Modified high performance liquid chromatographic method for the identification of chlordiazepoxide in animal blood

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ABSTRACT

Objective: A method for simultaneous determination of chlordiazepoxide in rat plasma using liquid-liquid extraction followed by high performance liquid chromatography (HPLC) is described.

Methods: The analytes were separated employing a LC-18 column (250mm×4.6mm,5µm) at ambient temperature using methanol and water (60 : 40 v/v) as a mobile phase at a flow rate 1.2 ml/min. Ultra violet (UV) detection was carried out at 254 nm. Employing liquid-liquid extraction (LLE), the best conditions were achieved with the extraction of 0.5 ml plasma using 7.5 ml deionized water, 0.5ml of 0.1M NaOH and 2.5 ml diethyl ether, the mixture was shaken for 15 min, centrifuged, and an aliquot of the ether phase evaporated off in a water bath at 30 °C. The residue was reconstituted with the mobile phase 50 µl followed by HPLC analysis.

Results and conclution: This method was validated for specificity and linearity with excellent correlation coefficient (r=0.99) showed their suitable applicability in order to examine chlordiazepoxide in rat plasma.

Key words: Chlordiazepoxide, chromatographic method, HPLC, rat plasma.

الخلاصة

الهدف: تضمنت الدراسة وصف لطريقة قياس تركيز دواء الـ chlordiazepoxide في مصل دم الجرذان بعد استخلاصه متضمنة طريقة الاستشراب السائل عالي الأداء.

طرق العمل: فصلت المحاليل باستخدام الطور الثابت من نوع C_{18} بدرجة حرارة الغرفة وباستخدام الطور المتحرك المتكون من المواد التالية ماء:ميثانول بنسبة 40:60:% بسرعة جريان 1.2 ملليلتر/دقيقة و حدد الطول الموجي لقياس التركيز على 270 نانوميتر . وكانت أفضل النتائج تم الحصول عليها بعد عملية الاستخلاص بانتقاء أفضل الظروف باستعمال 0.5 ملليلتر من مصل الدم مضاف إليه 7.5 ملليلترمن ماء خالي الايونات وإضافة 0.5 ملليلتر من 0.1 مولاري من هيدروكسيد الصوديوم و 2.5 ملليلتر داي إيثايل إيثر. وبعد مزج الخليط لمدة خمسة عشر دقيقة فصلت العينات وتم أخذ طبقة الايثر وبخرت بحمام مائي بدرجة حرارة 30 درجة مئوية ثم أضيف 50 مايكروليتر من مواد الطور المتحرك للمتبقي من عملية التبخير وتم حقنها بجهاز الاستشراب السائل عالي الأداء .

النتائج والاستنتاج: أظهرت هذه الطريقة ملأمتها العالية لقياس تركيز الدواء بمصل دم الجرذان كما أثبتت دقتها واستقامة قياساتها عند معامل ارتباط مقداره 0.99

Benzodiazepines (BZPs) are an important class of drugs commonly used as minor tranquillizers, hypnotics and muscle relaxants[1,2]. They are among the most frequently prescribed drugs for the treatment of anxiety, sleep disturbance and status epilepticus[3-5]. In addition, BZPs are used to relieve tension in the

preoperative period and to induce amnesia in surgical procedures[6].

Chlordiazepoxide, the prototype for the benzodiazepine compounds, has important effects in treating a variety of medical disorders [7]. Thus, extraction and identification of chlordiazepoxide in biological fluids is very important for forensic and clinical toxicology[8]. Various researchers have reported total plasma concentrations of chlordiazepoxide in relation to both clinical effect and toxicity, indicating that the therapeutic monitoring of this drug is important, but non of the articles dealt with animal blood samples[9].

Analytical methods include thin-layer chromatography, which tends to lack specificity, and gas chromatography, which often requires formation of derivatives for the determination of thermally labile chlordiazepoxide are not applicable for quantification. Hence, high-pressure liquid chromatography (HPLC) has become the most widely used analytical technique for the determination of chlordiazepoxide[10].

Analytical test method validated in this work for animal blood sample is completed to ensure that it is accurate, precise, specific, sensitive, reproducible and robust over the specified range that an analyte will be analyzed. Also a specific method for identification and quantification of chlordiazepoxide in biological fluids is described.

