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Ion-pair chromatography of inositol polyphosphates with N-methylimipramine

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SUMMARY

A novel counter-ion, N-methylimipramine, was synthesized and utilized in the separation of inositol phosphates by ion-pair chromatography. The structural identity of the counter-ion was documented by nuclear magnetic resonance spectroscopy. This counter-ion was capable of resolving inositol phosphates isocratically by reversed-phase high-performance liquid chromatography. Solvent polarity and ionic strength markedly affected the retention of the polyphosphorylated inositides. pH, however, was less significant in its effects. Injection of inositol trisphosphate paired to N-methylimipramine into a mobile phase containing tetrabutylammonium ions demonstrated free exchange of the inositide between the counter-ions. This counter-ion may therefore prove useful in defining empirically the mechanisms of ion-pair chromatography

INTRODUCTION

Ion-pair chromatography has provided a useful alternative to the separation of polyphosphorylated metabolites and pharmaceuticals by high-performance liquid chromatography (HPLC). Specifically, it has permitted the separation of both anionic and cationic compounds by reversed-phase chromatography. Such an approach provides several advantages over traditional ion-exchange methods including reproducibility, prolonged column life and most importantly the ability to exploit several variables in the optimization of resolution. These variables include solvent polarity, ionic strength, pH, temperature and the chemistry of the counter-ion employed. The specific mechanisms of ion-

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pair chromatography, however, are not well understood. This is due partly to the inability to easily monitor the concentration of the counter-ion in the mobile phase and thus provide empiric support for various models of ion-pairing.

We have recently reported the use of ion-pair chromatography in the separation of inositol phosphates by reversed-phase chromatography using tetrabutylammonium phosphate as a hetaeron [1]. We now report the synthesis and application of another quarternary amine, N-methylimipramine, as an alternative counter-ion for the separation of inositol polyphosphates. Because N-methylimipramine possess a high molar extinction coefficient it may be monitored on-line during the resolution of these important sugar phosphates.

EXPERIMENTAL

Imipramine hydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.); tetrabutylammonium phosphate, C^2HCl_3 and ^2H_2O were supplied by Aldrich (Milwaukee, WI, U.S.A.). Methyl bromide was supplied by Matheson Gas Products (Joliet, IL, U.S.A.). All other materials were HPLC grade. Tritiated inositol phosphate standards were obtained from New England Nuclear or Amersham.

Synthesis of N-methylimipramine

A 20-g amount of imipramine hydrochloride was dissolved in HPLC-grade water in a volume sufficient to fully dissolve the compound. The free amine was generated by the addition of 6 *M* sodium hydroxide. Formation of the free amine was evidenced by the development of an immiscible oily layer. Base was further added until no additional increase in this layer was evident. The solution was transferred to a separatory funnel and serially extracted into methylene chloride. The amine in methylene chloride was collected and dried partially under nitrogen at 50 °C and then completely in a Speed Vac concentrator (Savant, Farmingdale, NY, U.S.A.). The dried product was then transferred to an Erlenmeyer flask and dissolved in acetone.

The acetone solution was placed in an ice-cold water bath and methyl bromide was bubbled through resulting in the formation of a precipitate. This was continued with intermittent shaking for 10 min following the appearance of the precipitate to insure a more complete reaction. The reaction mixture was filtered through Whatman No. 1 paper and washed with four volumes of acetone. The precipitate was then dried at 40 °C.

The phosphate salt of N-methylimipramine was obtained by the addition of 0.2 *M* orthophosphoric acid to an equal volume of 0.4 *M* N-methylimipramine bromide. Silver oxide (6.2 g) was added to the solution and the mixture was shaken overnight at room temperature. The non-quaternized amine and silver bromide were removed by centrifugation of the solution for 5 min at 3000 *g*. The supernatant was removed and brought to pH 9.0 by the addition of 0.5 *M*

sodium phosphate. The free amine was serially extracted into methylene chloride until a constant partition ratio was obtained as monitored by UV absorbance at 300 nm. The aqueous layer containing N-methylimipramine phosphate was dried in a Speed Vac concentrator.

NMR spectroscopy

Proton magnetic resonance imaging of both imipramine and N-methylimipramine were performed on a Bruker spectrometer. Approximately 1 mg of each compound was scanned at 300 MHz in C^2HCl_3 and ^2H_2O, respectively.

