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DETERMINATION OF 7-AMINO-FLUNITRAZEPAM (Ro 20-1815) AND 7-AMINO-DESMETHYLFLUNITRAZEPAM (Ro 5-4650) IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

Y.C. SUMIRTAPURA, C. AUBERT, Ph. COASSOLO and J.P. CANO*

I.N.S.E.R.M. SC No. 16, Laboratoire de Pharmacocinétique et de Toxicocinétique, 27 Boulevard Jean Moulin, F-13385 Marseille (France)

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SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of 7-amino-flunitrazepam (Ro 20-1815) and 7-amino-desmethylflunitrazepam (Ro 5-4650) in plasma is described. After extraction with an organic solvent, the compounds and their internal standard (7-amino-methylclonazepam or Ro 5-3384) are derivatized with fluorescamine and chromatographed on a reversed-phase μBondapak C_{18} column using pH 8 buffer solution—acetonitrile (3:1) as mobile phase. The detection is performed by a fluorometer at excitation and emission wavelengths of 390 and 470 nm, respectively. The sensitivity limit is about 1 ng/ml of plasma for both 7-amino-flunitrazepam and 7-amino-desmethylflunitrazepam. The method has been applied to the determination of plasma levels of these substances during pharmacokinetic studies of flunitrazepam, desmethylflunitrazepam and 7-amino-flunitrazepam.

INTRODUCTION

Flunitrazepam is a benzodiazepine derivative which possesses all the activities typical of the class of compounds, namely anxiolytic, anti-convulsant, muscle-relaxant and central sedative effects which cause the onset of sleep, prolong the duration of sleep and tone down reactions to stress factors [1]. Its major metabolites found in the human plasma are 7-amino-flunitrazepam (Ro 20-1815, 7-AF) and desmethylflunitrazepam (Ro 5-4435), which are further metabolized to 7-amino-desmethylflunitrazepam (Ro 5-4650, 7-ADF) [2] (Fig. 1).

The determination of plasma levels of 7-AF and 7-ADF after administration of flunitrazepam, 7-amino-flunitrazepam or desmethylflunitrazepam provides important information/data for the psychopharmacological and

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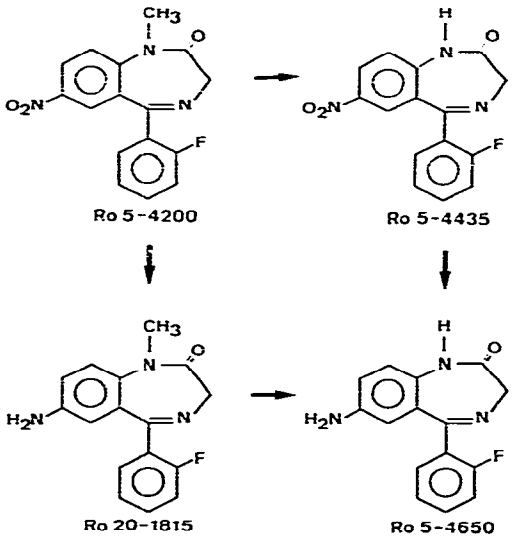

Fig. 1. Chemical structures of flunitrazepam and its major metabolites.

pharmacokinetic studies of these substances.

Several gas—liquid chromatographic methods are available for the determination of unchanged flunitrazepam and desmethylflunitrazepam [3—7], but 7-AF and 7-ADF cannot be measured by this technique at the concentrations that occur in human blood following therapeutic doses.

However, a thin-layer chromatographic assay for the determination of 7-AF and 7-ADF, involving derivatization with fluorescamine, has been developed by Haefelfinger [8]. Subsequently, the derivatized substances were quantified fluorometrically by direct scanning. The sensitivity limits reported were 1 ng/ml and 2 ng/ml of plasma for 7-AF and 7-ADF, respectively.

This paper describes a highly selective and sensitive high-performance liquid chromatographic method for the determination of 7-AF and 7-ADF involving fluorescamine derivatization and fluorescence detection. 7-Amino-methylclonazepam (Ro 5-3384, 7-AMC) was used as internal standard.

