Biochemical Aids in the Diagnosis of Parkinson's Disease

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ABSTRACT

Degeneration of dopamine (DA) containing neurons in the basal ganglia of Parkinsonian patients causes a reduction in cerebrospinal fluid (CSF) concentrations of the principal metabolite of dopamine, homovanillic acid (HVA). Not only are steady state HVA levels depressed, but there is substantial diminution in the rate of HVA accumulation in CSF during the oral administration of probenecid, a drug which inhibits HVA transport from the spinal fluid compartment. Two methods for the measurement of HVA in human CSF, employing fluorometry or gas liquid chromatography—mass spectroscopy, are described and the results compared in patients with Parkinsonism as well as other central nervous system disorders.

Introduction

Melanin containing neurons with cell bodies in the substantia nigra and axonal terminals in the corpus striatum characteristically degenerate in patients with Parkinson's disease. Dopamine occurs in high concentrations within these cells and probably serves as their neurotransmitter. As a consequence of the neuronal loss, brain levels of both DA and of its principal metabolite, HVA, are substantially reduced.

During the past decade, HVA levels in CSF have been widely used as an index of the functional state of central dopaminergic systems in man. The rationale for this usage derives from preclinical observations suggesting that HVA in CSF arises largely from central rather than peripheral metabolism, and that levels of HVA in the CSF pathways tend to correlate with those in the cerebral tissues. Recently, attempts to study the dynamic state of central DA metabolism have made use of CSF HVA measurements during probenecid treatment. Probenecid inhibits the active transport of HVA from the CSF compartment without significantly altering central DA levels or the diffusion of HVA from the cerebral parenchyma into CSF. Accordingly, the rate of HVA accumulation in CSF should reflect the rate of formation of this metabolite and thus the central turnover of the parent amine. Although the foregoing assumptions are not amenable to direct verification in man, it is known that drugs which augment DA formation enhance the probenecid-induced accumulation of HVA, while pharmaco-
logical agents which diminish DA synthesis depress the HVA rise induced by probenecid. It would thus appear that an aid to the biochemical diagnosis of Parkinson's disease might be based on the determination of HVA levels in CSF. Two techniques involving fluorometry or gas liquid chromatography-mass spectroscopy (GLC-MS) are currently in use for the measurement of HVA in human CSF.

**Fluorometric Method**

**Principle**

The CSF specimens are deproteinized with ZnSO₄ and NaOH. After centrifugation, the supernatant is acidified and the acid metabolites are extracted in EtAc. The metabolites are next back extracted into NaPO₄ buffer, pH 8.5, and oxidation is performed on one ml aliquots.

**Reagents and Solutions**

**PREPARED REAGENTS**

**Ascorbic Acid (0.2 percent).** Two hundred mg of ascorbic acid (reagent grade A.A.) are dissolved in 100 ml of distilled water.

**Zinc Sulfate (10 percent).** One hundred g of ZnSO₄ (ACS) are dissolved in distilled water and the volume made up to one liter. This solution can be kept for months.

**Sodium Hydroxide (10 percent).** One hundred g of NaOH (ACS) are dissolved in distilled water and the volume made up to one liter.

**Hydrochloric Acid (1 N).** Exactly 83.3 ml of concentrated HCl (ACS) are diluted to one liter with distilled water.

**Phosphate Buffer (0.5 M) pH 8.5.** Solution A: NaH₂PO₄ - H₂O (3.45 g) is dissolved in distilled water and the volume made up to 50 ml. Solution B: Na₂HPO₄ (7.098 g) is dissolved in distilled water and the volume made up to 100 ml. Solution A (5.1 ml) is mixed with solution B (100 ml). This buffer is made up immediately before use.

**Ammonium Hydroxide (1 N).** Precisely 135.1 ml of NH₄OH (ACS) are diluted to one liter with distilled water.

**Potassium Ferricyanide (0.01 percent).** Ten mg of K₃Fe₃(CN)₆ (ACS) are dissolved in 100 ml distilled water. This solution is made up fresh each time.

**Cysteine (1 percent).** One hundred mg of reagent grade cysteine are dissolved in 10 ml distilled water. This reagent is made up just before use.

**Sodium Chloride. Ethyl Acetate.** Spectroanalyzed grade.

**Standard Solutions**

**HVA Standard (1 mg per ml).** Chemically pure HVA (10 mg) is dissolved in 10 ml distilled water.

**Working HVA Standards.** Dilutions of the stock standard are prepared in concentrations of 1, 2, 3, 5, 10, 20 μg per ml, e.g. HVA (20 mg per ml) is made by diluting 200 μl of stock solution to 10 ml with distilled H₂O. One hundred μl of each solution is used to determine the standard curve.

