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Journal of Chromatography B, 856 (2007) 337–342
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Sensitive determination of trimetazidine in spiked human plasma by HPLC with fluorescence detection after pre-column derivatization with 9-fluorenylmethyl chloroformate

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Received 25 January 2007; accepted 22 June 2007
Available online 6 July 2007

Abstract

A high-performance liquid chromatographic method for the determination of trimetazidine dihydrochloride (TMZ) in spiked human plasma is described. The method is based on the pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) using the fluorimetric detection technique. Fluoxetine HCl (FLX) was used as internal standard. Both, TMZ and FLX were completely derivatized after heating at 50 °C for 20 min in borate buffer pH 8.0. Samples were analyzed by high performance liquid chromatography (HPLC) using Zorbax-TMS column (250 mm × 4.6 mm, i.d., 5 μm) and mobile phase consist of acetonitrile, methanol and 20 mM sodium acetate pH 4.7 (44:6:50; v/v/v). Fluorescence detector (FLD) was adjusted at excitation and emission wavelengths; 265 and 311 nm, respectively. The linearity of the method was in the range of 4.5–200 ng/ml. Limits of detection (LOD) and quantification (LOQ) were 1.5 and 4.5 ng/ml, respectively. Trimetazidine recovery was 96.5 ± 1.3% ($n = 6$; RSD = 2.1%).

Keywords: Trimetazidine; Fluoxetine; FMOC; Plasma; Chromatography

1. Introduction

Trimetazidine [1-(2,3,4-trimethoxybenzyl)-piperazine dihydrochloride] regulates ionic and extracellular exchanges, corrects ischemia-induced abnormal flow of ions across the cell membrane, and prevents cellular edema caused by anoxia [1–4]. The modified release TMZ tablet is used for the management of long-term angina pectoris [5]. Derivatives of 9-fluorenylmethyl chloroformate (FMOC-Cl) have been widely used for HPLC determination of a large variety of amines [6] but not applied for TMZ or FLX. Courte and Bromet described an HPLC method for the determination of TMZ with fluorescence detection [7]. The method was based on derivatization of TMZ with dansyl chloride. The calibration range of dansylated TMZ was from 10 to 500 ng/ml plasma. Other reported methods for determination of TMZ in biological fluids included gas chromatography–mass spectrometry (GC–MS) [8] and HPLC with UV detection at 240 nm [9]. In addition, TMZ has been determined using spectrophotometric methods based on charge transfer complexes formation using, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ), 7,7,8,8-tetracyano-quinodimethane (TCNQ), *p*-chloranil, and complexation with iron(III) chloride [10,11]. Other reported methods have been adopted for the analysis of TMZ in bulk or pharmaceutical dosage forms which include voltammetry and HPLC with UV detection [12–15]. With the exception of the HPLC-fluorescence procedure [7], all other analytical methods do not confer enough sensitivity for monitoring low TMZ levels in plasma. The British Pharmacopoeia cited an HPLC method with UV detection at 240 nm for purity testing of TMZ in bulk form [16].

Therefore, the current work was designed to develop a highly sensitive and selective procedure for determining TMZ in plasma using 9-fluorenylmethyl chloroformate as a derivatizing reagent and subsequent measurement using HPLC with fluorescence detection.

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doi:10.1016/j.jchromb.2007.06.036

2. Experimental

2.1. Chemicals and reagents

All solvents were of HPLC grade, Merck, Darmstadt, Germany. All other materials were of analytical grade. 9-Fluorenylmethyl chloroformate (97%) purchased from Sigma–Aldrich, Germany. Trimetazidine dihydrochloride was obtained as a gift from Servier Laboratories, France. Fluoxetine HCl [*N*-methyl-γ-[4-(trifluoromethyl)phenoxy]benzenepropanamine] was obtained as a gift from Eli Lilly Company, Indianapolis, IN, USA. Blank drug-free human plasma was obtained from the internal plasma bank of King Abdulaziz University Hospital, Jeddah, Saudi Arabia.

