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Simultaneous determination of flunitrazepam and its metabolites in plasma and urine by HPLC/DAD after solid phase extraction

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Abstract

A high performance liquid chromatography (HPLC) assay was developed for the determination of flunitrazepam (FNZ) and its metabolites in urine and plasma. The analytes and the internal standard (triazolam, TRZ) were extracted by Sep-Pak C18 SPE-cartridge and separated utilizing a 5 μm ChromSpher C8 glass column with a gradient mobile phase containing methanol and 0.125% (v/v) of isopropylamine in water. Diode array detection (DAD) was carried out at a monitoring wavelength of 240 nm and a reference wavelength of 550 nm. Standard curves were linear from their quantitation limits until 200 ng ml^{-1} urine or 250 ng ml^{-1} plasma for 7-amino-desmethyl-flunitrazepam (ADF), 7-amino-flunitrazepam (AF), 7-acetamino-flunitrazepam (ACF) and until 400 ng ml^{-1} urine or 500 ng ml^{-1} plasma for FNZ, 1-desmethyl-flunitrazepam (DF), and 3-hydroxyl-flunitrazepam (HF). The intraday and interday coefficients of variation ranged from 2.04 to 9.07% and from 2.64 to 14.10%, respectively in urine and from 5.13 to 8.60% and from 7.27 to 10.46%, respectively in plasma. The developed method is used in forensic toxicology and is also applicable to pharmacokinetic studies in man. © 1997 Elsevier Science B.V.

Keywords: Flunitrazepam; Metabolite; High performance liquid chromatography; Diode array detection; Solid phase extraction; Urine; Plasma

1. Introduction

Flunitrazepam (FNZ), Rohypnol^{R}, 5-(2-fluorophenyl)-1,3-dihydro-1- methyl-7-nitro-2H-1,4-benzodiazepin-2-one, is a benzodiazepine derivative and pharmacologically a full agonist whose hypnotic effect predominates over the sedative, anxiolytic, muscle relaxing and anticonvulsant effects, characteristics of benzodiazepines. It is used in many hospitals and its abuse and intoxication was reported by several authors [1–4].The equivalent oral dose of FNZ relating to 10 mg of diazepam, is 2 mg [5]. Following oral doses of 2 mg daily for 28 days given to seven subjects, a mean peak plasma concentration of 20 ng ml^{-1} (63.8 nmol l^{-1}) was reported [6]. According to the literature [7,8] toxic symptoms have been observed at serum concentrations of FNZ above 50 ng ml^{-1} (159.5 nmol l^{-1}).

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The still maintaining popularity of the benzodiazepines is mainly caused by their wide therapeutic index, minimal serious adverse reactions and the absence of undesirable autonomic nervous side effects especially when compared with formerly used anti-anxiety agents, e.g. meprobamate or phenobarbital [9]. The American Association of Poison Control Centers, in its 1994 annual report, cites benzodiazepines in third position (after analgesics and antidepressants) among the categories of substances with highest number of reported deaths [10].

Although benzodiazepines are well tolerated, prolonged regular use can lead to physical dependence which results from a kind of habituation, the functional counterparts of which become manifest during abstinence. With the long-acting benzodiazepines, this problem is less obvious, because of the delayed appearance of withdrawal symptoms. The severity of this syndrome is inversely related to the elimination half-life. There are indications that benzodiazepines with intermediate elimination half-lives, e.g. FNZ, lorazepam, bromazepam, are most frequently abused [11].

The structures of FNZ, its metabolites and triazolam (TRZ) are shown in Fig. 1. Abbreviations are explained in the paragraph of drug standards.

Numerous methods are currently available for the determination of FNZ in biological samples. Thin-layer chromatography (TLC) [12–14] (W. He, A. Heyndrickx, A two-dimensional thin-layer chromatography for determination of flunitrazepam and its metabolites, unpublished result), polarography [15], radioimmunoassay (RIA) [16], differential pulse voltametry (DPV) [17], and flow injection analysis (FIA) [18] methods have been described. By far the most of the published methods useful in clinical and forensic laboratories are utilizing gas chromatography (GC) [19–22] and high performance liquid chromatography (HPLC) [23–26].

This paper describes a developed HPLC method with a photo diode array detector (DAD) which facilitates either quantification or qualification of sample. The gradient elution provided a good separation of FNZ and its six metabolites. The solid phase extraction (SPE) was used for sample pretreatment, which may enable to extract large amount of sample in order to increase the sensitivity of determination.

