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Journal of Pharmaceutical and Biomedical Analysis 33 (2003) 1175–1180

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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Short communication

Specific determination of *myo*-inositol in multivitamin pharmaceutical preparations by a flow injection system using a *myo*-inositol dehydrogenase reactor coupled with a glucose eliminating enzyme reactor

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Received 31 March 2003; received in revised form 4 July 2003; accepted 9 July 2003

Abstract

A flow injection system for *myo*-inositol determination in multivitamin pharmaceutical preparations using two enzyme reactors was developed. *Myo*-inositol was detected using a fluorophotometer, to measure the fluorescence of NADH produced from NAD^+ by a *myo*-inositol dehydrogenase reactor (IDR) containing *myo*-inositol dehydrogenase immobilized on porous glass. Enhanced interference due to excess glucose included in a multivitamin pharmaceutical preparation as a sweetener was eliminated by a glucose eliminating reactor (GER) co-immobilized with three enzymes (glucose oxidase, mutarotase and catalase). The calibration coefficient for the standard curve was 0.9993 for *myo*-inositol detection in the range of 1–5 μg/ml. *Myo*-inositol was determined even in the presence of glucose concentrations of 140–420 μg/ml. The recovery of *myo*-inositol added to the multivitamin pharmaceutical preparation was 99.6% ($n = 9$).

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Keywords: *Myo*-inositol; Immobilized enzyme reactor; Flow injection analysis; Glucose elimination; Fluorometry; Multivitamins

1. Introduction

Although *myo*-inositol is widely distributed in nature, inositol biosynthesis in animals is restricted or insufficient. *Myo*-inositol is thus considered a growth factor and an indicator of abnormal metabolism or disease [1]. Eagle reported that lack of *myo*-inositol caused inhibition of prolif-

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0731-7085/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00427-8

eration and the death of cultivated human cells, and *myo*-inositol has come to be recognized as a kind of vitamin [2], because *myo*-inositol plays a vitamin-like role in vivo, and is used in multivitamin pharmaceutical preparations for nutritional supplementation with other water-soluble vitamins. Many researchers have been interested in the determination of *myo*-inositol, because its detection is extremely difficult. Numerous studies concerning analytical methods for this have been reported [3–7]. The most general analytical method is gas chromatography with high selectivity, but the sensitivity of this method is insufficient [6]. Analysis of *myo*-inositol in the multivitamin pharmaceutical preparations was also carried out by gas chromatography, which required silyl-derivatization by trimethylchlorosilane and hexamethyldisilazane [7]. This method required extraction of an inositol-derivative with an organic solvent, and therefore, was complicated for use in assay and not suitable ecologically. *Myo*-inositol dehydrogenase oxidizes *myo*-inositol to inosose in the presence of NAD^+. NAD^+ is reduced to NADH in the presence of equimolar amounts of inosose by enzymatic reaction. This conventional enzymatic determination using *myo*-inositol dehydrogenase is insufficient in sensitivity, since change in NAD^+ is detected by UV spectrophotometry [8]. A detection in enzymatic determination using *myo*-inositol dehydrogenase were improved by enzymatic cycling methods coupled with malate dehydrogenase [9] or coupled with lactate dehydorogenase and lactate oxidase [10]. The latter is a selective and sensitive flow injection method reported by Olsson et al., who used co-immobilized *myo*-inositol dehydrogenase, lactate dehydrogenase and lactate oxidase as a first enzyme reactor. *Myo*-inositol reacted to produce an equal amount of hydrogen peroxide. The hydrogen peroxide then oxidized hexacyanoferrate (II) to hexacyanoferrate (III) in the presence of a second reactor containing immobilized peroxidase. The hexacyanoferrate (III) was detected amperometrically. On the other hand, in this study, we prepared a simple immobilized *myo*-inositol dehydrogenase reactor (IDR) and have tried to determine fluorometrically NADH produced stoichiometrically in IDR. Glucose at low concentrations does not react with *myo*-inositol dehydrogenase, though the presence of glucose at high concentrations in a certain pharmaceutical product interferes an accurate determination of *myo*-inositol. Elimination of glucose was successfully achieved by pretreatment with a glucose eliminating enzyme reactor (GER), which contained co-immobilized glucose oxidase, mutarotase and catalase. Effectiveness of GER had reported by Olsson et al. for the determination of sucrose in the presence of glucose in a flow injection system with immobilized multi-enzyme reactors [11].

