

## Quantitative Determination of Diazepam in Serum Designed for Therapeutic Drug Monitoring

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A reversed-phase high-performance liquid chromatography (HPLC) procedure to quantify diazepam (D) in human serum in presence of its metabolites and fentanyl (F) was developed. After extraction diazepam has been analyzed on a liquid chromatograph equipped with a column RP-select B and an UV-VIS detector. Absorbances of D were measured at 230 nm. The diazepam standard curve was linear in the range 0.1–10.0  $\mu\text{g ml}^{-1}$ . 0.2 ml serum only was needed for the determination of D. Recovery of the drug added to serum was  $71 \pm 2\%$ . The method was applied for determination of D in patients of an Intensive Care Clinic. The concentrations found in the patients serum were in the range 0.5–4.5  $\mu\text{g ml}^{-1}$ . D metabolites: nordazepam (N), oxazepam (O) and signopam (S) have been identified by HPLC but their levels in blood have not been determined.

Metodę wysokosprawnej chromatografii cieczowej (HPLC) z odwróconym układem faz zastosowano do ilościowego oznaczania diazepam (D) w surowicy ludzkiej w obecności jego kilku metabolitów i fentanylu (F). Po ekstrakcji diazepam analizowano na chromatografii cieczowym wyposażonym w kolumnę RP-select B i detektor UV-VIS. Absorbancję diazepam mierzone przy 230 nm. Krzywa wzorcowa jest liniowa w przedziale stężeń 0.1–10.0  $\mu\text{g ml}^{-1}$ . Do oznaczenia D pobierano tylko 0.2 ml surowicy. Odzysk leku dodanego do surowicy wynosił  $71 \pm 2\%$ . Metodę stosowano do oznaczania poziomów D u pacjentów Kliniki Intensywnej Opieki Medycznej. Poziomy leku znajdowane u pacjentów mieściły się w przedziale 0.5–4.5  $\mu\text{g ml}^{-1}$ . Metabolity D: nordazepam (N), oxazepam (O), signopam (S) zidentyfikowano, lecz ich poziomów nie oznaczono.

Benzodiazepine derivative – diazepam (D) has been used as a sedative, anxiolytic and anticonvulsant agent for over three decades. Diazepam is completely metabolized

into compounds which are also pharmacologically active and applied in therapy [1–3]. Sedation using D is still common in Intensive Care Clinics [4].

Diazepam and its metabolites are usually assayed in biological fluids by chromatography. Gas–liquid chromatography (GLC) is a versatile technique in the analytical investigation of these drugs [1, 5, 6]. HPLC has been at the beginning an alternative to GLC but now HPLC is a major method of analysis of benzodiazepines. HPLC also offers the advantages of easy sample handling and volatile derivatives are not required for analysis. UV spectrophotometry is the most widely used detection method for these compounds [1–3, 7, 8].

This paper describes an application of reversed-phase HPLC for determination of diazepam in serum in presence of its three active metabolites and fentanyl (F).

## EXPERIMENTAL

### Patients

Determination of D was carried out in five patients from the Intensive Care Clinic of Karol Marcinkowski University of Medical Sciences in Poznań (age 34 to 68 years and body weight 65 to 70 kg). The patients were treated with D and F. The rate of i.v. infusion for D and F were in the range 5–20  $\text{mg h}^{-1}$  and 0.05–0.30  $\text{mg h}^{-1}$ , respectively (Table 1).

**Table 1.** Serum concentrations of diazepam after intravenous infusion of diazepam and fentanyl to patient O.A.

Duration of i.v. infusion h	i.v. infusion rate $\text{mg h}^{-1}$	D concentrations determined in serum $\mu\text{g ml}^{-1}$
0.00	20	—
0.16	20	1.24
1.50	20	1.15
6.50	20	2.72
7.50	10	—
9.50	10	2.49
21.50	10	3.41
25.50	10	1.75
32.50	10	4.55
35.50	10	4.41
46.50	10	2.16
223.00	15	—
223.50	15	1.60
224.50	5	—
225.50	5	1.25
244.00	5	2.75
247.50	15	—
249.50	15	1.17

Table 1 (continued)		
265.50	15	2.96
268.50	15	4.50
276.50	15	3.25
278.00	15	3.46
285.50	15	2.52
289.50	15	2.33
295.50	15	4.20
297.50	10	—
300.50	10	2.40
310.50	10	2.62

### Materials

Diazepam and oxazepam (Polfa, Poznań, Poland); nordazepam (F. Hoffmann–La Roche Ltd, Basel, Switzerland); signopam (temazepam) (Polfa, Tarchomin, Poland); prazepam (P) used as an internal standard (Gödecke AG, Freiburg, Germany); fentanyl citrate (Polfa, Warsaw, Poland). Methanol (Merck, Germany) HPLC grade; diethyl ether (Pronit, Pionki, Poland) and water, house distilled from glass. Sodium hydroxide (POCH, Gliwice, Poland), reagent grade, and 30 % hydrochloric acid (Z.O.CH. "Chemian", Raszyn, Poland) ultra reagent grade.

