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Quantitative analysis of *myo*-inositol in urine, blood and nutritional supplements by high-performance liquid chromatography tandem mass spectrometry

Kit-Yi Leung[a,1], Kevin Mills[b,1], Katie A. Burren[a], Andrew J. Copp[a], Nicholas D.E. Greene[a,*]

[a] *Neural Development Unit, Institute of Child Health, University College London, 30 Guilford Street, London, WC1N 1EH, United Kingdom*
[b] *Clinical and Molecular Genetics Unit, Institute of Child Health, University College London, 30 Guilford Street, London, WC1N 1EH, United Kingdom*

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ABSTRACT

Myo-inositol plays key physiological functions, necessitating development of methodology for quantification in biological matrices. Limitations of current mass spectrometry-based approaches include the need for a derivatisation step and/or sample clean-up. In addition, co-elution of glucose may cause ion suppression of *myo*-inositol signals, for example in blood or urine samples. We describe an HPLC–MS/MS method using a lead-form resin based column online to a triple quadrupole tandem mass spectrometer, which requires minimum sample preparation and no derivatisation. This method allows separation and selective detection of *myo*-inositol from other inositol stereoisomers. Importantly, inositol was also separated from hexose monosaccharides of the same molecular weight, including glucose, galactose, mannose and fructose. The inter- and intra-assay variability was determined for standard solutions and urine with inter-assay coefficient of variation (CV) of 1.1% and 3.5% respectively, while intra-assay CV was 2.3% and 3.6%. Urine and blood samples from normal individuals were analysed.

1. Introduction

Inositol, hexahydroxycyclohexane, is a six-carbon sugar alcohol. Among the nine stereoisomers *myo*-inositol is the predominant physiological form while *scyllo*-, *epi*-, *neo*-, D-*chiro*- and *muco*-inositols may be present in low quantities [1]. *Myo*-inositol is incorporated into phosphoinositides and inositol phosphates, which have multiple cellular functions as signalling mediators and regulators of membrane trafficking [2–4]. In addition, many proteins are bound to the cell surface by a glycosylphosphatidylinositol (GPI) linkage [5].

Inositol has been described as a vitamin of the B group, however, in addition to dietary sources, inositol can be synthesised *de novo* from glucose 6-phosphate by the action of *myo*-inositol 3-phosphate synthase [1]. Nevertheless, it appears likely that exogenous inositol is important during development, at least in mouse and rat embryos, as inositol deficiency results in development of neural tube defects [6]. Inositol has been tested as a pharmacological intervention for a variety of conditions including depression, panic disorder and obsessive compulsive disorder [7]. In addition, elevated levels of *myo*-inositol have been reported in urine of diabetics (both insulin-dependent and non-insulin-dependent) [8,9].

The potential application of inositol as a pharmacological agent necessitates usage of methods for assay of inositol in biological matrices. Quantification of *myo*-inositol has made use of enzymatic assay [10,11], magnetic resonance spectroscopy [12] and gas chromatography/mass spectrometry (GC–MS) [13–15]. As GC–MS requires a derivatisation step, HPLC–MS methods have also been used for assay of saliva and urine samples, to utilise the sensitivity of mass spectrometry without requirement for a derivatisation step [16]. In order to exploit the enhanced specificity of tandem mass spectrometry, an LC–MS/MS method has also been described for quantification of *myo*-inositol in brain homogenate [17]. The potential limitation of this method was the applicability to samples that may contain glucose, such as urine and plasma, due to suppression of the *myo*-inositol signal. In order to overcome potential interference by glucose we developed an LC–MS/MS method with online chromatography to separate *myo*-inositol from common hexose monosaccharides, including glucose, as well as other inositol epimers.

Chromatographic separation of carbohydrates presents a challenge owing to the wide range of compounds with similar chemical and physical properties. For accurate MS-based quantification it is desirable to resolve inositol from other monosaccharides and sugar alcohols that may suppress the MS signal. Separation of inositol epimers is also essential for their differential quantification. Resin-based columns offer one approach for separation of small carbohydrates. Elution is in descending order of molecular size, with mono- and di-saccharides retained for longer than oligosaccharides. As many monosaccharides and sugar alcohols do not differ

* Corresponding author. Tel.: +44 2079052217; fax: +44 2078314366.
E-mail address: n.greene@ich.ucl.ac.uk (N.D.E. Greene).
[1] These authors contributed equally to the study.

