Journal of Chromatography, 564 (1991) 330–337
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5629

Short Communication

Determination of diazepam and nordazepam in milk and plasma in the presence of oxazepam and temazepam

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(First received May 9th, 1990; revised manuscript received September 3rd, 1990)

ABSTRACT

For studies on the excretion of drugs into milk a sensitive high-performance liquid chromatographic assay was developed to quantitate diazepam and nordazepam in the milk and plasma of humans and rabbits in the presence of their major metabolites, oxazepam and temazepam. Flurazepam was used as an internal standard. The assay involves extractions with diethyl ether and an additional acid clean-up step. Chromatographic separation was achieved by a LiChrospher 60 RP-select B (5 μm) column and KH_2PO_4–acetonitrile (69:31, v/v) adjusted to pH 2.80 as a mobile phase. The same extraction and chromatographic conditions were suited to both types of samples, milk and plasma. The limits of determination using ultraviolet detection at 241 nm was for diazepam 20 ng/ml and for nordazepam 15 ng/ml. The absolute recoveries of diazepam, nordazepam and flurazepam in human milk were 84, 86 and 92% and in human plasma 97, 89 and 94%, respectively. The within- and between-day accuracy and precision for diazepam and nordazepam in milk and plasma at all concentrations tested (20–1500 ng/ml) were better than 8%. The high fat content which occurs in rabbit milk presented no limitation for the extraction of lipophilic diazepam: the method was successfully used to monitor milk and plasma concentrations of diazepam and nordazepam in lactating New Zealand White rabbits during 26-h infusions of diazepam (1.4 mg/h).

INTRODUCTION

For milk excretion studies a high-performance liquid chromatographic (HPLC) method was needed which allowed the quantitation of diazepam and nordazepam (N-desmethyldiazepam) in the presence of other occurring metabolites (oxazepam, temazepam) in both, human milk and plasma samples.

Although several methods are described in the literature [1–4] for the quantitation of benzodiazepines in various matrices, most of them use gas chromatography coupled with an electron-capture detector [5]. As noted previously many of these methods do not allow the simultaneous separation of the three main metabolites of diazepam (nordazepam, oxazepam and temazepam) [6]. Furthermore limitations occur either because of the adaptation of extraction procedures [7], the large sample volumes, the long retention times [8] or insufficient sensitivity [6].

0378-4347/91/$03.50

Using the experience gained while evaluating existing procedures a suitable HPLC method was developed with sensitivities down to 20 ng/ml for diazepam and 15 ng/ml for nordazepam. It assured good reproducibility and quantitative precision for these two compounds in the presence of other diazepam metabolites (oxazepam and temazepam) without the need to adjust the extraction or chromatographic procedure when switching between the two matrices, milk and plasma.

EXPERIMENTAL

Chemicals and reagents

Benzodiazepines were obtained from F. Hoffmann-La Roche (Basel, Switzerland). The reagents were purchased from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany) and were of analytical grade. All solvents could be used without distillation.

Extraction procedures

Milk and plasma. To a 500-μl milk or plasma sample 125 ng flurazepam (internal standard) in 25 μl water–acetonitrile (97.5:2.5, v/v) and 500 μl of 1/15 *M* phosphate buffer pH 7.4 are added. This mixture is extracted with 7 ml of diethyl ether for 15 min. The ether phase is acidified with 1 ml of 1.5 *M* hydrochloric acid and shaken for 10 min. The ether phase is decanted after freezing the water phase. The water phase is then made alkaline with 1 ml of 2 *M* sodium hydroxide and extracted with 7 ml of diethyl ether for 15 min. Finally, the ether phase is collected and evaporated at 37°C under a stream of nitrogen. The residue is dissolved in the mobile phase (see below).

Plasma. Because plasma contains less fat than milk, a simplified extraction method can be used. Benzodiazepines in the plasma–buffer mixture, prepared as above, can be extracted directly into 7 ml of diethyl ether by shaking the mixture for 15 min. The ether phase is again evaporated at 37°C under a stream of nitrogen and the residue is dissolved in the mobile phase.

Instrumentation and chromatographic conditions

The column used was a LiChrospher 60 RP-select B (5 μm) 125-4 (Merck No. 50829)) with a precolumn LiChrospher 60 RP-select B (5 μm) 25-4 (Merck No. 50937). The column was kept at 45°C with a Jones column heater (Jones Chromatography, Llanbradach, U.K.).

The mobile phase was 10 m*M* KH_2PO_4–acetonitrile (69:31, v/v) adjusted to pH 2.80 with H_3PO_4. We used a Kontron HPLC pump 420 (Kontron, Zürich, Switzerland) set to a flow-rate of 2.00 ml/min. The loop size was 50 μl. Samples were injected with a Kontron autosampler MSI T-660 (Kontron).

