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Short communication

Determination of metformin in human plasma by high-performance liquid chromatography

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Abstract

A simple, selective and sensitive high-performance liquid chromatographic method with spectrophotometric detection was developed for the determination of antihyperglycemic agent metformin in human plasma using a novel sample extraction procedure. Liquid–liquid extraction of metformin and ranitidine (as internal standard) from plasma samples was performed with 1-butanol/*n*-hexane (50:50, v/v) in alkaline condition followed by back-extraction into diluted acetic acid. Chromatography was carried out using a silica column (250 mm × 4.6 mm, 5 μm) under isocratic elution with acetonitrile-40 mM aqueous sodium dihydrogen phosphate (25:75, v/v), pH 6. The limit of quantification (LOQ) was 15.6 ng/ml and the calibration curves were linear up to 2000 ng/ml. The mean absolute recoveries for metformin and internal standard using the present extraction procedure were 98 and 95%, respectively. The intra- and inter-day coefficient of variation and percent error values of the assay method were all less than 8.3%.

Keywords: Metformin; Liquid–liquid extraction

1. Introduction

Metformin (Fig. 1) is an old drug with antihyperglycemic properties and one of most commonly prescribed medications for type II diabetes [1].

Numerous chromatographic methods are now available for metformin [2–20]. Extraction and clean-up of drugs from biological fluids are usually the first and most difficult step in bioanalysis due to the need to selectively remove interferences without significant analyte loss. Organic solvent extraction is a simple, specific and effective sample preparation method for most drugs, but it proved to be difficult because of the high polarity of metformin [2–7].

Protein precipitation has been the most common method used for plasma sample preparation for metformin [2–7]. However, this procedure is not specific and may not be effective in removing the endogenous or exogenous interferences. Moreover, the method may suffer from lack of sensitivity due to sample dilution. Ion-pair liquid–liquid [9,10] or ion-pair solid-phase [11] extraction have been developed to overcome the polarity of metformin. A solid-phase extraction [12], and ultrafiltration with column switching [13] have also been reported, but their reported sensitivity is low. Chemical derivatization of metformin used in GC [14,15] and some of HPLC [16,17] methods are time-consuming and tedious.

In recent years, HPLC–mass spectrometry methods following plasma deproteination are increasingly reported for metformin [18–20] to offer selectivity and sensitivity, however, they are not ideal for clinical purposes because the instrumentation is quite expensive and not always available in clinical laboratories.

In the present work, we describe a simple, rapid and efficient (in terms of recovery and removal of interfer-

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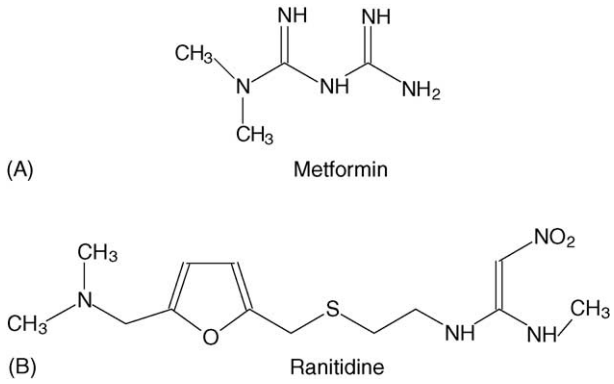

Fig. 1. Chemical structure of metformin (A) and ranitidine (B, internal standard).

ences) liquid–liquid extraction procedure for metformin from plasma. The method allows determination of metformin at low concentrations, while metformin is isocratically eluted in a reasonable time under a simple chromatographic condition.

2. Experimental

2.1. Reagents

Metformin HCl (purity of 99.3%) was prepared from Mahban Chemi Co. (Tehran, Iran). Ranitidine HCl (purity of 99.88, internal standard, Fig. 1B) was obtained from Shasun chemicals and drugs Co. (Cuddalore, India). Analytical grade phosphoric acid, acetic acid and HPLC grade methanol were purchased from E. Merck (Darmstadt, Germany). Analytical grade 1-butanol (n-butyl alcohol) was from BDH Chemicals (Poole, England). HPLC grade acetonitrile was purchased from Carlo Erba Reagenti (Rodano, Italy). All other reagents were of analytical grade.