2. Materials and methods

2.1. Liquid chromatography system and chromatographic conditions

A Hewlett Packard model 1090 M liquid chromatograph, equipped with a HP 1040 A DAD, was used. The system was controlled by a HP 79994 A HPLC work station which consists of HP 9000-300 computer, HP 9133 disc driver, HP 2225AB Thinkjet printer and HP 7440A Color Pro Graphics plotter. A Rheodyne injector with a 50 μl loop was fixed on the instrument. The column was a 100×3 mm i.d. ChromSep glass column packed with ChromSpher C8, 5 μm (Cat. No. 28262) protected by a 10×2 mm i.d. high efficiency reversed phase guard column (Cat. No. 28141) which was installed immediately before the analytical column by a ChromSep compressor, all from Chrompack (Antwerp, Belgium). The chromatographic condition was as follows: mobile phase A, methanol; mobile phase B, water containing 0.125% (v/v) of isopropylamine; gradient elution from 70 (v/v) to 45% (v/v) of B over a period of 8 min; and then isocratic elution of 45% (v/v) mobile phase B for 7 min; flow rate: 0.55ml min^{-1}; degassing with helium; column temperature, ambient; monitoring wavelength: 240 nm, band width: 10 nm with a reference wavelength: 550 nm, and band width 100 nm. After an equilibration time of about 60 min the first injection can be applied.

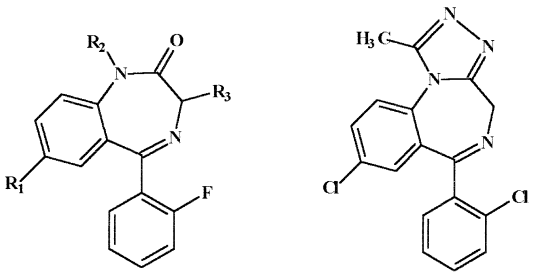

Fig. 1. Structures of FNZ, its metabolites and TRZ.

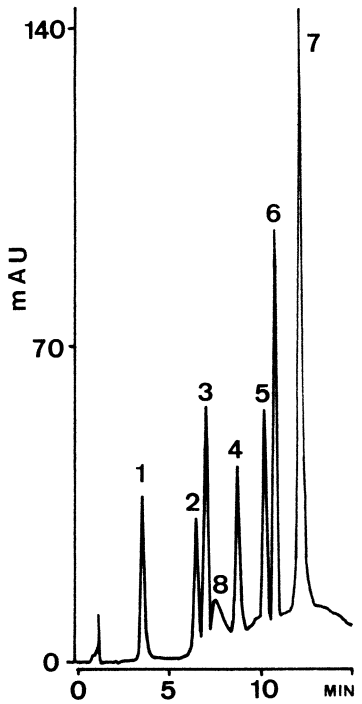

Fig. 2. Chromatogram of a test mixture detected at 240 nm: (1) ADF 100 ng; (2) AF 100 ng; (3) DF 200 ng; (4) ACF 100 ng; (5) HF 200 ng; (6) FNZ 200 ng; (7) TRZ 400 ng; (8) AHF 200 ng. Column: ChromSpher C8, 5 μm, 100 × 3 mm i.d.; gradient elution: methanol:water (0.125% isopropylamine) 30:70 (v/v) to 35:65 in 4 min, to 40:60 in 3 min, to 55:45 in 1 min; and then isocratic elution for 7 min; flow rate: 0.55 ml min^{-1}.

2.2. Reagents

2.2.1. Drug standards

FNZ 313.28 g mol^{-1}, 7-amino-flunitrazepam (AF) 283.30 g mol^{-1}, 7-amino-desmethyl- flunitrazepam (ADF) 269.27 g mol^{-1}, 7-acetamido-3-hydroxyl- flunitrazepam (AHF) 341.34 g mol^{-1}, 3-hydroxyl-flunitrazepam (HF) 329.28 g mol^{-1}, 1-desmethyl-flunitrazepam (DF) 299.26 g mol^{-1}, 7-acetamido-flunitrazepam (ACF) 325.34 g mol^{-1}, were gifts of Hoffmann-LaRoche (Basle, Switzerland and Brussel, Belgium). TRZ 343.21 g mol^{-1} was obtained from Upjohn (Kalamazoo, MI,).

