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Identification of *O*-Methyldopa in the Ventricular Fluid of Patients with Parkinson's Disease

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A newly developed liquid chromatographic system with a multiple electrode detector was used for quantification of neurochemical substances in ventricular fluid of patients with Parkinson's disease. During the analysis, an unknown peak was observed at almost the same high-performance liquid chromatography retention time as 5-hydroxytryptophan. Through the use of ion suppression techniques, voltammographic analysis and fast atom bombardment mass spectrometry, the compound was identified as *O*-methyldopa, the major metabolite of L-dopa.

INTRODUCTION

We have recently developed a computer-controlled liquid chromatographic system that uses multiple electrodes (abbreviated as LCMED) for simultaneous measurement of 20–30 different neurochemicals isolated from a single biological sample. The total analysis time is less than 30 min, with sensitivities in the range of 100–400 fmol.[1,2] Peak assignments are made on the basis of high-performance liquid chromatography (HPLC) retention time and electrochemical response at four different voltages in comparison with the results for authentic standards. While not affording the level of structural information available through mass spectrometry, LCMED has proved to be extremely valuable for the rapid and sensitive analysis of samples of biological origin.

In the course of a study involving analysis of multiple neurochemicals in ventricular fluid of patients with Parkinson's disease, we observed a prominent HPLC peak at essentially the same retention time as our standard 5-hydroxytryptophan (5-HTP). However, the relative responses of the four electrochemical detectors did not correspond to those from 5-HTP, indicating the presence of an unknown component. This report describes our use of continuous-flow fast atom bombardment liquid chromatography/mass spectrometry (CF FAB LC/MS) for identification of this substance as methoxyhydroxyphenylalanine (*O*-methyldopa, OMD).

Materials and methods

Ventricular cerebrospinal fluid (V-CSF). Samples (approximately 2 ml) from patients with either Parkinson's disease or essential tremor, obtained from the Department of Neurosurgery, Gunma University, Japan, were collected from the lateral ventricle by means of ventricular puncture during stereotactic selective thalamotomy, using xylocaine for local anaesthesia.[3] The CSF samples were immediately centrifuged at 1000 × *g* at 4 °C for 10 min and stored at −85 °C until analyzed.

Reagents. 3-Methoxy-4-hydroxyphenylalanine (3-*O*-methyldopa monohydrate, 3-OMD) was obtained from Dr. LeRoy Blank, Department of Chemistry and Biochemistry, University of Oklahoma (Norman, Oklahoma, USA). Other standard neurochemicals were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). All reagents for extraction and chromatography were of the highest purity available from commercial sources.

Sample preparation. One-millilitre aliquots of V-CSF were mixed with 0.1 ml of 0.8 N perchloric acid and passed through a 0.45 μm Millipore filter. The resulting filtrate was used for analysis without any subsequent purification.

HPLC system. The HPLC system with multiple electrochemical detectors (NEUBA®, Great Plains Laboratories Inc., Norman, Oklahoma, USA) used in this investigation for analysis of neurochemicals has been described in detail in previous reports.[1,2] The mobile phase for the analyses presented below was comprised of 0.1 M citrate buffer (pH 2.95) containing 0.05 mM ethylenediaminetetraacetic acid disodium salt, 0.344 mM sodium octylsulphate, 0.085% diethylamine and 0.5% acetonitrile, at a flow rate of 0.7 ml min^{-1}. HPLC separation was accomplished by means of a BAS PHASE II reversed-phase column (ODS, 100 × 3.2 mm, 3 μm; Bioanalytical Systems Inc., West Lafayette, Indiana, USA). The column temperature was maintained at 35 ± 1.5 °C by a BAS LC-22A column heater. The detector consisted of four glassy carbon electrodes arranged in series, with potentials (relative to a Ag/AgCl reference electrode) set at +0.5, +0.6, +0.7 and +0.8 V, respectively, starting with the electrode closest to the high-performance liquid chromatograph. The potentials of these working electrodes were regulated to within ±0.2 V by a single potentiostat unit. This potentiostat

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was also used for amplification of the current from each electrode. After conversion to voltage signals, a Macintosh II microcomputer was employed for data collection and subsequent data analysis. By means of a separate interface, this computer also controlled the autoinjector, allowing automated operation of the entire system.

