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Enzymatic Assay of *myo*-Inositol in Serum¹⁾

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Summary: An enzymatic spectrophotometric method for determination of *myo*-inositol in serum is described. The method is sensitive and rapid to perform without special equipment. Linearity between the amount of *myo*-inositol and absorbance was obtained in the range of 0.5 to 3 nmol *myo*-inositol per assay. The amounts of *myo*-inositol determined in sera from apparently healthy subjects agree well with gas chromatographic data.

Introduction

myo-Inositol, a cyclic isomer of *D*-glucose, occurs in plants, animals and human beings in its free form and bound in phospholipids and phytic acid. There is evidence that alterations in *myo*-inositol metabolism may play a role in a number of diseases such as diabetes mellitus, renal and hepatic dysfunctions and atherosclerosis (1, 2). Despite this fact, data on *myo*-inositol content and metabolism are relatively scarce due to a lack of methods that combine high sensitivity and easy performance. Up to now *myo*-inositol levels have been measured by microbiological (3, 4), spectrophotometric (5, 6), fluorimetric (7) and gas-chromatographic (8, 9) methods. The present work describes a sensitive enzymatic assay for *myo*-inositol, which does not need special equipment and is easy to perform. The results have been presented in part elsewhere (10).

Materials and Methods

Chemicals

myo-Inositol, bathophenanthroline disulphonic acid, phenazine methosulphate, *myo*-inositol dehydrogenase²⁾ and 3-(*N*-morpholino) propanesulphonic acid were from Sigma (Taufkirchen, FRG). Glucose oxidase and catalase were from Boehringer (Mannheim, FRG). All other chemicals were from Merck (Darmstadt, FRG).

Subjects

Serum was obtained from 69 apparently healthy subjects, from 25 persons with impaired kidney function (creatinine > 106 μ mol/l) and from 56 patients with normal kidney function (creatinine < 106 μ mol/l), including 18 diabetics, 15 and 7 patients suffering from malignant diseases and diseases of heart and circulation, respectively, and 16 patients with various other diseases.

Sample preparation

To 0.3 ml serum 0.005 ml 300 mmol/l Na₃N and 0.02 ml glucose oxidase reagent (100 mmol/l 3-(*N*-morpholino)propanesulphonic acid buffer, pH 7.0, containing 700 kU/l glucose oxidase²⁾ and 3500 kU/l catalase²⁾) were added. After incubation in stoppered micro-vessels at 37 °C overnight, samples were deproteinized with 0.3 ml 1 mol/l HClO₄ prior to centrifugation. The supernatant was neutralized with solid KHCO₃. KClO₄ was removed by centrifugation and the clear supernatant was analysed for *myo*-inositol content.

Assay of *myo*-inositol

To 0.05 ml of the sample were added 0.1 ml distilled water and 0.05 ml of a 2:2:1 mixture of 25 mmol/l NAD⁺ in 1 mol/l K⁺-phosphate buffer, pH 9.0, 20 mmol/l bathophenanthroline

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²⁾ Enzymes:

Catalase, hydrogen peroxide: hydrogen peroxide oxidoreductase (EC 1.11.1.6)

Glucose oxidase, β -*D*-glucose: oxygen oxidoreductase (EC 1.1.3.4)

myo-Inositol dehydrogenase, meso-inositol: NAD oxidoreductase (EC 1.1.1.18)

disulphonic acid in 4 mmol/l FeCl_3 and 63 $\mu\text{mol/l}$ phenazine methosulphate. The reaction was started by addition of 0.01 ml *myo*-inositol dehydrogenase in 20 mmol/l K^+ -phosphate buffer, pH 7.0 (corresponding to 50 mU). After incubation at 25 °C for 60 minutes, absorbance at 546 nm was determined. Three different blanks were necessary in the assay. Sample blanks were run without *myo*-inositol dehydrogenase. In reagent blanks the sample was replaced by neutralized HClO_4 , and *myo*-inositol dehydrogenase by 20 mmol/l K^+ -phosphate buffer, pH 7.0. Since *myo*-inositol dehydrogenase itself displayed absorbance at 546 nm, correction for enzyme addition was performed by omitting the sample from the reagent mixture. Samples were read against absorbance of *myo*-inositol dehydrogenase, while sample blanks were read against reagent blanks (tab. 1).