2.2. Equipment

The HPLC system consisting of an Alliance Waters separations module 2695, waters 2996 Photodiode array detector, and Waters 2475 multi λ fluorescence detector (Milford, MA, USA) was used. Column heater was set to $30 \pm 2\,^{\circ}C$. HPLC system control and data processing was performed by Empower software (Build 1154, Waters). Screw capped V-shaped vials 300-μl, with PTFE liners were used (Alltech, GmbH, Unterhaching, Germany). Heating oven (Heraeus, Kendro, Hanau, Germany) was adjusted at 50 °C. Calibrated digital micro-transfer pipettes 5–250 μl, Brand, Wertheim, Germany was used.

2.3. Chromatographic conditions

Analytes were separated isocratically on a Agilant Zorbax-TMS column (4.6 mm i.d. × 25 cm, 5 μ particle diameter) protected with Agilent Zorbax TMS pre-column (Agilent Technologies, Palo Alto, CA). The mobile phase was a mixture of acetonitrile, methanol and 20 mM sodium acetate buffer pH 4.7 (44/6/50, v/v/v) and the flow rate was 1 ml/min. The fluorescence detector was set at 265 and 311 nm as excitation and emission wavelengths, respectively. The column was washed after each 10 repetitive injections with a mobile system consisting of acetonitrile:methanol:water (40:40:10, v/v/v) for 30 min.

2.4. Derivatization reagent, internal standard solution and buffer solution

Ten milligrams of 9-fluorenylmethyl chloroformate (FMOC-Cl) was accurately weighed into 10-ml volumetric flask, dissolved in about 5 ml acetonitrile, and diluted to the volume with acetonitrile. A volume of 0.50 ml from this solution was further diluted to 10-ml with acetonitrile and 50 μl from final diluted solution was used for derivatization.

Internal standard solution was prepared by dissolving 10 mg of fluoxetine HCl (FLX) in 100 ml water. One milliliter from this solution was further diluted to 100 ml to give a final concentration of 1 μg/ml. A volume of 50 μl from this solution was used as an internal standard.

Borate buffer pH 8.0 ± 0.1 was prepared by mixing 100 ml of a solution containing 1.238 g of boric acid, 1.490 g of potassium chloride and 8.0 ml of 0.2 M sodium hydroxide. This mixture was then diluted to 400 ml with water and the pH was checked by calibrated pH-meter.

2.5. Standard solutions and quality control samples

Trimetazidine dihydrochloride (TMZ) 0.4 mg/ml aqueous solution was prepared and used for calibration and quality control samples preparation. Appropriate dilutions in water were prepared from this stock solution to obtain calibration standards in the range of 90–4000 ng/ml and quality control samples of 4.5, 100 and 200 ng/ml.

Dilutions for calibration standards were prepared daily, while quality control (QC) samples were prepared in plasma, divided in small aliquots and stored at $-20\,^{\circ}C$ until use. A sample volume of 1 ml of QC sample was extracted and analyzed at time intervals of 0, 10 and 30 days using FLX as an internal standard.

2.6. General derivatization procedure

A volume of 50 μl from the TMZ standard solution and 50 μl the FLX solution were transferred to an autosampler glass vial (capacity = 300-μl) using digital micropipette. A volume of 150 μl borate buffer pH 8.0 and 50 μl FMOC-Cl solutions were added. The vial was capped, swirled, and left to stand in hot air oven at 50 °C for 20 min. The vial was then cooled, and a volume of 10 μl was injected for HPLC analysis.

Blank experiments were carried out to identify any underivatized TMZ or FLX by HPLC-FLD by using water instead of sample solution and acetonitrile instead of FLX or FMOC-Cl solution.

