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Original Article

SIMPLE HPLC-UV METHOD FOR DETERMINATION OF METFORMIN IN HUMAN PLASMA AND ERYTHROCYTES APPLICATION TO THERAPEUTIC DRUG MONITORING

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ABSTRACT

Objective: The aim of this study was to develop a simple, rapid, efficient, cost effective and reproducible, stability indicating reverse phase high performance liquid chromatography method (RP-HPLC) for dosage of metformin in human plasma and erythrocytes.

Methods: In this method, the plasma or erythrocyte proteins were precipitated using Perchloric acid: acetonitrile (50 % v/v) mixture and the supernatant liquid were injected into the HPLC system. The separation was achieved with a symmetry C8 column with the mobile phase containing 10 % water and 90 % sodium dihydrogen phosphate buffer (5.8 mM), the pH was adjusted to 3.8 with Phosphoric acid. The temperature was elevated to 25 °C. The detection was done by a UV detector at 232 nm.

Results: The retention time was observed at around 4.412 min for metformin and 6.022 for lansoprazole an internal standard (IS). The response was linear over a range of 2-32µg ml-1, the coefficient of determination (r^2) was found to be (r^2 =0. 9988). The lowest limit of quantification and detection was 0.1 µg/ml and 0.3 µg/ml respectively. No endogenous substances were found to interfere with the peaks of the drug. The intra-day and inter-day coefficient of variations was 2.1 % or less for all the selected concentrations. The relative errors at all the studied concentrations were 3.5 % or less.

Conclusion: The HPLC method described in this article was simple, selective, reproducible, linear, and precise, it can be applied for therapeutic drug monitoring of metformin in human plasma and erythrocytes.

Keywords: Metformin, Method, Plasma, Erythrocytes, HPLC, C8 column.

INTRODUCTION

The necessity of drug monitoring in human plasma and erythrocytes of metformin is nowadays of great interest in pharmacology. Metformin (fig. 1) is an old drug with antihyperglycemic properties. It is one of the most commonly prescribed medications for the type II diabetes [1] indicated as an adjunct to diet and exercise, either as a single oral agent or in combination with sulfonylurea, alpha-glycosidase inhibitors, or insulin. Its adverse effects are generally tolerable and self-limiting.

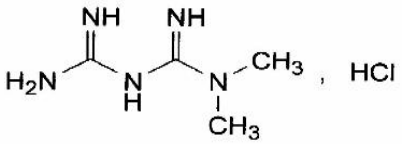

Fig. 1: **Chemical structure of metformin hydrochloride**

The targets tissues for these properties include both insulin-sensitive and non insulin-sensitive tissues. In the liver, the metformin can reduce gluconeogenesis and glycogenolysis. While in peripheral tissues it increases the glucose uptake and the oxidation among non-insulin sensitive tissues; it inhibits the glucose absorption and increases the glucose use in the small intestine. And finally in the erythrocytes, the metformin increases the non-insulin dependent glucose uptake as well as the glycogen levels [2], it has an absolute oral bioavailability of 40–60 %. The gastrointestinal absorption is complete at 6 h with a peak plasma concentrations reached after 2–3 h. Metformin pharmacokinetics can be described by a two-compartment open model, with a plasma elimination half-life of 2–6 h, which corresponds to rapid elimination from a central compartment (half-life), and a terminal elimination half-life of 8–20 h, corresponding to a slower elimination from a deep compartment (half-life) [3]. Metformin is stable, not metabolized, and is excreted in the urine at a rate of roughly 90 % in the 12h [2]. It is compatible with three-compartment model elimination, because it behaves as a strong base. It is almost completely ionized at the pH of plasma [4].

