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AUTOMATED CHROMATOGRAPHY OF AROMATIC ACIDS, ALDEHYDES AND ALCOHOLS WITH AN AMINO ACID ANALYZER

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SUMMARY

Chromatographic separations of a wide range of aromatic compounds (acids, aldehydes, alcohols) have been performed on a spherical cation-exchange resin with a sodium citrate–boric acid buffer, pH 4.53. The organic compounds were automatically determined in the eluate by measuring their light absorption at 280 mμ or their fluorescence at 315 mμ. The method is applicable to the determination of phenolic metabolites from tyrosine, DOPA, catecholamines or tryptophan in biological fluids and tissues.

INTRODUCTION

While organic acids are commonly separated by anion-exchange chromatography[1–9], we have recently demonstrated that acidic and neutral catabolites from dopamine or noradrenalin can be fractionated rapidly by cation-exchange chromatography[10]. This analysis has been performed on a spherical cation-exchange resin, which is used in commercial amino acid analyzers. The catecholamine metabolites were determined automatically in the eluate by measuring their light absorption at 280 mμ. This paper demonstrates that a wide range of aromatic acids, aldehydes and alcohols can be separated in a like manner. Since the new method is very rapid and organic compounds are not destroyed during the column chromatography, it may be of practical importance in the analysis of metabolites from aromatic amino acids or drugs like levodopa.

EXPERIMENTAL

Materials

The sources of materials were as follows: Cation-exchange resin PA-28 (spherical particles of a sulfonated styrene-divinyl-benzene, diameter 16 ± 6 μ) from Beckman Instruments GmbH, Munich, G.F.R.

3,4-Dihydroxyphenylacetic acid; 3-methoxy-4-hydroxyphenylacetic acid; 3,4-dihydroxymandelic acid; 3-methoxy-4-hydroxymandelic acid; 4-hydroxymandelic

acid; kynurenic acid; 5-hydroxyindole-3-acetic acid; caffeic acid; ferulic acid; and 3,4-dimethoxybenzoic acid were from Calbiochem, Los Angeles, Calif., U.S.A. 2,5-Dihydroxyphenylacetic acid; 4-hydroxyphenylacetic acid; 2-methoxymandelic acid; 3-methoxymandelic acid; 3,4-dihydroxyphenylalanine; tyrosine; 5-hydroxytryptophane; xanthurenic acid; 5-hydroxyanthranilic acid; 3,4-dihydroxyphenylglycol; 3-methoxy-4-hydroxyphenylglycol were from Sigma Chemical Company, St. Louis, U.S.A. 3-Hydroxybenzoic acid; 4-hydroxybenzoic acid; 2,6-dihydroxybenzoic acid; 2,4-dihydroxybenzoic acid; 3,5-dihydroxybenzoic acid; 3,4-dihydroxybenzoic acid; and 3-methoxy-4-hydroxybenzoic acid from Fluka AG, Buchs, Switzerland. 3,4-Dimethoxyphenylacetic acid; 4-methoxyphenylacetic acid; 4-methoxymandelic acid were from EGA-Chemie, Heidenheim, G.F.R. 3,4-Dihydroxyphenylethanol and 3-methoxy-4-hydroxyphenylethanol were from A. Kistner AB Fack, Göteborg, Sweden. 3,4-Dihydroxybenzaldehyde; 3-hydroxy-4-methoxybenzaldehyde; 3-methoxy-4-hydroxybenzaldehyde, 2-hydroxybenzoic acid; and 4 methoxybenzyl alcohol were from Dr. Th. Schuchardt AG, Munich, G.F.R. *threo*-3,4-Dibenzoxy-dopaserin, and *erythro*-3,4-dibenzoxy-dopaserin were from Alderich Chemical Company, Milwaukee, U.S.A.

3-O-Methyldopa was kindly supplied by Dr. A. PLETSCHER, Hoffmann-La Roche AG, Basel, Switzerland. 3-Hydroxy-4-methoxybenzoic acid was a gift from Prof. H. THOMAS, the Biochemical Dept. of the University of Ulm, G.F.R.

