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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CATECHOLAMINES AND THEIR CONGENERS WITH SIMPLE ACIDS AS ION-PAIRING REAGENTS

PAUL A. ASMUS and CURT R. FREED*

Division of Clinical Pharmacology, C237, University of Colorado Medical Center, 4200 East Ninth Avenue, Denver, Colo. 80262 (U.S.A.)

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SUMMARY

We have investigated the value of various common acids as ion-pairing reagents for high-efficiency separations of catecholamines and their metabolites in reversed-phase high-performance liquid chromatography. The retention of norepinephrine, α-methylnorepinephrine, dopamine, α-methyldopamine, L-dopa, α-methyldopa, dihydroxybenzylamine, epinephrine, carbidopa and DOPAC was measured in mobile phases composed of nitric, sulfuric, acetic and trichloroacetic acids at pH 2–5 and anion concentrations ranging from 5–500 m*M*. The solute capacity ratios were dependent on the hydrophobicity and concentration of the ion-pairing reagent and the pH of the mobile phase. Good retention, peak symmetry and high efficiency (3000 theoretical plates for 300 mm) was found for mobile phases composed of the strong inorganic acids and trichloroacetic acid. Chromatography was compared to that seen using the detergent sodium octylsulfate. Trichloroacetic acid gave retention and efficiency similar to sodium octylsulfate. These experiments show that simple acids can replace alkylsulfates as ion-pairing reagents for the separation of the catecholamines and their metabolites.

INTRODUCTION

The ionic nature of catecholamines and their metabolites make them suitable candidates for analysis by ion-exchange high-performance liquid chromatography (HPLC)[1–4]. While these methods provide adequate resolution for some applications, we felt that better separation efficiency would greatly enlarge the scope of HPLC for catecholamine analysis.

The introduction of reversed-phase ion-pairing chromatography (soap chromatography) by Knox and co-workers[5–8] and Wittmer *et al.*[9] has demonstrated the versatility of octadecyl columns for the chromatography of polar ionizable compounds. Knox and co-workers[6,8] found that a mobile phase composed of methanol–

* To whom correspondence should be addressed.

water containing a small concentration of sodium dodecyl sulphonate (SDS) gave good retention, efficiency and peak symmetry for the catecholamines and their metabolites. The packing material used in their study was an octadecylsilane-treated silica which had been further treated with trimethylsilane to react with any residual silanol groups on the surface. When they used simple acidic eluents in place of SDS, the catecholamines were not well retained and the peaks were broad.

However, a recent report by Molnár and Horváth[10] showed that a phosphate buffer mobile phase could be used to chromatograph the catecholamines on a commercially available non-polar stationary phase with good efficiency. Buffers composed of other acids were not tested, and it was of interest to us to determine the retention of catecholamines and their metabolites in acid buffer systems other than phosphoric acid. It was our hope to gain an empiric understanding of the retention process such that adequate retention and resolution could be maintained as the column capacity decreased without the addition of expensive *n*-alkyl sulfates to the mobile phase.

In this communication, we describe the chromatographic properties of the catechols on octadecylsilica columns with mobile phases composed of nitric, sulfuric, acetic and trichloroacetic acid. We have found two reversed-phase chromatographic systems for catecholamine separation which prolong the useful life of expensive octadecylsilica columns, and permit a difficult separation to be achieved for an extended period with minimum analysis time. The first system, using nitric acid, behaves with characteristics similar to hydrophobic chromatography, while the second, using trichloroacetic acid, appears to fit best an ion-pairing model. Our method bridges the gap between hydrophobic chromatography used by Molnár and Horváth[10] and the soap chromatography of Knox and co-workers[5–8], and introduces the use of cheap, readily available acids as ion-pairing reagents.

