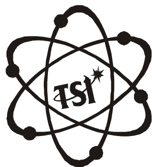

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Determination of ambroxol hydrochloride, guaifenesin and theophylline in their ternary mixtures and in the presence of excipients in different pharmaceutical dosage forms

Nada S.Abdelwahab

Analytical Chemistry Department, Faculty of Pharmacy, Benisuef University, (EGYPT)

E-mail : nadasayed2003@yahoo.com

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ABSTRACT

Determination of ternary mixtures of Ambroxol Hydrochloride (AMB), Guaifenesin (GU) and Theophylline (TH) with minimum sample pre-treatment and without analyte separation has been successfully achieved by using chemometric and RP-HPLC methods. The developed chemometric models are partial least squares (PLS) and genetic algorithm coupled with PLS (GA-PLS). Data of analysis were obtained from UV-VIS spectra of the studied drugs in different concentration ranges. These models have been successfully updated to be applied for determination of the proposed drugs in farcosolvin® syrup and in the presence of syrup excipients (methyl paraben). In the developed RP-HPLC method, chromatographic runs were performed on RP C18 analytical column with mobile phase comprising 0.05M phosphate buffer: methanol: acetonitrile: triethylamine solution in isocratic mode (63.5: 27.5: 9: 0.25%, by volume pH 5.5 with orthophosphoric acid) at a flow rate of 1.2 ml/ min. The analytes were detected and quantified at 220 nm. The method was optimized in order to obtain good resolution between the studied components and to prevent interference from methyl paraben. Method validation was performed with respect to ICH guidelines and the validation acceptance criteria were met in all cases. The proposed methods can be considered acceptable for the pharmaceutical quality control of the studied drugs in pharmaceutical capsules and syrup. The results obtained by the suggested chemometric methods for determination of the studied mixture in different pharmaceutical preparations were statistically compared to those obtained by applying the developed RP-HPLC one and no significant difference was found.

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KEYWORDS

Ambroxol;
Guaifenesin;
Theophylline;
Partial least squares;
Genetic algorithm;
RP-HPLC.

INTRODUCTION

Ambroxol Hydrochloride ((AMB) Figure 1A) has the IUPAC name 4-[(2-amino-3,5-dibromophenyl)methylamino]cyclohexan-1-ol[1], it is a pharmacologically active metabolite of bromhexine and a compound with potent mycolytic activity, for which it is used as an expectorant and bronchosecretolytic in therapeutics[2]. Guaifenesin ((GU) Figure 1B) has the IUPAC name (RS)-3-(2-methoxyphenoxy)propane-1,2-diol[1],

it has expectorant properties and is widely used in cough remedy formulations[3,4]. Also, it is used as an agent for reducing platelet adhesiveness, hypocholesteremic reagent, a muscle relaxant and a general anaesthetic for veterinary uses[5]. Theophylline ((TH) Figure 1C) has the IUPAC name 1,3-dimethyl-7H-purine-2,6-dione, it has maintained an important role as a potent and useful bronchodilator. However, the use of TH is often restricted by its narrow therapeutic range and various adverse effects occur when plasma levels exceed 20 μg / ml, so it is necessary to monitor its concentration in individual patients to ensure the maximum clinical response and to avoid undesirable side effects[6]. Combination of the AMB, GU and TH along with etofylline are indicated for the prophylaxis and relief of reversible bronchospasm associated with acute and chronic asthma, bronchitis and other chronic obstructive airway disease where reversible airway narrowing occurs[7].

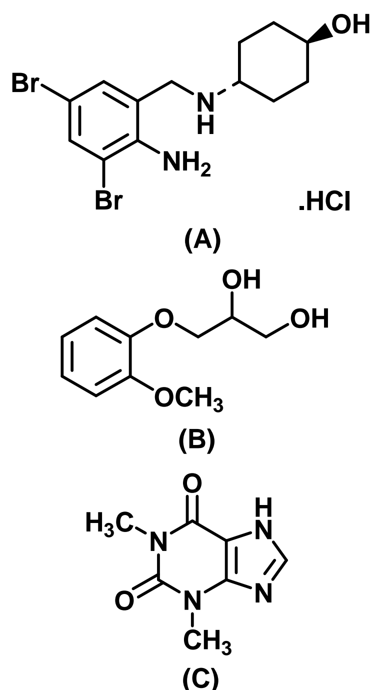

Figure 1 : Chemical structure of A- AMB, B- GU and C- TH.

Reviewing the literature, there are several reports that describes the analysis of each of AMB, GU and TH either alone or in combination with other components. Recently, AMB has been determined with other drugs by several techniques including TLC-Densitometric[8], HPLC[9-11] and HPLC/MS techniques[12,13]. Also different UV spectrophotometric[14,15] methods have been applied for its determination in tablets. On the other hand, GU with its combinations with other drugs have been analyzed by different HPLC[16,17] and spectrophotometric methods[18,19], including chemometrics. Ternary mixture of GU, salbutamol and dyphylline in oral formulations has been determined by micellar electrokinetic chromatographic technique[20], while GU along with antipyretics and analgesics in solid dosage forms were determined by powder X-ray diffraction method[21]. Several HPLC methods were currently described for quantification of TH and other components in different preparations and in urine samples[22-27], while HPTLC technique was used for its determination in blood[28]. Theophylline (TH) was among components that were quantified by micellar electrokinetic chromatographic methods[29,30] and it was determined alone by different voltametric techniques[31,32]. Also capillary electrophoretic methods have been recently developed for determination of some components including TH[33,34].

