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Determination of metformin in plasma by capillary electrophoresis using field-amplified sample stacking technique

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Abstract

A capillary electrophoresis method was described for the determination of metformin in human plasma based on the extraction of the ion-pair with bromothymol blue into chloroform. Phenformin was used as internal standard. Field-amplified sample stacking injection was employed with an electrokinetic injection voltage of 10 kV for 10 s. The running buffer was 0.1 *M* phosphate buffer (pH 2.5), running voltage was 20 kV and the UV absorbance detection was set at 195 nm. The limit of quantitation was 0.25 μg/ml. Linearity range of calibration curve was 0.25 to 3.5 μg/ml. Recoveries for three levels (0.25, 1 and 2 μg/ml) were 80.24%, 67.44% and 58.97% (n=5 for each level), respectively. The intra-day precisions for the three levels were 11.9%, 3.09% and 4.33% and the inter-day precisions were 12.4%, 4.57% and 4.94%, respectively. The concentrations of metformin hydrochloride in human plasma of eight volunteers were measured after orally administrating metformin enteric-capsule and tablet. © 1998 Elsevier Science B.V.

Keywords: Metformin

1. Introduction

Metformin (Fig. 1) is a biguanide antidiabetic agent used in the treatment of non-insulin-dependent diabetes. Owing to its weight-decreasing and serum lipid-normalizing effects, it has been especially recommended for obese patients [1]. The administration of radioactive labeled metformin indicated that no metabolism of the drug occurs in either humans [2] or animals [3].

Besides gas chromatographic methods [6–8], high-performance liquid chromatography (HPLC) has been used for the determination of metformin in

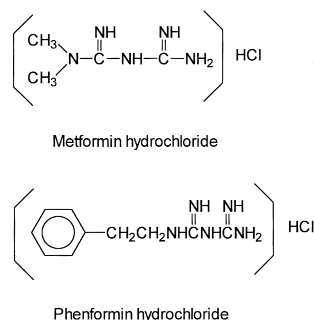

Fig. 1. Structures of metformin hydrochloride and phenformin hydrochloride (internal standard).

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biological fluids [4,9–13]. Recently, the determinations of drugs in biological fluids by capillary electrophoresis (CE) in fused-silica capillaries have been developed [14–17], as an attractive alternative to HPLC. However, no CE assay for metformin in plasma has yet been reported.

The main advantages of CE over HPLC are the much lower consumption for both reagents and time, much better mass-sensitive detection limit and high separation efficiency. Because of the small detective volume (nanoliters) in CE, the concentration sensitivity is typically 1- to 2-orders of magnitude lower than that in HPLC [18]. Using laser-induced fluorescence detection instead of ultraviolet absorbance detection can enhance sensitivity by as much as 3-orders of magnitude [18]. This approach requires expensive instrumentation and is limited to certain compounds which fluorescence at the few excitation wavelength provided by the various laser type available. Alternatively, sample stacking techniques applicable during or immediately after sample injection can provide comparable sensitivity without the need of special instrumentation [18,19]. A new sample-stacking technique termed head-column field-amplified sample stacking which is capable of enhancing the sensitivity >1000-fold has been developed [20].

The lack of information concerning the pharmacokinetic studies of metformin in human biological fluids is attributed to the difficulty in measuring very low concentration of the drug in biological fluids. In particular, metformin is a highly polar compound [4], its partition coefficient is 0.01 either in the octanol–water system or in methylene chloride–0.8 *M* NaOH system, 0.05 in chloroform–*tert*.-amyl alcohol–0.8 *M* NaOH system [5]. It is difficult to extract the drug directly from biological fluid by solvent–solvent extraction. The present sample pretreatment methods for HPLC assay were prederivatized with fluorescent agents prior to injection onto the column [12], solid-phase extraction [11], or by ion-pair extraction [4,13]. All these measures were not suitable for the on-column stacking CE analysis because the large amount of ions existed in the final sample solution. We reported here a field-amplified sample stacking (FASS) CE method, by using an ion-pair extracting method similar to Garrett and coworkers [5,21], which is more sensitive and specific, to the determination of the concentration of metformin in human plasma after oral administration of metformin tablet and its enteric-capsule. A bioavailability evaluation study was performed.

