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Selective isolation of in vitro phase II conjugates using a lipophilic anionic exchange solid phase extraction method

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Abstract

Identification, characterization and structure elucidation of human metabolites of drug candidates is crucial for the pharmaceutical industry to assess their activity against the therapeutic target of interest and potential toxicological effects. It often requires in vitro synthesis of microgram quantities of metabolites of interest with enzymatic preparations, pre-concentration of the reaction mixture by solid phase extraction (SPE), metabolite isolation using HPLC systems coupled to fraction collectors prior to nuclear magnetic resonance characterization. The method reported herein is a rapid and simple technique using solely off-line mixed phase anionic exchange lipophilic SPE cartridges to selectively isolate glucuronide and sulfate metabolites from their parent compound. This approach capitalizes on the p*K*a differences between the parent compound, devoided of acidic moieties, and the negatively charged glucuronide and/or sulfate metabolites. Once loaded on the SPE cartridge, the incubation mixture is washed successively with a basic aqueous solution, methanol to elute the non-anionic parent compounds, and then with an acidic methanolic solution to protonate and recover the phase II conjugates. Over 100 μg (>95% purity) of 17α-ethynylestradiol-3-glucuronide and 6-gingerol-4′-glucuronide were successfully isolated using this technique, as well as glucuronide and a sulfate conjugates of 1-{4′-[(1*R*)-2,2-difluoro-1-hydroxyethyl]biphenyl-4-yl}cyclopropanecarboxamide (DHBC) synthesized in-house. Their structures were confirmed by Ultra Performance Liquid Chromatography coupled to Quadrupole-Time of flight (UPLC-QTof) and nuclear magnetic resonance analysis.

Keywords: Phase II metabolite; Glucuronide conjugate; Sulfate conjugate; SPE; NMR; Accurate mass

1. Introduction

Metabolism plays a key role in the elimination of numerous xenobiotics, including therapeutic agents, leading to the formation of hydrophilic and polar metabolites usually excreted via biliary and/or urinary routes [1,2]. Oxidation, reduction, hydrolysis as well as glucuronide, sulfate and glutathione conjugations are the major phases I and II biotransformations involved in mammalian metabolism of xenobiotics. During the development of a drug candidate, it is of prime importance for the pharmaceutical industry to ensure that potential human metabolites identified in vitro using hepatocytes or sub-cellular systems are also observed in vitro or in vivo in pre-clinical safety species such as rat, dog or monkey [3–5]. Metabolites are typically characterized by high performance liquid chromatography coupled to photodiode array and mass spectrometric detectors (HPLC-PDA–MS/MS) to identify potential biotransformations involved in their formation [6–9]. However, analysis of MS/MS fragmentation patterns of unknown compounds usually provides limited structural information concerning the exact site of metabolism. Complete elucidation of the metabolite's structure is done by nuclear magnetic resonance (NMR) on microgram quantities of material, which can be generated by large scale in vitro biosynthesis. Alternatively, chemical synthesis of proposed structures of the unknown metabolite can be performed, which can be time and resource consuming. This material can then be used as an authentic standard for metabolite quantification in biological samples and will allow in vitro testing to

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assess its activity against the therapeutic target of interest, its toxicological effects, including the potential to cause drug–drug interactions via inhibition or inductions of cytochrome P450 (CYP) enzymes.

Common approaches for metabolite isolation involve large scale in vitro incubation and pre-concentration of the mixture using solid phase extraction (SPE) cartridges. Then, the metabolite of interest is typically isolated using either an HPLC-PDA or MS system coupled to a fraction collector [10]. More recently, the coupling of an HPLC system to an on-line SPE apparatus and an NMR spectrometer has been used to isolate natural products [11,12]. Despite the fact that they are well-accepted semi-automated methods, not all laboratories may have access to such instrumentation to perform metabolite isolation. Moreover, such methodologies require pre-concentration of incubation mixtures using SPE cartridges prior to injections on the HPLC system. This paper describes a simple approach for the selective isolation of phase II metabolites based on their p*K*a differences only using off-line mixed phase anionic exchange lipophilic SPE cartridges.

2. Experimental

2.1. Materials and chemicals

17α-Ethynylestradiol, 17α-ethynylestradiol-3-glucuronide, D-saccharic acid-1,4-lactone, uridine 5′-diphosphoglucuronic acid (UDPGA), alamethicin and hydrochloric acid were purchased from Sigma–Aldrich (Milwaukee, WI). Magnesium chloride, methanol (MeOH) and acetonitrile (ACN) HPLC grade were purchased from Fisher Scientific (Montreal, Canada). 6-Gingerol was supplied by Biomol International LP (Philadelphia, PA). 1-{4′-[(1*R*)-2,2-Difluoro-1-hydroxyethyl]biphenyl-4-yl}cyclopropanecarboxamide (DHBC) was synthesized at Merck Frosst. Formic acid and monobasic sodium phosphate were obtained from Anachemia (Montreal, Canada). Ammonium hydroxide 28.0–30.0% was obtained from A&C American Chemicals (Saint-Laurent, Canada). Sodium hydroxide (NaOH) 10N was purchased from J.T. Baker (Phillipsburg, NJ). Water was deionized using Milli-Q Plus from Millipore (Billerica, MA). Deuterated dimethyl sulphoxide and deuterium oxide (>99.9% atom D) from C/D/N Isotopes (Pointe-Claire, Canada) was used for NMR analysis.

