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DETERMINATION OF PARACETAMOL AND ITS METABOLITES IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ION-PAIR SYSTEMS

JOHN H. KNOX and JADWIGA JURAND

Wolfson Liquid Chromatography Unit, Department of Chemistry, University of Edinburgh, Edinburgh (Great Britain)

SUMMARY

Paracetamol (P) and its four main metabolites, the sulphate (S), glucuronide (G), cysteine (C) and mercapturic acid (M) conjugates, are separated on ODS/TMS silica using a standard eluent, water–methanol–formic acid (86:14:0.1, v/v/v), in the order S, G, P, C, M. On addition of the ion-pairing cations dioctylammonium (DOA) and tetrabutylammonium (TBA), the retention of S is vastly increased while those of G and M are substantially increased and that of C is reduced. The excessive retention of S and to a lesser extent that of M and G can be controlled by the addition of a suitable concentration of a co-ion such as nitrate. The order of elution is then G, C, P, M, S, although this can be varied by adjusting the amount of nitrate present.

Loading of DOA is slow as it is very strongly adsorbed and typical concentrations in the eluent are below 7 mg/l. Loading by TBA is rapid with typical concentrations being around 200 mg/l. The effects of added co-ions such as nitrate can be explained in terms of simple ion-pair equilibria. Equilibration with respect to added salts is rapid.

Application of the technique to analysis of therapeutic and overdose urines shows the presence of at least three additional metabolites, one of which is identified by mass spectrometry as most probably 3-methoxyparacetamol. Another appears to be a methoxymercapturic acid derivative. There is further evidence for a group of metabolites that elute unresolved from overdose urines as a broad band after the main metabolites.

INTRODUCTION

Paracetamol is widely used as an analgesic and, being very readily available, is one of the most widely used drugs in attempted suicides. The main result of a large overdose of paracetamol is not immediate death but severe liver damage, which may result in death some days later. It will be appreciated that for this reason the problem of paracetamol poisoning and its treatment is of great clinical importance.

The present knowledge of the metabolism of paracetamol[1–3] has been reviewed by Andrews *et al.*[1] and is summarised in Fig. 1. The major metabolites of paracetamol

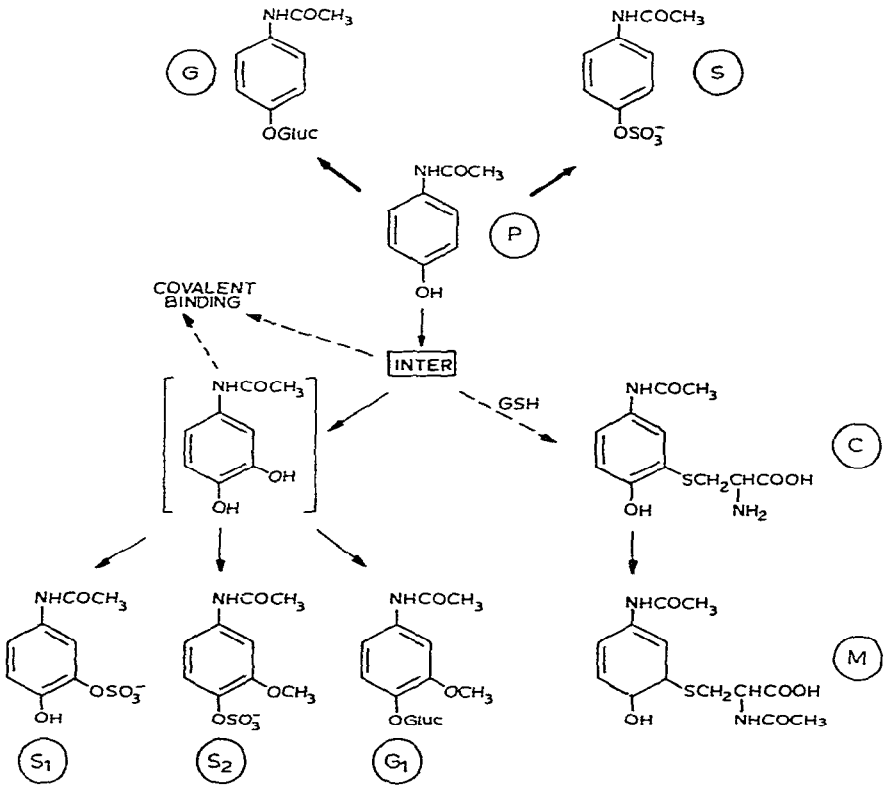

Fig. 1. Metabolism of paracetamol, after Andrews *et al.*[1]. The symbols P, S, G, C and M are used in the text to refer to paracetamol and its sulphate, glucuronide, cysteine and mercapturic acid conjugates, respectively.

(P) are the sulphate (S), the glucuronide (G), the cysteine (C) and the mercapturic acid (M) conjugates. There are several other minor metabolites in which P is substituted at the 3-position, often with a methoxy group. There are two main pathways leading to the final excretion of paracetamol. Direct conjugation to S and G accounts for about 80% of the excretion of paracetamol taken in small dosage, while oxidative pathways account for most of the remainder. Only a small proportion of the original paracetamol is excreted unchanged. The oxidative pathway is believed to involve the formation of a chemically reactive intermediate, which, under normal conditions (therapeutic dosage), rapidly complexes with reduced glutathione (GSH in Fig. 1) present in the liver cells. The GSH–paracetamol complex then degrades to C and M and is excreted. In hepatotoxic doses (suicidal overdoses), the GSH level is greatly reduced and the active intermediate can then bind covalently to hepatocyte protein, causing the liver damage which may be fatal[2,3].

Thus, when the rate of presentation of P to the liver exceeds the capacity of the liver to form the direct conjugates S and G, the oxidative path likewise becomes overloaded. C and M are formed in excess with concomitant liver damage.

