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Simultaneous determination of amitraz and its metabolite in human serum by monolithic silica spin column extraction and liquid chromatography–mass spectrometry

Takeshi Saito[a,*], Rie Yamamoto[a], Shigeaki Inoue[a], Izumi Kishiyama[b], Shota Miyazaki[b], Akihiro Nakamoto[c,d], Manami Nishida[d], Akira Namera[d], Sadaki Inokuchi[a]

[a] Department of Emergency and Critical Care Medicine, Tokai University School of Medicine, Isehara, Kanagawa 259-1143, Japan
[b] GL Sciences Inc., Japan
[c] Scientific Investigation Laboratory, Hiroshima Prefectural Police Headquarters, Japan
[d] Department of Legal Medicine, Graduate School of Biomedical Sciences, Hiroshima University, Japan

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ABSTRACT

A simple, rapid, sensitive, and specific liquid chromatography–mass spectrometry (LC–MS) method was developed and validated for the quantification of amitraz and its metabolite in human serum. Both the compounds were extracted using monolithic silica spin columns with acetonitrile. The chromatographic separation was performed on a reverse-phase C_{18} column with a mobile phase of 10 mM ammonium formate–acetonitrile. The protonated analyte was quantitated in positive ionization by mass spectrometry. The method was validated over the concentration range of 25–1000 ng/ml for amitraz and its metabolite in human serum. For both compounds, the limit of detection was 5 ng/ml. The method was applied to serum samples taken from an attempted suicide patient, and only small volumes of serum were required for the simultaneous determination of these compounds.

1. Introduction

Amitraz (*N*′-2,4-(dimethylphenyl)-*N*-[(2,4-dimethylphenyl) imino]methyl-*N*-methyl-methanimidamide) (Fig. 1) is a formamidine derivative widely used for the control of ticks and manage mites in animals. In the body, it is metabolized to *N*-2,4-(dimethylphenyl)-*N*′-methylformamidine (Fig. 1). Amitraz is associated with a number of side effects, the commonest being transient sedation or lethargy. Other side effects are bradycardia, hypothermia, hypotension, polyuria, vomiting, and hyperglycemia [1,2].

Chromatographic methods for the analysis of amitraz in plasma have been described in several previous publications [3–5]. Furthermore, Chou et al. reported a gas chromatograph–mass spectrometry (GC–MS) method for quantitatively measuring amitraz and amitraz metabolite in urine [6]. To the best of our knowledge, there has been no method reported in the literature for the simultaneous determination of amitraz and its metabolite using LC–MS.

In recent years, monolithic silica has been widely used in high-performance liquid chromatography (HPLC) columns [7,8]. Monolithic C_{18} silica differs from classical silica in that it consists of silica rods instead of particles. These monoliths provide a high surface area for adsorption of the analyte of interest. Variable pore size and porosity can be achieved based on concentration. Shintani et al. [9], for example, reported the extraction of phenol in water by using an in-tube solid-phase microextraction monolithic silica column.

As a new tool for sample preparation of compounds in biological materials, monolithic silica is packed into spin columns (Fig. 2). In these columns, the structure of the monolithic silica comprises the support body with a surface area per unit volume that is large in comparison with particle-type silica. The various preparation steps such as sample loading, washing, and the elution of target compounds can all be achieved by centrifugation of the spin column. Furthermore, this extraction method can prepare many samples simultaneously using only centrifugation, and without the need for evaporation. We have accordingly applied the characteristics of monoliths to an extraction column.

Hence, the main objective of this work was to develop a simple, sensitive, rapid, and reliable LC–MS method using spin column extraction for the simultaneous quantification of amitraz and its metabolite in human serum.

* Corresponding author. Tel.: +81 463 93 1121; fax: +81 463 95 5337.
E-mail address: saito@is.icc.u-tokai.ac.jp (T. Saito).

