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Simple development and validation of RP-HPLC and TLC- densitometric methods for the simultaneous determination of nadifloxacine and mometasone furoate in their binary mixture

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Abstract

The present work is concerned with development, optimization and validation of two chromatographic methods for the simultaneous determination of nadifloxacine (ND) and mometasone furoate (MF). The first developed method was RP-HPLC depended on chromatographic separation using Phenyl-hexyl column and a mobile phase composed of acetonitrile: acidified water with orthophosphoric acid up to (pH 2.5 ± 0.1) in the proportion of (65: 35 v/v) pumped at a flow rate of 1.25 mL min^{-1}. All measurements were performed with UV detection at 254 nm. The second method was TLC-densitometry, chromatographic separation was established on aluminum TLC plates pre-coated with silica gel GF254 as the stationary phase and chloroform: methanol: hexane: ethyl acetate: acetic acid (9: 1: 3: 3: 0.1, by volume) as the mobile phase followed by densitometric measurement of the separated bands at 254 nm. Validation of the suggested methods was successfully applied with respect to ICH guidelines. The proposed chromatographic methods were used to determine both drugs binary mixture in pure form and dosage form. The proposed methods give good linearity in the range 0.5–5.0 µg/mL and 0.5–40 µg/spot for HPLC and HPTLC methods, respectively. While MF standard solutions in the range 0.2–1.2 µg/mL and 0.5–3.8 µg/ spot for HPLC and HPTLC methods, respectively. The obtained results were statistically compared with those achieved by the reported methods, showing no significant difference with respect to accuracy and precision at p = 0.05.

Key words

Nadifloxacine, Mometasone furoate, HPLC, TLC-densitometry, pharmaceutical preparations

1. Introduction

Nadifloxacine (ND), chemically (RS)-7-fluoro-8-(4-hydroxypiperidin-1-yl)-12-methyl-4-oxo-1-azatricyclo[7.3.1.0]trideca-2,5,7,9(13)-tetraene-3-carboxylic acid **(Figure 1A)**, is considered the first potent topical fluoroquinolone for treatment of acne vulgaris and skin infections [1]. In addition, it has been shown to be effective against aerobic Gram-negative, Gram-positive antibacterial drug[2]. Mometasone furoate (MF), a glucocorticoid, chemically 9-chloro-17-(2-chloroacetyl)-11-hydroxy-10,13,16-trimethyl-3-oxo-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-17-yl] furan-2-carboxylate **(Figure 1B)**, is a corticosteroid drug, the anti-inflammatory actions of corticosteroids are thought to involve phospholipase A2 inhibitory proteins, lipocortins, which control the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes. Used for anti-inflammatory and antipruritic properties [3]. A mixture of ND and MF are available in cream dosage firms which topically applied for the treatment of dermatoses. Literature surveys revealed that ND is not official in any pharmacopoeia [3] while MF is an official drug in European Pharmacopoeia[4].The literature survey revealed several analytical methods have been reported for the determination of ND alone or in combinations with other drugs including, spectrophotometry [5, 6], HPTLC [7, 8]HPLC [9-11], Different methods were reported for determination of MF alone or in combinations with other drugs including, spectrophotometry, [12-14],TLC [8, 15]and HPLC [16-22] To the best of author's knowledge there is only one method has been reported for the determination of ND and MF in combinations using HPTLC technique [23].

The work in this paper was aimed to develop two selective, accurate and precise chromatographic methods for the simultaneous determination of ND and MF in topical cream.

2. Experimental

2.1. Instrumental

- HPLC system consisted of Agilent1260 (Agilent, USA) equipped with vacuum degasser, UV/visible detector-Model G 2489 A UV detector, and quaternary pump and 10 microliter

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loop auto sampler injector was used, Phenyl- hexyl column (250 mm × 4.6mm, 5µm) column, Sonicator Power Sonicator – Model 410 and data were recorded and analyzed by chemstation® software (Agilent, USA).

● Thin layer chromatography aluminum plates (20 × 20 cm, 0.25 mm layer thickness) pre-coated with silica gel 60F-254 was obtained from Merck (Darmstadt, Germany).

● Spectrodensitometric scanning was done using a Camag TLC Scanner Model 3 S/N 130319 and Win CATS 1.4.2 software (Muttenz, Switzerland). All measurements were performed in the reflectance/absorbance mode. The source of the light was deuterium and wolfram lamp.

