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Validated thin-layer chromatographic method for alternative and simultaneous determination of two anti-gout agents in their fixed dose combinations

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Abstract: A rapid, simple and sensitive thin-layer chromatography (TLC) spectrodensitometric method was developed for the simultaneous determination of colchicine and probenecid in their binary mixtures. The two drugs were quantitatively separated using silica gel 60 F_{254} as stationary phase and toluene–ethyl acetate–methanol–ammonia (30:20:20:0.1, v/v/v/v) as mobile phase with UV detection at 248 nm for both drugs and at 354 nm for colchicine alone. Both drugs were efficiently separated with R_f values of 0.33±0.03 and 0.60±0.03 for probenecid and colchicine, respectively. The linearity was found to be 16–320 and 120–2400 (ng/band) with quantitation limits of 17.59 and 225.82 ng/band for colchicine and probenecid, respectively, at 248 nm. At 354 nm, the linearity range of colchicine was found 16–240 ng/band with a quantitation limit of 54.03 ng/band. The experimental determination ranges were greatly extended with lower quantitation limits (15.60 and 116.13 ng/band for colchicine and probenecid, respectively at 248 nm, and 13.20 for colchicine at 354 nm) and correlation coefficients were improved when polynomial regression analysis was used. The quadratic model was found to be the best fit for all responses. The method has been validated according to the International Conference on Harmonization (ICH) guidelines providing good correlation coefficients (0.9997-0.9999) for both drugs, and it has been successfully applied in the determination of both drugs in their commercial dosage form without interference from excipients.

Keywords: TLC-Spectrodensitometric method; Simultaneous determination; Colchicine; Probenecid.

1 Introduction

Hyperuricemia is usually caused by either overproduction or under-excretion of urate. Crystals of monosodium urate monohydrate are formed and deposited in synovial fluid and various tissues leading to an inflammation in the affected joint.The ensuing exquisite pain, tenderness, erythema, and swelling constitute the clinical manifestations of acute inflammatory gouty arthritis. Treatements are devoted to alleviating the acute attack, preventing future attacks and lowering the concentration of urate in the plasma. Key therapeutic agents for gout include colchicine, allopurinol, and uricosuric agents, such as probenecid. Colchicine is one of the oldest available therapies for acute gout; the main indication for colchicine is in the prevention of recurrent gout, particularly in the early stages of antihyperuricemic therapy.

Uric acid is the only important endogenous compound whose excretion is known to be increased by probenecid [1]. Colchicine and probenecid (Figure 1) are officially listed in the Chinese, Japanese, British, United State and European Pharmacopoeias, which described several methods for their separate assay in bulk and dosage forms [2-6].

Several analytical methods have been developed for determination of colchicine and probenecid, including HPLC [7-22], TLC [8,23-25], GC [26-30], spectrophotometric [8,31-34], capillary electrophoretic [35,36], voltammetric [37-43], and fluorimetric [44,45] methods. Notably, only one HPLC method has been published for simultaneous determination of colchicine and probenecid in solid dosage form [7]. This method suffers from some major drawbacks such as inadequate sensitivity, tedious extraction procedures and the need for sophisticated and expensive instrumentation.

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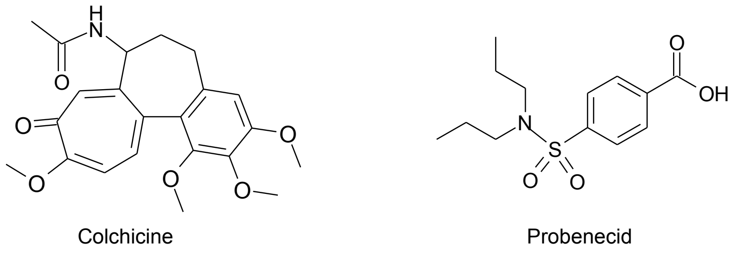

Figure 1: Chemical structures of the drugs under investigation.

To the best of the authors' knowledge, no TLC-densitometric method has been presented for simultaneous determination of colchicine and probenecid in their co-formulated fixed dose tablets until now. The design of a new, rapid, simple and highly sensitive TLC-spectrodensitometric method for their simultaneous determination was imperative. The proposed method presents a number of merits, including rapidity, simplicity, improved sensitivity and low cost. Furthermore, the developed TLC-spectrodensitometric method has the advantage of being more sensitive than other reported TLC and spectrophotometric methods.Relative to HPLC methods, the proposed TLC method has a shorter analysis time, is less expensive, has a large sample capacity per run, and uses small amounts of solvents, and it does not require extensive cleanup [46] because a new plate is utilized for each run. Hence, the proposed method can be used instead of published HPLC methods that require pre-extraction steps.

2 Experimental

2.1 Instrumentation

Sample application was done under a nitrogen stream using a 100 μL precision syringe (Hamilton, Bonaduz, Switzerland) in the form of bands utilizing a Linomat-V auto sampler and a CAMAG TLC Scanner 4 operated in the reflectance–absorbance mode, with winCATS version 1.4.10 software (CAMAG, Muttenz, Switzerland), with slit dimensions of 3×0.45mm and scanning speed of20 mm/s. The plates were developed in a twin-trough chamber (Sigma-Aldrich, St Louis, MO, USA).

2.2 Materials and Reagents

Colchicine and probenecid were kindly provided by Kahira Pharmaceuticals and Chemical Industries Co. and October Pharma Co., Cairo, Egypt, respectively. Their purities were found to be 99.2 and 98.8 (%) for colchicine and probenecid, respectively as determined by reported methods [23,24]. Silica gel 60 F_{254} plates (E. Merck, Darmstadt, Germany) were used. All solvents were of HPLC grade; methanol, ethyl acetate, toluene and ammonia were purchased from Sigma Aldrich, Steinheim, Germany. Goutyless® tablets were obtained from a local Egyptian market. Each tablet contains 0.5 mg of colchicine and 500 mg of probenecid, B.N: B01560216.

2.3 Standard Solutions

2.3.1 Stock Standard Solutions

Masses equivalent to 10.0 mg of colchicine and 150.0 mg of probenecid were transferred separately into 50 mL volumetric flasks then dissolved in 30 mL of methanol with the aid of ultra-sonication for 5 min.The volume was then adjusted to 50.0 mL with methanol to obtain colchicine and probenecid concentrations of 0.2 and 3.0 mg mL^{-1}, respectively. All solutions were kept in a refrigerator.

2.3.2 Working Standard Solutions

Mixed working solutions were obtained by further diluting of suitable volumes from each stock solution with methanol in separate 10.0 mL volumetric flasks to prepare concentrations in the range of 2.0–100.0 and 30.0–1500.0 (μg mL^{-1}) for colchicine and probenecid, respectively.

