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A simple microanalytical technique for the determination of paracetamol and its main metabolites in blood spots

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Abstract

The use of blood spot collection cards is a simple way to obtain specimens for analysis of drugs with a narrow therapeutic window. We describe the development and validation of a microanalytical technique for the determination of paracetamol and its glucuronide and sulphate metabolites from blood spots. The method is based on reversed phase high-performance liquid chromatography with ultraviolet detection. The limit of detection of the method is 600 pg on column for paracetamol. Intra- and inter-day precision of the determination of paracetamol was 7.1 and 3.2% respectively. The small volume of blood required (20 μl), combined with the simplicity of the analytical technique makes this a useful procedure for monitoring paracetamol concentrations. The method was applied to the analysis of blood spots taken from neonates being treated with paracetamol.

Keywords: HPLC; Paracetamol; Paracetamol sulphate; Paracetamol glucuronide; Neonates

1. Introduction

Paracetamol (4-acetamidophenol) is the most used analgesic and antipyretic drug in children and neonates [1]. Despite its common use, pharmacokinetic data about paracetamol is scarce, especially in young infants and neonates. Due to differences in their metabolism, there is special interest in the levels of paracetamol in neonates, particularly after multiple dosing [2–4].

In neonates, the volume of blood taken is limited by ethical considerations and thus a method requiring only small volumes of blood is desirable. The use of a collection card similar to the Guthrie paper card used for the sampling of small volumes of blood allows more frequent sampling while still complying with ethical guidelines of a maximum 1 ml/kg body weight for blood sampling from neonates. This paper describes the development and validation of a microanalytical technique for the determination of paracetamol and its glucuronide and sulphate conjugates in blood spots. The technique is based on high-performance liquid chromatography (HPLC) with ultraviolet detection. Due to the general availability of liquid chromatographs coupled with the

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simplicity of blood spot sampling the technique is well suited for the routine determination of blood levels of paracetamol and its main metabolites.

2. Materials and methods

2.1. Chemicals and reagents

Isolute ENV$^+$ solid phase extraction cartridges (3 ml, 500 mg) were purchased from IST (Mid Glamorgan, UK). Paracetamol and Hypersolv acetonitrile were obtained from BDH-Merck (Poole, Dorset, UK). Ammonium formate, 2-acetamidophenol and paracetamol glucuronide were purchased from Sigma-Aldrich (Poole, Dorset), and chlorosulfonic acid was purchased from Fluka (Poole, Dorset).

2.2. Paracetamol sulphate synthesis

Paracetamol sulphate was synthesised according to a published method [5]. The sulphate was purified by solid phase extraction using Isolute ENV$^+$ cartridges (3 m1, 500 mg). The cartridges were conditioned with 4 ml of methanol and 3 ml of water before loading the sample, which was dissolved in distilled water. The potassium chloride which was present in the paracetamol sulphate isolated from the reaction mixture was washed off with 3 ml of water and then the paracetamol sulphate was eluted with 6 m1 ($2 \times$ 3 m1) of methanol. The procedure was repeated twice for the effluent collected during sample loading in order to recover any paracetamol sulphate which had not been retained during the first loading steps. Purity and identity of the paracetamol sulphate were confirmed by HPLC, electrospray mass spectrometry and ^{1}H and ^{13}C NMR data. NMR spectra were acquired on a Bruker AMX 400 MHz spectrometer using deuterated DMSO as the solvent and the positive ion electrospray mass spectrum on a Thermo-Finnigan Automass multi LC-GC/MS. High resolution fast atom bombardment mass spectrometry (FAB-MS) was carried out using a JEOL 505HX instrument using the glycerol matrix as the calibrant.

^{1}H NMR (400 MHz, DMSO-d_6, δ from TMS): 1.99 (3H, *s*, COCH_3), 7.05 (2H, *d*, $J =$ 8.76 Hz, H-3, H-5), 7.41 (2H, *d*, $J = 8.84$ Hz, H-2, H-6), 9.96 (1H, *s*, N–*H*). ^{13}C NMR (100 MHz, DMSO-d_6, δ from TMS): 168.80 (*C*OCH$_3$), 149.05 (C-4), 135.16 (C-1), 121.31 (C-2, C-6), 120.20 (C-3, C-5), 24.06 (CO*C*H$_3$). Electrospray Mass Spectrometry: $m/z = 232$ [M + H]$^+$base peak, m/z 151 [M-S0$_3$H]$^+$. FAB-MS gave the elemental composition of [M + H]$^+$ as: $C_8H_9NO_5S$ with an error of 2.4 ppm.

