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DETERMINATION OF D-*myo*-1,2,6-INOSITOL TRISPHOSPHATE BY ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH POST-COLUMN LIGAND EXCHANGE AND FLUORESCENCE DETECTION

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SUMMARY

A detection system for D-*myo*-1,2,6-inositol trisphosphate (1,2,6-IP_3) has been developed which is based on the strong complexing properties of the phosphate groups and which includes a post-column ligand-exchange reaction. The weakly fluorescent complex of Fe^{III} with methylcalcein blue (MCB) is used as a reagent. Analytes that form a stronger complex with Fe^{III} than MCB cause a ligand-exchange reaction; the amount of strongly fluorescent MCB released during this reaction is proportional to the analyte concentration and can be measured sensitively by direct fluorescence detection. The influence of the pH, the reagent concentration, the Fe^{III} to MCB ratio, the content of organic modifier and the dimensions of the post-column reaction system on the sensitivity of the detection system are discussed. In combination with ion-pair reversed-phase liquid chromatography, a detection limit of 3–10 ng has been obtained for 1,2,6-IP_3. The present system has been combined with a preconcentration technique, based on ion-pair formation, which allows the determination of low-ppb concentrations of 1,2,6-IP_3.

INTRODUCTION

Interest in the biochemistry of inositol phosphates is increasing because of their important role in signal transmission in the living cell[1]. D-*myo*-1,4,5-Inositol trisphosphate (1,4,5-IP_3) was found to act as "second messenger", being released by hydrolysis of a cell membrane constituent, phosphatidylinositol-4,5-bisphosphate[1,2] after agonist stimulation. Another inositol trisphosphate isomer, D-*myo*-1,2,6-inositol trisphosphate[3] (1,2,6-IP_3), which has interesting pharmacological properties, is currently in a preclinical development phase at Perstorp Pharma (Perstorp, Sweden). The pharmacological properties of 1,2,6-IP_3 include inhibition of platelet thrombus formation,

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as shown by Sirén *et al.*[4], and prevention of complications of diabetes, as described by Renaud *et al.*[5]. The compound also shows antiinflammatory effects[6]. Unlike 1,4,5-IP_3, 1,2,6-IP_3 does not release calcium from intracellular stores[7].

Most data published on the determination of inositol phosphates are based on radioisotope techniques. They are applicable to cells and tissue samples, which are incubated with, *e.g.*, *myo*-[^{3}H]inositol, in order to obtain labelled inositol phosphates. These techniques have limitations as they do not provide absolute concentrations and are not suitable for the analysis of a large number of samples. In order to obtain and interpret the pharmacokinetics of, *e.g.*, 1,2,6-IP_3, the need for a direct method is apparent.

Several liquid chromatographic (LC) techniques have been developed for inositol phosphates in order to overcome the problems connected with radioactive labelling. The separation of the inositol phosphates is based on ion-exchange or ion-pairing principles[8–18]. However, the detection of inositol phosphates still remains the main problem for LC, as this type of compound is difficult to detect by conventional methods, such as with UV or electrochemical detectors. The low reactivity of the sugar-type OH groups limits the applicability of derivatization reactions for fluorescence detection. Post-column reaction detection systems developed so far are based on either enzymatic reactions with subsequent determination of the released inorganic phosphate[10,16] or inositol[19]. An alternative is the application of ligand-exchange reactions that make use of the strong complexing properties of the phosphate groups.

The latter principle was used in 1953 by Wade and Morgan[20] as a method to detect phosphate esters in paper chromatography. The reaction is based on the decrease of the colour intensity of the red Fe^{III}–sulphosalicylic acid complex at low pH when organic or inorganic phosphate-containing compounds are added. Cilliers and Van Niekerk[12] applied this principle to the LC detection of phytic acid (inositol hexakisphosphate), and Mayr[9] used the same ligand-exchange principle in a system based on coloured complexes of 4-(2-pyridylazo)resorcinol (PAR) with transition metal ions, such as holmium and ytterbium. Meek and Pietrzyk[11] developed a post-column indirect fluorescence detection system based on the quenching of the Al^{III}–morin fluorescence by phosphorus oxo acids. Detection limits as low as 15 ng were obtained for, *e.g.*, difluoro(methylene) phosphonic acid.

