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Simultaneous determination of levodopa and 3-*O*-methyldopa in human plasma by liquid chromatography with electrochemical detection

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Abstract

A simple and rapid assay is described for the simultaneous analysis of levodopa (L-DOPA) and 3-*O*-methyldopa (3-OMD) in human plasma samples, applying an ion-pair reversed-phase liquid chromatographic method with electrochemical detection, designed for clinical trials performed to study the effect of peripheral catechol-*O*-methyltransferase inhibitors on the metabolism of L-DOPA. After protein precipitation of 100 μl plasma sample aliquots with perchloric acid, the analytes are directly injected, separated within 10 min and simultaneously quantified down to 20 ng/ml by an electrochemical detector equipped with a dual-electrode system operating in redox mode eliminating effectively potential endogenous and exogenous interferences. The intra-assay precision for L-DOPA and 3-OMD was 1.34–6.54 and 3.90–5.50%, whereas the inter-assay precision was 2.09–7.69 and 4.16–9.90%, respectively. The recoveries were close to 90% for L-DOPA and almost 100% for 3-OMD. Satisfactory storage stability was achieved for up to 16 weeks at −70 °C by stabilizing plasma samples with antioxidants.

Keywords: Levodopa; 3-*O*-Methyldopa

1. Introduction

Levodopa (L-DOPA), the medication of choice for the treatment of Parkinson's disease, is principally metabolized by an aromatic amino acid decarboxylase (dopadecarboxylase, DDC) to dopamine (DA), and by catechol-*O*-methyltransferase (COMT) into 3-*O*-methyldopa (3-OMD). DA is rapidly deaminated by monoamine oxidase (MAO) into 3,4-dihydroxyphenylacetic acid (DOPAC), which subsequently is methylated to homovanillic acid (HVA) by COMT. When a peripherally acting DDC inhibitor such as carbidopa is used to improve the bioavailability of L-DOPA, *O*-methylation becomes a quantitatively even more important metabolic pathway [1]. Inhibition of the extracerebral COMT activity might thus improve further the bioavailability of L-DOPA [2]. In order to support the evaluation of efficacy by COMT inhibitors in clinical studies, many methods have been developed for the determination of L-DOPA and its metabolite 3-OMD, especially in the field of high-performance liquid chromatography with electrochemical detection (HPLC–ECD) [3–11].

In this paper, a simple and rapid method for the simultaneous analysis of levodopa and 3-*O*-methyldopa (3-OMD) using high-performance liquid chromatographic method with electrochemical detection is reported. The method was fully validated and provides a specific and reproducible assay with a small amount of human plasma. The samples can be deproteinized with perchloric acid and directly be injected to the chromatographic system without neutralization. The established dual-electrode system operating in redox mode eliminates effectively any potential endogenous and exogenous interferences and provides a highly selective and efficient approach in the field of catecholamine analysis.

2. Experimental

2.1. Chemicals and reagents

Levodopa, 3-*O*-methyldopa (3-OMD), *S*-(−)-carbidopa, (−)-epinephrine, 3-hydroxytyramine hydrochloride, 3,4-dihyroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylacetic acid and 5-hydroxytryptamine hydrochloride were purchased from Sigma (Tokyo, Japan) (Fig. 1).

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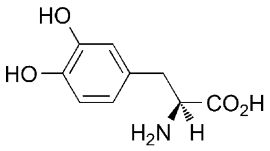
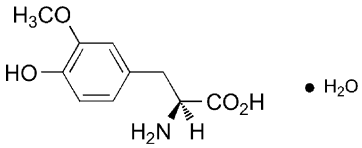


• L-3,4-Dihydroxyphenylalanine (L-DOPA) : $C_9H_{11}NO_4$
Molecular weight 197.2



• 3-Methoxy-L-tyrosine hydrate (3-OMD) : $C_{10}H_{13}NO_4 \cdot H_2O$
Molecular weight 229.2 (with hydrate)

Fig. 1. Chemical structures of L-DOPA and 3-OMD.

Methanol was of HPLC grade and obtained from Kanto Kagaku (Tokyo, Japan). Water was purified prior use by Milli-Q Ultrapure Water System of Millipore. Human blank plasma treated with disodium EDTA was purchased from New Drug Development Research Center Inc. (Tokyo, Japan). All other reagents were of analytical grade and ordered from Kanto Kagaku, Wako Pure Chemical Industries Ltd., and MC Medical (Tokyo, Japan).

