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Ion chromatographic determination of inositol in infant formulae and clinical products for enteral feeding

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Abstract

An ion chromatographic method is described for the determination of inositol in infant formula and products for enteral feeding. A two-step procedure for hydrolysis and extraction of total inositol has been developed, involving alkaline hydrolysis with 3 *M* potassium hydroxide and enzymatic dephosphorylation. Substances having a long chromatographic retention time were removed with an ion-exchange resin. Inositol was separated on a high-resolution ion-exchange column and detected by pulsed amperometric detection. Phytic acid interferes only slightly in the analysis. This method can be used for determination of total inositol in infant formulae, and enteral feeding products. The analytical method gave an average recovery of 94% from infant formula samples spiked with inositol and a recovery of 86±3% from products spiked with lecithin.

Keywords: Infant formulae; Inositol; Lecithin

1. Introduction

Inositol or cyclohexanehexol has nine isomers but only one, myo-inositol or *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol, is biologically active. The metabolically active form of myo-inositol is thought to be phosphatidylinositol [1]. Inositol is not considered to be a vitamin since there is no known requirement for it in human nutrition and it is synthesised endogenously in rather large amounts [1]. Nevertheless, its universal occurrence in phospholipids, its presence at high levels in human milk and its specific requirement in all studied human cell cultures, all point to its biochemical importance.

Inositol is quite abundant in foodstuffs and occurs in fruits, vegetables whole grains, meats and milk. It was recently reported [2] that the average diet in the USA provides about 1 g/day. It also occurs in relatively large amounts in cereals as its hexaphosphate-phytic acid. A major part of the inositol contained in phospholipids is bioavailable, but its bioavailability from phytic acid may be strongly dependent on the food matrix composition.

Inositol levels are supplemented in some commercially available infant formulae and clinical nutritional products. The current study is however concerned with the analysis of ''total inositol'' which is defined here as the sum of free inositol, inositol derived from lecithin and inositol monophosphate. Phytic acid is not included in this definition.

A range of analytical methods have already been published for determination of inositol: microbiologi-

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cal assay [3], gas chromatography (GC) [4–6], high-performance liquid chromatography (HPLC) [7–12] and ion chromatography [13,14], however these methods are not completely reliable and not fully validated for use with nutritional products. In particular, experience in our laboratories has shown that the microbiological assay using *Saccharomyces carlsbergensis* ATCC 9080 [3] is not sufficiently robust for routine analyses. Thus an investigation has been made of an ion chromatographic method with pulsed amperometric detection (IC–PAD), already established for sugars and alditol analysis, which could be a useful alternative to the microbiological assay.

Certain reports describe the determination of supplemental or free inositol, involving a simple aqueous extraction of certain food products [11,14]. Another approach is to hydrolyse the food product with hydrochloric acid to give ''total'' inositol, however it is rather difficult to completely hydrolyse phosphorylated forms of inositol to free inositol. A GC method [6] applicable to milk powders described the use of a HCl–methanol hydrolysis for 16 h at 85°C. However this sample preparation method was considered to be too long for routine analyses and an incomplete recovery of total inositol was obtained in our laboratory. In fact, Wang et al. [12] reported that a 60 h hydrolysis using 6 *M* HCl at 110°C was required to achieve a 100% liberation of inositol from inositol 1-phosphate.

Some initial investigations showed that the high concentrations of HCl–methanol used in acid hydrolytic procedures were incompatible with the ion chromatographic procedure. Thus, a different approach, involving alkaline hydrolysis of the food product followed by an alkaline phosphatase treatment was evaluated. The alkaline extracts of nutritional products containing total inositol are compatible with the sodium hydroxide eluent used for the ion chromatographic separation.

2. Experimental

2.1. Equipment

A four-place water bath with magnetic stirrers from Kuhn–Bieri (Bern, Switzerland) or a rotatory heating block from Scitec (Lausanne, Switzerland) was used for sample hydrolysis. A Dionex ion chromatograph, Model 4000 I, inert and metal free with a quaternary gradient pump and controller GP 50, an eluent degas system and an autosampler AS 50 were used. The PAD system was fitted with a gold working electrode cell, type ED 40.

