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DETERMINATION OF PARACETAMOL AND ITS METABOLITES IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING REVERSED-PHASE BONDED SUPPORTS

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SUMMARY

Paracetamol and its four main metabolites, the sulphate (S), glucuronide (G), cysteine (C) and mercapturic acid (M) conjugates, are readily separated in a synthetic mixture using slightly acidic aqueous alcoholic eluents (*e.g.* water–methanol–formic acid, 85:15:0.15, v/v/v) on either octadecyl silica (ODS silica) or octadecyl silica which has been further silanized to remove residual hydroxyl groups (ODS/TMS silica). The dependences of k' upon alcohol, acid and added salt concentrations are reported for both materials. The latter material gives the higher plate efficiencies and is much superior when applied to analysis of urines taken after therapeutic doses and overdoses of paracetamol.

At least four additional metabolites are reported in overdose urines. Mass spectrometric analysis (high and low resolution) has confirmed the identity of M and identified one of the additional metabolites as methoxyparacetamol. Mass spectra of the remaining additional metabolites enable major structural features to be deduced. One of these metabolites may be associated with liver damage.

INTRODUCTION

There is a growing interest in clinical studies of the metabolism of paracetamol, for the drug is readily available and is often misguidedly taken in overdose for suicidal purposes. Evidence from studies using thin-layer chromatography[1–3], conventional ion-exchange[4] and molecular sieve chromatography[5], and high-performance liquid chromatography (HPLC)[6–8] indicates the presence of at least four major metabolites whose formulae are given in Table I, although there is still conflicting opinion as to the identity of at least one of the metabolites, Mrochek *et al.*[6] suggesting that the mercapturic acid conjugate might in fact be a double cysteine–glucuronide conjugate. Other minor metabolites may well be present[2,3]. In the most recent study by HPLC[8] the excretion pattern of paracetamol and its four metabolites was detailed, and it was shown that virtually all the paracetamol from a therapeutic dose was excreted in 24 h.

While apparently safe in therapeutic dosage, paracetamol in overdose can

cause serious liver damage, which in severe cases can result in death. Detailed studies of its metabolism are therefore necessary in order to assess and assist in the development of the best methods of treatment. The importance of this problem, brought to our attention by Dr. L. Prescott of the Royal Infirmary, Edinburgh, coupled with the availability of new HPLC packing materials[9] and our recent experience in the use of additives in reversed-phase HPLC[10,11] prompted us to carry out a detailed study of the optimum mode of separation of paracetamol and its metabolites in synthetic mixtures and in urine, where numerous endogenous compounds would be expected to interfere.

In particular, the present study provides evidence for the superiority of octadecyl bonded silicas whose residual silanol groups have been extensively substituted by trimethylsilyl groups (ODS/TMS silica) over the more conventional ODS silicas, which have not been "capped" and possess a substantial fraction of unreacted silanol groups[9]. Data are also presented which indicate how the retention patterns of paracetamol and its metabolites may be altered by changing the composition of the eluent and by means of inorganic salts. In a forthcoming paper the effects of added detergents will be described[12]. Throughout the work identification of metabolites has been a major problem, but where possible solute peaks have been trapped out and the metabolite of interest has been submitted to mass spectroscopy. In this way additional metabolites have been detected in overdose urines. The identity of the material thought to be the mercapturic acid conjugate has now been fully confirmed. The study as a whole indicates that the problem of paracetamol metabolism is complex and that considerable further work will be required to elucidate the details.

EXPERIMENTAL —HPLC STUDIES

The high-performance liquid chromatograph consisted of the high-pressure gas-driven intensifier pump of a Du Pont 830 liquid chromatograph and a Cecil 212 UV spectrophotometer as detector. Columns and the septum injector were made according to designs developed by the Wolfson Liquid Chromatography Unit (WLCU) and Shandon Southern Products (Runcorn, Great Britain). Columns were 125 mm in length and 5 or 7 mm in bore. They were made of stainless steel, internally polished, and were terminated at the lower end by wire woven stainless-steel cloth, Type 325/2500 (Sankey Green Wire Weaving Co., Warrington, Great Britain) which will retain particles of 2 μm and above. Columns were operated at ambient temperature and injection volumes were in the range 1 to 30 μl.

Packing materials were based upon a 6-μm spherical silica gel developed in the WLCU (surface area 200 $m^2 \cdot g^{-1}$, pore diameter 10 nm), from which an ODS material was made by exhaustive reaction with octadecyltrichlorosilane. After hydrolysis this material, referred to below as ODS silica, gave identical results to Spherisorb ODS (Phase Separations, Queensferry, Great Britain). The ODS silica made in the WLCU contained a substantial number of silanol groups which had a significant adsorptive capacity for hydrophilic groups. These were effectively removed by exhaustive silanization to give what is designated below as ODS/TMS silica. This material is similar in chromatographic properties to ODS-Hypersil (Shandon Southern Products).

Mobile phases comprised methanol–water or isopropanol–water mixtures

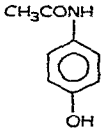
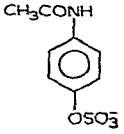
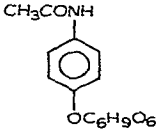
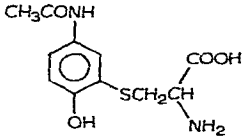
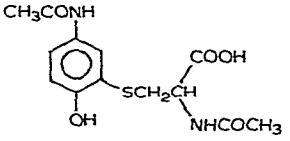
containing small proportions of formic or acetic acid with the further addition, in some cases, of inorganic salts.

Details of the formulae and sources of the paracetamol metabolites used as standards are shown in Table I.

TABLE I

REFERENCE COMPOUNDS AND SOURCES

Details of sources: RSA: Dr. R. S. Andrews, Sterling Winthrop & Co. Ltd., Research and Development Division, Newcastle upon Tyne, Great Britain; BR: Dr. B. Reynolds, McNeil Laboratories, Fort Washington, Pa., U.S.A.

Name	*Short symbol*	*Formula*	*Source*	
			Natural	*Synthetic*
Paracetamol	P			
Paracetamol sulphate	S		RSA	
Paracetamol glucuronide	G		RSA	
Paracetamol cysteine	C		RSA	BR
Paracetamol mercapturic acid	M		RSA	BR

RESULTS AND DISCUSSION

Choice of packing material

Paracetamol (P) and its four main metabolites, the sulphate (S), glucuronide (G), cysteine (C) and mercapturic acid (M) conjugates, are fairly easily separated by HPLC on short- or long-chain hydrocarbon bonded silicas with a variety of hydrophilic solvents in either acid or alkaline solution. Acidic solvents are, however, to be

preferred since hydrolysis of the conjugates is thereby avoided, and since the cysteine conjugate is rather strongly held on uncapped materials (*e.g.* ODS silica).