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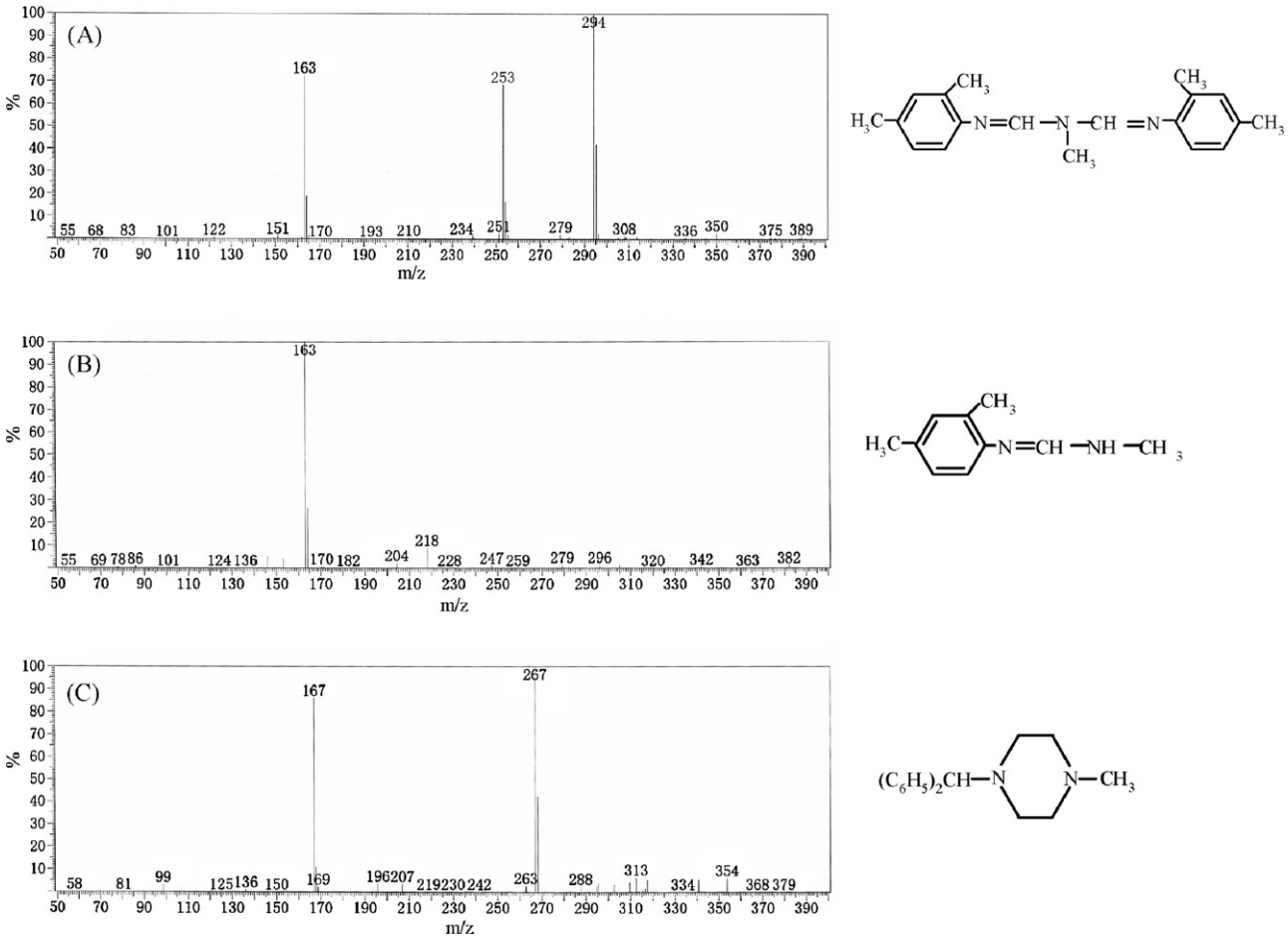

Fig. 1. Atmospheric pressure chemical ionization mass spectrum and chemical structures of amitraz (A), amitraz metabolite (B), and cyclizine (IS) (C).

