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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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ABSTRACT

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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 formulations

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 is based on the 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 colchicines and probenecid, respectively.

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

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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   mixtures.   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   centring   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 formulations 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 wavelength 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 centring. The sample solution was recorded in 1 cm quartz cells against solvent blank over the range 200–400 nm.

2.2. MATERIALS AND REAGENTS

Colchicine   and   probenecid   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   labelled   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

64 For HPLC. Working standard solutions (0.1 mg/ml) were prepared in methanol and standard solutions of colchicine and
65 probenecid containing concentration ranges of 5.00- 300.00 and 10.00 – 450.00 µg/ mL were prepared in methanol,
66 respectively [31].

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

69 For spectrophotometry. Working standard solutions (0.1 mg/ml) was prepared in ethanol. Standard solutions
70 containing concentration range of (36.0-200.0 µg/ mL) and (100.0-550.0 µg/ mL) for colchicine and probenecid,
71 respectively were prepared in ethanol [31].

72 2.3.3. Laboratory prepared mixtures

73 Different aliquots within calibration range from working colchicines solution in methanol (0.1 mg/ mL) were mixed
74 with aliquots within calibration ranges of working probenecid solution (0.1 mg/ mL) and volumes were completed with
75 suitable solvents for each method.

2.4. SAMPLE SOLUTION
77 **Colchicine** - 5 Goutyless ® tablets were weighed and crushed to a fine powder. An amount of powder equivalent
78 to 1 mg of colchicine and 1000 mg of probenecid was dissolved in 30 ml of water for HPLC and TLC and ethanol for the
79 spectrophotometric method. After sonication for 15 min the volume was then made up to the mark in a 50 ml volumetric
80 flask with the same solvent. Filtration was carried out using syringe filter to labelled concentration of 20 µg / mL colchicine.
81 Further dilution was done with methanol for HPLC and TLC or ethanol for spectrophotometric method.
82 **Probenecid**   - An amount of fine powder equivalent to 0.5 mg of colchicine and 500mg of probenecid was
83 dissolved in 70 ml of methanol for HPLC &TLC or ethanol for spectrophotometric method. The solution was sonicated for
84 15 min, made up to the mark in a 100- ml volumetric flask with the same solvent and filtered through filter paper to reach
85 a labelled concentration of 5 mg/ mL probenecid . Further probenecid dilution was carried out with the corresponding
86 solvent to obtain a solution a labelled to contain   100 µg / mL probenecid .

2.5. PROCEDURES

89 **HPLC method:** 100 µL injections from each solution were chromatographed as under conditions described previously"
90 2.1". The calibration curve was constructed by plotting the peak area against the corresponding drug concentration and
91 the regression equation was evaluated.

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

97 **Spectrophotometric method:** The spectra of the prepared standard solutions were scanned from 200 - 400 nm and
98 stored in the computer. For zero order method : The absorbance of colchicine at 349 nm was plotted against the
99 corresponding drug concentration and the regression equation was evaluated. For first derivative ratio (1DR): The stored
100 spectra of colchicine were divided by the spectrum of (10 µg/mL) of probenecid and the first derivative of the ratio
101 spectrum (1DR) was recorded using $\Delta\lambda = 8$ and scaling factor 1. Spectra of probenecid were divided by the spectrum of
102 (3.6 µg/ml) of colchicine and the first derivative of the ratio spectrum (1DR) was obtained using $\Delta\lambda = 4$ and scaling
103 factor= 1. The peak amplitude at 367.0 nm for colchicine and at 290.4 nm for probenecid were plotted against drug
104 concentration for derivative ratio method (1DR). Ratio difference (RD) was obtained by measuring the amplitude
105 difference between (385nm and 362.4 nm) for colchicine and between (270.0nm and 255.0 nm) for probenecid and the
106 difference was plotted against their corresponding drug concentration. For mean centering (MCR): The obtained spectra
107 of probenecid were mean cantered at 279.0 nm using (3.6 µg/mL) colchicine as divisor. Value obtained were plotted
108 against probenecid concentration.