Ambroxol (AMB) and GU have been determined together in mixtures with other drugs by HPLC[35] and micellar electokinetic capillary chromatographic[36] techniques, while the mixtures of GU and TH were analyzed by HPLC[37-41], derivative spectrophotometric[37,38] and chemometric[39,42] techniques. However the exhaustive literature survey reveals that there is only one report that described the analysis of the studied drugs in their quaternary mixtures with etofylline by using HPLC method[7]. This reported method was found to be time consuming (run time > 20 min) and of lower sensitivity (especially for GU) than the developed HPLC method, moreover, the reported chromatographic method has been performed at 30°c while the developed one has been carried out at room temperature. Also, it is the first time that the ternary mixture of AMB, GU and TH has been determined by spectrophotometric method in their different pharmaceutical preparations (capsules and syrup).

This work concerns PLS, GA-PLS and RP-HPLC methods for determination of ternary mixtures of AMB, GU and TH with highly overlapping UV absorption spectra. The simultaneous determination of such components in their available pharmaceutical dosage forms by convential, derivative and derivative ratio spectrophotometric methods is hindered by their strong spectral overlap and interference from syrup excipient (methyl paraben as recommended by the manufacturer). The suggested RP-HPLC and chemometric models (after

models update) can be used to overcome these problems. Also they are rapid, sensitive and suitable for routine determination of the studied components in their mixtures.

EXPERIMENTAL

Instruments

For the chemometric methods, Double beam UV-VIS spectrophotometer (Shimadzu, kyoto- Japan), model UV-1601 PC with 1cm quartz cells, connected IBM compatible computer. Matlab® version 6.5[43] was used for the proposed chemometric methods, the PLS was performed with PLS_Toolbox[44] for use with Matlab® 6.5.

For HPLC method, HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP controller, DGU-12 A Degasser and SPD-10 AVP UV-VIS detector, separation and quantitation were made on RP C18 column (250 × 4.6 mm i.d 4.6 µm particle size). The detector was set at 220 nm.

Samples

Pure samples

Pharmaceutical grade of AMB, GU and TH (PHARCO Pharmaceuticals, Alexandria, Egypt) were used and certified to contain 100.89, 99.7 and 99.1 %, respectively.

Pharmaceutical preparations

Farcosolvin® syrup (Batch No. 450 and 451) and farcosolvin® soft gelatin capsules (Batch No. 146) were manufactured by PHARCO Pharmaceuticals (Alexandria, Egypt). Trisolvin™ ® capsules (Batch No. 082161 A) were manufactured by GalaxoSmithKline S.A.E (El Salam City, Cairo, Egypt).

Chemicals and solvents

All chemicals and solvents used through this work (potassium dihydrogen phosphate, orthophosphoric acid, acetic acid and triethylamine) were of analytical grade and were purchased from El- NASR Pharmaceutical Chemicals Co., Abu- Zabaal, Cairo, Egypt. Methanol and acetonitrile were of HPLC grade (CHROMASOLVE®, Sigma-Aldrich Chemie GmbH, Germany). Deionized water (SEDICO Pharmaceuticals Co., Cairo, Egypt).

Solutions

- *Stock standard solutions of AMB, GU and TH* were prepared in methanol in the concentration of 1 mg /ml.
- *Working standard solutions of AMB, GU and TH* were prepared in methanol (for chemometric methods) and in methanol: acetonitrile (27.5: 9, v/v) (for HPLC method) in the concentration of 0.1 mg /ml.

PROCEDURE

Chemometric methods

Spectral characteristics

The absorption spectra of 10 µg /ml each of AMB, GU and TH, mixture of them contains (3: 6: 10 µg /ml) of them, respectively, and farcosolvin ® syrup (in the same mixture ratio) were recorded over the range of 200 - 350 nm using methanol as a blank, Figure 2.

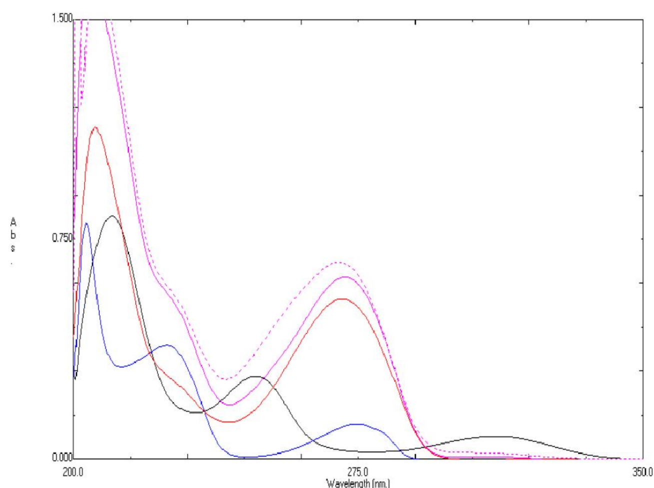

Figure 2 : Zero order absorption spectra of 10 µg /ml each of AMB (——), GU (——), TH (——), mixture of the three component (——) in the ratio (3: 6: 10 µg /ml), respectively and farcosolvin® syrup contains (---) (3: 6: 10 µg /ml) of each, respectively, using methanol as a solvent.

