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Application and validation of chemometrics-assisted spectrophotometry and liquid chromatography for the simultaneous determination of six-component pharmaceuticals

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Abstract

Three methods are developed for the simultaneous determination of theophylline anhydrous (TH), guaiphenesin (GP), diphenhydramine hydrochloride (DP), methylparaben (MP), propylparaben (PP) and sodium benzoate (BZ) in pharmaceutical syrup. The chromatographic method depends on a high performance liquid chromatographic separation on a reversed-phase C_{18} column at ambient temperature with mobile phase consisting of 25 mM KH_2PO_4, pH 3.2—acetonitrile (60:40, v/v). Quantitation was achieved with UV detection at 222 nm based on peak area. The other two chemometric methods applied were partial least squares (PLS-1) and principal component regression (PCR). These approaches were successfully applied to quantify the six components in the studied mixture using information included in the UV absorption spectra of appropriate solutions in the wavelength range of 220–270 nm with $\Delta\lambda = 0.4$ nm. The calibration PLS-1 and PCR models were evaluated by internal validation (prediction of compounds in its own designed training set of calibration), by cross-validation (obtaining statistical parameters that show the efficiency for a calibration fit model) and by external validation over synthetic and pharmaceutical preparation. The results of PLS-1 and PCR methods were compared with the HPLC method and a good agreement was found.

Keywords: Theophylline; Guaiphenesin; Diphenhydramine; Methylparaben; Propylparaben; Sodium benzoate

1. Introduction

Theophylline anhydrous (TH) is a xanthine bronchodilator, which is associated with guaiphenesin (GP), an expectorant and diphenhydramine hydrochloride (DP), an antitussive, antihistaminic and anticholinergic, in addition to methylparaben (MP), propylparaben (PP) and sodium benzoate (BZ), which are used as preservatives. This combination is used for treating acute chronic bronchitis. The UV absorption spectra of TH, GP, DP, MP, PP and BZ display considerable overlap, where the application of the conventional spectrophotometry and its direct derivative and derivative ratio technique failed to resolve it. No analytical method has been reported for the simultaneous determination of TH, GP, DP, MP, PP and BZ in a multicomponent mixture. Several analytical methods have been reported for the determination of TH or GP or DP or MP or PP or BZ in combination with other drugs, including, HPLC [1–21], micellar electrokienetic chromatography (MEKC) [22], spectrophotometry [23], HPLC-densitometry [24], TLC [25] and capillary electrophoresis [26,27].

The utility of chemometrics-assisted spectrophotometry based on PLS for multidetermination of drug combinations has been published for determination of TH with dyphylline and proxyphylline [28]; DP with MP, phenylephrine and naphazoline [29]; DP with phenylpropanolamine and paracetamol [30]. The five-component mixture of GP, acetaminophen, ρ-aminophenol, caffeine and chlorphenamine was determined using PLS [31] and PCR [32]. Application of orthogonal functions was used in determination of GP in presence of sulphadiazine [33] and DP and ephedrine hydrochloride [34].

In this paper, an HPLC method and two chemometric-assisted spectrophotometric methods based on the application of partial least squares and principal component calibrations are proposed for the resolution of the studied six-component mixture.

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2. Experimental

2.1. Instrumentation

A double-beam Shimadzu (Japan) UV–vis spectrophotometer, model UV-1601 PC equipped with 1 cm quartz cells and connected to an IBM compatible computer. HP 600 inkjet printer was used. The bundled software was UVPC personal spectroscopy software version 3.7 (Shimadzu). The spectral bandwidth was 2 nm and the wavelength scanning speed was 2800 nm min^{-1}. PLS and PCR analysis were carried out by using PLS-Toolbox software version 2.1—PC [35] for use with MATLAB5.

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12 A Degasser, Rheodyne 7725i injector with a 20 μl loop and a SPD-10AVP UV–vis detector, separation and quantitation were made on a 250 mm × 4.6 mm (i.d.) Shim-pack RP_{18} column (4.6 μm particle size). The detector was set at λ = 222 nm. Data acquisition was performed on class-VP software.

2.2. Materials and reagents

Pharmaceutical grade of TH, GP, DP, MP, PP and BZ were used and certified to contain 99.9, 99.8, 99.9, 99.7, 99.8 and 99.9%, respectively. Acetonitrile and methanol used were HPLC grade (BDH, Poole, UK). Potassium dihydrogen phosphate, hydrochloric and phosphoric acids used were analytical grade.

Tussipect-N® syrup (batch number 412112) (Misr Co. For Pharmaceutical Industries, Mataria, Cairo, Egypt) were used. Each 5 ml contains 46.65 mg of TH, 30 mg of GP, 4.15 mg of DP, 3 mg of MP, 1.5 mg of PP and 5 mg of BZ.

2.3. Procedure

2.3.1. HPLC method

The mobile phase was prepared by mixing 25 mM potassium dihydrogen phosphate (apparent pH was adjusted to 3.2 using phosphoric acid) and acetonitrile in a ratio of 60:40 (v/v). The flow rate was 2 ml min^{-1}. All determinations were performed at ambient temperature.

2.3.1.1. Standard solutions and calibration. Stock standard solutions of TH, GP, DP, MP, PP and BZ were prepared separately by dissolving 50, 60, 40, 60, 40 and 50 mg of TH, GP, DP, MP, PP and BZ, respectively, in 100 ml methanol. Further dilutions were made for HPLC method using the mobile phase to reach the concentration range of 5.0–33.0 μg ml^{-1} for TH, 3–21 μg ml^{-1} for GP, 1.2–4.0 μg ml^{-1} for DP, 0.3–3.0 μg ml^{-1} for MP, 0.4–2.0 μg ml^{-1} for PP and 0.5–4.0 μg ml^{-1} for BZ.

Triplicate 20 μl injections were made for each concentration and chromatographed under the specified conditions described previously. The peak area values were plotted against corresponding concentrations. Linear relationship was obtained.

2.3.2. Multivariate calibration

A calibration set of 25 samples was prepared in 0.1 M hydrochloric acid, applying a multilevel multifactor design [36] in which five levels of concentrations of TH, GP, DP, MP, PP and BZ were introduced. The levels were in the calibration range of 5.0–33.0 μg ml^{-1} for TH, 3–21 μg ml^{-1} for GP, 1.2–4.0 μg ml^{-1} for DP, 0.3–2.1 μg ml^{-1} for MP, 0.4–1.6 μg ml^{-1} for PP and 0.5–3.5 μg ml^{-1} for BZ (Table 1). The electronic UV absorption spectra for these samples were collected each 0.4 nm in the wavelength range of 220–270 nm. The computation was made in PLS-Toolbox software version 2.1.

