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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF GLUCURONIC ACID CONJUGATES AFTER DERIVATIZATION WITH 4-BROMOMETHYL-7-METHOXYCOUMARIN

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SUMMARY

In order to enhance the detection sensitivity of various glucuronic acid conjugates (phenol, menthol borneol, estrone and testosterone) in high-performance liquid chromatography (HPLC), the compounds were esterified with 4-bromomethyl-7-methoxycoumarin in the presence of potassium carbonate and 18-crown-6 in acetone. The resulting esters were chromatographed on either a normal-phase (NP) column (LiChrospher DIOL) with hexane–ethanol mixtures as eluents or a reversed-phase column (LiChrospher CH-18) with methanol–water mixtures. They were detected by UV spectrophotometry at 328 nm. The structure of the derivatives was confirmed by mass spectrometry by direct introduction and chemical ionization. Prior to this step, their isolation on a semi-preparative scale was performed by NP-HPLC. The extraction of the studied glucuronides from microsomal solutions was tested by ion-suppression and ion-pair liquid–liquid partition and liquid–solid chromatography (on octadecylsilica cartridges). Extraction and chromatographic data are discussed with regard to the determination of glucuronyltransferase activity towards the aglycones cited above.

INTRODUCTION

The need for a derivatization technique in the high-performance liquid chromatographic (HPLC) analysis of glucuronic acid conjugates has already been pointed out[1–3]. We have recently described the conversion of menthol glucuronide to an ester by use of 4-bromomethyl-7-methoxycoumarin (BrMmc) prior to chromatography[1].

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The resulting ester allows a detection sensitivity by UV spectrophotometry of 10 pmol injected in a derivatized extract from a microsomal solution.

Other selective and sensitive labelling reagents for glucuronides have been reported: N-(1-naphthyl)ethylenediamine (NED) and dansylcadaverine (Dns-C) were used to convert various glucuronides into fluorescent amides after activation of their acidic function with 2-bromo-1-methylpyridinium iodide[2]. The detection limit for the amide resulting from naphthol glucuronide and NED or Dns-C was found to be 50 and 100 fmol, respectively, by fluorimetry. Laser-induced fluorescence detection may lower this level to 100 amol for standards and 1–10 fmol for samples of biological fluids[3].

Another versatile technique for the determination of glucuronic acid conjugates has been devised[4]. It consists of a continuous-flow system which combines enzymatic hydrolysis (by β-glucuronidase, immobilized on a column) with lucigenin-induced chemiluminescence. The detection limit was 5 nmol for a standard of phenolglucuronide.

In this work, we have extended our previously described derivatization technique[1] to other glucuronic acid conjugates. Structural data of the resulting derivatives have been obtained by mass spectrometry after their isolation using a semi-preparative liquid chromatographic system. A general procedure for the extraction of free glucuronides from microsomal solutions prior to the derivatization step has been devised.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents were of analytical-reagent grade and were used without further treatment, except for acetone, which was dried over molecular sieves 3 Å (beads of size about 2 mm). HPLC-grade water was obtained with a Milli Q system (Millipore, Bedford, MA, U.S.A.). Menthol glucuronide ammonium salt and phenol, estrone and testosterone glucuronide sodium salts were obtained from Sigma (St. Louis, MO, U.S.A.). Borneol glucuronide was the synthetic material described previously[1]. Tetrabutylammonium bromide (TBA) was purchased from Aldrich (Strasbourg, France) and cethexonium bromide [hexadecyl(2-hydroxycyclohexyl)dimethylammonium bromide] was a generous gift from Clin-Midy (Paris, France). 4-Bromomethyl-7-methoxycoumarin (BrMmc) and 18-crown-6 were supplied by Fluka (Buchs, Switzerland).

