Journal of Chromatography, 182 (1980) 116—120
Biomedical Applications

CHROMBIO. 492

Note

Improved gas—liquid chromatographic method for the simultaneous determination of phenobarbital, phenytoin, carbamazepine and primidone in biological fluids

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(First received September 6th, 1979; revised manuscript received October 31st, 1979)

Among the techniques which have contributed to the present widespread determination of antiepileptic drugs, gas—liquid chromatography after flash-heater alkylation remains largely used by reason of its selectivity, sensitivity and reliability [1].

However, the analytical performances of flash-heater derivatization methods for phenobarbital (PB) and carbamazepine (CBZ) are questionable, since these two drugs decompose in the alkylating medium at high temperature. This problem can be overcome by using either an automatic injector [2—4], or internal standards structurally related to the drugs to be assayed. Such an appropriate internal standard is now often used for PB but, until now, was not available for CBZ.

Another problem encountered with CBZ determinations is the difficulty in achieving an efficient and specific extraction into the alkylating reagent. Interfering biological constituents can be eliminated by time-consuming clean-up procedures [2, 3, 5]. More conveniently, specificity can be improved by use of a nitrogen-selective detector [4, 6].

The method proposed here for the simultaneous determination of PB, phenytoin (DPH), CBZ and primidone (PM) in biological samples is an improvement on other published flash-heater methylation methods [4, 6, 7]. The two above-mentioned problems have been solved by the combination of appropriate internal standards (in particular, a new internal standard for CBZ), a modified rapid extraction procedure, and the use of a thermionic detector.

MATERIALS AND METHODS

Reagents and standards

Reagent grade dichloromethane, methanol and *n*-hexane were obtained from Merck (Darmstadt, G.F.R.). CBZ and 10-methoxycarbamazepine (MCBZ) were kindly supplied by Ciba-Geigy (Basel, Switzerland) and PM by Imperial Chemical Industries (Macclesfield, Great Britain). 5-Ethyl-5-*p*-tolylbarbituric acid (TEB), 4-methylprimidone (MPM) and 5-(*p*-methylphenyl)-5-hydantoin (TPH) were from Aldrich-Europe (Beerse, Belgium). PB and DPH meet the specifications of the United States Pharmacopeia (USP XVIII). MethElute® (trimethylphenylammonium hydroxide 0.2 mol/l methanol) was from Pierce Eurochemie (Rotterdam, The Netherlands).

The buffer used was 0.3 mol/l KH_2PO_4/Na_2HPO_4 (pH 6.7).

The extraction solvent was dichloromethane containing 4.5 mg of TEB, 7.5 mg of TPH, 11.0 mg of MCBZ and 5.0 mg of MPM per liter, as internal standards.

MethElute—methanol—water (2:2:1, v/v) was used as methylating solution. This solution was prepared daily.

Calibration serum. One volume of drug-free serum pool mixed with 0.025 vol. of a stock solution of drugs. This stock solution was prepared by dissolving PB, DPH, CBZ and PM in KOH (10 mol/l)—methanol—water (0.4:50:50, v/v). The following serum concentrations are obtained: 100 (PB), 60 (DPH), 30 (CBZ) and 60 (PM) μmol/l. The calibration serum, divided into 1-ml aliquots, was found to be stable for at least six months when stored at −30°.

Apparatus and operating conditions

An Intersmat IGC 120 DFL gas chromatograph equipped with a thermionic detector (alkali source: electrically heated rubidium glass bead) (Chelles les Coudreaux, France) was used. The glass column was 6 ft. × 0.4 in. O.D., packed with a mixture of equal volumes of 3% OV-1 and 3% OV-17 on 100—120 mesh Gas-Chrom Q. Separate packings were obtained from Applied Science Labs. (State College, Pa., U.S.A.). The injector temperature was 280°, the oven temperature 215° and the detector temperature 250°. The flow-rate of nitrogen (carrier gas) was 25 ml/min, of air 120 ml/min, and of hydrogen 1.2 ml/min. The attenuation was 4×10^{-9} A/mV.

Procedure

Into a 10-ml glass centrifuge tube, pipette 200 μl of sample (serum, plasma, saliva or cerebrospinal fluid) or of calibration serum, add 200 μl of buffer and 650 μl of extraction solvent. Shake on a Vortex-type mixer for 30 sec and centrifuge. With an Eppendorf pipette, transfer 500 μl of the organic layer into a stoppered conical glass centrifuge tube. Evaporate to dryness at 40° under a stream of nitrogen. Add 600 μl of *n*-hexane and 10 μl of methylating solution to the residue. Shake on a Vortex-type mixer for 30 sec and centrifuge for 2 min at 2000 *g*. Inject 1 μl of the lower layer into the chromatograph.

Quantitation

The following peak height ratios are calculated for each sample: PB/TEB,

DPH/TPH, CBZ/MCBZ and PM/MPM.

The concentration of each drug is calculated by comparing the ratio obtained for the unknown sample with that obtained for the calibration serum.

RESULTS AND DISCUSSION

A chromatogram of a calibration serum extract is shown in Fig. 1A. Retention times of the drugs and internal standards are: 106 (PB), 146 (TEB), 179 (CBZ), 204 (PM), 285 (MPM), 334 (MCBZ), 406 (DPH) and 570 (TPH) sec. A mixture of equal volumes of OV-1 and OV-17 has been selected for its excellent ability to separate these eight compounds without need of temperature programming.

