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Instantaneous determination of antihypertensive drugs utilizing TLC: Densitometric methods

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Abstract

An accurate, sensitive and time saving thin layer chromatography (TLC) - densitometric method has been developed and validated for determination of two Antihypertensive drug mixtures. Mixture one contains Spironolactone, Furosemide and Anthranilic acid while, mixture two contains Triamterene, Hydrochlorothiazide and chlorothiazide. Mixture 1 uses developing system consist of Hexane, Ethyl acetate and Glacial acetic acid (5ml – 5ml – 0.1ml) as developing system and 240 nm as scanning wavelength for separation and determination of Spironolactone, Furosemide and Anthranilic acid (metabolite of Furosemide) and mixture 2 uses developing system consist of Ethyl acetate, Methanol, Glacial acetic acid and Ammonium hydroxide (8ml - 1ml - 0.2ml - 0.3 ml) as developing system and 300nm as scanning wave length for separation and determination of Triamterene, Hydrochlorothiazide and Chlorothiazide (impurity of Hydrochlorothiazide).

This method was validated and shown to demonstrate good accuracy and precision according to the International Council for Harmonisation (ICH) guidelines.

Keywords: Anthranilic acid; Chlorothiazide; Furosemide; Hydrochlorothiazide; Spironolactone; Triamterene; TLC Densitometry

1. Introduction

Triamterene (TRI, 6-phenyl-2,4,7-triaminopteridine), has molecular formula of $C_{12}H_{11}N_7$ and its molecular weight is 253.269 g/mol. It is a relatively inefficient antihypertensive when used alone; hence it is used in combination with some potent diuretic (e.g. anthranilic acid or thiazide derivative) to give a synergistic action [1].

Hydrochlorothiazide (6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide -1,1-dioxide), is a “thiazide ” class diuretic. Its molecular formula is $C_7H_8ClN_3O_4S_2$ and its molecular weight is 297.728 g/mol.It increase the excretion of sodium, chloride and water[2].

Chlorothiazide (CZ) is 6-chloro-2H-1,2,4-benzothiadiazine-7-sulphonamide-1,1-dioxide. Its molecular formula is $C_7H_6ClN_3O_4S_2$ and molecular weight is 295.712 g/mol. It is considered a specified impurity for HCZ [2].

The literature review shows that Triametrene and Hydrochlorothiazide were determined in its bulk powder by derivative ratio spectrophotometry [3], liquid chromatography (LC) [1,4] and chemometric analysis[2].

Spironolactone is 7a-acetylthio-3-oxo-17a-pregn- 4-ene-21, 17-carbolactone. Its molecular formula is $C_{24}H_{32}O_4S$ and its molecular weight is 416.58 gm/mol. Spironolactone inhibits the effect of aldosterone by competing for intracellular

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aldosterone receptors in the distal tubule cells. This increases the excretion of water and sodium, while decreasing the excretion of potassium [5].

Furosemide is 4-chloro-2-(furan-2-ylmethylamino)- 5-sulfamoylbenzoic acid. Its molecular formula is $C_{12}H_{11}ClN_2O_5S$ and its molecular weight is 330.74 gm/mol. Furosemide, an Anthranilic acid derivative, is a potent diuretic as inhibits the active reabsorption of chloride, This diuretic is commonly used for the treatment of renal diseases, congestive heart failure and hypertension[5].

The literature review shows that FUR was determined in its bulk powder by liquid chromatography (LC) [6] and spectroscopy (Schiff's bases) [6],[7] . The binary mixture of SPIR and FUR was determined by some spectrophotometric methods (variable angle scanning fluorescence spectrophotometry [8], derivative ratio [9] and determined by reversed phase liquid chromatography [5, 10] .

Anthranilic acid; 2-aminobenzoic acid is an organic compound, its molecular formula is $C_6H_4(NH_2)(CO_2H)$ and its molecular weight is 137.138 g/mol.It is a substrate of enzyme anthranilate hydroxylase in benzoate degradation via hydroxylation pathway[11].