Liquid chromatography systems

The analytical HPLC system consisted of a ternary solvent-delivery pump (Model SP 8700; Spectra-Physics, Santa Clara, CA, U.S.A.), an injection valve with a 10-μl sample loop (Model 7125; Rheodyne, Cotati, CA, U.S.A.) and a UV–VIS detector (Model LC 313; Merck-Clevenot, Nogent-sur-Marne, France). The reversed-phase column, packed with LiChrospher 100 CH-18 (HIBAR R.T. 250-4, 5 μm) and the normal-phase column, packed with LiChrospher 100 DIOL (LiChrocart 250-4, 5 μm) were purchased from E. Merck (Darmstadt, F.G.R.). Guard columns (4 × 4 mm I.D.) packed, respectively, with LiChrospher 100 RP-18 and DIOL, were used in all chromatographic analyses. Mobile phases were filtered through a 0.6-μm

microfilter (Type HVLP; Millipore) deaerated by bubbling with helium and used at a flow-rate of 1.2 ml/min. Their composition is given in Table I. The spectrometric detector was set at 328 nm and the chromatograms were recorded at 20 ± 2°C, and all calculations were made with an integrator (Model 5020, Spectra-Physics).

The semi-preparative liquid chromatographic (LC) system included a low-pressure pump (Model Duramat, Merck-Clevenot), a three-way valve, fitted with a 2-ml syringe as injector, a glass column (310 × 25 mm I.D.) packed with LiChroprep DIOL (40–63 μm) (E. Merck) and a UV–visible spectrophotometer (Model Leres; Leres, Clamart, France) equipped with a flow-through cell of 200-μl volume and operating at 328 nm. Mobile phases (for compositions see Table I) were used at a flow-rate of 12 ml/min. Eluent fractions corresponding to the derivatives were collected, evaporated to dryness under a gentle stream of nitrogen and frozen (−20°C) until analysed by mass spectrometry.

TABLE I

CHROMATOGRAPHIC CONDITIONS AND CAPACITY FACTORS (k') CALCULATED FOR EACH DERIVATIVE (Mmc GLUCURONIDE ESTER) AND BrMmc IN BOTH NORMAL-PHASE AND REVERSED-PHASE LIQUID CHROMATOGRAPHY

Compound	*Chromatographic mode*					
	Reversed-phase		*Normal-phase*			
	Analytical, LiChrospher CH-18 (5 μm)		*Analytical, LiChrospher DIOL (5 μm)*		*Semi-preparative, LiChroprep DIOL (40–63 μm)*	
	Mobile phase, methanol–water (v/v)	k'	*Mobile phase, hexane–ethanol (v/v)*	k'	*Mobile phase, hexane–ethanol (v/v)*	k'
BrMmc	75:25	3.0	80:20	1.5	80:20	1.6
	70:30	3.4	70:30	1.2	70:30	1.1
	65:35	4.5	70:30	1.2	70:30	1.1
Mmc menthol glucuronide ester	75:25	7.3	80:20	6.0	80:20	6.0
Mmc borneol glucuronide ester	75:25	5.2	80:20	7.0	80:20	7.0
Mmc testosterone glucuronide ester	75:25	4.8	70:30	6.6	70:30	6.8
Mmc estrone glucoronide ester	70:30	8.0	70:30	7.6	70:30	8.3
Mmc phenol glucuronide ester	65:35	2.6	70:30	4.7	70:30	5.2

Mass spectra

All spectra were recorded on a quadrupole mass spectrometer (Ribermag R10-10C; Delsi, Suresnes, France) associated with a data system (Ribermag Sidar 111B; Delsi). The dried residues obtained from the semi-preparative chromatography were dissolved in acetone at a concentration of about 1 $\mu g/\mu l$ and 1–2 μl of this solution was deposited with a 10-μl syringe on the tungsten wire. After evaporation of the solvent at room temperature, the desorption–chemical ionization (D-CI) probe was introduced through the standard vacuum lock of the apparatus into the source. The intensity of the current in the emitter was programmed from 30 to 500 mA at a rate of 7 mA/s. Desorption generally occurred when the intensity reached 250–300 mA (corresponding to 150–200°C), The source temperature was about 140°C.

The pressure of the reactant gas (ammonia) was maintained at a constant value between 0.1 and 0.5 torr. All spectra were recorded with an integration time of 1 ms per mass unit. Between analyses, the filament was heated to 1200°C in order to clean the emitter.

