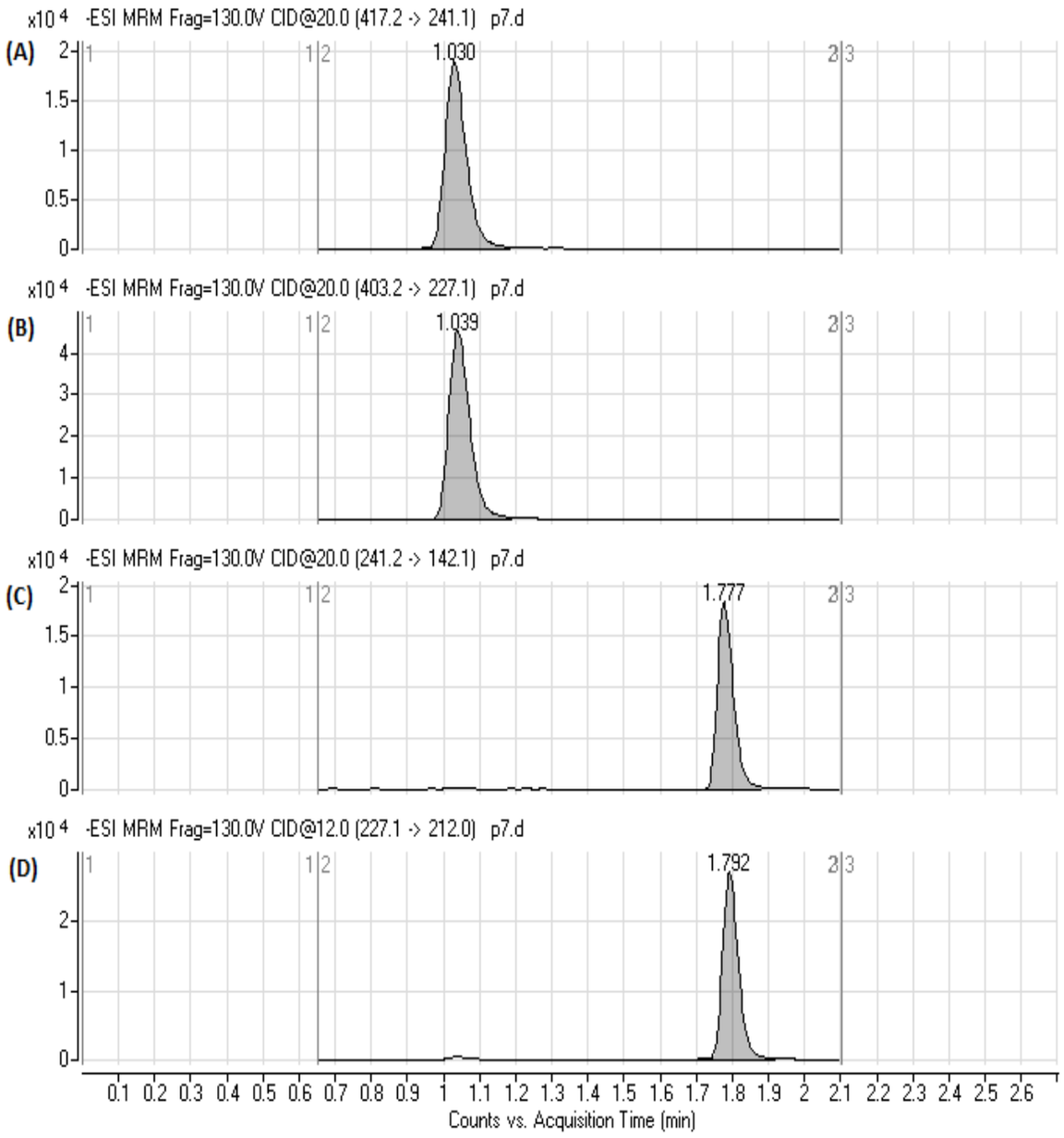

Figure 2. LC-MS/MS chromatogram in MRM mode for metabolite (BPAG) and parent bisphenol A (BPA) of a typical microsomal incubation. (A) mass transition for $BPAG_{d16}$ (internal standard for BPAG), (B) mass transition for BPAG, (C) mass transition for BPA_{d16} (internal standard for BPA), (D) mass transition for BPA. For analysis conditions refer to [4].

9.4. Metabolite stability

For drugs and metabolites that are unstable, with one converting to other, conditions used during sample preparation and analysis must be optimized in order to minimize such conversion and to achieve accurate quantification of the drug and metabolite. Common factors that affect drug and drug metabolite stability in biological matrices include temperature, light, pH, oxidation and enzymatic degradation [110]. Acyl glucuronides (O-ester conjugates) are probably the most commonly encountered problematic metabolites in bioanalysis. Acyl glucuronides are unstable and hydrolyze to release aglycone under neutral and alkaline conditions. Different acyl glucuronides have been shown to have different rates of hydrolysis. However, mildly acidic conditions (pH 3-5) should be the most desirable pH for minimizing the reaction in biological samples [59, 111]. Acyl glucuronides (1-O-acyl glucuronides) are also susceptible to internal migration under both, physiological and alkaline conditions. The rate of migration resulting in 2-, 3- and 4-O-acyl glucuronides increases with increasing pH and temperature. Such isomeric glucuronides are not susceptible to hydrolysis by ß-glucuronidases and may compromise the quantification of metabolites and total parent drug when indirect quantification approach via conjugate cleavage is applied [111]. Similar to O-glucuronides, N-glucuronides can be converted back to parent drug under acidic/basic/neutral pH conditions or at elevated sample processing temperature, but this instability is largely compound dependent. For example olanzapine glucuronide can be readily cleaved under acidic conditions, clozapine and cyclizine glucuronides are unstable at range pH 1-3 and doxepin glucuronide at pH 11 [112]. Lactone is another commonly unstable metabolite function group which may be converted to its open ring hydroxy acid drug. Lactone metabolites, such as atorvastatin metabolite, require optimization of the sample pH (typically 3-5) in order to minimize the hydrolysis of lactone metabolite back to parent drug [113].

