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DETERMINATION OF PHYTIC ACID IN URINE BY INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

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Introduction

Phytic acid (inositol hexakisphosphate) is a naturally occurring component in integral cereals and plant seeds[1]. A variety of benefits of phytic acid on human health are now recognized[2-4], including an interesting beneficial action on some pathological processes such as calcium urolithiasis by preventing, at very low concentrations, the development of renal solid concretions. Consequently it has been proposed as a promising drug to treat calcium oxalate and phosphate stone formers[5,6], since in humans between 1-10% of the total ingested phytic acid is excreted by the urine[7]. Accordingly, increasing the urinary excretion of phytic acid is recommended to diminish the risk of calcium oxalate and calcium phosphate kidney stone recurrence[8].

The growing interest in the biomedical implications of phytic acid and the inherent problems with its detection are reflected in the diverse analytical techniques used for its determination[9-14]. However, the sensitivity of most of the current methodologies does not permit to ordinary laboratories an easy application to determine phytic acid in biological fluids (as urine) due to the low levels found in such a complex matrix. Furthermore, previous methods for phytic acid determination in urine[13,14] are characterized by long, tedious sample treatment (purification, hydrolysis, derivatization, freeze-drying…) preceding the measurement of the analytical signal.

In this paper, a method for phytic acid determination in urine based on the purification of phytic acid by anion exchange and total phosphorus determination in the purified extract by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry), as a high sensitive detection system. Thanks to its low detection limit, ICP-MS as detection system allow for an accurate determination of low amounts of phytic acid in urine through total phosphorous quantification after an effective anion-exchange separation process from other phosphorous compounds present in the sample matrix, with no need of pre-concentration step even when using low amounts of sample (1 mL of urine).

Objective

The aim of this work is to provide a simple and accurate analytical procedure to easily determine phytic acid in urine, in contrast with time-consuming current methods.

Methods

Reagents and apparatus

All chemical used were of analytical-reagent grade. Myoinositol hexaphosphoric acid hexasodium salt from corn was purchased from Sigma (Steinheim, Germany). AG 1x8 200-400 mesh, chloride form, anion exchange resin was from Bio-Rad Laboratories (Hercules, CA, USA).

pH was measured using a Crison pH meter micropH 2002 (Barcelona, Spain) with a combined glass electrode. ICP-MS model PQ-Excell (Thermo Elemental, UK) was used for phytic acid determination by total phosphorus measurement.

Procedures

In order to improve the mechanical characteristics of the anion-exchange separation of phytic acid from phosphate and other phosphorus compounds present in the urine matrix, urine samples are filtered through a 0.45m µpore filter prior to the purification process. Then, 1.0 mL of filtered urine is transferred quantitatively to a solid phase extraction cartridge, prepared with 1.5 mL-syringe reservoir with

20 µm frits containing 0.2 g of AG 1x8 resin, which was conditioned with HCl 50 mmol L^{-1} before use. Phosphate and some other matrix components of urine are eluted with 50 mL of HCl 50 mmol L^{-1}. Finally the column is washed with 2 mL of HCl 2 mol L^{-1} to elute phytic acid. These purification processes are run by gravity (flow rate of 0.33 mL min^{-1}).

The 2 mL of purified extract are diluted to 10 mL in a volumetric flask to avoid problems related with total acid content in the ICP-MS system. The determination of phytic acid is then carried out through ^{31}P analysis of the purified extracts of phytic acid from urine by ICP-MS using ^{45}Sc (10 µg L^{-1}) as an internal standard.

Urine samples were analysed immediately after collection in thermostatic containers. Otherwise urine samples should be acidified with HCl to pH 3-4 to avoid precipitation and subsequent adsorption of phytic acid on the precipitates formed, which could lead to deficient recoveries of phytic acid from analysed samples.

Results

Study of the separation/recovery processes

The main problem of phytic acid determination in urine by ICP techniques lies in the presence of very low concentrations of phytic acid (10^{-6} mol L^{-1}) in a matrix rich in phosphate (10^{-2} mol L^{-1}).

Consequently, urinary phytic acid determination through phosphorus analysis by ICP-MS requires an extensive purification of phytic acid.

According to previous papers, the best results of purification and recovery of phytic acid from anionic columns are obtained using AG1-X8 columns, which show an extremely high selectivity for phytate and the capability of concentrating phytate from very diluted extracts[15].