The colorimetric or fluorescence methods described have the disadvantage that detection is based on the decrease in a steady signal, that is, the analyte signal is measured against a high background signal caused by the reagent. We have previously developed a direct fluorescence detection system based on a post-column ligand-exchange reaction which causes an increase in the signal against a low background[21]. In this paper, we report the application of this principle to the determination of 1,2,6-IP_3. In order to decrease the detection limit of 1,2,6-IP_3 further, we have also developed an ion-pairing preconcentration system that allows the introduction of larger sample volumes than direct with loop injections.

EXPERIMENTAL

Chemicals

All reagents were of analytical-reagent grade. The organic solvents and iron-

(III) nitrate were Baker (Deventer, The Netherlands) Analyzed Reagents. 1,2,6-IP_3 and inositol bisphosphate (a mixture of 1,2-IP_2 and 1,6-IP_2) was purchased from Perstorp Pharma, tetrapentyl- (TPABr) and tetrabutylammonium bromide (TBABr) from Kodak (Rochester, NY, U.S.A.), methylcalcein blue (MCB) from Janssen (Beerse, Belgium) and Tris buffer from Sigma (St. Louis, MO, U.S.A.). Stock solutions (1 m*M*) of iron(III) nitrate and MCB were prepared in 0.1 *M* nitric acid and methanol, respectively. The reagent solution (Fe:MCB = 1:3, $[Fe^{III}] = 6.6 \cdot 10^{-6}$ *M*) was prepared by adding iron(III) nitrate to a solution of MCB in methanol–20 m*M* Tris buffer (pH 7.0) (95:5, v/v).

Instrumentation

Experiments were carried out in an LC system consisting of a Waters Assoc. (Milford, MA, U.S.A.) Model 510 LC pump, a Valco six-port injection valve, a 200 × 4.6 mm I.D. stainless-steel separation column packed with 5-μm Hypersil ODS (Shandon Southern, Runcorn, U.K.) and a Kontron (Zürich, Switzerland) SFM 23 fluorescence detector (excitation wavelength 328 nm, emission wavelength 440 nm). The precolumn (10 × 4.6 mm I.D.), used for the preconcentration of 1,2,6-IP_3, was purchased from Chrompack (Middelburg, The Netherlands) and packed manually with a slurry of 10-μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.) in methanol. For preconcentration a Kontron 414 LC pump was used at a flow-rate of 2 ml/min. Samples were dissolved in the carrier solution [5 m*M* Tris buffer (pH 7.0)–2 m*M* TPABr] and injected onto the preconcentration column via a 1-ml loop. The washing solution was acetonitrile–10 m*M* Tris buffer (15:85, v/v) containing 1 m*M* TPABr. The LC mobile phase was acetonitrile–5 m*M* aqueous Tris buffer (pH 7.0) (37.5:62.5, v/v) containing 1 m*M* TPABr. The reagent pump was a laboratory-built syringe pump used at a flow-rate of 0.3 ml/min. Mixing of the eluent with the reagent solution was performed by using a T-type mixing union. The reaction coils (volume, 300 μl for heating and 180 μl for cooling) consisted of 0.3-mm I.D. PTFE tubing; the reaction temperature was 55°C.

RESULTS AND DISCUSSION

Optimization of the ligand-exchange reaction

The direct fluorescence detection method developed in our laboratory[21] is based on a post-column ligand-exchange reaction of organosulphur compounds, such as ethylenethiourea, cysteine or glutathione with the non-fluorescent Pd^{II}–calcein complex. The sulphur-containing ligands usually possess a higher affinity for Pd^{II} than oxygen- and nitrogen-containing ligands, such as calcein (Fig. 1). Therefore, this strongly fluorescing compound is released during the ligand-exchange reaction in an amount proportional to that of the analyte. The fluorescence detection of calcein is favoured by the low background of the reagent because palladium efficiently quenches the calcein fluorescence. However, this system cannot be applied to the detection of inositol phosphates, as the Pd^{II}–inositol trisphosphate complex is weaker than the Pd^{II}–calcein complex.

