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Reversed-phase high-performance liquid chromatography method for the determination of bemegride in serum and brain tissue: pharmacokinetics and brain distribution of an intraperitoneal subconvulsive dose in rats*

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Abstract: A simple and rapid HPLC method has been developed for the quantification of bemegride in serum and brain tissue, using *p*-methylphenobarbital as an internal standard. Serum and brain tissue homogenate samples were extracted with ethyl acetate and the evaporated and redissolved extracts injected into a reversed-phase column. The compounds were eluted with an acetonitrile–phosphate buffer mixture and monitored at 200 nm. A linear response was obtained in the range 1–40 μg ml^{-1} for serum and 1–40 μg g^{-1} for brain tissue. Within-day and between-day precisions were <5% and the analytical recovery >76.4%. This method has been used to investigate the kinetic profiles of the drug in serum and discrete areas of rat brain after intraperitoneal administration of a subconvulsive dose of bemegride (10 mg kg^{-1}). Peak concentrations occurred in the brain and serum at the same time (30 min), followed by a biphasic decay. The results also indicated the accumulation of the drug in the brain, with no significant differences ($p > 0.05$) in the impregnation of the different brain areas investigated.

Keywords: *Reversed-phase HPLC; bemegride; serum; brain tissue; pharmacokinetics; rats.*

Introduction

Bemegride (BMG, 4-ethyl-4-methyl-2,6-piperidinedione) is a central nervous system stimulant which has been used as a systemic chemoconvulsant [1]. Recently, it has been found that the evaluation of brain levels of pentylenetetrazol is useful in quantifying experimental non-convulsive models of epilepsy [2, 3]. As BMG was found to be capable of inducing better and longer lasting spike and wave discharges than pentylenetetrazol [4], the need arose for an accurate and precise method for the quantification of BMG in biological materials in order to facilitate basic biomedical research using this drug.

Several methods have been proposed for the identification of BMG in the presence of other drugs, for use in toxicological drug screening. These methods include thin-layer chromatography [5], gas–liquid chromatography [6, 7] and liquid chromatography [8]. However, these methods have not been specifically developed or evaluated for the quantification of the drug in biological materials. In addition, there is little information about the pharmacokinetic behaviour of the drug. Accordingly, a project was undertaken to develop a high-performance liquid chromatographic (HPLC) method for the determination of BMG in serum and brain tissue and to investigate the pharmacokinetics of BMG and its brain distribution in experimental animals.

This present report describes a rapid, sensitive and reproducible reversed-phase HPLC method for the quantification of BMG in small samples of serum and brain tissue. *p*-Methylphenobarbital [*p*-MPB, 5-ethyl-5-(4-methylphenyl)-2,4,6(1H,3H,5H)pyrimidionetrione] was used as an internal standard; the structures of the two compounds are shown in Fig. 1. The method has been used to investigate the pharmacokinetics and brain distribution of BMG after intraperitoneal (i.p.) administration of a single subconvulsive dose of the drug to rats.

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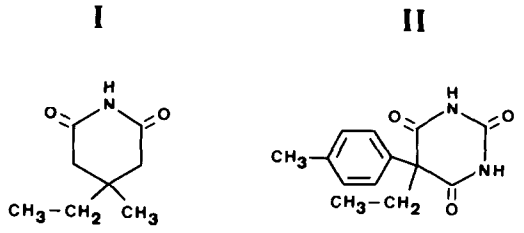

Figure 1
Structures of BMG (**I**) and *p*-MPB (**II**).

Materials and Methods

Apparatus

The liquid chromatograph consisted of a Model 620 solvent delivery system (Kontron AG, Zurich, Switzerland) equipped with a Model 7125 Rheodyne injection valve and a 20 μl sample loop, a Uvikon Model 720LC variable wavelength detector (Kontron AG), a Model 3390A plotting integrator (Hewlett-Packard, Avondale, PA, USA), and a Model 200 programmer (Kontron AG). Chromatographic separations were performed using a 250 × 4.6 mm i.d. stainless steel column packed with 5 μm Spherisorb ODS (Kontron AG), protected by a 50 × 4.6 mm i.d. guard-column filled with Co:Pell ODS (Whatman Inc., Clifton, NJ, USA).

