High-performance Liquid Chromatographic Determination of Paracetamol, Propyphenazone, and Caffeine in Pharmaceutical Formulations

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ABSTRACT

This study proposes a liquid chromatography (RPHPLC) method for quantitative analysis of the most widely prescribed combination of Paracetamol, Propyphenazone and Caffeine in tablet dosage form in presence of Rasagiline as an internal standard. The chromatography was performed on a Gracesmart C18 column (5μm, 250mm $\times$ 4.6mm i.d.) using the mixture of water and 2-propanol in the ratio 80:20 v/v as mobile phase. The pH of aqueous phase was adjusted to 3.0 with 1% o-phosphoric acid and a flow rate was maintained at 1.5ml/min. The analytes were detected by measuring the absorbance at 210nm. The chromatographic analysis time for each sample was kept about 20min as Rasagiline, Paracetamol, Caffeine and Propyphenazone were eluted at 1.63, 2.36, 3.17 and 11.22 min respectively. The calibration plots were obtained between 3–90μg/ml for Paracetamol, 1.5–45μg/ml for Propyphenazone and 0.5–15μg/ml for Caffeine. The method was validated in accordance with ICH norms and method was found to be specific, accurate, precise and easy to use for routine analysis.

Keywords: RPHPLC, Paracetamol, Propyphenazone, Caffeine, Rasagiline, Internal standard.

INTRODUCTION

Tablets containing Paracetamol (4-Hydroxyacetanilide, PCM), Propyphenazone (4-Isopropyl-2, 3-dimethyl-1-phenyl-3-pyrazolin-5-one, PP) and Caffeine (3, 7-Dihydro-1, 3, 7-trimethyl-1H-purine-2, 6-dione, CAF) (Figure 1)[1] are emerging as the most widely prescribed combination for pain relief. Few marketed formulations like DART (Juggat Pharma Ltd) and SARI-DON (Piramal HealthCare Ltd) tablets are available containing different proportions of PCM, PP and CAF. This combination causes reduction in the amount of prostaglandin where CAF is also known to increase the analgesic effect of PCM and PP, synergistically[2,3] and providing relief from the symptoms like headache, muscular aches, neuralgia, backache, joint pain, rheumatic pain, migraine, general pain, toothache and menstrual pain. The combination is also found to be effective in controlling fever originating from bacterial or viral infection. With limited side effects the combination is suitable for all ages.[4]

Currently, the reported methods for UV-Visible simultaneous estimation of PCM, PP and CAF in combined dosage form are a multiparameter-responding flow-through system with solid phase UV spectrophotometric detection,[5] derivative ratio spectra–zero crossing spectrophotometry and simultaneous estimation based on additivity of the absorbance of these drugs.[6] Various RPHPLC methods are reported for estimation PCM, PP and CAF individually or in binary mixture or in combination with other drugs.[7–22]
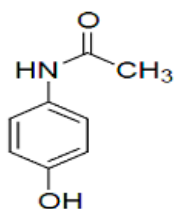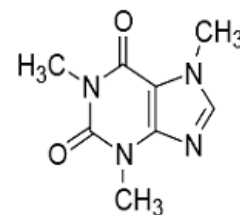
Figure 1: Chemical Structures of drugs.

Hence the attempts have been made to develop a simple, sensitive, validated RPHPLC method with internal standard for simultaneous determination of Paracetamol, Propyphenazone and Caffeine in pharmaceutical formulations. The method has been successfully used for quality-control analysis of the drugs and for other analytical purposes.

EXPERIMENTAL

Instrumentation

Analysis was performed with a Shimadzu (Japan) chromatograph equipped with an LC-10 AT solvent-delivery module, an SPD-10A UV–visible detector, and a Rheodyne model 7725 injector valve with 20-μL sample loop. The equipment was controlled by a PC workstation with Spinchrome CFR chromatography software.

Sartorius, 0.1mg sensitive Balance, RC SYSTEMS Ultrasonicator bath and Digisun electronics Digital pH meter (7007) were used during the development of an analytical method. Entire experiment was carried out in an air-conditioned instrument laboratory (20 ± 2°C).

Materials and reagents

Reference standards of Paracetamol, Propyphenazone and Caffeine were obtained from Juggat Pharma ltd, Bangalore, India while reference standard of Rasagiline was procured from Apotex Research Pvt Ltd, Bangalore, India. Pharmaceutical products (DART and SARIDON tablets) were obtained from the local pharmacy. The HPLC grade solvents like 2-propanol (Merck private limited, Mumbai), water and ortho-phosphoric acid (Spectrochem private limited) were used during the study.

