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Separation and enantioseparation of derivatized amino acids and biogenic amines by high-performance liquid chromatography with reversed and chiral stationary phases

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Abstract

This study demonstrates that an amino-β-cyclodextrin-bonded phase column exhibits enantioselectivity for various amino acid derivatives. Mixtures of methanol, acetonitrile, tetrahydrofuran or dioxan and triethylamine buffers (pH 4.0–7.0) were used as mobile phases. The effect of the mobile phase on the resolution process was studied by varying the mobile phase composition (type and percentage of organic modifiers, pH, and ionic strength of the buffer solution). The 1-octanol–water partition coefficients are calculated and tabulated for 16 derivatized amino acids. The chromatographic data for 42 pairs of derivatized amino acids resolved on the amino-β-cyclodextrin-bonded phase are summarised. The separation of adrenaline, noradrenaline and amphetamine on a novel vancomycin stationary phase is demonstrated.

Keywords: Enantiomer separation; Mobile phase composition; Octanol–water partition coefficients; Amino acids; Amines

1. Introduction

Amino acids and some amines and aminoalcohols are biologically active compounds. These analytes play an important role in living systems. Most amino acids are chiral. Separation of chiral amino acids is an analytical challenge of great relevance in many scientific fields, such as pharmaceutics, the study of food processes in human body, amino acid biochemistry, proteins and related areas of research, asymmetric syntheses in organic chemistry and the dating of archaeological materials [1–3]. Although previously only natural, L-series acids were available, D-amino acids are now becoming increasingly available as a result of being required as component materials of industrial interest [4].

Cyclodextrin-bonded stationary phases developed by Armstrong and Li [5] were successfully used for the enantiomeric resolution of amino acids. Cyclodextrins (CDs) are oligosaccharides, in which glucose units are joined together to form a toroidal structure with a hydrophobic cavity and hydrophilic exterior faces.

The main property of CDs, which allows them to influence chiral separation is their ability to form enantioselective inclusion complexes with guest molecules. It is believed that chiral recognition is caused by inclusion complex formation between the cavity of the CD and the hydrophobic moiety of the solute and by hydrogen bonding between the polar function groups of the solute in the vicinity of its chiral centre and hydroxyl groups of the CD [5,6].

Derivatizing CDs change their physicochemical properties as well as completion behaviour. Changing the length and type of the spacer arm of surface

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CDs could change the separation factor for some separations. Using amine or amide-bonded CDs is advantageous in separating several aromatic acid enantiomers [7,8]. This may be due to an additional interaction between the carboxylic acid groups of the enantiomeric acid and the basic nitrogen of the spacer arm.

Amino acids generally cannot be resolved using the native CD stationary phases. In this work, we report the new chiral stationary phase, amino-β-cyclodextrin, for resolving various derivatized amino acids. The chromatographic behaviour and the resolution of *N*-CBZ-, *N*-benzoyl-, dansyl-, FMOC-, *N*-phthalyl-, 2,4-DNP-, 3,5-DNPyr-, *N-tert.*-BOC-, OPA-amino acids on the amino-β-cyclodextrin column are demonstrated and discussed. The structures of substituents of amino acids are presented in Table 1. The structure of amino-β-cyclodextrin is shown in Fig. 1.

Retention and enantioseparation of analytes on CD chiral stationary phases depend on the hydrophobicity of the compounds studied. In this paper, various derivatized amino acids are compared for chiral separation as a function of their hydrophobicity.

Biogenic amines and aminoalcohols have a number of important functions in the control mechanism of living systems, and their activities are interesting not only when they are considered as nutrients, but also from the view point of overall biological activity. Some biologically active amines and aminoalcohols such as adrenaline, noradrenaline, ephedrine, and amphetamine are racemic compounds. It is very important to determine and separate this class of compound and their enantiomers.