PCR and PLS-1 models were applied to the UV absorption spectra of these mixtures using six latent variables for TH, GP, DP and BZ and seven latent variables for MP and PP by PLS-1. Seven principal components were used for PCR determination of each compound.

2.3.3. Pharmaceutical sample preparation

Five ml of the syrup equivalent to 46.65 mg of TH, 30.0 mg of GP, 4.15 mg of DP, 3.0 mg of MP, 1.5 mg of PP and 5.0 mg of BZ was diluted to 100 ml with methanol, further dilutions were made using 0.1 M hydrochloric acid (for spectrophotometric methods) or the mobile phase (for HPLC method) to reach the calibration range for each component. The general procedures for PCR, PLS-1 and HPLC methods described under calibration were followed and the concentration of each compound was calculated.

3. Results and discussion

3.1. Spectral features

Fig. 1 shows the UV absorption spectra of TH, GP, DP, MP, PP and BZ at their nominal concentrations in syrup. As can be seen, PP, DP, MP and BZ contribute very little to overall absorption of the sample; also, the absorption band of TH is extensively overlapped with GP, DP, MP, PP and BZ spectra. The simultaneous determination of TH, GP, DP, MP, PP and BZ in syrup by conventional, derivative and derivative ratio spectrophotometric methods is hindered by strong spectral overlap throughout the wavelength range. HPLC or multivariate calibration methods were necessary for such determination due to the presence of interference.

3.2. HPLC method

The developed HPLC method has been applied for simultaneous determination of TH, GP, DP, MP, PP and BZ. The mobile phase composition and pH of 25 mM potassium dihydrogen phosphate were studied and optimized. A satisfactory separation was obtained with a mobile phase composed of 25 mM potassium dihydrogen phosphate (apparent pH was adjusted to 3.2 using phosphoric acid) and acetonitrile (60:40, v/v). Increasing acetonitrile concentration to more than 50% led to overlapping of GP and DP. At lower acetonitrile concentration (<30%) separation occurred but with excessive delay for PP peak. Variation

Table 1
Concentration data for the different mixtures used in the calibration set and internal validation for the determination of TH, GP, DP, MP, PP and BZ using PLS-1 and PCR methods

Mixture composition ($\mu g\ ml^{-1}$)							Internal validation (percentage recovery)											
Mixture No.	TH	GP	DP	MP	PP	BZ	PLS-1						PCR					
							TH	GP	DP	MP	PP	BZ	TH	GP	DP	MP	PP	BZ
1	19	12	2.6	1.2	1	2	100.0	100.0	100.1	100.3	99.7	100.0	100.0	100.0	100.2	100.9	98.9	100.0
2	19	3	1.2	2.1	0.7	3.5	100.0	100.0	100.9	100.0	99.9	100.0	100.0	100.0	100.9	99.8	100.8	100.0
3	5	3	4	0.75	1.6	2	100.0	100.0	100.3	99.8	100.1	100.0	100.0	100.0	100.3	101.2	99.4	100.0
4	5	21	1.9	2.1	1	1.25	100.0	100.0	99.2	100.2	99.6	100.0	100.0	100.0	99.5	101.1	97.3	100.0
5	33	7.5	4	1.2	0.7	1.25	100.0	100.0	100.1	100.6	98.9	100.0	100.0	100.0	99.9	100.9	98.3	100.0
6	12	21	2.6	0.75	0.7	2.75	100.0	100.0	99.5	100.5	99.5	100.0	100.0	100.0	99.9	101.4	98.3	100.0
7	33	12	1.9	0.75	1.3	3.5	100.0	100.0	101.0	101.1	99.3	100.0	100.0	100.0	100.8	102.3	98.5	100.0
8	19	7.5	1.9	1.65	1.6	2.75	100.0	100.0	99.5	99.6	100.5	100.0	100.0	100.0	99.8	100.4	99.6	100.0
9	12	7.5	3.3	2.1	1.3	2	100.0	100.0	98.7	100.1	99.9	100.0	100.0	100.0	98.7	99.7	100.5	100.0
10	12	16.5	4	1.65	1	3.5	100.0	100.0	100.2	100.2	99.7	100.0	100.0	100.0	99.9	99.8	100.3	100.0
11	26	21	3.3	1.2	1.6	3.5	100.0	100.0	100.4	99.5	100.4	100.0	100.0	100.0	100.4	97.6	102.0	100.0
12	33	16.5	2.6	2.1	1.6	0.5	100.0	100.0	100.3	100.3	99.6	100.0	100.0	100.0	99.8	100.4	99.4	100.0
13	26	12	4	2.1	0.4	2.75	100.0	100.0	100.0	99.9	100.9	100.0	100.0	100.0	100.0	99.8	101.1	100.0
14	19	21	4	0.3	1.3	0.5	100.0	100.0	99.8	99.3	100.2	99.9	100.0	100.0	100.0	97.6	100.6	100.0
15	33	21	1.2	1.65	0.4	2	100.0	100.0	99.4	99.5	102.1	100.0	100.0	100.0	99.9	98.8	105.6	100.0
16	33	3	3.3	0.3	1	2.75	100.0	100.0	99.4	97.6	100.8	100.0	100.0	100.0	99.6	98.0	100.7	100.0
17	5	16.5	1.2	1.2	1.3	2.75	100.0	100.0	100.5	99.9	100.1	100.0	100.0	100.0	100.5	100.2	99.8	100.0
18	26	3	2.6	1.65	1.3	1.25	100.0	100.1	100.0	100.1	99.9	100.0	100.0	100.1	100.5	100.5	99.4	100.0
19	5	12	3.3	1.65	0.7	0.5	99.9	100.0	101.1	99.9	100.3	100.1	99.9	100.0	101.0	98.7	103.4	100.1
20	19	16.5	3.3	0.75	0.4	1.25	100.0	100.0	99.8	99.5	101.1	100.0	100.0	100.0	100.0	102.0	95.8	100.0
21	26	16.5	1.9	0.3	0.7	2	100.0	100.0	100.1	100.9	99.6	100.0	100.0	100.0	99.4	104.0	98.1	100.0
22	26	7.5	1.2	0.75	1	0.5	100.0	100.0	101.0	100.5	99.6	100.0	100.0	100.0	100.7	99.3	100.6	100.0
23	12	3	1.9	1.2	0.4	0.5	100.0	100.0	99.7	99.6	101.4	100.0	100.0	100.0	99.8	99.0	103.5	100.1
24	5	7.5	2.6	0.3	0.4	3.5	100.0	100.0	100.0	101.8	98.6	100.0	100.0	100.0	99.8	99.7	100.3	100.0
25	12	12	1.2	0.3	1.6	1.25	100.0	100.0	99.5	99.5	100.1	100.0	100.0	100.0	99.0	95.4	101.0	100.0
Mean[a]							100.0	100.0	100.0	100.0	100.1	100.0	100.0	100.0	100.0	99.9	100.1	100.0
S.D.[a]							0.02	0.02	0.59	0.76	0.77	0.03	0.02	0.02	0.55	1.76	2.05	0.02

