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Application of Stability Indicating TLC- Densitometric methods for the Determination of two Antihypertensive Drug Combinations.

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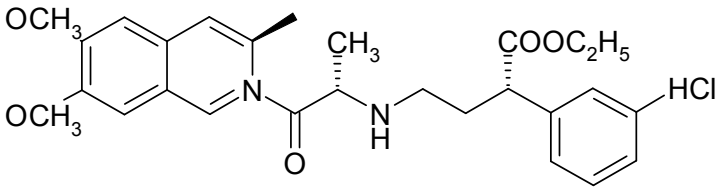
ABSTRACT

Introduction: Moexipril hydrochloride (Mox) and fosinopril (Fos), each of them in combination with hydrochlorothiazide are of the most commonly used antihypertensive combinations. Aim: To develop a stability indicating TLC densitometry for the determination of the drugs of interest. Materials and Methods: The degradation products were prepared by the alkaline hydrolysis of the intact drugs. TLC was carried out using silica gel F_{254} plates and a mobile phase of chloroform: ethyl acetate: methanol: triethyl amine in a ratio of (7.5: 4: 0.5: 0.1) and ethyl acetate: chloroform: methanol: formic acid in a ratio of 4.52: 4.5: 0.5: 0.1 (v/v/) followed by scanning of the developed chromatogram by a TLC scanner set at 276 nm and 220 nm respectively. Determination coefficients of calibration curves were found to be within the range of 0.9998-0.9999. Mixture of the investigated drugs and its degradates were analyzed using the proposed method with mean percentage recoveries of 99.10- 100.79%. Pharmaceutical dosage forms were assayed and found to give results of the same accuracy and reproducibility as the reported methods. Conclusion: The developed TLC methods were found successful in the stability indicating determination of the investigated drug and suitable for quality control purposes.

Key words: lymphoblastic leukemia, immunophenotypes, laboratory measurements

Introduction

Both moexipril hydrochloride (Mox) and fosinoril (Fos) (Figures 1a & 1b) are long acting orally active ACE inhibitors, they are the active ingredient used in treatment of hypertension. They can be used alone or in combination with HCTZ (Figures 1c) to increase its efficiency in antihypertensive therapy and to reduce the risk of damage to the kidneys, heart and other organs (Dart, 2004; Hochadel, 2009; Skolnik *et al.*, 2000; Fernandez *et al.*, 1994). Both drugs are liable to alkaline hydrolysis due to the cleavage of their ester linkage (El Shanawane *et al.*, 2008; Belal *et al.*, 2009; Ivanović *et al.*, 2004; Biljana *et al.*, 2008) leading to the formation of their degradates: moexiprilat (Mox-at) and fosinoprilat (Fos-at) respectively (Figures 2a & 2b). With regard to HCTZ, it undergoes both acid and alkaline hydrolysis to give formaldehyde and 6-chloro-2,4-disulfamoyl aniline (DSA) (Figure 2c) (Mollica *et al.*, 1969; Mollica *et al.*, 1971). Simple and rapid densitometric methods have been applied for identification and quantitative determination of some ACE inhibitors (Odović *et al.*, 2006; Odović *et al.*, 2009) in pharmaceutical preparations. In this work, stability indicating TLC methods was developed and validated for quantitative determination of the active ingredients in the complex mixture with their degradates.



(1a)

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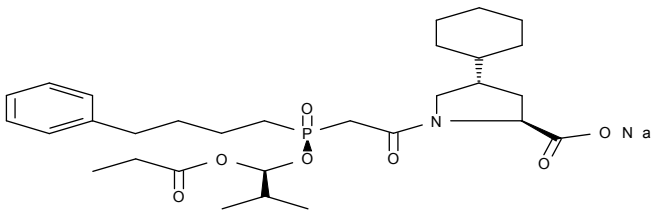
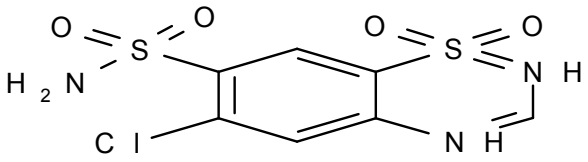
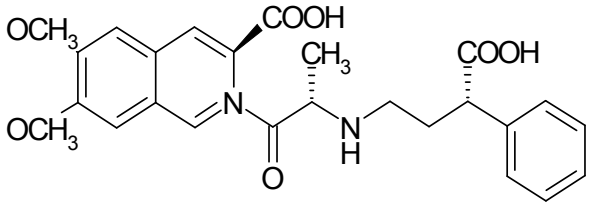