2.4 Chromatographic Conditions

Firstly, the silica gel G 60 F_{254} TLC plates were cut into 200 × 100 mm and 100 × 100 mm pieces and washed with methanol. Then, the working standards or sample solutions were applied to the plates as separate compact bands 15 mm from the bottom and 15 mm from the side edges of the plates with 4 mm band width. Twenty milliliters of mobile phase composed of toluene–ethyl acetate–methanol–ammonia 33% (30:20:20:0.1, v/v/v/v) was passed to the developing chamber, allowing the atmosphere to saturate with the mobile phase for 30 min at room temperature (25 ± 2°C). The plates were developed over a distance of 70 mm in an ascending manner, attained within 10 min, and then air-dried for 5 min. and scanned a deuterium/wolfram lamp at 248 nm for both drugs and at 354 nm for colchicine alone.

2.5 Sample Solution

Ten Gouty less® tablets were weighed and finely powdered in a mortar. A weight equivalent to 1.0 mg of colchicine and 1000.0 mg of probenecid was transferred into a 100-mL volumetric flask. Then the powder was dissolved in 80-mL methanol with sonication for 15 min. The volume was adjusted to 100-mL with methanol, and the solution was filtered to obtain colchicine and probenecid concentrations of 10.0 and 10000.0 (μg mL^{-1}), respectively (stock I). From stock I, aliquots of 4.0 mL were diluted to 10.0 mL with methanol to obtain colchicine and probenecid concentrations of 4.0 and 4000.0 (μg mL^{-1}), respectively (stock II). Aliquots of 1.0 mL of stock I were further diluted to 10.0 mL with methanol to obtain colchicine and probenecid concentrations of 1.0 and 1000.0 (μg mL^{-1}), respectively (stock III). From stock I, 1.0 mL was transferred quantitatively intoa 10.0 mLvolumetric flask, then 1.0 mL from the 250.0 μg mL^{-1} colchicine standard solution was added, and the volume was adjusted to 10.0 mL with the same solvent to obtain 26 μg mL^{-1}(1.0 μg mL^{-1} sample + 25.0 μg mL^{-1} standard) colchicine and 1000.0 μg mL^{-1} probenecid sample (stock IV). Then the procedure described under section 2.5 was applied.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results and Discussion

3.1 Spectral Analysis

Analysis the UV-absorption spectra of the two drugs under investigation demonstrated that both drugs have maximum absorptions at 248 nm, which provides a suitable wavelength for their simultaneous determination, while colchicine has another important maximum absorption at 354 nm (Figure 2). It was obvious that the spectra of both drugs in their binary mixtures completely overlapped in the region from 200 to 300 nm. Consequently, using traditional spectrophotometric method for their simultaneous detection is not simple.

The observation that at 354 nm probenecid has very little UV absorption, whilst colchicine has its maximum absorption at this wavelength, permits the solo detection of colchicine in the presence of probenecid with considerably good sensitivity. Hereinafter, an efficient separation was obtained by TLC, and each drug was detected at its specified R_f value either at 248 nm for both drugs or at 354 nm for colchicine only.

3.2 Optimization of Chromatographic Conditions

The mobile phase composition and the other parameters were optimized using the DOE approach. A tabulated Box–Behnken experimental design was built for efficient optimization of the experimental parameters (methanol and ammonia contents, saturation time and migration distance) and their obtained responses (peak areas and R_f values for both drugs) in 24 trial runs.

3.2.1 Mobile Phase Composition

Mobile phase selection was performed using controlled trial and error to obtain the most appropriate system. Several mobile phases of various compositions were tried, such as chloroform – methanol, toluene – methanol, chloroform– acetone and toluene – dichloromethane; in different proportions, these gave poor resolution or co-elution with the solvent front. Upon using toluene – ethyl acetate (6:2, v/v), a slight enhancement was obtained, where probenecid had an R_f value of 0.28 while colchicine stayed on the origin. With increasing solvent polarity using methanol in different ratios, it was noted that colchicine started to move from the origin. Consequently, toluene–ethyl acetate–methanol mixtures in different ratios and in the absence or presence of weakly acidic (acetic acid) or basic (ammonia) compounds were tested (Table 1). Upon using different compositions of this solvent system (12 optimized runs), it was noted that methanol and ammonia were the most effective additions (Figure 3). Moreover, using a toluene – ethyl acetate – methanol – ammonia system, especially in the ratios of (30:20:20:0.1, v/v/v/v), gave well-separated peaks with high areas and better shaped and symmetric bands for both drugs (Figure 4).

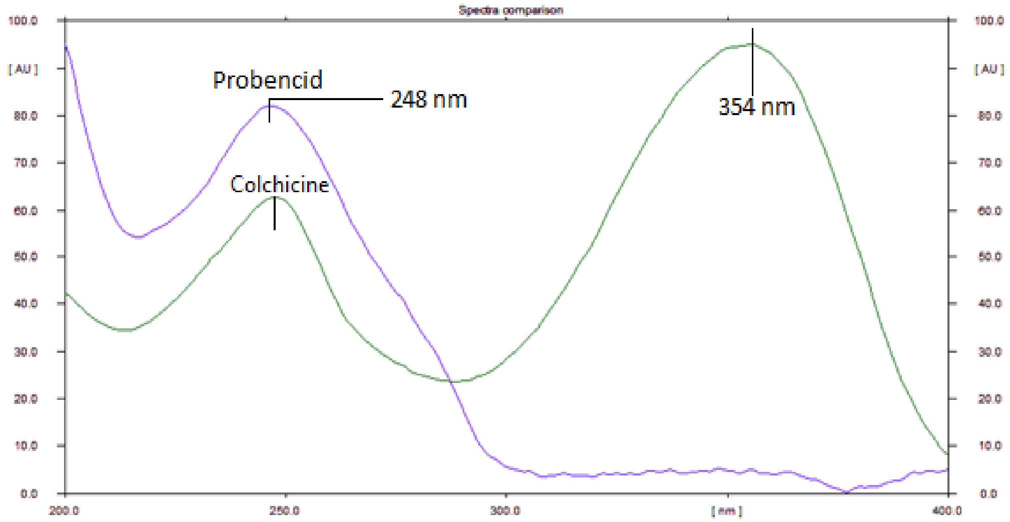

Figure 2: Comparison of spectra of colchicine (160 ng/band) and probenecid (600 ng/band).

Table 1: Effects of mobile phase solvent systems on retention factors and peak areas of colchicine and probenecid.

