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QUANTITATIVE ANALYSIS OF POLYOLS IN HUMAN PLASMA AND CEREBROSPINAL FLUID

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SUMMARY

We used gas chromatography in conjunction with flame ionization detection to quantitate nine polyols and aldo and keto sugars (as silyl derivatives) in human plasma and cerebrospinal fluid (CSF). Rhamnose, not found in CSF or plasma, was used as an internal standard with a lower limit of quantitation of 0.4 mg/l. CSF polyol and sugar concentrations (mean ± S.D.) in fourteen healthy subjects (age range 27.1–85.9 years) were: anhydroglucitol, 19.9 ± 5.3 mg/l; arabitol, 4.8 ± 0.9 mg/l; erythritol, 2.4 ± 0.5 mg/l; myoinositol, 28.6 ± 8.3 mg/l; ribitol, 1.6 ± 0.1 mg/l; fructose, 25.5 ± 11.1 mg/l; glucose, 587 ± 70 mg/l; glucitol, 7.7 ± 1.5 mg/l; and mannose, 10.6 ± 2.4 mg/l. The respective plasma concentrations were 30.6 ± 11.5, <0.4, 0.4 ± 0.2, 6.3 ± 2.6, <0.4, 23.4 ± 21.4, 897 ± 214, <0.4 and 13.7 ± 6.3 mg/l. Polyol CSF-to-plasma concentration ratios greater than 2 were observed for myoinositol, erythritol, arabitol, glucitol and ribitol, indicative of active accumulation or synthesis of these polyols within the central nervous system.

INTRODUCTION

Polyols are the reduction products of sugars. Their production by mammalian systems has been largely ignored with the exception of glycerol, inositol and sorbitol (glucitol), which are known to play central roles in human physiology and pathology [1–3]. Other polyols, although detectable in human tissue samples [4–8], have not been examined extensively. In a few limited studies it has been suggested that central nervous system myoinositol levels are

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increased in post-mortem brain and decreased in cerebrospinal fluid (CSF) in individuals diagnosed with Alzheimer's disease [1,9], while other studies indicate altered concentrations of CSF polyols in brain tumor patients [8] or serum polyols in invasive *Candida* infections [10–12].

We thought it of interest to establish polyol and keto and aldo sugar concentrations in CSF and plasma taken from healthy subjects. Although gas chromatographic (GC) methods are available to quantify a limited profile of polyols and aldo and keto sugars in CSF samples [7,8,13,14], assays are not available to analyze for a broad spectrum of these compounds in both CSF and plasma in large numbers of samples. We therefore developed a method with several advantages over existing assays. These improvements include: (1) simple and rapid sample preparation; (2) use of minimal sample volumes; (3) reproducible sample recovery; (4) introduction of internal standard directly into CSF and plasma specimens to compensate for differential matrix effects in CSF and plasma; and (5) virtually identical preparation and chromatography of CSF and plasma allowing accurate determination of relative CSF-to-plasma concentrations.

EXPERIMENTAL

Materials

D-Arabitol, isoerythritol, D-fructose, 1,5-anhydro-D-glucitol, D-glucitol, D-glucose, myoinositol, D-mannose, D-mannitol, L-rhamnose, ribitol, DL-threitol and xylitol were purchased from Sigma (St. Louis, MO, U.S.A.). Trimethylchlorosilane was obtained from Supelco (Bellefonte, PA, U.S.A.). Tri-Sil 'Z' was obtained from Pierce (Rockford, IL, U.S.A.). Chromatographic-grade hexane, methanol and acetonitrile were from Burdick and Jackson (Muskegon, MI, U.S.A.). [U-^{14}C]Erythritol (100 mCi/mmol), [U-^{14}C]D-glucitol (274 mCi/mmol) and [U-^{14}C]myoinositol (270 Ci/mmol) were from Amersham (Arlington Heights, IL, U.S.A.) and [U-^{14}C]D-fructose (265 mCi/mmol), [1-^{14}C]D-glucose (55 mCi/mmol) and [1-^{14}C]D-mannose (50 mCi/mmol) were from NEN-DuPont (Boston, MA, U.S.A.).

