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Determination of metformin in plasma using a new ion pair solid phase extraction technique and ion pair liquid chromatography

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Abstract

This article describes the development of the first ion pair solid phase extraction technique (IPSPE), which has been applied to the extraction of metformin from plasma samples. In addition an ion pair chromatographic method was developed for the specific HPLC determination of metformin. Several extraction and HPLC methods have been described previously for metformin, however, most of them did not solve the problems associated with the high polarity of this drug. Drug recovery in the developed method was found to be more than 98%. The limit of detection and limit of quantification was 3 and 5 ng/ml, respectively. The intraday and interday precision (measured by coefficient of variation, CV%) was always less than 9%. The accuracy (measured by relative error, R.E.%) was always less than 6.9%. Stability analysis showed that metformin is stable for at least 3 months when stored at −70 °C. The method has been applied to 150 patient samples as part of a medication adherence study.

Keywords: Metformin

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 coincides with the rapidly eluting endogenous substances from the plasma) and cannot be eluted using normal phase chromatography due to its very strong retention.

Three criteria must be met for the plasma metformin assay to be relevant and functional within clinical laboratories; firstly, the method should allow determination of metformin at low concentrations, secondly, sample pre-treatment should be simple, rapid and efficient (in terms of recovery and removal of interferences) and thirdly the choice of the chromatographic method should be such that metformin is eluted in a reasonable time, but well separated from the plasma front. Gas chromatographic methods and HPLC-mass spectrometry methods [4–7] are not ideal for clinical purposes because their procedures are time consuming and the instrumentation is not always available in clinical laboratories.

Several HPLC methods [2,3,8–17] have been used for the determination of metformin in biological fluids but none of them completely satisfy the aforementioned characteristics.

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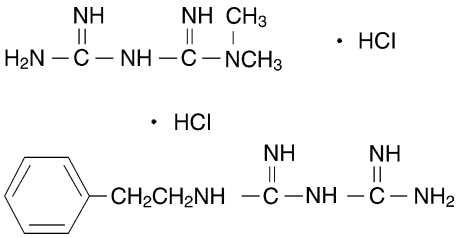

Fig. 1. Structures of metformin HCl (up) and phenformin HCl (down).

Previously described methods suffered from several disadvantages, such as lack of sensitivity [9,10,12,15,16] where the reported limit of quantification was 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 order to overcome the problem of difficulty in extraction of metformin, protein precipitation has been the most common method used for sample preparation [2,3,10,14,16], however, it has been reported that the use of this method sacrifices sensitivity [15] and that this method is not effective in removing the endogenous interferences [3,11]. Other authors solved the extraction problem by using ion pair extraction [11,18], where an ion pair reagent is complexed with metformin resulting in a complex that is less polar and which can be extracted using an organic solvent. Using this method these investigators were able to extract metformin from biological samples, however, the reported recoveries were 27% [11] and between 59% (2 μg/ml) and 80% (0.25 μg/ml) [18]. These methods involved solvent extraction which is tedious and time consuming.

Currently the most convenient method available for extraction of drugs from biological fluids is solid phase extraction (SPE) which has the advantages of being simple, rapid and effective in removing interferences, however, it is not possible to use conventional solid phase extraction for metformin as it will not be retained in the SPE column. Huupponen et al. [12] solved this by using C8 extraction cartridges, which are more polar than the conventional C_{18} units and can successfully retain the very polar metformin; the reported recovery was 92%, however, the LOQ was relatively high (50 ng/ml) and the retention time of metformin was 2.8 min (thereby allowing potential interference with endogenous substances).

