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Simultaneous Determination of Paracetamol, Propyphenazone and Caffeine in Presence of Paracetamol Impurities Using Dual-Mode Gradient HPLC and TLC Densitometry Methods

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Abstract

Two chromatographic methods were validated for the determination of the widely prescribed analgesic and antipyretic drug combination of paracetamol (PC) (recently integrated into the supportive treatment of COVID-19), propyphenazone (PZ) and caffeine (CF) in the presence of two PC impurities, namely 4-aminophenol and 4-nitrophenol. A "dual-mode" gradient high-performance liquid chromatography method was developed, where the separation was achieved via "dual-mode" gradient by changing both the ternary mobile phase composition (acetonitrile: methanol: water) and the flow rate. This enables a good resolution within a relatively shorter analysis time. The analysis was realized using Zorbax Eclipse XDB column C18, 5 μm (250 × 4.6 mm) and the UV detector was set at 220 nm. The other method is a thin-layer chromatography densitometry method, where the separation was achieved using a mobile phase composed of chloroform: toluene: ethyl acetate: methanol: acetic acid (6: 6: 1: 2: 0.1, by volume). Densitometric detection was performed at 220 nm on silica gel 60 F254 plates. The developed methods were fully validated as per the ICH guidelines and proved to be accurate, robust, specific and suitable for application as purity indicating methods for routine analysis of PC in pure form or in pharmaceuticals with PZ and CF in quality control laboratories.

Introduction

Non-steroidal anti-inflammatory drugs are amongst the most frequently prescribed drugs worldwide to treat a variety of pain-related conditions (1).

Paracetamol (PC) is N-(4-Hydroxyphenyl) acetamide has analgesic and antipyretic activities and recently described as the first line antipyretic in COVID19 symptomatic relief (2, 3). Propyphenazone

(PZ) is 1,2-Dihydro-1,5-dimethyl-4-(1-methylethyl)-2-phenyl-3H-pyrazol-3-one. It has the same analgesic and antipyretic properties as PC. They both are used for treatment of pain and fever. Caffeine (CF) is 3,7-Dihydro-1,3,7-trimethyl-1H-purine-2,6-dione. CF is CNS stimulant, inhibits the phosphodiesterase enzyme and has an antagonistic effect at central adenosine receptors (4). The

combination of the three drugs is prescribed for the treatment of mild fever and severe pain.

4-aminophenol, 4 AP, was considered as the main co-existing impurity of PC in pharmaceutical preparations originating from either synthesis or degradation (5, 6). As 4 AP is a pharmacologically active compound possessing nephrotoxic and teratogenic effects; therefore, its concentration should be strictly controlled (6). 4-nitrophenol, 4 NP, is the precursor of the 4 AP and is considered as a potential PC impurity (7).

Literature survey revealed several techniques for the analysis of the combination of the three drugs (8, 9, 10); however, impurity indicating studies as an example of the analysis of complex mixtures of multi-components need highly selective and sensitive techniques as high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Chromatographic methods are recommended to determine the intact drug in presence of its impurities.

Literature survey highlighted the importance and the contribution of chromatographic methods in the determination of these three drugs (11–15). However, no chromatographic methods were reported for the analysis of the ternary mixture CF, PZ and PC in the presence of potential PC impurities, namely 4 AP (Acetaminophen RCK) and 4 NP (Acetaminophen RCF).

The current work aims to develop selective and sensitive "dual-mode" gradient HPLC and TLC densitometry methods for simultaneous determination of the three drugs in presence of the two PC impurities, 4 AP and 4 NP.

HPLC and TLC methods were validated as per the International Conference on Harmonization (ICH) guidelines. One-way ANOVA statistical analysis was applied to compare results obtained from the two corresponding methods with a reported method.

Experimental

Instruments

HPLC apparatus

HPLC-UV detector (Agilent 1260 infinity) system consisted of a Quaternary pump (model G 711A, Quat pump VL 1260, Waldbronn-Germany) coupled to an ultraviolet multiple wavelength detector (model G7165 A, 1260 MWD) and Rheodyne injector (model 7225/7725I) equipped with 20 µL injector loop (Rohnert Park, CA.-USA). Open Lab CDS ChemStatiion® version A.01.05 software was used in data acquisition.

