ANALYTICAL CHARACTERIZATION OF FLUNITRAZEPAM

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Abstract

Supported by personal research and literature data, a comprehensive physical, chemical and analytical characterization of flunitrazepam is presented. It includes physical constants, mass, IR and UV spectra, thermal analysis data, acid-base properties in solution and most representative methods for the assay of flunitrazepam.

Rezumat

Este prezentată o caracterizare cuprinzătoare a flunitrazepamului din punct de vedere fizic şi chimico-analitic, pe baza cercetărilor proprii şi a datelor din literatură. Sunt cuprinse constante fizice, spectrele de masă, IR, UV analiza termică, proprietăți acido-bazice în soluție şi metodele de dozare reprezentative.

Keywords: flunitrazepam; analytical properties; assay methods

Introduction

Flunitrazepam, a psychotropic fluorinated nitro-benzodiazepine, was synthesized in 1963 in Hofmann La Roche Laboratories. Its hypnotic effect is very rapid and much more important than other actions, specific for all benzodiazepines, as anxiolytic, sedative and muscle–relaxant [1-3].

It is frequently used in the treament of clinically significant sleep disorders and as pre-medication in anaesthesia and intensive care [4], but during the last decades the use without prescription of flunitrazepam raised markedly, compared to other benzodiazepines. It was reported that, due to its inodore and tasteless solutions, flunitrazepam is used to reduce the defensing ability of rape or assault victims (*date rape drug*) [5, 6].

Due to its high therapeutic efficiency (5-15 μg/L active doses, while toxic effects occur at 50 μg/L or higher plasmatic levels [7]), rapid metabolisation and very low concentrations of major metabolites (7-amino-flunitrazepam and N-demethyl-flunitrazepam, highly selective and sensible methods, such as GC, HPLC, GC-MS, HPLC-MS&MS are used for

flunitrazepam detection and quantification in bulk, pharmaceutical dosage forms and biological liquids [8]).

The most important ways in flunitrazepam metabolism are N-demethylation, 3-hydroxilation and glucuronoconjugation, as well as reducing nitro moiety to amino, followed by acetylation (Fig. 1) [5].

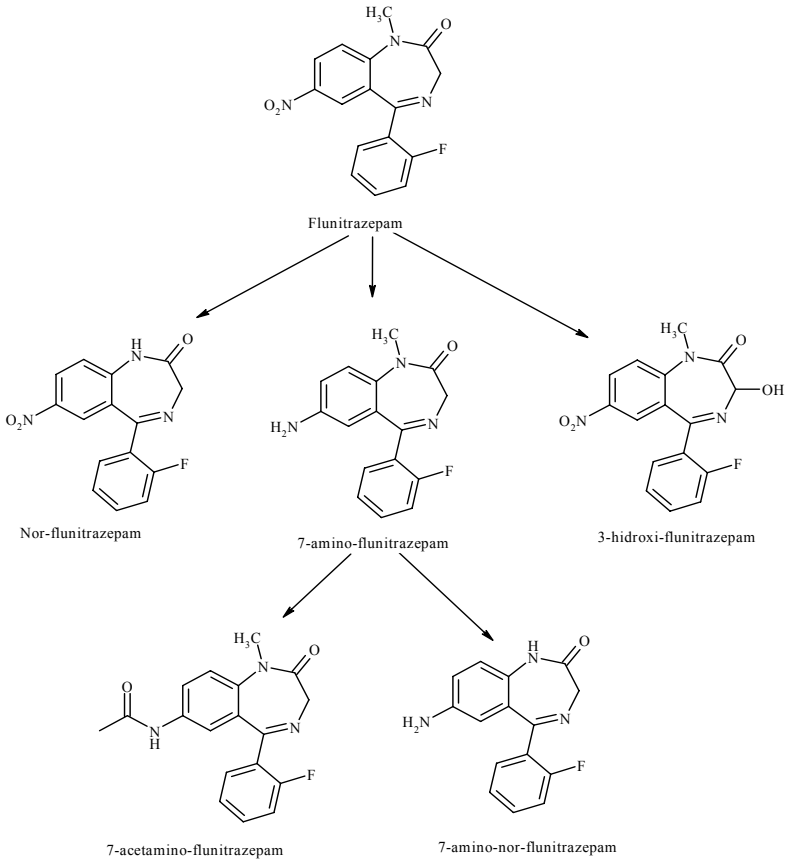


Figure 1
The metabolism of flunitrazepam

Description

Nomenclature

Chemical name:

5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepine-2-one

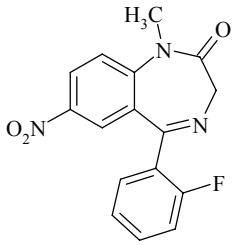
International nonproprietary name:

Flunitrazepamum

Proprietary names

Absint, Darkene, Flunimerck, Flunipam, Flupam, Fluscand, Fluserin, Flutraz, Hypnodorm, Hypnor, Insom, Narcozep, Rohipnol, Rohypnol, Roipnol, Ronal, Somnubene, Valsera [1].

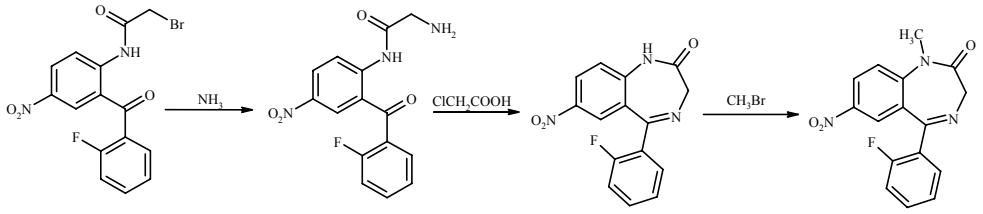
Structure, chemical formula and molecular mass



$C_{16}H_{12}FN_3O_3$; M_r= 313.3

Synthesis

One of the specific synthesis routes for flunitrazepam is presented further [8]:



Physical properties

Flunitrazepam is a white or slightly yellow crystalline substance. Different melting points were reported, depending on the solvent used for crystallization: slightly yellow needles, crystallized in dichloromethane-hexane, melt at 166-167°C, crystals obtained in acetonitrile and methanol have a melting point 170-172°C [10].

Flunitrazepam is practically insoluble in water, soluble in acetone, ethanol (1:172), methanol (1:100), chloroform (1:3) and diethyl-ether (1:300) [11].

IR spectrum

The IR absorption spectrum of flunitrazepam was recorded in a KBr disc (1mg substance in 400 mg KBr), using a Bruker spectrometer (Fig. 2).

The main absorption bands were attributed (Table I).

