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Short communication

Simultaneous determination of L-dopa and its prodrug (S)-4-(2-acetamido-3-ethoxy-3-oxopropyl)-1,2-phenylene diacetate in rat plasma by high-performance liquid chromatography–tandem mass spectrometry and its application in a pharmacokinetic study

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ABSTRACT

A sensitive, simple and rapid HPLC–MS/MS method has been developed and validated for the simultaneous determination of L-dopa and its prodrug (S)-4-(2-acetamido-3-ethoxy-3-oxopropyl)-1,2-phenylene diacetate (AEPD) in rat plasma in the present study. The analytes were separated on a C_{18} column (5 μm, 2.1 mm × 150 mm) with a security guard C_{18} column (5 μm, 4 mm × 20 mm) and a triple-quadrupole mass spectrometry equipped with an electrospray ionization (ESI) source was applied for detection. With α-methyldopa as internal standard, sample pretreatment involved in a one-step protein precipitation with 0.4 M perchloric acid. The method was linear over the concentration ranges of 50–5000 ng/ml for L-dopa and 12.5–2500 ng/ml for AEPD. The intra-day and inter-day relative standard deviations (RSD) were less than 15% and the relative errors (RE) were all within 15%. Finally, the method was successfully applied to support the pharmacokinetic study after L-dopa and its prodrug AEPD were orally administrated to the Sprague–Dawley rats, respectively.

1. Introduction

L-Dopa (3,4-dihydroxyphenyl-L-alanine) (Fig. 1A) is a precursor of dopamine (DA), which is deficient in the brains of patients suffering from the progressive disorder of the central nervous system (CNS) known as Parkinson's disease (PD). Peripheral administration of DA cannot be useful for the treatment of PD since DA is unable to cross the blood–brain barrier due to its hydrophilic nature and the absence of active transport mechanism [1]. Instead, L-dopa enters into the CNS through active transport and it is enzymatically decarboxylated in the brain giving rise to DA. Substitution therapy with L-dopa is, however, associated with a number of acute problems. The drug undergoes extensive decarboxylation to dopamine by amino acid decarboxylase in the gastrointestinal tract before entering the systemic circulation and is converted by catechol-O-methyltransferase into the inactive metabolite 3-O-methyldopa before crossing the blood–brain barrier. The main factors responsible for the poor bioavailability and the wide range of inter- and intra-patient variations of plasma levels are the drug's physical–chemical properties: low water and lipid solubility, resulting in unfavorable partition, and the high susceptibility to chemical and enzymatic degradation [2,3]. In order to improve the bioavailability the prodrug approach appeared to be the most promising and some L-dopa prodrugs have been prepared in an effort to solve these problems. An ideal prodrug of L-dopa should be soluble in water and in lipids, completely absorbed by the gastrointestinal tract without any chemical degradation or metabolism, and thus deliver intact L-dopa in the blood stream at a reproducible therapeutic level [4]. Following this idea, we synthesized (S)-4-(2-acetamido-3-ethoxy-3-oxopropyl)-1,2-phenylene diacetate (AEPD) (Fig. 1C) to improve the oral bioavailability of L-dopa. Therefore, a sensitive and accurate analytical method for the simultaneous determination of AEPD and L-dopa is required to support prodrug pharmacokinetic (PK) study. Although many methods have been developed for the determination of L-dopa in plasma, reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) [5–8] is currently used. In the present research, we applied a C_{18} column to determine simultaneously AEPD and L-dopa in rat plasma under the reversed-phase conditions. The developed HPLC–MS/MS method was more convenient comparing with these methods.

