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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF URINARY PARACETAMOL METABOLITES USING RADIALLY COMPRESSED COLUMNS

S.J. HART*, M.I. AGUILAR, K. HEALEY, M.C. SMAIL and I.C. CALDER

Department of Organic Chemistry, University of Melbourne, Parkville, Victoria, 3052 (Australia)

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SUMMARY

Methods have been adapted for the high-performance liquid chromatographic (HPLC) analysis of urinary paracetamol metabolites on radial compression columns. Enhanced resolution and decreased analysis time were two major advances. Various modifications to existing methods were made to counter the effect of the different C18 surface. Thus in ion suppression HPLC the addition of triethylamine at pH 3.0 (phosphate buffer) was necessary to block residual hydroxyl sites, while in ion-pair HPLC a higher tetrabutylammonium hydroxide concentration of 0.01 *M* at pH 5.0 was used to enhance selectivity. The methods were successfully applied to the study of the metabolism of paracetamol, its glutathione conjugate and 3-thiomethylparacetamol in Sprague—Dawley rats. 3-Thiomethylparacetamol sulphoxide and its glucuronide and sulphate conjugates were shown to be metabolites of both 3-thiomethylparacetamol and paracetamol. 3-Thiomethylparacetamol sulphate was unresolved from the sulphates of paracetamol and 3-methoxyparacetamol in ion-pair HPLC. This raises a previously unrecognised problem in which the peak normally attributed to paracetamol sulphate contains metabolites arising from an oxidative metabolic pathway. Elevated levels of 3-methoxyparacetamol conjugates were found in human overdose urine and to some extent in analgesic nephropathy.

INTRODUCTION

There has been great interest over the past few years in the development of chromatographic methods capable of separating paracetamol and its metabolites [1—4]. As methods have become more refined it has become possible to separate previously unidentified metabolites such as the conjugates of 3-thiomethylparacetamol [5], a metabolite which has previously been detected only by following enzymatic hydrolysis of urine [6]. The same workers [6] have unequivocally identified 3-thiomethylparacetamol sulphoxide

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as a further metabolite, presumably present in urine in conjugated form, although no high-performance liquid chromatographic (HPLC) analysis has been presented to date. Wilson et al. [7] have recently reported on the use of electrochemical detection for paracetamol and its major metabolites, the glucuronide and sulphate, as well as the unconjugated 3-methoxy-, 3-hydroxy- and 3-thiomethylparacetamols following enzymatic hydrolysis of urine containing conjugates of all these.

We have previously discussed the development of ion suppression and ion-pairing techniques for reversed-phase HPLC of urinary paracetamol [1]. Using stainless-steel columns packed with 10-μm particles of C18 reversed-phase silica, optimum solvent systems were developed with the emphasis on speed for the isocratic ion suppression and maximum resolution for the gradient programmed ion-pair methods, respectively. Regrettably the benefits of both high resolution and short analysis time (< 20 min) were not available within the one separation.

The introduction of radial compression technology [8] suggested that the same separations could be achieved quickly without any sacrifice in resolution. There was an immediate problem however; the packing in conventional stainless-steel columns is usually treated in situ with trimethylchlorosilane to cap residual silica hydroxyls on the C18 silica whereas this treatment is omitted for Radial Pak™ columns. Furthermore the percentage of C18 coating of the silica used for Radial Pak columns is greater than that used for stainless-steel columns which leads to increased k' values. Thus solvent systems have to be adapted to the different C18 surface and reoptimized to obtain a similar separation. Two such modifications are described in this paper.

The methods were applied in the continuing study of the metabolism of paracetamol and of 3-thiomethylparacetamol and paracetamol glutathione conjugate, and in the course of their development 3-thiomethylparacetamol sulphoxide and its glucuronide and sulphate conjugates were detected and identified as metabolites of paracetamol.

EXPERIMENTAL

Apparatus

A Spectra-Physics 3500B dual-pump liquid chromatograph fitted with a solvent programmer, a Waters Assoc. U6K loop injector and Model 440 dual-channel UV detector (filters 254 and 280 nm) were used for all HPLC analyses. Waters Assoc. Rad-Pak A columns were used (10 cm × 8 mm I.D., particle size 10 μm) and contained unsilanized C18 packing. These were operated in a Waters Assoc. radial compression module (RCM). Conventional stainless-steel μBondapak C18 columns (30 cm × 3.9 mm I.D., particle size 10 μm; Waters Assoc.) were used for comparison of selectivity and efficiency.

Solvents

Distilled water purified through a Milli Q ion-exchange system (Millipore) was obtained from the Marine Chemistry Laboratory (University of Melbourne, Australia). Methanol (AR) was used without further treatment.

Mobile phases

Water—methanol (1:1) was used to establish retentions of a test mixture of non-ionized paracetamol derivatives on both column types.

Ion suppression solvents

Initially 15% methanol in 0.05 *M* potassium phosphate buffer at pH 2.3 was used on Rad-Pak A columns. Subsequent modifications involved the addition of triethylamine (TEA) (AR, Aldrich, Milwaukee, WI, U.S.A.) at concentrations of 0.005 *M* and adjustment of pH with H_3PO_4 or KOH maintaining the phosphate concentration at 0.05 *M*.

