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Research Article

Optimization and validation of RP-HPLC method for the estimation of chlorzoxazone and paracetamol with its genotoxic impurity (4-amino phenol) in bulk and pharmaceutical drug product using PDA detector

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ABSTRACT

A simple, accurate, precise, reproducible RP-HPLC method has been developed for simultaneous estimation of chlorzoxazone (CZX) and paracetamol (PCM) with its genotoxic impurity, p-amino phenol (4-AP) in bulk and combined dosage form (tablet). The method was validated in compliance with ICH guidelines [1-2]. The LC separation was achieved on LiChrospher RP-18e (250X4.6mm), 5µm column at 279 nm in isocratic mode using mobile phase composition methanol: acetate buffer (70:30 v/v), pH adjusted to 5.5 by acetic acid. Flow rate employed was 1.0 ml/min. The retention time for paracetamol, chlorzoxazone and 4-amino phenol were found to be, 3.76, 6.20 and 2.75 minutes respectively. Linearity ranges for paracetamol, chlorzoxazone and 4-amino phenol were established in the range of 10-50 µg/ml, 10-50 µg/ml and 0.8-2.8 µg/ml respectively with correlation coefficient of 0.997, 0.996 and 0.999 respectively. The % recoveries for paracetamol, chlorzoxazone and 4-amino phenol impurity were found in range with relative standard deviation (RSD) less than 1. The LOD and LOQ were found to be 1.1483 and 3.4798 for paracetamol, 1.3890 and 4.2092 for chlorzoxazone and 0.01459 and 0.0442 for p-amino phenol respectively in µg/ml. The proposed method is successfully applied for the quantification of paracetamol, chlorzoxazone and 4-amino phenol impurity in bulk and formulations.

Key words: Chlorzoxazone, Paracetamol, 4-Amino phenol impurity, Photodiode array detector

INTRODUCTION

Synthesis of drug substances often involves the use of reactive reagents and hence, these reagents may be present in the final drug substances as impurities. Such chemically reactive impurities may have unwanted toxicities, including genotoxicity and carcinogenicity [3-5] and are to be controlled based on the maximum daily dose. Paracetamol(PCM), *N-(4-hydroxyphenyl)acetamide* (Fig.1)[6-8], also known as acetaminophen, is one of the popular non-steroidal anti-inflammatory drugs widely used for management of pain and fever in a variety of patients including children, pregnant women, the elderly and those with ostheoartheritis, simple headaches and non-inflammatory musculoskeletal conditions. Chlorzoxazone (CZX) is chemically, *5-chloro-2, 3-dihydro-1, 3 -benzoxazol-2-one* (Fig. 1), is a well known muscle relaxant drug[9]. It is official in United States Pharmacopoeia (USP). USP describe spectrophotometric method for its estimation[10]. Literature survey reveals HPLC and UV method for estimation of chlorzoxazone and paracetamol alone or in combination with other drug combination [11-17]. 4-aminophenol (4-AP)(Fig.1) is a degradation product of paracetamol or it may be originated from the synthesis; it is reported to have significant nephrotoxic and teratogenic effects[18], therefore its amount should be strictly controlled. It is limited to a low level of 0.005% in the drug substance by the European and British Pharmacopoeias.[19]. Under the conditions of high temperature and pH, paracetamol undergoes hydrolysis forming p-aminophenol [20,21]

Paracetamol and chlorzoxazone are frequently associated in pharmaceutical oral formulations. These active compounds have different polarity and, therefore chromatographic method development is cumbersome and is further complicated by the presence of impurities such as 4-aminophenol related to paracetamol.

