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Development and validation for high selective quantitative determination of metformin in human plasma by cation exchanging with normal-phase LC/MS/MS

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Abstract

An assay based on cation exchange solid-phase extraction and liquid chromatography-tandem mass spectrometry (LC/MS/MS) has been developed for the quantitative determination of metformin in human plasma. The analytical method consists of cation exchange solid-phase extraction (VersaPlate CBA) without any further evaporation/dissolution steps and cation exchange-based HPLC separation (Capcell Pak SCX column) with a normal-phase gradient system followed by semi-micro LC/MS/MS in positive ion selected reaction monitoring mode using electrospray ionization. The method exhibited excellent performance in terms of selectivity, robustness, short run time (7 min/sample) and simplicity of sample preparation.

The calibration range was 10–1000 ng/ml with 0.2 ml of plasma. Intra- and inter-day mean accuracies were within the ranges of 100.3–105.0% and 101.2–105.3%, respectively. Intra- and inter-day precisions were within the ranges of 0.8–1.9% and 1.5–8.6%, respectively. Mean absolute recovery was 67.0% for metformin. No apparent loss of metformin after extraction was observed in an autosampler at 10 °C for 24 h. Dilution of metformin by blank human plasma up to 20-fold was tested and revealed no impact on the results of determination. Furthermore, the method exhibited high selectivity, since no effect on metformin analysis was observed on comparison of samples with or without nateglinide and other agents in plasma. Results obtained with the method were also comparable to a published LC–UV method on cross-validation.

This method can be applied to various clinical pharmacokinetic studies of metformin.

Keywords: Cation exchange solid-phase extraction; Normal-phase; Cation exchange HPLC; LC/MS/MS; Metformin; NIDDM; Combination therapy

1. Introduction

Metformin (Fig. 1), an oral antihyperglycemic drug, is a biguanide agent that increases peripheral insulin sensitivity, inhibits hepatic gluconeogenesis, and reduces hepatic glucose production in patients with non-insulin-dependent diabetes mellitus (NIDDM) [1]. Recently, metformin has been administered in combination with glitazones, sulfonylureas and other agents for treatment of diabetics to achieve glycemic control [2–8]. In addition, since drug interaction studies with co-administered agents examine efficacy and safety, drug level monitoring of metformin is important in clinical development [9–12].

Several methods of analysis by HPLC have been published for the determination of metformin in human plasma using various separation modes, such as reverse-phase [13–16], ion-pair [17–19], cation exchange [20,21] and normal-phase [22,23]. However, these methods have exhibited low sensitivity [13,14,18,20,22] and required relatively large sample volumes [14–17,19], complicated sample preparation procedures including evaporation steps [13,16,17,19], long ana-

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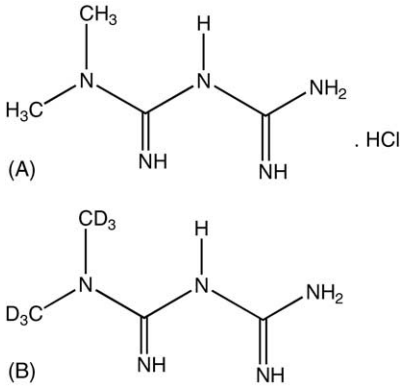


Fig. 1. Chemical structures of metformin (A) and [D_6]metformin (B) (internal standard).

lytical intervals [16,19,21], long analytical columns (at least 15 cm) and conventional mobile phase flow rates (at least 1 ml/min) [13–23].

Recently, two new methods for analysis of metformin in plasma using liquid chromatography-tandem mass spectrometry (LC/MS/MS) have been published [24,25]. Both involve protein precipitation without an evaporation step for sample preparation procedures and MS/MS detection. They include simple sample preparation procedures prior to injection into LC/MS/MS and short analytical intervals (3.4 min/sample [24] and 1–2 min/sample [25]). As a consequence, they achieve high throughput in metformin analysis. On the other hand, sample preparation by protein precipitation method lacks compound selectivity and robustness due to interference by any other substances. In addition, the LC/MS/MS methods have been insufficiently evaluated for interference by endogenous substances and/or concomitant drugs.

Taking selectivity into consideration, it is very important to make use of the physicochemical characteristics of metformin. Since metformin is a strongly basic compound (pK_a = 12.4), we attempted to use cation exchange procedures for both sample preparation and LC separation mode. Metformin exhibited favorable retention behavior with normal-phase mode in the cation exchange HPLC column, as well. This was a very useful characteristic for direct injection into the HPLC system after elution of solid-phase extraction (SPE) samples without evaporation or reconstitution steps.

We, therefore developed a new method based on cation exchange solid-phase extraction and LC/MS/MS by focusing on analyte selectivity. The method exhibited excellent performance in terms of selectivity, robustness, short run time of analysis (7 min/sample) and simplicity of sample preparation. The effects of fifteen compounds expected to be administered in combination with metformin were evaluated. The method was fully validated for use in therapeutic drug monitoring of metformin.

