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Application of a modified colorimetric enzyme assay to monitor plasma paracetamol levels following single oral doses to non-patient volunteers

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Abstract A modified enzyme-based colorimetric method has been used to determine plasma paracetamol profiles following single dose (2 × 500 mg) administration of three dosage forms to non-patient volunteers The assay is linear over the concentration range 0 15–60 μg ml^{-1} with a coefficient of variation of 9 1% at the 1 5 μg ml^{-1} level It is rapid, requiring small sample volumes, compares favourably with other techniques such as HPLC, and is not subject to interference from paracetamol metabolites and other drugs Administration of paracetamol as two different dosage forms, as a solid tablet and as a dispersible tablet, resulted in no statistically significant difference in pharmacokinetic parameters between treatments

Keywords *Paracetamol, enzyme assay, bioavailability study, colorimetry, aminophenol, indophenol dye*

Introduction

Acetaminophen (paracetamol), a non-steroidal *para*-substituted acylanilide, has long been used as an analgesic It is widely available in the UK without prescription, both alone and in combination, in numerous proprietary preparations

Of the several methods currently available for paracetamol measurement, gas–liquid chromatography [1] and high-performance liquid chromatography (HPLC) [2] have proved most accurate and reliable However, these methods involve lengthy solvent extractions and chromatographic procedures making quantitation of large numbers of samples very time consuming

Other methods based on colorimetric techniques, such as combination with 2,2-diphenyl-l-picrylhydrazine [3], nitrosation with nitrous acid [4] or acid hydrolysis to aminophenol [5–7] have been employed with limited success [8]

More recently, an enzyme-based colorimetric assay has been described [8–10] which is rapid, accurate and specific for paracetamol This assay is based on the use of an enzyme,

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specific for the amide bond of acylated aromatic amines such as paracetamol It cleaves the paracetamol molecule yielding *p*-aminophenol which reacts specifically with *o*-cresol in ammoniacal copper solution to produce a blue colour The assay is specific for the parent compound and does not detect paracetamol metabolites [8]

Previously, this technique has been employed in the quantitation of paracetamol following suspected overdosage [8–10] In this paper, we describe a modification of the colorimetric assay for the detection and quantitation of plasma paracetamol at levels applicable to single dose pharmacokinetic studies, i e 0 15–60 μg ml^{-1} The method was subsequently employed in a 12-volunteer, three-way crossover study to determine the comparative bioavailability of three paracetamol preparations, two plain tablet formulations and a dispersible tablet formulation

Experimental

1 *Measurement of paracetamol in plasma*

The concentration of unconjugated paracetamol in plasma was determined by a modified enzyme-based colorimetric assay

Chemicals and reagents

(i) Enzyme reagent — buffered aryl acylamide amidohydrolase dissolved in distilled water (approximately one enzyme unit/ml H_2O), where one unit of activity is defined as that amount of enzyme which catalyses the hydrolysis of 1 μmol *p*-nitroacetanilide/min at 30°C

(ii) Colour reagent A — o-cresol in distilled water (92 5 mM)

(iii) Colour reagent B — ammoniacal copper sulphate (4 mM $CuSO_4$, 91 4 mM NH_4Cl, 600 mM Na_2CO_3, 27 mM Tris)

(iv) Standard — paracetamol [2 0 mmol l^{-1} (302 μg ml^{-1}) in sodium acetate buffer, 50 mM, pH 5 0]

(v) Diluted standard — paracetamol [50 μl standard + 950 μl H_2O (15 1 μg ml^{-1})]

The reaction scheme for the formation of an indophenol dye from paracetamol is shown in Fig 1

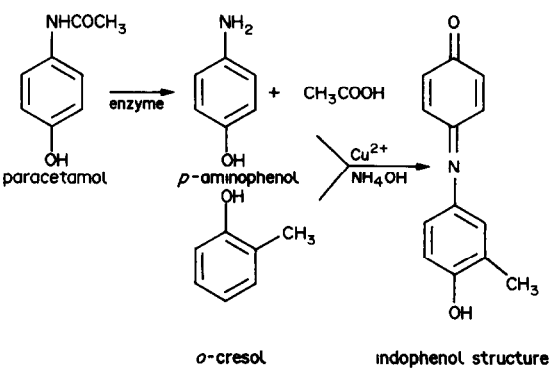

Figure 1
Reaction scheme for production of an indophenol dye by hydrolysis of paracetamol

2 *Procedure*

An aliquot (500 μl) of spiked, blank or sample plasma was placed into a clean Eppendorf centrifuge tube (1 5 ml) and 500 μl methanol (ice-cold) was added in order to precipitate protein The sample was vortex mixed for 30 s and subsequently centrifuged in a bench top micro-centrifuge for 30 min The supernatant (400 μl) was transferred to a clean Eppendorf tube and aryl acylamide amidohydrolase enzyme (200 μl) was added The tube was vortex mixed and incubated at room temperature for 2 min Colour reagent A (250 μl) followed by colour reagent B (250 μl) were added, the sample was vortex mixed and incubated at room temperature for at least 4 min The absorbance of the sample or standard was read against the blank at 615 nm on a Perkin–Elmer 552 UV/visible spectrophotometer