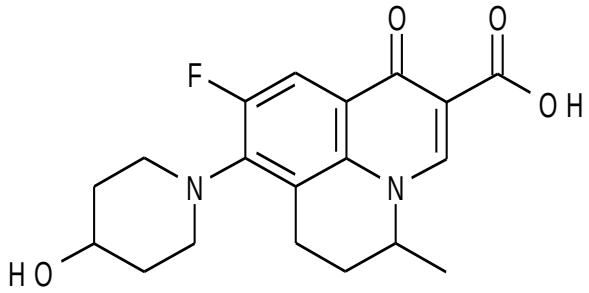

Figure 1A: Nadifloxacine

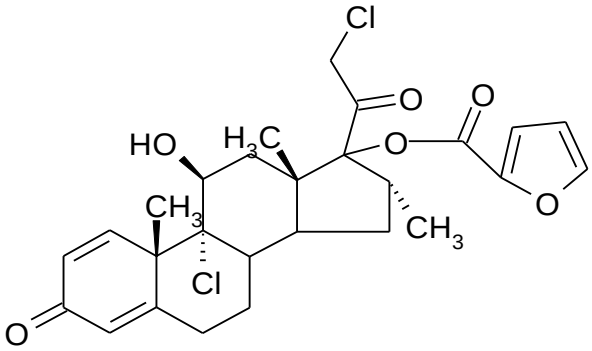

Figure 1B: Mometasone furoate

3. Materials and reagents

3.1. Pure standard

Nadifloxacine was kindly purchased from Pfizer pharmaceuticals Egypt S.A.E. (Cairo, Egypt), mometasone furoate was provided by Pharco Pharmaceutical, (Al Obour, Egypt.), claimed to contain 99.9% w/w and 99.5% w/w on dried basis for both ND and MF, respectively.

3.2. Pharmaceutical dosage form

Nadirest-M® cream was purchased from Labor ate Pharmaceuticals, India , labeled to contain (1% ND and 0.1% MF).

3.3. Chemicals and reagents

All chemicals used throughout the work were of analytical grade were used without further purification: Methanol (Merck, Darmstadt, Germany), chloroform (Sigma-Aldrich, Belgium), hexane (Adwic, Egypt), ethyl acetate (El-Nasr pharmaceutical chemical company, Egypt) and Acetic acid (Ardwic, Egypt), phosphoric acid (Ardwic, Egypt) and acetonitrile (Merck, Darmstadt, Germany).

3.4. Standard and working solutions

The primary stock solutions of ND and MF were prepared freshly and separately by dissolving 50.0 mg of each in 50.0 mL volumetric flasks (1.0 mg mL-1) and complete to the volume with methanol. Further dilutions were prepared by the appropriate dilution of the stock solutions with mobile phase reach the concentration ranges of 0.5-5 µg mL-1 for ND and 0.2-1.8 µg mL-1 for MF HPLC, TLC 0.8-40 µg/band for ND and 0.5-3.8 µg/band for MF.

4. Procedures

4.1. Chromatographic conditions

HPLC chromatographic separation of the binary mixture was performed using an isocratic elution based on a mobile phase consisting of acetonitrile and acidified water in the proportion of (65: 35 v/v) adjusted to pH 2.5 by orthophosphoric acid. The mobile phase was filtered through 0.45-µm membrane filter and degassed for 30 min in an ultrasonic bath prior to its use. The mobile phase was pumped through the phenyl-hexyl column at a flow rate 1.25 mL min−1. Analyses were performed at ambient temperature and detection was carried out at 254 nm. The injection volume was 10 µL. While in TLC samples of ND and MF were applied in the form of bands to (20 x 10 cm) TLC plates using Camag auto sampler. The bands were applied at l cm from the bottom edge of the plate and bandwidth was 6 mm. Triplicate applications were made for each solution. The chromatographic chamber was equilibrated with (chloroform: methanol: hexane: ethyl acetate: acetic acid) (9:1:3 :3: 0.1, by volume) for half an hour at room temperature. The approximate time of plate development was 10 min. The plates were then developed by ascending migration of the developing phase. The plates were removed, left to dry and the spots were visualized under UV lamp at 254 nm.