Materials and methods

Chemicals and Reagents

Purified free base of chlordiazepoxide for research purposes was provided by Nenava Drug Industry (NDI), Iraq .

All solvents used were HPLC grade, and all chemicals were analytical grade: HPLC-grade methanol (Scharlau/Spain) and deionised water NDI/Iraq. Analytical grade sodium hydroxide and diethyl ether were from GCC company, UK.

Instrumentation

The analyses were carried out using a chromatographic system from Shimadzu Corporation (Japan). This instrument consisted of a pump, a UV-visible detector, a system controller, and a manual injector. Software was used to control the LC system and data acquisition.

The simultaneous analysis of chlordiazepoxide was performed at room temperature on a C18 column (4.6 mm × 250 mm I.D.,5µm particle size) (GL Sciences Inc.) using methanol : water (60:40, v/v) as mobile phase at a flow rate of 1.2 ml/min. UV detector was operated at 254 nm. The mobile phase was filtered through a millipore membrane filter (0.45 µm)(Steril-R, USA) and degassed ultrasonically prior to use.

Preparation of stock solution and working standards

Stock solution of chlordiazepoxide was freshly prepared in mobile phase solution at the concentration of 10mg/10ml. Working standards of chlordiazepoxide were freshly prepared in the concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8 and 10µg/ml and made by the dilution of the stock solution with mobile phase.

Animals and sample preparation

Adult albino rats were used in this work that have been taken from animal house of the College of Veterinary Medicine, University of Mosul. This study was carried out on 5 animals (male and female), their weights were between 250-350 g. The work was done at laboratory of the College of Veterinary Medicine, University of Mosul.

One ml of blood samples were collected from healthy adult rats, not taking any kind of drug, in heparinized glass tubes, then one ml of blood samples were collected from each animal after 15, 30, 60 min of administration of therapeutic dose of chlordiazepoxide (50 mg/kg) was given by i.m. route to each animal[9].

The blood samples were centrifuged at 3000 rpm for 15 min and the plasma was frozen and stored at -20 °C, no longer than 72 h.

Extraction of the samples

A liquid-liquid extraction (LLE) in which plasma (0.5 ml) was mixed with, deionized water (0.75 ml) and sodium hydroxide 0.1M (0.5ml) in a stoppered test tube (15 ml). The mixture was extracted with diethyl ether (2.5 ml) by mechanically shaking for 15 min. The resultant mixture was centrifuged, and an aliquot of the ether phase (2 ml) transferred to a tapered test tube and the ether evaporated off in a water bath at 30 °C. The residue was reconstituted with the mobile phase (50 µl) used for the HPLC analysis. The test tube was vortex mixed and aliquots (20 µl) were injected on to the column of instrument[11].

Chromatographic conditions

Several chromatographic conditions, such as mobile phase, type of column and its length, mobile phase flow rate, temperature and volume of injection were studied to obtain a satisfactory chromatographic separation (good resolution and efficacy) for the compound. In addition, the total time required for the analysis was also an important factor because the analysis could be unfeasible since interfering compounds could elute close to the chlordiazepoxide, modifications were performed in order to reduce the analysis time.

Various solvents or mixture of solvents at different compositions were used to extract the chlordiazepoxide from rat plasma.

To optimize the HPLC parameters, several mobile phase compositions were tried like:

1. 0.5 M potassium dihydrogen phosphate (KH_2PO_4) : Methanol : Acetonitrile (40:40:20)[12].
2. Methanol : water (60:40)[13].

The flow rate was adjusted at 0.8 ml/min : chlordiazepoxide elute at 19.7min, then adjusted at 1 ml/min : it eluted at 15.8min, then finally adjusted at 1.2 ml/min: it eluted at 12.8min at ambient temperature.

Results

A satisfactory separation and good peak symmetry was found in a mixture of methanol:water in the ratio of 60:40%,v/v at a flow rate of 1.2ml/min. The optimum wavelength for detection was set at 254 nm at which much better detector response for drug was obtained. The retention

time was 12.8 min for chlordiazepoxide and no interferences were observed in formulation sample, also with a better reproducibility.