2.2.2. Stock solutions

Stock TRZ, FNZ and its metabolite solutions (1 or 2 mg ml^{-1}) were prepared by dissolving the required amount of standards in methanol. These solutions were stored at 4°C and were found stable for several months. Less concentrated solutions were prepared by dilution of the stock solutions with methanol:water, 50:50 (v/v). Stored also at 4°C for maximum 2 weeks.

2.2.3. Buffer

The buffer solution (pH 11) was prepared by dissolving 8.4 g sodium bicarbonate and 4.6 g sodium hydroxide in water to 1 l.

2.2.4. Solvents and chemicals

All solvents and chemicals were of HPLC grade or of analytical-reagent grade, obtained from Acros (Geel, Belgium), UCB (Gent, Belgium) and Merck (Darmstadt, Germany). The enzyme β-glucuronidase (EC 3.2.1.31 G0876) crude solution was purchased from Sigma (St. Louis, MO), which was stored frozen at −20°C in the dark. Water was de-ionized, doubly distilled and stored in glass container.

2.3. Preparation of drug-free urine and plasma

Drug-free urine was collected from FNZ free volunteers. Drug-free plasma was provided by the blood bank of the university hospital (Gent, Belgium). The plasma can also be obtained from whole blood by centrifugation at 1500 rpm for 5 min. The urine and plasma were pooled, extracted and after analysis, no peaks corresponding to FNZ and its six metabolites were observed.

As soon as possible after collection, the urine/plasma was separated into 5 ml/2ml aliquots and stored in 15 ml glass tubes at −20°C until analysis.

2.4. Solid phase extraction procedure

A Sep-Pak C18 cartridge (1g of 6ml capacity) was activated with 2 ml of methanol and washed with 5 ml of water. After that, buffered aqueous sample was added, and the sorbent was washed with two times 5 ml of water. The elution was

Table 1
Retention times and linear regression results of FNZ and its metabolites in urine and plasma

Compound	T_R (min)	Urine ($n=5$)			Plasma ($n=5$)		
		Slope ± SE[a]	Intercept ± SE[a]	r^2 [b]	Slope ± SE[a]	Intercept ± SE[a]	r^2 [b]
ADF	3.79	$(5.59 \pm 0.29)10^{-3}$	$(-7.27 \pm 5.01)10^{-3}$	0.9975	$(2.25 \pm 0.13)10^{-3}$	$(-0.90 \pm 2.04)10^{-3}$	0.9930
ACF	9.09	$(13.3 \pm 0.79)10^{-3}$	$(-9.73 \pm 8.52)10^{-3}$	0.9984	$(2.56 \pm 0.19)10^{-3}$	$(5.57 \pm 2.93)10^{-3}$	0.9923
AF	6.84	$(4.67 \pm 0.25)10^{-3}$	$(-1.22 \pm 3.63)10^{-3}$	0.9992	$(2.11 \pm 0.16)10^{-3}$	$(-2.43 \pm 2.37)10^{-3}$	0.9904
AHF	7.93	—	—	—	—	—	—
DF	7.35	$(2.88 \pm 0.10)10^{-3}$	$(-12.1 \pm 4.16)10^{-3}$	0.9967	$(1.18 \pm 0.11)10^{-3}$	$(2.16 \pm 1.78)10^{-3}$	0.9936
FNZ	11.09	$(3.91 \pm 0.17)10^{-3}$	$(-3.59 \pm 6.91)10^{-3}$	0.9963	$(22.18 \pm 0.22)10^{-3}$	$(16.7 \pm 3.14)10^{-3}$	0.9914
HF	10.49	$(0.16 \pm 0.03)10^{-3}$	$(7.56 \pm 2.69)10^{-4}$	0.9992	$(0.10 \pm 0.01)10^{-3}$	$(2.68 \pm 0.15)10^{-3}$	0.9916
TRZ	12.51	—	—	—	—	—	—

The calibration curve was described as $y = bx + a$; y is the ratio of peak area of compound to internal standard; x is the concentration of compound in ng ml^{-1}.
[a] SE, standard error.
[b] r^2, determination coefficient.

performed with 2 ml of chloroform at constant flow rate (2 ml min^{-1}). The collection tubes were centrifuged at 2500 rpm for 2 min, and the chloroform layer was evaporated to dryness under nitrogen at about 40°C. The residues were reconstituted with 60 μl of methanol prior to their injection into the liquid chromatographic system or the tubes were closed before stored at −20°C.

