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Dried blood spot liquid chromatography assay for therapeutic drug monitoring of metformin

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Abstract

The use of blood spot collection cards is a simple way to obtain specimens for analysis of drugs for the purpose of therapeutic drug monitoring, assessing adherence to medications and preventing toxicity in routine clinical setting. We describe the development and validation of a microanalytical technique for the determination of metformin from dried blood spots. The method is based on reversed phase high-performance liquid chromatography with ultraviolet detection. Drug recovery in the developed method was found to be more than 84%. The limits of detection and quantification were calculated to be to be 90 and 150 ng/ml, respectively. The intraday and interday precision (measured by CV%) was always less than 9%. The accuracy (measured by relative error, %) was always less than 12%. Stability analysis showed that metformin is stable for at least 2 months when stored at −70 °C. The small volume of blood required (10 μL), combined with the simplicity of the analytical technique makes this a useful procedure for monitoring metformin concentrations in routine clinical settings. The method is currently being applied to the analysis of blood spots taken from diabetic patients to assess adherence to medications and relationship between metformin level and metabolic control of diabetes.

Keywords: HPLC; Metformin; Blood spot; Therapeutic drug monitoring

1. Introduction

Metformin (Fig. 1) is one of the most commonly prescribed medications for type 2 diabetes and it is the drug of choice in obese diabetic patients [1]. Measuring the plasma concentration of metformin is important for studying the pharmacokinetics of this drug, for determination of patient adherence with prescribed therapy in diabetic patients and for general drug monitoring.

The most serious side effect associated with metformin is lactic acidosis, which may be related to the high circulating concentration of the drug [2]; it is recommended that plasma concentrations of metformin should be determined as a biological safety parameter in all treated type 2 diabetes patients [3]. Measuring plasma levels is also a direct method for measuring adherence of patients with a prescribed regimen of metformin.

In spite of the long-standing availability of metformin, there is relatively little published information on its pharmacokinetics in man. There are also limited data available regarding its disposition in different tissues or organs [2]. This is at least in part due to difficulties in analysing metformin which in turn is mainly due to its high polarity (octanol:water partition coefficient 0.01) which makes extraction very difficult. It also has a very short retention time when separated using reversed phase chromatography (and so may coincide with the rapidly eluting endogenous substances from the plasma) and cannot be eluted using normal phase chromatography due to its very strong retention. Gas chromatographic methods and HPLC-mass spectrometry methods [4–7] are not ideal for routine clinical purposes because their procedures are time consuming and the instrumentation is not always available in clinical laboratories.

Several HPLC methods [2,3,8–18] have been used for the determination of metformin in biological fluids. However, they suffered from several disadvantages, such as lack of sensitivity [9,10,12,15,16] where the reported limit of quantification was

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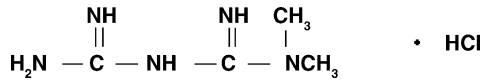

Fig. 1. Structure of metformin HCl.

above 50 ng/ml, the use of derivatisation and complex extraction procedures which are tedious and time consuming [8,17], and the use of ultrafiltration and a column switching system [15].

In a recent publication we developed a novel method for metformin extraction and chromatographic analysis utilizing solid phase extraction and ion-pair chromatography. The method proved to be reliable and simple and can be used successfully for pharmacokinetics analysis [19]. However, our main area of interest is to measure metformin level for clinical purposes such as therapeutic drug monitoring to prevent toxicity and to assess adherence to medications. The method we developed earlier was not successful in for routine clinical use for several reasons: first, although the procedure for sample preparation and solid phase extraction were simple in comparison to many published methodologies it was still not convenient enough for routine clinical use. Second, the requirement of solid phase extraction can be associated with high cost when used for routine clinic assessment. Third, the requirement of blood sample to be taken from patients every clinic visit can be inconvenient for patients.

