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HIGHLY SENSITIVE METHOD FOR THE QUANTITATION OF HOMOVANILLIC ACID IN CEREBROSPINAL FLUID

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SUMMARY

Homovanillic acid in minute samples (50–100 μl) of cerebrospinal fluid can be quantitated as its O-dimethylthiophosphinate methyl ester. The derivative is determined after glass capillary gas chromatography (GC) with high sensitivity by using a phosphorus-specific thermionic detector or by mass fragmentography, combined with a large sample volume split–splitless injector. The dimethylthiophosphinic esters show excellent stability against moisture and air. The precision of the overall procedure is 5.4% (GC) and 3.8% (GC–mass spectrometry). The method shows good linearity ($r = 0.9999$) over three orders of magnitude, from 500 pg to 500 ng. The lowest detectable concentration of homovanillic acid is 2–5 ng/ml. Concentrations of homovanillic acid determined in cerebrospinal fluid were 9.9–63.1 ng/ml ($n = 10$, $\bar{x} = 30.9$ ng/ml).

INTRODUCTION

Dopamine plays an important role in neurotransmission at the synaptic level. Homovanillic acid (HVA) is the major metabolite found in cerebrospinal fluid (CSF). The determination of these substances is of great interest for biochemical or pathobiochemical investigations, although not for diagnostic purposes at present.

In the past various methods have been described for the quantitative determination of HVA in CSF, including fluorimetry[1], gas chromatography[2,3] and gas chromatography–mass fragmentography[2,4–8]. The overall sensitivity of these methods, however, is poor so that a relatively large amount of CSF (0.5–3 ml) has to be analysed. Therefore, we intended to develop a more sensitive procedure for the determination of HVA, in order to minimize substantially the sample volume required.

For the derivatization of hydroxy compounds of biological interest we have used successfully dimethylthiophosphinic chloride, which leads to very stable and sensitively detectable O-dimethylthiophosphinates[9–12]. These derivatives can be detected with the alkali flame-ionization detector down to the femtogram level and to the lower picogram level with gas chromatography–mass spectrometry (GC–MS).

In this paper we describe the combination of a very sensitive detection method

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with a particular glass capillary sampling technique which allows the injection even of the complete sample, if desired[13,14].

EXPERIMENTAL

Reagents and materials

All solvents were of analytical-reagent grade and distilled prior to use. The reagents were of analytical purity, with the exception of dimethylthiophosphinic chloride, which was obtained from Riedel-de Haën (Seelze, G.F.R.) and used after distillation as a 0.25% (v/v) solution in dried diethyl ether. Triethylamine was used as a 1.25% (v/v) solution in the same solvent.

Methanolic hydrochloric acid (1 *M*) was prepared according to Islam and Darbre[15] by mixing 50 μl of acetyl chloride with 1 ml of methanol. As internal standards we used 3-hydroxyphenylacetic acid (Merck, Darmstadt, G.F.R.) or d_3-HVA (deuterated in the methoxy group), with a purity of more than 99% (Dr. Franzen Analysen-Technik, Bremen, G.F.R.).

Thin-layer chromatographic (TLC) plates (silica gel) were pre-washed with distilled methanol for 3 days.

Apparatus

A Model 3700 gas chromatograph (Varian, Palo Alto, CA, U.S.A.) was equipped with a split–splitless injector as described elsewhere[13,14]. Prior to the introduction of the sample the injector was cooled to room temperature. Volumes of 10–20 μl of the sample were injected slowly (1 μl/sec) with the splitting valve fully opened. After 30 sec, the splitting valve was closed and the interior of the injector heated immediately to 270°C. During these steps the temperature of the 25-m SE-30 column was maintained at 120°C. After 2 min the temperature programme (10°C/min) was run. For phosphorus detection a thermionic specific detector (TSD) was used (hydrogen, 4.5 ml/min; air, 175 ml/min; carrier gas, helium or nitrogen, 1 ml/min; He as make-up gas, 20 ml/min; bias voltage, 4.0 V; bead heating current, 500). The specificity under these conditions was 1000:1 for the dimethylthiophosphinate of homovanillic acid methyl ester compared with *n*-eicosane.