2.5. Studies in spiked urine and plasma samples

To 5 ml of urine, a certain amount of FNZ and its metabolites were added and the pH was adjusted to 5 with 1 M of acetic acid or 1 M of sodium acetate. A 0.2 ml of β-glucuronidase crude solution was added to each sample, stoppered and mixed well. The sample was then incubated at 37°C for 12 h. Thereafter, a 2 μg of internal standard was added. The urine sample was alkalized with 1.5 ml of buffer (pH 11), and applied to Sep-Pak cartridge column. The procedure was the same as mentioned above in 'SPE procedure'.

A 2 ml of plasma was spiked with FNZ, its metabolites, 2 μg of IS and 4 ml of acetonitrile (ACN) to precipitate the proteins. After vortex-mixing for 15 s, the sample was centrifuged at 1500 rpm for 10 min. The supernatant was then transferred to a clean tube and 10 ml of buffer (pH 11) was added to decrease the percentage content of ACN. The solution was mixed well on a vortex mixer and transferred on a Sep-Pak cartridge column.

3. Results

3.1. Chromatography

Under the described chromatographic conditions, FNZ and its metabolites are well separated. Note that at 7 min, a step gradient elution starts and so baseline increases. Fig. 2 shows the chromatographic separation of these compounds after injecting directly a mixture containing 100–200 ng per 50 μl of each of them. The retention times are shown on Table 1.

3.2. Linearity and assay detection limits

The linearity for FNZ, DF and HF was checked in the concentration range 25–500 ng ml^{-1} plasma, 20–400 ng ml^{-1} urine and for ADF, AF and ACF in the concentration range 12.5–250 ng ml^{-1} plasma and 10–200 ng ml^{-1} urine. Response ratio of peak areas between the corresponding compound and the internal standard versus theoretical concentration was fitted by

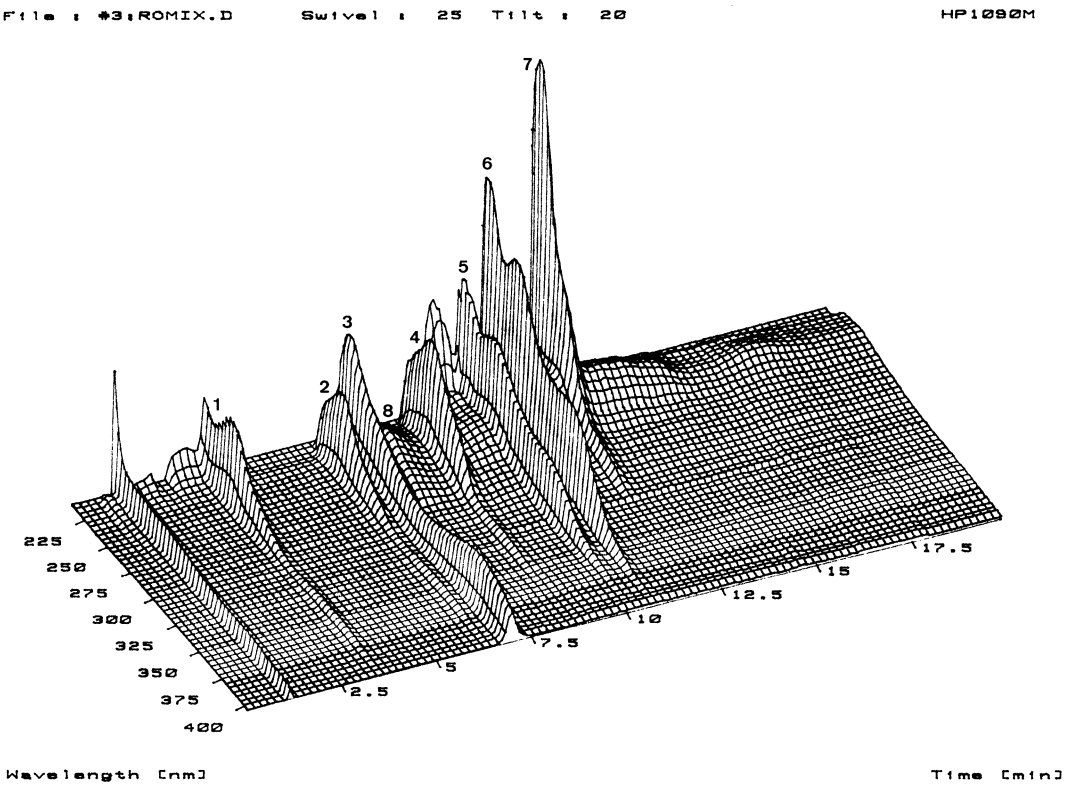

Fig. 3. The 3D chromatogram of FNZ and its metabolites obtained under the same conditions as in Fig. 2. UV detection over the range of 210–400 nm.

