Anal Bioanal Chem (2003) 377 : 1159–1164
DOI 10.1007/s00216-003-2185-6

ORIGINAL PAPER

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Chemometrics-assisted simultaneous determination of atenolol and chlorthalidone in synthetic binary mixtures and pharmaceutical dosage forms

Received: 16 March 2003 / Revised: 2 July 2003 / Accepted: 17 July 2003 / Published online: 3 September 2003

Abstract Resolution of binary mixtures of atenolol (ATE) and chlorthalidone (CTD) with minimum sample pre-treatment and without analyte separation has been successfully achieved, using a new and rapid method based on partial least squares (PLS1) analysis of UV spectral data. The simultaneous determination of both analytes was possible by PLS1 processing of sample absorbances between 255 and 300 nm for ATE and evaluation of absorbances in the 253–268 nm region for CTD. The mean recoveries for synthetic samples were 100.3±1.0% and 100.7±0.7% for ATE and CTD, respectively. Application of the proposed method to two commercial tablet preparations in the content uniformity test showed them to contain 103.5±0.8% and 104.9±1.8% ATE respectively, as well as 103.4±1.2% and 104.5±2.2% CTD. Use of this method also allowed the elaboration of dissolution profiles of the drugs in two commercial combined formulation products, through the simultaneous determination of both drugs during the dissolution test. At the dissolution time of 45 min specified by USP XXIV, both pharmaceutical formulations complied with the test.

Keywords Atenolol · Chlorthalidone · PLS1 · Dissolution · Chemometric method

Introduction

Hypertension is one of the most serious health problems faced by modern society; the disease is capable of silently and progressively affecting different organs, until damage is evident and irreversible, causing diminished quality of life and shortening life. Hypertension therapy must be individual, and in those cases not suited to single drug therapy, the addition of a second drug, usually in a fixed combination, is recommended [1]. The characteristics of these therapies demand strict quality control measures, including techniques for the determination of each of the active ingredients without interference of the others; this is continuously stimulating the development of new methods capable of fulfilling these requirements.

For routine analytical purposes, it is always of interest to establish methods capable of analysing a large number of samples in a short time period with due accuracy and precision. Spectroscopic techniques can generate large amounts of data within a short period of analysis; however, when coupled with chemometrics tools, the quality of the spectral information can be markedly increased, converting this combined technique into a powerful and highly convenient analytical tool.

The atenolol-chlorthalidone co-formulation (Fig. 1) is one of the most effective antihypertensive associations in the currently available therapeutic arsenal. Atenolol (CAS 29122-68-7), or 4-(2-hydroxy-3-[(1-methylethyl)amino] propyl) benzeneacetamide (ATE), is a widely employed β_1-selective adrenergic blocking agent which competitively blocks stimulation of the β-adrenergic receptors within the vascular smooth muscle and those of the myocardium, producing negative chronotropic and inotropic activity. On the other hand, chlorthalidone [CAS 77-36-1, 2-chloro-5-(2,3-dihydro-1-hydroxy-3-oxo-1*H*-isoindol-1-yl) benzenesulfonamide, CTD] is a phthalimide derivative of benzenesulfonamide, structurally and pharmacologically similar to the thiazide diuretics; the drug is practically insoluble in water and only slightly soluble in lower alcohols such as methanol and ethanol. Since ATE and CTD have complimentary but mechanistically different blood pressure lowering effects, this is a logical combination in the stepwise approach for managing hypertension [2], being also useful in treating fluid retention (oedema), stable angina and in preventing an often fatal heart attack

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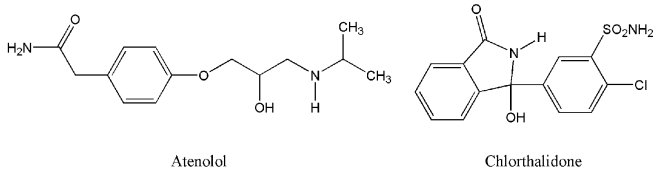

Fig. 1 Chemical structures of atenolol and chlorthalidone

repetition. ATE and CTD are official in the USP [3], BP [4] and Ph. Eur. [5] and the ATE-CTD combination in a 4:1 strength ratio is official in the USP [3].

