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Simultaneous high-performance liquid chromatographic assay of droperidol and flunitrazepam in human plasma

Application to haemodilution blood samples collected during clinical anaesthesia

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ABSTRACT

A simultaneous assay for droperidol and flunitrazepam by high-performance liquid chromatography has been developed and applied to blood samples collected during an acute normovolemic haemodilution under general anaesthesia. Haemodilution blood samples were stored at +4°C to be transfused, if required, to a patient during the post-surgical phase. A C_{18} Supelclean cartridge was used for solid-phase extraction, and the recoveries were 74% and 89%, respectively, for droperidol and flunitrazepam. Compounds were chromatographed on a C_{18} Novapak column at 250 nm, with a mobile phase of acetonitrile–10 m*M* ammonium acetate buffer (pH 6.7) (45:55, v/v). Nitrazepam was used as the internal standard. For both drugs, the assay was linear up to 500 μg/l, and the detection limits were 20 and 10 μg/l for droperidol and flunitrazepam, respectively, and their observed levels in haemodilution samples were 93 ± 82 μg/l and 76 ± 107 μg/l, respectively. Some of the values for flunitrazepam were higher than the minimal efficient concentration, defined as the plasma level observed at the time of the patient wakening from anaesthesia (12 ± 4 μg/l). According to our results, haemodilution sampling can be performed before induction of anaesthesia. When the blood is collected after the anaesthetic induction, it seems necessary to determine levels of the two drugs in haemodilution samples to avoid side-effects.

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INTRODUCTION

Droperidol (DP) and flunitrazepam (FZ) are compounds widely used in anaesthesiology. DP is a butyrophenone with sedative, anxiolytic and antiemetic properties, and FZ is a 1,4-benzodiazepine used for its very strong action, even at low concentration.

The extensive use of acute intentional normovolemic haemodilution (HDN) after induction of anaesthesia leads to the problem of the anaesthetic compounds entering the haemodilution samples. When an autotransfusion of this diluted blood is required after surgical procedure, side-effects can be expected with drugs like DP and FZ when at sufficient levels. Post-surgical anaesthetic phenomena occurring during autotransfusion have been described previously [1].

Our aim is to determine the pharmacokinetics of DP and FZ in plasma after anaesthesia induction, during and after blood sampling, and in the HDN samples, and to compare the last values with the lowest efficient concentrations (with which an anaesthetic effect is still observed), in order to assess the risk of side-effects of post-surgical transfusion.

Several high-performance liquid chromatographic (HPLC) methods for DP and FZ have been described [2–11]. This paper describes a rapid, sensitive and convenient HPLC assay for the simultaneous determination of FZ and DP.

EXPERIMENTAL

Materials and reagents

Droperidol (Droleptan) was kindly supplied by Janssen-Le Brun Labs. (Boulogne-Billancourt, France). Flunitrazepam (Narcozep) and nitrazepam (Mogadon) were kindly supplied by Roche (Neuilly/Seine, France). A solution containing 5 mg/l nitrazepam in methanol–water (50:50, v/v) was stored at +4°C, and used as the internal standard.

Methanol and acetonitrile, HPLC grade, were purchased from Farmitalia Carlo Erba (Milan, Italy). Distilled water and 0.15 *M* sodium chloride aqueous solution were purchased from Aguettant Labs. (Lyon, France), and ammonium acetate from Prolabo Society (Lyon, France). Two aqueous solutions of ammonium acetate containing 10 m*M* and 50 m*M*, respectively, were prepared and stored at +4°C.

Apparatus and chromatographic conditions

A Shimadzu LC-6A pump with a 20-μl injection loop (Rheodyne 7125) was used. Separation was performed on a 150 × 3.9 mm I.D. Novapak C_{18} (5 μm) column protected with a μBondapak C_{18} guard column, both obtained from Waters Chromatography Division (Milford, MA, USA). Detection was performed with an SPD-6A module detector from Shimadzu (Kyoto, Japan) and set at 250 nm. The flow-rate of the mobile phase was 1 ml/min.

