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COMPLETE SEPARATION OF URINARY METABOLITES OF PARACETAMOL AND SUBSTITUTED PARACETAMOLS BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic procedure has been developed for the separation of thirteen urinary metabolites of the analgesic drug paracetamol. The method involved the use of radially compressed columns packed with octadecylsilica with a particle diameter of 5 μm. Metabolites were chromatographed by linear gradient elution using an ion-pair solvent system composed of tetrabutylammonium hydroxide and Tris buffered to pH 5.0 with phosphoric acid, and acetonitrile as the organic solvent. Analyses can be performed at the rate of three per hour. This method enables the direct identification of sulphate and glucuronide conjugutes of 3-thiomethylparacetamol and 3-thiomethylparacetamol sulphoxide which have only previously been detected following enzyme hydrolysis of urine samples. The application of this fully optimised separation to the study of the metabolism of substituted paracetamols is also discussed.

INTRODUCTION

The mechanism by which paracetamol induces hepatic damage following acute administration is yet to be fully elucidated. However, the biotransformation of paracetamol in the liver has been well characterised in terms of the structural identification of the excreted metabolic products. As a result, the correlation between the chemical nature and the relative proportions of the excreted metabolites and the accompanying degree of toxicity has allowed a partial understanding of the biochemical events following the ingestion of paracetamol and their implications in the manifestation of hepatic toxicity. The monitoring of levels of uri-

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nary metabolites of paracetamol is therefore vital and only possible through the development of rapid and reliable methods by which the drug and its metabolites can be separated and quantitated.

Procedures that have been employed for the determination of paracetamol in biological samples include spectrophotometry, gas chromatography, gas chromatography–mass spectrometry, high-performance liquid chromatography (HPLC) and thin-layer chromatography [1]. Due to its speed, accuracy, precision, selectivity and high sensitivity, HPLC has become an indispensible tool for the analysis and monitoring of therapeutic agents in general, and has been particularly useful in the determination of paracetamol and its metabolites in various biological fluids. The remarkable powers of resolution associated with the use of HPLC offer particular advantages over other analytical methods, as examination of the metabolic profile of paracetamol shown in Fig. 1 reveals the formation of a large number of commonly observed metabolites which vary greatly in structure and solubility properties. These compounds which are tabulated in Table I range from the relatively non-polar unconjugated paracetamol derivatives to the corresponding highly water-soluble sulphate and glucuronide conjugates of paracetamol and its oxidised metabolites. Reversed-phase HPLC has offered the largest scope for the separation and quantitation of paracetamol metabolites. Chromatograms may be run with aqueous eluents, which allow direct application of biological fluids without preliminary extraction. However, normal conditions of reversed-phase chromatography involve the use of neutral eluents such as water–methanol, and as a result only those compounds which are non-ionic are retained [2]. This problem may be avoided by reducing the pH of the eluent so that the non-ionic free acid forms of the polar conjugates are retained by the column [2]. This technique, known as ion suppression, has only met with moderate success [3,4] in the separation of paracetamol metabolites as incomplete separation of glucuronide and sulphate conjugates was achieved.

While buffered ion-suppression solvents have since been used by others for the quantitation of paracetamol metabolites [5–10], insufficient resolution at the front of the chromatogram restricts the usefulness of the technique and workers have turned to ion-pair chromatography where a bulky non-polar cation is paired with the anionic polar conjugates [11]. The resultant ion pairs are well retained by the column, and Knox and Jurand [12] first applied this technique to the analysis of paracetamol metabolites using dioctylamine or tetrabutylammonium (TBA) ion as the pairing agent. Hart et al. [5] further developed the separation by using a solvent containing 0.005 *M* TBA, 0.01 *M* Tris and 0.005 *M* EDTA buffered to pH 7.2 with phosphoric acid. The retention times were optimised by the use of gradient elution with methanol as the eluting solvent. The gradient elution method was developed using conventional stainless-steel columns while the methods of isocratic ion suppression used by Hart et al. [5] were also adapted for use on the newly introduced radially compressed columns [14].

However, concurrent with this developmental work was the identification of the sulphate and glucuronide conjugates of 3-thiomethylparacetamol (SMeP) and 3-thiomethylparacetamol sulphoxide (SOP) as urinary metabolites of paracetamol [15]. This considerably complicated the metabolic profile and further

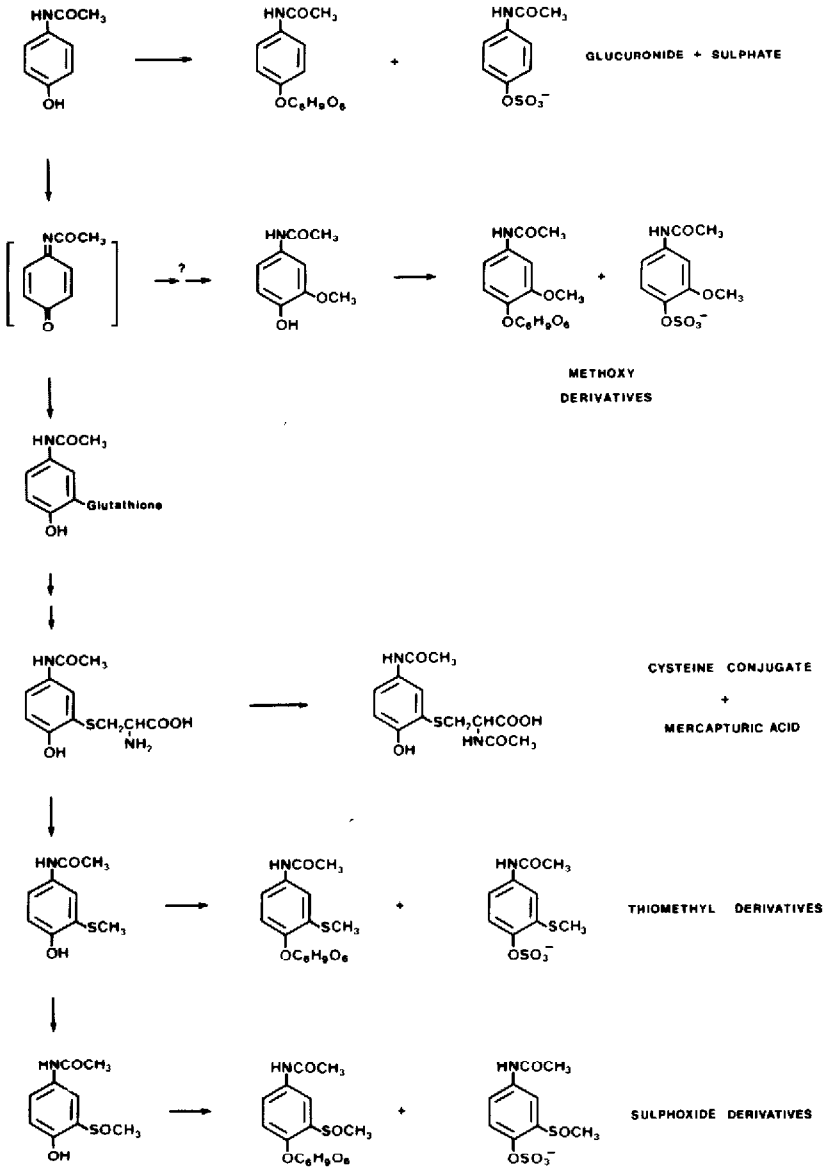

Fig. 1. Summary of the metabolic pathways of paracetamol.

