— Regular Article —

Monolithic Spin-column Extraction and GC-MS Method for the Assay of Eperisone in Human Serum

Takeshi Saito,*[,a] Takeshi Yamagiwa,[a] Yuko Yui,[b] Shota Miyazaki,[b] Akihiro Nakamoto,[c,d] Akira Namera,[d] and Sadaki Inokuchi[a]

[a]*Department of Emergency and Critical Care Medicine, Tokai University School of Medicine, Shimokasuya 143, Isehara 259–1143, Japan,* [b]*GL Sciences Inc., Sayamagahara 237–2, Iruma 358–0032, Japan,* [c]*Scientific Investigation Laboratory, Hiroshima Prefectural Police Headquarters, Kohnan 2–26–3, Naka-ku, Hiroshima 730–0825, Japan and* [d]*Department of Legal Medicine, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1–2–3, Minami-ku, Hiroshima 734–8551, Japan*

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A rapid gas chromatography-mass spectrometry (GC-MS) method was developed and validated for the analysis of eperisone in serum using monolithic spin-column extraction. The linear concentration range for eperisone was 2–2500 ng/ml. The limit of detection was found to be 0.5 ng/ml. The average extraction recovery range was 92.8–96.0%. The intra- and interday relative standard deviations (RSDs) of the concentrations were less than 12.6% and 12.5%, respectively. The accuracy of this method ranged from 95.0% to 98.3%. We successfully used this assay to analyze serum samples from an eperisone-overdose patient. Our method has some analytical advantages compared with previously reported gas chromatography (GC) and GC-MS methods, such as higher selectivity and sensitivity than GC with nitrogen-phosphorus detection and the avoidance of nonspecificity, the ability to use a smaller sample volume than that required for the GC-MS method, and a shorter sample preparation time than the previous solid-phase extraction (SPE) method.

Key words —— eperisone, monolithic spin-column extraction, GC-MS, validation, torsade de pointes

INTRODUCTION

Torsades de pointes refers to polymorphic ventricular tachycardia secondary to a prolonged QTc interval on an electrocardiogram.[1] A prolonged QTc interval can be a hereditary disorder, *e.g.*, the Jervell-Lange-Nielsen and Romano-Ward syndromes.[2] Other causes include electrolyte imbalance, antiarrhythmic medications, and cardiomyopathy. Thus far, several drug-induced torsade de pointes have been reported, involving overdoses of lithium,[3] tricyclic antidepressants,[4,5] phenothiazines,[6] and other drugs.[7] In most cases of drug-induced torsade de pointes, the drug is a known ion channel blocker for cardiac impulse-action potential transmission. The relatively large number of patients exposed to such drugs and the potentially fatal outcome make it important to investigate drug-related long QT syndrome. Moreover, women have a higher risk of developing it than men.[8] We recently saw a case of torsade de pointes induced by an overdose of eperisone hydrochloride. To the best of our knowledge, no other such cases have been reported.

Eperisone hydrochloride, also known as (2RS)-1-(4-ethylphenyl)-2-methyl-3-(piperidinyl)-1-propanone monohydrochloride (Fig. 1A), is an antispasmodic drug that relaxes both skeletal and the vascular smooth muscles. When used to treat spastic paralysis, it is administrated orally at a dose of 50–100 mg. According to the manufacturer, the therapeutic peak concentration of eperisone in plasma is approximately 7 ng/ml. In a pharmacokinetics study of eperisone, after the oral administration of 100 mg of eperisone hydrochroride, the maximum plasma concentration was 1.25 ng/ml; the mean terminal half-life ($t_{1/2}$) was 3.16 hr; the time to maximum concentration (T_{max}) was 1.38 hr; and the areas under the plasma concentration-time

*To whom correspondence should be addressed: Department of Emergency and Critical Care Medicine, Tokai University School of Medicine, Shimokasuya 143, Isehara 259–1143, Japan. Tel.: +81-463-93-1121; Fax: +81-463-95-5337; E-Mail: saito@is.icc.u-tokai.ac.jp

