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PROFILING OF ORGANIC ACIDS AND POLYOLS IN NERVES OF URAEMIC AND NON-URAEMIC PATIENTS

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SUMMARY

Organic acids, polyols and lipid-bound polyols in the cauda equina nerves of uraemic patients and non-uraemic patients were analysed with high-resolution gas chromatography–mass spectrometry. In the uraemic nervous tissue, the concentrations of myoinositol and 4-hydroxyphenylacetic acid were increased. Levulinic acid was first detected in the nervous tissue as a normal component. 1-Deoxyglucose and free and lipid phosphatide scylloinositol were detected in the nervous tissue as normal components.

INTRODUCTION

Uraemic polyneuropathy is one of the major problems in uraemic patients undergoing maintenance haemodialysis, since it starts with paraesthesia and then progresses to motor paralysis of the lower legs, causing difficulties in leading an ordinary life. Although the aetiology of uraemic polyneuropathy is unknown, speculation favours an as yet unidentified dialysable compound in uraemic serum as an important factor in the occurrence of uraemic polyneuropathy. Many hypotheses are reported as an aetiology of uraemic polyneuropathy, such as retention of guanidine compounds [1], unidentified middle-

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molecules [2] and myoinositol [3, 4]. The present authors have recently reported that the serum level and the urinary excretion of chiroinositol and scylloinositol as well as myoinositol were increased in uraemic patients [5].

The primary aim of this study is to determine if chiroinositol and scyllo-inositol are abnormal constituents of phosphoinositides in the nerves of uraemic patients. Since phosphoinositides are closely related to neural activity in the nerve, the abnormal phosphoinositides containing chiroinositol and scylloinositol instead of myoinositol may cause neural dysfunction, leading to uraemic polyneuropathy. The second aim is to determine if the concentrations of these free inositol isomers are increased in the nerves of uraemic patients, as observed in their urine and serum. Organic acids in the cauda equina nerves of uraemic patients were also analysed to detect abnormally increased levels of acids.

EXPERIMENTAL

Chemicals

Amberlite MB-3 resin was the product of Rohm and Haas (Philadelphia, PA, U.S.A.). *N,O*-Bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane were purchased from Pierce (Rockford, IL, U.S.A.). Adonitol, *p*-(*n*-amyl)-benzoic acid and levulinic acid were purchased from Sigma (St. Louis, MO, U.S.A.).

Samples

Small amounts of cauda equina nerve were obtained as autopsy samples from five patients with renal failure and five non-uraemic patients (two with pancreatic cancer, two with diabetes mellitus and one with cardiac failure). The samples were kept at −20°C prior to analysis.

Extraction of polyols and lipid-bound polyols

Slices (100 mg) of cauda equina nerves were homogenized with 1.9 ml of chloroform–methanol (2:1). After the addition of 8 ml of chloroform–methanol–water (2:1:0.126), the total lipid extracts were filtered. The filtrates were mixed with one fifth of their volumes of water. After centrifugation, as much of the upper phase as possible was removed and removal of its solutes was completed by rinsing the interface three times with 2 ml of methanol–water (1:1) in such a way as not to disturb the lower phase. The collected upper phases were dried on a rotary evaporator. After addition of 100 μg of adonitol as an internal standard, residues were deionized on Amberlite MB-3 columns (7 cm × 8 mm I.D.). Eluates were dried on a rotary evaporator and then over a nitrogen stream. Dried residues were assayed for free polyols.

The lower phases were dried on a rotary evaporator. After addition of 100 μg of adonitol as an internal standard, the dried residues were taken up in 1 ml of 6 *M* hydrochloric acid and heated at 110°C for 40 h. Hydrochloric acid was deionized over sodium hydroxide in a vacuum, and the residues were deionized on Amberlite MB-3 columns (7 cm × 8 mm I.D.). Eluates were dried on a rotary evaporator and over a nitrogen stream. Dried residues were assayed for lipid-bound polyols.

Samples were trimethylsilylated with 30 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide and 5 μl of trimethylchlorosilane at 60° C for 20 min; 2-μl aliquots of the samples were subjected to gas chromatography–mass spectrometry (GC–MS).

