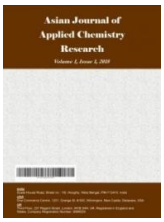

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HPLC, Densitometric and Spectrophotometric Methods for the Simultaneous Determination of Colchicine and Probenecid in Their Binary Mixture

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SAEM and SAAR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors SAEM and IAEM managed the analyses of the study. Author IAEM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To develop methods with complete validation according to ICH guidelines and to be applied for the determination of both drugs in laboratory prepared mixtures and in pharmaceutical formulation

Study Design: High performance liquid chromatography (HPLC), densitometric and different spectrophotometric methods (zero order, derivative ratio, ratio difference and mean centering) are developed for simultaneous determination of colchicine and probenecid in their combined pharmaceutical formulation.

Methodology: High performance liquid chromatography separation is developed using C18 column and methanol: ammonia (100: 1.5 v/v) as a mobile phase. The densitometric method based on the separation of both drugs using chloroform: methanol: ethyl acetate: water: ammonia (7: 5:2.5:0.5:0.5 by volume) as mobile phase and scanning λ at 254 nm. Zero order determination

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is based on measurement of colchicine absorbance at 349 nm. The first derivative ratio of peak amplitudes at 367 nm& at 290.4 nm and the ratio difference with the amplitude difference between (385 nm and 362.4 nm) and (270 nm and 255 nm) for colchicine and probenecid, respectively are developed for the determination of both drugs. Mean centering determination of probenecid is developed by measurement at 279 nm using 3.6 µg/mL of colchicine as a divisor.

Results: HPLC method was applied over the concentration ranges of 1.0-45.0 µg/mL & 0.5-30.0 , while densitometric method was linear over the concentration 0.15. 0-0.6 & 0.15-0.45 µg / band and spectrophotometric methods were linear over the concentration ranges 10.00-55.0 & 3.6-20.0 µg/mL for probenecid and colchicine, respectively.

Conclusion: Novel, simple and accurate method for the determination of colchicine and probenecid simultaneously in their binary mixture.

Keywords: Colchicine; probenecid; HPLC; densitometry; spectrophotometry.

1. INTRODUCTION

Colchicine;(S)-N-(5,6,7,9tetrahydro-1,2,3,10-tetramethoxy-9oxobenzol[a]heptalen-7-yl) acetamide) is an alkaloid contained in various species of colchicum and in other genera [1]. It is used in the relief of acute gout probably by reducing the inflammatory reaction to urate crystals [2].

Probenecid; (4-(Dipropylsulfamoyl) benzoic acid) [1] is a uricosuric agent used for the treatment of hyperureciemia associated with chronic gout, hyperuricemia caused by diuretic therapy and as adjunct to some antibacterial to reduce their renal tubular excretion [2]. It is used in combination with colchicine to treat chronic gouty arthritis when complicated by frequent recurrent acute attacks of gout. It inhibits the absorption of urate in the proximal convoluted tubule, thus increasing the urinary excretion of uric acid and decreasing serum urate levels [3].

The literature review revealed that numerous techniques have been applied for the analysis of probenecid in a single dosage form such as HPLC [4-7], TLC [8-9], spectrophotometric [10-13], capillary electrophoresis [14-15] and spectrofluremetriy [16]. Also, various techniques were reported for the single determination of colchicine as HPLC [17-20], TLC [21-23], spectrophotometry [24-25] and electrochemistry [26-28]. Only two chromatographic methods [29,30] has been reported for the determination of both drugs in binary mixture. Notably, the only reported HLC method [29] needs tedious sophisticated instrumentation and no published spectrophotometric method was developed for the determination of both drugs simultaneously until now. Therefore, it was valuable to develop simple and fast procedures which can be applied in quality control laboratories for the determination of both drugs simultaneously. In this work, spectrophotometric methods based on first derivative ratio, ratio difference and men centering was first applied for determination of both drug in binary mixture. Also two chromatographic methods, reversed-phase HPLC and densitometric methods are reported for the quantification of both drugs. These methods are applied to determine both drugs in commercial pharmaceutical formulation and in laboratory prepared mixtures.

2. MATERIALS AND METHODS

2.1 Instrumentation

The chromatographic HPLC (Agilent 1200 series, Germany) apparatus consists of an Agilent pump, equipped with a variable wavelength detector. The separation was performed using kromasil C18 column (250 mm × 4 mm) and the mobile phase “methanol: ammonia (100:1.5 v/v)” was pumped at a flow rate 1 mL/min after filtration and sonication. The detection wave length was 246 nm.

