Quim. Nova, Vol. 45, No. 7, 867-874, 2022 http://dx.doi.org/10.21577/0100-4042.20170913

Nota Técnica

ANALYSIS OF BENZODIAZEPINES IN PLASMA SAMPLES BY DLLME AND LC-DAD: CRITICAL ASPECTS, FLAWS AND ISSUES ENCOUNTERED – A DISCUSSION

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Recebido em 27/01/2022; aceito em 12/05/2022; publicado na web em 08/06/2022

Dispersive liquid-liquid microextraction (DLLME) is a fast and simple technique and applicable procedure for the analysis of xenobiotics in different biological matrices. We have developed a DLLME method for the determination of benzodiazepines in plasma samples using liquid chromatography-diode-array detection (LC-DAD). During method validation, relative standard deviation values of more than 20% were observed for both intra and inter-day associated with precision and accuracy. So, full method validation was unsuccessful. Critical aspects and flaws associated with DLLME for benzodiazepines determination in biological matrices has never been addressed. The majority of previous studies involving DLLME for benzodiazepines analysis either did not carry out a full method validation or they do not share any details regarding their full validation procedure. The current study has focused on the issues and pitfalls encountered upon method validation so that scientific groups worldwide can avoid unnecessary experiments and benefit from the present discussion. These observations reinforce the need to carry out such a process to guarantee the reliability of the results, even more so if one is intending its application for real case samples in the field of toxicological analysis. Indeed, full, and satisfactory method validation is paramount to provide adequate and trustworthy analytical results.

Keywords: DLLME; benzodiazepines; critical aspects; pitfalls; plasma; biological samples.

INTRODUCTION

Benzodiazepines are the most commonly prescribed drugs in the world to be used as anxiolytics, hypnotics, and anticonvulsants. This psychoactive class of substances is responsible for most intoxication cases in various different countries, including Brazil.[1-4] Toxicological analysis are often required and rather crucial when it comes to tackling cases such as driving under the influence (DUI) and drug-facilitated crimes (DFC) where the use of benzodiazepines may be employed.[1,5,6]

Considering individual toxicological analysis, to determine the presence or absence of these substances, different biological matrices may be required.[7,8] In order to do that, extraction procedures are necessary as a step prior to the chromatographic injection to promote a clean-up of the biological matrix and to avoid any major damage to the instrument and remaining consumables as well as to concentrate the analytes of interest so that their detection is enhanced.[9,10]

Among numerous extraction techniques, dispersive liquid-liquid microextraction (DLLME) stands out given its fast, simple, cost-effective characteristics and the fact that it can be applied to any laboratory setting.[11-14] DLLME usually involves a ternary system: an extraction solvent and a dispersive solvent are injected into an aqueous matrix quickly generating a cloud. After centrifugation, the system rapidly reaches an equilibrium state due to its large superficial area of extraction solvent drops and the sample itself.[15]

Considering the described DLLME features, urine samples may represent an ideal biological matrix for this procedure due to its large water content.[12,16-18] At the same time, this extraction technique may be considered a challenge for plasma samples.

Upon literature review, it was observed that most scientific articles involving DLLME and benzodiazepines determination in biological matrices do not present a full method validation containing proper statistical analysis.[7,12,13,16] Hence, there is a risk that these methods may not be reproducible consequently generating unreliable results. So far, these drawbacks have not been addressed in the scientific literature.

The goal of this study was to employ DLLME for the analysis of benzodiazepines in plasma samples considering a full method validation as well as to describe the limitations encountered, associated with this methodology and its purpose for this study. Additionally, critical aspects of DLLME in biological samples analysis, which have yet to be mentioned in the literature, were evaluated, discussed in presented in the current manuscript, in order to help other research groups avoid unnecessary future analytical essays.

MATERIAL AND METHODS

Acetonitrile, methanol, trimethylamine HPLC-grade were purchased from Tedia® (Fairfield, EUA). Reference standards of alprazolam, bromazepam, diazepam, nordiazepam and clonazepam were obtained from United States Pharmacopeia (USP) while internal standard diphenylamine (IS) was purchased from Sigma-Aldrich® (Sant Louis, EUA). For analysis, a Shimadzu® LC-Diode-Array Detection (DAD) (Kioto, Japan) was used and separations achieved with a C18 column Waters® XBridge (3.5 µm × 4.6 mm ID × 150 mm) (Milford, EUA) as stationary phase with pre-column using C18 cartridges (4 mm x 3mm).

Criteria for inclusion and exclusion

All peer-reviewed scientific articles available on benzodiazepines, DLLME and biological matrices were used as criteria for inclusion. Absence of ISSN indexing, references without authors, journalistic notes without scientific nature, impossibility of accessing the full article and manuscripts employing DLLME for benzodiazepines only in non-biological matrices were used as criteria for exclusion.

Development of chromatographic conditions

For stationary phase, a choice between two reversed-phase

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chromatographic columns C18 with the following specifications: 50 mm length × 2.1 mm inner diameter x 3.5 µm particle size and 150 mm length × 4.6 mm inner diameter × 3.5 µm particle size was made.

