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Article

Development and Validation of Method for the Determination of the Benzodiazepines Clonazepam, Clobazam and N-Desmethylclobazam in Serum by LC-MS/MS and its Application in Clinical Routine

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Clonazepam and clobazam are potent benzodiazepines derivatives that have been used primarily as anticonvulsants. Methods based on liquid chromatography tandem mass spectrometry (LC-MS/MS) are considered the gold standard in therapeutic monitoring. In this work a simple, rapid and sensitive LC-MS/MS method for the determination of clonazepam, clobazam and N-desmethylclobazam in serum was developed and validated. The method consisted in a simple liquid-liquid extraction, in which 200 µL of serum containing the internal standard (temazepam or stable isotopes of clonazepam and clobazam) were treated with ethyl acetate and subjected to LC-MS/MS analysis using positive electrospray ionization. Chromatographic separation was performed on a C18 column and isocratic mobile phase containing methanol:water:acetonitrile (50:30:20, v/v/v) with 0.05% of formic acid at 400 µL min^{-1}. The linear analytical range of the procedure was between 10.0 and 160.0 ng mL^{-1} for clonazepam, 25.0 and 525.0 ng mL^{-1} for clobazam and 100.0 and 5,000.0 ng mL^{-1} for N-desmethylclobazam. The sample dilution of 1:2 and 1:10 for clobazam/N-desmethylclobazam and 1:2 for clonazepam was validated for the samples that exceed the method linearity. Relative standard deviations for intra and inter-day precision were lower than 4.3% for clonazepam, 7.5% for N-desmethylclobazam and 9.7% for clobazam. The recovery range was between 95 and 109% for all analytes. The LC-MS/MS method has been developed and validated successfully for the quantitative analysis and therapeutic monitoring of these benzodiazepines. The method has been applied successfully in the clinical laboratory routine.

Keywords: Benzodiazepines, LC-MS/MS, Validation, Therapeutic Drug Monitoring.

INTRODUCTION

Benzodiazepines (BZD) are psychoactive drugs commonly used for treatment of anxiety, insomnia, and psychological disorders, and are the most frequently prescribed medications worldwide. Although overdoses can happen, BZD are considered relatively safe. However, its safety decreases when co-administered with alcohol, sedatives, antidepressants and neuroleptics [1,2].

Clobazam is a 1,5-benzodiazepine used successfully worldwide since 1970 as an anxiolytic and antiepileptic drug [3]. The activity of clobazam is attributed to both the parent drug and N-desmethylclobazam, one of its metabolite [4]. Clonazepam is a 1,4-benzodiazepine widely used as anticonvulsant agent and for treatment of epilepsy in adults and children [5]. Clonazepam is considered safe by addiction medicine specialists, but it has been frequently abused as a street drug [6].

The development of methods for quantifying the drugs concentrations in biological fluids made possible to study the relationship between drug dosage, drug concentration in body fluids and pharmacologic effects [7]. Therapeutic drug monitoring (TDM) is important for avoiding the adverse effects of drug interactions with other drugs as well as optimizing the dosage in individual patients [8].

Liquid chromatography tandem mass spectrometry (LC–MS/MS) is an increasingly important tool in TDM due to its great sensitivity and specificity compared to other techniques and the possibility that some

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of the methods can quantify multiple drugs simultaneously, which can be beneficial for patients that use more than one anticonvulsant [9]. Benzodiazepines have similar structure and they can coelute with another compound or metabolite mainly due to the administration of different drugs during a treatment. Thus, the specificity of LC-MS/MS is a differential factor in therapeutic monitoring of benzodiazepines.

The aim of this work was the development of a method for therapeutic monitoring of clonazepam, clobazam and N-desmethylclobazam in serum using LC-MS/MS and its application in clinical laboratory routine.

