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Normal-phase high-performance liquid chromatography with relay gradient elution

I. Description of the method

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Abstract

The utility of normal-phase HPLC with relay gradient elution covering the entire practical range of polarity from hexane through water is demonstrated. The separations have been carried out on a LiChrospher 100 Diol column. Analytes without appreciable UV absorbance have been detected by means of an evaporative light-scattering detector. The method presented is suitable for the classification of sample components according to their polarity. The potential utility of the technique in programs such as profiling, classification and screening of biological materials, metabolic studies and preparative-scale isolation is discussed.

1. Introduction

Although reversed-phase methods account for the majority of HPLC applications [1], recent developments indicate increasing interest in normal-phase separations. Reversed-phase and normal-phase HPLC frequently find applications for compounds in the range of non-polar to medium polarity. More recently, encouraging results have been reported in the application of normal-phase HPLC for the separation of some very polar compounds. A number of common carbohydrates have been successfully resolved [2] on a LiChrospher Diol column with an acetonitrile–water gradient. The resolution of proteins, peptides, oligonucleotides, carbohydrates, phosphorylated amino acids and underivatized amino acids by means of “hydrophilic-interaction”

chromatography on a PolyHydroxyethyl A column [3] has been reported. Several amino acids have also been resolved by means of subcritical fluid chromatography on LiChrospher CN and LiChrospher Diol columns with CO₂ modified with methanol, water, triethylamine and pyridine as mobile phase [4]. An early report [5] presents the theoretical aspects of “incremental gradient elution” capable of resolving mixtures of analytes covering a wide range of polarity. The method has been experimentally demonstrated using a column packed with Bio-Sil A silica gel and a series of twelve solvents to cover the polarity range from *n*-heptane to water in a run stretching over 7 h.

The properties of the common polar bonded-phase columns [6] and highly purified porous silica microspheres [7] have been extensively

studied and reported. The consistent performance of polar bonded-phase columns well documented for specific separations of compounds throughout a wide polarity range prompted the investigation to determine the possibility of their use in programs such as profiling, classification and screening of biological materials, metabolic studies and preparative-scale isolation. The present study describes a dynamic concept of chromatographic isolation based on normal-phase HPLC method with relay gradient elution suitable for sample constituents covering the entire practical polarity range from extremely non-polar lipophilic compounds through inorganic cations.

2. Experimental

2.1. Instrumentation and methodology

Reversed-phase separation of choline and acetylcholine has been carried out on a Shandon Hypercarb S (150 × 4.6 mm) column (Shandon Scientific, Cheshire, UK). The adsorbent in this column is graphitised carbon. Rainin Microsorb MV C₁₈ column (3 μm, 50 × 4.6 mm; Rainin, Woburn, MA, USA) has been used as pre-column.

Normal-phase separations have been performed on an E. Merck LiChrospher 100 Diol column (5 μm, 250 × 4 mm; EM Separations, Gibbstown, NJ, USA) with a modular HPLC instrument consisting of an HP-1050 quaternary solvent-delivery system, an HP 3390A integrator (both from Hewlett-Packard, San Fernando, CA, USA) and a Sédex 55 evaporative light-scattering detection (ELSD) system (Sédéré, Alfortville, France). The resolution of choline, acetylcholine and some inorganic cations by means of normal-phase chromatography has been the specific application in the extremely polar region studied in detail. The results have been obtained by means of binary gradient. For general applications covering the widest polarity range, the mobile phase is designed as a series of consecutive gradients, or relay gradient. The simplest relay gradient is starting with a program

from hexane (A) to EtOAc (B) and continuing from EtOAc (B) to 0.1% HCOOH in acetonitrile (C) and finishing from 0.1% HCOOH in acetonitrile (C) to 0.1% HCOOH in water (D). All sequences are linear gradients starting with 100% of the less polar component and changing to 100% of the more polar component. Pre-conditioning of the column for the next run is carried out by sequential washing the column with the pure solvents in the reverse order going stepwise from the more polar to the less polar solvent. Typically, depending on the solvent, washing for 5–12 min is required. The preconditioning procedure necessary for the column between runs has been empirically determined and is, with additional details of the experimental conditions, provided together with the illustrations in the Results and discussion section below.

