A simple assay of paracetamol based on dried blood spot suitable for paediatric pharmacokinetic studies

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Dried blood spots in Guthrie cards are a reliable means of blood sampling suitable for pharmacokinetic analysis in children. The aim of this study was to develop a simple and reliable bioanalytical method to measure the concentration of paracetamol in dried blood spots. Paracetamol was extracted from dry blood spots by precipitation using 30% perchloric acid and separated on Hichrom 3.5 µ C18 column. Detection was by a Waters 486 Tunable Absorbance Detector, at a wavelength of 244 nm. From five adult volunteers blood samples were collected as dried blood spots before and at 15, 30, 60, 120, 240 and 420 minutes after 1 g oral paracetamol was administered. C\(_{\text{max}}\), T\(_{\text{max}}\), t\(_{1/2}\) and CL/F were calculated using non-compartmental analysis. The standard curve for the method was linear in the range 0 – 100 µg ml\(^{-1}\). The LLOD and LLOQ were 550 pg and 1100 pg, respectively, on column. The median (min., max) C\(_{\text{max}}\), T\(_{\text{max}}\), t\(_{1/2}\) and CL/F, were 14.6 µg ml\(^{-1}\) (9, 19.1), 45 minutes (15, 60), 2.3 hours (1.1, 2.7) and 0.31 L kg\(^{-1}\) (0.21, 0.36), respectively. We report a simple assay for the analysis of paracetamol that can be used for monitoring blood concentration of paracetamol in young children.

**Keywords:** Bioanalytical, concentration, children, Guthrie, neonates

**INTRODUCTION**

Of all the biomaterials proposed for quantifying drugs and or their relevant metabolites for pharmacokinetic (PK) analysis, blood is the most suitable sample matrix (ICH, 1994; FDA, 1995). In children however, the total volume of blood to be withdrawn per trial is guided by recommendations that this should amount to no more than 3% of total body content (EMEA, 2006). An assay that utilizes less blood volume is desirable because reducing the volume of blood sampled per time would not only facilitate dense sampling required for traditional PK analysis, but would also meet the concerns of ethics committees and improve subject participation.

The search for a means of simplifying blood collection and minimising blood loss has led to the introduction of special filter papers for blood collection for the analysis of both endogenous and xenobiotic substance (Guthrie and Susi, 1963; Hoogtanders et al., 2007). Beyond acting as blood collecting devices, these special filter papers offer a volumetric function, the reliability of which has been demonstrated (Mei et al., 2001). The benefits of this collection method includes ease of handling, and more importantly, the possibility of including neonates and younger paediatric patients in research that require frequent blood sampling while ensuring low cumulative blood volumes. Gentamicin, theophyllin, tacrolimus and metformin are among drugs that have been successfully assayed using DBS, thus projecting DBS as a suitable bio-analytical method for therapeutic drug monitoring (Coombes et al., 1984; Mei et al., 2001; Aburuz et al., 2006). Motivated by the ability of blood collecting cards to minimize blood loss during research, Oliveira et al., (2002) developed and proposed a DBS assay for the analysis of paracetamol and its major metabolites for studies in neonates and younger children.
(Oliveira et al., 2002). However, this method used a laborious liquid-liquid extraction and also had a long run time, which was in excess of 12 minutes. These attributes limit analytical throughput and make desirable an assay which is simple, fast, less costly to run and yet suitable for PK studies in younger children.

Despite paracetamol being available for over a century and widely used as an analgesic and antipyretic in both adults and children, there are still unanswered questions on the use of the drug, especially in children (Gibb and Anderson, 2008). The need to adequately explain the effects of paracetamol on younger children justifies the quest for suitable micro analytical methods for paracetamol. The aim of this study was therefore to develop a simple and reliable bioanalytical method to measure the concentration of paracetamol in dried blood spots.