[a] Mean and S.D., percentage recovery from the added amount.

of pH of the 25 mM potassium dihydrogen phosphate resulted in maximum capacity factor (*K'*) value at pH 6.5. At pH 2.5–4.0 improved resolution for the six drugs was observed. However at pH 3.2 optimum resolution with reasonable retention time was observed. Quantitation based on peak area achieved with UV detection at 222 nm. The specificity of the HPLC method is illustrated in Fig. 2 where complete separation of the six compounds was noticed. The average retention time ± standard deviation for TH, GP, DP, BZ, MP and PP were found to be 1.5 ± 0.02, 2.0 ± 0.03, 2.5 ± 0.04, 2.9 ± 0.02, 3.5 ± 0.03 and 8.7 ± 0.02 min, respectively, for 10 replicates. The response of the TH is very high which is not affected by the dead volume. The HPLC chromatographic characteristics of the studied drugs were given in Table 2.

3.3. Multivariate calibration

3.3.1. Calibration matrix and selection of spectral zones for analysis by PLS-1 and PCR

The quality of multicomponent analysis is dependent on the wavelength range and spectral mode used. PLS procedures are designated to be full spectrum computational procedures, thus wavelength selection is seemingly unnecessary, and so all available wavelengths are often used. However, measurements from spectral wavelengths that are non-informative in a model degrade performance [37]. Original and reconstructed spectra of the calibration matrix were compared in order to select the range of wavelengths. The range was obtained by all regions in which the difference between each component of the mixture and the others was maximum. Besides, the regions in which each component of the mixture were best reconstructed were also considered. The spectral region between wavelengths 220 and 270 nm was selected for this purpose as it was that providing the greatest amount of information about the mixture components. This entailed using 126 experimental points per spectrum, as spectra were digitized at 0.4 nm intervals. In addition, wavelengths less than 220 nm were rejected due to the

Table 2
Chromatographic characteristics of TH, GP, DP, MP, PP and BZ

Compound	Retention time (min)	Capacity factor (*K'*)	Selectivity	Resolution
TH	1.5	0.81	1.74	1.54
GP	2.0	1.41	1.38	1.5
DP	2.5	1.95	1.31	1.67
BZ	2.9	2.55	1.23	1.82
MP	3.5	3.16	3	21.87
PP	8.7	9.48		

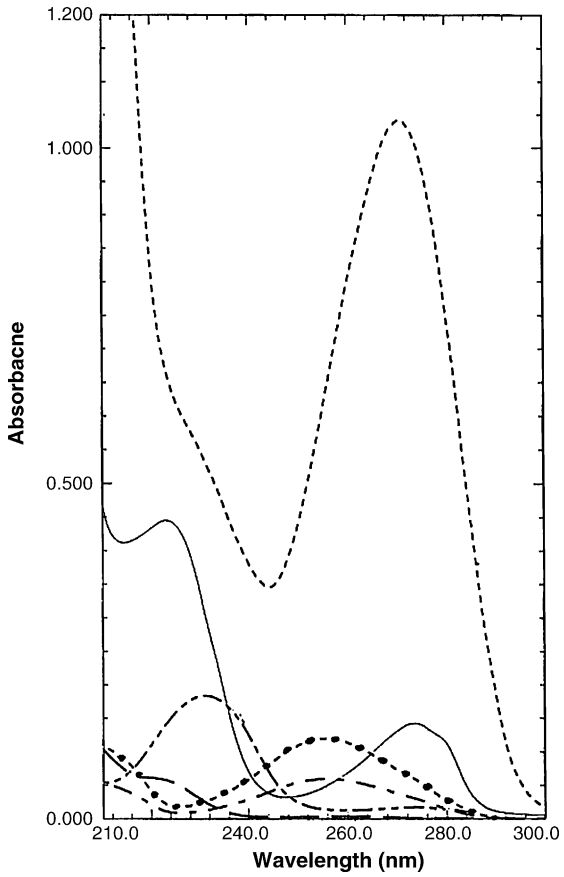

Fig. 1. UV absorption spectra of 18.7 μg ml^{-1} of TH (- - -), 12 μg ml^{-1} of GP (—), 1.7 μg ml^{-1} of DP (– – –), 1.2 μg ml^{-1} of MP (–●–●–), 0.6 μg ml^{-1} of PP (– – – – –) and 2 μg ml^{-1} of BZ (– – – –) in 0.1 M hydrochloric acid.

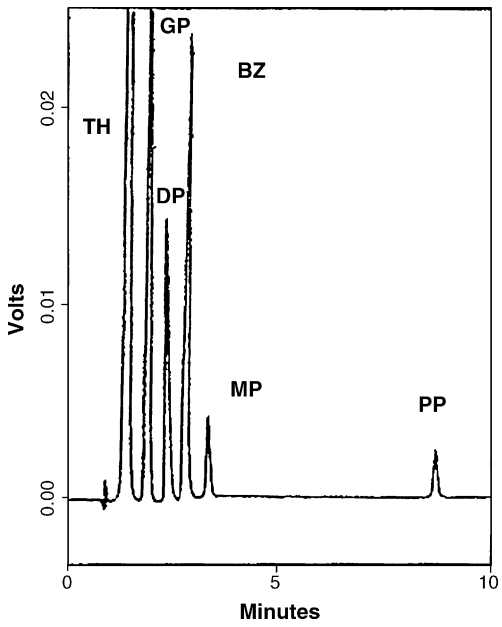

Fig. 2. HPLC chromatogram of 20 μl injection of syrup sample containing 18.66 μg ml^{-1} of TH, 12 μg ml^{-1} of GP, 1.66 μg ml^{-1} of DP, 1.2 μg ml^{-1} of MP, 0.6 μg ml^{-1} of PP and 2 μg ml^{-1} of BZ.

difference between the synthetic mixture and pharmaceutical syrup spectra. Wavelengths more than 270 nm were not used because DP has neglected absorption at the concentrations used in this region, so any absorbance values obtained at the wavelengths more than 270 nm would have introduced a significant amount of noise in the calibration matrix, thereby decreasing the precision.