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile and ammonium formate were purchased from Wako (Osaka, Japan). High-purity water was obtained from a Milli-Q water system (Millipore, Billerica, MA, USA). A solution of 10% ammonium formate was filtered before use through a 0.45-μm membrane (Millex, Millipore). HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Amitraz and its metabolite were both obtained from Wako. Cyclizine was purchased from Sigma–Aldrich Japan (Osaka, Japan) and used as the internal standard (IS) for amitraz and amitraz metabolite. Spin columns and C_{18}-bonded monolithic silica were purchased from GL Sciences (Saitama, Japan).

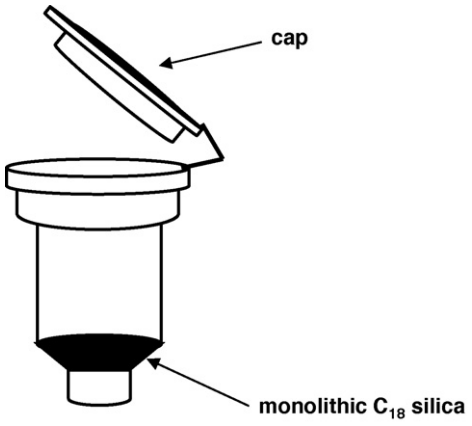

Fig. 2. Schema of a monolithic spin column.

2.2. Standard solutions

Separate solutions containing 1 mg/ml of amitraz, amitraz metabolite, and IS were prepared using acetonitrile. Amitraz and its metabolite solutions were further suitably diluted with the diluent to obtain working solutions of 10 and 1 μg/ml, respectively. The IS solution was further diluted to 10 μg/ml. All solutions were stored at −30 °C.

2.3. Calibration curves

A six-point standard calibration curve for amitraz and amitraz metabolite was prepared by spiking blank serum with appropriate amounts of the analytes and the IS. For both analytes, standard solutions were prepared with human serum to yield final concentrations of 25, 50, 100, 250, 500 and 1000 ng/ml. Three quality control (QC) samples were prepared at three concentration levels of (30, 300, and 900 ng/ml) for both the analytes. Calibration curves were plotted with the peak area ratio of the drug and IS on the *Y*-axis and concentration on the *X*-axis.

2.4. Sample preparation and extraction

Spin column extraction was used for the extraction of the compounds from the serum. Calibration standards and quality control samples were treated with 0.2 ml serum. Twenty-five microliters of IS (250 ng), calibrator, or QC sample and 0.3 ml water were added to each serum sample (0.2 ml), vortex mixed for 0.25 min, and then applied to the spin column.

The spin columns were conditioned by adding 0.2 ml acetonitrile to the column and then centrifuging at 2500 rpm for 1 min. Distilled water (0.2 ml) was then added, followed by a further centrifugation at 2500 rpm for 1 min. The samples were then applied to the conditioned spin columns and centrifuged at 2500 rpm for 5 min. Distilled water (0.2 ml) was then added to the columns and centrifuged at 2500 rpm for 1 min. Following this spin, 0.2 ml 5% methanol was added and centrifuged at 2500 rpm for 1 min. Finally, 100 μl acetonitrile was added to the columns and the residual compounds were eluted by centrifugation at 2500 rpm for 1 min. Ten-microliter samples were injected into the LC system.

2.5. LC–APCI-MS conditions

A Shimadzu 8000α single quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (Shimadzu Corp., Kyoto, Japan) and an LC 10 HPLC system (Shimadzu Corp., Tokyo, Japan) were used for LC–MS analysis. Isolation was performed using an XTerra MS C_{18} column (150 mm × 2.1 mm, 3.5 μm; Waters, Japan), and the column temperature was maintained at 50 °C. The mobile phase, containing 10 mM formic ammonium (A) and acetonitrile (B), was eluted according to the following gradient conditions: time = 0 min, 0% B; time = 2 min, 100%; time = 9.5 min, 100%; time = 10 min, 0%. The flow rate was set at 0.2 ml/min. The injection volume was 10 μl and the total run time was 10 min.