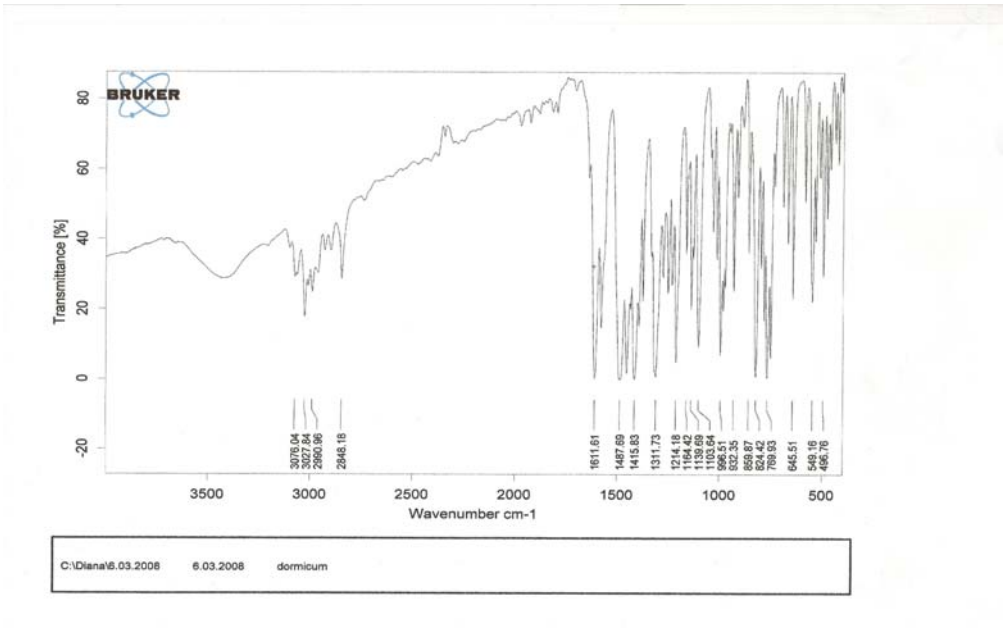


Figure 2
IR absorption spectrum for flunitrazepam

Table I
Main absorption bands in the IR absorption spectrum of flunitrazepam

Wave number (cm^{-1})	**Attribution**	**Notes**
3110	$\upsilon_{Caril-H}$ (aromatic ring)	
3081	$\upsilon_{Caril-H}$ (aromatic ring)	
3003	$\upsilon_{Caril-H}$ (aromatic ring)	
2952	$\upsilon_{asimCH3}$	
2922	$\upsilon_{asimCH2}$	
2860	υ_{simCH3}, υ_{simCH2}	
1686	$\upsilon_{C=O}$ amidic	
1523	$\upsilon_{asimNO2}$	
1336	υ_{simNO2}	
1610	$\upsilon_{C-C)aril}$	Possible overlapping of $\upsilon_{C=N\ endociclic}$
1581	$\upsilon_{C-C)aril}$	
1483	$\upsilon_{C-C)aril}$	

Mass spectrum

The most important ions, based on *m/z* ratio are: 285, 312, 313, 286, 266, 238, 294, 284; 7-amino-1-demethyl-flunitrazepam: 269, 240, 241, 268, 270, 107, 121, 213; 7-amino-flunitrazepam: 283, 44, 255, 282, 254, 284, 264, 256; demethyl-flunitrazepam: 298, 271, 299, 224, 272, 270, 252, 280 [11].

UV absorption spectrum

UV absorption spectra were recorded, both for chloroformic and ethanolic solutions, using a Perkin Elmer Lambda 2 UV-Vis spectrometer.

UV absorption spectrum of the midazolam solution prepared in chloroform shows two absorption maxima, at 255.5 and 315 nm ($A^{1\%}_{1cm}$ = 339.85) (Fig. 3), while the solution obtained with ethanol shows characteristic maxima at 219.5 nm ($A^{1\%}_{1cm}$ = 805), at 253 nm ($A^{1\%}_{1cm}$ = 528) and at 309 nm ($A^{1\%}_{1cm}$ = 338) (Fig. 4).

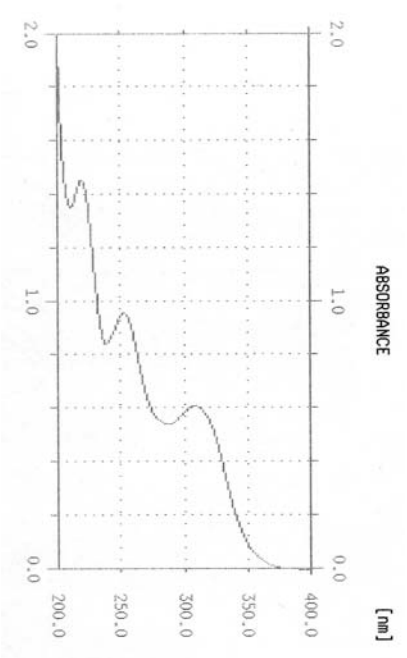


Figure 3
UV absorption spectrum for $1.2 \cdot 10^{-5}$ g/mL flunitrazepam solution, prepared with chlorophorm

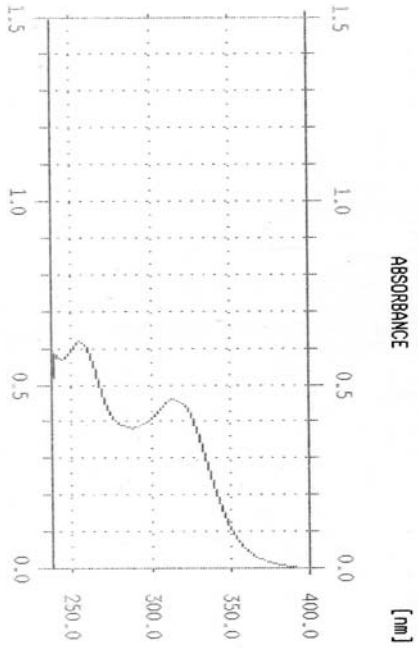


Figure 4
UV absorption spectrum for $1.8 \cdot 10^{-5}$g/mL flunitrazepam solution, prepared with ethanol

Thermal behaviour of flunitrazepam was investigated through TG, DTG and TDA, using a Nicolette equipment, aluminum crucible (a sample

of 4.87 mg); the temperature range was 20-700°C and 60-200°C, respectively, and the heating rate used was 10°C/min.

