Determination of Levodopa and Biogenic Amines in Urine Samples Using High-Performance Liquid Chromatography

Irena Baranowska* and **Joanna Płonka**

Department of Analytical and General Chemistry, Silesian University of Technology, 7 M.Strzody Str. 44-100 Gliwice, Poland

Abstract

A chromatographic system is developed for the separation and determination of levodopa, biogenic amines, and their metabolites from the catecholamines group: dopamine (DA), epinephrine (E), normetanephrine (NMN), metanephrine (MN), 3,4-dihydroxyphenylacetic acid (DOMA), 3-metoxy-4-hydroxyphenylglycol (MHPG), and homovanillic acid (HVA); and indoloamines group: serotonin (5HT) and 5-hydroxyindole-3-acetic acid (5HIAA) in urine. The limit of detection (LOD) and limit of quantitation (LOQ) are determined for all compounds with signal-to-noise ratio (S/N) of 3 and 10, respectively. LOD 10 (ng/mL) and LOQ 30 (ng/mL) are determined for L-DOPA, DOMA, E, NMN, DA, MN, and MHPG, as well as LOD 8 (ng/mL) and LOQ 24 (ng/mL) for HVA, 5HT, and 5HIAA. A fluorescence detector is used. Gradient elution with acetate buffer (pH = 4.66) with methanol is applied. In urine samples from patients treated with levodopa, the following concentrations (µg/mL) of analytes are determined: L-DOPA 3.73–46.80, DOMA 1.43–28.43, E 0.83–13.57, NMN 2.58–8.81, DA 24.07–62.11, MN 0.89–66.20, MHPG 6.72–63.64, 5HT 22.96–95.27, 5HIAA 1.45–14.77, and HVA 0.21–15.07.

Introduction

Biogenic amines are formed as a result of the decarboxylation of base and inactive amino acids. Those compounds can be divided into aliphatic amines (mono- and polyamines), heterocyclic amines (imidazole amines, indole amines), and catecholamines. Catecholamines include, among others, dopamine, epinephrine, and norepinephrine. Heterocyclic indoloamines include, among others, serotonin. Those amines are generated in the brain and are used by the human body as neurotransmitters.

Levodopa is a dopamine precursor and is used as a drug for Parkinson's Disease. Due to the role of levodopa, dopamine, serotonin, and their metabolites in human organisms, it is important to monitor concentrations of these amines in organic fluids. The determination of these compounds is done mainly by high-performance liquid chromatography (HPLC). HPLC methods for the determination of levodopa in different biological materials [e.g., serum (1–6,9), urine (6), brain (7), and skin cells (8)] have been described elsewhere. C8 (6) and C18 (1–3,5,7–9) packed columns have been used. Studies have been done in both isocratic (1–3,5–7,9) and gradient elution. DAD (7–8), fluorescent (3,5,8), and electrochemical (1–2,5–7,9) detectors have been used. Liquid chromatography–electrospray tandem mass spectrometry has been used as well (4). In the research conducted, levodopa was determined simultaneously with other compounds like carbidopa (1,6,9), dopamine (4,7–9), 3,4-dihydroxphenylacetic acid (8–9), 3-methoxytyramine (8), homovanillic acid (HVA) (8), S-adenosylmethionine (7), *S*-adenosylhomocysteine (7), 3-*O*-methyldopa (2,6,9), 3-*O*-carbidopa (6), and acetaminophen (3). A mixture of levodopa with L-tyrosine, dopamine, epinephrine, norepinephrine, 3,4-dihydroxybenzylamine, and 3,4-dihydroxphenylacetic acid was determined (5). Despite the simultaneous examination of a different combination of compounds, the total number of determined compounds never exceeded seven (5). Different sample preparation procedures of organic fluids were used. Serum samples were deproteinized by perchloric acid (1,3,9), then centrifuged and filtrated. The other method was isolation of catecholamines by an alumina adsorption procedure (5) or an alumina-based solid-phase sample preparation (4). For urine samples, a preparation mixture of HCl and $HClO_4$ was used for deproteinization (4), and then samples were centrifuged and filtrated.

The developed analytical procedure and the chromatographic system described in this paper allows one to simultaneously determine compounds, including levodopa, dopamine, serotonin, and their seven metabolites. Results from a literature review show that no chromatographic system which could determinate levodopa with all previously mentioned compounds has been developed. This new system obtains more indispensable information for the monitoring of levodopa therapy.