TLC densitometer

TLC densitometer system is composed of Camag Linomat 5 autosampler with Camag microsyringe 100 µL. Scanning speed and spraying rate were 20 mm/s and 10 s/mL, respectively. A Camag (Switzerland) TLC Scanner three densitometer model three equipped with WinCats® software version 1.4.2.8121.

Materials

CF, PZ and PC working standards were kindly supplied by Eva Pharma Co, Egypt; their purity was reported to be $99.35 \pm 1.68\%$, $100.09 \pm 0.88\%$ and $99.89 \pm 1.31\%$, respectively.

Stopain® Tablet manufactured by Eva Pharma Co, Egypt. Tablet was labeled to contain 50 mg CF, 150 mg PZ and 300 mg PC per tablet.

4 AP (Acetaminophen RCK) and 4 NP (Acetaminophen RCF) (16) were supplied by Sigma-Aldrich.

Methanol of HPLC grade (Fisher Scientific, UK) and acetonitrile of HPLC grade)Fisher Scientific, UK).

Chloroform, toluene, ethyl acetate, methanol and acetic acid used in TLC method were of analytical grade (Adwic Co, Egypt).

Chromatographic conditions

HPLC method

Chromatographic separation was performed on Zorbax® Eclipse XDB column C18 (250 mm × 4.6 mm, 5 µm) Agilent technologies, USA. The mobile phase was acetonitrile, methanol and water and the elution was performed by programming both the component ratios and the flow rate of the mobile phase. UV-detector was set at 220 nm and the separation was carried out at room temperature. Prior to injection, the column was conditioned with the mobile phase for 30 min. An injection volume of 20 µL of each sample was loaded onto the analytical column. The separation was performed under the mentioned chromatographic conditions.

TLC method

The mobile phase preferred for chromatographic separation was prepared containing chloroform: toluene: ethyl acetate: methanol: acetic acid (6: 6: 1: 2: 0.1, by volume). Separation was done on TLC aluminum sheet silica gel 60 F_{254} plates (10 × 20 cm) (Merck, Germany). Mobile phase moved a distance of 8 cm. Spots were scanned using a UV lamp at 220 nm for densitometric determination. Samples were accurately spotted onto 10 × 20 cm TLC plates, using Camag Linomat autosampler with 100 µL microsyringe. Bands were 6 mm length, 10.5 mm apart from each other and 15 mm from the bottom edge of the plate. The plates were developed by ascending chromatography to a distance of 8 cm from the spotting line at room temperature, in a chromatographic chamber previously saturated with a mobile phase for 60 min. The plates were left 30 min at room temperature to dry then bands were scanned at 220 nm in the absorption mode at scanning speed 20 mm s^{-1}.

Procedures

Solvent is consisted of methanol in water (1: 9 by volume).

Validation procedure

The two chromatographic methods were validated regarding linearity, accuracy, precision, specificity, limit of detection (LOD), limit of quantitation (LOQ), robustness and system suitability test.

Linearity. Stock standard solutions (1,000 µg mL^{-1}) of each drug were prepared in the previously mentioned solvent.

For HPLC method, working standard solutions were prepared from these stocks by suitable dilution with the mobile phase giving a final concentration range of 10–100 µg mL^{-1}, 20–400 µg mL^{-1} and 20–600 µg mL^{-1} for CF, PZ and PC, respectively. A triplicate of 20 µL was injected from each concentration into HPLC. The calibration curves were constructed using the linear regression method by plotting the concentrations of each drug versus their corresponding peak areas to prove linearity.

For TLC method, aliquots from each standard stock solution (1,000 µg mL^{-1}) were accurately spotted onto 10 × 20 cm TLC plates to deliver 4–24 µg spot^{-1}, 5–30 µg spot^{-1} and 5–30 µg spot^{-1} for CF, PZ and PC, respectively, using Camag Linomat autosampler with

100 µL microsyringe. The calibration curves were constructed relating the area under the peak versus the corresponding concentration of each standard and the corresponding linear regression equation was obtained.

Accuracy. The accuracy was expressed as (mean of percentage recoveries ± SD) assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range.

In TLC method, the spotted quantities were 4, 12 and 20 µg per spot for CF and 5, 15 and 25 µg per spot for both PZ and PC. For HPLC method, the concentrations involved were 20, 50 and 90 µg mL^{-1} for CF, 40, 100 and 300 µg mL^{-1} for PZ and 50, 200 and 500 µg mL^{-1} for PC. The previously mentioned procedures were performed for the analysis of the different concentrations and the concentrations were calculated using the corresponding linear regression equation.