Chromatography

All separations were performed in water jacketed glass columns (0.9 × 60 cm) of a modified amino acid analyzer of the Unichrom type (Beckman Instruments GmbH, Munich, G.F.R.). A schematic diagram of the chromatographic system is shown in Fig. 1. Columns were filled up to 55 cm with PA 28 resin equilibrated with buffer, pH 3.28. The aromatic compounds at levels of 60 μg to 600 μg, dissolved in 4 ml 0.4 *N* $HClO_4$, were forced into the resin with about 10 atm nitrogen pressure followed in a like manner by two 1 ml washes of buffer, pH 2.2. Then the head space above the resin was filled with buffer, pH 3.28, and elution was carried out as follows: buffer, pH 3.28, for 15 min; Na-citrate–boric acid buffer, pH 4.53, for 100–500 min; and finally with 0.2 *N* NaOH for 60 min. The flow rate was adjusted to 50 ml/h; the

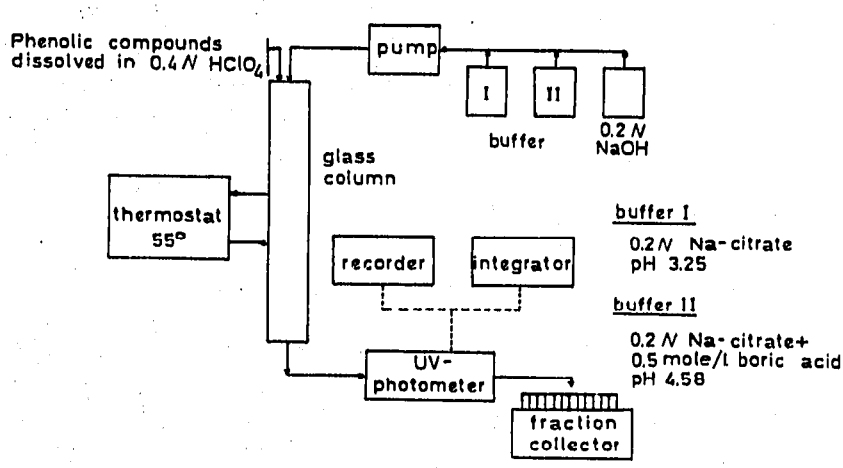

Fig. 1. Schematic diagram of the chromatographic system.

pressure varied between 15 and 20 atm, and the column temperature was 55°. The best separations of aromatic compounds were obtained with 50 mm liquid head space above the resin bed.

Analysis of the column effluent

The eluate from the column was passed through a quartz flow cell (optical path length 10 mm, volume 0.1 ml) of a UV-spectrophotometer (Photometer type DB, Beckman Instruments GmbH, Munich, G.F.R.), and the optical density was measured at 280 mμ. The output of the spectrophotometer was recorded on a laboratory potentiometric recorder and was fed into a digital integrator (Model 125, Beckman Instruments GmbH, Munich, G.F.R.) which automatically prints out the area under each peak. In some experiments the effluent of the flow cell was fractionated by an automatic fraction collector and the fluorescence of each fraction was determined. All fluorometric determinations were performed in an Aminco-Bowman spectrophotofluorometer.

Preparation of buffers

Buffer, pH 2.2. This consists of 19.6 g Na-citrate·$2H_2O$, 5 ml thiodiglycol, 1 g Brij-35, 1 ml *n*-caprylic acid, 850 ml distilled water and 19.6 ml HCl (37%, w/w). The solution was titrated with 6 *N* HCl to pH 2.2 and made up to 1 l.

Na-citrate buffer, pH 3.28. 392.2 g Na-citrate·$2H_2O$, 100 ml thiodiglycol, 20 g Brij-35, 2 ml *n*-caprylic acid, 18 l distilled water and 246.5 ml HCl (37%, w/w) were all mixed together and the solution was adjusted to pH 3.28 and made up to 20 l.

