Egypt J. Anal. Chem., Vol. 21, PP. 30-40 (2012)

Simultaneous Densitometric Determination of some Binary Corticosteroid Mixtures

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Summary:A thin-layer chromatographic method has been developed and validated for simultaneous determination of three binary mixtures. Betamethasone valerate was found with clioquinol in mixture (I) and with fusidic acid in mixture (II), while mixture (III) contains hydrocortisone acetate with fusidic acid. For achieving good separation, developing systems consisting of methanol:chloroform (9:1 v/v) for mixture (I) and methanol:chloroform (0.5:9.5, v/v) for mixtures (II) and (III) were chosen. The densitometric determination of each drug was carried at wavelengths, 240, 282, 245 and 253 nm for betamethasone valerate, clioquinol, fusidic acid and hydrocortisone acetate, respectively. The calibration curves were valid over the concentration range of 1-42 µg/spot and 2–65 µg/spot for betamethasone valerate and clioquinol in mixture (I) and 1-45 µg/spot, 5-50 µg/spot and 5–60 µg/spot for betamethasone valerate, hydrocortisone acetate and fusidic acid in mixtures (II) and (III), respectively. The methods were validated according to USP guidelines. The proposed methods were successfully applied for the simultaneous determination of these compounds in their laboratory prepared mixtures and drug products with good accuracy and precision. The results obtained were statistically compared with those obtained by the official ones.

Introduction

Betamethasone-valerate-[9-fluoro-11β, 21-dihydroxy-16β-methyl-3, 20-dioxopregna-1,4-dien-17-yl pentanoate] and hydrocortisone acetate [11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate][(1)] are corticosteroid drugs with both glucocorticoid and minralocorticoid activity. They have potent anti-inflammatory and immunosuppressive effects[(2)]. Clioquinol is [5-Chloro-7-iodoquinolin-8-ol][(1)], It has an antifungal and antiprotozoal effect. The drugs have been found to have activity against

both viral and protozoal infections[(2)]. Fusidic acid is *ent*-(17*Z*)-16α-(acetyloxy)-3β, 11β-

dihydroxy-4β, 8, 14-trimethyl-18-nor-5β, 10α-cholesta-17(20), 24-dien-21-oic acid[1]. It is an antimicrobial substance produced by the growth of certain strains of *Fusidium coccineum* or by any other means[2].

Different methods were published for their determination such as spectrop hotometric methods[3-5], thin layer chromatography[6-10], HPLC[11-18] and GC[19-21].

To the best of the author's knowledge, the suggested procedures have not been investigated before. Only TLC method [22] was reported for determination of betamethasone valerate and clioquinol mixture (I) which lakes sensitivity, simplicity of mobile phase and time consuming.

Due to the importance of simultaneous determination of binary mixtures in quality control work, we focused on developing simple, sensitive and accurate TLC–densitometric method for the simultaneous determination of betamethasone valerate, clioquinol, fusidic acid and hydrocortisone acetate in their binary mixtures at the same plate. The factors affecting best separation such as mobile phase composition, wavelength and time of jar conditioning were studied. The proposed methods were validated according to USP guidelines.[23]

Experimental

Instrumentation

SHIMADZU CS-9301 PC dual wavelength flying spot scanning densitometer, TLC plates (10 x 10 cm) with 0.25 mm thickness silica gel F_{254}, (E. Merck), UV short wavelength 254 nm Lamp (Desaga. Germany) and Chromatographic tanks (15x 20x 30 cm)

Materials and reagents

All chemicals and solvents were of analytical grade.