2. Experimental

2.1. Materials

All reagents were of analytical grade, include chloroform (Beijing Chemical Reagents Company); orthophosphoric acid, dipotassium hydrogenphosphate and potassium dihydrogenphosphate (all from Beijing Hongxing Chemical Factory); bromthymol blue (Tianjin Institute of Pharmaceutical Industry). Acetonitrile was of HPLC grade (Beijing Changhua Fine Chemical Factory).

Metformin hydrochloride and its enteric-capsule (250 mg/capsule) were provided by the Institute of Materia Medica, Chinese Academy of Medical Science. Metformin hydrochloride tablet (Diaformin, 500 mg/tablet) was manufactured by Alphapharm (Brisbane, Austrlia). Phenformin (internal standard, I.S.) was extracted from phenformin hydrochloride tablets (Beijing Pharmaceutical Factory).

2.2. Instrumentation

CE analysis was performed on a SpectraPhoresis 1000 CE system with Focus detection (Thermal Separation Products, San Jose, CA, USA) and an IBM 350-450DX2 personal computer with PC 1000 Ver. 3.0 software. The electrophoretic separation was performed on a fused-silica capillary of 40 cm (32.5 cm effective length)×50 μm I.D. (Polymicro Technologics, Phoenix, AZ, USA). The new capillary was conditioned with 1 *M* NaOH for 20 min at 60°C, followed with 0.1 *M* NaOH for 5 min at 60°C and water for 5 min at 20°C prior to use. After each run, the capillary was treated with 0.1 *M* NaOH and then with water for 1 min, respectively, then rinsed by running buffer for 5 min before the next run. Before electrokinetic injection the inlet end of the capillary was dipped in water and then moved to the sample vial. The sample solution was introduced by the electrokinetic method at 10 kV voltage for 10 s, the

anode was on the injection side and the injection current was about 14–20 μA. The running voltage was 20 kV and the average current was about 68 μA. The capillary temperature was controlled at 20°C and the detection wavelength was set at 195 nm. The running buffer was 0.1 *M* phosphate buffer (pH 2.5).

2.3. Stock solutions

Metformin and phenformin (I.S.) stock solutions as their hydrochlorides were prepared at 1 mg/ml with water. All above solutions were stable when stored at 4°C for one month. The stock solution was diluted to 40 μg/ml with water to form standard solution before use. All the data of metformin presented were calculated as its hydrochloride.

Bromthymol blue (BTB) solution (10^{-2} *M*) was prepared by dissolving 62 mg in 2 ml of 0.1 *M* NaOH. The final volume was brought up to 10 ml with distilled water and sonication. The solution was then adjusted to pH 7.8 with concentrated sodium hydroxide or hydrochloride acid if necessary. The 0.4 *M* phosphate buffer (pH 7.8) was prepared by dissolving 5.52 g $NaH_2PO_4 \cdot H_2O$ in 21 ml of 2 *M* NaOH. After adjusting to the final pH with saturated sodium hydroxide, the final volume was brought to 100 ml with water.

2.4. Extraction procedure

A 0.1-ml plasma sample containing a suitable volume of standard solution was spiked with 5 μl of 40 μg/ml I.S. solution and 0.3 ml of acetonitrile were added. The mixture was thoroughly mixed and then centrifuged at 12 000 *g* (15 000 rpm) for 1 min. The supernatant (almost all) was transferred to another plastic tube then evaporated to dryness under vacuum at 70°C, cooled down the plastic tube to ambient temperature. The residue was redissolved in 50 μl of pH 7.8 phosphate buffer, 5 μl of 10^{-2} *M* BTB solution added and mixed, then extracted twice with 1 ml of chloroform for 1 min, respectively. The organic and aqueous phases were separated by centrifugation at 12 000 *g* for 2 min. The combined chloroform portions were evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 100 μl of 200 μ*M* H_3PO_4 for assay.

