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Short communication

Liquid chromatographic assay in plasma of one of the members of a new series of anticonvulsants: D,L-3-hydroxy-3-ethyl-3-phenylpropionamide

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Abstract

A method for the simultaneous determination of HEPP (D,L-3-hydroxy-3-ethyl-3-phenylpropionamide), a member of a new homologous series of phenylamide-derivative anticonvulsants, with six other antiepileptic drugs (ethosuximide, primidone, phenobarbital, phenytoin, carbamazepine and clonazepam) in plasma by high-performance liquid chromatography is described. These drugs are extracted from plasma by adding an equal volume of acetonitrile. An aliquot of the extract is then injected on a reversed-phase column with a acetonitrile–methanol–phosphate buffer mobile phase. The total time required for the whole analytical process, including the plasma pretreatment and chromatography, is approximately 30 min. The assay method is simple, rapid and reproducible, and therefore considered suitable for routine use in clinical investigations monitoring HEPP simultaneously with common antiepileptic drugs.

Keywords: D,L-3-Hydroxy-3-ethyl-3-phenylpropionamide; Ethosuximide; Primidone; Phenobarbital; Phenytoin; Carbamazepine; Clonazepam

1. Introduction

Previous studies have demonstrated that the phenylamide derivatives D,L-4-hydroxy-4-ethyl-4-phenylbutyramide (HEPB) and its analogues D,L-3-hydroxy-3-ethyl-3-phenylpropionamide (HEPP) and D,L-2-hydroxy-2-ethyl-2-phenylacetamide (HEPA) cover a wide spectrum of antiepileptic action [1]. The chemical structure of the three anticonvulsants is shown in Fig. 1. Since this is a homologous series of anticonvulsants, the only difference lies in the length of the carbon chain. The experimental anticonvulsants have shown a very low incidence of toxic effect in mice, both in the acute and subchronic studies [2]. Of the experimental anticonvulsants, HEPP is the least neurotoxic and, therefore, it has been selected for the necessary safety and effectiveness studies. Furthermore, HEPP is a powerful anticonvulsant in amygdaline kindling [3], in the GABA-abstinence syndrome and in an animal model for generalized absence seizures [4]. The GABA-withdrawal syndrome is considered a model for

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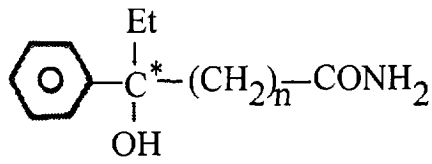

n	Compound
0	HEPA
1	HEPP
2	HEPB

Fig. 1. Structures of the phenylamine-derivative anticonvulsants.

intractable epilepsy because it does not respond to any known antiepileptic drug. However, HEPP is effective against this type of seizures. Other reports have shown HEPP to be a promising anticonvulsant for clinical experimentation [2,4].

In order to perform metabolic, pharmacokinetic and bioavailability studies, the physicochemical characteristics of the experimental compounds have to be known. Also, a sensitive analytical method is required for their detection, so that it can ultimately also be used to determine the serum levels of the drug in animals as well as in humans.

This paper describes a high-performance liquid chromatography method for the determination of HEPP in plasma. The method is simple, allows for several daily determinations and detects the experimental drug, even in the presence of the six most frequently used antiepileptic drugs (AEDs).

2. Experimental

2.1. Reagents, standards and experimental compound

Acetonitrile and methanol (HPLC grade) and phosphate salt (reagent grade) were purchased from Merck (Darmstadt, Germany). Ethosuximide, primidone, phenobarbital, phenytoin, carbamazepine and clonazepam were purchased from Sigma (St. Louis, MO, USA). HEPA (used as internal standard) and HEPP were synthesized by Dr. G. Carvajal et al. [1]. A stock solution containing HEPP and each of all standard AEDs was prepared in methanol, this solution was further diluted with the mobile phase to the required concentration of the drug. Another stock solution of HEPA (5 μg/ml) was dissolved in acetonitrile and both were stored at 4°C.

