DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Nifuroxazide and Drotaverine Hydrochloride in Pharmaceutical Preparations by Three Independent Analytical Methods

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Three new, different, simple, sensitive, and accurate methods were developed for quantitative determination of nifuroxazide (I) and drotaverine hydrochloride (II) in a binary mixture. The first method was spectrophotometry, which allowed determination of I in the presence of II using a zero-order spectrum with an analytically useful maximum at 364.5 nm that obeyed Beer's law over a concentration range of 2–10 μg/mL with mean percentage recovery of 100.08 ± 0.61. Determination of II in presence of I was obtained by second derivative spectrophotometry at 243.6 nm, which obeyed Beer's law over a concentration range of 2–10 μg/mL with mean recovery of 99.82 ± 1.46%. The second method was spectrodensitometry, with which both drugs were separated on a silica gel plate using chloroform–acetone–methanol–glacial acetic acid (6 + 3 + 0.9 + 0.1) as the mobile phase and ultraviolet (UV) detection at 365 nm over a concentration range of 0.2–1 μg/band for both drugs, with mean recoveries of 99.99 ± 0.15 and 100.00 ± 0.34% for I and II, respectively. The third method was reversed-phase liquid chromatography using acetonitrile–water (40 + 60, v/v; adjusted to pH 2.55 with orthophosphoric acid) as the mobile phase and pentoxifylline as the internal standard at a flow rate of 1 mL/min with UV detection at 285 nm at ambient temperature over a concentration range of 2–10 μg/mL for both drugs, with mean recoveries of 100.24 ± 1.51 and 100.08 ± 0.78% for I and II, respectively. The proposed methods were checked using laboratory-prepared mixtures and were successfully applied for the analysis of pharmaceutical formulations containing the above drugs with no interference from other dosage form additives. The validity of the suggested procedures was further assessed by applying the standard addition technique which was found to be satisfactory, and the percentage recoveries obtained were in accordance with those given by the EVA Pharma reference spectrophotometric method.

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Nifuroxazide (I) and drotaverine hydrochloride (II) are formulated together in the form of Drotazide® capsule, which is used for treatment of spasmodic diarrhea. Nifuroxazide, 4-hydroxybenzoic acid [(5-nitro-2-(furanyl) methylene] hydrazide (1), is used for treatment of acute and chronic diarrhea, gastroenteritis, and colitis. Drotaverine, 1-(3,4-diethoxybezylidene)-6,7-diethoxy-1,2,3,4-tetraisohydroquinoline (2), is a hydrochloride salt used as an effective spasmolytic drug. Structures of the drugs are shown in Figure 1.

I was determined earlier by various methods, including nonaqueous titration (1), aqueous titration (3), voltammetry (4–8), spectrophotometry (8, 9), colorimetry (10), high-performance liquid chromatography (HPLC) in pharmaceutical formulations (10), and HPLC in biological fluids (11). II was also determined by several methods, including electrochemistry (12), spectrophotometry (13–15), and HPLC (16–19).

The main task of this work is to establish rapid, simple, and accurate methods for the analysis of I and II in their binary mixture that can be used for their routine and quality control analysis in raw materials and pharmaceutical formulations.

Experimental

Apparatus

(**a**) *Spectrophotometer.*—UV-Vis 1601 PC with 1 cm quartz cuvets (Shimadzu Corp., Japan). The following parameters were used: scan speed, fast; $\Delta\lambda = 4$ [for second derivative (D2) method]; scaling factor = 100 (for D2 method).

(**b**) *UV lamp.*—254 nm.

(**c**) *Thin-layer chomatography (TLC) plates.*—20 × 20 cm, coated with silica gel 60 (Merck KGaA, Darmstadt, Germany).

(**d**) *Densitometer.*—TLC Scanner 3 (Camag, Muttenz, Switzerland).

(**e**) *Sample applicator for TLC.*—Linomat IV with 100 μL syringe (Camag).

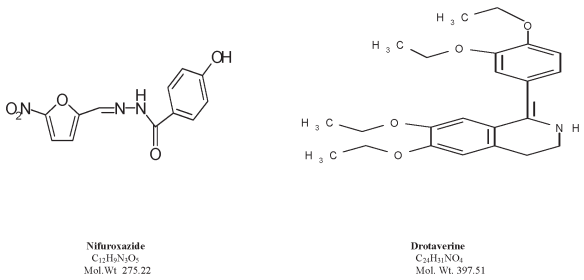

Figure 1. Chemical structure, molecular weight, and molecular formula of nifuroxazide and drotaverine.

