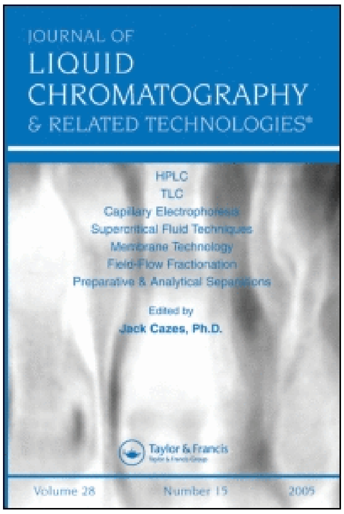

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DETERMINATION OF VALPROIC ACID SALTS IN PHARMACEUTICAL PREPARATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH COULOMETRIC ELECTROCHEMICAL DETECTION

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ABSTRACT

A highly sensitive and selective method for the determination of valproic acid salts in pharmaceutical tablet formulations by a high performance liquid chromatography method with coulometric electrochemical detection is described. The pre-column derivatizations of valproic acid and the internal standard (n-hexanoic acid) were carried out using 1-(2,5-dihydroxyphenyl)-2-bromoethanone as an electroactive labeling reagent. The resulting electroactive esters were separated by isocratic elution of a 3 µm Adsorbosphere ODS column with methanol-acetonitrile-acetate buffer eluent.

The compounds were detected by a porous graphite electrode set at an oxidation potential of + 0.45 V. The analytical method developed in this study is suitable for quality control assays of complex pharmaceutical formulations containing valproic acid or its salts.

INTRODUCTION

Since its introduction in the United States in 1964, valproic acid (VA) has become one of the most frequently prescribed anticonvulsivants in both pediatric and adult patients. VA has been found to be effective in treating a number of seizure types and is frequently used as a part of multi-drug therapy in patients with mixed seizure disorders.[1] In addition, VA has been used for psychiatric conditions such as maniac-depressive psychosis and tardive diskynesia.[2-3]

VA is available in a variety of oral dosage forms such as tablet, capsule, and drop solution. In addition to the acid form, the drug may be given as a sodium salt (sodium valproate), magnesium salt (magnesium valproate), or as a mixture of equal portions of VA and sodium valproate (divalproex sodium).

Several assay methods have been described for the analysis of VA in pharmaceutical and biological material, including immunoassay[4-5] and gas-chromatography procedures (GC) and high performance liquid chromatography (HPLC)[6-9] or gas-chromatography-mass spectrometry (GC-MS).[10] The GC-MS methods are suitable for simultaneous determination of VA and its metabolites. The common disadvantage of gas-chromatography based methods are the lengthy and the cumbersome multiple-step procedure for sample preparation, such as solvent extraction, evaporation, and derivatization that result in a loss of sample and poor reproducibility. In the enzyme immunoassay the VA determination at low concentrations shows a poor precision and the VA concentration shows a hyperbolic rather than a linear relationship to the change in absorbance. In addition, such a method is unable to analyze VA and its metabolites simultaneously.[4]

The HPLC technique is the method of choice due to its precision and simplicity. Direct detection of VA is troublesome due to the lack of chromophoric and fluorescent groups and, therefore, short UV wavelengths must be used. This results in a deficiency of sensitivity and in an increase of the interference from matrix components. In order to improve the sensitivity and the selectivity for the HPLC determination of VA, derivatization procedures, using 4-bromomethyl-7-methoxycoumarin (BrMMC) with fluorescence detection, have been developed.[11-14]

HPLC coupled with electrochemical detection (ECD) represents a very sensitive technique for the determination of many important substances. Moreover, enhanced selectivity is obtained by HPLC-ECD because of the limited number of substances that undergo redox reactions under certain conditions.[15] In a previous paper we synthesized 1-(2,5-dihydroxyphenyl)-2-bromoethanone (2,5-DBE), a new electro-chemical probe, useful for the electrogenic labeling of a carboxylic acid in HPLC analysis with electrochemical detection.[16]

In the present paper we describe a highly sensitive and selective HPLC-ECD method with isocratic elution for the determination of VA after pre-column derivatization with 2,5-DBE. For this purpose we established and optimized a novel reaction of derivatization of VA and n-hexanoic acid (HA) (used as internal standard), to form electroactive esters estimable by HPLC coupled with ECD. Moreover, the applicability of this procedure to the assay of VA in different pharmaceutical formulations was investigated and so were several factors that might influence the final results of the method.