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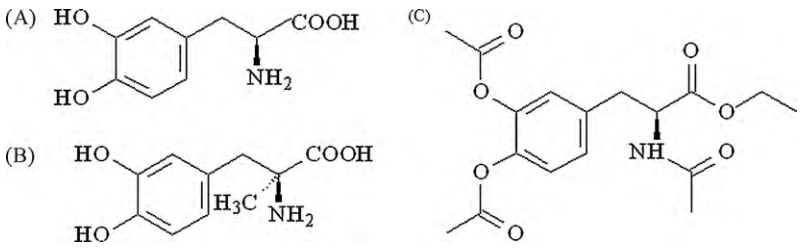

Fig. 1. Chemical structures of L-dopa (A), α-methyldopa (B) and AEPD (C).

2. Experimental

2.1. Chemicals and reagents

L-Dopa (99.6% of purity) and α-methyldopa (internal standard, IS 99.4% of purity) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). AEPD (99.8% of purity) was synthesized in Department of Medicinal Chemistry, Guangxi Medical University (Nanning, China). Acetonitrile and methanol of HPLC grade were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium acetate was obtained from Kelong Chemical (Chengdu, China). Formic acid was purchased from Concord Chemical (Tianjin, China). Water was purified by redistillation and filtered through a 0.22 μm membrane filter before use.

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

The separation was performed on a waters 2695 separation module (Waters Corp., Milford, MA, USA). A XTerra® ms C_{18} (5 μm, 2.1 mm × 150 mm) column and security guard column Phenomenex C_{18} (5 μm, 4 mm × 20 mm) were employed for separation. A gradient elution programme was conducted for chromatographic separation with the mobile phase A (10 mM ammonium acetate solution containing 0.1% formic acid, pH 3.8), and the mobile phase B (acetonitrile) as follows: 0–5.0 min (99% A), 5.01–6.0 min (99% → 30% A), 6.01–12.0 min (30% A), 12.01–13.0 min (30% → 99% A), 13.01–16.0 min (99% A). The flow rate was 0.25 ml/min and column temperature was 30 °C, sample introduction to the mass spectrometer was stopped for 0–1.5 min, 3.0–10.0 min and 13.0–16.0 min using a switching valve after injection into the system. Injection wash solvents were methanol:water (50:50, v/v).

2.2.2. Mass spectrometric conditions

A triple-quadrupole tandem mass spectrometer (Micromass® Quattro micro TM API mass spectrometer, Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) interface was used for analytic detection. The ESI source was set in positive ionization mode. High purity argon was used as the collision gas at a pressure of approximately 3.8×10^{-3} mbar. The optimal MS parameters were as follows: capillary 3.2 kV, source temperature 120 °C and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 650 and 100 l/h, respectively. Cone voltage was 24, 25 and 28 V for L-dopa, α-methyldopa and AEPD, respectively. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 198 → 152 with collision energy (CE) of 15 eV for L-dopa, m/z 212 → 139 with CE of 15 eV for α-methyldopa and m/z 352.01 → 194.2 with CE of 28 eV for AEPD (dwell time of 0.2 s). All data collected in centroid mode were acquired and processed using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standards and quality control samples

Stock standard solutions of L-dopa and AEPD were prepared by dissolving approximate 10 mg of accurately weighted substance in 100 ml of 0.4 M perchloric acid. And the solutions were then serially diluted with 0.4 M perchloric acid to provide working standard solutions of desired concentrations. The IS (10.0 mg) was dissolved and diluted with 0.4 M perchloric acid to yield a stock solution with a concentration of 1.0 mg/ml, which was further diluted with 0.4 M perchloric acid yielding an IS working solution at concentration of 5.0 μg/ml. All the solutions were stored at 4 °C and brought to room temperature before use. Calibration standards were prepared daily by spiking appropriate working standard solutions (50 μl of L-dopa and 50 μl of AEPD) to 100 μl of blank plasma giving L-dopa concentrations of 25, 50, 100, 500, 1000, 3000 and 5000 ng/ml AEPD concentrations of 12.5, 25, 50, 250, 500, 1500 and 2500 ng/ml. The quality control (QC) samples were prepared at low, middle and high concentrations in the same way.