Therefore, our objective in the present study was to develop a method for therapeutic drug monitoring of metformin using a more simple technique; the dried blood spot assays. The method will be useful in particular for adherence and toxicity monitoring. This method reduces the time requirement for sample preparation required for solid phase extraction; reduces the cost associated with the use of solid phase extraction cartridges and more convenient for patients as only blood spot will be collected. The only anticipated disadvantage associated with this method is the low sensitivity which makes it less suitable for pharmacokinetic studies.

2. Experimental

2.1. Materials

Metformin HCl (>99%), salbutamol (>99%) (Fig. 1), potassium dihydrogenphosphate (KH_2PO_4), sodium dodecyl sulphate salt (sodium lauryl sulphate, SDS), were purchased from Sigma (USA). Acetonitrile and methanol were of HPLC grade and purchased from Tedia Company (USA). Membrane filters F-450 0.45 μm were obtained from Gelman Laboratory (Portsmouth, UK). Blank blood for method development was donated from volunteers.

2.2. Standards

Salbutamol was used as an internal standard as it has similar polar properties as metformin. Stock solutions of the internal standard were prepared by dissolving 0.01 g in 100 ml of water then it was further diluted with water (1:10). When preparing the samples for extraction 20 μl of this stock was added to the extraction mixture (1 ml methanol and the filter paper) to produce an internal standard concentration of 200 ng/ml.

Standard stock solution of metformin HCl was prepared by dissolving 0.05 g of metformin HCl in 100 ml water. From this stock 1 ml was taken and further diluted with water to 10 ml, this was further diluted with water to produce different stock solutions. Twenty microliters of the stock solutions were added to 0.98 ml human whole blood aliquots to yield final concentrations of 300, 600, 1000, 2000, 3000 and 5000 ng/ml. These concentrations were chosen as they cover the actual plasma concentrations obtained in patients receiving metformin therapeutically.

2.3. Sample preparation and extraction

Thirty microliters of the prepared spiked blood standards were spotted directly onto Guthrie type cards (Whatman, UK) so that the filter paper was filled on both sides (at least 10 μl is required to produce 6 mm spot). The spots were labelled with the date and time of collection, allowed to dry overnight at room temperature and were stored in plastic bags at room temperature until assay. The average diameter of the 30 μl of blood spots was 9.5 mm. A linear relationship was obtained between the volume of blood and the diameter of blood spot (linearity range 5–100 μm; diameter (mm) = 0.17 × blood volume (μl) + 4.3; $r = 0.999$).

Metformin was extracted from the dried blood spots by punching out one spot using a hole puncher (6 mm). The punched 6 mm paper disks were then added to 1 ml 60% methanol (and 20 μl internal standard was added) and vortex mixed for 90 s. The spot was then discarded and the extract air-dried under a stream of air at 38 °C (it was made sure that all the extract was removed from the spot using a micropipette). The extract was redissolved in 0.2 ml of the mobile phase with duplicate 0.1 aliquots for assay. Blood spots (at least 10 μl is required) were taken from volunteers by finger-prick with a lancet. These were prepared and extracted as detailed above.

2.4. Chromatography

The chromatographic system consisted of Shimadzu SIL-10ADVP autosampler, LC-10ADVP pump, DGE-14A degasser, SCL-10ADVP controller and SDP-M10ADVP phtotodiode array detector. The separation was performed using a Waters Symmetry C_{18} analytical column (150 mm × 4.6 mm, 5 μ; Waters, USA) preceded by a matching Symmetry C guard column (Waters, USA). Data recording was carried out using Shimadzu Class VP System.

The mobile phase was pumped at a flow rate of 0.5 ml/min and consisted of 2 mM sodium dodecyl sulphate, acetonitrile (37.5%) and potassium dihydrogenphosphate (62.5%) (from 0.02 M buffer to produce a final buffer concentration of 0.0125 M). The pH of the mobile phase was adjusted at 7.3 using NaOH. The prepared mobile phase was filtered through a 0.45 μm Millipore filter and degassed ultrasonically before use. The detector wavelength was set at 236 nm for metformin analysis. The total time required for the extraction and analysis of 20

samples was approximately 3 h (which is 40% saving compared to our previously published paper).

