Urol Res (2012) 40:663–669
DOI 10.1007/s00240-012-0473-3

ORIGINAL PAPER

A simple and rapid colorimetric method for determination of phytate in urine

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Received: 27 October 2011 / Accepted: 24 March 2012 / Published online: 5 April 2012

Abstract Phytate is a natural product present in urine and biological fluids that is associated with health benefits, such as the prevention of calcium renal stone formation. The available methods for phytate analysis in urine all require elaborate instrumentation and cannot be routinely applied in clinical laboratories. Here, we describe a simple procedure for urinary phytate determination, employing colorimetric detection. Our method requires purification and preconcentration of phytate via solid-phase extraction prior to colorimetric detection employing Fe(III)–thiocyanate. The working linear range of the assay is 0–5 μM phytate. The limit of detection is 0.055 μM. The relative standard deviation obtained upon assay of samples containing 2 μM phytate was 3.5 %. Several urine samples were analyzed using an alternative method based on the detection of phosphorus; the results of the two assays were comparable. Our novel method of phytate analysis in human urine is simple, rapid (3 h for 10 samples), accurate, precise, reliable, and highly sensitive. The assay can be run in most analytical laboratories and does not require sophisticated instrumentation.

Keywords Phytate · Urinary levels · Solid-phase extraction · Colorimetric method

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Introduction

Phytate is a natural product normally present in the human diet. Although phytate has been classified as an antinutrient because, during gastrointestinal passage, the molecule may interfere with the absorption of some essential trace elements and minerals [1–3], important beneficial effects of phytate have recently been described. These include both antioxidant and anticancer activities [4, 5]. Phytate inhibits calcium salt crystallization, prevents renal stone formation, and reduces the extent of other forms of pathological calcification [6–8]. Phytate also has positive effects on blood glucose and cholesterol levels [9, 10]. The roles played by phytate in human nutrition and health continue to be intensively studied [11].

Determination of phytate levels in biological samples, particularly urine, presents several challenges. Phytate is present at very low concentrations (of the order of 1 μM [12]); the urine sample matrix is highly variable; and no simple sensitive phytate quantitation method is available. The current methods of urinary phytate determination require sophisticated instrumentation and pretreatments including purification, hydrolysis, evaporation, or digestion. It is difficult to routinely employ such procedures.

Sample pretreatment principally involves solid-phase extraction (SPE) to separate phytate from other urinary components.

Most detection methods for phytate employ one of two approaches based on the properties of the phosphate moiety [13–15] or procedures that detect inositol [16, 17]. Both methods require hydrolysis of the molecule. No direct photometric reaction of phytate is available because the molecule has no absorption bands in the UV–visible light region and no reagent reacts directly with phytate to form a colored compound. Thus, photometric methods for phytate

determination are indirect, and are all based on a side reaction of phytate, normally complexation of a metal, which decreases the absorption of a colored form of that metal upon reaction with a specific reagent [18]. Photometric methods allowing determination of phytate without hydrolysis of the molecule have been successfully applied to measure phytate levels in cereals, roots, and biological specimens [18–29]. However, with the exception of assays employing YCl_3–[4-(2-pyridylazo)resorcinol] (PAR), such tests are not adequately sensitive to detect phytate in biological samples in the absence of significant preconcentration.

To overcome these obstacles, new strategies are necessary. Both a sensitive and reliable photometric method for phytate detection, and an effective sample pretreatment for purification and preconcentration of phytate from urine are needed. The method also needs to be less tedious to better adapt to clinical laboratory routine.

Inhibition of calcium salts crystallization and prevention of renal stone formation through dietary phytate have been described [6, 11]. Due to the interest of phytate in renal lithiasis, in this paper we present a fast, simple, and reliable method for phytate determination in human urinary samples.

Materials and methods

Apparatus

Absorption was measured using a Microplate Spectrophotometer Powerwave XS (Biotek Instruments, Inc., Winoosk, VT, USA).

