Gas Chromatographic Determination of Homovanillic Acid in Human Cerebrospinal Fluid by Electron Capture Detection and by Mass Fragmentography with a Deuterated Internal Standard

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Two highly sensitive and specific methods for quantitative determination of 4-hydroxy-3-methoxyphenyl acetic acid (HVA) in cerebrospinal fluid (CSF) have been developed. After gas chromatographic separation, the HVA heptafluorobutyrylmethyl ester derivative has been analyzed by either electron capture detection (ECD) or by using the multiple ion detector of a LKB 9000 mass spectrometer (MS). 4-Hydroxy-3-methoxyphenylpropionic acid was used as an internal standard for the gas chromatographic–ECD method and the three deuterium methyl ester of HVA for the gas chromatographic–MS method. The standard deviations are 7% and 10%, respectively, in the 400–500 pmole/ml range. The main advantages of these procedures lie in increased sensitivity permitting analysis in smaller volumes (0.5–2 ml) of CSF, and increased specificity.

THE FLUORIMETRIC METHOD (*1*, *2*) for measuring homovanillic acid (HVA) in cerebrospinal fluid (CSF) has found widespread clinical acceptance. However, in spite of recent modifications (*3–7*), the method has several drawbacks in the light of present analytical technology. The main disadvantages are insufficient sensitivity, requiring large sample volumes, and possibly also the lack of specificity in concentrations normally found in human and animal CSF.

In view of the inherent specificity of gas–liquid chromatographic (GLC) analytical methods and the availability of increasingly sensitive and selective ancillary detection systems, we have explored the possibilities for GLC analysis of HVA and related compounds in biological fluids. Perfluoroacylation yielded derivatives with satisfactory properties for electron capture (EC) (*8–10*) as well as mass spectrometric analysis (*10*). In the present paper we describe two gas phase procedures for sensitive and specific analysis of HVA in human CSF. One method is based on EC detection. The other method makes use of quantitative multiple ion analysis (*11*, *12*) and a derivative labeled with a stable isotope as an internal standard (*13*, *14*).

EXPERIMENTAL

Instrumental Conditions. A Varian Model 204 gas chromatograph was fitted with columns of 2500 × 3 mm glass packed with 1% OV-1 on 100–120 mesh Gas Chrom Q (Applied Science Labs, State College, Pa.). The electron capture detector had a 250-μCi tritium foil as the electron source. The flow rate of the carrier gas (nitrogen) was 25–30 ml/min and the pressure was 3 kg/cm^2. Temperatures were: injection port, 170 °C; column, 140 °C; detector, 230 °C.

For the mass spectrometric determinations, we used a LKB Model 9000 combined gas chromatograph–mass spectrometer equipped with a multiple ion detector and a 1% SE 30 column. The flow rate of the carrier gas (helium) was 20–25 ml/min. Temperatures were: column, 170 °C; flash heater, 210 °C; ion source, 290 °C. The trap current was 60 μA and the energy of electrons was 22.5 eV. The column supports were coated with the liquid phase using the fluidization technique. Columns were conditioned at maximum temperature for 2 days.

Materials. 4-Hydroxy-3-methoxyphenyl-propionic acid (HVA-CH_2) was prepared from 4-hydroxy-3-methoxycinnamic acid ≥98% trans, mp 166–170 °C (Fluka AG. Buchs, S:t Gallen, Switzerland) by catalytic hydrogenation in room temperature and at atmospheric pressure. Ethanol was used as solvent and 10% Pd on carbon as a catalyst. The product was recrystallized twice from toluene, mp 90–91 °C (lit. 89–90 °C). The HVA heptafluorobutyryl methyl ester derivative gave a single peak on gas–liquid chromatography and the mass spectrum of this derivative gave a molecular ion with a *m/e* value of 406 (base peak). Fragments at 375, 346, and 333 are in agreement with the proposed structure.