As for the mobile phase, the following solvent combinations were evaluated: ultrapure water in pH 9 with trimethylamine/methanol/acetonitrile (63:19:18); ultrapure water/methanol (43:57) with 250 µL of trimethylamine in pH 6; ultrapure water/methanol (43:57) with 250 µL of trimethylamine in pH 8; methanol/perchloric acid (100 mL + 10 µL); ultrapure water/acetonitrile (53:47); ultrapure water/methanol (40:60); ultrapure water/methanol (35:65); ultrapure water /acetonitrile (40:60) and methanol/50 mmol L^{-1} ammonium acetate solution (60:40). Flow rate variations between 0.2-0.5 mL min^{-1} for the 50 mm columns and 0.5-1.5 mL min^{-1} for the 150 mm columns in both isocratic and gradient mode were also investigated.

Development of DLLME methodology for benzodiazepines in plasma samples

A DLLME method for the determination of benzodiazepines in plasma samples was developed using a LC-DAD with an isocratic elution consisting of a ultrapure water in pH 9 with trimethylamine: methanol:acetonitrile (63:19:18) mobile phase. Prior to any DLLME optimization steps, it was necessary to evaluate the biological matrix of choice (serum; plasma EDTA; plasma citrated or plasma heparinized) and a deproteinization step with methanol; ethanol; acetonitrile; acetone; trichloroacetic acid (10 and 20%); chloridric acid or trifluoroacetic acid.

Subsequently, DLLME optimization was performed via the following steps and their respective parameters: studies on extraction solvent (hexane, toluene, chloroform, dichloromethane and ethyl acetate) and dispersive solvent (methanol, acetonitrile, isopropanol, tetrahydrofuran (THF), ethanol and acetone); volume of extraction and dispersive solvents (100-500 µL; 400-800 µL, respectively); salting-out effect (0-3%); agitation by vortex with 2800 rpm and ultrasonic bath with 40 KHz frequency at 35 °C (0-60 seconds; 30-300 seconds, respectively); pH behavior (8-11); time and centrifugation speed (2-10 minutes; 2000-10000 rpm).

After DLLME optimization, validation of the method was performed according to the Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens from the United Nations Office on Drugs and Crime (UNODC) guidelines. The parameters evaluated were the following: limit of detection (LoD), limit of quantification (LoQ), specificity/selectivity, recovery, linearity, intra and inter-day precision and accuracy.[19] To access both carryover effect and dilution integrity, the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology guideline was used.[20] The internal standard diphenylamine was added at a concentration of 250 ng/mL.

Determination of benzodiazepines in plasma samples by DLLME

The DLLME procedure developed under this study was performed using 500 µL of citrated plasma, 250 ng/mL of diphenylamine and adjusted to pH 10 with a solution of NaOH 50%. Thus, 2.5% of NaCl (w/v) was added into the tube and homogenized using a vortex for 30 seconds. After that, 400 µL of chloroform and 700 µL of acetonitrile were simultaneously injected and the tube was closed and placed in the ultrasonic bath for 1 minute. Subsequently, the tube was centrifugated at 10000 rpm (Eppendorf® 5427 R model) for 10 minutes and the organic fraction (inferior phase) was transferred to a vial and dried under constant air flow without heat. For reconstitution of the dried extract, 30 µL of mobile phase were used and finally 20 µL were injected into instrument. Wavelengths of 254 nm for alprazolam, diazepam and nordiazepam; 320 nm for clonazepam and 280 nm for IS were used. The chromatograms obtained from the application of DLLME for benzodiazepines analysis in plasma samples according to the developed method, can be seen in the following Figure 1.

