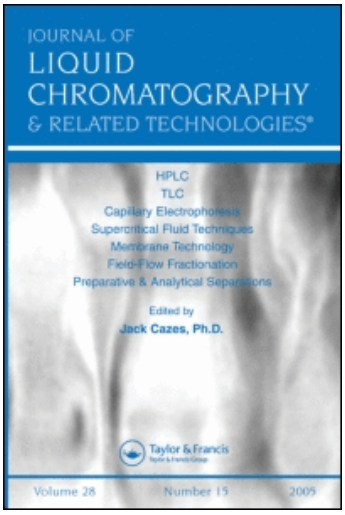

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

The High-Pressure Liquid Chromatographic Determination of Chlordiazepoxide and Its N-Demethyl Metabolite in Mouse Brain

Hebe B. Greizerstein[a]; Iris G. McLaughlin[a]
[a] Division of Alcoholism and Alcohol Abuse, Research Institute on Alcoholism N.Y.S., Buffalo, New York

To cite this Article Greizerstein, Hebe B. and McLaughlin, Iris G.(1980) 'The High-Pressure Liquid Chromatographic Determination of Chlordiazepoxide and Its N-Demethyl Metabolite in Mouse Brain', Journal of Liquid Chromatography & Related Technologies, 3: 7, 1023 — 1030
To link to this Article: DOI: 10.1080/01483918008060211
URL: http://dx.doi.org/10.1080/01483918008060211

JOURNAL OF LIQUID CHROMATOGRAPHY, 3(7), 1023-1030 (1980)

THE HIGH-PRESSURE LIQUID CHROMATOGRAPHIC DETERMINATION OF CHLORDIAZEPOXIDE AND ITS N-DEMETHYL METABOLITE IN MOUSE BRAIN

Hebe B. Greizerstein and Iris G. McLaughlin
Research Institute on Alcoholism
N.Y.S. Division of Alcoholism and Alcohol Abuse
1021 Main Street
Buffalo, New York 14203

ABSTRACT

This report presents a simple and accurate method for the analyses of chlordiazepoxide and its N-demethyl metabolite in small tissue samples such as mouse brains. The procedure involves the addition of diazepam as the internal standard, homogenization of the mixture with 0.01 N NaOH, and extraction with heptane containing 1.5% (v/v) iso-amyl alcohol. After evaporation of the organic solvent, the residue is dissolved in methanol and the compounds separated by reverse phase high pressure liquid chromatography with 66% (v/v) methanol in water as eluant in isocratic conditions. The analysis is linear for concentrations ranging from 0.5 to 50 ng/mg brain for chlordiazepoxide and the metabolite. The method was applied to the study of the distribution of chlordiazepoxide and the N-demethyl metabolite to the brain of mice receiving intraperitoneally 10 mg/kg chlordiazepoxide HCl.

INTRODUCTION

The use of chlordiazepoxide (7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide) in clinical practice has become increasingly important and, as a result, various methods

have been developed for the determination of its levels for both therapeutic and toxic situations (1-9). Extensive studies using small animals have been hindered by the absence of adequate sensitive analytical procedures to detect the drug and its metabolites in small samples. Since the ongoing research in our laboratory required the determination of these compounds at low concentrations in a small volume of blood samples, a sensitive method was developed using high pressure liquid chromatography (10). One of the shortcomings of such method was that due to the use of chlorpromazine as the internal standard, to obtain chromatographic separation of the compounds phosphate buffer (pH = 8.0) was required causing the rapid deterioration of the chromatographic columns. The present report presents an improved method for determination of chlordiazepoxide and its N-demethylmetabolite in small tissue samples using diazepam as the internal standard. The procedure involves a simple extraction followed by the separation of the compounds by reverse phase liquid chromatography with methanol-water as the eluant solution. This method has been applied to determine the concentration of these substances in brain of mice injected with chlordiazepoxide HCl.

METHODS

Apparatus

The chromatographic determinations were performed in a Waters Associates Liquid Chromatograph equipped with a Model 440 Absorbance Detector (254 nm wavelength), a U6K Universal injector, a constant flow pump, and a 10 mV recorder. A μ Bondapak C_{18} column, 4 mm i.d. x 30 cm long (Waters Associates) was used for the separation. The eluant consisted of a solution of 66% (v/v) methanol in water at a flow rate of 2 ml/min.

Reagents

All the solvents and chemicals used were reagent grade. The drug standards, chlordiazepoxide and diazepam were purchased from Applied Science Laboratories. Hoffman-LaRoche provided the N-demethyl metabolite and the chlordiazepoxide HCl.

Sensitivity and Linearity

Standard solutions of chlordiazepoxide and N-demethyl chlordiazepoxide in methanol were prepared in concentrations ranging between 10.0 and 0.1 µg/ml and containing 1.0 µg/ml diazepam (internal standard). Aliquots (10 µl) of these solutions were injected into the chromatograph.

