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REVERSED-PHASE ION-PAIR PARTION CHROMATOGRAPHY OF BIOGENIC CATECHOLAMINES AND THEIR α-METHYL HOMOLOGUES WITH TRIBUTYLPHOSPHATE AS STATIONARY PHASE

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SUMMARY

Ion-pair partition chromatography is applied to the separation of the biogenic catecholamines and their α-methyl homologues. A useful selectivity has been obtained using an adduct-forming organic stationary phase (tributylphosphate). The retention of the compounds can be regulated easily by means of the concentration of the counter-ion (the perchlorate ion) in the mobile phase. The selectivity for separation of amines from amino acids can be influenced by changing the pH of the aqueous phase. The phase system shows a good long-term stability and reproducibility with respect to the capacity ratios and the efficiency.

INTRODUCTION

A chromatographic phase system based on ion-pair partitioning has been developed to separate the biogenic catecholamines and their α-methyl homologues. The latter occur in biological samples after the administration of the hypotensive agent α-methyldopa. The separation and determination of the biogenic catecholamines has been described in literature. However, when the α-methyl homologues are also present, phase systems based on ion-exchange fail through lack of selectivity.[1] Phase systems based on a stationary phase of reversed-phase material and a mobile phase to which a lipophilic counter-ion, such as dodecylsulphate, is added[2] have some drawbacks. It appeared impossible to achieve a good separation of the closely related compounds adrenaline and α-methylnoradrenaline, the most important metabolite of α-methyldopa.

Moreover, such phase systems give rise to problems with respect to their stability. A gradual decrease of the capacity ratios is observed. To improve the stability of such phase systems an eluent without, or with only a very small concentra-

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tion of, the counter-ion can be used after loading of the column[3]. Separation of the catecholamines can also be achieved on octadecyl-silica and acid buffer solutions as mobile phases, but the lifetime of the columns is short under such conditions[4]. Straight phase systems based on ion-pair partition chromatography show good possibilities for the separation of catecholamines[5]. However, the organic solvent used as mobile phase is less well suited for an electrochemical detection system, which is needed to detect the small amounts of amines present in biological samples. Reversed-phase ion-pair partition systems based on the use of pentanol as the stationary phase can also separate catecholamines, but they show no selectivity towards the separation of primary amines and their N-methyl derivatives, *e.g.* noradrenaline and adrenaline[6].

This paper describes a reversed-phase ion-pair partition system based on tributylphosphate as stationary phase on LiChrosorb RP-8 as support material and aqueous phosphate buffers as mobile phases with perchlorate as counter-ion. Phase systems based on tributylphosphate as stationary phase in partition chromatography were introduced previously and applied to the separation of hydrophilic carboxylic acids[7].

EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from custom-made and commercially available parts and consisted of a constant-flow pump (Constametric I, LDC, Riviera Beach, FL, U.S.A.), a thermostatted eluent reservoir, a Bourdon type manometer, an injection system (U6K, Waters Assoc., Milford, MA, U.S.A.), a stainless steel column (100 × 2.8 mm I.D.) and an amperometric detection system. The amperometric detection system consisted of a so-called wall-jet detector cell unit (E.D.T., London, Great Britain) in combination with a polarograph used as potentiostat (E-310, Bruker, Karlsruhe, G.F.R.). The detector cell was modified by replacement of the Ag/AgCl reference electrode by a home-made saturated calomel electrode. The complete chromatographic system and the detector cell were placed in an air-heated cabinet.

Chemicals and reagents

The amines used as chromatographic reference substances are listed in Table I. All other chemicals and solvents were of analytical or reagent grade and were used without further purification. Tributylphosphate was obtained from Aldrich (Milwaukee, WI, U.S.A.). The chromatographic support material LiChrosorb RP-8 (particle diameter, 10 μm) was obtained from Merck (Darmstadt, G.F.R.). Water was purified by a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.). The mobile phase was prepared by mixing perchloric acid solution (11.8 M) and phosphoric acid solution (1.00 M). After dilution with water the solution was brought to the desired pH with a concentrated solution of sodium hydroxide and diluted to the desired concentration of the counter-ion and phosphate buffer. Before use the mobile phase was saturated with tributylphosphate.

