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Published in:
Clinica Chimica Acta

DOI:
10.1016/0009-8981(76)90310-7

Published: 01/01/1976

Document Version
Publisher's PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:

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Link to publication

Citation for published version (APA):

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QUANTITATIVE DETERMINATION OF UNDERIVATIZED ANTI-CONVULSANT DRUGS BY HIGH RESOLUTION GAS CHROMATOGRAPHY WITH SUPPORT-COATED OPEN TUBULAR COLUMNS

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(Received May 19th, 1976)

Summary

A method is described to prepare routinely high resolution support-coated open tubular (SCOT) columns. A siliceous support material (Cab-O-Sil) is deactivated with benzyltriphenylphosphonium chloride and deposited on the inside wall of a glass capillary column. After additional coating with a polar stationary phase (OV-225) a number of anticonvulsant drugs can be analyzed without prior derivatization.

The columns described show high plate numbers and do not deteriorate in use. The repeatability of the gas chromatographic analysis is better than 1.2% ($\sigma_{rel}$). The minimum detectable quantity is of the order of $10^{-10}$ g.

An improved procedure for isolating the drugs from serum is given, which results in an extraction recovery of better than 90% for the drugs of interest.

Introduction

In the treatment of chronic diseases such as epilepsy, which may require administration of drugs during a long period of time, estimation of the achieved serum concentration of the drug is necessary.

Individual differences in speed of absorption, renal clearance, drug conversion rate and "drug resistance" are other factors that have to be allowed for the individual patient.

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From the analytical point of view, according to Ekins (private communication) up to now there have been two main approaches used in the estimation of anticonvulsant drugs; the one is by use of an excess of reagents the other is by use of a very limited amount of reagent and is called “saturation analysis”. Examples of the first type of analysis are widely used. The most common procedure of this type are preseparation of the compound under estimation followed by an end point determination e.g. spectrophotometry or chromatographic detection. These methods have been extensively reviewed by several authors [1–4]. The various types of saturation analysis e.g. radio- and enzyme-immunochemical methods also seem to be promising.

In our opinion however the main arguments in favour of saturation analysis, namely its sensitivity and selectivity, should be weighted against the fact that severe interference from metabolites may occur, and so in cases where multiple medication is prescribed the method of choice is gas chromatography.

In gas chromatography most often packed columns are used; derivatization is necessary to improve the peak shape and to prevent irreversible adsorption of the anticonvulsant drugs to the support material. See e.g. Cremers and Verheesen [5].

Recently Horning and coworkers [6] described a technique based on selective ion detection using a gas chromatograph-mass spectrometer-computer system, after derivatization of the drugs. Although this method is highly specific and sensitive, the high price prohibits its use in routine analysis.

In gas chromatography the performance of micropacked and open tubular columns largely surpasses that of packed columns in separation power, speed of analysis and sensitivity [7].

Driessen and Emonds [8] introduced micropacked columns in the field of anticonvulsant drugs. By the use of mixed stationary phases (OV-225 and OV-17) a number of compounds could be analyzed without prior derivatization.

Disadvantages are the relatively large area of support material and the short life times of these columns as experienced in our laboratories.

Open tubular columns due to their high value of the phase ratio \( V_G/V_L \) (\( V_G \) and \( V_L \) are, respectively, the volumes of the gas phase and stationary phase of the column) can be used at lower temperatures. Open tubular columns contain very little support material and therefore possess favourable adsorption properties.

It was believed that glass open tubular columns after deactivation of the column wall and coated with polar stationary phases would strongly reduce undesirable adsorption effects, and give optimal conditions for the quantitative analysis of underivatized anticonvulsant drugs.

There are two types of open tubular columns. In the classical columns the liquid phase is present in the form of a thin film coated on the inside wall of the tubing: wall-coated open tubular (WCOT) columns.

In the second type of column, the so-called support-coated open tubular (SCOT) column, micron size porous support particles precoated with the liquid phase are deposited on the inside tube wall.

Glass WCOT columns can only be prepared satisfactorily with a very limited choice of apolar stationary phases. To make a polar phase spread and adhere, the glass wall should receive a special pre-treatment prior to coating. One line
followed in research is the etching of glass wall with e.g. gaseous hydrogen chloride [9]. The resulting rough surface, however, usually contains many active sites for adsorption.