2. Experimental

2.1. Materials and reagents

Metformin (Fig. 1) was purchased from Sigma Chemical (St. Louis, MO, USA), and internal standard (Fig. 1, [D_6]metformin) was obtained from Novartis Pharmaceutical Corporation (East Hanover, NJ, USA). Methanol (HPLC grade), acetonitrile (HPLC grade) and ammonium acetate (Reagent grade) were purchased from Kanto Kagaku (Tokyo, Japan) and acetic acid (Reagent grade) from Wako Pure Chemicals Industries (Osaka, Japan). Water was deionized and purified on a Millipore water purification system (Bedford, MA, USA). Human heparinized plasma was purchased from New Drug Development Research Center (Iwamizawa, Japan).

Nateglinide, fluvastatin, cyclosporine A, LAF237[1], DPP728[1], valsartan and imatinib mesylate were supplied by Novartis Pharma AG (Basel, Switzerland). Glibeneclamide was purchased from Wako Pure Chemicals Industries (Osaka, Japan). Atorvastatin was purchased from Kishida Chemical Co., Ltd (Osaka, Japan). Hydrochlorothiazide was purchased from Sigma Chemical (St. Louis, MO, USA). Losartan was synthesized by Novartis Pharma K.K. (Tsukuba, Japan). Candesartan was purified from a pharmaceutical product by Novartis Pharma K.K. Pravastatin and simvastatin were purified from pharmaceutical products by Chemical Evaluation and Research Institute, Japan (Tokyo, Japan). The simvastatin acid form was obtained by hydrolysis of simvastatin at the Chemical Evaluation and Research Institute, Japan.

2.2. Instrumentation

The HPLC system consisted of PU-980 pumps, DG-980-50 degasser, CO-965 column oven, HV-992-016-port valve and HG-1580-32 dynamic mixer with Borwin 1.50 system controller (JASCO, Tokyo, Japan) and HTS-PAL with Cycle composer 1.5.0 (CTC analytics AG, Zwingen, Switzerland). The mass spectrometer was a TSQ700 triple quadrapole with electrospray interface (Thermo Electron Corporation, San Jose, CA, USA). Data were acquired by ICIS (version 8.2.1, Thermo Electron Corporation) and processed by Xcalibur (version 1.0.1, Thermo Electron Corporation) (Fig. 2).

2.3. Chromatographic conditions

The custom-made HPLC column (2.0 mm × 20 mm, 5 μm) was a Capcell Pak SCX UG80 from Shiseido (Tokyo, Japan). Column temperature was set to 60 °C. Mobile phases were prepared for each of four pumps. Pump A was a 15 mM ammonium acetate solution, pump B acetonitrile, pump C acetonitrile/15 mM ammonium acetate (90:10, v/v) and pump D methanol/H_2O (50:50, v/v).

[1] Products developed by Novartis Pharmaceutical Corporation (East Hanover, NJ, USA) for Type II diabetes treatment [26].

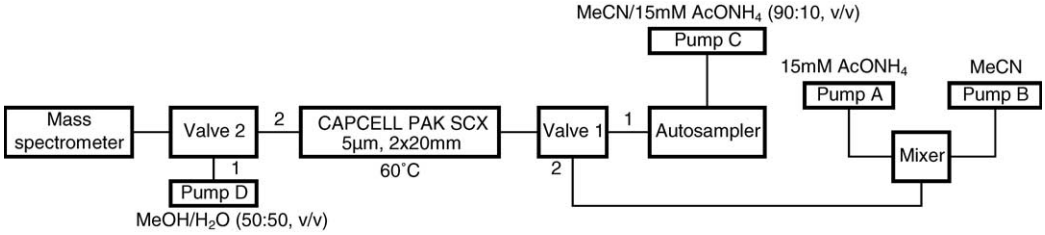


Fig. 2. Established HPLC system.

Chromatography was performed with a gradient system (Table 1). Total run time was 7 min for each injection.

2.4. MS/MS detection

Precursor ions for the analyte and internal standard were determined from mass spectra obtained during infusion of neat solution into TSQ700. Using an electrospray ion source (ESI), the mass spectrometer was operated in positive ionization mode. Under these conditions, the analytes yielded predominantly protonated molecular ions at *m/z* 130 for metformin and *m/z* 136 for internal standard ([D_6]metformin). Each of the precursor ion was subjected to collision-induced dissociation to determine the resulting product ions. The product ion spectra for metformin and internal standard are shown in Fig. 3. The primary fragments for metformin and internal standard were attributed to loss of the same fragment pattern. Product ions resulting from this fragmentation were chosen for MS/MS detection of each of the analytes at *m/z* 71 (metformin) and *m/z* 77 (internal standard). Interface-independent instrument parameters were optimized during infusion of a solution of metformin through the ESI interface with the initial HPLC mobile phase of the gradient system. The electron multiplier was set to 1200 V.