Sample for densitometric method was applied by an automatic sample applicator provided with 100 µL syringe to TLC plates precoated with Silica Gel60F254, 10x20 cm (Merck, Germany) and scanning by COMAG TLC scanner combined with WINCATS software (CAMAG, Switzerland) with scanning speed of 20 mm/ s.

A dual-beam UV-visible spectrophotometer [Shimadzu, Japan] model UV-1601 PC. Shimadzu UV- PROB version 2.32 and MATLAB®, version 7.0.124704 were used to process the absorbance, the derivative spectra and mean centering. The sample solutions were recorded in 1 cm quartz cells against solvent blank over the range 200–400 nm.

2.2 Materials and Reagents

Colchicine ($C_{22}H_{25}NO_6$) and probenecid ($C_{13}H_{19}NO_4S$) were kindly supplied by Pharaonia Pharmaceutical Co. and October Pharm Co. Cairo, Egypt, respectively. Their purities were found to be 99.7% and 99.5% for colchicine and probenecid, respectively referred to the reported methods [7,19]. Goutyless ® tablet labeled to contain 0.5 mg colchicine and 500 mg probenecid and was purchased from October Pharma, Cairo, Egypt. Ethanol and methanol, chloroform and ethyl acetate were of chromatographic grade (Fisher scientific, USA). Water was doubly distilled.

2.3 Standard Solutions

2.3.1 Stock standard solutions

Stock standard solutions of colchicine and probenecid (1 mg/mL) were prepared in methanol for (HPLC and TLC methods) and in ethanol for spectrophotometric method.

2.3.2 Working standard solutions

For HPLC: Working standard solutions (0.1 mg/ml) were prepared in methanol and standard solutions ocolchicine and probenecid containing concentration ranges of 5.00- 300.00 and 10.00 – 450.00 µg/ mL were prepared in methanol, respectively.

For densitometry: Working standard solutions (0.1 mg/ml) were prepared in methanol. Standard solutions equivalent to (75.0-225.0 µg/ mL) and (75.0-300.0 µg / mL) for colchicine and probancid were prepared in methanol.

For spectrophotometry: Working standard solutions (0.1 mg/ml) were prepared in ethanol. Standard solutions containing concentration ranges of (36.0-200.0 µg/ mL) and (100.0-550.0 µg/ mL) for colchicine and probenecid, respectively were prepared in ethanol.

2.3.3 Laboratory prepared mixtures

Different aliquots within calibration ranges from working colchicine solutions in methanol (0.1 mg/ mL) were mixed with aliquots within calibration ranges of working probenecid solutions (0.1 mg/ mL) and volumes were completed with suitable solvents for each method.

2.4 Sample Solution

Colchicine: 5 Goutyless ® tablets were weighed and crushed to a fine powder. An amount of powder equivalent to 1 mg of colchicine and 1000 mg of probenecid was dissolved in 30 ml of water for HPLC and TLC and ethanol for spectrophotometric method. After sonication for 15 min the volume was then made up to the mark in a 50 ml- volumetric flask with the same solvent. Filtration was carried out using syringe filter to labeled concentration of 20 µg / mL colchicine. Further dilution was done with methanol for HPLC and TLC or ethanol for spectrophotometric method.

Probenecid: An amount of fine powder equivalent to 0.5 mg of colchicine and 500 mg of probenecid was dissolved in 70 ml of methanol for HPLC &TLC or ethanol for spectrophotometric method. The solution was sonicated for 15 min, made up to the mark in a 100- ml volumetric flask with the same solvent and filtered through filter paper to reach a labeled concentration of 5 mg/ mL probenecid. Further probenecid dilution was carried out with the corresponding solvent to obtain a solution a labeled to contain 100 µg / mL probenecid.

2.5 Procedures

HPLC method: 100 µL injections from each solution were chromatographed as under conditions described previously" 2.1". The calibration curve was constructed by plotting the peak area against the corresponding drug concentration and the regression equation was evaluated.

Densitometric method: 20 µL of each solution was applied to a TLC plate (20 × 10 cm) and spotted as bands of 6 mm width, 5 mm interval and 2 cm from the bottom. The plate was developed for distance of 9 cm in chromatography tank presaturated with the mobile phase of chloroform: methanol: ethyl acetate: water: ammonia (7: 5: 2.5: 0.5: 0.5 by volume) for 30 min, then it was scanned at 254 nm. The calibration curve representing the recorded area under the peak against drug concentration in µg /spot was plotted and the regression equation was evaluated.