Interestingly, however, in spite of the ample use of this combined medicine, only a few methods have been reported for the simultaneous determination of its active principles; these comprise reverse phase HPLC, with C_8 [6] and C_{18} [7] columns and a recently disclosed derivative UV spectrophotometric procedure [8]. On the other hand, some papers make reference to the simultaneous determination of ATE and CTD in human plasma by HPLC, with application to pharmacokinetic studies [9] and the analysis of chlorthalidone in human plasma by reversed-phase micellar liquid chromatography without interference of atenolol [10]; the HPLC analysis of ATE employing CTD as internal standard has also been recorded [11].

Notably, only one chemometrics-assisted method for the simultaneous determination of both drugs in their pharmacologically useful combination has been reported to date, employing Principal Component Regression without variable selection [12]. This prompted us to disclose our results, consisting of a new and accurate method for the simultaneous determination of ATE and CTD in synthetic samples and pharmaceutical formulations, employing the Partial Least Squares algorithm with one dependent variable (PLS1) of their spectral data in selected portions of the ultraviolet region. The method is simple, fast and amenable for routine analysis.

Experimental

Reagents, stock solutions and commercial tablets

All experiments were performed with AR-grade chemicals and the water used was double distilled. USP-grade atenolol and chlorthalidone were employed. Stock solutions of ATE (5.005 g L^{-1}) and CTD (1.250 g L^{-1}) were prepared by exact weighing and dissolution of the drugs in MeOH. Working solutions were prepared daily by 1:5 dilution of the stock solutions with 0.01 N HCl. Commercial oral dosage forms, declared to contain 100 mg ATE and 25 mg CTD and non-absorbing excipients (starch, lactose, magnesium stearate), were acquired in local drugstores.

Synthetic samples

25 calibration samples were prepared in 25-mL flasks by dilution of appropriate amounts of working solutions of ATE and CTD with 0.01 N HCl. The concentration range of the analytes in the calibration samples was 36–260 mg L^{-1} for ATE and 15–65 mg L^{-1} for CTD.

Three sets of 9 validation samples, each covering the concentration range 64–220 mg L^{-1} for ATE and 15–55 mg L^{-1} for CTD, and a set of six synthetic samples containing CTD and ATE in a 4:1 relationship were prepared in 25-mL flasks by appropriate dilution of the standard solutions with 0.01 N HCl. All of the samples were then submitted for analysis under the conditions described in the "Apparatus, hardware and software" section.

Analysis of tablet formulations

Ten tablets of ATE-CTD co-formulation (two different brands) were accurately weighed, ground to fine powder and a sample of the powder (41–44 mg), equivalent to approximately 10 mg ATE and 2.5 mg CTD, was accurately weighed and transferred into a 10-mL volumetric flask. 5 mL of MeOH was added and the mixture was stirred for 30 min at room temperature, when it was diluted to volume with 0.01 N HCl and mixed. A 9-mL aliquot was centrifuged 10 min at 2500 rpm in order to separate the insoluble excipients, and 2.0 mL of the clear supernatant were placed in a 10-mL volumetric flask, completed to the mark with 0.01 N HCl and mixed. This sample was then submitted for analysis under the conditions described in the "Apparatus, hardware and software" section.

Dissolution Profiles

Dissolution profiles were acquired following the USP procedure, with the aid of a Hanson SRS 8 Plus dissolutor, configured as USP apparatus II (paddle). Dissolution was carried out at 37 °C in 0.01 N HCl dissolution medium (900 mL), at a stirring speed of 50 rpm. A series of 10-mL samples were taken from each dissolution vessel at pre-specified times (with solvent reposition), filtered through 20 μm filters and submitted to spectral scanning and subsequent PLS1 analysis under the conditions described in the "Apparatus, hardware and software" section.

