Journal of Chromatography, 499 (1990) 411–421
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 786

REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY OF GLUCURONIDES

RETENTION AND SELECTIVITY

MORGAN STEFANSSON and DOUGLAS WESTERLUND*
Department of Analytical and Pharmaceutical Chemistry, Uppsala University Biomedical Center, P.O. Box 574, S-751 23 Uppsala (Sweden)

SUMMARY

The parameters that influence the retention and selectivity of glucuronides in reversed-phase ion-pair liquid chromatography were investigated. The extent of ion-pair retention was dependent on the degree of substitution of the ammonium counter ion used and increased in the order 4- < 2- < 3- < 1-substituted, comparing counter ions with the same number of carbon atoms. The retentions and selectivities were influenced by the type of buffer system, solid phase and organic modifier used. The selectivities obtained were so high that a urine sample spiked with four glucuronides could be analysed by a direct injection technique and UV detection (254 nm).

INTRODUCTION

Glucuronides are metabolic conjugation reaction products[1], *i.e.* phase II metabolites, and for a long time have been considered to be inactive end products. However, later work suggested[1] that some compounds do form pharmacologically active conjugates or may be hydrolysed to reform the active parent compound.

The glucuronic acid that is covalently coupled to the parent compound by UDP–glucuronyltransferases transforms the parent compound to a considerably more polar and als ionizable compound, pK_a 3–4[1], making bioanalysis more difficult.

By using a hydrophobic ammonium compound as counter ion[2], the glucuronides can be retained as ion pairs in reversed-phase (RP) high-performance liquid chromatographic (HPLC) systems. This technique made the simultaneous and selective analysis of glucuronides and parent compounds possible when direct injections of incubated homogenates (liver microsomes) and UDP–glucuronyltransferase preparations were performed[3].

In this study, the retention and selectivity of glucuronides and parent compounds were investigated using different organic modifiers and solid phases. The character of the buffer system and the amine used as counter ion were shown to be efficient parameters in the regulation of retention and selectivity. By choosing appropriate conditions, direct injection of a spiked urine sample for UV detection of four glucuronides was possible.

0021-9673/90/$03.50

EXPERIMENTAL

Instrumentation

An Altex Model 100 A HPLC pump, Rheodyne 7125 injector with a 20-μl loop, Waters Assoc. Model 440 UV detector, Kipp & Zonen BD 40 recorder and HETO Type 02 PT 923 water-bath (Birkerød, Denmark) for thermostating the chromatographic system were used. The pH measurements were made with a Metrohm (Herisau, Switzerland) 632 pH meter.

Chemicals and solid phases

2-Aminophenyl-β-D-glucuronide, 4- nitrophenyl-β-D-glucuronide, phenyl-β-D-glucuronide, 2-aminophenol, 4-nitrophenol and 2-(N-morpholino)ethanesulphonic acid were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of HPLC or analytical-reagent grade and were used without further purification.

The column packings were Partisil-10 ODS (10 μm), 5% carbon loading (Whatman, Maidstone, U.K.), TSK-gel LS 410 (5 μm), 24% carbon loading (Toyo Soda, Tokyo, Japan), Nucleosil C_{18} (5 μm), 14.5% carbon loading, Nucleosil CN (10 μm) (Machery-Nagel, Düren, F.R.G.), and PRP-1 (10 μm) (Hamilton, Bonaduz, Switzerland).

Column preparation and chromatographic conditions

The TSK-gel, Partisil-10 ODS, Nucleosil C_{18} and CN were packed into stainless-steel columns (50 × 4.6 mm I.D.) with a slurry medium containing methanol–propanol (6:4, v/v), followed by 200 ml of methanol. The PRP-1 column was packed with a slurry medium containing 2.5% sodium chloride, 10% glycerol and a small amount of acetone in water, followed by 200 ml of slurry medium and 100 ml of water. The column dimensions were 100 × 4.6 mm I.D.