2. Experimental

2.1. Chemicals and methods

Amino acids and other compounds were obtained from Sigma (St Louis, MO, USA) or Aldrich (Milwaukee, WI, USA). HPLC-grade methanol (MeOH), acetonitrile (ACN), 2-propanol (2-Pr), and ethanol (EtOH) were purchased from Fisher (Tustin, CA, USA). Water was deionized by passing distilled water through a Barnstead water purification system. OPA-amino acids were produced by derivatization with *o*-phthalaldehyde (OPA) and 2-mercaptoethanol (2-ME) according to a procedure described previously [9].

2.2. Instruments

A Shimadzu LC-10A chromatograph (Shimadzu, Kyoto, Japan) was used in this study. A variable-wavelength detector, Model SPD-10AV (Shimadzu), was also used and interfaced with a CR601 Chromatopac (Shimadzu) data system. All derivatized amino acids were detected at 254 nm.

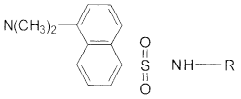
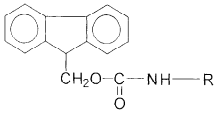
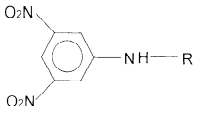
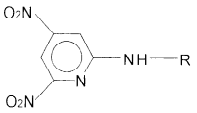
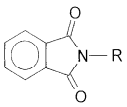
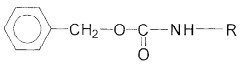
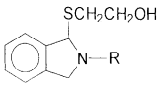
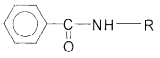
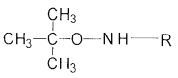
An amino-β-cyclodextrin column (250×4.6 mm I.D., 10-μm particle diameter) was obtained from the Centre of Bioengineering (Moscow, Russia). Another two columns (250×4.6 mm I.D., 5-μm particle diameter) were obtained from Advanced Separation Technologies (Whippanny, NJ, USA). The first was named ''Chirobiotic T'' and the second ''Chirobiotic V''. Another column (100×3.0 mm I.D., 10-μm particle diameter) packed with Silasorb C_{18} (Lachema, Neratovice, Czech Republic) was produced in our laboratory.

3. Results and discussion

From our experience of working with a β-CD bonded phase column, no enantiomers of underivatized amino acids can be separated with a β-CD column. It is believed that the size of an unsubstituted amino acid is too small to bind tightly with the CD cavity to form a strong inclusion complex [10–12], which is a prerequisite for chiral recognition.

The 42 pairs of derivatized amino acids were resolved on the amino-β-cyclodextrin bonded phase. The chromatographic data are summarised in Table 2. It was found that the L-enantiomer is eluted first. Mixtures of methanol, acetonitrile, tetrahydrofuran or dioxan and triethylamine buffers (pH 4.0–7.0) were used as mobile phases. The effect of the mobile phase on the resolution process was studied by varying the mobile phase composition (type and percentage of organic modifiers, pH and ionic

Table 1
The substituents of amino acids

Structure	Legend
	Dansyl
	FMOC
	3,5-DNP
	2,4-DNPyr
	*N*-Phthalyl
	*N*-CBZ
	OPA
	*N*-Benzoyl
	*N-tert.*-BOC

R, amino acid.

strength of buffer). In all cases, the operating conditions were: temperature ~22 °C, flow-rate of 1 ml/min, and a UV detector wavelength of 254 or 230 nm. All compounds had analogous retention behaviour when methanol was used as the organic modifier.

3.1. The influence of partition coefficient of derivatized amino acids (1-octanol–water) on retention and chiral recognition on amino-β-cyclodextrin