a least-squares linear regression to the equation: response ratio $(y) = \text{slope}(b) \times \text{concentration}(x) + \text{intercept}(a)$.

The calibration curves (Table 1) were obtained from spiked urine and plasma samples at five different added compound concentrations with $n = 5$ determinations for each point. The determination coefficient (r^2) of the calibration lines and the day to day standard error in the slopes and the intercepts are also given.

The limit of detection (qualitative), L_d, and the limit of quantitation, L_q, were obtained by use of the slope (b) and the standard error of the intercept (SE_a) of the regression line [27]. The limit of detection, calculated from $y - a = 3SE_a$ and $y - a = b \cdot L_d$, for ADF, AF, HF, DF, ACF and FNZ was $L_d = 2.72$, 3.37, 4.50, 4.53, 3.43, 4.32 ng ml^{-1} plasma and 2.69, 2.33, 5.04, 4.33, 1.92, 5.30 ng ml^{-1} urine, respectively.

The limit of quantitation, calculated from $y - a = 10SE_a$ and $y - a = b \cdot L_q$, was $L_q = 9.07$ (33.68), 11.23 (39.64), 15.00 (45.55), 15.08 (50.39), 11.45 (35.19), 14.40 (45.97) ng ml^{-1}(nmol l^{-1}) plasma and 8.96 (33.28), 7.77 (27.43), 16.81 (51.05), 14.44 (48.25), 6.41 (19.70), 17.67 (56.40) ng ml^{-1} (nmol l^{-1}) urine, respectively.

The compound of AHF (peak 8 in Figs. 2 and 3), has very weak UV absorption at 240 nm. If it is necessary to be quantified, a more sensitive wavelength has to be selected, i.e. 248 nm.

3.3. Precision

Precision of a quantitative method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings. It is measured by injecting repeatedly a ready-made sample pool and expressed as coefficient of variation of the results.

Table 2
Precision of the determination

Compound	Concentration in urine	Urine CV (%)		Concentration in plasma	Plasma CV (%)	
	ng ml^{-1} (nmol l^{-1})	Intraday ($n=6$)	Interday ($n=5$)	ng ml^{-1} (nmol l^{-1})	Intraday ($n=6$)	Interday ($n=5$)
ADF	50 (185.5)	3.09	5.44	125 (463.75)	5.13	7.27
ACF	50 (153.5)	3.02	3.04	125 (383.75)	8.45	10.46
AF	50 (176.5)	3.53	3.90	125 (441.25)	6.30	9.42
DF	100 (334.0)	3.27	3.94	250 (835.00)	6.45	8.53
FNZ	100 (319.0)	2.04	2.64	250 (797.50)	6.50	9.23
HF	100 (304.0)	9.07	14.10	250 (760.00)	8.60	9.32

The intraday precision (or repeatability) was evaluated by replicate analysis ($n=6$) of pooled urine (containing 50 ng ml^{-1} for ADF, AF and ACF, or 100 ng ml^{-1} for HF, DF and FNZ) and pooled plasma (containing 125 ng ml^{-1} for ADF, AF and ACF or 250 ng ml^{-1} for HF, DF and FNZ). The analysis was carried out in one laboratory by one operator, using the same reagents and instruments over a relatively short time span.

The interday precision (or reproducibility) is defined as the long-term variability of the measurement process which here was determined from the same pooled urine and plasma samples as above, analyzed on 5 different days over a 2 week period. The CVs are shown in Table 2.

