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Variation of $InsP_4$,$InsP_5$ and $InsP_6$ levels in tissues and biological fluids depending on dietary phytate

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Abstract

Due to the increasing interest of $InsP_6$ on human health, the aim of this paper is to compare the levels of highly phosphorilated inositols ($InsP_4$, $InsP_5$ and $InsP_6$) in organs and biological fluids of rats and to study the influence of the presence and absence of $InsP_6$ in diets. Thus, for this purpose, the variation of $InsP_4$, $InsP_5$ and $InsP_6$ levels in organs and biological fluids of rats submitted to two different diets were studied. In the AIN-76A diet no $InsP_6$ was present, yet the other was a 1% $InsP_6$ modified diet (AIN-76A + 1% $InsP_6$). The highest $InsP_4$, $InsP_5$ and $InsP_6$ levels were found to be 10-fold superior in the brain than those found in the kidney. When the $InsP_6$ was eliminated from the diet, the $InsP_6$ levels decreased dramatically (97.2% in kidney, 89.8% in brain, 100% in bone, 90.5% in plasma and 98.1% in urine), the $InsP_5$ levels showed an important decrease (61.2% in kidney, 45.5% in brain, 28.1% in bone, 30% in plasma and 88.6% in urine) and the $InsP_4$ levels in organs only changed slightly. From these results, it can be deduced that the majority of $InsP_6$ present in the organism is of dietary origin and its endogenous synthesis is not important. According to the results, it can be evidenced that the endogenous synthesis of $InsP_5$ can occur, besides $InsP_6$ can be transformed by enzymatic dephosphorilation in $InsP_5$. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Inositol tetraphosphate; Inositol pentaphosphate; Inositol hexaphosphate; $InsP_6$

1. Introduction

Phytate ($InsP_6$) is an abundant component of plant seeds [1], which are known as a phosphate store [2], resulting the unique biological function attributed to $InsP_6$ for a long time. However, recent studies have demonstrated that $InsP_6$ is found in all animal cells [3,4] and in biological fluids [5]. The reasons for the occurrence of $InsP_6$ in animal cells and fluids are not totally understood. Nevertheless, interesting beneficial effects of $InsP_6$ on human health have been pointed out. In this aspect it is necessary to remark that $InsP_6$ is an important antioxidant [6], protects against cancer [7,8] and prevents pathological calcifications such as renal calculi [9,10] and tissue calcifications [11].

Although many of the diverse pathways of the inositol phosphate metabolism have been described and the $InsP_6$ synthesis in the vegetables cells is known [12–17], there is controversy about the $InsP_6$ synthesis in the animal cells, and the pathways of $InsP_6$ *de novo* formation in such cases is not established [18]. So using cultures of different cell types treated by [^{3}H]-inositol in the medium, it was found, that in such conditions radioactive $InsP_6$ was formed, yet radio-active inositol incorporation to $InsP_6$ pool was very slow. Thus it took more than a week to reach the equilibrium. Nevertheless the metabolic pathways for $InsP_4$ and $InsP_5$ synthesis *de novo* in animal cells has already been established [19–21]. In all cases Ins-(1,4,5)-P_3 was identified as a precursor. Though several $InsP_4$ and $InsP_5$ isomers have been found in animals tissues [3], the Ins-(1,3,4,5,6)-P_5 was the predominant isomer of $InsP_5$ [4] and Ins-(1,3,4,5)-P_4, Ins-(1,3,4,6)-P_4, Ins-(1,4,5,6)-P_4 and Ins-(3,4,5,6)-P_4 were the predominant isomers of $InsP_4$.[18]

The goal of this paper is to compare the levels of $InsP_4$, $InsP_5$ and $InsP_6$ in organs and biological fluids of rats and to study the effects of the presence or absence of the dietary $InsP_6$ on such levels, hence evaluating the possibility of the diet's $InsP_6$ acting as a source of $InsP_4$ and $InsP_5$ *in vivo*, since several phosphatase enzymes with a high affinity for $InsP_6$ were previously found in animals [22]. Kidney was selected for this study for $InsP_6$ and others inositol phos-