Extraction study of glucuronides

Glucuronides were extracted from aqueous solutions of standards or from microsomal solutions having the previously described composition[1]. In the latter instance, they were spiked with known amounts of glucuronide standards and the extraction was carried out without the incubation.

Three extraction techniques were tested, as follows.

(1) Liquid–liquid partition of the acids was carried out by adding 1.5 ml of 0.1 *M* phosphate buffer (pH 2.5) to 0.5 ml of a solution containing 5–30 μg of glucuronide and extracting twice with 5 ml of diethyl ether with vigorous shaking for 10 min at maximum speed on a mechanical shaker (Yamato SA-31; Bioblock, Strasbourg, France). The organic layers were collected, dehydrated over sodium sulphate and evaporated to dryness under nitrogen below 40°C. The dried residues were derivatized as described in the next section.

(2) Ion-pair extraction was achieved in a similar way, except for the use of a 0.1 *M* phosphate buffer (pH 6.5) and an organic phase consisting of 2 ml of a 10^{-3} *M* TBA or cethexonium solution in chloroform. Only one extraction was performed.

(3) Liquid–solid extraction was carried out with an octadecylsilica cartridge (Sep-Pak C_{18}; Waters-Millipore, Milford, MA, U.S.A.). A 1-ml volume of the glucuronide solution was acidified with 0.5 ml of acetic acid (20%, v/v) and transferred to a cartridge that had been pre-wetted with 5 ml of methanol–water (4:1). After 15 min, the cartridge was washed with 3 ml of HPLC-grade water and residual water was removed by flushing with air by means of a syringe. The conjugates were eluted with 3 ml of methanol containing 0.1% (v/v) of triethylamine. The elutate was evaporated to dryness under nitrogen.

In all instances an internal standard was used, chosen according to the glucuronide studied: borneol glucuronide for menthol glucuronide, testosterone glucuronide for estrone glucuronide and *vice versa*. After extraction and before derivatization, 0.2 ml of 0.2 mg/ml glucuronide solution in acetone was added in order to study the recovery of the other glucuronide tested for extraction. For biochemical studies, microsomal solutions were directly spiked with the internal standard before extraction.

Derivatization procedures

For analytical purposes and extraction studies, the derivatization conditions were as follows: the dried residue (containing up to 30 μg of glucuronide) was dissolved in a mixture of 0.08 ml of N,N-dimethylformamide and 0.5 ml of acetone. Then, 0.2 ml of 1.5 mg/ml BrMmc and 0.1 ml of 1 mg/ml 18-crown-6 solutions in acetone and 20 mg of potassium carbonate were added. The reaction mixture was heated at 70°C for 30 min, then cooled in ice and an aliquot was injected into the HPLC system. The optimization of this procedure has been discussed previously[1].

For semi-preparative isolation, 1 mg of each glucuronide was mixed with 2 mg of BrMmc, 2 mg of 18-crown-6 and 20 mg of potassium carbonate in acetone (total volume, 4 ml). The solution was evaporated to dryness under nitrogen and the residue was dissolved in 0.5 ml of N,N-dimethylformamide–acetone (25:75, v/v). This final solution was injected into the semi-preparative LC system.

RESULTS AND DISCUSSION

Chromatographic conditions

Derivatives of BrMmc and organic acids of biological interest (fatty acids[5,6], prostaglandins[7] and a drug metabolite[8] are usually chromatographed on RP columns by isocratic[5,7,8] or gradient[6] elution. With an octadecylsilica column it is necessary to increase the water concentration in the mobile phases in order to elute the more polar esters more slowly. Consequently, many peaks may appear close to that of the excess reagent. They are assumed to be by-products of the derivatization reaction or esters of endogenous compounds extracted from the biological matrix. This is a disadvantage which is more critical when the analyte is eluted before the reagent itself, as is illustrated for the phenol glucuronide derivative, which exhibits a shorter retention time than BrMmc, in contrast to the other glucuronide esters tested in this work (see Table I). Accurate quantitation is difficult under such conditions. Therefore, we have developed an NP system, using an hydrophilic modified silica (LiChrospher DIOL) column. The main advantage of such a stationary phase is that it is less sensitive than bare silica to the residual water in the organic solvents used as mobile phases and thus gives a better day-to-day reproducibility. With this system, the excess reagent is efficiently separated from the phenol glucuronide derivative by increasing the capacity factor of the latter (see Table I for chromatographic data). The NP- and RP-HPLC systems described were selected on the basis of the polarity of the glucuronide ester in comparison with that of the derivatizing reagent. Choosing either of these HPLC systems obviates the need for more complicated HPLC systems, where two columns (ion-exchange and reversed-phase) are connected with a switching device in order to divert the excess reagent[2,3].