Building the calibration models

Training set of 15 laboratory prepared mixtures with different concentrations of AMB, GU and TH were prepared by dilution of their respective working standard solutions (0.1 mg /ml) with methanol in the concentration ranges of 1.5 - 5.5, 4 -12 and 2 - 10 µg /ml for AMB, GU and TH, respectively. These mixtures were prepared according to multilevel multifactor experimental design[45],

TABLE 1. The UV absorption spectra of these mixtures were recorded over the wavelength range of 200 – 350 nm with 0.4 nm interval, then transferred to Matlab®6.5 for subsequent data analysis and the calibration models (PLS and GA-PLS) were then constructed.

TABLE 1 : Concentrations of ambroxol, guaifenesin and theophylline in the calibration and validation sets.

Sample No.	AMB (µg /ml)	GU (µg /ml)	TH (µg /ml)
1*	5.5	4.0	8.0
2	3.5	8.0	6.0
3	3.5	4.0	2.0
4	1.5	4.0	10.0
5*	1.5	10.0	2.0
6*	4.5	4.0	6.0
7	1.5	12.0	4.0
8*	2.5	8.0	8.0
9	5.5	6.0	10.0
10	2.5	12.0	6.0
11	5.5	8.0	4.0
12	3.5	6.0	4.0
13	2.5	6.0	8.0
14*	3.5	10.0	8.0
15	2.5	10.0	10.0
16*	4.5	10.0	4.0
17	4.5	12.0	8.0
18	5.5	10.0	6.0
19*	4.5	6.0	2.0
20*	2.5	4.0	4.0
21	4.5	8.0	10.0
22	3.5	12.0	10.0
23	5.5	12.0	2.0
24*	1.5	6.0	6.0
25*	2.5	8.0	2.0

*** samples used for model validation.**

Assay of validation set

The absorption spectra of a validation set consisting of 10 different laboratory prepared mixtures prepared in the same way as the calibration set, TABLE 1, were recorded in the range of 200 – 350 nm. The concentrations of each component were calculated using the optimized PLS and GA-PLS calibration models.

Model update (for farcosolvin® syrup)

In order to perform the model update, the calibration set was augmented with different samples of farcosolvin® syrup containing known amounts of AMB, GU and TH. One to six samples containing different concentrations of the studied drugs were added to the initial calibration set and the predictive ability of the updated models was checked using an external validation samples of farcosolvin® syrup in the concentration ranges of 1.5 – 3, 3 – 6 and 5 – 10 µg /ml for AMB, GU and TH, respectively (their concentrations were previously determined using the developed HPLC method). The RMSEP (root mean squares error of prediction) values were calculated for each component using the developed models with different number of added updating samples.

HPLC method

Chromatographic conditions

Chromatographic analysis was performed in isocratic mode. Mobile phase consisted of 0.05 M phosphate buffer: methanol: acetonitrile: triethylamine (63.5: 27.5: 9: 0.25%, by volume), being pumped at a flow rate of 1.2 ml /min. Samples were injected manually as 20 µl and detection wavelength was 220 nm. Total run time was 11 min. all experiments were performed at room temperature and the total peak height was used to quantify the studied drugs.

Method validation

The developed HPLC method was validated according to USP requirements[41] and ICH guidelines[46]. *Linearity* of the detector response with the concentrations of the studied drugs was evaluated using different standard solutions of pure drugs. Working standard solutions each of AMB, GU and TH were diluted with mixture of methanol: acetonitrile (27.5: 9, v/v) to concentration ranges of 5 – 50, 5 – 50 and 3 - 30 µg /ml, respectively. Triplicate injections were made for each concentration and then the integrated peak height was plotted versus the corresponding concentration for construction of calibration curves and regression analysis. *Specificity* was evaluated by comparison of representative chromatograms of samples containing possible interfering substances (e.g. syrup excipients) and samples containing the studied drugs. *Accuracy* of the method was calculated as the percentage recoveries of blind samples of pure AMB, GU and TH. It was further assured by application of standard addition technique, by addition of

known amounts of pure drugs to known concentrations of the pharmaceutical preparations and then analyzing the prepared mixtures. *Precision* was assessed as RSD% at different levels; *repeatability* was evaluated by the analysis of three different concentrations of pure drugs (10, 20 and 25 µg /ml) in triplicates on the same day and *intermediate precision* by repeating the studies seven times on four consecutive days. *Limits of detection (LOD) and quantitation (LOQ)* were calculated from the standard deviation (δ) of the response and the slope of the calibration curve (S) in accordance to the following equations: LOD= 3.3 (δ/S) and LOQ= 10 (δ/S). *Robustness* was evaluated by small variation in triethylamine concentration in the prepared mobile phase (0.2 and 0.35 %) and by small variation in the mobile phase flow rate (1.15 and 1.25 ml/min). *System suitability test (SST) parameters* were performed during the development and optimization of the method as well as through the validation procedure. SST parameters include capacity factor (k'), selectivity factor (α), resolution (Rs), column efficiency (number of theoretical plates, N) and tailing factor.

