Journal of Chromatography, 209 (1981) 67–76
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,620

RAPID MICRO-METHOD FOR THE MEASUREMENT OF PARACETAMOL IN BLOOD PLASMA OR SERUM USING GAS–LIQUID CHROMATOGRAPHY WITH FLAME-IONISATION DETECTION

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(Received December 29th, 1980)

SUMMARY

A simple method for the measurement of plasma paracetamol concentrations using a novel extraction/acetylation procedure prior to gas–liquid chromatographic analysis has been developed. The sample (100 μl) is vortex-mixed for 30 sec with 0.067 mole/l phosphate buffer, pH 7.4 (50 μl), internal standard solution (N-butyryl-*p*-aminophenol (200 mg/l) in chloroform) (50 μl) and "acetylation reagent" (acetic anhydride–N-methylimidazole (catalyst)–chloroform, 5:1:30) (20 μl). After centrifugation at 9950 *g* for 3 min, a portion (5 μl) of the resulting extract is analysed on a 1.5 m × 4 mm I.D. glass column packed with 3% (w/w) C_{87} hydrocarbon (Apolane-87) on Chromosorb W HP, 100–120 mesh, maintained at 235°C. A specimen together with a quality control sample can be analysed, in duplicate, within 20 min. The limit of accurate measurement of the assay is 10 mg/l, and few potential sources of interference have been identified. The method has advantages of speed and reproducibility over other gas–liquid chromatographic procedures and, in addition, of selectivity over spectrophotometric techniques. The procedure provides a useful alternative to liquid chromatographic methods for emergency paracetamol measurements.

INTRODUCTION

The rapid measurement of plasma paracetamol (acetaminophen) concentrations can be important in the diagnosis and management of acute paracetamol poisoning[1], especially with respect to cases presenting more than 12 h after ingestion of the drug[2]. The available methods have been reviewed by Wiener[3]. Chromatographic techniques have advantages of selectivity and small sample requirement when compared with spectrophotometric methods, and a rapid high-performance liquid chromatographic assay has been described recently[4].

The method presented here for the measurement of plasma paracetamol concentrations involves the formation of the acetyl derivatives of paracetamol and of the internal standard, N-butyryl-*p*-aminophenol, prior to gas–liquid chromatographic (GLC) analysis[5]. However, studies with N-methylimidazole (*ca.* 360 times more efficient than pyridine in the catalysis of the acetylation of isopropanol[6]) have prompt-

0021-9673/81/0000–0000/S02.50

ed the simplification of the procedure to the extent that the extraction and derivatisation can be accomplished in a single step.

EXPERIMENTAL

Materials and reagents

Paracetamol and N-butyryl-*p*-aminophenol were obtained from Sigma London (Gillingham, Great Britain) and from Winthrop (Surbiton, Great Britain), respectively. The latter compound was used as a 200 mg/l solution in chloroform (analytical-reagent grade) which was stable for at least 3 months if stored at 4°C in the absence of light. The "acetylating reagent" was a mixture of acetic anhydride (analytical-reagent grade)–N-methylimidazole (Aldrich, Gillingham, Great Britain)–chloroform (5:1:30), and was prepared daily. Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate (both analytical reagent grade) buffer, 0.067 mole/l, pH 7.4 ("phosphate buffer") was prepared in distilled water.

Gas–liquid chromatography

A Pye 104 gas chromatograph equipped with dual flame-ionisation detectors was used, and integration of peak areas was performed using a Hewlett-Packard 3352 data system. The column oven temperature was 235°C and the detector oven temperature was 300°C; injection port heaters were not employed. The column, a 1.5 m × 4 mm I.D. glass tube, was silanised by treatment with 2% dichlorodimethylsilane in toluene for 8 h, rinsed with methanol and dried at 100°C prior to packing with 3% (w/w) C_{87} hydrocarbon (Apolane-87) on Chromosorb W HP, 100–120 mesh, purchased ready-prepared from Pierce and Warriner (Chester, Great Britain). The carrier-gas (nitrogen) flow-rate was 40 ml/min, and the hydrogen and oxygen inlet pressures were 15 and 10 p.s.i., respectively.