Thus, anion exchange chromatography may be a useful technique to effectively separate phytic acid from inorganic phosphate. Separation is pH-dependant: phytic acid is strongly dissociated even at low pH values (pKa1 = -0.15, pKa2 = 0.41, pKa3 = 0.85, pKa4 = 1.84)[16] and is strongly retained in the resin, whereas phosphate, which has a lower dissociation at low pH values (pKa1 = 2.12, pKa2 = 7.20, pKa3 = 12.36)[17] is relatively easily eluted from the resin by using diluted HCl.

Optimisation of the phytic acid/phosphate separation and phytic acid recovery processes was carried out by using phosphate and phytic acid standards dissolved in water. Some of the results obtained after carrying out the separation/recovery process are summarized in Table I. 50 mL of eluent A (HCl 50 mmol L^{-1}) are required for the total elution of inorganic phosphate without significant elution of phytic acid and 2 mL HCl 2.0 mol L^{-1} are required as eluent B for the subsequent elution of the retained phytic acid.

Table I. Optimisation of phytic acid separation/recovery process.

Phosphate (mg L^{-1})	Eluent A [a]	Eluent B	Phytic acid		
			Added (mg L^{-1})	Found (mg L^{-1})	Recovery (%)
-	50 mL	5.0 mL HCl 0.4 mol L^{-1}	4.00	0.98	24.5
-	50 mL	2.0 mL HCl 1.0 mol L^{-1}	4.00	2.65	66.3
-	50 mL	1.0 mL HCl 2.0 mol L^{-1}	4.00	3.20	80.0
-	50 mL	1.5 mL HCl 2.0 mol L^{-1}	4.00	3.69	92.3
-	10 mL	2.0 mL HCl 2.0 mol L^{-1}	4.00	3.98	99.5
-	30 mL	2.0 mL HCl 2.0 mol L^{-1}	4.00	4.00	100.0
-	50 mL	2.0 mL HCl 2.0 mol L^{-1}	4.00	4.01	100.3
1500	30 mL	2.0 mL HCl 2.0 mol L^{-1}	-	0.02	-
2500	30 mL	2.0 mL HCl 2.0 mol L^{-1}	-	0.05	-
3500	30 mL	2.0 mL HCl 2.0 mol L^{-1}	-	0.08	-
1500	50 mL	2.0 mL HCl 2.0 mol L^{-1}	-	0.00	-
2500	50 mL	2.0 mL HCl 2.0 mol L^{-1}	-	0.01	-
3500	50 mL	2.0 mL HCl 2.0 mol L^{-1}	-	0.00	-

[a] Eluent A was HCl 50 mmol L^{-1}

Some real urine samples were analysed in presence and in absence of phytic acid spikes (Table II). Our results of phytic acid recovery from real urine samples show recoveries ranging from 98-102%, indicating a good accuracy in the phytic acid determination without the need of pre-treatment other than filtering urine samples through 0.45µm pore filters.

Table II. Analysis of some human urine samples (mean±standard error of prediction; three determinations).

Sample	Phytic acid		
	Added (mg L^{-1})	Found (mg L^{-1})	Recovery (%)
A	-	1.05±0.04	-
	1.12	2.19±0.03	101.8
B	-	1.59±0.04	-
	0.97	2.54±0.03	97.8
C	-	0.86±0.05	-
	0.97	1.82±0.04	99.2

Analytical features of the method

Linearity and Limit of Detection

Linear range (0.02-0.6 mg phytic acid L^{-1}) and limit of detection (5 µg L^{-1} phytic acid) are adequate for analysis of usual amounts of phytic acid in urine (0.2 to 6.0 mg L^{-1})[13,14].

Study of Interferences

Urine samples present a complex matrix that contains potentially interfering species. Main interferences could be consequence of high amounts of inorganic phosphate and/or pyrophosphate in the urine samples that could difficult the separation process of phytic acid from other sources of phosphorus in the urine samples. pH of urine samples is another factor that could possibly affect the recovery of phytic acid from samples. Thus, the effect of phosphate, pyrophosphate and pH have been studied (Table III). A good recovery was obtained for almost all samples analysed, except for those with the highest phosphate spike and highest pH, due to precipitation of calcium phosphates from urine and subsequent adsorption/occlusion of phytic acid in the

precipitates formed, indicating that phosphate, pyrophosphate and pH do not interfere the phytic acid determination, even with values clearly exceeding the physiological urinary range. However, if a precipitate forms from urine, deficient recoveries of phytic acid from analysed samples will be obtained. Thus, acidification with HCl to pH 3-4 to avoid precipitation from urine is greatly recommended, especially if sample is not analysed immediately after collection.

