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Short communication

A modified simple and rapid reversed phase liquid chromatographic method for quantification of diazepam and nordiazepam in plasma

J. Atta-Politou [a],*, M. Parissi-Poulou [b], A. Dona [a], A. Koutselinis [a]

[a] *Department of Forensic Medicine and Toxicology, School of Medicine, University of Athens, M. Asias-Goudi, Athens 11527, Greece*
[b] *Department of Pharmacy, Division of Pharmaceutical Chemistry, Zografou-Kouponia, Athens 15771, Greece*

1. Introduction

Diazepam is still the most commonly used benzodiazepine, despite the large number of derivatives available [1–5]. Diazepam undergoes extensive biotranformation in the liver, mainly through demethylation and hydroxylation [2]. The major degradation products are *N*-desmethyldiazepam (nordiazepam) and 3-hydroxydiazepam (temazepam) both of which are degraded to oxazepam [3]. The parent compound and nordiazepam are the major measurable plasma components following single and chronic oral administration of diazepam in man [3]. Nordiazepam can be detected as early as 15 min following oral diazepam administration and tends to accumulate in the course of chronic treatment [3]. For an effective dosage scheduling, it is im-

portant to monitor the concentration of diazepam and nordiazepam in plasma [4,5].

Previously reported analytical methods for the quantification of diazepam and nordiazepam in plasma have been based on gas chromatography [6–10], as well as normal and reversed-phase high performance liquid chromatography [8–20]. Benzodiazepine immunoassays have also been reported [21,22].

This report describes a simple, specific and accurate reversed-phase liquid chromatographic method, which allows quantification of diazepam and nordiazepam in plasma.

The method described in this report is an extrapolation of a previously reported method developed by our group [23] concerning quantification of alprazolam and a-hydroxy-alprazolam in plasma, which was modified in several aspects (internal standard, mobile phase composition, calibration curve range) for the pur-

* Corresponding author: Tel./fax: + 30-1-770-6868.

poses of this study. The method includes a simple mobile phase, in which no pH adjustment is needed, as well as a one step rapid extraction procedure. It offers convenience and rapidity in analysis, in comparison with previously reported liquid chromatographic methods, for quantification of diazepam and nordiazepam in plasma. The method was successfully applied in an in vivo study.

2. Materials and methods

2.1. Apparatus

Chromatography was performed with an HPLC system consisting of a Jasco (Japan Spectroscopic Co. Ltd.) Model 880 PU pump, fitted with a model 880-2 Ternary Gradient Unit, which was used under isocratic conditions on manual mode. The system fitted with a Model 7125 manual injector (Cotati, Rheodyne, CA, USA) and a 50 μl sample loop. A Jasco Model 875 UV variable wavelength UV/Vis detector was operated at 230 nm. Samples were chromatographed on a 25 cm × 4 mm (i.d) Lichrospher RP-18 (MZ Analysentechnic D-6500 Mainz) reversed-phase column containing 5 μm octadecyl (C_{18}) silane as the sorbent. A Hewlett Packard HP 3394A integrator was used to record chromatograms, at peak height mode (chart speed 0.5 cm min^{-1}).

A Millipore filtration system (Millipore, Bedford, MA, USA) with type HV Millipore filters (pore size 0.45 μm) was used, for degassing the mobile phase under vacuum. A Fisher isotherm dry bath Model 145 was used for the evaporation of extraction solvents.

2.2. Reagents and chemicals.

Analytical grade sodium borate was obtained from Serva. Acetonitrile and water were HPLC grade (Lichrosolv®) and were obtained from Merck. Dichloromethane and n-pentane were analytical grade and were obtained from Ferak, Berlin. Alprazolam was a gift from Upjohn (Kalamazoo MI, USA). Diazepam and nordiazepam were gifts from Roche Pharmaceuticals (Nutley, NJ, USA).

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile:water 45:55 v/v. A flow rate of 1.0 ml min^{-1} was used at ambient temperature, resulting in a pressure of about 158 kg cm^{-2}. Mobile phases were degassed by vacuum through filtration, after mixing. The column effluent was monitored at 230 nm with the detector set at 0.016 absorbance units full-scale (Att. 4).

2.4. Standards for calibration graphs

Stock standard solutions of diazepam, nordiazepam and alprazolam (internal standard) were prepared in methanol to give final concentrations of 1 mg ml^{-1}. Standards stored at 4°C have been stable for 8 months.