MATERIALS AND METHODS

Reagents and standards

All aqueous solutions were prepared with double-distilled water. Dipotassium hydrogen phosphate, sodium hydroxide, and pH 8 and pH 10 buffer solutions were obtained from Merck (Darmstadt, G.F.R.). Sulfuric acid "Ultrex" was provided by J.T. Baker Co. (Phillipsburg, NJ, U.S.A.). Acetonitrile, RS grade, and isoamyl alcohol, RPE grade, were purchased from Carlo Erba (Milan, Italy). Acetone, diethyl ether and hexane (Pestipur grade) were obtained from Solvant-Documentation-Synthèse Co. (Peypin, France). Methanol was from Prolabo (Paris, France).

Fluorescamine solution (0.5 %, w/v) was prepared by dissolving a quantity of the solid substance (Fluram®, Hoffmann-La Roche, Basel, Switzerland) in acetone.

Standard substances of 7-amino-flunitrazepam, 7-amino-desmethylflunitrazepam and 7-amino-methylclonazepam were provided by Hoffmann-La Roche. Individual standard stock solutions (1 mg/ml) were prepared in acetone—methanol (1:1). Working solutions (varying from 0.01 to 10 ng/μl) were obtained by dilution with acetone—hexane (1:4).

Extraction and derivatization

Into a 20-ml glass-stoppered centrifuge tube, 20—500 ng of internal standard (7-AMC), 0.5—4 ml of plasma, 2 ml of pH 10 buffer solution and 10 ml of diethyl ether containing 1% of isoamyl alcohol were added. (The quantity of the internal standard added was about two times higher than that of 7-AF or 7-ADF to be analyzed; plasma volumes of 0.5, 1, 2, and 4 ml were used for plasma drug concentrations above 50 ng/ml, between 5 and 50 ng/ml, between 1 and 5 ng/ml and lower than 1 ng/ml, respectively.) The mixture was shaken on a three-dimensional shaker (type EM 4; Desaga, Heidelberg, G.F.R.) for 15 min and centrifuged for 5 min at 3750 *g*. The organic layer was transferred into another centrifuge tube containing 2 ml of 0.5 *M* sulfuric acid. The tube was shaken for 10 min and centrifuged for 5 min. The organic layer was discarded without removing any of the acid phase which was then adjusted to pH 9—10 with 1 *M* sodium hydroxide solution containing 1 *M* dipotassium hydrogen phosphate. The mixture was extracted with 10 ml of diethyl ether by shaking for 10 min and centrifuging for 5 min. The organic phase was transferred into another tube and evaporated to dryness in a 45°C water-bath under vacuum and a stream of nitrogen. To the residue, 100 μl of the mobile phase and 20 μl of 0.5% fluorescamine solution were added and the tube was mixed on a Vortex mixer for 60 sec. An appropriate volume of the aliquot was injected onto the column. (The volume to be injected was determined according to the detector sensitivity range used and the estimated drug concentration contained in the sample, to obtain an optimum detector response; with the detector we used, this could vary from 10 to 100 μl.)

Chromatographic conditions

A Waters Model M 45 pump and a Waters Model 6000A pump were used with a Waters Model 660 solvent programmer (Waters Assoc., Milford, MA, U.S.A.). One pump (A) contained pH 8 buffer solution (obtained by diluting 20 ml of concentrated pH 8 buffer solution to 1 l with double distilled water) and the other pump (B) contained acetonitrile. The solvent programmer was set to 74—75% of A and 26—25% of B for an isocratic mode. (The percentage of acetonitrile varied from 25 to 26%. It was adjusted to optimize the separation and the retention times of the compounds analyzed.) The mobile phase flow-rate was 2.5 ml/min.

The chromatography was performed on a 30 cm × 4 mm μBondapak C_{18} reversed-phase column (10 μm, Waters) at ambient temperature.

The detection was carried out with a Schoeffel Model GM 970 fluorometer (Schoeffel, Westwood, NJ, U.S.A.) at an excitation wavelength of 390 nm and an emission wavelength of 470 nm.