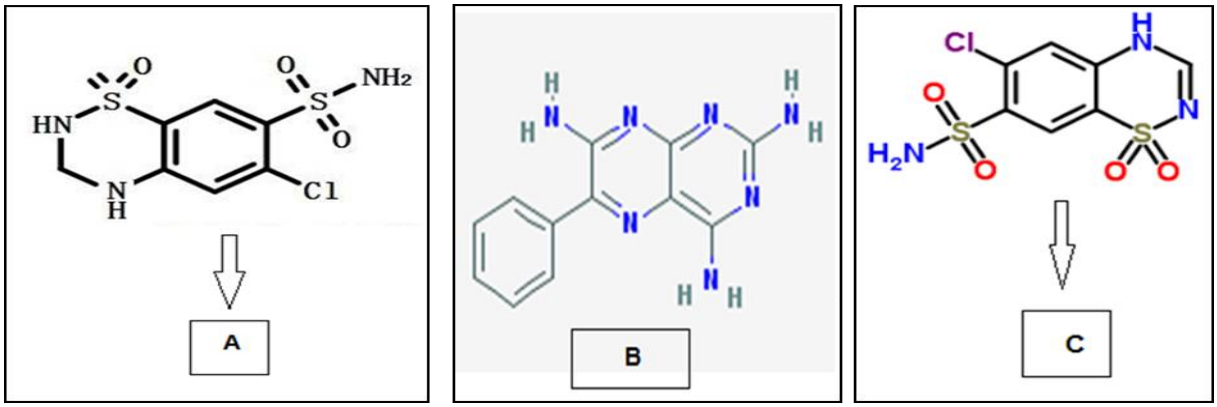

Figure 1 (A) The chemical structure of Hydrochlorothiazide, (B) the chemical structure of Triamterene and (C)the chemical structure of Chlorothiazide

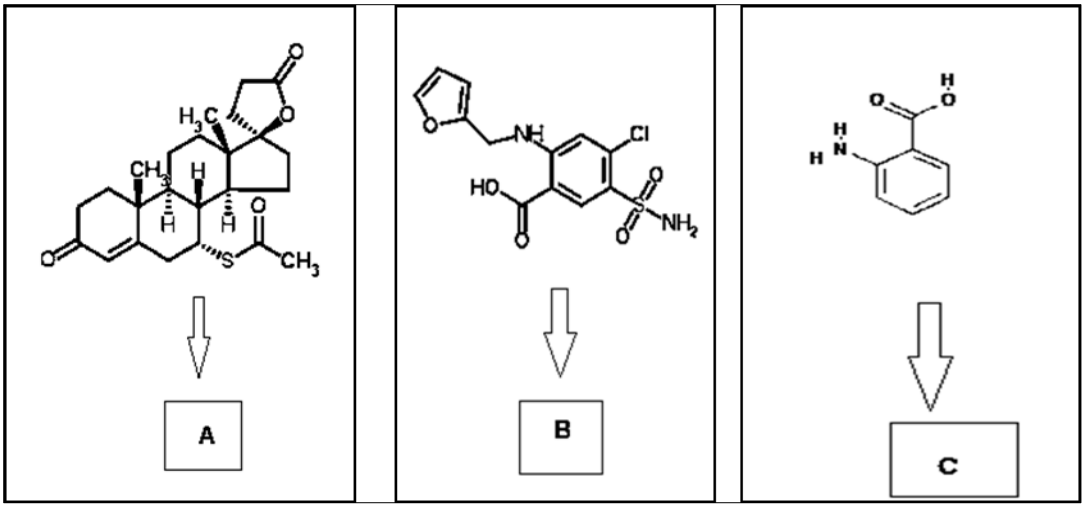

Figure 2 (A) The chemical structure of Spironolactone, [B] The chemical structure of Furosemide and (C) The chemical structure of Anthranilic acid

The aim of this work is to develop and validate a new analytical method for determination of the two tertiary mixture Triamterene, Hydrochlorothiazide and Chlorothiazide as well as Spironolactone, Furosemide and Anthranilic acid in bulk powder and pharmaceutical dosage form without interference or prior separation.

2. Materials and method

2.1. Instruments

A TLC scanner densitometer (Camag, Muttenz, Switzerland) is used with the following requirements : slit dimensions, 5x0.2 mm; scanning speed, 20 mm/s; spraying rate, 10/mL; data resolution: 100 mm/step. Pre-coated silica gel aluminum plates (20x10 cm; 60 F254) were obtained from Fluka, Sigma-Aldrich Chemie Gmbh, Germany. The sample applicator for TLC was a Linomat IV with a 100mL syringe (Camag, Muttenz, Switzerland).

