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Quantitative analysis of levodopa, carbidopa and methyldopa in human plasma samples using HPLC-DAD combined with second-order calibration based on alternating trilinear decomposition algorithm

Shu-Fang Li, Hai-Long Wu*, Yong-Jie Yu, Yuan-Na Li, Jin-Fang Nie, Hai-Yan Fu, Ru-Qin Yu

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China

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ABSTRACT

An HPLC method combined with second-order calibration based on alternating trilinear decomposition (ATLD) algorithm has been developed for the quantitative analysis of levodopa (LVD), carbidopa (CBD) and methyldopa (MTD) in human plasma samples. Prior to the analysis of the analytes by ATLD algorithm, three time regions of chromatograms were selected purposely for each analyte to avoid serious collinearity. Although the spectra of these analytes were similar and interferents coeluted with the analytes studied in biological samples, good recoveries of the analytes could be obtained with HPLC-DAD coupled with second-order calibration based on ATLD algorithm, additional benefits are decreasing times of analysis and less solvent consumption. The average recoveries achieved from ATLD with the factor number of 3 ($N = 3$) were 100.1 ± 2.1, 96.8 ± 1.7 and $104.2 \pm 2.6\%$ for LVD, CBD and MTD, respectively. In addition, elliptical joint confidence region (EJCR) tests as well as figures of merit (FOM) were employed to evaluate the accuracy of the method.

1. Introduction

Levodopa [(*S*)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid, LVD] is a precursor of the neurotransmitter dopamine, widely used in the clinical treatment of Parkinson's disease [1]. It could be converted to dopamine by DOPA decarboxylase and capable of crossing the protective blood-brain barrier, whereas dopamine itself cannot. To avoid adverse reactions caused by elevated levels of dopamine in peripheral tissues, LVD is often administered in combination with carbidopa [(2*S*)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropanoic acid, CBD], an inhibitor of the decarboxylase enzyme, which does not cross the blood-brain barrier. Methyldopa [(*S*)-2-amino-3-(3,4-dihydroxyphenyl)-2-methyl-propanoic acid, MTD], which is an old antihypertensive agent, is converted to 1-methyl dopamine and 1-methyl norepinephrine [2]. In addition, the United State Pharmacopoeia (USP) specifies MTD as one of the most important impurities in the analysis of levodopa–carbidopa (LVD–CBD) combination formulation [3]. Table 1 displays the chemical structures of LVD, CBD and MTD.

Changes in the concentration of these drugs in the body may influence the bioavailability and biopharmaceutical properties of the pharmaceutical preparation and, subsequently, their magnitude of action. Thus, the determination of LVD and its inhibitors and impurities in biological fluids have an essential role in the diagnostics of diseases related to them. Their simultaneous determination in pharmaceutical preparations and biological fluids are commonly carried out by using high performance liquid chromatography (HPLC) [4–9], capillary electrophoresis (CE) [10,11], and spectrophotometry [12–16]. Methods utilizing synchronous fluorescence [17] or NMR spectroscopy [18] have also been developed. The detection limit for the kinetic-spectrophotometric method reported by Chamsaz et al. [15] was 0.14, 0.07 and 0.12 $\mu g\,mL^{-1}$ for CBD, MTD and LVD, respectively. The detection limit obtained from the NMR spectroscopy analysis [18] was estimated as 4.2, 1.7 and 1.6 $\mu g\,mL^{-1}$ for LVD, CBD and MTD, respectively. The recovery studies performed on human serum samples ranged from about 82–96% with relative standard deviations of <4%. Most of the spectrophotometric methods are chemometrics-assisted spectrophotometric ones for the analysis of these analytes in pharmaceutical preparations. These methods are commonly based on the different kinetic behavior of oxidation because of their serious overlapping spectra. In addition, the experimental conditions should be carefully optimized. Most of HPLC methods are using high performance liquid chromatography with electrochemical detection (HPLC-ED) for their analysis in the biological fluids. Muzzi et al. [9] have also utilized HPLC with fluorescence

* Corresponding author. Tel.: +86 731 88821818; fax: +86 731 88821818.
E-mail address: hlwu@hnu.cn (H.-L. Wu).

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detection after derivatization with a fluorescent reagent for the detection of LVD and its main derivatives in the serum samples. To our knowledge, the simultaneous determination of LVD, CBD and MTD by using high performance liquid chromatography with diode array detection (HPLC-DAD) has not been reported in the literature.

In recent years, a growing number of papers in multi-way data analysis [19–29] involved the use of second-order data generated by HPLC-DAD. Second-order instruments involving separation are ideally suited for the analysis of complex samples and are frequently used as powerful tools for chemical analysis. Even under the best experimental conditions, the probability of peak overlap in chromatographic separations can become quite severe, especially for highly complex samples. In these cases, we could resort to the application of second-order calibration methods, which might light a new avenue to utilize "mathematical separation" to replace the "physical or chemical separation" of uncalibrated background or interferences by the chemometrical separation of their signals. In contrast to other traditional measurement techniques, these methods allow for accurate quantification of the analytes of interest even in the presence of uncalibrated interference, which is known as "second-order advantage" [30]. Several applications [19,21,23,26,28,29] of HPLC-DAD data in combination with second-order calibration based on alternating trilinear decomposition (ATLD) algorithm have been reported for the determination of some analytes of interest in complex samples and even for removal of three-dimensional background drift in comprehensive two-dimensional (2D) liquid chromatography coupled with diode array detection (LC × LC-DAD) data [31].