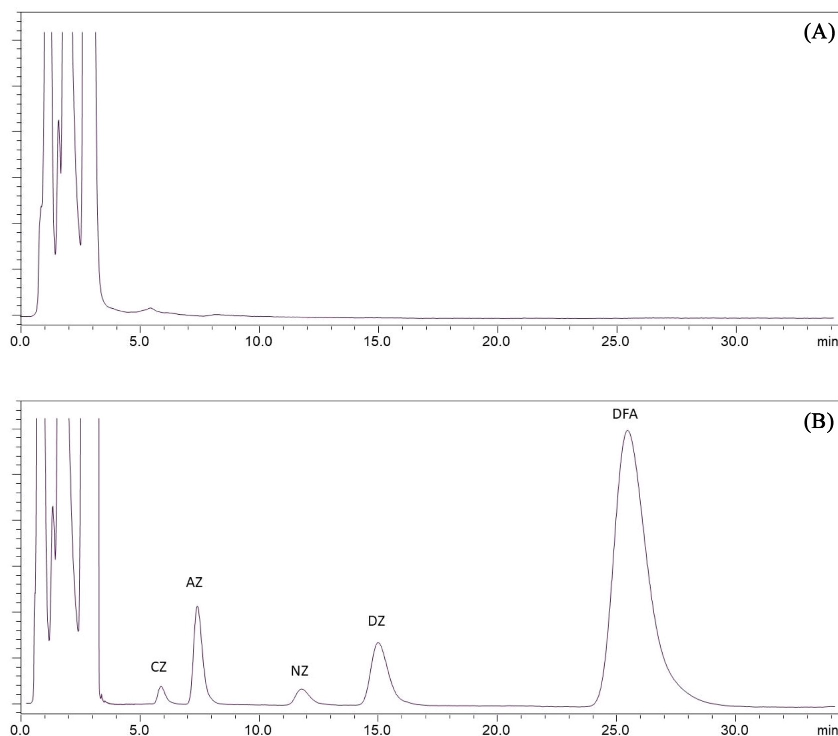

Figure 1. *Chromatograms obtained from the application of the optimized DLLME method for the determination of benzodiazepines in plasma samples. A) Blank plasma sample; B) Fortified plasma sample with the relevant benzodiapines and internal standard (IS). Retention times for clonazepam (CZ), alprazolam (AZ), nordiazepam (NZ), diazepam (DZ) and the IS diphenylamine (DFA) can be seen at 5.5, 7.3, 11.5, 14 and 26 min, respectively, at 254 nm*

Stationary phase consisted of a C18 column (150 mm length x 4.6 mm inner diameter x 3.5 µm particle size) while ultrapure water in pH 9 with trimethylamine/methanol/acetonitrile (63:19:18) was used as mobile phase at a flow rate 1 mL/min in isocratic mode.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Among all mobile phases tested, ultrapure water at pH 9 with triethylamine/methanol/acetonitrile in proportions of 63:19:18 yielded the best results for alprazolam, bromazepam, clonazepam, nordiazepam and diazepam in isocratic mode. Initially, mobile phase evaluations used a C18 column of 150 mm length x 4.6 mm inner diameter x 3.5 µm particle size as stationary phase and from all flow rates evaluated (0.5-1.5 mL/min) that of 1 mL/min was chosen due to its promising results. Posteriorly, a C18 columns with 50 mm length x 2.1 mm inner diameter x 3.5 µm particle size was evaluated.

Despite time reduction and mobile phase consumption, the 50 mm length x 2.1 mm inner diameter x 3.5 µm particle size column was unsable to separate all benzodiazepines standards, regardless of the mobile phase tested. In fact, we have observed co-elution between clonazepam and alprazolam as well as diazepam and nordiazepam in isocratic mode, regardless of the flow rate tested (0.2-0.5 mL min^{-1}). Thus, the length of the chromatographic column does influence the separation capacity of these compounds under the conditions tested.

Gradient mode analyses were evaluated in both analytical columns; however, co-elution problems continued in the smaller column. In addition, it was observed an excessively long period for rebalancing the initial condition necessary between injections to stabilize the chromatographic system (≥ 15 min), making it unfeasible for method validation. Therefore, the C18 column comprising of 3.5 µm x 4.6 mm inner diameter x 150 mm was established as stationary phase and separation was achieved in isocratic mode at flow rate of 1 mL min^{-1}.

Optimization of DLLME for benzodiazepines in plasma samples

Considering the composition of whole blood, serum and plasma, the matrix effect observed will be different according to the sample under study.[21-24] According to the DLLME procedure and taking into account biological matrix complexity, plasma was chosen as a biological matrix given that benzodiazepines are linked to plasma proteins due to their characteristic of high binding rate (above 70-90%). For toxicological analysis, a sample pre-treatment procedure is essential to remove endogenous interferences from the matrix.[25]

Deproteinization studies were carried out using methanol, ethanol, acetonitrile, acetone, trichloroacetic acid (10 and 20%) and hydrochloric acid with trifluoroacetic acid in a pool of serum samples; EDTA plasma; citrate plasma; heparin plasma and citrate, phosphate, dextrose, adenine (CPDA-1) plasma. Among all samples tested, citrate plasma treated with chloridric acid and trifluoroacetic acid showed the best conditions for deproteinization with minor presence of endogenous interferences.

However, this step has revealed significant loss of analytes in the supernatant, making it very challenging to quantify benzodiazepines by LC-DAD. Most likely, acidic hydrolysis was responsible for the conversion of 1,4-benzodiapines into 2-aminobenzophenones derivates as previously described in the literature.[26-28] For that reason, it was decided to directly perform DLLME in the plasma samples without any prior deproteinization step.

There are many aspects to be improved in the DLLME procedure, such as the extractor/dispersing solvents and their respective volumes, pH matrix, salting-out effect, agitation (if necessary), among others. In fact, DLLME is considered a challenge for complex matrices as described by Xiong and Hu.[29]

During experimental design, traditional extractor solvents (chloroform, hexane, ethyl acetate, dichloromethane and toluene) and low-density solvents (1-octanol, 1-nonanol, 1-decanol, 1-undecanol, 1-dodecanol and dihexyl ether) were evaluated as extractor solvent. Considering the coefficient partition of the target benzodiazepines in this study (Log P 2.4-3.1), an extractor solvent with similar Log P would represent a higher extraction capacity. For this reason, low-density solvents available in the method should be chosen based exclusively on their coefficient partition (Log P 2.58 – 4.55).