Precision. For determination of repeatability (RSD), the intraday precision studies were performed by analysis of three concentrations within the specified range for each standard repeated three times within the day. The interday precision (RSD) was done over three concentrations within the specified range for each standard repeated three times in three successive days.

For HPLC method, the three concentrations tested were 30, 50 and 60 µg mL^{-1} for CF, 60, 80 and 100 µg mL^{-1} for PZ and 100, 200 and 300 µg mL^{-1} for PC. For TLC method, quantities of 8, 12 and 16 µg of CF, 10, 15 and 20 µg of PZ and 15, 20 and 25 µg of PC were accurately spotted onto TLC plates for the determination of precision.

The previously mentioned procedures were involved in the analysis of the different concentrations.

Specificity. Working standard solutions of each impurity (4 AP and 4 NP) were prepared in 10% methanol in water (v/v) solution at a concentration (1,000 µg mL^{-1}). Separation of laboratory prepared mixtures containing different ratios of CF, PZ, PC, 4 AP and 4 NP was achieved using the previously mentioned procedures of both methods. R_F values, t_R values and resolution were obtained to evaluate the separation of the studied compounds and express the specificity of both methods.

Limit of detection and limit of quantitation. The LOD and LOQ of both methods were determined for CF, PZ and PC. After the construction of each calibration curve, the obtained data were involved in the determination of LOD and LOQ using the following equations:

LOD = 3.3 × SD of intercept/slope.
LOQ = 10 × SD of intercept/slope.

LOD values were verified practically where the founded concentrations for each component were prepared in triplicates and tested to investigate signal to noise ratio. The same was done for LOQ values and the signal to noise ratio was investigated.

Robustness and system suitability testing. Robustness was evaluated in terms of RSD after application of small variation in time of mobile phase saturation and mobile phase composition for TLC method and mobile phase flow rate, mobile phase composition and detector wavelength for HPLC method.

The system suitability test parameters were calculated regarding tailing factor, theoretical plate number, resolution and selectivity factor.

Application to pharmaceutical formulation procedure
Twenty tablets were weighted to calculate the average weight then the tablets were grinded. The average weight of the grinded powder (equivalent to 50 mg of CF, 150 of PZ and 300 mg of PC) was transferred into a 250 mL volumetric flask, 200 mL of 10% methanol in water (v/v) solution were added into the flask and sonicated for 15 min then filtered into new 250 mL volumetric flask. The residue was washed using 5 mL of previously mentioned solution for three consecutive times, then completed to final volume with the same solvent.

HPLC method. The previously prepared solution was five-fold diluted using the mobile phase before injection into the instrument at an injection volume of 20 µL. The separation was performed under the mentioned chromatographic conditions.

TLC method. Twenty microliters from the prepared solution was spotted on the TLC plate. Development and quantification were performed under the mentioned chromatographic conditions.

The standard addition technique was performed for both methods via the recovery assessment that was carried out on the pharmaceuticals instead of preparing placebos. Therefore, known accurate amounts of each standard substance were spiked into the pharmaceutical formulation to obtain three different levels of addition for each analyte.

Results

The current study aims to develop two chromatographic methods for the determination of CF, PZ and PC in presence of PC impurities, in the absence of a reported impurity indicating methods.

Method development and optimization
After the evaluation of several solvent compositions, the solvent used was 10% methanol in water (v/v) solution, at which standards and PC impurities exhibited good solubility.

In the HPLC method, a "dual-mode" gradient was applied to improve resolution and shorten the analysis time (1). Moreover, the ternary mobile phase systems allowed better separations compared to their binary counterparts (17).

Optimum separation was achieved using a mobile phase consisting of methanol: acetonitrile: water. The percent of each component and the flow rate were changed with time, as described in Table I. Figure 1a and b shows representative chromatograms obtained with the standards without and with impurities.

In the TLC method, a mobile phase composed of chloroform: toluene: ethyl acetate: methanol: acetic acid (6: 6: 1: 2: 0.1, by volume) was recommended to obtain satisfactory chromatographic separation between CF, PZ and PC without and with PC impurities as represented in Figure 2a and b and Supplementary Figure S1.