The unchanged weight of the sample until 185.6°C (TG and DTG curves, Fig. 5) shows thermal stability of this substance.

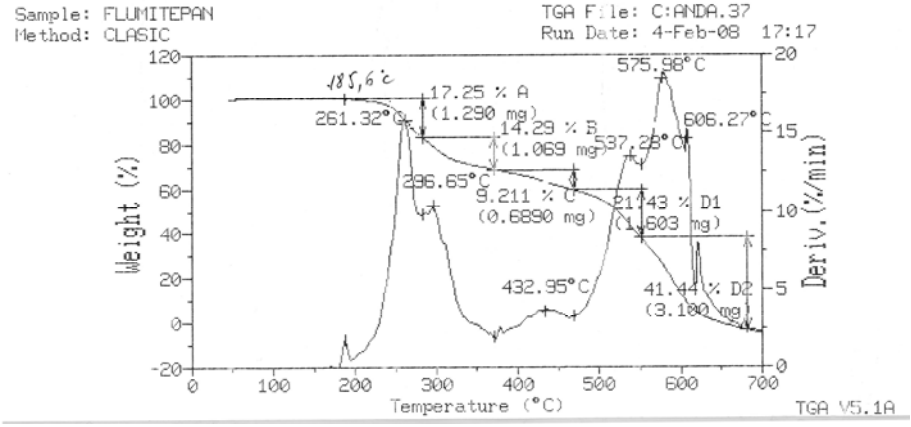


Figure 5
TG and DTG curves for flunitrazepam

The endothermal peak on the DSC curve indicates the melting of flunitrazepam at 170.9°C, with a 95.05J/g thermal effect (Fig. 6).

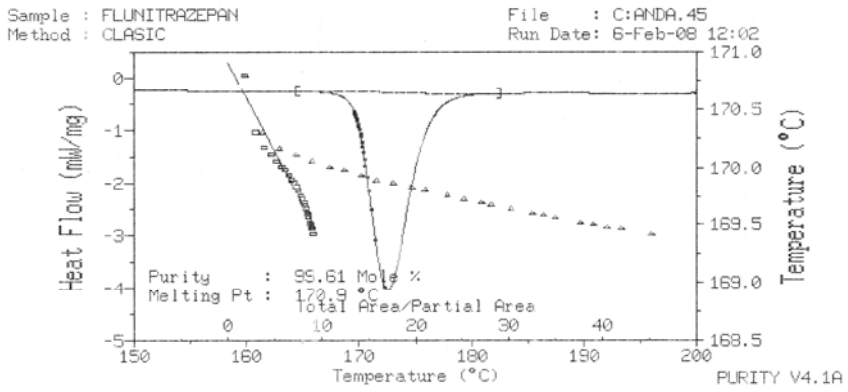


Figure 6
DSC curve for flunitrazepam

The thermal behaviour of flunitrazepam (TG, DTG and DSC curves) shows also its stability to oxidation.

Partition coefficient

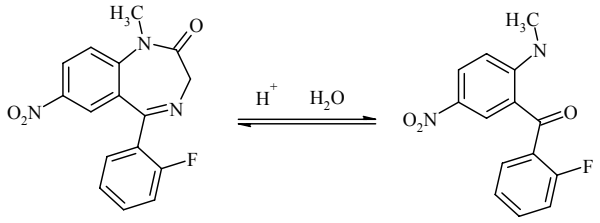
Partition coefficient of flunitrazepam in a *n*-octanol/water system ($K_{octanol/water}$), at room temperature is aproximatively $1.3 \cdot 10^2$ [11]. Distribution coefficients at membrane level were also determined as follows: sinaptic membrane (P = 18.5±0.8) [16] and heterogenous vesicle-like systems phosphatidyl-choline/water [17].

Chemical properties

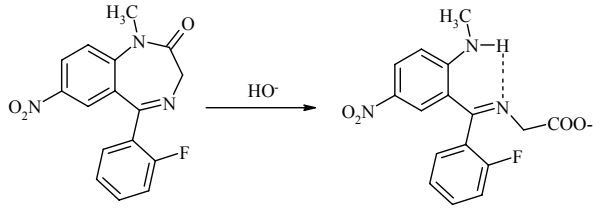
Acid-base properties in solutions

Flunitrazepam is a very weak base, due to the nitrogen atom in position 4 only, as the other atom in position 1 is methyl substituted. The electron withdrawing fluorine substituent on the phenyl ring has a diminishing effect on flunitrazepam protonation ability, $K_p = 1.32 \cdot 10^{-2}$ [13].