Ion-pair solvents

An aqueous solution of tetrabutylammonium (TBA) hydroxide (0.4 *M* solution, Eastman-Kodak, Rochester, NY, U.S.A.) was diluted to 0.005 *M* or 0.01 *M* in water or water—methanol (1:1). The pH was adjusted with H_3PO_4 or with Tris (tris-hydroxymethylaminomethylaminomethane) (Sigma, St. Louis, MO, U.S.A.) and H_3PO_4. In some cases ethylenediaminotetraacetic acid disodium salt (EDTA) was added to the eluent at a concentration of 0.005 *M*. Gradient programming was used to enhance resolution. Details of gradient programming on μBondapak C18 columns have been given previously [1]. Program times on Rad-Pak A columns were generally shorter as a result of faster flow-rates on these columns. Typically, program times of ca. 9–12 min were used.

Standard compounds

Paracetamol was obtained from Aldrich. 3-Methylcholanthrene (3MC) was purchased from Sigma. 3-Methoxyparacetamol (3OMeP) was synthesised in our laboratory. 3-Thiomethylparacetamol (3SMeP) was prepared synthetically [5] and contained no trace of paracetamol by HPLC. Paracetamol 3-glutathione conjugate [5-(5′-acetamido-2′-hydroxyphenyl)-L-glutathione (PSG)] was synthesised by the general method of nucleophilic addition of a sulphhydryl group, in this case L-glutathione, to the quinoneimine of paracetamol [9,10]. Paracetamol 3-cysteinylglycine conjugate [3-(5-acetamido-2-hydroxyphenylthio)alanylglycine) (PCG)] was prepared analogously to the PSG conjugate [10].

Urine samples

These were obtained from Sprague—Dawley albino rats following administration of paracetamol, 3-thiomethylparacetamol, 3-methoxyparacetamol or synthesised paracetamol glutathione conjugate. Details of the method of administration and urine collection have been given elsewhere [11]. In some cases rats were pre-treated with 3-methylcholanthrene, a known cytochrome P450 mixed function oxidase inducer, for two days prior to administration of paracetamol. Samples of human urine containing metabolites from a paracetamol overdose were kindly supplied by Dr. G. Duggin, Royal Prince Alfred Hospital, Sydney, Australia. A sample of human urine containing metabolites of paracetamol from a patient with suspected analgesic nephropathy was kindly supplied by St. Vincent's Hospital, Melbourne,

Australia. Urine from a healthy person was collected following the ingestion of a normal therapeutic dose of paracetamol (1 g). Urine samples were filtered through 0.5-μm cellulose filters (Millipore, Bedford, MA, U.S.A.) following the addition of methanol up to 20%. Filtered urine samples were kept frozen at −20°C in acid-washed screw-capped vials until ready for analysis.

Perfusion urines were obtained by techniques previously described [10—13] where paracetamol glutathione conjugate was added to the perfusion medium at a concentration of 1.6 m*M*. Perfusion urines were injected without further treatment onto the HPLC column because of their small volumes.

Bile duct cannulation

Female Sprague—Dawley rats (250—300 g) were anaesthetised with diethyl ether. A midline incision was made and the right kidney was exposed. The ureter was cannulated (Portex tubing, size PP10) and the tubing held with two ligatures around the ureter. The common bile duct was then exposed and cannulated. The incision was closed and the animals placed in restraining cages. A single dose of paracetamol (15.0 mmol/kg) was administered as a slurry (15 g per 100 ml) by oral gavage.

Samples of urine and bile were collected with a fraction collection at 2-h intervals over 24 h and immediately frozen. Animals were then killed. The urine and bile were analysed for paracetamol metabolites.

RESULTS AND DISCUSSION

k' Values

Apart from the obvious mechanical differences in using radially compressed columns, the packing presents major differences by comparison with μBondapak C18 columns. The higher percentage C18 loading on the particles causes difficulty in wetting and thus it is necessary to first treat the column thoroughly with methanol before lowering the methanol concentration. With a given solvent composition it is found that k' values on the Rad-Pak A columns are greater than on μBondapak C18 columns as illustrated by paracetamol,

TABLE I

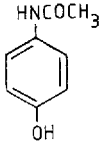
COMPARISON OF k' VALUES AND EFFICIENCIES ON μBONDAPAK C18 AND RADIAL PAK COLUMNS

Solvent: methanol—water (1:1).

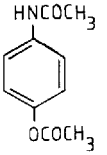
Solute	μBondapak C18			Rad-Pak A				
	Flow-rate			Flow-rate				
	2 ml/min			2 ml/min			4 ml/min	
	k'	Nf	Nf/t*	k'	Nf	Nf/t	Nf	Nf/t
Phenacetin	1.79	1069	4.6	2.78	1138	3.6	807	5.5
Paracetamol acetate	0.87	421	2.7	1.26	653	3.4	373	4.2
Paracetamol	0.14	–	–	0.29	–	–	–	–

*Nf/t = number of effective plates per second.

