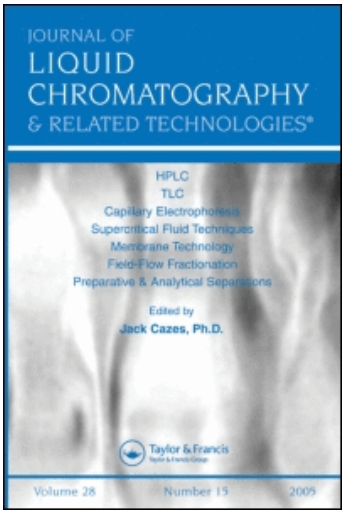

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 31 August 2001

To cite this Article Luis, Ma Luz , Blanco, Domingo , Arias, Juan J. , Jiménez, Francisco , Jiménez, Ana I. and Fraga, José M. G.(2001) 'CAPILLARY ZONE ELECTROPHORESIS ANALYSIS OF ANTI-EPILEPTIC DRUGS', Journal of Liquid Chromatography & Related Technologies, 24: 13, 1921 — 1928
To link to this Article: DOI: 10.1081/JLC-100104434
URL: http://dx.doi.org/10.1081/JLC-100104434

J. LIQ. CHROM. & REL. TECHNOL., 24(13), 1921–1928 (2001)

CAPILLARY ZONE ELECTROPHORESIS ANALYSIS OF ANTI-EPILEPTIC DRUGS

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ABSTRACT

This paper describes the development of a simple, reliable, reproducible, and fast method, based on capillary zone electrophoresis with diode array detection, for the separation and determination of two antiepileptic drugs. Phenytoin (DPH) and phenobarbital (PB) were separated in 4 min by using a carrier containing glycine 50 mM, pH 10.5. The limit of detection was 0.1 $\mu g\ mL^{-1}$ for both compounds. Satisfactory reproducibility was obtained for migration times (<1.4%) and corrected peak areas (<6.9%) in the analysis of pharmaceutical formulations. The average recoveries obtained, which ranged between 100 and 108%, testify to the accuracy of the proposed method.

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INTRODUCTION

Phenytoin (DPH) has been widely used in the treatment of patients with epilepsy, convulsions, and partial seizures, and phenobarbital (PB) is an effective anticonvulsant for the treatment of partial seizures.[1,2] Both compounds are frequently prescribed in combination,[3] and the dosage is very important since long-term anticonvulsant treatment produces abnormal bone mineral metabolism,[4] and other biochemical dysfunctions.[5,6]

Many techniques have been developed for antiepileptic analysis, including high-performance liquid chromatography,[7-12] gas chromatography,[13] supercritical fluid chromatography,[14] multivariate spectrometry,[15] and inmunochemical techniques.[16-18] Now, due to its different separation mode and much faster analysis times, capillary electrophoresis is widely accepted as a powerful new tool for drug analyses. In fact, phenobarbital has been analysed by capillary zone electroforesis (CZE) and micellar electrochromatography (MECC).[19,20]

This paper reports the CZE separation and quantification of phenytoin and phenobarbital in a pharmaceutical formulation using UV detection. The influence of the nature of the buffer on migration time and separation of antiepilectic drugs was evaluated. Limits of detection, linearity, reproducibility, and recoveries are presented for these analytes.

EXPERIMENTAL

Apparatus

A Hewlett-Packard (Waldbronn, Germany) three-dimensional capillary electrophoresis system was used in all measurements. The fused silica capillary (48.5 cm x 50 μm i.d., effective length 40.0 cm) was supplied by Hewlett-Packard.

Chemicals

Phenytoin was obtained from Sigma (St. Louis, MO, USA) and phenobarbital was purchased from Aldrich (Milwauke, Wisconsin, USA). TRIS, Phosphate and boric acid were obtained from MERCK (Darmstadt, Germany), Glycine and MES were purchased from Sigma (St. Louis, MO, USA). All were of analytical-reagent grade.

Milli-Q water (Millipore, Milford, MA, USA) was used throughout all the experiments.

The pharmaceutical formulation "Epilantín" was provided by Otsuka pharmaceutical, S.A. (Barcelona, Spain).

Sample and Buffer Preparation

The standard phenytoin and phenobarbital were dissolved in ethanol. The buffers were prepared by dissolving the required amount of glycine in Milli-Q water, and subsequently adding 1M NaOH to the former solution (for pH 10.5). Samples and buffers were filtered through a 0.45 μm syringe filter prior to use.

