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Quantification of conjugated metabolites of drugs in biological matrices after the hydrolysis with β-glucuronidase and sufatase: a review of bio-analytical methods

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ABSTRACT: Glucuronidation and sulfation represent two major pathways in phase II drug metabolism in humans and other mammalian species. The great majority of drugs, for example, polyphenols, flavonoids and anthraquinones, could be transformed into sulfated and glucuronidated conjugates simultaneously and extensively *in vivo*. The pharmacological activities of drug conjugations are normally decreased compared with those of their free forms. However, some drug conjugates may either bear biological activities themselves or serve as excellent sources of biologically active compounds. As the bioactivities of drugs are thought to be relevant to the kinetics of their conjugates, it is essential to study the pharmacokinetic behaviors of the conjugates in more detail. Unfortunately, the free forms of drugs cannot be detected directly in most cases if their glucuronides and sulfates are the predominant forms in biological samples. Nevertheless, an initial enzymatic hydrolysis step using β-glucuronidase and/or sulfatase is usually performed to convert the glucuronidated and/or sulfated conjugates to their free forms prior to the extraction, purification and other subsequent analysis steps in the literature. This review provides fundamental information on drug metabolism pathways, the bio-analytical strategies for the quantification of various drug conjugates, and the applications of the analytical methods to pharmacokinetic studies. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: conjugated metabolites; β-glucuronidase; sulfatase; hydrolysis; pharmacokinetics; review

Introduction

Compounds like drugs and other xenobiotics have various pharmacological and toxic effects, which can be influenced by the processes of absorption, metabolism and elimination after being administered into humans or other mammals. Drugs moving into, through, and out of the body can be illustrated by pharmacokinetic processes, that is, absorption, distribution, metabolism and excretion, which determine the concentrations of drugs *in vivo*. This has been a hot research area in the last two decades, with a large number of innovative and excellent researches published.

Drug metabolism mainly consists of phase I and phase II reactions. Phase I reactions comprise oxidation, reduction, hydrolysis, cyclization and decyclization with the incorporation of oxygen or the removal of hydrogen. In this process, a functional group (e.g. -OH, -SH, $-NH_2$, -NH, -COOH) is often introduced to the molecules of the parent drugs. Nevertheless, phase II reactions are usually known as conjugation reactions, and the drugs are basically conjugated with glucuronic acids, sulfonates, glutathione and amino acids. The reactions of the two phases are interrelated. The role of phase I reactions is to modify a foreign substance to make it susceptible to conjugation with a highly polar species via phase II reactions. However, if a drug can undergo phase II metabolism directly, phase I metabolism may make a minor contribution to the drug clearance.

The determination of drug concentrations in plasma, urine or bile is the most important step ahead of any subsequent work in pharmacokinetic researches. The quantification in complex biological matrices is challenged by two influence factors: (1) a lack of analytical methods sensitive enough for the determination of low drug concentrations in biological samples; and

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Abbreviations used: B3G, buprenorphine-3-β-D-glucuronide; DAD, diode array detecor; ECD, electrochemical detector; FLD, fluorescence detector; GLU, β-glucuronidase; LLE, liquid–liquid extraction; M1, 1-[4-aminomethyl-4-(3-chlorophenyl)-cyclohexyl]-tetrahydro-pyrimidin-2-one; M-7-S, maackiain-sulfate; M-7-G, maackiain-glucuronide; SULF, sulfatase; PPT, protein precipitation; SIM, selected ion monitoring; SPE, solid-phase extraction; UDPGA, uridine-5′-diphospho-α-D-glucuronic acid; UV, ultraviolet absorption.

(2) the nonexistence of the free forms of some drugs that are transformed into other metabolites by different metabolic pathways *in vivo*.

To solve the former problem, some state-of-the-art analytical apparatus has been extensively utilized in bio-analytical studies. The quantification can be accomplished using high-performance liquid chromatography (HPLC) coupled with different detectors such as ultraviolet absorption (UV) or a diode array detector (DAD). Developments in detection techniques and column chemistry have dramatically improved the detection sensitivity and selectivity, and shortened the analysis cycles. The two best examples are the utilization of HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) and ultraperformance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS). The latter coupling technique provides even shorter chromatographic cycle and higher sensitivity than conventional analytical methodologies, and is suitable for the analysis of a wide variety of samples in pharmacokinetic studies.

Although highly sensitive analytical techniques are available, detection will still be impeded when the drug under study is metabolized to other forms immediately and extensively. In such cases, direct analysis cannot be performed even if a highly sensitive detection method is employed, since there is no original drug molecule existing in biological samples. Previous studies reported that drugs such as flavones, polyphenols and anthraquinones were transformed into sulfated and glucuronidated conjugates extensively after the administration, and the free drug forms could not be found in the body (Hou *et al*., 2011; Shia *et al*., 2011a, b). Interestingly, it is worth noting that some sulfated and glucuronidated conjugates may also have pharmacological activities (Fang *et al*., 2003). The quantification of the sulfated and glucuronidated conjugates of drugs could be done alternatively to reflect the rate of absorption, metabolism and elimination of their free drug forms. Unfortunately, direct determination of the conjugated metabolites is usually not able to be carried out owing to the absence of reference standards. Nevertheless, β-glucuronidase (GLU) and sulfatase (SULF) have been wildly utilized to hydrolyze the sulfated and glucuronidated conjugates into the original drug forms for final quantification. This methodology exhibits high feasibility and practicability, and has been extensively applied to pharmacokinetic studies.

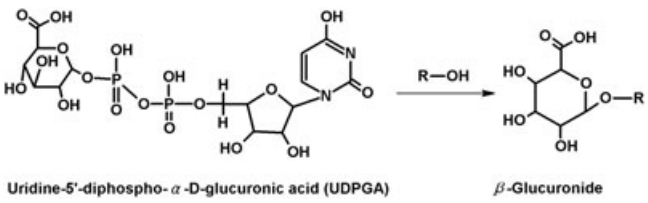

Figure 1. The glucuronidation of a substrate with a hydroxyl group utilizing uridine-5′-diphospho-α-D-glucuronic acid (UDPGA) as a cofactor.

Scope

This review aims to give a comprehensive overview of bio-analytical methods for the quantification of various drug conjugates by the hydrolysis using GLU and/or SULF. The content of this review article includes three major aspects as follows: (1) an introduction to drug metabolism pathways, especially glucuronidation and sulfation; (2) a summary of bio-analytical methods for the quantification of various drug conjugates by hydrolysis with GLU and SULF (e.g. hydrolysis conditions for sulfated and glucuronidated conjugates, such as the source and the concentration of the enzyme, pH, temperature, incubation time, chromatographic condition and detection system), and application to pharmacokinetic studies; and (3) discussion of relevant bio-analytical strategies and considerations for method development.