Apparatus, hardware and software

All spectrophotometric measurements were carried out with a Jasco V-530 double beam spectrophotometer, running spectrophotometric software provided by Jasco. Samples were measured in paired quartz cells of 10 mm path-length. Spectra were acquired at a scan speed of 100 nm min^{-1} with a fixed slit width of 2 nm, over the wavelength range 250–300 nm at intervals of 1 nm (51 data points/spectrum) against a blank of solvent. Spectra were saved in ASCII format, transferred to a PC Pentium II 466 MHz microcomputer and then transformed into Matlab 5.3 (Mathworks, Inc.) readable files, for subsequent manipulation.

Selection of the most appropriate wavelength intervals for calibration/prediction was carried out by means of a minimum PRESS search through a variable size moving-window [13], while PLS1 data evaluation was performed with an in-house set of routines [14] written for Matlab according to [15] and [16]. Spectra were mean-centred for calibration and prediction.

Statistical analysis of data and curve fitting were carried out with the aid of the programs Origin v. 6.0 (Microcal™) and SPSS v. 9.0 (SPSS, Inc.).

Results and discussion

Spectrometric measurements

The proposed method is based on the PLS1 analysis of UV spectral data from the analytes. Fig. 2 shows the absorption spectra of ATE (200 mg L^{-1}), CTD (50 mg L^{-1}), and a 4:1 mixture of both in 0.01 N HCl, in the 250–300 nm interval. This wavelength range was selected on account of the spectral characteristics exhibited by both drugs below 250 nm and their lack of absorption at wavelengths longer than 300 nm [12]. CTD shows maxima at 275 and 283 nm, while ATE exhibits a maximum at 274 nm, with a shoulder

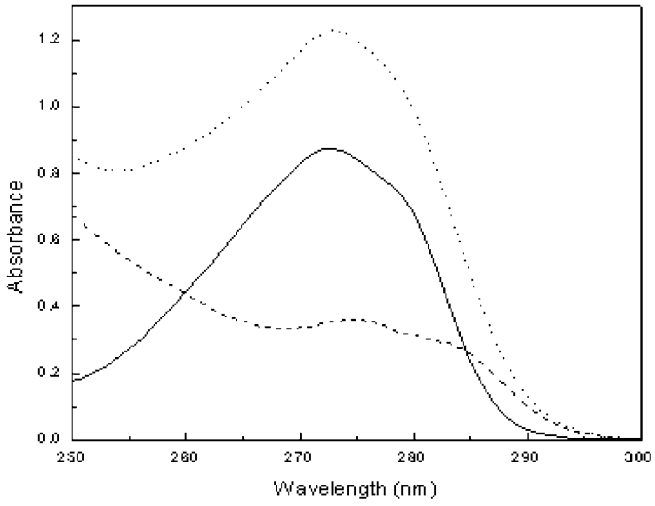

Fig. 2 Electronic absorption spectra of the analytes in the 250–300 nm region. (—) ATE, 200 mg L^{-1};(- - -) CTD, 50 mg L^{-1}; (.....) mixture of ATE (200 mg L^{-1}) and CTD (50 mg L^{-1}) in 0.01 N HCl

at 282 nm. It is evident that spectra strongly overlap, making difficult the simultaneous determination of both drugs by classical methodology due to their mutual interference.

A derivative spectroscopy method has been proposed for the resolution of this drug association [8]; however, we found it unsuitable for the analysis of ATE-CTD samples taken in the early phases of the dissolution experiments, when drug concentration in the samples is low, due to signal loss after derivation. Therefore, we expected that the use of multivariate calibration could be a better resource to circumvent spectral overlapping and mutual interference problems. This strategy is gaining popularity as a tool for the resolution of mixtures, and in recent years it has been applied to optical [17] as well as electrochemical [18] and other signals [19], allowing the resolution of complex mixtures of analytes without the need for their previous separation. Among the multivariate calibration methods, the PLS1 algorithm, a factor-based method working with overdetermined systems, is one of the most widely employed for that purpose.