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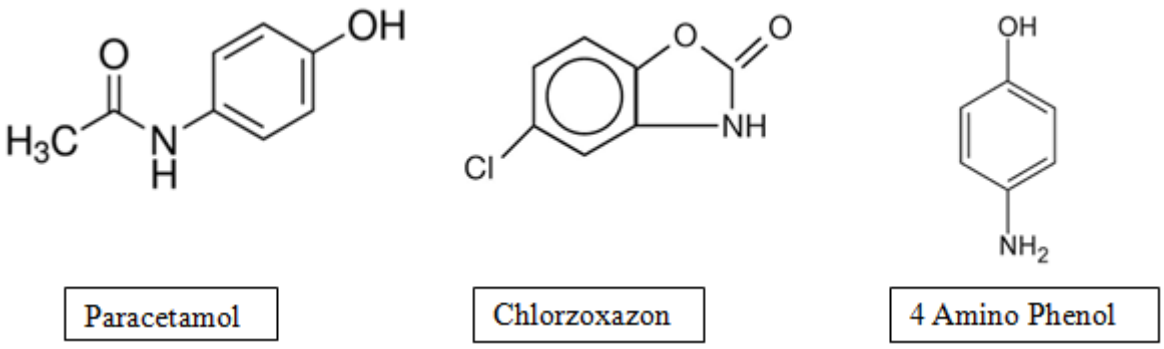

Figure 1: Chemical structure of paracetamol, chlorzoxazone and 4 amino phenol

The dosage forms also contain excipients, some of which may interfere with the analysis of the active ingredients. No single method is reported to determine the active ingredients quantitatively in this combination with a check on p-amino phenol (genotoxic impurity) in formulation.

2 Experimental

2.1 Instrumentation

The LC system consisted of Waters 600E Controller HPLC system equipped with degasser and coupled with photo diode-array detector (PDA 2998). The system connected to software Empower 2 for controlling the instrumentation and processing the data to generate the result. The injection volume was set to 20 µl and the separation was carried out on LiChrospher RP-18 e (250X4.6 mm) with particle size of 5µm. The pH meter used was digital pH meter DPH-115PM.

2.2 Material and methods

Analytically pure samples of Chlorzoxazone and paracetamol were received as gift samples from Auro Laboratories Ltd, Mumbai and Ipca Laboratories Pvt. Ltd. Ratlam (M.P.). 4-Amino phenol was purchased from Sigma Aldrich. HPLC grade methanol, water and acetic acid were purchased from Merck India Pvt Ltd. Sodium acetate was purchased from SD Fines chemicals, Bombay. Tablet containing 325 mg paracetamol and 250 mg chlorzoxazone (Myospaz, Win Medicare Pvt. Ltd) was purchased from local drug market.

2.3 Preparation of mobile Phase

Accurately measured quantity of sodium acetate was dissolved in 200 ml of HPLC grade water to give 20 mM concentration. The pH of acetate buffer was adjusted by 20 mM acetic acid solution using a pH meter. A mixture of buffer and methanol (30:70) was prepared and sonicated for 20 minutes. The solution mixture was filtered through whatman filter paper (0.45µm) and used as mobile phase.

2.4 Preparation of standard stock solution

A 100 mg of standard PCM, CZX and 4 -AP were accurately weighed and transferred separately to 100 ml volumetric flasks and dissolved in 15 ml methanol. The flask was sonicated for 20 minutes and volume was made up to the mark with mobile phase to give solutions of 1mg/ml.

2.5 Preparation of sample solution

Twenty tablets were weighed individually and the average weight of the single tablet was calculated. Powder equivalents to 32.5 mg PCM, 25 mg CZX was accurately weighed and transferred to 100 ml volumetric flask. 15 ml of methanol was added to same volumetric flask and sonicated for 20 minutes. The flask was shaken and volume was made up to the mark with mobile phase. The above solution was filtered through whatman filter paper (0.45µm). Further 1 ml of this solution was transferred to volumetric flask of 10 ml capacity. Volume was made up to the mark with the mobile phase to give a solution containing 32.5 µg/ml of PCM and 25 µg/ml of CZX. This solution was again sonicated for 5 minutes.

3 Method optimization

During preliminary investigations of chromatographic behavior of chlorzoxazone, paracetamol and 4-amino phenol, the influence of mobile phase composition (% of methanol, buffer and pH) was investigated. Retention time, capacity

factor and resolution were chosen as dependent variable. Mobile phase (methanol: buffer) solution in 3 different volume ratio of 20:80, 60:40 and 80:20 were used at pH 3, 5.4 and 6.8. The flow rate was used at 1.0 ml/min and the column temperature was maintained at 30±5 ◦C. The total chromatographic run time is 10 minutes with an additional 10 minutes of column re-equilibration time between each injection. The solution samples were analyzed using a photo-diode array (PDA) detector covering the range of 200–400 nm Because of similar structure paracetamol and 4-aminophenol (process-related impurity) have similar retention behavior and capacity factor therefore result in poor separation.