2.2. Rat liver microsomes, human intestine microsomes and rat hepatocytes preparation

Liver microsomes were prepared from fresh Sprague–Dawley rat livers, as described in the literature [13,14], whereas human intestine microsomes were supplied by Xenotech (Lenexa, KS). Hepatocytes were prepared following a two-step collagenase perfusion of a fresh rat liver, as previously described [15]. The viability of the cells was determined by tryptan blue uptake. Preparations showing cell viability under 80% were discarded [15,23].

2.3. In vitro incubations

Stock solutions of 17α-ethynylestradiol, 6-gingerol and DHBC were prepared in 50% acetonitrile/50% water at a final concentration of 10 mM. UDPGA buffer was prepared by adding 0.254 g of magnesium chloride, 0.402 g of D-saccharic acid-1,4-lactone, 0.806 g of sodium phosphate monobasic and 0.852 g of UDPGA in 100 mL of deionized water, and the pH was adjusted to 7.4 using NaOH 10N. All solutions were stored at 4°C.

The small-scale microsomal incubations were performed in UDPGA buffer containing 1 mg/mL of rat liver or 0.25 mg/mL of human intestine microsomal proteins, and 125 μg/mL alamethacine. Following a 15 min pre-incubation at 4 °C, the reaction was started by the addition of 17α-ethynylestradiol or 6-gingerol at a final concentration of 100 μM. Control incubations in phosphate buffer instead of UDPGA buffer as well as blank incubations were also performed in parallel. After 1 h incubation at 37 °C, samples were quenched with one volume of 2% (v/v) formic acid/acetonitrile to each tube. The samples were then vortexed and centrifuged at 16,100 × *g* for 10 min. Supernatants were diluted 1:1 with H_2O prior to HPLC-PDA–MS analysis.

Similar experimental conditions were used for large-scale production of the phase II metabolites of 17α-ethynylestradiol and 6-gingerol, except that larger incubation volumes were used in 125 mL sterile plastic Erlenmeyer flasks. For 17α-ethynylestradiol, the incubation mixture contained 30 mg of rat liver microsomes, 0.9 mg of 17α-ethynylestradiol, and 3 mg of alamethicine in 30 mL of UDPGA buffer. In the case of 6-gingerol, 3.75 mg of human intestinal microsomes, 0.45 mg of 6-gingerol, 1.5 mg of alamethicine were added to 15 mL of UDPGA buffer. Following 1–2 h incubation, samples were kept at −80 °C pending metabolite isolation.

For small-scale hepatocytes incubations, 0.5 mL of 2×10^6 cells/mL in Krebs-Henseleit buffer (pH 7.4, with 3 g/L Hepes added) (Sigma, St. Louis, MO) were pre-incubated for 20 min at 37 °C under 95%:5% O_2:CO_2 atmosphere (BOC Gases; Montreal, Canada) in a 48-well plate. A final concentration of 50 μM of DHBC (2.5 μL of a 10 mM stock solution in acetonitrile) were added to each well and the incubation was conducted at 37 °C under 95%:5% O_2:CO_2 for two hours. The incubations were then quenched with one volume of acetonitrile. Controls included quenched incubations spiked with the parent and a blank incubation. The quenched samples were transferred into Eppendorf tubes, vortexed, and centrifuged at 16,100 × *g* for 10 min. Supernatants were diluted 1:1 with water prior to HPLC-PDA–MS analysis.

For the large-scale hepatocyte incubation, a protocol similar to that described above was used except that a total volume of 100 mL of 4×10^6 cells/mL was incubated in a 125 mL sterile plastic Erlenmayer flask and 50 μM of DHBC was added after a 20 min pre-incubation period. After 2 h incubation, the solution was stored at −80 °C pending the metabolite isolation.

UGT involvement in the glucuronidation of DHBC was determined as described for liver microsomal incubations except that 0.25 mg/mL of recombinant uridine 5′-diphosphoglucuronosyltransferase (rUGT) 1A1, 1A3, 1A4, 1A6, 1A7,

1A8, 1A9, 1A10, 2B4, 2B7, 2B15 or 2B17 microsomal proteins was used (BD Biosciences; San Jose, CA).

