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Short communication

Gas chromatographic–mass spectrometric method for the determination of flurazepam and its major metabolites in mouse and rat plasma

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Abstract

A capillary gas chromatographic–negative chemical ionization (NCI) mass spectrometric method for the determination of flurazepam and its metabolites N-1-hydroxyethyl-flurazepam and N-1-desalkyl-flurazepam in mouse and rat plasma was described. Derivatization of the metabolites of flurazepam with BSTFA allowed a highly stable, accurate, and sensitive GC–MS analysis. The use of a single internal standard (halazepam) for the quantification of all compounds saved cost and time. The detection limits were 0.1 ng/ml for N-hydroxyethyl-flurazepam-TMS ($M_r = 404$), 0.5 ng/ml for desalkyl-flurazepam-TMS ($M_r = 360$), and 0.5 ng/ml for flurazepam ($M_r = 387$) with an injection volume of 1 μl at a signal-to-noise ratio greater than 5. The quantitation limit was set to 10 ng/ml for all compounds.

1. Introduction

Flurazepam [7-chloro-1-(2-diethylaminoethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one dihydrochloride] has been used as a sedative, hypnotic drug. Although numerous papers have been published on the analysis of flurazepam and its metabolites in human plasma and urine [1–4], no studies have been described for mouse plasma using a GC–MS method.

Dose and toxicity studies of drugs have usually been carried out in animals such as mouse or rat. Since the quantity of mouse (rat) plasma available for analysis is smaller than the amount of human plasma, only small (100–200 μl) samples of mouse plasma can be obtained. Thus a much more sensitive (5–10 times) technique is needed for the analysis of flurazepam in mouse plasma samples than in human plasma samples (usually 1 ml plasma). Moreover, mouse plasma contains a number of compounds that might give peaks in column liquid and gas chromatograms with the same retention time as the analytes resulting in serious interference problem. The high selectivity and sensitivity of GC–MS may obviate these problems.

2. Experimental

2.1. Chemicals

All pure drug compounds: N-desalkyl-flurazepam (Ro 05-3367, lot No. 3-28-67), N-1-

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hydroxyethyl-flurazepam (Ro 07-2750, lot No. 3925-108), flurazepam (Ro 05-6901, lot No. 238-101) and halazepam (Ro 08-6728, lot No. 5375-99) were generously supplied by Hoffman La Roche (Nutley, NJ, USA). BSTFA with 1% TMCS [N,O-bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane] was purchased from Pierce (Rockford, IL, USA). Mouse plasma (Lot No. 041593) and rat plasma (Lot No. 91092) were purchased from Rockland (Gilbertsville, PA, USA). All other chemicals were of the best analytical grade available.

The extraction solvent was prepared by mixing 700 ml of toluene and 300 ml of dichloromethane in a 1-l glass stoppered bottle. Borate buffer solution (1.0 *M*, pH 10.0) was prepared by titrating 1 *M* boric acid and 1 *M* potassium chloride solution with 1 *M* sodium carbonate to pH 10.0.

2.2. Sample preparation

Flurazepam was dosed to mice (and rats) at 5 mg, 15 mg, and 50 mg per kg body weight. Plasma samples were taken at specific time intervals after dosing. The change in the amount of flurazepam, and the metabolites N-1-hydroxyethyl-flurazepam and N-desalkyl-flurazepam in the plasma samples as a function of time will show the rate of metabolism of flurazepam.

A 200-μl volume of mouse (rat) plasma was pipetted into a test tube. To each of these samples, 25 μl of internal standard working solution were added. The sample was extracted by adding 1.0 ml of borate buffer (pH 10.0) and 5.0 ml of extraction solution (toluene–dichloromethane, 70:30, v/v). The tubes were capped and vortex-mixed (vigorous shaking and rotation) for 15 min. The samples were centrifuged for 10 min at 750 *g*. The organic layer which contained flurazepam (1), and its two metabolites hydroxyethyl-flurazepam (2) and desalkyl-flurazepam (3) was decanted into a clean tube. The solvent was evaporated to dryness under a stream of nitrogen at 50°C for 40 min in a Zymark Turbo Vap Evaporator (Hopkinton, MA, USA). A 60-μl volume of BSTFA with 1% TMCS was added and the test tube was capped and heated for 30 min at 50°C in a water bath for the reaction to take place. The hydroxyl and amine functional groups of compounds (2) and (3) in respectively were derivatized. An aliquot of this solution was analyzed by GC–MS NCI.