MATERIALS AND METHODS

Chemicals

Clonazepam, clobazam, temazepam, and the stable isotopes clonazepam-d_4 and clobazam-8-chloro isomer-$^{13}C_6$, used as internal standard, were obtained from Cerilliant (Round Rock, TX, USA). N-desmethylclobazam was obtained from LGC Standards (Luckenwalde, Germany). Ethyl acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid, ammonium hydroxide 25%, HPLC-grade acetonitrile and HPLC-grade methanol were supplied by Merck (Darmstadt, Germany).

Internal standards, calibrators and controls

Stock solutions of clonazepam and clobazam at a concentration of 0.1 mg mL^{-1} were prepared in methanol and the working solutions were prepared in methanol:water (80:20, v/v). Stock solution of N-desmethylclobazam at a concentration of 0.1 mg mL^{-1} was prepared in acetonitrile and the working solutions were prepared in acetonitrile:water (80:20, v/v). Stock solutions of clonazepam-d_4 and clobazam-8-chloro isomer-$^{13}C_6$ at a concentration of 0.01 mg mL^{-1} and temazepam at a concentration of 0.1 mg mL^{-1} were prepared in methanol and the working solutions were prepared in acetonitrile.

Calibration curve and quality controls were prepared in the same biological matrix as samples by spiking the matrix with the working solutions of clonazepam, clobazam and N-desmethylclobazam to obtain the same concentrations approved in the linearity test and covering the linear range, respectively.

Instrumentation

Clonazepam, clobazam and N-desmethylclobazam were determined using a Quattro Micro tandem mass spectrometer triple quadrupole analyzer with positive electrospray ionization (Waters, Milford, MA) and an HPLC Alliance HT System (Waters, Milford, MA).

The chromatographic parameters were optimized performing one injection after another until achieving the best result. The mobile phase composition was varied as to obtain better response and chromatographic profile, and a reduced run time. Therefore, methanol, acetonitrile, water, ammonium hydroxide, formic acid and acetic acid were tested in different proportions. The column temperature and injection volume were also varied.

The mass spectrometer conditions were optimized by direct infusion of a solution containing 1.0 µg mL^{-1} of each analyte separately in an aqueous solution of methanol 50%, in order to provide greater analyte response.

Sample preparation

Two hundred microliters of serum were spiked with 50 µL of internal standard (temazepam or stable isotopes of clonazepam and clobazam). The pH was adjusted to 9.0 with ammonium hydroxide 5% and then submitted to stirring for 5 s. 1,000 µL of ethyl acetate were added and the mixture was submitted to a vigorous stirring for 40 s using a vortex. It was centrifuged (5 min at 14,000 rpm) and the supernatant was transferred to a 5 mL test tube, and evaporated with a vacuum concentrator. The extract was reconstituted with 200 µL of acetonitrile:water (80:20, v/v) and 10 µL were injected in the chromatographic system.

Method linearity and limits

The linearity was evaluated using a serum pool spiked in concentrations of 10.0; 40.0; 70.0; 100.0; 130.0 and 160.0 ng mL^{-1} for clonazepam, 25.0, 125.0, 225.0, 325.0, 425.0 and 525.0 ng mL^{-1} for clobazam and 100.0; 1,000.0; 2,000.0; 3,000.0; 4,000.0 and 5,000.0 ng mL^{-1} for N-desmethylclobazam. The calibration curve levels including blank and zero level (blank spiked with internal standard) were extracted in triplicate and injected in simplicate.

The concentration of the lower and upper limit of calibration was proposed based in a reference value of these benzodiazepines in serum, and the linearity was assessed using ANOVA, Jackknife test for removing the outliers, and Brown-Forsythe test for evaluating residuals homoscedasticity [10].