2.2. Pure standards

Standard SPIR and FUR were kindly supplied by Amoun pharmaceutical company (Cairo, Egypt) with claimed purities of 99.6 % and 99.7 %, respectively, according to manufacturer certificates of analysis.

Standard TRI and HCZ with claimed purities of 99.88% and 99.98% respectively according to manufacturer certificate were kindly supplied by Egyptian International Pharmaceutical Industries Co SAE (EIPICO) (Nasr city _ Egypt).

While Anthranilic acid and Chlorothiazide (IMP) were obtained from (Sigma -Aldrich Chemie GmbH, Germany) with claimed purity of 98 % according to the manufacturer certificates of analysis.

2.3. Pharmaceutical dosage forms

Lasilactone® tablets (batch No. 7EG007) were manufactured by Amoun pharmaceutical company. Each tablet is claimed to contain 100 mg of SPIR and 20mg of FUR.

Dyazide ® tablets (batch No. 622102E) were manufactured by Amoun pharmaceutical company Co.SAE (Cairo _ Egypt). Each tablet is claimed to contain 37.5 mg of TRI and 25 mg of HCZ.

2.4. Chemicals and reagents

All reagents and chemicals used were of analytical grade and were used without further purification. They included:

- Methanol analar (Central Drug House Ltd., India)
- Hexane, ethyl acetate, ammonium hydroxide and glacial acetic acid (Al-Nasr Pharmaceutical chemicals company, Abu Zaabal cairo – Egypt).

2.5. Prepared solutions

- Stock solutions of SPIR, FUR, ANTH, TRI, HCZ and CZ were prepared in methanol. They were prepared by dissolving 0.025gm of each drug in 25ml of methanol in six separate calibrated flasks.
- Working solutions of the six drugs containing 100 µg/mL of each drug were prepared separately by diluting 2.5 mL of each stock solution to 25ml by methanol.

2.6. Methodology

2.6.1. Linearity and construction of calibration curves

Accurate aliquots were taken from working solutions of SPIR, FUR, ANTH, TRI, HCZ and CZ and transferred to six separate series of 10 mL volumetric flasks and diluted with methanol. A 10µl aliquots of each solution was spotted as band of 5mm width on TLC plates to obtain concentration ranges of (0.5-2µg), (0.7-2µg), (0.1-1µg), (0.3-3 µg), (0.2-2 µg) and (0.1-0.9 µg) respectively .

The peak areas were recorded using a scanning wavelength of 240 nm and 300 nm for the two antihypertensive mixtures respectively. Calibration curves were constructed by plotting the integrated peak area versus the concentration in µg / band for each compound and the regression equations were computed.

2.6.2. Analysis of laboratory prepared mixtures

For mixture 1: an accurate aliquots were transferred from SPIR, FUR and ANTH working solutions into a series of 10ml volumetric flasks completed to volume with methanol and mixed well to obtain tertiary mixtures of different ratios. The same previous steps were followed for determination of mixture 2 (TRI, HCZ and CZ).

2.6.3. Application to pharmaceutical formulations

Ten tablets of Lasilactone ® were weighed, powdered and mixed well; an accurate weight of the powdered tablets equivalent to 100 mg of SPIR and 20 mg of FUR was transferred into 100 mL volumetric flask. 75 mL of methanol was added and sonicated for 20 min, complete to the volume then filtered to obtain stock solution of 1 mg/mL of SPIR. From which sample working solution of 100 µg/mL was prepared by diluting.The procedure under linearity for each drug was followed and concentrations of SPIR and FUR were calculated using the previously computed regression equation.

Standard addition technique has been carried out by spiking the pharmaceutical formulation by known amount of standard drug powder. The recovery of the added standards was then calculated after applying the proposed methods.

The same previous steps were followed for determination of TRI and HCZ in Dyazide ® tablets.

3. Results and discussion

The TLC densitometric technique was successfully applied for the determination of SPIR, FUR and ANTH mixture as well as TRI, HCZ and CZ mixture in pure form, mixtures and dosage forms. This technique offers a simple way to quantify directly on TLC plate by measuring the optical density of the separated bands, in order to obtain optimum separation among the studied drugs. Different trials have been carried out to reach the optimum developing system, scanning wavelength, band dimension and slit dimension.