An antiepileptic tablet was ground to a homogeneous powder and dissolved with water containing 40% ethanol in an ultrasonic bath for 20 minutes. Then, the mixture was diluted to a final concentration of 50 and 25 mg/L for DPH and PB, respectively, and filtered through a 0.45 μm syringe filter and injected into the system.

Analytical Conditions

The capillary was preconditioned for 15 min with 1 M NaOH before the first run and then for 5 min with 0.1 M NaOH and for 6 min with run buffer prior to each subsequent run, since reproducibility and peak shape in capillary electrophoresis (CE) are sensitive to the state of the inner wall of the capillary. When the antiepileptic tablet extract was injected, an increase of migration times was observed probably due to interferences from the excipients, in our opinion from the starch content of antiepileptic tablet. In this case, after each injection, the capillary was washed with 0.5 M NaOH for 8 min and run buffer for 5 min.

The injection was carried out by pressure (hydrodynamic) at 50 mbar for 3.7 s (aprox. 5.9 nL). In order to ensure area reproducibility and a quantitative injection of the sample, subsequent injections in the same conditions of the run buffer was necessary.

The standard separation conditions were voltage 25 kV (positive polarity), capillary temperature 20°C, and a 50 mM glycine run buffer at pH 10.5.

RESULTS AND DISCUSSION

Separation Conditions

Initially, it is necessary to optimise the pH because it is important to have the analytes in their respective ionic forms. Furthermore, pH influences the magnitude

of EOF, which is directed towards the negative end of the fused-silica capillary. For the simultaneous determination of anions (and also cations) in CZE in a reasonable time when positive polarity was used, it was necessary to force an electroosmotic flow (EOF) towards the detector at a rate, which exceeded the migration rate of the anions. For anion analyses it was also possible to reverse the polarity.

Three buffers, namely Tris-Mes, phosphate-borate, and glycine were tested at different pHs and separation voltages. The resulting plots of observed current *vs* applied voltage for these buffers showed a deviation from linearity above 20 kV for the phosphate-borate buffer. Ohm's law plots for the Tris-Mes and glycine buffers were linear up to 25 kV.

Fig. 1 shows electropherograms of DPH and PB in these carriers at different pHs and different polarities for the best pH. As can be seen in Table 1, the maximum separation efficiency (theoretical plates) was obtained with 50 mM glycine at pH 10.5 and positive polarity. The separation run time does not exceed 4 min, and an optimum separation could be achieved (Fig. 1C).

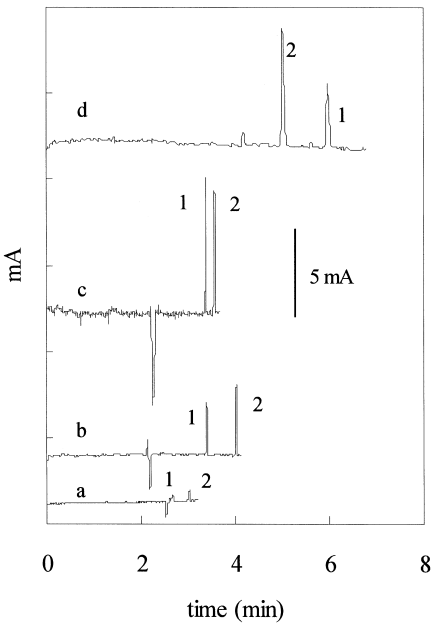

Figure 1. Electropherograms of a mixture of phenytoin (1) and phenobarbital (2) with different carrier electrolytes. a: Tris-Mes (50 mM pH 7.0, 25 KV), b: phosphate-borate (50 mM pH 9.0, 20 KV); c: glycine (50 mM, pH 10.5, 25 KV, positive polarity); d: glycine (50 mM, pH 10.5, -25 KV, negative polarity). Capillary temperature 20°C. Detection at 230 nm.

Table 1. Effect of Run Buffer and Polarity on Separation Efficiency

Run Buffer	N_{DPH}	N_{PB}
TRIS-MES, pH 7.0	5.1E4	8.9E4
Phosphate-borate, pH 9.0	1.9E5	1.5E5
Glycine, pH 10.5 (positive polarity)	1.9E5	2.5E5
Glycine, pH 10.5 (negative polarity)	4.2E4	4.8E4

N= number of theoretical plates.

The suggested analytical conditions were, therefore, analysis temperature 20°C, voltage 25 kV, and a 50 mM glycine pH=10.5 run buffer.

