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Application of HPLC and HPTLC-Densitometry for the Simultaneous Determination of Camylofin and Diclofenac in Pharmaceutical Dosage Form

[1]Nishitkumar S. Patel, [2]Vrijeshkumar P. Gandh, I, [3]Rajendra S. Mehta, [4]Kashyap K. Bhatt

[1]Analytical Development Laboratory, Relax Pharmaceuticals, Makarpura, Vadodara 390010, Gujarat, India
[2]Sardar Patel University, A.R.College of Pharmacy and G.H.Patel Institute of Pharmacy, PO Box No. 19, Vallabh Vidyanagar 388120, Gujarat, Inida
[3]Sardar Patel University, A.R.College of Pharmacy and G.H.Patel Institute of Pharmacy, PO Box No. 19, Vallabh Vidyanagar 388120, Gujarat, Inida
[4]Sardar Patel University, Indukaka Ipcowala College of Pharmacy, New Vallabh Vidyanagar 388121, Gujarat, India

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ABSTRACT

Simple and accurate liquid chromatographic (HPLC) and thin layer chromatographic (HPTLC-densitometry) methods for simultaneous determination of camylofin dihydrochloride (CAM) and diclofenac potassium (DIC) in tablets were elaborated. The first method was based on isocratic reversed phase liquid chromatography by using RP ODS-C18 column with a mobile phase consisting of acetonitrile:25mM potassium dihydrogen phosphate (80:20, v/v) containing 0.1% v/v acetic acid adjusted to pH 7 with triethylamine at a flow rate of 1.5 mL/min and UV detection at 215 nm. The calibration curve of camylofin and diclofenac was linear in the range of 50-300 µg/mL (r>0.999) and 5-30 µg/mL (r>0.999), respectively. The second thin layer chromatographic method employed by using pre-coated silica gel G60–F254 aluminum sheet using mobile phase chloroform:ethyl acetate:methanol:ammonia (5:3:2:0.1,v/v) and quantitation was achieved using spectrodensitometrically at 215 nm. The calibration curve of camylofin and diclofenac was linear in the range of 1500-9000 ng/spot (r>0.999) and 150-900 ng/spot (r>0.999), respectively. The validity of the methods was confirmed using the recovery studies, precision and limit of detection. Both techniques were applied successfully for the analysis of camylofin and diclofenac in tablets form. The results obtained from both procedures were statistically compared using the Student's-t and F-variance ratio tests.

Key words: Camylofin, Diclofenac, HPLC, HPTLC, Determination

INTRODUCTION

Camylofin dihydrochloride, Isopentyl 2-(2-diethylaminoethylamino)-2-phenylacetate dihydrochloride (Figure 1a) belongs to the group of spasmolytic, anticholinergic and gastrointestinal sedative [1]. Camylofin is used as an antispasmodic, usually in combination with diclofenac, paracetamol and nimesulide [2] .Diclofenac potassium, [2-(2,6-Dichloroanilino)phenyl]acetic acid, Potassium salt (Figure 1b) is a widely used non steroidal anti-inflammatory drug of the phenylacetic acid class. As a potent inhibitor of the prostaglandin synthesis it has antipyretic, analgesic and anti-inflammatory activities [3-6]. The binary mixture of camylofin and diclofenac is used as anticholinergic and anti-inflammatory agents. CAM bulk drug and formulations are not official in any pharmacopoeia where as DIC bulk drug and formulations are official in British Pharmacopoeia, 2007 and USP30NF23.

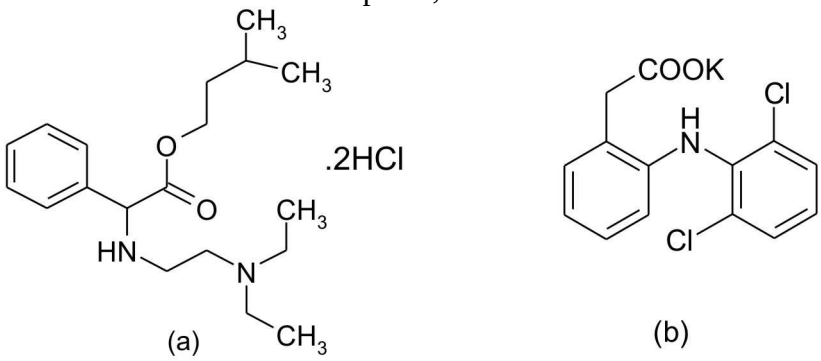

Figure 1. Chemical structure of CAM (a) and DIC (b).

A RP–HPLC method for estimation of CAM in spasmogenic tablets, combination with caffeine, ergotamine tartrate, propyphenazonemecloxamine [7], in tablets using an atomic absorption spectrometric method [8] and in tablets and suppositories using gas chromatography method [9]. Several analytical assays have been published sofar for the quantification of diclofenac in differentmatrices such as plasma, urine, human aqueoushumor and pharmaceutical formulations. They include gas chromatography with electron capture detection [10,11], gas chromatography–mass spectrometry [12], liquid chromatography with UV detection [13–19], with mass detection [20,21], with capillary electrophoresis [21-23] and spectrophotometry in combined dosage form [24, 25].