2.2. Solvents and chemicals

All chromatographic solvents have been purchased from EM Science (Gibbstown, NJ, USA) and are of OmniSolv quality. Inorganic salts have also been purchased from the same source. Other chemicals have been purchased from Aldrich (Milwaukee, WI, USA) and Sigma (St. Louis, MO, USA). The sample solutions used for reversed-phase chromatography have been dissolved in water. Samples for normal-phase chromatography have been dissolved in *n*-propanol or dimethylformamide both containing 20% of water. The amounts injected have been in the range of 1.5–6 μg per component.

3. Results and discussion

There is ample selection of reversed-phase and normal-phase HPLC methods published in the literature for the separation of compounds of low to medium polarity. Finding generally applicable separation methods for compounds in the polar to extremely polar range has historically been a more difficult problem. It is fair to assume that once a satisfactory general procedure for separating polar classes of compounds had been found, the extension of the method to the

medium to non-polar range would be relatively simple. Ion-exchange chromatography can be ruled out as a general method due to the inherent specificity of resins. The performance of reversed-phase media have limitations particularly in the extremely polar region. The limitations are not only resolution-related. While choline and acetylcholine are completely resolved on a Shandon Hypercarb S column (Fig. 1), the method cannot be used routinely for samples also containing much less polar compounds due to demanding column clean-up procedures specified by Shandon Scientific. The retention of non-polar organic molecules on activated carbon is so strong that column performance can be lost after only a few injections of real (e.g. biological) samples.

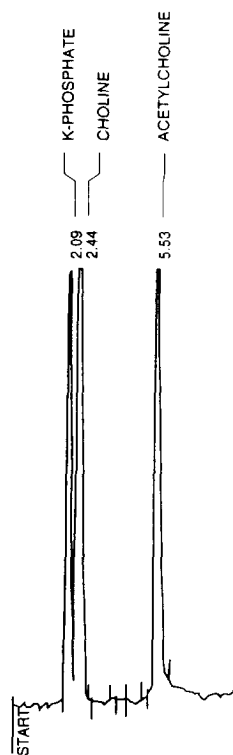


Fig. 1. Separation on a Shandon Hypercarb S column (150×4.6 mm) with Rainin Microsorb MV C_{18} column ($3 \mu\text{m}$, 50×4.6 mm) as precolumn. Mobile phase: 0.2% TFA in water. Flow-rate: 0.9 ml/min. In all figures, the numbers at the peaks indicate retention times in min.

On the LiChrospher 100 Diol column, not only is the resolution of acetylcholine and choline complete, but some common inorganic cations are also fully resolved (Fig. 2). This quite remarkable normal-phase column performance in the extremely polar region has never been demonstrated with any reversed-phase system. As a matter of fact, the dynamic range presented here cannot be matched by all known reversed-phase systems combined. It is not surprising at all that the elution profile of the same cations is drastically changing when trifluoroacetic acid (TFA) is substituted with HCOOH (Fig. 3). The characteristics of the counter ion greatly influence the polarity of the ion pair. The non-polar TFA facilitates the elution of cations at much lower water content of the mobile phase and with better resolution than the highly polar

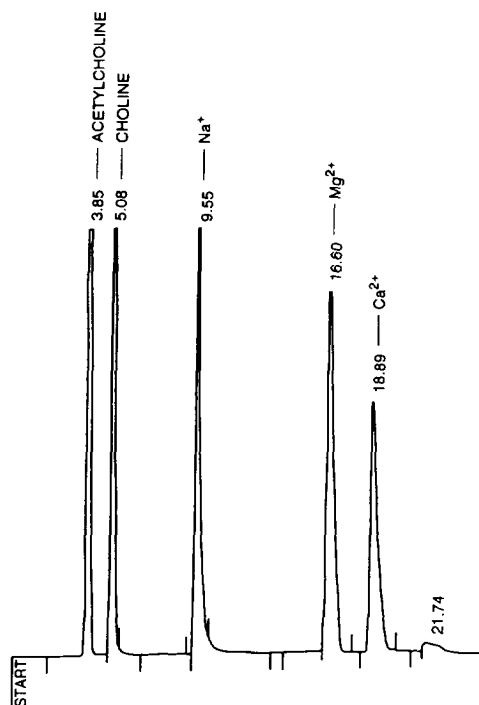


Fig. 2. Separation on an E. Merck LiChrospher 100 Diol column ($5 \mu\text{m}$, 250×4 mm). Gradient elution with solvents 0.1% TFA in MeCN (A) and 0.1% TFA in water (B). The gradient program is as follows: 0–1 min: 10% B; 1–18 min: 10→30% B; 18–20 min: 30→100% B; 20–24.5 min: 100% B. Flow-rate: 0.9 ml/min.