4.2. Construction of calibration curve

The standard solutions were prepared by dilution of the stock standard solution with mobile phase to reach a concentration range 0.50 – 5.00 µg mL-1 for ND and 0.20 - 1.80 µg mL-1 for MF in HPLC, while in TLC the concentration of ND 0.80 – 40.00 µg/band and 0.50 – 3.80 µg/band MF. 10.0 µL of each drug were injected in triplicates for each concentration and run under the above described conditions. The calibration plots were constructed and regression equation was derived through plotting the peak against each corresponding concentration.

4.3. Application to pharmaceutical formulations

1.0 g of Nadirest-M® cream was accurately measured equivalent 0.1% w/w of MF and 1.0% w/w of ND, transferred into 100 mL volumetric flask followed by addition of 50 mL methanol. Sonication for the resulted solution for 20 minutes and the volume was completed to the mark with mobile phase. Filtration of the solution using filter paper 0.45 mm (Millipore, Milford, MA) to remove excipients, and 1.0 mL was spiked for

further dilution to 10.0 mL with methanol. The resultant sample solution was used for chromatographic development. The aforementioned general analytical procedures were completed and the concentrations of ND and MF were computed from corresponding regression equations.

5. Results and discussion

5.1. RP-HPLC method

To the best of authors' knowledge, this is the first simultaneous determination of ND and MF in their topical preparations by using RP-HPLC method. Many attempts have been done to obtain the most suitable mobile phases for chromatographic separation such as water: acetonitrile, water: methanol at different flow rates and with different ratios. Water was acidified with orthophosphoric acid solution with different ratios and at different strengths. Lastly, a mobile phase consisting of (acetonitrile: acidified water with orthophosphoric acid (pH 2.5) in the percentage of (65: 35 %v/v). In addition, various reversed phase columns, trials were done successfully using a phenyl-hexyl column and UV detection at 254 nm at a flow rate of 1.25 mL min_{-1} to obtain a stable baseline. **(Figure 2)** illustrated that ND and MF were separated clearly and at reasonable retention times 3.59 min and 9.14 min, the corresponding peaks were developed sharply for ND and MF, respectively. Standard solution of ND and MF were prepared calibration as described above in order for determine of the linearity of LC detection response. The linearity of the drugs under study was confirmed by plotting a relative peak area versus concentrations and linear relation was achieved. The Linear regression equation was derived for ND and MF

PA = 161683 C + 117941 r = 0.9999 (ND)

PA = 62088 C + 18388 r = 0.9999 (MF)

Where C is the corresponding drug concentration in µg mL^{-1}, PA is the relative peak area and r is the correlation coefficient.

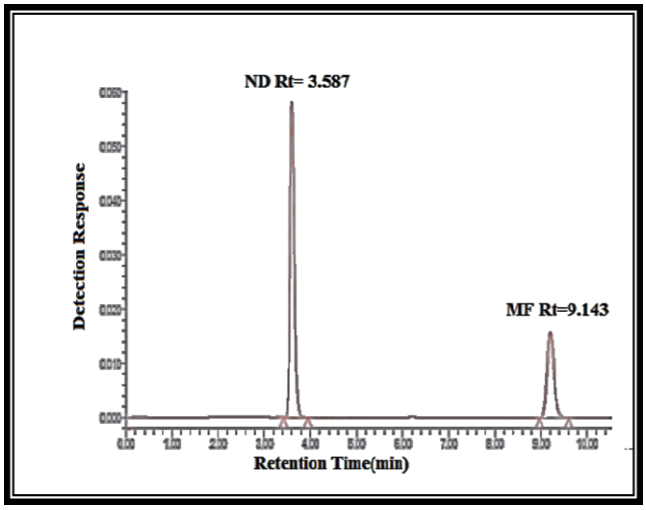

Figure 2: HPLC chromatogram of ND (Rt = 3.590) and MF (Rt = 9.250) using a phenyl- hexyle column (250 mm × 4.6mm, 5µm), mobile phase of acetonitrile: acidified water with orthophosphoric acid (pH 2.5) in the proportion (65: 35 by volume) at flow rate of 1.25ml/min at 254 nm.