Na-citrate–boric acid buffer, pH 4.53. This is the same as for buffer, pH 3.28, but with the addition of 167.5 ml HCl (37%, w/w) and 600 g boric acid and adjustment to pH 4.53.

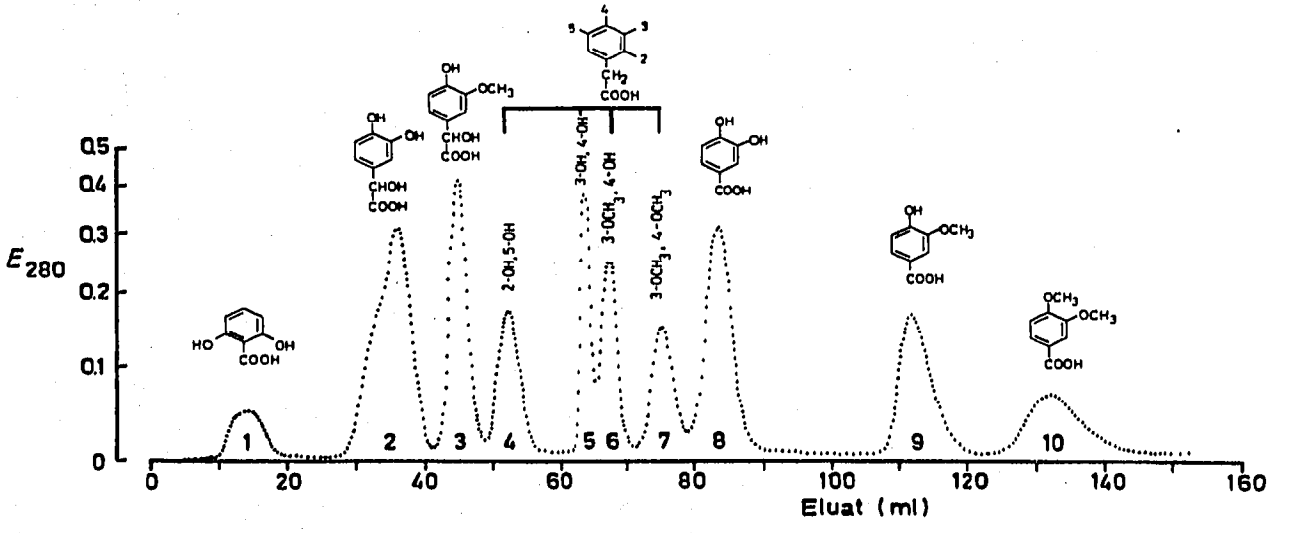

Fig. 2. Separation of ten phenolic acids by cation exchange chromatography. Conditions: column, 0.9 × 60 cm; resin, 16 ± 6 μ PA-28; eluent, 0–15 min Na citrate buffer, pH 3.28, 15–150 min Na-citrate–boric acid buffer, pH 4.53; flow rate, 50 ml/h; pressure 15–20 atm; column temperature, 55°. 1 = 2,6-Dihydroxybenzoic acid (200 μg); 2 = 3,4-dihydroxymandelic acid (600 μg); 3 = 3-methoxy-4-hydroxymandelic acid (400 μg); 4 = homogentisic acid (200 μg); 5 = homoprotocatechuic acid (200 μg); 6 = homovanillic acid (200 μg); 7 = homoveratric acid (200 μg); 8 = protocatechuic acid (200 μg); 9 = vanillic acid (200 μg); 10 = veratric acid (200 μg).

All water used for buffer formulation was deionized on a mixed bed resin and distilled in a quartz double distillation apparatus.