These chosen solvents were also applied to other DLLME studies involving drugs.[30-33] However, in the present study, this approach has failed due to the incompatibility of the mobile phase and the extracted portion (higher viscosity compared to traditional solvents). Consequently, we have observed an increase of the HPLC system pressure throughout a vast sequence of injections, making the chromatographic analysis not viable. In fact, low density solvents have a high temperature volatilization, which hinders the drying step and future resuspension. Therefore, the application of low-density solvents for DLLME would be suitable for Gas Chromatography (GC) analyses based on the injection volume and capillary column injection port temperature.

Among the traditional solvents found, toluene (Log P 2.73) showed the largest capacity of extraction for benzodiazepines, however, the high coefficient of variation between replicates and a rapid saturation of the pre-column was observed due to the carrying of endogenous interferents from the plasma matrix. As consequence, there was an increase in the cost of chromatograph consumables, in addition to the possible damage to the column and the instrument itself. Given that, chloroform was the extractor solvent of choice, obtaining satisfactory extraction efficiency and it also did not present the aforementioned negative effects.

Acetone was also evaluated along with THF, acetonitrile, ethanol, methanol, and isopropanol as dispersing solvent as well as the absence of dispersing solvent. The results showed that THF had the least extraction rate due to its acidic characteristic and it was incompatible with the mobile phase at pH 9.0. Among the dispersing solvents, acetonitrile was the solvent that best dispersed the extracting solvent in the plasma matrix, possibly allowing plasma deproteinization. Consequently, there was an increase of surface area and cloud point formation.

The choice of adequate pH values directly affects the selectivity of the method, allowing for non-ionized molecules to be captured by the extractor solvent, therefore improving the recovery rates of the analytes at the detriment of other compounds. A pH value of 10 was chosen, considering its high uniformity among all benzodiazepines analyzed. In fact, clonazepam has shown better results at this pH, which is extremely useful given that its dosage and therapeutic range are the lowest amongst all benzodiazepines evaluated.

As for the salting-out effect, the best extraction condition consisted of the addition of 2.5% NaCl (w/v). The ionic strength promoted an improvement of the extraction. Salt concentrations above 2.5% decreased the DLLME capacity to target benzodiazepines. Fernández and collaborators have added 40% NaCl (w/v) during the analysis of seven benzodiazepines in plasma samples, but their metabolites have not been studied.[13] Mashayekhi and Khalilian determined that the addition of salt had no significant effect on DLLME for three benzodiazepines in urine and plasma samples.[7] For liquid phase microextraction (LPME), Bairros and collaborators have

verified an increased efficiency when employing 10% NaCl (w/v) for nine benzodiazepines and 20% NaCl (w/v) for aminobenzodiazepines in urine samples.[34] These examples demonstrate that the salting-out effect has variabilities depending on the extraction procedure, matrix sample and specific benzodiazepines.

Initially, chemical dispersion strategy between extractor and dispersive solvents was evaluated, considering the traditional DLLME.[11,29] However, it was not possible to perform this procedure under such conditions, possibly due to the characteristics of the plasma samples, which are considered a matrix of greater complexity compared to urine samples and other aqueous matrices, avoiding the formation of the cloudy effect.[29] Therefore, we chose to systematically evaluate the effect of vortex agitation and ultrasonic bath, as previously highlighted in the scientific literature.[14,35-38]

The application of ultrasonic bath was also evaluated in the present study and compared to vortex homogenization. This study was carried out by homogenizing the samples by vortex or ultrasonic bath after simultaneous injection of the extractor and disperser solvents. It was found that 1 minute of ultrasonic bath did show greater extraction efficiency compared to that of vortex. Ultrasonic bath in DLLME is indeed described as a tool which increases the dispersion of the extractor solvent, improving efficiency and decreasing the extraction time by cavitation process when compared to the traditional DLLME.[35,37,38]

Perhaps the vibration from the ultrasonic bath revealed that there was a better dissolution of the emulsion present between the two phases, improving the transfer of analytes to the cloudy solution. Similar explanation was described by Meng and collaborators in urine samples for four new benzodiazepines (diclazepam, etizolam, flubromazepam and phenazepam).[18] However, over time, extraction solvent losses may occur due to the volatilization of chloroform in our experiment.

The volumes of the disperser and extractor solvents were also optimized in this study and efficiency variations were observed depending on the analyte under study. It was seen that volumes of 700 and 800 µL of dispersing solvent obtained similar results. Therefore, we opted for the addition of the smaller volume (700 µL). Extractor solvent volumes above 400 µL showed a worsening of the extraction rate, possibly due to the difficulty in generating a cloudy solution, which is essential for DLLME.

The last parameters evaluated was centrifugation (speed and time). These parameters influenced the sharpness of the phase separation at an appropriate time. The experiments revealed that 10 minutes was the time required for total phase separation at a rotation of 10.000 rpm. These parameters are crucial because the conditions provided a better separation of both aqueous and organic phases. It is important to mention that speeds below 10.000 rpm did not allow for the appropriate capture of the organic phase, hindering the reproducibility of the technique. A summary of the data from all optimized parameters can be seen in Figure 2.

Method validation

Once optimization was completed, a full validation of the method was performed according to the previously mentioned UNODC guidelines. All parameters were evaluated in sextuplicate for each concentration. Bromazepam had to be removed from the dispersing and extractor solvents evaluation and it was not validated given that the chromatographic peak showed matrix interference at the beginning of the chromatographic run.