The retention and separation of compounds under the mobile phase(methanol:buffer) ratio of 20:80, 60:40 at different pH and temperature was not achieved therefore the pH and ionic strength changes at mobile phase composition of 20:80 60:40 do not have any significant impact on separation of the compounds. At mobile phase ratio of 80:20(methanol:buffer solution) and pH of 5.4 all three compounds showed reasonable separation hence further optimization was done at above condition by fine-tuning of the pH mobile phases compositions, flow rates to obtain the final optimum elution profile of the method.

4 Analytical Method validation

Method Validation

The method was validated for linearity, accuracy and intra-day and inter-day precision, and robustness in accordance with ICH guidelines.

4.1 Linearity and Range

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

Linearity were established in the concentration range of 10-50 µg/ml for PCM and CZX and 0.8-2.8 µg /ml for 4-AP. Accurately measured working standard solutions of PCM and CZX and 4-AP were transferred to a series of 10 ml of volumetric flasks and diluted to the mark with mobile phase. Aliquots (20 µl) of each solution were injected under the operating chromatographic condition. All the chromatograms were repeated for 5 times. Calibration curves were constructed by plotting average area versus concentrations for all the three compounds.

4.2 Method precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. It provides an indication of random error results and was expressed as Coefficient of Variance (CV).

Precision of the method was performed as intraday and inter day study. Precision of the method was studied by determining the assay and analyzing corresponding responses six times on the same day (intraday) and different days (inter day) Standard solution mixture containing PCM, CZX and 4-AP were prepared in test concentration. The precision of the instrument was checked by repeatedly injecting mix standard solution of PCM, CZX and 4-AP under the same chromatographic condition and measurements of peak area done. Percentage relative standard deviation (RSD) or coefficient of variation (CV) should not be more than 2 %.

4.3 Limit of detection and Limit of quantification

The detection limit (LOD) of an individual analytical procedure is the lowest amount of analyte in the sample which can be detected but not necessarily quantified as an exact value. The quantitation limit (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOD and LOQ of drugs can be calculated using the following equations designated by International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$

$LOQ = 10 \times \sigma/S$

Where, σ = the standard deviation of the response and S = slope of the calibration curve.

4.4 Accuracy (Recovery studies)

Accuracy of an analytical procedure is the closeness of agreement between the conventional true value or an accepted reference value and the value found. The accuracy was determined by

adding a known amount of standard drug to the fixed amount of pre-analyzed tablet solution. % Recovery was calculated by standard addition method which was performed at 80%, 100% and 120% level. The % recovery and % RSD were calculated.

5 Result and Discussion

Appropriate solution concentration of each drug (PCM, CZX and 4 AP) was scanned over the range of 200 to 400 nm in spectrum mode. The overlay spectra (fig. 2) of these 3 drugs showed optimum absorbance at 279 nm. This wavelength was used for detection of PCM, CZX and 4 AP. The system containing methanol and buffer solution (20 mM sodium acetate, pH 5.5 adjusted with acetic acid) in the ratio70:30 gave well resolved peaks. The retention time were found to be 2.75 minutes for 4 AP, 3.76 minutes, for PCM and 6.20 minutes for CZX (fig 3).

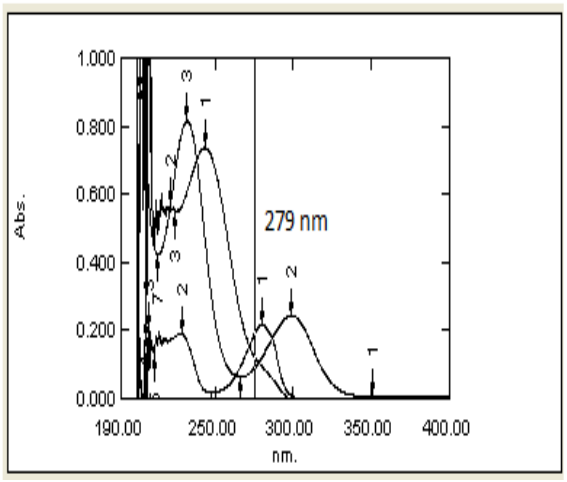

Figure 2: Overlay spectra of paracetamol, chlorzoxazone and 4-amino phenol

Table 1: Optimized condition for estimation of paracetamol, chlorzoxazone and p- amino phenol