Chromatographic conditions

Reversed-phase chromatography was performed using Gracesmart C_{18} column (5μm, 250mm×4.6mm i.d.) as a stationary phase and the mixture of water (pH 3.0 adjusted with 1% o-phosphoric acid) and 2-propanol (80:20 v/v) as mobile phase. The mobile phase was filtered through 0.2μm Supor200 membrane and degassed by sonication before use. The flow rate of mobile phase was maintained at 1.5ml/min. After equilibration of column with the mobile phase indicated by a stable baseline, aliquots of sample (20μl) were injected and the total run time was kept for 20min. All analytes including internal standards were monitored at 210nm with a detection sensitivity of 0.1000Aufs.

Preparation of calibration curves

The stock solution was prepared by dissolving accurately weighed 60, 30 and 10mg of PCM, PP and CAF in 100 ml HPLC grade water to get concentration of 600μg/ml, 300μg/ml and 100μg/ml respectively. From this stock solution 5ml was diluted to 50ml with HPLC grade water to get sub stock of concentration of 60, 30 and 10μg/ml of PCM, PP and CAF respectively. Standard stock solution of RASA was prepared by dissolving 10mg in 100ml of HPLC grade water to get a concentration of 100μg/ml. Standards were freshly prepared on the day of analysis. Using stock, sub stock and RASA stock solution, serial dilutions in the concentration range of 3:1.5:0.5, 6:3:1, 12:6:2, 18:9:3, 24:12:4, 30:15:5, 60:30:10, 90:45:15, and 120:60:20μg/ml of PCM, PP and CAF with 10μg/ml of RASA (IS) were prepared and filtered through 0.45μm cellulose acetate filter membrane (Sartorius AG) and the chromatographic analysis was performed. The results are as shown in a table 1.

Table 1: Statistical Data of PCM, PP and CAF			
Parameters	**PCM**	**PP**	**CAF**
Linear Range (μg/ml)	3–90	1.5–45	0.5–15
Standard regression equations (n = 6)	Y = 0.1945x− 0.0152	Y = 0.1136x + 0.0404	Y = 0.4425x − 0.0174
Slope	0.1945	0.1136	0.4425
Intercept	0.0152	0.0404	0.0174
Correlation coefficient (r^2)	0.9996	0.9998	0.9999
Limit of Detection (μg/ml)	0.2749	0.3206	0.0447
Limit of Quantitation (μg/ml)	0.8330	0.9715	0.1354

Table 2: Statistical Analysis of Linearity

Analyte	Parameter	Sum of square	Degree of freedom	Mean of square	F	p-value
			ANOVA			
PCM	Regression	0.08508	5	0.01701	0.00050	1
PP		0.02369	5	0.00473	0.00164	1
CAF		0.04911	5	0.00982	0.00202	0.9999
PCM	Residual	1619.21	48	33.7336		
PP		138.138	48	2.87789		
CAF		232.738	48	4.84872		
PCM	Total	1619.30	53			
PP		138.162	53			
CAF		232.787	53			
PCM	Multiple R			0.99978		
PP				0.99989		
CAF				0.99994		
PCM	R square			0.99956		
PP				0.99978		
CAF				0.99989		
PCM	Adjusted R square			0.99949		
PP				0.99975		
CAF				0.99988		
PCM	Standard Error			0.13001		
PP				0.02659		
CAF				0.02402		

Analyte	Parameter	Coefficient	Standard Error	t-statistic	p-value	Lower 95%	Upper 95%
			Multiple regression analysis				
PCM	Intercept	−0.0152	0.0600	−0.253	0.8068	−0.1572	0.1267
PP		0.0403	0.0122	3.2873	0.0133	0.0113	0.0694
CAF		−0.0174	0.0111	−1.5718	0.1599	−0.0436	0.0088
PCM	Slope	0.1944	0.0015	126.301	5.14E-13	0.1908	0.1981
PP		0.1136	0.0006	180.351	4.25E-14	0.1121	0.1151
CAF		0.4424	0.0017	259.103	3.37E-15	0.4384	0.4465

Validation procedure

Method validation was carried in accordance to the International Conference on Harmonization (ICH) guidelines with respect to linearity, precision, accuracy, sensitivity and robustness.