Samples

Healthy volunteers, ten males and four females (age, mean ± S.D, 49.6 ± 20.5 years; range 27.1–85.9 years), selected for the absence of major medical and neurological illnesses, were medication-free for at least two weeks prior to a study. Subjects were fasted for 12 h immediately before collection of plasma and CSF samples. Venous blood samples were withdrawn immediately before CSF samples were collected, centrifuged, and the resultant plasma was aliquoted. CSF was collected by lumbar puncture by a standardized procedure [15] on the in-patient unit of the Laboratory of Neurosciences, National Institute on Aging, National Institutes of Health (Bethesda, MD, U.S.A.). All

CSF and plasma samples were frozen immediately and stored at −70°C until analyzed.

A standard stock solution for analysis of CSF samples was prepared with the following concentrations of polyols and sugars: isoerythritol (20 mg/l), D-arabitol (20 mg/l), ribitol (10 mg/l), D-mannitol (40 mg/l), D-mannose (50 mg/l), D-fructose (60 mg/l), 1,5-anhydro-D-glucitol (80 mg/l), D-glucitol (40 mg/l), D-glucose (1500 mg/l), DL-threitol (5 mg/l), myoinositol (100 mg/l) and xylitol (5 mg/l) using triple-distilled water. Three additional standards were prepared by diluting the stock solution 1:1, 1:4 and 1:8 with triple-distilled water. For plasma samples the standards were prepared as above, however, the stock solution contained more D-glucose (2000 mg/l) and less myoinositol (20 mg/l) in accord with the known plasma concentrations of these substances. Calibration curves obtained for each batch of samples were linear with correlations greater than 0.99. To assess the within- and between-day reproducibility of the assay, five samples of the same pooled CSF or plasma specimen were assayed with each run of CSF or plasma, respectively.

Borosilicate glass test tubes (100 mm×13 mm) were treated with 3 ml of 5% (w/v) trimethylchlorosilane in hexane for 30 min at 75°C. Unreacted reagent was removed and the tubes were rinsed with methanol. Aliquots (100 μl) of standards, CSF or plasma were placed in the silanized tubes with 20 μl of rhamnose (50 μg/ml in water) as an internal standard. Methanol (2 ml) was added to each tube and the samples were vortexed and centrifuged at 2000 *g* for 20 min. Supernatants were transferred to silanized tubes and dried in vacuo. Tri-Sil 'Z' (150 μl) was added to each sample and the samples were sonicated in a bath sonicator for 5 min, prior to heating for 1 h at 75°C. Hexane (400 μl) and acetonitrile (250 μl) were added to the samples, which then were vortexed and centrifuged at 2000 *g* for 5 min. The hexane phase was transferred to vials for automatic injection of a 5-μl aliquot onto the column operated in a splitless mode. Reaction and extraction efficiencies from water, determined for rhamnose and radiolabeled polyols and sugars, were greater than 96% in all cases. Fractional recoveries of polyols and sugars from CSF and plasma relative to the internal standard were not equivalent, necessitating correction of the concentration determined to 100% recovery prior to calculation of plasma-to-CSF ratios. Recovery of rhamnose, erythritol, fructose, glucitol, glucose, myoinositol and mannose from plasma was 59, 75, 45, 48, 72, 60 and 65%, respectively, and from CSF 87, 88, 88, 92, 92, 93 and 84%, respectively. Reported concentrations are corrected to 100% recovery.

Chromatography

GC was performed on a Hewlett-Packard 5880A gas chromatograph equipped with a cross-linked methyl silicone capillary column (25 m×0.31 mm I.D., 0.52 μm film thickness), using a flame ionization detector and a 7672A automatic sampler (Hewlett-Packard, Palo Alto, CA, U.S.A.). The gas chromatograph

was fitted with a Hewlett-Packard 5880A integrator to acquire peak retention time and quantitation. Helium was used as the carrier gas. Chromatographic injector and detector temperatures were 250 and 300°C, respectively. The following temperature program was performed: from 50 to 140°C at 10°C/min, then 140 to 190°C at 1°C/min and finally to 260°C at 30°C/min. Gas chromatography–mass spectrometry (GC–MS) was performed on a Hewlett-Packard MS 5970 mass-selective ion detector coupled with a Hewlett-Packard GC 5890 gas chromatograph. Mass spectra and peak retention times were compared with those of authentic standards and used to confirm peak identity.

RESULTS

Separation of thirteen polyols and aldo and keto sugars was examined (Fig. 1). Peak separation to baseline was achieved for all thirteen compounds in the standard mixture. Retention times were reproducible with coefficients of variation of 0.1% or less for all polyols except glucose. Retention times of polyols and aldo and keto sugars present in tissue samples showed similar reproducibility with coefficients of variation less than 0.2% (Table I).

