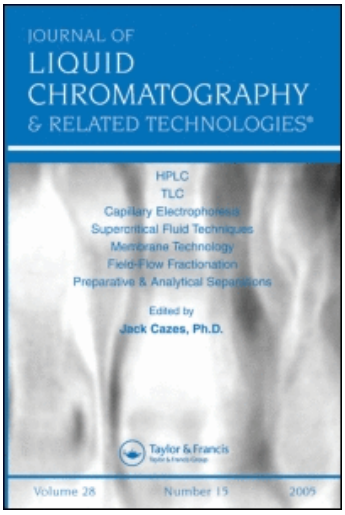

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An HPLC Determination of Trimetazidine in Human Plasma Using Liquid-Liquid Extraction for Sample Clean-Up

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Abstract: Trimetazidine dihydrochloride has been used as an antianginal drug that possess protective properties against ischemia-induced damage to heart. A simple and sensitive analytical method of trimetazidine dihydrochloride in human plasma by using high performance liquid chromatography (HPLC) was developed. The method employs a liquid-liquid extraction for isolation and sample concentration, followed by reversed-phase liquid chromatography (RPLC) analysis using ultraviolet (UV) detection at 207 nm. Analytes were extracted from plasma samples that previously were mixed with 300 μL saturated K_2CO_3 solution into an ethyl acetate phase. HPLC separation was accomplished at 40°C on a reversed-phase column using a mobile phase, 15% acetonitrile in 50 mM potassium dihydrogen phosphate and phosphoric acid (pH = 4.0), at a flow-rate of 1.0 mL/min. The linear range of the method was between 10- and 150 ng/mL of tirmetazidine dihydrochloride in human plasma and the quantification limit was 10 ng/mL. The intra- and inter-day relative standard deviation (RSD) were less than 7.6% and the accuracy was in the range of 98–107%. Extraction recoveries ranged from 71.5 to 84.6% and the C.V. values showed between 1.7 and 10.4% in the same concentration range. This method has been sought for carrying out pharmacokinetic studies and for assessing bioavailabiltiy. Such a method would be ideally suitable for pharmacokinetic studies in human volunteers after oral administration of different types of dosages of the drug.

Keywords: Trimetazidine dihydrochloride, Liquid-liquid extraction, HPLC, UV detection, Human plasma, Pharmacokinetics

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INTRODUCTION

Trimetazidine dihydrochloride (1-[2,3,4-trimethoxybenzyl]-piperazine dihydrochloride), the first known 3-ketoacyl coenzyme A thiolase inhibitor,[1] is an antianginal drug that has been used in ischemia-induced heart damage.[2] In recent years, many reports have appeared on the pharmacological mode of action for trimetazidine dihydrochloride. The drug exerts anti-ischemic properties without affecting myocardial oxygen consumption and blood supply,[3] protects erythrocytes against free oxygen radicals,[4] regulates high sodium and calcium levels occurring under ischemic conditions,[5,6] and, thus, has effects on patients with diabetes and ischemic cardiomyopathy.[7]

Recently, oral dosage forms have been rationally designed to have controlled release relative to their parent drug.[8,9] Thus, a simple but sensitive method that is available for the drug assay in human plasma is required for pharmacokinetic studies of the different dosage forms.

A variety of analytical methods for the estimation of trimetazidine dihydrochloride in biological fluids or pharmaceutical formulations have appeared. These include UV-visible spectrometry,[10] GC-MS,[11] GC-NPD,[9] adsorptive stripping square-wave voltammetry,[12] chemiluminogenic method,[13] and HPTLC.[14] But, currently available HPLC methods for the drug-assay in biological fluids are coupled with fluorescence[15] or ultraviolet (UV) detection.[8]

Trimetazidine has UV maxima at 205 nm and, hence, chromatographic separations of the analyte from polar interferences in human plasma are obviously not easy, because the endogenous materials are not completely removed by deproteination using solvents, or by other means. HPLC analysis using a fluorescence detector is performed after fluorescence labeling of the analyte. But, above-mentioned methods entail elaborate work-up procedures or use internal standards that are not easily obtainable.