The lower limit of quantification (LOQ) in this work was defined as the lowest point of calibration curve, and it should have relative standard deviation lower than 20% and accuracy within 80 and 120% of the nominal concentration [11]. Therefore, the LOQ was prepared in sextuplicate in three different days by three analysts. The limit of detection (LOD) was determined according to equation 1, where SD is the standard deviation of the intercepts of 5 calibration curves prepared in different days, and slope is these 5 curves slopes average.

$$\mathrm{LOD} = \frac{(3 \times \mathrm{SD})}{\mathrm{slope}} \qquad (1)$$

Precision, accuracy and analyte recovery

The precision, accuracy and analyte recovery were evaluated with a serum pool spiked in three different concentrations covering the linear range for each analyte and analyzed in sextuplicate. The concentrations evaluated were 30.0; 80.0 and 120.0 ng mL^{-1} for clonazepam, 75.0, 250.0, and 400.0 ng mL^{-1} for clobazam and 300.0, 2,500.0 and 3,800.0 ng mL^{-1} for N-desmethylclobazam.

The relative recovery was determined according to equation 2, where C_F is the concentration determined in fortified sample, C_U is the concentration determined in unfortified sample and C_A is the concentration of analyte added.

$$\mathrm{recovery}\ (\%) = \frac{(C_F - C_U)}{C_A} \times 100 \qquad (2)$$

Intra-day precision was determined by analysis of each level extracted in sextuplicate and processed within the same day of the preparation. Inter-day precision was assessed by determining the same levels of the controls above and prepared in three different days by three analysts. In this test, each analyst was responsible for preparing, injecting and analyzing data.

The accuracy was evaluated by the agreement between C_A and C_F, and was expressed by percentage.

The results of accuracy and recovery are acceptable if between 85% and 115% and the precision is acceptable if the relative standard deviation was lower than ±15% [11].

The sample dilution of 1:2 and 1:10 for clobazam/N-desmethylclobazam and 1:2 for clonazepam were also assayed. The criteria for approving the dilution were the same of the precision, accuracy and recovery.

Cross-talk and carry-over

The chromatographic system was tested in order to verify occurrence of cross-talk and carry-over. Cross-talk occurs when there is detection cross between analyte and internal standard, identical production or unintentionally monitored fragment ions [12], whereas carry-over occurs when the analytes present in a first sample are still detected in the injection of the subsequent sample [13].

In order to perform the test, two LLQ (lower limit of quantitation), four blank, two LLQ, two ULQ (upper limit of quantification), two blank, two ULQ, four blank, two ULQ without internal standard, and two zero were extracted and injected one at a time and sequentially.

Selectivity

Sample blank was analyzed and its chromatogram was compared with the lower limit of quantification of each analyte in order to check the endogenous interferences at the retention time of clonazepam, clobazam, N-desmethylclobazam, and internal standard.

Method comparison studies

The comparability of the methodologies LC-MS/MS and HPLC-UV was evaluated using 18 human serum samples contaminated with different concentrations of clonazepam, clobazam and N-desmethylclobazam. The HPLC-UV analysis for comparison study was performed by another clinical laboratory. The method performance using temazepam or clonazepam-d_4 and clobazam-chloro-isomer-$^{13}C_6$ as internal standards was evaluated by analysis of 19 human serum samples for clonazepam, and 31 human serum samples for clobazam and N-desmethylclobazam. The results were compared using a paired *t*-test with 95% confidence interval. The tests were performed in a supplemental data analysis tool in Microsoft Excel 2013 program.

RESULTS AND DISCUSSION

A LC-MS/MS method was developed and validated for determination of clonazepam, clobazam and N-desmethylclobazam in serum.

The precursor ions were monitored as $[M + H]^+$ for all analytes and quantitative data were obtained by selected reaction monitoring (SRM). The monitored ions were 316→270 (*m/z*) for clonazepam, 320→274 (*m/z*) for clonazepam-d_4, 301→259 (*m/z*) for clobazam, 307→265 (*m/z*) for clobazam-$^{13}C_6$, 287→245 (*m/z*) for N-desmethylclobazam and 301→255 (*m/z*) for temazepam. The mass spectrometric parameters selected were: source temperature of 120 ºC, desolvation temperature of 400 ºC, desolvation gas flow of 600 L h^{-1} and capillary voltage of 3,0 kV. The monitored ions and corresponding fragments voltages are listed in Table I.

