

BENZODIAZEPINES 2 in serum and/or in plasma by UV – FAST – Code Z35610
(Nordiazepam, Clobazam, Diazepam, Midazolam and Nitrazepam)

INTRODUCTION

The main effects of benzodiazepines are hypnotic, anxiolytic, anticonvulsant, myorelaxant and amnesic.

There is a large range in potency and elimination rates between different benzodiazepines.

The action of GABA is allosterically modulated by benzodiazepines which interact with the GABA-A receptor complex. Flumazenil is a potent benzodiazepine antagonist that competitively blocks the central effects of benzodiazepines.

Long-term treatment with benzodiazepines induces drug-dependence.

It should be pointed out that the intensity the action of benzodiazepines also depends on the production of active metabolites. Benzodiazepines are composed of a benzene ring fused to a seven membered diazepine ring, a 5-aryl substituent and a 1,4-diazepine ring. Modifications in the structure of the rings give compounds with different properties. Due to their molecular hydrophobic structure, all benzodiazepines are completely absorbed orally and distributed. There is rapid uptake of benzodiazepines into the brain and in all perfused tissues. They bind to plasma proteins and are metabolised in the liver. Benzodiazepine metabolism involves modification and/or removal of substituents, producing desalkylated compounds at position 1 and hydroxylation at position 3, with formation of less hydrophobic compounds. These metabolites are conjugated with glucuronic acid and excreted by urine.



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This product fulfills all the requirements of Directive 98/79/EC on in vitro diagnostic medical devices (IVD). The declaration of conformity is available upon request.

N° 003	Benzodiazepines 2 in plasma/serum by UV - FAST	09/2024
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TECHNICAL FEATURES

Principle of the Method:

Benzodiazepines are analysed after serum/plasma Clean-up Columns purification. After addition of a stabilization reagent, the solution is directly injected into HPLC system.

Recovery: 100 %

Sensitivity (LLOD):

<i>Nordiazepam</i> :	3,33 µg/l
<i>Clobazam</i> :	3,33 µg/l
<i>Diazepam</i> :	6,66 µg/l
<i>Midazolam</i> :	3,3 µg/l
<i>Nitrazepam</i> :	3,3 µg/l

Minimum concentration analyzable (LLOQ):

<i>Nordiazepam</i> :	10 µg/l
<i>Clobazam</i> :	10 µg/l
<i>Diazepam</i> :	20 µg/l
<i>Midazolam</i> :	10 µg/l
<i>Nitrazepam</i> :	10 µg/l

Linearity:

<i>Nordiazepam</i> :	10 – 5.000 µg/l
<i>Clobazam</i> :	10 – 5.000 µg/l
<i>Diazepam</i> :	20 – 5.000 µg/l
<i>Midazolam</i> :	10 – 5.000 µg/l
<i>Nitrazepam</i> :	10 – 1.000 µg/l

Therapeutic Range of the Method:

<i>Nordiazepam</i> :	100 – 2.000 µg/l
<i>Clobazam</i> :	200 – 1.000 µg/l
<i>Diazepam</i> :	100 – 1.500 µg/l
<i>Midazolam</i> :	100 – 1.000 µg/l
<i>Nitrazepam</i> :	20 – 120 µg/l

Accuracy intra serie (relative error %)

Nordiazepam :

C1	C2
50 µg/L	312,5 µg/L
4,99%	3,65%

Accuracy inter serie (relative error %)

Nordiazepam :

Ci	Cs
50 µg/L	312,5 µg/L
6,56%	4,91%

Reproducibility intra serie (coefficient of variation %)

Nordiazepam :

C LLOQ	Cm	Cs
10 µg/L	125 µg/L	625 µg/L
7,41%	2,68%	2,35%

Reproducibility inter serie (coefficient of variation %) Nordiazepam :

C LLOQ	Cm	Cs
10 µg/L	15 µg/L	80 µg/L
9,59%	3,09%	2,98%

Coefficient of correlation R2 + Dev. Std

Nordiazepam 0,9990 + 0,0007

Accuracy intra serie (relative error %) Clobazam :

C1	C2
50 µg/L	312,5 µg/L
3,08%	1,61%

Accuracy inter serie (relative error %) Clobazam :