Quantification was achieved with UV detection at 254nm based on the peak area. Better resolution of the peaks with clear base line separation is found as shown in Table 1, Figure 1 and 2.

Table 1. Optimized chromatographic conditions for estimation of chlordiazepoxide

Mobile phase	Methanol : water 60:40%,v/v
Pump mode	Isocratic
Diluent	Mobile phase
Column	C18 column(4.6 × 250mm, 5µm)
Column temp.	Ambient
Wavelength	254nm
Injection volume	20 µl
Flow rate	1.2ml/min
Run time	15min
Retention time	12.8min

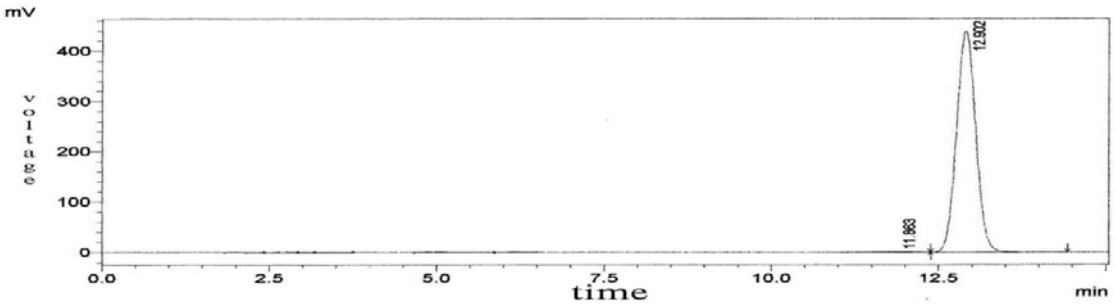

Figure 1. Chromatograms of validation of the method for chlordiazepoxide

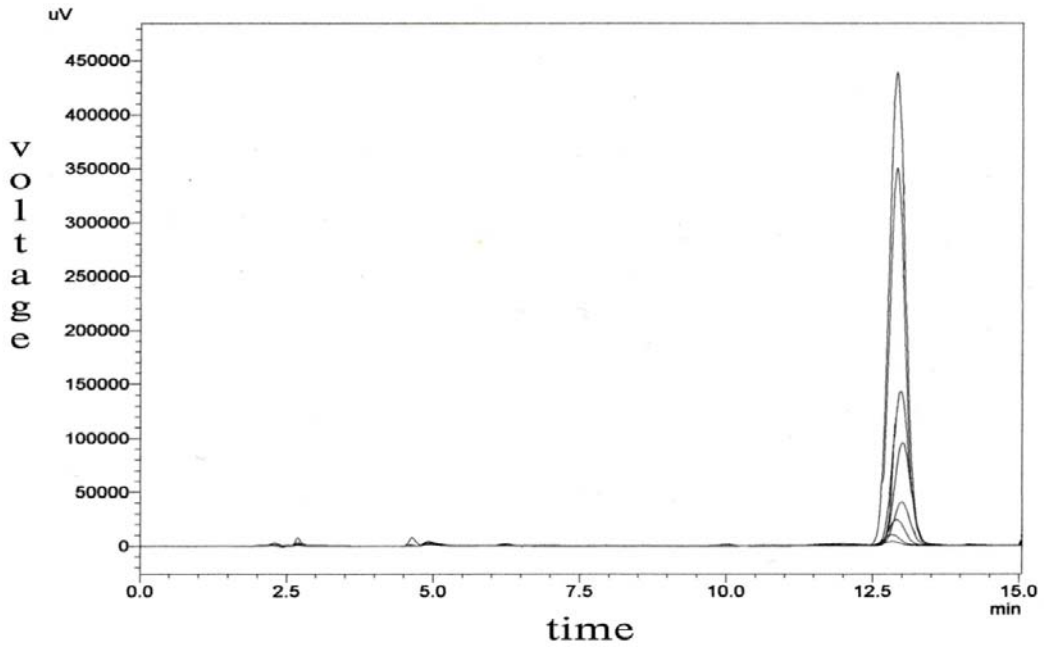

Figure 2. Chromatograms of standards solutions with different concentrations of chlordiazepoxide

Specificity

The specificity of method was performed by comparing the chromatograms of blank, standard and sample. It was found that there is no endogenous interference and also found good correlation between the retention times of standard and sample are shown in Table 2, Figure 3,4 and 5.