Reagents and solutions

All chemicals were of analytical reagent grade. Potassium thiocyanate, sodium persulfate, Fe(III) nitrate, sodium chlorate, and NaCl were purchased from Sigma (St. Louis, MO, USA). Ultra-pure deionized water from a Milli-Q system was used to prepare all solutions. Anion exchange resin AG 1-X8 (chloride form; 200–400 mesh) was purchased from Bio-Rad Laboratories (Richmond, CA, USA).

Phytic acid dipotassium salt (Sigma P5681, MW = 736.22 g/mol) was used to prepare standard phytate solutions.

Phytate colorimetric determination method

Purification and preconcentration

Urine samples (20 mL), acidified with HCl to pH 3, were diluted with 20 mL of deionized water and quantitatively transferred to 100 mL beakers each containing 0.5 g AG1-X8 resin without previous conditioning. During the following 15 min, each mixture was stirred several times, using a glass rod, to facilitate absorption of phytate to the resin. Resin and urine were transferred to an empty 20 mL SPE tube with a frit and urine was passed through to separate it from the resin and was discarded. The resin was washed with 120 mL 50 mM HCl and 5 mL of deionized water. Finally, phytate was eluted into 4 mL of 2 M NaCl. The elution was performed in four steps, employing 1 mL of elution solvent in each step, maintaining the resin and each 1 mL portion in contact during 5 min, and stirring it at least five times with a glass rod. The final 4 mL of eluate was collected into a single tube and the solution was mixed prior to phytate quantification.

Colorimetric detection

The reagent (R) used for phytate quantification was prepared daily, just before use, by mixing equal volumes of two solutions (DI and DII), prepared as follows. DI was a mix of 8 mL KSCN (5 M) with 2 mL glycine/HCl buffer (0.5 M; pH 2). DII was a mix of 5 mL of $Fe(NO_3)_3$ (5 mM) prepared in HNO_3 (5 mM) with 2.5 mL of $NaClO_3$ (0.4 M) and 5 mL sodium persulfate (75 mM).

Phytate standards in the range 5–25 μM were prepared daily in NaCl (2 M).

The assays were conducted in 96-well plates after mixing of 50 μL R and 300 μL of a standard or an eluate. Absorbance was measured at 460 nm 5 min after mixing.

Phytate ICP-AES determination method [13]

Five milliliters of fresh urine (acidified with HCl 1:1 to pH 3–4) was transferred to a column (inner diameter 4 mm) containing 0.2 g of anion exchange resin AG1-X8. The first eluate was discarded, and then the column was washed with 50 ml of 50 mM HCl. The second eluate was discarded. Then, phytate was eluted with 3 ml of 2 M HNO_3. The determination of phytate was carried out by direct phosphorus analysis of this last eluate using the ICP-AES and appropriate calibration graph.

Study of interferences

Compounds potentially interfering with colorimetric detection were studied. Phosphate and sulfate were present in synthetic urine (Table 1). Ascorbic acid, uric acid, oxalate, citrate, and pyrophosphate were added at concentrations in their normal urinary ranges, to synthetic urine (Table 2) without and containing 2 μM phytate, and subjected to the above-indicated phytate colorimetric determination method.

Table 1 Composition of synthetic urine

Solution A (mM)		Solution B (mM)	
$Na_2SO_4 \cdot 10H_2O$	19.34	$NaH_2PO_4 \cdot 2H_2O$	15.45
$MgSO_4 \cdot 7H_2O$	5.93	$Na_2HPO_4 \cdot 12H_2O$	15.64
NH_4Cl	86.73	NaCl	223.08
KCl	162.60		
$CaCl_2$	7.00		

Synthetic urine was obtained by mixing equal volumes of solutions A and B

Table 2 Study of interferences

Added substance	Concentration (mM)	Phytate added (μM)	Phytate found (μM)
None		0	0.029
		2	2.076
Phosphate	15.0	0	0.040
		2	1.987
Oxalate	0.30	0	0.019
		2	1.962
Citrate	1.00	0	0.014
		2	1.988
Oxalate + citrate	0.30 + 1.00	0	0.032
		2	2.007
Uric acid	2.38	0	0.100
		2	1.870
Oxalate + uric acid	0.30 + 2.38	0	0.128
		2	2.140
Pyrophosphate	0.06	0	0.050
		2	2.107

