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Simultaneous determination of cortisol and prednisolone in body fluids by using HPLC–DAD coupled with second-order calibration based on alternating trilinear decomposition

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Abstract

A novel method for simultaneous determination of cortisol and prednisolone in body fluids has been developed in this paper. Three-way data recorded by high-performance liquid chromatography with a diode array detector (HPLC–DAD) have been analyzed by second-order calibration based on the alternating trilinear decomposition (ATLD) algorithm. The chemometric methodology selected exploits the second-order advantage of the three-way data arrays, which allows one to obtain concentrations of individual calibrated analytes even in the presence of interferences not present in the calibration samples (e.g. background in urine or plasma). It was applied to simultaneous determination of cortisol and prednisolone in both plasma and urine samples. Though the chromatographic and spectral peaks of the analytes were heavily overlapped and interferents coeluted with the compounds studied, good recoveries of the analytes could be obtained with HPLC–DAD coupled with second-order calibration based on ATLD. Sample preparation was based on solvent extraction (SE), and quantification can be carried out with simple mobile phase. The time required for the quantification process is shorter than other methods.

Keywords: Second-order calibration; HPLC–DAD; Alternating trilinear decomposition; Cortisol; Prednisolone; Plasma; Urine

1. Introduction

Glucocorticoids are important in predicting against shock, stress, inflammation, etc. [1]. Cortisol excreted from the adrenocortex with a circadian rhythm is one of the most important glucocorticoids. It plays an important role in human physiology, and is being a useful marker for the diagnosis under pathologic conditions. The determination of cortisol is commonly used for proper diagnosis of adrenal function. Prednisolone is another widely used antiinflammatory and immunosuppressive agent [2]. The main side effect of prednisolone is the suppression of plasma and urinary cortisol [3].

Simultaneous determination of cortisol and prednisolone in both urine and plasma is useful for monitoring cortisol and prednisolone therapy, helping physicians to exclude the risk of subtherapeutic concentrations and assess overdose in the case of non-compliance with prescribed therapy.

There are many methods for the determination of cortisol and prednisolone, e.g. radio immuno assay (RIA) [4,5], LC-mass spectrometry or LC-tandem mass spectrometry [6–15], micellar electrokinetic capillary chromatography (MECC) [16,17], and fluorimetric derivation before HPLC analysis [18,19]. Most of these methods need internal standards [12,15], and most of them are only validated for urine [4,20,21] or plasma analysis [8,22–26]. Due to the structural similarity between cortisol and prednisolone (Fig. 1), it is not surprising that the two analytes have similar spectral profiles and close retention times. For the separation of these analytes, multi-component mobile phases [27] or gradient elution [12,14] could be used, and often long elution times for cortisol and prednisolone of up to 20 min were required [12,22,23].

In order to eliminate proteins from the sample and to concentrate the analytes, sample extraction is required before chromatographic separation. The common methods in sample extraction are solid phase extraction (SPE) [16,27] and solvent extraction (SE) [14]. These extraction processes are usually not selective enough, and often a number of interfering components are co-extracted with the interested analytes. If the compounds studied

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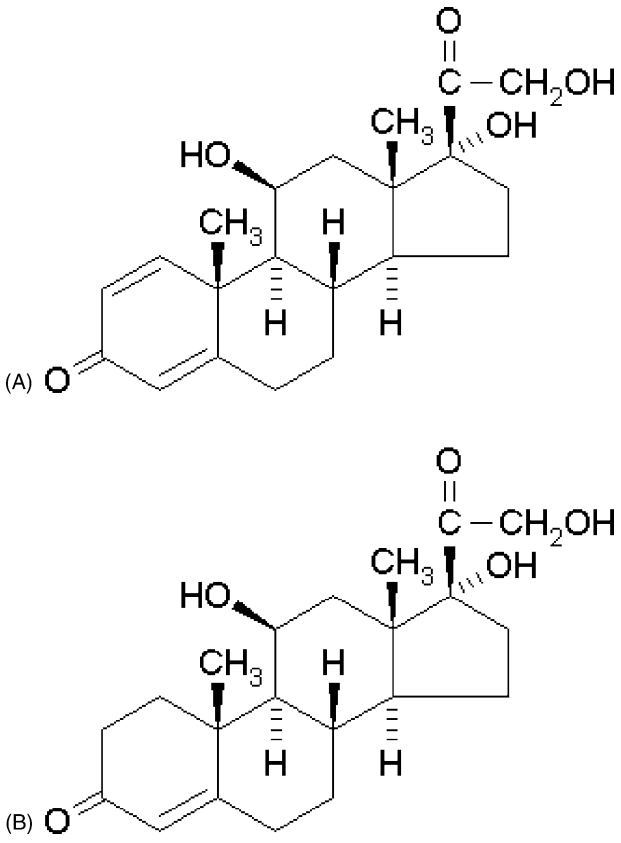