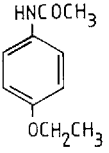
its O-acetate and phenacetin which provide a convenient series of non-ionic compounds of decreasing polarity to monitor k' values in water–methanol mixtures (Table I).



Paracetamol



Paracetamol acetate



Phenacetin

Likewise in a buffered solvent system containing 15% methanol the retention of paracetamol was greater on the Rad-Pak A column (Table II).

TABLE II

COMPARISON OF k' VALUES AND EFFICIENCIES ON μBONDAPAK C18 AND RADIAL PAK COLUMNS

Solvent: 15% methanol in 0.05 *M* potassium phosphate buffer, pH 2.8.

Solute	μBondapak C18 (2 ml/min)		Rad-Pak A (4 ml/min)	
	k'	Nf	k'	Nf
Paracetamol	1.85	985	4.1	2309

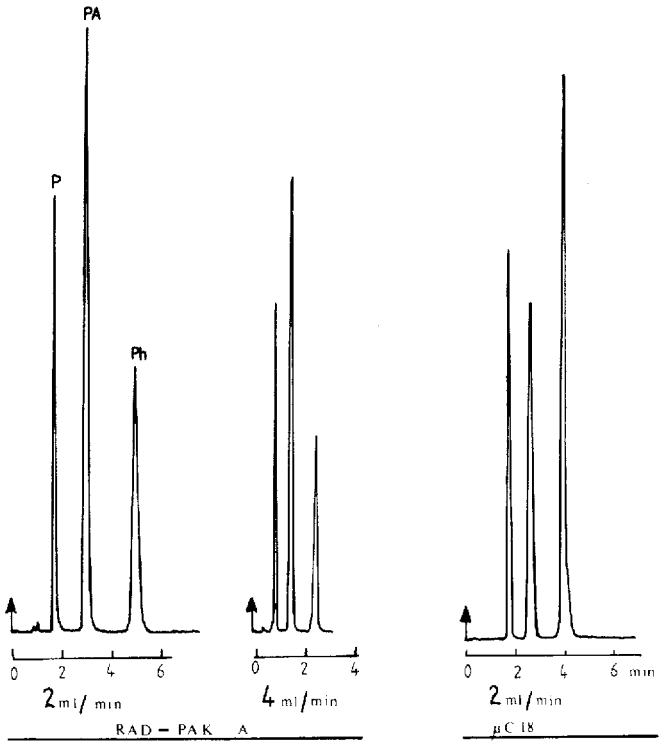


Fig. 1. Resolution of the test mixture, paracetamol (P), paracetamol acetate (PA) and phenacetin (Ph) on Rad-Pak A and μBondapak C18 columns using methanol–water (1:1) at the flow-rates indicated.

Efficiency

Tables I and II also list the efficiency of each column at different flow-rates. The effective number of plates per column (Nf) is calculated as $5.54 \times \left[\frac{t_r - t_0}{W_{\frac{1}{2}}h}\right]^2$. At a flow-rate of 2 ml/min on both columns (this represents a lower linear flow on the 8-mm I.D. Rad-Pak A column) similar efficiencies are obtained for a given k' value and the elution time for a given compound is approximately the same, e.g. 5 min for phenacetin. As the flow-rate on the Rad-Pak A column is increased to 4 ml/min the elution time is halved and although the efficiency drops off it is still competitive with conventional columns at 2 ml/min for peaks where k' is greater than 2. It is not possible to increase the flow-rate on μBondapak C18 columns much past 2 ml/min because the pressure drop becomes excessive. Fig. 1 shows the resolution of the test mixture on the two columns at different flow-rates. It is possible to achieve greater resolution on both the μBondapak C18 columns and Rad-Pak A columns at low flow-rates but such separations would be unnecessarily long.

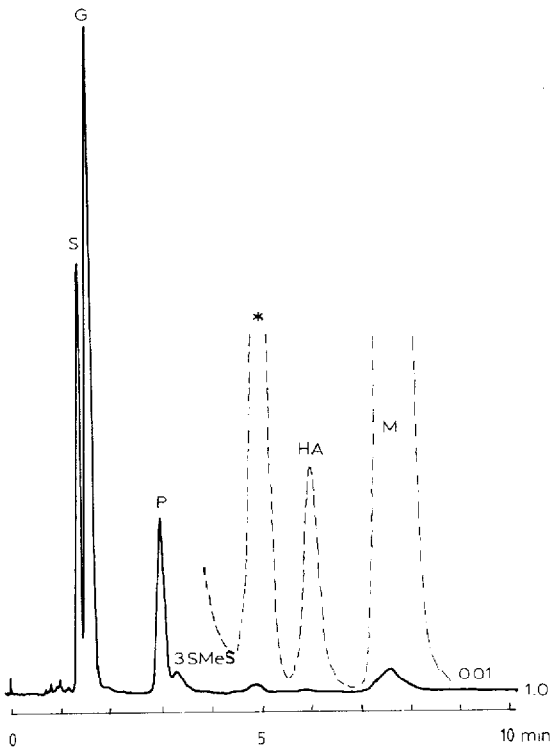


Fig. 2. Ion suppression HPLC of paracetamol metabolites on a Rad-Pak A column. Eluent, 15% methanol in 0.05 M phosphate buffer, pH 2.3; flow-rate, 4 ml/min; detector 254 nm at two sensitivities as indicated. Peaks: S = paracetamol sulphate; G = paracetamol glucuronide; P = paracetamol; 3SMeS = 3-thiomethylparacetamol sulphate; HA = hippuric acid (endogenous urine constituent); M = paracetamol mercapturic acid; (*) composite peak subsequently identified as a mixture of 3-thiomethylparacetamol sulphoxide and other metabolites (see text).

