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EVALUATION OF A VERSATILE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM USING CETHEXONIUM BROMIDE AS ION-PAIRING REAGENT FOR THE ANALYSIS OF GLUCURONIC ACID CONJUGATES

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SUMMARY

An ion-pair high-performance liquid chromatographic technique has been developed to reduce the elution times of both glucuronic acid conjugates and their free aglycones. The chromatographic system combines the use of a reversed-phase column (LiChrospher CH-18; 5 μm) and a mobile phase of methanol (70–80%)–0.01 *M* phosphate buffer (pH 6.0) containing 2.5 m*M* cethexonium bromide as counter-ion at a flow-rate of 1 ml min^{-1}. The hydrophobicity of this quaternary ammonium ion-pairing reagent and the high content of the organic modifier in the mobile phase provide close and short elution times for a wide structural variety of compounds (i.e. alcohols, phenols, steroids, carboxylic acids) and their conjugates with glucuronic acid (capacity factors lower than 7.5), without compromising the selectivity with respect to endogenous compounds of the microsomal incubation medium and urine. Advantages of cethexonium bromide over conventional tetrabutylammonium salts are clearly demonstrated, and the described system was applied to the simultaneous quantitation of clofibric acid and its acylglucuronide in human urine and validated for a pharmacogenetics purpose.

INTRODUCTION

Glucuronidation is an important metabolic conjugation pathway, occurring in mammals during the metabolism of both xenobiotics (drugs, pollutants)

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and endogenous compounds (hormones, neuromediators, bile acids). The enzyme involved (UDPglucuronosyltransferase; EC 2.4.1.17) exhibits a very broad substrate specificity, a multiple localization (mainly in the liver but also in other sites such as kidney, intestine, lung, adrenal glands, brain, heart and skin), differences in its perinatal development and variability on inducibility and activation by a wide range of compounds [1]. Moreover, genetically based polymorphism in the glucuronidation of foreign substances has not yet been demonstrated.

So, the investigation of the enzyme mechanism implies numerous in vitro and in vivo glucuronidation assays that require fast, general, selective and sensitive analytical methods. Among the techniques devoted to this purpose, those based on the analysis of the intact glucuronides are more advisable and many direct high-performance liquid chromatographic (HPLC) assays have been reported and reviewed [2,3]. Pre-column derivatization of glucuronides [4–6] provides more sensitive detection and has to be performed when the corresponding aglycones have no UV–visible spectral properties. Moreover, this analytical approach looks interesting since it can be easily extended to this class of conjugates. However, HPLC techniques in which detection relies on the same physicochemical properties of the glucuronide and its aglycone are still necessary, because they are more simple and they permit the measurement of the concentration ratio of glucuronide to aglycone.

Previously described chromatographic methods have some disadvantages. They have been elaborated on an individual basis and hence require quite different elution conditions for each conjugate–aglycone couple measured. Also, the reversed-phase systems require long analysis times (1 h or more) for the elution of apolar aglycones, such as naphthol and testosterone [7]; this decreases the number of assays that can be performed per day.

Several methods have been described to overcome these limiting factors. A more polar stationary phase can be combined with gradient elution, but the overall analysis time remains longer than 1 h [8]. A two-column switching technique shortens the elution time of the aglycone [9], but needs more material and increases the cost of analysis.

We have investigated the use of a hydrophobic quaternary ammonium compound as ion-pairing reagent in a reversed-phase HPLC system, and compared with that of tetrabutylammonium bromide its efficiency at separating glucuronic acid conjugates and their aglycones with close and short retention times. The possibility of using the technique for in vivo and in vitro glucuronidation measurements was tested.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents were analytical-reagent grade and used without further purification. Tetrabutylammonium (TBA) bromide was purchased

from Aldrich (Strasbourg, France) and cethexonium bromide [hexadecyl-(2-hydroxycyclohexyl)dimethylammonium bromide] from Cooper (Melun, France). Uridine diphosphoglucuronic acid (UDPGA) sodium salt and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) were obtained from Boehringer (Mannheim, F.R.G.). Triton X-100, Tris, clofibric acid, β-glucuronidase from bovine liver source, ($\pm$)-menthol glucuronide ammonium salt, 4-nitrophenol, phenol, phenolphthaleine, androsterone, estrone and testosterone glucuronide sodium salts were purchased from Sigma (St. Louis, MO, U.S.A.). Clofibrate tablets (Lipavlon® 500) were supplied by ICI-Pharma (Cergy, France).