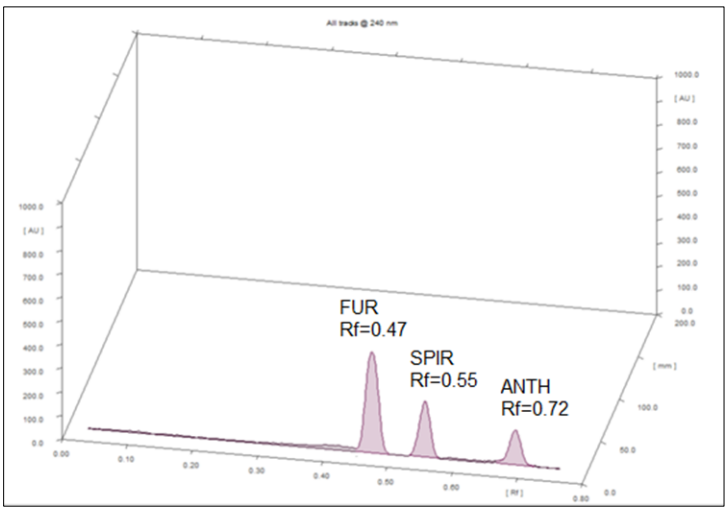

Figure 3 TLC densitogram of mixture of Spironolactone, Furosemide and Anthranilic acid using developing system of hexane: ethylacetate: acetic acid (5:5:0.1) by volume

The regression equations were computed and found to be:

- $A_{SPIR} = 0.5053C + 0.2477$ r = 0.9998
- $A_{FUR} = 0.5258C + 0.377$ r = 0.9998
- $A_{ANTH} = 0.5113C + 0.0099$ r = 0.9999

Different developing systems with different ratios have been tested for determination of mixture 1 using different solvents mixtures such as Hexane : Methanol, Chloroform: Methanol and hexane : Methanol: Acetic acid also different developing systems with different ratios have been tested for determination of mixture 2 using different solvents mixtures such as Ethyl acetate : Methanol,Chloroform : Methanol, Toluene : Methanol : Acetic acid and Ethyl acetate : Methanol: Acetic acid : Ammonia.

The best results concerning both chromatographic separation, peak symmetry and linearity were obtained upon using the system (hexane – ethyl acetate - acetic acid (5:5:0.1) by volume).

The obtained R_f values were 0.55, 0.47 and 0.72 for SPIR, FUR and ANTH, respectively.

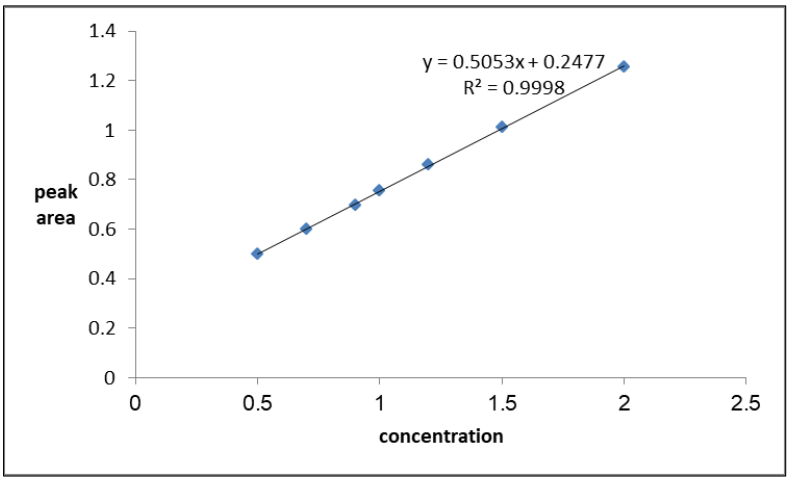

Figure 4 Calibration curve relating the peak area of TLC peaks of SPIR to its concentration in µg / band

