ANALYTICA CHIMICA ACTA 605 (2007) 185–191

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/aca

Fluorimetric determination of phytic acid in urine based on replacement reaction

Yingyu Chen[a], *Jingwen Chen*[b,*], *Kang Ma*[a], *Shuhong Cao*[b], *Xiaoqing Chen*[a,*]

[a] *School of Chemistry and Chemical Engineering, Central South University, Changsha 410083, PR China*

[b] *School of Chemical and Biological Engineering, Yancheng Institute of Technology, Yancheng 224003, PR China*

ARTICLE INFO

Article history:
Received 19 June 2007
Received in revised form
23 October 2007
Accepted 23 October 2007
Published on line 4 November 2007

Keywords:
Determination
Fluorescence
Phytic acid
Replacement reaction

ABSTRACT

A sensitive fluorimetric method for determination of phytic acid in human urine samples was described. The method was based on a fluorimetric replacement reaction, in which the added phytic acid replaced the Cu^{2+} ion from Cu^{2+}–gelatin complex, liberating the fluorescent gelatin molecule. The fluorescence of the solution was accordingly recovered proportionally to the amount of the foreign phytic acid. The excitation wavelength was 273.5 nm and the characteristic emission wavelength was 305.0 nm, respectively. The calibration graph was obtained by plotting the recovered fluorescent intensity at maximum 305.0 nm against the added standard phytic acid, and was divided into two sections. One section was linear over the range of 0.40–2.40 mg L^{-1} with a linear regression equation of $I_f = -0.895 + 15.146c$ ($R^2 > 0.9993$), and the other over the range of 2.40–9.20 mg L^{-1} with a linear regression equation of $I_f = -29.526 + 26.113c$ ($R^2 > 0.9996$), respectively. The relative standard deviation (R.S.D.) at 95% confidence degree for a 2.0 mg L^{-1} of standard phytic acid within 1 month was less than 1.26% ($n = 5$), indicating the procedure is reproducible. The detection and the quantification limits of phytic acid were estimated to be 0.23 and 0.40 mg L^{-1}, respectively. The proposed method was applied to the determination of phytic acid in urine samples and the found concentrations of phytic acid in urine were in the range of 0.49–0.75 mg L^{-1} with recoveries of 96.2–108.8%. Comparison of the obtained results with the reported HPLC was performed, indicating the proposed method was reliable.

1. Introduction

Phytic acid, *myo*-inositol hexaphosphoric acid ($InsP_6$), a fully phosphorylated form of inositol [1], is a naturally occurring component and the principal storage source of phosphorus in many plants, such as cereals, soybeans, legumes, oil seeds, pollens, nuts, fruits and vegetables [2–4]. In $InsP_6$ molecule, there are 12 replaceable protons, among which six are strongly ($pK_a < 3.5$) and six are weakly ($pK_a = 4.6$–10) dissociated [5]. Therefore, $InsP_6$ can be deprotonated and exists as a series of polyanions over a wide pH range [6]. Moreover, owing to the presence of the six phosphate groups in very close proximity [7], $InsP_6$, including its deprotonated species, possesses the highest affinity among its lower phosphorylated entities for chelating metal ions such as Fe^{2+}, Fe^{3+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+} and Ca^{2+}, as well as proteins and starch [7,8]. Among

* *Corresponding authors*. Tel.: +86 515 8298394; fax: +86 515 8298394.
E-mail addresses: jwchen@ycit.edu.cn (J. Chen), xqchen@mail.csu.edu.cn (X. Chen).
0003-2670/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2007.10.041

the common metal ions, $InsP_6$ has the highest affinity for Cu^{2+} near neutral pH in aqueous solution [9]. $InsP_6$ binds with the essential elements in human body, forming complexes with low solubility at physiological pH [9,10], and thus potentially influences their bioavailability [11]. Hence, $InsP_6$ has long been considered solely as an antinutrient and related studies attracted a great deal of interest in human nutrition [12]. However, a variety of epidemiological studies on living organisms demonstrated that $InsP_6$ and its lower phosphorylated forms have beneficial functions. For example, it could reduce the risk of cancers [13–15], heart disease, diabetes, and renal calculi [11,16,17]. In addition, $InsP_6$ can also bind potentially toxic mineral elements such as Cd^{2+} and Pb^{2+} in human body, and thus influences their toxicity and facilitates their excretion [18].