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in molecular weight, further separation relies on other properties such as conformation and configuration. Choice of specific column chemistry depends on the carbohydrates to be analysed and in the current study we made use of a lead-form resin column with reported high resolution and specificity for monosaccharides. The LC–MS/MS method was tested for applicability to urine and plasma samples, as well as samples derived from inositol-containing pharmaceutical and nutritional supplements.

2. Materials and methods

2.1. Materials

Inositol epimers (*myo*-, *allo*-, *muco*-, D-*chiro*, L-*chiro*- and *scyllo*-), fructose, galactose, glucose and mannose were purchased from Sigma–Aldrich (Dorset, UK). The internal standard, [$^2H_6$]-*myo*-inositol (MID_6), was purchased from Cambridge Isotope Laboratories, Inc. (MA, USA). Solvents and water were all of LC–MS grade (Fisher Scientific UK Ltd., UK). Inositol food supplement (HB710) caplets (650 mg) were purchased from Holland & Barrett, UK. *Myo*-inositol tablets (500 mg) were manufactured by University College London Hospitals NHS Foundation Trust. In addition to *myo*-inositol, caplets and tablets contain bulking agent (starch), firming agent (povidone) and anti-caking agents (magnesium stearate).

2.2. Preparation of samples

2.2.1. Inositols, sugars and tablets

All inositols and sugars were dissolved in HPLC-grade water for LC–MS/MS analysis. For analysis of *myo*-inositol containing food supplements or pharmaceutical preparations, powder equivalent to one average weight of inositol tablet (from homogenous powder mix of 20 tablets) was dissolved in water, allowed to stand for excipient solids to settle, filtered through Whatman Grade 2V filter paper and diluted 20-fold with water (to a nominal concentration of 100 mg/l).

2.2.2. Urine

Neat urine was spiked with 10 μM [$^2H_6$]-*myo*-inositol and diluted with an equal volume of HPLC-grade water. The sample was centrifuged in a microfuge at maximum speed for 5 min and the supernatant was transferred to a sample vial for LC–MS/MS analysis. Creatinine levels were measured in a 1/20 dilution of urine in HPLC water, with addition of [$^2H_3$]-creatinine to 5 mM concentration. Creatinine was quantified by LC–MS/MS using established methods [18].

2.2.3. Plasma

Whole blood was collected into 4 ml potassium/EDTA-vacutainers and plasma was obtained after centrifugation at 3000 rpm at 4 °C. Proteins were precipitated from the plasma by addition of 2 volumes of acetonitrile, containing [$^2H_6$]-*myo*-inositol to give a final concentration of 10 μM. The protein precipitate was removed by centrifugation and the supernatant was transferred to eppendorfs for lyophilisation. Samples were resuspended in HPLC water and further centrifuged before transferring into sample vials for LC–MS/MS analysis.

2.3. HPLC

Separation of samples and standards was performed using a 2795XE high performance liquid chromatography (HPLC) unit with a column oven and a solvent divert valve (Waters, UK). For inositol analysis, separation was performed on a SUPELCOGEL Pb (300 × 7.8 mm; 5 μm; Supelco, UK) column, with an injection volume of 40 μl and the column temperature set at 60 °C. The HPLC protocol consisted of an isocratic gradient of 95% dH_2O: 5% acetonitrile over a 55 min period. The flow rate was 0.5 ml/min and was diverted to waste for the first 10 min after sample injection, to minimise accumulation of endogenous compounds on the ionisation source. For creatinine quantification, separation was performed on a pentafluorophenylpropyl (PFPP)-bonded silica column (Discovery HS F5; 50 mm × 2.1 mm (i.d.); 5 μm bead size; Supelco, Sigma–Aldrich), with an injection volume of 20 μl and column temperature set at 30 °C. Creatinine was eluted using 4 mM ammonium acetate (pH5.0) which was replaced with a gradient of 5–100% methanol over 10 min at a flow rate of 0.5 ml/min.