(**f**) *Liquid chromatograph*.—Shimadzu LC10AD supplied with a Shimadzu SPD-10 UV-Vis detector and Phenomenex C18 (25 cm × 4.6 mm id, 5 μm particle size) column.

Materials

(**a**) *Authentic samples*.—I (Batch No. 20040501) and II (Batch No. 77673) were kindly supplied by EVA Pharma for Pharmaceuticals and Medical Appliances (Giza, Egypt). Their purity was 99.53 and 99.2%, respectively, according to the company analysis certificate. Pentoxifylline (Batch No. 0208000237) was kindly supplied by ALKAN Pharma (6th October City, Egypt).

Market Samples

Drotazide capsules.—Batch No. 405294; each capsule was labeled to contain 200 mg I and 40 mg II (EVA Pharma for Pharmaceuticals and Medical Appliances).

Chemicals and Reagents

(**a**) *Acetonitrile*.—HPLC grade (LAB-SCAN, Dublin, Ireland).

(**b**) *Deionized water*.—SEDICO Pharmaceutical Co. (6th October City, Egypt).

(**c**) *Phosphoric acid, chloroform, acetone, methanol, absolute ethanol, and glacial acetic acid*.—EL-NASR Pharmaceutical Chemicals Co. (Abu-Zabaal, Cairo, Egypt).

Note: All chemicals were analytical grade. Solvents used for the spectrophotometric method were spectroscopic grade, and those for the HPLC method were HPLC grade.

HPLC Conditions

The mobile phase was prepared by mixing acetonitrile and deionized water in a ratio of 40 + 60, and then the pH was adjusted to 2.55 with orthophosphoric acid. The mobile phase was filtered using 0.45 μm membrane filters, and degassed by ultrasonication for 20 min. The flow rate was 1 mL/min, and the injection volume was 20 μL. All determinations were performed at ambient temperature (20–25°C).

Preparation of Standards

(**a**) *Spectrophotometric and spectrodensitometric methods*.—For both I and II: Weigh accurately 50 mg pure powder, transfer into a 100 mL volumetric flask (protected from light due to photosensitivity; 20), add 75 mL absolute ethanol, shake well, and dilute to volume with absolute ethanol to prepare a 500 μg/mL stock solution. Then transfer an accurately measured 20 mL volume of the stock solution into a 100 mL volumetric flask (protected from light) and dilute to volume with absolute ethanol to prepare a 100 μg/mL working solution.

(**b**) *HPLC method*.—For I, II, and the internal standard: Proceed as above but use acetonitrile–deionized water (40 + 60, v/v) as solvent.

Spectrophotometric Method

(**a**) *Spectral characteristics of I and II*.—One mL each of I and II working solutions were separately transferred into a 10 mL volumetric flask (protected from light) and diluted to volume with absolute ethanol. The absorbance (Figure 2) and the D2 spectrum (Figure 3) were recorded for each solution using absolute ethanol as the blank.

(**b**) *Linearity and construction of calibration graphs*.—For I: Accurately measured aliquots ranging from 0.2 to 1 mL of I working solution were separately transferred into a series of 10 mL volumetric flasks (protected from light) and diluted to volume with absolute ethanol. The calibration graph was obtained by plotting the absorbance at 364.5 nm vs the corresponding concentration, and the regression equation was computed. For II: The procedure for I was followed for II, but the calibration graph was obtained by plotting the peak amplitude of the D2 spectrum at 243.6 nm vs the corresponding concentration, and the regression equation was computed.

(**c**) *Analysis of the laboratory-prepared mixtures*.—Into a series of 10 mL volumetric flasks (protected from light), transfer 0.6, 1, 0.6, 0.2, 1, 0.4, and 0.8 mL of I working solution plus 0.6, 0.6, 1, 1, 0.2, 0.8, and 0.4 mL of II working solution, respectively, and dilute to volume with absolute ethanol. The absorbance of each mixture was recorded in the zero-order spectrum (at 364.5 nm) and the peak amplitude in the D2 spectrum (at 243.6 nm); the concentrations of I and II were obtained by applying in the corresponding regression equation.

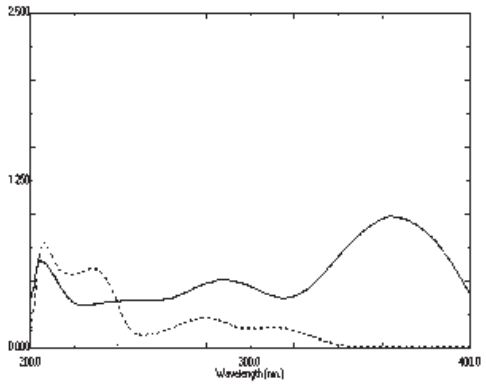

Figure 2. Zero-order spectrum of 10 μg/mL nifuroxazide (straight line) and 10 μg/mL drotaverine hydrochloride (dotted line).

