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QUANTITATION OF THE BENZODIAZEPINE ANTAGONIST FLUMAZENIL IN HUMAN PLASMA BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A gas chromatographic–mass spectrometric procedure has been developed for the quantitation of the benzodiazepine antagonist flumazenil in human plasma. The assay utilizes an extraction at alkaline pH with benzene–dichloroethane (80 20), selective ion monitoring, isobutane positive-ion chemical ionization mass spectrometry and stable isotope dilution. The method has been used to measure plasma concentrations of flumazenil in over 1500 clinical samples over a range of 0.5–200 ng/ml (using 2 ml of plasma).

INTRODUCTION

The imidazobenzodiazepinone flumazenil belongs to a class of benzodiazepine antagonists which exhibit little benzodiazepine activity [1]. The structures of flumazenil (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*][1,4]benzodiazepine-3-carboxylate) and its trideuterated stable isotope analogue are shown in Fig. 1.

Gas chromatographic (GC) [2,3] and high-performance liquid chromatographic (HPLC) [4,5] methods have been reported for this compound which has already been approved for clinical use in Europe. Currently clinical trials with flumazenil are being carried out in the U.S. as part of an NDA program. The assay reported here represents an improvement over previous assays in specificity and throughput. To date the assay has been used to analyze approximately 1500 plasma samples from subjects receiving doses ranging from 3 to 0.4 mg.

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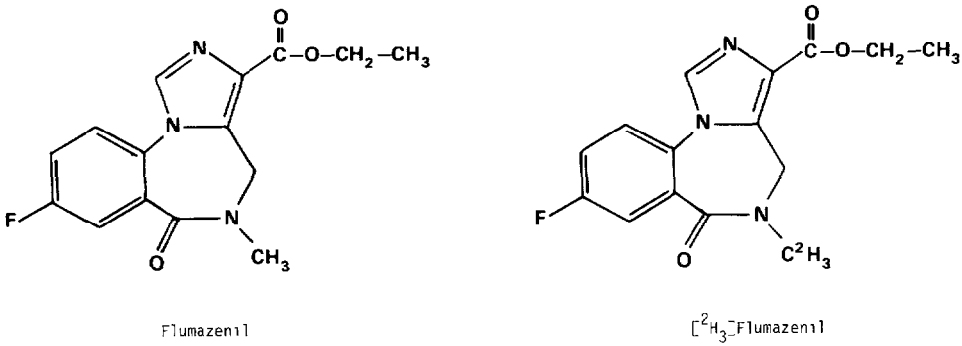

Fig. 1. Structures of flumazenil and [$^2H_3$]flumazenil.

EXPERIMENTAL

Materials

Flumazenil was obtained from the Quality Control Department of Hoffmann-La Roche (Nutley, NJ, U.S.A.). [$^2H_3$]Flumazenil was synthesized from the N-desmethyl compound by Dr. Y. Liu of the Isotope Synthesis Group at Hoffmann-La Roche using C^2H_3I and standard synthetic procedures. [^{14}C]Flumazenil (50.1 μCi/mg) was synthesized using standard radiochemical procedures by Dr. N. Flueck at F. Hoffmann-La Roche (Basle, Switzerland). All reagents were of analytical or UV grade. Culture tubes (15 ml, Pyrex 9826) with Teflon®-lined screw caps (Pyrex 8061) were treated with Siliclad® (Clay Adams, Parsippany, NJ, U.S.A.) if new. Disposable borosilicate culture tubes (Fisher Scientific, Fairlawn, NJ, U.S.A.; 75 mm×10 mm) were also treated with Siliclad. All glassware was rinsed with methanol before use.

Stock and calibration solutions

A stock solution of flumazenil was prepared at a concentration of 1 mg/ml in dimethylformamide (DMF). The reference standard ([$^2H_3$]flumazenil) solution was prepared at a concentration of 1 mg/ml in DMF. All solutions were stored at −20°C.

Calibration solutions were prepared by appropriate dilution with DMF of the stock solution to give final concentrations of 200, 50, 10, 2 ng or 0.5 ng per 50 μl. Similarly, the stock reference standard solution was diluted to give a final concentration of 100 ng per 50 μl.