Application to pharmaceutical preparations

Farcosolvin® syrup

Accurate volume of farcosolvin® syrup equivalent to 100 mg TH, 60 mg GU and 30 mg AMB was transferred into 100-ml calibrated measuring flask and the volume was then completed using methanol.

Farcosolvin® and trisolvin® capsules

The content of twenty capsules each of farcosolvin® and trisolvin® were separately emptied and weighed. An accurately weighted portion equivalent to 100 mg GU, 60 mg TH and 30 mg AMB of each dosage form was separately transferred into 100-ml calibrated measuring flask and then 75 ml methanol was added. The prepared solutions were sonicated for 30 minutes, the volume was completed with the same solvent and the solutions were then filtered.

Appropriate dilutions of the prepared solutions were made to prepare their working solutions (0.1 mg /ml) and then the proposed methods were followed.

Standard addition technique: it was carried out to assess the validity of the suggested methods. It was carried out by addition of accurately measured amounts of pure AMB, TH and GU, in the levels of 80, 100 and 120%, to each of the prepared dosage forms.

RESULTS AND DESCUSSION

The electronic absorption spectra of AMB, GU and TH are shown in Figure 2. As can be seen, the spectra of the studied components are overlapped with each other and with the syrup excipients (i.e. methyl paraben), thus these components can not be measured in the presence of each other and in the presence of methyl paraben by a simple calibration procedure without prior separation. To best of our knowledge, there is only one report that described a RP-HPLC method for determination of the studied drugs in their mixtures and in their liquid dosage form, these reported method was found to be time consuming and of low sensitivity (especially for GU). Moreover, there is no other previous report for the spectrophotometric determination of the proposed drugs in presence of methyl paraben by chemometric methods. In this study, PLS, GA-PLS and RP-HPLC methods have been successfully applied for determination of AMB, GU and TH in their mixtures and in different pharmaceutical preparations without interference from methyl paraben.

Chemometric methods

Multivariate calibration methods allow extracting analytical information from the full spectra providing simultaneous determination of several components in the sample. Moreover, these techniques permit rapid analytical response with minimum sample preparation, reasonable accuracy and precision without separation procedure. So they can be considered for routine analysis of drugs in their formulations[47].

PLS model

PLS has become the most frequently used method for simultaneous calibration because high performance calibration models are obtained, while the software is not only available, but also easily implemented[48,49].

For construction of calibration and validation sets, five levels three factors experimental design was used[45] and 25 mixtures of the studied drugs were prepared, TABLE 1. The absorbance of these solutions in the range of 200 - 350 nm was recorded, the range of 215.2 – 326.8 nm was taken and the spectral data acquisition

was taken with 0.4 nm intervals. This produced spectral data matrix of 25 rows representing different samples and 280 column representing wavelengths (25 × 280). Fifteen samples were chosen and used for calibration and the other ten samples were used for validation. In order to apply PLS model to the recorded data, it was autoscaled and validated with random selection of subset, each subset was consisted of five splits and iterated twice. To choose the optimum number of significant latent variables (LVs), Haaland and Thomas's criterion[48] was used. The optimum number of LVs described by the developing PLS and GA-PLS was found to be four.

GA-PLS model

Although PLS is usually considered as a full spectrum method, literature shows a growing tendency to perform variable selection before multivariate regression in order to improve its predicting ability. Consequently, in practice wavelength selection continues to be the process of interest because a selection procedure that optimize the prediction capacity will lead to those wavelengths for which the analyte of interest absorbs while its absorbance is different from other analytes[50]. Genetic algorithms (GAs) is one of wavelength selection methods in multivariate calibration models[51,52]. GAs has been shown to solve the optimization problem by exploring all regions of potential solutions and exponentially exploiting promising area through mutations, cross over and selection applied to individuals in the population. Complete discussion of GAs can be found in references[53-55].

To improve the GAs based wavelength selection procedure, several parameters were adjusted (in initial trials two settings were chosen for each variable, maximum and minimum value), keeping number of subsets, maximum number of LVs and number of iterations at constant values. Blackett-Burman's[56] design for the seven assigned variables was applied in which all parameters were changed together in order to determine the optimum parameters setting for GAs model. The GA configuration that gave the best fitness value with minimum number of LVs was selected for each run. The percent improvement in RMSEP of GA-PLS relative to PLS for each parameters setting was calculated[57], TABLE 2. The optimum configuration used in the study is shown in TABLE 3. Genetic algorithms allows for the concentration matrix to be used, however only the first component is optimized during the process of wavelength selection (PLS1). So the same procedure was repeated for each component. It was found that when GU was used for optimization, the RMSEP value for each component was improved, TABLE 4, and so the selected wavelengths can be used for adequate calibration of the other two components.

