Journal of Chromatography, 393 (1987) 454–458
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 494

Note

Separation and analysis of fluorescent derivatives of myo-inositol and myo-inositol 2-phosphate by high-performance liquid chromatography

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(First received October 31st, 1986; revised manuscript received February 10th, 1987)

Myo-inositol, myo-inositol phosphates and their glycerolipid derivatives serve a number of biological functions. As phosphoinositides they are important components of membrane structure. As myo-inositol pentakis-phosphates they alter hemoglobin oxygen affinity in avian and reptilian erythrocytes[1]. As inositol trisphosphates and possibily as inositol phosphate glucosamines they may serve as intracellular effectors for hormone action[2]. Evaluating the role of inositol derivatives as intracellular signals has proved to be difficult because of the small amount present in cells and the lack of convenient quantitative analyses of inositol and inositol phosphates[3,4]. Thus much of the information covering the role of inositol phosphates as cellular mediators has been inferred from radio-labelling studies with ^{3}H-myo-inositol.

The present studies were initiated to apply the high degree of resolution available with high-performance liquid chromatography (HPLC) and the sensitivity of fluorescence detection to the analysis of myo-inositol and myo-inositol phosphate. Previous studies have employed isatoic anhydride to prepare fluorescent anthraniloyl derivatives of nucleotides and nucleosides containing available ribose hydroxyl groups[5,6]. As inositol and inositol phosphates have multiple hydroxyl groups available for reaction with isatoic anhydride, we chose this strategy for preparation of fluorescent derivatives of myo-inositol and myo-inositol 2-phosphate. In the present report we show that fluorescent products can be readily prepared in good yields and resolved by HPLC on reversed-phase and NH_2 columns under isocratic conditions.

MATERIALS AND METHODS

Reagents

Myo-inositol and myo-inositol 2-phosphate were purchased from Sigma (St. Louis, MO, U.S.A.). Isatoic anhydride, pyridine, dimethylsulfoxide and triethylamine were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC-grade solvents were supplied by J. T. Baker (Philipsburg, NJ, U.S.A.).

0021-9673/87/$03.50

Formation of inositol derivatives

Reactions were conducted in a variety of solvents including water, dimethylsulfoxide and pyridine. Typically inositol and inositol-2-phosphate were dissolved in water or other solvents and isatoic anhydride dissolved in aprotic solvent containing triethylamine. Suitable aliquots of each mixture were combined and heated at 60°C for 1 h.

Chromatographic analyses

HPLC analysis was performed on Varian Model 5000 high-performance liquid chromatograph (Varian, Palo Alto, CA, U.S.A.). The detector used was a Varian Fluorochrome with excitation at 360 nm and emission above 420 nm. Separations were carried out on a 250 mm × 4.6 mm I.D. Econosphere 5 μm reversed-phase column (Alltech, Deerfield, IL, U.S.A.) and on a 250 mm × 4.6 mm I.D. Econosphere 5 μm NH_2 column (Alltech). The progress of reactions was screened by developement on reversed-phase thin-layer chromatographic (TLC) plates with the same mobile phases used for HPLC.

RESULTS AND DISCUSSION

The presumed reaction between myo-inositol and isatoic anhydride is shown in Fig. 1. Since myo-inositol and myo-inositol 2-phosphate have many available groups for derivatization, it is predicted that heterogeneous mixtures of products would be formed differing in regard to the position and the number of derivative groups.

Fig. 2 are chromatograms of a reaction of inositol with excess isatoic anhydride resolved on reversed-phase (C_8) and amino (NH_2) columns. On the reversed-phase

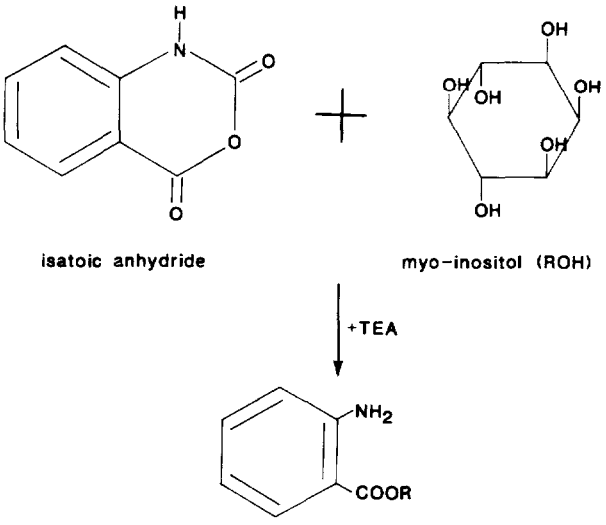

Fig. 1. Proposed reaction scheme for myo-inositol and isatoic anhydride. It is presumed that in the presence of base catalyst, triethylamine (TEA), the isatoic anhydride reacts with one or more hydroxyl groups to produce anthaniloyl derivatives and carbon dioxide.