The chromatography on this system of a derivatised extract of an aqueous solution of paracetamol to which N-butyryl-*p*-aminophenol had been added is illustrated in Fig. 1. The retention times on this system of some compounds extracted under the conditions of the assay, measured relative to the internal standard, are given in Table I.

Sample preparation

Plasma or serum (100 μl), internal standard solution (50 μl), phosphate buffer (50 μl) and acetylating reagent (20 μl) were added to a small test (Dreyer) tube (Poulten, Selfe and Lee, Wickford, Great Britain). Hamilton gas-tight luer-fitting glass syringes (2.5, 2.5 and 1.0 ml, respectively) fitted with Hamilton repeating mechanisms and stainless steel needles were used in the addition of these latter volumes. The contents of the tube were vortex-mixed for 30 sec and the tube was centrifuged at 9950 *g* for 3 min in an Eppendorf centrifuge 5412 (Anderman, East Molesey, Great Britain) which was modified to accept Dreyer tubes by drilling-out the 0.4-ml test-tube centrifuge adapters. Subsequently, a portion (3–5 μl) of the chloroform layer was taken and injected onto the gas chromatographic column using a syringe fitted with an 11.5-cm needle. The whole procedure was carried out at room temperature, normally 22°C.

Duplicate sample analyses were performed and the mean results taken.

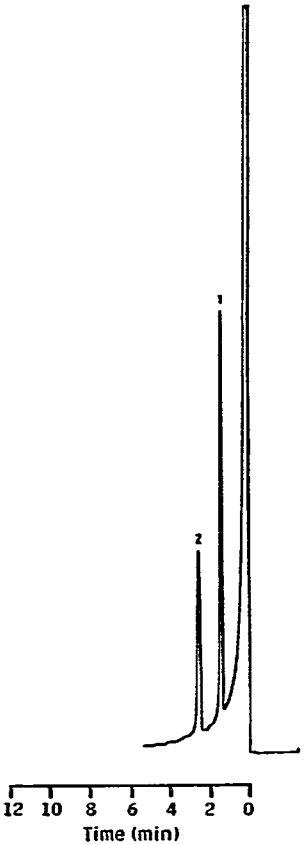

Fig. 1. Chromatogram obtained on analysis of an extract of an aqueous solution containing paracetamol (200 mg/l) on the C_{87} hydrocarbon system; 3-μl injection. The initial N-butyryl-*p*-aminophenol concentration was 200 m/l. Peaks: 1 = acetylated paracetamol; 2 = acetylated N-butyryl-*p*-aminophenol. See text for chromatographic conditions.

Instrument calibration

Solutions containing paracetamol at concentrations of 20, 50, 100, 200, 300 and 400 mg/l were prepared in distilled water by dilution of a 1 g/l paracetamol solution in methanol. These solutions were stable for at least 3 months if stored at 4°C in the absence of light. On analysis of these solutions, the ratio of the peak area of acetylated paracetamol to that of the internal standard was linear (Fig. 2). The calibration gradient normally obtained was 0.008 l/mg, with an intercept of −0.086.

RESULTS AND DISCUSSION

Choice of extraction conditions

Although some procedures involving the GLC analysis of underivatised paracetamol have been described, the majority of published methods incorporate a derivatisation step prior to chromatographic assay[3]. However, these derivatisations were not only time-consuming in themselves, but also required prior solvent extrac-

TABLE I
RETENTION TIMES OF ACETYLATED PARACETAMOL, N-BUTYRYL-*p*-AMINOPHENOL AND SOME OTHER COMPOUNDS ON TWO-COLUMN SYSTEMS
See text for chromatographic conditions.