Analysis of urinary paracetamol metabolites by ion suppression HPLC

The direct application of the ion suppression system devised for μBondapak C18 columns [1] to a Rad-Pak A column gave a different result (Fig. 2). Sulphates eluted early resulting in poor resolution of paracetamol metabolites. Addition of TEA, as recommended by the manufacturers, increased sulphate retentions (Fig. 3) presumably by an ion-pair reaction while other compounds eluted earlier since active adsorption sites on the column were now masked. Resolution was still not acceptable since paracetamol glucuronide (G) and paracetamol sulphate (S) were too close together, a criticism of earlier ion suppression methods, and 3-thiomethylparacetamol sulphate (3SMeS) and hippuric acid (HA) were unresolved. Variation of the three parameters, pH, methanol concentration and TEA concentration, enabled a wide range of

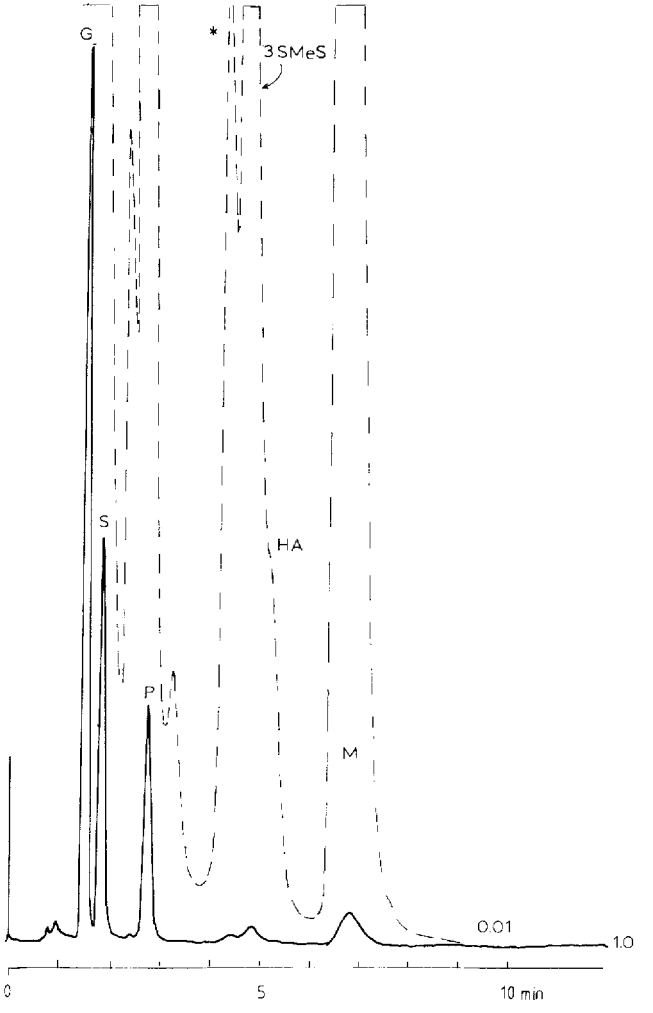


Fig. 3. The effect of 0.005 *M* TEA on ion suppression HPLC of paracetamol metabolites. Eluent, 0.005 *M* TEA, 15% methanol in 0.05 *M* potassium phosphate buffer, pH 2.3; flow-rate, 4 ml/min; detector 254 nm at two sensitivities as indicated. For peak identification see Fig. 2.

