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Determination of the enaminone DM5, an anti-epileptic agent, in mouse plasma and brain tissue by high-performance liquid chromatography with ultraviolet detection

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Abstract

Enaminone derivatives of the 4-carbomethoxy-5-methylcyclohexane-1,3-dione series represent a new and potentially active series of compounds for the treatment of Epilepsy. Enaminone esters have been previously evaluated as compounds with potent oral anticonvulsant activity similar to class 1 anticonvulsants phenytoin, carbamazepine, and lamotrigine. DM5, a member of this class with –Cl in the para-substituted position, has been assessed to have the most potent pharmacological activity (ED_{50}) in both the mouse and rat. A selective and specific high-performance liquid chromatography method was developed to quantitate DM5 in plasma and brain tissue in mice. Reverse phase chromatography with ultraviolet ($\lambda = 307$ nm) detection was utilized to quantitate eluate. A C_{18} analytical column was used and the mobile phase consisted of acetonitrile and 0.05 *M* NaH_2PO_4 buffer (60:40; v/v). Liquid–liquid extraction with ether was used to extract the DM5 from plasma or brain homogenates. DM5 and carbamazepine (internal standard) eluted at ~6.0 and 9.0 min without any interfering peaks. The calibration curves were found to be linear ($r \geq 0.9999$) in the range of 0.1–5.0 μg/ml or μg/g. Intra-run precision's were in all in the range of 90%. The absolute recovery of the analyte in brain and plasma samples was ≤90%. The valid method accurately quantified DM5 in plasma and brain tissue samples collected from a pharmacokinetic study consisting of an intravenous bolus in the tail vein of wild type and genetically altered mice.

Keywords: Enaminone

1. Introduction

Enaminone anticonvulsant derivatives of the 4-carbomethoxy-5-methylcyclohexame-1,3-dione series, recently synthesized by Scott et al. [1–4], represent a new and potentially active series of compounds for the treatment of generalized tonic-clonic and complex partial seizures. Compounds in this series maintain the general cyclic structure of enaminone esters, however differ in both para and meta-substituted chemical groups (–Cl, –F, –Br, $–OCH_3$, $–NO_2$, $–CF_3$, OCF_3, and –CN). Enaminone esters have been previously evaluated as compounds with potent oral anticonvulsant activity at the volt-

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age-dependent sodium channel binding site in a manner similar to class 1 anticonvulsants phenytoin, carbamazepine, and lamotrigine. Preliminary pharmacological studies suggested that DM5 (Fig. 1) was the most promising enaminone analog in this aniline series in both the rat and mouse. DM5 was active intraperitoneally (ip) in mice (ED_{50} 26.2 mg/kg) and orally (po) in rats (ED_{50} 5.8 mg/kg) and compared favorably to phenytoin under the same test conditions (ED_{50} 6.5 and 23.2 mg/kg, respectively) [1–4].

Enaminone esters have been evaluated for their permeability and transport characteristics across the blood–brain barrier (BBB) for elucidation of the physiochemical characteristics pertinent to flux into the CNS [5]. The utilization of a widely accepted in vitro model of the BBB, Bovine Brain Microvessel Endothelial Cells (BBMECs) assessing permeability of these analogs across cell monolayers revealed that a multidrug resistant protein (MDR) might be responsible for efflux of these agents. Further analysis of the transport of enaminone compounds in the presence of possible MDR proteins (i.e. P-glycoprotein or MRP) revealed that Pgp may be responsible for efflux. To better evaluate the mechanisms by which enaminone analogs permeate the BBB in vivo, a genetically altered mouse model originally developed by Shinkel et al., [6] was employed. These mice lack both genes for the expression of Pgp at the BBB and in other tissues as well. To evaluate the role Pgp at the BBB may play on the transport and pharmacokinetic distribution of DM5 in both mouse plasma and brain tissue, a sensitive and specific analytical method to quantitate DM5 was developed.

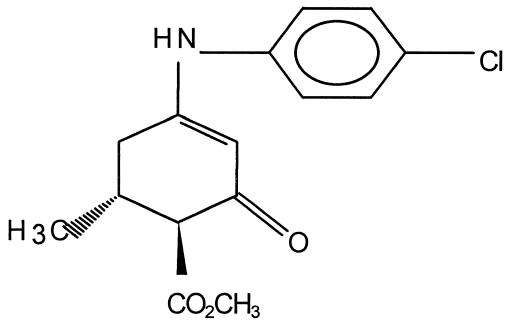

Fig. 1. Enaminone chemical structure of DM5.

