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Determination of flumazenil in plasma by gas chromatography–negative ion chemical ionization mass spectrometry

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Abstract

A gas chromatographic–negative ion chemical ionization mass spectrometric (GC–NCI-MS) method for the determination of flumazenil in plasma is described. The GC of flumazenil (M_r 303) is considered to be difficult as it is readily adsorbed in the GC column. Therefore, preconditioning the GC column with reconstituted extract from plasma and Silyl-8 was required to cover the active sites on the column. Monitoring the maximum mass peak (m/z 275) of the flumazenil resulted in a tenfold enhancement of sensitivity and signal-to-noise ratio (concentration = 1 ng/ml). Isotopically labeled flumazenil-d_3 (M_r 306, m/z 278) was used as the internal standard. The detection limit for flumazenil was found to be 0.1 ng/ml with an injection volume of 2 μl. The signal-to-noise ratio was about 10. The routine quantification limit was set at 2 ng/ml for dog plasma and 1 ng/ml for human plasma. The sample volumes in both instances were 1 ml.

1. Introduction

Flumazenil (8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylic acid ethyl ester) is a benzodiazepine antagonist drug. The structure of flumazenil and isotopically labeled flumazenil-d_3, used as an internal standard, are shown in Fig. 1. Flumazenil can bind to the active sites of the column and therefore its GC analysis is difficult. A derivatization technique cannot be used to enhance the sensitivity and improve the peak shape as there is no specific functional group or active hydrogen in flumazenil that could be derivatized. This is a great challenge for the GC–MS technique of flumazenil.

Flumazenil is fragmented (or the molecule is rearranged) in negative chemical ionization mass spectrometry (NCI-MS) and the maximum peak is at m/z 275. Monitoring the ions of m/z 275 for

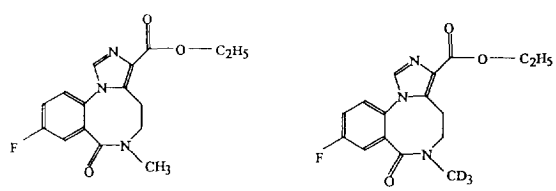

Fig. 1. Structures of flumazenil and flumazenil-d_3.

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flumazenil and m/z 278 for flumazenil-d_3 enhanced the sensitivity to 0.1 ng/ml and the signal-to-noise ratio (S/N) to 10 (concentration = 1 ng/ml). This is the greatest advantage of our method compared with previous GC–MS methods [1,2], in both of which the monitoring ion was at m/z 303 for flumazenil and the sensitivities were 1 ng/ml (sample volume 2 ml) and 5 ng/ml (sample volume 3 ml) for flumazenil. Also, it has a higher sensitivity than the method of Kintz and Mangin [3], in which the monitoring ion was at m/z 229 for flumazenil and the limit of detection was over 1 ng/ml. HPLC methods for the determination of flumazenil have been published [4,5]. However, the sensitivity and selectivity were much lower compared with capillary column GC–MS. Our method has a higher sensitivity over all other methods for testing flumazenil.

Isotopically labeled flumazenil-d_3 was made by replacing three hydrogen atoms with deuterium (see Fig. 1). Therefore, it is identical in structure and chemical properties with flumazenil. Also, it is the best choice for an internal standard, except that preparing this isotopic compound is very expensive. The experiment showed that the stability of flumazenil-d_3 was good; it was not changed for at least 2 years.

The high resolution with a capillary GC column and the selection of ions to be monitored can exclude interferences from the components of biological matrices. Therefore, the proposed method is suitable for the determination of flumazenil in various biological matrices such as dog, human or other plasma.

2. Experimental

2.1. Chemicals

The pure drug compounds flumazenil, (Ro 15-1788/001, log No. 17214-252-1) and flumazenil-d_3 (Ro 15-1788/701, lot No. 18065-215) were generously supplied by Hoffman-La Roche (Nutley, NJ, USA). N,N-Dimethylformamide was purchased from Aldrich (Milwaukee, WI, USA). Dog plasma, lot No. 033194, and human plasma, lot No. KP34764, were obtained from Rockland (Gilbertsville, PA, USA). Toluene, dichloromethane (Baker Analyzed HPLC Reagent) and sodium borate were purchased from J.T. Baker (Phillipsburg, NJ, USA). Heptane was the product of MCB Manufacturing Chemists (Cincinnati, OH, USA). Silyl-8 was obtained from Pierce (Rockford, IL, USA).

2.2. Sample preparation

A 1-ml volume of plasma was pipetted into a test-tube. To each of these samples, 50 μl of internal standard working solution (100 ng/ml) were added. The sample was extracted by adding 2.0 ml of saturated sodium borate buffer solution (pH 10) and 6.0 ml of extraction solution [toluene–dichloromethane (70:30, v/v)]. The tube was capped and vigorously shaken and rotated for 20 min. The sample was centrifuged for 10 min at 750 g and then put in refrigerator at −20°C for 30 min. The organic layer, which contained flumazenil, was transferred into a clean tube and evaporated to dryness at 60°C under nitrogen. The residue was reconstituted with 50 μl of heptane. An aliquot of the reconstituted sample was analyzed by GC–NCI-MS.

