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Original article

Development and validation of stability indicating HPLC and HPTLC methods for determination of sulpiride and mebeverine hydrochloride in combination

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ABSTRACT

Validated sensitive and highly selective stability indicating methods are adopted for simultaneous quantitative determination of sulpiride and mebeverine hydrochloride in presence of their reported impurities and hydrolytic degradates whether in pure forms or in pharmaceutical formulation.

The first method is High Performance Liquid Chromatography, where the mixture of sulpiride and mebeverine hydrochloride together with the reported interferents plus metopimazine as internal standard are separated on a reversed phase cyano column (5 μm ps, 250 mm × 4.6 id) using acetonitrile: water (70:30 v/v) adjusted to pH = 7 as a mobile phase. The drugs were detected at 221 nm over a concentration range of 5–40 μg ml^{-1} and 5–60 μg ml^{-1} with mean percentage recoveries 99.75% (S.D. 0.910) and 99.99% (S.D. 0.450) for sulpiride and mebeverine hydrochloride respectively.

The second method is High Performance Thin Layer Chromatography, where sulpiride and mebeverine hydrochloride are separated on silica gel HPTLC F_{254} plates using absolute ethanol:methylene chloride: triethyl amine (7:3:0.2 by volume) as mobile phase and scanning of the separated bands at 221 nm over a concentration range of 0.4–1.4 and 0.2–1.6 μg $band^{-1}$ with mean percentage recoveries 101.01% (S.D. 1.991) and 100.40% (S.D. 1.868) for sulpiride and mebeverine hydrochloride respectively.

1. Introduction

Sulpiride (SUL); N-(1-ethylpyrrolidin-2-ylmethyl)-2-methoxy-5-sulphamoylbenzamide, is a substituted benzamide used in the management of schizophrenia with antipsychotic, antidepressant and antiemetic activity [1]. Mebeverine hydrochloride (MEB); 3,4-dimethoxybenzoic acid 4-[ethyl [2-(4-methoxyphenyl)-1-methyl-ethyl] amino] butylester, is reported as an active antispasmodic drug [1]. The combination of the two drugs is used to treat gastrointestinal and colic spasms as a consequence of psychosomatic manifestation of nervous tension, mental stress or anxiety. The chemical structures, molecular weights and molecular formulae are shown in Fig. 1. SUL and MEB are determined by pharmacopoeial and non pharmacopoeial methods. Both SUL and MEB are assayed in the British pharmacopoeia via non-aqueous titration [2]. The non pharmacopoeial methods used for determination of SUL include spectrophotometry [3,4], electrochemistry [5–12], fluorimetry [13–16], TLC-densitometry [17,18], HPLC [19–35], LC-MS [36–41], radio-immuno assay [42,43], ion exchange chromatography [44] and capillary electrochromatography [45] while those mentioned for MEB include spectrophotometry [46–52], electrochemistry [53–55], HPTLC [56,57], HPLC [58–64] and LC-MS [65–68].

SUL and MEB were analyzed in their binary mixture via derivative spectrophotometry [69–71], TLC-densitometry [71], HPLC [71] and chemometric techniques (CLS) [72]. MEB is very liable to hydrolytic degradation [64] where it is degraded in the alkaline and acidic solutions into veratic acid and 4-(ethyl [2-(4-methoxyphenyl)-1-methylethyl] amino) butan-1-ol (mebOH) Fig. 2, while SUL has been analyzed before in presence of its major impurities [17]; 2-aminomethyl-1-ethylpyrrolidine (sulam) and 2-methoxy-5-sulfamoyl benzoic acid methyl ester (sules), Fig. 2. However, no analytical method has been published for the simultaneous analysis of this combination in presence of the reported degradation products and the impurities, whether in pure forms or in the pharmaceutical preparation, which became the aim of this work.