(1b)

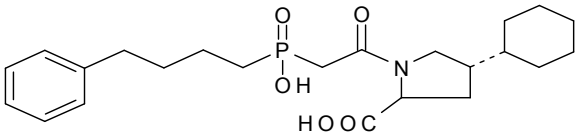


(1c)

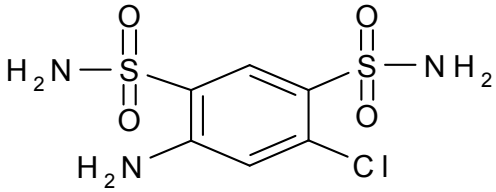
Fig. 1. Structural formula of Mox (1a), Fos (1b) and HCTZ (1c).



(2a)



(2b)



(2c)

Fig. 2. Structural formula of Mox (2a), Fos (2b) and HCTZ (2c).

Experimental

Instrumentation

- TLC scanner 3 densitometer (Comag, Muttenz, Switzerland)
- Sampel applicator for TLC linomat IV with 100 µl syringe (Camage, Muttenz, Switzerland).
- TLC aluminium plates (20x20 cm) precoated with 0.25 mm silica gel 60 F254 (Fluka, Germany).
- UV lamp with short wavelength (USA).

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Samples and Reagents

Samples

Raw materials:

- Mox certified to contain 99.90% by the manufacturer method was kindly supplied by Mina pharm, Egypt.
- Fos and HCTZ certified to contain 99.8% and 101.3% respectively by the manufacturer method were kindly supplied by Bristol Myers Squib, Egypt.

Market samples:

- A Commercial pharmaceutical formulation (Fempress® plus) produced by (actavis Deutschland GmbH and Co.Kg, Germany, Batch no. 0003599), each tablet is labeled to contain 15 mg/25 mg of Mox/ HCTZ combination was obtained.
- A commercial pharmaceutical preparation (Monozide® tablets) manufactured by (Bristol Myers Squib, Egypt, Batch no. L 93731), each tablet is labeled to contain 20 mg/12.5 mg Fos/HCTZ was obtained from the local market.

Reagents:

All chemicals and reagents used were of pure analytical grade ethylacetate, chloroform, methanol, formic acid and 33% ammonia solution (Sigma – Aldrich Co., USA).

Standard solutions

Standard stock solution of Mox, HCTZ and DSA:

Accurately weigh a portion equivalent to 500.0 mg of Mox, Fos, HCTZ and DSA, transfer into separate 100 ml volumetric flasks, dissolved in the minimum amount of methanol, sonicate for 10 minutes and complete the volume to the mark with the same solvent to give standard stock solutions of concentration 5.0 $mg.ml^{-1}$.

Standard stock solutions of Mox-at and Fos-at:

Mix a portion equivalent to 500.0 mg & 100.0 mg of Mox & Fos respectively with NaOH at concentrations of (3.0 M & 1.0 M) respectively, heat under reflux at 100°C, then cool the solutions and neutralize with a calculated volume of HCl. Filter the obtained precipitate, wash, transfer quantitatively into 100 ml volumetric flask and dissolve in the least amount of methanol. Then complete the volume to the mark with distilled water to give standard stock solution of concentration 5.0 $mg.ml^{-1}$ & 1.0 $mg.ml^{-1}$.