Selective hydrolysis of the glucuronide and sulfate conjugates formed in hepatocytes was carried out using β-glucuronidase enzyme and chemical solvolysis conditions, respectively, as previously reported [15].

2.4. Phase II metabolite isolation using mixed phase anion exchange SPE

Mixed phase anion exchange SPE Oasis MAX 6cc cartridges were purchased from Waters (Milford, MA). They were conditioned using 5× 5 mL of 2% formic acid in methanol, 5× 5 mL of methanol, 3× 5 mL of 5% ammonium hydroxide and 3× 5 mL of water. Large-scale incubation was thawed at room temperature and centrifuged 10 min at 4300 g (Haraeus Instruments, Megafuge 1.0R, USA). The supernatant was removed and kept separately and ∼5% (v/v) acetonitrile, relative to the original incubation volume, was added to the precipitate. The mixture was vortexed and centrifuged as described above. Both aqueous and organic supernatants were pooled and loaded on conditioned Oasis MAX 6cc SPE cartridges. The cartridges were washed sequentially with 20× 5 mL 5% ammonium hydroxide, 20× 5 mL methanol, 5× 5 mL of 2% formic acid in methanol and, for sulfate conjugate, with 5× 5 mL of 1N HCl in methanol:water (9:1) under a slight vacuum. Each fraction was collected separately and diluted 1:1 with water prior to HPLC-PDA–MS analysis. Fractions with the metabolite of interest and a purity >95%, based on UV traces between 210 and 400 nm, were pooled and dried using a Genevac EZ-2 personal evaporator (Ipswich, UK) and analyzed by NMR.

2.5. Apparatus and conditions

The HPLC-PDA–MS system consisted of an HPLC Waters 2790 Alliance HT and a Waters 996 photodiode array detector coupled with a Quattro Ultima triple quadrupole mass spectrometer (Manchester, England) equipped with an electrospray source. An HPLC column YMC ODS-A, 150 mm × 4.6 mm, 5 μm, from Waters (Milford, MA) was used at 40 °C and a flow rate of 1 mL/min for the analysis of 17α-ethynylestradiol, DHBC and their phase II conjugates. The mobile phase consisted of 0.1% formic $acid_{(aq)}$ (buffer B) and 0.1% formic acid in acetonitrile (buffer A). The gradient for 17α-ethynylestradiol analysis was 10–60% of buffer A over 17 min, ramped to 90% of buffer A in 1 min, then held at 90% buffer A for 4 min. The gradient for DHBC analysis was 10–90% of buffer A over 19 min, then held at 90% buffer A for 3 min. An injection volume of 75 μL was used. For the analysis of 6-gingerol, the mobile phase was also composed of 0.1% formic $acid_{(aq)}$ (buffer B) and 0.1% formic acid in acetonitrile (buffer A) but the elution gradient was from 0 to 100% buffer A over 30 min, and then isocratic at 100% buffer A for 5 min. A 0.3 mL/min flow rate was used and the HPLC system was equipped with a Luna C_{18} 150 mm × 2.1 mm, 3 μm, column from Phenomenex (Torrance, CA) kept at 40 °C for enhanced separation between the two potential glucuronide conjugates. The PDA detector was set to acquire from 210 to 400 nm. The mass spectrometer was operated in ESI positive/negative mode based on the compound, the capillary voltage was set at 3.5/−3.5 kV, the cone at 30/−30 V, the extractor at 1/−1 V, the source temperature at 120 °C, the desolvation temperature at 350 °C, the desolvation flow at 458 L/h, the nebulizer at 21 L/h and data were acquired in full scan mode between *m/z* 100 and 900.

The metabolite characterization was done by accurate mass measurement and NMR. For exact mass measurement, a Micromass QTof Premier from Waters (Manchester, UK) coupled to a Waters Acquity UPLC was used. The QTof was operated in ESI negative mode, the capillary voltage was set at −3.0 kV, the cone at −30 V, the extractor at −4 V, the ion guide at −1 V, the collision energy at 5 eV, the source temperature at 120 °C, the desolvation temperature at 350 °C, the desolvation flow at 800 L/h, the nebulizer at 20 L/h and data were acquired using a scan time of 0.45 s and a *m/z* range of 100–1200. Leucine-Enkephalin $(M-H)^-$ (*m/z* 554.2615) was used for the lock mass of the lock spray. A UPLC column BEH C_{18}, 100 mm × 2.1 mm, 1.7 μm, from Waters (Milford, MA) was used at 50 °C with a flow rate of 0.4 mL/min. The mobile phase consisted of 0.1% formic $acid_{(aq)}$ (buffer B) and 0.1% formic acid in acetonitrile (buffer A). The gradient for all analysis was 5–90% of buffer A over 2 min, then held at 90% buffer A for 2 min. An injection volume of 5 μL was used. Each isolated metabolite had masses which were in accordance within 10 ppm with their theoretical mass. NMR spectra were acquired on a Varian Inova 600 spectrometer equipped with a 5 mm HCN PFG Chili-Probe (cryogenic) and operating at 599.935 MHz for ^1H NMR. ^{13}C NMR data were acquired by ^1H observed gHSQC and gHMBC experiments (150.865 MHz equivalent). Samples were dissolved in 200 μL deuterated dimethyl sulphoxide with 5 μL deuterium oxide and transferred to 3 mm (335-PP) NMR tubes from Wilmad. ^1H and ^{13}C chemical shifts were referenced to the residual solvent peak (δ 2.50 ppm/29.92 ppm for dimethyl sulphoxide). ACD/CNMR Predictor v.8.09 software was used for simulation of ^{13}C NMR reference spectra.