2-Naphthol glucuronide and (−)-borneol glucuronide were synthesized by a Helferich reaction using stannic chloride as the catalyst. Cyclohexanol glucuronide was the synthetic material resulting from a Koenigs–Knorr reaction using mercuric cyanide as the catalyst. The IR and NMR data of the synthetic glucuronides were established, and their purity (over 98%) was determined by potentiometric titration [10].

Stock solutions of the standard glucuronides were prepared at a concentration of 0.1 mg ml^{-1} in the mobile phase and stored at −20°C.

Since the other glucuronides considered in this work were not commercially available, or their syntheses were difficult to carry out, they were obtained from a biological source as described below.

Biosynthesis of glucuronides

In vitro incubation. Hepatic microsomes of mature male Wistar rats were prepared by a conventional ultracentrifugation technique [11]. Their protein content was measured by the method of Lowry et al. [12] with bovine serum albumin as the standard. They were frozen at −20°C and used within four weeks. Microsomal fractions were diluted with 75 m*M* Tris–HCl buffer (pH 7.0) containing 5 m*M* magnesium chloride to a final protein concentration of 5 mg ml^{-1}. Triton X-100 was used as activating reagent for glucuronidation of phenolic aglycones, CHAPS for that of steroids and digitonin for that of carboxylic acids. They were added to the microsomal suspension in an optimal detergent-to-protein ratio of 0.4, 0.8 and 1.0 (w/w), respectively. The mixture was allowed to stand for 20 min at 0°C to complete full activation. The activated microsomes were diluted with Tris–HCl–$MgCl_2$ buffer to obtain a protein concentration of 1 mg per tube, and 35 μl of a 100 m*M* aqueous solution of UDPGA were added. The mixture was pre-incubated in a shaking water-bath at 37.0 ± 0.1°C for 5 min before addition of the aglycone, previously dissolved in ethanol–water (40:60, v/v) in the case of phenols and steroids or in dimethyl sulphoxide for carboxylic acids. The reaction volume was adjusted to 1.0 ml with Tris–HCl–$MgCl_2$ buffer, and the incubation step was carried out at 37.0 ± 0.1°C for 20 min. The reaction was stopped by adding 0.15 ml of 0.15 *M* hydrochloric acid and transferring the flask to an ice-bath.

In vivo source. Human urine samples were collected from healthy male and female subjects over an 8-h period after a single oral dose of 500 mg of clofibrate taken at 11 p.m. The volumes of the samples were measured and their pH values recorded (5.5–6.5); aliquots were stored at −20°C.

Identification of glucuronic acid conjugates

All the conjugates obtained from biological sources were identified by comparison of the chromatogram with those resulting from either a urine blank or a microsomal suspension incubated without aglycone and from enzymic hydrolysis. This latter operation was performed by dissolving 3000 Fishman units of β-glucuronidase in 1.0 ml of 0.1 *M* phosphate buffer (pH 5.0); this solution was added to 1.0 ml of urine or microsomal suspension resulting from incubation. The mixture was kept at 37.0 ± 0.1°C for 24 h. The β-glucuronidase-resistant isomers of acylglucuronides were identified by alkaline hydrolysis: 1 ml of sample was mixed with 1 ml of 1.0 *M* sodium hydroxide and incubated at 37°C for 4 h. The same extraction procedure was performed before and after the enzymic or chemical hydrolysis step, and the disappearance of the glucuronide peaks on the chromatogram was observed.

Extraction of glucuronides from biological media

Glucuronides were extracted either by a liquid–solid technique on octadecylsilica cartridges, as previously described [5], or by liquid–liquid partition as follows. Microsomal suspensions were acidified by adding 1 ml of 0.1 *M* phosphate buffer (pH 2.0), and the urine samples were diluted twenty-fold in the same buffer. The resulting solutions were extracted twice by ethyl acetate or diethyl ether (organic/aqueous phase volume ratio of 2). After centrifugation for 5 min at 5000 *g*, the organic layers were pooled and evaporated to dryness below 30°C under nitrogen. The dried extracts were dissolved in the mobile phase (0.2–1.0 ml) before injection in the HPLC system.