Two major drug metabolism pathways: glucuronidation and sulfation

Glucuronidation

Glucuronidation mainly occurs with two important cofactors: (1) glucuronosyl transferases and (2) uridine-5′-diphospho-α-D-glucuronic acid (UDPGA). The mechanism involves a nucleophilic attack of the oxygen nucleophiles (*R*-OH) or other atoms, such as nitrogen, sulfur and even carbon in the substrate molecule on the cofactor (Fig. 1), leading to *O*-, *N*-, *S*- and *C*-glucuronides, respectively (Uetrecht and Trager, 2007). The most common drug substrates are those containing hydroxyl groups in their structures, such as alcohols, phenols and carboxylic acids, which are easily attacked by glucuronic acid to form glucuronidated conjugates. The pharmacokinetic behaviors of polyphenols including flavonoids (e.g. baicalein and wogonin), anthraquinones (e.g. aloe-emodin, emodin and chrysophanol) and phenols (e.g. acetaminophen) are subjected to extensive conjugating metabolism to form *O*-glucuronides (Shia *et al*., 2010; Neirinckx *et al*., 2010; Shia *et al*., 2011a, b). In addition, researches on some uncommon glucuronides including *N*-glucuronide, *N*-carbamoyl glucuronide and *C*-glucuronide have also been reported. Hiller *et al*. (1999) observed the formation of retigabine *N*-glucuronide (Fig. 2) in humans and dogs. Another report investigated that a *N*-carbamoyl glucuronide conjugate was found in rat bile and urine after the administration of 1-[4-aminomethyl-4-(3-chlorophenyl)-cyclohexyl]-tetrahydro-pyrimidin-2-one (M1) (Gunduz *et al*., 2010). The conjugation at

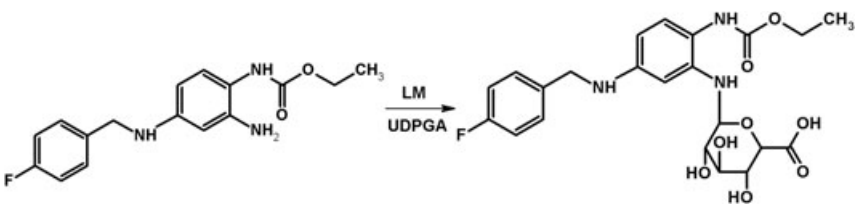

Figure 2. The glucuronidation of retigabine to form retigabine *N*-glucuronide.

the carbamic acid group resulted in the formation of *N*-carbamoyl glucuronide, which is illustrated in Fig. 3, and carbamic acid was formed when carbon dioxide reacted with the primary or secondary amine of M1. *C*-Glucuronidation, another uncommon metabolic pathway, is observed in the metabolism of sulfinpyrazone in humans. The conjugates (i.e. sulfinpyrazone β-D-glucuronide) are formed by the direct attachment of pyrazolidine ring to glucuronic acid via a *C*–C bond (Kerdpin *et al.*, 2006; Aarbakke, 1978).

Sulfation

The sulfotransferases belong to an emerging superfamily of enzymes that catalyze the transfer of SO_3^- to hydroxyl or phenolic groups on susceptible substrates, or the nitrogen atom of *N*-substituted aryl or alicyclic compounds, or pyridine *N*-oxides, through the sulfating cofactor, that is, 3′-phosphoadenosine-5′-phosphosulfate (Fig. 4). In most cases, substrates with hydroxyl groups like polyphenols (e.g. epicatechin, 6-, 8- and 10-gingerols; Vaidyanathan and Walle, 2002; Yu *et al.*, 2011), flavonoids (e.g. resveratrol, baicalein, wogonin, quercetin and catechins) and isoflavones (e.g. genistein, daidzein, glycitin, puerarin and biochanin A) could be transformed to sulfated and glucuronidated conjugates easily, simultaneously and extensively *in vivo* (Walle, 2011; Shia *et al.*, 2011a, b; Baranowska and Magiera, 2011; Zhang *et al.*, 2003). A rare observation of the sulfonation of *N*-hydroxy-2-acetylaminofluorine, which has a *N*-substituted aryl moiety, has been reported (Lewis *et al.*, 2000). In addition, some other drugs have also been reported to be metabolized to glucuronide/sulfate conjugates that contain both glucuronide and sulfated moieties. For instance, the metabolites of bisphenol A consist of monoglucuronide (major metabolite), sulfated conjugates and glucuronided/sulfated conjugates (minor metabolites; Pritchett *et al.*, 2002).

Glucuronidation and sulfation of drugs

Since glucuronidation and sulfation occur rapidly and extensively after the administration, the sulfated and glucuronidated conjugates of drugs are basically the major drug metabolites in biological samples. *In vivo* and *in vitro* studies demonstrated that diosmetin undergoes a rapid glucuronidation to form different glucuronides in rats. Four glucuronides were identified in rat blood after an oral administration of 100 mg/kg of diosmetin suspension (Campanero *et al.*, 2010). Only trace-level diosmetin

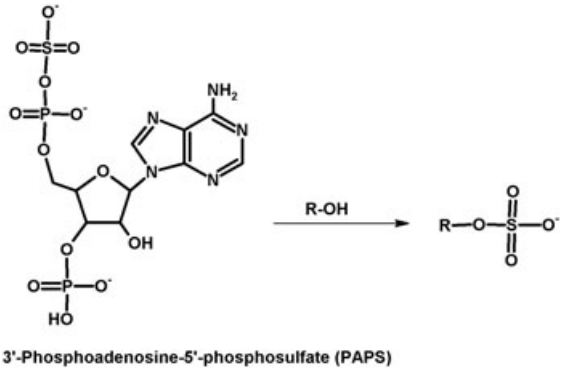

Figure 4. The sulfation of a substrate with a hydroxyl group utilizing PAPS(3′-phosphoadenosine-5′-phosphosulfate) as a cofactor.

(<5 ng/mL) was detected in human plasma without the treatment of GLU/SULF. The metabolism study of isoflavones from soy milk revealed that glucuronides were the main isoflavone metabolites in women (Zhang *et al.*, 2003). The amounts of daidzein and genistein glucuronides were 73 ± 4 and 71 ± 5% of the total amounts of daidzein and genistein excreted in urine, and 62 ± 4 and 53 ± 6% of those present in plasma, respectively. However, the drug amounts in free forms were 4 ± 1 and 5 ± 1% of the total amounts of daidzein and genistein in urine, and 18 ± 2 and 26 ± 7% of those present in plasma, respectively. The study showed that only about one-fifth of the circulating isoflavones was in the free drug form. Further research showed that, after an oral dose of 2.0 g of ginger extracts, all 6-gingerol, 8-gingerol and a majority of 10-gingerol and 6-shogaol were present as either the glucuronidated conjugates or sulfated conjugates, and only a small amount of 10-gingerol and 6-shogaol existed in the free forms in human plasma (Yu *et al.*, 2011).

The process of conjugation, which usually decreases pharmacological activities, normally adds a charge to the drug, thus making the drug more polar and facilitating its renal excretion. However, some reports have revealed that the conjugates themselves have biological activities, or serve as excellent sources of biologically active compounds, or are prone to act on target cells. For example, the conjugated metabolites of polyphenols, including emodin, baicalein, wogonin, aloe-emodin, rhein, chrysophanol, quercetin and morin, exhibited various bioactivities such as anti-inflammatory and antioxidant activities (Fang *et al.*, 2003; Shia *et al.*, 2009, 2010, 2011a, b; Shirai *et al.*, 2006;

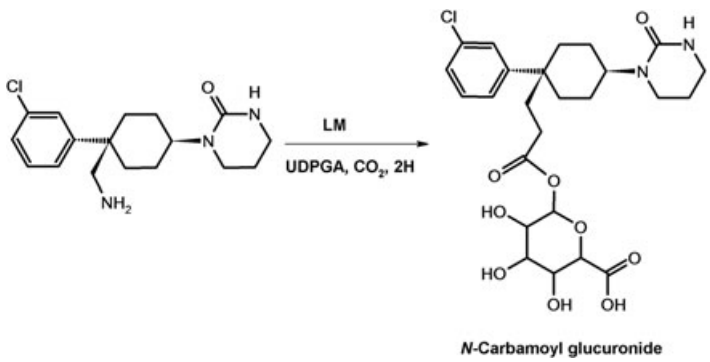

Figure 3. Formation of *N*-cabamoyl glucuronide by incubation with liver microsomes and UDPGA under a CO_2-rich atmosphere.