To our knowledge, there is no method reported for the simultaneous quantification of CAM and DIC in tablets. The present work presents two new methods for simultaneous determination of CAM and DIC in tablets using reversed phase HPLC and HPTLC-densitometry. The two proposed methods are simple, reduce the duration of the analysis and suitable for routine determination of the two drugs.

MATERIALS AND METHODS

2.1. Apparatus

(a)*HPLC system*.- The HPLC system was equipped with 250 binary gradient pump Series 200(Perkin Elmer, USA), a Rheodyne model 7125 injector with a 20 µl loop(Cotati, CA) and 235 diode array UV-Visible detector (Perkin Elmer, USA). HPLC separation was achieved on a RP ODS C18 column (250 mm X 4 mm i.d., 5-µm particle size) (Perkin Elmer, USA) and RP Luna C18 column (250 mm X 4 mm i.d., 5-µm particle size) (Phenomenex, USA). The

analytical column was maintained at 27±°C temperature and data was acquired and processed using Total Chrome HPLC software (Perkin Elmer, USA).

(b) *HPTLC system*.-The HPTLC workstation comprised of Linomat 5 semiautomatic sampleapplicator (Camag, Switzerland) equipped with 100 µl Hamilton syringe (Bonaduz, Switzerland); TLC Scanner 3 densitometric evaluation of thin layer chromatograms (Camag, Switzerland) equipped with mercury, tungsten and deuterium lamp for scanning of TLC plate. The separation was achieved on thin layer plates of silica gel aluminium Plate 60 F-254 (20 cm×10 cm) with 250µm thickness (E. Merck, Mumbai, India). Spectrodensitometric scanning was performed in the reflectance-absorbance mode and operated by winCATS 3.15 software (Camag, Switzerland).The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm.

(c) *Analytical balance.*- BP211D (Sartorius Gottingen AG, Germany)

(d) *Ultrasonicator*.-Ultrasonix TEC-4, (RoopTelesonic, Mumbai, India)

2.2. Reagents and standards

All chemicals and reagents were of analytical or HPLC grade. Standard pharmaceutical samples of CAM and DIC were supplied by the Analytical Development laboratory, M/s Relax pharmaceuticals (Vadodara, India); HPLC grade acetonitrile, chloroform, methanol and triethylamine were obtained from E. Merck (India) Ltd (Mumbai, India); analytical grade ethyl acetate and potassium dihydrogen phosphate from E. Merck (India) Ltd (Mumbai, India); analytical grade acetic acid and ammonia from S. D. Fine Chem. Pvt. Ltd., (Mumbai, India); Naylaflo 0.2µm membrane filter, Pall corporation (Newyork,USA). Triple distilled water was obtained from an all quartz apparatus. All glassware were washed with detergent, rinsed thoroughly with triple distilled water and dried prior to use.

Commercial pharmaceutical preparation ANASPAS TABLET (Khandelwal Laboratories Pvt. Ltd, India) with label claim values of 50 mg CAM and 50 mg DIC per tablet were analyzed.

2.3. Chromatographic conditions

(a) *HPLC method*

Different mobile phases were tested in order to find the best conditions for separating both the drugs simultaneously. The optimal composition of the mobile phase was determined to be a mixture of acetonitrile:25mM potassium dihydrogen phosphate (80:20, v/v) containing 0.1% v/v acetic acid adjusted to pH 7 with triethylamine. The mobile was filtered through a Naylaflo 0.2µm membrane filter and degassed using ultrasonicator. Chromatography was performed using 20 µL injection volume at a flow rate of 1.5 mL/min and the elution was monitored at 215 nm. The average retention time (R_t) for the CAM and DIC were 5.29 and 1.78 min, respectively.

(b) *HPTLC-densitometry*

Solution of CAM and DIC were applied on thin layer plates of silica gel aluminium plate 60 F-254 (10 cm×10 cm) by means of a Linomat5semiautomatic sample applicator. The plates were prewashed by methanol and activated at 110 °C for 5 min prior to chromatography. A constant application rate of 0.1 µL/sec was employed and space between two bands was 5 mm. The slit dimension was kept at 5mm×0.45mm and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The Solvent system used was Chloroform : Ethyl Acetate : Methanol : Ammonia (5:3:2:0.1, v/v/v/v)and 15 mL of mobile phase was used per chromatography at 27± 3°C. Linear ascending development was carried out in twin trough glass chamber (Camag, Switzerland) (Dimensions: length×width×height = 12 cm×4.7 cm×12.5 cm). It was saturated

(lined on the two bigger sides with filter paper that had been soaked thoroughly with the mobile phase) and the chromatoplate development was carried out in dark with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (27°C ± 3). The length of chromatogram run was 9 cm approximately 35 min and air dried. Spectrodensitometric scanning was performed in the reflectance-absorbance mode at 215 nm for all measurements and operated by winCATS 3.15 software (Camag, Switzerland). Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light.

2.4. Preparation of CAM and DIC Standard Stock Solutions

Stock standard solution was prepared by dissolving accurately weighed 500 mg standard CAM and 50 mg of standard DIC in to a 50 mL volumetric flask, dissolved and diluted to the mark with a dilutent (Mobile phase for HPLC and methanol for HPTLC-densitometry). 2.5 mL of this solution was further diluted to 50 mL with a diluent.

