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SIMULTANEOUS DETERMINATION OF ELEVEN ANTIEPILEPTIC COMPOUNDS IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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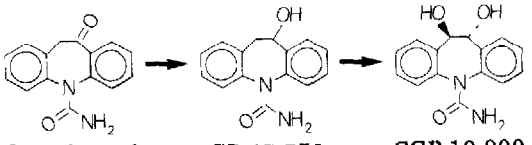
SUMMARY

A high-performance liquid chromatographic method for the simultaneous determination of carbamazepine, CGP 10 000 (a common metabolite of carbamazepine and oxcarbazepine), desmethylmephenytoin, 10,11-epoxycarbamazepine, ethosuximide, GP 47 779 (the active metabolite from oxcarbazepine, a new drug made by Ciba-Geigy), mephenytoin, phenylethylmalonamide, primidone, pheneturide, phenobarbital and phenytoin is described. The serum is extracted with ethyl acetate at pH 3.9 and the dried extract is dissolved in 70% ethanol in water and an aliquot is injected into a Hewlett-Packard 1084 B liquid chromatograph. A reversed-phase (RP-8) column is used with acetonitrile and water as the mobile phase. The eluted drugs are detected at 207 nm. The recovery of the compounds varies from 76% to 95% with the day-to-day precision (C.V.) between 3.8% and 9.8%, the within-day precision between 1.8% and 5.8% and run-to-run precision between 1.0% and 2.6%.

INTRODUCTION

Several high-performance liquid chromatographic (HPLC) methods for the determination of antiepileptic drugs have been published. None includes in a simultaneous program all the drugs routinely analysed in our laboratory. Riedmann et al. [1] described in 1981 the use of HPLC for the measurement of eight antiepileptic drugs and metabolites. Not included were GP 47 779 (Fig. 1), mephenytoin, desmethylmephenytoin and pheneturide. The peak they



Oxcarbazepine GP 47 779 CGP 10 000

Fig. 1. Biotransformation of oxcarbazepine

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named "carbamazepine metabolite" must probably correspond to CGP 10 000 (Fig. 1). Their chromatographic separation time is about 25 min. During the preparation of this paper, Kabra et al. [2] published a fast liquid chromatographic method for five antiepileptic drugs. The chromatography is completed in less than 2.5 min. But in this short time it is not possible to detect simultaneously phenylethylmalonamide, mephenytoin and desmethylmephenytoin. The retention time of 10,11-epoxycarbamazepine is very close to that of the internal standard. GP 47 779, CGP 10 000 and pheneturide are not mentioned. Although CGP 10 000 appears in serum in concentrations between 19% and 220% (mean 52%, n = 19) of the carbamazepine concentration, and therefore could cause serious interference, it is not mentioned in recently published simultaneous methods including carbamazepine [2, 3].

The present paper describes our HPLC procedure which has been used routinely in our laboratory for two and a half years. The chromatography of the eleven antiepileptic substances and CGP 10 000 is completed in about 15 min.

MATERIALS AND METHODS

Apparatus

The high-performance liquid chromatograph is a Hewlett-Packard Model 1084 B, equipped with a variable-wavelength detector (190—600 nm). The reversed-phase column, LiChrosorb RP-8, particle size 10 μm, 25 × 0.4 cm (Merck), is gradient-eluted with 18% to 23% acetonitrile in water for 6 min and then isocratically for 9 min until the wash program starts. After 16 min the rate of acetonitrile is 80% and remains so for 2.5 min. After 19.5 min acetonitrile has returned to 18% and after 2 min of equilibration the chromatograph is ready for the next injection. Total time is 21.5 min. The flow-rate is 2.4 ml/min, the column temperature 35°C and the column effluent is monitored at 207 nm.

The diluter used is a Hamilton digital diluter; the Gerhardt shaker with a modified tube holder is suitable for horizontally shaking 35 tubes simultaneously.

