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Rapid liquid chromatographic assay for the determination of acetaminophen in plasma after propacetamol administration: application to pharmacokinetic studies

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Abstract

A simple method for the rapid estimation of acetaminophen in plasma is described here. *p*-Propionamidophenol was used as internal standard. The assay involved a single ethyl acetate extraction and liquid chromatographic analysis at a wavelength of 242 nm using a reversed-phase encapped column, with a mobile phase of acetonitrile and 0.005 M potassium dihydrogen phosphate adjusted at pH 3.00. The limit of quantitation of acetaminophen by this method was 0.05 μg ml^{-1}, only 0.1 ml of the plasma sample was required for the determination. The calibration graph was linear from 0.05 to 100 μg ml^{-1}. Intra and inter-day precision (CV) did not exceed 8.93%. Mean recoveries of 90.31% with a CV of 1.38% were obtained. Applicability of the method was demonstrated by a pharmacokinetic study in normal volunteers who received 2 mg propacetamol.

Keywords: Column liquid chromatography; Paracetamol in plasma; Acetaminophen in plasma; Pharmacokinetics

1. Introduction

Acetaminophen, *N*-acetyl-*p*-aminophenol, (Fig. 1) is a synthetic non-opiate derivative of *p*-aminophenol which produces analgesia and antipyresis by a mechanism similar to that of salicylates. In therapeutic doses it is a very safe analgesic, but in overdosage may cause severe hepatic necrosis [1,2]. Since the reported or estimated quantity of the drug ingested is not a reliable guide to the therapeutic management of the overdose, plasma acetaminophen concentration levels should be determined as soon as possible. They are used in conjunction with developed nomograms [3] to estimate the potential of hepatotoxicity and determine the necessity of *N*-acetylcysteine therapy. Early treatment, beyond 15 h after drug ingestion, with *N*-acetylcysteine, an

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agent which facilitate glutathione synthesis, effectively prevent liver damage [4].

Acetaminophen is used extensively in the treatment of mild to moderate pain and fever. It has been used in the treatment of pain in combination with aspirin, caffeine, opiates and/or other agents. Acetaminophen–opiates combination produces greater analgesic effect than the produced by either acetaminophen or higher doses of the opiate alone [2]. Treatment of pain often requires parenteral administration of analgesics. However, acetaminophen is not available for parenteral use. Contrary to the latter propacetamol HCl can be easily solubilized and is suitable for parenteral administration [5,6].

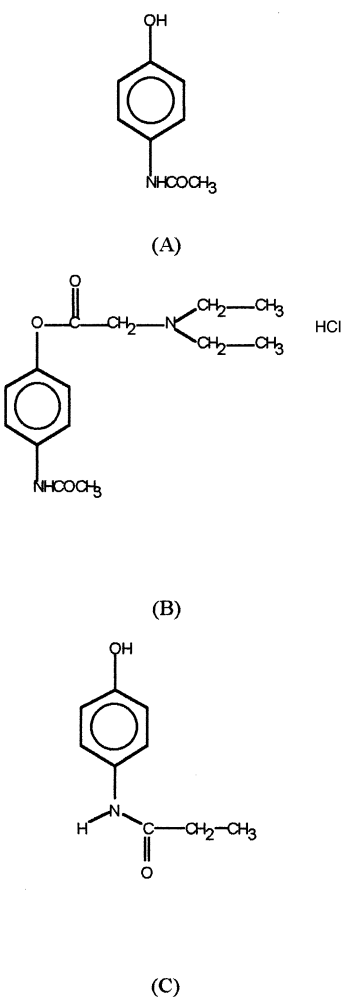

Fig. 1. Chemical structures of (A) acetaminophen, (B) propacetamol, and (C) *p*-Propionamidophenol (IS).

Propacetamol, the diethylamino-acetic ester of acetaminophen, (Fig. 1) is a prodrug that is very quickly and quantitatively hydrolysed into acetaminophen by plasma esterases within 7 min after intravenous injection [5,6]. With the exception of absorption, propacetamol exhibits similar disposition kinetics when given by either the intravenous route as propacetamol or by mouth as conventional tablets [6].