2.5. Assay characteristics for method validation

2.5.1. Specificity

To demonstrate the specificity of the method blank blood spots from five different sources, spiked blood spots and blood spots from volunteers who were taking metformin were analysed. Representative chromatograms were generated to show that other components that could be present in the sample matrix are resolved from the parent analyte.

2.5.2. Standard curve and linearity

A standard curve was prepared on each day of a 5 day validation period; the slope, the intercept and the correlation coefficient were determined. For calculation of the standard curve a plot of peak area ratios against concentrations were used.

2.5.3. Accuracy and precision

Intraday precision, interday precision and the accuracy were calculated from data obtained during a 5 day validation. Three concentrations were chosen from the high, medium and low range of the standard curve (500, 1500 and 4000 ng/ml). Samples spiked at these three concentrations were analysed on each day of the 5 day validation ($n = 5$ at each concentration). Precision was expressed as the coefficient of variation (CV%). Accuracy was expressed as the mean relative error (RE%). A precision (CV%) $\leq$15% and an accuracy (RE%) $\leq$15% are acceptable [20].

2.5.4. Limit of quantification (LOQ) and limit of detection (LOD)

LOD and LOQ are both defined in terms of the mean and standard deviation (S.D.) from a series of blank samples. To assess LOD and LOQ for the method, ten independent blank blood spots were analysed and absorbance signals were recorded at the chromatographic retention times for metformin. The LOD was expressed as the analyte concentration corresponding to the sample blank value plus 3 standard deviations. LOQ was expressed as the analyte concentration corresponding to the sample blank value plus 5 standard deviations [21]. Precision and accuracy at the LOQ were determined.

2.5.5. Recovery

The recovery was calculated by comparing the peak heats obtained after extraction of metformin from blood spots spiked with know concentrations of metformin and comparing those with peak heights obtained from solutions of metformin containing the same concentrations as the spiked blood spots. The following formula was used: recovery = (peak height for extracted analyte/peak height for solution of analyte) 100%. The recovery of metformin was determined at three concentrations (500, 1500 and 4000 ng/ml) ($n = 6$ at each concentration). The recovery of the internal standard (salbutamol) was determined at the concentration used which was 200 ng/ml.

2.5.6. Stability

The stability of samples during storage was determined by analysing blood spots containing metformin at the level of 500 and 1500 ng/ml ($n = 6$) twice with a 2 month interval in-between (after freezing at $-70\,^{\circ}C$). The mean ($\pm$S.D.) values of the ratios between the two measurements were determined.

2.6. Concentration–time profile

A single metformin dose of 850 mg was given to a volunteer and metformin blood spot level was then measured for the next 12 h.

2.7. Calculations

Standard regression curve analysis was computed using Class VP Software without forcing through zero. Means and standard deviation were calculated using EXCEL® software (Microsoft Corporation, USA).

3. Results

3.1. Optimisation of metformin extraction from DBSs

In order to improve extraction of metformin from the DBS several issues were investigated: the extraction efficiency was tested with 10–100% methanol ($n = 5$), the effect of vortex mixing period (30–90 s, $n = 5$) was also determined. The best conditions are the ones which produce the highest recovery.

The recoveries obtained when testing different percentages of methanol ($n = 5$) were close (within 5% range); however 60% methanol produced the highest recovery. The longer the vortex mixing time the higher the recovery obtained, the best recovery was obtained when vortex mixing for 90 s. Therefore, 60% methanol and 90 s vortex mixing were selected as the optimal conditions and used for the validation study.

3.2. Validation

3.2.1. Specificity

Under the assay conditions described above, metformin and salbutamol were well resolved with retention times of 5.4 and 6.7 min, respectively. The method exhibits selectivity for endogenous compounds as no interfering peaks were observed in the same chromatographic windows in the blank chromatogram (10 different blanks were examined) (Figs. 2–4). Fig. 2 illustrates the chromatogram obtained from a blank DBS while Fig. 3 is for a spiked DBS. Fig. 4 is for a DBS from a volunteer who took 850 mg metformin orally.