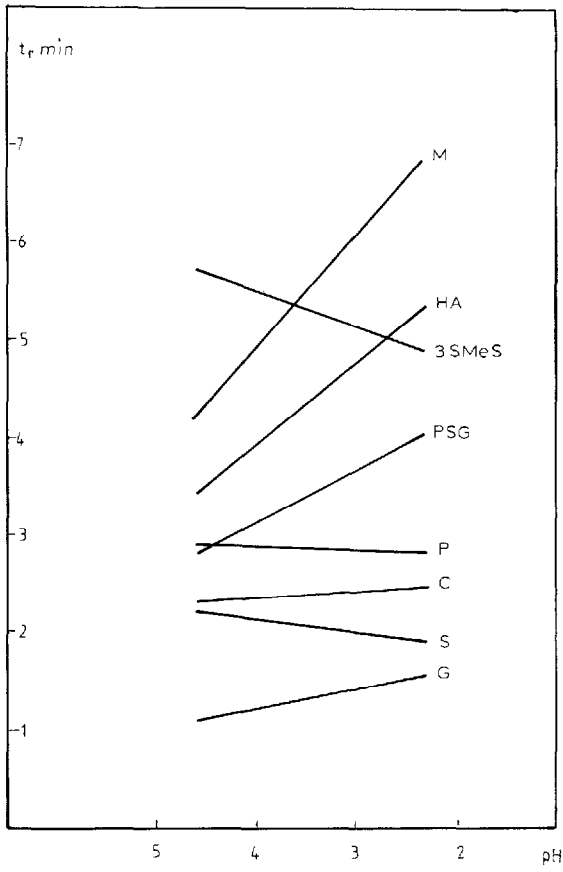


Fig. 4. Effect of pH on the selectivity of the separation of paracetamol metabolites by ion suppression HPLC on a Rad-Pak A column. Eluent, 0.005 *M* TEA, 15% methanol in 0.05 *M* potassium phosphate buffer, pH varied; flow-rate, 4 ml/min. For abbreviations see Fig. 2; PSG = paracetamol glutathione conjugate.

separation alternatives. Increasing methanol concentration past 15% was unfavourable since all metabolites eluted too quickly. Therefore it was more profitable to adjust pH and TEA concentration.

The effect of changing pH on a relatively new column is shown in Fig. 4 where it is noted that sulphates behave differently to the other metabolites. A TEA concentration of 0.005 *M* (0.07%) pH 3.2 was chosen as optimum.

A number of urine samples containing different proportions of various metabolites were run in this system so that detailed information on retention times could be obtained. These are tabulated in Table III. Metabolites of 3-thiomethylparacetamol were obtained from animals administered the parent compound [5] and the same metabolites were observed in animals induced with 3-methylcholanthrene and receiving high doses of paracetamol. Metabolites of 3-methoxyparacetamol were likewise obtained by the administration of the parent compound to Sprague—Dawley rats. The presence of these metabolites was noted in a sample of human urine obtained from a patient with analgesic nephropathy (Fig. 5a). The levels of 3-methoxypara-

TABLE III

RETENTION TIMES FOR PARACETAMOL METABOLITES IN ION SUPPRESSION CHROMATOGRAPHY

HPLC conditions as for Fig. 5.

Metabolite	Abbreviation	Retention time (min)
Paracetamol glucuronide	PG	1.2
Paracetamol sulphate	PS	1.9
3-Methoxyparacetamol glucuronide	3OMeG	2.2
Paracetamol cysteine conjugate	C	2.2
3-Methoxyparacetamol sulphate	3OMeS	2.4
Paracetamol	P	2.6
Paracetamol cysteinylglycine conjugate	PCG	2.6
Paracetamol glutathione conjugate	PSG	3.2
3-Thiomethylparacetamol glucuronide	3SMePG	3.6
3-Thiomethylparacetamol sulphoxide	3SOMeP	4.1
3-Methoxyparacetamol	3OMeP	4.2
Hippuric acid	HA	4.6
3-Thiomethylparacetamol sulphate	3SMePS	5.0
Paracetamol mercapturic acid	M	5.8
3-Thiomethylparacetamol	3SMeP	>8.0

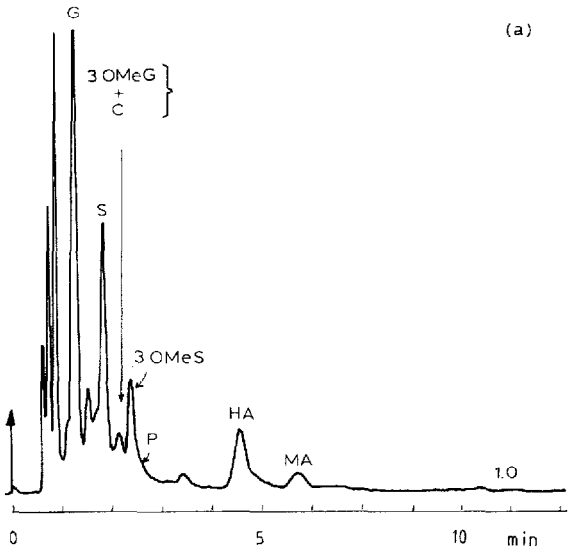
cetamol metabolites are slightly exaggerated by comparison with the metabolism of a therapeutic dose of paracetamol in a healthy volunteer (Fig. 5b).

The same chromatographic system was used successfully to monitor the metabolism of paracetamol glutathione conjugate both in vivo, in bile-cannulated animals and in the isolated perfused kidney [12,13]. While the major metabolites in vivo were the cysteine and mercapturic acid, perfusion urines and bile samples yielded an intermediate metabolite in the breakdown of the glutathione conjugate, that is paracetamol cysteinylglycine. The latter compound was prepared synthetically and found to run at the same retention time as paracetamol. Increasing the TEA concentration enabled resolution from P but care was needed to avoid the metabolite co-eluting with C.

It became apparent that the ion suppression separation was not adequate for the large number of metabolites now being studied. Furthermore, there was an inherent lack of stability in the system with the injection of a large number of urine samples and especially perfusion urines. The graph of t_R versus pH is quite different on an old column (Fig. 6) compared to a new one (Fig. 4). Sulphates were most particularly affected suggesting that urinary amine constituents became bound to the reversed-phase packing thus creating an anion exchange surface to which sulphates are strongly attracted at low pH. The end of useful column life occurred when the metabolite pairs S-C and 3SMePS-M coalesced.