Previous chromatographic studies have, until very recently, been carried out mainly by thin-layer chromatography[1,2,4]. Mrochek *et al.*[5], using high-performance

ion-exchange chromatography, found methoxy derivatives of S and G but surprisingly was unable to detect M. More recently, Howie *et al.*[6] applied high-performance liquid chromatography (HPLC) to the determination of P and its four metabolites and showed how the excretion patterns from normal and overdoses of paracetamol differed. In our own recent study[7], we were able to extend this work and to identify the metabolite M with certainty using combined HPLC and mass spectrometry. We also identified a new metabolite, 3-methoxyparacetamol, present in about the same amount as unchanged paracetamol, while an entirely new metabolite was found as an impurity in a sample of M obtained from an animal study. This compound was a homologue of M, and contained one more CH_2 group in the side-chain. Subsequent work has, however, provided no evidence that this metabolite is present in human urines taken after dosage of paracetamol. In this paper it is denoted by Z.

Evidently HPLC is a powerful but as yet little exploited method for studying the metabolism of paracetamol and of other drugs.

Evidence is now accumulating fairly rapidly that reversed-phase packing materials are particularly useful for urine analysis as urine samples can be injected directly into the chromatographic column without affecting the efficiency and general performance of the column[5–8]. In our own previous work[7,8] it was shown that fully capped or silanized silicas (subsequently called ODS/TMS silica) provide much better resolution with such samples than uncapped or partially silanized silicas (for example, ODS silica, which still contains a number of accessible silanol groups that can adsorb hydrophilic functional groups). However, conventional reversed-phase chromatography, even with ODS/TMS silicas, while excellent for the retention and resolution of the more lipophilic metabolites, is less satisfactory for the fully ionized sulphates. These elute with the solvent front and so are unresolved; they also overlap with endogenous material and so cannot be accurately quantitated. In view of the likely presence of methoxy derivatives of paracetamol sulphate that would arise from the oxidative pathway, we therefore decided to examine methods of extending the retention of the sulphates and in particular the effects of addition of ion-pairing agents such as cationic detergents and other hydrophobic cations. These would be expected to form ion pairs, which, being much less hydrophilic, would be retained by the reversed-phase column packing material[10].

The successful use of soap or ion-pair chromatography firstly requires prior equilibration of the column with the soap or ion-pairing agent. When the soap or ion-pairing agent is very lipophilic, it is strongly adsorbed by the column packing and consequently the concentration in the eluent has to be kept very low. Under these circumstances, loading and equilibration of the column may require the passage of a large volume of eluent. We therefore considered carefully the loading procedure required to produce stable elution conditions.

Ion-pair chromatography, particularly the form known as soap chromatography, has a formal resemblance to ion-exchange chromatography in that the retention of solutes is generally reduced by the addition of counter ions having the same charge as the solute ions being separated. Thus, when solute ions, S^-, are added to an eluent which contains pairing ions, P^+, and counter ions, C^-, the following equilibrium is set up:

$$PC_{org} + S^-_{aq} \rightleftharpoons PS_{org} + C^-_{aq} \qquad (1)$$

where PC_{org} and PS_{org} are ion pairs present in the organic phase and S^-_{aq} and C^-_{aq} are the solute and counter ions, respectively, present in the aqueous phase. Whether $[PC_{org}]$ is constant or not when $[P^+]$ and/or $[C^-]$ are altered depends upon the state of the following equilibrium:

$$PC_{org} \rightleftharpoons P^+_{aq} + C^-_{aq} \quad (2)$$

When P^+ is strongly adsorbed, the bonded surface layer of, say, ODS/TMS silica is readily saturated and $[PC_{org}]$ becomes more or less constant. In this instance the situation is similar to that with a fixed-site ion exchanger (*e.g.*, an ion-exchange resin) and eqn. 1 predicts that k' will be inversely proportional to $[C^-_{aq}]$. If, however, P^+ is weakly adsorbed then $[PC_{org}]$ is proportional to $[P^+]$ $[C^-]$. With $[P^+]$ constant, eqn. 2 predicts $[PC_{org}] \propto [C^-_{aq}]$ and so from eqn. 1 k' is independent of $[C^-_{aq}]$. In practice, an intermediate situation is expected to exist where, with strongly adsorbed ion-pairing agents such as soaps, k' is strongly dependent on $[C^-_{aq}]$, while with weakly adsorbed ion-pairing agents, k' should show only a weak dependence on $[C^-_{aq}]$.

In this study we have explored these relationships using a strongly adsorbed soap, dioctylamine (DOA), and a weakly adsorbed ion-pairing agent, tetrabutylammonium (TBA).

EXPERIMENTAL

The high-performance liquid chromatographs were home-assembled and used either a pneumatic intensifier pump (Haskel Engineering Co., Burbank, Calif., U.S.A.) or a reciprocating piston pump (Orlita, Giessen, G.F.R., Type DMP 1515). Detection was effected either by a variable-wavelength UV photometer (Cecil Instruments, Cambridge, Great Britain, Type CE212) or a fixed-wavelength UV photometer (DuPont Instruments, Wilmington, Del., U.S.A. Type 832). Columns and injectors were of the pattern developed by the Wolfson Liquid Chromatography Unit and Shandon Southern Products Ltd., (Runcorn, Great Britain). Columns were of length 125 mm and I.D. 5 mm, with internally polished walls.

Packing materials were prepared from 7-μm spherical silica gel made in the Wolfson Liquid Chromatography Unit and subsequently treated with octadecyltrichlorosilane to provide ODS silica. This material was capped by exhaustive silanization to provide ODS/TMS silica. The authors are indebted to Dr. A. Pryde for the preparation of these materials.