Thin-layer chromatography

Thin-layer chromatography (TLC) of the precursor and product was performed on silica gel glass backed plates which were preactivated at 80°C (Sigma). A mobile phase consisting of 0.5 *M* sodium bromide in methanol–chloroform (80:20, v/v) was employed [2]. The products were detected by exposure of the plates to UV light.

High-performance liquid chromatography

Chromatographic analyses were carried out on a Waters system consisting of a 501 pump, a U6K injector and a 481 Lamda-Max UV spectrophotometer. Radiochemical measurements were conducted on a Beckman 171 radioisotope detector. Separations of tritiated inositol phosphates were performed on a Supelcosil LC18 (250 mm × 4.8 mm I.D.) 5-μm reversed-phase column (Supelco, Bellefonte, PA, U.S.A.). A C_{18} guard-column insert was utilized as well.

RESULTS

Product identification

Fig. 1 displays the structure of the quaternary amine, N-methylimipramine. Fig. 2A displays the NMR spectrum of imipramine hydrochloride in C^2HCl_3

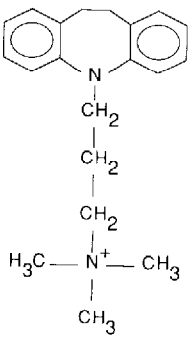
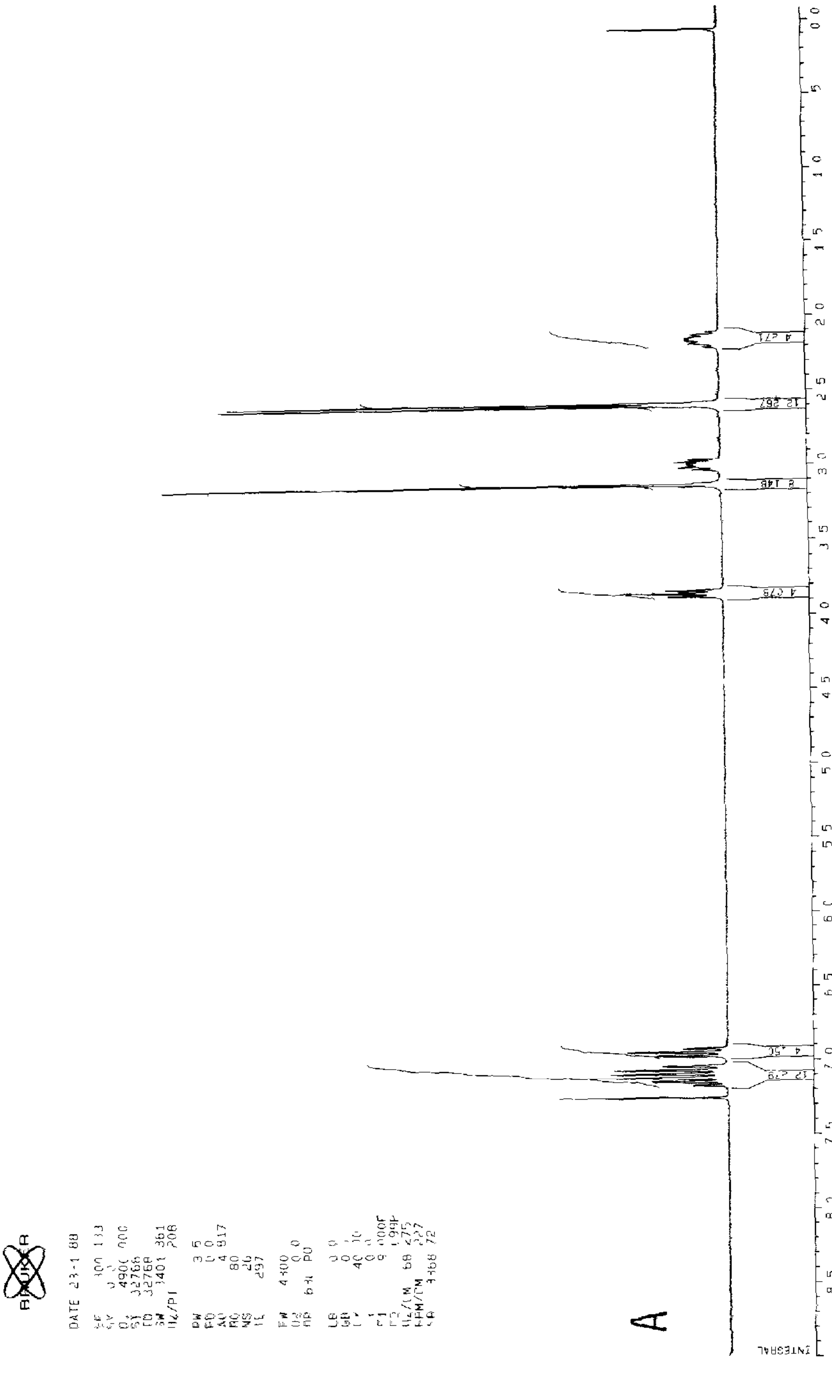


