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Identification of drug glucuronides in human urine by RP-HPLC after derivatization

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Abstract: A method for the identification of four types of drug glucuronides in human urine is presented. The approach involves solid-phase extraction (C_{18} columns) from acidified human urine and subsequent methylation and acetylation of the extracted drug glucuronides to triacetyl methyl derivatives. These derivatives were identified by RP-HPLC by comparison with synthesized authentic reference compounds. The scope of the method was demonstrated by identification of glucuronides formed by metabolism of clofibrate, phenazone, disulfiram and sulfamethoxazole in urine samples of two male volunteers.

Keywords: *Derivatives of drug glucuronides; RP-HPLC; clofibrate; phenazone; disulfiram; sulfamethoxazole.*

Introduction

Glucuronic acid conjugates of xenobiotics as polar, thermolabile and non-volatile substances represent an intricate challenge for analysts in the biomedical sciences. Due to their physicochemical properties glucuronides cannot be analysed by GLC without prior derivatization. Consequently, this necessity mitigates against the use of GC–MS as a sensitive method in the screening of urine samples for glucuronic acid metabolites.

Therefore, the identification of glucuronides in routine analysis has hitherto depended predominantly on indirect methods involving the enzymatic or chemical cleavage of the glucuronides followed by identification of the aglycone thus formed. Thereby the glucuronic acid moiety escapes analytical detection.

This way of proceeding suffers from three major drawbacks:

(i) The identity of the postulated glucuronides remains uncertain, especially if several functional groups capable of coupling to activated glucuronic acid are present in the drug moiety.

(ii) As it is generally necessary to remove the free aglycone from the sample by solvent extraction before cleavage of the conjugates, misinterpretations might arise for those glucuronides which can be easily extracted by organic solvents [1, 2].

(iii) The cleavage procedure itself can be the source of unforeseen problems, for example, standard β-glucuronidase does not cleave C-glucuronides [3] and certain S- [4] and N-glucuronides [5–7]. Furthermore, β-glucuronidases of different origin differ in their stereospecifity [8]. Chemical hydrolysis of glucuronides also requires painstakingly optimized conditions for hydrolysis depending on their chemical structure [9–10].

To overcome the above problems, a different approach to the analytical identification of glucuronic acid conjugates was sought. Representatives of ether-, ester-, N- and S-glucuronides were prepared as triacetyl methyl (TAM) derivatives (Fig. 1). RP-HPLC assays were developed for the identification of these derivatives and applied to metabolite analysis in the urine of healthy volunteers who had received thereapeutic doses of phenazone (PZ), clofibrate (CLO), disulfiram (DSM), and sulfamethoxazole (SMX).

Experimental

Apparatus

The HPLC-system consisted of an HPLC pump model 590 (Waters, Eschborn,

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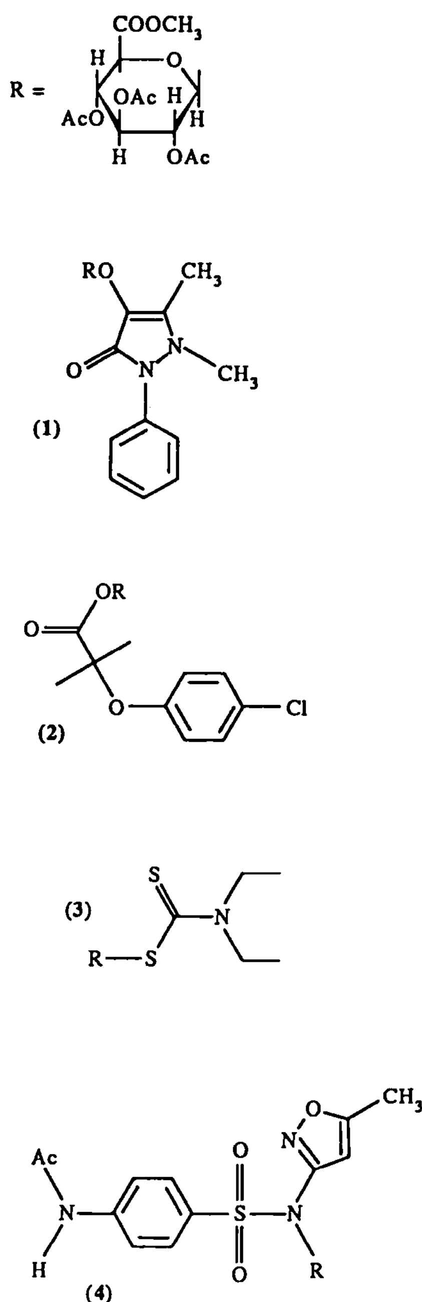