Prior to analysis, the systems were equilibrated with at least 100 ml of mobile phase. The mobile phases were prepared by dissolving the counter ion, as base or salt, in the organic modifier. The buffer phase and organic modifier were then mixed together and degassed in an ultrasonic bath. The mobile phase flow-rate was 1.0 ml/min at 25.0°C (thermostated water-bath). The volume of the mobile phase in the column, V_m, was obtained from the front disturbance in the chromatogram by injection of water and monitoring the UV signal at 254 nm. The capacity ratio, k', was calculated from $k' = (V_r - V_m)/V_m$, where V_r is the retention volume. The solutes were dissolved in deionized water (Millipore) and stored at −20°C before analysis.

Retention models

In this study it is assumed that the general retention models evaluated according to the ion-pair adsorption mechanism[2] are valid. No efforts have been made to calculate the equilibrium constants involved, the reason being that in most experiments the data points are too few in relation to the large number of constants present in the equations. The qualitative discussions are based on the following equations:

Uncharged solute (S):

$$k'_S = \frac{qK^0K_D}{1 + K_{BQ}[B^-]\,[Q^+]} \tag{1}$$

Anion (G^-):

$$k_{G^-} = \frac{qK^0[K_D a_H + K_{GQ}[Q^+]K'_a]}{(K'_a + a_H)(1 + K_{BQ}[B^-][Q^+])} \quad (2)$$

Cation (HA^+):

$$k'_{HA^+} = \frac{qK^0 [K_D K'_a + K_{HAB}[B^-]a_H]}{(K'_a + a_H)(1 + K_{BQ}[B^-][Q^+])} \quad (3)$$

Zwitterion ($^+Z^-$):

$$k'_{^+Z^-} = \frac{qK^0 K_{BZQ}[B^-][Q^+]}{1 + K_{BQ}[B^-][Q^+]} \quad (4)$$

The retention of a zwitterion will change with pH. At low pH it will distribute as cationic solute (eqn. 3), whereas at high pH it becomes anionic (eqn. 2).

In these equations:

K^0 = capacity of the solid phase;
q = phase ratio (the ratio of the solid phase in grams to the volume of the mobile phase in millilitres);
K_D = distribution constant for an uncharged solute;
K_{GQ} = stoichiometric distribution constant for the ion pair;
K_{BQ} = stoichiometric distribution constant for the ion pair of an anionic buffer component and an ammonium counter ion;
K_{HAB} = stoichiometric distribution constant for the ion pair of a cationic solute and an anionic buffer component;
K_{BZQ} = stoichiometric distribution constant for the ion-pair complex of zwitterionic solute, ammonium cation and anionic buffer component;
K'_a = acid dissociation constant;
a_H = hydrogen activity

The equations are valid on the assumptions that (i) a linear distribution isotherm, *i.e.*, a constant capacity factor independent of concentration, prevails, (ii) a zwitterionic solute distributes as an ion pair, not as uncharged species, and (iii) the solid phase has one active site of adsorption.

RESULTS AND DISCUSSION

Influence of pH

In Fig. 1 the logarithm of the retention is plotted *versus* pH for three glucuronides differing in character by the substituents on the phenyl ring. At low pH two of the glucuronides are retained as uncharged acids (eqn. 1), but as the pH is increased the retention increases as the glucuronides become charged and they are retained as ion pairs (eqn. 2) with dodecylethyldimethylammonium (DDEDA). The additional decrease in retention at low pH for 2-aminophenyl-β-D-glucuronide is caused by the protonation of the aromatic amino function ($pK_a \approx 5$) (*cf.*, eqn. 3). The

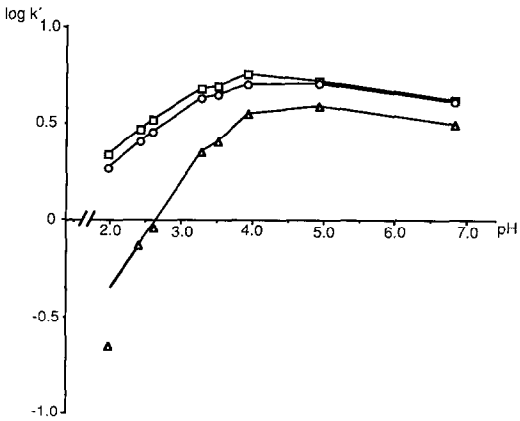

Fig. 1. Log k' *versus* pH. Column, TSK-Gel C_{18} (5 μm), 50 × 4.6 mm I.D.; mobile phase, phosphate buffer (μ = 0.1), 50% (v/v) methanol and 20.0 m*M* DDEDA-Br. ○ = Phenyl-β-D-glucuronide; △ = 2-aminophenyl-β-D-glucuronide; □ = 4-nitrophenyl-β-D-glucuronide.