The exact choice of packing material and eluent becomes much more critical when the separation of these compounds from urine is required using direct injection of urine since numerous endogenous materials can then interfere. In these circumstances the highest resolution is required along with the earliest possible elution of endogenous materials. Because packing materials possessing longer hydrocarbon groups (for example, C_{18} rather than C_8 or C_2) bear a heavier loading of organic material and so give longer retention of metabolite under similar conditions, it was decided at an early stage to use octadecyl materials in preference to the shorter-chain materials available.

Comparative experiments with ODS and ODS/TMS silicas revealed substantial differences in retention times, order of elution and efficiency of separation. Two typical chromatograms are shown in Fig. 1. Strikingly the cysteine metabolite is more strongly retained than the mercapturate with ODS silica and less strongly retained with ODS/TMS silica. With the former it also gives a very much wider peak. This difference is thought to be due to the interaction of the basic amino group of C with the acidic silanol groups of the uncapped ODS silica. These differences could, however, arise from there being different optimal elution conditions for the two packing materials, and accordingly a more detailed study of the effects of composition on both retention and plate efficiency was undertaken.

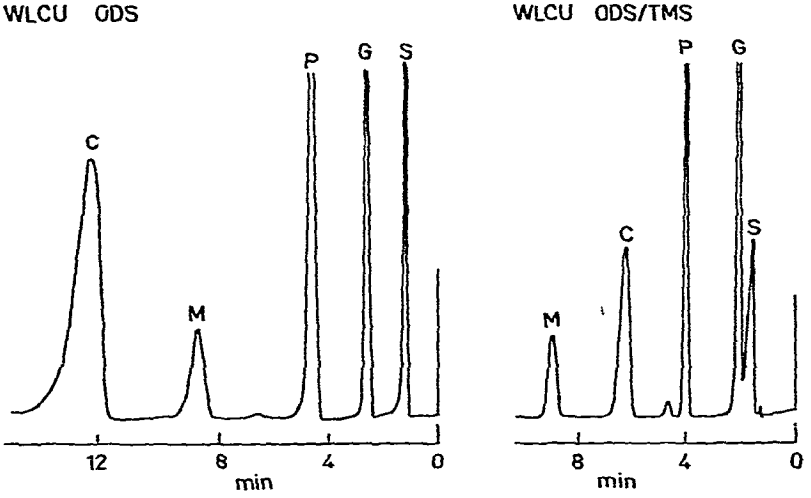


Fig. 1. Comparative chromatograms of reference compounds using ODS/TMS silica (WLCU) and ODS silica (WLCU). Eluent: water–methanol–formic acid (86:14:0.1, v/v/v). Detector: UV, 254 nm, 0.1 a.u.f.s.

Effect of alcohol content

The effect on the column capacity ratio, k', for paracetamol and its metabolites resulting from change in the water to alcohol ratio is shown in Fig. 2 for methanol–water mixtures containing 0.1% of formic acid.

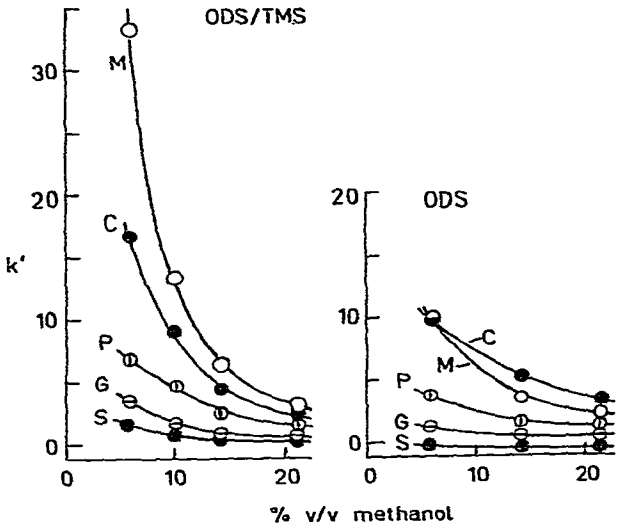


Fig. 2. Effect of methanol concentration on k' for ODS/TMS and ODS silicas (WLCU). Eluent: aqueous methanol containing 0.1% (v/v) formic acid.

k' values are similar at high alcohol contents but higher with the ODS/TMS silica at low alcohol contents. The opposite order of elution of C and M on the two materials is confirmed and applies at methanol concentrations above 6.5%, at which concentration both C and M have the same retention time on ODS silica. At lower concentration of methanol, the order of elution of C and M is probably the same but the peak for C on ODS-silica is now very wide and asymmetric. The significantly higher retention of the solutes on ODS/TMS silica at low alcohol contents is difficult to explain since the carbon content of this material is only about 20% greater than that of the ODS silica from which it is made. It would appear that the silanol groups present in ODS silica being strongly hydrophilic may make the surface layer less attractive to the lipophilic groups in the solutes.

With the ODS/TMS material the resolution of the different solutes is significantly improved by working at lower alcohol content, but with the ODS material there is an optimum methanol concentration of about 14% which gives the best selectivity, although this is never as good as that obtainable with the ODS/TMS material.

Comparative chromatograms for the two materials using the same eluent have been given for synthetic mixtures in Fig. 1. Fig. 3 shows comparative chromatograms for control urine samples on the two phases. Again these show the marked superiority of the ODS/TMS material in that much better resolution of endogenous components is achieved. This evident qualitative superiority is confirmed quantitatively by the plate heights for the various solutes under different conditions listed in Table II. Under virtually all conditions higher plate numbers are obtained with the ODS/TMS material than with the ODS material. Improvement in plate count for the ODS material can, however, be obtained by the addition of low percentages of intermediate polarity solvents such as ethyl acetate. This has little effect on the plate count of the more efficient ODS/TMS material, as shown in Table III.