Figure 1
Chemical structures of the synthesized triacetyl methyl glucuronide derivatives. (1) 4-hydroxy PZ glucuronide derivative: 2,3,4-tri-O-acetyl-1-O-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazole-4-yl)-β-D-glucopyran-methyl-uronate. (2) CLOA glucuronide derivative: 2,3,4-tri-O-acetyl-1-O-[2-(4-chlorophenoxyl)-2-methyl-propionyl]-β-D-glucopyran-methyl-uronate. (3) diethyl dithiocarbaminate glucuronide derivative: 2,3,4-tri-O-acetyl-1-S-(diethyl-thio-carbamoyl)-β-D-1-thio-glucopyran-methyl-uronate. (4) N-4-acetyl SMX N-1 glucuronide derivative: 2,3,4-tri-O-acetyl-1-{[4-(acetylamino)-phenyl-sulphonyl]-(5-methyl-isoxazole-3-yl)-amino}-β-D-1-desoxy-gluco-pyran-methyl-uronate.

Germany), a UV–vis filter photometer (Knauer, Berlin, Germany) and a one-channel recorder (Knauer, Germany). The system was equipped with an injector model 7125 (Rheodyne, Cotati, CA, USA) with 20 μl loop. The column was a 250 × 4 mm C_{18} column (Eurospher 80-5 Vertex column, Knauer, Germany). Low pressure chromatography was performed with a Duramat 80 diaphragm metering pump (ProMinent, Heidelberg, Germany) coupled to a Lobar Lichroprep Si 60 C column (440 × 37 mm; Merck, Germany) and a fraction collector 660 (Büchi, Flawil, Switzerland).

^{1}H-NMR spectra (90 MHz) were recorded with a Bruker WH 90 NMR spectrometer and 300 MHz ^{1}H-NMR spectra with a Bruker AC 300 NMR spectrometer. EI-Mass spectra (90 eV) were obtained on a Finnigan-MAT 212 mass spectrometer, IR- and UV-spectra were measured with a Perkin–Elmer IR spectrophotometer 299 and a Beckman DU-70 spectrophotometer, respectively.

Chemicals

Chemicals of the highest available purity were purchased from Aldrich (Steinheim, Germany) or Fluka (Buchs, Switzerland) to be used for the synthesis of reference compounds. Technical grade solvents for synthesis were dried and redistilled prior to use. Acetanhydride and pyridine of p.a. quality were bought from Merck (Darmstadt, Germany). 4-Dimethylaminopyridine was obtained from Aldrich. Ethereal diazomethane solution (approximately 0.5%) was prepared from Diazald using the Diazald-Kit (Aldrich). HPLC-grade water was prepared from demineralized water with the Millipore Milli-Q-Ultrapurification system. Acetonitrile Lichrosolv Gradient Grade was bought from Merck.