Table I. Mass spectrometric parameters for each benzodiazepine

Benzodiazepine	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
Clonazepam	316	270	0.20	40	25
Clonazepam-d_4	320	274	0.20	40	25
Clobazam	301	259	0.20	35	20
Clobazam-$^{13}C_6$	307	265	0.20	35	20
N-desmethylclobazam	287	245	0.20	35	20
Temazepam	301	255	0.20	25	20

Chromatographic performance was obtained with a Symmetry C18 column (75 mm x 4.6 mm x 3.5 µm particle size) (Waters, Milford, MA) using an isocratic mobile phase consisting of methanol:water:acetonitrile (50:30:20, v/v/v) with 0.05% of formic acid pumped at a flow rate of 400 µL min^{-1}. The column was maintained at 30 °C and the method had a chromatographic running time of approximately 6.0 min. The benzodiazepines were separated with a good resolution and no significant effects of carry-over and cross-talk were observed.

The comparability of using different internal standards, as described in the Material and Methods section, presented a good correlation between temazepam and the stable isotopes of clonazepam and clobazam.

The Pearson correlation, which measures the correlation between two variables, was greater than 0.98 for all analytes. The calculated Student's *t*-factor (t_{calc}) was 0.38 for clonazepam, 0.42 for clobazam and 0.40 for N-desmethylclobazam. The critical Student`s *t*-factor (t_{crit}) was 2.09 for clonazepam and 2.03 for clobazam and N-desmethylclobazam with a confidence interval of 95%. The value of t_{calc} is less than t_{crit}, therefore we accept the null hypothesis indicating that there is no significant difference between the use of stable isotopes of clonazepam and clobazam, and temazepam as internal standard. Thus, we can conclude that these internal standards have the same performance. However, temazepam cannot be applied as internal standard for patients that use temazepam or diazepam as medicine because temazepam is one of the metabolites of diazepam [14] and this fact restricts the application of the method. Due to this, the labelled isotopes were selected as internal standard for this clinical application.

The chromatogram of a serum sample containing clonazepam, clobazam and N-desmethylclobazam performed with internal standard temazepam is showed in Figure 1(a) and the chromatogram of the analytes above, performed with stable isotopes as internal standard, is showed in Figure 1(b).