2. Results and discussion

2.1. HPLC results

A sensitive, accurate and highly selective isocratic HPLC method is implemented in our work for analysis of SUL and MEB in

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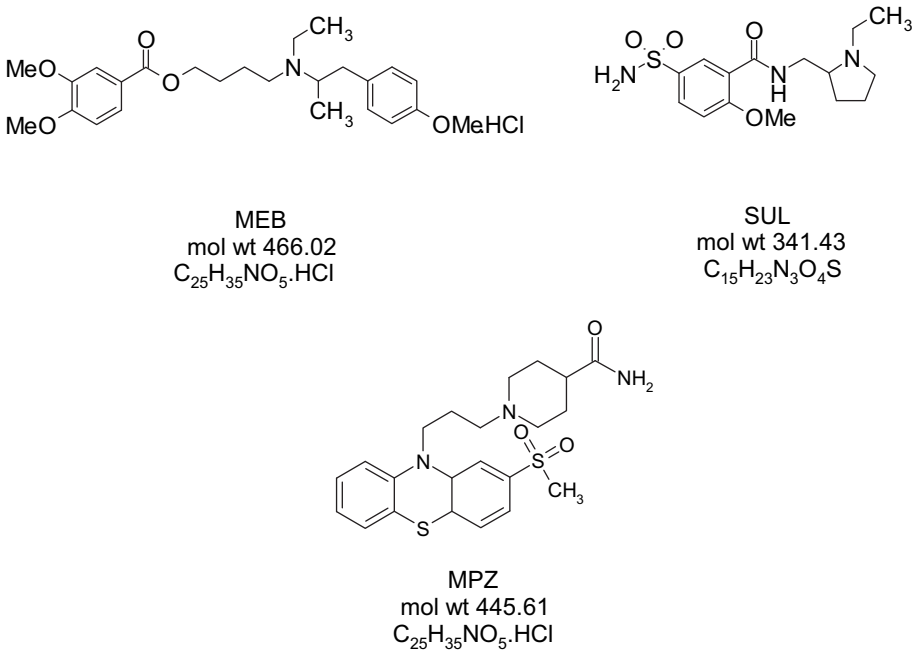

Fig. 1. Chemical structure, molecular weight and molecular formula of SUL, MEB and MPZ (internal standard).

combination and in presence of the reported impurities and degradation products, using acetonitrile: water (70:30 v/v) adjusted to pH = 7 as a mobile phase, with retention times of 1.52, 1.86, 4.41, 5.23, 5.74 and 6.62 min for veratic acid, sules, MPZ, SUL, mebOH and MEB respectively, Fig. 3. The retention times for SUL, MEB and MPZ come between 4 and 7 min which grants speed to the routine analysis of the main drugs with accuracy and extra selectivity compared to the previously published method [71]. Sulam is a non UV absorbing compound with no active chromophores (Fig. 2), hence couldn't be cited to the chromatogram, and is not expected to interfere with the peak area of any of the drugs of interest. Veratic acid and sules peaks elute very close to the solvent front and injection peak and no guarantee they could be quantifiable under the presented conditions. The method has the advantage of using an internal standard which compensates for any error that may occur due to baseline drift or fluctuations in the readings of the UV detector.

The calibration graphs for MEB and SUL were constructed by plotting the peak area ratio (drug peak area/internal standard peak area) for both MEB and SUL versus their corresponding concentrations respectively. Recording of peak area ratio of drugs of interest to the internal standard peak area compensates for errors that may occur due to baseline drift or fluctuations in the UV detector's readings. The regression equations were calculated as:

$$Y = 0.1596X + 0.2875,\ r = 0.9999 \text{ for SUL},$$

$$Y = 0.1439X + 0.4001,\ r = 0.9999 \text{ for MEB},$$

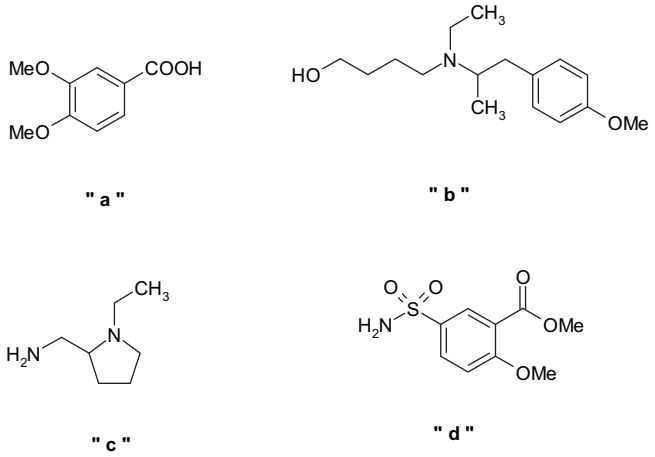

Fig. 2. The chemical structures of MEB degradation products [a – veratic acid (mol wt = 182), b – 4-(ethyl [2-(4-methoxyphenyl)-1-methylethyl] amino) butan-1-ol (mol wt = 265)] and the major impurities associated with SUL [c – 2-aminomethyl-1-ethyl pyrrloidine (mol wt = 128), d – 2-methoxy-5-sulfamoyl benzoic acid methyl ester (mol wt = 245)].