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phates importance as crystallization inhibitors [9,10]. It is clear that such inhibitory capacity was related to the ability of the corresponding inositol phosphate to interact with calcium sites on the crystal surface through the phosphate groups, consequently the most effective inhibitory action must correspond to $InsP_6$ and $InsP_5$. For similar reasons, bone was also selected. Thus, cristallization inhibitors are also crystal dissolution inhibitors and due to the affinity of phosphate compound by hydroxiapatite [23], it is important to know the highest phosphorilated inositol status in bone. Finally, due to the higher amounts of $InsP_6$ found in brain [29], to know the levels of the other upper inositol phosphates in such organ was considered interesting. Moreover, it is also known that $InsP_6$ and $InsP_5$ are present in virtually all mammalian cells in higher amounts than any other inositol polyphosphates [3,24] and it is clear that inositol-1,4,5-trisphosphate is a second messenger which regulates intracellular calcium both by mobilizing calcium from internal stores and, probably indirectly, by stimulating calcium entry [25]. Ins-(1,3,4,5)-P_4 was also implied in the regulation of cellular calcium flux [25,26]. Thus, whereas $InsP_6$ and $InsP_5$ comprise the bulk of the inositol phosphate content of mammalian cells and it seems that these compounds are metabolically lethargic, the lower inositol phosphates are clearly implied in cell signaling processes and consequently important intracellular changes will occur as a function of the corresponding stimulus. On the other hand our experimental methodology infers the use of a HPLC system to separate the different inositol phosphates. The analysis, after enzymatic hydrolysis, of total myo-inositol present in the collected fractions was performed by derivatization and gas chromatography [27]. Consequently it is not possible to discern the different isomers of each inositol phosphate. For all the mentioned reasons we have evaluated only $InsP_6$, $InsP_5$ and $InsP_4$ levels in the selected organs and biological fluids.

2. Materials and methods

2.1. Animals and diets

Female Wistar rats (21 days-old) from Harlan Ibérica S.L. (Barcelona, Spain) were acclimated to our animal house of 7 days and kept on diet and tap water ad libitum. Every rat was housed in a cage at a temperature 23 ± 1°C and relative humidity of 50% with 12-h on-off light cycle. The animals were assigned randomly to two groups of three rats each one.

The diets used were AIN-76A (Harland Tekland, Wisconsin. U.S.A.; composition water 5.8%, proteins 20.3%, carbohydrate 65%, lipids 5%, cellulose 5% and ash 4.7%), a purified diet in which phytate is absent, and AIN-76A modified diet (AIN-76A + 1% $InsP_6$), to which phytate dodecasodium salt from corn (Sigma-Aldrich, Madrid, Spain) was added to obtain 1g/100g (Harland Tekland, Wisconsin. U.S.A.). Each rat was fed one of the two different diets for 12 weeks.

On the final day of the experiment 24-h urine was collected by housing the rats in different metabolic cages (Tecniplast Gazzada s.a.r.l., Italy) and the next day the animals were anesthetized with pentobarbital (50 mg/kg, i.p.), sacrificed, and kidneys, brain, bone (femur) and blood were removed.

The procedures used in this experiment were made according the Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes.

2.2. Reagents

All chemicals were of analytical grade. Granular activated carbon (100 mesh) and Na_2EDTA were purchased from Panreac (Spain), phytic acid (from corn), scyllo-inositol, myo-inositol, myo-inositol-(1,3,4,6)-tetraphosphate, myo-inositol-(1,3,4,5,6)-pentaphosphate, pyridine (anhydrous), hexane, methanol, chloroform, isopropanol, sodium hydroxide and trichloroacetic acid were from Sigma (MO, USA).

Derivation chemicals, 1,1,1,3,3,3-hexamethildisilazane and chlorotrimethylsilane, were purchased from Aldrich (Germany). Crude phytase from *Aspergillus ficcum*, 3.5 units/mg specific activity was from Sigma. A suspension containing 1.0 mg crude/ml was prepared in 3 mM HCl solution with magnetic stirring.