2.2. Preparation of standard solutions

Standard solutions for L-DOPA and 3-OMD were individually prepared at concentrations of 500 μg/ml in 0.01 M hydrochloric acid containing 0.1% of sodium metabisulfite and 0.01% of disodium EDTA. Stock solutions were mixed at aliquots of 1:1 (v/v) resulting in final concentrations of 250 μg/ml for each analyte. Above described solutions were protected from light, stored at 4 °C and used on same day for preparations of spiking solutions for calibration and quality control (QC) samples. All preparation steps for calibration and QC samples were processed independently by different laboratory personnel.

2.3. Spiking procedure for calibration and QC samples

Mixtures of L-DOPA and 3-OMD designated for calibration or QC purposes, containing 250 μg/ml of each analyte, were diluted appropriately with 0.01 M hydrochloric acid containing 0.1% of sodium metabisulfite and 0.01% of disodium EDTA to prepare freshly working standard solutions. These individual solutions were added as 1% spikes to pooled human blank plasma, resulting in final concentrations of 2000, 1000, 500, 100, 50, 35 and 19 ng/ml for calibration, and 1800, 800, 120, 40 and 20 ng/ml for QC for both, L-DOPA and 3-OMD. Spiked plasma pools were dispensed accurately into Eppendorf tubes as 100 μl aliquots and stored frozen at −70 °C until use.

2.4. Sample preparation

Human plasma samples, to which 50 μl of a 10% sodium metabisulfite solution per ml had been added before freezing, were stored at −70 °C until required for analysis. Plasma samples, calibration and QC samples were thawed at room temperature. Immediately after thawing, 100 μl aliquots of samples were processed in polypropylene tubes on ice–water bath, by adding initially 10 μl of 0.01 M hydrochloric acid and 10 μl of ethylene glycol–triethylamine (95:5, v/v) with swift vortex mixing. Then the protein precipitation was performed by adding slowly 100 μl of 0.7 M perchloric acid containing 0.5% sodium metabisulfite and 0.05% disodium EDTA under continuous vortex mixing. The samples were thereafter kept in the ice–water bath for 10 min, followed by a repeated vortex mixing and centrifugation at 1700 *g* for 10 min at 4 °C. The supernatants were carefully transferred into injector vials and applied to the chromatographic system.

2.5. Instrumentation and chromatographic conditions

The HPLC system consisted of a JASCO CO. PU-980 pump, a JASCO AS-950 injector, a JASCO DG-980-50 3-line degasser and a JASCO solvent mixing module HG-980-30. The chromatographic column HR80T 3 μm (4.6 mm i.d. × 80 mm) purchased from ESA Inc., was preceded by an Opti-Guard C18 column (1 mm) from Optimize Technologies Inc., and kept at an analysis temperature of 50 °C by a JASCO CO-965 column oven. Injector and pump were linked to a JASCO LCSS-900 controller.

The system was connected to an ESA Coulochem II detector operated in DC mode (applied potential held constant) and equipped with in line placed ESA Model 5010 (Electrode 1/2) and 5011 (Electrode 3/4) dual-electrode analytical cells operating in redox mode. The potential of the first electrode was set at +100 mV, second electrode at +400 mV, third electrode at +50 mV, forth electrode at −350 mV and cells were set to 1 μA with filter values of 5 s. To protect analytical cells from any occurring solid particle accumulation, column and analytical cells were preceded by ESA graphite filter elements.

The signals of second and forth electrode were monitored by a Waters Corp., data acquisition and processing system with Millennium32 software V3.05 and for each sample analysis two chromatograms were simultaneously obtained.

The aqueous mobile phase contained (per l) 7.8 g of sodium dihydrogenphosphate dihydrate, 50 mg of 1-heptanesulphonic acid sodium salt, 50 μl reagent MB (antiseptic) and pH 2.6 was adjusted with phosphoric acid. The flow-rate during the data acquisition window from 0 to 10 min was kept constant at 1.5 ml/min, followed by a column washout cycle with an increased flow rate at 2.0 ml/min for another 10 min and resulting in a sample injection cycle of 22 min.

