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Research Article

TLC Method for Simultaneous Quantification of Chlorzoxazone, Paracetamol, Famotidine and Diclofenac Potassium in their Combined Dosage Form

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Abstract

A sensitive, selective and precise high performance thin layer chromatographic method has been developed for the estimation of chlorzoxazone, paracetamol, diclofenac potassium and famotidine in their pharmaceutical dosage form. TLC aluminum plates pre-coated with silica gel 60F_{254} used as the stationary phase, while chloroform: methanol: ethyl acetate: hexane: ammonia (10:2.5:1.5:1:0.1, v/v/v/v/v) used as mobile phase. The RF values were observed 0.74±0.01, 0.52±0.01, 0.30±0.01 and 0.14±0.01 for chlorzoxazone, paracetamol, diclofenac potassium and famotidine respectively. The densitometric analysis was carried out in absorbance mode at 282 nm. The method was linear in the range of 250-1500 ng/band for chlorzoxazone, diclofenac potassium and famotidine and 500-3000 ng/band for paracetamol and method was validated as per ICH guideline. The limit of detection and limit of quantitation were found to be 35.98 ng/spot and 109.05 ng/band respectively for chlorzoxazone, 99.74 ng/band and 302.25 ng/band respectively for paracetamol, 58.63 ng/band and 177.69 ng/band respectively for diclofenac and 50.93 ng/band and 154.35 ng/band respectively for famotidine. The proposed method was successfully applied to the quantification of chlorzoxazone, paracetamol, diclofenac potassium and famotidine in their pharmaceutical dosage form.

Keywords: Chlorzoxazone; Paracetamol; Diclofenac potassium; Famotidine; High performance thin layer chromatography; Validation

Abbreviations

CLZ: Chlorzoxazone; PCM: Paracetamol; DCL: Diclofenac Potassium; FAM: Famotidine; HPTLC: High Performance Thin Layer Chromatography

Introduction

Chlorzoxazone (CLZ) is chemically 5-chloro-2,3-dihydro-1,3-benzoxazol-2-one. The empirical formula of CLZ is $C_7H_4ClNO_2$ and a molecular weight is 169.56 g/mol. It is NSAID. It inhibits multisynaptic reflex a.c. involved in producing and maintaining skeletal muscle spasm. Paracetamol (PCM) is chemically N-(4-hydroxyphenyl) acetamide. The empirical formula for PCM is $C_8H_9NO_2$ and a molecular weight is 151.163 g/mol. It inhibiting both isoforms of cyclooxygenase, COX-1, COX-2 and COX-3 enzymes involved in Prostaglandin (PG) synthesis. Diclofenac Potassium (DCL) is chemically 2-{2-[(2,6-dichlorophenyl)amino]phenyl}acetic acid and empirical formula of DCL is $C_{14}H_{11}Cl_2NO_2$ and molecular weight is 318.13g/mol. It inhibition of leukocyte migration and the enzyme cylooxygenase (COX-1 and COX-2), leading to the peripheral inhibition of prostaglandin synthesis. Famotidine (FAM) is chemically 3-[({2-[(diaminomethylidene)amino]-1,3-thiazol-4-yl}methyl)sulfanyl]-N' sulfamoylpropanimidamide and empirical formula for FAM is $C_8H_{15}N_7O_2S_3$ and molecular weight is 337.44 g/mol. It is competitive histamine H_2-receptor antagonist and inhibits many of the isoenzymes of the hepatic CYP450 enzyme system.

The combined dosage form of CLZ, PCM, DCL and FAM is used as muscle relaxant. CLZ, PCM, DCL and FAM are official in United State Pharmacopoeia and British Pharmacopoeia. Official method has been reported for CLZ, PCM, DCL and FAM in United State Pharmacopoeia and British Pharmacopoeia. Some of UV, HPLC, TLC methods has been reported for the estimation of CLZ, PCM, DCL and FAM alone and with other drug combination. Till date no HPTLC method has been reported for the quantification of CLZ, PCM, DCL and FAM in their combined dosage form [1-27]. In comparison to LC and LC-MS/MS methods, HPTLC method is considered to be a good alternative and it should be widely explored as an important tool in routine drug analysis. A major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase. This reduces the time and cost of analysis.