2.3. Sample treatment for $InsP_4$, $InsP_5$ and $InsP_6$ determination

Treatment for urine. Urine was acidified with HCl to pH 3–4. The sample was purified using a chromatographic column with 0.5 g of activated carbon. 3 ml were lyophilized (Cryodos, Telstar, Barcelona, Spain) and reconstituted by 0.5 ml of water. The method continued as in the procedure described below.

Treatment for plasma. Whole blood in 6 U.I. mL^{-1} heparin was centrifuged at 3,500 rpm for 15 min. 1.5 ml of supernatant were treated with 0.15 ml of 0.1 M Na_2EDTA and 0.3 ml of 1 M trichloroacetic acid. Then the suspension was centrifuged and the supernatant was treated with 1 ml of chloroform:methanol (2:1). The water phase was lyophilized and reconstituted by 0.5 ml of water. This solution was used to determine the $InsP_4$, $InsP_5$ and $InsP_6$ according the procedure described below.

Treatment for kidney and brain. Tissues once extracted were frozen at -20°C to reduce any metabolic activity. For analysis they were lyophilized and pulverised to a uniform blend. 50 mg of tissue was homogenized in 2 ml of water using a Ultra-Turrax homogenizer (20 s at 13,500 rpm, three times). 0.25 ml of 0.1 M EDTA were added, and the mixture was stirred for 1 h. Afterwards 0.2 ml of 1 M trichloroacetic

acid were added to denaturalise protein. Solid phase was separated by centrifugation at 3,500 rpm for 5 min. The supernatant was treated with 1 ml of chloroform:methanol (2:1). The organic phase was discarded and the water phase was lyophilized. The solid residue was reconstituted with 0.5 ml of water. This solution was taken to carry out the analysis according the procedures described below.

Treatment for bone. 100 mg of sample, pulverised to a uniform blend, was shaken with 0.5 ml of 12 M HCl for 3 h. Then 0.2 ml of the suspension was lyophilized and reconstituted with 0.5 ml of water. The method continued as in the procedure described below.

2.4. $InsP_4$, $InsP_5$ and $InsP_6$ determination

The determination of $InsP_4$, $InsP_5$ and $InsP_6$ levels in organs and biological fluids is based in the separation of different inositol phosphate using a HPLC system, and analysis, after enzymatic hydrolysis, of total myo-inositol present in the collected fractions by gas chromatography.

The separation was carried out with a Omnipac Pax-100 anion-exchange column (25 cm × 4 mm i.d.; Dionex) equipped with a Omnipac Pax-100 (8 μm) pre-column and an anion suppressor (ASRS-I 4 mm). The anion suppressor was used to decrease the ionic strength. The separation was performed using an isocratic elution using as a solvent: 122 mM NaOH solution prepared in 4% isopropanol. A 200 μl constant volume injection was used throughout. The flow-rate was 1 ml/min and the anion suppressor was continually regenerated with 50 mM sulfuric acid solution (10 ml/min). The run time of the chromatogram was of 40 min. The eluent recollected during the firsts 18 min was discarded, afterwards fractions of 3 ml was collected to recover the $InsP_4$, $InsP_5$ and $InsP_6$.

The collected fractions were frozen at -20°C and lyophilized. The residue was reconstituted by 1 ml of 3 mM HCl. Then, 0.1 ml of the phytase enzyme suspension were added to carry out the hydrolysis of inositol phosphate. The solution was maintained 1 h at 37°C. Scyllo-inositol (in aqueous solution) was added to the vial (internal standard). Then it was frozen and lyophilized. The residue was reconstituted by 1 ml of pyridine and 0.2 ml of hexamethyldisilazane and 0.7 ml of chlorotrimethylsilane were added. After reaction the excess of reagents and organic solvent were blown off in a stream of nitrogen. The solid residue was extracted with 2 ml of hexane. The obtained solution was evaporated and the residue reconstituted in 200 μl of hexane. 1 μl of this solution was injected in a gas chromatograph doted with mass spectrometry (Shimadzu QP-5000 gas chromatograph using fused silica capillary column SPB-20 (Supelco) and He as carrier). The calibration graph for myo-inositol determination was obtained from peak height corresponding to a silylated compounds of scyllo- and myo-inositol.