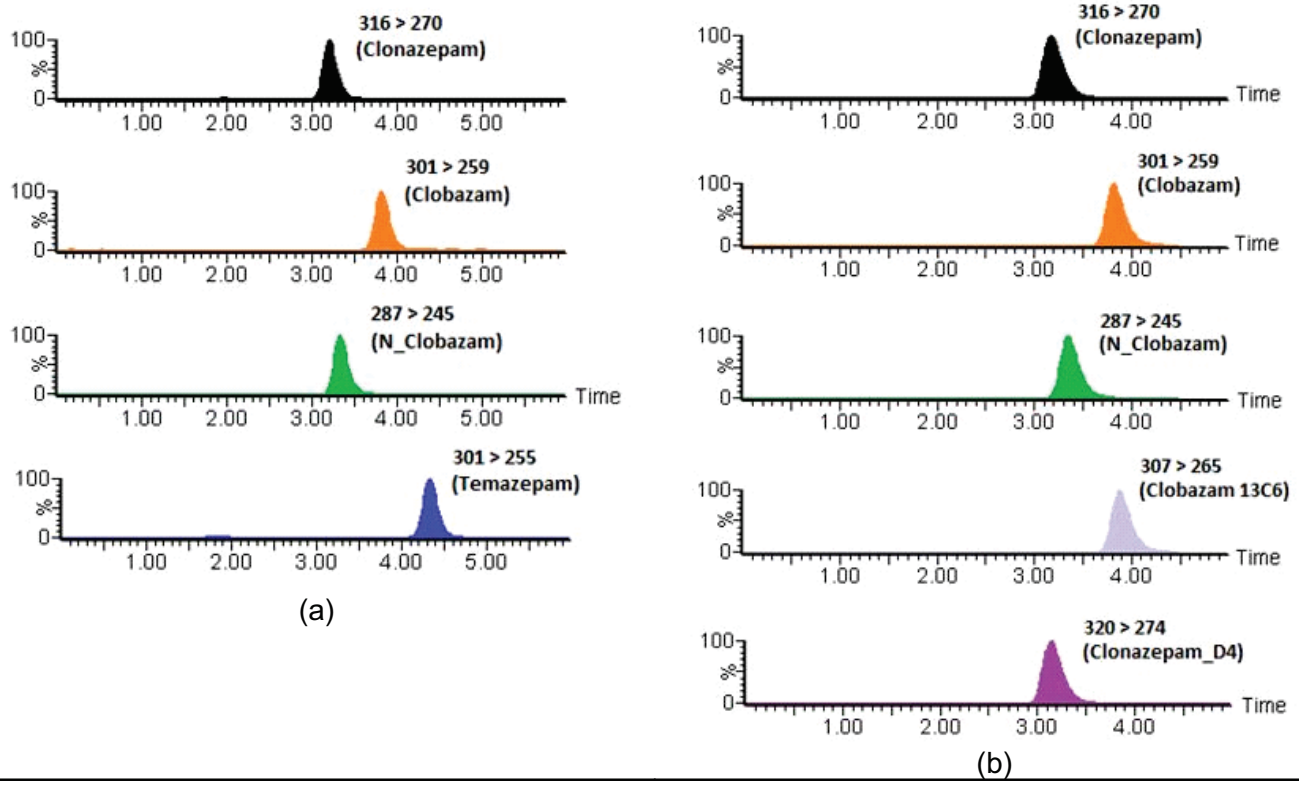

Figure 1. Chromatogram of clonazepam, clobazam and N-desmethylclobazam in human serum using (a) temazepam as internal standard and (b) stable isotope as internal standards.

The sample extraction established in this work was very simple, robust and economic. The method was linear for all analytes in the ranges studied and it allows quantifying high concentration of N-desmethylclobazam. The dilutions 1:2 and 1:10 for clobazam/N-desmethylclobazam and 1:2 for clonazepam were assessed and successfully validated. This allowed us to quantify samples until 320.0 ng mL^{-1} for clonazepam, 5,250.0 ng mL^{-1} for clobazam and 50,000.0 ng mL^{-1} for N-desmethylclobazam with precision and accuracy acceptable. The correlation coefficient, parameters of calibration curves, and the detection limit determined according to equation 1 are described in Table II.

Table II. Linearity parameters of clonazepam, clobazam and N-desmethylclobazam

Analyte	Slope	Intercept	Correlation Coefficient	Linear Range (ng mL^{-1})	LOQ (ng mL^{-1})	LOD (ng mL^{-1})
Clonazepam	0.023358	0.046095	0.997786	10.0 – 160.0	10.0	0.8
Clobazam	0.016541	-0.038457	0.998564	25.0 – 525.0	25.0	3.9
N-desmethylclobazam	0.009999	-0.095916	0.993020	100.0 – 5,000.0	100.0	34.8

Several papers in literature have reported assays for BZD by LC-MS/MS in conventional matrices. Although many of them provide more analytes like other BZD, antiepileptic and anticonvulsant drugs, they almost always focus in applying to forensic cases and not in TDM. Therefore, few works provide simultaneous analysis of active metabolites like N-desmethylclobazam [8, 15-17]. In our bibliographic search, we found three papers performing clonazepam, clobazam and N-desmethylclobazam by LC-MS/MS simultaneously in plasma [8], in serum [16] and whole blood [17], being only one performed in serum [16]. Other works performed with forensic objective do it only with clonazepam [18] or clobazam/clonazepam and do not perform N-desmethylclobazam [19-21]. A comparison between similar reported papers is described in Table III.