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram obtained in the analysis of the mixture of ten phenolic acids. This mixture, containing 200–600 μg of each acid, was separated in about 3 h. 2,6-Dihydroxybenzoic acid, a rather strong acid (dissociation constant 5.0×10^{-2}), was eluted first, then the mandelic acids—3,4-dihydroxymandelic acid and 3-methoxy-4-hydroxymandelic acid. Phenolic derivatives of phenylacetic acid were in general eluted ahead of the corresponding benzoic acids. Even related compounds, from the chemical point of view, like 2,5-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid or vanillic acid and isovanillic acid (11 and 12 in Fig. 5), are well separated. A typical example of the fractionation of phenol alcohols and phenol aldehydes is shown in Fig. 3. Aldehydes are retained for longer by the resin than alcohols.

The elution times of fifty aromatic compounds are reported in Table I. It

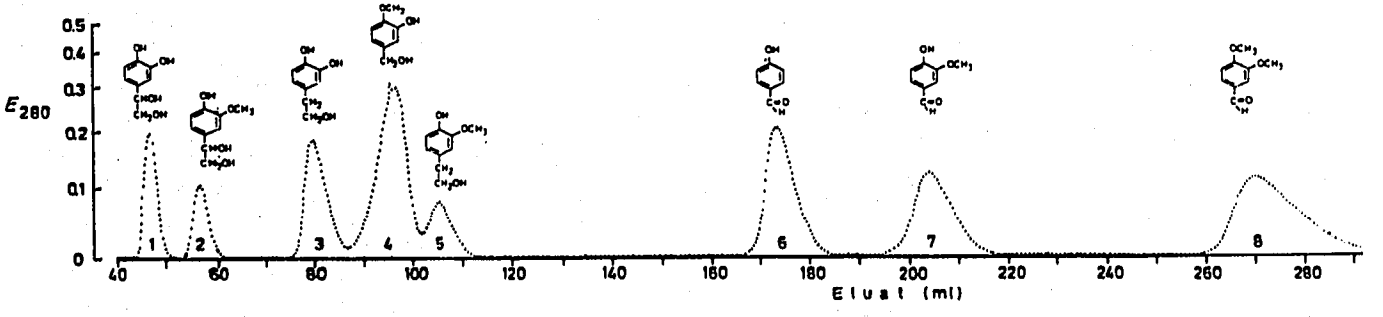

Fig. 3. Separation of five phenolic alcohols and three phenolic aldehydes by cation exchange chromatography. For conditions see Fig. 2. 1 = 3,4-Dihydroxyphenylglycol (200 μg); 2 = 3-methoxy-4-hydroxyphenylglycol (200 μg); 3 = 3,4-dihydroxyphenylethanol (200 μg); 4 = 3-hydroxy-4-methoxybenzyl alcohol (100 μg); 5 = 3-methoxy-4-hydroxyphenylethanol (200 μg); 6 = 4-hydroxybenzaldehyde (60 μg); 7 = vanillin (100 μg); 8 = veratraldehyde (200 μg).

would seem that more compounds may be separated by our chromatographic system than listed in this table, since Table I contains only those substances related to our biochemical problems and detectable by UV absorption. It can be seen from Table I that some relationship between the chemical structure of a compound and its elution time can be demonstrated. The different retention times of the various hydroxybenzoic acids are good examples of the influence of the position of the phenolic hydroxyl group on the elution pattern. In monohydroxybenzoic acids retention time increases in the sequence 2-OH < 3-OH < 4-OH. This order is still preserved in dihydroxybenzoic acids and in methoxy-hydroxybenzoic acids. In accordance with this, the retention time of 3-hydroxy-4-methoxybenzoic acid (isovanillic acid) is significantly shorter than that of 4-hydroxy-3-methoxybenzoic acid (vanillic acid).

Dissociation constants taken from a handbook[11] are indicated in Table I for some compounds, since we presumed a relationship between the dissociation and retention on the PA-28 column. A vague relationship of this kind can be seen in the group of the hydroxybenzoic acids. As a rule weak acids are retained by the column

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ELUTION TIME OF VARIOUS AROMATIC COMPOUNDS

For chromatographic conditions see Fig. 2.

