DRUG FORMULATIONS AND CLINICAL METHODS

Nonextractive Procedure and Precolumn Derivatization with 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole for Trace Determination of Trimetazidine in Plasma by High-Performance Liquid Chromatography with Fluorescence Detection

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A highly sensitive high-performance liquid chromatographic method with fluorescence detection has been developed and validated in a single laboratory for the trace determination of trimetazidine (TMZ) in human plasma. Fluoxetine (FLX) was used as the internal standard. TMZ and FLX were isolated from plasma by protein precipitation with acetonitrile and derivatized by heating with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole in pH 8 borate buffer at 70°C for 30 min. Separations were performed in the isocratic mode on a Nucleosil CN column with the mobile phase acetonitrile–10 mM sodium acetate buffer (pH 3.5)–methanol (47 + 47 + 6, v/v/v) at a flow rate of 1.0 mL/min. The derivatized samples were excited at 470 nm and monitored at an emission wavelength of 530 nm. Under the optimum chromatographic conditions, a linear relationship with a good correlation coefficient (r = 0.9997, n = 5) was obtained for the peak area ratio of TMZ to FLX and for TMZ concentrations of 1–120 ng/mL. The proposed method has the lowest limits of detection and quantitation reported to date for the determination of TMZ in plasma with values of 0.3 and 0.95 ng/mL, respectively. The values for intra- and interassay precision were satisfactory; the relative standard deviations were ≤4.04%. The accuracy of the method was demonstrated; the recoveries of TMZ from spiked human plasma were 98.13–102.83 ± 0.2–4.04%. The method has high throughput because of its simple sample preparation procedure and short run time (<10 min). The results demonstrated that the proposed method would have great value when applied in pharmacokinetic studies for TMZ.

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Trimetazidine (TMZ), 1-[(2,3,4-trimethoxyphenyl) methyl]piperazine, is a clinically effective antianginal agent (1). In several studies, TMZ was shown to improve the ergometric exercise capacity and total work output of patients with effort angina (2, 3), reduce attack frequency in patients with chronic stable angina (4), and increase effort tolerance in angina patients (5). The antianginal efficacy of TMZ is comparable to that of propranolol, but TMZ does not reduce cardiac rate-pressure product or coronary blood flow (6). In addition, the therapeutic efficacy of TMZ is comparable to that of nifedipine, but TMZ is devoid of vasodilator activity and alteration of hemodynamic parameters (7). In combination with diltiazem, synergestic antianginal effects were demonstrated (8). Modified-release TMZ tablets are used for the treatment of long-term angina pectoris (9). Because of these clinical successes, TMZ has become unique among the antianginal agents, and it has been clinically used throughout many countries worldwide (10, 11).

Analytical methods for the determination of TMZ in pharmaceutical formulations include spectrophotometry (12–15), spectrofluorometry (16), adsorptive stripping voltammetry (17), high-performance thin-layer chromatography (18), and high-performance liquid chromatography (HPLC; 19). These methods do not have adequate sensitivity for measuring TMZ in plasma. The first attempt to determine TMZ in plasma was made by using HPLC coupled with fluorescence detection after derivatization with dansyl chloride (20). The calibration range of dansylated TMZ was 10–500 ng/mL plasma. Gas chromatographic determination of TMZ in plasma was achieved after its liquid–liquid extraction and subsequent derivatization with *N*-(*t*-butylsilyl)-*N*-methyltrifluoroacetamide (21). HPLC with UV detection was successfully used for quantitation of TMZ in blood and urine after its isolation on Toxi-tubes (22). HPLC with electrochemical detection was described as an alternative for sensitive determination of TMZ in plasma (23). A chemiluminometric method was proposed for determination of TMZ in biological fluids, based on its reaction with potassium permanganate (24). LC/mass spectrometry (LC/MS) was reported for quantitation of TMZ in plasma samples (25). The sample preparation procedures involved liquid–liquid extraction with

