Pergamon

0731-7085(94)00070-0

*Journal of Pharmaceutical & Biomedical Analysis*, Vol. 12, No. 11, pp. 1417–1423, 1994

0731-7085/94 $7.00 + 0.00

# A selective fluorimetric method for the determination of some 1,4-benzodiazepine drugs containing a hydroxyl group at C-3

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**Abstract**: A highly selective and sensitive fluorimetric method was developed for the determination of four 1,4-benzodiazepine drugs containing a hydroxyl group at carbon 3, namely oxazepam, lorazepam, cinolazepam and temazepam. The method is highly specific because other benzodiazepinee lacking the hydroxyl group at C-3 do not react similarly and hence do not interfere. The proposed method involves reduction of the target compound using Zn°/HCl at room temperature with the formation of a highly fluorescent derivative within 15 min. The different experimental parameters were carefully studied and incorporated into the procedure. Under the described conditions, the proposed method is applicable over the concentration range of 0.1–1.2 μg ml$^{-1}$ for both temazepam and cinolazepam, and 0.2–2.5 and 1–8 μg ml$^{-1}$ for oxazepam and lorazepam respectively. The recoveries of the title compounds from spiked urine ranged from 90.0 to 92.0% and for serum from 94.1 to 95.4% with a limit of detection ($S/N$ = 2) of 4 ng ml$^{-1}$ for all drugs. The mechanism of the fluorimetric reaction is discussed.

**Keywords**: *Benzodiazepines; pharmaceutical preparations; biological fluids; fluorimetry.*

## Introduction

1,4-Benzodiazepine tranquilizers are extensively used as sedatives, hypnotics and anticonvulsants for the relief of insomnia, frequent nocturnal awakenings and/or early morning awakenings [1].

Many analytical methods have been reported for their determination in pharmaceutical preparations and in biological fluids. A good guide to the work published on the determination of benzodiazepines can be found in two excellent books written by Schutz [2, 3].

Oxazepam has been determined by acid-base titration [4], densitometry [5], spectrophotometry [6, 7], fluorimetry [8], polarography [9–10], G.C. [12] and HPLC [13, 14].

Temazepam has been determined by acid–base titration [4], spectrophotometry [7], polarography [11] and HPLC [13, 15]. Lorazepam has been determined by adsorptive stripping voltammetry [16, 17], polarography [10, 11], fluorimetry [8], G.C. [12] and HPLC [18]. Cinolazepam has been determined polarographically [19].

The present paper describes a selective and highly sensitive method for the determination of some 1,4-benzodiazepine drugs. The method is based on the reduction of the target compounds with Zn°/HCl to form highly fluorescent derivatives. The method has been applied to the determination of these drugs in biological fluids and in pharmaceutical formulations. The results obtained compare favourably with those obtained by the traditional method [20].

## Experimental

### *Apparatus*

An Aminco-Bowman model $J_4$-9860 spectrofluorimeter was used with the excitation and emission slit controls set at 5 mm. The measurements were performed using a 1-cm quartz cell.

### *Reagents and materials*

All the chemicals were of analytical reagents grade, and the solvents were of spectroscopic grade. Pure drug samples were kindly supplied by different manufacturers and their purity was

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**Table 1**
Performance data for the fluorimetric determination of pure samples of the studied 1,4-benzodiazepin

| Compound | Excitation wavelength (nm) | Emission wavelength (nm) | Range ($\mu g\ ml^{-1}$) | Slope | Intercept | Correlation coefficient |
|---|---|---|---|---|---|---|
| Oxazepam | 368 | 472 | 0.2–2.5 | 0.0342 | 0.015 | 0.999 |
| Lorazepam | 368 | 472 | 1.0–8.0 | 0.0902 | 0.0318 | 0.999 |
| Cinolazepam | 368 | 480 | 0.1–1.2 | 0.0133 | 0.0024 | 0.999 |
| Temazepam | 368 | 480 | 0.1–1.2 | 0.0143 | −0.0019 | 0.999 |

*Each result is the average of nine separate determinations.