Ci	Cs
50 µg/L	312,5 µg/L
5,71%	3,89%

Reproducibility intra serie (coefficient of variation %) Clobazam :

C LLOQ	Cm	Cs
10 µg/L	125 µg/L	625 µg/L
8,71%	4,86%	3,51%

Reproducibility inter serie (coefficient of variation %) Clobazam :

C LLOQ	Cm	Cs
10 µg/L	125 µg/L	625 µg/L
15,62%	5,44%	3,67%

Coefficient of correlation R2 + Dev. Std

Clobazam 0,9975 + 0,0008

Accuracy intra serie (relative error %) Diazepam :

C1	C2
50 µg/L	312,5 µg/L
5,61%	3,03%

Accuracy inter serie (relative error %) Diazepam :

Ci	Cs
50 µg/L	312,5 µg/L
6,46%	3,18%

Reproducibility intra serie (coefficient of variation %) Diazepam :

C LLOQ	Cm	Cs
20 µg/L	125 µg/L	625 µg/L
8,56%	6,14%	2,58%

Reproducibility inter serie (coefficient of variation %) Diazepam :

C LLOQ	Cm	Cs
20 µg/L	125 µg/L	625 µg/L
7,78%	6,26%	4,23%

Coefficient of correlation R2 + Dev. Std

Diazepam 0,9958 + 0,0022

Accuracy intra serie (relative error %) Midazolam:

C1	C2
50 µg/L	312,5 µg/L
3,93%	2,81%

Accuracy inter serie (relative error %) Midazolam:

Ci	Cs
50 µg/L	312,5 µg/L
6,00%	3,25%

Reproducibility intra serie (coefficient of variation %) Midazolam :

C LLOQ	Cm	Cs
10 µg/L	125 µg/L	625 µg/L
6,22%	4,56%	2,36%

Reproducibility inter serie (coefficient of variation %) Midazolam :

C LLOQ	Cm	Cs
10 µg/L	125 µg/L	625 µg/L
6,80%	4,83%	2,00%

Coefficient of correlation R2 + Dev. Std

Midazolam 0,9976 + 0,0014

**Accuracy intra serie (relative error %)
Nitrazepam:**

C1	C2
20 µg/L	125 µg/L
9,52%	4,07%

**Accuracy inter serie (relative error %)
Nitrazepam :**

Ci	Cs
20 µg/L	125 µg/L
6,86%	2,99%

Reproducibility intra serie (coefficient of variation %) Nitrazepam :

C LLOQ	Cm	Cs
10 µg/L	50 µg/L	250 µg/L
10,59%	5,01%	3,41%

Reproducibility inter serie (coefficient of variation %) Nitrazepam :

C LLOQ	Cm	Cs
10 µg/L	50 µg/L	250 µg/L
8,68%	5,24%	3,89%

Coefficient of correlation R2 + Dev. Std

Nitrazepam 0,9976 + 0,0003

<u>Components of the kit :</u>	All the components are ready-to-use. Stability: 3 years at 2-8 °C.
Reagent A – Conditioning Solution N° 1, 1 x 50 ml	
Reagent B – Conditioning Solution N° 2, 1 x 50 ml	
Reagent C – Diluting Solution, 1 x 50 ml	
Reagent D – Wash Solution, 1 x 100 ml	
Reagent E – Eluting Solution, 1 x 30 ml	
Reagent F – Stabilization Solution, 1 x 20 ml	
Reagent G – Internal Standard Solution, 1 x 5 ml	
Reagent H – Test Solution, 1 x 1 ml	<u>See Warnings</u>
Calibrator lyophil. In serum, 1 x 5 ml	<u>Store at – 20 °C</u>
Reagent M – Mobile Phase, 3 x 500 ml	<u>Code Z35016 (packed separately – see data sheets)</u>
Clean-up Columns, 100 pcs	

Minimum Instrumental equipment required:

Isocratic HPLC System with loop of 10 µl.
Spectrophotometric Detector $\lambda=242$ nm
Chromatograms Recorder

Optional Equipment:

Autosampler.
Operational Computer

Whole Blood Collection Procedure:

Take 3 ml of whole blood in a test tube without gel or with EDTA as anticoagulant. Centrifuge at 4000 rpm for 5 minutes. Separate the serum and store at – 20 °C. Stable 4 weeks.