Voltammographic analysis. Hydrodynamic voltammograms for each compound were acquired by means of the HPLC system described above. The applied potentials ranged from +0.4 V to +1.1 V; intervals of +0.1 V were employed.

CF FAB LC/MS. Mass spectral analysis of the unknown substance isolated from the V-CSF of a patient with Parkinson's disease was accomplished as previously described.[4,5] Basically, the system comprised a high-performance liquid chromatography (LC100 gradient system, Yokogawa Co Ltd, Tokyo, Japan) interfaced via a CF-FAB probe (FRIT-FAB, JEOL Ltd, Tokyo, Japan) to a JEOL model JMS-AX505W mass spectrometer in combination with a JEOL model JMS-OA 5000 data system. Mass spectrometric conditions were as follows: ion source temperature, 50 °C; accelerating voltage, 3 kV; conversion dynode voltage, −10 kV; electron multiplier voltage, 1.2 kV; resolution, 1000. A JEOL atom gun was used with xenon (5 keV) for FAB from the surface of the flow FAB probe. As is customary for CF FAB mass spectrometry, a small amount of glycerol (1%) was added to the HPLC mobile phase.

RESULTS AND DISCUSSION

The results for HPLC separation and detection by the four-electrode system for standard neurochemicals (a), components isolated from V-CSF of a patient with Parkinson's disease (b) and V-CSF from a patient with essential tremor (c) are shown in Fig. 1. An internal standard, 3,4-dihydroxybenzylamine (DHBA), was included in each sample. Peak assignments were made on the basis of HPLC retention time and agreement of the four-electrode response in comparison with standard neurochemicals. As can be seen from Fig. 1(b) and (c), the V-CSF of the patient with Parkinson's disease contained four components in common with the V-CSF of the patient with essential tremor. 3,4-Dihydroxyphenylalanine (DOPA) and dopamine (DA) were only detected in the former sample. Assignment of the peaks for these substances was readily accomplished by comparing their HPLC retention times and amperometric response characteristics with those of the standards illustrated in Fig. 1(a). The peak eluting at approximately 13.5 min (b), however, could not be identified on this basis. Although this retention time was essentially the same as that of 5-HTP, the relative responses of the four electrodes were clearly different (Table 1).

In order to obtain more information about the nature of the unknown substance, its HPLC elution behaviour and ion suppression characteristics[6] over a narrow range of mobile phase pH values (2.3–2.95) were compared with other neurochemicals. The results in Fig. 2

