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Determination of chlordiazepoxide in mouse 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–NCIMS) method for the determination of chlordiazepoxide (Librium) in mouse plasma is described. Chlordiazepoxide was not suitable for GC analysis because of its thermal instability. The specific derivatization technique described permits the analysis using GC–MS with high sensitivity and selectivity. The use of halazepam as an internal standard instead of an isotopic internal standard decreases the cost and time of the analysis. The detection limit for chlordiazepoxide was 0.1 ng/ml with an injection volume of 1 μl at a signal-to-noise ratio > 5. The limit of quantification was 5 ng/ml.

1. Introduction

Chlordiazepoxide (7 - chloro - N - methyl - 5-phenyl-3*H* - 1,4-benzodiazepin - 2-amine - 4-oxide) is used as an anxiolytic and tranquillizer drug. In our laboratory the determination of chlordiazepoxide in mouse plasma was first attempted using HPLC [1]. Since there was a serious interference peak at the same retention time as chlordiazepoxide and the sensitivity of detection was not high enough (250 ng/ml with UV detection), an alternative procedure was needed.

Although a number of HPLC methods and LC–MS methods [2,3] for the determination of chlordiazepoxide in human serum or urine have been published, they are not applicable to mouse plasma. The use of GC–MS to determine chlordiazepoxide has not been developed because it is unstable [4]. However, the GC–MS technique has the specific advantages of higher sensitivity and selectivity than other methods such as HPLC and LC–MS. This feature is particularly important for the determination of the drug in mouse plasma, as the amount of sample available is limited (100–200 μl). Hence a much more sensitive technique (5–10 times) was required to determine chlordiazepoxide in mouse plasma than in human plasma samples (normally 1 ml of plasma). Also, much higher selectivity was achieved to exclude the interference from the components of mouse plasma by capillary column GC.

Drugs such as chlordiazepoxide are thermally unstable and will decompose in GC analysis. This was a great challenge to the GC–MS technique. This problem was solved by deri-

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vatization. The most important feature of the proposed method was the derivatization of chlordiazepoxide with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS), which made the GC–MS technique possible.

2. Experimental

2.1. Chemicals

The pure drug compounds chlordiazepoxide (Ro 05-0690, Lot 501088) and halazepam (Ro 08-6728, Lot 5375-99) were generously supplied by Hoffman-La Roche (Nutley, NJ, USA). BSTFA containing 1% of TMCS was purchased from Pierce (Rockford, IL, USA). Mouse plasma, Lot 041593, and rat plasma, Lot 91092, were obtained from Rockland (Gilbertsville, PA, USA). All other chemicals were of the highest 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

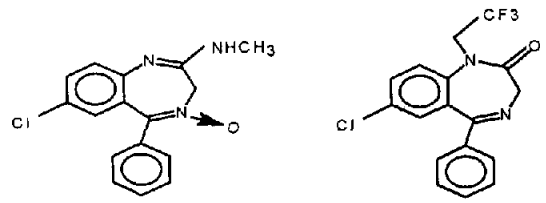
Chlordiazepoxide was administered to mice at 10, 30 and 100 mg per kg body mass. After dosing, plasma samples were removed from the mice at specific time intervals. The measurement of the level of chlordiazepoxide in the mouse plasma may indicate the rate of metabolism of chlordiazepoxide.

Volume of 200 μl of mouse (rat) plasma were pipetted into test-tubes. To each of these samples, 25 μl of internal standard working solution (50 ng/ml) were added. The sample was extracted by adding 1.0 ml of borate buffer solution (pH 10.0) and 5.0 ml of extraction solution [toluene–dichloromethane (70:30, v/v)]. The tube was capped and vortex mixed (vigorous shaking and rotation) for 15 min, then the sample was centrifuged for 10 min at 750 *g*. The organic layer which contained chlordiazepoxide was evaporated to dryness under nitrogen and the residue was allowed to react with BSTFA–1% TMCS for 30 min at 90°C, then cooled and the reaction was continued for another 36 h at ambient temperature. The amine hydrogen of the free base of chlordiazepoxide was derivatized. After the derivatization, an aliquot of the sample was analysed by GC with negative-ion chemical ionization MS detection (GC–NCIMS). Halazepam was used as an internal standard in the assay because of its stability and its similar structure (Fig. 1).