The extraction method used for each glucuronide studied is indicated in Table I.

Chromatographic conditions

The HPLC system consisted of a ternary solvent delivery pump (Model SP 8700; Spectra Physics, Santa Clara, CA, U.S.A.), an injection valve fitted with a 10-μl sample loop (Model 7125; Rheodyne, Cotati, CA, U.S.A.) and an integrator (Model SP 4290; Spectra Physics). Spectrophotometric detection was performed with a UV–visible variable-wavelength detector (Model LC 871; Pye Unicam, Cambridge, U.K.) and conductimetric detection with a Tridet detector (Perkin Elmer, CT, U.S.A.)

Analytical columns (Hibar, RT, 250 mm × 4 mm I.D., E. Merck, Darmstadt, F.R.G.) were prepacked with LiChrospher CH-18 (5 μm) and guard columns (4 mm × 4 mm I.D.) with LiChrospher 100 RP-18 (5 μm).

TABLE I

CAPACITY FACTORS OF VARIOUS AGLYCONES AND THEIR GLUCURONIDES

Column, LiChrospher CH-18 (5 μm; 250 mm × 4 mm I.D.); mobile phase, methanol–0.01 *M* phosphate buffer (pH 6.0) containing 2.5 m*M* cethexonium bromide; flow-rate, 1 ml min^{-1}.

Compound	Class	Capacity factors (k')[a]		Detection mode	Source[b]	Extraction mode[c]
		Aglycone	Conjugate			
Cyclohexanol	Alcohol	N.D.[d]	3.4 (2)		S	-
(−)-Borneol		N.D.	4.9 (2)	Conductimetric	S	-
(±)-Menthol		N.D.	6.9 (2)		S	-
Morphine	Aromatic	0.6	1.0 (2)	UV 254/220 nm	B	L-S
Acetaminophen		0.05	1.5 (2)	254 nm	B	L-L (EA)
Phenol		0.3	1.8 (2)	254 nm	S	-
Phenolphthalein		0.3	1 9 (2)	254 nm	S	-
4-Hydroxyphenytoin		0.3	2 1 (1)	254 nm	B	L-L (EA)
4-Hydroxyphenobarbital		0.3	2 5 (1)	254 nm	B	L-L (EA)
2-Naphthol		0.9	3.6 (2)	254 nm	S	-
					B	L-L (EA) or L-S
Thymol		1.9	4.9 (2)	254 nm	B	L-L (DE)
Estrone	Steroid	1.3	3.5 (2)	220 nm	S	-
Testosterone		1.3	3.9 (2)	220 nm	S or B	L-S
Androsterone		1.6	4.3 (2)	220 nm	S	-
Flurbiprofen	Carboxylic	4.1	2 7 (3)	273 nm	B	L-L (EA)
Pirprofen	acid	3.9	2 9 (3)	273 nm	B	L-L (EA)
Ibuprofen		5.0	3 8 (3)	278 nm	B	L-L (DE)
Clofibric acid		5.8	4.2 (2)	224 nm	B	L-L (EA)

[a]Methanol content of mobile phase: (1) 70%; (2) 75%; (3) 80%

[b]Sources. S, synthetic; B, biological.

[c]L-S, liquid-solid; L-L, liquid-liquid; EA, ethyl acetate; DE, diethyl ether.

[d]N.D., not detected.

The mobile phase was either acetonitrile (25–35%, v/v)–0.01 *M* phosphate buffer (pH 6.0) containing 5 m*M* TBA, or methanol (70–80%, v/v)–0.01 *M* phosphate buffer (pH 6.0) containing 2.5 m*M* cethexonium bromide. They were filtered through a 0.45-μm microfilter (Sartorius, Palaiseau, France) and used at a flow-rate of 1 ml min^{-1}.

Chromatograms were recorded at 20 ± 2°C, and the void volume of the column was measured by injecting a sodium chloride solution and using conductimetric detection.

The column was equilibrated by flushing with 60 ml of mobile phase, and the HPLC system was rinsed with 100 ml of methanol–ethanol–water (50:25:25, v/v) to prevent any blockage of the pump and column when cethexonium bromide was used.