Hydrophobicity of organic compounds is probably

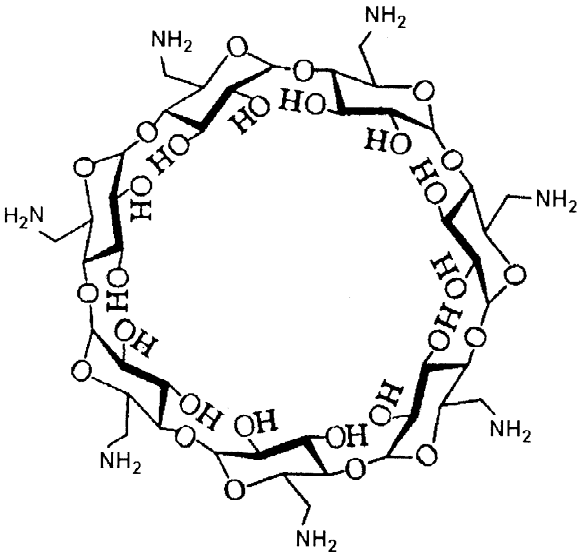


Fig. 1. Structure of amino-β-cyclodextrin.

one of the most informative physicochemical properties in chemistry and widely used in analysis of quantitative structure–activity relationships (QSARs) for pharmaceutical, environmental, and biochemical applications [13]. The partition coefficient, defined for dilute solutions as the molar concentration ratio ($P=C_o/C_w$) for a single species between the organic and aqueous phases at equilibrium, is a useful parameter for presenting the hydrophobicity of a substance [14]. The log *P* value for the 1-octanol–water system is widely used as a structure descriptor in QSARs.

Many methods for estimating log *P*, experimental as well as computational, are described in the literature [13]. Most computational techniques are ''fragment constant'' methods, in which a structure is divided into previously defined fragments, and the corresponding contributions are summed together to yield the final log *P* estimate. In this paper, we used the Advanced Chemistry Development, 2001 (ACD/LogD version 4.5) computer program. Log *P* is a partition coefficient for a neutral molecule between the organic and aqueous phases, but on the estimation log *P* for large organic molecules, such as derivatized amino acids, the form of the compound under certain pH conditions (log *D*) should be taken into consideration. Log *P* and log *D* (pH 4.0) for some derivatized amino acids in the 1-octanol–water system are listed in Tables 3 and 4.

This is not surprising since it had been previously reported that the majority of studied substituents could tightly bind to the β-CD cavity and interact with aminogroups of the β-CD cavity. The relative retention factors of *N*-CBZ-amino acids on amino-β-cyclodextrin are:

N-CBZ-alanine (1.41*) < *N*-CBZ-valine (2.29*) < *N*-CBZ-methionine (2.36*) < *N*-CBZ-leucine (2.82*) < *N*-CBZ-phenylalanine (3.20*) < *N*-CBZ-tyrosine (2.51*) < *N*-CBZ-tryptophan (3.19*)

where values with asterisks are the calculated partition coefficients under pH 4.0 conditions (log *D*).

The data obtained show that the solute retention depends on its hydrophobicity. The strong retention of *N*-CBZ-tyrosine is due to the hydroxyl group in the benzene ring of the analyte, which interacts with the aminogroup of the β-CD cavity. The results of this study show that the various substituents of amino acids play an important role in retention and chiral recognition. The relative retention factors of derivatized valines on amino-β-cyclodextrin are:

N-tert.-BOC-valine (−0.16*) < OPA-valine (0.21*) < *N*-benzoyl-valine (1.06*) < *N*-phthalyl-valine (1.90*) < 2,4-DNP-valine (2.15*) < 3,5-DNPyr-valine (0.99*) < *N*-CBZ-valine (2.29*) < dansyl-valine (2.60*) < FMOC-valine (4.06*)

A comparison of the retention and partition coefficients of derivatized valines indicates that the analyte retention depends on its hydrophobicity. The introduction of a 3,5-DNPyr-substituent into the amino acid molecule provides a strong binding site due to a second nitrogen in the aromatic ring, but log *D* is not high. As can be seen from Table 3, the enantio-recognition ability of the amino-β-cyclodextrin column is higher for *N*-CBZ-, dansyl-, OPA- and *N-tert*.-BOC-amino acids. Fig. 2 shows the enantioseparation of dansyl-DL-leucine.