Calibration curves are linear up to at least 350 (PB), 200 (DPH), 85 (CBZ) and 120 (PM) μmol/l of serum.

Owing to the short procedure time (about 30 min for an overall analysis of one sample), one analyst can easily process 30 samples per day.

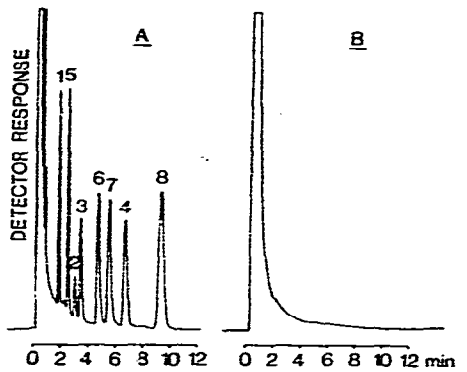

Fig. 1. (A) Gas chromatogram of a calibration serum containing PB (1), CBZ (2), PM (3) and DPH (4). Internal standards are TEB (5), MPM (6), MCBZ (7) and TPH (8). (B) Gas chromatogram of a drug-free serum extracted without internal standards.

Extraction procedure

To achieve a good recovery of CBZ into the methylating reagent, the procedures already published include evaporation of the organic extract and reconstitution of the residue with a methylating reagent [2—5, 7—9] or with methanol [6]. However, these procedures do not offer the advantages of the alkaline *extraction*, wherein a smaller volume of methylating reagent can be used, thus ensuring a greater sensitivity. For this reason, we first reconstitute the dried residue with *n*-hexane, and then add the methylating solution.

The composition of the methylating solution has been selected in order to achieve a good compromise between (a) complete derivatization of DPH, (b) complete breakdown of CBZ into iminostilbene, and (c) minimal methylation of iminostilbene [3]. DPH, CBZ and their respective internal standards are recorded each as a single peak.

Precision

Repeatability and reproducibility have been estimated from duplicates of patients' sera analysed under routine conditions. The results (coefficients of variation, C.V., %) are given in Table I, together with the limits of detection.

For the quantitation of each drug, we have selected the most appropriate internal standard, i.e. the respective *p*-methyl derivatives of PB, DPH and PM, and the 10-methoxy derivative of CBZ. This last substance was first introduced by Schwertner et al. [10] for the determination of CBZ as its N-pentafluorobenzamide. Table II demonstrates the importance of a good selection of the internal standards to ensure the greatest precision. For example, repeatability of the CBZ determination is 4.2% when calculated with MCBZ as internal standard, whereas the C.V. ranges from 9.6 to 16.9% when calculated by reference to the three other internal standards. Dudley [11] has also emphasized the usefulness of multiple internal standards to improve the precision of flash-heater alkylation methods.

TABLE I

PRECISION AND DETECTION LIMITS

	PB	DPH	CBZ	PM
Within-day C.V. (%)*	3.6 (2*n*=64)	3.7 (2*n*=62)	4.2 (2*n*=72)	3.4 (2*n*=58)
Between-day C.V. (%)*	3.2 (2*n*=86)	5.3 (2*n*=76)	4.5 (2*n*=62)	3.0 (2*n*=60)
Detection limit (μmol/l)	0.2	0.5	2.0	0.5

*Coefficient of variation on duplicates.

TABLE II

WITHIN-DAY COEFFICIENTS OF VARIATION CALCULATED ON PATIENTS' SERA ANALYSED IN DUPLICATE

For each drug, the precision has been calculated by reference to each of the four internal standards.

Internal standard	Drug			
	PB (2*n*=64)	DPH (2*n*=62)	CBZ (2*n*=72)	PM (2*n*=58)
TEB	3.6	11.5	16.9	11.8
TPH	10.4	3.7	11.8	6.3
MCBZ	14.7	13.5	4.2	10.8
MPM	11.7	7.7	9.6	3.4

Specificity and accuracy

The possible interference of several drugs and drug metabolites has been tested. Valproic acid, trimethadione, paramethadione, phenacemide, phenylethylmalonamide, ethylphenacemide, ethosuximide, methsuximide, phensuximide, ethotoin, mephenytoin and 5-ethyl-5-phenylhydantoin elute before PB and do not interfere. No peak is recorded within 30 min for sulthiame. 10,11-Epoxycarbamazepine is decomposed to several compounds which give no mea-

surable peaks. Mephobarbital yields the same N,N-dimethyl derivative as PB. Caffeine appears on chromatograms of some patients' sera. This substance has a retention time of 126 sec and does not interfere.

No peaks are recorded on chromatograms of drug-free sera extracted without internal standards (Fig. 1B). Serum constituents are recorded when a flame ionization detector is used instead of a thermionic detector [3, 7—9]. One of these observed peaks is not resolved from TEB, and thus interferes with the determination of PB. Some authors avoid this problem by tedious clean-up of the organic extract [2, 3]. We have chosen the alternative of using a thermionic detector. Being highly specific for substances containing nitrogen or phosphorus [12], this detector can be used in conjunction with our rapid extraction procedure.

Our participation in the St. Bartholomew's Hospital Quality Control Scheme [13] has enabled us to check the reliability of our method. At the present time, we have analysed eleven interlaboratory quality-control samples. None of our results fell outside the mean ± 1 S.D. limits of the accepted values reported by the participating laboratories.

ACKNOWLEDGEMENT

We gratefully acknowledge Mrs. J. Genin-Ramakers for her excellent technical assistance.

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