2.4. Plasma sample preparation

Mobile phase (100 μl) and 100 μl of plasma were pipetted to the 1.5 ml polypropylene micro-centrifuge tube. To each tube, 100 μl of IS solution and 100 μl of 0.4 M perchloric acid were added. The mixture was vertex-mixed for 60 s and centrifuged at 12,000 rpm and 4 °C for 10 min. The supernatant was transferred to an autosampler vial where 20 μl was injected into the HPLC–MS/MS system for analysis.

2.5. Method validation

Selectivity was assessed by comparing chromatograms of six different batches of blank rat plasma with the corresponding spiked rat plasma. Linearity was assessed by weighted ($1/x^2$) analysis of six different calibration curves. Intra- and inter-day precision (the relative standard deviation, RSD) and accuracy (the relative error, RE) were determined by analysis of low, medium, and high QC samples ($n = 6$) on three different days. The matrix effect was investigated by comparing the peak areas of analytes in the post-extraction spiked blank plasma at low and high concentrations with those of the corresponding standard solutions. The extraction recovery was determined by comparing the mean peak areas of six extracted samples at low, medium, and high QC concentrations with the mean peak areas of spike-after-extraction samples. The stability was assessed by analyzing replicates ($n = 6$) of low and high QC samples during the sample storage and processing procedures. The freeze–thaw stability was determined after three freeze–thaw cycles. Post-preparation stability was estimated by analyzing QC samples at 24 h at 4 °C. Six aliquots of QC samples were stored at −20 °C for 60 days and at ambient temperature for 4 h to determine long-term and short-term stability, respectively.

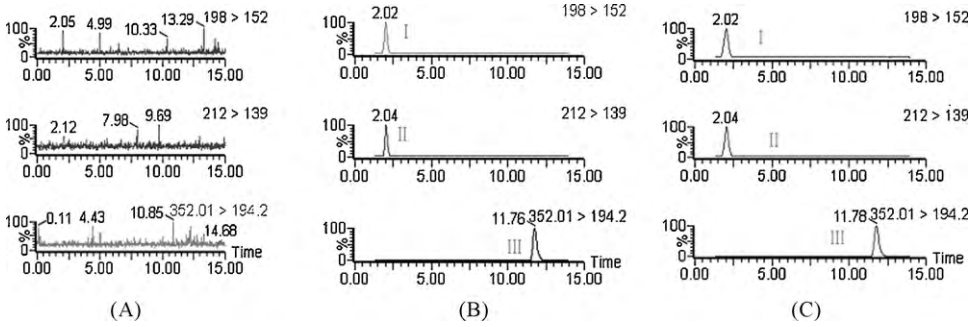

Fig. 2. Representative MRM chromatograms of L-dopa(I), α-methyldopa (IS,II) and AEPD (III) in rat plasmas: (A) a blank rat plasma sample; (B) a blank rat plasma sample spiked with L-dopa (200 ng/ml), AEPD (100 ng/ml), and α-methyldopa (5.0 μg/ml); (C) a rat plasma sample following 20 min after an oral dose of AEPD at 48 mg/kg (calculated as L-dopa) to a Sprague–Dawley rat.

Table 1
Accuracy and precision for the analysis of L-dopa and AEPD in rat plasma (in three validation days, six replicates at each concentration level per day).

Concentration (ng/ml)		RSD (%)		Relative error (%)
Added	Found (mean ± SD)	Intra-day	Inter-day	
L-Dopa				
100.0	102.4 ± 5.0	2.0	5.0	2.4
1000.0	1023.9 ± 52.9	4.1	5.2	2.4
3000.0	2925.1 ± 157.9	3.9	5.9	−2.5
AEPD				
50.0	50.7 ± 4.5	9.1	13.5	1.4
500.0	497.8 ± 19.8	4.1	4.4	−0.4
1500.0	1495.9 ± 90.7	3.7	6.2	−0.3

Table 2
Stability data of L-dopa and AEPD in rat plasma under different conditions ($n = 6$).