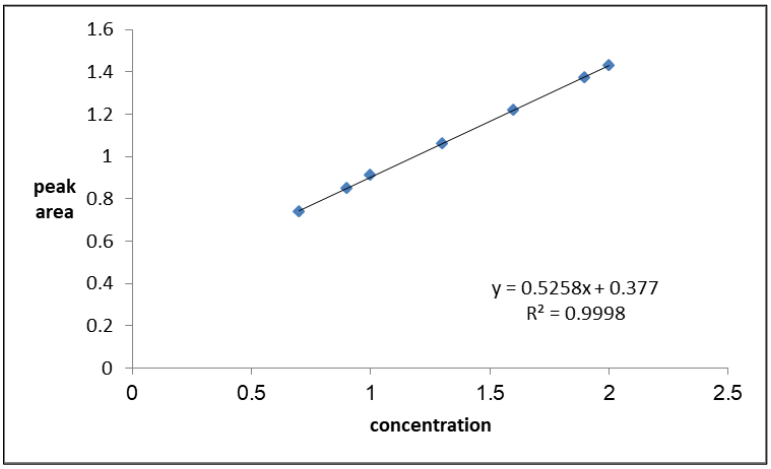

Figure 5 Calibration curve relating the peak area of TLC peaks of FUR to its concentration in µg / band

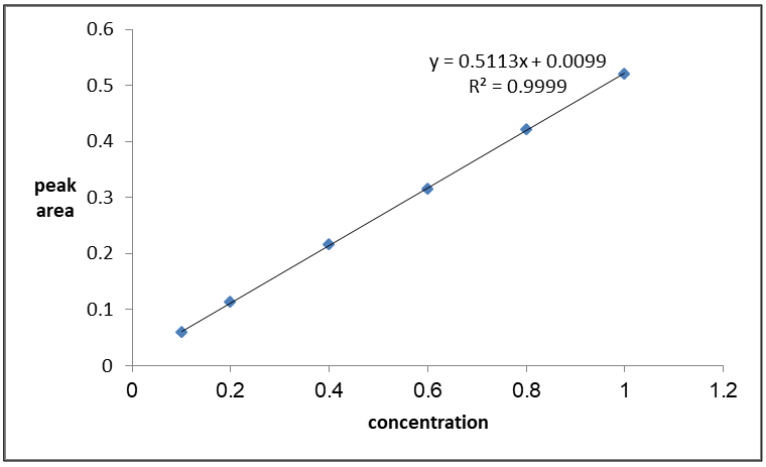

Figure 6 Calibration curve relating the peak area of TLC peaks of ANTH to its concentration in µg / band

The best results concerning both chromatographic separation and peak symmetry for mixture 2 were obtained upon using the system ethyl acetate, methanol, acetic acid and ammonia (8:1:0.2:0.3) by volume.

Which have good R_f values for these drugs where R_f values were 0.25, 0.74 and 0.62 for TRI, HCZ and CZ, respectively.

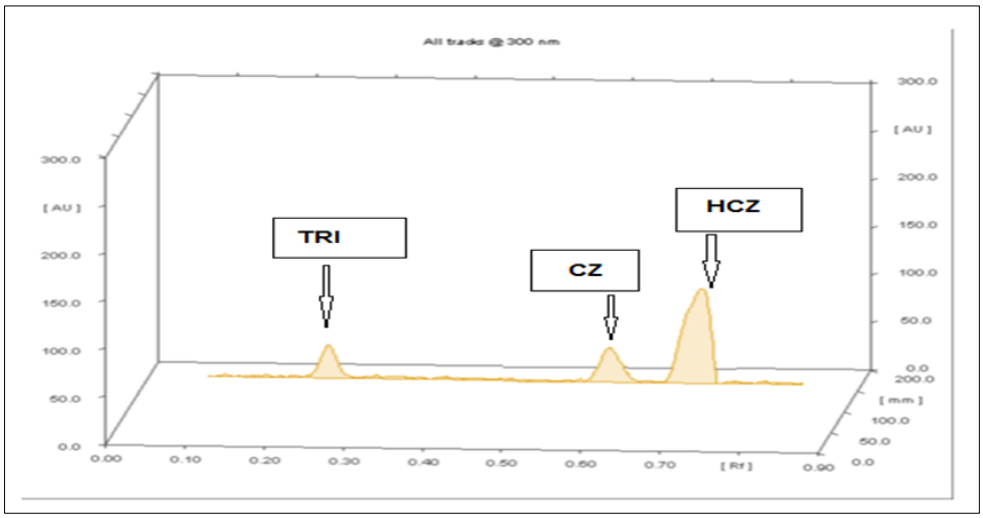

Figure 7 TLC densitogram of mixture of Triamterene, Hydrochlorothiazide and Chlorothiazide using developing system of Ethyl acetate: Methanol: Acetic acid : Ammonium hydroxide (8 : 1 : 0.2 : 0.3) by volume

Different scanning wavelength such as 210nm, 225nm, 240nm and 300 nm were tried but 240 nm for mixture 1and 300 nm for mixture 2 were the best scanning wavelength which showed high sensitivity with minimum noise.