Table I. Dual-Mode Gradient of HPLC Method for the Separation of CF, PZ, PC, 4 AP and 4 NP

Time (min)	Acetonitrile %	Methanol %	H_2O %	Flow rate (mL min^{-1})
0–2.3	25	25	50	2
2.3–2.7	15	15	70	2
2.7–6	15	15	70	2
6–6.2	20	30	50	2.5
6.2–8.4	20	30	50	2.5
8.4–9.4	0	50	50	2
9.4–14	0	50	50	2
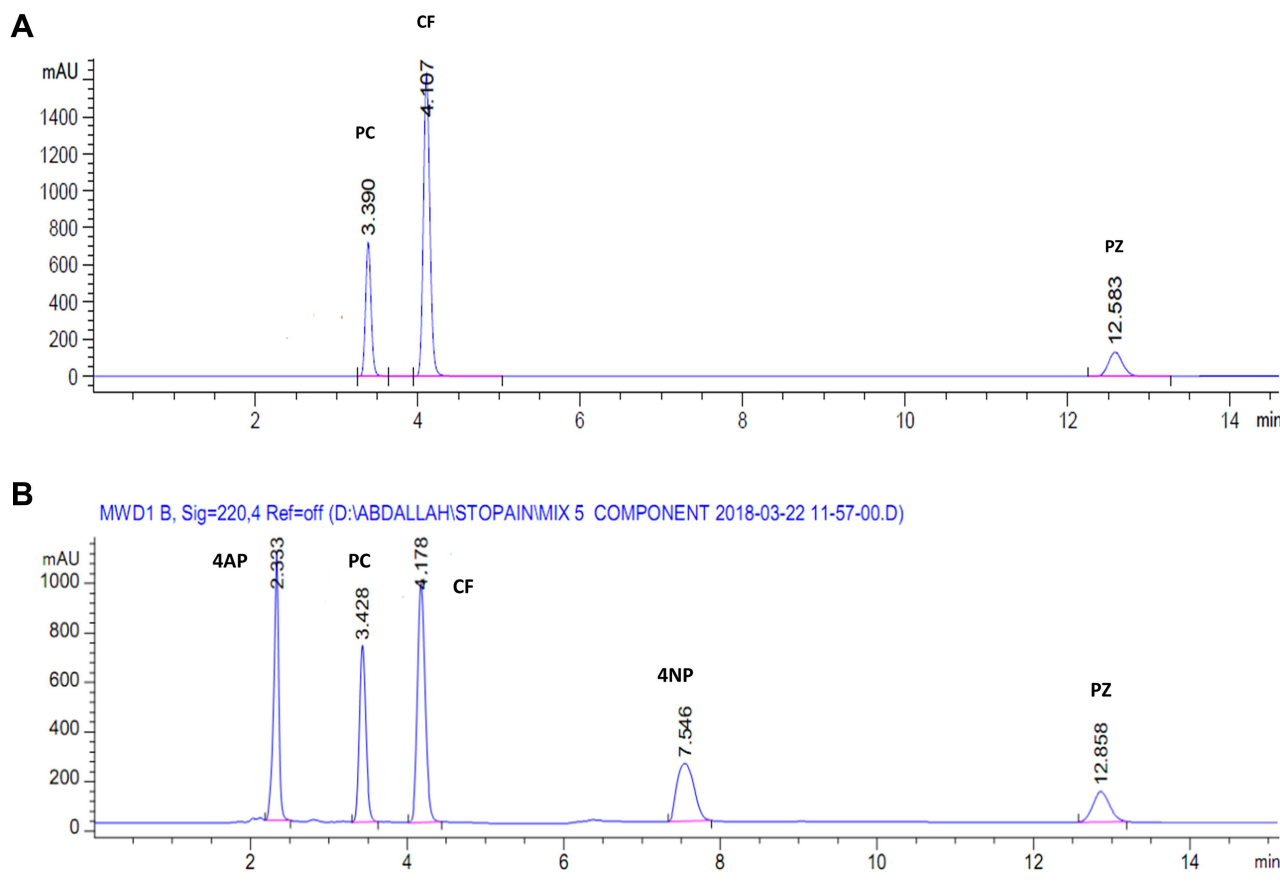
Figure 1. (a) HPLC chromatogram of PC (50 µgmL^{-1}), CF (75 µgmL^{-1}) and PZ (40 µgmL^{-1}) using specified chromatographic conditions. (b) HPLC chromatogram of 4-amio phenol (50 µgmL^{-1}), PC (50 µgmL^{-1}), CF (50 µgmL^{-1}), 4 nitrophenol (50 µgmL^{-1}) and PZ (50 µgmL^{-1}) using specified chromatographic conditions.