2.3. Method of analysis

Analysis was performed on an HP 5890 II gas chromatograph with an HP-5 column (12 m × 0.20 mm ID, 0.33-μm film thickness) and HP 5989 MS Engine equipped with a Model 7673 autosampler, a Model 59940 ChemStation and a Model 39944C (Rev. C. 10.0) HP-UX ChemSystem (Hewlett-Packard, Palo Alto, CA, USA).

The GC carrier gas was helium with a column head pressure of 20 kPa at about 25°C and the flow-rate was maintained at 1 ml/min. The injector temperature was 300°C. The oven temperature program was set from an initial temperature of 130°C to a final temperature of 300°C at a step rate of 30°C/min. With splitless injection, the injection volume was 2 μl. The running time was about 7 min. The mass spec-

trometer was set in the negative ion chemical ionization (NCI) and selected-ion monitoring (SIM) acquisition mode, monitoring ions at m/z 275 and 278. The temperatures of the ion source and quadrupole were 150 and 100°C, respectively. The energy of ionizing electrons and the voltage of the electron multiplier were set at 230 eV and 2500 V, respectively. The CI gas was methane and the best operating pressure was found to be 0.25–0.3 kPa.

2.4. Quantitative analysis

Quantification was based on the ratio of the peak height of flumazenil to that of flumazenil-d_3. The accuracy and reproducibility of the method were demonstrated by the results of duplicate analyses of control blank plasma spiked with flumazenil at six different concentrations and the internal standard and also of quality control samples.

Calibration graph

Standard samples for calibration were prepared, in duplicate, by placing 50 μl of the respective working solution (containing 2, 5, 10, 50, 100 and 200 ng of flumazenil for dog plasma and 1, 4, 20, 100, 200 and 400 ng of flumazenil for human plasma), 50 μl of the internal standard working solution (containing 100 ng of flumazenil-d_3) and 1000 μl of control blank plasma in a test-tube. The dynamic ranges for the calibration graph were 2–200 ng/ml for dog plasma and 1–400 ng/ml for human plasma. The final concentrations of flumazenil for each point on the calibration graph were 2, 5, 10, 50, 100 and 200 ng/ml for dog plasma and 1, 4, 20, 100, 200 and 400 ng/ml for human plasma. The

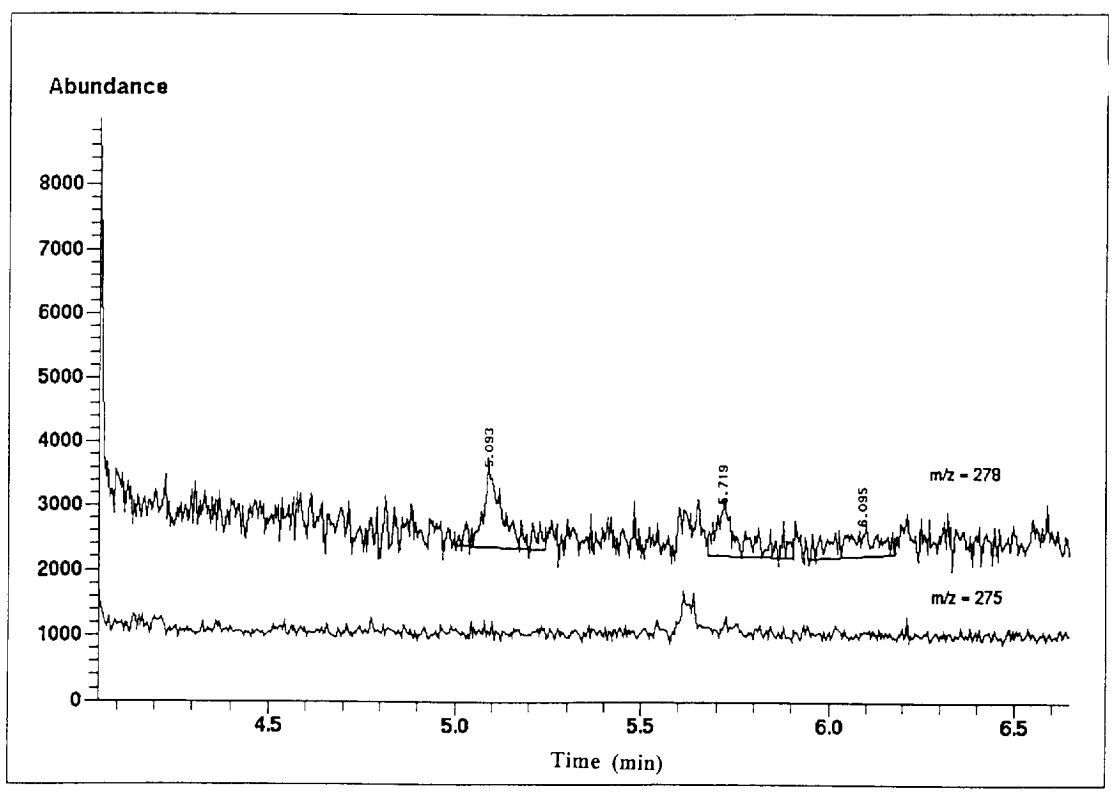

Fig. 2. Selected-ion chromatogram of blank dog plasma. Peaks with t_R = 5.093 and 5.715 min (both at m/z 278) are from the components of dog plasma.

concentration of the internal standard flumazenil-d_3 was 100 ng/ml in all samples.