By analogy with univariate calibration (in which a property – such as the concentration of the analyte in a set of standards – is mathematically related to the absorbance of these standards at a given wavelength), multivariate calibration is the process of constructing a mathematical model relating a property of a given analyte (in a series of reference samples) to the characteristics of their multivariate signals, such as their absorbances at several wavelengths. Analogously, in quantitative applications, the calibration stage of multivariate chemometric methods is followed by a prediction step, in which the results of the calibration model are used to determine the components' concentrations in the unknowns from their multivariate signals. The fundamentals of the PLS1 algorithm have been reviewed and extensively discussed elsewhere [20].

Interestingly, as a multivariate method PLS1 is advantageous and appealing for the determination of analytes in complex matrices, because calibration does not require knowledge of the concentrations of all other components except the analyte of interest. However, interferences should be present in the calibration set in order to be modelled and taken into account during the prediction step.

Table 1 UV-PLS1 analysis of ATE and CTD in 0.01 N HCl; statistical parameters for the calibration models

Parameter of interest[a]	Atenolol	Chlorthalidone
Optimum spectral range (nm)	255–300	253–268
Concentration range (mg L^{-1})	36–260	15–65
Number of PLS Factors	2	2
PRESS[(mg L^{-1})^2]	50	7.3
RMSD(mg L^{-1})	1.41	0.54
REC (%)	0.95	1.05
r^2	0.9997	0.9993
Selectivity	0.46	0.47
Sensitivity (SEN)	0.009	0.018
Analytical sensitivity [(γ), L mg^{-1}]	0.80	1.72
Minimum concentration difference ([(γ^{-1}), mg L^{-1}]	1.26	0.58
Limit of quantitation (mg L^{-1})	5.2	2.5

[a] $\mathrm{PRESS}=\sum(C_{\mathrm{pred}}-C_{\mathrm{act}})^2$, $\mathrm{RMSD}=[(1/N)\sum(C_{\mathrm{pred}}-C_{\mathrm{act}})^2]^{1/2}$, $\mathrm{REC}\,\%=(100/C_{\mathrm{mean}})[(1/N)\sum(C_{\mathrm{pred}}-C_{\mathrm{act}})^2]^{1/2}$, $r^2=1-[\sum(C_{\mathrm{pred}}-C_{\mathrm{act}})^2/\sum(C_{\mathrm{act}}-C_{\mathrm{mean}})^2]$, where C_{mean} is the average component concentration in the N calibration mixtures, sensitivity$=1/\|b_k\|$, where b_k is the final regression coefficients vector for component k, and $\gamma=(\mathrm{SEN}/\sigma_o)$, where σ_o is the standard deviation of the blank. Selectivity $= 1/(\|b_k\|\,\|AC/C^{\mathrm{T}}C\,\mathrm{i}|)$, where A and C are the mean centred absorbance (within the region of interest) and concentration data blocks, respectively.

Calibration model

As required, in order to carry out the proposed determinations, a calibration model was built and validated. Table 1 summarises the most relevant results of the calibration models, which were constructed with PLS1 regression on a calibration set of 25 spectra of samples with known contents of ATE and CTD, in the form of a two-component 5-level full-factorial (5^2) design. As data pre-treatment, all UV spectra were mean-centred before modelling.