Quantitation of clofibric acid and its glucuronide in urine

For simultaneous quantitation of clofibric acid and its glucuronide, we determined the wavelength at which the extinction coefficients of the cited com-

pounds were equal. The ratio of their peak areas was calculated for different wavelengths, ranging from 220 to 235 nm. For this purpose, urine samples of five volunteers, who had ingested a single 500-mg oral dose of clofibrate, were collected after 8 h and pooled. Several effluent fractions corresponding to the main glucuronide peak were collected and hydrolysed successively by enzymic and alkaline methods. The ratio of clofibryl glucuronide to clofibric acid obtained by hydrolysis was 1.00 ± 0.02 at a wavelength of 224 nm. A calibration curve was then established by using blank urine spiked with clofibric acid in the concentration range 5–200 $\mu g\ ml^{-1}$ and with 2-naphthol glucuronide (40 $\mu g\ ml^{-1}$) as internal standard.

RESULTS AND DISCUSSION

Choice of the counter ion and optimization of mobile phases

The carboxylic acid group on the sugar moiety of glucuronides has a dissociation constant (pK_a) close to 3.5. Thus the pH of the mobile phase used in combination with ion-exchange [13] or reversed-phase [14,15] columns is often the most important factor. With the latter stationary phases, ion suppression can be brought about by acidifying the eluent with trifluoroacetic acid [9] in order to increase the capacity factors of the glucuronides. However, an ion-pairing system appears more attractive for this purpose [14,15], and the usual counter ion is TBA. The elution of aglycones relies only on their respective polarities, except for morphine and its conjugate whose free amino group is associated with an anionic ion-pairing reagent such as sodium dodecylsulphate [16].

The weak content of organic modifier in mobile phases incorporating TBA does not allow the fast elution of apolar aglycones, such as naphthol and testosterone. Different authors have incorporated into eluents more hydrophobic quaternary ammonium compounds than TBA in order to chromatograph polar carboxylic acids, e.g. cetyltrimethylammonium for glycyrrhizinic and glycyr-

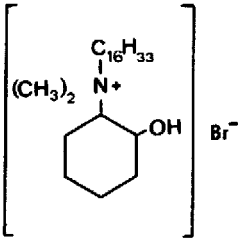

Fig. 1. Structure of cethexonium bromide.

rhetic acids [17], and tetraoctylammonium for cephalosporins [18]. We selected cethexonium bromide (Fig. 1) from among several drugs possessing a quaternary ammonium group, because of its strong hydrophobicity. This com-