Fig. 1. Chemical structures of prednisolone (A) and cortisol (B).

are to be quantified by simple chromatographic separation, these interferences have to be separated from the analytes. However, in most occasions, complete separation is hard to achieve or the separation time is too long.

In these cases, we could turn to the application of second-order calibration algorithms [28–34], which do not require complete separation of the analytes. Examples of these methods applied in HPLC–DAD analysis are alternating trilinear decomposition algorithm (ATLD) [31], generalized rank annihilation method (GRAM) [35,36], parallel factor analysis (PARAFAC) [37] and multivariate curve resolution–alternating least squares (MCR–ALS) [38,39]. They make quantification possible even in the presence of unknown interferences. This property has been called the "the second-order advantage" [40].

In the present paper, second-order calibration based on ATLD is employed to determine the concentration of cortisol and prednisolone in body fluids from second-order HPLC–DAD data. In this analysis, solvent extraction was used in sample preparation, since it is simple and convenient. A simple mobile phase consisting of methanol and water was used. Though heavily overlapped chromatographic peaks of the analytes and interferences were obtained and the spectra of these species were also overlapped, the powerful ATLD algorithm can resolve the overlapped peaks into corresponding chromatographic, spectral and concentration profiles even in the presence of unknown coeluting interferences.

2. Theory

The ATLD algorithm [31] is an alternative one for decomposition of three-way data arrays, which is an improvement of the traditional PARAFAC 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. The ATLD-based second-order calibration exploited the second-order advantage making the calibration possible even in the presence of interferences that are not present in the calibration samples, so it can provide satisfactory concentration estimates [41–43].

Second-order data are usually produced from hyphenated instruments such as HPLC–DAD and excitation/emission spectrofluorometer. Suppose a given sample produces a data matrix of size $I \times J$ or second-order array, where I and J denote the number of data points in the first and second dimensions, respectively. In the case of HPLC–DAD, I is the number of elution time data points and J is the number of wavelengths. If K samples, consisting of calibration samples and prediction samples, are stacked, a three-way data array $\underline{\mathbf{X}}$ is obtained with dimensions $I \times J \times K$. A trilinear model for such a three-way array $\underline{\mathbf{X}}$ has the form

$$x_{ijk} = \sum_{n=1}^{N} a_{in} b_{jn} c_{kn} + e_{ijk} \quad (i = 2, \ldots, I; \quad j = 1, 2, \ldots, J; \quad k = 1, 2, \ldots, K) \tag{1}$$

where x_{ijk} is the element (i,j,k) of $\underline{\mathbf{X}}$, N denotes the number of factors, which should correspond to the total number of detectable species, including component(s) of interest and background as well as unknown interferences; e_{ijk} is the element of an $I \times J \times K$ three-way residual array $\underline{\mathbf{E}}$; a_{in} is the element (i, n) of an $I \times N$ matrix $\mathbf{A}$ corresponding to elution profiles of N species; b_{jn} is the element (j, n) of a $J \times N$ matrix $\mathbf{B}$ corresponding to relative sensitivity of N species at different wavelengths; c_{kn} is the element (k, n) of a $K \times N$ matrix $\mathbf{C}$ corresponding to relative concentrations of N species in K.

The objective function to be minimized is the sum of the squares of the elements of the residual matrices, it may be written as

$$\sigma_1 = \sum_{k=1}^{K} \left\| \mathbf{X}_{\cdot\cdot k} - \mathbf{A}\,\mathrm{diag}(c_{(k)})\mathbf{B}^{\mathrm{T}} \right\|_F^2 \tag{2}$$

owing to the cyclic symmetry property of the trilinear model, (2) may also be represented as

$$\sigma_2 = \sum_{i=1}^{I} \left\| \mathbf{X}_{i\cdot\cdot} - \mathbf{B}\,\mathrm{diag}(\mathbf{a}_{(i)})\mathbf{C}^{\mathrm{T}} \right\|_F^2 \tag{3}$$

$$\sigma_3 = \sum_{j=1}^{J} \left\| \mathbf{X}_{\cdot j\cdot} - \mathbf{C}\,\mathrm{diag}(\mathbf{b}_j)\mathbf{A}^{\mathrm{T}} \right\|_F^2 \tag{4}$$

where $||\cdot||_F$ and diag$(\cdot)$ denote the Frobenius matrix norm and the diagonal matrix of order $N \times N$, respectively. $\mathbf{X}_{i\cdot\cdot}$, $\mathbf{X}_{\cdot j\cdot}$ and