Wavelength selection has proven to be critical for attaining optimum prediction ability, due to the fact that not all wavelengths in the spectra carry information of the same quality. Therefore, a minimum PRESS-guided search of the best wavelength interval was implemented. PRESS (Prediction Residual Error Sum of Squares), calculated as in Eqn. 1, is a measure of the quality of fitness of the predicted concentration results (C_{pred}) to the data (C_{act}).

$$\mathrm{PRESS} = \sum(C_{\mathrm{pred}}-C_{\mathrm{act}})^2 \quad (1)$$

After wavelength interval selection, the calibration model for ATE consisted of 46 (255–300 nm) variables and 25 samples, while the CTD calibration matrix contained 16 (253–

Table 2 Percentage of variance captured by the different PLS factors

Drug Factors	Atenolol			Chlorthalidone		
	X-Block	Y-Block	PRESS	X-Block	Y-Block	PRESS
1	96.153	83.894	25325	91.293	42.511	4487.0
2	99.993	99.971	50	99.999	99.923	7.3
3	99.998	99.971	64	99.999	99.925	8.7

Table 3 Accuracy and precision data for the spectrophotometric-PLS-1 simultaneous determination of Atenolol and Chlorthalidone

Parameter	Atenolol			Chlorthalidone		
Concentration range (mg l^{-1})	64.0–220.0			15.0–55.0		
Number of measurements	9	9	9	9	9	9
Mean recovery (%)	102.0	102.4	102.1	100.3	101.1	100.5
Standard deviation (S.D.)	1.3	1.3	1.4	1.0	1.4	1.2
Overall mean recovery (%)	102.1			100.6		
Overall S.D. (%)	1.3			1.2		
Between-groups variation[a]	0.58			3.01		
Within-groups variation[a]	42.4			37.2		
F-ratio[b]	0.16			0.97		

[a] Sources of variation in the ANOVA test; [b] Between-groups and within-groups degrees of freedom are 2 and 24, respectively. $F_{(0.95,2,24)}$=3.40.

Table 4 Simultaneous spectrophotometric-chemometric determination of Atenolol and Chlorthalidone in synthetic binary mixtures and commercial pharmaceutical preparations. Comparison with a reference method

Parameter[a]	Atenolol			Chlorthalidone		
	Synthetic	Brand 1[b]	Brand 2[b]	Synthetic	Brand 1[b]	Brand 2[b]
Mean recovery (%)	100.7	103.5	104.9	100.3	103.4	104.5
Mean recovery (mg)	100.7	103.5	104.9	25.1	25.8	26.1
S.D. (mg)	0.7	0.8	1.9	0.2	0.3	0.5
R.S.D. (%)	0.7	0.8	1.8	1.0	1.2	2.2
S.E. (mg)	0.3	0.3	0.8	0.1	0.1	0.2
Confidence limit[c]	0.5	0.7	1.5	0.2	0.2	0.4
Mean recovery (%)[d]	–	105.9	104.8	–	105.0	102.9
S.D.	–	1.8	0.9	–	1.0	2.1
t_{calcd}[e]	–	2.98	0.12	–	2.51	1.29

[a] For six replicates; S.D.=standard deviation; R.S.D.=relative standard deviation; S.E.=standard error; [b] Tablets were declared to contain (on the label) 100 mg ATE and 25 mg CTD; [c] Expressed in mg/tablet. P=0.05; [d] Results of the reference method; [e] $t_{(0.99,10)}$=3.17.

268 nm) wavelengths for the same 25 samples. Models were internally validated employing the well-known leave-one-out procedure, and the number of latent variables for prediction was chosen in agreement with the Haaland-Thomas criterion [21]. Both calibration models required two factors for optimum prediction; as shown in Table 2, these factors explained more than 99.9% of the variation in the X and Y data blocks of both analytes.

The high quality of the models was evident from the relationship between actual and predicted values observed over the examined linear ranges of both analytes [$ATE_{pred} = 0.002(\pm 0.005) + 1.00(\pm 0.01) \times ATE_{act}$ and $CTD_{pred} = 0.0001(\pm 0.0009) + 0.999(\pm 0.004) \times CTD_{act}$]. In addition, the squares of the correlation coefficients (r^2), which indicate the quality of the straight lines that fit the data, were 0.9997 and 0.9993 for ATE and CTD respectively. Notably, the limits of quantitation of the proposed method for both analytes, calculated as 10 times the standard deviation of the blank [5], were found to be below the ranges of interest. Also, the relative error of prediction during calibration (REC), an indication of the predictive ability of the models, remained around 1% for both analytes. In addition, analytical figures of merit obtained for the models also supported their quality.