2.4. Mass spectrometry

The HPLC was coupled to a triple quadrupole tandem mass spectrometer (MicroMass Quattro; Waters, Manchester, UK) operating in negative-ion mode using the following settings: capillary 3.2 kV, source temperature 150 °C, desolvation temperature 350 °C, cone voltage 20 V, cone gas flow rate 90 l/h and desolvation gas flow rate 900 l/h. The final multiple reaction monitoring (MRM) transitions were as follows: inositol, 178.8 → 86.4; [$^2H_6$]-*myo*-inositol, 184.9 → 88.5, with cone voltage 35 V and 30 V, and collision energy 18 V and 20 V, respectively. For creatinine, MRM transitions were as follows: creatinine, 113.7 > 43.9; [$^2H_3$]-creatinine, 116.7 > 46.9. The MRM mode was used for quantification and data were acquired and processed using MassLynx software (Waters, UK).

3. Results

3.1. Mass spectra

Following the direct infusion of *myo*-inositol and [$^2H_6$]-*myo*-inositol into the MS/MS, a greater signal to noise ratio was apparent using negative ion mode compared with positive ion mode (data not shown). Thus, MS/MS conditions were optimised for the predicted [Inositol-H]$^-$ deprotonated molecule of *m*/*z* 178.8 for *myo*-inositol and *m*/*z* 184.9 for $^2H_6$-*myo*-inositol (Fig. 1). Of note, the [$^2H_6$]-*myo*-inositol internal standard is deuterated on the carbon backbone. Therefore, the deprotonated molecule (precursor ion) corresponds to a loss of hydrogen ([M−H]$^-$) as opposed to a loss of deuterium ([M−D]$^-$). Product ion spectra for *myo*-inositol and [$^2H_6$]-*myo*-inositol yielded specific fragment ions of *m*/*z* 86.4 and 88.5, respectively (Fig. 1). Although the most abundant product ions for *myo*-inositol and [$^2H_6$]-*myo*-inositol were *m*/*z* 160.8 and 166.8, respectively (Fig. 1), further analysis of this reaction (178.8 → 160.8) in urine samples demonstrated poor signal intensity, peak shape and reproducibility compared to the more specific reaction (178.8 → 86.4). Thus, the final MRM transitions were as follows: inositol, 178.8 → 86.4; [$^2H_6$]-*myo*-inositol, 184.9 → 88.5.

3.2. Chromatography

Despite the specificity provided by tandem mass spectrometry, in preliminary experiments significant suppression of the *myo*-inositol signal was observed due to co-elution with glucose (using either an Amide column or a Chiral AGP column as reported [17]). We therefore applied HPLC using a lead-form resin based column (SUPELCOGEL Pb; Supelco Bulletin 887B), which provided the ability to resolve *myo*-inositol from glucose and other hexose monosaccharides of the same molecular mass including fructose, galactose and mannose (Fig. 2). Separation of glucose and *myo*-inositol was confirmed using plasma samples in which both are present. Inositol epimers cannot be separately analysed on the basis of mass spectra, but by HPLC *myo*-inositol could be resolved from *scyllo*- and *muco*-inositol, as well as L- and D-*chiro*-inositol (Fig. 3).