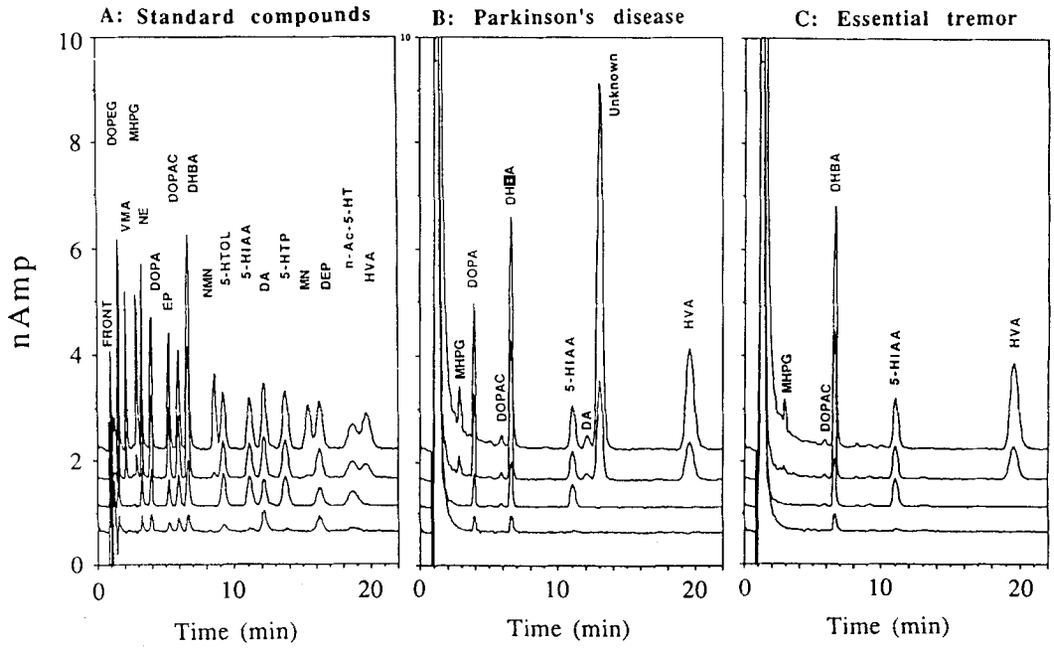

Figure 1. HPLC chromatograms with detection by four serial electrodes: (a) standard neurochemically relevant compounds; (b) components isolated from the V-CSF of a patient with Parkinson's disease; (c) components isolated from the V-CSF of a patient with essential tremor. Abbreviations: DOPEG, 3,4-dihydroxyphenylglycol; VMA, vanillylmandelic acid: MHPG, 3-methoxy-4-hydroxyphenylglycol; NE, norepinephrine (noradrenaline); DOPA, 3,4-dihydroxyphenylalanine; EP, epinephrine (adrenaline); DOPAC, 3,4-dihydroxyphenylacetic acid; DHBA, 3,4-dihydroxybenzylamine; NMN, normetanephrine; 5-HTOL, 5-hydroxytryptophol; 5-HIAA, 5-hydroxyindoleacetic acid; DA, dopamine; 5-HTP, 5-hydroxytryptophan; MN, metanephrine; DEP, deoxyepinephrine; nAC-5-HT, *N*-acetyl-5-hydroxytryptamine; HVA, homovanillic acid.

Table 1. Electrochemical response observed during LCMED analysis of 5-HTP and an unknown compound isolated from the V-CSF of a patient with Parkinson's disease

Compound	RT[b] (min)	Relative response[a] E1 (+0.5 V)	E2 (+0.6 V)	E3 (+0.7 V)	E4 (+0.8 V)
5-HTP	13.8	3.9 ± 0.2	52.2 ± 1.6	66.4 ± 1.4	100
Component in V-CSF	13.5	0	0	28.3 ± 0.5	100

[a] Values represent the mean ● SD for 3 separate determinations. For each analysis, the response at E4 was set to be 100%.
[b] HPLC conditions are described in 'Methods'.

show that the retention of amines (DA; norepinephrine (noradrenaline) NE; tyramine, TYM), acids (homovanillic acid, HVA; 3,4-dihydroxyphenylacetic acid, DOPAC), and neutrals (5-hydroxytryptophol, 5-HTOL; 3,4-dihydroxyphenylglycol, DOPEG) were either unaffected or minimally increased at slightly elevated pH, whereas amino acids (5-HTP; tyrosine, TYR; DOPA) tended to elute earlier at higher pH. The unknown substance in Parkinsonian V-CSF behaved essentially the same as 5-HTP, indicating similarities between the unknown component and α-amino acids.

Voltammographic analysis of the unknown substance was undertaken as a means of identifying electrochemically active functional groups. Comparisons were made with the following types of standards: catechol (NE), vanil (HVA), indole (5-HTP) and phenol (TYR). From the results shown in Fig. 3, it can be seen that the voltammogram of the unknown substance was quite similar to that of the vanil standard HVA. Furthermore, the indole exhibited a characteristic di-sigmoidal curve, distinguishing it from other chemical groups.

In order to obtain structural information about the unknown substance, CF FAB LC MS analysis was performed. The resulting mass spectrum is shown in Fig. 4(a). From the 92 amu mass difference between the two most intense peaks in the spectrum, it was concluded that the base peak at m/z 212 was $[M + H]^+$ and the ion at m/z 304, $[M + H + \text{glycerol}]^+$.