Extraction of organic acids

Slices (500 mg) of cauda equina nerves were homogenized with small amounts of water. After the addition of 250 μg of *p*-(*n*-amyl)benzoic acid as an internal standard, the homogenates were deproteinized with 2 ml of cold ethanol, and centrifuged at 25 000 *g* for 10 min. Supernatants were removed, and precipitates were washed with 2 ml of cold ethanol and centrifuged again. The collected supernatants were dried on a rotary evaporator, dissolved with 1 ml of water, acidified to pH 1 with 1 *M* hydrochloric acid and saturated with sodium chloride. Organic acids were extracted three times with 3 ml of ethyl acetate. The organic phases were dehydrated over anhydrous sodium sulphate and dried over a nitrogen stream. Residues were assayed for organic acids.

Samples were trimethylsilylated with 90 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide and 10 μl of trimethylchlorosilane at 60° C for 20 min; 2-μl aliquots of the samples were subjected to GC–MS.

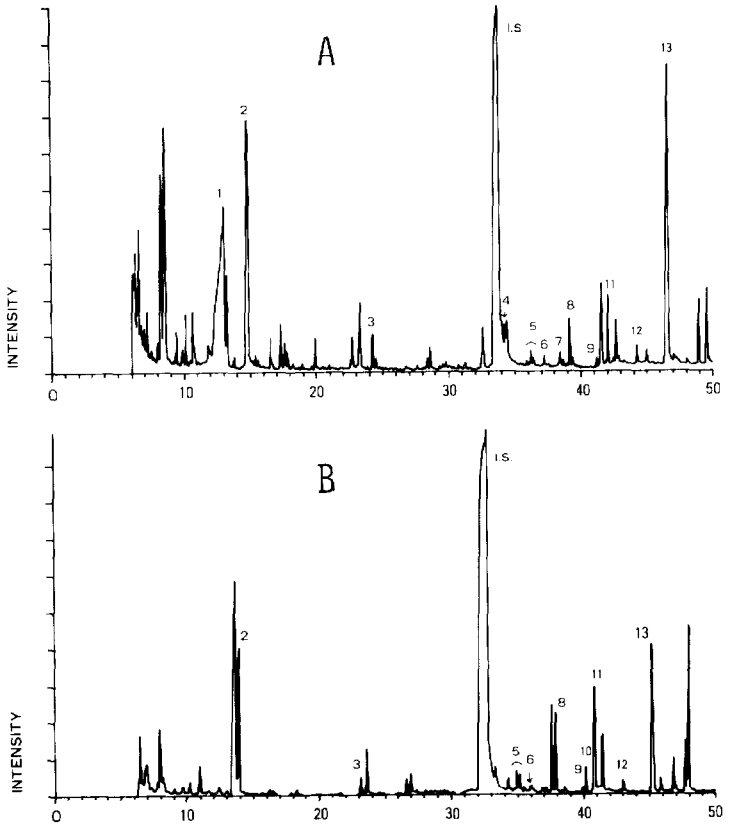

Fig. 1. Gas chromatograms of free polyols in the cauda equina nerves of a patient with renal failure (A) and a non-uraemic patient with pancreatic cancer (B). Peaks: 1 = urea, 2 = glycerol, 3 = erythritol, 5 = fructose, 6 = 1-deoxyglucose, 8 = α-glucose, 9 = mannitol, 10 = sorbitol, 11 = β-glucose, 12 = scylloinositol, 13 = myoinositol.

Gas chromatography– mass spectrometry

A Hewlett-Packard 5710A gas chromatograph was combined with a double-focusing mass spectrometer (JMS D 300, JEOL). Data were stored and processed by a JMA 2000 on-line data system (JEOL). The gas chromatograph was equipped with an OV-101 open tubular glass capillary column (30 m × 0.25 mm I.D.) and a splitless injector. The column temperature was programmed from 120 to 270°C at 3°C/min for the analysis of polyols and from 130 to 270°C at 3°C/min for organic acids. Electron-impact (EI) mass spectra were recorded at an ionizing energy of 70 eV, an ionization current of 300 μA and an accelerating voltage of 3 kV.

RESULTS

Free polyols in cauda equina nerve

Fig. 1 shows the gas chromatograms of polyols in the cauda equina nerves of a patient with renal failure and a non-uraemic patient with pancreatic cancer. Identification of the peaks was performed by comparing their mass