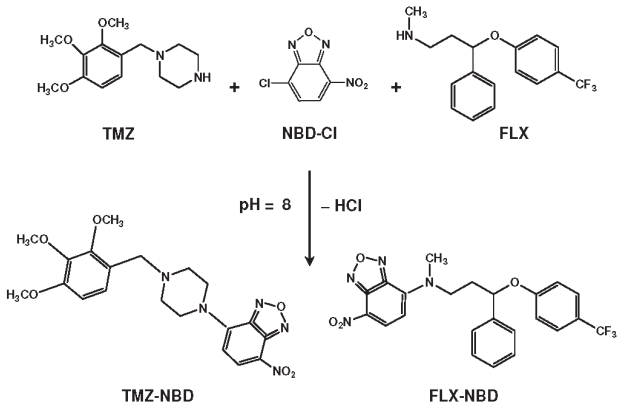

Figure 1. Scheme for the reaction of trimetazidine (TMZ) and fluoxetine (FLX) with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD–Cl).

hexane–dichloromethane followed by derivatization with acetic anhydride. The acetylated TMZ was back-extracted into an aqueous medium before its injection into the LC system. In a subsequent work, Ding et al. (26) described a LC/electrospray ionization-MS (LC/ESI-MS) method for quantifying plasma levels of TMZ without prederivatization after extraction of TMZ from plasma with cyclohexane–diethyl ether. Some researchers (25, 26) claimed high throughput for their methods but these methods were still handicapped with the multiple extraction procedures involved in pretreatment of the samples. Therefore, the overall analytical procedures were time-consuming. Recently, LC/atmospheric pressure chemical ionization tandem mass spectrometry (LC/APCI-MS/MS) with nonextractive pretreatment procedures was used to determine TMZ in plasma samples (27). However, the cost and complexity of MS/MS instrumentation limit the availability of these instrument systems in clinical laboratories. The LC/APCI-MS/MS assay is highly sensitive with a limit of quantitation (LOQ) of 1.5 ng/mL; however, some pharmacokinetic studies showed that some of the human plasma levels of TMZ in the terminal elimination phase were <1.5 ng/mL (26). For these reasons, the development of a more sensitive method with a simple pretreatment procedure was required for proper evaluation of the pharmacokinetics of TMZ in humans.

Fluorescence-based HPLC has been used as a sensitive and less costly alternative to LC/MS. The previously reported HPLC-fluorescence methods for detection of TMZ (20, 28) were not adequate for determination of TMZ in its initial and elimination pharmacokinetic phases. In addition, sample preparation was cumbersome, involving a liquid–liquid extraction before analysis. The present work describes the development and validation of a highly sensitive HPLC method with fluorescence detection with an LOQ of 0.95 ng/mL. The method involves a very simple nonextractive isolation of TMZ from plasma samples by using protein precipitation with acetonitrile followed by derivatization with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD–Cl). The method was successfully applied to the determination of TMZ in spiked human plasma samples.

Experimental

Chromatographic System

The HPLC apparatus consisted of a Shimadzu system (Shimadzu Corp., Kyoto, Japan) equipped with an LC-10AD VP pump with an FCV-10AL VP low-pressure flow-control valve, an SCL-10A VP system controller, a Rheodyne 7725 injection valve with a 20 μL loop, SPD-10A VP, a UV-Vis detector, and an RF-10A XL fluorescence detector. The chromatographic separations were performed on a Nucleosil CN analytical column (250 × 3.9 mm id, 5 μm particle diameter) manufactured by Macherey-Nagel GmbH (Düren, Germany). The column temperature was kept constant at 25 ± 2°C. Separations were performed in the isocratic mode. The mobile phase consisted of acetonitrile–10 mM sodium acetate buffer (adjusted to pH 3.5 with 100% acetic acid)–methanol (47 + 47 + 6, v/v/v). The mobile phase was filtered by a Millipore vacuum filter system equipped with a 0.45 μm filter and degassed by an ultrasonic bath and by bubbling helium gas. The flow rate was 1.0 mL/min. The sample injection volume was 20 μL. The fluorescence detector was set at 470 nm as the excitation wavelength and 530 nm as the emission wavelength. System control and data acquisition were performed by Shimadzu CLASS-VP software, Version 5.032. The ratio of the peak area of TMZ to that of the internal standard (IS), fluoxetine (FLX), was used for quantitation.