A standard mobile phase was used, consisting of water–methanol–formic acid (86:14:0.1, v/v/v) to which ion-pairing agents, in particular dioctylamine (DOA) and tetrabutylammonium hydroxide (TBAOH), were added.

For analytical purposes, 1–5-μl samples of urine were injected by microsyringe directly into the column. For mass spectral analysis (carried out on an MS902 AEI double-focusing mass spectrometer), 30-μl samples were injected. The fraction of eluate containing the metabolite to be identified was then collected and evaporated to dryness under vacuum. The residue was then shaken with 30-μl of methanol containing about 0.1 % of formic acid and the resulting solution taken up by a porous mass spectrometer probe. The excess of solvent was allowed to evaporate. Mean-

while, any remaining residue was treated with 15 μl of chloroform and the resulting solution taken up on the probe, which, after evaporation of the chloroform, was inserted into the mass spectrometer source at 200°. Mass spectra were then recorded at various temperatures up to 300°.

Reference samples of the four major paracetamol metabolites, S, G, C and M, were prepared by Dr. K. S. Andrews (Sterling Winthrop, Newcastle-upon-Tyne, Great Britain) and kindly made available to us by Dr. L. Prescott of the Edinburgh Royal Infirmary.

RESULTS AND DISCUSSION

Choice of ion-pairing agent

In a qualitative initial survey, the effects on retention of cationic and anionic detergents gave the results summarized in Table I. Both types of detergent caused slight increases in the retention of the weakly ionized solutes, G, P and M, whereas they caused opposite effects on the strongly ionized sulphate (present as an anion) and cysteine conjugates (present as a cation in acidic solution). The detergents cause strong increases in the retention of solute ions of opposite charge and decreases in the retention of solute ions of like charge. As our main object was to improve the retention of the sulphate conjugates, further work was concentrated on the effects of cationic detergents, particularly DOA and TBA.

TABLE I

EFFECT OF ION-PAIRING AGENTS ON THE RETENTION OF PARACETAMOL AND METABOLITES IN REVERSED-PHASE HPLC

on-pairing agent		*Metabolite**				
Type	*Name*	*S*	*G*	*P*	*M*	*C*
Anionic	Sodium lauryl sulphate	d	0	0	0	I
Cationic	Cetyltrimethylammonium	I	i	i	i	D
	Dioctylammonium	I	i	0	i	D
	Tetrabutylammonium	i	0	0	i	d

* i = slight increase; d = slight decrease; 0 = no clear change; I = large increase; D = large decrease.

The dramatic effect of the addition of DOA even at very low concentrations is shown by the two comparative chromatograms in Fig. 2. The addition of potassium nitrate when DOA was present was necessary in order to prevent excessive retention of S, as explained in the Introduction and described quantitatively below. Peak sharpness is similar in both chromatograms, indicating that the presence of the ion-pairing agent together with a suitable concentration of counter ion has no effect on column performance.

Column loading and equilibration

The initial survey showed that very low concentrations of detergents had a profound effect on retention, especially of S. It was observed that the retention increased more or less linearly with the amount of eluent passed. A detailed study

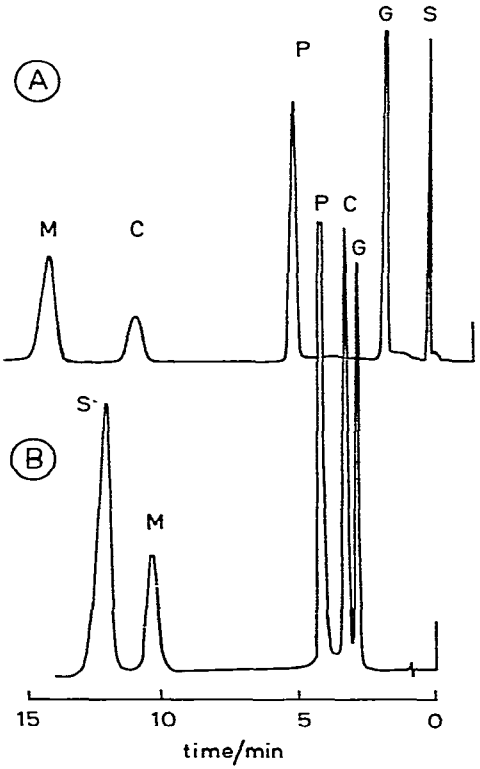

Fig. 2. (A) Separation of standard mixture of P, S, G, C and M by reversed-phase chromatography on ODS/TMS silica using water–methanol–formic acid (86:14:0.1, v/v/v) as eluent. (B) Separation by soap chromatography using same eluent containing 0.7 mg/l of dioctylamine (DOA) and 3 g/l of KNO_3. Detector: UV, 242 nm, 0.2 a.u.f.s.

of the equilibration process was therefore carried out using DOA as the ion-pairing agent. Both ODS and ODS/TMS silicas were examined and the results are presented in Fig. 3. Using ODS/TMS silica, the k' values for S, M and G increase linearly with the amount of DOA passed into the column until about 19 mg has been passed, after which k' appears to remain constant at least for G at around 20, the k' values of S and M by this time being too large to determine. The k' value for C decreases to a constant value at about the same point. With ODS silica, the process is less clear and the changes in k' for S, M and G are about one fifth as great. Furthermore, the peaks become broad and unsymmetrical when more than 7 mg of DOA has passed into the column. We believe that this poor performance of ODS silica in soap chromatography results from the presence of residual silanol groups that interact adversely with the ionized N^+ of the DOA, making the formation of ion pairs a slow process, possibly requiring the desorption of the amine and its rotation away from the surface before the N^+ can interact with a negatively charged centre.