3. Results and discussion

3.1. Method developed

3.1.1. Extraction

In FASS, the stacking efficiency depended on the ratio of resistivity of the sample solution and the running buffer [19,20]. This means that decreasing the conductivity of sample solution could increase the stacking efficiency with a high sensitivity. However, with given samples such as plasma, this is not a controllable factor because there are a lot of ions, proteins etc., in the samples. When similar injection and analytical conditions were applied to analysis, ineffective stacking and unsatisfactory chromatograms were obtained. Therefore, it was determined that the plasma should be deionized. Ionic substances, proteins and most of other interfering components can be removed simultaneously.

Acetonitrile was selected as a protein precipitant. Other classical protein precipitants such as trichloroacetic acid, perchloric acid and some heavy metal cations listed in Ref. [24] were also tested, but it was difficult to adjust the pH value of supernatant to 7.8, a large volume of aqueous phase resulted. Methanol was also tested, but the efficiency of precipitating is less than that of acetonitrile.

Organic solvent extraction is a simple and effective method, but it proved to be difficult because of the high polarity of metformin. The partition coefficient of metformin for the methylene chloride–water system had been determined as a function of sodium hydroxide concentration and it showed that metformin could not be extracted from water by methylene chloride even in the hydroxide solution [21]. The extraction of metformin from the basified solution by a series of organic solvents was tested, metformin could not be extracted even in a very strong sodium hydroxide solution (1 *M*). So, the ion-pair extraction method was taken into account. Garrett and coworkers [5,21] reported an ion-pair extraction method for the determining of three biguanides using a spectrophotometric method. An acid dye bromthymol blue was used and the ion-pair was extracted by use of dichloromethane, then the organic phase was siphoned into another tube. A volume of tetrabutylammonium hydroxide was added as a counter cation to metformin, then metformin was back-ex-

tracted in aqueous phase. Keal and Somogyi [4] reported a modified method in determining metformin in plasma and in urine by HPLC. Although the recovery was low, it showed only a small degree of variability and was deemed acceptable considering the low concentration which could be measured and the small inter- and intra-day assay variations. Liu et al. [13] developed another ion-pair extraction method where sodium dodecyl sulfate (SDS) was used as the ion-pair reagent to determine the concentration of metformin in human plasma by HPLC. However, all of these methods were not fully suitable for the field-amplified sample stacking injection in CE analysis because a high conductivity of the sample matrix was produced by the ionic substances in the final injecting solution. Comparatively, the method proposed by Garrett and coworkers [5,21] was better than the others.

The method of extraction described here was a modification of the Garrett and coworkers method [5,21]. The original studies were reported by Schill et al. [22,23] in which the concentration of bromthymol blue and the pH for maximum ion-pair formation is determined by the physicochemical characteristics and concentration of the organic cation for metformin. The pH value is one of the major factors effecting the extraction efficiency. It was reported that in conventional ion-pair extraction methods, where bromthymol blue was used as the anionic dye, the lower limit of the extraction pH is 7.5, below that pH the interference due to the background of bromthymol blue is too high [22,23]. The reason is probably that the dye could be excessively extracted into the organic phase in its molecular form at a pH value near or smaller than pK_a (7.18 [5]). For the extraction from the biological fluids, some endogenous components would be associated with BTB and then extracted to the final solution. The pH of the buffer solution was studied at pH 7.3, 7.5, 7.8 and 9.1. At the low pH values (7.3 and 7.5), there were some endogenous peaks interfering with metformin at the pH 7.3 buffer (Fig. 3c). At high pH values, the recovery decreased sharply. Possibly the ion-pair extraction constant is decreasing at high pH value. The recovery of a 2 μg/ml concentration of metformin at pH values of 7.5, 7.8, 9.1 was tested, the results were 51.2%, 68.6% and 4%, respectively. A pH 7.8 buffer was chosen in our study.

