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Determination of Clotiazepam in the Plasma Using Gas Chromatography/Mass Spectrometry with an Ion-Trap Detector and its Application to Pharmacokinetics in Healthy Volunteers

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ABSTRACT – A method determining the plasma concentration of clotiazepam was developed by using gas chromatography/mass spectrometry with an ion-trap detector and was validated for applying pharmacokinetics to human volunteers orally taken 5 mg dose of clotiazepam. The detection limit was 1 ng/ml and the limit of quantitation was 5 ng/ml. Intra-day reproducibility and accuracy bias % were less than 8.2 and 10.2% with inter-day variations for those being within 7.0 and 13.8%, respectively. The recovery of clotiazepam was higher than 87%. The principal pharmacokinetic parameters were determined from the plasma concentration-time plot by non-compartmental or two-compartmental analysis. In non-compartmental analysis, the elimination half-life of 10.4 hr and the area under the curve of 651.3 ng·hr/ml were determined, and the maximal concentration (158.6 ng/ml) in the plasma was obtained at 0.56 hr post-dose. The developed method can be appropriate to apply pharmacokinetics and bioequivalence of clotiazepam.

Key words – Clotiazepam, Gas chromatography/mass spectrometry (GC/MS) with ion-trap detector, Human volunteers, Pharmacokinetics, Bioequivalence

Various kinds of 1,4-benzodiazepine derivatives have been investigated as tranquilizers, antidepressants, anticonvulsants, muscle relaxants and sedatives. Clotiazepam (1-methyl-5-o-chlorophenyl-7-ethyl-1,2-dihydro-3H-thieno-[2,3-e],[1,4]-diazepine-2-one) belongs to the thienodiazepines class, which differ from benzodiazepines only in having a thiophene nucleus, as opposed to a benzene nucleus bound to the diazepine.[1)]

Its pharmacological effects are exerted by binding of γ-aminobutyric acid to its receptor on the cell membranes, resulting in the transport of chloride ions. The influx of chloride ions causes a small hyperpolarization that moves postsynaptic potential away from its firing threshold and thus inhibits formation of action potentials. Clotiazepam binds to specific and high affinity sites on the cell membrane that are separated from the receptor for γ-aminobutyric acid. This results in enhancing the affinity of GABA receptors for this neurotransmitters, leading the enhanced hyperpolarization and further inhibition of neuronal firing.[2-3)]

Several methods have been reported for the determination of clotiazepam in biological fluids such as gas chromatography (GC)/electron-capture detector or GC/nitrogen-phosphorus detector,[4-5)] high-performance liquid chromatography/diode array detector,[6)] thin-layer densitometry,[7)] and GC/mass spectrometry.[8)] Recently, the method analyzing 22 benzodiazepines including clotiazepam in blood or urine spiked authentic standards by GC/ion-trap tandem mass spectrometry was reported by Pirnay *et al.*[9)] They extracted clotiazepam using a cartridge and prepared its trimethylsilyl-derivatives with N,O-bis (trimethylsilyl)trifluoroacetamide prior to analysis. However, they made no attempt in the determination of clotiazepam in clinical samples.[9)]

A single 5 mg dose of clotiazepam was orally administered to 3 healthy volunteers and its half-life was reported to be 6.5 hr. Its main metabolites were known to be hydroxy- and desmethyl-clotiazeapam and were highly bound to plasma protein.[5)] The volume of distribution, total clearance and terminal half-life were determined to be 2.57 L/kg, 15 ml/min/kg and 10.2 hr, respectively, in human administered 5 mg dose of clotiazepam.[10)] However, the pharmacokinetic parameters of clotiazepam are not studied extensively and further pharmacokinetic analysis is necessary due to reobserved variations between the parameters. This current work was conducted to develop and validate analytical method of clotiazepam by using gas chromatography/ion-trap detector, and the method

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was applied to the pharmacokinetic analysis in human healthy volunteers orally taken 5 mg clotiazepam.

