The Gas Chromatographic Determination of Anticonvulsant Drugs in Serum

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ABSTRACT

A simple and accurate method is described for the quantitative determination of trimethadione, paramethadione, ethosuximide, metharbital, methsuximide, phensuximide, mephenytoin, ethotoin, primidone and diazepam in serum. Gas chromatography, with temperature programming, is employed and each of the drugs or any combination of them may be assayed on a single specimen during a single rapid determination on 3 percent OV-17 following chloroform extraction. Retention times relative to an internal standard (methyl myristate) are given. The recovery of the drugs is from 77 to 100 percent, the instrument response is linear for each drug, and the coefficients of variation are from 3 to 9 percent.

Introduction

A recurring problem in the management of seizure disorders is the adjustment of drug dosage to achieve a balance between seizure control and toxicity. It is recognized that this is facilitated when the levels of the involved drugs in the patient's serum can be determined. The two most commonly used seizure control drugs are diphenylhydantoin and phenobarbital. Methods for determining serum levels of these agents are well established. A procedure is reported for the quantitative analysis for ten other seizure control drugs in serum, using a single extraction and injection on a vapor phase chromatograph.

The ten drugs are, in increasing order of retention time in our chromatographic system, trimethadione, paramethadione, ethosuximide, metharbital, methsuximide, phensuximide, mephenytoin, ethotoin, primidone and diazepam. Barbital appears following metharbital, and methyl myristate, the internal standard, follows barbital. In figure 1 are shown the structures of the main classes of antiseizure drugs. Diazepam, a benzodiazepine derivative, shows little resemblance to these in a flat drawing, but in three dimensional conformation bears a startling resemblance to diphenylhydantoin.

Individual gas chromatographic methods have been reported for several of these drugs. However, the capability of handling all such analyses with a single system obviates maintaining multiple columns and
HYDANTOINS

DIPHENYLHYDANTOIN
ETHOTOIN
MEPHENYTOIN

BARBITURATES

PHENOBARBITAL
BARBITAL
PRIMIDONE (Carbonyl oxygen at (2) is replaced by H₂)
METHARBITAL

OXAZOLIDINEDIONES

TRIMETHADIONE
PARAMETHADIONE

SUCCINIMIDES

ETHOSUXIMIDE
METHSUXIMIDE
PHENSUXIMIDE

Figure 1. Structure of main classes of antiseizure drugs.

instrument conditions. Further, the savings in time when several specimens are to be analyzed or, as is often the case, when more than one of the drugs is to be determined, are obvious. Diphenylhydantoin and phenobarbital are strongly column absorbed and will produce no peak under the stated conditions if present even up to a level of 5 mg per dl.

There is also a more subtle advantage in this multiple drug analysis in that certain of these drugs, while being useful agents in their own right, are metabolites of other drugs. Thus, primidone metabolizes to phenobarbital and metharbital metabolizes almost completely to barbital.

Reagents

A six foot × 2 mm ID silanized glass column packed with 3 percent OV-17 on silanized 80/100 Chromosorb W was employed.*

Chloroform and methanol were reagent grade and used without further purification.

Methyl myristate was purchased.*

Acetate buffer, 0.2 M, pH 5.0.

Standard Solutions

The drugs used in the standard were pure samples and free of filler.

The standard solution contained 1 mg per ml of each of the drugs, and 0.25 mg per ml of methyl myristate in methanol. This is used for calibration of peak area, retention time and in the instrument linearity study. It was also used to spike sera for the recovery studies.

* Supelco, Bellefonte, PA 16823.
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**Apparatus**

A Bendix model 2500 four column, four flame ionization detector gas chromatograph was employed.† Hydrogen was supplied by an Elhygen hydrogen generator.‡ The instrument readout was a Honeywell Model 194 dual pen recorder§ and an Autolab Model 6300 digital integrator.|| Air and helium carrier gas were from Airco¶ and were used without further drying.

**Procedures**

To one ml of serum in a 50 ml conical tube are added one ml of acetate buffer and 10 ml of chloroform. The resulting mixture is shaken thoroughly and centrifuged. Of the chloroform extract, 8 ml are recovered and evaporated to a volume of 0.2 to 0.5 ml under a stream of nitrogen at room temperature, after which it is quantitatively transferred to a 2 ml disposable culture tube and evaporated to dryness. The residue is taken up in 25 μl of 0.25 mg/ml solution of methyl myristate in methanol, thus yielding a solution ready for injection.

The volatility of trimethadione, paramethadione, and ethosuximide precludes the application of heat during the solvent evaporation procedure.

Injections of 2 to 4 μl of sample are made. The injection port temperature is maintained at 260°, with the transfer zone at 275° and the detector block at 300°. The carrier gas flow rate is 50 ml per min (uncorrected).