Reagents and standards

The buffer is 500 g of ammonium sulfate and 41.4 g of $NaH_2PO_4 \cdot H_2O$ dissolved in 1000 ml of water, pH 3.9 [4].

The internal standard is 100 mg of hexobarbital in 100 ml of ethyl acetate, the working solution 11 ml of internal standard in 2.5 l of ethyl acetate (Merck).

Acetonitrile and ethanol are LiChrosolv reagents from Merck. The water used in the mobile phase is first distilled and then filtered through a Millipore filter type AA with a pore size of 0.8 μm.

The composition of the drug standard mixture is given in Table I. It is stable for at least half a year at room temperature.

Procedure

To 1000 μl of buffer in a 12-ml Pyrex screw-top tube are added 500 μl of

TABLE I

DRUG STANDARD MIXTURE

Drug	Substance concentration in ethanol (mg per 200 ml)	Concentration in serum (40 μl of standard in 500 μl of serum) (μmol/l)
Caffeine	8.01	16.5
Carbamazepine (Ciba-Geigy)	17.04	28.8
CGP 10 000 (Ciba-Geigy)	10.38	15.3
Desmethylmephenytoin (Aldrich)	30.82	60.3
10,11-Epoxycarbamazepine (Ciba-Geigy)	7.14	11.3
Ethosuximide (Park-Davis)	77.65	220.0
GP 47 779 (Ciba-Geigy)	27.74	43.6
Mephenytoin (Sandoz)	16.68	30.5
Phenylethylmalonamide (Imperial Chemical Industries)	19.65	38.1
Primidone (Imperial Chemical Industries)	21.08	38.6
Pheneturide (Sapos)	25.02	48.5
Phenobarbital (Aldrich)	44.84	77.3
Phenytoin (Aldrich)	35.76	56.6

serum with 5 ml of working solution (internal standard) using a Hamilton diluter. For calibration 40 μl of the drug standard are placed in a tube to which drug-free serum is added. The tubes are shaken for 10 min and centrifuged (2500 *g*) for 10 min. The supernatant is decanted into a tube and evaporated to dryness at 37°C by means of a direct air stream. The residue is dissolved in 400 μl of 70% ethanol in water and 10 μl are injected into the liquid chromatograph.

RESULTS

Calibration

The liquid chromatograph is calibrated with a standard containing concentrations within the therapeutic range, with the exception of 10,11-epoxycarbamazepine and mephenytoin. Caffeine is included in the drug standard but is not measured. It is there just to make the standard realistic, as most patients have this compound. Fig. 2 shows a chromatogram of the drug standard which was added to drug-free serum and extracted as a patient serum.

Recovery

The recovery of the drugs is shown in Table II. Due to the high solubility of ethosuximide in water, only 76% is extracted. The recovery of the other compounds ranges from 89% to 95%.

Precision

Table III shows the precision of the method. The only drug with a relatively poor day-to-day precision (C.V. = 9.8%) is ethosuximide. To compensate for its high C.V. we are running serum samples with ethosuximide twice and use