EXPERIMENTAL

Apparatus

The HPLC apparatus consisted of 2 Model 510 pumps, a Model 712 WISP auto-injector, and an electrochemical detector (Model 5100A Coulchem; ESA, Bedford, MA, USA) which consisted of a control module and an analytical cell (Model 5010) containing 2 on-line porous graphite coulometric electrodes. The analysis was performed in the oxidative mode. The ED sensitivity range and response time were set at 100 nA and 10 s respectively. Data from the detector were collected and elaborated by a computer using Maxima 820 software (Waters Assoc., Milford, MA, USA). The mobile phase was filtered through a GS-type filter (0.22 μ, Millipore, Bedford, MA, USA) and degassed on-line with a model ERC-331 1 solvent degasser (Erma, Tokyo, Japan).

Mass Spectra were recorded on a VG7070 spectrometer at 70 ev. IR spectra were obtained on a Perkin Elmer 1600 Fourier Transformed spectrometer as KBr disks. The ^{1}H-NMR spectra were recorded at 300 MHz on a Varian Gemini spectrometer in $CDCl_3$ with tetramethylsilane as internal standard. Elemental analysis for C, H, N was obtained on a Carlo Erba analyzer (Milano, Italy) and agreed with theoretical values within ± 0.4%. UV absorption spectra were recorded on a Uvikon 860 spectrometer (Kontron, Zurich, Switzerland) in a MeOH solution. Analytical thin layer chromatography (TLC) was performed on Merck 60 F_{254} silica gel plates.

Chemicals

2,5-dihydroxyacetophenone, and phenyltrimethylammonium bromide tribromide were obtained from Fluka (Buch, Switzerland). VA and HA were purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol, acetonitrile and water were obtained from Carlo Erba (Milano, Italy), other chemicals used were of reagent grade or better.

Drugs

Commercial tablet formulations containing sodium valproate (Depakin® 200 and 500 mg tablets by Laboratories Sanofi Pharma, France) and magnesium valproate (Depamag® 200 and 500 mg tablets by Sigma Tau, Italy) were purchased at a local pharmacy.

Synthesis of Electroactive Derivatization Reagent and Standards

1-(2,5-dihyroxyphenyl)-2-bromoethanone (2,5-DBE)

The derivatization reagent 2,5-DBE was synthesized as previously reported.[16] Briefly, phenyltrimethylammonium bromide tribromide (PTMABr$_3$, (6.6 mmol) was slowly added to a solution of 2,5-dihydroxyacetophenone (2,5-DAP), (6.6 mmol) in 20 mL of dry THF. The mixture was stirred overnight at room temperature (21°C) and checked by TLC with eluent cyclohexane:ethylacetate (7:3, v/v). The precipitate that formed was removed by vacuum filtration and purified by flash chromatography (cyclohexane:ethylacetate 7:3 v/v) giving 960 mg of 2,5-DBE (yield 63%). UV: 255nm, ε (M^{-1} cm^{-1}) 10356. IR (KBr) cm^{-1} 3335 (OH), 1620 (C=O). ^{1}H-NMR ($CDCl_3$) δ11.4 (s, 1H OH); 11.1 (1H, OH); 7.3-6.7 (m, 3H, ArH); 4.4 (s, 2H CH_2). MS (m/z): 232 (M^+ + 2), 230, 150, 136, 108.

1-(2 ,5-dihyroxyphenyl)-2-ethanone-2-valproate (2 ,5-DE-VE)

2,5-DBE (0.347 mmol) was added to a solution of VA (1.04 mmol) in 8 mL of dry CHCN containing 110 μl of triethylamine (1.08 mmol), and heated at 70° C for 4 hours. The mixture was diluted with 20 mL of H_2O and extracted three times with diethyl ether. The organic layer was washed with saturated $NaHCO_3$, then with H_2O, dried (Na_2SO_4), evaporated, and purified by flash chromatography (hexane:ethyl acetate, 1:1, v/v). IR (KBr, cm^{-1}) 3335 (OH), 2930 (CH_2), 1719 (CO), 1660 (Ph-CO); MS (m/z): 294(M^+), 168, 158, 137,127,99. ^{1}H-NMR ($CDCl_3$, δ) 7.09 (m, 5H; ArH and OH), 5.28 (s, 2H, CO-CH_2-COO), 2.31(m, 1H, CH), 1.81-1.38 (m, 41-1, CH_2), 0.94 (m, 6H, CH_3).