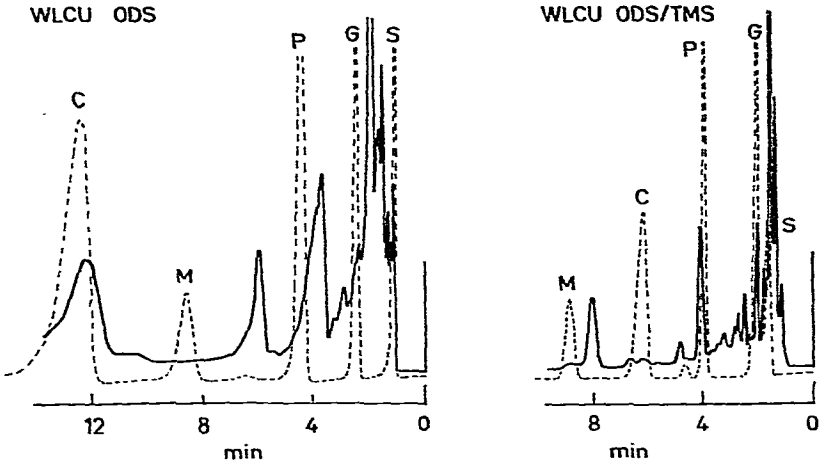


Fig. 3. Comparative chromatograms of normal urines using ODS/TMS and ODS silicas (WLCU). Eluent: water–methanol–formic acid (86:14:0.1, v/v/v). Broken lines show chromatograms of standards as given in Fig. 2.

TABLE II

EFFECT OF METHANOL CONTENT IN MOBILE PHASE ON THE NUMBER OF THEORETICAL PLATES IN THE SEPARATION OF PARACETAMOL METABOLITES ON ODS AND ODS/TMS COLUMNS

Column	*% of methanol in the solution of 0.1% formic acid*	*S*	*G*	*P*	*M*	*C*
ODS	6.5	600	400	1360	200	200
	14.0	720	1660	1460	1230	920
	21.0	3350	1950	2420	3160	1070
ODS/TMS	6.5	400	1430	2400	1340	200
	14.0	330	3200	4150	4200	1300
	21.0	570	3600	3600	4200	2400

TABLE III

EFFECT OF ADDITION OF SMALL QUANTITIES OF ETHYL ACETATE TO THE MOBILE PHASE ON THE NUMBER OF THEORETICAL PLATES FOR PARACETAMOL AND ITS METABOLITES

A: Before addition; B: after addition of 0.4% ethyl acetate. Mobile phase: water–methanol–formic acid (85.9:14:0.1, v/v/v).

Packing		*S*	*G*	*P*	*M*	*C*
ODS	(A)	720	1660	1460	1230	920
	(B)	3000	4500	2900	3160	740
ODS/TMS	(A)	330	3200	6400	4200	1300
	(B)	350	4700	5500	3900	1200

Effect of acid content

In the absence of acid the retention of paracetamol metabolites is slight and peaks are poorly shaped. Low concentrations of acid substantially increase retention, but as shown in Fig. 4 there appears to be a maximum in k' as the concentration of formic or acetic acid increases. This occurs with both supports, but the maxima appear at higher concentrations with ODS silica than with ODS/TMS silica. For formic acid the maximum k' occurs at below 0.05% (v/v) with ODS/TMS silica and at about 0.15% with ODS silica. For acetic acid the maxima occur below 0.5% with the former and at about 1.0% with the latter. In the range 0.1 to 0.3% of formic acid with ODS/TMS silica k' is essentially independent of acid concentration. Table IV shows that formic acid may give slightly improved efficiency over acetic acid and confirms the generally better performance of the ODS/TMS material.

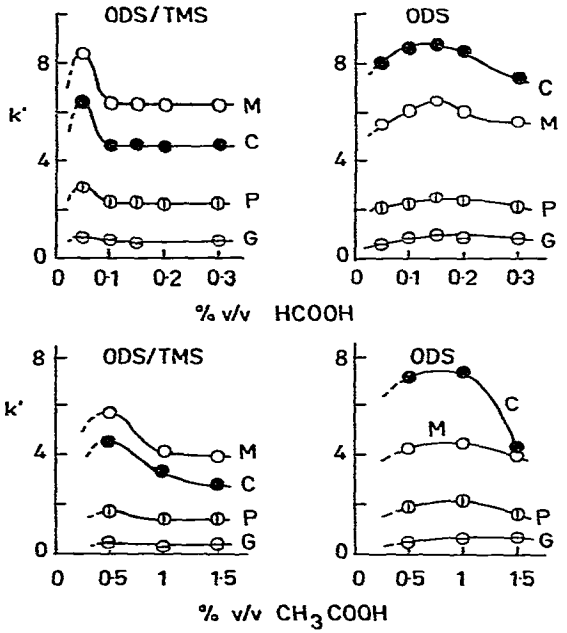


Fig. 4. Effect of acid concentration on k' for ODS/TMS and ODS silicas (WLCU). Eluent: water–methanol (86–14, v/v).

Relative performance of ODS and ODS/TMS materials

The data presented above indicate without doubt that the completely blocked reversed-phase material ODS/TMS silica performs better in terms of plate efficiency than the unblocked material ODS silica. This is most probably due to the tendency for the ODS material to retain solutes by a dual mechanism involving both the octadecyl groups and the silanol groups. The blocked material has the following main advantages in the present analysis: (1) It provides a higher degree of selectivity for paracetamol and its metabolites. (2) It can be operated with high efficiency at lower alcohol content and under these conditions the greatest selectivity can be obtained.

TABLE IV

EFFECT OF THE ACIDITY OF THE MOBILE PHASE ON THE NUMBER OF THEORETICAL PLATES IN THE SEPARATION OF PARACETAMOL AND ITS METABOLITES ON ODS AND ODS/TMS SILICA

Column	*% of acid in the 14% methanol–water eluent*	*N*				
		S	*G*	*P*	*M*	*C*
A. Formic acid						
ODS	0.05	550	1000	700	650	450
	0.10	700	1700	1500	1200	900
	0.20	700	1600	1600	1000	600
ODS/TMS	0.05	1600	1700	1600	1100	800
	0.10	300	3200	6400	4200	1300
	0.20	400	1900	3100	3000	1900
	0.30	1600	1600	4500	2600	1500
B. Acetic acid						
ODS	0.5	1100	1600	2900	1800	1300
	1.0	800	2000	3100	1200	2400
	1.5	900	1600	1600	900	900
ODS/TMS	0.5	1600	1900	2500	4500	1700
	1.0	700	1900	4300	2700	800
	1.5	1200	1600	3000	2600	500

(3) In the analysis of urine samples the higher plate efficiencies obtainable with the ODS/TMS material enables metabolites to be much more cleanly resolved from endogenous materials.

A general weakness of the methods so far described is that the strongly ionized sulphate is very weakly retained on both materials (indeed, it appears to be partially excluded by the ODS material). For a detailed study of the sulphate metabolite there is a need to increase its retention. As shown in the next section, this can be achieved by the addition of salts to the eluent.