Table 2. Specificity study (Retention time of blank, standard and sample)

Name of the solution	Retention time in Min (Rt)
Blank	No peak
Standard	12.8
Sample	12.8

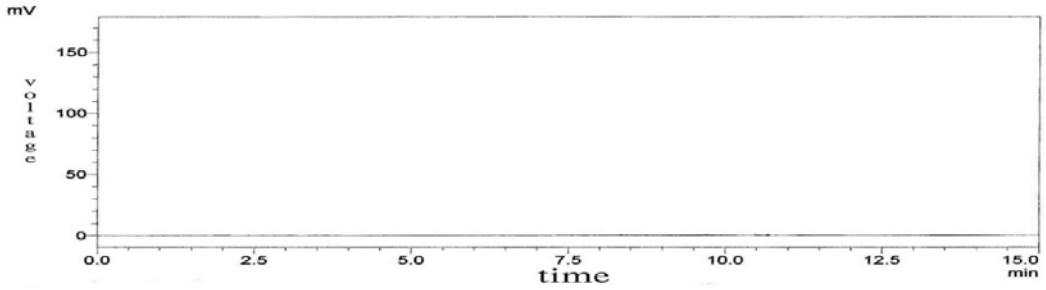

Figure 3. Chromatogram of blank

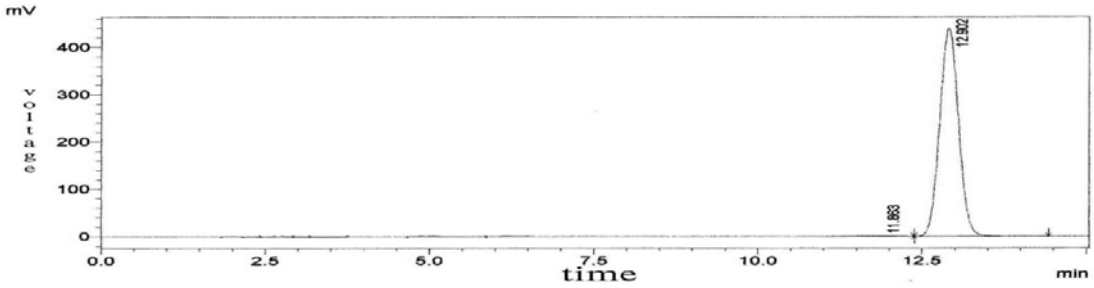

Figure 4. Chromatogram of standard chlordiazepoxide

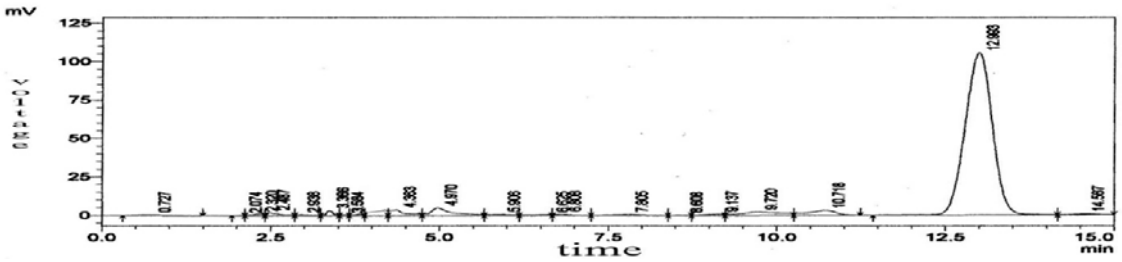

Figure 5. Chromatogram of sample

Linearity

Linearity was performed by preparing standard solutions of chlordiazepoxide at different concentration levels including working concentration mentioned above. Twenty microliters of each concentration was injected in duplicate into the HPLC system. The response was read at 254 nm and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas were calculated and linearity plot of concentrations over the mean peak areas were constructed. The regression of the plot was computed by least square regression method. Linearity results were presented in Table 3, calibration plot was shown in Figure 6 and calibration plots of the samples was shown in Figure 7.