Procedure

Chromatographic conditions:

Perform the analysis on 20x20 cm TLC aluminium plates precoated with 0.25 mm silica gel 60 F_{254} and apply the samples to the TLC plates as bands using sample applicator for TLC, linomat IV with 100µl syringe leaving a space of 15 mm from the bottom edge of the plate and 6 mm between each band. Presaturate the chromatographic tank with the developing system for one hour then develop the plates by ascending chromatography using:

- *Mobile Phase (A) for the separation of Mox, HCTZ and their degradates:* composed of ethyl acetate: chloroform: methanol: ammonia solution in a ratio of 6: 3: 1: 0.2 (v/v).
- *Mobile Phase (B) for the separation of Fos, HCTZ and their degradates:* composed of ethyl acetate: chloroform: methanol: formic acid in a ratio of 4.52: 4.5: 0.5: 0.1 (v/v)

as developing systems to a distance of about 19.0 cm ,then air dry the plates at room temperature, detect under UV lamp and scan at 276 nm and 220 nm for systems A & B respectively under the following experimental conditions:

- Source of radiation: deuterium lamp.
- Slit dimensions: 6.00 x 0.45 mm.
- Scanning speed: 20 mm/s
- Band width: 6.0 mm
- Data resolution: 100 µm/step.
- Result output: chromatograms and integrated peak areas.
-

Method validation

Linearity

Method (A) for stability indicating assay of Mox & HCTZ:

Accurately transfer different aliquots (0.25-2.5 mg) of Mox and (0.1-1.7 mg) of HCTZ from their standard solutions into 10 ml volumetric flasks and dilute to the volume with methanol.

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Method (B) for stability indicating assay of Fos & HCTZ:

Accurately transfer different aliquots (0.50-4.0 $mg.ml^{-1}$) of Fos and (0.50-1.9 mg) of HCTZ were from their standards tock solutions into 10-ml volumetric flasks and dilute to the volume with methanol.

Apply 10.0 µl of each of the prepared solutions to the TLC plates (20x20 cm) as bands following the previously mentioned chromatographic conditions. Scan the bands at the previously selected wavelengths. Construct calibration curves by plotting the peak height/1000 versus the corresponding concentrations and the regression equations were then computed.

Accuracy

Apply the previously mentioned procedures under linearity for different concentrations of the investigated drugs. Calculate the concentrations from their corresponding regression equations. Then calculate the mean percentage recoveries and standard deviations.

Precision

Intraday precision (repeatability):

Use the previously mentioned procedures under linearity for the analysis of freshly prepared solutions in triplicate of concentrations (0.25, 1.25, 2.5 $mg.ml^{-1}$) & (0.1, 0.9, 1.7 $mg.ml^{-1}$) of Mox & HCTZ respectively for method (A) and (0.5, 2.0, 4.0 $mg.ml^{-1}$) & (0.50, 1.30, 1.90 $mg.ml^{-1}$) of Fos & HCTZ respectively for method (B) for the determination of intraday precision (n=3) and then calculate the relative standard deviations (R.S.D%).

Interday precision (intermediate precision):

Use the previously mentioned procedures under linearity for the analysis of freshly prepared solutions in triplicate of the previously mentioned concentrations for three consecutive days for the determination of interday precision (n=3) and then calculate the relative standard deviations (R.S.D%).

Selectivity

Prepare a laboratory prepared mixture containing 1.0 $mg.ml^{-1}$ of each of the four components of the two investigated mixtures and apply to the TLC chromatographic plate. After drying, conduct densitometric analysis at the predetermined wavelength. Determine the concentrations of the investigated drugs by referring to their corresponding regression equations. Then calculate the percentage recoveries and the standard deviations.

Application to pharmaceutical preparations

Accurately weigh twenty tablets of Fempress® plus & Monozide® tablets, then finely ground in a mortar. In one volumetric flask, extract a portion of the powder equivalent to 15 mg/25 mg of Mox/HCTZ with the minimum amount of methanol. In another two volumetric flasks, repeat the same procedure but using an amount of powder equivalent to 100.0 mg of each of Fos and HCTZ. Then sonicate for 30 minutes, centrifuge for 10 minutes, filter the precipitate through a micropore filter, wash into a 100 ml volumetric flask and make up the volume to the mark with the same solvent to provide a stock solution of concentration 150/250 $\mu g/ml^{-1}$ of Mox/HCTZ & 100 $\mu g/ml^{-1}$ of each of Fos &HCTZ respectively. Make an appropriate dilution to prepare the working solution and then apply the proposed method. Calculate the concentrations of the investigated drugs from their corresponding regression equations and the mean percentage recoveries.

Validation by standard addition technique

Perform this study by adding known amounts of the studied compounds from their working standard solutions to a known concentration of the commercial preparation. Then chromatograph the resulting mixtures by adopting the procedures mentioned under linearity.