Because of the high polarity of metformin it is rapidly eluted from reversed phase columns, even if the mobile phase has a very low organic content. Even with the use of a more polar phenyl column [12] the retention time is still not optimal. Yuen and Peh [16] and Cheng and Chou [2] used Cyano and Si columns, respectively; the reported retention times for metformin were more than 5 min, however, the methods were limited by the use of protein precipitation in the sample preparation and the sensitivity of the first method is low (60 ng/ml). The use of cation exchange columns can solve the retention problem of metformin [3,9,15], however, these published methods are limited mainly by the tedious and time consuming sample preparation methods and/or by poor sensitivities which were 200,100, and 20 ng/ml, respectively.

In the present study a sensitive, rapid and specific method was developed for SPE and HPLC determination of metformin.

2. Experimental

2.1. Materials

Metformin HCl (>99%), phenformin HCl (>98%) (Fig. 1), potassium dihydrogenphosphate (KH_2PO_4), sodium dodecyl sulphate salt (SDS; sodium lauryl sulphate), decane sulfonic acid sodium salt and heptanesulfonic acid sodium salt were purchased from Sigma (Poole, England). Acetonitrile and methanol were of HPLC grade and purchased from Romil (Cambridge, UK). Membrane filters F-450 0.45 μm were obtained from Gelman Laboratory (Portsmouth, UK). Solid phase extraction cartridges [Oasis® HLB and MCX cartridges (1 ml, 30 mg)] were purchased from Waters (AGB, Belfast). Extraction was carried on a Waters extraction manifold. Blank blood was donated from Northern Ireland Blood Transfusion Centre.

2.2. Standards

Phenformin HCl was used as an internal standard. Stock solutions of the internal standard were prepared by dissolving 0.016 g in 100 ml of water (initially dissolved in a few drops of methanol) then it was further diluted with water (1:40). When preparing the samples for extraction 50 μl of this stock was added to 1 ml of plasma to produce an internal standard concentration of 200 ng/ml plasma.

Standards stock solutions were prepared by dissolving 0.08 g of metformin HCl in 100 ml methanol. From this stock 0.2 ml was taken and further diluted with water to 10 ml (first working standard with a concentration of 16 μg/ml); this was further diluted with water to produce the remaining working standards. Aliquots (50 μl) of the aqueous solution of the internal standard (phenformin HCl) and 0.125 ml of metformin HCl working standards were added to 1 ml aliquots of plasma resulting in the following concentrations 50, 100, 250, 500,1000, and 2000 ng/ml plasma. This procedure was used in order to use the lowest possible amount of methanol as the presence of methanol significantly affects the recovery of metformin. These concentrations were chosen as they cover the actual plasma concentrations obtained in patients receiving metformin HCl therapeutically.

2.3. Sample preparation and extraction

Patients' blood samples were collected into glass tubes containing EDTA and centrifuged at 3000 rpm (1610 × *g*)

for 15 min. The separated plasma was kept frozen at −70 °C until analysis. To 1 ml of patient plasma samples, 50 μl of the internal standard solution and 0.125 ml of water were added, vortex mixed for 30 s and centrifuged at 14000 rpm (17,500 × *g*) for 5 min before extraction to prevent blockage of the cartridges.

The samples and standards were prepared as detailed above and then extracted using the developed ion pair solid phase extraction (IPSPE) technique utilising Oasis® HLB cartridges (1 ml, 30 mg), which were connected to Waters extraction vacuum manifold as follows:

1. Condition 1: 1 ml methanol followed by 1 ml of water.
2. Condition 2: 1 ml aqueous solution of 2 mM/l sodium dodecyl sulphate (ion pair reagent).
3. Load: 1.175 ml spiked plasma (as prepared above).
4. Wash: 1 ml 30% methanol.
5. Elute: 1 ml methanol.
6. Evaporate eluent under nitrogen stream, reconstitute in 350 μl of the mobile phase and inject 150 μl onto the HPLC column.

This extraction procedure was developed by following a series of experiments in an effort to extract metformin from plasma with good recovery.