Recovery

Mice not receiving the drug were sacrificed by cervical dislocation; the brains were immediately removed and frozen in liquid nitrogen. To each brain 10 µl of 0.1 mg/ml diazepam in methanol and 10 µl of chlordiazepoxide and of N-demethyl metabolite in methanol solutions were added. The concentration of the compounds of interest were varied to obtain total amounts of 1.0 to 10.0 µg each. The standards were then extracted as described below. The recovery rates were calculated by comparison with a standard solution of the compounds in methanol.

Extraction Procedure

The frozen brains were homogenized after addition of 10 µl of 0.1 mg/ml diazepam in methanol and 5 ml 0.01 N NaOH. The homogenate was centrifuged 30 minutes at 20,000 r.p.m.; 4 ml of the supernatant was removed. This solution was extracted with 10 ml of heptane containing 1.5% (v/v) iso-amyl alcohol. The organic layer was evaporated to dryness under a stream of

nitrogen. The residue was dissolved in 50 µl methanol, and an aliquot (10 µl) was injected in the chromatograph. The use of phosphate or TRIS buffer to achieve a pH of 8 prior to the extraction step was not satisfactory; they produced erratic results during the chromatographic separation.

Analyses of Mouse Brains

Male BALB/6J mice (28-32 g in weight) were purchased from Jackson Laboratories, Bar Harbor, Maine. The animals were housed in individual cages for a week, and fasted for 20 hours prior to the experiment. A dose of 10 mg/kg chlordiazepoxide HCl in isotonic saline was administered i.p. to 30 mice. Groups of 6 animals were sacrificed after 15, 30, 60, 90, and 120 minutes, the brains were immediately removed, frozen in liquid nitrogen, and kept at -80° until the time for analyses. Brain standards were extracted and analyzed simultaneously with the samples. The concentrations of the compounds in the brain samples were determined using the brain standards as the calibrating solutions; this procedure eliminated the need for the correction due to recovery losses. All samples and standards were kept in the dark to prevent decomposition and they were analyzed in duplicate.

Calculations

The peaks on the chromatogram were identified by their retention times relative to that corresponding to the internal standard. Further confirmation of their identity was carried out by GC-MS analysis of brain extracts and standard solutions. The concentration of the drugs was calculated on the basis of the ratio of their peak heights to that of the diazepam internal standard.

RESULTS AND DISCUSSION

Sensitivity and Linearity

The lowest amount of chlordiazepoxide and its N-demethyl-metabolite injected in the chromatograph measured was 1.25 nanograms in 10 µl of methanol.

The detector response curve for both compounds was linear in the range of 1.0 to 10.0 µg/ml for chlordiazepoxide ($y = 0.002 + 0.193\ x$, corr. coeff. 0.977) and N-demethyl metabolite ($y = 0.025 + 0.104\ x$, corr. coeff. 0.999).

Recovery

The recovery rates calculated on the basis of the amount of the compounds measured after extraction from control brains and compared with standard methanol solutions were 80.5 ± 4.6, 70.7 ± 3.7, and 82.3 ± 3.2% for chlordiazepoxide, N-demethyl metabolite, and diazepam, respectively.

Brain Determinations

Control brains did not show interfering peaks in the chromatographic analyses, although some peaks with shorter retention times were present. A typical chromatogram of the brain extraction from a mouse receiving 10 mg/kg chlordiaze-poxide HCl is shown in figure 1. The small peaks were due to endogenous substances present both in the brains of controls and experimental mice. The retention times were 3.43, 4.13, and 5.05 minutes for N-demethylmetabolite, chlordiazepoxide, and diazepam respectively, and the time allowed between injections was 10 minutes. The concentrations of the drug and its metabolite in the brain of mice as a function of time is illustrated in figure 2. As we reported earlier, the lactam metabolite was not detected under the conditions of our extraction procedures.

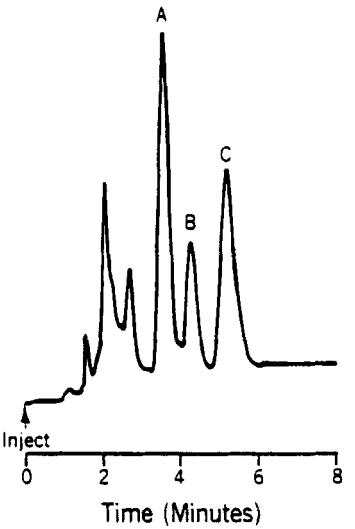

FIGURE 1. Chromatogram of a mouse brain extract injected with chlordiazepoxide HCl. A: N-demethyl metabolite, B: chlordiazepoxide, and C: diazepam.