Calibration curves

Standard calibration curves (ratios of 7-AF-fluorescamine or 7-ADF-fluorescamine to 7-AMC-fluorescamine peak areas versus concentrations of 7-AF or 7-ADF) were obtained by analyses of blank plasma samples to which known quantities of 7-AF and 7-ADF were added together with a constant amount of internal standard. The areas of the chromatographic peaks were calculated by electronic digital integration (Hewlett-Packard 3352 B Data System).

The quantities of 7-AF and of 7-ADF used for the establishment of the calibration curves were of the same magnitude as the concentrations of the compounds contained in the samples. For both compounds there was good assay linearity over the concentrations range tested (2.5—500 ng/ml of plasma) with correlation coefficients of > 0.999. Moreover, the regression curves practically passed through the origin.

RESULTS AND DISCUSSION

Derivatization

The quantity of reagent required for complete derivatization of 7-AF, 7-ADF and 7-AMC at concentrations below 500 ng/ml of plasma has been investigated. For this purpose, a set of mixtures, containing 500 ng of 7-AF, 500 ng of 7-ADF, 500 ng of 7-AMC and the extract of 4 ml of plasma blank were derivatized with 20 μl of fluorescamine solutions of various concentrations (0.25, 0.5, 1, 2.5, 5 and 10 μg/μl) using 100 μl of mobile phase as reaction medium. For each mixture, an identical volume of aliquot was injected onto the column and fluorescence was continuously recorded.

As can be seen in Fig. 2, the peak heights of fluorescamine derivatives of 7-AF, 7-ADF and 7-AMC are constant in the presence of fluorescamine at concentrations of 2.5 μg/μl and above. Consequently, we used routinely 20 μl of 0.5% (w/v) reagent solution for the quantitative determination of 7-AF and 7-ADF in our pharmacokinetic studies.

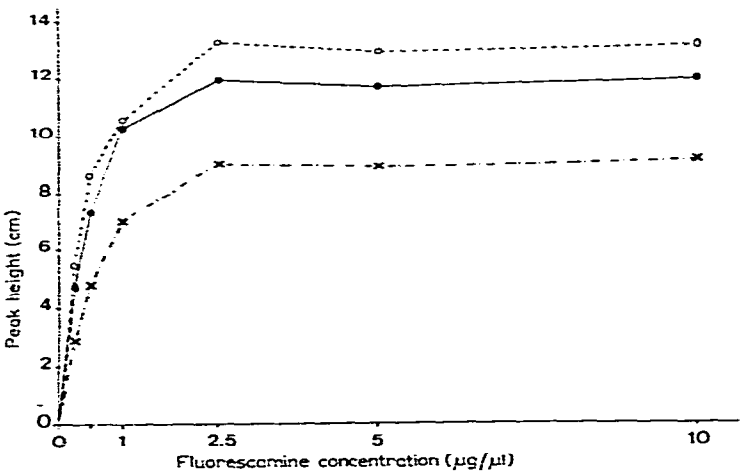

Fig. 2. Evolution of signal intensity observed for 7-AF-fluorescamine (•), 7-ADF-fluorescamine (○) and 7-AMC-fluorescamine (×) as a function of fluorescamine concentration.

Overall recoveries

Extraction characteristics of 7-AF and 7-ADF have been studied. Both compounds were quantitatively extracted with diethyl ether from an aqueous phase of pH 10 and they were quantitatively back-extracted into 0.5 *M* sulfuric acid from this solvent. The overall recoveries of 7-AF and of 7-ADF from plasma samples after complete extraction by this method were about 70% and 50%, respectively. These recoveries could be improved by performing twice extractions at the first step of extraction described above. In this case, the first portion of the ether phases must be concentrated.

Sensitivity

Using fluorescence detection and derivatization with fluorescamine, both 7-AF and 7-ADF can be detected at picogram levels. By this method, a relatively great volume of plasma sample can be utilized. Using 4 ml of the sample the sensitivity limit is about 0.5—1 ng/ml for both 7-AF and 7-ADF.