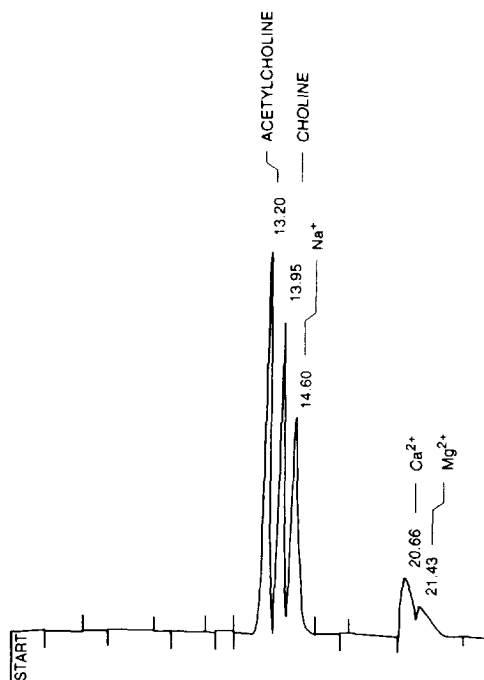


Fig. 3. Separation on the same column as in Fig. 2. Gradient elution with solvents 0.1% HCOOH in MeCN (A) and 0.1% HCOOH in water (B). The gradient program is as follows: 0–1 min: 20% B; 1–15 min: 20→100% B; 15–24.5 min: 100% B. Flow-rate: 0.9 ml/min.

HCOOH does. However, for a general method designed to classify compounds according to their polarity, HCOOH-containing mobile phases are found more suitable than their TFA-containing counterparts. Although the resolution for cationic species is sacrificed to some extent, the relative polarities of the sample components are more realistically represented when the inherent polarity of cations is not suppressed by using a non-polar counter ion. A classification procedure designed with a broad scope in mind necessitates such a sacrifice. This allows the cations to be pushed beyond other polar classes of organic compounds such as carbohydrates and amino acids. Even the most polar quaternary ammonium compounds are still being eluted before the inorganic cations. All cations discussed here were used in the chloride form. During the chromatographic run, Cl^- is dis-

placed by the HCOO^- anion. Cl^- and many other common organic and inorganic anions form volatile acids when protonated and are not detected by ELSD under the conditions used here. Therefore, the separation of anions will be discussed in a separate report.

A mixture of arbitrarily selected solutes used as polarity markers allows the classification of the components of actual samples according to their polarity. A chromatogram of a test mixture obtained by means of a simple relay gradient elution illustrates the wide dynamic range of the method (Fig. 4). The origin of some minor peaks (e.g. at 10.66, 40.44 and 49.65 min) is currently unknown. It may be the result of incomplete equilibria between the stationary and the mobile phases, limited solubility in the mobile phase, etc. Minor peaks are partially eliminated by overlapping the EtOAc to acetonitrile and acetonitrile to aqueous cycles (Fig. 5). This modi-

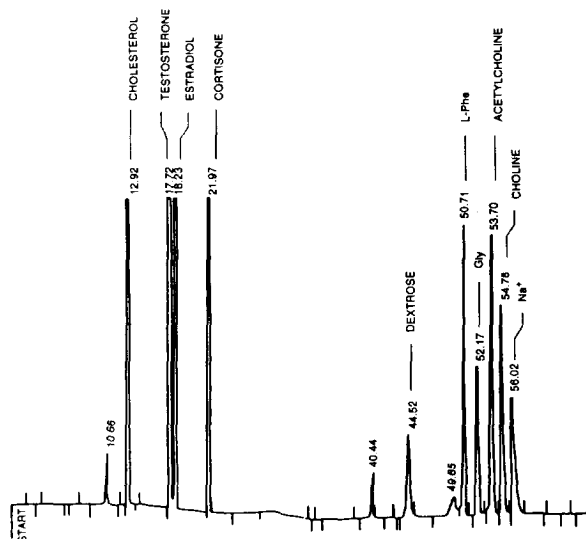


Fig. 4. Separation on the same column as Fig. 2. Relay gradient elution with solvents hexane (A), EtOAc (B), 0.1% HCOOH in MeCN (C) and 0.1% HCOOH in water (D). The gradient program is as follows: 0–5 min: 100% A; 5–20 min: 100→0% A and 0→100% B, 0% C and D; 20–25 min: 100% B; 25–30 min: 100→0% B and 0→100% C, 0% A and D; 30–35 min: 100% C; 35–60 min: 100→0% C and 0→100% D, 0% A and B; 60–65 min: 100% D.