After optimization of the LC/MS/MS system, ionization of analytes was carried out using the following settings: capillary temperature: 225 °C; spray voltage, 4.5 kV; sheath gas (N_2) pressure, 90 psi; auxiliary gas (N_2) flow, 30 ml/min; collision energy, −26 V and collision gas (Ar) pressure, ca. 4.2 m Torr. The scan time for each analyte was set to 1.0 s.

2.5. Preparation of calibration standards (CS) and quality control (QC) samples

Metformin stock solutions for CS samples were prepared in methanol/H_2O (50:50, v/v). CS samples were prepared by spiking respective stock solutions in blank human plasma at concentrations of 10, 20, 50, 100, 200, 500 and 1000 ng/ml. CS samples were prepared from a blank plasma pool. Metformin stock solution for QC was prepared separately and QC plasma samples were prepared at 10, 25, 75, 150 and 900 ng/ml in the same manner as for plasma standard. QC samples were prepared from different matrix pools on each day of analysis.

All prepared plasma samples were stored at −15 °C or below and all prepared stock solutions were stored at ca. 4 °C.

Table 1
Gradient program and valve switching timing

Time (min)	Pump A[a](%)	Pump B[b](%)	Pump A/B flow rate (ml/min)	Pump C[c] flow rate (ml/min)	Pump D[d] flow rate (ml/min)	Valve position V1 and V2	Function
0.0	30	70	0.0	1	0.25	1æ1	Sample load by pump C
0.9	30	70	0.5	1	0.25		
1.0	30	70	0.5	1	0.25	2æ1	Sample load by pump A/B
1.1	30	70	0.5	1	0.25		
1.2	30	70	0.5	0	0.25		Pump C stop
2.0	30	70	0.5	0	0.25		Reduce pump A/B flow rate
2.1	30	70	0.3	0	0.25		Start linear gradient
3.5			0.3	0	0.25	2æ2	Eluent from column into MS interface
5.5	50	50	0.3	0	0.25		
5.6	50	50	0.5	0	0.25	2æ1	Washing column
6.3	50	50	0.5	1	0.25		
6.4	30	70	0.5	1	0.25	1æ1	Reset to starting position
6.9	30	70	0.5	1	0.25		
7.0	30	70	0.0	1	0.25		

[a] 15 mM $AcONH_4$.
[b] MeCN.
[c] MeCN/15 mM $AcONH_4$ (90:10, v/v).
[d] MeOH/H_2O (50:50, v/v).

