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Dispersive Liquid-Liquid Micro-Extraction as a Sample Preparation Method for Clonazepam Analysis in Water Samples and Pharmaceutical Preparations

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A B S T R A C T

Dispersive liquid–liquid micro–extraction (DLLME) technique was successfully used as a sample preparation method for the determination of clonazepam in pharmaceutical preparations and water samples. In this method, a suitable mixture of methanol (disperser solvent) and chloroform (extraction solvent) was injected rapidly into a conical test tube that contained an aqueous solution of clonazepam. After centrifuging, phase separation was performed by sedimenting the fine droplets of the micro–extraction solvent on the bottom of a test tube (about 100 μL) and then the absorbance of the enriched extracted phase was determined at the absorption wavelength of clonazepam (307 nm). Some important parameters such as, the type and volume of extraction and dispersive solvents as well as the extraction time were investigated and optimized. Under the optimum conditions, the calibration graph was linear over the range of 0.95 to 18.94 μg/mL of clonazepam with a limit of detection of 0.06 μg/ml.

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Introduction

Clonazepam, 5–(2–chlorphenyl)–1,3–dihydro–7–nitro–2H–1,4–benzodiazepine–2-one (Scheme 1), is a benzodiazepine drug. Benzodiazepine drugs have hypnotic, tranquillizing, anticonvulsantand muscle relaxant properties. They are the drugs of choice for treatments of anxiety, sleep disorders, status epilepticus and insomnia [1, 2]. Because of these properties they are frequently encountered in clinical and forensic casework samples involving drug overdoses. Clonazepam has a $t^{1/2}$of 18–60 h. Nearly 2% of the dose is excreted in urine as unaltered drug [3].Furthermore, the residuals of pharmaceuticals in surface as well as underground waters may induce serious damages to the human life. Different analytical methods have been used for the determination of benzodiazepines based on high performance liquid chromatography [4-6], voltammetry [7, 8], gas chromatography [9, 10],flow injection analysis [11] and spectrophotometry[12, 13].

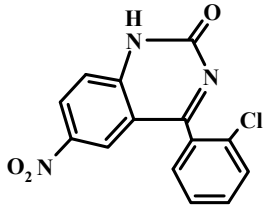
Trace analysis of many toxic chemicals is required; therefore, preconcentration step prior to the determination methods have been developed during past decades. The sample preconcentration step typically consists of the extraction of the components of interest from a sample matrix. Recent research activities are being focused on the development of efficient, economical, and miniaturized sample preparation methods. Consequently, among other methods, solid-phase microextraction (SPME) [14-16], solvent micro-extraction (SME) [17, 18], liquid–liquid micro-extraction(LLME) [19], liquid-phase micro-extraction (LPME) [20, 21] and single drop micro-extraction (SDME) [22, 23] have been used extensively.

Dispersive liquid–liquid microextraction (DLLME) was demonstrated by Assadi and co-workers previously [24] as a simple and efficient preconcentration method. In this method, an appropriate mixture of the extraction solvent and the disperser solventis injected into the aqueous sample by a syringe to form a cloudy solution. The cloudy state results from the formation of fine droplets of the extraction solvent which are dispersedin the sample solution. The cloudy solution will be centrifuged and the fine droplets sediment at the bottom of a conical test tube. Determination of analytes in the remainedphase can be performed by instrumental techniques.

Simplicity of the operation, speed, low sample volume, low cost, high recovery and high enhancement factor are some advantages of DLLME. The performance of DLLME was illustrated with the determination of polycyclic aromatic hydrocarbons (PAHs) [24], organoph-osphorus pesticides (OPPs) [25], chlorobenzenes [26], trihalomethanes (THMs) [27], chlorophenols [28], selenium [29] and cadmium [30] in water samples.DLLME has been used as a preconcentration step in many significant determinations of organic and inorganic compounds [31-35] as well.

The increasing attention, on pharmaceutical residues as potential pollutants in water samples [36-38], is due tothe fact that they often have similar physico-chemical behaviour than other harmful xenobiotics which are persistent or produce adverse effects. In addition, by contrast with regulated pollutants, which often have longer environmental half-lives, its continuous introduction in the environment may make them “pseudopersistents”.

The present paper describes a simple and reliable extractive-spectrophotometric method for the determination of clonazepam using DLLME. To the best of our knowledge, thisstudy may be the first report describing the application of DLLME spectrophotometric method for determination of the drug in water samples. The effects of various experimental parameters, such as the kind and volume of extraction and disperser solvents, pH of thesample solution, extraction time and salt effect have been optimized.



