Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 9, pp. 777–779, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

Short Communication

High-performance liquid chromatographic method for the determination of CHEB, a convulsant barbiturate, in rat biological fluids/tissue

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Keywords: *Reversed-phase LC; UV-detection; CHEB; convulsant barbiturate; biological fluids.*

Introduction

CHEB [5-(2-cyclohexylidene-ethyl)-5-ethyl barbituric acid; Fig. 1] is a synthetic CNS excitatory (or stimulatory) barbiturate. CHEB is a convulsant producing seizures/convulsions in animals [1, 2]. It is postulated to act via the picrotoxin sensitive site at the benzodiazepine–γABA–ionophore receptor complex [3] and may therefore serve as an appropriate experimental tool for examining perturbation(s) to this receptor system *in vivo*.

Currently, CNS depressant barbiturates are routinely assayed using reversed-phase LC methods [4] but no such assays are available for the convulsant barbiturates. In particular, no analytical method is currently available for CHEB quantitation either in solution or in biological fluids or tissues. Thus there is a need for a simple assay procedure for CHEB determination.

This paper describes a reliable reversed-phase HPLC method for the analysis of CHEB, especially applicable to routine measurement of convulsant (or neurotoxic) concentrations in experimental animals (rats). The analyses are performed by direct injection of cerebrospinal fluid (CSF) samples or by injection of serum or brain samples after deproteinization with acetonitrile and employs UV-detection at 210 nm.

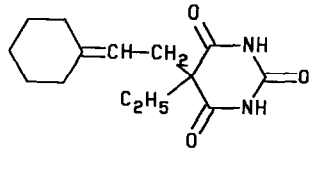

Figure 1
Chemical structure of CHEB.

Experimental

Reagents, chemicals and standards

CHEB (as the free acid) was a gift from Dr H. Downes (University of Oregon, Portland, USA) and was used as received without further purification. The internal standard, 5-(*p*-methylphenyl)-5-phenylhydantoin, MPPH, was purchased from Sigma (St Louis, MO, USA). All other reagents were of analytical grade or better and were purchased from Ajax Chemicals (Sydney, Australia). All the solvents were HPLC-grade.

Stock solution (0.5 mg ml^{-1}) of CHEB was prepared in acetonitrile and kept at +4°C. Standard solutions for determination of linearity of detector response and for spiking blank samples were then prepared from this stock by dilution, also with acetonitrile. Blank rat CSF, serum or brain tissue were then spiked with CHEB in the concentration range 1–10 μg ml^{-1} or 1–10 μg g^{-1}. A stock solution of the internal standard (MPPH), 0.1 mg ml^{-1} was prepared in water containing ~10% methanol.

Instruments and equipment

A Shimadzu isocratic HPLC system consisting of the following Model 6A components was used: a solvent delivery unit with a single plunger reciprocating pump; a variable wavelength detector set at 210 nm; an auto-sampler

driven by a system controller and a Chromatopac Model CR3A integrator operated at attenuation 5 or 32 mV full scale (Shimadzu Corp., Kyoto, Japan).

Chromatography

CHEB was separated on a stainless steel analytical column, 300 × 4.1 mm, i.d., filled with 10 μm Versapack C_{18} packing material (Alltech, Deerfield, IL, USA) using a mobile phase consisting of methanol and aqueous 5 mM KH_2PO_4 (50:50, v/v), flow rate 1.5 ml min^{-1}.

Preparation of biological samples

Prior to analysis, the samples were stored at −20°C. After thawing, 100 μl of serum (rat, blank or spiked with 10 μl of the standard solutions) was mixed with IS (5 μl, 0.1 mg ml^{-1}) and 200 μl acetonitrile for deproteinization and vortexed for 30 s. After centrifugation at approx. 12 800*g* for 2 min the supernatant (50 μl) was used for chromatographic analysis. For CSF, a smaller (25 μl) sample was used without deproteinization and 20 μl was injected onto the column. Brain CHEB was analysed in 0.5 g cerebral tissue following homogenization with 50 μl standards, 25 μl IS and 1 ml acetonitrile followed by centrifugation and chromatography of a 50-μl aliquot of the supernatant. The volumes of the various solutions utilized in the brain assay are five times higher than in the serum assay since 0.5 g brain tissue was used compared to only 100 μl of serum.

Results and Discussion

CHEB possesses insignificant UV absorption at wavelengths higher than 230 nm, thus the analyses were performed with the detector wavelength set at 210 nm. Using a C_{18} column, CHEB was efficiently separated from serum and brain components as shown in Fig. 2. Direct injection of deproteinized serum/brain samples were not accompanied by any loss of analytical column life or performance nevertheless a guard column was used throughout.