Accuracy and precision

The accuracy of the method was determined by the evaluation of phytic acid recoveries from 18 first-morning urine samples analysed by contrast with aliquots of spiked amounts of phytic acid. Recoveries ranged from 96 to 104%, indicating good accuracy for the proposed method.

RSD values of 4.6, 2.8 and 2.0% were obtained from five replicates of real samples that, according to this procedure, contained 0.34, 0.90 and 1.59 mg phytic acid L^{-1}, respectively.

Table III. Study of interferences. Determination of phytic acid in presence of phosphate, pyrophosphate and pH exceeding their usual physiologic ranges. Original sample contained 1225 mg L^{-1} of total P and pH 5.38. Normal physiologic values: phosphate (<1100mg·day^{-1}), pyrophosphate (3.5-7.0 mg·day^{-1}), pH (5.5-6.5), normal diuresis (800-1400 mL).

Phosphate added (mg L^{-1})	Pyrophosphate added (mg L^{-1})	pH	Phytic acid	
			Found (mg L^{-1})	Recovery (%)
-	-	5.38	0.93	-
1500	-	-	0.94	101.2
3000	-	-	0.91	98.0
4500	-	-	0.92	99.0
6000	-	-	0.94	101.2
7500	-	-	0.88	94.7
-	12.5	-	0.96	103.3
-	25.0	-	0.96	103.3
-	37.5	-	0.95	102.3
-	50.0	-	0.91	98.0
-	-	3.90	0.91	98.0
-	-	4.98	0.95	102.3
-	-	6.06	0.96	103.3
-	-	7.00	0.83	89.3

Application of the method to urolithiasic patients

Applicability of the developed method was demonstrated by determining the phytic acid content of first-morning urine samples of healthy people as well as oxalocalcic lithiasic patients. Results collected in Table IV suggest that low urinary phytic acid concentration and low urinary phytic acid/calcium molar concentration ratios values are related with oxalocalcic urolithiasis. Thus, the proposed method can have a promising application in the prediction of risk to develop calcium urolithiasis illness.

Table IV. Phytic acid concentration in first-morning urine of oxalocalcic lithiasic patients and healthy controls. Values are represented as mean ± SD.

	Healthy controls	Oxalocalcic lithiasic patients
Individuals	**8**	**7**
pH	**5.67±0.14**	**5.57±0.20**
Ca (mg L^{-1})	**165±50**	**210±100**
IP6 (mg L^{-1})	**1.19±0.36**	**0.82±0.27 ***
IP6 / Ca ($x10^5$)	**45±16**	**24±13 ***

*** Significantly different from healthy controls by Student's t-test ($p < 0.05$)**

Conclusions

The applicability of ICP-MS facilities on the determination of phytic acid in urine has been demonstrated. The lack of selectivity of ICP-MS measurement for phytic acid in urine is satisfactorily overcome by an extraordinarily selective anion-exchange purification process of phytic acid from other phosphorus components present in urine matrix.

In contrast, ICP-MS gives excellent sensibility and low detection limits for accurate determination of phytic acid through ^{31}P measurement, even when low amounts of sample are used. The good agreement obtained between the phytic acid content in spiked aliquots and the values found in natural urines, demonstrate the suitability of the proposed analytical procedure.

Main characteristics of the method are its simplicity, low sample requirement, relatively high sample throughput (10 to 6 minutes per sample for runs between 50 to 100 samples, respectively), and high selectivity and sensibility. Thus, the present method provides the best alternative to current methods suitable for phytic acid determination in biological samples (such as urine), especially when considering their scarcity and long sample pre-treatments (including several steps for purification, hydrolysis, derivatization, etc.).

This method can contribute to elucidate the action of phytic acid in urolithiasis and the possible relation of low phytic acid content in human samples with renal lithiasis. Accordingly, the developed method could be easily used by clinical chemists to establish normal and pathological levels of phytic acid in urine for a rational diagnostic or treatment of the urolithiasic illness.

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