The predictive ability of the suggested models was evaluated by plotting the known concentrations versus predicted ones for each component for each model

TABLE 2 : Plackett-Burman design for GAs parameter settings.

Trial	Assigned variables (A-G)							Unassigned variables (H-K)				Percent Improvement
	A	B	C	D	E	F	G	H	I	J	K	
1	-	-	-	-	-	-	-	-	-	-	-	-27.02
2	+	-	+	+	+	-	-	-	+	-	+	-43.24
3	-	+	+	+	-	-	-	+	-	+	+	-10.81
4	+	+	+	-	-	-	+	-	+	+	-	8.11
5	+	+	-	-	-	+	-	+	+	-	+	-62.16
6	+	-	-	-	+	-	+	+	-	+	+	-5.41
7	-	-	-	+	-	+	+	-	+	+	+	6.76
8*	-	-	+	-	+	+	-	+	+	+	-	16.22
9	-	+	-	+	+	-	+	+	+	-	-	-32.43
10	+	-	+	+	-	+	+	+	-	-	-	-70.27
11	-	+	+	-	+	+	+	-	-	-	+	5.41
12	+	+	-	+	+	+	-	-	-	+	-	5.41

note: a-population size, b-% wavelengths at initiation, c-maximum generations, d-% at convergence, e- mutation rate, f-crossover type and g- window width. percent improvement- percentage improvement of RMSEP p of GA-PlS in comparison with that of PLS.
*** the chosen parameter setting used for building the developed GA-PLS model.**

TABLE 3 : Levels of GAs parameters setting.

Parameters	Low (-)	High (+)	Chosen level
Population size	16	40	40
% Wavelength used at initiation	10	50	50
Maximum generations	25	100	100
% at convergence	10	100	10
Window width	2	5	2
Mutation rate	0.001	0.005	0.001
Crossover type	single	double	single
Number of subsets	---	---	5
Maximum no. of latent variables	---	---	2
Number of iterations	---	---	2

TABLE 4 : Percentage improvement upon using the three components in GAs optimization.

Compound used for optimization	Improvement %		
	AMB	GU	TH
AMB	16.22	18.48	-1.50
GU*	13.50	15.20	1.50
TH	-32.40	-4.00	8.33

* compound chosen for GA-PLS optimization.

and the statistical parameters of the regression equations are summarized in TABLE 5. To further access the predictive ability of the models, they were applied to an external validation set where good results and low RMSEP values were obtained, TABLE 6. The developed models were successfully applied for determination of AMB, GU and TH in farcosolvin® and trisolvin® capsules, TABLE 6. As shown in Figure 2, there is a degree of spectral overlap among the studied drugs and methyl paraben, thus we have explored the use of models update in order to remove the interference from methyl paraben.

Models update

Multivariate calibration models can be updated by including samples containing the new source of data variance to the existing calibration set and the concentrations of the new samples are added to the existing concentration matrix. The minimal number of samples needed to efficiently update the developed models must be accurately determined and the influence of number of samples added to the calibration set on RMSEP was studied for each developed multivariate calibration model. The number of different samples added shows a large impact on the predictive ability of the updated models, where for AMB the RMSEP of the models updated decreases from 1.623 and 2.067 for the initial models to 0.029 and 0.051 for the updated PLS and GA-PLS models, respectively, which represents an improvement of 98.2 and 97.5 % for the two methods, respectively. While for GU the RMSEP of the models updated decreases from 0.651 and 0.372 to 0.096 and 0.093 which represents an improvement of 85.3 and 75 % for the two methods, respectively. The RMSEP of TH in the updated models decreases from 1.25 and 1.23 to 0.067 and 0.096 which represents an improvement of 94.6 and 92.2 % for the

TABLE 5 : Regression and analytical parameters of the proposed methods for determination of ambroxol, guaifenesin and theophylline.

Parameters	Chemometric methods						RP-HPLC method		
	AMB		GU		TH		AMB	GU	TH
	PLS	GA-PLS	PLS	GA-PLS	PLS	GA-PLS			
Linearity									
Range	1.5 – 5.5 µg /ml		4 - 12 µg /ml		2 - 10 µg /ml		3 – 50 µg /ml	5 – 50 µg /ml	3 – 35 µg /ml
Slope	0.9927	0.9947	1.0028	1.0024	0.9821	0.9821	0.0091	0.0176	0.0353
Intercept	0.0012	0.0049	0.0973	0.0749	0.0757	0.0677	0.0003	0.0009	-0.0176
(r)	0.9998	0.9998	0.9997	0.9997	0.9998	0.9998	0.9997	0.9997	0.9997
Accuracy							100.58	100.04	100.20
Precision									
Repeatability							0.958	0.897	0.699
Intermediate precision							1.005	0.957	1.001
LOD							0.80 µg /ml	1.50 µg /ml	0.50 µg /ml
LOQ							2.42 µg /ml	4.55 µg /ml	1.52 µg /ml

r: the correlation coefficient.

two methods, respectively. Four samples are found to be necessary to perform an efficient update of the developed PLS and GA-PLS models. The results of analysis of farcosolvin® syrup (of different batch number) by the updated models and its statistical comparison with those obtained by applying the developed HPLC method are presented in TABLE 6, which shows that the updated models can be successfully applied for determination of the studied mixture in syrup with good accuracy and precision without reconstruction of the calibration set.