MATERIALS AND METHODS

Chemicals and reagents
Paracetamol (>99% purity), 2-acetamidophenol (>97% purity), ammonium formate (>98% purity), triethylamine, acetic acid and perchloric acid (HClO₄) were all purchased from Sigma-Aldrich (Dorset, England, UK). Methanol and acetonitrile were of HPLC grade and purchased from Fisher Scientific (Loughborough, UK). Blood collecting cards (Figure 1) were supplied by Whatman, Gmbh, Germany and blank whole blood was obtained from a healthy adult volunteer.

Standards and solutions

As an internal standard, a stock solution of 2-acetamidophenol was prepared by dissolving 0.003 g of 2-acetamidophenol powder in 10 ml of acetonitrile. One millilitre (1 ml) of the resulting solution was diluted in 10 ml of acetonitrile to yield a 30 µg ml⁻¹ solution for routine use.

A 20 mM ammonium formate (pH 3.5) was prepared for chromatographic separation by weighing 1.26 g of ammonium formate crystals into a one-litre capacity beaker with a magnetic stirrer. To this was added 800 ml of distilled water (Milli-Q Integral). While stirring the solution was buffered with 100% concentrated acetic acid to pH 3.5 using a pH meter. The acidified solution was then transferred to a one-litre volumetric flask and with distilled water the volume was brought up to 1 litre.

Dried blood spot standards were prepared from standard paracetamol concentrations. For this a stock solution of paracetamol was prepared by dissolving 0.05 g of paracetamol powder in 10 ml of ammonium formate buffer (pH 3.5). Standard paracetamol concentrations from 0 to 5000 µg ml⁻¹ were prepared taking appropriate volumes of stock solution and buffer. Twenty micro-litres of each of these standards were added to 980 µl of blank whole blood to give a standard whole blood concentration range: 0 to 100 µg ml⁻¹. Standard DBS were prepared by transferring 30 µl of spiked whole blood onto Guthrie cards. To avoid bacterial growth due to moisture, blood-spotted papers were dried thoroughly for three hours on a non-absorbent surface at 22ºC in a cupboard away from light. Dried papers were then packed in plastic bags and stored at 4ºC in the dark until analysed.

Acidified triethylamine solution, 0.1% constituted the aqueous component of the chromatographic mobile phase and was prepared by transferring 1 ml of 100% triethylamine solution to a one-litre capacity beaker containing 800 ml of distilled water in a fume cupboard. The mixture was acidified to pH 3.5 with 100% acetic acid using a pH meter and the volume brought to 1 litre in a volumetric flask.

Sample preparation and extraction

![Figure 1: Blood collecting card indicating dried spots filled in a circle 10mm in diameter](image-url)
Sample collection
For the purposes of validation, 5 ml of blood was withdrawn from a peripheral vein of a healthy adult volunteer into a heparinised tube (Lithium Heparin, Teklab Ltd, UK) with subsequent spotting of 30 µl of the blood on a Guthrie card (Whatman 903, Whatman, Gmbh, Germany), which was then dried and stored at 4°C away from light. The volunteer then ingested 1 g of paracetamol and the sampling procedure repeated 30 minutes post dose. Approval for volunteer inclusion in the study was obtained from the North of Scotland Research Ethics Committees.

Volume of blood in dried blood spot
To determine the volume of blood in the 6 mm disc punched out of the dried blood spot, volumes of whole blood from 1 to 50 µl were transferred to blood collecting cards with an automatic micropipette. Filter paper was soaked to the other side with blood, and the spots thoroughly dried overnight. The diameters of the dried blood spots were measured with a Dual Scale Vernier Caliper (Mitutoyo, Japan). A calibration curve was then constructed by plotting volume against diameter. The volume of spotted blood was regressed on the diameter of the circle formed from the spots and the equation of the relationship between the diameter and the volume of spotted blood was established as:

Diameter = 2.0034 x (volume)^0.4557, R² = 0.9957

For the 6 mm diameter, calculated volume was 11 µl, representing the volume of blood contained in a punched out paper disc. This volume was used in the calculation of the concentrations of analytes in the blood spot samples throughout the study.