In acidic solutions hydrolysis to respective benzophenone occurs. At pH lower than 1, hydrolysis is complete; when the solution is neutralized, the ring closes again, as follows:



When the methanolic solution of flunitrazepam reacts with aqueous NaOH 8.5% solution, an intense yellow colour occurs, due to iminic derivative hydrolysis, as follows:



Assay methods

Assay of constituting elements

The quantitative determination of carbon, nitrogen and hydrogen in the structure of flunitrazepam was performed using a Perkin Elmer 2400 series II CHNS/O elemental analyser. The obtained values, compared to the theoretical ones, are presented in Table II:

Table II
The results in the assay of constituting elements

	C	H	N
Calculated	61.34	3.86	13.41
Found	60.98	3.92	13.84

Protometric titration in anhydrous medium

Due to its basic character, flunitrazepam can be quantified, using a mixture of anhydrous acetic acid and acetic anhydride as titration medium, in the presence of perchloric acid. The equivalence point is determined potentiometerically [12].

Spectrometric assay in visible

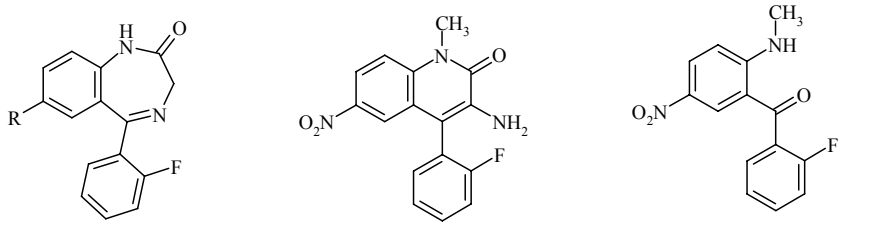
The spectrometric methods in visible quoted in literature are indirect procedures to determine flunitrazepam, through derivation, in the presence of different reagents. Thus a simple and precise method for the determination of some benzodiazepines as diazepam, prazepam and flunitrazepam is based on the reaction with 1,3-dinitrobenzene in the presence of tetraethylamonium hydroxide. The resulted purple solution has a characteristic absorption maximum at 590 nm [14].

Chromatographic determination

Thin layer chromatographic (TLC) methods

Flunitrazepam was quantified and identified through thin layer chromatography and densitometry, using kieselgel GF254 as a stationary phase and a mixture of chloroform and acetone 9/1 (v/v) as a mobile phase.

In the European Pharmacopoeia, 4th Edition [12], the flunitrazepam monograph indicates an official TLC method for the assay and identification of flunitrazepam and related substances. Main chemically related impurities of flunitrazepam are presented in Fig. 7.



R : $-NH_2$

7-amino-5-(2-fluorophenyl)-1,3-dihydro--2H-1,4-benzodiazepine-2-one

R : $-NO_2$

5-(2-fluorophenyl)-7-nitro-1,3-dihydro--2H-1,4-benzodiazepine-2-one

3-amino-4-(2-fluorophenyl)-1-methyl--6-nitroquinoline--2(1H)-one

(2-fluorophenyl)[2-(methylamino)--5-nitrophenyl]methanone

Figure 7
Chemically related impurities of flunitrazepam

The detection of flunitrazepam is made by comparing the sample chromatogram to the chromatogram of the reference substance. As adsorbent, silica gel GF_{254} is used, and a mixture ethyl-acetate/nitromethan 15/85 (v/v) is the mobile phase.

A TLC method for the assay of flunitrazepam and its metabolites in urine is quoted; after hydrolysis, fluorescent acridinic derivatives resulted are detected. The main interfering species are diclofenac, carbamazepine and tricyclic anti-depressants [15].

Gas-chromatographic methods

Flunitrazepam in various biological materials was determined by gas-chromatography [18- 20]. Thus, it was determined in plasma and urine, after solid phase microextraction (DL_{urine} 0.01 to 0.45 μmol/L and DL_{plasma} 0.01-0.48 μmol/L), in blood, with its N-desmethylated metabolite, using electron-capture detection (QL 0.5-1.0 ng/mL).

