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Determination of Paracetamol (Acetaminophen) in Blood and Plasma Using High Performance Liquid Chromatography with Dual Electrode Coulometric Quantification in the Redox Mode

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A simple, but sensitive, micro-assay for paracetamol in blood and plasma is described. Samples were prepared by precipitation with trichloroacetic assay solution and the drug quantified by reversed phase liquid chromatography with dual electrode electrochemical detection in the redox mode (oxidation at +0.25 V followed by reduction at −0.15 V). The limit of detection was 0.1 mg/L for a 10 μL sample of blood or plasma, the coefficient of variation at this concentration being 11.4%. The method was applied to a study of gastric emptying in patients before and after cardiac surgery.

INTRODUCTION

Paracetamol may be assayed as part of the management of paracetamol overdose, when estimations of glucuronide, sulphate, cysteine and mercapturic acid conjugates may be useful (Howie *et al.*, 1977; Wilson *et al.*, 1982). The drug has also been used as a marker of intestinal absorption (Nimmo *et al.*, 1973) and under these circumstances it is the concentration of the parent compound that is important. The assay described here was developed primarily for the latter purpose. Paracetamol has been determined using gas chromatography with flame ionization detection as its trimethylsilyl (Prescott, 1971a) or acetyl derivatives (Prescott, 1971b). Liquid chromatography with ultraviolet detection at 248–250 nm has been described (Howie *et al.*, 1977; Wilson *et al.*, 1982). The advantage of electrochemical (amperometric) detection has been demonstrated (Miner and Kissinger, 1979; Wilson *et al.* 1982); indeed paracetamol must have been one of the first drugs to be quantified by high performance liquid chromatography (HPLC) with electrochemical detection (Riggin *et al.*, 1975). This was the approach chosen for the present study. The requirement was for a rapid assay using small volumes of sample. As the method was to be applied to patients undergoing cardiac surgery, who would be receiving a number of other drugs (Table 1), dual electrode coulometric detection was chosen for its selectivity. This selectivity allowed a simple sample preparation, while the increased sensitivity meant that samples as small as 10 μL could be used.

EXPERIMENTAL

Materials. Paracetamol, 4-aminophenol hydrochloride, propionic acid anhydride and trichloroacetic acid were purchased from Sigma Chemical Company (Poole, UK). The internal standard was prepared by shaking 4-aminophenol (1.5 g) in water (15 mL) with propionic acid anhydride (1 mL) for 60 min. The solid (0.5 g) was filtered and dried. Crystallization from ethyl acetate gave off-white plates, m.p. 175–178 °C. The structure was confirmed by proton NMR. HPLC grade acetonitrile was from Fisons Scientific Apparatus (Loughborough, UK).

Blood samples, by finger prick puncture, were from a healthy male volunteer (42 years old) who had received an oral dose (1 g, 2 tablets) of Paracetamol B.P. Plasma samples were from healthy volunteers (during method development) and from 14 patients (13 male) requiring cardiopulmonary bypass for coronary artery bypass grafting, valve replacement or both. Patients were given test doses (1 g in 100 mL water) of soluble paracetamol the day before and the day following surgery. Blood samples were taken aseptically from an indwelling cannula, predose and at intervals for up to 8 h postdose. The protocol was approved by the Tower Hamlets Ethics Committee and the subjects gave their informed consent. Plasma for the preparation of standard solutions was provided by the National Blood Transfusion Service (Brentwood, UK).

Liquid chromatography. An Ultrasphere ODS column, 150 × 4.6 mm i.d. (Beckman, High Wycombe, UK) was used. The eluent, 7% (w/v) acetonitrile in 0.01 M phosphoric acid, was pumped at 1 mL/min using an SA6410B pump with a built-in SSI membrane pulse dampener (Severn Analytical, Shefford, UK). Samples were introduced via a Kontron MSI660 autosampler fitted with a 50 μL sample loop. An ESA (Environmental Science Associates) Coulochem Model 5100 detector with dual graphite electrodes (5010 Cell) was used (Severn Analytical). The electrodes were set at +0.25 V and −0.15 V, relative to the reference electrodes. The detector outputs were connected in parallel to a two-channel analogue recorder and a two-channel 16BIT A/D converter board mounted in an Amstrad 1512 or Elonex PC 88M computer. The board and integration software (JCL 6000) were supplied by Jones Chromatography (Hengoed, Wales).