Full-scan data acquisition was performed over the range *m*/*z* 50–400. The mass spectrometer was operated in selected ion monitoring (SIM) positive ion mode, under the conditions defined by the mass characterization. High-purity nitrogen gas was used as a nebulizer gas, set at 2.5 l/min. The instrumental parameters of APCI were as follows: vaporizer temperature, 400 °C; curve desolvation line (CDL) temperature, 250 °C; CDL voltage, −40 V; deflector voltage, 20 V; interface voltage, 11.9 μA. The MS was operated to generate at *m*/*z* 294, 163, and 253 for amitraz, *m*/*z* 163 for amitraz metabolite, and *m*/*z* 267 for the IS (Fig. 1). The ions of *m*/*z* 294, *m*/*z* 163, and *m*/*z* 267 were the most abundant and were selected for quantitation of amitraz, amitraz metabolite, and IS, respectively. Instrument control, data acquisition, and analysis were performed using Class 8000 version 1.2 data system software.

2.6. Validation

The accuracy, precision, recovery and stability of the analytical method were validated in accordance with the US Food and Drug Administration guidelines [10].

2.7. Linearity and LLOQ

In order to establish linearity, a series of calibration standards were prepared by analyzing known concentrations of amitraz, its metabolite, and the IS added to drug-free human serum. The lowest concentration on the standard curve with a detector response five times greater than the drug-free (blank) human serum was considered to be the limit of quantification (LOQ). The analyte peak in the LOQ sample should be identifiable, discrete, and reproducible with a precision of 20% and an accuracy of 80–120%.

2.8. Accuracy and precision

The intra-day precision and accuracy of the assay were evaluated by running three validation batches on three separate days. Each batch consisted of one set of calibration standards and five replicates of three QC samples. The inter-day precision and accuracy were also assessed in a similar manner. A comparison was made between the obtained values and the experimental values. Precision was expressed as percentage relative standard deviation (% RSD). The mean value of accuracy should be within 15% of the actual value except at the LOQ, where it should not deviate by more than 20%. The precision determined at each concentration level should not exceed 15% RSD except for the LOQ, where it should not exceed 20% RSD.

2.9. Extraction recovery and matrix effect

Three QC solutions of amitraz, its metabolite, and the IS (250 ng) were added to the blank serum (0.2 ml). Three QC samples of amitraz and its metabolite were then extracted. After extraction, the samples were injected into the LC–MS system. The method recovery was determined by comparing the peak-area ratios of amitraz or its metabolite with the IS of the extracted samples, which were calculated from the respective calibration curves.

2.10. Stability

The stability of amitraz and its metabolite in serum was studied at room temperature (20–24 °C) for 48 h, 4 °C for 1 week, −30 °C for 4 weeks, and three freeze–thaw cycles. Three triplicates of QC samples at the three concentration levels were processed and stored in 1.5-ml centrifuge tubes, then assayed in order to assess processed sample stability. The stability of aqueous standard solutions was also evaluated. Stability is considered acceptable if the mean value is within 15% of the theoretical value at each concentration.

2.11. Application

A 38-year-old man who had lost consciousness was admitted to the emergency department of our University Hospital. Serum, urine, and stomach washing solution samples were collected at this time for toxicological analysis. Toxicological screening of gastric contents by GC–MS revealed the presence of amitraz pesticide. The serum sample was stored at −30 °C prior to analysis, and was subsequently prepared according to the sample preparation method described above. Sample concentrations were quantified using a standard curve.

3. Results

Typical chromatograms of blank serum, spiked serum, and extracts obtained from the amitraz-poisoned patient are shown in Figs. 3 and 4. A good selectivity was demonstrated, since the peaks of three compounds tested, were co-eluted with the retention times of amitraz, its metabolite, and the IS.

A good linearity was obtained after the simultaneous analysis of amitraz and its metabolite in serum. The data for the linearity studies are expressed as the intercept, the slope of the linear function as a mean, the standard error of the mean (SEM), and the linear correlation coefficient (r^2). For amitraz over the concentration range 25–1000 ng/ml, the intercept was 0.1614 (SEM: 0.0015); the slope, 0.0036 (SEM: 0.0035); and r^2 0.994. Similarly, for amitraz metabolite over the same concentration range, the intercept was 0.1557 (SEM: 0.0013); the slope, 0.004 (SEM: 0.0031); and r^2 0.999.