A multilevel multifactor design [36] in which five levels of concentrations of TH, GP, DP, MP, PP and BZ were introduced. The levels were in the range of 5.0–33.0 μg ml^{-1} for TH, 3–21 μg ml^{-1} for GP, 1.2–4.0 μg ml^{-1} for DP, 0.3–2.1 μg ml^{-1} for MP, 0.4–1.6 μg ml^{-1} for PP and 0.5–3.5 μg ml^{-1} for BZ (Table 1). A calibration set consisting of 25 samples was used.

3.3.2. Selection of the optimum number of factors

An appropriate choice of the number of principal components or factors is necessary for PCR and PLS-1 calibrations. The number of factors should account as much as possible for the experimental data without resulting in over fitting. Various criteria have been developed to select the optimum number [38]. Cross-validation methods leaving out one sample at a time was employed [39]. The predicted concentrations were compared with the known concentrations of the compounds in each calibration sample. The root mean squares error of cross validation

Table 3
RMSECV and statistical parameter values for simultaneous determination of TH, GP, DP, MP, PP and BZ using PLS-1 and PCR methods

Item	Method	Compound					
		TH	GP	DP	MP	PP	BZ
RMSECV	PLS-1	1.59×10^{-3}	1.53×10^{-3}	2.01×10^{-2}	2.04×10^{-2}	2.25×10^{-2}	3.48×10^{-4}
	PCR	1.59×10^{-3}	1.57×10^{-3}	1.91×10^{-2}	2.07×10^{-2}	2.29×10^{-2}	3.27×10^{-4}
Intercept	PLS-1	-4.0×10^{-6}	1.33×10^{-6}	5.61×10^{-4}	5.87×10^{-5}	1.47×10^{-4}	-2.0×10^{-5}
	PCR	1.43×10^{-6}	-1.07×10^{-5}	4.75×10^{-4}	4.95×10^{-4}	1.13×10^{-3}	-1.33×10^{-5}
Slope	PLS-1	1.0000	0.9999	0.9998	0.9999	0.9998	1.0000
	PCR	0.9999	1.0000	0.9998	0.9996	0.9989	1.0000
r	PLS-1	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999
	PCR	0.9999	0.9999	0.9999	0.9998	0.9995	0.9999
S.E. of intercept	PLS-1	4.67×10^{-4}	4.79×10^{-4}	8.56×10^{-3}	2.0×10^{-3}	2.64×10^{-3}	1.01×10^{-4}
	PCR	4.61×10^{-4}	4.62×10^{-4}	7.69×10^{-3}	5.72×10^{-3}	7.57×10^{-3}	8.89×10^{-5}
S.E. of slope	PLS-1	2.18×10^{-5}	3.53×10^{-5}	3.08×10^{-3}	1.47×10^{-3}	2.43×10^{-3}	4.46×10^{-5}
	PCR	2.15×10^{-5}	3.40×10^{-5}	2.76×10^{-3}	4.21×10^{-3}	6.97×10^{-3}	3.93×10^{-5}

Table 4
Determination of TH, GP, DP, MP, PP and BZ in commercial syrup using the proposed methods

Sample No.	Concentration ($\mu g\,ml^{-1}$)						Recovery percentage																	
	TH	GP	DP	MP	PP	BZ	PLS-1						PCR						HPLC					
							TH	GP	DP	MP	PP	BZ	TH	GP	DP	MP	PP	BZ	TH	GP	DP	MP	PP	BZ
1	13.995	9.0	1.245	0.9	0.45	1.5	101.8	97.8	97.7	99.3	101.0	100.0	101.8	97.8	97.7	98.5	102.8	100.0	100.7	100.3	99.2	98.2	98.1	100.2
2	15.55	10.0	1.383	1.0	0.5	1.667	101.9	100.0	99.8	100.2	99.7	100.0	101.9	100.0	99.5	100.6	98.6	100.0	101.2	99.1	98.3	98.1	99.2	99.9
3	18.66	12.0	1.66	1.2	0.6	2.0	101.3	99.1	98.2	99.0	102.3	100.1	101.3	99.1	98.1	98.5	103.6	100.1	100.6	100.1	98.4	97.9	100.1	100.4
4	23.325	15.0	2.075	1.5	0.75	2.5	101.3	99.9	99.9	97.9	102.0	100.4	101.3	99.9	100.1	98.0	101.8	100.4	100.3	99.7	99.3	98.1	98.1	100.6
5	27.99	18.0	2.49	1.8	0.9	3.0	100.8	99.9	99.4	100.3	99.4	100.0	100.8	99.9	99.5	100.2	99.7	100.0	101.3	99.8	98.6	99.4	100.9	99.9
Mean[a]							101.4	99.3	99.0	99.3	100.9	100.1	101.4	99.4	99.0	99.2	101.3	100.1	100.8	99.8	98.8	98.3	99.3	100.2
S.D.[a]							0.44	0.93	0.99	0.98	1.31	0.17	0.44	0.93	1.03	1.16	2.10	0.17	0.42	0.46	0.46	0.60	1.23	0.31
t[b]							2.21	1.08	0.41	1.95	1.99	0.63	2.21	1.08	0.40	1.54	1.84	0.63						
F[b]							1.10	4.09	4.63	2.67	1.13	3.32	1.10	4.09	5.01	3.74	2.91	3.32						

[a] Mean and S.D., percentage recovery from the label claim amount.
[b] Theoretical values for t and F at $P = 0.05$ are 2.31 and 6.39, respectively.