Determination of the chromatographic parameters

The capacity ratio k'_i of a compound i was determined from its retention time

TABLE I

STRUCTURES OF THE CHROMATOGRAPHIC REFERENCE SUBSTANCES

Structure	R_1	R_2	R_3	Name	Origin
R_1O—(ring)—CH_2–C(R)(COOH)–NH–R_3; HO—	H	H	H	DOPA	Fluka, Buchs, Switzerland
	H	CH_3	H	α-Methyldopa	M.S.D., West Point, PA, U.S.A.
R_1O—(ring)—CH_2–CH(R_2)–NH–R_3; HO—	H	H	H	Dopamine	Fluka
	H	CH_3	H	α-Methyldopamine	Synthesized[8]*
	CH_3	H	H	3-Methoxytyramine	Aldrich, Beerse, Belgium
	H	H	CH_3	Epinine	Synthesized[9]*
R_1O—(ring)—CH(OH)–CH(R_2)–NH–R_3; HO—	H	H	H	Noradrenaline	Fluka
	H	CH_3	H	α-Methylnoradrenaline	Sterling Winthrop, New York, NY, U.S.A.
	CH_3	H	H	Normetanephrine	Sigma, St. Louis, MO, U.S.A.
	H	H	CH_3	Adrenaline	O.P.G., Utrecht, The Netherlands
	CH_3	H	CH_3	Metanephrine	Sigma

* The syntheses were performed by the Department of Pharmacochemistry, Subfaculty of Pharmacy, Gorlaeus Laboratories.

t_{Rt} and the retention time of an unretarded compound, t_{R0}, for which potassium iodide was used. The theoretical plate height for a compound was calculated from its retention time and the peak width at 0.61 of the peak height. The porosity, ε_m, was calculated from the retention volume of the unretarded compound (V_m) and the volume of the empty column (V_0) by $\varepsilon_m = V_m/V_0$[10].

Chromatography

The columns were packed using a slurry technique with tetrachloromethane as dispersing solvent and n-hexane as displacing solvent. After washing the column with 25 ml of ethanol and 10 ml of water the mobile phase was pumped through the column and the column was loaded in situ by injection of the stationary phase[7]. The column was considered to be maximally loaded at a porosity of $\varepsilon_m = 0.46 \pm 0.01$[10]. The volume of the stationary phase (V_s) was calculated from the difference in porosity before and after loading of the column, and was usually 0.115 ± 0.005 ml. In order to obtain a stable system the mobile phase was saturated with the stationary phase, but no precolumn was used in the experiments. To prevent demixing of the mobile phase in the column caused by a slight rise of temperature the eluent reservoir was thermostatted at 298°K and the cabinet with the chromatograph and detector at 297°K. It should be noted that the solubility of tributylphosphate in water decreases with increasing temperature.

THEORETICAL

Many ion-pair extraction procedures involving adduct formation with tributylphosphate (S) are described in literature. This technique has been applied to ion-pair extractions of metal ions with perchlorate counter-ions (X^-) or of inorganic anions with hydronium ions (H_3O^+) as counter-ions, and the extraction of perchloric acid is

a well known process[11]. These ion-pairs dissociate in the organic phase[12,13]. The ion-pair distribution of a protonated amine (BH$^+$) with perchlorate counter-ions can be described in a similar way, taking into account ion-pair extraction and possible ion-pair dissociation in the organic phase:

$$BH_{aq}^+ + X_{aq}^- + nS_{org} \overset{K_{ex}}{\rightleftharpoons} BHXS_{n,org} \tag{1}$$

$$BHXS_{n,org} \overset{K_{diss}}{\rightleftharpoons} BHS_{n,org}^+ + X_{org}^- \tag{2}$$

where K_{ex} and K_{diss} are the equilibrium constants.

Distribution of the amine as such hardly occurs. Catecholamines such as dopamine are in ionic form in an aqueous solution at any pH. The pK_a values of dopamine are[14]: for the first phenol function 8.85; for the amine function 10.3.