Extraction procedure

A Superclean LC-18 cartridge and an elution apparatus, Visiprep from Supelco (Bellefonte, PA, USA) were used for liquid–solid extraction. The cartridge was conditioned twice with 2 ml of methanol, then 2 ml of distilled water and 2 ml of 50 m*M* ammonium acetate aqueous solution (pH 6.9). Then 1 ml of plasma sample was applied, the cartridge was washed three times with 1 ml of 0.15 *M* sodium chloride solution, the remaining saline solution was removed by 50 μl of methanol and discarded. FZ and DP were eluted with 2 ml of acetonitrile collected in 10-ml conical glass tubes. The organic phase was evaporated under a stream of nitrogen in a water-bath at 60°C. The dry residue was dissolved in 100 μl of pure acetonitrile. The extracts were filtered on Millex 0.45 μm (Millipore SJHV 004NS), before injection (10 μl).

Calibration graph

Standard solutions containing 25, 50, 100, 250 and 500 μg/l DP and FZ were prepared by dilution of a 5 mg/l methanol stock solution, in a drug-free pool of plasma. These preparations were processed as described in *Extraction procedure*. The peak-area ratios of DP and FZ to NZ were used to plot the calibration curve, using a Shimadzu C-R6A integrator.

The plasma standard solutions were stored at −20°C for 1 month.

Patients and protocols

All patients were selected in the Department of Stomatology of the Centre Hospitalier Lyon-Sud, within the age range 15–35. Equal numbers of males and females took part.

Protocol 1: pharmacokinetic study. Ten patients were treated by i.v. injection of 0.15 ± 0.04 mg/kg DP and 0.04 ± $5 \cdot 10^{-3}$ mg/kg FZ. Heparinized blood was collected at 5, 10, 20, 30, 45, 60, 120 and 180 min after anaesthetic induction, then centrifuged at 1000 *g*. The separated plasma aliquots were stored at −20°C.

Protocol 2: determination of the minimal efficient concentration (MEC). Blood samples (5 ml) from ten patients were collected immediately on waking. Wakefulness was controlled by clinical signs. Blood was centrifuged at 1000 *g*, and the separated plasma aliquots were stored at −20°C.

Protocol 3: haemodilution sample concentration study. Twenty patients were included in this study: 5–40 min after the anaesthesia induction, 0.3–0.5 l of haemodiluted blood was sampled. After homogenization by gentle shaking at +4°C for 15 min, 5 ml of the blood was sampled then centrifuged for 5 min at 1000 *g*.

RESULTS

Chromatographic conditions

The chromatographic behaviour of DP and FZ was examined by using a mobile phase of acetonitrile with an aqueous fraction containing various amounts of ammonium acetate (Table I). When the ammonium acetate concentration was increased, the resolution between DP and FZ/NZ decreased. Thus, a low molarity of ammonium acetate in the mobile phase produces better chromatographic behaviour of the neuroleptic drugs (Table I). This phenomenon was previously described by Curry *et al.* [4] with haloperidol.

Use of methanol instead of acetonitrile considerably modified the retention time of DP (25 min). Three columns packed with different bonded phases were tested with the same mobile phase (Table II). As a result, the Novapak C_{18} column from Waters Chromatography Division was selected.

Extraction procedure

Liquid–liquid and solid–liquid extraction procedures are compared in Table III [4,11–14]. Though DP is highly soluble in diethyl ether, better results were obtained with a solid–liquid extraction procedure using the C_{18} cartridge. The C_{18} solid phase, conditioned with ammonium acetate, allowed satisfactory recovery of the three compounds, and was less time-consuming than liquid–liquid extraction (Fig. 1).

Precision and recovery

The means and coefficients of variation calculated from ten assays with concentrations in the range 50–200 μg/l for FZ and 100–200 μg/l for DP are shown in Table IV.

TABLE I

COMPARISON OF MOBILE PHASES

Chromatographic conditions: injection of 10 μl of methanol solution containing DP and FZ (250 μg/l); flow-rate, 1 ml/min; attenuation, 0.02 a.u.f.s.; detection wavelength, 250 nm; stationary phase, C_{18} Novapak (Waters); organic modifier, 50% acetonitrile.