PREANALYTICAL PROCEDURE

Pipette in a tube :

- 350 µl of H₂O HPLC grade
- 100 µl of **Reagent H – Test Solution**
- 50 µl of **Reagent G – Internal Standard Solution**

Vortex for 10 seconds

INJECTION :

Inject 10 µl of this solution in the chromatographic system.

Verify that the Test Solution has retention time similar to fig. 1. If the Test is all right you can start with the analytical procedure; if not, check the functionality of the analytical system.

Important : Don't use this solution to calibrate!

ANALYTICAL PROCEDURE

STEP 1 : Prepare Clean up columns for each Sample, Calibrator and Controls.

- Condition clean up columns by fluxing 0,5 ml of **Reagent A - Conditioning Sol. 1** and don't let the columns be dry
- Condition clean up columns by fluxing 0,5 ml of **Reagent B - Conditioning Sol. 2** and don't let the columns be dry

STEP 2 : Dispense in a tube:

	Sample	Calibrator	Control
Sample	500 µl		
Calibrator		500 µl	
Control			500 µl
Reagent C – Diluting Solution	500 µl	500 µl	500 µl
Reagent G – Internal Standard	50 µl	50 µl	50 µl

Vortex for 10 seconds

STEP 3 : Dispense the Samples and Calibration Standard previously prepared into Clean-up Columns.

Don't let the columns be dry!

STEP 4 : Wash Clean-up Columns with 1 ml of **Reagent D – Wash Solution**

STEP 5 : Elute with 300 µl of **Reagent E – Eluting Sol.**

STEP 6 : Add at eluate 200 µl of **Reagent F – Stabilization Sol.**

Vortex for 10 seconds

N.B.: at this step, the sample is stable 7 days at 2-8 °C

INJECTION :

- Inject 10 µl of the eluate into the chromatographic system.

BENZODIAZEPINES 2 - Warnings

REAGENT H : TEST SOLUTION

BENZODIAZEPINES	
Nordiazepam	20.000 µg/l
Clobazam	10.000 µg/l
Diazepam	20.000 µg/l
Midazolam	10.000 µg/l
Nitrazepam	2.600 µg/l

SPECTROPHOTOMETRIC DETECTOR PARAMETERS

λ	242 nm
GAIN	0,001 AUFS
INTEGRATION TIME	10 seconds

HPLC COLUMN PROTECTION

To save the analytical column Reverse Phase POROSHELL C18 4,6 x 50 mm, 2,7 µm, the use of Javelin Colfilters (1 x 10 pcs.) cod. S90199511 is obligatory.

HPLC COLUMN CONDITIONING

Install a new analytical C₁₈ Reversed-Phase column POROSHELL C18 4,6 x 50 mm, 2,7 µm. Disconnect the detector and filter 30 ml of **H₂O : Acetonitrile or Methanol (20 : 80 v/v)** solution and subsequently 30 ml of **H₂O : Acetonitrile or Methanol (80 : 20 v/v)** solution, set flow at 1,0 ml/min. **Don't recycle the washing solutions.** Condition the column with the mobile phase at a flow of 1,4 ml/min. For 15 min. Finally inject the Chemical Standard and verify the quality of the HPLC run.

It is NOT possible to do analysis at recycling phase. If room temperature is > 20 °C store the Mobile Phase at 2-8 °C between an analytical session and another.

COLUMN CLEANING

Disconnect the detector. Flux 15 ml of a solution of **H₂O : Acetonitrile (20 : 80 v/v)** set flow at 1,4 ml/min and discharge. Store the column in a solution of **H₂O : Acetonitrile (20 : 80 v/v)**. **It is recommended to wash the column at the end of each analytical session.**

INJECTION NEEDLE WASHING

Flux 15 ml of a solution of **H₂O : Acetonitrile (80 : 20 v/v)** set flow at 1,4 ml/min.