The distribution of an amino acid (HBH$^+$), such as DOPA, will be influenced by the dissociation of the carboxylic group (pK_a 2.2), when the pH of the aqueous phase is raised:

$$HBH_{aq}^+ \overset{K_a}{\rightleftharpoons} {}^-HB_{aq}^+ + H_{aq}^+ \tag{3}$$
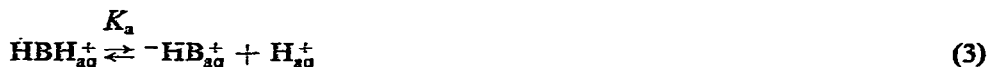
From the different equilibria an expression describing the distribution ratio of an amine ion-pair can be derived:

$$D = \frac{[BHSX_n]_{org} + [BHS_n^+]_{org}}{[BH^+]_{aq}} = K_{ex}[X^-]_{aq}\,[S]_{org}^n\,(1 + K_{diss}/[X^-]_{org}) \tag{4}$$

The following expression is obtained for the distribution ratio of an amino acid as ion-pair:

$$D = (K_{ex}[X^-]_{aq}\,[S]_{org}^n\,(1 + K_{diss}/[X^-]_{org}))/(1 + K_a/a_{H^+,aq}) \tag{5}$$

where K is the acid dissociation constant and $a_{H^+,aq}$ is the hydrogen activity.

Dissociation of the ion-pair in the organic phase is a common side-reaction in ion-pair partition systems[15]. According to eqn. 4, this phenomenon leads to non-linear distribution isotherms (since $[X^-]_{org}$ originates from the sample ion-pair) resulting in tailing peaks in reversed-phase chromatography unless other dissociating ion-pairs, with a common counter-ion, are extracted in high concentrations[6]. In the present case any possible dissociation is held constant due to the presence in the organic phase of perchlorate ions from extracted perchloric acid, which is known to be highly dissociated in tributylphosphate[13]. Under such conditions $[X^-]_{org}$ is governed by the perchloric acid distribution and is not influenced by the low sample concentrations (1–500 ng were injected), and a linear distribution isotherm is obtained.

The relationship between the distribution ratio (D) and the chromatographic retention is given by the expression for the capacity ratio:

$$k' = D\,(V_s/V_m) \tag{6}$$

in which V_s/V_m represents the phase ratio of the stationary and the mobile phases.

RESULTS AND DISCUSSION

In order to obtain information about the distribution phenomena and to determine optimum conditions for the required separation, the influence on the retention behaviour of the counter-ion concentration, the pH and the temperature have been investigated.

The concentration of the counter-ion in the mobile phase is an important parameter in reversed-phase ion-pair chromatography. According to eqns. 4, 5 and 6, an increase of the counter-ion concentration should lead to a linear increase of the capacity ratios, if the quotient $K_{diss}/[X^-]_{org}$ is much less than unity or is kept constant. Fig. 1 demonstrates the high increase in capacity ratios which was obtained on increasing the concentration of perchlorate ions in the mobile phase at constant pH. A deviation from linearity at low concentrations of counter-ion is observed, as well as an intercept. The intercept can be due to distribution to the stationary phase or the support material of the phosphate ion-pairs of the samples. The deviation from linearity can depend on the increase of the ionic strength, which occurs by the addition of perchlorate ions and also on a change in the degree dissociation of the sample ion-pairs, *i.e.* the factor $(1 + K_{diss}/[X^-]_{org})$, which can be caused by extracted perchloric acid. The dissociation constant, K_{diss}, for the dopamine–perchlorate ion-pair in aqueous saturated tributylphosphate has been determined by conductivity measurements and appeared to be $5 \cdot 10^{-4}$ (ref. 17).
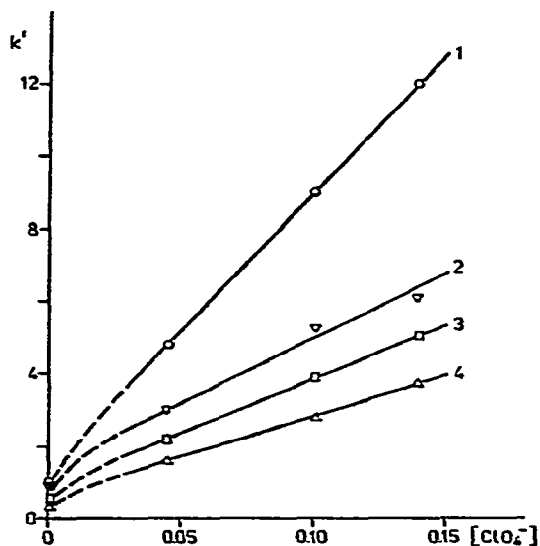

Fig. 1. Capacity ratio and counter-ion concentration. Mobile phase: phosphate buffer 0.05 M, pH = 2.10 saturated with tributylphosphate. Stationary phase: tributylphosphate on LiChrosorb RP-8 (10 μm). Compounds: 1 = dopamine; 2 = DOPA; 3 = noradrenaline; 4 = adrenaline.