2.7. Calibration curves

2.7.1. Standard solutions for calibration curve

Six standard solutions of TMZ were prepared spanning the range 0.09–4.00 μg/ml. A volume of 50 μl from each TMZ concentration with 50 μl of FLX solution were derivatized as described under general derivatization procedure. A volume of 10 μl was injected for HPLC analysis. The percentage of peak area ratio of FMOC-TMZ to FMOC-FLX was plotted versus the TMZ concentration in pg/μl of the final dilution. The calibration curve was constructed using a least-square regression equation for the calculation of the slope, intercept and correlation coefficient. The calibration range was 15–667 ng/ml of the final injected solution.

2.7.2. Calibration curve of plasma extract

Six serial spiked plasma samples were prepared to contain TMZ spanning the range of 4.5–200 ng/ml. A volume of 1 ml from each spiked plasma sample was extracted and derivatized as described in Section 2.8. The calibration curve was produced by linear regression of percentage of peak-area ratios (FMOC-TMZ to FMOC-FLX) against their respective concentrations in picogram per microliter of the final dilution. The calibration curve was constructed using a least-square regression equation for the calculation of the slope, intercept and correlation

coefficient. The regression line was used to calculate the concentrations of TMZ in spiked plasma based on the percentage of peak-area ratios.

2.8. Extraction and derivatization of spiked plasma

A volume of 1 ml human plasma was transferred to 10-ml centrifuge tube and spiked with 50 μl TMZ standard solution (90–4000 ng/ml). This mixture was vortexed for 30 s, a volume of 100 μl 2 M sodium hydroxide was added, vortexed for 30 s, and extracted three times by vortexing each time with 5 ml of *n*-hexane-dichloromethane (3:1, v/v) for 15 min. The organic phases were separated, collected and evaporated under gentle stream of nitrogen gas. Before the last evaporation, 50 μl of FLX solution (internal standard, 1 ng/μl) was added and dried. The residue was reconstituted in 150 μl borate buffer pH 8.0, 100 μl water and 50 μl FMOC-Cl solutions. The tube was capped, vortexed for 2 min and left to stand in a hot air oven at 50 °C for 20 min. Then the tube content was cooled, transferred to 300 μl autosampler vial with pasture pipette and a volume of 10 μl was injected for HPLC analysis. Blank experiment was carried out using 1 ml control plasma spiked with 50 μl water instead of TMZ solution.

2.9. Calculations

The peaks eluted at 9.09 and 12.08 min are corresponding to derivatized fluoxetine (FMOC-FLX) and trimetazidine (FMOC-TMZ), respectively. The peak-area ratio of FMOC-TMZ to FMOC-FLX was used to calculate the amount of TMZ as ng/ml from the calibration curve. The calculated amount of TMZ was multiplied by 0.30 to get the concentration as ng/ml plasma, considering the percentage recovery factor of 100/96.5.

3. Results and discussion

The UV spectrum of TMZ in acidic pH is characterized by two λmax at 231 and 269 nm and A (1%, 1-cm) value of 424 [17]. This absorptivity does not confer adequate sensitivity for the determination of TMZ in biological fluids. It was necessary to develop a more sensitive procedure for determination of TMZ in plasma. The ratio of the response factor of derivatized TMZ monitored by HPLC-fluorescence to underivatized TMZ monitored by HPLC-UV was 7400.0 ± 5.2. The proposed reaction pathway via formation of the fluorescent reaction product is shown in Fig. 1.

3.1. Chromatographic variables

To obtain the best overall chromatographic conditions, the mobile phase was optimized by examining the effect of pH, content of acetonitrile or methanol, and the effect of counter ions (including tetrabutylammonium bromide, and *n*-hexanesulfonic acid sodium salt). Other chromatographic variables were investigated including column temperature, column type, flow rate and the setting of the gradient elution program. The optimal chromatographic conditions were achieved as described in the experimental part. The fluorescence response of TMZ was decreased or abolished upon using any of the above mentioned counter ions. Methanol was used to give better resolution and symmetric peaks.

