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Determination of metformin in plasma by high-performance liquid chromatography after ultrafiltration

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Abstract

A rapid high-performance liquid chromatography (HPLC) method was developed for determination of metformin, an oral antidiabetic agent, in plasma. Sample preparation entailed a 30-min centrifugation of plasma through a micron filter with direct injection of the protein-free ultrafiltrate into an HPLC system consisting of a cation-exchange extraction column (7.5×4.6 mm), a column switching valve, and a cation-exchange analytical column (250×4.6 mm). The eluent was monitored at 232 nm. Metformin was well resolved at a retention time of about 5 min. There was less than 2% loss of metformin during ultrafiltration and good linearity was established from 0.10 to 40 mg/l of metformin hydrochloride. The lower limit of quantitation was about 0.05 mg/l, at which concentration the signal-to-noise was above 10. The intra- and inter-assay coefficients of variation at plasma concentrations of metformin hydrochloride between 0.25 and 25 mg/l were typically 0.8–1.4% and 3.5–6.4%, respectively. This method offers a rapid sample preparation time and achieves excellent sensitivity without resorting to extraction and evaporation techniques.

Keywords: Metformin

1. Introduction

Metformin is used to lower blood glucose in patients with non-insulin-dependent diabetes [1]. A typical treatment schedule requires metformin to by taken with meals, and a total daily dose of three to four 500-mg tablets or two to three 850-mg tablets [1]. Maximal plasma concentrations of metformin after an oral dose of 500 to 1000 mg are typically 1 to 2 mg/l [1]. However, in patients with lactic acidosis and other pathological conditions such as renal impairment, a significant accumulation of metformin may occur and plasma concentrations of over 50 mg/l have been reported [2].

Several methods for measurement of plasma metformin have been developed, most of which have been based on high-performance liquid chromatography (HPLC) following some form of protein precipitation or solid-phase extraction of plasma [3–7]. Methods that use dilution with a protein precipitating agent are fast but sacrifice sensitivity. Methods that use solvent extraction and evaporation are time consuming. Since metformin does not bind to plasma proteins [8], plasma ultrafiltrates should have the same concentration as metformin in plasma.

This paper describes a new HPLC method, which offers rapid sample preparation using ultrafiltration

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and achieves excellent sensitivity without resorting to extraction and evaporation techniques.

2. Experimental

2.1. Chemicals

Metformin hydrochloride was purchased from Sigma (St. Louis, MO, USA). Centrifree Micropartition Devices (Amicon, Beverly, MA, USA) were used for ultrafiltration of plasma. Ammonium phosphate was from Mallinckrodt (Paris, KY, USA). Pooled EDTA plasma, pooled heparinized plasma, and pooled serum were from Lab Serums (Fort Lauderdale, FL, USA).

2.2. Instrumentation

The HPLC system consisted of a Kratos Spectroflow 400 pump (Kratos Analytical, Ramsey, NJ, USA), a Waters 590 pump (Waters, Marlborough, MA, USA), a Waters 712 WISP autosampler, and Waters 680 gradient controller. The extraction column was a Whatman SCX precolumn (7.5×4.6 mm I.D., 10 μm) purchased from Alltech (Deerfield, IL, USA). The analytical column was a Whatman SCX column (250×4.6 mm I.D., 10 μm) from Whatman (Clifton, NJ, USA). A Rheodyne 7076 pneumatically actuated switching valve and 7163 solenoid (Rheodyne, Cotati, CA, USA) was used to perform the column switching. The analytical column was connected to a Kratos Spectroflow 783 Programmable Absorbance Detector (Kratos Analytical). The detector was connected to a Perkin Elmer series 900 serial interface A/D box and an IBM compatible PC with the Perkin Elmer TurboChrom™ version 3.3 (4B11) chromatographic software (Perkin Elmer, Norwalk, CT, USA).

2.3. Sample preparation

2.3.1. Ultrafiltration

Aliquots of 300 μl plasma or serum were filtered by centrifugation at 2000×*g* for 30 min using Centrifree Micropartition Devices. Aliquots of 50 μl of the filtrate were injected onto the HPLC system.

2.3.2. Plasma samples

For generation of standard curves, solutions of metformin hydrochloride were prepared in water (Type II or better) and 100 μl of each solution was added to 900 μl of pooled plasma (plasma refers to EDTA plasma throughout the text unless otherwise stated) to give final plasma concentrations of metformin hydrochloride from 0.1 to 40 mg/l. Aliquots of the prepared plasma samples were stored at −70°C. A complete standard curve was generated with each analytical run.