2.5. Statistics

Values in *figures* are expressed as mean ± SE. The Student *t*-test was used to assess differences of means. The SPSS for the Windows program was used for statistical computations. A probability of $P < 0.05$ was used for assessing statistical significance.

3. Results

The chromatograms of the standards containing $InsP_4$, $InsP_5$ and $InsP_6$ are shown in Figure 1. As can be deduced from these results, the $InsP_4$ is present in the fractions 6–7 (Figure 1A), $InsP_5$ in fractions 7–9 (Figure 1B) and $InsP_6$ in the fractions 9–11 (Figure 1C). The chromatograms obtained from organs (brain, kidney and bone) and biological fluids (blood and urine) of the rats treated with AIN-76A and the rats treated with AIN-76A + 1% $InsP_6$ diet are shown in Figures 2–6. The estimated amounts of each inositol poliphosphate in each case appear in Table 1. As can be seen, in kidney, using a diet containing normal $InsP_6$ amounts, the $InsP_6$ levels were double than $InsP_5$ which were similar to $InsP_4$. When rats were fed with a diet without $InsP_6$ (AIN-76A diet), $InsP_6$ practically disappeared, $InsP_5$ levels were reduced to half and $InsP_4$ practically was not affected. In brain and using AIN-76A + 1% $InsP_6$ diet, the $InsP_6$, $InsP_5$ and $InsP_4$ levels were 10-fold superior to those detected in kidney and bone. $InsP_6$ levels were 5 times superior to $InsP_5$ which were double than $InsP_4$. Using the AIN-76A diet, $InsP_6$ decreased a 90%, $InsP_5$ levels were reduced by half and $InsP_4$ was practically unaffected. Moreover in bone and by using AIN-76A + 1% $InsP_6$ diet, $InsP_6$ levels were 30 times superior to $InsP_5$, which resulted similar to $InsP_4$. By using the AIN-76A diet, whereas $InsP_5$ and $InsP_4$ levels were slightly affected, $InsP_6$ remained undetectable.

In urine and plasma the most abundant inositol poliphosphate was $InsP_6$ ($InsP_6$ levels were 10 times superior to $InsP_5$) and when $InsP_6$ was eliminated from the diet its levels decreased 50 times in urine and 10 times in plasma.

Thus, in general when the $InsP_6$ was present in the diet (AIN-76A + 1%$InsP_6$), $InsP_6$ levels were notably higher than $InsP_4$ and $InsP_5$ levels. Thus in organs, the minimum difference between the $InsP_6$ and $InsP_5$ levels was observed in kidney and the maximum difference was observed in bone. The highest $InsP_4$, $InsP_5$ and $InsP_6$ levels were found in the brain and were 10-fold superior to those detected in the kidney. When the rat was fed with a diet without $InsP_6$ (AIN-76A diet), whereas the $InsP_6$ levels decreased dramatically, the $InsP_5$ levels decreased less (50% as maximum) and the $InsP_4$ levels only changed slightly. It is interesting to observe how the $InsP_4$ levels were of the same order for the two groups of rats.