Standard solutions of final concentration of 10 μg ml^{-1} were prepared by diluting the stock standards 100-fold with distilled water. An aqueous reference solution containing both diazepam and nordiazepam to final concentration 10 μg ml^{-1} was prepared from stock standard solutions of each compound. Working solutions were prepared containing 0.2, 0.4, 0.8, 1.0, 1.2, 1.6 and 2 μg ml^{-1} of diazepam and nordiazepam by appropriate dilutions of the reference solution with water. Plasma standards for calibration curves were prepared by spiking 1.0 ml aliquots of pooled drug free plasma with 100 μl of the above mentioned working solutions, to make diazepam and nordiazepam plasma standards ranging from 20 to 200 ng ml^{-1}. A working solution of internal standard (0.8 μg ml^{-1}) was prepared from the 10 μg ml^{-1} aqueous solution.

Calibration graphs of the recovered standards were prepared for each day of analysis to establish linearity and reproducibility of the HPLC system. Graphs were constructed of the peak height ratio of each compound to internal standard against drug concentration.

2.5. Extraction procedure

In 10 ml conical glass tube with a glass stopper, 1.0 ml of plasma, 100 μl of internal standard aqueous solution 0.8 μg ml^{-1} (80 ng), and 0.5 ml

of saturated solution of sodium borate buffer (pH 9.3) were added and mixed briefly. Each sample was extracted with 5.0 ml of dichloromethane:*n*-pentane (4:6 v/v) on vortex for 60 s at speed 4 (Vortex-Genie, Model K-550 GE, Scientific Ind. Springfield, MA). The sample was centrifuged for 5 min at 2000 rpm. The upper (organic) layer was then transferred into a 10 ml conical glass tube and evaporated to dryness in a 50°C dry bath under a gentle stream of nitrogen. The residue was reconstituted in 100 µl of mobile phase and an aliquot of about 70 µl was injected onto the HPLC system.

2.6. Analytical variables

Extraction recovery was calculated at 10, 80, 120, 200 ng ml^{-1} spiked plasma samples by comparing the peak heights from extracted samples with those obtained from a direct injection of the corresponding unextracted standards dissolved in mobile phase.

Within-run and between-run precision was determined by extracting plasma supplemented with diazepam and nordiazepam to 10 and 80 ng ml^{-1}.

2.7. In vivo study

Twelve healthy female subjects aged 26–50 years old, who were not receiving any other medication, were included in the study. Two single oral doses of diazepam ranging from 5 to 10 mg each were administered to them: the first late at night and the second 8 h later, early in the morning. From all subjects blood samples were collected prior to first dose, to serve as blank samples, and 2 h after the second dose, in unstoppered tubes containing sodium heparin as anticoagulant. Samples were centrifuged immediately after collection and plasma stored at −20°C until analysis. Subjects included in the study were divided in three groups according to the dosage regimens administered. First group (nine subjects): two single doses of 5 mg each. Second group (one subject): two single doses of 7.5 mg each. Third group (two subjects): two single doses of 10 mg each.

3. Results

Retention times of alprazolam (internal standard), nordiazepam and diazepam were 5.8, 7.7 and 12.3 min, respectively. Fig. 1(a) shows a chromatogram obtained from a direct injection of an aqueous test solution containing 0.8 µg ml^{-1} of each of the above mentioned compounds (injected volume 50 µl corresponding to 40 ng of each compound). Fig. 1(b) shows a chromatogram obtained from an extracted drug free plasma while Fig. 2(a) of an extracted drug free plasma supplemented with diazepam, nordiazepam and internal standard to 80 ng ml^{-1} each. Fig. 2(b) shows a chromatogram obtained from extracted plasma of a patient receiving two single doses of diazepam. No interfering peaks were observed in several samples of drug free plasma.

3.1. Recovery

Extraction recovery data, as well as reproducibility of recovery (CV%), from plasma samples supplemented with diazepam and nordiazepam to 10, 80, 120, 200 ng ml^{-1} are referred in Table 1 (means of five experiments).

3.2. Linearity

The peak height ratios for diazepam/internal standard and nordiazepam/internal standard were linearly related to plasma concentrations of diazepam and nordiazepam, respectively, from 20 to at least 200 ng ml^{-1}.