A change in the degree of dissociation of the dopamine–perchlorate ion-pair in the presence of perchloric acid requires a perchlorate ion concentration in the organic phase of *ca.* $5 \cdot 10^{-3}$ M, which seems possible owing to the high distribution ratio and dissociation of perchloric acid in tributylphosphate[11].

In order to investigate the possibility of influencing the selectivity of the phase system, the pH of the mobile phase was varied from 1.75 to 4.85 at constant counter-ion concentration, and the capacity ratios of amines and amino acids were measured. The results are represented in Fig. 2, a plot of log k' vs. pH. As a consequence of the dissociation of the carboxylic group of the amino acids, the capacity ratios of these compounds decrease when the pH of the mobile phase is raised above the pK_a value (2.2). The slope of the curve of log k' vs. the pH of the mobile phase comes close to a value of -1. If $a_{H^+,ap} \ll K_a'$ and $[X^-]_{org} \gg K_{diss}$, eqn. 5 reduces to:

$$D = K_{ex}[X^-]_{aq} [S]_{org}^n \frac{a_{H^+,aq}}{K_a} \qquad (7)$$

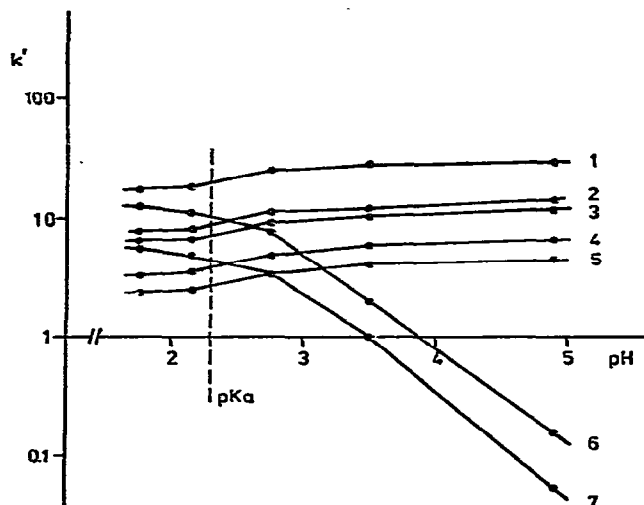or log D (and hence log k') is proportional to $-$pH.

Fig. 2. Capacity ratio and pH. Mobile phase: phosphate buffer 0.05 M, with perchlorate as counter-ion 0.10 M, saturated with tributylphosphate. Stationary phase: tributylphosphate on Li-Chrosorb RP-8 (10 μm). Compounds: 1 = α-methyldopamine; 2 = dopamine; 3 = α-methylnorad-renaline; 4 = noradrenaline; 5 = adrenaline; 6 = α-methyldopa; 7 = DOPA. pK_a is the dissocia-tion constant of the carboxylic group of the amino acids.

The increase of the capacity ratios of the amines, when the pH of the mobile phase is raised, is probably caused by an interaction with the support material. Even when no perchlorate is present in the mobile phase, a relatively large capacity ratio for the amines is observed when the pH of the mobile phase is raised. This increase is different for different comparable support materials.

As mentioned above, the temperature was kept constant at 298°K for the eluent reservoir and at 297°K for the cabinet with the chromatograph. In almost all experiments the chromatograph operated at a flow-rate of 0.50 ml/min, corresponding to a linear velocity of *ca.* 3 mm/sec, which caused a pressure drop of *ca.* 7.5 MPa. As a consequence of frictional forces, the temperature of the mobile phase will rise