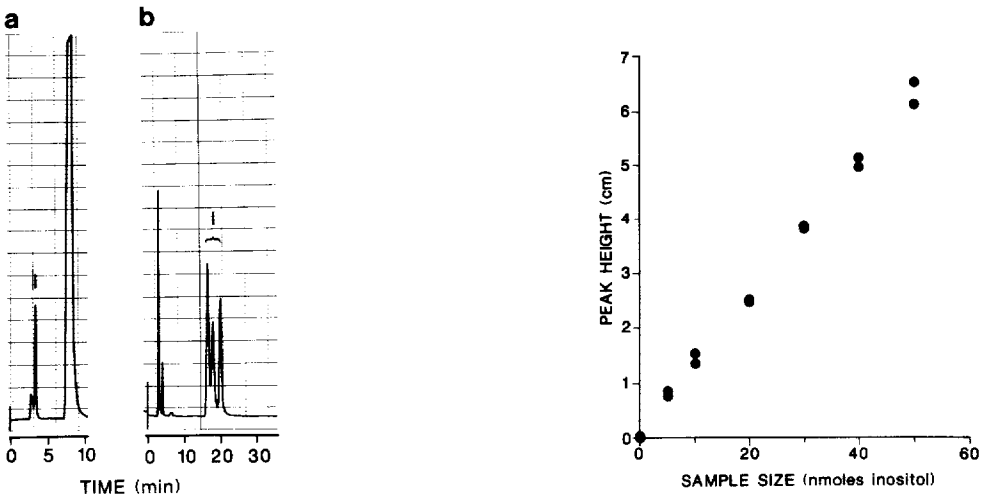

Fig. 2. Left panel: (a) chromatogram of myo-inositol derivative of inositol on the C_8 reversed-phase column. Mobile phase, acetonitrile–10 m*M* potassium dihydrogen phosphate, pH 4.0 (20:80); flow-rate, 1 ml/min. The major product (peak I) has a retention time of 3.7 min. (b) Chromatogram of myo-inositol derivative on NH_2 column. Mobile phase, acetonitrile–water (90:10); flow-rate, 1 ml/min. The major products (peak group I) appear as three peaks with retention times of 15–20 min. Right panel: curve relating fluorescent intensity (obtained from peak heights) to amount of myo-inositol present in reaction mixtures. Separate reactions were conducted with 0.1–1.0 μmoles myo-inositol. Identical aliquots were chromatographed on the C_8 column and peak heights calculated. Each pair of points represents values from two separate injections.

C_8 column one major product peak was noted. It ran with a retention time of 3.7 min just behind a fluorescent side product peak. When chromatographed on the amino column, three distinct peaks were resolved indicating, as might be expected, that a heterogenous product was formed.

When a series of reactions were conducted with increasing amounts of myo-inositol and the product peak heights were calculated from the reversed-phase chromatograms, a linear relationship between myo-inositol and peak height was apparent (Fig. 2 right panel). In these reaction mixtures 10–100 μl aliquots of an aqueous 10 m*M* myo-inositol solution were added to 100 μl of 10 m*M* isatoic anhydride in dimethyl sulfoxide (DMSO). A volume of 10 μl of triethylamine was added to each sample and the final reaction volume brought to 210 μl by the addition of water. The samples were heated to 60°C for 1 h then diluted 1:10 in the mobile phase. The sample size for the HPLC injections was 100 μl.

Using the relative standard deviation of measured peak heights for ten replicate samples the precision of the assay was determined to be 6%. The relative error in the predicted concentration of standard samples, not used in the calculation of calibration curves, was used as the measure of accuracy of the procedure and was routinely found to be a 4–8%. The detection limit given as the mean of the blank plus three times the standard deviation of the blank was determined to be 0.1 nmoles. Although these studies were conducted with nanomolar amounts of myo-inositol, the detection of much smaller amounts of myo-inositol should be feasible with more sensitive fluorescence detection and smaller reaction volumes.