Yang *et al*., 2006; Yoshino *et al*., 2011). The effects of intravenous and oral metabolites of emodin against AAPH-induced hemolysis were higher than those of emodin (Shia *et al*., 2010). The conjugated metabolite of morin was 1000-fold more potent than its free form in anti-inflammatory activities (Fang *et al*., 2003). Daidzein-7,4′-di-*O*-sulfate competitively inhibited sterol SULF in hamster liver microsomes, whereas daidzein did not (Wong and Keung, 1997). Similarly, sulfated conjugates of endogenous steroids are thought to possess biological activity and to be an important source of free cellular steroids after hydrolysis by SULF (Shelnutt *et al*., 2002). It is possible, therefore, that sulfated drugs are active *in vivo* or are the primary sources of drugs in the original forms after enzymatic hydrolysis in target tissues. Genistein glucuronides may also be active *in vivo* because they have been shown to have weak estrogenic activity and can activate human natural killer cells *in vitro* (Zhang *et al*., 1999). Thus, the biological importance of the drug conjugates may be multifaceted, ranging from the inactivation form of drugs for easy excretion from the body to the regulation of specific biological processes either by direct action or by serving as an immediate source of drugs in free form within target tissues. As the bioactivities of drugs are thought to be connected to the kinetics of the conjugates, it is essential to study the pharmacokinetic behavior of the conjugates in more detail.

Bio-analytical methods

A great many quantification methods for glucuronidated and sulfated conjugates in biological matrices (e.g. plasma, urine, bile) and tissue homogenates have been reported. A straight way to quantify drug concentrations in biological samples is the utilization of its authentic reference standard. However, since authentic reference standards of the conjugated metabolites are normally not available, direct quantification is usually not feasible.

There are only a few reports on the preparation of conjugated metabolites and their application to quantifications in biological samples. Menozzi-Smarrito *et al*. (2011) reported the synthesis of two potential metabolites of 5-*O*-feruloylquinic acid, that is, 4′-sulfate and 4′-*O*-glucuronide conjugates. The synthetic reference standards were used for the identification of the metabolites of 5-*O*-feruloylqinic acid in *in vitro* incubations using human recombinant uridine 5′-diphosphoglucuronosyltransferases and sulfotransferases. However, this study did not mention the pharmacokinetics of the conjugates. Gao *et al*. (2011) developed a sensitive and reproducible UPLC-MS/MS method which simultaneously quantified maackiain and its phase II metabolites, maackiain-sulfate (M-7-S) and maackiain-glucuronide (M-7-G), in plasma. The reference standards of the conjugates were prepared by M-7-G and M-7-S, which were biosynthesized by the incubation of liver S9 fraction.

Treatment with GLU and/or SULF has been widely used to obtain the free drug forms by the hydrolysis of glucuronidated and/or sulfated conjugates for the purpose of quantifying drug concentrations in biological matrices. Since a variety of drug conjugates, that is, glucuronides, sulfates, diglucuronides, disulfates and mixed sulfa-glucuronides, can be formed in biological matrices, the advantage of the indirect determination method lies in the fact that it is not necessary to attempt the analysis of all of the possible drug conjugates above when all of the reference standards are not available.

Hydrolysis of glucuronidated and sulfated conjugates

The hydrolysis of the glucuronidated and sulfated conjugates can be carried out by enzymatic or nonspecific chemical incubation. Chemical hydrolysis usually refers to acid hydrolysis and base hydrolysis. For acid hydrolysis, samples are generally treated with hydrochloric acid by heating at 70 °C for 1 h in a thermostatic water bath. Under such drastic conditions, however, unstable target analytes cannot always be quantified accurately and completely (Alvarez-Sánchez *et al*., 2009). Owing to the fact that base hydrolysis cannot always be achieved, and acid hydrolysis usually results in extensive degradation, enzymatic hydrolysis, a relatively mild hydrolytic procedure, is feasible to keep samples stable under physiological conditions (Elsohly *et al*., 2005).

GLU and SULF are the major deconjugating enzymes used in the cleavage of glucuronidates and sulfates. GLU, the enzyme that is innately found in many tissues, such as the intestines and body fluids in humans and other mammalian species, is responsible for the hydrolysis of glucuronidated conjugates of drugs such as flavonoids and anthraquinones by the cleavage of the *C*–O bond. It was used to hydrolyze sulfinpyrazone *C*-glucuronidation (Kerdpin *et al*., 2006). SULF, the enzyme that is able to cleave sulfate esters in biological systems, plays a key role in regulating the sulfation states of molecules that determine various physiological functions. The reactions between sulfate monoesters and the nucleophiles, such as acid, base and enzyme, in aqueous solutions are basically through either *S*–O bond cleavage or *C*–O bond cleavage, with the former being the commonest bond cleavage in SULF catalyzed hydrolysis (Edwards *et al*., 2012).

The utilization of a mixture of GLU/SULF to hydrolyze glucuronidated and sulfated conjugates has been successfully applied for the quantification of drugs in plasma. The hydrolysis procedure often begins with the addition of GLU/SULF solution to biological samples with 100 μL of 1 mol/L of sodium acetate buffer (pH 5) and 0.1 mol/L of ascorbic acid solution subsequently. The mixture is then incubated at 37 °C for a certain period of time (Shia *et al*., 2010; Ding *et al*., 2012). The optimization of the parameters of enzymatic hydrolysis includes the choice of the amount and different sources of enzymes, various incubation periods and pH values of incubations.

Several enzymes are commercially available from various organisms. For example, GLU could be obtained from bovine liver, *Helix pomatia* (*H. pomatia*), *Escherichia coli* (*E. coli*) and *Patella vulgate* (*P. vulgate*) (Elsohly *et al*., 2005), whereas SULF can be extracted from *H. pomatia*, *Abalone entrails* and *Aerobacter aerogenes* (Nakamura *et al*., 2011). Previous studies indicated that there are remarkable differences in the hydrolysis activities of these enzymes from different sources. Elsohly *et al*. (2005) reported the optimum enzymatic condition for the hydrolysis of buprenorphine-3-β-D-glucuronide (B3G). Urine fortified with synthetic B3G was hydrolyzed with GLU from different sources, including *H. pomatia*, *E.coli* and *P. vulgata*. It was evident that the hydrolysis effects on B3G by various enzymes were different, and GLU extracted from *H. pomatia* provided the

Table 1. Summary of different enzymatic hydrolysis conditions for sulfated and glucuronidated conjugates in biological samples