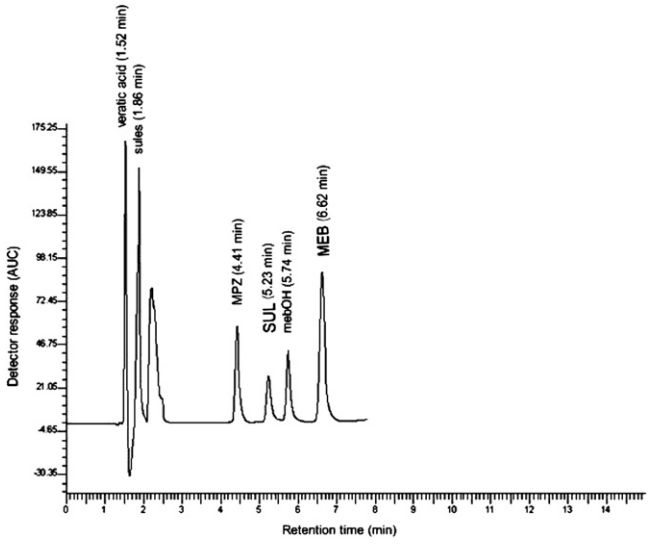

Fig. 3. HPLC chromatogram of 10 μg ml^{-1} of veratic acid, sules, MPZ, SUL, mebOH and MEB (1.52, 1.86, 4.41, 5.23, 5.74 and 6.62 min, respectively) Note: sulam shows no active chromphores, hence not detected at 221 nm.

where Y is the peak area ratio, X is the concentration in μg ml^{-1} and r is the correlation coefficient, Table 1.

2.2. HPTLC results

This method offers high sensitivity and selectivity for analysis of SUL and MEB in presence of the reported interferants using absolute ethanol:methylene chloride:triethyl amine (7:3:0.2 by volume) as mobile phase, where the good separation is shown by the difference in the retention factor (R_f) values of sulam (R_f = 0.057 ± 0.01), veratic acid (R_f = 0.316 ± 0.01), SUL (R_f = 0.423 ± 0.01), mebOH (R_f = 0.511 ± 0.01), MEB (R_f = 0.617 ± 0.01) and sules (R_f = 0.713 ± 0.01); Fig. 4.

Sulam (R_f = 0.057 ± 0.01) is a non UV absorbing substance, but when the plate is sprayed with ninhydrin solution (100 mg ninhydrin dissolved in 5 ml ethanol and 1 ml acetic acid) and heated at 80 °C for 10 min, a violet spot appear which can be scanned at 500 nm [17].

In the presented work, samples are applied as bands using TLC Linomat IV sampler with 100 μl syringe (Camag), where bands have several advantages over spots as proved in the literature [73].

A linear correlation was obtained between the peak area/10,000 (Y) and the corresponding concentration (X in μg band^{-1}) Table 1, where the regression equations were calculated as:

$$Y = 0.5322X + 0.1707,\ r = 0.9993 \text{ for SUL},$$

$$Y = 0.5838X + 0.1396,\ r = 0.9994 \text{ for MEB}$$

Results obtained by applying the proposed HPLC and HPTLC methods showed that the concentration of SUL and MEB can be simultaneously determined in prepared mixtures with mean percentage recoveries of 99.87% (0.867) and 99.79% (0.969) respectively, for the HPLC method, Table 2A, and 99.34% (2.296) and 100.69% (2.010) respectively for the HPTLC method; Table 3A.

The proposed methods have been applied to assay SUL and MEB in Colona® tablets (labeled to contain 25 mg SUL and 100 mg MEB per tablet). The validity of the method was further assessed by applying the standard addition technique, Tables 2B and 3B. The results obtained indicate no interference from dosage form additives present with the studied mixture.