When PLS model was preceded by GA wavelength selection, the prediction of AMB, GU and TH was improved as described by the decrease in RMSEP values relative to the developed PLS model.

TABLE 6 : Determination of the studied drugs in the laboratory prepared mixtures (L.P.) and different pharmaceutical preparations by the proposed methods and statistical comparison with the developed RP-HPLC method.

Parameters	Chemometric methods						RP-HPLC method		
	AMB		GU		TH		AMB	GU	TH
	PLS	GA-PLS	PLS	GA-PLS	PLS	GA-PLS			
L.P. Mixtures[a]	99.32 ± 1.096	99.69 ± 0.893	98.70 ± 1.039	99.03 ± 0.976	100.21 ± 1.438	99.98 ± 1.258			
RMSEP	0.037	0.032	0.099	0.084	0.065	0.064			
Farcosolvin® Syrup [b] (B.N.451)	97.80 ± 0.379	98.60 ± 1.856	98.03 ± 1.063	97.33 ± 1.073	96.76 ± 0.522	97.70 ± 0.464	99.05 ± 0.906	97.80 ± 1.213	98.31 ± 1.067
Standard addition[a]	102.29 ± 0.958	102.02 ± 1.415	101.52 ± 1.810	99.73 ± 3.012	99.74 ± 1.613	100.51 ± 0.794	101.09 ± 1.005	98.45 ± 0.968	99.83 ± 1.540
Degree of freedom	8	8	8	8	8	8			
F-test (6.388)[c]	5.702	1.234	1.302	1.278	4.168	5.293			
Farcosolvin ® capsules [b] (B.N.146)	105.93 ± 0.829	106.33 ± 1.291	103.42 ± 1.013	103.50 ± 1.965	101.80 ± 0.322	101.67 ± 0.709	108.30 ± 1.362	105.47 ± 1.326	102.07 ± 0.795
Standard addition[a]	98.31 ± 0.906	102.18 ± 1.076	101.20 ± 1.697	101.46 ± 0.238	100.46 ± 1.111	99.37 ± 1.266	100.99 ± 1.549	102.05 ± 0.965	99.87 ± 1.839
Degree of freedom	8	8	8	8	8	8			
F-test (6.388) [c]	2.697	1.188	1.711	2.197	6.083	1.260			
Trisolvin ® capsules[b] (B.N.082161 A)	102.00 ± 1.369	101.33 ± 1.068	100.80 ± 1.217	99.80 ± 1.472	95.33 ± 1.076	96.17 ± 1.157	102.22 ± 1.323	100.97 ± 1.621	96.64 ± 1.423
Standard addition[a]	101.94 ± 0.587	101.59 ± 1.294	101.55 ± 1.058	101.27 ± 0.551	101.64 ± 1.365	100.53 ± 1.446	102.56 ± 1.054	100.99 ± 1.598	101.45 ± 0.859
Degree of freedom	8	8	8	8	8	8			
F-test (6.388)[c]	1.072	1.536	1.773	1.212	1.752	1.514			

RP-HPLC method

For analytical purpose, it is always of interest to establish methods of analysis that need short time period with acceptable accuracy and precision. In this study a convenient and efficient RP-HPLC method was developed for determination of AMB, GU and TH in their ternary mixtures and in different pharmaceutical preparations in the presence of methyl paraben with high sensitivity, short analysis time and without sample pretreatment.

Method development and optimization

Prior to the validation step, the hereby proposed method was developed in order to provide a simple and optimum procedure with reduced time and cost of analysis. The initial run was performed using the reported HPLC method[7] where long run time was needed (> 20 min). Moreover, scanning at 235 nm gave poor sensitivity for each the studied drugs (especially GU). Different parameters were then manipulated to obtain an acceptable resolution between the three studied drugs and methyl paraben, reduce the analysis time, enhance the quantitation limit of the method and satisfy the HPLC system suitability.

Influence of organic modifier and its ration

The optimization was started with 0.05 M phosphate buffer: acetonitrile (70: 30, v/v pH 3.8 with acetic acid) which gave a strongly tailed peak for AMB after long time (> 18 min), also changing acetonitrile ratio (60:40 and 50:50, v/v pH 3.8) did not improve AMB peak. The next step is to replace acetonitrile with methanol which is reported to influence the retention characteristics of basic solutes (such as AMB) and also affect the dissociation of both buffer and basic

solutes[58]. Different ratios of buffer: methanol were tested (85:15, 70:30 and 50:50, v/v pH 3.8) where in all cases improvement of AMB separation was observed but with poor resolution among TH and GU peaks. Combinations of methanol and acetonitrile along with the phosphate buffer in different ratios were then tried (59:40:1, 63.5:18.5:18 and 63.5:27.5:9, by volume pH 3.8) by volume where reasonable chromatographic separation of TH, GU and AMB were obtained on using buffer: methanol: acetonitrile (63.5: 27.5: 9, by volume pH 3.8 with acetic acid) but with the same value for tailing factor of AMB peak.