2.3. Method of analysis

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

The GC carrier gas was helium with a column head pressure 30 kPa at about 25°C and the flow-rate was maintained at 1 ml/min. The injector temperature was 280°C, the oven temperature was programmed from 180 to 300°C at 30°C/min, the sample volume was 1 μl and the run time was about 7 min. The mass was set in the negative-ion chemical ionization (NCI) and selected-ion monitoring (SIM) acquisition mode, monitoring ions at m/z 352 and 281.5. The temperatures of the source and quadrupoles were 150 and 100°C, respectively. The ionizing voltage and electron multiplier were set at 230



[1] CHLORDIAZEPOXIDE [2] HALAZEPAM

Fig. 1. Structures of chlordiazepoxide and halazepam.

eV and 2000 V, respectively. The chemical ionization gas was methane and the optimum operating pressure was found to be 0.173–0.25 kPa.

2.4. Quantitative analysis

Determination was based on the ratio of peak height of the analyte to that of halazepam. The accuracy and reproducibility of the method were demonstrated by the results of duplicate analyses of control mouse plasma spiked with the analyte at six different concentrations and the internal standard.

Calibration

A calibration graph was obtained, in duplicate, by adding 25 μl of the respective working solution (containing 1, 4, 10, 20, 40 and 100 ng of the analyte), 25 μl of the internal standard working solution and 200 μl of control mouse plasma to a test-tube. The final concentrations of the analyte (S1–S6) were 5, 20, 50, 100, 200 and 500 ng/ml, respectively, and 6.25 ng/ml for the internal standard halazepam.

Quality assurance (QA) samples

Quality assurance (QA) samples (a set of known concentrations of analyte in plasma samples) were used to check the calibration graph in compliance with good laboratory practice (GLP) and run in duplicate at three different concentration levels (QA_L, QA_M, QA_H) for each experiment in order to ensure the accuracy of the analysis. The concentrations of QA samples were 30 ng/ml of analyte for QA_L, 150 ng/ml for QA_M and 300 ng/ml for QA_H.

Calculations

The peak height of chlordiazepoxide was measured and its ratio to that of halazepam (internal standard) was calculated. The calibration graph for the analyte 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, y is the peak-height ratio of analyte to internal standard found for an experimental sample and 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) (by G.L. Rathsmill, PennComp, PA, USA).

3. Results and discussion

3.1. Chromatography

Fig. 2 shows a typical GC–MS trace for blank mouse plasma with the internal standard halazepam. The interference peak of mouse plasma that appeared before the halazepam at the same m/z of 352 as halazepam was excluded in this chromatogram. A similar chromatogram was observed in the analysis of flurazepam [5]. Fig. 3 shows the GC–MS of chlordiazepoxide and the internal standard halazepam. The concentration of chlordiazepoxide was 20 ng/ml and the injection volume was 1 μl.

3.2. Derivatization

The hydrogen of the NH group would form hydrogen bondings with the active surface of the column matrix resulting in peak tailing, asymmetry and low sensitivity. The molecular peak M (m/z 298) was not the largest one as is usual in an NCI mass spectrogram; instead, the m/z 282 ion gave the largest peak. This could be interpreted that the oxygen and the hydrogen connected to nitrogen were lost in the mass source with the NCI gas (see Fig. 4). A similar basic peak due to $[MH - O]^+$ of chlordiazepoxide was observed by Sato et al. [2,3].

Trifluoroacetic anhydride (TFAA) is commonly used to derivatize the NH group. However, we found that TFAA or pentafluorobenzoyl chloride (PFB) were unsuitable for derivatization of chlordiazepoxide as no peaks were observed in the m/z 200–500. It is possible that