The regression equations were computed and found to be:

A_{TRI} = 0.2685C + 0.0697 r = 0.9999
A_{HCZ} = 0.4715C + 0.054 r = 0.9999
A_{CZ} = 0.7085C + 0.0966 r = 0.9999

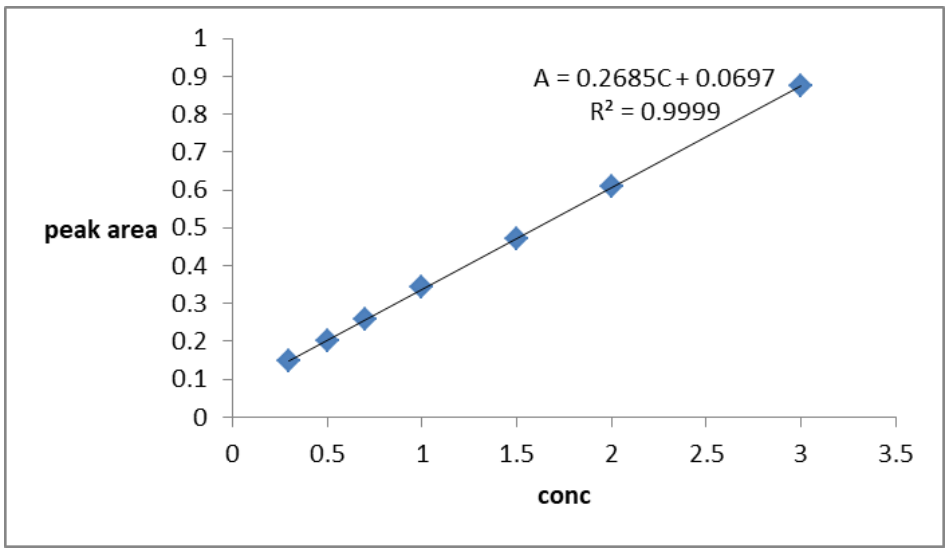

Figure 8 Calibration curve relating the peak area of TLC peaks of TRI to its concentration in µg / band

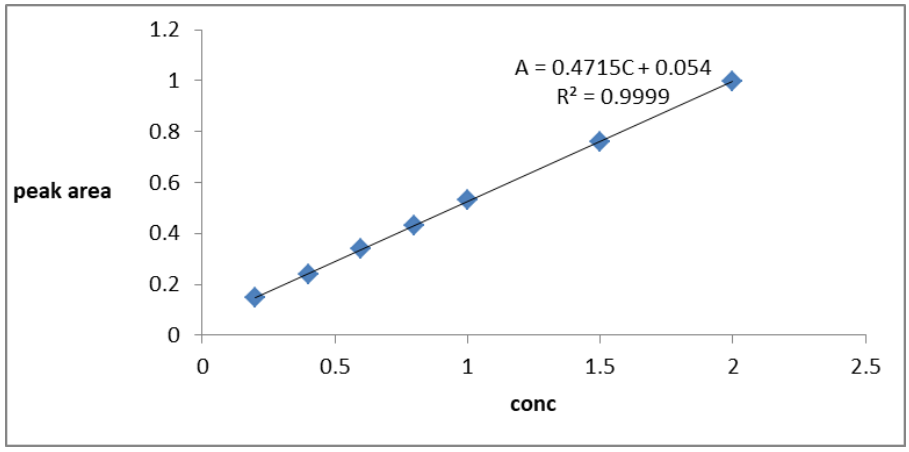

Figure 9 Calibration curve relating the peak area of TLC peaks of HCZ to its concentration in µg / band

