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REVERSED-PHASE CHROMATOGRAPHY OF URINARY METABOLITES OF PARACETAMOL USING ION SUPPRESSION AND ION PAIRING

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SUMMARY

High-performance liquid chromatography (HPLC) has proven particularly useful for the study of paracetamol metabolism. Two alternative methods were developed using reversed-phase C_{18} columns. A rapid ion suppression technique was used for the analysis of free paracetamol, paracetamol mercapturic acid and cysteine conjugate in urine samples obtained from isolated perfused rat kidney preparations, which has conveniently demonstrated the oxidative metabolic capacity of the kidney towards paracetamol. A somewhat longer, but higher resolution, ion-pair HPLC procedure was developed for the analysis of paracetamol metabolites in urine samples from experimental animals. The ion-pairing solvent was composed of tetrabutylammonium hydroxide, Tris and EDTA buffered to pH 7.2 with phosphoric acid. Gradient programming was further used to enhance resolution. Using this system two new metabolites, the sulphate and glucuronide conjugates of 3-thiomethylparacetamol were detected and routinely determined along with other known paracetamol metabolites, viz. free paracetamol, paracetamol sulphate, glucuronide, mercapturic acid, and cysteine conjugates, 3-methoxyparacetamol glucuronide and sulphate, *p*-aminophenol and its O-glucuronide and O-sulphate conjugates. Phenolic O-substituted glucuronide and sulphate conjugates of N-hydroxyparacetamol were also separated.

INTRODUCTION

The toxicity of many drugs is a result of their biotransformation to toxic metabolites and this is well illustrated by the metabolism of paracetamol [1]. To understand the mechanisms involved it is useful to correlate the pattern of excreted metabolites with the toxicity in experimental animals which are chosen so as to exhibit variations in both the site and extent of toxicity. Having chosen specific animal models it is then essential to develop rapid and reliable methods by which the drug and its metabolites may be separated and quantitated.

When one considers the array of known paracetamol metabolites [2—7] (Fig. 1) it is obvious that many different compound types are present: highly

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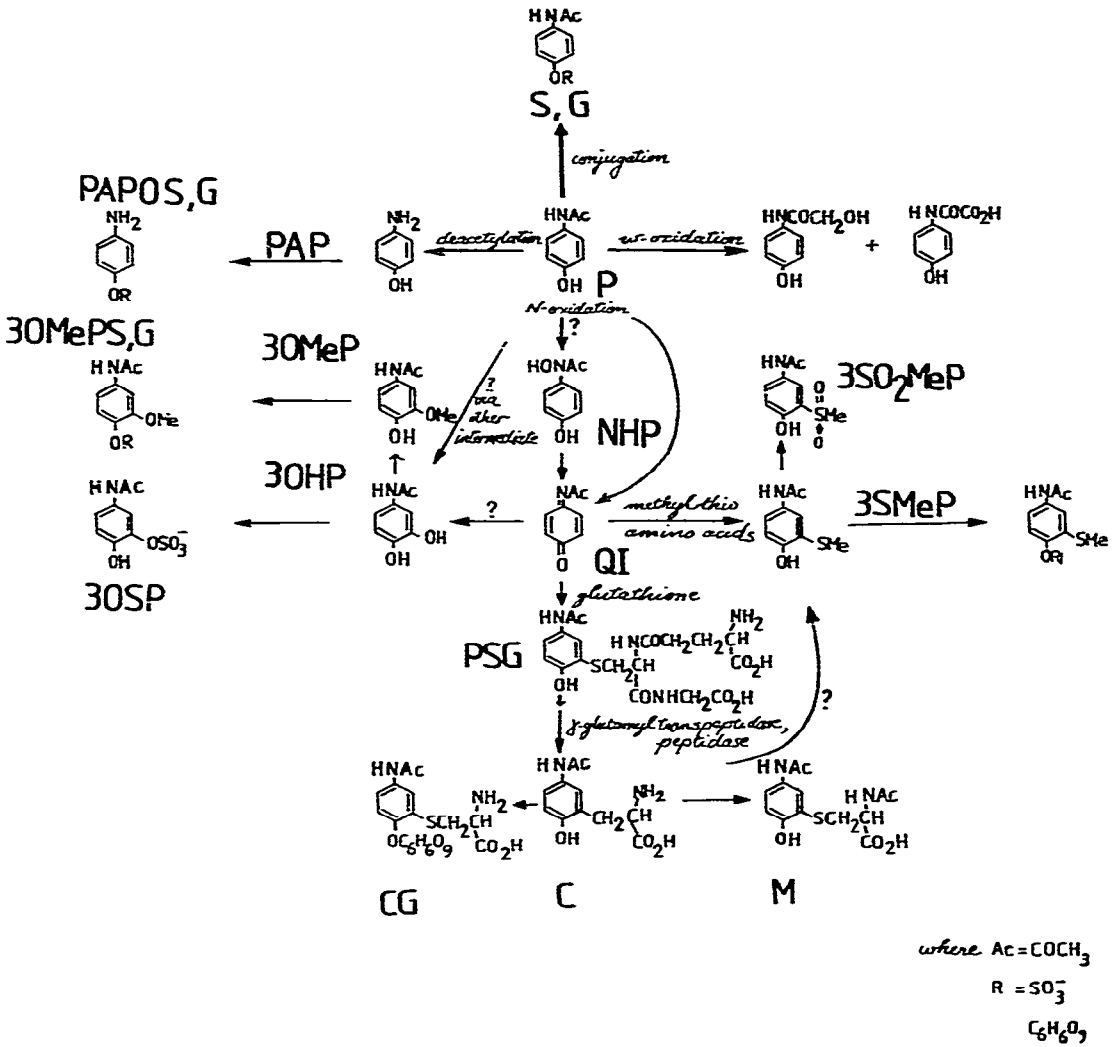


Fig. 1. Paracetamol metabolites. Major excretory products are paracetamol sulphate potassium salt (S), paracetamol-β-D-glucuronic acid (G), paracetamol (P), paracetamol mercapturic acid (M), and paracetamol cysteine conjugate (C) [2—5]. 3-Hydroxylated metabolites, 30MePS, 30SP and 30MePG have been reported by Andrews et al. [4]. Glycolyl and oxanilic acid derivatives formed by ω-oxidation have also been observed [6]. 3-Thiomethyl-substituted paracetamol (3SMeP) has been observed both as the sulphone ($3SO_2MeP$) [3] and conjugated with glucuronic acid and/or sulphate [7]. The double conjugate CG was also postulated [5]. Conjugates of *p*-aminophenol (PAPOS, PAPOG) would be expected if deacetylation occurs.

water soluble conjugates of paracetamol and its oxidized metabolites, the relatively less polar nonconjugated products including N-hydroxyparacetamol [8] which has been considered until recently to be the precursor of the reactive intermediate, N-acetyl-*p*-benzoquinone imine [9], and finally amino acid conjugates derived from glutathione condensation with the reactive intermediate [10]. In order to separate all of these compound types in one separation,

reversed-phase high-performance liquid chromatography (HPLC) offered the largest scope over conventional methods of thin-layer chromatography (TLC) and ion-exchange chromatography. Bonded reversed-phase packings were most suitable since columns packed with these materials are eluted with aqueous eluents. This allows direct application of biological fluids without preliminary extraction. However under typical conditions of reversed-phase chromatography using neutral eluents, e.g. water—methanol mixtures, only those compounds which are non-ionic are retained [11].