Preparation of reference compounds

4-Hydroxy PZ glucuronide derivative (Fig. 1, formula (1); 2,3,4-tri-O-acetyl-1-O-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazole-4-yl)-β-D-glucopyran-methyl-uronate) was synthesized as follows:

In a 100 ml three-neck flask equipped with a thermometer, nitrogen inlet, mercury bubbler and septum 2.5 mmol 1,2,3,4-tetra-O-acetyl-β-D-glucopyran-methyluronate, prepared according to [11], and 2 g mortared, freshly activated molecular sieve (4Å) were suspended in 15 ml dry dichloromethane. $SnCl_4$ (0.2 ml) was added via septum inlet. After stirring for

10 min at room temperature under nitrogen atmosphere the mixture was cooled to $-15°C$ and stirred for a further 30 min. 4-Hydroxy PZ (5 mmol), prepared according to [12], in 20 ml dry dichloromethane was gradually injected via the septum inlet over a period of 40 min. After 6 h the reaction mixture was allowed to warm to room temperature. When the reaction came to a standstill, which was monitored by TLC (SiO_2, solvent mixture dichloromethane–methanol 98:2, v/v) the molecular sieve was removed and the filtrate poured into 30 ml of saturated $NaHCO_3$ solution. After extraction with dichloromethane and washing with water the product was purified by low pressure chromatography (solvent mixture dichloromethane–methanol, 98:2, v/v, flow rate 5.8 ml min^{-1}).

m.p.: 233–234°C (uncorr.). $C_{24}H_{28}N_2O_{11}$ (520.5). *Calc.*: C 55.4, H 5.42, N 5.4, O 33.8; *Found*: C 55.2, H 5.42, N 5.3, O 33.9. *IR* (KBr): 1760 (s, C=O), 1685 cm^{-1} (s, amide). *UV* (acetonitrile): max (logϵ) = 206 (4.04), 240 (3.98), 278 nm (3.98). *EI-MS* (90 eV): *m/z* (rel. intens.) = 520 (0.8%, $M^{\cdot+}$), 317 (14%, $M^{\cdot+}$-aglycon), 257* (18%), 215* (10%), 204 (40%, 4-hydroxy PZ), 197* (18%), 155* (70%), 127* (30%), 56 (100%, $H_3CN{=}C{-}CH_3^+$). *Glucuronic acid moiety (cf. ref. 13). *^1H-NMR* (300 MHz; TMSi; $CDCl_3$): δ (ppm) = 7.37–7.40 (m) and 7.25–7.30 (m) AA'BB'C-system (5 aromatic H), 5.18–5.38 (m, 4 H, glucuronic acid H-1, H-2, H-3, H-4) ABCD-system, 4.04 (d, $J_{4,5}$ = 9.75 Hz, glucuronic acid H-5), 3.73 (s, 3 H, $COOCH_3$), 2.97 (s, 3 H, N- CH_3), 2.22 (s, 3 H, CH_3), 2.12 (s, 3 H, CH_3COO), 2.03 (s, 3 H, CH_3COO), 2.02 (s, 3 H, CH_3COO).

Clofibric acid (CLOA) glucuronide derivative (Fig. 1, formula (2); 2,3,4-tri-O-acetyl-1-O-[2-(4-chlorophenoxy)-2-methylpropionyl]-β-D-glucopyran-methyl-uronate) was prepared according to Rothley and Oelschläger [14]. Structure was confirmed by carrying out the same analytical procedures as described for 4-hydroxy PZ glucuronide derivative.

Diethyl dithiocarbamate glucuronide derivative (Fig. 1, formula (3); 2,3,4-tri-O-acetyl-1-S-(diethyl-thiocarbamoyl)-β-D-1-thio-glucopyran-methyl-uronate) was prepared according to ref. 15. Structure was confirmed by the same analytical procedures as described above.

N-4-Acetyl-SMX-N-1-glucuronide derivative (Fig. 1, formula (4); 2,3,4-tri-O-acetyl-1-{[4-(acetylamino)phenyl-sulphonyl]-(5-methyl-isoxazole-3-yl)-amino}-β-D-1-desoxy-glucopyran-methyl-uronate) was synthesized as described by Ahmed and Powell [16]. Structure was confirmed as described for 4-hydroxy PZ glucuronide.