diminished the resolving power of the chromatographic system. The sulphate and glucuronide conjugates of SMeP (SMeS and SMeG, respectively) were resolved using isocratic ion-suppression systems but the oxidised derivative SOP and the corresponding sulphate (SOPS) and glucuronide (SOPG) were found to elute with other major metabolites. Furthermore, the position of 3-methoxyparacetamol (OMeP) and its sulphate (OMeS) and glucuronide (OMeG) conjugates is also important due to their presence in human urines. Once again these were found to coelute or run very closely to other paracetamol metabolites.

TABLE I

SUMMARY OF URINARY METABOLITES OF PARACETAMOL

Metabolite	Abbreviation
Paracetamol glucuronide	G
Paracetamol cysteine conjugate	C
Paracetamol	P
3-Methoxyparacetamol	OMeP
3-Methoxyparacetamol glucuronide	OMeG
3-Thiomethylparacetamol sulphoxide	SOP
3-Thiomethylparacetamol sulphoxide glucuronide	SOPG
3-Thiomethylparacetamol glucuronide	SMeG
Paracetamol mercapturic acid	M
Hippuric acid	HA
Paracetamol sulphate	S
3-Thiomethylparacetamol sulphoxide sulphate	SOPS
3-Methoxyparacetamol sulphate	OMeS
3-Thiomethylparacetamol sulphate	SMeS

At this stage, the application of ion-pair gradient elution was the obvious course to follow, and this paper outlines the development of this system to enable the complete separation of all paracetamol metabolites using reversed-phase radially compressed columns. The application of the final separation to the study of the metabolism of substituted paracetamols is also discussed.

EXPERIMENTAL

Instrumentation

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 and 5 μm; 10 cm×5 mm I.D., particle size 10 μm) and contained unsilanized C_{18} packing. These were operated in a Waters Assoc. radial compression module (RCM-100), fitted with a Waters Assoc. C_{18} Guard-Pak precolumn insert. Conventional stainless-steel μBondpak C_{18} columns (30 cm×3.9 mm I.D., 10 μm particle size Waters Assoc.) were also used.

Solvents

Distilled water was purified through a Milli-Q ion-exchange system (Millipore, Bedford, MA, U.S.A.) Methanol (AR, BDH Australia) was used without further treatment. Acetonitrile (HPLC grade) was obtained from Waters Assoc.

Mobile phase

The final ion-pair solvent optimised for the separation of paracetamol metabolites on 5-μm particle columns consisted of an aqueous solution of 0.02 *M* TBA

hydroxide (various sources) and 0.01 *M* Tris (Tris-hydroxymethylaminomethylaminomethane) (Sigma, St. Louis, MO, U.S.A.) in water or water–acetonitrile (1:1). The pH was adjusted to 5.0 with phosphoric acid. The sample was injected with 15% acetonitrile passing through the column at a flow-rate of 2.0 ml/min. After a delay of 1 min the acetonitrile concentration was increased linearly to 50% over 11 min, immediately after which the column was returned to 15% acetonitrile. 6 min elapsed before the next sample was injected.

The gradient system was modified for separation of substituted paracetamols by eliminating the initial delay and linearly increasing the acetonitrile concentration from 10 to 50% over 12 min. The solvent pH was adjusted to 3.5 for the analysis of 3-methylparacetamol metabolites while 3-fluoro-, 3-chloro- and 2-methylparacetamol metabolites were analysed at pH 4 [16]. For the analysis of 3,5,- 2,6- and 2,3-dimethylparacetamol metabolites pH 5 was used.

Standard compounds

Paracetamol was obtained from Aldrich. All substituted analogues of paracetamol and their metabolites were synthesised in our laboratory as described elsewhere [5,16,17]. Following positive identification of the metabolites, urine samples obtained from Swiss mice following administration of paracetamol or its analogues were used as a mixed standard for the determination of retention times. All 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.

RESULTS AND DISCUSSION

Use of radially compressed columns

To meet the needs of current liquid chromatographic applications, the HPLC column must be highly reliable, efficient and reproducible. Unfortunately, the approaches to satisfying this range of needs are often contradictory and result in compromises. For example, in the quest for higher efficiencies, small particle size packings have been employed, but while higher efficiencies have been achieved, it has been at the expense of column reliability, economy and operating pressure. Smaller particle diameter packings are more difficult to pack into reproducible columns. The resulting columns cost more and operate at higher back-pressures because of reduced bed permeability.

Radial compression has been an innovative approach to the formation of the HPLC column which avoids these limitations without compromising performances [14]. The basic aspects of column formation are dealt with by radial compression. The first is reproducibly forming a homogeneous packed bed and the second is eliminating wall effects which refer to the loss of column efficiency caused by channels that inevitably are present between packing particles in the vicinity of the column wall and between the packed bed and the wall. In the radial compression technique, the column wall is made of a flexible material. External radial pressure is applied causing the wall to conform to the packed bed, creating

a more uniform structure throughout, and eliminating channels along the wall. In addition, these radial forces cause particles throughout the entire cross-section of the bed to move together, closing up any voids or channels that exist in the main body of the packed bed.

The original ion-pair system previously developed [5] for use on stainless-steel columns was therefore applied directly to the radially compressed system initially without modification. This entailed a solvent composition of 0.005 *M* TBA, 0.001 *M* Tris and 0.005 *M* EDTA buffered to pH 7.2 with phosphoric acid. Operating at a flow-rate of 2 ml/min, and after a delay of 4 min, the methanol concentration was increased linearly from 0 to 50% over 18 min and then returned to 0% over 5 min. Approximately 1 h was required in total for sample analysis and column reequilibration. Different selectivities, which were reflected in altered retention times, were obtained as shown in Fig. 2 and were due to the change in the packing material. Overall, retention times were reduced and the resolution between various metabolites deteriorated. In addition, the thiomethyl and sulphoxide derivatives of paracetamol had to be incorporated into the separation following their identification as urinary metabolites of paracetamol [15]. The retention times of these compounds were obtained by running urine samples containing SMeP and SOP metabolites. It was found that SOP, OMeG and paracetamol ran together, and there was poor resolution between hippuric acid (HA), a commonly observed urinary component and the paracetamol mercapturic acid (M), and also SMeP and paracetamol sulphate (S). Furthermore, SOPG and the paracetamol cysteine conjugate (C) coeluted as did SOPS, S and OMeS. Detection of chromatograms at two different wavelengths, 254 and 280 nm, allows a wavelength ratio to be determined which is characteristic for each compound. Identification and quantitation of two metabolites within one peak can therefore be made as the observed wavelength ratio will be the sum of the individual contributions from each compound, which will also be proportional to the relative amounts of the coeluting compounds. It is not possible to use this technique, however, when three metabolites coelute because complex mathematical computations would be required to determine the individual contributions to the observed ratio.