Experimental

Reagents

Standard solutions (1 mg/mL) in 0.1M HCl with $Na_2S_2O_3$ (5 g/L) of levodopa (L-DOPA), dopamine (DA), epinephrine (E),

* Author to whom correspondence should be addressed: email irena.baranowska@polsl.pl.

normetanephrine (NMN), metanephrine (MN), 3,4-dihydroxyphenylacetic acid (DOMA), 3-metoxy-4-hydroxyphenyl-glycol (MHPG), homovanillic acid (HVA), serotonin (5HT), and 5-hydroxyindole-3-acetic acid (5HIAA) (Sigma-Aldrich, St. Louis, MO) were prepared. Solutions were prepared from 50 mg of each standard, dissolved in 50 mL of acid solution, and diluted to obtain a concentration of 1 (µg/mL), 2 (µg/mL), or more. A methanol and acetate buffer (pH = 4.66) of HPLC grade used in this work for mobile phase preparation was purchased from Merck (Darmstadt, Germany).

HPLC conditions

HPLC analyses were performed using a Merck-Hitachi chromatograph equipped with a L-6200A pump and an L-7480 fluorescent detector (Merck, Germany). Chromatographic separations were carried out on a LiChroCARD Purospher column RP-18e, 125 × 3 mm, particle size 5 µm, accompanied with a LiChroCARD 4- × 4-mm precolumn packed with LiChrospher 100 RP-18, particle size 5 µm (Merck, Germany) at room temperature. A gradient elution with acetate buffer (pH = 4.66) (A) and methanol (B) was applied. A similar chromatographic system has been used for the determination of biogenic amines only (10). In this work, the following elements have been changed: chromatographic column (RP-18 Superspher 100 was used before), gradient elution, flow rate, and temperature. The gradient elution is shown in Table I.

Table I. Gradient Elution Parameters*

Time (min)	A (%)	B (%)	Flow rate (mL/min)
0	100	0	0.5
3	100	0	0.5
8	90	10	0.5
23	65	35	0.5
24	65	35	0.8
30	100	0	1.0

*A = Acetate buffer (pH = 4.66) and B = methanol.

Calibration curve

A calibration curve was prepared for all the analytes in the urine matrix. Urine samples for the calibration curves were taken from healthy people not treated with levodopa. The preparation procedure for the urine samples was the same as that described in the "Urine samples" section. The calibration curve for levodopa was prepared for solutions in the range of 0.05–2 (µg/mL), and in the range of 0.06–2 (µg/mL) for biogenic amines. The number of experimental points taken for regression was $n = 6$. Every analyte was injected thrice. The volume of the solution in each injection was 20 µL. The limit of detection (LOD) and limit of quantitation (LOQ) were determined.

Urine samples

Urine samples from patients with Parkinson's Disease were examined. Urine samples were collected four times within a 24 h period after applying levodopa and subsequently frozen at –20°C. The volume of all samples was 150 mL. The first sample was urine collected in the morning; 4 h after the last dose of levodopa (350 mg) was applied. The patient took the first dose of the drug (150 mg), and the urine sample was collected after 20 min. Immediately after sample collection, the patient took the second dose of levodopa (150 mg). Consecutively, the third urine sample was collected after 20 min, and the third dose of the drug (100 mg) was applied. The fourth urine sample was collected 1 h after application of the last dose of levodopa. The same sample collection procedure was used for three different days. Before assay, the urine sample was defrosted at room temperature for 1/2 h. After defrosting, 0.2 mL of a urine sample was acidified with 4 mL of perchloric acid (0.2M) and centrifuged for 10 min (2500 rpm). The examined material was filtrated using a Bakerbond nylon filter with 0.45 µm pores. The applied method is a modification of a published method where 0.25 mL of $HClO_4$ (0.2M) was added to a sample (3). The application of different centrifuging parameters and increasing acid volume in these examinations eliminated interferences from the matrix. The volume of the filtrated solution in every injection was 20 µL. Volume was taken three consecutive times from every urine sample, and subsequently prepared and analyzed. Each of the resulting supernatant was injected three times; therefore, the standard deviation was calculated for $n = 9$.