**Table 2**
Determination of the studied 1,4-benzodiazepines in 5 ml serum samples

| Oxazepam | | Lorazepam | | Cinolazepam | | Temazepam | |
|---|---|---|---|---|---|---|---|
| Taken ($\mu g\ ml^{-1}$) | Found % | Taken ($\mu g\ ml^{-1}$) | Found % | Taken ($\mu g\ ml^{-1}$) | Found % | Taken ($\mu g\ ml^{-1}$) | Found % |
| 0.20 | 92.9 | 1 | 90.9 | 0.10 | 86.7 | 0.10 | 92.9 |
| 0.40 | 92.9 | 2 | 93.2 | 0.20 | 93.3 | 0.20 | 89.3 |
| 0.60 | 96.2 | 3 | 93.9 | 0.30 | 93.3 | 0.30 | 92.8 |
| 0.80 | 95.2 | 4 | 95.5 | 0.40 | 94.9 | 0.40 | 94.6 |
| 1.20 | 95.3 | 5 | 97.2 | 0.50 | 94.4 | 0.50 | 94.4 |
| 1.60 | 95.5 | 6 | 97.7 | 0.60 | 95.0 | 0.60 | 95.3 |
| 2.00 | 97.1 | 7 | 96.8 | 0.70 | 97.3 | 0.80 | 96.4 |
| 2.40 | 97.0 | 8 | 97.8 | 0.80 | 97.8 | 1.00 | 96.4 |
| | | | | | | 1.20 | 97.2 |
| $\bar{x}$ | 95.3 | | 95.4 | | 94.1 | | 94.4 |

checked using the usual methods [20]. Pharmaceutical preparations containing the studied compounds were obtained from commercial sources.

*Standard preparation*

Twenty five milligrams of each drug were dissolved in 100 ml methanol and serial dilutions with distilled water were made, to cover the working range (Table 1).

*Calibration graph*

Aliquot volumes of the standard solution were transferred into a series of 25-ml volumetric flasks. Ten millilitres of distilled water, 120 mg of zinc powder and 3 ml 10% (v/v) hydrochloric acid were added. After 15 min, volume was completed with distilled water. After filtering as necessary into a dry flask, the fluorescence at the specified excitation and emission wavelength of each fluorophore was measured (Table 1). The blank used was 3 ml of 10% (v/v) HCl + 22 ml distilled water. The concentration vs the %RI was plotted to obtain the standard calibration graph. Alternatively, one could calculate the linear regression equations.

*Procedure for dosage forms*

After either weighing and pulverizing 20 tablets or mixing the contents of 20 capsules, an accurately weighed amount of the powder, equivalent to 25.0 mg of each drug was transferred into a 100-ml volumetric flask. After extraction with 3 × 30 ml of methanol, the

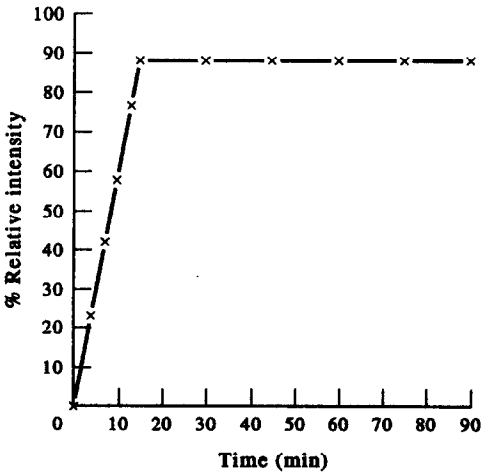



**Figure 1**
Effect of time on the stability of the fluorescent product of oxazepam (2.5 $\mu g\ ml^{-1}$).

mixture was filtered into a 100-ml volumetric flask. The flask and filter were then washed and the volume completed with methanol. Aliquot volumes of the filtrate were transferred into a series of 25-ml volumetric flasks and the procedure described under the calibration graph section was performed.

*Assay of drugs in spiked human serum*

Five millilitres of human serum were spiked with the studied compounds separately so that the final concentration was in the range cited in Table 2. The compound was extracted as follows: 5 ml of serum were transferred into a separatory funnel, to which 5 ml of saturated potassium chloride solution were added and extracted with 3 × 10 ml of toluene–methylene chloride (90:10, v/v) and completed to 50 ml with the same solvent. Twenty five millilitres of the organic layer were transferred to a rotary evaporator and evaporated to dryness. The residue was dissolved in 5 ml of methanol and the procedure as described under the calibration graph section was performed.

*Assay of the drugs in spiked human urine*

Five millilitres of human urine were spiked with the studied compounds separately, so that the final concentration was in the range cited in Table 3. The compounds were extracted as follows: 5 ml of urine were transferred into a separatory funnel, to which 0.5 ml of 0.5 N sodium chloride and 1.0 ml of 1 M phosphate buffer (pH 11) were added and extracted using 3 × 10 ml of diethyl ether. Centrifugation at 1500 rpm for 3 min was performed. The organic layer was evaporated to dryness. The residue was dissolved in 5 ml of methanol and the procedure as described under the calibration graph section was performed.