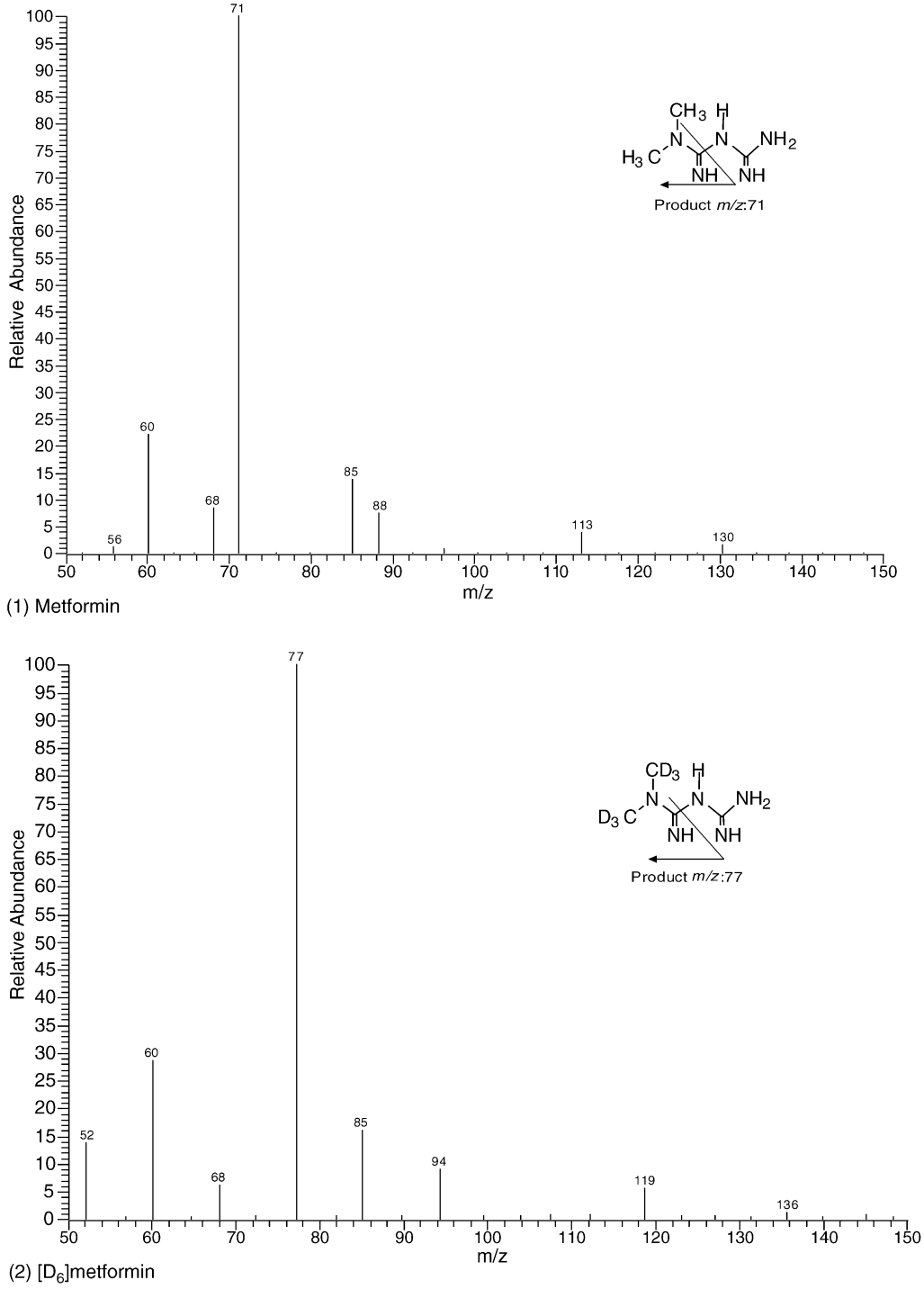


Fig. 3. Product ion mass spectra of protonated molecule $[M+H]^+$ from (1) metformin at *m/z* 130 and (2) $[D_6]$metformin (IS) at *m/z* 136.

2.6. QC samples with other agents

Metformin QC samples with other agents (Nateglinide, Pravastatin, Fluvastatin, Simvastatin, Simvastatin acid form, Atorvastatin, Glibenclamide, Cyclosporin A, LAF237, DPP728, Valsartan, Losartan, Candesartan, Hydrochlorothiazide and Imatinib mesylate) were prepared by spiking the metformin (concentrations: 25, 150 and 900 ng/ml) and working solutions of each agent (concentration: 1000 ng/ml except for Nateglinide; final Nateglinide concentrations were 10, 100, 1000 and 20,000 ng/ml) into blank human plasma.

2.7. Dilution test samples

Dilution test samples were prepared by spiking respective working solutions into blank human plasma. The following

concentrations were used for dilution tests: 900 (the highest concentration of QC sample), 2000 and 5000 ng/ml.

2.8. Sample preparation

A 0.2 ml aliquot of each plasma sample was transferred to a 96-well polypropylene block. A 0.5 ml of internal standard solution (100 ng/ml of [D_6]metformin in H_2O) was added to each well. A VersaPlate 96-well CBA (50 mg, Varian) was conditioned with 1 ml of methanol and 1 ml of H_2O, and then the plasma samples with internal standard were loaded onto 96-well extraction plates that were washed with 1 ml of H_2O and 0.5 ml of methanol and eluted with 1 ml of methanol containing 2.5% acetic acid. A 50 μl of extracted sample was directly applied to LC/MS/MS without any further evaporation/dissolution step.

2.9. Validation procedure

The method was validated for accuracy, precision, sensitivity, specificity, calibration curve range, and reproducibility according to the FDA guideline for validation of bioanalytical methods [27].

2.9.1. Calibration curve and linearity

A seven-point calibration curve was constructed by plotting peak area ratio (y) of metformin to the internal standard versus metformin concentrations (x). Analysis of calibration standard (CS) samples at each concentration was performed in duplicate. Results for blank samples were not used as part of the calibration curve. Slope, intercept and correlation coefficient were calculated as regression parameters by weighted ($1/x^2$) linear regression with Xculibur software.

2.9.2. Intra-day and inter-day accuracy and precision

Precision and accuracy were evaluated by determining the metformin concentrations in six replicates of QC samples at five different concentrations daily for three separate days. Each run consisted of calibration standards, blank plasma samples with and without internal standard in duplicate and QC samples in six replicates.

2.9.3. Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) was evaluated by spiking metformin at a concentration of 10 ng/ml.

2.9.4. Specificity

The specificity of this method was investigated by preparing and analyzing six individual human blank plasma samples. Specificity was assessed by comparing the mean apparent signal for metformin and for [D_6]metformin ($n = 5$) in blank samples with the mean signal obtained for samples spiked ($n = 6$) with a concentration of metformin at LLOQ, and [D_6]metformin at the working concentration.

2.9.5. Recovery

The absolute extraction recovery was assessed by comparison of the responses obtained from extracted spiked human samples and that from extracted human blank plasma sample spiked with the analyte, and given as the ratio of the mean peak areas obtained for metformin and [D_6]metformin. Recovery of metformin from plasma was evaluated at three different concentrations (each $n = 4$) of 25, 150 and 900 ng/ml. Recovery of internal standard was evaluated using a concentration of 100 ng/ml, which was used in the assay.