Materials and Methods

Chemicals and reagents

Clotiazepam authentic standard and tablets (5 mg) were donated from Daewoong Pharmaceutical Co. (Seoul, Korea). Diazepam was obtained from Myeongin Pharmaceutical Co. (Seoul, Korea). Analytical grade of diethyl ether and methanol was purchased from J. T. Baker (Phillipsburg, NJ, USA). The other agents used for clotiazepam analysis were of analytical grade.

GC/MS Instruments

Clotiazepam concentrations in plasma were determined by using a GC/MS (Trace GC/Polaris Q, Thermo Finnigan, Austin, TX, USA) equipped with an ion-trap detector. An autosampler (AI 3000) was loaded on it. The instrument and data handling were operated by the support of Chemstation X-Calibur (Thermo Finnigan, Austin, TX, USA). Ultra-1 capillary column (17 m length × 0.2 mm inner diameter × 0.33 μm film thickness; Agilent Technologies, Palo Alto, CA, USA) was used. Oven temperature was set initially on 120°C, increased by 30°C per min to 220°C for 1 min, and finally increased by 10°C per min to 300°C, at which staying for 2 min. Inlet and transfer line temperature were 310 and 300°C, respectively. A splitless mode was selected. Ion source temperature was 250°C. Helium (99.999%) was used as carrier gas at a flow rate of 0.8 ml/min. The detector was applied in the electron impact (EI) mode, being equivalent to ionization energy of 70 eV.

Study subjects

Male volunteers who submitted the agreement to attend this project were medically examined and 8 healthy volunteers were selected by a medical doctor in Bestian Hospital (Seoul, Korea), based on clinical examination including seropathological (hemoglobin, hematocrit, WBC, platelet), serochemical (blood urea nitrogen, creatinine, total protein, albumin, SGOT, SGPT, total bilirubin, cholesterol, glucose fasting, alkaline phosphatase), and urological (specific gravity, color, pH, sugar, albumin, bilirubin, RBC, WBC) data. The subjects were instructed not to take any medicine for at least 1 week prior to and during the study period. Informed consent was completed by the subjects after explanation of the nature and aims of the work. They were accommodated to the same place one day before collecting the blood. They were fasted overnight before administration of the tablet. The study protocol was approved by the Institutional Review Board of Korea Institute of Science and Technology and by Korea Food and Drug Administration (KFDA).

Oral administration of a clotiazepam tablet to human volunteers

A 21-gauge scalp-vein set was established on the arm vein of the volunteers, and 8 ml blood for blank sample was collected. According to the prescription directed by a doctor, a tablet of clotiazepam (5 mg) was orally taken to the designated group at random design (8 volunteers) with 150 ml of drinking water. No food was allowed until 4 hr after dose administration. Lunch and dinner were provided to volunteers according to a time schedule. Beverages and caffeine were not allowed during the study. Blood was taken into a heparin-treated Vacutainer tube (Becton Dikinson, Rutherford, NJ, USA) at 0.33, 0.67, 0.5, 1, 1.5, 2, 4, 8, 12, 24 and 36 hr after the oral administration. The time interval of blood sampling between volunteers was 2 min to consider blood collection time. The blood was centrifuged to obtain plasma at 4°C. The plasma was stored at -70°C until analyzed.

Preparation of the calibration curve of clotiazepam

To a 15 ml centrifuging tube, 1 ml of the thawed blank plasma was added. And the various concentrations of clotiazepam were spiked to make the final concentration of 0, 2, 5, 10, 20, 50, 100 and 200 ng/ml. Diazepam of 200 ng (10 μg /ml, 20 μl) was added to the tube as internal standard. And one half ml of sodium carbonate (1 M, pH 9.5) was added. After the tube was mechanically mixed on a vortex-mixer (Maxi Mix II, Thermolyne Co., Dubuque, IA, USA), 5 ml of diethyl ether was added. The tube was vigorously shaken on a shaker (SM-25, Edmund Buhler, Germany) for 20 min and the organic layer was separated at 900 g for 10 min by centrifugation (Triac, Clay Adams, Rutherford, NJ, USA) and by freezing at -30°C in a deep freezer (Ecoline RE112, Laüda, Germany). The organic layer was transferred to another tube and evaporated on an evaporator under vacuum condition. The residue was dissolved in 100 μl of methanol. The solution of 2 μl was applied to the GC/MS system. The calibration curve was prepared from the area ratios of the ion chromatogram of clotiazepam to diazepam, and inter- and intra-day precision and accuracy were obtained.