Scheme 1. Chemical structure of clonazepam

Materials and Methods

Instrumentation

A Hewlett-Packard 8453 diode-array spectroph-otometer controlled by computer and equipped with a quartz micro-cell (1.0 mm path length) was used for UV-Visible spectra acquisition.A Jenway 3345 pH/mV meter using a combined glass electrode was used for pH measurements of the experimental solutions. A Denley bench centrifuge model BS400 (Denley Instruments Ltd., Billingshurst, UK) was used to accelerate the phase separation.

Reagent and solutions

All reagents used were of analytical reagent grade. Doubly distilled water was used throughout the experiment. Pure clonazepam powder was purchased from Sun Pharma (India). Clonazepam tablets containing 2.0 mg clonazepam were purchased from a local market.All solvents used were obtained from Merck (Darmstadt, Germany). A stock solution of clonazepam (158µg/ml) was prepared in 10 % (v/v) methanol and stored in the dark at 4°C. Working standard solutions were prepared freshly by appropriate dilution of the stock solution.

Effect of experimental variables

In order to optimize the experimental conditions, a variable-at-a-time method was used. For example, to investigate the effect of pH on the recovery of clonazepam, the pH of the solution was varied between runs, other parameters were kept constant. The extent of extraction, which was directly proportional to the absorbance of the sedimented phase, was measured and plotted against the different values of the variable.

Calibration curve

Different standard solutions of clonazepam (5 – 500 µM) were prepared by dilution of the stock solution. A calibration curve was constructed by measuring the absorbance of the solutions at 307 nm. Then, each of the sampleswerepreconcentrated by DLLME and the absorbance of the sedimented phase was measured. Using the new data, another calibration curve was plotted and compared with that without DLLME.

Assay procedure for clonazepam tabletsand water samples

Tablets: Ten clonazepam tablets (2mg/tablet) were weighed and the average mass per tablet was determined. An amount of the powder equivalent to 2 mgof clonazepam was accurately weighed and dissolved in 10% (v/v) methanol by sonication. The solution was filtered through a filter paper (Whatman No.6) into a 25 mL volumetric calibrated flask and diluted to the volume with ultra pure water to achieve a final concentration of 79 µg/mL.A sample solution was prepared (2.84 µg clonazepam/mL) based on the labeled amount and examined for the drug content by using DLLME as a preconcentration step.

Water samples

Two water samples were collected from tap and spring sources. In order to evaluate the usefulness of the method for the analysis of clonazepam residual in water samples, known amounts of the drug (in the range of the calibration curve) were spiked to the samples and after DLLME, the absorbance of the sedimented phase was measured at 307 nm.

Dispersive liquid-liquid micro–extraction procedure

An appropriate volume (2.3 ml) of methanol, as disperser solvent, containing 300µl of chloroform, as extracting solvent, was injected rapidly into a sample solution (clonazepam 3.16 mg/Land sodium chloride, 0.1 mol/L). Acloudy solution containing fine droplets was rapidly produced, in which clonazepam was extracted. The mixture was centrifuged at 4000 rpm for 2 min and the dispersed fine droplets of chloroform were settled.The supernatant aqueous phase was readily decanted with a Pasteur pipette.The remained organic phase was diluted to 200µL with chloroform and the absorbance was measured at 307 nm against a reagent blank.Figure 1 shows the absorption spectrum of clonazepam in chloroform. Although clonazepam has a higher molar absorptivity at 250 nm, and a higher sensitivity will be obtainable, due to the interferences at this region, 307 nm was selected for the measurement of the absorbance of the sedimented phase. The volume of the sedimented phase was determined to be about 100 µL.

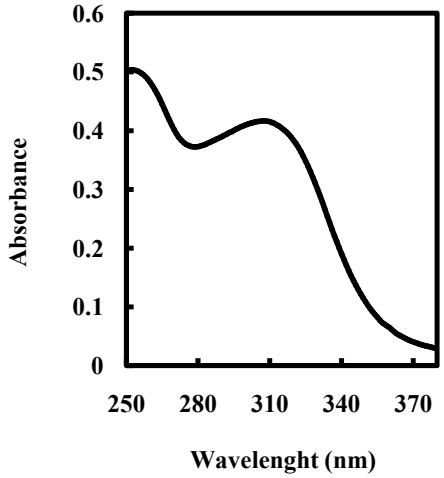


Fig.1. Absorption spectrum of clonazepam in chloroform after DLLME. Initial concentration of clonazepam was 9 µM.