Analysis of urinary paracetamol metabolites by ion-pair HPLC

The increased complexity of paracetamol metabolism made it clear that an ion-pair reagent with a higher log E_{QX} [14] than TEA would be more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(a)
3 OMeG
+
C
S
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HA
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MA
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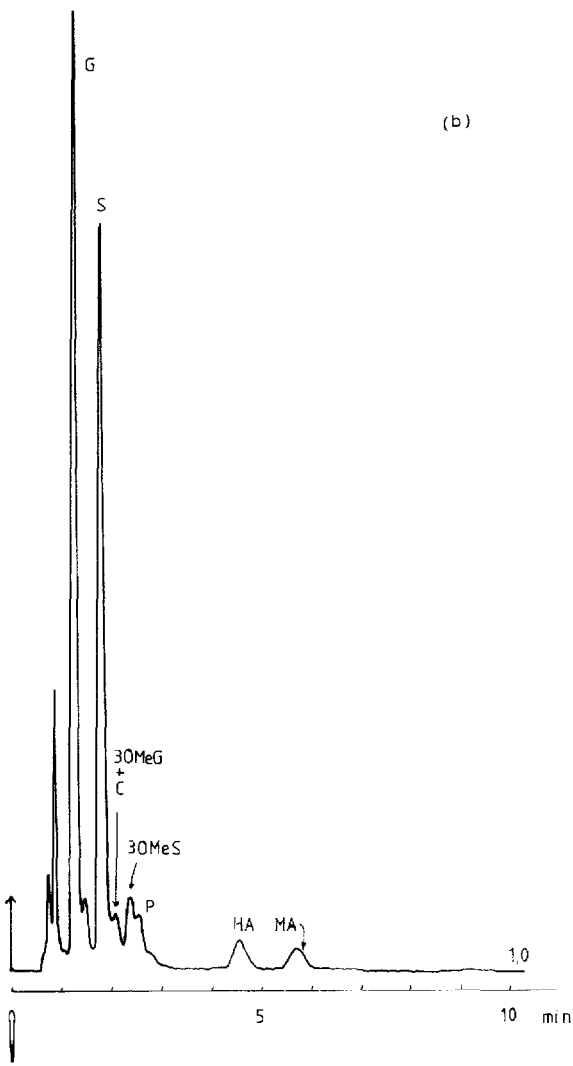
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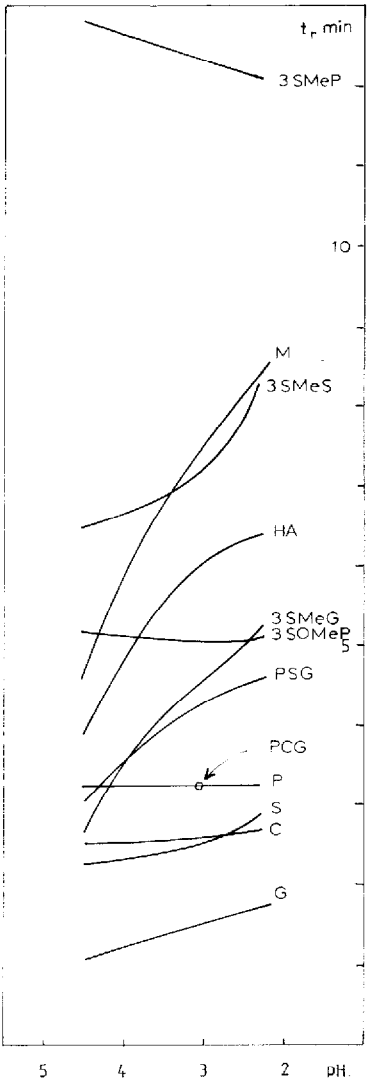

Fig. 6. Effect of pH on the selectivity of the separation of paracetamol metabolites by ion suppression HPLC on a Rad-Pak A column. Conditions as for Fig. 4 except that the experiment repeated on an older column subjected to numerous urine injections. For abbreviations see Table III.

successful in providing the necessary selectivity. Initially TBA at 0.005 *M*, pH 5.0 in 20% methanol was used. Gradient programming was necessary because the retention of 3SMePS was prohibitively long (60 min) and poor resolution occurred between HA, M and S. The solvent system 0.005 *M* TBA,

Fig. 5. Ion suppression HPLC of urinary paracetamol metabolites on a Rad-Pak A column. Eluent, 0.005 *M* TEA, 15% methanol in 0.05 *M* potassium phosphate buffer, pH 3.2; flow-rate 4 ml/min; detector 254 nm, two sensitivities as indicated. (a) Sample of human urine following a therapeutic dose of paracetamol from a patient with suspected analgesic nephropathy. (b) Sample of human urine from a healthy volunteer who ingested 1 g of paracetamol (i.e. therapeutic dose). For abbreviations see Table III.

0.01 *M* Tris, 0.005 *M* EDTA at pH 7.2 with gradient programming as previously developed for stainless-steel μBondapak C18 columns [1] was compared with the same solvent on a Rad-Pak A column and a different result was obtained. In addition to this expected change in selectivity the new metabolites had to be taken into account and it was found that insufficient resolution was available to separate the greater number of metabolites.

