International Journal of Advanced Research in Chemical Science
Volume 7, Issue 6, 2020, PP 7-16
ISSN No.: 2349-0403
DOI: https:// doi.org/10.20431/2349-0403.0706002
www.arcjournals.org

HPLC Method for Simultaneous Determination of Ascorbic acid, Phenylephrine, Paracetamol, Caffeine in Their Pure and Dosage Forms

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Abstract: *An isocratic HPLC method has been developed for determination of ascorbic acid, phenylephrine, paracetamol, and caffeine in their pure and tablet forms. Separation was carried out at room temperature on an Kinetex 2.6 □ C18 100A (4.6 mm × 100 mm) column using a mobile phase of 0.05 M potassium dihydrogen phosphate buffer (pH 3.50 by ortho-phosphoric acid): acetonitrile: methanol (70:20:10). The flow rate was 1 mL/min, maximum absorption was measured at 220 nm and linearity was in the range of 1-50 µg/mL for all drugs. The retention times of ascorbic acid, phenylephrine, paracetamol and caffeine were reported to be 1.83, 2.94, 3.74 and 5.13 minutes, respectively, indicating a very short analysis time compared with other reported methods. Also, limits of detection were reported to be 0.76, 0.82, 0.47 and 0.24 µg/mL for ascorbic acid, phenylephrine, paracetamol, and caffeine respectively, showing a high degree of the method sensitivity. The proposed method was validated in terms of linearity, accuracy, precision and robustness according to ICH guidelines and results were compared statistically with reference methods in respect of precision and accuracy.*

Keywords: *HPLC; ascorbic acid; phenylephrine; paracetamol; caffeine; ICH guidelines*

1. INTRODUCTION

Ascorbic acid (ASC), is chemically (2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one (Figure 1). It is a water-soluble vitamin (Vitamin C) and synthesized by eukaryotes only. It was isolate d from the adrenal cortex by Albert Szent-Györgyi in 1928 [1]. It is found naturally in certain foods, a dded to others, and available as a dietary supplement. Unlike most animals, humans cannot endogenou sly synthesize ASC, so it is an important dietary compound [2]. ASC is an essential component of plan t and animal antioxidant systems which can be characterized as complex redox networks with mutual i nteractions and synergistic effects, including metabolites and enzymes [3]. ASC is necessary for the bi ogenesis of collagen, L-carnitine, cholesterol as well as some neurotransmitters [4]. It also has the pote ntial involvement in cancer and cardiovascular diseases [5-7]. The effects of vitamin C in the treatmen t of ocular disease was investigated, illustrating that ascorbate influences cataract development [8]. Hi gh-performance liquid chromatography (HPLC) [9-13], thin layer chromatography (TLC) [14-17] and spectrophotometric methods [18-20] were developed for the quantification of ASC in pharmaceutical a nd biological samples.

Phenylepherine (PHE), is chemically (1R,2S)- respectively (1S,2R)-2-methylamino-1-phenylpropan-1 -ol (Figure 1). PHE is α1-adrenergic receptor agonist; it is a potent, synthetic, sympathomimetic eleme nt that has been reported to have significant vasoconstrictor confidants when intravenously dosed. PH E appears to have no effect on the heart's beta receptor cells. When given intravenously, it blunts the h eart rate but increases the stroke output thereby causing diastolic and systolic pressures to rise [21]. PH E is a drug used for nasal congestion, hypertension, sinusitis and rhinitis [22]. PHE is now the preferre d vasopressor during the elective caesarean section but it can trigger bradycardia reflex [23]. PHE was determined by several methods such as HPLC [24-26], TLC [27-30], spectrophotometric [31-33] and s pectrofluorimetric methods [34].

Paracetamol (PAR), is chemically N-(4-hydroxyphenyl)acetamide, (Figure 1). As an analgesic and ant ipyretic agent, PAR or acetaminophen is commonly used [35]. It is a safe and effective analgesic agen t used to treat viral and bacterial infections as well as to alleviate pain associated with migraine, backa che, cephalagra and post-operative pain fevers [36]. HPLC methods were developed for the quantificat

ion of PAR [37-40]. Also, TLC methods were used in several experiments for the evaluation of PAR [41,42]. In addition, highly sensitive UV spectrophotometric methods were used for the characterizatio n of PAR in different samples [38,43,44].