Mass fragmentography was performed with a Varian-MAT 311A double-focusing mass spectrometer which was combined with a Model 1400 gas chromatograph (Varian-MAT, Bremen, G.F.R.) and equipped with the same injector system as described above. The glass capillary column (25 m SE-30) was coupled to the ion source of the mass spectrometer with an open split-type connection[16], which consisted of a glass capillary (50 cm × 0.15 mm I.D.). A four-channel hardware multiple-ion detection unit (MID) was operated at a resolving power of $R = 2000$ with an ionization energy of 70 eV and an emission current of 2 mA.

For the elution of the separated derivative zones from the TLC plates we used an Eluchrom (Camag, Muttenz, Switzerland).

Extraction of HVA and derivatization

A solution of 2 ng of 3-hydroxyphenylacetic acid (for GC) or d_3-HVA (for GC–MS) in 100 μl of water is added to 50–100 μl of CSF. The CSF is extracted according to Sjöquist and Ānggård[2] once with 400 μl and then with 500 μl of ethyl

acetate in conical polypropylene tubes. The solvent is evaporated in a stream of dry nitrogen. The residue is dissolved in 25 μl of methanol and methylated by the addition of 25 μl of the freshly prepared reagent over a 1.5-h period at ambient temperature. The reaction is stopped by the addition of 20 mg sodium hydrogen carbonate over a 2-min period. Subsequently the supernatant is transferred, the hydrogen carbonate washed with 50 μl of methanol and the combined solutions evaporated under nitrogen. The residue is allowed to react with 50 μl of dimethylthiophosphinic chloride solution and 50 μl of triethylamine solution at 50°C for 30 min. The reaction mixture is evaporated with a stream of nitrogen. The residue is dissolved in 50 μl methanol and 20 mg of sodium hydrogen carbonate are added. The methanolysis of the excess of reagent occurs within 30 min at 50°C.

Gas chromatographic determination

The supernatant is transferred quantitatively to a TLC plate. The plate is developed with dichloromethane–methanol–4 *M* ammonia solution (50:20:3). The solvent front migrates 8 cm. After drying, the TLC plate is cut 1.5 cm below the solvent front and the upper part containing the derivative developed in the second solvent system (chloroform–ethyl acetate, 9:1). The zone containing the dimethylthiophosphinates (R_F 0.52) is eluted with 600 μl of methanol with the Eluchrom and, after evaporation, dissolved in 50 μl of ethyl acetate and a 10–20-μl volume is injected into the gas chromatograph.

Mass fragmentography determination

The supernatant is transferred quantitatively with methanol into another polypropylene tube and evaporated with a stream of nitrogen. The residue is dissolved in 50 μl of ethyl acetate and 10–20 μl are injected into the GC–MS system without any purification step. The HVA derivative is detected via the molecular ion at *m/e* 288.0585 and the deuterated HVA derivative at *m/e* 291.0773. The exact tuning of these fine masses was performed with the perfluorokerosene ion at *m/e* 292.9825, which served as reference peak.

RESULTS AND DISCUSSION

Derivative formation

Aromatic hydroxy acids can be methylated selectively in the carboxylic group without any attack on the phenolic hydroxy group as described under Experimental. Karoum *et al.*[7] reported similar results under different reaction conditions. Another reagent, boron trichloride–methanol, was used by Gelpi *et al.*[17]. Sjöquist and Änggård[2] have shown that the use of short reaction times allows the selective formation of homovanillic acid methyl ester even with diazomethane. However, it is known that this reagent usually leads to inhomogeneous products for this type of multifunctional compound.

Dimethylthiophosphinates of aromatic hydroxy compounds are formed easily with dimethylthiophosphinic chloride in the presence of triethylamine[12] in the picogram range (Fig. 1). After 3 min the reaction was complete, yielding a uniform product, as we could expect from our previous results with similar functional groups[9–11]. Acetonitrile, diethyl ether, carbon tetrachloride, tetrahydrofuran and dioxane were

checked for their suitability for the derivatization reaction. With respect to the stability of the derivative and the time of derivative formation, diethyl ether was found to be the best.

