Organic Nitrogen-Selective Detector Used in Gas-Chromatographic Determination of Some Anticonvulsant and Barbiturate Drugs in Plasma and Tissues

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The specific nitrogen flame-ionization detector was used for the rapid simultaneous determination of eight anticonvulsant drugs and one drug metabolite in plasma and for three of the drugs in tissues. The system of analysis allows the occasional rapid estimation of barbiturate in plasma. No derivative formation is required and the whole range of drugs can be determined in a single diethyl ether extract of plasma in about 20 min.

The principal contribution of gas-liquid chromatography to clinical chemistry has been in the areas of steroid biochemistry and clinical toxicology. As most drugs contain at least one nitrogen atom, it appeared likely that a detector that has increased sensitivity and selectivity for organic nitrogen would bring improvements to established toxicological procedures. For example, the rapid analysis for basic drugs in urine by Donike et al. (1, 2) at the 20th Olympic Games first demonstrated in practical terms how the rapid and specific determination of stimulant drugs can be improved by use of the nitrogen detector.

In the clinical laboratory, one of the fastest growing areas is the monitoring of anticonvulsant concentrations in the blood of epileptic patients. In our laboratory the number of such plasma samples for analysis has increased from 378 in 1970 to 1713 in 1973, most of them from other hospitals.

This assay, as a routine determination, done on a dedicated gas chromatograph with the nitrogen-selective detector, has been in use at least 5 days a week, for longer than 18 months.

To analyze as many anticonvulsant drugs as possible on one particular column system and to provide a service for occasional barbiturate analyses, we now use the selective nitrogen flame detector. This modified detector is so specific and sensitive that we need only small plasma sample volumes (e.g., 0.2 ml).

Table 1 shows some anticonvulsant drugs that can be determined in a single plasma extract, although not all of them can be assayed simultaneously. For example, ethotoin and pheneturide cannot be determined in the same sample, a compound assumed to be an ethotoin metabolite is not completely separated from carbamazepine, and mesantoin elutes in the same position as one of the internal standards. In practice, however, these problems are of minor importance. No patient has yet been studied who is receiving both pheneturide and ethotoin. Where mesantoin has been analyzed, it has been possible to select another drug for use as internal standard in place of heptabarbital; cyclobarbital is the standard of choice in this case.

Phenytoin was first chromatographed with the aid of the nitrogen detector by Riedmann (3), using on-column methylation with a solution of tetramethylvammonium hydroxide. This type of analysis was repeated by Goudie and Burnett (4) in their analyses of phenobarbital, primidone, and phenytoin and by Brachet-Liermann et al. (5) for determination of phenobarbital and phenytoin. Both Riedmann (3) and Brachet-Liermann et al. (5) used the same make of nitrogen detector that we used in this study. With this particular design, one can “tune” the detector in order to eliminate endogenous compounds isolated from plasma that do not possess at least one nitrogen atom. However, Goudie and Burnett (4) found it necessary to extract plasma samples with dichloromethane after a pre-extraction with cyclohexane to remove “lips and other compounds.” All the previous

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Table 1. Drugs That Can Be Determined in a Single Extract of Plasma

<table>
<thead>
<tr>
<th>Anticonvulsant drugs</th>
<th>Therapeutic plasma concns, mg/dl</th>
<th>Range</th>
<th>Mean</th>
<th>CV, %a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td></td>
<td>0.4–4.2</td>
<td>1.7</td>
<td>3.9</td>
</tr>
<tr>
<td>Primidone</td>
<td></td>
<td>0.2–1.4</td>
<td>0.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Phenytoin</td>
<td></td>
<td>0.3–1.6</td>
<td>0.7</td>
<td>3.2</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td></td>
<td>0 –1.4</td>
<td>0.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Ethotoin</td>
<td></td>
<td>0.6–5.2</td>
<td>2.6</td>
<td>5.2</td>
</tr>
<tr>
<td>Ethosuximide</td>
<td></td>
<td>0.8–8.5</td>
<td>3.2</td>
<td>6.0</td>
</tr>
<tr>
<td>Phenturidene</td>
<td></td>
<td>0.3–1.5</td>
<td>0.6</td>
<td>5.3</td>
</tr>
<tr>
<td>Mesanind</td>
<td></td>
<td>1.5–6.0</td>
<td>3.3</td>
<td>7.5</td>
</tr>
<tr>
<td>Phenyl ethyl malondiamide</td>
<td></td>
<td>0.1–0.6</td>
<td>0.3</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*D Determined by individual analysis of 10 identical samples of horse serum, to which the drug was added 10 μg/ml (except phenobarbital, 20 μg/ml).

a Diphenylhydantoin.

b Ethylphenacemide.

c Also mephenytoin and methion are generic names.