3. Results and discussion

The stationary phase of the Oasis MAX SPE cartridges involves two retention mechanisms, based on anionic–cationic and reverse-phase interactions with analytes. In general, formation of phase II metabolites leads to addition of a polar moiety containing an anionic function. Using a mixed phase anionic exchange lipophilic stationary phase, a selective isolation of the neutral or cationic parent compound and their phase II anionic metabolites (e.g. glucuronide and sulfate conjugates) could be performed by capitalizing on the lack of ionic interaction of the parent with the cationic stationary phase. Once loaded on the column, the parent compound can be eluted using an organic solvent such as methanol, whereas the glucuronide or sulfate conjugates, due to their ionic interactions with the positively charged tertiary amines of the stationary phase, will stay on the column. The conjugates can then be selectively eluted in the uncharged state using acidified organic solvents. This procedure

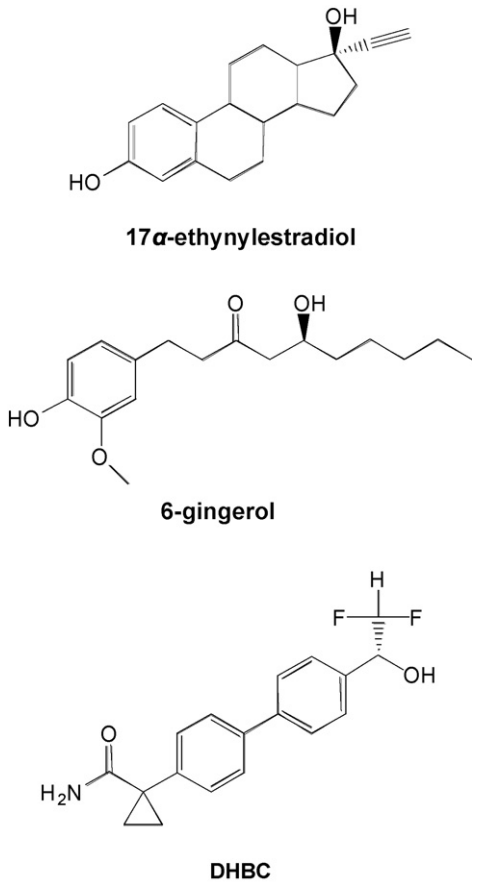

Fig. 1. Chemical structures of 17α-ethynylestradiol, 6-gingerol and DHBC.

was first tested on 17α-ethynylestradiol and 6-gingerol (Fig. 1), both known to undergo phase II metabolism, leading to formation of glucuronide conjugates [16–18] and then applied to the selective isolation of both glucuronide and sulfate conjugates of DHBC, an in-house discovery compound.

17α-Ethynylestradiol is a semi-synthetic estrogen which is a key active ingredient of contraceptive pills. Major metabolites of 17α-ethynylestradiol reported in the literature are 2-hydroxylation, 3-sulfation, 3 and 17-glucuronidation products [19,20]. Considering that 17α-ethynylestradiol leads to phase II metabolism and that authentic standards of the phase II conjugates are commercially available, it was selected as the first prototypical compound to test the selective isolation method using mixed phase anionic SPE cartridges. Due to their ease of availability and low cost, rat liver sub-cellular fractions were selected to generate microgram quantities of phase II metabolites of ethylnylestradiol. In rat liver microsomal incubations under glucuronidation conditions, extents of metabolism of 27% were obtained and one glucuronide conjugate showing a characteristic +176 u shift relative to the parent compound was observed by HPLC-PDA–MS/MS (Fig. 2). This metabolite was confirmed to be the 17-ethylnylestradiol-3-glucuronide when co-injected with the authentic standard. An in vitro large-scale incubation of 17α-ethynylestradiol was performed under glucuronidation conditions and a 23% turn-over was observed. After 2 h, the incubation mixture was centrifuged and the protein pellet washed with acetonitrile to recover maximal amount of material. Once pooled with aqueous supernatant, the acetonitrile content represented <5% (v/v) of total volume in order to avoid retention issues on the SPE cartridges. The solution was then loaded onto anionic Oasis MAX cartridges, which were then washed with an ammonium hydroxide aqueous solution to deprotonate the carboxylic acid function of the glucuronide metabolite, allowing an ionic interaction with the stationary phase to take place. No parent or metabolite was present in the load flow-through or the aqueous ammonium hydroxide washes. 17α-Ethynylestradiol was eluted off the cartridge using methanol, whereas the glucuronide conjugate was recovered in a separate fraction by protonating its carboxylic acidic moiety with a 2% formic acid solution in methanol, thus removing the anionic interactions with the stationary phase. Typical HPLC-PDA traces of the incubation mixture, methanol and 2% formic acid methanolic SPE fractions are shown at Fig. 2. These results confirmed that selective isolation of 17α-ethynylestradiol and its glucuronide can be achieved with mixed phase anionic SPE stationary phase. All fractions of 17α-ethynylestradiol-3-glucuronide with a purity >95% were pooled together and evaporated to dryness to provide enough material for NMR characterization (>150 μg), representing approximately 39% of the expected amount. The NMR data of the isolated 17α-ethynylestradiol-3-glucuronide were in