In the mass spectrometric analysis, the D_3-methyl ester of homovanillic acid was used as the internal standard. Two hundred milligrams of HVA (Kistner AB, Gothenburg, Sweden) was dissolved in 10 ml of CD_3OD (isotopic purity 99%, Merck Sharp & Dohme, Darmstadt, W. Germany) +0.25 ml acetyl chloride. The reaction was allowed to proceed overnight at room temperature and then evaporated to dryness. One ml of water was added, the pH was adjusted to 7 and the solution extracted three times with 2 ml of ethyl acetate. Evaporation of the solvent yielded crystalline HVA-CD_3, mp 42–43 °C.

The HFB-derivative of HVA-CD_3 gave a single peak on GLC analysis. The mass spectrum is shown in Figure 1. The molecular ion peak at *m/e* 395 is also the base peak. Major peaks are also observed at *m/e* 333 and 198 resulting from loss of a carbomethoxy (62) and a heptafluorobutyryl (197) group, respectively. The *m/e* 392 to 395 ratio in the HVA-CD_3-HFB was 0.003. HVA-7-^{3}H (specific activity 1.05 Ci/mmole) was a custom synthesis from New England Nuclear, Boston, Mass. The radiopurity was controlled by thin layer chromatography on silica gel (toluene:HOAc:H_2O 70:36:1.5) of the free acid and radiogas chromatography of

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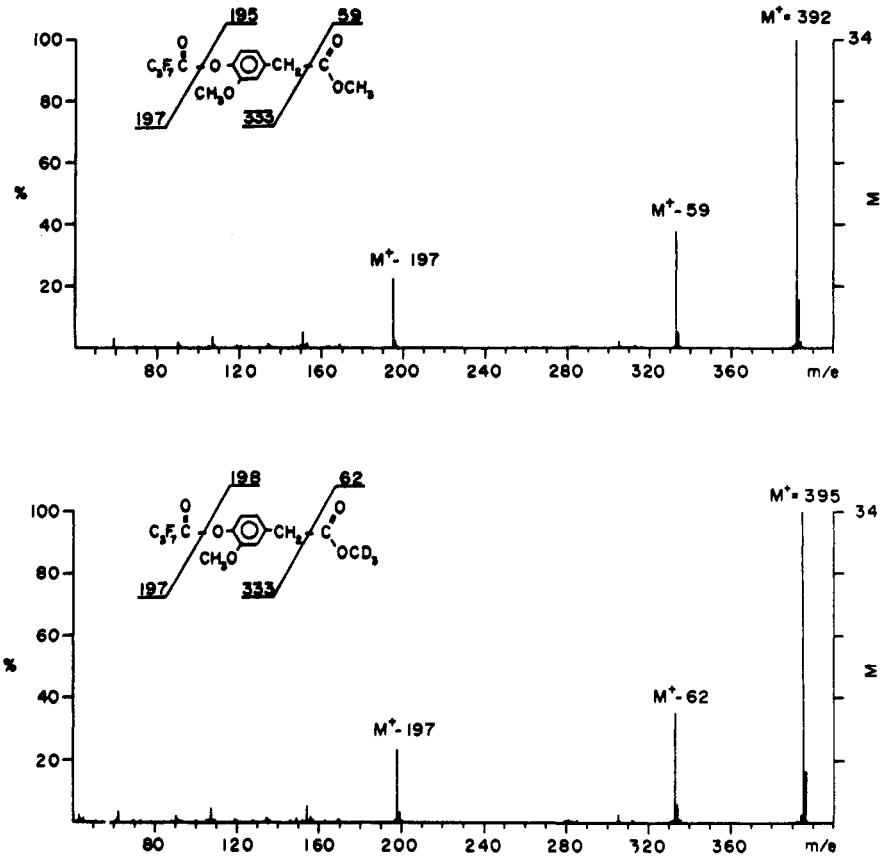

Figure 1. Mass spectra for heptafluorobutyryl derivative of the protium (top) and the deuterium (bottom) methyl ester of HVA

the methyl ester HFB-derivative. Only one peak of radioactivity cochromatographing with the inactive compound was observed. The radiopurity was therefore assessed to be in excess of 99%.

