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Short communication

Determination of myo-inositol in biological samples by liquid chromatography–mass spectrometry

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Abstract

Due to the absence of HPLC methods to determine myo-inositol using mass detection and considering its sensitivity and selectivity, a high performance liquid chromatography-mass spectrometry method for the analysis of myo-inositol is described and applied to its direct determination in urine and saliva samples. Successful resolution of myo-inositol and its related substances was achieved with a stationary phase Aminex HPX-87C Column with milli-Q water as mobile phase and 5 mM ammonium acetate added post-column. The detector counted positive ions by monitoring $m/z = 198$, which corresponds to the myo-inositol adduct with ammonium cation. Urine and saliva samples were previously purified by passing through an anion-exchange resin. Concentrations as low as 138 and 461 μg/l in saliva and urine could be respectively quantified. Intra-day R.S.D. ranged from 0.83 to 1.02%, whereas inter-day R.S.D. was between 1.54 and 3.58%.
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1. Introduction

Myo-inositol plays a key role in an important intracellular signalling pathway, thus it is a key intermediate of the phosphatidyl-inositol cycle [1]. An altered myo-inositol metabolism has been associated with neural tube defects [2,3]. Cerebrospinal fluid inositol has been reported as decreased in depression and it seems that myo-inositol has therapeutic effects in psychiatry [4,5]. It is also known that several inositol polyphosphates are involved with calcium mobilization, moreover myo-inositol hexakisphosphate exhibits notable effects on preventing calcium oxalate and phosphate pathological crystallization [6,7]. For all these aspects, the determination of myo-inositol in biological samples could be a parameter of great interest.

Methods of analysis of myo-inositol can be classified in different groups. Enzymatic methods [8–11] use a myo-inositol dehydrogenase to oxidize myo-inositol. This reaction is NAD^+-dependent and NADH can be reoxidized with oxalacetate and malate dehydrogenase, measuring the resultant malate fluorimetrically [8]. Myo-inositol dehydrogenase can also be screened [10]. If reoxidation of NADH is performed with iodonitrotetrazolium chloride and diaphorase, the resultant formazan can be measured spectrophotometrically [11].

HPLC with refractive index or photometric detection [12] can be used. Recently, a new HPLC method has been published [13]; pulsed amperometric detection is used and no prior derivatization is requested.

Gas cromatography (GC) [14–16] gives better sensitivity than HPLC methods but requires previous derivatization to volatile compounds. Myo-inositol can be determined as trifluoroacetyl derivative [14] or hexa-O-trimethylsilyl ethers [15,16].

In this paper, we present a very sensitive HPLC/MS method which requires a small sample volume (an ordinary inositol urinary determination requires 20 ml of sample) and no prior complex purification or derivatization (thus reducing the sampling time compared with GC methods).

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2. Experimental

2.1. Chemicals

All chemicals were of analytical-reagent grade. The anion-exchange resin AG 1-X8 (200–400 mesh) was purchased from Bio-Rad (Hercules, CA, USA), myo-inositol (*cis*-1,2,3,5-*trans*-4,6-hexahydroxycyclohexane) from Fluka (Buchs, CH, Switzerland) and ammonium acetate from Probus (Badalona, Barcelona, Spain). Solutions were prepared with Milli-Q (18.2 MΩ cm) distilled-deionised water and filtered through 0.45 μm pore filters from Sugelabor S.A. (Madrid).

2.2. Apparatus

Analysis of myo-inositol was performed with an Agilent 1100 Series LC/MSD system. Chromatographic separations were performed at ambient temperature on a Aminex HPX-87C Column (300 mm × 7.8 mm i.d.), 9 μm particle size (supplied by Bio-Rad) with a 30 mm × 4.6 mm i.d. micro-guard cartridge (slurry-packed with aminex resin, supplied by Bio-Rad). The mobile phase (milli-Q water) was delivered at a flow rate of 0.6 and 0.2 ml/min of 5 mM ammonium acetate were mixed post-column with the mobile phase by using a three-way T mixing chamber. Mass spectral identification of myo-inositol was carried out with an electrospray ionization interface and a quadrupole mass analyzer. The mobile phase was nebulized by nitrogen gas at 350 °C, with a flow rate of 11 ml/min, into an electrospray mass analyzer. The detector counted positive ions with selected ion monitor (SIM) mode, by monitoring $m/z = 198$, which corresponds to the myo-inositol adduct with ammonium cation, the most abundant pseudo molecular ion. The nebulization pressure used was 413.7 kPa and the fragmentor voltage 85 V. Capillary voltage was 4000 V.