Human studies

Two healthy male volunteers received 1 g PZ, 1 g CLO, 500 mg DSM or 800 mg SMX orally. Drugs were administered at 2 week intervals during which each drug was completely eliminated from the body. Urine was collected for 12 h (PZ, SMX) or 24 h (CLO, DSM).

Sample preparation and derivatization

Aliquots (10 ml) of pooled urine samples collected 12 or 24 h after drug administration were adjusted to pH 2 with 6 N HCl. The acidified urine was extracted using the SPE-10 system with C_{18} columns (Bakerbond 500 mg; Baker, Phillipsburg, NJ, USA). The columns were conditioned with methanol and 0.1% HCl. After extraction of the urine the columns were washed with 5 ml 0.1% HCl. Samples were eluted with 0.5 ml methanol, the methanol was evaporated under a stream of nitrogen. The dry extracts were derivatized with 1 ml 0.5% ethereal diazomethane solution and left for 20 min at room temperature. Excessive diazomethane was blown off with nitrogen. A mixture consisting of 0.2 ml acetanhydride, 0.2 ml pyridine and 4 mg N,N-dimethylaminopyridine was added to each sample. After 5 h at room temperature 5 ml H_2O was added to samples. Samples were extracted with 3 × 3 ml diethyl ether, the combined organic extracts were washed with 3 × 3 ml 0.1 N HCl, dried over sodium sulphate and evaporated under a stream of nitrogen. Samples were reconstituted in 1 ml of the appropriate HPLC solvent. Aliquots of 20 μl were analysed by HPLC.

Chromatographic conditions

All HPLC separations were performed isocratically at 20°C, with a flow rate of 2.0 ml min^{-1}, a 20 μl injection volume and detection wavelength of 280 nm, except for CLOA glucuronide derivative, for which the detector was set at 220 nm.

The solvent systems consisted of acetonitrile–water at v/v ratios of 35:65 for 4-hydroxy PZ glucuronide derivative, 50:50 for CLOA glucuronide derivative, 40:60 for N-4-acetyl SMX N-1-GLUC derivative and 45:55

for N,N-diethyl dithiocarbaminate glucuronide derivative.

Results and Discussion

This report describes the feasibility of replacing the cleavage procedures routinely used in the identification of glucuronides with a procedure in which the entire glucuronide molecule is determined.

The drugs for which this method was investigated here were the analgesic PZ, the hypolipidaemic agent CLO, DSM, an agent predominantly used in the treatment of chronic alcoholism and the bacteriostatic SMX. All of these drugs generate glucuronides in humans, the first three after metabolic transformation via phase I pathways. PZ is metabolized to, among other species, 4-hydroxy PZ [17], CLO undergoes rapid hydrolytic cleavage of its ester bond [18] and DSM is reduced to N,N-diethyl dithiocarbaminate [19]. 4-Hydroxy PZ forms an ether-linked glucuronide [20], CLOA generates an ester-linked glucuronide [18] and N,N-diethyl dithiocarbaminate furnishes an S-linked glucuronide [19]. SMX is glucuronidated mainly at the N-1 position [21].

Suitable reference substances for these metabolites were synthesized as TAM derivatives (Fig. 1). Products obtained were characterized as β-D-glucuronides by EI-MS [13, 22–25] and ^{1}H-NMR [26–30]. In case of the SMX-N-1 glucuronide, N-4-acetyl-SMX-N-1 TAM glucuronide was prepared (Fig. 1, formula (4)).