Compound	*Retention time (relative to N-butyryl-p-aminophenol)*	
	C_{87} hydrocarbon system	*SP-2250 system*
Ethosuximide	0.19	0.15
Barbitone	0.32	0.27
Methyprylone	0.36	0.32
Ibuprofen	0.40	(0.21 and 0.29)
Allobarbitone	0.41	0.35
Aprobarbitone	0.45	0.37
Meprobamate	0.47	0.71
Butobarbitone	0.48	0.39
Allylbarbitone	0.50	0.38
Methsuximide	0.50	0.37
Amylobarbitone	0.54	0.43
Phenacetin	0.55	0.51
Paracetamol (acetylated)	0.59	0.68
Chlorpropamide	0.60	0.37
Pentobarbitone	0.63	0.48
Quinalbarbitone	0.68	0.55
Ethotoin	0.76	0.87
Methoin	0.77	0.76
"Glutethimide metabolite"	0.84	1.58
Phenylethylmalondiamide	0.85	1.19
Brallobarbitone	0.87	0.85
Hexobarbitone	0.91	0.79
Caffeine	0.93	0.92
Glutethimide	0.96	0.82
Lignocaine	1.00	0.73
Phenazone	1.00	0.95
N-Butyryl-*p*-aminophenol (acetylated)	1.00	1.00
Amidopyrine	1.08	1.00
Thiopentone	1.09	0.68
Diphenhydramine	1.12	0.67
Propylphenazone	1.18	1.07
"Glutethimide metabolite"	1.20	1.00
Phenobarbitone	1.22	1.26
Cyclobarbitone	1.29	1.17
Orphenadrine	1.36	0.83
Heptabarbitone	1.74	1.57
Cyclizine	2.21	1.25

tion and concentration steps, rendering the procedures too lengthy for emergency use. The introduction[6] of N-methylimidazole as both catalyst and proton-accepting base in place of pyridine in the catalysis of acetylation reactions prompted the present work. N-Methylimidazole, a liquid at room temperature, is not only a more efficient catalyst than pyridine but is also widely available, non-toxic and miscible with water.

Although it was thought that a large excess of acetic anhydride would be required if the derivatisation of paracetamol was attempted in the presence of water,

