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Short communication

Rapid and simple high-performance liquid chromatographic assay for the determination of metformin in human plasma and breast milk

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Abstract

A rapid and simple high-performance liquid chromatographic (HPLC) assay for the determination of metformin in human plasma and breast milk is described. After proteins were precipitated with acetonitrile, metformin and the internal standard buformin were resolved on a cation-exchange column and detected by UV detection at 236 nm. Standard curves were linear over the concentration range 20.0–4000 μg/l. Intra- and inter-day coefficients of variation were <9.0% and the limit of quantification was around 20 μg/l.

Keywords: Metformin

1. Introduction

With the increasing popularity of breast-feeding, the distribution of drugs and environmental chemicals into human milk has been of clinical interest. Although breast milk is the optimal food for babies, the young breast-fed child may be exposed to drugs during maternal drug therapy. It is important to know the extent of drug transfer into human breast milk in order to assess drug safety during the lactation period.

The biguanide metformin is an oral antihyperglycaemic agent widely used in the management of type-2 diabetes, a common disease that combines defects of both insulin secretion and insulin action. Metformin offers advantages over alternative antihyperglycaemic agents that include negligible potential for hypoglycaemic episodes and the ability to reduce hyperinsulinaemia [1]. Metformin is also being used in the management of polycystic ovary syndrome where it may reduce insulin resistance and hyperinsulinaemia, and may ultimately reduce hyperandrogenism and decrease menstrual cycle abnormalities [2,3]. The use of metformin for this condition, and the increasing prevalence of type-2 diabetes in developed countries suggests that there is likely to be increasing use of metformin amongst pre-menopausal women.

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The pharmacokinetic properties of metformin in patients with type-2 diabetes and in healthy volunteers have been described previously [4,5]. However, nothing is known about the pharmacokinetics of metformin in breast-feeding mothers, their infants and in breast milk. Generally, it has been recommended that metformin is avoided in breast-feeding because its safety has not been established. However, we are aware of instances locally and internationally where metformin has been used during the puerperal period. Therefore, it is essential to have good information regarding metformin's transfer into breast milk.

Several methods for measuring metformin in human plasma or urine have been developed [6–11]. To our knowledge, no assay for measuring metformin in human breast milk has been reported. The aim of this work was to develop a HPLC method for measuring metformin in human plasma and breast milk that could be used in the research of metformin distribution in human milk.

2. Experimental

2.1. Reagents

Metformin hydrochloride and the internal standard, buformin, were kindly donated by Glaxo (Gunnels Wood Road, Stevenage, Hertfordshire, UK). Tetramethylammonium hydroxide (1.0 *M* solution in water) was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). HPLC-grade acetonitrile was purchased from BDH (Poole, UK). Distilled, deionised water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). The human plasma used as the assay blank and for the preparation of standards was obtained from Canterbury Health Laboratories (Christchurch, New Zealand). The human breast milk, used as the assay blank and for the preparation of standards, was kindly donated by a healthy volunteer.

2.2. Chromatography

The HPLC system consisted of a Kontron HPLC pump 420, a Kontron Autosampler 460 (Kontron Instruments, Watford, UK), a cation-exchange Nucleosil 5 μm SA 100A 30×4.00 mm I.D. guard column and a cation-exchange Nucleosil 5 μm SA 100A 250×4.00 mm I.D. column (Phenomenex, Torrance, CA, USA). Detection was via a UVIKON 735LC UV detector operated at 236 nm (Kontron Instruments Ltd., Watford, UK). Data was collected and analysed by the Kontron AT Data System 450 vers 3.01 (Kontron Instruments, Watford, UK). The mobile phase was a mixture of a tetramethylammonium phosphate buffer, pH 3.7 (made by adjusting the pH of 100 mmol/l tetramethylammonium hydroxide to 3.7 with 1 mol/l H_3PO_4) and acetonitrile (80:20, v/v). The mobile phase was filtered through a 0.45-μm filter and degassed under vacuum before use. The flow-rate was 1.0 ml/min and the system was operated at ambient temperature.

2.3. Standards

A standard stock solution of metformin (400 mg/l) was prepared by dissolving 12.82 mg of metformin hydrochloride in 25 ml of a 1:1 mixture of methanol and water. The working standards of metformin (200, 625, 1250, 2500, 5000, 10 000, 20 000 and 40 000 μg/l) were prepared by diluting the stock solution with a 1:1 mixture of methanol and water. Plasma or milk standards were freshly prepared for each analytical run by diluting 0.1 ml of each working standard with 0.9 ml of blank plasma or milk, giving a metformin calibration range of 20.0–4000 μg/l. The stock internal standard buformin solution (1000 mg/l) was prepared by dissolving 10 mg of buformin in 10 ml of methanol. A working solution of the internal standard (10 mg/l) was prepared by diluting 100 μl of the stock solution to 10 ml with methanol.