Authors	Objective analytes	Type of enzymes	Amount of the enzymes utilized	pH value	Temperature (°C)	Incubation time (h)
Baranowska and Magiera, 2011	Glucuronides and sulfates of five isoflavones and seven flavonoids	GLU/SULF (crude solution from *H. pomatia*, type HP-2, G7017, Sigma)	40 μL for 2.5 mL urine sample	4.66	37	18
Plomley *et al.*, 2011	Glucuronides and sulfates of *S*-equol	GLU/SULF (from *H. pomatia*, type H-5, Sigma)	1 mL for 50 μL urine or plasma sample	5	37	2
Teekachunhatean *et al.*, 2011	Glucuronides and sulfates of daidzein and genistein	GLU/SULF (crude solution from *H. pomatia*, type H-2, G0876, Sigma)	0.25 mL for 125 μL plasma sample	—	37	15–18
Yu *et al.*, 2011	Glucuronides and sulfates of 6-, 8- and 10-gingerols and 6-shogaol	GLU (type IX-A from *E. coli*, Sigma)SULF (type H-1 from *H. pomatia*, Sigma)	10 μL GLU (500 units) and 10 μL SULF (60 units) for 100 μL plasma sample	pH 6.8 for glucuronides; pH 5.0 for sulfates	37	1
Hou *et al.*, 2011	Glucuronides and sulfates of baicalein and wogonin	SULF (type H-1,14400 units/g, from *H. pomatia*, containing 574000 unit/g of GLU) GLU (type B-1, 666400 units/g, from bovine liver)	150 μL SULF for 300 μL serum sample 150 μL GLU for 300 μL serum sample	5	37	10 min (SULF) 1 h (GLU)
Wang and Morris, 2005	Glucuronides and sulfates of quercetin	GLU (type B-10, from bovine liver, 10,400 units/mg, solid, Sigma) SULF (type VI, 4.9 mg/mL, 3.9 units/mg, Sigma)	5 μL GLU and 8μL SULF for 25 μL human plasma sample; 10 μL GLU and 20μL SULF for 25 μL human urine sample	5	37	1
Lin *et al.*, 2012	Glucuronides and sulfates of resveratrol and emodin	GLU (type B-1, from bovine liver, Sigma) SULF (type H-1 from *H. pomatia*, Sigma)	200 μL GLU for 100 μL plasma sample for glucuronides; 200 μL of SULF for 100 μL plasma sample for both glucuronides and sulfates	5	37	4 (GLU) 1 (SULF)
Hoffmann *et al.*, 2010	Glucuronides and sulfates of oestrone	SULF (type H-1 from *H. pomatia*, Sigma)	300 units SULF for 5 mL urine sample	5	37	3
Lévèques *et al.*, 2012	Glucuronides and sulfates of hesperetin	GLU (type H-1 from *H. pomatia*, Sigma)	5 μL GLU/SULF (crude preparation) for 100 μL urine sample	5.5	37	24
Campanero *et al.*, 2010	Glucuronides and sulfates of diosmin	GLU (type HP-2S from *H. pomatia*, 106,000 units/mL, Sigma)	50 μL GLU for 5 mL plasma sample	5	37	2
Shelnutt, *et al.*, 2000	Glucuronides and sulfates of of genistein and daidzein	GLU (type B-1 from bovine liver) SULF (type V, from *H. pomatia*)	SULF (100units), or GLU (1000 units), or SULF/GLU (100/1000 units)	—	37	3
Shia *et al.*, 2010	Glucuronides and sulfates of emodin and ω-hydroxy emodin	GLU (type B-1, from bovine liver) SULF (type H-1, from *H. pomotia*, containing 14,000 units/g of SULF and 498,800 units/g of GLU)	100 μL GLU for 100 μL serum sample for glucuronides 100 μL SULF for both glucuronides and sulfates	5	37	4 (GLU) 2 (SULF)

—:Not mentioned

fastest hydrolysis rate for B3G, with the hydrolysis extents being 96% at 60 °C and 79% at 37 °C after a 4 h incubation. In contrast, the hydrolysis reaction was completed at 37 °C after a 16 h incubation using the GLU extracted from *E. coli* and *H. pomatia*. The GLU obtained from *P. vulgata* could only hydrolyze 26% of the total amount of B3G after a 16 h incubation at 37 °C and 35% of that of B3G after a 4 h incubation at 60 °C. This research also proved that the enzyme activity was influenced by incubation temperature. The duration required to complete the hydrolysis of B3G by the enzyme extracted from *H. pomatia* at 60 °C was only 4 h. However, the duration required at 37 °C was as long as 16 h.

The rate of hydrolysis could also be influenced by different types of biological matrices. Taylor *et al.* (2005) reported that all of the phytoestrogen conjugates investigated in their research were speculated to be hydrolyzed completely within 2 h in urine, whereas a 16 h hydrolysis cycle was required in plasma. Such discrepancies between the hydrolysis rates in urine and in plasma implied that the enzymatic hydrolysis is predominately regulated by the types of conjugates and the presence of potential interfering substances in the complex biological matrices.

To sum up, the enzyme activity for hydrolysis is influenced by different types of enzymes from various sources, temperatures, pH values, incubation durations, concentrations of the enzyme and features of different biological matrices. Table 1 summarizes various hydrolysis conditions for drug conjugates in plasma and in urine from the literature.

Sample extraction and purification

Owing to the existence of endogenous substances and the low level of analytes, biological matrices need to be cleaned up and concentrated prior to any further analysis. Proteins, lipids, salts and glucose in biological samples may interfere with chromatographic separation, block the chromatographic column with high back-pressure and shorten the column life. The most widely used techniques for sample extraction, purification and enrichment include liquid–liquid extraction (LLE), solid-phase extraction (SPE) and protein precipitation (PPT).

Liquid–liquid extraction

LLE is a flexible extracting method for the separation of compounds according to their solubilities in two immiscible solvents, which usually consist of one aqueous phase and one organic phase. LLE can offer parallel preparations of a large number of samples, and is less expensive than SPE. However, LLE also has some inevitable problems: (1) frequent occurrence of emulsification; (2) consumption of large amounts of organic solvents; (3) time-consuming sample-concentrating steps; and (4) risks of sample loss and cross-contamination owing to the selection of inappropriate solvents. The optimization of a LLE method includes the selection of appropriate organic solvents, the volume of extraction solvents and the number of times of extraction. For example, in the quantification of diosmin and diosmetin in human plasma, the use of diethyl ether, cyclohexane or *tert*-butyl methyl ether was evaluated. As a result, *tert*-butyl methyl ether in acidic medium proved to be the best extraction solution based on the highest extraction recoveries, negligible matrix effects and the absence of endogenous interference in HPLC chromatograms (Campanero *et al.*, 2010). Taxifolin was also extracted from plasma by LLE after the hydrolysis with GLU and SULF, using biochanin A as an internal standard (IS). Ethyl acetate, diethyl ether, chloroform, acetonitrile, methanol, acetone and water–ethanol (1:1) were screened to select the best solvent for extracting taxifolin from rabbit plasma. The results showed that ethyl acetate gave the highest extraction recoveries without any significant interference from endogenous substances in this study (Pozharitskaya *et al.*, 2009).

Protein precipitation

PPT is used to concentrate and fractionate the target product from biological matrices. A common operation procedure is mixing the biological samples with three or four volumes of acid or methanol prior to centrifugation. The supernatant so obtained is then evaporated under nitrogen flow to dryness before being reconstituted with a certain amount of a solvent such as methanol, acetonitrile and mobile phase. Wang and Morris (2005) reported a method of precipitating plasma proteins for the quantification of quercetin and conjugated quercetin metabolites in human plasma using two volumes of acetone. Lin *et al.* (2012) investigated the pharmacokinetic behavior and tissue distribution of resveratrol, emodin and their corresponding metabolites after the intake of *Polygonum cuspidatum* (PC) in rats, using methanol as the extraction solution for PPT. Furthermore, the method validation results revealed that the use of perchloric acid for PPT would neither affect recovery nor cause any detectable matrix effects (Liu *et al.*, 2012; César *et al.*, 2011). The operation of PPT seems to be much easier than that of LLE and SPE. However, researchers still need to evaluate the implications of matrix effect for the proposed PPT method.

Solid-phase extraction

SPE has become an increasingly powerful sample preparation technique in bio-analytical studies, and the possibility of emulsification, incomplete phase separation and sample loss associated with LLE can be eliminated. SPE is more efficient in removing endogenous substances such as proteins, lipids and glucose than LLE and PPT. Kanaze *et al.* (2004) reported a clean-up method employing Sep-Pak C_{18} cartridges that effectively eliminated the interfering peaks and resulted in high recoveries for the quantification of hesperetin and naringenin in urine samples. Lévèques *et al.* (2012) published the application of SPE cartridges, and the recoveries using different sorbents and elution protocols were tested. As a result, the utilization of Waters Oasis® MAX 96-Well Plate 30 μm (30 mg) SPE cartridges yielded the highest recoveries and the best precision. Magiera *et al.* (2011) reported an extraction method using Waters Oasis® HLB cartridges with a mixture of *tert*-butyl methyl ether–methanol–formic acid (4.5:4.5:1) as the mobile phase to extract β-blockers, flavonoids, isoflavones and their metabolites in human urine.