An ion-exchange column, MA1 (Dionex) with guard column was used. Data acquisition and instrument control was performed by PeakNet software.

2.2. Products

A range of commercial milk- and soya-based infant formulas, commercial products for enteral feeding and lecithins were used for the method development and validation.

2.3. Reagents

Myo-inositol, myo-inositol 1-monophosphate and alkaline phosphatase (from bovine intestinal mucosa) were purchased from Sigma. Methanol, HCl, sodium hydroxide and potassium hydroxide were from Merck. Anion-exchange resin, chloride form, AG 1X8 200–400 mesh was purchased from Bio-Rad. Takadiastase enzyme preparation, Serva Co. Soy lecithin mixed reference standard was obtained from Spectral Service (Köln, Germany). Water was purified using a Milli-Q water purification system from Millipore.

2.4. Procedure

2.4.1. Preparation of standard solutions

Aqueous standard solutions of inositol were prepared by dilution of inositol to concentrations of 1–16 μg/ml.

2.4.2. Alkaline hydrolysis and dephosphorylation of samples

A 20-g amount of infant formula or enteral product was dissolved in 100 ml water. An aliquot (5 ml) was pipetted into a 25-ml glass tubes with screw cap.

If the sample contained starch, 10 mg Takadiastase was added and the sample was incubated in a water bath at 40°C for 30 min.

Potassium hydroxide pellets (2 g) were added and dissolved by swirling. Methanol (10 ml) was added

and the tube was closed and mixed by shaking and then heated in a water bath or in a rotatory heating block at 80°C for 1 h. The contents of the tube were mixed from time to time.

After hydrolysis, the tube was cooled to room temperature and the contents were transferred to a volumetric flask with about 15 ml water. A 3-ml volume of conc. HCl (37%) was added. The volume was made up to 50 ml with water, mixed and filtered.

A 25-ml portion of the filtrate was pipetted into a 50-ml beaker and the pH was adjusted to 9.8 with 1 *M* NaOH solution. This solution was transferred into a volumetric flask and the volume made to 50 ml with water. An aliquot of this solution (5 ml) was pipetted into a test tube and 0.5 ml phosphatase solution 200 IU/ml was added. The solution was mixed and incubated at 37°C for 1 h.

2.4.3. Ion-exchange clean-up

A cartridge body was filled with anion-exchange resin (Bio-Rad, AG 1X8). The filled cartridge was washed with 10 ml of 1 *M* NaOH solution and then with 5 ml of 0.1 *M* NaOH solution. Sample hydrolysate solution (2 ml) was pipetted into the cartridge and eluted with 0.1 *M* NaOH solution. The eluent was collected in a 5-ml volumetric flask, the elution was stopped when the mark is reached. The eluent was filtered though a 0.45-μm membrane filter and injected into the ion chromatograph.

2.5. Ion chromatographic analysis

The following conditions were used: column: Carbopak MA1 (Dionex), 250×4 mm; mobile phase: isocratic 1.0 *M* NaOH; column temperature: ambient; flow-rate: 0.4 ml/min; injection volume: 25 μl. PAD conditions [15]were as follows:

Time (min)	Potential (V)
0	0.05
0.20	0.05
0.40	0.05
0.41	0.70
0.60	0.70
0.61	−0.10
1.00	−0.10

3. Results and discussion

3.1. Sample preparation

The liberation of inositol was incomplete after a 1 h alkaline hydrolysis with 3 *M* KOH, thus a second step involving an enzymatic dephosphorylation with a non-specific phosphatase was evaluated. Inositol monophosphate was completely hydrolysed after 30 min (Fig. 1). This phosphatase also liberates free inositol from phytic acid but at a much slower rate. After 1 h less than 10% of the total available inositol was released from phytic acid.

3.2. Extract clean-up

After hydrolysis, the product solutions show a multitude of peaks on IC–PAD analysis. The retention time of inositol is about 7–8 min but other matrix components may have retention times above 60 min. In order to shorten the run time, the sample extract was purified through a cartridge containing an anion-exchange resin (AG 1X8). This retained all the major components having long retention times. Therefore it is possible to operate with run-times less than 30 min without interferences from the previous run.