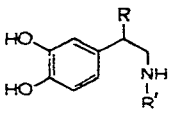
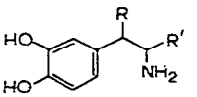
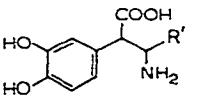
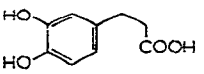
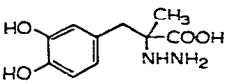
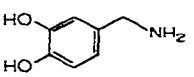
EXPERIMENTAL

All experiments were performed using a Model 6000 solvent delivery system (Waters Assoc., Milford, Mass., U.S.A.) with a Model U6K sample injector (Waters Assoc.) and a thin-layer electrochemical detector (Model LC-10, Bioanalytical Systems, West Lafayette, Ind., U.S.A.). The detector potential was set at +0.5 V *vs.* a Ag–AgCl reference electrode. The column used was a μBondapak C_{18} reversed-phase column (300 × 3.9 mm, Waters Assoc.) operated at a flow-rate of 2 ml/min.

The mobile phases were prepared from reagent-grade sulfuric acid, nitric acid, glacial acetic acid or trichloroacetic acid and were adjusted to the appropriate pH with saturated sodium hydroxide. The pH of the mobile phase was the same before and after passage through the column. Chromatographic conditions are given in the figure legends. The authentic catecholamine standards are listed in Table I, along with their structures. Norepinephrine (NE) was obtained from Sigma (St. Louis, Mo., U.S.A.). Epinephrine (EPI), L-3,4-dihydroxyphenylalanine (L-dopa), 3,4-dihydroxyphenylacetic acid (DOPAC), and dihydroxybenzylamine (DHBA) were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). Dopamine–HCl (DA) was from Calbiochem (Los Angeles, Calif., U.S.A.) and carbidopa (CD), α-methyldopa (α-MD) and α-methylnorepinephrine (α-MNE) were generously supplied by Merck Sharp & Dohme (Rahway, N.J., U.S.A.). α-Methyldopamine–HCl (α-MDA) was synthesized in the laboratory of Dr. Neal Castagnoli at the University of California at San Fran-

TABLE I

COMPOUNDS STUDIED

Compound	*Abbreviation*	*Formula*	*R*	*R'*
Catecholamines				
Norepinephrine	NE		OH	H
Dopamine	DA		H	H
Epinephrine	EPI		OH	CH_3
α-Methyl derivatives				
α-Methylnorepinephrine	α-MNE		OH	CH_3
α-Methyldopamine	α-MDA		H	H_3
Catechol amino acids				
L-Dopa	LD			H
α-Methyldopa	α-MD			CH_3
Others				
Dihydroxyphenylacetic acid	DOPAC			
Carbidopa	CD			
Dihydroxybenzylamine	DHBA			

cisco. All solutions were prepared from triple distilled water, with the final distillation over alkaline permanganate.

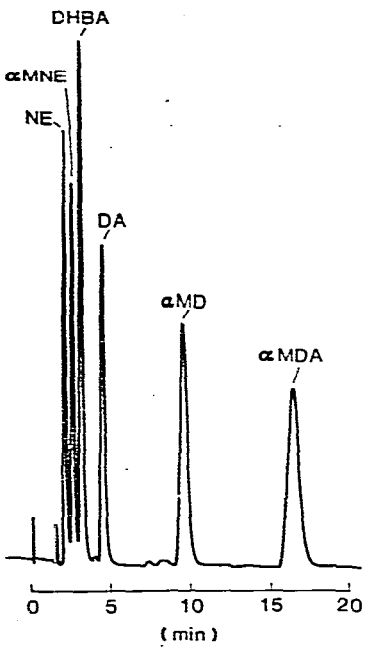
The retention of the chromatographic peaks was expressed as the capacity ratio, k, which was calculated by the following formula: $k = (t_R - t_0)/t_0$, where t_0 and t_R are the retention of an unretained solute and the solute in question, respectively. Retention times were measured with a Hewlett-Packard Model 9815A electronic integrator. The column's dead time, t_0, was measured by noting the first baseline disturbance after 50 μl of 0.1 M nitric acid was injected.

RESULTS AND DISCUSSION

Inorganic acid mobile phases

A mobile phase composed of 0.1 M nitric acid titrated to pH 3.0 was used for the separation of selected catechol compounds shown in Fig. 1. Good separation, peak symmetry and retention of these very polar compounds were obtained.