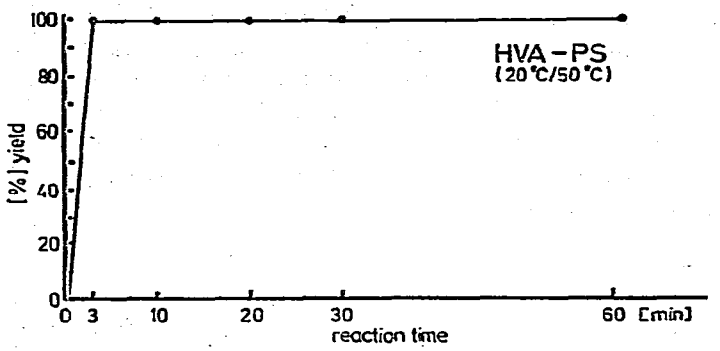

Fig. 1. Kinetics of the reaction of dimethylthiophosphinic chloride with homovanillic acid methyl ester at 50°C (methylation at 20°C).

In order to obtain complete derivatization even with crude biological material, we extended the reaction time to 30 min.

Removal of interfering by-products

Owing to the high sensitivity of the TSD in the GC procedure, a TLC step is necessary for the removal of phosphorus-containing by-products. They consist mainly of dimethylthiophosphinic anhydride, dimethylthiophosphinic methyl ester generated by the methanolysis, and dimethylthiophosphinic amides originating from trace impurities in the triethylamine used. It is of great advantage that both the HVA and 3-hydroxyphenylacetic acid derivative have the same R_F value in the solvent systems described.

Properties of the derivatives

The dimethylthiophosphinate of HVA showed excellent stability against moisture and air compared with the perfluoro derivatives. The latter are easily de-

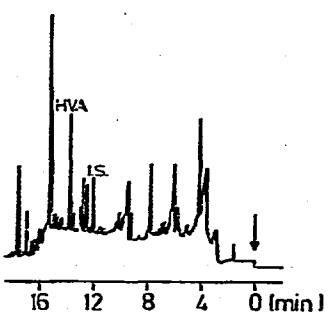

Fig. 2. Glass capillary gas chromatogram of homovanillic acid (HVA) and 3-hydroxyphenylacetic acid (internal standard, I.S.) as their dimethylthiophosphinate methyl esters from 50 μl of cerebrospinal fluid. The sample volume injected was 10 μl.

stroyed by trace amounts of water. Another advantage of our compounds is the reduced volatility. This allows us to use the injection system described with minimal loss of material.

Gas chromatographic properties and detection limit

The derivative of iso-HVA could be well separated from that of HVA. The peaks were sharp and showed little tailing on the SE-30 column (Fig. 2).

The detection limit for the dimethylthiophosphinates of HVA methyl ester and 3-hydroxyphenylacetic acid methyl ester was 250 fg, with a 3-fold signal-to-noise ratio. The lowest detectable concentrations of HVA were 3–5 ng/ml with a sample volume of 50–100 μl of CSF.

Gas chromatographic–mass fragmentographic determination

It is one of the benefits of the combined GC–MS technique that the time-consuming and laborious purification steps can be omitted. Because we were working with extremely minute amounts of substance, close to the limit of the sensitivity, we were forced, however, to enhance the mass spectrometric resolution from 500 to 2000 (Fig. 3), which increases the specificity of the detection dramatically.

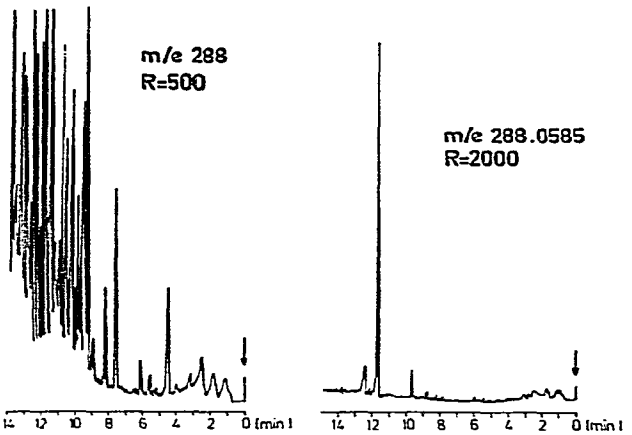

Fig. 3. Comparison of the detection of derivatized homovanillic acid from cerebrospinal fluid without a TLC purification step with low-resolution mass fragmentography (R = 500, *m/e* 288) (left-hand side) and high-resolution mass fragmentography (R = 2000, *m/e* 288.0585) (right-hand side).