Analytical Performance

Table 2 summarises the quantitative analytical parameters of CZE separation of the antiepileptic drugs. As can be seen, satisfactory reproducibility for corrected areas was obtained for 6 pharmaceutical sample injections. These range from 4.58 to 6.88 relative standard deviation (RSD).

To measure repeatability and reproducibility of the method, we prepared two series of three samples for each one, analysis of variance obtained values of 1.03 and 7.49 for F of DPH and PB, respectively, less than F critical, 7.71, for both compounds.

Calibration graphs for DPH and PB showed good correlation between peak areas and drug concentrations, with regression coefficients above 0.999 in both cases. Linearity of the calibration graphs was also checked with two different statistical tests (the Fisher and the lack-of-fit tests). For the Fisher test, the values obtained were always higher than the tabulated values (α=0.05), thus linearity

Table 2. Analytical Characteristics of the Proposed Method

	Migration Time (min)	Migration Time RSD % (n=11)	Peak Area RSD% (n=6)
Phenytoin	3.35	1.34	4.58
Phenobarbital	3.54	1.43	6.88

RSD = relative standard deviation.

Table 3. Parameters of Regression

	b_0[a]	b_1	r	LOD[b] (μg mL^{-1})
Phenytoin	0	1.59 ± 4.06E-2	0.9997	0.1
Phenobarbital	0	1.48 ± 3.38E-2	0.9997	0.1

[a] Accepted null hypothesis intercept is zero.
[b] LOD = Limit of detection based on a signal to noise ratio of 3.

was demonstrated. In the lack-of-fit test, the calculated values were lower than the tabulated ones, and the hypothesis was, therefore, also demonstrated.

The limits of detection were 0.1 μg mL^{-1} for both compounds. These were determined by injecting serial dilutions of a concentrated standard mixture, followed by the preparation of calibration plots (peak height *vs* concentration injected), which were extrapolated to a signal-to-noise ratio (S/N) of 3 to assign the detection limits.

Each drug in the sample was identified by matching the migration time with the standard and by using a spectral library search. Quantification was carried out by calibration with standard drug solutions. Peak areas were corrected for the retention time.

Recovery experiments were performed in order to study the accuracy of the method. Known amounts of each analyte were added to a variety of samples and the resulting spiked samples were subjected to the entire analytical sequence. Each solute was spiked at three different concentrations and recoveries were calculated on the basis of the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses

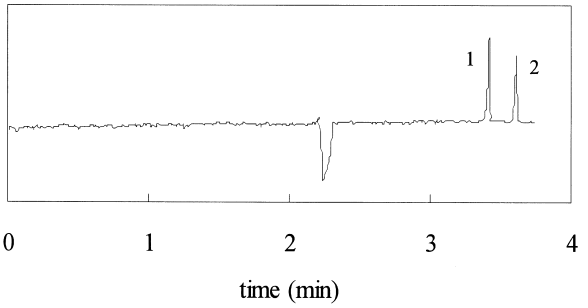

Figure 2. Electropherograms of a pharmaceutical formulation. Phenytoin (1) phenobarbital (2). Conditions as in Fig. 1 C.

Table 4. Results of the Pharmaceutical Formulation Analysis

	Found Concentration[a] ($\mu g\ mL^{-1}$)	Declared Concentration ($\mu g\ mL^{-1}$)
Phenytoin	104 ± 2	100
Phenobarbital	45 ± 1	50

[a] Mean of three determinations.

were carried out in triplicate. The average recoveries obtained which ranged between 100 and the 108%, testify to the accuracy of the proposed method.

Figure 2 shows an electropherogram of Epilantín tablets, and Table 4 shows the results of the analysis. We found that the phenobarbital content declared by the producer was higher than the one we found, but recoveries obtained demonstrate the accuracy of the proposed method.

CONCLUSIONS

This paper proposed a rapid and reproducible method for the determination of antiepileptic drugs. The method was satisfactorily applied to the analysis of a pharmaceutical formulation. The limits of detection of the method were in the low $\mu g\ mL^{-1}$ range and showed a high level of precision. Furthermore, the analysis time is very short (<4 min).

ACKNOWLEDGMENT

The authors wish to acknowledge the financial support of this work by the Ministerio de Educación y Cultura (DGICyT, research project no. PB98/0439).

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Received November 9, 2000 Manuscript 5443
Accepted December 5, 2000