The content of BTB is another factor. Although the calculated recovery of metformin-spiked plasma in the concentration range from 1.60 to $16.60\cdot10^{-5}$ *M* was 98.5%, and in the concentration range 4.00 to $20.00\cdot10^{-5}$ *M* was 100%, the experimental recovery was 52.9% and 40.3%, respectively [21]. The effect on the recovery by the content of BTB was studied at 0.6, 1, 2 and 4 m*M* of BTB in pH 7.8 buffer containing 50 and 100 ng of metformin per 50 μl. At a concentration of 0.6 m*M* BTB, the recovery and reproducibility were poor, especially when the concentration of metformin was at 100 ng/50 μl. Probably BTB was not sufficient to form ion-pair with metformin. When the concentration of BTB was 1 m*M*, high recovery and precision were obtained. But at a concentration of BTB of 2 or 4 m*M*, the organic extraction gave no peaks in the electropherograms due to an excessive amount of BTB extracted into the organic phase. When 0.1 ml of 200 μ*M* H_3PO_4 was added, it was difficult to dissociate metformin from the ion-pair. Although increasing H_3PO_4 could dissociate the ion-pair, the contents of ions were also increased, consequently the conductivity of sample solution was increased and the efficiency of FASS was decreased. Experimentally, the peak heights of both metformin and I.S. were decreased so sharply that the peaks became almost undetectable. In this study, 1 m*M* BTB was chosen for high recovery and precision.

3.1.2. Stability of metformin and I.S. during dryness at 70°C

The test solution was prepared by placing 5 μl of 40 μg/ml of metformin standard solution and 5 μl 40 μg/ml of I.S. solution in a plastic tube, adding 0.1 ml of water and 0.3 ml of acetonitrile, then vortex mixing before drying under vacuum at 70°C. A 50-μl volume of pH 7.8 buffer and a 5-μl volume of 10^{-2} *M* BTB were added to the residue, and the extraction procedure as described in Section 2.4 was followed. The control solution was prepared by using 0.1 ml of water and 0.3 ml of acetonitrile and then vacuum drying at 70°C. The same volumes of metformin standard solution and I.S. solution as mentioned above were added to the residue, then 40 μl of pH 7.8 buffer and 5 μl of 10^{-2} *M* BTB were added and extraction was carried out (see Section 2.4). The results of the analytes' contents in the test solution compared with those in the control solution

were 93.2%, relative standard deviation (R.S.D.) 6.8% ($n=5$) for metformin and 95.6%, R.S.D.=5.7% ($n=5$) for I.S., respectively. It was clear that metformin and I.S. were stable under these conditions.

3.1.3. CE conditions

Both metformin and phenformin (I.S.) are highly polar compounds, and their mobilities are mainly influenced by pH value of running buffer. The pH range from 2.5 to 7 was studied. At low pH value, the metformin, I.S. and an endogenous peak were completely separated. Upon increasing the pH value, the migration times shortened and the separations of the I.S. and the endogenous substance were poor. Fig. 2 shows that the mobility (μ_{eff}) was influenced by pH. A typical electropherogram is shown in Fig. 3.

3.2. Linearity, limit of quantitation and recovery

3.2.1. Linearity and limit of quantitation (LOQ)

Linearity of the calibration curve was assessed over a concentration range of 0.2 to 5 μg/ml of plasma. Control plasma samples were spiked to give concentrations of 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 μg/ml of metformin, 2 μg/ml of I.S. was added to each sample prior to extraction. The linearity of standard curve was confirmed by plotting the ratio of metformin and I.S. peak areas versus the concentrations of metformin. A straight line obtained in the concentration range of 0.2 to 3.5 μg/ml was $y=0.8212c+0.0017$ with a correlation coefficient of 0.9995, where y is the ratio of metformin to I.S. peak areas and c is the concentration of metformin in μg/ml. The standard error of the calibration curve was 3.41%.