Table II. Retention Factors and Calibration Curve Parameters ($n = 6$)

Compound	k′ (SD)	Slope	Intercept	R^2	LOD (ng/mL)	LOQ (ng/mL)
DOMA	1.82 (0.01)	4000000	105965	0.999	10	30
L-DOPA	3.52 (0.03)	10000000	1550046	0.999	10	30
E	5.22 (0.01)	10000000	114242	0.999	10	30
NMN	7.69 (0.02)	10000000	–15649	0.999	10	30
DA	11.03 (0.01)	7000000	–172373	0.999	10	30
MN	12.14 (0.03)	8000000	85890	0.999	10	30
MHPG	14.35 (0.01)	6000000	162062	0.999	10	30
5HT	18.00 (0.01)	2000000	–138216	0.999	8	24
5HIAA	21.20 (0.01)	30000000	–70000000	0.997	8	24
HVA	27.28 (0.01)	9000000	1000000	0.999	8	24

HPLC analysis

HPLC assay of urine samples was performed using the previously described chromatographic system. Detection was performed with the use of a fluorescent detector at an excitation wavelength of λ_{EX} = 285 nm and an emission wavelength of λ_{EM} = 315 nm.

Results and Discussion

A chromatographic system was developed for the separation and determination of levodopa and nine biogenic amines: DA, E, NMN, MN, DOMA, MHPG, 5HT, 5HIAA, and HVA. The total

Table III. Within-day and Between-day Precision for Examined Compounds*

Compound	Input (µg/mL)	Within-day Measured (µg/mL)	Within-day SD (µg/mL)	Within-day CV (%)	Within-day Recovery (%)	Between-day Measured (µg/mL)	Between-day SD (µg/mL)	Between-day CV (%)	Between-day Recovery (%)
L-DOPA	0.10	0.08	0.02	1.52	83.3	0.09	0.02	1.58	91.7
	0.20	0.18	0.01	0.17	87.5	0.19	0.01	0.56	95.8
	0.30	0.26	0.03	3.29	86.1	0.29	0.04	4.21	97.2
DA	0.10	0.08	0.02	1.54	82.3	0.09	0.05	5.36	94.1
	0.20	0.18	0.01	0.76	90.9	0.19	0.01	0.01	97.0
	0.30	0.28	0.03	3.08	91.8	0.29	0.01	0.96	98.0
DOMA	0.20	0.15	0.02	1.66	73.7	0.14	0.05	5.20	68.4
	0.40	0.36	0.01	1.40	89.2	0.32	0.01	0.49	81.1
	0.60	0.56	0.04	4.14	96.4	0.57	0.01	1.07	94.5
E	0.10	0.09	0.02	2.38	90.9	0.09	0.02	2.44	90.9
	0.20	0.15	0.01	1.42	76.2	0.14	0.03	2.78	71.4
	0.30	0.22	0.02	1.78	74.2	0.29	0.01	1.14	96.8
NMN	0.20	0.19	0.01	1.29	95.2	0.19	0.06	6.45	95.2
	0.40	0.34	0.03	3.41	85.4	0.33	0.01	1.02	82.9
	0.60	0.45	0.06	5.66	75.0	0.46	0.02	1.63	76.7
NM	0.20	0.17	0.03	3.54	84.0	0.18	0.05	5.15	88.0
	0.40	0.36	0.03	2.74	89.9	0.33	0.06	6.25	83.7
	0.60	0.58	0.03	3.13	95.8	0.50	0.03	2.99	83.3
MHPG	0.10	0.08	0.05	4.95	80.0	0.09	0.05	4.67	86.7
	0.20	0.19	0.05	5.00	96.7	0.17	0.04	4.44	86.7
	0.30	0.28	0.05	4.89	93.2	0.29	0.04	4.29	95.4
5HT	0.10	0.10	0.05	5.01	99.9	0.09	0.06	5.94	91.7
	0.20	0.17	0.01	1.27	87.0	0.17	0.06	6.04	82.6
	0.30	0.28	0.03	3.19	94.1	0.26	0.04	4.48	88.2
5HIAA	0.20	0.19	0.04	4.09	94.7	0.18	0.02	2.11	89.5
	0.40	0.30	0.02	1.85	75.7	0.37	0.02	2.31	92.0
	0.60	0.57	0.06	5.81	94.5	0.50	0.03	2.89	83.6
HVA	0.10	0.08	0.06	5.96	80.0	0.09	0.05	5.34	93.3
	0.20	0.17	0.04	4.44	83.3	0.18	0.03	2.97	90.0
	0.30	0.25	0.01	0.26	81.8	0.28	0.01	1.27	93.2

* Number of replicates $n = 9$

Table IV. Levodopa and Biogenic Amines Content in the Urine Samples of Patients with Parkinson's Disease