Considering the present study and a full validation protocol, a distinct internal standard should be applied. Among all internal standards evaluated (medazepam, diphenylamine, etidocaine, bupivacaine, tetracaine, benzocaine and ethylmorfine), only medazepam and diphenylamine presented the appropriate conditions. Medazepam was discarded due to the high increase in the total run time as well as its consequent large delay in re-establishing the initial chromatographic conditions for gradient procedure, making the toxicological analysis unfeasible. Therefore, diphenylamine was chosen as an appropriate internal standard.

Linearity was performed from the limit of quantification for each analyte up until 1500 ng/mL. Calibration curves and their respective determination coefficients were obtained as follows: $y=16{,}077x+1188{,}5$; $r^2= 0{,}9983$ for clonazepam; $y=913{,}03x-35597$, $r^2=0{,}9992$ for alprazolam; $y=144{,}61x+15409$, $r^2=0{,}9928$ for nordiazepam and $y=269{,}92x+51751$, $r^2=0{,}9936$ for diazepam.

The specificity/selectivity parameter has proven that the method did not show interferences in plasma samples and prescribed drugs tested. Regarding the intra and inter-day precision, clonazepam did not show reproducibility during the three consecutive days tested while diazepam and nordiazepam were not reproducible in at least one concentration tested, which would invalidate the method. The inaccuracy showed these analytes was outside the range of 80 to 120% in at least one concentration tested, as specified by the UNODC guidelines. Recovery rates for all analytes varied, however according to the guidelines, this is not considered as an indicative of method validation failure.[19]

No memory effect was observed when evaluating six blank replicates after a 1500 ng/mL sample injection. Finally, the dilution integrity has shown that a 25 times diluted sample (160 ng/mL) from a benzodiazepine pool at a concentration of 4 µg/mL containing an internal standard which a variation of 20% is accepted to guarantee the fidelity of the result.[20] In our results, nordiazepam showed unacceptable value for precision parameter while accuracy parameter did not reach the required values for clonazepam, diazepam and nordiazepam.

Despite the fact that diphenylamine has provided the best results, the application of six replicates during the validation of the method for each concentration, as recommended by the validation guideline followed, showed high standard deviation among the replicates for accuracy, intra and inter-day precision assays. This behavior was not observed during the optimization steps.[19] Considering these guidelines, it was not possible to obtain a completely successful validation of the method proposed. All discussed parameters for the validation of benzodiazepines in a plasma sample can be seen in Table 1.

The first study of DLLME being applied to the analysis of benzodiazepines was performed by Melwanki and colleagues and aimed to determine 7-aminoflunitrazepam (log P=1.3) by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) using conventional DLLME.[12] Since then, other DLLME studies were performed for the study of benzodiazepines in biological and non-biological matrices with different strategies.

DLLME studies reported in the literature using HPLC-UV in plasma samples, showed 2.7 ng mL^{-1} as limit of detection (LoD), a linearity range of 9-100 ng mL^{-1} and 94.3% recovery for chlordiazepoxide only.[39] Mashayekhi and Khalilian, reported a linearity range of 2.5-500 ng mL^{-1} for both alprazolam and oxazepam and 1-500 ng mL^{-1} for diazepam. LoD values were of 0.7 ng mL^{-1} for alprazolam and oxazepam and 0.4 ng mL^{-1} for diazepam, limit of quantification (LoQ) was 2.5 ng mL^{-1} for alprazolam and oxazepam and 1.5 ng mL^{-1} for diazepam with a recovery of 86%, 84% and 90% for alprazolam, oxazepam and diazepam, respectively.[7]

From all of the DLLME studies involving benzodiazepines discussed in this manuscript, Fernández,[13] Fisichella[35] and De Boeck[8] and their respective co-authors were the only research groups able