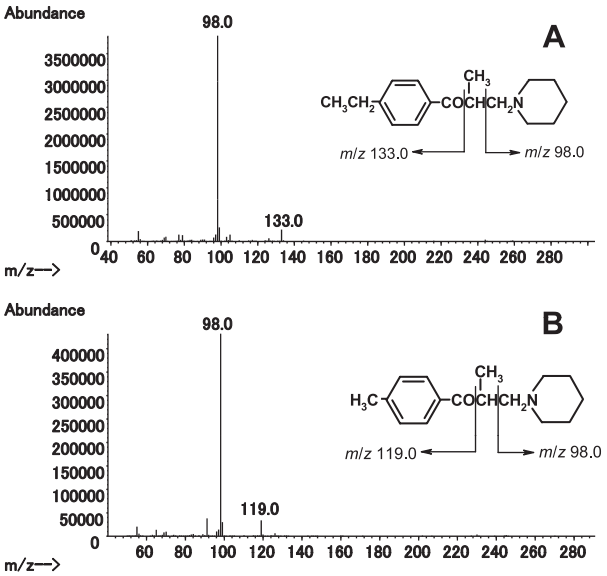

Fig. 1. Chemical Structure and Mass Spectra of Eperisone (A) and the Tolperisone (IS) (B), Obtained in Full-scan Mode

curves ($AUC_{0-12\,hr}$ and $AUC_{0-\infty}$) were 3.85 ± 1.89 and $4.21 \pm 2.13\,ng\,ml^{-1}\,h^{-1}$, respectively.[9] Loss of consciousness, seizures, apnea, and ventricular tachycardia were observed in 1 patient with eperisone overdose,[10] where the serum concentration of eperisone was found to be 162.93 ng/ml. This concentration was more than 20 times the peak therapeutic concentration. The ventricular tachycardia observed in this rare occurrence of eperisone overdose prompted the theory that high doses of eperisone may cause torsade de pointes. To investigate the possible link between eperisone and torsade de pointes, a rapid analytical method is required to measure the toxic level of eperisone during a toxicological analysis.

The extraction of eperisone hydrochloride from tablets with an analysis using liquid chromatography with mass spectrometry (LC-MS) has previously been described.[11] The liquid-liquid extraction of eperisone from plasma with LC-MS has also been described.[9,12] Linear concentration ranges of 0.01–10 ng/ml and 0.02–20 ng/ml have been achieved using this method.[9,12] Although these methods are most suitable for pharmacokinetics studies, these linear ranges are much lower than toxic levels.[10] Moreover, due to its high cost at present, LC-MS is not as popular as GC-MS in Japanese forensic or clinical toxicology. Therefore, in Japan, the GC-MS method is preferred over the LC-MS method for analyses. The use of solid-phase extraction (SPE) with GC-MS has also been reported for the analysis of eperisone in plasma samples.[13] The reported lower limit of detection (LOD) with this method was 0.2 ng/ml when 2.5 ml of plasma was used: however, this sample volume was not appropriate for clinicotoxological analysis. At least 5 ml of whole blood is needed for an eperisone analysis. Although GC with nitrogen-phosphorus detection (GC-NPD) method has been reported,[14] its specificity is not sufficient, as compared to that of GC-MS.

Monolithic spin-column extraction, involving sample loading, washing, and elution of target compounds, has been accomplished by the centrifugation of a spin column, with multiple samples processed simultaneously. Recently, we reported the HPLC-diode array detection and GC-MS detection of drugs in biological matrices using this spin column.[15,16] In this study, we developed a rapid, simple, and specific GC-MS method that uses monolithic spin-column extraction to determine the eperisone in human serum for toxicological screening. We also performed a validation test and used this method to analyze the eperisone in a serum sample obtained from an eperisone overdose patient with torsade de pointes.

MATERIALS AND METHODS

Eperisone hydrochloride, methanol, and acetonitrile (HPLC grade) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), while tolperisone hydrochloride was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and the derivatization mixture of *N*-methyl-*N*-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) + 1% *tert*-butyldimethylchlorosilane (tBDMCS) was obtained from Thermo Scientific (Rockford, IL, U.S.A.). The monolithic spin-extraction column (MonoSpin® C_{18}) was purchased from GL Science (Tokyo, Japan). The surface of a monolithic silica gel was chemically modified to contain octadecyl groups (C_{18}) and added to the spin column. Blank serum samples were collected from volunteers after obtaining informed consent and were used to prepare serum standards for validating this method. Blank serum samples were obtained after centrifugation of the volunteers' whole blood.

