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Note

Improved gas—liquid chromatography—electron-capture detection technique for the determination of paracetamol in human plasma and urine

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Paracetamol is an easily available analgesic. Thus, poisoning by paracetamol is still a relatively common occurrence [1]. A quantitative, rapid, selective and sensitive method for the determination of paracetamol in biological fluids is essential for investigations such as poisoning due to acute overdose, research into factors affecting drug absorption in the gastro-intestinal tract when the drug is used as a marker, and studies on pharmacokinetics and bioavailability of this drug in various innovative dosage forms. A number of quantitative methods for the estimation of this drug in plasma or urine samples have been described but none have been ideal. The generally accepted gas—liquid chromatographic methods [2,3] suffer from interference peaks which often mask analytical peaks or prolong analysis time [4,5]. Hackett and Dusci [6] reported an improved procedure which involved column extraction with diethyl ether, acetylation and estimation by GLC with flame ionization detection. The extraction procedure can be time-consuming and the lowest limit for quantitation is about 5 μg/ml in a 2-ml sample of plasma which, however, is adequate for detecting paracetamol poisoning.

This paper reports a specific and sensitive GLC—electron-capture detection assay for paracetamol in human plasma and urine samples based on the method of assay for indomethacin in biological fluids previously described [7]. A preliminary report of the procedure was presented at the Pharmaceutical Conference [8].

METHODS

Apparatus

A Pye Series 104 gas chromatograph was used, fitted with a nickel 63 electron-capture detector and linked to a chart recorder (Hitachi Model 156). The detector was operated at a temperature of 330°, with a nitrogen purge flow-rate of 11.0 ml/min. A coiled glass column (1.5 m × 4 mm I.D.) packed with 3%

SP2100 coated on Supelcoport 100—120 mesh (Phase Separation, Queensferry, Great Britain) was used. The column was conditioned at 300° for 48 h and silanised in situ with 2 × 10 µl of hexamethyl disilane (HMDS) before use. The injector temperature was 300° and the oven temperature was 200°. White spot nitrogen was used as carrier gas at a flow-rate of 50 ml/min.

Centrifuge tubes (10 ml and 20 ml) with well-fitting screw caps were obtained from Sovirel (A.V. Howes, Great Britain); 10-µl SGE microsyringes from Chromatography Services (Wirral, Great Britain); Pipetman microsyringes from Anachem (Bedford, Great Britain), and for thin-layer chromatography, TLC plates coated with 0.3 mm silica gel, Merck GF254 from Merck (Darmstadt, G.F.R.).

Materials

The following chemicals were used: acetone, dichloromethane, diethyl ether, methanol and *n*-hexane (all of Analar grade and freshly redistilled); Sörensen phosphate buffer (1 *M*) pH 7.4; Analar ammonium sulphate and potassium carbonate (diethyl ether washed and dried); pentafluorobenzyl bromide (Pierce, Rockford, Ill., U.S.A.) stock solution for use was prepared by diluting the supplied material 1:1000 (v/v) in acetone; paracetamol (BDH, Poole, Great Britain), *n*-butyryl-*p*-aminophenol, the internal standard (R & R Lab., Hollywood, Calif., U.S.A.).

Treatment of glassware

It was found that all glassware should be cleaned and silanised before use according to the following procedure in order to eliminate impurities which might interfere with analytical peaks and minimise the loss of drugs which might absorb on to the wall of glassware. Evaporation tubes and screw-capped centrifuge tubes were cleaned in a 2% solution of RBS 25 (Chemical Concentrates (RBS), London, Great Britain) in water by soaking overnight, then rinsed with methanol (commercial grade) and hot tap water and then distilled water several times. The tubes were then dried in an oven at 105°. After cooling at room temperature, all the tubes were then silanised by rinsing with a 3% solution of HMDS in redistilled chloroform. They were then dried at 250° overnight. Silanisation of tubes should be repeated when necessary at monthly intervals.