This problem is avoided by modifying the eluents used so as to render the ionic conjugates non-polar. This can be achieved in two ways. Firstly by pairing the anionic conjugate with a bulky non-polar cation such as tetrabutylammonium ion (TBA). Ion pairs so formed are well retained by the column and excellent selectivity can be obtained by the choice and concentration of pairing ion [12]. Alternatively the pH of the eluent may simply be reduced so that the non-ionic free acid forms of the conjugates are retained by the column [11]. The latter technique is known as ion suppression.

Buckpitt et al. [13] have illustrated all three reversed-phase HPLC separations for the specific separation of 3-substituted paracetamol metabolites: paracetamol mercapturic acid (M), paracetamol cysteine conjugate (C) and the glutathione conjugate (PSG). These separations while useful for the specific study described, were limited for the full range of urinary metabolites.

Howie et al. [14] and Knox and Jurand [15] used an ion suppression solvent of dilute acetic acid solution containing 15% methanol to investigate urinary excretion of paracetamol metabolites over a 24-h period. While they concluded that excellent separation was achieved the early section of their chromatograms allowed little definition of glucuronide and sulphate conjugates from endogenous urine components. Thus quantitative estimation of these particular conjugates was made difficult. In their study, Knox and Jurand [15] confirmed the presence of 3-methoxyparacetamol sulphate as a human paracetamol metabolite [4]. However, separation of the sulphate conjugates of paracetamol and its 3-methoxy analogue was insufficient, so the authors turned their attention to ion-pairing reagents [16]. Dioctylamine and tetrabutylammonium were both evaluated as pairing ions. Separations with these additives were a major improvement over those using ion suppression because increased retention of paracetamol sulphate enabled the resolution of other possible sulphate metabolites. However, the separation of paracetamol-β-D-glucuronic acid (G) and C was not entirely satisfactory at the front of the chromatograms particularly with the TBA columns where endogenous urine constituents appear to be difficult to resolve from the metabolites.

In the present study the techniques of ion suppression and ion-pairing reversed-phase HPLC for urinary paracetamol metabolites have been further investigated and conditions optimized to obtain maximum separation of the major metabolites.

EXPERIMENTAL

Apparatus

A Spectra-Physics 3500B dual-pump liquid chromatograph fitted with a

solvent programmer, a Waters Assoc. U6K loop injector and an Aerograph Variscan variable-wavelength ultraviolet—visible detector were used for all HPLC analyses.

Commercially packed columns (30 cm × 3.9 mm I.D.; particle size, 10 μm) containing microporous bonded phase support, μBondapak C_{18} were purchased from Waters Assoc.

Solvents

Water, doubly distilled and deionized, was obtained from Marine Chemistry Laboratory (University of Melbourne, Australia). Methanol (AR) was used without further treatment.

Mobile phases

Flow-rates of 2 ml/min were used throughout.

Ion suppression HPLC. Two alternative systems were used. (a) Solvent was prepared according to Howie et al. [14], i.e. 1% acetic acid—methanol—ethyl acetate (900:150:1.0). (b) Methanol was added at 15 volumes to 85 volumes of 0.05 *M* phosphoric acid. The pH of the solvent mixture was adjusted to the required value using 10% potassium hydroxide (AR). After studying a range of pH values a final pH of 2.8—2.85 was chosen for routine analysis.

Ion-pair HPLC. An aqueous solution of tetrabutylammonium (TBA) hydroxide (0.4 *M*, Eastman-Kodak, Rochester, NY, U.S.A.) was diluted to 0.005 *M* and neutralized to pH 7.2 with phosphoric acid (AR). Addition of 0.01 *M* Tris (tris-hydroxymethylaminomethane) (Sigma, St. Louis, MO, U.S.A.) and 0.005 *M* disodium EDTA (ethylenediaminotetraacetic acid disodium salt) was also made to control the selectivity of the separation. Various concentrations of methanol ranging from 0—50% were added to the above solution to control retention times.

Gradient elution ion-pair HPLC

In order to obtain all paracetamol metabolites on a single chromatogram with satisfactory resolution, it was necessary to use gradient elution. Two solvents were prepared: Solvent A = 0% methanol in water containing 0.005 *M* TBA, 0.010 *M* Tris, 0.005 *M* EDTA, pH 7.2 (H_3PO_4), and Solvent B = as for A, but containing 50% methanol.

With solvent A passing through the column at a flow-rate of 2 ml/min the sample was injected. After a delay time of 4 min, solvent B was introduced at a linear rate of 5.6%/min over 18 min. The column was held at 100% solvent B for a minimum of 10 min or until the next sample was ready for analysis. The column was then returned to 0% methanol at a rate of 20%/min over 5 min. After an equilibration period of 18—25 min, the next sample was injected. Approximately 1 h was required in total for sample analysis and column re-equilibration.

Column regeneration

For periods when the HPLC system was not in use, buffers were washed from the pumps and the column with water followed by dilute methanol solution. After the injection of more than 30—50 urine samples on reversed-phase

columns, non-polar impurities were removed by eluting the column with a neutral water—methanol solution in which the methanol concentration was gradually increased to 100%.

Standard compounds

Table I lists authentic drug conjugates which were synthesised and used to establish HPLC conditions. The glucuronides were prepared by the reaction of methyl (tri-O-acetyl-α-D-glucupyranosyl bromide) uronate [17] and the appropriate phenol in the presence of silver carbonate and subsequent transformation of the substituents and protecting groups was achieved by standard methods [18, 19]. The sulphates were prepared as potassium salts by standard methods from the corresponding phenols [20].

TABLE I

SYNTHETIC DRUG CONJUGATES

Conjugate	m.p. (°C)	Symbol
β-Phenyl-D-glucuronic acid	159—161	PhG
β-Quinolyl-D-glucuronic acid	107—109	QG
Paracetamol-β-D-glucuronic acid	260 d	G
p-Aminophenyl-β-D-glucuronic acid	220 d	PAPOG
Potassium phenyl sulphate		PhS
Paracetamol sulphate potassium salt		S
p-Aminophenyl-N-sulphate		PAPNS
p-Aminophenyl-O-sulphate		PAPOS

N-Hydroxyparacetamol (NHP) was chromatographically pure [10]. Paracetamol was obtained from Aldrich (Milwaukee, WI, U.S.A.). Paracetamol mercapturic acid (M) and paracetamol cysteine conjugate (C) were synthesized by reaction of N-acetylcysteine and cysteine respectively dissolved in phosphate buffer, pH 7.2, with N-hydroxyparacetamol at 22°C [10]. The reaction was allowed to proceed for several hours and was monitored by the disappearance of the peak for N-hydroxyparacetamol and the appearance of the paracetamol conjugates by ion-pair HPLC. The filtered reaction mixtures were used directly as standards. *p*-Nitrosophenol was observed as a by-product of the reactions. *p*-Aminophenol hydrochloride (AR) (PAP) was recrystallized.