An injection volume of 20 μl was applied to the system. Injector needle and sample loop were automatically

conditioned with methanol–water (1:1, v/v) after every injection.

2.6. Calibration curves

A seven-point standard curve (each concentration in duplicate) of peak heights versus plasma concentrations for L-DOPA and 3-OMD, covering each the range from 19 to 2000 ng/ml were daily established by using weighted [1/*x*] least-squares linear regression analysis method.

2.7. Recovery

The recovery from human plasma was determined at three different concentrations ($n = 3$ each) for L-DOPA and 3-OMD by comparing the peak heights obtained from analysis of spiked human plasma samples with known concentrations of L-DOPA and 3-OMD (1800, 800 and 20 ng/ml) versus absolute recovery solutions without plasma deriving from reference solutions with subsequent dilution to identical target concentrations of 1800, 800 and 20 ng/ml with 0.01 mol/l HCl. Regardless of either plasma content or replacement by 0.01 mol/l HCl, 100 μl sample aliquots were subjected to above described protein precipitation procedure and analysis on the HPLC system.

2.8. Selectivity and specificity

Blank plasma samples obtained from six healthy volunteers were assayed to check for interference and to particularly evaluate the extend of endogenously occurring L-DOPA and 3-OMD.

To investigate the retention time of some other potentially interfering L-DOPA related catechol metabolites and drugs, stock solutions of *S*-(−)-carbidopa, (−)-epinephrine, dopamine, DOPAC, 4-hydroxy-3-methoxyphenylacetic acid (HVA) and serotonin were prepared individually at concentrations of 50 μg/ml. Working solutions of 0.5 μg/ml were identically obtained with 0.01 M hydrochloric acid containing 0.1% of sodium metabisulfite and 0.01% of disodium EDTA. In addition, a mixture of all above mentioned compounds including L-DOPA and 3-OMD was prepared at a working concentration of 0.5 μg/ml accordingly.

2.9. Precision and accuracy

The precision of the established method was assessed by calculating the intra-day and inter-day coefficients of variation (CV), using human plasma spiked with five different concentrations (1800, 800, 120, 40 and 20 ng/ml) of L-DOPA and 3-OMD, respectively.

To estimate the intra-day CV, replicated spiked QC samples ($n = 8$) were analyzed and concentrations were calculated by using the appropriate daily established standard curve.

The inter-day CV was calculated by performing a replicate analysis ($n = 8$, 6 and 6) for each concentration of L-DOPA and 3-OMD, respectively, on three consecutive days.

The accuracy was evaluated by calculating the relative error on the total number of assayed samples ($n = 20$) for all assessed concentrations of L-DOPA and 3-OMD, respectively.

The lower limit of quantification (LLOQ) was chosen to be the concentration of the lowest QC sample concentration with an acceptable limit of variance (at LLOQ 20%, above LLOQ 15%).

2.10. Stability

Stability of L-DOPA and 3-OMD in human plasma was assessed with QC samples (1800, 800 and 20 ng/ml, each con-