Quality control samples

Quality control (QC) samples (a set of samples with known concentration of flumazenil in plasma) were run in duplicate at three different concentration levels (QC_L, QC_M, QC_H) for each experiment in order to assure the accuracy of analysis. The concentrations of flumazenil in the QC_L, QC_M and QC_H samples were 8, 60 and 150 ng/ml, respectively, for the analysis of dog plasma and 3, 60 and 300 ng/ml, respectively, for the analysis of human plasma.

Calculations

The peak height of flumazenil was measured and its ratio to that height of flumazenil-d_3 (internal standard) was calculated. The calibration graph for flumazenil was generated by weighted non-linear regression ($1/Y^2$), which had also been used successfully in the determination of flurazepam and chlordiazepoxide [6,7]. The concentrations of flumazenil in the experimental samples were calculated using the equation

$$X = (A - YC)/(YB - 1)$$

where X = the concentration of flumazenil in ng/ml and Y = the peak-height ratio of flumazenil to the internal standard flumazenil-d_3 found for an experimental sample. A, B and C are constants generated by the Drug Metabolism Laboratory Information Manager System (DM-LIMS) (by G.L. Rathsmill, PennComp, PA, USA).

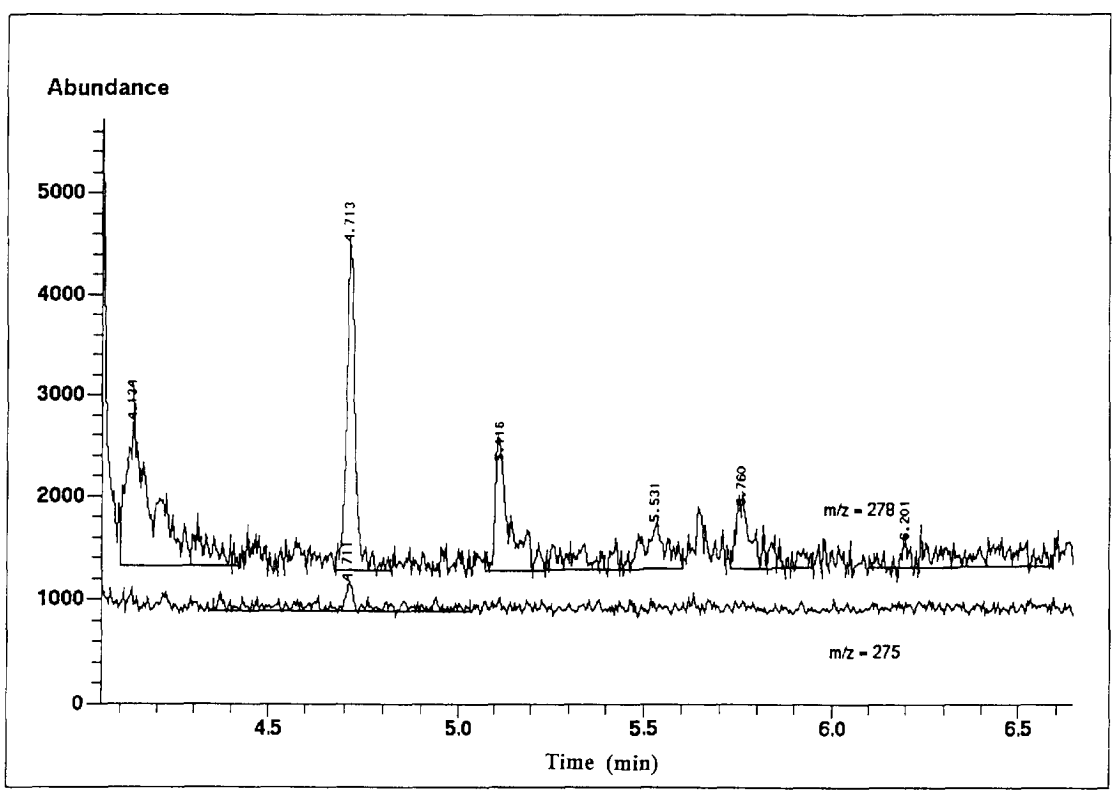

Fig. 3. Selected-ion chromatogram of blank human plasma. Peaks with t_R = 4.713, 5.416, 5.531 and 5.760 min (all of them at m/z = 278) are from the components of human plasma.

3. Results and discussion

3.1. Chromatography

Figs. 2 and 3 show chromatograms of blank dog plasma and human plasma, respectively. There are more ingredient peaks in human plasma than in dog plasma at m/z 278.