Despite poor lipid solubility resulting from this markedly polar nature, a certain proportion of the drug enters the cells [3]. Studies in normal and diabetic mice have indeed shown that metformin may accumulate in many tissues, at concentrations greater than, those of plasma. The greatest accumulation has been observed in the gastrointestinal tract, where concentration has been shown to reach 10-100 times those of plasma [5]. The Metformin quantification in plasma and erythrocytes samples is required for therapeutic drug monitoring [6]. Several HPLC methods have been used for this purpose. The techniques used are chromatography-tandem mass spectrometry (LC-MS-MS), reversed-phase HPLC [**7**], ion-pair HPLC [8**,** 6**,** 9] and cation-exchange HPLC [10**,** 11]. UV including DAD [12] detection has been described.

The therapeutic use of metformin lasted for more than 40 y, there is still little information on its accumulation by various tissues in humans. The measurement of metformin is now available in human plasma and erythrocytes with HPLC, we examined whether this assay could also provide useful information on the treatment of metformin in type 2 diabetes and contribute to the discussion on the relationship between metformin and lactic acidosis. To explore the clinical implications, we reviewed all patients in whom metformin concentration was determined in plasma and erythrocytes.

In the present work, we describe a simple, rapid and efficient (in terms of recovery, accuracy and precision, removal of interferences) liquid-liquid extraction procedure for metformin from plasma and

erythrocytes. The method allows determination of metformin at low concentrations, while metformin is isocratically eluted in a reasonable time under a simple chromatographic condition.

MATERIALS AND METHODS

Reagents and chemicals

The working standard of Metformin HCl (purity of 99.1 %, fig. 1) and lansoprazole (purity of 99.1 %, internal standard, were obtained from Sigma-Aldrich (St. Louis, MO, USA), sodium di-hydrogen phosphate 1-hydrate (NaH2PO4. H_2O) Reag. Ph Eur, M= 137, 99, acetonitrile from Merck KGaA, Germany, Perchloric Acid 70 %, Sigma-Aldrich C=11.63M.

Mobile phase preparation

The HPLC grade solvents were used for the preparation of mobile phase, isocratic elution of mobile phase comprising of water: Phosphate buffer in the ratio (10:90, v/v). Phosphates Buffer was prepared as follows of sodium dihydrogen phosphate Buffer (from 118.9M buffer to produce a final buffer concentration 57.5 mM) the pH was adjusted to 3.8 with H3PO4 10 %. The contents of the mobile phase were filtered before use through a 0.45µm membrane filter; the mobile phase was degassed and pumped from the solvent reservoir to the column at a flow rate of 0.8 ml/min. Injection volumes were 50 µl. The HPLC column was kept at ambient temperature.

Calibration curve and standard solution

Stock solution of metformin hydrochloride was prepared by dissolving 5 mg of metformin hydrochloride in methanol (1 mg/ml) and the final volume was made to 100 µl with the same solvent. Similarly lansoprazol, the internal standard, was also prepared by dissolving 5 mg in 5ml of methanol (1 mg/ml). From The stock solution of metformin a standard range is prepared for concentrations 2, 4, 8, 16, 32 µg/ml, the dilutions are performed by using a pool of serum from individuals not receiving metformin. These concentrations were chosen as they cover the actual plasma concentrations obtained in patients receiving metformin therapeutically.

Instrument and chromatographic system

The analysis was performed on a Shimadzu chromatography system (Kyoto, Japan) equipped with an LC-20AD solvent delivery pump, SPD-M20A ultraviolet detector (operated at 232 nm, (fig. 2), C-R8A integrator and a CTO-6A column heater. The samples were applied by a reading 7725 loop injector with an effective volume of 100 µl. Waters Spherisorb S5W symmetry C8 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. Vortex-2 Genies and Mini spin plus (Eppendorf) were used to vortex and centrifuge plasma and erythrocytes samples respectively.