$\mathbf{X}_{..k}$ represent the horizontal, lateral and frontal slices of three-way data array $\underline{\mathbf{X}}$, respectively.

Using the objective functions mentioned above, the ATLD algorithm minimizes alternately one of the three objective 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

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

$$\mathbf{b}_{(j)}^{\mathrm{T}} = \mathrm{diagm}(\mathbf{C}^{+}\mathbf{X}_{.j.}(\mathbf{A}^{\mathrm{T}})^{+}),\ j = 1, \ldots, J \tag{6}$$

$$\mathbf{c}_{(k)}^{\mathrm{T}} = \mathrm{diagm}(\mathbf{A}^{+}\mathbf{X}_{..k}(\mathbf{B}^{\mathrm{T}})^{+})k = 1, \ldots, K \tag{7}$$

where diagm(·) 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.

Using the Moore–Penrose generalized inverses based on singular value decomposition, the ATLD algorithm has the property of being insensitive to the estimated component numbers and fast convergence. For detail information of ATLD algorithm, one could refer to [31].

3. Experimental

3.1. Reagents

Cortisol and Prednisolone were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). Standard solutions of each compound were prepared in methanol at a concentration of 10 μg/ml and stored at 4 °C. All the working solutions were prepared by diluting these standard solutions. Methanol and acetonitrile were of HPLC grade. Analytical grade ethyl acetate was used in the extraction procedure. Ultra pure water was prepared using a Milli-Q water purification system (Aquapro, China).

3.2. Instrumentation

Analysis was performed using an Agilent-1100 liquid chromatographic system (Agilent Technologies, USA), which consisted of a degasser, a pump, a manual injector provided with a 20 μl loop, a column oven and a diode array detector (DAD). The separation was carried out using a Hypersil-ODS analytical column (125 mm × 4.0 mm, 5.0 μm; Agilent Technologies, USA). In the extraction procedure, a TG16-II Tabletop centrifuge was used, and the extraction solvent was evaporated in a W201B constant-temp heading bath (Shanghai). All algorithms were programmed in Matlab environment.

3.3. Chromatography

The mobile phase was isocratic and consisted of methanol (60% (v/v)) and water (40% (v/v)), pumped at a flow rate of 1.0 ml/min with sample injection volume of 20 μl. The column temperature was set at 25 °C. Photometric detection was performed in the range 200–380 nm, with a spectral interval of 2 nm.

3.4. Sample handling and extraction procedure

Cortisol free plasma and urine samples were prepared in the way as described in [27]. Then plasma tubes were thawed, to 1 ml of the individual plasma samples, an appropriate amount of working solution was added. Each sample was mixed with 6 ml of ethyl acetate and conditioned at room temperature for 10 min and then centrifuged at 1789 × g for 20 min. The layers were allowed to separate, and the upper ethyl acetate layer was transferred to another tube. The aqueous layer was extracted once more with another 1 ml of ethyl acetate, mixed, centrifuged and separated in the same way. The combined ethyl acetate extract was dried under nitrogen at 40 °C and reconstituted with methanol to 250 μl. An aliquot of 20 μl was injected into the HPLC column.

Urine samples were stored immediately after collection at −70 °C until analysis. First, urine tubes were thawed and centrifuged at 644 × g for 15 min. To 1 ml of individual urine sample, an appropriate amount of working solution was added. Then the samples were treated in the same way as the plasma samples described above.