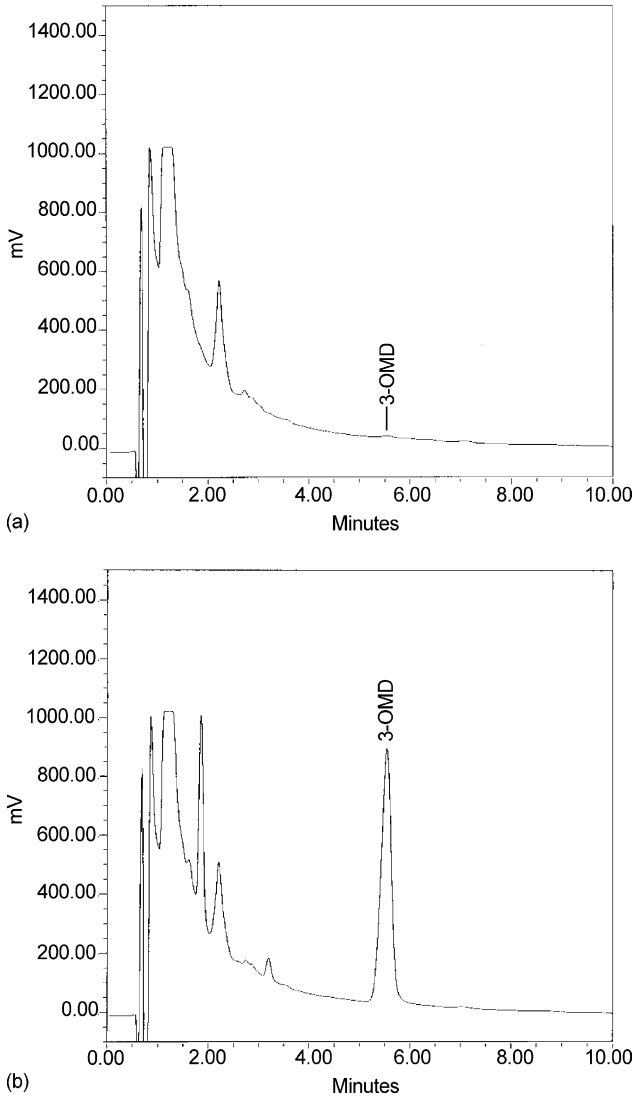


Fig. 2. (a) A typical chromatogram of a human blank plasma sample, monitored at +400 mV at second electrode in series. (b) A typical chromatogram of 3-OMD (1800 ng/ml) spiked to human plasma, monitored at +400 mV at second electrode in series.

centration $n = 3$) stored at −70 °C for up to 16 weeks. Reference solutions for corresponding calibration curves were prepared freshly on each day of measurement (days 1, 21, 56 and 112).

Freeze-thaw stability of L-DOPA and 3-OMD in human plasma was investigated with QC samples (1800, 800 and 20 ng/ml, each concentration $n = 3$) exposed to three cycles of freezing-thawing versus regularly treated QC samples.

Stability of L-DOPA and 3-OMD in protein-precipitated QC samples (20, 800 and 1800 ng/ml, each $n = 8$) was analyzed by storing processed samples in the injector of the chromatographic system at approximately 4 °C for a period of up to 22 h.

2.11. Effect of dilution

The effect of dilution was evaluated by analysis of QC samples (1800 ng/ml) for both L-DOPA and 3-OMD by two- and eight-fold dilutions ($n = 3$ for each dilution) with human blank plasma.

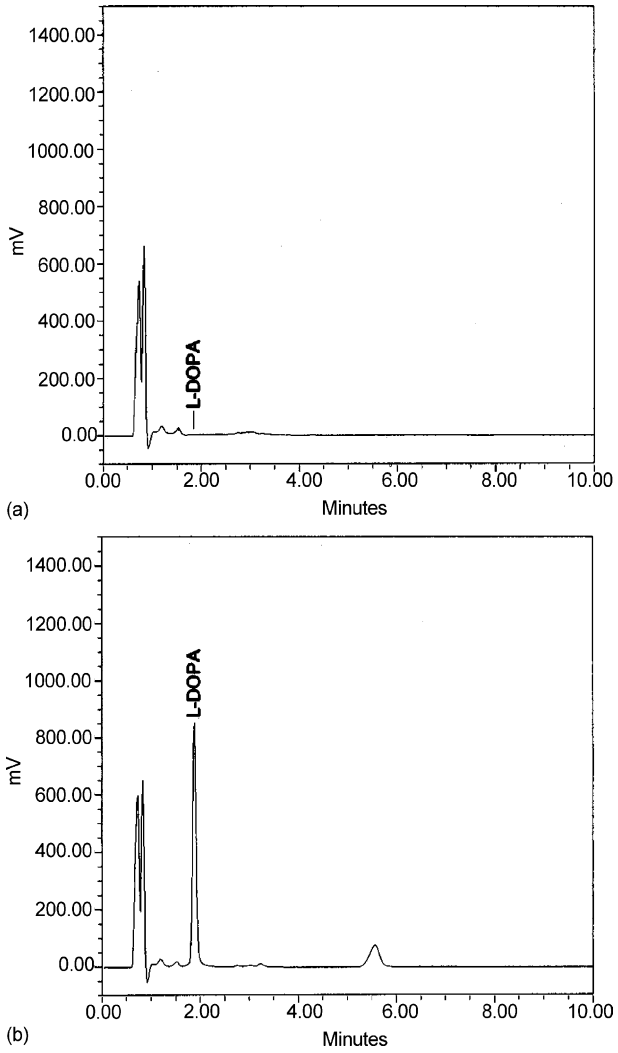


Fig. 3. (a) A typical chromatogram of a human blank plasma sample, monitored at −350 mV at fourth electrode in series. (b) A typical chromatogram of L-DOPA (1800 ng/ml) spiked to human plasma, monitored at −350 mV at fourth electrode in series.