In this paper, HPLC-DAD coupled with second-order calibration based on ATLD algorithm has been applied to simultaneously determine LVD, CBD, and MTD in human plasma samples in the selected region of retention time for them. There are two main difficulties arose in identifying and quantifying the analytes in this work. Firstly, the chromatographic peaks of the matrix interferences overlapped with those of the analytes. The second-order calibration method based on ATLD algorithm solved this problem. Secondly, the spectra of the analytes are almost parallel and serious overlapped in the useful spectral range. The selection of the retention time domain for each analyte is crucial to avoid the collinearity problem in the second-order calibration. Furthermore, under the condition of a retention time shift, the second-order chromatographic standardization [32] was applied to align the data sets and to get better analytical figures of merit. With the second-order calibration method, one can simplify the sample preparation procedure only by using a convenient solvent extraction with methanol and employ a simple mobile phase in an isocratic mode. The time and resources required for the quantification process is shorter than other methods.

2. Theory

2.1. ATLD algorithm

The ATLD algorithm [19] is a decomposition method for three-way data arrays, which is an improvement of the traditional PARAFAC (parallel factor analysis) algorithm without any constraints. It is based on an alternating least squares principle and an improved iterative procedure that utilizes the Moore-Penrose generalized inverse obtained by singular value decomposition. When the data follows the famous trilinear component model which has been widely accepted owing to its consistency with Beer's law in chemistry, ATLD can make the calibration possible even in the presence of interferences that are absent in the calibration samples.

A trilinear model for second-order calibration is depicted as follows:

$$x_{ijk} = \sum_{n=1}^{N} a_{in} b_{jn} c_{kn} + e_{ijk}$$

$$(i = 1, 2, \ldots, I;\ j = 1, 2, \ldots, J;\ k = 1, 2, \ldots, K), \quad (1)$$

where N denotes the number of factors, which should correspond to the total number of detectable species, consisting of the component(s) of interest and the background as well as unknown interferences. In the case of HPLC-DAD system, I denotes the number of elution time data points and J is the number of wavelengths, respectively. If the K samples, consisting of the calibration samples and the predication samples, are stacked, a three-way trilinear array of data $\mathbf{X}$ is obtained with dimensions of $I \times J \times K$. x_{ijk} is the element of $\mathbf{X}$ and e_{ijk} is the element of an $I \times J \times K$ three-way residual array $\mathbf{E}$. a_{in} is the element of an $I \times N$ matrix $\mathbf{A}$ corresponding to elution profiles; b_{jn} is the element of a $J \times N$ matrix $\mathbf{B}$ corresponding to spectral profiles; c_{kn} is the element of a $K \times N$ matrix $\mathbf{C}$ corresponding to relative concentrations.

The loss functions to be minimized are the sum of squares of the residual matrices, which may be written as

$$\sigma_1(\mathbf{A}) = \sum_{i=1}^{I} ||\boldsymbol{X}_{i..} - \boldsymbol{B}\ diag(\boldsymbol{a}_{(i)})\boldsymbol{C}^T||_F^2, \quad (2)$$

$$\sigma_2(\boldsymbol{B}) = \sum_{j=1}^{J} ||\boldsymbol{X}_{.j.} - \boldsymbol{C}\ diag(\boldsymbol{b}_{(j)})\boldsymbol{A}^T||_F^2, \quad (3)$$

$$\sigma_3(\boldsymbol{C}) = \sum_{k=1}^{K} ||\boldsymbol{X}_{..k} - \boldsymbol{A}\ diag(\boldsymbol{c}_{(k)})\boldsymbol{B}^T||_F^2, \quad (4)$$

where $||\bullet||_F^2$ denotes the Frobenius matrix norm. Eqs. (2)–(4) can be considered to be equivalent to each other owing to the cyclic symmetry the property of trilinear model. The ATLD algorithm minimizes alternately one of the above-mentioned loss functions over **C** on fixed **A** and **B**, then over **A** on fixed **B** and **C**, and then over **B** on fixed **C** and **A**. The updates for **A**, **B** and **C** from (2)–(4), based on the least squares principle, are

$$\mathbf{a}_{(i)}^T = diagm(\mathbf{B}^+\mathbf{X}_{i..}(\mathbf{C}^T)^+) \quad (i = 1, 2, \ldots, I) \quad (5)$$

$$\boldsymbol{b}_{(j)}^T = diagm(\boldsymbol{C}^+\boldsymbol{X}_{.j.}(\boldsymbol{A}^T)^+) \quad (j = 1, 2, \ldots, J) \quad (6)$$