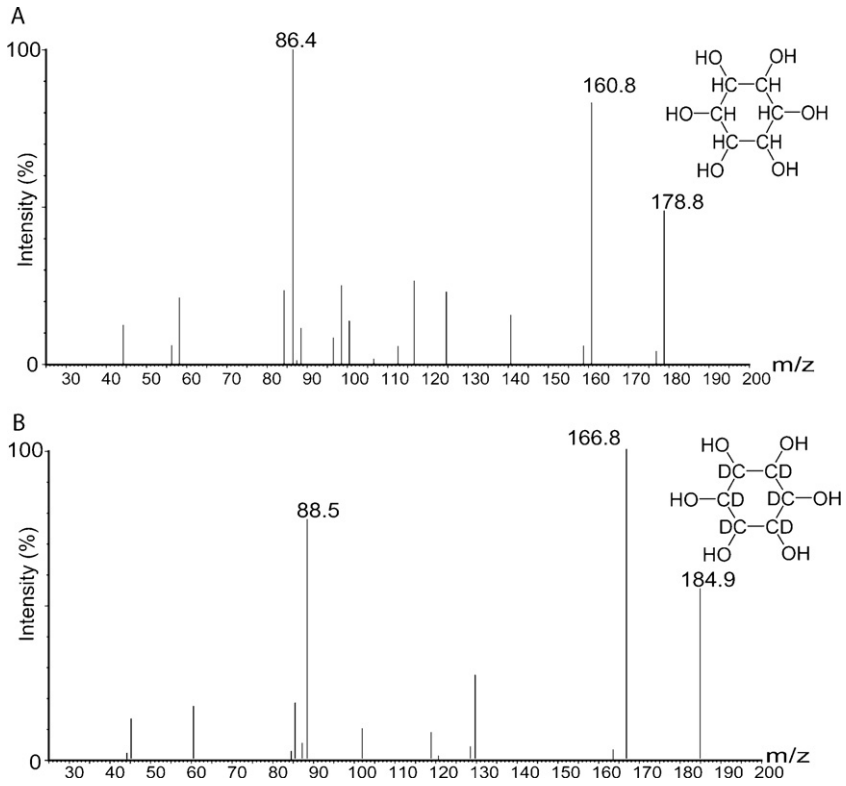

Fig. 1. Product ion spectra of deprotonated molecules used for quantification of *myo*-inositol. Precursor ions were *m*/*z* 178.8 for *myo*-inositol (A) and 184.9 for [$^2H_6$]-*myo*-inositol (B). The MS/MS conditions were optimised to favour transition to product ions at *m*/*z* 86.4 and 88.5.

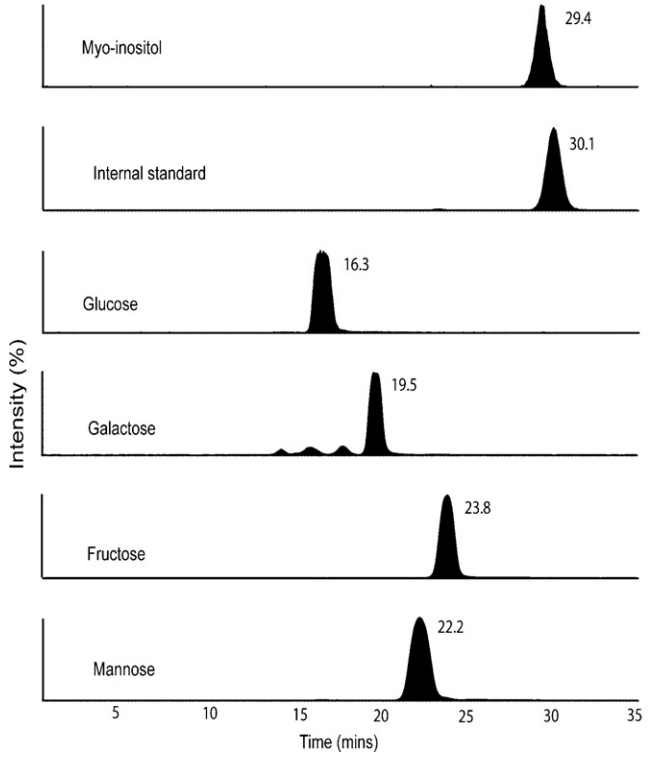

Fig. 2. LC–MS/MS chromatograms obtained in MRM mode for hexose sugars. Representative chromatograms are shown for aqueous standards of *myo*-inositol (unlabelled and [$^2H_6$]-labelled), glucose, galactose, fructose and mannose. MRM transitions were 179.33 > 88.97 for hexose sugars, 179.34 > 86.90 for *myo*-inositol and 185.34 > 88.90 for [$^2H_6$]-*myo*-inositol. Retention time is indicated for each peak. Elution of *myo*-inositol occurred 13 min later than for glucose.

3.3. Linearity and variability

The internal standard [$^2H_6$]-*myo*-inositol, was used for quantification of *myo*-inositol in urine and plasma as well as samples derived from inositol-containing tablets. Calibration curves were linear throughout a concentration range of 0–1000 μM in either aqueous solution or a matrix of urine (Fig. 4A and B). This range of concentrations was found to be suitable for the analysis of individual urine and blood samples. The coefficient of linear correlation (r^2) was 0.9995 and 0.9966 in aqueous and urine matrices, respectively. Generation of further calibration curves for concentrations of 0–50 μM showed that detection was achievable at least down to 2.5 μM inositol in aqueous and urine matrix.