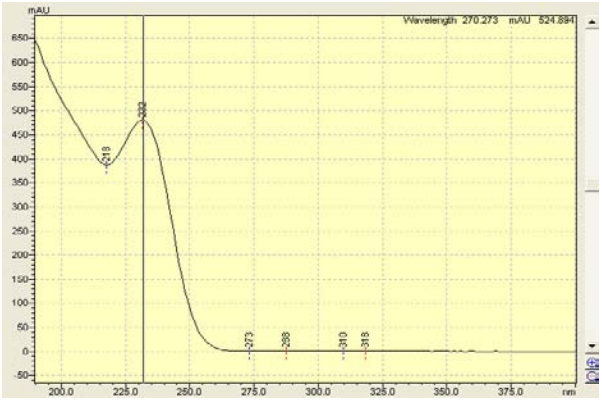

Fig. 2: **The optimum wavelength for detection was used at 232 nm**

Sample preparation and extraction

A 500 µl volume of the plasma or erythrocytes from individuals not receiving metformin was transferred to 1.5 ml polypropylene micro centrifuge tube. Extraction was performed by adding an internal standard 50 µl equal to 10µg of lansoprazol deproteinized with acetonitrile 400 µl to the tube and shaking for 60s, after centrifugation at 10000g for 10 min, the whole organic layer was separated into another tube. Then, 200 µl of Perchloric acid 10 % was added. The mixture was vortex–mixed and centrifuged at 10000g for 5 min. The organic phase was injected into the exchange column.

RESULTS

The developed HPLC method was optimized for the analysis of metformin hydrochloride in human plasma and erythrocytes. Different mobile phases were tested to find the best condition to quantify metformin hydrochloride in plasma. Different ratios of methanol, acetonitrile, and methanol and potassium dihydrogen phosphate were tried and the optimum mobile phase was finalized. Then the method was validated for selectivity, linearity, limit of quantification, accuracy, precision and recovery as per the international guidelines (FDA guideline, 2001).

Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For plasma and erythrocytes samples were chromato graphed to check for endogenous components which might interfere with metformin hydrochloride. Spiked plasma and erythrocytes samples representing a low (2µg/ml), medium (8µg/ml) and high (32µg/ml) metformin hydrochloride concentration was analyzed to verify the selectivity of the method of analysis. The peak of metformin hydrochloride did not interfere with any endogenous components. There was also a very good resolution between the peaks of drug (table 1).

Linearity

Linearity was determined separately for metformin by plotting peak area against concentration. From these calibration plots it was clear that the response was a linear function of concentration over the range 2–32µg/ml for Metformin hydrochloride as shown in graphs (fig. 3). The linear regression equations for metformin, y = 1.35911881E-05x+0.8668161359 (r2 =0. 9988), where X is corresponding (peak area) and Y by the concentration.

The response was determined to be linear over the range of 2 µg/ml to 32 µg/ml for metformin. The solutions were injected into HPLC system. Each of the concentration was injected in triplicate to get reproducible response. The run time was 4.41 min and the peak areas were measured (table 1). The calibration curve was plotted as concentration of the respective drug versus the response at each level. The purposed method was evaluated by its correlation coefficient and intercept value calculated by statistical study. They were represented by the linear regression equation (fig. 3).

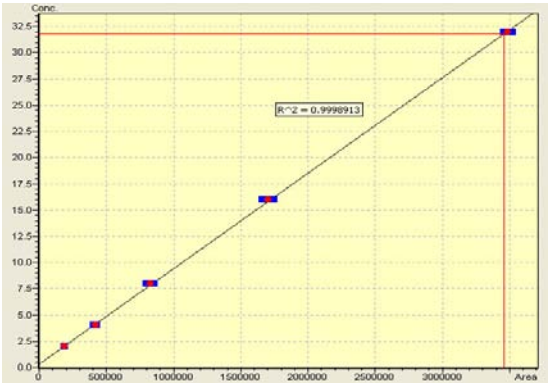

Fig. 3: HPLC Linearity graph for metformin (r^2=0.99980)

Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

The limit of detection (LLOD) and limit of quantification (LLOQ) of the developed method was determined by injecting progressively low concentrations of the standard solutions using the developed

RP-HPLC method. The LLOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The LLOD for Metformin Hydrochloride was found to be 0.3µg/ml; The LOQ is the smallest concentration of the analyte, which gives a response that can be accurately quantified (signal to noise ratio of 10). The LLOQ was 0.1µg/ml for Metformin Hydrochloride.