109 **Statistical analysis:**

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

3. RESULTS AND DISCUSSION

HPLC method

As there was only one very tedious HPLC method was reported [29] for the determination of colchicine and probenecid in the 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 conditions, retention times were found to be 1.917 and 2.848 for: probenecid and colchicine, respectively, (Figure 2) 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].

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

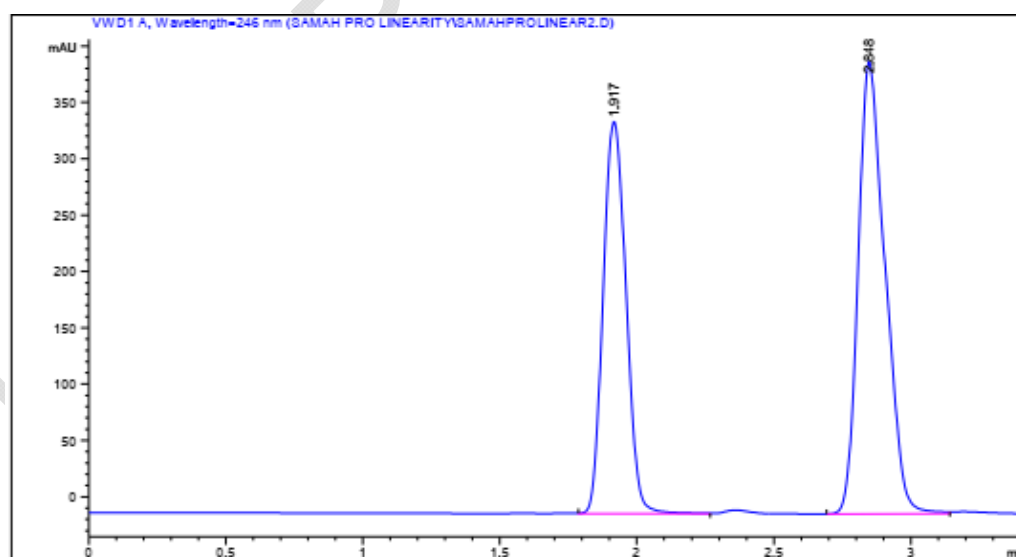| Parameters	Obtained value		
	probenecid	Colchicine	Reference value
Retention time	Rt=1.917	Rt=2.848	
capacity factor (K´)	0.92	1.86	0.5-10 is acceptable
Selectivity factor (α)	2.01		> 1
Resolution factor(R)	5.53		R >2
tailing factor (T)	1.130	1.282	Not more than 2
symmetry	0.84	0.64	0.5-1
Number of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

Figure 2: HPLC chromatogram of   laboratory mixture of probenecid Rt = 1.917 and Colchicine Rt= 2.818 at 246 nm.

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 . <mark>The mobile phase</mark> composition (chloroform: methanol: ethyl acetate: water 7:5:2.5:0.5 by volume), the valuable separation was recorded but with slight closed Rf . 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 colchicin and probenecid , respectively, (Figure 3). 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
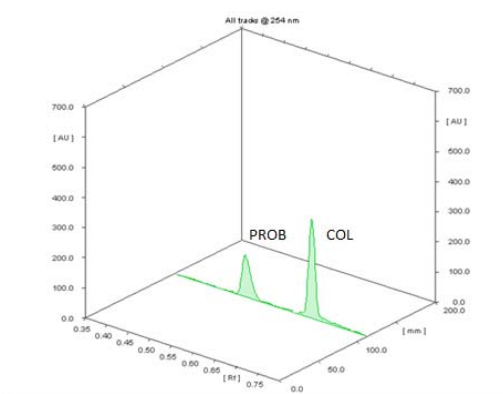
two wavelengths to be measured.