Mobile phase system	R_f Value Colchicine	Probenecid	Peak area at 248 nm Colchicine (80 ng/band)	Probenecid (1200 ng/band)	Notes
1- Toluene–ethyl acetate–methanol (30:20:5, v/v)	0.14	0.34	1908.89	9809.33	Streaking with poor R_f value for colchicine.
2- Toluene–ethyl acetate– methanol (30:20: 7, v/v)	0.18	0.40	1970.55	10056.20	Streaked bands for colchicine
3- Toluene–ethyl acetate– methanol (20:20:10, v/v)	0.50	0.40	2030.22	10100.07	Poor resolution; small difference in R_f values.
4- Toluene–ethyl acetate– methanol (30:20:20, v/v)	0.35	0.54	2219.40	10180.20	Broad bands and asymmetric peaks for both drugs.
5- Toluene–ethyl acetate– methanol– Acetic acid (30:20:20:0.1, v/v)	0.36	0.56	2110.30	10211.5 4	Compact and sharp peaks for probenecid,but broad, tailed bands for colchicine.
6-Toluene – ethyl acetate – methanol – ammonia (30:20:20:0.1, v/v)	0.63	0.33	2273.70	10193.70	Reversed order of elution with well-separated, better shaped and more symmetric peaks for both drugs

3.2.2 Saturation Time

The degree of vapor saturation must be the same in all studies to attain reproducible results [47]; therefore, we systematically altered saturation times to determine the effect on replication.Upon varying saturation times from 10 to 40 min, it was noted that at 25 min, good results were obtained. Accordingly, to obtain the most robust results, 30 min was used as a saturation time (Figure 5).

3.2.3 Migration Distance and Development Time

The migration distance throughout the study was standardized. To obtain efficient separation and good R_f values, therefore, 70 mm from the origin was selected as a migration distance for optimum separation of both drugs. This distance was attained within approximately 10 min (Figure 5).

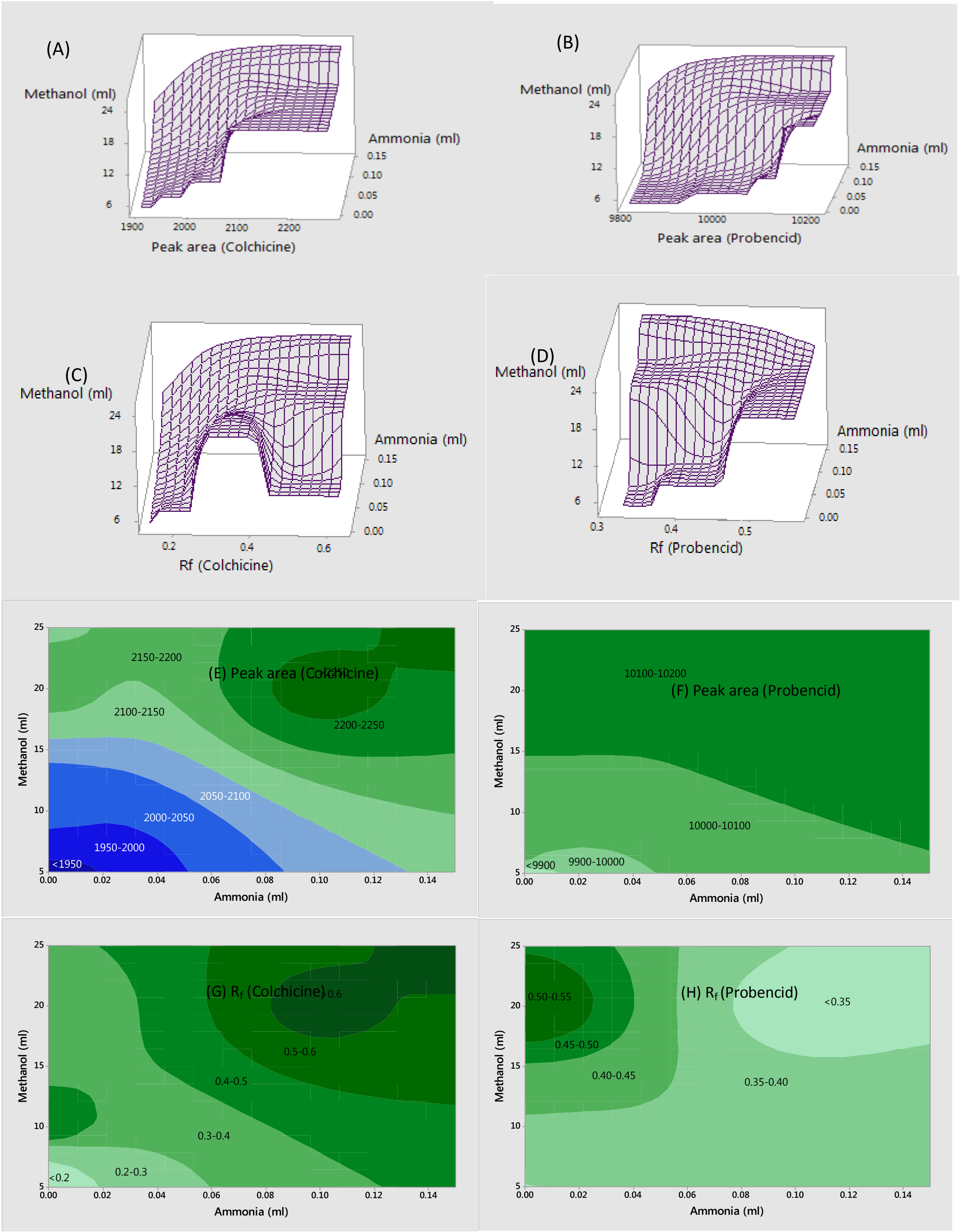

Figure 3: Surface and contour plots of peak area and R_f values for both colchicine and probenecid against methanol and ammonia contents of the solvent system.