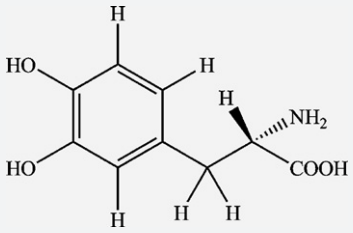
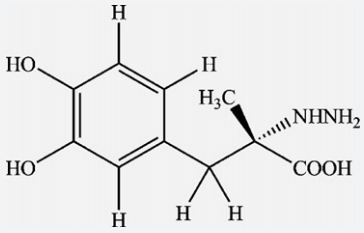
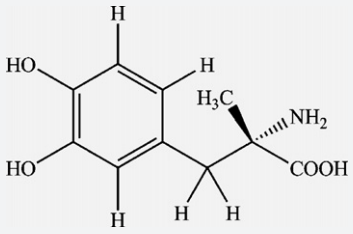
$$\boldsymbol{c}_{(k)}^T = diagm(\boldsymbol{A}^+\boldsymbol{X}_{..k}(\boldsymbol{B}^T)^+) \quad (k = 1, 2, \ldots, K) \quad (7)$$

here $diagm(\bullet)$ denotes a column N-vector whose elements are diagonal elements of a square matrix. In each iteration cycle, **A** and **B** are normalized column-wise to unit length. With the aid of the resolved profile matrices, the analyte(s) concentrations can be obtained by regression of the appropriate column of **C** corresponding to each analyte against its standard concentrations, which is second-order calibration. ATLD holds the fastest convergence, which is attributed to the operation based on sliced matrices with less size and two other major strategies. One is the truncated least squares method which uses the tolerance to truncate the small singular values in the singular value decomposition, the other is the operation of selecting diagonal elements which also makes ATLD retain trilinearity property indeed and be insensitive to the component number. The advantages have been reviewed by Fleming and Kowalski [33].

2.2. Figures of merit

The estimation of analytical figures of merit (FOM), such as sensitivity (SEN), selectivity (SEL) and limit of detection (LOD), are used

Table 1
Chemical structures and retention time domain examined of levodopa (LVD), carbidopa (CBD) and methyldopa (MTD); t_1 is the first scan analysed and t_2 the last one. The value between parentheses is the retention time of each analyte in minutes.

Analyte	Structure	t_1–t_2 (t_{Ret}), min
Levodopa (LVD)		3.07–3.34 (3.2)
Carbidopa (CBD)		3.54–3.81 (3.66)
Methyldopa (MTD)		3.37–3.60 (3.46)

herein to investigate the performance of the developed method. In second-order calibration, the idea of FOM is easy to be comprehended by resorting to the useful concept of net analyte signal (NAS) [34], which is defined as the part of the signal that relates uniquely to the analyte of interest. Different approaches for the calculation of the FOM have been extensively discussed in the literature [35,36].

The sensitivity is estimated as the NAS at unit concentration, and the selectivity is the ratio between the sensitivity and the total signal. The following equations can be obtained to estimate the SEN and SEL in this presently studied case:

$$\mathrm{SEN} = k\{[(\boldsymbol{A}^T\boldsymbol{A})^{-1}]_{nn}[(\boldsymbol{B}^T\boldsymbol{B})^{-1}]_{nn}\}^{-1/2}, \quad (8)$$

$$\mathrm{SEL} = \{[(\boldsymbol{A}^T\boldsymbol{A})^{-1}]_{nn}[(\boldsymbol{B}^T\boldsymbol{B})^{-1}]_{nn}\}^{-1/2}, \quad (9)$$

where nn designates the (n, n) element of a matrix and k is the total signal for component n at unit concentration, which is also a parameter for converting scores to concentrations.

The limit of detection (LOD) [37] can be calculated as LOD = 3.3s(0), where s(0) is the standard deviation in the predicted concentration estimated for three different background blank plasma samples in the ATLD algorithms. The limit of quantification (LOQ) is computed as LOD = 10s(0).

3. Experimental

3.1. Reagents and chemicals

Levodopa (LVD), carbidopa (CBD) and methyldopa (MTD) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Changsha, China). Drug free human plasma was received from the National Blood Center (Changsha, China).

Stock solutions of LVD (68 μg mL^{-1}), CBD (126 μg mL^{-1}) and MTD (64.5 μg mL^{-1}) were prepared by accurately weighing the required amounts of the corresponding compounds, and dissolving with 0.01 M hydrochloric acid solution. All solutions were protected from light and stored in a refrigerator at 4–5 °C. Under these conditions, the solutions were stable for 15 days.

Appropriate LVD, CBD and MTD working solutions of different concentrations were prepared by diluting stock solutions with mobile phase solution (methanol (25%, v/v) and 0.002 mol L^{-1} KH_2PO_4 (pH 5) solution (75% (v/v))). Methanol used for HPLC-DAD measurements and solution preparation was of HPLC grade. Ultrapure water was prepared with a Milli-Q water purification system (Millipore, USA).

3.2. Instrumentation and chromatographic conditions

The HPLC system used was an LC-20AT liquid chromatographic system (Shimadzu Corporation, Japan), which consists of a degasser, four pumps, a manual injector provided with a 20 μL loop, a column oven and a SPD-M20A diode array detector (DAD). The separation was carried out in a Hypersil-ODS analytical column (125 mm × 4.0 mm, 5.0 μm, Shimadzu, Japan). The LC solution software was used for controlling the instrument, data acquisition, and data interpretation. In the sample preparation procedure, a centrifuge (Sigma, Germany) and ultrasonic instrument (China) were used.