The aim of the present work was to develop an sensitive, accurate, repeatable and specific HPTLC method for the determination of CLZ, PCM, DCL and FAM both as a bulk drug and in formulation. There is no HPTLC method reported for the quantification of CLZ, PCM, DCL and FAM in combination so attempt have been made to develop High Performance Thin Layer Chromatographic method. The proposed method was validated according to ICH guidelines [28] and its updated international convention.

Experimental

HPTLC instrument

The samples were applied in the form of a bands of width 8 mm

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with a Camag 100 µl sample syringe (Hamilton, Switzerland) using Camag Linomat 5 (Switzerland) sample applicator on pre-coated silica gel aluminum plate 60 F_{254}(10x10 cm^2 with 0.2 mm thickness, E. Merck, Germany). Camag TLC scanner 4 was used for the densitometric scanning.

Chemicals and reagents

Analytically pure CLZ, PCM, DCL and FAM from Sun pharmaceutical industry ltd., Vadodara, India were obtained as gift samples. Methanol (AR grade) of SRL Private Ltd. and chloroform of Chemdyes Corporation (AR grade) were used. Ammonia and hexane of Chiti-Chem Corporation (AR grade) were used. Ethyl acetate of Astron Chemicals (AR grade) Tablet formulation fastran MR (Horizon biocauticals Pvt. Ltd.) containing 500 mg of PCM, 250 mg CLZ, 50 mg DCL and 10 mg FAM was procured from local pharmacy.

Chromatographic System

Sample application

Standards and formulation samples of CLZ, PCM, DCL and FAM were applied on the HPTLC plates in the form of narrow bands of 6 mm length, 10 mm from the bottom and left edge and with 9 mm distance between two bands. Samples were applied under a continuous stream of nitrogen gas.

Mobile phase and development

Plates were developed using a mobile phase consisting of chloroform: methanol: ethyl acetate: hexane: ammonia (10:2.5:1.5:1:0.1, v/v/v/v/v). Linear ascending development was carried out in a twin-trough glass chamber equilibrated with the mobile phase vapors for 30 min at 25±2^{0}C. Ten milliliters of the mobile phase (5 ml in the trough containing the plate and 5 ml in the other trough) was used for each development and was allowed to migrate a distance of 80 mm, sample application rate is 200 nl/sec. After development, the HPTLC plates were dried completely using continuous stream of nitrogen.

Densitometric analysis

Densitometric scanning was performed in the absorbance mode under control by winCATS planar chromatography software. The source of radiation was the deuterium lamp and bands were scanned at 282 nm. The slit dimensions were 6 mm length and 0.45 mm width, with a scanning rate of 20 mm/s. Concentrations of the compound were determined from the intensity of diffusely reflected light and evaluated as peak areas against concentrations using a linear regression equation.

Preparation of standard stock solution

PCM (10 mg), CLZ (5 mg), DCL (5mg) and FAM (5mg) were accurately weighed and transferred to 10 ml volumetric flasks and dissolved in few ml of methanol. Volumes were made up to the mark with methanol to yield a solution containing 1000 µg/ml of PCM and 500 µg/ml of CLZ, DCL and FAM. Aliquot from the stock solutions of PCM, CLZ, DCL and FAM were appropriately diluted with mobile phase to obtain working standard of 100 µg/ml of PCM and 50 µg/ml of CLZ, DCL and FAM respectively.

Validation

Validation of the developed HPTLC method was carried out according to International Conference on Harmonization (ICH) guidelines Q2 (R1) for specificity, sensitivity, accuracy, precision, repeatability and robustness [28].

Linearity of calibration curves

Linearity of the method was evaluated by constructing calibration curves at six concentration levels over a range of 500-3000 ng/band for PCM and 250-1500 ng/band for CLZ, DCL and FAM by applying 5 µl to 30 µl from stock solution has been applied on HPTLC plate using sample applicator. The calibration curves were developed by plotting peak area versus concentration (n=6) with the help of the winCATS software.