Radioactively labelled paracetamol

Ring-labelled [^{14}C]paracetamol of specific activity 9.263 μCi/mmol and ring-labelled [^{3}H]paracetamol of specific activity 12 μCi/mg were administered to rats for the collection of radioactively labelled paracetamol metabolites.

Sample preparation

Standard solutions. Standard compounds were dissolved in double distilled deionized water and filtered through 0.5-μm cellulose Millipore membrane filters using a stainless-steel Swinney holder attached to a Luer-lok hypodermic syringe.

Urine samples. These were obtained from Sprague-Dawley albino and Gunn

rats following administration of paracetamol or N-hydroxyparacetamol. Urines were filtered through 0.5-μm cellulose filters held in washable plastic Swinney holders which were also fitted with borosilicate microfibre glass prefilters (Type AP25; Millipore, Bedford, MA, U.S.A.). Samples greater than 2 ml were undiluted whereas smaller volumes were first mixed with water to a known volume before filtration. Filtered urine samples were kept frozen at —20°C in acid-washed screw-capped vials (3 ml capacity) until ready for analysis. Injection volumes varied according to the concentration of metabolites expected in the urine. Typically 1—40 μl of urine were injected for gradient ion-pair HPLC. There was no necessity to add ion-pair reagent to the sample before injection onto the column as ion-pair equilibrium was quickly obtained.

Perfusion urines. These were injected without any treatment [21]. Injections (100 μl) were made in order to determine mercapturic acid. This was necessary because of the limit of sensitivity of the detector (0.1 a.u.f.s.). A 5-μl injection of the same sample was made to determine the larger paracetamol peak.

HPLC analyses of radioactive metabolites

A 0—6 h collection of urine from a female Sprague-Dawley rat given 1 mmol/kg [^{14}C]paracetamol, specific activity 9.263 μCi/mmol, intravenously (i.v.) was used to obtain a radioactive chromatogram of simple paracetamol metabolites. Further metabolites were discerned in the 6—24 h urine from a heterozygous Gunn rat given 2 mmol/kg i.v. [^{3}H]paracetamol of specific activity 1800 μCi/mmol.

Urine samples were separated by HPLC and fractions collected for scintillation counting to positively identify paracetamol metabolites. The column eluent following sample injection was collected in 0.4-ml fractions using an LKB Ultro-Rac 7000 fraction collector filled with plastic scintillation vials. Scintillation fluid (5 ml) (2,5-diphenyloxazole (4 g, Beckman, Fullerton, CA, U.S.A.) dissolved in a small quantity of toluene plus 333 ml Triton X-100 diluted to 1 l with toluene) was added to each vial using an Oxford pipettor. Fraction numbers greater than 100 collected from gradient elution ion-pair separations contained excess methanol which caused turbidity in the scintillation fluid and a consequent decrease in counting efficiency. This was overcome by the addition of 100 μl of water to these fractions. Radioactivity was determined in a Beckman LS-133 liquid scintillation counter. Counts were corrected for quench using calibration graphs. A radioactive chromatogram was thus constructed by plotting number of counts per minute (dpm) versus fraction number, i.e. elution volume.

Thin-layer chromatography

The two-dimensional TLC method of Andrews et al. [4] was used to separate metabolites in urine from rats given both paracetamol and N-hydroxyparacetamol. Spots assigned to paracetamol metabolites were removed from the plates, extracted with phosphate buffer, filtered and separated by ion-pair HPLC to confirm retention times of metabolites in the latter system. Similarly, the cysteine—N-hydroxyparacetamol reaction mixture was run on TLC plates and the fraction ascribed to cysteine conjugate rechromatographed by HPLC.

Rechromatography of separated fractions

Metabolites collected from previous HPLC or TLC separations were reinjected into the chromatograph via the U6K injector. Up to 2 ml of dilute solution could be reinjected, thus solvent evaporation proved to be unnecessary. Samples from TLC plates were scraped off, extracted into a suitable solvent and filtered through membrane filters before injection onto the column. Fractions derived from HPLC columns did not require filtration.

RESULTS AND DISCUSSION

Ion suppression chromatography

Initially an unbuffered ion suppression solvent [1% acetic acid—methanol—ethyl acetate (90:15:0.1)] was used. Synthetic paracetamol conjugates and paracetamol metabolites in a standard urine sample were well retained by the column and a good separation resulted (Fig. 2), although it was different to that reported with the same solvent system [14]. In that case C moved much later than all other metabolites and there was little resolution between S and G. Endogenous urine constituents interfered in this section of the chromatogram (Fig. 2). M was well resolved from all other components. Continued use

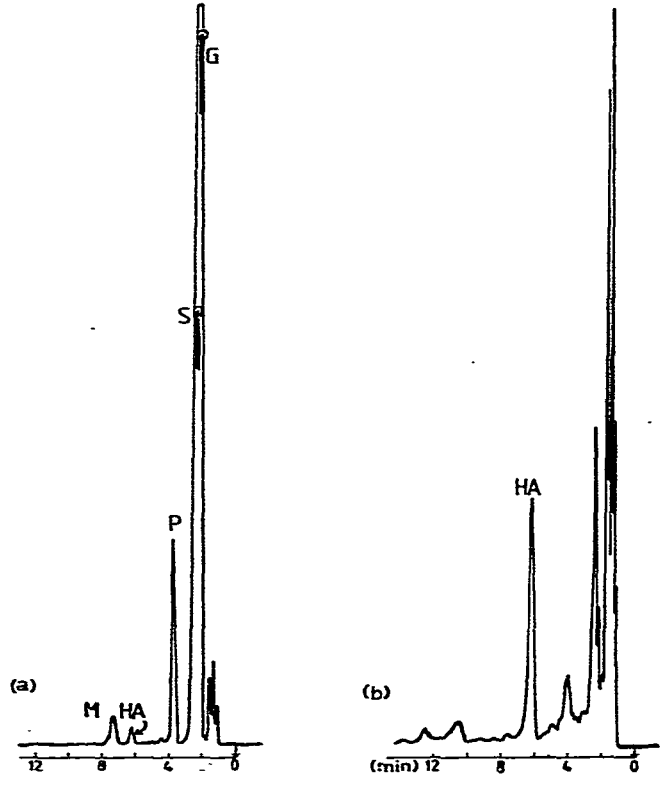
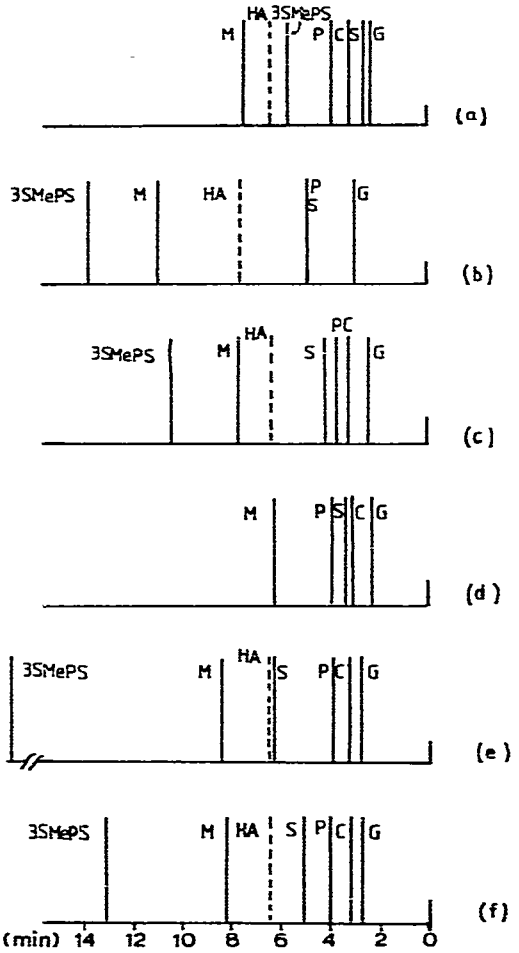