Effect of salt additions

The effect on retention of the addition of various salts to the eluent is shown in Table V. The major features are as follows. The addition of more or less of any salt increases the retention of the sulphate conjugate substantially. For example, when using ODS/TMS silica and an eluent containing 10% methanol and 0.1% formic acid, the addition of KH_2PO_4 at a concentration of 0.1 *M* increases the k' of S from zero to 0.8. It is then eluted after the glucuronide. The retention of the cysteine conjugate on the other hand is reduced especially when ODS silica is used. This is perhaps not unexpected since the cysteine conjugate appears to be retained on ODS silica mainly by interaction with the silanol groups. This presumably occurs through affinity of the $-NH_3^+$ group with the –SiOH or $-SiO^-$ groups of the silica. The presence of a significant concentration of cations, especially divalent cations, is expected to reduce this interaction by direct competition for the silanol groups. The k' values for the other metabolites are much less affected. The order of decreasing effectiveness of the various salts for both S and C is

$$CdSO_4 > MgSO_4 > KH_2PO_4 > Na_2SO_4$$

TABLE V

EFFECT OF INORGANIC SALTS PRESENT IN THE ELUENT ON THE k' VALUES OF PARACETAMOL AND ITS METABOLITES

Column packing	*Mobile phase*	*Salt added*	*Molar conc.*	k'				
				S	*G*	*P*	*M*	*C*
ODS WLCU 484	water–methanol–acetic acid (85.1:14:0.9)	KH_2PO_4	0.0	0.03	0.50	1.50	2.30	4.60
			0.06	0.42	0.43	1.92	2.86	1.71
			0.10	0.80	0.43	1.67	2.67	1.14
			0.2	1.10	0.46	1.67	2.47	0.93
Spherisorb ODS S10	water–methanol–acetic acid (85.1:14:0.9)	$CdSO_4$	0.0	0.0	0.83	1.93	2.87	6.20
			0.004	0.31	0.82	1.91	3.00	
			0.02	0.50	0.51	1.85	3.00	1.85
			0.06	0.57	0.57	1.47	2.85	1.40
		$MgSO_4$	0.04	0.38	0.69	1.84	3.00	2.38
		Na_2SO_4	0.09	0.38	0.76	1.75	3.10	2.18
ODS/TMS WLCU 503	water–methanol–formic acid (89.9:10:0.1)	KH_2PO_4	0.0	0.18	1.33	3.67	14.10	10.90
			0.1	1.00	1.25	3.26	10.13	10.40
			0.2	1.50	1.25	3.26	9.60	10.40

While the decrease in k' of the cysteine conjugate on ODS silica may be associated with the saturation of the silanol groups by cations added to the eluent, the increase in retention of the sulphate is more likely to be associated with the formation of sulphate ion-pairs which are more strongly retained by the bonded hydrocarbon groups than the dissociated sulphate.

Broadly the effect of salt addition is to render the elution pattern of paracetamol and its four metabolites more compact. This is illustrated by typical chromatograms shown in Fig. 5. The addition of salts can undoubtedly be beneficial for fine tuning of the elution pattern.

Application to analysis of urines taken after therapeutic doses of paracetamol

When using capped reversed-phase packings it is possible to obtain excellent chromatograms from direct injection of small urine samples (1–5 μl). In our experiments urine samples were obtained from a volunteer who had received a therapeutic dose of 1 g of paracetamol. The samples were taken at intervals of a few hours up to 24 h after ingestion. Fig. 6 shows chromatograms of a control and from specimens taken at 1.5 h, 7 h and 21 h after ingestion. The maximum excretion rate is around 7 h after ingestion. After 21 h the bulk of the dose has been excreted, but the presence of metabolites is still evident. The chromatograms show that all five components are clearly distinguishable from endogenous components as early as 1.5 h and as late as 21 h after ingestion. There is some overlap of S with an endogenous component, but the error deriving from this overlap and an even smaller overlap of the glucuronide is minimal for both of these components are present at relatively high concentration and are, of course, eluted as extremely narrow peaks. Addition of salt could have been employed to move the sulphate peak nearer to the glucuronide peak and so avoid the small interfering endogenous peak, but with the very large excess of the sulphate this did not seem necessary. For a more detailed study of the sulphate

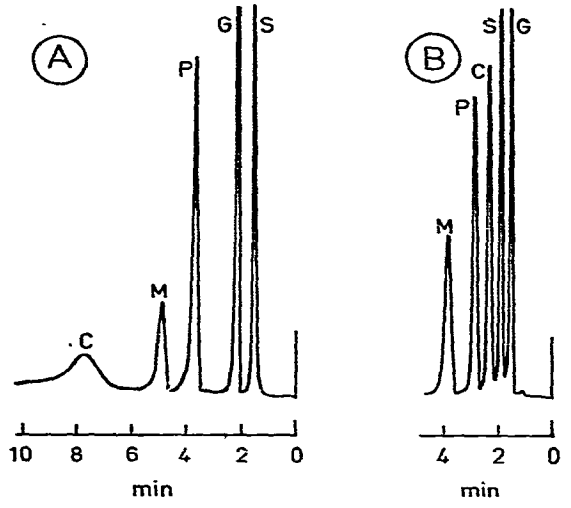


Fig. 5. Effect of added salt on elution of reference compounds using ODS silica (WLCU). Eluent: (A) water–methanol–acetic acid–ethyl acetate (86:14:0.8:0.5, v/v/v/v); (B) as for A but containing 0.1 mole/l of KH_2PO_4. Sensitivity, 0.1 a.u.f.s.

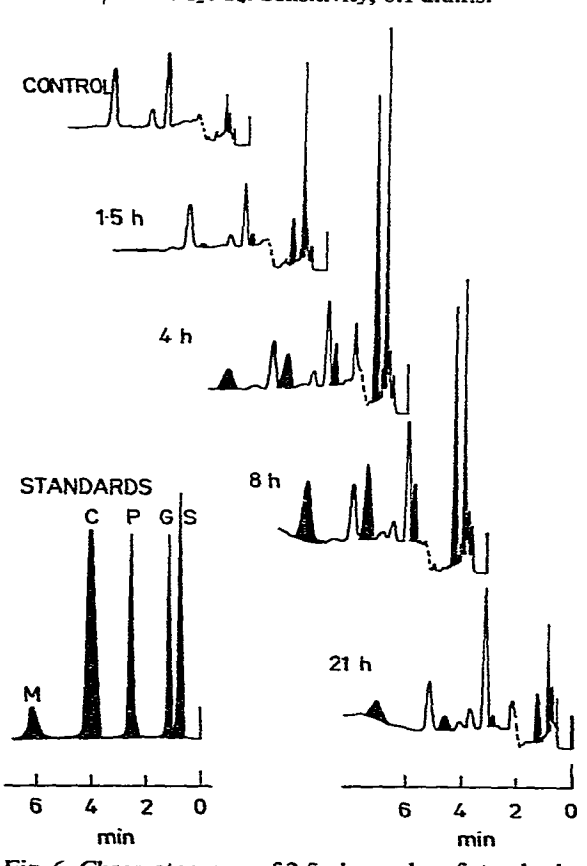


Fig. 6. Chromatograms of 2.5-μl samples of standards and of urines taken at intervals after ingestion of a therapeutic dose (1 g) of paracetamol. Packing material ODS/TMS silica (WLCU). Eluent: water–methanol–formic acid–ethyl acetate (94.5:6.5:0.1:0.5, v/v/v/v). Sensitivities, 0.5 a.u.f.s. initially; 0.05 a.u.f.s. after approx. 2 min. The peaks due to the paracetamol metabolites are shown in black.