Levodopa (L-DOPA, molecular weight 197) is widely used for therapy of Parkinson's disease;[7,8] this drug was present in the V-CSF of the patient sample as seen in

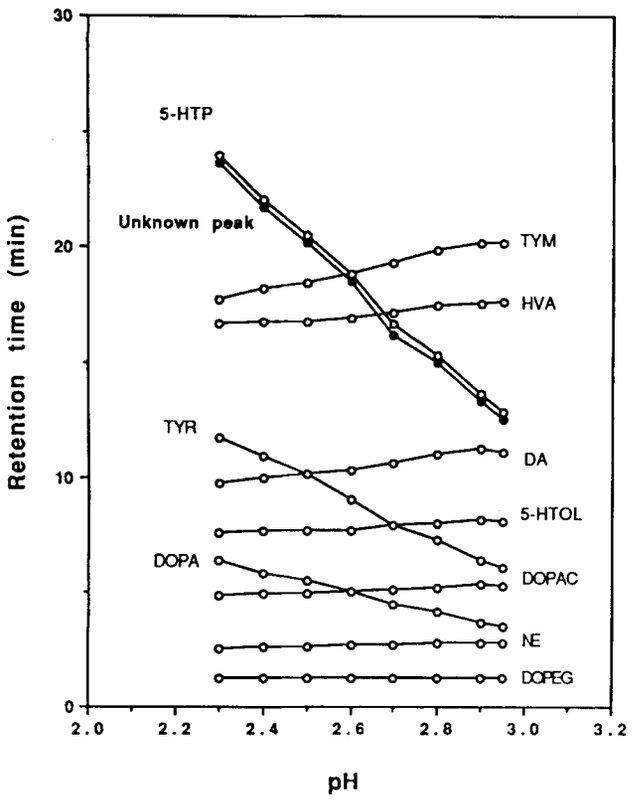

Figure 2. The effect of pH on the HPLC retention time of the unknown component isolated from the V-CSF of a patient with Parkinson's disease as compared to standards representing; amino acids (5-HTP, 5-hydroxytryptophan; TYR, tyrosine; DOPA, 3,4-dihydroxyphenylalanine), amines (TYM, tyramine; DA, dopamine; NE, norepinephrine (noradrenaline), acids (HVA, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid) and neutrals (5-HTOL, 5-hydroxytryptophol; DOPEG, 3-4-dihydroxyphenylglycol). HPLC conditions are described in 'Methods'.

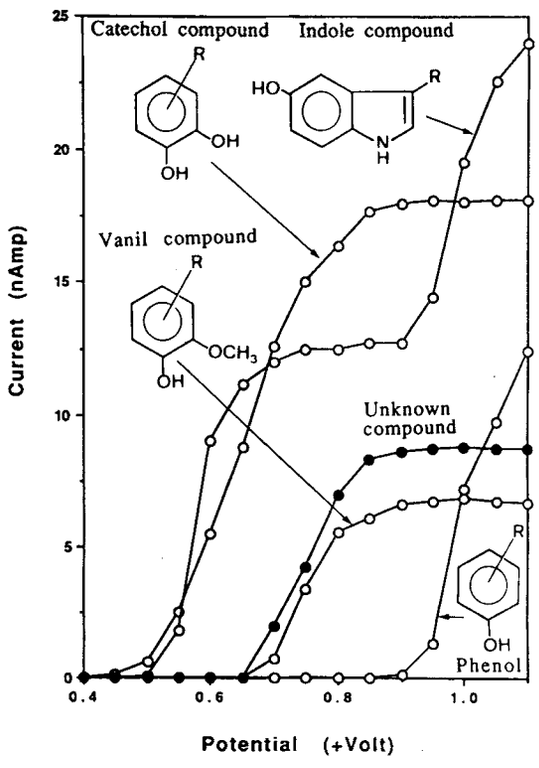

Figure 3. Voltammographic analysis of the unknown component isolated from the V-CSF of a patient with Parkinson's disease as compared to the following standards: catechol (NE, norepinephrine (noradrenaline)), vanil (HVA, homovanillic acid), phenol (TYR, tyrosine) and indole (5-HTP, 5-hydroxytryptophan). HPLC conditions are described in 'Methods'.