Heptafluorobutyric anhydride (HFBA) obtained from Pierce Chemical Co., Rockford, Ill., was distilled before use. The purity of the ethyl acetate is critical for the method using EC detection. Ethylacetate, nanograde (Mallinckrodt Chemical Co., St. Louis, Mo.) proved superior to all other quality grades of ethyl acetate examined by us. Diazomethane was prepared in diethyl ether from *p*-toluenesulfonylmethyl nitrosoamide (Fluka AG, Buchs, S:t Gallen, Switzerland) using ethyleneglycolmonoethyl ether as solvent.

CSF was collected from diagnostic lumbar and ventricular punctures. After acidification to pH 2–3, the pooled CSF was stored at −20 °C.

GLC–ECD Analysis. An outline of the procedure is presented in Figure 2. To 2 ml of CSF was added 1.00 × 10^5 dpm (7.8 ng = 43 pmole) of ^{3}H-HVA and 0.50 μg of HVA-CH_2. The pH was adjusted to 7 and the CSF was extracted twice with 2 ml of ethyl acetate and once with 2 ml of toluene. These extracts were discarded. The pH of the aqueous residue was adjusted to 2–3 with a few drops of dilute HCl and extraction was continued with 2 ml of toluene (discarded). HVA and related aromatic acids were extracted with 2 ml of ethyl acetate repeated three times. The combined ethyl acetate phases were reduced to dryness under vacuo. All extractions were performed in glass stoppered acid washed test tubes with tapered ends.

The residue was dissolved in 0.2 ml of ethyl acetate and mixed. Diazomethane in ether was added, the contents were mixed, and the diazomethane was removed by a stream of nitrogen within 1 min. The dry residue was dissolved in 50 μl of ethyl acetate and 50 μl of heptafluorobutyric acid anhydride. After 30 min at room temperature, the reagent and solvent were removed in a fume hood.

The derivative of HVA was purified by chromatography on lipophilic Sephadex (*15*) using heptane as solvent. The column dimensions were 0.4 × 40 cm. Bed volume was 2.3 ml. The flow rate through the column was 0.1 ml/min. Fractions of 0.5 ml were collected and radioactivity was counted in aliquots. The radioactive fraction and 2 ml of effluent appearing before it were combined and evaporated to dryness under N_2 (Figure 3). The residue was dissolved in 200 μl of ethyl acetate and 2–4 μl were injected into the gas chromatograph equipped with a ^{3}H electron capture detector (Figure 4).

The amount of HVA present in the original sample was calculated from peak height ratios using a standard curve and a known amount of added internal standard.

GLC–MS Analysis. To 2.00 ml of CSF was added 0.50 × 10^5 dpm (3.9 ng = 22 pmole) of ^{3}H-labeled HVA. After mixing, the acids were extracted as described above. The recovery of ^{3}H-HVA was measured in each sample after addition of 27 nmoles of D_3-methyl-HVA. The mixture was treated with diazomethane and heptafluorobutyric anhydride as described above. The D_3-methyl-HVA was added at this stage to compensate if any methylation of phenolic hydroxyl groups would occur in spite of the short exposure to diazomethane. No ester interchange was found under these conditions. The derivatives were dissolved in 50 μl of ethyl acetate containing 2% heptafluorobutyric acid anhydride and 1 μl was injected into the GLC–MS instrument. The mass spectrometer was focused alternately on the molecular ion of the HVA-derivative at *m/e* value of 392 (see Figure 1), and on the deuterated methyl ester HFB-derivative at 395, using the multiple ion detector. The intensity of these ions formed by the electron impact on the gas chromatographic effluent was continuously recorded. The peak height