Phenyl ethyl malondiamide was a generous gift from Dr. H. Schafer, Desitin-Werke, Hamburg, West Germany.

Standards

The pure drugs were dissolved in methanol (1 mg/ml) and 2-ml aliquots stored at 4 °C.

Standard curves were prepared by adding 5, 10, 25, and 50 μg, respectively, to 1-ml samples of drug-free pooled plasma. The plasma was extracted and chromatographed in the manner described below, to ascertain if there are any interfering peaks. The internal standards were added to the standard plasma samples and also to the unknown samples in amounts equivalent to 20 μg of heptabarbital and 10 μg of methyl phenyl phenylhydantoin per milliliter of plasma. These particular concentrations were chosen because the methyl phenyl phenylhydantoin is used as the internal standard for the drugs primidone and phenytoin, whereas heptabarbital is used as the internal standard for all the other drugs except mesantoin.

Extraction

Plasma. Plasma, 0.2 ml, containing, respectively, 4 and 2 μg of heptabarbital and methyl phenyl phenylhydantoin, was extracted with 5 ml of diethyl ether by shaking for 2 min. Sufficient solid ammonium sulfate (about 200 mg) was added to the mixture to saturate the aqueous layer and the shaking was repeated. It is important that the drugs are extracted into the ether before the ammonium sulfate is added, because the resulting plasma protein precipitation may result in the co-precipitation of some of the more strongly bound compounds. The addition of ammonium sulfate makes it unnecessary to dry the separated ether phase.

The ether layer was poured directly into a glass centrifuge tube and evaporated in a water bath at 50 °C. The residue was reconstituted in 25 μl of methanol, then 1 to 2 μl of this solution was injected into the gas chromatograph.

The 0.2-ml plasma sample is a convenient size for use in this type of analysis; however, the assay is sufficiently sensitive to permit use of as little as 50 μl of plasma.

Tissues. Tissue samples (0.2–1 g) were mixed with the two internal standards in the proportion of 20 μg of heptabarbital and 10 μg of methyl phenyl phenylhydantoin to 1 g of solid sample, and the whole mixture was sonicated for 30 s in 10 ml of ethanol. The solution was filtered through Whatman No. 1 filter paper, then evaporated in a rotary evaporator at 40 °C, the residue dissolved in 10 ml of chloroform, and the extraction continued as described previously (7).

Gas Chromatography

The Series 5750 Gas Chromatography (Hewlett-Packard, Palo Alto, Calif. 94304), equipped with dual flame-ionization detectors, one of them modified
with the Model 1516A Nitrogen Detector (Hewlett-Packard), was used throughout this study. The 120-cm glass column, 2 mm i.d., was packed with 1.5% WG-11 on Gas Chrom Q 80-100 mesh (Applied Science Laboratories, State College, Pa. 16801). The column merely requires conditioning by a programmed heating period from 100 to 275 °C at 4 °C/min, then maintaining this temperature for 5 min. After the conditioning, this phase is stable for as long as 14 months, especially with helium or argon as the carrier gas. When not in actual use, the column is maintained at 100 °C with a carrier gas flow of about 10 ml/min. The condition of the column can be readily determined by the separation of the three compounds heptabarbital, phenobarbital, and carbamazepine. Chromatography of carbamazepine in a single direct ether extract is particularly advantageous, in spite of comments (8), unsupported by evidence, that high operating temperatures lead to rapid column breakdown.

Figure 1 shows the seven compounds plus two internal standards that are determined in a plasma extract. The most common determination, shown in Figure 2, is of the four compounds, phenyl ethyl malondiamide, phenobarbital, primidone, and phenytoin.

**Gas-chromatographic selectivity.** The nitrogen flame-ionization detector responds proportionally to the percent nitrogen content of the organic compound, independent of its chemical structure (9). The combined characteristics of selectivity, sensitivity, with the linear range of the normal flame ionization detector make the nitrogen detector the detector of choice for analysis of organic nitrogen-containing compounds.