The biological samples may sometimes have to be cleaned-up and purified with the combination of the two or three above methods. Plomley *et al.* (2011) reported that the recovery of *S*-equol in the SPE method alone was only 30%. However, the recovery increased dramatically to 85% when the methods of PPT and SPE were combined. Owing to the propensity for *S*-equol to undergo protein binding, it was essential to perform a protein precipitation prior to the subsequent processing by SPE.

Table 2. Brief descriptions of various bio-analytical methods for the quantification of glucuronides and sulfates after the hydrolysis by GLU and/or SULF

Authors	Analytes	Method/conditions	Validation parameters	Applicable conclusions
Kanaze *et al.*, 2004	Hesperetin and naringenin (total free and conjugated forms)	System: HPLC-VWD Column: Macherey Nagel Nucleosil C_8 (250 × 4.6 mm i.d., 5 μm particle size)	Specificity: no interfering peaks for hesperetin, naringenin and IS Linearity range: 0.5–10 μg/mL ($r^2 > 0.999$ for both analytes)	The method is suitable for the quantitative determination of total (free and conjugated) hesperetin and naringenin in urine samples obtained in pharmacokinetic studies in humans, after the oral administration of their flavanone glycosides, i.e. hesperidin and naringin
	IS: 7-ethoxycoumarin	Guard column: (20 × 4.6 mm i.d.) dry packed with pellicular ODS material Oven: 45 °C	LOD: 50 ng/mL for both analytes Intra- and inter-day precisions: <8.2% for hesperetin and <7.8% for naringenin	
		Mobile phase: methanol–water–acetic acid (40:58:2; isocratical elution)	Inter-day accuracies: >94.9% for hesperetin and > 95.5% for naringenin	
		Flow rate: 0.9 mL/min Injection volume: 20 μL Detection wavelength: 288 nm	Stability: stable through all three freeze–thaw cycles and stable for 24 h at room temperature	
Pozharitskaya *et al.*, 2009	Taxifolin (total free and conjugated forms)	System: HPLC-DAD. Column: Luna C_{18} (150 × 4.6 mm i.d.,5 μm)	Specificity: no endogenously interference peaks for both of taxifolin and IS Linearity: 0.03–5 mg/mL ($r = 0.9997$)	As taxifolin is easy to be conjugated with sulfate and glucuronic acid, the concen tration of taxifolin in rabbit plasma was determined with the aim of enzymatic hydrolysis. This method was successfully applied to the pharmacokinetic study of taxifolin lipid solution after an oral administration
		Guard column: (2.0 mm) with the same sorbent as Luna C_{18} Oven: room temperature	LOD and LOQ: 0.03 and 0.11 μg/mL, respectively. Intra- and inter-day precisions: within 5%.	
		Mobile phase: gradient elution using 0.03% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B): 80% A–60% A (0–10 min), 60% A–0% A (10–20 min), 0% A–80% A (20– 25 min) Flow rate: 1 mL/min Detection wavelength: 290 nm	Mean extraction recovery: ≥82.0% for taxifolin and 85.0% for IS Stability: frozen samples were stable after being stored at −20 °C for 30 days and stable after three freeze–thaw cycles	
	IS: biochanin A	Retention time: 7.9 min (taxifolin) and 18.3 min (IS)		
Hou *et al.*, 2011	Baicalein and wogonin (the glucuronidated and sulfated forms)	System: HPLC-UV Column: Apollo® 100 RP_{18e} column (250 × 4.6 mm i.d., 5 μm)	For serum samples: Linearity: 0.3–20 μg/mL for baicalein and 0.2–10 μg/mL for wogonin.	This method was used to investigate the pharmacokinetics and tissue distribution of flavonoids and their

	IS: ethyl paraben	Mobile phase: an isocratic elution using 0.1% phosphoric acid in water (solvent A) and acetonitrile (solvent B): (50:50, v/v) for serum sample; gradient elution for tissue sample: 65% A (0–20 min), 65 A–55% A (20–23 min), 55% A (23–35 min), 55% A–30% A (35–40 min), 30% A (40–45 min), 30% A–65% A (45–50 min), 65%A (50–60 min) Flow rate: 1.0 mL/min Injection volume: 20 μL Detection wavelength: 270 nm	Intra- and inter-day accuracy: relative errors (REs) <15.0% Intra- and inter-day precision: coefficients of variation (CVs) <8.6% Recovery: 90.2–93.4% for baicalein and 73.9–84.0% for wogonin For tissue homogenates: Linearity: the same as in serum samples Intra- and inter-day accuracy and precision: all CVs in intra- and inter-day analysis of various tissue homogenates were <8.6 and <9.7%, and the REs were <13.9 and <11.0% Recovery: 69–93% for baicalein and 91–113% for wogonin from the lung, liver, and kidney	metabolites in rats after repeated dosing of a SR (Scutellariae Radix) decoction. The glucuronides/sulfates of baicalein and wogonin were exclusively present in circulation, whereas their free forms were present in the lung, liver and kidney
Shia *et al.*, 2010	Emodin and ω-hydroxyemodin (ω-OHE) (total free and conjugated forms) IS: 2-methylanthraquinone	System: HPLC-MS Column: Luna C_{18} column (150 × 1.0 mm i.d., 5 μm) Mobile phase: acetonitrile–0.1% formic acid (40:60; isocratic elution) Flow rate: 0.1 mL/min Selected ion monitoring (SIM) parameters: [M − H] of 269 for emodin, 445 for emodin glucuronides, 285 for ω-OHE, 365 for ω-OHE sulfates and 461 for ω-OHE glucuronides	Linearity: 0.3–80.0 μg/mL for emodin and 0.6–10.0 μg/mL for ω-OHE ($r = 0.999$) Precision: CVs < 17% Accuracy: REs < 20% LLOQ: 0.3 and 0.6 μg/mL for emodin and ω-OHE, respectively Recovery: 99.7–104.7% for emodin, and 91.5–104.1% for ω-OHE	Concentration of emodin, ω- OHE, emodin glucuronides, and ω-OHE sulfates/glucuronides were assayed by HPLC before and after the hydrolysis with SULF and GLU
Baranowska and Magiera, 2011	Genistein (GT), daidzein (DA), glycitin (GLY), puerarin (PUR), biochanin A (BIO), (±)-catechin [(±)-CA)] (−)-epicatechin [(−)-EC], rutin (RUT), hesperidin (HSD), neohesperidin (NHSD), quercitrin (QUR) and hesperetin (HST) (total free and	System: UPLC-UV Column: Hypersil Gold column (50 × 2.1 mm i.d., 1.9 μm) Oven: 25 °C Mobile phase: a gradient elution using 0.05% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B): 95% A–80% A (0–0.5 min), 60% A (0.5–2.0 min), 60% A–10% A (2.0–3.5 min) Injection volume: 2 μL	Linearity: 0.5–10 μg/mL for (±)-CA and (−)-EC; 0.05–5 μg/mL for RUT and QUR; 0.1–10 μg/mL for HSD, NHSD and HST; 0.04–3 μg/mL for PUR, DA and BIO; 0.02–1.5 μg/mL for GLY and 0.07–3 μg/mL for GT ($r^2 > 0.9992$ for all analytes)	After being hydrolyzed by pure GLU and SULF, the concentrations of five isoflavones (i.e. genistein, daidzein, glycitin, puerarin, and biochanin A) and seven flavonoids [i.e. (±)-catechin, (−)-epicatechin, rutin, hesperidin, neohesperidin, quercitrin, and hesperetin] in human urine could be determined by using this method

(Continues)

Table 2. (Continued)