External Validation

The calibration models were validated with three external validation sets of 9 samples each, which were analysed on three separate occasions after the calibration model had been made. This test was carried out in order to check stability of each calibration model over time in terms of accuracy and precision, which should ensure its usefulness for later analysis of samples. The results, contained in Table 3, show that the proposed method is accurate for both analytes, with mean recovery values close to 100%; it is also precise and repeatable, as evidenced by the results of the ANOVA test carried out on the three external validation sets.

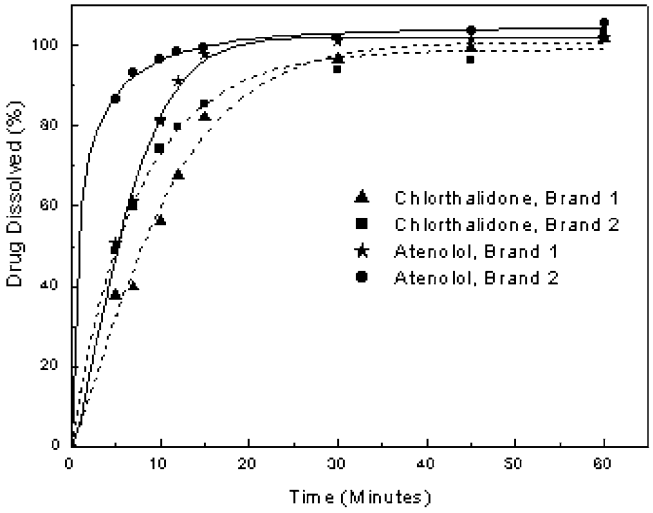

Fig. 3 Dissolution profiles of CTD and ATE in two combined commercial formulations, as determined by the UV-PLS1 method

Applications: Content uniformity and dissolution tests of commercially available solid oral dosage formulations

Content uniformity and drug dissolution profiles of tablets are two relevant quality parameters of these solid pharmaceutical dosage forms. Therefore, the proposed method was applied to the determination of ATE and CTD in two commercial pharmaceutical tablet preparations. As shown in Table 4, tablets of both tested brandscomplied with the declared amounts of their pharmacologically active drugs. Very good standard deviation, relative standard deviation and standard error values were observed, and analysis of the residual spectra indicated that no interference was produced by excipients in the sample. In addition, for the sake of comparison, synthetic samples maintaining the concentration ratio of both drugs (4:1) were simultaneously analysed, showing near quantitative mean recovery values. It can be concluded that despite CTD (the less concentrated analyte) determinations yielded slightly higher data dispersion than those of ATE, the precision of the determinations was very good in all cases.

The accuracy of the procedure was assessed by comparison with results provided by a method described in the literature [7]. As shown in Table 4, a paired *t*-test demonstrated that recovery values from both procedures for both analytes of interest were not statistically different.

The usefulness of the proposed method was also challenged, through its application to the construction of dissolution profiles of both drugs in their co-formulation. Dissolution testing is an increasingly important technique within the pharmaceutical industry because it provides valuable information on batch conformity, being an *in vitro* measurement of the bioavailability of the active component(s) from the formulation. The test can also be employed as evidence that certain process variables are under control, and to evaluate the effects of formulation changes upon drug release. The technique is therefore routinely used for quality control purposes as well as during formulation development.

However, dissolution data acquisition and construction of dissolution profiles is a laborious, repetitive, and often time consuming process, especially in the case of mixtures of analytes; therefore, much effort toward automation and increase of analytical speed has been reported, including the use of automated sample management [22], fiber optic technology [23] and diode array detection [24], usually combined with derivative spectroscopy [25], which have shown to favourably compete with HPLC in the determination of the dissolution profile of binary mixtures [26, 27]. There are only scattered references to chemometric methods with minimum sample pre-treatment and high sample throughput being associated with the dissolution test. Ourselves [28], Wehner [12], and the groups of Chen [23] and Dinç [29], among others, have recently shown the ability of multivariate calibration algorithms to solve drug mixtures during dissolution.