Caffeine (CAF), is chemically 1,3,7-trimethyl-1H-purine-2,6 (3H,7H)-dione (Figure 1). It is a natural a lkaloid commonly used in the food industry as the most beneficial psychostimulant for motor stimulati on, mood improvement, information processing and cognitive / motor output in beverages or foods. C AF can have both positive and negative effects on the health [45]. It is on the one hand, powerful stim ulant on the central nervous system and also induces the cardiac muscle. On the other hand, its high le vels have visible gastrointestinal tract irritation and may induce anxiety, insomnia, irritability and head aches resulting from the "caffeinism" syndrome [45]. Several analytical methods such as HPLC [46-49] and MS-detection [50], capillary electrophoresis (CE) with UV-detection [51], gas chromatograph w ith flame ionization detection (GC-FID) [52], TLC [53-55], voltammetry [56], synchronous fluorescen ce [57] and spectrophotometric method [58, 59] had been developed for the determination of CAF in s everal matrices.

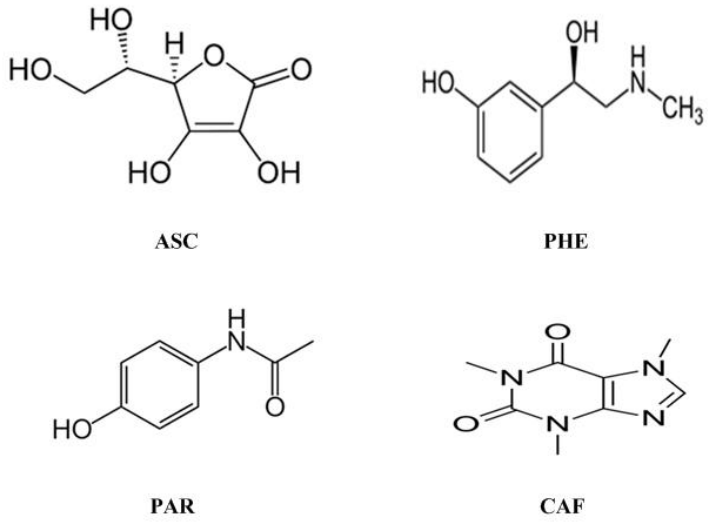

Figure1. *Chemical structures of ascorbic acid (ASC), phenylephrine (PHE), paracetamol (PAR), caffeine (CAF)*

To the best of our knowledge, there is a reported method [13] for the simultaneous chromatographic se paration of ASC, PHE, PAR, and CAF but it some limitations were reported in respect of high linearit y range and LODs. As such, the present work introduces a simple, rapid, reproducible and very sensiti ve chromatographic method with lower LOD values that has been established and validated for the det ermination of ASC, PHE, PAR, and CAF in their pure forms and in their tablet dosage form according to ICH guidelines [60].

2. Materials and Methods

2.1. Instrumentation

High performance liquid chromatography (HPLC) apparatus is equipped with Surveyor quaternary pu mp with Intel vacuum degasser (Agelint 1100), a Surveyor autosampler plus (Thermo Scientific Co., U SA), Kinetex 2.6 µ C_{18} 100A (4.6 mm × 100 mm) column (Thermo Scientific Co. USA), Autosampler vials 1.8 mL screw cap (Thermo Scientific, USA), and Surveyor photodiode array detector (PDA) (Th ermo Scientific Co. USA). A computer with a software chromo quest 5 (Surveyor Thermo Scientific C o. USA), has been used for data collection and analysis,.Consort P400® digital pH-meter was used for pH adjustment.

2.2. Chemicals and Reagents

All solvents and reagents were of HPLC analytical grade. Acetonitrile and methanol HPLC grade were supplied by Fischer scientific (Loughborough, UK), while ortho-phosphoric acid was purchased from Merck (Darmstadt, Germany) and water used in all the experiments was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA). Standard powders of ASC,PHE, PAR and CAF were kindl y supplied by EIPICO (Egypt) .

2.3. Chromatographic Conditions

HPLC was connected with Kinetex 2.6 µ C_{18} 100A (4.6 mm × 100 mm) column as a stationery phase. A mixture of 0.05 M potassium dihydrogen phosphate buffer (pH 3.50 by ortho-phosphoric acid), acet onitrile, methanol a ratio of 70:20:10 (v/v/v) was freshly prepared and used as an isocratic mobile phas e. The mobile phase was pumped at a flow rate of 1 mL/min. The injection volume was 10 µL and the column was maintained at ambient temperature while the eluent was monitored at 220 nm. All chroma tographic conditions are illustrated in Table 1.