We developed a flow injection system composed of GER and IDR in series and performed *myo*-inositol analysis of multivitamin pharmaceutical preparations in which glucose was present in 140-fold excess amount compared with *myo*-inositol.

2. Experimental

2.1. Materials

Myo-inositol and catalase (EC 1.11.1.6, lyophilized powder, 5000–15 000 U/mg) from bovine liver were purchased from Wako Pure Chemical (Tokyo, Japan). *Myo*-inositol dehydrogenase (*myo*-inositol: NAD^+ 2-oxidoreductase, EC 1.1.1.18, lyophilized powder, 25–50 U/mg protein) from *Enterobacter aerogenes* was obtained from Sigma (St. Louis, MO, USA). Glucose oxidase (β-D-Glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, lyophilized powder, more than 300 U/mg protein) from *Aspergillus niger* and β-diphosphopyridine nucleotide, oxidized form (β-NAD^+) from yeast, were purchased from Wako Pure Chemical. Mutarotase (EC 5.1.3.3, lyophilized powder, more than 1500 U/mg) from porcine kidney was obtained from Biozyme Laboratories, LTD (San Diego, CA, USA). Aminopropyl controlled-pore glass (aminopropyl-CPG, 1400 Å pore diameter, 120–200 mesh) was purchased from CPG (Lincoln, Park, NJ, USA). A pre-formulation of “Multivitamin Pharmaceutical Drink” was prepared as follows. The pre-formulation included 50 mg *myo*-inositol in 100 ml, riboflavin phosphates, pyridoxine hydrochloride,

nicotinamide, aminoethylsulfonic acid and caffeine as other ingredients, and glucose, fructose and sorbitol as sweeteners. The concentration ratio of *myo*-inositol and glucose in pre-formulation was designed to be 1:140.

2.2. Preparation of enzyme reactors

The immobilization of *myo*-inositol dehydrogenase and preparation of IDR were based on a previous paper, which described preparation and characterization of immobilized acid phosphatase used an enzyme reactor [12]. Fifty units of *myo*-inositol dehydrogenase were immobilized by covalent coupling with glutaraldehyde to aminopropyl controlled-pore glass (aminopropyl-CPG). Immobilized *myo*-inositol dehydrogenase was packed into a stainless steel column (50 × 4 mm I.D.), and used as IDR. GER, i.e. a co-immobilized reactor of glucose oxidase, mutarotase and catalse, was prepared according to a previous paper by Olsson et al. [11]. Aminopropyl-CPG 0.5 g (wet weight) was activated for 1 h with phosphate buffer (pH 7.0; 0.1 M) including 2.5% glutaraldehyde under reduced pressure, and then washed with 500 ml of water on the glass filter. The glutaraldehyde-activated glass was transferred to the reaction container, and three enzymes, i.e. 8000 U of glucose oxidase, 10 000 U of mutarotase and 230 000 U of catalase in 5 ml of phosphate buffer (pH 7.0; 0.1 M), were added. The reaction was allowed to continue by shaking occasionally overnight at 5 °C, and then filtered on the glass filter. The gel was transferred to the reaction container again, and subsequently a cocktail solution containing three enzymes, i.e. 8000 U of glucose oxidase, 10 000 U of mutarotase and 230 000 U of catalase in 5 ml of phosphate buffer (pH 7.0; 0.1 M), that was freshly prepared was added. The reaction was allowed to continue by shaking occasionally for 4 h at room temperature. The gel of co-immobilized enzymes was washed with 500 ml of phosphate buffer (pH 7.0; 0.1 M) and further treated with 500 ml of Tris–HCl buffer (pH 8.5; 0.4 M) to block the residual active aldehyde on the glass beads. The preparation was packed into a stainless steel column (250 × 2.1 mm I.D.) and used as GER.

2.3. Preparation of sample solution and standard solution

Myo-inositol standard was dried at 105 °C for 4 h. The standard of *myo*-inositol was prepared by dissolution in phosphate buffer (pH 7.0; 0.1 M) at the required concentrations.

Removal of riboflavin phosphates and pyridoxine hydrochloride with fluorescence from sample solution in the multivitamin pharmaceutical preparations was performed as follows. Riboflavin phosphates and pyridoxine hydrochloride were eliminated using an octadecyl-silicagel cartridge (C_{18} cartridge, Sep-pak Vac 6 cc, Waters, Milford MA), and a strong-cation exchange cartridge (SCX cartridge, BOND ELUT LRC-SCX, 500MG, Varian, Middelburg, Netherlands), respectively. The two cartridges were connected in series, and were equilibrated with 10 ml of methanol and 10 ml of water, respectively, before use.