2.5. Preparation of sample solutions

To determine the content of CAM and DIC simultaneously in tablets, the twenty tablets were weighed, their mean weight determined and they were finely powdered and powder equivalent to 50 mg CAM and 50 mg DIC was weighed. Then equivalent weight of the drug was transferred into a 50 mL volumetric flask containing 20 mLdiluent (Mobile phase for HPLC and methanol for HPTLC-densitometry), sonicated for 15 min and diluted to 50 mL with mobile phase.Filtered 2.5 mL of this solution was transferred to 50 mL volumetric flask containing 4.5 mL standard CAM solution containing concentration 500 µg/mL and diluted to 50 mL with diluent.The resulting solution containing 500 µg/mL of CAM and 50µg/mL of DIC.

2.6. Method Validation

The aim of method validation was to confirm that the present methods were suitable for its intended purpose as described in ICH guidelines Q2A and Q2B [26]. The described method has been extensively validated in terms of linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ), specificity, robustness and system suitability. The accuracy was expressed in terms of percent recovery of the known amount of the standard drugs added to the known amount of the pharmaceutical dosage forms. The precision (% relative standard deviation) was expressed with respect to the intra-day and inter-day variation in the expected drug concentrations. After validation, the developed methods have been applied to pharmaceutical dosage forms containing CAM and DIC and compared statistically.

(a) *Calibration curve (linearity) of the HPLC method*.-Calibration curves were constructed by plotting peak area vs. concentration of CAM and DIC, and the regression equations were calculated. The calibration curves were plotted over the concentration range 50-300 and 5-30 µg/mL of working solution of CAM and DIC, respectively, prepared by diluting standard stock solution. Triplicate 20 µL injections were made six times for each concentration for CAM and DIC, respectively and chromatographed under the conditions described above.The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

(b) *Calibration curve (linearity) of the HPTLC-densitometry method*.- Calibration curve were plotted over the concentration range of 1500–9000 and 150–900 ng/spot for CAM and DIC, respectively. Accurately prepared mixed standard stock solutions of CAM and DIC (3.0, 6.0, 9.0, 12.0, 15.0 and 18.0 µl) were spotted on the TLC plate to obtain final concentration. Each concentration was spotted six times on the TLC plate. The plate was developed on previously described chromatographic conditions. The calibration curves were constructed by plotting peak

areas vs. concentration with the help of winCATS 3.15 software. Area were plotted against the corresponding concentrations to obtain the calibration graphs.

(c) *Accuracy (%Recovery).*-For both methods recovery studies was carried out by applying the methods to drug sample to which known amount of CAM and DIC corresponding to 50, 100 and 150% of label claim had been added (standard addition method). Known amounts of standard solutions of CAM (100, 200 and 300 µg/mL) and DIC (10, 20 and 30 µg/mL) for the HPLC method and CAM (2250, 4500 and 6750 ng/spot) and DIC (225, 450 and 675 ng/spot) for HPTLC-densitometry method were added to prequantified sample solutions of tablet dosage forms.The amounts of CAM and DIC were estimated by applying these values to the regression equation of the calibration curve. At each level of the amount six determinations were performed and the results obtained were compared with expected results.

(d) *System precision (Repeatability).*-System repeatability was determined by six replicate applications and six times measurement of a sample solution of CAM (200 µg/mL) and DIC (20 µg/mL) for the HPLC method and by scanning of the same spot (n=6) of CAM (4500 ng/spot) and DIC (450 ng/spot)for the HPTLC-densitometry. The repeatability of sample application and measurement of peak area for active compound were expressed in terms ofrelative standard deviation (RSD).

(e) *Intermediate Precision (reproducibility).*-The intra-day and inter-day precisions of the proposed methods were determined by analyzing mixed standard solution of CAM and DIC for 3 times on the same day and on 3 different days over period of 1 week for 3 different concentration of CAM (150, 200 and 250 µg/mL) and DIC (15, 20 and 25 µg/mL) for the HPLC method and CAM (3000, 4500 and 6000 ng/spot) and DIC (300, 450 and 600 ng/spot) for the HPTLC-densitometry. The results are reported in terms of relative standard deviation (RSD).

(f) *Limit of detection (LOD) and limit of quantitation(LOQ).*-The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for the determination of impurities and/or degradation products.ICH guideline (26) describes several approaches to determine the detection and quantitation limits. These include visual evaluation, signal-to-noise ratio and the use of standard deviation of the response and the slope of the calibration curve. In the present study, the LOD and LOQ were based on the third approach and were calculated according to the 3.3σ/S and 10σ/S criterions, respectively; where σ is the standard deviation of the peak area and s is the slope of the corresponding calibration curve.

(g) *Specificity*- Specificity can be described as the capability of the method to accurately measure the response of the analyzed compound with no interferences originating from sample matrix. High percentage recovery observed with assay samples of pharmaceutical dosage forms, including standard addition experiments, indicates that the proposed method was not affected by interferences from excipients used in formulations. The excipients hydroxypropylcellulose, mannitol microcrystalline cellulose, lactose monohydrate, talc and aerosil (Signet Ltd. Mumbai, India) were spiked into a preweighed quantity of drugs to assess the specificity of the methods. The peak area was measured to determine the quantity of drugs.

