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A NOVEL VALIDATION HPTLC METHOD FOR THE QUANTITATIVE DETERMINATION OF CLOFAZIMINE

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ABSTRACT

A novel, highly sensitive high-performance thin-layer chromatography (HPTLC) method for analysis of clofazimine (CFZ) was developed and validated. Separation was achieved on aluminum plates pre-coated with silica gel 60 F_{254} using mobile phase toluene/ethyl acetate/methanol/glacial acetic acid (6:3:1:0.1, v/v/v/v) at room temperature (25 ± 2 ºC) for CFZ. The analytes were quantified spectrodensitometrically at 286 nm. The calibration curve was linear (r^2 = 0.9974 ± 0.0012). Various parameters like limit of detection (LOD) and quantification (LOQ), accuracy (% DEV), precision (%RSD) and robustness of the quantification method were assessed. Also the method was employed to estimate drug levels in biological fluids and to study *ex-vivo* permeation profile of simple ointment of CFZ through rat skin. The method was found to be simple, rapid, accurate, reproducible, cost-effective and finds suitability in estimating drug levels in biological tissues and fluids and for routine analysis in bulk and pharmaceutical dosage forms. The primary advantage of the proposed procedures is the sensitivity, which surpasses the sensitivity of previously reported procedures

KEYWORDS: **HPTLC, Validation, Quantitative analysis, Clofazimine and Leprosy.**

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INTRODUCTION

CFZ is a substituted iminophenazine bright-red dye originally developed in 1954 for the treatment of tuberculosis [1]. CFZ is mainly indicated in the treatment of lepromatous leprosy and erythema nodosum leprosum, and has been included as an anti-leprosy medicine in the current WHO Model Lists of Essential Medicines [2,3]. Currently, CFZ is given to patients in oral regimens of 100 to 300 mg, one to three times a day for 3 months to 4 years, depending on the disease and the response of the patients [4]. In humans, the absorption of orally administered drug varies considerably (45 % – 62 %) depending on whether the drug is taken with or without food [5]. CFZ is excreted slowly, largely in the unchanged form [6]. CFZ passes into breast milk too. Being lipophilic, the drug tends to remain for a long time in human tissues (fat, skin lymph nodes, intestines, adrenal glands and spleen) and less than 1 % of metabolites are recovered in the urine within a 24 h period [7]. The drug concentrates in macrophages, and serum levels are often low or undetectable [8]. So, to assess the CFZ levels in plasma, different tissues, urine and milk, a very sensitive and accurate method is required. Various previously reported techniques for estimation of CFZ *i.e.* colorimetric [9], fluorometric [10], spectrophotometric detection and high performance liquid chromatography (HPLC) methods [11-15] are associated with drawbacks like lack of sensitivity, more time consumption and high cost. Therefore, considering the significance of the sensitivity and reliability of HPTLC technique, an effort is made for the first time to develop a highly sensitive HPTLC method useful for determination of CFZ in skin tissue, plasma and milk. In addition, the developed method is used for estimation of drug in skin permeation studies.

MATERIALS AND METHODS

CFZ was received as a free gift sample from Sangrose Labs Pvt Ltd. (Kerela, India). Toluene, Ethyl acetate, methanol and glacial acetic acid were analytical grade purity (Merck, Mumbai, India). Milk (Verka, Milkfed, India) with protein and fat content of 3.3 % w/w and 1.5 % w/w, respectively, was procured from local market. All chemicals and reagents were used without further purification. Male Wistar rat weighing 232 g, bred in the Central Animal House facility of Panjab University, Chandigarh was used (IAEC/221).

(i) HPTLC Instrumentation

A Camag HPTLC system (Muttenz, Switzerland) was used comprising an automatic Camag TLC sampler 4 fitted with a 100 μl syringe, Camag TLC plate heater III, Camag UV cabinet and Camag TLC scanner 3 (deuterium lamp). The Camag TLC Scanner 3 was equipped with user-friendly WinCATS planar chromatography manager software (version 1.4.2) installed on a personal computer. The twin-trough glass chambers (20 cm × 10 cm, Camag, Muttenz, Switzerland) with tight fitting lids and pre-coated silica gel aluminium plates 60 F_{254} (20 cm × 10 cm with 0.2 mm thickness, Merck, Darmstadt, Germany) were used.