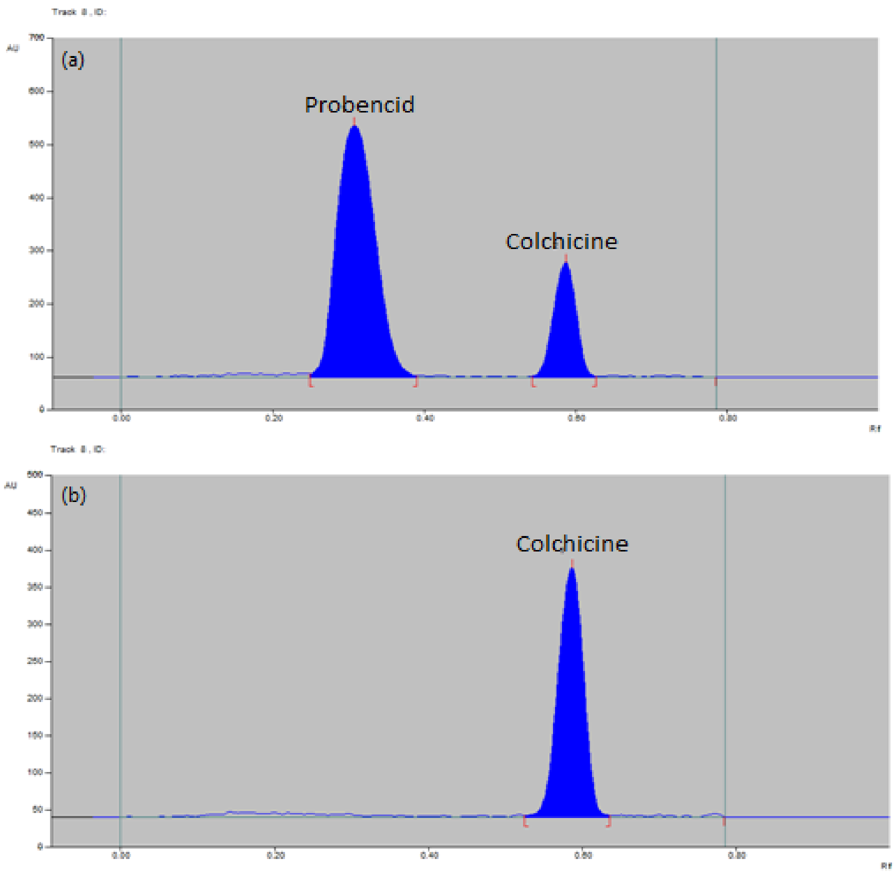

Figure 4: Two-dimensional TLC-densitogram of colchicine and probenecid at 160 and 2400 ng/band, respectively, using the optimized solvent system (toluene – ethyl acetate – methanol – ammonia, (30:20:20:0.1, v/v/v/v)); (a) Measured at 248 nm and (b) measured at 354 nm.

After optimization of all variables that affect separation, it was noted that the best R_f values to quantify probenecid and colchicine were 0.33 ± 0.03 and 0.60 ± 0.03, respectively. The wavelength of 248 nm was found to be suitable for determination of both drugs simultaneously. While this wavelength is optimal for probenecid but not for colchicine, the wavelength of 354 nm is λ_{max} for colchicine, and probenecid showed no absorption at this wavelength (spectra comparison, Figure 2). Herein, using of scanner four in the present work allowing the determination of the studied drugs at their optimal wavelengths (using dual wavelengths) simultaneously with higher sensitivity and saving efforts, time and cost. Three dimensional densitograms recorded in the multi-wavelengths were presented (Figure 1S, supplementary information).

3.3 Method Validation

The proposed method validation was performed in conformance with ICH guidelines [48]

3.3.1 Linearity and Range

Eight concentrations, with three replicates of each concentration, of colchicine and probenecid were selected to create the calibration graphs (Figures 1S and 2S). At 248 nm, the selected concentration ranges were 16–400 and 120–6000 ng/band for colchicine and probenecid, respectively (Figure 6). At 354 nm, a range of 16–400 ng/band was selected for colchicine (Figure 7). For validation following ICH guidelines, determination of linearity ranges is required. Therefore, both linear and polynomial models were constructed. It was noted that the second order polynomial plot produced a much better fit and wider ranges (Table 2) than that of the linear model. Using the linear model, at 248 nm, r values of 0.9998 and 0.9989 were found for colchicine and probenecid, respectively, and r^2 values were 0.9996 and 0.9978 for the two compounds.At 354 nm, an r value of 0.9962 and r^2 value of 0.9924 were found for colchicine. Using the second order polynomial fit, at 248 nm, an r value of 0.9999 and r2 of 0.9998 were found for both compounds. At 354 nm

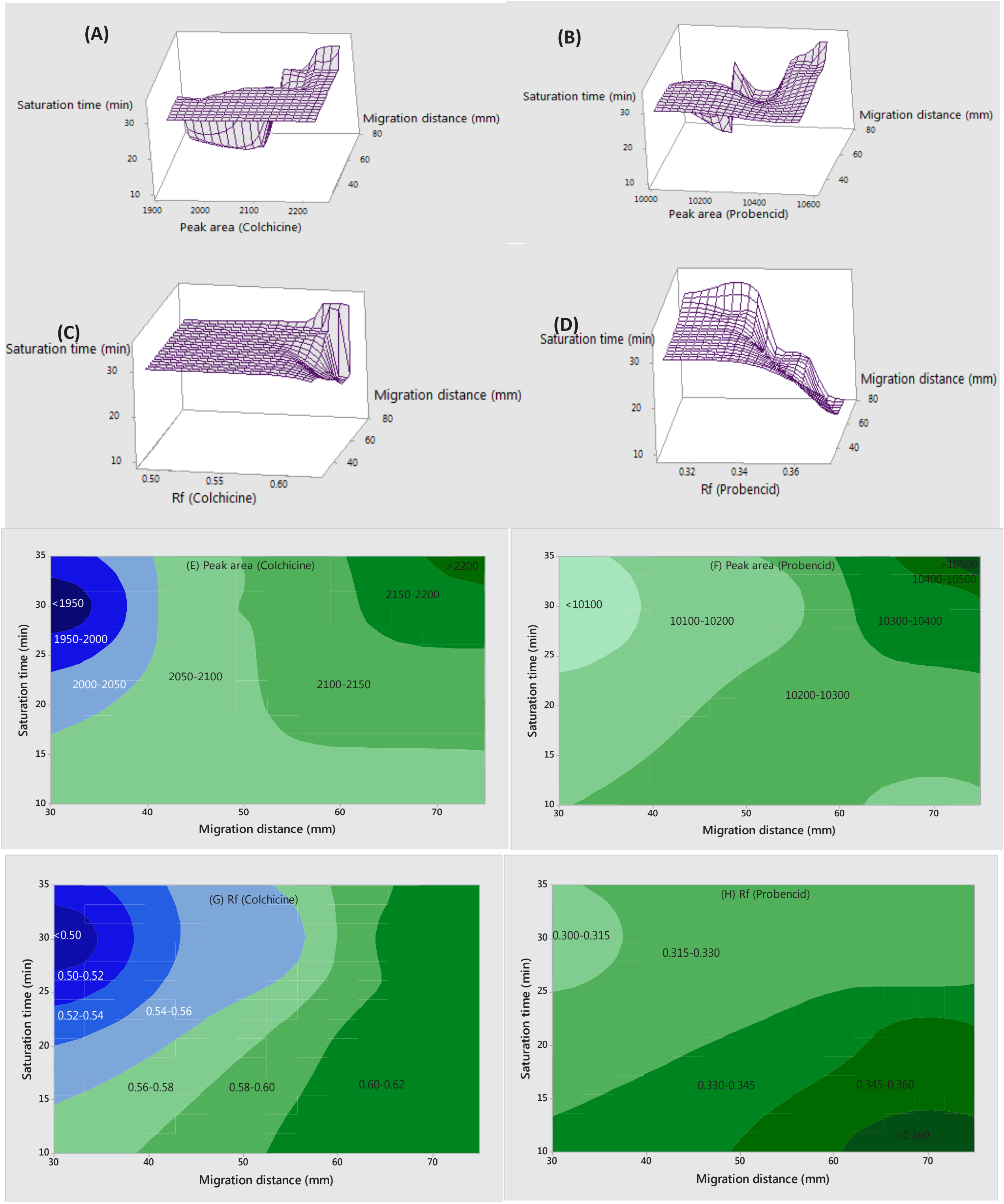

Figure 5: Surface and contour plots of peak area and Rf values for both colchicine and probenecid against saturation time and migration distance.