Table 5
Characteristic parameters of the calibration equations for the proposed HPLC method for simultaneous determination of TH, GP, DP, MP, PP and BZ

Parameters	TH	GP	DP	MP	PP	BZ
Calibration range ($\mu g\,ml^{-1}$)	5.0–33.0	3–21	1.2–4.0	0.3–3.0	0.4–2	0.5–4.0
Detection limit ($\mu g\,ml^{-1}$)	1.59×10^{-2}	1.90×10^{-2}	2.38×10^{-2}	2.14×10^{-2}	2.40×10^{-2}	2.02×10^{-2}
Quantitation limit ($\mu g\,ml^{-1}$)	5.30×10^{-2}	6.33×10^{-2}	7.93×10^{-2}	7.13×10^{-2}	8.0×10^{-2}	6.73×10^{-2}
Regression equation (Y)[a]						
Slope (b)	35.03×10^{3}	23.10×10^{3}	28.59×10^{3}	18.51×10^{3}	17.05×10^{3}	47.60×10^{3}
Standard deviation of the slope (S_b)	2.80×10^{2}	2.19×10^{2}	3.42×10^{2}	1.99×10^{2}	2.06×10^{2}	4.83×10^{2}
Relative standard deviation of the slope (%)	0.80	0.95	1.20	1.08	1.21	1.01
Confidence limit of the slope[b]	34.76×10^{3}–35.30×10^{3}	22.89×10^{3}–23.31×10^{3}	28.25×10^{3}–28.92×10^{3}	18.32×10^{3}–18.70×10^{3}	16.85×10^{3}–17.25×10^{3}	47.13×10^{3}–48.07×10^{3}
Intercept (a)	-1.07×10^{3}	0.53×10^{2}	0.23×10^{3}	$-0.0.03 \times 10^{3}$	0.05×10^{3}	0.55×10^{3}
Standard deviation of the intercept (S_a)	5.85×10^{3}	2.94×10^{3}	0.63×10^{3}	0.27×10^{3}	0.14×10^{3}	1.08×10^{3}
Confidence limit of the intercept[b]	(-6.75×10^{3})–4.62×10^{3}	(-2.81×10^{3})–2.91×10^{3}	(-0.39×10^{3})–0.85×10^{3}	(-0.29×10^{3})–0.23×10^{3}	(-0.09×10^{3})–0.18×10^{3}	(-0.50×10^{3})–1.60×10^{3}
Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999
Standard error of estimation	2.62×10^{3}	1.32×10^{3}	0.28×10^{3}	0.20×10^{3}	0.06×10^{3}	0.48×10^{3}

[a] $Y = a + bC$, where C is the concentration of compound in $\mu g\,ml^{-1}$ and Y is the peak area.
[b] 95% Confidence limit.

Table 6
Analysis of variance for repeatability and intermediate precision for TH, GP, DP, MP, PP and BZ using the proposed HPLC, PLS-1 and PCR methods

Drug	Concentration level ($\mu g\,ml^{-1}$)	Sources of variance	Sum of squares			DF	MS			*F*-ratio			*P*-value		
			HPLC	PLS-1	PCR		HPLC	PLS-1	PCR	HPLC	PLS-1	PCR	HPLC	PLS-1	PCR
TH	5.0	Between	1.42	2.11	2.11	7	0.20	0.30	0.30	0.64	1.25	1.25	0.72	0.38	0.38
		Within	2.56	1.94	1.94	8	0.32	0.24	0.24						
		Total	3.98	4.05	4.05	15									
TH	19.0	Between	0.83	2.63	2.63	7	0.12	0.38	0.38	0.21	0.89	0.89	0.97	0.56	0.56
		Within	4.50	3.40	3.40	8	0.59	0.42	0.42						
		Total	5.33	6.03	6.03	15									
TH	33.0	Between	1.08	0.27	0.27	7	0.15	0.04	0.04	1.11	1.15	1.15	0.44	0.42	0.42
		Within	1.11	0.27	0.27	8	0.14	0.33	0.33						
		Total	2.19	0.54	0.54	15									
GP	3	Between	3.01	1.36	0.57	7	0.43	0.19	0.08	1.28	1.15	0.70	0.37	0.42	0.67
		Within	2.69	1.35	0.92	8	0.34	0.17	0.12						
		Total	5.70	2.71	1.49	15									
GP	12	Between	1.11	1.40	2.95	7	0.15	0.20	0.42	1.11	0.96	2.55	0.44	0.52	0.11
		Within	1.11	1.67	1.32	8	0.14	0.21	0.17						
		Total	2.22	3.07	4.27	15									
GP	21	Between	1.10	0.49	0.73	7	0.16	0.07	0.10	0.22	0.74	0.92	0.97	0.65	0.54
		Within	5.82	0.75	0.90	8	0.73	0.09	0.11						
		Total	6.92	1.24	1.63	15									
DP	1.2	Between	3.89	2.27	0.73	7	0.56	0.32	0.10	1.51	0.94	0.73	0.29	0.53	0.65
		Within	2.94	2.77	1.13	8	0.37	0.35	0.14						
		Total	6.83	5.04	1.86	15									
DP	2.6	Between	0.78	0.32	2.06	7	0.11	0.05	0.29	0.16	0.17	1.45	0.99	0.99	0.31
		Within	5.71	2.16	1.62	8	0.71	0.27	0.20						
		Total	6.49	2.48	3.68	15									
DP	4.0	Between	1.37	0.99	1.06	7	0.20	0.14	0.15	0.58	0.52	0.54	0.76	0.80	0.78
		Within	2.70	2.18	2.24	8	0.34	0.27	0.28						
		Total	4.07	3.17	3.30	15									
MP	0.3	Between	2.15	2.39	2.96	7	0.31	0.34	0.42	0.65	1.04	1.36	0.71	0.47	0.34
		Within	3.80	2.63	2.49	8	0.47	0.33	0.31						
		Total	5.95	5.02	5.45	15									
MP	1.2	Between	1.08	1.01	2.21	7	0.15	0.14	0.32	1.11	0.96	1.70	0.44	0.52	0.24
		Within	1.11	1.20	1.49	8	0.14	0.15	0.19						
		Total	2.19	2.20	3.70	15									
MP	2.1	Between	1.24	1.34	2.28	7	0.18	0.19	0.33	0.44	0.42	2.01	0.85	0.86	0.17
		Within	3.20	3.62	1.30	8	0.40	0.45	0.16						
		Total	4.44	4.96	3.58	15									
PP	0.4	Between	0.62	1.40	2.88	7	0.09	0.20	0.41	1.86	0.96	1.95	0.20	0.52	0.18
		Within	0.38	1.67	1.69	8	0.05	0.21	0.21						
		Total	1.00	3.07	4.57	15									
PP	1.0	Between	2.25	0.72	2.98	7	0.32	0.10	0.43	1.68	0.28	2.46	0.24	0.94	0.12
		Within	1.53	2.91	1.38	8	0.19	0.36	0.17						
		Total	3.78	3.63	4.36	15									
PP	1.6	Between	2.66	2.70	1.29	7	0.38	0.39	0.18	1.96	1.34	1.05	0.18	0.34	0.47
		Within	1.55	2.30	1.40	8	0.19	0.29	0.17						
		Total	4.21	5.00	2.69	15									
BZ	0.5	Between	2.71	2.47	0.56	7	0.39	0.35	0.08	1.52	1.44	0.34	0.28	0.31	0.91
		Within	2.03	1.96	1.89	8	0.25	0.24	0.24						
		Total	4.74	4.43	2.45	15									
BZ	2	Between	1.89	1.55	0.93	7	0.27	0.22	0.13	1.08	0.55	0.99	0.45	0.78	0.50
		Within	1.20	3.23	1.07	8	0.25	0.40	0.13						
		Total	3.09	4.78	2.00	15									
BZ	3.5	Between	0.43	0.36	0.23	7	0.06	0.05	0.03	0.10	0.69	0.75	1.00	0.68	0.64
		Within	5.01	0.59	0.35	8	0.63	0.07	0.04						
		Total	5.44	0.95	0.58	15									

DF is the degree of freedom and MS is the mean square. The critical value of *F*-ratio is 3.5 and *P*-value is 0.05.