S. No.	Parameter	Specification
1	Instrument	Waters 600E Controller
2	Column	LiChrospher RP-18e (250X4.6mm)
3	Particle size	5 µm
4	Detector	2998 PDA detector(Waters)
5	Wavelength	279 nm
6	Mobile phase	Methanol: Acetate buffer(70:30)
7	pH	5.5
8	Flow rate	1ml/min.
9	Run time	10 minutes
10	Temperature	Room Temperature
11	Injection volume	20 µl

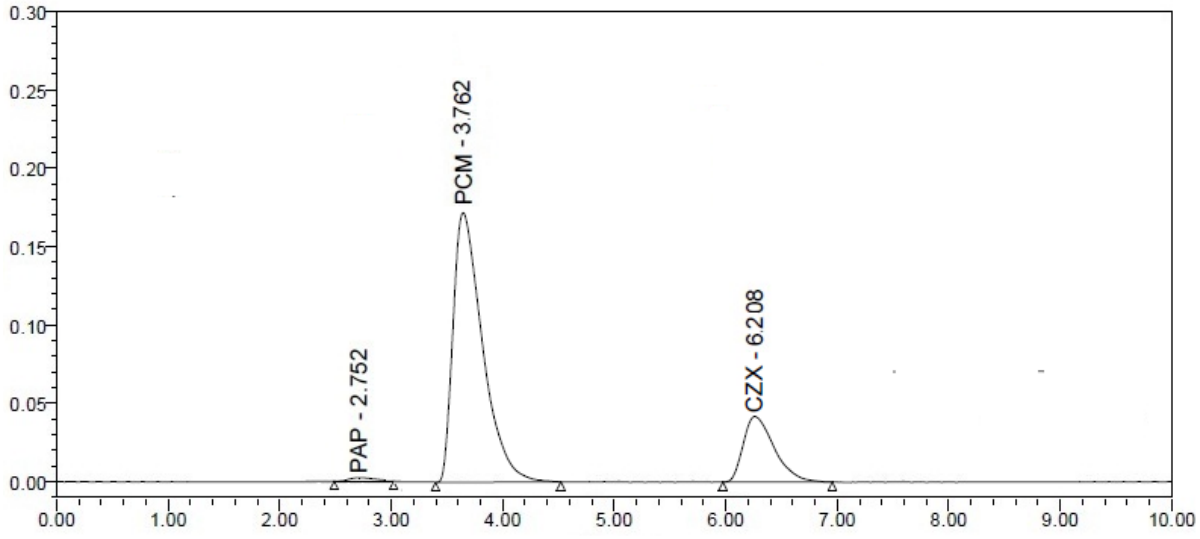

Figure 3: Chromatogram of mixed standard solution of PCM, CZX and 4-AP

Table 2:

	Peak Name	RT	Area	USP Plate	Symmetry	USP Tailing	USP Resolution
1	PAP	2.752	24367	2109	1.78	1.78	
2	PCM	3.762	4884596	2567	1.79	1.79	1.713998
3	CZX	6.208	781042	3894	1.55	1.55	3.424432

	K Prime
1	1.752005
2	2.762004
3	5.208027

5.1 Analytical method validation

Linearity and Range

The method was found to be linear at the concentration range of 10-50 µg/ml for PCM and CZX and 0.8-2.4 for 4 amino phenols. Calibration data for PCM, CZX and 4 amino phenol are shown in Table 2-4 respectively. The calibration curves for PCM, CZX and 4 amino phenol were prepared by plotting area and concentration (Fig. 4-6).