Fig. 2. Ion suppression HPLC of (a) paracetamol metabolites in rat urine and (b) control rat urine. Eluent, 1% aqueous acetic acid—methanol—ethyl acetate (90:15:0.1). Detector, UV, 254 nm, 1.0 a.u.f.s. Peak HA is endogenous urine constituent hippuric acid.

of this solvent failed to give reproducible retentions. These changes were dependent upon the presence of residual ion-pairing salts and changes in pH (Fig. 3b—d). Thus a buffered solvent was prepared according to Molnár and Horváth [22] at pH 2.1. Retentions became longer in this system (Fig. 3e). Optimization of selectivity and retention was achieved by observing variations of retention time with pH (Fig. 4). This was a useful experiment since it clearly differentiated between ionic species such as S, G and M and neutral compounds such as paracetamol. This aided in the identification of a previous-



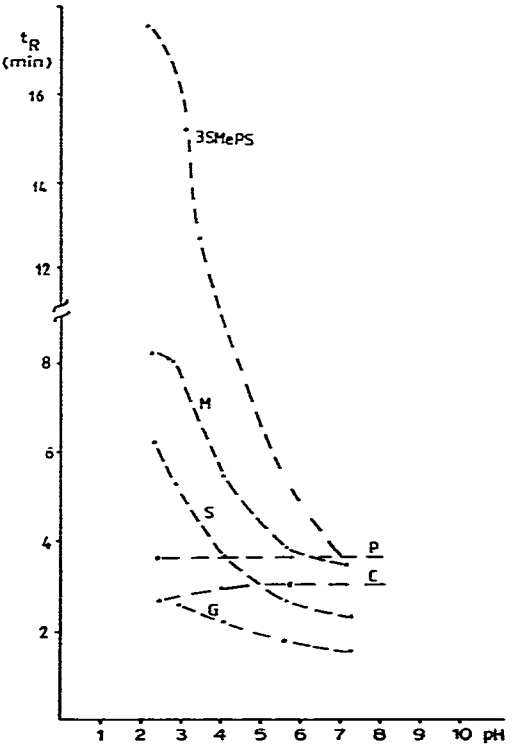


Fig. 3. Change in retention of paracetamol metabolites in ion suppression HPLC with different eluent: (a) as in Fig. 2; (b) as in (a) following the use of TBA phosphate; (c) freshly prepared solvent as in (a) and long column washing to eliminate residual pairing and buffer ions; (d) as in (a) plus 0.1 *M* KH_2PO_4; (e) 15% methanol in 0.05 *M* potassium phosphate buffer (pH 2.1), eluent pH 2.4; (f) as in (e), eluent pH 2.85.

Fig. 4. Change in retention time in ion suppression chromatography versus pH. Eluent, 15% methanol in potassium phosphate buffer (0.05 *M* re phosphate).

ly unidentified metabolite 3-thiomethylparacetamol sulphate (3SMePS) [23]. Fifteen per cent methanol in 0.05 *M* phosphate buffer (pH eluent 2.85) (Fig. 3f) was eventually chosen as a standard eluent for ion suppression chromatography and was used quite successfully to analyse a number of whole animal urine samples and perfusion urines.

There are various advantages and disadvantages in routinely using an ion suppression method. With whole urine samples, G, S, C and P moved very close together making it difficult to estimate each of these metabolites individually especially since endogenous urine constituents eluted close to them also. In whole urine samples G and S represent a large proportion of the drug and large peaks in this section of the chromatogram easily became overloaded, masking other constituents.

By contrast urines from isolated perfused kidneys [21] contained only small amounts of G and S and thus were well suited to ion suppression chromatography. An interesting aspect of the perfusion study was the detection of M and C formed by the kidney. In the absence of large quantities of G and S, the glutathione-derived conjugates were easily monitored as was the proportion of free drug recovered (Fig. 5). In the context of kidney metabolism, ion suppression chromatography was extremely valuable, the separations being completed in 10—15 min without solvent programming and column regeneration, thus enabling the analysis of many samples in a single day. The technique was further refined with the use of a dual-wavelength detector set at two different sensitivities for 254 nm, to simultaneously monitor the small levels of

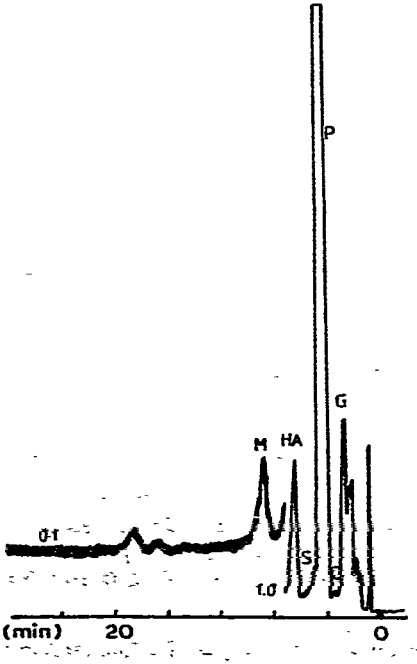


Fig. 5. Ion suppression chromatography of perfusion urine. Eluent, 15% methanol in potassium phosphate buffer (0.05 *M*), pH 2.85. Detector, UV, 254 nm; sensitivity as indicated.

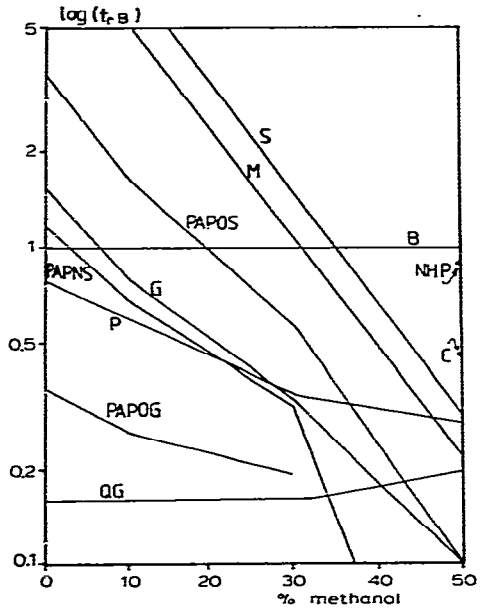
M and C, and the relatively high concentrations of P [24].

In the case of whole animal urine samples such a rapid separation, while desirable, was not so important. Rather, slightly longer separation times could be tolerated, provided the resolution was superior to the ion suppression method. The logical choice for these samples was thus ion-pair partition chromatography on reversed-phase columns.