The mobile phase consisted of methanol (25%, v/v) and 0.002 mol L^{-1} KH_2PO_4 (pH 5) solution (75%, v/v) in an isocratic mode, which was pumped at a flow rate of 1.0 mL min^{-1} with 20 μL injection volume. The solvents were filtered daily through

Table 2
Concentrations of levodopa (LVD), carbidopa (CBD) and methyldopa (MTD) in calibration and prediction samples.

Sample	Added concentration ($\mu g\,mL^{-1}$)		
	LVD	CBD	MTD
Calibration samples			
C1	0.000	5.670	0.000
C2	2.720	5.040	3.225
C3	3.400	4.410	6.450
C4	4.080	3.780	3.870
C5	4.760	3.150	5.805
C6	5.780	2.520	4.515
C7	6.800	0.000	0.000
C8	0.000	0.000	5.160
Prediction samples			
P1	3.808	5.166	3.612
P2	4.080	4.914	5.676
P3	4.352	4.662	4.128
P4	4.624	4.410	4.386
P5	4.896	4.158	4.902
P6	5.168	3.906	5.160
P7	5.440	3.654	4.644
P8	5.712	3.402	3.870
P9	5.984	3.150	5.418
P10	6.256	2.898	5.934

a 0.45 μm cellulose membrane filter before use. The column temperature was set at 30 °C. Photometric detection was performed in the range of 190–400 nm, with a spectral resolution of 1.2 nm. Data was obtained over an integration period of 640 ms per spectrum. In the MATLAB environment all homemade programs were written and further used for data analysis. All calculations were carried out on a microcomputer under the Windows XP operating system.

3.3. Sample preparation

Two hundred and fifty microliters of plasma samples were mixed with 750 μL methanol. The mixture was vortexed for 1 min and then centrifuged at 12,000 rpm for 10 min in a centrifuge at 10 °C. The supernatant was transferred into a 10-mL volumetric flask and diluted to the mark with the mobile phase. Then the solution was filtered through a 0.45 μm cellulose syringe filter and an aliquot of 20 μL was injected into HPLC system.

3.4. Calibration samples

A calibration set of 10 samples (C1–C8) was constructed. The concentrations were randomly selected, covering the linear range of concentrations and avoiding the collinearity between analytes. The levels corresponded to values in the linear range of 0–7.00 $\mu g\,mL^{-1}$ for LVD, 0–6.00 $\mu g\,mL^{-1}$ for is CBD and 0–7.00 $\mu g\,mL^{-1}$ for MTD. Concentrations of each analyte in the calibration samples are listed in Table 2. Duplicate analysis was performed for each sample, and HPLC-DAD was measured in random order according to the sample number.

3.5. Prediction samples

Ten plasma samples (P1–P10), each 250 μL in volume, were spiked with suitable amounts of standard LVD, CBD and MTD solutions, and treated as explained in Section 3.3. The final analyte concentrations of prediction samples were within the calibration concentration range and also listed in Table 2. Duplicate analysis was performed for each sample, and HPLC-DAD was measured in random order according to the sample number.

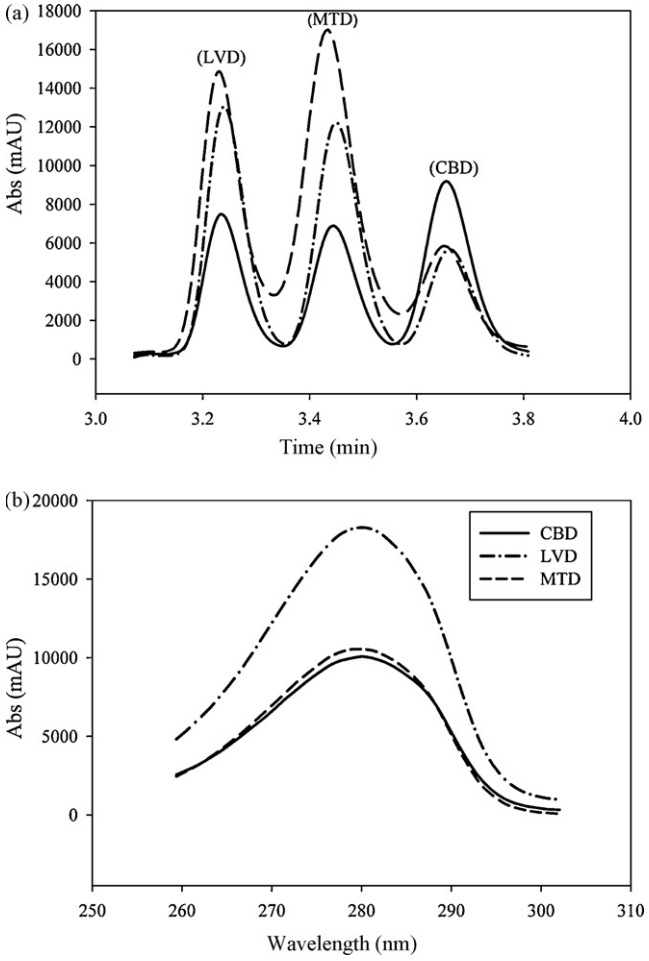


Fig. 1. (a) Chromatograms at 280 nm for samples C2 (solid line), C5 (dash-dot line), and P9 (dashed line); (b) UV spectra of levodopa (LVD) (dash-dot line), carbidopa (CBD) (solid line) and methyldopa (MTD) (dashed line) in corresponding samples C7, C1 and C8, respectively.

