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Short communication

# Rapid and simple chromatographic method for the determination of diazepam and its major metabolites in human plasma and urine

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**Abstract**

A simple, rapid, sensitive and selective HPLC method has been developed for the analysis of diazepam (DZP) and its major metabolites, *N*-desmethyldiazepam (DMDZP), temazepam (TZP) and oxazepam (OZP), in plasma and urine, using clonazepam (CZP) as the internal standard and chloroform as the extracting solvent, with a 10 ng/ml limit of quantitation for the four assayed drugs, and an average (±S.D.) recovery of 87.7±6.46%, 92.9±5.31%, 91.4±4.01% and 91.7±2.68% for DZP, DMDZP, TZP and OZP, respectively (from plasma), and 89.6±2.26%, 90±4.24%, 87.45±0.64% and 94.50±0.71% for DZP, DMDZP, TZP and OZP, respectively (from urine). The method has also proved to be selective and reproducible.


*Keywords:* Diazepam; *N*-Desmethyldiazepam; Temazepam; Oxazepam

## 1. Introduction

Diazepam (DZP) (Fig. 1) is a widely used benzodiazepen as hypnotic, sedative and muscle relaxant. It is metabolised by the liver cytochromes to three major metabolites; *N*-desmethyldiazepam (DMDZP), temazepam (TZP) and oxazepam (OZP) [1], which is conjugated and excreted mainly as a glucuoronide in urine (Fig. 2).

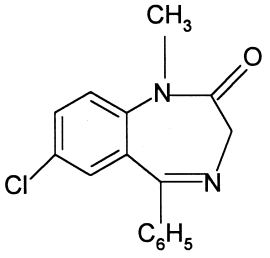


Fig. 1. Structure of diazepam.

Several methods have been reported for the determination of DZP and its metabolites, using high-performance liquid chromatography (HPLC)–UV [2–5], gas chromatography–electron-capture detection (GC–ECD) [6–8], and /or thin-layer chromatography (TLC) [9]. They usually involve multiple extraction steps, especially when dealing with urine, are time consuming, and in the case of GC require high temperature which is not suitable for the thermolabile metabolites OZP and TZP.

In this paper we report a rapid chromatographic method for the determination of DZP and its metabolites in plasma and urine.

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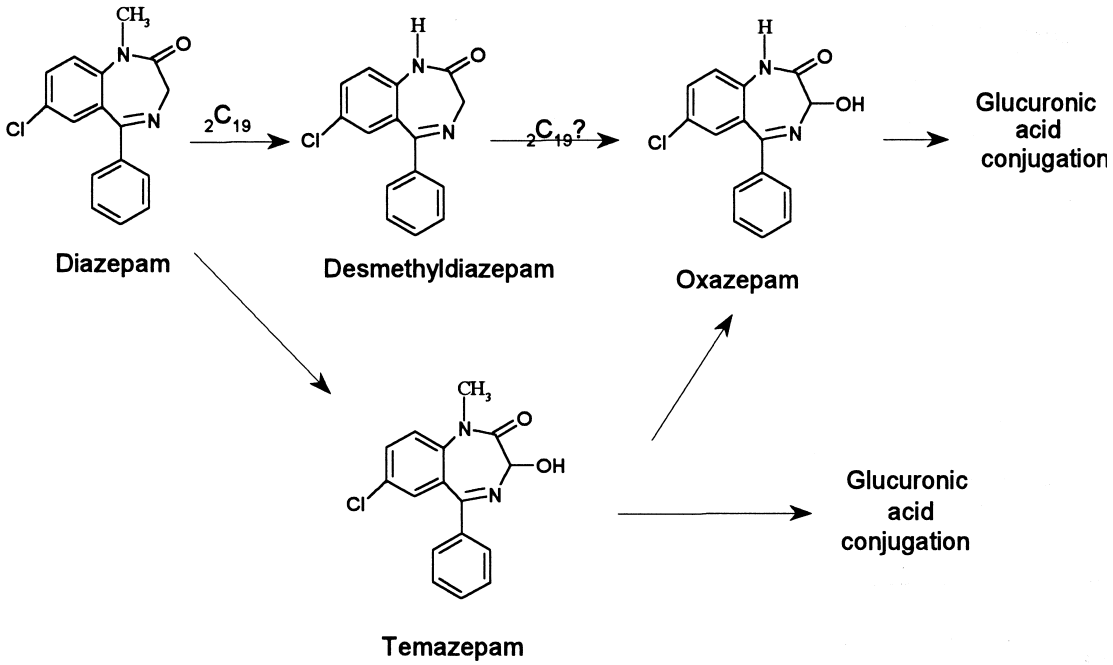


Fig. 2. Metabolic pathway of DZP.