AP exhibited relatively high affinity toward the polar silica and showed good separation from CF, PZ, PC and 4 NP

Method validation

Validation was carried out according to ICH guidelines (18) and the assay validation parameters were computed, as shown in Table II.

Linearity

Linearity was achieved for in the range of 10–100 µg mL^{-1} CF, 20–400 µg mL^{-1} PZ and 20–500 µg mL^{-1} PC for HPLC method and 4–24 µg spot^{-1} CF, 5–30 µg spot^{-1} PZ and 5–30 µg spot^{-1} PC for TLC method, Figure 3. Calibration curves were constructed and equations of linear regressions were obtained for the two methods.

Accuracy

In TLC method, accuracy was evaluated via the mean percent recovery and standard deviation. Mean ± SD was found to be 100.73 ± 1.09, 99.92 ± 0.84 and 101.22 ± 0.67 for CF, PZ and PC, respectively. Whereas, the HPLC method was 99.17 ± 1.66 for CF, 101.46 ± 0.98 for PZ and 101.08 ± 1.31 for PC.

Precision

In repeatability testing, RSD of CF was 0.77 in TLC method and 1.23 in HPLC method while RSD of PZ was 1.53 in TLC method and 0.97 in HPLC method and that of PC was 0.81 in TLC method and 1.32 in HPLC method.

Upon examination of interday precision, RSD was found to be 1.92 for CF, 1.12 for PZ and 0.64 for PC in TLC method and 1.44,