Extraction of analytes from paper
Paracetamol was extracted from the Guthrie cards by sampling paper discs with a paper perforator, 6 mm internal diameter, into a micro Eppendorf. Ammonium formate buffer and internal standard were added and vortexed for 2 minutes. Perchloric acid, 30% was added in the ratio 1 part of acid to 9 parts of test mixture, to precipitate proteins. Samples were then centrifuged at 13000 g for 5 minutes after which 20 µl of the supernatant was injected for chromatographic analysis.

Analytical system
Chromatographic analysis was performed by reverse phase high-performance liquid chromatography (RP- HPLC) with ultraviolet detection. Samples were introduced on Hichrom 3.5 µ C18 (100 x 4.6 mm) column (Hichrom Ltd, Reading UK), using a Gilson 231 autosampler (Anachem Ltd. Luton, UK). Samples were eluted with an isocratic mobile phase consisting of methanol / 0.1% triethylamine (TEA), pH 3.5 in a composition 20/80 %. The mobile phase was delivered with Gilson 302 pumps (Anachem Ltd. Luton, UK), at a flow rate of 0.8 ml/minute. In other to avoid back pressure, pump pressures were always checked to ensure that the pressure was not above 2.6 pounds per square inch (psi) at the first sample injection. Before each set of chromatographic run both methanol and 0.1% TEA were degassed with helium gas for 15 minutes to free the eluents of air bubbles. Chromatographic detection was carried out on a Waters 486 Tunable Absorbance Detector (Waters Ltd. Elstree, UK), fixing the detection wavelength at 244 nm. A guard cartridge, Hichrom HI-3.5 C18-10C (Hichrom Ltd, Reading UK) was used pre-column to prolong column life.

Method validation
Method performance characteristics namely: specificity, standard curve and linearity, accuracy and precision, limit of quantification (LOQ) and limit of detection (LOD), recovery, and stability of analytes were demonstrated according to the ICH guidelines (ICH 2001).

Specificity
To 920 µl of whole blood, 20 µl each of 100 µg ml⁻¹ of paracetamol, 2-acetamidophenol, caffeine and dimethylxanthine (a major metabolite of caffeine) were added. Thirty microlitres of this whole blood mixture was spotted on the Guthrie cards in duplicate. A blank whole blood and whole blood from a volunteer 30 minutes after 1 g oral paracetamol...
were also spotted. All spots were dried and extracted by the acid precipitation method described above. A chromatogram for each sample was generated by injecting 20 µl of extract into the HPLC system to assess the resolution of components present in the sample matrices.

**Standard curve and linearity**
The standard DBS concentrations prepared earlier were used for the construction of a calibration curve. Ten microliters (300ng) of 2-acetamidophenol was added to punched out discs and extraction proceeded by acid precipitation as described previously. Twenty microlitres supernatant was injected into the HPLC system. A standard linear curve was constructed by regressing peak area ratio [paracetamol:2-acetamidophenol] against known DBS concentrations. From this curve, the slope, the intercept and the coefficient of determination were obtained. This procedure was repeated on three separate days to give a four-day validation period.

**Accuracy and precision**
To determine accuracy, five replicates of DBS quality control (QC) samples containing paracetamol at the high, medium and low concentrations from the range 0-30 µg ml⁻¹ were extracted as previously described. These samples were analysed using the developed method and accuracy assessed as percentage relative errors. Precision was assessed as the percentage coefficient of variation (CV%) for each of the high, medium and low concentrations of paracetamol. Intra-day (within day) precision was evaluated by analysing five QC samples prepared on the same day, whilst a set of QC samples were analysed on five different days for inter-day (between assay) precision. A CV% less than 20 % was accepted as reliable precision.