To overcome these sample preparation constraints, major modifications were necessary in the clean-up procedure to permit analysis of human plasma. We used a liquid-liquid extraction method that requires minimal sample handling, providing a more sensitive and selective analysis. The method was optimized for trace determination of trimetazidine dihydrochloride in human plasma. The structures of tirmetazidine and lidocaine used as an internal standard are shown in Figure 1.

Trimetazidine was administered to eight healthy human volunteers at a single dose of two tablets (40 mg). Blood samples were drawn off over a 24 h period, before (time 0) and after administration at specific intervals, respectively. The plasma concentration of trimetazidine was determined by the present method and validated for the pharmacokinetic studies.

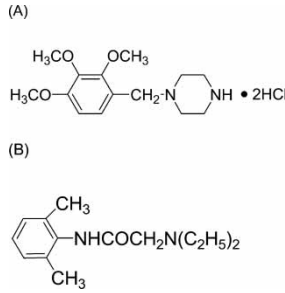

Figure 1. The structure of trimetazidine dihydrochroride (1-[2,3,4-trimethoxybenzyl]-piperazine dihydrochloride) (A) and lidocaine (2-(diethylamino)-*N*-(2,6-dimethyl phenyl) acetamide) (B).

EXPERIMENTAL

Materials

Trimetazidine dihydrochloride (99.0–101.0% purity) was supplied by the Boehringer-Ingelheim, Korea Pharmaceutical Co., LTD (Seoul, Korea). Lidocaine was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and ethyl acetate were of HPLC grade (Fisher Scientific Co., Fairlawn, NJ, USA). All other chemicals and reagents were of analytical-reagent grade and used without further purification. Water was passed through Milli-RO4 and Milli-Q water purification systems (Milipore Co., Bedford, USA). Mobile phase was filtered through a 0.2 μm membrane filter (Phenomenex, CA, USA) and ultrasonically degassed prior to use. Micro-tubes (2.0 mL) were purchased from Axygen (CA, USA).

Standard Solutions

Stock solutions of trimetazidine dihydrochloride (100 μg/mL) and lidocain (10 μg/mL) were prepared in methanol and stored at 4°C. Standard solutions were prepared by serially diluting the stock solutions with methanol to required concentrations before use. Calibration standards were prepared with drug-free human plasma, spiked with appropriate standard solutions that have the final concentrations; 0 (blank), 10, 20, 50, 100, and 150 ng/mL trimetzidine in plasma, respectively.

Sample Preparation

A 500 μL aliquot of human plasma was accurately measured into a 2-mL micro-tube with a teflon-lined cap, followed by the addition of 100 μL of

internal standard (lidocaine, 1 μg/mL) and 300 μL of saturated K_2CO_3 solution. The sample mixture was stirred for 5 min by means of a vortex mixer and then ethyl acetate (1 mL) was added to the sample mixture, followed by additional vortex mixing for 5 min and centrifuged at 5,000 × g for 1 min. The resulting supernatant liquid was transferred to another tube and evaporated to dryness using a speed-vacuum concentrator (Hanil Co. Ltd., Seoul, Korea). The residue was reconstituted in 200 μL of freshly prepared mobile phase and then, a 100-μL aliquot was injected onto the HPLC.

Chromatography

The HPLC system consisted of a Jasco PU-980 pump coupled with a Jasco UV-975 UV/VIS intelligent detector. The system was equipped with an L-7200 autosampler (Hitachi, Tokyo, Japan), a column heater (CH-150, Eldex Lab. Inc., CA, USA), and Ds-CHROM chromato-integrator (Donam instruments Inc., Seoul, Korea). Chromatographic separation was performed at 40°C on an analytical RP-column (Luna C18, 250 × 4.6-mm i.d., 5 μm, Phenomenex; Torrance, CA, USA), preceded by a guard column (4 cm × 3-mm i.d.) packed with the same packing material (Phenomenex; Torrance, CA, USA). Mobile phase consisted of 15% acetonitrile in 50 mM potassium dihydrogen phosphate (pH was adjusted to 4.0 by 1 M phosphoric acid), and was delivered at the flow rate of 1.0 mL/min.