We found that the complex stability of 1,2,6-IP_3 with different metal ions at pH 7, obtained by potentiometric titration, increases in the order $Hg^{II} < Ca^{II} < Pb^{II} < Ni^{II} < Cr^{III} \approx Zn^{II} \approx Cd^{II} < Al^{III} < Fe^{III}$. This suggests Al^{III} or Fe^{III} as the metal ions

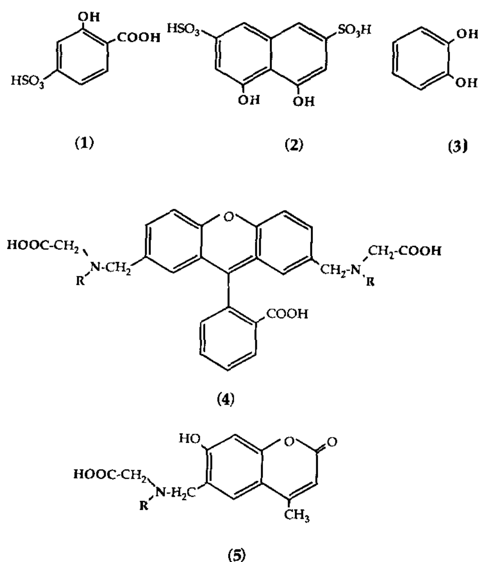

Fig. 1. Ligands investigated with regard to their use in a post-column ligand-exchange detection system. 1 = Sulphosalicylic acid; 2 = chromotropic acid; 3 = catechol; 4 = calcein (R = CH_2COOH) or methylcalcein (R = CH_3); 5 = calcein blue (R = CH_2COOH) or methylcalcein blue (R = CH_3).

of choice. Preliminary experiments showed that Al^{III} does not efficiently quench the fluorescence of the ligands investigated (see Fig. 1). In further work, we therefore investigated only the corresponding Fe^{III} complexes. The fluorescence of suitable ligands should efficiently be quenched by Fe^{III}, and 1,2,6-IP_3 should form a stronger complex with Fe^{III} than the ligand of choice. We found that sulphosalicylic acid, chromotropic acid and MCB meet these requirements. Ligands with an iminodiacetic acid (*e.g.*, calcein and calcein blue) or an *o*-dihydroxy (catechol) group form complexes with Fe^{III} that are too strong, while the fluorescence of methylcalcein is hardly quenched by Fe^{III}. Table I shows the optimum pH values for the ligand fluorescence and the ligand-exchange reaction with 1,2,6-IP_3 and the detection limits obtained in flow-injection analysis (FIA). MCB (λ_{exc} = 370 nm, λ_{em} = 440 nm) is clearly the most

TABLE I

PH RANGE FOR FLUORESCENCE AND LIGAND-EXCHANGE REACTION AND DETECTION LIMIT FOR THE 1,2,6-IP_3–Fe^{III} LIGAND REACTION FOR DIFFERENT LIGANDS

Ligand[a]	*pH range for*			*Detection limit (FIA) (ng)*
	Fluorescence	*Maximum fluorescence*	*Ligand exchange*	
SSA	>4.0	>4.0	1.0–4.5	138
CTA	3.5–5.5	3.5–4.0	1.0–5.0	18
MCB	4.0–10.0	6.0–7.0	1.0–8.0	3

[a] SSA = Sulphosalicylic acid; CTA = chromotropic acid; MCB = methylcalcein blue.

favourable ligand regarding the compatibility of the optimum pH values of ligand exchange and fluorescence and the sensitivity of the detection system. All further experiments were therefore carried out with the system Fe^{III}–MCB.