A

B
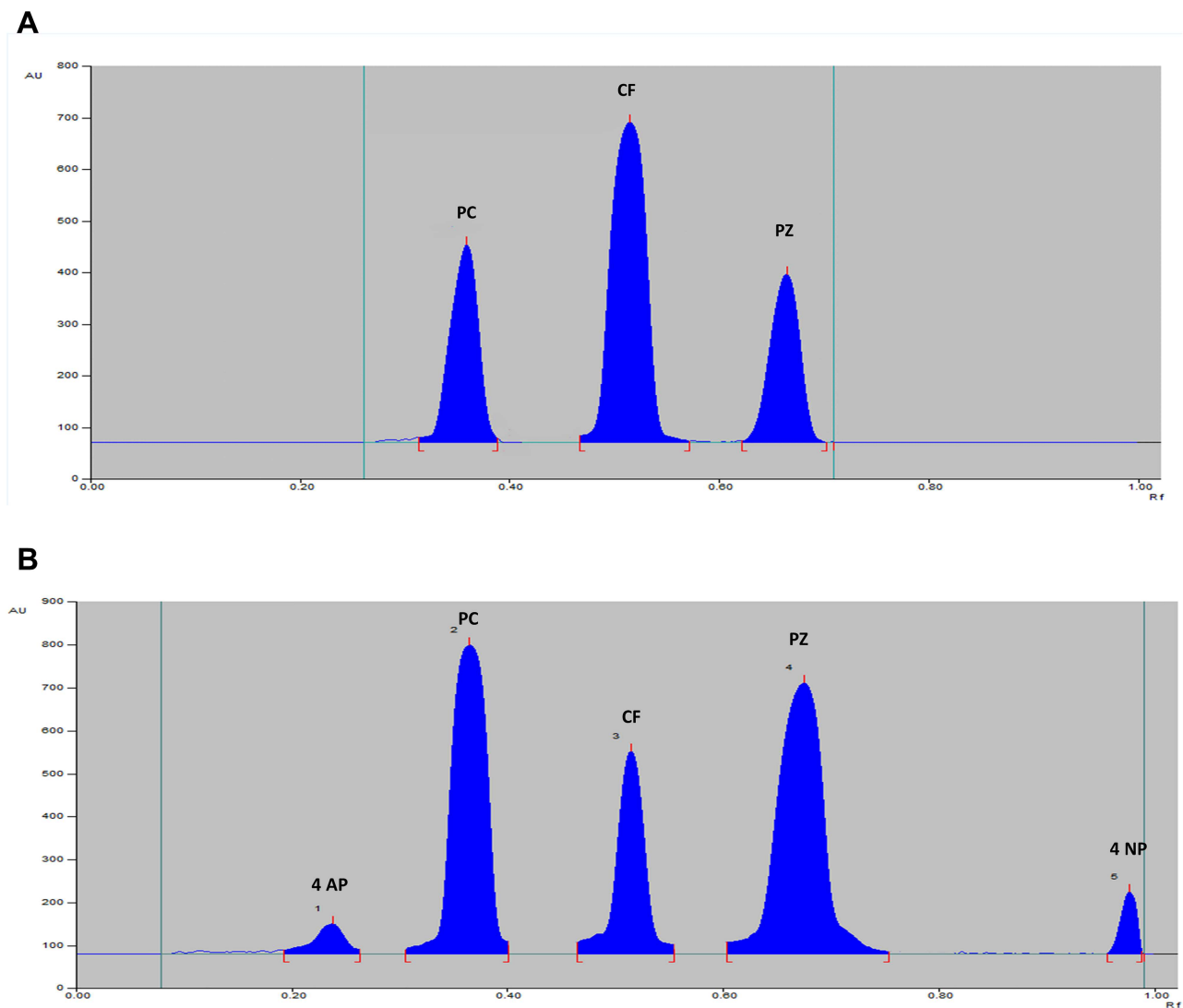
Figure 2. (a) Two dimensions TLC chromatogram showing separation of PC (5 μg spot-1, RF: 0.36), CF (24 μg spot-1, RF: 0.52) and PZ (5 μg spot-1, RF: 0.64). (b) Two-dimensions TLC chromatogram showing separation of PC (20 μg spot-1, RF: 0.36), CF (20 μg spot-1, RF: 0.52) and PZ (20 μg spot-1, RF: 0.65) from the two paracetamol impurities (4-amino phenol and 4-nitro phenol) at RF: 0.22 and 0.97, respectively.
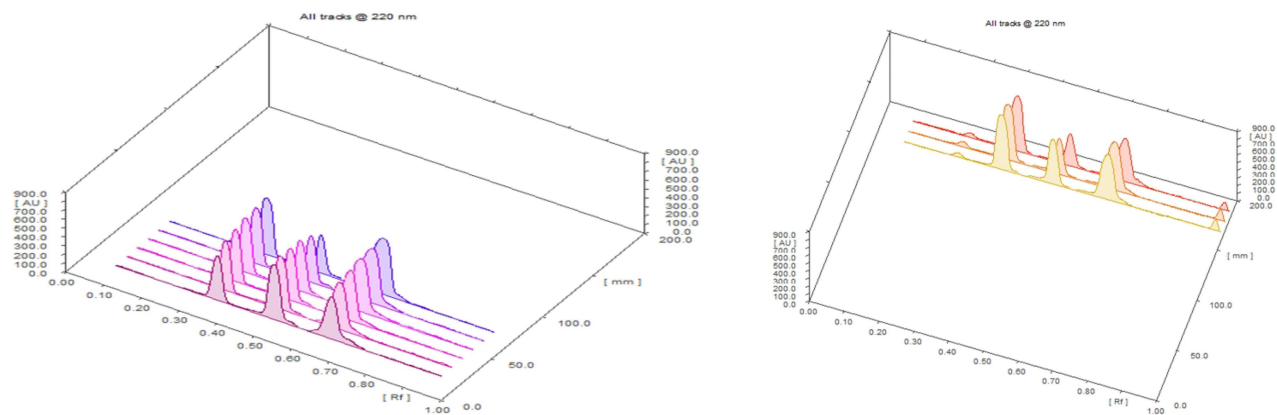
Figure 3. Three-dimensions TLC chromatogram showing separation of PC in range (5–30 μg spot-1), CF in range (4-24 μg spot-1) and PZ in range (5–30 μg spot-1) at RF: 0.36, 0.52 and 0.65, respectively.