Acetaminophen can be estimated in biological fluids by colorimetric [7], spectrophotometric, GC and GC-MS [8–10], thin layer chromatographic and liquid chromatographic methods [11–30].

Colorimetric and spectrophotometric methods may give rise to gross overestimates of the true concentration, because these methods are based on unspecific acid hydrolysis of the drug to 4-aminophenol without prior solvent extraction [7]. Acetaminophen metabolites, acid-labile acetaminophen conjugates, are also hydrolysed to 4-aminophenol in acidic conditions giving gross overestimates of the true free acetaminophen concentration.

Gas chromatographic methods have also been employed for acetaminophen analysis. These methods are specific and allow acetaminophen quantitation below 0.1 μg ml^{-1}, using 100 μl-samples. Nevertheless, samples must be derivatised, previous chromatographic analyses, to convert acetaminophen into a volatile derivative.

The preferred analytical method to emergency estimation of the plasma acetaminophen concentration is high-performance liquid chromatography (HPLC). Several assays for acetaminophen using HPLC are suitable for analysis in pharmaceutical preparations, and others are suitable for analysis in biological fluids at therapeutic to toxic concentrations of the drugs [11–15,17–19]. Such methods are, however, not enough sensitive when sub-therapeutic drug levels must be measured as frequently occurs in pharmacokinetic studies. Methods reporting the sensitivity necessary to quantify pharmacokinetics acetaminophen levels accurately have also been published [16,20,21]. Most of these methods used HPLC in conjunction with electrochemical detection, solid-phase extrac-

tion and sample extraction procedures that involve multiple time-consuming organic extractions and solvent evaporation steps [17–20]. Only a few of these methods [16,21,29] are available for rapid measuring the large number of samples that have to be analysed in a pharmacokinetic study. All of these procedures require at least 1 ml of plasma. Consistently, pharmacokinetic studies after acetaminophen-drug combinations have not been completed, as plasma samples of 3–4 ml cannot be repeatedly obtained from human subjects.

One method [16] is available for measuring acetaminophen at toxic and pharmacokinetic levels. Acetaminophen plasma concentrations above 100 μg ml^{-1} at 4 h and 50 μg ml^{-1} at 12 h after drug ingestion cause hepatotoxicity [4]. Acetaminophen plasma concentrations below 0.1 μg ml^{-1} was found 12 h after 325-mg oral doses of acetaminophen.

This paper reports a rapid, sensitive and selective method for the quantitation of acetaminophen plasma levels over a 0.05–100-μg ml^{-1} range, which may be used when working with small samples.

2. Experimental

2.1. Chemicals and reagents

Acetaminophen and. *p*-Propionamidophenol (Fig. 1) were supplied by the Chemical Section of UPSA (Rueil Malmaison, France). Acetonitrile HPLC-grade, methanol HPLC-grade, ethyl acetate, *ortho*-Phosphoric acid 85%, sodium hydroxide, potassium dihydrogen phosphate, potassium monohydrogen phosphate were analytical grade from Merck (Darmstadt, Germany).

2.2. Instrumentation

A Hewlett Packard high performance liquid chromatograph (Waldbronn, Germany), equipped with a HP 1050 quaternary pump; a HP 1050 autosampler and a HP 1050 diode-array detector operating at 242 nm were used. The computer programs used were Chemstation 3D® for chromatographic analysis, and SPSS® for statistic analysis.

2.3. Chromatographic conditions

A reversed-phase, 250×4 mm encapped column packed with 5 μm C_8 silica reversed-phase particles was used (Lichrospher 60 RP-select B). This column was obtained from Merck (Darmstadt, Germany). A guard column, 4×4 mm packed with the same C_8 material, was also used. Separations were conducted using a mobile phase of acetonitrile-potassium dihydrogen phosphate (pH 3; 0.005 M) (20:80, v/v). Mobile phase was filtered though a 0.45-μm pore-size membrane filter. The flow-rate was 1 ml min^{-1}. The analytical and guard columns were operated at 20 ± 3°C.