1-(2,5-dihydroxyphenyl)-2-ethanone-2-hexanoate (2,5-DE-HE)

2,5-DBE (0.340 mmol) was added to a solution of HA (1.02 mmol) in 8 mL of dry CHCN containing 110 μl of triethylamine (1.06 mmol), and heated at 70°C for 4 hours. The mixture was diluted with 20 mL of H_2O and was extracted three times with diethyl ether. The organic layer was washed with saturated $NaHCO_3$, then with H_2O, dried (Na_2SO_4), evaporated and purified by flash chromatography (hexane:ethyl acetate, 1:1, v/v). IR (KBr, cm^{-1}) 3445 (OH) 1722 (CO), 1657 (Ph-CO); MS (m/z) 266 (M$^+$), 235 168, 150,137,109,99. ^{1}H-NMR ($CDCl_3$, δ) 7.04 (m, 5H, ArH e OH), 5.32 (s, 2H, CO-CH_2-COO), 2.51 (m, 2H, COO-CH_2), 1.85-1.28 (m, 6H, CH_2), 0.96 (m 3H, CH_3).

Standard Solutions

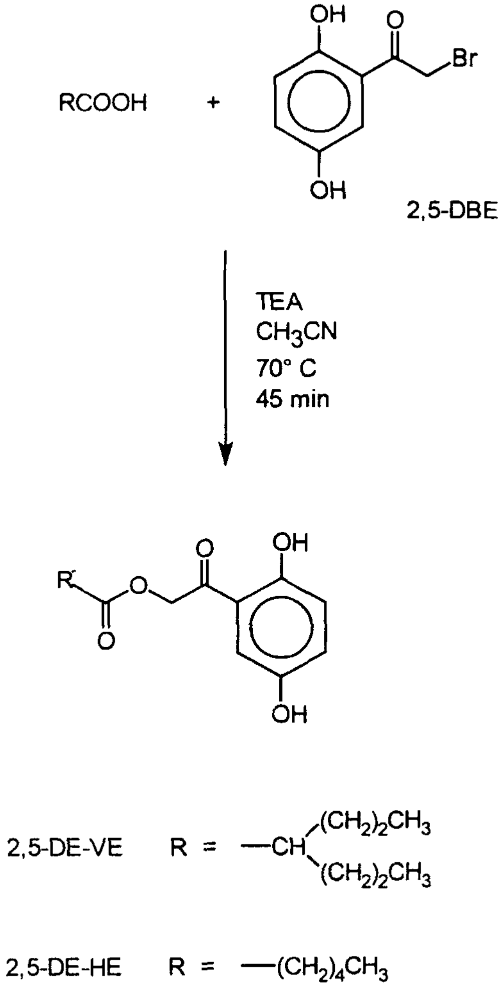
Standard solutions in the concentration range 50-200 ng/mL were prepared by accurately diluting, with acetonitrile, known amounts of a stock solution containing 200 μg/mL of VA and an appropriate amount of internal standard.

Analysis of Pharmaceutical Formulations

Individual commercial tablets were powdered and a known amount was transferred to a 50 mL volumetric flask along with 25 mL of acetonitrile. The tablet powder was dispersed by mixing in an ultrasonic bath for 10 min and a suitable amount of internal standard (HA) was added, finally the mixture was diluted to volume with acetonitrile. An appropriate amount of the resulting suspension was filtered through a 0.45 μm membrane filter (Millipore) and 1 mL was utilized for the derivatization procedure. In order to check that no interfering peaks were present in the pharmaceutical tablets matrix, a blank mixture in acetonitrile containing the tablets excipients was prepared and subjected to the derivatization procedure.