metabolite it would be desirable to reduce the alcohol content and to add salts to improve its retention and possibly elute it after the glucuronide (see Table V) or to use soap chromatography, as discussed in a forthcoming paper[12].

The general trend of excretion of metabolites and their proportions is in close agreement with that found by Howie *et al.*[7,8]. However, in this study the presence of the mercapturate was certainly detectable at least 24 h after ingestion.

Application to analysis of urines taken from patients suffering from overdoses of paracetamol

Urine specimens taken from five patients who had ingested overdoses of paracetamol were kindly supplied by Dr. Prescott of the Royal Infirmary, Edinburgh, Great Britain. These urines contained very large quantities of paracetamol and its metabolites, the overdoses being probably in excess of 20 g. In these cases, the peaks arising from the endogenous components are insignificant compared to those arising from the paracetamol and its metabolites, and very much smaller samples of urine were injected (normally 1 μl). Problems did, however, arise in the separation of the expected paracetamol metabolites because of the considerable number of additional constituents in these pathological urines which were not present in the normal control urines. In order to obtain good resolution of all components it was necessary to use very much lower alcohol contents, and the chromatogram shown in Fig. 7, for example, was obtained using 1% aqueous isopropanol containing 0.15% formic acid.

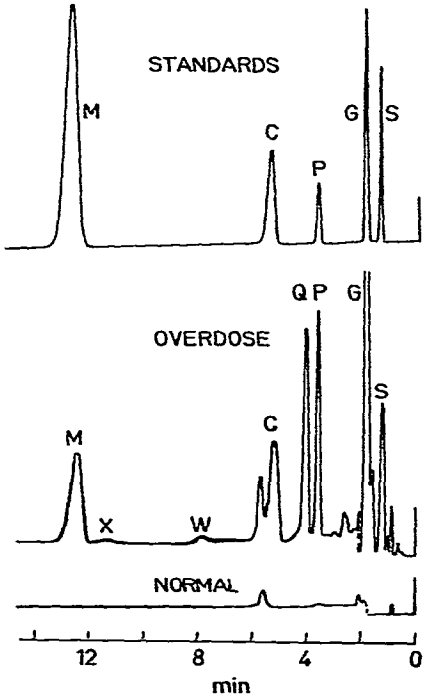


Fig. 7. Chromatogram of standards, normal urine and urine from a case of severe overdose of paracetamol. Samples, 1 μl. Eluent: water–isopropanol–formic acid (99:1.0:0.15, v/v/v). Detector: 242 nm, 1.0 a.u.f.s. Note: peaks Q, W and X are due to additional metabolites in the overdose sample.

This and other chromatograms show that in addition to the expected five compounds, there are three additional components not present in the control urines. These are marked Q, W and X on the figure. In addition a further component Y, with a much longer retention than that of M can be eluted under conditions of low alcohol content as a broad asymmetric peak. The peak is "flat topped", which suggests that it arises from a material which either precipitates out in the eluent, or is very strongly adsorbed and subsequently decomposed slowly to a less strongly retained solute.

Compound Q is eluted between P and C. Its retention behaviour was examined using aqueous methanol mixtures as eluent. With 14% methanol Q is eluted before paracetamol, and with 7% methanol it eluted after paracetamol. This implies that it is more lipophilic than paracetamol. Salt addition had exactly the same effect on Q as on paracetamol. These trends are consistent with the idea that this compound is a paracetamol metabolite which still contains the hydroxyl group but in addition is substituted in the ring by a methoxy group.

Further attempts to identify the additional peaks in the pathological urines were carried out by mass spectrometry, having trapped out the relevant peak and evaporated the bulk of the eluent. The opportunity was also taken to establish the identity of the metabolite generally identified as the mercapturate, but which Mrochek *et al.*[6] had failed to detect.

MASS SPECTROMETRIC STUDIES

Mass spectrometric studies were carried out with an A.E.I. Type MS 902 mass spectrometer in both the low- and high-resolution modes, precise mass numbers being obtained for selected ions (to 10^{-4} mass numbers). Mass spectra for reference compounds and compounds separated by HPLC from urine samples are presented in Tables VI and VII, respectively. High-resolution mass analysis (HRMA) results for selected ions are given in Table VIII. Fig. 8 indicates the presumed breakdown patterns of the different compounds studied. Mass numbers of sections of the molecules which are considered to be related to observed mass numbers (usually with addition or loss of one or two H atoms) are shown underlined.

Reference compounds

The results presented in Table VI show that the mass spectra of all paracetamol metabolites show the parent ion of paracetamol ($m/e = 151$) (excepting only C), the ion of *p*-aminophenol ($m/e = 109$) and the $COCH_3^+$ ion ($m/e = 43$). The origin of these ions is shown in Fig. 8.

The mass spectra of S and G show no parent ions (expected $m/e = 230$ and 327, respectively). S shows characteristic ions with $m/e = 80$ and 81 corresponding most likely to SO_3^+ and SO_3H^+. The ion $m/e = 193$ is not derivable from the structure of S and may arise from an impurity.

The mass spectrum of G contains the ion $m/e = 176$ which HRMA shows to have the formula $C_6H_8O_6$, that of glucuronic acid α-lactone. The ion $m/e = 192$ may arise from initial breaking of the aromatic carbon to oxygen bond (Fig. 8).

The mass spectrum of C again shows no parent ion (expected $m/e = 270$) which confirms previous observations[6]. The ion at $m/e = 226$ arises by loss of CO_2 and was previously reported[6]. The major ion at $m/e = 183$ indicates loss of the side

TABLE VI

MASS SPECTRA OF REFERENCE COMPOUNDS (*m/e* VALUES)

A: Source temperature 180–200°; B: source temperature 200–220°.