Mobile phase pH	50% Ammonium acetate buffer (m*M*)	Retention time (min)			Efficiency, *N* (plates/m)		Resolution, R_s
		DP	FZ	NZ	DP	FZ	
6.7	10	4.8	3.5	2.5	9585	12 714	2.60
6.8	20	4.5	3.6	2.7	8199	12 449	1.45
6.85	30	4.0	3.6	2.6	7047	12 250	1.00
6.9	50	3.5	3.3	2.2	4984	10 308	0.40

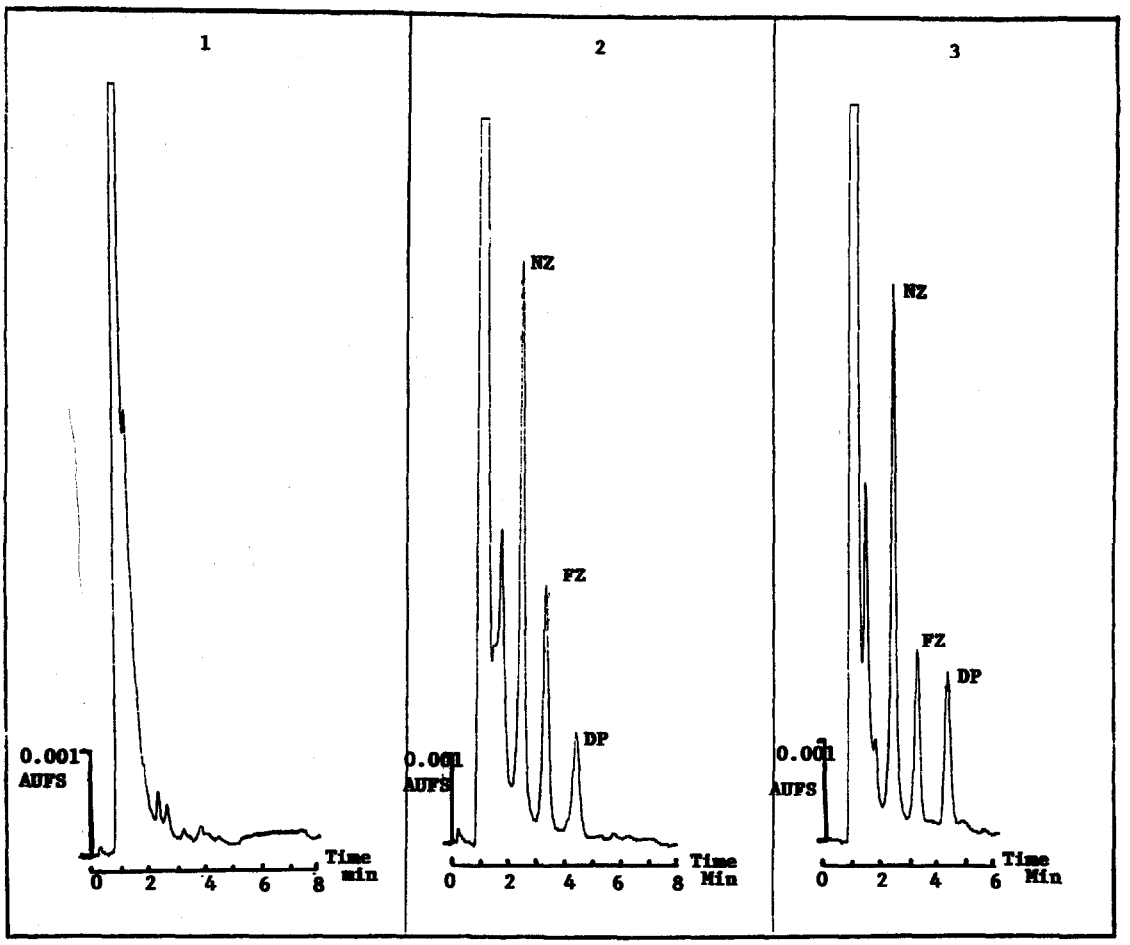

Fig. 1. Chromatograms of (1) drug-free plasma (10 μl), (2) plasma spiked with FZ and DP (100 μg/l) and NZ (250 μg/l) and (3) sample from patient by anaesthesized intravenous bolus of 2.5 mg of FZ and 10 mg of DP, haemodiluted 20 min after anaesthesia; the calculated concentrations in this patient were 70 μg/l and 135 μg/l for FZ and DP, respectively. Detector wavelength, 250 nm; attenuation, 0.01 a.u.f.s.