Determination of phytate when applying the phytate colorimetric determination method (see "Materials and methods") to synthetic urine containing 0 or 2 μM phytate, to which interfering substances where added. Synthetic urine contained 15.5 mM of phosphate and 12.5 mM of sulfate

Assay validation

Linearity and detection limit

Triplicates of six standards of phytate in NaCl 2 M in the range from 0.0 to 25.0 μM, prepared with adequate dilution of 1.0 mM phytate (0.7378 g/L of phytic acid dipotassium salt, MW = 736.22 g/mol) were used to study linearity.

Limit of detection and limit of quantification were calculated as three and ten times the standard deviation of the blank reagent for phytate concentration ($n = 3$), respectively.

Precision and recovery evaluation

The intra-day precision of the method was evaluated by analysis of two concentrations of phytate (1 and 2 μM) prepared by adding corresponding volumes of 1.0 mM phytate stock solution in synthetic urine (Table 1). Five replicates were assayed each day, and the mean, standard deviation, and relative standard deviation (RSD or CV) were calculated.

Inter-day precision was calculated by analyzing the same samples (five replicates) on each of four different days; again, the mean, standard deviation, and relative standard deviation were calculated.

Results and discussion

Colorimetric detection

Colorimetric detection of phytate in the absence of hydrolysis is based on the ability of phytate to complex metals; this decreases the color intensity of a dye–metal complex because of competition between phytate and the dye. This is the principle of phytate detection using Fe(III)–sulfosalicylic acid [18–21], Fe(III)–thiocyanate [25, 26], or YCl_3–PAR [27–29] complexes.

The three systems described above were used in efforts to detect phytate in urine. Detection based on reaction of phytate with Fe(III)–sulfosalicylic acid was improved, and was linear in the range 0–40 μM phytate. However, the absorbance values were low. Also, the slight yellow-brownish color of human urine eluates increased absorbance values, thus erroneously lowering the phytate concentrations detected. Each eluate color reflected the original color of the particular urine sample and could not be controlled. For this reason, we discarded this phytate detection system.

Detection based on reaction of phytate with YCl_3–PAR was also improved upon the addition of a buffer and a surfactant. However, intra-day absorbance varied considerably and high CVs were observed when replicates of the same standard were assayed. The reaction was very sensitive to phytate, being linear in the range 0–6 μM. Unfortunately, PAR forms colored complexes with a wide variety of metal ions and, although the YCl_3–PAR detection of phytate is highly sensitive it suffers from some instability and high day by day variations.

Finally, we chose a procedure based on formation of an Fe(III)–thiocyanate complex. To avoid reduction of Fe(III), an oxidant such as sodium persulfate, was added at a level sufficiently low to prevent reaction with thiocyanate.

We observed that, even in the presence of an oxidant, absorbance decreased at a low rate, and the decrease was higher in samples as compared to standards. For this reason, only a short time (5 min) was allowed to elapse prior to absorbance measurement.

In an effort to avoid the potential Fe(III)-reducing effect of Cl^-, solutions containing sodium salts of non-reducing anions (NO_3^-, ClO_3^-, SO_4^-) were tested as eluents instead of NaCl 2 M, following the purification and preconcentration process described in "Materials and methods". Obtained phytate recovery was very low (from 50 to 60 %) for these three compounds. Thus, 2 M NaCl was chosen as the elution solvent.

SPE variables

Optimization of phytate purification and preconcentration and recovery was achieved by dissolving known amounts of phytate in synthetic urine.

During previous experiences to determine phytate in urine using SPE in column format, in some cases, depending on urine composition and content of organic matter, partial clogging of the column was observed, that led to an uncontrolled sample flow rate and much longer times for sample passage. For that reason, the batch format was chosen and, although SPE tubes are used for the wash and elution steps, in no case it can be considered as a column format since the resin is not packed. The SPE tubes are used because they are the easiest systems to perform wash and elution steps simultaneously for several samples.