Chemicals and Materials

TMZ was obtained as a gift from Servier Laboratories (Orléans, France). FLX was obtained as a gift from Eli Lilly and Co. (Indianapolis, IN). NBD–Cl was purchased from Sigma Chemical Co. (St. Louis, MO). Human plasma samples were collected from a normal healthy volunteer at King

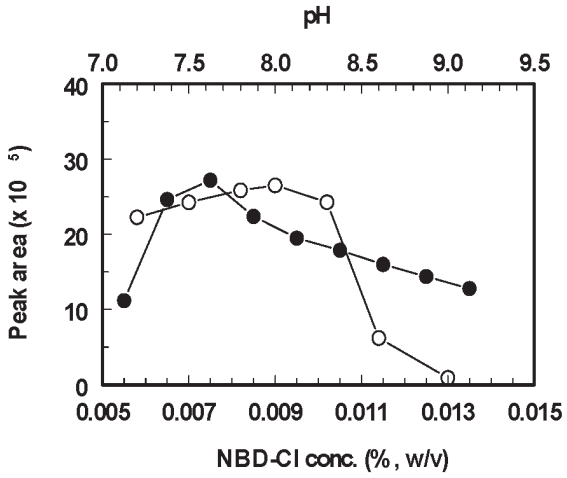

Figure 2. Effect of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD–Cl) concentration (●) and pH (○) on the derivatization of trimetazidine with NBD–Cl.

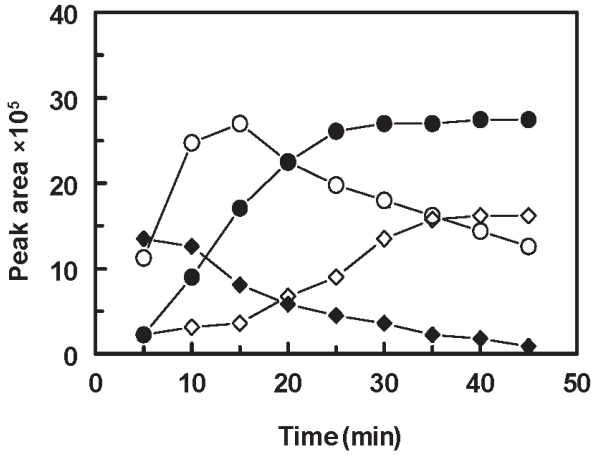

Figure 3. Effect of temperature and heating time on the derivatization of trimetazidine with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD–Cl). The investigated temperatures (°C) were 60 (◇), 70 (●), 80 (○), and 90 (◆).

Khalid University Hospital (Riyadh, Saudi Arabia), and they were stored in a freezer at –20°C until analysis. All solvents were HPLC grade (Merck, Darmstadt, Germany). All other materials were analytical grade.

Preparation of Solutions

(**a**) *TMZ standard solution.*—An accurately weighed amount (25 mg) of TMZ was quantitatively transferred to a 50 mL calibrated flask and dissolved in 30 mL distilled water; the contents of the flask were diluted to volume with the same solvent to produce a stock solution of 0.5 mg/mL. This stock solution was further diluted with water to obtain a working standard solution of 200 ng/mL.

(**b**) *FLX IS solution.*—An accurately weighed amount of FLX (25 mg) was quantitatively transferred to a 50 mL calibrated flask and dissolved in 30 mL distilled water; the contents of the flask were diluted to volume with the same solvent to produce a stock solution of 0.5 mg/mL. This stock solution was further diluted with water to obtain a working standard solution of 2.5 μg/mL.

(**c**) *NBD–Cl derivatizing reagent.*—An accurately weighed amount of NBD–Cl (25 mg) was quantitatively transferred to a 50 mL calibrated flask and dissolved in 5 mL methanol; the contents of the flask were diluted to volume with the same solvent to produce a stock solution of 0.05% (w/v). The solution was freshly prepared daily and protected from light during use.