Halazepam (4) was used as internal standard. The internal standard working solution was prepared at the concentration of 20 ng/ml. The structures of all compounds are shown in Fig. 1. Fig. 2 shows the derivatization of N-hydroxyethyl flurazepam and N-desalkyl-flurazepam with BSTFA and 1% TMCS.

2.3. Apparatus and instrumental parameters

Analysis was performed on an HP 5890 II gas chromatograph with a HP-5 column (25 m × 0.25 mm I.D., 0.33 μm film thickness) and HP 5989 MS Engine equipped with a 7673 autosampler, 59940 ChemStation and 59944C (Rev. C.10.0) HP-UX ChemSystem (Hewlett-Packard, NJ, USA).

Helium was used as carrier gas with a column head pressure of 30 KPa at *ca.* 25°C and the flow-rate was maintained at 1 ml/min. Injector temperature was 280°C. Oven temperature was programmed from 180–300°C at a step rate of 30°C/min. Injection volume was 1 μl. Run time was *ca.* 7 min. The mass was set at the negative-

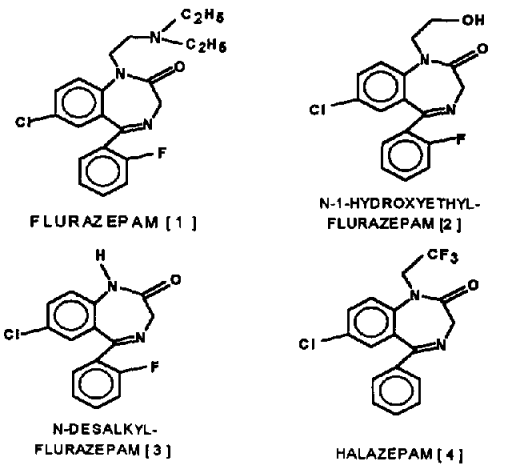

Fig. 1. The structures of flurazepam (1) and its metabolites (2,3) and halazepam (4).

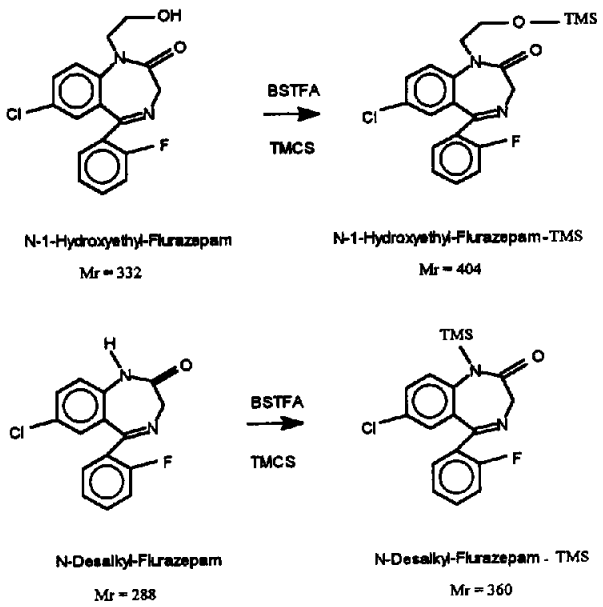

Fig. 2. Derivatization of N-hydroxyethyl-flurazepam and N-desalkyl-flurazepam with BSTFA [N,O-bis(trimethyl)trifluoroacetamide] and 1% TMCS (trimethyl-chlorosilane).

ion chemical ionization (NCI) and selected-ion monitoring (SIM) acquisition mode: monitoring ions at M_r = 352, 360, 404 and 387. The temperatures of source and quadrupoles were 150°C and 100°C, respectively. The electrical multiplier (EM) was set at *ca.* 2000 V during routine analysis. The chemical ionization gas (CI gas) was methane and the optimum operating pressure was found to be 0.173–0.25 kPa.

2.4. Quantitative analysis

Quantitation was based on the ratio of peak height of each analyte to that of the internal standard halazepam. The accuracy and reproducibility of the method were determined from the results of duplicate analyses of control mouse (rat) plasma spiked with the three analytes at six different concentrations and the internal standard.

Calibration standard curve

A calibration curve was generated, in duplicate, by adding 25 μl of the respective working solution, 25 μl of the internal standard working solution, and 200 μl of control mouse (rat) plasma in a test tube. The final concentrations of the analytes were 10, 20, 50, 100, 200, 400 ng/ml, respectively for each analyte and 2.5 ng/ml for the internal standard halazepam.

