Journal of Chromatography, 530 (1990) 222–225
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5348

Letter to the Editor

Liquid–liquid extraction and analysis of paracetamol (acetaminophen) and its major metabolites in biological fluids by reversed-phase ion-pair chromatography

Sir,

Paracetamol (acetaminophen; N-acetyl-*p*-aminophenol), at therapeutic doses, is a safe and effective analgesic; 80–90% of the drug is metabolised by conjugation to paracetamol glucuronide (60%) and paracetamol sulphate (30%) which are then excreted in the urine [1]. Current assays for paracetamol and its metabolites in biological fluids involve either direct injection of urine samples or protein precipitation of plasma samples, prior to injection [2–8]. However, we found that direct injection of samples did not always give an adequate resolution between the compounds of interest and endogenous, interfering compounds. We describe a new assay method which involves ion-pair liquid–liquid extraction, with subsequent detection and quantitation by reversed-phase ion-pair isocratic high-performance liquid chromatography (HPLC).

EXPERIMENTAL

High-performance liquid chromatography

The chromatographic system employed a Waters pump (Model 510), a Waters sample processor (Model 710B), a Waters data module (Model H730), a Pye Unicam variable-wavelength UV detector and a reversed-phase C_{18} steel column (5 μm particle size, 15 cm × 4 mm I.D.; FSA Laboratory Supplies, Loughborough, U.K.). Filtration of solvents was carried out using a Pyrex holder, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.).

All tests were carried out at ambient room temperature (approximately 20°C). Solvents were mixed to the required volume after being filtered through a 0.2-μm Millipore filter, and the mobile phase was degassed by sonication under vacuum. The column was equilibriated with the mobile phase for at least 30 min prior to analysis of samples. The mobile phase was methanol–1% acetic acid, containing tetrabutylammonium dihydrogenphosphate (TBA) (500 μM) and potassium sulphate (20 mM) (18:82, v/v) at a flow-rate of 1.2 ml/min (pressure 80 bar). Paracetamol, paracetamol glucuronide and paracetamol sulphate were detected at 254 nm.

0378-4347/90/$03.50

Reagents

Paracetamol glucuronide and paracetamol sulphate were generously donated by Sterling Winthrop (Alnwick, U.K.). Paracetamol and β-hydroxyethyltheophylline, which was used as the internal standard (I.S.), were purchased from Sigma (St. Louis, MO U.S.A.). Methanol (HPLC grade) was supplied by FSA Lab. (Loughborough, U.K.), TBA (1.0 *M* solution) by Aldrich (Gillingham, U.K.) and potassium sulphate and glacial acetic acid by BDH (Poole, U.K.). Water was distilled and deionized using a nanopure water system (Nanopure II, Barnstead, Fisons, U.K.).

Sample preparation

Samples of plasma or urine (200 μl) were spiked with paracetamol, paracetamol glucuronide, paracetamol sulphate and I.S. TBA (0.2 ml) and ammonium sulphate (0.2 g) were added, followed by mixing for 60 s. Paracetamol, its metabolites and the internal standard were extracted with 3 ml of chloroform–isopropanol (50:50) by mixing for 60 s. After centrifugation (5 min at 2500 *g*), 2 ml of the organic layer were transferred and evaporated to dryness at 35°C under a stream of nitrogen gas. The residue was redissolved in 200 μl of the mobile phase by mixing for 60 s. Quantitaion of paracetamol and its metabolites was achieved by sample/I.S. peak-area ratio method. A standard curve for each compound was prepared. The extraction efficiency was determined by extraction of test samples and the addition of the I.S. followed by analysis.

RESULTS AND DISCUSSION

The elution patterns for plasma and urine samples, using the extraction method, and that of the supernatant obtained after protein precipitation of plasma with trichloroacetic acid are shown in Fig. 1. The mean recovery for paracetamol, paracetamol sulphate and paracetamol glucuronide was in excess of 88% (coefficient of variation $<12\%$) over the range of the standard curve (5.0–100 μg/ml) when compared to the direct injection of standard samples dissolved in water. The limit of detection was 0.1 μg/ml for paracetamol and its metabolites. There was some degree of interference from the peaks due to endogenous compounds with peaks due to paracetamol and its metabolites in samples of urine and plasma, using the extraction method. However, this did not result in any significant overestimation of paracetamol and its metabolites as was evident from precision and percentage recovery for these compounds.