(h) *Robustness.*-To evaluate HPLC method robustness a few parameters were deliberately varied. The parameters included variation of C18 columns from different manufacturers, flow rate, pH of mobile phase, detection wavelength, column temperature and percentage of acetonitrile in the mobile phase. Two analytical columns, One ODS C18 column (Perkin Elmer, USA) and the other Luna C18 column (Phenomenex, USA), were used during the experiment.

For HPTLC–densitometry method,by introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition were tried and chromatograms were run. The amount of mobile phase, temperature was varied in the range of ±5%. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 min. Spot stability, the time the sample is left to stand on the solvent prior to chromatographic development can influence the stability of separated spots and are required to be investigated for validation. Spot stability was observed by performing 2-dimensional HPTLC development using the same mobile phase (27). Solvent stability of drugs were studied in mobile phase and methanol for HPLC and HPTLC-densitometry, respectively.

(I) Stability of drugs in diluents was studied for 24 h at ambient temperature.

2.6. Analysis of CAM and DIC in Tablet Dosage Forms

The response of tablet dosage forms were measured at 215 nm for quantitation of CAM and DIC by using HPLC and HPTLC-densitometry methods as described above. The amounts of CAM and DIC present in sample solution were determined by applying values of peak area to the regression equations of the calibration curve.

RESULTS AND DISCUSSION

3.1 HPLC method

To optimize the HPLC parameters, several mobile phase composition were tried. Initially various ratio of methanol and water was triedfor each drug individually but satisfactorily peak was not found. Then methanol was replaced by acetonitrile in the same ratio but splitting was observed for both peaks. Then acetonitrile and phosphate buffer pH 7 were tried in the ratio of 60:40 v/v. Again the peaks for both drugs showed splitting. Then above mobile phase in different ratios were tried along with change in pH from 3.0 to 5.0 with the help of acetic acid. But the peak for DIC showed slight negative absorbance and splitting . To rectify it changing pH to 7 by triethylamine both drugs showed typical peak nature and peaks were symmetrical at 215 nm (Figure 2.). Resolutionfor both peaks was more than 2but the asymmetric factor was not satisfactory. To improve the peak asymmetric factor and sharpness of two peaks ratio of acetonitrile:25mM potassium dihydrogen phosphate (80:20, v/v) containing 0.1% v/v acetic acid adjusted to pH 7 with triethylamine at flow rate 1.5 mL/min and this ratio was selected for validation purpose (Figure3). The system suitability test parameters are shown in Table 1.

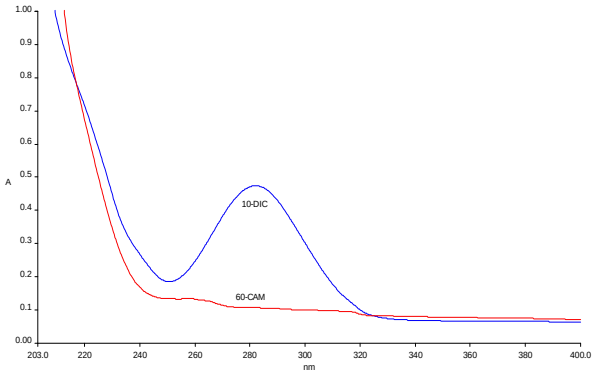

Figure 2. Overlain spectrum of CAM (60 µg/mL) and DIC (10 µg/mL) in HPLC Mobile phase taken on UV–vis spectrophotometer from 200-400 nm (Series 1700, Shimadzu, Japan).

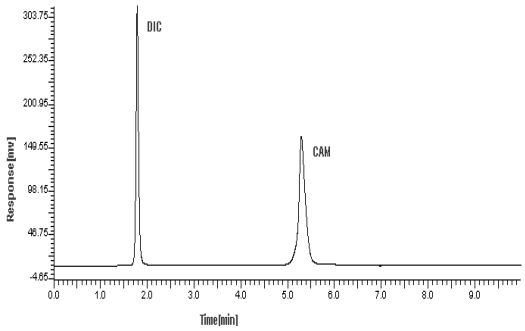

Figure 3. Chromatogram obtained with the mixture of standard CAM (250 µg/mL); (Rt: 5.25 ± 0.083) and DIC (25 µg/mL); (Rt: 1.76 ± 0.029), measured at 215 nm, acetonitrile:25mM potassium dihydrogen phosphate (80:20, v/v) containing 0.1% v/v acetic acid adjusted to pH 7 with triethylamine as mobile phase, at flow rate 1.5 mL/min.