Sample extracts were spiked with inositol in order to check for its irreversible retention on the anion-exchange resin used for purification. Recoveries of about 95% were obtained demonstrating that any retention losses on the cartridge are low.

3.3. Recovery of inositol

An average recovery of 94% was obtained from samples of infant formula spiked with inositol corresponding to 50 mg/100 g product before the saponification step. This procedure is useful for estimating losses of inositol during saponification, irreversible adsorption on the ion-exchange resin and the chromatographic step but not for the incomplete liberation of inositol during saponification and enzymatic hydrolysis. For this reason, the recovery of inositol was also measured by spiking with a reference lecithin; and in this case the recovery of inositol was usually about 86±3%. This low recovery may be

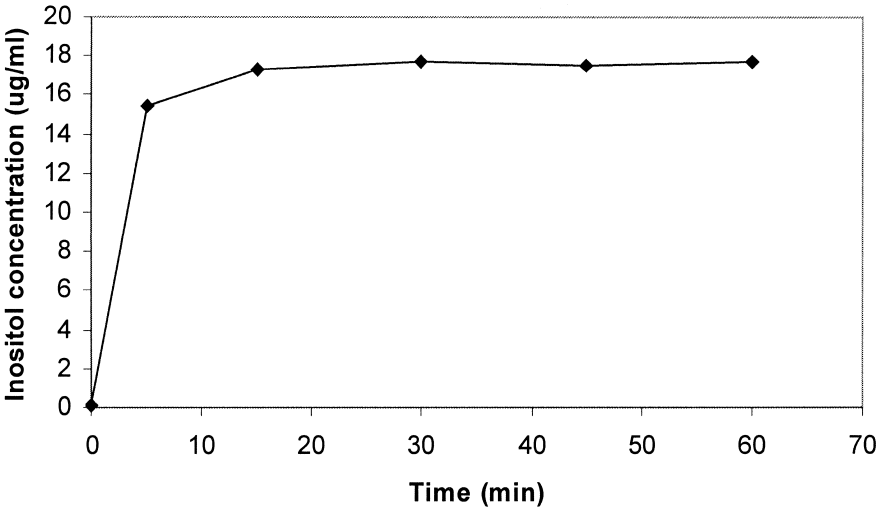

Fig. 1. Kinetics of dephosphorylation of inositol monophosphate with alkaline phosphatase at 42°C, pH 9.8.

due to incomplete saponification or incomplete dephosphorylation. In some cases, however, a recovery of about 95% was obtained. Probably the sample matrix has an effect on the hydrolysis recovery.

Inositol was determined in different samples of lecithin and in some cases compared with the phosphatidyinositol content determined by HPLC. For example, a soya lecithin containing 8.59% phosphatidylinositol and 0.4% lysophosphatidylinositol should theoretically give after hydrolysis 1968 mg/100 g of inositol. The result obtained by IC–PAD was 1784 mg/100 g which corresponds to a 91% recovery.

3.4. Chromatographic interferences

During IC–PAD analysis of certain infant formula and enteral products interfering peaks co-eluted with