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Table 1. A summary of additional medication

Adrenaline	Allopurinol	Amiodarone	Atenolol
Atropine	Azathioprine	Benzylpenicillin	Cefuroxime
Codeine	Digoxin	Diltiazam	Dopamine
Enalapril	Fentanyl	Flucloxacillin	Frusemide
Gentamicin	Glyceryl trinitrate	Heparin	Hyoscine
Isoniazid	Isoprenaline	Isosorbide mononitrate	Iorazepam
Lignocaine	Metoclopramide	Metoprolol	Midazolam
Minoxidil	Nifedipine	Omnopon	Pethidine
Prednisolone	Propofol	Salbutamol	Thiopentone

For the determination of the current–voltage curves the column was removed and an Altex Model 210 valve with a 6 mL (nominal) sample loop fitted between the pump and the detector. Paracetamol solution (2.5 mg/L in eluent) was injected and the scanning facility used to vary the potential at the first electrode (0–0.5 V, relative to the Pd reference electrode). The signal was stored as an ASCII file. Control scans were obtained using paracetamol-free eluent. The control scan was subtracted from the paracetamol signal to produce a typical sigmoidal current–voltage curve (Figure 1). The procedure was repeated scanning the second electrode (0.1 to −0.4 V) while the first electrode was set at +0.3 V.

Sample preparation. Initially, calibration samples were prepared in the range 10–0.1 mg/L using blood bank plasma. Plasma or blood (10, 50 or 100 μL) was added to 10 volumes of 5% (w/v) trichloroacetic acid solution containing 0.5 mg/L of internal standard in either a 250 μL or 1.5 mL polythene centrifuge tube, depending on the size of sample. In later experiments the calibration range was extended to 40 mg/L and the concentration of internal standard increased to 2 mg/L. The capped tubes were agitated on a Vortex Genie 2 for 60 s and centrifuged at 12,000× g for 60 s. The supernatant was transferred to 250 μL tubes which were closed with plastic autosampler caps (8-PEP1, Chromacol, London, UK) before they were placed in the autosampler.

Intra-assay precision and accuracy were determined from replicate assays ($n = 8$) of known plasma solutions at 10, 1 and 0.1 mg/L, and by replicate assays ($n = 5$) of samples from a healthy volunteer. Inter-assay precision and accuracy were determined from assays of plasma containing 30, 3 and 0.3 mg/L on separate days.

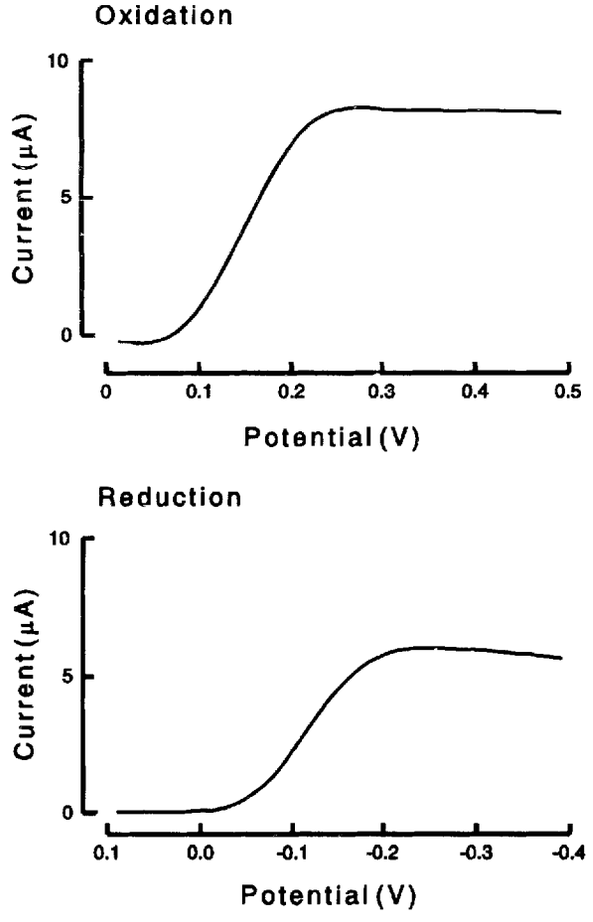

Figure 1. Current–voltage curves for the oxidation of paracetamol at the first electrode (top) and reduction of the product at the second electrode with the potential of the first electrode maintained at +0.3 V relative to the Pd reference electrode (bottom).