Results and Discussion

A one-variable-at-a-time optimization strategy was used in order to evaluatethe experimental parameters that affect the dispersive liquid–liquid microextraction of clonazepam. The concentration of clonazepam was 3.16 mg/L throughout the optimization experiments.

Effect of type and volume of the extraction solvent

The selection of a suitable extraction solvent is very important for the DLLME process. The extracting solvent has to meet some properties such as:low solubility in water, extraction capability of the analyte and have ahigher densitythan water. Hence, carbon tetrachloride (density, 1.59 g/mL) and chloroform (density, 1.48 g/mL) were selected as extracting solvents in this work.The obtained recoveries for clonazepam were higher in the case ofchloroform, therefore, $CHCl_3$ was selected as the extraction solvent.

In order to study the effect of the volume of chloroform on the extraction efficiency, different volumes of $CHCl_3$ and a constant volume of the dispersive solvent (1500µL of methanol) were used. As the volume of chloroform increased (up to 300 µL), more clonazepam was extracted; after which, due to the increase in the volume of the sedimented phase, the concentration of the extracted clonazepam decreased (Fig. 2). Based on these observations, a volume of 300 µLof chloroform was used for further experiments.

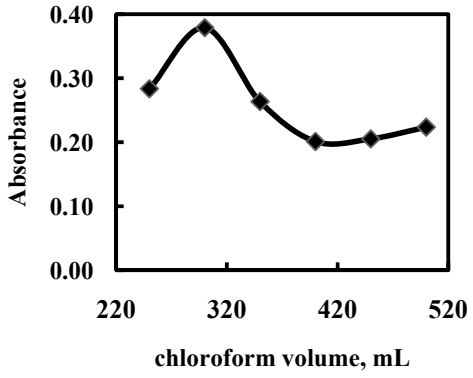


Fig. 2. Effect of chloroform volume on the efficiency of the extraction of clonazepam. Other experimental conditions: [Clonazepam] = 3.16 mg/L; Sample volume = 8 mL; V_{MeOH}= 1500 µL); [NaCl] = 0.1 M; Centrifuge rate = 2000 rpm; Centrifuge time = 2 min.; λ_{max} = 307 nm.

Effect of type and volume of the disperser solvent

Miscibility of disperser solvent in organic phase (extraction solvent) and aqueous phase is the main point for selection of disperser solvent [22].Therefore, it candisperse extraction solvent into very fine droplets in aqueous phase.

Methanol and ethanol were examined as dispersers.The maximum extraction efficiency of DLLME was obtained by using methanol as the disperser solvent.

The volume of disperser solvent is one of the importantfactors to be considered in DLLME. When the volume of the dispersive solvent is increased, two competitive processes occur. Oneis the increase in the solubility of analytes in the aqueous phase leading to diminished partition of the polar compounds into chloroform droplets. On the contrary, an increased volume of the partitioned methanol into chloroform phase leads to increase in theextraction efficiency. To obtain the optimized volume of methanol, various volumes of methanol (0.5–2.2 mL) containing 300 µL of chloroform were used in DLLME experiments. The results showed that the extraction efficiency increased with increase of the volume of methanol up to 2 mL (Fig. 3). Reduction in the extraction efficiency, due to enhanced solubility of clonazepam in aqueous phase, was observed when the volume of methanol exceeded 2 mL.

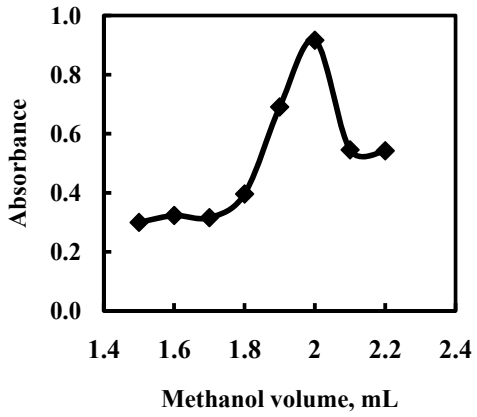


Fig. 3. Effect of methanol volume on the efficiency of clonazepam extraction. Other experimental conditions are as in Fig. 2.

