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The gas-liquid chromatographic estimation of phenacetin and paracetamol in plasma and urine

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Methods are described for the gas-liquid chromatographic estimation of phenacetin and paracetamol in plasma and free and conjugated paracetamol in urine. *p*-Chloracetanilide and *p*-bromacetanilide were used as internal standards. The drugs were extracted with ethyl acetate and before chromatography were converted to trimethylsilyl derivatives with *N*,*O*-bis(trimethylsilyl)acetamide (simultaneous assay of phenacetin and free paracetamol in plasma), or *N*-trimethylsilylimidazole (assay of paracetamol alone). Phenacetin was extracted with chloroform and chromatographed directly. Paracetamol glucuronide and sulphate were hydrolysed enzymatically to the parent compound before extraction. Recovery of added phenacetin and paracetamol in plasma at concentrations of 1–100 μg/ml was complete, and the limit of detection of the drugs in plasma was 0·05 μg/ml.

Existing methods for the estimation of phenacetin and its metabolite paracetamol in plasma and urine are non-specific, time consuming or lack sensitivity (Brodie & Axelrod, 1948, 1949; Welch & Conney, 1965; Büch, Pfleger & Rüdiger, 1967; Cummings, King & Martin, 1967; Routh, Shane & others, 1968). A gas-liquid chromatographic method providing a more sensitive and specific technique for the assay of these drugs either separately or together in biological fluids is now described. The gas-liquid chromatography of paracetamol has been reported by Klutch & Bordun (1968), but in the present studies the drug could not be chromatographed directly in low concentrations without peak tailing and absorption losses. It was therefore converted to trimethylsilyl (TMS) derivatives using *N*-trimethylsilylimidazole (TMSI) or *N*, *O*-bis(trimethylsilyl)acetamide (BSA). TMSI is a powerful silylating reagent which is selective for -OH groups (Pierce, 1968) and paracetamol is converted to the TMS ether derivative. BSA reacts with both -NH- and -OH groups (Klebe, Finkbeiner & White, 1966; Pierce, 1968) and mono- and di- TMS derivatives are formed with phenacetin and paracetamol respectively. *p*-Chloracetanilide and *p*-bromacetanilide were used as internal standards. The gas-liquid chromatographic properties of some phenacetin metabolites and related compounds were also investigated.

MATERIALS AND METHODS

Hewlett Packard Model 5755B and Pye Series 104 Model 14 gas chromatographs with flame ionization detectors were used in conjunction with Moseley Model 7128A and Leeds & Northrup Speedomax "W" recorders respectively. Argon or nitrogen was used as the carrier gas at a flow rate of 50 ml/min. Hydrogen and air flow rates were approximately 40 and 400 ml/min respectively. The colmns were glass, $\frac{1}{4}$ inch diameter and 5 or 6 ft in length. Pyridine, TMSI and BSA (Pierce) were obtained

from Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Flintshire, and prepared column packings (Applied Science) from Field Instruments Ltd., Orchard Road, Richmond, Surrey.

Preparation of TMS derivatives

TMSI, BSA and anhydrous pyridine were stored separately under nitrogen in capped vials and standard precautions were taken to avoid hydrolysis of the TMS derivatives (Pierce, 1968). All transfers were made with clean dry microsyringes.

The reactions were carried out in glass vials, 36 × 14 mm with capillary end-bulbs of about 30 μl capacity (Glass Appliances Ltd., Holburn Street, Aberdeen). The vials were flushed with nitrogen, sealed with silicone rubber caps and 15 μl of a pyridine solution of the compound or extract under investigation injected through the cap into the end-bulb. An equal volume of the silylating reagent was added and the solutions mixed thoroughly with the syringe. The tubes were left at room temperature (20°) for 15–30 min and 2–3 μl aliquots were injected directly into the chromatograph.

Assay of free paracetamol in plasma and urine

Plasma or urine samples (2·0 ml) containing up to 40 μg of paracetamol and 1M phosphate buffer pH 8·0 (1·0 ml) were extracted in stoppered glass tubes with redistilled ethyl acetate (5·0 ml) containing 3·0 μg/ml of *p*-chloracetanilide. The tubes were shaken mechanically for 20 min and then centrifuged (vigorous shaking causes emulsions). The organic phase only was transferred to 15 ml tapered glass centrifuge tubes and evaporated to dryness on a rotary vacuum evaporator with the tubes immersed in water at 26°. The residue in the tips of the tubes was dissolved in 15 μl of anhydrous pyridine with the aid of a vortex mixer, transferred to reaction vials and 15 μl of TMSI added as described above. Samples containing 20–200 μg/ml of free paracetamol were extracted with ethyl acetate containing 30 μg/ml of *p*-chloracetanilide. Appropriate dilutions of more concentrated samples were made before analysis. The columns were packed with 10% OV17 on 80/100 mesh Gaschrom Q and run at 200°.