Storage conditions	Concentration (ng/ml)		
	Drug	Added	Found (mean ± SD)
Three freeze–thaw cycles	L-Dopa	100.0	102.0 ± 5.7
		3000.0	3060.8 ± 96.7
	AEDP	50.0	52.7 ± 4.9
		1500.0	1515.8 ± 33.9
Long-term (−20 °C for 60 days)	L-Dopa	100.0	100.8 ± 10.5
		3000.0	3034.8 ± 103.5
	AEDP	50.0	51.3 ± 4.4
		1500.0	1501.5 ± 20.0
Short-term(room temperature for 4 h)	L-Dopa	100.0	101.3 ± 3.8
		3000.0	3043.8 ± 141.1
	AEDP	50.0	52.0 ± 5.4
		1500.0	1506.8 ± 40.6
Post-preparative (4 °C for 24 h)	L-Dopa	100.0	95.5 ± 4.5
		3000.0	3016.7 ± 50.1
	AEDP	50.0	50.8 ± 4.4
		1500.0	1514.3 ± 37.9

2.6. Pharmacokinetic (PK) study in rats

Male Sprague–Dawley rats weighing from 200 to 250 g were used for PK study. All animal experiments were performed in accordance with institutional guide lines and were approved by the University Committee on Use and Care of Animals, Guangxi Medical University. The aqueous solutions of L-dopa and AEPD were separately administrated to 12 rats by gavage at 48 mg/kg (all calculated as L-dopa). Serial blood samples (0.2 ml) were obtained at 5, 10, 20, 30, 60, 90, 120, 180, 240, 300 and 360 min after oral administration separately. All samples were placed into heparinized tubes. After centrifugation at 15,000 rpm and 4 °C for 15 min, plasma was collected and frozen at −20 °C until analysis. The maximum plasma concentrations (C_{max}) and their times (T_{max}) were noted directly from the measured data. The elimination rate constant (k_e) was calculated by linear regression of the terminal points in semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve (AUC_{0-t}) to the last measurable plasma concentration (C_t) was calculated using the linear trapezoidal rule.

3. Results and discussion

3.1. Method development

The ESI source provided a better response over the APCI source for the two analytes, especially for AEPD. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[M+H]^+$ m/z 198, 352.01 and 212 for L-dopa, AEPD and IS, respectively. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain highest intensity of protonated molecules of the two compounds and IS. The product ion scan spectra showed high abundance fragment ions at m/z 152, 194.2 and 139 for L-dopa, AEPD and IS, respectively. The collision gas pressure and collision energy of collision-induced decomposition (CID) were optimized for maximum response of the fragmentation of the two compounds. Multiple reaction monitoring (MRM) using the precursor → product ion transition of m/z 198 → 152 for L-dopa, m/z 352.01 → 194.2 for AEPD and m/z 212 → 139 for IS. The protein precipitation using methanol or acetonitrile was commonly employed, but during our exploration, we found that this protein precipitation method resulted in a bad extraction recovery for L-dopa and IS. After referencing Kazuo Igarashi's method [9], we found using 0.4 M perchloric acid could solve this problem.

3.2. Method validation

Fig. 2 shows the typical chromatograms of a blank, a spiked plasma sample with L-dopa (100 ng/ml), AEPD (50 ng/ml) and the internal standard (5.0 μg/ml), a plasma sample from a rat after an oral administration of AEPD. No interference from the endogenous compound with the analytes and the internal standard was detected. The matrix effects calculated were in the range of −9.0% to 8.5%, which was within the acceptable limits. Calibration curves were obtained between the mass responses and the plasma concentration over the range of 50–5000 ng/ml for L-dopa and 12.5–2500 ng/ml for AEPD. The typical regression equation were as follows: L-dopa, $Y = 2.25 \times 10^{-4}X + 1.06 \times 10^{-2}$; AEPD, $Y = 7.53 \times 10^{-3}X - 6.02$, where Y is the peak area ratio of L-dopa or AEPD to the internal standard, X is the concentration of L-dopa or AEPD (ng/ml). All correlation coefficients (r)