Accuracy

Accuracy is closeness of the test results obtained by the method to the true value and should be established across specified range of analytical Procedure. The accuracy of the method was determined by calculating recoveries of PCM, CLZ, DCL and FAM by method of standard additions respectively. Known amount of PCM (0, 500, 1000, 1500 ng/spot) and AML (0, 250, 500, 750 ng/spot) were taken from the working standard solutions (100 µg/ml of PCM and 50 µg/ml of CLZ, DCL and FAM respectively). It was added to a pre quantified sample and the amount of PCM, CLZ, DCL and FAM were estimated by measuring the peak area and by fitting these values to the straight-line equation of calibration curve. The proposed acceptance criteria for the accuracy studies are ranges from 95-105%.

Precision

Precision was evaluated in terms of intraday and intraday precisions. Working standard solutions of 100 µg/ml of PCM and 50 µg/ml of CLZ, DCL and FAM, were prepared and used for the precision study. Intraday precision was determined by analyzing sample solutions of PCM, CLZ, DCL and FAM at three levels covering low, medium and high concentrations of the calibration curve three times on the same day. Intraday precision was determined by analyzing sample solutions of PCM, CLZ, DCL and FAM at three levels covering low, medium and high concentrations over a period of 3 days. The peak areas obtained were used to calculate mean and RSD values. Less than 5% RSD values indicate that the method is precise.

Specificity

Specificity is the ability to assess unequivocally the analytes in the presence of components which may be expected to be present. The specificity of the method was ascertained by analyzing PCM, CLZ, DCL and FAM in presence of excipients commonly used for tablet formulations. The bands of PCM, CLZ, DCL and FAM were confirmed by comparing RF values and respective spectra of sample with those of standards. The peak purity of PCM, CLZ, DCL and FAM was assured by comparing the spectra at three different levels, that is, peak start, peak apex and peak end positions.

Sensitivity

Sensitivity of the method was determined with respect to LOD and LOQ. Noise was determined by scanning a blank band (methanol) six times. LOD was calculated as 3 times the noise level and LOQ was calculated as 10 times the noise level.

Robustness

Small changes in the chamber saturation time, solvent migration

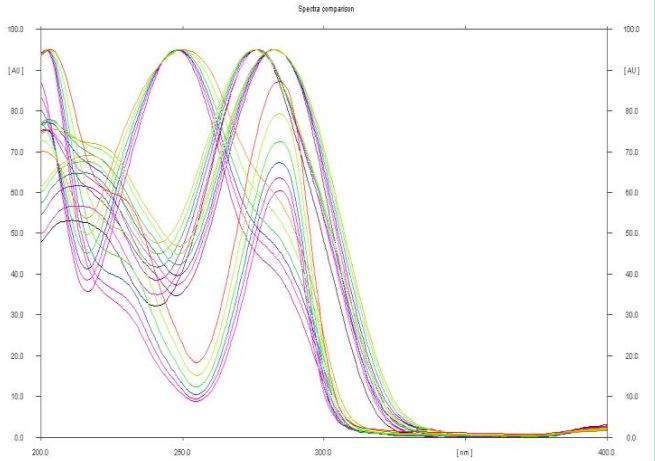

Figure 1: Overlain UV spectra of PCM, CLZ, FAM and DCL (200-400 nm).

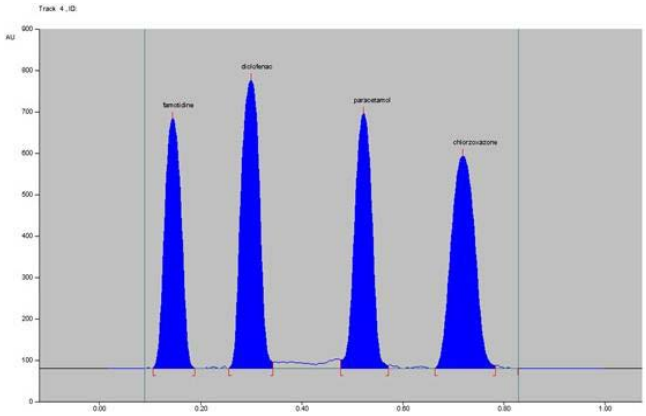

Figure 2: Densitogram of CLZ, PCM, DCL and FAM using mobile phase chloroform: methanol: ethyl acetate: hexane: ammonia (10:2.5:1.5:1:0.1, v/v/v/v/v).