(**d**) *Borate buffer solution.*—Accurately weighed amounts of 1.238 g boric acid and 1.490 g potassium chloride were dissolved in 100 mL distilled water. A volume of 8.0 mL 0.2 M NaOH was added, and the mixture was diluted to 400 mL with distilled water. The pH of the solution was adjusted to 8 ± 0.1 by using a calibrated pH meter (Microprocessor pH meter BT-500, Boeco, Germany).

General Derivatization Procedure

TMZ standard solution (400 μL) and FLX solution (400 μL) were transferred to a screw-cap reaction tube by using a micropipet. A volume of 800 μL borate buffer, pH 8.0, and 300 μL NBD–Cl solution were added. The tube was capped, the contents were swirled, and the tube was left to stand in a thermostatically controlled water bath (MLW type, Memmert GmbH Co., Schwabach, Germany) at 70°C for 30 min. The tube was cooled rapidly, and a volume of 100 μL HCl was added. A 20 μL aliquot of the resulting solution was injected into the HPLC system.

Sample Preparation and Construction of Calibration Graph

The calibration standard samples were prepared by spiking blank human plasma with TMZ to yield final concentrations of 1, 2, 4, 10, 20, 40, 80, 100, and 120 ng/mL. A 1 mL aliquot of each sample was mixed with an equal volume of acetonitrile, and the mixture was mixed on a Vortex mixer for 30 s and centrifuged for 20 min at 13 000 rpm by a Biofuge Pico centrifuge (Heraeus Instruments, Langenselbold, Germany). The supernatants were isolated and manipulated as described under the general derivatization procedure, and 20 μL was injected into the HPLC system. An 8-point calibration graph was constructed by plotting the peak area ratio of TMZ to FLX versus TMZ concentration (x). Analysis of calibration samples at each concentration was performed in triplicate. Slope, intercept, and correlation coefficient (r) were calculated as regression parameters by linear regression. The linear regression equation was used to calculate the concentrations of TMZ in spiked plasma, based on ratios of their peak areas.

Quality Control Samples

The quality control (QC) plasma samples analyzed for determination of the accuracy and precision of the method were independently prepared at low (2 ng/mL), medium (40 ng/mL), and high (100 ng/mL) concentrations in the same manner as the calibration standards and stored at –20°C until use. A 1 mL aliquot was thawed and analyzed at 3 time intervals (0, 8, and 21 days), with FLX as the IS. The system suitability parameters were evaluated each working day.

Results and Discussion

Design and Strategy for Assay Development

TMZ contains a weakly absorbing chromphore in its chemical structure; its absorbance (1%, 1 cm) value is 424 at 231 nm (29). This weak absorptivity does not confer adequate sensitivity for the determination of TMZ in plasma without preconcentration of the samples, particularly at the initial and elimination pharmacokinetic phases (22). Therefore, a prederivatization procedure was necessary to enhance the sensitivity. NBD–Cl is an activated halide derivative that has been used as a fluorogenic reagent for the determination of amines (30–32). In a previous study, Darwish (15) demonstrated the reactivity of NBD–Cl with TMZ via its