Linearity and Range

The linearity of analytical method for PCM, PP and CAF were determined statistically as shown in table 2 and by studying linearity plot as response vs log concentration. Parallel horizontal lines were drawn on the graph corresponding to 95 percent and 105 percent of the horizontal line. The method is linear up to the point where plotted relative response line intersects the 95 percent line. (Fig 2, 3 and 4)

Precision

The precision commonly expressed as the standard deviation or relative standard deviation (coefficient of variation). It was performed as explained below.

Intra-day precision

Intra-day precision was evaluated by analysis of three concentration of PCM, PP and CAF (6:3:1, 30:15:5 and 60:30:10μg/ml) at three different time intervals under the same experimental conditions on the same day.
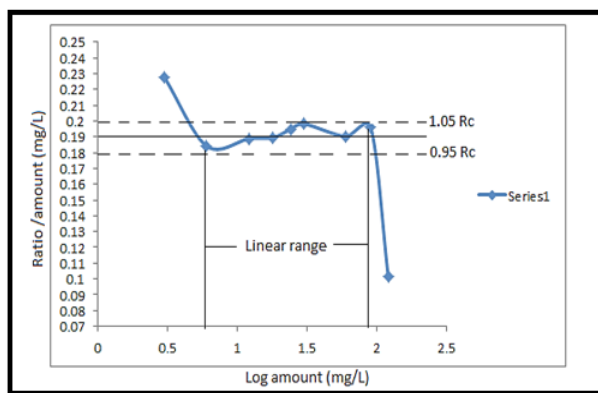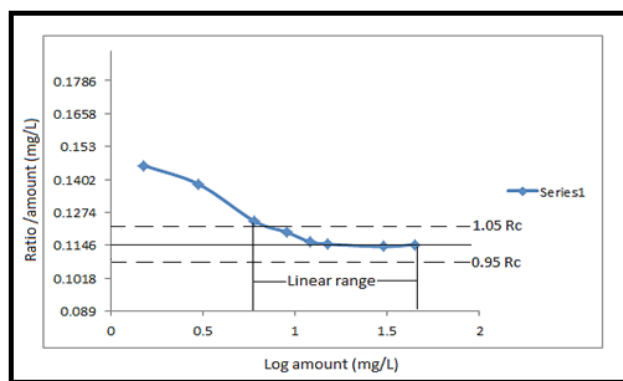
Figure 2: Graph for Linearity Study of PCM.

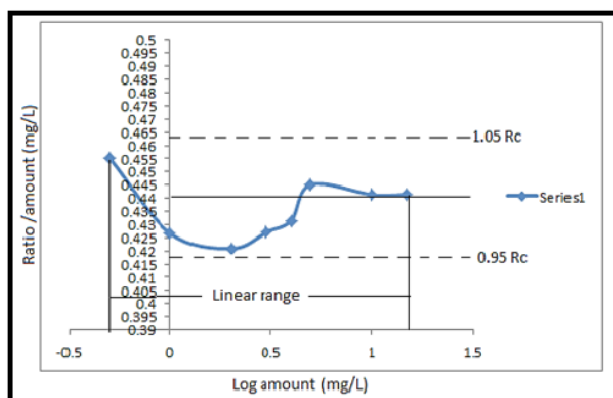**Figure 3:** Graph for Linearity Study of PP.

Figure 4: Graph for Linearity Study of CAF.

Inter-day precision

Intermediate precision (inter-day precision) was determined by analysing three concentration of PCM, PP and CAF (6:3:1, 30:15:5 and 60:30:10 µg/ml) on three consecutive days.

Accuracy/Recovery

For the determination of the accuracy, 20 tablets were weighed and powdered and analysis of the same was carried out in three sets. To study the accuracy of the proposed method, recovery studies were carried out by standard addition of drug at three different levels (50%, 100% and 150%). A known amount of PCM, PP and CAF were added to pre-analysed tablet powder and percentage recovery of each drug was calculated and is as given in table 3.

Sensitivity

Sensitivity of the method was determined by means of the detection limit (LOD) and quantification limit (LOQ). Calculations for LOD and LOQ were based on the standard deviation of the intercept from the calibration curve (σ) and the slope of curve (S), using the equation LOD = $3.3 \times \sigma/S$ and the equation LOQ = $10 \times \sigma/S$.