At this stage therefore, only one analysis was still completed per hour, which was considered unsatisfactory. Although the gradient time was only 22 min, a further 36 min elapsed before the column was reequilibrated. The large rise in the baseline which occurred during the program was removed by the omission of EDTA from the solvent. Following this modification, however, the cysteine conjugate tailed badly as shown in Fig. 3a. This was due to interaction of the amino acid moiety with the column, which demonstrated the role of EDTA as a deactivator of chelating sites on the column. In addition, EDTA apparently competed with the water-soluble metabolites in ion-pair formation with TBA, as all retention times were generally decreased. Furthermore, the removal of EDTA caused paracetamol and its glucuronide conjugate (G) to be unresolved and there was little resolution between SMeP, M, S and HA. The peak shape of C was improved by lowering the solvent pH which suppressed the zwitterionic nature of the compound. The resulting chromatogram at pH 5 is shown in Fig. 3b. C was found to

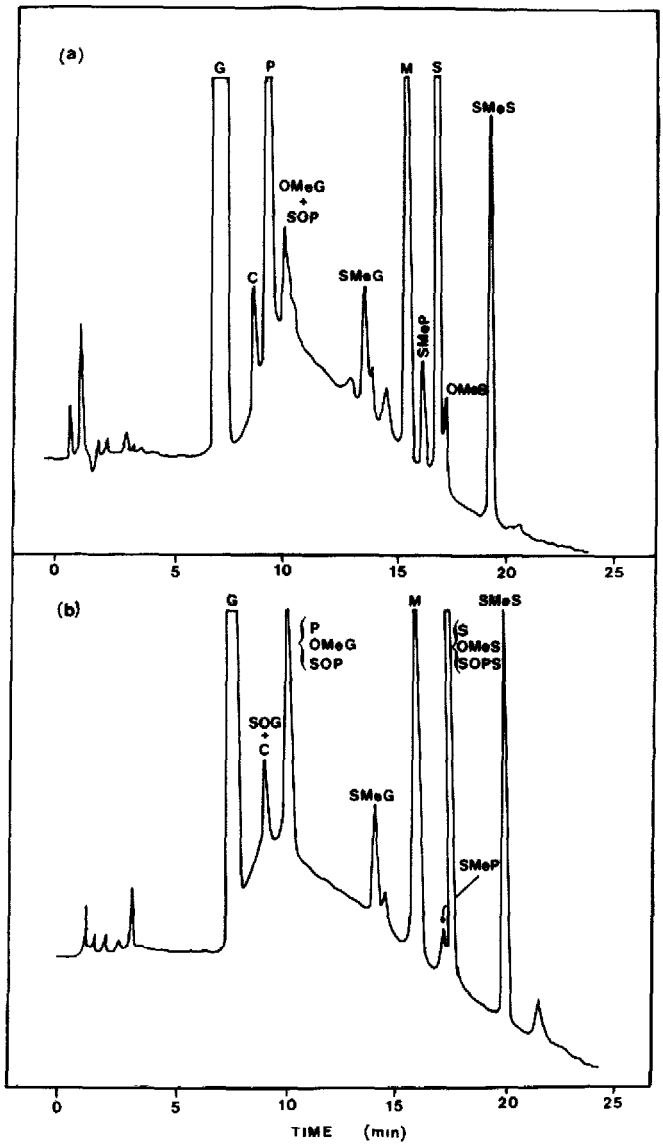

Fig. 2. Effect of column radial compression on gradient ion-pair HPLC of urinary paracetamol metabolites. Columns: (a) stainless-steel μBondapak C_{18}, (b) Radial Pak C_{18}; solvent: 0.005 *M* TBA, 0.01 *M* Tris, 0.005 *M* EDTA, pH 7.2 phosphoric acid programmed from 0 to 50% methanol over 18 min after 4 min delay; flow-rate: 2 ml/min; detector: 254 nm, 1.0 a.u.f.s. Sample: mixed urine sample from female Swiss mouse treated with paracetamol, 3-methoxyparacetamol or 3-thiomethylparacetamol. See Table I for identification of metabolites.

elute as a sharp peak but had a shorter retention time and was unresolved from paracetamol. In addition, the pH change caused all glucuronides to have longer retention times and caused SMeG and SMeP to be unresolved, while M and S continually ran closely together. However, an acidic pH was required for reliable estimations of C to be made as it is an important marker of oxidative metabolism.

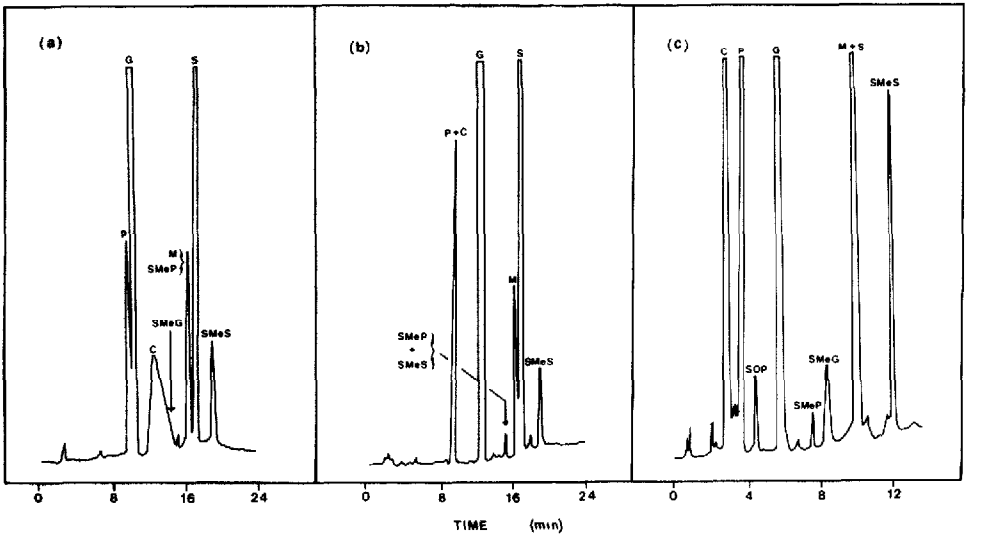

Fig. 3. Optimisation of gradient ion-pair separation of urinary paracetamol metabolites. Chromatographic conditions as in Fig. 2b except: (a) EDTA removed, (b) EDTA removed and pH adjusted to 5.0 (phosphoric acid), (c) as in (b) plus programmed from 10 to 50% methanol over 9 min and no delay; flow-rate, 4 ml/min.

pH 5 was found to be optimum as more acidic conditions caused all retention times to be shorter and resolution to deteriorate further, and higher pH values resulted in the peak tailing of C.

