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Quantification of Glucuronide Metabolites in Biological Matrices by LC-MS/MS

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1. Introduction

For many drugs and xenobiotics glucuronidation is the major metabolic pathway of detoxification. There, a highly hydrophilic glucuronic acid is coupled to aliphatic / aromatic alcohols, thiols or carboxylic acids or even amines. Although the glucuronidation is usually considered a deactivation reaction, electrophilic acyl-glucuronides can be formed from a number of acidic drugs, such as diclofenac, zomepirac, diflunisal, valproate, ibuprofen, gemfibrozil and clofibrate, among others. Acyl-glucuronides have been linked with idiosyncratic adverse drug reactions due to the chemical reactivity towards plasma and tissue proteins and nucleic acids (Shipkova, Armstrong et al. 2003). Even the most common ether O-glucuronides, which are generally pharmacologically inactive, can be cleaved back to the active parent compounds inside the human body or in the in the environment. Similarly, some N-glucuronides have been linked to bladder cancer due to their fast deconjugation which releases the toxic aglycone inside the bladder. Therefore, on many occasions in drug metabolism studies or in toxicology, it is necessary or prudent to detect and to quantify glucuronide metabolites in biological samples.

2. Overview of glucuronide quantification approaches

In the past, the majority of the quantification methods for glucuronides involved an enzymatic (β-glucuronidase) or an acid/base hydrolysis of the glucuronide ether bond prior to HPLC analysis, mainly because the hydrophilic glucuronides were difficult to separate from the interfering matrix components (Kadi and Hefnawy 2009). Nowadays, the highly selective and sensitive triple quadrupole LC-MS/MS instruments enable us to directly measure the glucuronides without the need to de-conjugate the glucuronide moiety before the analysis. This offers some significant benefits: quicker sample preparation and better accuracy and precision, as the glucuronide hydrolysis may be incomplete or the aglycon is unstable in hydrolytic conditions or binds non-specifically to the β-glucuronidase or matrix components (Kadi and Hefnawy 2009). Furthermore, the glucuronide moiety can be bound to different nucleophilic functional groups on the parent aglycone, giving rise to different glucuronide structural isomers, which can then be selectively quantified. Differentiation among glucuronide isomers might be important as they can have profoundly different pharmacological activity, for example the morphine-6-glucuronide has a many times greater affinity towards the μ-opioid receptor than the morphine-3-glucuronide or the morphine-6,3-diglucuronide (Loser, Meyer et al. 1996).

On the other hand, a direct measurement of the glucuronides in biological samples requires the use of their authentic analytical standards for the preparation of the calibration and the quality control samples in an appropriate matrix. Quite often, these analytical standards are not easily obtainable and even if they are commercially available, a few milligrams may cost up to several thousand of US Dollars or even more. Their isotope labeled analogs (for use as internal standards) can cost even more. To overcome this difficulty, several possibilities exist (Soars, Mattiuz et al. 2002; Sanceau, Larouche et al. 2007). The first is a chemical synthesis of the glucuronide standards, which can be performed either by a glucopyranoside synthesis or by the use of glucuronide donors or glucuronide precursors and often involves a several step reaction, which is time-consuming and the glucuronide yield is often very low (Yu, Zhu et al. 2000; Burkon and Somoza 2008). The second approach is an enzymatic synthesis of the glucuronides, where the aglycone is incubated with uridine diphosphoglucuronosyl transferases (UGTs) in the presence of a co-factor uridine-diphospho-glucuronic acid (UDPGA). Recombinant human UGTs or human microsomes can be used in these incubations in order to obtain the same glucuronide isomers as present in human body fluids (Trontelj, Bogataj et al. 2007). Some alternative methods to microsome incubations have also been described where the UGT enzymes are adsorbed or covalently linked onto silica or to a monolithic carrier surface. There, the enzymes can be re-used many times or can continuously catalyze the glucuronidation reaction by a substrate flow-through technique called immobilized enzyme reactors (IMER) (Kim and Wainer 2005).

The third approach is a bioproduction process, where specially modified strains of bacteria (for example *Streptomyces sp*.) can be used to produce large amounts of certain glucuronides (Trdan, Roskar et al. 2011). However, the latter approach needs an extensive clean-up and purification procedures in order to obtain high purity analytical standards (Briggs, Baker et al. 1999). The fourth approach is the semi-quantitative method where one aliquot of the sample containing the glucuronides is subjected to a quantitative beta-glucuronidase cleavage and the other aliquot is not. After the analysis of first and second aliquot, the glucuronide *response factor* is calculated as the ratio between the glucuronide signal from the second aliquot and the rise of the signal of aglycon in the first aliquot (originating from the cleaved glucuronide). Then, in subsequent analyses, the glucuronide *response factor* is used to quantify the glucuronides without the β-glucuronidase treatment and by using the calibration curve of the easily obtainable aglycone (Sanceau, Larouche et al. 2007; Farrell, Poquet et al. 2011; Trdan, Roskar et al. 2011). In such scenarios, a careful study of the possible relative matrix effects on glucuronides is warranted. The semi-quantitative approach can also be used if a radiolabeled parent is available by correlating the radioactive responses from the radiolabeled metabolite and parent to the corresponding LC-MS/MS responses (Yu, Chen et al. 2007). If a radiolabeled parent is not available, the labeled glucuronide can be synthesized by microsomes using a radiolabeled UDP-glucuronic acid (UDP-[U-^{14}C]-GlcA) (Maurel 2010). The semi-quantitative approach is based on the assumption that the concentration-response relationship for glucuronide is linear. However, on many occasions in LC-MS analyses, this is not the case. Therefore, it is advisable to check the response factors across the expected glucuronide concentration range (Trdan, Roskar et al. 2011) .

3. Sample preparation techniques

This step is often the most crucial in the whole analysis. The choice of the sample preparation method is based on the glucuronide concentration, the available sample

volume, the matrix type and the analyte stability. In this section, the most commonly used methods for sample preparation are presented, including: deconjugation with β-glucuronidase, solid and liquid extractions, protein precipitation, on-line SPE and others. In order to ensure adequate stability of the glucuronides prone to hydrolysis or acyl-group migration, a proper sample collection / storage / buffering is required. Therefore, the stability considerations are discussed as well.

3.1 Deconjugation with β-glucuronidase

This is not a complete sample preparation procedure; it is just one of the optional steps within the sample handling workflow which is used to liberate the aglycone from glucuronide in order to quantify it more easily. Historically, the β-glucuronidase treatment was the most commonly used approach to quantify glucuronides of xenobiotics in biological samples (Kadi and Hefnawy 2009). However, this procedure has some drawbacks and considerations that have to be taken into account.

First, not all glucuronides can be cleaved by the commercially available enzymes, such as the β-glucuronidase from *E. coli* or *Helix pomatia*, bovine liver glucuronidase etc. Some N-glucuronides may remain quite stable in such incubation mixtures and require a chemical cleavage with, for example, hydrazine hydrate (Frandsen 2007) or a hydrolysis with a dilute acid. On the other hand, the acyl (ester) glucuronides can be easily cleaved by a dilute base (0.1-1.0 M NaOH) with mild heating (Wen, Stern et al. 2006; Zhang, Zhu et al. 2008). β-glucuronidases isolated from different species may have different enzyme activities toward various substrates and even for structural isomers of glucuronides (Choi, Ha et al. 2010). In addition, some acyl-glucuronides or their isomers formed after acyl-migration can be β-glucuronidase stable and require a basic hydrolysis (with 1M NaOH) (Wen, Stern et al. 2006). Furthermore, β-glucuronidases isolated from mollusks (like *Helix pomatia* or *Patella vulgata*) may posses a significant sulfatase activity as well. Note that molluskan β-glucuronidases may be less effective in hydrolyzing ether glucuronides than E. coli β-glucuronidases (Smith and Athanaselis 2005).

Secondly, in most cases, there is at least some amount of aglycone present in the starting material. Therefore, the measured aglycone concentration after deconjugation has to be subtracted by the initial aglycone concentration in order to quantify the amount of present glucuronide. Of course, in such calculations, it is necessary to use the molar concentrations and not mass/volume concentrations because the molecular mass of the freed aglycone is lower by 176 amu compared to the mono glucuronide in positive ESI, which corresponds to the loss of monodehydrated glucuronic acid.

Thirdly, the binding of glucuronides to the matrix components can significantly reduce the β-glucuronidase cleavage efficiency. For example, a complete cleavage of raloxifene glucuronides dissolved in aqueous solution of acetate buffer takes only a few minutes if not seconds, but if plasma proteins are present, the glucuronides can be >95% bound, and consequently, it can take up to even 24-48 hours to completely deconjugate the same amount of glucuronides (*author's own experience, data not published*). Similarly, when performing a glucuronide quantification in tissue samples, the β-glucuronidase may not have a complete access to all subcellular compartments, such as microsomes, resulting in an underestimation of glucuronides. In such instances, better results may be obtained after an acid hydrolysis (Gu, Laly et al. 2005). Additionally, some β-glucuronidases may posses other catalytic

properties as well, like for example steroid conversion with preparations from *H. pomatia* (Gomes, Meredith et al. 2009).

Alternatively, instead of deconjugation with beta glucuronidases, also a methanolysis of glucuronides can be used, which frees both glucuronide and sulfate conjugates. The downsides of this approach are an increase in baseline noise in mass spectrometer and a poor efficiency of deconjugation (Cooper, Currie et al. 2001; Yu, Ho et al. 2005).

Therefore, if the β-glucuronidase treatment turns out to be effective on the studied glucuronide within its sample matrix, then this should be the method of choice for its deconjugation. The most common procedure for a β-glucuronidase treatment is as follows.

3.1.1 Sample pre-treatment

Biological samples, such as urine, plasma or feces are simply diluted in 1:1 (volume/volume) ratio with purified water or buffer. Note that the buffer can be added later on as well. A feces sample has to be homogenized prior to the dilution step. If an internal standard is to be used, this is the correct moment for its addition. However, in some instances, for example, if a deuterated glucuronide internal standard is not available and another glucuronide or a structural isomer or a compound with similar retention factor is used instead, then it may be beneficial to add the internal standard solution *after* the extraction procedure in order to minimize the IS content variability. In such cases, the aim of internal standard addition is not to correct for the sample preparation inconsistencies, but rather to correct the possible matrix effects. Furthermore, if an internal standard is not a deuterated or a C-14 labeled analogue, then it may as well be extracted differently than the analyte, thereby increasing the method variation (RSD). Rarely, the samples are extracted or treated with an organic solvent prior to the β-glucuronidase treatment. This shortens the cleavage incubation time as the protein binding is usually eliminated by the extraction with an organic solvent. However, the organic solvent must be evaporated before the enzyme is added to the sample.

3.1.2 Sample buffering

The pH of incubation buffer may be important for the reaction velocity and the selectivity towards the glucuronides or the sulfates. Quite high buffer concentrations can be used (0.1-1.0 M). The optimal pH range depends on the enzyme source. If molluskan β-glucuronidase is used, then the pH should be 4.0 - 4.5 for maximal glucuronidase activity. However, if maximal sulfatase activity is desired, then the pH should be 6.5 (Tephly, Green et al. 1997). If bovine liver glucuronidase is used, then the maximal glucuronidase and sulfatase activity is achieved at pH 4.4 (Himeno, Hashiguchi et al. 1974). If *E. coli* glucuronidase is used, then there should be no sulfatase activity and the maximal velocity is achieved at pH range of 6-7 (DongHyun, YoungHo et al. 1995). The selection of buffer salts depends on the desired pH range. For acidic buffers, acetate buffer is most commonly used, for more neutral pH values, phosphate or carbonate buffers can be used.