Synthesis and characterisation of fluoro derivatives

The pentafluorobenzyl derivatives of paracetamol and its internal marker, *n*-butyryl-*p*-aminophenol were synthesised as follows: paracetamol or its internal marker ($1.6 \cdot 10^{-4}$ mole, 25 mg) was dissolved in re-distilled acetone (2.0 ml) in a micro round bottom flask (5.0 ml). Pentafluorobenzyl bromide ($3 \cdot 10^{-4}$ mole, 50 µl) and anhydrous potassium carbonate ($3.5 \cdot 10^{-4}$ mole, 50 mg) were added. The mixture was heated under reflux for 5 h at 50°. After cooling, the acetone in the reaction mixture was removed by a gentle stream of nitrogen. Distilled water (1.0 ml) was added to dissolve the potassium carbonate and the derivative was extracted into diethyl ether and subsequently recrystallised in acetone.

Paracetamol, *n*-butyryl-*p*-aminophenol and their pentafluorobenzyl deriva-

tives were characterised by thin-layer chromatography, GLC, infra-red spectroscopy and melting point determination.

Procedure for determination of paracetamol in biofluids

Duplicate samples of 0.5 ml of plasma or urine were pipetted into 20-ml glass centrifuge tubes and diluted with 0.5 ml of distilled water; 50 μl of internal marker (500 ng) solution, 1.0 ml of phosphate buffer (pH 7.4) and 1.0 g of ammonium sulphate were added. The tubes were each vortex mixed for 10 sec and 10 ml of an extracting solvent mixture [diethyl ether—dichloromethane (4:1)] were added. The tubes were then shaken by an automatic shaker (40 rpm) for 10 min followed by centrifugation at 1500 *g* for 10 min.

The organic layer was carefully transferred to a clean 10-ml screw-capped tube and evaporated to dryness at 45° under a stream of nitrogen. The walls of the tubes were then carefully rinsed using acetone (about 1.0 ml) which was subsequently evaporated to dryness. To each tube was added 25 mg of potassium carbonate and 0.5 ml of the stock solution of pentafluorobenzyl bromide. Pentafluorobenzylation was carried out in the well screw-capped tube at 60° for 30 min using a thermostatic water-bath. The reaction tubes were carefully agitated every 10 min during this period. The excess derivatising agent was then evaporated off under a stream of nitrogen at room temperature. To each tube was added 0.5 ml phosphate buffer and 0.5 ml *n*-hexane. The tubes were then mixed on an automatic shaker at a speed of 40 rpm for 10 min. An aliquot of the *n*-hexane layer (0.5—1.0 μl) was injected onto the gas chromatography column.

Standard solutions of paracetamol and its internal marker were prepared in methanol. These were diluted to give a series of solutions in plasma or urine (1 ml) covering the concentration range 50—1000 ng in a 0.5-ml sample of plasma or urine. The solutions were then analysed as described in the procedure, and the peak height ratios were plotted against the corresponding concentrations. The recovery of paracetamol from plasma or urine using the present extraction condition was well documented [9].

Reproducibility was checked by performing an anlysis on plasma samples containing paracetamol at concentrations of 50 and 500 ng per 0.5-ml sample.

The procedure was used to determine paracetamol concentrations in plasma and urine after a single oral dose of 1.0 g paracetamol (2 Panadol tablets of 0.5 g) was given to a healthy male subject.

RESULTS AND DISCUSSION

Table I summarises the characteristics of paracetamol, its internal marker *n*-butyryl-*p*-aminophenol and their pentafluorobenzyl derivatives. The melting points, thin-layer chromatographic R_F values, retention times of GLC in both electron-capture and flame ionization detection indicate that the pentafluorobenzylation reaction is successful. The absence of OH-stretching in the IR spectra of the derivatised paracetamol and internal marker also supports the view that the derivatisation procedure is correct although it was not possible to carry out a GC—mass spectrometry analysis on the corresponding peak.

Optimal formation of pentafluorobenzyl derivatives was found to occur at a

TABLE I

CHARACTERISTICS OF PARACETAMOL, *n*-BUTYRYL-*p*-AMINOPHENOL AND THEIR PENTAFLUOROBENZYL DERIVATIVES

Compound	Melting point (°C)	TLC* R_F	GLC retention time (min) using 10% Apiezon at 240°**		GLC retention time (min) using 3% SP2100 at 200°	
			FID	ECD	FID	ECD
Paracetamol	172	0.79	3.4 (TP)	–	1.8 (TP)	–
n-Butyryl-*p*-aminophenol	143	0.90	5.6 (TP)	–	3.4 (TP)	–
PFB-paracetamol	193	0.89	4.0	4.0	2.4	2.4
PFB-*n*-butyryl-*p*-aminophenol	162	0.98	6.2	6.2	4.6	4.6

*TLC system consists of silica gel plate (Merck GF 254) and solvent composition: ethyl-acetate—methanol—water—acetic acid (60:30:9:1) and detection using UV light and Dragendorff reagent.