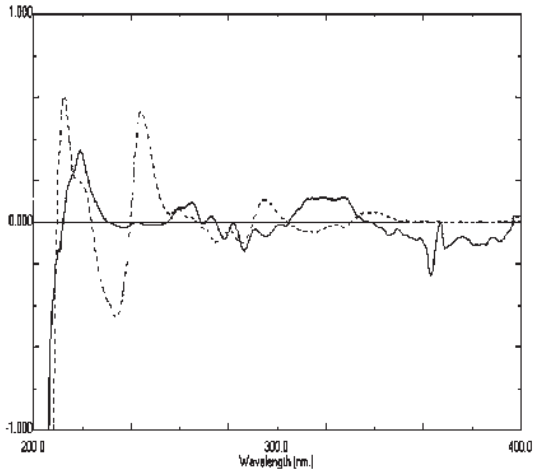

Figure 3. Second derivative spectrum of 10 μg/mL nifuroxazide (straight line) and 10 μg/mL drotaverine hydrochloride (dotted line).

(**d**) *Application to the pharmaceutical preparation (Drotazide capsule for I and II).*—The content of 10 Drotazide capsules was accurately weighed and well mixed. Then the powder equivalent to 50 mg of each of I and II was weighed, transferred into a 250 mL beaker (protected from light) separately, extracted with 75 mL absolute ethanol, filtered into a 100 mL volumetric flask, and diluted to volume with absolute ethanol. An accurately measured 20 mL volume of the stock solution was transferred into a 100 mL volumetric flask (protected from light) and diluted to volume with absolute ethanol. The procedure detailed in the *Spectrophotometric Method*, (**b**), for each of I and II was then followed.

Note: When performing the standard addition technique, the powder content of the capsule and that of the authentic drug are mixed well together before proceeding with the above-mentioned procedure.

Table 1. Determination of I and II in laboratory-prepared mixtures (A) and application of the standard addition technique for a pharmaceutical preparation (Drotazide capsule; B) by the spectrophotometric method

A: Laboratory-prepared mixtures

	Nifuroxazide (I)			Drotaverine (II)		
Mixture No.	Taken, μg/mL	Found, μg/mL[a]	Found, %	Taken, μg/mL	Found, μg/mL[a]	Found, %
1 (1:1)	6	6.057	100.95	6	5.946	99.10
2 (5:3)	10	10.245	102.45	6	5.965	99.42
3 (3:5)	6	6.201	103.35	10	10.111	101.11
4 (1:5)	2	1.982	99.10	10	9.977	99.77
5 (5:1)[b]	10	10.163	101.63	2	2.031	101.53
6 (1:2)	4	4.129	103.22	8	7.866	98.32
7 (2:1)	8	8.141	101.76	4	4.027	100.67
Mean ± SD[c]			101.78 ± 1.47			99.99 ± 1.16
Drotazide capsule	4	4.096[d]	102.40	4	4.353[d]	108.83

B: Standard addition technique

Drotazide capsule	Pure compound x added, μg/mL[e]	Pure compound x found, μg/mL[a]	Found, %	Pure compound y added, μg/mL[f]	Pure compound y found, μg/mL[a]	Found, %
	3	2.993	99.76	3	3.001	100.01
	4	3.943	98.57	4	3.939	98.01
	5	4.923	98.47	5	4.971	99.42
	6	6.031	100.51	6	6.008	100.13
Mean ± SD			99.33 ± 0.98			99.51 ± 0.76

[a] Average of 3 experiments.
[b] Ratio present in Drotazide capsules.
[c] SD = Standard deviation.
[d] Average of 6 experiments.
[e] x = Nifuroxazide (I).
[f] y = Drotaverine (II).