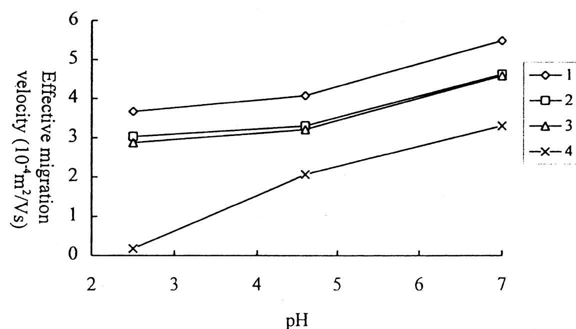

Fig. 2. Effect of the pH on peak resolution. Peaks: 1=phenformin, internal standard; 2=endogenous peak in plasma; 3=metformin; 4=EOF. Column: 40 cm (32.5 cm effective length)×50 μm I.D. fused-silica capillary. Buffer: 0.1 M phosphate buffer (pH 2.5). Conditions: 20 kV, 20°C, detection at 195 nm.

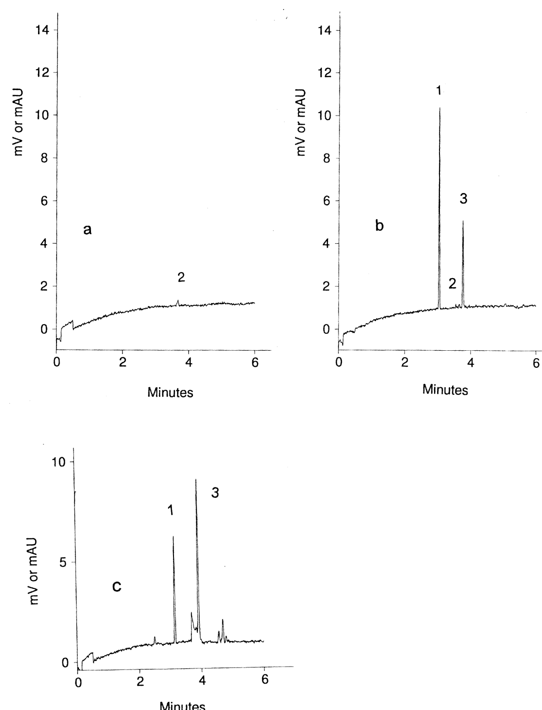

Fig. 3. Electropherogram of an extract of (a) a blank plasma sample, (b) a plasma sample from a subject 2 h after oral administration of 1 g of metformin hydrochloride. Peaks and conditions as in Fig. 1. (c) Shows some endogenous peaks in the extract with a pH 7.3 buffer. Conditions and peaks 1, 3 are as in Fig. 2, other peaks are due to substances in the plasma.

The limit of quantitation (LOQ) is defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy and precision. At the LOQ, an acceptable precision value of ≤20% R.S.D. was obtained from a set of measured concentrations [25]. In this study, the measured LOQ was 0.25 μg/ml, R.S.D.=15.6% ($n=5$). The limit of determination was 0.1 μg/ml ($S/N=3$).

The limit of determination depended on the final sample matrix. If the standard metformin solution diluted with 200 μM H_3PO_4 (without extraction) was tested, the measured limit of determination approached 10 ng/ml. But for the ion-pair extraction, some endogenous ionic substances and other salts could be transferred into the extractive by use of a medium polar organic solvent such as chloroform.

The conductivity of the background of final sample solution was higher than that of an unextracted metformin solution. The FASS efficiency was reduced, but it was still satisfactory for the determination.