2.12. Impact of replacing parts of instrumentation

Two different lots of the ESA Model 5010 (S/N6877A and S/N7135A) and 5011 (10741H/L and 11330H/L) dual-electrode analytical cells, and chromatographic columns HR80T 3 μm (4.6 mm i.d. × 80 mm, serial nos. 1934 and 1936) were compared by including the lot testing into the overall inter-day analysis of the method validation.

3. Results and discussion

3.1. Selectivity and specificity

Optimal electrochemical detector responses with lowest interference were found at an oxidation potential of +400 mV for 3-OMD. By applying a relatively weak but effective oxidation potential of +100 mV at first electrode in series, eliminating any potential compounds with lower oxidation potential than 3-OMD, further enhancement of the assay selectivity was achieved (Fig. 2).

Considering the rather rapid chromatographic elution of L-DOPA in the periphery of common front-peak patterns, optimal selectivity was reached by implementing a reduction potential of −350 mV for quantification at fourth electrode in series (Fig. 3).

The analysis of various human blank plasma revealed no major interferences at the retention time of L-DOPA and 3-OMD. The lower limits of quantification were set at

Table 1
Corresponding peak height ratios of L-DOPA and 3-OMD, compared with the lower limit of quantification

Human blank plasma[a]	Human blank plasma peak height (μV)	Mean peak height at LLOQ[b] (μV)	Ratio peak height[c]
L-DOPA			
No. 1	503	7070 ($n = 8$)	0.0711
No. 2	300		0.0424
No. 3	608		0.0860
No. 4	471		0.0666
No. 5	454		0.0642
No. 6	308		0.0436
3-OMD			
No. 1	4757	14376 ($n = 8$)	0.331
No. 2	5041		0.351
No. 3	3703		0.258
No. 4	4566		0.318
No. 5	5257		0.366
No. 6	4385		0.305

[a] Human blank plasma of different origin.
[b] Lower limit of quantification (LLOQ) = 20 ng/ml.
[c] Peak height of human blank plasma/mean peak height at LLOQ.

Table 2
Typical retention times of the analytes and some potentially interfering compounds

Test substance		Typical RT (min)	
		L-DOPA[a]	3-OMD[b]
L-3,4-Dihydroxyphenylalanine (L-DOPA)	$C_9H_{11}NO_4$	1.9	1.9
3-Methoxy-L-tyrosine hydrate (3-OMD)	$C_{10}H_{13}NO_4{\cdot}H_2O$	5.6	5.6
S-[−]-α-Hydrazino-3,4-dihydroxy-2-methylbenzenepropanoic acid (*S*-(−)-carbidopa)	$C_{10}H_{14}N_2O_4$	6.0	6.0
[−]-Adrenalin ((−)-epinephrine)	$C_9H_{13}NO_3$	2.2	2.2
3-Hydroxytyramine hydrochloride (DOPAMINE)	$C_8H_{11}NO_2{\cdot}HCl$	3.9	3.9
3,4-Dihydroxyphenylacetic acid (DOPAC)	$C_8H_8O_4$	4.8	4.7
4-Hydroxy-3-methoxyphenylacetic acid (HVA)	$C_9H_{10}O_4$	16.3	16.2
Serotonin (5-hydroxytryptamine hydrochloride)	$C_{10}H_{12}N_2O{\cdot}HCl$	11.5	11.4

[a] Analytical cell; MODEL 5011 electrode no. 4: −350 mV (detection of L-DOPA).
[b] Analytical cell; MODEL 5010 electrode no. 2: +400 mV (detection of 3-OMD).

20 ng/ml for both analytes and proofed to be well above expected endogenously occurring concentrations. Corresponding peak height ratios compared with the lower limit of quantification (20 ng/ml), obtained for each L-DOPA and 3-OMD, were below 0.0860 and 0.366, respectively. Similar data based on chosen LLOQ at 20 ng/ml were also reported by other authors [1]. Despite the occurrence of endogenous 3-OMD in human blank plasma, at LLOQ there was still in reproducible manner a good precision and acceptable accuracy obtained (Table 1).