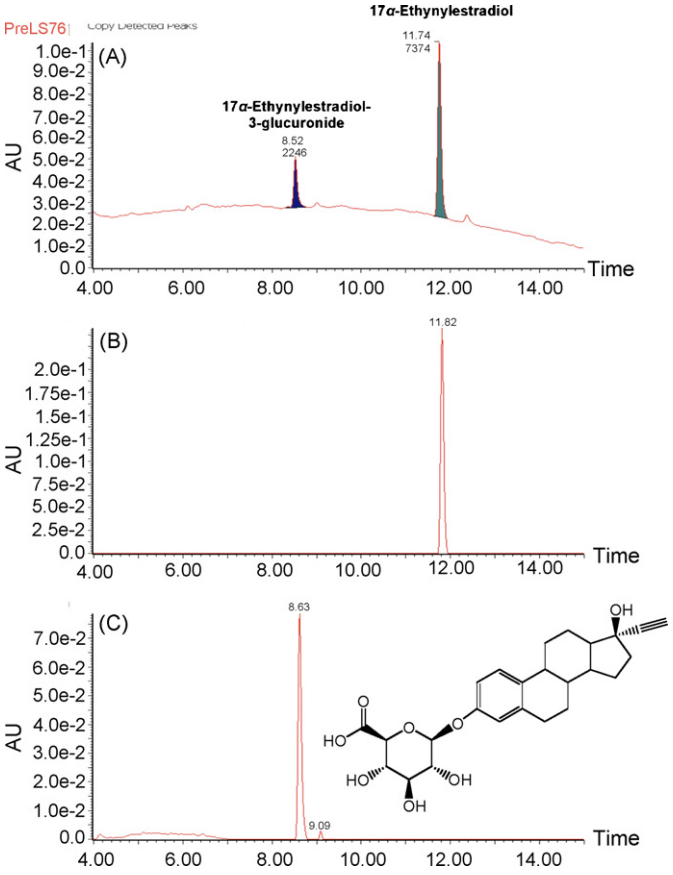

Fig. 2. Chromatographic profiles of rat liver microsomal incubations of 17α-ethynylestradiol (A) prior to the load on the mixed phase anionic exchange SPE cartridge, as well as methanol (B) and 2% (v/v) formic acid:methanol (C) eluates of the SPE cartridge.