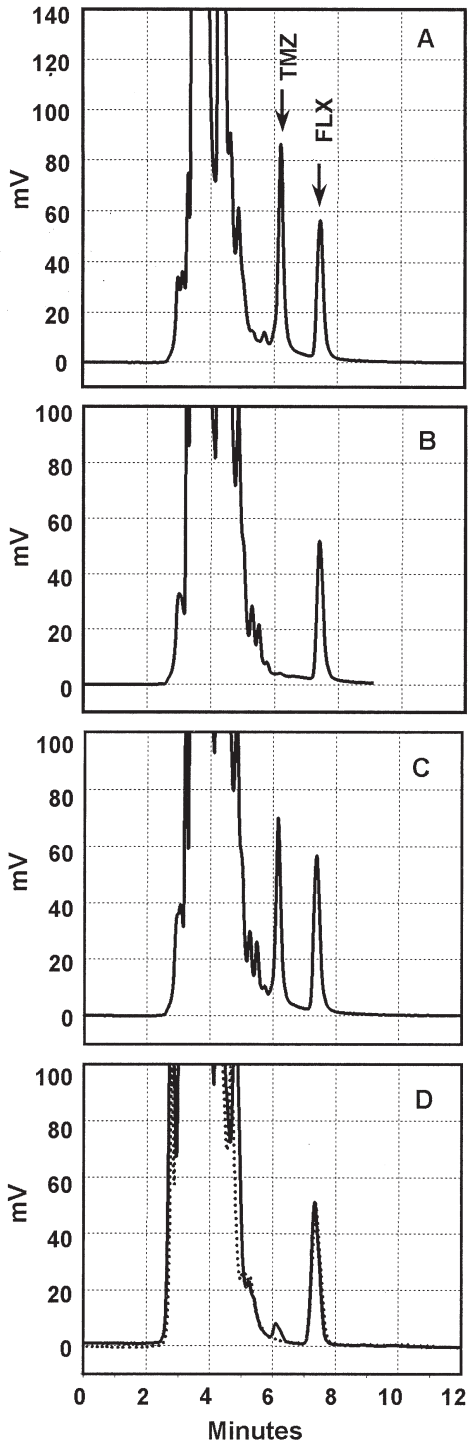

Figure 4. Representative chromatograms for (A) standard solution of trimetazidine (TMZ; 25 ng/mL) and fluoxetine (FLX; internal standard, 0.5 μg/mL), (B) blank human plasma with FLX (0.5 μg/mL), (C) plasma spiked with TMZ (25 ng/mL) and FLX (0.5 μg/mL), and (D) blank plasma (dotted line) and the same plasma sample spiked with TMZ (1 ng/mL) and FLX (0.5 μg/mL; solid line); mV is the detector response in millivolts.

secondary amino group (N–H of piperazine); the scheme for the reaction is given in Figure 1. The excitation and emission spectra of the TMZ–NBD–Cl derivative were obtained with an RF-5301 PC spectrofluorometer (Shimadzu Corp.); the derivative was found to be fluorescent and to exhibit the highest fluorescence intensity at λ_{ex} of 470 nm and λ_{em} of 530 nm. The present study was undertaken to use this reaction for precolumn derivatization in the development of a sensitive HPLC method with fluorescence detection for the determination of TMZ in plasma. In our preliminary experiments, derivatized and underivatized TMZ were concurrently monitored by fluorescence (at λ_{ex} of 470 nm and λ_{em} of 530 nm) and UV detectors (at 225 nm). It was found that the ratio of the response factor of derivatized TMZ to that of the underivatized fraction was ~150 000. The following sections describe the optimization of the experimental assay variables and validation of the assay performance.

Method Development

(**a**) *Optimization of derivatization procedure.*—Our experiments for optimization of the derivatization procedure were initiated by using acetonitrile–10 mM sodium acetate buffer, pH 3.5 (50 + 50, v/v) as the mobile phase. Because of the presence of labile chloride in the chemical structure of NBD–Cl, daily preparation of a fresh solution is recommended. Optimum conditions of the reaction with respect to concentration of the NBD–Cl reagent, pH of the reaction medium, type of buffer used, temperature, heating time, and diluting solvent were investigated. The choice of conditions and range of investigation were based on our cumulative experience and previous investigations described by our laboratory for derivatization with NBD–Cl (33–35). In order to select the optimum NBD–Cl concentration, the reaction was performed by using various concentrations (0.01–2%, w/v) of NBD–Cl reagent. The maximum detector signal was obtained when the concentration of NBD–Cl reagent in the reaction solution was 0.0075%, w/v (300 μL 0.05%; Figure 2); therefore, this concentration was considered optimum and used in all subsequent experiments. In order to generate the nucleophile from TMZ, the reaction should be performed in an alkaline medium. The dependence of the reaction on the pH of the reaction medium was studied in the pH range of 7.2–9 by using buffer solutions at these pH values. The results indicated that the maximum detector signal was obtained at pH 8 ± 0.1 (Figure 2). Different buffer systems (borate, phosphate, and carbonate) at pH 8 were tested. The highest signals were obtained when the reaction was performed with borate buffer. With carbonate buffer, precipitation of white colloid occurred upon addition of NBD–Cl reagent solution. This was attributed to the weak solubility of the inorganic buffer components in the organic solvent. With phosphate buffer, nonreproducible results and/or weak sensitivities were observed. Studies for optimization of the ionic strength of the borate buffer revealed that the optimum concentration was 0.2 M. To determine the temperature and time required for completion of the reaction, the derivatization reaction