Chemicals

BMG and *p*-MPB were obtained from Aldrich-Chemie (Steinheim, FRG). Acetonitrile and methanol were of HPLC grade from Romil Chemicals Ltd (Shepshed, Leicestershire, UK). Ethyl acetate was LiChrosolv grade from Merck (Darmstadt, FRG). HPLC-grade water was prepared with the Milli RO/Q water purification system (Millipore Corp., Bedford, MA, USA). All other chemicals were of analytical grade from Merck.

Standards

Stock solutions of BMG and *p*-MPB were prepared by dissolving 10 mg of each compound in 10 ml of methanol. These solutions are stable for at least 6 months when stored at 4°C in screw-cap vials with Teflon faced rubber liners (Alltech Associates, Deerfield, IL, USA).

Five different calibration standards were prepared by spiking serum or brain tissue homogenates with the appropriate volume of the BMG stock solution, to cover the range of concentrations 1–40 μg ml^{-1} (serum) or μg g^{-1} (brain tissue).

A working internal standard solution was prepared daily in water to give a concentration of 16 μg ml^{-1} of *p*-MPB.

Animals and drug administration

The experiments were performed on 50 adult Wistar male rats weighing 300–350 g. All the animals received an i.p. aqueous solution of BMG as a single bolus injection (10 mg kg^{-1}). The administered volume was 0.5 ml. Then, groups of five rats were sacrificed at 5, 15 and 30 min and 1, 2, 4, 6, 8, 10 and 12 h after drug administration.

Sampling of serum and brain areas

After rapid decapitation, the trunk blood was collected from the neck wound, and the brain was rapidly removed and frozen at −20°C until assayed. Later, serum was separated by centrifugation and frozen for subsequent analysis. Prior to analysis, brain areas (cerebral cortex, midbrain, cerebellum) were dissected according to the procedure of Glowinski and Iversen [9].

Serum extraction

To 200 μl of serum or calibration standard in a screw-cap centrifuge tube, 100 μl of the working internal standard solution were added, followed by 2.5 ml of ethyl acetate. After vortex-mixing for 1 min and centrifugation at 2800*g* for 5 min, the organic layer was transferred into a glass culture tube and evaporated to dryness at room temperature in a Sped Vac concentrator (Savant Instruments Inc., Farmingdale, NY, USA). The residue was then reconstituted in 50 μl of methanol, and 20 μl of this solution were injected into the chromatograph.

Brain tissue extraction

Three hundred milligrams of brain tissue were homogenized with 1.5 ml of distilled water in a Potter S all-glass homogenizer (B. Braun Melsungen AG, Melsungen, FRG). One millilitre of the homogenate was transferred into a screw-cap centrifuge tube, followed by the addition of 100 μl of the working internal standard solution and 5 ml of ethyl acetate. After vortex mixing for 1 min and centrifugation at 2800*g* for 5 min, the organic phase was transferred into a glass culture tube and evaporated to dryness at room

temperature in a Speed Vac concentrator. The residue was then redissolved in 50 μl of methanol, and 20 μl of this solution were injected into the chromatograph.

Chromatographic conditions

The mobile phase was a mixture (28:72, v/v) of acetonitrile and 0.15 mM potassium phosphate buffer pH 4.8 (adjusted with 0.9 M phosphoric acid). This mixture was filtered through a 0.5 μm filter type HVLP 04700 (Millipore Corp.) and degassed under reduced pressure before use. Chromatography was performed at room temperature with a flow rate of 2 ml min^{-1}. The column effluent was monitored at 200 nm and quantitation was based on the peak height ratio of BMG to the internal standard.

Pharmacokinetic analysis

Serum and brain (cerebral cortex, midbrain, cerebellum) concentrations were analysed by the IGPHARM program for kinetic studies [10] on a Model 50 IBM PS/2 computer according to a two-compartment model.

The following pharmacokinetic parameters were calculated: constants of absorption or invasion (k_a), of distribution (α) and of disposition (β); microconstants of elimination (k_{10}) and of compartment-transfer (k_{12}, k_{21}) and the area under the curve (AUC).

Results and Discussion

Method evaluation

Figure 2 shows typical chromatograms obtained in the analysis of serum and brain tissue homogenates from both drug-free and drug-treated animals. As can be seen, resolution between the drug and internal standard, and between analytes and endogenous components, was satisfactory. No interference was found in either serum and brain tissue homogenate blanks or in predose samples from the rats assayed during the pharmacokinetic study. It was also shown that an i.p. injection of the vehicle alone had no effect on the chromatographic profiles of both blanks. The retention times for serum and brain tissue analyses were approximately 4 min for the analyte and 9 min for the internal standard.

