Liquid Chromatography in the Analysis of Neurotransmitters and Alkaloids

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Abstract

Several thin-layer chromatographic (TLC) and high-performance liquid chromatographic (HPLC) systems for the determination of biogenic amine neurotransmitters, alkaloids, and the metabolites of these two groups of compounds are developed. The possibility of the interference of these compounds is investigated. In all the systems developed for neurotransmitters, the retention of alkaloids is examined and vice versa. In TLC because of different visualization procedures (a dyeing reagent for alkaloids and visible light for neurotransmitters), compounds of both the groups may be determined simultaneously without the risk of interference. In HPLC, similarly, the possibility of false diagnosis through simultaneously employing the fluorimetric and diode-array detectors can be excluded.

Introduction

The determination of biogenic amine neurotransmitters (i.e., dopamine (DA), adrenaline, noradrenaline, and serotonin (5HT)) and their metabolites is of great importance in the clinical prognosis and pharmacotherapy of many diseases. As we reported earlier (1), alkaloids present in body fluids together with their metabolites as a result of diet or medication may interfere with neurotransmitters producing false diagnosis during chromatographic analysis. Both the groups of compounds have been analyzed with the use of thin-layer chromatography (TLC) (2–4) and reversed-phase (RP) high-performance liquid chromatography (HPLC) (5–9), but there are no published reports devoted to the problem of interference between them. This is the subject of this work.

Previously, we developed various liquid chromatographic systems for the separation of neurotransmitters, alkaloids, and their derivatives (1). In this study we have produced several new TLC systems achieving better separation of the compounds of interest and separating some metabolites of neurotransmitters not examined earlier. Besides, in all the systems developed for neurotransmitters, we have investigated the retention of alkaloids and vice versa. Moreover, we have developed isocratic and gradient RP-HPLC systems with fluorimetric (FL) and spectrophotometric (diode-array, DAD) detection for the simultaneous analysis of the compounds of interest.

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Experimental

Materials

Standard solutions (1 mg/mL) in 0.1M HCl were prepared from neurotransmitters (i.e., DA, epinephrine (E), norepinephrine (NE), and 5HT) and their metabolites methanephrine (MN), normethanephrine (NMN), 3,4-dihydroxyphenylacetic acid (DOPAC), vanilly1mandelic acid (VMA), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5HIAA), 3,4-dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenylglycol (DHPG), 3-methoxy-4-hydroxyphenylglycol (MHPG), and 3-methoxytyramine (3MT) (Sigma Chemicals Co., St. Louis, MO). Methanol solutions (1 mg/mL) of caffeine (CAFF), theobromine (THBR), and nicotine (NIC) (Sigma) and cotinine (COT) (Aldrich Chemicals Co., Milwaukee, WI) were prepared.

Water solutions (1 mg/mL) were prepared with theophylline (THPH) (Sigma), 1,7-dimethylxanthine (1.7DMX), 1,3-dimethyluric acid (1.3DMU), 7-methylxanthine (7MX), 1-methylxanthine (1MX), and 3-methylxanthine (3MX) (Aldrich).

Methods

TLC

TLC was performed on 20- × 20-cm glass plates precoated with 0.25-mm layers of Kieselgel G (SG), aluminum oxide ($A1_2O_3$), or Kieselguhr G (KG) (E. Merck, Darmstadt, Germany).

The plates were developed with various mobile phases: chloroform–methanol–acetic acid (7:2:1, v/v, mobile phase A); chloroform–propan-2-ol–acetic acid (7:2:1, v/v, mobile phase B) (7.5:2:0.5, v/v, mobile phase C); propan-2-ol–*n*-hexane (30:70, v/v, mobile phase D) (20:80, v/v, mobile phase E); chloroform–acetone (30:30, v/v, mobile phase F); toluene–propan-2-ol–acetic acid (16:5:2, v/v, mobile phase G) (17:5:1, v/v, mobile phase H); ethyl acetate–propan-2-ol–0.1M NH_4OH (80:25:16, v/v, mobile phase I); and ethyl acetate–methanol–0.1M NH_4OH (80:25:10, v/v, mobile phase J).

Alkaloids and their metabolites were either examined under UV light or visualized with an iron (III) chloride (5 g)–iodine (2 g) mixture in 50 mL of acetone and 50 mL of 20% aqueous tartaric acid solution (10).