4. Results and discussion

4.1. General consideration

In this experiment, simple mobile phase of methanol–0.002 mol L^{-1} KH_2PO_4 (pH 5) solution was selected. In order to determine the best ratio to be used, different ratio of methanol/0.002 mol L^{-1} KH_2PO_4 (pH 5) solution were tested. Taking into account the elution time, the peak profile and the spectral character, a ratio of methanol/0.002 mol L^{-1} KH_2PO_4 (pH 5) solution (25:75, v/v) was chosen. With this composition of mobile phase, the retention times of levodopa (LVD), carbidopa (CBD) and methyldopa (MTD) were 3.20, 3.66 and 3.46 min, respectively.

Fig. 1(a) shows the chromatographic profiles recorded at 280 nm of samples C2, C5 and P9. As can be seen, the separation of these analytes is basically achieved in the calibration samples C2 and C5. However, there is apparently coelution problem in the prediction sample P9, because of the interferences derived from biological matrix. Great effort may be necessary to optimize the chromatographic conditions of the separation for the problem (the overlap of the analytes with matrix interferences), but a complicated chromatographic condition means more time and resources would be consumed. Alternatively, one can resort to the application of multichannel detector (such as diode array detection, DAD) coupled with the second-order calibration method based on ATLD algo-

rithm, which can give satisfactory estimates of analytes in complex samples.

It is important to note that second-order calibration requires that the data show the property of trilinearity, which can be lost if the chromatographic retention time are not exactly reproducible. In this paper, necessary mathematical alignment would be made in those chromatographic data which existed peak misalignments. In addition, the collinearity problem is also very crucial for data analysis of second-order calibration. The absorption spectra of LVD, CBD and MTD in corresponding pure samples C7, C1 and C8 are shown in Fig. 1(b). It can be observed that the maximum UV absorption wavelengths of these analytes are λ_{max} = 280 nm and the absorption spectra of them are almost parallel and a serious overlapping is present in the useful spectral range (260–302 nm). This hindered the application of second-order calibration based on ATLD algorithm for the quantitative analysis of the analytes.

Since the retention times of LVD, CBD and MTD were 3.24, 3.66 and 3.46 min, respectively, we can selected three time domains from the raw data (Table 1), i.e., in the range of 3.07–3.34, 3.54–3.81 and 3.37–3.60 min for LVD, CBD and MTD, respectively. So the determination of LVD, CBD and MTD was exactly a single analyte determination in their own elution time domain, as well as eliminated the possibility of collinearity problem, finally realized the simultaneous determination of the analytes.

4.2. Quantitative analysis of LVD, CBD and MTD

As discussed above, an identical wavelength range of 260–302 nm ($\Delta\lambda$ = 1.2 nm) and three time domains 3.07–3.34 min (Δt = 640 ms), 3.54–3.81 (Δt = 640 ms) and 3.37–3.60 min (Δt = 640 ms) were adopted for the analytes. Three-way data generated from HPLC-DAD (ultraviolet-visible spectra registered at different elution times) were arranged in three tensors with dimensions $26 \times 36 \times 18$, $26 \times 36 \times 18$ and $23 \times 36 \times 18$ for LVD, CBD and MTD, respectively. The first dimension refers to the chromatographic mode (number of scans, the time domain examined for each analyte is detailed in the last column of Table 1), the second one to the spectral mode (number of wavelengths) and the third one to the sample mode (number of calibration samples and prediction samples).

In the calibration samples, it was observed that single analyte respond in the selected regions, with almost no interference from other sample component. Nevertheless, it was obvious that interference coeluted with each analyte studied in corresponding regions in human plasma samples. Under such circumstance, we turn to use second-order calibration based on ATLD which allows the concentration of individual components to be correctly estimated even in the presence of uncalibrated interferences. Although the ATLD algorithm is insensitive to the component number, it can perform well with more acceptable calibration and resolution and give better result when the most appropriate factors have been adopted. A three-component models (N = 3) was built for each analyte, refer to the suggestion of the core consistency diagnostic [38] analysis. One factor is modeled for each analyte, one is corresponding to the plasma, and the remaining one factor is mainly fit the noise or nonlinearity effects in the data array, which is regarded as background. The contribution from background takes a very small part, whose values in the resolved relative concentration profiles are almost equal to zeros.