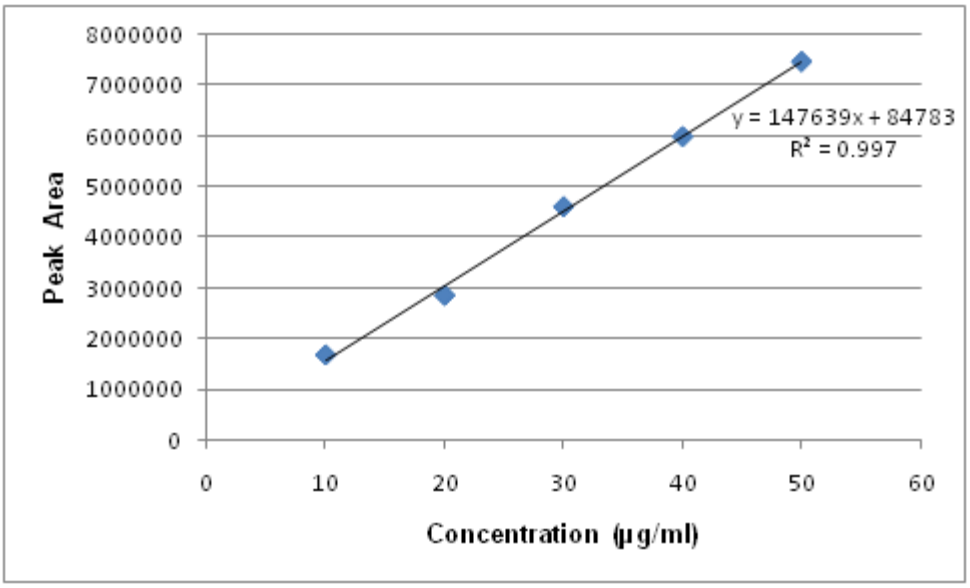

Figure 4: Calibration curve of paracetamol

Table 3 Peak area of paracetamol

Concentration (µg/ml)	Mean Area ± SD	% RSD
10	1677213±469.2736	0.027979
20	2854472±425.9734	0.014923
30	4604300±573.7983	0.012462
40	5987484±760.5837	0.012702
50	7469487±884.1101	0.011836

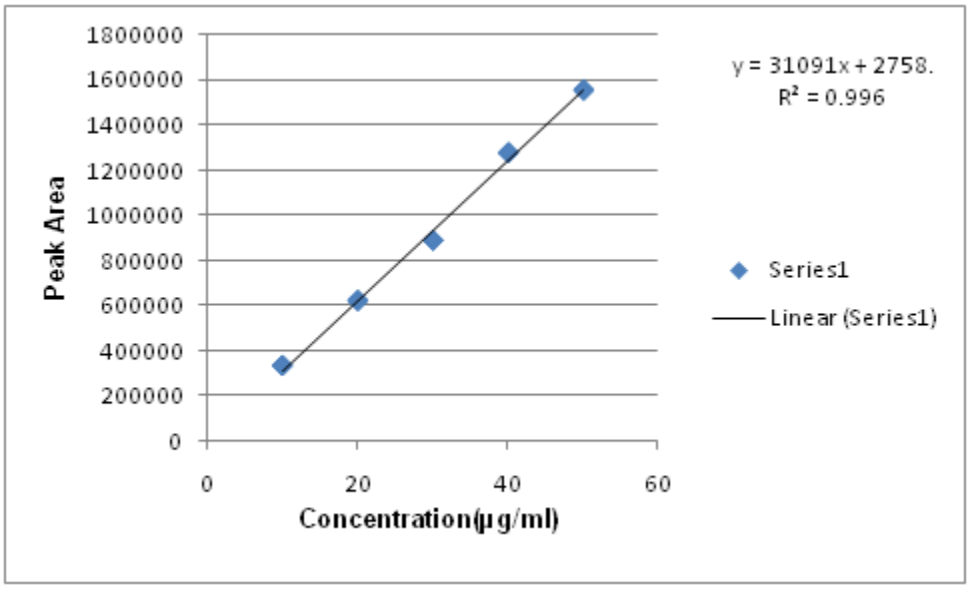

Figure 5: Calibration curve of chlorzoxazone

Table 4: Peak area of chlorzoxazone

Concentration (µg/ml)	Mean Area ± SD	% RSD
10	331772.2±343.4148	0.103509
20	620901.6±723.5622	0.116534
30	888645.8±607.514	0.068364
40	1278693±1075.756	0.084129
50	1557424±1021.269	0.065574