**Lower limit of detection and lower limit of quantification**
To assess the lower limit of detection (LLOD) and lower limit of quantification (LLOQ), 10 different blank blood spots were analysed and absorbance signals at the retention times for paracetamol recorded. The LLOD was expressed as the paracetamol concentration that corresponded to blank value plus 3 standard deviations. LLOQ was determined as the paracetamol concentration that corresponded to blank value plus 5 standard deviations. Precision and accuracy at the LLOQ were also determined as described above. A CV% less than 15% and 20% were accepted as reliable for LLOD and LLOQ, respectively.

**Recovery**
Five replicates of DBS samples containing paracetamol at the LLOQ were extracted as previously described and 20 µl injected for chromatography analysis. For comparison, 20µl of parallel paracetamol buffer concentration was also injected onto the chromatographic column. Recovery was determined by comparing the chromatographic peak heights of extracted paracetamol from spiked blood spot to peak heights of paracetamol solutions. Peak height of extracted paracetamol expressed as a percentage of peak height of paracetamol solution was taken as the Recovery:

\[
\text{% recovery} = \frac{\text{peak height for extracted paracetamol}}{\text{peak height for paracetamol solution}} \times 100
\]

The recovery of 2-acetamidophenol (the internal standard) was determined at the concentration (30 µg ml⁻¹) used in the chromatographic analysis and replacing the peak height of paracetamol with peak height of the internal standard.

**Stability during storage**
A batch of DBS containing paracetamol at 3 and 30 µg ml⁻¹ was prepared and a set analysed immediately. A set of samples from the batch was stored at 25°C over a period of 1 month when six replicates of each concentration were analysed against a freshly prepared standard curve and the mean and standard deviation of the ratios of the different measurements determined.

**Clinical application**
As part of the validation, an in vivo experiment was conducted by applying the method to study the PK of oral paracetamol in five adult volunteers. Following ethics approval 2 ml of blood was obtained from a peripheral vein of five healthy male adult volunteers before and at 15, 30, 60, 120, 240 and...
420 minutes after 1 g oral paracetamol. DBS were prepared, extracted and analysed as described earlier.

**Pharmacokinetic analysis**
Maximum paracetamol concentration (C_{max}) and the time to reach this concentration (t_{max}) were taken directly from the observed concentration-time data for blood for each individual. All other PK parameters were calculated using the standard non-compartmental analysis, with a computer programme (EXCEL, Microsoft Inc.). Paracetamol elimination rate constant (k_{e}) was estimated in each individual by log linear regression of terminal concentration-time data. Terminal half-life (t_{1/2}) was calculated as ln2/k_{e}. Area under the concentration-time curves from the time of drug administration until the last quantified sample (AUC_{0-t}) were calculated using the linear trapezoidal method for the ascending and log-linear trapezoidal rule for the descending phase of the curves. AUC_{t-\infty} was estimated by dividing the last predicted concentration data by the elimination rate constant. AUC_{0-\infty} was calculated as the sum of AUC_{0-t} and AUC_{t-\infty}. Oral clearance (CL/F) was calculated from the relationship, Dose/AUC_{0-\infty}.

**RESULTS**

**Method validation experiment**

**Specificity**
Figure 2 represents chromatograms of DBS extracts from a blank blood (A), a spiked blood (B) and volunteer blood (C). Paracetamol and the 2-acetamidophenol were well resolved from each other and from endogenous blood components. Caffeine and its major metabolite, dimethylxanthine, which are potential interfering compounds, were also resolved from both paracetamol and 2-acetamidophenol. The mean retention time for paracetamol was 3.5 ± 0.01 min (mean ± SD, n = 5); and for 2-acetamidophenol, 6.4 ± 0.01 min (mean ± SD, n = 5). These retention times informed the setting of a reasonable chromatographic sample runtime of 8 minutes.

**Standard curves and linearity**

A plot of the peak area ratio against concentration was best described by a linear regression line in the range 0 - 100 µg ml^{-1} with a small negative intercept on the y axis (y = 0.0014x-0.0011). As the standard error of the intercept was 0.0010 (virtually equal to the intercept), the decision was made to allow a zero intercept for the curve. Evaluation of the goodness of fit revealed heteroscedasticity (increasing absolute error with increasing concentration) in the data and hence a weighting factor of 1/x^2 was applied to improve predictive performance of the curve (where x was concentration).