3.1.3 Sample incubation

The sample incubations are usually performed in at least two parallels. Sometimes, a separate incubation of a commercially obtainable substrate (for example 4-methylumbelliferone glucuronide) in the same matrix is used to monitor the β-glucuronidase activity (Blount, Milgram et al. 2000). Each incubation should contain 200 -

25 000 units of β-glucuronidase per 1 mL of incubation medium (Gu, Laly et al. 2005; Belanger, Caron et al. 2009). Note that vendors define the glucuronidase activity in Roy or in Fishman units, where 1 Fishman unit releases 1 μg phenolphthalein from phenolphthalein-β-glucuronide in 1 h at 38°C. Typically, the incubations are left in a thermostated shaker for 1-5 hours at 37-60°C. For slow deconjugation reactions, 24 hours and longer incubations are needed in order to quantitatively deconjugate all the present glucuronides. It is noteworthy, that an excess of enzyme may lead to incomplete deconjugation, therefore it is important to determine the appropriate incubation conditions for the specified conjugate in a given sample matrix (Gomes, Meredith et al. 2009).

3.1.4 Reaction termination and final sample preparation

Prior to the analysis, the proteins have to be denatured and removed. This is usually accomplished by a subsequent liquid-liquid extraction with an organic solvent like ethyl acetate, cyclohexane, diethyl ether, methyl tert-butyl ether. Note that if the extraction solvent is highly non-polar, like cyclohexane alone, then it may fail to extract the possibly remaining hydrophilic glucuronide from the sample, resulting in an unnoticed glucuronide left-over and consequently its under prediction. Therefore, a combination of organic solvents can be used, like for example, ethyl acetate/diethyl ether (1:1, vol/vol), in order to extract both, aglycone and glucuronide. Alternatively, a solid phase extraction can be used (Weinmann, Vogt et al. 2000; Stolker, Niesing et al. 2004). After the protein removal, a pH re-adjustment may be necessary in order to gain an acceptable recovery, especially for basic aglycones, which tend to be protonated at acidic pHs of β-glucuronidase incubations. After evaporating the organic solvent, the samples are reconstituted in a solvent similar to mobile phase and injected into LC-MS/MS. Alternatively, instead of a liquid-liquid extraction, it is also possible to denature the proteins with an equal volume of ice-cold acetonitrile, or by adding approximately 1/10 of incubation volume of trichloroacetic acid solution (TCA) (10% vol/vol) (Lu, Palmer et al. 2003), followed by a brief vortex mixing, centrifugation (15 min at 3 000 – 15 000 × *g*) and injection of the supernatant into LC-MS/MS. Such sample preparations procedures, without an extraction, allow a confident detection of the possibly still remaining glucuronides; however, the analyte responses may be lower due to the sample dilution and the peak broadening, in contrast to the liquid-liquid and solid phase extractions, where the analytes can be concentrated many times and reconstituted in a weaker elution solvent.

3.2 Liquid-liquid extraction

Sometimes, a liquid-liquid extraction (LLE) may be preferable over a solid phase extraction (see paragraph 3.3) because of its greater simplicity and applicability to almost all laboratories and low cost (Jemal, Ouyang et al. 2010). However, the extraction of glucuronides into a lipophilic organic solvent can be difficult because of the glucuronide's highly polar and hydrophilic nature (both the glucuronic acid and hydroxyl groups can be ionized). In order to de-ionize the glucuronic acid moiety, acidic conditions should be ensured during the extraction. For example, if 1 mL urine sample is to be extracted, then some 200 μL of 1 M HCl should be used to sufficiently protonate the glucuronic acid (pKa 3.1-3.2) (Paradkar 2008). Note that if extraction of both glucuronide and aglycone is required, then acidic extraction conditions may diminish the extraction efficiency of the basic aglycones. The extraction efficiency for both types of analytes may be improved if a

combination of organic solvents is used (see subheading 3.1.4 for details and references). The choice of the extraction solvents may have a strong impact on the matrix effects later in LC-MS analysis. Methyl-tert-butyl ether (MTBE) and n-butyl chloride seem to extract only negligible amounts of lyso-phosphatidyl choline, which is the matrix component that elutes early from a reverse phase (RP) column and tends to interfere with ionization of drugs and their metabolites (Jemal, Ouyang et al. 2010). However, both solvents are highly non-polar and the recovery of glucuronides is expected to be low. Nevertheless, if aglycone should be extracted after a β-glucuronidase treatment, then these two solvents can be used successfully for that purpose.

3.3 Solid Phase Extraction (SPE)

SPE has become a very popular sample preparation technique in bioanalysis due to low sample volume requirements, low organic solvents consumption, high recovery, good reproducibility, easy automation, high speed and high throughput. There are also some drawbacks, like a possible low recovery of hydrophilic analytes (some glucuronides included), column blockage due to sample viscosity or precipitation, analyte instability in elution solvent, and lot-lot variability in column packaging (Telepchak, August et al. 2010).

The principle mechanism of separation in a SPE is similar to analytical chromatography, i.e. the analytes in sample solution bind with a greater affinity to the solid surface than the matrix components which are washed away. Afterwards, a stronger elution solvent is used to elute the analytes from the solid surface and collect them for further analysis (HPLC, LC-MS/MS).

The main goal while developing a SPE method is to achieve the highest possible reproducibility, good matrix clean-up and a high recovery, if possible. The retention of glucuronides on a solid phase sorbent can be facilitated by a number of mechanisms, from hydrophobic to ion-pairing. The chemistry and hydro-lipophilic properties of the aglycone play a major role. There are a number of possible combinations of solid phases / eluents and the analyst should choose the right combination that gives the best performance for the samples to be analyzed. A good starting point is the sorbent selection kit or the method development kit, which is offered by the majority of leading SPE cartridge producers (like Phenomenex, Waters, Agilent, Varian, Supelco, Macherey-Nagel, Thermo, etc.). Usually, in such a package, there are at least four different cartridge chemistries: strong cation exchange, a weak cation exchange, a strong anion exchange and a weak anion exchange. There are also classical reversed phases available, like C-18, C-8, C-4 and polymeric phases for example divinylbenzene and N-vinylpyrrolidone. A mixed-mode SPE of glucuronides is also possible, where an ionized analyte binds to an ion-exchange sorbent due to its charged state whereas neutrals can be retained at the same time due to their lipophilic interactions with the polymeric or reversed phase part of the sorbent. The manufacturers also propose the generic condition/wash/elution solvent protocols. After subjecting the pre-spiked, post-spiked, and solvent-only samples to various sorbents (in replicates), the subsequent LC-MS/MS analysis shows which sorbent chemistry gives the highest and the most reproducible recovery (RE) and can also show the matrix effects (ME). See the paragraph 5.5.

In glucuronide extraction with SPE, special attention should be paid to the washing step if the analytes are retained by a reverse phase mechanism. Too high percentage of organic solvent can easily wash the hydrophilic glucuronides into waste (author's own experience, data not published). If silica based or polymeric reversed phase cartridges are used,

decreasing the methanol content in the elution solvent can reduce the elution of phospholipids which can cause unwanted matrix effects later in LC-MS. On the other hand, 100% acetonitrile as an elution solvent drastically minimized the phospholipid elution on silica based cartridges (Lahaie, Mess et al. 2010). If endogenous glucuronides are present in "blank" samples , like for instance steroid glucuronides, then it is necessary to spike the blanks with labeled glucuronides for matrix effect and recovery calculation (Pozo, Van Eenoo et al. 2008). The glucuronides can be retained to various extents on a reversed phase (Trontelj, Bogataj et al. 2007), on a strong anion-exchange (Pu, McKinney et al. 2004), on a mixed-mode (Kakimoto, Toriba et al. 2008) and even on strong and weak cation exchange sorbents (Okura, Komiyama et al. 2007), depending on the ionization and lipophilic properties of the aglycone. Even highly polar glucuronides and sulfates, like paracetamol conjugates can have a high recovery on mixed-mode weak anion exchange sorbents like Waters Oasis WAX (Sunkara and Wells 2010). Some lipophilic conjugates, like steroid glucuronides and sulfates may not be eluted fully with only methanol and may require addition of ion-pairing reagents like 5 mM triethylamine (Isobe, Serizawa et al. 2006). It is noteworthy that the ion-exchange or mixed mode SPE is preferred over the reversed phase mechanism of retention since the former two methods can include a rigorous washing of the cartridge with a strong solvent, for example 100% methanol or ethyl acetate, without any detrimental effect on the analyte recovery while producing the lowest possible matrix effects (Chambers, Wagrowski-Diehl et al. 2007). With the mixed mode anion-exchange SPE sorbents it is even possible to separate not only the conjugates from the parent compounds, but also to differentiate between the various types of conjugates, like glucuronides from sulfates (Fontanals, Marcé et al. 2010).

3.4 Protein precipitation

Protein presence in the samples can cause significant problems in glucuronide analysis. First, the glucuronides can be more than 50% protein bound (Hochner-Celnikier 1999; Burkon and Somoza 2008) which may negatively impact the absolute method recovery. In high throughput analysis, there is a tendency to minimize the sample preparation steps. Protein precipitation (PP) is one of such fast and easy-to-automate procedures. Therefore, PP is quite a popular sample preparation technique, especially for plasma samples and *in vitro* incubations, where this step terminates the enzyme reactions as well (Rosenfeld 2004).

However, samples obtained with a PP procedure may contain higher amounts of problematic matrix components than samples after a SPE or LLE. Furthermore, chromatography problems may arise, since the percentage of organic solvent in the sample is higher, worse peak shape can be expected, shorter column life-time, worse separation and lower responses due to the sample dilution with a precipitating agent. In addition, more frequent ESI spray chamber and capillary cleaning may be required (Bakhtiar and Majumdar ; Elbarbry and Shoker 2007), (author's experience). Most importantly, worse matrix effects can be expected with PP than with either LLE or SPE. The PP is most commonly accomplished with an addition of an organic solvent in 3:1 volume ratio to the sample. The most commonly used organic solvents are acetonitrile or methanol, which should precipitate at least 99% of the proteins. Acidification of the samples is another effective alternative to the organic solvents; a 6:10 ratio of 10% TCA should precipitate more than 99% of the proteins, however the resulting drop in pH can cause analyte instability. A 1:1 (vol/vol) addition of 2 M perchloric acid to a plasma sample precipitates the proteins but

may not release all of the protein bound glucuronides and may not ensure the stabilization of acyl-glucuronides despite the acidic pH. Better results have been obtained with an addition of 1:2 (vol/vol) 15% metaphosphoric acid to a plasma sample (de Loor, Naesens et al. 2008). Addition of metal ions (like zinc sulfate) or hydrophilic salts (like ammonium sulfate) is used less frequently to precipitate the proteins in LC-MS analyses (Rosenfeld 2004). After the precipitation, a centrifugation is used to separate the liquid supernatant from the proteins. Centrifugal field of 15.000 × *g* for 10 minutes is usually enough for complete sedimentation. Supernatant can be directly injected or evaporated and then reconstituted.

3.5 Hybrid SPE

A novel sample clean-up procedure has been recently introduced, the Hybrid SPE, which should combine the advantages of both the protein precipitation and SPE. It is said to be quick, simple and should provide extracts almost free of phospholipids, one of the main culprits for matrix effects. The proteins in samples are precipitated with a triple volume of acetonitrile, then passed through the hybrid SPE cartridges, and directly injected into the LC-MS. The hybrid SPE cartridges should firmly bind phospholipids and eliminate them from the sample. Unfortunately, the glucuronides can bind to the same sorbent very efficiently; therefore the glucuronide recovery may be close to 0% (author's own experience with haloperidol glucuronide, (Silvestro, Gheorghe et al. 2011)). Hence, this method may be used in applications, where a complete elimination of glucuronide is needed.