Fig. 4 shows a selected-ion chromatogram of blank dog plasma with internal standard flumazenil-d_3. It is evident that a trace amount of flumazenil is present in the flumazenil-d_3. The amount of flumazenil in flumazenil-d_3 was calculated to be about 1.7% according to the peak-height ratio of flumazenil to flumazenil-d_3. However, this purity was suitable for quantitative analysis when the quantification limit was set at $\geq$1 ng/ml.

Fig. 5 shows a mass spectrum of a standard sample in human plasma. The concentrations of flumazenil and flumazenil-d_3 were 400 and 100 ng/ml, respectively. The mass spectrometer was set in the NCI and scan acquisition mode. The mass scan range was from m/z 200 to 350. The highest peak was at m/z 275 for flumazenil. The peak at m/z 278 was due to flumazenil-d_3.

Fig. 6 shows a selected-ion chromatogram of a standard sample in human plasma. The concentrations of flumazenil and flumazenil-d_3 were 200 and 100 ng/ml, respectively. Ions with m/z 303, 306, 275 and 278 were monitored simultaneously. The results showed that these ions appeared at the same retention time, indicating that the molecular fragmentation occurred in the ion source. A better sensitivity and higher signal-to-noise ratio were achieved by monitoring the ions at m/z 275 and 278 rather than those at m/z 303 and 306. Fig. 7 shows a typical selected-ion

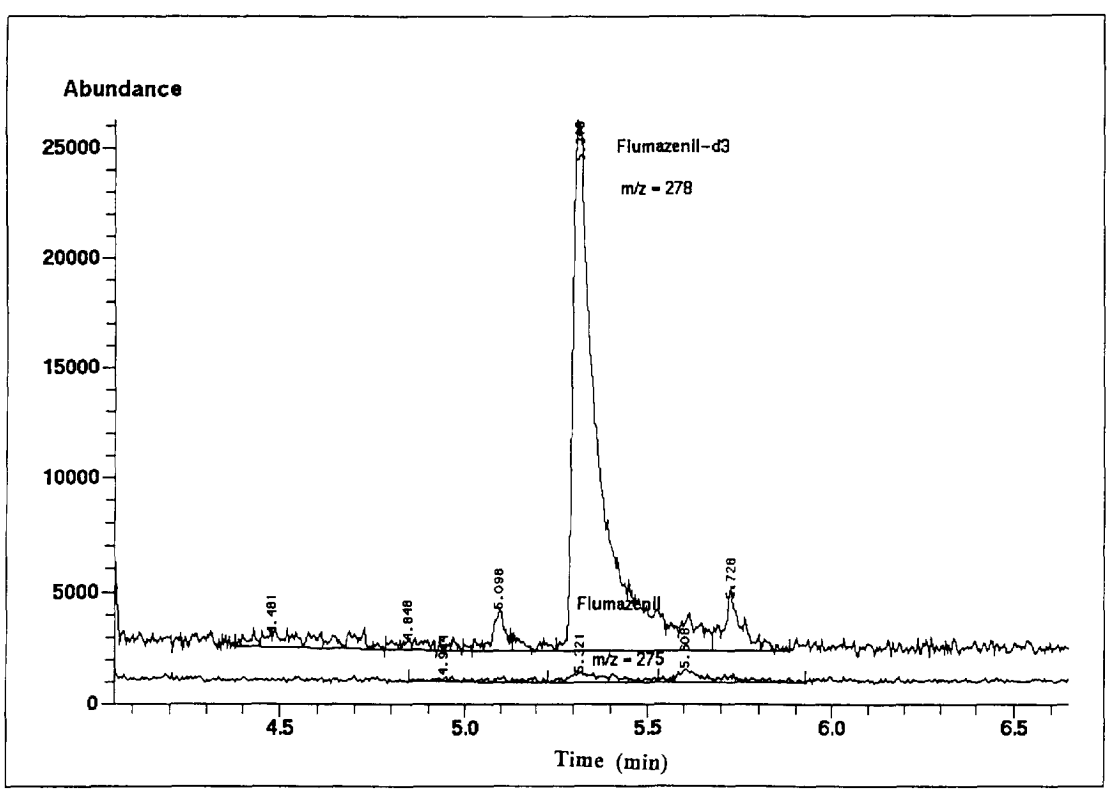

Fig. 4. Selected-ion chromatogram of blank dog plasma with flumazenil-d_3. The small peak with t_R = 5.321 min is due to flumazenil as an impurity in flumazenil-d_3, which is about 98.3% pure.