Efforts were then made to reduce the separation time by increasing the flow-rate from 2 to 4 ml/min and halving the gradient time from 18 to 9 min. At this stage, the first metabolites were not eluted until 5 min had elapsed. Removal of the initial program delay of 4 min and commencing the program at 10% methanol rather than 0% resulted in a more even distribution of metabolites and an overall analysis time of 20 min as shown in Fig. 3c. Furthermore, only M and S remained to be fully resolved. Doubling the TBA concentration was found to accomplish this without affecting the resolution thus far attained. Further optimisation of the separation was achieved by adjusting the gradient time from 9 to 12 min. Following a reequilibration time of 9 min, samples could be completed in just over 20 min thereby increasing the rate of analysis by three.

Overall, the use of radially compressed columns with a larger internal diameter allows the use of higher flow-rates, which decreases overall analysis times. In so doing, however, column efficiencies can be diminished, and the ion-pair gradient system was assessed on the newly introduced radially-compressed columns with an internal diameter of 5 mm. With identical packing material, selectivities should be directly comparable. Furthermore the smaller diameter would allow a lower flow-rate in order to operate at a comparable linear velocity, thereby increasing efficiencies and sensitivity. A flow-rate of 2.4 ml/min and a reduced gradient time of 8 min was adopted, with the result shown in Fig. 4. The methanol concentra-

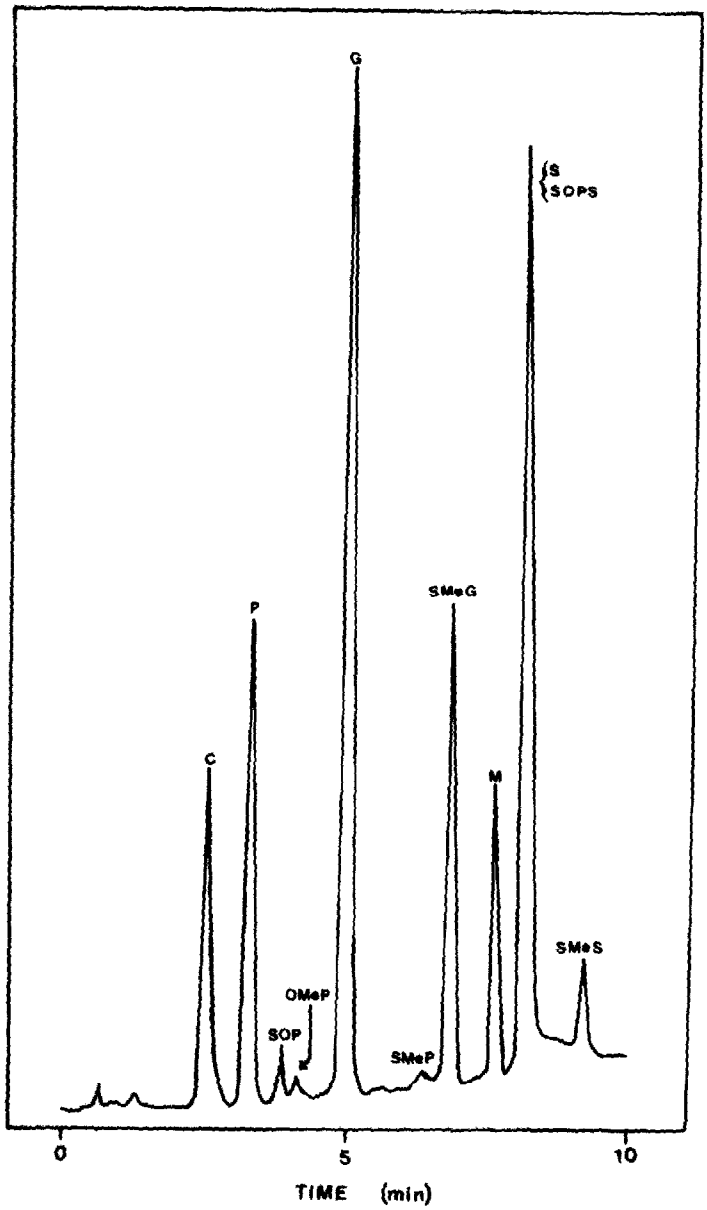

Fig. 4. Effect of decreased column diameter on the separation of urinary paracetamol metabolites. Column: Radial-Pak C_{18}, 10 cm×5 mm I.D., 10 μm particle diameter; solvent: 0.01 *M* TBA, 0.01 *M* Tris, pH 5.0 (phosphoric acid) programmed from 10 to 50% methanol over 8 min; flow-rate: 2.4 ml/min.

tion was held at 50% for 3 min followed by a reequilibration time of 3 min. The separation thus far developed was subsequently used for various metabolic studies of paracetamol and thiomethylparacetamol, and had reached a point where the metabolites eluted as three distinct groups. The unconjugated paracetamol, SOP and OMeP eluted first, followed by their respective glucuronides and thirdly the sulphates ran towards the end of the chrom-

unconjugated compounds and M eluted between the glucuronides and sulphates. Furthermore, analysis time was reduced from 22 to 15 min.

Nevertheless, the resolution of S, OMeS and SOPS had yet to be attained, and it was decided to test the effectiveness of acetonitrile as the organic solvent. Acetonitrile causes lower back-pressures and has a different selectivity to methanol through its increased solvating properties. The result of direct substitution of acetonitrile for methanol as the organic solvent is shown in Fig. 5, where SOPS was resolved from S and OMeS. Although SOPS had previously been known as a urinary metabolite of paracetamol, its detection was only possible following enzyme hydrolysis. The separation of SOPS now enabled, for the first time, direct quantitation to be made in the presence of all other metabolites. The use of acetonitrile also increased the sensitivity of the separation through a decrease in the bandwidths for all metabolites. However, all compounds had shorter retention times which caused a deterioration in resolution between the earlier eluting metabolites and necessitated further development of the system. The extent of ion-pairing is related to the degree of ionisation of the particular compound, which would also influence the respective elution characteristics of each metabolite. This suggests that variation of solvent pH may produce a more satisfactory separation and the results at pH 3, 5 and 7 are given in Fig. 6. Very poor resolution was observed at pH 3 as a number of metabolites eluted closely to one another, while separation at pH 7 also caused a slight deterioration in resolution and also caused C to elute as an asymetric peak. The most acceptable resolution of all metabolites was obtained at pH 5, with the order of elution of free phenols, glucuronides and sulphates preserved, and therefore provided the widest scope for further development.