Optimization of conditions for ion-pair chromatography

TBA was used as the pairing ion since it covered a large range of log E_{QX} values [25] suitable for those metabolites likely to be encountered. Various synthetic paracetamol and *p*-aminophenol conjugates (Table I) were chosen to optimize concentrations of the various solvent components. The complete range of ion-paired conjugates could not be eluted at a single methanol concentration. For example, under conditions whereby P and G were only just resolved at around $k' = 2$—3, S had a k' value of more than 20. Therefore retention times for conjugates were also determined at different methanol concentrations.

Fig. 6 shows the retention data (relative to benzyl alcohol) obtained using the simplest composition of 0.005 *M* TBA only in the mobile phase. The pH was adjusted to 7.2 with phosphoric acid; however, little buffering capacity was evident and pH control was limited. There was quite acceptable resolution



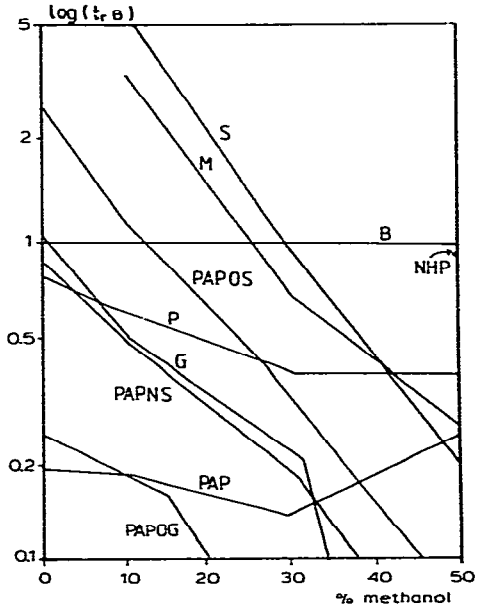
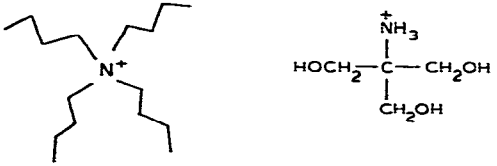


Fig. 6. Relative retention of synthetic paracetamol derivatives in ion-pair HPLC. Solvent, 0—50% methanol in water containing 0.005 *M* TBA adjusted to pH 7.2 with phosphoric acid. k' for benzyl alcohol (B) at 0, 10, 30 and 50% methanol are 7.0, 4.8, 2.0 and 0.8 respectively.

Fig. 7. Relative retentions of synthetic paracetamol derivatives in ion pair HPLC. Solvent as in Fig. 6, but also containing 0.01 *M* Tris. k' for benzyl alcohol (B) at 0, 10, 30 and 50% methanol are 6.2, 4.6, 2.0 and 0.7 respectively.

of all of the conjugates although retentions for M and S were very long. An endogenous urine constituent, hippuric acid, was unresolved from M under these conditions. A further disadvantage was that C and NHP could only be eluted at methanol concentrations of at least 50%, that is, after all the other conjugates. Their peak shapes were also quite poor and inadequate for analytical purposes.

In order to increase buffering capacity Tris was used. This was found to give selectivity superior to simple sodium or potassium phosphate buffers. In Fig. 7, the effect of added Tris is shown. Two concentrations were used, 0.005 *M* and 0.01 *M*. As there was little difference in retention and selectivity between the two concentrations, the higher level of Tris was chosen since it provided greater buffering capacity. Addition of Tris altered the elution pattern quite significantly. By inspection of the structures of TBA and protonated Tris ion,



it would be expected that ion pairs formed with Tris would be more water soluble and hence less strongly retained on the column. Under these conditions endogenous urine constituents in a control urine sample did not interfere with paracetamol conjugates, in particular M. Unfortunately the decrease in retention for paired conjugates meant that resolution between P, G and PAPNS had diminished. NHP and C still did not chromatograph well.

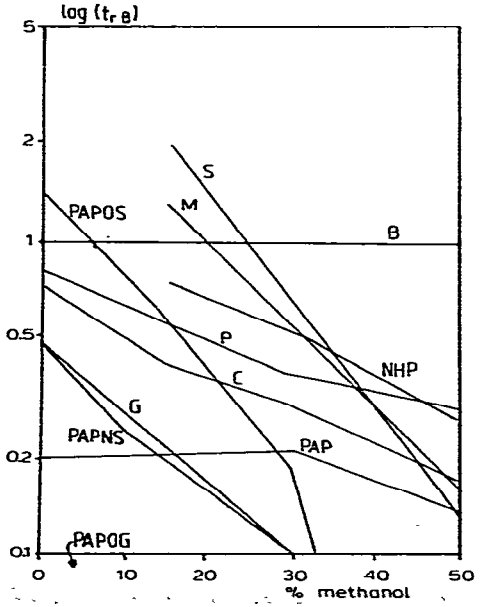


Fig. 8. Relative retentions of synthetic paracetamol derivatives in ion pair HPLC. Solvent as in Fig. 7, but also containing 0.005 *M* EDTA. *k'* for benzyl alcohol (B) at 0, 15, 30 and 50% methanol are 5.9, 3.3, 1.8 and 0.7 respectively.

The effect of introducing 0.005 *M* EDTA into the mobile phase is shown in Fig. 8. EDTA was also incorporated since it prevented excessive tailing of N-hydroxyparacetamol (unpublished work in this laboratory). Retentions for the conjugates once again decreased suggesting involvement of EDTA in ion pairing. The effect was beneficial in many respects. Much less methanol was required to elute S and M, G now moved sufficiently ahead of P to improve the resolution of these two compounds, although now G coincided exactly with *p*-aminophenol-N-sulphate. Loss of resolution for this pair of conjugates however was considered to be less important since improved resolution of the paracetamol metabolites had been achieved and *p*-aminophenol has not been observed as a quantitatively significant metabolite of paracetamol [26]. Perhaps the most beneficial result was that NHP and C eluted at all concentrations of methanol. It appeared that this solvent composition offered the best separation and was used for all future urine analysis.

Gradient programming

Urine samples were tested with the standardized ion-pair reagent at 10% methanol. S appeared as a broad peak taking approximately 20 min to elute, while the early part of the chromatogram was moderately well resolved (Fig. 9a). When the methanol concentration was increased to 20% (Fig. 9b) the sulphate conjugate appeared as a sharp peak at a shorter retention, and 3SMePS which had not appeared at 10% methanol was now detected. These benefits