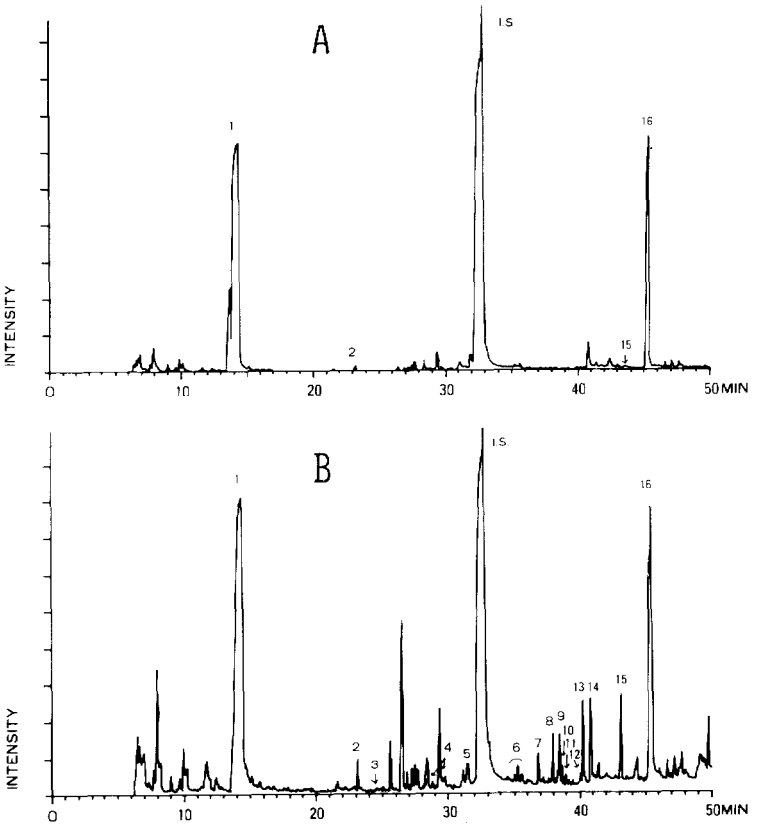

Fig. 2. Gas chromatograms of lipid-bound polyols in the cauda equina nerves of a patient with renal failure (A) and a non-uraemic patient with pancreatic cancer (B). Peaks: 1 = glycerol; 2 = erythritol; 3 = 5-deoxyxylitol or 5-deoxyarabitol; 4 = arabinose; 5 = arabitol; 6 = fructose; 7, 10 = galactose; 8 = α-glucose; 11 = neoinositol; 12 = mannitol; 13 = sorbitol; 15 = scylloinositol; 16 = myoinositol.

spectra and retention times with those of authentic compounds or with reference to the literature. In the uraemic nerve, the peak of myoinositol was higher than in the cancerous nerve. Unexpectedly, chiroinositol was not detected in the uraemic nerve, although it had been detected in the uraemic serum at an increased level as indicated in a previous report [5]. Scylloinositol and 1-deoxyglucose were detected in the nerves of all patients.

Lipid-bound polyols in cauda equina nerve

Fig. 2 shows the gas chromatograms of lipid-bound polyols in the cauda equina nerves of a patient with renal failure and a non-uraemic patient with pancreatic cancer. Scylloinositol was detected in the nerves and considered to be a normal component of phosphoinositides. Chiroinositol was not detected in the nerves. Neoinositol was detected in the nerves at a minor level, but 1-deoxyglucose was not detected therein as a lipid-bound form.

Concentration of myoinositol in cauda equina nerve

Table I shows the concentrations of free and lipid phosphatide myoinositol in the cauda equina nerves of uraemic and non-uraemic patients. The concentration of free myoinositol in the nerves of uraemic patients was increased as compared with non-uraemic patients, whereas the concentration of lipid phosphatide myoinositol was decreased in comparison thereto.

Organic acids in cauda equina nerve

Fig. 3 shows the gas chromatograms of organic acids in the cauda equina nerves of a patient with renal failure and a non-uraemic patient with pancreatic cancer. In all uraemic nerves, the concentrations of phosphoric acid and 4-hydroxyphenylacetic acid were increased as compared with the non-uraemic nerves. Fig. 4 (lower spectrum) presents the EI mass spectrum of peak 3 in the gas chromatograms. The chemical-ionization (CI) mass spectrum of the peak indicated that the molecular ion of the compound was 188. The ion at m/z 173 is due to $(M - CH_3)^+$, while that at m/z 145 is due to $(M - CH_3CO)^+$. Since the trimethylsilyl (TMS) derivative of authentic levulinic acid and peak 3 in Fig. 4 exhibited identical retention times on the gas chromatograms and identical EI mass spectra (Fig. 4), peak 3 was identified as levulinic acid. Levulinic acid was first detected in the nerves of all patients as a normal component. However, 4-hydroxyvaleric acid, a reductive form of levulinic acid,

TABLE I

CONCENTRATION OF MYOINOSITOL IN CAUDA EQUINA NERVES IN URAEMIC AND NON-URAEMIC PATIENTS

	Concentration* (mean ± S.D., $n = 5$)	
	Free myoinositol	Lipid phosphatide myoinositol
Uraemic patients	0.39 ± 0.20	0.088 ± 0.094
Non-uraemic patients	0.13 ± 0.084	0.17 ± 0.18

*Peak-area ratio to 50 μg of adonitol (internal standard) per 0.1 g of wet nervous tissue.