In addition to the poor resolution between paracetamol, SOP and OMeP, and S and OMeS, the retention time of C was very close to endogenous urinary constituents at the front of the chromatogram. Reducing the flow-rate from 2.4 to 1.6 ml/min and increasing the gradient time from 8 to 12 min alleviated the latter problem and resolved OMeP, but SOP and paracetamol and S and OMeS were still unresolved. Increasing the initial concentration of acetonitrile from 10 to 15% resulted in the separation of SOP and paracetamol, but also coincided with decreased retention times with C again eluting close to early endogenous peaks. A 1-min delay followed by a linear gradient from 15 to 50% acetonitrile over 11 min was found to give optimum retentions and resolution. With a further 6 min required for column reequilibration, the analysis time was again 18 min, but a superior separation had been established, with greatly improved sensitivity and resolution.

The separation developed to this point was once again used extensively for the analysis of urine samples obtained from animals administered paracetamol. The problem of resolution between S and OMeS could be overcome by determining UV absorbance ratios. It was found, however, that as the column aged following increasing numbers of urine analyses, retention times decreased resulting in the early elution of C with urinary constituents. Furthermore, retention times of the early eluting metabolites were not reproducible on each new column installed.

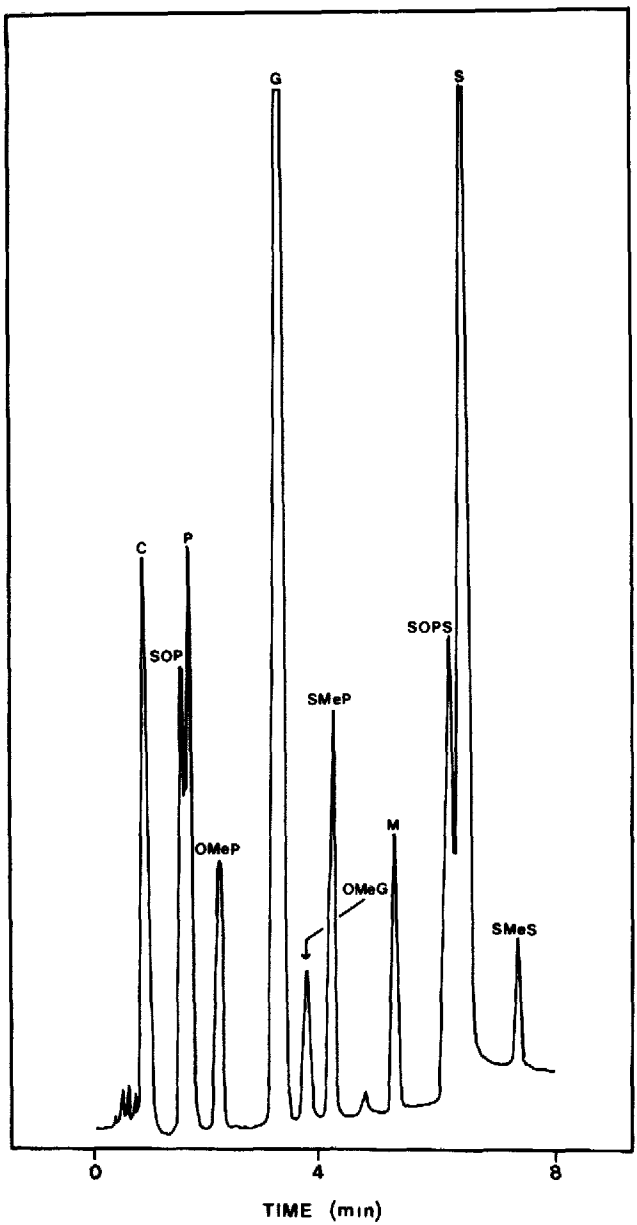

Fig. 5. Effect of acetonitrile as the organic modifier on the separation of urinary paracetamol metabolites. Chromatographic conditions as in Fig. 4 with acetonitrile as organic solvent.

Therefore, columns which caused the relatively early elution of C, SOP and paracetamol left little room for deterioration in column performance and resulted in inaccurate quantitation of low levels of C. The increasing requirement for improved resolution has led to the introduction of smaller-particle-size packings of 5 and 3 μm. Smaller particle size increases the total surface area of the chromatographic surface allowing a higher degree of mass transfer to occur, resulting in improved resolution. As previously discussed smaller particle diameter packings

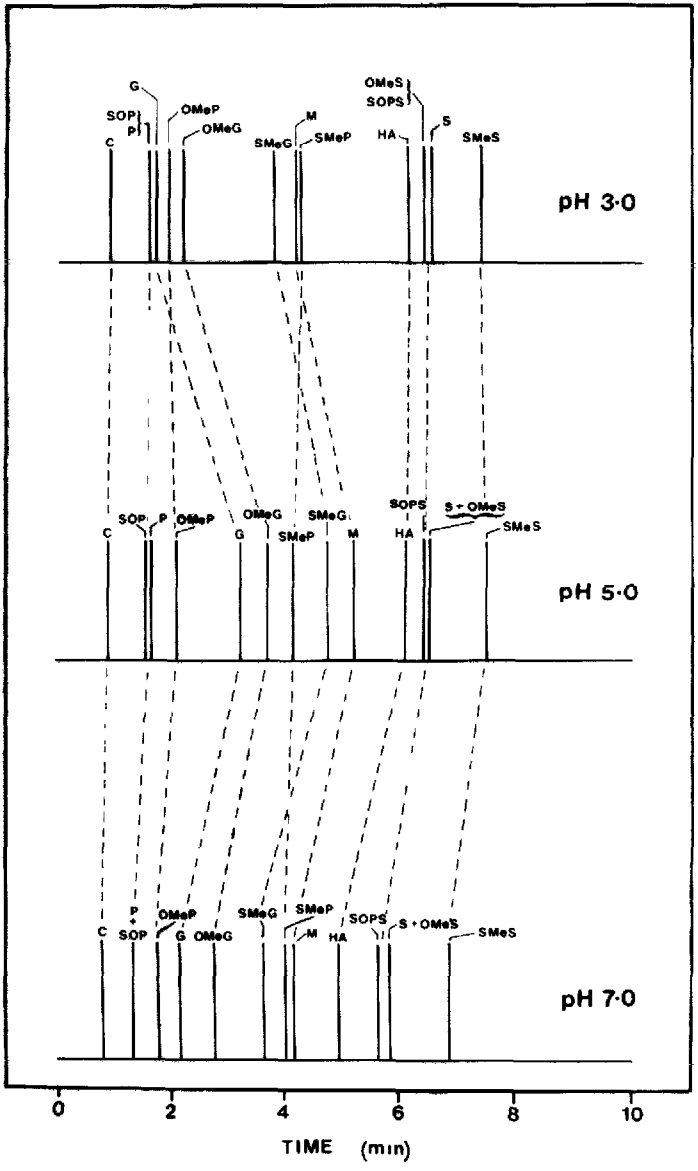

Fig. 6. Effect of pH on the separation of urinary paracetamol metabolites. Chromatographic conditions as in Fig. 5 with pH as indicated.

are more difficult to pack into reproducible columns and operate at higher back-pressures because of reduced bed permeability. It thus remained to investigate the effectiveness of the 5-μm radial compression columns as this technology enables a homogeneously packed bed to be obtained, and the 8-mm internal diameter allows the operating back-pressure to be well within the limits of the instrumentation.