## 2. Experimental

### 2.1. Reagents and materials

DZP, DMDZP, TZP and OZP reference standards were purchased from Sigma, clonazepam (CZP) the internal standard (I.S.) was a kind gift from the Jordanian Ministry of Health. HPLC grade acetonitrile, methanol, chloroform and pro-analysis ethanol and potassium dihydrogenphosphate were purchased from E. Merck (Darmstadt, Germany).

### 2.2. Extraction procedure

To 0.50-ml plasma or urine, add 5.0 ml chloroform (containing CZP in a concentration of 68.4–114.0 ng/ml as I.S.), and vortex for 30 s. After centrifugation at 5000 *g* for 5.0 min, refrigerate for ~10.0 min, decant the organic layer, evaporate under a stream of nitrogen with gentle heat, and reconstitute with 0.50 ml mobile phase, of which 100-μl will be injected onto a $C_{18}$ column connected to a HPLC–UV system as described in Section 2.3. (In the case of urine, pH adjustment to ~5.0 by acetate buffer pH 5.50 is needed before extraction).

### 2.3. High-performance liquid chromatography

A Varian Star Model 9002 HPLC system with a Varian Star Model 9100 autosampler, and a Varian Star Model 9050 UV detector set at 232 nm were used with a Hypersil BDS RP-$_{18}$ (25.0 cm×4.0 mm), 5.0 μm particle size column, and a 5.0 μm BDS $C_{18}$ guard column to separate DZP from its major metabolites and matrix interfering peaks, and I.S.

The mobile phase was methanol–acetonitrile–potassium dihydrogenphosphate buffer, 0.05 *M* (50:10:40, v/v), with a pH of ~3.50 and a flow-rate of 1.2 ml/min.

The data was collected using a Varian star chromatography workstation.

### 2.4. Standard solutions preparation

A 100 μg/ml standard solution was prepared by dissolving ~5 mg of each of DZP, DMDZP, TZP, OZP and CZP in 50 ml ethanol to make up the stock solutions, which were used for preparation of the quality control samples (concentrations; 60, 100 and 200 ng/ml), as well as for validation purposes.

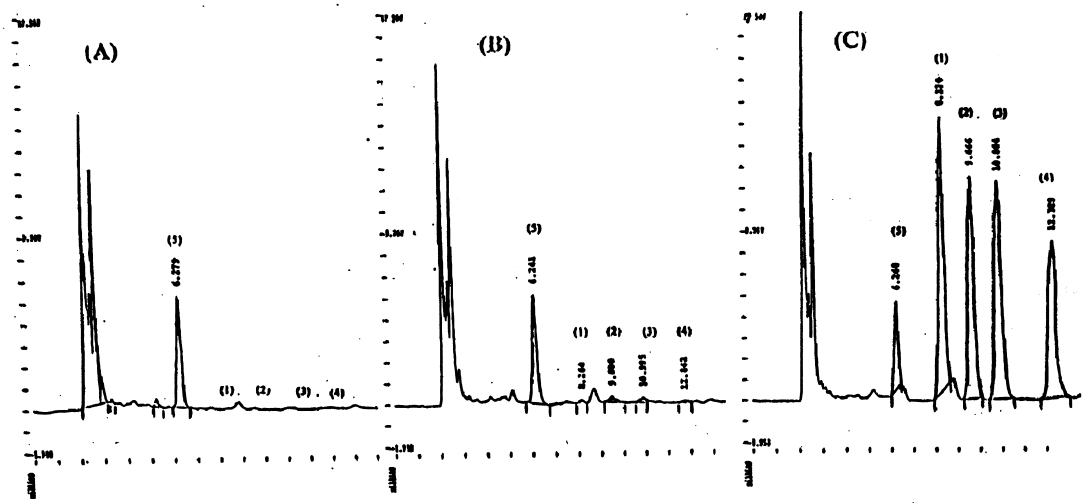


Fig. 3. Representative chromatograms of (A) blank plasma extract, (B) spiked plasma extract containing 10 ng/ml of each of (1) OZP, (2) TZP, (3) DMDZP, (4) DZP, with 648 ng/ml (5) CZP, (C) spiked plasma extract containing 1000 ng/ml of each of compounds (1–4), with 648 ng/ml (5) CZP.