Table III. Comparative table with similar reported papers

Reference		THIS WORK	[16]	[8]	[17]
Matrix		Serum	Serum	Plasma	Whole blood
Extraction		Liquid-Liquid	SPE	Precipitation	SPE
Clonazepam	Linearity (ng mL^{-1})	10.0 – 160.0	10.0 – 500.0	10.0 – 50.0	1.0 – 1000.0
	Accuracy (%)	95.1 - 105.0	90.1 - 97.7	-	72.0
	RSD (%)	3.5	4.0	-	6.0
Clobazam	Linearity (ng mL^{-1})	25.0 – 525.0	5.0 – 500.0	50.0 - 250.0	2.0 – 1000.0
	Accuracy (%)	96.3 - 109.0	91.3 - 100.1	-	82.0
	RSD (%)	5.3	4.6	-	4.0
N-Desmethyl clobazam	Linearity (ng mL^{-1})	100.0 – 5000.0	5.0 – 500.0	250.0 – 1250.0	10.0 – 500.0
	Accuracy (%)	100.9 - 107.2	92.1 - 98.7	-	82.0
	RSD (%)	4.5	3.5	-	6.0

A previously reported method analyzed 21 BZD and metabolites in serum by LC-MS/MS and showed low LOQ and good recovery [16]. However, they used solid phase extraction (SPE) for sample preparation, which is more expensive than LLE, and alleged that their method can be applied for TDM although the linearity (5.0 – 500.0 ng mL^{-1}) is below the therapeutic window for N-desmethylclobazam (300.0 – 3,000.0 ng mL^{-1}) [7].

The same is observed in another study, which analyses 33 BZD, metabolites and benzodiazepine-like substances in whole blood by SPE and LC-MS/MS [17]. The authors report a low linearity range for TDM of N-desmethylclobazam (10.0 – 500.0 ng mL^{-1}) and suggest repeating the analysis after sample dilution for those out of linearity, which makes analysis more laborious and expensive. Another reported method analyses clonazepam, clobazam and N-desmethylclobazam and other antiepileptic drugs in plasma by LC-MS/MS [8]. They use protein precipitation with methanol and although their method is simple and fast to execute, they did not reach linearity acceptable for TDM.

In our development, we tested sample extraction by precipitation with acetonitrile and, initially, it showed good results. However, the durability of the chromatographic column was lower, only 200 injections, and makes the analysis economically infeasible due to the high cost of the column. The liquid-liquid extraction with diethyl ether described for clonazepam analyses [22] and liquid-liquid extraction with ethyl acetate were tested. Both showed good recovery, however the extraction with ethyl acetate was selected considering the precision and easiness to work. The results for intra and inter-day precision presented in Table IV were below 10% for all analytes.

Table IV. Intra and inter-day precision and recovery of the LC–MS/MS method for clonazepam, clobazam and N-desmethylclobazam in serum.