Assay of total free and conjugated paracetamol in urine

Urine samples (0·5 ml), 0·2M sodium acetate buffer pH 5·0 (0·5 ml) and glusulase* (0·1 ml) (Endo Laboratories, Garden City, N.Y., USA) were mixed in stoppered glass tubes and incubated at 37° for 16 h. Phosphate, 0·2M, pH 9·9 (2·0 ml) was then added and the mixture extracted with ethyl acetate (5·0 ml) containing 30 μg/ml of *p*-chloracetanilide and assayed as described for free paracetamol. Preliminary dilutions were made if the urine contained more than 1000 μg/ml of total paracetamol. Hydrolysis of paracetamol conjugates in urine with glusulase has been shown to give a significantly higher recovery of free paracetamol than hydrolysis with hydrochloric acid (Prescott, 1969).

Simultaneous assay of phenacetin and free paracetamol in plasma

The assay was the same as that for free paracetamol except that *p*-bromacetanilide was used as the internal standard. BSA was used as the silylating reagent, and all three compounds were converted to TMS derivatives. The column was packed with either 5% OV1 or 5% UC W98 on 80/100 mesh Gaschrom Q, and run at 160°.

* Glusulase contains 100 000 units of β-glucuronidase and 50 000 units of aryl sulphatase per ml.

Assay of phenacetin alone in plasma

Phenacetin was extracted as for free paracetamol in plasma using chloroform containing 3 μg/ml of *p*-bromacetanilide. The extract was evaporated to dryness and the residue redissolved in 20 μl of chloroform. Aliquots were injected directly into the gas chromatograph. The column was packed with 3% XE 60 on 80–100 mesh Gaschrom Q and run at 160°. Other suitable liquid phases were: 3% QF1, diethyleneglycol succinate, HI-EFF 8BP or Carbowax 20M, 10% OV17 and 10% SE 52 plus 1% HI-EFF 8BP.

Calibration

Aqueous solutions containing known amounts of the drugs were run through the entire procedures and the ratios of the peak heights of the drugs (or their TMS derivatives) to those of the internal standards were plotted against the concentrations of phenacetin and paracetamol. Paracetamol was used for the standardization of the assay for total free and conjugated paracetamol as the glucuronide and sulphate were not available. Linear working curves relating the peak height ratios and drug concentrations were obtained over the range 1–100 μg/ml. The concentration of the drugs in plasma or urine can be determined from previously constructed calibration graphs, but a more accurate result may be calculated from the peak height ratio of a standard solution of the drug run with the samples since there were minor day-to-day variations in peak height ratios.

RESULTS

Aniline, acetanilide, *p*-phenetidine, *p*-aminophenol and phenacetin were all chromatographed directly without difficulty on Gaschrom Q coated with 3% XE 60, 3% HI-EFF 8 BP, 5% Carbowax 20M, 10% OV17 or mixed phases consisting of 10% SE 52 plus 1% HI-EFF 8BP and 0·5% SE 30 plus 0·5% Carbowax 20M. *N*-Methylacetanilide, acet-*p*-toluidide, *p*-chloracetanilde and *p*-bromacetanilide can be used as internal standards. With the exception of *N*-methylacetanilide, all of these compounds were converted to TMS derivatives by treatment with BSA and could then be chromatographed on "non-polar" liquid phases such as OV1 and UC W98. With TMSI, selective silylation of the -OH groups of paracetamol, 2-hydroxyphenacetin and *p*-aminophenol occurred, although with the latter compound derivative formation was apparently incomplete. Paracetamol could not be chromatographed directly on any of the columns without significant peak tailing. The retention times of the different compounds and their TMS derivatives relative to *p*-aminophenol on four different liquid phases are listed in Table 1. In general, normal human plasma and urine samples run through the procedures did not show interfering peaks at the attenuation used for the most sensitive assays. A large extraneous peak eluting after paracetamol-TMS was sometimes encountered with plasma, thus delaying the injection of subsequent samples. Spurious (but non-interfering) peaks were also traced to impurities in some batches of TMSI and ethyl acetate. Chromatograms of extracts of plasma and urine from individuals taking therapeutic doses of paracetamol are shown in Fig. 1 (a, b and c).

Recovery studies

The results of 10–20 analyses of samples of plasma and urine containing known amounts of added phenacetin and paracetamol are shown in Table 2. Except for the

Table 1. *Relative retention of some aromatic amine derivatives (p-aminophenol = 1·00).*