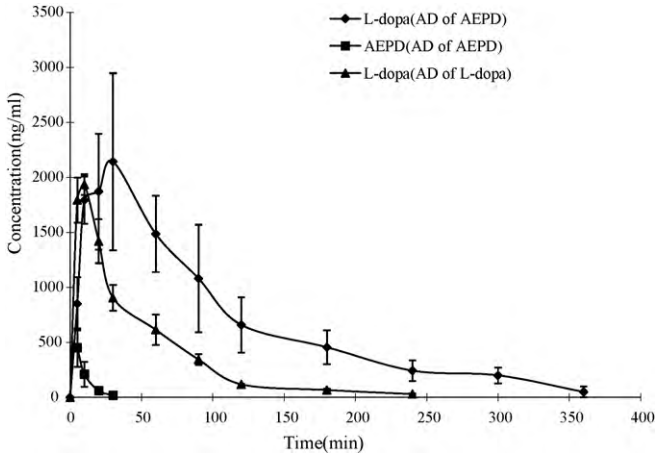

Fig. 3. Mean plasma concentration–time profiles of L-dopa and AEPD in the Sprague–Dawley rats. (♦): L-dopa and (■): AEPD following oral administration of AEPD to 12 rats (48 mg/kg, calculated as L-dopa); (▲): L-dopa following oral administration of L-dopa to 12 rats (48 mg/kg).

exceeded 0.99, showing a good linearity over the concentration range. The lower limit of quantitation (LLOQ) was 25 ng/ml for L-dopa and 12.5 ng/ml for AEPD in rat plasma. The intra- and inter-day precision and accuracy for L-dopa and AEPD at three QC concentration levels were shown in Table 1. The results indicated that all the values were within the acceptable range of 15% and the method is accurate and precise. The mean extraction recovery were 85.78 ± 4.10%, 87.63 ± 5.67%, and 92.40 ± 3.55% for L-dopa at 100, 1000, and 3000 ng/ml, and 86.93 ± 5.30%, 89.63 ± 12.18%, and 86.32 ± 5.41% for AEPD at 50, 500, and 1500 ng/ml, respectively. The mean recovery of the internal standard was 84.72 ± 3.69%. Stability results in Table 2 demonstrated that L-dopa and AEPD were stable in rat plasma under the indicated conditions.

3.3. Application to PK study in the Sprague–Dawley rats

This validated method was successfully applied to PK studies of L-dopa and AEPD following oral administration of L-dopa and AEPD to 12 Sprague–Dawley rats at 48 mg/kg (all calculated as L-dopa), respectively. Mean plasma concentration–time curve of L-dopa and AEPD in single does study is shown in Fig. 3. Since AEPD was rapidly hydrolyzed into L-dopa by the esterase in vivo, its concentration in plasma was low. It is clear that oral administration of AEPD is able to enhance oral bioavailability of L-dopa. The maximum plasma concentration (C_{max}) was 1980.7 ± 538.5 and 1936.6 ± 114.6 ng/ml, the time of maximum plasma concentration (T_{max}) was 24.5 ± 3.5 and 4.5 ± 0.8 min, the area under the plasma concentration–time curve from 0 h to the time of last measurable concentration (AUC_{0-t}) was 217,158.9 ± 70,832.1 and 94,469.5 ± 7183.0 ng/ml min, the half-life of drug elimination at the terminal phase ($t_{1/2}$) was 56.5 ± 14.4 and 30.6 ± 1.6 h for L-dopa after administration of AEPD and L-dopa after administration of L-dopa, respectively.

4. Conclusion

A sensitive, simple and rapid HPLC–MS/MS method was developed for the simultaneous analysis of L-dopa and AEPD in single does study in rat plasma. It has been successfully applied to the PK study of L-dopa and AEPD in the rat.

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