The inter- and intra-assay variability was initially determined using a *myo*-inositol standard solution (Table 1). For intra-assay variability, a known 277 μmol of *myo*-inositol (50 mg/l), was

Table 1
Repeatibility and reproducibility of *myo*-inositol quantification. Intra- (within experiment) and inter-assay variability was determined for aqueous standard solutions, samples derived from inositol-containing tablets (pharmaceutical preparation) and urine samples. MS runs for determination of inter-assay variability were performed on 6 separate days, such that the 25 runs for aqueous standards were divided between these days.

Sample	No. of MS runs	Mean concentration of *myo*-inositol (μM ± S.D.)	CV (%)
Aqueous standards			
Intra-assay	24	589.8 ± 6.5	1.1
Inter-assay	25	590.2 ± 13.6	2.3
Pharmaceutical preparation			
Intra-assay	6	446.4 ± 5.7	1.3
Inter-assay	6	458.1 ± 1.1	2.4
Urine sample			
Intra-assay	5	388.4 ± 14.0	3.6
Inter-assay	6	403.4 ± 14.3	3.5

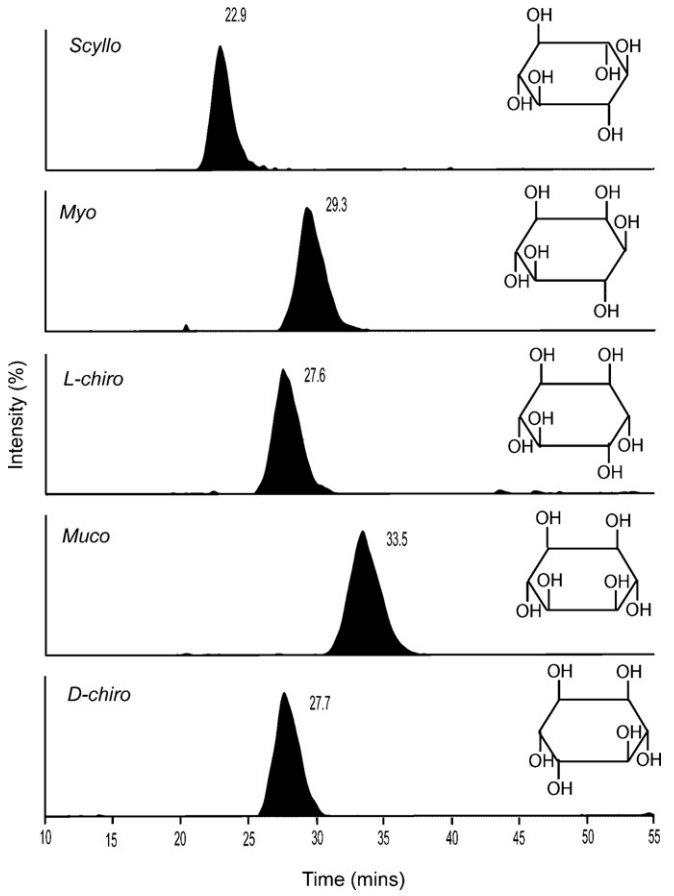

Fig. 3. LC–MS/MS chromatograms obtained in MRM mode for inositol epimers. Representative chromatograms are shown for standard solutions of *scyllo*, *myo*, D- and L-*chiro* and *muco*-inositol. The SRM transition for MS/MS detection was 179.34 > 86.90. The structure and retention time for each epimer is indicated. Using the Pb column *myo*-inositol could be clearly resolved from *scyllo*- and *muco*-inositol, while there was partial overlap of *myo*- and *chiro*-peaks. D- and L-*chiro*-inositol were not resolved.

repeatedly analysed ($n = 24$) over a continuous period, with a CV of 1.1%. The inter-assay variability for standard solution was determined by six separate experiments over an 18 day period ($n = 25$ assays in total), with overall CV of 2.3% (for individual experiments, CV = 0.34–3.01%). Repeated analysis of samples prepared from *myo*-inositol containing pharmaceutical preparations and urine samples confirmed the repeatability and reproducibility of the method (Table 1).