2.2. Apparatus

HPLC was carried out on a Varian 5000 chromatograph (Varian Instruments, Palo Alto, CA, USA) with a Rheodyne Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, USA) and a UV-50 variable-wavelength ultraviolet detector (Varian Instruments).

Analysis were performed on a Ultrasphere C_{18} (150 × 4.6 mm I.D., 5 μm particle size). Detector output was recorded at 10 mU with a Varian Model 9176 recorder (Varian Instruments). Data were processed using a Spectra Physics Model SP 4270 integrator (Spectra Physics, San Jose, CA, USA). Other equipment included 1.5-ml stopped conical polypropylene test tubes (Sarstedt) a bench-top vortex-type mixer and a centrifuge (Model TJ-6, Beckman Instruments, Palo Alto, CA, USA).

2.3. Assay procedure

Each plasma standard (500 μl) was added to a 1.5-ml Sarstedt centrifuge tube containing an equal volume of acetonitrile and 2 μg of the internal standard, mixed for 30 s, and then centrifuged at 2700 *g* for 5 min in order to separate the acetonitrile phase from the bottom layer.

The chromatographic conditions were set as follows: column temperature, 25°C; mobile phase, methanol–acetonitrile–phosphate buffer 10 m*M*, pH 7.4 (35:15:50, v/v); flow-rate, 1.0 ml/min; wavelength, 219 nm.

2.4. Quantitation

Plasma standard was prepared by adding known amounts of AEDs to pooled/drug-free plasma at the

required concentration for each of the calibration curves. A calibration curve for each drug was obtained by calculating the peak-area ratio of analyte/internal standard.

Solutions of all seven compounds in drug-free plasma were assayed in triplicate. The concentration ranges employed were 0.1–50 μg/ml for ethosuximide, primidone, phenobarbital, phenytoin and carbamazepine, and 0.05–50 μg/ml for clonazepam. In all cases, separate experiments showed that the calibration curves were linear in at least the clinical concentration ranges. Linear regression analyses were performed for each drug relative to the internal standard.

Following the optimization of the chromatographic conditions and validation of the assay, it was used for preliminary detection in rat plasma. Representative chromatograms obtained at 15 min after the intraperitoneal (i.p.) administration of 50 mg/kg HEPP are shown in Fig. 2D.

3. Results

3.1. Chromatography

Under the chromatographic conditions used, ethosuximide, primidone, phenobarbital, phenytoin, carbamazepine, clonazepam, HEPA and HEPP all showed symmetrical peaks. Fig. 2A shows a typical chromatogram for the standard for each drug. No interfering peak was observed when the blank plasma extract was analyzed (Fig. 2B). Fig. 2C is a chromatogram from a standard plasma sample containing known quantities of six AEDs (ethosuximide (20 μg/ml), primidone (5 μg/ml) and HEPA (20 μg/ml), HEPP (20 μg/ml), phenobarbital (15 μg/ml), phenytoin (10 μg/ml), carbamazepine (4 μg/ml) and clonazepam (4 μg/ml)).

Whilst the purpose of this report is not to study the pharmacokinetics in animals, and since our results in rats coincide with those previously reported [11], we show a representative determination of HEPP in samples of rat plasma, 15 min after the administration of AED (50 mg/kg body weight). This time lapse was chosen, since at this time the maximal concentration of HEPP is reached.

3.2. Analytical variables

3.2.1. Precision

Repeated analyses of plasma specimens, each containing known concentrations of the drugs under study were performed. Table 1 shows that the coefficient of variation (C.V.) values for the within-day precision ranged from 1.03 to 3.43 ($n=5$), and those of the between-day precision from 1.34 to 7.75 ($n=10$).