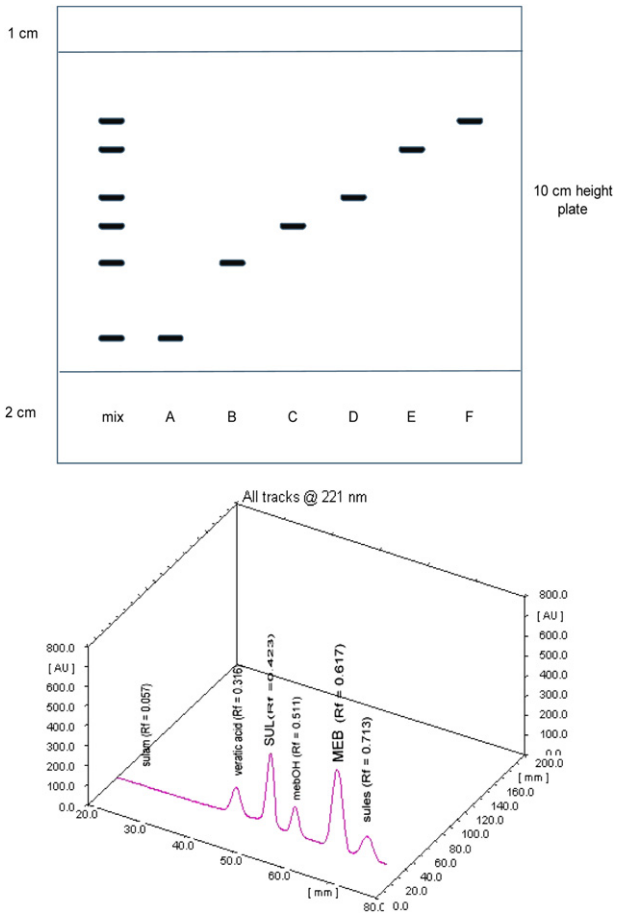

Fig. 4. Thin layer chromatogram of showing a separated mixture of A-sulam (R_f = 0.057 ± 0.01), B-veratic acid (R_f = 0.316 ± 0.01), C-SUL (R_f = 0.423 ± 0.01), D-mebOH (R_f = 0.511 ± 0.01), E-MEB (R_f = 0.617 ± 0.01) and F-sules (R_f = 0.713 ± 0.01) after scanning at 221 nm using absolute ethanol:methylene chloride:triethyl amine (7:3:0.2 v/v) as mobile phase. N.B the 3D graph is (mixture no. 3 [track 3] in the laboratory prepared mixtures' series).

Table 1
Assay parameters and method validation obtained by applying the proposed HPTLC method for the determination of SUL and MEB in binary mixture.

Parameters	Compound	HPLC method	HPTLC method
Calibration range	SUL	5–40 μg ml^{-1}	0.4–1.4 μg band^{-1}
	MEB	5–60 μg ml^{-1}	0.2–1.6 μg band^{-1}
Detection limit (LOD)[a]	SUL	0.857 μg ml^{-1}	0.02 μg band^{-1}
	MEB	1.117 μg ml^{-1}	0.04 μg band^{-1}
Quantitation limit (LOQ)[b]	SUL	2.596 μg ml^{-1}	0.3 μg band^{-1}
	MEB	3.386 μg ml^{-1}	0.2 μg band^{-1}
Slope	SUL	0.1596	0.5322
	MEB	0.1439	0.5838
Intercept	SUL	0.2875	0.1707
	MEB	0.4001	0.1396
Mean	SUL	99.74	99.89
	MEB	99.95	99.65
S.D	SUL	0.910	2.686
	MEB	0.372	2.344
Coefficient of variation	SUL	0.0091	0.027
	MEB	0.0037	0.024
Correlation coefficient(r)	SUL	0.9999	0.9993
	MEB	0.9999	0.9994
RSD%[b]	SUL	1.156–1.367	2.170–1.273
	MEB	0.854–0.770	1.816–1.030
RSD%[b]	SUL	1.771–2.212	2.424–2.101
	MEB	1.368–1.637	2.568–1.576

[a] LOD and LOQ were determined via calculations for HPLC method and experimentally for HPTLC method [75].
[b] RSD%[a], RSD%[b]: the intra-day, inter-day relative standard deviation for the precision testing samples.