The results obtained in this study show that the

Table 2
Retention factor (k') and separation factor (α) of some derivatized amino acid enantiomers on amino-β-cyclodextrin chiral stationary phase

Analyte	k[a]	α	Mobile phase
N-CBZ-DL-alanine	5.7	1.13	MeOH–1% TEAA[b] buffer pH 4.0 (10:90)
N-CBZ-DL-methionine	8.4	1.16	
N-CBZ-DL-norleucine	6.9	1.18	
N-CBZ-DL-leucine	9.3	1.26	
N-CBZ-DL-valine	6.2	1.17	
N-Benzoyl-DL-valine	3.9	1.13	
N-Benzoyl-DL-phenylalanine	7.4	1.10	
Dansyl-DL-norleucine	12.8	1.27	
N-CBZ-DL-phenylalanine	6.2	1.12	MeOH–1% TEAA buffer pH 4.0 (20:80)
N-CBZ-DL-tyrosine	7.5	1.10	
N-CBZ-DL-tryptophan	10.0	1.17	
N-Phthalyl-DL-valine	5.9	1.13	
3,5-DNP-DL-valine	5.0	1.08	
3,5-DNP-DL-norvaline	9.0	1.10	
3,5-DNP-DL-methionine	10.2	1.10	
3,5-DNP-DL-ethionine	12.1	1.08	
3,5-DNP-DL-norleucine	9.6	1.08	
3,5-DNP-DL-citruline	6.9	1.10	
2,4-DNPyr-valine	5.5	1.02	
2,4-DNPyr-DL-leucine	4.7	1.16	
Dansyl-DL-leucine	12.2	1.65	MeOH–1% TEAA buffer pH 4.0 (30:70)
Dansyl-DL-norvaline	6.3	1.12	
Dansyl-DL-valine	8.8	1.27	
Dansyl-DL-phenylalanine	14.9	1.09	
FMOC-DL-valine	5.2	1.18	
FMOC-DL-leucine	5.4	1.09	
N-tert.-BOC-DL-tryptophan	9.5	1.12	MeOH–1% TEAA buffer pH 4.0 (5:95)
N-tert.-BOC-DL-phenylalanine	6.2	1.24	
N-tert.-BOC-methyl-DL-phenylalanine	4.7	1.18	
N-tert.-BOC-DL-valine	3.7	1.20	
N-tert.-BOC-DL-alanine	4.2	1.44	
N-tert.-BOC-methyl-DL-alanine	3.0	1.37	
N-tert.-BOC-phenyl-DL-alanine	6.0	1.22	
OPA-DL-valine	3.8	1.21	MeOH–1% TEAA buffer pH 7.0 (10:90)
OPA-DL-leucine	5.8	1.23	
OPA-DL-isoleucine	8.1	1.32	
OPA-DL-norleucine	4.9	1.08	
OPA-DL-threonine	1.9	1.19	
OPA-DL-aspartic acid	7.6	1.20	
OPA-DL-glutamic acid	8.4	1.18	
OPA-DL-phenylalanine	4.2	1.37	MeOH–1% TEAA buffer pH 7.0 (20:80)
OPA-DL-tyrosine	7.0	1.50	

[a] Retention factor of the first eluted enantiomer.
[b] 1% triethylamine acetate.

Table 3
The calculated partition coefficients of *N*-CBZ-amino acids for the 1-octanol–water system

Analyte	Log P^a	Log D^b
N-CBZ-DL-alanine	1.71±0.55	1.41
N-CBZ-DL-valine	2.59±0.55	2.29
N-CBZ-DL-methionine	2.77±0.59	2.36
N-CBZ-DL-tyrosine	2.84±0.56	2.51
N-CBZ-DL-leucine	3.12±0.55	2.82
N-CBZ-DL-tryptophan	3.50±0.57	3.19
N-CBZ-DL-phenylalanine	3.58±0.56	3.20

[a] The partition coefficients for the neutral molecule.
[b] The partition coefficients under pH 4.0 conditions.