A Merck–Hitachi L-4200 UV detector (Hitachi, Tokyo, Japan) was used to monitor the column effluent. The absorption was measured at 241 nm. Peak heights were measured with a Spectra-Physics SP4200 integrator (Spectra-Physics, San Jose, CA, U.S.A.).

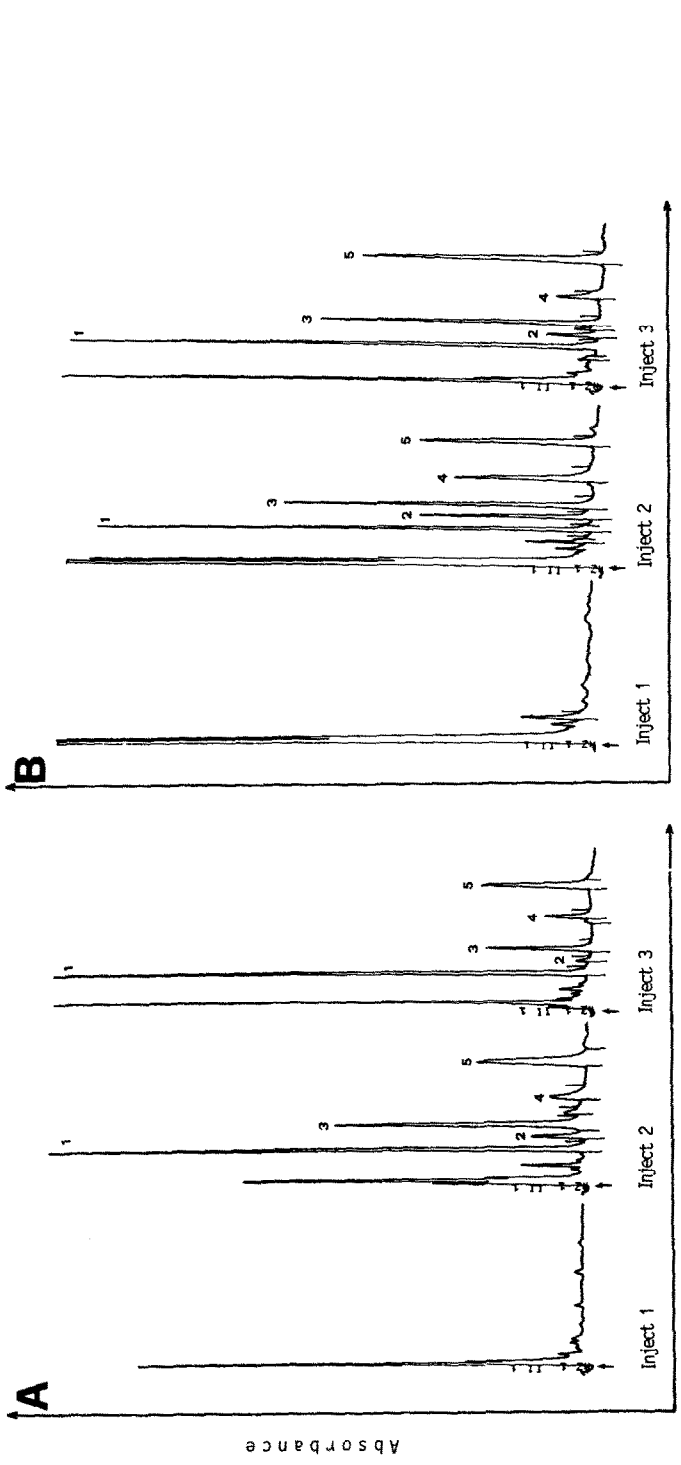

Fig. 1. (A) Chromatograms of extracts of (1) human blank milk, (2) standard mixture of benzodiazepines in human milk (each component about 100 ng/ml) and (3) milk of a NZW rabbit 24 h after a diazepam dose. Dosing scheme: injection of 2 mg/kg diazepam, followed by a continuous infusion of 1.4 mg/h over 26 h. (B) Chromatograms of extracts of (1) human blank plasma, (2) standard mixture of benzodiazepines in human plasma (each component about 100 ng/ml) and (3) plasma of a NZW rabbit 24 h after a diazepam dose (same dosing scheme as in A). Peaks: 1 = flurazepam (retention time = 3.0 min); 2 = oxazepam (3.8 min); 3 = nordazepam (4.5 min); 4 = temazepam (6.3 min); 5 = diazepam (8.6 min).

Determination of extraction ratio and accuracy

The extraction ratios of diazepam, its metabolites (nordazepam, oxazepam and temazepam) and flurazepam (internal standard) were estimated by comparing the peak heights of the extracted spiked milk and plasma samples (50, 100 and 1000 ng/ml, respectively) with those of directly injected standard solutions dissolved in the mobile phase. All determinations were carried out in triplicate.