Plasma samples

Blood was drawn into Vacutainer® tubes (Becton-Dickinson, Type BD 6470) containing sodium fluoride and potassium oxalate. The tubes were then centrifuged, and the plasma was transferred into siliconized glass scintillation vials. The use of the proper Vacutainer tubes containing an esterase inhibitor (so-

dium fluoride or equivalent) is essential. The samples were stored at −17°C until analyzed.

Two quality assurance (Q.A.) samples were prepared by fortifying drug-free sodium fluoride-treated plasma to concentrations of 5 and 50 ng/ml. Enough Q.A. sample was prepared so that duplicate 2-ml aliquots could be analyzed along with each set of samples over the course of all the analyses. The Q.A. sample was also used to determine medium-term stability of the drug in plasma and to check the reproducibility of the assay. Both Q.A. samples were stored at −20°C.

Calibration standards

Five calibration standards and both Q.A. samples were analyzed in duplicate along with each set of experimental samples. The calibration standards contained 1, 4, 10, 100 and 400 ng of flumazenil in 2 ml of drug-free plasma.

Extraction method

The plasma sample was thawed, and 2.0 ml were transferred into a 15-ml culture tube. A 100-μl volume (100 ng) of the reference standard solution was added, and the mixture was vortexed. To make the solution alkaline, 50 μl of 1 *M* sodium hydroxide solution was added to each tube, and the mixture was vortexed for 10 s. Each sample was extracted with 5 ml of benzene–ethylene dichloride (80:20) by shaking for 10 min at room temperature. After centrifuging for 5 min at 2000 *g*, the organic phase was transferred to a disposable glass tube. The samples were evaporated to dryness under dry nitrogen in a water bath (N-Evap, Organomation) set to 45–50°C. The residue was redissolved in 50 μl of butyl acetate, then transferred to a Chrompack crimp vial containing a 200-μl glass insert.

Instrumentation

Gas chromatograph. A Carlo Erba Model 4200 gas chromatograph was equipped with a 2 m × 2 mm I.D. borosilicate glass column packed with 3% SP-2250 (Supelco, Bellefonte, PA, U.S.A.). Prior to use, the column was conditioned with Silyl-8 (Pierce Chemicals) and a concentrated ethyl acetate extract of drug-free plasma. Hydrogen (5.4 bar, Liquid Carbonic, 99% purity) was used as the carrier gas. The injector, oven and transfer line temperatures were 325, 290 and 250°C, respectively. The gas chromatograph was equipped with a divert, and the gas chromatographic–mass spectrometric (GC–MS) interface utilized a jet separator. A 10-μl aliquot of the sample was injected onto the column.

Mass spectrometer. A Kratos (Manchester, U.K.) MS50 mass spectrometer was equipped with a VG (Vacuum Generators, Manchester, U.K.) DIGMID (digital multiple ion detector). The MS50 was tuned for maximum sensitivity and typically operated at a resolution of 7000 or better. The protonated molec-

ular ions of flumazenil (m/z 304) and [$^2H_3$]flumazenil (m/z 307) generated by isobutane (Liquid Carbonic, 99% purity) positive-ion chemical ionization (PICI) were monitored using the protonated molecular ion of tris(trifluoromethyl)-s-triazine (m/z 286) as the reference lock.

Data collection and analysis. The DIGMID output was interfaced to QSIMPS (quantitative selected ion monitoring processing system), a collection of hardware and software designed to collect and process SIM data and print out the information in report form. The QSIMPS has been described in detail elsewhere [6,7].