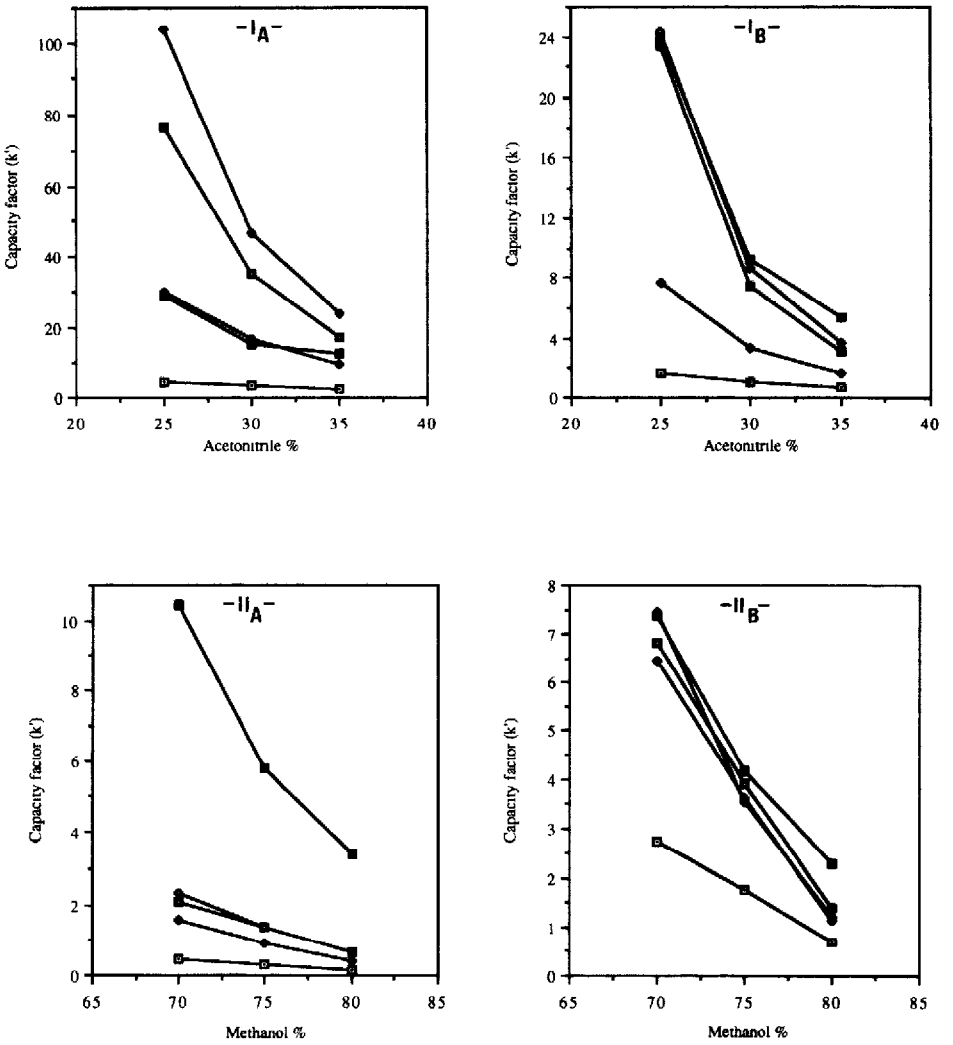

Fig. 2. Relationship between the capacity factors (k') of glucuronides and their aglycones and the content of organic modifier in the mobile phases. (I) Mobile phase, acetonitrile–0.01 *M* phosphate buffer (pH 6.0)+5 m*M* tetrabutylammonium bromide. (II) Mobile phase, methanol–0.01 *M* phosphate buffer (pH 6.0)+2.5 *M* cethexonium bromide. Flow-rate, 1 ml min^{-1}; column, LiChrospher CH-18 (5 μm; 250 mm×4 mm I.D.). (A) (⊡) Phenol; (♦) 2-naphthol; (□) testosterone; (◇) estrone; (■) clofibric acid. (B) (⊡) Phenol glucuronide; (♦) 2-naphthol glucuronide; (□) testosterone glucuronide; (◇) estrone glucuronide; (■) clofibryl glucuronide.

pound, which is known for its antiseptic properties, fulfills the requirements of the present analytical application since it exhibits no UV absorption.

Cethexonium is almost insoluble in acetonitrile, but more soluble in methanol and other alcohols. Acetonitrile–2-propanol was tested as the organic modifier in the mobile phase, but it was difficult to optimize their proportion. Methanol was preferred, and when its content in eluents including cethexonium as ion-pairing reagent was in the range 70–80% the capacity factors of tested glucuronides were close to those obtained with TBA-containing mobile phases. Moreover, the elution times of aglycones were considerably shorter than with eluents incorporating 25–35% of acetonitrile and TBA (Fig. 2).

All the alcoholic, phenolic and steroidal aglycones were eluted even faster than their respective glucuronide. Carboxylic acids and their acyl glucuronides both possess a free acid group, and so their elution order was inverted. The capacity factors of a wide variety of compounds (Table I) demonstrate that the chromatographic system is quite versatile and only needs the optimization of methanol percentage in a narrow range.

In vivo glucuronidation measurements of clofibric acid

Clofibrate is an hypolipidaemic drug whose free acid form (clofibric acid) is pharmacologically active [19]. The main metabolite found in urine is the corresponding acyl glucuronide [20]. Previously described HPLC techniques devoted to the measurement of clofibric acid and its conjugate in biological fluids are based on reversed-phase systems, including acidified mobile phases [21–24]. Eluents containing TBA as counter-ion have been reported for other acyl glucuronides, e.g. zomepirac [25] and tolmetin [26] conjugates. Under the selected chromatographic conditions (Fig. 3), the retention times of 2-naphthol glucuronide (internal standard), clofibryl glucuronide and clofibric acid were 9.5, 12 and 15 min, respectively, with a resolution between vicinal peaks better than 1.5 and full selectivity versus endogenous peaks. For comparison, another mobile phase (CH_3CN–H_2O–CH_3COOH, 33:66.5:0.5, v/v) was tested on the same column [LiChrospher CH-18 (5 μm); 250 mm×4 mm I.D.]; clofibryl glucuronide and its aglycone were eluted within 8 and 30 min, which doubled the overall analysis time.