3.4. Accuracy/recovery

The accuracy of the method is defined as the degree of agreement of test results generated by the method to the true value. The analytical recovery was used to assess the accuracy and it was measured by spiking drug-free urine and plasma samples with known concentrations of the standards. After the extraction of the analyte from the matrix and injection onto the analytical instrument, its response was compared with the response of the standard injected directly to the column, which gives the analytical recovery. The internal standard was added to the final injection solvent just before injection. The recovery of internal standard was evaluated using FNZ as internal standard. As it can be seen in Table 3, initially the recoveries in urine were better than in plasma, but the latter results improved after de-proteinization.

3.5. Selectivity

Selectivity of an analytical method is its ability to measure accurately an analyte in the presence of interference that may be expected to be present in the sample matrix.

In Fig. 4, where we examined the chromatographic blanks from urine and plasma, in the expected time windows of the analytes we found a lack of response for $n=10$ independent sources of the same matrix.

Also peak homogeneity could be demonstrated by plotting the absorbance ratio, or by calculating the area ratio, of two signals acquired at two different wavelengths.

This method also allows the determination of other benzodiazepines. The most of them will be eluted later than 10 min by this gradient program except bromazepam, clonazepam and chlordiazepoxide with retention times 8.61, 7.55 and 9.27 min, respectively.

4. Discussion

FNZ has only two pharmacologically active metabolites, AF and DF which appear in blood or plasma [28]. The urinary excretion of unchanged FNZ is less than 1%. To be able to

Table 3
Percentage absolute recoveries of FNZ and its metabolites in urine and plasma by SPE

Compound	Concentration in urine (ng ml^{-1})	Recovery (%)		Concentration in plasma (ng ml^{-1})	Recovery (%)			
		Urine % ± SE[a]	*n*		Plasma % ± SE[a]	*n*	Plasma[b] % ± SE[a]	*n*
ADF	10 20 50 100 200	74.69 ± 4.43	20	12.5 25 50 100 250	41.35 ± 3.24	20	77.51 ± 4.51	20
ACF	10 20 50 100 200	84.42 ± 3.76	20	12.5 25 50 100 250	46.79 ± 4.68	20	75.61 ± 3.05	20
AF	10 20 50 100 200	105.02 ± 4.33	20	12.5 25 50 100 250	61.15 ± 7.05	20	92.05 ± 3.67	20
DF	20 40 100 200 400	99.27 ± 7.18	20	25 50 100 250 500	59.30 ± 3.59	20	86.11 ± 2.73	20
FNZ	20 40 100 200 400	83.88 ± 3.13	20	25 50 100 250 500	65.25 ± 4.39	20	96.73 ± 2.19	20
HF	20 40 100 200 400	64.29 ± 7.23	15	25 50 100 250 500	35.74 ± 3.78	15	72.39 ± 4.38	15
TRZ	400	96.62 ± 6.61	20	1000	41.26 ± 3.03	20	88.29 ± 5.02	20

[a] Standard error.
[b] With de-proteinization.

correlate the effects or symptoms with the plasma concentrations we must take into account not only the main compound but also its active metabolites. It has to be kept in mind that only the free-drug is pharmacologically active.

In urine, the metabolites of FNZ are present mainly as glucuronide conjugates which have high polarity. Usually two hydrolysis procedures, acidic or enzymatic, can be used on the conjugates prior to their extraction. Benzodiazepines are converted to benzophenones by acid hydrolysis and this may give difficulties to identify the parent compounds. Hence the enzymatic hydrolysis was chosen to obtain the free benzodiazepines at the optimal conditions of pH 5 and at 37°C for about 12 h.

Benzodiazepines are known to bind the protein albumin but mainly on α-glycoprotein. About 77–80% of the absorbed FNZ is bound to plasma proteins [29]. However organic solvents miscible with water, e.g. ACN can lower the solubility of proteins, precipitate them from aqueous solutions and render them removable by centrifugation [30]. This influence can be seen on Table 3.

In this SPE method, chloroform was chosen for elution in order to be easily separated from the aqueous phase. The extract was found clean and containing less hydrophilic impurities compared to a methanol extract (compare Figs. 4 and 5). However, it decreased the recoveries of AHF and HF.

Chromatographic methods have the advantage to detect and to quantitate the whole spectrum of effective compounds and metabolites in a single run under identical conditions. Besides an improved interpretation, unexpected drugs can be detected and incorrect declarations concerning medications can be corrected. HPLC offers the advantage to separate and quantitate the compounds without derivatization and at low temperature, that is without the risk of decomposition.