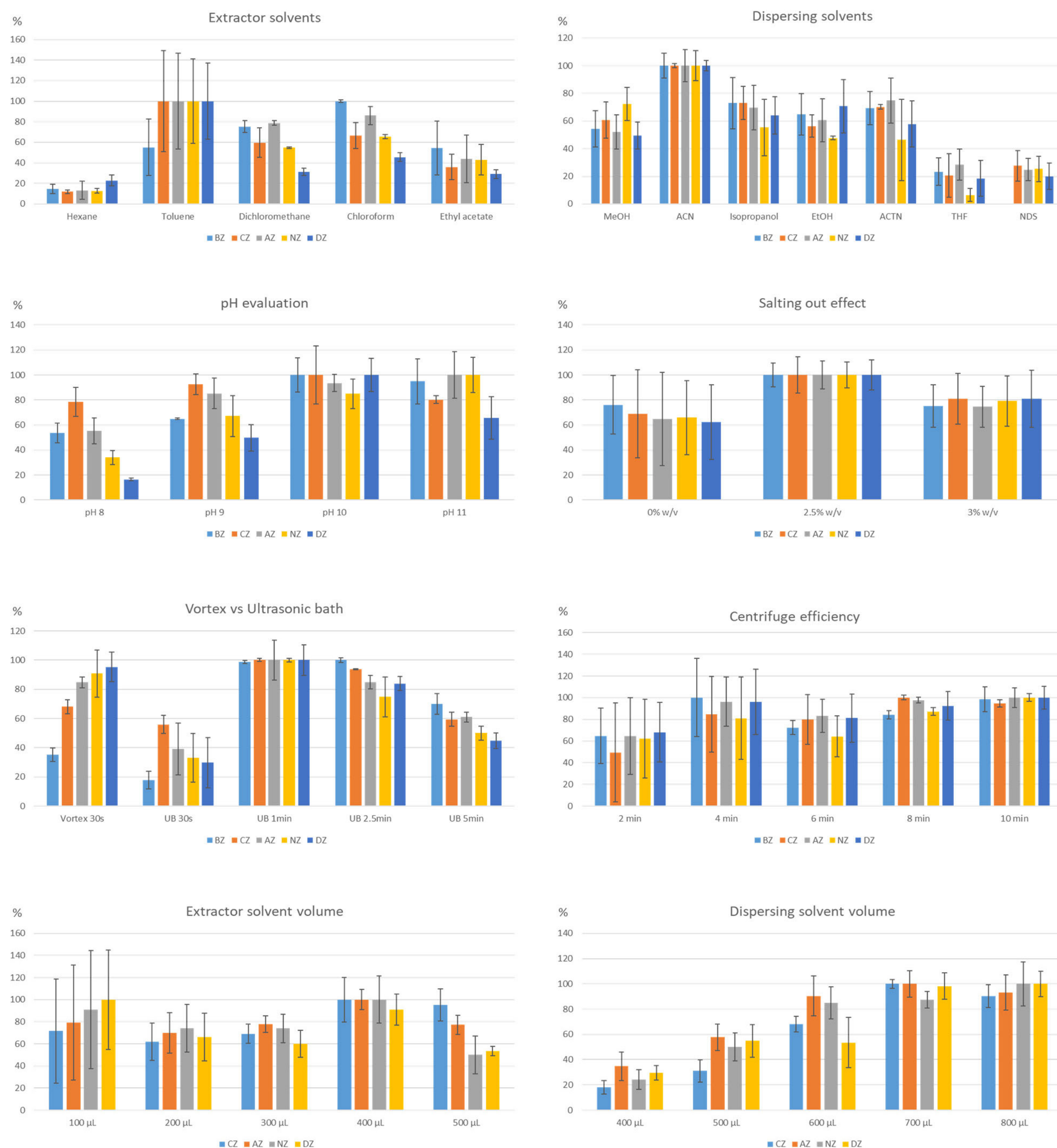

Figure 2. *Optimization of DLLME for benzodiazepines in plasma samples. AZ - alprazolam; BZ - bromazepam; CZ - clonazepam; DZ - diazepam; ND - nordiazepam; MeOH - methanol; ACN - acetonitrile; EtOH - ethanol; ACTN - acetone; THF - tetrahydrofuran; NDS - no dispersing solvent; UB - ultrasonic bath. Vortex with 2800 rpm and ultrasonic bath with 40 KHz frequency at 35°C. Centrifugation speed (10000 rpm)*

to perform a full validation with application of the method to real samples. Fernández and collaborators investigated DLLME in plasma samples using an LC-DAD and did not follow a specific validation guideline. In fact, the parameters described by the authors used triplicates for each concentration evaluated during validation. For intra and inter-precision, they have stated the use of two different concentrations.[13]

Fisichella and De Boeck and their respective collaborators validated their methods in whole blood by LC-MS/MS.[8,35] In spite of Fisichella et al. not following a specific validation guideline, all parameters were explained in the manuscript and performed in different concentrations with five replicates for each level. Nevertheless, two-way ANOVA was not used to evaluate intra and inter-day precision.[35]

De Boeck and co-authors have successfully followed internationally accepted validation guidelines for bioanalytical methods for the determination of benzodiazepines and benzodiazepine-like hypnotics, including the addition of statistical resources. Hence, this is the only study that reports the phenomenon of heteroscedasticity, ensuring greater safety in the quantification of analytes.[8] Additionally, De Boeck et al. applied ionic liquids-DLLME to whole blood to determine multiple benzodiazepines to be used in 11 *postmortem*

Table 1. Validation parameters of the developed method for the determination of benzodiazepines in plasma samples (six replicates for each point), including recovery; LoD; LoQ; intra and inter-day precision; accuracy and dilution integrity

Parameters	Alprazolam	Clonazepam	Diazepam	Nordiazepam
Recovery (%)				
C1	94.53	116.54	49.04	37.11
C2	80.52	65.28	256.06	74.16
C3	157.23	124.84	143.44	138.89
LoD (ng mL^{-1})	40	50	50	50
LoQ (ng mL^{-1})	50	50	60	60
Intra-day precision (RSD %)				
C1	2.12	31.28	23.85	17.71
C2	5.50	36.21	13.09	25.93
C3	10.82	39.42	21.74	17.13
Inter-day precision (RSD %)				
C1	4.57	49.97	32.22	82.20
C2	9.27	23.34	2.70	42.69
C3	8.21	47.43	6.98	9.62
Accuracy (%)				
C1	133.04	97.50	63.97	46.61
C2	86.26	76.09	99.17	93.96
C3	82.72	75.30	84.79	90.06
Dilution integrity				
Precision (RSD%)				
25 times (160 ng mL^{-1})	5.35	17.95	12.89	25.16
Accuracy (%)				
25 times (160 ng mL^{-1})	86.17	59.09	42.41	22.85