Hence, three three-component ATLD models (N = 3) were performed, one for each analyte. The chromatographic profiles as well as spectral profiles of the analytes and concentration information were resolved from the decomposition of the three-way data array obtained for both the calibration and predicted samples by using the ATLD (Figs. 2–4) with a factor number of three (N = 3). Figs. 2–4 show the resolved chromatographic profiles with the relative

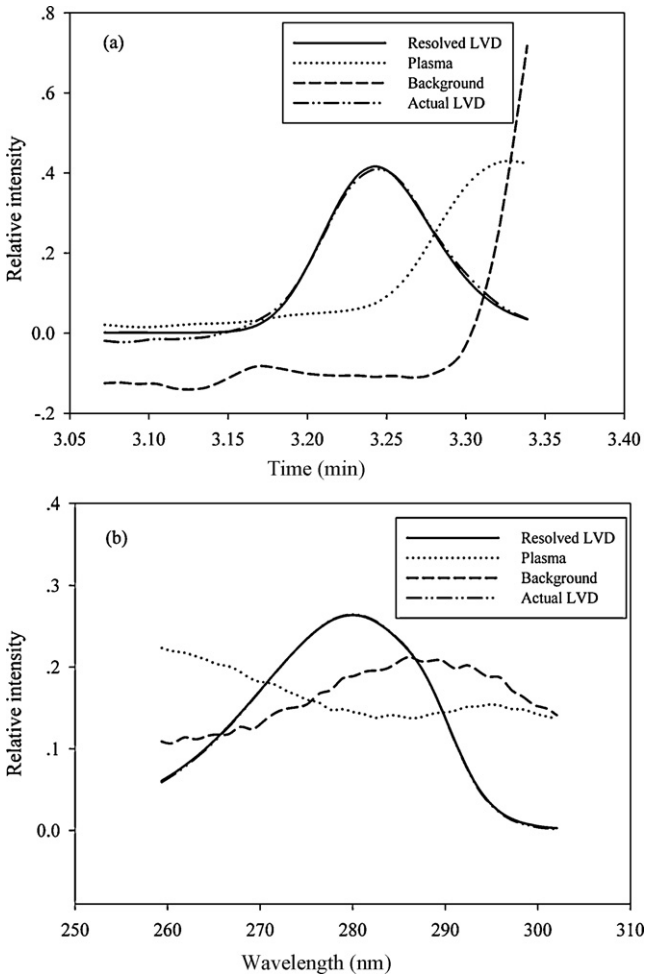


Fig. 2. (a) Chromatographic profiles, normalized to unit length, obtained from ATLD when the number of factors was chosen as three for the spiked plasma samples and actual elution profile of levodopa (LVD). (b) Spectral profiles, normalized to unit length, obtained from ATLD when the number of factors was chosen as three for the spiked plasma samples and actual spectral profile of levodopa (LVD).

absorbance intensity as a function of time and the corresponding spectral profiles with the relative absorbance intensity as a function of wavelength in plasma samples together with the actual ones. The loadings associated with the chromatographic mode were shown in Figs. 2(a), 3(a) and 4(a), and the loadings associated with the spectral mode were shown in Figs. 2(b), 3(b) and 4(b). These chromatographic profiles as well as spectral profiles were collected into matrices **A** and **B**, respectively. In Figs. 2–4, the solid lines represent the loadings of the LVD, CBD and MTD, respectively; the dotted lines represent the loadings for an inherent interference deriving from human plasma; and the dash lines represent the background consist of noise and nonlinearity effects. Besides, the dash-dot-dot lines denote the actual profiles. It can be appreciated that the resolved profiles are quite similar to the actual ones, which confirms the accuracy and reliability of the proposed strategy, and satisfactory predicted results of the analyte concentrations maybe expected.

With the aid of the resolved profile matrices, the analyte(s) concentrations can be obtained by a linear-regression of the appropriate column of absolute concentration modes (relative concentrations matrix **C**) corresponding to each analyte against its standard concentrations in spiked plasma samples, which is second-order calibration. The prediction results for LVD, CBD and MTD using second-order calibration based on the ATLD algorithm with N = 3 were summarized in Table 3. For LVD, CBD and MTD, the average predicted recoveries gained from ATLD are 100.1 ± 2.1,