2.3. Optimization of the analytical methods

For the HPLC method, different mobile and stationary phases were tried, where the best separation was achieved by the cyano column after several trials to adjust the organic strength and the pH of the mobile phase where the optimum mobile phase was acetonitrile: water (70:30 v/v). The cyano column used in the literature before [71] was 15 cm length with 10 μm particle size and 3.9 mm internal diameter, while the one used in our study is longer (25 cm) with smaller particle size (5 μm) and wider internal diameter (4.6 mm); to offer a better opportunity for the mixture to separate. Triethyl amine was added which prevented tailing of SUL peak and then the pH was adjusted to 7 by adding 40% phosphoric acid which in turn prevented the tailing of MEB that is present as hydrochloride salt and helped as well to offer an optimum pH for separation. Several flow rates were tried and the best was found 1.4 ml min^{-1}. Deciding a suitable internal standard based on similarity in chemical structure to the two main drugs was not feasible due to diversity in their structures and hence several random drug internal standards were tried amongst which MPZ (Fig. 1) proved to be optimum being well resolved from other peaks and at a suitable

Table 2
Determination of SUL and MEB in laboratory prepared mixtures (A) and application of the standard addition technique for the pharmaceutical preparation (Colona® tablets) (B) by the HPLC method.

A: Laboratory prepared mixtures							
Mixture No.	Ratio by volume [SUL:MEB:sulam:sules:mebOH:veratic acid]	SUL			MEB		
		Taken ($\mu g\ ml^{-1}$)	Found[a] ($\mu g\ ml^{-1}$)	Recovery %	Taken ($\mu g\ ml^{-1}$)	Found[a] ($\mu g\ ml^{-1}$)	Recovery%
1	0.5:2:0.2:0.4:1.3:0.8[b]	5	4.94	98.80	20	19.80	99.00
2	2:0.5:0.8:1.5:0.4:0.2	20	20.03	100.15	5	4.99	99.80
3	1:2:0.4:0.8:1.3:0.8	10	9.90	99.00	20	19.76	98.80
4	2:1:0.8:1.5:0.7:0.4	20	20.14	100.70	10	10.16	101.60
5	1:3:0.4:0.8:2:1.2	10	9.91	99.10	30	30.03	100.30
6	3:1:1.2:2.2:0.7:0.4	30	30.20	100.67	10	9.99	99.90
7	2:2:0.8:1.5:1.3:0.8	20	20.13	100.65	20	19.82	99.10
Mean% (S.D)				99.87 (0.867)			99.79 (0.969)

B: Standard addition technique						
	Claimed taken ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Found[c]%	Pure added ($\mu g\ ml^{-1}$)	Pure found[a] ($\mu g\ ml^{-1}$)	Recovery%
SUL	10	9.92	99.20	10	10.22	102.20
				20	20.28	101.40
				30	28.89	99.63
Mean% (S.D)						101.08 (1.315)
MEB	10	9.97	99.70	10	10.02	100.20
				20	19.91	99.55
				30	30.33	101.10
Mean% (S.D)						100.28 (0.778)

[a] Average of 3 experiments.
[b] The ratio present in Colona® tablets.
[c] Average of 6 experiments.

retention time (4.41 min) closest to the two main drugs of interest (SUL and MEB at 5.23 and 6.62 min respectively) Fig. 3.

For the HPTLC method, studying the optimum parameters for maximum separation was carried out by trying different developing systems with different ratios where complete separation of the 6 components' mixture was achieved by using absolute ethanol:methylene chloride:triethyl amine (7:3:0.2 by volume). Different scanning wavelengths were tried – depending on literature – like 365, 254, 240 and 221 nm of which 221 nm offers the highest sensitivity for both SUL and MEB in the same scan. The optimum band width chosen – taking into consideration the range of concentrations applied and number of tracks – was 6 mm and the best interspaces between bands were 5 mm. Moreover (6 mm × 0.60 mm, macro), proved to be the slit dimensions of choice which provides highest sensitivity for both drugs in the same scan.