TABLE II

COMPARISON OF STATIONARY PHASES

	MPLC C_8 (Brownlee Labs.)	Novapak C_{18} (Waters)	Hypersil C_{18} (Touzart & Matignon)
Length (mm)	100	150	250
I.D. (mm)	4.6	3.9	4.6
Particle size (μm)	5	5	5
Efficiency, N (plates DP/m)	3700	23 080	4167
Efficiency, N (plates FZ/m)	2630	20 540	4167
Resolution, R_s	2.53	2.60	0.36

TABLE III

COMPARISON OF THE SIMULTANEOUS EXTRACTION PROCEDURES FOR DP AND FZ

Ref.	Extraction conditions	Absolute recovery (%)		
		DP	FZ	NZ
Liquid–liquid extraction				
12	Chloroform, NaOH, pH 11.0	62	87	85
4	Diethyl ether, phosphate, pH 7.4	79	58	59
11	Hexane, phosphate, pH 7.4	64	66	70
Solid–liquid extraction				
14	C_{18} Supelclean, borate, pH 9.5	12	90	91
13	C_{18} Supelclean, acetate, pH 6.9	74	74	95
13[a]	C_{18} Supelclean, water	8	79	86

[a] Modified ref. 13.

Interfering compounds

Benzodiazepines such as clonazepam, alprazolam and triazolam can be extracted and separated by this method. Their retention times are similar to those of DP and FZ, but they are never administered simultaneously with the two compounds. Atropine, frequently used in premedication, does not interfere with DP and FZ, its retention time being shorter (1.5 min). Other associated anaesthetics, phenoperidine and pancuronium, are neither extracted nor chromatographed by this method.

Pharmacokinetics

Fig. 2 shows the pharmacokinetic curves of DP and FZ. This study demonstrates that haemodilution sampling starts during distribution with a very short half-life and at a high concentration.

Minimal efficient concentration

Mean plasma concentrations immediately after the patient awoke were 29 ± 16 and 12 ± 4 μg/l for DP and FZ, respectively. In accordance with the detection limit (20 and 10 μg/l for DP and FZ, respectively), no narcotic effect was observed when FZ levels were below 12 ± 4 μg/l.

Haemodilution sampling level

The DP and FZ levels in the first sample were 93 ± 80 μg/l (C.V. 94%) and 76 ± 107 μg/l (C.V. 113%). The time from the end of induction and the beginning of haemodilution was 15 ± 20 min. This result showed the importance of the haemodilution time. The stability of the two compounds in the haemodilution samples stored at +4°C was studied for 10 days (the maximum duration of storage of the blood). The DP and FZ levels decreased considerably. After 7 days, *ca.* 50% of the initial concentration disappeared, yet the binding to red cells was *ca.* 5%. So, only degradation of the compounds can explain this slow decrease in haemodilution samples.

TABLE IV

PRECISION AND RECOVERY

Compound	Concentration (μg/l)	Reproducibility		Recovery[a], mean values (n = 20) (%)
		Intraday C.V. (%) (n = 10)	Interday C.V. (%) (n = 10)	
FZ	50	8.5	7.9	85 ± 5
	200	5.5	4.1	89 ± 3
DP	100	6.8	9.8	65 ± 5
	200	6.6	4.5	74 ± 4

[a] Recoveries calculated from the ratio to NZ (100 μg/l).