3.2.2. Standard curve and linearity

The standard curve was determined on each day of the 5-day validation period, the slope (0.0415 ± 0.002), the intercept (1.39 ± 0.3) and the correlation coefficient were determined ($\geq$0.99). The relationship between peak area ratio

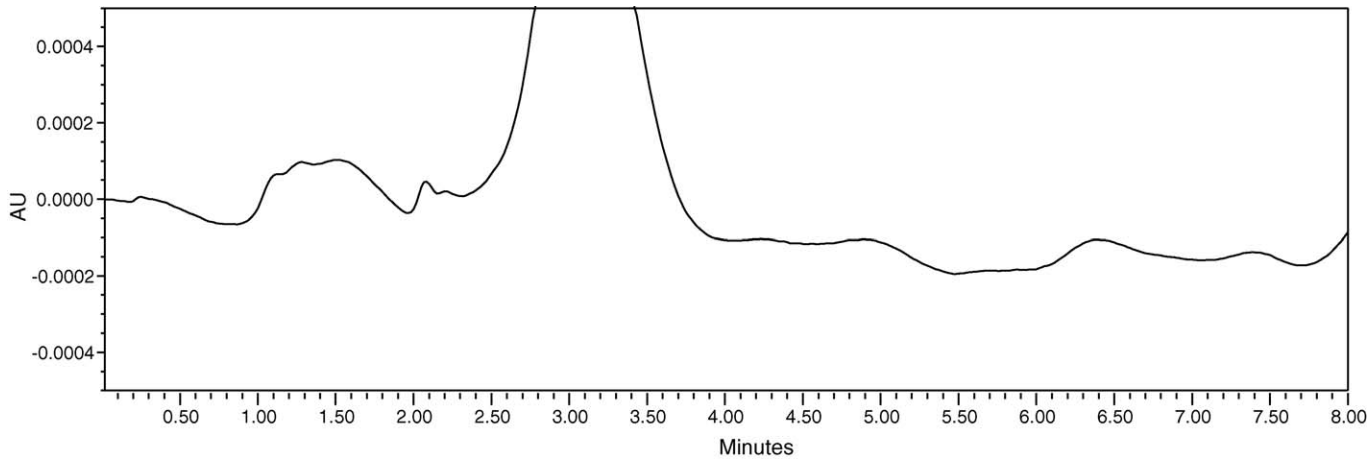

Fig. 2. Chromatogram showing the result of extraction of a blank DBS.