slight decrease in retention with increasing pH (> 5) is probably caused by the change in buffer composition with pH. At higher pH a higher concentration of divalent phosphate anion will be present. This anion may compete more efficiently for the hydrophobic counter ion than the monovalent ion (see *Influence of buffer components in the mobile phase*, below).

Influence of organic modifier

The retention and the selectivity (defined as the ratio $k'_{\text{parent compound}} : k'_{\text{glucuronide}}$) of glucuronides and their parent compounds were investigated using methanol, acetonitrile and tetrahydrofuran as organic modifiers (Table I). The percentage of organic modifier were chosen in order to give about the same magnitude of the capacity ratios. The influence on the selectivity differs depending on the structure of the parent compound. For the simple monoprotolytic compounds, phenol and

TABLE I

INFLUENCE OF ORGANIC MODIFIER

Column, Nucleosil C_{18} (5 μm), 50 × 4.6 mm I.D.; mobile phase: phosphate buffer (pH 4.95, μ = 0.1), 20.0 m*M* DDEDA and modifier [methanol, acetonitrile (ACN) or tetrahydrofuran (THF)].

Substrate	*50% Methanol*		*25% ACN*		*22% THF*	
	k'	α^a	k'	α^a	k'	α^a
Phenyl-β-D-glucuronide	5.21	0.58	4.79	1.23	3.73	2.64
Phenol	3.01		5.90		9.83	
2-Aminophenyl-β-D-glucuronide	3.93	0.32	3.37	0.56	2.96	0.93
2-Aminophenol	1.25		1.88		2.75	
4-Nitrophenyl-β-D-glucuronide	5.28	1.14	7.50	1.84	9.44	2.64
4-Nitrophenol	6.04		13.8		24.9	

[a] $\alpha = k'_{\text{parent compound}}/k'_{\text{glucuronide}}$.

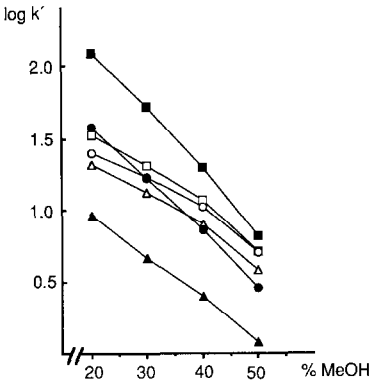

Fig. 2. Log k' *versus* methanol concentration. Column, TSK-Gel C_{18} (5 μm), 50 × 4.6 mm I.D.; mobile phase, phosphate buffer (pH 4.95, μ = 0.1), 20.0 m*M* DDEDA-Br and methanol (MeOH). ○ = Phenyl-β-D-glucuronide; △ = 2-aminophenyl-β-D-glucuronide; □ = 4-nitrophenyl-β-D-glucuronide; ● = phenol; ▲ = 2-aminophenol; ■ = 4-nitrophenol.

4-nitrophenol, the selectivity increases on changing from methanol to acetonitrile to tetrahydrofuran.

This indicates that a hydrogen-accepting solvent is to be preferred when a high selectivity is needed for such compounds. On the other hand, for 2-aminophenol the selectivity decreases with a change in solvents in the same direction. However, using pH as a parameter, the selectivity can easily be modified. The retentions for the parent compounds and the 4-nitrophenyl-β-D-glucuronide increase on changing the solvent from methanol to acetonitrile to tetrahydrofuran, whereas they decrease for the remaining two compounds.

No general guidelines can be drawn from these limited studies, but they indicate that each pair of compounds has to be studied separately regarding retention and selectivity effects.