Post-column parameters

Several parameters that influence the post-column ligand-exchange reaction were investigated by FIA. The mobile phase pumped with the LC pump was the same as the ion-pair LC eluent.

pH. pH has a strong influence on (i) the complexing strength of 1,2,6-IP_3, as it determines the degree of its ionization; (ii) the formation of hydroxo complexes of Fe^{III}, which interferes with the formation of Fe–MCB above pH 8 and results in a large increase in the background fluorescence; (iii) the fluorescence of MCB, which has its maximum at pH 6–8, and (iv) the stability of 1,2,6-IP_3 (see below). Batch experiments showed that the ligand-exchange reaction between 1,2,6-IP_3 and Fe–MCB proceeds at a pH as low as 1, but the pH range suitable for the ligand-exchange reaction and fluorescence detection lies between 4 and 8. Owing to the instability of 1,2,6-IP_3 at pH 4–5 (see below), a pH of 7.0, obtained with Tris buffer, was chosen for the post-column reaction system.

Organic modifier. The type and content of organic modifier have a strong influence on the fluorescence of MCB, whereas the ligand-exchange reaction between Fe–MCB and 1,2,6-IP_3 is not affected. In aqueous solutions MCB fluoresces only weakly, and a considerable increase in fluorescence is observed if the content of organic modifier, *e.g.*, methanol, is increased above 50%. At a content of 70% methanol the fluorescence of MCB reaches its maximum. As optimum LC conditions were obtained with a mobile phase containing 37.5% acetonitrile, the total, *i.e.*, the post-column, content of organic modifier was increased to about 50% by preparing Fe–MCB in 95% (v/v) methanol and mixing the eluent and reagent solution at a flow-rate ratio of 1.0:0.3.

Molar ratio of Fe^{III} and MCB. The molar ratio of Fe^{III} and MCB has a strong influence on both the 1,2,6-IP_3 response and the fluorescence background and, therefore, on the signal-to-noise ratio. It is obvious that the 1,2,6-IP_3 response will decrease if an excess of Fe^{III} is present in the reagent solution, as the analyte will then complex the free Fe^{3+} ions before reacting with Fe–MCB. An excess of MCB, on the other hand, will increase the noise level caused by background fluorescence and therefore increase the detection limit of 1,2,6-IP_3.

Fig. 2 shows the dependence of both the 1,2,6-IP_3 response and the background fluorescence on the MCB:Fe ratio measured by means of FIA experiments. The response factor for 1,2,6-IP_3 increases steadily with increasing MCB:Fe ratio and reaches a more or less constant level at a ratio of 3.5:1. The fluorescence background starts to increase significantly at an MCB:Fe ratio higher than 3:1. The presence of free MCB then leads, as expected, to a higher fluorescence background. An MCB:Fe ratio of 3:1 was chosen for further experiments.

Reaction time and temperature and concentration of Fe–MCB. Fig. 3 shows the dependence of the response factor of 1,2,6-IP_3 on the reaction temperature for three different reaction coil volumes at a reagent (*i.e.*, Fe^{3+}) concentration of $6.6 \cdot 10^{-6}$ *M*. The reaction times (total flow-rate 1.3 ml/min) are 8.3, 13.8, and 23.0 s for the 180-, 300- and 500-μl coils, respectively. Fig. 3 shows that the optimum temperature is *ca.*

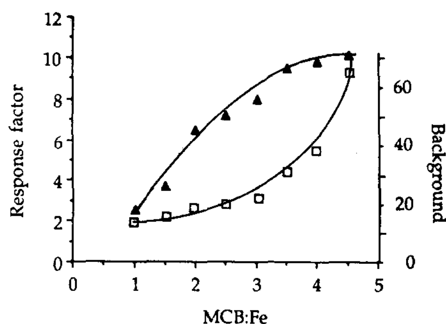

Fig. 2. Dependence of (▲) the response factor for 1,2,6-IP_3 and (□) the fluorescence background on the Fe:MCB molar ratio.