3.5. Analytical procedure

3.5.1. Simultaneous determination of prednisolone and cortisol in urine

Mixtures with varying concentrations of prednisolone and cortisol were analyzed by HPLC–DAD, and calibration and prediction data sets were constructed. A seven-sample set was built for calibration with ATLD. The levels correspond to values in the range 0.0–450.0 ng/ml for prednisolone and 0.0–400.0 ng/ml for cortisol. Eight prediction samples were built with analyte concentrations within the calibration range. The first four were artificial samples prepared in methanol containing only prednisolone and cortisol. And the other four of the eight prediction samples were urine samples with known amount of the two analytes to testify the performance of ATLD algorithm when the sample contains complicated species that were not present in the calibration set. These urine samples were treated before chromatographic analysis in the same way as described in Section 3.4. Table 1 lists the concentrations of each analyte in the calibration and prediction samples. The calibration and prediction sets samples were concentrated by four times as the urine samples did. Samples of 20 μl were injected into the chromatographic system and analyzed under conditions as described in Section 3.3. A duplicate analysis was performed for each sample.

3.5.2. Quantification of prednisolone in plasma in the presence of cortisol

The determination of predinisone in plasma was done by the proposed method. Table 2 lists the concentrations of the samples. In the calibration sample, cortisol was not added, but it was added in the prediction samples acting as an interferent. Four of the seven prediction samples were plasma samples, the rest were artificial samples. The plasma samples were treated before chromatography separation in the same way as described in Section 3.4. And duplicate analysis was performed for each sample.

Table 1
Calibration and prediction sets for simultaneous determination of prednisolone and cortisol in urine

Sample number	Added concentrations (ng/ml)	
	Prednisolone	Cortisol
Calibration sample		
1	50.0	350.0
2	100.0	300.0
3	200.0	200.0
4	300.0	150.0
5	400.0	100.0
6	450.0	50.0
7	0.0	400.0
Prediction sample		
1	75.0	250.0
2	150.0	200.0
3	250.0	150.0
4	350.0	100.0
5[a]	0.0	0.0
6[a]	75.0	250.0
7[a]	125.0	175.0
8[a]	200.0	175.0

[a] Prepared using urine as the matrix.

3.5.3. Quantification of cortisol in urine in the presence of prednisolone

In this section, cortisol in urine in the presence of prednisolone was determined. Samples with varying concentrations of analytes were analyzed by HPLC–DAD, and calibration and prediction data sets were constructed. Table 3 lists the concentrations of each calibration and prediction sample. Prednisolone was only added in the prediction samples acting as an interferent. And the last four of the prediction samples were urine samples. Before chromatography analysis, these urine samples were treated in the same way as described in Section 3.4 and also the calibration and prediction samples were concentrated. A duplicate analysis was performed for each sample.

Table 2
Calibration and prediction sets for quantification of prednisolone in plasma in the presence of cortisol

Sample number	Added concentrations (ng/ml)	
	Prednisolone	Cortisol
Calibration sample		
1	50.0	–
2	100.0	–
3	200.0	–
4	400.0	–
5	450.0	–
Prediction sample		
1	100.0	200.0
2	200.0	150.0
3	275.0	100.0
4[a]	0.0	0.0
5[a]	125.0	100.0
6[a]	150.0	100.0
7[a]	200.0	150.0

[a] Prepared using plasma as the matrix.

Table 3
Calibration and prediction sets for quantification of cortisol in urine in the presence of prednisolone

Sample number	Added concentrations (ng/ml)	
	Prednisolone	Cortisol
Calibration sample		
1	–	50.0
2	–	100.0
3	–	200.0
4	–	300.0
5	–	350.0
6	–	400.0
Prediction sample		
1	275.0	125.0
2	200.0	175.0
3	125.0	200.0
4	150.0	250.0
5[a]	0.0	0.0
6[a]	250.0	100.0
7[a]	200.0	150.0
8[a]	150.0	250.0

[a] Prepared using urine as the matrix.

4. Results and discussion

4.1. Optimisation of the experimental condition

In this experiment, simple mobile phase of methanol–water was selected. In order to determine the best ratio to be used, three different ratio of methanol/water were tested, 60:40, 50:50 and 40:60 (v/v). It had been shown that with a methanol/water ratio of 60:40 (v/v), the retention times of prednisolone and cortisol were 2.32 min and 2.36 min, respectively, the chromatographic and spectral peaks of their mixture were completely overlapped, apparently only one chromatographic peak could be seen; with a methanol/water ratio of 50:50 (v/v), the retention times were 4.55 min and 4.68 min respectively, and the overlapping peak of their mixture was not improved much, still only one chromatographic peak could be seen; and with a methanol/water ratio of 40:60 (v/v), the elution times of the two substances both reached as long as 12.6 min, the chromatographic peaks of their compounds were still heavily overlapped and floating peak tails appeared. Taking into account the elution time and the peak profile, a methanol/water ratio of 60:40 was chosen. Also, different temperatures of 25 °C and 30 °C were tried, considering the peak profile and the retention time, 25 °C was selected. In [12], it was reported that modified LC conditions with less polar mobile phase and higher column temperature (30 °C) were beneficial for achieving better separation. However, in this experiment, with the mobile phase of methanol–water, the two substances could not be separated thoroughly even in less polar mobile phase and at relatively high temperature.

