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Note

Performing high salt concentration gradient elution ion-exchange separations using thermospray mass spectrometry

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Thermospray liquid chromatography (LC)–mass spectrometry (thermospray) can be performed by vaporizing the liquid chromatograph effluent with a resistance-heated capillary vaporizer within the mass spectrometer[1]. The greatest sensitivity is obtained when the capillary is heated to a temperature such that complete vaporization of the LC effluent occurs at a temperature at the capillary exit that produces neither unvaporized droplets nor a superheated dry vapor[1]. In gradient elution ion-exchange separations with thermospray analysis the ionic strength is changed with volatile salts such as ammonium formate or ammonium acetate. As an example of interest to us, in ion-exchange separations of inositol phosphates the salt concentration has to be raised to 0.8 *M* to elute the most highly phosphorylated member of this group of compounds, inositol hexaphosphate. However, an ammonium acetate concentration of 0.8 *M* causes frequent clogging of the vaporizer reducing its useful life and interrupting analyses. A simple way to avoid that problem is to add, postcolumn, a gradient in the reverse sense of that of the eluting gradient, enabling the thermospray process to be performed with an isocratic salt solution of half the concentration of the eluting buffer. This has the additional benefit of increased stability of the thermospray process and of the resulting ion intensities by not having to program the vaporizer temperature.

EXPERIMENTAL

Two methods have been used to generate a gradient that is the reverse of the separation gradient. In both cases the high-pressure separation pumping system consisted of a pair of Shimadzu Model LC-6A pumps with gradient controller (Shimadzu, Kyoto, Japan). The counter-gradient was developed in two ways: (1) using two sets of HPLC pumps, each with a gradient programmer, and (2) generating the reverse gradient with a two-reservoir low-pressure gradient mixer that supplies a single high-performance LC pump. In both cases the mixing tee used was a Valco (Houston, TX, U.S.A.) 1/16-in. tee.

Method 1 (Fig. 1)

Two Waters Model M-6000A HPLC pumps (Fig. 1, pumps 3 and 4) and Model 660 gradient forming programmer (Waters, Milford, MA, U.S.A.) were used

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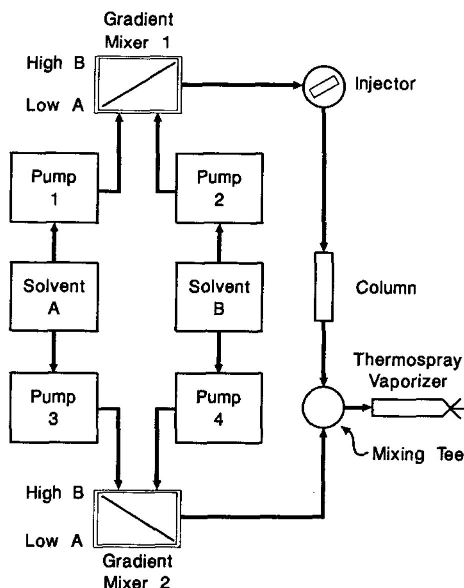

Fig. 1. Diagram of the pumping arrangement for isocratic thermospray mass spectrometry with a high ionic strength gradient elution separation.

drawing eluent from the same reservoirs that supplied the separation gradient, however, generating the gradient from high to low concentration and matched in time and volume with the separation gradient.

Method 2

The arrangement differs from that of Fig. 1 in that the counter gradient was developed at ambient pressure and supplied to a single Waters M-6000A pump. The linear gradient was supplied by a 100-ml concentric cylinder linear gradient former (Pace Model PGM-1; Isolab, Akron, OH, U.S.A.). The counter-gradient volume and the pumping rate were matched with that of the separation gradient.

Inositol phosphate mixture

A mixture of *myo*-inositol monophosphates (IP_1s) and polyphosphates (IP_2s, IP_3s, etc.) was prepared by dissolving 6.5 g of sodium phytate ($Na_{12}IP_6$) (Sigma, St. Louis, MO, U.S.A.) in 75 ml of water, adjusting the pH to 9.2 with glacial acetic acid and heating under reflux for 48 h. The mixture was found, by high-voltage paper electrophoresis[2], to contain all of the IP_ns except IP_6. A small amount of $InsP_6$ was added to give a mixture of all of the $InsP_n$s.

Separation of the myo-*inositol phosphate mixture*

Separations were carried out on either of two resin-based anion-exchange columns; Mono-Q, 50 mm × 5 mm (Pharmacia LKB, Piscataway, NJ, U.S.A.) or Shodex DEAE-825, 75 mm × 8 mm (Showa Denko, New York, NY, U.S.A.). In each case the separation gradient was from 0.1 *M* to 0.8 *M* aqueous ammonium formate with a flow-rate of 0.8 ml/min.

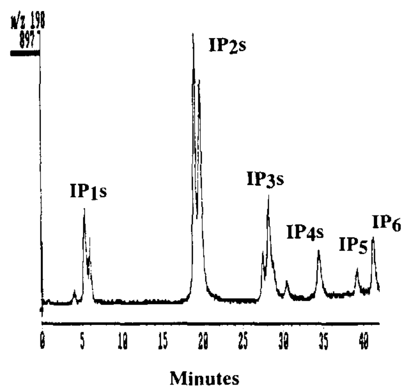

Fig. 2. Separation of inositol phosphates (IP_1s = *myo*-inositol monophosphates, IP_2s the bisphosphates, etc.) with a linear gradient of ammonium acetate from 0.1 *M* (at zero time) to 0.8 *M* (at 40 min) and thermospray LC–mass spectrometric detection. The separation was monitored by following *m/z* 198, *i.e.*, $[MNH_4]^+$ of unphosphorylated *myo*-inositol that is produced from each of the inositol phosphates during the thermospray process.

RESULTS

The separation of the inositol phosphate mixture on the mono-Q column is shown in Fig. 2. The counter-gradient in this case was provided by method 1. The thermospray process results in the hydrolysis of phosphomonoesters, thus the only positive ion of significant intensity seen in the thermospray mass spectrum of any of the IPs is *m/z* 198, *i.e.*, ammoniated inositol; $[MNH_4]^+$ (ref. 3). Since the rates of hydrolysis of the different species differ, the amount of inositol produced decreases with increasing degrees of phosphorylation and the ion intensity is thus not proportional to the inositol polyphosphate concentration. The several peaks in the elution regions noted in Fig. 2 (*e.g.*, IP_1s, IP_2s, etc.) are reproducible. The extent to which they are due to phosphate positional isomers of *myo*-inositol, or to inositol with different numbers of phosphate moieties, has not yet been determined. With the system used, the thermospray mass spectrometry for the entire process takes place in 0.4 *M* ammonium formate, a concentration that results in no vaporizer clogging.

The advantages of method 1 are flexibility: both linear and exponential gradients can be accommodated, only limited by the capabilities of the two programmers; the total volume of eluent is readily set by the pumping rate; the end point of a run remains 0.4 *M* without operator attention. Method 2, while inexpensive, constrains one to linear gradients (or to matching the shapes of exponential volume-dilution gradients) and the volume of eluent for a separation must be known in advance. In the latter case the operator must terminate the introduction of effluent to the instrument otherwise, at the point where the volume-dilution system empties its reservoirs, the solvent entering the mass spectrometer becomes the high-concentration eluent. An alternative not tested by us is to use two low-pressure gradient formers each supplying a single high-performance LC pump.

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