3.2.2. Recovery

The absolute analytical recovery from plasma of the seven drugs was obtained as follows: the AEDs were added to drug-free plasma samples to achieve the midpoint concentration as given in Table 1 and then they were analyzed by HPLC. Exact aliquots of the acetonitrile extract were then injected into the column and peak areas measured. Absolute recovery was calculated by comparing these peak areas with the peak areas obtained by the direct injection of pure drug standards. Absolute recoveries of AEDs were 101.42, 99.2, 97.6, 95.6, 93.6, 96.8 and 99.6% for ethosuximide, primidone, carbamazepine, phenytoin, clonazepam, phenobarbital and HEPP, respectively.

3.2.3. Linearity and sensitivity

Plasma standards were prepared containing various known amounts of each drug. A constant amount of the internal standard was added to each sample. Concentration and peak-height ratios correlated linearly with each other for all AEDs examined.

In addition, all the calibration curves passed through the origin (Fig. 3). The sensitivity was such that the compounds can be detected at plasma concentrations as low as 0.1 μg/ml for HEPP and at least as low as 0.05 μg/ml for the other six AEDs.

3.2.4. Stability

In order to determine the stability of HEPP in buffered solution and human plasma, 5.0 μg/ml of this drug was added to the buffered solution or serum, in several tubes, vigorously shaken and stored at room temperature or at 4°C for one to eight days. At the allotted times the internal standard was added to each tube, the HEPP was extracted and immediately thereafter the chromatographic analysis was