We conclude from the loading experiments that for successful soap chromatography it is essential to use a fully silanized support. From Fig. 3A we conclude that with ODS/TMS silica all of the DOA that is passed into the column is initially adsorbed so that a front of DOA moves down the column. When the front emerges,

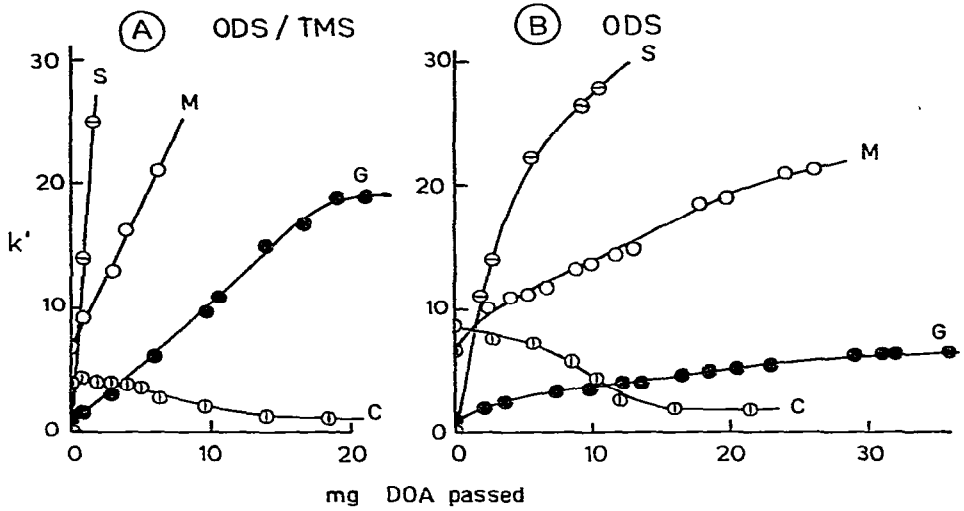

Fig. 3. Changes in k' of paracetamol and metabolites during loading of column with DOA from a solution containing 7 mg/l of DOA. Column packing: (A) ODS/TMS silica; (B) ODS silica.

the k' values become constant. On the basis of this hypothesis, we deduce that the column containing about 1.7 g of ODS/TMS silica adsorbs 19 mg of DOA when in equilibrium with eluent containing 7 mg/l of DOA. No corresponding deduction can be made for ODS silica as there appears to be no sharp transition from a period of linear increase in k' to a constant value, although the amount that must be passed to bring about equilibration is certainly not less than 20 mg.

Further evidence that a true equilibrium state is reached in the loading experiment using ODS/TMS silica is provided by Fig. 4, which shows a sequence of experiments. A column containing an untreated sample of ODS/TMS silica was first loaded by passage of about 3 l of eluent containing 7 mg/l of DOA. The k' value increased to a constant value of about 20 when 19 mg of DOA had been passed into the column. This column was then washed with progressively more dilute eluents and ultimately with eluent containing no DOA. The k' value for G then decreased to about 8. Eluent containing 7 mg/l of DOA was again passed into the column and k' increased from 8 to about 20, where it stabilized after passage of 1.8 l of eluent containing 12 mg of DOA. It can be noted from these loading experiments (a) that k' increases more or less linearly with the amount of DOA passed, (b) that when 19 mg of DOA have passed, the k' value of G has increased from 1 to 20, (c) that when sufficient DOA has been washed out of the column to reduce k' to 8, it is necessary to add only 12 mg of DOA in order to restore the k' value to 20, and (d) k' is not increased beyond 20 by passing more DOA into the column.

We can therefore conclude that k' is proportional to the weight of DOA adsorbed by the column and that with an eluent containing 7 mg/l of DOA, 19 mg of DOA are adsorbed by about 1.7 g of ODS/TMS silica.

An alternative way to bring the column to the equilibrium state is to overload the column with DOA and subsequently to elute the excess. The curve labelled "deloading" in Fig. 4 shows how k' varies subsequent to the addition of a fairly

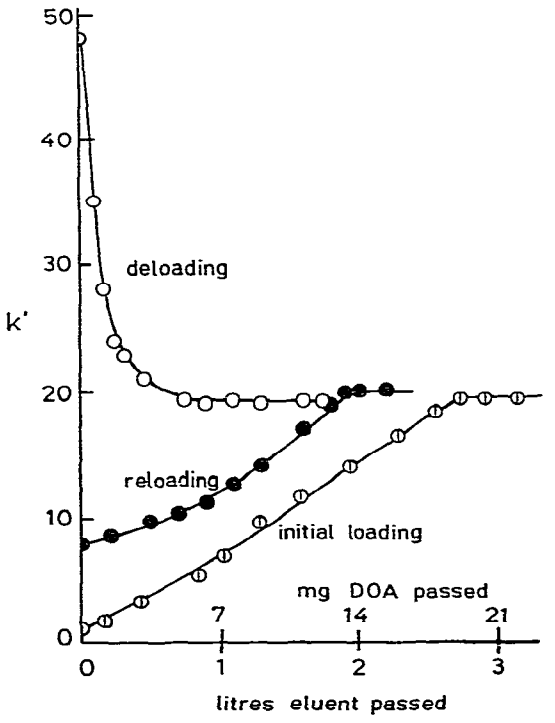

Fig. 4. Establishment of stable elution conditions by various procedures using ODS/TMS silica. Standard eluent containing 7 mg/l of DOA; solute G.

concentrated solution which contained *in toto* about 50 mg of DOA. The k' value now decreases but again stabilizes at a value around 20. It is noted that the volume of eluent that must be passed to equilibrate the column using the deloading procedure is about 800 ml compared with nearly 3000 ml when using the loading procedure. The deloading procedure is therefore to be preferred. The final value of k' is unaffected by the method used to equilibrate the column.