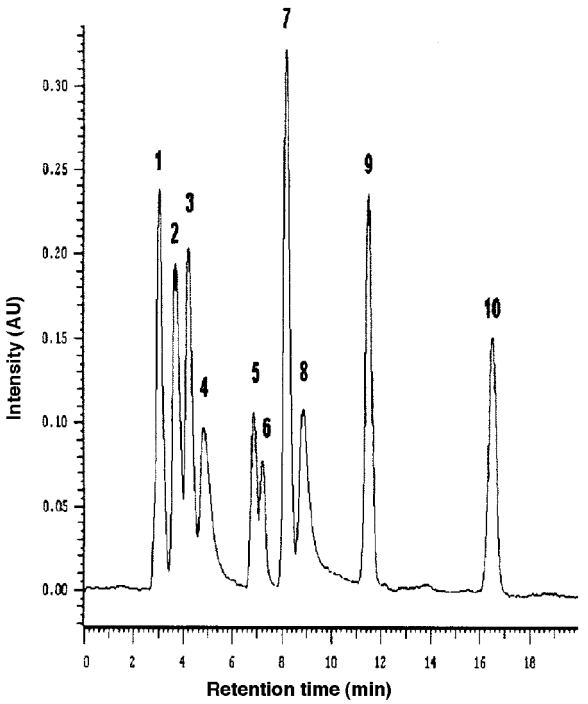

Figure 1. Chromatogram of biogenic amines and their derivatives in an HPLC–FL system (RP18 Superspher100 column, 250 × 4 mm, acetate buffer (pH 4.66)–methanol (90:10, v/v) as the mobile phase, 0.8-mL/min flow rate, and 40°C): DOMA, 1; VMA, 2; DHPG, 3; DA, 4; DOPAC, 5; MHPG, 6; 5HT, 7; 3MT, 8; 5HIAA, 9; and HVA, 10.

E and NE were examined under UV light. Other biogenic amines and their metabolites appeared visible.

HPLC

A Merck-Hitachi L4500 A chromatograph equipped with an octadecylsilane Superspher100 column (250 × 4 mm) and FL and DAD detectors were applied to the HPLC investigations. Buffer

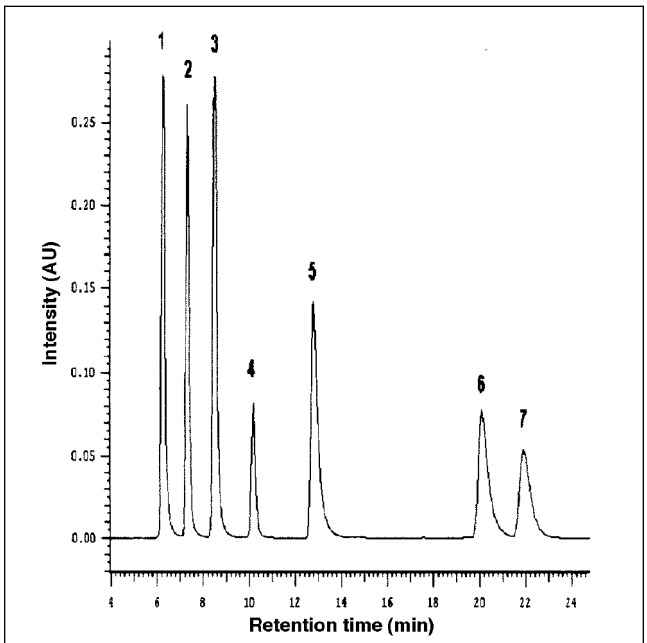

Figure 2. Chromatogram of xanthines and their derivatives in an HPLC–DAD system (RP-18 Superspher100 column, 250 × 4 mm, acetate buffer (pH 4.66)–methanol (90:10, v/v) as the mobile phase, 0.8 mL/min flow rate, and 40°C): 7MX, 1; 3MX, 2; 1MX, 3; 1.3DMU, 4; THBR, 5; 1.7DMX, 6; and THPH, 7.