Fig. 1. Structure of N-methylimipramine.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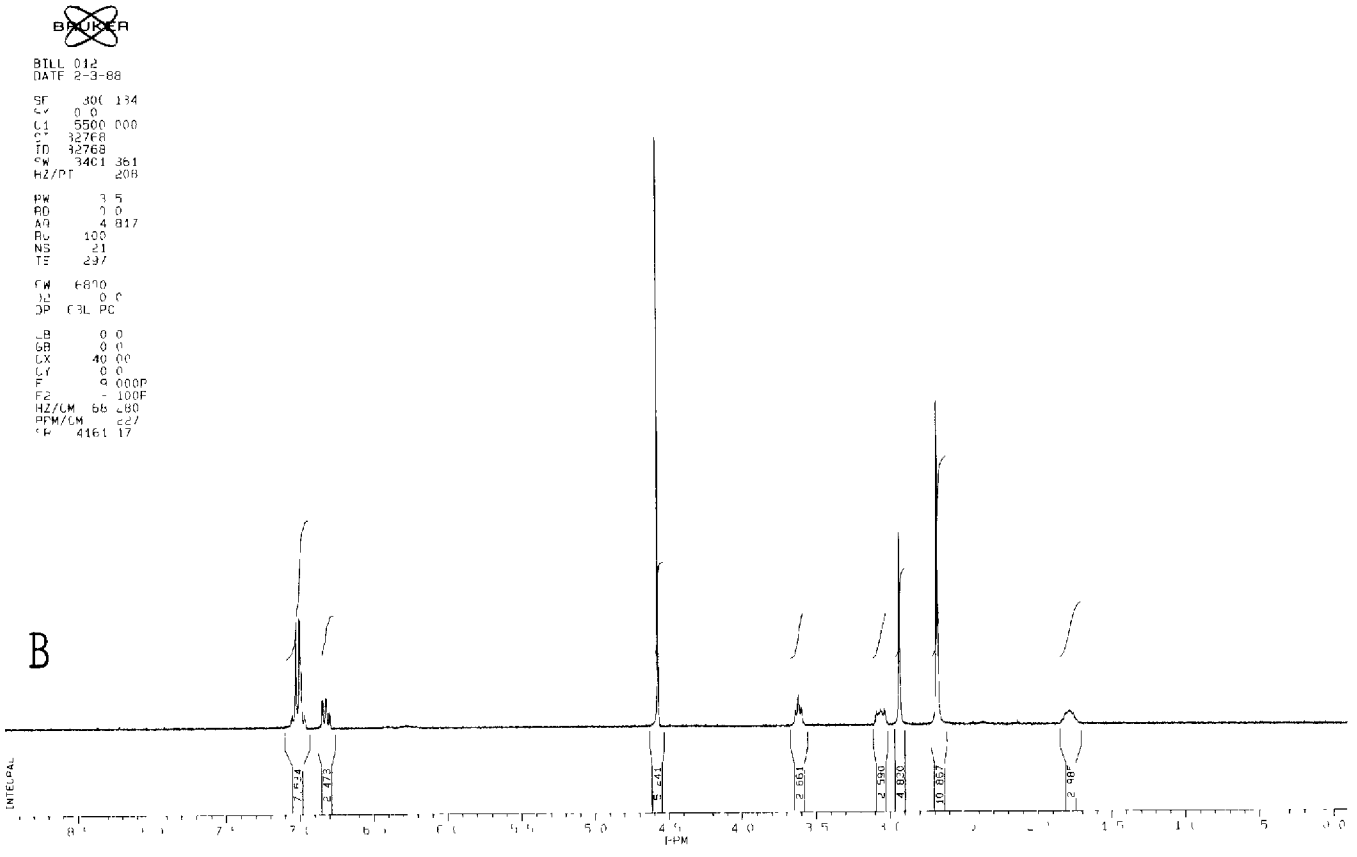