In this instance any TLC separation can be avoided. A mass fragmentogram of a 50-μl CSF specimen is shown in Fig. 4. With the lower resolution, identification of the interesting peaks is impossible and a TLC step is needed.

The use of d_3-HVA as internal standard allows perfect control of the whole method (Fig. 5).

The overall detection limit for a 50–100-μl sample is 1–2 ng/ml.

Linearity and reproducibility

The amount of HVA applied showed a strong correlation with the observed peak area of the derivative (Fig. 6). Calculation of the correlation coefficient of the GC reference curve gave a value of 0.9999 for a range from 500 pg to 500 ng.

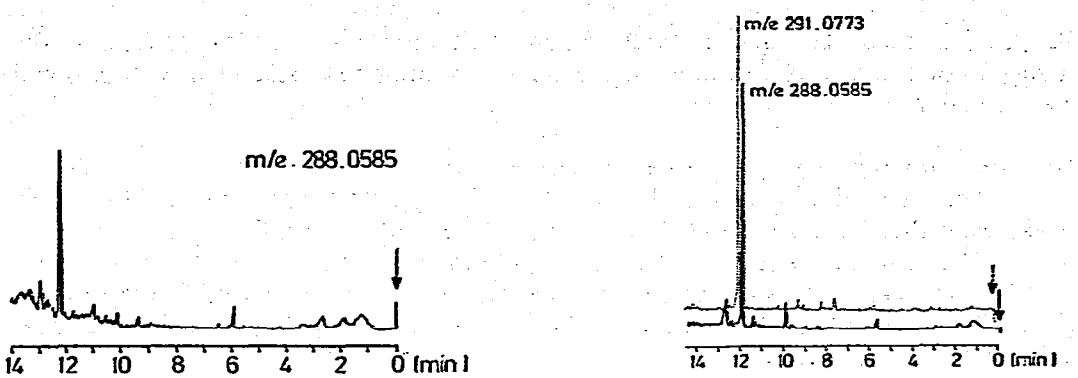

Fig. 4. Mass fragmentogram of a 50-μl cerebrospinal fluid specimen. The peak represents 50 pg of HVA on the column.

Fig. 5. Mass fragmentograms of the HVA derivative (*m/e* 288.0585) and the internal standard d_5-HVA derivative (*m/e* 291.0773) of a 100-μl cerebrospinal fluid specimen.

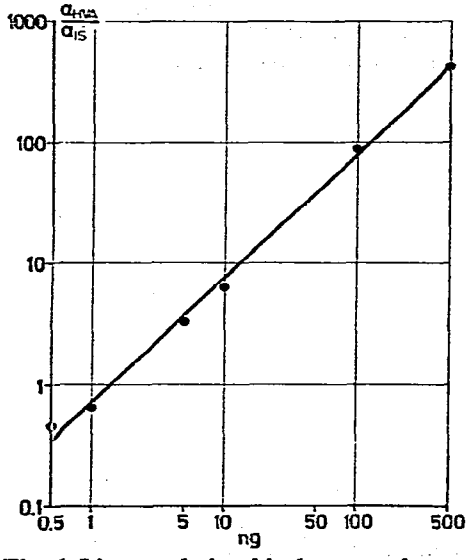

Fig. 6. Linear relationship between the amount of HVA applied and the ratio of the peak areas (HVA *vs.* internal standard) determined by gas chromatography (thermionic detector).

The linearity of the mass fragmentographic method was checked for HVA concentrations from 10 to 60 ng/ml in CSF. The correlation coefficient was 0.9996.

The precision was determined with lumbar CSF containing 38.5 ng/ml of HVA (mean of eight determinations). The reproducibility was 5.4% for the GC procedure and 3.8% ($n = 10$, $\bar{x} = 35.2$ ng/ml) for mass fragmentography. The mean recovery of the overall procedure was about 40% (GC) and 60% (GC–MS).

Concentrations found in lumbar CSF

The concentrations of HVA found in CSF collected from lumbar diagnostic punctures were 9.9–63.1 ng/ml ($n = 10$, $\bar{x} = 30.9$ ng/ml). This agrees well with the values reported by Takahashi *et al.*[8].

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