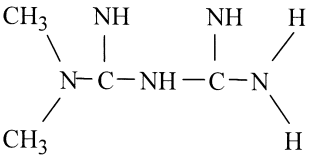
Bulk metformin plasma or milk standards for freeze–thaw stability determinations were prepared in single 10-ml aliquots in the following concentrations: 62.5, 250, 1000 and 4000 μg/l. Metformin plasma or milk quality control (QC) standards were prepared in the same concentrations as the bulk standards and stored in multiple 0.5-ml aliquots for assay with each analytical run. Both bulk standards and QC standards were stored at −30°C until analysed. QC standards were discarded once thawed and analysed.

2.4. Sample preparation

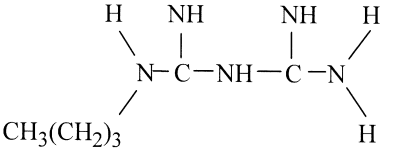
The buformin internal standard, 20 μl of 10 mg/l, was added to 0.2 ml of blank, standard, quality control or unknown sample of plasma or milk. The mixture was vortexed briefly and 200 μl of acetonitrile was added to precipitate the proteins. The mixture was vortexed for 30 s, centrifuged at 15 000 *g* for 15 min and 50 μl of clear supernatant was injected into the HPLC system.

2.5. Validation

The standard curve was a plot of the peak area ratios of metformin–buformin versus the corresponding concentrations of metformin in the standard curve samples. The linearity of the standard curve was evaluated using least-squares linear regression analysis. To determine recovery of metformin at concentrations of 62.5, 250, 1000 and 4000 μg/l and of buformin at the concentration used in the assay (200 ng/0.2 ml plasma or milk) from plasma or milk, an identical set of standards prepared in the mobile phase was analysed. Absolute recoveries at each concentration were measured by comparing the response of pre-treated standards with the response of standards which had not been subjected to sample pre-treatment. Intra- and inter-day coefficients of variation of the assay were determined by the analysis of six QC samples at each concentration on the same day and of one QC sample at each concentration on 6 different days, respectively. The limit of quantification for this assay is defined as the lowest concentration of metformin that can be detected with an intra- and inter-day coefficient of variation of $<20\%$ ($N=5$) and a mean value of $<20\%$ deviation from the spiked value.



Metformin



Buformin

Fig. 1. Chemical structures of metformin and buformin.

3. Results and discussion

3.1. Chromatography

Metformin and the internal standard, buformin, are both biguanides. They are structurally very similar, although metformin has a short methyl side chain as compared with buformin (Fig. 1). Under the chromatographic conditions employed, the retention times were 9.0 and 15.0 min for metformin and the internal standard, buformin, respectively (Fig. 2). The two peaks were free of interference from any peaks present in the plasma or milk blank. Changes in pH value, tetramethylammonium hydroxide concentration and percentage of acetonitrile in the mobile phase were found to have a profound influence on the retention time and peak shape of the chromatographed compounds. With higher tetramethylammonium hydroxide concentration and/or percentage of acetonitrile, the retention time decreased and the peak shape improved. At lower pH, the retention time increased and the peak shape widened. With a mobile phase containing 20% acetonitrile, optimum retention and peak shape were achieved at pH 3.7 and 100 mmol/l tetramethylammonium hydroxide.

3.2. Linearity and limit of quantification

Both plasma and milk standard curves of metformin were linear ($r^2>0.99$) over the concentration range 20.0–4000 μg/l. The intercept with the *y*-axis was not significantly different from zero. The limit of quantification of metformin was around 20 μg/l in both plasma and milk.

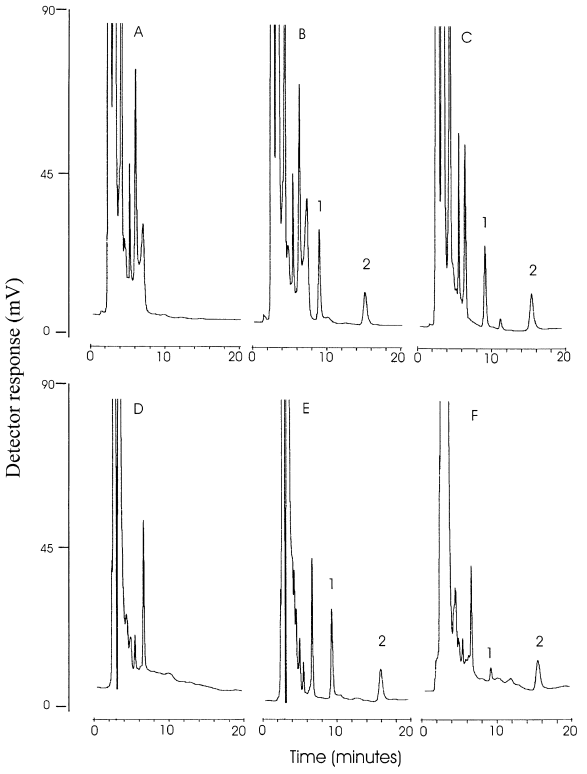


Fig. 2. HPLC chromatograms obtained for (A) blank plasma sample, (B) plasma sample spiked with metformin at 1000 μg/l, (C) plasma sample from a patient containing 648 μg/l metformin 2 h after oral administration of 500 mg of metformin, (D) blank breast milk sample, (E) breast milk sample spiked with metformin at 1000 μg/l and (F) breast milk sample from a patient containing 168 μg/l metformin 2 h after oral administration of 500 mg of metformin. Peaks: (1) Metformin; (2) buformin.