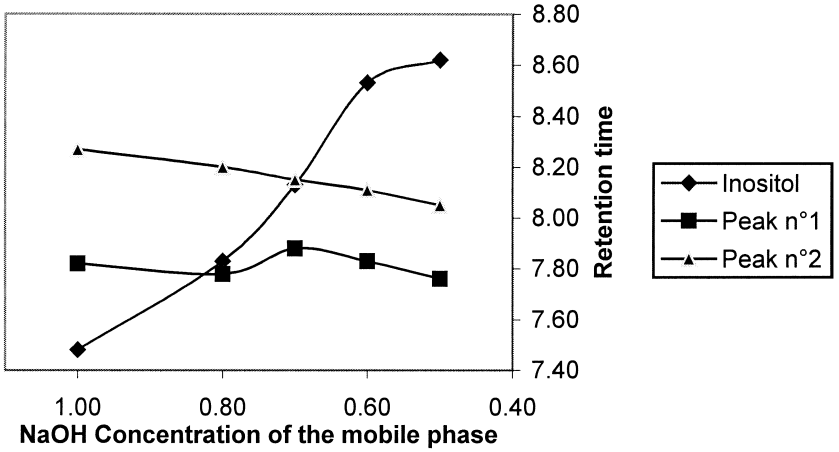

Fig. 2. Variation of the retention time of inositol as a function of the NaOH concentration in the mobile phase. Peaks 1 and 2 are potential interfering peaks occurring in milk- or soya-based infant formulas, cereal-based products and products for enteral feeding.

the inositol peak. However it is possible to improve the efficiency of the chromatographic separation by modifying the sodium hydroxide concentration (Fig. 2) of the mobile phase, e.g., 0.5 *M* NaOH. In case of doubt, two different mobile phases (0.5 and 1.0 *M*) can be used systematically. Some typical chromatograms are shown in Fig. 3.

3.5. Influence of phytic acid on the analysis

Soya-based products may contain phytic acid. The action of the phosphatase used in the sample preparation is not specific, therefore, the liberation of inositol from phytic acid may occur. Nevertheless it takes considerably longer than inositol monophos-