The relationship between the concentration of BMG and its peak height ratios relative to the internal standard was linear over the concentration ranges: 1–40 μg ml^{-1} for serum and 1–40 μg g^{-1} for brain tissue. Correlation coefficients (r^2) were generally greater than 0.99 for calibration curves.

The within-day precision for the serum and brain tissue assay was assessed by using five samples spiked with BMG at three different concentrations. These samples were prepared and analysed on the same day. The between-

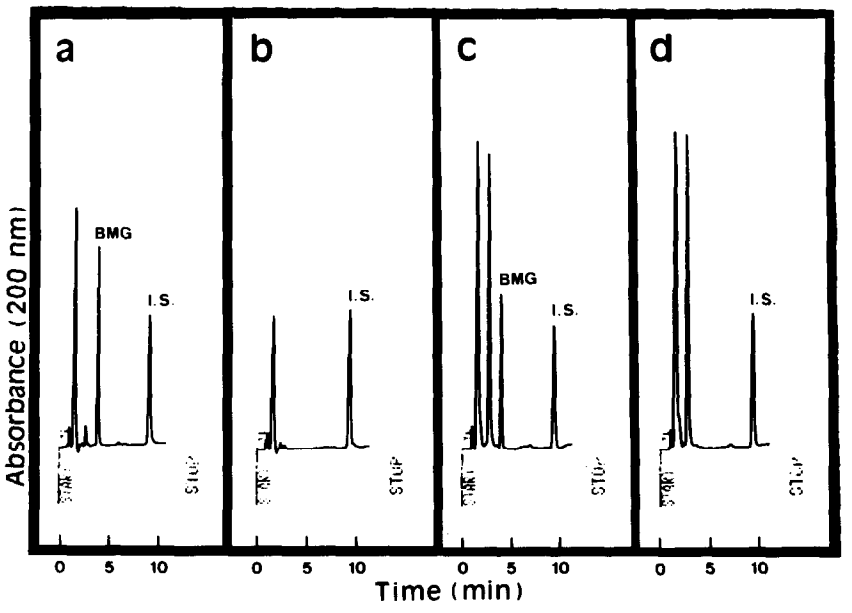

Figure 2
Chromatograms of (a) extract of a serum sample from a treated rat containing 12.4 μg ml^{-1} of BMG, (b) extract of a rat serum blank, (c) extract of a brain tissue homogenate sample from a treated rat containing 15.5 μg g^{-1} of BMG, and (d) extract of a rat brain tissue homogenate blank. Peaks: BMG = bemegride; IS = internal standard. Attenuation was 2^6.

Table 1
Within-day and between-day precision and recovery of the assay of BMG in spiked serum and brain tissue homogenates

Nominal concentration	Within-day (n = 5)		Between-day (n = 5)		
	Mean concentration found	RSD (%)	Mean concentration found	RSD (%)	Recovery (%)
		Serum*			
1.0	1.0	3.2	0.9	4.1	91.6
10.0	9.9	2.7	9.8	3.0	87.9
40.0	39.5	1.4	38.6	2.3	84.7
		Brain tissue homogenates†			
1.0	0.9	3.7	0.9	4.6	88.5
10.0	9.6	2.8	9.3	3.3	83.7
40.0	39.7	1.8	38.9	2.1	76.4

*Concentrations are expressed in $\mu g\ ml^{-1}$.
†Concentrations are expressed in $\mu g\ g^{-1}$.

day precision was assessed by using five samples spiked with BMG at the same concentrations as mentioned above. These samples were analysed on five different days. Drug-free sera and brain tissue homogenates from Wistar rats were used. As shown in Table 1, the precision and accuracy were characterized by a relative standard deviation (RSD) lower than 5%.

The recovery of BMG from serum and brain tissue was determined in the following way. Blank serum and brain tissue homogenates were spiked with BMG at three different concentrations. Then, a sample from each pool was analysed by our procedure, adding the internal standard after the extraction. Finally, analytical recovery was calculated by comparing these peak height ratios with those obtained by direct injection of the same amounts of BMG and internal standard in water. As Table 1 shows, recovery was greater than 84.7% for serum and 76.4% for brain tissue homogenates. For drug extraction, the following solvents were tested: dichloromethane, chloroform, diethyl ether, tert-butylmethyl ether, diisopropyl ether and ethyl acetate. Although they all yielded clean chromatograms, the greatest extraction efficiency was obtained with ethyl acetate.

