Citrimalic Acid in Cerebrospinal Fluid of Patients with Bacterial Meningitis

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Cerebrospinal fluid (CSF) from uninfected patients and from patients with bacterial and viral meningitis was analyzed by gas–liquid chromatography, with use of a flame ionization detector, and by gas chromatography–mass spectrometry. The resulting profiles were consistent and reproducible. Hydroxy acids were the compounds found in greatest abundance in both normal and infected CSF. Control experiments to establish the sensitivity and efficiency of the extraction and derivatization methods are also presented. Constituents of CSF from patients with bacterial meningitis differed quantitatively and qualitatively from those of CSF from uninfected patients or patients with nonbacterial infections. CSF from seven of eight patients with bacterial meningitis contained citrimalic acid, a compound not previously identified in either normal or infected CSF. The implications of these findings are discussed.

Additional Keyphrases: gas chromatography • nervous-system disorders

Disorders of the central nervous system are expressed clinically by a nonspecific set of symptoms and signs, including changes in mental status, loss of neurological functions, seizures, and signs of increased intracranial pressure. Such diseases may be caused by traumatic, infectious, metabolic, degenerative, hypoxic–ischemic, toxic, and immunologic insults. In many instances the specific diagnosis is not determined until late in the course of disease, when it is too late to reverse clinical deterioration. Rapid determination of specific etiology is particularly important in infection of the central nervous system. As therapy for viral disease has become available, it has become important not only to distinguish bacterial from viral disease but also to determine which specific virus is involved in a given infection (1, 2).

The traditional methods of viral diagnosis involve isolation of the virus in tissue culture from the appropriate site or a serological assay. Rapid diagnosis is not usually possible, however, with either one of these two techniques. Methods for detection of viral antigens in cerebrospinal fluid (CSF), which would result in rapid diagnosis in an illness caused by a specific virus, are in the process of being developed, but thus far have not been proven useful in clinical settings (2).

Gas chromatography (GC) of CSF has been reported to be useful in the diagnosis of neurological diseases (3–7). Waterbury and Pearce analyzed CSF by GC with a flame ionization detector (GC-FID) and by combined gas chromatography–mass spectrometry (GCMS). Malcolm and Leonards, using GC-FID, analyzed CSF from normal newborns and infants and from those with disease of the central nervous system (4).

Brooks and coworkers used frequency-pulsed electron-capture gas–liquid chromatography to analyze CSF and CSF from patients with infections, including nonbacterial meningitis (5, 7, 8). The GC patterns from patients with tuberculous, viral, or fungal meningitis reportedly were different, although most of the unique compounds were not identified by mass spectrometry. This technique, although very sensitive, has certain drawbacks for biomedical work. In comparison with FID, results are linearly related to concentration over a far narrower range, and sensitivity to contamination and temperature changes makes reproducible results technically more difficult than with an FID. These factors have probably contributed to the more widespread acceptance and availability of gas chromatographs with FIDs. If such an apparatus were sufficiently sensitive to allow differentiation among the various causes of meningitis and encephalitis it would make GC clinically useful in this setting.

In this study, we used a gas chromatograph equipped with a FID to analyze CSF from patients with and without infections of the central nervous system. Compounds with only slightly different retention characteristics were resolved by using a capillary column of fused silica. In this first report, we describe GC analysis of the CSF from 23 patients without infections who underwent myelograms and present preliminary data on several patients with either bacterial or viral meningitis. Control experiments have been performed to determine which of the compounds previously reported to be in CSF are detected by our method, and at what concentrations. Representative samples have also been analyzed by GCMS to identify unambiguously the compounds present in both normal and infected CSF. Citrimalic acid, a compound not previously reported to be present in CSF, was found in patients with bacterial meningitis.

Materials and Methods

Reagents

All reagents were purchased "distilled in glass," and were used directly without further purification. N-O-Bis(trimethylsilyl)trifluoroacetamide containing 1 g of trimethylchlorosilane per deciliter (reagent A) was obtained from Regis Scientific, Morton Grove, IL.

Nonanoic acid, palmitic acid, homovanillic acid, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, 3-methoxy-4-hydroxyphenylethanol, α-hydroxyisovaleric acid, citric acid, and citrimalic acid were from Sigma Chemical Co, St. Louis, MO.