Spectrophotometric method: The spectra of the prepared standard solutions were scanned from 200 - 400 nm and stored in the computer. For zero order method: The absorbance of

colchicine at 349 nm was plotted against the corresponding drug concentration and the regression equation was evaluated. For first derivative ratio (1DR): The stored spectra of colchicine were divided by the spectrum of (10 µg/mL) of probenecid and the first derivative of the ratio spectrum (1DR) was recorded using Δλ = 8 and scaling factor 1. Spectra of probenecid were divided by the spectrum of (3.6 µg/ml) of colchicine and the first derivative of the ratio spectrum (1DR) was obtained using Δλ = 4 and scaling factor= 1. The peak amplitude at 367.0 nm for colchicine and at 290.4 nm for probenecid were plotted against drug concentration for derivative ratio method (1DR). Ratio difference (RD) was obtained by measuring the amplitude difference between (385nm and 362.4 nm) for colchicine and between (270.0nm and 255.0 nm) for probenecid and the difference was plotted against their corresponding drug concentration. For mean centering (MCR): The obtained spectra of probenecid were mean cantered at 279.0 nm using (3.6 µg/mL) colchicine as divisor. Value obtained were plotted against probenecid concentration.

2.6 Statistical Analysis

Statistical comparison was conducted based on the preliminary dataset which showed both calculated t and F ratio by using statistical tools.

3. RESULTS AND DISCUSSION

3.1 HPLC Method

As there was only one very tedious HPLC method was reported [29] for the determination of colchicine and probenecid in mixture so this reversed phase HPLC method was developed to provide simple and fast procedure for the analysis of the mixture in quality control laboratories. Different mobile phase systems composed of variable solvents with different ratios were tested and the best resolution was achieved by using methanol: ammonia (100:1.5 v/v) as a mobile phase which was pumped with flow rate 1ml/min. The best separation with the good tailing factor of the peaks and highest no of theoretical plates was achieved by using kromasil C18 column (250 x 4 mm) and detection wavelength at 246 nm. By using the selected chromatographic condition, retention times were found to be 1.917 and 2.848 for: probenecid and colchicine, respectively, Fig. 1 and the results of system suitability is shown at Table 1. These retention times are shorter than retention times for the reported one" 2.4 nm and 4.3 nm for colchicine and probenecid, respectively [29].

3.2 Densitometric Method

The TLC-Densitometric technique was successfully applied for simultaneous determination of colchicine and probenecid mixture. Developing systems of different composition and ratios were tested; as chloroform: acetone, ethylacetate: methanol: ammonia, chloroform: methanol and chloroform : methanol: ethyl acetate. The use of mobile phase composition (chloroform: methanol: ethyl acetate: water 7:5:2.5:0.5 by volume) result in a separation with slight closed R_f. Addition of ammonia to the mobile phase (chloroform: methanol: ethyl acetate: water: ammonia 7:5:2.5:0.5:0.5 by volume) gave well separated symmetry bands at Rf 0.53 and 0.69 for colchicine and probenecid, respectively, Fig. 2. Different scanning wavelengths were tested (246 nm, 254nm and 348nm) and 254 nm was found to be the most suitable wave length for the detection of both drugs rather than the reported method [30], which required two wavelengths to be measured.

3.3 Spectrophotometric Methods

As no spectrophotometric method was reported for the determination of two drugs simultaneously up till now, development of several

Table 1. System suitability data for HPLC for determination of probenecid and colchicine

Parameters	Obtained value		
	Probenecid	Colchicine	Reference value
Retention time	Rt=1.917	Rt=2.848	
capacity factor (K´)	0.92	1.86	0.5-10 is acceptable
Selectivity factor (α)	2.01		> 1
Resolution factor(R)	5.53		R >2
tailing factor (T)	1.130	1.282	Not more than 2
symmetry	0.84	0.64	(0.5-1)
Number of plates	2446	3931	The higher the more efficient the column