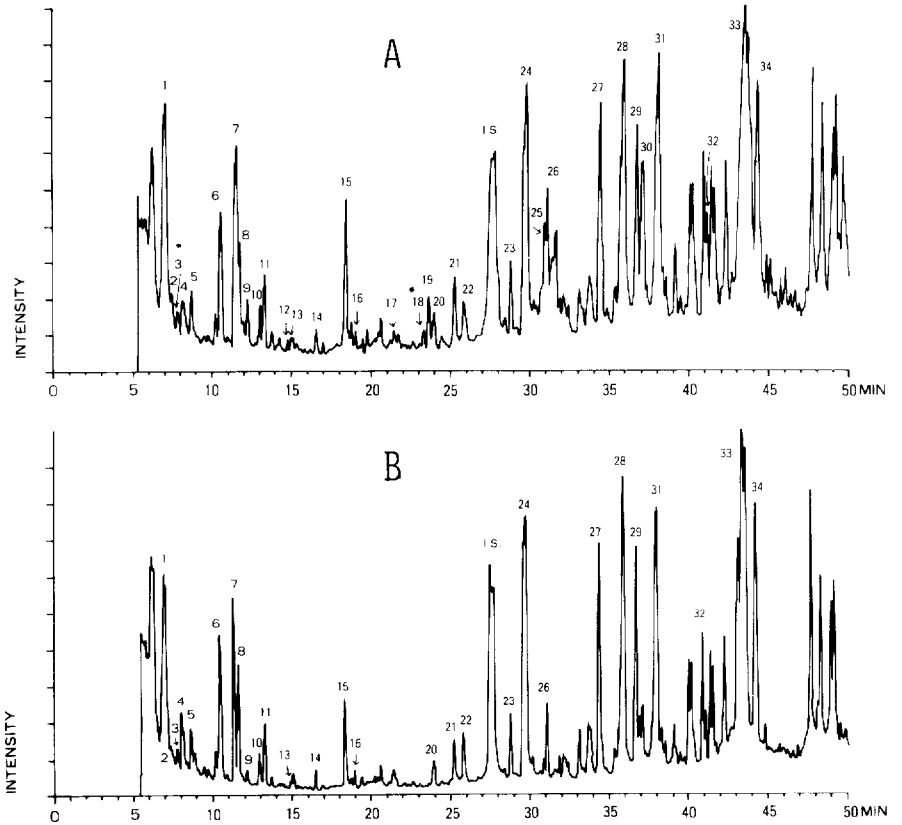

Fig. 3. Gas chromatograms of organic acids in the cauda equina nerves of a patient with renal failure (A) and a non-uraemic patient with pancreatic cancer (B). Peaks: 1 = lactic acid; 2 = pyruvic acid; 3 = levulinic acid; 4 = 2-hydroxybutyric acid; 5 = 3-hydroxybutyric acid; 6 = diethyleneglycol (artifact); 7 = phosphoric acid; 9 = succinic acid; 10 = uracil; 11 = fumaric acid; 12 = lactic acid lactate; 14 = 2-deoxytetronic acid; 15 = malic acid; 16 = pyroglutamic acid; 17 = 2-hydroxyglutaric acid; 18 = 4-hydroxyphenylacetic acid; 20 = lauric acid; 23 = α-glycerophosphate; 25 = citric acid; 26 = myristic acid; 30 = palmitoleic acid; 31 = palmitic acid; 32 = heptadecanoic acid; 33 = oleic acid; 34 = stearic acid.

could not be found in the nervous tissue. Lactic acid lactate was detected in the nerves of uraemic and non-uraemic patients.

DISCUSSION

Retention of myoinositol in uraemic blood has been considered a possible cause of uraemic polyneuropathy [3, 4]. Myoinositol is a normal constituent of phosphoinositides, such as monophosphoinositide, diphosphoinositide and triphosphoinositide, which are closely related to neural activity in the nervous tissue. Since the catabolism of myoinositol in the renal tissue is a major mechanism for the disposition of endogenously synthesized myoinositol or diet intake of myoinositol [6, 7], the urinary excretion and the serum level of myoinositol are increased in uraemic patients.