Results:

The results of assay validation parameters of proposed methods including linearity, accuracy, precision, selectivity, application to pharmaceutical preparation and standard addition techniques are listed in tables (1-2). They were all found within the acceptable limits.

Table 1. Results of assay validation parameters obtained by applying the proposed TLC-densitometric method for determination of Mox and HCTZ.

Parameters	Mox	HCTZ
Linearity	0.25-2.5mg.ml-1	0.1-0.7 mg.ml-1
Correlation coefficient (r)	0.9998	0.9998
Slope	7288.112665	27969.91846
Intercept	1088.206233	19614.63087
Standard error of the slope	33.03934159	1380.4099926
Confidence limit of the slope	7211.923807-7364.301524	27642.63084-28297.20609

Confidence limit of the intercept	51.25093082	143.617737
Confidence limit of the intercept	970.0213749-1206.391092	19275.02889-19954.23285
Standard error of estimation	75.02368069	214.4238386
Accuracy (mean±SD)	100.62±0.416	100.04±1.075
Selectivity	100.50±1.250	99.06±1.852
Precision (R.S.D.%)		
Repeatability*	0.432	1.285
Intermediate precision*	0.585	1.364
Application to pharmaceutical preparation	100.42±0.424	99.70±0.958
Standard addition technique	100.34±0.406	99.38±0.799
Student's t-test (2.306)	2.255	2.281
F-value (6.388)	5.024	2.972

* *The intra-day and inter-day relative standard deviations of the average of concentrations 0.25, 1.25, 2.5 mg.ml-1 for Mox.*
* *The intra-day and inter-day relative standard deviations of the average of concentrations 0.1, 0.9, 1.7 mg.ml-1 for HCTZ.*

Table 2. Results of assay validation parameters obtained by applying the proposed TLC-densitometric method for determination of Fos and HCTZ.

Parameters	Fos	HCTZ
Linearity	0.25-2.5mg.ml-1	0.1-0.7 mg.ml-1
Correlation coefficient (r)	0.9998	0.9998
Slope	7288.112665	27969.91846
Intercept	1088.206233	19614.63087
Standard error of the slope	33.03934159	1380.4099926
Confidence limit of the slope	7211.923807-7364.301524	27642.63084-28297.20609
Confidence limit of the intercept	51.25093082	143.617737
Confidence limit of the intercept	970.0213749-1206.391092	19275.02889-19954.23285
Standard error of estimation	75.02368069	214.4238386
Accuracy (mean±SD)	100.62±0.416	100.04±1.075
Selectivity	100.32±1.336	99.54±1.730
Precision (R.S.D.%)		
Repeatability*	0.432	1.285
Intermediate precision*	0.585	1.364
Application to pharmaceutical preparation	100.76±0.653	99.56±0.516
Standard addition technique	100.25±0.978	99.37±0.860
Student's t-test (2.306)	0.426	1.057
F-value (6.388)	1.528	5.863

* *The intra-day and inter-day relative standard deviations of the average of concentrations 0.5, 2.0, 4.0 mg.ml-1 for Fos.*
* *The intra-day and inter-day relative standard deviations of the average of concentrations 0.1, 0.9, 1.7 mg.ml-1 for HCTZ.*

Discussion

Quantitative TLC in situ scanning densitometry is rapidly gaining wide acceptance in pharmaceutical analysis (Aboul-Enein *et al.*, 1994; Agbaba *et al.*, 1999; Ling *et al.*, 1989). This is because of its simplicity, accuracy, cost effectiveness and the possibility of simultaneous determination of a number of samples on a single TLC plate because of all of the above mentioned reasons, TLC-UV densitometry is considered a promising alternative for HPLC in resolution and in turn analysis of complex drug mixtures as mixture of Mox, HCTZ and their degradation products (Miller *et al.*, 2000).Initially, trial experiments were performed in a view to select a suitable mobile phase for the accurate estimation of studied drugs without interference from their degradation products.