2.9.6. Stability

The stability of metformin in stock solutions (MeOH/H_2O, 50/50) has already been reported, with no apparent loss after 8 months of storage at 4 °C observed [21]. Metformin in human plasma was also stable, with no apparent loss after 7 months of storage at −20 °C and five freeze–thaw cycles [20,21]. We, therefore performed additional stability testing of the extract on an autosampler (10 °C, 24 h).

3. Results and discussion

3.1. Development of the method

Since metformin is a strongly basic compound (pK_a = 12.4), the method was developed utilizing this particular characteristic for achievement of high selectivity and robustness.

Metformin is poorly retained in reverse-phase HPLC mode even under a basic mobile phase. However, we found that metformin had weak normal-phase retention properties in some reverse-phase columns with a bonded phase in which a polar functional group was embedded in the ligand's alkyl chain, such as XTerra RP (Waters, Milford, MA, USA) and Inertsil ODS-EP (GL Science, Tokyo, Japan). The stationary phase of these columns might have contributed to retention of the analyte. Based on these results and the reported normal-phase analytical method [22,23], we also tested normal-phase HPLC columns, but low analyte reproducibility on chromatography, including peak retention and peak shape, was observed in this mode.

Cation exchange-mode was then tested based on previous reports [20,21]. In this mode, a polymer-based HPLC column (Capcell Pak SCX, Shiseido, Tokyo, Japan) proved to be more robust than other silica-based columns, such as Partisil-10 SCX (Whatman, Clifton, NJ, USA), Nucleosil 100-5SA (Macherey-Nagel, Duren, Germany) and Zorbax 300-SCX (Agilent, Palo Alto, CA, USA). Analyte retention time was shortened by using these silica-based columns within 100 samples. On the other hand, the chromatographic response of Capcell-Pak was not changed at least 200 samples (200 samples were the limit for a single analytical run for the ICIS softwear used). Since metformin is strongly retained in cation exchange mode, we examined conditions of the mobile phase, such as buffer concentration, pH and organic phase ratio as well as the column length required for achievement of ap-

propriate retention time and peak shape on chromatograms. Metformin exhibited normal-phase retention in that mode, as well. This characteristic was very useful for direct injection into the HPLC system after elution of solid-phase extraction (SPE) samples by 2.5% acetic acid in methanol without evaporation and reconstitution steps, because the eluted SPE samples in normal-phase mode could be concentrated on the front of the analytical column. As a result, the injection volume of the samples extracted from SPE could be increased without loss of chromatographic response. We confirmed that this extract had a good peak shape on the chromatogram for injections up to 100 μl. In addition, the analyte with high organic solvent ratio exhibited increased ionization efficiency. Analytical sensitivity in LC/MS/MS detection of metformin could be achieved based on these results. This normal-phase LC approach was devised referring to Naidong's report [28].

Isocratic-mode testing yielded insufficient separation in avoiding interference from ion suppression material. However, the gradient mode required a longer run time than the isocratic mode. For achievement of shorter run time and separation, we examined the Mallet approach [29], i.e., use of a third pump for column conditioning and sample loading into the HPLC system. Furthermore, a rather high background level on the MS chromatogram was observed. Eluent derived from the column was not introduced into LC/MS/MS, and instead methanol/H_2O (1:1, v/v) using pump D was directed into the ion source of MS with a six-port valve to reduce background noise level. The final analytical system and gradient program are shown in Fig. 2 and Table 1, respectively.

We selected SPE with cation exchange mode as the procedure for sample preparation because it is highly selective for basic chemicals. We thus attempted to optimize the method to increase recovery and avoid ion suppression by selecting the wash solvent and elution volume. We thus, devised a procedure without evaporation/reconstitution steps by combination with the above-described normal-phase HPLC separation mode.

Estimation of the lower limit of quantification (LLOQ) of metformin was performed in our study with extracted human plasma samples. Although, it was possible to established a high sensitive method using TSQ (API II) LC/MS/MS instrument (Thermo Electron Corporation, data not shown) instead of TSQ700, 10 ng/ml of sample was set as the LLOQ and was quite sufficient for therapeutic monitoring of metformin based on the results of clinical pharmacokinetic determinations [30]. Typical SRM chromatograms of blank with IS sample and at the LLOQ concentration sample are shown in Fig. 4.

3.2. Absolute extraction recovery and matrix effect

The absolute extraction recovery was assessed by comparison of the responses obtained from analysis of extracted spiked human samples and that from extracted human plasma sample spiked with the analyte after extraction. The absolute extraction recoveries for metformin and [D_6]metformin were 67.0% (throughout the concentration range) and 69.2% (at working concentration), respectively.

The matrix effect was assessed by comparison of the responses obtained from analysis of extracted blank human samples spiked with the analyte after extraction and neat reference solutions. The matrix effects for metformin and [D_6]metformin were 97.8% (throughout the concentration range) and 97.7% (at working concentration), respectively.