Plasma collection device

The plasma collection device experiments were performed using human blood as follows. Fresh blood ($\sim$50 ml) was obtained using Vacutainer (B-D 6470) tubes. A 25-ml volume of blood was fortified with flumazenil to give a final concentration of 30 ng/ml. The remaining blood was used to generate a blood calibration curve. Aliquots (4.0 ml) of the fortified blood were transferred to three B-D 6470 Vacutainers and three glass tubes. After shaking gently for about 30 min, a 1.0 ml aliquot was removed from each tube, extracted and analyzed by GC–MS using a blood calibration curve. The remaining blood samples were centrifuged at 4000 *g* and 4 °C for 30 min to obtain plasma. A 1.0-ml plasma sample was removed from each of the six tubes, extracted and analyzed by GC–MS using a plasma calibration curve.

Recovery experiments

The percentage recovery of [^{14}C]flumazenil from plasma was determined by the following method. A 30-ml pool of drug-free plasma was fortified with [^{14}C]flumazenil and thoroughly mixed, achieving a concentration of 8500 dpm/ml (77 ng/ml). Six 2-ml aliquots were extracted according to the extraction method described in an earlier section. The organic phase was transferred to scintillation vials, evaporated to dryness, and the residue analyzed by liquid scintillation counting (LSC). The remainder of the fortified plasma was aliquoted into scintillation vials and analyzed by LSC. The percentage recovery was calculated by comparing the dpm of six extracted samples with unextracted samples, using the following equation:

$$\text{recovery} = \frac{\text{dpm in extracts of fortified plasma}}{\text{dpm in fortified plasma}} \times 100\%$$

Stability experiments

The benchtop stability of flumazenil in plasma was determined by comparing the concentrations of six experimental samples allowed to set at room temperature for 0, 3 and 6 h. The experimental samples were 2.0-ml aliquots of the 50 ng/ml Q.A. sample stored at -20 °C. The long-term stability at -20 °C was

determined by duplicate determinations of the same Q.A. sample over a period of three months.

The stability of the calibration solutions was determined by the following method. Aliquots (2 ml) of five calibration solutions (200, 50, 10, 5 or 2 ng per 50 μl) were allowed to remain at room temperature for 0, 7, 14 or 28 days. These solutions were then used to fortify drug-free plasma samples (1.0 ml). A 50-ng amount of the reference standard was added to all samples which were then extracted and analyzed by GC–MS.

RESULTS AND DISCUSSION

Fig. 2 shows the isobutane PICI mass spectrum of flumazenil. The base peak (m/z 304) represents the protonated molecular ion of the analyte. Similarly, the base peak in the isobutane PICI mass spectrum of [$^2H_3$]flumazenil is m/z 307.

Fig. 3 is an example of selected ion current profiles from an extract of a 0.75-

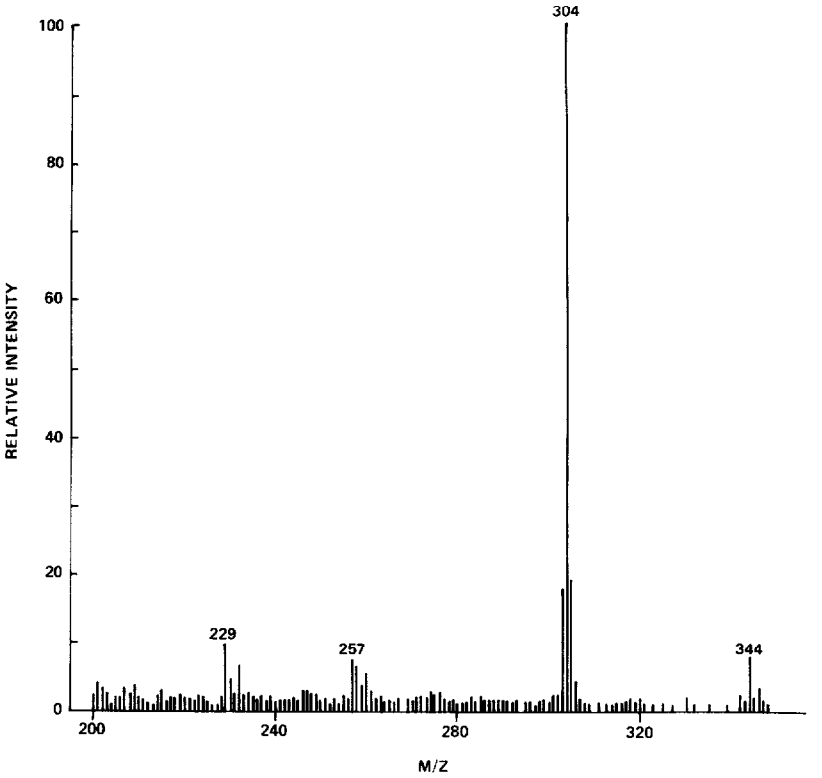

Fig. 2. Isobutane PICI mass spectrum of flumazenil. The base peak, m/z 304, is the protonated molecular ion.