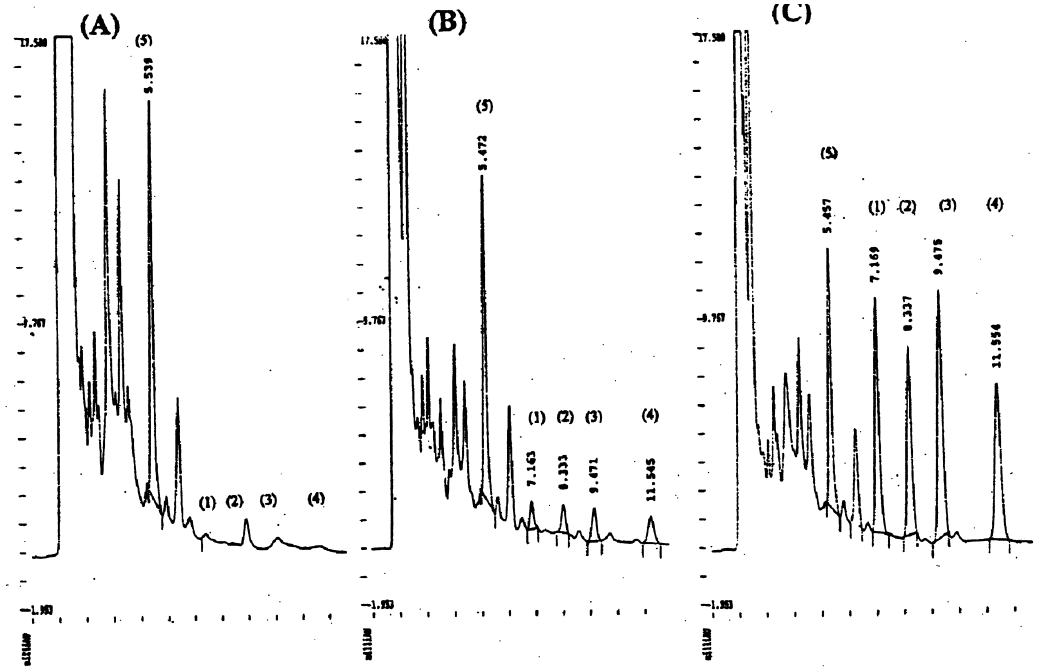


Fig. 4. Representative chromatograms of (A) blank urine extract, (B) spiked urine extract containing 60 ng/ml of each of (1) OZP, (2) TZP, (3) DMDZP, (4) DZP, with 1140 ng/ml (5) CZP, (C) spiked plasma extract containing 400 ng/ml of each of compounds (1–4), with 1140 ng/ml (5) CZP.

Table 1
Retention times of CZP, OZP, TZP, DMDZP and DZP, assayed by the aforedescribed HPLC system

| Drug | Retention time (min) | |
|---|---|---|
| | Plasma | Urine |
| Clonazepam | 6.26 | 5.47 |
| Oxazepam | 8.30 | 7.16 |
| Temazepam | 9.70 | 8.34 |
| *N*-Desmethyl diazepam | 10.90 | 9.47 |
| Diazepam | 12.80 | 11.55 |

## 3. Results

### 3.1. Retention times

Representative chromatograms of DZP, and its major metabolites in spiked plasma and urine, together with their blanks are shown in Figs. 3 and 4, respectively. The corresponding retention times are shown in Table 1.

