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RAPID ANALYSIS OF SORBITOL, GALACTITOL, MANNITOL AND MYOINOSITOL MIXTURES FROM BIOLOGICAL SOURCES

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SUMMARY

The three commonly found hexitols mannitol, sorbitol and galactitol are well separated from each other and from myoinositol by gas chromatography as their butylboronate derivative on Dexsil-400, on a 1:1 mixture of OV-1 and OV-17, or on a DB-17 fused-silica capillary column. The method allows all four substances to be measured by autosampling electron ionization gas chromatography–mass spectrometry (GC–MS) in small tissue samples at organ concentrations as small as 5 μmol/kg wet mass in less than 4 min. Comparisons were made to determine the relative sensitivity of GC–MS and other detection methods. The order of sensitivity was electron ionization GC–MS > chemical ionization GC–MS > flame photometric detection using a boron-selective filter > hydrogen flame ionization detection.

INTRODUCTION

Hyperglycemia associated with diabetes results in the accumulation of sorbitol in many organs, a result of the action of the enzyme aldose reductase on glucose (for a review see ref. 1). A similar condition results in experimental and, presumably, natural galactosemia where galactitol accumulates [2]. In many organs the accumulation of these hexitols is accompanied by a loss of tissue inositol which is thought to be a predisposing feature of some or all of the associated pathologic conditions [3]: The causes and the consequences of the increases in concentration of the hexitols and the loss of inositol have been a matter of speculation for many years. Among the complications of diabetes thought to be related to these changes are peripheral neuropathy [4], changes

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in lens permeability that lead to cataracts [5] and increases in vascular permeability and blood flow in many tissues [6–8]. The accumulation of the hexitols has been shown to be arrested in organs of experimental animals and in red blood cells of humans by the use of aldose reductase inhibitors. In many cases the loss of inositol is also moderated. In experimental animals the pathologic alterations associated with these changes are often diminished by the aldose reductase inhibitors.

The measurement of inositols and hexitols in tissues has commonly made use of gas chromatography (GC) of their trimethylsilyl (TMS) or acetyl derivatives. From the analytical viewpoint the disadvantage of these derivatives is that the three hexitols of interest cannot be rapidly separated from each other and from myoinositol, glucose, galactose or other tissue constituents. For example, it has been reported that a 25-m bonded-phase methyl silicone capillary column requires elution times of 25 min for the separation of the TMS derivative of the three hexitols and inositol from each other and from other substances in nerve [9], urine and other tissues [10]. In another study it was shown that elution times of over 50 min were required to separate these hexitols as their acetates [11]. An advantage of the alkylboronates is the structural specificity of the derivative, i.e., requiring pairs of hydroxyls, and the formation of intense mass spectral ions for gas chromatographic–mass spectrometric (GC–MS) applications.

The use of the alkylboronates for the GC of the substrates of interest has been described [12], but chromatographic conditions have not previously been characterized that achieve their complete separation. We describe here the separation and quantitative analysis of all four substrates from biological extracts in chromatographic runs of 4 min duration.

EXPERIMENTAL

Sample preparation

Tissue samples were typically prepared as follows. Tissue (ca. 50 mg) was removed, weighed, internal standard added in water, and the sample was then heated at 100°C for 15 min. A 100-μl volume of 0.3 *M* zinc sulfate was then added, followed by an equivalent of barium hydroxide which was added to precipitate any residual protein. The samples were centrifuged at low speed and an aliquot of the supernatant was then lyophilized. The residue was then derivatized.

Derivatization

Dried standards and tissue samples were allowed to react for 24 h at room temperature with a solution of butylboronic acid (Sigma, St. Louis, MO, U.S.A.) in dry pyridine (100 mg/ml). The volume of reagent was typically 100–200 μl for extracts from tissue samples of from 5–100 mg original wet mass.

Gas chromatography

Glass columns (1 m×4 mm I.D.) were packed with 3% Dexsil-400 (Supelco, Bellefonte, PA, U.S.A.) or with 1:1 3% OV-1/OV-17 (Supelco) (mixed or serially packed). Using isothermal conditions with helium as carrier gas the methylene unit values of the four substrates on the Dexsil column at a temperature of 210°C were: mannitol, 22.29; sorbitol, 22.69; galactitol, 23.16; myoinositol, 23.63. Similar retentions and elution order were observed on the mixed-phase packed column. With a DB-17 fused-silica column (15 m×0.25 mm I.D., 0.25 μm film; J&W Scientific, Folsom, CA, U.S.A.) operating at a head pressure of 20 kPa and a column temperature of 208°C on a Hewlett Packard 5970 GC–MS system, the methylene unit values were: mannitol, 24.23; sorbitol, 24.79; myoinositol, 25.34; galactitol, 25.53.