(ii) Chromatographic conditions

The plates were prewashed with methanol and activated at 75 ± 0.5 ºC for 10 min using Camag TLC plate heater III to remove elutable components, if any and a clean layer background for visual evaluation be produced when scanning. The slit dimensions were kept at 5 mm x 0.45 mm and the scanning speed of 20 mm/s was employed. The data resolution was 100 μm/step. The 6 mm wide bands were applied on the TLC plates, 10 mm from the bottom, by means of a pressurized nitrogen gas (150 kg/cm^2) through automatic TLC sampler. The distance between the tracks varied depending on the type of analysis. The application parameters were identical for all the analyses performed. Then, the plates were conditioned for 10 min at room temperature (25 ± 2 ºC). Mobile phase consisted of toluene/ethyl acetate/methanol/glacial acetic acid (6:3:1:0.1, v/v/v/v). A total volume of 20 ml,

mobile phase was utilized for the development of each TLC plate. The development chambers were previously saturated with the mobile phase for 10 min at room temperature (25 ± 2 ºC) and relative humidity (55 ± 5 %). The chromatogram was run for 80 mm in length (migration time of 20 min). Subsequent to the development, plates were dried at 60 ± 0.5 ºC before densitometry analysis. Densitometry scanning of the CFZ was performed at 286 nm in absorption mode. This wavelength was selected through preliminary experiments as it corresponded to the near absorption maxima of CFZ. CFZ was quantified from the intensity of diffusely reflected light and evaluated by retention factor (R_F) values and UV spectrum along with the peak area measurement.

(iii) Preparation of calibration standards

A stock solution of CFZ (10 µg/ml) was prepared in chloroform. Different volumes of the stock solution, 2, 3, 4, 5, 6 and 8 µl were applied as band (6 mm) to the TLC plate (20 cm × 10 cm) leading to calibration standards of 20, 30, 40, 50, 60 and 80 ng/band of CFZ respectively. The calibration standards were prepared freshly in triplicate for each chromatographic application. The calibration curve was plotted by using data of peak area against the corresponding amount per band which was treated by least-square regression analysis.

(iv) Preparation of biological samples

Blood sample was collected in heparinized tubes under slight ether anesthesia from tail vein of male wistar rat and then sacrificed by decapitation. From the blood portion, plasma was separated by ultracentrifugation at 1000 rpm for 10 min at 4 ºC. Also, the rat skin was excised and hairs were removed from isolated skin. A piece weighing 1 g of freshly excised defatted abdominal skin of rat was cut into pieces, kept in 10 ml double distilled water at 4 °C for 24 h with intermittent ultra-sonication to ensure maximum extraction of any biological substance and any leachable matter from skin that may interfere during drug analysis [16].

(v) Analysis of biological samples

To assess system suitability and to quantify CFZ in biological samples, an accurately measured (1 ml) CFZ-chloroform solution (10 µg/ml) was added to equal volume of rat blood plasma, defatted abdominal skin homogenate (1 % w/v in water) and milk, respectively. The mixtures obtained were sonicated in a bath sonicator for 30 min intermittently. Then, organic layer from each mixture was separated by ultra-centrifugation at 4 ± 0.5 ºC and analyzed after application on TLC plates with corresponding blank treated solutions (chloroform without CFZ) and standard solution. All the respective samples were analyzed separately by HPTLC as described above to check the interference of extracted biological substance(s) from skin, plasma or milk, if any, to estimate accurate levels of CFZ extracted from study involving skin tissue.

(vi) Specificity

The specificity of the method was ascertained by analyzing standard CFZ solution and biological samples. The band for CFZ in samples was confirmed by comparing the R_F and spectra of the band with that of standard. The peak purity of CFZ was assessed by comparing the peak start (S), peak apex (M) and peak end (E) positions of the band in the spectra.

(vii) Accuracy and precision

Quality control stock samples were prepared daily in chloroform in concentrations of 10 µg/ml as described above for the calibration standard. From this quality control stock solution of 10 µg/ml, 2 µl was applied on the TLC plate in order to result in a concentration of 20 ng/band as limit of quantification. From the same solution, 4 µl and 8 µl were applied so as to result a final concentration of 40 ng/band (intermediate concentration) and 80 ng/band (highest concentration), respectively. Measurement of peak area was carried out at three different concentration levels (20, 40 and
80 ng/band) by applying in hexaplicate on the same day and repeated on three separate occasions in triplicate to determine the intra-day and inter-day accuracy and precision,

respectively. The area obtained from each set of quality control sample containing CFZ was evaluated from the calibration curve to calculate concentration in the band. The intra and inter-day accuracy and precision of the assay were assessed by the average relative percentage deviation (% DEV) from the nominal concentration and the relative standard deviation (% RSD) values, respectively, based on the reported guidelines [17-19].