Table 3
Determination of SUL and MEB in laboratory prepared mixtures (A) and application of the standard addition technique for the pharmaceutical preparation (Colona® tablets) (B) by the HPTLC method.

A: Laboratory prepared mixtures							
Mixture No.	Ratio by volume [SUL:MEB:sulam:sules:mebOH:veratic acid]	SUL			MEB		
		Taken ($\mu g\ band^{-1}$)	Found[a] ($\mu g\ band^{-1}$)	% Found	Taken ($\mu g\ band^{-1}$)	Found[a] ($\mu g\ band^{-1}$)	% Found
1	1:4:0.4:0.6:2.5:1.5[b]	0.4	0.399	99.75	1.6	1.960	99.38
2	4:1:1.5:2.7:0.5:0.3	1.2	1.183	98.58	0.3	0.305	101.67
3	1:2:0.4:0.8:1.3:0.8	0.6	0.603	100.50	1.2	1.202	100.17
4	2:1:0.8:1.5:0.7:0.4	1.2	1.177	98.08	0.6	0.621	103.50
5	1:3:0.4:0.7:2:1.2	0.4	0.387	96.75	1.2	1.234	102.83
6	3:1:1.2:2.2:0.7:0.4	1.2	1.245	103.75	0.4	0.396	99.00
7	1:1:0.4:0.7:0.7:0.4	0.8	0.784	98.00	0.8	0.786	98.25
Mean% (S.D)				99.34 (2.296)			100.69 (2.010)

B: Standard addition technique						
	Taken ($\mu g\ band^{-1}$)	Found ($\mu g\ band^{-1}$)	Found[c] %	Pure added ($\mu g\ band^{-1}$)	Pure found[a] ($\mu g\ band^{-1}$)	% Recovery
SUL	0.6	0.595	99.17	0.4	0.409	102.25
				0.6	0.594	99.00
				0.8	0.799	99.88
Mean % (S.D)						100.38 (1.681)
MEB	0.6	0.597	99.50	0.4	0.407	101.75
				0.6	0.602	100.33
				0.8	0.798	99.75
Mean % (S.D)						100.61 (1.029)

[a] Average of 3 experiments.
[b] The ratio present in Colona® tablets.
[c] Average of 6 experiments.

Table 4
Statistical analysis of parameters required for system suitability testing of the proposed HPLC method.

Parameter	Obtained value		Reference value
	SUL	MEB	
Resolution (Rs)	1.5886		$R > 0.8$
Relative retention (α)	1.4744		>1
Tailing factor (T)	1.0870	1.0500	$T = 1$ for a typical symmetric peak
Capacity factor (K′)	1.2739	1.8783	1–10 acceptable
Number of Theoretical plates (N)	893.1559	636.0004	Increases with efficiency of the separation
HETP (cm/plate)	0.0280	0.0393	The smaller the value, the higher the column efficiency

2.4. Validation of the analytical methods

For the HPLC method, linearity was established by analyzing five concentrations of SUL and seven concentrations of MEB ranging between 5–40 and 5–60 μg ml^{-1} respectively, by plotting the peak area ratio against the corresponding concentration, while for the HPTLC method, linearity was evaluated by analyzing five concentrations ranging between 0.4–1.4 and 0.2–1.6 μg $band^{-1}$ for SUL and MEB respectively, by plotting peak area/10,000 against the corresponding concentration. Linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value; Table 1.The calibration range for the two proposed methods was established through considerations of the practical range required and the concentrations of SUL and MEB present in the pharmaceutical product to give accurate, precise and linear results; Table 1.

To estimate precision of the methods, repeatability of the results for concentrations of 10 and 30 μg ml^{-1} for the HPLC method and 0.6, 1.2 μg $band^{-1}$ for the HPTLC method were performed by 3 replicate determinations to estimate intra-day variation and 7 replicate determinations on different 4 days to estimate inter-day variation. Then the coefficient of variation at these concentration levels was calculated; Table 1.

Detection and quantitation limits were determined via calculations for the HPLC method [74], while for the HPTLC method they were determined experimentally [75]; Table 1.

Selectivity of the methods was achieved by analysis of different laboratory prepared mixtures of SUL and MEB in combination with the reported degradation products and impurities within the calibration range of the two main drugs, where satisfactory results were shown; Tables 2A and 3A.

