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GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF CARBAMAZEPINE AND PHENYLETHYLMALONAMIDE IN PLASMA AFTER REACTION WITH DIMETHYLFORMAMIDE DIMETHYLACETAL

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SUMMARY

A previously published procedure for the gas chromatographic analysis of carbamazepine has been modified and expanded to allow simultaneous determination of phenylethylmalonamide, a metabolite of primidone. Internal standards that closely resemble each compound are used, and derivatives are made by reaction with dimethylformamide dimethylacetal. This change of internal standard for carbamazepine and the use of a commercial, pretested column-packing material eliminate the major pitfalls of the original method.

INTRODUCTION

In the early 1970s, the dimethylformamide dialkylacetals were investigated for analytical use in derivatization of fatty acids and amino acids [1, 2]. Although these original reports indicated that reactions were easy, fast, and quantitative, the reagents were never used for actual assays in biological systems. Apparently problems with reagent purity and multiple products became evident after further study.

The reagent was later found to undergo rapid quantitative reaction with primary amides, and this property was used in the quantitation of carbamazepine (now one of the primary antiepileptic drugs) in plasma [3, 4]. Structural studies of the derivatives of carbamazepine, 10,11-dihydrocarbamazepine, and

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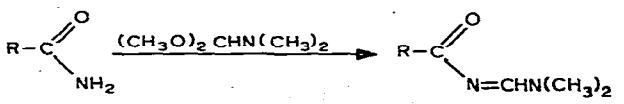
Cyheptamide (dibenzo[*a*,*d*] [1,4] cycloheptadiene-5-carboxamide) [5] showed that the reaction product was formed by coupling of the amide nitrogen through the acetal linkage of the reagent, to give an N-dimethylaminomethylene structure.



This method has been improved and expanded by changing the internal standard for carbamazepine to a compound that is easily synthesized in one step and more closely resembles carbamazepine than does Cyheptamide, the standard previously used. The column packing has been changed to one that is commercially available and has a guaranteed performance. Finally, a new internal standard, *p*-methylphenylethylmalonamide, has been incorporated to allow simple, accurate quantitation of phenylethylmalonamide (PEMA), one of the metabolites of primidone.

Since the initial studies of Gallagher et al. [6] and Baumel et al. [7, 8], almost no clinical information about PEMA has been published. This can be directly related to the scarcity of analytical information. The early techniques [8—10], which were developed for brief clinical studies, were long, involved, and unsuited to routine use. The most promising method published to date [11] involves a long extraction and uses an internal standard that is chemically unrelated to most of the drugs determined. Use of the usual internal standards is impossible because the drugs are chromatographed as trimethylsilyl derivatives, and under most conditions these phenytoin and carbamazepine derivatives are unresolved from the carbamazepine and phenytoin internal standards, respectively.

The present method, along with reliable on-column methylation techniques will allow rapid, accurate quantitation of all the major antiepileptic drugs and their metabolites that are present in plasma. With these two basic techniques, compounds are grouped rationally according to functional groups and concentration, and are analyzed as valid derivatives rather than as degradation products.

MATERIALS AND METHODS

Apparatus

A Varian Model 2100 gas chromatograph equipped with flame ionization detectors was used for this study. The column (91.5 cm × 2 mm I.D.) was a glass U-tube, packed with 3% OV-225 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The injection port, column oven, and detector were heated to 250°, 235°, and 270°, respectively. Nitrogen carrier gas was adjusted to about 70 ml/min to give a retention time for the carbamazepine derivative of 3.0 ± 0.2 min.

The mass spectra for the derivative of phenylethylmalonamide were run on a DuPont Model 490-F single-focusing mass spectrometer, operated in the

electron impact mode. Samples were probe-distilled directly into the ionization chamber. The ion source was operated at 235° with an ionizing voltage of 70 eV.

Reagents

Dimethylformamide dimethylacetal (DMF-DMA) (Aldrich, Milwaukee, Wisc., U.S.A.) was redistilled through a 15-cm distillation column before use. The product was collected between 105° and 107° in an amber bottle, which was then closed with a PTFE-lined screw cap and stored in the refrigerator. Carbon disulfilde was also redistilled and stored in the same type of bottle. All solvents and chemicals were analytical reagent grade. Carbamazepine was obtained as 200-mg tablets of Tegretol (Geigy Pharmaceuticals, Ardsley, N.Y., U.S.A.) and recrystallized from isopropanol to give a product that melted at 191—192°. The internal standard for carbamazepine, 10,11-dihydrocarbamazepine, was made by the procedure of Palmer et al. [12] and was recrystallized from isopropanol to give a product that melted at 200—202°. Phenylethylmalonamide* and *p*-methylphenylethylmalonamide** were used as received. The internal standard solution is made by dissolving about 1.5 mg of 10,11-dihydrocarbamazepine and 2.5 mg of *p*-methylphenylethylmalonamide in 10 ml of methanol and quantitatively adding this solution to 190 ml of 0.1 *M* ascorbic acid. The solution is stored under refrigeration in an amber bottle with a PTFE-lined screw cap and is stable for at least two months.