Table 2: Quantitative analytical parameters of linear and polynomial regression analysis for the studied drugs by the proposed TLC-spectrodensitometric method .[a]

Parameter	**Colchicine**		**Probenecid**
	λ_{max}(354 nm)	**λ_{max}(248 nm)**	**λ_{max}(248 nm)**
Linear regression model			
Experimental linearity range (ng/band)	16 - 240	16 - 320	120 - 2400
Slope (b)[a] ± SD[b]	27.71±1.21	15.51±0.15	6.35±0.21
Intercept (a)[a] ±SD[b]	431.08±149.70	30.11±27.28	857.10±143.30
Determination coefficient (r^2)	0.9924	0.9996	0.9978
Correlation coefficient (r)	0.9962	0.9998	0.9989
LOD[b] (ng/band)	17.83	5.81	74.52
LOQ[b] (ng/band)	54.03	17.59	225.82
polynomial regression model			
Full studied range (ng/band)	16 - 400	16 - 320	120 - 6000
Coefficient x ±SD[b]	34.79±1.01	14.73±0.58	7.24±0.16
Coefficient x^2 ±SD[b]	-0.028±0.0025	0.0023±0.0016	-0.0006±4.2005
Intercept (a)[a] ±SD[b]	225.91±45.82	64.47±22.99	657.12±84.08
Determination coefficient (r^2)	0.9994	0.9998	0.9998
Correlation coefficient (r)	0.9997	0.9999	0.9999
LOD[b] (ng/band)	4.35	5.15	38.32
LOQ[b] (ng/band)	13.20	15.60	116.13

[a] Average of six replicates.

[b] Limit of detection (LOD), Limit of quantitation (LOQ), Standard deviation (SD).

using the polynomial fit, r was 0.9997 and r^2 was 0.9994 for colchicine.Taken together, these values, as depicted in Table 2, indicate high correlation for the assay, especially in the case of the second-order polynomial fit.

3.3.2 Detection and Quantification Limits (LOD and LOQ)

LOD and LOQ were calculated using 3.3 σ/S for LOD and 10 σ/S for LOQ. The results (Table 2) demonstrated that the method has good sensitivity for the determination of both drugs, especially when the polynomial fit is utilized. According to the results, and considering the very low concentrations of colchicine in comparison to probenecid in their fixed dose commercial combinations (probenecid is roughly 1000-fold more concentrated) [6], it became clear that the polynomial model results in optimal calculations, as it gives wider concentration ranges, higher r values and smaller LOQ values, which enables the simultaneous determination of both drugs in their fixed dose combinations. As a result of this analysis, polynomial equation data were found to be optimal for all calculations (Table 2).

3.3.3 Precision

Intra-day precision (repeatability) was determined by analyzing three concentrations in six replicates (80, 160 and 240 ng/band for colchicine and 1200, 2400 and 3600 ng/band for probenecid).Inter-day precision (intermediate precision) for both drugs was evaluated by analyzing the same three concentrations on three different days.The results (Table 3) demonstrated that RSD did not exceed 2.30%, which confirms high precision of the proposed method (Figures 3S and 4S).

3.3.4 Accuracy

The accuracy of the developed method was evaluated by its application to a tablet containing both colchicine and probenecid, which was spiked with known amounts of standard colchicine and probenecid.Spiked amounts covered low, medium and high ranges of the calibration graphs. The results (Table 4) showed that the recovery ranged from 96.71±1.01% to 102.43±0.12%, which supports the high accuracy of the proposed method.

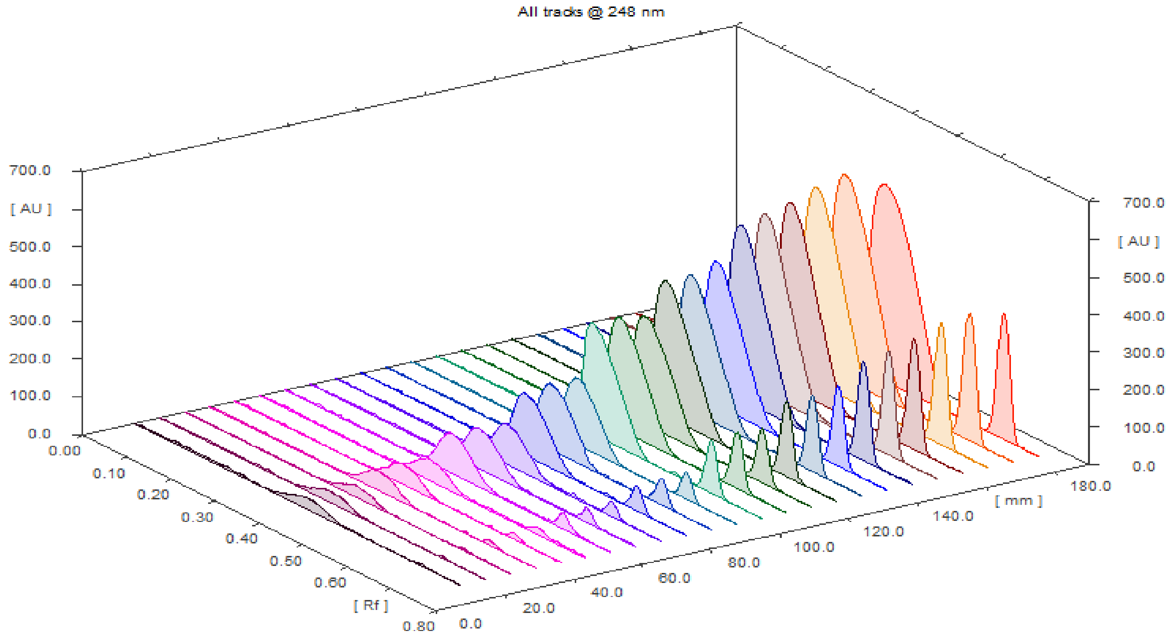

Figure 6: Three-dimensional chromatogram for calibrations of binary mixtures of colchicine and probenecid at concentrations ranging from 16 to 400 ng/band and from 120 to 6000 ng/band for colchicine and probenecid, respectively, at 248 nm.