3.6 Direct injection of biological samples

The recent advances in increasing the sensitivity of triple quad mass spectrometers for 10 times and more made it possible to directly inject some liquid biological samples (like for example urine or saliva) into LC-MS/MS systems almost without any sample pre-treatment. Actually, at least the particulates have to be removed from the samples somehow prior to their injection to prevent injector fluid path blockage, column deterioration and pressure build-up. Narrow bore UHPLC capillaries and columns are especially sensitive to particulate impurities because of their finer pore diameters and dense inlet filters. Therefore, at least some sort of sample pre-treatment is necessary, like diluting the samples 1:10 with water in combination with filtering through 0.2 µm pore filters or centrifugation (McMaster 2007; Kaklamanos, Theodoridis et al. 2011). Such an approach without any form of extraction greatly reduces the time needed for an analysis; however the method and system robustness parameters should be carefully monitored. Furthermore, the majority of biological samples may contain at least some amount of proteins, which may pass through the aforementioned filters and precipitate later on the column or in the tubing when the percentage of organic modifier increases. This difficulty can be overcome by two approaches. First, an on-line SPE can be used to wash the endogenous impurities from the sample into waste before they can reach and contaminate the analytical column and mass spectrometer (see subheading 3.6 (On line-SPE) for details). Second, the samples can be incubated with proteases in order to degrade the proteins into peptides which are no longer a threat to a LC-MS system (Yu, Ho et al. 2005).

3.7 On-line SPE

On-line SPE is a system which allows injecting crude samples or diluted samples onto an extraction column, which is first washed with a weak solvent to waste to remove the salts

and proteins and then eluted with a stronger solvent onto the analytical HPLC or UHPLC column. The key to this separation procedure is the turboflow chromatography. Sometimes, it is also designated as a size-exclusion chromatography or a restricted access material (RAM) or a high turbulence liquid chromatography (HTLC). This process takes place in a short extraction column (for example 30x1, 25x4 mm) with RP silica or polymeric based stationary phase with large particles and pores (25-50 μm) for greater robustness (Bentayeb, Batlle et al. 2008). After the injection, the flow rate is quite high (1-5 mL/min), so the proteins and other macromolecules cannot enter the pores of the stationary phase and are quickly washed away into waste. Smaller molecules however can enter the pores and are therefore retained until a stronger solvent elutes them (usually in reverse flow direction) onto the analytical column. A dilution step in a high pressure mixing tee may be employed during the transfer of the analytes from the extraction onto the analytical column in order to concentrate the analytes in a narrow band on analytical column for narrower peak shape, resulting in better sensitivity (Ye, Kuklenyik et al. 2005). The hardware setup for such an on-line SPE is usually a modified HPLC with a larger sample loop (injection volumes are usually 10-900 μL), an additional binary or quaternary pump, and an additional six- or ten-port valve. If a 10-port valve is used, two extraction columns can be used at the same time to increase throughput; this allows a combination of sample loading, washing, equilibration and elution steps simultaneously (Ferreirós Bouzas, Dresen et al. 2009). Typically, on-line SPE columns can withstand a few hundred injections of diluted plasma or urine samples, it depends on the sample matrix and injection volume (Rosenfeld 2004). Care must be taken to disrupt the protein binding prior to the injection; otherwise the recovery may be diminished. Therefore, some authors use a protein precipitation step, followed by evaporation and reconstitution before the samples are injected into an on-line SPE (Ferreirós Bouzas, Dresen et al. 2009)

3.8 Dried blood spots

Dried blood spots (DBS) and dried plasma spots (DPS) are novel approaches for quantitative determination of analytes in systemic circulation. Very low sample volumes are needed (as low as 15 μL up to 100 μL) for a successful quantitative HPLC-MS/MS method (Spooner, Lad et al. 2009). Plasma or blood is applied onto a paper sorbent; after drying, a punch from DBS (or DPS) sample is extracted with a solvent, which is injected in HPLC-MS/MS. This method offers some significant benefits over the classical sample preparation techniques: very low volume of plasma or blood is needed, which minimizes the patient burden (finger prick or heel prick sampling instead of venous cannula) and is very appropriate for animal and pediatric studies; the stability/transport/storage of samples are a lot simpler because no refrigeration is needed and in case of DBS, also no centrifugation at the site of the clinical trial is required. Furthermore, when the stability of paracetamol glucuronide and sulfate was studied in DBS samples, no noticeable degradation and paracetamol formation was observed (Spooner, Lad et al. 2009). This technique can be automated for high throughput analysis in pharmaceutical drug discovery and clinical trials (Barfield and Wheller 2010). Apart from classical HPLC-ESI-MS configuration, some direct DPS analysis tools have been introduced, such as the thin layer chromatography MS interface (CAMAG) which does not require punching of DPS samples (Abu-Rabie and Spooner 2009), direct elution or on-line desorption (Deglon, Thomas et al. 2009), desorption electrospray ionization (DESI) and direct analysis in real time (DART) (Takats, Wiseman et al. 2004). It should be emphasized however, that all the presented direct DPS analysis techniques without any

chromatographic separation are principally unsuitable for glucuronide analysis due to the possible in-source deconjugation of glucuronides or sulfates or even N-oxides back to their parent molecules and ion channel cross-talk (Wong, Pham et al. 2010).

3.9 Glucuronide derivatization

Some of the more hydrophilic glucuronide conjugates (like paracetamol-glucuronide) exhibit a poor ionization either due to their hard-to-ionize aglycones or due to the slow desolvation caused by the low percentage of organic solvent in mobile phase composition required for their chromatographic retention. In such instances, two approaches may be explored to overcome the low glucuronide signals. First, the hydrophilic interaction chromatography (HILIC) or a Hypercarb™ column may be a good option to increase the percentage of organic solvent for elution of glucuronides (see subheading 4.4). Secondly, after the extraction, the glucuronides can be derivatized at their carboxylic moiety using an ethereal diazomethane in methanol. Methyl esters are formed at room temperature. Afterwards, the samples are dried and reconstituted with methanol and injected into LC-APCI-MS, where a positive or negative ionization mode can be used to detect either $[M+H]^+$ or $[M+O_2]^-$ ions, respectively (Ohta, Kawakami et al. 2003).

3.10 Glucuronide stability issues

The stability of any type of glucuronide (O-, N-, S-, acyl-) should be thoroughly investigated at all stages of the analysis: sample acquisition and storage, during the sample preparation and after the sample preparation (autosampler time included). It has to be emphasized that even if the glucuronides are quantified indirectly, with β-glucuronidase treatment, the stability of glucuronides is crucial prior to this step, otherwise the glucuronides may be underestimated and the aglycone overestimated. The stability study is especially important for acyl- and N-glucuronides, which can be highly labile compounds in slightly alkaline and even neutral solutions (Mullangi, Bhamidipati et al. 2005). Even acidic additives like perchloric acid may not provide a good-enough stabilization of the acyl-glucuronides. With acyl-glucuronides, either a hydrolysis or an acyl migration can occur. Acyl migration is a process, where the aglycone is being transferred from the C-1 on glucuronide ring to C-2, C-3, C-4 carbon atoms. Such isomeric glucuronides become more stable towards β-glucuronidases and require alkaline conditions for their hydrolysis. Usually, the samples are acidified if acyl-glucuronides are present. Low temperature, such as 4°C may play a major role for acyl-glucuronide stability (Tan, Jin et al. 2009). Plasma and tissue samples may contain endogenous β-glucuronidases which may compromise even O-glucuronide stability.

4. Chromatographic methods

It is imperative to achieve a good chromatographic separation of conjugates (glucuronides) not just from the solvent peak but also from their parent molecules, otherwise they can de-conjugate in MS ion source (ESI, APCI) or in the collision cell (ion channel cross-talk), thereby increasing the signal of the parent, which may cause its false over-estimation (Naidong, Lee et al. 1999). This is why Jemal et al. suggest to use an incurred plasma sample (plasma after dose which still contains metabolites) during the method development in order to ascertain that the developed method for a drug is not compromised by their presence either by chromatographic co-elution and channel cross-talk or by deconjugation

during the sample handling (Jemal, Ouyang et al. 2010). The glucuronides often prove to be difficult analytes for HPLC separations. First, in most instances, they are rather hydrophilic, and therefore they tend to elute near the peak of the solvent or the hydrophilic matrix components. At the same time, the strongest ion suppression is often observed at the beginning of the chromatographic run. Secondly, if more than one glucuronide structural isomers are present in a sample, i.e. the glucuronide acid moiety can be bound to different nucleophilic centers on the aglycon, then the chromatographic separation may be difficult and requires a careful gradient elution (Trontelj, Bogataj et al. 2007; Trdan, Roskar et al. 2011). Furthermore, the chromatographic separation of glucuronide structural isomers is crucial, as they share the same parent mass to charge ratio and the fragmentation pattern; therefore a tandem mass analyzer cannot distinguish among the different co-eluting isomers. Ultra high pressure liquid chromatography (UHPLC) seems to offer some significant advantages over the classical HPLC in terms of higher selectivity towards the glucuronide isomers, a greater sensitivity and a higher speed of analysis (Korfmacher 2009).

4.1 Reversed phase chromatography

Reversed phase HPLC and UHPLC on C-18 columns has been used quite successfully for glucuronide separation (Kemp, Fan et al. 2002; Trontelj, Bogataj et al. 2007; Trdan, Roskar et al. 2011). The mobile phase is usually neutral to slightly acidic (pH 2.5-6) and the buffers used are 0.01 – 0.1% formic or acetic acid. 2-5 mM ammonium acetate with or without acetic acid addition may also be used. The organic phase is usually acetonitrile because in comparison to methanol, acetonitrile may provide a better peak shape of basic compounds and glucuronides, a better resolution among the structural glucuronide isomers and a higher MS response (author's own experience). Usually, a gradient is started at a very low percentage of organic modifier (2-10%) in order to assure an adequate retention of the glucuronides and a good separation from the hydrophilic matrix components (Kemp, Fan et al. 2002; Fayet, Béguin et al. 2009). Ballistic gradients are not optimal for glucuronide HPLC methods if the samples may also contain the parent molecule or glucuronide isomers. Ion channel cross-talk and in-source deconjugation, can both cause inaccuracy in quantification of the parent if it is not adequately separated from conjugates (Mei and Morrison 2009). Furthermore, greater matrix effects may also be observed in fast or ballistic gradients due to the poorer separation of analytes from the matrix components (Chambers, Wagrowski-Diehl et al. 2007). A more efficient chromatographic separation of analytes from the endogenous or other interfering components, such as drug vehicles, PPG, PEG or even over-the-counter medicines like analgesics, may be achieved with UHPLC columns resulting in lesser matrix effects (Marín, Gracia-Lor et al. 2009).

4.2 Ion-pairing reagents

Although ion-pairing reagents have proven to be beneficial for glucuronide retention (Yau, Vathsala et al. 2004), such techniques have rarely been used in LC-MS analyses due to the limited compatibility of ion-pairing reagents with ionization and desolvatation in LC-MS interface. A successful and robust application of hexylamine as an ion pairing reagent together with a careful control of mobile phase pH has been described for acidic metabolites retention and detection with LC-negative-ESI-MS. In this case, no interference was observed from hexylamine as it is a volatile reagent and ionizes only in the positive mode (Coulier, Bas et al. 2006). With a similar ion pairing reagent, dimethylhexylamine, a switch from the

negative to the positive ESI is possible, which increases the sensitivity and selectivity of the method (Pruvost, Théodoro et al. 2008). Interestingly, even the addition of ion-pairing reagents just to the sample solution or to a reconstitution solvent (and not to the mobile phase) may significantly affect the retention of polar analytes. For example, a retention time of methadone has been doubled when the heptafluorobutanoic acid (HFBA) had been added to the reconstitution solvent after the extraction. Moreover, the addition of this ion-pairing reagent significantly reduced the carry-over, probably because it reduced the attachment of the analyte to LC surfaces (Gao, Bhoopathy et al. 2006).

