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Quantification of clotiazepam in human plasma by gas chromatography–mass spectrometry

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Abstract

An analytical procedure was developed and validated for the quantification of clotiazepam in human plasma. After subjecting plasma samples to solid-phase extraction, the extract was evaporated and the residue re-constituted. An aliquot of the mixture was injected onto a gas chromatography–mass spectrometry system. The detector response was linear for clotiazepam concentrations in the range of 5–200 ng/ml. Intra- and inter-day precision for the assay over the concentration range was below 13.1 and 13.5%, and the accuracy ranged between 99.0–107.9% and 92.4–101.3%, respectively. The drug was found to be stable under various processing conditions used. The method is applicable to human pharmacokinetic studies of clotiazepam.

Keywords: Clotiazepam; Gas chromatography–mass spectrometry; Pharmacokinetics

1. Introduction

Clotiazepam (Fig. 1A), a thienodiazepine derivative, is clinically useful [1–3] because of its effectiveness in reducing the symptoms such as anxiety, restlessness, irritation and tension headaches. In addition to the clinical applications, the drug has been reported to be effective in the treatment of functional cardiovascular diseases and digestive disorders [4]. The pharmacokinetic characteristics of the drug include a relatively short biological half-life [5], the primary route of elimination via hepatic metabolism and a lack of interactions with drugs, such as cimetidine, oral contraceptives, alcohol, and isoniazid [6].

Earlier methods for the quantification of clotiazapam were primarily based on gas chromatographic assays with electron-capture detection (GC/ECD) [5,7], nitrogen–phosphorus detection (GC/NPD) [7], mass spectrometry (GC–MS) [8] and GC–MS/MS [9]. Most of these methods, however, do not appear to be directly applicable to the characterization of clotiazepam pharmacokinetics in the systemic circulation because they do not provide sufficient assay sensitivity [7–11] and/or adequate assay validation results [8–11]. In the literature, high performance liquid chromatography assays for clotiazepam was also described although these methods [10–12] do not appear to provide sufficient sensitivity. Currently, a validated assay of clotiazepam for the human pharmacokinetic study does not appear to be available.

The objective of this study, therefore, was to develop and validate a method for the assay of clotiazepam in human plasma using gas chromatography–mass spectrometry. Since previous assays for clotiazepam were not directly intended for the pharmacokinetic studies of the drug in human subjects, the applicability of the assay was also studied.

2. Experimental

2.1. Chemicals and reagents

Pure clotiazepam and diazepam (i.e., the internal standard of this study) were obtained from Myung In Pharm. Co., Ltd. (Seoul, South Korea). The purity of the compounds was rated as 100.19% for clotiazepam and 100.12% for diazepam. All other reagents were obtained either from Fisher Scientific (Pittsburgh, PA, USA) or from Sigma (St. Louis, MO, USA),

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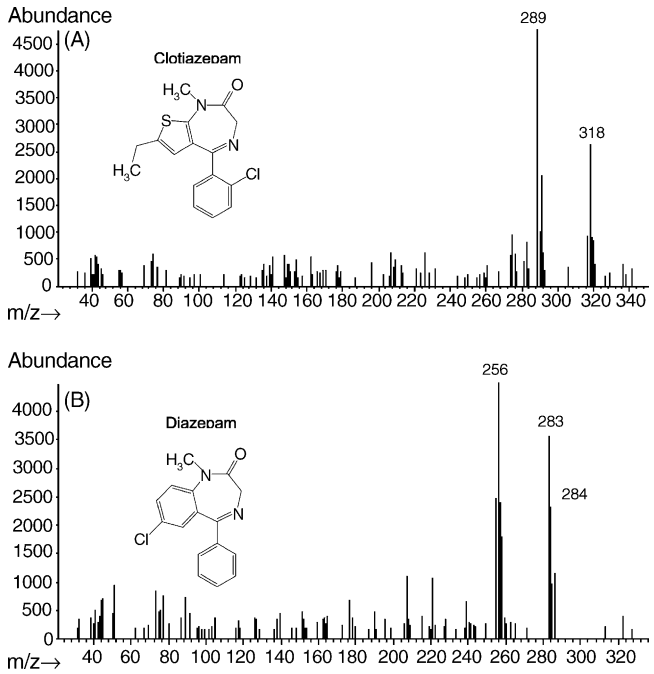

Fig. 1. Chemical structures and full scan mass spectra of (A) clotiazepam and (B) diazepam (internal standard).

and used without further purification. Rize® tablets [5 mg as clotiazepam, Dae Woong Pharm. Co. Ltd (Seoul, South Korea)] were also used in the study.