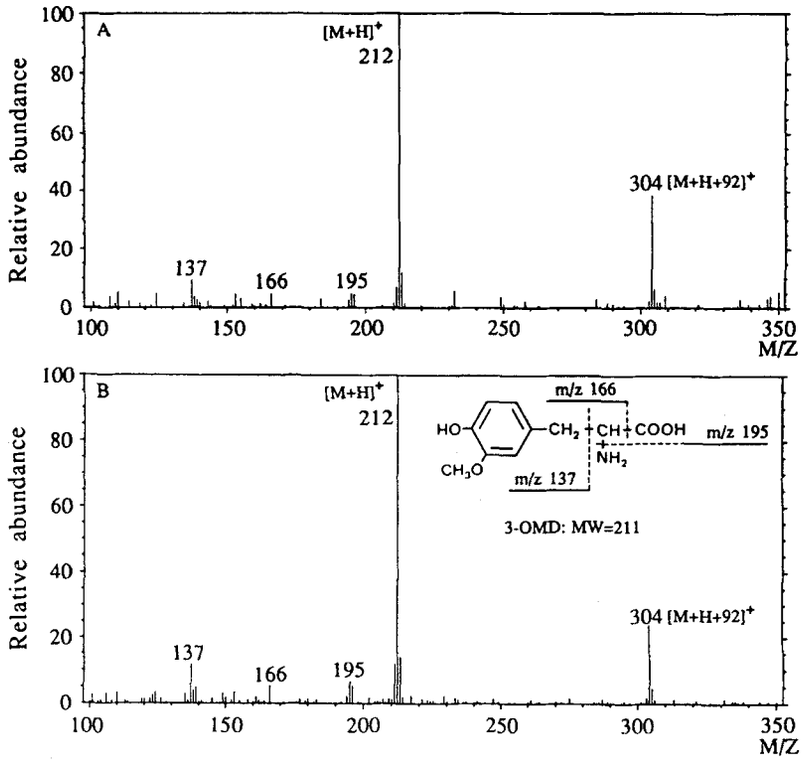

Figure 4. *CF FAB mass spectra: (a) the unknown component isolated from the V-CSF of a patient with Parkinson's disease; (b) standard 3-O-methyldopa (3-OMD). Mass spectral conditions are described in 'Methods'.*

the LCMED tracing shown in Fig. 1(b). In view of the fact that the unknown component had an apparent molecular weight of 211 and exhibited structural similarities to amino acids and vanil compounds, it was postulated that the unknown substance was *O*-methyldopa (OMD), the major metabolite of L-dopa.[9,10] In order to, confirm the identity of the unknown component, a CF FAB mass spectrum of an authentic standard of 3-OMD was recorded (Fig. 4(b)). Comparison of panels (a) and (b) in Fig. 4 show that the spectra are essentially identical. In addition, the HPLC retention times of the two analytes were the same. Proposed cleavages involved in the fragmentation are illustrated in Fig. 4(b), but full elucidation of the mechanism of their formation has not been undertaken. Additional corroboration that the unknown substance was OMD is afforded by the fact that the LCMED tracing of the unknown substance matched that of standard 3-OMD. From the information available in the present study it was not possible to deduce the location of the *O*-methyl group; however, the *O*-methyl substitution is most likely at the 3-position in view of the known metabolism of L-DOPA.[9,10]

Thus, the results of mass spectral and voltammographic analyses in combination with studies of HPLC retention behaviour clearly showed that the unknown compound isolated from the V-CSF of a patient with Parkinson's disease was OMD. We have now used LCMED to quantify the level of OMD in V-CSF from 12 patients with Parkinson's disease; we found 313.5 $\pm$ 103.8 ng ml^{-1} (mean $\pm$ SD) as compared to 1.5 $\pm$ 1.0 ng ml^{-1} in 7 control patients.[2]

During HPLC analysis of clinical samples, it is not uncommon for unknown peaks to be observed. Identification is often made by comparing the retention behaviour of the unknown component with authentic standards appropriate for the biological sample. However, this could lead to an incorrect assignment, and such a mis-identification could have critical clinical consequences. For example, as mentioned above, 3-OMD is known to be a major metabolite of L-DOPA used for therapy of Parkinson's disease. The *O*-methyl metabolite has a much longer biological half-life (12–13 h) than L-DOPA (30 min), and it has been speculated that 3-OMD might be a pharmacologically important precursor of dopamine.[10] Based on retention time alone, the unknown component isolated from V-CSF of the Parkinson's disease patient might have been identified as 5-HTP instead of 3-OMD. The results of the present study demonstrate the value of the four-series amperometric LC detection system as an aid to mass spectrometry for identification of neurochemically important compounds.

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