As expected, the retentions of all the compounds decrease with increasing concentration of methanol in the mobile phase (Fig. 2), but there are no linear relationships. For the phenol pair there is even a retention reversal at methanol concentrations higher than 30% when the glucuronide has the highest retention. This is another illustration of the complex retention mechanism, which is to be expected considering the many equilibria prevailing in systems of this kind. A change in the concentration of the organic modifier in the mobile phase leads to several changes. When the concentration of methanol is increased, there is a non-linear decrease in the adsorption of a quaternary ammonium counter ion[4], and there is also a non-linear increase in the solvating ability of the mobile phase. Recent studies[5] on solute retention in methanol–water mixtures showed that the "free" (*i.e.*, not associated with water) methanol concentration increases non-linearly with increasing methanol content. The relative adsorption of buffer components and solutes may change on increasing the content of organic modifier. Finally, the possibility of solutes being retained by more than one site of adsorption on the solid phase must be taken into account[6]. With this background, it is obvious that a linear relationship between k' and the concentration of organic modifier in the mobile phase is not to be expected in this kind of system.

TABLE II

INFLUENCE OF SOLID PHASE

Mobile phase, phosphate buffer (pH 4.95, $\mu = 0.1$), 20.0 m*M* DDEDA and 50% (v/v) methanol.

Substrate	*Nucleosil-CN*[a]		*PRP-1*		*Partisil ODS*[b]		*Nucleosil* C_{18}[c]		*TSK-GEL*[d]	
	k'	α	k'	α	k'	α	k'	α	k'	α
Phenyl-β-D-glucuronide	5.50	0.90	5.84	1.85	3.29	0.51	5.21	0.58	4.95	0.58
Phenol	4.94		10.8		1.68		3.01		2.90	
2-Aminophenyl-β-D-glucuronide	4.58	0.56	4.32	0.73	2.73	0.32	3.93	0.32	3.80	0.32
2-Aminophenol	2.56		3.16		0.86		1.25		1.20	
4-Nitrophenyl-β-D-glucuronide	9.41	1.34	9.42	3.60	3.41	1.40	5.28	1.14	5.10	1.31
4-Nitrophenol	12.6		33.9		4.77		6.04		6.68	

[a] 30% methanol.
[b] Carbon load 5%.
[c] Carbon load 14.5%.
[d] Carbon load 24%.

Influence of the solid phase

The retention and selectivity were investigated using different solid phases (Table II). The character of the solid phase may have an influence on many parameters involved in the retention eqns. 1–4: the capacity (K^0), the equilibrum constants (K_{GQ}, K_D, etc.) and the phase ratio (q). The selectivities obtained for different carbon loadings (5, 14.5 and 24%) are fairly constant whereas the retentions increase up to the level of 14.5% (Nucleosil). A further increase in carbon content does not increase the retention except for 4-nitrophenol. The different solid phases were obtained from different suppliers and the methods used to produce the solid phases may differ, including the character of the basic silica used as raw material. However, non-derivatized silanol groups and steric hindrance may also play a part in the total retention[7].

For the PRP-1 phase, which is highly hydrophobic and has π–π interaction possibilities, the glucuronides are relatively less retained compared with their parent compounds. The cyano phase gave lower retentions in general, and the methanol concentration in the mobile phase had to be decreased. This might prove useful with a coupled column system, assuming that the glucuronide is retained on a precolumn of the nitrile type during the clean-up step and later transferred on-line to the analytical column by changing the mobile phase.