In this study, a simple extraction method with *n*-hexane:dichloromethane (3:1, v/v) was used for sample preparation, since it gave the highest %recovery (96.5%) with minimal interfering peaks due to endogenous plasma substances. Initially, a trials of liquid–liquid extraction process was performed using chloroform, ethyl acetate, or ether after spiking and made alkaline with 0.1 ml 2 M NaOH. However, these trials were not reproducible, and the extraction recovery was less than 20%. Several protein precipitating solvents such as acetonitrile, methanol, or acetone were also tested for sample preparation. The results showed that deproteinization using acetonitrile gave poor recovery (64%) in addition to co-extraction of plasma endogenous substances. In the present study, fluoxetine hydrochloride was chosen as the internal standard because it is commercially available and it is quantitatively derivatized by

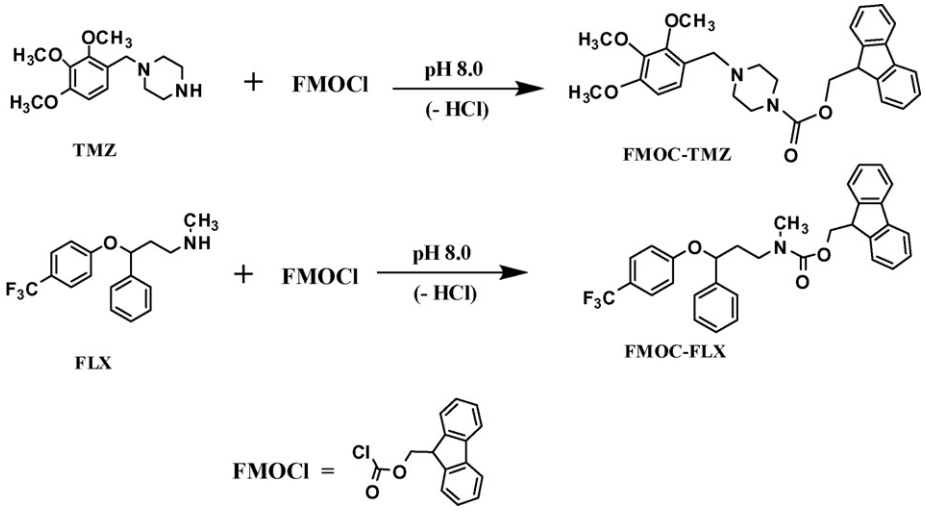

Fig. 1. Proposed reaction pathway of FMOC-Cl with both TMZ and FLX.

FMOC-Cl under the same conditions required for TMZ. More important, the derivatized FLX has the same excitation and emission fluorescence maxima, and has shown good resolution and good chromatographic profile with the applied chromatographic conditions. Two additional C18 columns were tried including; Agilent Zorbax-C18 Eclipse XDB (4.6 mm i.d. × 25 cm length, 5 μm particle diameter) and μ-Bondapak C18 column (3.9 mm i.d. × 300 mm length, 5 μm particle diameter). Upon using these columns, the derivatized FLX was early eluted at 3.5 min with far separation of TMZ at 12.0 min. The endogenous plasma constituents were co-eluted with the investigated drug. Representative chromatograms of the extraction of blank plasma or spiked plasma are shown in Fig. 2 using Agilent-TMS C18 column. No interferences with endogenous substances were seen.

3.2. Optimization of derivatization procedure

The completeness of derivatization was investigated at different temperatures (25, 40, 50, 60, and 70 °C) and different reaction time. Both TMZ and FLX were completely derivatized in borate buffer pH 8.0 after heating at 50 °C for 10 min. Among borate buffer pH 7.5–9.0, no significant difference of peak area was observed, thus borate buffer pH 8.0 was selected. The same results were obtained upon using 150 μl or 200 μl of borate buffer solution with total volume of reaction mixture of 300 μl. The peak area of the derivatized TMZ and derivatized FLX reached a maximum after 10 min and tends to decrease after 40 min. Therefore, the derivatization time of 20 min was selected. The amount of derivatization reagent was also studied. The reagent concentration was investigated using spiked plasma. The amount of 2.5 μg of reagent was sufficient for complete derivatization of TMZ (≤0.5 μg), FLX (≤0.5 μg) and plasma extract. The underivatized TMZ or FLX was monitored by HPLC-UV at high concentration levels.