2.2. Preparation of standard solutions

Clotiazepam or diazepam was weighed and dissolved in methanol to prepare a stock solution (1 mg/ml) for the drug and an internal standard. Working standard solutions for clotiazepam were prepared by serial dilution with methanol to obtain the desired concentration range. The working standard solution for the internal standard (5 μg/ml) was prepared by dilution of the stock solution with methanol. All clotiazepam and diazepam solutions were stored at 4 °C. An aliquot (20 μl) of the working stock solution was added to 980 μl of blank human plasma to prepare standard clotiazepam plasma samples (5, 10, 20, 50, 100, or 200 ng/ml, all final concentrations).

2.3. Extraction

Solid-phase extraction was used to extract the drug and the internal standard. A C-18 cartridge (Strata C18-E, Phenomenex, Torrance, CA, USA) was preconditioned by the sequential addition of methanol (1 ml) and water (1 ml). A 20 μl aliquot of the internal standard solution (diazepam, 5 μg/ml in methanol) was added to the standard clotiazepam plasma samples. The mixture was then loaded on the cartridge and the column washed with 3 ml of water. The analyte was eluted with 1 ml of methanol; the eluent was then evaporated to dryness under a stream of nitrogen on a thermal dryer (Dry thermo bath MG-2100, Eyela, Tokyo, Japan). The residue was reconstituted by the addition of 50 μl of methanol and an aliquot (3 μl) of the mixture were injected onto a gas chromatography–mass spectrometry system (see below).

2.4. Instruments

GC–MS system consisted of an HP 6890 gas chromatographic system (Hewlett Packard, Avondale, CA, USA) and an HP 5973 mass selective detector. The separation of clotiazepam and diazepam from endogenous substances was performed on a fused-silica capillary column [HP-5MS, (5%-phenyl)-methylpolysiloxane, 30 m length × 0.25 mm internal diameter, 0.25 μm film thickness, Hewlett Packard]. The temperatures of the injector, source, quadruple and transfer line were set at 250, 250, 100, and 290 °C, respectively. Helium, at a flow rate of 1 ml/min, was used as the carrier gas. The ionizing energy was adjusted to 70 eV. The split mode with a split ratio of 10:1 was used in the study. Upon the introduction of the sample to the chromatography, the oven temperature was increased from 150 to 210 °C at a rate of 30 °C/min, then to 270 °C at a rate of 8 °C/min, and was thereafter maintained at 270 °C for 0.5 min. The primary ion species for clotiazepam and the internal standard, the two most abundant ion species for the drug (i.e., *m/z* 289 and 318), and the major ion species for the internal standard (i.e., *m/z* 283) were chosen for selected ion monitoring.

2.5. Validation of assay method

2.5.1. Specificity

Interference by endogenous compounds was assessed by comparison of the ion chromatograms for standard clotiazepam, drug-free plasma, plasma spiked with clotiazepam, plasma spiked with diazepam as the internal standard, plasma spiked with clotiazepam and diazepam, and plasma obtained from subjects after an oral administration of clotiazepam (i.e., 1 h after an oral administration of clotiazepam at the dose of 5 mg)

2.5.2. Sensitivity

In this study, the limit of quantification (LOQ) was defined as the concentration required to yield a precision of less than 20% (relative standard deviation, R.S.D.) and an accuracy between 80 and 120% of the theoretical value. In addition, the signal-to-noise ratio for the LOQ sample was determined.

2.5.3. Linearity

The linearity of the assay was assessed for standard plasma samples in the concentration range of 5–200 ng/ml. The sample was processed as described above (see Section 2.3) and analyzed for the drug and the internal standard. The peak area ratios of clotiazepam to diazepam were determined and calibration curves constructed.

2.5.4. Precision and accuracy

The intra- and inter-assay relative standard deviation and standard errors of the mean were evaluated for the precision and accuracy of the assay by determination multiple batches of calibration samples. For the intra-day validation, five sets of calibration samples having concentration levels (5–200 ng/ml; 5, 10,

20, 50, 100, or 200 ng/ml) were assayed in one day. For inter-day validation, five sets of calibration samples having the concentration levels were determined on five different days. Accuracy was determined by comparing the calculated concentration from the calibration curve to known concentration.