HPLC PARAMETERS

LOOP	10 µl
FLOW	1,2 ml/minute
PRESSURE	About 160 bar

ACCESSORIES AND CONSUMABLES

CODE	DESCRIPTION	PACKAGING
Z35016	Calibrator in serum for Benzodiazepines 2	4 x 5 ml
Z35019	Control in serum for Benzodiazepines 2 – Levels 1 and 2	2 x 5 x 5 ml
Z699975902	Poroshell 120 EC-C 18 (50 x 4,6mm -2,7 um) Analytical Column	1 Pc
S90199511	Javelin Colfilters	1 x 10 Pcs
S29057U	Standard glass vials of 2 ml with screw cap	1 x 100 Pcs

BENZODIAZEPINES 2 IN SERUM AND/OR IN PLASMA

(Reference Chromatograms)

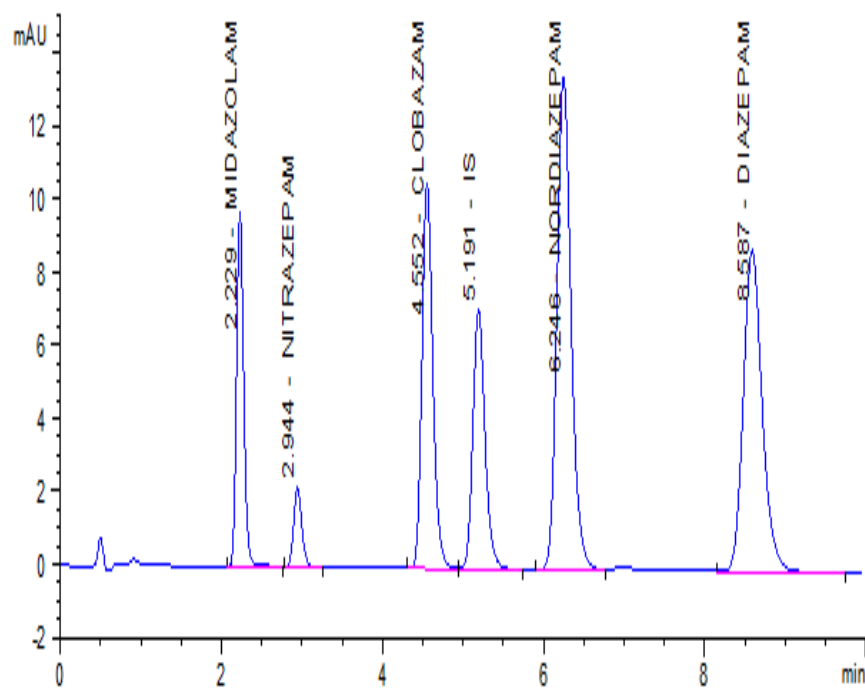


Fig. 1: Test Solution

R.T.	2.22	Midazolam
R.T.	2.94	Nitrazepam
R.T.	4.55	Clobazam
R.T.	5.19	Internal Standard
R.T.	6.24	Nordiazepam
R.T.	8.58	Diazepam