Table 2. Determination of I and II in laboratory-prepared mixtures (A) and application of the standard addition technique for a pharmaceutical preparation (Drotazide capsule; B) by the spectrodensitometric method

A: Laboratory-prepared mixtures

	Nifuroxazide (I)			Drotaverine (II)		
Mixture No.	Taken, μg/band	Found, μg/band[a]	Found, %	Taken, μg/band	Found, μg/band[a]	Found, %
1 (1:1)	0.3	0.299	99.95	0.3	0.303	100.97
2 (5:3)	0.5	0.496	99.16	0.3	0.303	101.16
3 (3:5)	0.3	0.301	100.37	0.5	0.508	101.53
4 (1:5)	0.2	0.205	102.25	1	1.001	100.15
5 (5:1)[b]	1	0.990	98.99	0.2	0.197	98.35
6 (1:2)	0.2	0.206	102.80	0.4	0.397	99.25
7 (2:1)	0.4	0.404	101.05	0.2	0.199	99.35
Mean ± SD[c]			100.59 ± 1.47			100.11 ± 1.17
Drotazide capsule	0.4	0.411[d]	102.78	0.4	0.444	111.07

B: Standard addition technique

Drotazide capsule	Pure compound x added, μg/band[e]	Pure compound x found, μg/band[a]	Found, %	Pure compound y added, μg/band[f]	Pure compound y found, μg/band[a]	Found, %
	0.3	0.297	98.90	0.3	0.299	99.64
	0.4	0.407	101.83	0.4	0.392	98.09
	0.5	0.507	101.43	0.5	0.501	100.22
	0.6	0.590	98.28	0.6	0.596	99.28
Mean ± SD			100.11 ± 1.78			100.3 ± 0.90

[a] Average of 3 experiments.
[b] Ratio present in Drotazide capsules.
[c] SD = Standard deviation.
[d] Average of 6 experiments.
[e] x = Nifuroxazide (I).
[f] y = Drotaverine (II).

Spectrodensitometric Method

(**a**) *Linearity and construction of calibration graphs for I and II*.— 2, 4, 6, 8, and 10 μL of I working solution were applied to a TLC plate as bands using the Camag TLC applicator. The plate was developed in a chromatographic tank previously saturated with the mobile phase, chloroform–acetone–methanol–glacial acetic acid (6 + 3 + 0.9 + 0.1, v/v), by ascending chromatography at room temperature in the dark. The I and II bands were scanned at 365 nm. The peak areas were recorded, the calibration curves were constructed by plotting the peak area vs the corresponding concentration, and the regression equation for each was computed.

(**b**) *Assay of laboratory-prepared mixtures*.—Into light-protected test tubes, mix 1, 5, 3, 1, 5, 1, and 2 mL of I working solution with 1, 3, 5, 5, 1, 2, and 1 mL of II working solution, respectively. Apply 6, 8, 8, 12, 12, 6, and 6 μL of these prepared mixtures to the silica gel plate and proceed as mentioned under *Spectrodensitometric Method*, (**a**). The concentration of each drug was calculated from the corresponding regression equation.

(**c**) *Application to pharmaceutical preparation (Drotazide capsule) for I and II*.—The procedure of *Spectrophotometric Method*, (**d**), was followed up to "...mixed well together before proceeding with the above-mentioned procedure." Then proceed as described for *Spectrodensitometric Method*, (**a**).

Reversed-Phase High-Perfromance Liquid Chromatographic (RP-HPLC) Method

(**a**) *Linearity and construction of calibration curves for I and II*.—Accurate aliquots equivalent to 0.2–1 mL of each of I and II working solutions were transferred into a series of 10 mL volumetric flasks (protected from light) separately, and each was mixed with 0.5 mL of the working solution of pentoxifylline. The dilution was completed with acetonitrile–deionized water (40 + 60, v/v). Triplicate 20 μL injections were made for each concentration. Chromatograms were recorded with a flow rate of 1 mL/min and UV detection of the effluent at 285 nm. The peak areas for I, II, and