one or two degrees[18]. At high linear velocities the temperature will rise above 298°K, and if no precautions are taken the mobile phase will demix. The influence of the linear velocity on the theoretical plate height has been measured, and the results for a few compounds are represented in Fig. 3.
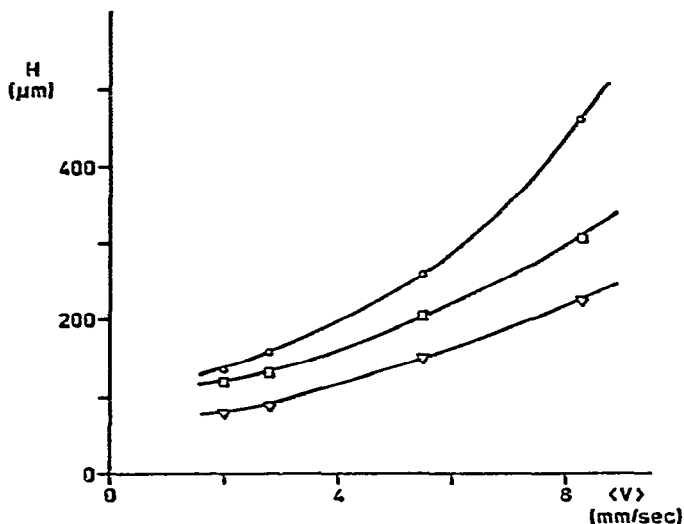

Fig. 3. Theoretical plate height and linear velocity of the mobile phase. Mobile phase: phosphate buffer 0.05 M, pH = 2.10 with perchlorate counter-ion 0.10 M saturated with tributylphosphate. Stationary phase: tributylphosphate on LiChrosorb RP-8 (10 μm). Compounds: ○, adrenaline; □, DOPA; △, dopamine.

Since the distribution properties in liquid–liquid phase systems are known to be dependent on temperature, the influence of an increase of 10°K on the capacity ratios has been measured. The influence is about the same for all compounds, i.e. there is a decrease of the capacity ratios (k'_{298}/k'_{308} = 1.6), so the selectivity is not influenced by temperature.

The phase system shows a remarkable selectivity with respect to the presence of an extra methyl group in the molecule. Introduction of a methyl group at the α-position of the amines results in an increase of the capacity ratio, which is expected. Introduction of a methyl group to form 3-O-methyl derivatives (the metanephrines, biotransformation products of the biogenic catecholamines) has only a small influence on the capacity ratios. Methylation of the nitrogen atom even results in a decrease of the capacity ratios (Table II).

Reversed-phase ion-pair chromatography allows the application of large volumes of samples by using a high counter-ion concentration in the sample solution. Because of the large capacity ratio during the injection the compounds are concentrated on the top of the column[16]. As a consequence of the large injection volume, the retention times of the compounds will increase. This retention time, $t_{R,observed}$, can be calculated from the injection time, the capacity ratio at high counter-ion concentration (k'_1), the capacity ratio at low counter-ion concentration (k'_2) and the distances

TABLE II

CAPACITY RATIOS OF THE BIOGENIC CATECHOLAMINES AND THE CORRE-
SPONDING METHYL DERIVATIVES

Chromatographic conditions: stationary phase, tributylphosphate on LiChrosorb RP-8 (10 μm); mobile phase, phosphate buffer 0.05 M, pH 2.10 with perchlorate counter-ion 0.10 M and saturated with tributylphosphate.

	k' amines	k' α-methyl derivatives	k' 3-O-methyl derivatives	k' N-methyl derivatives
DOPA	4.9	11.0	—	—
Dopamine	8.3	17.5	7.6	5.0
Noradrenaline	3.5	7.0	3.4	2.5

migrated at these counter-ion concentrations. For the retention time of the median of the compound, the maximum of a symmetrical peak at the end of the column, eqn. 8 is valid, assuming symmetrical compound zones, a step change of the capacity ratios and a plug injection:

$$t_{R,\text{observed}} = \frac{t_{\text{inj}}}{2} + t_1 + \frac{L - z}{\langle v \rangle}(1 + k_2')$$ (8)

The three terms represent, in order, the time elapsed from the start of the injection until the median of the sample enters the column, the time during which the median of the compound migrates with a high counter-ion concentration from the top of the column to the point where the eluent front reaches the median, and the time during which the median of the compound migrates with the low counter-ion concentration. The symbols are as follows: z is the distance migrated by the median of the compound at high counter-ion concentration; $\langle v \rangle$ is the linear velocity of the eluent; L is the length of the column; and t_{inj} is the injection volume/flow. For the median of the compound, t_2 is given by eqn. 9:

$$t_1 = z(1 + k_1')/\langle v \rangle$$ (9)

For the eluent front the same time interval is given by eqn. 10:

$$t_1 = \frac{t_{\text{inj}}}{2} + \frac{z}{\langle v \rangle}$$ (10)