Table1. *Chromatographic Conditions for the proposed method*

Parameters	Conditions
Column	Kinetex 2.6 µ C_{18} 100A (4.6 mm × 100 mm)
Mobile phase	0.05 M potassium dihydrogen phosphate buffer (pH 3.50 using ortho-phosphoric acid): acetonitrile: methanol (70:20:10, v/v/v)
UV detection, nm	220
Flow rate, mL/min	1
Injected volume, µL	10
Temperature	Ambient

2.4. Preparation of Standard Stock Solution and Construction of Calibration Curves

Standard stock solution of ASC, PHE, PAR and CAF (100 µg/mL) was prepared by dissolving 10 mg of each pure drug in 100 mL water. Then, Standard solution was diluted by methanol to get final conc entrations of 1, 5, 10, 15, 20 and 50 µg/mL for all drugs for construction of calibration plots. The mixt ure was injected in triplicate and chromatographed under the previously mentioned conditions. A linea r relationship was obtained when average drug standard peak areas were plotted against the correspond ing concentrations for each drug and regression equations were computed.

2.5. Pharmaceutical Preparations

10 tablets (6 mg ASC, 0.5mg PHE, 40 mg PAR and 3.5 CAF) were weighed and finely powdered. An accurately weighed portion from the powdered tablets equivalent to the average concentration of one t ablet was transferred into a 100 mL volumetric flask. 80 mL of water were added and sonicated for 20 minutes then the volume was completed with diluent to 100 mL and filtered. Further dilution was perf ormed to obtain the required concentration range of the drug mixture.

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions

Several trials were done to obtain the optimized chromatographic condition for determination of ASC, PHE, PAR, and CAF. First, chromatographic detection was performed at 220, 215, and 210 nm using a PDA detector and the optimal wavelength was set at 220 nm. Second trials were carried out by changi ng mobile phase composition to reach the optimum separation with good resolution where the mobile phase 0.05 M potassium dihydrogen phosphate buffer (pH 3.50 by ortho-phosphoric acid), acetonitrile , methanol at a ratio of 70:20:10, v/v/v was chosen as the optimum one based on faster separation and good peak resolution. Final trials were carried out to show the effect of different flow rates and optima l separation was achieved at a flow rate of 1 mL/min. Under these conditions, ASC, PHE, PAR, and C AF in pure form were separated and eluted at 1.83, 2.94, 3.74 and 5.13 minutes, respectively as illustra ted in Figure 2(A) and in dosage form as illustrated in Figure 2(B). However, the optimum mobile pha se showed symmetrical peaks ($0.86 < T < 1.22$), capacity factor ($1 < k < 10$), resolution > 2 and theore tical plates > 2000 which are in agreement with the CDER values recommendation [61]. Table 2 show s all system suitability parameters of the proposed HPLC method for simultaneous determination of th e four drugs in pure form.