**Precision and accuracy**
The results for the precision and accuracy of the method are presented in Table 1, where it is shown that the method had acceptable intra-day and inter-day precision; with all CV% less than the upper limit of 20% recommended by the relevant guidelines (FDA 2001; ICH, 2005). Intra-day and inter-

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**Figure 2. Chromatograms of extracted DBS from:**
(A) blank blood; (B) spiked blood of concentration 2µg/ml each of paracetamol, 2-acetamidophenol, caffeine and dimethylxanthine; (C) volunteer blood after 1 gram of oral paracetamol. E - blood components, P - paracetamol, IS - 2-acetamidophenol, Dx - dimethylxanthine, C - caffeine
Table 1: Precision and accuracy

<table>
<thead>
<tr>
<th>Nominal Concentration</th>
<th>Precision</th>
<th>Mean relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±S.D</td>
<td>CV %</td>
</tr>
<tr>
<td>Intraday</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.3 ± 0.4</td>
<td>11.7</td>
</tr>
<tr>
<td>10</td>
<td>9.2 ± 0.8</td>
<td>10.5</td>
</tr>
<tr>
<td>30</td>
<td>30.2 ± 0.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Interday</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.8 ± 0.4</td>
<td>14.3</td>
</tr>
<tr>
<td>10</td>
<td>8.8 ± 0.8</td>
<td>10.8</td>
</tr>
<tr>
<td>30</td>
<td>29.9 ± 0.5</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Accuracy was measured by percentage mean relative error and nominal concentration was in microgrammes/ml.

Day accuracy was also satisfactory, as the mean relative errors were all less than 20%.

Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)
The LLOD and LLOQ were 550 pg and 1100 pg on column, corresponding to 0.25 and 0.5 µg ml⁻¹, respectively. The intra-assay percentage coefficient of variation for LLOD and LLOQ were 15 and 3%, respectively.

Recovery
The recovery of paracetamol and the internal standard yielded 84 and 101% respective recoveries. The intra-assay variations for the extractions were from 4.5 - 7.5% and show that they were precise as they were all below the 20% upper limit recommended by the ICH guidelines.

Stability
Stability data were assessed based on the ratio of concentrations at the different periods of measurements. The ratios were close to unity indicating paracetamol stability in DBS during storage at room temperature for at least 28 days (Table 2).

Figure 3 is a representative blood concentration–time profile obtained from DBS from a volunteer after ingesting 1 gram of paracetamol and where a peak at 15 minutes post dose can be seen. The concentration then declined over the 8 hour blood collection period depicting the elimination phase of the administered drug.

Table 2: Stability data of DBS containing 3 and 30 µg ml⁻¹ of paracetamol, stored at 25°C for 28 days.

<table>
<thead>
<tr>
<th>Days after storage</th>
<th>Mean concentration ± S.D.</th>
<th>Mean concentration ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5 ± 0.1</td>
<td>24.9 ± 0.9</td>
</tr>
<tr>
<td>28</td>
<td>2.4 ± 0.3</td>
<td>24.6 ± 2.3</td>
</tr>
<tr>
<td>Mean ratio</td>
<td>1.04</td>
<td>1.01</td>
</tr>
</tbody>
</table>

DISCUSSION
The lack of suitable bioanalytical methods to support the conduct of PK studies in young children is often cited among factors that contribute to inadequate data on paediatric medicines. The recommendation by ethics committees, that not more than 2.4 ml kg⁻¹ body weight of blood should be withdrawn from a study participant during a period of four
Table 3: Pharmacokinetic parameters of paracetamol in 5 adult volunteers after 1 g of oral paracetamol