5.2. TLC- Densitometric method

Planar chromatography with accurate determination of the samples and computer controlled quantification and evaluation of the established chromatograms has been represented to be a reliable technique for quantitative drug and for purity control. Regarding the TLC technique, the opposite polarity for both drugs made the separation extremely critical (The MF was non-polar while ND was highly polar). Initial method development was directed to choice the most proper mobile phase for the adequate separation of ND and MF such as methanol: chloroform (2:8, v/v), methanol: water: ammonia (9:0.5, 0.5 v/v) and ethyl acetate: hexane: chloroform (3:3:4, v/v/v) but tailing accompanied by bad resolution was observed. Band characteristic was enhanced by adding acetonitrile to the previous mobile phase and ethyl acetate was added to minimize fronting which was observed in ND. Finally, the good TLC separation was obtained when using the mobile phase chloroform: methanol: hexane: ethyl acetate: acetic acid (9: 1: 3: 3: 0.1, by volume), which gave a sharp and symmetrical peak. Bands were observed and well defined at 0.26 ± 0.02 and 0.75 ± 0.02 for ND and MF, respectively as shown in **(Figure 3)**. The spots were separated successfully and scanned discretely on the same plate at λ254 for ND and MF as shown in **(Figure 4)**. The relationship between the peak area of the spot and the concentration of each drug ND and MF was determined. The data of drug concentration versus peak area was established by linear least square regression analysis at wavelength λ_{254} nm and the concentrations corresponding to ND and MF were in the over the range 0.8–40 µg/band and 0.5–3.8 µg/band for ND and MF, respectively:

A = 307.9 C+ 318.8 r=0.9999 (for ND)

A = 988.8 C+203.14 r =0.9999 (for MF)

Where C is the corresponding concentration in µg/band, A is the integrated peak area and r is the correlation coefficient.

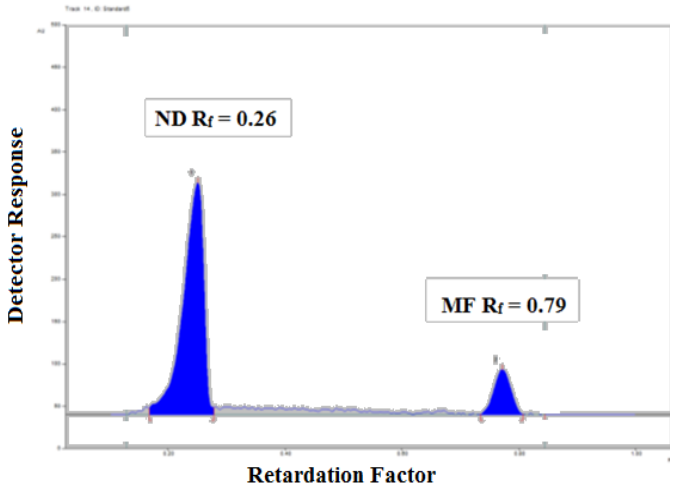

Figure 3: TLC chromatogram of nadifloxacine (R_f = 0.26) and mometasone furoate (R_f = 0.79) using a mobile phase of chloroform: methanol: hexane: ethyl acetate: acetic acid (9:1:3:3:0.1, by volume) and detection at 254 nm.

5.3. System suitability

U.S. Pharmacopeia (USP) States that tests which considered as an integral part of LC methods [24]. It is useful to confirm that the reproducibility and the resolution of the any chromatographic system for the separation and determination to be feasible. For first we used HPLC method to confirm the resolution (Rs), capacity factor (K'), column efficiency (N) reproducibility and selectivity factor (α) of the system. All system suitability parameters were calculated as shown in (Table 1).

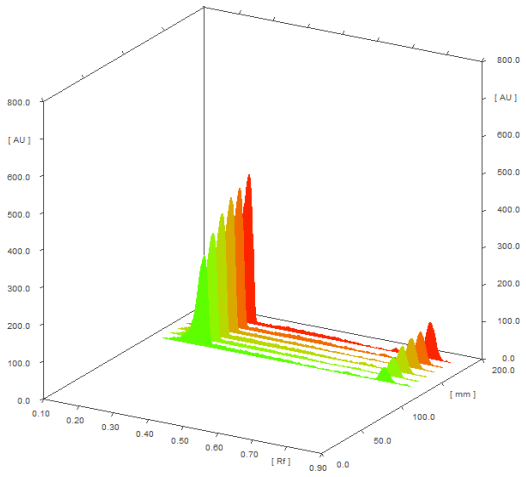

Figure 4: Scanning profile of the TLC chromatogram of nadifloxacine 0.80 –40.00 µg/band) and mometasone furoate (0.50 – 3.80 µg /band) at 254.0 nm.

5.4. Method validation

The suggested methods were validated according to the ICH guideline [25].The technique used was validated for parameters such as linearity, system suitability, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy and selectivity.