The relatively high efficiency (2100 theoretical plates) and good peak symmetry ($A_s = 1.1$) may be due to good surface coverage by octadecylsilane which minimizes



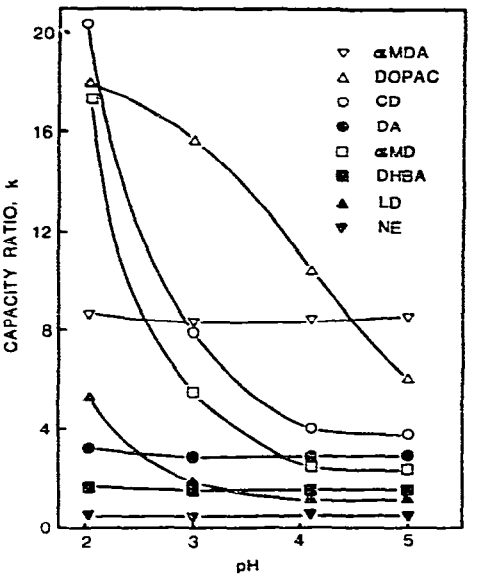


Fig. 1. Chromatogram of standard catechols with a nitric acid mobile phase. Mobile phase: 0.1 *M* HNO_3–$NaNO_3$, pH 3.00. Catechol abbreviations: NE = norepinephrine; α-MNE = α-methylnorepinephrine; DHBA = dihydroxybenzylamine; DA = dopamine; α-MD = α-methyldopa; α-MDA = α-methyldopamine.

Fig. 2. Effect of the pH of nitric acid mobile phase on the capacity ratio, *k*, of standard catechols. Mobile phase: 0.1 *M* HNO_3–$NaNO_3$. Catechol abbreviations as in Fig. 1, with the addition of DOPAC = dihydroxyphenylacetic acid; CD = carbidopa; LD = L-dopa.

the ion-exchange and hydrogen-bonding interactions between the catecholamines and the residual silanol groups on the silica backbone.

By measuring retention as a function of pH, we were able to optimize mobile phase pH and to obtain some information about retention mechanisms. Data from this type of experiment for 0.1 *M* nitric acid are shown in Fig. 2. The figure shows the retention behavior of the catecholamines NE, DHBA, DA, α-MDA, as well as the amino acids L-Dopa and α-MD, the carboxylic acid, DOPAC and the acid hydrazine, CD. The capacity factors of the catecholamines are constant over the pH 2–5 range. This is expected since their pK_a values are larger than 5.0 and therefore they carry the same charge from pH 2 to 5. Since retention on octadecylsilica is due to the hydrophobicity of the solute[10], the relative retention of the catecholamines can be understood by examination of their structures. NE is the most polar of the catecholamines studied and therefore has the smallest capacity ratio. DA is more hydrophobic than NE and is retained longer. The addition of an α-methyl group to NE and DA increases hydrophobicity and accounts for the increased retention of α-MNE and α-MDA.

The catechol amino acids, L-dopa and α-MD, and carbidopa, show a drastic drop in retention when the mobile phase pH is raised from 2 to 5. The deprotonation of the carboxylic acid and the formation of the very polar zwitterionic species causes the retention of these compounds to decrease. The curve shape for DOPAC com-

pares to the curve obtained in aqueous phosphate buffer[10]. Since the retention of these compounds is very sensitive to mobile phase pH, the experiment was repeated with the same results.

Table II demonstrates that the retention of these catechols using 0.1 *M* sulfuric acid is qualitatively similar to that seen for a nitric acid mobile phase. However, the solutes have slightly smaller capacity ratios in sulfuric acid, which suggests that the sulfate anion experiences less retention than the nitrate anion on reversed-phase packing materials.

TABLE II

RETENTION OF SELECTED CATECHOLS IN VARIOUS ACID MOBILE PHASES

All acids were 0.1 *M*, pH 3.0.