55°C for all the reaction coils tested. The response factors for the 300- and 500-μl reaction coils at 55°C are only slightly different, and a further increase in the reaction time by choosing lower flow-rates or higher reaction-coil volumes did not result in higher response factors; this indicates that the ligand-exchange reaction is virtually complete under these conditions. Decreasing the reagent concentration in order to reduce the background fluorescence caused a decrease in the reaction rate. At a reagent concentration of $1.6 \cdot 10^{-6}$ M the reaction-coil volume had to be increased to 700 μl and the temperature to 60°C in order to obtain similar response factors to those obtained with $6.6 \cdot 10^{-6}$ M at 300 μl and 55°C, but the detector noise was three times lower. A further decrease in the reagent concentration required longer reaction times, *i.e.*, a higher reaction-coil volume, which led to increased peak broadening and did not result in a lower detection limit for 1,2,6-IP_3.

Analytical data

Using a reagent concentration of $6.6 \cdot 10^{-6} M$, the detection limit for 1,2,6-IP_3 in ion-pair LC amounted to 10 ng (signal-to-noise ratio = 3:1). The detector response was linear ($r > 0.999$, $n = 4$) up to 1000 ng 1,2,6-IP_3. The relative standard deviation for 10-μl injections of 50 ng of 1,2,6-IP_3 was less than 2% ($n = 6$). If the reagent concentration was decreased to $1.6 \cdot 10^{-6}$ M, the detector response was linear only up to 100 ng, whereas the detection limit could be decreased to 3 ng.

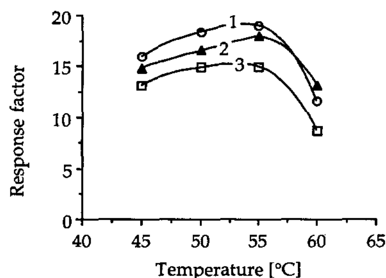

Fig. 3. Dependence of the response factor of 1,2,6-IP_3 on the reaction temperature for reaction-coil volumes of (1) 500, (2) 300 and (3) 180 μl. Reaction-coil volume used for cooling, 180 μl.

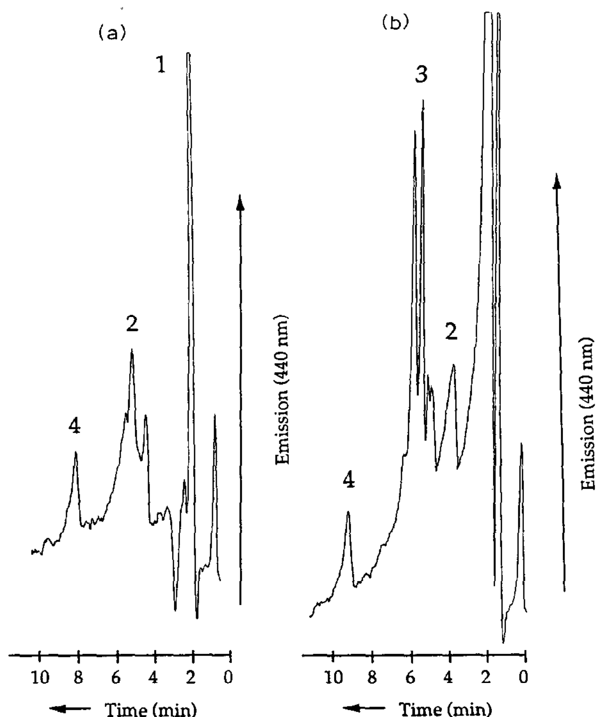

Fig. 4. (a) Chromatogram of a standard solution containing 10 ppb of 1,2,6-IP_3 and 1 mM sodium phosphate. Peaks: 1 = phosphate; 2 = TPABr impurities; 4 = 1,2,6-IP_3. Conditions: preconcentration to 1 ml; washing of the precolumn with 5 ml of acetonitrile–10 mM Tris buffer (15:85, v/v) containing 1 mM TPABr. For other conditions, see Experimental. (b) Chromatogram of a standard solution containing 10 ppb of 1,2,6-IP_3 and 100 ppb of IP_2. Peaks: 2 = TPABr impurities; 3 = IP_2; 4 = 1,2,6-IP_3. Conditions: preconcentration to 1 ml; no washing step. For other conditions, see Experimental.