Influence of buffer components in the mobile phase

According to the retention models many parameters may influence the retention of solutes. For charged solutes a hydrophobic counter ion often dominates the retention. However, if its concentration is kept constant, other parameters such as the concentration of buffer components may have an influence on the retention. This is demonstrated in Fig. 3, where two commonly used buffer systems, citrate and phosphate, plus a zwitterionic buffer system, 2-(N-morpholino)ethanesulphonic acid (MES), were compared at pH 4.95. Phosphate and citrate had an ionic strength of 0.1, equivalent to 98.0 and 35.0 mM, respectively. MES was 50.0 mM and the pH was adjusted by addition of sodium hydroxide. There is a dramatic effect on glucuronide retention and the selectivity depending on the buffer used. The parent compounds are

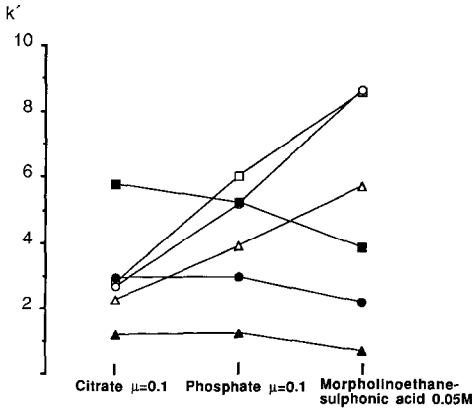

Fig. 3. Variation of k' with buffer system. Column, Nucleosil C_{18} (5 μm), 50 × 4.6 mm I.D.; mobile phase, buffer (pH 4.95) in 50% (v/v) methanol and 20.0 mM DDEDA-Br. Solutes as in Fig. 2.

only slightly affected; MES buffer gives the lowest retentions. At this pH there are differences in the concentrations of the monovalent anions of citrate and phosphate and also MES, being 21.3, 97.5 and 3.0 m*M*, respectively.

The character and concentration of the buffer components determine their influence on the retention of the solutes. The citrate buffer, which gives the lowest retentions of the glucuronides, contains in addition to the monovalent anion about the same concentration of divalent anion. Divalent buffer ions may have a higher competing ability than a monovalent ion for the hydrophobic counter ion. Hence, the MES buffer exhibits the lowest competing ability and gives the highest retentions for the glucuronides. Undoubtedly, large selectivity differences can be obtained by choosing an appropriate buffer system, as will be demonstrated below.

Influence of counter ion in the mobile phase

In RP ion-pair LC the distribution of an ion pair is governed by its hydrophobicity and interaction with the solid phase. Ammonium compounds of similar size, but differing in carbon chains and substitution at the nitrogen, were compared as counter ions (Fig. 4). Clearly, the existence of one long chain favours retention rather than several short chains (compare decylamine, dipentylamine and tetrapropylammonium). Also, nonyltrimethylammonium gives a higher retention than tetrapropylammonium. Dimethyloctylamine and octylamine both have the same main chain but differ in nitrogen substitution. At 50 m*M* they give approximately the same retention for 2-aminophenyl-β-D-glucuronide, although octylamine contains two carbons less and hence is less hydrophobic. Apparently, the increased polarity (low degree of substitution at the nitrogen) counteracts the decreased hydrophobicity by an increased interaction in ion-pair formation with the glucuronic moiety and/or the solid phase, probably by hydrogen bonding.

In order to obtain a high retention, the counter ion should be a primary amine with a long carbon chain. Unfortunately, the solubility in water-based mobile phases is

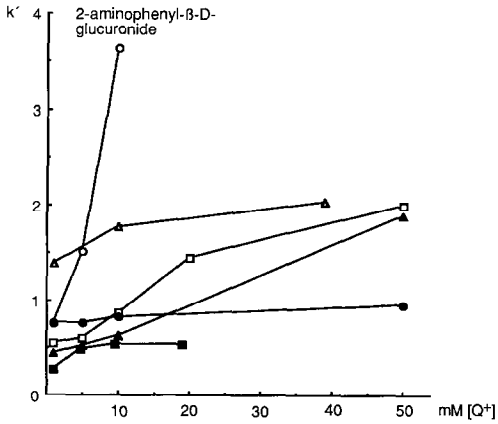

Fig. 4. Variation of k' for 2-aminophenyl-β-D-glucuronide with Q^+ concentration. Column, Nucleosil C_{18} (5 μm), 50 × 4.6 mm I.D.; mobile phase, phosphate buffer (pH 4.95, μ = 0.1), 25% (v/v) acetonitrile and Q. ○ = Decylamine · HCl; △ = nonyltrimethylammonium · Br; □ = dimethyloctylamine; ▲ = octylamine · HCl; ● = dipentylamine; ■ = tetrapropylammonium · OH.