Authors	Analytes	Method/conditions	Validation parameters	Applicable conclusions
	conjugated forms of these compounds above)	Detection wavelengths: 254 nm for RUT, QUR, PUR, GLY, DA, GT and BIO, and 280 nm for (±)-CA, (−)-EC, HSD, NHSD and HST	LOD: <107.0 ng/mL for all analytes Accuracy and precision: REs and CVs are all lower than 3.90%	
Wang and Morris, 2005	Quercetin (the free and conjugated forms) IS: fisetin	System: LC-MS/MS Column: C_{12} column (250 × 2.1 mm i.d., 5 μm) Mobile phase: acetonitrile–0.2% formic acid in water (40:60; isocratical elution) Flow rate: 200 μL/min Injection volume: 10 μL MRM transitions: *m/z* 301.1 → 151.1 for quercetin and *m/z* 285.0 → 135.1 for IS (in negative mode)	Linearity: 1–800 ng/mL for plasma samples and 50–2000 ng/mL for urine samples ($r > 0.99$) LOD: 100 and 500 pg/mL in plasma and urine, respectively LLOQ: 500 and 1000 pg/mL in plasma and urine, respectively Precision: CVs <11% for inter- and intra-day analysis Intra- and inter-day accuracy: REs are within ±15% of known concentrations	The method was successfully utilized to determine the concentrations of both quercetin and conjugated quercetin after enzymatic hydrolysis with GLU/SULF in human plasma and urine samples after the ingestion of 500 mg of quercetin
Campanero *et al.*, 2010	Diosmin and diosmetin (total free and conjugated forms) IS: 7-ethoxycoumarin	System: LC coupled with a MSD Ion Trap XCT Plus detector Column: C_{18} column (150 × 3 mm i.d., 5 μm) Mobile phase: methanol–1% formic acid in water (58:42; isocratical elution) Flow rate: 0.5 mL/min MRM transitions: *m/z* 609.0 → 463.0 for diosmin, *m/z* 301.2 → 286.1 for diosmetin and *m/z* 191 → 162.9 for IS	Linearity: 0.25–500 ng/mL for diosmin ($r = 0.9971$) and diosmetin ($r = 0.9959$) Recovery: 85.2, 87.75 and 67.18% for diosmin, diosmetin and IS, respectively Selectivity: the matrix effect was less than 2.65, 3.22 and 2.87% for diosmin, diosmetin and the IS, respectively Within- and between-day precisions: 1.79–8.77, and 3.75–12.01% for diosmin; 4.05–12.54 and 3.75–12.01% for diosmetin, respectively Stability: stable in plasma samples stored at −20 °C for 3 months; in processed samples left at 4 °C over 48 h; in samples through three freeze–thaw cycles (−80 °C); and in plasma samples and stock solutions at 18 °C over 24 h	The applicability of this method was demonstrated by the determination of diosmin and diosmetin in plasma samples. In addition, diosmin could be detected in human plasma with the aid of GLU/SULF treatment of plasma samples
Yu *et al.*, 2011	6-Gingerols, 8-gingerols, 10-gingerols and 6-shogaol (their free forms and their glucuronidated and sulfated forms) IS: pelargonic acid vanillylamide (PAV)	System: HPLC-M/MS	Linearity: 5–2000 ng/mL ($r > 0.998$ for all analytes) LLOQ: 5 ng/mL for 6-, 8- and 10-gingerols and 2 ng/mL for 6-shogaol	The sensitive LC-MS/MS method has been established and applied for the pharmacokinetic analysis of 6-, 8- and 10-gingerols and 6-shogaol and their corresponding metabolites by using GLU and SULF

			Extraction recovery: 84.4–97.6% for 6-gingerol, 81.7–94.7% for 8-gingerol, 80.4–92.2% for 10-gingerol, and 81.8–93.6% for 6-shogaol, respectively Intra- and inter-day accuracy: Res: −7.3–10.4 and −9.4–9.8%, respectively	
		Column: Agilent Zorbax StableBond C_{18} column (50 × 4.6 mm i.d., 1.8 μm) Mobile phase: a gradient elution using 0.1% formic acid in water (solvent A) and acetonitrile (solvent B): 38%A (0–1.5 min), 38% A–0% A (1.5–3.4 min), 0% A–38% A (3.4–3.5 min) Injection volume: 2 μL MRM transitions: *m/z* 295.2 → 137.1 for 6-gingerol, *m/z* 323.2 → 137.1 for 8-gingerol, *m/z* 351.2 → 137.1 for 10-gingerol, *m/z* 277.2 → 137.1 for 6-shogaol, and *m/z* 294.2 → 137.1 for IS (in ESI positive ion mode)	Intra- and inter-day precision: CVs 0.9–10.9 and 2.0–12.4%, respectively.	
Plomley *et al.*, 2011	*S*-equol (the free and conjugated forms)	System: HPLC-MS/MS Column: Chiracel OJ-H supercritical fluid column (50 × 4.6 mm i.d., 5 μm)	Selectivity: no significant interfering peaks for both *S*-equol and equol-d_4 Linearity: 2.5–2500 ng/mL in plasma and 2.5–2000 ng/mL in urine ($r^2 > 0.98$)	The HPLC-MS/MS method for the determination of unconjugated and total *S*-equol in human plasma and urine was used to determine the pharmacokinetics of unconjugated and conjugated *S*-equol in normal subjects after an oral dose of 20 mg of *S*-equol
		Mobile phase: ethanol–hexane (60:40) for human urine, and ethanol–hexane (68:32) for human plasma (both in isocratical elution) Flow rate: 0.75 and 0.90 mL/min for urine and plasma samples, respectively	Intra- and inter-accuracy and precision: CVs were within 15% and REs were within ±15% of the theoretical concentration of *S*-equol	
	IS: racemic equol-d_4	MRM transitions: *m/z* 241 → 121for *S*-equol and *m/z* 245 → 123 for IS		
Jing *et al.*, 2011	Bisphenol A (BPA), nonylphenol (NP) and octylphenol (OP) (their free forms and their glucuronidated and sulfated forms)	System: UPLC- MS/MS Column: Acquity UPLC™ BEH C_{18} column (100 × 2.1 mm i.d., 1.7 μm) Oven: 40 °C	Linearity: 1.00–500 ng/mL for all of BPA, NP and OP in human urine samples ($r^2 > 0.999$ for all analytes) LOD: 0.10, 0.10 and 0.15 ng/mL for BPA, NP and OP, respectively	The method was used to quantify both free and glucuronidated and sulfated forms of BPA, NP and OP by the hydrolysis with GLU and SULF. Results of 60 urine samples collected from healthy people

(Continues)

Table 2. (Continued)