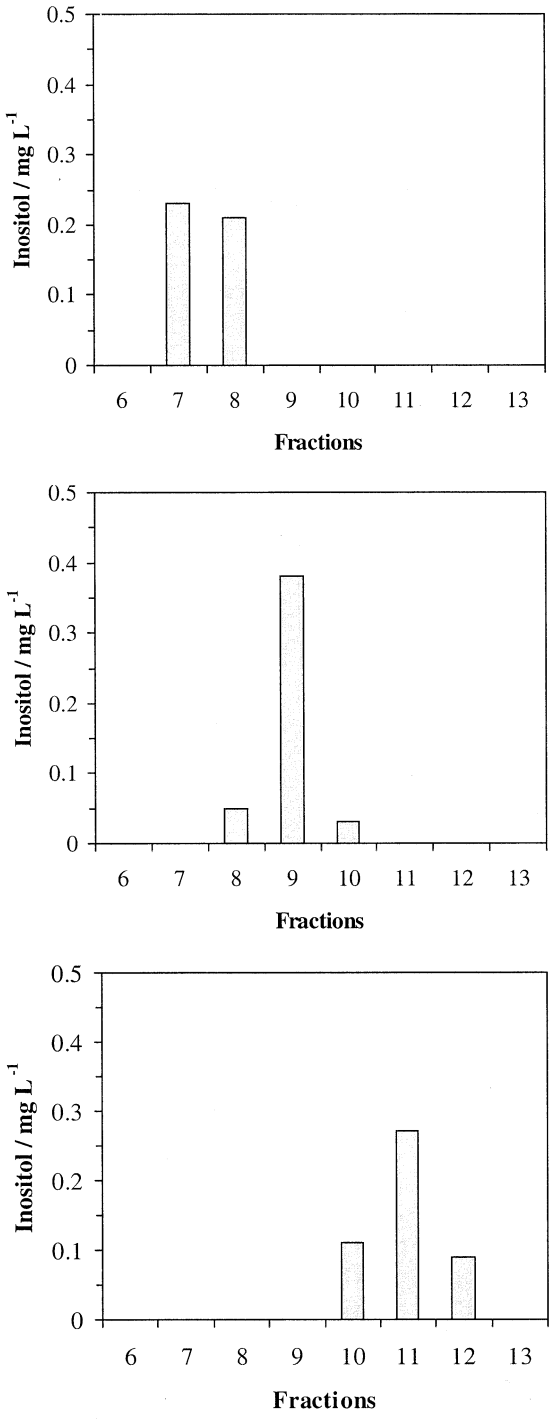

Fig. 1. Chromatograms corresponding to solutions of: a)- 0.5 mg L^{-1} myo-inositol-(1,3,4,6)-tetrakisphosphate; b)- 0.5 mg L^{-1} myo-inositol-(1,3,4,5,6)-pentakisphosphate; c)- 0.5 mg L^{-1} myo-inositol-hexakisphosphate or phytate. Concentrations referred to initial solutions.

4. Discussion

As can be deduced from the results section, $InsP_6$ levels clearly depended on their dietary intake. This was in accordance to previously reported data [28,29]. After 12 weeks of consuming a purified diet in which $InsP_6$ was practically

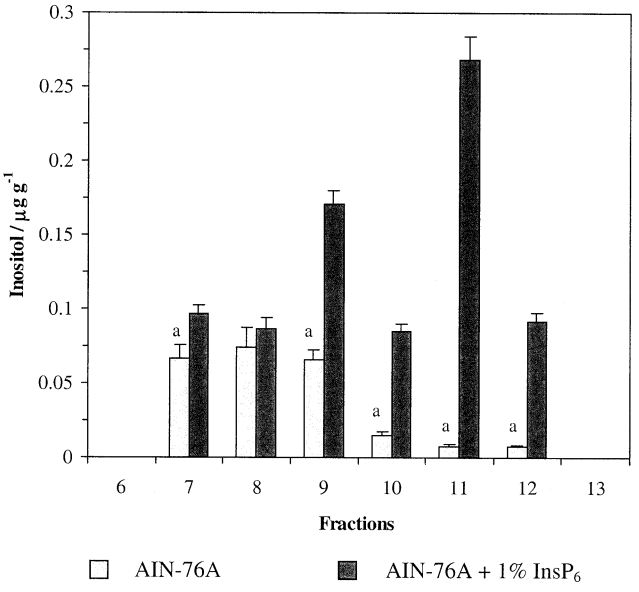

Fig. 2. Chromatogram obtained from kidney samples of animals fed with 1% $InsP_6$ (AIN-76A + 1% $InsP_6$) diet and rats fed with a diet without $InsP_6$ (AIN-76A). The results are expressed as amount of inositol in every fraction referred to dry weight of tissue ± SEM. $^aP < 0.05$ *v.s.* AIN-76A + 1% $InsP_6$ group.