4.2. Extraction

Both ethyl acetate and acetonitrile were tried as the extraction solvent. The recoveries of prednisolone and cortisol extracted with the two solvents were compared, with ethyl acetate, the

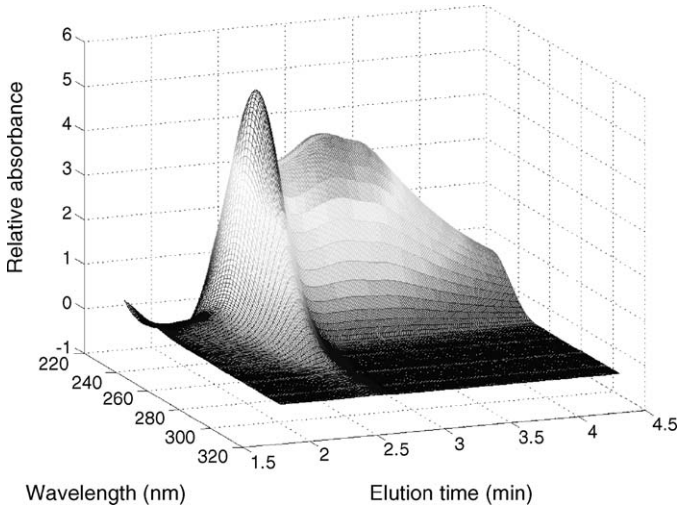

Fig. 2. Three-dimensional plot of the HPLC–DAD data for urine sample 7[a]. The absorbance is shown as a function of wavelength and retention time.

average recoveries of prednisolone and cortisol were 95% and 92% respectively, but the average recoveries of below 70% for both analytes were obtained with acetonitrile, so ethyl acetate was selected for extraction.

4.3. Data analysis

Due to the similar property of prednisolone and cortisol, their peaks are overlapped; owing to the complexity of the sample and the low selectivity of the detector, the urine and plasma samples exhibit several peaks, which were probably originated from species coeluted with the compounds of interest (Fig. 2). Under such circumstance we turn to ATLD, which can give satisfactory estimates of analytes in complex samples.

In the simultaneous determination of prednisolone and cortisol in urine, the response data were taken over an elution time range of 1.79–4.33 min ($\Delta t = 1/150$ min) and a wavelength range of 200–318 nm ($\Delta\lambda = 2$ nm), the recorded data were combined into a $381 \times 50 \times 11$ data array, here the number 11 corresponds to seven calibration samples plus four urine samples in the prediction set. The three-way data matrix obtained was then decomposed with ATLD, a four-component model ($N = 4$) was built, as the core consistency diagnostic [44] suggested $N = 4$ was the best choice. No scaling or centring was done before modeling.

As an example, a three-dimensional plot of the absorbance as a function of retention time and wavelength in the HPLC–DAD data for urine sample 7[a] in Table 1, is shown in Fig. 2. As can be seen, the peaks of prednisolone and cortisol are heavily overlapped, and the urine peaks coeluted with the analytes. In Fig. 3, the loadings from the ATLD decomposition of data for samples (calibration samples and urine samples in prediction set) in Table 1 are shown. The loadings related to the chromatographic profiles are shown in Fig. 3A (a1, a2, a3, a4), the spectral profiles in Fig. 3B (b1, b2, b3, b4). The notation of loadings refers to that used in Eq. (1). In Fig. 3A, loadings a1, a2, and a3 represent the chromatographic profiles of prednisolone, cortisol and urine respectively, loading a4 represents the background. The spectral profiles of the two analytes in urine are shown in Fig. 3B, where loadings b1, b2, b3, and b4 represent prednisolone, cortisol, urine and the background respectively. In looking at chromatograms and spectra of the mixture in Fig. 2 and its pure component chromatograms and pure spectra in Fig. 3, one can judge the difficulty in analyzing such mixture samples.