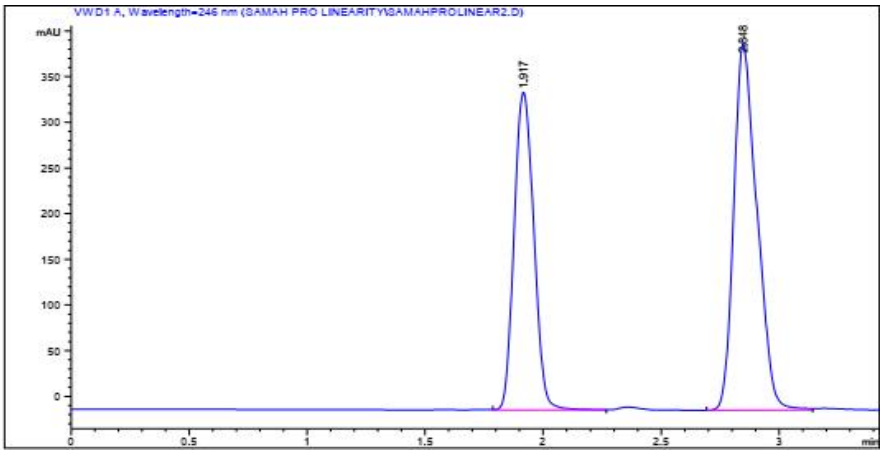

Fig. 1. HPLC chromatogram of laboratory mixture of probenecid Rt = 1.917 and Colchicine Rt= 2.818 at 246 nm

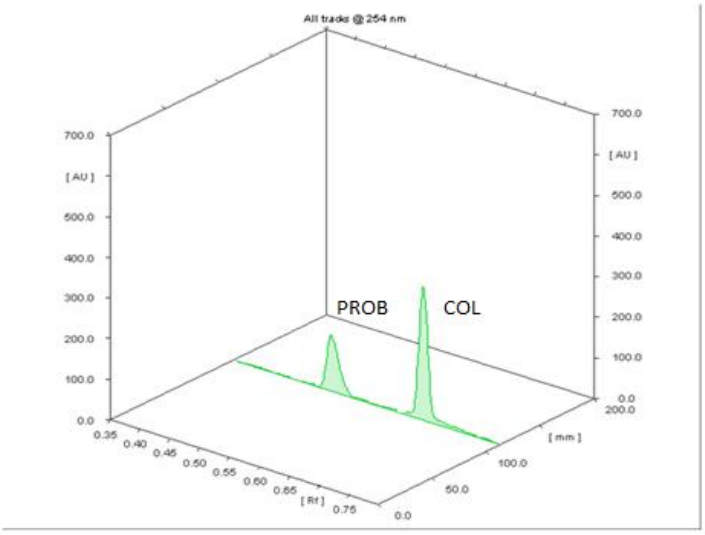

Fig. 2. Densitometric chromatogram of mixture of colchicine and probenecid

spectrophotometric methods is significant for fast and easy determination of mixture in quality laboratories.

3.3.1 Zero order

The zero-order absorbance spectra of colchicine and probenecid showed obvious overlapping but the extended part in colchicine spectra allowed its determination at 349 nm in presence of probenecid, Fig. 3.

3.3.2 First Derivative Ratio (1DR)

This method depends on division of the mixture's spectrum by the spectrum of one of the two component. Then the derivative ratio spectrum of that mixture will be independent on that divisor and the other component can be determined with no interference [31]. Different parameters were studied such as concentration of divisor, wavelength and the wavelength increment over which the derivative of the ratio spectra derivative is obtained (Δλ). The sharpest and best peak amplitude were achieved using Δλ=8 for colchicine and Δλ =4 for probenecid. Different concentrations of colchicine (3.6, 10 and 20 µg/mL) and of probenecid (10, 30 and 55 µg/mL) were tested as a divisor, the minimum noises in ratio spectra and the best recoveries were shown at the concentrations 3.6 µg/mL of colchicine and 10 µg/mL of probenecid. There was a reasonable

linearity at wavelengths 315, 338, 367, 377, 388 nm for colchicine and 258, 270, 275, 290.4 nm for probenecid but the best recoveries were at 367.2 nm and 290.4nm for colchicine and probenecid, respectively, Figs. (4a,4b).

3.3.3 Ratio difference (RD)

It has the ability of solving severely overlapped spectra without prior separation with high degree of simplicity, accuracy and reproducibility [31]. It can be carried out at any two wavelengths throughout the whole ratio spectrum, where no contribution of the overlapped component in the amplitude difference at any wavelength couples [32]. As shown in Figs. 5a, 5b, (385 nm-352.4 nm) and (270 nm -255nm) were the chosen as amplitude differences for colchicine and probenecid, respectively where linear correlations against the corresponding concentrations of both drugs were obtained.

3.3.4 Mean cantering

The ratio spectra are obtained, after which the constant is removed by mean cantering of the ratio spectra [33]. Probenecid concentration is determined by measuring the amplitude of mean cantered peak at 279 nm, Fig. 6.

3.4 Method Validation

Validation of the methods was carried out according to the ICH recommendation [34].