2.3. Sample preparation and standards

Urine samples were purified by passing 1 ml of the sample through an anion-exchange resin (0.2 g of AG 1-X8 resin packed into a 16 mm × 5 mm chromatographic column) in order to retain ionic compounds and thus reducing ionic strenght. The column was washed with 2 ml of distilled water and the eluted solution was diluted until a total volum of 10 ml before injection. 50 μl of this solution were injected in the HPLC/MS system.

Saliva samples were purified by passing 1 ml of the sample through an anion-exchange resin (0.2 g of AG 1-X8 resin packed into a 16 mm × 5 mm chromatographic column) in order to retain ionic compounds and thus reducing ionic strength. The column was washed with 1.5 ml of distilled water and the eluted solution was diluted until a total volum of 3 ml before injection. Fifty microlitres of this solution were injected in the HPLC/MS system.

Standards were prepared from aqueous solutions, in the same way as urine and saliva samples and were prepared immediately before use.

3. Results and discussion

Due to the absence of HPLC methods to determine myo-inositol using mass detection and considering that the sensitivity and selectivity of this type of detection makes the procedure specially attractive for biological samples with complex matrix as urine and saliva, the LC-MS system was selected to develop the presented procedure.

Successful separation of myo-inositol from its related substances was achieved with a stationary phase Aminex HPX-87C Column with milli-Q water as mobile phase and 5 mM ammonium acetate added post-column (Fig. 1). As can be appreciated, the first peak obtained corresponds to non retained substances. Working with glucose standards, two peaks were obtained (probably corresponding to α-glucose and β-glucose). These peaks are labelled. The second of them has the same retention time as scyllo-inositol, which was assigned by coincidence of retention times with standards. Published data on inositol isomers in urine concluded that the two major components are myo- and chiro-inositol

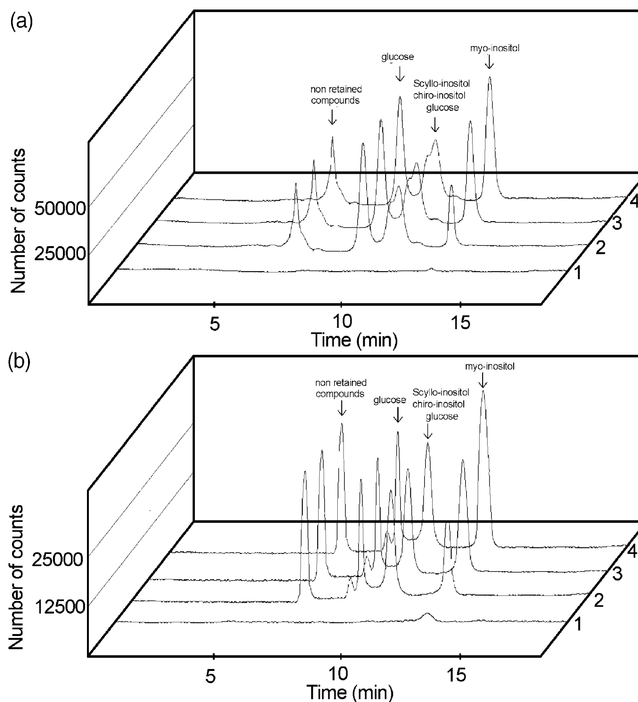

Fig. 1. (a) Typical SIM chromatogram at $m/z = 198$ of a urine sample. Obtained working as described in the Section 2. (1) Standard at the LOQ concentration level (2) Urine sample. (3) Urine sample + 10 mg/l myo-inositol. (4) Urine sample + 15 mg/l myo-inositol. (b) Typical SIM chromatogram at $m/z = 198$ of a saliva sample. Obtained working as described in the Section 2. (1) Standard at the LOQ concentration level (2) Saliva sample. (3) Saliva sample + 1.5 mg/l myo-inositol. (4) Saliva sample + 3 mg/l myo-inositol.

Table 1
Analytical figures of merit of the method

Calibration line[a]	
Slope ± S.D.	21514.8 ± 180.6
Intercept ± S.D.	101.6 ± 68.7
R^2	0.9997
$S_{y/x}$	99.3
Linear working range (mg myo-inositol l^{-1})	0–1.0
Limit of detection[b] (ng myo-inositol)	0.69
Limit of quantification[c] (ng myo-inositol)	2.31

[a] $y = \text{Slope} \times x + \text{intercept}$; x = myo-inositol concentration expressed as mg l^{-1}, $n = 5$.
[b] Calculated as $3S_{y/x}$/sensitivity.
[c] Calculated as $10S_{y/x}$/sensitivity.

[17]. Considering the very similar stereochemistry of scyllo- and chiro-inositol (1,3,5/2,4,6-hexahydroxycyclohexane and 1,2,5/3,4,6-hexahydroxycyclohexane) an almost identical polarity can be assumed, and both isomers (scyllo- and chiro-) are expected to have the same retention time (unfortunately chiro-inositol is not commercially available). Probably other inositol isomers and sugars appear with the same retention time. The last peak corresponds to myo-inositol.