Due to both potentially detrimental and beneficial effect of $InsP_6$, it is very necessary to develop a reliable method for the determination of $InsP_6$ in order to make a valuable evaluation on its metabolism in human body. Studies have demonstrated that most urinary $InsP_6$ originates from the diet though human self can also synthesize this compound [19]. Therefore, investigations on the content of $InsP_6$ in urine sample can provide information for its dietary intake and metabolism in human body [20]. The analysis of $InsP_6$ in various matrices was usually performed by means of chromatography [21–23] and capillary isotachophoresis [24]. Among them, ion pair chromatography [21,25] and high-performance ion chromatography [26] are the commonly used methods, in which most are capable of simultaneous separation and determination of $InsP_6$ and its lower phosphorylated species. Other methods are based on the direct/indirect determination of phosphorus [27] or inositol [28] in $InsP_6$ or its quantitative hydrolytic products by spectrophotometry [29], NMR spectroscopy [30] and inductively coupled plasma atomic emission spectrometry [31]. However, limited reports are available on the quantification of $InsP_6$ by means of fluorescent method. Based on the activation effect of $InsP_6$ on the oxidation of 2,2′-dipyridyl ketone hydrazone catalysed by Cu^{2+} ion and the property that the oxidation product is highly fluorescent, March et al. [32] first developed a fluorimetric measurement procedure for $InsP_6$ in human urine and food samples. A strategy based on a replacement reaction, in which a metal complex is dissociated proportionally to the added amount of $InsP_6$, was proposed to be an alternative for the determination of $InsP_6$ [33]. Similarly, a fluorimetric detection system for inositol 1,2,6-triphosphate, a lower phosphorylated compound of $InsP_6$, was also developed by using the stoichiometric replacement of Fe^{3+} in the fluorescent Fe^{3+}-methylcalcein complex by inositol 1,2,6-triphosphate [34].

Gelatin is a naturally occurring denatured collagen with high molecular weight and rigid-chain similar to some of rigid-chain synthetic polymers [35]. It is water-soluble and can emit intrinsic blue fluorescence that was attributed to the formation of dimeric species of tyrosine in the gelatin network [36]. Gelatin was extensively used as emulsifier, stabilizer, and film forming binder [37], however, limited research was reported in analytic application [38]. It was found that the fluorescence of gelatin can be quenched by Cu^{2+}, and then recovered proportionally by addition of $InsP_6$. By this means, a simple procedure for the determination of $InsP_6$ in urine was developed in this paper.

2. Experimental

2.1. Apparatus

The fluorescence spectra were recorded on a JASCO FP-6500 spectrofluorometer equipped with a thermostated compartment using 1.0 cm quartz cuvette. The pH measurements were carried out on a PHS-3C Exact Digital pH meter equipped with Phonix Ag–AgCl reference electrode (Cole–Parmer Instrument Co.), which was calibrated with standard pH buffers. The control experiments were carried out on a Dionex 680 reverse phase HPLC system with a photo diode array detector. A CN column (25 cm × 4.6 mm i.d.) with 5 μm cyanopropylsilica packing material (Spherisorb) was used in the separation.

2.2. Reagents

Sodium $InsP_6$ (dodecasodium salt hydrate from corn) was purchased from Sigma. The purity of the sodium $InsP_6$ was assessed by NMR spectroscopy and its concentration was verified by total phosphorus analysis after complete ashing of the sample. Gelatin (acid extracted from porcine skin) was obtained commercially from Sigma and trihydroxymethylaminomethane (Tris) from Fluka. A working solution of gelatin (3.0 mg mL^{-1}) was prepared by dissolving a weighted gelatin in water at 40 °C. An aqueous standard stock solution of dodecasodium $InsP_6$ (94.16 mg L^{-1}) was prepared and stored in refrigerator prior to use. $CuCl_2$ solution (30 mmol L^{-1}) was prepared to quench the fluorescence of gelatin. Tris–HCl buffer (15 mmol L^{-1}, pH 7.40) was used to maintain the solution pH value throughout the fluorescent titrations. The cations examined in the disturbance experiments were all chloride salts and the anions were all sodium compounds. *n*-Methyl piperazine buffer, sodium nitrate, $FeCl_3{\cdot}6H_2O$ and 5-sulfosalicylic acid were obtained commercially and used in the determination of $InsP_6$ in human urine by HPLC method. All reagents and solvents were of analytical grade and used without further purification unless otherwise noted. The ultra pure deionized water purified by Milli-Q system was used in the HPLC analysis. All other aqueous solutions were prepared using newly doubly-distilled and deionized water. The AG1-X8 anion-exchange resin (chloride form, 200–400 mesh) and activated carbon granular (100 mesh) were purchased from Shanghai Chemical Co. (China).

2.3. Fluorimetric titration

A solution of 3.00 mL gelatin (3.0 mg mL^{-1}) containing 15 mmol L^{-1} Tris buffer (pH 7.40) was transferred to a quartz cell, and then an appropriate aliquot of Cu^{2+} solution (30 mmol L^{-1}) was added. The resultant mixture was allowed to equilibrate for 5 min at ambient temperature and scanned on the fluorophotometer in the range of 280–400 nm with excitation wavelength at 273.5 nm. The spectral bandwidths of excitation and emission slits were both set at 5.0 nm. The fluorescence of the solution was recorded and the fluorescent