3.3. Specificity

The specificity of the analytical method was investigated by analyzing six individual human blank plasma samples. The acceptance criteria for metformin was mean interference per batch ≤20% of signal at LLOQ, and that for [D_6]metformin was mean interference per batch ≤5% of signal at working concentration.

No interference peak was detected for metformin or IS.

3.4. Method validation

3.4.1. Calibration curves and linearity

A calibration curve was established on each validation day.

The calibration curve was linear over the concentration range of 10–1000 ng/ml of metformin in human plasma with a coefficient of determination (r^2) ≥0.9994. The average slope and intercept of regression equations were 0.00412113 (%CV: 3.1) and 0.00334867 (%CV: 6.9), respectively. Linearity was found to be quite satisfactory and reproducible with time.

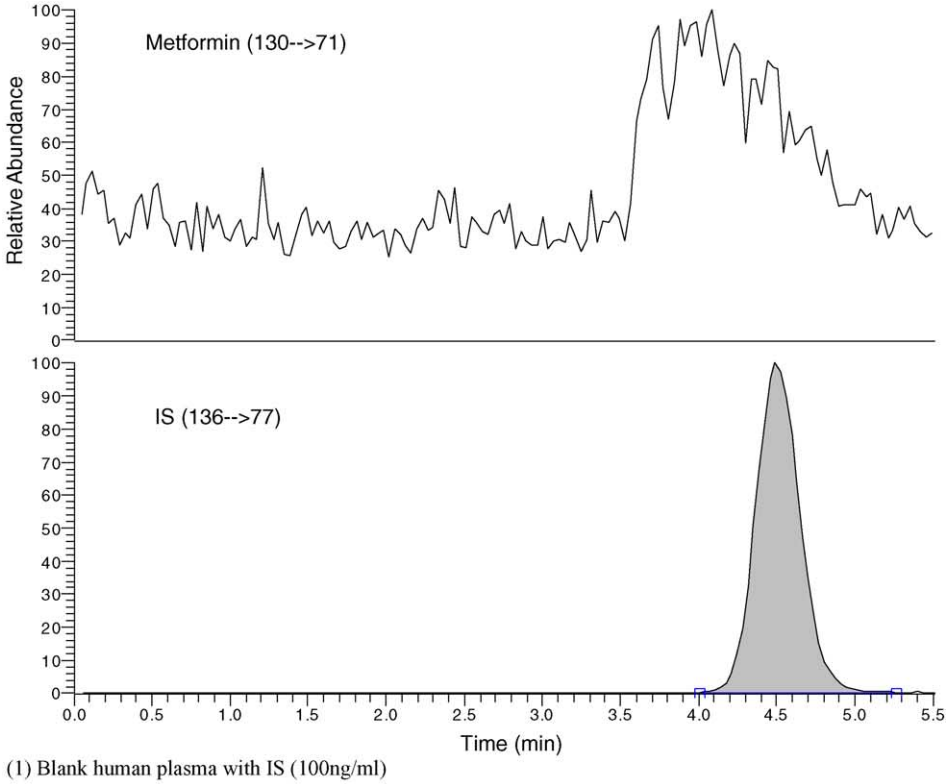
3.4.2. Accuracy, precision, and LLOQ

The precision and accuracy data for QCs are summarized in Table 2. For QCs at 10 ng/ml (LLOQ), intra-assay mean accuracy and precision values were 105.0 and 1.9%, respectively, while above LLOQ they were 100.3–102.8% and 0.8–1.2%, respectively. Inter-assay mean accuracy and

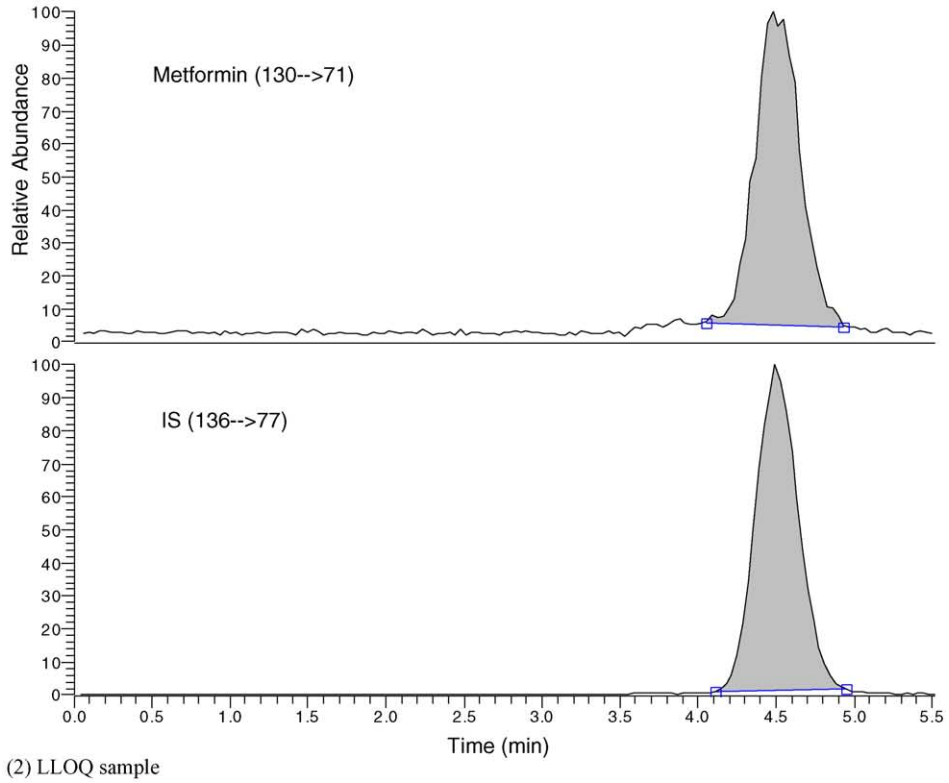
Table 2
Intra- and inter-day accuracy and precision of the method in human plasma