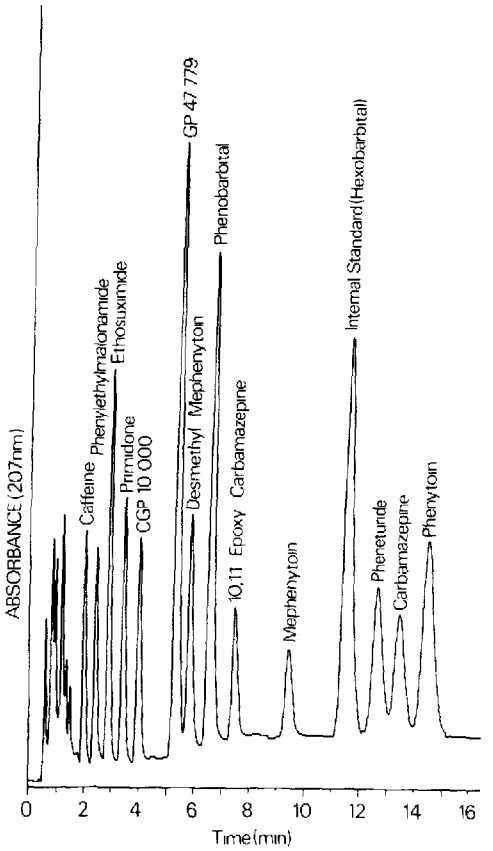

Fig. 2. Chromatographic separation of the drug standard which was added to drug-free serum and extracted as a patient serum. The concentrations are those listed in Table I.

TABLE II

RECOVERY OF DRUGS ADDED TO SERUM

Drug	Concentration (μmol/l)	Mean recovery* (%)
Carbamazepine	38.2	94
CGP 10 000	15.3	89
Desmethylmephenytoin	64.4	94
10,11-Epoxycarbamazepine	16.9	93
Ethosuximide	313.0	76
GP 47 779	43.8	93
Hexobarbital	169.3	90
Mephenytoin	37.8	92
Phenylethylmalonamide	38.0	95
Primidone	40.5	95
Pheneturide	54.5	90
Phenobarbital	76.7	94
Phenytoin	59.1	89

*$n = 7$.

TABLE III

PRECISION OF THE METHOD

n = 30 in every case.

Drug	Range ± S.D. (μmol/l)	C.V. (%)
Day-to-day		
Carbamazepine*	21.7 ± 1.4	6.4
CGP 10 000**	15.4 ± 0.8	5.2
Desmethylmephenytoin**	61.2 ± 2.8	4.6
10,11-Epoxycarbamazepine**	11.6 ± 1.0	8.5
Ethosuximide*	364.4 ± 35.6	9.8
GP 47 779**	44.4 ± 1.7	3.9
Mephenytoin**	30.4 ± 2.2	7.1
Phenylethylmalonamide**	38.7 ± 2.8	7.2
Primidone*	47.7 ± 2.7	5.6
Pheneturide**	73.1 ± 2.5	3.5
Phenobarbital*	66.7 ± 2.5	3.8
Phenytoin*	41.9 ± 1.6	3.8
Within-day		
Carbamazepine*	21.6 ± 1.0	4.6
Ethosuximide*	370.5 ± 21.7	5.8
Primidone*	48.5 ± 1.2	2.4
Phenobarbital*	68.2 ± 1.2	1.8
Phenytoin*	40.5 ± 0.9	2.3
Run-to-run		
Carbamazepine***	28.1 ± 0.7	2.5
Ethosuximide***	391.1 ± 4.6	1.2
Primidone***	38.5 ± 1.0	2.6
Phenobarbital***	91.3 ± 1.5	1.6
Phenytoin***	63.4 ± 0.6	1.0

*Control from Laboratoires Biotrol, Paris, France.
**Serum pool.
***Seronorm Pharmaca, Nyegaard, Norway.

the mean value as a result, when the two results are inside our within-day C.V.

Linearity

The linearity of the method is guaranteed to at least five times the upper therapeutic level.

Interferences

Retention times of the elven antiepileptic substances and other compounds which are also extracted by this method are shown in Table IV. Since methsuximide has a short half-life of 2—4 h [5], and therefore low serum concentrations, we monitor desmethylmethsuximide. The latter has a retention time equal to that of 10,11-epoxycarbamazepine. A selective extraction of 10,11-epoxycarbamazepine from serum in hexane at a pH of 2.0 eliminates this