**Special Apparatus**

A spectrophotofluorometer is used.

**Procedure**

The method is based on that of Anden, Roos and Werdinius as modified by Gerbode and Bowers. Ten ml CSF specimens are collected by lumbar puncture in tubes containing 20 mg ascorbic acid and immediately frozen to −20°C until assayed.

Six ml aliquots are deproteinized by adding 1.5 ml of 10 percent ZnSO₄ and 0.3 ml of 10 percent NaOH. This is centrifuged at 4°C for 20 minutes at 15,000 rpm. The supernatants are poured into tubes con-

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* Aminco Bowman, American Instrument Co, Inc, Silver Spring MD 20910
taining 2 ml of 1 N HCl, 6 g NaCl and 10 ml of EtAc. The samples are shaken vigorously for 15 minutes and then centrifuged at 2,500 rpm for one minute. Nine ml aliquots of the organic phase containing the acid metabolites are shaken with 2.3 ml of 0.5 M Na-P04 buffer, pH 8.5. After centrifugation, the EtAc fraction is removed by aspiration and one ml aliquots of the aqueous phase are taken for both sample and blank oxidation.

Precisely 0.2 ml of NH4OH is added to both samples and blanks. Oxidation is performed on the samples by adding 0.05 ml of K3Fe(CN)6. Two minutes later, the reaction is stopped with the addition of 0.05 ml cysteine. Blanks are treated in the same manner, but cysteine is added before the oxidation step. After 10 minutes, both sample and blank are read in the fluorometer at activation and fluorescence wavelengths of 320 nm and 420 nm, respectively. The fluorescent product remains stable for several hours.

A standard curve is determined by assaying along with CSF specimens a set of duplicate samples for each concentration of authentic HVA made up in 6 ml of 0.2 percent ascorbic acid.

Calculation

Levels of HVA present in CSF specimens can be obtained from the standard curve.

Discussion

The standard curve is non-linear for HVA values below about 30 ng per ml. Despite the use of scrupulously clean glassware and deionized or quartz double-distilled water, residual impurities may interfere with the fluorometric assay at the lower limits of its sensitivity. In addition, certain acid metabolites have been found which can be dimerized with HVA to form nonfluorescent compounds. The resulting quench would largely influence fluorescent readings at the lower concentrations and thus explain the observed non-linearity below 30 ng per ml.

Gas Liquid Chromatography-Mass Spectroscopy

Principle

An internal standard of deuterated (D) HVA is added to all samples to establish recovery. Four additional samples are made up with (D) HVA. Two of the four are needed to determine the amount of protium (H) HVA present as a contaminant in the deuterated standard. By adding 100 ng (H) HVA to the remaining two tubes, it is possible to quantify the amount of (D) internal standard.

All samples are initially passed through an alumina column to remove catechol contaminants, e.g. dihydroxyphenylacetic acid. The acid metabolites are then extracted into EtAc and dried down. They are next reacted with diazomethane, followed by trifluoroacetic anhydride, to produce a volatile derivative. The resulting trifluoroacetates of the methyl ester of HVA are separated from contaminants by GLC and quantified by MS.

Reagents and Solutions

PREPARED REAGENTS

Diazomethane (4.4 x 10^-3 M). The concentration of diazomethane* is determined by reacting a two ml aliquot with a known amount of benzoic acid in ether until the solution is colorless and an excess amount of acid is present. The excess benzoic acid is titrated with 0.1 N NaOH. The remaining diazomethane solution is then diluted to 4.4 x 10^-3 M and stored at 0°.

Aluminum Oxide, Woelm Neutral, Activity Grade 1. Two liters of 2 N HCl are added to 500 g alumina, stirred constantly and heated to 90° to 100° for 45 minutes. After the alumina is allowed to settle for 10 minutes, the supernatant and fines are decanted. The procedure is repeated twice with heating at 70° for 15 minutes.

* Diazald, Aldrich Chemical, No. D2, 800-0.
Precipitate is washed (15 to 20 times) with distilled water until the supernatant is approximately pH 3.4. The precipitate is transferred into a large evaporating dish and allowed to dry overnight at 250° to 300°. The alumina is stored in a warm oven.

Trifluoroacetic Anhydride. This should be of the highest purity.