3.4.1 Linearity

Good linearity was obtained over the concentration ranges of 0.5-30.0 µg/mL colchicine 1.0-45.0 µg/mL probenecid "for HPLC method", 0.15-0.45 µg / band colchicine & 0.15. 0-0.6 µg / band probenecid "for densitometric method" and 3.6-20.0 µg/mL colchicine &10.00-55.0 µg/mL probenecid " for spectrophotometric methods". Regression parameters were summarized in Table 2.

3.4.2 Accuracy

The accuracy of the proposed methods was studied by analysis of three different concentrations of each pure sample drug within the linearity ranges and the concentrations were calculated from the corresponding regression equations. Further assessment of accuracy is done by application of standard addition technique. It expressed as mean R% and RSD%, Tables 2 showed acceptable results for accuracy.

3.4.3 Precision

Repeatability and intermediate precision were determined by analyzing three different concentrations of probenecid and colchicine three times on a single day and on three consecutive days, by the proposed methods. Intraday RSD% was ranged between 0.72, 1.37 and 0.09, 1.05 while inter day RSD% range was1.21, 1.86 and 0.45, 1.72 for colchicine and probenecid, Table 2.

3.4.5 Selectivity

Selectivity of proposed methods was evaluated by the determination of different synthetic laboratory prepared mixtures containing different ratios of probenecid and colchicine within the linearity range. Satisfactory recoveries ranged between 98.68±1.96 and 100.96±1.24 for colchicine and 99.05±0.82, 100.41±2.16 for probenecid were obtained, Table 3.

3.4.6 Analysis of pharmaceutical formulation

The proposed methods were applied for the determination of the cited drugs in their combined dosage form to study the interference effect of the added excipients. As the ratio of probenecid and colchicine in Goutyless® tablet is 1000:1 thus, the determination is carried out by preparing two separate dilutions for each drug. Although separate formulation dilutions were prepared but the very high probenecid concentration still making a problem on TLC plate and HPLC column. This was overcome by changing the solvent used in the first dilution. Water was used as solvent where probenecid was insoluble and colchicine was very soluble. Table 4 showed recoveries percent results, the represented data support good recoveries of two standards from mixture of tablets diluted in 2 solvents. These data prove simultaneous determination of two drugs in mixture and valuable application of standard addition technique. Statistical comparison of the results obtained by the proposed methods and a reported one [29] showed that both calculated t and F ratio were less than the theoretical ones indicating that there was no significant difference between two methods, Table 4.