2. Experimental

2.1. Materials and reagents

DM5 was synthesized and donated by Kenneth R. Scott (Howard University, Washington, DC). Carbamazepine (CBZ; internal standard) was purchased from Sigma Chemical Co. (St. Louis, MO), sodium phosphate monobasic was obtained from Fisher Scientific (Newark, DE), and dimethyl sulfoxide (DMSO) from Gibco (Grand Island, NY). Acetonitrile and methyl-butyl ether was also purchased from Fisher Scientific. All chemicals and solvents were ACS analytical grade or HPLC grade. Deionized water was prepared by an ultrapure water system Pyrosystem Plus® (Hydro, Research Triangle Park, NC).

2.2. Instrumentation

The plasma and brain samples were analyzed by an HPLC system consisting of a Model 515 liquid chromatograph (Waters-Millipore, Milford, MA) supplied with a 717 Waters autosampler. Solutes were detected with a model 486 Waters UV detector ($\lambda = 307$). A model 3390A Hewlett-Packard (Rockville, MD) integrator recorded chromatograms. All samples were run on a reverse phase ODS C_{18} column (250×4.6 mm, 5 μm; Phenomenex, IL) fitted with a Supelguard™ 5 μm LC-18, 2 cm cartridge guard column (Supelco, Belfont, PA).

The buffer component (0.05 *M* NaH_2PO_4) of the mobile phase was prepared with deionized water and the pH was adjusted to 7.0. The mobile phase consisted of acetonitrile and 0.05 *M* NaH_2PO_4 buffer (60:40, v/v). The mobile phase was filtered through a 0.45 μm nylon filter and degassed under ultrasound and vacuum for 30 min. The mobile phase was delivered at a flow-rate of 1.0 ml/min.

2.3. Preparation of plasma and brain standards

Mice were sacrificed via CO_2 asphyxiation and either plasma was collected via heart puncture or brain was immediately excised for the preparation of standards. DM5 standards were prepared separately from a stock solution (100 μg/ml) in 5% DMSO and PBS and added to plasma standards (0.5 ml) at

the following concentrations: 0.1, 0.2, 0.5, 1, 2, and 5 μg/ml. Appropriate volumes (150 μl) of the stock solutions were added to each plasma or brain sample homogenate to obtain the desired concentration.

2.4. Extraction procedure

Thirty μl of the internal standard (CBZ; 5 μg/ml) solution was added to plasma samples/standards or to brain homogenates samples/standards. One ml of ether was added to either plasma or brain samples. The samples were vortexed briefly (1–2 min) and another 1 ml of ether was added. After the second vortexing, samples or standards were transferred to −70°C freezer for 5–10 min to freeze the aqueous phase. The organic phase was then decanted into 20 ml conical test tubes. The samples were evaporated at 37°C under a gentle stream of nitrogen. The resulting residue was reconstituted in 0.5 ml of mobile phase, vortexed and centrifuged. The supernatant was transferred to micro-vials (150 μl) and 30 μl of standard/sample was injected onto the HPLC.

2.5. Assay validation

2.5.1. Linearity and range

The linear detector response for the assay was tested as follows. Three determinations ($n=3$) from a minimum of six concentration levels (0.1, 0.2, 0.5, 1, 2, and 5 μg/ml or μg/g) of the analyte were made for both plasma and brain. Detector response was correlated against analyte concentration by least-squares regression. A weight of $1/y$ was utilized to determine slopes, intercepts, and correlation coefficients. The minimum acceptable coefficient to establish linearity was set at 0.95 a priori.

2.5.2. Method precision and percent recovery

Precision of the assay was established by analysis of three replicates ($n=3$) of a analyte standards at the following concentrations: 0.1, 0.2, 0.5, 1, 2, and 5 μg/ml. To determine intra-day precision of the assay, replicate ($n=3$) samples (plasma and brain) of five different concentrations were analyzed. To determine inter-day precision, replicate brain and plasma samples ($n=3$) were analyzed on three different days. The percent relative standard deviations (RSDs) of the assay results were determined.