To optimize the use of AG1-X8 resin, preliminary tests employing different amounts of resin were performed using 50 mL samples of synthetic urine containing 1 μM phytate, and different wash volumes of HCl (50 mM) (Fig. 1). When either 1.0 or 1.5 g AG1-X8 was employed, the

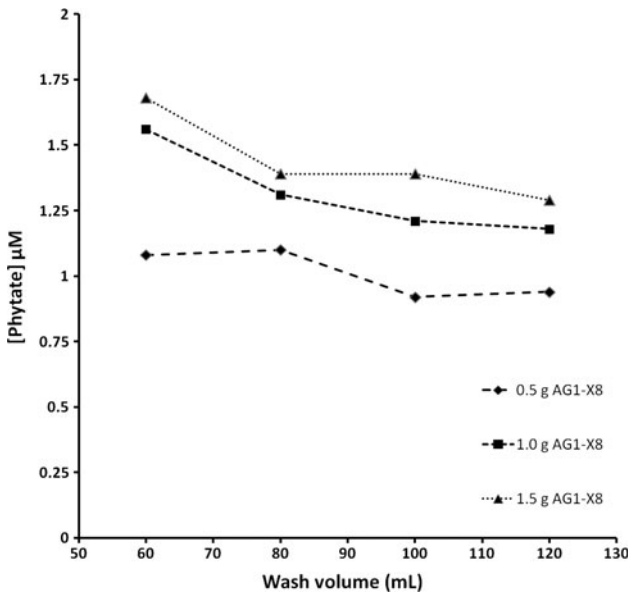

Fig. 1 Measured concentrations of phytate in 50 mL of synthetic urine containing 1 μM phytate using different amounts of AG1-X8 resin and various wash volumes of HCl 50 mM. (Elution with four 1-mL portions of NaCl 2 M)

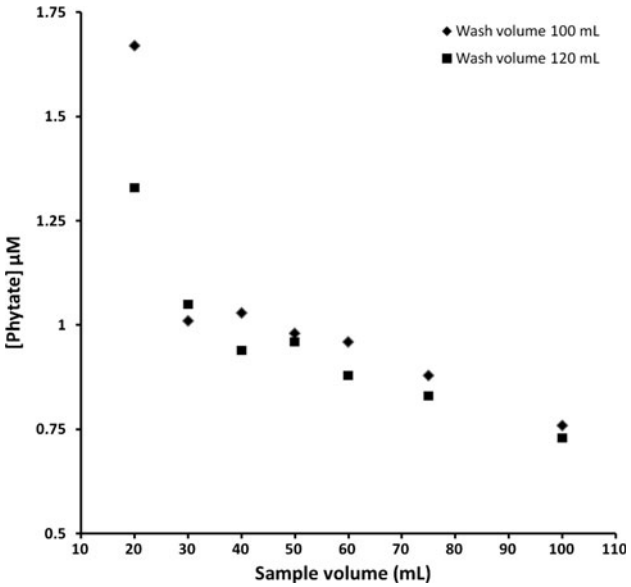

Fig. 2 Measured concentrations of phytate in different synthetic urine volumes containing 1 μM phytate, and using different wash volumes of HCl 50 mM. (500 mg AG1-X8, elution with four 1-mL portions of NaCl 2 M)

Table 3 Use of different urinary sample volumes for determination of phytate levels [500 mg AG1-X8 resin; wash volume 120 mL of HCl (50 mM) + 5 mL distilled water; elution volume 4 mL NaCl (2 M)]

Sample	Sample volume (mL)	Phytate		
		Added (μmol/L)	Found (μmol/L)	Recovery (%)
Urine 1	40		1.00	
	40	1.0	1.85	85
	50		0.87	
	50	1.0	1.65	78
	60		0.61	
	60	1.0	1.71	110
Urine 2	30		1.26	
	30	1.0	2.04	78
	40		0.84	
	40	1.0	1.71	87
	50		0.74	
	50	1.0	1.82	107
Urine 3	20		1.70	
	20	1.0	2.28	58
	30		1.77	
	30	1.0	2.38	61
	40		1.44	
	40	1.0	1.97	53

phytate concentration determined was higher than that actually present, indicating that interfering substances were adsorbed by the resin and the wash volume used was