The ion-pair gradient system was therefore applied to the 5-μm columns. The

only modification made in the first instance was to increase the flow-rate from 1.6 to 2.4 ml/min to compensate for the wider column bore. Retention times were similar to those observed with the 10-μm columns but peaks were much sharper resulting in improved resolution and sensitivity. S and OMeS were still unresolved, however, which may be a result of the large TBA ion masking any effects the methyl group may have on the chromatographic behaviour of the metabolite. Conversely, the TBA concentration may have been insufficient to produce respective ion pairs of different extraction constants to allow resolution by the column. Hence, halving the TBA concentration did not separate the pair and resulted in poor resolution of all metabolites, whereas doubling the concentration did indeed separate S and OMeS. The final chromatogram is shown in Fig. 7. Interestingly, S eluted after OMeS, whereas paracetamol and G eluted before OMeP and OMeG, respectively. The higher TBA concentration also increased the retention times of all metabolites. A decreased flow-rate of 2 ml/min was also used and as a result, C eluted after the urinary peaks, but each analysis could still be completed in 18 min using identical gradient conditions as previously described.

Separation of urinary metabolites of substituted paracetamols

A number of studies involving chemically modified paracetamols have been carried out [16,17] to determine the effect of blocking potential sites of metabolism in the paracetamol molecule. In the course of these studies, the analogues were administered to experimental animals at varying doses, and urine samples obtained in the usual manner. Chromatographic methods were therefore required for the analysis of the respective metabolites.

The substituted paracetamols which were studied are tabulated in Table II and exhibited varying polarities, but were all less water-soluble than paracetamol itself, as the structures in Fig. 8 indicate. A gradient elution profile of paracetamol and 2-methyl-, 3-methyl-, 3-fluoro-, 3-chloro-, 2,3-dimethyl, 3,5-dimethyl, 2,6-dimethyl and 3-thiomethylparacetamol is shown in Fig. 9. It was found that the urines obtained from animals dosed with these compounds contained glucuronide and sulphate peaks which, by comparison with the respective paracetamol metabolites, eluted later by a factor proportional to the effect of the substituent on the retention of the parent paracetamol molecule [16,17].

The most exhaustive study was carried out with 3-methylparacetamol (3MeP) [17], and provided the model for all other analogues, both metabolically and chromatographically. The detailed analysis of the metabolic pathways of 3MeP has been discussed elsewhere [17]. It has been established that the methyl analogue undergoes similar biotransformation to that observed for paracetamol, and a summary of the metabolites is given in Table III. The urine samples obtained at various stages were analysed by HPLC using the particular ion-pair gradient system in current use at the time. The elution order of the metabolites of 3MeP was found to be comparable to the respective paracetamol analogues except that 3-methylparacetamol mercapturic acid (3MeM) eluted after 3-methylparacetamol sulphate (3MeS).

Following the adoption of the 5 mm I.D. columns, the identical solvent used

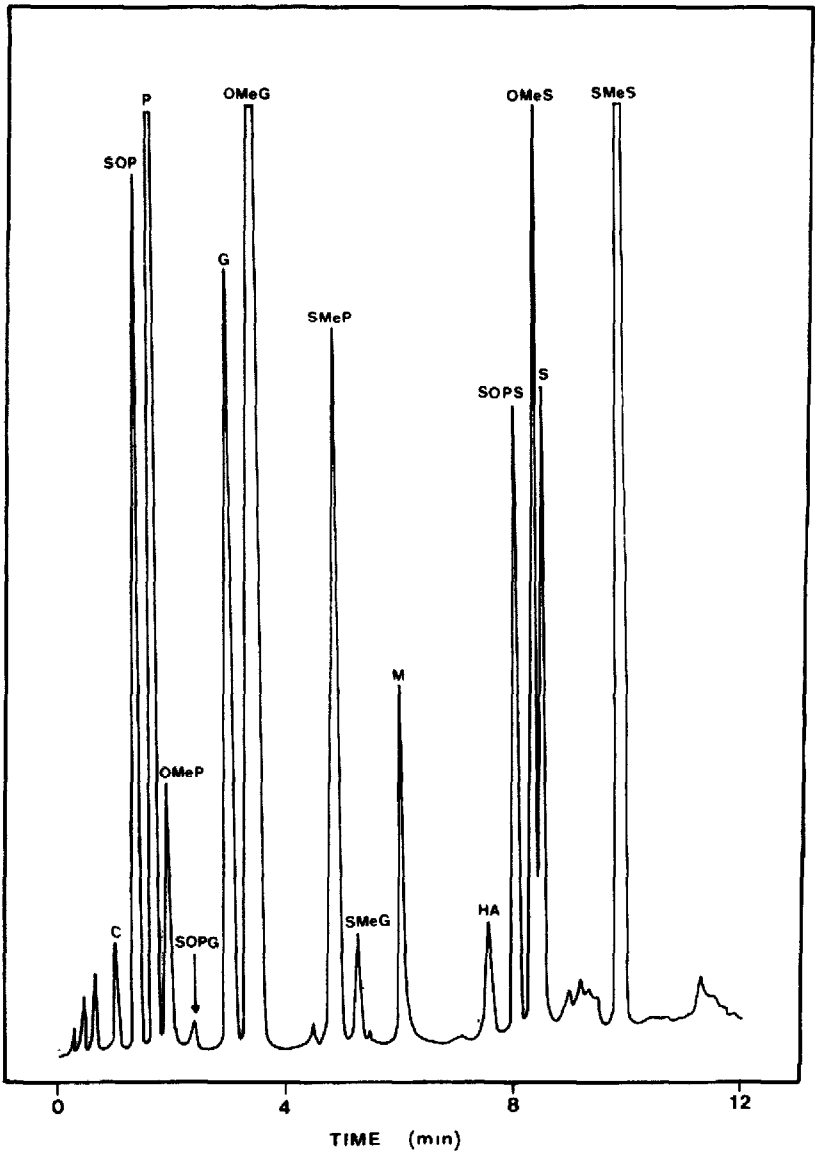

Fig. 7. Final optimisation of the gradient-programmed ion-pair separation of urinary paracetamol metabolites using a Radial-Pak C_{18} column, 10 cm × 8 mm I.D., 5 μm particle diameter. Solvent: 0.02 *M* TBA, 0.01 *M* Tris, pH 5.0 (phosphoric acid) programmed from 15 to 50% acetonitrile over 11 min after an initial delay of 1 min; flow-rate: 2 ml/min; detector: 254 nm, 1.0 a.u.f.s. See Table I and Fig. 1 for identification of metabolites.