Quality control (QC) samples

Quality control (QC) samples (a set of samples with known concentration) (QC_L, QC_M, QC_H, where L, M, and H denote low, medium, and high concentration) were used to check the standard curve in compliance with Good Laboratory Practice (GLP) and run in duplicate at three different concentration levels for each run. The concentrations of the QC samples in this experiment were 35 ng/ml of each analyte for QC_L, 150 ng/ml of each analyte for QC_M, and 300 ng/ml of each analyte for QC_H.

Calculations

The peak heights of flurazepam, N-hydroxyethyl-flurazepam and N-desalkyl-flurazepam were measured, and their ratios to the halazepam (internal standard) peak height were calculated. The calibration curve for the analytes was generated by weighted nonlinear regression ($1/y^2$). Concentrations of analyte in the experimental samples were calculated using the equation, $x = (a - yc)/(yb - 1)$ where x is the concentration of analyte in ng/ml and y is the peak-height ratio of analyte to internal standard found for an experimental sample; a, b and c are constants generated by the nonlinear regression analysis of the internal standard calibration data.

The regression and calculation were performed using the Drug Metabolism Laboratory Information Manager System (DM-LIMS) (developed by Gary Lee Rathsmill, PennComp, PA, USA)

3. Results and discussion

3.1. Chromatography

Fig. 3 shows the GC–MS chromatogram of blank mouse plasma spiked with the internal

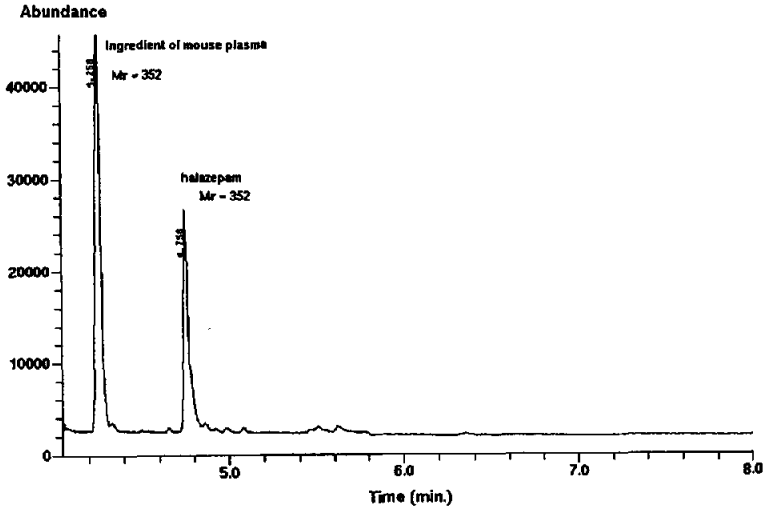

Fig. 3. GC–MS chromatogram of blank mouse plasma with halazepam. The first peak (t_R = 4.258) is from a component of mouse plasma.

standard halazepam; the first peak is from a component of mouse plasma with M_r 352, which is the same as that of halazepam. Fig. 4 shows the GC–MS chromatogram of flurazepam, N-desalkyl-flurazepam-TMS, and N-hydroxyethyl-flurazepam-TMS at a concentration of 50 ng/ml each and halazepam at concentration of 2.5 ng/ml with an injection volume of 2 μl. The chromatography shows the separation of the three analytes and the absence of peaks from other mouse plasma components. Fig. 5 shows a mouse plasma sample 10 h after administration of a dose of 50 mg/kg. The major metabolite of flurazepam in mouse was N-desalkyl-flurazepam.