Urinary excretion and the serum level of both chiroinositol and

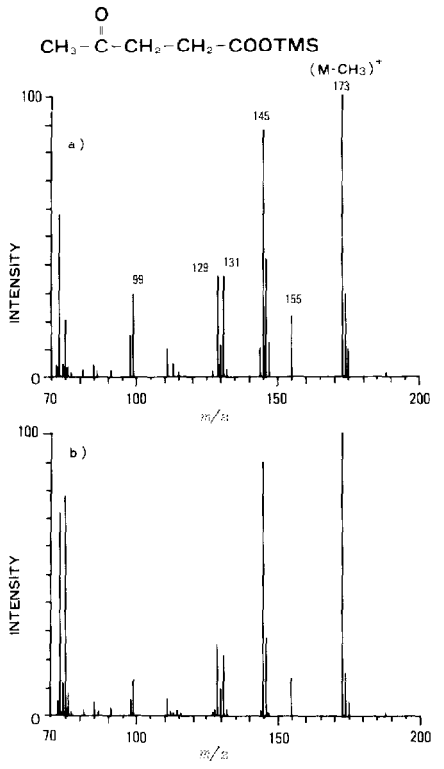

Fig. 4. Electron-impact mass spectra of trimethylsilylated levulinic acid (upper spectrum) and of peak 3 (lower spectrum) in the gas chromatograms of Fig. 3.

scylloinositol were also found to be increased in uraemic patients by the present authors [5]. In the uraemic nerves, only the concentration of free myoinositol was increased, whereas that of lipid phosphatide myoinositol was decreased. Free and lipid phosphatide scylloinositol was detected in the uraemic and non-uraemic nerves at a minor level, but free or lipid phosphatide chiroinositol could not be detected therein. These results suggest that chiro-inositol and scylloinositol are not related to neural dysfunction in uraemic patients, but that a metabolic disorder of myoinositol is presumably related to the neural dysfunction in uraemic patients.

Scylloinositol and myoinositol are metabolically interrelated. Myoinositol, myoinose-2 and scylloinositol were detected in the organs of rat and rabbit [8]. Scylloinositol is postulated to be formed by the reduction of myoinose-2, which derives from dehydrogenation of myoinositol. Free scylloinositol is reported to be detected in several nervous tissues [8]. Scylloinositol was first detected in the nervous tissue as lipid phosphatide inositol. Neoinositol was also detected at a very minor level as lipid phosphatide inositol.

1-Deoxyglucose was first detected in the cerebrospinal fluid by Pitkänen [9]. The levels of this compound in the cerebrospinal fluid [10] and serum [5, 10] were reported to be low in uraemic patients. 1-Deoxyglucose was detected in the cauda equina nerves of all patients only as a free form.

An increased level of 4-hydroxyphenylacetic acid in the cauda equina nerve

was observed in uraemic patients. 4-Hydroxyphenylacetic acid is known to be one of the organic acids most accumulated in uraemic serum [11]. The retention of the compound in uraemic nerves seems to be due to its diffusion from uraemic blood into nervous tissue. 4-Hydroxyphenylacetic acid as well as other phenolic acids was shown to inhibit several cerebral enzymes in vitro [12]. Retention of 4-hydroxyphenylacetic acid in uraemic nerves may be related to the pathogenesis of uraemic polyneuropathy. Further study is needed to elucidate the adverse effect of 4-hydroxyphenylacetic acid accumulated in uraemic nerves.

Ketting et al. [13] detected lactic acid lactate in the urine of patients screened for inherited metabolic disease. In the gas chromatograms, lactic acid lactate was observed as two peaks: the L,L and/or D,D form as well as the D,L and/or L,D enantiomer. The occurrence of lactic acid lactate containing D-lactate suggested that it was an artifact formed from bacterial D-lactate. However, the authors detected lactic acid lactate as one peak in the gas chromatogram of the acid fraction of the nervous tissue. It seemed to be an artifact due to a non-enzymatic condensation of lactic acid during lactic acidosis at death or during the extraction procedure.

Levulinic acid was first identified in the nerves of all patients, which suggested it to be a normal component of the nervous tissue. The metabolic pathway of the acid is unknown at present, and further study is needed to clarify its metabolic significance in nervous tissue.

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