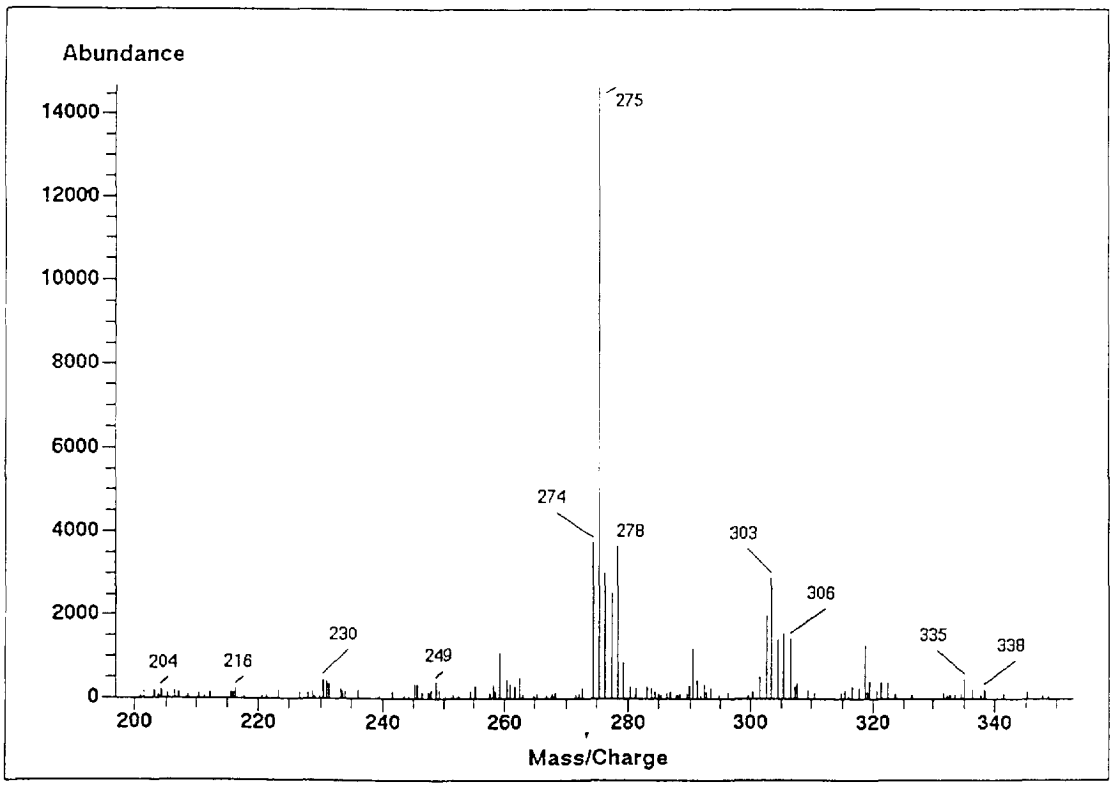

Fig. 5. Mass spectrum of standard sample with concentrations of flumazenil and flumazenil-d_3 of 400 of 100 ng/ml, respectively. The maximum peak (*m/z* 275) is the result of molecular fragmentation of flumazenil.

chromatogram for flumazenil (concentration 10 ng/ml) and the internal standard flumazenil-d_3 (concentration 100 ng/ml) in human plasma. Fig. 8 shows a representative selected-ion chromatogram of dog plasma 12 h after dosing with 200 mg flumazenil. The concentration of flumazenil was found to be 47.8 ng/ml in this sample.

3.2. Fragmentation of flumazenil in the mass spectrometer

It is evident from the spectrum in Fig. 5 that the fragmentation process must occur in the ion source, as the four ions appeared in chromatogram simultaneously. If the fragmentation had occurred in the GC column, there would have been differences in the retention times of these ions.

In the ion source, when the reagent gas methane is bombarded with high-energy electrons from the filament, the following reaction occurs:

$$CH_4 + e\ (230\ eV) \rightarrow CH_4^+ + e^-(\text{thermal})$$

The thermal electrons have lower energy levels than the electrons from the filament. It is these thermal (slow) electrons that react with the analyte molecules [8]. The electron attachment is the basic mechanism for generating negative ions. It is also favorable for molecules that have heteroatoms, e.g., nitrogen, oxygen or halogens, to follow this type of reaction.

As flumazenil contains three nitrogens, three oxygens and one fluorine, it is strongly favorable for electron attachment by capturing a thermal electron to give [flumazenil]$^-$ (*m/z* 303), which was seen in mass spectrum (see Fig. 9). It mostly fragments in the ion source and the maximum peak was found at *m/z* 275. A possible mechanism of this process is shown in Fig. 9.