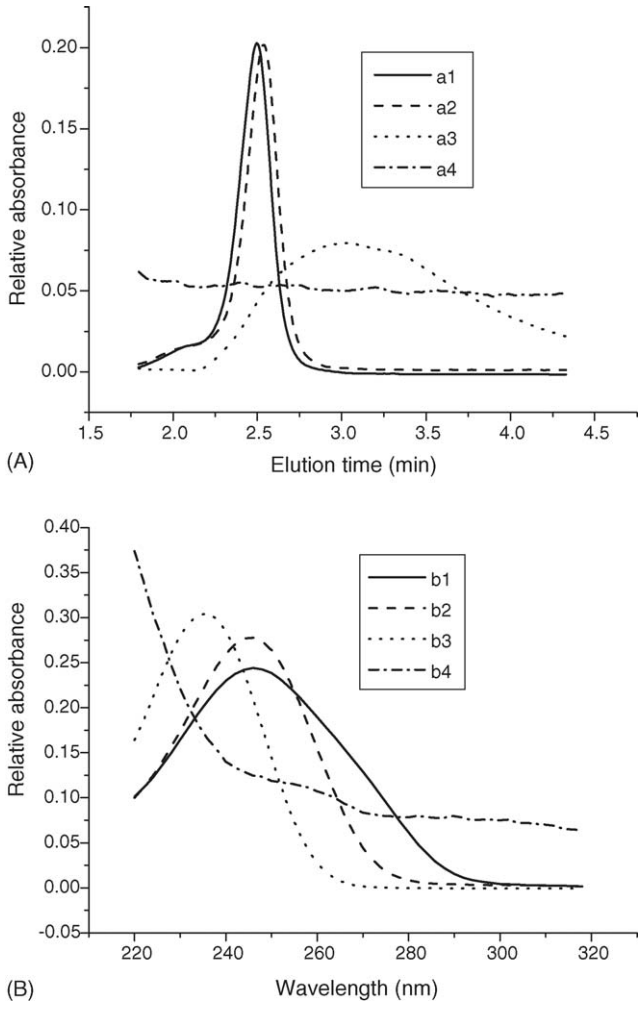

Fig. 3. (A) Chromatographic profiles, normalized to unit length, as found by ATLD for the urine samples in Table 1, loadings a1, a2, a3 and a4 represent prednisolone, cortisol, urine, and the background respectively. (B) Spectral profiles, normalized to unit length, as found by ATLD, loadings b1, b2, b3 and b4 represent prednisolone, cortisol, urine, and the background respectively.

With ATLD algorithm, good results for the artificial samples are obtained which are not displayed in detail. The predicted concentrations and recoveries of prednisolone and cortisol in urine samples are shown in Table 4. The fluctuation of the recoveries for prednisolone and cortisol seems to be due to the extracting process. The ATLD decomposition with four components was capable to provide good estimates of concentrations of the two analytes and their spectral profiles despite the fact that the two analytes were heavily overlapped and unkown interferent also existed in the urine samples.

Table 4
ATLD results for urine or plasma samples

Sample number	Predicted concentrations (ng/ml)		Recoveries (%)	
	Prednisolone	Cortisol	Prednisolone	Cortisol
6[a]	73.4 (75.0)[d]	221.1 (250.0)	97.9	88.4
7[a]	126.0 (125.0)	167.6 (175.0)	100.8	95.8
8[a]	193.6 (200.0)	160.1 (175.0)	96.8	91.5
5[b]	126.7 (125.0)		101.4	
6[b]	138.7 (150.0)		92.5	
7[b]	196.1 (200.0)		98.1	
6[c]		90.4 (100.0)		90.4
7[c]		155.5 (150.0)		103.6
8[c]		261.2 (250.0)		104.4

6[a], 7[a] and 8[a] are the urine samples in Table 1; 5[b], 6[b] and 7[b] are the plasma samples in Table 2; 6[c], 7[c] and 8[c] are the urine samples in Table 3.
[d] The real concentrations in parentheses.