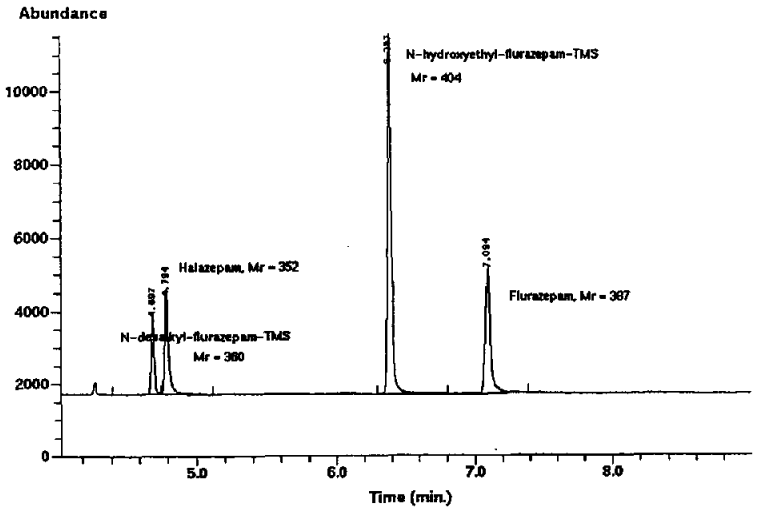

Fig. 4. GC–MS chromatogram of flurazepam, N-desalkyl-flurazepam-TMS, N-hydroxyethyl-flurazepam-TMS and halazepam. The analyte concentrations were 50 ng/ml, injection volume was 2 μl.

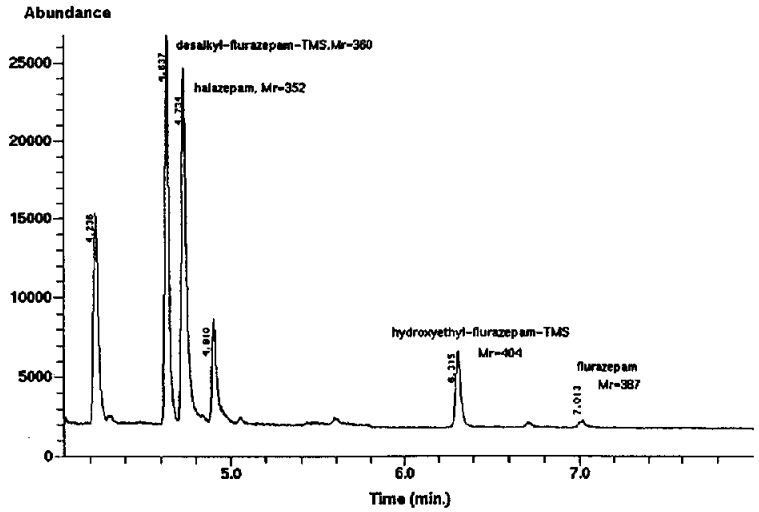

Fig. 5. GC–MS chromatogram of mouse plasma sample taken 10 h after administration of a 50 mg/kg dose of flurazepam. The major metabolite of flurazepam in mouse is N-desalkyl-flurazepam.

3.2. Accuracy and reproducibility

Three hundred samples each of mouse plasma and rat plasma were analyzed by this method. The results showed that N-1-desalkyl-flurazepam is the major metabolite and that its half-life in rat plasma is shorter than in mouse plasma [5].

Tables 1 and 2 show the data of the calibration standards and 5 replicates of the QC samples for hydroxyethyl-flurazepam. The results showed that the regression correlation coefficients were higher than 0.99. The accuracy of the analysis was *ca.* 10%, and the reproducibility was 2–12%. The average recovery for the QC samples was in the range of 90–110% of the theoretical value. This result was conform the Good Laboratory Practice (GLP). Similar data were simultaneously obtained for desalkyl-flurazepam and flurazepam and showed essentially the same accuracy. For 906 samples, all data fulfilled the same criteria as stated above and were all conform the GLP.

3.3. Limit of detection and limit of quantitation

The detection imits for our procedure were found to be 0.1 ng/ml for N-hydroxyethyl flurazepam, 0.5 ng/ml for desalkyl-flurazepam and flurazepam, with injection volume of 1 μl. The signal-to-noise ratio is greater than 5. The quantitation limit was set to 10 ng/ml for all compounds. This assured consistent results, even if the sensitivity decreased because of contamina-

Table 1
Pre-study assay evaluation for hydroxyethyl-flurazepam: calibration and QC samples

Sample ID/ replicate	Calibration concentration (ng/ml)	Concentration found (ng/ml)	Error (%)
STD-1/1	10.0	10.0	0
STD-1/2	10.0	11.0	10
STD-2/1	20.0	17.0	−15
STD-2/2	20.0	19.0	−5
STD-3/1	50.0	48.0	−4
STD-3/2	50.0	50.0	0
STD-4/1	100	104	4
STD-4/2	100	111	11
STD-5/1	200	214	7
STD-5/2	200	218	9
STD-6/1	400	390	−3
STD-6/2	400	386	−4
r^2	0.994		

Table 2
QC sample results for hydroxyethyl-flurazepam ($n = 5$)

Sample	Theoretical concentration (ng/ml)	Concentration (mean ± S.D.) (ng/ml)	%Theoretical
QC_L	35.0	35.0 ± 2.12	100.0
QC_M	150	161.6 ± 3.78	107.7
QC_H	300	326.6 ± 11.8	108.8

tion of the mass source and GC column caused by the large number of samples analyzed.