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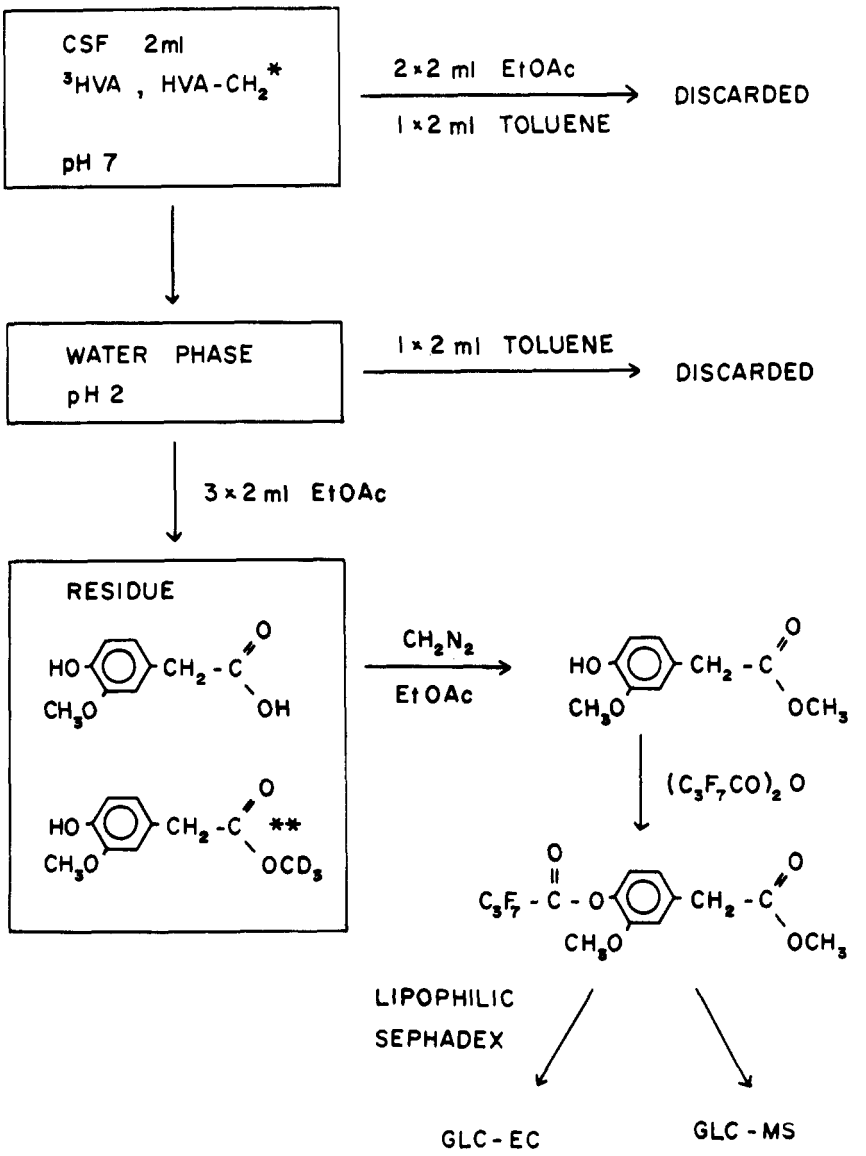

Figure 2. A flow diagram showing the methods for determination of HVA in human CSF