Reference samples and market samples:

Betamethasone valerate (BV) was kindly provided by *GlaxoSmithKline*, its purity was found to be 99.91± 0.61 according to the official spectrophotometric method[1]. Clioquinol (CQ) was kindly provided by *GlaxoSmithKline*, its purity was found to be 99.96± 0.51 according to the official non aqueous titration method[1] and Betnovate-C cream batch number (083758) nominally containing betamethasone valerate and clioquinol (0.1:3% w/w) was purchased from local market, while Fusidic acid (FA)

was kindly provided by *Minapharm*, its purity was found to be 99.84± 0.43 according to the official titrimetric method[(1)] and Fucicort cream batch number (9HE1807) labeled to contain betamethasone valerate and fusidic acid (0.1:2% w/w) was purchased from local market. Hydrocortisone acetate (HA) was kindly provided by *Pharaonia* pharmaceuticals, its purity was found to be 99.84±0.43 according to the official spectrophotometric method[(1)] and Fusi-zone cream batch number (0311057) nominally containing hydrocortisone acetate and fusidic acid (1:2% w/w) was purchased from local market.

Solvents:

Chloroform (EL-Nasr Pharmaceutical Chemical Co.) and Methanol (Riedel – dehaën).

Standard stock solutions:

Solutions of 0.5 mg ml^{-1} of BV and HA and 1 mg ml^{-1} of CQ and FA were prepared by dissolving 50 mg of each of BV and HA and 100 mg of each of CQ and FA in 100 ml volumetric flask and completed with methanol to volume. The standard stock solutions were subsequently used to prepare working solutions in methanol. All solutions were stored in refrigerator at 4 ºC.

Procedures

Preparation of calibration curves

Ten µl of working solutions in the range for betamethaone valerate, (0.1–4.2 ug mL^{-1}) and (0.5–6.5 ug mL^{-1}) for clioquinol in mixture (I), betamethaone valerate (0.1–4.5 ug mL^{-1}) and fusidic acid (0.5–6 ug mL^{-1}) in mixture (II), hydrocortisone acetate (0.5–5 ug mL^{-1}) and fusidic acid (0.5–6 ug mL^{-1}) in mixture (III), were applied to the TLC plates as spots. The spots are separated by a distance of 1 cm apart from each other and 2 cm from the bottom. The development chamber was saturated with mobile phase for 10 min. The plates were developed in the ascending way at ambient temperature using methanol: chloroform (9:1 v/v) for mixture (I) and methanol: chloroform (0.5:9.5 v/v) for mixtures (II) and (III) after developing over a distance of 10cm, the plates were air dried and scanned densitometrically at 240 nm, 253 nm, 282 nm and 245 nm for BV, HA; CQ and FA respectively. Calibration curves were prepared by plotting the recorded peaks area versus the corresponding concentrations and the regression equations were computed for each drug.

Laboratory prepared mixtures

In a series of 10 ml measuring flasks, aliquots of BV, CQ, FA and HA from their standard stock solutions were transferred to prepare mixtures containing different ratios (1:30), (5:5), (20:10) and (10:20) for mixture (I), (2:40), (5:5), (5:10) and (20:10) for mixture (II) and (2:4), (5:5), (5:10) and (20:10) for mixture (III) of the two drugs in each mixture. The mixtures were assayed as mentioned under "preparation of calibration curve". The content of each drug was calculated from its corresponding regression equation.

Determination of the cited drugs in creams

An accurate weight of 40 g of betnovate-c cream equivalent to 1:30 BV and CQ, respectively; fusicort cream equivalent to 1:20 BV and FA, and fusizone cream equivalent to 1:2 HA and FA, respectively were dissolved in 50 ml methanol with gentle heating at 50ºC using water bath. The mixtures were cooled by putting on ice and then centrifuged at 250 rpm for 10 min., the supernatant of each mixture was taken. The volumes were completed to the mark with methanol in 50 ml volumetric flask; appropriate dilutions for each mixture were prepared and assayed as previously mentioned under "preparation of calibration curve". The same experiments were repeated applying the standard addition technique. The content of each drug was calculated from its corresponding regression equation.

Results and Discussion

Thin layer chromatography is known to be one of the simplest chromatographic separation techniques. The densitometry technique could be used for the assay of drugs in mixtures, due to the high power of resolution of TLC.[24]

It has many applications in the field of pharmaceutical studies including stability, drug impurities, pharmacokinetic, enantiomeric purity and drug monitoring in biological fluids.[25-31]

In this work, TLC-densitometric method for the simultaneous determination of three binary mixtures is described; the method is based on the difference in R_f values of each drug in its mixture.