Extraction Recoveries

Extraction recoveries of trimetazidine (0.1 μg/mL) from human plasma (500 μL) according to three different solvents (diethylether, ethylacetate, and acetonitrile), along with the spiked amount of alkaline salt (saturated K_2CO_3) were examined. Extraction was performed by the proposed method. The recovery was calculated by comparing the peak areas of the plasma spiked with standard with the ones from direct injection of 100 μL of trimetazidine dihydrochloride standard (100 ng/mL).

Method Validation

Batches consisting of five calibration standards at different concentrations were analyzed on three different days to complete the method validation. In each batch, QC samples at 10, 20, 50, 100, and 150 ng/mL were assayed in sets of four replicates to evaluate the intra- and inter-day precision and accuracy.

Pharmacokinetic Study

Pharmacokinetic studies were carried out on eight healthy male volunteers aged from 20 to 25 years. The protocol was approved by the Korean Food and Drug Administration (KFDA) beforehand, and the volunteers gave a formal written consent to participate in the study. A physical and biological examination was carried out before and after the completion of the experiment. Two tablets (40 mg) of trimetazidine dihydrochloride (Vastinan tab^{R}, Boehringer Ingelheim, Korea) were given after an overnight fasting with 200 mL of tap water. Food was allowed for 6 hr, 12 hr, 24 hr, and 36 hr after the drug intake. After each administration, blood samples were drawn into venoject heparin containing tubes just prior to and up to 36 hr after administration. After centrifugation at 9000 × g for 10 min at 4°C, the plasma was deep frozen at −70°C before beinghe assayed. A non-compartmental pharmacokinetic analysis was performed on plasma concentrations using PCNONLIN software. Levels lower than the quantification limit were taken as zero. Maximum plasma concentration (C_{max}) and the corresponding time to C_{max} (T_{max}) were obtained through direct observation of plasma concentration-time curves. The area under the plasma concentration-time curves from time zero to the time of the last quantifiable concentration ($AUC_{0\text{-last}}$) was calculated by the trapezoidal rule, whereas $AUC_{0\text{-}\infty}$ was calculated according to $AUC_{0\text{-}\infty} = AUC_{0\text{-last}} + C_{last}/k$, where k is the slope of the terminal phase of the plasma concentration curve using log-transformed concentrations and C_{last} is the last concentration higher than the quantification limit. Plasma half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k$.

RESULTS

Chromatography

Figure 2 shows representative chromatograms of a drug-free human plasma (A), a human plasma sample 2 hr after oral administration of two tablets of trimetazidine (95 ng/mL) (C), and human plasma spiked with trimetazidine (10 ng/mL) and internal standard (200 ng/mL) (B). The retention time of trimetazidine dihydrochloride and internal standard were 7.4 and 12.9 min, respectively, and the total run time was within 15 min. Trimetazidine dihydrochloride was identified by comparing its retention time, spectral data, and quantification from the calibration curve based on the peak area ratio of analyte to internal standard. The internal standard, lidocaine, was selected because it was well separated on the chromatogram not only from the drug but also from other extraneous peaks with the judicious choice of HPLC conditions. In addition, it is commercially available. The isocratic

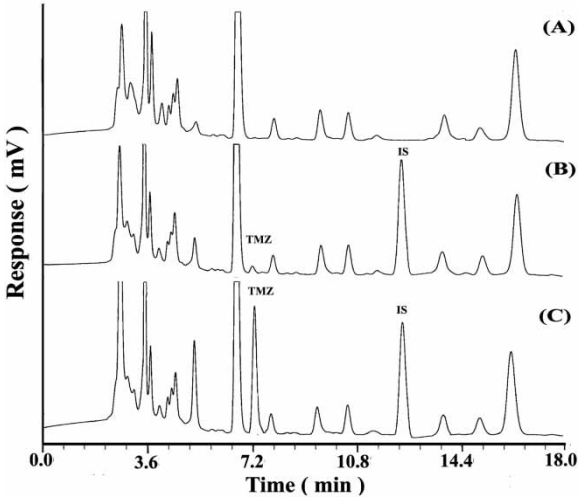

Figure 2. Typical chromatograms of a human plasma containing trimetazidine dihydrochloride and an internal standard (lidocain): (A) A drug-free human plasma (0.5 mL, blank plasma), (B) human plasma (0.5 mL) containing 0.01 μg/mL of trimetazidine dihydrochloride and 1.0 μg/mL of lidocaine, (C) human plasma (0.5 mL), 2 hr after oral administration of two tablets with 40 mg trimetazidine dihydrochloride (98.4 ng/mL).

elution with the mobile phase afforded base-line resolved separation of analyte and internal standard from different endogenous peaks in human plasma.