By carrying out the equilibration by the deloading procedure for eluents that contain different concentrations of DOA, the plot of k' *versus* concentrations of DOA in the eluent shown in Fig. 5 was obtained. This can be translated directly into an approximate adsorption isotherm if it is assumed that k' is proportional to the amount of DOA adsorbed. It may be noted that when eluent containing no DOA was passed through the column, k' decreased increasingly slowly and appeared to reach a stationary value of about 8, corresponding to about 8 mg of DOA adsorbed by the packing. Undoubtedly this does not represent a true equilibrium value; its apparent constancy is simply an indication of how strongly DOA is adsorbed by the material. This point is therefore not on the adsorption isotherm.

The loading experiments with DOA show that equilibrium at low DOA concentrations requires the passage of large volumes of eluent, particularly when the concentration of DOA in eluent is reduced, and that a deloading procedure is always preferable. This comes about because the adsorption isotherm is strongly concave to

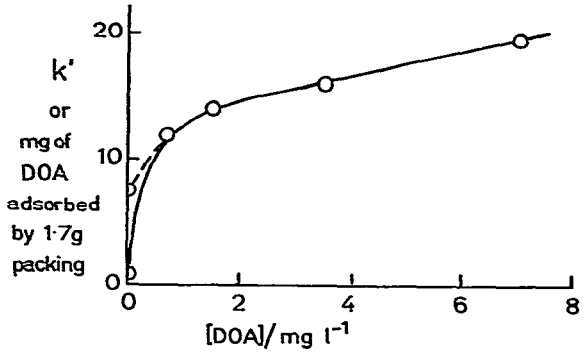

Fig. 5. Dependence of k' on concentration of DOA in eluent and corresponding adsorption isotherm for DOA (see text for explanation). Packing, ODS/TMS silica.

the concentration axis (Fig. 5). The concavity also explains why, during loading, a front of DOA appears to move gradually down the column.

If very low content of DOA is required on the column, *e.g.*, less than 8 mg under our conditions, the appropriate amount of DOA should be loaded directly from a fairly dilute solution (*e.g.*, 7 mg/l) and then eluent initially containing no DOA should be passed and recycled.

TBA, in contrast to DOA, is much less lipophilic and so is relatively weakly adsorbed. In order to obtain extended retention of the acidic conjugates S, G and M, the concentration of TBA in the eluent must be very much higher than that of DOA for corresponding retention. This is demonstrated in Fig. 6, which shows the change in k' values as eluent containing 280 mg/l of tetrabutylammonium hydroxide

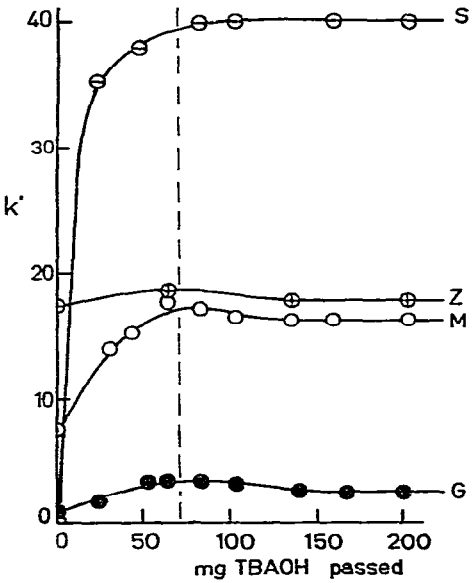

Fig 6. Change in k' during loading of column with TBA from standard eluent containing 280 mg/l of TBAOH. Packing, ODS/TMS silica.

(TBAOH) is passed into a column of ODS/TMS silica. Equilibration is clearly rapid and the column is equilibrated after 300 ml of eluent have been passed. The weak maximum in the k' values of G, M and Z is not explained. The k' values of P and C are unchanged on addition of TBA, as is the k' of Z, the homologue of the mercapturic acid conjugate. Even with TBA at 280 mg/l, the final increases in k' are 5–10 times lower than when the eluent contains 7 mg/l of DOA, indicating the much weaker bonding in the ion pairs formed by TBA. Presumably this large difference results from the shielding of the N^+ atom in the $(tert.\text{-}C_4H_9)_4N^+$ ion compared with the ready accessibility of the N^+ atom in the $(C_8H_{17})_2NH_2^+$ ion. The effect of TBA concentration on retention with equilibrated columns shown in Fig. 7 may be compared with the effect of DOA shown in Fig. 5. The curves for G and Z, the least acidic of the metabolites, show maxima while the curves for M and S become flat at the higher concentrations of TBA. Similar effects were observed by Knox and Laird[10] in their study of the retention of sulphonic acids in the presence of cetyltrimethylammonium, where the maxima were considered to result from solubilization or ion-pair formation in the eluent. Addition of TBA at concentrations higher than $3 \cdot 10^{-2}$ M caused deterioration in column efficiency, a further indication of complexities in the partitioning system.

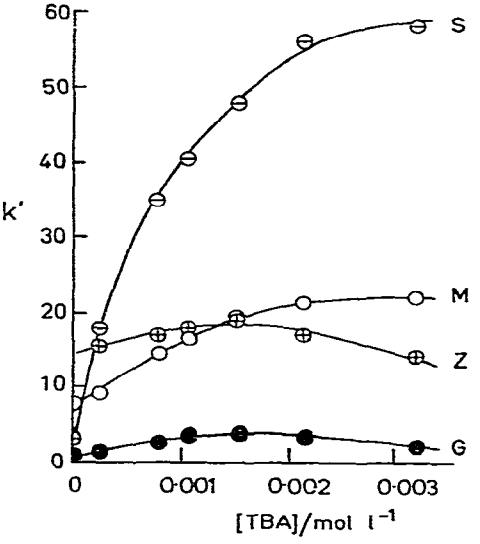

Fig. 7. Dependence of k' on concentration of TBAOH at equilibrium. For identity of Z, see text. Packing, ODS/TMS silica. Standard eluent.