3.3. Recoveries

The absolute recoveries of metformin from plasma and breast milk determined at concentrations of 62.5, 250, 1000 and 4000 μg/l were similar and consistent. The mean±SD absolute recoveries of metformin were 93.7±0.76% from plasma (N=4 plasma extracts at each concentration) and 93.9±1.00% from breast milk (N=4 breast milk extracts at each concentration), respectively. The mean±SD absolute recoveries of the internal standard, buformin, at the concentrations employed were 93.3±1.97% from plasma (N=4 plasma extracts) and 93.9±1.00% from breast milk (N=4 breast milk extracts), respectively.

3.4. Stability of metformin

In order to assess the effects of freezing and thawing on metformin, bulk metformin plasma and breast milk standards at 62.5, 250, 1000 and 4000 μg/l were subjected to four freeze–thaw cycles before analysis. The mean values (N=4) measured at each concentration were ≤4.0% deviation of the spiked values for both plasma and breast milk samples. Metformin was found to be stable in plasma and breast milk for at least four freeze–thaw cycles when stored at −30°C. In addition, QC samples of plasma and breast milk were stored at −30°C for 7 months. After 7 months of storage, samples at each concentration were analysed and the metformin values remained stable (<9.0% deviation of the spiked values). Standard stock solution of metformin was shown to remain stable for at least 8 months at 4°C.

3.5. Accuracy and precision

The precision and accuracy of the method were determined by intra- and inter-day assay variance (Tables 1 and 2). The intra-day coefficients of variation were less than 7.0% and the inter-day coefficients of variation were less than 9.0% for both

Table 1
Intra- and inter-day assay variance of the determination of plasma metformin

Type of variance	Sample	Concentration spiked (μg/l)	Concentration found (μg/l) (mean±SD)	C.V. (%)
Intra-day (N=6)	QC1	62.5	62.9±4.4	7.0
	QC2	250	260±6.3	2.4
	QC3	1000	1030±40.0	3.9
	QC4	4000	4130±190	4.7
Inter-day (N=6)	QC1	62.5	63±3.5	6.0
	QC2	250	250±22.0	8.9
	QC3	1000	1000±52.0	5.2
	QC4	4000	3900±240	6.2

Table 2
Intra- and inter-day assay variance of the determination of milk metformin

Type of variance	Sample	Concentration spiked (μg/l)	Concentration found (μg/l) (Mean±SD)	C.V. (%)
Intra-day	QC1	62.5	62.9±1.8	2.9
(*N*=6)	QC2	250	250±7.5	3.0
	QC3	1000	1000±42.0	4.2
	QC4	4000	4140±84	2.0
Inter-day	QC1	62.5	62.8±3.9	6.2
(*N*=6)	QC2	250	240±13.0	5.5
	QC3	1000	1050±22.0	2.1
	QC4	4000	4080±160	3.9

plasma and breast milk assays at all concentrations of the QC samples.

3.6. Application of the assay

The assay is currently being used in a clinical study to investigate metformin's distribution in human milk. The plasma and milk concentration–time profiles for a typical patient at steady state are shown in Fig. 3.

4. Conclusions

The HPLC method described has been validated and is currently being used to analyse metformin concentrations in human plasma and breast milk after oral administration of metformin in breastfeeding mothers. The method has proven to be simple, rapid, sensitive and specific.

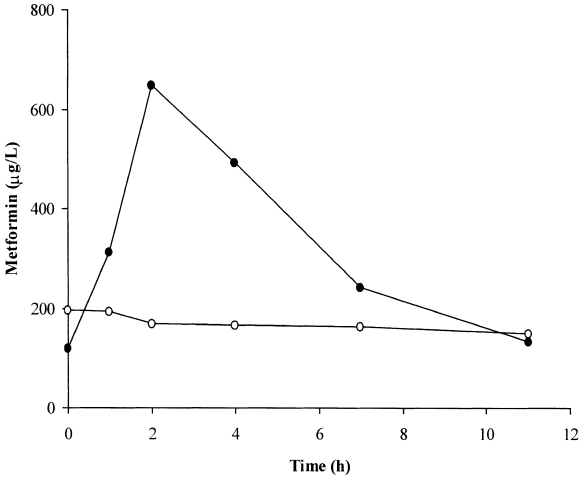


Fig. 3. Steady-state metformin concentrations in plasma and breast milk over an 11-h dosing interval in a patient on a dose of 500 mg twice daily. (●) Concentration of metformin in plasma, (○) concentration of metformin in breast milk.

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