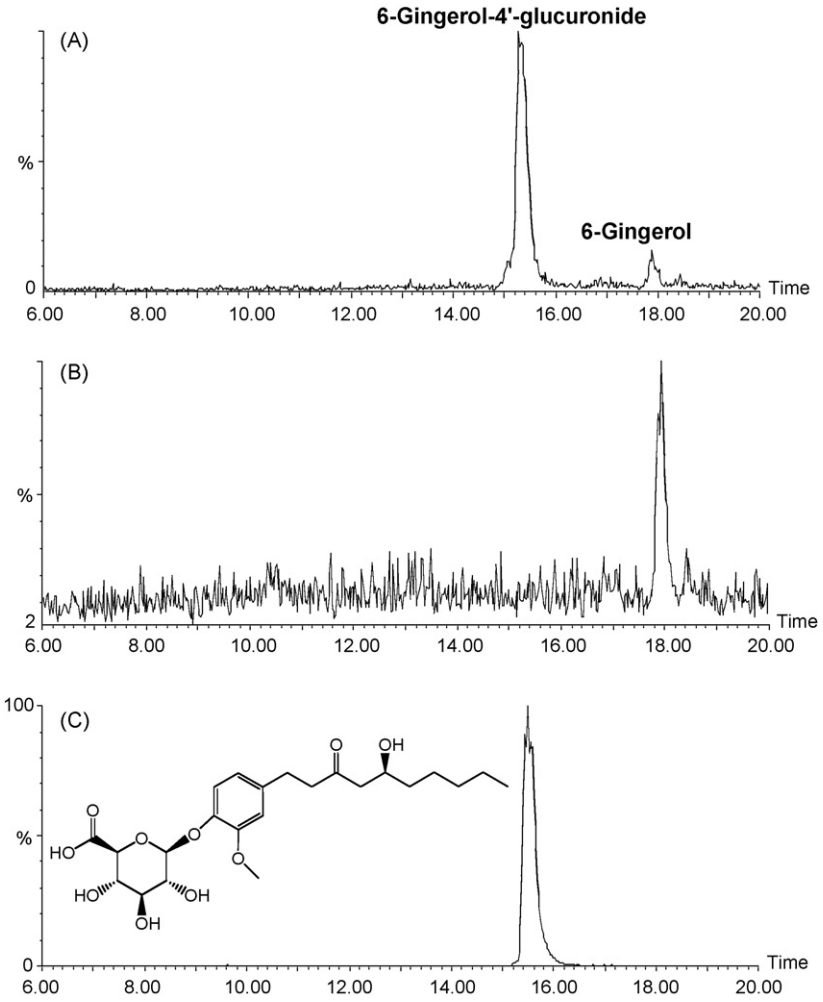

Fig. 3. Chromatographic profiles of human intestine microsomal incubations of 6-gingerol (A) prior to the load on the mixed phase anionic exchange SPE cartridge, as well as methanol (B) and 2% (v/v) formic acid:methanol (C) eluates of the SPE cartridge.

good agreement with those of the authentic standard and literature [21].

This procedure was used to isolate a phase II metabolite of 6-gingerol, the major compound of the ginger pungent principle [18]. Interestingly, the 6-gingerol was shown to undergo glucuronidation both on its phenolic and aliphatic alcohol functions. Human UGT1A9 would be implicated in the aliphatic alcohol glucuronidation, whereas the phenolic glucuronide would be selectively formed by UGT1A1, UGT1A3, as well as in human intestinal microsomes, which have minimal UGT1A9 activity. Following an in vitro incubation in presence of human intestine microsomes under glucuronidation conditions, only one metabolite with a +176 u mass difference relative to 6-gingerol was detected by HPLC–MS/MS, as reported in the literature [18]. A 37% turn-over after 1 h incubation was obtained, providing sufficient conversion for scale up and metabolite isolation. The protocol developed for 17α-ethynylestradiol-3-glucuronide isolation was used for the isolation of the phase II conjugate of 6-gingerol. As expected, the 6-gingerol, bearing no acidic function, eluted in the methanol fraction whereas the glucuronide conjugate was recovered using a 2% formic acid methanolic solution (Fig. 3). Over 100 μg of metabolite (>95% purity) was isolated, representing approximately 38% of the expected amount, and NMR characterization were in good accordance with the literature results and confirmed its structure to be 6-gingerol-4′-glucuronide [17,18].

After all the preliminary work done with 17α-ethynylestradiol and 6-gingerol, the procedure was then applied to isolate phase II metabolites of an in-house Merck Frosst discovery compound, DHBC. When incubated in presence of fresh rat hepatocytes, two majors phase II metabolites were identified by HPLC–MS/MS as glucuronide (+176 u) and sulfate (+80 u) conjugates. Upon incubation in presence of β-glucuronidase enzyme, the putative glucuronide conjugate was selectively converted back to DHBC. UGT's 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17 were involved in the formation of the glucuronide conjugate of DHBC, at extents ranging from 0.2 to 2.5%. Under chemical solvolysis conditions, the putative sulfate conjugate was hydrolyzed back to the parent compound whereas the glucuronide conjugate remained intact.

In vitro large-scale incubation of DHBC in presence of rat hepatocytes lead to formation of 54 and 45% of glucuronide and sulfate conjugates, respectively, based on relative UV response