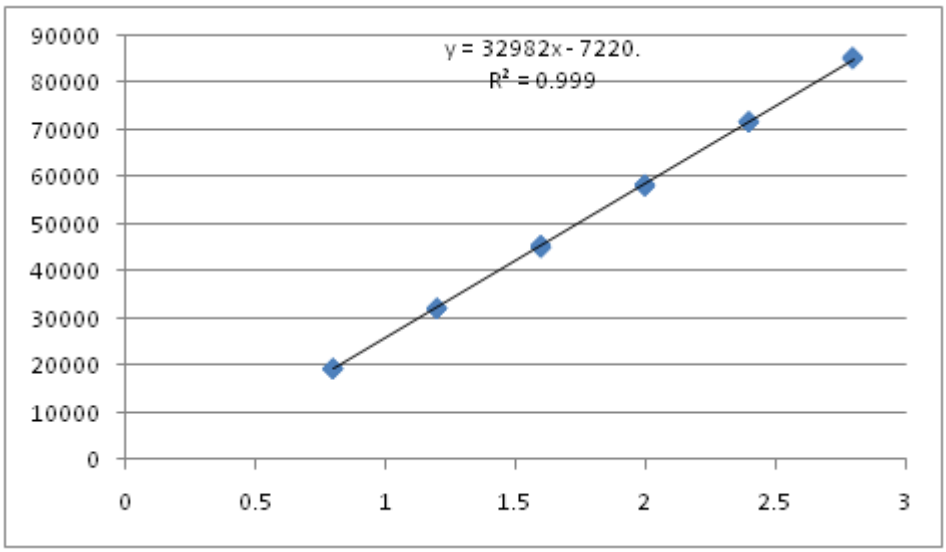

Figure 6: Calibration curve of 4- amino phenol

Table 5: Peak area of 4- amino phenol

Concentration (µg/ml)	Mean Area ± SD	% RSD
0.8	19460±103.9471	0.534158
1.2	32265.2±89.92052	0.278692
1.6	45421±264.3492	0.581998
2	58404±191.4536	0.327809
2.4	71912.4±94.92523	0.132001
2.8	85425.6±181.6241	0.212611

Precision

The low %RSD value (table 5) of intraday precision (0.17237 for PCM, 0.5064for CZX and 0.9593 for 4-AP) and intraday precision (0.2028 for PCM, 0.5774 for CZX and 1.1172 for 4-AP) at 279 nm, reveal that proposed method is precise.

Table 6: Intraday and Interday precision study of PCM, CZX and 4 Amino phenol

	Concentration	Intraday		Inter day	
Drug	(µg/ml)	Mean Area ± SD	% RSD	Mean Area± SD	% RSD
Paracetamol	32.5	4881579±8414.362	0.17237	4882803±9905.265	0.20286
Chlorzoxazone	25	783320.3±3966.871	0.506417	782456±4518.466	0.577472
4-AP	1	25128.17±241.0622	0.95933	24956±278.8283	1.11728

The limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for PCM, CZX and 4-AP were found to be 1.148364 and 3.4798, 1.3890 and 4.20921 and 0.014595 and 0.044227 respectively in µg/ml. These data show that method is sensitive for the determination of PCM, CZX and 4-AP.

Accuracy

The recovery experiment was performed by the standard addition method. The mean recoveries for PCM, CZX and 4-AP were found in the range respectively (Table 06). The results of recovery studies indicate that the proposed method is highly accurate. The proposed validated method was successfully applied to determine PCM, CZX and 4-AP in synthetic mixture.

Table 7: Recovery study of PCM, CZX and 4-AP

Amount of Paracetamol, (µg/ml)								
S No	% Drug in sample	% Std drug added	% Test Concentration	Total drug recovered	% Recovery	Mean	±SD	% RSD
1	80 (26 µg)	0	80 (26 µg)	25.96	99.8461	99.7820	±0.2937	0.2943
	80 (26 µg)	0	80 (26 µg)	25.86	99.4615			
	80 (26 µg)	0	80 (26 µg)	26.01	100.0384			
2	80 (26 µg)	20(6.5 µg)	100(32.5 µg)	32.47	99.9076	99.9692	±0.0814	0.0814
	80 (26 µg)	20(6.5 µg)	100(32.5 µg)	32.48	99.9384			
	80 (26 µg)	20(6.5 µg)	100(32.5 µg)	32.52	100.061			
3	80 (26 µg)	40(13 µg)	120(39 µg)	39.04	100.1025	99.9829	±0.1068	0.1068