Procedure

In a culture tube (13 × 100 mm) having a PTFE-lined screw cap, 1 ml of plasma is combined with 0.50 ml of the internal standard, 0.5 ml of a 1 *M* K_3PO_4 solution and 3 ml of benzene—ethyl acetate (3:2, v/v). The mixture is shaken for 10 min and centrifuged to separate the layers. The organic phase is then transferred to a 5-ml Mini-vial (Alltech Assoc. Arlington Heights, Ill., U.S.A.) and evaporated at 50° under a stream of dry, filtered air or nitrogen. The residue is allowed to react for 10 min at 100° in a closed vial with 50 µl of DMF-DMA. The vial is then set aside to cool. Immediately before sample injection, the reagent is evaporated at room temperature under an air or nitrogen stream and the residue is dissolved in 25 µl of carbon disulfide. With a clean, dry syringe, about 1 µl is injected into the gas chromatograph.

RESULTS AND DISCUSSION

After publication of the method on carbamazepine determination by derivatization with DMF-DMA [3], several problems arose as people tried to duplicate the work. Initially, the main problem was the layered liquid phase column of OV-1 over OV-210. This was not a mixed liquid phase, but each phase was coated separately on the solid support to retain its individual advantage. The OV-210, which was solution-coated on the bare solid support, gave good resolution of the peaks of interest and minimum tailing. The OV-1,

*Supplied by Dr. C.E. Pippinger.
**Supplied by Dr. Kenneth H. Dudley.

which acted to greatly decrease the retention times of the peaks, was layered over the OV-210 by the pan-coating technique. For this step, a solvent (toluene) was used that would not dissolve the layer of OV-210 already on the solid support. A second problem arose later when Cyheptamide, the internal standard, became unavailable. The purposes of this work were (i) to expand the method to include phenylethylmalonamide, the primary amide metabolite of primidone, and (ii) to make the method routinely available by using internal standards that could be easily made or bought, and by using a commercial column packing of guaranteed performance.

Without any change in the basic method, these goals were all reached by changing the column packing to 3% OV-225 on 100—120 mesh Gas-Chrom Q, which is available as a pretested packing material, by substituting 10,11-dihydrocarbamazepine for Cyheptamide as the internal standard for carbamazepine, and by incorporating *p*-methylphenylethylmalonamide as a new internal standard for PEMA. Unlike the Cyheptamide derivative, the dihydrocarbamazepine derivative is stable in carbon disulphide, and it is easily made by a simple catalytic hydrogenation procedure [12]. The internal standard for PEMA is commercially available (Aldrich; Cat. No. 19,496-4).

The chromatograms in Fig. 1 show extraction of plasma from a patient on a regimen of phenobarbital (60 mg/day), primidone (500 mg/day), ethosuximide (750 mg/day), and carbamazepine (600 mg/day) and of drug-free plasma. The barbiturate, hydantoin, and succinimide anticonvulsants, when reacted with DMF-DMA, generally give multiple products, which elute within 1.5 min under the present conditions. Measured drug concentrations in the patient's plasma were 58.4, 8.2, 17.7, 5.91, and 8.97 μg/ml for phenobarbital, primidone, ethosuximide, carbamazepine, and PEMA, respectively. Pheno-

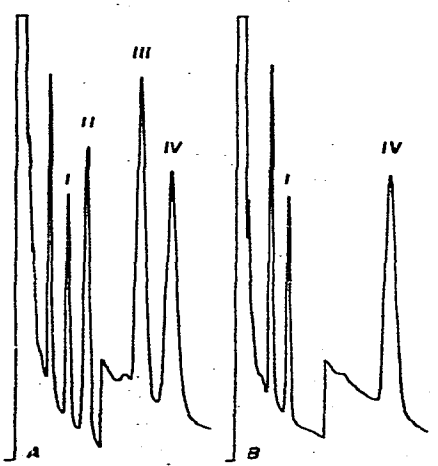
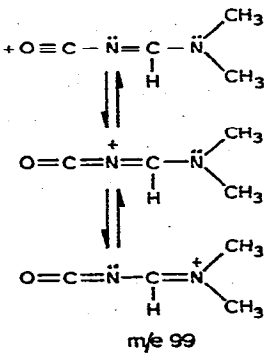