RESULTS AND DISCUSSION

Being phenolic, paracetamol was readily oxidized at the first electrode, E1, and the oxidation product was reduced at the second, E2. The polarographic waves are shown in Fig. 1. As the Coulochem electrometer has a third output, E1–E2, and because the signal from E2 is negative with respect to E1 this effectively adds the signals to produce a larger signal. Any of the outputs can be used for quantification, so that if, for example, there is a problem with one channel, another can be used. The ratio of the responses in each channel can be used to give an indication of peak purity. The output for the oxidation and reduction signal for paracetamol and the internal standard are shown in Fig. 2. Use of a paracetamol homologue as internal standard was considered preferable to using a readily available, but more chemically distant, alternative. Although the yield was low, the method of preparation was simpler than previous methods (Horvitz and Jatlow, 1977; Palmer, 1986) and could be carried out in any analytical laboratory. The produce prior to crystallization was

Table 2. Intra-assay precision and accuracy ($n = 8$)

Concentration (mg/L)	Found (mg/L)	Accuracy (%)	RSD (%)
10	9.91	−0.9	0.86 (0.9)[a]
1.0	0.98	−2.0	4.12 (4.4)
0.1	0.10	0.0	11.4 (18.9)

[a] Numbers in parentheses all values of RSD calculated without internal standard.

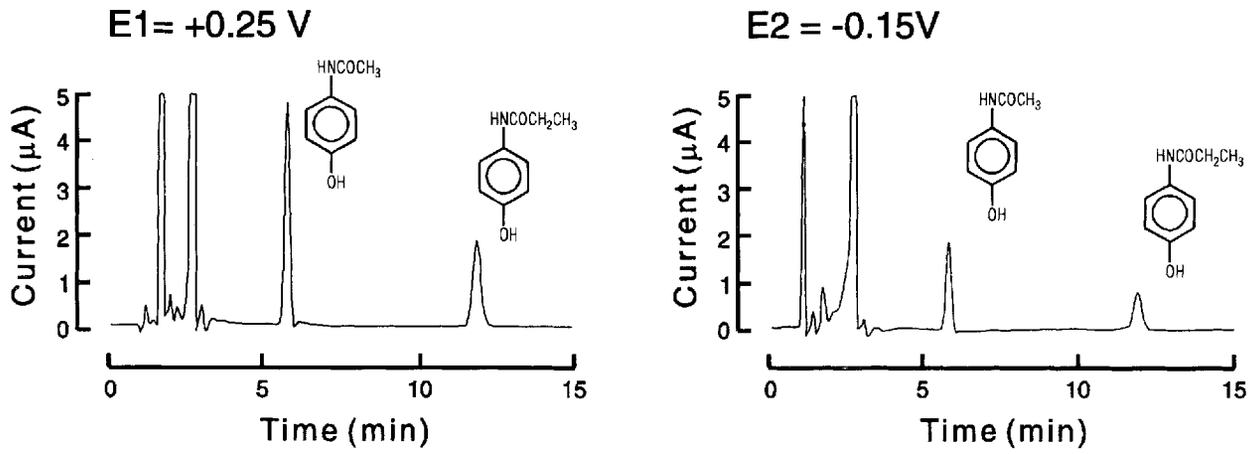

Figure 2. Chromatogram of paracetamol and internal standard showing the output from the first (E1) and second electrode (E2).

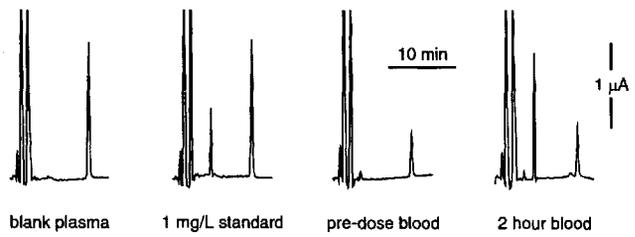

Figure 3. Chromatograms from samples prepared by adding 10 μL of biological fluid to 100 μL of trichloroacetic acid solution.

pure enough for use as an internal standard. Typical of chromatograms of plasma standards and blood samples from a volunteer are shown in Fig. 3. The results using whole blood are presented in Fig. 4.

The calibration curves of paracetamol:internal standard peak-height ratios versus known concentrations were linear over the range 40–0.2 mg/L; typically, correlation coefficients were >0.998 and sometimes >0.9999. Intra-assay coefficients of variation and accuracy are presented in Table 2. When the intra-assay coefficients were calculated without reference to the internal standard, they were larger, particularly at the lowest concentration when the CV increased from 11. 4 to 18.9%. Figure 5 gives an indication of intra-batch precision using unknown samples. The relative standard deviations were <4% apart from at one time-point, for which the value was 10%. The between-batch relative standard deviations were <4% and 30 and 3 mg/L but were somewhat greater at 0.3 mg/L (Table 3). Apart from the predose samples only 2 (out of 114) samples from the absorption study were below 0.3 mg/L.