Figure 3: Densitometric chromatogram of mixture of colchicine   and probenecid

Spectrophotometric methods As no spectrophotometric method was reported for the determination of two drugs simultaneously up till now, the development of several spectrophotometric methods is significant for fast and easy determination of mixture in quality laboratories.

 -**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, Figure (4).
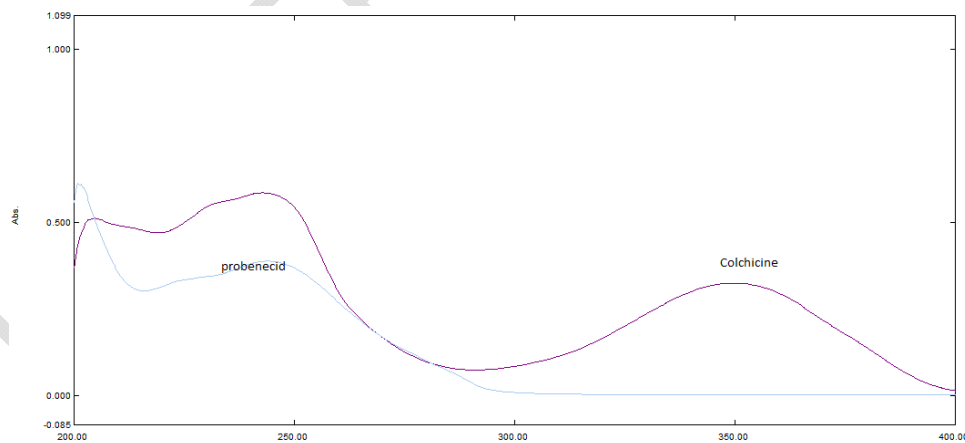

Figure 4: Absorption spectra of probenecid and colchicine in ethanol

-**First derivative ratio (1DR) -**This method depends on the 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 [32]. Different parameters were studied such as the 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 was 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 55μg/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, figure (5a,5b).
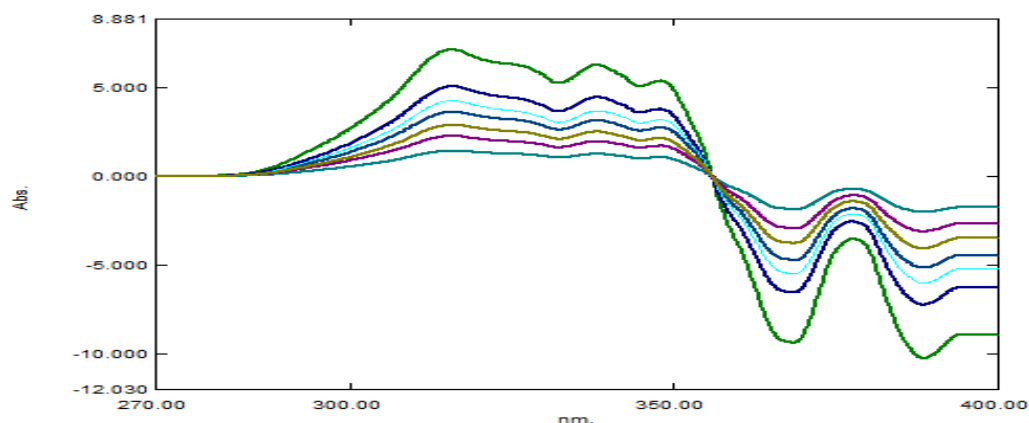