Robustness

Robustness of the method was evaluated by the analysis of drug solution under different experimental conditions such as pH of the mobile phase and flow rate. The flow rate was varied by ±0.045ml/min (3%) and pH of the mobile phase was changed by ±0.09 units (3%). Their effects on the retention time (*t*R), tailing factor (*T*) and resolution of the peaks (*R*) were studied. These facts suggest that the method did not change with time and experimental conditions. However, it could be noted that organic composition of the mobile phase can influence the method performance.

Specificity

The peak purity of PCM, PP and CAF were assessed by comparing the chromatogram of standard solutions and sample solutions. The retention time of standard drugs compared with that of sample solution. Good correlation was obtained between the retention time of standard and sample of PCM, PP and CAF as shown in fig 5.

Assay in formulation

Twenty tablets (DART Tablet contains 300, 150 and 50mg of PCM, PP and CAF respectively,) were weighed and powdered. An amount of tablet powder equivalent to 30mg of PCM, 15mg PP and 5mg CAF were accurately weighed and transferred it to a 100ml volumetric flask. This mixture was sonicated for 10min. in few ml of HPLC grade water for complete extraction of the drugs and made up to the volume with the same solvent. The resulting solution was filtered through whatman filter paper no. 41. From the above solution 1ml was transferred to a 10 ml volumetric flask along with 1ml of RASA solution (100µg/ml) and made up the volume with the mobile phase to furnish PCM, PP, CAF and RASA of

Table 3: Summary of Recovery studies (DART TABLETS)						
Drug	Pre-analysed Sample Solution (µg/ml)	Amount of Drug Added* (µg/ml)	Total Amount Found (µg/ml)*	Amount of Drug* Recovered (µg/ml)	%Recovery*	%RSD
PCM	30	15	44.97	14.97	99.7277	0.1422
		30	59.81	29.81	99.3823	0.6095
		45	74.7	44.7	99.3332	0.3299
PP	15	7.5	22.47	7.47	99.6982	0.6411
		15	29.94	14.94	99.5656	0.0511
		22.5	37.61	22.61	100.4997	0.3227
CAF	5	2.5	7.49	2.49	99.6279	0.4078
		5	9.97	4.97	99.5538	0.4373
		7.5	12.45	7.45	99.3241	0.3317

*n=3

Table 4: Robustness of the method							
Parameter	Analyte	pH (n=3)			Flow rate (ml/min) (n=3)		
		2.91	3.0	3.09	1.455	1.5	1.545
R^a	RASA	–	5.666	–	–	5.666	–
	PCM and CAF	3.641	3.889	3.424	3.831	3.889	3.852
	CAF and PP	11.71	18.872	17.966	19.511	18.872	19.323
T^b	PCM	1.474	1.450	1.70	1.50	1.450	1.368
	PP	1.474	1.250	1.267	1.598	1.250	1.429
	CAF	2.065	2.079	1.969	2.133	2.079	2.034
R_t^{c*} (min.)	PCM	2.347	2.360	2.333	2.337	2.360	2.293
	PP	10.84	11.223	10.930	11.18	11.223	11.170
	CAF	3.067	3.120	3.020	3.227	3.120	3.137
	RASA	-	1.653	-	-	1.653	-
N^d	RASA	-	2620	-	-	2620	-
	PCM	4393	4916	4357	4737	4916	4697
	PP	5689	5934	5290	5870	5934	5666
	CAF	2291	2233	2125	2253	2233	2305

[a]Resolution factor, [b]Tailing factor, [c]Retention time, [d]Number of theoretical plates Specificity

concentrations 30, 15, 5 and 10µg/ml, respectively. This solution was filtered through 0.45µm cellulose acetate filter membrane and chromatographic analysis was performed for sample and standard solutions in similar experimental condition and obtained results are as shown in table no. 5.