Frequently, flunitrazepam detection in gas-chromatographic methods is mass-spectrometric. Flunitrazepam was determined in urine, after solid phase or liquid-liquid extraction, with its metabolites (3-hydroxi-flunitrazepam, 7-amino-flunitrazepam, 7-amino-3-hydroxi-flunitrazepam, desmethyl-flunitrazepam, 3-hydroxi-desmethyl-flunitrazepam, for the latter ones DL is lower than 1μg/L), after hydrolysis with glucuronidase, using oxazepam-d_5 as internal standard, with 7-amino-flunitrazepam, after extraction with organic solvent, followed by derivatization with methyl-bis-trifluoroacetamide, with its metabolites, 7-amino-flunitrazepam and N-desmethyl-flunitrazepam, after solid phase extraction, with 7-amino-flunitrazepam and desmethyl-flunitrazepam, after enzymatic hydrolysis with β-glucuronidase, followed by solid phase extraction and using N-methyl-clonazepam as internal standard, with a detection limit from 13 to 30 ng/mL. In serum, flunitrazepam and 7-amino-flunitrazepam were simultaneously determined through GC-MS/MS, after derivatization of 7-amino-flunitrazepam using trifluoracetic anhydride]. Several benzodiazepines, among them flunitrazepam, and their hydroxylated and desmethylated metabolites were determined in plasma (DL 1.5 ng/mL for benzodiazepines) after extraction using butyl acetate at pH 9; hydroxylated metabolites were derivatized with N,O-bis(trimethylsilyltrifluoroacetamide). Flunitrazepam and its metabolites were determined in blood and dry blood stains, after solid phase extraction and derivatization with pentafluoropropionic anhydride and silylation followed by the reaction with N(tbutyldimethyl-silyl)-N-methyltrifluoroacetamide, flunitrazepam and its major metabolite 7-amino-flunitrazepam in blood, after acidic hydrolysis, extraction and derivatization

using heptafluorobutirate, followed by the resulted benzophenones derivatization. Flunitrazepam and a large number of psychotropic drugs were determined in oral fluids, using as internal standards deuterated analogues of morfine, 3,4-methylendioxymetamfetamine, 11-nor-9-carboxi-delta-9-tetrahydrocanabinol and clonazepam, after solid phase extraction. Flunitrazepam was determined in hair after decontamination with methylen chloride, incubation in Sorensen buffer pH 7.6, using diazepam as internal standard, followed by liquid extraction with ethylic ether/chlorophorm 80/20 and derivatization with heptafluorobutylaldehide or with heptafluorobutiric anhydride.

Though sensibility and sensitivity of the GC-MS methods are suitable for flunitrazepam detection and assay, their major limit is a longer time for the analysis due to the prior metabolites derivatization.

HPLC methods

HPLC methods in flunitrazepam detection and assay are very numerous, especially for biologic fluids, as they allow simultaneous separation of flunitrazepam and its metabolites, usually without any derivatization [21- 24].

The specificity of the HPLC methods using UV detection (Table III) is lower than that of the methods using mass-spectrometric detection.

HPLC-MS methods having a higher sensitivity and sensibility are more suitable for flunitrazepam and its metabolites detection in biologic fluids. The most reprezentative HPLC-MS methods are presented in Table IV.

Recently, there have been reported micellar electrokinetic chromatographic methods. Thus, flunitrazepam and 3 of its major metabolites were separated through electrokinetic chromatography, using capillary columns, 25 kV; detection 220 nm and the microemulsion system consisted of octan 70mM, 1-buthanol 800 mM, SDS 80 mM and borate buffer 10 mM, pH 9. The effect of the mobile phase composition, pH, applied field and temperature were studied. Eight benzodiazepines, among them flunitrazepam, were separated through capillary electrokinetic chromatography, using tetraborate buffer 25 mM (pH 9.5), SDS 50 mM and methanol (at least 12%, organic modifier). This method was used in order to study benzodiazepines stability in acidic media.

It was also used a scanning method coupled with MECK to determine flunitrazepam and its metabolites 7-amino-flunitrazepam and N-desmethyl-flunitrazepam. A 25 mM borate buffer pH 9.5, 50 mM cetyltrimethylamonium bromide and MeOH 30% were used. DL for flunitrazepam and its 2 metabolites were, respectively, 13.4; 5.6; 12.9 ng/mL.