Ratio of triethylamine

Tailing of AMB peak may probably be explained by the establishment of interactions between the amine group of AMB (as a basic component) and silanol groups of the stationary phase[59]. There are many possible ways for suppressing these interactions, among them is the addition of "silanol blockers' "[58] e.g. triethylamine (TEA) to the mobile phase. TEA provides a competing amine that can also strongly bond to free silanol groups in the stationary phase inhibiting or at least reducing AMB interaction with these groups. Different ratios of triethylamine were tested (0.1, 0.2, 0.25 and 0.3% of the mobile phase). Acceptable and stable tailing factor was obtained when the percentage of triethylamine was above 0.2% of the prepared mobile phase volume. Finally mobile phase consisting of buffer: methanol: acetonitrile: triethylamine (63.5: 27.5: 9: 0.25%, by volume pH 5 with acetic acid) was chosen for the chromatographic separation.

pH of the mobile phase

The molecular structures of AMB, GU and TH, Figure 1, imply that AMB is the only component of the studied mixture that contains functional group (amine group) which ionizes and reflects the impact on separation with pH variation. The retention time and the resolution of both TH and GU were almostly unchanged upon using different pH values. On the other hand, AMB showed dramatic change in its retention time with pH variation which affected its chromatographic separation from methyl paraben. AMB, being basic compound, is more retained in RP-HPLC in its molecular or unionized form (i.e. high pH value of the mobile phase)[60], so when different pH values were tested (3, 3.8, 4.5, 5.5, 7 and 8 pH). The best resolution with reasonable retention time of AMB and methyl paraben was obtained at pH 5.5.

Different reports realized that better models are obtained when the pH in the mobile phase is considered instead of aqueous pH of the buffer[61,62], so pH of 5.5 was adjusted for the prepared mobile phase as a whole. In addition, acetic and phosphoric acids used for adjusting the mobile phase pH were tested. Using acetic acid gave good resolution even at lower pH value (pH 3.8) but with poor sensitivity for all of the studied drugs, while on using phosphoric acid the sensitivity was improved but with bad resolution among AMB and methyl paraben at pH values < 5. Phosphoric acid was used to adjust the mobile phase pH to 5.5 which resulted in good chromatographic separation and good sensitivity.

Optimization of mobile phase flow rate and scanning wavelength

The mobile phase was pumped at different flow rates (1, 1.2, 1.3 and 1.5 ml /min) where optimum separation with reasonable time of analysis was obtained with flow rate of 1.2 ml/min. Scanning the produced chromatograms at different wavelengths (220, 235 and 254 nm) was also carried out where scanning at 220 nm showed good sensitivity for all of the studied drugs.

After an extensive study, the method has been finalized on RP-C18 column (250×4.6 mm, 5 μm particle size) using mobile phase of 0.05 M phosphate buffer: methanol: acetonitrile: triethylamine (63.5: 27.5: 9: 0.25%, by volume pH 5.5 with phosphoric acid), flow rate of 1.2 ml/min and detection at 220 nm for all components. Typical HPLC chromatograms, Figures 3, 4 represent the satisfactory separation between TH (3.15 min), GU (4.5 min), methyl paraben (6.38 min) and AMB (8.9 min).

Method validation

Linearity

Under optimum chromatographic conditions, linear relationships existed between the mean integrated peaks height and the corresponding concentrations for each of AMB, GU and TH. The values of correlation coefficients were close to unity indicating good linearity, the characteristic parameters for the regression equations obtained by least square treatment of the results are summarized in TABLE 5.

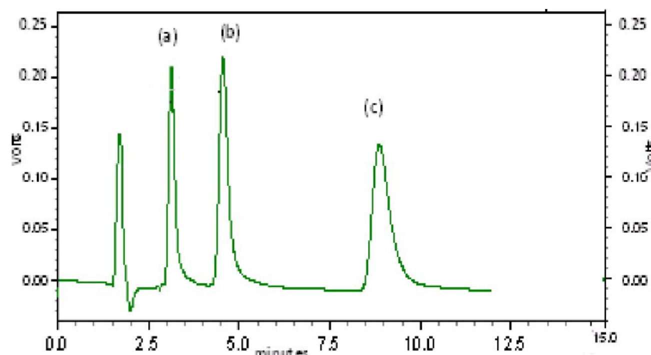

Figure 3 : HPLC chromatograme of 20 µg /ml of (a) TH, 50 µg /ml (b) GU and 50 µg /ml (c) AMB.