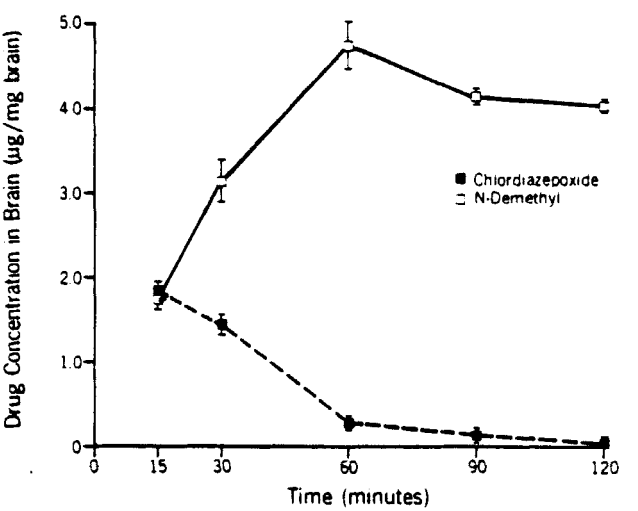

FIGURE 2. Drug concentration curves in brain of mice after i.p. administration of 10 mg/kg chlordiazepoxide HCl. Each point represents the mean value of 6 samples ± S.E.M.

It has been reported that benzodiazepines can suffer degradation or chemical changes during gas chromatographic analyses (11) due to the high temperatures required for the separation of the substances. One of the advantages of liquid chromatography is that the determinations were carried out at room temperature. In addition, we had taken the precaution of keeping all solutions and samples in the dark.

Although we do not present the data, this method can be used in the analyses of blood samples. The modification over the previous procedure (10) being the addition of diazepam, followed by the extraction step as described earlier and the separation of the compounds under the chromatographic conditions of this report. This modified method is now routinely used in our laboratory to measure the drug and its N-demethylmetabolite in 50 µl blood samples, as well as in brain samples.

An important result of the changes in the chromatographic determination is the elimination of the need of a second pump and a solvent programmer system making the procedure accessible to laboratories with limited resources.

Currently, we are developing a method based on two extractions at different pH values to measure chlordiazepoxide, the N-demethyl and the lactam metabolites in small blood and tissue samples.

ACKNOWLEDGMENTS

The authors wish to extend thanks to Dr. W.E. Scott of Hoffman-LaRoche for the supply of N-demethyl chlordiazepoxide and chlordiazepoxide HCl.

REFERENCES

1. Clifford, J.M. and Smyth, W.F.: The Determination of Some 1,4-Benzodiazepines and Their Metabolites in Body Fluids. Analyst 99: 241 (1974).

2. Hailey, D.M.: Chromatography of the 1,4-Benzodiazepines. Journal of Chromatography 98: 527 (1974).

3. Hackman, M.R.; Brooks, M.A.; de Silva, J.A.F.; Ma, T.S.: Determination of Chlordiazepoxide Hydrochloride (Librium) and Its Major Metabolites in Plasma by Differential Pulse Polarography. Analytical Chemistry 46: 1075 (1974).

4. Baselt, R.C. and Stewart, C.B.: Toxicological Determination of Benzodiazepines in Biological Fluids and Tissues by Flame-Ionization Gas Chromatography. Journal of Analytical Toxicology 1: 10 (1977).

5. Osselton, M.D.; Hammond, M.D.; and Twitchett, P.J.: The Extraction and Analysis of Benzodiazepines in Tissues by Enzyme Digestion and High-Performance Liquid Chromatography. Journal of Pharmacy and Pharmacology 29: 460 (1977).

6. Strojny, N.; Bratin, K.; Brooks, M.A.; and de Silva, J.A.F.: Determination of Chlordiazepoxide, Diazepam, and Their Major Metabolites in Blood or Plasma by Spectrophotodensitometry. Journal of Chromatography 143: 363 (1977).

7. Butterfield, A.G.; Matsui, F.F.; Smith, S.J.; and Sears, R.W.: High-Performance Liquid Chromatographic Determination of Chlordiazepoxide and Major Related Impurities in Pharmaceuticals. Journal of Pharmaceutical Sciences 66: 684 (1977).

8. Sun, S.R.: Quantitative Determination of Chlordiazepoxide and Its Metabolites in Serum by Fluorescence TLC-Densitometry. Journal of Pharmaceutical Sciences 67: 639 (1978).

9. Skellern, G.G.; Meier, J.; Knight, B.I.; and Whiting, B.: The Application of HPLC to the Determination of Some 1,4-Benzodiazepines and their Metabolites in Plasma. British Journal of Clinical Pharmacology 5: 483 (1978).

10. Greizerstein, H.B. and Wojtowicz, C.: Simultaneous Determination of Chlordiazepoxide and Its N-Demethyl Metabolite in 50-µl Blood Samples by High Pressure Liquid Chromatography. Analytical Chemistry 49: 2235 (1977).

11. Sadee, W.; Van Der Kleijn, E.: Thermolysis of 1,4-Benzodiazepines during Gas Chromatography and Mass Spectroscopy. Journal of Pharmacological Sciences 60: 135 (1971).