2.4. Spiking procedure

A stock solution of acetaminophen (1 mg ml^{-1}) was prepared by dissolving 50 mg of acetaminophen in 50 ml of pure water. Seven standard solutions (0.1, 0.3, 5, 50, 80, 50 and 100 μg ml^{-1}) were made by further dilution of the stock solution with appropriate volumes of pure water.

The internal standard stock solution of *p*-Propionamidophenol (1 mg ml^{-1}) was prepared in water by dissolving 50 mg of *p*-Propionamidophenol standard in 50 ml of pure water. The internal standard solution (20 μg ml^{-1}) was prepared by diluting the stock solution with pure water.

Standards and stock solutions of acetaminophen, and *p*-Propionamidophenol were stored at 4°C.

Plasma standard solutions for calibration curve were prepared by adding 50 μl of each acetaminophen standard solution to 0.1 ml drug-free plasma. Plasma solutions had the following concentrations of acetaminophen in each plasma sample: 0.05, 0.150, 2.5, 25, 40, 70 and 100 μg ml^{-1}.

2.5. Extraction of plasma samples

Human plasma (0.1 ml) was pipetted into a 15-ml glass tube and was spiked with 25 μl of internal standard solution (20 μg ml^{-1}), 0.2 ml of

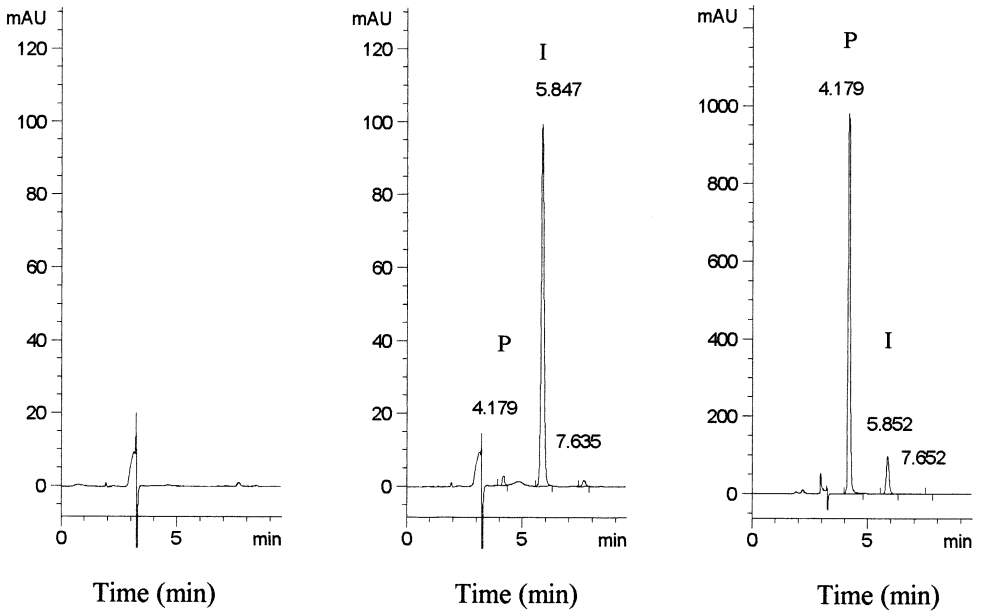

Fig. 2. Chromatograms resulting from the analysis of blank human plasma (A), and human plasma samples (0.10 and 36.49 μg ml^{-1}) obtained at 24 h (B) and 0.25 h (C), from a subject who received a single intravenous infusion of Pro-Dafalgan® (2 g). *p*-Propionamidophenol; P, acetaminophen.

pure water, and 0.1 ml of phosphate buffer (pH 7.4, 2.5 M). The samples were extracted with 2.5 ml of ethyl acetate using a vortex mixer for 30 s. The organic layer was separated after centrifugation at $2000 \times g$ for 15 min. The separated organic layer was evaporated to dryness at 55°C under reduced pressure (Rotary evaporator, Model 4322000, Labconco, USA). The residue was reconstituted in 200 μl of pure water and vortex-mixed for 60 s. A 100-μl aliquot was then injected onto HPLC column.