From eqns. 9 and 10 an expression can be calculated for z:

$$z = \frac{t_{\text{inj}}}{2} \cdot \frac{\langle v \rangle}{k_1'}$$ (11)

Combination of eqns. 8, 9 and 11 gives a relationship for the observed retention time:

$$t_{R,\text{observed}} = \mathrm{s}_{\text{inj}}\left(1 - \frac{k_2'}{2k_1'}\right) + t_R$$ (12)

where

$$t_R = L(1 + k_2')/\langle v \rangle$$ (13)

The influence of the injection volume at different counter-ion concentrations on peak broadening and retention times has been investigated. The influence of the injection volume on peak broadening is represented in a plot of σ_V vs. injection volume (Fig. 4); σ_V was determined from half the peak width at 0.61 of the peak height and the flow-rate. The calculated (eqn. 12) and measured retention times obtained under the conditions given in Fig. 4 agree within 2%, where the k' values at high counter-ion concentration were obtained by extrapolation of the results represented in Fig. 1. The results show that peak broadening resulting from use of large volumes of samples can be reduced when using high counter-ion concentrations in the sample solution without disturbing the chromatographic system.
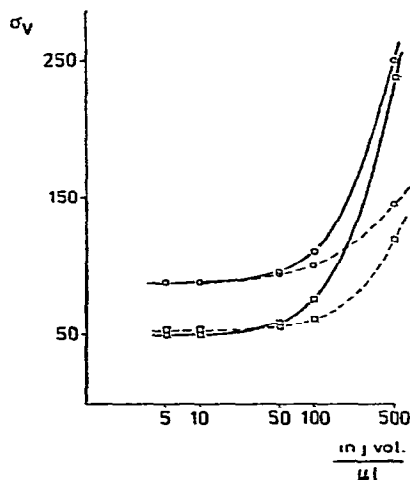

Fig. 4. Peak broadening and injection volume at different counter-ion concentrations of the sample for dopamine (O) and noradrenaline (□). Mobile phase: phosphate buffer 0.05 M, pH = 2.10 with perchlorate counter-ion 0.10 M saturated with tributylphosphate. Stationary phase: tributylphosphate on LiChrosorb RP-8 (10 μm). Samples dissolved in phosphate buffers 0.05 M, pH = 2.10 and $[ClO_4^-] = 0.10$ M (O—O) or $[ClO_4^-] = 0.50$ mol/l (O— —O).

CONCLUSIONS

The phase system described is flexible, which means that conditions can be varied easily to obtain the required retention and selectivity. The desired separation has been achieved by proper choice of the counter-ion concentration and pH, in conformity with the proposed retention model for ion-pair distribution. The separation of the compounds of interest is shown in Fig. 5. The least retained compound (adrenaline) has a capacity ratio of 2.4, and the most retained compound (α-methyldopamine) a capacity ratio of 17.5. The system is reproducible and no problems with respect to its stability have been observed. The phase system is in use now for the determination of α-methyldopa and its metabolites after their isolation, and more than 400 biogenic samples have been run without affecting the chromatographic properties of the system. The lifetime of the support material under the conditions described is at least 6 months.
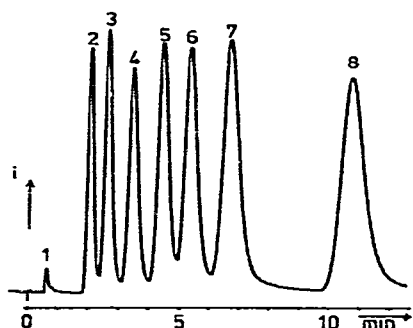
Fig. 5. Separation of a test mixture of the compounds of interest. Mobile phase: phosphate buffer 0.05 M, pH = 2.19 with perchlorate counter-ion 0.10 M saturated with tributylphosphate. Stationary phase: tributylphosphate on LiChrosorb RP-8 (10 μm). Compounds (100–200 ng injected): 1 = front peak; 2 = adrenaline; 3 = noradrenaline; 4 = DOPA; 5 = α-methylnoradrenaline; 6 = dopamine; 7 = α-methyldopa; 8 = α-methyldopamine. Flow-rate, 0.50 ml/min; pressure, 7.5 MPa; detection, amperometric at +0.75 V vs. saturated calomel electrode; sensitivity, 500 nA full-scale deflection.

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