5.5. Linearity and range

According to the above-mentioned described experimental parameters, we constructed standard calibration curves for each drug by plotting relative peak areas against concentration. The linear regression equation parameters and the linearity ranges for each drug are mentioned in (Table 2). Calibration curves were constructed using a series of ND standard solutions in the range 0.5–5.0 µg/mL and 0.5–40 µg/spot for HPLC and HPTLC methods, respectively. While MF standard solutions in the range 0.2–1.2 µg/mL and 0.5–3.8 µg/ spot for HPLC and HPTLC methods, respectively. A linear relationship was constructed between the peak amplitude in the recorded areas in the HPLC and TLC methods versus the corresponding concentrations of the drug. The linear regression equation was calculated from triplicate run. Table 2 showed linearity range, slope, intercept and Correlation coefficient.

Table 1 : System Suitability Parameters for the RP-HPLC and HPTLC Methods.

parameter	TLC		HPLC		Reference
	ND	MF	ND	MF	
Retention time (Rt) [min]			3.5	9.14	
Retardation factor (Rf)	0.26	0.79			
Tailing factor (T)	0.99	1.07	1.17	1.12	T = 1 for a typical symmetric peak
Capacity factor (K0)			2.61	8.23	1 < K' < 10
Selectivity factor (a)	4.6		2.15	3.16	α > 1
Resolution factor (Rs)	7		7.40	23.14	$R_s \geq 2$
Column efficiency (N)			7649.499	14229.401	Increase with efficiency of the separation(N > 2000)
HETPa [mm]			0.032	0.017	The smaller the value the higher the column efficiency

HETPa= height equivalent to theoretical plates (length of column in mm/N).

5.9. Analysis of marketed formulation

Table 2 : Validation parameters of the proposed HPLC and TLC-spectrodensitometric method for the determination of nadifloxacine and mometasone furoate in pure sample

parameter	HPLC		TLC	
	ND	MF	ND	MF
Range [a]	0.5–5 (µg/ml)	0.2–1.8 (µg/ml)	0.8–40 (µg/band)	0.5–3.8 (ng/band)
Slope	161683	62088	307.90	307.90
Intercept	117941	18388	318.80	203.14
SE of the slope	535.620	289.822	307.46	980.28
SE of the intercept	1661.717	292.706	318.65	209.95
Correlation coefficient(r)	0.9999	0.9999	0.9999	0.9999
LOD	0.046	0.018	0.23	0.18
LOQ	0.141	0.056	0.71	0.65
Accuracy (mean ± SD) [b]	99.27 ± 0.954	98.88± 0.625	99.83 ± 0.387	99.27 ± 0.719
Robustness(mean ± SD) [b]	99.95 ± 0.770	99.70 ± 0.531	98.79 ± 0.635	100.05 ± 0.462
Precision				
Repeatability (%RSD) [c]	0.889	0.484	0.996	0.749
Intermediate precision (%RSD) [c]	1.018	0.476	0.829	0.553

[a] Concentration in µg/ml for HPLC and ng/band for TLC.
[b] Mean ± standard deviation for three determinations
[c] % relative standard deviation

5.6. Accuracy

The accuracy of the two methods used was applied successfully to quantify the drug in pure form and pharmaceutical product. This study was achieved by standard addition method to which a known concentration of ND and MF combination tables. we added about 50%, 100% and 150% of the label claim and mixed well then the powder was extracted and analyzed by chromatogram as described under section (calibration), (Table 2).

5.7. Precision

Intraday and Interday precision were assessed using three concentrations and three replicates of each concentration. The calculated relative standard deviation values were found to be small below 2 % indicating good repeatability and reliability of the proposed method. The results and their statistical analysis were summarized in (Table 2).

5.8. Robustness

The method robustness was evaluated by interchange some parameters such as organic phase ratio of mobile phase, column oven temperature and pH. These small deliberate variations unaffected the capacity. The developed methods were robust, as shown in (Table 2).

The simultaneous determination of ND and MF in Nadirest-M® cream was applied successfully by using the proposed methods without previous separation and without interference of the existing excipients. 1.0 gram of cream which equivalent to 1.0% w/w of ND and 0.1% w/w of MF was accurately measured and transferred into 100 mL volumetric flask then add of 30 mL methanol. After that sonicate the solution for 30 min. and complete the volume with methanol to the mark and sonicate again for 10 min. 1 mL was taken and diluted to 10 mL methanol after filtration by using whatman paper 0.45 µm. The efficacy of the proposed methods were confirmed by replicate analysis of the pharmaceutical product and the obtained results are statistically evaluated (Table 3).