(RMSECV) was calculated for each method as follows:

$$\text{RMSECV} = \sqrt{\frac{\text{PRESS}}{n}}$$

where n is the number of training samples and:

$$\text{PRESS} = \sum (Y_{\text{pred}} - Y_{\text{true}})^2$$

where Y_{pred} and Y_{true} are predicted and true concentrations in $\mu g\,ml^{-1}$, respectively.

The RMSECV was used as a diagnostic test for examining the errors in the predicted concentrations. It indicates both of the precision and accuracy of predictions. It was recalculated upon addition of each new factor to the PLS-1 and PCR models.

The optimum number of factors was selected by following the criterion of Haaland and Thomas [40]. The selected model was that with the fewest number of factors such that its RMSECV was not significantly greater than that for the model, which yielded the lowest RMSECV. A number of factors of six latent variables for TH, GP, DP and BZ and seven latent variables for MP and PP were found to be optimum by PLS-1. Seven principal components were optimum for PCR determination of each compound. Plotting the actual known concentrations against the predicted concentrations performed the evaluation of the predictive abilities of the models. The obtained results are shown in Table 3. A satisfactory correlation coefficient (r) value was obtained for each compound in the training set by PLS-1 and PCR optimized models indicating good predictive abilities of the models. The RMSECV obtained by optimizing the calibration matrix of the absorption spectra for the PLS-1 and PCR methods are shown in Table 3 indicating good accuracy and precision.

3.3.3. PLS-1 versus PCR

Recent studies involving the use of multivariate calibration techniques for the multicomponent resolution of UV–vis data revealed no significant difference between the PLS and PCR predictions. However, if one considers its theoretical advantages and optimal performance over a wide range of conditions, PLS-1 is the method of choice [41].

3.4. Analysis of pharmaceutical syrup

The proposed PLS-1, PCR and HPLC methods were applied to the simultaneous determination of TH, GP, DP, MP, PP and BZ in commercial syrup. Five replicates determination were made. Satisfactory results were obtained for each compound in good agreement with label claims (Table 4). No published method has been reported for simultaneous determination of the six components of this mixture. So that the results of the proposed PLS-1 and PCR methods were compared with those of the proposed HPLC method. Statistical comparison between the results was performed with regards to accuracy and precision using Student's t-test and F-ratio at 95% confidence level (Table 4). There is no significant difference between the results.

The syrup contains sugar, saccharin, propylene glycol, strawberry, tuttifruity, carnosin, citric acid and sodium citrate as syrup excipients. The syrup sample was diluted 1:500 with mobile

Table 7
Determination of TH, GP, DP, MP, PP and BZ in synthetic mixtures using the proposed methods

Mixture No.	Concentration						Recovery percentage																	
	TH	GP	DP	MP	PP	BZ	PLS-1						PCR						HPLC					
							TH	GP	DP	MP	PP	BZ	TH	GP	DP	MP	PP	BZ	TH	GP	DP	MP	PP	BZ
1	9	3	1.7	0.6	0.9	1.5	100.0	100.2	100.6	98.8	100.6	99.9	100.0	100.2	100.6	98.2	101.4	99.9	100.2	99.8	100.1	99.7	100.5	99.9
2	14	12	2.8	0.9	1	0.5	100.0	100.0	99.8	99.2	99.3	100.0	100.0	100.0	99.8	98.8	99.5	100.0	99.9	99.9	99.8	100.3	99.4	100.2
3	19	6	1.2	2.1	1.4	2.5	99.8	100.4	100.1	99.6	99.0	99.8	99.8	100.4	100.0	99.8	98.9	99.9	99.8	100.2	99.9	101.4	98.7	100.6
4	23	18	4	1.6	0.8	1	100.0	100.0	100.3	98.7	100.1	99.9	100.0	100.0	100.2	98.5	100.9	99.9	100.4	100.1	99.6	98.8	100.3	99.8
5	28	9	2.6	1.5	1.6	0.5	99.6	100.0	99.8	100.3	98.7	100.0	99.6	100.0	99.8	100.1	99.2	100.0	99.7	100.4	100.3	99.9	99.1	99.7
6	5	15	3.3	1.8	1.2	2	100.0	100.1	99.6	98.4	100.6	100.2	100.0	100.1	99.7	98.1	98.4	100.1	100.6	99.6	99.5	99.2	99.9	100.5
7	33	6	1.9	1.2	0.7	3	99.7	100.1	100.4	99.7	98.9	100.0	99.7	100.1	100.1	99.4	101.5	100.0	100.2	99.8	99.4	100.6	101.2	100.1
8	14	9	2	1.8	1	3.5	99.9	100.2	100.2	100.4	98.5	99.9	99.9	100.2	100.3	100.6	99.1	99.9	99.9	100.4	100.3	99.6	100.2	99.9
9	19	3	1.9	0.9	0.7	2	100.0	100.5	99.7	98.3	99.3	99.8	100.0	100.5	99.8	98.5	99.1	99.9	100.1	101.2	99.4	100.4	99.8	100.3
Mean[a]							99.9	100.2	100.1	99.3	99.4	99.9	99.9	100.2	100.0	99.1	99.8	100.0	100.1	100.2	99.8	100.0	99.9	100.1
S.D.[a]							0.14	0.18	0.34	0.78	0.80	0.13	0.14	0.18	0.30	0.90	1.16	0.07	0.29	0.48	0.36	0.78	0.76	0.31

[a] Mean and S.D., percentage recovery from the added amount.