Effect of addition of salt

Theory backed up by preliminary experiments showed that the excessive retention of strongly ionized solutes (*e.g.*, S), which resulted from addition of even trace amounts of DOA, could be reduced to manageable proportions by the addition of salts containing ions that would compete with the solute ions. It is clear from Fig. 3 that the retention of both S and M in the presence of eluent containing 7 mg/l of DOA is so large that soap addition alone provides no solution to the problem of assaying these metabolites, although with addition of TBA the retention of both

solutes is acceptable. However, here the peaks tend to be broad. Accordingly, the effect on retention of the addition of formate and nitrate was examined initially. As the effect of nitrate addition was found to be greater, the detailed study was restricted to the effect of addition of potassium nitrate.

Fig. 8 shows the dependence of k' on the concentration of added potassium nitrate from 10^{-3} to $6 \cdot 10^{-2}$ M; k' is plotted against $M/[KNO_3]$ in order to reveal whether the linear dependence predicted by theory is in fact obtained. The eluents contained either 0.7 mg/l of DOA or 280 mg/l of TBAOH. An important property of the retention in the presence of salts was the rapid stabilization of retention after a change in salt concentration, which contrasted with the much slower stabilization when the concentration of either DOA or (to a lesser extent) TBA was altered.

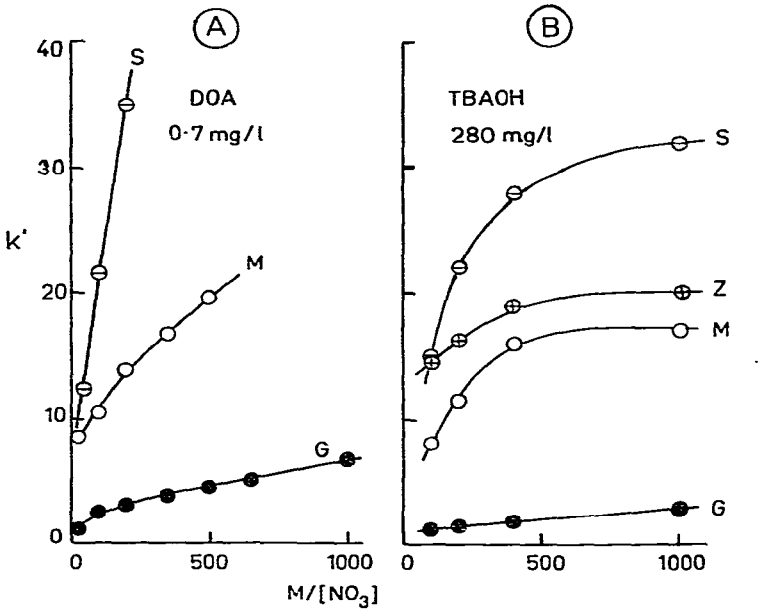

Fig. 8. Dependence of k' on concentration when KNO_3 is added to standard eluent containing (A) 0.7 mg/l of DOA and (B) 280 mg/l of TBAOH. Packing, ODS/TMS silica.

When using DOA as ion-pairing agent, k' shows a more or less linear dependence upon $M/[KNO_3]$, as predicted for a strongly adsorbed agent, whereas with TBA the dependence is non-linear, especially at low concentrations of potassium nitrate when the adsorption of the TBA is least.

It now becomes clear that in order to obtain optimal resolution of paracetamol and its metabolites, the eluent composition must be carefully adapted to the particular application under study. This adaptation should be carried out after choice of a suitable ion-pairing agent by the addition of sufficient amount of a salt to reduce k' of the most strongly affected solutes to acceptable values.

If the degree of retention of the less polar metabolites (*e.g.*, P, G) is inadequate, then the proportion of methanol in the eluent must be reduced or methanol replaced with another water-soluble material.

Our conclusions can be summarized as follows:

(1) DOA is strongly adsorbed by ODS/TMS silica from an eluent consisting of water–methanol–formic acid (86:14:0.1, v/v/v) and shows a strong ion-pairing tendency with ionized or partially ionized solutes.

(2) Column equilibration with DOA requires the passage of a large volume of eluent and is best achieved by a deloading procedure.

(3) DOA adsorbed by ODS/TMS silica behaves analogously to a fixed-site ion exchanger in respect of added salts. Indeed, it can be regarded as a dynamically renewable ion-exchange material.

(4) To obtain reasonable values of k' for M and S using DOA as pairing agent it is necessary to add a salt such as potassium nitrate. Suitable operating conditions are as follows: DOA in eluent, 0.7–7 mg/l (giving 4–10 mg of DOA adsorbed per cubic centimetre of packing); potassium nitrate concentration, $2 \cdot 10^{-3}$–$2 \cdot 10^{-2}$ M.

(5) TBA is weakly adsorbed by ODS/TMS silica and shows a weak ion-pairing tendency. Column equilibration is rapid.

(6) The mode of action of TBA is best interpreted in terms of the partitioning of solute–TBA ion pairs into the bonded layer. Addition of salt is not necessary in order to obtain reasonable k' values for S and M but can be used for fine alterations to the elution pattern. Optimal TBA concentrations are 100–200 mg/l.

(7) The chromatographic efficiency in ion-pair chromatography using DOA or TBA is similar to that in simple reversed-phase chromatography if a fully silanized packing material such as ODS/TMS silica is used.

Paracetamol and metabolites in urines

The basic study reported above indicated how the k' values of paracetamol and its four main metabolites can be adjusted. When analysing urine samples, it is necessary to make such adjustments so that the compounds of interest are not masked by endogenous compounds, and to provide optimal resolution of relevant compounds.