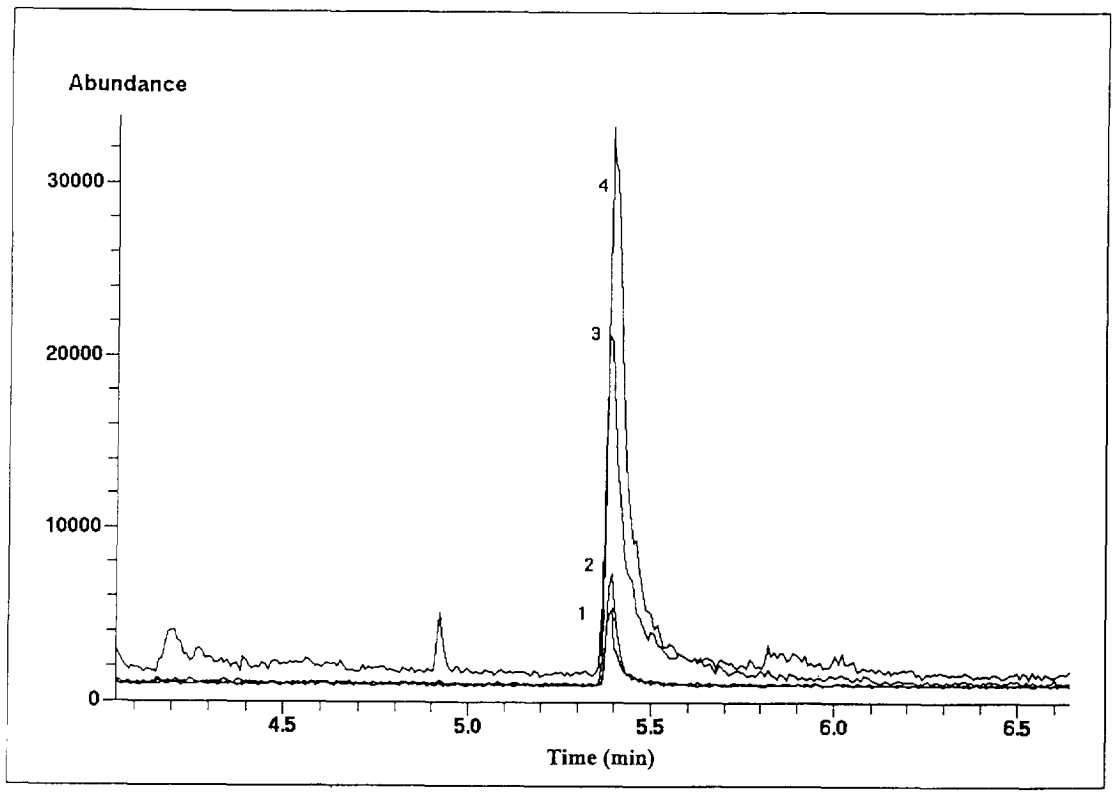

Fig. 6. Selected-ion chromatogram of a standard sample of flumazenil and flumazenil-d_3 in human plasma with concentrations of 200 and 100 ng/ml, respectively. The four ions at *m/z* 306, 303, 278 and 275 were monitored simultaneously and are shown as peaks 1, 2, 3 and 4, respectively, on the chromatogram.

Table 1
Calibration results for flumazenil in dog plasma

Sample ID/ replicate	Calibration concentration (ng/ml)	Concentration found (ng/ml)	Mean concentration (ng/ml)	Error (%)
STD-1/1	2.00	1.90	2.10	5.0
STD-1/2		2.30		
STD-2/1	5.00	4.80	4.60	−8.0
STD-2/2		4.40		
STD-3/1	10.00	9.60	10.20	2.0
STD-3/2		10.80		
STD-4/1	50.0	49.50	51.60	3.2
STD-4/2		52.70		
STD-5/1	100.0	101.8	102.3	2.3
STD-5/2		102.7		
STD-6/1	200.0	197.7	197.2	−1.4
STD-6/2		196.6		

$r^2 = 0.9976$.