Typical chromatograms of drug-free serum and brain supernatants and serum/brain taken from a rat pre-treated with CHEB to a

Table 1
Elution times for CHEB, MPPH (internal standard) and some potential barbiturate interference

Compound	Retention time t_R (min)
CHEB	9.5
MPPH (IS)	7.0
Barbitone	2.6
Phenobarbitone	3.3
Butabarbitone	4.2
Amylobarbitone	5.6
Pentobarbitone	6.0
Secobarbitone	7.4
Thiopentone	8.6

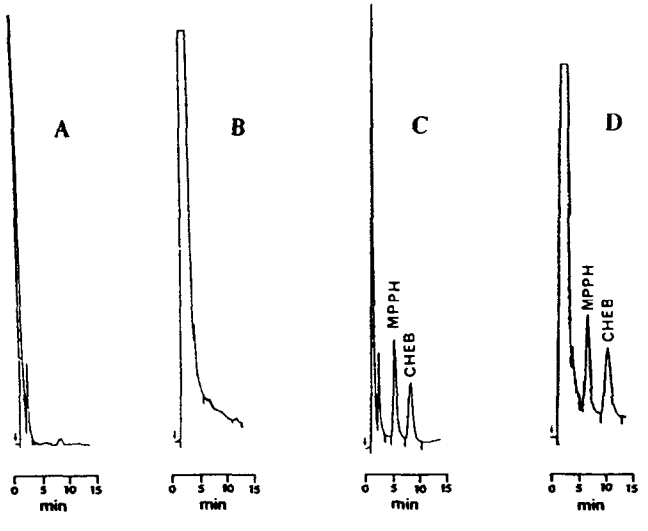

Figure 2
Chromatographic determination of CHEB after direct injection of deproteinized serum and brain supernatants. Conditions: Versapack C_{18}, 300 × 4.1 mm, i.d. Eluent: methanol–5 mM KH_2PO_4 (50:50, v/v). Flow rate: 1.5 ml min^{-1}; UV detection at 210 nm. (A) "Pre-dose" or blank rat serum, (B) blank rat brain, (C) and (D) rat serum and brain respectively at onset of maximal seizure induced with CHEB. Retention time 9.5 min for CHEB and 7.0 min for IS, MPPH; concentrations 8.3 μg ml^{-1} and 10.1 μg g^{-1} respectively.

Table 2
Typical standard curves for CHEB: linear regression of the spiked concentration (X) vs the peak area ratio (Y); number of replicates (n) = 3; mean values ±SD

Fluid/tissue	Intercept	Slope	r
Serum	−0.0173 ± 0.0161	0.1070 ± 0.0059	0.999 ± 0.0011
CSF	−0.0086 ± 0.0057	0.1030 ± 0.0014	0.999 ± 0.0006
Brain	−0.0295 ± 0.0318	0.1101 ± 0.0057	0.997 ± 0.003

maximal seizure and containing CHEB and added IS are shown in Fig. 2. A good separation was obtained within 10 min. CSF chromatograms, though not presented here also gave clean separations. Table 1 lists the retention times for CHEB; the IS and some potential interferents. As seen there is unlikely to be interference from other barbiturates tested.

Quantitation of CHEB was achieved by comparing its peak area relative to that for the IS in rat samples to those for known samples from the standard curve. Standard curves from serum, CSF and brain were prepared in the concentration range 1–10 μg ml^{-1} or 1–10 μg g^{-1} and were consistently linear from all fluids/tissue. Data from typical curves are shown in Table 2. The relative standard deviation (RSD) of the slopes from the three biological matrices ranged from 1.4 to 5.2%. Based on a signal-to-noise ratio of 2:1, the practical limit of detection for CHEB was found to be about 0.4 μg ml^{-1} (or μg g^{-1}) using either a 100-μl sample of serum or a 0.5-g sample of brain tissue. CSF assay gave a similar value despite a smaller (25 μl) volume being used for the assay since there are no dilution effects attributable to the addition of protein precipitant solution as with the other two assays. Serum and brain samples spiked with 10 μg ml^{-1} (μg g^{-1}) CHEB were analysed on three separate days. Accuracy and precision data from these assays are presented in Table 3. The recovery of the drug from plasma using deproteinization with acetonitrile was also examined at 10 μg ml^{-1} and was found to be 97.4 ± 3.6% (n = 4) compared to water samples.

Table 3
Accuracy and precision for the determination of CHEB in spiked rat serum and brain tissue

Spiked value (μg ml^{-1} or μg g^{-1})	Assay value	RSD (%)	Accuracy (%)	n
Serum 10.0	9.87 ± 0.36	3.7	98.7	3
Brain 10.0	9.76 ± 0.47	4.8	97.6	3

In conclusion, a selective and simple reversed-phase HPLC method has been developed for the convulsant barbiturate, CHEB, in serum, CSF and brain tissue, suitable for neurotoxic studies in rats and presumably other animal species.

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[Received for review 20 September 1990; revised manuscript received 25 March 1991]