2.4. Chromatography

The chromatographic system consisted of Shimadzu LC-GA pump, Waters 712 WISP autosampler and UV absorbance detector (LDC 12 Milton Roy, Riviera Beach, USA). The separation was performed using a Discovery C_{18} Supelco analytical column (250 mm × 4.6 mm, 5 μ; Sigma, Poole, England). A Supelco Discovery guard column (20 mm × 4 mm, 5 μ; Sigma, Poole, England) was used. Data recording was carried out using the Shimadzu Class VP system.

The mobile phase was pumped at a flow rate of 1 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.

These chromatographic conditions were developed by following a series of experiments in an effort to elute metformin at a retention time that is suitable for bioanalysis. The total time required for the extraction and analysis of 20 samples was approximately 5 h.

2.5. Assay characteristics for method validation

2.5.1. Specificity

To demonstrate the specificity of the method, blank plasma, spiked plasma samples and plasma samples from patients who were prescribed metformin therapeutically were analysed. Representative chromatograms were generated to show that the extraneous peaks were 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 height ratios against concentration were used.

2.5.3. Accuracy and precision

Intraday precision, interday precision and the accuracy were calculated from data obtained during a five day validation. Three concentrations were chosen from the high medium and low range of the standard curve (100, 400 and 1500 ng/ml). Plasma 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 (R.E.%). A precision (CV%) ≤15% and an accuracy (R.E.%) ≤15% are acceptable [19].

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

Ten independent blank plasma samples were measured singly. 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 [20].

2.5.5. Recovery

The recovery of metformin was determined at three concentrations (100, 400 and 1500 ng/ml) ($n = 6$ at each concentration). The recovery of the internal standard (phenformin) 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 12 spiked plasma samples twice (at two concentrations 100 and 400 ng/ml, $n = 6$ at each concentration) with a three month interval between (after freezing at −70 °C). The mean (±S.D.) values of the ratios between the two measurements were determined.

2.6. 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 and discussion

3.1. Optimisation of the mobile phase

Initially a mobile phase consisting of 0.01 M potassium dihydrogen orthophosphate and acetonitrile (40:60, pH 7) was tested. The retention time for metformin was 2.8 min which was not suitable for analysis of biological samples due to potential interferences with the plasma front. Different concentrations of acetonitrile and different pHs did not make significant changes in the elution pattern of metformin. Hence, a reversed phase ion-pair chromatographic (IPC) method was tested to improve the separation and retention of metformin. Ion pair chromatography (IPC) is a method of improving separation of charged analytes especially for strong acids and bases such as metformin.

Both heptane sulfonic and decane sulfonic acid did provide the suitable retention even when using different pH or organic phase contents. Further work on the optimisation of the mobile phase for use with plasma resulted in the following mobile phase being selected for plasma assays: pH: 7.3, 2 mM sodium dodecyl sulphate, acetonitrile 37.5%, potassium dihydrogenphosphate (62.5%) (from 0.02 M buffer to produce a final buffer concentration of 0.0125 M).

3.2. Optimisation of the extraction techniques

Due to the very high polarity of metformin it is not possible to extract it from biological fluids using an organic solvent or conventional SPE. Oasis® HLB cartridges are packed with a water wettable polymeric sorbent that allows greater flexibility in processing samples since it can dry out during the extraction procedure without diminishing its ability to retain analytes, thereby overcoming the drying out problem of the conventional silica based SPE cartridges [21].

Extraction of metformin using Oasis® HLB resulted in a poor recovery of 10.0 ± 0.1%. Oasis® MCX extraction cartridges contain a mixed-mode polymeric patented sorbent with reversed phase and cation-exchange functionalities. The major difference between Oasis HLB and MCX sorbents is the high selectivity of the MCX sorbent for basic compounds due to the cation exchange group. When metformin was extracted using Oasis® MCX it was not eluted from the cartridges. Metformin is a highly polar, basic compound with five nitrogen atoms in its structure. Hence, when applied to the cartridges metformin will bind to the through several bonds. It is likely that the strong binding led to the poor recovery.