2.5.5. Recovery

When it was necessary to determine the absolute recovery for the extraction procedure, the peak area ratio was compared with or without the use of the extraction procedure. For the case of the determination in the absence of extraction, the standard clotiazepam solution (1 ml) in methanol was serially diluted with methanol to give samples having three concentrations (i.e., 5, 50, and 200 ng/ml, final concentrations) containing the internal standard (i.e., 100 ng/ml, final concentration). The standard samples were evaporated to dryness under a stream of nitrogen on a thermal dryer. The residue was then re-constituted by the addition of 50 μl of methanol and an aliquot (3 μl) of the mixture was injected onto the GC–MS system. For the case of samples that were extracted, an aliquot (20 μl) of the standard clotiazepam solution in methanol was added to blank human plasma (980 μl) to give three concentrations (i.e., 5, 50, and 200 ng/ml, final concentrations) containing the internal standard (i.e., 100 ng/ml, final concentration). The residue was then subjected to solid-phase extraction and the eluent evaporated, as described previously. The residue was re-constituted in methanol (50 μl) and an aliquot (3 μl) injected onto the GC–MS system. Peak area ratios for the drug to the internal standard were calculated and the absolute recovery determined by the dividing the assay parameter in the presence of the extraction to that in the absence of the extraction. In this study, quadruplicated sets for each concentration level were prepared and determined.

2.5.6. Stability

When it was necessary, the stabilities of clotiazepam and/or the internal standard were examined for various storage conditions. In the case of stability assessment after a freeze-thaw cycle, quality control (QC) samples (i.e., 5, 50, and 200 ng/ml in human plasma) were subjected to three freeze–thaw cycles. The sample was processed and assayed for clotiazepam. The assay parameter of the samples was compared with that for a freshly prepared sample. To examine short- and long-term stabilities, QC samples were stored either at room temperature for 24 h or at −70 °C for 1 month. The stored samples were processed as described previously and assayed for clotiazepam. The assay parameter for the stored samples was compared with that of a freshly prepared sample. In the case of post-preparative stability in an autosampler after extraction, QC samples were processed and an aliquot of the mixture was added to fresh autosampler vials. The vials were then transferred to the autosampler and held under these conditions for 12 h. The sample was then analyzed for clotiazepam. The assay parameter of the samples was compared with that for the freshly prepared sample. In the case of the stability of the drug and the internal standard in the stock solution, a stock solution (5, 50, and 200 ng/ml for clotiazepam; 5 μg/ml for the internal standard) was stored for 6 h at room temperature under daylight conditions, and then for 1 week at 4 °C. The assay parameter was compared with that of a freshly prepared sample. Triplicate sets for each concentration level (i.e., 5, 50, or 200 ng/ml) were prepared and determined in these experiments.

2.6. Pharmacokinetic studies of clotiazepam

Prior to the study, the protocol was reviewed and approved by the institutional review board of the College of Pharmacy, Seoul National University and by Korea Food and Drug Administration (KFDA). Korean male volunteers who submitted the agreements to attend this project were medically examined and eight healthy volunteers were selected (25.6 ± 2.5 years; 74.9 ± 9.3 kg; 178 ± 4.6 cm) for pharmacokinetics study for clotiazepam. All subjects fasted for least 10 h before the administration of the drug. Each volunteer received a single Rize® tablet (5 mg as clotiazepam) with 240 ml of water and was subjected to further fasting up to 4 h after the administration. They abstained from consuming of alcohol or xanthine-containing foods and beverages during the study.

Blood samples (8 ml) were withdrawn from the forearm vein before the oral administration and at 5, 15, 30 min, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 36 h after the oral administration, transferred to a Vacutainer® (10 ml, Becton Dickinson, NJ, USA) tubes, and centrifuged at $3000 \times g$ for 10 min. After centrifugation, the plasma samples were separated and stored at −70 °C prior to analysis. One ml of the plasma sample was used for clotiazepam assay.

Pharmacokinetic parameters were calculated by non-compartment analysis of the plasma concentration-time curve data using WinNonlin software (Ver 3.1; Pharsight Corporation, CA, USA). The peak concentration (C_{max}) and time to reach C_{max} (T_{max}) were determined by inspection of individual plasma concentration-time profiles for clotiazepam. The area under the plasma concentration–time curve ($AUC_{0\rightarrow t}$) was calculated by the linear trapezoidal method from 0 to 36 h. The area under the plasma concentration–time curve from zero to time infinity ($AUC_{0\rightarrow\infty}$) was calculated as $AUC_{0\rightarrow t} + C_t/\lambda$, where C_t is the last measured concentration and λ represent the slope of the terminal phase. The terminal half-life ($t_{1/2}$) was calculated to be $0.693/\lambda$.