Preparation of Standard and Internal Standards —— The primary stock solutions of the analyte and internal standards (IS) (tolperisone) were

prepared in methanol (1 mg/ml) and stored at 4°C. Eperisone was diluted in methanol to obtain 0.1, 1, 10, and 100 μg/ml working stock solutions, and on the day of analysis, these were used to prepare standards for the calibration curve control (CC). Another set of working stock solutions with the same concentrations was prepared as the quality control (QC) samples. These stock solutions, CC and QC, remained stable for 3 months at 4°C. The CC and QC working stock solutions of eperisone were individually spiked into blank serum as the CC and QC samples. The IS working stock solution (1 μg/ml) was prepared in methanol using a primary stock solution (1 mg/ml). Calibration standards were prepared each day by spiking 0.2 ml of pooled blank serum with 0.1, 1.0, 10, and 100 μg/ml working solutions of eperisone and 10 μl of 1 μg/ml IS. To determine the precision, accuracy, and detection limits, samples were prepared by spiking the blank serum to obtain 2 ng/ml (the lower limit of quantitation, LOQ), 20 ng/ml (low QC), 200 ng/ml (medium QC), and 2000 ng/ml (high QC).

GC-MS Conditions —— GC-MS was carried out using an Agilent 6890N GC system (Agilent Technologies, Santa Clara, CA, U.S.A.) equipped with an Agilent 5975B mass-selective detector. An HP-5MS capillary column [30 m × 0.25 mm internal diameter (i.d.), 0.25 μm film thickness; J & W Scientific, Folsom, CA, U.S.A.] crosslinked with 5% phenyl and 95% methylpolysiloxane was used with helium (99.999% grade purity) as the carrier gas. The helium flow rate was maintained at 1 ml/min using an electronic pressure control. The sample was injected at an inlet temperature of 250°C in the splitless mode. The following sequence of temperatures was used in the chromatographic oven: 100°C for 3 min, followed by an increase of 20°C/min up to 300°C, after which the temperature was maintained at 300°C for 1 min. The chromatographic run was completed in 14 min. The mass spectra were obtained at 70 eV in the electron ionization (EI) mode. The source and quadrupole temperatures were maintained at 230°C and 150°C, respectively. The solvent delay was set 10.0 min. The eperisone and tolperisone were quantified in the selected-ion monitoring (SIM) mode and monitored at *m/z* 98.0.

Sample Preparation —— The IS solution (10 μl) and distilled water (0.2 ml) were added to 0.2 ml of a serum sample and a sample or analyte was extracted using the method described below.

The monolithic spin columns were conditioned by adding 0.2 ml of acetonitrile and centrifuging at 2500 × *g* for 30 s. Distilled water (0.2 ml) was then added to each column, and they were centrifuged at 2500 × *g* for 30 s. The samples were then placed in the conditioned spin columns, and these were centrifuged at 2500 × *g* for 1 min. Then, 0.2 ml of 5% methanol (5% concentrated methanol in water) was added to each spin column, and they were centrifuged at 8000 × *g* for 5 min. Finally, the analyte was eluted using 25 μl of acetonitrile, collected in a 10-ml glass test tube, and centrifuged at 2500 × *g* for 1 min. We added 25 μl MTBSTFA + 1% tBDMCS to the collected eluent. The mixture was then vortexed for 15 s at room temperature, and 1 μl of this sample was injected into the GC-MS system.

Validation Procedures —— We performed a full validation according to the United States Food and Drug Administration (U.S. FDA) guidelines for the assay of serum samples.[17] The selectivity, linearity, sensitivity, accuracy, precision, recovery, and stability of the analytical method were validated.