For Method (A) for stability indicating assay of Mox & HCTZ:

The main problems faced during the separation procedure was the separation of HCTZ from DSA due to the great similarity in their structures and their physicochemical properties and the strong retention of Mox at on silica gel. Selection of the best system started with mixtures of (chloroform: methanol) and (ethyl acetate: methanol) in different ratios which are typical mobile phase components in NPTLC. It was found that high ratios of CHCl3 leads to better resolution of HCTZ and DSA but increased retention and low Rf values of Mox and Mox at whereas high ratios of ethyl acetate increase migration of Mox but deteriorates the resolution of HCTZ and its degradate therefore a combination of the three solvents was tried in different ratios for optimum separation and acceptable Rf values and ammonia was added as a trial to elute Mox at from the site of application. In all instances, it was established that Mox at exhibit stronger retention i.e., they have low Rf values. This distinction in the chromatographic behaviour of Mox and its degradate results from the difference in their interaction with silica gel namely due to the presence of two carboxylic groups in the molecule of the degradate, their specific interaction with silica gel via hydrogen bonds are much stronger than that of the corresponding ACE inhibitor containing only one carboxylic group.

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Among the different mobile phase combination tested, (ethyl acetate: chloroform: methanol: ammonia) in a ratio of (7.5: 4: 0.5: 0.1) which gave compact, sharp and symmetrical peaks with Rf values of 0.18±0.018, 0.44±0.017, 0.58±0.02 and 0.03±0.013 for Mox, HCTZ, DSA and Mox at respectively at the wavelength of 276 **nm. The representative densitograms are given in figures (3-5).**

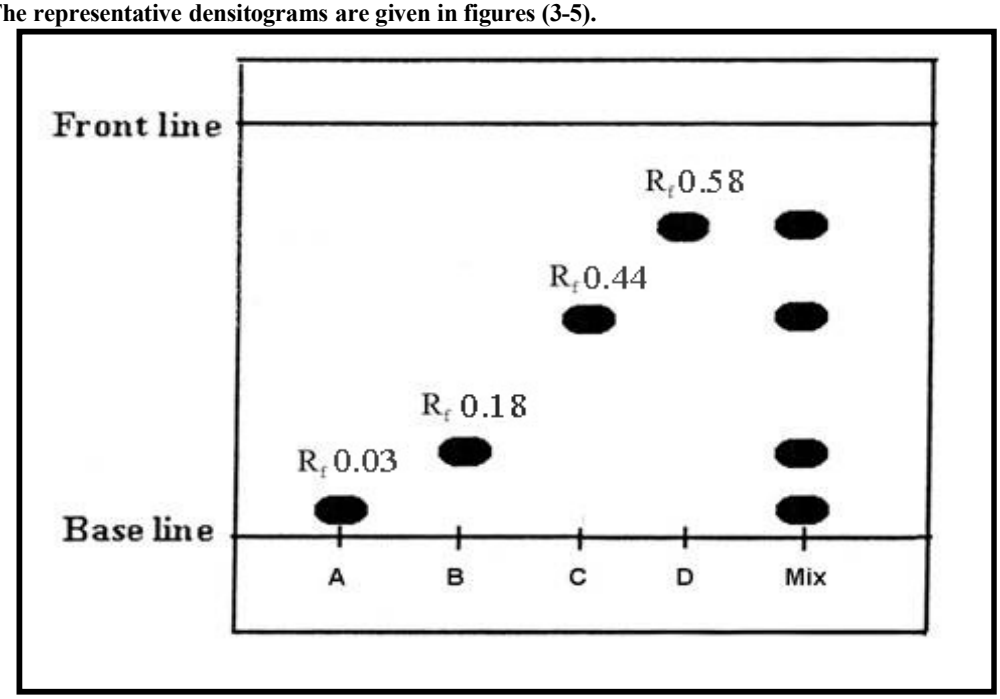
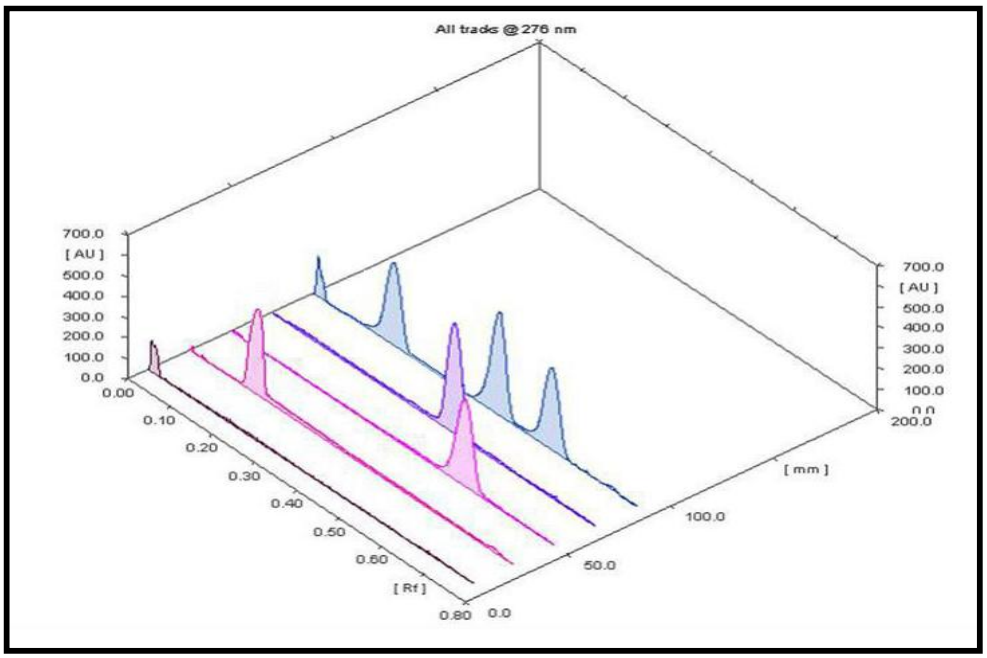