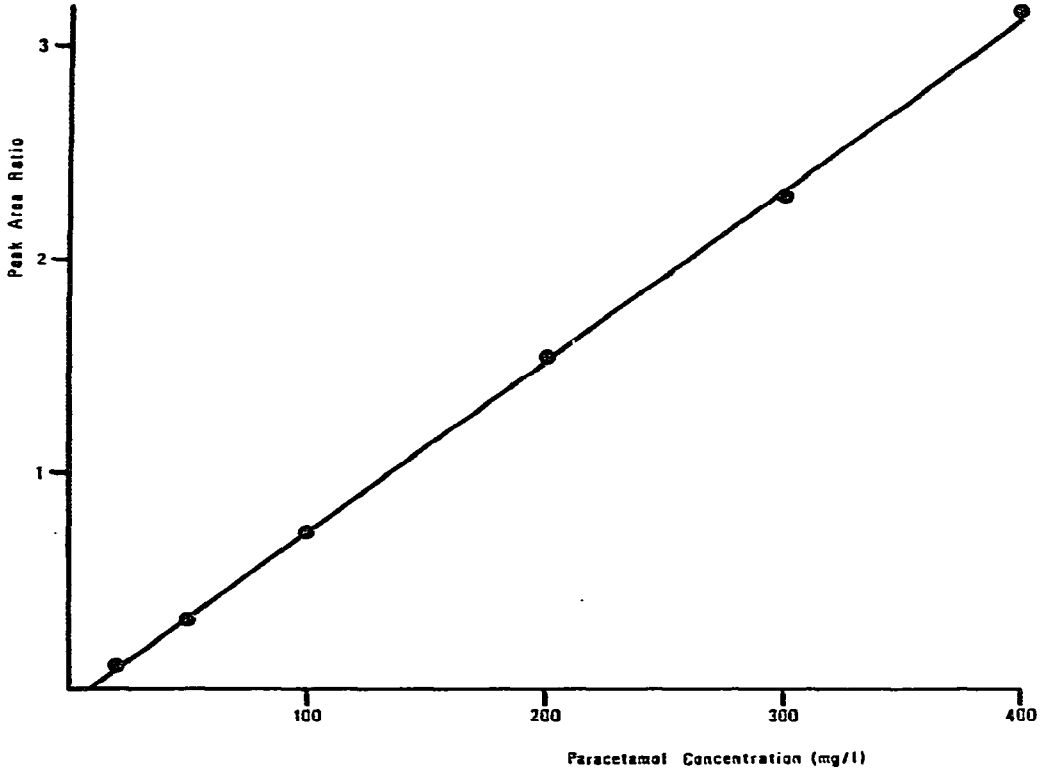

Fig. 2. Calibration graph of peak area ratio of acetylated paracetamol to internal standard against paracetamol concentration in the aqueous calibration standards.

the direct use of an acetic anhydride–catalyst–chloroform mixture has allowed the simple extraction procedure to be used. In practice, it was found that only relatively small amounts of both acetic anhydride and N-methylimidazole were required, and this had the advantage of restricting the size of the "solvent front" obtained on GLC analysis of the extract to that normally associated with the injection of chloroform alone (Fig. 1). Phosphate buffer was used in the assay in order to yield a final extraction mixture with a pH of *ca.* 6. On analysis of extracts of standard aqueous paracetamol solutions, both paracetamol and the internal standard gave rise to sharp, symmetrical peaks (Fig. 1), suggesting that the acetylated derivatives had been formed. Analysis of these standard solutions performed without the addition of acetic anhydride did not give rise to any peaks, whereas treatment of both paracetamol and N-butyryl-*p*-aminophenol with N-methylimidazole–acetic anhydride (2:1, v/v) for 10 min at room temperature gave rise to compounds with retention times identical with those obtained on the direct analysis of aqueous paracetamol solutions (Table I).

Recovery studies

Standard solutions containing paracetamol at concentrations of 50, 100 and 250 mg/l were prepared in heparinised bovine plasma by dilution of a 1 g/l methanolic

solution of the drug. The apparent recoveries of acetylated drug were 94.9 ± 1.1, 94.5 ± 1.3 and 95.8 ± 0.4 (S.E.M.)%, respectively (n = 10 at each concentration), when compared with the aqueous calibration solutions. (*N.B.* The latter solutions were preferred for routine use in view of their greater stability with respect to plasma standards[7].)

Sources of interference

No endogenous sources of interference have been observed. The chromatogram obtained on analysis of a specimen of drug-free human plasma is illustrated in Fig. 3, and that obtained using a specimen from a patient who had ingested paracetamol is shown in Fig. 4. Analyses undertaken without the addition of the internal