Application —— A 67-year-old man was found unresponsive near his car, and he was transferred to our hospital. At admission, his score was 3 on the Glasgow Coma Scale [a neurological scale in which patients are scored between 3 (deep coma or death) and 15 (fully awake)], and his hemodynamic parameters were normal. No information on his medication history was available. Routine toxicological serum screening was performed using LC-MS, and 110.8 ng/ml of triazolam was found in his serum. Additionally, his QT interval gradually prolonged to 820 ms, with the manifestation of torsades de pointes after hospitalization. We therefore initiated percutaneous cardiopulmonary support and warfarinization treatment. Finally, the patient became alert, but he experienced an episode of torsades de pointes. He stated that he had ingested a few hundred tablets of Myonal® (eperisone hydrochloride; 50 mg tablets) to commit suicide. His QT interval had almost returned to the reference range on 6 days.

RESULTS AND DISCUSSION

MS Analysis

Figure 1 shows the mass spectral patterns of eperisone and IS with tBDMCS. Although the base peak for eperisone and IS was at *m/z* 98.0, its molecular ion peak could not be clearly observed. Moreover, the fragment ion peaks of eperisone and IS were very weak (< 10%). The low abundance or

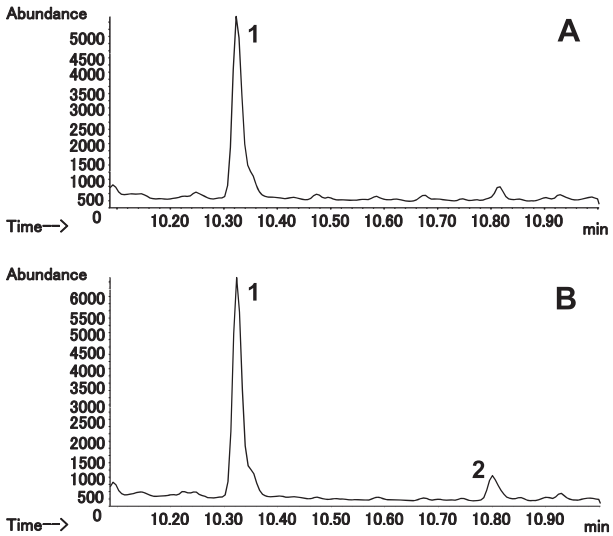

Fig. 2. Signal Chromatogram Obtained in the SIM (*m/z* 98.0) Mode by Extraction with a Monolithic Spin Column from Blank Serum Spiked with the IS (A) and Blank Serum Spiked with a Lower QC (2 ng/ml) and the IS (B)

Detected are: 1 = tolperisone (IS), and 2 = eperisone.

absence of molecular ion is a typical characteristic of eperisone in GC-MS.[13] Therefore, eperisone and IS were monitored at *m/z* 98.0. Because eperisone and IS are monitored by only one ion, and this is a low weight ion, we need to be more careful when analyzing a low concentration of eperisone.

Figure 2 shows typical chromatograms for the blank serum spiked with the IS and the blank serum spiked with eperisone and the IS at the LOQ (2 ng/ml). No significant interference was found at the IS and eperisone retention times, which were 10.33 and 10.80 min, respectively.

Sensitivity and Mass Spectral Pattern

Although underivatized eperisone and IS were detected using GC-MS with a splitless injection, a wide peak width and high LOD were obtained. No difference was noted between the mass fragments of authentic eperisone and IS (without the derivatization reagent) and the sample derivatized with MTBSTFA in 1% tBDMCS. Hence, eperisone and IS were not derivatized by MTBSTFA in 1% tBDMCS. However, the eperisone and IS detection sensitivity increased by more than 4 times after injecting a sample derivatized with MTBSTFA in 1% tBDMCS. First, we speculate that the difference in the GC-MS response was the result of solvent recondensation in the column inlet during splitless injection. The recondensation of the solvent strongly increases the rate of transfer into the column and improves the shape of the peak. However, one may also get a concentration effect if the analytes are very high boiling, but this is not the case. The split splitless injector conditions may not have been optimal. Although eperisone and IS were not derivatized with MTBSTFA in 1% tBDMCS, we used it to achieve eperisone and IS sensitivity. Therefore, to achieve complete dehydration, acetonitrile was added after centrifugation at 8000 rpm. Centrifugation is essential to remove the water from a monolithic spin column.