Table1. System suitability parameters of HPLC method

Parameters	Value		Comments
	CAM	DIC	
Calibration range (µg/mL)	50-300	5-30	
Retention time (min)	5.25± 0.083	1.76± 0.029	± Standard deviation
Asymmetric factor	0.848	1.25	Calculated by B/A[a]
Theoretical plates	4813.17	3781.58	Column efficiency
Resolution	7.97	7.97	Calculated by 2(t2-t1)/w2+w1[b]
Injection repeatability (RSD[c], n[d]=6)	0.0119793	0.02649	RSD of area for six injections

[a] A= Distance from the front side of the peak to the peak apex, B= distance from the apex to the peak, both measured at 10% of peak height.
[b] t= Retention times of the peaks; w= baseline widths of the peaks.
[c]RSD= Relative standard deviation, %
[d] n= Number of determination

Table 2. System suitability test parameters of HPTLC-densitometry method

Parameters	CAM	DIC
Retention factor	0.53	0.23
Peak purity	0.9991	0.9993
Calibration range (ng/spot)	1500-9000	150-900
Injection repeatability (RSD[a], n[b]=6)	0.121	0.859

[a] RSD= Relative standard deviation, %
[b] n= Number of determination

3.2. HPTLC-densitometry method

Optimization of HPTLC–densitometry method

Initially chloroform and methanol in the ratio of 5:5 (v/v) and different ratio were tried for both drugs simultaneously. The spots were not developed properly and dragging was observed. Then toluene, ethyl acetate and methanol in the ratio of 3:3:4 (v/v/v) was tried. The developed spots were diffused and R_f was near to solvent front. Then 0.1 mL of ammonia was added to Chloroform and methanol in the ratio of 7:3 (v/v). Total dragging of the spots from the point of sample application was observed. Then 0.1 mL of ammonia was added to chloroform, ethyl acetate and methanol in the ratio of 3:3:4 (v/v/v). The spots developed were dense, compact and typical peak nature for both CAM and DIC were observed but resolution between them was less.

To improve the resolution, the volume of chloroform was increased by 1 mL and that of methanol was reduced by 1 mL. Ultimately mobile phase consisting of chloroform:ethylacetate:methanol:ammonia (5:3:2:0.1, v/v/v/v) gave good resolution. Both the peaks were symmetrical in nature and no tailing was observed when plates were scanned at 215 nm (Figure 4.). The chamber was saturated with the mobile phase for 30 min at room temperature (Figure 5.). The system suitability test parameters are shown in Table 2.

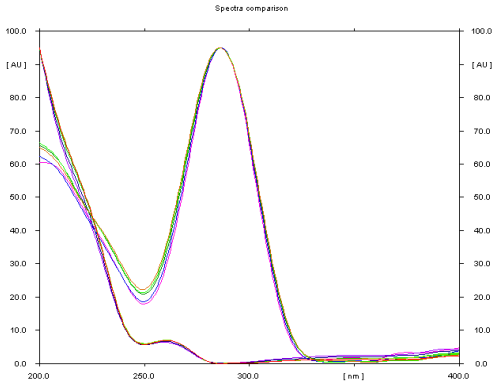

Figure 4. In situ overlain spectra of CAM and DIC measured from 200 to 400 nm.

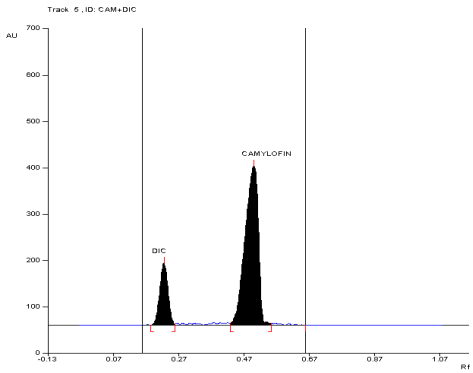

Figure 5. Spectrodensitogram of standard CAM (6000 ng/spot); peak 2 (Rf: 0.53±0.03) and DIC (600 ng/spot); peak 1 (Rf: 0.23±0.02), measured at 215 nm, mobile phase chloroform:ethyl acetate:methanol:ammonia (5:3:2:0.1, v/v/v/v).

3.3. Validation of the Proposed Methods

Linearity.- CAM showed good correlation coefficient in concentration range of 50–300µg/mL(r = 0.9993) and 1500-9000 ng/spot (r = 0.9991) for HPLC and HPTLC-densitometry, respectively, where as DIC in the concentration range of 5–30 µg/mL(r = 0.9994) and 150–900 ng/spot (r = 0.9998) for HPLC and HPTLC-densitometry, respectively. For both methods the linearity of calibration graphs and adherence of the system to Beer's law was validated by high value of correlation coefficient. Data of the regression analysis are summarized in Table 3.

Accuracy.-Both the proposed methods when used for extraction and subsequent estimation of CAM and DIC from pharmaceutical dosage form after spiking with standard additional drug afforded recovery of 98–102% and mean recovery for CAM and DIC from the marketed formulation were 99.70 ± 0.90 and 99.94 ± 0.41for CAM and DIC, respectively by HPLC method and 99.96 ± 0.13and 99.92 ± 0.09for CAM and DIC, respectively by HPTLC-densitometry method(Table 8). The data are presented in Table 4a and b show the excellent recoveries of the added standard drugs and validate the good accuracy of both methods.

System precision (repeatability).- The repeatability of measurement of peak area were expressed in terms of RSD and were found to be 0.037 and 0.026 for CAM and DIC, respectively using HPLC method and 0.121 and 0.859 for CAM and DIC, respectively using HPTLC-densitometry method.