3.3. Development of the new ion pair solid phase extraction technique (Oasis HLB 1 ml, 30 mg)

Several researchers were able to solvent extract metformin by using ion pair extraction (IPE) [11,18], however, the reported recoveries were low. It was hypothesised that if this solvent IPE could be coupled with SPE it would save the time and labour involved with liquid extraction and it may improve the recovery. Based on this hypothesis the concept of IPSPE was developed. It was postulated that if HLB cartridges were equilibrated with an ion pair reagent (IPR) (by passing 1 ml of IPR solution through the cartridge) the IPR will be retained in the cartridge by a hydrophobic interaction. Upon loading the sample metformin will form a complex with the retained IPR which then could be easily eluted using methanol. To test this theory a solution of IPR (SDS, 2 mM) in water was prepared and passed through the HLB cartridges before sample loading. A solution of metformin was prepared in water (100 μl taken from a methanolic stock solution and added to 1 ml with water to given concentration of 1000 ng/ml water) and applied to the cartridge. No loss of metformin occurred during loading with a negligible loss of metformin during washing with 30% methanol. The recovery for metformin was more than 99% after elution with methanol.

The developed IPSPE was proved to be effective in the extraction of metformin from spiked solutions. Further experiments were then conducted on the extraction from plasma samples.

The result of these experiments led to the conclusion that the recovery of metformin from plasma was dependent on the concentration of methanol that was used for preparing the standard solutions. Hence, the plasma samples were prepared so that they contained a very low amount of methanol (see standards in Section 2). Using this procedure a recovery similar to that obtained from extraction of standard solutions was achieved. The best washing concentration (at the washing step) was found to be 30% methanol. The samples should be loaded without addition of acid, as this reduced the recovery by decreasing the ionisation of the ion pair reagent. Furthermore, it was determined that plasma samples must be centrifuged before SPE to prevent them from blocking the cartridges. The washing step should be one step only and followed by the elution step (i.e. not two washing steps) as it was observed that the ion pair reagent is eluted when using two washing steps.

3.4. Validation

3.4.1. Specificity

Fig. 2 illustrates the chromatogram obtained from a blank plasma sample while Fig. 3 is for a spiked plasma sample. Fig. 4 is for a plasma sample from a patient who was taking prescribed metformin. From the chromatograms it is clear that there is no endogenous interfering substances and that the retention times of metformin and the internal standard.

3.4.2. Standard curve and linearity

The standard curve was determined on each day of the 5-day validation period, the slope (0.5647 ± 0.0277), the intercept (0.1756 ± 0.0520) and the correlation coefficient was determined (≥0.997). The re-