Plasma paracetamol concentrations were calculated from the following equation

$$\frac{\text{sample absorbance–blank absorbance}}{\text{standard absorbance–blank absorbance}} \times \text{standard concentration } (\mu g\ ml^{-1}) = \text{sample concentration } (\mu g\ ml^{-1})$$

A standard of 0 755 μg ml^{-1} gave an absorbance of 0 042 This was used in the calculation of all paracetamol concentrations

$$\frac{\text{sample absorbance–blank absorbance}}{0\ 042} \times 0\ 755 = \text{sample concentration } (\mu g\ ml^{-1})$$

or

$$\text{sample absorbance–blank absorbance} \times 17\ 97619 = \text{sample concentration } (\mu g\ ml^{-1})$$

All dilution factors cancel out since the standard is assayed under the same conditions as the sample

In the event of the plasma samples being highly pigmented or lipaemic, a sample blank was set up replacing enzyme reagent with distilled water and adding colour reagents as usual

3 *Calibration and accuracy of the analytical method*

A calibration line with 95% confidence limits was constructed from five individual sets of data obtained over the range 0 15–60 μg ml^{-1} A linear calibration line was obtained after logarithmic transformation of the data (Fig 2) Using the data from all calibration points, the coefficient of variation at 1 51 μg ml^{-1} of paracetamol was 9 12%, and at 3 02 μg ml^{-1} was 9 11% The integrity of the calibration data was assured by the assay of two quality control samples spiked with 1 51 and 3 02 μg ml^{-1} paracetamol, respectively, with each batch of samples

4 *Clinical study*

The study was of a randomized, single-dose, three-treatment crossover design and for which 12 non-patient volunteers (age range 18–40 years, weight within 15% of ideal body weight) were selected

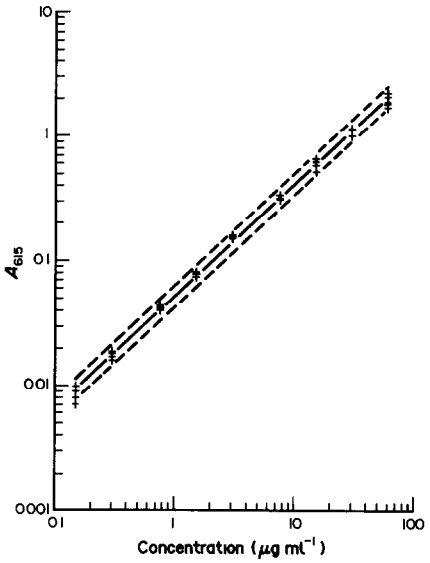

Figure 2
Paracetamol calibration curve Absorbance (615 nm) versus paracetamol concentration (μg ml^{-1}) Number of coefficients = 2, ---, 95% confidence limits

Each volunteer received by oral administration either tablet A (2 × 500 mg paracetamol) plus 150 ml tap water, tablet B (2 × 500 mg paracetamol) dispersed in 150 ml tap water, or tablet C (2 × 500 mg paracetamol) plus 150 ml tap water There was at least a 2-week interval between treatments

Blood samples (10 ml) were obtained through an indwelling venous cannula in a peripheral vein before and at 0 167, 0 333, 0 5, 0 75, 1 0, 1 5, 2, 3, 4, 6, 8, 10 and 12 h after drug administration The sample was immediately transferred to a siliconized lithium heparin tube and gently mixed After centrifugation (3000 rpm for 15 min) the supernatant plasma was separated and stored deep frozen (−20°C) until required for analysis

5 *Pharmacokinetics and statistics*

The areas under the plasma concentration versus time curves were estimated from $t = 0$ to $t = \infty$ using the trapezoidal rule with correction for terminal half-life The terminal half-life was determined by a least-squares regression analysis of five data points including and beyond 1 h after dosing

The statistical significance of any differences in pharmacokinetic parameters between the three dosage forms was tested by analysis of variance using a residual effects model with logarithmic transformation where appropriate The following parameters were tested on individual data peak plasma level (C_{max}), time to peak (t_{max}), terminal half-life ($t_{½}$) and area under the curve ($AUC_{0-\infty}$)

Results

1 *Interference of the assay by endogenous* p-*aminophenol*

To establish the absence of endogenous *p*-aminophenol in the plasma samples, a number of samples were analysed replacing amidase enzyme with water Absorbances were subsequently compared to those obtained with the same samples in the presence of enzyme (Table 1) It is clear from these results that there is no interference of the assay

Table 1
Comparison of absorbances obtained for the same plasma samples with the addition of the amidase enzyme and replacing enzyme with water

Sample	Absorbance (615 nm) with addition of H_2O	Absorbance (615 nm) with addition of amidase enzyme	Paracetamol concentration ($\mu g\ ml^{-1}$)
1	0 001	0 021	0 35
2	0 002	0 045	0 75
3	−0 001	0 053	1 01
4	0 001	0 105	2 01
5	−0 003	0 157	3 06