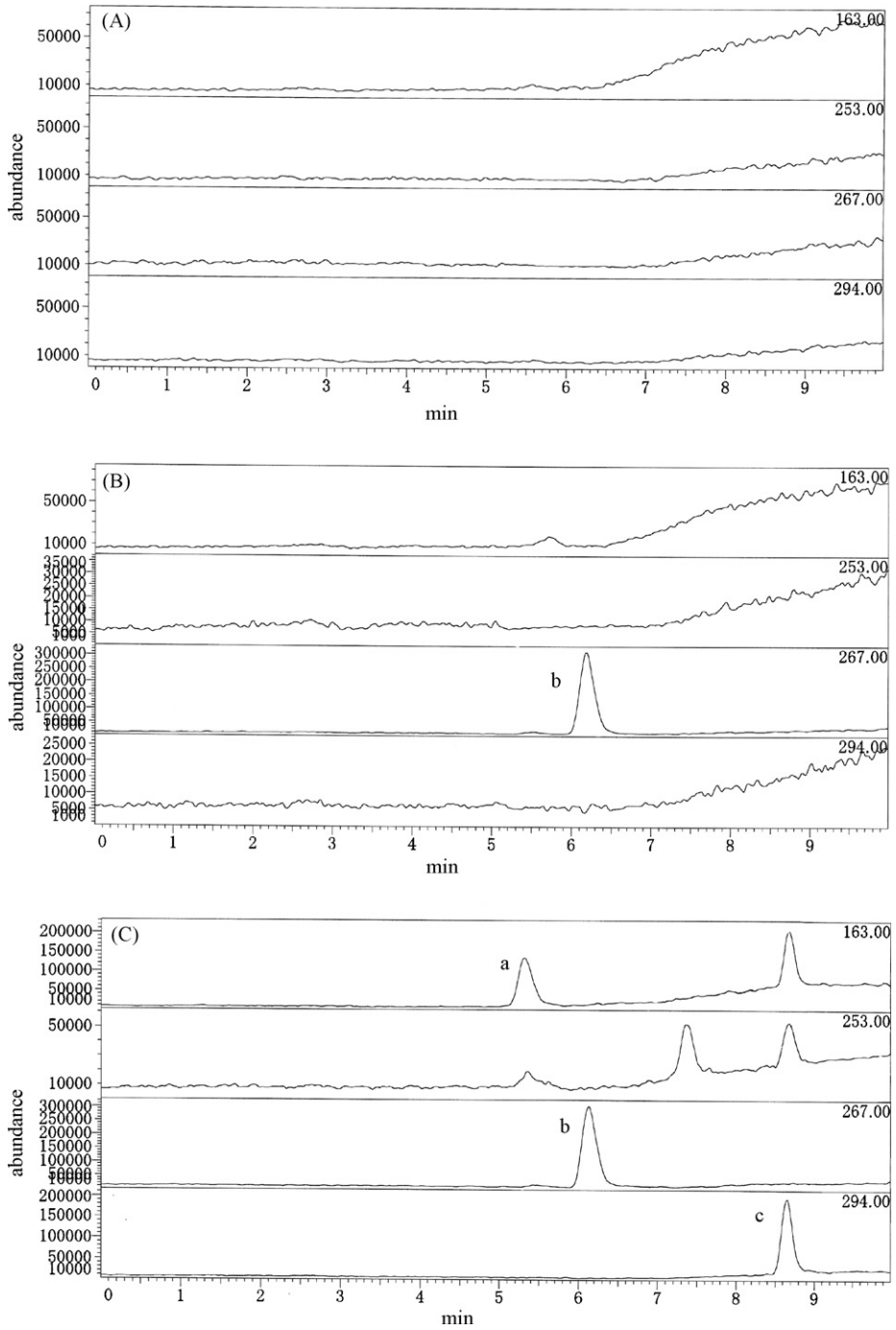

Fig. 3. LC–MS-SIM chromatograms obtained by monolithic silica spin column extraction of (A) blank human serum; (B) blank human serum with internal standard (IS); (C) blank human serum with medium QC (amitraz, 300 ng/ml and its metabolite, 300 ng/ml) and IS. (a) amitraz metabolite; (b) cyclizine (IS); (c) amitraz.