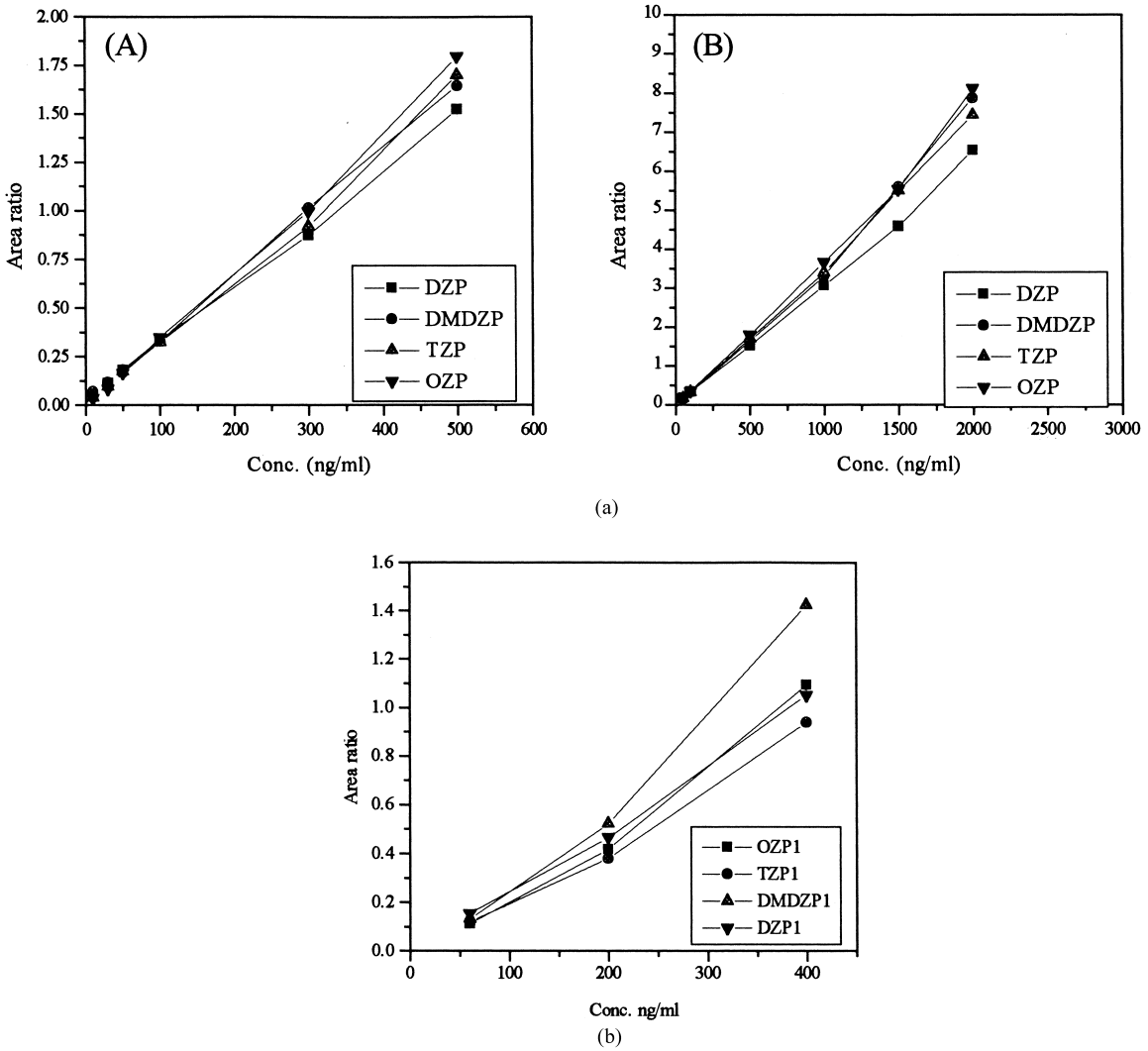


Fig. 5. (a) Calibration curves (A) 0–500 ng/ml and (B) 0–2000 ng/ml for DZP, DMDZP, TZP and OZP in plasma. Correlation coefficients of 0.9993, 0.9997, 0.9982 and 0.9990, for the four drugs, in (A) and 0.9988, 0.9968, 0.9992 and 0.9977, in (B) were obtained, respectively. (b) Calibration curves (0–400 ng/ml) for DZP, DMDZP, TZP and OZP in urine. Correlation coefficients of 0.9973, 0.9923, 0.9944 and 0.9935 for the four drugs, respectively.