Extraction efficiency was determined by comparing replicate ($n=9$) peak height ratios (PHRs) of extracted plasma and brain samples vs. unextracted water standard for the 0.1, 0.5, 2, and 5 μg/ml concentrations. The percent recovery was determined by the following equation:

$$\%\text{Recovery} = \frac{\text{Peak Height Ratio (plasma or brain standard)}}{\text{Peak Height Ratio (water standard)}} \quad (1)$$

2.6. Accuracy

Method standards in the concentration range of 0.2–5.0 μg/ml from three different runs performed over several days were utilized to evaluate accuracy of the assay method for both plasma and brain samples. The means of the three runs were calculated and compared to the spiked value to determine the percentage difference between the mean and the spiked value (amount added). The percentage relative error was determined by the following equation:

$$\%\text{relative error (ER)} = \frac{[\text{mean} - \text{spiked}]}{[\text{spiked}]} \times 100 \quad (2)$$

2.7. Pharmacokinetic study

A study was designed to investigate the pharmacokinetics and brain distribution of DM5 in FVB wild type mdr*1*ab (+/+) and knockout mice (−/−) (Taconic Laboratories, NY). The protocol was approved by the University of Maryland, School of Pharmacy IACUC. Both wild type and knockout male mice approximately 4–6 weeks of age (wt.= 20±5 g) were administered a 12.5 mg/kg intravenous bolus (injection volume 0.2 ml/kg) via the tail vein. Cohorts of 3 animals were sacrificed by CO_2 asphyxiation at the following time points: 15, 30, 60, 90 min and at 2, 4, 8, and 12 h. Plasma and brain tissue samples were collected, flash frozen, and stored at −70°C until analysis.

3. Results and discussion

3.1. Resolution

Fig. 2A and B represent chromatograms of extracted blank mouse plasma and a calibration stan-

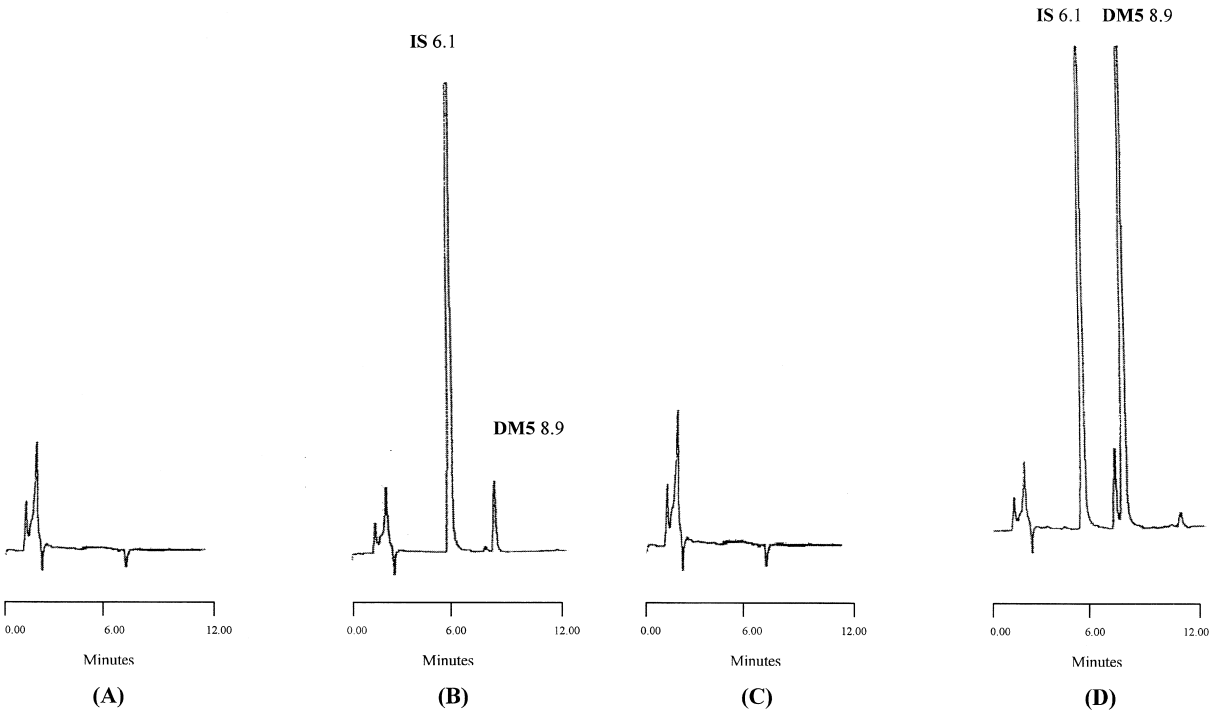

Fig. 2. Extracted mouse plasma chromatograms: (A) extracted blank mouse plasma; (B) extracted mouse plasma standard (0.1 μg/ml); (C) extracted blank mouse plasma pre-dose sample; and (D) extracted mouse plasma at 60 min (1.0 μg/ml).

dard containing DM5 at 0.1 μg/ml with internal standard (CBZ; 5 μg/ml) respectively. Fig. 2C is a representative chromatogram of an extracted pre-dose mouse plasma sample and Fig. 2D displays a chromatogram from the 60 min sample after intravenous administration (≅1.0 μg/ml). The assay was found to be specific for DM5 and no interfering peaks from degradation products due to metabolism were detected. Additionally, the peaks of DM5 and the internal standard (CBZ) were sufficiently separated with typical retention times of approximately 6 min for CBZ and 9 min for DM5.