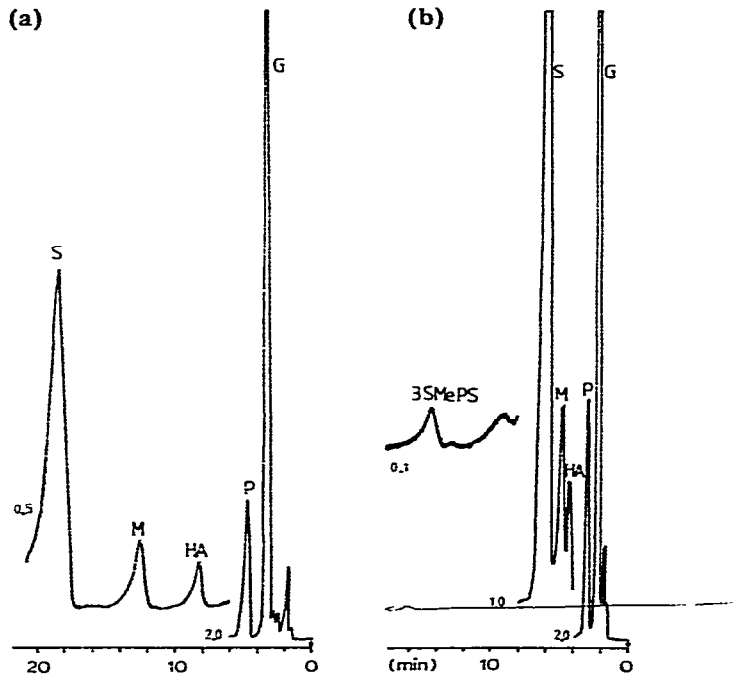


Fig. 9. Isocratic ion-pair HPLC of urinary paracetamol metabolites. Eluent (a) 10% methanol and (b) 20% methanol in standard ion-pair reagent, i.e., 0.005 *M* TBA—0.01 *M* Tris—0.005 *M* EDTA buffered to pH 7.2 with phosphoric acid. Detector, UV, 254 nm, sensitivity as indicated.

were overshadowed however by crowding at the beginning of the chromatogram which was poorly resolved.

Thus it was clear that solvent programming with increasing concentration of methanol was necessary. This was quite possible since no stationary phase was used on the column [27—31]. By reference to Fig. 8 it was noted that adequate resolution was maintained at 0% methanol and all conjugates were eluted with 50% methanol. Therefore these solvent concentrations were chosen as initial and final values respectively. After some trial and error a gradient programme as outlined in Experimental was adopted with samples run every hour.

Identification and quantitation of paracetamol metabolites

Fig. 10 illustrates a relatively simple metabolic profile following the administration of a low dose of paracetamol. P, S, G, M and *p*-aminophenol-O-sulphate were observed and identified by comparison with standard compounds. Further proof of identity was obtained when the urine from a rat given radioactively labelled paracetamol was analysed (Fig. 11a) and compared

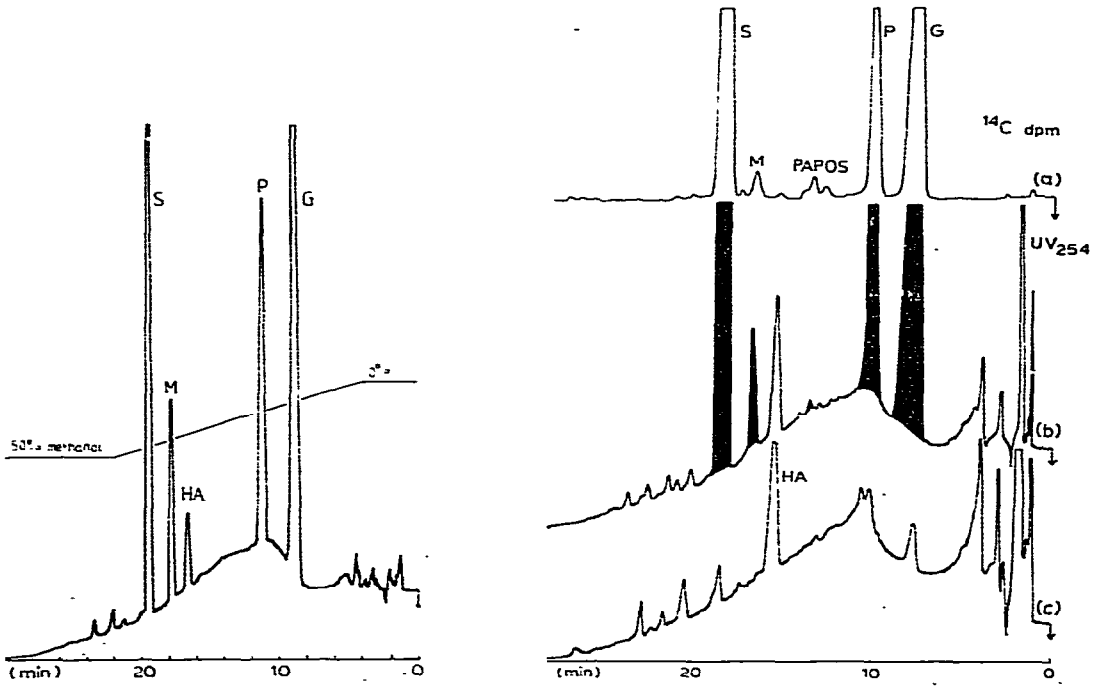


Fig. 10. Solvent programmed ion-pair HPLC of urinary paracetamol metabolites. Eluent, standard ion-pair reagent linearly programmed from 0—50% methanol over 18 min after an initial hold time of 4 min. Detector, UV, 254 nm, 1.0 a.u.f.s. Sample, 6-h urine collection from a female Sprague-Dawley rat following the intravenous administration of 3 mmol/kg body weight of paracetamol.

Fig. 11. Identification of paracetamol derived metabolites using radioactive detection. HPLC conditions as in Fig. 10. Detection, (a) ^{14}C-counts per 0.4-ml fraction; (b) UV detection at 254 nm, 1.0 a.u.f.s. Urine samples (a) and (b) from a heterozygous Gunn rat given 2 mmol/kg body weight [^{14}C]paracetamol, (c) control rat urine, i.e. no paracetamol administered.

with the conventional 254-nm chromatogram (Fig. 11b). The chromatogram of control urine from a rat which was not given paracetamol is shown for comparison (Fig. 11c). It can be seen that there is little interference from endogenous constituents.

The radioactive chromatogram served a second purpose since it enabled the calculation of response factors for each metabolite. A formula was derived for the response factor by comparison of the 254-nm peak area with the number of counts per peak as calculated from the radioactive chromatogram.

$$\text{Response factor} = \frac{\dfrac{\text{number of counts per peak}}{\text{peak area}_{254/1.0}} \times \dfrac{\text{mmol paracetamol administered}}{\text{total counts administered}}}{} \times 10^6$$

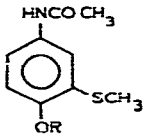
Units for the response factor nm per cm^2 peak area at 254 nm, sensitivity 1.0 a.u.f.s. Once the response factor for each metabolite was determined, this value was used to calculate the mass of the same metabolite, and hence percentage of the dose excreted in any subsequent chromatogram. Table II lists response factors for all the metabolites recovered in this study.