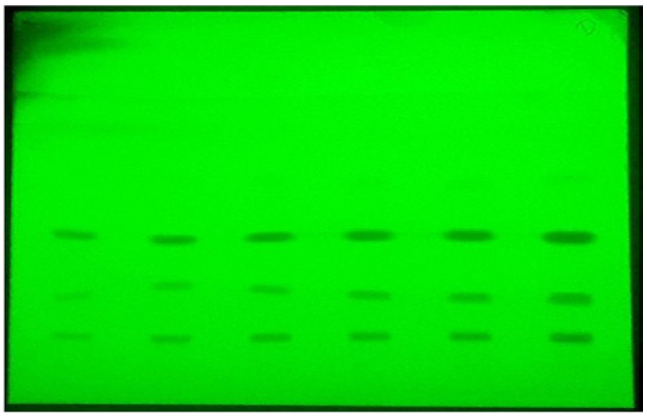

Figure 3: Photograph of developed HPTLC plate.

distance and mobile phase composition were introduced and the effects on the results were examined. Robustness of the method was determined in triplicate at a concentration level of 2000 ng/band for PCM and 1000 ng/band for DCL and 750 ng/band for CLZ and FAM. The mean and RSD of peak areas were calculated.

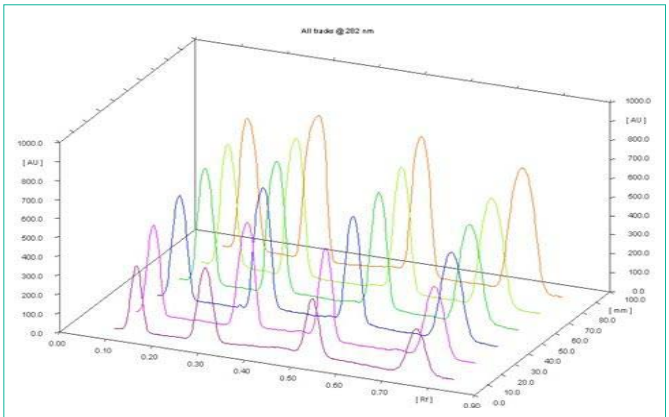

Figure 4: Three dimensional overlay of HPTLC densitograms of calibration bands of CLZ, PCM, DCL and FAM.

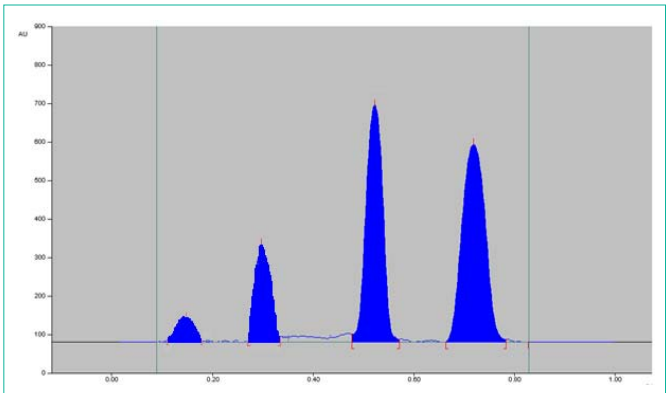

Figure 5: Densitogram of analysis of marketed formulation of PCM (1500 ng/spot), CLZ (750 ng/spot), FAM (300 ng/spot) and DCL (1500 ng/spot).

Analysis of marketed formulations

Twenty tablets were weighed accurately and finely powdered. Tablet powder equivalent to 500 mg of PCM, 250 mg of CLZ, 50 mg of DCL and 10 mg of FAM was accurately weighed and transferred to a 100 ml volumetric flask. A few ml (40 ml) of methanol was added to the above flask and flask was sonicated for 15 min. The solution was filtered using what man filter paper No. 41 in another 100 ml volumetric flask and make up the volume up to the mark with the methanol.

A solution containing 300 ng/band FAM and 1500 ng/band DCL were injected as per the above chromatographic conditions and peak areas were recorded. Appropriate volume of the aliquot was transferred to a 10 ml volumetric flask and the volume was made up to the mark with the mobile phase to obtain a solution containing 750 ng/band CLZ and 1500 ng/band PCM. The quantifications were carried out by keeping these values to the straight line equation of calibration curve.