Evaluation of Interference

No peaks for endogenous compounds were observed at the retention times for the eperisone and IS in the blank serum extracts. This absence of endogenous interference at the retention times of the compounds clearly demonstrates the high selectivity of the method.

Linearity and Calibration Curve

A calibration curve was obtained by plotting the peak-area ratio of the eperisone to IS against the respective concentrations. The final concentrations of the calibration standards plotted on the calibration curve were 2, 5, 10, 25, 100, 500, 1000, and 2500 ng/ml. The results were fitted to a linear regression analysis model using $1/X^2$ as the weighting factor. It was necessary for the calibration curve to have a correlation coefficient (r) of 0.995 or better. The acceptance criterion for each back-calculated standard concentration was ± 15% deviation from the nominal value, except at the LOQ, where it was set at ± 20%.[17]

The serum calibration curve was constructed using 8 calibration standards (2–2500 ng/ml). Excellent linearity was obtained within this range. The calibration curve was drawn by determining the best fit of the peak-area ratios (peak area analyte/peak area IS) versus the concentration and fitted to $y = mx + c$ using the weighing factor ($1/X^2$). The regression equation for eperisone was $y = 0.0132x - 0.0285$. The average regression ($n = 3$) was found to be ≥ 0.996. The overall linearity was acceptable for the quantification of eperisone in serum. The accuracy of the mean value of the back-calculated concentrations from 3 calibration curves was in the range of 95.0–98.3, whereas the % of the relative standard deviation (RSD) values were in the range of 7.9–10.5 (Table 1).

Table 1. Validation Data of Eperisone from Human Serum

		2 ng/ml	20 ng/ml	200 ng/ml	2000 ng/ml
Accuracy (%)		95.0	98.3	97.0	98.3
Precision RSD (%)		10.5	9.9	7.9	10.0
Intra-day	calculated conc.	1.9 ± 0.2	19.8 ± 2.5	195.8 ± 17.4	2028.1 ± 108.8
	RSD (%)[a)]	11.2	12.6	8.9	5.4
Inter-day	calculated conc.	2.0 ± 0.1	19.9 ± 0.7	201.0 ± 3.3	2008.7 ± 28.4
	RSD (%)[b)]	4.6	3.4	1.7	1.4
Extraction recovery (%)[c)]		93.4 ± 11.7	96.0 ± 9.5	92.8 ± 7.3	93.6 ± 9.4

conc.: concentration. *a*) Intra-day accuracy and precision results were obtained from six replicate samples ($n = 6$) for each concentration of the analyte analyzed on a single day. *b*) Inter-day accuracy and precision results were measured using a minimum of five determinations per concentration of the analyte on three separate days. *c*) Data are expressed as mean ± S.D.

Lower LOD and LOQ

The sensitivity was expressed in terms of the LOQ, which is defined as the injected amount that results in a peak with a height at least 10 times higher than the baseline noise level, and in terms of the LOD, with a peak height to baseline ratio of 3 : 1. The LOQ is acceptable if the analyte peak response is identifiable and reproducible with a precision of 20% and an accuracy of 80–120%.

The LOD was found to be 0.5 ng/ml. The lowest concentration with an RSD of less than 20% was considered to be the LOQ, and was determined to be 2 ng/ml.

Accuracy and Precision

The intraday assay precision and accuracy were estimated by analyzing 6 replicates at 4 different QC levels, *i.e.*, 2, 20, 200, and 2000 ng/ml. The interassay precision was determined by analyzing the 4 QC samples during 3 different runs. The criteria for the acceptability of the data were: accuracy within ± 15% of the standard deviation (S.D.) from the nominal values and precision within ± 15% of RSD, except for the LOQ, where the criterion was ± 20% for both the accuracy and precision.[17)]

The accuracy and precision data for the intra- and interday serum samples and the recovery analysis data are presented in Table 1. The intra- and interassay precisions were satisfactory at the 4 different concentrations, *i.e.*, all of the values were less than 12.5% for eperisone, which was acceptable and feasible for the purpose of this study. The intra- and interday assay values were found to be within the accepted variable limits.