Table 4 Use of different urinary sample dilutions for determination of phytate levels [500 mg AG1-X8 resin; wash volume 120 mL of HCl (50 mM) + 5 mL distilled water; elution volume four 1-mL portions of NaCl (2 M)]

Sample	Sample volume (mL)	Dilution	Phytate		
			Added (μmol/L)	Found (μmol/L)	Recovery (%)
Urine 4	40	None		1.12	
	40	None	1.0	1.50	38
	20	1/2		1.41	
	20	1/2	1.0	2.12	71
Urine 5	40	None		0.69	
	40	None	1.0	1.31	62
	20	1/2		0.82	
	20	1/2	1.0	1.53	71
Urine 6	40	None		0.14	
	40	None	1.0	0.82	67
	20	1/2		0.42	
	20	1/2	1.0	1.20	78
Urine 7	40	None		1.20	
	40	None	1.0	2.10	90
	20	1/2		1.91	
	20	1/2	1.0	2.79	88
Urine 8	40	None		1.34	
	40	None	1.0	2.09	75
	20	1/2		1.99	
	20	1/2	1.0	2.73	74

Table 5 Features of the colorimetric detection of phytate

Parameter	Result
λ_{max} (nm)	460
Calibration range (μM)	0–25
Regression equation	$y = -(0.0252 \pm 0.0014)x + (1.16 \pm 0.06)$
Correlation coefficient	0.998 ± 0.001

insufficient to remove such materials. Thus, 0.5 g of the resin was chosen as optimal. Next, all of sample, wash, and elution volumes were simultaneously studied. The minimum elution volume was determined by stepwise elution with 1 mL amounts of solvent, and was 4 mL of 2 M NaCl. The optimal sample and wash volumes are shown in Fig. 2; these were determined using synthetic urine containing 1 μM phytate. A sample volume of 30–60 mL and a wash volume of 120 mL were optimal for phytate determination in synthetic urine samples.

Application of the method to human urine samples revealed that lower urine volumes, and a 1/2 dilution, were optimal (Tables 3, 4); this was because the total ionic concentration of real urine samples was in some instances much higher than that of synthetic urine. Thus, although recovery improved when a greater sample volume was used, the phytate concentration obtained was higher at a lower sample volume (Table 3), indicating that separation was compromised by an excess of ions. Therefore, human urine samples were diluted in efforts to optimize phytate determination (Table 4). Such dilution either improved or did not change the extent of recovery of added phytate, although, in all instances, an increase in the observed phytate concentration was noted.

As human urine composition is very variable, and in light of the results obtained, the final selected sample volume for phytate determination was 20 mL of urine, diluted 1/2.

Study of interferences

Compounds interfering with colorimetric detection were studied, with a focus on substances normally present in urine that have the capacity to complex Fe(III) or that possess redox activity. Sulfate did not interfere with the reaction. Thus, each of ascorbic acid, uric acid, oxalate, citrate, phosphate, and pyrophosphate were added, at different concentrations, to synthetic urine without and with 2 μM phytate. The results are shown in Table 2.

As can be seen, in all instances, the obtained results were equivalent to those found for synthetic urine without the potentially interfering substances. Thus, the applied SPE effectively removed all interfering substances.