The slopes of eight calibration curves of diazepam in plasma, prepared over a period of 2 months, had a CV of 5.6%. The average regression equation was: $y_1 = 0.00802(\pm 0.00045)x_1 + 0.072(\pm 0.029)$, where y_1 = peak height ratio of diazepam/internal standard and x_1 = plasma concentration of diazepam (ng ml^{-1}). The correlation coefficients for each standard curve constructed invariably exceeded 0.998 and intercepts were all close to zero and not statistically significant.

The slopes of eight calibration curves of nor-

diazepam in plasma, prepared over a period of 2 months, had a CV of 6.5%. The average regression equation was: $y_2 = 0.01460(\pm 0.00095)x_2 + 0.020(\pm 0.042)$, where y_2 = peak height ratio of nordiazepam/internal standard and x_2 = plasma concentration of nordiazepam (ng ml^{-1}). The correlation coefficients for each standard curve constructed invariably exceeded 0.995 and intercepts were all close to zero and not statistically significant.

The lower limit of detection was 1 ng ml^{-1} for diazepam and nordiazepam, while the lower limit of quantification was 2 ng ml^{-1} for both compounds.
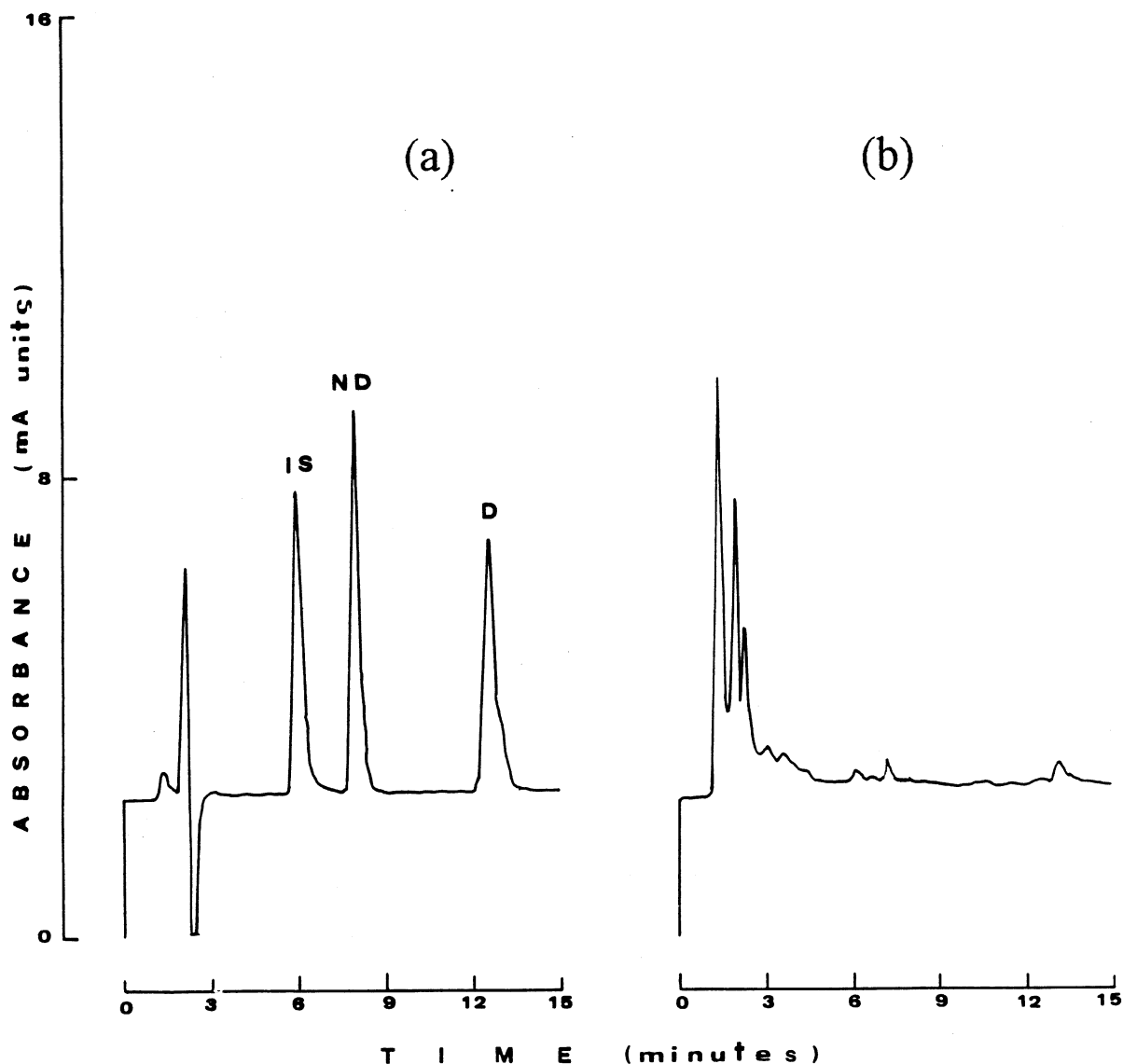