	80 (26 µg)	40(13 µg)	120(39 µg)	38.98	99.9487			
	80 (26 µg)	40(13 µg)	120(39 µg)	38.96	99.9874			

Amount of Chlorzoxazone, µg/ml

1	80 (20 µg)	0	80 (20 µg)	19.9864	99.932	100.08	±0.1337	0.1336
	80 (20 µg)	0	80 (20 µg)	20.0257	100.1285	2		
	80 (20 µg)	0	80 (20 µg)	20.0375	100.1875			
2	80 (20 µg)	20(5 µg)	100(25 µg)	25.0321	100.1284	100.043	±0.1387	0.1387
	80 (20 µg)	20(5 µg)	100(25 µg)	25.0296	100.1184			
	80 (20 µg)	20(5 µg)	100(25 µg)	24.9708	99.8832			
3	80 (20 µg)	40(10 µg)	120(30 µg)	29.9742	99.914	99.8824	±0.0273	0.0274
	80 (20 µg)	40(10 µg)	120(30 µg)	29.9605	99.8683			
	80 (20 µg)	40(10 µg)	120(30 µg)	29.9595	99.865			

Amount of 4 Amino phenol, µg/ml

	Impurity added, µg/ml	**Impurity µg/ml**	**Recovered**	**% Recovery**	**±SD**	**%RSD**
1	0.8	0.8028		100.35	0.83638	0.836903
	0.8	0.8039		100.4875		
	0.8	0.7918		98.975		
2	1.2	1.213		101.0833	0.538602	0.533916
	1.2	1.2032		100.2666		
	1.2	1.2154		101.2833		
3	1.6	1.6123		100.7687	0.955991	0.95625
	1.6	1.5826		98.9125		
	1.6	1.6038		100.2375		

System Suitability

System suitability parameters obtained shows that the chromatographic conditions are appropriate for separation and quantification of compounds.

Table 8: System suitability result under optimized condition

Parameters	4-AP	Paracetamol	Chlorzoxazone
Capacity factor(k')	1.752	2.762	5.208
Precision(RSD)	< 1	<1	<1
Resolution(R_s)	-	1.71	3.42
Tailing Factor(T)	1.78	1.79	1.55
Plate count(N)	2109	2567	3894

5.2 Assay of marketed formulation

Two Sample solutions of marketed tablet formulation were prepared to give test concentration. One of the solutions is spiked with 4 amino phenol impurity and the proposed validated method was applied successfully to determine PCM, CZX and 4 -AP in spiked and unspiked samples.