Table II. Assay Validation Table of the Proposed Methods for the Determination of CF, PZ and PC as per the ICH Guidelines

Parameter	TLC			HPLC		
	CF	PZ	PC	CF	PZ	PC
Accuracy[a]	100.73 ± 1.09	99.92 ± 0.84	101.22 ± 0.67	99.17 ± 1.66	101.46 ± 0.98	101.08 ± 1.31
Precision						
Repeatability[b] (%RSD)	0.77	1.53	0.81	1.23	0.97	1.32
Intermediate Precision[c] (%RSD)	1.92	1.12	0.64	1.44	1.22	1.56
Linearity						
Correlation Coefficient	0.9992	0.9994	0.9991	0.9995	0.9993	0.9992
Slope	1,315.0842	1,527.0258	1,264.3691	61.5296	19.8574	34.1023
Intercept	5,891.1464	5,684.4189	4,638.2217	19.6058	−82.7323	323.0196
Range	4–24 μg spot^{-1}	5–30 μg spot^{-1}	5–30 μg spot^{-1}	10–100 μg mL^{-1}	20–400 μg mL^{-1}	20–600 μg mL^{-1}
LOD	1.21 μg spot^{-1}	1.59 μg spot^{-1}	1.50 μg spot^{-1}	3.0976 μg mL^{-1}	5.1928 μg mL^{-1}	6.5660 μg mL^{-1}
LOQ	3.67 μg spot^{-1}	4.83 μg spot^{-1}	4.54 μg spot^{-1}	9.3869 μg mL^{-1}	15.7360 μg mL^{-1}	19.8971 μg mL^{-1}

[a]Accuracy (mean of percentage recoveries ± SD) assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range. [b]The intraday ($n = 3$), RSD on three concentrations within the specified range for each standard repeated three times within the day. [c]The interday ($n = 3$), RSD on three concentrations within the specified range for each standard repeated three times in three successive days.

Table III. Parameters Associated With Robustness Assessment

Method		TLC						HPLC							
Variable parameter		Time of mobile phase saturation (min)			Mobile phase composition (%, CHCl$_3$)			Mobile phase flow rate (mL min^{-1})			Mobile phase composition (%, methanol)			Detector wavelength (nm)	
Degree of variation		45 ± 5			40 ± 2			2 ± 0.1			25 ± 1			220 ± 4	
Measured value	R$_F$ value							t$_R$ value							
	CF	0.56	0.52	0.55	0.52	0.52	0.54	0.54	0.52	0.50	4.11	4.12	4.17	4.14 4.12	4.12
	PZ	0.63	0.64	0.64	0.61	0.64	0.69	0.66	0.64	0.60	12.65	12.80	12.54	12.78 12.80	12.81
	PC	0.36	0.36	0.38	0.35	0.36	0.37	0.38	0.36	0.33	3.39	3.41	3.42	3.39 3.41	3.40
RSD (%)	CF	3.83			2.19			3.85			0.78			0.28	
	PZ	0.91			6.25			4.82			1.03			0.12	
	PC	3.15			2.78			7.06			0.45			0.29	

1.22 and 1.56 for CF, PZ and PC, respectively, in HPLC method. The obtained data suggest that both methods are accurate and precise.

Specificity
Also, results obtained from the analysis of the laboratory prepared mixtures indicate that both methods are specific. A good separation of CF, PZ and PC from PC impurities was obtained as represented in Figure 1b, Figure 2b and Supplementary Figure S1. In TLC method, separation of PC (R$_F$: 0.36), CF (R$_F$: 0.52) and PZ (R$_F$: 0.65) from the two PC impurities 4 AP and 4 NP (at R$_F$: 0.22 and 0.97, respectively) was achieved. In HPLC method, the values of t_R were 2.333, 3.428, 4.178, 7.546 and 12.858 min for 4 AP, PC, CF, 4 NP and PZ; respectively. A good resolution between the components was obtained using the specified chromatographic conditions where resolution (Rs) values were found to be 4.38 for 4 AP relative to PC, 2.30 for PC relative to CF, 7.48 for CF relative to 4 NP and 10.08 for 4 NP relative to PZ.

Limit of detection and limit of quantitation
LOD and LOQ which are limit of detection and limit of quantitation were calculated for both methods as mentioned before and results were represented in Table II.

The calculated LOD and LOQ values were verified practically where the signal to noise ratio of each component was found to be at least 3 for LOD and at least 10 for LOQ.

Robustness and system suitability testing
Robustness was assessed by evaluating the influence of the application of small changes on chromatographic conditions as mobile phase flow rate (2 ± 0.1 mL/min), percentage of mobile phase components (methanol, 25 ± 1%), and wavelength (220 ± 4 nm) of the detector in the HPLC method. For TLC method, small variation in time required for saturation with the mobile phase (45 ± 5 min), and the percentage of mobile phase components (chloroform, 40 ± 2%) were performed. The investigated parameters did not exhibit considerable differences in results regarding RSD, indicating that both methods were robust as represented in Table III.