Precision (RSD) and accuracy (DEV) were calculated by the following equations:

RSD = (standard deviation/ average calculated concentration) × 100

DEV = (1 − average calculated concentration/nominal concentration) × 100

The method validation was performed following the reported procedures in the literature [17, 20].

(viii) Limit of quantification and limit of detection

In order to determine detection and quantification limits, CFZ solutions of 20, 40 and 80 ng/band were applied in triplicate. The amount of CFZ by spot versus average response (peak area) was graphed and the equation for this curve was determined, thereby obtaining an estimate of the target response: ybl. The ybl value corresponds to the intersection of the curve. Subsequently, a second curve was graphed showing the amount of CFZ by spot versus the standard deviation of the responses. From the equation of this curve, we obtained an estimate of the standard deviation for target: sbl, which corresponds to the intersection of this curve. Detection and quantification limits were calculated by means of the equations: detection limit = (ybl + 3 sbl)/*b*; quantification limit = (ybl + 10 sbl)/*b*, where "*b*" corresponds to the slope obtained in the linearity study of the method [21, 22].

(ix) Robustness of the method

Time from spotting of CFZ on TLC plate to development of the plate and time from development of plate to scanning was varied as 10 and 30 min. Robustness of the method was checked at two different concentration levels of 20 and 40 ng/band.

(x) Repeatability

In order to study the influence of different plates and the effect of vehicle on the chromatography of CFZ, repeatability studies were performed with quality control samples. These samples at two different concentration levels (80 and 100 ng/band) were applied as 12 tracks on three different TLC plate on the same day. The repeatability of the method was considered to be acceptable if on each plate bands are identical with respect to position and intensity and form parallel lines on the chromatogram. The R_F value for CFZ on
the three plates should not vary more than 0.02 [20].

(xi) Stability

The quality control sample (10 μg/ml) was prepared and stored in tight capped volumetric flask at room temperature for 6 h. The solution was then spotted (20 ng/band) on the TLC plate and analyzed by method demonstrated in the presence of any additional peak. Similarly, band stability was analyzed by keeping the resolved plate and inspecting at intervals of 10 and 60 min.

(xii) Analysis of marketed formulation

To determine the amount of CFZ in capsules (Lamprene® labeled claim: 50 mg CFZ per capsule), the contents of one capsule were dissolved in chloroform to obtain 33.33 ng/ml of CFZ. From this solution, 2 µl was applied on TLC plate followed by development and scanning as described earlier.

(xiii) Ex-vivo skin permeation studies

Experiments were run in Franz diffusion cells (PermeGear, Inc., PA, USA) having a receptor compartment volume of 30 ml and available diffusion area of 3.14 cm^2. Skin permeation studies were carried out in Franz diffusion cells maintained at 32 ± 0.5 °C with receiver compartment containing ethanolic dilute acetic acid (1:1). Freshly excised hairless defatted abdominal skin of rat was mounted with dermis facing the receiver cell.

Then 1 g drug loaded simple ointment B.P. (0.2% w/w) was applied on to skin surface non-occlusively[23]. Ethanolic dilute acetic acid (1:1) was used as receptor media. The cell contents were stirred by externally driven magnetic bar at 500 rpm and were maintained at temperature of 32 ± 1˚C by circulating water through an external jacket of the cell. An aliquot of 2 ml was periodically withdrawn at suitable time intervals from the sampling arm of receptor chamber. Fresh diffusion media was simultaneously replaced in the receptor chamber. Samples were analyzed at the end of 24 h by the method described above.

(xiv) Statistical analysis

Confidence limits were calculated with the help of Graph Pad Prism version 5 software (trial version).

RESULTS AND DISCUSSION

1. Optimization of chromatogram

The TLC procedure was optimized with a view to quantify CFZ in biological samples. Different solvents such as chloroform, acetic acid, dichloromethane, n-propanol, n-butanol, liquid ammonia, methanol, ethyl acetate, acetonitrile and toluene were investigated to get a well-defined and stable peak of CFZ. The selected optimized mobile phase comprised of toluene/ethyl acetate/methanol/acetic acid (6:3:1:0.1 v/v/v/v). Well defined bands at R_F value of 0.25 ± 0.02 (figure 1) were obtained when the chamber was saturated with mobile phase for 10 min at room temperature.