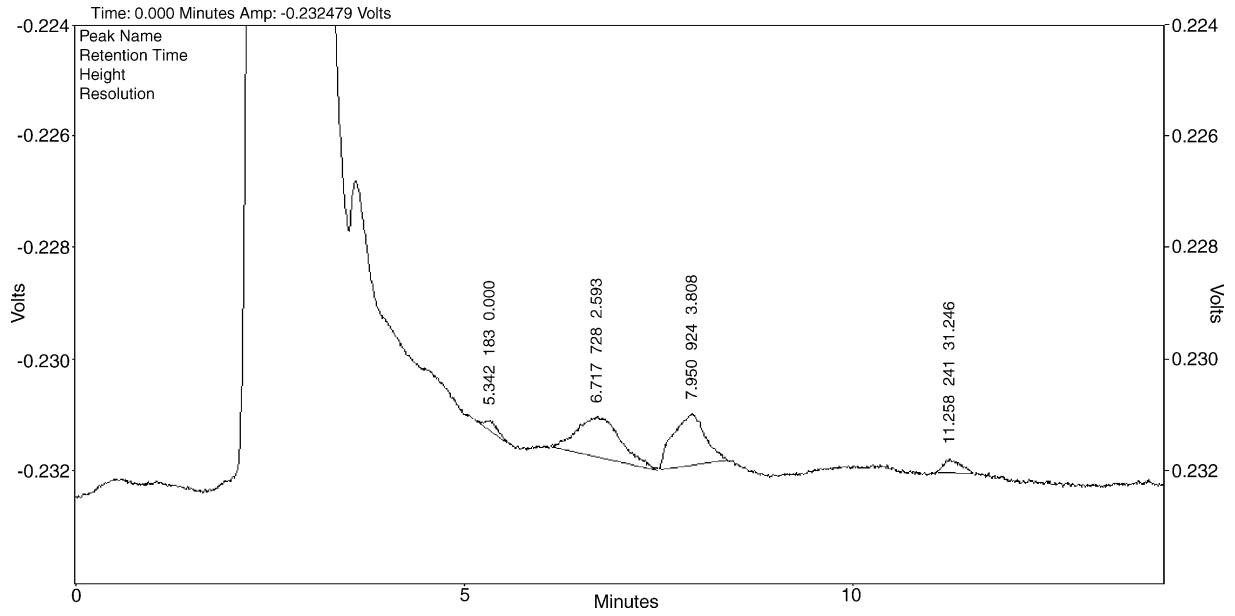

Fig. 2. Chromatogram showing the result of extraction of a blank plasma sample.

lationship between peak height ratio and concentration were linear within the studied concentration range (50–2000 ng/ml).

3.4.3. Accuracy and precision

The values obtained for intraday and interday precision and accuracy during the 5-day validation for plasma are shown in Table 1. All values for accuracy and precision were within recommended limits. Intraday precision ranged between 1.5 and 4.5% whereas the interday precision was between 2.4 and 8.9%. The intraday mean error was between 0.4 and 6.9% whereas the interday mean error was between 0.4 and −3.5%.

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

The LOD and the LOQ for metformin were calculated to be 3.0 and 5.0 ng/ml, respectively. Concentrations down to 5 ng/ml were detected with accepted accuracy and precision using this method (Fig. 5 and Table 1).