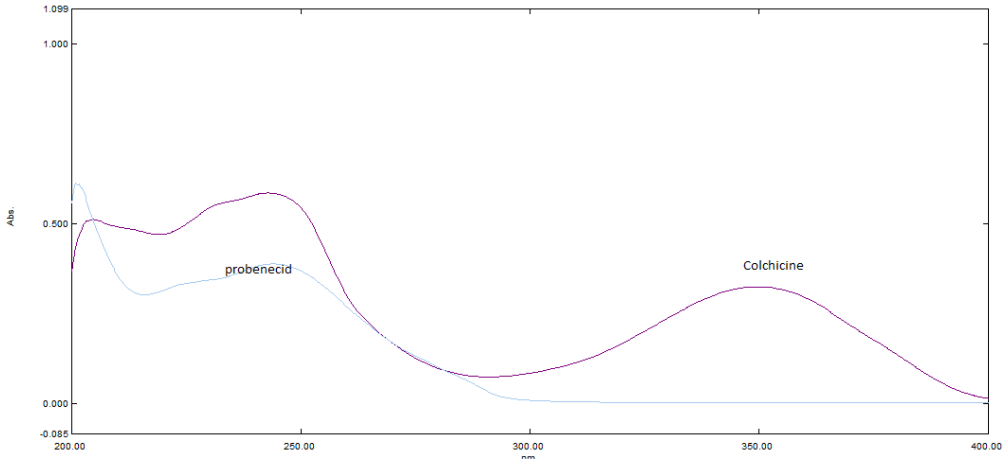

Fig. 3. Absorption spectra of probenecid and colchicine in ethanol

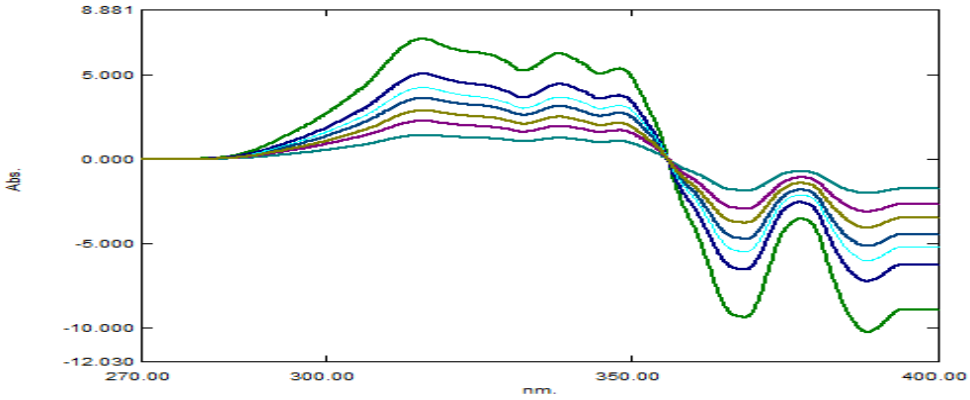

Fig. 4a. First derivative of ratio spectra of colchicine (3.60-20.00 µg/ml) using 10.00 µg /ml probenecid as divisor and ethanol as blank.

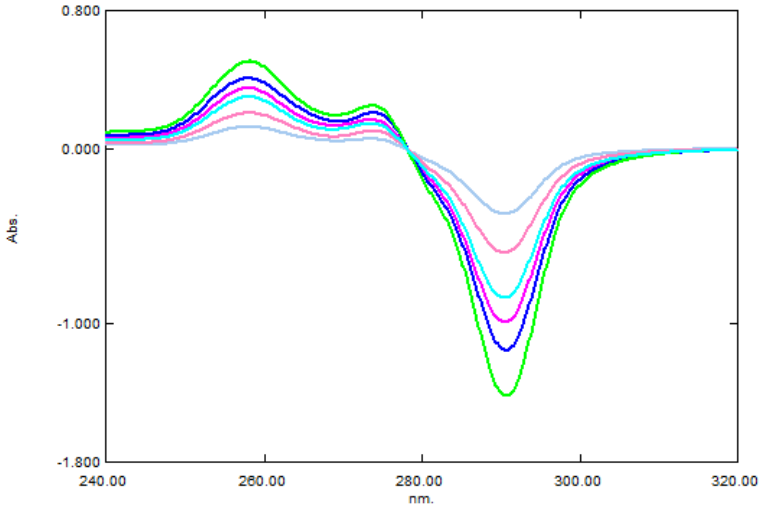

Fig. 4b. First derivative of ratio spectra of probenecid (10.00-55.00µg/mL) using 3.60 µg /ml colchicine as divisor and ethanol as blank

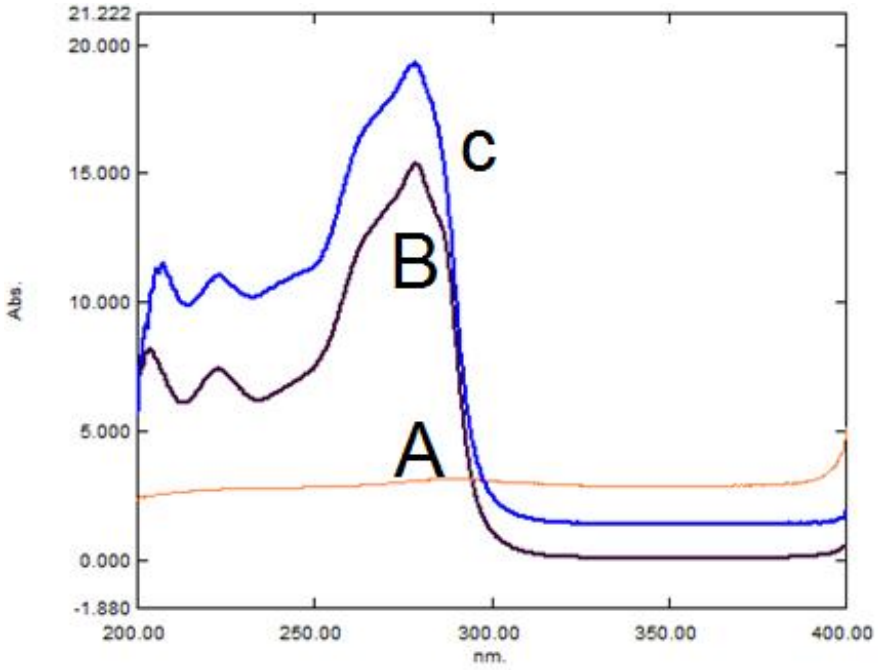

Fig. 5a. Ratio spectra of probenecid (55.00 µg/ml) A, colchicine (10.00 µg/ml) B, their mixture C using 3.6 µg/ml colchicine as divisor and ethanol as blank