Representative chromatograms of polyols and sugars extracted from CSF

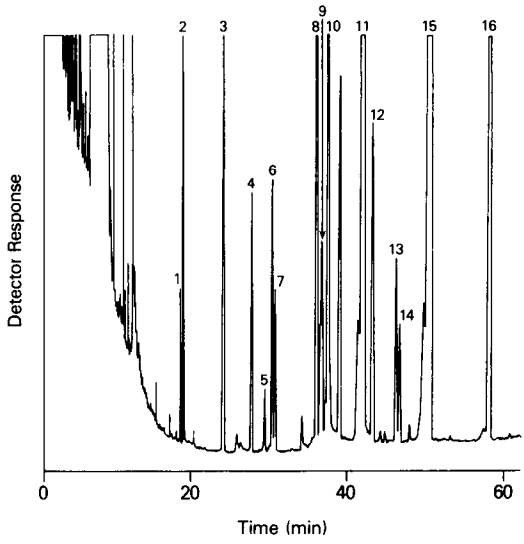

Fig. 1. Chromatogram of aldo and keto sugars and polyols as silyl derivatives from a 1:1 dilution of a CSF standard polyol mixture. The concentration of each polyol is given in the Experimental section. Peaks: 1 = threitol; 2 = erythritol; 3 and 4 = internal standard (rhamnose); 5 = xylitol; 6 = arabitol; 7 = ribitol; 8 and 12 = mannose; 9 = fructose; 10 = anhydroglucitol; 11 and 15 = glucose; 13 = mannitol; 14 = glucitol; 16 = myoinositol.

TABLE I

PEAK RETENTION TIMES FOR POLYOLS AND ALDO AND KETO SUGARS

Retention times of polyols and aldo and keto sugars were determined from standards prepared with each tissue sample run.

Carbohydrate	Retention time (mean ± S.D.) (min)	
	Within-day ($n=5$)	Between-day ($n=4$)
Threitol	18.49 ± 0.01	18.49 ± 0.01
Erythritol	18.87 ± 0.01	18.86 ± 0.01
Rhamnose I	24.07 ± 0.02	24.06 ± 0.02
Rhamnose II	27.68 ± 0.02	27.67 ± 0.02
Xylitol	29.36 ± 0.02	29.36 ± 0.02
Arabitol	30.31 ± 0.02	30.30 ± 0.02
Ribitol	30.68 ± 0.02	30.67 ± 0.02
Mannose I	35.96 ± 0.02	35.95 ± 0.02
Mannose II	43.09 ± 0.02	43.07 ± 0.03
Fructose	36.62 ± 0.01	36.62 ± 0.02
Anhydroglucitol	37.41 ± 0.02	37.40 ± 0.02
Glucose I	42.01 ± 0.11	42.01 ± 0.11
Glucose II	50.60 ± 0.17	50.59 ± 0.16
Mannitol	46.14 ± 0.02	46.12 ± 0.03
Glucitol	46.60 ± 0.01	46.58 ± 0.03
Myoinositol	58.17 ± 0.05	58.13 ± 0.06

and plasma samples are presented in Fig. 2A and B, respectively. Of the nine polyols and sugars identified in tissue samples, sufficient separation for peak-area integration was achieved for all except glucitol, which was not completely resolved from an unidentified peak. L-Rhamnose, selected as an internal standard, is not present in normal CSF or plasma, but has chemical properties similar to the sugars and polyols normally present. After silization the L-rhamnose, detected as two peaks, was well resolved from the other aldo and keto sugars, polyols and unidentified peaks present in tissue samples. For quantitative analysis, the areas of both rhamnose peaks were measured and the sum was determined.