Fig. 3. Thin layer chromatogram of (a) Mox.HCl, (bs) HCTZ, (c) DSA, (D) Mox-at using ethyl acetate; chloroform: methanol: ammonia solution in a ratio of 6: 3:1: 0.2 (v/v) as a developing system.



(b)

Fig. 4. Thin layer chromatogram (3D) of (a) Mox.HCl, (b) HCTZ, (c) DSA, (D) Mox-at using ethyl acetate; chloroform: methanol: ammonia solution in a ratio of 6: 3:1: 0.2 (v/v) as a developing system.

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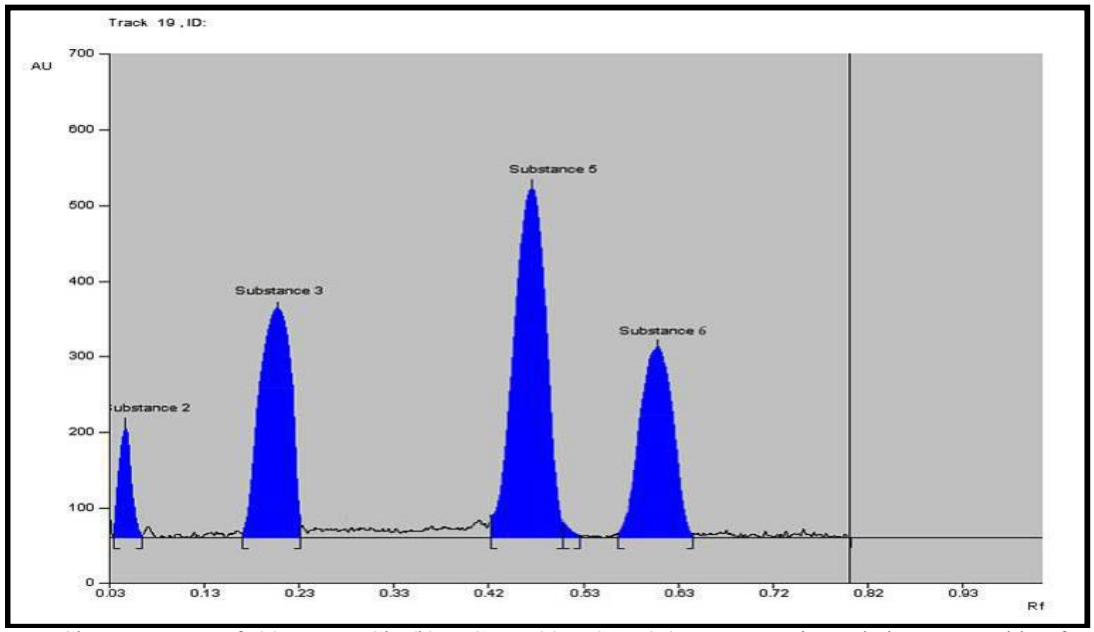

Fig 5. Chromatogram of (a) Mox.HCl, (b) HCTZ, (c) DSA, (D) Mox-at using ethyl acetate; chloroform: methanol: ammonia solution in a ratio of 6: 3:1: 0.2 (v/v) as a developing system.