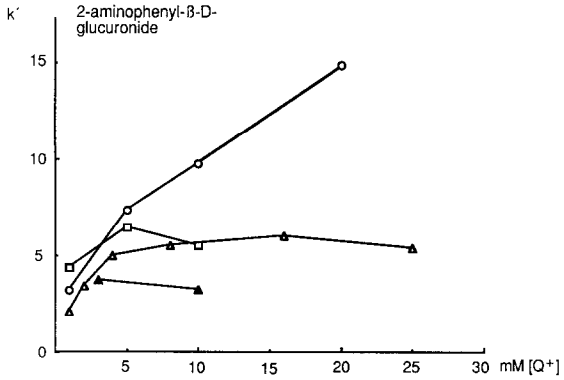

Fig. 5. As Fig. 4. ○ = Dodecylamine · HCl; □ = hexadecyltrimethylammonium · Br; △ = dimethyldodecylamine; ▲ = tetrahexylammonium · Br.

low for this kind of compound and further one long carbon chain favours micelle formation. Differently substituted ammonium compounds with as many methylene groups as possible preventing micelle formation were tested as counter ions (Fig. 5). Again, the primary amine, dodecylamine, gives the highest retention. Fig. 6 shows the retention for three glucuronides and their parent compounds when using dodecylamine in combination with the zwitterionic MES buffer, 40% methanol and 5% tetrahydrofuran as organic modifiers. The retention can be varied over a wide range and suitable selectivities can be selected by varying the counter-ion concentration. The parent compounds have all capacity factors below 4 at all dodecylamine concentrations.

Direct injection of spiked urine sample

A chromatogram from a direct injection of a urine sample, spiked with four

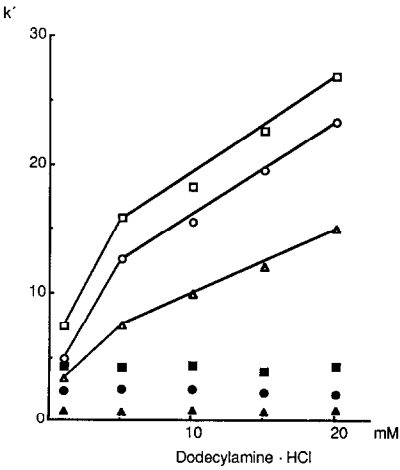

Fig. 6. Variation of k' with dodecylamine concentration. Column, Nucleosil C_{18} (5 μm), 50 × 4.6 mm I.D.; mobile phase, MES buffer (0.05 *M*, pH 4.95), 40% (v/v) methanol, 5% (v/v) tetrahydrofuran and dodecylamine · HCl. Solutes as in Fig. 2.