The pH of the mobile phase was found to be crucially important for the chromatographic resolution of L-DOPA and 3-OMD and taking into consideration several potentially interfering compounds. As a result, pH 2.6 was found to provide optimal selectivity. Typical retention times of both analytes and selected potentially interfering compounds are herewith reported (Table 2).

In conclusion, the presence of listed compounds in human plasma did not influence the determination of L-DOPA and 3-OMD.

3.2. Calibration curves

Calibration curves established on six different days with L-DOPA and 3-OMD spiked to human plasma demonstrated good and reproducible linearity over the selected concentration range from 19 up to 2000 ng/ml, with coefficient of determination r^2 above 0.991 for L-DOPA and above 0.999 for 3-OMD. A weighting factor of $1/x$ was generally used to improve homogeneity of variance. During the development

Table 3
Intra- and Inter-day accuracy and precision of L-DOPA and 3-OMD spiked to human plasma

	Nominal concentration (ng/ml)				
	20.0	40.0	120	800	1800
Intra-day[a] accuracy and precision of L-DOPA spiked to human plasma					
Measured concentrated mean (ng/ml)	23.6	39.8	115	811	1900
Standard deviation (ng/ml)	0.309	1.29	7.51	11.3	36.3
Accuracy (%)	118	99.6	95.7	102	105
CV (%)	1.34	3.37	6.54	1.51	1.97
Intra-day[a] accuracy and precision of 3-OMD spiked to human plasma					
Measured concentrated mean (ng/ml)	20.5	40.3	119	817	1900
Standard deviation (ng/ml)	1.00	1.58	6.47	32.6	86.0
Accuracy (%)	103	101	99.4	102	105
CV (%)	4.95	4.04	5.50	3.90	4.73
Inter-day[b] accuracy and precision on three consecutive days of L-DOPA spiked to human plasma					
Measured concentrated mean (ng/ml)	21.7	37.7	112	853	1880
Standard deviation (ng/ml)	1.68	2.10	6.06	42.4	39.5
Accuracy (%)	109	94.3	93.4	107	104
CV (%)	7.69	5.62	5.40	4.91	2.09
Inter-day[b] accuracy and precision on three consecutive days of 3-OMD spiked to human plasma					
Measured concentrated mean (ng/ml)	20.0	38.8	115	793	1830
Standard deviation (ng/ml)	1.94	3.47	7.30	33.6	79.5
Accuracy (%)	100	97.2	96.0	99.2	102
CV (%)	9.90	8.98	6.41	4.16	4.41

[a] Each concentration $n = 8$.
[b] Each concentration $n = 20$.

of the assay, it was observed that optimal determination of coefficients were obtained by applying $1/x$ weighting, hence with focus also for a good fit at the lower end of the calibration curve.

3.3. Recovery

Absolute recoveries of L-DOPA and 3-OMD spiked to human plasma at 20, 800 and 1800 ng/ml concentrations ($n = 3$ each) ranged between 87.4–89.6 and 100–109%, respectively. Highly reproducible recoveries were achieved by adding triethylamine and ethylene glycol into the perchloric acid precipitation procedure, based on the assumption of changing the protein binding sites with the protonated amine groups and catechol-moieties of the analytes.

3.4. Precision and accuracy

The method was validated with spiked human plasma at five different concentrations, distributed over the entire quantification range, resulted in satisfactory intra-assay precision for both L-DOPA and 3-OMD. Comparably inter-assay precision underlined also the general soundness in terms of reproducibility (Table 3).

3.5. Stability

Because of their catechol structure, analytes are susceptible to oxidation. Satisfactory storage stability for up to 16 weeks at −70 °C was achieved by adding sodium metabisulfite and EDTA as antioxidants to human plasma samples, without any detrimental impact observed on the selected coulometric detection mode (Table 4).

Stability was also confirmed by storing processed plasma samples in the injector of the chromatographic system for a period of up to 22 h at approximately 4 °C. Over the entire timeframe observed concentrations of L-DOPA and 3-OMD ranged between 93.7–114 and 99.4–119%, respectively, without any obvious trend of concentration changes over time.

