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CATECHOLAMINES AND RELATED COMPOUNDS

EFFECT OF SUBSTITUENTS ON RETENTION IN REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

The capacity factors of 32 compounds were measured on octadecyl-silica columns by using neat aqueous phosphate buffer, pH 2.1, as the eluent. The tabulated results allow the estimation of the effect of various substituents on the retention of catecholamines and related compounds under similar chromatographic conditions.

INTRODUCTION

With the sophisticated instrumentation presently available, liquid chromatography has become a precision microanalytical tool. Recent developments in column technology gave rise to novel high efficiency columns made with so-called bonded phases, which contain an organic moiety covalently bound to the surface of 5- or 10-μm silica particles. The most widely used bonded phases are those with hydrocarbonaceous functions such as octadecyl groups and the technique in which such nonpolar stationary phases are used is commonly referred to as reversed-phase chromatography. The popularity of reversed-phase chromatography is mainly due to the simplicity of the chromatographic system as well as the wide variety of sample mixtures which can be conveniently analyzed by this technique.

Recently the method has been successfully employed for the analysis of catecholamines and related substances [1, 2]. These compounds have wide ranging physiological significance [3, 4] and their analysis is of great interest

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in the life sciences and medicine. For this reason we present the retention values of 32 compounds and attempt to quantify the effect of various substituents on their retention. The results can facilitate the prediction of their elution order in reversed-phase chromatography as well as the identification of certain peaks on the chromatogram of mixtures containing such substances.

EXPERIMENTAL

A Perkin-Elmer (Norwalk, Conn., U.S.A.) Model 601 high-pressure liquid chromatograph with a Rheodyne (Berkeley, Calif., U.S.A.) Model 7010 sample injector and a Schoeffel (Westwood, N.J., U.S.A.) Model 770 variable wavelength UV detector was used. Chromatograms were obtained at 200 nm detector setting with a Model 54 (Perkin-Elmer) strip-chart recorder. All measurements were carried out by using 5-μm LiChrosorb RP-18 columns (Rainin, Boston, Mass., U.S.A.). The No. 316 stainless steel columns were 250 × 6.4 mm O.D. × 4.6 mm I.D.

All experiments were carried out with 0.1 *M* phosphate buffer, pH 2.1, by isocratic elution at a flow-rate of 2 ml/min. Reagent grade H_3PO_4 and KH_2PO_4 were supplied by Fisher Scientific (Pittsburgh, Pa., U.S.A.). The column temperature was maintained at 70° by using the oven of the liquid chromatograph. The column inlet pressure was 160 bar (2200 p.s.i.).

The samples were supplied by Sigma (St. Louis, Mo., U.S.A.) and Aldrich (Milwaukee, Wisc., U.S.A.). Stock solutions of the substances were made in the eluent. The amount of the individual substances in the 10-μl samples injected into the chromatograph was about 1 μg. The elution time of an unsorbed solute, t_M, was measured as described previously [5]. The retention time of the solutes, t_R, was evaluated at the peak maxima as the peaks were almost symmetrical. The capacity factors, k, were calculated by the following relationship

$$k = (t_R - t_M)/t_M$$

The relative retention values, α, were obtained as the ratios of the pertinent capacity factors.

RESULTS AND DISCUSSION

The chromatograms in Fig. 1 illustrate the speed and efficiency of the chromatographic system used in this study.

In our experience, the chromatographic conditions stated are particularly advantageous for the separation of mixtures containing compounds described here. The employment of 5-μm octadecyl-silica columns at elevated temperatures offers relatively high efficiency even with neat aqueous eluents. At elevated temperatures the column inlet pressure does not exceed the practical limits even at relatively high flow-rates. The small particle size of the stationary phase and the high linear flow-velocity of the eluent together afford high speed and efficiency.