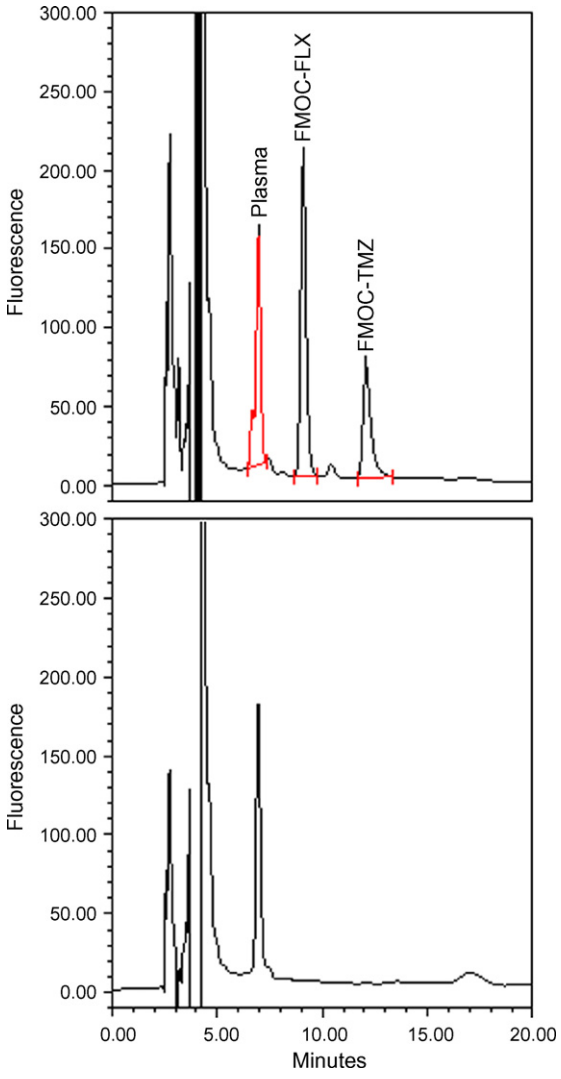

Fig. 2. Representative chromatograms of derivatized; TMZ-spiked plasma extract, 23 ng/ml (upper) and blank plasma (bottom).

3.3. Selectivity, precision, and performance parameters

The precision of %recoveries of TMZ and FLX from plasma were calculated from the corresponding calibration curve of each TMZ and FLX. The calculated %recoveries of TMZ and FLX from plasma were, 96.5.0 ± 1.3% ($n = 6$; RSD = 2.1%) and 100 ± 0.05% ($n = 6$; RSD = 0.02%), respectively.

The blank experiments carried out in aqueous and plasma extract solutions showed the peaks due to the derivatized biogenic materials and absence of any peaks at the retention time of interest. Typical chromatograms obtained from blank plasma and TMZ spiked plasma are shown (Fig. 2). This figure showed a complete separation of derivatized TMZ and FLX from reagent and endogenous plasma constituents. The chromatographic performance parameters of the TMZ and FLX are presented in Table 1.

3.4. Linearity and sensitivity

A linear fluorescence-HPLC response of peak areas of TMZ was observed over the range, 15–667 ng/ml of the injected solution which was equivalent to 4.5–200 ng/ml of drug spiked in

Table 1
Chromatographic parameters[a] of TMZ spiked in human plasma, 23 ng/ml, $n = 6$

Name	RT, min (RSD)	Area (RSD)	Width (min)	k'	R (RSD)	α	As (RSD)
Closest plasma peak	6.95 (1.30)	Not considered	0.30	2.47			Not considered
FMOC-FLX	9.09 (0.01)	3450930 (0.55)	0.56	3.54	4.96 (0.01)	1.43	1.12 (0.02)
FMOC-TMZ	12.08 (0.05)	1769835 (0.15)	0.60	5.04	5.16 (0.01)	1.42	1.23 (0.11)

[a] k', capacity factor; α, selectivity coefficient; R, resolution; and As, peak asymmetry.