Table 1. Chromatographic parameters of trimetazidine (TMZ) added to human plasma

Parameter	Value
Retention time of TMZ, min	6.14 ± 0.08
Retention time of IS[a], fluoxetine, min	7.32 ± 0.13
Capacity factor of TMZ, K′, min	1.047
Resolution, TMZ and IS peaks	1.9549
Peak asymmetry	1.072
Number of theoretical plates	1412
Height equivalent to theoretical plate, μm	177.054

[a] IS = Internal standard.

was performed at various temperatures, and the induced signals were monitored at different time intervals. The results indicated that the reaction was dependent on temperature, and complete reactions were achieved after 15 and 30 min at 80 and 70°C, respectively (Figure 3). The signals at 80°C were not reproducible; thus further experiments were performed at 70°C. As the results show (Figure 3), the maximum fluorescence intensities of the final solutions at 90°C were lower than those obtained at lower temperatures, and a rapid progressive decrease in the readings was observed as the reaction time increased. This was attributed to the degradation of the reagent at high temperatures. This observation was coincident with the results reported by Aktas et al. (36). Under these conditions, significantly high fluorescence background was also observed. This was attributed to the hydrolysis of NBD–Cl to the corresponding hydroxy derivative, namely, 7-hydroxy-4-nitrobenzo-2-oxa-1,3-diazole (NBD–OH; 37). The fluorescence of NBD–OH was quenched by decreasing the pH of the reaction medium to <1 (38). Therefore, acidification of the reaction mixture before its injection into the HPLC system was necessary to remarkably decrease the background signal; the reaction product was not affected, and thus the sensitivity was ultimately increased. The amount of HCl required for acidification was found to be 0.1 mL per 2 mL reaction volume.

(**b**) *Optimization of chromatographic conditions.*—Separation was achieved in the isocratic mode. The separation of TMZ and FLX could be adjusted by changing the composition of the mobile phase. Several experiments have shown that the use of an appropriate ratio of sodium acetate buffer solution in the mobile phase may improve the chromatographic peaks (39–41). Reciprocal variations of sodium acetate and acetonitrile contents in the mobile phase from 40 + 60 to 64 + 36 (v/v) were used. These proportions produced retention times of 5.2–6.7 and 5.5–10.7 min for TMZ and FLX, respectively. Both TMZ and FLX were retained longer on the column as the aqueous content of the mobile phase increased; however, FLX was affected more than TMZ. The best resolution was achieved with aqueous sodium acetate–acetonitrile was (47 + 53, v/v). It was found that the addition of methanol in the mobile phase (at 6% on expense of acetonitrile) gave better resolution and symmetric peaks. Different concentrations of sodium acetate buffer solution at 10, 20, and 30 mM were tested in the mobile phase. The results showed that the lowest concentration of 10 mM sodium acetate buffer was able to improve the shapes of the chromatographic peaks of both TMZ and FLX, resulting in more symmetric peaks. The acidic pH of the mobile phase was beneficial in reducing the significantly high fluorescence background as mentioned above. In the present study, FLX was chosen as the IS because it is quantitatively derivatized by NBD–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 a good chromatographic profile under the chromatographic conditions applied. Another reversed-phase column was tested: a μ-Novapak C_{18} column, 150 mm × 3.9 mm id, 5 μm particle diameter (Waters, Milford, MA). With this column and the optimized mobile system, acetonitrile–10 mM sodium acetate (pH 3.5)–methanol (47 + 47 + 6, v/v/v), TMZ was eluted from the column at 4.4 min; however, FLX was not eluted within the run time (10 min). Therefore, the Nucleosil CN column was selected for all subsequent work. Under these chromatographic conditions, the run time for analysis of the sample was 10 min, and the retention times were 6.14 ± 0.08 and 7.32 ± 0.13 min (n = 5) for TMZ and FLX, respectively (Figure 4A).