Flame photometric detection

Using a Varian dual-flame photometric detector (FPD) [13] with a 518-nm narrow bandpass filter and with oxygen instead of air as the combustion gas for both flames, boron is selectively detected by its strong emission at that wavelength.

Gas chromatography–mass spectrometry

Evaluation of chemical ionization (CI) MS was carried out on a Finnigan Model 3200 instrument using either methane or ammonia as reagent gas and with on-column injection using packed glass columns. Evaluation of electron ionization (EI) MS was performed on a Hewlett Packard 5970 GC–MS system with autosampler. Both packed and capillary columns have been used with the HP 5970 instrument. The packed columns were used with a glass jet separator on the HP 5970 and the capillary columns with direct connection to the EI source. EI was carried out at 70 eV. The capillary columns were used with a silanized 6 mm O.D., 4 mm I.D. glass injection port liner fitted with a loose plug of glass wool. The injection port pressure was 20 kPa, resulting in a flow-rate of 1.1 ml/min. A split ratio of 10:1 was used. The internal standards evaluated for the MS analyses were [$^{2}H_{6}$]myoinositol (Merck Isotopes, Rahway, NJ, U.S.A.) and [$^{13}C_{6}$]sorbitol (Cambridge Isotope Labs., Woburn, MA, U.S.A.).

RESULTS AND DISCUSSION

Chromatographic separation

Fig. 1 shows the kind of separation achieved on a 15 m×0.25 mm I.D. DB-17 fused-silica capillary column and Fig. 3 on a 1.8 m×4 mm I.D. glass column packed with 3% Dexsil-400. The peculiar broadening of the chromatographic peak of the tris-butylboronate derivative of sorbitol on the capillary column is not seen on either the Dexsil or the mixed-phase packed chromatography col-

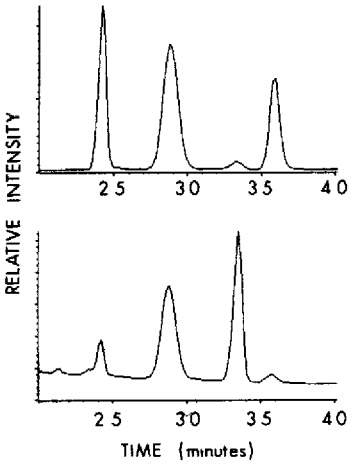

Fig. 1. Selected-ion monitoring electron ionization GC–MS trace of *m/z* 127 produced from the tris-butylboronate derivative of (in order of elution) mannitol, sorbitol, myoinositol and galactitol. Chromatography was carried out on a DB-17 fused-silica column (15 m×0.25 mm I.D., 0.25 μm film thickness) (split injection, 4 mm I.D. injection port liner). The upper trace is from a mixed standard containing 73, 130, 132 and 126 pmol of each substance, respectively. The low intensity of the inositol peak is because *m/z* 127 is of low abundance in the mass spectrum of the butylboronate of myoinositol which is measured at *m/z* 139 (and which is not shown in the figure). The lower trace is of normal rat sciatic nerve and was obtained at ten times greater amplification than the upper trace. The concentrations of the substances in the tissue were: mannitol, 14 nmol/kg (wet weight); sorbitol, 70.6 nmol/kg; myoinositol, 4980 nmol/kg, galactitol, 8.0 nmol/kg. In both examples the tris-butylboronate of sorbitol is unexpectedly wide for its elution time. This is seen consistently and only on the capillary column (e.g., see Fig. 3).

umns. When the sorbitol derivative is chromatographed on the capillary column at a temperature that gives a retention time of 30 min the peak still shows no asymmetry that would suggest the presence of more than one component. Mass spectra taken across the peak show no evidence of the presence of another substance or of boronate conformational isomers. We could not detect any features in the mass spectra of the three hexitols that revealed any structural differences that would explain this property. Both commercial D-sorbitol and the metabolic product of the action of aldose reductase on D-glucose show the same characteristic. Derivatized galactitol from both sources similarly has a narrower peak width than sorbitol, even though it elutes later.

Gas chromatography with flame ionization or flame photometric detection

The choice of detector is mainly dictated by the sensitivity requirements of the analysis and by the complexity of the mixture to be analyzed. In general, the FPD is about 1.7-fold more sensitive than the flame ionization detector (FID). For example, 80 pmol of sorbitol butylboronate can be detected with a signal-to-noise ratio of 21:1 with an FID and 38:1 with the FPD using the 518-

nm filter. The sensitivity of the FPD with the normal phosphorus filter (530 nm) is about half that of the FID, but does retain the advantage of boron selectivity, being half-way between two major emission wavelengths of BO_2 (518.0 and 547.6 nm). Use of air as the combustion/scavenging gas with the FPD abolished the detector response toward boron for practical purposes.