Nominal (ng/ml)	Mean observed (ng/ml)	Precision (%)	Mean accuracy (%)
Intra-assay (n = 6)			
10	10.5	1.9	105.0
25	25.7	1.2	102.8
75	76.1	0.9	101.5
150	152	0.7	101.3
900	903	0.8	100.3
Inter-asay (n = 18)			
10	10.3	2.9	103.0
25	25.4	1.6	101.6
75	79.0	8.6	105.3
150	153	2.6	102.0
900	911	1.5	101.2

All the mean accuracies were calculated against their nominal concentrations.



(1) Blank human plasma with IS (100ng/ml)



(2) LLOQ sample

Fig. 4. Representative mass chromatograms of (1) blank human plasma with IS (100 ng/ml) and (2) human plasma spiked with metformin (10 ng/ml) and IS (100 ng/ml).

Table 3
Evaluation for dilution with blank human plasma ($n = 3$)

Nominal (ng/ml)	Dilution factor	Mean accuracy (%)
900	2	102.0
	5	101.9
2000	5	98.5
	10	100.5
5000	10	99.0
	20	101.8

All the mean accuracies were calculated against their nominal concentrations.

precision at LLOQ were 103.0 and 2.9%, respectively, and above LLOQ, 101.2–105.3% and 1.5–8.6%, respectively.

3.4.3. Dilution

Up to 20-fold of metformin by blank human plasma was tested with spiked samples exceeding the upper limit of the calibration curve and samples with the highest concentration of QC (Table 3). The mean accuracy for diluted samples was 98.5–102.0%.

3.4.4. Processed sample stability

Processed validation samples at low, middle and high concentrations were kept at 10 °C for 24 h and then reanalyzed and quantified against calibration curves obtained using freshly prepared standards. The mean accuracy and precision for determination of metformin in these extracted and stored samples were 96.0–104.0% and 0.6–4.2%, respectively (Table 4). These results demonstrated that extracted samples can be analyzed after storage in an autosampler at 10 °C for at least 24 h.

3.4.5. Effects of other agents on metformin determination

Metformin QC samples spiked with various other agents into plasma were prepared.

Table 4
Stability in extract on the autosampler at 10 °C ($n = 4$)

Nominal (ng/ml)	Mean observed (ng/ml)	Precision (%)	Mean accuracy(%)
25	26.0	4.2	104.0
150	145	0.7	96.7
900	864	0.6	96.0

All the mean accuracies were calculated against their nominal concentrations.

Nateglinide, glibeneclamide, LAF237 and DPP728 are type II diabetic drugs, and likely to be co-administered with metformin. Statins are cholesterol-lowering agents, which inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. Sartans are specific angiotensin II antagonists acting on the AT1 receptor subtype. Cyclosporine A is an immunosuppressive agent. Hydrochlorothiazide is a diuretic and antihypertensive. Imatinib mesylate is an inhibitor of the receptor tyrosine kinases. These agents as well are likely to be co-administered with many drugs, including metformin.

The mean accuracy and precision of metformin determination for all samples were 96.7–102.7% and 0.5–7.9%, respectively (Table 5). No effect of other agents was observed on metformin determination and no interfering peak was found in any chromatograms of metformin and IS for any co-spiked samples due to the high selectivity of the analytical method, we devised.