Compound	*k values*					
	HNO_3	*H_2SO_4*	*CH_3COOH*	*H_3PO_4*	*TCA*	*Octylsulfate**
NE	0.4	0.3	0.2	0.3	1.8	2.5
α-MNE	0.8	0.6	0.3	0.6	3.2	4.7
EPI	1.0	0.7	0.4	0.7	4.3	5.7
DHBA	1.2	0.8	0.5	0.8	5.1	7.9
LD	1.4	1.3	0.9	1.2	3.7	3.7
DA	2.2	1.5	0.9	1.5	9.5	15.3
α-MD	3.7	3.3	1.8	3.1	11.3	—
CD	5.3	4.7	2.6	4.4	—	16.3
α-MDA	6.2	4.2	2.4	4.3	27.7	—
DOPAC	11.2	11.2	7.4	11.2	10.3	8.4

* 0.5 m*M* sodium octylsulfate in 0.1 *M* HNO_3.

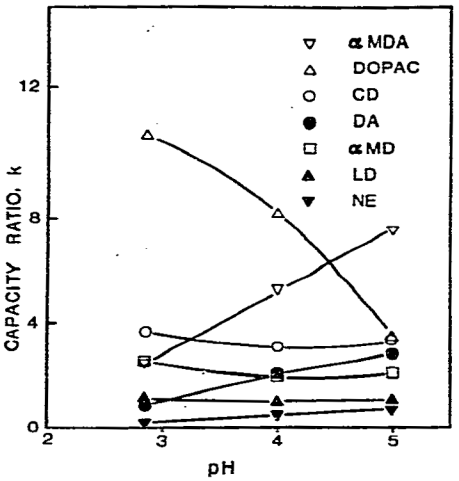
Organic acid mobile phases

The retention of catechols with 0.1 *M* acetic acid as the mobile phase is shown in Fig. 3. As the pH is raised from 2.8 to 5, there is a threefold increase in catecholamine retention. This behavior is probably due to an increase in acetate anion available for ion-pairing at higher pH.

The pH *versus* capacity ratio curves for the amino acids and CD in acetic acid are slightly concave with a minimum at pH 4. This may be due to a combination of two opposing effects. The increasing acetate ion concentration and the decreasing hydrophobicity with increasing pH tend to cancel each other and result in the observed curves.

Evidence for competition between the acetic acid and solute for the octadecyl groups in the stationary phase is the observation that DOPAC, which cannot be retained by an ion-pairing mechanism, has a smaller capacity ratio in acetic acid than in nitric or sulfuric acid. Acetic acid was rejected as a useful, general purpose, ion-pairing reagent because at pH 3.0 the retention of catecholamines is small due to the low concentration of acetate. At higher pH, tailing peaks and low efficiency were found for unknown reasons. A previous paper demonstrates the problems with acetic acid as mobile phase[11].

Fig. 4 shows the improved separation of selected catechols obtained with a mobile phase of 0.1 *M* trichloroacetic acid (TCA) at pH 3.0. Precise quantitation of



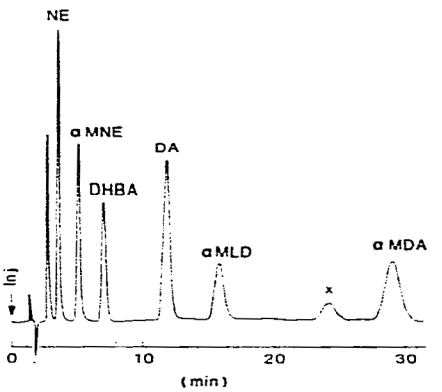


Fig. 3. Effect of the pH of acetic acid mobile phases on the capacity ratio, k, of standard catechols. Mobile phase: 0.1 M acetic acid–sodium acetate. Because of the relatively high pK_a of acetic acid, the lowest attainable pH is 2.8. Abbreviations as in Fig. 2.

Fig. 4. Chromatogram of standard catechols with a trichloroacetic acid mobile phase. Mobile phase: 0.1 *M* TCA–NaTCA, pH 3.00. Catechol abbreviations as in Fig. 1 (X is an unknown impurity).

these compounds is possible due to the very clean separation and good peak symmetry.