### Apparatus

Liquid chromatograph Isochrom (Spectra Physics) with UV–VIS detector; 250×4.6 mm stainless steel column packed with 5 µm LiChrosorb RP-select B and a guard column with 30–40 µm Perisorb RP-18 (Merck, Germany). The absorbance output (0.5 AUFS) of the detector was connected to a recorder, (Radelkis, Budapest, Hungary). Full range recorder spans of 2–100 mV were used to provide on-scale peaks. Shaker type 357 (Elpan, Lubawa, Poland); microshaker type 326m (Marki/Warszawa, Poland).

### Chromatographic conditions

A mixture of methanol–water [70:30 (V/V)] was filtered through a 0.45 µm nylon-66 membrane filter and deaerated by argon. The mobile phase was pumped at the rate of 0.5 ml min<sup>-1</sup> with the pressure of 12 MPa at 20°C. UV detection was carried out at 230 nm.

### Stock solutions and standards

Stock solutions containing 0.2; 0.5; 1.0; 2.0; 5.0; 10.0 and 20.0 µg ml<sup>-1</sup> D and 15.0 µg ml<sup>-1</sup> P in methanol were prepared and they were stable more than one month when refrigerated. One hundred µl of each standard solution was transferred to conical flask (25 ml). To each flask 0.2 ml of serum was added after which the flasks were closed and vortex-mixed for 1 minute. The resulting serum based standards containing 0.1; 0.25; 0.5; 1.0; 2.5; 5.0; 10.0 µg ml<sup>-1</sup> D and 7.5 µg ml<sup>-1</sup> P were processed according to the procedure described below.

### Procedures

To each 0.2 ml of serum standard 2 ml of 0.1 ml l<sup>-1</sup> sodium hydroxide and 6 ml of ethyl ether were added. After shaking for 10 minutes and centrifuging for 3 minutes, most of the ether layer was transferred to a clean flask and 3 ml of 3 mol l<sup>-1</sup> hydrochloric acid were added. After shaking for 10 minutes and centrifuging for 3 minutes the ether layer was rejected. The aqueous layer was alkalinized with 1.5 ml of 2.5 mol l<sup>-1</sup> sodium hydroxide and 6 ml of diethyl ether were added. After extraction the ether layer was transferred to a dry tube and was evaporated to dryness under gentle nitrogen flow. The residue was reconstituted in 150 µl of the mobile phase and 20 µl was injected onto the column.

Each sample was injected in duplicate. Peak height ratios of D to P were plotted *versus* D concentration in  $\mu\text{g ml}^{-1}$ , and the resulting calibration curve was used to calculate the plasma concentration of unknown samples.

#### Precision determinations of D in serum

Five patients' serum samples were processed in the same manner, as standards except that to each 0.2 ml serum 100  $\mu\text{l}$  of methanol containing 15  $\mu\text{g ml}^{-1}$  of prazepam were added. D mean concentration and standard error mean (SEM) were calculated.

#### D recovery

To each of five 0.2 ml serum samples 100  $\mu\text{l}$  of 10  $\mu\text{g ml}^{-1}$  of D stock solution were added. The resulting serum based standards were processed according to the above extraction procedure with the exception that to the last ether extract 100  $\mu\text{l}$  of 15  $\mu\text{g ml}^{-1}$  of P were added. Five blank sera were also extracted according to the same procedure with the exception that to the last ether extract 100  $\mu\text{l}$  of the stock solution containing 10  $\mu\text{g ml}^{-1}$  D and 15  $\mu\text{g ml}^{-1}$  of P were added. Mean peak height ratio of D to P for the blank sera was considered as a recovery of 100 %.

#### Identification of F and D metabolites

150  $\mu\text{l}$  of a solution containing 10  $\text{mg ml}^{-1}$  each oxazepam, signopam and nordazepam and 50  $\mu\text{g ml}^{-1}$  of F was used to reconstitute a D serum residue of a patient and was injected onto the column. It was observed if the peaks of D metabolites and F were enriched.

## RESULTS AND DISCUSSION

A bromazepam extraction procedure described by Klotz [9] was applied for D extraction from human serum. Mobile phase constituents were taken from the literature [1, 10], but its composition was adjusted to have the best separation of D, its metabolites, P and F. A serious problem to overcome was to find a suitable internal standard. Carbamazepine [1] and nitrazepam could not be used, because they did not dissolve in hydrochloric acid needed for the serum extraction procedure. Oxazepam, signopam and nordazepam are D metabolites [1, 2]. Chlordiazepoxide retention time is almost identical to that of nordiazepam. So, finally P has been chosen as an internal standard, since it gives a good response at the working wavelength and does not interfere with any of the compounds analyzed in patients sera.

