Reverse-Phase Chromatography of Polar Biological Substances: Separation of Catechol Compounds by High-Performance Liquid Chromatography

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Catecholamines and their metabolites have been separated isocratically by reverse-phase chromatography with aqueous (no organic solvent admixed) eluents. Unlike ion-exchange or ion-pair chromatography, mixtures of both acidic and basic substances can be separated in a single chromatographic run, because the retention is governed by hydrophobic interactions between the nonpolar moiety of the solute molecules and the octadecyl-silica stationary phase. The relative retention values strongly depend on the pH of the eluent, which governs the degree of dissociation of ionogenic solutes. The reproducibility of the results and the stability and efficiency of the chromatographic systems make this approach particularly attractive for use in clinical analysis.

Additional Keyphrases: separation of acids and bases in a single run • catecholamines and metabolically related compounds • reverse-phase chromatography with aqueous eluents

Nonpolar bonded phases with hydro-organic eluents have been increasingly used in high-performance liquid chromatography for the separation of substances that are sparingly soluble or insoluble in water. Because the eluent is more polar than the stationary phase, the term “reverse-phase chromatography,” originally suggested by Howard and Martin (1), is used to describe this technique. In HPLC the stationary phase is most commonly a finely divided organo-silica with octadecyl moieties covalently bound to the solid surface, and aqueous methanol or acetonitrile are used as the mobile phase. The popularity of the method rests with its relative simplicity and reproducibility (2), which are also responsible for the recent application of reverse-phase chromatography in the analysis of mixtures of various biological substances.

In this study we demonstrate by the results obtained with the separation of catecholamines and their metabolites that octadecyl-silica columns can also be used successfully with neat (i.e., not admixed with organic solvent) aqueous eluents in HPLC and that reverse-phase chromatography is suitable for the separation and analysis of polar substances as well. This approach represents an alternative not only to the usual chromatographic techniques, in which a polar sorbent and a less polar eluent are used, but also to ion-exchange chromatography, which is most widely used to separate polar biological substances.

The need for rapid analysis of catecholamines and their derivatives arises from their important biological role (3, 4). Moreover, the clinical significance of these substances is increasingly recognized and a suitable chromatographic technique could serve as a powerful tool for clinical analysis to provide valuable diagnostic information. At present, fluorescent analysis (5) is used most commonly to estimate certain urinary catecholamines, and paper chromatography is used to determine HVA and VMA (6). Ion-exchange chromatography has been frequently used for such separations (7–9), but this technique and other column-chromatographic methods have not been used routinely in clinical laboratories. The main impediment in using column chromatography for this is the very low physiological concentration of these substances, which precludes their analysis with the conventional ultraviolet or fluorometric detectors. However, the electrochemical detector recently introduced by Kissinger et al. (10, 11) offers a highly sensitive way to detect catechol compounds and appears to be eminently suitable for their analysis. Thus, further progress requires the development of fast, reliable, and simple separation methods.

Potentially, HPLC offers the necessary speed and efficiency. It does not involve the formation of volatile derivatives from polar substances such as the catecholamines and their metabolites, as is required in gas chromatography (12, 13). Nevertheless, the chromatographic systems used so far have not been fully sat-
isfactory. Cation- or anion-exchange chromatography is applicable only to the separation of basic or acidic sample components, respectively. The recently introduced ion-pair chromatography (14) shows the same discriminatory effect, because both techniques strongly involve coulombic interactions in the chromatographic process. Whereas the speed and efficiency of the latter technique is superior as compared with conventional ion-exchange columns, the inherent instability of liquid–liquid chromatographic systems makes this approach less desirable in routine analytical work.

Here, we demonstrate that reverse-phase chromatography, which involves hydrophobic rather than coulombic or hydrogen bonding interactions between the solutes and the stationary phase, allows one rapidly to separate mixtures of biogenic amines and their acidic metabolites in a single chromatographic run. In view of the previously mentioned advantages of this technique our approach may be useful for routine analysis of other low-molecular-weight biological substances.