Method validation

Within (intra-) and between (inter-) days precision and accuracy (bias %) were calculated from repeated analysis (n = 5) of

clotiazepam added in blank plasma, respectively. The limit of detection is a parameter of limit tests and may be determined as the smallest quantity of analyte that is expected to produce a response which is significantly different from that of a blank. The limit of quantitation may be defined as the smallest quantity of analyte which can be determined with acceptable precision and accuracy. The limit of quantitation was determined by diluting successively the lowest point of calibration and by performing within and between precision and accuracy tests (less than 20%). The recovery of clotiazepam was calculated by comparing the ion chromatogram area ratios of the clotiazepam to internal standard obtained after extraction to those of the standard compound.

Preparation of plasma samples

One ml of the thawed plasma obtained from healthy human volunteers was added to the 15 ml centrifuging tubes, followed by addition of internal standard diazepam (10 μg/ml, 20 μl). The tube was treated as described above. Based on the calibration curve of clotiazepam, the plasma concentrations of clotiazepam were determined from peak area ratios of clotiazepam to diazepam.

Pharmacokinetic analysis

Pharmacokinetic parameters were determined from the time-plasma concentrations of clotiazepam by non-compartmental or two-compartmental analysis by using WinNonlin software (Scientific Consulting Inc., Cary, NC, USA).

In non-compartmental analysis, the highest concentration (C_{max}) and the time to reach the highest concentration (T_{max}) were read directly from the time-plasma concentration curves of clotiazepam. The area under the curve of time-plasma concentrations of clotiazepam until the last sampling time ($AUC_{0\rightarrow last}$) was determined by the equation of $AUC_{0\rightarrow inf} = AUC_{0\rightarrow last} + C_{last}/\beta$, where β is the slope of the terminal phase of the time-log plasma concentration curve and C_{last} is the concentration at the last sampling time.[11]

In two-compartmental analysis, pharmacokinetic parameters were obtained from the equation $C(t) = A \exp(-\alpha t) + B \exp(-\beta t)$, where α is the distribution rate constant, β is the terminal elimination constant, k_{10} is an elimination rate constant, and k_{21} and k_{12} are rate micro-constants for the transfer of a drug between two compartments.

Results

Specificity

Scan spectra of clotiazepam and its internal standard diazepam are shown in Figure 1. The base peak of clotiazepam was m/z 289 ($M^+-C_2H_5$) and its molecular ion (M^+) was m/z 318. The other ions for clotiazepam were m/z 275, 291 and 320. The base peak for diazepam was m/z 256, and the ion with very similar abundance to the base peak was observed at m/z 221. The molecular ion (M^+) of diazepam is shown at m/z 284. Three ions each (m/z 289, 291, and 318 for clotiazepam; m/z 221, 256, and 283 for diazepam) were selected for the confirmation and quantitation of clotiazepam or diazepam. The ion selected for clotiazepam quantitation among these ions was m/z 289, and for diazepam m/z 256. Typical ion chromatograms for clotiazepam are shown in Figure 2. The retention times of clotiazepam and diazepam were 6.35 and 5.83 min, respectively. No interfering peaks were found.