Fig. 3A and B are representative chromatograms of extracted blank mouse brain tissue and a calibration standard containing DM5 at 1.0 μg/g with the internal standard carbamazepine (5 μg/ml) respectively. Fig. 3C and D are chromatograms of an extracted pre-dose brain tissue sample and from a 60 min sample after intravenous administration of DM5. Again, the assay for DM5 in brain tissue was found to be specific with no interfering peaks from either metabolites, internal standard or brain tissue. There was sufficient separation of DM5 and the CBZ with typical retention times of approximately 6 min for CBZ and 9 min for DM5.

3.2. Extraction recovery

After comparison of the PHRs of extracted plasma/brain tissue standards and water standards, the extraction recovery was found to be in the range of 96–99.8% for plasma and 90–96% for brain tissue.

3.3. Validation assay precision

The standard curves for DM5 showed linearity over the selected concentration range (0.1–5.0 μg/ml) for both plasma and brain tissue with consistent slopes and excellent correlation coefficients ($r \geq$

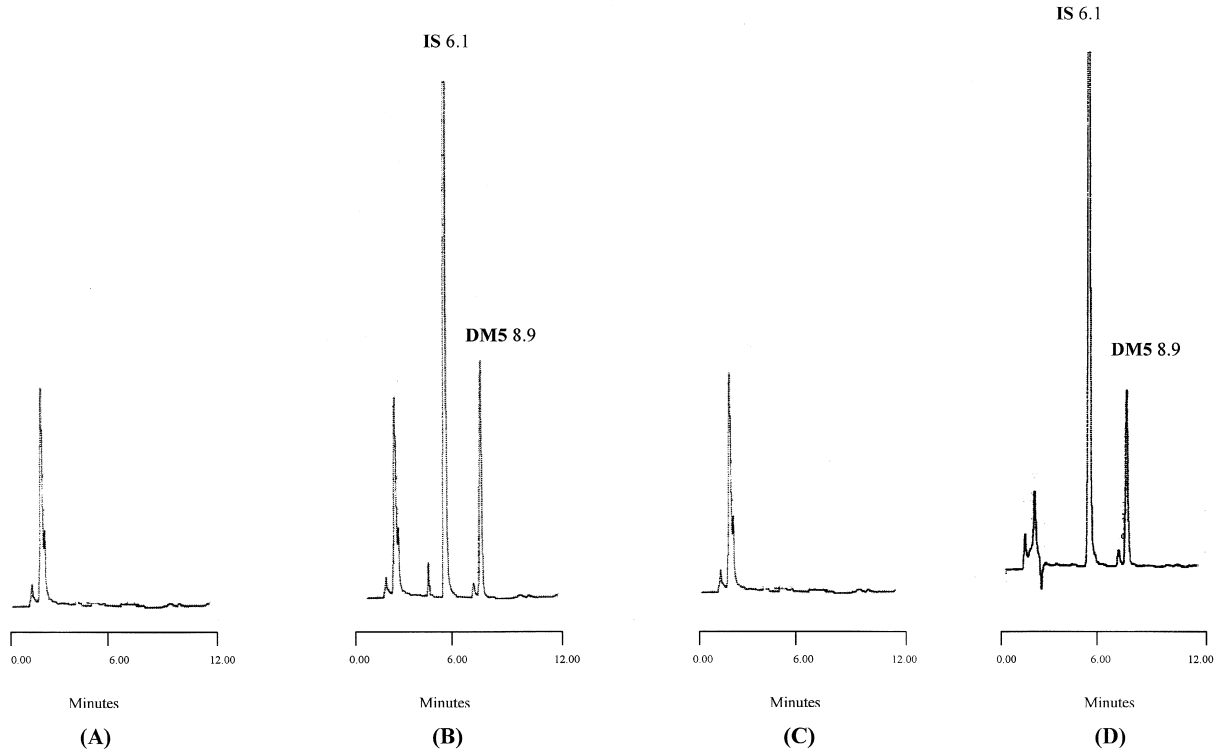

Fig. 3. Extracted mouse brain tissue chromatograms: (A) extracted blank mouse brain tissue; (B) extracted mouse brain tissue sample at 60 min (1.0 μg/g); (C) extracted blank mouse tissue; and (D) extracted mouse brain tissue standard (0.2 μg/g).