*Calculation*

The content of the drugs in the sample solutions were calculated either from previously plotted calibration graphs or from linear regression equations (Table 1).

*Isolation of the reduction product*

About 50.0 mg of lorazepam were allowed

**Table 3**
Determination of the 1,4-benzodiazepines in 5 ml urine samples

| Oxazepam | | Lorazepam | | Cinolazepam | | Temazepam | |
|---|---|---|---|---|---|---|---|
| Taken ($\mu g\ ml^{-1}$) | Found % | Taken ($\mu g\ ml^{-1}$) | Found % | Taken ($\mu g\ ml^{-1}$) | Found % | Taken ($\mu g\ ml^{-1}$) | Found % |
| 0.20 | 92.9 | 1 | 90.9 | 0.10 | 86.7 | 0.10 | 92.9 |
| 0.40 | 89.9 | 2 | 90.9 | 0.20 | 90.0 | 0.20 | 92.9 |
| 0.60 | 90.9 | 3 | 87.9 | 0.30 | 88.9 | 0.30 | 92.9 |
| 0.80 | 91.1 | 4 | 92.0 | 0.40 | 91.5 | 0.40 | 91.1 |
| 1.20 | 90.5 | 5 | 89.9 | 0.50 | 89.9 | 0.60 | 90.1 |
| 1.60 | 91.1 | 6 | 90.8 | 0.60 | 91.7 | 0.80 | 93.0 |
| 2.00 | 90.0 | 7 | 91.6 | 0.70 | 90.7 | 1.00 | 91.1 |
| 2.40 | 91.1 | 8 | 91.0 | 0.80 | 90.6 | 1.20 | 91.7 |
| $\bar{x}$ | 90.8 | | 90.6 | | 90.0 | | 92.0 |

**Table 4**
Application of the proposed method for the determination of title compounds in raw materials

| Compound | Proposed method % Found ± SD | Reported methods*†‡ % Found ± SD |
|---|---|---|
| Oxazepam | 100.0 ± 0.62 | 100.6 ± 0.41 |
| Lorazepam | 99.9 ± 0.42 | 100.0 ± 0.47 |
| Temazepam | 100.1 ± 0.53 | 100.3 ± 0.88 |
| Cinolazepam | 99.9 ± 0.59 | 98.7 ± 1.57 |

*Note*: Each result is the average of nine separate determinations.
*Official method [20] for oxazepam and temazepam.
†Reference method [15] for temazepam.
‡Reference method [19] for cinolazepam.