Small changes in the ammonium acetate concentration did not produce any major changes in the detected signal of myo-inositol and related substances, demonstrating the robustness of the method.

There was a linear relationship between detector response and concentration over a concentration range of 0–10 mg/l for myo-nositol; however, the working range for this method was 0–1.0 mg/l (Table 1). The analysis must be performed during the 12 h after standard or sample preparation due to the instability of myo-inositol aqueous solutions. Thus, the stability of a standard aqueous solution of 1 mg/l was not superior to 24 h (at 48 h the loss of sample at room temperature was approximately of 50% and at 96 h the sample was totally destroyed). Due to the instability of aqueous solutions of myo-inositol, urine and saliva samples and standard solutions were storaged frozen at −20 °C.

The limit of detection of myo-inositol (calculated as $3\,S_{y/x}$/sensitivity) was 0.69 ng, while the limit of quantification (calculated as $10\,S_{y/x}$/sensitivity) was 2.31 ng. Thus, taking into account the dilution performed with saliva and urine samples and considering the fact that 50 μl of either standard or sample were injected, concentrations as low as 138 and 461 μg/l saliva and urine could be respectively quantified. These are very reduced amounts compared with those normally found in such samples.

As can be seen in Table 1, precision is also acceptable. The relative standard desviations of some selected standards ranged between 0.83 and 1.02% ($n = 5$) when analyzed at the same day, and between 1.54 and 3.58% ($n = 5$) when analyzed at different days, thus showing a good repeatability and reproducibility.

Table 2
Direct and adition standard analysis of several urine and saliva samples

Urine samples (myo-inositol)			Saliva samples (myo-inositol)		
Added	Found	Recovery (%)	Added	Found	Recovery (%)
Sample 1					
0	12.8		0	0.54	
10	21.8	90.4	1.5	1.99	97.1
15	24.1	75.8	3	3.44	96.7
Sample 2					
0	18.7		0	0.93	
10	29.3	106.3	1.5	2.51	104.8
15	34.3	104.2	3	4.03	103.3
Sample 3					
0	8.3		0	1.85	
10	15.8	75.1	1.5	3.51	110.0
15	19.2	72.4	3	5.14	109.5
Sample 4					
0	12.1		0	0.50	
10	20.6	84.3	1.5	1.83	88.9
20	29.2	85.6	3	3.59	103.1
Sample 5					
0	6.4		0	0.57	
10	15.0	86.0	1.5	1.95	92.0
20	23.6	85.8	3	3.42	95.1

Results are expressed as mg/l of myo-inositol.

The proposed HPLC/MS method has been used in the analysis of several urine and saliva samples, and recovery tests of added standard have been carried out. Results are summarized in Table 2. As can be seen, a satisfactory recovery was accomplished in all saliva samples and some urines, considering that recoveries between 80 and 120% are acceptable. Nevertheless, several urine samples showed low recovery values. In this way, three calibration lines were obtained, one working with standards and two more working with a pooled sample of urine and saliva, respectively, and adding standards at different concentration levels. Working with saliva, the slope of the obtained regression line ($y = 21536.3x + 6478$, $R^2 = 0.9975$, $n = 5$) did not show statistical differences with a 95% confidence level, compared with the obtained regression line when working with standards (see Table 1). However, the slope of the obtained line ($y = 18631.8x + 25450$, $R^2 = 0.9983$, $n = 5$) showed statistical differences with a 95% confidence level. This finding indicates that, depending on the urine matrix composition, a negative error could be obtained, but considering the linearity of the signal obtained by addition of standards, the standard addition method could be used in urine analysis to avoid such error.

Finally, to ensure the suitability of the proposed methodology determination of myo-inositol of several urine and saliva samples was compared using a different analytical procedure based on gas chromatography-mass spectrometry [14]. Results are shown in Table 3. As can be seen, there was a good agreement between present and GC/MS procedures, confirming the applicability of the method.

Table 3
Myo-inositol analysis of human urine and saliva samples applying two different analytical methods

Sample	Method GC-MS [14]	Method LC-MS (this paper)
Urine 1	11.8	11.6
Urine 2	14.9	14.1
Urine 3	7.1	7.5
Urine 4	14.9	15.3
Saliva 1	1.09	1.05
Saliva 2	0.35	0.54
Saliva 3	0.86	0.93
Saliva 4	0.09	0.13
Saliva 5	2.00	1.85

Results are expressed as mg/l of myo-inositol. In urine samples, the standard addition method was used. Both methods were comparable with a 95% confidence level. Regression graph for these samples was $y = 0.9842x + 0.0833$, $R^2 = 0.9969$, where y represents obtained concentration by the present method and x concentration by GC-MS method.

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