Distilled diethyl ether could not be stored over one month, because it produced some extra peaks. Then, its distillation was needed once again. Hydrochloric acid of reagent grade produced a peak of identical retention time to D. To crossover that problem an ultra pure hydrochloric acid was applied. So-called back extraction procedure was needed to get rid of some disturbing endogenous serum ingredients. A wavelength of 230 nm has been chosen for D in the mobile phase applied. However, it is the mobile phase chosen dependent and ranges from 228–254 nm [1–3, 11]. The HPLC procedure proposed provides a good separation mode for D, its metabolites, F and P as an internal standard (Fig. 1). The retention times of D and P are 11.5 and 15.0 min, respectively. The peaks at 8, 9, 10, and 23 min were identified with oxazepam, signopam, nordazepam and F, respectively (Fig. 2).

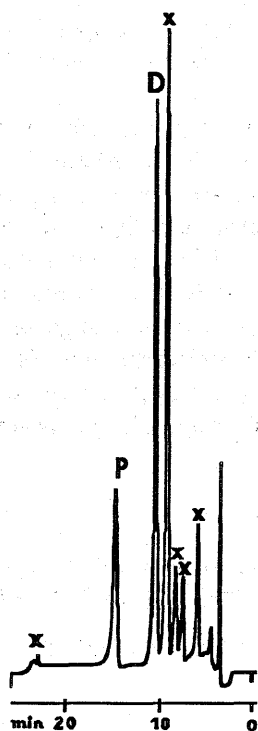


Figure 1. Chromatogram of diazepam (D) and fentanyl (F) extracted from serum; X - unidentified peaks of fentanyl and diazepam metabolites, P - prazepam (internal standard)

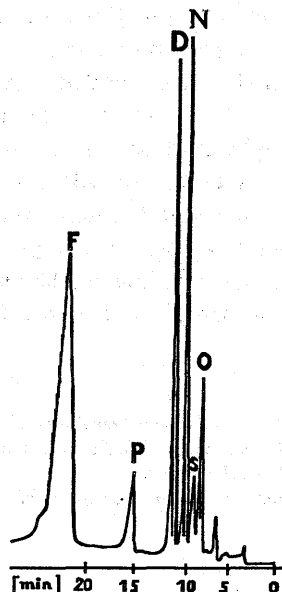


Figure 2. Chromatogram of diazepam (D) and fentanyl (F) extracted from serum and supplemented with fentanyl (F), oxazepam (O), signopam (temazepam) (S) and nordazepam (N) for identification of unknown peaks. P - prazepam (internal standard)

Calibration curves of peak height ratio *versus* concentration were linear over the concentration range of  $0.1\text{--}10.0\text{ }\mu\text{g ml}^{-1}$  and the intercept was essentially zero. The correlation coefficient for D determinations was 0.999. Mean calibration curve was described by regression equation  $y = (1.28 \pm 0.09)x$ .

Standard error mean (SEM) for 5 determinations of D in serum at its concentration  $1.62 \pm 0.04\text{ }\mu\text{g ml}^{-1}$  was equal 6 % and typical for other drug assays in biological fluids [11, 12]. The recovery of D was relatively high ( $71 \pm 2\%$ ) in spite of the back extraction procedure needed.

The method worked out was used for determination of diazepam in serum of patients of an Intensive Care Clinic treated with D and F for sedation needed (Table 1). Sensitivity of the method developed ( $100\text{ ng ml}^{-1}$ ) was sufficient taking into consideration the therapeutic D level ( $0.5\text{--}2.5\text{ }\mu\text{g ml}^{-1}$ ) [13]. The results obtained

(0.5–4.5  $\mu\text{g ml}^{-1}$ , Table 1) did not considerably exceed the therapeutic level needed. It should be considered that those patients suffer usually from severe diseases and a certain excess of D in serum is warranted.

The method developed has some advantages if compared with other procedures [1–3, 7]. Up to now, a method of determination of diazepam in serum in presence of fentanyl has not been published. A special RP-select-B column, designed for bases, was for the first time applied and gave a good separation of D, its metabolites and F. Their retention times are even shorter than obtained in other methods [1–3, 7]. It should be emphasized that those retention times were received at relatively slow rate (0.5  $\text{ml min}^{-1}$ ) of the mobile phase. A very practical advantage of the method proposed is also a small volume of serum (0.2 ml) needed for analysis.

The reversed-phase HPLC procedure worked out is sensitive, selective, and reproducible and therefore can be applied for monitoring of D and its active metabolites in patients serum treated with D.

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*Free of charge drug samples supplied by their producers are also greatly appreciated.*

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