Fig. 1. Chromatograms obtained from: (a) a direct injection of an aqueous test solution containing 0.8 μg ml^{-1} of diazepam, nordiazepam and alprazolam (IS) (injected volume 50 μl, corresponding to 40 ng of each compound); (b) an extracted drug free plasma. Diazepam (D), nordiazepam (ND), alprazolam (IS), internal standard.
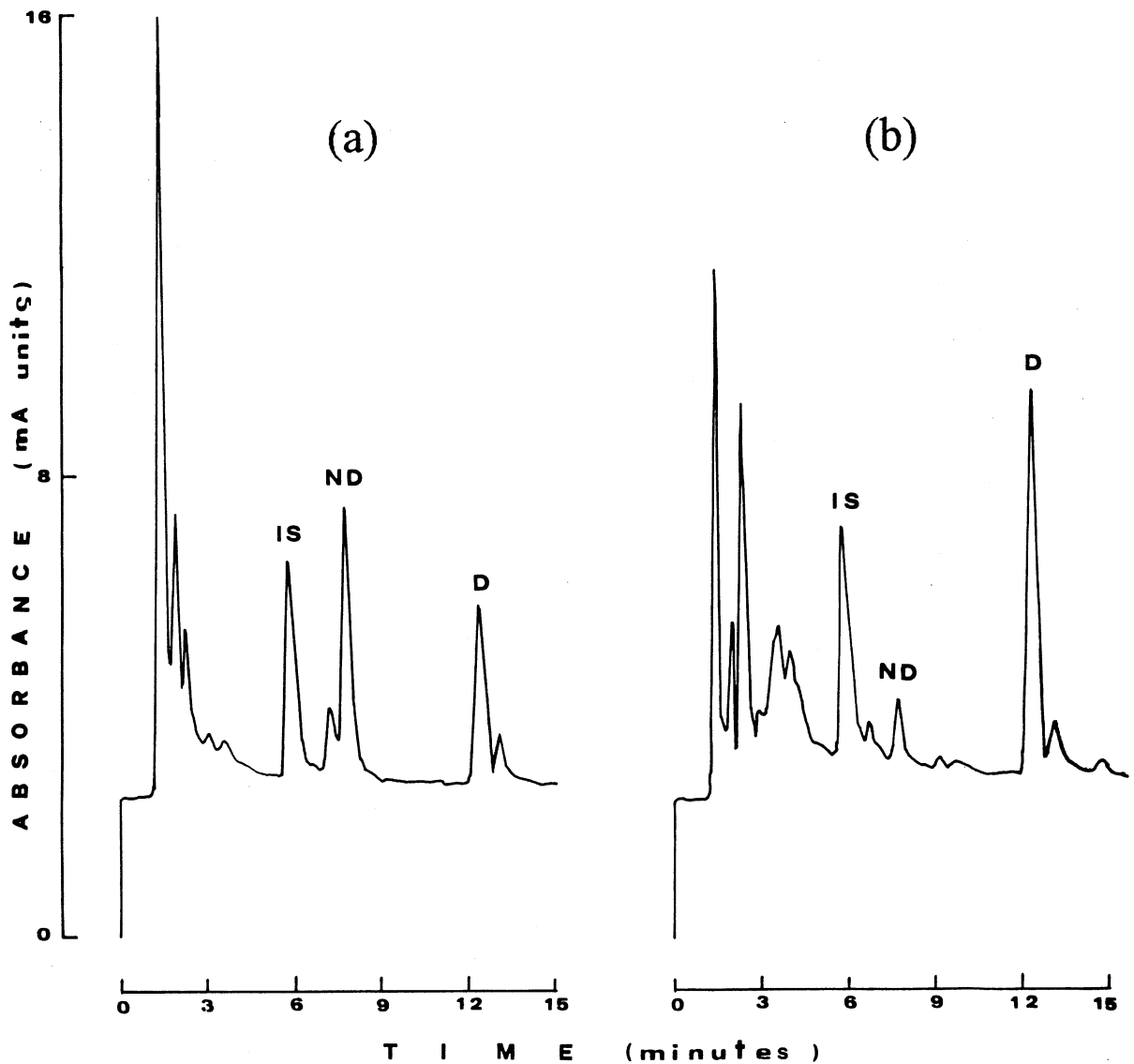
Fig. 2. Chromatograms obtained from: (a) an extracted drug free plasma supplemented with diazepam, nordiazepam and alprazolam (IS) to 80 ng ml^{-1} each; (b) an extracted plasma sample of a patient receiving two single doses of diazepam (5 mg each), collected 2 h post second dose. Plasma diazepam concentration = 173.1 ng ml^{-1}; plasma nordiazepam concentration = 17.1 ng ml^{-1}. Diazepam (D), nordiazepam (ND), alprazolam (IS), internal standard.