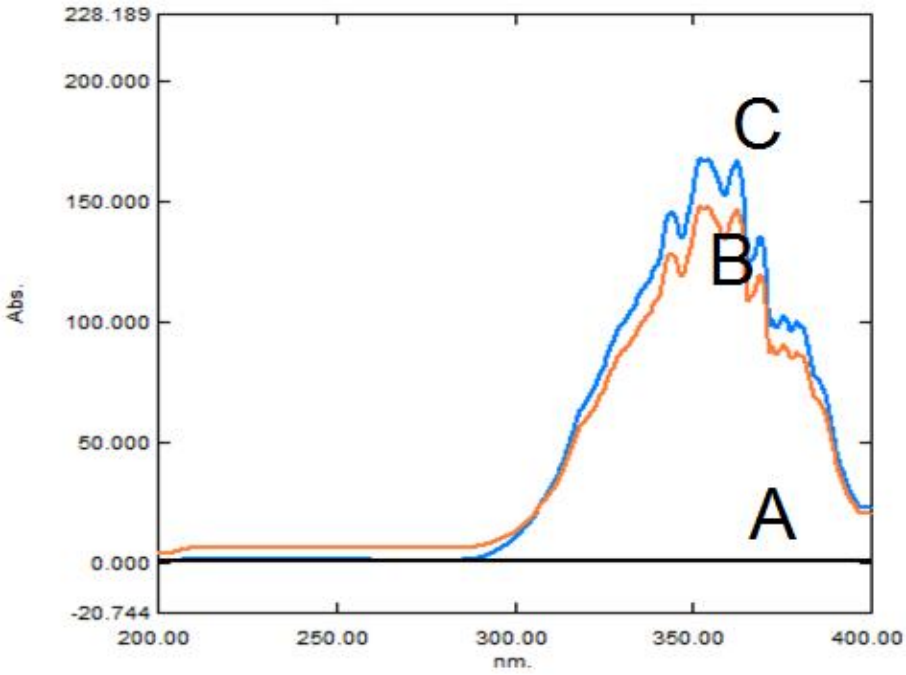

Fig. 5b. Ratio spectra of probenecid (55.00 µg/ml) A, colchicine (10.00 µg/ml) B, their mixture C of the same concentration using 10 µg/ml of probenecid as divisor and ethanol as blank

Table 2. Regression and assay validation parameters by the proposed methods

	Probenecid					Colchicine				
	HPLC	**DR1**	**RD**	**MC**	**TLC**	**HPLC**	**DR**	**RD**	**ZO**	**TLC**
λmax (nm)	244	290.4	270-255	279	254	246	367	385-352.4	349	254
Linearity range (µg/ml)	1-45	10-55	10-55	10-55	0.15-0. 60*	0.5-30	3.6-20	3.6-20	3.6-20	0.15-0.45*
Slope	264.52	0.0294	0.1031	0.2337	5725.1	413.64	0.5468	9.7642	0.0322	8056.7
Intercept	77.409	-0.0337	-0.191	-0.326	495.07	52.266	0.0178	1.7992	0.0014	985.98
Correlation coefficient (r2)	0.9999	0.9995	0.9998	0.9998	0.9994	0.9998	1.0	0.9999	0.9999	0.9992
Accuracy (mean±SD)	99.12±2.74	99.26±0.95	98.34±0.29	99.50±1.65	100.11±2.37	100.16±0.99	99.89±062	99.44±049	100.36±0.83	98.19±0.1.50
Precision (RSD %)										
Interday	0.97	0.91	1.37	0.78	0.72	0.29	0.09	0.47	0.19	1.05
Intraday	0.95	1.21	1.40	1.86	1.43	0.45	0.93	1.49	0.72	1.72

**is µg/band*

Table 3. Determination of probenecid and colchicine in their laboratory prepared mixtures using the proposed methods