Authors	Analytes	Method/conditions	Validation parameters	Applicable conclusions
		Mobile phase: a gradient elution using methanol (solvent A) and water (solvent B): 35–90% A (0–2 min), 90–96% A (2.0–2.5 min), 96–97% A (2.5–5.0 min), 97–100% A (5.0–5.1 min), 100% A (5.1–6.0 min), 100–35% A (6.0–9.0 min) Flow rate: 0.3 mL/min Sample temperature: 10 °C Injection volume: 10 μL MRM transitions: *m/z* 227 → 212 for BPA, *m/z* 219 → 133 for NP, and *m/z* 205 → 106 for OP	Average recovery: >85% in human urine samples at the spiked concentration of 1, 10 and 50 ng/mL (RSD < 10%)	showed that BPA, NP and OP appeared to be prevalent in human urine
Hoffmann *et al.*, 2010	Oestrone (E_1), 17β-oestradiol (E_2), oestriol (E_3) and their biologically active metabolites, 2-methoxyoestrone (2-$MeOE_1$), 2-hydroxyoestradiol (2-OHE_2), 16-ketooestradiol (16-OE_2), 16-epioestriol (16-epiE_3) and testosterone (T) (their free forms and their glucuronidated and sulfated forms) IS: [2,4-$^2H_2$]E_1, [2,4,16,16-$^2H_4$]E_2, [2,4,17-$^2H_3$]E_3, [16,16,17-$^2H_3$]T, [1,4,16,16-$^2H_4$]2-$MeOE_1$, [1,4,16,16,17-$^2H_5$] 2-OHE_2, [2,4,15,15, 17-$^2H_5$] 16-OE_2 and [2,4-$^2H_2$]16-epiE_3	System: GC-MS Column: Optima-1-MS fused silica capillary column (25 m × 0.2 mm i.d., 0.10 μm film thickness) Carrier gas: helium Flow rate: 1 mL/min Injector temperature: 270 °C Oven: 80 °C(hold 2 min), to 190 °C at 20 °C/min ramp, then to 240 °C at	Linearity: 50 pg/mL to 50 ng/mL for E_1 and 16-epiE_3, 25 pg/mL to 25 ng/mL for E2, E_3, 16-OE_2, T and 2-$MeOE_1$, and 6.25 pg/mL to 6.25 ng/mL for 2-OHE_2 (r^2 0.999–1.0). LOD: 0.025 ng/mL for E_2, 2-OHE_2, 16-OE_2, T and E_1, 0.05 ng/mL for 2-$MeOE_1$, 0.2 ng/mL for 16-epiE_3 and 0.25 ng/mL for E_3 Precision: intra-day CVs from 1.00 to 1.92%, and inter-day CVs from 0.65 to 2.21%. Accuracy: REs between 9.60% (for 2-OHE_2) and −10.23% (for E_3) for low concentration spiking level, and between 11.63% (for 2-OHE_2) and −5.38% (for E_3) for medium concentration spiking level	A practical assay based on GC-MS to measure E_1, E_2 and E_3 and their biologically active metabolites 2-MOE_1, 16-OE_2, 16-epiE_3 and 2-OHE_2 and T in human urine after the enzymatic hydrolysis was developed

		2.5 °C/min ramp, and then 40 °C (hold 5 min), to 120 °C at 10 °C/min ramp, then to 300 °C at 40 °C/min ramp and hold for 6 min Detection: SIM mode with the energy of the electron beam of 70 eV; the target and qualifier ions were selected by the intensity of their ions, whereby the highest signal was defined as the target ion		
Jin *et al.*, 2004.	Quercetin (the free and conjugated forms) IS: luteolin	System: HPLC-ECD Column: Capcell Pak C_{18} UG 120 microbore ODS column (150 × 1.0 mm i.d., 3 μm) Oven: 40 °C Mobile phase: methanol–water mixture (4:6, v/v) containing 0.5% phosphoric acid (isocratical elution) Flow rate: 25 μL /min Detection potential: +0.5 V vs Ag–AgCl	Linearity: 1.5–750 pg (r = 0.999) LOD: 0.3 pg	Determination of quercetin in human plasma was carried out using semi-micro HPLC coupled with electrochemical detection after the hydrolysis of quercetin glucuronides and sulfates

Detecting methods

Analytical techniques for the quantification of glucuronidates and sulfates in biological samples indirectly after the hydrolysis with GLU and SULF have been developed extensively. The analysis is commonly performed on HPLC coupled with various types of detectors, such as a UV detector (Lai *et al.*, 2003; Teekachunhatean *et al.*, 2011), a fluorescence detector (FLD; Chetiyanukornkul *et al.*, 2006; Shia *et al.*, 2011a, b), an electrochemical detector (ECD; Azuma *et al.*, 2003; Jin *et al.*, 2004), a mass detector (MS; Shelnutt *et al.*, 2000) or a tandem mass detector (MS/MS; Wang and Morris, 2005; Campanero *et al.*, 2010). Gas chromatography coupled with mass spectrometry (GC-MS) is also a popular technique, which sometimes involves derivatization steps for the purpose of enhancing detection sensitivity (Magiera *et al.*, 2011; Feng *et al.*, 2001).

All of the above analytical methods have their own characteristics. HPLC-UV is the most frequently used analytical technique in quantification analysis, whereas the sensitivity is not high enough for the analysis of low-level analytes in plasma. HPLC-FLD is able to provide high sensitivity for quantification of analytes with fluorescence characteristics. However, this method is frequently interfered with by sample matrices. HPLC-ECD, although providing high detection sensitivity, normally requires strict maintenance to keep the system running.

Taking low-level analytes in biological samples into account, normally in the concentration ranges of tens of nangrams to micrograms per milliliter, highly sensitive methods with easy maintenance are required. HPLC-MS is considered to be a powerful tool for the quantitative determination of analytes in biological samples owing to its high selectivity, sensitivity, robustness and sample through-put. Moreover, HPLC-MS^n allows the sequential fragmentations of a given molecular ion, which provides substantial information for the identification of the selected molecule based on the fragmentation patterns. This information is very useful to differentiate compounds with similar chromatographic and UV spectral features. Recently, in order to meet the increasing demand for simpler and high-throughput methods for sample analysis, commercially available UPLC has proved to be one of the most promising developments in rapid chromatographic separation with the aim of reducing analysis durations and maintaining good efficiency at the mean time (Baranowska and Magiera, 2011). To develop a practical quantification method, a complete validation should be performed according to the US Food and Drug Administration guidelines (US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research and Centre for Veterinary Medicine, 2001), including selectivity, sensitivity, interference check, linearity, precision and accuracy, recovery, matrix effect, cross-specificity, stability and dilution integrity (Campanero *et al.*, 2010). In order to make it more beneficial to the readership, we have provided a concise compilation of the validation details and the applications of various analytical methods (detection systems/chromatographic conditions) in Table 2.

Applications to pharmacokinetic studies

The enzymetic hydrolysis converting the conjugated drugs to the free forms has been extensively applied to pharmacokinetic research. Plomley *et al.* (2011) reported an HPLC-MS/MS method for the determination of total *S*-equol (i.e. the summation of

conjugated and unconjugated forms) in human plasma and urine for a full description of its pharmacokinetic profiles. Results indicated that *S*-equol is highly conjugated *in vivo* with <1% of the unconjugated forms present. Unconjugated *S*-equol (total urinary excretion) and $F_{(0-24)}$ (calculated fraction of administered dose excreted) in urine were 11,736 ng and 0.0586%, respectively, while total *S*-equol and $F_{(0-24)}$ were 202,579 ng and 1.0123%, respectively. Campanero *et al.* (2010) developed a sensitive HPLC-MS/MS method that has been validated for a simultaneous determination of diosmin and diosmetin in human plasma. This method was suitable for the development of pharmacokinetic studies after food intake or oral administration of pharmaceutical preparations containing diosmin. The results showed that, whether the plasma samples were treated with GLU/SULF or not, diosmin was not detected in human plasma. Interestingly, diosmetin was measurable at the first sampling point (0.33 h) in most of the volunteers after the treatment with GLU. This phenomenon can be explained by diosmin being hydrolyzed by enzymes in intestinal microflora before diosmetin, that is, the free form of diosmin, is absorbed in intestines. Diosmin is subsequently absorbed and transformed to the sulfated and glucuronidated conjugates into the systemic circulation.

Lin *et al.* (2012) investigated the dose-dependent pharmacokinetics and tissue distribution of resveratrol and emodin after oral administration of PC in rats. To determine the concentrations of resveratrol and emodinin in tissue homogenates, samples were analyzed before and after the treatments with GLU and SULF, respectively. After the oral administration of PC extract, the highest concentration of sulfated/glucuronidated conjugates of resveratrol and emodinin were detected at the first sampling point (10 min), suggesting that the absorbed resveratrol and emodinin were rapidly and extensively converted into their sulfated/glucuronidated conjugates. The tissue distribution analysis revealed that the glucuronides/sulfates were the only form of emodin and resveratrol present in rat plasma. The glucuronides/sulfates of resveratrol were the major forms in rat liver, kidney, lung and heart, and those of emodin were also the major forms in rat kidney and lung. Nevertheless, a considerable amount of emodin free form was found in rat liver. Neither the free forms nor the conjugated metabolites of resveratrol and emodin were detected in rat brain (Fig. 5).