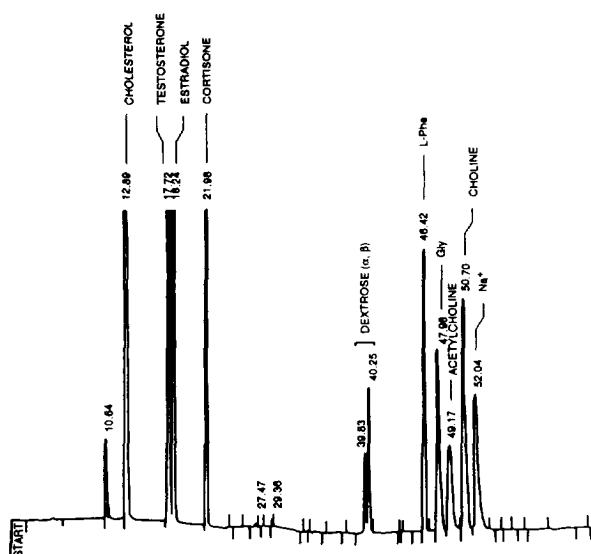


Fig. 5. Separation on the same column as Fig. 2. Relay gradient elution with solvents hexane (A), EtOAc (B), 0.1% HCOOH in MeCN (C) and 0.1% HCOOH in water (D). The gradient program is as follows: 0–5 min: 100% A; 5–20 min: 100 → 0% A and 0 → 100% B, 0% C and D; 20–25 min: 100% B; 25–30 min: 100 → 10% B, 0 → 80% C, 0 → 10% D, 0% A; 30–35 min: 10 → 0% B, 80% C, 10 → 20% D, 0% A; 35–60 min: 80 → 0% C, 20 → 100% D, 0% A and B; 60–65 min: 100% D.

fication also resulted in the resolution of dextrose to its α and β anomers previously accomplished on a Nucleosil OH column (Macherey-Nagel) [2].

The reliability of the method is understandably an important concern. The most relevant measure of reliability in this case being a separation method is the reproducibility of retention times. The prerequisite to acceptable reproducibility of retention times is obviously strict control over the key parameters such as the condition of the column, the quality of the solvents and the accuracy of the gradient program. While some parameters are mostly dependent on the instrumentation chosen, the development of a suitable gradient program and the restoration of the column to a standardized starting condition are completely the operator's responsibility. Assuming that the stationary phase is sufficiently stable, its initial condition at the beginning of a run

depends on the pre-equilibration of the column. This is particularly true when following 0.1% aqueous HCOOH as mobile phase, the column has to be prepared for hexane as the initial eluent. For obtaining reproducible retention times, the pre-equilibration consists of washing with MeCN, EtOAc and hexane, in that order, for 10, 5 and 12 min, respectively. Changing the duration of the wash cycles results in predictable changes of the retention times. Shorter wash cycles may cause insufficient displacement of residues of polar solvents from the column leading to significantly shorter retention times and poor resolution. Extending the wash cycles may cause much smaller (less than 1.0%) increase of the retention times. The results of the latter case are still useful for the classification of the fractions according to their polarity, but constitute poor utilization of instrument time. Also predictable is the effect of any polar solvent used for dissolving the samples for injection, particularly in the non-polar portion of the relay gradient. Adherence to standard HPLC practices and the time cycles specified assure reproducibility of retention times comparable to that of any HPLC method as shown by the data summarized in Table 1. The slight discrepancies between long term and short term reproducibilities of the retention times are a good indication of the stability and reliability of both the system and the method. It should be noted, however, that the instrument including the column has been regularly used during the four months for numerous separations, some of them unrelated to this work. In any long-term study, factors such as gradual deterioration of the column packing, contaminants retained, changes in the mechanical properties of the resin, dead space in the column, etc. would readily explain inferior reproducibility compared to the same in short term studies. The results summarized in Table 1 show no evidence of any of these factors.