Fig. 5 shows the effects of pH on capacity ratios using a mobile phase of 0.1 *M* TCA. The retention of the catecholamines is much greater than with any of the other acids tested and this strong retention is likely due to the high affinity of the tricholoro-

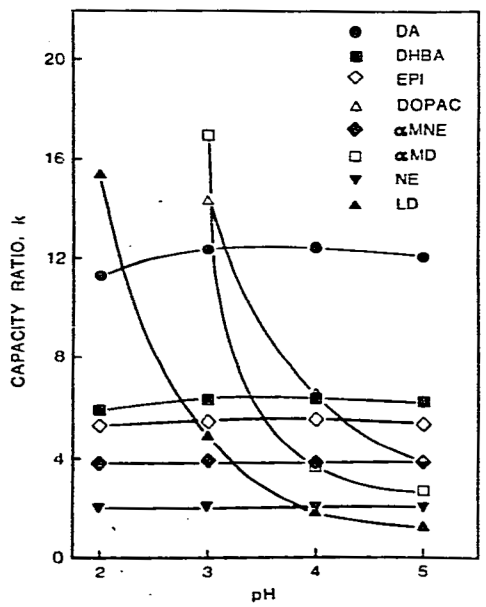


Fig. 5. Effect of the pH of the trichloroacetic acid mobile phase on the capacity ratio, k, of standard catechols. Mobile phase: 0.1 *M* TCA–NaTCA. Catechol abbreviations: as in Fig. 2, with the addition of EPI = epinephrine.

acetate ion for the reversed-phase packing material. Since TCA is a strong acid (pK_a 0.70), the trichloroacetate anion is present at a constant concentration over the pH range 2–5 and no changes in catecholamine retention was observed over this pH range. The catechol amino acids have the expected increase in retention at lower pH.

The retention of DOPAC is less in a mobile phase composed of TCA than in HNO_3, and the shape of the curve is concave upwards instead of sigmoidal. The reduced retention in TCA suggests that TCA offers more competition for DOPAC binding to the stationary phase than does nitric acid. We have no explanation for the change in curve shape.

A summary of the retention data for these compounds measured at constant pH is shown in Table II. In addition to HNO_3, H_2SO_4, acetic acid and TCA, we present data obtained using sodium octylsulfate as the ion-pairing reagent. The retention for all catechols except DOPAC is somewhat greater in a mobile phase composed of sodium octylsulfate, but the high cost of this detergent makes it less desirable as a mobile phase. Also, using an acid mobile phase, equilibrium is attained much faster than with the larger alkylsulfates; therefore, the "start-up" time for a catecholamine assay procedure is minimized when TCA or HNO_3 is used as the ion-pairing reagent[14].

Maintaining a catechol separation

The loss of capacity with time in liquid–solid adsorption chromatography is a well-known problem[12]. It is our experience that with reversed-phase liquid chromatography using aqueous mobile phases, the column capacity steadily decreases with time. We think this is because of two problems. (1) in aqueous mobile phases, trace organics are very strongly retained on the column and interfere with the normal equilibrium between the two chromatographic phases and (2) hydrolysis of octadecyl molecules from the surface of the packing material occurs and physical loss of column material results.

The measurement of naturally occurring catecholamines, their synthetic analogues, and their metabolites in brain tissue and plasma is under investigation in our laboratory. Since we are interested in measuring a large number of trace catecholamines in small tissue samples, efficient separation and sensitive detection is imperative. Because of the high cost of C_{18} columns, it is important to have a flexible chromatographic system to maintain catechol separations and prolong column life. We have found that a mobile phase composed of 0.1 *M* nitric acid will give good separations with minimal analysis time on a new column. After a period of use as column capacity is lost, a switch to a TCA mobile phase will regain resolution by increasing retention. Regeneration of the column by washing off the strongly retained organic compounds with methanol partially restores retention, but the progressive loss of stationary phase is irreversible and necessitates the change to TCA. Details of this assay are reported elsewhere[14].