Selectivity

In this study, fluorescamine was chosen as derivatizing agent for 7-AF, 7-ADF and 7-AMC (internal standard) for several reasons: high reactivity, sensitivity and particularly for its high selectivity. Only primary amine compounds can react and produce an intensely fluorescent derivative with fluorescamine, while fluorescamine by itself is not fluorescent [9, 10]. Moreover, by performing back-extraction into an acidic medium, the interfering substances were limited to the basic compounds.

Chromatograms obtained from human and monkey plasma blanks show that no endogenous substances interfered with the compounds of interest.

By the present method, 7-AF and 7-ADF can be quantitatively analyzed in the presence of 7-amino derivatives of clonazepam and nitrazepam. The retention times of the five derivatized substances were about as follows: 7-ADF-fluorescamine (7-ADF-Fl), 4.5 min; 7-amino-clonazepam-Fl and 7-amino-nitrazepam-Fl, 6 min; 7-AF-Fl, 9.2 min; and 7-AMC-Fl, 13.6 min (Fig. 3). 7-Acetamido-flunitrazepam (Ro 20-9800), another possible metabolite of 7-AF, is not detected by the proposed method; we did not notice the acid hydrolysis of Ro 20-9800 to 7-AF during the extraction described above, especially during the back-extraction into 0.5 *M* sulfuric acid.

Furthermore, the selectivity of this assay has been tested with plasma of intensive care unit patients receiving several other drugs frequently administered to such patients [7]. Fig. 4 illustrates a representative chromatogram obtained from a patient in an intensive care unit.

Reproducibility

The reproducibility of the assay was determined at concentrations ranging from 1 to 500 ng/ml for 7-AF and from 1.25 to 50 ng/ml for 7-ADF, by assaying six plasma samples at each concentration on the same day. The results obtained (expressed as variation coefficient) are presented in Table I. The variation coefficients within the range tested varied from 3 to 9.8%.