Using pooled CSF and pooled plasma samples chromatographed with each assay batch, the reproducibility of the assay for quantitating polyols and sugars was examined. Within- and between-day reproducibility for polyols and sugars from pooled plasma and CSF samples are presented as coefficients of variation (C.V.) (Table II). The within-day variability was less than the between-day variability for all the compounds examined. Analysis of CSF samples yielded overall better reproducibility and recovery (see above) than did that of plasma

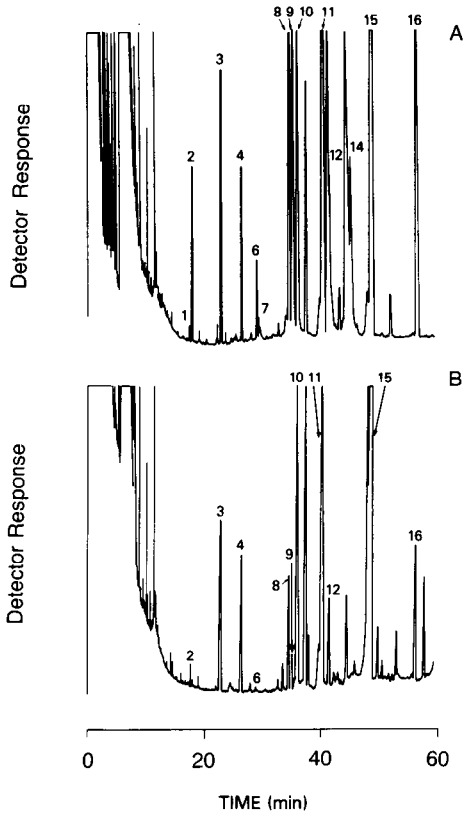

Fig. 2. Chromatograms of aldo and keto sugars and polyols as silyl derivatives from (A) CSF and (B) plasma. Peaks: 1 = threitol; 2 = erythritol; 3 and 4 = internal standard (rhamnose); 6 = arabitol; 7 = ribitol; 8 and 12 = mannose; 9 = fructose; 10 = anhydroglucitol; 11 and 15 = glucose; 14 = glucitol; 16 = myoinositol.

samples, possibly due to the higher protein content of plasma matrix. Precision of the assay determined with tissue samples, however, was more than adequate for measuring sugars and polyols in plasma or CSF at concentrations greater than 0.4 mg/l, the lower limit of quantitation with this assay.

Concentrations of polyols and sugars in CSF (Table III) and plasma (Table IV) from fourteen healthy subjects are presented and compared with published values. In general, our results are in good agreement with published values. However, the CSF concentration of ribitol was a notable exception, being one third of the published CSF levels [7,14]. Although we could readily detect glucitol in CSF, as noted above, it was not completely resolved from an unidentified peak present in some tissue samples (see Fig. 2). The concentration

TABLE II

REPRODUCIBILITY FOR QUANTITATING POLYOLS AND ALDO AND KETO SUGARS FROM POOLED CSF SAMPLES

Pooled CSF was prepared by combining CSF from five healthy subjects.

Carbohydrate	Within-day ($n=5$)		Between-day ($n=3$)	
	Mean concentration (mg/l)	C.V. (%)	Mean concentration (mg/l)	C.V. (%)
Erythritol	4.1	9	3.7	20
Arabitol	4.9	8	4.8	13
Ribitol	1.6	6	1.6	8
Mannose	12.6	11	12.2	22
Fructose	23.3	9	19.7	20
Anhydroglucitol	20.2	8	19.8	12
Glucose	543	3	613	19
Glucitol	6.0	5	6.7	10
Myoinositol	45.2	5	44.1	10

TABLE III

CSF CONCENTRATIONS OF POLYOLS AND ALDO AND KETO SUGARS FOR HEALTHY SUBJECTS

Healthy subjects used in this study (ten males and four females) ranged in age from 27.1 to 85.9 years; mean ± S.D. = 49.6 ± 20.5 years.

Carbohydrate	Concentration (mean ± S.D.) (mg/l)	Range of previously reported values[a] (mg/l)
Erythritol	2.4 ± 0.9	3.5-5.5 [14]
Arabitol	4.8 ± 0.9	4.4 [13]
Ribitol	1.6 ± 0.1	3.0-3.5 [7,14]
Mannose	10.1 ± 2.3	6.8-13 [7,8,14]
Fructose	25.5 ± 11.1	7.6-47 [7,8,14,19]
Anhydroglucitol	19.4 ± 5.3	16.9-53 [7,13,14]
Glucose	618 ± 83	526-789 [7,8,14,19]
Glucitol	7.7 ± 1.5	0.2-7 [7,8,13,14,19]
Myoinositol	33.0 ± 4.6	20-54 [7,8,13,14]

[a]Values are ranges of means when reported; references are provided in square brackets.