For Method (B) for stability indicating assay of Mox & HCTZ:

Owing to the difficulty of the stability indicating assay of Fos and HCTZ in presence of their degradates which necessitates the use of specific column, elevated temperature and in some cases, for saving the analysis time, applying sophisticated HPLC techniques as gradient elution which requires expensive instrumentation.

TLC densitometry offers a magical alternative for the time consuming or expensive HPLC technique where it can provide a 15 to 30 minute analysis time as an alternative for a 2 hour using HPLC. The separation procedure was considered challenging because the investigated compounds are structurally different with extremely different lipophilicity and polarity.

Preliminary experiments involving mobile phase systems of (ethyl acetate: methanol) and (chloroform: methanol) were conducted, ratios of ethyl acetate or chloroform which produce good resolution of HCTZ and DSA causes Fos to be coeluted with solvent front therefore arises the use of a ternary mobile phase system of ethyl acetate, chloroform, methanol. Acetic acid was added to reduce the interaction of acidic groups with the silica surface especially unreacted silanol groups on the silica gel, but then was replaced by formic acid which was found more efficient. Finally chromatography was performed on 20x20 silica gel plates employing a mobile phase of (ethylacetate : CHCl3: methanol: formic acid) in a ratio of (4.5: 4.5: 0.5: 0.1) with densitometric scanning at 220 nm which established complete separation of all of the studied compounds with R_f of 0.75±0.019, 0.29±0.012, 0.41±0.019 and 0.05±0.009 of Fos, HCTZ, DSA and Fos-at which is strongly retained on silica and eluted with extensive tailing which is considered the only limitation of the proposed method (figures 6-8).

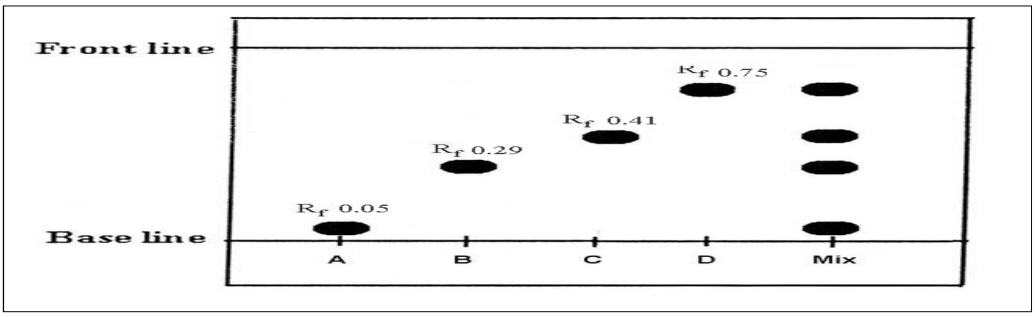

Fig. 6. Thin layer chromatogram of (a) Fos, (b) HCTZ, (c) DSA, (D) Fos-at using ethyl acetate; chloroform: methanol: ammonia solution in a ratio of 6: 3:1: 0.2 (v/v) as a developing system.