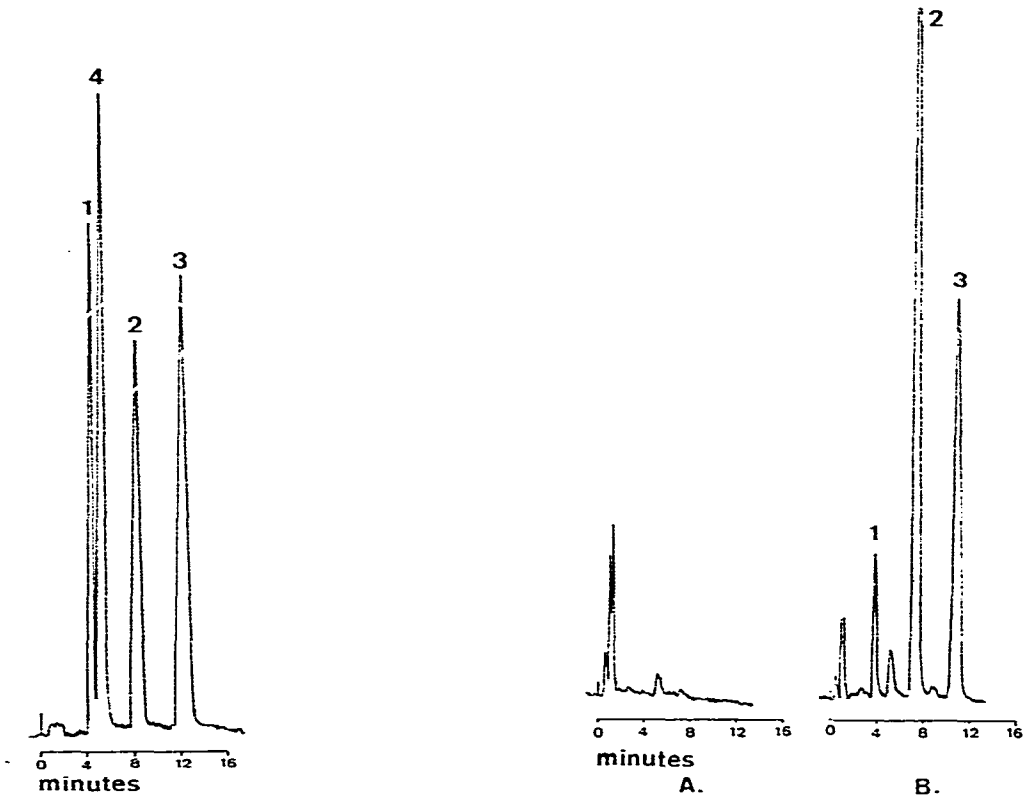

Fig. 3. HPLC separation of fluorescamine derivatives of 7-ADF (1), 7-amino-clonazepam (4), 7-amino-nitrazepam (4), 7-AF (2) and 7-AMC (3).

Fig. 4. Chromatograms of (A) plasma of a patient receiving diazepam, droleptan, dopamine and other drugs, 5 min prior to flunitrazepam administration, and (B) plasma of the same patient 8 h following the ninth intravenous injection of 2 mg of flunitrazepam (injections at time 0, 0.33, 2, 6, 10, 14, 22, 30 and 38 h) with addition of 15 ng/ml of 7-AMC. (Amount of plasma used = 2 ml; volume of the last aliquot injected = 50 μl; detector sensitivity range = 0.2 μA.) 1 = 7-ADF-Fl; 2 = 7-AF-Fl; 3 = 7-AMC-Fl.

TABLE I

REPRODUCIBILITY OF THE ASSAY

Compound	Concentration (ng/ml)	Amount of plasma used (ml)	Volume of the last aliquot injected (μl)	Coefficient of variation (%)
Ro 20-1815	1	4	100	4.7
	5	2	50	6.2
	50	1	25	5.0
	500	0.5	10	3.0
Ro 5-4650	1.25	4	100	9.8
	5	2	50	6.6
	50	1	25	7.9

Application to biological samples

The present method has been successfully applied to the assay of plasma levels of 7-AF and 7-ADF in man and monkey during pharmacokinetic studies of flunitrazepam, desmethylflunitrazepam and 7-amino-flunitrazepam.

The plasma level—time course of 7-AF and of 7-ADF following a single intramuscular dose of 1.2 mg/kg 7-AF in one monkey is presented in Fig. 5. In this study we observed significant differences between the pharmacokinetic behaviour of 7-amino-flunitrazepam in man from that observed in rhesus monkey. The drug clearance in this monkey is found to be approximately 0.94 l/h per kg of body weight. This value is about 20 times higher than that obtained in the human subject (0.05 l/h per kg). The elimination half-lives of

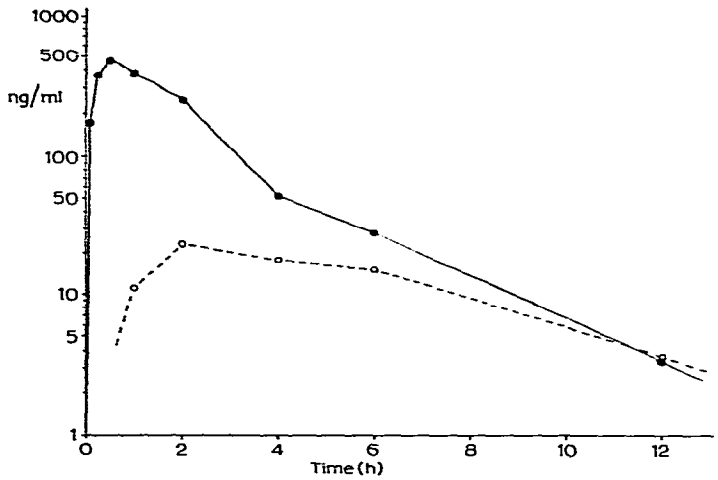

Fig. 5. Plasma level—time course of 7-AF (•) and 7-ADF (○) in a monkey after a single intramuscular dose of 1.2 mg/kg 7-AF.