Figure (5a): First derivative of ratio spectra of colchicine (3.60-20.00 μg/ml) using 10.00 μg /ml probenecid as divisor and ethanol as blank.
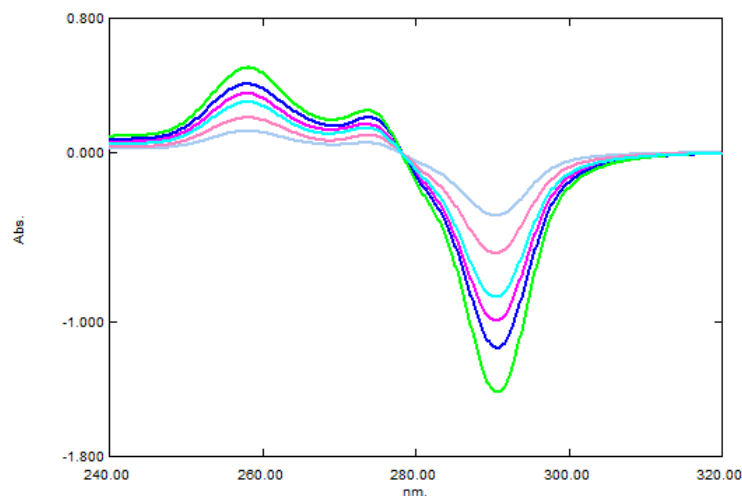

Figure (5b): First derivative of ratio spectra of probenecid (10.00-55.00μg/mL) using 3.60 μg /ml colchicine as divisor and ethanol as blank.

-Ratio difference (RD): It has the ability to solve severely overlapped spectra without prior separation with a 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 [34]. As shown in Figure (6a, 6b), (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.

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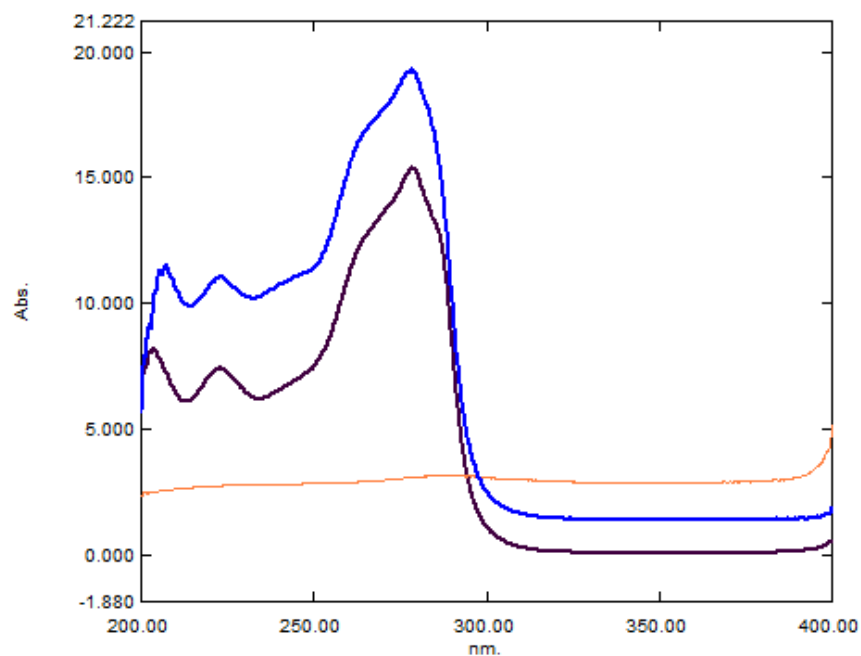
Figure (6a): Ratio spectra of probenecid (---) (55.00 µg/ml), colchicine (----(10.00 µg/ml), their mixture using 3.6 µg/ml colchicine as divisor and   ethanol as blank.