Direct injection of diluted urine could not be applied to routine analysis since late-eluting peaks appeared on the chromatogram, so sample pretreatment was needed. The liquid–liquid extraction technique gives over 95% recovery from urine for the three solutes. The characterization of conjugate peaks was performed by both enzymic and chemical hydrolysis of urine. After reaction with β-glucuronidase, the main glucuronide peak disappeared and simultaneously the peak surface of free clofibric acid increased. No change was observed for the other twin small peaks, which were close to the previously identified glucuronide peak. This implies that corresponding compounds were insensitive to the enzyme action. Their peaks were suppressed by alkaline hydrolysis, which

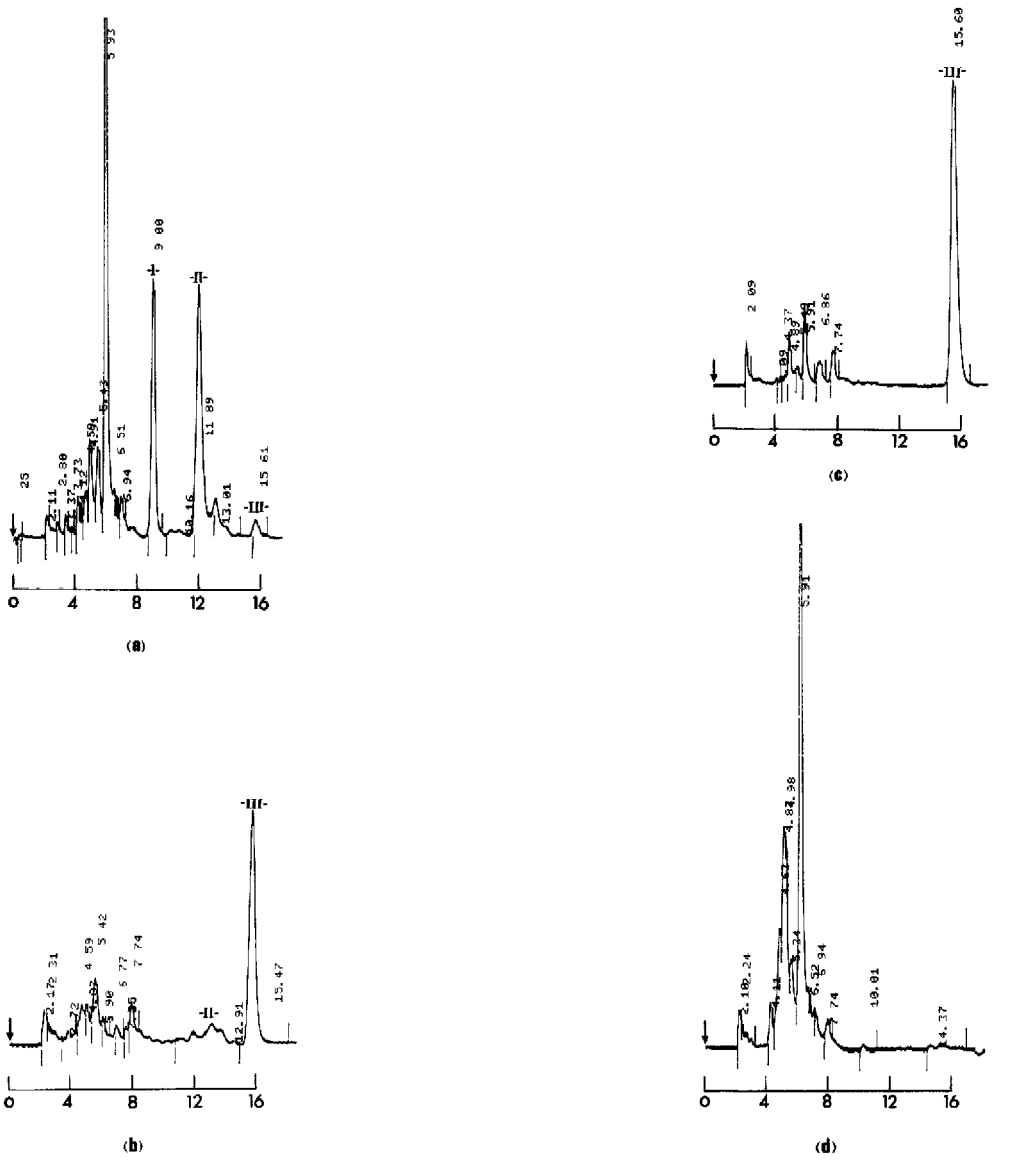

Fig. 3. Typical chromatograms obtained for clofibric acid and its acyl glucuronide extracted from the urine of a healthy subject 8 h after ingestion of a single 500-mg oral dose of clofibrate. (a) 2-Naphthol glucuronide (I) used as internal standard, clofibryl glucuronide (II) and clofibric acid (III). (b) Same sample as in (a), hydrolysed by β-glucuronidase over a 24-h period. (c) Same sample as in (a), hydrolysed in 0.5 *M* sodium hydroxide. (d) Blank corresponding to a urine extract of a subject not treated with clofibrate. Chromatographic conditions: LiChrospher 100 CH-18 (5 μm; 250 mm$\times$4 mm I.D.) column; mobile phase, methanol–0.01 *M* phosphate buffer, pH 6.0 (75:25, v/v), plus 2.5 m*M* cethexonium bromide; flow-rate, 1 ml min^{-1}; detection wavelength, 224 nm.

suggests the formation in human urine of isomers resulting from intramolecular rearrangement of the ester glucuronide [27]. These acyl isomers are known to be β-glucuronidase-resistant metabolites, and they have been previously separated by a thin-layer chromatographic method [28]. In this latter report, urine fractions collected from twelve subjects were analysed, and total clofibric acid was recovered as follows: an average of 54.4% as 1-O-acyl glucuronide, 5.1% as free clofibric acid and thus 40.4% as alkaline-labile isomers. In the present work, a mean value of 28% of conjugate was recovered as metabolites resistant to β-glucuronidase.