Table IV. System Suitability Parameters for CF, PZ and PC in the Proposed HPLC Method

Parameter	Obtained value							Recommended value[a]
	CF	PZ	PC	4AP and PC	PC and CF	CF and 4NP	4NP and PZ	
Tailing factor (T)	1.2	1.1	1.1					≤ 2
Number of theoretical plates (N)	3,247	2,460	2,950					>2000
Resolution (Rs)				4.38	2.30	7.48	10.08	>2
Selectivity factor (α)				1.47	1.218	1.81	1.84	>1

[a]Values defined by FDA Center of Drug Evaluation and Research's reviewer guidance on validation of chromatographic methods (November 1994) [19].

Table V. Analysis of Pharmaceutical Preparation and Application of Standard Addition Technique

Drug/method	TLC (Recovery % $\pm$ SD)	HPLC (Recovery % $\pm$ SD)
CF (Stopain® tablet)	101.86 ± 1.67	99.52 ± 2.07
PZ (Stopain® tablet)	101.12 ± 1.44	101.34 ± 0.66
PC (Stopain® tablet)	100.94 ± 0.15	102.11 ± 0.85
CF (Standard addition technique)[a]	99.81 ± 1.46	100.39 ± 1.02
PZ (Standard addition technique)[a]	101.36 ± 0.42	100.49 ± 1.24
PC (Standard addition technique)[a]	99.86 ± 0.67	101.17 ± 0.22

[a](Mean $\pm$ SD) estimated using nine determinations over three concentration levels covering the specified range.

Table VI. One-Way ANOVA Statistical Analysis Within 95% Confidence Interval on Recovery Percentage Data Obtained From Reported Method and Application of the Two Corresponding Methods on Pharmaceutical Preparation

One way ANOVA					
Dependent variable: recovery percentage data					
Source	Sum of squares	df[a]	Mean square	F [b]	P-value
CF					
Between groups[c]	0.479	2	0.240	0.161 (5.14[d])	0.855
Within groups	8.946	6	1.491		
Total	9.425	8			
PZ					
Between groups[c]	0.044	2	0.022	0.014 (5.14[d])	0.986
Within groups	9.154	6	1.526		
Total	9.197	8			
PC					
Between groups[c]	3.679	2	1.839	1.799 (5.14[d])	0.244
Within groups	6.135	6	1.022		
Total	9.814	8			

[a]Degrees of freedom. [b]F is the ratio of mean square to error mean square. [c]Between reported method (RP-HPLC-UV method) [21] and the two corresponding methods. [d]The tabulated value of F.

System suitability parameters including resolution of peaks, tailing factor, number of theoretical plates and selectivity factor was computed for the proposed HPLC method and successfully fulfilled FDA recommendations [19], as shown in Table IV.

Assay of pharmaceuticals

The methods were applied for the determination of PC, PZ and CF in Stopain® tablets, then, standard addition technique was applied to assay the validity of the proposed methods to determine PC, PZ and CF selectively in the presence of formulation additives and excipients, where satisfactory results were obtained, as shown in Table V.

Statistical comparison

One-way ANOVA statistical comparison at 95% confidence interval was performed [20] on the recovery percent results obtained from the application of the two proposed methods on the pharmaceutical dosage form, as shown in Table VI. Comparison proved that there was no significant difference between the proposed methods and the reported RP-HPLC-UV method [21]. The described methods can

be used for accurate assessment of CF, PZ and PC in their ternary mixtures and in pharmaceutical preparations.

Discussion

The current study aims to develop two chromatographic methods for the determination of PC, PZ and CF in the presence of PC impurities, namely 4 AP and 4 NP, in the absence of a reported impurity indicating methods. Both methods were optimized and a good separation of PC, PZ and CF from impurities was achieved. Results of validation parameters reveal that the two methods are accurate, precise, linear, specific and robust.

Conclusion

Two accurate, precise and selective impurity indicating chromatographic methods were developed for the determination of PC, PZ and CF without interference from PC impurities. The methods were validated as per the ICH guidelines. Both methods were found suitable to be used as impurity indicating methods for determination of PC, PZ and CF in pharmaceutical preparations in quality control laboratories. HPLC method is time-saving method while the TLC method provides an inexpensive technique.

Supplementary data

Supplementary data mentioned in the text are available to subscribers in *CHRSHI* online.

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