3.4. Nonlinear regression

In our experiments, we found that weighted nonlinear regression analysis for the construction of the calibration curves gave the most consistent and reproducible results even though there was no concrete theoretical basis for this model. Moreover, we consistently obtained excellent results with our quality control samples. This supported the mathematical analysis even though the curve was nonlinear. Another advantage of this model is that it becomes a linear regression when $b = 0$. If the standard curve had been linear, b would have been zero or it would have changed along with a and c to get the best fitting. In our experiments b ranged from 0 to 0.1.

3.5. Derivatization

Analysis of the two metabolites of flurazepam showed that the peaks of hydroxyethyl-flurazepam ($M_r = 332$) and desalkyl flurazepam ($M_r = 288$) were broad and asymmetrical and the sensitivity was also very low (detection limit > 100 ng/ml). This implied that the O–H or N–H group of these two compounds may show interaction with the active groups of the column material by forming hydrogen bonds. This would lower the volatility and result in the low sensitivity of the GC–MS analysis.

Therefore, substitution of the hydrogen in the O–H and N–H groups with non-polar groups through derivatization will decrease this interaction with concomitant enhancement of the sensitivity of the GC–MS analyses. Derivatization of these metabolites was successfully performed with BSTFA [N,O-bis(trimethyl)trifluoro-acetamide] and 1% TMCS (trimethylchlorosilane). Both the O–H group in N-1-hydroxyethyl-flurazepam and the N–H group in N-desalkyl-flurazepam reacted with BSTFA and 1% TMCS and their M_rs became 404 and 360, respectively (see Fig. 2). The sensitivity of the detection of hydroxyethyl-flurazepam was enhanced from 100 ng/ml to 0.1 ng/ml (*ca.* 1000 times) after derivatization.

After *ca.* 2000 injections and analyses, the column only needed to be cleaned once with methanol and ethyl acetate. The stability of the derivatized samples was high; they lasted more than one week.

Several parameters were varied in order to find the optimum derivatization conditions, in particular the reaction temperature (25–90°C), the heating time (10–90 min), and the volume of the BSTFA used (50–100 μl). The optimum reaction efficiency was obtained with the conditions described above, *i.e.* 60 μl BSTFA and heating at 50°C for 30 min.

The present method measures not only flurazepam but also its metabolites N-1-hydroxyethyl-flurazepam and N-1-desalkyl-flurazepam simultaneously. The derivatization technique was used in our method only to solve the problem of analyzing these metabolites by GC–MS and gain advantage over previously published methods.

4. Conclusions

As stated in the Introduction, the low quantity of sample available and the serious analytical interferences occurring in mouse plasma (rat plasma contains fewer interferences than mouse plasma) require a highly sensitive and selective analytical technique. The interference problem was solved by using a long GC capillary column (> 25 m) and the SIM (selected-ion monitoring) mode of the mass spectrometer. The high separation capability of the long GC capillary column made the interfering peaks appear far from the analyte peaks. Monitoring only the masses of the compounds of interest (SIM mode) eliminated the interfering peaks even if they appeared at the same retention time of the analytes. The exclusion of the interferences occurring in mouse plasma made it possible to reach a much lower detection limit.

Some drugs have a low thermal stability and will decompose during GC analysis. This is a big challenge to the GC–MS technique. On the other hand, high interference and low sensitivity present serious problems in HPLC. Solutions to both problems were presented in the method described. The results showed the accuracy to be 10% and the reproducibility to be 2–12%.

Moreover the present method provides a way to use only one suitable internal standard for the quantitative analysis of several analytes by GC–MS. This saves money and time compared with the synthesis of isotopic compounds usually used as internal standards in GC–MS analysis. Usually, each analyte needs its isotopic compound (replacing some hydrogens with deuteriums) as the internal standard and three isotopic compounds are required for three analytes. Because these isotopic compounds are not easily synthesized and more difficult to purify they are rather expensive. The results of our experiments show that essentially the same accuracy may be reached using a well-chosen internal standard instead of an isotopic internal standard.

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