Method Validation

(**a**) *Selectivity, linearity, limit of detection (LOD), and LOQ.*—The selectivity of the method was evaluated by performing blank experiments with the mobile phase and different batches of blank human plasma to identify the reagent peaks and the peaks due to the derivatized biogenic plasma components. Typical chromatograms obtained for blank plasma (containing FLX at 0.5 μg/mL) and plasma spiked with the same concentration of FLX and with TMZ (25 ng/mL) are shown in Figure 4, B and C, respectively. The chromatogram showed complete separation of TMZ and FLX from the reagent and endogenous plasma constituents. The chromatographic performance parameters of TMZ and FLX are shown in Table 1.

Under the above optimum conditions, a linear relationship with a good correlation coefficient (r = 0.9997, n = 5) was found between the peak area ratio of TMZ to FLX (Y) versus TMZ concentration (X) in the range of 1–120 ng/mL. The response factor of TMZ was 390 000 ± 5% peak area unit per each 1 ng/mL injected. The mean regression equation [mean ± standard deviation (SD), n = 5] of the calibration graph obtained from 8 points was Y = 0.0034 (± 0.0014) + 0.0146 (±0.0003) X. The relative standard deviation (RSD) for the slopes of the calibration graphs was 1.93% (n = 5). The LOD and LOQ were calculated according to the International Conference on Harmonization guidelines for validation of analytical procedures based on the SD of the response and the

Table 2. Accuracy for the determination of trimetazidine in spiked human plasma

	Intra-assay		Interassay	
Nominal concn, ng/mL	Measured concn, ng/mL	Recovery ± RSD, %	Measured concn, ng/mL	Recovery ± RSD, %
10	9.83	98.32 ± 2.82	9.24	92.39 ± 4.04
20	19.81	99.05 ± 0.98	20.57	102.83 ± 1.99
30	30.57	101.89 ± 2.37	30.02	100.07 ± 3.01
40	39.92	99.81 ± 0.22	40.50	101.25 ± 2.77
50	50.27	100.55 ± 1.25	49.07	98.13 ± 1.45
80	79.66	99.57 ± 0.33	79.75	99.69 ± 0.20
100	99.69	99.69 ± 0.23	99.87	99.87 ± 0.25

slope of the calibration graph (42) as follows: LOD or LOQ = $\kappa\sigma/S$, where κ = 3.3 for LOD and 10 for LOQ, σ is the SD of the response, and S is the slope of the calibration graph. In calculations for 5 replicate experimental injections, the LOD and LOQ were 0.3 and 0.95 ng/mL, respectively, and the RSD values were ≤2%.

(**b**) *Precision.*—Intra-assay precision was studied at 3 concentration levels (2, 40, and 100 ng/mL) of TMZ and 0.5 at the μg/mL level for FLX. Six aliquots at each concentration level were injected into the HPLC system. The RSD of the ratios between the TMZ and FLX peak areas were 0.59, 0.45, and 0.2% for 2, 40, and 100 ng/mL, respectively. The RSD calculated for the values of the peak areas corresponding to the IS during the study was 5.77% (n = 18). The RSD values calculated for the retention times corresponding to the chromatographic peaks were 0.81 and 0.75% for TMZ and FLX, respectively.

Interassay precision was evaluated on 5 different days at the same concentration levels for spiked plasma samples freshly prepared daily. The RSD of the ratios between the TMZ and FLX peak areas were 4.39, 1.77, and 0.79% for 2, 40, and 100 ng/mL, respectively. The RSD values calculated for the retention times corresponding to the chromatographic peaks were 1.33 and 1.78% for TMZ and FLX, respectively.