Although most work in reversed-phase chromatography is performed with hydro-organic eluents, we have found [1, 6] that the use of neat aqueous

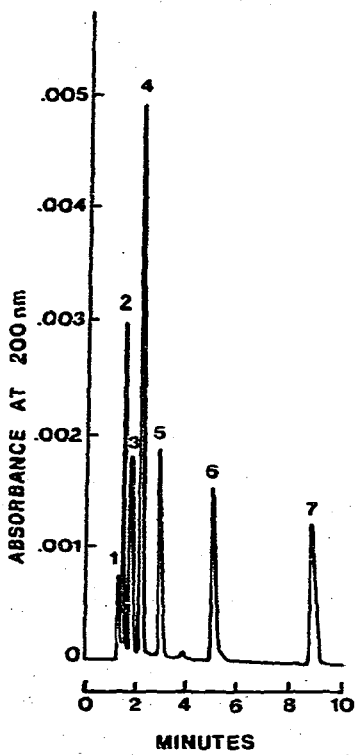
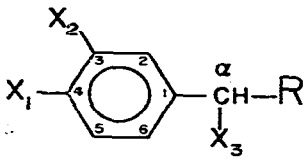


Fig. 1. Typical chromatogram of some compounds investigated. Column, 5-μm LiChrosorb RP 18, 25 × 0.64 cm O.D. × 0.46 cm I.D.; eluent, 0.1 *M* phosphate buffer, pH 2.1; flow-rate, 2 ml/min; temp, 70°, inlet pressure, 160 bar. The elution order of the substances, whose symbols are given in Table I, is as follows: 1 = NE; 2 = E; 3 = DA; 4 = TA; 5 = PEOA; 6 = PEA; 7 = DMDA.

eluents offer certain advantages in the separation of such polar biological substances. In the present study neat aqueous phosphate buffer (without any organic solvent) was used also to provide a meaningful reference framework for the retention values. At the pH of the eluent (2.1) the amino groups in the solute molecules were fully protonated, whereas for the most part the dissociation of carboxyl groups was suppressed. Under such conditions, fairly symmetrical peaks could be obtained. Octadecyl-silica was employed for two reasons. First of all, among the commercially available hydrocarbonaceous bonded phases octadecyl-silica is used most widely. Second, under otherwise fixed conditions the retention values have been found to be the greatest on such a stationary phase having a high carbon content and for the separation of such relatively polar substances a strongly-retentive column is required.

The structure of most substances under investigation can be described by the general formula given in Fig. 2. The symbols used for these compounds, the substituents of each substance according to the structure given in Fig. 2 and their capacity factors, which have been measured under conditions stated in the experimental section, are listed in Table I.

In Tables II—IV the effect of various substituents on the retention is shown.



R = —COOH

or

R = —CH(β)—X5
X4

Fig. 2. Generalized structural formula of the substances investigated.

In each case the capacity factor of both the parent compound and that of a derivative, in which a hydrogen is replaced by the substituent are listed. For each solute pair the relative retention of the derivative with respect to its parent compound, α_{SP}, together with the corresponding log α_{SP} value are also given. The data offer an overview on the effect of the various substituents on the retention of this type of compound.

The results can be broadly interpreted in terms of the solvophobic theory which has been successfully applied to reversed-phase chromatography [6—8]. According to this approach, solute retention is governed by the balance of two solvent effects on the reversible association of the solute with the hydrocarbonaceous functions of the stationary phase. The reduction in the molecular surface area upon binding and the increase in surface tension of the eluent augment retention due to the free energy change associated with the cavity effect [6]. On the other hand, an increase in the energy of interaction between the solute and the solvent reduces binding. This interaction can be broken down to van der Waal's interaction, which is primarily dependent on the molecular size of the solute and solvent, and electrostatic interactions. The latter are determined by the dipole moment and/or the charges of the molecule. With our solutes of similar molecular dimensions and with a fixed eluent, the magnitude of relative retention is primarily affected by the "polarity" of the respective substituents which play a major role in determining the energy of solute interaction with water. Consequently, the degree of dissociation of ionogenic groups in the solute molecules has also a great influence on retention in such chromatographic systems. This effect has been analyzed both theoretically and experimentally [7].