Extraction Recovery

Two factors affecting the recovery, extraction solvents, and the spiked amount of the alkaline salt (saturated K_2CO_3 solution) with plasma samples, were investigated with the same protocol and optimized for the clean-up procedure. As shown in Figure 3, the recovery was about 70% when acetonitrile was used to precipitate proteins from human plasma regards of amount of spiked salt; but several endogenous peaks appeared with UV 207 nm. Although, use of ethylacetate gave extremely low (~10%) recovery from plasma samples, the drug extraction improved depending on the amount of the alkaline spiked salt used. However, adding more than 300 μL of saturated K_2CO_3 solution formed an emulsion, which led to problems with layer separation. Consequently, solvent extraction of analytes, simultaneously with removal of endogenous interferences in human plasma by using ethylacetate, was efficiently achieved by adding 300 μL saturated K_2CO_3 solution to the plasma samples.

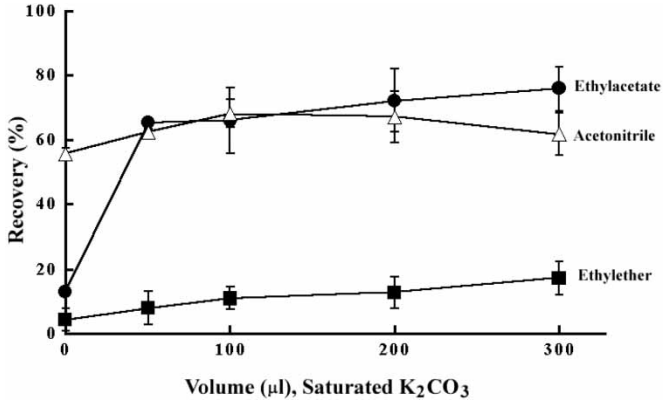

Figure 3. Recoveries of trimetazidine from human plasma depending on the solvents used for extraction together with the amount of sat. K_2CO_3 solution spiked with (n = 4).

Table 1 shows that extraction recoveries ranged from 71.5 to 84.6% with their C.V. values of 1.7–10.4% in four different concentrations from 10 to 150 ng/mL of trimetazidine dihydrochloride.

Linearity

The calibration curve constructed for the determination of the drug exhibited good linearity by plotting the peak area ratio of the analyte to the internal standard in the concentration range between 10 and 150 ng/mL trimetazidine dihydrochloride in plasma (r = 0.9994).

Sensitivity

The limit of detection (LOD) was determined to be 5 ng/mL as defined by the concentration of analyte, giving a signal to noise ratio of 3 : 1. The limit of quantification (LOQ) was 10 ng/mL as defined by the lowest concentration

Table 1. Extraction recovery of trimetazidine dihydrochloride from human plasma (n = 7)

Spiked concentration (ng/mL)	Recovery (%)	R.S.D. (%)
10	71.5	3.1
20	79.6	10.4
50	80.0	6.4
100	77.2	4.9
150	84.6	1.7

in linear range that can be detected with variation within 6.7%. The relative standard deviation (RSD) of seven replicate determinations was in the range 0.55–7.63%.

Precision and Accuracy

The intra-day assay variations were determined by analyzing plasma samples spiked with 10, 20, 50, 100, and 150 ng/mL of trimetazidine dihydrochloride. The inter-day assay variations were determined by analyzing plasma samples spiked with the same amount in duplicates, on four separate days. In both cases, accuracy was within the range, 92.2–101.5% and their RSD values were less then 7.6% over the above concentrations (Table 2).

Pharmacokinetic Study

Figure 4 shows the mean plasma concentration time profiles of trimetazidine, after oral administration of the trimetazidine dihydrochloride formulations (two tablets, 40 mg) to eight human volunteers. The pharmacokinetic parameters of trimetazidine after oral administration are summarized in Table 3. The mean terminal half-life ($t_{1/2}$) was 1.93 hr; maximum plasma concentration (C_{max}) was 105.37 ng/mL; time to maximum concentration (T_{max}) was 1.5 hr; the area under the plasma concentration-time curves, the $AUC_{0\text{-}36}$ and $AUC_{0\text{-}\infty}$, were 1276.2 ± 122.93 and 1387.9 ± 124.96 ng/mL/hr, respectively.