Compound	*Elution time (min)*	*Compound*	*Elution time (min)*	K_{diss}[a]
Hydroxyphenylacetic acids		Hydroxybenzoic acids		
2,5-Dihydroxy	70	2,6-Dihydroxy	20	5.0×10^{-3}
4-Hydroxy	78	2-Hydroxy	73	2.0×10^{-3}
3,4-Dihydroxy	80	2,4-Dihydroxy	74	5.2×10^{-4}
3-Methoxy-4-hydroxy	85	3,5-Dihydroxy	88	9.1×10^{-5}
3,4-Dimethoxy	93	3-Hydroxy	91	4.4×10^{-5}
4-Methoxy	98	3,4-Dihydroxy	106	3.3×10^{-5}
		4-Hydroxy	142	1.1×10^{-4}
Hydroxymandelic acids		3-Hydroxy-4-methoxy	138	3.2×10^{-5}
3,4-Dihydroxy	40	3-Methoxy-4-hydroxy	150	3.0×10^{-5}
3-Methoxy-4-hydroxy	60	3,4-Dimethoxy	160	3.6×10^{-5}
4-Hydroxy	62			
2-Methoxy	63	Hydroxybenzaldehydes		
3-Methoxy	68	3,4-Dihydroxy	115	2.8×10^{-8}
4-Methoxy	72	4-Hydroxy	214	2.2×10^{-9}
		3-Hydroxy-4-methoxy	245	
Amino acids		3-Methoxy-4-hydroxy	258	4.0×10^{-8}
threo-Dopaserine	68			
erythro-Dopaserine	72	Phenol alcohols		
DOPA	90	3,4-Dihydroxyphenylglycol	53	
Tyrosine	100	3-Methoxy-4-hydroxyphenyl glycol	66	
3-O-Methyldopa	117	3,4-Dihydroxyphenylethanol	93	
5-Hydroxytryptophane	198	3-Methoxy-4-hydroxyphenyl-ethanol	126	
Tryptophane	285	3-Hydroxy-4-methoxybenzyl-alcohol	101	
Other aromatic acids		4-methoxybenzylalcohol	140	
4-Hydroxyphenyllactic acid	67			
Xanthurenic acid	72			
Kynurenic acid	136			
5-Hydroxyindole-3-acetic acid	146			
5-Hydroxyanthranilic acid	158			
4-Hydroxyphenylpyruvic acid	209			
Caffeic acid	320			
Urocanic acid	346			
Ferulic acid	417			

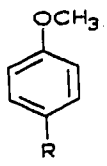
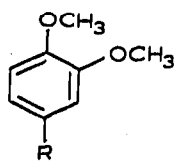
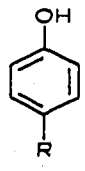
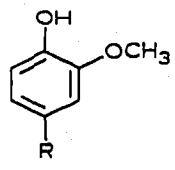
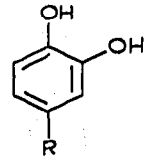
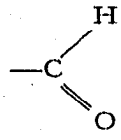
[a] Dissociation constants in aqueous solution at 20° or 25°. All values are taken from ref. 11.

for longer than stronger ones. But a comparison of aldehydes with the benzoic acids in Table I immediately demonstrates that dissociation cannot be of great importance for this kind of separation. The dissociation constants of aldehydes are much smaller than those of acids, for example, protocatechaldehyde has a K_{diss} of 2.8×10^{-8} and protocatechuic acid has a K_{diss} of 3.3×10^{-5}, while their retention times shown in Table I are rather similar, 115 min for protocatechaldehyde and 106 min for protocatechuic acid.