3.4. Urine and plasma analysis

Myo-inositol was present at measurable quantities in all urine samples analysed. In some samples a second peak was also detected. The retention time for this peak most closely matched that of scyllo-inositol as reported previously [16], but we cannot exclude the possibility that this peak could correspond to the *chiro* epimer [8]. Thus, *myo*-inositol was the most abundant inositol epimer present in urine, as expected. To determine the precision for analysis of urine samples, replicate experiments were performed containing 10 μM internal standard [$^2H_6$]-*myo*-inositol. The intra-assay coefficient of variation (CV) was 3.6% and the inter-assay CV was 3.5% (Table 1).

Myo-inositol was quantified in samples of urine and blood plasma collected from healthy volunteers (Table 2). *Myo*-inositol levels in urine were normalised to the concentration of creatinine. Creatinine is produced at a constant rate by the body and is excreted from the body entirely by the kidneys, and the measurement of creatinine in urine therefore acts as an index of urinary excretion rate. This provides a means to normalise the concentration of metabolites in urine and *myo*-inositol was thus expressed as units per mmol of creatinine

Inositol is reputedly a very stable molecule. To assess the stability of inositol in urine samples, samples were collected from four

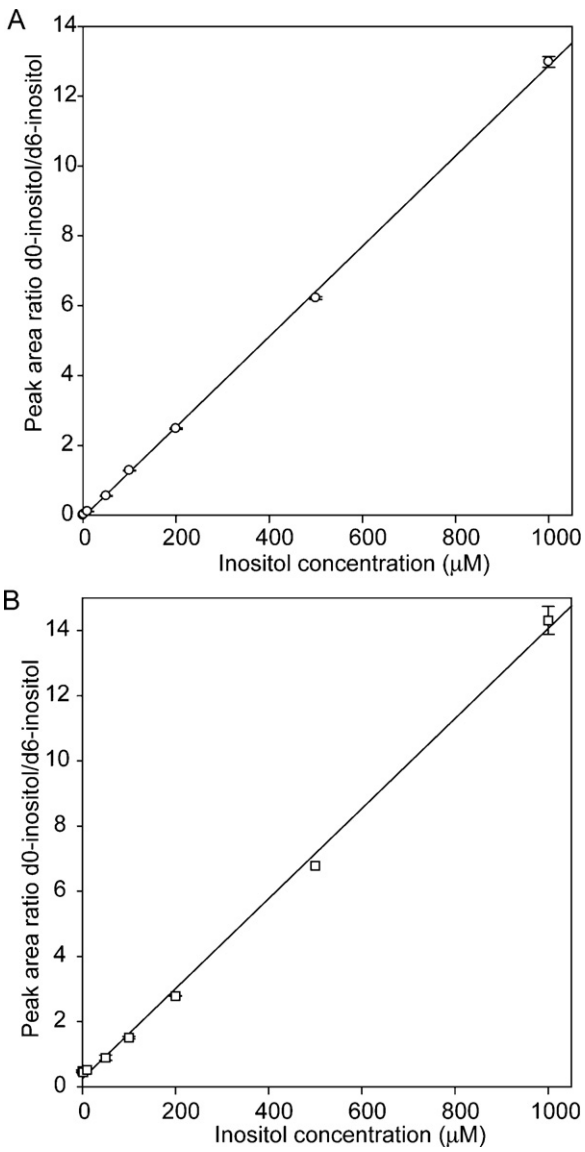

Fig. 4. Linearity of *myo*-inositol assay. Calibration curves were generated using (A) standard aqueous solutions, and (B) urine spiked with known amounts of *myo*-inositol (the *y*-intercept reflects the endogenous inositol). Data are given as mean ± standard deviation for each solution. The response is linear with a coefficient of linear correlation (r^2) of 0.999 for aqueous and 0.997 for urine matrices, respectively.

Table 2
Assay of *myo*-inositol in urine and plasma in control subjects. LC–MS/MS was used to determine levels of *myo*-inositol in urine and plasma of 10 control subjects (healthy adult volunteers). Inositol concentration in urine was normalised to concentration of creatinine. The mean value is given together with the range of values observed among the individuals tested.

