Determination of anticonvulsants in serum A-42.3

Key words

Instrumental HPTLC - quantitative analysis - densitometry (absorbance) - therapeutic drug monitoring - anticonvulsants - PEMA - primidone - phenobarbital - phenytoin - barbital - butethal - talbutal - amobarbital - pentobarbital - secobarbital - carbamazepine - mephobarbital

Scope

The above mentioned anticonvulsants can be analyzed solely or combined by this procedure. It is demonstrated by primidone as follows.

Primidone is used in therapy for control of complex partial psychomotor seizures. It is rapidly metabolized to phenobarbital and 2-ethyl-2-phenylmalonamide (PEMA). Patients are usually monitored for both phenobarbital and primidone serum levels. Previous studies [1] have reported coefficients of variation for primidone as high as ± 25% when analyzed by HPLC and GC. This could be due to the UV absorbance characteristics and polarity of the compound. The precision of the HPTLC method presented here is about ± 2% for primidone, phenobarbital and PEMA in serum.

The anticonvulsants are extracted with dichloromethane - isopropanol and then concentrated. The residue is taken up in dichlormethanol - methanol and separated on silica gel. It is quantitatively determined by absorbance measurement at 215 nm.

The method was developed by T.J. Siek, Analytical Bio - Chemistries Inc., Feasterville, PA.

Advantages of using HPTLC for this analytical task

- Simple sample preparation
- Low consumption of solvents
- Very acceptable precision and accuracy
- Short analysis time
- Determination of drug and metabolites simultaneously

Chemicals

Water dist.
Methanol
Dichloromethane
Isopropanol
Ethyl acetate
Ammonium hydroxide conc.
Standards: primidone, phenobarbital, 2-ethyl-2-phenylmalonamid (PEMA)

Standard solutions

Dissolve 10 mg primidone, 4 mg phenobarbital, and 4 mg PEMA and fill up each to 100 mL with methanol. Dilute 1:20, 1:10, 1:5, 1:2.5, 1:1.7 with dist. water.

Sample preparation

- Vortex 2.0 mL dichloromethane - isopropanol 19:1 with 0.4 mL serum for 15-30 s*.
- Centrifuge for 5 min and discard the aqueous layer.
- Transfer 1 mL of the organic phase to a disposable glass tube and evaporate to dryness on a water bath (60-70°C).
- Take up the residue in 25 µL dichloromethane - isopropanol 19:1.This solution is applied directly onto the HPTLC layer.

Layer

HPTLC plates silica gel Merck 60 F_{254}, 20x10 cm.

Sample application

Apply 1 µL spotwise with CAMAG Automatic TLC Sampler III, track distance 5 mm, distance from lower edge 8 mm, distance from left edge 15 mm = 35 applications per plate.

Application scheme:

S1	U1	U2	S2	U3	U4	S3	U5	U6	S4	U5	U6	S5	... 1.0 µL/spot	
5			10			20			40			60	ng Phenobarbital/spot	(mg/L)
2			4			8			16			24	ng Primidone/spot	(mg/L)
2			4			8			16			24	ng PEMA/spot	(mg/L)

S = standard, U = sample

* As internal standard for volume correction 20 µL of a 0.02% methanolic solution of mephobarbital can be added.

Chromatography

In a CAMAG Twin Trough Chamber 20x10 cm with dichloromethane - ethyl acetate - methanol - concentrated ammonium hydroxide 18:16:3:1; condition the plate prior to chromatography with developing solvent plus three drops of concentrated ammonium hydroxide for 10 min; migration distance 80 mm.

☞ *Enhanced detection and quantification of PEMA and primidone in the presence of other barbiturates may be achieved by the acidification of the solvent system dichloromethane - ethyl acetate - methanol - 0.1N HCL 90:80:15:2.*

Densitometric evaluation

With CAMAG TLC Scanner and CATS evaluation software; scanning by absorbance at 215 nm. Evaluation via peak height; repeatability (n=10) for both phenobarbital and primidone was found to be ± 2%.

Results

Serum samples	Phenobarbital	Primidone [mg/L]	PEMA
#1750-1	15.6	13.5	6.0
#1750-2	15.2	12.4	5.4
#2770-1	1.2	3.9	-
#2770-2	0.9	4.0	-
#2770-3	0.8	3.8	-