To determine the specificity of the method, the relative retention times of other drugs (and metabolites) that might be co-administered

Table 2
Relative retention times (RRT) of some drugs under the chromatographic conditions described in the text

Compound	RRT
Phenylethylmalonamide	0.31
p-Hydroxyphenobarbital	0.33
Ethosuximide	0.34
trans-10,11-Dihydro-10,11-dihydroxycarbamazepine	0.36
Primidone	0.37
Pentylenetetrazol	0.42
BMG	0.44
Picrotin	0.54
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	0.56
Phenobarbital	0.62
Carbamazepine-10,11-epoxide	0.80
Picrotoxinin	0.81
p-MPB (internal standard)	1.00
Pentobarbital	1.11
Phenytoin	1.36
Carbamazepine	1.62
Clonazepam	ND
Diazepam	ND
Flurazepam	ND
Nitrazepam	ND
Oxazepam	ND

ND = peak not observed within 20 min.

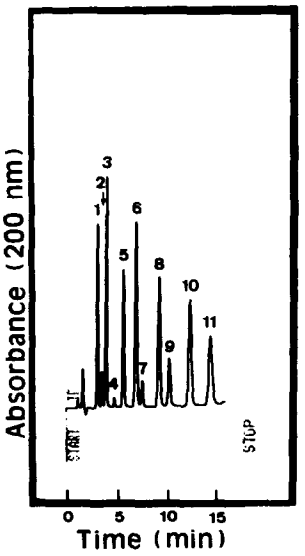

Figure 3
Chromatographic separation of some of the most representative potentially interfering compounds in the assay of BMG. Peaks: 1 = primidone; 2 = pentylenetetrazol; 3 = BMG; 4 = picrotin; 5 = phenobarbital; 6 = carbamazepine-10,11-epoxide; 7 = picrotoxinin; 8 = internal standard; 9 = pentobarbital; 10 = phenytoin; 11 = carbamazepine. Each peak represents 64 ng of each drug. Attenuation was 2^6.

with BMG in biomedical research on epilepsy were determined. However, none of the compounds tested interfered (Table 2). Figure 3 shows the chromatogram obtained from a mixture of the most representative potentially interfering compounds.

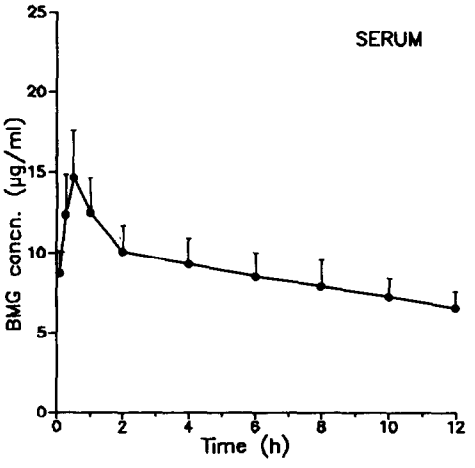

Figure 4
Serum and brain concentrations of BMG after intraperitoneal administration of a single bolus injection of 10 mg kg^{-1} of BMG to rats. Each point represents the mean ±SD of five animals.