Recovery

The recovery of eperisone after the spin-column extraction procedure was determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 6$) with those of the analytes from the postextraction serum standard (spiked serum samples after extraction). The recovery of eperisone was determined at 4 QC concentrations.

The recovery values for the pre- and postextraction serum standards were calculated and compared for eperisone concentrations of 2, 20, 200, and 2000 ng/ml, and the absolute recovery values were determined to be 93.4, 96.0, 92.8, and 93.6%, respectively (Table 1).

Stability

The stability of the serum standards was evaluated by assaying the analyte during sample collection, handling, after short-term storage at room temperature for 48 hr, storage in the refrigerator at 4°C for 1 week, storage in the freezer at −30°C for 4 weeks, and after 3 freeze-thaw cycles. The frozen samples were kept at room temperature for 8 hr until completely thawed. The samples were then refrozen for 16 hr under the same conditions. This freeze-thaw cycle was repeated twice, and the sample was analyzed after the third cycle. The stability of the samples after 24 hr was evaluated by repeated injection. Samples were considered to be stable if the assay values were within the acceptable limits of accuracy (*i.e.*, ± 15% S.D.) and precision (*i.e.*, ± 15% RSD).

The stability data (extraction; $n = 3$) are summarized in Table 2. This study examined the stability of eperisone in the serum and in an autosampler vial. The stability of eperisone in an autosampler vial stored at room temperature for 24 hr was examined by repeated assays of the same sample, and these results were compared with those for the freshly prepared standard. No significant changes were detected in the samples.

Table 2. Stability of Eperisone in Human Serum

Nominal concentration of eperisone (ng/ml)	Room temp. (24°C) for 48 hr	4°C for 1 week	Freeze-thaw (3 cycles)	−30°C for 4 weeks	Repeated injection after 24 hr (%)[a)]
2 ng/ml[b)]	N.D.	detected[c)]	detected[c)]	detected[c)]	detected[c)]
mean %	—	—	—	—	—
%RSD	—	—	—	—	—
20 ng/ml[b)]	5.4 ± 1.8	16.3 ± 0.8	19.8 ± 2.6	16.2 ± 2.3	106.3 ± 0.5
mean %	27.0	81.5	99.0	81.0	
%RSD	33.8	5.9	15.5	13.7	0.5
200 ng/ml[b)]	52.9 ± 17.4	163.9 ± 6.0	176.2 ± 12.4	178.6 ± 30.7	103.7 ± 2.9
mean%	26.5	82.0	88.1	89.3	
%RSD	32.9	3.7	7.0	17.2	2.8
2000 ng/ml[b)]	790.0 ± 96.0	1632.0 ± 57.2	1787.3 ± 129.9	1755.3 ± 166.8	101.3 ± 1.6
mean%	39.5	81.6	89.4	87.8	
%RSD	37.5	3.5	7.3	9.5	1.5

n = 3 each. N.D. = not detected. *a*) These data were compared with data of the first injection data and calculated as mean ± S.D. *b*) Detected concentrations (ng/ml) were calculated as mean ± S.D. without repeated injection data. *c*) Although these peaks were detected, quantitation of all values were less than 2 ng/ml.

The short-term stability of the compounds in the serum was tested by storing samples in a refrigerator at 4°C for 1 week, after which the samples were extracted and compared with a freshly extracted sample. The concentrations calculated from the calibration curve showed that degradation occurred at 4 QC concentrations. Moreover, the stability test for the compound in the serum revealed that the compound was not stable after 48 hr at room temperature. However, the long-term stability test revealed that the serum samples spiked with the different concentrations of the compound were stable for 4 weeks when stored at −30°C, excluding the low QC samples (2 and 20 ng/ml). Further, there was no significant decrease in the serum concentrations after the samples were exposed to 3 freeze/thaw cycles, except for the low QC sample (2 ng/ml), and the mean recovery ranged from 88.1% to 99.0%.