The within-day accuracy and precision of determination was assessed for both compounds, diazepam and nordazepam, by analyzing in triplicate samples spiked at four different concentrations (around 20, 50, 500 and 1500 ng/ml). This procedure was repeated on three different days to obtain, in addition, a measure of between-day assay performance.

Calibration curve

The concentrations of diazepam and nordazepam in unknown samples were calculated by relating the peak-height ratios of drug to internal standard to the calibration curves prepared on the same day. Calibration curves were obtained by analyzing seven spiked plasma or milk samples containing concentrations of both drugs between 20 and 1600 ng/ml. The linear regression analysis of the calibration curves was weighed $1/y^2$.

RESULTS AND DISCUSSION

In the analytical procedures which have been described, the absolute recoveries of diazepam, nordazepam, flurazepam, oxazepam and temazepam from human milk were 84, 86, 33, 29 and 29% and from human plasma (single-step procedure) 97, 89, 94, 85 and 85%, respectively. Oxazepam and temazepam have very low pK_a values and the lowest octanol/water partition coefficient when compared to the other compounds. This may explain the lower extraction ratios of these two drugs in milk.

The retention times for flurazepam (internal standard), oxazepam, nordazepam, temazepam and diazepam were 3.0, 3.8, 4.5, 6.3 and 8.6 min, respectively. Typical chromatograms of these benzodiazepines which have been extracted from plasma and milk are shown in Fig. 1. The form of all the peaks in the chromatograms was symmetric and showed only a minimal tailing. Each peak was separated down to the baseline.

The calibration curves obtained were linear within the concentration range tested for both, the diazepam and nordazepam compound. The limit of determination for diazepam was 20 ng/ml and for nordazepam 15 ng/ml [coefficient of variation (C.V.) $< 11\%$].

The within-day accuracy and precision for both compounds, in plasma and milk at all tested concentrations, was better than 8%. The between-day precision for quantitating both compounds in plasma and milk was excellent with a C.V. in replicate analyses of less than 7% (Tables I and II).

TABLE I

WITHIN- AND BETWEEN-DAY ASSAY PERFORMANCE OF NORDAZEPAM (N) AND DIAZEPAM (D) IN MILK

Precision is expressed as the coefficient of variation of repeated analysis. Accuracy is expressed as the percentage difference of measured and actual values.

Concentration added (ng/ml)	Concentration found (ng/ml)		Precision (%)		Accuracy (%)	
	N	D	N	D	N	D
Within-day						
Day 1						
20	19.8	18.7	1.9	2.3	−1.2	−6.4
50	51.0	47.4	1.6	4.6	2.0	−5.2
450	472.3	442.8	3.3	3.6	5.0	−1.6
1500	1529.6	1408.5	4.1	3.2	2.0	−6.1
Day 2						
20	20.4	20.2	3.9	4.3	2.2	0.8
50	50.5	48.9	2.6	5.5	0.9	−2.3
450	457.5	427.7	2.9	3.5	1.7	−5.0
1500	1515.4	1387.9	2.4	3.1	1.0	−7.5
Day 3						
20	20.2	19.1	1.0	5.3	1.0	−4.4
50	51.5	49.5	1.3	0.8	2.9	−1.0
450	457.7	431.2	2.0	2.8	1.7	−4.2
1500	1524.9	1411.6	0.8	0.7	1.7	−5.9
Between-day						
20	20.1	19.4	2.7	5.0	0.7	−3.4
50	51.0	48.6	1.9	4.0	2.0	−2.8
450	463.1	434.7	2.9	3.2	2.9	−3.4
1500	1524.9	1411.6	0.8	0.7	1.7	−5.9

The single-step extraction procedure can be used if only plasma samples have to be quantitated and sufficiently clean extracts are usually obtained. This extraction procedure requires less time than the double extraction procedure required for milk. If plasma samples show unsatisfactory baselines with the single-step extraction procedure, they can be worked-up like milk with the additional acid extraction step which results in a less noisy baseline during chromatography. To gain time and avoid the evaporation step micro back-extraction into acid could also be considered for acid stable compounds.

When extracting diazepam and nordazepam from milk it is important to keep the pH close to 7.4 during the first extraction step. If the pH rises to higher levels (pH > 9) the extraction ratio was found to decrease drastically. Casein, a major milk protein, is very soluble in an alkaline aqueous medium and a change in

TABLE II

WITHIN- AND BETWEEN-DAY ASSAY PERFORMANCE OF NORDAZEPAM (N) AND DIAZEPAM (D) IN PLASMA

Precision is expressed as the coefficient of variation of repeated analysis. Accuracy is expressed as the percentage difference of measured and actual values.