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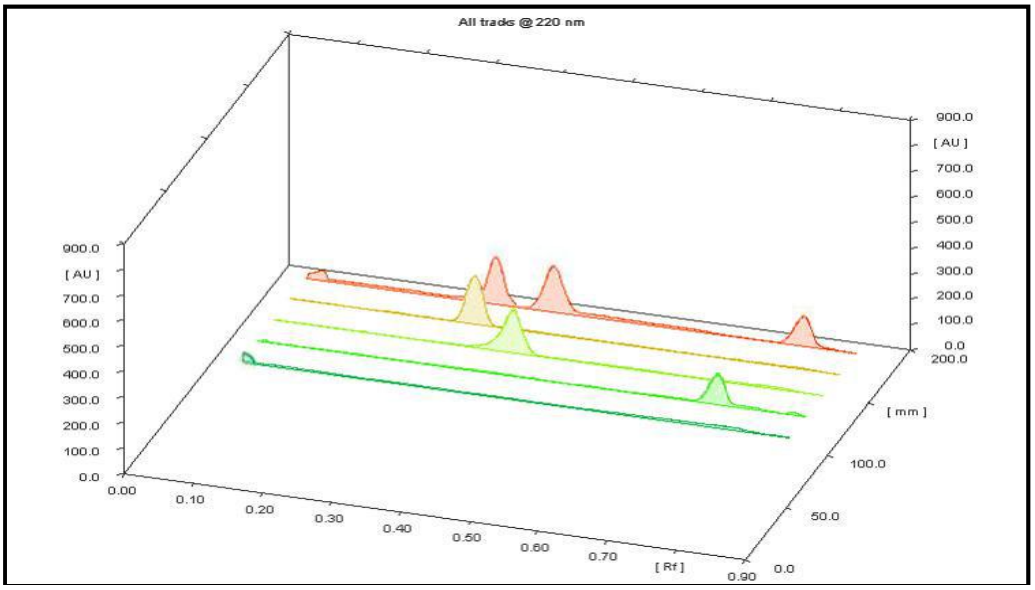

Figure 7. Thin layer chromatogram (3D) of (a) Fos, (b) HCTZ, (c) DSA, (D) Fos-at using ethyl acetate; chloroform: methanol: ammonia solution in a ratio of 6: 3:1: 0.2 (v/v) as a developing system.

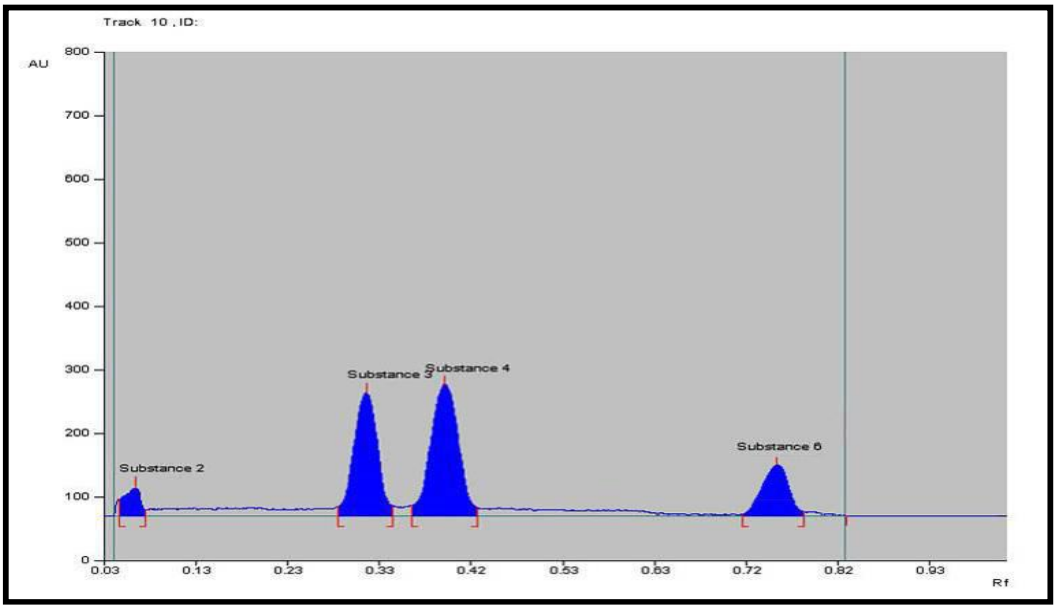

Figure 8. Chromatogram of (a) Fos, (b) HCTZ, (c) DSA, (D) Fos-at using ethyl acetate; chloroform: methanol: ammonia solution in a ratio of 6: 3:1: 0.2 (v/v) as a developing system.

Conclusion

The developed densitometric methods were found to be simple, rapid, selective and suitable for simultaneous analysis of antihypertensive drug mixtures in synthetic mixtures with their degradation products as well as in pharmaceutical formulations. They also utilized the merit of applying several sample spots on TLC plate which may be advantageous for regulatory quality control laboratories.

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