Intermediate precision (reproducibility).-The intermediate precision of the methods were assessed by carrying out determinations of three different concentrations (high, medium and low) of CAM and DIC both on intra-day and inter-day. TheRSD values of intra-day were 0.24-0.99 and 0.66-1.45% for CAM and DIC using HPLC method, respectively and 0.37-0.89 and 0.34-0.67% for CAM and DIC using HPTLC-densitometry method, respectively. The RSD values of inter-day were 0.42-0.97 and 0.77-1.22% for CAM and DIC using HPLC method, respectively and 0.56-0.87 and 0.31-0.68% for CAM and DIC using HPTLC-densitometry method, respectively. The low RSD value indicating good precision and there was no significant difference between the assays which were tested using the both methods on the same day or different days. The intra-day and inter-day precision has been depicted in Table 5.

Limit of Detection and Limit of Quantitation.-The signal to noise ratios 3:1 and 10:1 were considered as LOD and LOQ, respectively. LOD for CAM and DIC were found to be 6.763and 0.036 µg/mL, respectively for HPLC method and 90.23 and 11.29 ng/spot, respectively, for HPTLC-densitometry method. LOQ for CAM and DIC were found to be 22.319and 0.122 µg/mL, respectively for HPLC method and 298.25 and 37.26 ng/spot, respectively, for HPTLC-densitometry method (Table 3.). These data show that both methods are sensitive for the determination of CAM and DIC.

Specificity.-The specificity of the HPLC method is illustrated in (Figure 6.) where complete separation of CAM and DIC were noticed in presence of tablet excipients. The average retention time ± standard deviation for CAM and DIC were found to be 5.29 ± 0.071 and 1.78 ± 0.046 min, respectively, for six replicates. The peaks obtained were sharp and have clear baseline separation.

For HPTLC–densitometry method. The peak purity of CAM and DIC were assessed by comparing their respective spectra at peak start, peak apex and peak end positions of the spot. Good correlation (r = 0.9991 and 0.9993) was obtained for sample spectra of CAM and DIC, respectively (table 2). Hence, the methods were found to be specific for estimation of CAM and DIC.

Robustness.-For HPLC method, each factor selected (except columns from different manufacturers) to examine were charged at three levels (−1, 0 and 1). One factor at the time was changed to estimate the effect. Thus, replicate injections (n = 6) of mixed standard solution at three concentration levels were performed under small changes of six chromatographic parameters (factors). Results, presented in Table 6a indicate that the selected factors remained unaffected by small variations of these parameters. The results from the two columns indicated that there is no significant difference between the results from the two columns. Insignificant differences in peak resolution, asymmetric factor and less variability in retention time were observed. For HPTLC-densitometry method, the standard deviation of peak areas was calculated for each parameter in HPTLC-densitometry and RSD was found to be less than 2 %. The low values of RSD as shown in Table 6b indicated robustness of the method. Hence, the methods were found to be robust for simultaneous determination of CAM and DIC.

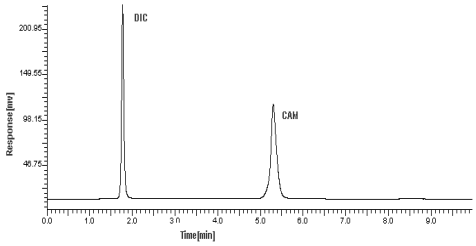

Figure 6. Chromatogram of CAM (200 µg/mL) and DIC (20 µg/mL) in presence of common tablet excipients, measured at 215 nm.

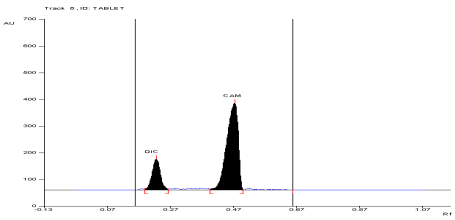

Figure 7. Chromatogram of sample CAM (4500ng/spot) and DIC (450 ng/spot) measured at 215 nm, mobile phase chloroform:ethyl acetate:methanol:ammonia (5:3:2:0.1, v/v/v/v).

Table 3. Linear regration data for calibration curve (n=6)

Parameters	HPLC		HPTLC-densitometry	
	CAM	DIC	CAM	DIC
Linear Range	50-300 (µg/mL)	5-30 (µg/mL)	1500-9000 (ng/spot)	150-900 (ng/spot)
Slope ± S.D.	11745 ± 154.91	90325 ± 1109.22	1.2826 ± 0.061	3.4753 ± 0.144
Intercept ± S.D.	29797 ± 301.66	116.17 ±21.59	689.69 ± 307.64	118.52 ± 71.88
Correlation coefficient (r)	0.9993	0.9994	0.9991	0.9998
Limit of Detection	6.763 (µg/mL)	0.036(µg/mL)	90.23(ng/spot)	11.29 (ng/spot)
Limit of Quantitation	22.319 (µg/mL)	0.122 (µg/mL)	298.25(ng/spot)	37.26(ng/spot)

Table 4 a and b. Standard addition technique for determination of CAM (a) and (b) DIC by HPLC and HPTLC densitometry method.