The same procedure was applied for analysis of SARI-DON tablets containing 250, 150 and 50mg of PCM, PP and CAF respectively (Piramal Healthcare Ltd, Bangalore) where chromatographic analysis of sample was performed of concentration 25, 15 and 5µg/ml of PCM, PP and CAF respectively and the results are given in table no. 5.
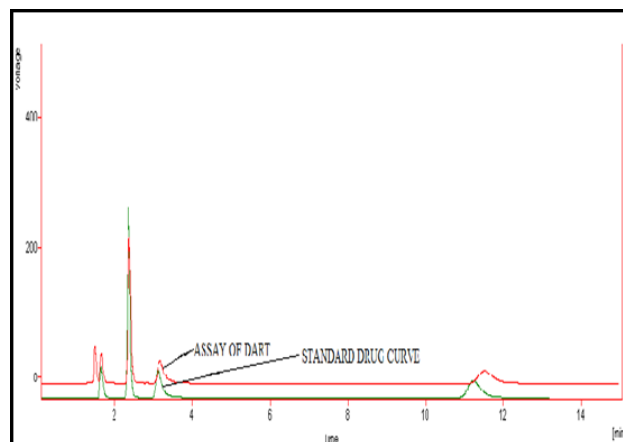

Figure 5: Overlay Chromatogram (3-D View) of Sample and Standard for Specificity Study.

Table 5: Assay of Dart and Saridon tablets (n=6)				
Brand name	Component	Label claim (mg)	Mean amount found (mg)	Amount found (%) ± RSD
DART TABLETS	PCM	300	299.97	99.99 ± 0.8533
	PP	150	150.18	100.12 ± 0.5707
	CAF	50	50.28	100.56 ± 0.9036
SARIDON TABLETS	PCM	250	251.98	100.79 ± 1.0552
	PP	150	150.30	100.20 ± 0.6535
	CAF	50	50.31	100.63 ± 0.8755

RESULTS AND DISCUSSION

Development and optimization of the chromatographic method

Column chemistry, solvent selectivity (solvent type), solvent strength (volume fraction of organic solvent(s) in the mobile phase), additive strength, detection wavelength and flow rate were varied to determine the chromatographic conditions giving the best separation. The mobile phase conditions were optimised so the peak from the first-eluting compound did not interfere with those from the solvent or excipients.

The chromatographic conditions were adjusted to provide the best performance of the assay. For system optimization the important parameters such as type and concentration of organic solvents, ultra-violet detection, pH, mobile phase and flow rate were investigated.

Detection wavelength

To investigate the appropriate wavelength for simultaneous determination of PCM, PP CAF and RASA, the solutions of drugs in the mobile phase were scanned by UV–Visible spectrophotometry (Shimadzu, Japan; model UV-1700) in the range 200–400 nm. From the overlain UV spectra, suitable wavelength choices considered for monitoring analytes were 210, 245 and 260nm. Detection of the mixture of analytes and IS in the mobile phase was carried out at 210, 245 and 260nm. Considering the response and the sensitivity of RASA, 210nm was selected as the most appropriate wavelength for analysis as shown in Figure 6.

Effect of mobile phase composition

RP-TLC of PCM, PP and CAF using water: 2-propanol, methanol: water and acetonitrile: water at different proportions were tried to select the mobile phase composition for further HPLC method development. It was observed that the water: 2-propanol showed better separation among the tried compositions.

Different proportions of water: 2-propanol (90:10, 80:20, 70:30, 60:40, and 50:50v/v) were tested on HPLC. As 80:20 proportions of water: 2-propanol showed better resolution (R) among PCM, PP, CAF and RASA with good peak symmetry, this composition of the mobile phase was finalised. (figure 7)

Effect of pH

Different pH values of the mobile phase were checked to establish the optimum separation and highest analytical sensitivity for PCM, PP, CAF and RASA. The pH values tested were from 2.5 to 7.0. Finally, the best results were obtained by aqueous pH adjusted to 3 using 1% ortho-phosphoric acid.

Effect of flow rate

Different mobile phase flow rates (0.5, 1.0, 1.2 and 1.5 ml/min) were investigated. The optimum flow rate for which the column plate number (N) was maximum, with the best resolution between all components and lower asymmetry of peaks was found to be 1.5ml/min.

Method validation

System suitability

System suitability was performed to confirm that the equipment was appropriate for the analysis to be performed. The test was carried out by making six replicate injections of a standard solution containing 30μg/ml PCM, 15μg/ml PP, 5μg/ml CAF and 100μg/ml RASA (IS) and analysing each solute for their peak area, theoretical plates (N), resolution (R) and tailing factor (T). The results of system suitability in comparison with the required limits are shown in table no 6.