As shown in Table II, the attachment of hydroxyl groups to the aromatic ring reduces retention in such a way that the effect of the first hydroxyl group is significantly greater than that of the second. On the other hand the replacement of a hydrogen on the aromatic ring by a methoxy group brings about an increase in the size of the molecule by this "unpolar" group. The effect is, of course, much more pronounced on retention when a polar hydroxyl group is replaced by a methoxy group.

Table III shows that the replacement of a hydrogen on the α-carbon atom by a hydroxyl group greatly reduced the retention. In view of the solvophobic

theory, this effect is likely to be caused by drastic changes in the molecular properties such as the dipole moment [6]. With the last four solute pairs (R = COOH) the change in relative retention is significantly greater than in the other cases. The introduction of an α-hydroxyl group into carboxylic acids

TABLE I.

STRUCTURE AND CAPACITY FACTOR OF THE SUBSTANCES INVESTIGATED

The meaning of the substituents is given in Fig. 2.

Symbol	Name	Structure				Capacity factor
		X_1	X_2	X_3	R	k
AMINES						
NE	Norepinephrine	OH	OH	OH	CH_2NH_2	0.145
OCT	Octopamine	OH	H	OH	CH_2NH_2	0.26
E	Epinephrine	OH	OH	OH	CH_2NHCH_3	0.28
NMET	Normetanephrine	OH	OCH_3	OH	CH_2NH_2	0.48
SYN	Synephrine	OH	H	OH	$CH(CH_3)NH_2$	0.51
DA	Dopamine	OH	OH	H	CH_2NH_2	0.56
MET	Metanephrine	OH	OCH_3	OH	CH_2NHCH_3	0.93
TA	Tyramine	OH	H	H	CH_2NH_2	0.96
PEOA	Phenylethanolamine	H	H	OH	CH_2NH_2	1.03
3MDA	3-O-Methyldopamine	OH	OCH_3	H	CH_2NH_2	1.86
PEA	Phenylethylamine	H	H	H	CH_2NH_2	4.06
NIE	Norisoephedrine	H	H	NH_2	$CH(OH)CH_3$	5.17
EPH	Ephedrine	H	H	OH	$CH(CH_3)NHCH_3$	6.76
DMDA	Dimethyldopamine	OCH_3	OCH_3	H	CH_2NH_2	9.09
ACIDS						
DOMA	Dihydroxymandelic	OH	OH	OH	COOH	0.51
POMA	*p*-Hydroxymandelic	OH	H	OH	COOH	0.87
MOMA	*m*-Hydroxymandelic	H	OH	OH	COOH	1.66
VMA	Vanilmandelic	OH	OCH_3	OH	COOH	1.69
DOBA*	3,4-Dihydroxybenzoic	OH	OH	—	—	2.66
DOPAC	Dihydroxyphenylacetic	OH	OH	H	COOH	4.42
MA	Mandelic	H	H	OH	COOH	5.57
POPAC	*p*-Hydroxyphenylacetic	OH	H	H	COOH	7.57
VA*	Vanillic	OH	OCH_3	—	—	9.13
HVA	Homovanillic	OH	OCH_3	H	COOH	14.67
PAC	Phenylacetic	H	H	H	COOH	47.80
AMINO ACIDS						
DOPS	Dihydroxyphenylserine	OH	OH	OH	$CH(NH_2)COOH$	0.15
DOPA	Dihydroxyphenylalanine	OH	OH	H	$CH(NH_2)COOH$	0.57
TYR	Tyrosine	OH	H	H	$CH(NH_2)COOH$	0.98
PSER	Phenylserine	H	H	OH	$CH(NH_2)COOH$	1.04
3MDOPA	3-O-Methyl-DOPA	OH	OCH_3	H	$CH(NH_2)COOH$	1.88
PHE	Phenylalanine	H	H	H	$CH(NH_2)COOH$	3.85
TRP*	Tryptophane	—	—	—	—	7.18

*The general formula in Fig. 2 is not applicable to the structure of these compounds.

generally results in a reduction of the pK_a values due to the above mentioned effect. Thus, the mandelic acid derivatives are likely to more dissociated at the eluent pH than the corresponding parent compounds, which are phenylacetic acid derivatives, and this also could contribute to the observed large decrease in relative retention.