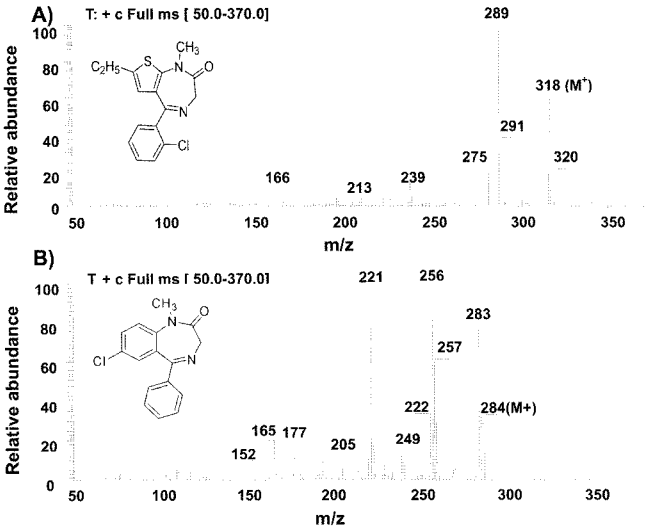

Figure 1–Mass spectra of clotiazepam (A) and the internal standard diazepam (B) that were obtained by GC/MS with ion-trap detector. The molecular ions were indicated as M^+.

Precision and accuracy

The validation data about precision and accuracy of clotiazepam were summarized in Table I. The signal to noise ratios for 1 and 5 ng/ml clotiazepam were 3.23 ± 0.75 (n = 5) and 17.00 ± 4.49 (n = 5), respectively (data not shown). Therefore, the detection limit of clotiazepam was decided to be 1 ng/ml, at which the signal to noise ratio was more than 3. The limit of quantitation was 5 ng/ml, at which it satisfies the analytical criteria that is defined as the lowest concentration yielding precision of less than 20% of coefficient variation and accuracy between 80 and 120% of the theoretical value (Table I). At 5-200 ng/ml of clotiazepam as shown in Table I, intra- and inter-day precision were less than 8.6%, and the bias % for intra- and inter-day accuracy were less than 13.8%.

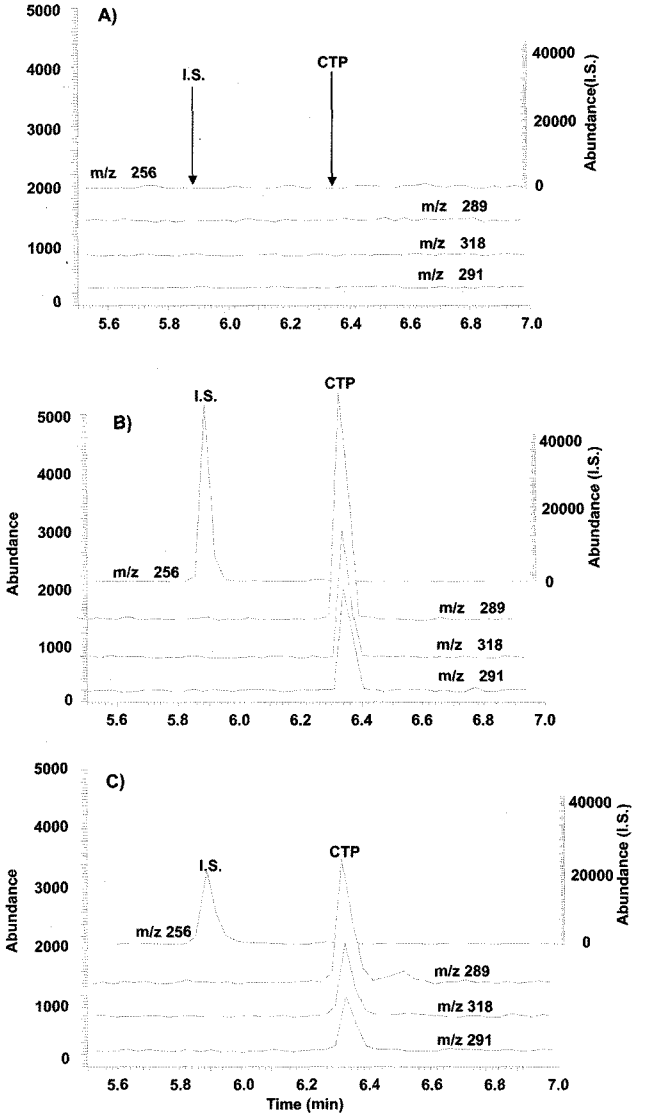

Figure 2–The ion chromatograms of clotiazepam obtained by selected ion storage mode of GC/MS with an ion-trap detector, showing the ion chromatograms at m/z 289, 291, and 318 for clotiazepam (CTP, 6.35 min) and at m/z 256 for internal standard (I.S., 5.83 min). Ion chromatograms obtained from blank (A), 25 ng/ml of clotiazepam spiked into blank plasma (B) and the plasma sample 0.5 hr after administration (C) are shown. No interfering peak was found. The ion of m/z 289 was used for quantitation of the plasma concentration of clotiazepam.