absent, $InsP_6$ levels decreased to very low values in all the studied organs and biological fluids and appeared inferior to $InsP_5$ levels. This clearly demonstrated that the endogenous synthesis of $InsP_6$ is not important and the majority of $InsP_6$ present in the organism have a dietary origin. On the other hand, $InsP_5$ levels were also clearly affected by the oral

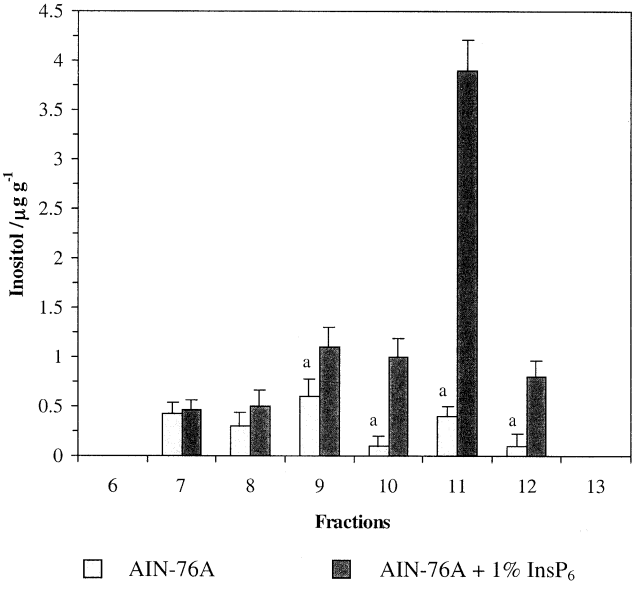

Fig. 3. Chromatogram obtained from brain samples of animals fed with 1% $InsP_6$ (AIN-76A + 1% $InsP_6$) diet and rats fed with a diet without $InsP_6$ (AIN-76A). The results are expressed as amount of inositol in every fractions referred to dry weight of tissue ± SEM. $^aP < 0.05$ *v.s.* AIN-76A + 1% $InsP_6$ group.

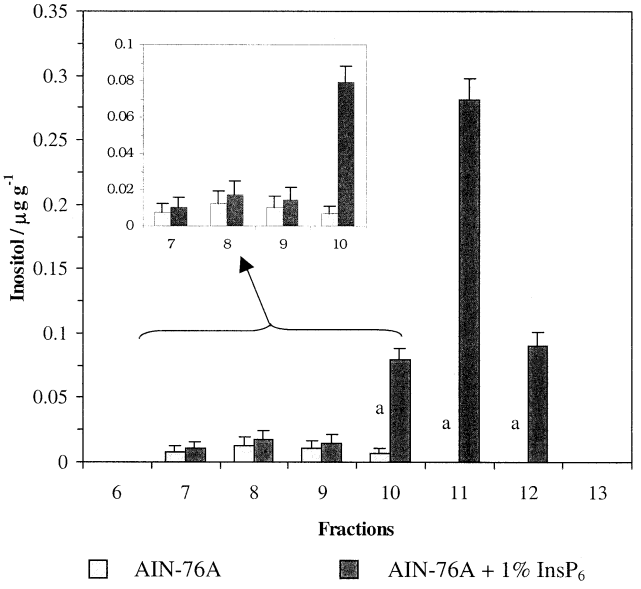

Fig. 4. Chromatogram obtained from bone samples of animals fed with 1% $InsP_6$ (AIN-76A + 1% $InsP_6$) diet and rats submitted a diet without $InsP_6$ (AIN-76A). The results are expressed as amount of inositol in every fractions referred to dry weight of tissue ± SEM. $^aP < 0.05$ *v.s.* AIN-76A + 1% $InsP_6$ group.