Carboxylic acid glucuronides are more unstable than alcoholic or phenolic glucuronides; they easily undergo hydrolysis or intramolecular rearrangement at mild pH [29]. For clofibric acid conjugate, the acyl migration mainly occurs at pH values higher than 7.0 at 37°C [27]. Otherwise, any nucleophilic solvent can be applied in an intramolecular transesterification and a degradation of the acyl glucuronide. For example, the stability of the zomepirac conjugate has been studied as a function of pH at 37°C [30]. When a buffer of pH 7.4 was used with 50% methanol, the half-life of the glucuronide reached 0.16 h and was 2.8 times shorter than the value obtained with the buffer alone. For these reasons, the stability of the ester glucuronide of clofibric acid was tested under our assay conditions. At room temperature, aliquots of a same urine extract dissolved in the mobile phase were injected into the HPLC system at different times over a 5-h period. No significant change was observed in the ratio of the clofibryl glucuronide/internal standard peak areas.

The use of the isobestic point of the UV absorption curves of both measured compounds as the detection wavelength ($\lambda = 224$ nm) permitted their simultaneous monitoring in urine with only one calibration curve, established with clofibric acid as the standard added to the biological fluid. The resulting equation of the regression straight line was $y = 0.0048x - 0.0044$, with a correlation coefficient of 0.999 and a coefficient of variation of 3.6% ($n = 5$) for the lowest measured concentration. The HPLC procedure was applied to the investigation of possible genetically based differences occurring in the glucuronidation of clofibrate in a human population [31].

CONCLUSION

The HPLC system described in this paper provides a versatile and fast method for glucuronidation measurement. The use of cethexonium bromide instead of TBA salts as a hydrophobic ion-pairing reagent, with a high content of methanol in the mobile phase, decreases the retention times of apolar aglycones, which also elute close to their conjugates. The short resulting analysis times do not preclude selectivity versus other components of microsomal suspensions and urine and will permit numerous assays for biochemical and pharmacogenetic purposes.

Moreover, the similar capacity factors obtained for each aglycone–glucuronide couple allow the choice of their isobestic point as the detection wavelength without loss of precision occurring with late-eluting peaks. This advantage is particularly helpful when a glucuronide standard is difficult to obtain by chemical synthesis.

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