TABLE IV

PLASMA CONCENTRATIONS OF POLYOLS AND ALDO AND KETO SUGARS FOR HEALTHY SUBJECTS

Carbohydrate	Concentration (mean ± S.D.) (mg/l)	Range of previously reported values[a] (mg/l)
Erythritol	0.5 ± 0.2	0.45 [5]
Arabitol	< 0.4	0-0.76 [5,10,11,13]
Ribitol	< 0.4	0.06 [5]
Mannose	15.1 ± 7.0	4.4-9.7 [8,19]
Fructose	18.2 ± 16.6	1.4-4.7 [8,19]
Anhydroglucitol	30.6 ± 11.47	21 [13]
Glucose	1095 ± 261	700-940 [1,8,19]
Glucitol	< 0.4	0-1.8 [5,6,8,13,19]
Myoinositol	6.5 ± 2.7	3-6.5 (6,8,13,19]

[a]Values are ranges of means when reported; references are provided in square brackets.

TABLE V

CSF-TO-PLASMA CONCENTRATION RATIOS OF POLYOLS AND SUGARS FOR HEALTHY SUBJECTS

Carbohydrate	Concentration ratio[a] (mean ± S.D.)	Ratio calculated from literature values[b]
Erythritol	6.9 ± 1.5	–
Arabitol	> 10	> 5 [13]
Ribitol	> 4	–
Mannose	0.8 ± 0.2	0.7 [8,19]
Fructose	1.3 ± 0.2	7 [19], 30 [8]
Anhydroglucitol	0.7 ± 0.1	0.8 [13]
Glucose	0.7 ± 0.1	0.6 [8], 0.65 [19]
Glucitol	> 10	3.7 [8], > 2.4 [13]
Myoinositol	5.2 ± 1.4	4.7 [8], 6.5 [13,19]

[a]The CSF-to-plasma concentration ratio for each subject was log transformed prior to calculating the mean ± S.D. ratio reported above.
[b]References for data are provided in square brackets.

of glucitol, calculated by peak-height analysis, however, is similar to previously reported values for human CSF [7,8,13,14].

The ratio of the concentration of a substance in the CSF as compared to its concentration in plasma is an important index of whether the substance in the CSF is derived mainly from brain [13]. Simple filtration would yield CSF-to-plasma concentration ratios of 1.0 or less. Ratios greater than 2 strongly suggest either active transport into or biosynthesis within the central nervous

system. To determine these values for the polyols and sugars, CSF and plasma samples were collected from subjects at the same time and were assayed under identical conditions. The CSF-to-plasma ratios for individual sugars and polyols are presented in Table V. Myoinositol and erythritol had ratios exceeding 2. The CSF-to-plasma ratios for arabitol and glucitol were very high (exceeding 10). This is because the plasma concentration of both arabitol and glucitol was below our limit of quantitation of 0.4 mg/l, while mean CSF concentrations exceeded 4 mg/l. The low arabitol and glucitol plasma concentrations are consistent with those reported by others assaying two- to ten-fold larger sample volumes and/or using selected ion monitoring [5,6,11,13].

DISCUSSION

GC of sugars and polyols in CSF and plasma is possible after they are derivatized to volatile forms. Silization is one of several analytical methods applied to polyol separation. This technique produces anomers, which can be readily resolved by capillary GC into discrete peaks [14]. We used N-trimethylsilylimidazole, which reacts with hydroxyl functional groups but not with aliphatic primary amine groups [15], to yield volatile polyols and sugars. Previous procedures [4,6,10] have relied on filtration and evaporation steps to separate the derivatized polyols and aldo and keto sugars from the matrix and unreacted derivatizing reagents. We used a one-step extraction with hexane and acetonitrile. These solvents are immiscible and the silyl derivatives partition into the hexane phase, whereas excess silyl reagent and organic matrix remain in the acetonitrile phase. The procedure, which is rapid and simple, decreases the loss of sample, which can occur during the filtration and concentration steps after derivatization. Additionally, the silyl derivatives are stable in hexane.

The above described method with subsequent GC separation is a simple, rapid and reproducible procedure for multi-component analysis of polyols and aldo and keto sugars from small samples of plasma and CSF. Its use should facilitate further investigation of central nervous system polyol metabolism in clinical and animal experimental systems where sample volume, particularly of CSF, may be limiting. As polyols are reduction products of sugars, knowledge of their CSF concentrations (especially those with CSF-to-plasma ratios greater than 2 indicating central production or transport) may provide insight into intermediary brain carbohydrate metabolism in health and disease.

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