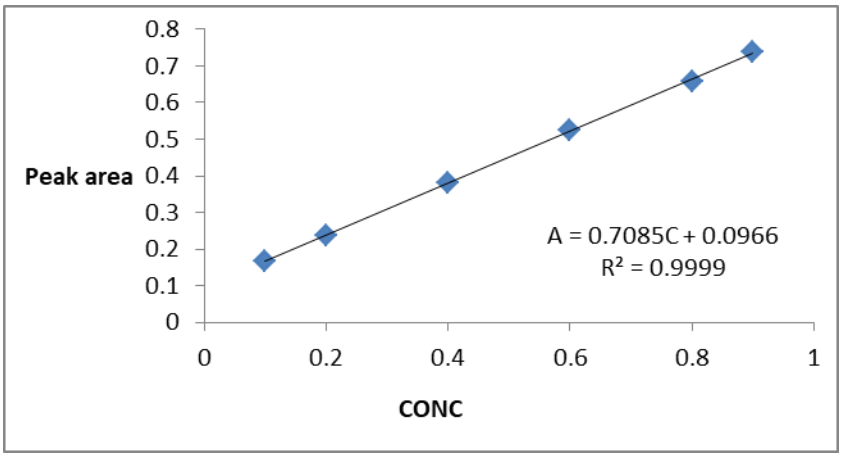

Figure 10 Calibration curve relating the peak area of TLC peaks of CZ to its concentration in µg / band

3.1. Method validation

Validation of the methods was carried out according to ICH recommendation

3.1.1. Linearity and range

The linearity of the proposed methods was evaluated by analyzing different concentrations of SPIR, FUR, ANTH, TRI,HCZ and CZ. It was evident in the range of 0.5-2 µg/ml, 0.7-2 µg/ml and 0.1-1 µg/ml for SPIR, FUR and ANTH respectively and 0.2-2 µg/ml, 0.3-3 µg/ml and 0.1-0.9 µg/ml for HCZ, TRI and CZ respectively. The values of correlation coefficients were close to unity indicating good linearity. The regression parameters like the slope, intercept and the correlation coefficient were calculated and are presented in Table 1.

Good linearity is evident from the closeness of the correlation coefficient values to 1 and low values of intercepts.

3.1.2. Accuracy

The accuracy of the developed methods were computed as percentage recoveries of pure samples of the interested studied drugs. The concentration of each drug was computed from its corresponding regression equations, Table 1.

Accuracy was further estimated through application of the standard addition technique to lasilactone ® and Dyazide ® tablets, where good recoveries were obtained revealing that there was no interference from excipients, Table 2.

3.1.3. Precision

- Repeatability: 3 concentrations of each drug of them were analyzed three times intra-daily using the proposed methods. Good results and acceptable standard deviations (SDs) were obtained, Table 1.
- Intermediate precision: The previous steps were repeated inter-daily on three different days for the analysis of the chosen concentrations. Good results and acceptable SDs were obtained and presented in Table 1.

Table 1 Assay parameter and method validation for the determination of pure sample of SPIR, FUR, ANTH, TRI, HCZ and CZ by the proposed TLC – densitometric methods

	Mixture 1			**Mixture 2**		
Parameters	**SPIR**	**FUR**	**ANTH**	**TRI**	**HCZ**	**CZ**
Range (µg/band)	0.5 – 2	0.7 – 2	0.1 – 1	0.3-3	0.2-2	0.1-0.9
Slope	0.505	0.525	0.511	0.268	0.4715	0.7085
Intercept	0.247	0.377	0.009	0.069	0.054	0.096
Correlation coefficient	0.9998	0.9998	0.9999	0.9999	0.9999	0.9999
Accuracy Mean ±SD	99.94 ±0.62	99.96±0.79	99.83±1.01	99.71±1.41	99.81±0.98	99.98±0.612
Precision						
Repeatability[a]	2.03	1.75	2.11	0.919	0.598	0.454
Intermediate precision[b]	2.15	1.88	2.21	1.1	0.717	0.545

[a] The intraday (n=3), average of three different concentrations repeated three times daily; [b] The interday (n=3), average of three different concentrations repeated three times in three successive days.

4. Determination of SPIR, FUR, TRI and HCZ in Pharmaceutical preparation using the TLC – densitometric methods

The developed TLC – densitometric methods were also applied for determination of SPIR and FUR in Lasilactone ® tablets and for determination of TRI and HCZ in Dyazide ® tablets.