To evaluate the proposed method for the elaboration of dissolution profiles of ATE and CTD, the dissolution step was implemented following the USP directives with regards to dissolution media, apparatus type and stirring rate. For the analytical step with the proposed multivariate method, several preliminary assays were initially performed in order to check the variation of the absorbance values during dissolution, with the aim of optimising the sample dilution. Work was then carried out with the commercial formulations, and the results are graphically shown in Fig. 3.

Acceptable levels of data dispersion among the vessels were obtained; RSD values were slightly higher (6–8%) for the first 4 data points, belonging to the ascending part of the profiles, than for the last 4 data points (1–4%), and small differences in time during sample withdrawal were probably a relevant contributing factor to this dispersion. The dissolution data of both drugs in the formulations

Table 5 Weibull function fit of dissolution data for commercial preparations containing the ATE-CTD co-formulation[a]

Parameter	Brand 1		Brand 2	
	ATE	CTD	ATE	CTD
A	102±1.2	101.0±2.1	105±2	99±2
B	1.4±0.1	1.2±0.1	0.48±0.15	1.0±0.1
k	0.143±0.004	0.091±0.004	0.69±0.09	0.136±0.006
N	54	54	54	54
r^2	0.9746	0.9524	0.9701	0.9687
χ^2	28.6245	55.3443	31.9546	30.8878

[a] Dissolved drug (%) = $A(1–e^{-(kt)^{\wedge}B})$ was fitted with 9 data points; time units are minutes; $\chi^2_{(0.95,50)} = 65.5048$.

were statistically adjusted to the Weibull distribution function [22, 30, 31, 32] of Eqn. 2 below, showing excellent fits, as summarised in Table 5.

$$\text{Dissolved drug}(\%) = A\left(1\text{-}e^{-(kt)\cdot B}\right) \quad (2)$$

In this equation, parameter A refers to the maximum amount of drug dissolved, while B and k relate to the slope of the climbing section of the dissolution profiles and the overall shape of the curve.

Different dissolution rates for both drugs were observed in the formulations tested; CTD, the less soluble drug, was the slowest to dissolve. Interestingly, differences in ATE dissolution between the brands tested were also observed during the first 10–12 minutes of the dissolution experiment. Nevertheless, both formulations complied with the USP requirements, being the percentages of dissolved drugs after the officially stipulated 45 min of stirring time, greater than their respective Q (and Q+5) values (Q=70% for CTD and Q=80% for ATE).

Conclusions

A new method based on PLS1 analysis of UV spectra was developed for the determination of ATE and CTD in synthetic samples and pharmaceutical dosage forms. The proposed procedure allowed the simultaneous determination of the contents of the active principles in ATE-CTD tablets, where comparison with a reference procedure indicated that no statistically different drug recovery values were obtained for both analytes in the two brands analysed. This new method was also successfully employed for the simultaneous acquisition of the dissolution profiles of each member of this combination of co-formulated drugs, whose spectra are strongly overlapping under the dissolution conditions officially recommended by the USP. Compared to other procedures available for this determination, the proposed method, which is characterised by minimum sample pre-treatment, has high sample throughput and requires inexpensive apparatus and reagents. It therefore provides a fast, accurate and convenient alternative for the simultaneous determination of ATE and CTD in routine quality control of their pharmaceutical formulations.

Acknowledgements The authors thank UNR for financial support, Dr. Alejandro C. Olivieri for providing access to the Jasco V-530 UV-Vis spectrophotometer, Droguería Prest for the donation of atenolol, and Dr. Lucio Jeroncic for the kind provision of chlorthalidone. T.S.K. is also grateful to CONICET.

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