	Intra-assay (n=6)			Inter-assay (n=18)		
	Mean (ng mL^{-1})	Recovery (%)	RSD (%)	Mean (ng mL^{-1})	Recovery (%)	RSD (%)
Clonazepam	29.0	96.7	2.0	29.3	100.3	3.2
	30.2	99.2	3.0			
	28.6	105.0	1.7			
	79.4	100.7	1.5	78.1	100.2	2.9
	79.1	98.9	2.6			
	75.7	101.1	1.8			
	126.0	95.4	0.9	120.5	95.0	4.3
	121.4	94.6	1.6			
	114.1	95.1	0.9			
Clobazam	73.5	98.0	3.2	72.9	97.2	4.8
	72.2	96.3	7.7			
	73.0	97.3	3.0			
	251.6	100.7	5.2	254.7	101.9	4.8
	248.1	99.3	5.0			
	264.2	105.7	1.2			
	388.0	97.0	4.6	406.0	101.5	6.2
	394.1	98.5	3.3			
	436.0	109.0	1.5			
N-desmethylclobazam	315.6	105.2	3.9	317.2	105.0	4.6
	315.4	103.1	6.7			
	320.5	106.8	3.3			
	2523.4	100.9	3.5	2558.1	102.3	4.3
	2538.4	101.5	5.9			
	2612.4	104.5	2.8			
	3927.1	103.3	2.8	4012.7	104.8	4.7
	3950.6	104.0	2.8			
	4160.2	107.2	5.7			

The average ranges of recovery of a spiked serum were 95.1 – 105.0% for clonazepam, 96.3 – 109.0% for clobazam and 100.9 – 107.2% for N-desmethylclobazam.

The relative standard deviation for the LOQ was between 1.8 and 9.3% and the accuracy was between 87.3 and 116.9% for all analytes.

The LC-MS/MS method developed in this work was compared with a reference HPLC-UV assay (n = 18) by Passing & Bablok method [23]. The graphical dispersion, the corresponding regression equation and the correlation coefficient for clonazepam, clobazam and N-desmethylclobazam were showed in Figure 2. The Pearson correlation was greater than 0.99 for all analytes suggesting that the methods are equivalent.

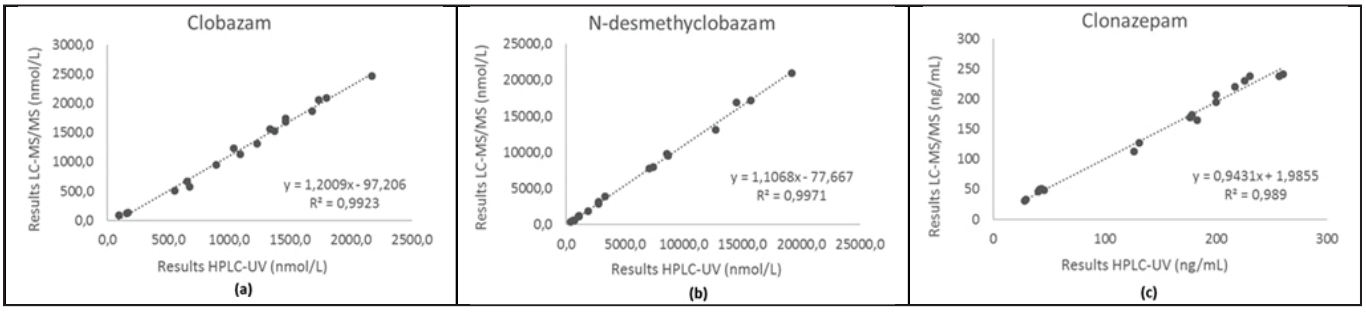

Figure 2. Graphical dispersion for HPLC-UV and LC-MS/MS methods for clobazam (a), N-desmethylclobazam (b) and clonazepam (c).

The method has been applied successfully in the clinical laboratory routine. So far, more than 1000 samples for clonazepam, clobazam and N-desmethylclobazam were analyzed.

CONCLUSIONS

The parameters linearity, selectivity, repeatability, intermediate precision, accuracy, quantification limit and detection limit evaluated in the validation step were successfully determined. The developed method has high selectivity, demands a simple extraction procedure and short consumption of solvent. The results showed that this analytical methodology can be applied in clinical routine as a reference technique to drug therapeutic monitoring of clonazepam, clobazam and N-desmethyclobazam ensuring quality in the exam report. Despite the good correlation of temazepam with the stable isotopes, its use should be carefully checked for every single blood sample before the analysis avoiding incorrect results.

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