The multivitamin pharmaceutical preparation was diluted to 10-fold with citric acid (20 mM). A 2 ml aliquot of the diluted solution was applied to the equilibrated cartridges described above, and then *myo*-inositol in cartridges was eluted with 15 ml of citric acid (20 mM). The eluate was diluted with phosphate buffer (pH 7.0; 0.1 M) to the required concentration.

2.4. Apparatus and procedures

A schematic diagram of the flow-injection system is shown in Fig. 1. A system controller (Shimadzu SCL-10A) controlled two Shimadzu LC-10A pumps (Kyoto, Japan), which pumped phosphate buffer (pH 7.0; 0.1 M) and Tris–HCl buffer (pH 8.5; 0.4 M) containing 4 mM NAD^+, respectively. Sample solutions containing a specific amount of *myo*-inositol were injected through an automatic sample injector (Simadzu SIL-10A) with a sample volume of 20 μl. The sample solution was carried via a carrier stream containing phosphate buffer (pH 7.0; 0.1 M), and the flow was then passed through the GER. The sample solution reacted with GER was then introduced to a static mixer (Non-Metal static mixer, 250 μl volume, GL-Science, Tokyo), and mixed with

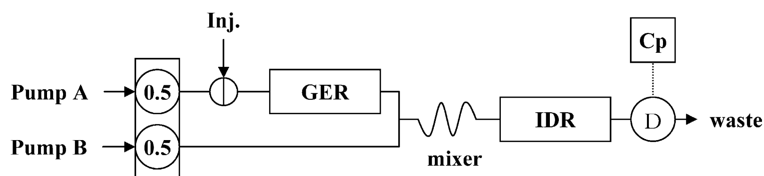

Fig. 1. Flow path used throughout the study. GER, glucose eliminating reactor; IDR, inositol dehydrogenase reactor; Pump A, phosphate buffer (pH 7.0; 0.1 M); Pump B, Tris–HCl buffer (pH 8.5; 0.4 M) containing 4 mM NAD^+; inj., automatic sample injector; D, fluorescent detector (Ex. 340 nm, Em. 460 nm); Cp, computing integrator.

another stream including Tris–HCl buffer (pH 8.5; 0.4 M) containing 4 mM NAD^+. The sample containing *myo*-inositol and NAD^+ was then introduced to IDR. The NADH produced depending on the amounts of *myo*-inositol was detected by fluorescence (Ex: 340 nm, Em: 460 nm) with a fluorophotometer (1100 series, Agilent Technologies, Waldbronn, Germany). The signal data were recorded on a Chromatopac CR-7A integrator (Shimadzu).

3. Results and discussion

3.1. Detection of inositol

The standard solutions at various concentrations were determined by this flow injection system. Typical signals of the standard samples are shown in Fig. 2. The limit of detection (S/N = 3) of this system was 0.01 μg/ml. The sensitivity of the present method was increased about 100-fold compared with UV-detection [8], and was equivalent to the enzymatic cycling method reported by Olsson et al. [10]. The correlation coefficient for signal height versus concentration of *myo*-inositol preparation was 0.9993. For application of this system, we used the standard curve method with three different concentrations of standard solutions.

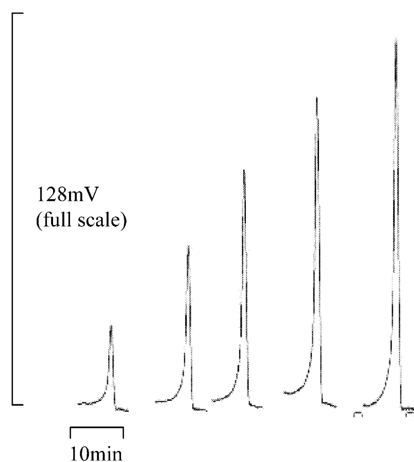

Fig. 2. Typical signals for *myo*-inositol standards. Concentrations from left to right: 0.996, 1.992, 2.998, 3.984 and 4.980 μg/ml.