Table 3. Linearity Results

Levels	Concentration of chlordiazepoxide in (µg/ml)	Mean peak area (mV)
1	0.125	91570.4
2	0.25	173699.1
3	0.5	469904.1
4	1	847053.6
5	2	2032980
6	4	2883865
7	8	7221231
8	10	8674700
Range:0.125 to 10	Slope Intercept Correlation coefficient	873892.8 -27121.7 0.9935

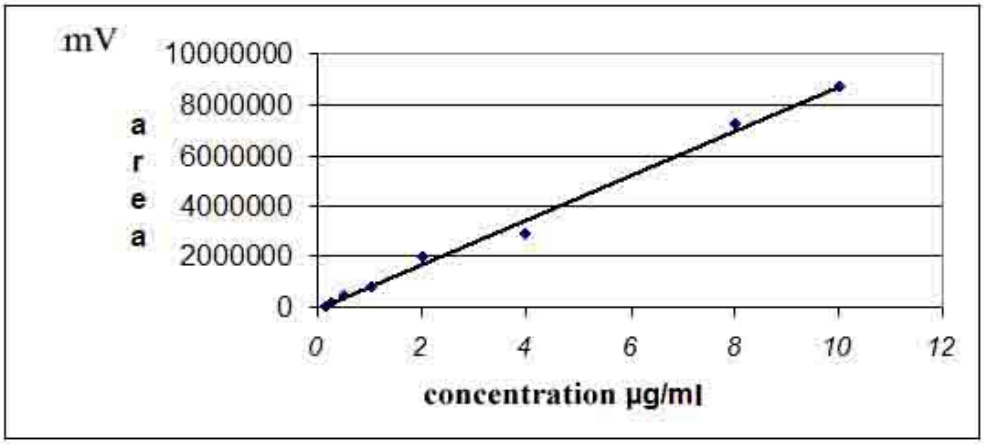

Figure 6. Calibration plot for chlordiazepoxide standards on X axis concentration (µg/ml) and on Y axis peak area (mV)

