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Note

Novel method of sample preparation for the determination of paracetamol in plasma by high-performance liquid chromatography with electrochemical detection

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Paracetamol (APAP) is a widely used mild analgesic. Many methods for its determination at therapeutic and toxic levels in plasma or serum have been published [1—6]. Such methods are, however, not sufficiently sensitive when sub-therapeutic drug levels are to be measured. Methods reporting the sensitivity necessary to quantify sub-therapeutic drug levels accurately have also been published [7—9]. Sample preparation and analysis times are, however, unacceptably long when many samples are to be analysed by these methods. The combination of a large number of samples with the need to measure sub-therapeutic levels of APAP frequently occurs in pharmacokinetic studies. A new method was therefore developed to meet the conflicting requirements of maximal sensitivity and minimal analysis time.

High-performance liquid chromatography (HPLC) with electrochemical detection (ED) is an accepted method for the determination of trace amounts of APAP [10, 11]. This paper reports a novel method of sample preparation which in conjunction with HPLC—ED allows accurate and precise quantification of APAP in plasma at low levels (10 ng/ml) whilst keeping sample preparation and analysis time to a minimum.

EXPERIMENTAL

Materials

Ethyl acetate, 1,1,2-trichlorotrifluoroethane (TCTFE), sodium acetate, ammonium acetate, glacial acetic acid and sodium hydroxide were all obtained

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from BDH (Poole, U.K.). Methanol was obtained from Fisons (Loughborough, U.K.). All chemicals were AnalaR grade with the exceptions of TCTFE which was GPR grade and methanol which was HPLC grade. APAP was obtained commercially.

N-Propionyl-*p*-aminophenol (PPAP) was synthesised from *p*-aminophenol and propionic anhydride by a modification of the method reported by Horvitz and Jatlow [3]. *p*-Aminophenol hydrochloride (2.9 g) and sodium propionate (2.0 g) were dissolved in ca. 25 ml water. Propionic anhydride (3.0 ml) was added and the mixture was stirred for 10 min. The pH of the mixture was adjusted to 6—7 with concentrated sodium hydroxide solution saturated with sodium chloride. The product was extracted twice with 50-ml portions of ethyl acetate. These were combined and dried by filtering through anhydrous sodium sulphate. The ethyl acetate was evaporated under a stream of air with gentle heating (50°C). The residue was recrystallised twice from water/industrial methylated spirits. Decolourising charcoal was used to remove any faint colouration. The product was a chromatographically homogeneous colourless fine crystalline material.

Sample preparation

Plasma (1.0 ml) and ethyl acetate (3.5 ml) were pipetted into a 15-ml test tube containing 100 μl PPAP solution. The concentration of PPAP was dependent upon the expected concentration of APAP. For expected APAP concentrations of < 2, 2–10 and > 10 μg/ml, concentrations of 2, 5 and 25 μg/ml PPAP, respectively, were used. The tube was vortex-mixed for 15 s and then centrifuged for 5 min at 1000 *g*. The organic phase (3.0 ml) was transferred to a fresh test tube containing 3.0 ml of TCTFE and 250 μl of 0.001 *M* sodium acetate adjusted to pH 12. The tube was again vortex-mixed for 15 s and centrifuged for 5 min at 1000 *g*. The aqueous phase (150 μl) was transferred to a 250-μl glass vial containing 20 μl of 10% (v/v) acetic acid.

Standards were prepared using 1.0-ml aliquots of appropriately spiked drug-free plasma which were then treated as described above.

Chromatography

The mobile phase was 0.1 *M* sodium acetate and 0.001 *M* ammonium acetate—methanol (77:23) adjusted to pH 4.75 with glacial acetic acid. It was degassed in an ultrasonic bath before use.