Linearity

The linearity of clotiazepam calibration curve was determined by the linear least-square regression. The relative coefficient (r^2) was 0.9993 with the equation of $y = 0.0091\ x + 0.0157$ at the range between 5 and 200 ng/ml of clotiazepam, showing a good linearity as shown in Figure 3.

Recovery

Recovery of clotiazepam was determined to be 87.7-105.6% with CV% of 1.9-13.3. The mean recovery between 5 and 20 ng/ml clotiazepam was about 98.8% (Table II). These data indicate that diethyl ether is a good selective solvent for the

Table I–*Intra- and Inter-day Precision and Accuracy for the Determination of Clotiazepam in the Plasma of Human Volunteers*

Concentrations of clotiazepam (ng/ml)	Precision (CV%)		Accuracy (bias %)	
	Intra-day	Inter-day	Intra-day	Inter-day
5	8.2	7.0	-10.2	13.8
10	5.6	8.6	-4.0	3.2
20	3.3	7.3	3.0	-0.1
50	3.5	4.5	5.1	-9.9
100	2.0	7.7	1.9	-2.9
200	2.4	1.1	-1.8	1.2

Each value for intra (within)- and inter (between)- days precision and accuracy was obtained from 5 repeated experiments; The CV% is determined from (S.D./mean × 100); The accuracy bias % is determined from [100-{(the observed concentration/the theoretical concentration) × 100}].

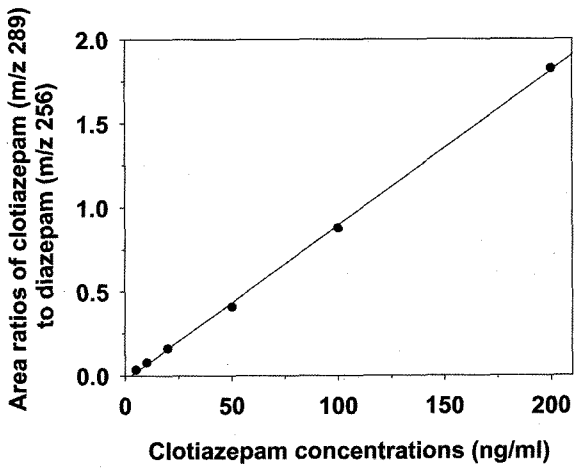

Figure 3–The standard curve of clotiazepam. The curve was prepared from ion chromatogram area ratios of clotiazepam (m/z 289) to diazepam (m/z 256) after known amounts (5-200 ng) of clotiazepam and a fixed amount of internal standard diazepam (200 ng) were added to 1 ml of blank human plasma. The standard curve showed good linearity ($y=0.0091\ x+ 0.0157$, $r^2=0.9993$). Each point in the curve was the mean value of ion chromatogram area ratios (n=10).

Table II–*Recovery of Clotiazepam*

Concentrations of clotiazepam (ng/ml)	Recovery (%, mean ± SD)[a]	CV (%)
5	105.6 ± 14.0	13.3
10	87.7 ± 1.7	1.9
20	103.0 ± 5.5	5.5

[a]Each value was obtained from 3 repeated experiments.