Results and Discussion

Optimization of the mobile phase

To develop the HPTLC method for analysis of PCM, CLZ, DCL and FAM in the pharmaceutical dosage form for routine analysis, selection of the mobile phase was carried out on the basis of polarity. A mobile phase that would give a dense and compact band with an

Table 1: Regression analysis of calibration curve.

Parameters	CLZ	PCM	DCL	FAM
Linearity range (ng/spot)	250-1500	500-3000	250-1500	250-1500
Slope	14.3	5.7	10.9	10.8
Standard deviation of slope	0.21	0.03	0.22	0.30
Intercept	4619.6	4953.4	9033.6	4310.6
Standard deviation of intercept	155.71	171.73	193.72	166.82
Correlation coefficient	0.995	0.997	0.995	0.996

Table 2: Summary of validation parameter.

Parameters	CLZ	PCM	DCL	FAM
RF	0.74	0.52	0.29	0.14
Detection limit (ng/band)	35.98	99.74	58.63	50.93
Quantitation limit (ng/band)	109.05	302.25	177.69	154.35
Accuracy (%)	98.81-99.57	98.55-99.88	99.22-100.27	98.94-99.55
Intra-day (n=3) (% RSD)	1.18-1.249	0.94-1.31	1.33-1.60	0.82-1.06
Inter-day (n=3) (% RSD)	1.71-1.89	1.45-1.68	1.44-1.83	1.57-1.93
Repeatability study (n=6) (% RSD)	1.77-1.96	1.77-1.86	1.71-1.85	1.58-1.83

Table 3: Robustness Study of proposed method.

Parameters	PCM (2000 ng/band)		CLZ (1000 ng/band)		FAM (1000 ng/band)		DCL (1000 ng/band)	
	RF mean±SD	%RSD	RF mean±SD	%RSD	RF mean±SD	%RSD	RF mean±SD	%RSD
Chamber saturation time: 20 min	0.50±0.005	1.13	0.72±0.005	0.79	0.13±0.005	4.22	0.28±0.005	2.03
Chamber saturation time: 40 min	0.51±0.005	1.11	0.73±0.005	0.78	0.14±0.005	4.02	0.30±0.55	1.90
Wave length 280 nm	0.52±0.0	0.0	0.74±0.005	0.77	0.14±0.00	0.00	0.3±0	0.0
Wave length 284 nm	0.52±0.0	0.0	0.74±0.005	0.77	0.14±0.00	0.00	0.30±0.005	1.90
chloroform:methanol:ethayl acetate:hexane:ammonia (9:3.5:1.5:1:0.1, v/v/v/v/v)	0.52±0.005	1.09	0.75±0.005	0.76	0.14±0.005	4.02	0.31±0.005	1.84
chloroform:methanol:ethaylacetate:hexane:ammonia (11:2:1:1:0.1, v/v/v/v/v)	0.51±0.005	1.12	0.72±0.005	0.79	0.13±0.005	4.3	0.29±0	0

Table 4: Accuracy study of the proposed method.

% Level	Amount Added (ng/band)				Amount Recovered (ng/band) (n=3)				% Recovered±S.D.			
	PCM	CLZ	DCL	FAM	PCM	CLZ	FAM	DCL	% PCM	%CLZ	%FAM	%DCL
0	1000 + 0	500+0			994.48	495.47	496.73	501.38	99.44±1.38	99.09±0.58	99.34±1.19	100.27±0.52
50	1000+500	500+250			1498.81	747.89	744.73	749.28	99.88±0.92	99.57±0.66	98.94±0.93	99.85±0.38
100	1000+1000	500+500			1985.55	997.13	997.75	998.99	98.55±0.62	99.42±0.51	99.55±1.03	99.79±0.96
150	1000+1500	500+750			2489.83	1244.06	1245.07	1246.10	98.98±0.75	98.81±0.87	99.01±1.13	99.22±0.85