For determining the precision of the assay, four lots of plasma samples were prepared corresponding to concentrations of metformin hydrochloride of 0.25, 1, 2, and 25.6 mg/l. To determine the lower limit of quantitation, pooled plasma from five different healthy subjects was used to prepare five lots of plasma spiked with 0.05 mg/l of metformin hydrochloride and each lot of plasma was assayed in duplicate.

For determination of the stability of metformin in plasma, two lots of plasma were prepared corresponding to 0.3 and 1.2 mg/l of metformin hydrochloride. Aliquots were stored at −20°C and −70°C. Stability of metformin in EDTA plasma, heparinized plasma and serum at room temperature was assessed by preparing EDTA plasma, heparinized plasma, and serum with metformin hydrochloride corresponding to 0.3 and 1.2 mg/l. Aliquots were analyzed after 24 and 48 h at room temperature. The effects of repeated freezing and thawing of plasma was tested using plasma containing 0.4 and 0.8 mg/l of metformin hydrochloride.

2.4. Chromatographic analysis

The column switching system is depicted in Fig. 1. A short extraction column was used to further purify the plasma ultrafiltrates prior to injection onto the analytical HPLC column. The mobile phase for the extraction column consisted of 0.05 mol/l ammonium phosphate and was delivered by pump B at a flow-rate of 1.0 ml/min. Fig. 1A depicts the column set up during sample injection and purification on the extraction column. The fraction containing metformin was transferred from the extraction column to the analytical column by a mobile phase of 0.4 mol/l ammonium phosphate delivered by pump A at a

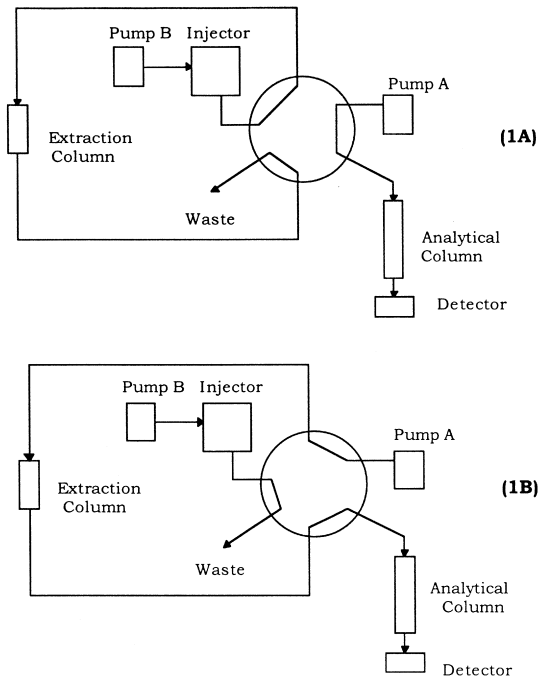

Fig. 1. Schematic Picture of the Column Switching System during the injection phase (1A) and during the analytical phase (1B).

flow-rate of 2.0 ml/min for 0.5 min (Fig. 1B). After this the same mobile phase was used for continued separation of metformin on the analytical column using a flow-rate 2.0 ml/min. Under these conditions metformin eluted at about 5 min. The extraction column was then re-equilibrated with about 10 ml of 0.05 mol/l ammonium phosphate prior to injection of the next sample. The column switching events are listed in Table 1. The time intervals for the column switching were carefully chosen by first performing chromatographic analysis of metformin using only the extraction column to ensure that no losses of metformin occurred during the extraction phase.

2.5. Statistics

Results are presented as mean±S.D..

3. Results and discussion

Typical chromatograms of blank plasma and plasma samples spiked with 0.05 and 0.8 mg/l of metformin hydrochloride are shown in Fig. 2. Chromatograms of plasma samples obtained from a subject after administration of metformin are shown in Fig. 3. Metformin was well resolved from peaks caused by endogenous constituents in the plasma filtrates.

Standard curves were generated by plotting peak heights versus the concentrations of metformin hydrochloride. The standard curves were linear from 0.1 to 40 mg/l with a mean 5.6% deviation from the nominal concentration (e.g., $y=4235x+108$, $r=1.000$).