STANDARD SOLUTION

(D) HVA Standard. This solution is purchased commercially. *

Special Apparatus

A LKB-9000 GSC-MS equipped with an alternating voltage accelerator for multiple ion monitoring is used. The class column, 6 ft x 2 mm i.d., is filled with 3 percent OV-17 on 100 to 200 mesh Supelcoport. † The column temperature should be 150°, with the flow rate for the carrier gas (helium) 15 ml per min, and the electron energy 70 eV.

Procedure

The CSF is collected and stored as described in the fluorometric procedure. Specimens of 0.3 to 3.0 ml are brought to a total volume of 5 ml with distilled water. In addition, four tubes containing 5 ml 0.2 percent ascorbic acid are prepared. One hundred ng (H) HVA are added to two of the four tubes. To all samples 0.1 ml 5 percent EDTA and 10 to 25 ng (D) HVA in 0.1 ml H2O are added.

Samples are adjusted to pH 8.5 with 2 ml 2 M Tris buffer, pH 8.5, before loading onto alumina columns. Organic acids are washed off with 2 ml of 0.2 M Tris buffer, pH 8.5, followed by 3 ml H2O. The combined effluent and wash are acidified to pH 1 with HCl, saturated with NaCl and extracted by shaking 10 minutes with 25 ml EtAc. After centrifugation, the organic phase is transferred to a tube containing anhydrous Na2SO4 and shaken to remove any water present. Following centrifugation, EtAc is removed and evaporated to dryness in vacuo. The residue is redissolved in about 5 ml methanol and again evaporated in vacuo until no odor of acetic acid can be detected. The final residue is dissolved in 1 ml EtAc and transferred to a glass-stoppered tube where it is cooled in dry ice and acetone to −80°.

Both (H) and (D) HVA are converted to their methyl esters by the addition of 0.1 ml of diazomethane. The solvent and excess diazomethane are then evaporated in a stream of N2. The resulting products are redissolved in 0.5 ml EtAc and reacted with 50 µl trifluoroacetic anhydride at room temperature. After one hr, the mixture is evaporated under N2 or in a vacuum desiccator. The residue is redissolved in 25 to 100 µl EtAc and 2 to 4 µl of this mixture are injected into the GLC-MS system. The retention time for the (D) and (H) HVA derivatives is three min 50 sec.

With the use of the multiple ion detector, the magnetic field is held constant at 292 m/e for the (H) form of the methyl ester trifluoro derivative of HVA. To focus on the (D) form at 295 m/e the accelerating voltage is alternated by 1.027 percent. From the resulting tracings of peak heights, it is possible to calculate the amount of endogenous (H) (HVA) present.

Calculation

With each series of assays, the undiluted (D) standard is examined for contamination by (H) HVA. Although the amount of the (H) form is relatively small (usually about 5 percent), this correction is necessary when low endogenous HVA levels are to be measured. Two samples containing (D) standard plus a known amount of (H) HVA are assayed in each series since very dilute HVA solutions tend to deteriorate even when frozen.

After the amount of (D) HVA added is determined, the amount of endogenous (H)
HVA can be calculated using the (general) formula: (ng of (H) HVA in (D) standard + endogenous HVA)/ng of (D) HVA added = peak height (H) HVA/peak height (D) HVA.

Discussion

There is a linear relationship between the amount of (H) HVA and the (H)/(D) ratio found by the mass spectrometer. Sample and standard size should be chosen to give an (H)/(D) ratio in the range of 0.1 to 10. It is preferable to have the (D) form in lesser quantity to minimize the amount of (H) contamination introduced by the internal standard. Since each sample has its own internal standard, variations in recovery rate are automatically corrected. As little as one ng of (H) can be assayed when 10 to 20 ng of the (D) standard are used.

Catechols are retained on an alumina column to eliminate dihydroxyphenylacetic acid which would be converted to HVA by diazomethane. Conditions of the diazomethane reaction are controlled to minimize methylation of the 4-hydroxyl group of HVA. This group reacts with trifluoroacetic anhydride to form the derivative assayed.

Values of HVA obtained by GLC-MS average about 35 percent higher than those obtained fluorometrically (figure 1). This discrepancy might reflect the presence of contaminants which can combine with HVA to form nonfluorescent compounds. Another possibility is the loss of HVA when the CSF specimens are deprotenized with ZnSO₄ and NaOH.

GLC-MS is substantially more sensitive and specific than the fluorometric method, thus requiring less CSF for analysis. The procedures involved are reasonably simple, allowing a relatively large number of samples to be assayed at one time. The sensitivity of the GLC-MS method is limited by the amount of (H) present in the (D) internal standard, which would increase the apparent endogenous HVA concentration. It is possible to detect 1 ng of HVA in CSF by mass fragmentography but only 30 ng per ml by fluorometry. In the fluorometric procedure concentrations from about 10 to 30 ng per ml can only be estimated from the lower portion of the standard curve through the use of a linear approximation.