3. Results and discussion

3.1. Identification of primary ions for clotiazepam and diazepam

To determine the primary ion species for clotiazepam and diazepam, full mass spectra of the drug and the internal standard were obtained (Fig. 1). The primary ions (Fig. 1A) for clotiazepam were found to be *m/z* 318 (molecular ion) and *m/z* 289 (fragment ion). In comparison, the primary ions for diazepam (Fig. 1B), the internal standard, were *m/z* 283 (molecular ion) and *m/z* 256 (fragment ion). These mass spectra were similar to previously reported spectra from the literature [8,13,14].

In this study, diazepam was used as the internal standard for the clotiazepam assay. In principle, deuterium labeled

Table 1
Precision and accuracy of clotiazepam in human plasma ($n = 5$)

Concentration (ng/ml)	Precision (%)		Accuracy (%)	
	Intra-day ($n = 5$)	Inter-day ($n = 5$)	Intra-day ($n = 5$)	Inter-day ($n = 5$)
5	13.1	13.5	102.7	92.4
10	6.9	5.9	107.9	97.0
20	8.0	3.6	99.0	99.8
50	9.6	5.4	99.1	100.5
100	9.2	2.9	99.1	101.3
200	3.3	1.0	100.1	99.8

clotiazepam would be the best internal standard for the assay. Unfortunately, however, the benzodiazepine with the stable isotope labeling is not readily synthesized nor commercially available. In the literature, diazepam has been used as an internal standard for clotiazepam assay in the literature [5] since the compound is most closely analogous to clotiazepam. Therefore, we chose to use diazepam as the internal standard in our study.

3.2. Specificity

Based on the chromatograms of various samples, a reproducible chromatographic separation between clotiazepam and diazepam was achieved (Fig. 2). The chromatogram for a blank human plasma sample indicates that no interfering peak occurs at the retention time for the drug and the internal standard. When chromatograms were obtained for standard solutions of clotiazepam and diazepam, the retention times for clotiazepam and diazepam were approximately 8.5 and 7.9 min, respectively. The retention times were consistent with those obtained for standard plasma samples and plasma samples obtained from human subjects after an oral administration of the drug at a dose of 5 mg. In addition, the mass spectral peaks for the drug and the internal standard were comparable (data not shown) to those obtained previously. Collectively, these observations indicate that the specificity of the assay is adequate.

3.3. Linearity

The calibration curves for clotiazepam in human plasma were linear over the clotiazepam concentration range of 5–200 ng/ml. Using the linear least squares regression, the calibration line was (mean ± standard deviation) $y = (0.0169 \pm 0.000648)x - (0.00052 \pm 0.022)$ with $r^2 = 0.9985 \pm 0.0021$ (y, the peak area ratio; x, the concentration of clotiazepam in the plasma and r, the correlation coefficient). A statistical analysis indicated that the correlation coefficient is highly significant ($p < 0.001$) and the intercept is not different from zero. Therefore, these observations indicate that the GC–MS response is directly proportional to the clotiazepam concentration in plasma and that the assay is linear.

3.4. Precision and accuracy

A summary of intra- and inter-day precision/accuracy is listed in Table 1. In general, the precision and accuracy data were