Fig. 2. (A) NMR spectrum of imipramine in C^2HCl_3. The doublet at 2.60 ppm represents the dimethylamino group of the precursor. (B) NMR spectrum of N-methylimipramine phosphate in ^2H_2O. The trimethylamino group is represented by a singlet at 2.65 ppm.

following its conversion to the free amine. The dimethylamino function is represented as a doublet with a chemical shift of 2.60 ppm. Predictably, this functional group is the only one of the starting material to have a doublet; the assignment of this peak is therefore unequivocal. Fig. 2B represents the spectrum of N-methylimipramine phosphate in ^2H_2O. Here the doublet has been converted to a singlet as predicted for the formation of a trimethylamine. The area of this peak, when corrected for the number of protons present, remains constant in comparison to the other functional groups present. This is consistent with the formation of a single product.

Thin-layer chromatography

Fig. 3 displays the TLC separation of imipramine hydrochloride and of the hydroxide and phosphate salts of N-methylimipramine. Lane A shows the re-

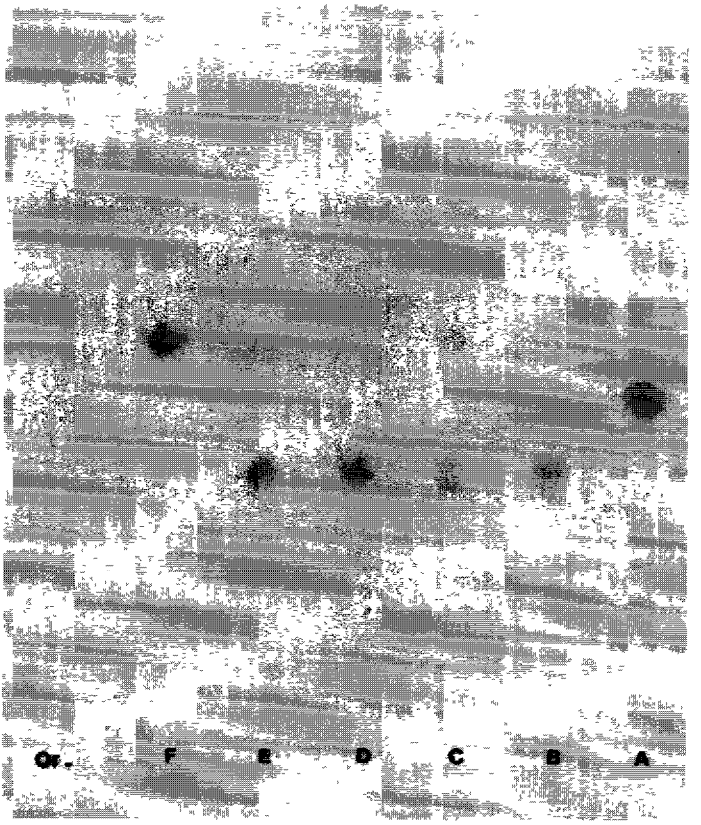

Fig. 3. Thin-layer chromatogram of imipramine and N-methylimipramine. (A) Imipramine hydrochloride; (B) N-methylimipramine phosphate; (C) N-methylimipramine hydroxide; (D) and (E) sequential back extractions of the methylene chloride phase demonstrating the removal of unreacted imipramine; (F) the residual product in the organic phase probably imipramine in its free tertiary form.

tention of 5 μl of 0.01 *M* imipramine hydrochloride in water following exposure to UV light. Lanes B and C display the retention of N-methylimipramine phosphate and hydroxide, respectively. The R_F values for imipramine and the N-methylimipramine salts were 0.69 and 0.56, respectively. An impurity with a larger R_F value was observed following the first aqueous extraction when water was substituted for phosphoric acid. This was demonstrated to be unreacted substrate by sequential aqueous extractions of the chloroform phase (lanes D and E) and subsequent spotting of the remaining material in the organic phase (lane F). The unreacted free amine displayed a significantly longer retention than did the chloride salt of imipramine or the N-methylimipramine.