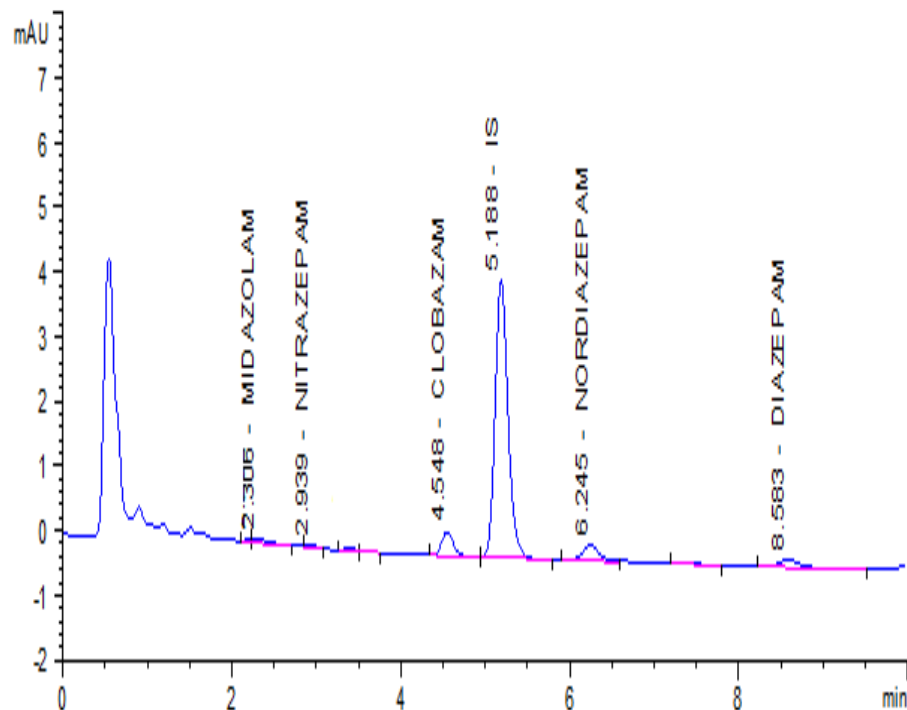


Fig. 2: Serum Control Low

R.T.	2.30	Midazolam	96,5 µg/L
R.T.	2.93	Nitrazepam	19,0 µg/L
R.T.	4.54	Clobazam	111,9 µg/L
R.T.	5.18	Internal Standard	
R.T.	6.24	Nordiazepam	110,4 µg/L
R.T.	8.58	Diazepam	98,1 µg/L

BENZODIAZEPINES 2 IN SERUM AND/OR IN PLASMA

(Reference Chromatograms)

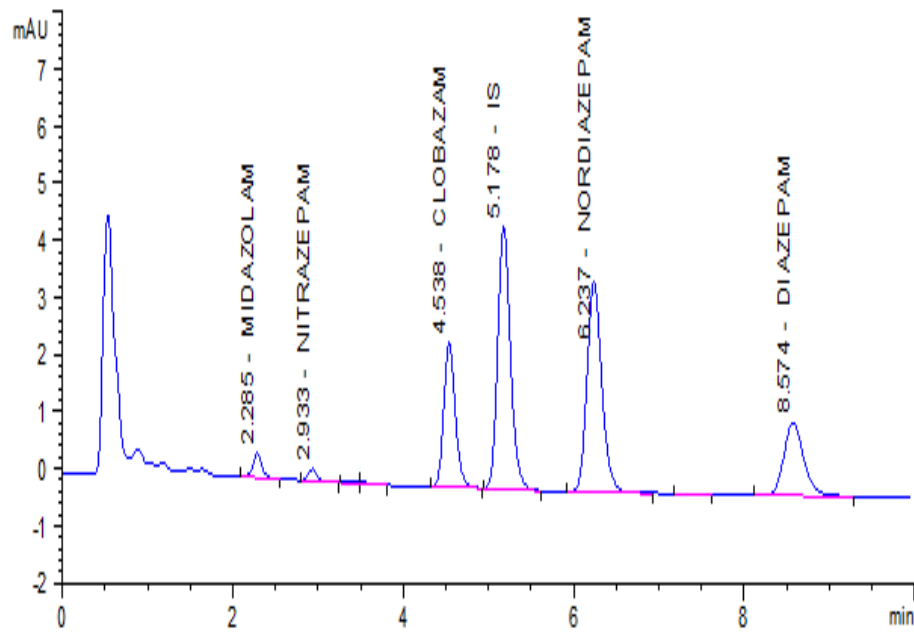


Fig. 3: Serum Calibrator

R.T.	2.28	Midazolam	645,1 µg/L
R.T.	2.93	Nitrazepam	71,8 µg/L
R.T.	4.53	Clobazam	681,4 µg/L
R.T.	5.17	Internal Standard	
R.T.	6.23	Nordiazepam	1346,3 µg/L
R.T.	8.57	Diazepam	1109,9 µg/L