Therefore our attention was directed to SCOT columns where small particles of a support material, showing favourable adsorption properties, deposited on the inside column wall promote the spreading of the polar stationary phase.

Materials and methods

Reagents

All chemicals are of analytical grade. Ethylacetate (Baker), diethyl ether (Baker), digitonine (BDH), sodium sulphate (Merck), ammonium sulphate (Merck).

Drugs and standard compounds

These are given in Table I.

Isolation of drugs

Pipet 100 µl of serum into a thoroughly cleaned glass tube. Add 10 µl ethylacetate containing 1 µg cyheptamide (internal standard) or 10 µl ethylacetate containing 1 µg of each compound to be estimated, if the absolute calibration method is used.

Add subsequently 3 mg digitonin powder and 25 µl of a saturated ammonium sulphate solution in water to the mixture. The tube is heated for 5 minutes at 50°C. The mixture is extracted three times with 1.5 ml of freshly distilled diethyl ether. The collected ether fractions are evaporated to dryness and the residue is redissolved in 100 µl ethylacetate, already containing 2 µg tetracosanoic acid methylester.

Finally a sample of 1 µl is transferred to the injection system. The sensitivity of the total system using SCOT columns is such that in research problems the initial sample volume can easily be reduced by a factor of ten.

Gas chromatography

Preparation of SCOT-columns with a polar (OV-225) stationary phase

Glass capillaries of 0.4 mm i.d. are drawn from Pyrex tubing (and subsequently coiled) by a home built drawing machine; lengths up to 15 m are used; the coil diameter is 10 cm. In this work the column walls remain untreated; after

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**TABLE I**

Numbers refer to chromatogram Fig. 4.

1. Tetracosanoic acid methylester (Merck)
2. Phenobarbital(5-ethyl-5-phenylbarbituric acid) (Katwijk)
3. Cyheptamide(10,11-dihy dro[5H]dibenzo[a,d]-cycloheptene-5-carboxamide) (Mycofarm)
4. Carbamazepine(5H-dibenzo(b,f)azepine-5-carboxamide) (Ciba)
5. Primidone(6-ethyl-dihydro-5-phenyl-4,6-(1H,5H) pyrimidinedione) (Katwijk)
6. Phenytoin(5,5-diphenyl-2,4-imidazolidinedione) (Katwijk)
drawing, the columns are only flushed with dry acetone and carbon tetrachloride.

The inside of the column tubing is now coated following a modified two-step dynamic coating procedure according to German et al. [10]. Fine particles of a siliceous support Cab-O-Sil (Packard-Becker, Delft, Holland) are used; the particle size is smaller than 10 μm.

In this method a stable suspension of the support particles has to be formed. First the surface of the Cab-O-Sil is deactivated by adding 0.3 g Cab-O-Sil to 3 ml of a solution of 1% (g/g) benzyltriphenylphosphonium chloride (Aldrich Europe) in methylene chloride. After 10 min the solvent is removed by centrifugation. The residue is now washed with 3 ml of methylene chloride (2 times) again centrifuged and then dried at 80°C.

The deactivated Cab-O-Sil particles are now suspended in a solution of the polar stationary phase OV-225, a 25% phenyl and 25% cyanopropyl substituted methylpolysiloxane (Packard Becker, Delft, Holland) in carbon tetrachloride.

The mixture consists of 0.5 g Cab-O-Sil; 0.3 g OV-225 and 50 ml carbon tetrachloride. The suspension is homogenized in an ultrasonic bath, the particle size of the Cab-O-Sil treated in this way is smaller than 10 μm.

In the first coating step about 30 cm of the column is filled with carbon tetrachloride immediately followed by a plug of 25% of the column length of the suspension.

These plugs are now passed through the column at a speed of approximately 5 cm/s under nitrogen pressure. By means of a "pig-tail" dummy column, a rise in the flow rate of the solution when leaving the column is prevented.

As soon as the plugs have left the tail column the nitrogen pressure is controlled in such a way as to give a nitrogen flow of 10 ml/min and the column is for one hour left at room temperature for drying. After mounting in the gas chromatograph the column is now programmed at a rate of 5°C/min up to 250°C and left at this temperature for 2 h. It is essential that during the first coating step the column is placed in an ultrasonic bath.