for the separation of paracetamol metabolites caused 3MeP and 3MePG to run together. Previous experience demonstrated that varying the pH would solve this problem, and the separations at pH 3, 4 and 5 are shown in Fig. 10. The two metabolites in question were separated at pH 3, but 3-methyl-5-thiomethylparacetamol (3SMeP) eluted with HA. Optimum separation was thus obtained at

TABLE II

SUMMARY OF THE SUBSTITUTED ANALOGUES OF PARACETAMOL

Analogue	Abbreviation
Paracetamol	P
2-Methylparacetamol	2
2,6-Dimethylparacetamol	2,6
3-Fluoroparacetamol	F
3-Methylparacetamol	3
2,3-Dimethylparacetamol	2,3
3,5-Dimethylparacetamol	3,5
3-Thiomethylparacetamol	SMe
3-Chloroparacetamol	Cl

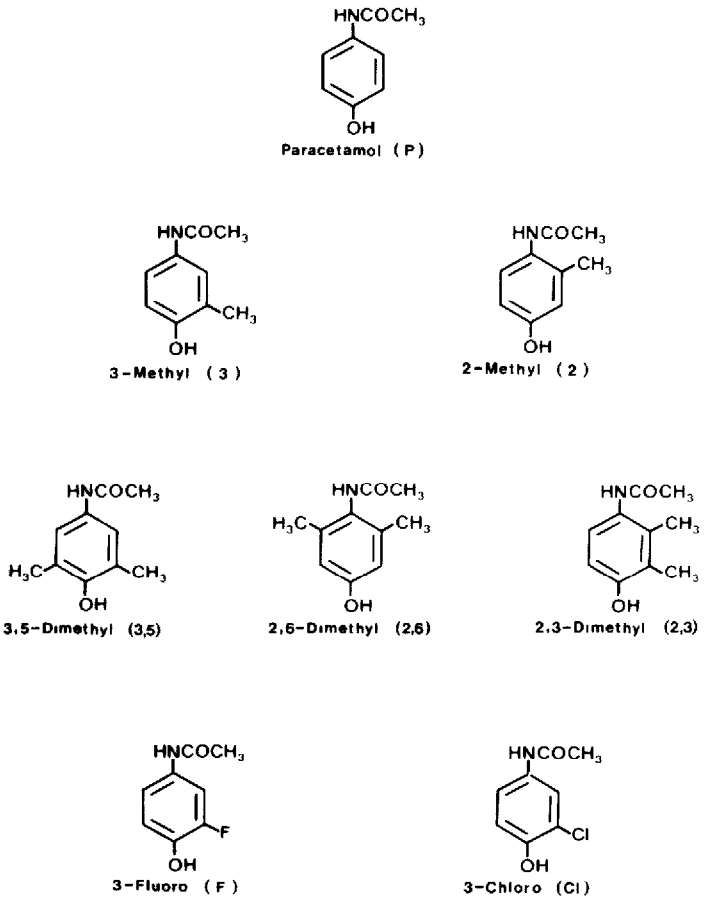

Fig. 8. Substituted analogues of paracetamol.

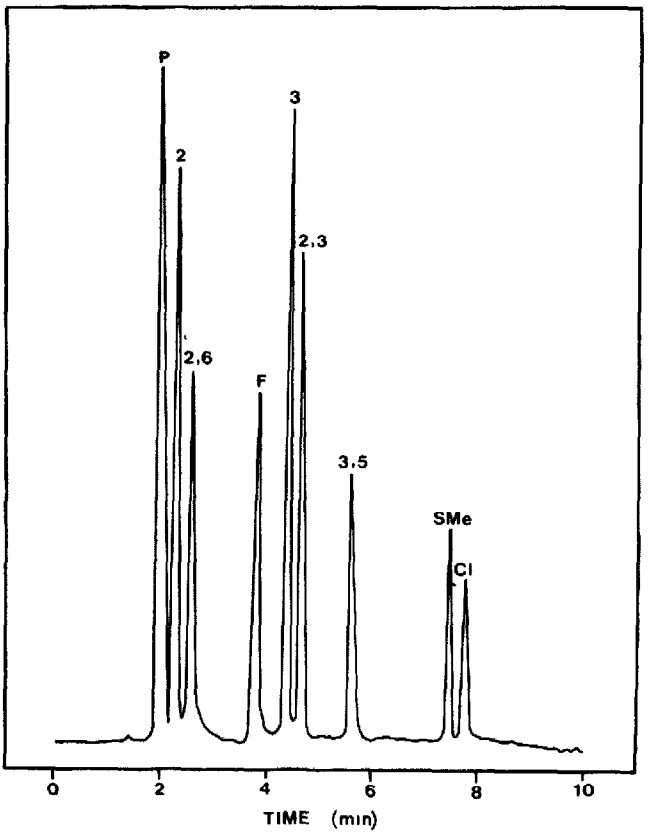

Fig. 9. Solvent-programmed ion-pair separation of paracetamol and its structural analogues. Chromatographic conditions as in Fig. 7 except gradient from 10 to 50% acetonitrile over 12 min without initial delay. See Table II for identification of analogues.

pH 4.0. The same lack of resolution between 3MeP and 3-methylparacetamol glucuronide (3MeG) was encountered following the of use the 5-μm columns. Once again, varying the pH resulted in total separation of all possible metabolites

TABLE III

SUMMARY OF THE URINARY METABOLITES OF 3-METHYLPARACETAMOL

Metabolite	Abbreviation
3-Hydroxymethylparacetamol	CH_2OH
3-Methylparacetamol cysteine conjugate	3MeC
3-Methylparacetamol	3MeP
3-Methylparacetamol glucuronide	3MeG
3-Methyl-5-thiomethylparacetamol	3SMeP
Hippuric acid	HA
3-Carboxyparacetamol	COOH
3-Methylparacetamol mercapturic acid	3MeM
3-Methylparacetamol sulphate	3MeS

