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Separation of substrates and closely related glucuronide metabolites using various chromatographic modes

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ABSTRACT

The aim of this study was to assess the retention and selectivity of a cocktail of 10 substrates of uridine diphosphate glucuronosyltransferase enzymes (UGTs) and their respective glucuronides using four chromatographic approaches. For this purpose, seven different stationary phases were employed in reversed phase liquid chromatography (RPLC), two in hydrophilic interaction liquid chromatography (HILIC), one in aqueous normal phase chromatography (ANPC) and four in subcritical fluid chromatography (SFC). Highly orthogonal separations were achieved with these chromatographic modes. Hydrophobic interactions mainly governed the retention of the substrates and their polar glucuronides in RPLC despite the use of different chemical stationary phase bonding, involving additional possible interactions. In ANPC, atypical separations and poor peak shapes were observed with the selected compounds. In HILIC and SFC conditions, the metabolites were more retained than the substrates because of the polarity increase related to the glucuronic acid moiety. For the latter, a very high proportion of organic solvent (up to 80%) was required to elute the glucuronides that often displayed poor peak shapes. Finally, the selectivity of nine chromatographic systems was compared for the separation of isomeric and diastereoisomeric compounds. The stationary phases used in RPLC mode were more selective towards the two positional isomers of morphine glucuronides since they possess distinct lipophilicity. HILIC and SFC columns were found to be promising for the separation of a critical diastereoisomers pair, namely epitestosterone-glucuronide and testosterone-glucuronide.

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1. Introduction

The uridine diphosphate (UDP) glucuronosyltransferases (UGTs) are the most important phase II metabolizing enzymes, since glucuronidation represents more than 35% of conjugation reactions [1]. The UGT isoforms are distributed in 3 subfamilies, UGT1A, 2A and 2B. They are mainly expressed in the liver but are also present in the intestines, kidney or lungs for instance. They catalyze the covalent linkage of a highly polar group, namely the glucuronic acid derived from the cofactor UDP-glucuronic acid (UDPGA), to a substrate containing nucleophilic heteroatom by an S_N2 substitution mechanism [2]. *O*-, *N*-, *S*- and *C*-Glucuronides are produced from functional groups such as alcohols, phenols, carboxylic acids, amines, amides, thiols and acidic carbons [3]. UGTs are responsible of the biotransformation of many endogenous compounds including bile acids, bilirubin, hydrosteroids, as well as exogenous substrates including pollutants, dietary compounds [4], drugs (anti-cancer agents, nonsteroidal anti-inflammatory drugs, and opioids) and their phase I metabolites [5]. The resulting metabolites are more hydrophilic than the parent drug, thus enhancing their excretion into urine or bile. Moreover, one substrate is often metabolized by several isoforms, since they have distinct but overlapping substrate selectivities. The glucuronidation is therefore considered as one of the main biological detoxification pathway, although some metabolites may have biological activity [6].

The UGTs are regioselective enzymes. For instance, Robotham and Brodbelt [7] demonstrated that the 7-OH position of some flavones was preferentially glucuronidated, but never the 5-OH position. Another example is the conjugation of raloxifene at the position 6 and 4′ by UGT1A1 and 1A8 whereas UGT1A10 produces only raloxifene-4′-glucuronide [8]. Stereoselectivity of drug glucuronidation has been illustrated by many publications, such as Court et al. [9] who observed glucuronidation of *S*-oxazepam only by 2B15 isoform, whereas the *R*-enantiomer was conjugated by UGT1A9 and 2B7. Racemic carvedilol is metabolized to two diastereoisomeric glucuronides, the *R*-diasteroisomer by the isoenzymes 1A1 and 2B4, and the *S*-diasteroisomer by 2B4 and 2B7 [10]. Since glucuronidation can occur at different sites of the sub-

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strate (isomeric glucuronides) or stereoselectively at the same site (diatereoisomeric glucuronides), the metabolites are isobars and can provide the same product ion mass spectra [11]. Therefore, highly efficient chromatographic methods are required since such compounds are not easily separated by tandem mass spectrometry (MS). Some papers highlighted the challenging separation of drugs and closely related molecules by comparing different chromatographic modes and stationary phases [12–15]. The separation of the pair substrate/metabolite is also crucial, because deconjugation can occur in the electrospray ionization source, leading to the overestimation of the substrate in the case of co-eluting peaks [16,17].

The reversed phase liquid chromatography (RPLC) has been widely used for glucuronides analysis [18–21]. and in particular on highly lipophilic stationary phase chemistry such as C_8 [22–25] and C_{18} materials [26–29]. Since glucuronides are polar molecules with acidic pK_a of around 3, acidic mobile phases are generally used in RPLC to obtain hydrophobic interactions and thereby enhance the retention. Columns with polar end-capping or polar functional group embedded in the alkyl chain are more hydrophilic, thus both improving the retention of polar compounds and providing different selectivities compared to conventional C18 phases [30,31].

Hydrophilic interaction liquid chromatography (HILIC) is based on the partition of a solute between the mobile phase (>60% organic solvent) and a water layer at the surface of a polar stationary phase [32,33]. The retention is mainly governed by hydrogen bonding, ionic and dipole–dipole interactions [34]. HILIC is thereby dedicated to the analysis of polar analytes, such as glucuroconjugates, as illustrated in previous papers [35–39].