Derivatization Procedure

The derivatization was achieved by adding an appropriate amount of a stock solution of 2,5-DBE (600 μg/mL) and triethylamine (390 μg/mL in CH_3CN anhydrous to 1 mL of the solutions containing VA (tablet samples or standards or blank). The molar ratio between 2,5-DBE and VA was fixed at 10:1. The reaction mixture was heated at 70°C for 45 min. After cooling 50 μL of the reaction mixture was withdrawn, diluted to the 50 mL with the eluent and analyzed by HPLC.



Scheme 1. Derivatization reaction of VA and HA with 2,5-DBE to give the electroactive esters 2,5-DE-VE and 2,5-DE-HE.

Optimization of the Derivatization Reaction

To investigate optimum conditions for derivatization, solutions containing both VA (6.0 µg/mL) and HA (5.50 µg/mL) were incubated at 50, 70 and 90°C and at appropriate time intervals samples were taken and analyzed immediately by HPLC.

Chromatographic Conditions

Derivatized samples of 5 µL were injected into a 3 µm Adsorbosphere column (100x4.6 mm; Alltech, Deerfield, IL, USA) fitted with a guard column (Hypersil ODS RP C18, 5 µm, 4x4 mm; Policonsult, Rome, Italy). Separations were performed with a mobile phase of methanol:acetonitrile:sodium acetate buffer 0.1 M pH 6.5 (55:20:25 v/v/v) at room temperature (21°C) with a flow rate of 1.0 mL/min and internal standardization was used.

Optimization of Electrochemical Detection

In order to optimize the detection of the electroactive esters several parameters were examined such as oxidation potential, hydrodynamic voltammograms, pH, and the ionic strength of the eluent.

RESULTS AND DISCUSSION

Optimization of Derivatization Procedure

Scheme 1 represents the VA and HA reaction of esterification with 2,5-DBE to give the electroactive esters 2,5-DE-VE and 2,5-DE-HE by nucleophilic substitution. The reaction conditions were optimized with respect to high electroactive derivative yield, short reaction time, and clean chromatograms.

At 50°C the reaction was incomplete after 100 min whereas at 90°C additional HPLC peaks were observed suggesting the reagent or the ester derivatives were undergoing some decomposition. The time course of the derivatization for both VA and HA at 70°C is shown in Figure 1. For sensitivity analysis at low concentrations of VA and HA the optimal heating time for the derivatization reaction was concluded to be 45 min for both acids. The electroactive esters remain stable in the reaction mixture up to 72 hours after the optimum.

Chromatography

Chromatographic separations were carried out under reverse phase conditions on a 3 µm Adsorbosphere column using a ternary eluent, methanol:acetonitrile:sodium acetate 0.1 M (55:20:25 v/v/v), at a flow rate of 1 mL/min with an injected volume of 5 µL.

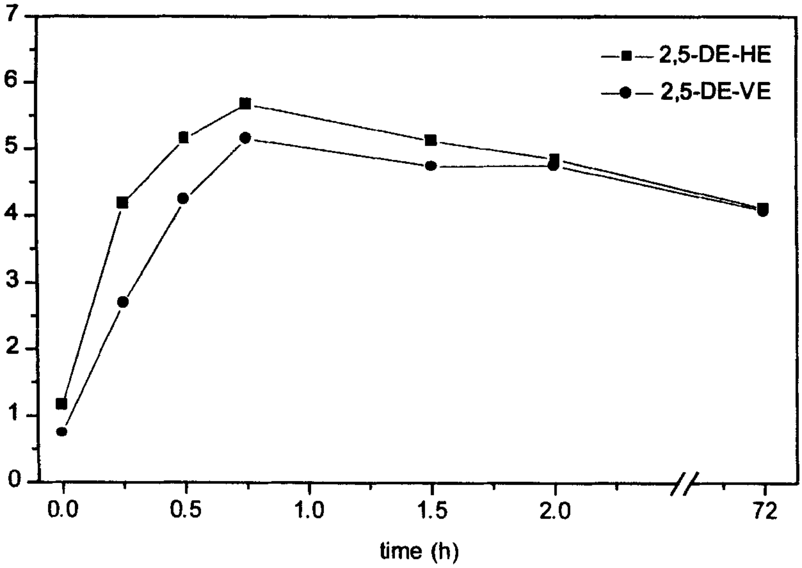


Figure 1. Time course of the derivatized esters 2,5-DE-VE and 2,5-DE-HE in the optimization of derivatization study.