2.2. Instrumentation

The analyses were performed on a Shimadzu chromatographic system (Kyoto, Japan) equipped with an LC-6A solvent delivery pump, SPD-10AVP ultraviolet detector (operated at 234 nm), C-R8A integrator and a CTO-6A column heater. The samples were applied by a Rheodyne 7725 loop injector with an effective volume of 100 μl. A Waters Spherisorb S5W column (250 mm × 4.6 mm i.d.; 5 μm particle size) with a Waters Spherisorb S5W guard column (30 mm × 4.6 mm i.d.) were used for the chromatographic separation. The mobile phase comprised of acetonitrile-40 mM aqueous sodium dihydrogen phosphate (25:75, v/v), pH 6. Analyses were run at flow rate of 1.3 ml/min at 50 °C.

2.3. Standard solutions

Standard solutions of metformin and ranitidine were prepared by dissolving 5 mg of each in 50 ml of 90% methanol and stored at −5 °C. The internal standard stock solution was diluted in methanol to produce a final concentration of 5 μg/ml. Working solutions for metformin were prepared daily in methanol producing 100, 50, 25, 12.5, 6.25, 31.25, 15.625 and 7.8127 μg/ml.

2.4. Calibration curves and quantitation

Plasma standards for calibration curves were prepared by spiking different samples of 1 ml drug-free plasma each with 20 μl of one of the above mentioned metformin working standards to produce 2000, 1000, 500, 250, 125, 62.5, 31.25 and 15.6 ng/ml. They were shaken for 2 min, and then stored at least 15 min at room temperature before use. The prepared calibration standards and quality control standards (100 μl) were pipetted into 1.5 ml polypropylene tubes and stored at −20 °C pending analysis. In each run, a plasma blank sample was also analyzed, but result for blank samples were not used as part of the calibration curves.

Calibration curves were constructed by plotting peak height ratio (y) of metformin to the internal standard versus metformin concentrations (x). A linear regression was used for quantitation.

2.5. Extraction procedure

A 100 μl volume of plasma was transferred to a 1.5 ml polypropylene microcentrifuge tube. Extraction was performed by adding internal standard (20 μl, equal to 100 ng ranitidine), 100 μl of 8 M NaOH and 1.3 ml of 1-butanol/*n*-hexane (50:50, v/v) to the tube and shaking for 2 min. After centrifugation at 11300 g for 2 min, the whole organic layer was separated and transferred into another tube. Then, 100 μl of 1% acetic acid was added. The mixture was vortex-mixed and centrifuged for 2 min. The organic phase was removed and a 50 μl volume of aqueous phase was injected into the chromatograph.

2.6. Assay validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the samples containing metformin and internal standards with those obtained from blank samples. Besides calibration standards, additional standards were prepared for the determination of intra-day ($n = 5$) and inter-day ($n = 5$) of the assay accuracy and precision. The absolute recoveries ($n = 5$) was calculated by comparing peak heights obtained from prepared sample extracts with those found by direct injection of drug solution made in 1% acetic acid adjusted to pH 5.5 with concentrated NaOH at the same concentration.

The limit of quantification (LOQ) was estimated by analyzing metformin at low concentrations of the calibration curves. The LOQ was defined as a concentration level where accuracy and precision were still better than 10%. To determine the limit of detection (LOD), lower plasma

concentrations than the lower end of the calibration curves were used. The LOD was then defined as the concentration which caused a signal three times the noise (S/N = 3/1).

3. Results and discussion

3.1. Method development

Various separation modes, such as reversed-phase [12,19,20], ion-pair [6,9,11], cation exchange [5,7,13] and normal phase chromatography on silica column with a mobile phase normally associated with reversed phase type columns [3,4] have been reported for metformin. In the present study, retention of metformin was examined on C18, C8, CN and silica columns with mobile phases consisted of a mixture of phosphate buffer and acetonitrile. The metformin retention was greater on more polar columns. The order of Sil > CN > C8 > C18 in ability to retain metformin was observed and therefore a silica column was used for chromatographic separation.

A simple buffered acetonitrile mobile phase was found appropriate for the separation and both metformin and internal standard showed acceptable peak symmetry and retention. The addition of an organic amine, such as triethylamine or an ion-pair reagent to the mobile phase was not necessary and therefore was avoided.