Table 2
Recovery of DZP and its major metabolites from spiked plasma and urine

| Drug | Added concentration (ng/ml) | Mean recovery (%) | C.V. (%) | No. of replicates |
|---|---|---|---|---|
| *Plasma* | | | | |
| DZP | 106 | 90.6 | 3.9 | 4 |
| | 530 | 80.3 | 5.9 | 4 |
| | 1060 | 90.6 | 8.4 | 4 |
| DMDZP | 104 | 87.7 | 6.2 | 4 |
| | 520 | 92.6 | 6.6 | 4 |
| | 1040 | 98.3 | 5.0 | 4 |
| TZP | 102 | 92.5 | 5.8 | 5 |
| | 510 | 94.8 | 6.0 | 5 |
| | 1020 | 87.0 | 7.1 | 4 |
| OZP | 108 | 94.7 | 4.9 | 5 |
| | 540 | 89.5 | 8.3 | 5 |
| | 1080 | 91.0 | 3.8 | 4 |
| *Urine* | | | | |
| DZP | 228 | 89.6 | 2.5 | 2 |
| DMDZP | 260 | 90.0 | 4.7 | 2 |
| TZP | 200 | 87.5 | 0.7 | 2 |
| OZP | 212 | 94.5 | 0.8 | 2 |

Table 3
Intra-day, inter-day precision and accuracy of DZP and its major metabolites from spiked plasma

| Drug | Added concentration (ng/ml) | Mean concentration (ng/ml) | Intra-day C.V. (%) | No. of replicates |
|---|---|---|---|---|
| *Intra-day* | | | | |
| DZP | 106 | 105.2 | 2.4 | 4 |
| | 530 | 434.4 | 7.1 | 4 |
| | 1060 | 945.2 | 5.0 | 5 |
| DMDZP | 104 | 99.2 | 3.5 | 4 |
| | 520 | 106.5 | 3.9 | 3 |
| | 1040 | 1013.2 | 3.8 | 5 |
| TZP | 102 | 94.4 | 5.8 | 5 |
| | 510 | 511.4 | 6.0 | 5 |
| | 1020 | 1021.0 | 3.7 | 5 |
| OZP | 108 | 111.9 | 8.9 | 3 |
| | 540 | 526.7 | 5.4 | 4 |
| | 1080 | 1084.2 | 3.4 | 5 |
| *Inter-day* | | | | |
| DZP | 106 | 101.5 | 7.5 | 12 |
| | 530 | 425.3 | 5.1 | 12 |
| | 1060 | 909.1 | 9.3 | 13 |
| DMDZP | 104 | 102.1 | 12.6 | 11 |
| | 520 | 494.5 | 6.5 | 11 |
| | 1040 | 1044.7 | 5.0 | 14 |
| TZP | 102 | 98.4 | 11.5 | 11 |
| | 510 | 494.0 | 5.5 | 14 |
| | 1020 | 998.7 | 8.5 | 14 |
| OZP | 108 | 100.7 | 10.1 | 12 |
| | 540 | 519.4 | 8.3 | 14 |
| | 1080 | 1071.4 | 6.4 | 14 |

### 3.2. Calibration and limits of quantitation

Two calibration curves were constructed in plasma in the concentration range 0–500 ng/ml and 0–2000 ng/ml, respectively as shown in Fig. 5a, with 10 ng/ml as the limit of detection. Another calibration curve was constructed in urine between 0 and 400 ng/ml, Fig. 5b.

### 3.3. Recovery

The % recovery of DZP, and its major metabolites from plasma, ranged from 80.3 to 98.3%, and from 87.5 to 94.5% from urine, respectively (Table 2).

### 3.4. Accuracy and precision

The accuracy and precision of this method were assessed from spiked plasma using 21 samples at three different concentrations, extracted and analysed during three consecutive days, with 2.4–7.1%, 3.5–3.9%, 3.7–6.0% and 3.4–8.9% as intra-day coefficient of variation (C.V.) for DZP, DMDZP, TZP and OZP, respectively, at 100, 500 and 1000 ng/ml. Inter-day C.V.s ranged from 5.1 to 7.5%, 5.0 to 12.6%, 5.5 to 11.5% and 6.4 to 8.3% for DZP, DMDZP, TZP and OZP, respectively, at 100, 500, and 1000 ng/ml, as shown in Table 3.

## 4. Discussion and conclusion

The previously reported methods for the analysis of DZP and its major metabolites in biological fluids were unsatisfactory because: most of them are time consuming, involving multiple steps of extraction and pre-assay preparations, low recovery and lack of selectivity and sensitivity.

The present method requires neither multiple extraction steps, nor the use of large volumes of organic solvents. Furthermore it is precise, accurate, selective and sensitive. Hence it can be concluded that this method is satisfactory for routine analysis of DZP and its metabolites in biological fluids.

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