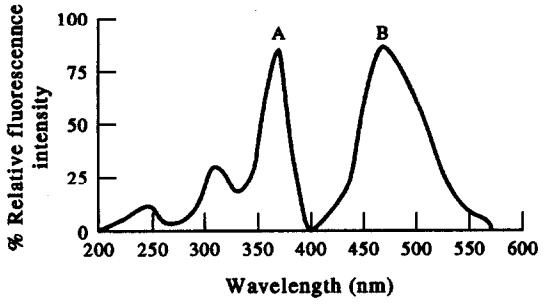
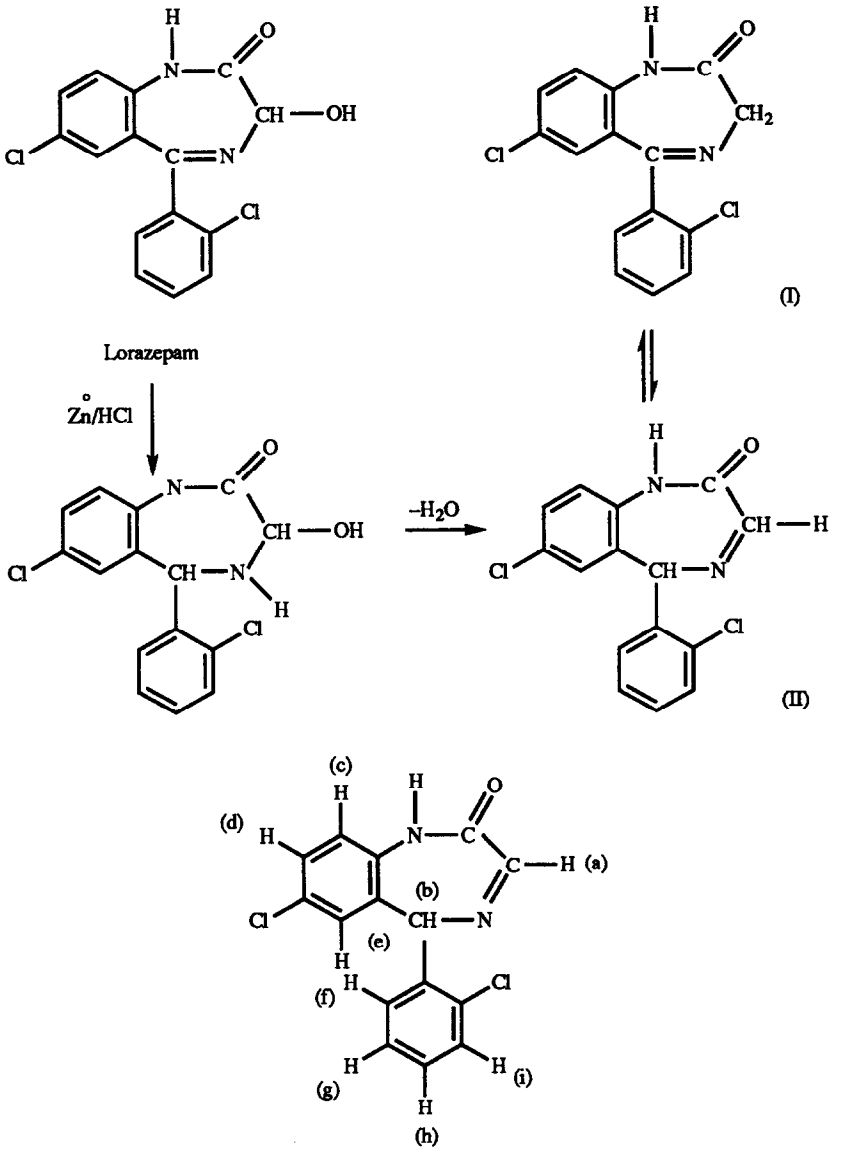



**Figure 2**
Excitation (A) and emission (B) fluorescence spectra of oxazepam fluorophore (2.5 μg $ml^{-1}$).

to react with the zinc/hydrochloric acid reagent for about 30 min. After filtering, the solution was rendered alkaline with ammonia, extracted with 2 × 10 ml of chloroform. The chloroform was evaporated to dryness under nitrogen gas. The residue was extracted with methanol, to which 1.0 gm of charcoal was added. The reduction production was then filtered and crystallized from methanol.

## Results and Discussion

During studies on the polarographic reduction of benzodiazepine drugs [19] it was




**Scheme 1**
Reduction of lorazepam using Zn°/HCl.

noticed that the reduction products of only those members containing a hydroxyl group at C-3 are fluorescent. This observation led to the present study.

The experimental conditions affecting the development and stability of the fluorophores produced were carefully studied, 120 ± 20 mg of zinc powder and 3.2 ± 0.2 ml of 10% (v/v) hydrochloric acid were found to produce the maximum fluorescence intensity. The fluorophores produced were developed within 15 min and remained stable for more than 2 h at room temperature (Fig. 1). Typical excitation and emission spectra of oxazepam as a model example are shown in Fig. 2. Table 1 summarizes the performance data for the deter-

**Table 5**
Application of the standard addition technique to the determination of the studied 1,4-benzodiazepin compounds in dosage forms