In the quantification of prednisolone in plasma, cortisol which acted as an interferent was only added in the prediction samples. Three-way data array of $250 \times 50 \times 9$ was obtained. Also, the ATLD model was built with $N = 4$ as suggested by the core consistency analysis. In Fig. 4, the original chromatogram of plasma sample 7[b] is depicted, the first peak is the peak of plasma, and the second one is the peak of prednisolone and the interferent. As can be seen, the chromatographic profiles of prednisolone and the interferent completely merged into each other. In such a situation, biased results might be obtained with conventional analytical method. However, good estimation could be obtained with ATLD decomposition. The loadings from the ATLD decomposition of the data are shown in Fig. 5. Loadings related to the chromatographic profiles are shown in Fig. 5A (a1, a2, a3, a4), and the spectral profiles in Fig. 5B (b1, b2, b3, b4). In this figure, loadings a1, a2, a3 and a4 denote the chromatographic profiles of prednisolone, cortisol, plasma and background, respectively; and loadings b1, b2, b3, and b4 denote the corresponding spectral profiles. As can be seen, though the peak of prednisolone overlapped with the interferent, their respective peaks were obtained after the ATLD treatment. The predicted concentrations and recoveries of prednisolone in plasma are shown in Table 4.

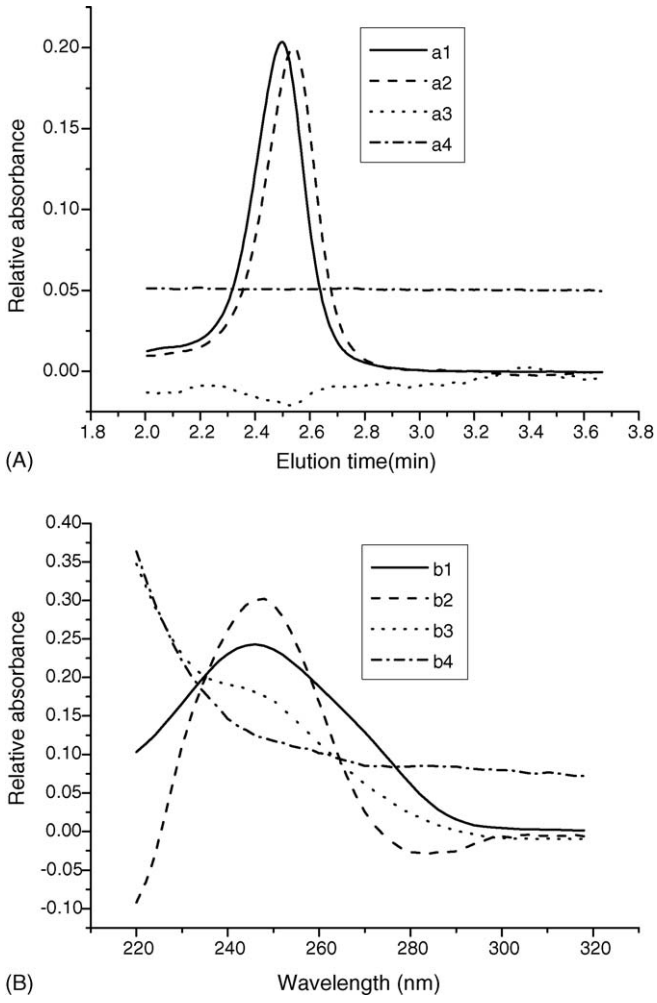

Fig. 5. (A) Chromatographic profiles, normalized to unit length, as found by ATLD for the plasma samples in Table 2, loadings a1, a2, a3 and a4 represent prednisolone, cortisol, plasma, and the background respectively. (B) Spectral profiles, normalized to unit length, as found by ATLD, loadings b1, b2, b3 and b4 represent prednisolone, cortisol, plasma, and the background respectively.

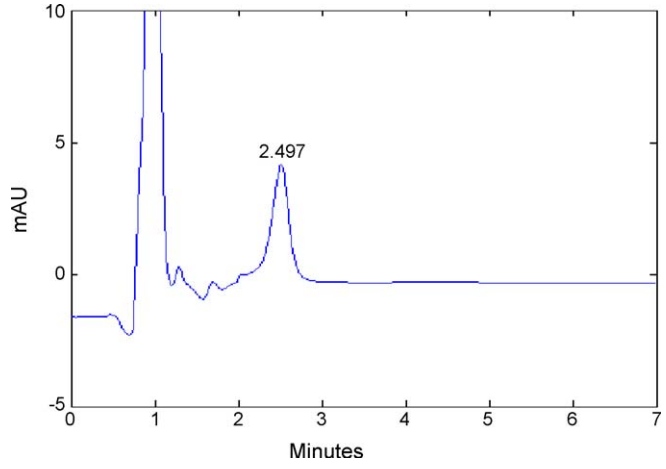

Fig. 4. Chromatogram of plasma sample 7[b].