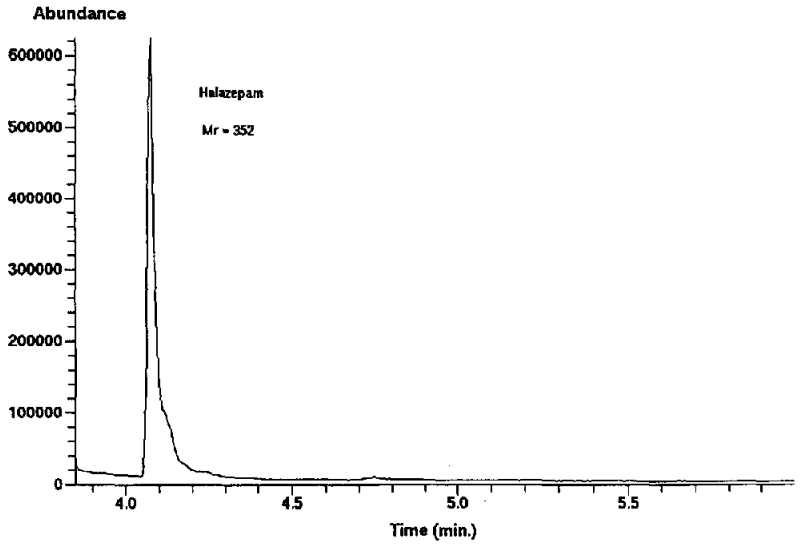


Fig. 2. GC–MS of blank mouse plasma containing halazepam. The peak before the halazepam due to the components of mouse plasma was excluded.

chlordiazepoxide was not stable and was fragmented in the reaction with TFAA or PFB and the mass of the fragments was less than 200.

BSTFA–1% TMCS was used for derivatization. After derivatization, GC–MS showed a remarkable improvement. The peak at m/z 282 was very sharp and the detection limit was enhanced over 1000-fold. The peak of $[M+TMS]^-$ (m/z 370) was very small compared with that at m/z 282. This could be because the TMS group was bonded to chlordiazepoxide through the GC column and lost together with the

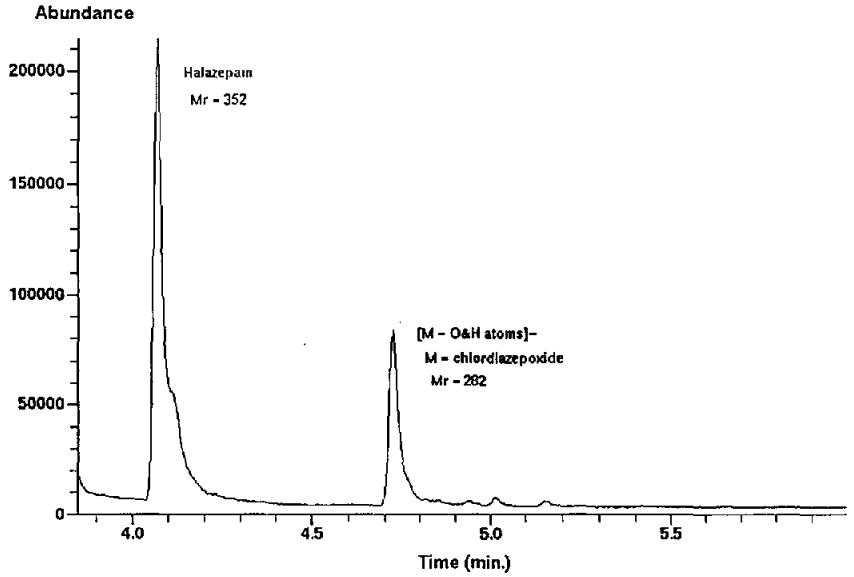


Fig. 3. GC–MS of chlordiazepoxide and halazepam. The concentration of chlordiazepoxide was 20 ng/ml and the injection volume was 1 μl.

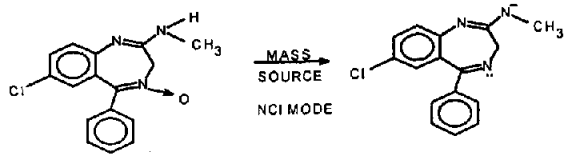


Fig. 4. Mechanism of ionization of chlordiazepoxide in the NCI mass spectrometer.

oxygen in the ion source with NCI gas (see Fig. 5). As the peak at m/z 282 was the largest and most stable, it was used as the basic peak for quantitative analysis. The results also showed that the polarity of the NH bond of chlordiazepoxide is weak so it requires a longer reaction time with the derivatizing agent. It should be pointed out that it would not shorten the reaction time if the reaction were carried out at 90°C for longer than 30 min, nor would a better result be obtained if the reaction were initiated at ambient temperature. The best result was obtained as described by carrying out the reaction for 30 min at 90°C, then continued for two nights (over 36 h) at ambient temperature.

The results showed that after derivatization, the sensitivity for the detection of chlordiazepoxide was enhanced from 500 to 0.1 ng/ml.