| Preparation | Proposed method: Claimed amount taken μg ml$^{-1}$ | Pure pwd added μg ml$^{-1}$ | Total amount found μg ml$^{-1}$ | Found % | Official method*†‡ Found% |
|---|---|---|---|---|---|
| §Persumbrax tablets | 0.2 | 0.0 | 0.199 | 100.0 | 100.0 |
| (oxazepam 10 mg/tablet) | 0.4 | 0.6 | 0.98 | 98.9 | 100.0 |
| | 0.8 | 0.6 | 1.41 | 100.7 | 98.5 |
| | 1.0 | 0.6 | 1.59 | 99.4 | 101.4 |
| | 1.5 | 0.6 | 2.12 | 100.9 | 100.0 |
| Mean | | | | 99.9 | 100.0 |
| ‖SD | | | | 0.76 | 0.92 |
| Trango-alupent tablets | 0.2 | 0.0 | 0.198 | 99.1 | 99.2 |
| (oxazepam 10 mg/ml/tablet) | 0.6 | 0.6 | 1.21 | 100.8 | 100.9 |
| | 0.8 | 0.6 | 1.40 | 100.0 | 99.7 |
| | 1.0 | 0.6 | 1.60 | 100.0 | 100.5 |
| | 1.5 | 0.6 | 2.11 | 100.5 | 99.6 |
| Mean | | | | 100.1 | 100.0 |
| ¶SD | | | | 0.58 | 0.56 |
| Ativan tablets | 1.0 | 0.0 | 0.997 | 99.7 | 99.7 |
| (lorazepam 2 mg/tablet) | 2.0 | 2.0 | 4.03 | 100.9 | 100.6 |
| | 3.0 | 2.0 | 4.98 | 99.8 | 100.1 |
| | 4.0 | 2.0 | 5.94 | 99.0 | 99.3 |
| | 5.0 | 2.0 | 7.03 | 100.4 | 100.2 |
| Mean | | | | 100.0 | 99.8 |
| **SD | | | | 0.64 | 0.44 |
| Levanexol capsules | 0.2 | 0.0 | 0.199 | 100.0 | 99.8 |
| (temazepam 10 mg/tablet) | 0.3 | 0.4 | 0.69 | 100.0 | 99.8 |
| | 0.4 | 0.4 | 0.79 | 99.5 | 100.3 |
| | 0.5 | 0.4 | 0.91 | 100.7 | 100.5 |
| | 0.6 | 0.4 | 0.99 | 99.3 | 100.6 |
| Mean | | | | 99.9 | 100.2 |
| SD | | | | 0.47 | 0.42 |
| Cinolazepam tablets | 0.2 | 0.0 | 0.199 | 99.7 | 99.6 |
| (cinolazepam 40 mg/tablet) | 0.3 | 0.4 | 0.70 | 100.6 | 98.6 |
| | 0.4 | 0.4 | 0.79 | 99.3 | 97.5 |
| | 0.5 | 0.4 | 0.90 | 100.4 | 98.9 |
| | 0.6 | 0.4 | 0.99 | 99.2 | 98.7 |
| Mean | | | | 99.8 | 98.7 |
| SD | | | | 0.59 | 0.57 |

*Official method [2] for the first three preparations.
†Reference method [15] for the fourth preparations.
‡Reference method [19] for the fifth preparations.
§‖Boehringer Ingelheim, Co., Germany.
¶Wyeth Co., USA.
**Cid. Co. Cairo, Egypt, under licence from Carlo Erba.

mination of the compounds studied by the proposed method.

To test the validity of the method, it was applied to the determination of pure samples of the title compounds. The results, summarized in Table 4, show that the proposed method is satisfactorily accurate and precise compared with the traditional method [20]. The proposed method was further applied to the determination of the studied drugs in dosage forms. Drugs commonly co-formulated with the studied compounds, such as dipyridamol and orciprenaline sulphate, did not interfere with the determination, indicating the high selectivity of the proposed method. The results in Table 5 are in accordance with those obtained with the traditional method [20] with regard to the accuracy and precision as shown by statistical analysis of the data [21].

Analysis of human serum and urine samples spiked with the title compounds was conducted using the proposed method. The results summarized in Tables 2 and 3 showed that no interference arose from endogenous compounds.

The proposal mechanism of the reduction process is illustrated in Scheme I.

The reaction of lorazepam with zinc powder in the presence of 10% (v/v) hydrochloric acid resulted in the formation of a 1,4-benzodiazepine-2-one derivative, form I; however, interconversion may occur to give form II as the predominant tautomer (the more thermodynamically stable one). This was confirmed by I.R. H-NMr and MS spectral data of the product. The I.R. (KBr disc) spectrum indicates the presence of C=O amide stretching at 1680 $cm^{-1}$. H-NMR spectrum ($CDCL_a$): $\sigma$ 5.65 (s,1 H,a-proton), $\sigma$ 6.6(s,1 H,b-proton), $\sigma$ 6.95, (d,1H, c-proton), $\sigma$ 7.2–7.45 (m,5H,Ar-H,e-i), $\sigma$ 7.6 (d,1H,d-proton) and $\sigma$ 8.1 (bs,1H,NH-proton). The spectrum also showed additional signals at $\sigma$ 1.2 indicating the presence of $H_2O$ in $CDCL_3$, and at $\sigma$ 1.5 and 3.5 due to the presence of methanol which is used as crystallization solvent Fig. 3.