TABLE II

RESPONSE FACTORS FOR PARACETAMOL METABOLITES

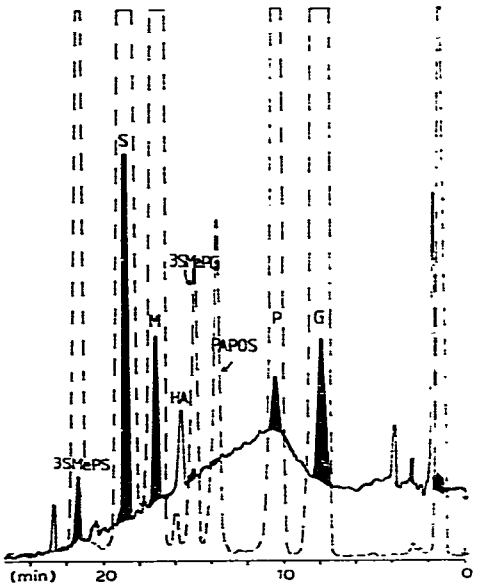
Metabolite	Response factor nmol cm^{-2} (254/1.0)
Paracetamol	23.5
Paracetamol glucuronide	21.5
Paracetamol sulphate	19.5
Paracetamol mercapturic acid	20.0
3-Thiomethylparacetamol sulphate	18.5
3-Thiomethylparacetamol glucuronide	21.5
Cysteine conjugate	20.0
p-Aminophenol-O-sulphate	150.0

Inspection of the radioactive chromatogram of the urine from a different species of rat given ^3H-radiolabelled paracetamol (Fig. 12) revealed the presence of another new metabolite, 3-thiomethylparacetamol glucuronide (3SMePG) as well as 3SMePS. The isolation and identification of these new metabolites is described elsewhere [23]. They were found routinely in animals receiving high



where $R = SO_3^-$ $C_6H_6O_9$

doses of paracetamol [26]. Previously 3-thiomethylparacetamol has only been isolated from urine after treatment with glucuronidase—aryl sulphatase enzymes [7].



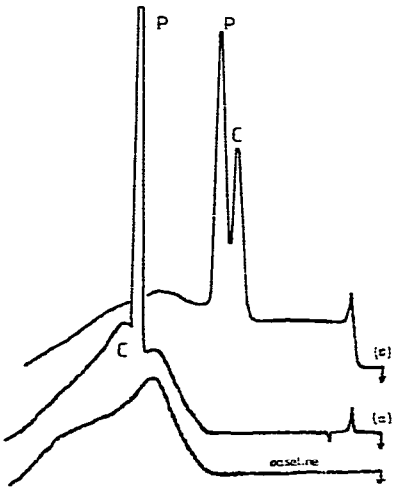


Fig. 12. Chromatograms of urinary [^{3}H]paracetamol metabolites from a heterozygous Gunn rat given 2 mmol/kg body weight paracetamol. HPLC conditions as in Fig. 10. Detection, solid line, UV, 254 nm, 1.0 a.u.f.s.; broken line, ^{3}H-counts per 0.4-ml fraction.

Fig. 13. Effect of column age on recovery of paracetamol cysteine conjugate, C. HPLC conditions as in Fig. 10; (a) new column, (b) old column.

Cysteine conjugate proved to be an elusive metabolite and was originally expected to be present in rat urine as a metabolite of paracetamol. It was not found in rats given paracetamol, but was found however in rats given relatively large quantities of N-hydroxyparacetamol. A spot assigned to C in two-dimensional TLC was rechromatographed in the ion-pair system. It eluted near paracetamol; however, unless present in sufficiently large quantities, it was difficult to discern, especially since at this point in the chromatogram the baseline changed under the solvent programming conditions. Recovery of cysteine conjugate was improved with older EDTA-saturated columns (Fig. 13) and this is consistent with the ability of amino acids to coordinate with metal ions or silica hydroxyls of the column packing. Ion suppression HPLC or two-dimensional TLC [4] were used as alternatives to the ion-pair method for detection of C. These systems demonstrated that over very wide ranges of paracetamol administered to rats, cysteine conjugate was not present as a metabolite. On the other hand in the urines from mice or humans it was observed in significant quantities [9]. It was noted that cysteine conjugate is relatively non-polar and in fact elutes close to paracetamol in neutral reversed-phase chromatography (Fig. 14a). By contrast the N-acetylated cysteine conjugate, that is paracetamol mercapturic acid, eluted with other water soluble conjugates, which included the two 3-thiomethylparacetamol conjugates, under these conditions. Rechromatography of fractions collected under neutral conditions by ion pair chromatography confirmed their identity (Fig. 14b).

Sulphate and glucuronide metabolites of 3-methoxyparacetamol were not

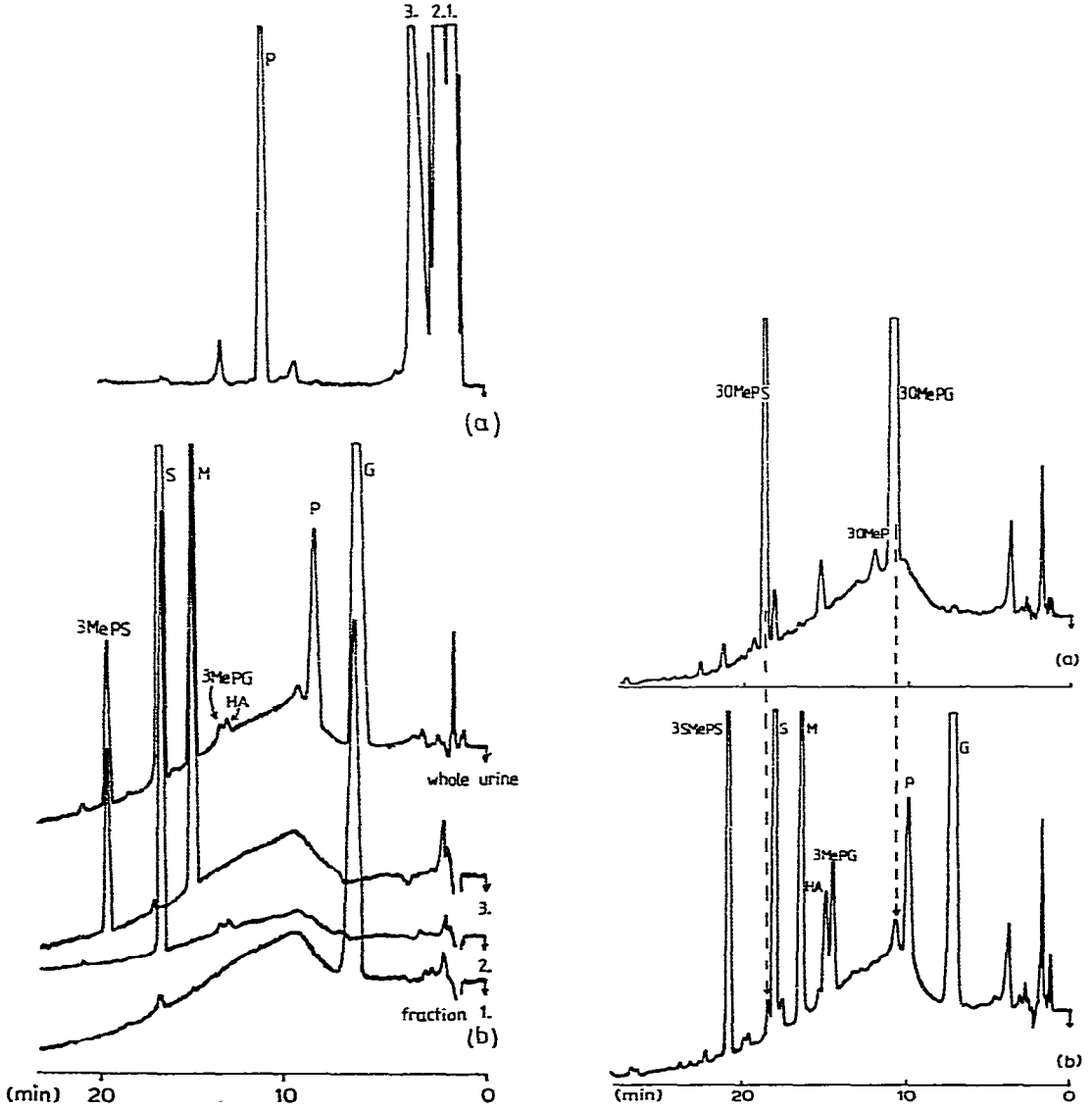


Fig. 14. (a) Neutral reversed-phase chromatograms of urinary paracetamol metabolites. Eluent, water programmed from 0—50% methanol linearly over 18 min after 4 min delay. Three water-soluble fractions 1, 2 and 3 were collected and rechromatographed (b) by standard ion-pair gradient programming as in Fig. 10.