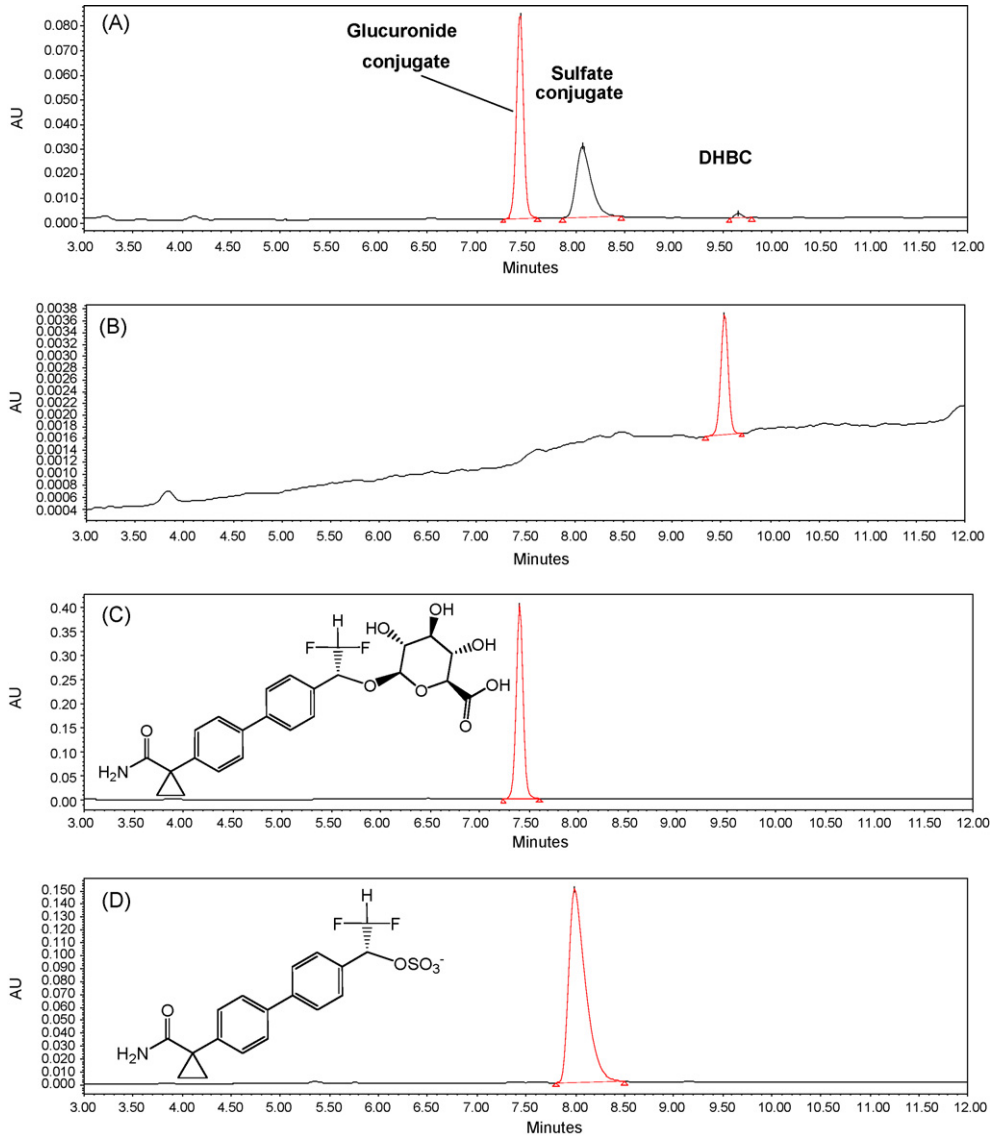

Fig. 4. Chromatographic profiles of rat hepatocyte incubations of DHBC (A) prior to the load on the mixed phase anionic exchange SPE cartridge, as well as methanol (B), 2% (v/v) formic acid:methanol (C) and 1N HCl 9/1 methanol:water (D) eluates of the SPE cartridge.

(Fig. 4). The selective isolation of each of the phase II metabolites was performed solely based on their p*K*a differences: less than −1, and 2.7 for the sulfate and glucuronide conjugates, respectively, as predicted using ACDlabs, v8.0. As previously described, the supernatant of the in vitro incubation was loaded onto Oasis MAX cartridges and washed with ammonium hydroxide aqueous solution to convert analytes to their anionic form. DHBC and its glucuronide conjugate were then successively isolated in separate fractions using methanol and 2% formic acid methanolic solutions, respectively. Due to its low p*K*a, the sulfate conjugate remained in an anionic state in presence of 2% formic acid (pH ∼ 2.5) and was retained on the cartridge. A 1N HCl methanolic solution was necessary to elute off the sulfate conjugate from the stationary phase. However, a rapid conversion of the sulfate conjugate to DHBC in the eluate was observed by HPLC within hours. The use of high concentrations of HCl in methanol to hydrolyze phase II metabolites (methanolysis) has been previously reported in the literature and shown to be an alternative to Helix Pomatia enzymatic preparations [22]. In order to prevent methanolysis of the sulfate back to the parent compound under these harsh conditions, 10% of water was added to the 1N HCl methanol solution, which prevented chemical hydrolysis of the DHBC sulfate over 48 h. Once isolated, the sulfate conjugate was shown by UPLC-QTof to be stable upon evaporation and reconstitution in deuterated DMSO. As for 17α-ethynylestradiol and 6-gingerol, the procedure allowed isolation and full characterization of the glucuronide and the sulfate conjugates of DHBC by NMR. The recovery was sufficient to allow NMR characterization.