Mass spectrometry

Both EI and CI MS were considered from the standpoint of the choice of internal standard and of maximizing sensitivity, which is necessitated by the low levels of alditols in control tissues.

The EI spectra of the tris-butylboronates of the hexitols are all similar, with the most intense ion at *m/z* 127 (Fig. 2, I). Labeling studies show that the ion is principally derived from carbon atoms 1, 2 and 5, 6 [14]. In EI MS of myoinositol the most intense ion in the spectrum of the tris-butylboronate derivative is found at *m/z* 139 (Fig. 2, II), the positional origin of which is not known. Thus the best choices for internal standard in EI MS are the fully labeled hexitols or inositol.

CI MS of myoinositol tris-butylboronate and of the hexitol tris-butylboronates produces predominantly pseudomolecular ions thus allowing the use of internal standards that are not uniformly labeled. In the positive ion CI spectra of the hexitol butylboronates, using ammonia as reagent gas, the most intense peak is $[MNH_4]^+$ (*m/z* 398); with methane as reagent gas the base peak is MH^+ (*m/z* 381). With negative ion detection and ammonia as reagent gas the hexitols form $[MNH_3]^-$ (*m/z* 397). With methane, *m/z* 397 is also formed, but with much lower intensity. Exclusion of ammonia from the mass spectrometer suggested that *m/z* 397 in methane CI is probably MOH^-. The spectra of myoinositol butylboronate have pseudomolecular ions $[MNH_4]^+$ (*m/z* 396) and MH^+ (*m/z* 379) with ammonia and methane, respectively. In negative ions, ammonia gives $[MNH_3]^-$ (*m/z* 395) and methane produces what again appears to be MOH^- (*m/z* 395), again of low intensity.

Quantitative gas chromatography–mass spectrometry

CI GC–MS of the four substrates was found to give less intense ions than EI GC–MS. On the EI HP 5970 the signal-to-noise ratio was 5:1 for 0.77 pmol of hexitol butylboronate injected (i.e., 0.077 pmol on-column with the 10:1 split

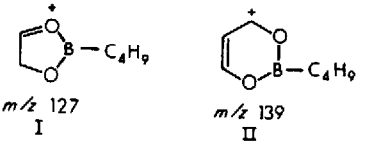

Fig. 2. Structures of the most intense ions in the EI spectra of the tris-butylboronates of hexitols (I) and myoinositol (II).

ratio). Thus a calculated signal-to-noise ratio for 10 pmol on-column would be 650:1. Table I shows the signal-to-noise ratios with which 10 pmol of the substrates of interest can be measured using CI GC–MS on a packed glass column (splitless injection). An approximation of the relative sensitivity of EI and CI can be made by comparing the EI GC–MS result above with the results in Table I. Signal-to-noise measurements made to compare capillary EI GC–MS with the flame detectors (which were run with packed columns) showed the flame detectors to be similar to CI GC–MS in sensitivity. Efforts to measure the hexitols in control tissues using the flame detectors gave unreliable results due to the poor sensitivity.

With packed columns, a 5-μl wash of butylboronic acid derivatizing reagent had to be injected between samples to eliminate analyte carry-over (ghosting) from one analysis to the next. As an additional measure to reduce carry-over the samples were run in groups with the low- and high-concentration individuals separated. With the capillary column, a 1-μl wash was used between samples. While ghosting on the capillary column was less than on the packed columns the samples were still run in groups with standards randomly mixed through the set. Injection port liners were changed daily.

With EI GC–MS, using the HP 5970 with the DB-17 column, the standard curve for the three hexitols and myoinositol was linear to about 1 pmol injected when [$^2H_6$]myoinositol was used as the internal standard.

Routine GC–MS analysis of tissue samples was carried out using EI with

TABLE I

SENSITIVITY BY CHEMICAL IONIZATION GC–MS OF 10-pmol SAMPLES OF HEXITOL AND MYOINOSITOL BUTYLBORONATES

Chemical ionization GC–MS using packed glass chromatography columns was performed on a Finnigan 3300 with methane or ammonia as reagent gases. Positive and negative pseudomolecular ions were detected. Samples of the derivatized substrates were diluted until a measure of the signal-to-noise ratio could be obtained using selected-ion monitoring and the signal-to-noise ratio for 10 pmol was then calculated. The positive ions used in the measurements of the hexitol butaneboronates were: with ammonia *m/z* 398 (MNH_4^+) and with methane *m/z* 381 (MH^+). With negative-ion detection *m/z* 397 is monitored with both ammonia (MNH_3^-) and with methane (MOH^-). Myoinositol butaneboronate was measured in positive ions at *m/z* 396 (ammonia, MNH_4^+) and at *m/z* 379 (methane, MH^+) and in negative ions at *m/z* 395 (which is MNH_3^- with ammonia and MOH^- with methane). The chromatographic conditions were similar to those shown in Fig. 3.