Effect of pH

The effect of pH of aqueous solution on DLLME extraction of clonazepam was studied over the range of 2.0–12.0 (Fig. 4). The results reveal that the absorbance is initially increased by rising pH to 6 and then decrease at higher pH values. According to the reported pK_a values [39] for clonazepam (1.5, 10.5), the drug that is protonated at highly acidic solutions (Scheme 2), becomes neutral at higher pH values. It seems reasonable that at pH 6, clonazepam is mainly in its neutral deprotonated form which is more likely to be extracted into organic phase. At alkaline conditions, the enolic form of the drug is stabilized which is relatively weakly extracted due to the presence of charge on the molecule.

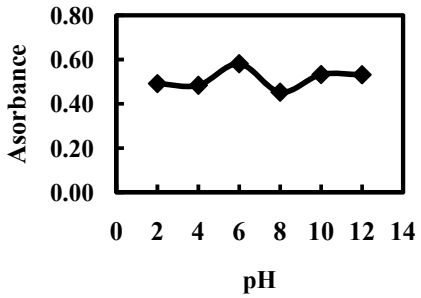
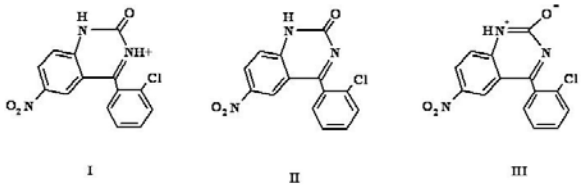


Fig. 4.Effect of pH on the efficiency of clonazepam extraction. Other experimental conditions are as in Fig 2.



Scheme 2. Clonazepam in: I, acidic; II, Methanolic; III, Alkaline solutions [13].

Effect of salt concentration

The addition of salt often improves extraction of analytes in conventional liquid–liquid micro–extraction, as a result of the salting-out effect. Various experiments were performed by adding different amounts of sodium chloride (0 –75 mg).
As was mentioned, at pH 6, clonazepam is mainly in neutral form and its extraction is not expected to be sensitive to ionic strength. But the solubility of extraction solvent (chloroform) in aqueous phase is dependent on salt concentration [25], therefore, the volume of sedimented phase as well as enrichment factor varies. Up to 45 mg of NaCl (in 8.0 mL of the mixture), the solubility of chloroform decreased and clonazepam was extracted to a proper volume of the sedimented phase, after which, due to the increased solubility of chloroform in water, the volume of the optimized sedimented phase and, as a result, the efficiency of clonazepam extraction decreased.

Effect of extraction time

Mass-transfer is a time-dependent process. The extraction time is defined as the time interval between the addition of the mixture of dispersive solvent (methanol) and extraction solvent (chloroform) to the sample andthe start of centrifugation. The effect of extraction time was studied over the time range between 0 - 10 min. The results indicated that the extraction time almost had no impact on the extraction recoveries. It was revealed that after the formation of a cloudy state of the solution, the surface area between extraction solvent and aqueous sample phase is infinitely large. In such a case, the equilibrium state can be achieved quickly and therefore the extraction time required could be very short [22]. Unlike SDME and SPME, the establishment of the equilibrium in DLLME was not a time- consuming step. On the other hand, there is a possibility for the SDME and SPME techniques not to reach equilibrium.The short extraction time is one of the remarkable advantages of the DLLME technique. In this method, 2 min was selected as the extraction time.

Effect of centrifugation rate

A series of identical solutions were tested in DLLME experiment at various rates of centrifugation. The rate of centrifugation was adjusted at 1000, 2000, 3000, 4000, 5000 and 6000 rpm for 5 minutes. The concentration of clona-zepam in sedimented phase increased slowly with increasing the rate to 4000 rpm andafter that, it was approximately constant. Therefore, 4000 rpm was selected as the optimum centrifugation rate.