Glucuronides generated metabolically from the drugs were identified by HPLC analysis in the urine of two humans after solid-phase extraction and derivatization of urine samples. Figures 2–5 show the chromatograms of deriv-

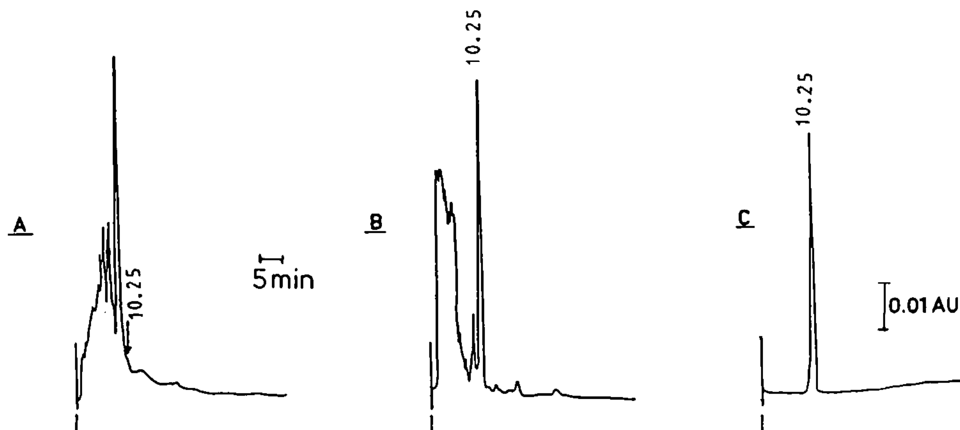

Figure 2
Typical HPLC traces of extracted and derivatized urine samples (A) before administration of 1 g PZ, (B) after administration of 1 g PZ, and (C) authentical reference: 4-hydroxy PZ glucuronide derivative (840 μg ml^{-1}). For chromatographic conditions see Experimental section. I = Injection, AU = absorption units.

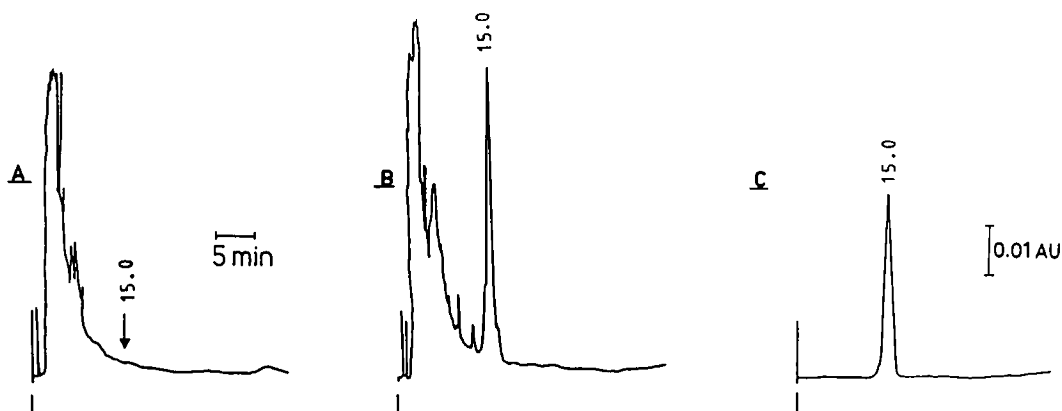

Figure 3
Typical HPLC traces of extracted and derivatized urine samples (A) before administration of 1 g CLO, (B) after administration of 1 g CLO, and (C) authentic reference: CLOA glucuronide derivative (900 μg ml^{-1}). For chromatographic conditions see Experimental section. I = Injection, AU = absorption units.