Table 2
Within and between day precision and accuracy for determination of TMZ in spiked plasma

Nominal concentration (ng/ml)	Within-day			Between-day		
	Observed concentration[a] (ng/ml)	CV[b] (%)	Relative error (%)	Observed concentration[a] (ng/ml)	CV[b] (%)	Relative error (%)
4.5	4.46 (0.05)	1.1	−0.8	4.47 (0.08)	1.8	−0.7
100	99.12 (0.12)	0.1	−0.9	98.80 (0.16)	0.2	−1.2
200	198.00 (1.32)	0.7	−1.0	197.6 (1.44)	0.7	−1.2

[a] Mean (standard deviation), $n = 5$.
[b] CV, coefficient of variation.

plasma. The squared regression coefficient was 0.9995, and values of the slope and intercept were 0.2425 and 0.02, respectively. The response factor of TMZ was 22290 peak-area unit per each one pictogram injected. The lower limit of quantitation (LOQ) was estimated by satisfying two criteria: the S/N ratio was not less than 10 and the percent relative standard deviation (%RSD) of five replicate injections of the LOQ solution was less than 6%. LOQ of TMZ was 4.5 ± 0.02 ng/ml, and the limit of detection (LOD) value was 1.5 ± 0.21 ng/ml.

3.5. Precision and accuracy

Within- and between-day precision and accuracy were evaluated by analyzing five replicates of quality control samples at three different concentrations of TMZ (Table 2). Precision was expressed as the coefficient of variation, though accuracy was presented as a relative percentage error = [(observed concentration − nominal concentration)/nominal concentration] × 100 (%). Within- and between-day relative standard deviations were less than 1.1% and 1.8%, respectively. Accuracy was within 1.5% when compared with nominal concentrations. The results indicate that the method is reliable, reproducible, and accurate. The system suitability solution containing both FMOC-TMZ and FMOC-FLX was analyzed after standing time of 0, 2, 5, and 10 h, in autosampler. The data of the derivatized products revealed enough precision and stability. The relative %errors of the peak areas (last injected sample to the first one) were negligible. Also, the chromatographic data showed no further foreign peaks.

3.6. Recovery and stability

Recovery was calculated by comparing the peak areas obtained from the quality control (QC) samples to those from the standard solutions containing the same amount of TMZ. The mean recoveries of TMZ from plasma performed at three representative concentrations of 4.5, 100.0, and 200.0 ng/ml were 94.6% [relative standard deviation (RSD) = 1.7%], 96.7% (RSD = 1.2%), and 98.1% (RSD = 1.0%), respectively. The stability of TMZ in human plasma was investigated through three freeze–thaw cycles of the QC samples during the storing period of 1 month at −20 °C. TMZ was considered to be stable in human plasma after three freeze–thaw cycles. The mean concentrations following this storage period were 100.1% (RSD = 1.1%), 96.5% (RSD = 2.0%), and 97.4% (RSD = 2.6%) of TMZ concentrations in freshly prepared samples, respectively. Results of the stability experiments indicated that TMZ in the plasma samples was stable for at least 1 month when stored at −20 °C.

4. Conclusion

The described HPLC method is simple, specific, and sufficiently sensitive for the analysis of TMZ in human plasma after derivatization with 9-fluorenylmethyl chloroformate. The simple extraction process for sample preparation and HPLC analyses with only a 15-min run time enhanced the efficiency of the procedure. The developed method is also characterized by adequate selectivity, enough %recovery and precision.

Acknowledgement

We would like to thank the pharmaceutical companies (Servier Laboratories, Eli Lilly Company) for supplying the reference analytes.

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