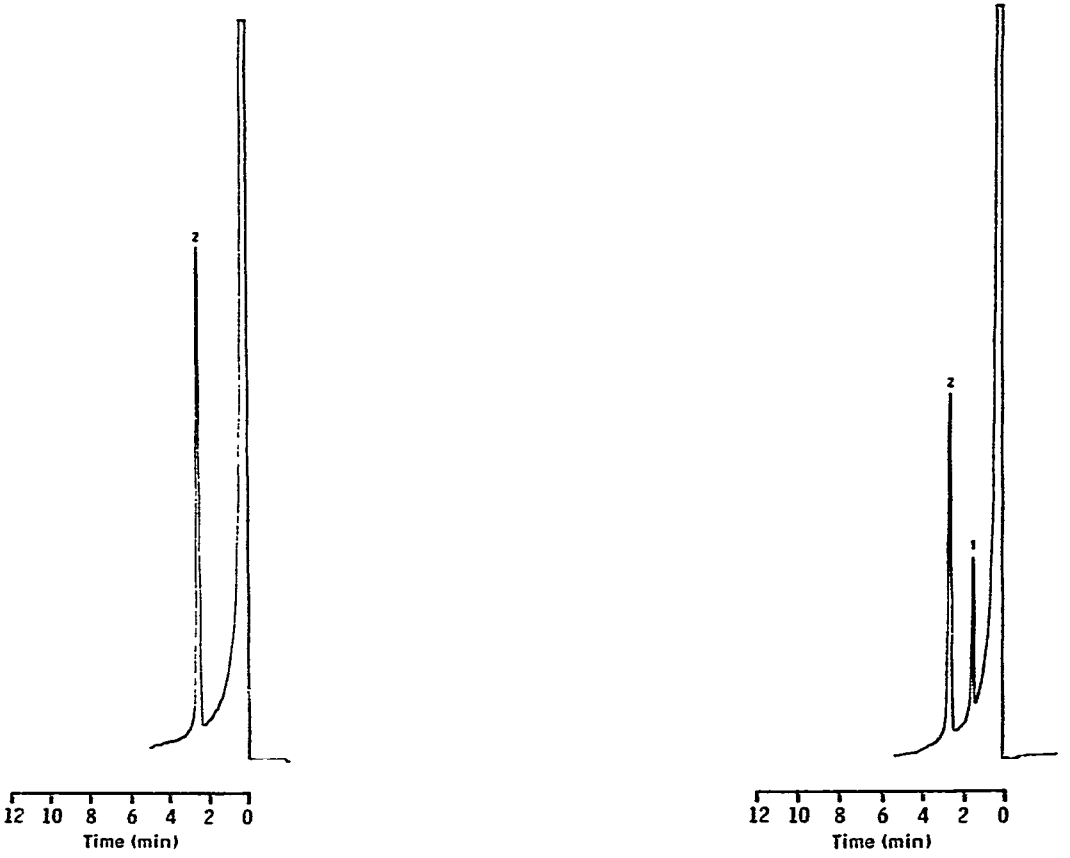

Fig. 3. Chromatogram obtained on analysis of an extract of drug-free human plasma on the C_{87} hydrocarbon system; 5-μl injection. The initial N-butyryl-*p*-aminophenol concentration was 200 mg/l, and peak 2 is acetylated N-butyryl-*p*-aminophenol.

Fig. 4. Chromatogram obtained on analysis of an extract of plasma from a patient who had ingested paracetamol on the C_{87} hydrocarbon system; 4-μl injection. The initial N-butyryl-*p*-aminophenol concentration was 200 mg/l, and the plasma paracetamol concentration was found to be 44 mg/l. Peaks: 1 = acetylated paracetamol; 2 = acetylated N-butyryl-*p*-aminophenol.

standard have not revealed the presence of endogenous compounds which could interfere with this latter substance. Analyses of specimens obtained *post mortem* have given analogous results.

Assessment of potential interference from other drugs or exogenous agents is complicated by the need to consider not only drugs directly extracted under the conditions of the assay but also additional compounds which could be acetylated/extracted. However, the range of compounds involved is probably no greater than with methods incorporating separate extraction and derivatisation steps[5]. A further consideration is that the concentrations of paracetamol attained following overdosage are relatively high (in our experience up to 1 g/l) when compared with most centrally-acting agents such as barbiturates, glutethimide or methaqualone[8], suggesting that only compounds potentially occurring at plasma concentrations of the order of 10 mg/l or greater need be considered. Finally, if the presence of interfering compounds is suspected, analysis of specimens without addition of the acetylating reagent but using pH 4.5 buffer[8] provides a simple means of differentiating between compounds extracted directly under the conditions of the assay and those, such as paracetamol and N-butyryl-*p*-aminophenol, which have to be acetylated prior to chromatographic analysis.