*S (230)**	*G (327)*	*C (270)*	*M (312)*				
			Synthetic	*Batch 1 urine*	*Batch 2 urine*	*Batch 1 purified HPLC*	*Batch 1 compound Z*
A/B	*A*	*A*	*B*	*B*	*B*	*B*	*B*
43	43	43	43	43	43	43	
	44	44			44	44	44
			45				
				57	57(A)§§		
80							
81							
			87	87	87	87	
							101
				105			
		108	(108)	108	108	108	
109	109	109	109	109	109	109	109
				111	111	111	111(A)
			129	129**	129	129	
			130	130**	130	130	
						140	
		141	141	141	141	141	
							143
151	151		(151)	151	151	151	151
			154				
				155*	155	155	155
	176**						
		183	183	183	183	183	183
	192						
193							
		(197)***		197		(197)	197
		226					
		230§					
		231§					
			253	253**	253	253	
							264
				267**			267
		273§					
			312	312**	312	312	
				326**			326
			332**				
		364	(364)**				
			(382)				
				(402)			

* *m/e* for parent ion in brackets.

** HRMA carried out, see Table VIII.

*** *m/e* values in parenthesis indicate low-intensity peak.

§ These ions recorded by Reynolds were ascribed to a benzyloxy derivative.

§§ Only present under condition A.

TABLE VII

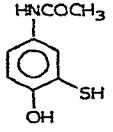
MASS SPECTRA (*m/e* VALUES) OF COMPOUNDS SEPARATED BY HPLC FROM OVERDOSE URINES

Q	*W*	*X*	*Y*	*M*	*Purified M Batch 1*
				41	
43				43	43
44		44			44
		45			
		46			
			59		
		60			
			80		
			81		
		87			87
				103	
105					108
109	109	109	109	109	109
					111
		113(A)**			
	118				
124			124		
				129	129
				130	130
	134				
139*			139		
	140		140	140	140
			141	141	141
	142				
	147				
151	151	151	151		151
	155	155	155		155
165					
181*		181	181*		
		183	183	183	183
			194		
	197	197	197		
				253	253
				312*	312

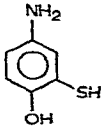
* HRMA carried out, see Table VIII.

** Only present at a source temperature of 180–200°.

chain leaving only a sulphhydryl group (compound I) and the ion $m/e = 141$ arises by further loss of $COCH_2$ giving compound II.



I



II

Some ions of high molecular weight were also observed and these are discussed later.

TABLE VIII

HIGH-RESOLUTION MASS ANALYSIS OF SELECTED IONS

Source	*m/e*	*Measured m/e*	*Formula*	*True m/e**	$10^4 \times (m/e)_{exp} - (m/e)_{true}$
M	326	326.0927	$C_{14}H_{18}N_2O_5S$	326.0936	−9
batch	312	312.0778	$C_{13}H_{16}N_2O_5S$	312.0780	−2
1 TLC	267	267.0554	$C_{12}H_{13}NO_4S$	267.0565	−11
	253	253.0401	$C_{11}H_{11}NO_4S$	253.0409	−8
	197	197.0508	$C_9H_{11}NO_2S$	197.0510	−2
	155	155.0406	C_7H_9NOS	155.0405	+1
	130	130.0498	$C_5H_8NO_3$	130.0504	−6
	129	129.0427	$C_5H_7NO_3$	129.0426	+1
M	364	364.0533	$C_{16}H_{16}N_2O_4S_2$	364.0551	−18
synthetic	332	332.0769	$C_{15}H_{16}N_4OS_2$	332.0765	+4
G	176	176.0318	$C_6H_8O_6$	176.0321	−3
Q	181	181.0735	$C_9H_{11}NO_3$	181.0739	−4
	139	139.0633	$C_7H_9NO_2$	139.0633	0
Y	181	181.0723	$C_9H_{11}NO_3$	181.0739	−16
		181.0730			−9

* Calculation:

Atom	*Mass*	$10^4 \times$ *Difference from integral m/e*
^{12}C	12.000000	0
^{1}H	1.007825	+78
^{14}N	14.00307	+30
^{16}O	15.99491	−51
^{32}S	31.97207	−289

M was the only metabolite showing a parent ion ($m/e = 312$), but its appearance was transitory being present only for a minute or so when the sample was introduced into the mass spectrometer source at 200°. No previous mass spectrum of M has been described, to our knowledge, and as noted earlier there has been some doubt as to whether this metabolite is indeed the mercapturic acid conjugate of paracetamol. HMRA has now shown that the ion at $m/e = 312$ is beyond any possible doubt that of $C_{13}H_{16}N_2O_5S^+$, the empirical formula of M. HRMA also showed that the ion $m/e = 253$ corresponded to M having lost the group NH_2COCH_3 ($m/e = 59$) (see Table VIII and Fig. 8). The ions with $m/e = 183$ and 141, identical to those obtained from C, probably correspond to compounds I and II. HRMA also showed that the ions at $m/e = 129$ and 130 correspond to the side chain of M with the break being at the S–C bond. Additional ions, observed at $m/e = 197$ and 155, were identified by HRMA as having the empirical formulae of compounds I and II with the addition of CH_2. As shown below they arise from a contaminant in the sample of M.

The low-resolution mass spectrum and the high-resolution mass analysis results prove without any possible doubt the identity of the mercapturic acid metabolite as obtained from two natural sources (overdose urine samples) and one synthetic sample.

Comparison of the mass spectra of the synthetic sample of M with that of Batch 1 of the naturally obtained material show that the latter contained a number of additional ions occurring at *m/e* values of 14 higher than corresponding ions present in all samples of M. These ions have *m/e* values of 155, 197, 267 and 326. HRMA