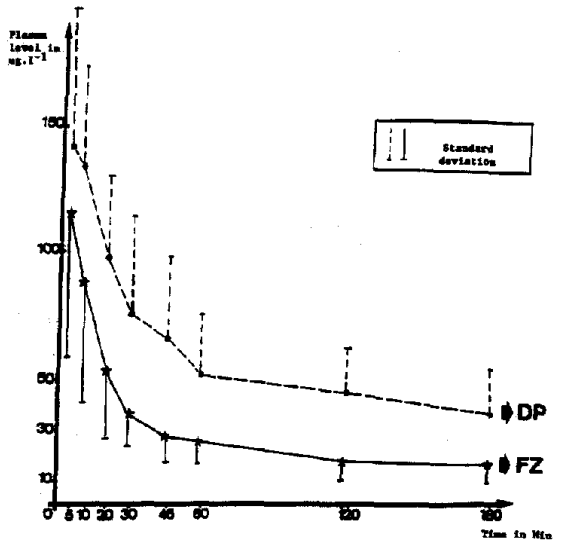

Fig. 2. Plasma levels of DP (dashed curve) and FZ (continuous curve). Ten patients were treated by i.v. injection of 0.15 ± 0.04 mg/kg DP and 0.04 ± 5.10^{-3} mg/kg FZ. Blood was sampled at 5, 10, 20, 30, 45, 60, 120 and 180 min after anaesthesia.

DISCUSSION

Extraction procedure

The solid-phase extraction allowed rapid and simultaneous extraction of DP and FZ. Liquid–liquid extraction procedures were also tested but required more steps and were time-consuming [2–4]. Nevertheless, the most important limitation in liquid–solid extraction occurs when the plasma contains high lipid levels. The conditioning of the cartridge is most important. After various attempts, the ammonium acetate buffer proved to be most effective. This extraction method can also be performed with some other benzodiazepines, such as clonazepam and triazolam.

Chromatographic conditions

The mobile phase we propose, 10 m*M* ammonium acetate (pH 6.7)–acetonitrile (55:45, v/v) gave good column efficiency (9600 and 12 700 theoretical plates/m for DP and FZ, respectively), and a good resolution factor (2.60). Its low molarity and neutral pH prolong the life of the column.

The molarity of ammonium acetate affects the chromatographic behaviour of DP, but not of FZ (Table I). Similar effects were observed for other butyrophenones by Curry *et al.* [4]. Also, the use of acetonitrile instead of methanol yields sharper and more symmetrical peaks, and thus a better resolution factor. This method allows rapid and sensitive assay of DP and FZ simultaneously.

Plasma pharmacokinetics

Our results were similar to those obtained in previous work [15,16]. They show that haemodilution sampling is best performed during the first distribution phase, when the plasma levels of the drugs are high but rapidly decreasing. Thus, small changes in either the time or the duration of sampling will produce the observed variations of DP and FZ levels in heamodilution samples.

Minimal efficient concentration study

This study was important for FZ. This compound gave a rapid and fast hypnotic effect at small doses [15]. The experimental values show that hypnotic effect was still obtained when the plasma level of FZ was very low (12 ± 4 μg/l). For half of the patients (n = 10), this level was below the detection limit (10 μg/l). Contrary to the results of Amrein [18], Richardson and Manford [19] and Cano [20], but in accord with Lader [17], our result suggests that the plasma concentration is not related to the clinical effect. However, some authors have evaluated the MEC levels at the beginning of narcosis instead of during the awakening phase. Nevertheless, the concept of an MEC needs to be studied in plasma samples although, of course, the observation would better made at the site of drug action.

Anaesthetic concentration in haemodilution samples

Results obtained with haemodilution samples show large variations (94% and 113% for DP and FZ, respectively), with mean levels of 93 and 76 μg/l for DP and FZ, respectively. Thus, some values were significantly higher than the observed MEC (12 ± 4 μg/l). This wide variation was produced essentially by two factors: the time elapsed

since drug injection and the duration of blood sampling, as described above. Our results confirm such a hypothesis.

So, the transfusion of large volumes of blood (0.5–2 l) allows simultaneous administration of DP and FZ at levels possibly higher than (or at least equal to) the *in vivo* MEC, in spite of their dilution. Of course, other phenomena (tissue distribution, elimination and the duration of the transfusion) may reduce DP and FZ levels, but are not considered.

Consequently, postsurgical autotransfusion by haemodilution samples containing anaesthetic drugs requires special medical assistance and the determination of drug concentrations both in haemodilution samples and in the plasma of the transfused patient.

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