Off-line liquid chromatography–mass spectrometry (LC–MS) of Mmc-glucuronide esters

Direct introduction of a sample into a mass spectrometer requires compounds of great purity. For a study of the products resulting from the esterification of glucuronic acid conjugates and BrMmc, it was important to eliminate excess reagent. LC can easily achieve this separation. We first used RP chromatography and freeze-dried the collected fractions containing the derivatives. However, hydrolysis

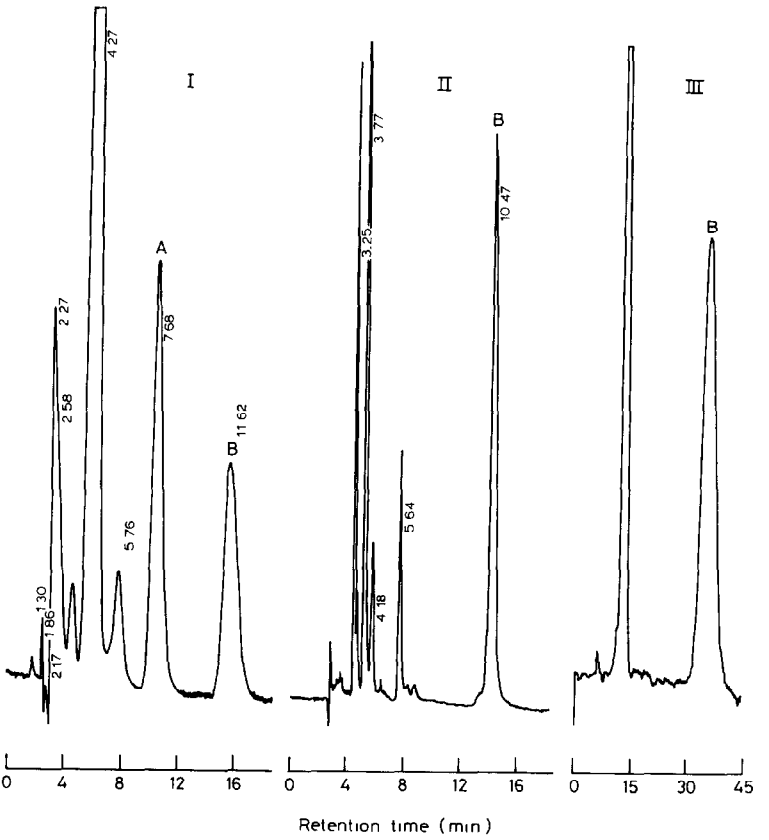

Fig. 1. Typical chromatograms obtained for derivatized testosterone (A) or menthol (B) glucuronide with BrMmc in the HPLC (I, II) or semi-preparative (III) system. I, II, Extracts of microsomal solution obtained by liquid–solid chromatography and derivatized were injected on to (I) a LiChrospher CH-18 (5 μm) column (250 × 4 mm I.D.) and eluted with methanol–water (3:1)(II) on to a LiChrospher DIOL (5 μm) column (250 × 4 mm I.D.) and eluted with hexane–ethanol (4:1). About 0.25 μg of each adduct was injected. Flow-rate, 1.2 ml/min. III, Semi-preparative scale isolation of ester (1 mg injected) carried out on a Lobar LiChroprep DIOL (40–63 μm) column, eluted with hexane–ethanol (4:1) at a flow-rate of 12 ml/min. Detection at 328 nm in all instances.

of the ester bond occurs during this operation, resulting in a poor recovery of the adduct. We prefer NP chromatography, because solvents are more easily removed. In order to increase the amount of derivatives collected, a semi-preparative LC apparatus was employed, including the same stationary phase as in NP-HPLC. No major selectivity decrease was observed compared with the analytical system. The chromatographic conditions and data are indicated in Table I and typical chromatograms are shown in Fig. 1.