180
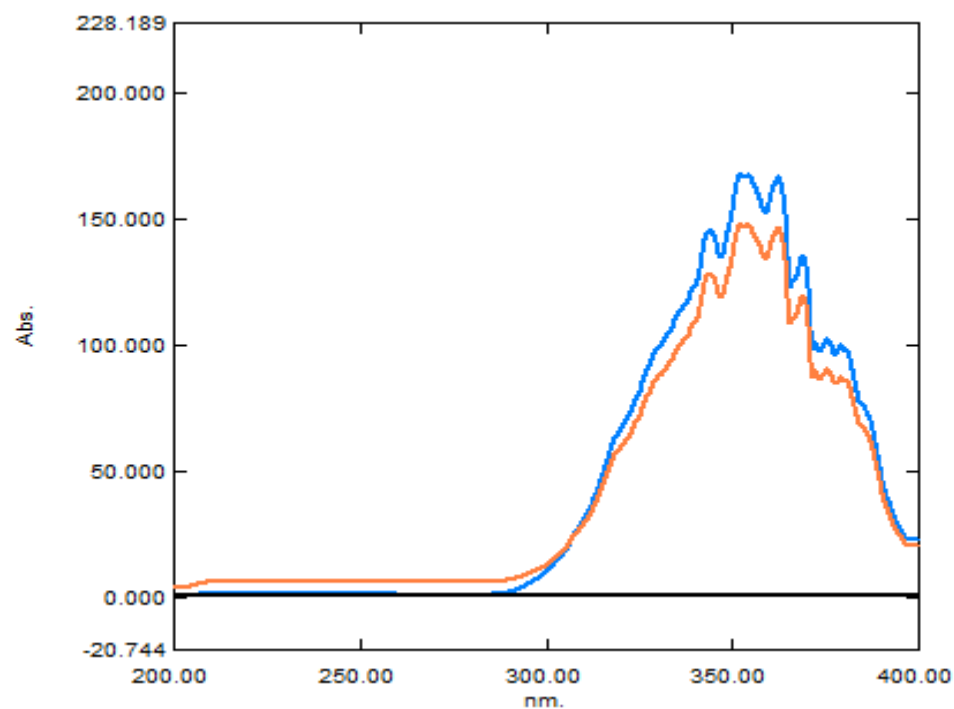
Figure (6b): Ratio spectra of probenecid (55.00 µg/ml), colchicine (10.00 µg/ml), their mixture of the same concentration using 10 µg/ml of probenecid as divisor and ethanol as blank.

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185 -**Mean cantering:** The ratio spectra are obtained, after which the constant is removed by mean centring of the ratio
186 spectra [33]. Probenecid concentration is determined by measuring the amplitude of mean cantered peak at 279 nm,
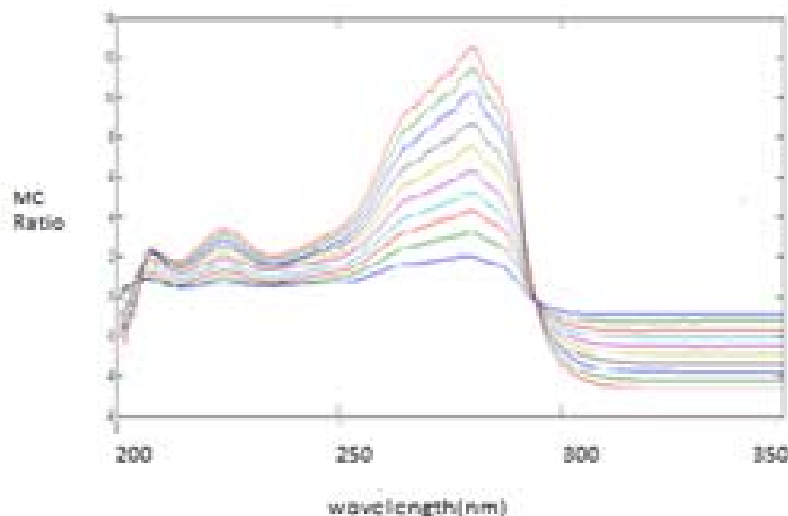187 figure (7).