Upon introduction of a charged ammonium group into the molecule, retention decreases considerably as shown by the examples at the top of Table IV. In the next section the retention values for zwitterionic amino acids and the corresponding amines are compared. It is seen that at pH 2.1 the relative retention of these solute pairs is very close to unity. As expected the reten-

TABLE II

EFFECT OF RING SUBSTITUTION (X_1, X_2) ON THE CAPACITY FACTORS OF THE SUBSTITUTED DERIVATIVE, S, AND THE PARENT COMPOUND, P, AND ON THE RELATIVE RETENTION, α_{SP}

S	P	k_S	k_P	α_{SP}	$\log \alpha_{SP}$
X_1 = OH	X_1 = H				
TA	PEA	0.96	3.90	0.25	−0.61
OCT	PEOA	0.26	1.03	0.25	−0.60
TYR	PHE	0.98	3.85	0.25	−0.59
POMA	MA	0.87	5.57	0.16	−0.81
POPAC	PAC	7.57	47.80	0.16	−0.80
X_2 = OH	X_2 = H				
NE	OCT	0.145	0.26	0.56	−0.25
DA	TA	0.56	0.96	0.58	−0.23
DOPA	TYR	0.57	0.98	0.58	−0.24
DOMA	POMA	0.51	0.87	0.59	−0.23
MOMA	MA	1.66	5.57	0.30	−0.53
DOPAC	POPAC	4.42	7.57	0.58	−0.23
X_2 = OCH_3	X_2 = H				
NMET	OCT	0.48	0.26	1.85	0.27
VMA	POMA	1.69	0.87	1.94	0.29
3MDA	TA	1.86	0.96	1.94	0.29
3MDOPA	TYR	1.88	0.98	1.92	0.28
HVA	POPAC	14.67	7.57	1.94	0.29
X_2 = OCH_3	X_2 = OH				
NMET	NE	0.48	0.145	3.31	0.52
MET	E	0.93	0.28	3.32	0.52
VMA	DOMA	1.69	0.51	3.31	0.52
3MDA	DA	1.86	0.56	3.32	0.52
3MDOPA	DOPA	1.88	0.57	3.30	0.52
HVA	DOPAC	14.67	4.42	3.32	0.52

TABLE III

EFFECT OF SUBSTITUTION (X_3) AT THE α-CARBON ATOM ON RETENTION

S	P	k_S	k_P	α_{SP}	$\log \alpha_{SP}$
X_3 = OH	X_3 = H				
NE	DA	0.145	0.56	0.26	—0.59
DOPS	DOPA	0.15	0.57	0.26	—0.58
OCT	TA	0.26	0.96	0.27	—0.57
PSER	PA	1.04	3.85	0.27	—0.57
NMET	3MDA	0.48	1.86	0.26	—0.59
PEOA	PEA	1.03	4.06	0.25	—0.60
DOMA	DOPAC	0.51	4.42	0.115	—0.938
POMA	POPAC	0.87	7.57	0.115	—0.940
VMA	HVA	1.69	14.67	0.115	—0.939
MA	PAC	5.57	47.80	0.117	—0.934