Fig. 3 shows reversed-phase chromatograms obtained from reaction of isatoic anhydride with commerical prepartions of myo-inositol 2-phosphate. One major product peak (labelled as III), was strongly retained on the reversed-phase column under mobile phase conditions used for the separation of the myo-inositol derivative (Fig. 3a). Two other fluorescent peaks (labelled as I and II) were also apparent. These products varied inconsistantly as a function of the myo-inositol 2-phosphate added. Neither of these peaks chromatographed with the derivatives of myo-inositol and therefore they do not represent a myo-inositol contamination. The major derivative of myo-inositol 2-phosphate also resolved with a short retention time when chromatographed with a mobile phase of acetonitrile–potassium dihydrogen phosphate, pH 4.0 (50:50) (Fig. 3b). The precision, accuracy, and detection limit for the myo-inositol 2-phosphate reaction were found to be the same as for the myo-inositol reaction.

Paradoxically the anthraniloyl derivative of myo-inositol 2-phosphate was much more strongly retained on the reversed-phase column than were the derivatives of the less polar myo-inositol. This might reflect the possibility that the presence of the phosphate group enhances the derivatization so that under identical reaction conditions more hydroxyl groups on the inositol phosphate are reacted and the products are thus much less polar. Alternatively it may be that the amino group of the anthranilic ester coordinates with the phosphate group of the myo-inositol so that intramolecular ion pairing results. Identification of the structure of the derivative will be necessary to establish the reason for this somewhat anomalous behavior.

The method presented in this report provides a simple and sensitive means of quanitating myo-inositol (Fig. 2, right panel). Hitherto, studies have relied upon gas chromatography of trimethylsilyl derivatives[3]. Reports by others have described separation of inositol phosphates by anion-exchange HPLC, and recently Meek[4]

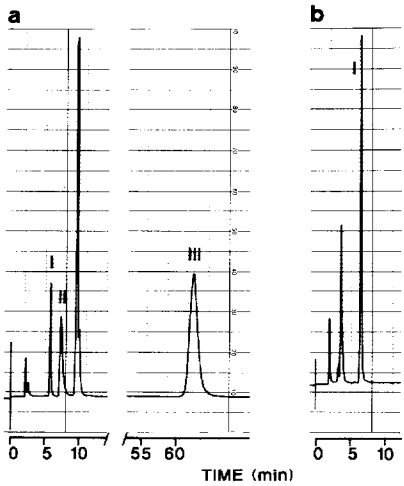

Fig. 3. (a) Chromatogram of myo-inositol 2-phosphate derivative on the C_8 reversed-phase column. Mobile phase, acetonitrile–10 m*M* potassium dihydrogen phosphate, pH 4.0 (18:82); flow-rate, 1 ml/min. Peaks I and II appear to be derivatives of contaminants. The major product, peak III appears as a symmetrical peak, strongly retained on the column. (b) As in (a) but mobile phase acetonitrile–potassium dihydrogen phosphate, pH 4.0 (50:50). Major product peak is labelled I.

described a post-column detection method whereby the inositol phosphates were hydrolysed by immobilized alkaline phosphatase and inorganic phosphate detected by formation of molybdate complexes. This post-column detection method however lacks sensitivity and exhibits interference by heavy metal cations. A combination of the techniques for separation of inositol phosphates by anion exchange followed by enzymatic dephosphorylation and quantitation of inositol as fluorescent derivatives may offer advantages of sensitivity and lack of interference for quantitation of inositol polyphosphates. Furthermore, since myo-inositol tris- and tetra-phosphates have available hydroxyl groups it is possible that the present method could be adapted for derivatization of these inositol polyphosphates prior to separation by HPLC.

REFERENCES

1 L. F. Johnson and M. E. Tate, *Can. J. Chem.*, 47 (1969) 63.
2 A. R. Saltiel, J. A. Fox, P. Seline and P. Cuatracasus, *Science (Washington, D.C.)*, 233 (1986) 967.
3 S. E. Rittenhouse and J. P. Sasson, *J. Biol. Chem.*, 260 (1985) 8657.
4 J. L. Meek, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 4162.
5 T. Hiratsuka, *J. Biol. Chem.*, 257 (1982) 13354.
6 T. Hiratsuka, *Biochim. Biophys. Acta*, 742 (1983) 496.