188

189 **Figure (7): Mean centered ratio spectra of probenecid (10.00-55.00 µg /ml) using 3.6.0 µg/ ml of colchicine as a**
190 **divisor and ethanol as blank.**

191
192 **Method validation**
193 Validation of the methods was carried out according to the ICH recommendation [35]

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195 <u>**Linearity**</u>
196 Good linearity was obtained in the concentration ranges of 1.0-45.0 µg/mL & 0.5-30.0 " HPLC method", 0.15. 0-0.6 &
197 0.15-0.45 µg / band "densitometric method" and 10.00-55.0 & 3.6-20.0 µg/mL "spectrophotometric methods" for
198 colchicines and probenecid, respectively. Regression parameters were summarized in Table (2).
199
200 **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	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

(mean±SD)										
Precision (RSD%) Interday Intraday	0.97 0.95	0.91 1.21	1.37 1.40	0.78 1.86	0.72 1.43	0.29 0.45	0.09 0.93	0.47 1.49	0.19 0.72	1.05 1.72

201 ***is μg/band**

202
203 **Accuracy**
204 The accuracy of the proposed methods was studied by analysis of three different concentrations of each pure sample
205 drug within the linearity ranges and the concentrations were calculated from the corresponding regression equations.
206 Further assessment of accuracy is done by application of standard addition technique. It expressed as mean R% and
207 RSD%, Tables (2) showed acceptable results for accuracy.
208 **Precision**
209 Repeatability and intermediate precision were determined by analyzing three different concentrations of probenecid and
210 colchicine three times on a single day and three consecutive days, by the proposed methods. Intraday RSD% was ranged
211 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 colchicines and
212 probenecid , Table (2).
213 **Selectivity**
214 Selectivity of proposed methods was evaluated by the determination of different synthetic laboratory prepared mixtures
215 containing different ratios of probenecid   and colchicine within the linearity range. Satisfactory recoveries ranged between
216 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).
217
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219 **Table 3. Determination of probenecid and colchicine in their mixtures using the proposed methods**

		Probenecid (A)					Colchicine (B)				
(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

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 a 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 was less than the theoretical ones indicating that there was no significant difference between two methods (Table 4).

Table 4: Determination of probenecid and colchicine in pharmaceutical formulation by the proposed methods and comparison with the manufacture method (43)

Probenecid	HPLC	TLC	DR	RD	MC	Reported [35]
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(43)
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	

238 *The theoretical t- and F- values at P=0.05 were 2.31 and 6.39; respectively*.

239 The proposed method presented numerous advantages, such as enhanced sensitivity, better recovery, shorter analysis time,
240 lower expense, larger sample capacities, need for lower amounts of solvents and simpler cleanup relative to other
241 chromatographic methods [36, 37]. The chromatographic approach is highly sensitive and selective, enables simultaneous
242 determination, however, these methods requires expensive equipment and specific sample preparation [38].

4. CONCLUSION

246 It is concluded that the study enlighten a novel, simple and accurate spectrophotometric methods for the determination of
247 colchicine and probenecid simultaneously in their binary mixture was developed. Moreover, an alternative RP- HPLC and
248 densitometric methods were developed to overcome the disadvantages of the reported HPLC method which required pre-
249 extraction. The proposed methods are simple, accurate, precise, specific, and low cost; hence, they can be used for
250 routine analysis. Separate formulation dilutions were prepared but the very high probenecid concentration still making a
251 problem on TLC plate and HPLC column.

COMPETING INTERESTS

255 Authors have declared that no competing interests exist

AUTHORS' CONTRIBUTIONS

259 'Author A' designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the
260 manuscript. 'Author B' and 'Author C' managed the analyses of the study. 'Author C' managed the literature searches……
261 All authors read and approved the final manuscript."