Table I. Retention Times of Biogenic Amines, Their Derivatives, and Xanthines in the System Developed for Biogenic Amines

System	VMA	DA	DOPAC	5HT	5HIAA	HVA	NE	E	MN	NMN	DHPG	MHPG	3MT	DOMA	Xanthines
SG/A	0.31	0.21	0.54	0.25	0.65	0.88	0.11	0.17	0.31	0.25					THBR (0.57), COT (0.86), CAFF (0.87)
SG/B	0.21	0.05	0.57	0.07	0.66	0.92	0.02	0.04	0.09	0.06					NIC (0.11), CAFF(0.94)
KG/B	0.79	0.38	0.99	0.80	0.99	0.99	0.33	0.42	0.83	0.36	0.89	0.97	0.67	0.70	CAFF, COT, THPH, THBR, 1.7DMX, 1MX, 1.3DMU (~0.97); 7MX(0.91);NIC(0.46)
KG/C	0.63	0.31	0.94		0.99	0.99			0.71	0.37	0.85	0.97	0.70		CAFF,COT,THPH,THBR, 1.7DMX(~0.97); 7MX(0.91);NIC(0.64)
Al_2O_3/D	0.27	0.03	0.03	0.97	0.75	0.83	0.03	0.03	0.95	0.95					NIC,COT,CAFF,THPH(0.98)
KG/D	0.16	0.28	0.46	0	0.56	0.67	0.10	0.05	0.69	0.55	0.73	0.97	0.74	0.13	CAFF,NIC,COT,THPH(~0.97); THBR,1.7DMX,1MX(~0.92); 7MX(0.73)
SG/G	0.87	0.04	0.65	0.11		0.69	0.02	0.03	0.15	0.16					NIC(0.02),COT(0.19)
KG/G	0.85	0.43	0.92		0.97	0.98	0.32	0.39	0.89	0.50	0.89	0.96	0.81	0.73	CAFF,COT,THPH,THBR, 1.7DMX,1MX,1.3DMU (~0.97);7MX(0.93);NIC(0.44)
KG/H	0.73	0.28	0.93		0.97	0.99	0.18	0.36	0.85	0.35	0.84	0.94	0.83	0.41	CAFF,COT,THPH,THBR, 1.7DMX,1MX(~0.98);7MX(0.91)
SG/I		0.11	0.81	0.16		0.81	0.06	0.07	0.14	0.20					NIC(0.05);THPH(0.79)
SG/J	0.84	0.30	0.69	0.36	0.73	0.72	0.23	0.24	0.43	0.42					–

acetate (pH 4.66) and MeOH with a flow rate of 0.8 mL/min were used as the components of the mobile phase. The measurements were performed at temperatures of 13°C and 40°C. A 10-ng sample of each compound was introduced into the chromatographic column.

Sample preparation for HPLC

Twelve healthy volunteers who were university students provided 24-h urine samples. There were five men and seven women, and the mean age was 23 years old with a range of 21 to 25.

Fresh urine samples with the pH adjusted to 6 were vortexed at 10,000 rpm for 5 min. Then, the pH was adjusted to 8.5 with 2M ammonium hydroxide. BAKERBOND spe Octadecyl (C_{18}) columns (J.T. Baker Inc., Phillisburg, NJ) were conditioned with 2- × 1-mL methanol followed by 2- × 1-mL ammonium chloride–0.5% ethylenediaminetetraacetic acid (pH 8.5). After adding

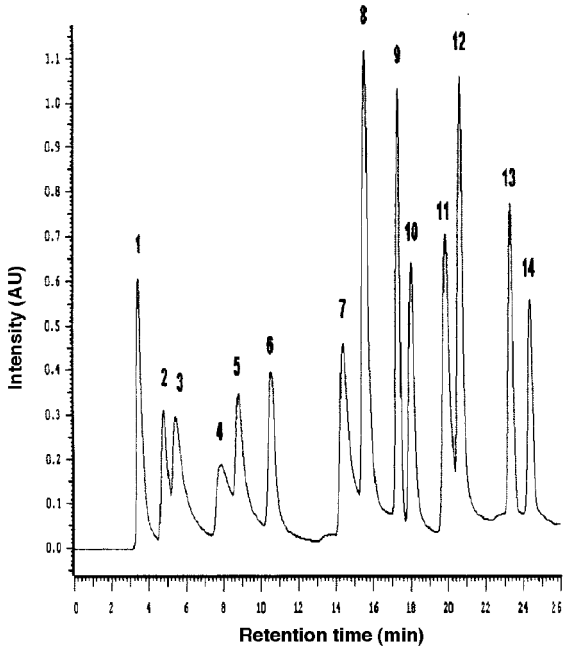

Figure 3. Chromatogram of biogenic amines and their derivatives in the gradient system described in the text: DOMA, 1; NE, 2; VMA, 3; E, 4; DHPG, 5; NMN, 6; DA, 7; MN, 8; MHPG, 9; DOPAC, 10; 3MT, 11; 5HT, 12; 5HIAA, 13; and HVA, 14.