To determine the total losses of metformin during ultrafiltration due to protein binding and/or binding to the filter, a standard curve was prepared by spiking increasing amounts of metformin hydrochloride into plasma protein-free filtrates which were directly injected onto the HPLC system. When metformin hydrochloride was added to plasma in concentrations from 0.1 to 3.2 mg/l and measured against the standard curve prepared in the protein-free filtrate, the recovery was 98.5%±5.7. The same

Table 1
Column switching events[a]

Time (min)	Event
0.0	Injection onto extraction column using pump B. Pump A always on.
0.6	Column switch, elution of compound from extraction column onto analytical column using pump A.
1.1	Column switch, separation continued on analytical column using pump A. Extraction column re-equilibrated with 0.05 mol/l ammonium phosphate using pump B.
12.0	Next sample injected.

[a] The switching events are given for a flow-rate of 1.0 ml/min and 2.0 ml/min for pump A and pump B, respectively.

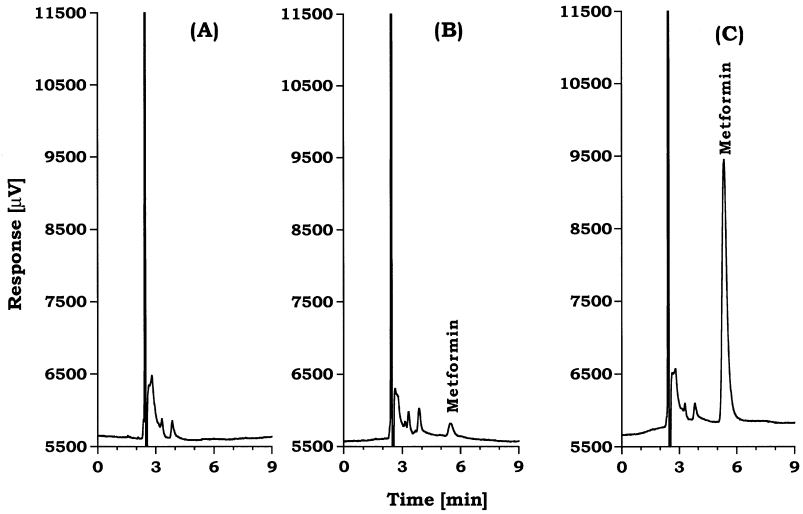

Fig. 2. Chromatograms of ultrafiltrates of blank plasma (A) and blank plasma spiked with 0.05 mg/l (B) and 0.8 mg/l (C) of metformin hydrochloride.

recovery of metformin (98.4%±2.0) was obtained when protein-free filtrates were spiked with metformin hydrochloride and filtered a second time, indicating that binding of metformin to the filter is less than 2% and accounts for the minor losses of metformin when filtering plasma.

The lack of protein binding was further demonstrated when samples from patients on metformin were assayed both after acetonitrile extraction and after ultrafiltration. The results showed an average 3.6% higher concentration of metformin after ultrafiltration compared with the acetonitrile extraction

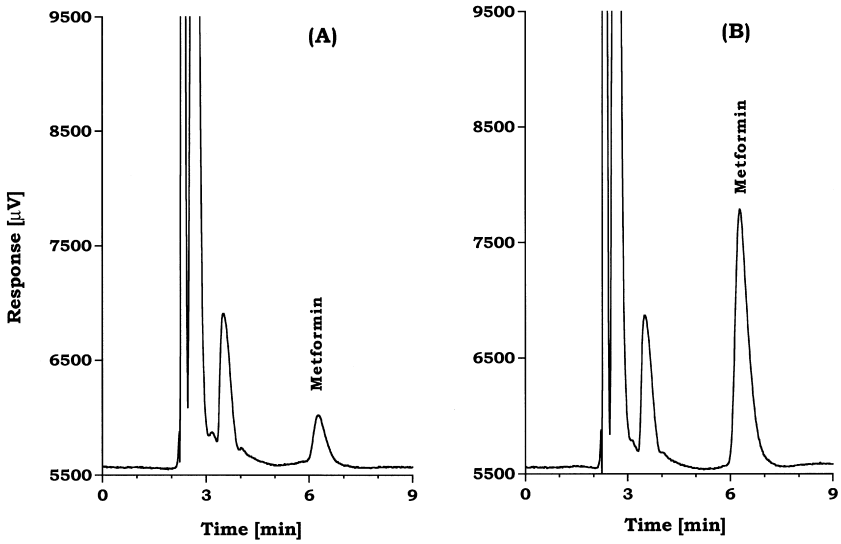

Fig. 3. Chromatograms of ultrafiltrates of plasma samples obtained from a subject before (A) and 3 h (B) after administration of 500 mg of metformin. Sample A was obtained 14 h after the previous dose of metformin. The concentration of metformin was 0.25 and 1.20 mg/l in sample A and B, respectively.