The chromatographic system consisted of a Model 2150 pump (LKB, Selsdon, U.K.) with the eluent flow-rate set at 2.0 ml/min, an ISS-100 auto-injector (Perkin-Elmer, Beaconsfield, U.K.), an LCA 15 electrochemical detector (EDT Research, London, U.K.) and a 3388A integrator (Hewlett-Packard, Wokingham, U.K.). The column was Hypersil 5 ODS (5 μm), 100 mm × 4.6 mm (HPLC Technology, Altrincham, U.K.). The electrochemical detector was operated in the oxidation mode at an applied potential of +0.80 V versus a silver/silver chloride reference electrode. A back-pressure regulator was placed in the waste line from the electrochemical cell to reduce pulsing and prevent bubble formation in the cell. The setting of the pressure regulator was optimised to suit the chromatographic conditions. The injection volume varied between 10 and 25 μl and was adjusted to suit the expected APAP concentration.

RESULTS

Typical chromatograms of blank plasma, blank plasma spiked with APAP and PPAP and plasma taken from a volunteer 5 min after intravenous dosing with 200 mg APAP are shown in Fig. 1. In drug-free plasma samples there were no peaks observed in the position of either APAP or PPAP. The analyte and internal standard peaks were well defined with retention times of 1.4 min (capacity factor $k' = 0.78$) and 2.2 min ($k' = 1.78$), respectively.

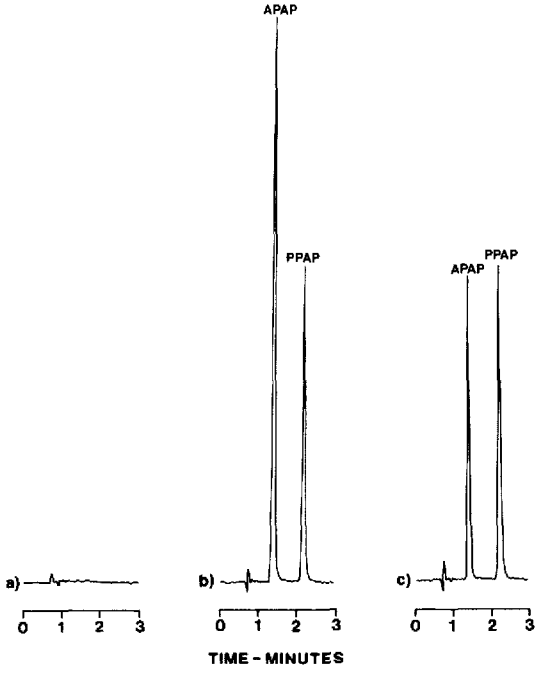

Fig. 1. Chromatograms of (a) blank plasma, (b) plasma spiked with 7 μg/ml APAP and 5 μg/ml PPAP and (c) plasma taken from a volunteer 5 min after administration of 200 mg APAP intravenously prepared as described in the text.

The linearity of the method was evaluated by spiking drug-free plasma with APAP at eighteen concentrations over the range 20 ng/ml to 40 μg/ml. The least-squares correlation coefficient of the resulting plot of detector response versus plasma concentration was 0.9998. Assay precision was determined by spiking five different drug-free plasma samples at each of three levels. The coefficients of variation of the determined concentrations for samples containing 7.20, 3.80 and 0.10 μg/ml APAP were 0.84, 2.42 and 4.15%, respectively.

The following drugs, all of which are on occasion co-administered with APAP, were screened for interference: salicylamide, naproxen, codeine, chlorpheniramine maleate, phenylephrine, phenylpropanolamine, caffeine, dextromethorphan, noscapine, ephedrine, thiamine and nicotinamide. At concentrations of 100 μg/ml no interfering peaks were found.

DISCUSSION

The HPLC methods most frequently reported for the analysis of APAP in plasma involve extraction of the analyte from plasma into ethyl acetate. The solvent is then evaporated and the residue reconstituted in a small volume of eluent for injection. A number of interfering compounds are co-extracted with the APAP when plasma samples are prepared in this way for HPLC—ED. These interferences largely negate any potential increase in sensitivity available from the ED system.