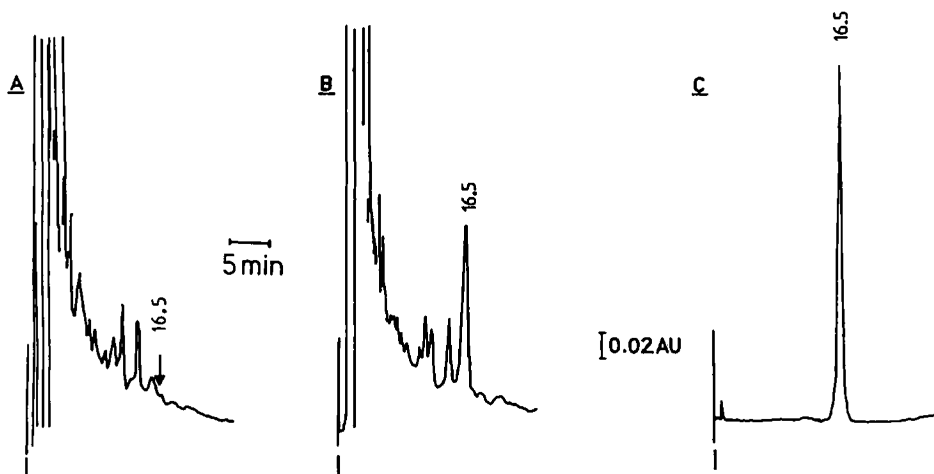

Figure 4
Typical HPLC traces of extracted and derivatized urine samples (A) before administration of 500 mg DMS, (B) after administration of 500 mg DSM, and (C) authentic reference: N,N-diethyl dithiocarbaminate glucuronide derivative (210 μg ml^{-1}). For chromatographic conditions see Experimental section. I = Injection, AU = absorption units.

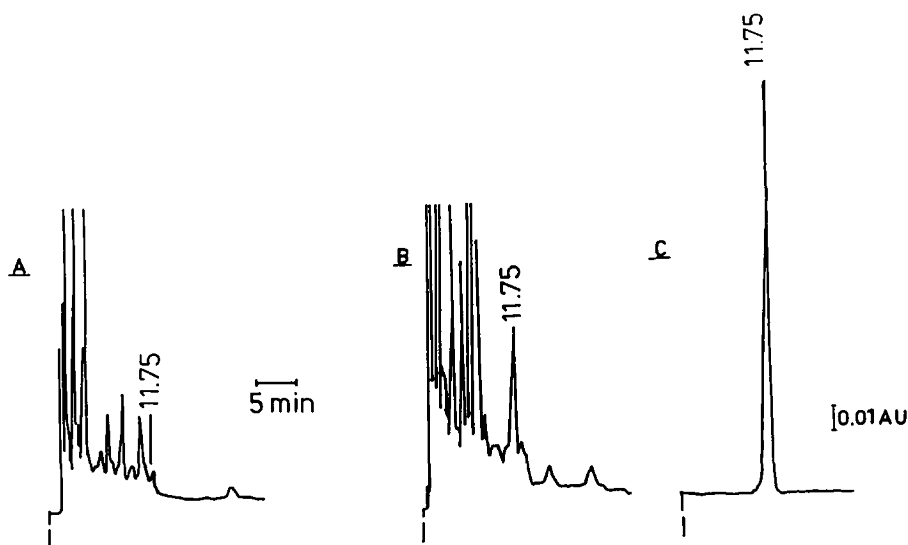

Figure 5
Typical HPLC traces of extracted and derivatized urine samples (A) before administration of 800 mg SMX, (B) after administration of 800 mg SMX, and (C) authentic reference: N-4 acetyl SMX N-1 glucuronide derivative (210 μg ml^{-1}). For chromatographic conditions see Experimental section. I = Injection, AU = absorption units.

atized urine extracts and of the synthesized standards.

The glucuronic acid moiety provides only a weak UV absorption in the range around 200 nm, therefore the UV characteristics of the drug moiety determine the suitable detection wavelengths for the conjugates. Peak purity was ensured by investigation of samples at both 220 and 280 nm and by analysis of spiked samples and blanks (results not shown).

The derivatization procedure transforms the genuinely acidic or amphoteric glucuronides to neutral, relatively lipophilic compounds which can be separated easily by isocratic RP-HPLC from physiological urine constituents. The use of TAM derivatives considerably simplifies the structural elucidation of glucuronides by MS. However, caution has to be exerted in the interpretation of GC analysis of TAM glucuronides because they partially decompose on the column, and are therefore not suitable for GC–MS. However, the HPLC system could be scaled up to provide milligram quantities of isolated glucuronides that could then be subjected to MS analysis, if required.

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