Table III

The characteristics of chromatographic systems used to determine flunitrazepam in different types of biological samples through HPLC with UV detection

Analites	Matrix	Sample preparation	Column	Mobile phase	Detection	Internal standard	DL	QL
1	2	3	4	5	6	7	8	9
Flunitrazepam	Plasma, urine	$ZnSO_4$ deproteination	C_{18}	MeCN/MeOH/phosphate buffer pH3 1/25/6,5	260 nm			
Flunitrazepam	Plasma	The internal standard is added in a basic solution, extraction with ethylic ether/methylen chloride 2/1 (v/v)	Novapak C18	Acidic buffer /MeCN/MeOH 64/23/13	242 nm	Norprazepam		
Flunitrazepam	plasma		Novapak C18	Water/MeCN/TEA 700/300/4, adjusted to pH 7.5 with H_3PO_4			5 ng/mL	
Flunitrazepam, 7-amino-flunitrazepam, desmethyl-flunitrazepam, 7-amino-desmethylflunitrazepam, 3-hydroxi-flunitrazepam	Plasma	Extraction with ethylic ether/chlorophorm 80/20, after adjusting the pH to 9.5	RSIL CN		242 nm	Prazepam	2.5-5 µg/L	10 µg/L
Flunitrazepam and metabolites	Plasma and urine	SPE	Chromspher C18	Gradient elution A: MeOH B: isopropilamine in water 0.125%	240 nm	triazolam		
Flunitrazepam, nor-flunitrazepam, 7-amino-flunitrazepam, 7-amido-flunitrazepam	Serum, urine, plasma		Lichrospher 60 RP Select B	MeCN/ Phosphate buffer 0.02M pH 2 36/64	254 nm	Metilclonazepam	1 ng/mL	
Flunitrazepam	Blood, serum	Adjusting to basic pH, extraction using 1-chlorobutan	Semimicrocolumn C8		220 nm	Metilclonazepam		

(Continued)

Table III (Continued)

1	2	3	4	5	6	7	8	9
Flunitrazepam	Blood	Deproteination using a mixture of MeOH/MeCN 50/5, centrifuge, organic phase drying and sample solution is prepared using mobile phase as diluent	Semimicrocolumn C8 Lichrospher Select B	Phosphate buffer 20 mM pH2.2 MeCN 70/30	254 nm			
Flunitrazepam	Human hair	The sample is incubated 2 hours at 45 C, a mixture of MeOH and NH_3 is added, sonication, centrifuge and methanolic phase is dried out and the residue is dissolved in mobile phase	Semimicrocolumn C8 Lichrospher Select B	Phosphate buffer 20 mM pH2.2 MeCN 94/6	254 nm			
Flunitrazepam	Serum	Serum with internal standard + phosphate buffer 0.1M pH 6, hydrolysis with β-glucuronidase, 2 hours incubation at 55 C, solid phase extraction	C2	Gradient elution A:TP 0.02M pH 2.5/ B: MeCN min / A / B 0 / 85 / 15 10 / 65 / 35 20 / 50 / 50 30 / 50 / 50	UV	Lorazepam		

(Continued)

Table III (Continued)

1	2	3	4	5	6	7	8	9
Flunitrazepam	Blood	Extraction using a mixture of *n*-hexan/ethyl acetate 7/3	LichroCAR T125-4 Alusphere RP Select B	Gradient elution A:NaOH 0.0125M in MeOH B: aqueous NaOH 0.0125M Min A B 0 10 90 5 10 90 20 90 10 25 90 10	230 nm 242 nm			
Flunitrazepam, clonazepam, diazepam, midazolam, oxazepam	Plasma, serum	Direct injection of the sample in the Biotrap 1500MS (hidrophobic polimer) column, after washing with phosphate buffer/MeCN, (*column switching technique*)	Lichrospher Select B C8	MeCN/phosphate buffer				
Flunitrazepam	Plasma	Preconcentration on different columns (*column switching technique*)	Lichrospher Select B RP8	MeCN/ phosphate buffer 20 mM pH 2.1 35/65				
Flunitrazepam	blood	Liquid extraction using n-butyl-chloride	Chromolith^{TM} RP performance 18a	Phosphate buffer 35 mM pH 2.1/MeCN 70/30				
Flunirazepam, simultaneously with droperidol	Plasma	SPE	C18 Novapak	MeCN/CH_3COONH_4 10 mM (pH 6.7) 45/55	250 nm	nitrazepam	10 µg/L (flunitrazepam)	