0.9999 and 0.999) respectively throughout the validation runs. The intra-day and inter-day precision data for DM5 in mouse plasma is listed in Table 1. The intra-day %RSD was 3.5% or less where the inter-day precision was 4% or less. Table 1 also presents the precision data for DM5 in brain. The RSD intra-day was 8.0% or less and the inter-day precision was <5.0%. Table 2 presents the intra- and inter run accuracy data for both plasma and brain analysis. The relative intra-day and inter-day error ranged from −2.1 to 4.2% for DM5 in plasma and −2.6 to 7.2% in brain tissue.

Table 1
Intra-day and Inter-day assay precision for DM5 in mouse plasma and brain

Concentration (μg/ml or μg/g)	Intra-run precision (%RSD)		Inter-run precision (%RSD)	
	Plasma	Brain	Plasma	Brain
0.1	3.3	6.2	3.5	4.8
0.2	3.0	4.7	4.0	2.1
0.5	1.1	1.4	0.5	1.5
1	0.8	1.2	0.3	1.0
2	0.4	3.4	0.5	2.1
5	0.2	7.2	0.2	2.2

Table 2
Intra-day and Inter-day assay accuracy for DM5 in mouse plasma and brain

Concentration (μg/ml or μg/g)	Intra-run accuracy (% RE)		Inter-run accuracy (%RE)	
	Plasma	Brain	Plasma	Brain
0.1	3.2	6.3	1.0	5.5
0.2	2.8	3.1	4.2	7.2
0.5	−1.6	2.8	−1.0	0.9
1	−2.1	−2.6	0.7	0.3
2	1.0	1.5	0.2	1.1
5	−0.4	−0.5	−0.1	−0.3

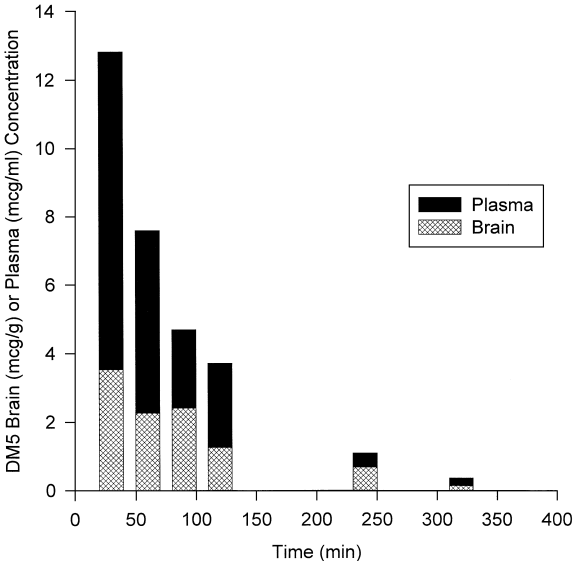

Fig. 4. DM5 Plasma and Brain concentration in mice after a single i.v. bolus dose of 12.5 mg.

3.4. Pharmacokinetic study

Fig. 4 presents a brain and tissue concentration vs. time profile for DM5 in mice after single intravenous administration of 12.5 mg/kg. This profile highlights the sensitivity of the assay method as well as its utility since it is possible to follow the time course of DM5 in plasma and brain samples after a single intravenous dose. In addition, we examined the stability of plasma and brain samples spiked with DM5. We observed no degradation of DM5 in plasma or brain samples over a 24 h period when stored at room temperature. Further, the assay allowed for the comparison of DM5 disposition profiles in wild type and knockout mice.

4. Conclusion

In summary, a valid, specific analytical method has been developed for a new potent anticonvulsant agent, DM5 with high recovery in both plasma and brain. The method employs reverse phase HPLC, ultraviolet detection and liquid–liquid extraction. The ease of sample preparation will allow for rapid analysis of samples from tissue distribution studies and pharmacokinetic studies. The liquid–liquid extraction procedure used provides purer extracts, permitting greater sensitivity to be achieved. This analytical method has been used to examine the pharmacokinetic disposition of DM5 after single dose administration and will be used for additional studies examining the disposition, and brain distribution properties of DM5 and other potent enaminone agents.

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