N-oxides are also unstable in solutions and biological samples during sample preparation, especially under strong acidic or basic conditions. Light exposure may further accelerate the decomposition of these metabolites. Other compounds are also susceptible to photodegradation such as, catechols, nisoldipine, rifampin and their metabolites and should be protected from light during sample preparation and analysis [112].

10. Conclusion

Current drug discovery efforts have been focused on identification of drug metabolism and pharmacokinetic issues at the earliest possible stage in order to reduce the attrition rate of drug candidates during the developmental phase. Metabolic fate of drugs can be responsible for problems associated with their bioavailability, interindividual variability, drug-drug interactions, pharmacologic activity or the toxicity. Different *in vitro* methods, from subcelullar to organ range, and *in vivo* studies are applied for the clarification of drug metabolism. Among them microsomes and hepatocytes are the most frequently utilized *in vitro* models in drug metabolic profiling and drug interaction studies. For the

successful monitoring of the drug metabolism, suitable bioanalytical methods have to be developed and validated. Liquid chromatography coupled with mass spectrometry has become the most powerful analytical tool for identification and quantification of drug metabolites.

The known identity of metabolites is the prerequisite for a suitable metabolic assessment of drugs. Appropriate LC-MS instrumentation is clearly critical to both, detection and structural elucidation. Tandem mass spectrometry instruments are beside their key role for metabolite quantification also well suited for qualitative purposes as they enable different scan possibilities (constant neutral loss, precursor ion, product ion) for structural characterization of metabolites. However, the drive to more versatile and powerful instruments which can perform intelligent data dependent experiments and accurate mass measurements has led to newer high resolution mass analyzers, such as Q-TOF instruments, which now dominate the metabolite identification field.

Direct quantification of metabolites in biological samples is the most appropriate approach, but also others approaches such as indirect quantification through parent drug after metabolite hydrolysis or quantification supported by using response factors may be used which primary depends on the availability of suitable authentic standards. Analytical methods for metabolite quantification are based on liquid chromatography or other separation techniques coupled with various detector systems where LC-MS/MS plays predominant role in bioassays for pharmacokinetic and metabolism studies due to its inherent specificity, sensitivity and speed.

In order to support metabolism experiments in a timely manner, the use of high throughput methods for the analysis of drugs and their metabolites in biological samples has become an essential part, especially in the most time consuming sample preparation. Trend is going toward semi automated off-line sample treatment in 96-well plate format or on-line SPE after direct injection of samples. Additionally, other high throughput approaches can be introduced. Ultra-high performance liquid chromatography with small particles and monolithic chromatography offer improvements in speed, resolution and sensitivity compared to conventional chromatographic techniques. Hydrophilic interaction chromatography (HILIC) or other specialized columns suitable for polar metabolites are emerging as a valuable supplement to classical reversed phase chromatography.

The main advantage of LC-MS/MS allows development of high throughput methods with little or no sample preparation and minimal chromatographic retention. However, matrix effect may have a significant impact on such analyses. Matrix effect issue is frequently underestimated and should be adequately addressed. Not without reason, matrix effect have been called the Achilles heel of quantitative LC-ESI-MS/MS [103]. The use of stable isotope labeled analog as internal standard is the most efficient way to reduce matrix effect. But normally, additional approaches to reduce or eliminate matrix effect are needed.

Abbreviations

DMPK, drug metabolism and pharmacokinetic; CYP, cytochrome P450; UGT, UDP-glucuronosyltransferase; NAT, N-acetyltransferase; GST, glutathione-S-transferase; SULT, sulfotransferase; UDPGA, uridine diphospoglucuronic acid; PAPS, phospohoadenosine phosphosulfate; HLM, human liver microsomes; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; CE, capillary electrophoresis; LC-NMR, liquid chromatography-nuclear magnetic resonance; HPLC, high performance liquid chromatography; UHPLC, ultra-high performance liquid chromatography; HILIC, hydrophilic interaction chromatography; PGC, porous graphic carbon; RAD, radioactivity detector; MS, mass spectrometry; MS/MS, tandem mass spectrometry; API, atmospheric pressure ionization; ESI, electrospay ionization; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; QQQ, triple quadrupole; IT, ion trap; QTrap, triple quadrupole-linear ion trap; TOF, time of flight; Q-TOF, triple quadrupole-time of flight; FT-ICR, fourier transform-ion cyclotron resonance; IM-MS, ion mobility mass spectrometry; MRM, multiple reaction monitoring; SRM, selected reaction monitoring; SIM, single ion monitoring; CNL, constant neutral loss scan; PI, precursor ion scan; PP, protein precipitation; LLE, liquid-liquid extraction; SPE, solid-phase extraction; RAM, restricted access materials; ME, matrix effect; IS, internal standard.

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