Table 8
Application of standard addition technique to the analysis of TH, GP, DP, MP, PP and BZ by the proposed methods

Sample no.	TH					GP					DP				
	Concentration ($\mu g\,ml^{-1}$)		% found of added			Concentration ($\mu g\,ml^{-1}$)		% found of added			Concentration ($\mu g\,ml^{-1}$)		% found of added		
	Claimed	Added	PLS-1	PCR	HPLC	Claimed	Added	PLS-1	PCR	HPLC	Claimed	Added	PLS-1	PCR	HPLC
1	13.995	5.0	100.0	100.0	100.0	9.0	9	100.4	100.4	99.9	1.245	1.2	99.8	99.9	100.2
2	13.995	9.0	99.9	99.9	100.3	9.0	10	99.9	99.9	100.3	1.245	1.6	100.4	100.4	99.9
3	13.995	12.0	99.6	99.6	99.7	9.0	10.5	98.9	98.9	100.2	1.245	2	99.6	99.6	99.7
4	13.995	16.0	99.4	99.4	100.4	9.0	11	100.2	100.2	99.8	1.245	2.4	100.2	100.1	100.4
5	13.995	19.0	100.1	100.1	99.9	9.0	12	99.9	99.9	99.7	1.245	2.7	100.4	100.3	100.6
Mean[a]			99.8	99.8	100.1			99.9	99.9	100.1			100.1	100.1	100.1
S.D.[a]			0.29	0.29	0.28			0.58	0.58	0.31			0.36	0.32	0.36
Sample no.	MP					PP					BZ				
	Concentration ($\mu g\,ml^{-1}$)		% found of added			Concentration ($\mu g\,ml^{-1}$)		% found of added			Concentration ($\mu g\,ml^{-1}$)		% found of added		
	Claimed	Added	PLS-1	PCR	HPLC	Claimed	Added	PLS-1	PCR	HPLC	Claimed	Added	PLS-1	PCR	HPLC
1	0.9	0.3	98.1	97.8	100.1	0.45	0.4	100.9	101.9	100.2	1.5	0.5	99.8	99.8	100.7
2	0.9	0.4	98.6	98.5	100.4	0.45	0.5	100.5	100.7	99.9	1.5	1	100.1	100.1	99.7
3	0.9	0.6	100.2	101.2	99.8	0.45	0.7	99.3	99.7	99.2	1.5	1.5	100.2	100.3	99.6
4	0.9	0.9	100.1	100.3	99.6	0.45	0.8	98.7	98.3	100.5	1.5	1.7	99.7	99.8	99.4
5	0.9	1.2	99.2	99.0	99.4	0.45	1.1	100.3	100.5	98.8	1.5	2	99.6	99.9	100.6
Mean[a]			99.2	99.4	99.9			99.9	100.2	99.9			99.9	100.0	100.1
S.D.[a]			0.92	1.38	0.40			0.91	1.33	0.73			0.26	0.22	0.58

[a] Mean and S.D., percentage recovery from the added amount.

phase or 0.1 M hydrochloric acid for HPLC and spectrophotometric methods, respectively. At this dilution, there is no interference from excipients for both methods. The PLS-1 and PCR methods can be used for very complex mixtures since only knowledge of constituents of interest is required and they can be used to predict samples with constituents not present in the original calibration mixtures [42].

3.5. Validation of the methods

3.5.1. Linearity

The linearity of the HPLC method for determination of TH, GP, DP, MP, PP and BZ was evaluated by analysing a series of different concentrations of each compound. In this study seven concentrations were chosen, ranging between 5.0 and 33.0 $\mu g\,ml^{-1}$ for TH, 3 and 21 $\mu g\,ml^{-1}$ for GP, 1.2 and 4.0 $\mu g\,ml^{-1}$ for DP, 0.3 and 3.0 $\mu g\,ml^{-1}$ for MP, 0.4 and 2.0 $\mu g\,ml^{-1}$ for PP and 0.5 and 4.0 $\mu g\,ml^{-1}$ for BZ. Each concentration was repeated three times; this approach will provide information on the variation in peak area between samples of same concentration. The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value, which was not statistically ($P < 0.05$) different from zero (Table 5). Characteristic parameters for regression equations of the HPLC method obtained by least squares treatment of the results were given in Table 5.

3.5.2. Precision

For evaluation of the precision estimates, repeatability and intermediate precision were performed at three concentration levels for each compound. The data for each concentration level were evaluated by one-way ANOVA. A 8 days × 2 replicates design was performed. Statistical comparison of the results was performed using the *P*-value of the *F*-test (Table 6). Three univariate analyses of variance for each concentration level were made. Since the *P*-value of the *F*-test is always greater than 0.05, there is no statistically significant difference between the mean results obtained from one level of day to another at the 95% confidence level.

3.5.3. Range

The calibration range was established through consideration of the practical range necessary, according to each compound concentration present in pharmaceutical product, to give accurate, precise and linear results. The calibration range of the proposed HPLC method was given in Table 5.

3.5.4. Detection and quantitation limits

According to ICH recommendations [43] the approach based on the S.D. of the response and the slope was used for determining the detection and quantitation limits. The theoretical values were assessed practically and given in Table 5.

3.5.5. Selectivity

Methods selectivity was achieved by preparing nine synthetic mixtures of the studied drugs at various concentrations within the linearity range for HPLC. The external validation of the PLS-1 and PCR models was achieved over set of nine synthetic mixtures of the six components. The concentrations of TH, GP, DP, MP, PP and BZ were falling within the ranges of calibration matrix. The synthetic mixtures were analyzed according to the previous procedures described under the proposed methods. Satisfactory results were obtained (Table 7), indicating the high selectivity of the proposed methods for simultaneous determination of TH, GP, DP, MP, PP and BZ.

3.5.6. Accuracy

This study was performed by addition of known amounts of the studied compounds to a known concentration of the commercial pharmaceutical syrup (standard addition method). The resulting mixtures were analyzed and the results obtained were compared with the expected results. The excellent recoveries of standard addition method (Table 8) suggested good accuracy of the proposed methods.

3.5.7. Robustness

Variation of pH of the 25 mM potassium dihydrogen phosphate of the mobile phase by ±0.1, variation of the percentage of organic solvent by ±1% did not have significant effect on chromatographic resolution in HPLC method. Variation of strength of hydrochloric acid by ±0.02 M did not have significant effect on chemometric methods.

3.5.8. Stability

The studied compound solutions in the mobile phase or 0.1 M hydrochloric acid exhibited no chromatographic or absorbance changes for 4 h when kept at room temperature, and for 10 h when stored refrigerated at 5 °C.

4. Conclusion

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. Spectrophotometric 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.