HPLC separation

Fig. 4 displays the separation of myo-[2-^{3}H]inositol mono-, bis-, tris- and tetrakisphosphates utilizing an ideal mobile phase consisting of 0.003 *M* N-methylimipramine, 0.03 *M* potassium dihydrogenphosphate and 22% acetonitrile, pH 3.5, at a flow-rate of 0.75 ml/min. Resolution of all standards was apparent with complete elution of inositol tetrakisphosphate in 30 min. The contribution of mobile phase composition was more formally assessed and is displayed in Fig. 5. Here the observed capacity ratio (k') was measured as a function of solvent polarity (panel A), pH (panel B), ionic strength (panel C) and counter-ion concentration (panel D). All three factors affected the retention of the tritiated standards. As for tetrabutylammonium phosphate, the retention of inositol monophosphate was resistent to any manipulation. In contrast to the tetrabutylammonium counter-ion, however, the effect of pH was less apparent in the present study.

Fig. 6 displays the chromatographic profile of inositol phosphates from MDCK cells prelabelled with 4 μCi myo-[2-^{3}H]inositol for 48 h and stimulated with 10^{-7} *M* bradykinin.

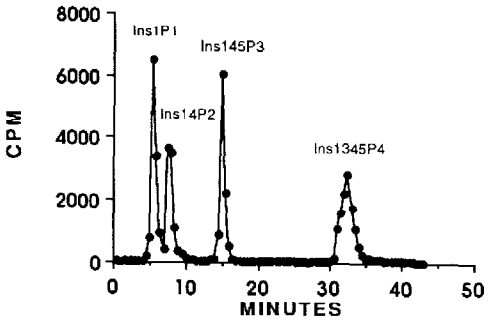

Fig. 4. Separation of myo-[2-^{3}H]inositol phosphate standards in a mobile phase consisting of 0.003 *M* N-methylimipramine, 0.03 *M* potassium dihydrogenphosphate and 22% acetonitrile, pH 3.5. The flow-rate was 0.75 ml/min.

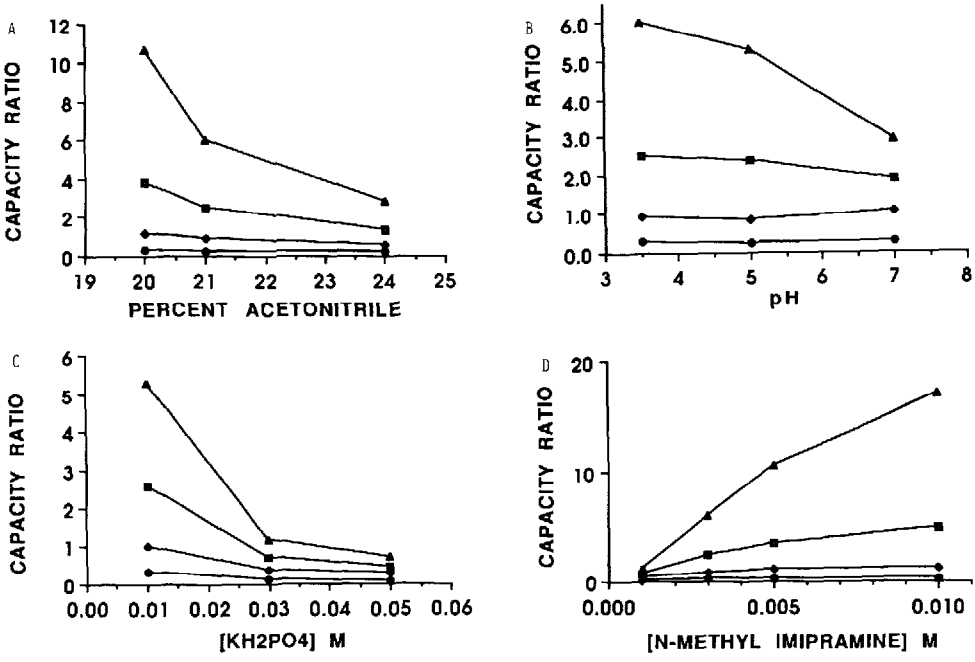

Fig. 5. Observed capacity ratios for inositol phosphate standards as a function of solvent polarity (A), pH (B), ionic strength (C) and counter-ion concentration (D) (▲) Myoinositol 1,3,4,5-tetrakisphosphate; (■) myoinositol 1,4,5-trisphosphate; (♦) myoinositol 1,4-bisphosphate; (●) myoinositol 1-monophosphate.