It is known that the combination of HPLC with DAD is considered as a highly effective screening method. Criterion for identification of the analyte is that the maximum absorption wavelength in the UV spectrum of the analyte should be the same as that of the standard material within a margin determined by the resolution of the detection system which for DAD is typically within $\pm$2nm. The use of the photo DAD gives also the advantage of identifying the analytes both by retention time and UV spectrum (Fig. 3).

The bonded silica sorbent product in the analytical column, is stable within a pH range of approximately 2–8. Siloxane linkages are cleaved below pH 2 while at pH above 8 silica substrate is susceptible to dissolution in aqueous solutions. Nonetheless, in practice bonded silica may be used in a pH above 8, since degradation of the sorbent is a finite process and if a guard column packed with microparticulate silica is included to saturate the eluent before it enters the analytical column. A guard column can considerably prolong the life-time of the analytical column [31,32].

The mobile phase containing no buffer but isopropylamine for the modification of pH can avoid some problems which are often happening in HPLC assay when the buffer is used, such as crystal formation in connecting tubing and detector cell, as well as damage of the pump seals. By this mobile phase, the analytical column can run for 400–500 injections with a stable retention time and acceptable column efficiency.

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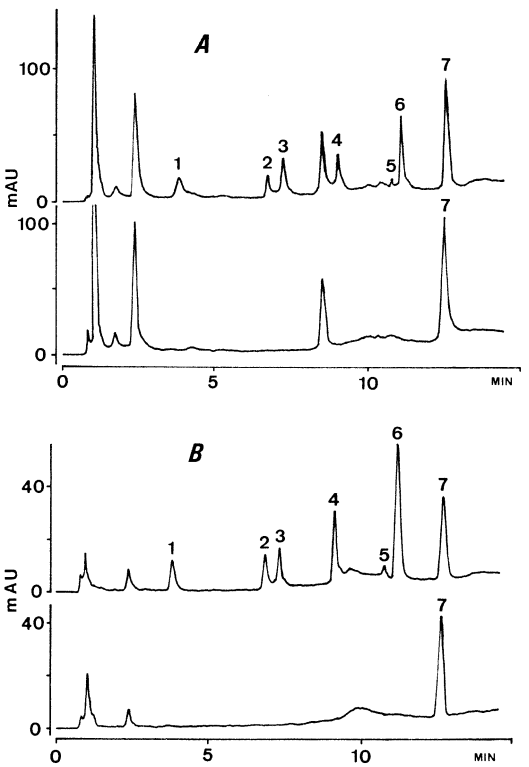

Fig. 4. HPLC separation of FNZ and its metabolites of 100–200 ng in urine (A) and plasma (B) after Sep-Pak C18/chloroform extraction. (1) ADF; (2) AF; (3) DF; (4) ACF; (5) HF; (6) FNZ; (7) TRZ. Chromatographic condition: the same as in Fig. 2. (A) Chromatograms of spiked urine (top) and blank urine (bottom) after enzymatic hydrolysis and SPE. (B) Chromatograms of spiked plasma (top) and blank plasma (bottom) after SPE.

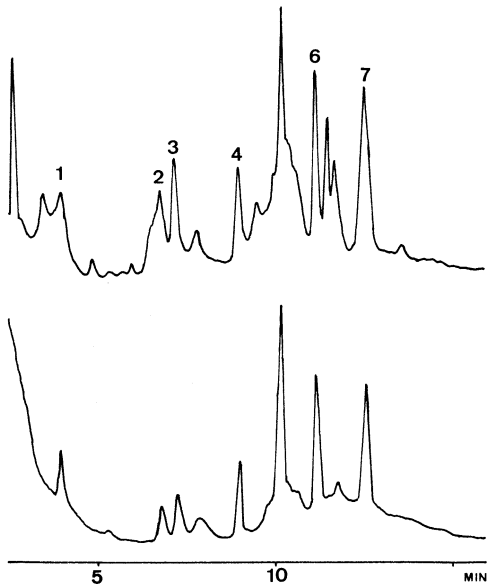

Fig. 5. Chromatograms of 100–200 ng of FNZ and its metabolites spiked in urine (bottom) and plasma (top) after Sep-Pak C18/methanol extraction. (1) ADF; (2) AF; (3) DF; (4) ACF; (5) HF; (6) FNZ; (7) TRZ. Chromatographic condition: the same as in Fig. 2.

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