It has been the experience of this laboratory that after using the column for other methods, a complete cycle of the relay gradient also including the standard pretreatment adequately restores the system to its reliable operating condition. For practical reasons and for reasons of

Table 1
Retention times of the test compounds under the conditions given in Fig. 4

Fraction	t_R (min)		
	Initial values	Five consecutive runs after four months	
		Average	Range
Cholesterol	12.92	13.23	13.22–13.25
Testosterone	17.72	17.71	17.70–17.74
Estradiol	18.23	18.22	18.21–18.26
Cortisone	21.97	21.90	21.88–21.93
Dextrose	44.52	44.46	44.41–44.55
L-Phe	50.71	50.87	50.84–50.92
Gly	52.17	52.37	52.34–52.44
Acetylcholine	53.70	53.94	53.87–53.99
Choline	54.78	55.04	54.99–55.11
Na ⁺	56.02	56.29	56.23–56.37

assuring standardization of the conditions to the fullest extent possible, the pre-equilibration cycle for every run is included in the gradient program of the previous run. The results of long-term as well as short-term reproducibility summarized in the table fully satisfy if not exceed the criteria of reliability required within the scope of the intended use of the method presented.

The primary goal of this report is to describe the methodology suitable for the initial and general classification of complex mixtures according to the polarity of their components. A detailed analysis of the theoretical aspects and the understanding of the phenomena underlying the method of separation according to polarity require additional studies. However, it appears reasonable to point out similarities and differences between previous studies and the present work. Firstly, one run in the current method is completed in 65 min as opposed to 7 h for the method based on incremental gradient elution [5]. With the re-equilibration time of the column of ca. 30 min in the current method, the total turn-around time is approximately 1.5 h. Furthermore, the polarity range covered appears considerably wider in the present method with only four solvents used as opposed to twelve solvents needed for the incremental gradient elution method. As a matter of fact, Scott and

Kucera [5] suggested, that incremental gradient elution would require even more than twelve solvents for additional improvement of resolution. Their projection was based on theoretical considerations using GC with temperature programming as model. The validity of the same model is obviously limited when applied to the interpretation of the results presented here. Considering the stationary and mobile phases used in the present study, the key phenomena responsible for the resolution are more likely to be related to those reported in connection with the concept of hydrophilic-interaction chromatography [3] and with polar bonded phases [6].

ELSD has been found very useful as a general mass detection method [2,4,8–10] particularly with analytes not detectable with spectrophotometric detectors. It also allows the freedom of solvent selection not possible with UV detectors and gradient programs not compatible with refractive index detectors. ELSD certainly has its scope and limitations when used in analytical work, as do all other detection methods. The emphasis in this report is placed on chromatographic performance rather than on examining various aspects of quantitation by means of ELSD.

The technique described here is envisioned to be used primarily as the first step in a series of

classification and preparative isolation methods. The general procedure using the relay gradient elution method covering the entire practical range of polarity facilitates the separation of mixtures into classes defined by the polarity markers. The second steps may then be designed for specific separations within the narrow range of polarity such as non-polar lipids, organic compounds with low to medium polarity, polar organic compounds such as carbohydrates, amino acids, quaternary ammonium ions, etc. One example of a specific separation is shown in Fig. 1 and Fig. 2 involving extremely polar organic and inorganic cations. In this scenario, ELSD fulfils the expectations of the chromatographer by locating the components of the mixtures to be separated. Calibration curves can be established [2] if quantitation is the objective of the work. Reports on applications in specific areas will be published in subsequent papers.

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References

- [1] R.E. Majors, *LC·GC*, 9 (1991) 686.
- [2] M. Lafosse, B. Herbreteau, M. Dreux and L. Morin-Allory, *J. Chromatogr.*, 472 (1989) 209.
- [3] A.J. Alpert, *J. Chromatogr.*, 499 (1990) 177.
- [4] V. Camel, D. Thiébaud and M. Caude, *J. Chromatogr.*, 605 (1992) 95.
- [5] R.P.W. Scott and P. Kucera, *Anal. Chem.*, 45 (1973) 749.
- [6] A.W. Salotto, E.L. Weiser, K.P. Caffey, R.L. Carty, S.C. Racine and L.R. Snyder, *J. Chromatogr.*, 498 (1990) 55.
- [7] J.J. Kirkland, C.H. Dilks, Jr. and J.J. DeStefano, *J. Chromatogr.*, 635 (1993) 19.
- [8] P. Carraud, D. Thiebaut, M. Caude, R. Rosset, M. Lafosse and M. Dreux, *J. Chromatogr. Sci.*, 25 (1987) 395.
- [9] D. Nizery, D. Thiébaud, M. Caude, R. Rosset, M. Lafosse and M. Dreux, *J. Chromatogr.*, 467 (1989) 49.
- [10] M. Dreux and M. Lafosse, *SPECTRA 2000*, 153 (1990) 25.