A typical chromatogram with electrochemical detection for the standards is shown in Figure 2. The analysis was complete within 6 min and the retention times were 2.20 min for 2,5-DBE, 3.11 min for 2,5-DE-HE (internal standard), and 4.20 min for 2,5-DE-VE, at concentrations of 0.81, 1.6, and 2.2 nmol/mL respectively. This chromatographic system gave a complete and rapid baseline resolution of 2,5-DE-VE, 2,5-DE-HE, and the derivatization reagent 2,5-DBE and, therefore, was adopted in the following pharmaceutical formulation analysis studies.

Linearity and Detection Limit

The linearity of response was examined for both the electroactive esters 2,5-DE-VE and 2,5-DE-HE in the range 50-200 ng/mL. The correlation coefficients of the linear regression of the standard curves were consistently greater than 0.99. The detection limit was determined by analyzing progressively lower concentrations of the electroactive esters and were found to be 0.82 and 0.74 pmol/mL for 2,5-DE-VE and 2,5-DE-HE, respectively, for a signal/noise ratio of 5:1 (n = 5) with an injected volume of 5 μL.

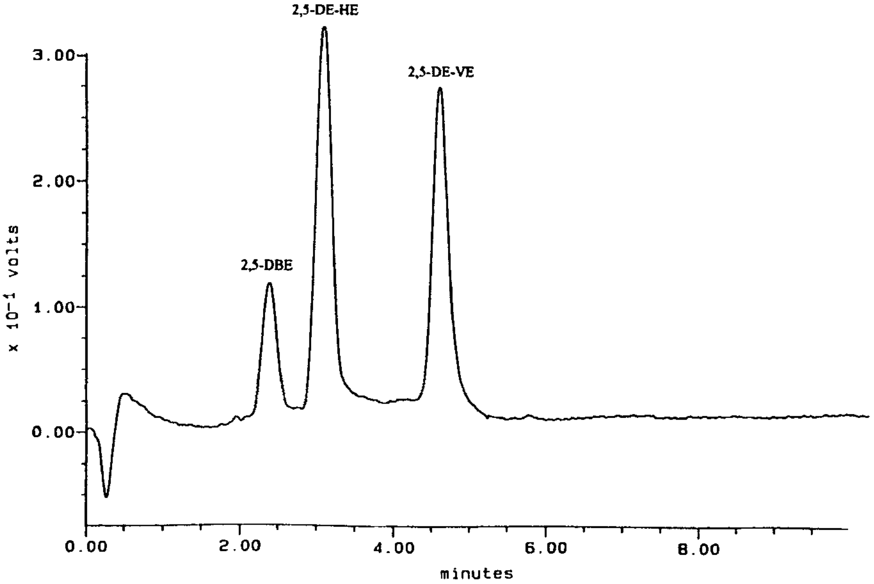


Figure 2. Typical HPLC chromatogram of a standard mixture of derivatization reagent, 2,5-DBE (2.20 min), and the electroactive esters 2,5-DE-VE (3.11 min) and 2,5-DE-HE (4.20 min).

The sensitivity found for electroactive ester of VA with ECD was higher than the UV direct method and the procedure based on pre-column derivatization with BrMMC, that ranged in micromolar and nanomolar intervals, respectively.[5,17]

Accuracy and Precision

The accuracy of the assay was determined by repetitive analysis of blank excipients mixture spiked with 50, 100 and 200 ng/mL of VA standard. The accuracy of the assay was determined by comparing the measured concentration to its true value.

The reproducibility of the method was evaluated by replicate analysis of the above mentioned blank formulation spiked with a known amount of VA standard and was expressed as RSD. The obtained results are reported in Table 1.

Table 1

Accuracy and Precision in the Analysis of VA

True Conc. (ng/mL)	Accuracy[a] Mean ± SD	RSD[a] (%)
50	98.5 ± 3.25	3.12
100	99.2 ± 2.93	2.62
200	101.2 ± 1.58	2.25

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[a] n = 6.