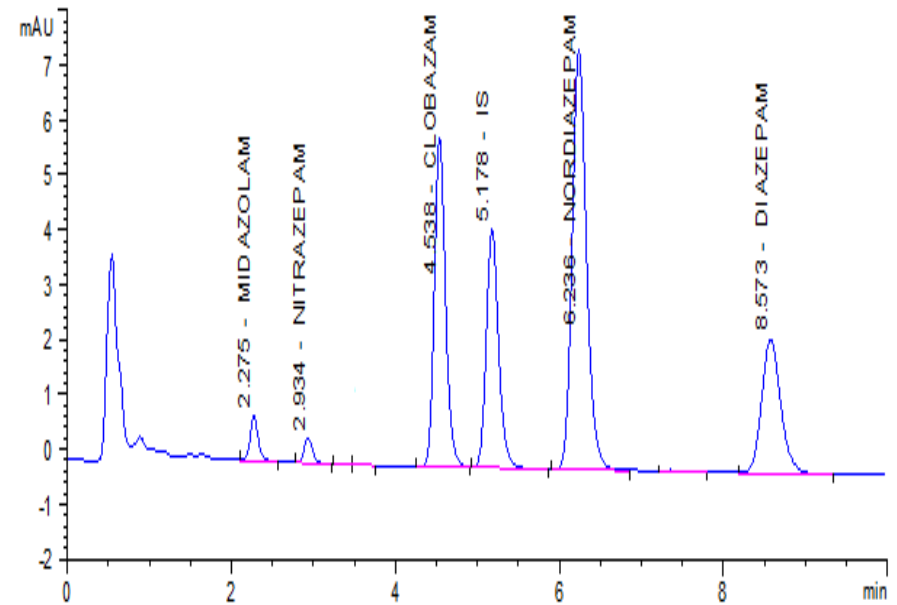


Fig. 4: Serum Control High

R.T.	2.27	Midazolam	1191,3 µg/L
R.T.	2.93	Nitrazepam	143,1 µg/L
R.T.	4.53	Clobazam	1561,7 µg/L
R.T.	5.17	Internal Standard	
R.T.	6.23	Nordiazepam	2789,2 µg/L
R.T.	8.57	Diazepam	2212,7 µg/L

BENZODIAZEPINES 2 IN SERUM AND/OR IN PLASMA

(Reference Chromatograms)

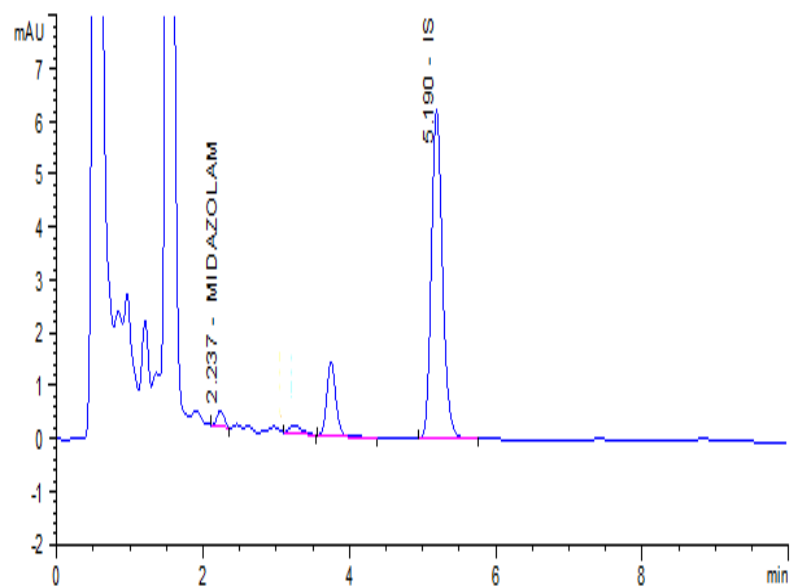


Fig. 5:	Sample of Midazolam			
	R.T.	2.23	Midazolam	301,4 µg/L
	R.T.	5.19	Internal Standard	