and a good overall correlation ($r=0.988$, $n=9$) between the results obtained using the two different sample preparation techniques. However, the ultrafiltration method achieves superior sensitivity because the samples remain undiluted.

The lower limit of quantitation was about 0.05 mg/l of metformin hydrochloride (see Fig. 2B), at which concentration repeated analysis ($n=10$) showed a 7% deviation from the nominal concentration. With a signal-to-noise ratio at this concentration of 13:1 and with a coefficient of variation of 1.9% this assay is as sensitive and precise as the one reported by Huupponen et al. [7]. At concentrations of metformin hydrochloride below 0.05 mg/l the standard curve showed a slight change in slope and intercept. Intra- and inter-assay coefficients of variation (C.V.), based on measured plasma concentrations of metformin hydrochloride between 0.25 and 25.6 mg/l were 0.8–1.4% and 3.5–6.4%, respectively (Table 2). These data prove that the method is sensitive enough to accurately measure metformin at the lowest steady-state trough concentrations, which were reported to be 0.257 mg/l at a dose of 500 mg q.8 h [6]. At the same time the dynamic range of the method can go as high as 40 mg/l without diluting the samples. Such high concentrations are occasionally seen in renally-impaired patients [2].

When metformin hydrochloride was spiked into EDTA plasma, heparinized plasma and serum to generate standard curves, the slopes and intercepts were essentially identical (EDTA plasma: $y=4868x-115$; heparinized plasma: $y=4755x-145$; and serum: $y=4672x-99$). Virtually the same assay results were obtained irrespective of whether EDTA plasma, heparinized plasma or serum was used (Table 3).

Table 2
Intra-assay ($n=6$) and inter-assay ($n=5$[a]) variation for HPLC analysis of metformin hydrochloride in human plasma

Parameter	Nominal concentration (mg/l)			
	0.25	1.0	2.0	25.6
Grand mean (mg/l)	0.21	0.96	1.96	25.47
Deviation (%)	−14.4	−4.5	−2.1	0.5
Intra-assay variation, C.V. (%)	1.3	1.3	1.4	1.4
Inter-assay variation, C.V. (%)	6.4	3.5	3.7	4.7[b]

[a] Inter-assay variation based on 5 independent runs with $n=3$ per run.
[b] Inter-assay variation based on 3 independent runs with $n=6$ per run.

Table 3
Stability of metformin in human EDTA plasma, heparinized plasma, and serum at room temperature

Matrix	Time (h)	Metformin hydrochloride (mg/l)	Metformin hydrochloride (mg/l)
EDTA plasma	0	0.30	1.21
	24	0.30	1.17
	48	0.30	1.18
Heparinized plasma	0	0.30	1.20
	24	0.31	1.17
	48	0.31	1.16
Serum	0	0.31	1.19
	24	0.30	1.18
	48	0.31	1.20

Metformin in EDTA plasma, heparinized plasma, and serum was stable at room temperature for up to 48 h (Table 3). Metformin stored in plasma at −20°C and −70°C was stable for at least eight months with less than 2% change in the plasma concentrations. Repeated freezing and thawing of plasma had no effect on the measured concentrations of metformin (Table 4).

In conclusion, we report the development of a rugged, rapid and highly sensitive HPLC method for the measurement of metformin in plasma. It is suitable for monitoring the entire range of steady-state metformin concentrations achieved in patients. The use of a short extraction column and the column switching technique removed a significant portion of the material in the solvent front without significantly changing the overall run time. This maintains the analytical column at optimal performance over hundreds of analytical runs.

Table 4
Repeated freezing and thawing of plasma metformin

Freeze/thaw cycle	Metformin hydrochloride (mg/l)	
	Sample A	Sample B
Baseline	0.42	0.85
1	0.43	0.84
2	0.42	0.83
3	0.42	0.85
4	0.42	0.83
5	0.41	0.86

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