This procedure is a rapid approach to selectively isolate glucuronide and sulfate conjugates from in vitro incubation mixture, and it minimizes the necessity to use fraction collector system. However, it can be used solely for phase II metabolites of neutral or cationic parents, or in cases where the parent compound and its conjugate would have p*K*a's differing by at least 2 units. Similarly, multiple glucuronide conjugates formed in a single

incubation mixture could not be isolated in individual fractions. However, since SPE is necessary prior to automated fraction collection methodology, it would be of interest to use this selective approach to segregate non-anionic compounds and phase I metabolites from anionic phase II metabolites, thus providing an additional chromatographic dimension prior to HPLC fraction collection and simplifying peak isolation from complex incubation mixtures. This method could also be used as a sample purification and pre-concentration method for the quantification of analytes in complex biological matrices such as bile and urine.

4. Conclusion

A new and rapid alternative to on-line isolation procedure for phase II in vitro metabolites was developed using Waters Oasis MAX SPE cartridges, containing a stationary phase with both anionic exchange and reverse phase retention mechanisms. This approach capitalizes on the fact that glucuronide and sulfate conjugates, but not their corresponding parent compounds, are negative charged, thus allowing selective elution of the parent, glucuronide and sulfate conjugates in separate and distinct fractions. This procedure has been applied successfully to isolate sufficient amounts of 17α-ethynylestradiol-3-glucuronide, 6-gingerol-4′-glucuronide and the glucuronide and the sulfate conjugate of DHBC, a Merck Frosst discovery compound, to allow complete NMR characterization.

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References

[1] Y. Chen, M. Monshouwer, W.L. Fitch, Pharma. Res. 24 (2007) 248.
[2] G.G. Gibson, P. Skett, Introduction to Drug Metabolism, 3rd ed., Nelson Thornes, 2001, pp. 1–256.
[3] E. Tanaka, T. Nakamura, S. Inomata, K. Honda, Basic Clin. Pharmacol. Toxicol. 98 (2006) 181.
[4] C. Lu, P. Li, R. Gallegos, V. Uttamsingh, C.Q. Xia, G.T. Miwa, S.K. Balani, L.-S. Gan, Drug Metab. Dispos. 34 (2006) 1600.
[5] M.J. Gomez-Lechon, J.V. Castell, M.T. Donato, Chem. Biol. Interact. 168 (2007) 30.
[6] G. Wei, S. Xiao, R. Lu, C. Liu, J. Chromatogr. B 830 (2006) 120.
[7] J. Gan, T.W. Harper, M.-M. Hsueh, Q. Qu, W.G. Humphreys, Chem. Res. Toxicol. 18 (2005) 896.
[8] A.E. Nassar, S.M. Bjorge, D.Y. Lee, Anal. Chem. 75 (2003) 785.
[9] J. Castro-Perez, R. Plumb, J.H. Granger, I. Beattie, K. Joncour, A. Wright, Rapid Commun. Mass Spectrom. 19 (2005) 843.
[10] Y. Ishii, H. Mukoyama, M. Ohtawa, Drug Metab. Dispos. 22 (1994) 79.
[11] M. Sandvoss, B. Bardsley, T.L. Beck, E. Lee-Smith, S.E. North, P.J. Moore, A.J. Edwards, R.J. Smith, Magn. Reson. Chem. 43 (2005) 762.
[12] D. Sørensen, A. Raditsis, L.A. Trimble, B.A. Blackwell, M. Sumarah, J.D. Miller, J. Nat. Prod. 70 (2007) 121.
[13] A.Y. Lu, W. Levin, Biochem. Biophys. Res. Commun. 46 (1972) 1334.
[14] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[15] J.F. Levesque, M. Gaudreault, R. Houle, N. Chauret, J. Chromatogr. B 780 (2002) 145.
[16] M.G. Soars, B.J. Ring, S.A. Wrighton, Drug Metab. Dispos. 31 (2003) 762.
[17] T. Nakazawa, K. Ohsawa, Life Sci. 70 (2002) 2165.
[18] E. Pfeiffer, F.F. Heuschmid, S. Kranz, M. Metzler, J. Agric. Food Chem. 54 (2006) 8769.
[19] T. Shiraga, T. Niwa, Y. Ohno, A. Kagayama, Biol. Pharm. Bull. 27 (2004) 1900.
[20] E. Pfeiffer, C.R. Treiling, S.I. Hoehle, M. Metzler, Carcinogenesis 26 (2005) 2172.
[21] M.G. Soars, E.L. Mattiuz, D.A. Jackson, P. Kulanthaivel, W.J. Ehlhardt, S.A. Wrighton, J. Pharmacol. Toxicol. Methods 476 (2002) 161.
[22] P.W. Tang, D.L. Crone, Anal. Biochem. 182 (1989) 289.
[23] J.M. Silva, S.H. Day, D.A. Nicoll-Griffith, Chem. Biol. Interact. 121 (1999) 49.