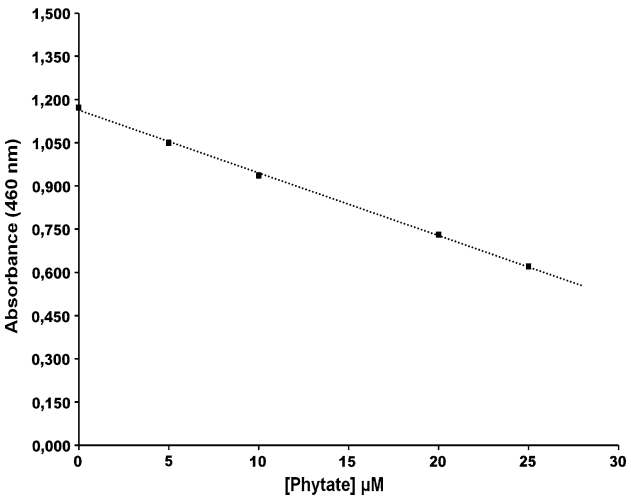

Fig. 3 Calibration graph for phytate using Fe-SCN method, following the procedure indicated in "Materials and methods". Phytate standards were prepared in NaCl 2 M with adequate dilution of 1.0 mM phytate (0.7378 g/L of phytic acid dipotassium salt, MW = 736.22 g/mol). Each point represents the mean of three determinations

Characteristics of the method

Various features of the colorimetric detection of phytate are shown in Table 5. Calibration graph is presented in Fig. 3; the limit of detection is 0.275 μM. Considering that preconcentration was employed, the limit of detection is 0.055 μM phytate in urine and the limit of quantification 0.440 μM.

Intra- and inter-day precision values were obtained, and a recovery study was conducted, using solutions of 1 and 2 μM phytate in synthetic urine (see "Materials and methods"). The results are shown in Table 6.

Table 6 Analytical characteristics of the phytate colorimetric determination method (see "Materials and methods") using solutions of 1 and 2 μM phytate in synthetic urine

	Mean phytate concentration found (μM)	SD	Recovery (%)	CV
Intraday ($n = 5$) (1 μM)	0.97	0.07	97	7.05
Intraday ($n = 5$) (2 μM)	1.89	0.07	95	3.56
Interday ($n = 4$) (1 μM)	0.97	0.06	97	6.65
Interday ($n = 4$) (2 μM)	1.91	0.05	96	2.48

Table 7 Analysis of phytate in human urine employing the developed phytate colorimetric determination method and the phytate ICP-AES determination method [13] (see "Materials and methods")

Sample	Phytate colorimetric determination method (μmol/L)	Phytate ICP-AES determination method (μmol/L)
Urine 9	1.92 ± 0.02	1.46 ± 0.39
Urine 10	1.51 ± 0.04	1.99 ± 0.43
Urine 11	1.61 ± 0.03	1.46 ± 0.24
Urine 12	1.11 ± 0.05	1.04 ± 0.30
Urine 13	1.08 ± 0.04	1.25 ± 0.69
Urine 14	0.88 ± 0.02	1.17 ± 0.21
Urine 15	0.62 ± 0.06	0.48 ± 0.39

Values expressed as means ± SDs, $n = 3$

Phytate determination in human urine

Finally, the utility of the method was checked by determining phytate levels in human urine samples in comparison with another method that employs ICP-AES phosphorus detection for phytate quantitation [13] (Table 7). It is important to note that the system of quantification of phytate using either assay differs. Our novel method measures phytate exploiting the ability of phytate to complex Fe(III), whereas the alternative assay quantifies phytate by measuring the levels of phytate phosphate groups. The results of the two methods were in good agreement (Table 7).

Conclusions

All methods used to analyze phytate in urine have their own advantages and disadvantages. Our procedure has important advantages over other methods, including simplicity, the short time required for analysis (about 3 h for 10 samples), and the lack of any need for elaborate instrumentation. The proposed method for phytate analysis in human urine is reliable and accurate, and can be routinely employed by most analytical laboratories. In conclusion, the proposed method is simple, rapid, selective, accurate, precise, and highly sensitive.

Acknowledgments This work was supported by Fundació Barceló (Ref. no. 1458/2007) and project grant CTQ2010-18271/PPQ from the Ministerio de Ciencia e Innovación (Gobierno de España). I.G. expresses her appreciation to the Conselleria d'Innovació i Energia del Govern de les Illes Balears for the award of a fellowship supporting her work.

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