3.3. Precision and accuracy

Within-run CV was 8.97% and 9.62% for diazepam and nordiazepam, respectively, at 10 ng ml^{-1} spiked plasma ($n = 10$) with a mean concentration $\pm$ SD (ng ml^{-1}) of 10.92 $\pm$ 0.98 (relative error, $E_r = 0.092$ and relative range, $R_r = 0.27$) and 11.12 $\pm$ 1.07 ($E_r = 0.112$, $R_r = 0.29$), correspondingly. Between-run CV was 9.5% and 10.2% for diazepam and nordiazepam, respectively, at 10 ng ml^{-1} spiked plasma ($n = 10$) over a period of 2 months.

Within-run CV was 3.42% and 5.15% for diazepam and nordiazepam, respectively, at 80 ng

Table 1
Extraction recoveries and coefficient of variation (CV%) of alprazolam, nordiazepam and diazepam from spiked plasma samples

Spiked plasma standards (ng ml^{-1})	Alprazolam[a]		Nordiazepam		Diazepam	
	Rec.% $\pm$ SD	CV%	Rec.% $\pm$ SD	CV%	Rec.% $\pm$ SD	CV%
10 ($n = 5$)	90.5 $\pm$ 3.1	3.4	85.3 $\pm$ 3.4	4.0	84.8 $\pm$ 3.8	4.5
80 ($n = 5$)	91.2 $\pm$ 2.6	2.8	88.1 $\pm$ 2.5	2.8	83.4 $\pm$ 3.0	3.6
120 ($n = 5$)	92.0 $\pm$ 2.7	2.9	87.3 $\pm$ 3.0	3.4	82.8 $\pm$ 2.0	2.4
200 ($n = 5$)	90.8 $\pm$ 3.2	3.5	86.8 $\pm$ 3.2	3.7	84.0 $\pm$ 2.5	3.0

[a] All spiked plasma standards contained alprazolam (internal standard) to 80 ng ml^{-1}.

ml^{-1} spiked plasma ($n = 10$) with a mean concentration $\pm$ SD (ng ml^{-1}) of 83.14 $\pm$ 2.84 ($E_r = 0.039$ and $R_r = 0.114$) and 84.20 $\pm$ 4.34 ($E_r = 0.052$, $R_r = 0.142$), correspondingly. Between-run CV was 4.6% and 6% for diazepam and nordiazepam, respectively, at 80 ng ml^{-1} spiked plasma ($n = 10$) over a period of 2 months.

3.4. Interferences

Twenty-one compounds were studied for possible interference, including several drugs that might be administered to anxious or depressed patients (Table 2). Triazolam, a triazolobenzodiazepine, cannot be separated from alprazolam by the described method but in clinical practice, it is not administered with diazepam.

3.5. In vivo study

Table 3 shows diazepam and nordiazepam plasma concentrations in 12 subjects included in the study.

4. Discussion

A great number of analytical methods, using high performance liquid chromatography, have been published for quantification of diazepam and nordiazepam in plasma [8–18], which have been extensively reviewed [17]. Reported procedures for sample preparation have been based on solvent extraction from alkaline media [8–12,18] or solid phase extraction [15–17,19,20]. Although most of these methods are sensitive and accurate, they are time consuming, especially during the extraction procedure. This makes them impractical when large-scale human studies are required which involve the analysis of a great number of specimens on a routine basis in order to demonstrate bioequivalence of formulations or in clinical pharmacokinetic studies.