A comparative study of the use of HPLC and multivariate calibration (PLS-1 and PCR) methods for the resolution of six-component mixture of TH, GP, DP, MP, PP and BZ has been accomplished, showing that multivariate calibration methods provide, with adequate software support, a clear example of the high resolving power of this technique. Although the HPLC method is more specific than the multivariate calibration methods, it needs expensive equipment and materials. Multivariate calibration methods are less expensive by comparison and they do not require sophisticated instrumentation and any prior separation step. The proposed HPLC, PLS-1 and PCR methods were found to be suitable for the routine determination of TH, GP, DP, MP, PP and BZ in pharmaceutical syrup.

References

[1] M.H. Abdel-Hay, M.S. El-Din, M.A. Abuirjeie, Analyst 117 (1992) 157–160.
[2] M.S. Bispo, M.C.C. Veloso, H.L.C. Pinheiro, R.F.S. de Oliveira, J.O.N. Reis, J.B. de Andrade, J. Chromatogr. Sci. 40 (2002) 45–48.
[3] D. Boberic-Borojevic, D. Radulovic, D. Ivanovic, P. Ristic, J. Pharm. Biomed. Anal. 21 (1999) 15–22.
[4] A. Meyer, T. Ngiruwonsanga, G. Henze, Fresen. J. Anal. Chem. 356 (1996) 284–287.
[5] J. Hu, Z. Wang, W. Weng, Yaowu Fenxi Zazhi 10 (1990) 97–99.
[6] K.P.R. Shenoy, K.S. Krishnamurthy, I. Vasundhara, Indian Drugs 38 (2001) 428–432.
[7] M. Vasudevan, S. Ravisankar, A. Sathiyanarayanan, R.S. Chandan, J. Pharm. Biomed. Anal. 24 (2000) 25–31.
[8] M. Vasudevan, S. Ravisankar, M. George, J. Ravi, Indian Drugs 37 (2000) 489–492.
[9] Y.Q. Li, Z.X. Tian, W. Zhao, J.H. Zhu, Yaowu Fenxi Zazhi 20 (2000) 97–98.
[10] L.A. Shervington, Anal. Lett. 30 (1997) 927–944.
[11] T. Chen, J.R. Pacifico, R.E. Daly, J. Chromatogr. Sci. 26 (1988) 636–639.
[12] M.L. Qi, P. Wang, J. Chen, Chromatographia 60 (2004) 105–108.
[13] V.V. Vaidya, M. Khanolkar, J.N. Gadre, Indian Drugs 38 (2001) 16–20.
[14] L. Carnevale, J. Pharm. Sci. 72 (1983) 196–198.
[15] I.I. Hewala, Anal. Lett. 27 (1994) 71–93.
[16] T.D. Wilson, W.G. Jump, W.C. Neumann, T. San Martin, J. Chromatogr. 641 (1993) 241–248.
[17] C. Barbas, A. Garcia, L. Saavedra, M. Castro, J. Chromatogr. A 870 (2000) 97–103.
[18] J.P. Surmann, M. Glienke, Fresen. Z. Anal. Chem. 332 (1988) 820.
[19] M.L. Qi, P. Wang, L. Zhou, Y. Sun, Chromatographia 58 (2003) 183–186.
[20] H. Luo, L.J. Wang, J. Wang, Yaowu Fenxi Zazhi 22 (2002) 222–224.
[21] I. Caraballo, M. Fernandez Arevalo, M.-A. Holgado, J. Alvarez Fuentes, A.-M. Rabasco, Drug Dev. Ind. Pharm. 21 (1995) 605–613.
[22] X.H. Xu, J.T. Stewart, J. Liq. Chromatogr. Relat. Technol. 23 (2000) 1–13.
[23] A. Sachan, P. Trivedi, Indian Drugs 36 (1999) 735–738.
[24] H. Tomankova, M. Vasatova, Cesk. Farm. 37 (1988) 255–259.
[25] H. Tomankova, M. Vasatova, Pharmazie 44 (1989) 197–198.
[26] A.F. Marchesini, M.R. Williner, V.E. Mantovani, J.C. Robles, H.C. Goicoechea, J. Pharm. Biomed. Anal. 31 (2003) 39–46.
[27] M.R. Gomez, R.A. Olsina, L.D. Martinez, M.F. Silva, J. Pharm. Biomed. Anal. 30 (2002) 791–799.
[28] H. Iturriaga, J. Coello, S. Maspoch, M. Porcel, Anal. Bioanal. Chem. 374 (2002) 33–38.
[29] H.C. Goicoechea, A.C. Olivieri, Anal. Chim. Acta 453 (2002) 289–300.
[30] H.C. Goicoechea, A.C. Olivieri, J. Pharm. Biomed. Anal. 20 (1999) 255–261.
[31] L.Q. Zhang, X.H. Wu, X. Tang, X.L. Zhu, W.T. Su, Fenxi Kexue Xuebao 18 (2002) 318–320.
[32] L.Q. Zhang, X.H. Wu, X. Tang, X.L. Zhu, W.T. Su, Guangpuxue Yu Guangpu Fenxi 22 (2002) 427–429.
[33] F.A. El-Yazbi, M.A. Korany, Spectrosc. Lett. 18 (1985) 543–553.
[34] M.A. Korany, M. Bedair, F.A. El-Yazbi, Analyst 111 (1986) 41–44.
[35] B.M. Wise, N.B. Gallagher, PLS-Toolbox Version 2.1, Eigenvector Research Inc., 830 Wapato Lake road, Manson, WA 98831, 2000.
[36] R.G. Brereton, Analyst 122 (1997) 1521–1529.
[37] M. Blanco, J. Coello, F. Gonzalez, H. Iturriaga, S. Maspoch, J. Pharm. Sci. 82 (1993) 834–837.
[38] H. Martens, T. Naes, Multivariate Calibration, Wiley, Chichester, 1989.
[39] A. Espinosa-Mansilla, F. Salinas, I. De Orbe Paya, Anal. Chim. Acta 313 (1995) 103–112.
[40] D.M. Haaland, E.V. Thomas, Anal. Chem. 60 (1988) 1193–1202.
[41] J.A. Murillo Pulgarin, A. Alanon Molina, P. Fernandez Lopez, Anal. Chim. Acta 449 (2001) 179–187.
[42] K.R. Beebe, J.P. Randy, Seasholtz, Chemometric: A Practical Guide, Wiley/Interscience, New York, 1998.
[43] The European Agency for the Evaluation of Medical Products. ICH Topic Q2B Note for Guidance on Validation of Analytical Procedures: Methodology GPMP/ICH/281/95, 1996.