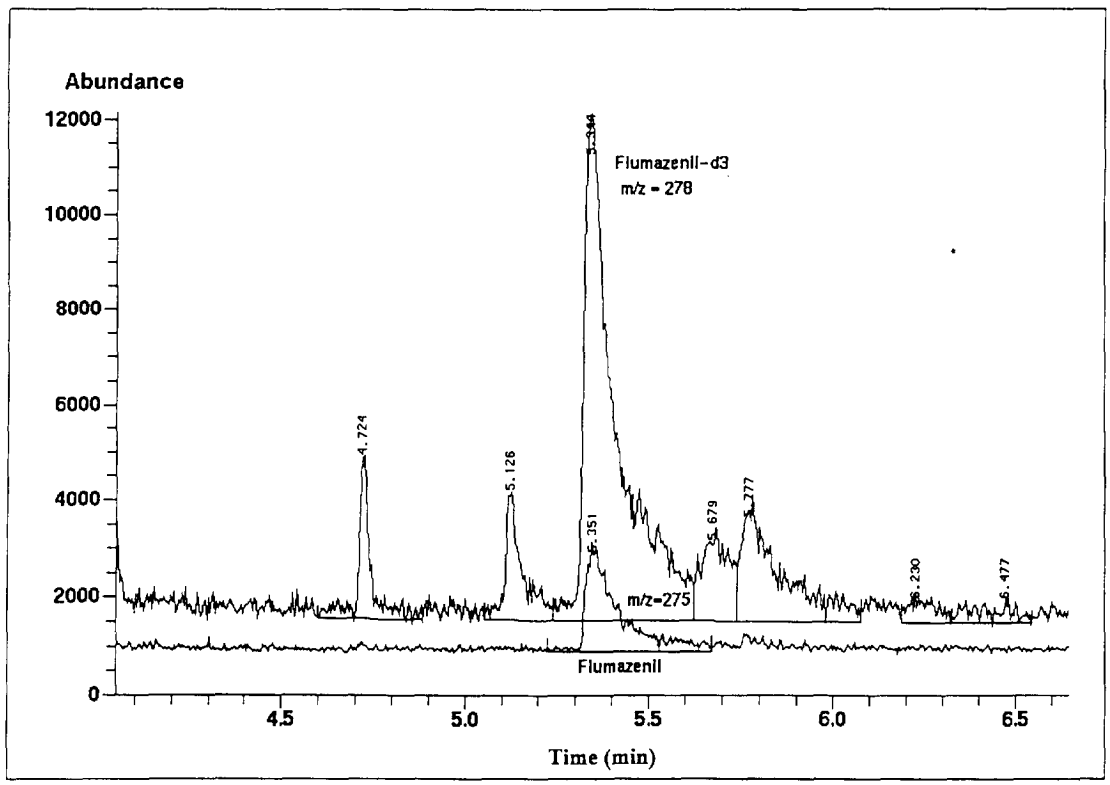

Fig. 7. Selected-ion chromatogram of a standard sample of flumazenil (t_R = 5.351 min, concentration = 10 ng/ml) and flumazenil-d_3 (t_R = 5.344 min, concentration = 100 ng/ml).

$CH_4 + C_2H_5^+ \rightarrow C_3H_8 + H^+$ is only one of the possible reactions with the reagent gas methane. Actually, there are a number of reactions in the reagent gas and many positive and negative ions in the ion source simultaneously.

From the experiment, the peak intensities of the *m/z* 275 ion for flumazenil and the *m/z* 278 ion for flumazenil-d_3 were the maximum in the mass spectrum. Therefore, it can deduced that $[M]^-$ (in Fig. 9) is the most stable ion in the ion source with methane as reagent gas. In addition to the sensitivity, our experiments showed that very good accuracy and reproducibility could be achieved for the determination of flumazenil and flumazenil-d_3 based on measurements of these two ions.

Our experiments also showed that the use of heptane as reconstitution solvent will result in fewer fragments for flumazenil in GC–MS than the use of toluene as the solvent. It also gave

Table 2
QC sample results for flumazenil in dog plasma (n = 5)

Sample	Theoretical concentration (ng/ml)	Concentration (mean ± S.D.) (ng/ml)	Recovery (%)
QC_L	8.0	7.98 ± 0.881	99.75
QC_M	60.0	61.68 ± 2.81	102.80
QC_H	150	153.16 ± 4.56	102.10

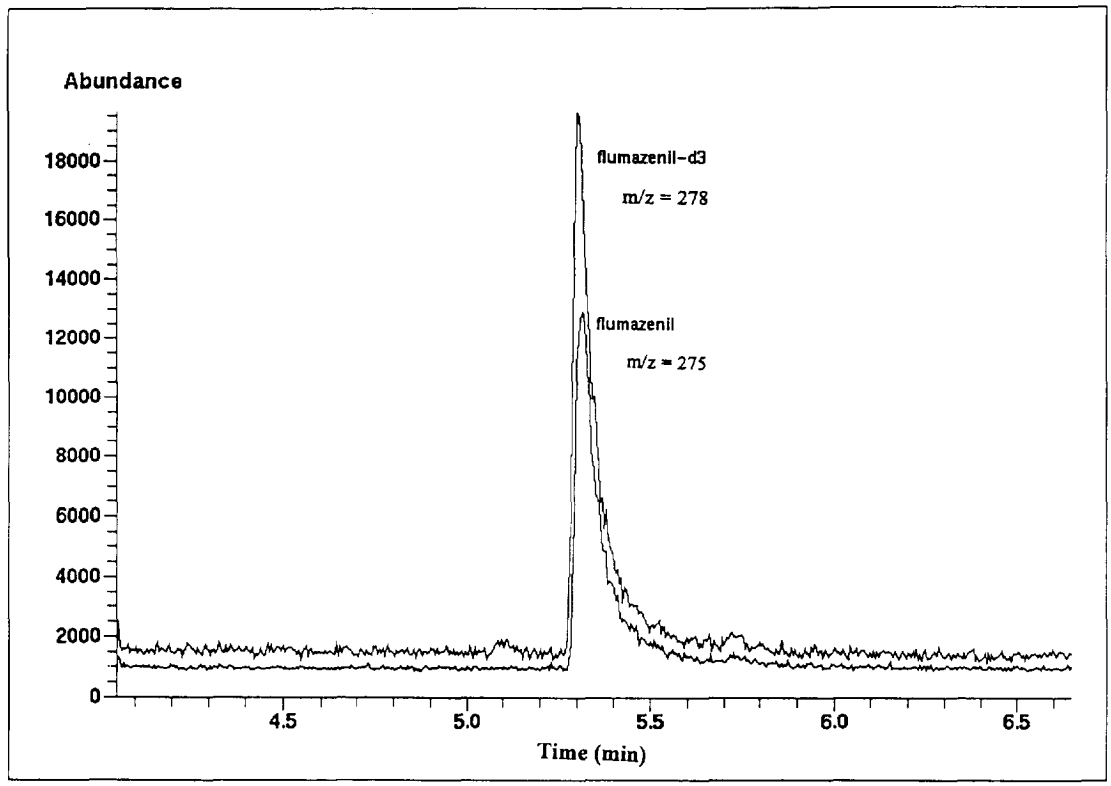

Fig. 8. Selected-ion chromatogram of a dog plasma sample 12 h after dosing with 200 mg of flumazenil. Concentration of flumazenil 47.8 ng/ml.