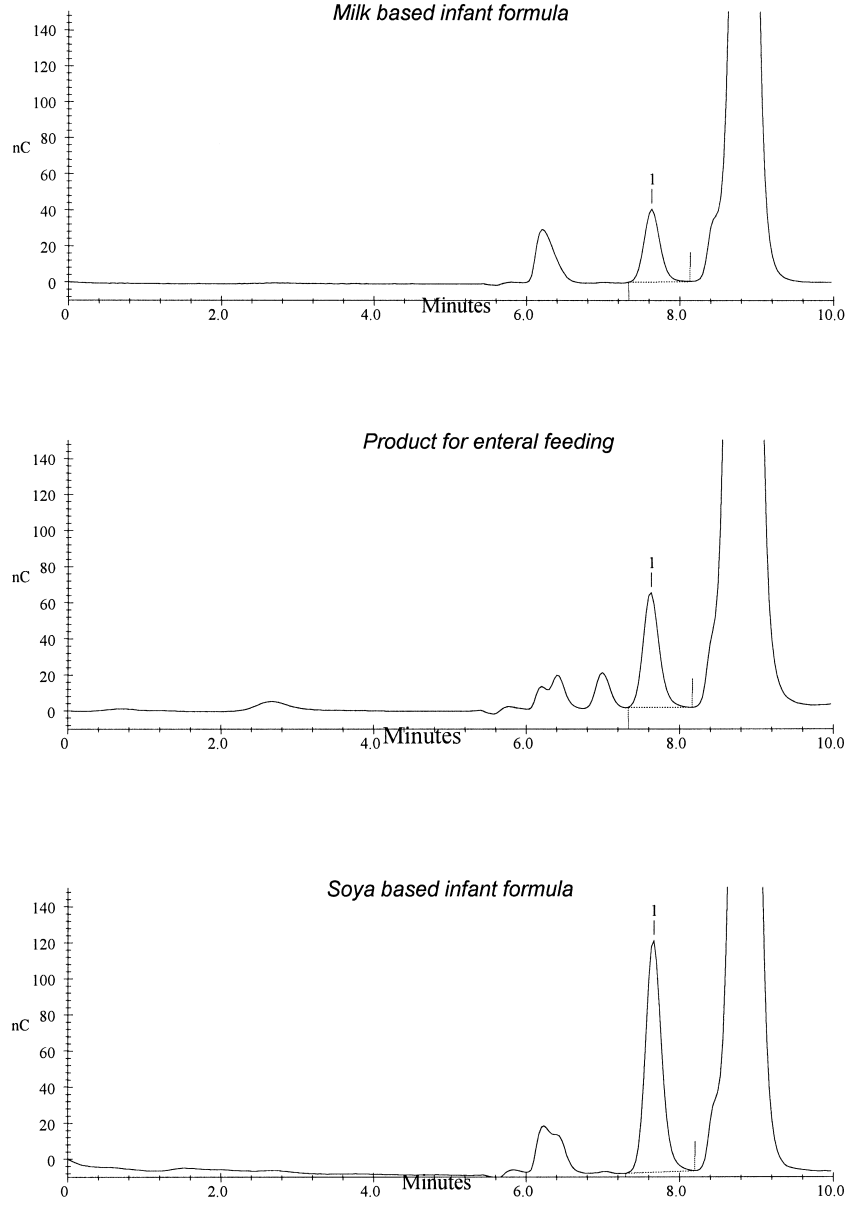

Fig. 3. Examples of chromatograms for milk-based infant formula, product for enteral feeding and soya-based infant formulae. Peak 1=inositol.

Table 1
Repeatability of inositol measurements by IC–PAD

Product	No. samples analysed	Range of inositol values (mg/100 g)	Mean SD_r (95% confidence level)
Milk-based infant formulae	28	30–150	2.5
Soya-based infant formulae	4	35–150	2.8
Clinical products for enteral feeding	9	8–185	2.9

phate because six phosphates must be hydrolysed. Therefore, with a 1-h hydrolysis, which is normally sufficient to entirely hydrolyse inositol monophosphate, less than 10% of the total inositol from phytic acid was liberated.

Experiments were carried out to test if phytic acid could be eliminated by treatment of the sample solution before hydrolysis with an ion-exchange resin. The recovery of inositol from a milk powder spiked with phytic acid (spiking: equivalent to 50 mg inositol per 100 g), without ion-exchange resin, was low (about 13% with a phosphatase hydrolysis of 20 h). Probably the major part of phytic acid was eliminated by precipitation during the pH adjustment and filtration after alkaline hydrolysis. Thus phytic acid does not appear to be of major importance as an interference in this determination.

3.6. Repeatability and reproducibility of results

Forty-one samples (soya and milk powders and products for enteral feeding) were analysed in duplicate with an inositol content in the range of 8–185 mg/100 g (Table 1). The difference between duplicate results were between 0 and 7 mg/100 g and did not vary significantly as a function of the inositol content; the median difference was 1. The maximal deviation (7 mg/100 g) corresponds to a RSD_r of 5.8%.

The pseudo-reproducibility (same operator, same apparatus but different days) was evaluated by analysing 13 milk-based products in duplicate. Again, the deviations were found to be independent of the inositol concentration; for contents between 39 and 64 mg/100 g, reproducibilities were found between 1 and 7 mg/100 g. The median of the reproducibility values was 2 mg/100 g and the highest deviation (only one point=7) corresponds to a relative reproducibility (RSD_R) of 15.6%.

3.7. Comparison with other methods

A comparison was made (Table 2), of results from IC–PAD, microbiological assay [3] and a laboratory GC method. The microbiological assay gave systematically lower results because the liberation of total inositol by acid hydrolysis [sample in 3 *M* HCl–methanol (1:1), 6 h at 100°C under reflux] is incomplete and the microorganism (*Saccharomyces carlsbergensis* ATCC 9080) cannot utilise inositol

Table 2
Comparison of analyses of inositol by three different methods

Product	IC–PAD (mg/100 g)	Microbiology (mg/100 g)	GC (mg/100 g)
Dietetic milk powder 1	56	38	60
Dietetic milk powder 2	42	27	40
Dietetic milk powder 3	48	34	47
Dietetic milk powder 4	61	51	67
Dietetic milk powder 5	130	112	
Dietetic milk powder 6	134	105	
Dietetic milk powder 7	120	107	

monophosphate. Hydrolyses using higher concentrations of acid are not compatible with the microbiological growth and the results for inositol were even lower. The results from the GC method are close to those from the IC–PAD method. The GC method uses an acid-hydrolysis step: heating samples in 6 *M* HCl at 100°C for 16 h.

4. Conclusion

A new method for determination of total inositol in infant formulae based and clinical nutrition products has been developed and validated. It involves ion chromatographic separation with PAD. Total inositol is liberated by a two-step alkaline and alkaline dephosphorylation procedure.

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