		Probenecid					Colchicine				
(A)	**(B)**	**HPLC**	**DR1**	**RD**	**MC**	**TLC**	**HPLC**	**DR**	**RD**	**ZO**	**TLC**
15.3	1		102.5	97.22	98.39	-		102.98	101.27	98.43	-
5.5	1		98.18	102.08	99.72	-		99.03	98.94	100.12	-
2.75	1		101.25	98.32	99.42			99.61	98.94	99.90	-
1	1	100.85	-	-	-	100.35	98.36	100.26	100.60	101.68	100.45
1	2	100.39	-	-	-	99.2	99.83	102.01	99.41	100.83	101.13
2	1	101.63	-	-	-	104.78	97.83	100.73	99.83	101.98	96.31
3.67	1		97.30	101.82	97.75			102.08	97.92	101.77	-
4	1		98.78	102.43	99.20	99.49		100.97	97.85	100.71	97.02
11.11	1		99.11	100.58	99.84	-		100.93	101.28	100.55	-
4	3		-	-	-	101.35		-	-		99.12
1	3		-	-	-	96.02		-	-		98.35
90	1	98.99					102.86				
3	2	97.58					99.53				
Mean ±SD		99.89±1.61	99.52±1.96	100.41±2.16	99.05±0.82	100.2±2.87	99.68±1.96	100.96±1.24	99.56±1.30	100.66±1.11	98.73±1.89

(A) Ratio of probenecid in the mixture; (B)Ratio of colchicine in the mixture

Table 4. Determination of probenecid and colchicine in pharmaceutical formulation by the proposed methods and comparison with the manufacture method [29]

Probenecid	HPLC	TLC	DR	RD	MC	Reported [29]
Mean ±SD	98.18±2.00*	100.32±1.5	99.37±2.04	99.09±1.94	100.71±1.28	100.28±2.25
Variance	4.01	2.25	4.17	3.78	1.65	5.07
Test number	5	5	5	5	5	5
t-test	1.56	0.04	0.67	0.89	0.37	-
F-ratio	0.79	0.44	0.82	0.74	0.32	-
Standard addition	100.72±1.92	103.05±0.57	98.79±1.73	100.32±1.93	98.97± 1.27	-
Colchicine	**HPLC**	**TLC**	**DR**	**RD**	**ZO**	**Reported [29]**
Mean ±SD	99.29±1.58*	98.02±1.76	100.28±1.86	99.19±1.51	102.14±0.73	99.61± 2.48
Variance	2.51	3.11	3.46	2.26	0.53	6.15
Test number	5	5	5	5	5	5
t-test	0.24	1.17	0.48	0.33	2.19	-
F-ratio	0.41	0.51	0.56	0.37	0.09	-`
Standard addition	99.80±1.41	101.18±0.53	100.79±0.65	100.43±1.47	101.06±1.75	

The theoretical t- and F- values at P=0.05 were 2.31 and 6.39; respectively.
**All values shows the recovery values*

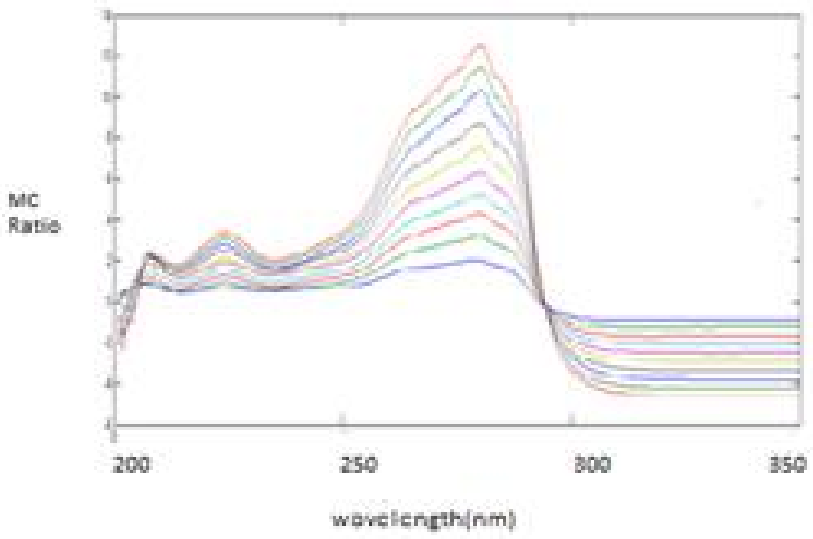

Fig. 6. Mean centered ratio spectra of probenecid (10.00-55.00 µg /ml) using 3.6.0 µg/ ml of colchicine as a divisor and ethanol as blank

4. CONCLUSION

Although the mixture of colchicine and probenecid was present in market many years ago, there is no publish spectrophotometric method for the determination this mixture simultaneously and only one reported very sophisticated HPLC method. The novelty of this work is to provide many accurate and simple spectrophotometric methods for the determination of this mixture simultaneously. Moreover an alternative RP- HPLC and densitometric methods were developed to overcome the disadvantages of the reported HPLC method which required pre-extraction. The proposed methods are simple, accurate, precise, specific, and low cost. Hence, they can be used for routine analysis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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