Table IV

The characteristics of chromatographic systems used to determine flunitrazepam in different types of biological samples through HPLC with MS detection

Analites	Matrix	Sample preparation	Column	Mobile phase	Internal standard	DL	QL
1	2	3	4	5	6	7	8
Flunitrazepam, 7-amino-flunitrazepam, N-desmethyl flunitrazepam, 3-hydroxi-flunitrazepam	blood	SPE	ODS	MeCN/HCOONa 50 mM pH 3 45/55	Flunitrazepam d_3, 7-amino-flunitrazepam d_3	0,2 µg/L (flunitrazepam, 7-amino-flunitrazepam) 1 µg/L (N-desmethyl-flunitrazepam, 3-hydroxi-flunitrazepam)	
Flunitrazepam	Blood, urine	Enzimatic hydrolysis of urine: blood	C18	$MeOH/H_2O/NH_3$ 60/40/0,03			
Flunitrazepam, 7-amino-flunitrazepam, 7-acetamino-flunitrazepam, desmethyl flunitrazepam	Plasma	Column Biotrap 1500MS (polimer hidrofobic)	Lichrospher RP-18 ADS	Isocratic elution MeCN/phosphate buffer 20 mM pH 2.1 35/65			
Flunitrazepam, 7-amino-flunitrazepam, N-desmethyl flunitrazepam	Plasma	SPE	Simmetry C18	Gradient elution A: HCOOH 0.1% B: MeCN	Flunitrazepam d_3	0.25 µg/L (flunitrazepam) 2 µg/L (N-desmethyl-flunitrazepam 0.5 µg/L (7-amino-flunitrazepam)	

(Continued)

Table III (Continued)

1	2	3	4	5	6	7	8
Flunitrazepam, 7-amino-flunitrazepam, N-desmethyl M flunitrazepam, 3-hydroxi-flunitrazepam	Plasma, urine	SPE	RP	Isocratic elution using *salt free eluent*	7-amino-flunitrazepam d_3, desmethyl-flunitrazepam d_3	0.025 ng/mL (flunitrazepam, 7-amino-flunitrazepam) 0,040 ng/mL (N-desmehyl-flunitrazepam 0.200 ng/mL (3-hydroxi-flunitrazepam)	
Flunitrazepam, 7-amino-flunitrazepam	Urine	SPE	C18	Mobile phase and flow rate gradient elution A: NH_3 1.5% B: NH_3 1.5% in MeOH C: MeCN/H_2O 5/95 D: MeCN/H_2O 95/5		1-3 ng/mL (flunitrazepam and 7-amino-flunitrazepam)	

Several benzodiazepines in serum (among them flunitrazepam) were determined on C8 and C18 columns, using mobile phases containing SDS and buthanol or penthanol as organic modifier. This method was compared with the conventional one, using a mixture methanol/water 5/5 as mobile phase; MLC is more convenient, because it does not require separate extraction procedures, serum samples are injected directly.

Other electrophoretic methods are presented in Table V.

Table V

The characteristics of electrophoretic methods used to determine flunitrazepam

Analites	Matrix	Sample preparation	Column	Buffer	Applied potential
Flunitrazepam			Capillary	Borate buffer 25 mM-MeOH 20%-SDS 100M, Phosphate buffer 50 mM pH 2.35, Borate buffer 50 mM pH 9.24	20 kV 20 kV 12 kV
Flunitrazepam	urine	SPE	Capillary, covered with poliacrylamide		
Flunitrazepam (pharmaceutical dosage forms)			Capillary covered with SiO_2	Borate buffer 15 mM, pH 9.2-SDS 35 mM-aqueous sodium deoxicolat 35 M	25 kV

Flunitrazepam was quantified through a radiometric method, using an improved radio-receptor for benzodiazepines. A MultiScreen® Assay System was used in order to filter the sample, consisting in a 96 well plate, sealed with glass fiber at the bottom; this system allows both incubation and analyte filtration. Filters were broke after filtration, for quantitative determination of linked radio-marked [^{3}H] flunitrazepam [26].

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