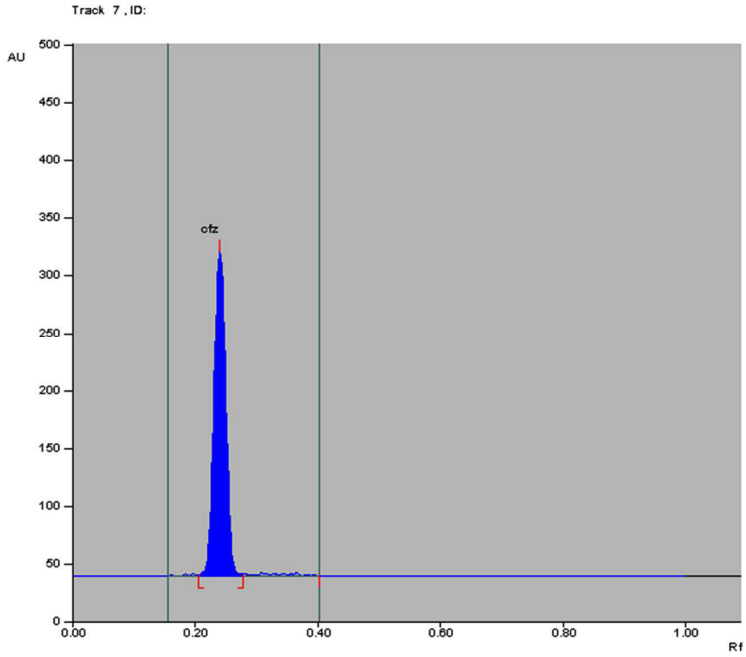


Figure 1
Chromatogram of standard CFZ (20 ng/band): peak (R_F = 0.25 ± 0.02), mobile phase: toluene-ethyl acetate–methanol–acetic acid (6:3:1:0.1)

2. Linearity and calibration curves

The developed HPTLC method for estimation of CFZ showed a good correlation coefficient (r^2 ± SD = 0.9974 ± 0.0012) in concentration range of 20–80 ng/band with respect to the peak area (figure 2). The data for the triplicates of the standard samples are shown in table 1. The linearity of calibration graphs was indicated by the high value of correlation coefficient and the standard deviation for the intercept value was 3 %. No significant difference was observed between the slopes of standard curves ($p > 0.05$).

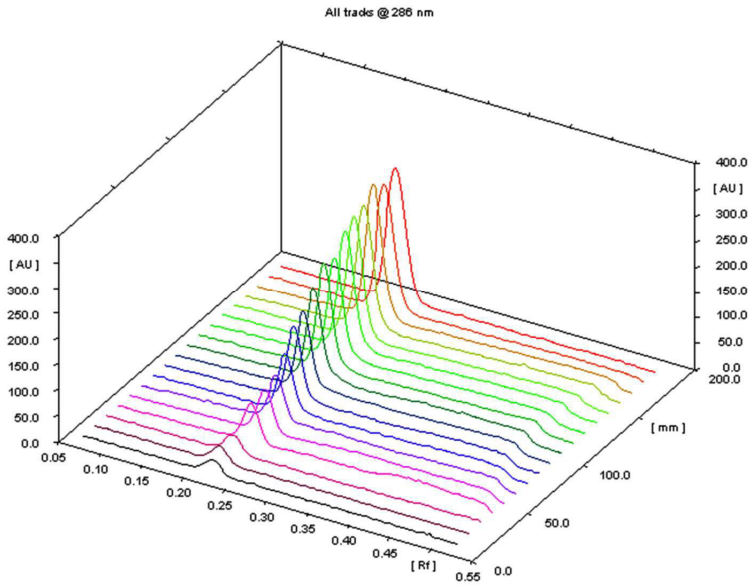


Figure 2
Three-dimensional image of the HPTLC densitograms of the calibration samples of CFZ at 286 nm.

3. Accuracy and Precision

The accuracy and precision of the results for inter-day and intra-day quality control samples were within an acceptable level with % DEV and % RSD values as presented in table 2. The reproducibility of the method was evaluated by studying these samples on three different occasions in triplicate.

4. Limit of detection and quantification

Detection and quantification limits with signal-to-noise ratios of 3:1 and 10:1 were considered. The LOD and LOQ were found to be 2.59 and 7.86 ng/band, respectively, which indicates excellent sensitivity of the method.