2.3. Sample collection preparation

Samples were taken from an indwelling arterial cannula placed for continuous blood pressure monitoring of neonates and infants in a paediatric intensive care unit. Single drops of blood were collected on Guthrie-type cards (Whatman, UK). For the purposes of calibration and development of the method drops of blood were taken from volunteers by finger-prick with a lancet. Paper discs (7 mm) were sampled from the blood spots with a hole puncher. The punched paper disks were transferred to 3.5 ml aluminium lined screw-capped vials and 200 μl of 20 mM ammonium formate buffer pH 3.5 was added. The samples were vortexed until blood was extracted from the paper (1–2 min). The internal standard (200 ng of 2-acetamidophenol) was added, and the samples were then mixed with 3 ml of acetonitrile. After brief vortexing to precipitate the proteins, samples were centrifuged ($3500 \times g$ for 5 min) and the supernatant transferred to another 3.5 ml vial. The solvent was evaporated to dryness under a stream of nitrogen and the residue was redissolved in 200 μl of 20 mM ammonium formate buffer pH 3.5. The sample was transferred to an autosampler vial fitted with a 200 μl glass insert and 20 μl was injected into the HPLC.

The volume of blood contained in the punched blood spot disks was determined by pipetting known volumes of blood (from 1 to 50 μl) onto the paper cards with an automatic pipette. The diameter of the blood spots were

then measured and a calibration curve constructed. A power equation (see below) was fitted and the equation used to determine the volume of blood contained in the punched disks used for analysis, which had a fixed diameter of 7 mm. This procedure gave a figure of 15.84 μl of blood

$$\text{Diameter of spot} = 2.0624 \times \text{volume of blood}^{0.4423}$$

$$(R^2 = 0.996).$$

The relationship between blood volume pipetted onto the cards and the diameter of the blood spot was linear from 10 to 50 μl. When a linear regression line was fitted between these points and the equation of the line used to calculate the volume of blood contained in 7 mm disks, the figure was 16.33 μl of blood. The volume of 16 μl of blood was thus used for calculating the paracetamol concentration in all the samples analysed.

2.4. Calibration solutions

Blood spots were collected from volunteers that were not receiving treatment with paracetamol. Punched paper disks were transferred to 3.5 ml aluminium lined screw-capped vials and solutions of standards (all prepared in acetonitrile) were spiked onto the paper discs. The solvent was evaporated to dryness under a stream of nitrogen and 200 μl of 20 mM ammonium formate buffer pH 3.5 was added. The samples were then extracted as described above for sample preparation.

2.5. HPLC analysis

HPLC was carried out using a Thermoseparations Spectra Series P4000 gradient pump coupled with a Spectra System UV 6000 LP photodiode array detector and a Thermoseparations AS1000 autosampler. The detector was set to scan from 200 to 500 nm and had a discrete channel set at 254 nm, which was the wavelength used for quantification. Separation was achieved using a Hypersil C18 column (75 × 4.6 mm, 3 μm). The mobile phase consisted of 20 mM ammonium formate buffer pH 3.5 (A) and methanol (B). The conditions of the gradient are specified in Table 1.

Precision of the method was estimated by analysing samples prepared by spiking blank blood spots with each analyte. The blood spots were extracted as described above for 'sample collection and preparation'. Intra-day precision was evaluated by analysing a series of samples prepared and analysed on the same day, while inter-day precision analyses were done with samples prepared and analysed on separate days (over a total period of 2 weeks). RSDs of less than 15% were considered satisfactory.

The limit of detection was based a peak height 3X the largest baseline fluctuation in mAu in a 1 min window around the elution time of the analyte in an analytical blank.