The novel feature of this method is the way in which a useful amount of analyte is extracted from plasma without co-extracting interfering endogenous compounds. After extraction with ethyl acetate in the conventional manner, a volume of TCTFE is added to the extraction medium together with a small quantity of alkaline water. The TCTFE fulfils two purposes. Firstly, it appears to decrease the solubility of APAP in the ethyl acetate, thereby increasing the efficiency of the back-extraction process. Secondly, it makes the organic phase more dense than water. The aqueous phase therefore floats on top of the organic phase after the two have been mixed and centrifuged. This facilitates its removal and subsequent analysis. Although the absolute efficiency of the extraction process is only about 50% for both APAP and PPAP, the increase in sensitivity of the method more than outweighs the greater extraction efficiency of conventional methods. This is largely due to the virtual absence of peaks in the chromatograms of drug-free plasma samples, as shown in Fig. 1a.

The oxidation potential of +0.8 V at the working electrode was chosen after preparation of hydrodynamic voltammograms for APAP and PPAP. The operating potential is well above the half-wave potential for both APAP and PPAP. This together with their very similar electrochemical properties, helps to minimise the temperature dependence of the detector [3].

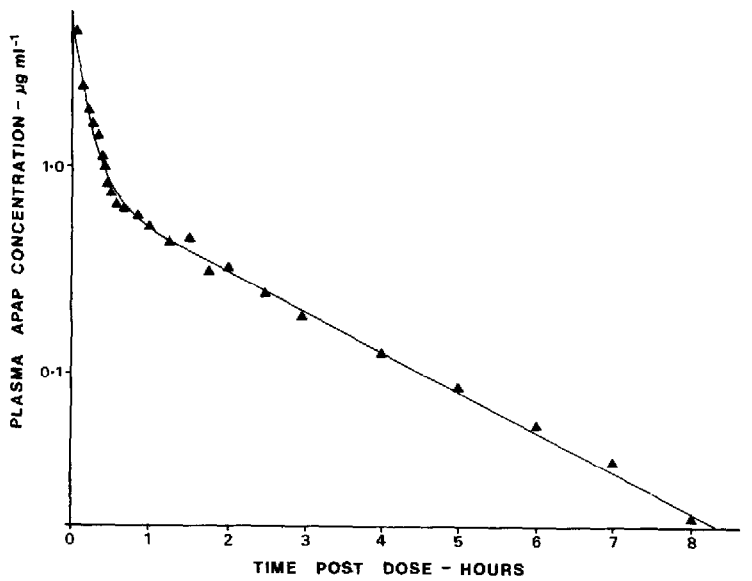

Fig 2. Plasma APAP profile following a 100-mg intravenous APAP dose.

The only problem encountered during the analysis of samples was coating of the glassy carbon electrode. This led to a reduction in sensitivity which, although not invalidating the method, would have made it considerably less useful. Electrode coating is a recognised problem [12—14] and is frequently attributed to polymerisation of the analyte or other compounds on the glassy carbon electrode surface. A review of relevant literature suggested several possible methods to reverse the passivation of the glassy carbon electrode, one of which proved to be both practical and efficient [13]. The electrode was removed from the cell and treated with a CrO_3—H_2SO_4 solution for 30 s, then thoroughly rinsed with distilled water and replaced. Experience has shown that such treatment is necessary about only every 500 samples.

A typical application of the method described here would be the determination of the plasma APAP concentration—time profile in a volunteer following intravenous dosing with 100 mg APAP. In such a case the plasma APAP concentration would not be expected to exceed 5 μg/ml, and for the majority of an 8-h time course would be less than 0.5 μg/ml. Use of the method described above allows this type of profile to be followed accurately and precisely whilst keeping sample preparation to a minimum. A profile obtained in this manner is shown in Fig. 2.

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