TABLE IV

EFFECT OF SUBSTITUTION (R) AT THE β-CARBON ATOM

S	P	k_S	k_P	α_{SP}	$\log \alpha_{SP}$
R = $CH(NH_2)COOH$	R = CH_2COOH				
DOPA	DOPAC	0.57	4.42	0.129	—0.890
TYR	POPAC	0.98	7.57	0.129	—0.890
PHE	PAC	3.85	47.80	0.081	—1.092
PSER	MA	1.04	5.57	0.187	—0.729
3MDOPA	HVA	1.86	14.67	0.127	—0.897
R = $CH(NH_2)COOH$	R = CH_2NH_2				
DOPA	DA	0.57	0.56	1.02	0.01
TYR	TA	0.98	0.96	1.02	0.01
3MDOPA	3MDA	1.88	1.86	1.01	0.00
PSER	PEOA	1.04	1.03	1.01	0.00
DOPS	NE	0.15	0.145	1.03	0.01
PHE	PEA	3.85	4.06	0.95	—0.02
R = CH_2NH-CH_3	R = CH_2NH_2				
E	NE	0.28	0.145	1.93	0.29
MET	NMET	0.93	0.48	1.94	0.29
R = $CH(CH_3)NH_2$	R = CH_2NH_2				
SYN	OCT	0.51	0.26	1.96	0.29

tion increases upon substituting a hydrogen, which is attached to the nitrogen or carbon, by a methyl group, as shown in the two lower sections of Table IV. Similarly an intercalating methylene group also increases the retention of a homologue with respect to that of the parent substance as demonstrated in Table V. Nevertheless, the log α value for the methylene group is only about half the log k increment found for aliphatic α-amino acids under similar chromatographic conditions [9].

The relative retention values, which express the effect of a given substituent, are surprisingly consistent. In most cases, however, the magnitude of the α-values is determined by the charge on the molecules, i.e., α_{SP} of a given substituent is not the same for neutral molecules as for those containing an ammonium group. Such an effect of ionization is expected in view of the different behavior of monopoles, dipoles and zwitterions [7].

In order to illustrate further the effect of a charged amino group on the retention, the data are arranged in a different way in Table VI. In compounds A the substituent R on the α-carbon atom is invariably a carboxylic group whereas in substances B the moiety R contains a primary amino group which is assumed to be fully protonated at the pH of the eluent. Thus, the relative retention values, α_{AB}, give an indication of the effect of replacing a fully ionized substituent by a carboxyl group which can be more or less dissociated under the conditions of the experiment. The pK_a values for the carboxylic groups of the compounds under investigation fall in the range 2—4.5 [10]. The acids having a hydroxyl group on the α-carbon (X_3 = OH) have generally a lower pK_a, therefore, a higher degree of dissociation than those in which X_3 = H at the eluent pH 2.1 The effect of the ionization of a carboxyl group on the retention of weak acids has extensively been investigated [7] and the findings have been corroborated in a study on the effect of pH on the retention of amino acids [9]. Full protonation of carboxylic groups having pK_a values below 3 requires an eluent pH significantly lower than 2 and it is not recommended to use such acidic eluents in commercially available liquid chromatographs. According to the theory [7] the increase in the relative retention should be greater when a largely undissociated carboxyl group is replaced by a substituent containing a protonated amino group than that occurs upon the replacement of a largely dissociated carboxyl group. Indeed the relative retention values, with two exceptions, are quite consistent when arranged in two groups depending on the substituent X_3, as seen in Table VI. Nevertheless, the relatively large effect observed with substances having a hydroxyl group on the α-carbon atom cannot be fully accounted for by the greater

TABLE V

EFFECT OF A 1-CH_2-GROUP ON THE RETENTION

Substituent	S	P	k_S	k_P	α_{SP}	log α_{SP}
—CH_2—	HVA	VA	14.67	9.13	1.61	0.21
	DOPAC	DOBA	4.42	2.66	1.66	0.22

dissociation of the adjacent carboxyl group. A more detailed analysis regarding the changes in the dipole moments upon introduction of such a substituent is required for a satisfactory theoretical interpretation [7, 8].