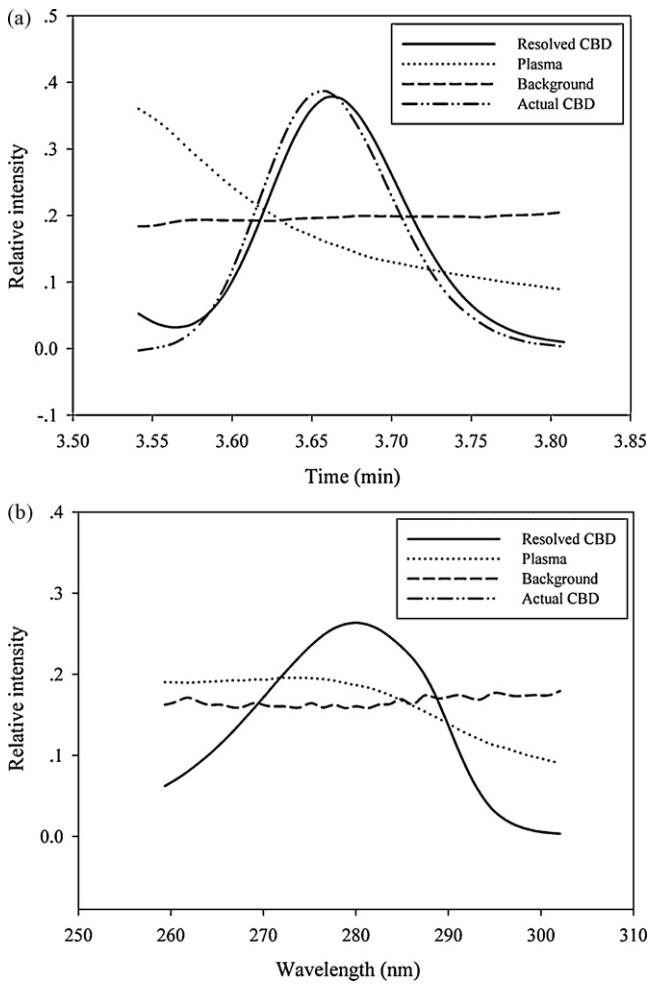


Fig. 3. (a) Chromatographic profiles, normalized to unit length, obtained from ATLD when the number of factors was chosen as three for the spiked plasma samples and actual elution profile of carbidopa (CBD). (b) Spectral profiles, normalized to unit length, obtained from ATLD when the number of factors was chosen as three for the spiked plasma samples and actual spectral profile of carbidopa (CBD).

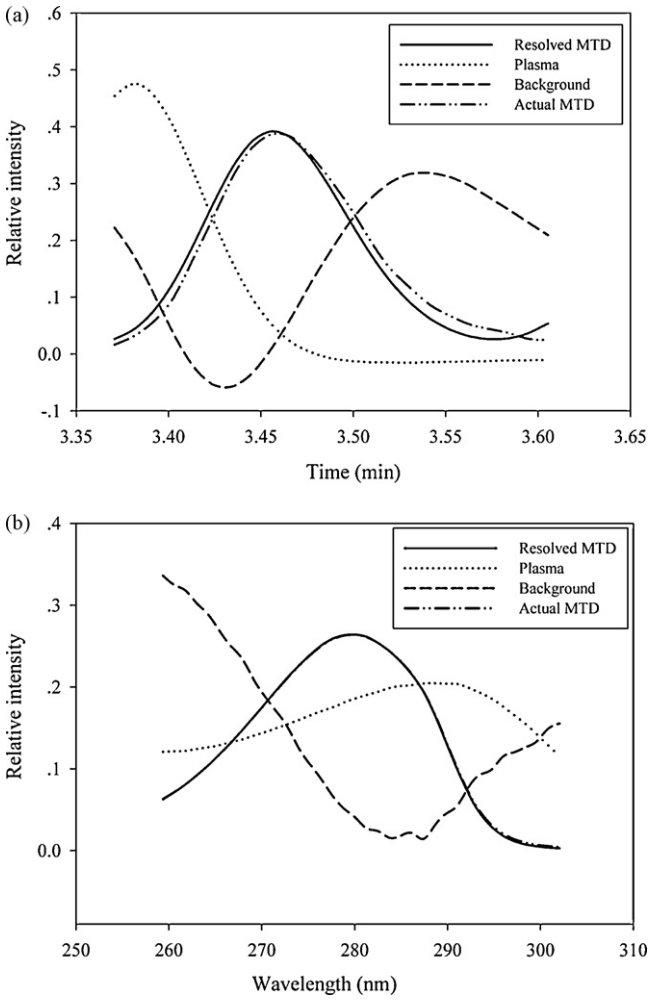


Fig. 4. (a) Chromatographic profiles, normalized to unit length, obtained from ATLD when the number of factors was chosen as three for the spiked plasma samples and actual elution profile of methyldopa (MTD). (b) Spectral profiles, normalized to unit length, obtained from ATLD when the number of factors was chosen as three for the spiked plasma samples and actual spectral profile of methyldopa (MTD).

96.8 ± 1.7 and $104.2 \pm 2.6\%$, respectively. The correlation coefficients of LVD, CBD and MTD are 0.9974, 0.9980 and 0.9989. The results of the root-mean-square error of prediction (RMSEP) obtained from the three analytes are 0.1337 $\mu g\,mL^{-1}$ for LVD, 0.1475 $\mu g\,mL^{-1}$ for CBD and 0.2374 $\mu g\,mL^{-1}$ for MTD. These results indicate that the second-order calibration based on ATLD algorithm provides for the analysis with a satisfactory prediction capacity to quantitatively determinate LVD, CBD and MTD in human plasma samples.

Table 3
Resolved concentrations and recoveries obtained by applying ATLD[a] analysis ($N = 3$) to human plasma samples spiked with levodopa (LVD), carbidopa (CBD) and methyldopa (MTD).

Sample	LVD ($\mu g\,mL^{-1}$)		CBD ($\mu g\,mL^{-1}$)		MTD ($\mu g\,mL^{-1}$)	
	Added	Predicted	Added	Predicted	Added	Predicted
P1	3.808	3.775[b] (99.1)	5.166	5.046 (97.7)	3.612	3.710 (102.7)
P2	4.080	4.167 (102.1)	4.914	4.895 (99.6)	5.676	5.923 (104.4)
P3	4.352	4.225 (97.1)	4.662	4.425 (94.9)	4.128	4.408 (106.8)
P4	4.624	4.632 (100.2)	4.410	4.366 (99.0)	4.386	4.772 (108.8)
P5	4.896	4.828 (98.6)	4.158	3.967 (95.4)	4.902	5.231 (106.7)
P6	5.168	4.951 (95.8)	3.906	3.645 (93.3)	5.160	5.311 (102.9)
P7	5.440	5.584 (102.6)	3.654	3.609 (98.8)	4.644	4.981 (107.3)
P8	5.712	5.667 (99.2)	3.402	3.281 (96.4)	3.870	4.007 (103.5)
P9	5.984	6.122 (102.3)	3.150	3.015 (95.7)	5.418	5.376 (99.2)
P10	6.256	6.502 (103.9)	2.898	2.830 (97.6)	5.934	5.916 (99.7)
Average recovery (%)		100.1 ± 2.1[c]		96.8 ± 1.7[c]		104.2 ± 2.6[c]

[a] In all cases, the number of components for the analysis of each analyte is three.
[b] Denotes the recoveries in the parentheses.
[c] Denotes standard deviation of the recoveries.