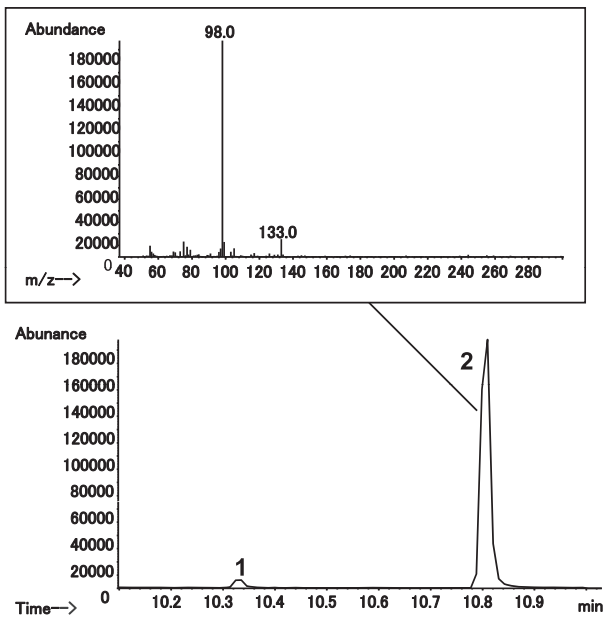

Fig. 3. SIM and SCAN Mode Signal Chromatogram Extracted by Monolithic Spin Column from Serum Diluted 10 Times with IS Obtained from an Eperisone Overdosed Patient at Hospitalization

Detected are: 1 = tolperisone (IS), and 2 = eperisone.

Application

The serum samples were collected from the patient every 6 hr after hospitalization. No urine samples were collected for toxicological analyses. All the serum samples were kept in the freezer at −30°C for 20 days until just before the analyses. The GC-MS analysis proved the presence of a very large amount of eperisone (Fig. 3). The eperisone concentrations in the serum samples are presented in Fig. 4. These concentrations were almost unchanged according to the stability study (Table 2). The highest concentration of eperisone was observed at the time of the patient's arrival at the hospital: 15.3 μg/ml. This sample was analyzed after being diluted 10 times. Although the eperisone concentration decreased to 1.9 μg/ml 17 hr after hospitalization, this is still 270 times higher than the therapeutic concentration.[10)] More data are required to determine the therapeutic levels of eperisone; however, this result can be explained by the possibility that eperisone might have induced torsade de pointes. Moreover, the QT interval normalized with the decrease in

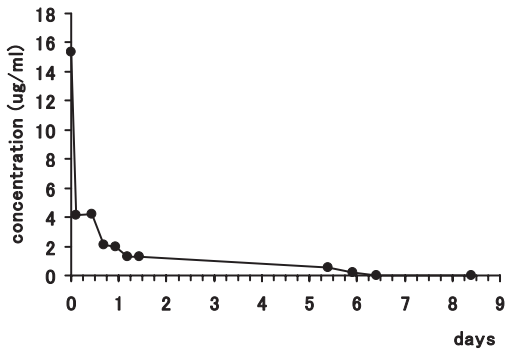

Fig. 4. The Time Course of Eperisone Concentrations in the Serum of the Eperisone Overdose Patient

eperisone concentration. Eperisone was not detected 7 days after hospitalization.

Comparison with Other Analytical Methods

When compared with previously reported GC and GC-MS methods, our method has some analytical advantages, such as higher selectivity and sensitivity than GC-NPD[14] and the avoidance of nonspecificity, the ability to use a smaller sample volume than that required for the GC-MS method,[13] and a shorter sample preparation time than the previous SPE method.[13,14] Although extraction methods are necessary to evaporate the elution solvent, evaporation after extraction or derivatization may lead to the loss of some volatile compounds. However, the sample preparation time can be reduced by monolithic spin-column extraction. Thus, one important advantage of this method is that it does not require dry conditions, incubation, or heating for derivatization. The monolithic spin-column extraction and GC-MS analysis made it possible to separate the eperisone and IS within 30 min. We identified 2 limitations of this method compared to the previous LC-MS methods.[9,12] These limitations were related to the LOD and nonrequirement of derivatized samples. Although the sensitivity of our method is suitable for the quantification of toxic levels of eperisone, it is not suitable for the therapeutic concentration of eperisone. Side effects such as torsade de pointes are probably induced by extraordinary high concentrations of eperisone in serum, as compared to the therapeutic concentrations. Therefore, a rapid analytical method is necessary for toxicological screening.

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