A statistical comparison was done between the results obtained from the proposed method and the reported HPTLC method [23]. By calculated t test and F value the results indicating that the values are less than the tabulated ones, revealing that there is the no significant difference between the proposed and reported methods with respect to precision and accuracy. The suggested methods were validated by further application; the standard addition technique was done, as shown in (Table 4).

6. Conclusion

The suggested HPLC and TLC chromatographic methods provided cost-effective, accurate, simple and reproducible

Table 3: Application of the proposed HPLC and TLC-Spectrodensitometric method for the determination of nadifloxacine and mometasone furoate in Nadirest-M® and a results obtained by applying standard addition technique

Claimed concentration HPLC µg ml^{-1}		Claimed concentration TLC µg /spot		mean ± SD				Standard addition technique													
				HPLC method		TLC method		HPLC method						TLC method						HPLC method		TLC method	
								taken conc. µg ml^{-1}		Added conc. µg ml^{-1}		Found conc µg ml^{-1}		taken conc. µg /band		Added conc. µg band		Found conc µg /band		Recovery %		Recovery %	
ND	MF	ND	MF	ND	MF	ND	MF	ND	MF	ND	MF	ND	MF	ND	MF	ND	MF	ND	MF	ND	MF	ND	MF	ND	MF
2	0.2	20	2	99.71 ± 0.284	99.94 ± 0.531	99.58 ± 0.723	99.56 ± 0.665	2	-	1	-	0.99	-	20	-	4	-	3.98	-	99.90	-	99.67	-
								2	-	1.5	-	1.49	-	20	-	6	-	5.89	-	99.93	-	98.31	-
								2	-	2	-	2.01	-	20	-	8	-	7.99	-	100.5	-	99.91	-
								-	0.2	-	0.4	-	0.39	-	2	-	1.6	-	1.60	-	99.72	-	1.60
								-	0.2	-	0.8	-	0.8	-	2	-	2	-	1.98	-	100.3	-	1.98
								-	0.2	-	1	-	0.98	-	2	-	2.4	-	2.39	-	98.88	-	2.39
Mean																				100.1	99.65	99.3	99.77
SD																				0.337	0.749	0.861	0.376
RSD																				0.336	0.751	0.867	0.377

Table 4: Statistical comparison of the results obtained by the proposed HPLC and TLC- Spectrodensitometry method and a reported HPTLC method for the analysis of nadifloxacine and mometasone furoate

Parameter	HPLC method		TLC method		Reference method*	
	ND	MF	ND	MF	ND	MF
Mean	99.27	99.88	99.83	100.27	99.52	100.87
SD	0.954	0.625	0.387	0.719	0.659	0.987
Variance	0.910	0.391	0.150	0.517	0.434	0.974
n	9.00	9.00	9.00	9.00	9.00	9.00
Student's t-test	0.175 (2.120)*	0.684 (2.120)*	0.500 (2.120)*	0.379 (2.120)*		
F-value	2.09 (3.44)*	2.49 (3.44)*	2.900 (3.44)*	1.884 (3.44)*		

* The values in parentheses are the corresponding tabulated values at P = 0.05.

** The stationary phase was Merck precoated silica gel aluminum plate 60 F254 using dichloromethane: diethyl ether: ammonia: methanol: ethyl acetate (6: 3: 0.2: 1.75: 3.5, by volume) as mobile phase at 254 nm.

quantitative study for simultaneous estimation of ND and MF in their admixtures and pharmaceutical product. The suggested TLC-densitometric method is more sensitive rather than HPLC. It has the advantages of the use of minimal volume of solvents, very short run time and large sample capacity. Meanwhile, HPLC technique provides a good resolution between the different active constituent within suitable time of analysis and it is highly specific.

7. Availability of data and material

All detailed data and equations are included in the result and discussion section and also any other samples and information of the compounds are available from the authors.

8. Competing interests

All authors have no conflict of interest, no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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10. Authors' contributions

All authors contributed sufficiently and equally in this work, there have been no involvements that might raise the question of bias in the work reported or conclusions and all authors agreed to publish the work in this journal.

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