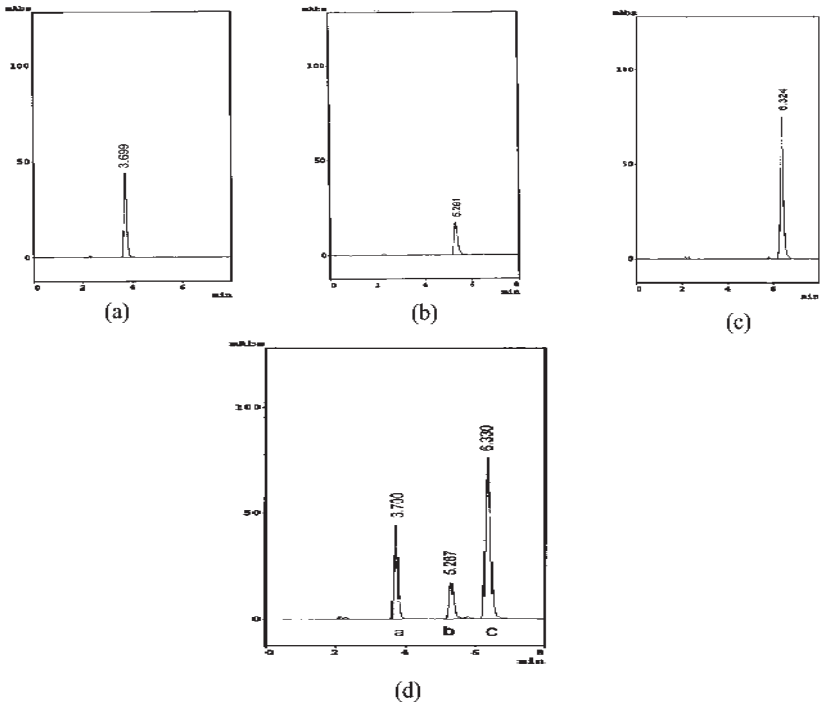

Figure 4. HPLC chromatograms of (a) pentixifylline, 10 μg/mL; (b) II, 10 μg/mL; (c) I, 10 μg/mL; and (d) mixture of a, b, and c.

pentoxifylline were recorded, and the peak area ratio was calculated for each solution. The calibration graph was obtained by plotting the peak area ratio vs the corresponding concentration, and the regression equation was computed.

(**b**) *Analysis of laboratory-prepared mixtures*.—Into a series of 10 mL volumetric flasks (protected from light), transfer 1, 1, 0.6, 0.2, 1, 0.4, and 0.8 mL of I working solution, plus 1, 0.6, 1, 1, 0.2, 0.8, and 0.4 mL of II working solution respectively. Add to each of these mixtures 0.5 mL pentoxifylline working solution and dilute to volume with acetonitrile–deionized water (40 + 60, v/v). Proceed as under *RP-HPLC Method*, (**a**), from "Triplicate 20 μL injections were made for each ..." The concentration of I and II in each mixture was calculated from the corresponding regression equation.

(**c**) *Application to pharmaceutical preparation (Drotazide capsule) for I and II.*—The procedure of *Spectrophotometric Method*, (**d**), was followed up to "...mixed well together before proceeding with the above-mentioned procedure." Then proceed as described for *RP-HPLC Method*, (**a**).

Results and Discussion

This work deals with the determination of I and II mixture in Drotazide capsules by 3 simple, applicable methods.

Spectrophotometric Method

A rapid, simple, and low-cost spectrophotometric method based upon measuring the zero-order spectrum to determine I at 364.5 nm was developed with good selectivity, sensitivity, accuracy, and without interference of II, as shown in Figure 2. A linear correlation was obtained between the absorbance and the corresponding concentration in the range of 2–10 μg/mL, from which the linear regression equation was computed as:

$$Y = 0.0979X + 0.001,\ r = 0.9999$$

where Y is the absorbance at 364.5 nm, X is the concentration in μg/mL, and r is the correlation coefficient. The zero-order spectrum and the first derivative spectrum (D1) of II showed overlap with that of I at its maximum absorbance wavelength, while the D2 spectrum was used to determine II at 243.6 nm, as shown in Figure 3. A linear correlation was obtained between the peak amplitude at 243.6 nm and the corresponding concentration in the range of 2–10 μg/mL, from which the linear regression equation was computed as:

$$Y = 0.0521X + 0.0032,\ r = 0.9998$$

where Y is the peak amplitude of the second spectrum at 243.6 nm, X is the concentration in μg/mL, and r is the correlation coefficient.

To optimize the D2 method for the determination of II in the presence of I, the influence of different variables was studied. Different smoothing factor ($\Delta\lambda$) values given by the program (2, 4, and 8) were tried; smoothing factor = 4 showed a suitable signal-to-noise ratio, and the spectra showed good resolution. Different scaling factor values (10, 100, and 1000) were tried; scaling factor = 100 was suitable to enlarge the signal of II to facilitate its measurement and to diminish the error in reading the signal.

