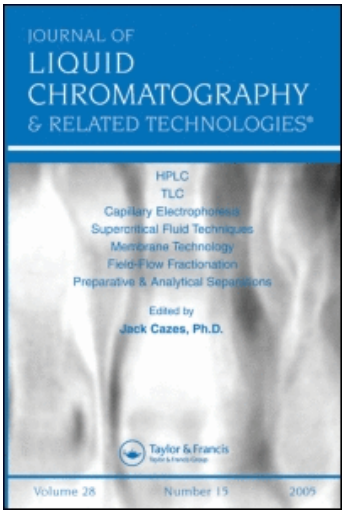

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Simultaneous Determination of Ethosuximide, Phenobarbital, Pheny-Toin, and Carbamazepine in Brain Tissue by HPLC

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To cite this Article Soto-Otero, Ramón , Méndez-Alvarez, Estefania and Sierra-Marcuño, Germán(1985) 'Simultaneous Determination of Ethosuximide, Phenobarbital, Pheny-Toin, and Carbamazepine in Brain Tissue by HPLC', Journal of Liquid Chromatography & Related Technologies, 8: 4, 753 — 763
To link to this Article: DOI: 10.1080/01483918508067116
URL: http://dx.doi.org/10.1080/01483918508067116

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(4), 753-763 (1985)

SIMULTANEOUS DETERMINATION OF ETHOSUXIMIDE, PHENOBARBITAL, PHENYTOIN, AND CARBAMAZEPINE IN BRAIN TISSUE BY HPLC

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ABSTRACT

A simple and rapid method was evaluated for the simultaneous determination in brain tissue of ethosuximide, phenobarbital, phenytoin, and carbamazepine. The drugs were simultaneously extracted at low pH using dichloromethane as extraction solvent in the presence of an excess of ammonium sulfate. High-performance liquid chromatography was performed on a Spherisorb 5ODS column, with a mobile phase of acetonitrile/methanol/phosphate buffer pH 4.0 (21:24:55 by vol), and monitored at 195 nm. The internal standard was 5-(p-tolyl)-5-phenylhydantoin. The sensitivity, precision, and reproducibility of the assay were all found to be acceptable for the simultaneous determination of the above described drugs in small samples of brain tissue.

0148-3919/85/0804-0753$3.50/0

INTRODUCTION

The determination of brain levels of antiepileptic drugs is of special interest since their site of action lies within the brain and knowing their levels makes it possible to investigate the kinetics of the penetration and accumulation of these drugs and their possible alterations (1,2). The implications of their distribution in pathological brain tissue are also of clinical interest (3). Recently, it has been suggested that the preferential accumulation of drugs in tumour tissue may play a role in the natural evolution of the tumour (4). However, despite the importance of these data and the advantages of liquid chromatography over other techniques (5), there is little in the literature on the application of HPLC to the determination of levels of antiepileptic drugs in the brain and, moreover, published methods are not well documented (6-9), giving no data on accuracy, precision, and column life. Very recently, a well evaluated method was reported (10), but it only permitted the determination of phenobarbital or phenytoin because it used 5-(p-hydroxyphenyl)-5-phenylhydantoin and phenobarbital as internal standards.

In a previous paper (11) we reported a simple and rapid HPLC method for the simultaneous determination of ethosuximide, phenobarbital, phenytoin, and carbamazepine in plasma, and, in view of the good results obtained and the present state of methods for investigating antiepileptic drug levels in the brain, we decided to apply the technique to brain tissue. In the present study we evaluated the simultaneous determination of those drugs in brain tissue as a follow-up to the previous report.

MATERIALS AND METHODS

Apparatus

We used a Kontron HPLC system Model 620 (Zurich, Switzerland) coupled to a Uvikon variable-wavelength UV-VIS detector Model 720LC (Kontron AG), a Kontron programmer Model 200, and a Hewlett-Packard integrator Model 3390A (Avondale, PA 19311). The analytical column was a 25cm x 4.6mm I.D. Spherisorb 5ODS (Kontron AG), protected by a 5cm x 4.6mm I.D. pre-column packed with Co:Pell ODS (Whatman, Clifton, NJ 07014). Samples were injected using a Rheodyne injector Model 7125 fitted with a 20 µl loop.

Reagents and Drugs

Acetonitrile and methanol were of HPLC grade (Fisher Scientific, Fair Lawn, NJ 07410). Dichloromethane was LiChrosolv^{R} (Merck, Darmstadt, F.R.G.). All inorganic chemicals were of A.R. grade (Merck). HPLC grade water was prepared with the Norganic water purification system (Millipore, Bedford, MA 01730). All pure drugs used were donated by pharmaceutical companies: ethosuximide from Sustancia-Parke Davis (Barcelona, Spain), phenobarbital and phenytoin from Bayer (Barcelona, Spain), and carbamazepine from Ciba-Geigy (Barcelona, Spain). 5-(p-tolyl)-5-phenylhydantoin (internal standard) was purchased from Aldrich-Chemie (Steinheim, F.R.G.).