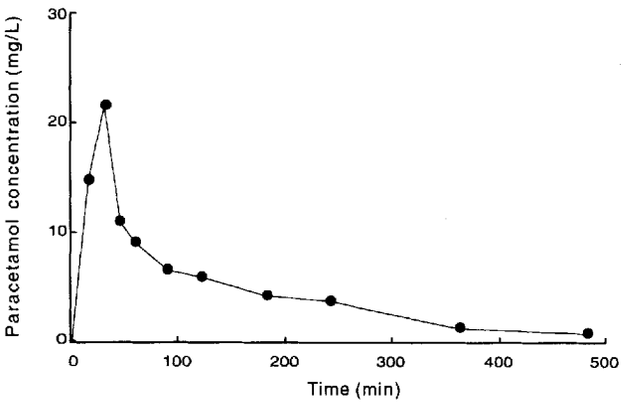

Figure 4. Blood concentrations of paracetamol after a single oral dose.

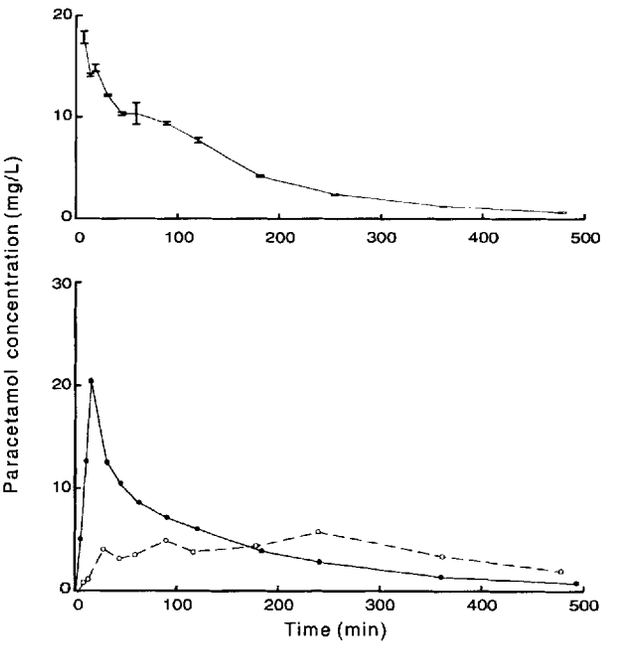

Figure 5. Plasma concentrations following 1 g oral dose of paracetamol. Top: mean concentrations ± SD for replicate ($n=5$) analysis of samples from a healthy volunteer. Bottom: samples from a patient (69 kg, 71 y) before (●) and after (○) cardiac surgery.

Application of the method

Peak plasma concentrations (C_{max}) and the areas under the concentration–time curves, 0–8 h (AUC) were reduced after surgery compared with preoperative controls in every subject. Similarly, within-subject comparisons of the time of the peak concentration

Table 3. Between-batch precision and accuracy ($n=5$)

Concentration (mg/L)	Found (mg/L)	Accuracy (%)	RSD (%)
30	29.0	−3.3	3.7
3.0	2.96	−1.2	3.6
0.3	0.32	6.8	15.8

(T_{max}) showed that it was delayed following surgery (Fig. 5). Comparison of mean data showed that the differences were highly significant ($p<0.001$). Mean pre-operation values ±SEM of T_{max}, C_{max} and AUC were 13.8±2.0 min, 24.2±1.8 mg/L and 2822±172 mg.h/L, respectively, whereas the corresponding post-op values were 214±41 min, 4.9±0.8 mg/L and 1524±195 mg.h/L. One subject vomited during the post-op sample collection period but omission of these results in no way altered the conclusions.

There was no evidence of interference by any of the additional drugs (or their metabolites) that subjects were taking or that were administered during or after surgery. This is in part due to the low oxidation and reduction potentials required for paracetamol and in part due to the fact that some of the more electroactive compounds (dopamine) would be present in low concentrations and have very short half-times. The phenolic groups on the opioids require potentials in excess of +0.5 V and would be expected to be in much lower concentrations and have markedly different chromatographic properties.

CONCLUSION

Liquid chromatography with electrochemical detection provides a convenient, sensitive and selective assay of paracetamol in situations where other drugs may be present.

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