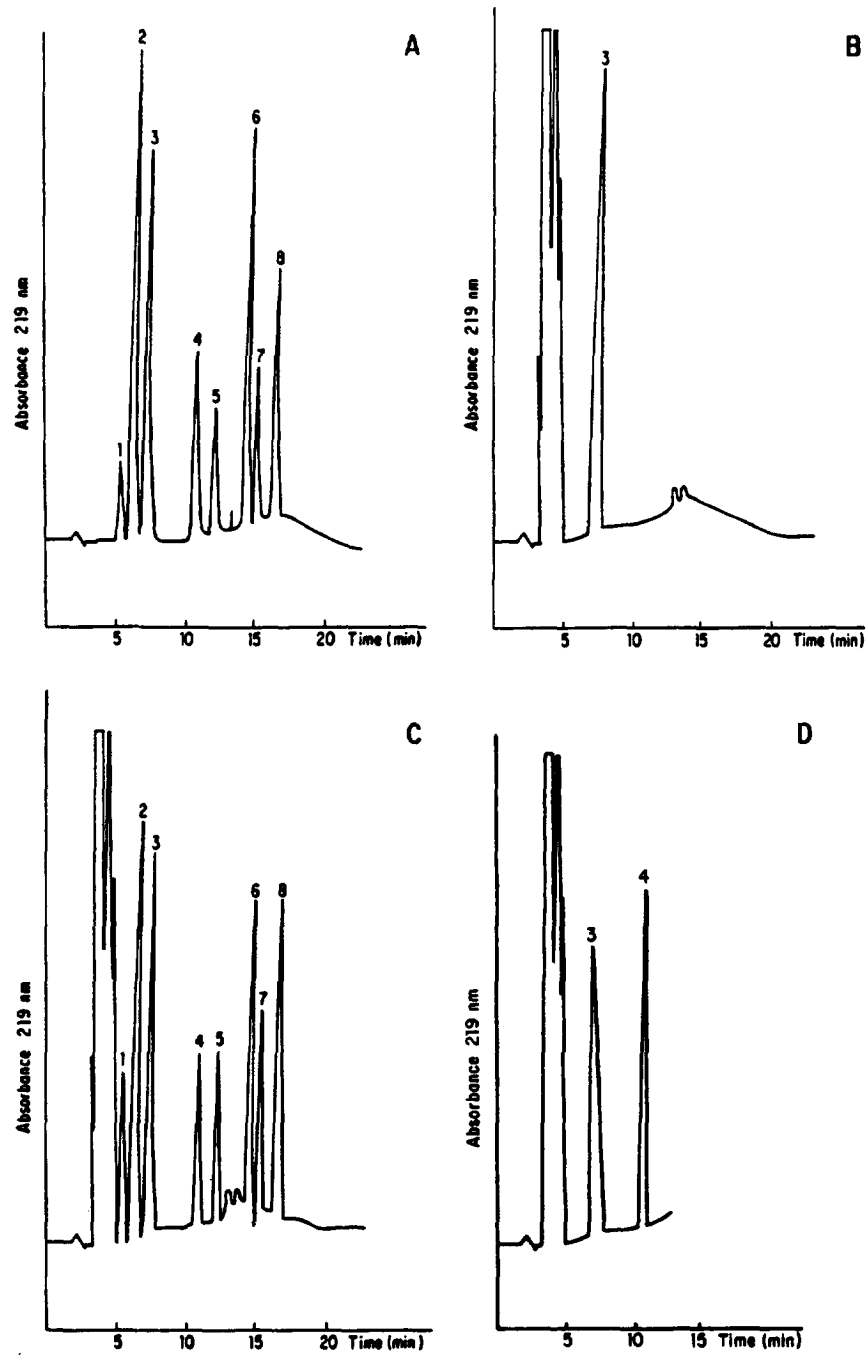

Fig. 2. Chromatograms obtained from: (A) a standard mixture of HEPP (0.2 μg), ethosuximide (0.2 μg), primidone (0.05 μg), phenobarbital (0.15 μg), phenytoin (0.1 μg), carbamazepine (0.05 μg), clonazepam (0.05 μg) and internal standard (0.2 μg) without extraction; (B) blank plasma; (C) 500 μl of plasma spiked with HEPP and AEDs as in (A), (D) from rat plasma 15 min after a single i.p. administration of 50 mg/kg HEPP, with extraction according to the procedure.

Table 1
Precision of the antiepileptic drug assay in serum

Concentration (μg/ml)	μg/ml±S.D.[a]	Between-day C.V. (%)[b] (n=10)	Within-day C.V. (%) (n=5)
Ethosuximide			
10	10.21±0.21	2.1	1.84
25	25.61±0.52	2.06	1.29
50	50.29±1.11	2.221	1.66
100	101.14±2.03	2.01	1.95
150	151.29±2.78	1.84	1.73
Primidone			
2.5	2.61±0.13	5.01	2.21
5.0	5.76±0.28	4.83	2.43
10	10.95±0.43	3.95	3.17
25	26.13±0.80	3.06	1.26
50	54.12±1.35	2.51	1.43
HEPP			
2.5	2.41±0.12	4.93	3.11
10	10.36±0.32	3.02	2.77
25	25.61±0.76	2.97	2.42
50	52.14±1.36	2.61	1.58
100	106.18±2.87	2.71	1.99
Phenobarbital			
5	5.34±0.24	4.57	3.43
10	10.17±0.41	4.15	3.17
25	25.61±0.40	1.69	2.69
50	51.19±0.69	1.34	1.52
100	100.4±3.62	3.61	1.05
Carbamazepine			
2.5	2.71±0.18	6.73	2.96
5	5.61±0.28	5.17	3.13
10	11.06±0.35	3.24	1.59
25	24.98±1.15	4.68	1.07
50	56.13±2.80	5.05	2.61
Phenytoin			
2.5	2.33±0.13	5.50	2.12
5	5.84±0.25	4.29	2.16
10	10.96±0.36	3.26	1.75
25	24.62±2.03	8.23	1.88
50	54.34±3.30	6.02	1.94
Clonazepam			
2.5	2.61±0.10	3.86	2.69
5	5.43±0.22	4.02	2.70
10	11.06±0.32	2.86	1.35
25	26.14±0.75	2.83	1.03
50	54.32±4.23	7.75	2.68

[a] Standard deviation.
[b] Coefficient of variation. HEPA was used as internal standard.

Table 2
Stability of HEPP in serum

Time (days)	Detection (mean±S.D.) (μg/ml)	Degradation (%)
Room temperature		
0	5.02±0.217	0
2	4.99±0.219	0.4
4	4.98±0.321	0.6
6	5.03±0.204	0.6
8	5.02±0.316	0.8
4°C		
0	5.00±0.210	0
2	5.01±0.235	0
4	4.98±0.196	0.2
6	4.98±0.301	0.2
8	5.01±0.209	0.4

Analyzed concentration 5.0 μg/ml, $n=3$ each day.