Various alterations to the solvent system were made in order to improve resolution. Dropping the pH to 5.0 enabled the cysteine conjugate to be eluted as a symmetrical peak without the need to add EDTA to the solvent. Omission of the EDTA caused all glucuronides, M and HA to elute much later thus illustrating its role not simply as a column deactivator but also as a competitive ion-pair substrate for TBA at the expense of the more water-

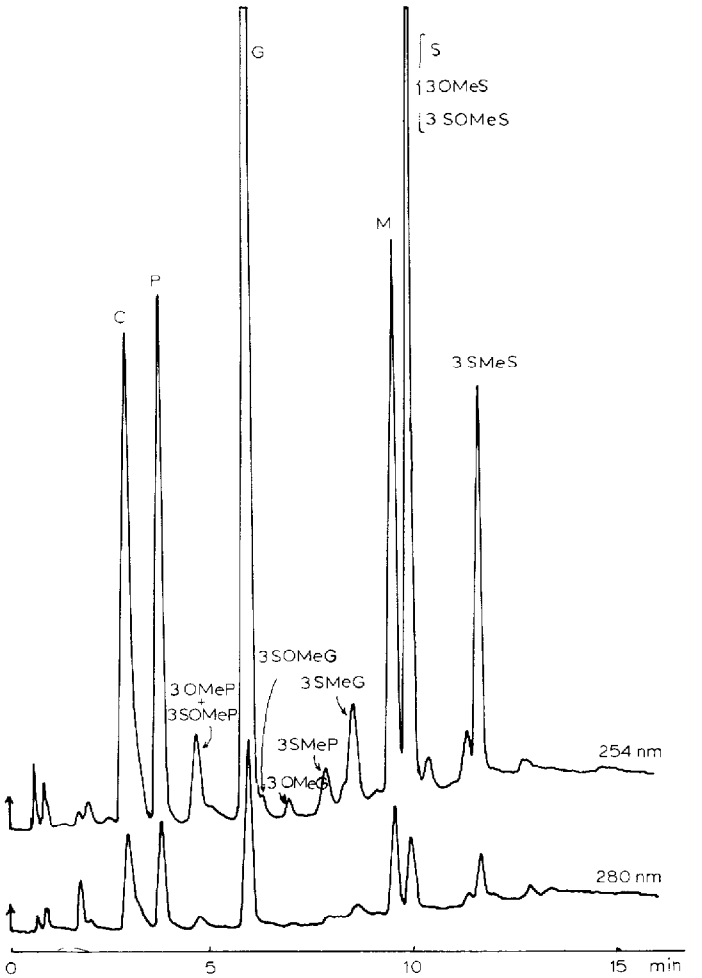

Fig. 7. Optimum separation of urinary paracetamol metabolites by ion-pair HPLC on Rad-Pak A column. Conditions: solvent, 0.01 *M* TBA, 0.01 *M* Tris, pH 5.0 (H_3PO_4) programmed from 10—50% methanol over 12 min; flow-rate, 4 ml/min; detector: 254 nm, 1.0 a.u.f.s.; 280 nm, 0.5 a.u.f.s.; sample, urine from Sprague—Dawley rat pretreated with 3-methylcholanthrene and given 15 mmol/kg paracetamol. Urine sample spiked with C.

soluble anions. Doubling the TBA concentration ensured that glucuronides were moved well past their parent compounds thus enhancing overall selectivity and resolution. Further fine tuning of the separation was achieved by adjusting the initial methanol concentration and the gradient sweep time. The optimized separation is shown in Fig. 7. A re-equilibration time of 9 min has been found to be ample thus allowing the analysis of a sample in 20 min. The previous gradient programmed separation took 1 h [1].

The method has so far proved useful in the study of paracetamol metabolism. In particular it has allowed the isolation and spectroscopic identification of the conjugates of 3SOMeP following Sephadex LH-20 separation of urine containing 3-thiomethylparacetamol metabolites and enabled the identification of these compounds as products of paracetamol metabolism (unpublished results). 3SOMeP has been previously reported only as the free compound in enzymatically hydrolysed urines [6]. The most significant finding was the presence of the metabolite 3SOMeS and the difficulty of its separation from S. In previous studies the peak assigned to S in paracetamol urines has always been assumed to be the result of non-toxic metabolism. From our investigations