2.6. Application of the method

Twelve healthy volunteers received a single intravenous infusion of 2 g propacetamol (Pro-Dafalgan®, UPSA, Rueil Malmaison, France) over 15 min. The dosage used corresponded to 1 g acetaminophen. Venous blood samples were withdrawn and plasma fractions were separated immediately prior to dosing and at 5, 15, 30, 45 min, 1, 2, 4, 6, 8, 12 and 24 h after dosing, using heparin tubes. Plasma samples were stored frozen (-40°C until analyses).

3. Results

3.1. Chromatography

Acetaminophen and *p*-Propionamidophenol (IS) had a retention time of 4.14 ± 0.12 and 5.8 ± 0.10 min, respectively, under the chromatographic conditions described. Fig. 2 shows chromatograms of drug free-plasma, and a typical subject's plasma chromatograms 0.25 and 24 h post-infusion dose.

3.2. Extraction efficiency

Recoveries of acetaminophen and *p*-Propionamidophenol from spiked samples were determined by comparing the peak areas of plasma obtained from freshly prepared sample extracts at low, medium and high concentration levels, and submitted to the sample preparation procedure; with those obtained from analysis of corresponding directly injected standards ($n = 3$). The extraction recoveries of acetaminophen and *p*-Propionamidophenol in plasma were $90.3 \pm 1.38\%$ and $93.1 \pm 3.97\%$.

3.3. Selectivity of the assay

The selectivity of the assay was examined in relation to interference from endogenous substances and acetaminophen metabolites, in drug-free plasma. The selectivity of the assay was determined by individual analysis of blank plasma from 24 different subjects, with and without internal standard. Under these chromatographic conditions no endogenous sources of interference were observed in plasma, and the resolution between acetaminophen and IS was satisfactory (Fig. 2). Neither lipophilic nor lipophobic acetaminophen metabolites were extracted with highly polar extracting solvents, such as ethyl acetate, when samples were diluted with saturated buffer solutions.

The specificity of the assay was confirmed by the absence of co-eluting peaks when other drugs, such as butalbital, codeine, caffeine, dextromethorphan, diphenhydramine, ketorolac, phenylephrine, propacetamol, salycilates, theophylline; and tramadol, morphine, codeine and their metabolites were injected with acetaminophen. These drugs did not interfere with the quantitation of acetaminophen at concentrations achieved in plasma after therapeutic dose administration by intravenous route.

3.4. Sensitivity of the assay

Detection limit (LOD) of the assay method was determined by analysis of the peak baseline noise in ten blank samples. LOD was calculated as three times the variation in measure response, and was 0.01 μg ml^{-1}. The estimated limit of quantification (LOQ) was calculated as ten times the variation in measure response. LOQ was 0.03 μg ml^{-1}. This LOQ was confirmed for plasma, in separate experiment, using calibrators with nominal concentration of 0.03 μg ml^{-1}. The mean assay result was 0.032 μg ml^{-1} ($n = 5$) for plasma, with a CV of 2.23%.

3.5. Linearity of the assay

Calibration curves were determined by least squares linear regression analysis (weighting $1/\chi^2$). The assays exhibited linearity between the response (y) (peak-area ratio of acetaminophen over the internal standard) and the corresponding concentration of acetaminophen (x), over the 0.05–100 μg ml^{-1} range in plasma (typical equation: $y = 0.0021x + 0.0025$). The linearity of the relationship between peak area ratio and acetaminophen concentration is demonstrated by the correlation coefficients obtained for the regression lines. The correlation coefficients of all standard curves were greater than or equal to 0.999.

Moreover, a linearity test, by comparing calculated standard points to the nominal ones, was carried out on calibration curves in order to confirm the linearity and to test the quality of the fitting. The assays exhibited linearity ($r > 0.999$), with a slope near to unit (calculated t value was 198.633) and an intercept (calculated t value was 0.019) not statistically different from zero. The critical t value was 0.49 ($P = 0.01$).