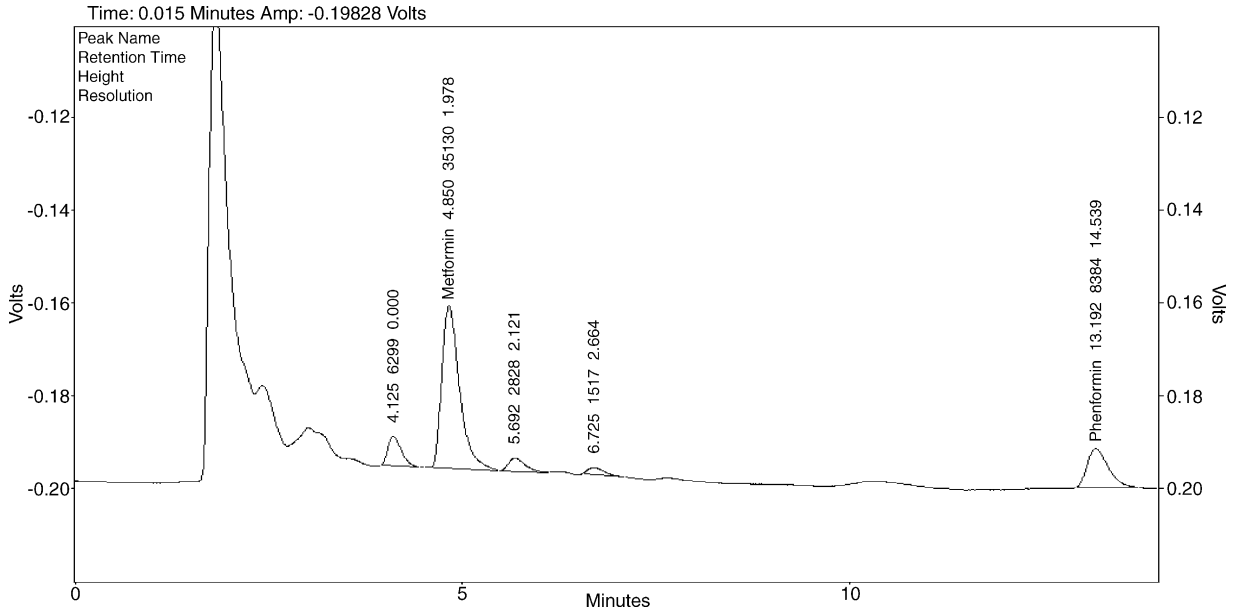

Fig. 3. Chromatogram showing the result of extraction of spiked plasma showing metformin and phenformin (metformin 500 ng/ml and phenformin 200 ng/ml).

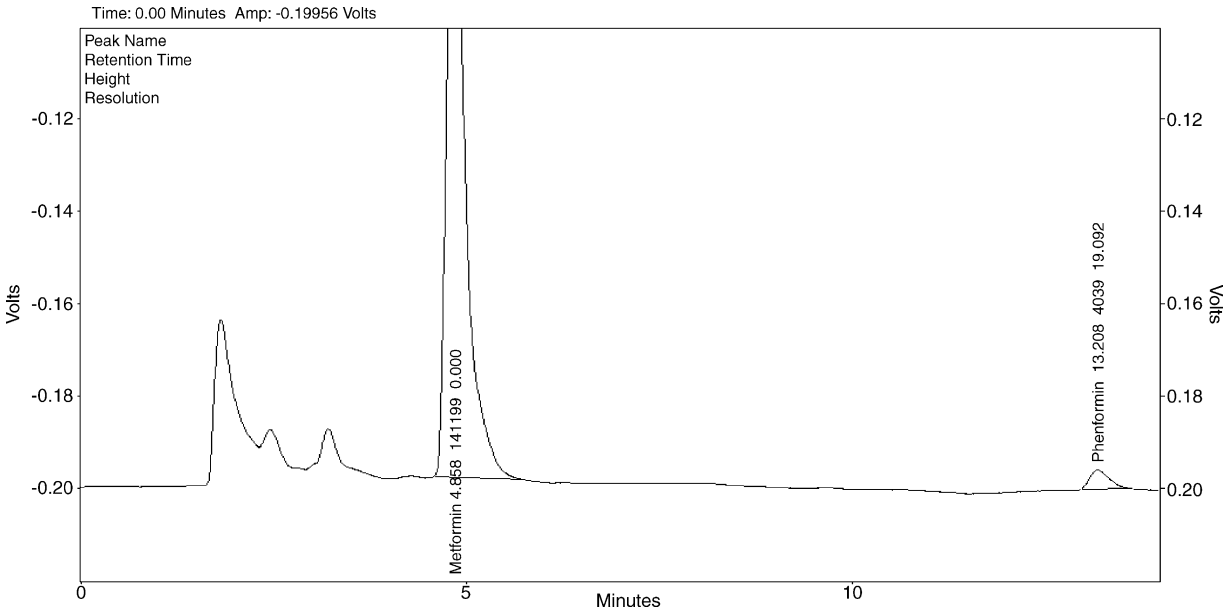

Fig. 4. Chromatogram showing the result of extraction of a plasma sample from a patient who was receiving 850 mg metformin twice daily showing metformin and phenformin (metformin 3985 ng/ml and phenformin 200 ng/ml).