Standards

Stock solutions of each drug were prepared in methanol to give a concentration of 1 mg/ml.

Standards were prepared in brain tissue homogenates of Wistar rats at five different levels containing the appropriate amounts at the following concentrations in brain tissue (µg/g): ethosuximide 10, 25, 50, 100, 150; phenobarbital 5, 10, 20, 40, 80; pheny-

toin 2.5, 5, 10, 20, 40, and carbamazepine 2, 4, 8, 12, 20.

Internal standard solution (60 µg/ml) was prepared daily in water.

Mobile Phase

The mobile phase was a mixture of acetonitrile, methanol, and phosphate buffer pH 4.0 (21:24:55 by vol). The phosphate buffer was prepared by adding 150 µl 1M KPO_4H_2 to 1000 ml of water and adjusting to pH 4.0 with 0.9M H_3PO_4. Prior to use the mobile phase was degassed by filtration through a 0.45 µm FHUP filter (Millipore).

Procedure

Three hundred mg of brain tissue were homogenized with 1.5 ml of water to a smooth consistency in a glass pestle tube and 1 ml was then transferred to a glass-stoppered centrifuge tube to which 100 µl of the internal standard solution was added, followed by 3 drops of 6N HCl. After mixing, 5 ml of dichloromethane were added and the tube mixed again and an excess of crystalline ammonium sulfate was added. After mixing and centrifuging, the upper aqueous layer was discarded and the organic phase transferred to a conical glass tube. Four ml of the organic phase were pipetted to a new conical glass tube and evaporated to dryness at 42°C under a gentle stream of air.

The dryness residue was dissolved in 50 µl of methanol and 20 µl injected into the chromatograph, maintaining a flow rate of 3ml/min with the detector at a wavelength of 195nm. Chromatography was performed at room temperature.

Quantitation was based on peak height ratio of each drug to the internal standard.

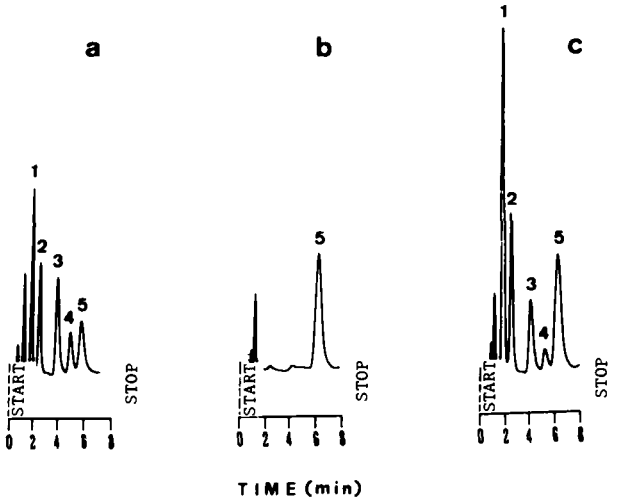

FIGURE 1. a) Chromatogram from the injection of 1 µg of each ethosuximide (peak 1), phenobarbital (peak 2), phenytoin (peak 3), carbamazepine (peak 4), and internal standard (peak 5) in 20 µl of methanol. b) Chromatogram of an extract from a blank brain homogenate. c) Chromatogram of an extract from a blank brain homogenate spiked with 10 µg/ml ethosuximide, 4 µg/ml phenobarbital, 2 µg/ml phenytoin, and 1.6 µg/ml carbamazepine.

RESULTS

Figure 1 shows typical chromatograms obtained with the reported procedure from a test mixture, from an extract of a blank brain homogenate, and from an extract of a spiked brain homogenate. Note the good resolution and the absence of interfering peaks of brain components.

Figure 2 shows chromatograms obtained from extracts of cortex matter from two rats treated intraperitoneally once a day with two different combined antiepileptic drug regimens for ten days.

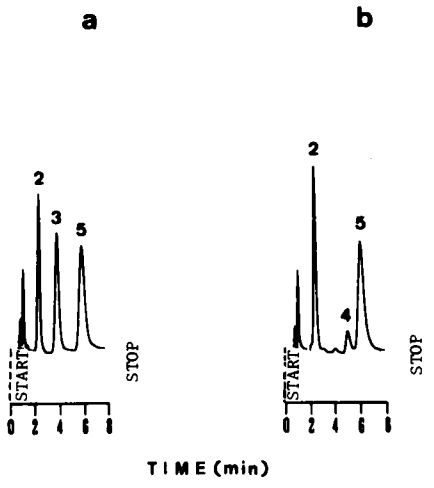

FIGURE 2. a) Chromatogram of an extract from a brain homogenate of cortex matter from a rat intraperitoneally treated with a single dose of phenobarbital and phenytoin for 10 days. Concentrations were 20.8 µg/g phenobarbital and 17.1 µg/g phenytoin. b) Chromatogram of an extract from a brain homogenate of cortex matter from a rat intraperitoneally treated with a single dose of phenobarbital and carbamazepine for 10 days. Concentrations were 22.9 µg/g phenobarbital and 9.5 µg/g carbamazepine.