4.3 Other reversed stationary phases

Apart from the classic RP stationary phases, like the C-18 or C-8, phenyl group containing sorbents can be used in order to enhance the retention of mainly aromatic analytes through the formation of PI-PI bonds in addition to the regular mechanisms of RP chromatography (adsorption, partition, lipo- and solvophilic interactions) (Rafferty, Zhang et al. 2007). Additional selectivity enhancement may be observed when the percentage of methanol is increased in the organic modifier phase (in combination with acetonitrile), since the methanol can increase the formation of the aforementioned PI-PI interactions. Therefore, phenyl phases, such as the phenyl-hexyl, phenyl-butyl, biphenyl, pentafluorophenyl can be used to effectively increase the retention of hydrophilic aromatic metabolites, like for example paracetamol-glucuronide and sulfate (Xiong, Jin et al. 2010), or cyanidine-glucuside-glucuronides (Marczylo, Cooke et al. 2009). Phenyl columns have been successfully used for more lipophilic metabolites as well, like for example cannabinoid glucuronides (Mazur, Lichti et al. 2009).

4.4 Zwitter ionic and hydrophilic interaction chromatography (HILIC)

HILIC is a mode of separation similar to the normal phase chromatography, but it can be performed on an ordinary RP HPLC instrument using a HILIC column and a mobile phase similar to the RP chromatography. In HILIC, the stationary phase is polar and the mobile phase is an aqueous/polar organic solvent, where water (or buffer) plays the role of a stronger eluting solvent. The order of peaks eluting from a HILIC column is reverse compared to RP chromatography, and glucuronides elute after their aglycones. Therefore, HILIC can be used primarily for the increased retention and separation of highly polar analytes. For example, a successful application of HILIC mechanism for morphine glucuronides separation and ionization has been described more than a decade ago (Naidong, Lee et al. 1999). The retention mechanism in underivatized silica is not only hydrophilic, but also hydrophobic and ion-exchange, which may cause a mixed-mode retention, and is sometimes difficult to predict (Nguyen and Schug 2008). In derivatized silica with a zwitterionic bonded phase (commercially known as ZIC-HILIC), there is no ion-exchange mechanism of retention (the zwitterions in stationary phase cancel each other out and there is no net charge of ion-exchange (Ahuja and Dong 2005)). For some phase I metabolites and parent compounds that are highly hydrophobic or less polar than glucuronides, the HILIC column may not provide enough retention and these compounds may be eluted close to the solvent peak. To overcome this issue, a dual column orthogonal approach has been described by (Qin, Zhao et al. 2008), where the estrogens have been derivatized with dansyl chloride (for better ionization) and separated on a RP column, while the polar estrogen conjugates were separated on a ZIC-HILIC column. In step one, the

sample is divided into two fractions: polar and non-polar: first, both columns are in series, and a sample is injected onto a RP column; the minimally retained polar conjugates are eluted from a RP column onto a HILIC column for subsequent separation in the next step, while the non-polar derivatized estrogens remain parked on the RP column. In step two, the mobile phase flows only through the HILIC column, where the polar analytes are separated and eluted to MS. In step three, the flow is directed only through the RP column, where the non-polars are separated and eluted to MS. This 2D orthogonal approach combines the benefits of both RP and HILIC methods and provides an excellent sensitivity (Thomas, Déglon et al. 2010).

5. Mass spectrometry

Glucuronides can be analyzed by LC-MS/MS in a number of different MS fragmentation experiments. The analyst will choose an appropriate MS experiment based on the purpose of the study.

5.1 Ionization of glucuronides

Before an analyte can enter a mass spectrometer and produce a signal, it must be efficiently ionized first. The majority of documented methods for glucuronide LC-MS detection employ the electrospray ionization (ESI) as it is the softest and the most suitable method for ionization without the in-source fragmentation of the relatively weak glucuronide ether or ester bonds (Kadi and Hefnawy 2009). On the other hand, sometimes it is desired to achieve an in-source fragmentation in order to record just one Selected Reaction Monitoring (SRM) trace for the glucuronide and the parent to achieve a better sensitivity (Felli, Martello et al. 2011), (Schwartz, Desai et al. 2006). Of course, in such cases it is imperative to achieve a good chromatographic separation from the parent. The conjugate metabolites (glucuronides included) are usually ionized in positive ionization with electrospray (+ESI) (Keski-Hynnilä, Kurkela et al. 2002; Levsen, Schiebel et al. 2005). Generally, the glucuronides should not be problematic also in the negative ionization mode due to the presence of the acidic carboxylic group on glucuronic moiety with the reported pKa of around 3 (Farrell, Poquet et al. 2011). However, the negative ionization is often less sensitive than the positive one. Furthermore, fragmentation mechanisms in negative ion mode are frequently hard to predict and understand. Moreover, in negative ionization the specificity may be worse than in the positive mode (Pruvost, Théodoro et al. 2008). On many occasions, it is therefore advisable to try to develop a method for glucuronide quantification using the positive ionization mode, especially if the aglycone is a proton-acceptor (a weak base). In positive-ion mode, an abundant fragment $[M + H - 176]+$ is detected in most cases after a glucuronide fragmentation; the neutral loss of 176 Da corresponds to a monodehydrated glucuronic acid. Acyl- or benzyl glucuronides, however, may undergo a loss of 194 Da (glucuronic acid) from the pseudo- or quasi-molecular ions, either in addition to the loss of 176 Da or even exclusively (Levsen, Schiebel et al. 2005).

Sometimes however, the positive ionization mode cannot be used if the aglycone is not a base or does not accept a proton or any other positive adduct-forming ions, like for example Na^+, K^+, NH_4^+ etc. In such cases, it is necessary to use the negative ionization or switch to another type of ionization. If analyte derivatization has been employed to increase the ionization efficiency like for example with steroids or with paracetamol glucuronide (Ohta,

Kawakami et al. 2003), then the negative ionization mode may be even superior in sensitivity to the positive mode because the noise in negative mode may be lower, thereby increasing the signal/noise ratio (Higashi and Shimada 2004).

Atmospheric pressure chemical ionization (APCI) interface may sometimes be beneficial over the ESI if the principally lower sensitivity of APCI is still adequate for the intended study. The APCI may be less prone to matrix effects due to plasma components and salts (Schwartz, Desai et al. 2006), especially if isotope labeled internal standard for glucuronide is not available (Matuszewski, Constanzer et al. 1998; Matuszewski, Constanzer et al. 2003). Furthermore, APCI seems to have a wider dynamic response range than ESI (Ramanathan, Comezoglu et al. 2009).

Novel LC-MS interfaces include APPI (atmospheric photo ionization, which is similar to APCI and may be important for less polar analytes; further, nanospray ionization (NSI) coupled with nano flow LC may provide a superior limit of quantification and reduced matrix effects (Ramanathan, Comezoglu et al. 2009)

5.2 Mass spectrometry operation modes for glucuronide detection

Tandem MS is the key principle for metabolite structure elucidation and quantification (Clarke, Rindgen et al. 2001). There are many layouts and types of mass filters or analyzers for selecting the parent and daughter ions and the collision cells. For example just to name a few major types of mass analyzers: quadrupole/linear ion trap, triple stage quadrupole (QQQ), time of flight (TOF), hybrid instruments (QTOF, QTRAP) and others. For detection and quantification of glucuronides in complex biological matrices, the triple quadrupole mass spectrometer is usually the instrument of choice due to the high selectivity and superior sensitivity (Mullangi, Bhamidipati et al. 2005). Therefore, our brief review will focus on mass experiments performed typically in a QQQ type of instrument.

Precursor ion scan mode is usually used at the start of conjugate identification; aglycone is selected as the product by Q3 mass filter and Q1 is scanning for the possible conjugates that may give rise to the selected parent fragment after a collision induced dissociation (CID). Such an experiment may reveal for example glucuronides, glucosides, sulfates and glutathione conjugates with mass shifts of m/z 176, 80, 162, 305 Da, respectively (Levsen, Schiebel et al. 2005; Ramanathan, Comezoglu et al. 2009).

Constant neutral-loss experiment is the most suitable mode experiment for searching the selected or expected conjugates in a sample as it detects the loss of a neutral fragment. Here, both Q1 and Q3 are scanning at the same time, with a pre-selected mass shift between them. Therefore, the analyst does not need to know the exact mass of the conjugate or its parent (it may undergo an unknown phase I metabolic conversion prior to the conjugation). The result of such an experiment is the *m/z* of the parent molecule before the CID and the resulting mass shift of 176 Da (for glucuronic acid), similar to the precursor ion scan experiment, described in the upper paragraph. In neutral loss experiment and in positive ionization mode, the glucuronides will produce a nominal shift of *m/z* 176 (176.0321 on accurate mass analyzers) (Ramanathan, Comezoglu et al. 2009). In negative ionization, the glucuronides produce ions at *m/z* 175 (anhydrous glucuronic acid) and 113 (a fragment of glucuronic acid) (Fay 2010).

Product ion scan experiment is performed on a single pre-selected ion in Q1, which is fragmented in the collision cell and daughter ions are scanned by Q3. The result of such an experiment is a full scan product ion spectrum, which may also contain unfragmented parent ion (figure 1).

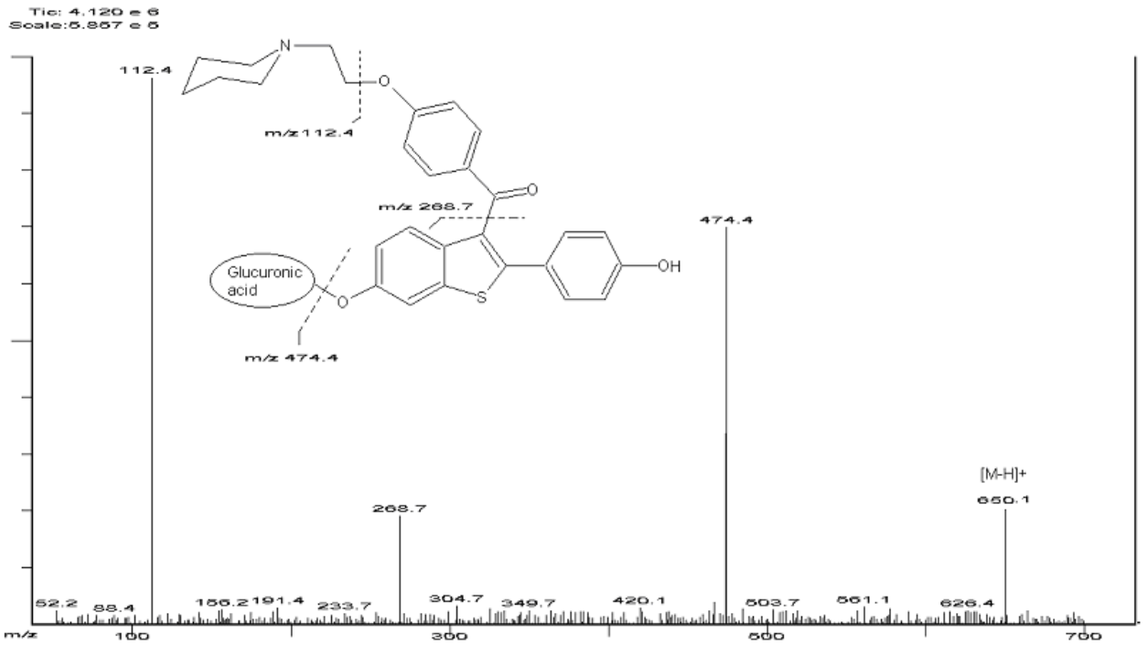


Fig. 1. A product ion scan from raloxifene-6-glucuronide (*m/z* 650.1), aglycone fragment (*m/z* 474.4), phenyl benzotiophene ring (*m/z* 268.7) and N-ethyl-piperidine fragment (*m/z* 112) (Trontelj, Bogataj et al. 2007). With permission.