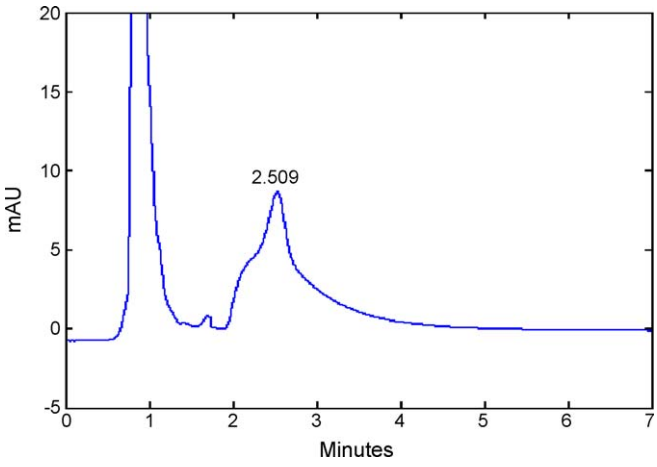

Fig. 6. Chromatogram of urine sample 8[c].

Table 5
Analytical figures of merit[a] for the determination of prednisolone and cortisol both in urine and plama by ATLD

	Urine		Plasma
	Prednisolone	Cortisol	Prednisolone
SEN (ml/ng)	0.155	0.143	0.261
SEL	0.036	0.034	0.055
Linear range (ng/ml)	0.0–450.0	0.0–400.0	0.0–450.0
R	0.994	0.991	0.997
RMSEP (ng/ml)	3.85	9.08	6.97
LOD (ng/ml)	1.7	0.8	1.0
LOQ (ng/ml)	5.2	2.5	3.0

[a] SEN (sensitivity) is estimated as the net analyte signal at unit concentration, SEL (selectivity) is computed as the ratio between the sensitivity and the total signal [45], *R* is the correlation coefficient, RMSEP is the root-mean-square error of predication, LOD (limit of detection) = 3.3 s(0) where s(0) is the standard deviation in the predicted concentration of the analyte of interest in a blank sample[46], LOQ (limit of quantification) = 10 s(0).

For the quantification of cortisol in urine in the presence of prednisolone, three-way data array of $381 \times 50 \times 10$ was obtained. The ATLD decomposition was performed with $N = 4$. Fig. 6 depicts the chromatogram of urine sample 8[c] in Table 3, the first peak is the component of urine, as can be seen, the second peak is low and wide, actually it is due to the overlapped peaks of cortisol, interferent and some components of urine. In this analysis, one of the urine peaks that affect the measurement of cortisol is also regarded as an interferent. After ATLD decomposition, the estimated concentrations of the prediction samples together with the recoveries of cortisol in urine are shown in Table 4. The loadings related to the chromatographic and spectral profiles are similar to those in Fig. 3. This analysis further proved that ATLD algorithm is a useful tool for the quantification of analytes in complex samples containing more than one interferent.

4.4. Method validation

The study based on ATLD calibration also furnishes analytical figures of merit. Table 5 summaries the analytical figures of merit including sensitivity, selectivity, linear range, correlation coefficient, root-mean-square error of predication (RMSEP), limit of detection (LOD) and limit of quantification (LOQ). As can be seen, the LOD and LOQ for prednisolone in urine were 1.7 and 5.2 ng/ml, respectively. The LOD and LOQ for cortisol in urine were calculated to be 0.8 and 2.5 ng/ml, respectively. And in plasma, the LOD and LOQ for prednisolone were 1.0 and 3.0 ng/ml, respectively.

5. Conclusions

This study shows that ATLD is a powerful chemometric tool for resolving heavily overlapped peaks into their pure chromatographic, spectral and concentration profiles even in complicated systems such as plasma and urine samples. This is a demonstration of the second-order advantage, which makes the calibration in the presence of unknown interferences feasible. For the determination of analytes in plasma and urine samples with heavily overlapped peaks and interferences, the ATLD can provide satisfactory concentration estimates. In the present work, a new method with simple sample disposal and short analysis time has been done. This method, which did not require complete separation of the analytes, demonstrated good accuracy and recoveries, and is free from interferences, can be used as an alternative method in routine analysis. The method is especially convenient for analysis of a number of samples, without the need to use internal standards. It has been proved that ATLD is an effective data treatment tool for a chromatographic system, which really improves the separation capability of the system by mathematic manipulations with substantially reduced time and cost.

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