Recovery was calculated by comparing peak areas obtained for each analyte in samples prepared by spiking blank blood spots with peak areas obtained for samples of buffer spiked with

Table 1
Gradient conditions for HPLC analysis

Time (min)	20 mM ammonium formate pH 3.5 (%, v/v)	Methanol (%, v/v)	Flow (ml/min)
0.0	96.0	4.0	0.8
5.0	96.0	4.0	0.8
15.0	46.0	54.0	0.8
16.0	10.0	90.0	1.0
18.0	10.0	90.0	1.0
19.0	96.0	4.0	0.8
24.0	96.0	4.0	0.8

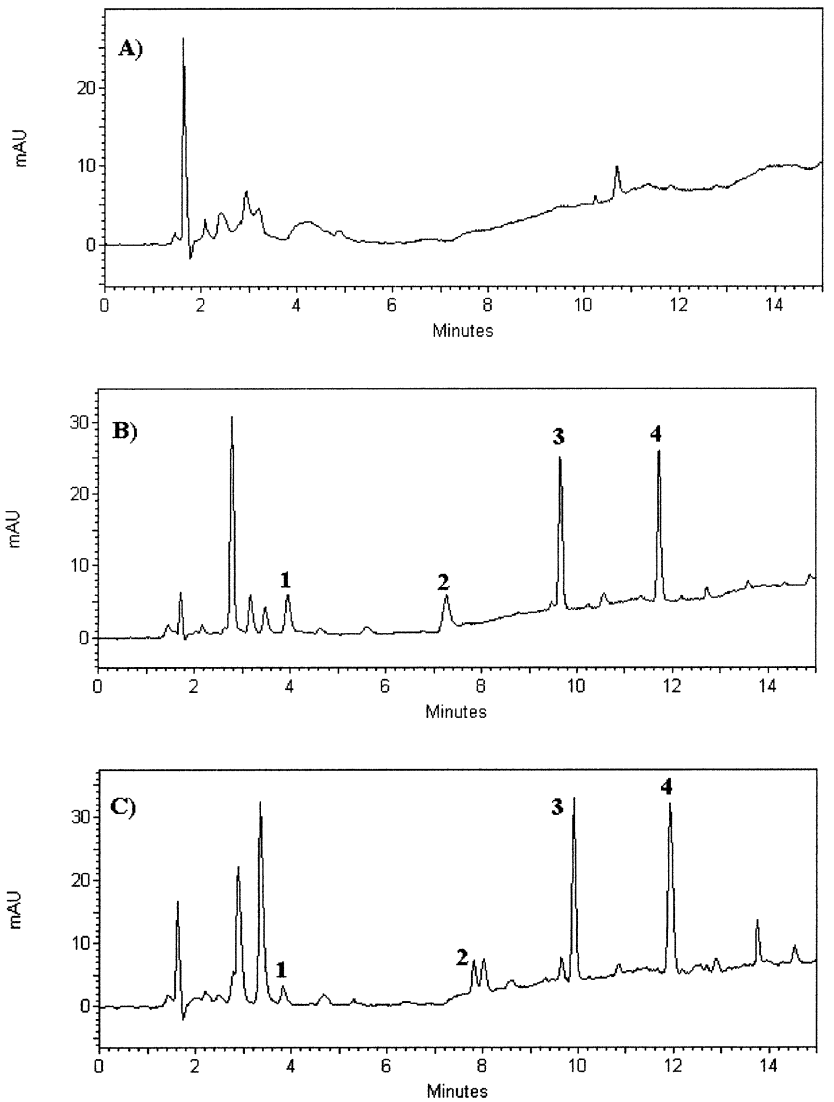

Fig. 1. HPLC chromatograms showing separation of the analytes. (A) Sample prepared from a blank bloodspot. (B) A calibration sample containing 600 ng/ml of paracetamol glucuronide (peak labelled 1), 600 ng/ml of paracetamol sulphate (peak labelled 2), and 300 ng/ml of paracetamol (peak labelled 3) extracted from a spiked blood spot. (C) A patient sample. The internal standard (2-acetamidophenol) is labelled as 4 in the chromatograms. Detection by UV at 254 nm. For HPLC gradient conditions see Table 1.

the same amount of the analytes. Paracetamol glucuronide and paracetamol sulphate were spiked at a concentration of 200 ng/ml, paracetamol was spiked at a concentration of 80 ng/ml, and 2-acetamidophenol at a concentration of 1 μg/ml. Recoveries above 80% were considered satisfactory.