Accuracy and precision

The accuracy of the analytical method describes the closeness of the mean test results obtained by the method to the true value of the analyte whereas precision is the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. For accuracy the mean value of three concentrations should be within 15 % of the actual value and for the precision the coefficient of variation (CV) should not exceed 15 % at each concentration level (FDA guideline, 2001). The intra-day and inter-day degree of precision and accuracy of the method is expressed as coefficient of variation and relative error respectively (table 1). The intra-day and inter-day coefficient of variation was 2.1 % or less for all the selected concentrations. The relative errors at all the studied concentrations were 3.5 % or less. These data indicate a considerable degree of precision and accuracy of the method both during the analytical run and between different runs. The relative error shows that the method is remarkably accurate which ensures that reliable results are obtained.

Recovery

The chromatographic retention times were 4.42 min for metformin and 6.022 for internal standard (IS), the mean r^2 for plasma and erythrocytes curve 0.9998, the CV of intra and inter-day assessments for both matrices were less than 4 % (table 2 and 3), Mean inter-day error in human plasma was less than 2 % (table 1), in erythrocytes mean percentage error was up to 2 % (table3). Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. A single stage extraction using 200 µl of 70 % m/m Perchloric acid was used for protein precipitation of 500 µl of plasma and erythrocytes.

This minimal volume does not dilute the drug which ultimately improved the sensitivity of this technique. This method gave a good recovery with a minimal time for extraction (table 2). The metformin was found to be stable during storage and during all steps of the analytical method. For the protein extraction from erythrocytes, acetonitrile, trichloroacetic acid and methanol were also tried but the protein precipitation was not complete and there were interfering peaks as well (Acetonitrile + Methanol, acetonitrile + trichloroacetic acid). The ratio of erythrocytes and acetonitrile was 1:1.5. Methanol was also used in the same ratio. As mentioned above, the precipitation of erythrocytes proteins with organic solvents was insufficient. Increasing the volume of organic solvent would dilute the drug in the sample and adversely affect the sensitivity. Previous methods that used a protein precipitation or liquid–liquid extraction procedure with a column specifically designed for reversed phase chromatography used an injection volume of 100 µl or more. So the modification of the previous method [1] by using Perchloric acid reduced the dilution of the sample and also the volume of injection (100 µl) but the method still remained sensitive.

Table 1: The average and standard deviation, Relative standard deviation inter-day precision and Coefficient of Variance data for the measurement of metformin in human plasma (n=3)

Concentration	Con. calculated n=3		Std. deviation		% CV		% Relative error	
added (µg/ml)	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
2µg/ml	2.019	1.999	0.0471	0.0721	2.335	3.61	-0.95	0.05
4µg/ml	4.097	4.2	0.1119	0.1123	2.73	2.674	-2.425	-5
8µg/ml	7.902	7.951	0.2575	0.1536	3.25	1.932	1.225	0.612
16µg/ml	16.037	15.879	0.4029	0.4078	2.51	2.568	-0.231	0.756
32µg/ml	32.294	32.217	0.4816	0.4632	1.49	1.437	-0.918	-0.678

Table 2: Recovry of metformine hydrochloride at three concentrations (n=3)

Con. added (ug/ml)	Mean recovery±SD	%RSD
4	102.41±2.78	2.71
8	98.77±3.22	3.26
32	100.91±1.50	1.48