In the second coating step additional stationary phase is coated on the surface layer of Cab-O-Sil.

A plug of 25% of the column length of a solution of 5% OV-225 in toluene is forced through the column at a speed of 2–5 cm/s; again a dummy "pig-tail" column is used. When the liquid plug has left the latter column, the pressure is controlled to give a nitrogen flow rate of approximately 5 ml/min. The column is left at room temperature under these conditions for one hour and then conditioned in the gas chromatograph with the following temperature program: First at a rate of 2°C/min up to 250°C, isothermal at 250°C for 3 h, followed by an isothermal run at 230°C for 12 h. During this program the nitrogen flow rate is again 5 ml/min.

Instrumental conditions

In this study and for routine purposes both a Packard Becker model 421 and a Pye Unicam gas chromatograph model 104 are used. An all-glass solid injector as depicted in Fig. 1 replaces the standard injection systems; a full description of this direct sample introduction system as well as the column connections is given elsewhere [11,12].
Fig. 1. All glass "moving needle" system for the direct introduction of high boiling compounds on to open tubular columns.

Fig. 2. The relationship between $H$ and the average linear carrier gas velocity $U$ for a $5 \text{ m} \times 0.4 \text{ mm I.D.}$ SCOT column. Column temperature $210^\circ\text{C}$; carrier gas nitrogen; sample quantity 20 ng/compound.
For a 5-m column the column head pressure was set at 0.15 atm giving a flow rate of 0.9 ml/min nitrogen. The temperature settings were as follows: injection port 270°C; detector 300°C; column 210°C.

Column performance

Columns coated following the procedure as described above typically show approximately 1500 theoretical plates/m at an optimal linear gas velocity of approx. 20 cm/s (see Fig. 2). These figures are obtained for underivatized samples of both primidone, capacity ratio \( k' = 27.0 \), and cyheptamide, capacity ratio 14.2.

For the measurement of the \( H-U \) curve, the column temperature is kept at 210°C, the sample quantity for both compounds is 20 ng, and nitrogen is used as the carrier gas.

Comparison with retention data obtained on micro packed columns for the same compounds, stationary phase and temperature, shows that a phase ratio \( V_G/V_L \) of our SCOT columns is of the order of 250—300. These figures are appreciably larger than in most SCOT columns described in literature. In fact sim-

Fig. 3. Scanning electron micrograph of a SCOT column coated with Cab-O-Sil and OV-225. Magnification: 2000 ×.
ilar numbers are stated for wall coated open tubular columns. This suggest a very thin layer of porous particles evenly distributed on the column wall. This is proven by a scanning electron microscope photograph of a treated column wall (Fig. 3). The Cab-O-Sil particles promote the spreading of OV-225 on the column wall. Without pretreatment pyrex cannot be coated with polar stationary phases; the film originally formed will break up and form droplets.

The high phase ratio, as formed in our columns, dictates the use of lower column temperatures in order to get reasonable values for the capacity ratios \( k' \) of the sample compounds. This has two obvious advantages: less possibility for decomposition of thermolabile compounds and less column bleeding.

In fact SCOT columns have now been used in our laboratories for over 9 months without any reduction in column performance. This compares very favourably with the lifetime of micropacked columns used in our laboratories for the same analysis.

Experimental results

Qualitative analysis

Fig. 4 shows a chromatogram obtained from a human serum. The numbers refer to Table I, peaks 5 and 8 originate from unknown compounds in the serum.

Table II gives the retention data relative to the internal standard cyheptamide.

Quantitative analysis

A. Calibration

Fig. 5 shows a calibration curve obtained following the direct calibration method (peak area versus amount of component). Using this calibration graph corrections have to be made for the aliquot injected and the overall recovery of added internal standards. The straight lines obtained pass through the origin of the axis system proving that no irreversible adsorption occurs. The relative standard deviation of the data points (10 measurements) is approx. 2% for a sample size of 20 ng/compound. Peak areas are determined by measuring peak height \( \times \) width at half height.

A chromatogram showing the optimal separation of some anticonvulsants and standard compounds is given in Fig. 6. Also here the numbers refer to Table I.

Using the method of internal standardization (standard compound cyheptamide) relative standard deviations in quantitative analysis of better than 1.2% are found for the drugs of interest. The calibration curves then depict the peak area ratio of compound to cyheptamide versus concentration.