The LOQ and LOD values for both amitraz and amitraz metabolite were 10 and 5 ng/ml, respectively. Good results were obtained for accuracy, intra-day and inter-day precision, and recovery (Table 1).

The extraction recovery was determined by comparing the peak-area ratios of amitraz and its metabolite with the IS of the extracted samples with the peak-area ratios obtained from direct injection of a standard solution containing the same concentration of amitraz, or its metabolite and the IS. The extract recovery of the assay was 91.2–105% (Table 1, $n = 5$).

Table 2 summarizes the results of a stability study carried out under various conditions. Both the analytes were found to be unstable at room temperature for 48 h and at 4 °C for 1 week in human serum. The three QC samples of both the analytes remained unaffected at −30 °C for 1 month. Moreover, the freeze–thaw stability results demonstrated that amitraz and its metabolite are stable for at least three freeze–thaw cycles. The percentage of accuracy obtained was over 95.5% and 92.2% for amitraz and its metabolite, respectively. The stability results indicated that human serum samples could be thawed and refrozen without compromising the integrity of the samples.

The working solutions of amitraz metabolite and the IS were stable and the deviation was less than ±2%. There was little degradation in the solutions even after 30 days.

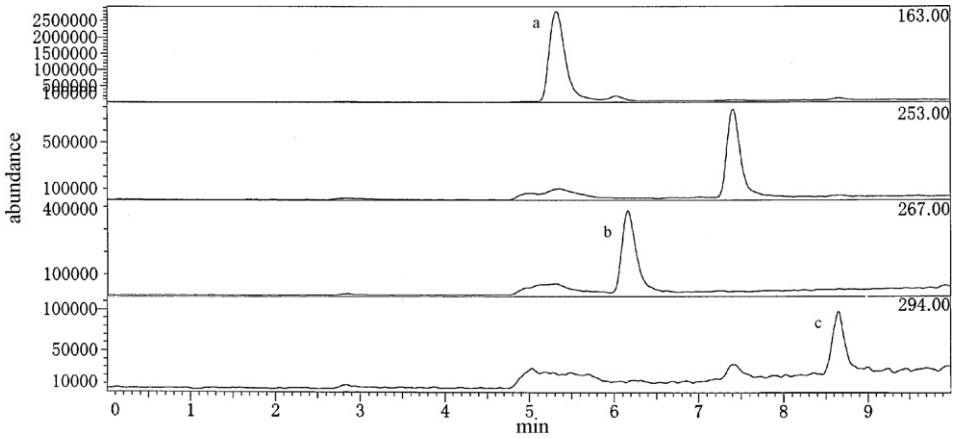

Fig. 4. LC–MS-SIM chromatograms obtained by monolithic silica spin column extraction of serum from an amitraz-poisoned patient with IS. (a) amitraz metabolite, (b) IS, and (c) amitraz.

Table 1
Validation characteristics of amitraz and its metabolite in human serum

Nominal concentration (ng/ml)		30 ($n=5$)	300 ($n=5$)	900 ($n=5$)
Accuracy (%)	Amitraz	91.71	92.23	97.47
	Metabolite	96.28	94.82	98.25
Precision (RSD%)	Amitraz	9.82	8.64	10.37
	Metabolite	8.74	9.46	9.76
Intra-day (RSD%)[a]	Amitraz	6.3	5.8	8.1
	Metabolite	5.3	6.8	7.4
Inter-day (RSD%)[b]	Amitraz	7.4	8.2	9.3
	Metabolite	6.2	7.7	8.9
Extraction recovery (%)[c]	Amitraz	91.2 ± 3.6	93.7 ± 4.3	96.1 ± 5.2
	Metabolite	94.3 ± 3.7	94.6 ± 5.1	105.0 ± 2.4

[a] Intra-day accuracy and precision results were obtained from five duplicate samples ($n=5$) for each concentration of the analyte analyzed on a single day.
[b] Inter-day accuracy and precision results were obtained by analyzing five duplicate samples for each concentration of the analyte on three separate days.
[c] Data are expressed as mean ± SD.