The oven temperature is 100° at injection, and is maintained for ten minutes, during which trimethadione, paramethadione, and ethosuximide are eluted. It is then programmed at 3° per min up to 225°.

Linearity studies were performed by making injections of 1, 2, 3, and 5 μl of each drug and plotting the peak areas obtained vs the amount injected.

Recovery studies were carried out at the 3 mg per dl and 5 mg per dl level of drug from spiked human serum samples shown to be originally free of drugs.

**Calculation**

For each drug:

\[
\text{mg per dl} = \frac{\text{peak area of drug in sample}}{\text{peak area of methyl myristate}} \times \frac{\text{peak area of drug in std.}}{\text{peak area of methyl myristate}} \times C \times \frac{25 \mu l}{0.8 \text{ ml}} \times 0.1
\]

where \( C = \) concentration of standard in μg per μl.

**Results**

No component of normal, drug-free serum interferes with any of the drugs determined in the above method, although a final serum peak with a long retention time appears after the final drug. Retention times of the ten antiseizure drugs, plus barbital, are shown in table I relative to methyl myristate as the internal standard. Separation is complete, and all peaks are symmetrical with the exception of that from ethosuximide, which shows very slight tailing, and barbital, which shows moderate tailing. In figure 2 are results of an injection of chloroform standard con-
Figure 2. Chromatographic scan of standard plus added internal standard and barbital. Peaks represent 2 µg of each substance, except 5 µg of barbital.

Figure represents photograph of actual recorder output from two columns operating simultaneously, one pen scale having been traced over in India ink for clarity. One set of peaks has been numbered to correspond with listing in table I. Peak number 7 is methyl myristate, the internal standard. Total time span of figure is 50 minutes.

The results of the recovery studies are shown in table II. The recoveries shown are averages of at least four studies done at each of two concentrations in serum, 3 mg per dl and 5 mg per dl. They are all quite close to quantitative. In our laboratory, tests results using the above procedure are used uncorrected.

The results of the instrument linearity study are shown in figures 4 and 5. The

<table>
<thead>
<tr>
<th>% Recovery</th>
<th>CV*</th>
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<tbody>
<tr>
<td>1. Trimethadione</td>
<td>75.0</td>
</tr>
<tr>
<td>2. Paramethadione</td>
<td>82.7</td>
</tr>
<tr>
<td>3. Ethosuximide</td>
<td>84.6</td>
</tr>
<tr>
<td>4. Metharbital</td>
<td>87.6</td>
</tr>
<tr>
<td>5. Methsuximide</td>
<td>90.2</td>
</tr>
<tr>
<td>6. Phensuximide</td>
<td>90.2</td>
</tr>
<tr>
<td>7. Mephenytoin</td>
<td>93.6</td>
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<tr>
<td>8. Ethotoin</td>
<td>91.7</td>
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<tr>
<td>9. Primidone</td>
<td>84.3</td>
</tr>
<tr>
<td>10. Diazepam</td>
<td>100.7</td>
</tr>
</tbody>
</table>

* Based on eight serial determinations.

Figure 3. Chromatographic scan resulting from analysis of a clean human serum sample to which all drugs have been added at a level of 3 mg per dl, except barbital at 8 mg per dl.

Figures represent photograph of actual recorder output, traced over in India ink for clarity. Final peak is a normal human serum component. Peaks have been numbered to correspond with listing in table I. Peak number 7 is methyl myristate, the internal standard. Peak number 13 is the long retention time serum component. Total time span of figure is 80 minutes.
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Figure 5. Peak area vs micrograms injected for trimethadione, phenytoin, diazepam, and metharbital.

response of the instrument to each of the drugs is linear at least to 5 μg, and each of the linearity curves intercepts quite close to the origin, indicating minimal column absorption.

The coefficients of variation, as shown in table II, are based on at least eight determinations of each drug in a serum pool, with assays performed by two people over a period of four days.

Sources of Interference

Phenobarbital and diphenylhydantoin do not interfere (vida supra). Other barbiturates, glutethimide, methyprylon and methaqualone, will give peaks within the retention time of the drugs covered in the procedure discussed. However, it is rare for these to be used in cases of seizure disorder. As with any such procedure, it is prudent to be aware of the extraction and chromatographic behavior of all drugs which the monitored patient is taking.

The therapeutic serum level of valium is one to two orders of magnitude less than that of the other drugs in the scheme. However, valium shows very little irreversible column absorption, has good sensitivity in the flame ionization detector and can be successfully determined by the procedure described.

The procedure described is a rapid, specific and precise method for the analysis for antiseizure drugs in serum. It provides a measure of practicality unobtainable when individual assays must be maintained and employed to cover all the drugs used in epilepsy control.

References