Compound	Determined (µg/mL)* Day 1 Urine1	Day 1 Urine2	Day 1 Urine3	Day 1 Urine4	Day 2 Urine1a	Day 2 Urine2a	Day 2 Urine3a	Day 2 Urine4a
L-DOPA	6.71 ± 0.02	8.23 ± 0.04	8.58 ± 0.08	46.80 ± 0.05	7.77 ± 0.03	24.21 ± 0.07	34.38 ± 0.01	40.11 ± 0.03
DA	39.36 ± 0.12	43.86 ± 0.15	28.11 ± 0.45	40.12 ± 0.19	26.06 ± 0.04	24.07 ± 0.05	58.51 ± 0.02	62.11 ± 0.02
DOMA	14.90 ± 0.68	28.43 ± 0.03	3.17 ± 0.40	8.26 ± 0.12	n/f	n/f	14.02 ± 0.18	n/f
E	7.96 ± 0.13	8.80 ± 0.05	n/f	n/f	6.67 ± 0.11	12.95 ± 0.01	6.76 ± 0.20	1.43 ± 0.08
NMN	6.64 ± 0.40	n/f	n/f	5.04 ± 0.13	n/f	n/f	5.02 ± 0.53	8.81 ± 0.04
MN	18.61 ± 0.07	23.43 ± 0.04	n/f	n/f	9.74 ± 0.10	18.72 ± 0.37	23.38 ± 0.02	27.57 ± 0.02
MHPG	25.32 ± 0.02	28.38 ± 0.25	6.72 ± 0.01	15.58 ± 0.01	n/f	38.16 ± 0.41	n/f	7.21 ± 0.04
5HT	22.96 ± 0.11	25.41 ± 0.12	n/f	76.52 ± 0.26	n/f	33.90 ± 0.31	n/f	n/f
5HIAA	5.50 ± 0.01	7.01 ± 0.01	2.03 ± 0.01	n/f	4.57 ± 0.07	4.41 ± 0.01	13.85 ± 0.05	14.77 ± 0.05
HVA	n/f	n/f	n/f	n/f	n/f	n/f	13.22 ± 0.01	15.07 ± 0.02

* n/f = not found; number of replicates $n = 9$.

time of each assay was under 30 min. A chromatogram of a mixture of all analytes in quantities of 2 ng up to 5 ng in 20 µL is shown in Figure 1. All examined compounds were well-separated. Because reverse-phase chromatography was used, compounds with a lower polarity eluted with higher retention times. For example, DOMA has two hydroxyl groups at the ring and a carboxyl group; therefore, it is the most polar compound and was eluted first. MHPG has only one hydroxyl group at the ring and a $–CH_2OH$ group instead of a carboxyl one. This results in retention times eight times longer than DOMA because of the weak polarity of the examined compound. Therefore, chemical affinity to the C18 phase is greater.

Table IV. (Continued) Levodopa and Biogenic Amines Content in the Urine Samples of Patients with Parkinson's Disease

	Determined (µg/mL)*			
	Day 3			
Compound	Urine1b	Urine2b	Urine3b	Urine4b
L-DOPA	3.73 ± 0.05	15.24 ± 0.01	23.11 ± 0.08	33.48 ± 0.03
DA	27.75 ± 0.19	39.89 ± 0.17	44.61 ± 0.02	56.57 ± 0.37
DOMA	n/f	1.66 ± 0.02	1.43 ± 0.01	n/f
E	13.57 ± 0.93	3.24 ± 0.03	0.83 ± 0.01	1.00 ± 0.01
NMN	n/f	n/f	2.58 ± 0.06	n/f
MN	51.44 ± 0.28	66.20 ± 0.03	16.00 ± 0.04	0.89 ± 0.01
MHPG	n/f	n/f	24.90 ± 0.166	3.64 ± 0.16
5HT	n/f	n/f	29.48 ± 0.13	95.27 ± 0.85
5HIAA	n/f	1.45 ± 0.02	7.73 ± 0.02	n/f
HVA	n/f	n/f	n/f	0.21 ± 0.01

*n/f = not found; number of replicates n = 9.

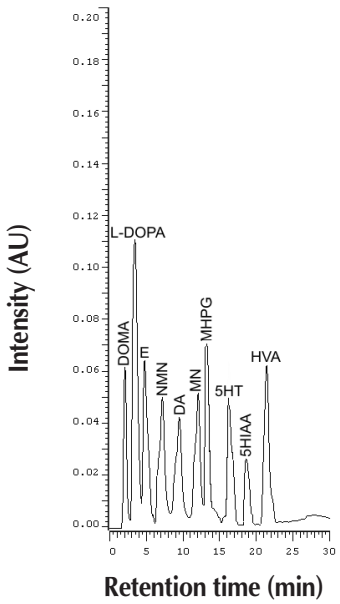

Figure 1. Chromatogram of a mixture of levodopa and biogenic amines.