Since metformin is a strongly basic and polar compound, we used vigorous alkaline condition to extract it to a suitable organic solvent. In the present study, the extractability of metformin from plasma was tested in recovery experiments using different 1-butanol/*n*-hexane mixtures, different ratios of extraction solvent to sample volume and different sodium hydroxide concentrations. Evaporation of extraction solvent was not tried. Instead, back-extraction into different aqueous mediums was tested and finally 1% acetic acid was selected. It was observed that the metformin extractability is dependent to the percent of 1-butanol in *n*-hexane, sodium hydroxide concentration and the ratio of extraction solvent to sample volume. It must be noticed that the pH of 1% acetic acid increases to 5.5 following back-extraction, which is caused by dissolved NaOH in extraction solvent.

Structurally related biguanides, such as propylbiguanide [5,9], buformin [7], and phenformin [10–12,19] used as internal standard, were not available in our study. Moreover, the retention time reported for phenformin is two- [12] or three-fold [11] higher than that for metformin which prolongs the total run time for assay. Atenolol [3] was also not suitable. In the present study, ranitidine was used as internal standard as it offered high and reproducible recovery like metformin and suitable retention time.

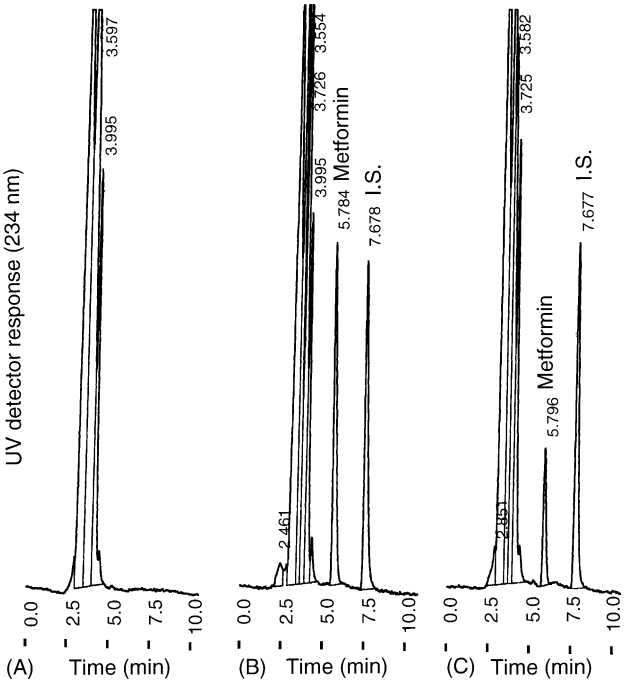

Fig. 2. Representative chromatogram of (A) a blank plasma; (B) plasma spiked with 500 ng/ml metformin; (C) a volunteer samples containing 188.2 ng/ml of metformin, 12 h after oral administration of 500 mg tablet of metformin (attenuation = 3, chart speed = 4 mm/min).

3.2. Method validation

Representative chromatograms of drug-free plasma, plasma spiked with metformin and a volunteer sample collected after oral dosing with metformin are shown in Fig. 2. The retention times for metformin and the internal standard were 5.8 and 7.7 min, respectively. No interfering peaks from the endogenous plasma components were observed in the retention time of metformin or internal standard. In addition, no late-eluting peak was observed and new injection could be done in every 9 min.

The calibration curves were linear over the concentration range of 15.6–2000 ng/ml in human plasma, with a correla-

Table 1
The intra- and inter-day precision and accuracy, and recovery data for the measurement of metformin in human plasma ($n = 5$)

Nominal concentration (ng/ml)	Recovery (%)	Intra-day			Inter-day		
		Mean ± S.D.	Precision (%)	Accuracy (%)	Mean ± S.D.	Precision (%)	Accuracy (%)
15.6	100.9 ± 7.2	16.1 ± 0.95	5.9	3.2	14.4 ± 1.2	8.3	−7.7
62.5	96.1 ± 4.4	60.6 ± 3.1	5.1	−3	59.3 ± 4.2	7.1	−5.1
500	98.8 ± 1.8	505.3 ± 7.2	1.4	1.1	510.5 ± 14.9	2.9	2.1
2000	97.5. ± 3.7	1978 ± 15.8	0.8	−1.1	2005.1 ± 25.3	1.3	0.25

tion coefficient greater than 0.999. The LOQ was 15.6 ng/ml and the LOD was about 5 ng/ml. The values obtained for intra-day and inter-day 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. Intra-day precision ranged between 0.8 and 5.9% whereas the inter-day precision was between 1.3 and 8.3%. The intra-day mean error was between −3 and 3.2% whereas the inter-day mean error was between −7.7 and 2.1%. The mean absolute recoveries for metformin and internal standard using the present extraction procedure were 98 and 95%, respectively.

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