Sample Treatment

We did several preliminary control experiments. We prepared and analyzed mixtures of model compounds, including long-chain fatty acids, hydroxy acids, and amines. Our results suggested that a simplification of the method of Dalgliesh et al. (9) would be most useful for the extraction and derivatization of CSF constituents. In their procedure NaCl is added to the sample before extraction and the
organic phase is dehydrated with either magnesium sulfate or sodium sulfate before derivatization. Neither of these steps increased the extraction efficiency in our control experiments.

CSF was obtained from patients undergoing myelograms and from the bacteriology and hematology laboratories. Most of the patients underwent myelograms before surgery for scoliosis or kyphosis. Most were between 3 and 25 years old. All samples were obtained for other examinations and the GC analyses reported herein were done on the CSF remaining after those tests were performed.6

The samples were refrigerated and centrifuged (1000 × g, 5 min) as soon as possible, usually within an hour of the procedure, and the supernates were stored at −20 °C. Immediate refrigeration is not crucial; in control experiments, samples kept at room temperature for as long as 2 h before centrifugation showed the same GC profiles as samples that had been promptly refrigerated and centrifuged.

Nonanoic acid (5 µg, in either chloroform or 0.1 mol/L NaOH) was added to samples before extraction, as the internal standard. Samples (0.5–2.0 mL) were acidified to pH 1 with concentrated HCl and extracted three times with 2-mL portions of ethyl acetate and three times with 2-mL portions of diethyl ether. This procedure effectively extracts most acidic and neutral metabolites (9). The extracts were combined and evaporated under a stream of dry nitrogen, and the residue was dissolved in 25 µL of reagent A and 10 µL of pyridine. The samples were reacted at 45 °C for 30 min, then diluted to 100 µL with hexane. This method involves only one transfer of the sample, thus minimizing loss.

GC and GCMS

Aliquots of the silylated CSF extracts (1.0 to 3.0 µL) were gas-chromatographed on a 30-m DB-1 fused silica capillary column (J&W Scientific, Rancho Cordova, CA) in a Hewlett-Packard Model 5840A gas chromatograph equipped with a splitless injector and an FID. The temperature was held at 50 °C for 4.0 min, then linearly programmed from 50 to 320 °C at 10 °C/min. The flow rate of the carrier gas, helium, was 1 mL/min. The GCMS computer system consists of a Varian Model 3700 gas chromatograph modified for direct coupling of the capillary column to the ionization source of a Finnigan-MAT 312 double-focusing mass spectrometer operating in electron impact mode with an ionization potential of 70 eV (source temperature, 220 °C; filament current, 1 mA; carrier gas flow rate, 1 mL/min). A Finnigan-MAT SS-200 data system controls the instrument and acquires, processes, and stores the data. For these experiments the mass spectrometer was scanned from m/z 40 to m/z 800 at a 2-s repetition rate.

Results

CSF was obtained from 23 noninfected patients who were undergoing myelograms for either diagnosis of structural lesions or prior to surgery for scoliosis or kyphosis. The cell count and the protein and glucose concentrations of the fluid were within normal limits. When CSF was extracted and derivatized as described above and analyzed by GC, patterns of peaks were consistent and reproducible for all 23 of the CSF extracts. Results for three representative samples are shown in Figure 1; the components identified by GCMS are listed in Table 1. Figures 1A–C are "total ionization profiles," reconstructions of the gas chromatograms obtained during analysis of these samples by GCMS (10). Each of the compounds listed in Table 1 and most of the currently unidentified constituents were consistently present in these samples. The major peaks in the total ionization profiles of the CSF of normal patients were produced by hydroxy fatty acids.

Our results differ from those reported earlier. Waterbury and Pearce (3) and Malcolm and Leonards (4) both identified long-chain carboxylic acids as the predominant compounds in their gas chromatograms of CSF. Brooks et al. similarly found several carboxylic acids present in normal CSF (7). As seen in Figure 1 and Table 1, we did not find carboxylic acids to be major components of normal CSF. In particular, palmitic acid, although always present in the normal CSF, was never a prominent peak.