Optimization of Detection

Several parameters were examined in order to optimize the electrochemical detection of the electroactive compound synthesized in this study. Under the chromatographic conditions mentioned above the electroactive ester derivatives responded to the ED oxidation potential higher than +0.2 V. Enhanced responses for all electroactive compounds were obtained as the working electrode potential was increased from +0.2 to +0.6 V. With additional applied potential no further increase in the peak height occurred and a rise in the background current was observed.

Electroactive properties of the compounds 2,5-DBE, 2-5-DE-VE, and 2,5-DE-HE (internal standard) were also examined by their hydrodynamic voltammograms (Figure 3). Inspection of data reported in Figure 3 shows that increasing the applied potential from +0.2 V to +0.6 V the detector response was enhanced for all the electroactive compounds. For potentials higher than +0.45 the detector responses for the internal standard 2,5-DE-HE was progressively lower from 4.5 nA at +0.45 V to 4.0 nA at +0.6 V, with an 11.10 % decrease. Whereas for the derivatization reagent and for the 2,5-DBE-VE the detector response was amplified for both compounds but was almost three fold higher for the electroactive reagent with respect to the electroactive esters. In fact, we recorded a 4.17 % (from 2.4 to 2.5 nA) and 12.12 % (from 3.3 to 3.7 nA) increase in the detector response for 2,5-DE-VE and 2,5-DBE, respectively.

For the analysis of the marketed pharmaceutical formulation we used a molar excess of derivatization reagent in order to obtain the maximum yield in electroactive esters and therefore the highest sensitivity. Consequently in the chromatograms of pharmaceutical formulations we found a strong peak corresponding to the derivatization reagent 2,5-DBE.

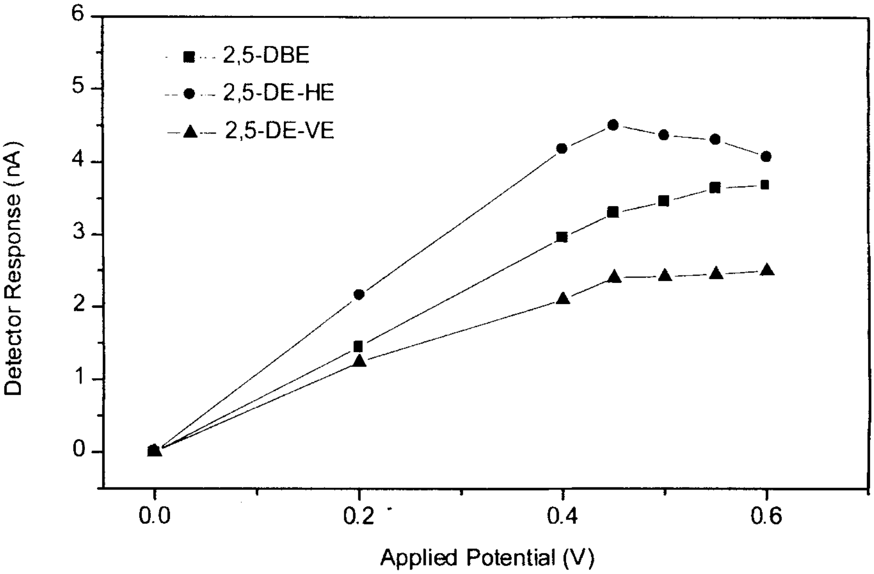

Figure 3. Hydrodynamic voltammograms of the electroactive compounds 2,5-DBE, 2,5-DE-VE, and 2,5-DE-HE.

Taken together these results suggest that the best potential was +0.45; because in this way we obtained a sensitive analysis of the electroactive esters of VA and HA and, at the same time, the 2,5-DBE peak area was reduced, with respect to +0.60 V, and did not interfere with the elution of the electroactive ester peaks.

The ECD performance was markedly influenced by the ionic strength but not by the pH of the mobile phase. With increasing concentrations of the sodium acetate buffer from 0.05 to 0.10 M an increase in the electrochemical detector response of 3 % was observed. No significant improvement in the detector response was achieved by further increasing the buffer molarity that was consequently fixed at 0.1 M and the pH at 6.50.

Analysis of Pharmaceutical Formulations

VA has a poor detectability in the ultraviolet range and therefore the HPLC analysis with UV detection is difficult. In general, when compounds have a very low ultraviolet absorbance, one would attempt to derivatize them for detection enhancement and/or to evaporate the extract for their enrichment.