3.3. Accuracy and reproducibility

For 450 samples (including standard and QA samples), 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 QA samples were within 20% and on average the results for QA samples were 90–110% of the theoretical values. These results were calculated by the DM-LIMS and were in compliance with GLP.

Table 1 gives the results for a typical pre-study assay evaluation for calibration and Table 2 gives the results for the determination of chlordiazepoxide in QA samples.

3.4. Sensitivity

The results showed that the detection limit can be as low as 0.1 ng/ml with an injection volume of 1 μl. The limit of quantification was set at 5 ng/ml in routine analysis. This ensured consistent results, even if the sensitivity decreased because of contamination of the mass source or the GC column as a result of large numbers of analyses.

3.5. Non-linear regression

We found that the weighted non-linear regression analysis for the construction of the calibration graph gave the most consistent and reproducible results even though there was no concrete theoretical basis for this mathematical model. Moreover, we consistently obtained excellent results with our QA samples. This supported our mathematical analysis even though the graph was non-linear. Another advantage of this model was the inclusion of linear regression that started regression at $A = 1$, $B = 0$ and $C =$

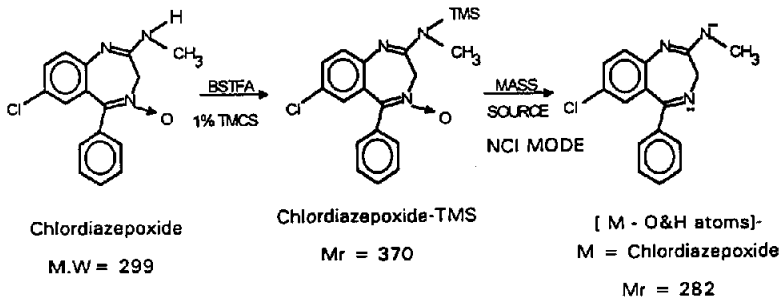


Fig. 5. Reaction of chlordiazepoxide with BSTFA–1% TMCS and the mechanism of the ionization of the reaction product in the NCI mass spectrometer.

Table 1
Pre-study assay evaluation: calibration results for chlordiazepoxide

Sample No.	Replicate	Calibration concentration (ng/ml)	Concentration found (ng/ml)	(Found − added)/ added (%)
1	1	5.00	4.90	−2.0
	2	5.00	5.10	2.0
2	1	20.00	20.20	1.0
	2	20.00	20.10	0.5
3	1	50.00	53.70	7.4
	2	50.00	50.50	1.0
4	1	100.0	94.50	−5.5
	2	100.0	96.70	−3.3
5	1	200.0	202.0	1.3
	2	200.0	200.0	0.2
6	1	500.0	477.9	−4.4
	2	500.0	530.0	6.0

$r^2 = 0.9935$.

8.5. If the calibration graph were linear, B would be 0 or it would change along with the A and C values to give the best fit. In our experiments, we found B values in the range of 0–0.01, which means that the calibration curves were very close to linearity. A similar method of analysis was employed in the determination of flurazepam and its major metabolites by GC–MS [5].

4. Conclusion

The advantages of the high sensitivity and separation ability of GC–MS were utilized in the determination of chlordiazepoxide in mouse plasma. The specific derivatization technique applied to chlordiazepoxide made GC–MS analysis possible.

The method also provided a way to choose a normal compound instead of an isotopic compound as an internal standard. This saves money and time in synthesizing isotopic compound standards [5].

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Table 2
QA sample results for chlordiazepoxide

Sample	Theoretical concentration (ng/ml)	Concentration found (mean ± S.D., $n = 5$) (ng/ml)	Percentage of theoretical
QA1	30.00	27.82 ± 2.501	92.73
QA2	150.0	149.2 ± 13.27	99.44
QA3	300.0	320.9 ± 12.64	106.98

References

[1] R. Gill, B. Law and J.P. Girrs, *J. Chromatogr.*, 356 (1986) 37–46.
[2] K. Sato, H. Hattori, C.M. Moore and Y. Kastsumata, *Huchudoku*, 10 (1992) 23–30.
[3] K. Sato, H. Hattori, C.M. Moore and Y. Kastsumata, *Huchudoku*, 10 (1992) 128–129.
[4] C. Lau, S. Dolan and M. Tang, *J. Chromatogr.*, 416 (1987) 212–214.
[5] D. Song, S. Chang and K. Kohlhof, *J. Chromatogr.*, 658 (1994) 142.