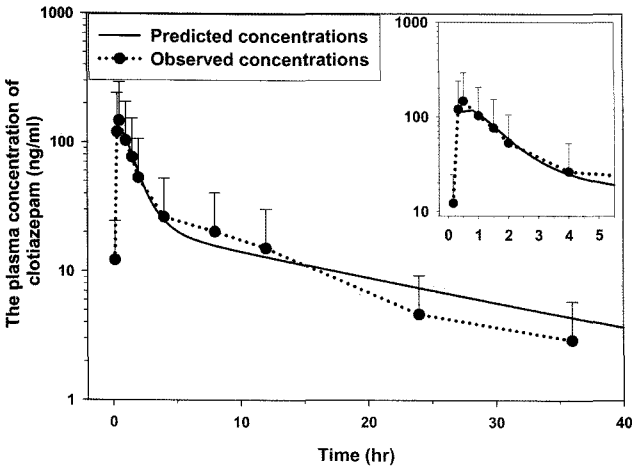

Figure 4–The time-plasma concentration curves after oral administration of a 5 mg tablet of clotiazepam to 8 healthy volunteers. Each point was represented mean ± S.D. of 8 persons. The time-plasma concentrations were curve-fitted by the two-compartment model (solid line). The curves till 5 hr are showed in the inset.

extraction of clotiazepam at alkaline pH.

Pharamcokinetic analysis of clotiazepam

A clotiazepam tablet (5 mg) was given to 8 healthy male volunteers and blood samples were collected according to the scheduled time interval as described in Materials and Methods. The mean time-plasma concentration curves of clotiazepam are shown in Figure 4 and its pharmacokinetic parameters were calculated by non-compartmental or two-compartmental analysis of the plasma concentration-time plot as shown in Table III and Table IV, respectively.

In non-compartmental analysis, the ratio of $AUC_{t=36hr}$ to AUC_{∞} was 89.3%, indicating that blood sampling times were appropriately designed. The maximum concentration (C_{max}) was 158.6 ng/ml, which was obtained at 0.56 hr (T_{max}) after the oral administration of a 5 mg clotiazepam tablet to volunteers. Its mean half-life was about 10.4 hr.

In two-compartmental analysis, the terminal half-life was determined to be 16.3 ± 4.3 hr. The other parameters of C_{max}, T_{max} and AUC_{∞} were very similar to the values obtained from the non-compartment model (Table III and Table IV). Large variation of Vd/f value was observed between non-compartment and two-compartment model, and this may have resulted from the variation of the rate constants obtained from each compartment.

Discussion

This work describes the application of GC/MS/ion-trap detector to the trace quantitation of clotiazepam in the plasma after oral administration of clotiazepam to healthy male volunteers for the pharmacokinetic analysis. Our study showed that the precision and accuracy data for the determination of clotiazepam in the human plasma satisfied the analytical criteria (Table I).

The selectivity and specificity of GC and HPLC methods are not sufficient for unequivocal identification of a compound. In this case, the GC/MS method is a good choice as it improves sensitivity and selectivity over existing analytical methods as well as identification of target compounds. However, most of the GC/MS methods were conducted by using GC/mass selective detector. Bioanalytical applications of the ion-trap detector as a selective detector for gas chromatography are somewhat limited. Various applications of the ion-trap detector to forensics and drug-abuse testing have been reported,[12-13] and the practical comparison of the ion-trap detector performance to a

Table III–*Pharmacokinetic Parameters of Clotiazepam Determined by Non-compartmental Analysis in 8 Healthy Male Volunteers Receiving a 5 mg Tablet of Clotiazepam*

Pharmacokintic parameters[a]	Mean ± SD (n=8)
$AUC_{t=36\,hr}$, ng · hr/ml	581.4 ± 202.9
AUC_{∞}, ng · hr/ml	651.3 ± 232.5
C_{max}, ng/ml	158.6 ± 44.5
T_{max}, hr	0.56 ± 0.3
K_e, hr^{-1}	0.075 ± 0.029
$t_{1/2}$, hr	10.4 ± 3.4
CL/f, L/min	8.6 ± 3.1
Vd/f, L/kg	119.2 ± 31.7

The parameters were determined by non-compartmental analysis [11], as described in Materials and Methods.