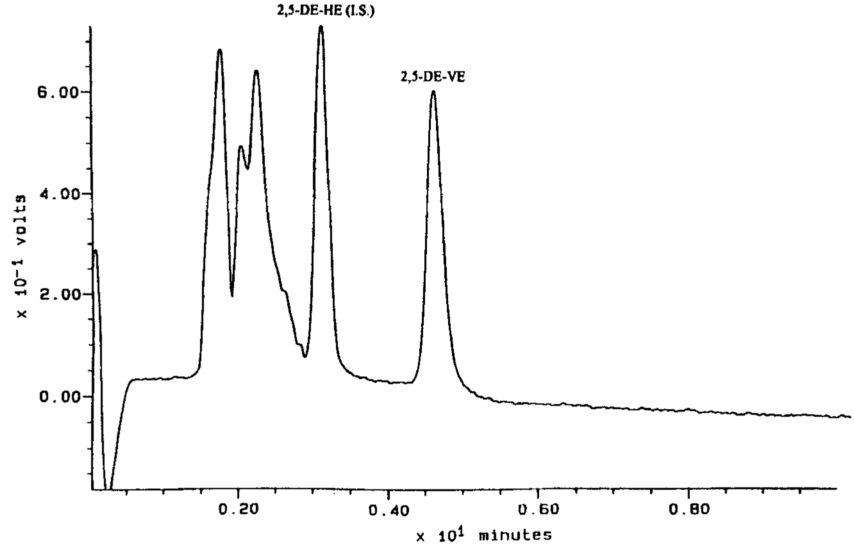

Figure 4. Representative chromatogram of a tablet formulation.

Table 2

HPLC-ECD Assay Results for VA in Pharmaceutical Marketed Tablet Formulations

Commerical Preparation	Label Claim (mg)	% Found + RSD (n = 6)
Depakin Tablet	200[a]	99.7 ± 0.3
Depakin Tablet	500[a]	101.0 + 0.8
Depamag Tablet	200[b]	101.3 ± 1.4
Depamag Tablet	500[b]	101.2 ± 1.1

[a] sodium valproate
[b] magnesium valproate

Since VA is a volatile compound,[17-18] extreme care must be taken to avoid losses due to volatilization when concentrating the extract. Obviously the volatility of VA precludes any evaporation procedure. HPLC in conjunction with a pre-column chemical derivatization, using the electroactive-labeling reagent 2,5-DBE, constitutes an effective approach to overcome the problem.

The HPLC-ECD method developed in this study was applied to the assay of VA in pharmaceutical dosage forms. Four different commercially available VA tablet formulations were subjected to the derivatization and HPLC analysis. The high sensitivity achieved by ECD permitted an accurate quantification of the drug present in the pharmaceutical preparations. Figure 4 shows a representative chromatogram of a tablet formulation. The results are presented in Table 2 and were found to be in good agreement with the label claim and demonstrated the precision of the method. No interfering peaks were observed in the blank excipients mixture subjected to derivatization and HPLC analysis.

CONCLUSION

Although HPLC is a versatile technique for the analysis of drug in complex matrices, such as biological or pharmaceutical, the poor ultraviolet absorbency of VA makes the direct HPLC separation and quantification difficult due to the interfering substances. The preferred method for a HPLC analysis of VA in complex matrices is the direct conversion into a detectable compound without complicated steps to isolate it.

The derivatization of VA and HA acid with the electroactive labeling reagent 2,5-DBE yields stable and highly sensitive electroactive esters which are easily quantifiable by the HPLC-ECD technique. The applied potential of +0.45 V permits the selective oxidation of the electroactive esters without interference from the excipients present in the pharmaceutical matrix because of the limited number of substances that can undergo redox reactions under these conditions.

This HPLC-ECD method offers a means of enhancing the selectivity and sensitivity of conventional HPLC-UV analysis of the poorly absorbing valproic acid. In conclusion, the high selectively, good accuracy, reproducibility, and sensitivity of the HPLC-ECD technique developed in this study makes it suitable for quality control assays of complex pharmaceutical formulations containing VA and its salt. In addition the methods proposed may be suitable with some modification to in vivo quantification of VA.

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