No interference from exogenous compounds has been observed during the routine operation of the assay over a nine-month period, during which time specimens from patients who had ingested or been treated with a wide variety of compounds were analysed. (*N.B.* Whenever possible, the identification of paracetamol in plasma specimens was confirmed by a colour test performed by addition of *o*-cresol–ammonia to a hydrolysed urine specimen[9].) In particular, interference from salicylate, diflunisal, ibuprofen and benorylate or from metabolites of paracetamol or chlormethiazole was not observed. Of the drugs which were extracted and chromatographed under the conditions of the assay (Table I), several (including phenacetin) present potential sources of interference. (*N.B.* Some additional compounds, such as phenytoin, primidone, carbamazepine, azapropazone, methaqualone and the benzodiazepines eluted at retention times greater than 1.80 relative to acetylated N-butytyl-*p*-aminophenol.) Phenacetin was resolved from paracetamol on a second relatively polar column system, 3% SP-2250 on Chromosorb W HP, 80–100 mesh (Chromatography Services, Hoylake, Great Britain). The retention times of the compounds under study on this second system are given in Table I, and the separation of acetylated paracetamol and N-butyryl-*p*-aminophenol is illustrated in Fig. 5. The chromatographic conditions employed for this second system were identical with those used for the C_{87} hydrocarbon system.

Interference from "cresol B.P." (a mixture of *o*-, *m*- and *p*-cresol) and "chlorocresol B.P." (4-chloro-3-methylphenol) did not occur. These compounds are used as preservatives in mucous heparin solutions and, as reported by Pitts[10], contamination from the solution containing "cresol B.P." especially can cause serious interference in the widely-used spectrophotometric paracetamol assay of Glynn and Kendal[11]. Contamination of this type is possible if a specimen is drawn initially into a heparinised blood–gas syringe, and may not be recognised unless the absorption spectrum of the final reaction product is examined. Similarly, potential interference from salicylate in this latter method, although not of great magnitude, necessitates either examination of the final absorption spectrum or simultaneous salicylate measurement[3]. As with all

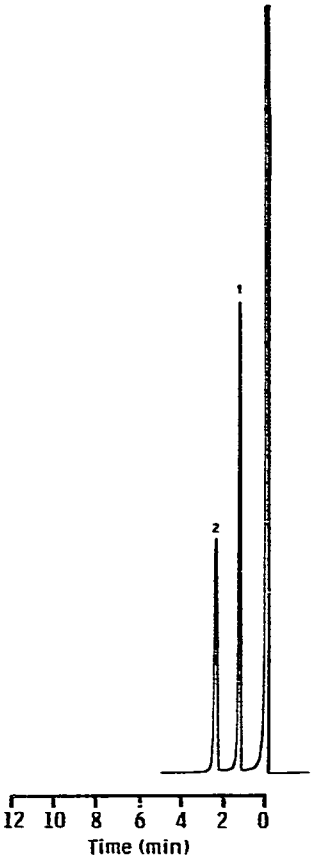

Fig. 5. Chromatogram obtained on analysis of an extract of an aqueous solution containing paracetamol (200 mg/l) on the SP-2250 system; 4-μl injection. The initial N-butyryl-*p*-aminophenol concentration was 200 mg/l. Peaks: 1 = acetylated paracetamol; 2 = acetylated N-butyryl-*p*-aminophenol. See text for chromatographic conditions.

spectrophotometric methods for plasma paracetamol assay, interference from this or other sources can lead to equivocal results, especially at relatively low plasma concentrations. This can be very important where the patient presents, or paracetamol overdose is suspected on clinical grounds, many hours after ingestion of the drug. In such cases, the diagnosis often rests upon the accurate measurement of the residual paracetamol plasma concentration.

Reproducibility

The intra-assay coefficients of variation (C.V.) obtained on replicate analysis of standard solutions of paracetamol prepared in expired blood-bank plasma[7] were 4.2% at 48 mg/l (n = 10) and 2.3% at 238 mg/l (n = 10).