Usually, the full scan mode does not provide sufficient sensitivity in a triple quadrupole MS for a proper glucuronide detection or quantification in a complex matrix (Fay 2010).

The Selected Reaction Monitoring (SRM) and the Multiple Reaction Monitoring (MRM) modes are the techniques of choice for selective and sensitive glucuronide quantification. Here the Q1 and Q3 are set at the selected *m/z* values for conjugate (glucuronide) and its parent molecule, respectively. Additional specificity for isobaric ions in MS detector can be provided by the high-field asymmetric waveform ion mobility spectrometry (FAIMS) module, which is inserted between the ion source and the high vacuum MS inlet. The principle of this additional separation technique is that the ion mobility at high electric fields can differ according to the shape of the ions. Therefore, FAIMS can separate isomeric or isobaric analytes that the LC and ordinary MS cannot (Hatsis and Kapron 2008). On a model acyl-glucuronide of ifetroban Xia et al. have demonstrated the use of FAIMS for locating the place where the acyl glucuronide is being fragmented to its parent inside the mass spectrometer (Xia and Jemal 2009). FAIMS method is also one of the confirmatory methods for detection of testosterone glucuronide in urine after a hydrolysis and immuno-affinity separation coupled to LC-MS/MS (Fong-Ha and et al. 2010). Guddat et al. reported a better interference removal with FAIMS in an anti-doping testing of human urine, which should effectively extend the post-dose detection time of androgen steroids and their glucuronides (Guddat, Thevis et al. 2009).

5.3 Other mass spectrometry approaches used in glucuronide characterization

The described mass experiments in previous subparagraph can be performed also on some other tandem instruments as well, like for example on ion traps, where the CID happens in time rather than in space, as in triple quads. The sensitivity of ion traps is usually lower

compared to QQQ instruments, however, they tend to be cheaper. Furthermore, with ion traps, MS^n experiments can be performed for possible structure elucidation.

QTRAP, which is a hybrid between a triple quad and an ion trap, can perform all the described tandem mass experiments and more – there is the possibility to perform MS^n experiments (multiple stages of fragmentation) which may be beneficial for glucuronide structure elucidation, but lacks the mass accuracy of a Q-TOF. Information dependent acquisition (IDA) can trigger the product ion scans (MS^2 and MS^3) when a certain ion intensity threshold is reached in full scan or neutral loss scan experiment. Therefore, IDA should shorten the cycle time and provide some structural information in a minimal number of analytical runs.

The mass spectrometers that enable accurate mass measurements in LC/MS are time-of-flight instruments and their hybrids (TOF), Q-TOF, TOF-TOF and Orbitrap or Fourier transform ion cyclotron resonance mass spectrometers (FTICRMS) (Tolonen, Turpeinen et al. 2009). It is noteworthy that an accurate mass instrument like Orbitrap can be used for quantification and glucuronide detection comparable to a middle range QQQ because of the Orbitrap's extreme selectivity due to its high resolution of 15000 and more (mass/FWHM) (Zhang, Yu et al. 2009).

Q-TOF instruments are also capable of tandem mass experiments. These hybrid systems offer an increased mass accuracy and mass resolution of TOF and provide a good fragmentation capability. Furthermore, full scan mode in TOF mass analyzers provides an increased sensitivity compared to a QQQ. Therefore, the product ion scan experiment on Q-TOF offers significant benefits in terms of accuracy and sensitivity, which may help in element and structural elucidation for isomeric glucuronides, like for example multiple estriol glucuronides (Lampinen-Salomonsson, Bondesson et al. 2006). On the other hand, precursor ion scan and neutral loss experiments cannot be performed on a Q-TOF (Hoffmann and Stroobant 2007).

A good example of complimentary approach by different mass spectrometers (QQQ, ion trap, Q-TOF) is presented on a complicated mixture of estriol glucuronide isomers and their structure elucidation (Lampinen-Salomonsson, Bondesson et al. 2006).

5.4 Data acquisition and ion channel cross-talk

For glucuronide quantification, the ion source settings are important for efficient ionization. However, the parameters of quadrupoles and detector in MRM mode are also important for LC-MS selectivity and especially, sensitivity. If analyte concentration level and sensitivity of the instrument permit it, the mass resolution of both Q1 and Q3 should be set to at least one unit (0,7-1.0) Da full width at half maximum (FWHM)). If the concentration level of glucuronides is very low, then increasing the mass window of both quadrupoles to up to 3 units may increase the signal intensity up to 10 times and more (author's own experience) without the equal increase in noise at the high *m/z* values as can be expected for glucuronide conjugates. However, the selectivity of the method may suffer. Nowadays, triple quadrupoles with enhanced resolution exist which offer a resolution of ≤0.1 FWHM and may provide a significant increase in selectivity from the interfering substances (like PPG, PEG) and a minimal loss in sensitivity (3 times lower signals, but lower noise as well) (Yang, Amad et al. 2002).

Another important parameter is the dwell time (the time at which quadrupole stays at one *m/z* value). The greater the dwell time, the better is the sensitivity and the slower rate of data

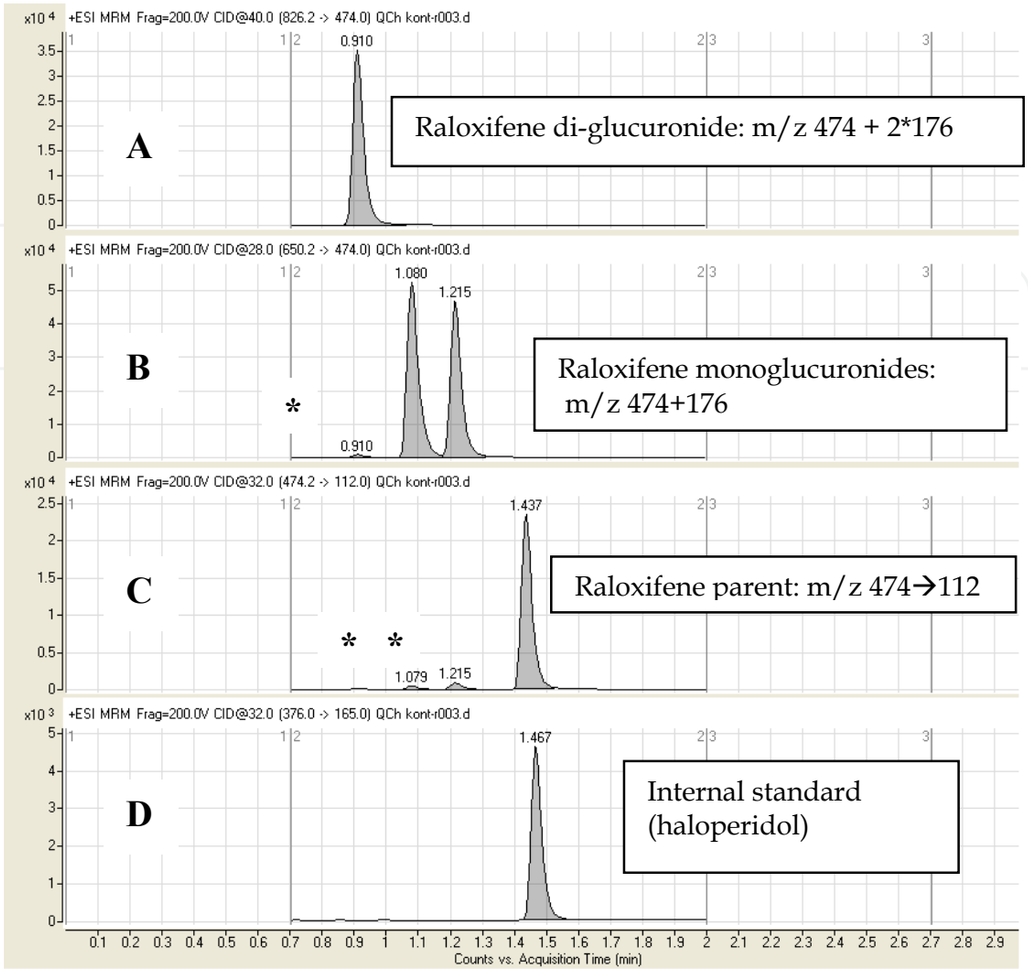


Fig. 2. MRM chromatogram of an extracted urine sample containing raloxifene di-glucuronide (trace A), two isobaric raloxifene monoglucuronides (trace B), parent raloxifene (trace C) and haloperidol (D) as an internal standard. Ion channel cross-talk is evident in traces B in C, where the little peaks in front of analytes (marked as stars *) present cross talk and originate from different analytes (producing the same daughter ion fragments).

acquisition. With shorter dwell times, the probability of ion-channel cross talk also increases. Ion-channel cross talk is a false positive signal in a SRM channel that originates from another analyte. A typical example is the signal of aglycone in the SRM trace of its conjugate (Figure 2, trace C with two peaks originating from cross-talk). Typical values of dwell times are 1 to 250 ms (Lee, Zhu et al. 2011). However, with modern UHPLC columns which can produce very high and narrow peaks (often less than 5 s wide at half height) (Jemal, Ouyang et al. 2010), the data acquisition rate should be fast enough to provide at least 10-15 points per peak. Not only the dwell time, also the cycle time (the summation of dwell times of all MRMs and pause times between them) is extremely important for data acquisition rate. Therefore, for the fastest cycle times with long-enough dwell times it is advisable to use the scheduled MRM mode if ultimate sensitivity is required. Again, an effective and robust chromatographic separation of analytes is vital. In glucuronide analysis, ion channel cross-talk is often observed even with high-end QQQ instruments where the commercial

brochures claim that there is no cross talk even at 1 ms dwell time (author's own experience, see figure 2). This however is of no real concern if the analytes are baseline separated chromatographically.

The detector voltage (or delta EMV as it is sometimes called) has also a big influence on the signal intensity. However, with increasing the detector voltage, the rise in background noise is almost certain as well. Furthermore, the dynamic range of the detector may be narrower with higher voltage and the lifetime of detector may be shortened by using high voltages over longer periods of time.

5.5 Matrix effects

The most problematic matrix components for LC-MS/MS analyses are supposed to be the phospholipids, which are rather lipophilic and therefore, late-eluting (Chambers, Wagrowski-Diehl et al. 2007). The glucuronides on the other hand, are usually more hydrophilic and fast eluting. Yet, they may still be very susceptible to interferences from the co-eluting matrix components (author's own in-house experiences, data not shown). Not only the phospholipids, but also earlier eluting lysophospholipids can cause substantial matrix effects (Xia and Jemal 2009), which may interfere with the quantification of drugs and their metabolites (Jemal, Ouyang et al. 2010). Especially difficult matrices are plasma and urine, where even after a LLE or SPE clean-up, major matrix effects can be observed.