Use of DOA. Three examples of the use of DOA in combination with potassium nitrate are shown in Figs. 9, 10 and 11. Fig. 9 shows chromatograms obtained from urine taken after a therapeutic dose of paracetamol. The concentrations of DOA and potassium nitrate were 0.7 mg/l and 2.5 g/l, respectively. Approximately 12 mg of DOA were adsorbed by the 2 ml of column packing. Figs. 10 and 11 show chromatograms obtained from urines taken from patients who had ingested an overdose of paracetamol. Lower concentrations of both DOA and potassium nitrate were used, the column containing approximately 3.5 and 2.0 mg of DOA, respectively. It is noted that while the slightly retained components C, G and P are not particularly well resolved, the selectivity of separation of the more strongly retained compounds M and S and related metabolites is generally excellent but appears to be optimal when the intermediate conditions are used (*i.e.*, those in Fig. 10). We have also noted that different types of ODS/TMS silica can exhibit substantially different retention behaviour.

Figs. 9–11 show the presence of several new metabolites, marked A, B and D. Metabolite A is thought to be methoxyparacetamol, which was previously found and identified using simple reversed-phase HPLC[7]. Metabolite B has not been identified. Metabolite D was collected and was initially thought to be one of the methoxy sulphates shown in Andrews *et al.*'s scheme (Fig. 1). However, mass spectrometry

showed ions at *m/e* values of 44, 86, 129, 139, 151, 181, (183), 197 and 312. No ions were found at *m/e* 80 and 81, characteristic of S. The ions at *m/e* 44 and 151 are typical of all of the paracetamol metabolites. The ions at *m/e* 129, 197 and 312 are characteristic of M, while that at *m/e* 197 is also characteristic of Z. The ions at *m/e* 139 and 181 are characteristic of methoxyparacetamol. It therefore seems that compound D is a paracetamol metabolite containing both mercapturic acid and methoxy groups. If this is correct, and if D is a single substance, as seems most likely, then approximately one third of the total mercapturic acid conjugates is present as a methoxy derivative.

Fig. 10 also shows a chromatogram of the standard sample of the mercapturic acid conjugate. In a previous paper[7], we showed that this material contained about 20% of an impurity Z, which was identified by mass spectrometry as a homologue of M containing an additional CH_2 group in the side-chain. An additional peak is also present in the chromatogram of the standard obtained with added DOA. This peak has again been identified by mass spectroscopy as the homologue of M. Compound Z does not appear to be found in more than trace amounts in human overdose cases.

Use of TBA. The separation of metabolites from an overdose urine with addition of TBA is shown in Fig. 12. The pattern is similar to that found with DOA, namely increased retention of S and M, decreased retention of C and the presence of peaks due to new metabolites. The labels A, B and D have been assigned, corresponding to the similarly labelled peaks in Figs. 10 and 11. When TBA is present, however, mass spectroscopic analysis proved unsuccessful owing to the large amount

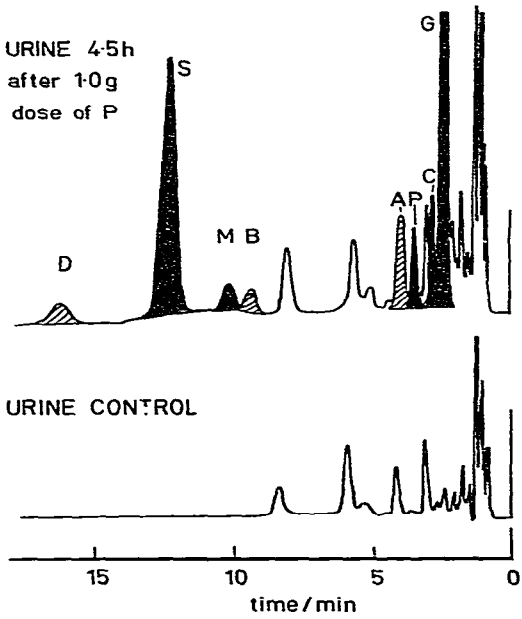

Fig. 9. Chromatogram of human urine taken 4.5 h after therapeutic dosage (1 g) of paracetamol. Standard eluent contained 0.7 mg/l of DOA and 2.5 g/l of KNO_3. Packing, ODS/TMS silica. Injection, 2 μl of urine. Detector: UV, 249 nm, 0.1 a.u.f.s. Major metabolites shown blacked out; additional metabolites shaded. Lower chromatogram is from a urine sample taken before dosage.