Freezing and thawing did not reveal any detrimental effect on the absolute concentrations of both analytes spiked to human plasma and investigated at 20, 800 and 1800 ng/ml. After completion of three freezing and thawing cycles, the measured concentrations of L-DOPA and 3-OMD still ranged between 99.7–101 and 101–104%, respectively.

In summary, these stability data clearly suggest that human plasma samples containing above described compounds could be handled under normal laboratory conditions without significant loss.

3.6. Effect of sample dilution

No dilution effect was observed on the absolute concentration of analytes by comparing non-diluted versus two- and eight-fold diluted samples with human blank plasma. The measured and back-calculated concentrations of L-DOPA and 3-OMD still ranged between 95.8–96.3 and 97.7–98.5%, respectively.

Table 4
Stability of L-DOPA and 3-OMD in human plasma stored at −70 °C

	Nominal concentration (ng/ml)		
	20.0	800	1800
L-DOPA			
Day 1			
Accuracy (%)	118	102	105
CV (%)	1.34	1.50	1.97
Day 21			
Accuracy (%)	106	95.5	100
CV (%)	1.64	0.894	1.62
Day 56			
Accuracy (%)	90.8	91.9	96.9
CV (%)	0.841	0.845	0.298
Day 118			
Accuracy (%)	90.0	91.9	96.8
CV (%)	0.962	1.37	0.656
3-OMD			
Day 1			
Accuracy (%)	103	102	105
CV (%)	4.96	3.90	4.72
Day 21			
Accuracy (%)	99.0	96.9	98.7
CV (%)	1.82	1.42	2.49
Day 56			
Accuracy (%)	85.5	95.9	97.8
CV (%)	4.22	1.13	1.12
Day 118			
Accuracy (%)	98.3	99.8	101
CV (%)	2.80	1.37	0.57

Each concentration $n = 3$.

3.7. Impact of replacing parts of the instrumentation

Two different lots of the ESA Model 5010 and 5011 dual-electrode analytical cells, and chromatographic columns HR80T 3 μm (4.6 mm i.d. × 80 mm) were successfully included into the inter-day analysis.

4. Conclusion

The herewith described and fully validated liquid chromatography method with electrochemical detection provides a specific and reproducible assay with a small amount of human plasma required (100 μl aliquots or less), contributing to a considerable reduction of blood taken from patients.

Human plasma samples can be deproteinized with perchloric acid and directly injected to the chromatographic system without further neutralization. The established dual-electrode system operating in redox mode eliminates effectively any potential endogenous and exogenous interferences and provides a highly selective and efficient approach in the field of catecholamine analysis. The method

was developed and validated with subsequent use for the analysis of Phase II clinical samples.

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References

[1] J.G. Nutt, J.H. Fellman, Clin. Neuropharmacol. 7 (1984) 35.
[2] P.T. Mannisto, S. Kaakkola, Trends Pharmacol. Sci. 10 (1989) 54.
[3] M.F. Beers, M. Stern, H. Hurtig, G. Melvin, A. Scarpa, J. Chromatogr. 336 (1984) 380.
[4] T. Ishimitsu, S. Hirose, J. Chromatogr. 337 (1985) 239.
[5] A. Baruzzi, M. Contin, F. Albani, R. Riva, J. Chromatogr. 375 (1986) 165.
[6] Y. Michotte, M. Moors, D. Deleu, P. Herregodts, G. Ebinger, J. Pharm. Biomed. Anal. 5 (1987) 659.
[7] D.C. Titus, T.F. August, K.C. Yeh, R. Eisenhandler, W.F. Bayne, D.G. Musson, J. Chromatogr. 534 (1990) 87.
[8] C. Lucarelli, P. Betto, G. Ricciarello, M. Gianbenedetti, C. Corradini, F. Stocchi, F. Belliardo, J. Chromatogr. 511 (1990) 167.
[9] G. Zurcher, M. Da Prada, J. Chromatogr. 530 (1990) 253.
[10] T. Wikberg, J. Pharm. Biomed. Anal. 9 (1991) 167.
[11] I. Rondelli, D. Acerbi, F. Mariotti, P. Ventura, J. Chromatogr. B 653 (1994) 17.