Calibration curves for each analyte were obtained for solutions of the concentrations previously specified. LOD ranged from 8 to 10 ng/mL, and LOQ ranged from 24 to 30 ng in 1 mL of sample. LOD for all compounds was determined for a signal-to-noise ratio (S/N) of 3, while LOQ was determined for an S/N of 10. Calibration curve parameters, retention factors, LOD, and LOQ for all analytes are listed in Table II.

Intra- and interday precision and accuracy of the applied method for three different additions of levodopa and other biogenic amines are presented in Table III. For these examinations, urine samples were taken from healthy people who had not been treated with levodopa. Those samples performed the function of a matrix. Examinations were repeated after one week. The applied sample preparation method achieved recoveries from 83 to 97% for the levodopa and from 80% to 99% for the biogenic amines. Only single values were lower: 68.4% and 73.7% for DOMA, 71.4% for E, and approximately 75% for NMN, E, and 5HIAA.

The contents of levodopa, dopamine, serotonin, and their metabolites in urine samples were determined from the calibration curves. Every urine sample was injected three times. The obtained results are presented in Table IV and V.

A concentration of the drug (L-DOPA) in organisms decreases 4–5 h after the dose is taken. The patient took the last dose of the drug before sleep on a particular day. The time between the last dose and the first dose on the following day was 4–5 h; therefore, the concentration of levodopa in the first sample on the following day was so low. This sample can be used, consequently, as the base for the results of the whole day. Following doses of the drug, increased signal levels of levodopa and dopamine were observed on the chromatograms. The increased level of dopamine is caused by the metabolism process of levodopa.

The concentration of dopamine in urine can be observed in

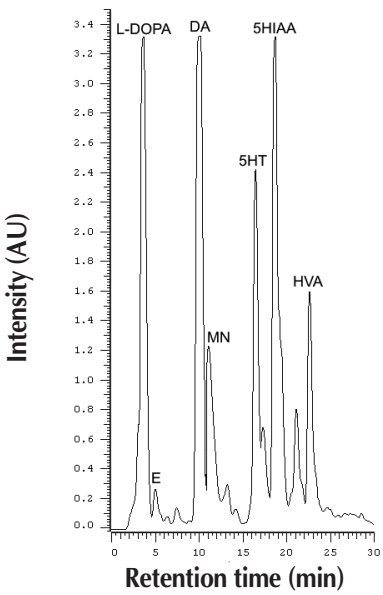

Figure 2. Chromatogram of a urine sample from a patient treated with levodopa.

the following chromatograms, as well as in Table IV. A comparison of the results from Table IV and V shows that the concentration of dopamine in urine samples from patients treated with levodopa were on the average 10 times higher than in urine samples from healthy people. The developed method allows one to adjust the levodopa dosage. For example, when levodopa and dopamine concentratiosn in urine are increasing and further levodopa dosage increasing has no positive influence on patient treatment, a therapist can introduce proper changes to the levodopa treatment. Simultaneous information about the other eight metabolites allows therapists more data about drug metabolism, to take into consideration the differences between particular patients.

In the examined urine samples of patients treated with levodopa, the following compounds were determined: 3,4-dihydroxyphenylacetic acid k′ = 1.81 (0.03); levodopa k′ = 3.33 (0.01); epinephrine k′ = 4.88 (0.01); normetanephrine k′ = 7.83 (0.01); dopamine k′ = 10.95 (0.03); metanephrine k′ = 11.57 (0.01); 3-metoxy-4-hydroxyphenyl-glycol k′ = 14.86 (0.01); serotonin k′ = 18.90 (0.01); 5-hydroxyindole-3-acetic acid k′ = 20.77 (0.01); and homovanillic acid k′ = 27.22 (0.01). In the examined urine samples of healthy people (not treated with levodopa), the following biogenic amines and their metabolites were determined: epinephrine k′ = 5.20 (0.01); normetanephrine k′ = 8.21 (0.01); dopamine k′ = 11.09 (0.02); serotonin k′ = 18.56 (0.01); 5-hydroxyindole-3-acetic acid k′ = 20.79 (0.01); and homovanillic acid k′ = 27.48 (0.01).