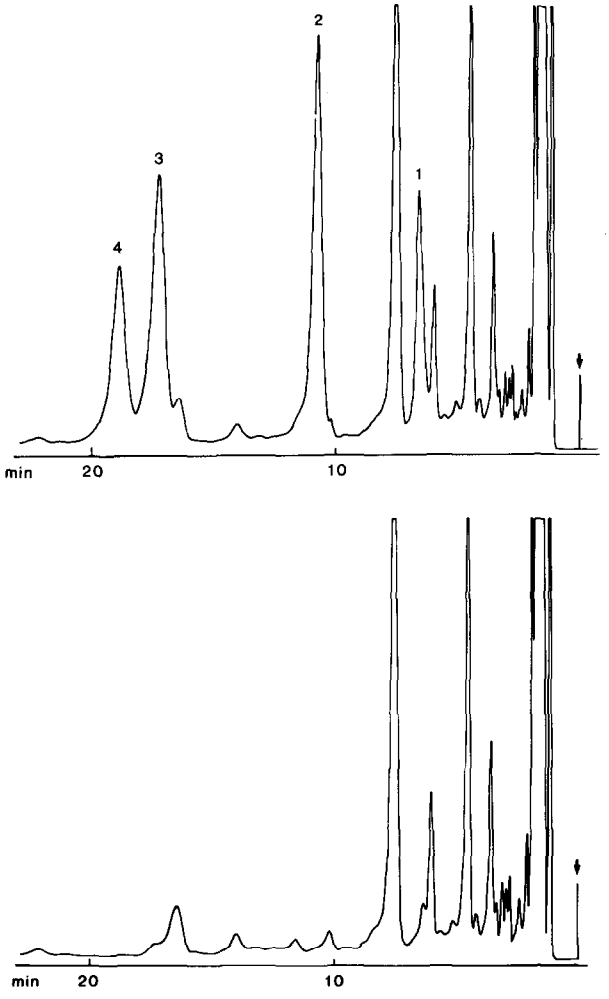

Fig. 7. Top chromatogram: 20-μl injection of a spiked urine sample containing 12–40 nmol of each glucuronide. Bottom chromatogram: Blank urine sample. The urine was filtered and diluted 5-fold before analysis. Column, Nucleosil C_{18} (5 μm), 150 × 4.6 mm I.D.; mobile phase, glycine buffer (0.05 *M*, pH 3.00), 50% (v/v) methanol and 20.0 m*M* dodecylamine · HCl. UV detection at 254 nm. Peaks: 1 = 2-aminophenyl-β-D-glucuronide; 2 = 8-hydroxyquinoline-β-D-glucuronide; 3 = 4-nitrophenyl-β-D-glucuronide; 4 = Phenyl-β-D-glucuronide.

glucuronides, together with the blank urine sample is shown in Fig. 7. In this instance a pH of 3.0 was used to suppress the ionization of acids, present in the urine, with pK_a values of 4–5. The zwitterion glycine was used as the buffer system. The small glycine probably acts as a strong dipole[8] which, in combination with its polarity, should minimize competition on the solid phase. Even the highly polar and charged 2-aminophenyl-β-D-glucuronide is well retained, showing the suitable selectivity obtained with this system.

CONCLUSIONS

RP ion-pair LC of glucuronides has been shown to be considerably influenced by many parameters. Suitable retention and selectivity can be obtained by appropriate choices of the buffer system, organic modifier, pH, solid phase and concentration and character of the counter ion. Of the counter ions tested, dodecylamine exhibited the greatest influence on retention, but also the concentration of organic modifier in the mobile phase had a large effect. On direct injection of a urine sample spiked with four glucuronides, the pH could successfully be utilized to suppress the ionization of endogenous acids present, hence improving the glucuronide selectivity. Character, concentration and charge are distinctive features for the choice of the buffer system. The introduction of zwitterionic buffer components decreased the competition for the counter ions, thereby promoting the ion-pair formation and retention of an anionic solute. When using coupled columns, a less hydrophobic precolumn should be used for the preconcentration of the glucuronide during the clean-up step. The glucuronide could then be desorbed by changing the mobile phase and transferred on-line to a more hydrophobic analytical column where it will be enriched on the top of the column. Several conjugation reaction products are possible, often present in low concentrations, which is why the enhanced selectivity and sensitivity possible with coupled column systems would make this a suitable technique for metabolic studies of polar and ionizable compounds.

REFERENCES

1 G. J. Dutton (Editor), *Glucuronic Acid. Free and combined,* Academic Press, New York, 1966.
2 A. Tilly Melin, Y. Askemark, K.-G. Wahlund and G. Schill, *Anal. Chem.*, 51 (1979) 976.
3 M. Stefansson and D. Westerlund, in preparation.
4 A. Bartha and Gy. Vigh, *J. Chromatogr.*, 260 (1983) 337.
5 E. D. Katz, C. H. Lochmüller and R. P. W. Scott, *Anal. Chem.*, 61 (1989) 349.
6 A. Tilly Melin, M. Ljungcrantz and G. Schill, *J. Chromatogr.*, 185 (1979) 225.
7 R. E. Majors, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives,* Vol. 1, Academic Press, New York, 1980, p. 77.
8 G. Scatchard and J. G. Kirkwood, *Phys. Z.*, 33 (1932) 297.