3.6. Accuracy of the assay

Accuracy of the assay method was defined as the percentage of the systematic error, which is calculated as deviation agreement between the measured value and the true value. To be acceptable, measures should be within $\pm 15\%$ at all concentrations.

Accuracy values in intra-day variation studies at low, medium and high concentrations of acetaminophen in plasma were within acceptable limits (Table 1).

Table 1
Accuracy of the HPLC method for determining acetaminophen concentrations in plasma samples

Accuracy ($n = 5$)		
Concentration added (μg ml^{-1})	Concentration found (Mean ± S.D.) (μg ml^{-1})	Accuracy (%)
0.05	0.049 ± 0.007	1.9
0.15	0.149 ± 0.005	0.8
40	40.1 ± 0.117	0.3
75	74.9 ± 0.175	0.1

Table 2
Between and within-day variability of the HPLC method for determining acetaminophen concentrations in plasma samples

Concentration added ($\mu g\ ml^{-1}$)	Between-day variability ($n = 6$)		Within-day variability ($n = 18$)	
	Concentration found (Mean ± S.D.) ($\mu g\ ml^{-1}$)	C.V. (%)	Concentration found (Mean ± S.D.) ($\mu g\ ml^{-1}$)	C.V. (%)
0.05	0.049 ± 0.007	8.93	0.052 ± 0.005	14.1
0.15	0.149 ± 0.005	6.66	0.148 ± 0.010	3.1
40	40.1 ± 0.117	3.24	39.5 ± 0.128	2.9
75	74.9 ± 0.175	2.20	74.2 ± 0.165	2.4

3.7. Precision of the method

Precision of a method was expressed as the percentage coefficient of variation (CV) of replicate measurements. To be acceptable, the measures should be within 15% at all concentrations.

In this work precision of the method was tested both within-day and between-day reproducibilities in plasma.

3.7.1. Within-day variability of the assay

Within-day variability of the assay method was determined by repeated analysis of four quality control samples at low, medium and high concentrations on the same day. The results are showed in Table 2. These data indicate that the assay method is reproducible within the same day.

3.7.2. Between-day variability of the assay

Between-day variability of the assay method was determined in method validation by repeated analysis of four quality control samples at low, medium and high concentrations on 6 different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided in aliquots that were stored at −20°C until analysis. The results are showed in Table 2. These data indicate that the assay method is reproducible within different days.

3.8. Stability of acetaminophen

The stability of the drug in frozen plasma (−20°C) over 24 months, plasma stored at room temperature (20 ± 3°C) over 6 h and plasma stored in the refrigerator (4°C) over 6 h were studied in previous manuscripts [27]. Acetaminophen was stable in all these conditions.

The stability of acetaminophen and internal standard in processed samples left at room temperature (20 ± 3°C) over 24 h was studied from laboratory quality-control set up for the drug at concentrations of 50 and 100 $\mu g\ ml^{-1}$, respectively. Acetaminophen and the internal standard were also stable in these conditions.

4. Discussion

This paper describes a rapid, sensitive and selective method for the determination of acetaminophen levels over a 0.05–100-$\mu g\ ml^{-1}$ concentration range. Several authors have reported that standards ranging from 5 to 400 $\mu g\ ml^{-1}$ [17,19] and 0.02–20 $\mu g\ ml^{-1}$ [21] gave a linear response. However, none of these reported methods allow acetaminophen quantitation in the concentration range 0.05–100 $\mu g\ ml^{-1}$ without sample size variations.

The analytical procedure described here, allows to utilise one only calibration range for acetaminophen plasma levels quantitation, as a result of weighted least-squares linear regression method use to adjust the peak-area ratios of acetaminophen and internal standard against the concentration of acetaminophen.