TABLE IV

RETENTION TIMES FOR SOME COMPOUNDS WHICH ARE EXTRACTED BY THE METHOD

Compound	Retention time (min)
Theobromine	1.6
Theophiline	1.7
Caffeine	2.1
Desethylethadione	2.2
Phenylethylmalonamide	2.5
Ethosuximide	3.0
Primidone	3.5
CGP 10 000	4.1
Sulthiam	4 2
GP 47 779	5.4
Sulfamethoxazole	5.4
Ethadione	5.9
Desmethylmephenytoin	5.9
Phenobarbital	6.6
Desmethylmethsuximide	7.5
10,11-Epoxycarbamazepine	7.5
Butalbital	8.8
Oxcarbazepine	9.2
Mephenytoin	9.5
Hexobarbital	11.6
Methsuximide	12 3
Pheneturide	12.7
Carbamazepine	13.5
Phenytoin	14.5
N_4-Acetylsulfamethoxazole	14.9

interfering peak for the determination of desmethylmethsuximide [6]. Sulfamethoxazole, one of the two components in Bactrim®, has the same retention time as GP 47 779. It is possible to see from the chromatogram whether the patient is taking Bactrim together with oxcarbazepine because a metabolite of one of the two substances in Bactrim gives a peak shortly before the common peak of GP 47 779 and sulfamethoxazole. It is also possible to verify the presence of sulfamethoxazole in a common peak with GP 47 779 by monitoring at 270 nm where sulfamethoxazole has an absorption maximum and GP 47 779 has no absorption. Patients who receive pheneturide have two unidentified peaks appearing in the chromatogram, which we are in the process of identifying.

DISCUSSION

Oxcarbazepine (Fig. 1), a new drug from Ciba-Geigy, has been in clinical trial for the last 8 years in our center. This drug has a very short half-life (~1 h) and therefore the main pharmacological effect is due to GP 47 779 [7] which we are measuring. Our liquid–liquid extraction may be more time-consuming than solid-phase extraction [2]; however, the advantages are that the mate-

rials used are less expensive, the extraction is done at a fixed pH and the concentrations of the drugs are increased, thus less volume has to be injected into the chromatograph. The time required for the preparation of the serum extract is reduced due to the fact that it is possible to decant the ethyl acetate extract directly into the tube where the evaporation takes place, thus avoiding time-consuming pipetting. The buffer used [4], saturated with ammonium sulfate, gives a baseline free from interfering peaks of substances naturally occurring in serum. Our serum extracts are evaporated at 37°C because of the low melting point (64—65°C) of ethosuximide. The other drugs can be heated to 60°C without any loss. With the selected wavelength of 207 nm, the drugs are not measured at their maximum absorption level but on a shoulder, while the maxima are below 200 nm, with the exception of carbamazepine, which has its maximal absorption at 215 and 285 nm. Thus our selected wavelength of 207 nm is a compromise between the carbamazepine maximum at 215 nm and the other substances with maxima below 200 nm. It is important that the chromatograph has gradient facilities. The five substances which elute at the beginning (Fig. 2) are well separated by 18% acetonitrile in water. An isocratic run with this percentage of acetonitrile would give phenytoin a retention time of 27.0 min instead of 14.5 min with the gradient suggested. A chromatographic run takes 21.5 min altogether, the last 6.5 min being a wash program. This is done because the ethyl acetate extract substances, although they do not interfere with the determination, shorten the life-span of the column. In the wash program the column is flushed with 80% acetonitrile in water 2.4 times the total volume of mobile phase within the column. A higher percentage of acetonitrile in the wash program does not seem to prolong the life-span of the column. With this wash program a column can last for half a year with a weekly load of about 200 samples. Only the filter in the inlet has to be replaced when the pressure exceeds about 250 bar. A new column has a pressure of around 100 bar with 2.4 ml/min of 18% acetonitrile in water. The life of the column can be somewhat extended by inverting it. In our experience a shorter column with 5-μm material has a shorter life. The pressure rises faster, resulting in asymmetric peaks and poor separation. But, in the long run, the advantages of shorter analysis time with less acetonitrile might outweigh the disadvantage of more frequent column changes.

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