Trace enrichment of 1,2,6-IP_3 via ion-pair formation

Precolumns are frequently applied in LC in order to increase the injection volume and also to achieve an on-line clean-up[22]. For highly ionized compounds, such as 1,2,6-IP_3, ion-exchange or ion-pairing techniques can be used for trace enrichment. The high capacity ratio of 1,2,6-IP_3 in ion-pair LC with TBABr or TPABr suggested the addition of alkylammonium-type counter ions to the sample in order to preconcentrate 1,2,6-IP_3 from aqueous solutions on C_{18}-bonded silica. The desorption of 1,2,6-IP_3 can occur by simply switching the preconcentration column to the analytical column.

By using a 10 × 4.0 mm I.D. precolumn packed with C_{18}-bonded silica, breakthrough volumes above 25 ml were obtained with both TBABr and TPABr at a 2 mM concentration. Counter-ion concentrations higher than 5 mM led to a significant decrease in recovery. At pH 7 and 2 mM TPABr the recovery for a 3-ml preconcentration of a solution containing 28 ppb (or $5.1 \cdot 10^{-8}$ M) 1,2,6-IP_3 was 95% with a relative standard deviation of 1% (n = 6). The detection limit for 1-ml preconcentrations was 5 ppb. For both direct injections and preconcentrations the pH of the sample solution had a large influence on the stability of 1,2,6-IP_3. At pH 4 the recov-

eries for 1,2,6-IP_3 were below 20%, probably owing to hydrolysis of the ester linkages. The recoveries increased steadily with increasing pH and reached a maximum at pH 7.

Owing to the strong retention of 1,2,6-IP_3 as a TPA ion pair on C_{18}-bonded silica, the precolumn can be washed with solutions having a low acetonitrile content (5–15%) in order to remove interfering components, such as inorganic phosphates, which are present in a large excess, *e.g.*, in plasma samples. We found that flushing the precolumn with 5 ml of acetonitrile–10 m*M* Tris buffer (15:85, v/v) containing 1 m*M* TPABr removed the inorganic phosphate present in the sample without losses of 1,2,6-IP_3.

A chromatogram for the trace enrichment of a standard solution of 10 ppb of 1,2,6-IP_3 in the presence of 1 m*M* sodium phosphate [preconcentration of 1 ml, washing with 5 ml of acetonitrile–10 m*M* Tris buffer (15:85, v/v) containing 1 m*M* TPABr] is shown in Fig. 4a. Fig. 4b demonstrates the ability of the present method to detect 1,2,6-IP_3 (10 ppb) in the presence of inositol bisphosphates (IP_2; 100 ppb) which are formed during the metabolic degradation of 1,2,6-IP_3 in blood. IP_2 is probably present as a mixture of isomers, which may explain the occurrence of two peaks. The slightly different retention times in Fig. 4a and b may be caused by slight changes in the LC eluent composition as a result of prolonged re-use. In both chromatograms, peak 2 has the same shape and size for injected blanks and standard solutions and therefore cannot be attributed to hydrolysis products of 1,2,6-IP_3.

CONCLUSIONS

The use of a post-column reaction detection system based on ligand exchange between phosphate ions and an Fe^{III}–MCB reagent with subsequent fluorescence monitoring allows the successful determination of low-nanogram amounts of 1,2,6-IP_3 with good reproducibility and low standard deviation. If the ion-pair LC–reaction detection system is combined with an on-line trace enrichment step based on ion-pair formation, preconcentration of 1-ml sample volumes permits a detection limit of *ca.* 5 ppb to be achieved.

Preliminary investigations have shown that the present detection technique is, as expected from results with other metal ions and ligands[9,11], generally applicable to the determination of organic phosphates, such as metabolites of 1,2,6-IP_3 (inositol mono- and bisphosphates). Currently the application of the method to the determination of 1,2,6-IP_3 in plasma is being investigated. Owing to the presence of a large number of phosphate-containing compounds in plasma (including inorganic phosphates), the optimization of the trace-enrichment system is of major concern in this work. Primarily, different washing steps for the removal of interfering anions are being investigated. The preconcentration of inositol phosphates on metal-loaded phases[22] will also be studied.

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