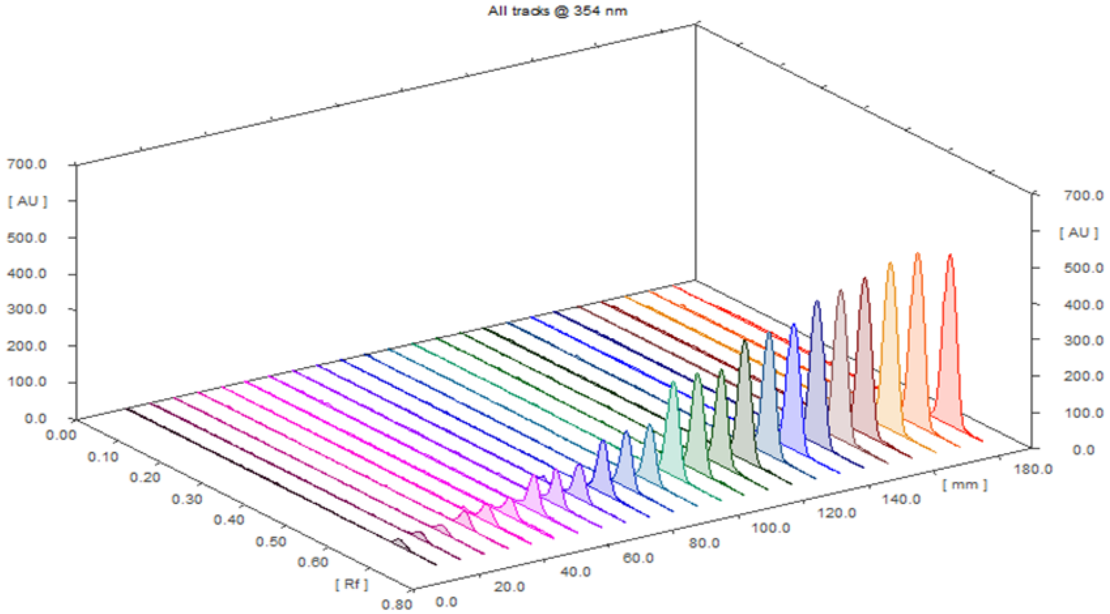

Figure 7: Three-dimensional chromatogram for calibration of colchicine ranging from 8 to 400 ng/ band at 354 nm.

3.3.5 Specificity and Selectivity

Upon comparison of the spectra of samples and standards for colchicine and probenecid at the peak start (s), peak apex (m) and peak end (e) positions of the band [49], the values were found to be very close to 1, as shown in Table 5, which indicates that all peaks from different peak sites were attributable to the investigated drugs. The recovery obtained upon dividing the response of samples by that of standards using the same concentration level 40 ng/band (colchicine) and 4000 ng/band (probenecid) for both sample and standard, were in the ranges of 97.78±1.37% to 98.29±2.86%, which indicates suitable peak purity and selectivity.

Table 3: Intra- and inter-day precision studies of the proposed TLC-spectrodensitometric method for the studied drugs.

Concentration (ng/band)	Intraday precision		Interday precision	
	Mean area[a]	RSD[a]	Mean area[b]	RSD[b]
Colchicine at 248 nm				
80	2363.40	1.40	2382.90	2.19
160	4182.03	0.17	4155.20	0.93
240	5754.10	0.414	5714.50	2.21
Colchicine at 354 nm				
80	4445.50	0.72	4368.40	1.34
160	7357.01	0.46	7242.30	2.26
240	10193.90	0.58	10113.06	1.92
Probenecid at 248 nm				
1200	10184.87	1.73	10264.30	2.30
2400	14922.33	0.77	14930.78	0.72
3600	19777.80	1.33	19936.07	1.90

[a] Average of five determinations within the same day.
[b] Average of fifteen determinations over three days.

Table 4: Recovery study of the proposed TLC-spectrodensitometric method for analysis of the investigated drugs.

Claimed taken (ng/band)	Standard drug added (ng/band)	Percentage recovery ± SD[a]	RSD
Colchicine at 248 nm			
20	50	97.02±2.02	2.08
20	100	96.71±1.01	1.04
20	150	100.74±0.05	0.05
Colchicine at 354 nm			
20	50	102.01±1.96	1.92
20	100	97.77±1.03	1.05
20	150	100.50±0.86	0.85
Probenecid at 248 nm			
1000	500	101.89±2.13	2.09
1000	1000	102.43±0.12	0.11
1000	1500	96.88±0.03	0.03

[a] Average of five determinations± standard deviation.

Moreover, the percentage recoveries obtained by applying the standard addition method were good (96.71±1.01 to 102.43±0.12 %) (Table 4), confirming the efficiency of the proposed method for simultaneous analysis of both drugs in pure form and combined dosage form and proving the accuracy and selectivity of the developed method. Furthermore, the R_f values at the start, maximum and end of the peaks were 0.58, 0.62 and 0.66 for standard colchicine and 0.58, 0.61 and 0.66 for colchicine in its sample solutions; for probenecid, the R_f values were 0.25, 0.31 and 0.41 for its standard solutions and 0.25, 32 and 0.41 for its sample solutions (Figure 8). The obtained results therefore demonstrated no significant differences in the R_f values at a variety of positions between the compared peaks.

Finally, by comparing an overlay of the spectra of both standard and sample solutions for both colchicine and probenecid, it was observed that all spectra of samples and standards were superimposable, as shown in Figure 5S.

Together, all of the obtained results proved the specificity and selectivity of the developed method for the simultaneous analysis of colchicine and probenecid in their binary mixtures and commercial pharmaceutical formulations.

3.3.6 Robustness

The robustness of the method was evaluated by studying the effects of small changes in experimental variables, such as mobile phase composition, development time and saturation time, on the peak area and R_f values. Thus, slight (in the range of 10%) but deliberate changes were made in the aforementioned parameters. The small values of RSD of peak areas along with the nearly similar R_f values, together with the percentage of recovery, which ranges from 96.65±1.10 to 102.56±1.93%, all supported the robustness of the proposed method for analysis of both drugs in their co-formulation (Table 6).

3.3.7 Solution stability

To evaluate the stability of colchicine and probenecid before chromatographic development, samples of colchicine and probenecid, with concentrations of 80ng/band and 1200 ng/band, respectively, were prepared and stored at room temperature for 1, 2 and 24 h. Then, densitograms were analyzed for the appearance of any additional bands or changes in peak intensities or positions. Notably, neither were new bands observed nor did band positions change, which indicates the stability of the studied over the analysis times.