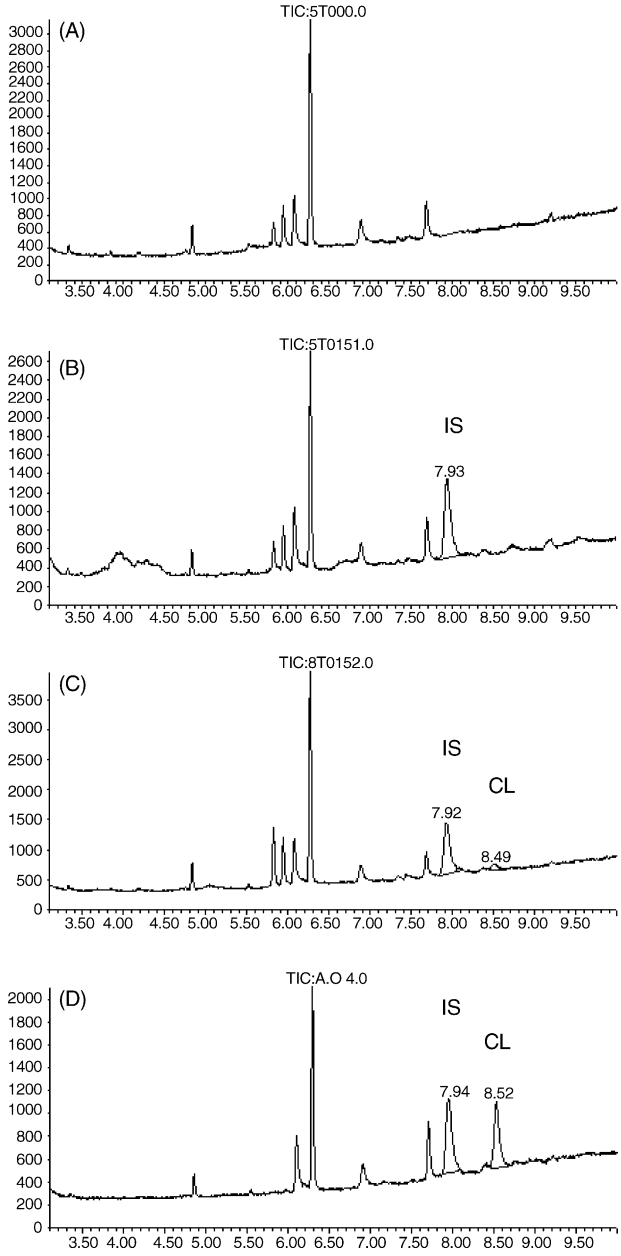

Fig. 2. Chromatograms of (A) blank human plasma, (B) blank human plasma spiked with internal standard (IS, diazepam 100 ng/ml), (C) blank human plasma spiked with 5 ng/ml (LOQ) of clotiazepam containing internal standard, and (D) a plasma from a volunteer at 1 h after a single oral administration of clotiazepam (5 mg) tablet.

Table 2
Absolute recoveries of clotiazepam from human plasma by solid-phase extraction method

Concentration (ng/ml)	Absolute recovery (%, mean ± S.D., $n = 4$)
5	91.6 ± 5.6
50	92.1 ± 1.8
200	95.5 ± 5.5

less than 13.5% and 92.4–107.9%, respectively. The intra-day accuracies for plasma were 99.0–107.9% while the intra-day precision as a relative standard deviation (R.S.D.) was less than 13.1%. The inter-day accuracies for plasma ranged from 92.4 to 101.3% and the precision was less than 13.5%. These results indicate that the accuracy and precision of the current assay are within the recommendations for the assay validation by "Guidance for Industry: Bioanalytical Method Validation (FDA, May 2001)" and that the reproducibility of the assay is adequate.

3.5. Sensitivity

The limit of quantification (LOQ) was determined as the concentration of drug to have an accuracy between 80 and 120% with a precision R.S.D. (%) of less than 20%. The precision and accuracy of the assay at the lowest concentration level (i.e., 5 ng/ml) for clotiazepam were within the limit (Table 1). Based on the precision/accuracy estimation, the limit of quantification for the current assay was set at 5 ng/ml. The signal-to-noise ratio for the 5 ng/ml standard sample (i.e., LOQ sample) was found to be approximately 5 (Fig. 2C).

3.6. Recovery

The extraction recoveries of clotiazepam were determined at low (5 ng/ml), medium (10 ng/ml), and high (100 ng/ml) concentrations using quadruplicate sets (Table 2). The absolute recoveries (in %) of clotiazepam from human plasma by solid-phase extraction were found to be 91.6 ± 5.6 for 5 ng/ml, 92.1 ± 1.8 for 50 ng/ml, 95.5 ± 5.5 for 200 ng/ml, respectively. Based on one-way ANOVA, the absolute recovery was not statistically different with respect to the clotiazepam concentration, indicating that the recovery is independent of the concentration of the drug.