X denotes internal standard used in the GLC–EC method. XX denotes the internal standard used in the GLC–MS method

ratios of the protium (392) and the deuterium (395) forms of the HVA-derivative were measured (Figure 5). The background ratio consisting of the contribution of protium from the deuterium-labeled and the tritium-labeled internal standards, 0.25 and 0.25%, respectively, was subtracted from this value. The background ratio was obtained by running samples of artificial CSF (*16*) containing the same amount of the internal standards through the procedure. The artificial CSF contained $NaH_2PO_4 \cdot 2H_2O$ (78 mg), $Na_2HPO_4 \cdot 2H_2O$ (45 mg), $MgCl_2 \cdot 6H_2O$ (81 mg), $CaCl_2$ (72 mg), KCl (222 mg), NaCl (7.19 g), $NaHCO_3$ (2.10 g), and human serum albumin (250 mg) per liter. The amount of HVA present in the ethyl acetate extract was obtained by entering the corrected protium–deuterium ratio into a standard curve constructed from known varying amounts of protium-HVA derivative in the presence of a fixed amount of deuterium-HVA derivative (see Figure 6). The amount of HVA present in the original sample was obtained by correcting for recoveries in the extraction step.

RESULTS

Analysis Using Electron Capture Detection. The principle of the analysis is based on the addition of HVA-CH_2 as an internal standard to the CSF sample, extraction and purification on lipophilic Sephadex, and finally quantitation by the GLC–ECD (electron capture detection) method. The purification of the HVA-Me-HFB derivative on lipophilic Sephadex proved to be an important step since it removed a great deal of background signal generated by other endogenous compounds and solvent impurities from the reactions with diazomethane and HFBA. A chromatogram of HVA-Me-HFB and HVA-CH_2-Me-HFB on lipophilic Sephadex is shown in Figure 3.

The precision of the method was evaluated by repetitive analysis of a pool of human CSF. The mean of 10 determinations was 0.46 nmole of HVA/ml of CSF with a standard deviation of ±7%. The recovery of 1.04 nmole of HVA added to 2 ml of CSF containing 0.35 nmole/ml was 95 ± 13% (n = 6).

The sensitivity was sufficiently high to permit analysis of HVA present in 2 ml of CSF. The GLC response from a sample containing 0.46 nmole/ml is shown in Figure 4. It is possible to increase sensitivity further by concentrating the final extract from 200 μl to 50 or 100 μl before the GLC analysis. This would permit the measurement in smaller volumes of CSF.

The specificity of the method was investigated using the same CSF pool with both the GLC–ECD and the GLC–MS

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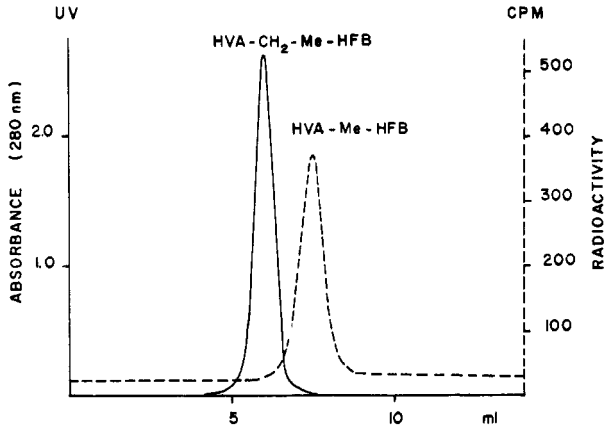

Figure 3. A column chromatogram showing the eluation of the derivatives made of the internal standard HVA-CH_2 and of HVA from the lipophilic Sephadex column