Satisfactory results were obtained, Table 2. The validity of the methods was further assessed by applying standard addition technique which also confirmed the accuracy of the proposed methods (Table 2).

4.1. System suitability testing

System suitability testing for HPTLC densitometric method was based on the concept that the equipment, electronic analytical operations and samples constitute an integral system that can be evaluated as whole.System suitability is used to ensure system performance before or during the analysis of the drugs. System suitability was checked by calculating the Capacity factor (K), Tailing factor (T), Selectivity factor (α) and Resolution (R) and the system was found to be suitable, as shown in table 3.

The results obtained by applying the proposed method were statistically compared with those obtained by applying the reported HPLC method for determination of Lasilacone® tablets and Dyazide ® tablets.

The values of the obtained F and T tests were less than the calculated ones confirming that the difference between the developed methods and the reported one is non-significant, Table 4.

Table 2 Assay result for the determination of SPIR, FUR, TRI and HCZ in Pharmaceutical preparation using the proposed TLC – densitometric methods

Pharmaceutical preparation																				
Lasilactone ®									**Dyazide®**											
SPIR					**FUR**				**TRI**					**HCZ**						
Taken (µg/band)	**Found (%)**	**Standard addition**			**TAKEN (µg/ml)**	**FOUND (%)**	**Standard addition**			**TAKEN (µg/ml)**	**FOUND (%±SD)**	**Standard addition**			**TAKEN (µg/ml)**	**FOUND (%±SD)**	**Standard addition**			
0.5	99.06	DF	0.5	99.87	1	102.6	DF	1	101.9	0.3	99.87	DF	0.3	98.8	0.2	101.9	DF	0.2	100.26	
		PURE	0.5				PURE	0.3				PURE	0.5				PURE	0.2		
1	109.8	DF	0.5	101.5	1.3	105.3	DF	1	102.1	0.5	102.8	DF	0.3	100.56	0.6	103.4	DF	0.2	101.49	
		PURE	0.7				PURE	0.6				PURE	0.7				PURE	0.6		
1	109.3	DF	0.5	98.88	1.6	109.4	DF	1	98.56	0.7	102.25	DF	0.3	100.64	1	104.9	DF	0.2	100.79	
		PURE	1				PURE	1				PURE	1				PURE	0.8		
Mean ± SD	106.08±6.08	100.08±1.32			Mean ± SD	105.7± 3.42	100.85±1.98			Mean ± SD	101.64±1.25	100±0.848			Mean ± SD	103.4±1.5	100.84±0.616			

Table 3 System suitability testing parameters of TLC- densitometric method

parameters	Obtained value					
	Mixture 1			Mixture 2		
	SPIR	FUR	ANTH	TRI	HCZ	CZ
Symmetry factor	0.80	0.97	0.70	1	1.33	1.5
Resolution (R_S)	1.63		1.73		4.27	6.66
Capacity factor (K')	0.85	1.12	0.40	3.54	0.17	0.72
Selectivity factor (α)	1.21		1.17		1.4	2.25

Table 4 Statistical comparison of the results obtained by the proposed methods and the established method

Items	Lasilactone ® tablet				Dyazide ® tablet			
	Developed method		Reported method [1]		Developed method		Reported method [2]	
	SPIR	FUR	SPIR	FUR	TRI	HCZ	TRI	HCZ
Mean	99.94	99.96	100.25	100.15	99.71	99.8	100.174	100.38
SD	0.62	0.79	0.70	0.45	1.41	0.98	0.957	0.575
N	6	6	6	6	6	6	6	6
Variance	0.43	0.75	0.50	0.20	1.998	0.968	0.917	0.331
Student T test (2.22) [a]	0.61	0.5			0.706	1.32		
F – value (5.05) [b]	1.15	3.6			2.179	2.92		

[a] figures in parentheses represent the corresponding tabulated values of T at P=0.05; [b] figures in parentheses represent the corresponding tabulated values of F at P=0.05

5. Conclusion

The developed methods have advantages of being simple, rapid, and cost effective, less tedious and time saving as compared to chromatographic techniques. These methods can be easily and conveniently adopted for routine quality control analysis of the two mixtures.

Compliance with ethical standards

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Disclosure of conflict of interest

The author are certify that they have no affiliations with or involvement in any organization or entity with financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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