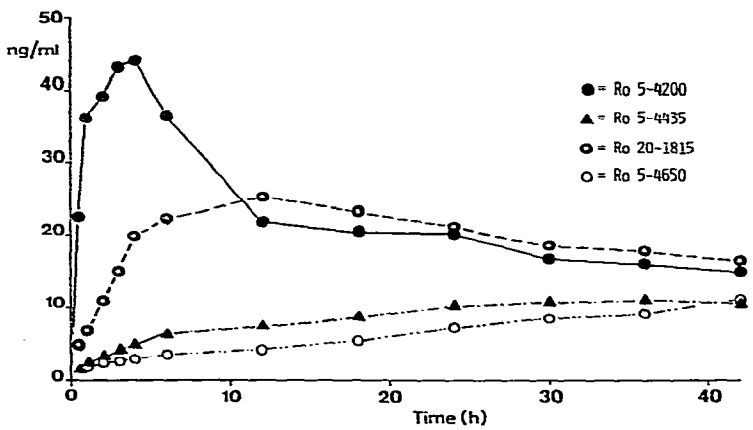

Fig. 6. Evolution of plasma concentrations of flunitrazepam and its major metabolites as a function of time in a patient in an intensive care unit during multiple intravenous administration of 2 mg of flunitrazepam (injections at time 0, 0.5, 1, 2, 3, 4, 6 h and then every 6 h).

the drug are about 2 h in the monkey and 13 h in man. Meanwhile, the plasma levels of the desmethyl metabolite (7-ADF) compared with those of the unchanged compound are negligible.

Fig. 6 shows the accumulation of 7-AF and 7-ADF plasma concentrations during a multiple intravenous administration of 2 mg of flunitrazepam to one patient in an intensive care unit (concentrations of unchanged flunitrazepam and of desmethylflunitrazepam presented in this figure were determined by a gas—liquid chromatographic method developed in our laboratory). As can be seen in this figure, during chronic administration of flunitrazepam, concentrations of 7-AF can slightly exceed those of the unaltered drug, while 7-ADF levels remain inferior.

Another matter of interest of this specific and sensitive method is that it allowed us to calculate the pharmacokinetic parameters of 7-AF after intravenous administration of 7-AF itself, to correlate plasma levels of the derivative to pharmacological responses and to estimate the extent of flunitrazepam biotransformation to this metabolite [11].

REFERENCES

1 W. Haefely, R. Cumin, A. Kulcsár, P. Polc and R. Schaffner, in W. Hügin, G. Hossli and M. Gemperle (Editors), Bisherige Erfahrungen mit "Rohypnol" (Flunitrazepam) in der Anästhesiologie und Intensivtherapie, Editiones Roche, Basel, 1976, p. 13.
2 G. Wendt, in W. Hügin, G. Hossli and M. Gemperle (Editors), Bisherige Erfahrungen mit "Rohypnol" (Flunitrazepam) in der Anästhesiologie und Intensivtherapie, Editiones Roche, Basel, 1976, p. 27.
3 J.A.F. de Silva, C.V. Puglisi and N. Muno, J. Pharm. Sci., 63 (1974) 520.
4 J.A.F. de Silva and I. Bekersky, J. Chromatogr., 99 (1974) 447.
5 J.P. Cano, J. Guintrand, C. Aubert and A. Viala, Arzneim.-Forsch., 27 (1977) 338.
6 D.B. Faber, R.M. Kok and E.M. Rempt-van Dijk, J. Chromatogr., 133 (1977) 319.
7 Y. Sumirtapura, C. Aubert and J.P. Cano, Arzneim.-Forsch., 32(I), 3 (1982) 252.
8 P. Haefelfinger, J. Chromatogr., 111 (1975) 323.
9 M. Weigele, S.L. DeBernardo, J.P. Tengi and W. Leimgruber, J. Amer. Chem. Soc., 94 (1972) 5927.
10 S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber and M. Weigele, Science, 178 (1972) 871.
11 Ch. Crevoisier, M. Eckert, W.H. Ziegler, J.P. Cano and Y. Sumirtapura, Paper presented at the IIIrd World Congress on Biological Psychiatry, Stockholm, 28 June—3 July 1981, Abstract F 1017.