For this combination of selectivity and sensitivity, the rubidium bromide crystal must be carefully "tuned" with respect to the detector flame. The common practice is to use a combination hydrocarbon and azobenzene injection, as proposed by the instrument manufacturer, but Donike and Stratmann (2) showed that use of a drug or drugs involved in the analysis is more convenient. Thus we adopt the procedure that involves the use of an ether extract of plasma along with heptabarbital, as illustrated in Figure 3.
Analytical Variables

Carbamazepin e and etho toin. Figure 4 shows the problem that occurs when these two compounds are analyzed together. Although etho toin can be determined in the normal way, a peak always appears on the side of the carbamazepine peak, which is presumed to be an etho toin metabolite, as it is found only in plasma samples from patients receiving etho toin. Back extraction into sodium hydroxide solution, as reported previously (7), eliminates this interfering peak.

Barbiturate drugs. The common barbiturates, butobarbital, amylobarbital, pentobarbital, and quinalbarbital, along with barbital, can be adequately resolved on this column. Figure 5 shows the technique used to identify a barbiturate. In Figure 5 (left-hand chromatogram) the sample is chromatographed alone to detect the presence of a suspected barbiturate drug. A standard of known composition is chromatographed, followed by the two mixed together. As Figure 6 illustrates, these barbiturate drugs chromatograph in the same region as pheneturide and etho toin; thus further confirmation of the barbiturate structure should be sought in the direct ether extract.

If the extract of plasma is back-extracted into sodium hydroxide solution (7), neither pheneturide nor etho toin is extracted into the sodium hydroxide solution, whereas all the barbiturates are. The current method does not separate these two compounds; however, the identification of an unknown peak with a retention time of etho toin or pheneturide is aided by the presence of a possible etho toin metabolite on the side of the carbamazepine peak.

We made several unsuccessful attempts to improve the sensitivity for carbamazepine. This particular compound undergoes acid-catalyzed degradation and decomposition when chromatographed on polar columns (10), and the phases WG-11 and SP-1000 are no exception. In spite of the reported lack of degradation of the compound on injection with ethanol as compared to methanol (10), in a comparative study involving a series of six independent determinations with each solvent, we found no significant difference.

Precision and accuracy. The precision of the assay was determined by analyzing independently 10 samples of horse serum containing 1 mg of the drugs per milliliter (except phenobarbital, of which 2 mg/100 ml was added). The coefficients of variation for each drug are given in Table 1. Assays of the compounds determined in the standard isothermal procedure have a much better precision than do those deter-
determined in the temperature-programmed chromatograms. We believe that the poorer precision of the temperature-programmed determinations results from the inability of gas chromatographs to reproduce exactly the identical temperature conditions from run to run.

The accuracy of the assay was checked by each of two types of control sample. One sample of commercial manufacture, containing phenobarbital and phenytoin, is included with each batch of samples. The other sample, a blind control sent by Dr. Alan Richens of St. Bartholomew's Hospital, London, to 44 different clinical laboratories throughout Europe, contains the three drugs phenobarbital, primidone, and phenytoin. The results for phenytoin and primidone with this system have always been within one standard deviation of the mean, those for phenobarbitone within two.

Recovery. Five drugs and the metabolite phenyl ethyl malondiamide are recovered in essentially quantitative amounts; however, the yields of the compounds ethotoin, pheneturide, and mesantoin were, respectively, 78, 84, and 80%. All recovery data were calculated on the basis of the two internal standards that were added to the plasma before the extraction; absolute recoveries were not measured.

Information of Clinical Interest

Therapeutic ranges. The therapeutic concentrations of several of these drugs have already been reported (11). However, further analyses now enable us to re-assess some of these ranges (Table 1). Here, the term “therapeutic” is defined as that concentration at which no toxic symptoms were reported by the clinician on his request form for that particular analysis. Although such a system is obviously open to various criticisms, particularly in view of the sparse clinical details often given, it is the only one available to clinical chemists. A zero value for carbamazepine is included, because we have investigated several cases in which the clinician is assured that the patient is taking the drug and yet none can actually be determined in the plasma. However, in two such patients the drug was detected in the urine, and we concluded that their plasma concentrations were below the sensitivity of the assay. This particular phenomenon appears to occur only with adults; the drug can always be detected in children, where there is good evidence that the patient is actually taking the drug.

Concentrations in tissues. Figure 7 shows results of a determination of the three drugs, phenobarbital, primidone, and phenytoin. The current method of extraction of the drugs, particularly from brain, is designed for these three compounds. Of the other
ple taken on an out-patient basis, while that particular patient is being seen in the out-patient department. This service is available because of the nitrogen detector. The latter is somewhat more demanding of the operator than the normal flame-ionization detector is, but it does not have the problems inherent in the easily contaminated electron-capture detector. The nitrogen detector has a great deal to offer routine clinical chemistry.

References