Aqueous normal phase chromatography (ANPC) performed with silica hydride (Si–H) stationary phase implies a dual mechanism: a RP mechanism, due to the aqueous composition of the mobile phase and a normal phase mechanism (increasing retention as the eluent becomes less polar, similar to HILIC) [40,41]. Therefore, such stationary phases can be employed both in RP and ANP chromatography, allowing the retention of hydrophobic and hydrophilic compounds [42]. For instance, Pesek et al. [43] compared two silica hydride stationary phases for the successful analysis of ethyl glucuronide contained in hair.

Subcritical fluid chromatography (SFC) is a re-visited green technique based on the use of a mobile phase containing a mixture of apolar CO_2 and polar organic solvent (e.g., methanol) and additives (e.g., ammonium formate) [44] for improving the analytes solubility [45]. Different retention mechanisms (e.g., dispersive interactions, hydrogen bonding, dipole–dipole interactions, and solvent adsorption on polar stationary phases) are involved, depending on the stationary phase bonding (i.e., polar or less polar), thus allowing the analysis of compounds with wide polarity range [46]. To date, only very few applications of SFC with glucuronides are available in the literature [14,47].

The aim of the present study was to compare the retention and selectivity of 10 UGT substrates and their respective glucuronides, covering log D_{pH3} range from −4.05 to 4.76 using 13 different stationary phases employed in various chromatographic modes (RPLC, HILIC, ANPC and SFC) coupled with MS/MS detection. Finally, the selectivities achieved on nine of these columns were assessed with a set of six critical compounds including isomeric and diastereoisomeric glucuronides.

2. Materials and methods

2.1. Chemicals

Azidothymidine (AZT), chenodeoxycholic acid (CDCA), dimethylsulfoxide, etoposide (ETO), isoferulic acid (IFA), serotonin (SER) hydrochloride, testosterone (TST), trifluoperazine (TFP) dihydrochloride, uridine diphosphate-glucuronic acid (UDPGA), ammonium formate and ammonium hydroxide were provided by Sigma–Aldrich (Steinheim, Germany). Levemedetomidine (LMT) was supplied by Toronto Research Chemicals Inc. (Toronto, Canada). Codeine (COD) hydrochloride, D,L-4-hydroxy-3-methoxymethamphetamine (HMMA) hydrochloride, morphine hydrochloride, morphine-3-glucuronide hydrate and morphine-6-glucuronide hydrate were obtained from Lipomed AG (Arlesheim, Switzerland). Testosterone-glucuronide, epitestosterone-glucuronide and etiocholanolone-glucuronide were supplied by Steraloids (Newport, RI, USA). Androsterone-glucuronide was obtained from LGC Standards (Molsheim, France). Formic acid was purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN), heptane, isopropanol and methanol (MeOH) of ULC-MS grade were provided by Biosolve (Valkenswaard, Netherlands). Pooled human liver microsomes (HLMs) from 20 donors and UGT reaction mixture-Solution B (250 mM Tris–HCl, 40 mM $MgCl_2$, 0.125 mg/mL alamethicin and 1.25% (v/v) methanol in water) were supplied by Corning (Amsterdam, The Netherlands). Ultra-pure water was purchased from a Milli-Q purification unit from Millipore (Belfort, NY, USA). Pressurized liquid CO_2 3.0 grade (99.9%) was obtained from PanGas (Dagmerstellen, Switzerland).

2.2. Samples

Stock solutions were prepared at different concentrations in methanol (COD, HMMA, CDCA, LMT, testosterone-glucuronide, epitestosterone-glucuronide, etiocholanolone-glucuronide and androsterone-glucuronide), in dimethylsulfoxide (ETO, IFA and TST) and in water (SER, TFP, AZT, morphine and its two glucuronides). The cocktail of substrates was prepared in water through dilutions of stock solutions and was 10-fold more concentrated than the final concentrations of the substrates in the incubation medium.

2.2.1. Cocktail of substrates: glucuronidation procedure

The HLMs (0.5 mg protein/mL) was mixed to the incubation medium containing buffer Tris–HCl pH 7.5 (50 mM), alamethicin (25 μg/mL) and $MgCl_2$ (8 mM), with the final concentrations given in brackets. After 10 min on ice, the cocktail of substrates was added and a pre-incubation of 10 min at 37 °C under agitation (400 rpm) was carried out. The reaction was then initiated with the addition of UDPGA cofactor (4 mM) for a total volume of 500 μL. The final organic solvent concentration did not exceed 2% (v/v). The final substrate concentrations were the following: ETO, IFA and AZT at 50 μM, LMT and TST at 2 μM, COD at 50 μM, CDCA at 24 μM, TFP at 5 μM, SER at 400 μM, and HMMA at 35 μM. The incubation proceeded under agitation (400 rpm) for 45 min at 37 °C, and was stopped by adding 500 μL of ACN to the reaction medium. The precipitated proteins were removed by centrifugation (10,000 rpm for 10 min). The supernatant was evaporated until dryness and the residue was reconstituted after a two-fold preconcentration step in 1:9 (v/v) ACN/water for RPLC, 7:3 (v/v) ACN/water for HILIC and ANPC, and 1:1 (v/v) ACN/water for SFC.

2.2.2. Test mixtures of isomeric and diastereoisomeric glucuronides

From the previous stock solutions, mixtures of steroid glucuronides (i.e., testosterone-glucuronide, epitestosterone-glucuronide, etiocholanolone-glucuronide and androsterone-glucuronide) at 0.1 mg/mL each was prepared in 1:9 (v/v) MeOH/water for RPLC, in 1:1:8 (v/v/v) MeOH/water/ACN for HILIC, and at 5 mg/mL in 7:3 (v/v) isopropanol/heptane for SFC. Mixture of morphine and its glucuronides (morphine, morphine-3-glucuronide and morphine-6-glucuronide) were prepared at

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