appropriate RF value for PCM, CLZ, DCL and FAM was desired. Various mobile phases such as acetone–methanol, methanol-chloroform acetic acid, methanol-toluene-ammonia, methanol-toluene-glacial acetic acid, toluene-ethyl acetate-methanol, methanol-acetonitrile-glacial acetic acid were evaluated in different proportions. A mobile consisting of chloroform: methanol: ethyl acetate: hexane: ammonia (10:2.5:1.5:1:0.1, v/v/v/v/v) gave good separation of PCM, CLZ, DCL and FAM from its matrix. It was also observed that chamber saturation time and solvent migration distance were crucial in the chromatographic separation. Therefore, chloroform: methanol: ethyl acetate: hexane: ammonia (10:2.5:1.5:1:0.1, v/v/v/v/v) mobile phase with a chamber saturation time of 30 min at 25^{0}C and solvent migration distance of 80 mm was used. Densitogram of PCM, CLZ, DCL and FAM, photograph of TLC plate and three dimensional overlays of HPTLC densitograms of calibration bands of PCM, CLZ, DCL and FAM are depicted in figures (Figures 1-4).

Validation

Linearity and calibration curves

The method was found to be linear for PCM in concentration range of 500-3000 ng/band (n=6) and for CLZ, DCL and FAM 250-1500 ng/band (n=6) respectively. (Figure 3) displays a three-dimensional overlay of HPTLC densitograms of the calibration bands of PCM, CLZ, FAM and DCL at 282 nm (Figure 1). The regression data shown in (Table 1) reveal a good linear relationship over the concentration range studied, demonstrating the suitability of the

Table 5: Assay results of marketed formulation.

Formulation	Drug	Amount Taken (ng/band)	Amount Found (ng/band) (n=3)	%Amount of drug found Mean±SD (n=3)
FASTRAN MR (Tablet)	PCM	1500	1482.33	98.82±0.69
	CLZ	750	743.66	99.15±1.13
	FAM	300	295.00	98.33±1.00
	DCL	1500	1485.33	99.02±0.85

method for analysis.

Accuracy

Accuracy was determined by the application of analytical procedure to recovery studies, where a known amount of standard is spiked into reanalyzed samples solutions. Results of the accuracy studies from excipients matrix are shown in table. Recovery values demonstrated the accuracy of the method in the desired range.

Precision

In all instances, RSD values were less than 2%, confirming the precision of the method. Repeatability of the scanning device was studied by applying and analyzing sample seven times. RSD was less than 2%, which was well below the instrumental specifications. Summary of validation parameters are shown in (Table 2). The RSD values obtained were less than 2%, which was under the acceptance criteria of ICH method validation guideline (<2%). The results indicated that the method is repeatable and reproducible.

Limit of detection and limit of quantification

Under the experimental conditions used, the lowest amount of drug that could be detected LOD was found to be 35.98 ng/band, 99.74 ng/band, 58.63 ng/band and 50.93 ng/band for PCM, CLZ, DCL and FAM respectively and LOQ was found to be 109.05 ng/band 302.25 ng/band, 177.69 ng/band and 154.35 ng/band for PCM, CLZ, DCL and FAM respectively. It indicate that the nanogram quantity of all the drugs can be estimated accurately and precisely which means that the method is sensitive.

Specificity

There was no interfering peak at the RF value of PCM, CLZ, DCL and FAM from excipients added in the synthetic formulation. In addition, there was no interference from excipients present in the commercial formulation, thereby confirming the specificity of the method.

Robustness

The low values of RSD obtained after introducing small, deliberate changes in parameters of the developed HPTLC method confirmed its robustness. The robustness data of the proposed method are shown in (Table 3).

Analysis of marketed formulation

Marketed formulation was analyzed using proposed method which gave percentage recovery of 98.46%, 98.26%, 98.09% and 99.01% for PCM, CLZ, DCL and FAM respectively. No interference from the excipients present in the marketed tablet formulation was observed. Assay results and densitogram of marketed formulation are shown in (Tables 4 & 5 & Figure 5).

Conclusion

A selective, sensitive, accurate and precise high performance thin layer chromatography method has been developed for the simultaneous identification and quantification of chlorzoxazone, paracetamol, famotidine and diclofenac potassium in their combined pharmaceutical dosage form. The method was successfully validated in accordance with ICH guidelines. It can be conveniently used for routine quality control analysis of chlorzoxazone, paracetamol, famotidine and diclofenac potassium in marketed tablet without any interference from excipients. The method might be used to determine the purity of drug available from various sources.

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