HPLC					HPTLC-densitometry				
Excess standard drug added to the sample (%)	Theoretical content (µg)	Recovery (%)	RSD	S.E	Excess standard drug added to the sample (%)	Theoretical content (ng)	Recovery (%)	RSD	S.E
(a) CAM									
0	200	99.09	0.97	0.98	0	4500	100.14	1.31	0.80
50	300	98.80	1.02	0.82	50	6750	99.82	0.79	0.50
100	400	100.18	1.24	0.48	100	9000	99.94	1.04	0.74
150	500	100.71	1.79	0.45	150	11250	99.92	1.33	0.97
(b) DIC									
0	20	100.45	0.81	0.63	0	450	99.83	0.87	0.61
50	30	100.00	1.34	0.78	50	675	99.85	1.03	0.82
100	40	99.45	1.13	0.72	100	900	100.02	1.25	0.83
150	50	99.84	1.13	0.97	150	1125	99.97	0.73	0.80

Table 5. Summary of intra-day and inter-day (intermediate precision) variability data for CAM and DIC using HPLC and HPTLC-densitometry method

Compound	HPLC			HPTLC-densitometry		
	Theoretical drug concentration (µg/mL)	Mean drug found (µg/mL ± S.D,[a])	RSD%	Theoretical drug concentration (ng/spot)	Mean drug found (ng/spot ± S.D.[a])	RSD%
CAM	Intra-day					
	150	149.58 ± 1.15	0.99	3000	2966.66 ± 11.88	0.40
	200	199.23 ± 0.97	0.48	4500	4447.21 ± 39.78	0.89
	250	248.88 ± 0.60	0.24	6000	5962.24 ± 21.84	0.37
	Inter-day					
	150	148.41 ± 0.91	0.62	3000	2968.00 ± 25.86	0.87
	200	197.44 ± 1.92	0.97	4500	4455.56 ± 33.94	0.76
	250	248.23 ± 1.04	0.42	6000	5953.24 ± 33.42	0.56
DIC	Intra-day					
	15	14.68 ± 0.10	0.66	300	296.83 ± 1.10	0.34
	20	19.70 ± 0.29	1.45	450	443.04 ± 2.95	0.67
	25	24.44 ± 0.22	0.89	600	594.47 ± 3.53	0.59
	Inter-day					
	15	14.71 ± 0.11	0.77	300	297.59 ± 2.02	0.68
	20	19.72 ± 0.24	1.22	450	442.72 ± 2.71	0.61
	25	24.27 ± 0.23	0.94	600	593.02 ± 1.85	0.31

[a] Mean value represents five different sample standards for each concentration

Table 6. Robustness (a) testing of HPLC and (b) evaluation of the HPTLC method (n=6)

Chromatographic changes		CAM		DIC		
Factor[d]	Level	*Rt*[e]	*T*[f]	*Rt*[e]	*T*[f]	*R*[g]
(a) Robustness evaluation of the HPLC method (n=6)						
A:pH of mobile phase						
6.90	-1	5.28	0.86	1.87	1.25	8.07
7.00	0	5.31	0.84	1.81	1.25	7.74
7.10	1	5.38	0.88	1.76	1.26	8.09
Mean ± S.D		5.32±0.05	0.86±0.02	1.81±0.06	1.25±0.01	7.97±0.20
B:% of acetonitrile in mobile phase						
78	-1	5.36	0.86	1.76	1.25	8.05
80	0	5.28	0.88	1.83	1.24	7.90
82	1	5.21	0.85	1.87	1.26	8.08
Mean ± S.D		5.28±0.08	0.86±0.01	1.82±0.06	1.25±0.01	8.01±0.10
C: wavelength of detection						
214 nm	-1	5.29	0.84	1.77	1.25	7.85
215 nm	0	5.25	0.83	1.88	1.23	7.72
216 nm	1	5.25	0.81	1.82	1.23	7.80
Mean ± S.D		5.26±0.02	0.83±0.02	1.82±0.05	1.24±0.02	7.79±0.07
D:Flow rate						
1.40	-1	5.38	0.88	1.76	1.29	5.38
1.50	0	5.29	0.87	1.82	1.24	5.29
1.60	1	5.27	0.85	1.88	1.27	5.27
Mean ± S.D		5.31±0.06	0.87±0.01	1.82±0.06	1.27±0.02	5.31±0.06
E:Column temperature						
25	-1	5.36	0.84	1.74	1.27	7.73
27	0	5.27	0.86	1.77	1.28	7.80
29	1	5.23	0.85	1.87	1.20	7.84
Mean ± S.D		5.29±0.07	0.85±0.01	1.79±0.07	1.25±0.04	7.79±0.06

F:Column from different manufacturer					
ODS C_{18}(Perkin Elmer)	5.31	0.87	1.74	1.24	7.94
Luna (Phenomenex)	5.24	0.82	1.86	1.24	7.78
Mean ± S.D	5.28±0.05	0.84±0.03	1.80±0.09	1.24±0.01	7.86±0.11

Parameter	CAM		DIC	
	SD[b] of peak area	RSD[b]	SD[b] of peak area	RSD[b]
(b) robustness of HPTLC-densitometry method				
Mobile phase concentration	1.28	1.08	1.45	1.47
Amount of mobile phase	1.11	1.35	1.66	1.13
Relative humidity	1.39	1.23	1.25	1.41
Temperature	0.84	0.80	0.87	0.94
Plate pretreatment	0.81	0.31	0.91	0.64
Time from spotting to chromatography	0.73	0.53	0.64	0.74