Recovery (RE) and matrix effect (ME) can be calculated from extraction experiments of blank matrix which is pre- and post- spiked by using equations 1 and 2 (Matuszewski, Constanzer et al. 2003), see also paragraph 3.2.

$$RE = \frac{\text{pre - spiked}}{\text{post - spiked}} * 100\% \qquad \text{Eq. 1}$$

$$ME = \left(1 - \frac{\text{post - spiked}}{\text{solvent - only}}\right) * 100\% \qquad \text{Eq. 2}$$

A similar method for calculation of the matrix effect and recovery can also be used with other types of extraction and protein precipitation. Note that the presented method implies the calculation of absolute matrix effect. However, much more important for bioanalytical quantification is the *relative* matrix effect, caused by interindividual variability in the sample matrix (Matuszewski, Constanzer et al. 2003). Therefore, at least 5 matrices from individual donors have to be used to asses the relative matrix effect either by a post spike method presented above or by comparing the slopes of calibration lines in those matrices. The coefficient of variation of slopes should not exceed 4-5% (Matuszewski, Constanzer et al. 2003). The use of an isotope labeled aglycone as an internal standard does not necessarily correct the matrix effect for glucuronide as well, because the retention times between both analytes are supposed to be different and the nature and/or concentration of the co-eluting matrix components are very likely different as well (Taylor 2005). Therefore, to *thoroughly* compensate the matrix effects for glucuronides, their labeled analogues should be used. Even with labeled internal standards, the analyst should be cautious, since the purity of a labeled standard may not be 100% and studies should be performed to confirm the isotope integrity of the label in a sample matrix and during the sample handling. Furthermore, the possible presence of an ion channel cross-talk between the analyte and the labeled standard

SRMs must be investigated as same fragments can be formed from the analyte and the internal standard (Chavez-Eng, Constanzer et al. 2002; Matuszewski, Constanzer et al. 2003). Alternatively, the β-glucuronidase approach (paragraph 3.1) can be used to transform the glucuronide into its parent molecule, for which an isotope labeled analogue may be available. If more than one glucuronide isomers are present in a sample then the β-glucuronidase approach cannot be used directly to quantify each of the present glucuronides. In such cases, the response factors for each glucuronide has to be determined based on isolated β-glucuronidase incubations for each isolated glucuronide. The matrix effects in such cases cannot be overcome with a simple use of the labeled analogue of the parent aglycone, because the quantification of glucuronides has to be performed directly with their MRMs and not *via* their degradation to parent aglycone.

6. Some selected examples

In this section, a brief overview of the current literature is given, regarding the use of a LC-tandem-MS instrument for the quantification of glucuronides in various samples from both *in vitro* and *in vivo* studies, such as microsome and tissue incubations and pharmacokinetic studies or forensic applications (Tables 1, 2).

Analyte	**Matrix**	**Sample preparation**	**separation technique**	**Ionization / type of MS**	**Range**	**Reference**
Nicotine-N glucuronide[D]	microsome incubation	PP with perchloric acid		(+) ESI / QQQ	10-1000 nM	(Guo, Zhou et al. 2011)
ethyl-glucuronide	human hair	water extraction followed by SPE on activated carbon	UHPLC	(-) ESI QQQ	2pg/g – 300 pg/g hair	(Kronstrand, Brinkhagen et al. 2011)
raloxifene O-glucuronides	human urine	RP polymeric SPE	UHPLC C18	(+)ESI / QQQ	1 -4800 nM*	(Trdan, Roskar et al. 2011)
morphine-O-glucuronides	human urine	RP (C18) SPE	HPLC-HILIC	(+)ESI / TOF	50-5000 µg/L	(Kolmonen, Leinonen et al. 2010)
sorafenib-O-glucuronide[D]	mouse plasma and liver homogenate	PP	HPLC C18	(+)ESI / QQQ	1-500 µg/L	(Sparidans, Vlaming et al. 2009)
rampiril-acyl-glucuronide	plasma	RP (C18) SPE	HPLC C18	(+)ESI / QQQ	1-500 µg/L	(Tan, Jin et al. 2009)
cediranib-N^+ -glucuronide[D]	micorosomes/ feces	PP / SPE	HPLC C18	(+)ESI / ion trap	?	(Lenz, Spear et al. 2010)
THC-acyl-glucuronide	blood, liver, muscle, urine, bile	ACN homogenization /LLE with ethylacetate/hexane	HPLC C18	(+)ESI	100-1000 µg/L	(Gronewold and Skopp 2011)

[D] signifies the use of a labeled internal standard for glucuronide
* The range was divided into two calibration curves

Table 1. Some selected recent examples of a direct glucuronide measurement approach. For descriptions of abbreviations, please see the text.

Analyte	Matrix	Sample preparation	separation technique	Ionization / type of MS	Range	Reference
SN-38-O-glucuronide	plasma	PP / β-glucuronid.	HPLC	(+) ESI QTRAP	0.5-200 µg/L	(Zhang, Dutschman et al. 2009)
ezetimibe-O-glucuronide	feces	LLE / tert butyl ether	HPLC C18	(-) APCI /QQQ	0.1-20 mg/L for total ezetimibe	(Oswald, Scheuch et al. 2006)
quercetine-O-glucuronide	rat brain tissue	β-glucuronid. / LLE (ethyl acetate)	HPLC C18	(-) ESI / QQQ	?	(Ishisaka, Ichikawa et al. 2011)
bisphenol A- O-glucuronide	human urine	β-glucuronid. / PP / On-line SPE	HPLC C18	(-) APCI / QTRAP	1-500 pg/µL	(Völkel, Kiranoglu et al. 2008)

Table 2. Some selected recent examples of an indirect glucuronide measurements (by deconjugation). For descriptions of abbreviations, please see the text.

7. Summary

The glucuronide metabolites are important both for toxicology and for pharmacokinetics of many drugs and xenobiotics. Even though glucuronides often lack the pharmacological activity, after de-conjugation, the free aglycone can regain that activity and may present an environmental burden. Furthermore, glucuronides may be used as markers of the past substance exposure, like for example the ethyl-glucuronide in body hair for alcohol abuse or the cannabinoid-glucuronides for cannabis abuse. Therefore, quantification of glucuronide metabolites in biological matrices may be very important. However, due to their highly hydrophilic nature, the separation of glucuronides from the matrix (body fluids, hair, and environmental samples) may be difficult. For that purpose, many sample preparation techniques can be used, including the liquid extraction, solid phase extraction, dried blood spots, protein precipitation, direct injection, on-line solid phase extraction and others. Furthermore, some glucuronides, such as acyl- and N-glucuronides can be highly unstable. Glucuronides can be measured either by a direct or an indirect approach. With the latter, the glucuronides are cleaved back to their parent aglycone by enzymes known as the β-glucuronidases, afterwards the freed aglycone is quantified according to its calibration curve. With direct measurement, unchanged glucuronides are quantified by their MRMs, where some significant benefits can be expected, namely, quicker sample preparation, better accuracy and precision, and selectivity towards glucuronide isomers. Tandem mass spectrometry is the corner stone for direct glucuronide quantification because it offers both exceptional selectivity and sensitivity. However, this approach requires the use of authentic glucuronide standards. This necessity can be avoided with the semi-quantitative approach, where the response factor for glucuronide is used to quantify it against the calibration curve for the parent. The recent advancement of mass spectrometers' speed and sensitivity coupled with higher mass accuracy promises an even easier measurement of glucuronides in complex biological matrices, allowing a faster method development, robust response, and quicker sample preparation. Notwithstanding the described improvements, the glucuronide identification and quantification in difficult matrices will still remain a challenge for the analyst.

8. References

Abu-Rabie, P. and N. Spooner (2009). "Direct quantitative bioanalysis of drugs in dried blood spot samples using a thin-layer chromatography mass spectrometer interface." *Anal Chem.* 81(24): 10275-10284.

Ahuja, S. and M. W. Dong (2005). *Handbook of pharmaceutical analysis by HPLC.* Amsterdam; Boston, Elsevier Academic Press.

Bakhtiar, R. and T. K. Majumdar "Tracking problems and possible solutions in the quantitative determination of small molecule drugs and metabolites in biological fluids using liquid chromatography-mass spectrometry." *Journal of Pharmacological and Toxicological Methods* 55(3): 227-243.

Barfield, M. and R. Wheller (2010). "Use of Dried Plasma Spots in the Determination of Pharmacokinetics in Clinical Studies: Validation of a Quantitative Bioanalytical Method." *Analytical Chemistry* 83(1): 118-124.

Belanger, A. S., P. Caron, et al. (2009). "Glucuronidation of the antiretroviral drug efavirenz by UGT2B7 and an in vitro investigation of drug-drug interaction with zidovudine." *Drug Metab Dispos.* 37(9): 1793-1796. Epub 2009 Jun 1791.

Bentayeb, K., R. Batlle, et al. (2008). "Determination of bile acids in human serum by on-line restricted access material-ultra high-performance liquid chromatography-mass spectrometry." *Journal of Chromatography B* 869(1-2): 1-8.

Blount, B. C., K. E. Milgram, et al. (2000). "Quantitative Detection of Eight Phthalate Metabolites in Human Urine Using HPLC−APCI-MS/MS." *Analytical Chemistry* 72(17): 4127-4134.

Briggs, B. S., P. J. Baker, et al. (1999). "Microbial process for preparation of glucuronides of raloxifene." *Journal of Industrial Microbiology & Biotechnology* 23(3): 194-197.

Burkon, A. and V. Somoza (2008). "Quantification of free and protein-bound trans-resveratrol metabolites and identification of trans-resveratrol-C/O-conjugated diglucuronides – Two novel resveratrol metabolites in human plasma." *Molecular Nutrition & Food Research* 52(5): 549-557.

Chambers, E., D. M. Wagrowski-Diehl, et al. (2007). "Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses." *Journal of Chromatography B* 852(1-2): 22-34.

Chambers, E., D. M. Wagrowski-Diehl, et al. (2007). "Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses." *J Chromatogr B Analyt Technol Biomed Life Sci* 852(1-2): 22-34.

Chavez-Eng, C. M., M. L. Constanzer, et al. (2002). "High-performance liquid chromatographic-tandem mass spectrometric evaluation and determination of stable isotope labeled analogs of rofecoxib in human plasma samples from oral bioavailability studies." *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* 767(1): 117-129.

Choi, R. J., I. J. Ha, et al. (2010). "Biotransformation of flavonoid-7-O-glucuronides by β-glucuronidases." *Natural Product Sciences* 16(1): 1-5.

Clarke, N. J., D. Rindgen, et al. (2001). "Peer Reviewed: Systematic LC/MS Metabolite Identification in Drug Discovery." *Analytical Chemistry* 73(15): 430 A-439 A.

Cooper, J., W. Currie, et al. (2001). "Comparison of the efficiences of enzymatic and chemical hydrolysis of (nortestosterone and diethylstilboestrol) glucuronides in bovine

urine." *Journal of Chromatography B: Biomedical Sciences and Applications* 757(2): 221-227.

Coulier, L., R. Bas, et al. (2006). "Simultaneous Quantitative Analysis of Metabolites Using Ion-Pair Liquid Chromatography−Electrospray Ionization Mass Spectrometry." *Analytical Chemistry* 78(18): 6573-6582.

de Loor, H., M. Naesens, et al. (2008). "Stability of mycophenolic acid and glucuronide metabolites in human plasma and the impact of deproteinization methodology." *Clinica Chimica Acta* 389(1-2): 87-92.

Deglon, J., A. Thomas, et al. (2009). "On-line desorption of dried blood spot: A novel approach for the direct LC/MS analysis of micro-whole blood samples." *J Pharm Biomed Anal.* 49(4): 1034-1039. Epub 2009 Feb 1013.