Experimental conditions such as mobile phase, wavelength and slit dimension were optimized to provide accurate, precise and reproducible results. Different developing systems were tried for the separation and quantitation of BV and CQ, BV and FA and HA and FA such as chloroform:methanol:acetonitrile, chloroform:methanol:toluene, chloroform:methanol:acetic acid and chloroform:methanol in different proportions. Best separation of mixture (I) was achieved using methanol:chloroform (9:1v/v) and for both mixtures (II) and (III) was methanol:chloroform (0.5:9.5 v/v). This good separation allows the simultaneous determination of each drug in its binary mixture without interference when scanned at, 240, 282, 245 and 253 nm for BV, CQ, FA and HA

Linear correlations were obtained between the peak area and drug concentration in the ranges 1-42 and 2–65 µg/spot for betamethasone valerate and clioquinol in mixture (I) and 1-45, 5-50 and 5–60 µg/spot for betamethasone valerate, hydrocortisone acetate and fusidic acid in mixtures (II) and (III), respectively. The proposed method is valid and applied for the determination of the drugs substances. The selectivity and specificity of the proposed method was proved by analysis of the laboratory prepared mixtures containing different ratios of the two drugs in the mixture. The method applied for creams analysis can be used without interference from creams additives such as cetomacrogol, cetostearyl alcohol, chlorochresol, liquid paraffin, sodium dihydrogenphosphate and white paraffin.

Method validation

Linearity range

Under the experimental conditions, Beer's plots for the drug show linear relationship with regression equations shown in table (1).

Accuracy

The accuracy of the proposed method was determined by investigating the percentage recoveries of six levels, of each, three times in the concentration ranges within Beer's plots as shown in table (1). The percentage relative standard deviation (% RSD) revealed high accuracy.

Repetability

The intraday precision was evaluated by assaying freshly prepared solutions in triplicate in the concentration range within the Beer's plots, the percentage relative standard deviation (% RSD) shown in table (1).

Intermediate precission

The inetrday precision was calculated by assaying freshly prepared solutions in triplicate in the concentration range within the Beer's plots, the percentage relative standard deviation are (% RSD) shown in table (1).

Table (1): Validation report on the proposed TLC – densitometric method for the determination of betamethasone valerate, clioquinol, fusidic acid and hydrocortisone acetate in drug substances.

Parameter	BV	CQ	BV	FA	HA
Linearity range (ug/spot)	1 – 42	2 – 65	1 – 45	5 – 60	5 – 50
Accuracy (Mean+RSD)	100.12 ±0.83	99.89 ±0.22	99.63 ±0.40	99.74 ±0.42	99.78 ±0.35
Regression equations					
Slope	0.537	0.345	0.485	0.288	0.289
Standard deviation of slope	0.007	0.007	0.008	0.006	0.008
Intercept	0.137	0.054	0.018	0.003	0.116
Standard deviation of intercept	0.169	0.293	0.201	0.214	0.252
Standard deviation of estimation	1.548	2.454	1.77	1.62	1.940
Correlation coefficient (r)	0.9996	0.9991	0.9995	0.9992	0.9984
Precision					
Intermediate precission [a] RSD%	0.33	0.12	0.36	0.09	0.11
Repetability [a] RSD%	0.36	0.28	0.39	0.31	0.09
LOD (ug/ spot)	0.196	0.157	0.112	0.207	0.171
LOQ (ug/ spot)	0.595	0.476	0.338	0.627	0.519

Robustness

The robustness of the method was determined by minor change in the experimental conditions such as minor change in the mobile phase constituents by ±0.1 ml of chloroform or methanol and change of the time conditioning 10±1 min, did not have significant effect on TLC chromatographic resolution.

Limits of detection and quantification

According to the USP guidelines the approach based on the response and slope was used for the determination of the detection and quantification limits. The theoretical values were assessed practically and given in table (1).