Linearity

The linearity of RP-HPLC method was determined from linearity plot as shown in fig. no 2, 3 and 4 for PCM, PP and CAF respectively and was found to be in the concentration ranges of 6–90μg/ml for PCM, 6–45μg/ml for PP and 0.5–15μg/ml for CAF with correlation coefficients of 0.9999, 0.9998 and 0.9999,
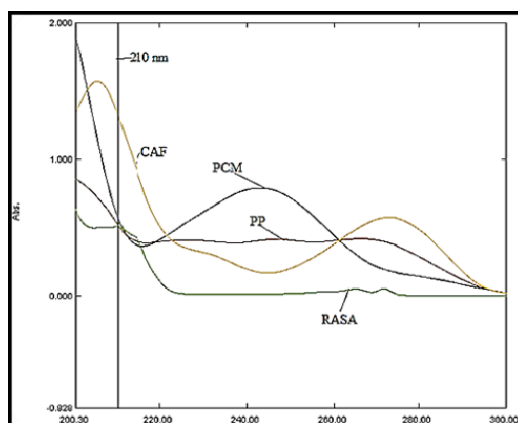
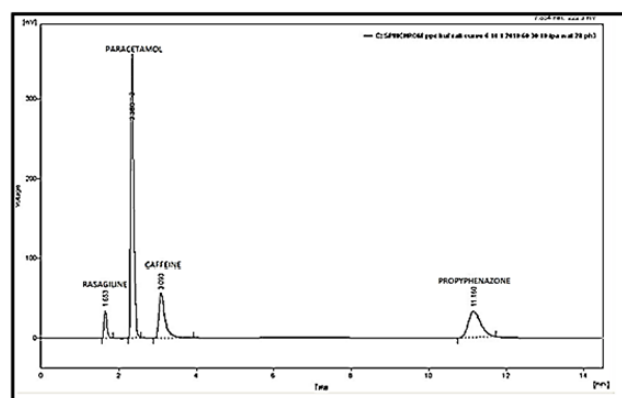**Figure 6:** Overlay Spectra of PCM, PP, CAF and RASA in Mobile Phase.

Figure 7: Chromatogram of standard drugs with IS.

Table 6: Summary of System Suitability Parameters of PCM, PP and CAF with RASA

Analyte	*R[a]	*N[b]	*T[c]	*RSD	
				R_t	Peak Area
PCM	5.666	4916	1.450	0.0066	0.086
PP	18.094	5934	1.250	0.1048	0.108
CAF	3.880	2233	2.079	0.0263	0.095
IS (RASA)	–	2620	1.765	0.0075	0.054
Required limits	R >2	N > 2000	T < 2.5	R.S.D. < 3%	

*n= 6
[a]Resolution factor, [b]Number of theoretical plates (per column), [c]Tailing factor

for PCM, PP and CAF respectively. This was further assured by statistical analysis ANOVA and results were shown in table no 2.

Precision

Three different concentrations of combination of PCM, PP and CAF (6:3:1, 30:15:5 and 60:30:10µg/ml) were selected for intra-day and inter-day precision. The RSD of the study was found to be less than 3%.

Accuracy

Accuracy by standard addition method was determined by calculating percentage recovery and percentage relative standard deviation. The results obtained (as shown in table no 3) indicate that recoveries were good, not less than 99% and percentage relative standard deviations were less than 1%.

Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were established by evaluating the minimum level at which the analyte could be readily detected and quantified with accuracy, respectively. LOD was found to be 0.2749µg/ml, 0.3206µg/ml and 0.0447µg/ml for PCM, PP and CAF respectively and LOQ was found to be 0.8330µg/ml, 0.9715µg/ml and 0.1354µg/ml for PCM, PP and CAF respectively as shown in table no 1.

Robustness

During the robustness study, peak symmetry (T) was maintained and the retention times were not significantly changed as shown in table no 6. These facts suggest that the method did not change with time and experimental conditions. However, it could be noted that organic composition of the mobile phase can influence the method performance.

CONCLUSION

Using Rasagiline as an internal standard, a reverse phase liquid chromatographic method was developed and validated for quantitative determination of PCM, PP and CAF in tablets. It was found that the peaks of drugs and IS were highly resolved and didn't overlap with the probable guessed excipients. The method was validated in terms of linearity, precision, accuracy, limit of detection, limit of quantification and robustness. A simple procedure for preparation of standard, sample and use of low quantity of the organic phase (20% 2-propanol) in the mobile phase making the analysis less expensive are the major advantages of the developed method.

The proposed RP-HPLC method was found to be simple, sensitive, accurate, precise, rapid and easy to apply for routine quality control analysis of Paracetamol,

Propyphenazone and Caffeine in pharmaceutical dosage form.

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