**Materials and Methods**

**Chromatograph**

We used a Model 601 liquid chromatograph with a Model LC 55 detector (Perkin-Elmer Corp., Norwalk, Conn. 06856) and a Rheodyne Model 7105 sampling valve (Rheodyne, Berkeley, Calif. 94710). The instrument was operated in the isocratic mode and the adsorbance of the eluent was monitored at 254 nm, with use of a Perkin-Elmer Model 56 strip-chart recorder. Chromatographic conditions are figure in the legends.

**Columns**

Most experiments were done with Partisil-1025 ODS columns (Whatman, Clifton, N. J. 07014), 0.46 cm (i.d.) X 25 cm, packed with octadecyl-silica particles of 10-μm av diameter. We observed slight variations in the efficiency and retention properties of columns from different lots. Octadecyl-silica stationary phases have been prepared (15) from 10- and 5-μm Partisil (Whatman) by reacting the silica gel with trichloro-octadecyl silane (Aldrich Chemical Co., Milwaukee, Wis. 53233) without subsequent treatment with trimethyl chlorosilane. Columns of the above dimensions have been packed by the isopyncnic slurry technique at 52 MN/m² (1500 atm). The chromatographic properties of the columns with the 10-μm particles were similar to those of the commercial columns, whereas retention values with the 5-μm octadecyl silica were greater under the same elution conditions.

**Eluents**

We used phosphate solutions in all experiments. Reagent-grade phosphoric acid, KH₂PO₄, and Na₂HPO₄ were purchased from Fisher Scientific Co., Pittsburgh, Pa. 15219. In certain experiments the ionic strength of the eluent was adjusted with KCl (Fisher, reagent grade). In studies where the pH of the eluent was varied, phosphate concentration was maintained at 0.1 mol/liter over the pH range investigated.

Type 316 stainless steel used in the instruments and for the column is not sufficiently resistant to halides. Therefore the instrument was exposed to KCl solutions only for the duration of the experiments and the system was purged with distilled water thereafter. The siliceous column material cannot withstand eluents that have a pH exceeding 8 without degradation. It appears that a high concentration of halides in the eluent also shortens the useful life of the column material if it is long exposed to such concentrations. On the other hand, both the stainless steel and the column material are remarkably resistant to phosphate solutions in the pH range 1.9 to 7.0.

**Samples**

All catecholamines and their derivatives were purchased from Aldrich Chemical Co. and the samples were prepared by mixing stock solutions of the individual components in distilled water. Urine samples were obtained from the clinical laboratory of Yale–New Haven Hospital.

In most chromatographic experiments, 20 μl of sample solution, containing 2 to 10 μg of the individual components, was injected.

**Evaluation of the Chromatograms**

The retention of the individual peaks is expressed by the retardation factor, k', which was evaluated from the chromatogram by using the following relationship: k' = (tR - t₀)/t₀, where t₀ and tR are the retention time for an unretarded solute and the solute in question, respectively (16).

**Results**

The chromatograms depicted in Figures 1 to 4 illustrate the separation of substances that are involved in certain metabolic pathways of neurotransmitters (4) and consequently include both acidic and basic compounds. Figure 1 shows the species that occur in the degradation of phenylalanine to norepinephrine. Because catechol-O-methyltransferase (EC 2.1.1.6) does not act in this pathway, the phenolic hydroxyls of the intermediates are not methylated. Thus, the order of elution reflects the increasing polarity of the substances produced successively in the metabolic process. Figure 2 shows the intermediates involved in the metabolic degradation of dopamine to HVA and homovanillic alcohol. In this case the mixture is composed of both catechol and 3-O-methyl-catechol compounds that have either acidic or basic functions, or both. The chromatogram in Figure 3 shows a similar picture for the metabolic pathway from dopamine to VMA.