An amount of 1 mg of each glucuronide ester was isolated with a single injection. The purity of the collected and evaporated fractions was tested in the HPLC system and was calculated to be greater than 98.0 ± 1.5%.

TABLE II

CHARACTERISTIC FRAGMENTS OF Mmc GLUCURONIDE ESTERS, OBTAINED BY MASS SPECTROMETRY WITH DESORPTION CHEMICAL IONIZATION (AMMONIA)

Ester of Mmc-glucuronide of	*m/z values**					
	A	*B*	*C*	*D*	*E*	*F*
Menthol	538	520	350	332	224	207
Borneol	536	518	348	330	224	207
Testosterone	670	652	482	464	224	207
Estrone	652	634	–	444	224	207
Phenol	460	442	288	270	224	207

* A = ester molecular weight (MW) + 18; B = ester MW; C = glucuronide MW + 18; D = glucuronide MW; E, F = parts of the chromophoric label.

For MS, chemical ionization (with ammonia) rather than the electron impact mode was chosen in order to produce an intense molecular peak and recognizable fragments. Two significant peaks (A and B in Table II) occurred at *m/z* values corresponding to the molecular weight (MW) of each Mmc glucuronide ester and to MW + 18. The fragments C and D correspond to the MW of each glucuronic acid conjugate moiety and to MW + 18. Other peaks (*m/z* 207 and 224) appear constantly in each derivative spectrum and are due to the chromophoric label. A typical mass spectrum of Mmc-menthol glucuronide ester is shown in Fig. 2.

Extraction study of glucuronides

The development of a general extraction procedure permitting the complete recovery of glucuronides from aqueous solutions or biological matrices is difficult

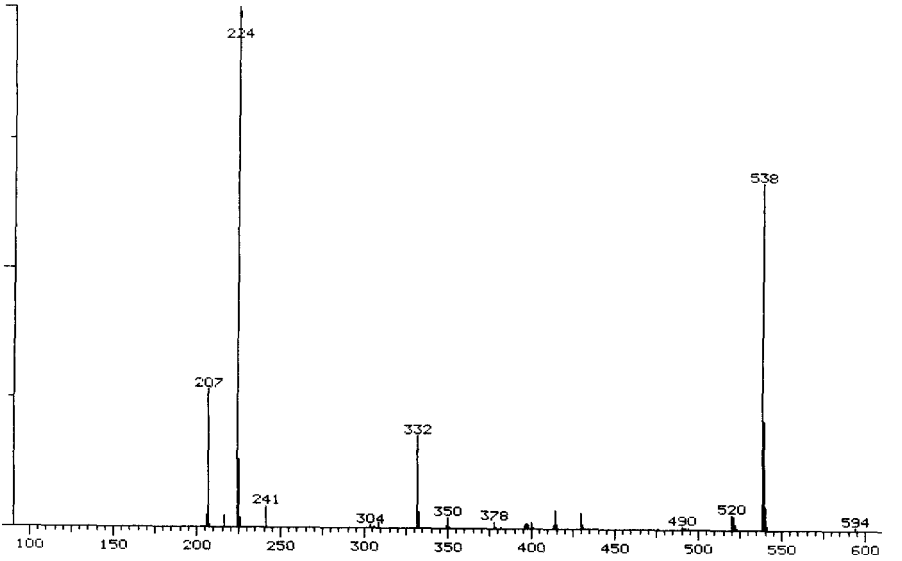

Fig. 2. Typical mass spectrum of Mmc-menthol glucuronide ester.