Limit of sensitivity

The limit of accurate measurement of the assay was 10 mg/l; the intra-assay C.V. at this concentration was 9.2% (n = 7).

External quality control scheme

Paracetamol solutions prepared in expired blood-bank plasma[7] were analysed by the present method over a nine-month period. There was a good correlation between the results obtained (mean = 195 mg/l, range 50–476 mg/l) and the weighed-in paracetamol value (mean = 192 mg/l; range 48–492 mg/l) ($r = 0.99$; $n = 27$). Linear regression analysis using the weighed-in value as the independent variable revealed a gradient of 1.06 and an intercept on the y-axis of −7.9 mg/l.

Catalyst concentration, extraction time and extract stability

In order to investigate the effect of such variables as the concentration of N-methylimidazole on the apparent recovery of acetylated paracetamol and internal standard, a second non-derivatisable standard was incorporated in the procedure. The compound chosen, *n*-tetracosane (C_{24}) (Koch-Light, Colnbrook, Great Britain), eluted at a retention time of 1.42 on the SP-2250 system relative to acetylated N-butyryl-*p*-aminophenol, and was used as a 1 g/l solution in chloroform (analytical-reagent grade). The investigations were performed using standard solutions of paracetamol (100 mg/l) prepared in water or expired blood-blank plasma. The extractions were performed as described previously in triplicate, except that *n*-tetracosane solution (20 μl) was added to each tube prior to vortex mixing and, in the first instance, changes were made in the amount of N-methylimidazole added. Peak-height measurements of both acetylated paracetamol and N-butyryl-*p*-aminophenol were performed relative to the peak height of *n*-tetracosane.

Use of an acetylating reagent consisting solely of acetic anhydride–chloroform (1:6) with an aqueous paracetamol solution did give rise to acetylated paracetamol and N-butyryl-*p*-aminophenol (mean peak height ratios 0.39 and 0.28, respectively), but these ratios were less than those obtained using the normal acetylating reagent (mean ratios 0.50 and 0.40, respectively) and there was greater variation in the individual results. Re-mixing and subsequent analysis of the extracts performed without the addition of the catalyst did give an increased yield of the acetylated compounds (mean ratios 0.46 and 0.34, respectively), but no major change was observed following this sequence using the normal extracts (mean ratios 0.48 and 0.41). Addition of N-methylimidazole at twice the normal concentration in a further experiment did not give rise to an increased yield of acetylated products (mean ratios 0.50 and 0.41, respectively).

Information on the adequacy of the 30-sec mixing period was obtained from the experiment above where the results of the re-mixing and re-analysis of normal extracts were quoted. The stability of these extracts was shown by re-analysis of the extracts after 8 h at ambient temperature, both before and after re-mixing, which gave identical results (mean ratios 0.48 and 0.41, respectively) to those given above. Analyses of the plasma paracetamol solution gave analogous results to those obtained with the aqueous standard with respect to extraction time and extract stability, given the slightly lower relative recovery of paracetamol from plasma as compared with aqueous solutions.

CONCLUSIONS

The method described here has been found to be suitable for the measurement

of the plasma paracetamol concentrations attained after overdosage with this compound. N-Methylimidazole is preferable to pyridine as a catalyst in the derivatisation procedure since the former compound is widely available and is also non-toxic, and thus the procedure can be carried out in the open laboratory. Neither extract concentration nor prolonged heating is employed, and only 200 μl of specimen are required for a duplicate analysis which can be completed, together with the analysis of a quality control specimen, within 20 min. The procedure is relatively specific, and offers a useful alternative to liquid chromatographic procedures for emergency plasma paracetamol measurements.

ACKNOWLEDGEMENTS

We thank Mr. J. Epton, Regional Toxicology Laboratory, Dudley Road Hospital, Birmingham, who supplied the quality control sera, Winthrop Laboratories for their gift of N-butyryl-*p*-aminophenol and Dr. B. Widdop, Poisons Unit, for criticism of the manuscript.

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