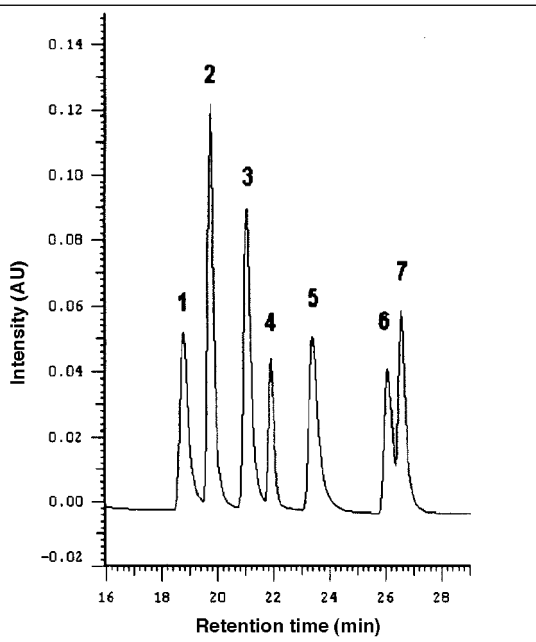

Figure 4. Chromatogram of xanthines and their derivatives in the gradient system described in the text: 7MX, 1; 3MX, 2; 1MX, 3; 1.3DMU, 4; THBR, 5; 1.7DMX, 6; and THPH, 7.

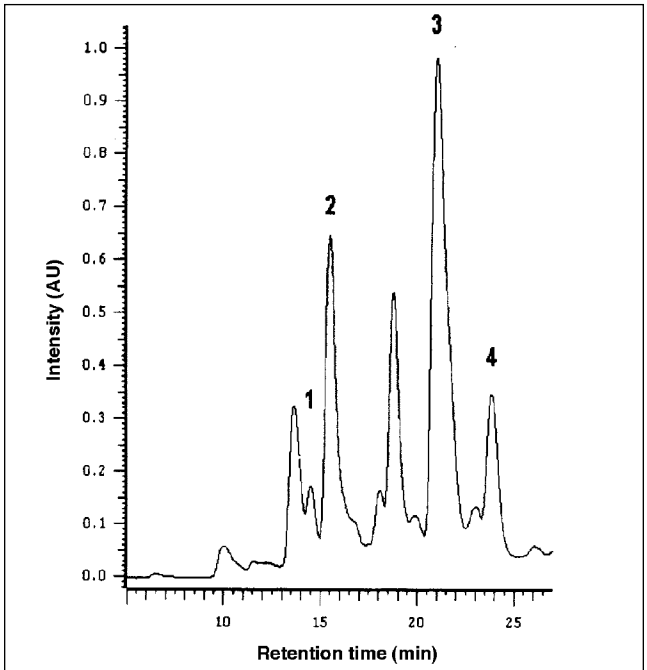

Figure 5. Biogenic amines and their derivatives determined in urine after the application of the J.T. Baker SPE sample pretreatment procedure and the developed HPLC gradient system: DA, 1; MN, 2; 5HT, 3; and 5HIAA, 4. The presence of these compounds in urine was confirmed by the analysis of urine spiked with standards.

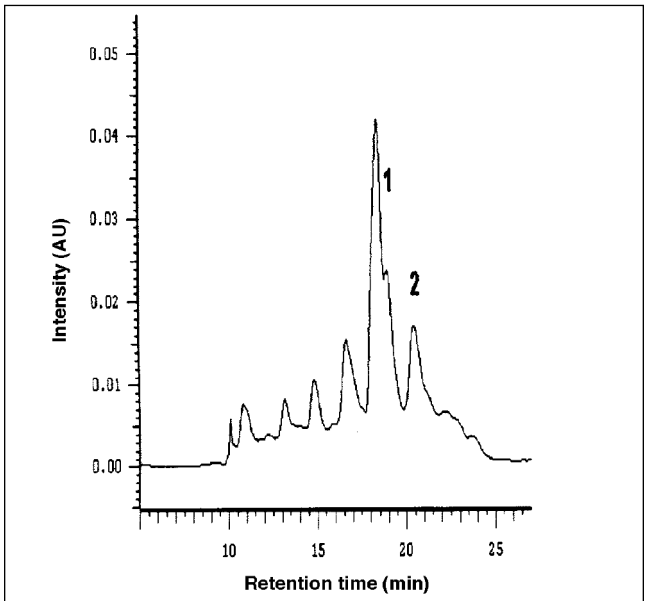

Figure 6. Methylxanthines determined in urine after the application of the J.T. Baker SPE sample pretreatment procedure and the developed HPLC gradient system: (1) 7MX and (2) 3MX. The presence of these compounds in urine was confirmed by the analysis of urine spiked with standards.