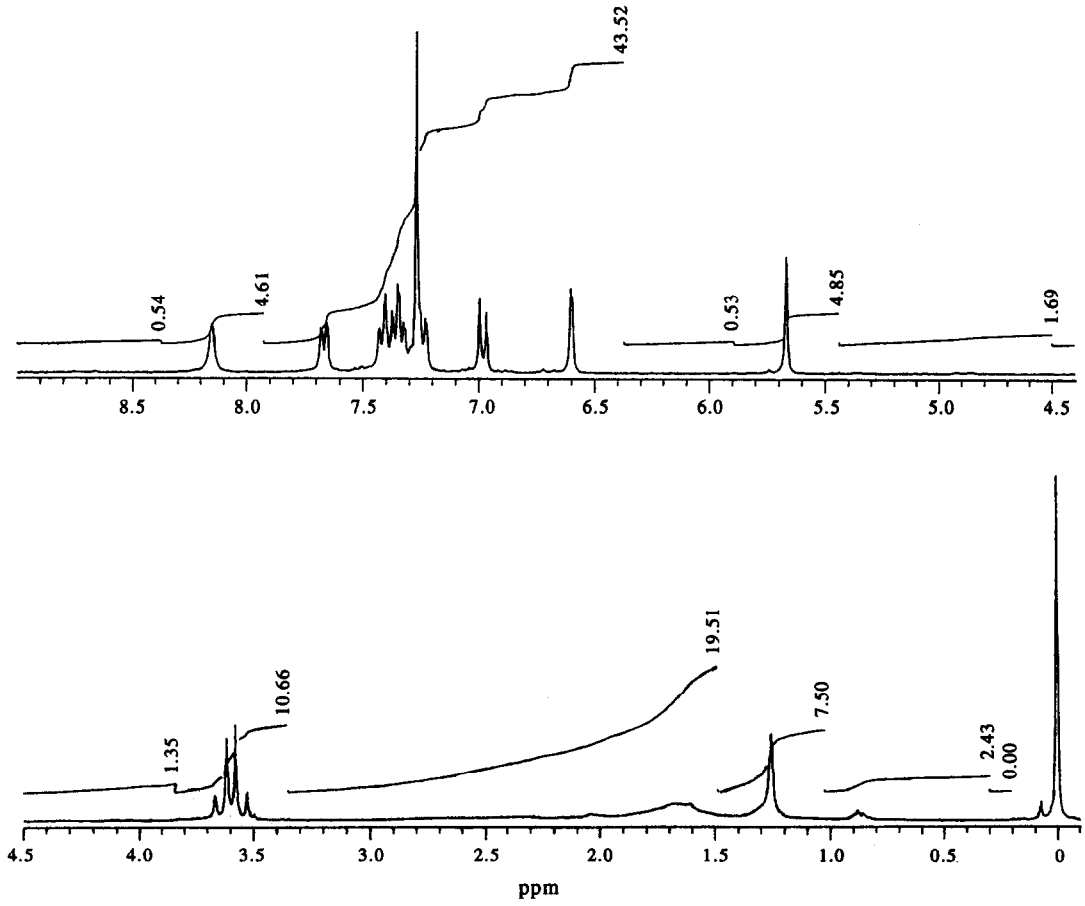



**Figure 3**
NMR-spectra of the reduction product of lorazepam.

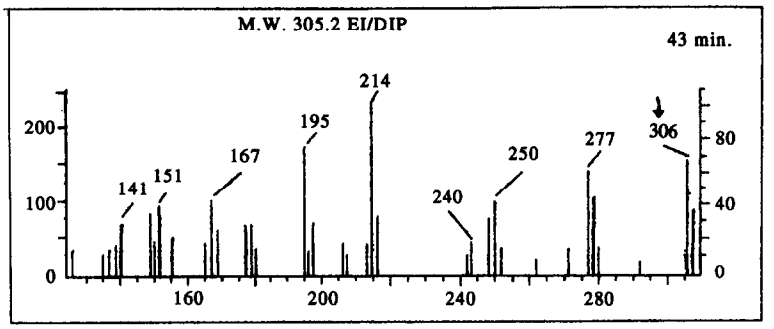



**Figure 4**
MS-spectra of the reduction product of lorazepam.

MS spectra of the compound indicated the presence of the main molecular ion peak $M^+$ 305.2 and M + 1306.2. It also showed peaks at m/e 277 ($M^+$-CO), m/e 250 (277-HCN) and at m/-e 214.5 (base peak, 250-Cl) Other peaks at m/e 195, 167, 151 and 141 indicated the rest of the fragmentation pattern of the compound (Fig. 4).

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*[Received for review 20 July 1993; revised manuscript received 22 April 1994]*