3.4 Pharmaceutical Sample Analysis

It was observed that in their fixed dose combination (Gouty less® tablets), colchicine and probenecid are found in a ratio of 1:1000, respectively. In other words, the concentration of colchicine is very low in comparison with

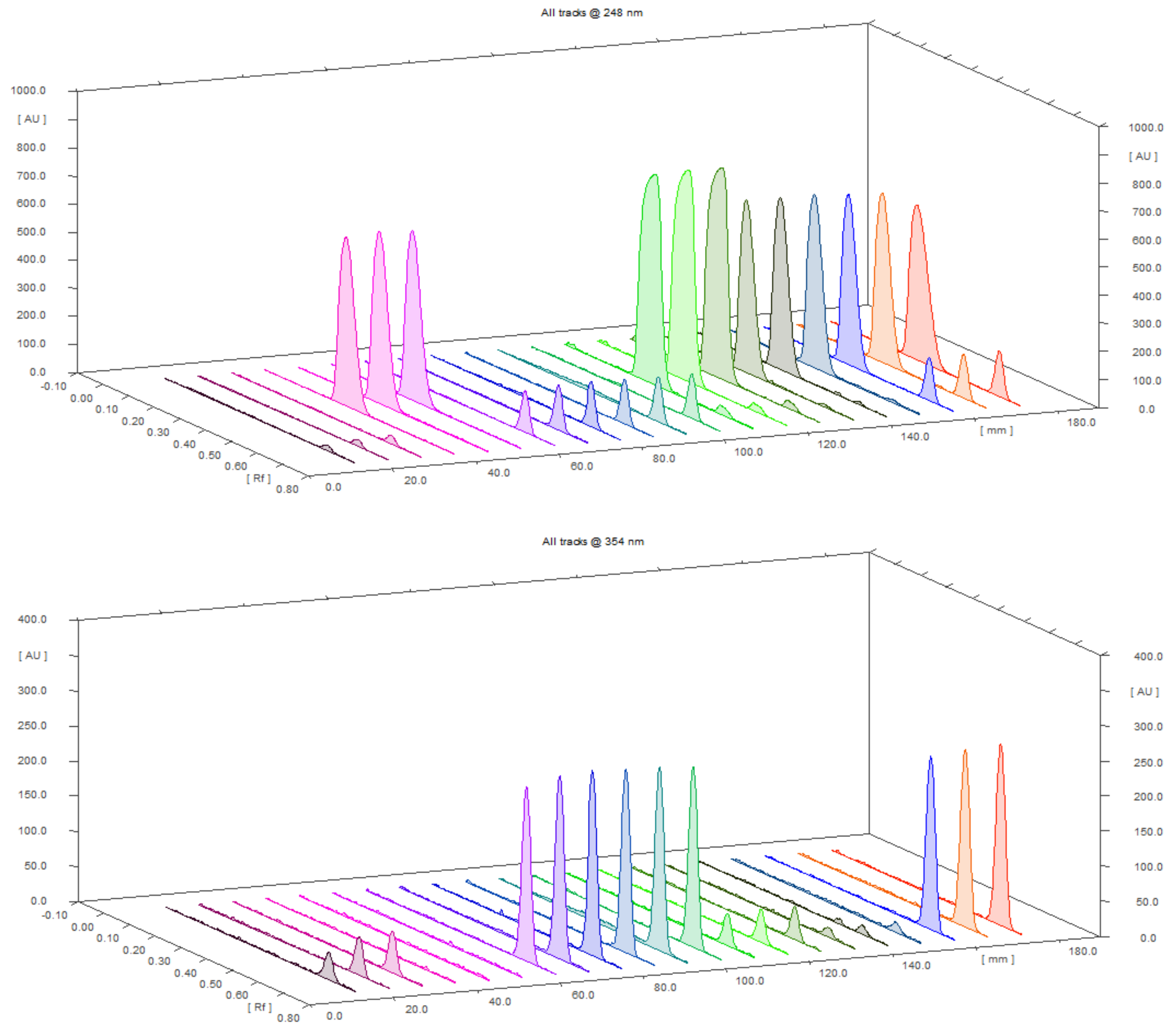

Figure 8: Three-dimensional TLC-densitograms to assess the application of colchicine and probenecid. Tracks 1-12 represent standard solutions, while tracks 13-21 represent the dosage form. Tracks 1, 2 and 3 include 16 ng/band standard colchicine; tracks 4, 5 and 6 include 4000 ng/band standard probenecid; tracks 7, 8 and 9 include 104 ng/band standard colchicine; tracks10, 11 and 12 contain 100 ng/band standard colchicine; tracks 13, 14 and 15 contain 16 ng/band dosage form colchicine and 16000 ng/band dosage form probenecid; tracks 16, 17 and 18 contain 4 ng/band dosage form colchicine and 4000 ng/band dosage form probenecid; and tracks 19, 20 and 21 contain 104 ng/band colchicine (4.0 ng/band dosage form+100.0 ng/band standard) and 4000 ng/band dosage form probenecid. Measurements were taken at 248nm (top panel) and 354nm (bottom panel).

Table 5: Peak purity and identity test for specificity evaluation of the proposed method for simultaneous determination of probenecid and colchicine.

Solutions	Concentration (ng/band)	Wavelength (nm)	r (s,m)[a]	r (m,e)[b]
Colchicine standard solution	40	354	0.9998	0.9999
Colchicine sample solution	40	354	0.9994	0.9998
Colchicine standard solution	104	248	0.9965	0.9999
Colchicine sample solution	104	248	0.9997	0.9950
Probenecid standard solution	4000	248	0.9985	0.9994
Probenecid sample solution	4000	248	0.9968	0.9989

[a] Correlation Coefficient from start to maximum position of the spectrum.

[b] Correlation Coefficient from maximum to end position of the spectrum.

Table 6: Robustness of the proposed TLC-spectrodensitometric method for determination of the studied drugs.