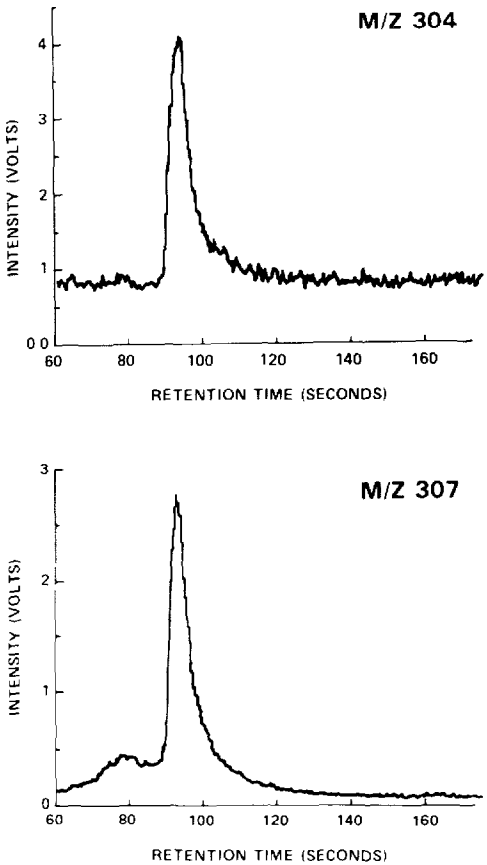

Fig. 3. Selected ion chromatograms from an extract of a 0.75-h post-dose plasma sample from a subject given a 5-min 1-mg intravenous infusion of flumazenil. The concentration of flumazenil in this sample was 4.8 ng/ml.

h post-dose sample from a healthy subject given a 1.0-mg intravenous dose of flumazenil. The concentration of flumazenil in this sample was 4.8 ng/ml. No interferences from other endogenous plasma components were observed.

In every experiment, each calibration concentration was analyzed in duplicate along with two duplicate Q.A. samples. For calibration curves used in several studies involving the assay of over 1500 plasma samples, the overall inter-assay precision was 2.5% and the overall intra-assay precision was 5.8%. Tables I and II summarize the inter- and intra-assay precisions determined from the analysis of calibration and Q.A. sample data. The inter-assay precision was estimated from the difference between the observed concentration and the concentration back-calculated from the regression line. The intra-assay precision was estimated from the ratio of the duplicate analyses, including the

TABLE I

SUMMARY OF INTER-ASSAY PRECISION OF FLUMAZENIL DETERMINED FROM THE ANALYSIS OF CALIBRATION DATA

The values in parentheses are relative standard deviations (R.S.D.); overall R.S.D. = 2.48%; overall R.S.D. at 0.5 ng/ml = 2.3%.

Added concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)			
	Study 1 (n = 11)	Study 2 (n = 4)	Study 3 (n = 6)	Study 4 (n = 5)
200	199.50 ± 1.77 (0.89)	199.67 ± 0.52 (0.26)	204.65 ± 8.93 (4.36)	201.92 ± 1.57 (0.78)
50	51.13 ± 1.31 (2.57)	50.58 ± 0.69 (1.36)	51.04 ± 1.92 (3.76)	48.96 ± 1.83 (3.74)
10	9.89 ± 0.31 (2.08)	9.87 ± 0.10 (1.01)	9.85 ± 0.28 (2.84)	9.99 ± 0.27 (2.74)
2	1.99 ± 0.10 (5.04)	2.03 ± 0.03 (1.35)	2 01 ± 0.09 (4.50)	2.05 ± 0.06 (2.88)
0 5	0.51 ± 0.02 (4.38)	0.50 ± 0.04 (0.86)	0.50 ± 0 01 (1 76)	N.A.[a]

[a]N.A. = not applicable.