Concentration added (ng/ml)	Concentration found (ng/ml)		Precision (%)		Accuracy (%)	
	N	D	N	D	N	D
Within-day						
Day 1						
20	20.1	19.9	0.8	1.4	0.4	−0.1
40	41.1	39.0	1.6	2.5	2.8	−2.6
400	420.18	407.0	2.9	6.0	5.1	1.8
1600	1573.9	1519.9	1.0	2.7	−1.6	−5.0
Day 2						
20	21.1	18.9	0.9	6.1	5.5	−5.4
40	41.3	39.0	0.9	6.3	3.1	−2.6
400	402.0	383.7	3.1	6.3	0.5	−4.1
1600	1553.3	1494.6	2.6	6.5	−2.9	−6.6
Day 3						
20	19.4	20.1	7.6	2.3	−3.1	0.3
40	41.0	40.7	3.0	0.7	2.5	1.6
400	425.2	422.6	1.2	1.7	6.3	5.7
1600	1575.4	1552.5	0.4	0.5	−1.5	−2.97
Between-day						
20	19.9	19.6	6.2	3.7	−0.4	−2.0
40	40.9	40.1	2.9	4.5	2.2	0.3
400	420.2	408.9	3.1	5.2	5.1	2.2
1600	1583.6	1531.7	2.0	2.9	−1.0	−4.3

extraction behavior is observed at elevated pH values. In the single-step plasma extraction procedure the pH is not a critical factor (pH 7–11). The extraction procedures described are similar to the methods reported by Klotz *et al.* [9]. To avoid the distillation of diethyl ether as recommended by these authors we tested diethyl ether with different stabilizers. 2,6-Di-*tert.*-butyl-*p*-cresol, as used in ether from Fluka (No. 31690), gave rise to a peak in the chromatogram with a similar retention time to that of nordazepam. In contrast sodium thiocarbaminate, as used in diethyl ether for fluorescence spectroscopy (Merck, No. 10162), did not interfere with the chromatogram and ether from this source was therefore preferred in our procedure. In our blood collection procedure heparin (Liquemin®, F. Hoffman-La Roche) was an indispensable anticoagulant. This compound eluted together with temazepam but this was of no importance in our studies.

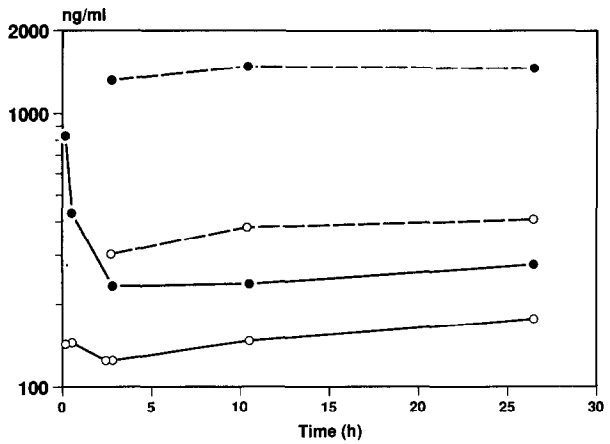

Fig. 2. Milk and plasma concentrations of diazepam (●) and nordazepam (○) in a lactating NZW rabbit over a 26-h observation period. The solid lines connect measured plasma and the dashed lines measured milk concentrations. Dosing scheme: injection of 2 mg/kg diazepam, followed by a continuous infusion of 1.4 mg/h over 26 h.

When developing the methods described above we used human plasma and milk. Additionally, we tested both extraction procedures on plasma and milk samples from the New Zealand White (NZW) rabbit and used the assay extensively for samples from this species. There were no problems in adapting our method for these biological fluids, although rabbit milk contains much more fat than human milk. In Fig. 2 plasma and milk concentrations are shown in a lactating NZW rabbit after an intravenous infusion of diazepam during 26 h (infusing rate 1.4 mg/h) which was preceded by a bolus dose of 2 mg per kg body weight. This example illustrates the versatility of the developed method.

Our specific application of the assay did not require us to quantitate the two diazepam metabolites, oxazepam and temazepam. The evaluation of linearity, accuracy and precision was therefore limited to diazepam and nordazepam. However, since the forms of all the peaks in the chromatograms were symmetric and without any significant tailing, the performance of the method can easily be characterized also for additional metabolites when their determination is required.

In conclusion, our results show that the method which has been presented to determine the concentrations of diazepam and nordazepam in the presence of oxazepam and temazepam in plasma and milk samples was reproducible and accurate. There was no need to adjust the extraction or the chromatographic procedure when switching between the two matrices, milk and plasma, unless use was made of a simplified extraction procedure with plasma.

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