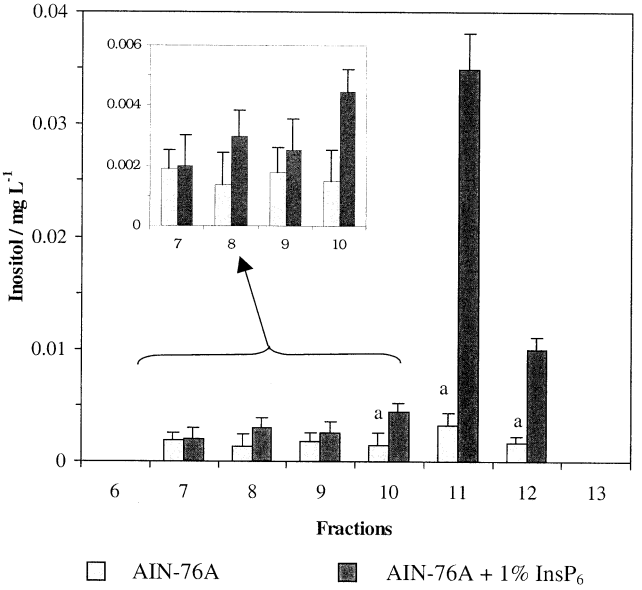

Fig. 6. Chromatogram obtained from plasma samples of animals fed with 1% $InsP_6$ (AIN-76A + 1% $InsP_6$) diet and rats fed with a diet without $InsP_6$ (AIN-76A). The results are expressed as concentration of inositol in every fractions expressed as concentration in the sample ± SEM. $^aP < 0.05$ *v.s.* AIN-76A + 1% $InsP_6$ group.

intake of phytate. Thus, $InsP_5$ levels, after removing $InsP_6$ from the diet, decreased around 50%, reaching levels higher than those seen by $InsP_6$, however it was similar to $InsP_4$. Hence, these results indicate that not only is the endogenous synthesis of $InsP_5$ in rat possible, according to the literature data [4], but the formation of $InsP_5$ by enzymatic dephosphorilation of $InsP_6$ can also take place. We believe that it is a reasonable hypothesize that $InsP_6$, besides preventing radical formation and pathological calcifications, as discussed in the Introduction, may also present a physiological function as precursor of $InsP_5$. On the other hand, as is previously explained, $InsP_5$ is a natural precursor of several $InsP_4$ and $InsP_3$, that regulate important physiological processes.

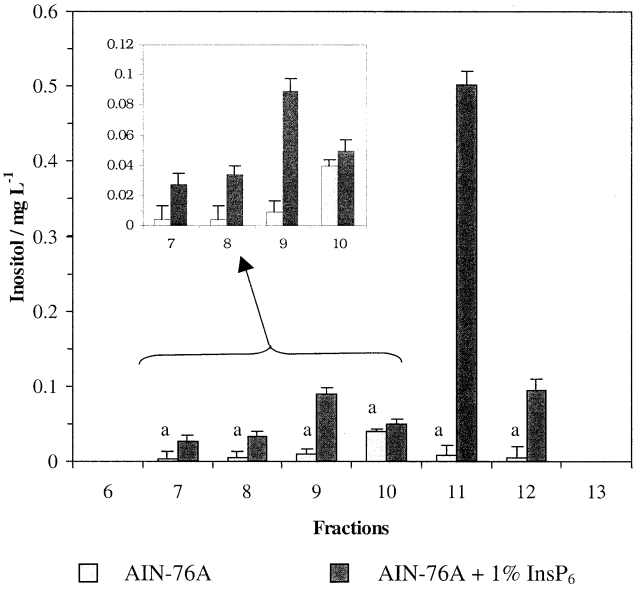

Fig. 5. Chromatogram obtained from urine samples of animals fed with 1% $InsP_6$ (AIN-76A + 1% $InsP_6$) diet and rats fed with a diet without $InsP_6$ (AIN-76A). The results are expressed as concentration of inositol in every fractions expressed as concentration in the sample ± SEM. $^aP < 0.05$ *v.s.* AIN-76A + 1% $InsP_6$ group.