REFERENCES

267 1- Parkway T , Rockville MD. The United States Pharmacopeial Convention, USP. 39 th. Ed, USA, 2016.
268 2- Sweetman,SC. The Complete Drug Reference,39th Ed., Pharmaceutical Press,London,UK, 2017.
269 3- https://pubchem.ncbi.nlm.nih.gov/compound/Probenecid #section=Top .
270 4- Patel SC, Chauhan PP, Patel NM , Shah Sardar SK. Novel isocratic RP-HPLC for simultaneous multicomponent
271 analysis of amoxicillin and Probenecid in pharmaceutical formulation. IJIPLS.2014;4(5): 28-36.
272 5- Nilam P, Paranjape DP. RP-HPLC method development and validation for simultaneous estimation of cefadroxil
273 and Probenecid in synthetic mixture .IJUPBS.2014;3(3):560-571.
274 6- Harle RK , Cowen T. Determination of Probenecid in serum by high -performance liquid chromatography .
275 Analyst .1978 ;103:492-496.
276 7- Jin-fang S, Yan-ling S , Xin Y. HPLC determination of ampicillin and Probenecid in their compound capsules. J.
277 of Pharma. Anal.2004;3:411- 413.
278 8- Dhaneshwar SR, Kadam SS , Sirisha V ; Development and validation of a HPTLC method for simultaneous
279 estimation of cefuroxime axetil and Probenecid . J Pharm Sci.2004;66 :278 - 282.
280 9- Al-Badr AA , El-Obeid HA. Probenecid, in Analytical Profiles of Drug Substances. Academic Press. 1981: 639.
281 10- Jain D, Jain DK , Trivedi P. Simultaneous Spectrophotometric Determination of Amoxycillin And Probenecid In
282 Tablet Dosage Form. Indian J. Pharm. Sci.1998:60 (5):318 -320.
283 11- Kurian T , Kurien J . Simultaneous multicomponent spectrophotometric analysis of ampicillin and Probenecid
284 in pharmaceutical formulation by derivative spectroscopy .H.J.D.Med.2011;3(2): 57 - 61.
285 12- Maheta PS, Patel PR, Parmar RR, Modasiya MMK , Dushyant A. Development and validation of derivative
286 spectroscopic method for simultaneous estimation of cefadroxil and Probenecid. IJPSN .2014;7(1): 2350 - 2355.
287 13- Kumar R, Nain P , kaur J. Development and validation of uv-visible spectroscopic method for estimation of
288 Probenecid in tablet dosage form. IJAR.2016; 4(5) :212 - 219.
289 14- Sun H , Wu Y. Effective separation and simultaneous determination of cefamandole and Probenecid in body
290 fluids by capillary zone electrophoresis with salicylic acid as an internal standard. Anal. Methods.2013; 5:6017 -
291 6022.

15- Sirén H, Shimmo R, Sipola P, Abenet S , Riekkola ML. Capillary electrophoresis of diuretics and Probenecid in methanol. J Chromatogr A.2008;(11): 1198 - 1199.

16- Cunningham RF, Israil ZH , Dayton PG ; New spectrophotofluorometric assay for Probenecid. J Pharm Sci.1978; 67(3): 434 - 436.

17- Nirmala K , Raju RR. A novel method development for validation and detection of colchicine drug by RP-HPLC. RJC;5(1): 106 - 111.

18- Joshi SA, Jalalpure SS, Kempwade AA, Peram MR. Development and Validation of HPLC Method to Determine Colchicine in Pharmaceutical Formulations and its Application for Analysis of Solid Lipid Nanoparticles. CPA.2018;14(1): 76 - 83.

19- Samanidou VF,. Sarantis GA , Papadoyannis IN. Development and validation of a rapid HPLC method for the direct determination of colchicine in pharmaceuticals and biological fluids. J Liq Chromatogr R T.2006;29(1/4): 1- 13.

20- Abdulbaqi IM, Darwis Y, Khan N , Loh GOK, A simple (HPLC–UV) method for the quantification of colchicine in bulk and ethosomal gel nano-formulation and its validation . Int J Pharm Pharm Sci.2017; 9(7) :72 - 78.

21- Sarg,TM, El-Domiaty MM , Bishr MM. Thin-layer chromatographic scanner, spectrophotometric and high-performance liquid chromatographic methods for the determination of colchicine . ANALYST .1989;114(5) :575 - 578.

22- Hadad GM, Badr JM, El-Nahriry K , Hassanean HA. Validated HPLC and HPTLC Methods for Simultaneous Determination of Colchicine and Khellin in Pharmaceutical Formulations. J Chromatogr Sci.2013;51(3): 258-265 .