TABLE 1
LINEAR REGRESSION DATA FOR CALIBRATION CURVES (n=3)

Parameters (Units)	TLC densitometry
Linearity range (ng/band)	20 – 80
Correlation coefficient (r^2) ± SD	0.9974 ± 0.0012
Slope ± SD	46.426 ± 3.792
Intercept ± SD	404.63 ± 29.85
R_f ± SD	0.25 ± 0.02
Confidence limit of slope[a]	40.843 – 51.107
Confidence limit of intercept[a]	340.61 – 519.20

[a] *95% confidence limit*

TABLE 2
PRECISION AND ACCURACY OF THE METHOD

Nominal Concentration (ng/band)	Intra-Day		Inter-Day	
	Precision (%RSD)	Accuracy (%Dev)	Precision (%RSD)	Accuracy (%Dev)
20	0.71	-1.90	3.42	-3.55
40	2.09	0.63	1.02	2.65
80	0.20	-0.63	1.90	0.33

5. Specificity

The peak purity of CFZ was assessed by comparing the spectra at peak start, peak apex and peak end position of the band, *i.e.*, r^2 (S, M) = 0.999 and r^2 (M, E) = 0.999. These correlation value indicate the ability of the method to separate and specifically detect CFZ from the sample solutions as described in sections 2.5.1 and 2.5.2. The overlaid spectra of CFZ standard and CFZ from various biological sample extracts (milk and plasma) and skin are given in figure 3 and figure 4, respectively. This signifies the competent way for the chromatographic estimation of CFZ in biological samples.

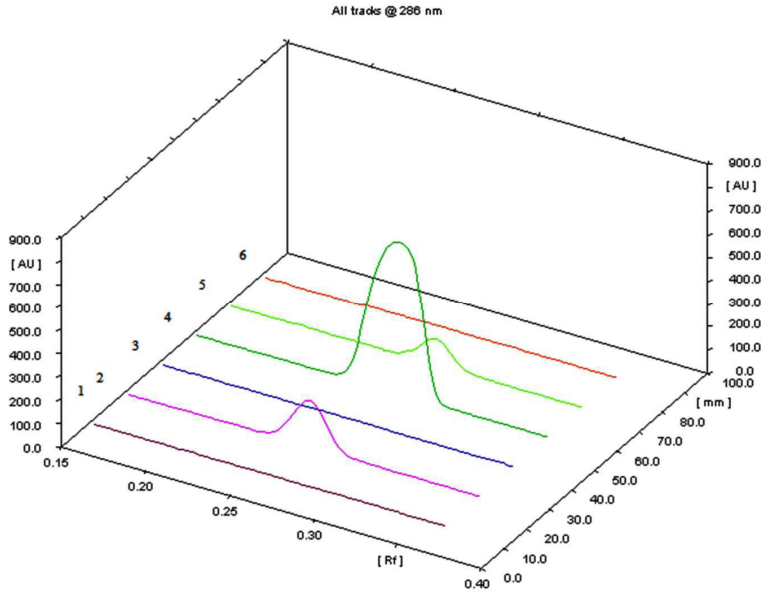


Figure 3

Representative three-dimensional densitogram of the TLC plate shown in numbered tracks represent the following chromatographic bands: (1) blank plasma; (2) CFZ in plasma; (3) milk; (4) CFZ in milk; (5) working standard of CFZ in chloroform (10 ng/band); and (6) chloroform

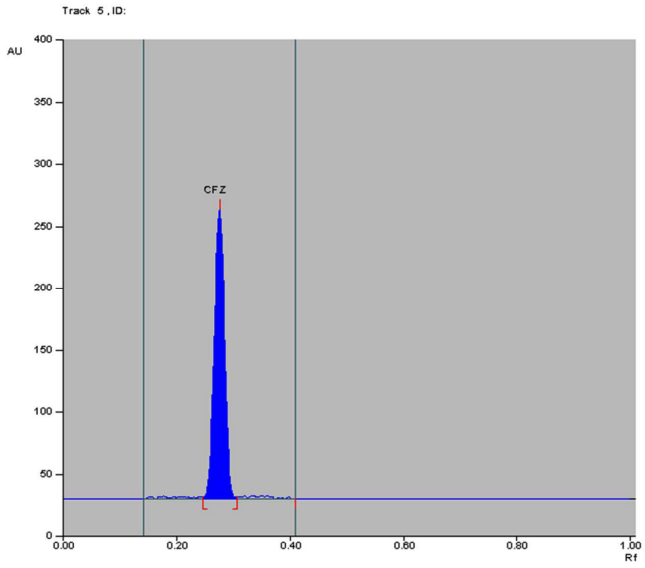


Figure 4

Chromatogram of CFZ from skin sample

5. Robustness of the method

Low % RSD value of 0.137 and 0.117 at 20 and 40 ng/band respectively, between area proved the robustness of the method indicating that CFZ is stable during analysis.