In this study several solvent mixtures were tested for the extraction of diazepam, nordiazepam and alprazolam (internal standard) from plasma samples, such as methyl *tert*-butyl ether, dichloromethane:*n*-pentane (4:6 v/v), ethylacetate:*n*-pentane (85:15 v/v and 40:60 v/v) and ether. Dichloromethane:*n*-pentane (4:6 v/v) was found suitable for the extraction; it appeared satisfactory extraction recovery (Table 1) and was rapidly evaporated at 50°C since both solvents of the mixture have very low boiling points. In previously reported methods, ethylacetate/hexane [10],

Table 2
Compounds studied for interference

Amitriptyline[c]	Imipramine[c]
Amobarbital[c]	Lorazepam[a]
Bromazepam[a]	Nitrazepam[c]
Caffeine[c]	Nortriptyline[c]
Carbamazepine[c]	Oxazepam[a]
Chlorpheniramine[c]	Pentobarbital[c]
Chlorpromazine[a]	Perphenazine[a]
Chlorthiazide[a]	Thioridazine[a]
Diphenylhydantoin[c]	Triazolam[b]
Haloperidol[c]	
Hexobarbital[c]	
Hydrochlorthiazide[a]	

[a] Not detected.
[b] Interfered, see text.
[c] Detected but not interfered.

Table 3
Plasma diazepam and nordiazepam concentrations of 12 subjects included in the study

1st oral dose (mg)	2nd oral dose (mg)	Total dose/weight $\times 100$	Plasma diazepam (ng ml^{-1})	Plasma nordiazepam (ng ml^{-1})
5	5	14.9	46.0	5.8
5	5	15.8	63.0	3.4
5	5	16.6	108.0	8.9
5	5	17.2	24.1	2.8
5	5	18.5	75.2	8.2
5	5	19.2	161.5	8.9
5	5	19.6	173.1	17.1
5	5	20.0	131.1	21.9
5	5	21.7	142.9	6.2
7.5	7.5	22.7	121.4	59.6
10	10	25.9	161.5	39.7
10	10	26.3	76.4	4.1

toluene/heptane/isoamyl alcohol [9], toluene [8] or toluene/heptane [18] have been used for the extraction step, which require much longer time for the evaporation step.

Good chromatography (i.e. peak shape, retention time) was achieved with acetonitrile:water 45:55 v/v mobile phase. The presence of phosphate buffer [8,9] or sodium acetate buffer [11] in the mobile phase, used in other methods has been avoided. Thus, pH adjustment of the mobile phase as well as wash out of the column with water at the end of the day are not needed. Additionally, analysis was performed at ambient temperature while other methods [11] include higher column temperature which is hazardous for the column. In the method developed in this study late eluting peaks were not observed.

Diazepam and nordiazepam are highly plasma protein bound, approximately 98% and 97%, respectively [3,24,25]. The procedure described in this methodology does not include protein removal from plasma samples as ultramicrofiltration or equilibrium dialysis, which could enable the determination of the free fraction of the drug in plasma [17]. Taking into account the high absolute extraction recoveries from spiked plasma samples achieved by this methodology (Table 1), we can suggest that total drug levels are estimated by the method developed in this study.

For the purposes of the in vivo study presented in this report, calibration curves of diazepam and

nordiazepam from 20 to 200 ng ml^{-1} were constructed. These curves were linear up to 400 ng ml^{-1}. The method can also be suitably applied for therapeutic diazepam and nordiazepam plasma levels during chronic administration [4,5,26,27] or for measuring toxic levels of diazepam and nordiazepam in acute overdoses [28], by extrapolating calibration curves at higher concentrations. In these cases the amount of internal standard has to be appropriately chosen.

Plasma diazepam and nordiazepam concentrations in 12 healthy female subjects included in this study, who received two single doses of diazepam ranging from 5 to 10 mg, have been determined successfully by this rapid assay and ranged from 24.1 to 173.1 ng ml^{-1}, and from 2.8 to 59.6 ng ml^{-1}, respectively, at 2 h post second dose. These plasma levels are in accord with plasma concentrations reported in relevant studies [4,29].

The technique presented is an optimization of existing methods, with respect to our laboratory needs, where, on a daily basis, a limited amount of different drugs (therapeutic drug monitoring or toxicology) is determined and therefore a rapid one step extraction procedure is needed.

In conclusion, the analytical methodology developed in this report is simple, rapid, accurate, sensitive and specific. It can be used for monitoring plasma diazepam and nordiazepam concentrations in clinical and pharmacokinetic studies. The potential applications of this technique in the

qualitative and quantitative analysis of other benzodiazepines, especially in the field of clinical, emergency and forensic toxicology, are currently under investigation.

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