3.2.2. Recovery

Generally, the recovery of a solute from human plasma is evaluated by comparing the peak area of the test solution which the solute was extracted from spiked plasma with that of the control solution which a standard solution was the same concentration of the test solution. It was not acceptable for this study to use this control solution because its background had not the same conductivity as that of the test solution. For FASS injection of CE analysis, there was a significant difference between the peak areas of the two solutions with the same concentration but different background, or in other words, differing conductivity of the two solutions. The higher the conductivity, the less the peak area. So it is important to maintain the same background matrix of the test solution and of the control solution to obtain a correct value of recovery.

The recovery of metformin from human plasma was evaluated by comparing the peak areas of the analytes in the recovery test samples and in the control samples. The recovery test samples were prepared by spiking control human plasma samples with metformin at three levels of 0.25, 1, 2 μg/ml (n=5 for each level). These samples were extracted as described in Section 2.4. The control samples were prepared by spiking a set of 0.1 ml of blank human plasma samples in plastic tubes, adding 0.3 ml of acetonitrile and vortex mixing and centrifuging at 12 000 g (15 000 rpm) for 1 min. The supernatant was transferred to another plastic tube and then was evaporated to dryness under vacuum at 70°C. Once the control samples were dried, appropriate amounts of analytes were added (same levels as the recovery test samples) and the final volume was made up to 100 μl with 200 μM H_3PO_4. Both test samples and the control samples were analyzed by CE. The results are presented in Table 1.

3.3. Precision and accuracy of the method

The intra-day and inter-day precision and accuracy study was carried out by analyzing quality control samples prepared by spiking control human plasma samples with standard solution of metformin at three level of 0.25, 1, 2 μg/ml. The samples were stored at −20°C. Intra-day precision and accuracy was carried out by analyzing a set of samples (n=5) at each of the three levels. An inter-day precision and accuracy study was carried out by analyzing three samples at each level on five days. These results are presented in Table 1. From these data, it is considered that metformin is stable in plasma at least for five days.

3.4. Application

Metformin hydrochloride enteric-capsule and tablet were administered orally to eight healthy volunteers. Blood samples were taken at time points up to 24 h. Plasma samples were separated and stored at −20°C, then assayed as described above. Results showed that the concentration in plasma at maximum concentration time (C_{max}) and the area under curve (AUC) of the two dosage forms were similar (Fig. 4), but times of maximum concentration of the plasma (t_{max}) are slightly different. The t_{max} value of the enteric-capsule was 1.5 h later than that of the tablet dosage form.

Table 1
Assay recovery, precision an accuracy for determination of metformin in human plasma

Spiked concentration (μg/ml)	Recovery (mean±S.D.) (%)	Intra-day precision and accuracy (%)	Inter-day precision and accuracy (%)
0.25	80.24±15.6	98.52±11.9	92.69±12.4
1.00	67.44±6.02	101.4±3.09	101.7±4.57
2.00	58.97±4.83	97.62±4.33	95.12±4.94

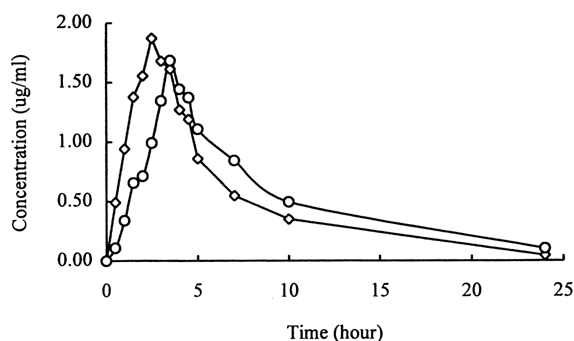

Fig. 4. Profile of human plasma metformin concentration vs. time after oral administration of 1 g of metformin hydrochloride enteric capsule and tablet to eight volunteers. Keys: (○) enteric-capsule, (◇) tablet.

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