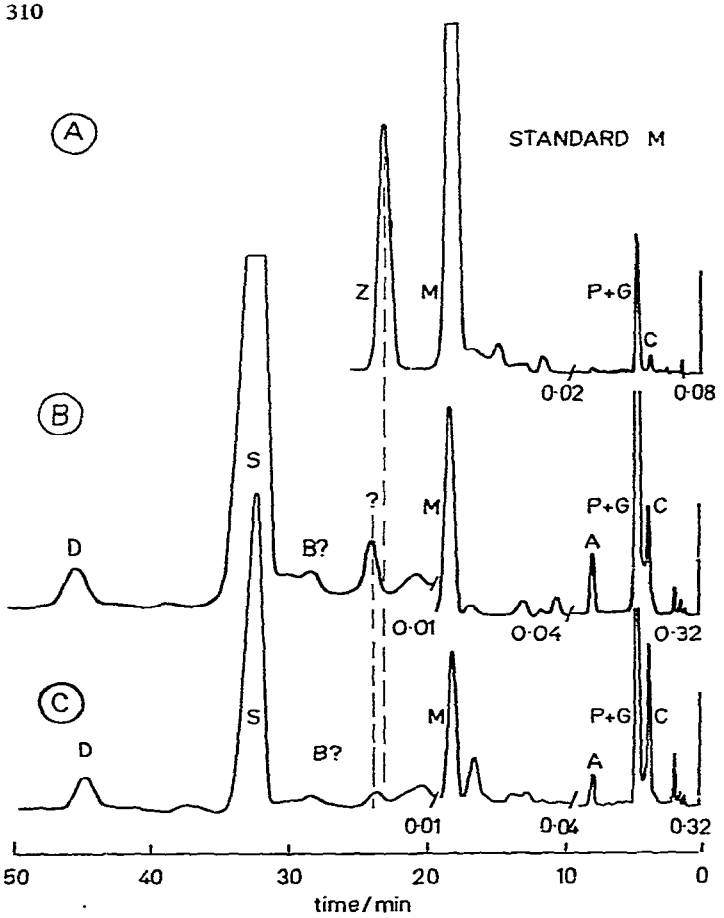

Fig. 10. Chromatograms of standard mercapturic acid (A) and of human urines (B) and (C) taken after severe overdoses of paracetamol. Standard eluent containing 400 mg/l of KNO_3 equilibrated by recycle with column containing 3 mg of DOA on 2 ml of ODS/TMS silica. Detector: UV, 254 nm, sensitivity as indicated. (B) Patient treated with cysteamine. (C) Patient treated with methionine. Sensitivity as shown on chromatograms.

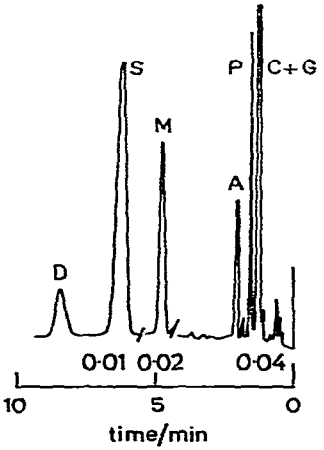

Fig. 11. As Fig. 10 but eluent contained 200 mg/l of KNO_3 and column contained 2 mg of DOA. Detector: 254 nm. Sensitivity as shown on chromatogram.

of pairing agent present in the eluent. For mass spectrometric identification DOA is to be preferred as pairing agent because of the very low concentration required in the eluent.

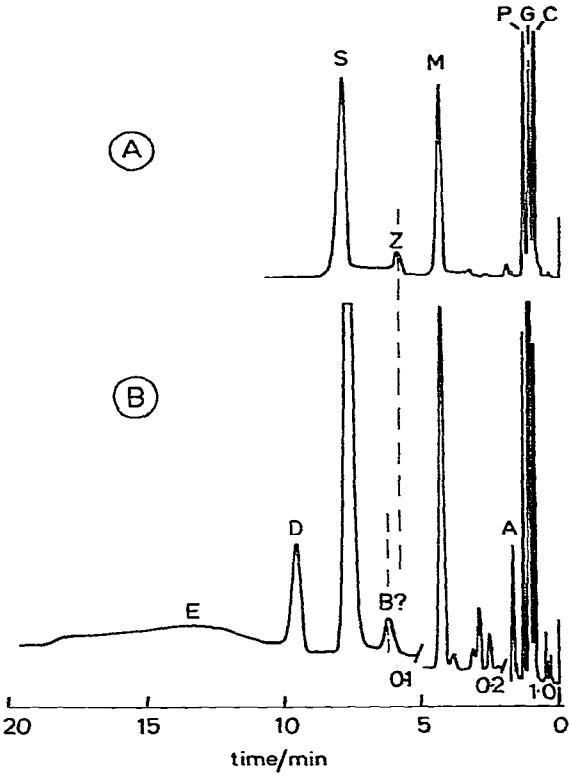

Fig. 12. Chromatogram of standards (A) and human urine (B) taken after severe overdose of paracetamol. Packing, ODS/TMS silica. Standard eluent contained 140 mg/l of TBAOH and 550 mg/l of KNO_3. Detector: UV, 242 nm, sensitivity as marked. Note additional metabolites and presence of low wide peak after peak D.

Fig. 12 illustrates a further feature of the analysis of overdose urines. When the TBA concentration exceeds about 280 mg/l, a large asymmetric peak is eluted shortly after M. This peak obscures S and the minor metabolites B and D. Elution of this material was encouraged by increasing the acidity (by adding formic acid) and by adding salt. By using lower concentrations of TBA, this peak (E) could be eluted at the end of the series. With successive injections of urine, the material E became more strongly retained and the top of the column became contaminated. Replacement of a few millimetres of packing restored the chromatogram to its previous appearance. The presence of a large asymmetric peak was reported in our previous study[7] using a simple reversed-phase system. Its mass spectrum indicated the presence of fragments corresponding to paracetamol itself, the mercapturic acid side-chain and methoxyparacetamol. We believe that peak E now reported using ion-pair chromatography may arise from the same group of compounds, although firm evidence of this is lacking.

Conclusions from urine analyses

Applications of the methods developed for the standard metabolites when applied to therapeutic and overdose urines show that either DOA or TBA can be used to great effect. Both produce elegant chromatograms that are similar in general appearance. TBA can be used in combination with added potassium nitrate over a concentration range of 100–200 mg/l while DOA must be used at exceedingly low concentrations. In general, TBA is the simpler to use but has the disadvantage that its concentration in the eluent makes it very difficult to identify new metabolites by mass spectrometry. Here DOA is to be preferred as its concentration in the eluent is negligible.

The study has shown that there are several new metabolites of paracetamol, of which only a few have been identified. Much work is still necessary before the full picture of the metabolism will be understood, especially in overdose cases. So far we have little evidence for any large amounts of metabolites that could be identified as the methoxy sulphates, S_1 and S_2, or the methoxy glucuronide, G_1, of the scheme of Andrews *et al.*, but the chromatograms are sufficiently complex that it is not possible at this early stage to be sure that these compounds are not present as minor metabolites.

In the future, we expect that HPLC will be able to make an important contribution to this field and it may be that major new insight will be obtained when the identity of the compound or group of compounds constituting the final wide peak is established.

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