Recovery C1, 50 ng/mL (alprazolam and clonazepam) and 60 ng/mL (nordiazepam and diazepam); Accuracy C1, 60 ng/mL (alprazolam and clonazepam) and 75 ng/mL (nordiazepam and diazepam). For other parameters, 130 ng/mL and 430 ng/mL for C2 and C3, respectively; RSD%, relative standard deviation %; 25 times, 4000 ng/mL.

cases. The efficiency of ionic liquids in this procedure was given by their properties of thermal stability, low vapor pressure and variable selectivity due to structural flexibility, showing a green chemistry technique with full validation.[36] A summary of the procedures which have used DLLME for the analysis of benzodiazepines, available in the scientific literature, can be seen in Table 2.

Other studies reported in this manuscript did not carry out an adequate full validation as it can be seen in Table 2. In fact, the vast majority of all DLLME studies were based on one up to three validation parameters with three replicates for each concentration. Therefore, there are no details available for the validation step throughout the manuscripts described here. During the validation of the proposed analytical methodology, according to the UNODC guidelines,[19] we observed that duplicates and triplicates for each parameter tested, indeed, did not show large coefficients of variation. However, there was an increase in the relative standard deviation (RSD%) when using six replicates, mainly for intra and inter-day precision as well as accuracy in the tested conditions.

It should also be highlighted that there was no statistical approaches described for the analysis of both intra and inter-day precision assay in the totality of the studies reported and summarized in Table 2, with the exception of Boeck *et al.*[8] In fact, there were studies which have not evaluated accuracy, which is a crucial method validation parameter.

In regard to linearity, the evaluation of homoscedasticity/heteroscedasticity was not described in most of the methodologies, mainly in larger linear ranges. The present work, we have also included the study of both dilution integrity and robustness parameters, which, similarly have not been shown in the reviewed literature for this topic.

Considering that, in intoxication cases it is not uncommon to encounter high benzodiazepine concentrations, possibly above the highest point of the studied calibration curve, the inclusion of the dilution integrity parameter at known levels (eg: 1:10; 1:5) is rather important and needed to ensure reliable results. As for robustness, given that it is a measure of the method's capacity to remain unaffected by small and deliberate variations, the risk of finding out that a given method does not fulfill this criteria, late in the validation process, may result in the need for it to be redeveloped and optimised.[44]

Despite the issues encountered in the validation process of the proposed method, this was evaluated using different LC-DAD models in different laboratories by different laboratory technicians and slight variations in mobile phase pH ($\pm$ 0.3) values based on data obtained from optimization studies of chromatographic conditions. In these assays, we did not observe significant changes in the retention time of the analytes as well as in the chromatographic response after the DLLME procedure.

Therefore, accuracy, precision and linearity parameters without the proper analysis as well as robustness test established by the validation guides may result in erroneous data in these methodologies. That is why, the reproducibility of analytical methodologies reported, to be used future laboratories worldwide, must be as transparent and reliable as possible.

Indeed, these observations reinforce the need to carry out such a process in order to guarantee the reliability of the results, even more