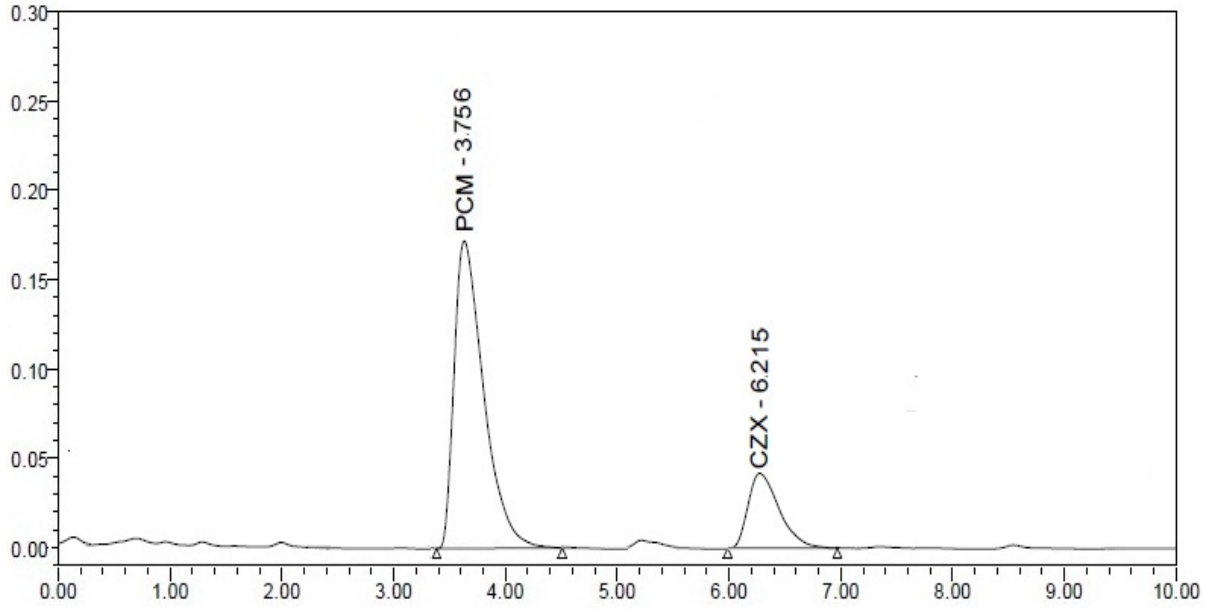

Figure 7: Chromatogram of tablet sample solution of PCM and CZX(unspiked)

Table 9: Assay result of tablet formulation unspiked

Drug/Impurity	Lable claimed/spiked	Amount	% purity	SD	%RSD
PCM	325	32.3785	99.62	0.1348	0.4166
CZX	250	25.281	101.24	0.1377	0.5448

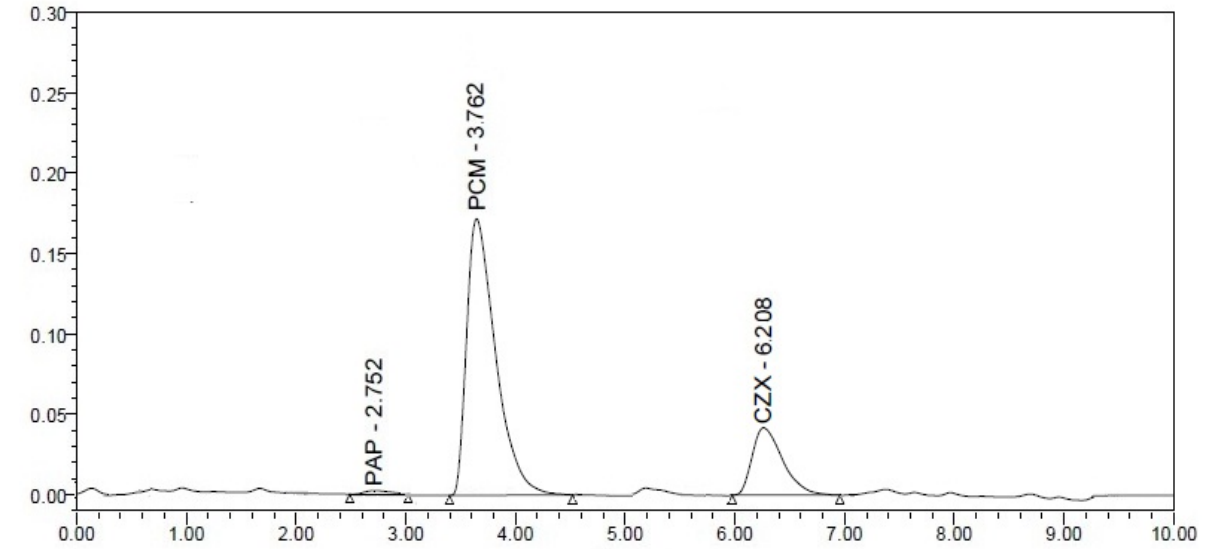

Figure 8: Chromatogram of tablet sample solution of PCM and CZX(spiked)

Table 9: Assay of tablet formulation spiked with 4- amino phenol impurity

Drug/Impurity	Lable claimed/spiked	Amount	% purity	SD	%RSD
PCM	325	32.4204	99.75	0.1018	o.3140
CZX	250	24.8635	99.45	0.1191	0.4790
4-AP	1	0.9836	98.36	0.0690	0.6995

Conclusion

No interference of the excipients with the retention time of drugs appeared (Figure 7,8); hence the proposed method is applicable for the routine simultaneous estimation of CZX , PCM and its impurity 4-AP

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