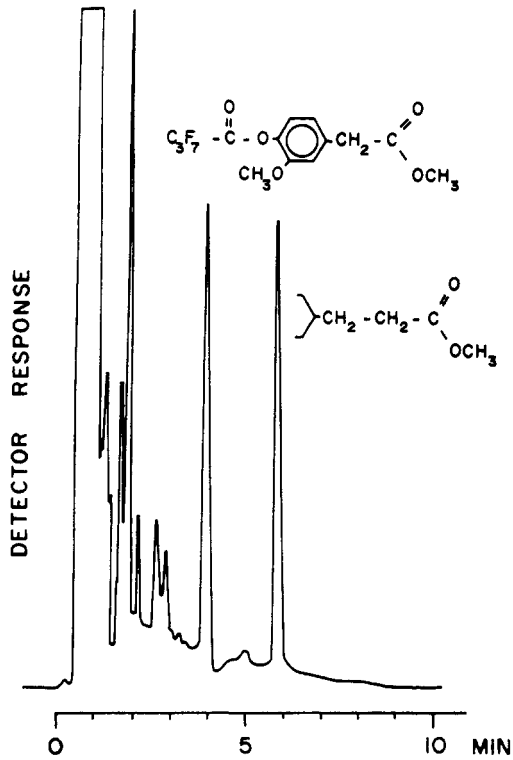

Figure 4. Gas–liquid chromatographic analysis of HVA in human CSF with electron capture detection, column 1% OV-1, temperature 130 °C. The amount of HVA in this sample was 0.46 nmole/ml

method described below. Using the latter method, a value of 0.48 nmole/ml ±10% (n = 17) was obtained on the CSF pool which gave a value of 0.46 nmole/ml ±7% by the GLC–ECD method. The good agreement between these inherently highly specific methods indicates that the responses recorded are due entirely to HVA.

Analysis Using Mass Spectrometric Detection. The principles of the method as outlined by Figure 2 involve the use of ^{3}H-labeled HVA to correct for recovery in the extraction step and the use of D_3-methyl-HVA as an internal standard in quantitative analysis by the mass spectrometer.

The precision was evaluated using repetitive analysis of a CSF pool. The mean value was 0.48 nmole/ml from 17

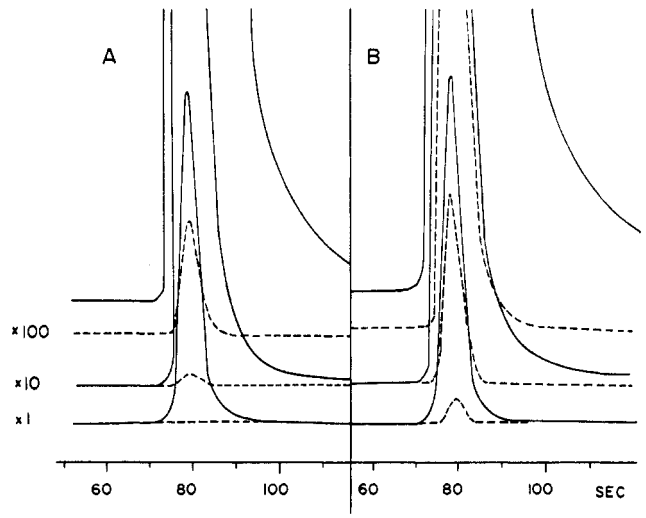

Figure 5. Mass fragmentograms obtained from the heptafluorobutyryl derivative of: *A*, the deuterated methyl ester of HVA that contained 0.3% of the protium form; *B*, a CSF sample containing 0.48 nmole/ml of HVA. The mass spectrometer was set to detect the molecular ions at *m/e* 392 (broken line) and *m/e* 395 (solid line)

analyses. The standard deviation was ±10%. The somewhat lower precision of the GLC–MS method as compared to the GLC–ECD method is most likely due to difficulties in attaining accurate focusing. It proved to be important to readjust the focusing by means of magnetic current after every fifth injection. Hopefully we will utilize a computer programmed focusing to minimize this error in the future. The recovery of 0.62 nmole of HVA ml added to the CSF pool was found to be 105 ± 2% (n = 4).

The sensitivity of the GC–MS method is very high and not fully utilized in the CSF analysis. Theoretically, the sensitivity-limiting factor is the amount of protium in the deuterium-labeled and tritium-labeled internal standards and the contribution of endogenous material to intensity at *m/e* value 392. The latter factor proved to be negligible under our experimental conditions. In the present method about 1 μl of a 50-μl sample was injected, representing about 0.5 nmole of the HVA-CD_3. With a protium contribution of 0.005 in the internal standards, the lower limit of measurement should be two times the background intensity. Figure 5 shows mass fragmentograms of the internal standard alone, as well as together with a CSF sample containing 0.48 nmole/ml.