1 mL of the urine sample, the columns were washed with 2- × 1-mL 0.2M ammonium chloride (pH 8.5) followed by 1 mL of ammonium chloride–methanol (80:20, pH 8.5) and air dried under vacuum for 2 min.

Catecholamines and their derivatives were eluted with 2- × 1-mL 0.08M acetic acid, vortexed to mix, and 20 µL of it was injected into HPLC for analysis.

Urine samples spiked with standards were treated with the same procedure.

A BAKERBOND spe-12G (J.T. Baker Inc.) with the application procedure and a Refrigerated Universal Centrifuge Z 323 K (Hermle Labortechnik GmbH, Wehingen,Germany) were employed during the sample preparation.

Results and Discussion

TLC

TLC systems for the separation of biogenic amines and their metabolites were developed. Appropriate retention times (R_t) are shown in Table I. In all of the systems, the retention of alkaloids and their metabolites was investigated. Xanthines (which have similar R_t values as neurotransmitters) are also displayed in this table. Other compounds of the group did not interfere with the biogenic amines and their metabolites.

Various TLC systems for the separation of alkaloids and their metabolites were also elaborated: (*a*) CAFF, NIC, COT, THPH, and THBR were separated and determined in an Al_2O_3/E system with retention times of 0.57, 0.86, 0.63, 0.22, and 0.06 min, respectively; (*b*) 1.7DMX, THBR, THPH, COT, and NIC were separated and determined in an Al_2O_3/F system with retention times of 0.01, 0.13, 0.18, 0.80, and 0.93 min, respectively; (*c*) NIC, COT, 7MX, 1.7DMX, and 1.3DMU were separated in a KG/G system with retention times of 0.02, 0.19, 0.28, 0.37, and 0.43 min, respectively.

The following biogenic amines had approximate R_t values similar to alkaloids and their derivatives in the developed systems: (*a*) MN (R_t = 0.04 min), MHPG (R_t = 0.04 min), and NMN (R_t = 0.05 min); (*b*) DHPG (R_t = 0.04 min) and MHPG (R_t = 0.012 min); and (*c*) E (R_t = 0.39 min) and DA (R_t = 0.43 min).

Other biogenic amines did not interfere in these systems.

HPLC

In the conditions previously described (1), a 250- × 4-mm RP-18 Superspher100 column; a buffer acetate (pH 4.66)–methanol (90:10, v/v) eluent at 40°C; and a fluorimetric detector (λ_{ex} = 285 nm and λ_{em} = 315 nm) were used after a slight decrease of the flow rate of the mobile phase (1 to 0.8 mL/min). Instead of only DA, 5HT, and their metabolites VMA, DOPAC, 5HIAA, and HVA, we separated four more catecholamine metabolites: DOMA, DHPG, MHPG, and 3MT. In this system retention times were 3.03 min for DOMA, 3.67 min for VMA, 4.20 min for DHPG, 4.80 min for DA, 6.82 min for DOPAC, 7.18 min for MHPG, 8.13 min for 5-HT, 8.82 min for 3MT, 11.45 min for 5HIAA, and 16.44 min for HVA. The chromatogram of the whole group of compounds is shown in Figure 1.

We also tested the system connected to FL and DAD detectors for the separation of xanthines. Thus, we obtained a very good separation of these compounds (Figure 2) with the following retention times: 6.39 min for 7MX, 7.33 min for 3MX, 8.53 min for 1MX, 10.19 min for 1.3DMU, 12.77 min for THBR, 20.05 min for 1.7DMX, and 22.20 min for THPH. The simultaneous use of DAD and FL detectors enabled us to determine xanthines in mixtures with neurotransmitters and their metabolites.