6. System repeatability

In repeatability studies, CFZ solution was prepared as described in section 2.5.1 and 2.6.5 was performed. In all 12 tracks identical peak was found with respect to position and also formed parallel lines on the TLC plate (figure 5). Further, % RSD of R_F values for CFZ on the three plates was < 0.01 %.

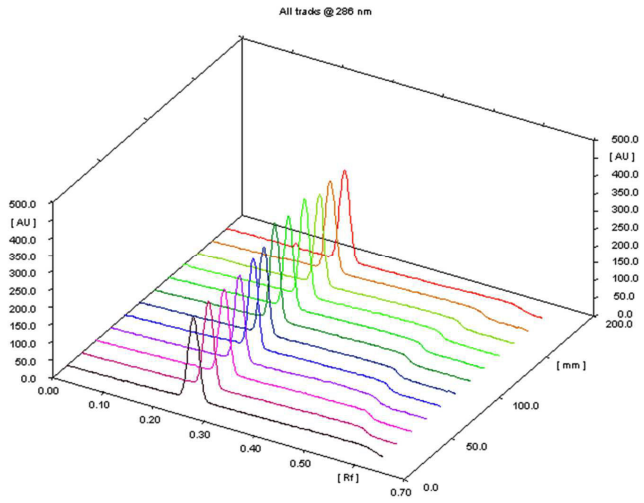


Figure 5
Representative three dimensional densitogram of CFZ (80 and 100 ng/band) applied in hexaplicate on TLC plate

7. Stability

No additional peak or significant deviation in peak area (% RSD) for concentration of 20 ng/band was observed on analysis, which indicated the sample solution were stable for analysis for a day.

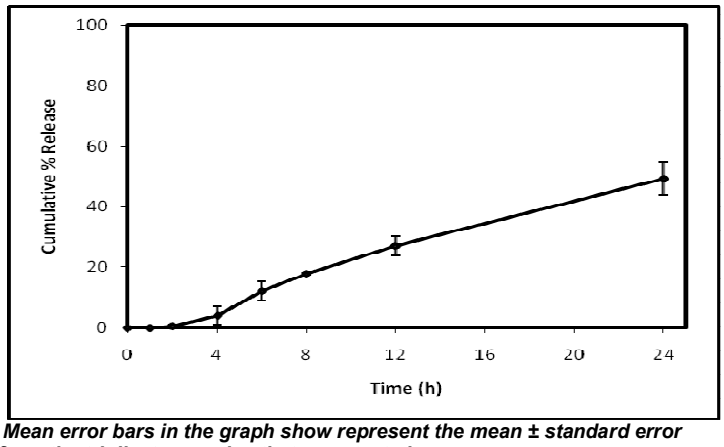
8. Analysis of CFZ in Formulation

Using the proposed chromatographic method, assay of CFZ in marketed formulation (Lamprene®, label claim: 50 mg CFZ per capsule, Novartis Pharma S.A.S, France) was carried out. A single peak at R_F 0.23 for CFZ was observed in the densitogram of the drug sample extracted from capsules. There was no interference from the excipients commonly present in the dosage form. Satisfactory results were obtained for drug in a good agreement with the label claim. The drug content was found to be 99.99 % with RSD percentage of 0.73. The low percentage of R.S.D. value indicated the suitability of this method for routine analysis of CFZ in pharmaceutical dosage forms.

9. Skin permeation studies

The method was also employed to check its utility in permeation studies of CFZ through rat skin. The results are as shown in graph 1. No interference during analysis due to skin components was encountered.

Graph 1
Ex- vivo skin permeation studies of CFZ



Mean error bars in the graph show represent the mean ± standard error from the triplicate samples that were tested.

The above mentioned HPTLC method is an attractive alternative for the estimation of CFZ in biological samples and otherwise without interference from other constituents. It is the first validated HPTLC method to the best of our knowledge. Statistical data analysis proves that the method is reproducible, precise and selective. Since this method has added advantage of robustness, accuracy, speed and low cost, it can be utilized both for qualitative as well as quantitative estimation of CFZ for routine purposes as well as to monitor skin permeation of drug, where high sensitivity is required. Further, this method can also be used for estimation of CFZ from biological samples.

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