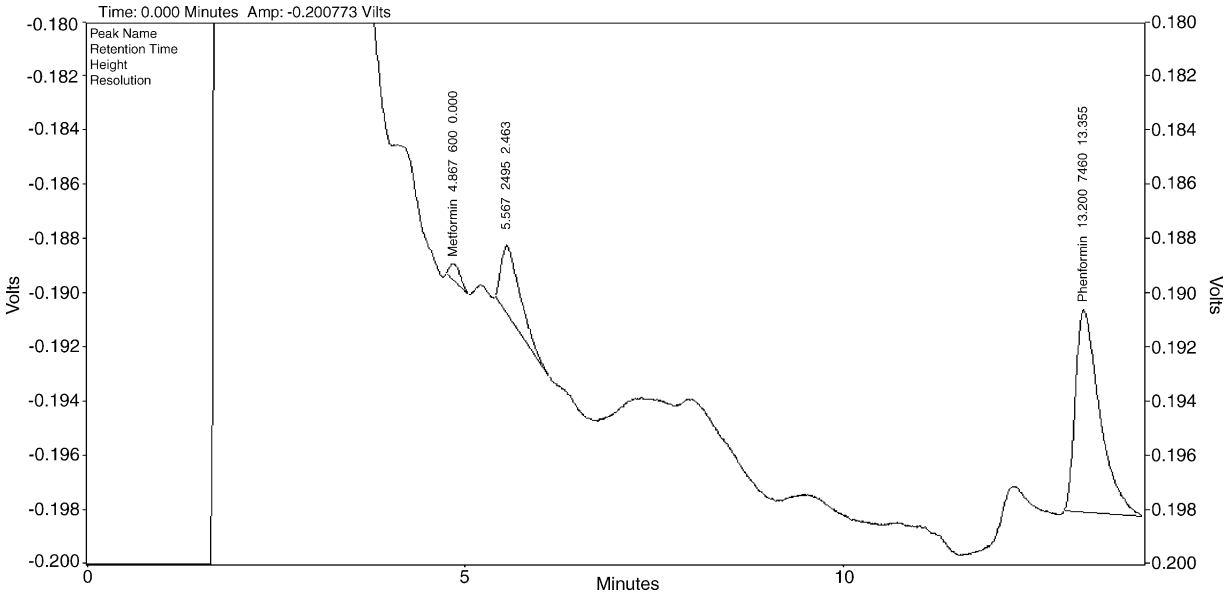

Fig. 5. Chromatogram showing the result of extraction of spiked plasma showing metformin and phenformin (metformin 5 ng/ml and phenformin 200 ng/ml).

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

Nominal concentration (ng/ml)	Precision		Accuracy
	Mean ± S.D.	CV%	Mean relative errors (%)
Intraday			
100	106.9 ± 4.2	3.9	6.9
400	398.6 ± 18.0	4.5	0.4
1500	1446.6 ± 21.7	1.5	−3.6
5 (LOQ)	5.8 ± 0.6	10.3	16.0
Interday			
100	103.3 ± 9.2	8.9	3.3
400	385.9 ± 29.5	7.6	−3.5
1500	1505.2 ± 36.5	2.4	0.4

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

Nominal concentration (ng/ml)	Mean ± S.D.	CV%
100	99.4 ± 5.8	5.8
400	100.5 ± 1.8	1.8
1500	97.9 ± 2.0	2.0

3.4.5. Recovery

Table 2 shows data for extraction recovery ($n = 6$). The recovery of metformin was between 97.9 and 100.5%.

3.4.6. Stability

The mean and standard deviation for the ratio between the two measurements carried at a 3-month interval (plasma stored at $-70\,^{\circ}C$) for metformin ($n = 12$) was 1.02 ± 0.03. This indicates that metformin is stable for at least 3 months when stored at $-70\,^{\circ}C$.

4. Conclusion

The use of ion pair chromatography using SDS as an ion pair reagent solved the problem of rapid elution of metformin. The IPSPE technique solved the problem of difficulty of extraction of metformin. This IPSPE provides a new technique that has the potential for extracting other highly polar compounds with high recovery. This method offers the high sensitivity and specificity required for pharmacokinetic studies. The method was successfully used in the analysis of 104 samples obtained from patients on metformin.

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