[a] *n=6.*
[b] *Average of three concentrations 150, 200 and 250 µg/mL and 15, 20 and 25 µg/mL for CAM and DIC, respectively.*
[c] *Average of three concentrations 3000, 4500 and 6000 ng/spot and 300, 450 and 600 ng/spot for CAM and DIC, respectively.*
[d] *Five factors were slightly changed at three levels (1, 0, −1); each time a factor was changed from level (0) the other factors remained at level (0).*
[e] *Retention time.*
[f] *Asymmetric factor.*
[g] *Resolution between CAM and DIC.*

3.5. Analysis of the Marketed formulation (CAM and DIC 50 mg/ Tablet)

(a) *For HPLC method.-*

The peaks at Retention time 5.29 min (for CAM) and 1.77 (for DIC) were observed in the chromatogram of the drug samples extracted from tablets. Experimental results of the amount of CAM and DIC in tablets, expressed as percentage of label claim were in good agreement with the label claims, thereby suggesting that there is no interference from any excipients, which are normally present in tablets. The drug content was found to be 99.40 ± 0.69 (RSD.of0.48) and 99.18 ± 0.56 (RSD. of 0.31) for CAM and DIC, respectively (Table 7.).

(b) *For HPTLC–densitometry method.-*

The spots at R_f0.53 (for CAM) and 0.23 (for DIC) were observed in the spectrodensitogram of the drug samples extracted from tablets (figure 7.). There was no interference from the excipients commonly present in the tablets. It may therefore be inferred that degradation of CAM and DIC had not occurred in the marketed formulations that were analyzed by this method as shown in Table 8. The low RSD value indicated the suitability of this method for routine analysis of CAM and DIC in pharmaceutical dosage form. Statistical evaluation was performed using Student's *t*-test and the *F*-ratio at 95% confidence level as shown in Table 7.

The data of summary of validation parameters are listed in Table 8.

Table 7. Applicability of the proposed methods for the determination of CAM and DIC in commercial tablets (n=5)

Parameters	HPLC		HPTLC-densitometry	
	CAM	DIC	CAM	DIC
Label claim (mg)	50	50	50	50
Drug content (%) ± S.D.	99.40 ± 0.69	99.18 ± 0.56	99.40 ± 0.69	99.04 ± 0.46
RSD	0.48	0.31	0.48	0.21
S.E.	0.28	0.22	0.28	0.19
t-value[a]	0.45	1.14		

[a] The theoretical *t*-values is equal to 2.77(*P*=0.05).

Table 8. Summary of validation parameters; statistical data for the calibration curves of CAM and DIC by HPLC and HPTLC-densitometry

Parameters	HPLC		HPTLC-densitometry	
	CAM	DIC	CAM	DIC
Linearity range	50 - 300 (µg/mL)	5 - 30 (µg/mL)	1500-9000 (ng/spot)	150-900 (ng/spot)
Limit of detection	6.763 (µg/mL)	0.036(µg/mL)	90.23(ng/spot)	11.29 (ng/spot)
Limit of quantitation	22.319 (µg/mL)	0.122 (µg/mL)	298.25(ng/spot)	37.26(ng/spot)
Recovery (% ± S.D.)	99.70 ± 0.90	99.94 ± 0.41	99.96 ± 0.13	99.92 ± 0.09
Precision (RSD)				
Repeatability of application (n=6)	0.037	0.026	0.121	0.859
Intraday (n=6)	0.57	1.00	0.55	0.53
Interday (n=6)	0.67	0.97	0.73	0.54
Robustness	Robustness	Robustness	Robustness	Robustness
Specificity	0.071	0.046	0.9991	0.9983
Solvent suitability	Suitable for 24 h	Suitable for 24 h	Suitable for 24 h	Suitable for 24 h

3.6. Comparison of the proposed methods (HPLC versus HPTLC)

Six different sampled of marketed tablet were taken for determined CAM and DIC simultaneously byHPLC and HPTLC-densitometry methods. Each sample was analyzed in duplicate. To test differences between the proposed HPLC and HPTLC-densitometry methods statistical tests were performed for the level of confidence 95% (P = 0.05). To test means (averages) a paired t-test was applied. The test removes any variations between samples [28]. The obtained value of t statistical value 0.45 for CAM and 1.14 for DIC waslower than two tail t critical value 2.77 (n=5), which leads to the conclusion that there is no significant difference between the means (Table7.).

The literature describes an HPLC method [6] for determination of CAM and HPLC [24] for determination of DIC in tablet dosage forms. The assay results obtained by these methods were used for statistical comparison to evaluate the validity of developed HPLC and HPTLC methods. The calculated F-value for CAM and DIC was found to be 0.97 and 1.20 for HPLC method, respectively and 1.48 and 2.77 for HPTLC-densitometry method, respectively. The calculated F-value were less than the tabulated F-value 3.89 at 95% (P=0.05) confidence level. Therefore, there was no significant difference among the two methods.

CONCLUSION

The proposed HPLC and HPTLC-densitometrymethods provide simple, accurate and reproducible quantitative analysis for simultaneous determination of CAM and DIC in tablets. Both the methods were validated as per ICH guidelines. Six real samples of tablets were determined by HPLC and HPTLC-densitometry methods and the results were correlated. Statistical tests indicate that the proposed HPLC and HPTLC-densitometry methods reduce the duration of analysis and appear to be equally suitable for routine determination of CAM and DIC simultaneously in pharmaceutical formulation.

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