DongHyun, K. I. M., J. I. N. YoungHo, et al. (1995). "Purification and Characterization of β-Glucuronidase from Escherichia coli HGU-3,a Human Intestinal Bacterium." *Biological & pharmaceutical bulletin* 18(9): 1184-1188 %U http://ci.nii.ac.jp/naid/110003641017/en/.

Elbarbry, F. A. and A. S. Shoker (2007). "Therapeutic drug measurement of mycophenolic acid derivatives in transplant patients." *Clinical Biochemistry* 40(11): 752-764.

Farrell, T., L. Poquet, et al. (2011). "Characterization of hydroxycinnamic acid glucuronide and sulfate conjugates by HPLC-DAD-MS2: Enhancing chromatographic quantification and application in Caco-2 cell metabolism." *Journal of Pharmaceutical and Biomedical Analysis* 55(5): 1245-1254.

Fay, L.-B. (2010). *Mass spectometry and nutrition*. [S.l.], Royal Soc Of Chemistry.

Fayet, A., A. Béguin, et al. (2009). "A LC-tandem MS assay for the simultaneous measurement of new antiretroviral agents: Raltegravir, maraviroc, darunavir, and etravirine." *Journal of Chromatography B* 877(11-12): 1057-1069.

Felli, M., S. Martello, et al. (2011). "LC-MS-MS method for simultaneous determination of THCCOOH and THCCOOH-glucuronide in urine: Application to workplace confirmation tests." *Forensic Science International* 204(1-3): 67-73.

Ferreirós Bouzas, N., S. Dresen, et al. (2009). "Determination of basic drugs of abuse in human serum by online extraction and LC–MS/MS." *Analytical and Bioanalytical Chemistry* 395(8): 2499-2507.

Fong-Ha, L. and et al. (2010). "Final report on CCQM-K69 key comparison: Testosterone glucuronide in human urine." *Metrologia* 47(1A): 08018.

Fontanals, N., R. M. Marcé, et al. (2010). "Mixed-mode ion-exchange polymeric sorbents: dual-phase materials that improve selectivity and capacity." *TrAC Trends in Analytical Chemistry* 29(7): 765-779.

Frandsen, H. (2007). "Deconjugation of N-glucuronide conjugated metabolites with hydrazine hydrate - Biomarkers for exposure to the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)." *Food and Chemical Toxicology* 45(5): 863-870.

Gao, S., S. Bhoopathy, et al. (2006). "Evaluation of volatile ion-pair reagents for the liquid chromatography-mass spectrometry analysis of polar compounds and its application to the determination of methadone in human plasma." *Journal of Pharmaceutical and Biomedical Analysis* 40(3): 679-688.

Gomes, R. L., W. Meredith, et al. (2009). "Analysis of conjugated steroid androgens: Deconjugation, derivatisation and associated issues." *Journal of Pharmaceutical and Biomedical Analysis* 49(5): 1133-1140.

Gronewold, A. and G. Skopp (2011). "A preliminary investigation on the distribution of cannabinoids in man." *Forensic Science International* 210(1-3): e7-e11.

Gu, L., M. Laly, et al. (2005). "Isoflavone Conjugates Are Underestimated in Tissues Using Enzymatic Hydrolysis." *Journal of Agricultural and Food Chemistry* 53(17): 6858-6863.

Guddat, S., M. Thevis, et al. (2009). "Application of FAIMS to anabolic androgenic steroids in sport drug testing." *Drug Testing and Analysis* 1(11-12): 545-553.

Guo, J., D. Zhou, et al. (2011). "Liquid chromatography-tandem mass spectrometry method for measurement of nicotine N-glucuronide: A marker for human UGT2B10 inhibition." *Journal of Pharmaceutical and Biomedical Analysis* 55(5): 964-971.

Hatsis, P. and J. T. Kapron (2008). "A review on the application of high-field asymmetric waveform ion mobility spectrometry (FAIMS) in drug discovery." *Rapid Communications in Mass Spectrometry* 22(5): 735-738.

Higashi, T. and K. Shimada (2004). "Derivatization of neutral steroids to enhance their detection characteristics in liquid chromatography–mass spectrometry." *Analytical and Bioanalytical Chemistry* 378(4): 875-882.

Himeno, M., Y. Hashiguchi, et al. (1974). "β-Glucuronidase of Bovine Liver." *Journal of Biochemistry* 76(6): 1243-1252.

Hochner-Celnikier, D. (1999). "Pharmacokinetics of raloxifene and its clinical application." *Eur J Obstet Gynecol Reprod Biol.* 85(1): 23-29.

Hoffmann, E. d. and V. Stroobant (2007). *Mass spectrometry : principles and applications*. Chichester, J. Wiley & Sons.

Ishisaka, A., S. Ichikawa, et al. (2011). "Accumulation of orally administered quercetin in brain tissue and its antioxidative effects in rats." *Free Radical Biology and Medicine* In Press, Corrected Proof.

Isobe, T., S. Serizawa, et al. (2006). "Horizontal distribution of steroid estrogens in surface sediments in Tokyo Bay." *Environmental Pollution* 144(2): 632-638.

Jemal, M., Z. Ouyang, et al. (2010). "Systematic LC-MS/MS bioanalytical method development that incorporates plasma phospholipids risk avoidance, usage of incurred sample and well thought-out chromatography." *Biomedical Chromatography* 24(1): 2-19.

Kadi, A. A. and M. M. Hefnawy (2009). "Biological Fluids: Glucuronides from LC/MS." *Encyclopedia of Chromatography, Third Edition*: 203 - 209.

Kakimoto, K., A. Toriba, et al. (2008). "Direct measurement of the glucuronide conjugate of 1-hydroxypyrene in human urine by using liquid chromatography with tandem mass spectrometry." *Journal of Chromatography B* 867(2): 259-263.

Kaklamanos, G., G. A. Theodoridis, et al. (2011). "Determination of anabolic steroids in bovine serum by liquid chromatography-tandem mass spectrometry." *Journal of Chromatography B* 879(2): 225-229.

Kemp, D. C., P. W. Fan, et al. (2002). "Characterization of raloxifene glucuronidation in vitro: contribution of intestinal metabolism to presystemic clearance." *Drug Metab Dispos.* 30(6): 694-700.

Keski-Hynnilä, H., M. Kurkela, et al. (2002). "Comparison of Electrospray, Atmospheric Pressure Chemical Ionization, and Atmospheric Pressure Photoionization in the

Identification of Apomorphine, Dobutamine, and Entacapone Phase II Metabolites in Biological Samples." *Analytical Chemistry* 74(14): 3449-3457.

Kim, H. S. and I. W. Wainer (2005). "The covalent immobilization of microsomal uridine diphospho-glucuronosyltransferase (UDPGT): Initial synthesis and characterization of an UDPGT immobilized enzyme reactor for the on-line study of glucuronidation." *Journal of Chromatography B* 823(2): 158-166.

Kolmonen, M., A. Leinonen, et al. (2010). "Hydrophilic interaction liquid chromatography and accurate mass measurement for quantification and confirmation of morphine, codeine and their glucuronide conjugates in human urine." *Journal of Chromatography B* 878(29): 2959-2966.

Korfmacher, W. A. (2009). "Using Mass Spectrometry for Drug Metabolism Studies." *Taylor and Francis*.

Kronstrand, R., L. Brinkhagen, et al. (2011). "Ethyl glucuronide in human hair after daily consumption of 16 or 32 g of ethanol for 3 months." *Forensic Science International* In Press, Corrected Proof.

Lahaie, M., J.-N. Mess, et al. (2010). "Elimination of LC–MS/MS matrix effect due to phospholipids using specific solid-phase extraction elution conditions." *Bioanalysis* 2(6): 1011-1021.

Lampinen-Salomonsson, M., U. Bondesson, et al. (2006). "Differentiation of estriol glucuronide isomers by chemical derivatization and electrospray tandem mass spectrometry." *Rapid Communications in Mass Spectrometry* 20(9): 1429-1440.

Lee, M. S., M. Zhu, et al. (2011). "Mass spectrometry in drug metabolism and disposition basic principles and applications." from http://site.ebrary.com/id/10456208.

Lenz, E. M., M. Spear, et al. (2010). "Characterisation and identification of the human N+-glucuronide metabolite of cediranib." *Journal of Pharmaceutical and Biomedical Analysis* 53(3): 526-536.

Levsen, K., H.-M. Schiebel, et al. (2005). "Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview." *Journal of Chromatography A* 1067(1-2): 55-72.

Loser, S. V., J. Meyer, et al. (1996). "Morphine-6-O-beta-D-glucuronide but not morphine-3-O-beta-D-glucuronide binds to mu-, delta- and kappa- specific opioid binding sites in cerebral membranes." *Naunyn Schmiedebergs Arch Pharmacol* 354(2): 192-197.

Lu, J., B. D. Palmer, et al. (2003). "Thalidomide Metabolites in Mice and Patients with Multiple Myeloma." *Clinical Cancer Research* 9(5): 1680-1688.

Marczylo, T., D. Cooke, et al. (2009). "Pharmacokinetics and metabolism of the putative cancer chemopreventive agent cyanidin-3-glucoside in mice." *Cancer Chemotherapy and Pharmacology* 64(6): 1261-1268.

Marín, J. M., E. Gracia-Lor, et al. (2009). "Application of ultra-high-pressure liquid chromatography-tandem mass spectrometry to the determination of multi-class pesticides in environmental and wastewater samples: Study of matrix effects." *Journal of Chromatography A* 1216(9): 1410-1420.

Matuszewski, B. K., M. L. Constanzer, et al. (1998). "Matrix Effect in Quantitative LC/MS/MS Analyses of Biological Fluids: A Method for Determination of Finasteride in Human Plasma at Picogram Per Milliliter Concentrations." *Analytical Chemistry* 70(5): 882-889.

Matuszewski, B. K., M. L. Constanzer, et al. (2003). "Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS." *Anal Chem.* 75(13): 3019-3030.

Maurel, P. E. S. F.-G., Michael W.H. Coughtrie, Mohamed Ouzzine and Jacques Magdalou (2010). Hepatocytes methods and protocols. New York, Humana: 309-326.

Mazur, A., C. F. Lichti, et al. (2009). "Characterization of human hepatic and extrahepatic UDP-glucuronosyltransferase enzymes involved in the metabolism of classic cannabinoids." *Drug Metab Dispos.* 37(7): 1496-1504. Epub 2009 Apr 1491.

McMaster, M. C. (2007). *HPLC : a practical user's guide*. Hoboken [NJ, USA], Wiley-Interscience.

Mei, H. and R. A. Morrison (2009). PK Principles and PK/PD Applications. *Using Mass Spectrometry for Drug Metabolism Studies, Second Edition*, CRC Press: 59-98.

Mullangi, R., R. K. Bhamidipati, et al. (2005). "Bioanalytical Aspects in Characterization and Quantification of Glucuronide Conjugates in Various Biological Matrices." *Current Pharmaceutical Analysis* 1(3): 251-264.

Naidong, W., J. W. Lee, et al. (1999). "Simultaneous assay of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human plasma using normal-phase liquid chromatography-tandem mass spectrometry with a silica column and an aqueous organic mobile phase." *Journal of Chromatography B: Biomedical Sciences and Applications* 735(2): 255-269.

Nguyen, H. P. and K. A. Schug (2008). "The advantages of ESI-MS detection in conjunction with HILIC mode separations: Fundamentals and applications." *Journal of Separation Science* 31(9): 1465-1480.

Ohta, M., N. Kawakami, et al. (2003). "Analysis of acetaminophen glucuronide conjugate accompanied by adduct ion production by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry." *Journal of Pharmaceutical and Biomedical Analysis* 30(6): 1759-1764.