Accuracy of the methods was assured by the standard addition technique, involving analysis of market samples (Colona® tablets) to which certain amounts of authentic SUL and MEB were added. The resulting mixtures were assayed and the results obtained for both drugs were compared to those expected. The good recoveries of the standard addition method suggest good accuracy of the proposed method; Tables 2B and 3B.

The proposed HPLC method proved robustness when we tried to induce minor deliberate changes in the organic strength (±1.5%) and the pH (±0.1 unit) of the mobile phase where the retention time of the peaks was not significantly affected (±0.01 min). For the HPTLC method, variation of the composition of the mobile phase by changing the concentration of absolute ethanol or methylene chloride by ±2% or the temperature (25 °C ± 2) did not have a significant effect on R_f values of the bands. However the concentration of triethyl amine proved to be critical where upon decreasing its percentage, dramatic changes in R_f values and resolution occur.

The proposed HPLC method showed ruggedness when we tried to transfer our model to another lab with another HPLC instrument (Shimadzu, Japan) in another city and running the analysis via another fellow analyst as well, where the same results and retention times were obtained (±0.01 min) proving no significant lab to lab, instrument to instrument, analyst to analyst and time to time variations and hence ruggedness of the method and transferability of the method to routine industrial pharmaceutical analysis.

System suitability testing for HPLC [75] was performed and the results are shown in Table 4.

3. Conclusion

The presented HPLC and HPTLC methods could provide highly selective quantitative stability indicating methods for the simultaneous analysis of SUL and MEB in presence of the degradation products of MEB and the major impurities of SUL, which represent an advantage over the previously published methods. The internal standard HPLC method offers extra selectivity for the analysis of SUL and MEB and allowing detection at a single wavelength for both drugs; saving time, though retaining sensitivity. The HPTLC method has the advantage of using HPTLC plates with smaller particle size and higher resolution ability, besides using one single scanning wavelength for the two drugs in the same run; saving time and effort.

The proposed methods were applied for the analysis of SUL and MEB in Colona® tablets, where the results of the proposed methods were statistically compared with the reference HPLC method [71]. The *t* and *F* values were computed and found less than the tabulated ones indicating no significant difference with respect to accuracy and precision; Table 5. Furthermore, statistical analysis using one way ANOVA (*F*-test) at $p < 0.05$ was conducted. *F* values were found for MEB = 1.857, while for SUL = 1.301, which are less than the tabulated *F* value = 3.682. The test ascertains that the proposed methods are as precise and accurate as the reference method and are comparable to one another.

The results obtained indicate that the introduced methods can be classified amongst highly selective and sensitive procedures. These merits suggest the use of the proposed method in routine and quality control analysis without interference of commonly encountered dosage form additives.

4. Experimental protocols

4.1. Apparatus

- Camag TLC Scanner 3 plus Camag TLC sampler Linomat IV supplied with 100 μl syringe for HPTLC-densitometric determinations (Camag, Muttenz, Switzerland).

Table 5
Statistical comparison of the results obtained by the proposed HPLC and HPTLC method in comparison to the reference HPLC method for the analysis of Colona® tablets (Batch No. 06533 labeled to contain 25 mg SUL and 100 mg MEB per tablet).

Parameters	HPLC method		HPTLC method		Reference method[b]	
Compound	MEB	SUL	MEB	SUL	MEB	SUL
Mean (%)	99.70	99.20	99.50	99.17	99.84	100.02
S.D	0.331	0.614	0.439	1.087	0.207	0.833
n	6	6	6	6	6	6
Student's *t*-test	0.637	1.938	1.564	1.521	(2.228)[a]	(2.228)[a]
F-test	2.546	1.842	4.474	1.703	(5.050)[a]	(5.050)[a]

[a] Figures between parentheses represent the corresponding tabulated values of *t* and *F* at $p = 0.05$.
[b] Reference method is HPLC [71].