Reagent gas	Signal-to-noise ratio			
	Mannitol	Sorbitol	Galactitol	Myoinositol
NH_3 (MNH_4^+)	6	12	8	3
CH_4 (MH^+)	22	12	18	31
NH_3 (MNH_3^-)	12	14	30	26
CH_4 (MOH^-)	4	3	4	3

[$^2H_6$]myoinositol as internal standard. Because of concern that deuterium-labeled myoinositol would not serve as a satisfactory internal standard for sorbitol measurements we compared recoveries of the two substrates from red blood cells using [$^{13}C_6$]sorbitol in one case and deuterated inositol in the other. Table II shows the results of one such experiment in which the measurement of inositol appears to benefit from the use of deuterated inositol as internal standard while the results of the sorbitol measurement are not consistently improved by using the labeled sorbitol. [$^2H_6$]inositol was therefore used in most experiments. The deuterated inositol has the advantage of a lesser analytical blank because inositol elutes well separated from the hexitols. An advantage of using deuterated inositol is that there is no background signal from the internal standard at the retention time of the hexitols, which results in a more sensitive analysis of the hexitols which, in control animals, are always present in very small concentrations.

Figs. 1 and 3 are examples of the EI GC–MS analysis of sciatic nerve and retina from control rats. The low intensity of the myoinositol peak results from the use of *m/z* 127 to measure the derivatized hexitols; that ion is only of low intensity in the spectrum of the inositol derivative which is measured using ion monitoring of *m/z* 139. Using tissue samples of 31–38 mg wet weight from rat and with [$^2H_6$]myoinositol as internal standard the measured amounts of inositol, mannitol and sorbitol were as follows (nmol/g, mean ± S.D., $n=6$): retina (control), myoinositol, 1749 ± 707; mannitol, 8.9 ± 3.2; sorbitol, 100 ± 30.5; retina (diabetic), myoinositol, 1313 ± 403; mannitol, 15.6 ± 7; sorbitol, 1124 ± 630. With sciatic nerve the results were: myoinositol (control),

TABLE II

COMPARISON OF INTERNAL STANDARDS FOR THE ANALYSIS OF MYOINOSITOL AND SORBITOL BUTYLBORONATES

Red blood cells from two rats (A and B) were incubated with either 50 m*M* glucose alone or with 50 m*M* glucose plus 1 m*M* myoinositol. The samples were divided and either [$^2H_6$]myoinositol or [$^{13}C_6$]sorbitol added as internal standard, extracted, derivatized with butylboronic acid and then analyzed by EI GC–MS for sorbitol and myoinositol.

Rat	Additions to samples	Concentration (nmol/g wet weight)			
		[$^{13}C_6$]Sorbitol		[$^2H_6$]Myoinositol	
		Myoinositol	Sorbitol	Myoinositol	Sorbitol
A	No additions	27.2	3.7	27.6	3.9
B	No additions	31.5	1.9	38.1	1.5
A	Glucose alone	29.1	131.9	32.1	133.8
B	Glucose alone	17.9	41.3	19.5	44.4
A	Glucose + inositol	48.3	111.7	55.1	111.5
B	Glucose + inositol	34.8	47.7	41.7	49.9

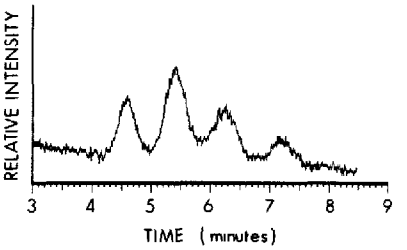

Fig. 3. Selected-ion monitoring electron ionization GC–MS trace of *m/z* 127 produced from the tris-butylboronate derivative of (in order of elution) mannitol, sorbitol, galactitol and myoinositol from normal rat retina. Chromatography was on a 2 m×4 mm I.D. glass column packed with 3% Dexsil-400. Note that, in contrast with Fig. 1, sorbitol has the expected peak width in this case and that the elution order of myoinositol and galactitol is reversed.

4780±437; mannitol, 17±2.5; sorbitol, 152±49.4; myoinositol (diabetic), 3675±650; mannitol, 25±6.3; sorbitol, 1324±787. Galactitol, if present, was below the detection limit, i.e., <2 nmol/g of tissue.

The method, as described, has been in use for over one year using GC–MS with an autosampler for most of that time. Extracts of lens, retina, sciatic nerve and erythrocytes have all been analyzed successfully, both from control and experimental animals. Consecutive analysis of up to 80 samples per day have been routinely carried out without operator attention during the analytical process.

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