<table>
<thead>
<tr>
<th>Volunteer ID</th>
<th>C_{max} (µg/ml)</th>
<th>T_{max} (min)</th>
<th>t_{1/2}(h)</th>
<th>CL/F (l/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.0</td>
<td>30</td>
<td>2.6</td>
<td>0.24</td>
</tr>
<tr>
<td>2</td>
<td>9.0</td>
<td>60</td>
<td>2.0</td>
<td>0.34</td>
</tr>
<tr>
<td>3</td>
<td>14.6</td>
<td>60</td>
<td>1.1</td>
<td>0.31</td>
</tr>
<tr>
<td>4</td>
<td>19.1</td>
<td>45</td>
<td>2.3</td>
<td>0.21</td>
</tr>
<tr>
<td>5</td>
<td>10.9</td>
<td>15</td>
<td>2.7</td>
<td>0.36</td>
</tr>
<tr>
<td>Median</td>
<td>14.6</td>
<td>45</td>
<td>2.3</td>
<td>0.31</td>
</tr>
<tr>
<td>(min,max)</td>
<td>(9,19.1)</td>
<td>(15,60)</td>
<td>(1.1,2.7)</td>
<td>(0.21,0.36)</td>
</tr>
</tbody>
</table>

Published values

\[ \text{Median} \pm \text{SD} \]

Key: * - Prescott et al., 1989; § - Haderslev et al., 1998; Φ - Shinoda et al., 2007; † - Bannwarth et al., 1992; ‡ - Yin et al., 2001; ♦ - Osborne et al., 1991. All published values are minimum and maximum, except for C_{max} which is mean ± s.d.

weeks (EMEA, 2006) has encouraged the development of analytical methods that utilise micro volumes of blood. The Guthrie card, as a volumetric sampling device, offers an opportunity to develop micro-analytical assays that require as little as 30 µl of blood in the form of DBSs and with the added advantage of not requiring controlled handling and frozen transport. DBS analysis has been proposed for the therapeutic monitoring of theophylline (Rattenbury and Tsanakas, 1988), metformin (Aburuz et al., 2006) and tacrolimus (Hoogtanders et al., 2007) and the current study for the quantification of paracetamol based on HPLC separation with UV detection supports this approach. The developed method, meets guideline criteria (ICH, 2001) and proved to be specific, accurate, precise and sensitive, thus demonstrating its suitability for PK studies in children. The method demonstrated acceptable specificity by providing resolution of paracetamol and the internal standard from the endogenous blood components.

The use of an isocratic mobile phase resulted in less varied retention times (S.D., 0.01), which made analyte identification more reliable and separation of analytes was achieved in less than 7 minutes compared to more than 12 minutes required for a similar previously published method (Oliveira et al., 2002). The longer retention times of the method described by Oliveira et al., (2002) were required in order to ensure resolution of the highly polar glucuronide and sulphate metabolites of paracetamol, whereas the current method was developed specifically to measure parent paracetamol, which under the chosen chromatographic conditions could be eluted in less than 4 minutes. Another previously published method for the measurement of paracetamol in plasma required a retention time in excess of 4 minutes (Campanero et al., 1999) and it is these reduced retention times which reduce sample run-times and improve sample throughput. The efficiency of the current method therefore emphasises the need to develop assays that are study-objective orientated. In conducting research in children it is useful to have as few as possible restrictions on research participants. As the developed method resolved caffeine and its major metabolites, the consumption of caffeine-containing beverages, which is popular among the population, would not be a restriction in studies using this assay.

The method had acceptable intra-day and inter-day accuracy and precision; all CV% were less than 15%, as recommended by guidelines (FDA 2001; ICH, 2005). Although the limit of quantification for the method was 0.5 µg/ ml, corresponding to 1100 pg on column, was greater than that reported in previous assays (al-Obaidy et al., 1995; Oliveira et al., 2002; Jensen et al., 2004), the sensitivity was still well within the requirements for the measurement of paracetamol blood concentration 8 hours post dose. When the recommendation that PK studies should measure analyte concentration to at least three times the terminal half-life of elimination (t_{1/2}) (Ette et al., 1995) is taken together with the reported t_{1/2} for paracetamol of 1-2.9 h (Bannwarth et al., 1992), blood sampling that covers 8 hours post dose should permit reliable paracetamol PK analyses using this method.