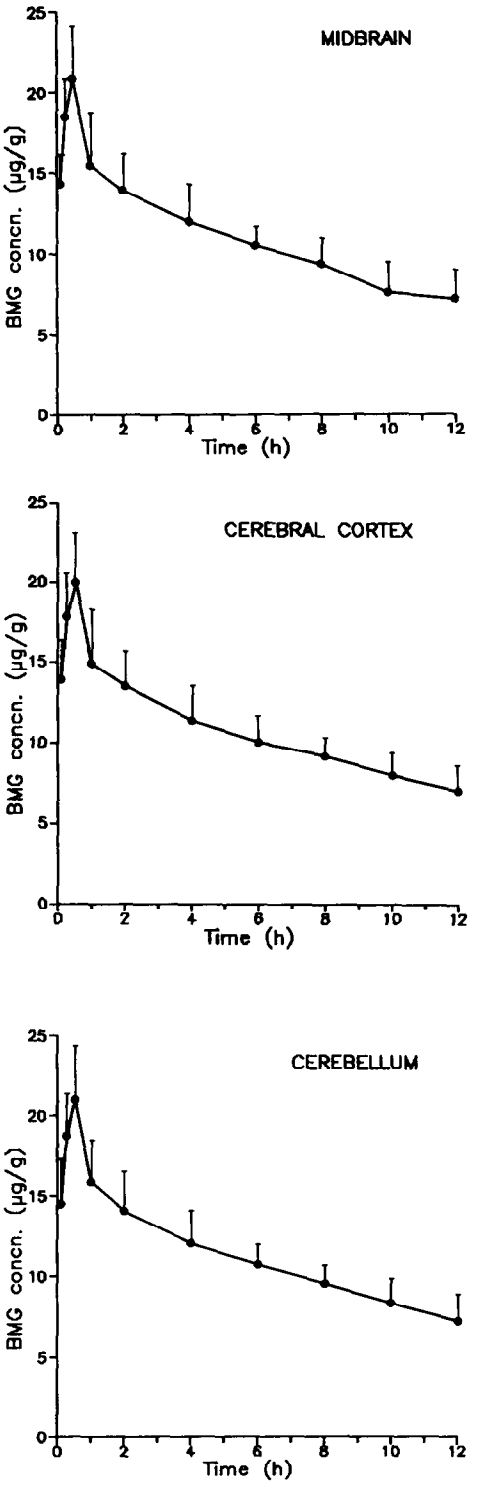

Figure 4
Continued.

Pharmacokinetics

Figure 4 shows the mean serum and brain (cerebral cortex, midbrain, cerebellum) concentrations of BMG after i.p. administration of a single dose of 10 mg kg^{-1} of drug to rats. As

Table 3
Pharmacokinetic parameters* after an i.p. bolus injection of 10 mg kg $^{-1}$ of BMG to rats

	Serum	Cerebral cortex	Midbrain	Cerebellum
k_a (h^{-1})	6.63 ± 1.09	6.30 ± 1.24	6.24 ± 1.21	6.13 ± 1.21
α (h^{-1})	0.242 ± 0.0212	0.559 ± 0.0536	0.587 ± 0.0537	0.592 ± 0.0572
β (h^{-1})	0.0422 ± 0.00368	0.0709 ± 0.00856	0.0698 ± 0.00879	0.0704 ± 0.00877
k_{10} (h^{-1})	0.0831 ± 0.0101	0.157 ± 0.0171	0.158 ± 0.0193	0.159 ± 0.0178
k_{12} (h^{-1})	0.0782 ± 0.00931	0.221 ± 0.0262	0.241 ± 0.0241	0.242 ± 0.0255
k_{21} (h^{-1})	0.123 ± 0.0140	0.253 ± 0.0277	0.260 ± 0.0264	0.262 ± 0.0250
k_{21}:k_{12}	1.57	1.14	1.08	1.08
AUC†	76.8 ± 9.30	102 ± 13.8	93.3 ± 14.1	100 ± 13.0
Brain/serum AUC ratio	—	1.33	1.25	1.30

*Data are mean ±SE values: 50 animals were used.
†Area under the curve, μg ml^{-1} h for serum and μg g^{-1} h for brain tissue.

can be seen, the serum concentration–time profile showed a relatively rapid absorption of the drug, reaching peak concentration (14.86 ± 1.81 μg ml^{-1}) at 30 min, followed by a rapid decrease from 30 min to 2 h and a slower decline during the subsequent 10 h. This kinetic behaviour indicates that the drug is metabolized or redistributed, or both, in two separate processes and conforms to at least a two-compartment model. The pharmacological parameters calculated from the concentration data are summarized in Table 3. Entry of BMG into the brain was rapid with peak concentrations (cerebral cortex 19.95 ± 2.88 μg g^{-1}, midbrain 20.82 ± 3.22 μg g^{-1}, cerebellum 20.97 ± 3.13 μg g^{-1}) all occurring at the same time (30 min), and also followed by a biphasic decay of brain concentrations (Fig. 4). From the pharmacokinetic results shown in Table 3 it is possible to deduce the presence of some accumulation phenomenon in the brain. However, the statistical comparison of the brain AUCs by Duncan's multiple range test [11] showed no significant differences ($p > 0.05$) in the impregnation of the different brain areas investigated. Finally, the ratio of k_{21}:k_{12} was >1 in all cases, which reflected a relatively free movement of the drug between blood and brain compartments.

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