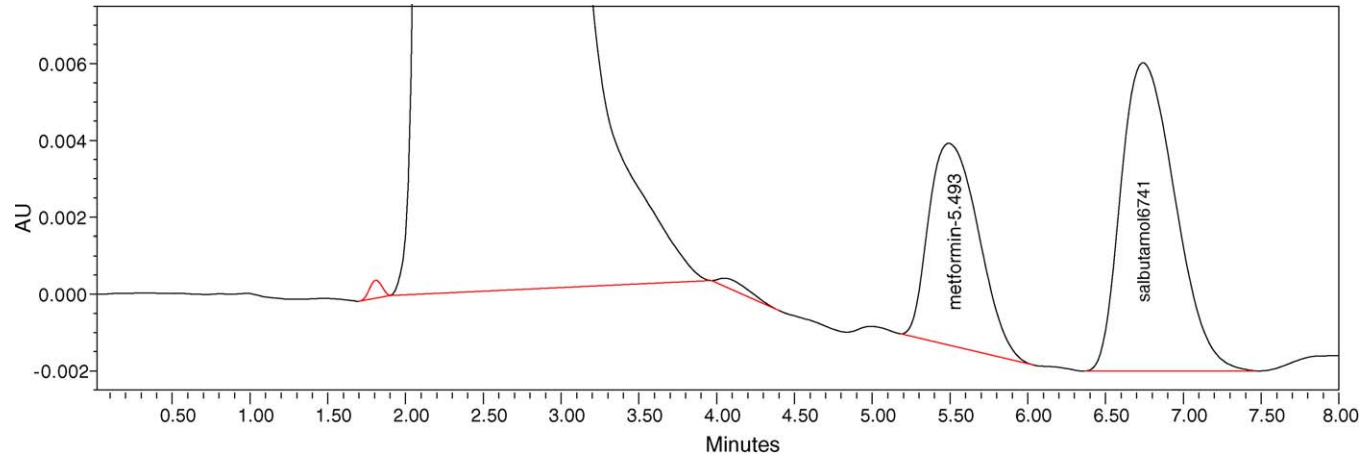

Fig. 3. Chromatogram showing the result of extraction of DBS spiked with metformin and salbutamol [metformin 3000 ng/ml, salbutamol 200 ng/ml].

and concentration were linear within the studied concentration range (300–5000 ng/ml). During clinical application of the method linearity was established for concentrations up to 10,000 ng/ml.

3.2.3. Accuracy and precision

The values obtained for intraday and interday precision and accuracy during the 5 day validation are shown in Table 1. All values for accuracy and precision were within recommended

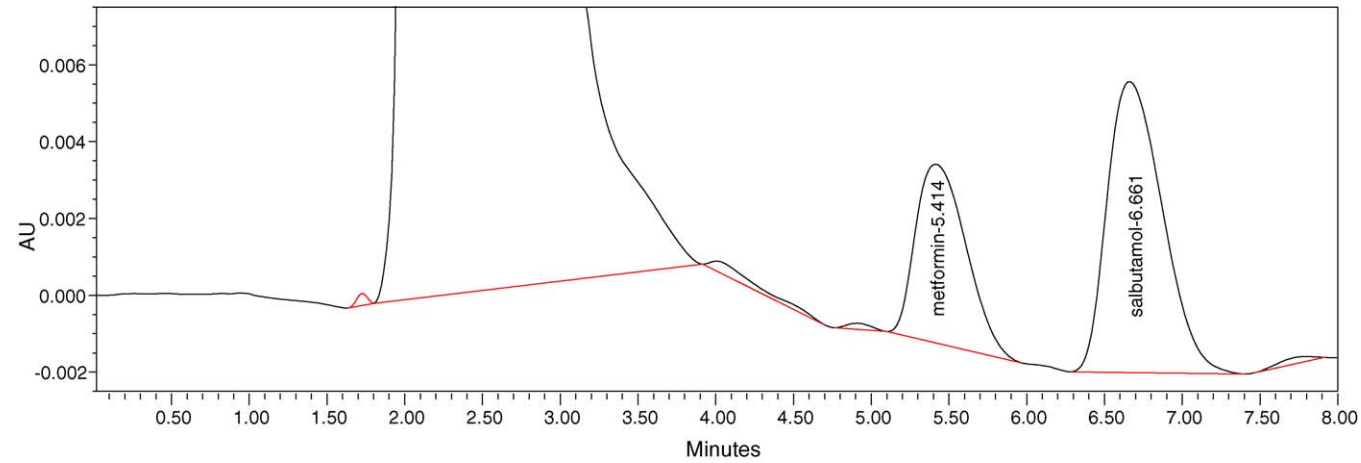

Fig. 4. Chromatogram showing the result of extraction of a DBS for volunteer who was ingested 1000 mg metformin showing metformin and salbutamol [metformin 2400 ng/ml, salbutamol 200 ng/ml].