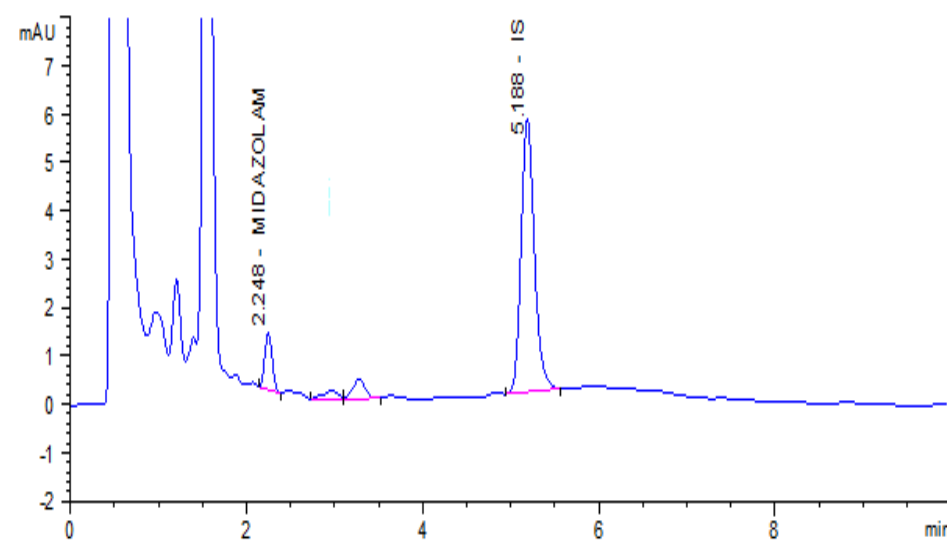


Fig. 6:	Sample of Midazolam			
	R.T.	2.24	Midazolam	1.062 µg/L
	R.T.	5.18	Internal Standard	

BENZODIAZEPINES 2 IN SERUM AND/OR IN PLASMA
(Reference Chromatograms)

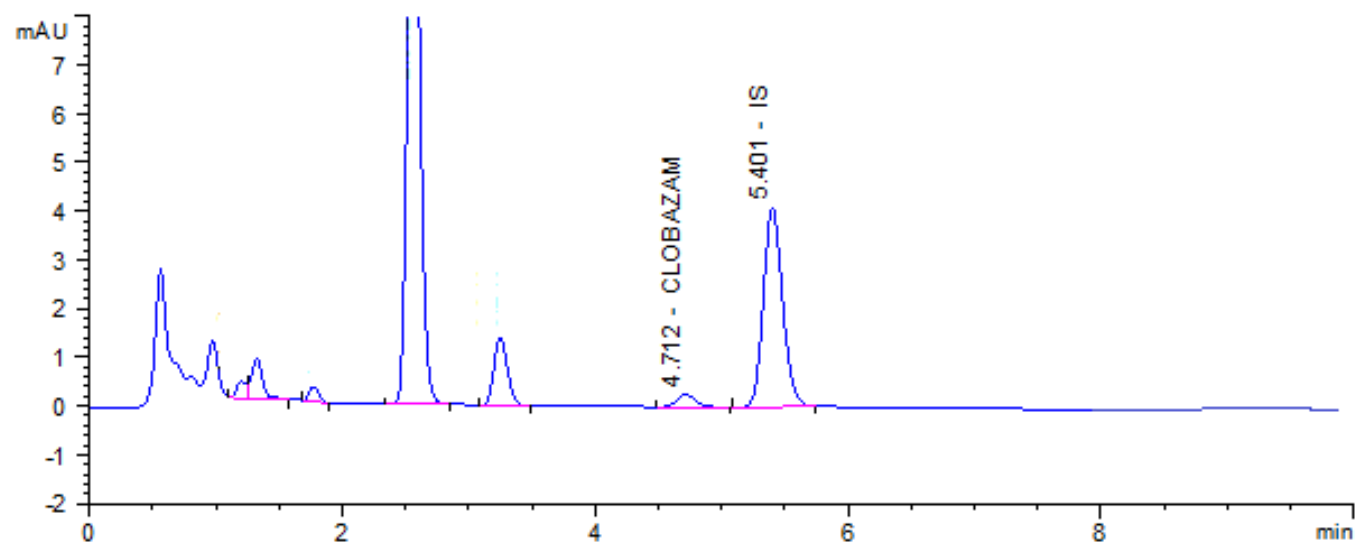


Fig. 7:	Sample of Clobazam		
	R.T. 4.71	Clobazam	102,64 µg/L
	R.T. 5.40	Internal Standard	