myo-Inositol content of samples was read from a calibration curve prepared from aqueous *myo*-inositol standards, treated like serum samples. Phenazine methosulphate, bathophenanthroline disulphonic acid and *myo*-inositol dehydrogenase solution were prepared freshly every day, while FeCl_3 was stable for one week at 4 °C.

Statistical significance was calculated by *Student's* t-test for unpaired data.

Tab. 1. Procedure for measurement of *myo*-inositol.

Pipette into cuvette	Sample	Sample blank	Reagent blank	Enzyme blank
Volume (ml)				
Reagent mixture*	0.05	0.05	0.05	0.05
Sample (supernatant after neutralization)	0.05	0.05	—	—
Neutralized HClO_4	—	—	0.05	0.05
Distilled water	0.1	0.1	0.1	0.1
Start reaction by addition of				
<i>myo</i> -Inositol dehydrogenase	0.01	—	—	0.01
K^+ -phosphate buffer pH 7.0	—	0.01	0.01	—
Incubate for 60 min at 25 °C. Read samples against enzyme blank and sample blanks against reagent blank (546 nm).				
Calculation of <i>myo</i> -inositol concentration in serum:				
$\Delta A = A_{\text{sample}} - A_{\text{sample blank}}$				
Read <i>myo</i> -inositol concentration/assay (<i>c</i>) from calibration curve.				
Serum <i>myo</i> -inositol ($\mu\text{mol/l}$) = $\frac{c(\text{nmol}) \times \text{dilution factor (2.08)}}{\text{Sample volume (ml)}}$				

* Reagent mixture consists of 2 volumes of 25 mmol/l NAD^+ in 1 mol/l K^+ -phosphate buffer, pH 9.0, 2 volumes of 20 mmol/l bathophenanthroline disulphonic acid in 4 mmol/l FeCl_3 and 1 volume of 63 $\mu\text{mol/l}$ phenazine methosulphate, prepared immediately before the assay.

Results and Discussion

Enzymatic determination of *myo*-inositol is based on the NAD^+ -dependent oxidation of *myo*-inositol by *myo*-inositol dehydrogenase. Since the reaction is inhibited by NADH accumulation (11, 12), the generated NADH is used to reduce Fe^{3+} -bathophenanthroline disulphonic acid to Fe^{2+} -bathophenanthroline disulphonic acid (13), which is measured spectrophotometrically by an increase in absorbance at 546 nm (fig. 1). Under the conditions of the assay described, oxidation of *myo*-inositol does not proceed to completion, but NADH formation is proportional to *myo*-inositol concentration (5, 6). *myo*-Inositol was therefore determined in a fixed-time kinetic assay. Thus a calibration curve for determination of *myo*-inositol content of samples was required.

Due to the high molar lineic absorbance of the ferrous bathophenanthroline disulphonic acid complex (2150 m^2/mol (14)), the sensitivity of the assay allowed *myo*-inositol measurements as low as 0.5 nmol/assay (corresponding to about 6 $\mu\text{mol/l}$) which is one order of magnitude lower than that described by Weissbach (5, 6). Figure 2 shows that the increase in absorbance was linear up to 3 nmol/assay. For human serum there was a linear relationship between the amount of serum employed and the amount of *myo*-inositol recovered (fig. 3). *myo*-Inositol, added to human serum, was recovered in high yield (tab. 2). As to reproducibility, intra-assay coefficients of variation for the whole serum procedure (including glucose oxidase treatment, deproteinization and neutralization) were 7.5% (mean value = 45 $\mu\text{mol/l}$, $n = 7$) and 7.3% (mean value = 70 $\mu\text{mol/l}$, $n = 9$), respectively, while the between-run coefficient of variation was 9.5% (mean value = 40 $\mu\text{mol/l}$, $n = 9$).