DISCUSSION

The present methods for measuring HVA afford several advantages over the fluorimetric. The sensitivity is greatly improved, making the collection of large CSF samples unnecessary.

Second, the specificity is greater. Preliminary experiments suggest that the HVA-levels obtained with our methods are higher than those obtained by fluorimetry. Although the reasons for this difference are not clear, it seems that the oxidative coupling reaction used in the generation of the fluorophore (*17*) may not be quantitative at normal levels of HVA. For example, *p*-hydroxy-phenyllactic acid, which occurs abun-

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dantly in normal CSF (*18*) may couple with HVA and thus give lower yields of the dimer of HVA.

Analysis of HVA by GLC with ECD has the necessary precision, specificity, and sensitivity for analysis of 1–2 ml samples of CSF. The method requires considerable care in its execution, and the solvent purity is critical. An experienced technician can analyze eight samples in two days. The cost of the instrumentation is also within reach of most analytical laboratories.

The mass spectrometric measurement of HVA has many attractive features, especially the unparalleled sensitivity and specificity. The specificity of the method rests on the unique capacity of the mass fragmentographic technique to distinguish between different molecules. Of the variety of endogenous compounds which may be present in the biological sample, only those which have the same retention time and generate fragments at the same *m/e* value as the HVA derivative would interfere with the analysis. It should be noted that our choice of derivative (HFB) confers a high specificity since its molecular ion is the base peak and also occurs at a relatively high *m/e* value, where background contributions would be expected to be low. In the CSF samples, essentially no peaks other than that due to HVA-ME-HFB were observed. A considerable advantage of the GLC-MS method is also its rapidity. In our laboratory, we routinely process 50 samples in two days. The first day is used for extractions and preparation of derivatives. The second is required for the mass spectrometric analysis.

HVA is the end product of dopamine metabolism. This amine is assumed to play an important role in neurological disorders such as parkinsonism (*19*) and Guy de la Tourettes syndrome (*20*). More recent evidence also suggests that the dopaminergic system in the brain mediates affective qualities of the mind such a mood elevation after amphetamine (*21*) and fear (*22*). The availability of new sensitive and specific

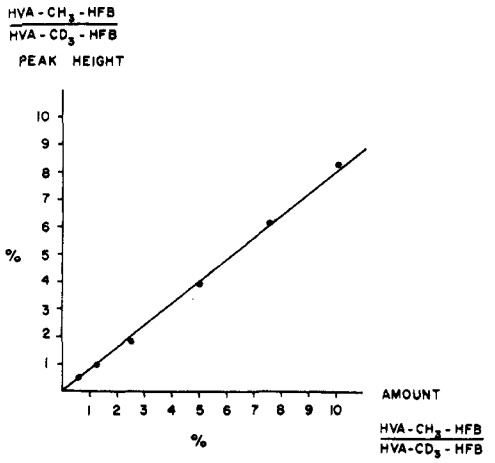

Figure 6. Standard curve for the GLC–MS method. The injected samples containing 0.25–5.0 nmole HVA-CH_3-HFB/ml and 50 nmole HVA-CD_3-HFB/ml. Peak height ratios ranging from 1–10% correspond to 0.2–2.1 nmole/ml HVA in the original CSF sample

methods for the analysis of HVA in human CSF, blood (*23*), urine (*24*), and tissues will aid further research into the role of dopamine in man.

ACKNOWLEDGMENT

We appreciate the help of Olle Marions at Södersjukhuset, Stockholm, for supplying us with CSF samples. The authors are indebted to Berit Holmberg for skillful technical assistance.

RECEIVED for review April 17, 1972. Accepted July 17, 1972. This study was supported by grants from the Delegation for Applied Medical Defense Research No 452, Swedish Medical Research Council No B72-40X-3560-01 and B72-14X-2211-06C.

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