An exemplary chromatogram of the urine sample of a patient treated with levodopa is shown in Figure 2. As can be noted, because of the insufficient separation of peaks, it is impossible to quantitatively determine dopamine and metanephrine in this particular example. To achieve satisfactory peak separation for quantitative analysis of those biogenic amines, it was necessary to dilute the urine sample. The differences between the retention times of the analytes in model samples and in the real urine sample are results of a matrix effect. The presence of particular analytes was confirmed by the standard addition method.

Table V. Biogenic Amines Content in the Urine Samples of Healthy People

	Determined (µg/mL)*		
Compound	**Urine1**	**Urine2**	**Urine3**
DA	3.16 ± 0.03	1.71 ± 0.01	3.22 ± 0.10
DOMA	n/f	n/f	n/f
E	1.28 ± 0.03	7.28 ± 0.01	3.44 ± 0.15
NMN	0.67 ± 0.05	n/f	n/f
MN	n/f	n/f	n/f
MHPG	13.81 ± 0.38	n/f	n/f
5HT	12.81 ± 0.11	26.56 ± 0.01	11.29 ± 0.08
5HIAA	4.03 ± 0.27	2.98 ± 0.04	2.36 ± 0.39
HVA	n/f	1.00 ± 0.04	n/f

* n/f = not found; number of replicates $n = 9$

The chromatographic system described in this paper allows the determination of levodopa, dopamine, serotonin, and their metabolites. A small amount of urine (0.2 mL) needed to perform the analysis, a fast and easy sample preparation, and convenient analysis time make this method applicable to the estimation of the content of levodopa and particular biogenic amines in the urine samples of patients with Parkinson's Disease.

Acknowledgments

This work was supported from science funds for scientific research for years 2007–2009, as Project No. N20402532/0738.

References

1. A. Tolokan, I. Klebovich, K. Balogh-Nemes, and G. Horvai. Automated determination of levodopa and carbidopa in plasma by high-performance liquid chromatography-electrochemical detection using an on-line flow injection analysis sample pretreatment unit. *J. Chromatogr. B* **698:** 201–207 (1997).
2. F. Balandini, E. Martignoni, C. Pacchetti, D. Desideri, D. Rivellini, and G. Nappi. Simultaneous determination of L-dopa and 3-O-methyldopa in human platelets and plasma using high-performance liquid chromatography with electrochemical detection. *J. Chromatogr. B* **700:** 278–282 (1997).
3. K. Hatori, T. Kondo, and Y. Mizuno. Levodopa absorption profile in Parkinson's disease: evidence to indicate qualitative difference from the control. *Parkinsonizm Relat D* **2:** 137–144 (1996).
4. W. Li, D.T. Rossi, and S.T. Fountain. Development and validation of a semi-automated method for L-dopa and dopamine in rat plasma using electrospray LC–MS–MS. *J. Pharm. Biomed. Anal.* **24:** 325–333 (2000).
5. S. Letellier, J.P. Garnier, J. Spy, and B. Bousquet. Determination of the L-dopa/L-tyrosine ratio in human plasma by high-performance liquid chromatography usefulness as a maker in metastatic malignant melanoma. *J. Chromatogr. B* **696:** 9–17 (1997).
6. K.A. Sagar and M.R. Smyth. Simultaneous determination of levodopa, carbidopa and their metabolites in human plasma and urine samples using LC-EC. *J. Pharm. Biomed. Anal.* **22:** 613–624 (2000).
7. X.X. Liu, K. Wilson, and C.G. Charlton. Effects of L-dopa treatment on methylation in mouse brain: implications for the side effects of L-dopa. *Life Sci.* **66:** 2277–2288 (2000).
8. H. Blessing, M. Bareiss, H. Zettlmeisl, J. Schwarz, and A. Storch. Catechol-O-methyltransferase inhibition protects against 3,4-dihydroxyphenylalanine (DOPA) toxicity in primary mesencephalic cultures: new insights into levodopa toxicity. *Neurochem. Int.* **42:** 139–151 (2003).
9. M. Karimi, J.L. Carl, S. Loftin, and J.S. Perlmutter. Modified high-performance liquid chromatography with electrochemical detection method for plasma measurement of levodopa, 3-*O*-methyldopa, dopamine, carbidopa and 3,4-dihydroxyphenyl acetic acid. *J. Chromatogr. B* **836:** 120–123 (2006).
10. I. Baranowska and M. Zydron. Liquid chromatography in the analysis of neurotransmitters and alkaloids. *J. Chromatogr. Sci.* **40:** 224–228 (2002).

Manuscript received October 2, 2006;
Revision received February 26, 2007.