Fig. 15. Chromatograms of urinary metabolites of (a) 3-methoxyparacetamol (30MeP) and (b) paracetamol. HPLC conditions as in Fig. 10.

ordinarily found in rat urines, although their occurrence has been mentioned by other workers, in particular from human urines, [4, 15, 16] (Fig. 1). As standards of 30MePS and 30MePG were unavailable, the free compound was administered to rats at a dose of 1.0 mmol/kg. Chromatography of the urine confirmed these compounds as the principal metabolites (Fig. 15). 30MePG ran just after paracetamol while 30MePS ran very close to S.

N-Hydroxyparacetamol metabolites

For some time N-hydroxylation [32—34] has been considered to be the initial metabolic step in the toxicity of paracetamol, although evidence is now accumulating to suggest that direct oxidation of paracetamol to the reactive N-acetyl-*p*-benzoquinone imine occurs [9]. In this context it was interesting to observe phenolic O-substituted conjugates of N-hydroxyparacetamol, i.e. N-hydroxyparacetamol-O-sulphate and -O-glucuronide in the urine of animals given N-hydroxyparacetamol. They were identified by their close retention to the analogous paracetamol conjugates and by their chromatographic behav-

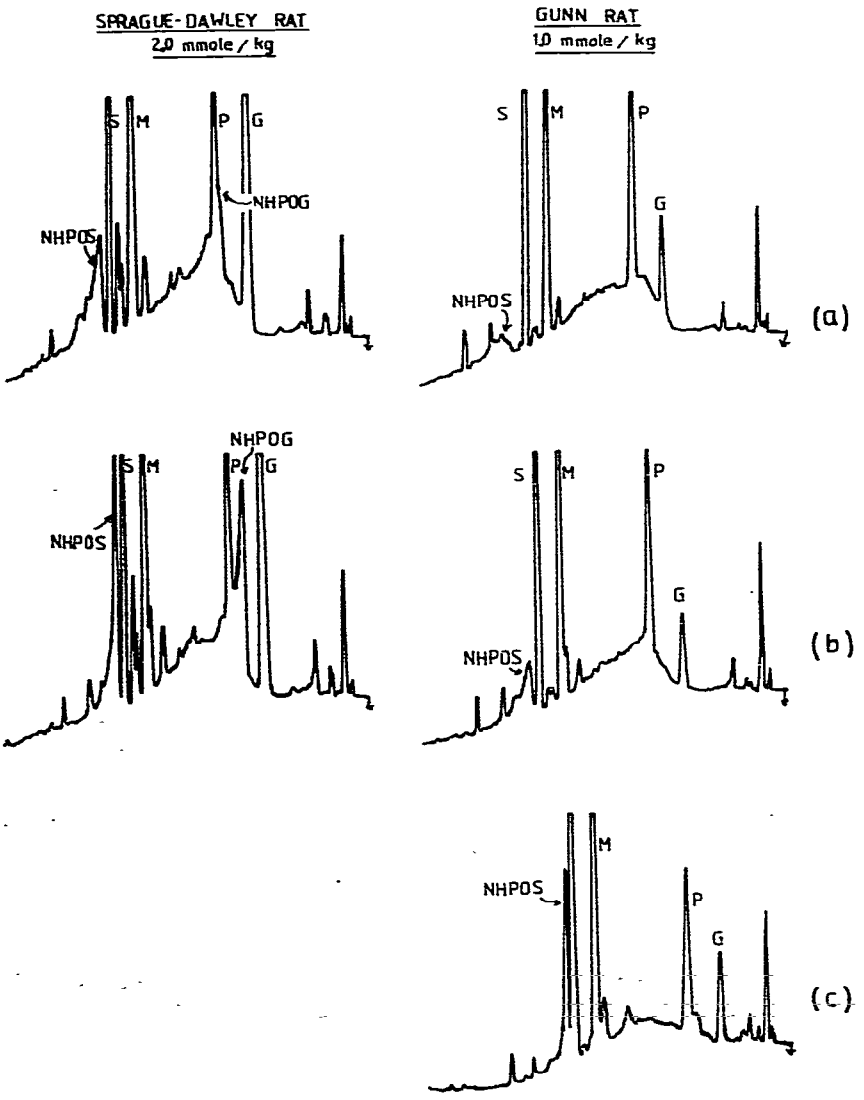
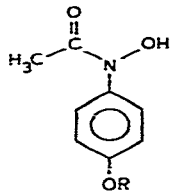
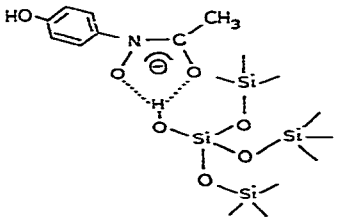


Fig. 16. Chromatograms of urinary N-hydroxyparacetamol metabolites from two different species of rat. HPLC conditions as in Fig. 10. Column conditions, (a) new column; (b) column partly deactivated with EDTA and (c) fully saturated with EDTA.



where R = $C_6H_6O_9$
= SO_3^-

iour, which was very similar to N-hydroxyparacetamol itself. That is, with a new column these peaks moved well after the analogous paracetamol conjugates and tailed badly (Fig. 16a) due to coordination with metal ions or silica hydroxyls of the column packing. As the column aged and became fur-



ther satured with EDTA the peaks eluted progressively earlier with increased sensitivity and decreased tailing (Fig. 16b, c). N-Hydroxyparacetamol glucuronide showed identical chromatographic properties to an authentic sample synthesized in this laboratory [9]. Interestingly with Gunn rats the peak ascribed to N-hydroxyparacetamol glucuronide virtually disappeared while the corresponding sulphate conjugate increased. Limited glucuronidation of paracetamol with Gunn rats is well known [35]. This observation further confirmed the occurrence of phenolic N-hydroxyparacetamol-O-conjugates. To date these compounds have not been detected in the urine from animals given paracetamol alone [9, 26].

CONCLUSION

The methods developed for the separation of paracetamol metabolites have opened a wide field of metabolic profiling universally applicable to a number of different species, and to biological fluids other than urine. The methods have been especially useful for the study of paracetamol metabolism in isolated organ and cell fraction preparations.

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