Control experiments were performed to rule out selective loss of palmitic and other carboxylic acids during extraction. In one set of experiments we added 1 µg each of myristic, 1210 CLINICAL CHEMISTRY, Vol. 30, No. 7, 1984
palmitic, and arachidic acid (C14:0, C16:0, and 20:0, respectively) to 1 mL of water, then extracted as usual with ethyl acetate and ether. These quantities of fatty acids were based on the work of Brooks et al. (7), who found from 0.5 to 1.0 μg of palmitic acid per milliliter of CSF. Peak areas in the gas chromatograms of samples extracted from water before derivatization were compared with those for samples that were simply evaporated and the residue derivatized. The results (Table 2) indicate that the yield of derivatized fatty acid is unaffected by extraction from water. However, longer-chain fatty acids such as arachidic acid are silylated in lower yield than shorter-chain acids. Clearly, myristic and palmitic acids are efficiently derivatized and extracted from water as concentrations comparable with those found in CSF, and at concentrations lower by as much as 10-fold than normally present. Approximately 1 ng of injected fatty acid may be readily observed by GC-FID under the conditions described here.

The relative efficiency of derivatization of fatty vs hydroxy acids was also compared for nonanoic, palmitic, and α-hydroxyisovaleric acids (data not shown). The peak areas of the fatty acids were within twofold the peak area for the hydroxy fatty acid; evidently selective extraction is not a notable problem.

CSF reportedly contains substances representing end products of neurotransmitter metabolism, including 5-hydroxyindoleacetic acid, homovanillic acid, 3-methoxy-4-hydroxyphenylethylenglycol, 3-methoxy-4-hydroxyphenylethanol, and 3,4-dihydroxyphenylacetic acid (3, 4). These compounds are said to be present in the range of 0–70 ng/mL in normal CSF (11–17). As shown in Table 1, we observed none of these five compounds in normal CSF. In further experiments, we mixed together 50 ng of each of these compounds, derivatized the mixture, and analyzed it by GC. About 5 to 10 ng of each substance had to be injected onto the GC before a signal was recorded. These compounds would not be detected by GC-FID in concentrations less than 200–400 ng/mL if only 1–2% of the sample were injected. All of these compounds could, however, be readily detected by GCMS analysis at much lower concentrations. These results suggest that GC-FID analysis would not be useful for analyzing these neurotransmitter metabolites in normal CSF, although GCMS would detect them at physiological concentrations.

CSF from patients with bacterial or viral meningitis was next analyzed by GC. For this preliminary study, CSF was obtained from four patients with Haemophilus influenzae meningitis, two patients with meningococcal meningitis, one patient with group B β-hemolytic streptococcal meningitis, one patient with Staphylococcus epidermidis meningitis, and four patients with aseptic meningitis (one echovirus, three culture-negative). Several of these samples were also analyzed by GCMS. Figure 2 depicts the total ionization profile of the CSF from a patient with H. influenzae meningitis analyzed by GCMS. In agreement with previous reports (7, 19), lactic acid, succinic acid, and 3-hydroxybutyric acid are much more prominent in CSF infected with H. influenzae.

In addition, we also find a major peak corresponding to the trimethylsilyl derivative of citramalic acid (Figure 3), a compound not previously observed in CSF. The retention time on GC and electron impact mass spectrum of a derivatized authentic standard were identical to that of the unknown, confirming the structure assignment. Citramalic acid was present in the CSF from seven of eight patients with bacterial meningitis, but not in the CSF of one patient with meningococcal meningitis or in that from patients with aseptic meningitis. CSF from two of the patients with aseptic meningitis contained >500 leukocytes per milliliter, suggesting that the presence of the citramalic acid does not simply signify inflammation. Citramalic acid was not found in normal CSF or in that of febrile patients without meningitis, but it was found in fluid obtained from one patient with neurofibromatosis and a CSF protein concentration of 12.7 g/L, who did not have meningitis at the time the CSF was analyzed.

**Discussion**

A profile of acidic and neutral metabolites in CSF from normal patients and from patients with meningitis has been presented. The major components seen in gas chromatograms of normal CSF were all hydroxy acids. As mentioned, this seems to be at variance with previously reported data. In the study of Waterbury and Pearce (3), components of CSF eluting early in the gas chromatogram were not shown. Hydroxy acids, which we found to be the major components, elute primarily in this portion of the gas chromatogram and therefore would not have been identified in their study.

![](image1)

**Figure 2.** Total ionization profile of a derivatized extract of CSF from a patient with *H. influenzae* meningitis

Glycerol, citramalic, and homovanillic acid (HVA) are labeled; all other peaks are identified in Table 1.