	Mean value (±S.E.)	Range of values
Urine (μM)	138.2 ± 27.5	21.2–287.9
Urine (μmol/mmol creatinine)	18.8 ± 2.9	4.5–38.7
Plasma (μM)	32.5 ± 1.5	26.8–43.0

individuals and each divided between four tubes, one of which was stored immediately at −20 °C, and the others kept at room temperature for 24, 48 or 142 h prior to storage at −20 °C. Subsequent analysis of the samples (in the same LC–MS/MS run) did not reveal significant changes in abundance of *myo*-inositol over 48 h. Evaluation of the inositol concentration at different time points as a function of the baseline value (time 0) showed that on average the concentration was 98.3% of baseline at 48 h, and 94.0% of baseline at 142 h. These data suggest that *myo*-inositol is stable in urine kept at room temperature for at least 48 h, without major decline in concentration even up to 6 days.

4. Discussion

In order to quantify inositol in biological material we developed an LC–MS/MS method to exploit the sensitivity and selectivity of tandem mass spectrometry and to avoid the need for a derivatisation step. The method reported here allows direct analysis of urine without the need for sample clean-up procedures and therefore minimises sample loss during processing. As reported previously, inositol and other hexose monosaccharides of the same molecular mass can be distinguished by MS/MS on the basis of differing fragmentation pattern. This results in product ions of differing mass, presumably due to the pyranoside structure of hexose monosaccharides compared with the hexahydroxycyclohexane structure of inositol [17].

Despite the specificity of MS/MS significant suppression of the *myo*-inositol signal by glucose necessitated use of appropriate chromatography to allow analysis of samples, such as blood and urine, in which both inositol and glucose may be present. We made use of a lead-form resin column that offers high resolution and selectivity for monosaccharides. Monosaccharides and sugar alcohols, including inositol, exhibit longer retention on the column, whereas larger carbohydrates cannot enter the resin pores, have less interaction with counter-ions and elute earlier (Supelco Bulletin 887B). We confirmed resolution of glucose, galactose, fructose, mannose and inositol over a 55 min run. Elution onto the MS at different times overcame signal suppression by co-eluting molecules. The run duration could be reduced using a shorter column (10 cm), which would likely allow separation of glucose and inositol but at the expense of resolution of different inositol epimers.

The approach that we describe differs from published methods in several respects. Use of LC–MS [16] has the drawback that molecules with the same ion mass cannot be distinguished, and we therefore applied MS/MS which provides increased selectivity. The method of Perello et al. [16] used an Aminex column which did allow separation of *myo*-inositol and glucose. However, this chromatography gave rise to two major glucose peaks, one of which co-eluted with *scyllo*-inositol. In addition, an initial anion-exchange resin purification step was used, which is not necessary with the current method. A previously reported LC–MS/MS method made use of an NH_2 column [17], which did not resolve inositol from glucose (or other monosaccharides and sugar alcohols). A chiral column (Chiral-AGP, Chromtech) was also used and shown to resolve four inositol epimers [17] but does not separate *myo*-inositol and glucose (our studies and confirmed by manufacturer). Although glucose is not detected using the MS/MS parameters for inositol, we find that co-eluting glucose can significantly suppress the inositol signal. Therefore, for accurate quantitative analysis of inositol in biological samples which contain glucose separation of inositol from monosaccharides is necessary.

In this study the concentration of *myo*-inositol in urine varied quite widely between individuals, even when normalised to creatinine. This variation may reflect differences in dietary intake of inositol as well as differential catabolism of inositol in the kidneys. In the same group of individuals the concentration of *myo*-inositol in plasma exhibited a smaller range (27–43 μM). Compared to previous studies of normal individuals, the mean urine concentration of around 140 μM that we observed is broadly similar to the 60–80 μM determined by LC–MS [16]. Similarly, the mean plasma concentration of 32 μM determined in the current study is comparable to the value of around 22 μM reported using GC–MS [19].

5. Conclusions

We have developed an LC–MS/MS method which allows quantification of *myo*-inositol in biological fluids such as blood and urine as well as in pharmaceutical preparations. Calibration curves in water or urine matrices were linear between 2.5 and 50 μM and analyses of intra- and inter-assay precision indicate that the method is robust and reproducible.

Acknowledgments

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