In the preparation of samples prior to introduction onto the chromatographic column, paracetamol has previously been extracted by liquid-liquid extraction...
(Campanero et al., 1999; Oliveira et al., 2002) or by solid phase extraction (Speed et al., 2001). The authors of these methods did not report the time required for this sample preparation. Although the acid precipitation method required cheaper reagents and was faster, because it did not require drying, it was less efficient (84%). All recoveries had CV% less than 8% further demonstrating acceptable accuracy as recommended by guidelines (FDA, 2001, ICH 2005).

The authors of these methods did not report the time required for this sample preparation. Although the acid precipitation method required cheaper reagents and was faster, because it did not require drying, it was less efficient (84%). All recoveries had CV% less than 8% further demonstrating acceptable accuracy as recommended by guidelines (FDA, 2001, ICH 2005).

The results for the stability experiments showed that paracetamol was stable in DBS for at least 4 weeks of storage at 25°C which was in agreement with a recent report (Spooner et al., 2009) which demonstrated that paracetamol was stable in DBS stored at room temperature even up to 113 days. Blood collecting filter papers have also demonstrated stability for at least three months when metformin DBS were stored at -70°C (Aburuz et al., 2006) and compound stability has been reported up to one month of storage at room temperature for different classes of organic compound including coumarins, carboxylic acids, imidazole and secondary amines (Beaudette and Bateman, 2009). This stability during storage confers on the DBS assay the qualities of ease of storage and transport.

The results of the volunteer PK studies of paracetamol indicated that the time to achieve maximum blood concentration after 1 g of oral paracetamol administration was 15 - 60 minutes, and the median of the maximum paracetamol concentration (C_{max}) was 14.6 µg ml\(^{-1}\). These findings agree with adult studies that suggest a time of between 10 and 60 minutes for paracetamol plasma concentration to peak (Haderslev et al., 1998; Shinoda et al., 2007). With the widely held view that paracetamol plasma therapeutic concentration is from 10-20 µg/ml, a C_{max} above 10 µg/ml obtained with this newly developed method was expected. The median elimination half-life of paracetamol obtained in the volunteer study was 2.3 h, which is within the range (1.1 – 2.9 h) reported in previous studies (Bannwarth et al., 1992; Yin et al., 2001). The median (min, max) values of 0.31 l/h/kg (0.21, 0.36) for oral clearance (CL/F) obtained also agree with previously reported values of 0.28 – 0.37 l/h/kg oral clearance in healthy adults (Osborne et al., 1991; Haderslev et al., 1998). All the PK parameters and measures determined with the developed method confirm the known PK of oral paracetamol in healthy adults, thus giving further credence to the performance of the method.

Conclusions
A simple and rapid micro-analytical method based on HPLC with UV detection for the measurement of paracetamol in dried blood spots was developed and validated. The method requires the withdrawal of only 30 µl of blood from a study participant, making it a suitable bio-analytical method to support PK studies of paracetamol in all paediatric age groups. This micro volume of blood also means that the method can be applied to both dense sampling, needed for traditional PK analysis, and sparse sampling for population PK analysis. The method employed an isocratic eluting mode which was able to resolve all analytes within 7 minutes, allowing sample run-time to be set at 8 minutes. This run-time should allow a rapid throughput, which together with the simple chromatographic equipment used should also reduce analytical costs.

Acknowledgement
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COMPETING INTERESTS
The authors declare that they have no competing interests.

REFERENCE

al-Obaidy S.S., Li Wan Po A., McKiernan P.J.,


Spooner N., Lad R. and Barfield M. (2009) Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioana-
Assay for paracetamol in dried blood spots

Mohammed et al.,

Analytical method. *Analytical chemistry* 81, 1557-1563.