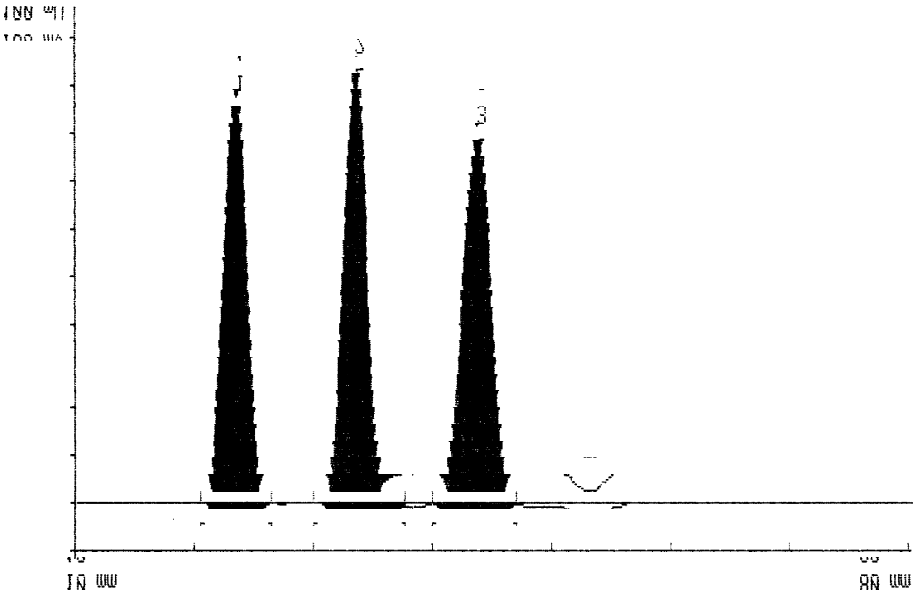

Fig. 1 Serum sample spiked with 250 ng phenobarbital (1), 250 ng phenytoin (2) and 200 ng mephobarbital (3).

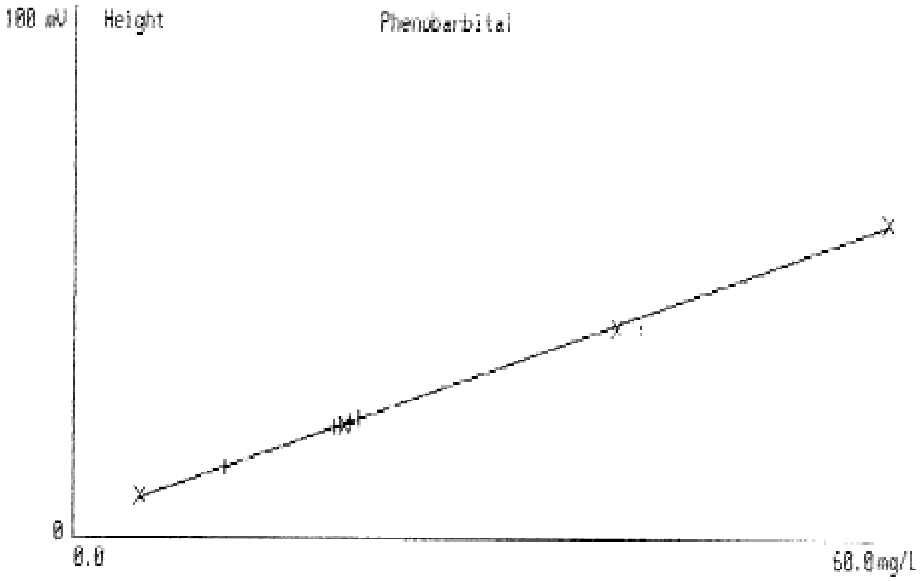

Fig. 2 Calibration curve for phenobarbital in human serum.

APPENDIX 1

Application to other anticonvulsants

Sample preparation, application and chromatography as described above may be used in the determination of other anticonvulsants listed below with minor changes. Since most anticonvulsants absorb strongly between 200 and 220 nm, adequate signals are achieved with quantities of between 25 and 250 ng applied to the plate and measured at 215 nm. Relative standard deviations of between ± 3% and ± 6% are achieved, which exceeds reported routine precision of GC or HPLC methods [1].

Anticonvulsant	R_F	Suitable Internal Standard (if necessary)
phenobarbital	0.20	talbutal, mephobarbital
phenytoin	0.23	talbutal, mephobarbital
primidone	0.27	mephobarbital
barbital	0.28	talbutal, mephobarbital
2-ethyl-2-phenylmalonamid	0.37	mephobarbital
butethal	0.38	phenobarbital, mephobarbital
talbutal	0.40	mephobarbital
amobarbital	0.41	phenobarbital, mephobarbital
pentobarbital	0.42	phenobarbital
secobarbital	0.43	phenobarbital
carbamazepine	0.44	nordiazepam
mephobarbital	0.45	talbutal

Comparison to other methods

Anticonvulsants are currently quantified in serum by many techniques including gas chromatography, liquid chromatography, fluorescence polarization immunoassay (FPIA), fluorescence immunoassay, radioimmunoassay, enzyme immunoassay, and nephelometry. FPIA is the most commonly used method in clinical laboratories in the United States.

The HPTLC method presented here offers a significant advantage over FPIA. While FPIA is very rapid for single analytes, each anticonvulsant must be separately determined and the metabolite concentrations are available in only a few instances by this method. For example, it is not possible to determine the concentration of PEMA by FPIA. Furthermore, some metabolites may cross-react in immunoassays and give inaccurate results for the parent drug.

The advantages of HPTLC over HPLC are cost-effectiveness, speed of analysis for batch samples, and the fact that extensive filtering of sample extracts is not necessary.

An additional advantage of HPTLC over GC is that no prechromatographic derivatization is necessary.

To summarize, HPTLC offers a chromatographic separation method for anticonvulsants which will provide specificity, accuracy, precision, and sensitivity comparable to HPLC and GC. For the more polar anticonvulsants precision superior to routine HPLC and GC is apparent [1].

Literature

[1] Summary Report for 1984, SPD 50P. Therapeutic Drug Monitoring, Report of 389 laboratories on primidone, U.S. Department of Health and Human Services, CDC, Atlanta, GA., February, 1985.