23- Bodoki E, Oprean R, Vlase L, Tamas M ,. Sandulescu R. Fast determination of colchicine by TLC-densitometry from pharmaceuticals and vegetal extracts. J Pharm Biomed Anal.2005;37:971 - 977.

24- Ajage RK , Kasture VS . Validated UV spectroscopic method for the estimation of three marker compounds in marketed polyherbal ayurvedic formulation .Der Pharmacia Lettre.2014;6(3):160 - 166 .

25- Verma P , Patial A . Development of UV spectrophotometric method for estimation of colchicine in phosphate buffer saline pH 6.4 . IRJP.2012;3(2) :87 - 89.

26- Zhang H. Electrochemistry and voltammetric determination of colchicine using an acetylene black-dihexadecyl hydrogen phosphate composite film modified glassy carbon electrode. j.bioelechem.2006;68(2) :197 - 201.

27- Wang F, Zhou J, Liu,Y. Wu S, Song G , Baoxian Y . Electrochemical oxidation behavior of colchicine on a graphene oxide-Nafion composite film modified glassy carbon electrode. RSC.2011;136: 3943 - 3949.

28- Stanković DM, Švorc L, Mariano JF, Ortner A , Kalcher K ; Electrochemical Determination of Natural Drug Colchicine in Pharmaceuticals and Human Serum Sample and its Interaction with DNA. Electroanalysis.2017;29:2276 - 2281.

29- Lo W , G. M. Krause; Simultaneous Determination of Probenecid and Colchicine in Solid Dosage Form by Reversed Phase High Performance Liquid Chromatography; Drug Dev. Ind. Pharm.1987; 13(1): 57 - 66.

30- Mohamed AI, Omar MA, Hammad M, Mohamed AA. Validated thin-layer chromatographic method for alternative and simultaneous determination of two anti-gout agents in their fixed dose combinations. Open Chemistry.2018; 16: 496–510 .

31- Bhagyalaxmi, S., Naresh, D., Kumar, G. V., & Haneef, M. A. 2017. Development and validation of analytical method for simultaneous estimation of Cephalexin and Probenecid in API and marketed formulation by RP-HPLC. International journal of novel trends in pharmaceutical sciences, 7(6), 192-196.

32- Lotfy M, Saleh SS, Hassan NY, Elgizawy SM. A Comparative Study of the Novel Ratio Difference Method versus Conventional Spectrophotometric Techniques for the Analysis of Binary Mixture with Overlapped Spectra. AJAC.2012; 3:761 - 769.

33- Elzanfaly ES, Saad AS , Abd-Elaleem AB. Simultaneous determination of retinoic acid and hydroquinone in skin ointment using spectrophotometric technique (ratio difference method). SPJ.2012;20(3) : 249 - 253.

34- Lotfy HM , Saleh SS. Recent development in ultraviolet spectrophotometry through the last decade. Int J Pharm Sci.2016;8(10):40 - 56.

35- International Conference on Harmonization (ICH). ICH Harmonized Tripartite Guideline. Topic Q2 (R1). Validation of Analytical Procedures: Text and Methodology; Geneva, Switzerland; 2005

36- Mohamed, A. M. I., Omar, M. A., Derayea, S. M., Hammad, M. A., & Mohamed, A. A. 2018. Validated thin-layer chromatographic method for alternative and simultaneous determination of two anti-gout agents in their fixed dose combinations. *Open Chemistry, 16*(1), 496-510.

37- United States Pharmacopeia 40. The National Formulary 35, US Pharmacopeial Convention: Washington, DC, 2017, 3552, 5834.

38- Stankovic D. M., Svorc L., Mariano J.F.M.L., Ortner A., Kalcher K., Electrochemical determination of natural drug colchicine in Pharmaceuticals and human serum Sample and its interaction with DNA, Electroanalysis, 2017, 29, 2276–2281.