Table 2. Use of DLLME for the determination of benzodiazepines

Matrix	Analytes	Instruments	Detectability (ng mL^{-1})	Extraction essay	Validation parameters	Reference
Urine	7-AF	LC-ES-MS/MS	0.025	DLLME	Reproducibility, linearity and limit of detection.	12
Urine	AZ, DZ, OZ	HPLC-UV	0.3- 0.7	DLLME	Linearity and limit of detection.	16
Plasma	AZ, BZ, CZ, DZ, LZ, LM and TT	UPLC-PDA	1.7 – 10.6	UA-DLME	Linearity (7 points in triplicate), selectivity (without description), limit of detection, limit of quantification, intra and inter-precision (2 concentrations), accuracy and recovery	13
Water, urine, plasma, and pills	CD	HPLC-UV	0.5	DLLME	Limit of detection, limit of quantification, linearity (7 points in triplicate), accuracy (2 concentrations)	39
Ultrapure water, tap water, fruit juice, urine	DZ, MZ and AZ	GC-FID	0.1- 0.2	SPE-DLLME	Linearity (triplicate for each point), limit of detection, limit of quantification.	40
Whole Blood	7-AC, 7-AF, 7-AN, α-HA, α-HE, AZ, BZ, CD, CB, CZ, DL, DF, DZ, EZ, FN, FB, HT, LZ, LM, MZ, NT, OZ, TZ and TO	UHPLC-MS/MS	2.0	UA-DLLME	Limit of detection, limit of quantification, specificity, linearity, accuracy (5 replicates for 3 points), intra and inter-day precision (5 replicates for 3 points for 3 three days), recovery and matrix effect.	35
Serum	NT and MZ	HPLC-MWD	0.017 and 0.086	UA-SEME-SFOD-DLLME	Linearity (10 levels in triplicate), limit of detection, limit of quantification, recovery, intra and inter-day precision	41
Urine	CD, EZ, TZ, MZ, CZ, LZ, LM and ME	CE-DAD	50- 100	DLLME-FASS	Linearity, limit of detection, limit of quantification, intra and inter-precision, accuracy, selectivity, recovery.	17
Urine, plasma	OZ, AZ and DZ	HPLC-UV	0.4- 0.7	SPE-DLLME	Linearity, limit of detection, limit of quantification, intra-day precision (2 levels in 5 replicates).	7
Pills, plasma and urine	CP, DZ, SR	HPLC-DAD	2.7	UA-DLLME-SFOD	Linearity, limit of detection, limit of quantification, intra-day precision (6 replicates for 3 leves).	42
Plasma, urine and water	AZ, DZ, FR and MZ	GC-μECD	0.005-0.01	BE-DLLME	Linearity, enrichment factor, limit of detection and quantification, intra and inter-precision, selectivity and matrix effect.	43
Whole blood	7-AF, AZ, BZ, CB, CZ, CT, DZ, EZ, LE, EI, FR, LM, MZ, OZ, PZ, TZ, TO, ZP and ZC	LC-ESI-MS/MS	0.003 – 4.74	IL-DLLME	Selectivity, linearity with heteroscedasticity study, accuracy, intra and inter-precision (5 days), limit of quantification, limit of detection, recovery, accuracy, matrix effect and stability.	8
Urine	FE, DC, FB and EI	GC-QQQ-MS	1 – 3	UA-LDS-DLLME	Selectivity, linearity, accuracy, intra and inter-precision, limit of quantification, limit of detection, recovery.	18
Whole blood	7-AF, AZ, BZ, CB, CZ, CT, DZ, EZ, LE, EI, FR, LM, MZ, OZ, PZ, TZ and TO	LC-ESI-MS/MS	0.003 – 4.74	IL-DLLME	It was based Boeck and co-authors method[8].	36
Citrated plasma	AZ, CZ, DZ and NZ	HPLC-DAD	40-50	UA-DLLME	Limit of detection, limit of quantification, selectivity, specificity, linearity, intra and inter-day precision, accuracy, recovery, robustness, memory effect and dilution integrity.	Proposed method

LC - Liquid chromatography, UV - Ultraviolet, MS - Mass spectrometer, CE - Capillary electrophoresis, μECD – micro electron capture detector, MEKC - Micellar electrokinetic chromatography, HPLC - High-performance liquid chromatography, GC - Gas chromatography, QQQ - Triple quadrupole, MWD - Multiple wavelength detector, ESI - Electrospray ionization, FID - Flame ionization detector, DAD or PDA - Diode-array detector or Photodiode-array detector , DLLME - Dispersive liquid-liquid microextraction (conventional), BE- Back extraction, UA - Ultrasonic-assisted, SPE - Solid phase extraction, SEME - Surfactant-enhanced emulsification microextraction, SFOD - Solidified floating organic drop microextraction, FASS - Field amplified sample stacking, IL - Ionic liquid, UA-LDS-DLLME - Ultrasonic-assisted low-density solvent dispersive liquid-liquid microextraction, MEPS - Microextraction by packed sorbent, CA - Cyclodextrin-assisted. 7-aminoclonazepam (7-AC), 7-aminoflunitrazepam (7-AF), 7-amino nitrazepam (7-AN), α-hydroxy alprazolam (α-HA), α-hydroxyethyl flurazepam (α-HE), alprazolam (AZ), bromazepam (BEZ), citalopram (CP), clobazam (CB), clonazepam (CZ), chlordiazepoxide (CD), clotiazolam (CT), diazepam (DZ), delorazepam (DL), desalkylflurazepam (DF), diclazepam (DC), estazolam (EZ), etizolam (EI), phenazepam (FE), flunitrazepam (FN), flurazepam (FR), flubromazepam (FB), hydroxytriazolam (HT), ethyl loflazepate (LE), lorazepam (LZ), lormetazepam (LM), medazepam (ME), midazolam (MZ), nitrazepam (NT), nordiazepam (NZ), oxazepam (OZ), prazepam (PZ), temazepam (TZ), tetrazepam (TT), and triazolam (TO).

so if one is intending its application for real case samples in the field of toxicological analysis.

CONCLUSIONS

It was not possible to achieve a full validation of the proposed methodology for the determination of low concentrations of benzodiazepines in plasma using DLLME by LC-DAD in accordance with both UNODC and SWGTOX guidelines. However, this is exactly where the strength of this study lies, given that it could avoid future unnecessary experiments for other scientific groups, reducing their time and cost.

In view of the critical aspects on DLLME for benzodiazepines, we have found that most of the methods developed did not apply full validation studies, which may compromise the results obtained, especially considering their application to real toxicological cases. Thus, we have concluded that a full method validation is paramount to provide adequate, reproducible, and trustworthy analytical results using the proposed methodology in this study.

ACKNOWLEDGMENTS

The authors would like to thank Coordination of Superior Level Staff Improvement (CAPES) for scholarship provided to Geovane de Almeida Saldanha.

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