Acetaminophen is a moderately water- and lipid-soluble weak organic acid with pK_a of 9.5, and is thus largely unionised over the physiological range of pH. In previous investigations normal- and reversed-phase packing materials were

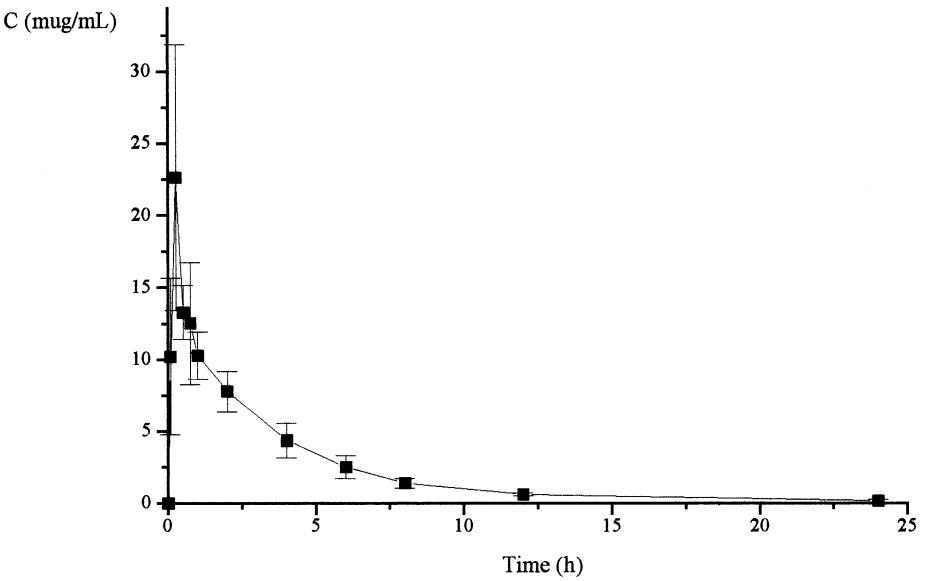

Fig. 3. Application of the method: mean plasma acetaminophen concentrations versus time after intravenous infusion of Pro-Dafalgan® to ten healthy volunteers (CV < 25%).

used for acetaminophen analysis. In these papers, it was shown that conventional reversed-phase packings provide much better resolution of acetaminophen and the more lipophilic metabolites than partially silanized silicas. The fully ionised metabolites elute with the solvent front and so are unresolved. In this work, a encapped C_8 reversed-phase packing has been used for specific determination of unconjugated acetaminophen.

In the present paper the drug and its internal standard are readily extracted into ethyl acetate at neutral pH with no special sample preparation. Although other organic extractants (dichloromethane, diethylether, acetonitrile and dichloromethane-ether-2-propanol) gave slightly cleaner extracts than ethyl acetate, the extraction efficiency was significantly higher. On the other hand, interfering compounds that are co-extracted with acetaminophen in this process are slightly water-soluble, and were not dissolved when plasma extracts were reconstituted with water.

Other sample clean-up procedures with only a protein precipitation step and solid-phase extraction are unsuitable for pharmacokinetic analysis because of insufficient sample clean-up, lack of sensitivity, or long analysis time.

In this work UV detection has been used. Although acetaminophen trace levels could be obtained with electrochemical detection in an order of magnitude below the capability of this detector, its sensitivity is limited when applied to the analysis of the drug in biological samples. The endogenous compounds that are co-extracted with acetaminophen are electrochemically reactive materials and have to be eliminated. For this reason, sample clean-up procedures involve at least two time consuming extraction steps, with the corresponding increment in analysis time.

5. Conclusions

In summary, the HPLC method described here permits the rapid determination of acetaminophen in 0.1 ml plasma samples from 0.05 to 100 μg ml^{-1}. Both the sensitivity and the precision of the method are good and no interfering peaks are seen in plasma.

This method is suitable for routine clinical monitoring of plasma levels in patients with hepatic failure, and for use in research studies involving pharmacokinetics and bioavailability. A

typical application of the method described here would be the determination of the plasma acetaminophen concentration-time profile in ten volunteers, as shown in Fig. 3.

This method is adaptable to an automatic sampling system, such that up to 100 samples can be analysed in a 24-h period. No loss in performance has been observed after 1000 plasma assays.

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