It should be noted that in obtaining these results an electronic integrator (Infotronics CRS-208) is used. Also here these repeatabilities are obtained for sample sizes of approx. 20 ng/compound.

B. Analysis of a reference serum

The analysis procedure as described before (isolation, and quantitative gas
Fig. 4. Gas chromatogram obtained from human serum. 1, C-24 methyl ester (external standard); 2, phenobarbital; 3, cyheptamide (internal standard); 4, carbamazepine; 5, unknown; 6, primidone; 8, unknown. Sensitivity: $1 \times 10^{-11}$ A f.s.d. Conditions as mentioned in Fig. 2.

TABLE II

<table>
<thead>
<tr>
<th>Relative Retention Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tetracosanoic acid methylester: 0.27</td>
</tr>
<tr>
<td>2. Phenobarbital: 0.40</td>
</tr>
<tr>
<td>3. Cyheptamide: 1.00</td>
</tr>
<tr>
<td>4. Carbamazepine: 1.27</td>
</tr>
<tr>
<td>6. Primidone: 1.96</td>
</tr>
<tr>
<td>7. Phenytoin: 2.48</td>
</tr>
</tbody>
</table>
Fig. 5. Calibration curves for quantitative analysis following the direct calibration method (see text).

Fig. 6. Chromatogram of standard mixture of antiepileptics and standards. 1, C24 ester; 2, phenobarbital; 3, cyheptamide; 4, carbamazepine; 6, primidone; 7, phenytoin. Sensitivity: $2 \times 10^{-11}$ A f.s.d. Conditions as mentioned in Fig. 2.
<table>
<thead>
<tr>
<th></th>
<th>PB(2)</th>
<th>PRI(6)</th>
<th>CAZ(4)</th>
<th>PH(7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assigned value</td>
<td>24.2</td>
<td>6.35</td>
<td>4.2</td>
<td>11.3</td>
</tr>
<tr>
<td>Experimental</td>
<td>22.72</td>
<td>6.03</td>
<td>3.75</td>
<td>10.61</td>
</tr>
<tr>
<td>Extraction</td>
<td>94%</td>
<td>95%</td>
<td>89%</td>
<td>94%</td>
</tr>
</tbody>
</table>

* Added anticonvulsant drugs in μg/ml.

Chromatographic analysis by the method of internal standardization was applied to a reference serum. This serum was obtained from Haagse Apotheek, P.O. Box 8100, Den Haag, The Netherlands (see also ref. 13).

In Table III assigned values of plasma concentrations of the anticonvulsant drugs under study are compared with our experimental data.

For phenytoin the extraction recovery was also measured by means of the isotope dilution method ($^{14}$C-labeled phenytoin, CIS, Gif-sur-Yvette, France). By this method an extraction recovery of 96 ± 2% was found.

**Detection limit**

Using the set up as described in this paper, absolute amounts of the drugs mentioned of the order of 0.1 ng can be detected in blood serum, except for phenobarbital, because of the large "solvent peak" at high sensitivities.

**Discussion**

Using SCOT columns of 5–10-m length plate numbers of 7500–15 000 can be routinely obtained.

Analysis times under optimal conditions vary between 15 and 30 min. Under these optimal conditions serum extracts are analyzed for interfering substances. If these compounds are well separated from the drugs of interest the carrier gas velocity can easily be increased by a factor of 3, yielding analysis times of 5 to 10 min for routine use.

During our experiments it was observed that in the isolation procedure the amount of digitonin (added to the serum to remove cholesterol which interferes in the chromatogram) is rather critical. Cholesterol is incompletely removed for low amounts of digitonin, whereas gel formation is observed if an excess of digitonin is added. In the latter case inclusion of the drugs occurs.

Standard procedures for the removal of cholesterol with digitonin usually prescribe boiling of the mixture [8]. In our experiments, however, it was found that much higher recoveries and a better repeatability were obtained at a temperature of approximately 50°C.

At this temperature a reaction time of 5 min is sufficient.

Using gas chromatographic analysis with SCOT columns the limiting factor in the obtainable accuracy and repeatability of the overall analysis is the isolation procedure. This is proven by both chromatographic and radiochemical measurements.
References

11. van den Berg, P.M.J. and Cox, Th. (1972) Chromatographia 6, 301