Table 4
The calculated partition coefficients of derivatized DL-valines for the 1-octanol–water system

Analyte	Log P^a	Log D^b
2,4-DNPyr-DL-valine	1.52±0.39	0.99
N-Benzoyl-DL-valine	1.54±0.54	1.06
N-tert.-BOC-DL-valine	2.20±0.52	−0.16
N-phthalyl-DL-valine	2.42±0.32	1.90
3,5-DNP-DL-valine	2.56±0.38	2.15
N-CBZ-DL-valine	2.59±0.55	2.29
OPA-DL-valine	2.60±0.53	0.21
Dansyl-DL-valine	3.52±0.41	2.60
FMOC-DL-valine	4.42±0.41	4.06

[a] The partition coefficients for the neutral molecule.
[b] The partition coefficients under pH 4.0 conditions.

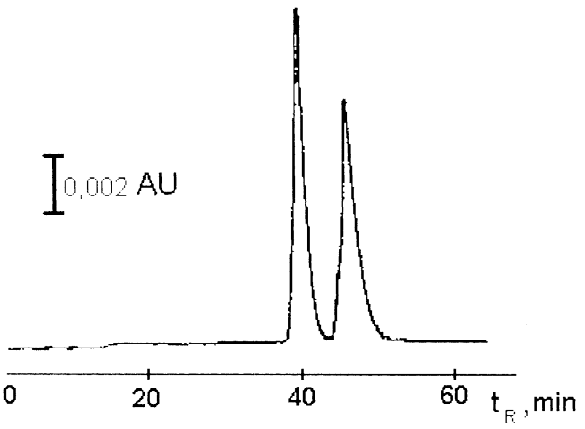


Fig. 2. Chromatogram of the enantiomeric separation of dansyl-DL-leucine. Mobile phase: MeOH–TEAA (1%, pH 4.0) (30:70, v/v), flow-rate, 1 ml/min. Column: 25 cm×0.46 cm I.D., amino-β-cyclodextrin. Injection volume: 20 μl; UV detection at 254 nm.

retention of solutes increases with log *D*, and enantioselectivity depends on the type of the substituent. It seems that the oxygen in the substituent structure around the chiral center plays an important role in hydrogen bonding; oxygen interacts with the protonated amino group at the surface of the amino-β-cyclodextrin column. It is true for *N*-CBZ-, dansyl- and *N-tert.*-BOC-amino acids.

It was found that the effect of the amino groups of amino-β-cyclodextrin resolves some non-aromatic derivatized amino acids—*N-tert.*-BOC-DL-valine, *N-tert.*-BOC-DL-alanine, *N-tert.*-BOC-methyl-DL-alanine. In fact, there are relatively few reports on the chromatographic resolution of *N-tert.*-BOC-amino acids on CD chiral stationary phases.

3.2. Determination and separation of biogenic amines and amino alcohols

Determination of biogenic amines in human blood serum is a difficult problem in analytical chemistry [15,16]. Creatinine is an indicator of different diseases; serotonine and histamine are pharmacologically active compounds and affect blood vessels and blood pressure. Histamine supervises an allergic mood of organisms.

Some biologically active compounds such as adrenaline, noradrenaline, ephedrine, and amphetamine exist as enantiomers. Ephedrine, adrenaline, and noradrenaline are stimulants and affect α- and β-adrenoreceptors of the cardiovascular system. L-Isomers of adrenaline and ephedrine are more active than D-isomers. Amphetamine is a stimulant of the central nervous system.

A procedure for the separation and determination of serotonine, creatinine, and histamine was developed by reversed-phase high-performance liquid chromatography in an isocratic-elution mode using UV detection. Retention of biogenic amines with different additives (H_3PO_4, CCl_3COOH, $NaClO_4$) in the mobile phase on Separon RP18 was studied. Table 5 shows the effect of the additives on selectivity and resolution. The separation of the biogenic amines is shown in Fig. 3.

The current interest in the resolution of optical isomers is due to the growing awareness of the role that the configuration plays in biological activity.