Table 3. Determination of I and II in laboratory-prepared mixtures (A) and application of the standard addition technique for a pharmaceutical preparation (Drotazide capsule; B) by the RP-HPLC method

A: Laboratory-prepared mixtures

	Nifuroxazide (I)			Drotaverine (II)		
Mixture No.	Taken, μg/mL	Found, μg/mL[a]	Found, %	Taken, μg/mL	Found, μg/mL[a]	Found, %
1 (1:1)	10	9.895	98.95	10	10.072	100.72
2 (5:3)	10	10.076	100.76	6	6.037	100.62
3 (3:5)	6	5.950	99.17	10	10.034	100.34
4 (1:5)	2	2.064	103.21	10	10.298	102.98
5 (5:1)[b]	10	10.168	101.68	2	1.972	98.61
6 (1:2)	4	4.017	100.43	8	8.125	101.56
7 (2:1)	8	8.036	100.45	4	4.088	102.20
Mean ± SD[c]			100.66 ± 1.46			101.00 ± 1.42
Drotazide capsule	4	4.069[d]	101.72	4	4.321[d]	108.02

B: Standard addition technique

Drotazide capsule	Pure compound x added, μg/mL[e]	Pure compound x found, μg/mL[a]	Found, %	Pure compound y added, μg/mL[f]	Pure compound y found, μg/mL[a]	Found, %
	3	2.977	99.23	3	3.033	101.05
	4	4.066	101.64	4	3.932	98.29
	5	5.003	100.06	5	5.128	102.56
	6	6.076	101.26	6	6.144	102.39
Mean ± SD			100.55 ± 1.11			101.08 ± 1.98

[a] Average of 3 experiments.
[b] Ratio present in Drotazide capsules.
[c] SD = Standard deviation.
[d] Average of 6 experiments.
[e] x = Nifuroxazide (I).
[f] y = Drotaverine (II).

The proposed method is valid for the simultaneous determination of I and II in different laboratory-prepared mixtures, with mean percentage recoveries of 101.78 ± 1.47 for I and 99.99 ± 1.16 for II (Table 1). It has been applied for the determination of the 2 drugs in Drotazide capsules, and the validity was further assessed by applying the standard addition technique (Table 1). The results obtained indicate that additives present in the capsule did not interfere with the studied mixtures.

Spectrodensitometric Method

This is a simple and sensitive method that is based on the difference in the R_f value of I (0.79) and II (0.46).

Study of the optimum parameters to achieve maximum separation was performed by trying different mobile phases, but complete separation of the 2 drugs was achieved by using chloroform–acetone–methanol–glacial acetic acid (6 + 3 + 0.9 + 0.1, v/v).

The separated bands of I and II can be scanned on the same plate at 365 nm. A linear correlation was obtained between the peak area and the corresponding concentration in the range of 0.2–1 μg/band for both. The regression equations were calculated as:

$$Y = 1.9872X + 0.4159, r = 0.9999 \quad \text{I}$$

$$Y = 1.0064X + 0.2234, r = 0.9999 \quad \text{II}$$

where Y is the peak area (1/10 000), X is the concentration in μg/band, and r is the correlation coefficient.

Results obtained by applying the spectrodensitometric method showed that the concentrations of I and II can be simultaneously determined in prepared mixtures with mean recoveries of 100.59 ± 1.47 and 100.11 ± 1.17%, respectively (Table 2).

The proposed method has been applied to the assay of I and II in Drotazide capsules. The validity of the method was further assessed by applying the standard addition technique (Table 2). The results obtained indicate that additives (lactose,

Table 4. Assay parameters and method validation obtained by applying the proposed methods for determination of I and II in binary mixtures

Parameters	Compound	Spectrophotometric method	Spectrodensitometric method	RP-HPLC method
Calibration range		2–10 μg/mL	0.2–1 μg/band	2–10 μg/mL
Detection limit[a]	I	0.8 μg/mL	0.05 μg/band	0.3 μg/mL
	II	1 μg/mL	0.1 μg/band	1 μg/mL
Quantitation limit[a]	I	2 μg/mL	0.2 μg/band	2 μg/mL
	II	2 μg/mL	0.2 μg/band	2 μg/mL
Slope	I	0.0979	1.9872	0.5044
	II	0.0521	1.0064	0.1008
Intercept	I	0.0010	0.4159	0.0030
	II	0.0032	0.2234	0.0331
Mean	I	100.08	99.99	100.24
	II	99.82	100.00	100.08
Standard deviation	I	0.61	0.15	1.51
	II	1.46	0.34	0.78
Variance	I	0.37	0.02	2.28
	II	2.13	0.11	0.61
Coefficient of variation	I	0.006	0.002	0.015
	II	0.015	0.003	0.008
Correlation coefficient (r)	I	0.9999	0.9999	0.9999
	II	0.9998	0.9999	0.9999
Intraday RSD, %[b]	I	0.15–0.53	2.34–1.66	0.78–0.13
	II	1.91–0.72	0.81–1.99	0.32–0.45
Interday RSD, %[b]	I	0.59–0.77	2.12–3.04	1.36–0.75
	II	1.14–1.37	1.83–3.02	1.15–0.88

[a] Limit of detection and limit of quantitation were determined experimentally.