As to specificity, serious interference was observed by glucose. Thus glucose concentrations of 5.5 and 27.7 mmol/l yielded values corresponding to 90 and 340 $\mu\text{mol/l}$ *myo*-inositol, respectively. Hence it is necessary to remove glucose by treatment of the samples with glucose oxidase prior to *myo*-inositol measurement. *myo*-Inositol in serum is stable against this treatment (data not shown). Ethanol (20 mmol/l) is slightly registered in the assay, seemingly corresponding to 7 $\mu\text{mol/l}$ *myo*-inositol.

No interference was found by the following substances (tested at 0.1 mmol/l): acetaldehyde, acetoacetate, N-acetylglucosamine, dihydroxyacetone, fructose, galactose, galactitol, glucosamine, glyceraldehyde, glycerol, β -hydroxybutyrate, mannose, mannitol, ribose, ribitol, sorbitol, xylose and xylitol. From these results it is reasonable to conclude that the assay is not subject to serious interference by substances pre-

Fig. 1. Enzymatic determination of *myo*-inositol (BPS = bathophenanthroline disulphonic acid) Fig. 2. Linear relationship between the amount of *myo*-inositol and absorbance at 546 nm ($\bar{x} \pm \text{SD}$, $n = 4$) Fig. 3. Linear relationship between *myo*-inositol found and the amount of serum employed

sent in serum, although the possibility could not be excluded that hitherto unknown substances will react in the assay.

Levels of *myo*-inositol in normal controls and in patients suffering from various diseases are summarized in table 3. The amount of *myo*-inositol in sera from apparently healthy subjects ranged from 12–

Tab. 2. Recovery of *myo*-inositol added to normal human serum.

<i>myo</i> -Inositol added ($\mu\text{mol/l}$)	n	<i>myo</i> -Inositol recovered mean ($\mu\text{mol/l}$)	Recovery (%)
50	4	55.3	111
100	6	111.3	111
150	6	155.2	103
200	6	198.4	99
250	6	247.7	99
300	6	302.2	101

Tab. 3. Serum *myo*-inositol levels in normal controls and in patients suffering from various diseases.

	n	Age (years)	<i>myo</i> -Inositol ($\mu\text{mol/l}$)			p
			$\bar{x}$	SD	Range	
Normal controls	69	19–62	31	9.3	12–53	
Patients with normal kidney function (creatinine < 106 $\mu\text{mol/l}$)						
– Diabetics	18	20–80	41	10.5	20–55	< 0.0005
– Malignant diseases	15	36–76	38	13.0	20–66	< 0.01
– Diseases of heart and circulation	7	47–85	34	13.2	18–51	n. s.
– Varia	16	21–81	33	11.8	13–61	n. s.
Patients with impaired kidney function (creatinine > 106 $\mu\text{mol/l}$)	25	25–86	218	152	51–598	< 0.0005

53 $\mu\text{mol/l}$ (mean value = 31 $\mu\text{mol/l}$). These values agree well with gas-chromatographic data (15–18). *myo*-Inositol levels are normally distributed and not dependent on sex or age.

In confirmation of reports from other laboratories (9, 15–17), *myo*-inositol was found to be markedly elevated in serum from patients with renal failure. Furthermore, in these patients a clear correlation was observed between *myo*-inositol and creatinine levels ($n = 25$, $r = 0.769$). A small but statistically significant increase in *myo*-inositol was also found in diabetics and patients suffering from malignant diseases.

Since the method described herein is sensitive and rapid to perform without special equipment, it may be useful not only for *myo*-inositol determination in serum, but also for the investigation of *myo*-inositol content and metabolism in biological fluids other than serum, or in tissues.

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