Table 5
Chromatographic parameters of biogenic amines on Separon RP18

Compound	k^a	α	R_s	Mobile phase
Creatinine	0	–	–	
Serotonine	0	–	–	ACN–water (40:60)
Histamine	–	–	–	
Creatinine	1.1			ACN–water–CCl_3COOH
Serotonine	1.7	1.4	1.2	(40:60:0.05)
Histamine	5.3	2.8	1.8	
Creatinine	1.9			ACN–water–CCl_3COOH
Serotonine	2.7	1.3	1.5	(40:60:0.1)
Histamine	6.6	2.1	2.3	
Creatinine	1.4			ACN–water–CCl_3COOH
Serotonine	2.3	1.5	1.5	(40:60:0.2)
Histamine	10.4	4.0	2.7	
Creatinine	2.0			ACN–water–H_3PO_4
Serotonine	2.3	1.2	0.8	(40:60:0.5)
Histamine	15.1	5.7	2.9	
Creatinine	2.7			ACN–water–$NaClO_4$
Serotonine	3.3	1.2	0.7	(40:60:0.1)
Histamine	6.6	1.9	1.4	

[a] Retention factor of the first eluted enantiomer.

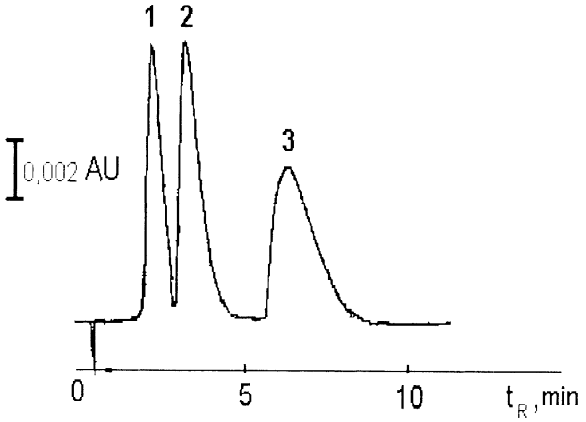


Fig. 3. Reversed-phase separation of biogenic amines: creatinine (1), serotonine (2), histamine (3). Mobile phase:, ACN–water–CCl_3COOH (40:60:0.1, v/v), flow-rate, 0.5 ml/min. Column: 10 cm×0.3 cm Separon RP18 (10-μm silica gel). Injection volume: 20 μl; UV detection at 254 nm.

Although chiral separations may be achieved by derivatizing the enantiomers with optically pure derivatizing agents, much of the work in chiral separations has been directed to the development of new chiral stationary phases [17].

The newest class of chiral selectors for liquid chromatography is the macrocyclic glycopeptides such as teicoplanin and vancomycin. Teicoplanin contains 23 chiral centers surrounding four pockets or cavities. Hydrogen donor and acceptor sites are readily available close to seven aromatic rings [3,18]. Vancomycin contains 18 chiral centers surrounding three cavities. Five aromatic ring structures bridge these strategic cavities [18].

The conditions for the reversed-phase HPLC separation of adrenaline and noradrenaline enantiomers on the teicoplanin chiral stationary phase using isocratic elution and UV detection were determined. Selectivity and optimization of reversed-phase separations are accomplished by controlling the amount of organic modifier to adjust the retention and type of buffer (TEAA, phosphate, acetate, and ammonium nitrate) and pH (3.5–7.0) to control selectivity. A variety of organic modifiers have been tested for their selectivity (MeOH, ACN, EtOH, 2-Pr).

Table 6 shows the retention, enantioselectivity, and enantioresolution of adrenaline, noradrenaline and ephedrine on a teicoplanin CSP (''Chirobiotic T''). Fig. 4 shows the enantioresolution of adrenaline.