Specificity

The specificity of the method was investigated by observing any interference encountered from the common creams additives. These expeients did not interfere with the proposed method.

Stability of the solution

The stability of standard and sample solutions of the cited drugs was evaluated using TLC method. The solutions were stored in tightly capped volumetric flask, protected from light, on laboratory bench and in refrigerator. Recovery of these solutions was cheked for 10 hrs with one hr intervals against freshly prepared solutions. It was found that the solutions

were stable if kept on the laboratory bench up to 8 hrs; and up to 4 days if kept in a refrigerator.

Method validation of drug products

The validity of the suggested method was assessed by applying the standard addition technique[32] by adding the drug to the previously analayzed pharmaceutical preparation. Statistical comparison of the results obtained by the proposed method with those obtained from potentiometric, titrimetric and spectrophotometric methods for mixtures (I), (II) and (III), respectively, showed that the recommended method is simple and sensitive without lack of accuracy and precision as shown in table (2).

Table (2): Results of assay obtained by applying the proposed TLC – densitometric method for the determination of betamethasone valerate, clioquinol, fusidic acid and hydrocortisone acetate.

Parameter	BV	CQ	BV	FA	HA	FA
Mobile phase	Methanol:chloroform (9:1 v/v)	Methanol:chloroform (9:1v/v)	Methanol:chloroform (0.5:9.5v/v)	Methanol:chloroform (0.5:9.5v/v)	Methanol:chloroform (0.5:9.5v/v)	
R_f of intact drug	0.67	0.58	0.66	0.59	0.45	
λ_{max} of measurement	240 nm	282 nm	240 nm	245 nm	253 nm	
1-Drug substances*	100.12 ± 0.83	99.89 ± 0.22	99.63 ± 0.0.40	99.74 ± 0.42	99.78 ± 0.35	
2-Laboratory prepared mixture*	99.72 ± 0.38	99.71 ± 0.18	99.75 ± 0.38	99.88 ± 0.13	99.76 ± 0.38	99.79 ± 0.17
3-Ddrug products*	99.75 ± 0.31	99.94 ± 0.02	99.49 ± 0.37	99.92 ± 0.04	99.86 ± 0.12	99.91 ± 0.11
4-Drug added*	99.78 ± 0.48	100.07 ± 0.22	99.40 ± 0.55	99.95 ± 0.16	99.90 ± 0.13	99.74± 0.42

* Means of 3 experiments ± S.D

The values of the calculated t-test and F-ratio at 95% confidence level are less than the tabulated ones which reveals that there is no significant difference with respect to accuracy and precision as shown in table (3).

Table (3): Tests of significance of the TLC method for the determination of betamethasone valerate, clioquinol, fusidic acid and hydrocortisone acetate in drug products.

Parameter	Betamethasone valerate		Clioquinol		Betamethasone valerate		Fusidic acid		Hydrocortisone acetate	
	TLC method	Official method [1]	TLC method	Official method [1]	TLC method	Official method [1]	TLC method	Official method [1]	TLC method	Official method [1]
Mean*	100.12	99.91	99.89	99.96	99.63	99.91	99.74	99.84	99.78	99.84
SD	0.83	0.61	0.22	0.50	0.40	0.61	0.42	0.43	0.35	0.43
SE	0.37	0.25	0.10	0.21	0.18	0.25	0.19	0.18	0.16	0.18
N	5	6	5	6	5	6	5	6	5	6
V	0.69	0.37	0.05	0.25	0.16	0.37	0.18	0.18	0.13	0.18
t-test	2.239	(2.262)**	2.294		1.882		2.120		2.205	
F-ratio	1.87	(5.19)**	5.00		2.31		1.00		1.38	

*Average of three experiments

** Theoretical values of t- and F- ratio at 95% confidence level.

Conclusion

The proposed TLC densitometric method was found to be suitable for identification and determination of betamethasone valerate and hydrocortisone acetate in presence of clioquinol and fusidic acid in their mixtures and drug products. It has the advantage of being simple, sensitive and suitable for routine analysis in quality control laboratories.

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