BENZODIAZEPINES					
N°	Active Principle Name	Commercial Name			
1	Chlordemethyldiazepam	1	EN		
2	Chlordiazepoxide	1	Endequil	8	Psicoterina
		2	Equibral	9	Reliberan
		3	Labican	10	Seren-vita
		4	Librium	11	Smail
		5	Lixin	12	Ansiacal
		6	Philcorium	13	Benzodiapin
		7	Psicofar		
3	Diazepam	1	Aliseum	16	Apozepam
		2	Ansioli	17	AspaserineB ₆ Tranq
		3	Avex	18	Atensine
		4	Eridan	19	Benzopin
		5	Noan	20	Betapam
		6	Qetinil	21	Calmaven
		7	Quievita	22	Calmigen
		8	Tranquirit	23	Complutine
		9	Valitran	24	Diaceplex
		10	Valium	25	Dialar
		11	Vatran	26	Diaquel
		12	Alupran	27	Diazemuls
		13	Aneurol	28	Ansium
		14	Antenex	29	Anxicalm
		15	Vivol		
4	Flunitrazepam	1	Rohypnol	6	Roipnol
		2	Rohipnol	7	Darkene
		3	Hypnodorm	8	Hypnor
		4	Narcozep	9	Noriel
		5	Valsera		
5	Lorazepam	1	Temesta	13	Lorans
		2	Tran-Qil	14	Tranqipam
		3	Somagerol	15	Novolorazem
		4	Pro-Dorm	16	Duralozam
		5	Orfidal	17	Idalprem
		6	Donix	18	Tolid
		7	Securit	19	Sedizepam
		8	Tavor	20	Almazine
		9	Somnium	21	Ativan
		10	Tè mesta	22	Quait
		11	Punktyl	23	Lorax
		12	Control	24	Laubeel
6	N-Demethyldiazepam	1	Madar		
7	Clobazam	1	Clarmyl	5	Sederlona
		2	Clopax	6	Urbadan
		3	Frisium	7	Urbanol
		4	Noiafren	8	Urbanyl

	Active Principle Name	Commercial Name			
8	Nitrazepam	1	Somnite	15	Ipersed
		2	Pelson	16	Mitidin
		3	Remnos	17	Mogadon
		4	Paxadorm	18	Surem
		5	Eatan-N	19	Mitidin
		6	Imeson	20	Dormo-Purem
		7	Insoma	21	Serenade
		8	Dormigen	22	Somnipar
		9	Nitrados	23	Ormodon
		10	Mogadan	24	Alodorm
		11	Somnibel-N	25	Apodorm
		12	Unisomnia	26	Arem
		13	Pelsonfilina	27	Persopir
		14	Novanox	28	Tri
9	Flurazepam	1	Staurodorm New		
10	Flurazepam Dihydrochloride	1	Dalmadorm	5	Midorm
		2	Dalmane	6	Novoflupam
		3	Dormodor	7	Remdue
		4	Irdal		
11	Flurazepam Monohydrochloride	1	Dalmadorm	5	Paxane
		2	Dalmane	6	Somnol
		3	Felison	7	Valdorm
		4	Flunox		
12	Clonazepam	1	Iktorivil	3	Rivotril
		2	Klonopin		
13	Delorazepam	1	Briantum	2	EN
14	Bromazepam	1	Bromazanil	8	Lexil
		2	Brozam	9	Lexomil
		3	Compendium	10	Lexotan
		4	Durazanil	11	Lexotanil
		5	Gityl	12	Neo-OPT
		6	Lectopam	13	Normoc
		7	Lexatin		
15	Lormetazepam	1	Ergocalm	4	Loretam
		2	Lembrol	5	Minias
		3	Loramet	6	Noctamid
16	Brotizolam	1	Lendormin	2	Lendormine
17	Midazolam	1	Dormicum	2	Hypnovel
18	Midazolam Hydrochloride	1	Dormicum	3	Versed
		2	Hypnovel		

	Active Principle Name	Commercial Name			
19	Midazolam Maleate	1	Dormicum		
20	Aprazolam	1	Tafil	4	Xanax
		2	Trankimazin	5	Xanor
		3	Valeans		
21	Oxazepam	1	Adumbram	6	Sedotin
		2	Isodin	7	Serpax
		3	Limbial	8	Serax
		4	Quen	9	Serenid
		5	Quilibrex		
22	Prazepam	1	Prazene	5	Mono-demetrin
		2	Centrax	6	Reapam
		3	Demetrin	7	Trepaidam
		4	Lysanxia		
23	Triazolam	1	Halcion	4	Novodorm
		2	Apo-Diazo	5	Songar
		3	Dumozolam		
24	Temazepam	1	Restoril	3	Levanxol
		2	Euhypnos		