Because catechols can readily be removed from their 3-O-methyl or 4-O-methyl derivatives by adsorption on alumina (17), there is a considerable interest in the separation of the acidic and basic methylated substances. Figure 4 illustrates that reverse-phase chromatography is eminently suitable for such a separation, which cannot be easily effected by ion-exchange chromatography. Figure 5 demonstrates that the tradi-
tionally difficult separation of the physiologically most important catecholamines also is readily done by reverse-phase chromatography, and that this technique offers an efficient alternative to cation-exchange chromatography. This chromatogram was obtained with use of the 5-μm octadecyl-silica column, which relatively strongly retarded the sample components even at pH 4.4, where they are dissociated. Figures 6 and 7 illustrate the effect of pH on the chromatographic retention of the substances of interest. The pH of the eluent was varied, but the ionic strength did not change significantly. Retention of the acidic compounds decreases and that of the basic substances increases with increasing pH of the eluent. Obviously the retention decreases with increasing dissociation of the ionogenic functions of the molecules. It is seen that the O-methylated derivatives are always more strongly retarded than are the corre...

Fig. 1. Chromatogram of intermediates of L-phenylalanine metabolism to norepinephrine
Column, Partisil 1025 ODS; eluent, 0.2 mol/liter phosphoric acid-potassium phosphate; pH, 1.9; flow rate, 0.5 ml/min; inlet pressure, 5.05 MN/m² (50 atm); temperature, 25 °C

Fig. 3. Chromatogram of the intermediates of dopamine metabolism to vanilmandelic acid
Column, Partisil 1025 ODS; eluent, 50 mmol/liter phosphoric acid-potassium phosphate; pH, 2.0; flow rate, 0.5 ml/min; inlet pressure, 5.05 MN/m² (50 atm); temperature, 23 °C

Fig. 2. Chromatogram of the intermediates of dopamine metabolism to homovanillic acid
Column, Partisil 1025 ODS; eluent, 50 mmol/liter potassium phosphate; pH, 4.6; flow rate, 2.0 ml/min; inlet pressure 20.2 MN/m² (200 atm); temperature, 22 °C

Fig. 4. Chromatogram of 3-O-methyl metabolites of dopamine
Column, Partisil 1025 ODS; eluent, 50 mmol/liter potassium phosphate; pH, 4.6; flow rate, 0.66 ml/min; inlet pressure 3.54 MN/m² (35 atm); temperature, 25 °C
The effect of ionic strength on retention also has been investigated. It is seen in Figure 8 that retardation increases with increasing salt concentration, in contrast to the effect of salt concentration in ion-exchange chromatography; this proves that ionic interactions between the solutes and stationary phase are negligible. On the other hand, the increase in values for the retardation factors with increasing salt concentration—an increase of about the same magnitude for all solutes investigated—implies that the retention is due to hydrophobic interaction.

Van't Hoff plots for different solutes are depicted in Figure 9. The slopes of the straight lines are very similar and it can be seen that the $k'$ values are approximately halved when the temperature is increased by 30 to 40 °C.
To explore the potential of this method for urine analysis, we chromatographed several acidified urine samples. As expected, the chromatograms obtained with either the native urine specimen or an ethyl acetate extract of it show that many components that absorb light at 254 nm are present. One such chromatogram is shown in Figure 10. The results indicate that reverse-phase chromatography is of potential use for rapid separation of the low-molecular-weight organic molecules in urine, but further refinement of the technique is necessary. Our interest is focused on the analysis of urinary VMA and HVA because preliminary studies indicate that reverse-phase chromatography could represent an appropriate alternative to the presently used paper chromatography.

Discussion

Relatively polar ionogenic substances can be separated by reverse-phase chromatography with neat aqueous eluents, and the peaks do not show excessive tailing even when the solutes are partially dissociated in the mobile phase. Because the separation is the result of hydrophobic interactions between the hydrocarbonaceous stationary phase and the hydrophobic moiety of the solute molecules, mixtures of acidic and basic compounds can be easily separated in a single chromatographic run when the pH of the eluent is appropriate.