The relative retention values presented here can be used to estimate the retention of this type of compound. Thus, the tentative identification of certain peaks on the chromatogram obtained under similar conditions is facilitated. The sample population is not large enough to establish a statistically acceptable quantitative structure-retention relation. Nevertheless, the data appear to be consistent enough to express quantitatively the effect of replacing a hydrogen by certain substituents on the retention. Such substituent parameters which are given by the appropriate log k increments, are designated by τ and listed in Table VII. The meaning of these parameters is similar to that of the π-values introduced by Hansch [11] for use in quantitative structure—activity relationships (QSAR), in medicinal chemistry and related areas. The π-values are obtained from partition coefficients measured in octanol—water system as opposed to our τ-values which come from chromatographic measurements by using a bonded hydrocarbonaceous stationary phase. Nevertheless, both phenomena are subject to similar linear free-energy relationships which have wide currency in physico-chemical investigations [12]. Indeed, the log k values of certain amino acids have been found to be co-linear with the logarithm of the corresponding partition coefficients in octanol-water [9]. Unfortunately, literature data are not available to compare the τ values listed in Table VII with the corresponding π values. On the other hand, a general dis-

TABLE VI

EFFECT OF REPLACEMENT OF R CONTAINING AN IONIZED AMINO GROUP BY A CARBOXYLIC GROUP

	A	B	k_A	$-k_B$	α_{AB}	$\log \alpha_{AB}$
R	COOH	$CH_2NH_3^+$				
X_3 = OH	POMA	OCT	0.87	0.26	3.35	0.53
	VMA	NMET	1.69	0.48	3.52	0.55
	DOMA	NE	0.51	0.145	3.52	0.55
X_3 = H	HVA	3MDA	14.8	1.86	7.96	0.90
	DOPAC	DA	4.42	0.56	7.89	0.90
	POPAC	TA	7.57	0.96	7.89	0.90
R	COOH	$CH(NH_3^+)COOH$				
X_3 = OH	DOMA	DOPS	0.51	0.15	3.40	0.53
	MA	PSER	5.57	1.04	5.36	0.73
X_3 = H	DOPAC	DOPA	4.42	0.57	7.75	0.89
	POPAC	TA	7.57	0.98	7.72	0.89
	HVA	3MDOPA	14.67	1.88	7.80	0.89
	PAC	PHE	47.8	3.85	12.42	1.09

TABLE VII

LIST OF THE LOG K INCREMENTS, τ, OBTAINED FOR THE EFFECT OF REPLACING A HYDROGEN BY A GIVEN SUBSTITUENT ON THE RELATIVE RETENTION.

Substituent	τ	Substituent	τ
4-OH	−0.80*	α-NH_3^+	−0.89
	0.60		
3-OH	−0.53***	β-NH_2^+-CH_3	0.29
	−0.24§		
3-OCH_3	0.52	β-CH_3	0.29
α-OH	−0.94*		
	−0.58**		

*no ionized amino group in the molecule
**ionized amino group in the molecule
***X_1 = H
§ X_1 = OH

cussion of the use of chromatographic data in this particular field is given in the review by Tomlinson [13].

In using the data presented here a caveat is necessary, however. As has already been pointed out, the extent of dissociation of ionogenic groups in the molecules has a great effect on the magnitude of both the capacity factors and the relative retentions. Since the degree of dissociation depends on the pK_a values of the ionogenic groups and the pH of the eluent, the data presented here rigorously apply only at pH 2.1. When chromatographic analysis is carried out at higher pH, the ionization of the acidic and basic groups may change. The concomitant changes in retention, however, can be estimated on the basis of earlier treatment [7]. That the extent of the dissociation of ionogenic groups in the molecule has to be taken into account is also apparent from the two different τ-values obtained for the same substituents in charged and neutral species. It should be kept in mind, therefore, that the pH of the medium plays an important role in determining not only the capacity factors but also the τ-values for ionogenic substances. On the other hand, a variety of non-polar bonded stationary phases, which differ in hydrocarbon chain length, carbon loading and silica support, are commercially available. The properties of the stationary phases have an effect on the optimum eluent composition for such separations and the relative retention values likely to be different in another chromatographic system.

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