Okura, T., N. Komiyama, et al. (2007). "Comparative measurement of spinal CSF microdialysate concentrations and concomitant antinociception of morphine and morphine-6[beta]-glucuronide in rats." *Life Sciences* 80(14): 1319-1326.

Oswald, S., E. Scheuch, et al. (2006). "A LC-MS/MS method to quantify the novel cholesterol lowering drug ezetimibe in human serum, urine and feces in healthy subjects genotyped for SLCO1B1." *Journal of Chromatography B* 830(1): 143-150.

Paradkar, A. R. (2008). "Biopharmaceutics and Pharmacokinetics."

Pozo, O. J., P. Van Eenoo, et al. (2008). "Direct quantification of steroid glucuronides in human urine by liquid chromatography-electrospray tandem mass spectrometry." *Journal of Chromatography A* 1183(1-2): 108-118.

Pruvost, A., F. Théodoro, et al. (2008). "Specificity enhancement with LC-positive ESI-MS/MS for the measurement of nucleotides: application to the quantitative determination of carbovir triphosphate, lamivudine triphosphate and tenofovir diphosphate in human peripheral blood mononuclear cells." *Journal of Mass Spectrometry* 43(2): 224-233.

Pu, F., A. R. McKinney, et al. (2004). "Direct detection of boldenone sulfate and glucuronide conjugates in horse urine by ion trap liquid chromatography-mass spectrometry." *Journal of Chromatography B* 813(1-2): 241-246.

Qin, F., Y. Y. Zhao, et al. (2008). "Column-switching reversed phase-hydrophilic interaction liquid chromatography/tandem mass spectrometry method for determination of free estrogens and their conjugates in river water." *Analytica Chimica Acta* 627(1): 91-98.

Rafferty, J. L., L. Zhang, et al. (2007). "Retention Mechanism in Reversed-Phase Liquid Chromatography: A Molecular Perspective." *Analytical Chemistry* 79(17): 6551-6558.

Ramanathan, R., S. N. Comezoglu, et al. (2009). Metabolite Identification Strategies and Procedures. *Using Mass Spectrometry for Drug Metabolism Studies, Second Edition*, CRC Press: 127-203.

Rosenfeld, J. M. (2004). *Sample preparation for hyphenated analytical techniques*. Oxford; Boca Raton, Blackwell ; CRC Press.

Sanceau, J. Y., D. Larouche, et al. (2007). "Synthesis and deuterium labelling of the pure selective estrogen receptor modulator (SERM) acolbifene glucuronides." *Journal of Labelled Compounds & Radiopharmaceuticals* 50(3-4): 197-206.

Schwartz, M. S., R. B. Desai, et al. (2006). "Determination of a prostaglandin D2 antagonist and its acyl glucuronide metabolite in human plasma by high performance liquid chromatography with tandem mass spectrometric detection--A lack of MS/MS selectivity between a glucuronide conjugate and a phase I metabolite." *Journal of Chromatography B* 837(1-2): 116-124.

Shipkova, M., V. W. Armstrong, et al. (2003). "Acyl Glucuronide Drug Metabolites: Toxicological and Analytical Implications." *Therapeutic Drug Monitoring* 25(1): 1-16.

Silvestro, L., M. Gheorghe, et al. (2011). "Development and validation of an HPLC–MS/MS method to quantify clopidogrel acyl glucuronide, clopidogrel acid metabolite, and clopidogrel in plasma samples avoiding analyte back-conversion." *Analytical and Bioanalytical Chemistry* 401(3): 1023-1034.

Smith, F. P. and S. A. Athanaselis (2005). *Handbook of forensic drug analysis*. Amsterdam; Boston, Elsevier Academic Press.

Soars, M. G., E. L. Mattiuz, et al. (2002). "Biosynthesis of drug glucuronides for use as authentic standards." *J Pharmacol Toxicol Methods* 47(3): 161-168.

Sparidans, R. W., M. L. H. Vlaming, et al. (2009). "Liquid chromatography-tandem mass spectrometric assay for sorafenib and sorafenib-glucuronide in mouse plasma and liver homogenate and identification of the glucuronide metabolite." *Journal of Chromatography B* 877(3): 269-276.

Spooner, N., R. Lad, et al. (2009). "Dried Blood Spots as a Sample Collection Technique for the Determination of Pharmacokinetics in Clinical Studies: Considerations for the Validation of a Quantitative Bioanalytical Method." *Analytical Chemistry* 81(4): 1557-1563.

Stolker, A. M., W. Niesing, et al. (2004). "Liquid chromatography with triple-quadrupole and quadrupole-time-of-flight mass spectrometry for the determination of micro-constituents – a comparison." *Analytical and Bioanalytical Chemistry* 378(7): 1754-1761.

Sunkara, M. and M. J. M. Wells (2010). "Phase II pharmaceutical metabolites acetaminophen glucuronide and acetaminophen sulfate in wastewater." *Environmental Chemistry* 7(1): 111-122.

Takats, Z., J. M. Wiseman, et al. (2004). "Mass spectrometry sampling under ambient conditions with desorption electrospray ionization." *Science*. 306(5695): 471-473.

Tan, A., W. Jin, et al. (2009). "Bioanalytical method development and validation using incurred samples--Simultaneous quantitation of ramipril and ramiprilat in human EDTA plasma by LC-MS/MS." *Journal of Chromatography B* 877(29): 3673-3680.

Taylor, P. J. (2005). "Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry." *Clinical Biochemistry* 38(4): 328-334.

Telepchak, M. J., T. F. August, et al., Eds. (2010). *Forensic and Clinical Applications of Solid Phase Extraction*. Totowa, New Jersey, Humana Press Inc.

Tephly, T. R., M. D. Green, et al. (1997). Metabolism of Endobiotics and Xenobiotics by UDP-Glucuronosyltransferase. *Advances in Pharmacology*. G. E. David S. Goldstein and M. Richard, Academic Press. Volume 42: 343-346.

Thomas, A., J. Déglon, et al. (2010). "On-line desorption of dried blood spots coupled to hydrophilic interaction/reversed-phase LC/MS/MS system for the simultaneous analysis of drugs and their polar metabolites." *Journal of Separation Science* 33(6-7): 873-879.

Tolonen, A., M. Turpeinen, et al. (2009). "Liquid chromatography-mass spectrometry in in vitro drug metabolite screening." *Drug Discovery Today* 14(3-4): 120-133.

Trdan, T., R. Roskar, et al. (2011). "Determination of raloxifene and its glucuronides in human urine by liquid chromatography-tandem mass spectrometry assay." *Journal of Chromatography B* 879(23): 2323-2331.

Trontelj, J., M. Bogataj, et al. (2007). "Development and validation of a liquid chromatography-tandem mass spectrometry assay for determination of raloxifene and its metabolites in human plasma." *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* 855(2): 220-227.

Völkel, W., M. Kiranoglu, et al. (2008). "Determination of free and total bisphenol A in human urine to assess daily uptake as a basis for a valid risk assessment." *Toxicology Letters* 179(3): 155-162.

Weinmann, W., S. Vogt, et al. (2000). "Simultaneous determination of THC-COOH and THC-COOH-glucuronide in urine samples by LC/MS/MS." *Forensic Science International* 113(1-3): 381-387.

Wen, Z., S. T. Stern, et al. (2006). "Structural Characterization of Anti-HIV Drug Candidate PA-457 [3-O-(3′,3′-Dimethylsuccinyl)-betulinic acid] and Its Acyl Glucuronides in Rat Bile and Evaluation of in Vitro Stability in Human and Animal Liver Microsomes and Plasma." *Drug Metabolism and Disposition* 34(9): 1436-1442.

Wong, P., R. Pham, et al. (2010). "Increasing efficiency for dried blood spot analysis: prospects for automation and simplified sample analysis." *Bioanalysis* 2(11): 1787-1789.

Xia, Y.-Q. and M. Jemal (2009). "High-Field Asymmetric Waveform Ion Mobility Spectrometry for Determining the Location of In-Source Collision-Induced Dissociation in Electrospray Ionization Mass Spectrometry." *Analytical Chemistry* 81(18): 7839-7843.

Xia, Y.-Q. and M. Jemal (2009). "Phospholipids in liquid chromatography/mass spectrometry bioanalysis: comparison of three tandem mass spectrometric techniques for monitoring plasma phospholipids, the effect of mobile phase composition on phospholipids elution and the association of phospholipids with matrix effects." *Rapid Communications in Mass Spectrometry* 23(14): 2125-2138.

Xiong, W. J., H. Jin, et al. (2010). "Evaluating human liver reserve function by measuring serum concentrations of phenacetin and its metabolites." *Journal of Digestive Diseases* 11(6): 358-363.

Yang, L., M. a. Amad, et al. (2002). "Investigation of an enhanced resolution triple quadrupole mass spectrometer for high-throughput liquid chromatography/ tandem mass spectrometry assays." *Rapid Communications in Mass Spectrometry* 16(21): 2060-2066.

Yau, W.-P., A. Vathsala, et al. (2004). "Simple reversed-phase ion-pair liquid chromatography assay for the simultaneous determination of mycophenolic acid and its glucuronide metabolite in human plasma and urine." *Journal of Chromatography B* 805(1): 101-112.

Ye, X., Z. Kuklenyik, et al. (2005). "Automated On-Line Column-Switching HPLC-MS/MS Method with Peak Focusing for the Determination of Nine Environmental Phenols in Urine." *Analytical Chemistry* 77(16): 5407-5413.

Yu, B., X. Zhu, et al. (2000). "A Novel and Effective Procedure for the Preparation of Glucuronides." *Organic Letters* 2(16): 2539-2541.

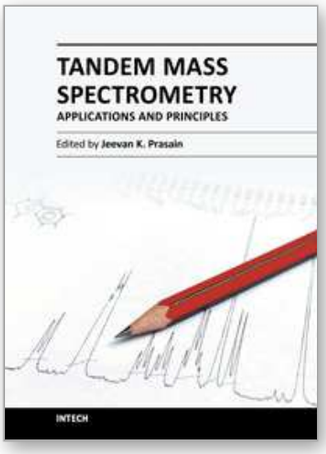
Yu, C., C. L. Chen, et al. (2007). "A rapid method for quantitatively estimating metabolites in human plasma in the absence of synthetic standards using a combination of liquid chromatography/mass spectrometry and radiometric detection." *Rapid Communications in Mass Spectrometry* 21(4): 497-502.

Yu, N. H., E. N. M. Ho, et al. (2005). "Screening of anabolic steroids in horse urine by liquid chromatography-tandem mass spectrometry." *Journal of Pharmaceutical and Biomedical Analysis* 37(5): 1031-1038.

Zhang, D., M. Zhu, et al. (2008). Drug metabolism in drug design and development : basic concepts and practice. Hoboken, Wiley-Interscience.

Zhang, N. R., S. Yu, et al. (2009). "Quantitation of small molecules using high-resolution accurate mass spectrometers – a different approach for analysis of biological samples." *Rapid Communications in Mass Spectrometry* 23(7): 1085-1094.

Zhang, W., G. E. Dutschman, et al. (2009). "Quantitation of Irinotecan and its two major metabolites using a liquid chromatography-electrospray ionization tandem mass spectrometric." *Journal of Chromatography B* 877(27): 3038-3044.



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Tandem Mass Spectrometry - Applications and Principles presents comprehensive coverage of theory, instrumentation and major applications of tandem mass spectrometry. The areas covered range from the analysis of drug metabolites, proteins and complex lipids to clinical diagnosis. This book serves multiple groups of audiences; professional (academic and industry), graduate students and general readers interested in the use of modern mass spectrometry in solving critical questions of chemical and biological sciences.

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