Due to the large differences in polarity between paracetamol and its metabolites, selection of a simple organic extraction solvent providing a high extraction coefficient for both paracetamol and its metabolites was impossible. Thus even at low pH, when both paracetamol and its metabolites are predominantly unionized, extration of the metabolites from the aquous to organic phase (chloroform–isopropanol, 95:5, v/v) solvent was very inefficient. Increasing the polarity of the

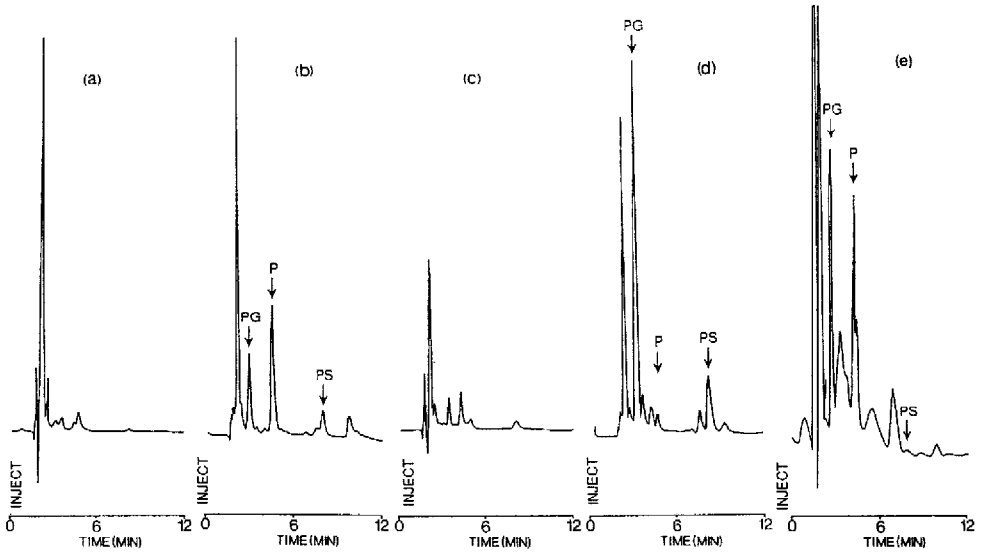

Fig. 1. Elution patterns following extraction for (a) a blank plasma sample, (b) a plasma sample from a subject 2 h after receiving 500 mg paracetamol intravenously, (c) a blank urine sample, (d) a urine sample from a subject 2 h after receiving 500 mg paracetamol intravenously and (e) the plasma supernatant (sample as in b) obtained by protein precipitation with trichloroacetic acid (20%, w/v). Trichloroacetic acid (100 μl) was added to 100 μl of plasma, followed by centrifugation for 5 min at 2500 *g*.

extraction solvent (ethyl acetate–chloroform–isopropanol, 40:40:20, v/v) did not result in a significant improvement on the extraction efficiency. Instead, it resulted in extraction of other interfering endogenous compounds.

Ion-pair extraction has been shown to be particularly efficient for polar and ionized compounds [9]. Therefore, the highly polar paracetamol glucuronide and paracetamol sulphate were extracted by means of an ion-pair extraction whilst paracetamol was extracted by normal liquid–liquid extraction. Paracetamol glucuronide and paracetamol sulphate form an ion-pair complex with TBA counter-ion which can be efficiently extracted by a chloroform–isopropanol (50:50, v/v) mixture. Extraction efficiency was improved by the addition of high concentrations of ammonium sulphate to produce a salting-out effect. The optimum concentration for TBA was found to be 0.1 *M*, and higher concentrations did not improve the extraction efficiency. Increasing the polarity of the extraction medium did not result in greater recoveries. Following extraction, samples were analysed by a reversed-phase ion-pair isocratic HPLC system. The addition of TBA counter-ion had a profound effect on the retention times of paracetamol and its metabolites, especially paracetamol sulphate, and the addition of a co-ion such as potassium sulphate was necessary in order to prevent the excessive retention of paracetamol sulphate. The optimum concentration of the TBA counter-ion in the presence of potassium sulphate was found to be 500 μmol/l, and increasing the

concentration of TBA above the optimum level resulted in longer retention times for paracetamol and its metabolites, particularly paracetamol sulphate.

The ion-pair extraction technique described has a high degree of selectivity and efficiency for paracetamol and its metabolites. This, together with ion-pair HPLC, provides an accurate and sensitive method of measuring paracetamol and its metabolites in various biological fluids.

Wolfson Unit of Clinical Pharmacology, F. KAMALI*
Department of Pharmacological Sciences, B. HERD
The University, Newcaste upon Tyne NE1 7RU (U.K.)

1 A.J. Cumming, M.L. King and B.K. Martin, *Br. J. Pharmacol. Chemother.*, 29 (1967) 150.
2 D. Howie, P.I. Adriaenssen and L.F. Prescott, *J. Pharm. Pharmacol.*, 29 (1977) 233.
3 P.I. Adriaenssen and L.F. Prescott, *Br. J. Clin. Pharmacol.*, 6 (1978) 87.
4 N. Watari, M. Iwai and N. Kaneniwa, *J. Pharmacokin. Biopharm.*, 11 (1983) 245.
5 P. Moldeus, *Biochem. Pharmacol.*, 27 (1978) 2859.
6 J. Knox and L. Jurand, *J. Chromatogr.*, 142 (1977) 651.
7 J.M. Wilson, J.T. Slattery, A.J. Forte and S.D. Nelson, *J. Chromatogr.*, 227 (1982) 453.
8 G. Ladds and K. Wilson, *J. Chromatogr.*, 414 (1987) 355.
9 G. Schill, *Separation Methods for Drugs and Their Related Organic Compounds*, Apothekar Societetan, Stockholm, 1978.

(First received February 5th, 1990; revised manuscript received April 2nd, 1990)