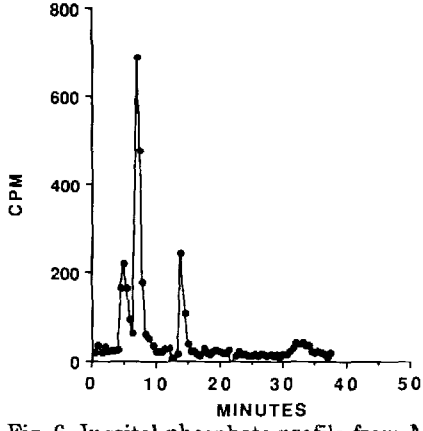

Fig. 6. Inositol phosphate profile from Madin-Darby canine kidney cells radiolabelled for 48 h with myo-[2-^{3}H]inositol and stimulated with 10^{-7} *M* bradykinin. Free inositol and inositol monophosphate isomers were eliminated by solid-phase extraction of a trichloroacetic acid cellular extract. A C_{18} Sep-Pak cartridge was preequilibrated with 0.003 *M* N-methylimipramine phosphate in water. The neutralized cellular extract was added to the column which was subsequently washed with 20 ml of water. The retained inositol phosphates were eluted with 100% acetonitrile, dried and resuspended in mobile phase prior to separation by HPLC. The chromatographic conditions were the same as in Fig. 4.

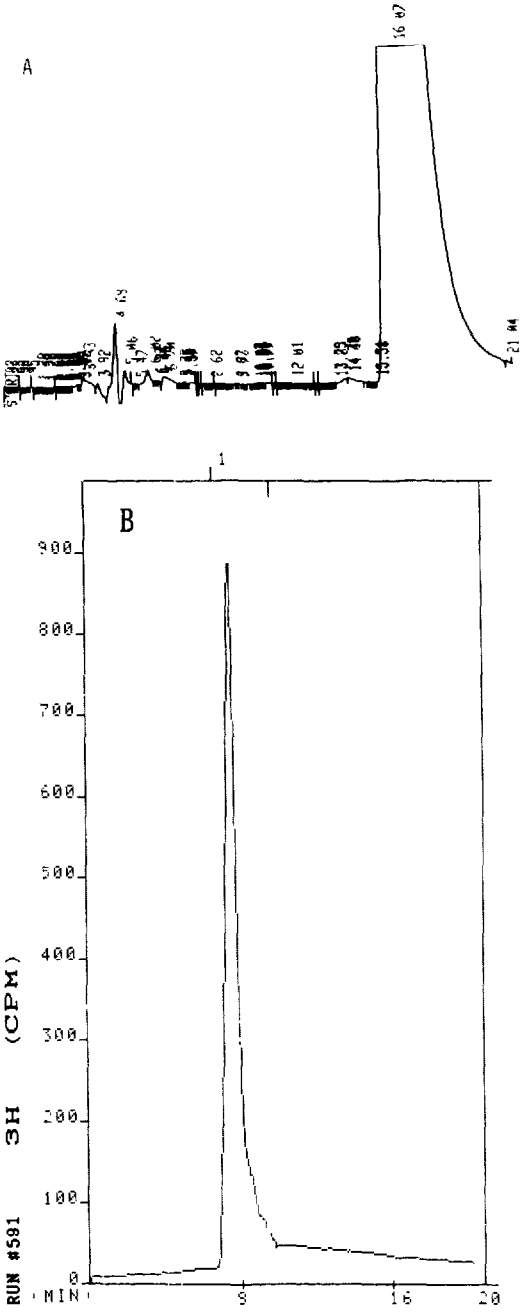

Fig. 7. Combined UV and radioisotopic measurements of inositol 1,4,5-trisphosphate and N-methylimipramine phosphate. Myo-[2-^{3}H]inositol 1,4,5-trisphosphate (5 nCi) was added to 5 nmol of unlabelled myoinositol 1,4,5-trisphosphate in a solution of 0.01 *M* N-methylimipramine phosphate and subjected to HPLC separation utilizing tetrabutylammonium phosphate as a counterion. (A) UV tracing at 300 nm. (B) Radioisotopic elution of the inositol trisphosphate standard.