Table 2. Precision (C.V.) and accuracy (relative error) for determination of trimetazidine dihydrochloride in spiked plasma

Trimetazidine spiked (ng/mL)	Concentration found (ng/mL)	R.S.D.(%)	Accuracy (%)[a]
Intra-day assay (n = 4)			
10	9.55	4.09	95.46 ± 2.90
20	18.43	4.24	92.17 ± 3.39
50	48.44	0.90	96.88 ± 0.82
100	100.05	1.13	100.05 ± 1.10
150	149.60	2.98	99.74 ± 2.92
Inter-day assay (n = 4)			
10	9.35	7.63	93.53 ± 5.26
20	19.25	3.28	96.26 ± 2.75
50	48.94	2.21	97.88 ± 2.05
100	99.47	0.55	99.47 ± 0.54
150	152.39	2.06	101.59 ± 2.05

[a] Mean ± S.D.

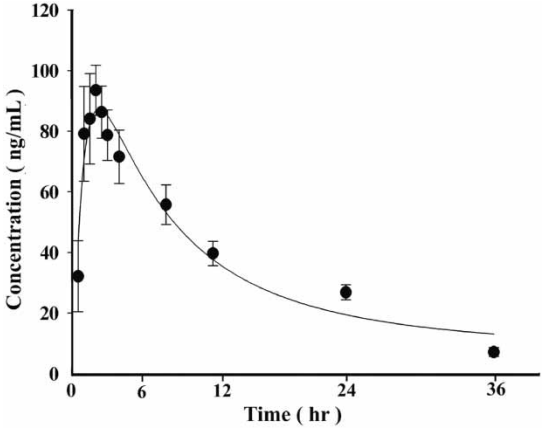

Figure 4. Human plasma concentration-time profile after oral administration of trimetazidine dihydrochloride (40 mg). Each point represents mean plus S.E. of eight volunteers.

DISCUSSION

HPLC analyses of drugs in plasma samples relying on UV detection are widely used owing to their ruggedness. However, UV detection at low wavelength often interferes with several polar materials originating from plasma matrices. In addition, potentially interfering substances can not be easily removed by a deproteination procedure using organic solvents.

To obtain pharmacokinetic profiles in human volunteers in this study, a liquid-liquid extraction method was developed for the clean-up in HPLC determination of trimetazidine in human plasma. Prior to solvent extraction, 300 μL of saturated K_2CO_3 solution was spiked with a plasma mixture and subsequently, the analyte was quantitatively extracted into the ethylacetate phase.

Table 3. Pharmacokinetic parameters of trimetazidine dihydrochloride after oral administration of two tablets (40 mg) in 8 healthy human subjects

Parameters	Mean ± S.E
C_{max} (ng/mL)	105.37 ± 10.75
T_{max} (hr)	1.94 ± 0.22
$AUC_{0\text{-}36\,h}$ (ng h/mL)	1276.22 ± 122.93
$AUC_{0\text{-}\infty}$ (ng h/mL)	1387.94 ± 124.96
$t_{1/2}$ (hr)	5.83 ± 0.43

The sample preparation step was capable of concentrating the sample and, at the same time, reducing the amount of interfering materials so as to prolong analytical column life. Accordingly, HPLC separation was base-line resolved and stabilized from low background interferences, which afforded adequate sensitivity, specificity, and reproducibility.

The pH of mobile phase has serious effects on the retention of trimetazidine dihydrochloride and lidocain; therefore it was required to strictly control the pH of the mobile phase. The present method revealed good sensitivity showing its LOD and LOQ values, 5 ng/mL and 10 ng/mL of trimetazidine dihydrochloride, respectively in the final solution. From the above results, it was concluded that the method is sensitive and reproducible for the determination of the plasma concentrations of trimetazidine, and therefore, is valuable for pharmacokinetic studies on different dosage forms of the drug.

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