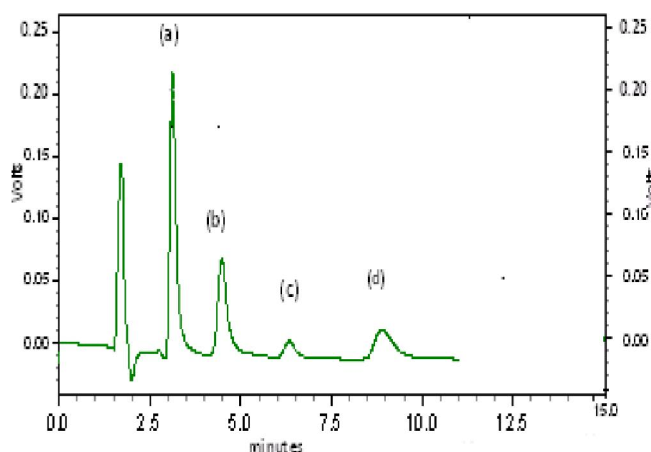

Figure 4 : HPLC chromatograme of farcosolvin® syrup contains 20 µg /ml of (a) TH, 12 µg /ml (b) GU, (c) methyl paraben and 6 µg /ml (d) AMB.

Specificity

It was proven as the retention times for each compound in recovery experiments were identical to those of standard solution, Figures 3, 4. Also, no peaks at the retention times of the studied drugs were observed, indicating absence of interference from methyl paraben.

Accuracy

Accuracy indicates the agreement between obtained results and those accepted as true, detailed results are presented in TABLE 5. Results of standard addition technique, TABLE 6, also confirm the accuracy of the method.

Precision

The results of intra-day and inter-day precision confirme good precision of the proposed RP-HPLC method, TABLE 5.

Limits of detection and quatitation

The lowest concentration at which an analyte can be detected (LOD) or quantified with acceptable accuracy and precision (LOQ). Results presented in TABLE 5, indicate that the method is sensitive for determination of the studied drugs.

Robustness

Robustness testing is useful in order to prove that typical variations to the method are negligible in the procedure outcome, being usually studied by deliberately changing critical parameters and monitoring possible alterations. The method showed no significant changes in its results concerning small but expectable variations of the mobile phase flow rate and ratio of triethylamine in the mobile phase. On the other hand, any small variation in the mobile phase pH showed dramatic change in retention time of AMB, affecting the resolution among AMB and methyl paraben, hence it was concluded that the method is sensitive to mobile phase pH.

System suitability tests (SST)

System suitability testes (SST), TABLE 7, confirmed that the chromatographic system was adequate for the analysis planned to be done. Also the calculated SST parameters were within the acceptance criteria for good HPLC practice (except the capacity factor for TH which can not be improved more than 0.8).

The proposed validated HPLC method has been applied for determination of AMB, GU and TH in farcosolvin® and trisolvin® capsules, TABLE 6. Also, it was optimized and applied for their determination in farcosolvin® syrup, TABLE 6, where satisfactory resolution from methyl paraben was obtained, Figures 3, 4. Statistical comparison (using F-test) of the results obtained by applying the proposed chemometric models for analysis of the three proposed drugs in different pharmaceutical preparations to those obtained by applying the proposed RP-HPLC method showed no significant difference between them regarding both accuracy and precision, TABLE 6.

CONCLUSION

The described methods provide convenient and efficient methods for the determination of AMB, GU and TH in different pharmaceutical formulations in the presence of methyl paraben. Spectrophotometric techniques when coupled with chemometric tools, the quality of

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TABLE 7 : Statistical analysis of parameters required for system suitability testing of RP- HPLC method.

Parameters	Obtained value				Reference value
	TH	GU	Syrup additive	AMB	
Resolution (Rs)	1.83	2.21	1.59		> 0.8
Relative retention (α)	1.94	1.68	1.53		>1
Tailing factor (T)	1.25	1.12	1.00	1.34	T = 1, for a typical symmetrical peak
Capacity factor (k')	0.81	1.57	2.65	4.04	1-10 acceptable
Number of theoretical plates (n)	4019	3600	7236	4979	Increase with the efficiency of the separation
HETP	6.22×10^{-3}	6.94×10^{-3}	3.45×10^{-3}	5.02×10^{-3}	The smaller the value the higher the column efficiency

RMSEP: root mean squares error of prediction; a: average of 3 determinations; b: average of 5 determinations; c: the values in the parenthesis are the corresponding theoretical values at p= 0.05; HETP = height equivalent to theoretical plates (cm/ plate)

spectral information can be markedly increased, converting this combined technique into a powerful and highly convenient analytical tool. The suggested multivariate methods are rapid, economic and do not require sophisticated instrumentation. It was found that GA-PLS is more accurate than PLS model, the superiority of GA-PLS is due to the wavelength selection in PLS calibration using genetic algorithm without loss of prediction capacity that provides useful information about the multi- component system. On the other hand, the developed HPLC method is more specific than the suggested chemometric models, but it needs expensive equipment and materials. Good results obtained by applying the proposed methods shows that they are free from interference of methyl paraben used in commercial formulation (after updating the chemometric methods and optimizing the RP-HPLC method), therefore no additional extraction or separation are required. The developed methods can be applied for routine analysis, quality control of mixtures and commercial preparations containing these three drugs.

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