Parameters	Percentage recovery ± SD[a]			R_f		
	Colchicine		Probenecid	Colchicine		Probenecid
	At 354 nm	At 248 nm	At 248 nm	At 354 nm	At 248 nm	At 248 nm
Mobile phase composition; Toluene–ethyl acetate–methanol–ammonia 33% (30:20:20:0.1,v/v/v/v) (Optimized)						
(a) 33:22:22:0.1, v/v/v/v	100.80 ±3.21	102.51±1.20	98.00±2.23	0.63	0.63	0.34
(b) 27:18:18:0.1, v/v/v/v	100.51±1.85	100.37±0.12	100.56±0.10	0.62	0.62	0.32
Saturation time; 30 Min. (Optimized)						
(a) 33 min.	100.57±1.04	98.89±3.31	102.10±1.43	0.60	0.60	0.32
(b) 27 min.	100.97±2.97	101.10±2.23	101.64±2.32	0.63	0.63	0.34
Migration distance;70 mm. (Optimized)						
(a) 77 mm.	102.40±1.71	102.56±1.93	99.23±0.89	0.63	0.63	0.33
(b) 63 mm.	96.65±1.10	97.80±1.14	100.38±2.34	0.60	0.60	0.32

[a] Average of six determinations at concentrations of 80 and 1200 (ng/band) for colchicine and probenecid, respectively.

Table 7: Determination of the studied drugs in their pharmaceutical dosage form using the proposed TLC-spectrodensitometric method.

Pharmaceutical dosage form	Contents (mg/tablet)	Percentage recovery ± SD[a] Proposed method		Reference methods	t-value[b]		F-value[b]	
Gouty less® tablets		At 354 nm	At 248 nm	At 350 nm[24] & at 266 nm[23]	At 354 nm	At 248 nm	At 354 nm	At 248 nm
	Colchicine (0.5)	98.29±2.86	98.22±1.46	97.52±1.68[24]	0.52	0.70	2.92	1.32
	Probenecid (500)	-	97.78±1.37	96.63±0.69[23]	-	1.67	-	3.92

[a] Average of six determinations ± standard deviation.
[b] Theoretical values at 95% confidence limit: *t*= 2.31, *F*= 6.39.

probenecid, which makes simultaneous determination of both drugs potentially difficult. To determine if the challenges presented by the differences in amounts would negate the usefulness of the proposed method, several tests of the method were performed. Firstly, we used 4 μL from stock II to obtain 16 ng/band of colchicine and 4 μL from stock III to obtain 4000 ng/band of probenecid. By application of the standard addition method for determination of colchicine, we used 4 μL from stock IV to obtain 104 ng/band for colchicine (4.0 ng/band samples + 100.0 ng/band standard colchicine) and 4000 ng/band for probenecid sample (Figure 8).The data obtained upon the analysis of colchicine and probenecid in either pure or pharmaceutical form (Gouty less® tablets) using the proposed TLC- spectrodensitometric method were compared with those results obtained by the reference methods (23,24).The calculated t- and F- values were lower for our method than for the tabulated results, indicating that there is no significant difference with reference to accuracy and precision (Table 7). Two dimensional TLC-densitograms of mixtures of colchicine and probenecid in fixed dosage forms are presented in Figure 9.

4 Conclusion

A novel, simple, selective and sensitive TLC-spectrodensitometric method has been developed for the simultaneous determination of colchicine and probenecid in their fixed dose combinations. The proposed method presented numerous advantages, such as enhanced sensitivity, better recovery, shorter analysis time, lower expense, larger sample capacities, need for lower amounts of solvents and simpler cleanup relative to other

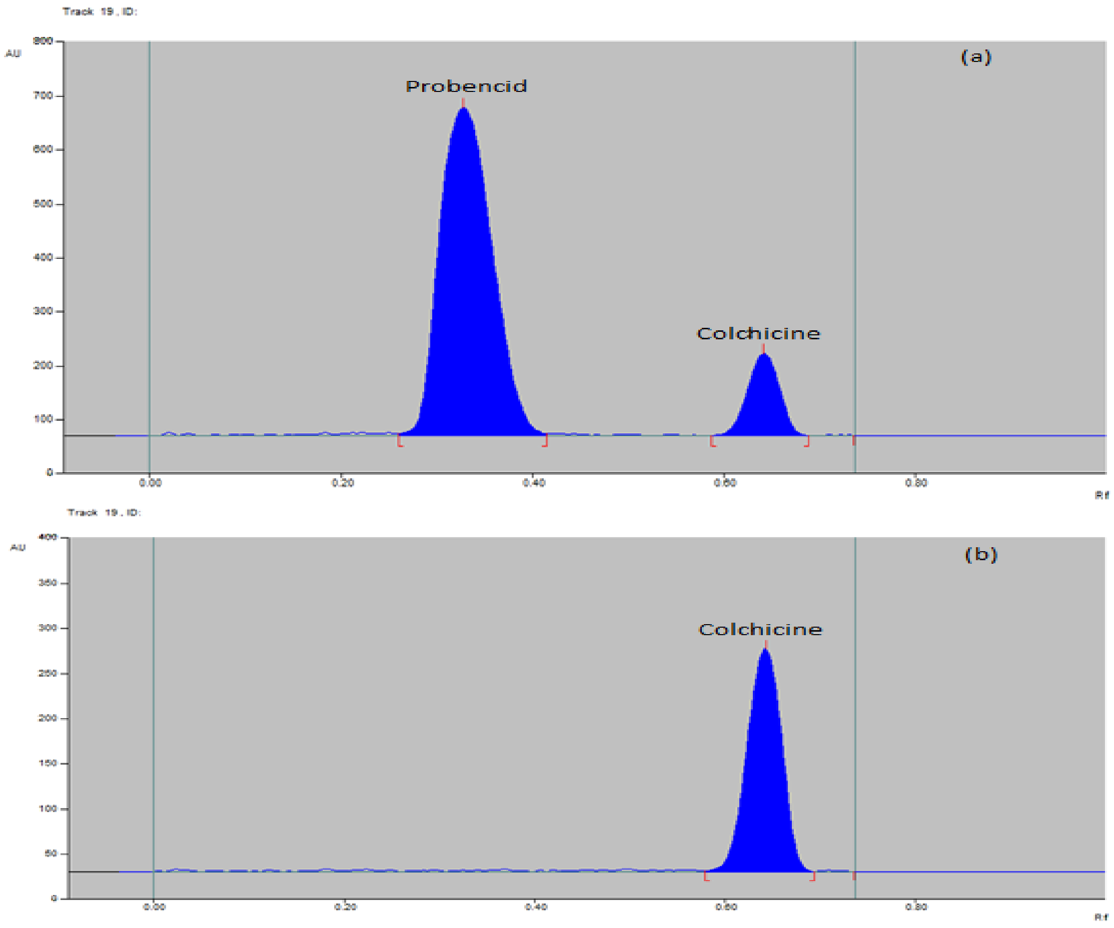

Figure 9: Two-dimentional TLC-densitogram of the dosage form extract containing colchicine [104 ng/band (4.0 ng/band dosage form+100.0 ng/band standard)] and probenecid (4000 ng/band), where (a) is measured at 248 nm and (b) at 354 nm.

chromatographic methods. The developed method can be considered to be a facile alternative to existing HPLC methods that require pre-extraction steps.

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