From Table I it is evident that those compounds which have particularly short retention times have several OH-groups in the benzene ring and several OH- or COOH-groups in the side chain. Table II is another illustration of this fact. In this table 3,4-dihydroxymandelic acid, which has two phenolic OH-groups in the benzene

TABLE II

INFLUENCE OF THE NUMBER OF HYDROXYL OR CARBOXYL GROUPS PER MOLECULE ON ITS RETENTION TIME ON A PA-28 COLUMN

OH or COOH groups in the side chain	*R*	*Aromatic OH-groups*				
		0		*1*		*2*
						
0		—	333	214	245	115
1	—COOH		160	142	146	106
	—CH_2—CH(—NH_2)—COOH	—	—	100	117	90
	—CH_2—CH_2OH	—	—	—	126	93
	—CH_2—COOH	98	93	78	85	80
	—CH(OH)—CH(NH_2)—COOH	—	—	—	—	68 (*threo-*) 72 (*erythro-*)
	—CH(OH)—CH_2OH	—	—		66	53
2	—CH(OH)—COOH	72	—	62	60	40
	—CH_2—CH(OH)—COOH	—	—	67	—	—

ring and one carboxylic group and one OH-group in the side chain, has the shortest retention time (40 min). 3,4-Dimethoxybenzaldehyde, which contains neither free phenolic OH-groups nor OH- or COOH-groups in the side chain, has the longest elution time. Thus, in general our chromatographic system seems to separate aromatic compounds according to their polarity.

The content of boric acid in the buffer is not critical, since many compounds listed in Table I can be separated by the Na citrate buffer, pH 4.53, even without boric acid.

Many substances, the separation of which is described in this paper, are rather unstable and they may be destroyed during the chromatographic separation. Therefore the percentage recovery was measured for the following compounds[10]: 3,4-dihydroxymandelic acid, 92%; 3-methoxy-4-hydroxymandelic acid, 100%; 3,4-dihydroxyphenylglycol, 83%; 3-methoxy-4-hydroxyphenylglycol, 97%; 3,4-dihydroxy-

phenylacetic acid, 90%; 3-methoxy-4-hydroxyphenylacetic acid, 100%; 3,4-dihydroxyphenylethanol, 100%; and 3-methoxy-4-hydroxyphenylethanol, 88%. Thus, from a practical point of view this chromatographic system has a recovery of around 100%.

Between 60 and 600 μg of each compound were separated in the chromatograms shown in Figs. 2 and 3. This rather high quantity is necessary as long as a UV flow cell is used as a detector. Much smaller quantities of each compound are sufficient, if their fluorescence is measured in the eluate. Aromatic compounds which contain mobile (π) electrons are normally fluorescent. Fluorescence of benzene derivatives depends on the freedom of the π-electrons. Hydroxylic groups or methoxylic groups enhance benzene fluorescence, carboxylic groups diminish it[12]. An example of the detection of aromatic compounds in the eluate by fluorescence measurements is given in Fig. 4. In this case 10–30 μg of each compound was applied on the column and the fluorescence of 1.5 ml fractions of the eluate was measured in a spectrofluorometer.

The chromatographic system described in this paper was initially developed to separate acidic and neutral metabolites of catecholamines. Fig. 5 gives a typical example of this kind of application. It demonstrates that the catecholamine precursor DOPA can easily be separated from all its neutral and acidic biological metabolites.