The separation of adrenaline, noradrenaline and amphetamine on vancomycin stationary phase is demonstrated in Fig. 5. The vancomycin chiral

Table 6
Chromatographic parameters of enantiomers of biogenic amines on teicoplanin CSP

Compound	k^a	α	R_s	Mobile phase
Adrenaline	5.4	1.8	2.0	EtOH–water (50:50)
Noradrenaline	5.2	1.7	1.9	
Ephedrine	0.7	1.2	1.2	MeOH–1% TEAA[b] buffer pH 4.5 (95:5)

[a] Retention factor of the first eluted enantiomer.
[b] 1% triethylamine acetate.

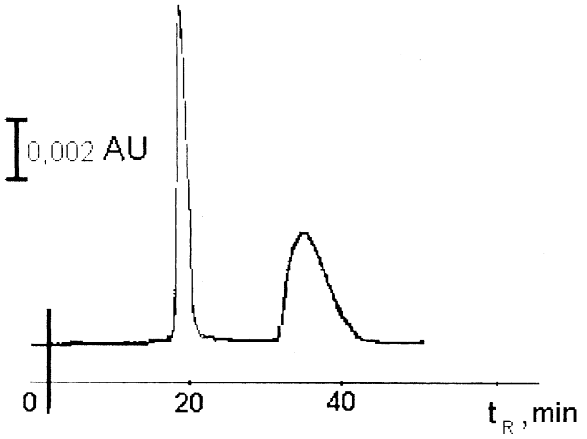


Fig. 4. Reversed-phase enantioseparation of adrenaline. Mobile phase: EtOH–water (50:50, v/v), flow-rate, 1.0 ml/min. Column: 25 cm×0.46 cm teicoplanin CSP (''Chirobiotic T''). Injection volume: 20 μl; UV detection at 230 nm.

stationary phase was used for enantioseparation of amphetamine in a polar-organic mode. Unfortunately, ephedrine and amphetamine enantiomers were not separated on this column with baseline resolution.

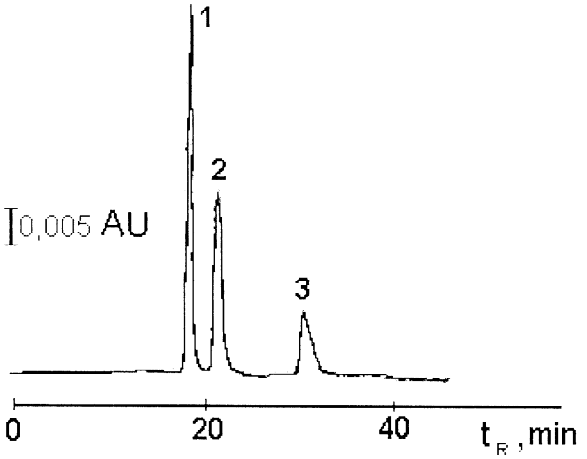


Fig. 5. Reversed-phase separation of noradrenaline (1), adrenaline (2) and amphetamine (3). Mobile phase: MeOH–TEAA (0.2%, pH 4.5) (20:80, v/v), flow-rate, 1 ml/min. Column: 25 cm×0.46 cm I.D., vancomycin CSP (''Chirobiotic V''). Injection volume: 20 μl; UV detection at 254 nm.

4. Conclusions

It has been demonstrated that the amino-β-cyclodextrin-bonded phase column exhibits enantioselectivity for the *N*-CBZ-, *N*-benzoyl-, dansyl-, FMOC-, *N*-phthalyl-, 2,4-DNP-, 3,5-DNPyr-, *N-tert.*-BOC-, and OPA-amino acid derivatives. The effect of the partition coefficients of analytes and the structural features of substituents of amino acids on the retention and resolution suggests that the inclusion complex formation between the CD cavity and the substituent, and the hydrogen bonding of oxygen around the chiral center of the solutes are the important factors in chiral recognition.

The separation of biogenic amines and amino alcohols and some its enantiomers are presented. In the polar-organic mode, enantiomers of the compounds studied are easily resolved on macrocyclic antibiotics ''Chirobiotic T'' and ''Chirobiotic V''.

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