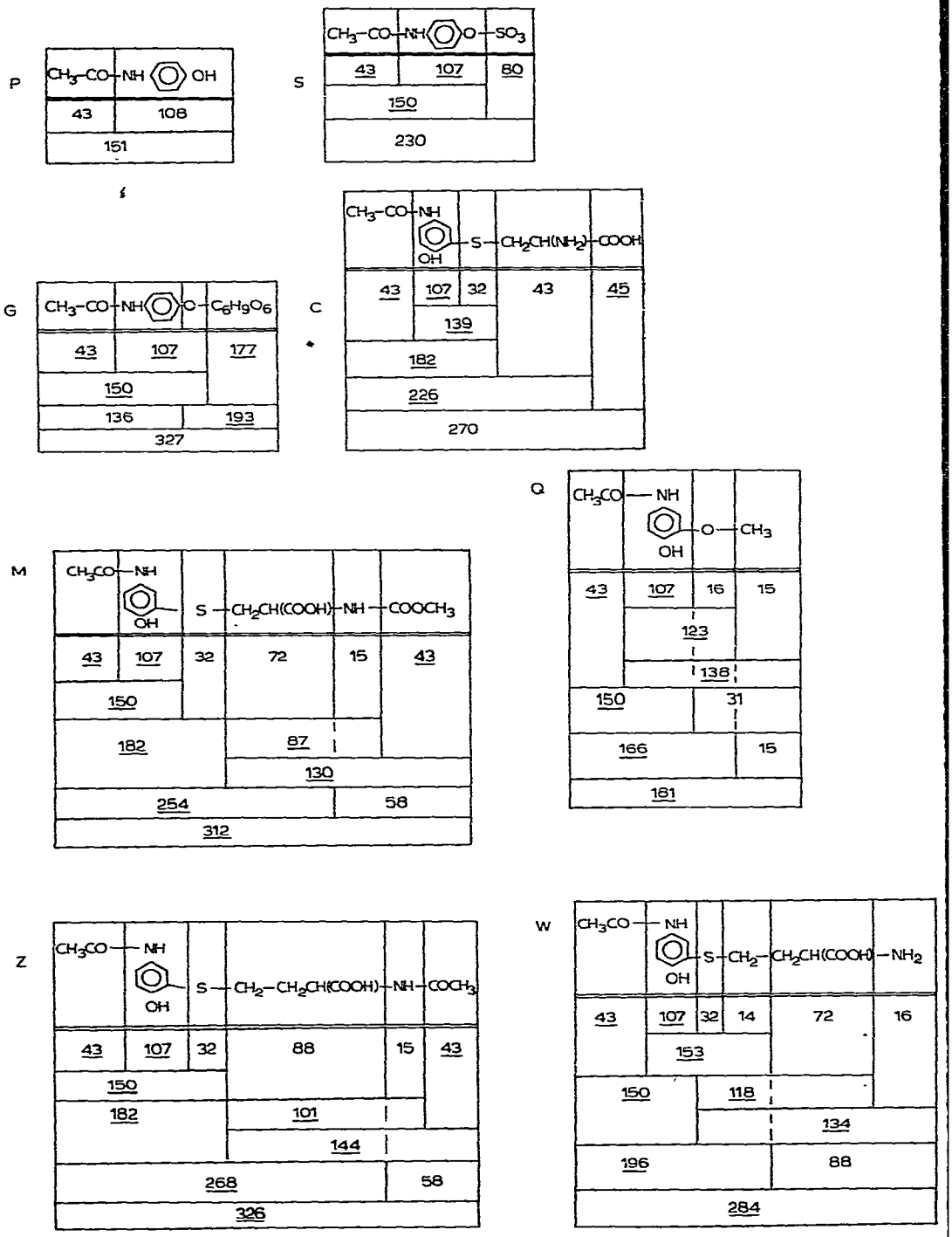


Fig. 8. Proposed breakdown patterns of paracetamol and metabolites. The mass numbers underlined are those most closely related to the *m/e* values of ions recorded in the mass spectra and listed in Tables VI–VIII. Differences of 1 or 2 hydrogen atoms between the mass numbers and *m/e* values are to be expected.

of the ion $m/e = 326$ shows this to have a formula corresponding to compound III, having one more CH_2 group than M itself.

The situation was clarified by purifying the natural samples (Batch 1) of M by HPLC. A typical preparative chromatogram is shown in Fig. 9 and indicates the presence of a number of impurities including paracetamol and a significant component, labelled Z, which elutes after M. After purification by HPLC, the mass spectrum of M showed much reduced peaks at $m/e = 155$, 197 and no peaks at 267 and 326, while the compound Z showed these peaks much enhanced. Whereas Z showed no peaks with $m/e = 81$, 129 or 130, it showed substantial peaks for $m/e = 101$ and 143 corresponding to side chain fragments containing an additional CH_2 group.

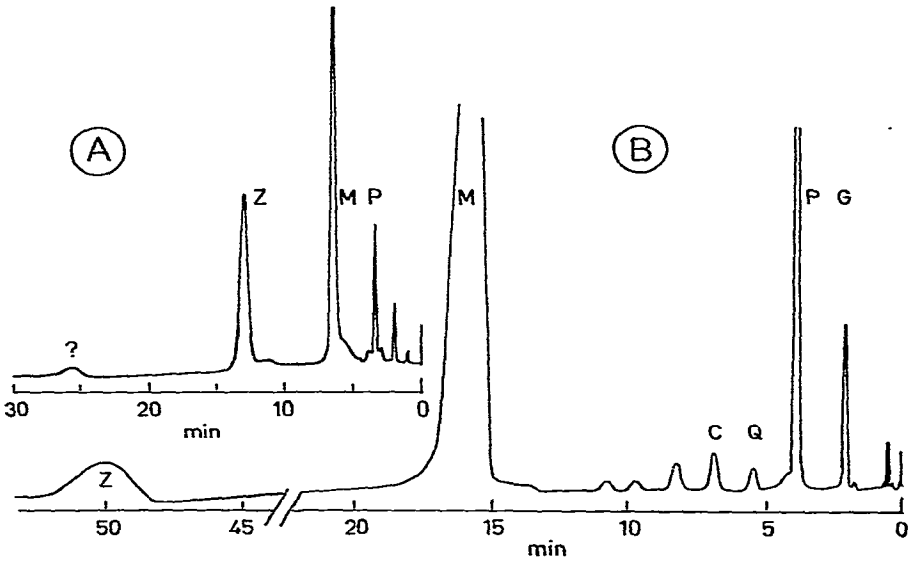
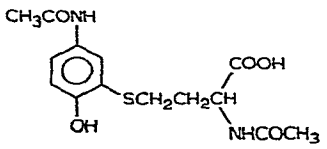


Fig. 9. Analytical (A) and preparative (B) chromatograms of standard sample of M (Batch 1). Packing material ODS/TMS silica (WLCU). Eluent: water–methanol–formic acid (A: 83:17:0.1, v/v/v) (B: 95:5:0.1, v/v/v). Sensitivity A: 0.1 for P and M, 0.02 a.u.f.s. for Z; B: 1.0 throughout. Wavelength A: 242 nm; B: 254 nm.

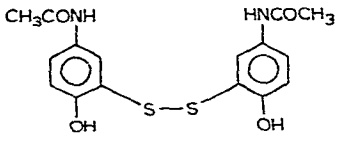
A small peak has been found in the chromatograms of all overdose urines with the same k' as Z but not positively identified as Z. Taken together there is therefore strong evidence that the natural samples of M were contaminated with a compound Z which has an additional CH_2 group in the side chain, the suggested formula being III, and that Z is present in overdose urines.