Temperature	3% XE 60 160°C	3% HI-EFF 8BP 180°C	10% OV17 200°C	10% OV17 200°C	5% OV1 155°C
	Without derivative formation			As TMS derivatives*	
Aniline	0·10	—	0·26	—	0·22
N-Methylacetanilide	0·36	0·20	1·01	—	0·33
p-Phenetidine	0·33	0·27	1·00	—	0·79
Acetanilide	1·49	0·91	1·60	—	0·33
p-Aminophenol	1·00	1·00	1·00	1·00†	1·00
Acet-*p*-toluidide	2·10	1·28	2·38	—	0·51
p-Chloracetanilide	4·67	2·90	3·75	—	0·73
p-Bromacetanilide	6·41	3·84	5·85	—	1·08
Phenacetin	7·64	4·95	5·80	—	1·29
Paracetamol	—	—	—	4·40	1·56
2-Hydroxyphenacetin	—	—	—	6·52	3·46
Approximate retention time of *p*-aminophenol (min)	2	5	3	4	10

* Compounds run on OV17 and OV1 were treated with TMSI and BSA respectively.
† Incomplete derivative formation.

assays of paracetamol alone, the recoveries were calculated using previously constructed calibration equations derived from the assay of aqueous solutions of the drugs in the same concentration ranges. The values presented for paracetamol alone were obtained using an aqueous standard run with the samples.

The recovery of both drugs from plasma was essentially complete, and the reproducibility of the assays good, down to concentrations of 1 μg/ml. The limits of detection in plasma are about 0·05 μg/ml. Phenacetin and paracetamol are likely to be taken in combination with acetylsalicylic acid, caffeine, codeine and barbiturates. Caffeine and codeine may be extracted from biological fluids under the present conditions, but salicylates and barbiturates will remain largely in the aqueous phase. These compounds all form TMS derivatives with BSA but do not interfere with the present methods.

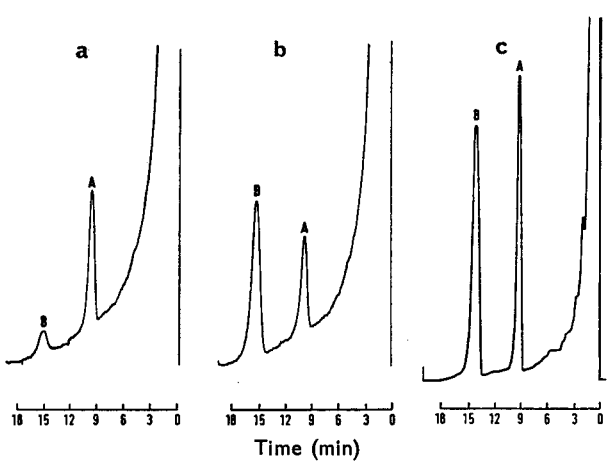

FIG. 1. Chromatograms of extracts of plasma obtained from a patient (a) 30 and (b) 90 min after administration of 1·5 g of paracetamol. The samples were assayed for free paracetamol and the measured concentrations were 1·6 μg/ml (a) and 16·5 μg/ml (b). (c) Chromatogram of an extract of hydrolysed urine obtained from a healthy subject 12–24 h after taking 1·5 g of paracetamol. The urine was assayed for total free and conjugated paracetamol and the measured concentration was 482 μg/ml. The internal standard in all three chromatograms was *p*-chloracetanilide (peak A) and paracetamol was chromatographed as the mono-TMS derivative (peak B).

Table 2. *Recovery of phenacetin and paracetamol added to normal human plasma or urine in replicate assays.*

Assay	Concentration or concentration range (μg/ml)	Mean recovery ± s.d. (%)
Phenacetin in plasma	20–100	101·5 ± 4·1
	1– 10	96·6 ± 10·4
Phenacetin and paracetamol together in plasma		
phenacetin	25–100	100·8 ± 3·0
paracetamol	25–100	102·6 ± 4·0
phenacetin	1– 10	99·7 ± 6·7
paracetamol	1– 10	99·6 ± 9·0
Paracetamol in plasma	10	100·1 ± 1·8
Paracetamol in urine	500	100·9 ± 2·8

DISCUSSION

The gas-liquid chromatographic assay of phenacetin and paracetamol compares favourably with other techniques in respect of sensitivity and reproducibility. Spectrophotometric methods are liable to interference by other aromatic amines or extractable drugs with ultraviolet absorption, and sensitivity is compromised by high blank values if preliminary thin-layer chromatographic separation is used. The inherent specificity of gas-liquid chromatographic analysis is further increased by the formation of TMS derivatives. Paracetamol can be identified with certainty by differential silylation and chromatography on columns of differing polarity.

A disadvantage is that the method can be more time consuming than other techniques, but on the other hand it is particularly useful to be able to estimate phenacetin and paracetamol together in a single analysis. A further drawback is that analytical precision depends on quantitative conversion to TMS derivatives which are readily hydrolysed by moisture, even in the presence of excess reagent. The N-TMS derivatives are more susceptible to hydrolysis than O-TMS derivatives and TMSI is therefore the preferred silylating reagent for the analysis of paracetamol alone. However, the solvent front elutes rather slowly after injection of TMSI, and this limits sensitivity. Minor decomposition of N-TMS derivatives can occur on ageing columns, but this seems to affect the drugs and the internal standards to a comparable degree.

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