3.7. Stability

The stability of clotiazepam was assessed for typical handling conditions (e.g., freeze–thaw and short-term temperature stability) and the stability of processed samples (Table 3). In general, the above parameters did not result in appreciable differences in the assay parameters and were 92.7–99.6% of those of the corresponding fresh samples. For the case of stability after a freeze–thaw cycle, the clotiazepam peak area ratio had negligible effect on this assay parameter (93.7–99.0%). For the case of short- and long-term stabilities, the response of the GC–MS system was comparable to that for the fresh sample (95.7–97.6% for a short-term during 24 h at room temperature, 92.7–96.3% for a long-term during 1 month at −70 °C, respectively). In the case of post-preparative stability in an autosampler, the quality control sample in the autosampler vials was stable during the 12 h period at room temperature after the extraction (97.4–98.6%). Finally, for the case of the storage of stock solutions of clotiazepam and diazepam (I.S.) at room temperature for 6 h under conditions of daylight and 4 °C for 1 week, no significant decrease in the clotiazepam and diazepam in peak areas (96.3–99.6% for clotiazepam, 96.7–98.9% for diazepam, respectively) were found. Therefore, these observations suggest that the drug and the internal standard are stable in storage or under sample handling conditions.

Table 3
Stability of samples ($n = 3$)

Concentration (ng/ml)	Stability (%)
Freeze and thaw stability	
5	93.7 ± 2.0
50	99.0 ± 2.6
200	96.7 ± 1.0
Short-term temperature stability (24 h at room temperature)	
5	97.6 ± 4.3
50	97.6 ± 5.3
200	95.7 ± 2.3
Long-term stability (1 month at −70 °C)	
5	93.0 ± 5.2
50	92.7 ± 2.5
200	96.3 ± 1.0
Post-preparative stability (12 h at room temperature)	
5	97.4 ± 2.8
50	98.6 ± 1.3
200	97.6 ± 2.2
Clotiazepam stock solution (6 h at room temperature)	
5	97.8 ± 1.8
50	99.6 ± 1.7
200	98.4 ± 1.3
Clotiazepam stock solution (1 week at 4 °C)	
5	96.3 ± 1.8
50	98.5 ± 2.2
200	98.9 ± 0.6
Diazepam (IS) stock solution (5 μg/ml)	
6 h at room temperature	98.9 ± 0.9
1 week at 4 °C	96.7 ± 2.8

3.8. Applicability of the assay to the pharmacokinetics study for clotiazepam

The feasibility of using the current assay in the pharmacokinetic characterization of clotiazepam involving human subjects was also examined. Eight healthy Korean male volunteers participated in the study. Fig. 3 shows the temporal profiles of clotiazepam levels in plasma after a single oral administration of a clotiazepam formulation (Rize® tablet, 5 mg as clotiazepam/tablet). The parameters estimated in the present study were found to be comparable to those reported in the literature (i.e., C_{max}, 100–160 ng/ml; T_{max}, 0.5–1.5 h; and $t_{1/2}$ for clotiazepam, 6.5–17.8 h; and AUC, 354–649 h × ng/ml, respectively) [5,15]. Therefore, collectively these observations

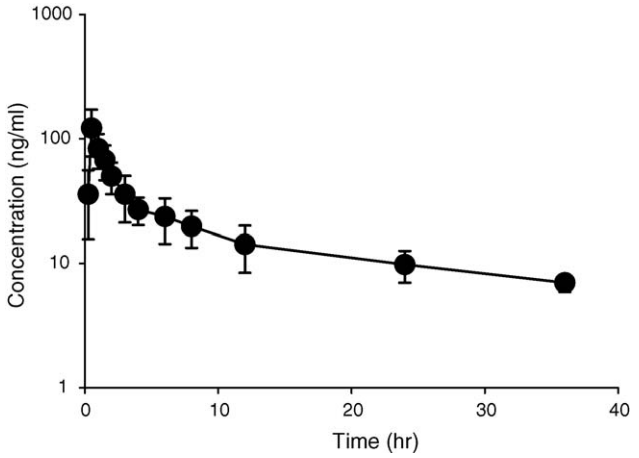

Fig. 3. Mean plasma concentration–time profiles of clotiazepam following a single oral administration of a clotiazepam (5 mg) tablet to eight healthy volunteers. Each value represents the mean ± S.D. of eight experiments.

suggest that the use of the current assay in pharmacokinetic studies is feasible.

4. Conclusions

A gas chromatography-mass spectrometry method (GC–MS) was developed for the determination of clotiazepam in human plasma. The method was found to have adequate sensitivity, reproducibility and specificity. Considering the fact the run time per sample may be limited to approximately 10 min, the throughput of the assay makes it amenable for studies of clotiazepam pharmacokinetics involving human subjects.

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