[b] Intraday and interday relative standard deviation (RSD) values of the concentrations of 4 and 8 μg/mL for spectrophotometric and HPLC methods, respectively, 0.4 and 0.8 μg/band for the spectrodensitometric method.

PVP, magnesium stearate, and talc) present do not interfere with analysis of the studied mixtures.

RP-HPLC Method

A simple, selective, accurate, and sensitive isocratic RP-HPLC method was adopted for the analysis of I and II in combination. To optimize the HPLC assay parameters, the effect of the acetonitrile–water composition and apparent pH of the mobile phase on the capacity factor were studied. A satisfactory separation could be obtained by using acetonitrile–water (40 + 60, v/v) adjusted to pH 2.55 with phosphoric acid (0.7 mL added to 250 mL acetonitrile–water mixture) as the mobile phase. Pentoxifylline was used as the internal standard. The flow rate was 1 mL/min, with UV detection of the effluent at 285 nm. The 3 drugs were dissolved in acetonitrile–water (40 + 60, v/v, HPLC grade) to avoid the appearance of solvent front peaks. A typical chromatogram is shown in Figure 4, where the retention time for pentoxifylline is 3.70 min, II is 5.28 min, and I is 6.33 min, with no peak interferences among the 3 drugs.

The calibration graphs for I and II were constructed by plotting the peak area ratio for I and II versus their corresponding concentrations, respectively; good linearity for both was found over the range 2–10 μg/mL. The regression equations were calculated as:

$$Y = 0.5044X + 0.0031,\ r = 0.9999 \qquad \text{I}$$

$$Y = 0.1008X + 0.0331,\ r = 0.9999 \qquad \text{II}$$

where Y is the peak area ratio, X is the concentration in μg/mL, and r is the correlation coefficient.

Results obtained by applying the RP–HPLC method showed that the concentrations of I and II can be simultaneously determined in prepared mixtures with mean percentage recoveries of 100.66 ± 1.46 and 101.00 ± 1.42, respectively (Table 3).

The method has the advantage of using an internal standard, which compensates for any error that may occur due

Table 5. Statistical analysis of parameters required for system suitability testing of the HPLC method

Parameter	Compound	Obtained value	Reference value
Resolution (R)		1.768	R > 0.8
Relative retention (α)		1.214	> 1
Tailing factor (T)	I	1	T = 1 for a typical
	II	1.08	symmetrical peak
Capacity factor (k′)	I	11.66	1–10 Acceptable
	II	9.57	
Column efficiency (N)	I	5762.50	Increases with
	II	5736.14	efficiency of the separation
HETP[a]	I	4.35×10^{-4}	The smaller the value
	II	4.33×10^{-4}	the higher the column efficiency

[a] HETP = Height of theoretical plate, cm/plate.

to baseline drift or fluctuations in the readings of the UV detector.

The proposed method has been applied to the assay of I and II in Drotazide capsules. The validity of the method was further assessed by applying the standard addition technique (Table 3). The results obtained indicate that additives present do not interfere with the studied mixtures.

Validation

(**a**) *Linearity.*—The linearity of the proposed methods was evaluated by analyzing 5 concentrations of both I and II ranging between 2–10 μg/mL (for the spectrophotometric and HPLC methods) and 0.2–1 μg/band (for the spectrodensitometric method). Each concentration was repeated 3 times. The assay was performed according to the experimental conditions previously described. The linearity of the calibration graphs and adherence of the system to Beer's law were validated by the high value of the correlation coefficient and the intercept value (Table 4).

(**b**) *Precision*.—For evaluation of precision, repeatability of the results for a concentration of 4 and 8 μg/mL (for the spectrophotometric and HPLC methods) and 0.4 and 0.8 μg/band (for the spectrodensitometric method), respectively, was evaluated by 3 replicate determinations to estimate intraday variation and 7 replicate determinations on 4 days to estimate interday variation. The coefficient of variation (CV) values at these concentration levels were calculated (Table 4).

(**c**) *Range*.—The calibration range was established through considerations of the practical range necessary according to the concentration of I and II present in the pharmaceutical product to give accurate, precise, and linear results (Table 4).