3.2. Elimination of glucose

Glucose at low concentrations dose not interfere with *myo*-inositol dehydrogenase activity, but glucose reacts slowly with this enzyme above 10^{-3} mol/l [8]. A certain pharmaceutical product contains glucose at high concentration, and therefore, in this study elimination of the glucose in pharmaceutical preparations was very important. Glucose elimination was successfully achieved in flow injection analysis by using a co-immobilized multienzyme system for the determination of sucrose [11,13,14]. In this flow injection system, the IDR was located downstream of GER, and therefore, was influenced by hydrogen peroxide produced by glucose oxidase. It was recognized that the signal height of *myo*-inositol detection was decreased with repeated use when the immobilized catalase was insufficient (data not shown). As shown in Section 2.2, the step of co-immobilization of glucose oxidase, mutarotase and catalase was well performed compared with that of previous papers [11,13,14]. As a result of an improvement, the GER obtained here was effective in eliminating glucose in high concentrations (140–420 μg/ml) shown in Table 1. Recoveries of *myo*-inositol (1–3 μg/ml) added to large amounts

Table 1
Recoveries of *myo*-inositol from solutions with glucose at high concentration

Myo-inositol added (μg/ml)	Glucose (μg/ml)	Intra-day assay		Inter-day assay (3 days)	
		Recovery (%)	Mean ± R.S.D. (%, $n = 3$)	Recovery (%)	Mean ± R.S.D. (%, $n = 3$)
1	140	98.3		98.3	
		102.1	99.3 ± 2.5	101.7	101.4 ± 3.0
		97.5		104.3	
2	280	99.3		99.3	
		98.8	99.1 ± 0.3	99.0	98.8 ± 0.6
		99.1		98.2	
3	420	100.2		100.2	
		100.7	100.0 ± 0.8	97.2	99.6 ± 2.1
		99.1		101.3	

Table 2
Validation data for determination of inositol in multivitamin pharmaceutical preparation

LOD (μg/ml)	Recovery and precision		
	Added (μg/ml)	Recovery ± R.S.D. (%, $n = 3$)	Mean values ± R.S.D. (%, $n = 9$)
0.01	2.237	101.3 ± 3.6	99.6 ± 3.1
	2.982	99.4 ± 2.9	
	3.976	98.1 ± 3.2	

LOD, limit of detection; R.S.D., relative standard deviation.

of glucose solutions (140–420 μg/ml) were nearly 100% on intra- and inter-day assay.

3.3. Determination of *myo*-inositol in multivitamin pharmaceutical preparation

A pharmaceutical preparation including *myo*-inositol at 50 mg/100 ml, glucose at 7000 mg/100 ml and other ingredients, i.e. thiamine, nicotinamide, riboflavin phosphates, pyridoxine hydrochloride, aminoethyl sulfonic acid and caffeine, was prepared. Since riboflavin phosphates and pyridoxine are fluorescent, the detection of NADH produced stoichiometrically from *myo*-inositol was interfered with in this system. Riboflavin phosphates and pyridoxine hydrochloride were easily removed by the C_{18} cartridge and SCX cartridge from sample solution. The limits of detection and recoveries of *myo*-inositol at three different concentrations from the pharmaceutical preparation are shown in Table 2. Recoveries of each concentration from the pharmaceutical formulation were nearly 100%.

4. Conclusion

There are two methods of efficient analysis of pharmaceutical products. One is simultaneous determination of all ingredients, which we reported previously [15]. The other is specific determination of a certain ingredient, as described in this paper. The present method increased specificity of *myo*-inositol detection by using two kinds of enzyme reactors, i.e. GER and IDR, and performs reliable determination of *myo*-inositol in multivitamin pharmaceutical preparations without chromatographic techniques. In this study, glucose elimination and removal of fluorescent vitamins were very important. Several studies on the elimination of glucose by a GER have been reported [11,13,14], but none on elimination of glucose present in more than 100-fold excess of the

analyte. The stability of IDR was increased, because hydrogen peroxide could be completely eliminated by immobilized catalase in GER. It should be pointed out that fluorometric detection yielded sufficient sensitivity, with a limit of detection of 0.01 μg/ml (S/N = 3). The sensitivity of this system was almost equivalent to the enzymatic cycling method reported by Olsson et al. [10]. The fluorescent vitamins were easily removed with applying a simple clean-up procedure. This is an important factor for the judgment in quality control tests of pharmaceutical productions. *Myo*-inositol in pharmaceutical preparations including glucose at extremely high concentrations can be determined fluorometrically with sufficient accuracy by inclusion of two reactors. The time required for one analysis was less than 10 min. This method is fast enough for routine analysis, unlike the gas chromatographic method.

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