TABLE II

SUMMARY OF INTRA-ASSAY PRECISION OF FLUMAZENIL DETERMINED FROM THE ANALYSIS OF CALIBRATION AND Q.A. SAMPLE DATA

The values in parentheses are relative standard deviations (R.S.D.); overall R.S.D. = 5.8%; overall R.S.D. at 0.5 ng/ml = 12.5%.

Added concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)			
	Study 1 (n = 11)	Study 2 (n = 4)	Study 3 (n = 6)	Study 4 (n = 5)
200	1.00 ± 0.04 (4.0)	1.02 ± 0.03 (3.0)	0.98 ± 0.09 (9.0)	0.98 ± 0.03 (3.1)
50	1.00 ± 0.03 (3.0)	1.00 ± 0.04 (4.0)	1.01 ± 0.03 (3.0)	1.05 ± 0.05 (4.8)
10	1.03 ± 0.04 (4.0)	1.06 ± 0.03 (3.0)	1.02 ± 0.08 (7.8)	0.99 ± 0.06 (6.1)
2	1.00 ± 0.10 (10.0)	1.03 ± 0.06 (6.0)	1.02 ± 0.06 (5.9)	1.06 ± 0.04 (3.8)
0.5	1.07 ± 0.15 (14.0)	1.12 ± 0.09 (8.0)	1.04 ± 0.16 (15.4)	N.A.[a]
Q.A. medium	0.95 ± 0.6 (6.3)	1.01 ± 0.03 (3.0)	1.04 ± 0.7 (6.7)	0.98 ± 0.07 (7.1)
Q.A. low	0.99 ± 0.4 (4.0)	0.98 ± 0.03 (3.1)	0.98 ± 0.4 (4.0)	1.02 ± 0.06 (5.9)

[a]N.A. = not applicable.

TABLE III

PLASMA COLLECTION DEVICE EXPERIMENTS

Values in parentheses are relative standard deviations (%).

Sample stored in	Concentration (ng/ml) (mean ± S.D., n = 3)	
	Blood	Plasma
Glass tube	31.5 ± 1.4 (4.4)	31.5 ± 1.0 (3.2)
Vacutainer (BD 6470)[a]	33.4 ± 1.1 (3.3)	29.5 ± 0.9 (3.1)

[a]Becton-Dickinson No. 6470 containing potassium oxalate and sodium fluoride.

TABLE IV

LONG-TERM STABILITY

Assay date	Flumazenil concentration (mean ± S.D.) (ng/ml)		Number of duplicate determinations
	Q.A. low	Q.A. high	
3/88	5.0 ± 0.3 (5.9)	53.6 ± 3.8 (7.1)	9
4/88	4.8 ± 0.2 (4.5)	51.0 ± 3.2 (6.4)	4
5/88	4.8 ± 0.2 (4.1)	50.6 ± 2.8 (5.6)	12
6/88	4.9 ± 0.02 (0.3)	49.9 ± 0.5 (0.9)	1
9/88	4.7 ± 0.1 (2.7)	47.0 ± 1.2 (2.7)	5

TABLE V

STABILITY OF FLUMAZENIL IN N,N-DIMETHYLFORMAMIDE MAINTAINED AT AMBIENT TEMPERATURE FOR UP TO 28 DAYS BEFORE ASSAY

Each value is the mean of two determinations. The values listed in parentheses are percentages relative to the appropriate day 0 ratio.