**GLC system reported by Chan and Highley [8]. TP = Tailing peak.

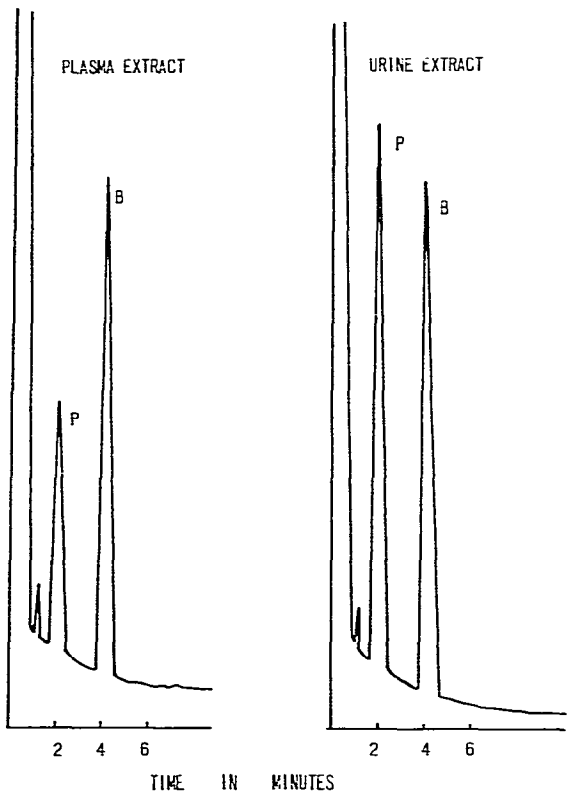

Fig. 1. Chromatograms of pentafluorobenzyl derivatives of paracetamol (P) and *n*-butyryl-*p*-aminophenol (B). Plasma extract: 200 ng per 0.5 ml; urine extract: 500 ng per 0.5 ml.

reaction temperature of 60° for a period of 30 min. This is in agreement with Walle's [10] suggestion of a reaction time of 5—120 min for the pentafluorobenzylation of barbituric acids and diphenylhydantoin.

In a previous report [8], 10% Apiezon L was used as a stationary phase for analysis. As the oven temperature was 240°, which is very close to the maximum recommended temperature for this stationary phase, column bleeding was observed. Further investigation suggested that 3% SP2100 on Supelcoport was considered satisfactory. This stationary phase has a high recommended temperature up to 375°.

Fig. 1 illustrates a typical GLC trace of paracetamol and *n*-butyryl-*p*-aminophenol, as their *o*-pentafluorobenzyl derivatives, extracted from both plasma and urine. Under the GLC conditions described the retention times of pentafluorobenzyl-paracetamol and pentafluorobenzyl-internal marker were 2.4 min and 4.6 min, respectively. The overall accuracy and reproducibility of the analytical procedure is satisfactory. The recoveries of paracetamol from plasma at 50 ng and 500 ng levels were 50.55 ± 2.41 ng and 494.66 ± 12.5 ng, respectively. The graph is linear over the range of 50 ng to 1000 ng per 0.5 ml of plasma and was found to be reproducible when repeated six times during the studies.

The present procedure provides a relatively selective and sensitive assay for the determination of paracetamol concentrations in biological fluids. Using this technique a pharmacokinetic study of an oral dose of Panadol (1 g) in a fasting subject was performed. The results obtained, i.e. an elimination $t_{1/2}$ of 2.69 h and a 24-h urinary recovery of 5.57% as the unchanged drug, compare favourably with previous reported data and indicate that the technique is reliable. Thus, this method has several advantages over those previously reported. Only a small sample of biological fluids (0.5—1.0 ml) is required for analysis. The high sensitivity of the assay allows a more thorough study of the fate of paracetamol in the body. The selectivity of the electron-capture detector gives a cleaner chromatogram and enables analysis of up to ten samples in an hour. A disadvantage is that all organic solvents used should be distilled twice.

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