(**d**) *Detection and quantitation limits*.—Both were determined experimentally. The detection limit is defined as the concentration of the analyte producing a peak area signal

Table 6. Statistical comparison of the results obtained by the 3 proposed methods and the reference method for Drotazide capsules (Batch No. 405294)

	Proposed methods							
	Spectrophotometric		Spectrodensitometric		RP-HPLC		Reference method[a]	
Parameters	I	II	I	II	I	II	I	II
Mean, %	102.40	108.83	102.78	111.07	101.72	108.02	101.31	108.19
SD	2.19	1.66	3.02	3.30	1.15	0.95	2.01	1.74
N	6	6	6	6	6	6	6	6
Variance	4.78	2.76	9.15	10.86	1.33	0.91	4.05	3.02
Student's *t*-test	0.90	0.65	0.99	1.89	0.43	0.21	(2.23)[b]	(2.23)[b]
F-test	1.18	1.09	2.26	3.60	3.05	3.32	(5.05)[b]	(5.05)[b]

[a] Reference method is the method adopted by EVA Pharma for Pharmaceuticals and Medical Appliances for the analysis of nifuroxazide and drotaverine hydrochloride in combination (spectrophotometry).

[b] Values in parentheses represent the corresponding tabulated values of *t* and *F* at *P* = 0.05.

Table 7. Statistical analysis[a] of the results obtained by applying the 3 proposed methods and the reference method for Drotazide capsules (Batch No. 405294) using 1-way ANOVA (*F*-test)

	Mean ± RSD, %[b]	
Method	Compound I	Compound II
Spectrophotometry	102.40 ± 2.13	108.83 ± 1.52
Spectrodensitometry	102.78 ± 2.94	111.07 ± 2.97
RP-HPLC	101.72 ± 1.13	108.02 ± 0.88
Reference method	101.31 ± 1.98	108.19 ± 1.61
F value[c]	0.54	2.70
P value[c]	0.66	0.07

[a] $n = 6$.

[b] RSD = Relative standard deviation.

[c] There was no significant difference between the methods using 1-way ANOVA (*F*-test), where *F* tabulated = 3.098 for both I and II, followed by the Dunnett test (*t*-test) at $P < 0.05$; the Dunnett test treats one group as the control (reference method) and compares all other groups against it.

that is at least twice that of the baseline noise measured from peak to peak. The quantitation limit is defined as the concentration of the analyte producing a signal that is at least 5 times the baseline noise (Table 4).

(**e**) *Selectivity*.—Selectivity of the methods was measured by analysis of different laboratory-prepared mixtures of both drugs within the linearity range. Satisfactory results are shown in Tables 1, 3, and 5.

(**f**) *Accuracy*.—Accuracy of the methods was assured by use of the standard addition technique, involving analysis of synthetic formulation samples (Drotazide capsule) containing 200 mg of I and 40 mg of II mixed with 110 mg excipients (lactose, PVP, magnesium stearate, and talc) to which certain amounts of authentic drugs were added. The resulting mixtures were assayed, and the results obtained for both drugs were compared to those expected. The good recoveries with the standard addition method (Tables 1–3) prove the good accuracy of the proposed methods.

(**g**) *Robustness*.—Variation of the pH of the HPLC mobile phase by ±0.1 unit and its organic strength by ±1.5% did not have a significant effect on the chromatographic resolution.

(**h**) *Stability*.—Both I and II showed no spectrophotometric or chromatographic changes when kept away from light for 2 days at room temperature (stability indicating method).

It is known that when I solutions are exposed to light, they exhibit spectral changes (9) due to the formation of a photoisomer (20).

(**i**) *System suitability testing for HPLC*.—*See* Table 5.

Conclusions

From the results obtained by applying the suggested procedures, it is obvious that they are applicable for the determination of I and II in binary mixtures without interference and with good sensitivity. They are superior to the French Pharmacopoeial method (1), which is a macroanalytical nonaqueous titration for determination of I. The 3 new methods take into consideration the photosensitivity of I, unlike most of the previously published methods.

The characteristics of the proposed methods are summarized in Table 4. The results of the proposed methods were statistically compared with the spectrophotometric reference method (adopted by EVA Pharma for Pharmaceuticals and Medical Appliances). The *t* and *F* values were computed by a Microsoft Excel program and found to be less than the tabulated values, indicating no significant differences with respect to accuracy and precision (Table 6).

Furthermore, statistical analysis of the results obtained by the proposed methods and the reference method were performed with the Microsoft Excel program using 1-way analysis of variance (ANOVA; *F*-test) followed by Dunnett's test (*t*-test) at $P < 0.05$. The test ascertained that the proposed methods are as precise and accurate as the reference method (Table 7).

The results obtained indicate that the proposed methods can be classified as being rapid, simple, and sensitive. These merits suggest the use of the proposed methods in routine and quality control analysis without interference from commonly encountered excipients and additives.

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