The chromatogram in Fig. 6A shows a separation of standard catechols on the same column and mobile phase as in Fig. 1. The column had been used for approximately 200 samples over a period of two months and early peaks were no longer adequately resolved. Fig. 6B shows the separation could be restored when the mobile phase was switched to TCA. The conditions for this chromatogram were chosen to provide adequate retention while minimizing analysis time.

Retention can be further increased by raising the concentration of TCA at

constant pH as shown in Fig. 7. There is a two-fold increase in retention when the TCA concentration is raised from 0.01 to 0.20 *M*. This experiment also provides additional evidence that TCA–catechol ion-pairs are the species retained. The curve shapes and retention are similar to those obtained for C_4–C_{10} alkyl sulfates[13]. DOPAC is less retained as the TCA concentration increases because no ion-pairing mechanism is possible and there is greater competition between the DOPAC and the similarly charged TCA for the stationary phase.

The same experiment was performed using pH 3.0 nitric acid at various concen-

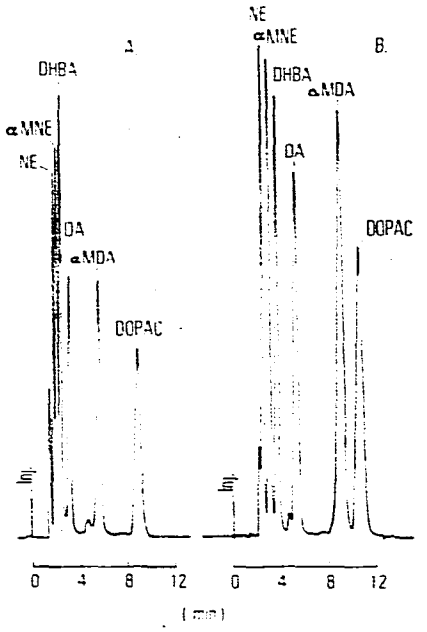


Fig. 6. Restoration of adequate retention on an old chromatographic column by changing mobile phase. Abbreviations as in Fig. 2. (A) Chromatogram obtained using same column and conditions as in Fig. 1 after two months of use with a nitric acid mobile phase. (B) Chromatogram obtained using same column after changing the mobile phase to 0.01 *M* TCA, pH 2.0.

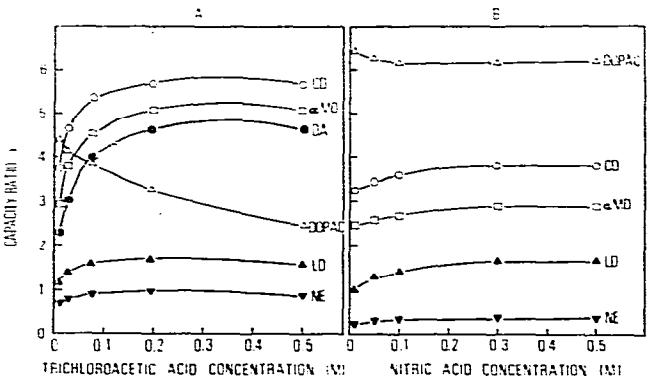


Fig. 7. Effect of mobile phase anion concentration on the capacity ratio, *k*, of standard catechols. Abbreviations as in Fig. 2. (A) Mobile phase: TCA–NaTCA, pH 3.0. (B) Mobile phase: HNO_3–$NaNO_3$, pH 3.0.

trations and the results are also shown in Fig. 7. The capacity factor dependence is much less than for TCA, indicating that the nitrate ion is a much weaker ion-pairing reagent than TCA.

CONCLUSIONS

(1) The catecholamines and their congeners show good retention and high efficiency chromatography on a non-polar C_{18} packing material with strong inorganic acids or trichloroacetic acid as ion-pairing mobile phases.

(2) A catechol separation can be maintained over a long period of time by using two different mobile phases. Nitric acid provides short retention times but adequate resolution on a new column. When column capacity is lost as the column ages, retention and resolution can be restored by switching the mobile phase to trichloroacetic acid.

(3) Retention is readily controlled by changes in the pH and concentration of the ion-pairing reagent.

ACKNOWLEDGEMENTS

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