Using the same column and detector we also developed a gradient HPLC system for the separation of neurotransmitters and their metabolites. The gradient was as follows: from 1 to 5 min, 100% buffer acetate (pH 4.66) was used; from 5 to 8 min, buffer acetate decreased in concentration to 90% (methanol increased in concentration to 10%); from 8 to 23 min, concentration of buffer acetate decreased to 65% (methanol concentration increased to 35%); and after 23 min, there was a return to initial conditions. The analysis was performed at 13°C, and the flow rate of the mobile phase was 0.8 mL/min.

The gradient system enabled us to separate neurotransmitters and their metabolites with the following retention times: 3.45 min for DOMA, 4.78 min for NE, 5.42 min for VMA, 7.81 min for E, 8.76 min for DHPG, 10.50 min for NMN, 14.35 min for DA, 15.49 min for MN, 17.25 min for MHPG, 17.98 min for DOPAC, 19.81 min for 3MT, 20.57 min for 5HT, 23.27 min for 5HIAA, and 24.31 min for HVA. The chromatogram is presented in Figure 3.

Xanthines were also separated in the gradient system with the following retention times (Figure 4): 18.75 min for 7MX, 19.71 min for 3MX, 21.01 min for 1MX, 21.87 min for 1.3DMU, 23.36 min for THBR, 26.03 min for 1.7DMX, and 26.53 min for THPH.

The linear dependence of the HPLC response on concentration can be expressed by the equation $y = ax$, where the values of a for the compounds of interest were 2.320 for DA, 9.831 for E, 1.611 for NE, 6.027 for 5HT, 1.210 for MN, 4.934 for NMN, 1.750 for DOPAC, 6.191 for VMA, 1.280 for HVA, 4.118 for 5HIAA, 5.433 for DOMA, 4.109 for DHPG, 6.762 for MHPG, 3.814 for 3MT, 0.012 for THBR, 0.022 for THPH, 0.011 for 1.7DMX, 0.008 for 1.3DMU, 0.016 for 7MX, 0.014 for 1MX, and 0.011 for 3MX.

We applied the developed HPLC gradient system to the determination of biogenic amines and their derivatives in the urine of healthy subjects. The urine samples were prepared according to the J.T. Baker application procedure (11). The presence of VMA, DA, MN, 5HT, 5HIAA, HVA, 7MX, and 3MX in the urine was confirmed through the analysis of urine spiked with standards. Spiking random urine samples with standards of 2 µg/mL gave a recovery of 81.2% for VMA, 97.2% for DA, 98.5% for MN, 95.1% for 5HT, 80.0% for 5HIAA, 66.3% for HVA, 80.1% for 7MX, and 79.2% for 3MX. The determined concentrations of biogenic amine neurotransmitters in 24-h urine samples of healthy volunteers ranged between 1.32 and 1.99 µmol for DA, 0.80 and 1.20 µmol for 5HT, 0.54 and 20.11 µmol for 5HIAA, 0.10 and 0.33 µmol for MN, 17.30 and 30.11 µmol for VMA, and 0.10 and 0.32 µmol for HVA. For concentrations of xanthines the range was 0.90 to 1.50 µg/mL for 7MX and 0.99 to 1.70 µg/mL for 3MX. These ranges are consistent with the literature (12,13). The obtained FL and DAD chromatograms are presented in Figure 5 and Figure 6, respectively. The results of the experiment heighten the advantages of using multidetector systems and the importance of testing every new method for this kind of interference.

We developed several TLC and HPLC systems for the determination of neurotransmitters, alkaloids, and metabolites of the two

groups of compounds. In all the systems developed for neurotransmitters, the retention of alkaloids was examined and vice versa. Both of the groups of compounds can be determined simultaneously without the risk of interference by the use of different detection procedures: in TLC a dyeing reagent for alkaloids and visible light for neurotransmitters and in HPLC the simultaneous employing of the FL (for observation of both the groups of compounds) and DAD (only for alkaloids) detectors.

It should be emphasized that although there are many studies concerning the analysis of biogenic amines, alkaloids, and their metabolites, both of these groups of compounds are not analyzed in the same chromatographic systems and none of these works has a good separation efficiency for such a set of biogenic amines and their metabolites.

The proposed TLC and HPLC (isocratic or gradient) systems can be employed for the analysis of body fluids.

Acknowledgments

This work was supported by the State Committee for Scientific Research (Poland), Project No. 4#T09A#08922.

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Manuscript accepted January 15, 2002.