3. Results

Fig. 1 shows chromatograms of a blank sample prepared from a blood spot (Fig. 1A), a calibration sample (Fig. 1B) prepared from a spiked blood spot and a sample from a patient (Fig. 1C). The chromatograms show that the analytes were

well resolved from other endogenous components of plasma. The retention times were 3.88 ± 0.08 min for paracetamol glucuronide, 7.14 ± 0.18 min for paracetamol sulphate, 9.56 ± 0.05 min for paracetamol and 11.64 ± 0.05 min (mean $\pm$ S.D., $n = 5$) for 2-acetamidophenol (internal standard).

The response of the detector was linear for all the analytes (Table 2) over the concentration range used during analysis of samples. Since the detector response was lower for the metabolites of paracetamol compared to paracetamol itself, the limit of detection for paracetamol sulphate and paracetamol glucuronide (2 ng on column) was higher than that of paracetamol (600 pg on column).

The recovery of the analytes by the extraction procedure was estimated by comparing the peak area obtained for the analytes spiked in blank blood spot samples with the area of the analytes spiked in buffer (Table 3). The recovery was good and reproducible for paracetamol and the internal standard (2-acetamidophenol). The more variable recoveries obtained for the metabolites are possibly a consequence of their polar nature with correspondingly lower solubility in the extraction solvent. The recovery of paracetamol glucuronide was the most variable of all, which is a reflection of both its high polarity and its short retention time, which makes it more susceptible to interference from early eluting peaks.

The precision of the method (Table 4) was evaluated by analysing samples spiked with standards in concentrations close to the limit of detection. The samples were prepared and analysed on the same day (Intra-day precision), or prepared and analysed in different days (over a period of 2 weeks) for estimating inter-day precision. The precision was reasonable for a microanalytical method, and can possibly be im-

Table 2
Calibration curve parameters for paracetamol, paracetamol sulphate and paracetamol glucuronide

	Paracetamol glucuronide (mean, RSD, $n = 3$)	Paracetamol sulphate (mean, RSD, $n = 3$)	Paracetamol (mean, RSD, $n = 3$)
Slope	0.0005166, 10.7%	0.0007606, 3.1%	0.0028357, 7.3%
r^2	0.9978, 0.1%	0.9949, 0.6%	0.9973, 0.2%
Range (ng)	160–4000	160–4000	40–2000

Table 3
Recovery of the analytes by the extraction procedure

	Paracetamol glucuronide (%)	Paracetamol sulphate (%)	Paracetamol (%)	2-acetamidophenol (internal standard) (%)
Replicate 1	105.6	91.1	94.4	90.2
Replicate 2	90.9	76.0	93.9	86.9
Replicate 3	86.5	75.3	92.8	90.3
Replicate 4	106.4	88.4	96.5	90.6
Replicate 5	96.08	100.3	92.6	99.2
Replicate 6	66.13	73.6	93.1	91.3
Average	91.9	84.1	93.9	91.5
RSD%	16.2	12.8	1.5	4.5

Values are percentage of recovery when compared with the average peak area of the analytes spiked in buffer. Paracetamol glucuronide and paracetamol sulphate were spiked at a concentration of 200 ng/ml, paracetamol was spiked at a concentration of 80 ng/ml, and 2-acetamidophenol at a concentration of 1 μg/ml.

Table 4
Intra- and inter-day precision of the method

	Paracetamol glucuronide (ng/ml)	Paracetamol sulphate (ng/ml)	Paracetamol (ng/ml)
Intra-day precision			
Replicate 1	145.3	160.5	39.9
Replicate 2	151.2	134.8	46.4
Replicate 3	150.7	142.1	41.6
Replicate 4	165.0	139.8	46.8
Replicate 5	155.2	190.1	42.2
Average	153.5	153.5	43.4
RSD%	4.8	14.8	7.1
Inter-day precision			
Replicate 1	162.9	126.4	42.1
Replicate 2	182.9	139.8	43.6
Replicate 3	209.9	132.2	41.2
Replicate 4	174.1	169.2	40.4
Average	182.4	141.9	41.8
RSD%	11.0	13.4	3.2

The samples were spiked with a nominal concentration of 160 ng/ml for paracetamol glucuronide, 160 ng/ml of paracetamol sulphate, and 40 ng/ml of paracetamol.