We have verified the linearity of the peak height ratios versus concentrations for all the drugs over the studied range. Table 1 shows the results obtained using a linear regression analysis.

The accuracy and precision of the method were determined by assaying brain homogenates containing known quantities of the drugs. As shown in Table 2, within-day precision (CV) ranged from 1.2% to 4.9%, and between-day precision (CV) ranged from 1.2% to 5.2%.

Recoveries were calculated by comparing the peak height ratios from extracted samples to which internal

standard was added prior to the injection with those obtained from direct injection of the same amount of each drug in methanol. As shown in Table 3, recoveries ranged from 87% to 96%.

DISCUSION

With the mobile phase composition reported we obtained a good resolution for all assayed drugs in a short period of time. The use of methanol was necessary in order to resolve carbamazepine from phenytoin. The acidic pH of the buffer in the mobile phase allows the peak tails to be reduced and maintains a constant absorbance.

We found that extraction with dichloromethane at low pH in the presence of an excess of ammonium sulfate allows a rapid precipitation of brain proteins and a complete elimination of all brain interfering compounds. The major advantage of dichloromethane is its easy evaporation at 42°C which avoids losses of ethosuximide (5).

TABLE 1

Regression Lines of the Standard Curves for the Four Antiepileptic Drugs

Drug	Slope	y-intercept	Correlation Coefficient
Ethosuximide	0.061	0.052	0.997
Phenobarbital	0.084	0.087	0.998
Phenytoin	0.073	0.011	0.999
Carbamazepine	0.026	0.002	0.999

Each standard curve was plotted from 35 points, which corresponded to 7 samples assayed for each standard.

TABLE 2

Accuracy and Precision of the Assay

Drug	Actual (μg/g)	Within-day ($n^{\cdot}$ =5) Mean measured (μg/g)	Within-day ($n^{\cdot}$ =5) CV (%)	Between-day ($n^{\cdot\cdot}$ =5) Mean measured (μg/g)	Between-day ($n^{\cdot\cdot}$ =5) CV (%)
Ethosuximide	10	10.2	4.9	10.8	5.2
	25	25.0	3.2	25.3	4.1
	50	50.4	2.9	50.0	3.2
	100	101.2	1.7	99.8	1.9
	150	151.7	1.6	150.4	1.2
Phenobarbital	5	4.9	3.8	4.8	3.1
	10	10.3	3.2	10.2	3.0
	20	20.3	3.1	20.6	2.8
	40	40.8	2.8	40.2	1.7
	80	81.0	2.2	80.7	1.3
Phenytoin	2.5	2.5	3.7	2.6	4.1
	5	5.1	3.2	4.8	3.8
	10	10.0	3.1	10.5	3.4
	20	20.4	2.7	19.8	2.4
	40	40.8	2.1	39.6	1.9
Carbamazepine	2	2.0	3.8	2.1	3.2
	4	4.1	3.4	4.2	3.0
	8	8.2	2.8	7.9	2.9
	12	12.0	2.6	11.8	2.3
	20	20.7	1.2	19.8	1.7

$n^{\cdot}$ = number of samples assayed in one day

$n^{\cdot\cdot}$ = number of days with one sample assayed every two days

TABLE 3

Analytical Recovery of Assayed Drugs from Brain Homogenate

Drug	Added (μg/g)	% Recovery
Ethosuximide	10	92
	25	92
	50	94
	100	96
	150	95
Phenobarbital	5	91
	10	91
	20	91
	40	92
	80	90
Phenytoin	2.5	87
	5	89
	10	89
	20	91
	40	92
Carbamazepine	2	88
	4	88
	8	88
	12	93
	20	94

The pre-column was used in order to avoid the accumulation of neutral lipids within the analytical column. We carried out a daily column cleanup and a replacement of pre-column packing every 30 to 40 working days. Under these conditions we observed no loss of resolution or increase in back pressure after ten months of regular use.

From the results obtained, the present method was considered to be applicable to the quantitative analysis of the drugs assayed in brain tissue, having

the following advantages over previously published methods: a) simultaneous determination of the four major antiepileptic drugs, thus permitting a study of the pharmacokinetic fates of those drugs in the brain when concomitantly administered; b) a rapid, single extraction procedure which eliminates all interfering components and extends the analytical column life, and c) the use of small samples of brain tissue, which enables one to work with discrete areas of brain from small animals like the rat in basic biomedical research.

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