Sources of Error

The specificity of the GLC-MS method, inherently higher than that of the fluorometric method, derives from the characteristic retention time on the GLC column and the formation of a specific molecular fragment. Only compounds having the same retention time as HVA as well as the same fragment m/e of 292 or 295 interfere with the analysis.

The 3-hydroxy-4-methoxyphenylacetic acid (iso-HVA) levels ranging up to 25 percent of total HVA concentrations are found in the CSF of non-parkinsonian patients. Since iso-HVA is not separated from HVA in the fluorometric procedure, it can react with HVA and interfere with fluorescent development. The methyl ester trifluoro derivatives of HVA and iso-HVA have different retention times, thus allowing their separation by GLC.

Spurious HVA values may also be ob-
tained from patients receiving drugs which affect DA metabolism. For example, L-dopa alters CSF levels of several catecholamine metabolites in addition to HVA. Some of these metabolites, such as dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid and 3-methoxy-4-hydroxymandelic acid, are known to lower HVA fluorescence possibly by forming nonfluorescent dimers with HVA. Dihydroxyphenylacetic acid can also react with diazomethane to yield authentic HVA and therefore must initially be removed by alumina. Moreover, O-methyl dopa and vanillacetic acid, which are known to effect HVA fluorescence, can be separated from HVA by GLC.

Normal Range

HVA levels in the lumbar spinal fluid of normal adults as determined by the fluorometric method usually range between 20 to 52 ng per ml. As already noted, values by the GLC-MS method tend to be somewhat higher.

Résumé of Clinical Interpretations

Both steady state levels (table I) and probenecid-induced accumulations of HVA (table II) are substantially reduced in the lumbar CSF of parkinsonian patients. Although it has been difficult to document any consistent relationship between clinical se-

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\begin{array}{|c|c|c|}
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\text{Number of Patients} & \text{HVA} \\
\hline
\text{Controls} & 25 & 36 ± 4.0 \\
\text{Parkinson's disease} & 30 & 10 ± 1.2* \\
\text{Parkinsonism-dementia} & 15 & 9 ± 2.7* \\
\text{Huntington's chorea} & 14 & 16 ± 3.3* \\
\text{Dystonia muscularum deformans} & 15 & 20 ± 3.3* \\
\text{Down's syndrome} & 9 & 56 ± 11.0 \\
\text{Amyotrophic lateral sclerosis} & 21 & 1.3 ± 2.4* \\
\hline
\end{array}
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*Values are the means ± SEM, expressed in ng per ml for untreated patients. 
$^*p < 0.001$. 
$^*$p < 0.01.

verity and reductions in steady state HVA values, a statistically significant inverse relationship has been demonstrated between the severity of parkinsonian akinesia or rigidity and the probenecid-induced accumulation of HVA in lumbar CSF.

Not all patients manifesting parkinsonian signs have abnormally depressed HVA levels and conversely some clinically normal individuals have very low values for the DA metabolite. The degree to which these observations reflect biological or methodological factors is unknown. Moreover, a marked depression in CSF content of HVA is not specific for Parkinson's disease. As may be seen in tables I and II, several other extrapyramidal disorders have similar findings.

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\begin{array}{|c|c|c|c|}
\hline
\text{Number of Patients} & \text{Baseline} & \text{Treatment} & \text{Difference} \\
\hline
\text{Controls} & 8 & 28 ± 6.8 & 194 ± 24 & 166 ± 24 \\
\text{Parkinson's disease} & 20 & 12 ± 1.7 & 73 ± 12 & 62 ± 11* \\
\text{Huntington's chorea} & 12 & 14 ± 5.0 & 115 ± 14 & 100 ± 14§ \\
\text{Dystonia muscularum deformans} & 8 & 14 ± 4.9 & 168 ± 22 & 154 ± 19 \\
\text{Amyotrophic lateral sclerosis} & 7 & 23 ± 7.7 & 129 ± 22 & 104 ± 16§ \\
\hline
\end{array}
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*Values are the means ± SEM expressed in ng per ml. Probenecid (2 g) was administered orally immediately after obtaining the baseline spinal fluid sample and again 3 and 6 hours later. The second lumbar puncture was performed 9 hours after the initial one. 
$^*p < 0.001$. 
$^*$p < 0.05.
References