Analytical performance

Table 1 summarizes the analytical characteristics of the optimized method, including linearrange, limit of detection, reproducibility and enhancement factor. Calibration graph (Fig 5a) was obtained afterDLLME of 8.0 mL of standard solutions of clonazepam. The sedimented phase (≈100 μL) was

diluted to 200 µL with chloroform and the absorbance was measured. Thelinear concentration rangewasfrom0.95 to 18.94µg/mL of clonazepamwithlinear regression equationas:

A = 0.153C + 0.003, **r^2= 0.998**,

Where Aistheabsorbance, C is theconcentrationof clonazepam(µg/mL) and r is correlationcoefficient.
The limit of detection (LOD) , calculated as three times the standard deviation of the blank measurement divided by the slope of the calibration curve ($3s_b/m$), was found to be 0.06 µg/ml.
For comparison, Fig. 5b shows the calibration curve for clonazepam before DLLME in aqueous solution. As is obvious, the sensitivity of the determination (slope of the regression line) is much lower than Fig. 5a. On the other hand, due to the low concentrations of clonazepam, the absorbances are in a low range accompanied with inherent uncertainty.
The enhancement factor was calculated as the ratio of the slope of the obtained linear calibration curve after DLLME (0.153) to the slope of the linear curve before extraction (0.002). An enhancementfactor of 76.5 was achieved. Compared to some previous reports on clonazepam analysis [11, 12], the present work shows good linear range and limit of detection.

Table 1. Analytical characteristics of spectrophotometric determination of clonazepam after DLLME.

Linear range (µg/mL)	0.95 – 18.94
r^2	0.998
Limit of detection (µg/mL)	0.06
Enhancement factor	76.5
Sample volume (mL)	8.0

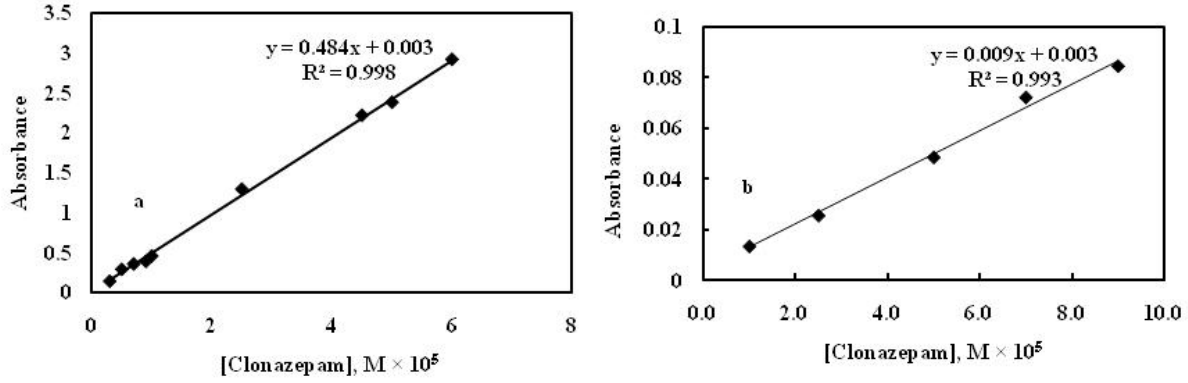


Fig.5. a) Calibration curve for spectrophotometric determination of clonazepam after DLLME. b) Before DLLME. Experimental conditions: Sample volume, 8.0 mL; V_{CHCl3} = 300 µL; V_{MeOH} = 300 µL; [NaCl] = 0.1 M; pH = 6; t = 2 min.; Centrifuge rate = 4000 rpm.

Analytical applications

Validity of the proposed method was checked by determining clonazepam in tablets, and by calculating the recovery of clonazepam that wasspiked to tap water and spring water (TaqBostanspring- Kermanshah). Recovery was calculated as the percent ratio of the drug concentration obtained by the proposed method to the expected one. The results obtained are given in Table 2, which show the usefulness of DLLME to concentrate very small amounts of clonazepam in different samples.

Table 2. Determination of clonazepam in real samples using DLLME.

Sample	Spiked, µg/mL	Found, µg/mL	Recovery[a] %	RSD%[b]
Tap water	2.21	2.27	102.7	2.66
	3.16	2.98	94.5	2.99
Spring water	2.21	2.26	102.3	2.92
	3.16	2.93	92.7	1.44
Tablet	Amount (µg/mL)	Found (µg/mL)		
	2.84	2.60	91.3	3.96

Conclusions

In order to determine trace amounts of clonazepam (from the benzodiazepine category) in pharmaceutical preparations and water samples, DLLME coupled to UV-Visible spectrophotometry was used. The effective parameters on DLLME such as, the kind and volume of extraction and dispersing solvents, salt concentration and time of centrifugation were optimized. Methanol and chloroform were selected as dispersive and extraction solvents, respectively. A preconcentration factor of 51 was obtained which shows the concentrating ability of the method. The analytical applications of the method in real samples were quite satisfactory.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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