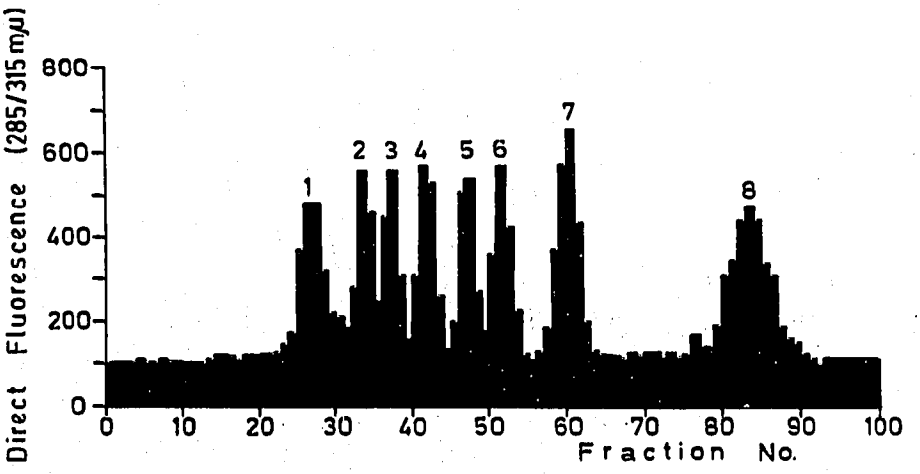


Fig. 4. Determination of aromatic compounds in PA-28 column eluate by measurement of the fluorescence at 315 mμ. Activation at 280 mμ: for chromatographic conditions see Fig. 2. 1 = 3,4-Dihydroxymandelic acid (30 μg); 2 = 3,4-dihydroxyphenylglycol (30 μg); 3 = 3-methoxy-4-hydroxymandelic acid (10 μg); 4 = 3-methoxy-4-hydroxyphenylglycol (10 μg); 5 = 3,4-dihydroxyphenylacetic acid (20 μg); 6 = 3-methoxy-4-hydroxyphenylacetic acid (10 μg); 7 = 3,4-dihydroxyphenylethanol (30 μg); 8 = 3-methoxy-4-hydroxyphenylethanol (10 μg).

This separation may even be of some practical interest, since BIRKMAYER AND HORNYKIEWICZ[13] as well as BARBEAU *et al.*[14] and more recently COTZIAS *et al.*[15] have demonstrated that Parkinson's disease can efficiently be treated with DOPA. Thus, DOPA metabolism in mammals was reinvestigated by several groups[16,17]. All these investigations suffered from the lack of efficient analytical methods for the separation of neutral and acidic metabolites from DOPA.

From a practical point of view another advantage of our separation procedure

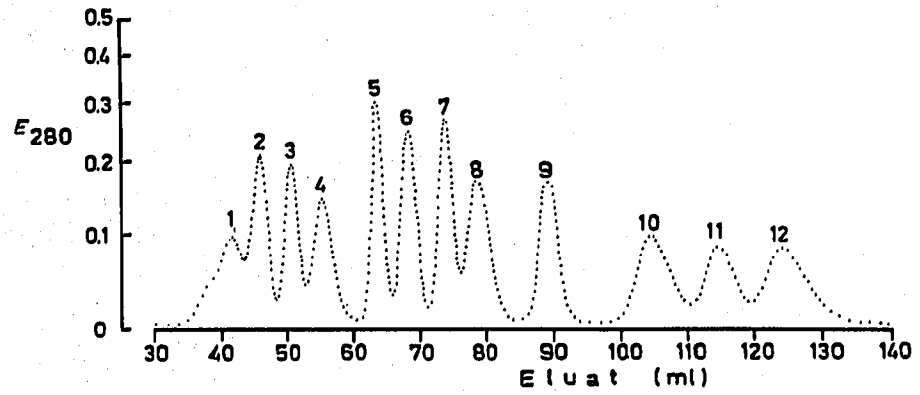


Fig. 5. Separation of twelve acidic and neutral metabolites from DOPA. For conditions see Fig. 2. 1 = 3,4-dihydroxymandelic acid (200 μg); 2 = 3,4-dihydroxyphenylglycol (200 μg); 3 = 3-methoxy-4-hydroxymandelic acid (200 μg); 4 = 3-methoxy-4-hydroxyphenylglycol (400 μg); 5 = 3,4-dihydroxyphenylacetic acid (200 μg); 6 = 3-methoxy-4-hydroxyphenylacetic acid (200 μg); 7 = 3,4-dihydroxyphenylethanol (200 μg); 8 = DOPA (200 μg); 9 = 3-O-methyldopa (200 μg); 10 = 3-methoxy-4-hydroxyphenylethanol (200 μg); 11 = isovanillic acid (400 μg); 12 = vanillic acid (100 μg).

is worth mentioning. In experiments on catecholamine metabolism metabolites are generally extracted from tissues by 0.4 N $HClO_4$. These perchloric acid extracts can be analyzed immediately without further treatment with our system.

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