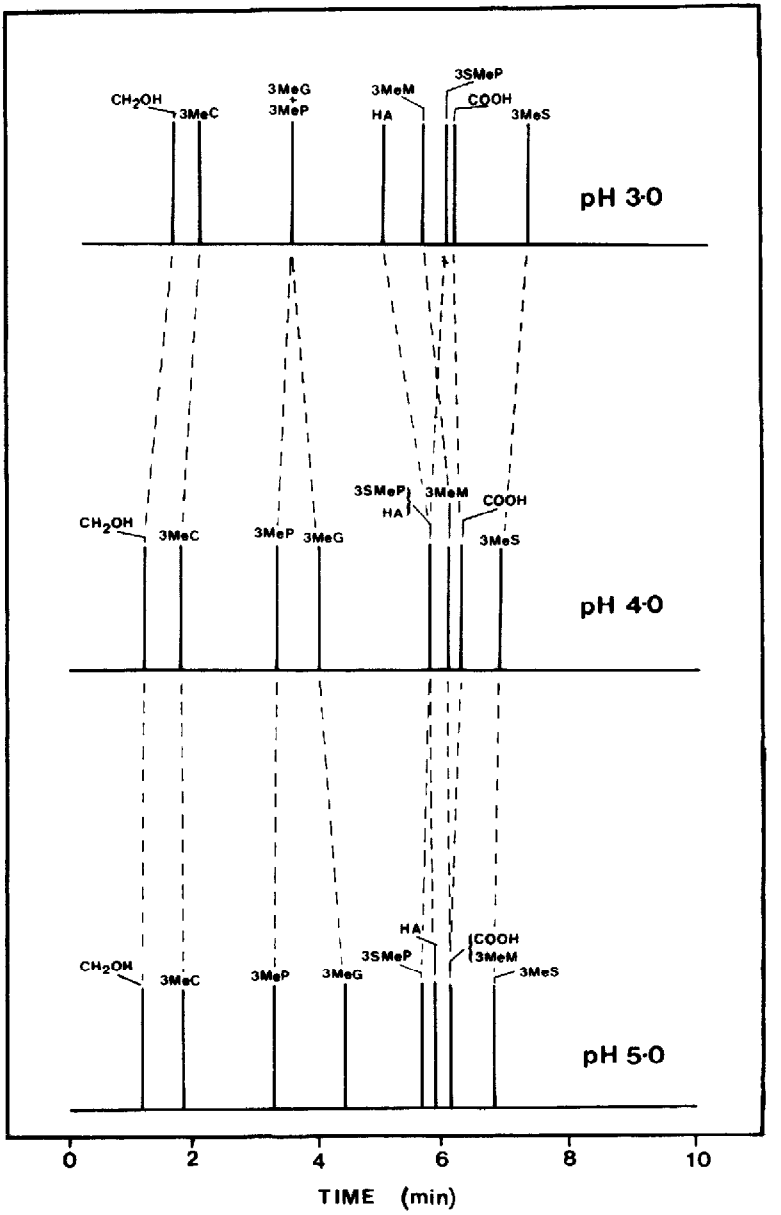

Fig. 10. Effect of pH on the separation of urinary metabolites of 3-methylparacetamol. Column: Radial-Pak C_{18}, 10 cm×5 mm I.D., 10 μm particle diameter; solvent: 0.01 *M* TBA, 0.01 *M* Tris, pH as indicated, programmed from 10 to 50% acetonitrile over 8 min; flow-rate: 2.4 ml/min; detector: 254 nm, 1.0 a.u.f.s. See Table III for identification of metabolites.

at pH 3.5 and is illustrated in Fig. 11. Similar problems of resolution associated with the urines obtained from animals dosed with 3-chloro-, 3-fluoro- and 2-methylparacetamol [17] were also solved in the same manner, and analyses of these samples were carried out at pH 4. Similarly, urines obtained from animals given each of the dimethylparacetamols were analysed at pH 5.

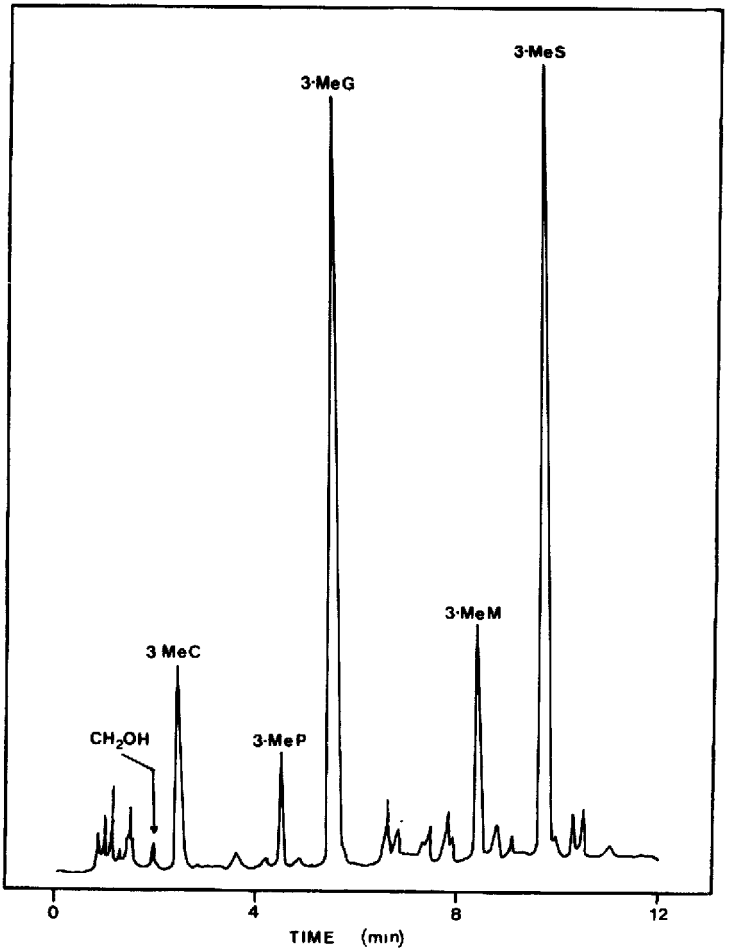

Fig. 11. Final optimisation of the gradient-programmed ion-pair separation of urinary metabolites of 3-methylparacetamol using Radial-Pak C_{18} columns (10 cm×8 mm I.D., 5 μm particle diameter). Solvent: 0.02 *M* TBA, 0.01 *M* Tris, pH 3.5 (phosphoric acid) programmed from 10 to 50% acetonitrile over 12 min; flow-rate: 2 ml/min; detector: 254 nm, 1.0 a.u.f.s. See Table II for identification of metabolites.

CONCLUSION

Comparison of Figs. 2 and 7 illustrate the marked improvement in the separation of paracetamol metabolites since those reported by earlier workers [5]. The current separation has resulted in the grouping together of the three main classes of metabolites that is, the parent compounds, the glucuronides and the sulphate conjugates, while the relative positions of the two amino acid conjugates also remain constant. This pattern of elution provides a qualitative identification of the nature of the metabolites which proved to be extremely useful in the chromatographic analysis of substituted paracetamols.

Every metabolite is not normally encountered in a particular biological sample,

but the capacity now exists to detect and directly quantify any paracetamol-derived metabolite in a single analysis. Previous to this, urine samples have had to be chromatographed in different solvent systems, or have been hydrolysed, either chemically or enzymatically, to simplify the chromatographic profile [6]. Sulphate and glucuronide conjugate levels were then determined by the increase in the amount of hydrolysis products. Following the development of the current reversed-phase HPLC system, however, the complete metabolic disposition of paracetamol can be rapidly assessed in any biological system, thereby enabling more efficient and accurate correlation with further toxicological data. The versatility of the ion-pair gradient system was also demonstrated by its use in the separation and quantitation of various substituted paracetamols and their metabolites. Detailed studies on the metabolic disposition of paracetamol and its structural analogues is therefore now greatly facilitated through the development of this universal chromatographic procedure and should provide the basis for a more rigorous approach to the investigations of paracetamol metabolism and hepatotoxicity.

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