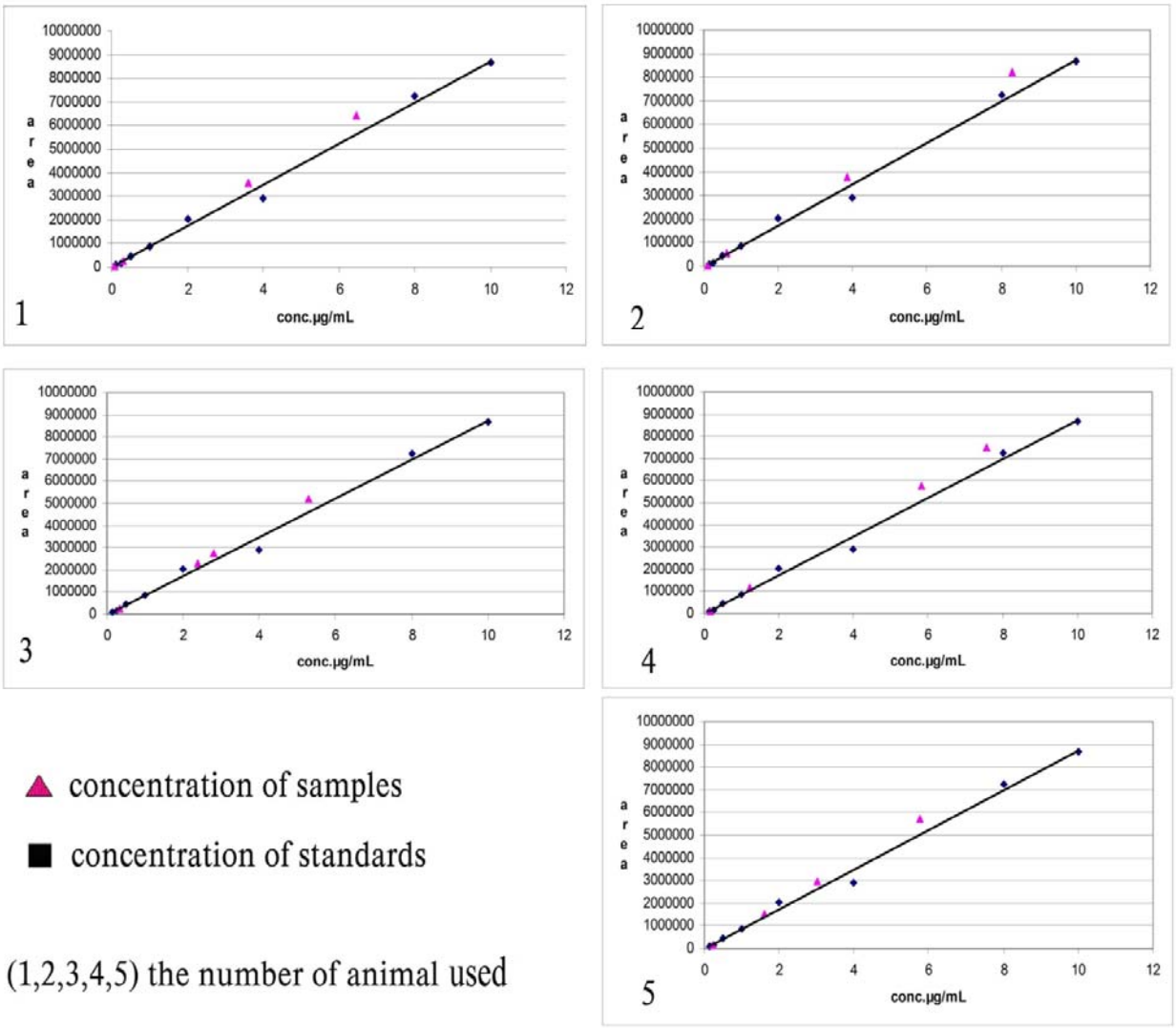

Figure 7. Calibration plots for chlordiazepoxide plasma concentration-area at (0,15,30,60min) after i.m. administration of the drug in 5 animals

- Calibration curves were found to be linear with correlation coefficient (0.9935), and the intercept and the slope values were found to be (-60500) and (1000000) respectively.

Discussion

HPLC combines many of the advantages of other methods by providing adequate separation of components at room temperature with quantification of the drug[11]. While analytical methods include thin-layer chromatography, tend to lack specificity, and gas chromatography, often requires formation of derivatives for the determination of thermally labile chlordiazepoxide[10].

HPLC method provides an assurance of reliability during normal use, and is sometime referred to as the process of providing documented evidence that the method dose what it is intended to do[14].

Chlordiazepoxide analysis in rat plasma by HPLC presents high detectability to allow detection of low quantities of analytes (µg/ml). HPLC with UV detection employing Liquid-Liquid Extraction conforms the employment of the technique in routine analysis.

The quantitative evaluation was carried out in rat plasma using chlordiazepoxide, the results showed that the data were accurate for all the 5 animals within the acceptance level of the standard concentrations at the quantitation limit.

Modification of a procedure described above gave good elution of the drug Figure 1.

Extraction procedure usually resulted in an extract which was free from interfering peaks Figure 5. Since the peaks were fairly symmetrical and peak areas were used as a measure of concentration.

Several papers have been described in the literature for the simplicity of analysis of benzodiazepaine from biological fluids by HPLC, Pongraveevongsa, et. al.[8] determine BZPs in human serum by HPLC with solid phase extraction. Skellern et al.[15] describe the application of HPLC in determination of some BZPs and their metabolites in human plasma. Borges, et. al.[1] investigate liquid-liquid extraction and solid-liquid extraction for determination of BZPs in human plasma by HPLC/UV, and Mergen, et. Al.[2] described a therapeutic drug monitoring of BZPs in human plasma and urine by HPLC.

The primary concern of this study was the simplicity of the method to be used in clinics or hospitals for both humans and animals. Therefore, rather than the method itself, this study stands out for its clinical application. This study showed that the dosage of medication should be adjusted carefully according to the analytical data relating to drug levels in plasma for each individual animal.

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