4. Application

Human serum sample from an amitraz poisoning case were analyzed and quantified by reference to a standard curve. The concentration of serum amitraz and its metabolite determined for samples collected from the patient were 96.2 and 2071 ng/ml, respectively (Fig. 4). However, since we could find no relevant information in the literature regarding amitraz and its derivative in human serum, we were unable to obtain any comparative data.

5. Discussion

Extraction methods for the determination of amitraz in plasma using solid-phase extraction have been reported in the literature [5,6]; however, no analytical procedure for the simultaneous extraction of amitraz and its metabolite in serum has been previously described.

The method we describe here for amitraz and its metabolite requires lower volumes of serum for the clean up of biological matrices. In addition, the elution of compounds from spin columns enabled us to use a lower volume of elution solvent. Moreover, the elution solvent could be injected into the LC–MS system without the risk of evaporation. Compared with other procedures reported in the literature, the present technique was found to be advantageous with respect to selectivity and sensitivity, guaranteed stability, precision, and accuracy.

We detected an unknown peak at m/z 253 at approximately 7.4 min. This peak was often detected in the calibrator sample and in the QC sample analysis. Thus, this unknown peak may represent a metabolite of amitraz. However, we were unable to clarify the origin of the peak.

In cases of acute poisoning resulting from an unidentified source, rapid toxicological screening is usually necessary. Consequently, a rapid and reliable assay method is essential when analysis is required in a short period of time. An ideal method should entail simple sample preparation, and sensitive and specific detection. Spin column extraction in conjunction with LC–MS is one such efficient analytical tool that meets most of the above criteria. We have accordingly applied a rapid and sensitive extraction method using a monolith spin column for microflu-

Table 2
Stability of amitraz and its metabolite in human serum

Nominal concentration (ng/ml)		30	300	900
Room temperature (48 h)	Amitraz	ND	ND	0.9 ± 0.4
	Amitraz metabolite	26.5 ± 8.3	28 ± 9.2	32 ± 7.8
4 °C, 1 week	Amitraz	44.3 ± 5.6	51.7 ± 6.2	47.3 ± 5.8
	Amitraz metabolite	57.4 ± 8.2	46.2 ± 5.9	71.4 ± 6.2
−30 °C, 4 weeks	Amitraz	97.3 ± 3.5	96.8 ± 4.2	98.3 ± 5.7
	Amitraz metabolite	98.2 ± 4.6	97.6 ± 5.7	98.4 ± 4.3
Freeze thaw (3 cycles)	Amitraz	96.4 ± 4.9	95.5 ± 5.2	98.0 ± 6.1
	Amitraz metabolite	92.2 ± 4.8	93.5 ± 6.1	99.0 ± 5.6

Data are expressed as percent accuracy $n=3$ each.

idic trapping and concentration of amitraz and its metabolite in serum.

6. Conclusion

A rapid and highly sensitive method for the determination of amitraz and its metabolite was developed using monolithic spin columns in conjunction with LC–MS. The method exhibits high throughput capability owing to the short time required for extraction. Efficient and rapid isolation of compounds is important in the field of analytical toxicology as well as in clinical and pharmaceutical studies. Using monolithic spin column extraction in conjunction with LC–MS analysis, amitraz and its metabolite were separated within 20 min. Both the analytes were found to be stable in human serum for 30 days when stored at −30 °C. A simple and convenient extraction procedure makes this method more feasible for amitraz and its metabolite. It is expected that this method could also be applied in clinical and toxicological studies.

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