Table 3: One day validation for assay of metformin in human erythrocytes, n=3

Nominal concentration of metformin HCl, µg/ml	mean±SD, µg/ml	CV %	% Relative error
2	1.99±0.07	3.61	0.5
4	4.1±0.02	2.67	-2.5
8	7.95±0.15	1.93	0.62
16	15.97±0.40	2.56	0.18
32	32.21±0.46	1.43	-0.65

DISCUSSION

Dosage metformin in plasma analysis has been used for therapeutic drug monitoring or other clinical purposes for theophyline, phenytion and aminoglycoside antibiotics, but this is the first report of its application to metformin monitoring in the plasma and erythrocytes. Various separation modes have been described for metformin, like reversed-phase [13-15], ion pair [8, 16, 6] cation exchange [11, 10, 17], and silica column with a mobile phase normally associated with reversed phase type columns [19, 20]. One study [20] has examined the retention of metformin on C18, C8, and silica columns with mobile phases consisted of a mixture of phosphate buffer and water, and it has shown a greater retention on more polar columns; which is the silica column in that case. Therefore a silica column has been used for the chromatographic separation. A simple buffered water mobile phase was found appropriate for the separation of metformin. 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 and erythrocytes was tested in recovery experiments using different acetonitrile and Perchloric acid 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 the mixture of acetonitrile and Perchloric acid was selected.

The HPLC was optimized with a view to develop the precise and stable assay method. Both the pure drug Metformin was run in different composition of mobile phases and different column types (Ultra Aqueous C18 25 cm x 4.6 mm i.d., 5 µm), (Atlantis C18 100 mm x 4.6 mm i.d., 5 µm) ODS (250 mm x4.6 mm, 5 µm). The mixture of Sodium Dihydrogen Phosphate Buffer, pH 3.8 (adjusted with H3PO410 %) and water (90:10 v/v) as mobile phase was found as optimal for obtaining well defined and resolve peaks at a flow rate of 0.8 ml/min at a column oven temperature 25 °C. The optimum wavelength for detection was used at 232 nm, at which best detector response gave sharp and symmetrical peaks with 4.412 min for Metformin. The typical chromatogram of sample solution is shown in (fig. 4). The percentage of individual drugs was calculated. The results of analysis are in agreement with the therapeutic interval of the molecule in question.

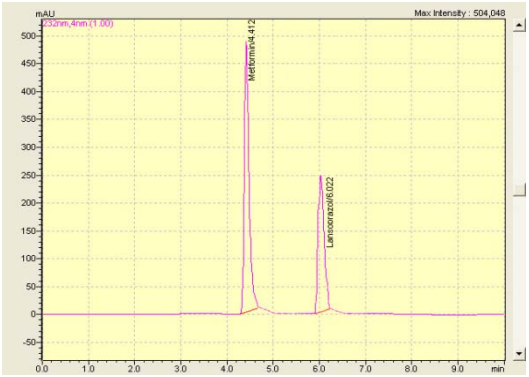

Fig. 4: HPLC-UV chromatogram obtained human plasma sample after metformin administration (retention time 4.41 min), and HPLC-UV chromatogram obtained for internal standard (6.022 min)

Under the operating conditions described previously, the average retention time of metformin was 4.412 min±0.12 min, with a perfectly symmetrical peak. The detection limit was 0.1 µg/ml, and the limit of quantification was 0.3 µg/ml. The linearity of the method was tested at concentrations ranging from 2 to 32µg/ml, with a regression coefficient r^2 = 0.9998. Each concentration was tested three times. There was no significant difference between the slopes of the regression lines: the continuous linearity allows us to use only one calibration range.

The recovery of plasma and erythrocyte metformin in patients was calculated by comparing the results obtained after deproteinization and after direct injection: it was of the order of 99.5 %. Deproteinization in acidic medium yielded a complete recovery, as it was previously shown [21, 22]. The choice of the chromatographic method HPLC- UV was perfectly justified in terms of both selectivity and removal of interferences. In fact, this method which has proved the most consistent with our assay, allowed us to have very good results, without any impurity (as shown below in the purity peak and a better separation and peak identification, as well as the HPLC in inverse phase with a still phase C l8 involved in a drastic reduction of the proportion of organic solvent, with the risk of denaturation of the still phase.