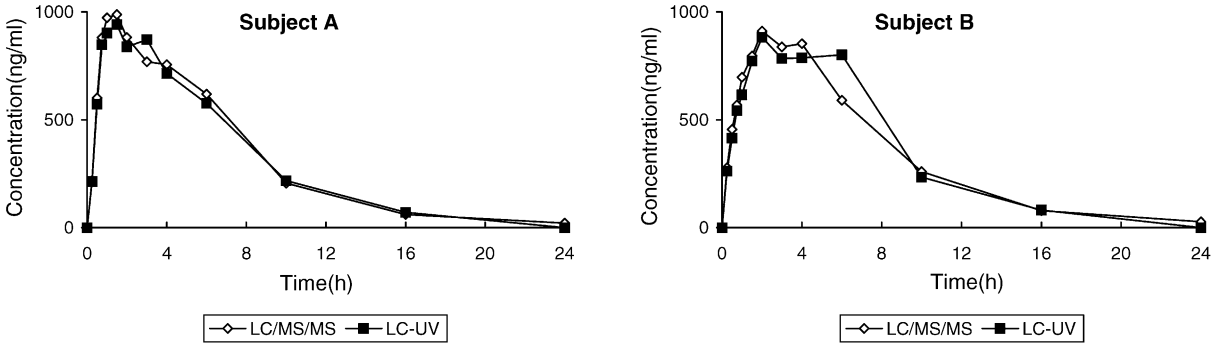
3.4.6. Comparison of LC–UV and LC/MS/MS with biological samples

The LC/MS/MS method, we devised and another previously reported method (LC–UV) [18] for metformin determination was compared using clinical samples from healthy subjects. We considered this LC–UV method appropriate for the purpose of comparison with our method because HPLC analysis was performed with cation exchange-mode, and because the plasma samples included both metformin and nateglinide.

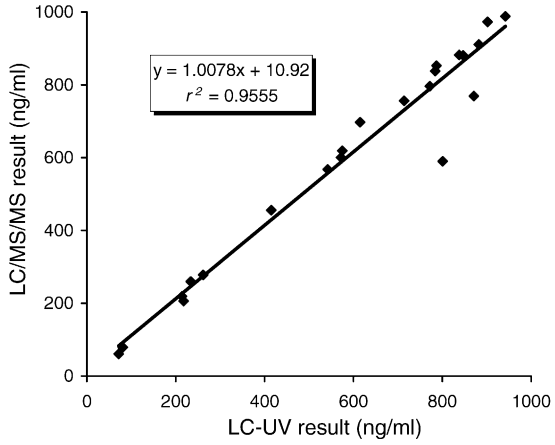
Table 5
Effect of other agents on metformin determination ($n = 4$)

Spiked agent	Agent concentration (ng/mL)	Metformin nominal concentration (ng/mL)	Metformin mean accuracy (%)	Precision (%)
Nateglinide	10, 100, 1000 and 20000	25, 150 and 900	96.7–100.8	0.5–2.4
Pravastatin	1000	25, 150 and 900	99.7–101.6	2.0–3.1
Fluvastatin	1000	25, 150 and 900	100.0–102.7	2.6–3.4
Simvastatin	1000	25, 150 and 900	98.0–102.7	1.3–2.6
Simvastatin acid form	1000	25, 150 and 900	97.6–102.0	0.7–3.3
Atorvastatin	1000	25, 150 and 900	98.8–102.0	1.3–1.6
Glibeneclamide	1000	25, 150 and 900	99.1–100.7	1.2–2.8
CyclosporineA	1000	25, 150 and 900	100.0–101.6	0.7–3.6
LAF237	1000	25, 150 and 900	98.0–100.7	1.3–2.9
DPP728	1000	25, 150 and 900	100.7–101.8	2.1–3.6
Valsartan	1000	25, 150 and 900	98.8–101.9	1.2–2.4
Losartan	1000	25, 150 and 900	100.0–102.2	0.5–2.8
Candesartan	1000	25, 150 and 900	100.4–102.0	0.8–7.6
Hydrochlorothiazide	1000	25, 150 and 900	98.0–101.2	1.3–7.9
Imatinib mesylate	1000	25, 150 and 900	97.3–100.8	2.1–4.8

All the mean accuracies were calculated against their nominal concentrations.



(1) Plasma concentration-time profiles



(2) Correlation between LC/MS/MS and LC-UV methods

Fig. 5. Plasma concentration-time profiles and correlation between LC/MS/MS and LC–UV methods.

Fig. 5 shows the plasma concentration-time profile and correlation of results obtained with LC–UV and our established method. The correlation of determination (r^2) was 0.9555 and the slope was 1.0078. The results of analysis thus matched well, and the two methods provided identical determination.

4. Conclusion

A semi-micro HPLC/ESI/MS/MS method for quantification of metformin in human plasma has been successfully developed and validated. The processed samples for LC/MS/MS analysis were prepared by cation exchange solid-phase extraction. The eluted samples were directly injected into the HPLC system without evaporation or reconstitution steps. The HPLC system consisted of two 6-port valves, four pumps and a cation exchanging column (Capcell Pak SCX, 2 mm × 20 mm), based on combination with cation exchange and normal-phase separation. Prior to MS detection, HPLC separation was performed in gradient mode. Detection was performed with positive selected reaction monitoring (SRM). The lower limit of quantification was set at 10 ng/ml using a 0.2 ml sample aliquot. The method exhibited excellent performance in terms of selectivity, robustness, short run time of analysis and simplicity of sample preparation, and it was fully validated for use in therapeutic drug monitoring of metformin. This method also yields results comparable to those obtained with a reported LC–UV method.

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