III

Since methionine, $CH_3SCH_2CH_2CH(COOH)NH_2$, is commonly used in the treatment of overdose cases it is possible that metabolite Z arises from such treatment, but it remains an open question as to whether Z arises *in vivo* or as a subsequent product formed during storage of our samples.

In the mass spectra of the reference samples of C and M several ions of high molecular weight were observed with $m/e = 332$, 364 and 382. HRMA was carried out on the first two of these ions (Table VIII) yielding the formulae $C_{15}H_{16}N_4OS_2$ and $C_{16}H_{16}N_2O_4S_2$. The second of these ions can be identified with reasonable confidence as compound IV.



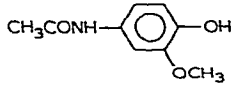
IV

The ion with $m/e = 332$ was present only in the synthetic M. It is less easily placed and cannot be constructed in any simple way by assembling fragments of either M or C. The problem is that to obtain 4 nitrogen atoms in the ion it would be necessary to incorporate 16 carbon atoms not 15. The presence of only a single oxygen atom does, however, suggest that the compound may be formed by elimination of water from the two phenolic hydroxyl groups.

Urine components

Fig. 7 shows three peaks in the overdose urine which are additional to those clearly present in normal urine. They are labelled Q, W and X, and their mass spectra are detailed in Table VII with HRMA results in Table VIII.

The mass spectrum of Q contains the ion with $m/e = 181$ which is identified by HRMA as having a formula corresponding to methoxyparacetamol (compound V).



V

The ion $m/e = 139$ has a formula corresponding to methoxy-*p*-aminophenol while the ion with $m/e = 124$ corresponds to this compound with loss of a further CH_2. There is evidence[2,3,6] that 3-methoxy derivatives of the sulphate and glucuronide conjugates are metabolites of paracetamol, but the uncoupled methoxyparacetamol has not been reported in the metabolic pathway. The chromatographic behaviour of Q is certainly not what is expected of a sulphate conjugate, but the absence of a parent ion in the mass spectrum is, of course, no evidence that Q is not a conjugate. On the other hand, the absence of an ion at $m/e = 176$ would suggest that Q is not the glucuronide. We therefore believe that Q is most probably a methoxy-substituted derivative of paracetamol as suggested in formula V.

Compound W is a minor metabolite whose mass spectrum shows the characteristic ions of paracetamol metabolites at $m/e = 109$ and 151. The ions at $m/e = 197$ and 284 suggest that W bears the same relationship to C as does Z to M, that is it is the cysteine metabolite containing an additional CH_2 group in the side chain. This is confirmed by the additional presence of the fragment with $m/e = 155$. The ions with $m/e = 118$ and 134 could correspond to side chain fragments but have no analogues in the mass spectra of M, C or Z (see Fig. 8).

Compound X is another minor metabolite which has not been fully identified. A characteristic ion at $m/e = 181$ suggests that it contains a methoxy group while the ions at $m/e = 155$ and 197 are characteristic of W and Z. X appears to contain the cysteine or mercapturic acid side chain and, in addition, a methoxy group.

Compound Y, as noted above, eluted as a wide asymmetric peak well after M. It is a major component in overdose urines roughly equivalent to M and C together. Y is eluted only when using eluents having a very low alcohol content and is thought to be a compound or group of compounds which are precipitated by alcohol, for example protein conjugates. The mass spectrum obtained from a collection of the entire peak is shown in Table VII. It contains characteristic ions for a paracetamol metabolite ($m/e = 43$, 109, 151); it contains ions characteristic of M and Z (m/e 141, 155, 183, 197). It contains the ion m/e 59 representing $NH–CO–CH_3^+$ as well as ions characteristic of Q, methoxyparacetamol, (m/e 181, 139, 124). The ion $m/e = 194$ has not been identified. Compound Y gives no ions corresponding to sulphur-containing side chains of M and Z ($m/e = 87$ and 101). The structure of Y cannot be deduced from the mass spectrum, but it is undoubtedly a major component in the samples of overdose urines available to us. At this stage, we cannot be certain that it arises *in vivo* as it could have been formed during storage through bacterial action.

The importance of Y, if formed *in vivo*, is that it may be more directly associated with liver damage in overdose cases than is M or C. It is, for example, possible that Y may be a further degradation product (in addition to M and C) of the product of reaction of the paracetamol intermediate with the glutathione within the liver cell[2,13,14], or it may be the degradation product of the conjugate formed when the intermediate reacts directly with liver protein. This occurs[15] when the glutathione level has fallen to 20–30% of its normal level. The chromatographic behaviour of Y supports the second suggestion, but until further work is carried out these suggestions must be regarded as highly speculative.

MAJOR CONCLUSIONS

(1) Excellent resolution of paracetamol and its metabolites is obtained on microparticulate reversed-phase packing materials. The order of elution is S, G, P, C, M or S, G, P, M, C. The sulphate metabolite is eluted close to the unretained solute.

(2) Optimum elution conditions are obtained by adjustment of the methanol content of the aqueous eluent. The addition of methanol reduces retention.

(3) The presence of acid, formic or acetic acid is necessary for good elution conditions. With concentrations of formic acid above about 0.1% and of acetic acid above about 1% there is little effect on k' values.

(4) The addition of salts reduces the k' of strongly retained metabolites but

increases the retention of S, thereby making the elution pattern on ODS/TMS silica more compact. Salt addition is valuable for fine tuning of the elution pattern.

(5) While good resolution of paracetamol and its metabolites can be obtained on ODS silica, much improved resolution is obtained if the ODS silica is further treated by vigorous silanization to remove residual hydroxyl groups, which tend to interact disadvantageously with basic functional groups.

(6) For analysis of urine samples the superiority of ODS/TMS silica over simple ODS silica is very marked.

(7) With ODS/TMS silica the excretion of paracetamol and its metabolites after therapeutic doses of paracetamol is readily followed for at least 24 h.

(8) Mass spectrometric analysis using selective high-resolution mass analysis for selected ions has given unequivocal proof of the identity of the mercapturic acid conjugate and excellent proof of identity of the other metabolites used as standards.

(9) The standard samples of M have been shown to contain an impurity having an additional CH_2 group in the side chain. It is not certain whether this is natural or formed during storage.

(10) Methoxyparacetamol has been identified as a metabolite in overdose cases. One major and two further minor metabolites have been observed. The mass spectrum of the major metabolite indicates the presence of $-OCH_3$ and $-SCH_3$ groups in the molecule.

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