(**c**) *Accuracy.*—Accuracy was determined by analyzing the quality control plasma samples spiked at different concentration levels (10–100 ng/mL) of TMZ and at the 0.5 μg/mL level of FLX. The values of the ratios between TMZ and FLX were then interpolated in the linear regression equation computed in the linearity study, to calculate the experimental concentration values. The accuracy was presented as a percent error (relative error), [(calculated concentration – nominal concentration)/nominal concentration × 100]. The accuracy ranged from 98.13 to 102.83% (± 0.2 to 4.04%; Table 2). These results indicated the accuracy of the method.

(**d**) *Robustness and ruggedness.*—In order to measure the extent of the robustness of the method, the most critical parameters were interchanged, while the other parameters were kept unchanged. The chromatographic parameters were interchanged within the range of 1–10% of the optimum recommended conditions. The parameters studied were the pH, the composition of the mobile phase, the percentage of methanol, the concentration and pH of the sodium acetate buffer, the pH of the derivatization reaction mixture, and the column temperature. The components of the chromatographic profile—including capacity factor (k′), retention time (RT), peak asymmetry, resolution, column efficiency, and peak width—were calculated and compared with those in Table 1. The results revealed that the method was robust with small changes in methanol content, concentration of sodium acetate in the mobile phase, and pH in the range of 3.2–3.7. With respect to the pH of the derivatization reaction, the results did not significantly change in the pH range of 7.8–8.2. However, increasing the pH value above 8.3 resulted in a dramatic decrease in the detector signal. Because increasing the column temperature generally decreased the k′ values, the column temperature must be maintained at 25 ± 5°C.

The ruggedness of the method was evaluated by applying the recommended analytical procedures to the same HPLC system by the 3 authors of the study (independently on different days) to the analysis of a series of TMZ samples. The RSD values for k′, RT, and peak area obtained by the 3 analysts were ≤2%.

(**e**) *Stability of TMZ in plasma.*—The stability of TMZ in plasma was studied under a variety of storage and handling conditions at low, medium, and high concentration levels (2, 40, and 100 ng/mL, respectively). Short-term temperature stability was assessed by analyzing 3 aliquots at each concentration level that were thawed at room temperature (25 ± 5°C) and kept at this temperature for 6 h. The freeze-thaw stability (–20°C in plasma) was checked through 3 cycles. Three aliquots at each concentration level were stored at –20°C for 24 h and thawed without assistance at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-thaw cycle was repeated 3 times, and the samples were then analyzed after the third cycle. The long-term stability was determined by analyzing 3 aliquots at each concentration level stored at –20°C for 30 days. The results revealed the stability

of TMZ under the tested conditions; the RSD values for the recovered TMZ were 0.38–3.64%.

The post-preparative stability of TMZ was tested by processing stock samples obtained from plasma samples spiked with TMZ at 40 ng/mL that were stored at room temperature on the bench top. The samples were assayed immediately and 1, 3, 6, 18, and 24 h after processing; the RSD values calculated for recovered TMZ were 0.25, 1.49, 0.78, 1.23, and 1.81%, respectively. These results indicated the stability of the derivatized TMZ and the suitability of the system for processing large numbers of samples in pharmacokinetic studies without negatively affecting the analytical results.

Conclusions

This paper describes the development of an HPLC method with fluorescence detection for trace determination of TMZ after its precolumn derivatization with NBD–Cl. The sample preparation procedure is very simple and robust because it does not involve the liquid–liquid extraction of TMZ. It is based only on protein precipitation with acetonitrile, followed by the derivatization reaction. The derivatized sample is directly injected into the HPLC system. The chromatographic separation is based on a reversed-phase mechanism performed with isocratic elution for a run time of only 10 min. The analytical results demonstrated that the proposed method is suitable for the accurate determination of TMZ in human plasma at concentrations as low as 0.95 ng/mL with a wide linear range. Compared with previously reported methods, this method provided the highest sensitivity in measuring very low concentrations of TMZ (with better accuracy and precision) in the initial and termination phases of its pharmacokinetic profile. The simple sample preparation procedure and the short run time added the advantage of higher throughput to the method. Although actual TMZ samples were not analyzed by the proposed method, the performance of the method indicated its applicability to combined pharmacokinetic studies and the bioavailability evaluation of TMZ in human subjects in the long elimination phase after oral administration of low therapeutic doses.

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