Finally, the ability of N-methylimipramine to exchange within the mobile phase with another quaternary amine was investigated. Fig. 7 displays the simultaneous spectrophotometric and radioisotopic chromatograms where 5 nCi of myo-[2-^{3}H]inositol 1,4,5-trisphosphate was added to 5 nmol of unlabelled myoinositol 1,4,5-trisphosphate in a solution of 0.01 *M* N-methylimipramine phosphate and injected into a mobile phase consisting of 0.04 *M* tetrabutylammonium phosphate, 0.025 *M* potassium dihydrogen phosphate and 21% acetonitrile (apparent pH 3.25). As observed here, the N-methylimipramine eluted both as a single peak and separately from the inositol trisphosphate with a retention of 16.22 min. Inositol 1,4,5-trisphosphate, on the other hand, eluted at 7.44 min presumably paired to tetrabutylammonium. Comparable elution profiles were observed for concentrations of N-methylimipramine varying between 0.001 and 0.01 *M*.

DISCUSSION

It is now commonly appreciated that the generation of inositol 1,4,5-trisphosphate is accompanied by its subsequent metabolism to a large number of additional inositol phosphates [3]. The ability to discern both the discrete pathways for metabolism and the physiological importance of individual metabolites has been limited in part by the chromatographic methods available for their resolution. Our group and others [1,4] have recently described the use of ion-pair chromatography for the resolution of polyphosphorylated inositides and have characterized in part the effects of pH, ionic strength and solvent polarity and the retention characteristics of the inositol phosphates. For many applications this method is comparable to anion-exchange-based HPLC and in many respects is technically superior [1]. The potential manipulation of pH, ionic strength and solvent polarity provide the investigator with a large number of potentially useful parameters to accomplish more ideal separations. Previous investigations in this area, however, have for the most part neglected the composition of the counter-ion as an additional variable for chromatographic separation.

The current study reports the synthesis and utilization of an alternative counter-ion, N-methylimipramine, for inositol phosphate separation. This compound was originally described for use in liquid–liquid chromatographic separations of singly charged anionic compounds which can be readily partitioned into organic phases [5]. The inositol phosphates are not amenable to a similar approach since they cannot be partitioned with this or other more hydrophobic counter-ions into an organic phase such as methylene chloride and pentanol (data not shown). Thus, in contrast to salicylates and to other organic anions, the potential use of N-methylimipramine or other fluorescent or UV-absorbing counter-ions for on-line quantitation of inositol phosphates appears limited. To our knowledge, however, N-methylimipramine has not been

reported for use in conventional solid-phase ion-pair chromatography. It is unique from the traditionally used alkylamines in that it possesses a high molar extinction coefficient and thus its concentration can be easily monitored within the mobile phase.

Lagerstrom and Theodorsen [5] reported the synthesis of N-methylimipramine but provided no documentation of its structure. NMR spectroscopy was utilized to provide confirmation of the synthesis of the quaternary amine. Protons corresponding to a chemical shift of 2.60 ppm were assigned to the methyl functions based on peak multiplicity, peak height, the proton number and electronegativity. The conversion of this peak from a doublet to a singlet was consistent with the formation of the N-methyl derivative.

TLC provides a rapid alternative for following the formation of the N-methyl species and follows an established method for the resolution of phenothiazine derivatives [2]. The retention characteristics were independent of the anion associated with the quaternary amine but were significantly different from both the chloride salt of imipramine and from non-ionized imipramine.

The retention characteristics of the inositol phosphates were comparable to those observed for the use of tetrabutylammonium phosphate as a counter-ion [1]. Both ionic strength and solvent polarity markedly changed the capacity ratios of the individual standards. However, variation of pH less of an effect on the retention of the standards than was observed with the alkylamine.

Finally, the comparative affinities of tetrabutylammonium phosphate and N-methylimipramine phosphate were evaluated by monitoring the elution of inositol trisphosphate and N-methylimipramine injected as an ion pair into a mobile phase containing tetrabutylammonium ions. Under these conditions it was observed that the inositol phosphate and the imipramine derivative eluted with markedly different retentions. This observation was compatible with the existence of free exchange of the inositol trisphosphate between the two counter-ions. This is consistent with the dynamic exchange model of ion-pairing which postulates the rapid exchange of the separated species between counter-ions as opposed to its continued association as a single ion-pair [6].

In summary, N-methylimipramine may be used as an alternative counter-ion for the isocratic separation of inositol phosphates by reversed-phase chromatography. Its optical properties make it a useful compound for further discerning the mechanisms of ion-pair chromatography.

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