Table 2
Quality control data for paracetamol determination by colorimetric enzyme assay

Actual concentration ($\mu g\ ml^{-1}$) $n = 36$	Mean concentration found ($\mu g\ ml^{-1}$)	CV (%)
Within assay		
1 51	1 43	2 30
3 02	2 86	0 99
Between assay		
1 51	1 43	5 72
3 02	2 85	3 46

by *p*-aminophenol, suggesting that *p*-aminophenol is not present in plasma as endogenous material or a metabolite of paracetamol Acetylsalicylic acid, salicylic acid, common metabolites of paracetamol, and other drugs, have previously been tested and shown not to interfere with the assay [8]

2 *Quality control of the assay*

A total of 504 samples plus quality control and duplicates were analysed over a 4-day period Accuracy of the assay was assured by duplicating one in six of the samples tested, in addition to analysing two quality control samples with every 14 samples assayed Table 2 shows the mean quality control data over the 4-day period The lowest ratio concentration observed/actual concentration was, 0 915 at 1 51 $\mu g\ ml^{-1}$ and 0 937 at 3 02 $\mu g\ ml^{-1}$, the highest ratio was 0 960 and 0 960 at 1 51 and 3 02 $\mu g\ ml^{-1}$, respectively These results indicated a good correlation between measured values and actual values throughout the study period

3 *Bioavailability study*

The mean data of plasma paracetamol concentration following dosing with the three preparations is shown in Fig 3 This data was used to determine (i) peak plasma concentration (C_{max}), (ii) time to peak (t_{max}), (iii) area under the plasma concentration versus time curve, calculated from $t = 0$ to $t = \infty$ ($AUC_{0-\infty}$) and (iv) elimination half-life ($t_{½}$), for each volunteer The pharmacokinetic parameters for treatments A, B and C are shown in Table 3 No significant differences in C_{max}, t_{max}, $t_{½}$ or $AUC_{0-\infty}$ were observed between treatments

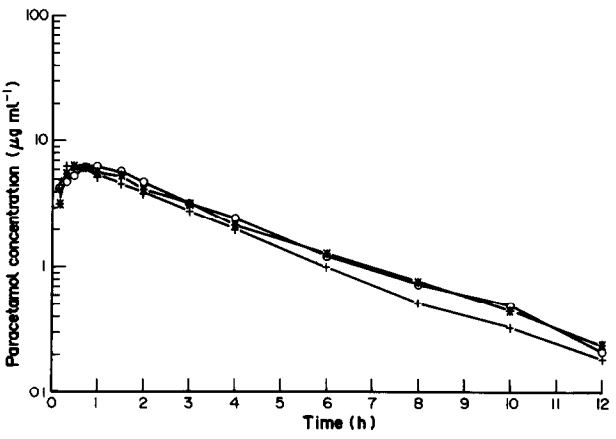

Figure 3
Mean data of plasma paracetamol levels after dosing with the three different formulations *, Treatment A, +, treatment B, O, treatment C

Table 3
Pharmacokinetic parameters for paracetamol after dosing with Treatment A, B and C

Treatment	Peak plasma concentration, C_{max} ($\mu g\ ml^{-1}$)	Time to peak, t_{max} (h)	Terminal half-life, $t_{1/2}$ (h)	$AUC_{0-\infty}$, ($\mu g\ ml^{-1}$ h)
A	7 439 ± 0 620	0 653 ± 0 102	2 186 ± 0 203	24 663 ± 1 629
B	7 671 ± 0 561	0 590 ± 140	2 297 ± 0 263	22 228 ± 1 453
C	8 067 ± 0 792	0 667 ± 0 117	2 138 ± 0 138	25 540 ± 1 855

Conclusions

A method for the determination of therapeutic plasma concentration of paracetamol is described The method, which involves enzymatic cleavage of paracetamol followed by coupling to *o*-cresol to produce a blue colour, had previously been used in monitoring patients suspected of paracetamol overdose [8] In this paper, a modification to the original assay has been successfully employed in a study to compare the bioavailability of three different paracetamol preparations The assay was sensitive to plasma paracetamol concentrations as low as 0 15 $\mu g\ ml^{-1}$ and exhibited no interference from paracetamol metabolites or endogenous compounds A comparison of the pharmacokinetics of paracetamol obtained with this technique agreed well with data previously published employing an HPLC method [11]

The observed values for $t_{1/2}$ in all three preparations agree with previously published values of 1 7–2 8 h [12]

Analysis of variance using a residual effects model showed no statistically significant difference (at the 95% confidence level) in half-life between volunteers, treatments or period Similar analysis of $AUC_{0-\infty}$ again showed no difference between volunteers, treatment or period There was no significant difference between treatments for maximum plasma concentration of paracetamol (C_{max}) or for the time required to attain this concentration (t_{max}) These results demonstrate no statistically significant difference

in the relative bioavailability of the three preparations as interpreted from measurements of the areas under the plasma concentration–time curves

In conclusion, a method for the determination of plasma paracetamol concentrations is described which facilitates accurate and rapid analysis of samples without the need for hazardous reagents or expensive equipment

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