Table 4
Statistical parameters and figures of merit for determination of levodopa (LVD), carbidopa (CBD) and methyldopa (MTD) in human plasma samples using second-order calibration based on ATLD algorithm.

Parameter	LVD	CBD	MTD
RMSEP ($\mu g\,mL^{-1}$)	0.1337	0.1475	0.2374
Linear range ($\mu g\,mL^{-1}$)	0.00–7.00	0.00–6.00	0.00–7.00
R^a	0.9974	0.9980	0.9989
SEN/AFU ($mL\,\mu g^{-1}$)	8.57×10^3	4.28×10^3	8.71×10^3
SEL	0.3604	0.2405	0.4344
LOD ($\mu g\,mL^{-1}$)	0.0531	0.0079	0.0805
LOQ ($\mu g\,mL^{-1}$)	0.161	0.024	0.244

[a] R is the correlation coefficient.

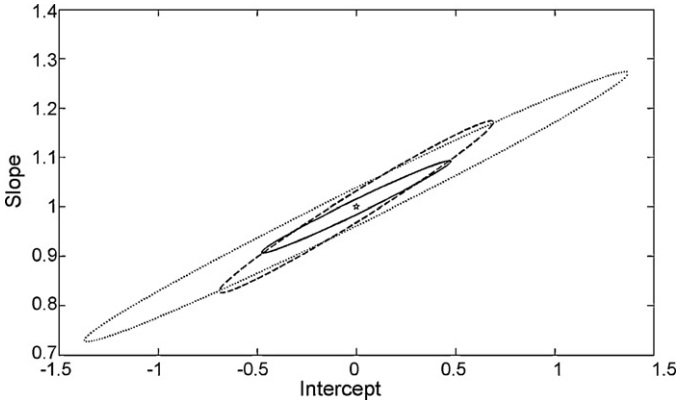


Fig. 5. EJCR plots for the application of the ATLD algorithm in the determination of levodopa (LVD) (solid line), carbidopa (CBD) (dashed line), and methyldopa (MTD) (dotted line) in human plasma samples. The pentacle indicates the ideal points (0, 1).

4.3. Method validation

For the sake of evaluating the performance of the developed method, some statistical parameters and analytical figures of merit including sensitivity (SEN), selectivity (SEL) as well as limit of detection (LOD) for the determination of LVD, CBD and MTD in human plasma samples using ATLD algorithm were investigated and summarized in Table 4. The root-mean-square error of prediction (RMSEP) can be calculated in terms of the formula as $\mathrm{RMSEP} = [(1/M)\sum_{m=1}^{M}(c_{act} - \hat{c}_{pred})^2]^{1/2}$, where M is the number of prediction samples, c_{act} and $\hat{c}_{pred}$ are the actual and predicted concentrations, respectively. It seems that the overall prediction ability of the ATLD algorithm is satisfactory. Moreover, in order to acquire further insight into the accuracy of the proposed algorithm, a linear-regression analysis of actual versus prediction concentration was applied [39]. The estimated intercept and slope were compared with their ideal values of 0 and 1, based on an elliptical joint confidence region (EJCR) test [40]. All EJCR plots contain the ideal point (0, 1), labeled with a pentacle (☆) in the experimental ellipse (Fig. 5). The elliptic size corresponding to MTD is bigger, but a high precision is shown by LVD and CBD. This fact further proves that the ATLD algorithm can give accurate results for the quantitative determinations of LVD, CBD and MTD in human plasma samples.

5. Conclusions

In the present work, we selected three time domains from the raw three-way data recorded by HPLC-DAD for levodopa (LVD), carbidopa (CBD) and methyldopa (MTD), so the determination of these analytes was exactly a single analyte determination in their own elution time domain, finally realized the simultaneous determination of the analytes. Though the chromatographic and spectral peaks between the analytes and biological matrix were heavily overlapped, ATLD could provide accurate concentration prediction together with good resolution of chromatographic and spectral profiles for the analytes of interest in complex system even in the presence of unknown and uncalibrated interferences, fully exploiting "second-order advantage" in the selected region. The results obtained indicate that the HPLC-DAD combined with second-order calibration methods based on ATLD algorithm is an attractive alternative strategy for the quantitative analysis of LVD, CBD and MTD in biological samples. The application in our work is only a limited example of the enormous potentiality of this strategy in the biomedical analytical fields, HPLC-DAD data in combination with ATLD holds great potential to be extended as a promising alternative for more applications in other fields, such as food, environmental analysis can also be imagined.

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