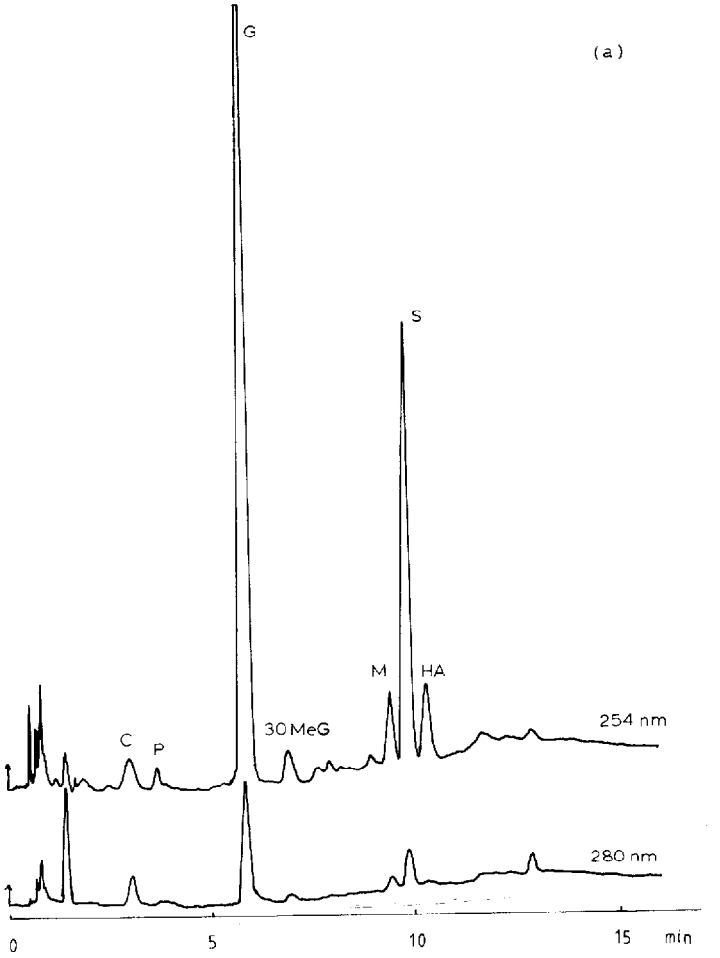

Fig. 8.

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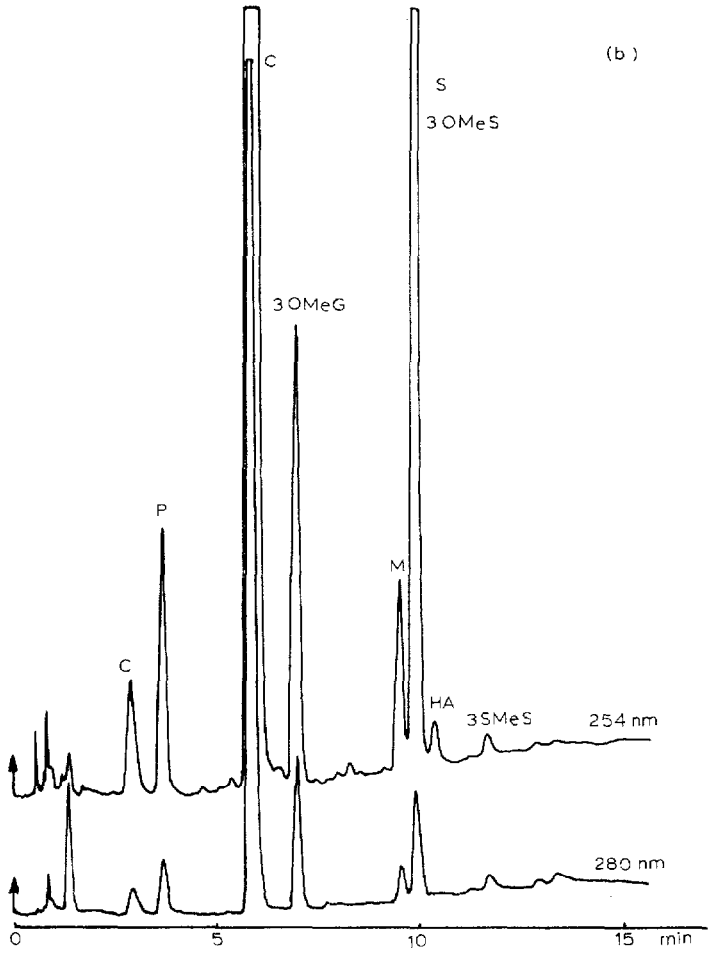

Fig. 8. Separation of human urinary paracetamol metabolites. Conditions as in Fig. 7. (a) Sample of urine taken following therapeutic administration of 1 g paracetamol to a healthy adult (same sample as shown in Fig. 5b). (b) Sample of urine taken following a fatal overdose of paracetamol. Note relatively high concentration of 3-methoxyparacetamol derivatives relative to M.

with experimental animals it appears that varying proportions of the 3SOMeS conjugate are produced following paracetamol administration as determined by UV ratio since in most of these cases 3-methoxyparacetamol glucuronide, and by inference its sulphate analogue, were absent. However, our work has shown that in other instances, e.g. in the study of human paracetamol metabolism, 3-methoxyparacetamol formation is significant as determined by UV ratio (Fig. 8). This creates a major problem when the trio of metabolites S, 3SOMeS, 3OMeS are all present as would be the case under conditions of human overdose.

The formation of 3-methoxy, 3-thiomethyl and 3-thiomethylsulphoxide derivatives of paracetamol appears to be inextricably linked to paracetamol-induced toxicity. Until a better separation of the three sulphate metabolites is obtained these results are presented to illustrate the complex nature of paracetamol metabolism.

ACKNOWLEDGEMENTS

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