The pH dependence of the retention values shown in Figures 6 and 7 can serve as a guide to select the optimum eluant pH for the isocratic separation of a particular mixture. As expected, retention values decrease with increasing dissociation of the solutes, which attenuates the magnitude of their hydrophobic interactions with the stationary phase. If the sample consists only of acidic components the eluant pH should be low, to suppress dissociation and obtain stronger retardation and better separation. The opposite is true for the separation of amino compounds. Retention also can be prolonged by increasing the ionic strength of the eluent, as shown in Figure 8, but this effect is not nearly as dramatic as that of pH change and its magnitude is similar for such closely related substances, because the slopes of the curves almost parallel one another. Therefore, the values for relative retention do not change significantly with ionic strength. The effect of temperature is such that the retention values rapidly decrease with increasing temperature, and again a comparison of the slopes obtained for the various substances indicates that relative retention values are not affected significantly by temperature.

Most results presented here have been obtained with commercially available reverse-phase columns. It is known, however, that the chromatographic behavior of such columns depends on the structure and particle size of the silica gel and the amount of covalently bound octadecyl moiety as well as on the method of its attachment. Consequently, different results can be expected when other column materials are used. Nevertheless, columns packed with octadecyl-silicas prepared in our laboratory showed retention properties not much different from those of the commercial columns.

We expected that the efficiency of the column would increase when the particle size of the stationary phase was reduced to 5 μm. As shown in Figure 5, however, columns containing such a material are not significantly more efficient; this result is attributed to the inadequate packing structure. Further work is required, therefore, to obtain a two- to threefold increase in column efficiency by using 5-μm instead of 10-μm particles and improved column-packing technology, which is currently under study in our laboratory. The retardation factors we obtained with our 5-μm particles were significantly higher than those attained with the 10-μm particles under identical conditions. Consequently, the early peaks were better separated.

It has been shown recently (18) that reverse-phase chromatography can also be used as "reverse ion-exchange chromatography," and this technique is termed

**Fig. 9. Effect of temperature on the retardation factor**
Column, Partisil 1025 ODS; eluent, 50 mmol/liter KH2PO4; flow rate, 1 ml/min. DA, dopamine; MET, metanephrine; MOPET, homovanillic alcohol; for other symbols see preceding Figures

**Fig. 10. Chromatogram of an acidified urine sample**
Column, Partisil 1025 ODS; eluent, 0.1 mol/liter phosphate buffer, pH 2.05, flow rate, 1 ml/min; inlet pressure, 10.1 MN/m² (100 atm); temperature, 25 °C; sample size, 10 μl, detector sensitivity, 0.1 AUFS
"paired-ion chromatography." In this method methanol/water mixtures containing strong long-chain aliphatic acids or bases are used as the eluent. This technique also represents a significant broadening of the scope of reverse-phase chromatography, but like ion-exchange chromatography it still can only be used efficiently to separate acidic or basic components in a single chromatographic run. In addition, the species that interact with the stationary phase are ion pairs formed from the sample components and the acid or base in the mobile phase. Because the heptane sulfonate or tributylammonium ions, which are frequently used in this method, are of relatively high molecular weight, the selectivity of the system for the separation of ion pairs with small molecules is expected to be inferior to the direct method presented here, even when only acidic or basic sample components are analyzed.

In the conventional mode, reverse-phase chromatography with aqueous methanol or acetonitrile as eluent has been extensively used to separate nonpolar biological substances. Our results show that chromatographic systems that involve a hydrophobic stationary phase have a much greater versatility than envisioned previously. In addition to the conventional technique and the recently introduced "reverse ion-exchange" chromatography, the present method can be used to separate biological substances directly with neat aqueous eluents. There are great practical advantages to using a nonswelling stationary phase, which yields stable columns, and aqueous eluents, which are readily available with high purity and are not poisonous or flammable. A major drawback of the siliceous column materials now in use is that they are not resistant to eluents having pH values higher than 7. Therefore development of hydrophobic phases on a support such as zirconia, which resists higher pH, is required to extend the operating range of the chromatographic system.

We think that with improved column technology the efficiency can be significantly increased when column materials having a particle size range of 4 to 5 μm can be used. Then short columns could yield rapid separations, and the sensitivity of analysis could be enhanced to meet the demanding requirements encountered in clinical analysis.

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References