TABLE III

RECOVERY FROM SAMPLES OF AQUEOUS STANDARD SOLUTIONS OF GLUCURONIDE* OR MICROSOMAL SOLUTIONS, SPIKED WITH STANDARDS** EXTRACTED WITH DIFFERENT LIQUID–LIQUID AND LIQUID–SOLID TECHNIQUES

Glucuronide of	*Recovery (%)*			
	Liquid–liquid extraction			*Liquid–solid extraction***
	*Partition of acid***	*Ion-pair extraction**		*Sep-Pak C_{18} cartridge*
		TBA	*Cethexonium*	
Menthol	80.90 ± 3.50	15.17 ± 0.75	69.80 ± 2.23	97.04 ± 4.27
Borneol	80.00 ± 3.52	10.00 ± 0.50	80.22 ± 2.55	98.77 ± 4.32
Testosterone	51.60 ± 2.22	<0.50	95.00 ± 3.00	96.00 ± 4.10
Estrone	50.73 ± 1.79	<0.50	90.00 ± 2.80	93.33 ± 4.10
Phenol	9.60 ± 0.43	11.16 ± 0.56	<0.50	7.36 ± 0.29

owing to differences in polarity between the various aglycones. Early methods relying on precipitation with heavy metal salts (zinc[9] or lead[10]) or with excess ammonium sulphate[11] give good results only at high concentrations. Liquid–liquid partition techniques may give good yields if the solvent is well matched with the polarity of the aglycone conjugated with glucuronic acid (see Table III, results for menthol and borneol glucuronides, extracted with diethyl ether). The use of more polar organic solvents in the case of less hydrophobic conjugates increases the recovery, *e.g.*, from 9 to 51% for the phenol glucuronide with ethyl acetate. Even butanol has been suggested[12], but the phase separation is difficult.

Ion-pair extraction may be a good alternative for resolving these problems[13,14] if the chosen counter ion is sufficiently hydrophobic and is used at high enough concentrations. Better recoveries are obtained with the use of cethexonium than with TBA, but the method is unsuccessful with phenol glucuronide (see Table III). This technique failed for all the glucuronides studied when applied to microsomal solutions. The unfavourable results were assumed to be due to the high chloride concentrations in the medium studied. This anion is also extracted and interferes in the derivatization reaction. This was studied in more detail previously[15]. Liquid–solid extraction has been investigated as an alternative method. Much has been accomplished with the synthetic polymeric sorbent Amberlite XAD-2, especially with steroid glucuronides[16,17], but poor results are obtained with terpenoid aglycones, because they are less adsorbed, and with aromatic aglycones, which are irreversibly adsorbed.

Recently, octadecylsilica cartridges have been demonstrated to give quantitative recoveries of glucuronides from plasma or urine at very low concentrations[2]. We have tested this technique and obtained excellent recoveries (see Table III) without interference from biological components, except with phenol glucuronide. The extraction procedure for the *in vitro* assay of this conjugate was a triple liquid–liquid extraction with ethyl acetate. The accuracy of the method, including the extraction, derivatization and HPLC, was a coefficient of variation of 4.4% ($n = 5$). The detec-

tion limit for a derivatized extract of a microsomal solution, spiked with menthol glucuronide and corresponding to a signal-to-noise ratio of 10, was 10 pmol of injected sample.

Calibration graphs obtained with this clean-up procedure exhibit good linearity. For instance, the equation for the calibration line corresponding to the determination of menthol glucuronide in microsomal solutions for six samples containing 5–30 μg/ml of the conjugate was $y = 0.0973\ (\pm\ 0.0043)\ x - 0.0674\ (\pm\ 0.0029)$ ($r = 0.994$).

Other new derivatization methods in which extraction and derivatization steps are carried out simultaneously are still to be investigated for glucuronides. Extractive alkylation of organic acids has been said to be promising for HPLC analysis[15,18]. The use of sorbents on which the derivatizing reagent is adsorbed may also be attractive[19].

CONCLUSION

The two main disadvantages of the direct HPLC of glucuronides are the loss of selectivity resulting from the low wavelength used in UV spectrophotometry in order to increase the sensitivity and the long analysis time required for the elution of the aglycone that is also present in biologicl samples and is much less polar. In our technique, the derivatization of the glucuronic acid conjugates is less time consuming and provides selective and sensitive UV detection. Combination of a liquid–solid extraction procedure with this HPLC technique is being used in our laboratory as a useful analytical method for the measurement of glucuronyltransferase activity towards various aglycones in *in vitro* assays.

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