As can be seen, $InsP_4$ levels were only slightly affected by the presence or absence of $InsP_6$ in the diet. Hence considering that several $InsP_4$ have cellular functions as secondary messagers [26,30], the cell must control the $InsP_4$ levels, likewise partly explaining why $InsP_4$ levels were similar for both rat groups, and that the adequate $InsP_4$ levels could be reached by endogenous synthesis.

Moreover, considering, as can be deduced from the results obtained here, that $InsP_6$ levels found in organs and biological fluids consuming a normal diet were notably higher than $InsP_4$ and $InsP_5$ levels, as a result the capacity to prevent pathological calcifications observed *in vivo* when the $InsP_6$ was administrated orally, [9,11], must be attributed to $InsP_6$ and not to the other inositol phosphates.

Iron dependent free radicals formation has been related to greater damage in brain isquemia [31] and the ability of $InsP_6$ to inhibit iron-catalyzed hydroxyl radical formation has been previously pointed out [6]. For these reasons, one of the possible roles of the higher $InsP_6$ levels found in brain could be a protective function as antioxidant due to its capacity to act as iron chelator, avoiding the lipidic membranes peroxidation. Obviously this is a hypothesis that needs further study and clarification.

Table 1
Estimated amounts of $InsP_4$, $InsP_5$ and $InsP_6$ in brain, kidney, bone (femur), urine and plasma of rats fed the AIN-76A diet and AIN-76A + 1% $InsP_6$ diet for 12 weeks

Group	AIN-76A diet			AIN-76A + 1% $InsP_6$ diet		
	$InsP_4$	$InsP_5$	$InsP_6$	$InsP_4$	$InsP_5$	$InsP_6$
Brain (μg/g)	2.28 ± 0.30	2.34 ± 0.55[a]	2.55 ± 0.37[a]	2.44 ± 0.28	4.29 ± 0.64	24.89 ± 1.14
Kidney (μ/g)	0.35 ± 0.03[a]	0.26 ± 0.02[a]	0.048 ± 0.005[a]	0.51 ± 0.02	0.67 ± 0.03	1.71 ± 0.06
Bone (μg/g)	0.041 ± 0.013	0.041 ± 0.020	ND[a]	0.056 ± 0.01	0.057 ± 0.02	1.79 ± 0.06
Urine (mg/L)	0.02 ± 0.03[a]	0.04 ± 0.02[a]	0.06 ± 0.04[a]	0.15 ± 0.02	0.35 ± 0.03	3.20 ± 0.06
Plasma (mg/L)	0.010 ± 0.002	0.007 ± 0.003	0.021 ± 0.004[a]	0.011 ± 0.003	0.010 ± 0.003	0.22 ± 0.01

The evaluation of the amounts of $InsP_4$, $InsP_5$ and $InsP_6$ have been carried out considering that the fraction 7 represents a 52.3% of the total amount of $InsP_4$ present in the sample, that the fraction 9 represents a 82.6% of the total amount of sample's $InsP_5$ and that the fraction 11 represents a 57.5% of the total amount of sample's $InsP_6$. Percentage of recovery (96.9% $InsP_4$, 98.7% $InsP_5$ and 99.1% $InsP_6$).

Values represent mean ± SEM for three rats per group.

[a] $P < 0.05$ *v.s.* AIN-76A + 1% $InsP_6$ group.

ND- non detectable.

Finally, the total depletion of $InsP_6$ in bone when animals were fed on AIN-76A diet, could be explained expeculating that $InsP_6$ in bone is mainly linked to hydroxiapatite and the contribution of cellular $InsP_6$ to the total $InsP_6$ pool would be small. For this reason $InsP_6$ could be easily removed as a consequence of bone renewal and $InsP_6$ desabsorption from hydroxiapatite surface.

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