Table 3
Calibration results for flumazenil in human plasma

Sample ID/ replicate	Calibration concentration (ng/ml)	Concentration found (ng/ml)	Mean concentration (ng/ml)	Error (%)
STD-1/1	1.00	0.90	0.95	−5.0
STD-1/2		1.0		
STD-2/1	4.00	4.60	4.10	2.5
STD-2/2		3.60		
STD-3/1	20.00	19.60	20.90	4.5
STD-3/2		22.20		
STD-4/1	100.0	98.80	97.05	−2.95
STD-4/2		95.30		
STD-5/1	200.0	189.1	198.4	−0.8
STD-5/2		207.7		
STD-6/1	400.0	411.7	400.8	0.2
STD-6/2		398.9		

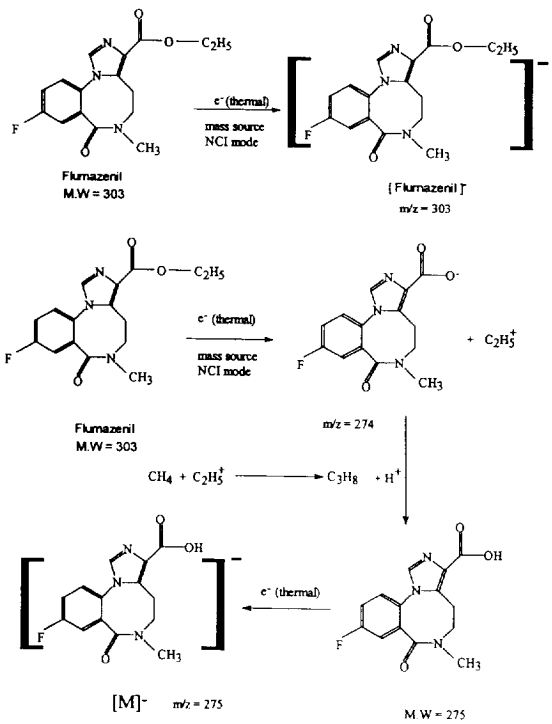

Fig. 9. Mechanism of negative-ion formation from flumazenil and the fragmentation process in the NCI mass spectrometer.

good sensitivity because the peak heights for the *m/z* 275, 278, 303 and 306 ions in the mass spectrum were higher than those using toluene as solvent.

3.3. Preconditioning of the GC column and sensitivity

As flumazenil could bind to the active sites of the column, it is very important to precondition the column before analysis. In our experiments, the preconditioning solution was prepared by extracting the blank plasma with ethyl acetate. This preconditioning solution contained many lipids that could cover the active sites of the column and the contact surface of the analytical system. Therefore, injecting Silyl-8 in addition to the preconditioning solution five times each with an injection volume of 2 μl before analysis for flumazenil minimizes surface adsorption with a concomitant increase in sensitivity. Our experiments showed that the detection limit for flumazenil was as low as 0.1 ng/ml (monitoring the *m/z* 275 ion), which was 100 times lower than using a column without preconditioning.

The quantification limit was set to 2 ng/ml in all our routine analyses for dog plasma and 1 ng/ml for human plasma. This assured consistent results, even if the sensitivity is decreased because of contamination of the ion source or the degradation of the GC column through prolonged usage.

3.4. Accuracy and reproducibility

For 300 dog plasma samples and 400 human plasma samples (including standard and QC samples), we found that the regression correlation coefficients for all the calibration graphs were over 0.99, the accuracy and precision of analysis were about 10%, the errors for all standard and QC samples were within 20% and the average values for all QC samples were in the range 90–110% of the theoretical value. These results were calculated by the Drug Metabolism Information Manager System (DM-LIMS).

Tables 1–4 give the data for a typical cali-

Table 4
QC sample results for flumazenil in human plasma ($n = 5$)

Sample	Theoretical concentration (ng/ml)	Concentration (mean ± S.D.) (ng/ml)	Recovery (%)
QC_L	3.0	3.0 ± 0.255	100
QC_M	60.0	59.08 ± 3.05	98.47
QC_H	300	314.02 ± 6.85	104.67

bration and the results for five replicates of each QC sample in dog and human plasma.

4. Conclusions

Drugs such as flumazenil that could bind to active sites of a GC column and lack a functional group suitable for derivatization are not suitable for GC analysis. This is a great challenge for the GC–MS technique. This problem was solved in the proposed method. Moreover, in this method, monitoring the maximum peaks of flumazenil and its isotopically labeled compound (*m/z* 275 and 278, respectively) enhanced the sensitivity and S/N tenfold.

The high sensitivity of the method made it possible to analyse large amounts of samples with low quantification limits. The use of a capillary GC column can separate the peaks from the plasma ingredients and make it available for various biological matrices. The use of flumazenil-d_3 as the internal standard assures the accuracy of quantitative analysis, as the isotopic compound is identical in structure and chemical properties with the analyte. The disadvantage is that such an isotopic compound is expensive and it is also difficult to synthesize and purify.

Acknowledgments

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