Concentration (ng per 50 μl)	*m/z* 304/307 ratio			
	Day 0	Day 7	Day 14	Day 28
200	5.5230 (100)	5.6628 (102.5)	5.8013 (105.0)	6.0524 (109.6)
50	1.4795 (100)	1.5884 (107.4)	1.6109 (108.9)	1.6126 (109.0)
10	0.3170 (100)	0.3330 (105.0)	0.3172 (100.1)	0.3338 (105.3)
5	0.1720 (100)	0.1724 (100.2)	0.1738 (101.0)	0.1728 (100.5)
2	0.0806 (100)	0.0782 (97.0)	0.0746 (92.6)	0.0803 (99.6)

Q.A. samples. At 0.5 ng/ml, the lower limit of quantitation, the inter- and intra-assay precision values were 2.3 and 12.5%, respectively.

The results of the plasma collection device experiments are shown in Table III. These data demonstrate that the flumazenil concentration measured in plasma was not biased by the collection device. However, experiments to determine the red blood cell to plasma partition ratio could not be done because the use of sodium fluoride caused some red blood cell lysis and leakage, which gave rise to an anomalously low hematocrit value.

The extraction recovery for flumazenil from 2.0 ml of human plasma was found to be 79.5 ± 3.5% at a concentration of 77 ng/ml.

Flumazenil was found to be stable in human plasma on the benchtop for at least 6 h. The mean (± S.D.) concentrations measured for flumazenil at 0, 3 and 6 h were 51.1 ± 0.9, 50.9 ± 1.0 and 50.7 ± 0.6 ng/ml, respectively. The long-term stability results are shown in Table IV. These results show that flumazenil is stable in human plasma stored at −20°C for at least six months. This agrees with the stability experiments reported by Timm and Zell [5].

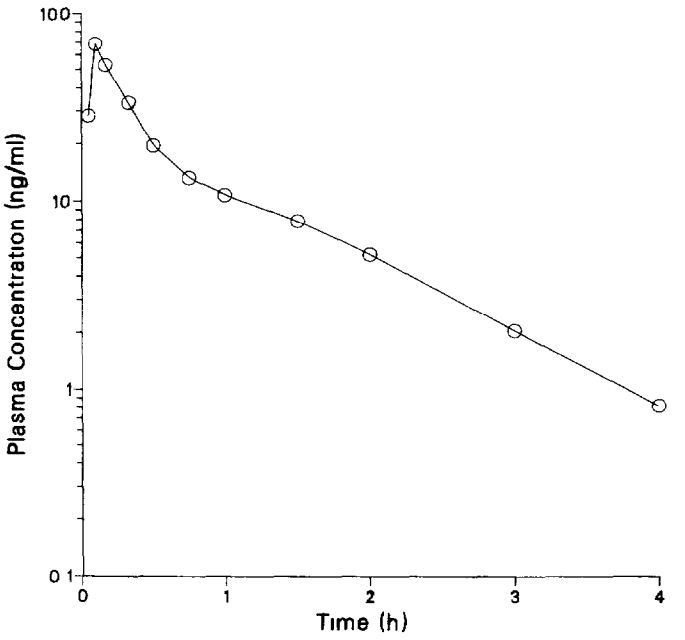

Fig. 4. Plasma concentration–time curve for a healthy subject given a single 2.0-mg intravenous dose of flumazenil.

Calibration solutions of flumazenil in DMF were found to be stable at room temperature for at least 28 days. The results are shown in Table V. The small differences in the m/z 304/307 ratios can be explained by small evaporative losses over the 28-day period.

Fig. 4 shows the concentration of flumazenil in the plasma of a healthy subject following a single 2.0-mg intravenous dose. The plasma concentration for the subject reached a maximum within 5 min of dosing and decreased with a half-life of about 45 min.

The assay has also been applied to the assay of plasma samples from dogs and rabbits used in toxicological experiments. It is not applicable to rat plasma without the additional use of dichlorvos as an additional esterase inhibitor [8].

CONCLUSION

A GC–MS procedure has been developed for the quantitation of flumazenil in human, dog and rabbit plasmas. The assay represents an improvement over previous HPLC and GC methods in terms of specificity and speed (sample analysis time on the order of 5 min).

Additionally, experiments have been done to determine the effect of collec-

tion device and storage containers, the percentage recovery from plasma and benchtop long-term stability of flumazenil.

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