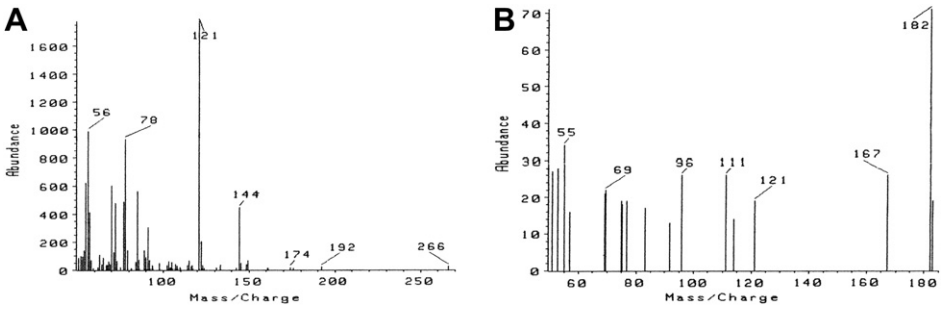

Fig. 5. The mass spectra of A – mebOH, B – veratic acid.

- Perkin Elmer series LC 200: UV/Vis pump, detector, autosampler and vacuum degasser, Perkin Elmer 600 series LINK chromatography interface and the instrument is operated by a software TotalChrom navigator-HPLC 200 (Massachusetts, USA).

4.2. Materials

4.2.1. Authentic and market samples

SUL (Batch No. 20061005), MEB (Batch No. MEB/009/02/07) and Colona® tablets (Batch No. 06533, labeled to contain 25 mg SUL and 100 mg MEB per tablet) were kindly supplied by RAMEDA (The Tenth of Ramadan) Co. for Pharmaceutical Industries and Diagnostic Reagents, 6th of October City, Egypt. SUL and MEB purity was found to be 99.90% and 99.45% respectively according to the company's analysis certificate.

4.2.2. Chemicals and reagents

- Acetonitrile – HPLC grade (Riedel-de Haen, Germany).
- Deionised water – SEDICO Pharmaceutical Co. (6th October City, Egypt).
- Absolute ethanol, phosphoric acid and methylene chloride are from (EL–NASR Pharmaceutical Chemicals Co., Cairo, Egypt).
- Triethyl amine (Riedel-de Haen, Germany).
- HPTLC plates (20 × 20 cm) coated with silica gel 60 F_{254} (1.05548.0001) (Merck KGaA, Darmstad, Germany).
- 2-methoxy-5-sulfamoyl benzoic acid methyl ester (CAS No. 33045-52-2) and 2-aminomethyl-1-ethylpyrrolidine (CAS No. 26116-12-1) were imported from Wuhan Yuancheng Technology Development Co. Ltd., China.

4.3. Preparation and separation of the hydrolytic degradation products of MEB

The literature was followed for preparation of veratic acid and mebOH (Fig. 2) [64], with some modification, where heating was carried out at 45 °C; unlike what stated in the literature (reflux for 3 h) to avoid the formation of methyl veratate and this was confirmed by TLC where two spots only of veratic acid and mebOH appeared after the complete disappearance of MEB spot.

The identity of veratic acid, mebOH and the major impurities of SUL [17]; sules and sulam were confirmed by mass spectra, Fig. 5.

4.4. Standard preparations

1000 μg ml^{-1} stock solutions of SUL, MEB, MPZ, veratic acid, mebOH, sules and sulam were prepared in absolute ethanol for the HPLC and HPTLC methods.

For the HPLC method, 100 μg/ml working solution for each was prepared in acetonitrile: water (70:30 v/v) solution, while 200 μg ml^{-1} working solution for each (excluding MPZ) was prepared in absolute ethanol for the HPTLC method.

4.5. HPLC conditions

The mobile phase was prepared by mixing acetonitrile and water in the ratio of 70:30 v/v. To each 100 ml of the mobile phase, 0.1 ml of triethylamine was added and then the pH was adjusted to 7 by adding 40% orthophosphoric acid. The analysis was conducted at 25 °C using a cyano column; Phenomenex Luna CN (5 μm ps, 250 mm × 4.6 id, Torrance, CA, USA), with a mobile phase flow rate of 1.4 ml/min and scanning the eluate at 221 nm which represents the wavelength that shows highest absorbance for both SUL and MEB and a suitable absorbance for MPZ (the internal standard). The mobile phase was filtered using 0.45 μm membrane filters. All the injections were run in three replicates and the injection volume was 20 μL.

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