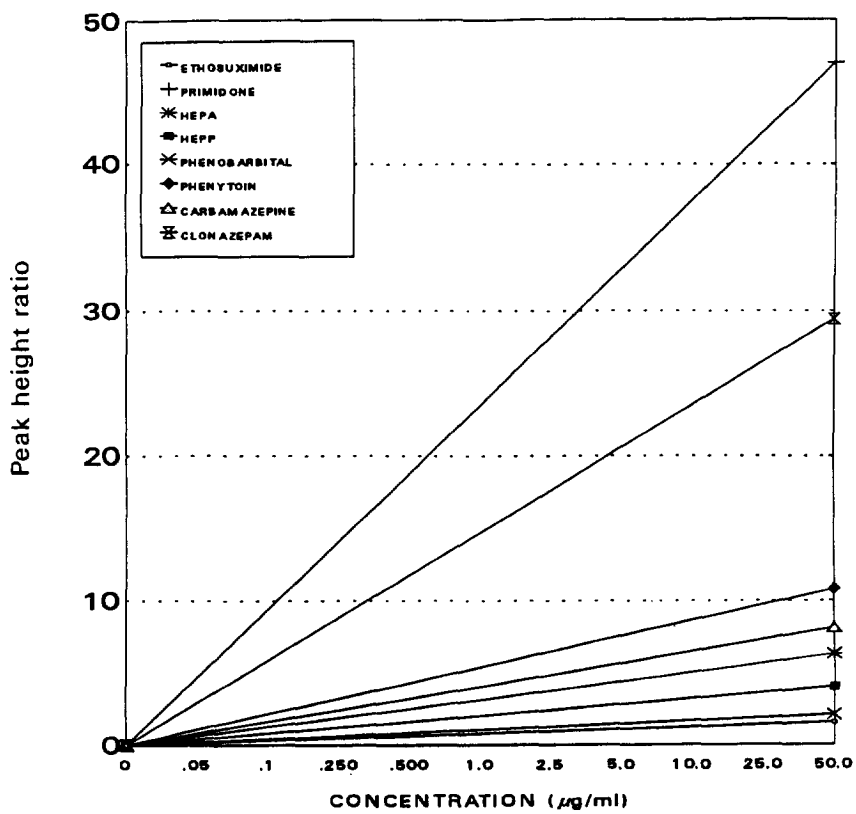

Fig. 3. Calibration curves for HEPP, ethosuximide, primidone, phenobarbital, phenytoin, carbamazepine and clonazepam in plasma. A three-point standard curve was prepared by plotting on the ordinate the ratio of each compound's peak height to that of the internal standard for each concentration. Linear regression analysis of calibration curve data indicated no significant deviation from linearity ($r=0.9966$–0.9995). In addition, intercept values did not significantly differ from zero.

carried out (Table 2). At the end of storage period HEPP remained chemically stable in both solutions and at both temperatures, and in addition, no degradation products were detected.

4. Conclusions

Various HPLC techniques for simultaneously determining the plasma concentrations of AEDs have been reported [5–10]. The extraction technique described here for assays in plasma is simple, rapid and reproducible enough for application of therapeutic monitoring of HEPP. The time required for the pretreatment procedure is 10 min and that for the chromatographic run is 20 min, resulting in a total of 30 min. The C.V. values for both within-day and between-day assays were within the performance limit required for medical management (Table 1) [12]. The absolute recovery of all the compounds from plasma was >93.6%. Linear calibration curves (r=0.9966–0.9995) were obtained for each compound. A simple, reproducible and adequate chromatographic method for HEPP determinations, even in the presence of the six most used AEDs, has been described.

At present, pharmacokinetic studies are being carried out on healthy volunteers (clinical Phase 1) at the National Neurological Institute of Mexico and will be the subject of a future publication.

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