![](image2)

**Figure 3.** Mass spectrum corresponding to the trimethylsilyl derivative of citramalic acid

The spectrum was produced during a GCMS experiment on a derivatized extract of CSF from a patient with *H. influenzae* meningitis (see Figure 2, scan no. 403)

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**Table 2. Efficiency of Extraction and Derivatization**

<table>
<thead>
<tr>
<th></th>
<th>Fatty acid</th>
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<tr>
<td></td>
<td>Myristic</td>
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<tr>
<td>0.1 μg</td>
<td>1.37</td>
</tr>
<tr>
<td>0.1 μg (extracted)</td>
<td>1.06</td>
</tr>
<tr>
<td>1 μg</td>
<td>1.02</td>
</tr>
<tr>
<td>1 μg (extracted)</td>
<td>0.98</td>
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</tbody>
</table>

0.1 or 1 μg of myristic, palmitic, and arachidic acid in chloroform was either directly derivatized and injected or extracted from water before derivatization and injection ('extracted'). For the 1 μg samples, 1% of the samples was injected; 3% of the 0.1 μg samples was injected. The data are expressed as signal, in arbitrary units, per nanogram injected.
Malcolm and Leonards (4) do present this portion of the gas chromatogram and identify lactic acid as a major peak; in fact, a closer analysis of their data shows that lactic acid is present in at least 10-fold higher concentration than palmitic acid in normal CSF. This is qualitatively consistent with our data. Thus, the apparent discrepancy between the earlier data and ours can be explained in part by differences in their presentation.

We performed several control experiments to establish the sensitivity of the GC to selected carboxylic and hydroxy acids. α-Hydroxyisovaleric acid, nonanoic acid, and palmitic acid were detected with equal efficiency after extraction from water and derivatization. Myristic and palmitic acids were readily detected, even at starting concentrations as low as 100 ng/mL (Table 2). From these data, about 100 ng of myristic or palmitic acid per milliliter of CSF would be easily detected by GC-FID, even if only 1–2% of the sample were injected onto the GC. Longer-chain fatty acids were not as efficiently extracted and detected as were shorter-chain fatty acids (Table 2).

Acidic and neutral metabolites of neurotransmitter metabolism such as homovanillic acid, 3-methoxy-4-hydroxyphenylethanol, 3-methoxy-4-hydroxyphenylethylamine, 3,4-dihydroxyphenylacetic acid, and 5-hydroxyindoleacetic acid were identified in CSF by Waterbury and Pearce (3) and Malcolm and Leonards (4). We did not observe these compounds in normal CSF in this study, although homovanillic was seen in CSF from patients with bacterial meningitis. 5-Hydroxyindoleacetic acid has been reported to be in normal CSF in concentrations of 20–35 ng/mL (12, 13, 18), 3-methoxy-4-hydroxyphenylethylamine at 10–25 ng/mL (11, 13, 18), homovanillic acid at 20–55 ng/mL (13–18), and 3,4-dihydroxyphenylacetic acid at <1.0 ng/mL (14).

In our control experiments, these compounds could not be detected by GC-FID at 50 ng/mL, although this concentration was readily detected by GCMS. The reason that these compounds could not be detected in normal CSF by GCMS is not known at present. In any case, neurotransmitter metabolites are not major constituents in a GC profile of normal CSF.

In this study, mass spectral data were used to identify the compounds separated by gas chromatography. The power of this technique is evident by the identification of a previously unidentified substance, citramalic acid, in the CSF of patients with bacterial meningitis. This compound is probably a degradation product of glutamate (20). Citramalic acid was found in the CSF of seven of eight patients with bacterial meningitis, but not in the CSF of uninfected patients or in patients with aseptic meningitis, two of whom had markedly high CSF cell counts. It was found in the CSF of one patient with CNS degenerative disease and greatly above-normal protein, for unknown reasons.

Citramalic acid appears to be present in the CSF of almost all patients with bacterial meningitis, regardless of etiology. The diagnostic dilemma in cases of bacterial meningitis occurs when the leukocyte count in CSF is very low on initial examination or when the patient has been treated with antibiotics before CSF cultures have been obtained, rendering culture results unreliable. In future studies, the CSF from such patients will be analyzed by GC for the presence of citramalic acid, to determine if this compound will be a useful marker for bacterial meningitis in these two settings.

This study suggests that GC-FID can be used to distinguish disease states since the compounds that are observed to vary in concentration are easily detectable at physiologically concentrations with readily available equipment and reagents. Access to GCMS will allow unambiguous identification of compounds associated with specific diseases of the central nervous system.

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References