Table IV–*Pharmacokinetic Parameters of Clotiazepam Obtained from 8 Healthy Male Volunteers Receiving a 5 mg Tablet of Clotiazepam*

Pharmacokinetic parameters[a]	Mean ± SD (n=8)
K_{10}, hr^{-1}	0.511 ± 0.220
K_{12}, hr^{-1}	0.986 ± 0.335
K_{21}, hr^{-1}	0.148 ± 0.063
Vd/F, L/kg	16.5 ± 5.3
A, ng/ml	175.7 ± 17.0
AUC, ng.hr/ml	685.1 ± 236.6
α, hr^{-1}	1.615 ± 0.509
$T_{1/2\alpha}$, hr	0.472 ± 0.160
B, ng/ml	22.6 ± 14.6
β, hr^{-1}	0.0433 ± 0.0128
$T_{1/2\beta}$, hr	16.3 ± 4.3
C_{max}, ng/ml	126.6 ± 35.0
T_{max}, hr	0.73 ± 0.25

The parameters were determined by two-compartmental analysis, in which the equation was expressed by $C(t)=A\exp(-\alpha t)+B\exp(-\beta t)$, as described in Materials and Methods.

quadrupole-based mass selective detector has been reported.[14-15]

Other groups have used ethyl ether/chloroform (80:20, v/v),[16] acetate/*n*-hexane (30:70, v/v),[17] dichloromethane/isopropanol/ethyl acetate (20:20:60, v/v/v),[8] and *tert*-butylmethyl ether[18] for liquid-liquid extraction of benzodiazepine drugs. And a few method used solid-phase extraction by C_{18} or C_8 cartridges.[4,19] The residue prepared by vaporizing the organic solvents was reacted with acetic anhydride or N,O-bis(trimethylsilyl) trifluoroacetamide for acetylation or trimethylsilylation derivatives, respectively, in order to improve the analytical sensitivity of clotiazepam.[8-9]

In this work, clotiazepam was extracted with 5 ml of diethyl ether from 1 ml plasma samples and no derivatives were prepared before the application to the instrument. However, our developed method results in high recovery of 88-106% at relatively low concentrations (Table II), and good sensitivity with 5 ng/ml as the limit of quantitation. Using this method, the plasma concentrations of clotiazepam were determined in human volunteers orally given a 5 mg clotiazepam tablet and its pharmacokinetic parameters were determined. To 3 human volunteers orally administered a single 5 mg dose of clotiazepam, the half-lives of parent form (6.5 hr) and its two metabolites (7.0 and 17.8 hr) were reported.[5] Ochs *et al.*, compared pharmacokinetic parameters between normal patients and patients with cirrhosis and renal failure.[10] In normal patients treated with a single 5 mg oral dose of clotiazepam, the volume of distribution, total clearance, and elimination half-life were 2.57 L/kg, 3.15 ml/min/kg, and 10 hr, respectively. In our study, the half-life of clotiazepam was determined to be 10.4 ± 3.4 hr, being similar to the data by Ochs *et al.*,[10] but 2 times higher than that reported by Arendt *et al.*[5] The variance of pharmacokinetic parameters may be due to difference of the pharmacokinetic model used. In our data, the terminal elimination phase was observed to occur at 4 hr postdose, giving the half-life of 10.4 hr by using a non-compartment model. However, if this phase is pointed to less than 4 hr, the half-life will be determined to be 6.2 ± 2.2 hr. And if two-compartment model is used, the terminal half-life is calculated to be 16.3 ± 4.3 hr. Therefore, pharmacokinetic parameters, in large part, are dependent on the type of the pharmacokinetic model used, the last sampling time, and the sensitivity of analytical methods. The plasma concentrations-time plots for clotiazepam are relatively well fitted to 2-compartment model in healthy human volunteers (Figure 4).

Conclusions

The analytical method of clotiazepam in human plasma was developed using GC/MS with an ion-trap detector rarely used, and validated for applying to pharmacokinetics after oral administration of 5 mg clotiazepam to 8 healthy male volunteers. This data showed that our method can be applicable to determining the plasma concentration of clotiazepam for pharmacokinetics and bioequivalence studies.

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