Table 1
Intraday and interday precision and accuracy data for assay of metformin in DBS ($n=5$)

Nominal concentration (ng/ml)	Precision		Accuracy , mean relative errors (%)
	Mean ± S.D.	CV%	
Intraday			
500	553.4 ± 29.9	5.4	10.7
1500	1560.3 ± 35.1	2.3	4.0
4000	4280.3 ± 196.8	4.6	7.0
LOQ	168.3 ± 20.5	12.2	12.2
Interday			
500	559.2 ± 37.4	6.7	11.8
1500	1615.2 ± 67.6	4.2	7.7
4000	4302.4 ± 372.3	8.7	7.6

Table 2
The recovery data for assay of metformin in plasma ($n=6$)

Nominal concentration (ng/ml)	Mean ± S.D.	CV%
500	84.0 ± 2.3	2.8
1500	84.5 ± 2.8	3.3
4000	86.5 ± 1.6	1.9

limits. Intraday precision ranged between 2.3 and 5.4% whereas the interday precision was between 4.2 and 8.7%. The intraday mean error was between 4.0 and 10.7% whereas the interday mean error was between 7.6 and 11.8%.

3.2.4. Limit of quantification (LOQ) and limit of detection (LOD)

The LOD and the LOQ for metformin were calculated to be to be 90 and 150 ng/ml, respectively. Concentrations down to 150 ng/ml were detected with accepted accuracy and precision using this method (Table 1).

3.2.5. Recovery

Table 2 shows data for extraction recovery ($n=6$). The recovery of metformin was between 84.0 and 86.5%.

3.2.6. Stability

The mean and standard deviation for the ratio between the two measurements carried at a 2 months interval (stored at −70 °C) for metformin ($n=6$) was 1.03 ± 0.04. This indicates that metformin is stable for at least 3 months when stored at −70 °C. In process stability was proven during the 5-day precision accuracy study where all samples were stored at room temperature.

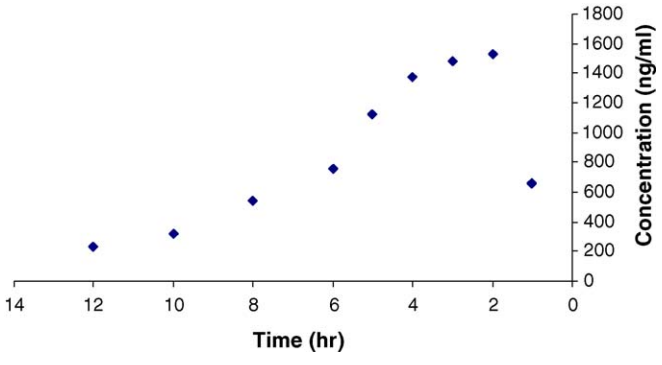

Fig. 5. Blood spot concentration-time profile of metformin in a healthy volunteer after a single 850 mg dose of metformin.

3.3. Concentration–time profile

Fig. 5 shows time profile of metformin concentration in blood spots taken from a volunteer after an oral dose of 850 mg.

4. Discussion and conclusions

Dried blood spot analysis has been used for therapeutic drug monitoring or other clinical purposes for theophyline, phenytion and aminoglycoside antibiotics [22–25] but this is the first report of its application to metofmrin monitoring. The methodology presented has combined the advantage of microsampling, inherent in blood collection on filter-paper, with an accurate, precise and selective liquid chromatography technique. The small volume of blood required (10 μl), combined with the simplicity of the analytical technique makes this a useful procedure for monitoring metformin concentrations in routine clinical settings.

The concept of monitoring metformin concentrations in blood using the DBS technique arises from the need to monitor metformin level routinely in diabetic patents. This routine measurement is important to assess adherence to medications and to prevent toxicity from metformin. Monitoring adherence is important to improve diabetic control and to prevent unneeded dose adjustment or adding more medications in those who have poor disease control due to poor adherence as the clinician may think that treatment was not effective. Therefore clinical pharmacist and physicians can conveniently take a blood spot at the time of patients' visits from those with poor disease control and check for metformin level to decide if patients are adherence or not. In a recent study we found that plasma concentrations of 84 patients on metformin ranged between 198 and 7479 ng/ml; thus confirming the applicability of the current method with out problems of low sensitivity. The method is currently being applied to the analysis of blood spots taken from diabetic patients to assess adherence to medications and relationship between metformin level and metabolic control of diabetes.

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