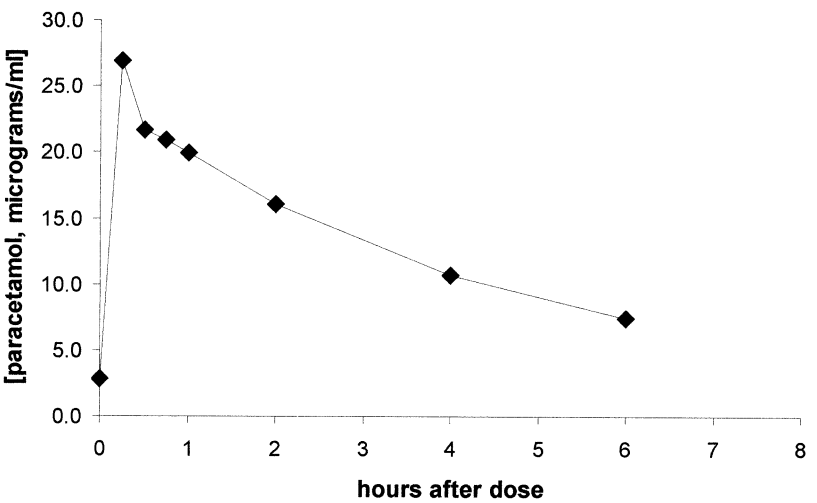

Fig. 2. Time profile of paracetamol concentration in blood spots taken from a neonate after a rectal dose of 20 mg/kg.

proved if a larger volume of sample were injected or if the sample was prepared in more concentrated form (for example by diluting it to a final volume of 100 μl, instead of 200 μl).

An example of the application of this method to the determination of paracetamol concentrations in blood spots from neonates is shown in Fig. 2.

4. Discussion

Although widely used as an analgesic and antipyretic, the pharmacokinetics of paracetamol in young infants and neonates is not fully understood. There are reports that paracetamol administered rectally in neonates at a dose of 20 mg/kg body weight results in subtherapeutic concentra-

tions [2,3]. Neonates are known to have a different metabolic profile, and in the case of paracetamol, sulphation is known to be the major route for metabolism [4]. A better understanding of the pharmacokinetics of paracetamol in neonates is necessary to ensure that paracetamol plasma concentrations do not reach toxic or fall to subtherapeutic levels, especially when multiple dosing is being used. The development of sensitive and selective methods for measuring paracetamol concentrations in biological fluids is an important prerequisite of this endeavour.

Most methods for determining paracetamol in biological samples use HPLC [6–8] or gas-chromatography [9–11]. Other methods include enzymatic and colorimetric techniques. Methods that use a small volume of blood are desirable in situations where the collection of larger volumes are not feasible, such as in the case of neonates, for which the volume of blood sampled is limited by ethical guidelines. Also, other features which are desirable are simplicity of sample preparation and general availability of the analytical instrument required for analysis. A recent method [12] makes use of only 10 μl of blood or plasma and relies on HPLC with electrochemical detection for quantification of paracetamol. The method described here makes use of HPLC with ultraviolet detection, which is widely available in biochemical laboratories. It has the advantage of requiring only a small sample volume (20 μl) combined with simple sample preparation and analysis. The analytes are well resolved from endogenous components from the blood (Fig. 1) and the method can be applied to detection of paracetamol and its metabolites. The short retention time of paracetamol glucuronide means that it is the analyte most prone to suffer interference from polar components eluting early in the run. However, in most cases resolution from interfering peaks was achieved. The use of new reversed phase columns that can run 100% aqueous mobile phases (through the use of stationary phases with polar end-capping functionalities) might improve retention of the glucuronide and improve precision of the method for this analyte.

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