The studies of the variability intra-day and inter-day show high values at low concentrations (CV % = 2.33 % to 2µg/ml) than in high concentrations (CV % = 1.49 % to 32µg/ml). The operating procedure we describe is best suited for the diagnosis of side effects, which is a part of the therapeutic monitoring.

The Plasma and erythrocyte concentration found in our patient was in the range 0µg/ml and 4µg/ml, however, it should be much higher on admission, knowing that the metformin plasma elimination half-life is short (plasma half-life: 2.7–4 h) [23, 24]. The normal therapeutic level of the plasmatic metformin is less than 1.34 mg/l, and is less than 1.65 mg/l in the erythrocytes cells [24-26]. The observation of the rate erythrocytes of the high metformin is an important element of the diagnosis. The suspicion of acidosis lactic associated with the metformin has to make practice its dosage.

The finding of a high plasmatic metformin level is an important element of diagnosis. The association of lactic acidosis with metformin should be dosed. But since only few Moroccan laboratories practice this assay, and since the result is generally obtained after several days, the concentration of metformin is rather part of a retrospective diagnosis. We talk about accumulation when the rates are higher than 5 mg/l [27-30, 25, 6, 2]. But this last point is not constant because there is genuine lactic acidosis in patients with normal plasma of metformin. The assay of erythrocyte metformin is more important because it allows to highlight a chronic overdose with deep tissue accumulation of the drug.

The volume of blood required (2 ml), combined with the simplicity of the analytical technique makes this useful procedure for monitoring metformin concentrations in routine clinical settings. The concept of monitoring metformin concentrations in blood using the HPLC-DAD technique arises from the need to monitor metformin level routinely in diabetic patients. This routine measurement is important to assess adherence to medications and to prevent toxicity from metformin. Monitoring adherence is important to improve diabetic control and to prevent unneeded dose adjustment or adding, more medications in those who have poor disease control due to poor adherence as the clinician may think that treatment was not effective.

Therefore clinical pharmacist and biologists can conveniently take a blood sample at the time of patients' visits from those with poor disease control and check for metformin level to decide if patients are adherence or not. In this study, we found that the plasma concentration of residual metformin in the plasma and erythrocytes of more than 150 patients ranged between 0µg/ml and 5µg/ml; thus confirming the applicability of the current method without problems of low sensitivity. The method is currently being applied to the analysis of sample blood taken from diabetic patients to assess adverse effects case of lactic acidosis and the relationship between the amount of metformin and the therapeutic range. The interpretation of the current results requires knowledge of each of the determinants of the concentration of metformin, metformin pharmacokinetics, and renal function and during metformin dosage, and the time between the late withdrawal of blood and the last administration metformin.

CONCLUSION

The choice HPLC-UV with a column SEMYTRY C8 suits thus perfectly for the dosage of the metformin in the sample of plasma and erythrocytes, bringing a big selectivity and allowing an ultra fast analysis: The metformin is retained at least by 5 min. The method which we propose is simple and linear in the range 2-32µg/ml. Is particularly well adapted to the diagnosis of the acute (sharp) poisonings by the metformin within the frame work of the therapeutic drug monitoring of the persons diabetics of type 2.

The validation of this method was proved to be simple, fast and reliable. The method was validated for its performance parameters Linearity, Repeatability, Accuracy, Precision, Ruggedness, and Robustness etc. The developed method offers several advantages in terms of simplicity in mobile phase, isocratic mode of elution and sample preparation steps and comparative short run time makes the method specific, repeatable and reliable for its intended use in simultaneous determination of Metformin in the human plasma and erythrocytes.

CONFLICT OF INTERESTS

Declared None

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