Neirinckx *et al.* (2010) reported that hepatic glucuronide and sulfate conjugation were the major elimination pathways for acetaminophen in ponies, dogs and pigs, as in humans. The calculated results of absolute bioavailability were significantly elevated from 39.0, 44.5 and 75.5% to 72.4, 100.5 and 102.2% in turkeys, dogs and pigs, respectively, after enzymatic hydrolysis of plasma samples with GLU/SULF. Without the application of enzymetic hydrolysis of the conjugate to drug, the pharmacokinetic determination of a drug that is rapidly and extensively conjugated *in vivo* seems impossible. However, employing GLU and SULF successfully conquers this difficulty and they have been extensively utilized in pharmacokinetic studies of glucuronided and sulfated conjugates, and could be used to reflect the absorption ratio of the free drug froms and the conjugated forms and the bio-availabilities of the objective drugs.

Discussion

Given the popularity of glucuronidated and sulfated conjugates formed *in vivo*, comprehensive attention has been paid to the quantification methods in biological matrices and their application to pharmacokinetic studies. Owing to the absence of authentic reference standards of most glucuronidated and sulfated conjugates, the widely used methods for quantitative analysis of these conjugates usually involve the treatment of biological samples with GLU and/or SULF in order to obtain free drug forms by hydrolysis, which could be further quantified by authentic reference standards. The enzymatic hydrolysis is conventionally carried out by the addition of an amount of GLU and SULF in the biological samples prior to a certain period of incubation. Different sources of enzymes, amounts of enzymes, pH values for incubation and incubation times are the main parameters that need to be optimized. The optimized conditions listed in Table 1 may give the readers some advice on the selection of parameters in their future studies.

Some innovative strategies providing increased efficiency and reduced time consumption of hydrolysis have also been reported. Alvarez-Sánchez *et al.* (2009) implemented an enzymatic hydrolysis method (GLU with the activity of SULF) for conjugated female steroid cleavage which was kinetically enhanced by ultrasonic energy in order to generate the free steroid forms. This enabled a dramatic reduction of sample preparation duration required (30 min) as compared with conventional protocols (12–18 h). Toennes and Maurer (1999) developed a fast and easy-to-handle procedure by immobilizing GLU and SULF into columns. The results revealed that the enzyme activity in this method was 45-fold higher than that in traditional enzymatic hydrolysis. This method combined the specificity of enzymatic hydrolysis with a speed comparable to acid hydrolysis, leading to a fast and gentle cleavage of the conjugation bonds. This

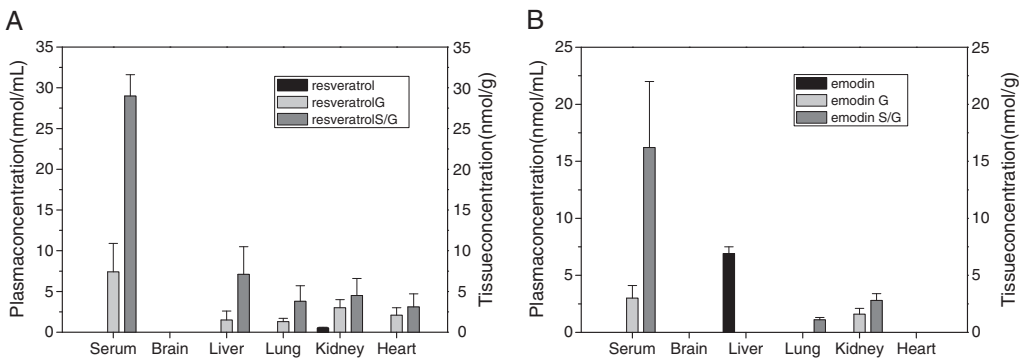

Figure 5. Mean (±SE) concentrations of (A) resveratrol, resveratrol glucuronides and resveratrol sulfates/glucuronides and (B) emodin, emodin glucuronides and emodin sulfates/glucuronides in plasma and different tissues after oral administration of seven doses of *Polygonum cuspidatum* (4 g/kg).

strategy particularly provides concepts for developing a new method for the hydrolysis of glucuronides and sulfates.

When we reviewed the literature on the quantification analysis of conjugates in blood/urine samples with the aim of enzymatic hydrolysis, two main approaches to the enzymatic treatment of biological samples could be differentiated. The first approach involves the treatment of samples with GLU (type H-1, from *H. pomatia*) containing some companion of SULF, which in turn acts as a secondary enzyme for the hydrolysis of glucuronidates. In this case, the free drug forms obtained by GLU are the summation of the hydrolysis results from both sulfates and glucuronides. Since SULF only hydrolyzes sulfates, the differences in the determination results between the treatment with GLU and those with SULF are used to calculate the real concentration of glucuronidated conjugates. The second approach is to treat biological samples with a combination of GLU and SULF. In this case, if all the sulfated and glucuronidated conjugates could be entirely converted to free drug forms, the concentration of the original forms of the drugs or other xenobiotics in biological matrices could be accurately determined.

Nevertheless, there are also some conjugates that are extremely resistant to GLU and SULF that could not be hydrolyzed completely. Saha *et al*. (2012) reported that the sulfated conjugates of epicatechin and methylepicatechin were extremely resistant to the commercially available SULF enzyme preparations. This finding is very important because the essence of the widespread practice of using enzymetic hydrolysis is to convert sulfated and glucuronidated forms of drugs and other xenobiotics to their free forms. If a study assumes that all the sulfated forms would be converted to the free forms without any validation, it is entirely possible that the subsequent estimates of total drugs appearance in biological samples would be substantially underestimated. To gain a maximum enzymatic hydrolysis rate is the goal of method optimization in such study. That is why the selection of the appropriate source of enzyme, and the concentration and pH value of the enzyme for hydrolysis, are the most important parameters that need to be optimized in the analytical method.

After hydrolysis by enzymes, the biological matrices need to be processed by a clean-up and concentration step prior to any further analysis. PPT is thought to be the easiest and cheapest option for purification, while the appropriate precipitating medium should be selected to avoid any matrix effects. LLE could provide opportunities for researchers to select different types of solvents or solvent mixtures, but inconsistent recoveries and the phenomenon of emulsification should be paid attention to. Furthermore, LLE consumes large amounts of organic solvents, and the concentration steps are time-consuming and tedious. As compared with other extraction techniques, SPE produces substantially purified extracts with low occurrence of significant matrix effects. The automation of SPE is feasible in spite of the high cost.

The quantification is commonly accomplished by HPLC coupled with various detectors such as UV, FLD, ECD, MS and MS/MS. Moreover, GC-MS is also a traditional method for bioanalytical quantification. HPLC-MS/MS and UPLC-MS/MS are powerful analytical techniques providing high sensitivity and selectivity with reduced analysis cycles and high efficiency. The selection of detection system depends on the characteristics of analytes, and the requirements for sensitivity and selectivity. The newly developed method should be fully validated based on the USFDA guidelines.

Conclusions

This review provides an in-depth coverage of the validated bioanalytical methods for the quantification of various drug conjugates by GLU and/or SULF hydrolysis, which convert sulfated and glucuronidated forms of drugs and other xenobiotics to their free forms for subsequent quantification by different analytical techniques. The indirect detection method, which solves the problems of the lacking of authentic reference standards of the conjugates, has been extensively applied to the pharmacokinetic studies.

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