Fig. 1. Chromatograms of extracts of plasma from a patient receiving carbamazepine and primidone (A) and a person receiving no drugs (B). Designated peaks are N-dimethylaminomethylene derivatives of 10,11-dihydrocarbamazepine (I), carbamazepine (II), phenylethylmalonamide (III), and p-methylphenylethylmalonamide (IV). Measured concentrations of carbamazepine and phenylethylmalonamide were 5.91 μg/ml and 8.97 μg/ml, respectively.

barbital, primidone, and ethosuximide were determined by a modification of the method of Perchalski and Wilder [14]. The discontinuity in the baseline between the carbamazepine and PEMA peaks indicates a fourfold decrease in attenuation.

The derivative of PEMA, formed by reaction with DMF-DMA, was subjected to electron impact mass spectrometry. As in the previous study of these derivatives [5], the base peak at *m/e* 99 was by far the major peak in the spectrum. The molecular ion peak was clearly visible (1.8%) at *m/e* 316, which indicated that both nitrogens of PEMA are derivatized to the N-dimethylaminomethylene structure. No peak was visible at *m/e* 261, which would have been characteristic of the singly derivatized molecule.

The high relative intensity of the peak at *m/e* 99 is probably due to resonance stabilization of the ion through the conjugated oxygen-nitrogen system.



This characteristic spectrum makes these derivatives ideally suited to single ion monitoring. A method based on this mass spectrometric technique could probably be developed and would require only about 10 μl of plasma.

Reproducibility of the technique was evaluated by extracting ten replicates of a plasma sample that contained 4.53 μg/ml and 7.17 μg/ml of carbamazepine and phenylethylmalonamide, respectively. The coefficient of variation (CV) of the carbamazepine results was 1.9% and that of the PEMA results was 2.2%. Two of these samples were run over the 90-min period after evaporation of the derivatizing reagent, and again at 21 h. The CVs of the carbamazepine results were 1.1% for 90 min and 1.2% over the 21 h. That of the PEMA results was 4% over 90 min. After 21 h the peaks for PEMA and its internal standard were barely detectable.

Quality control samples, containing low (2.33 and 3.53 μg/ml) and high (9.32 and 14.1 μg/ml) concentrations of carbamazepine and phenylethylmalonamide, respectively, were run daily. Over a 20-day period, results had CVs of 6% and 1.8% (means, 2.44 and 9.47 μg/ml) for the low and high carbamazepine samples, respectively; and 4% and 5% (means, 3.55 and 14.2 μg/ml) for the low and high PEMA samples, respectively.

Recovery of carbamazepine from plasma was 93.5 ± 2.4% over the range of 2—10 μg/ml, whereas that of phenylethylmalonamide was much less, at 41.0 ± 1.0% over the range of 3—15 μg/ml. Reduction of the aqueous volume by

cutting plasma, standard, and buffer volumes in half did not significantly increase the recovery of carbamazepine. Recovery of PEMA was increased to about 55%; however, the smaller sample size was not adequate for detection of low drug levels.

The extracting solvent used in the original method, benzene—ethyl acetate (4:1, v/v), gave a slightly lower recovery of carbamazepine; however, the recovery of PEMA was only about 25%. Various other lighter-than-water solvents and solvent mixtures — made from cyclopentane, benzene, ethyl acetate, ether, methylene chloride, and ethylene dichloride — were tried, but no combination enhanced the recovery of PEMA without introducing interferences.

Such recovery could not normally be tolerated because low recoveries generally result in poor precision and accuracy. In this case, however, use of an internal standard, which has exactly the same functional groups in the same relative positions as PEMA, compensates for the recovery problem and maintains a high level of precision for the method. Even with the low recovery, the limit of detection of PEMA is 0.5 μg/ml, and, as before, that of carbamazepine is 0.2 μg/ml.

A random series of samples from 21 patients who were receiving various doses of primidone were analyzed for primidone and PEMA. A plot of primidone concentration versus that of PEMA had a correlation coefficient of 0.888 and a slope of 0.779, in excellent agreement with the values of 0.814 and 0.727, respectively, reported by Gallagher and Baumel [13]. The ratio of concentrations of primidone to PEMA for these samples was 1.0 ± 0.4. This finding along with the relatively longer half-life of PEMA (about 32 h as opposed to 6 h for primidone [13] may indicate that the PEMA concentration is a more consistent gauge of the primidone activity than is primidone itself. Further studies are under way with a more controlled group of patients to determine if this ratio has some correlation with dosage, seizure control, or metabolic abnormalities.

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