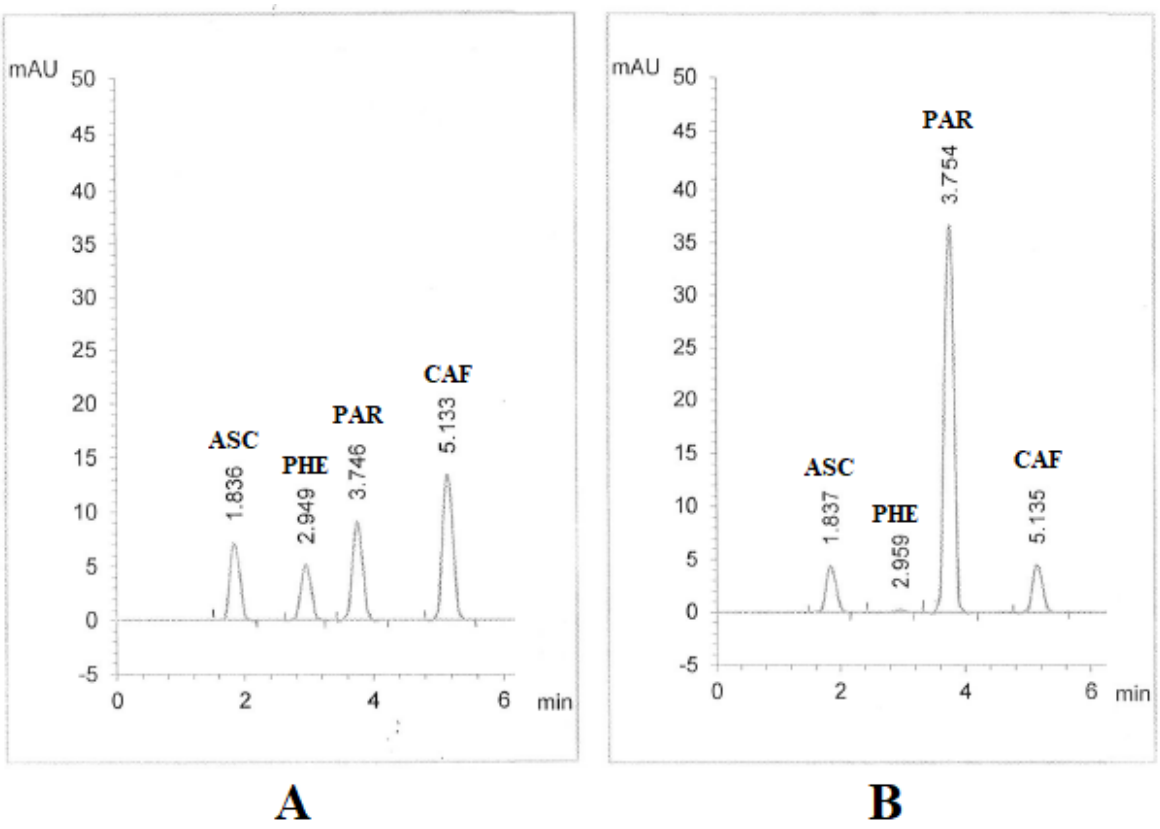

Figure2. *Typical HPLC chromatograms obtained from ASC, PHE, PAR and CAF using Kinetex 2.6 μ C_{18} 100A (4.6 mm × 100 mm) column in (A) pure form and (B) pharmaceutical formulation using mobile phase of 0.05 M potassium dihydrogen phosphate buffer (pH 3.50 by ortho-phosphoric acid): acetonitrile: methanol (70:20:10, v/v/v). Other chromatographic conditions are stated in Table 1*

Table2. *System suitability parameters for ascorbic acid (ASC), phenylephrine (PHE), paracetamol (PAR) and caffeine (CAF) in their pure form*

Parameters	ASC	PHE	PAR	CAF	Reference values [61]
Retention time, tr	1.86	2.92	3.37	5.16	
Capacity factor, k'	2.21	2.41	2.11	2.31	Accepted k' value (1-10)
Peak asymmetry (Tailing factor, T)	1.08	1.22	0.91	0.86	Accepted T value $\leq$ 2
Therotical plates, N	5886	6769	6233	7561	Accepted N value > 2000
Resolution, Rs	8.32	7.68	6.63	9.52	Accepted value > 2
Selectivity (Separation factor, α)	8.25	8.02	8.22	8.16	

3.2. Method Validation

The proposed method was validated according to ICH guidelines [61] in terms of specificity, linearity, precision, accuracy, robustness, limit of detection and limit of quantification.

3.2.1. Specificity

specificity, is the ability of an analytical method to distinguish the analyte from other chemicals in the sample. The specificity of the method was assessed by deliberately adding impurities into a sample co ntaining the analyte and testing how well the method can identify the analyte. It was found that there w as no interference due to excipients found in tablet formulation as seen in Figure 2(B).

3.2.2. Linearity

Six different concentrations of the drug mixture were specified for linearity studies in the range of 1-5 0 μg/mL for all drugs as seen in Table 3. A linear relationship was established by plotting concentratio ns against corresponding peak areas. The correlation coefficient was around 0.999 indicating good line arity as shown in Figure 3. Also, the regression equations were found to be y=5.086x + 1.093, y= 4.91 1x + 1.301, y=7.124x + 1.074, and y=8.15x -1.015, for ASC, PHE, PAR, and CAF respectively.

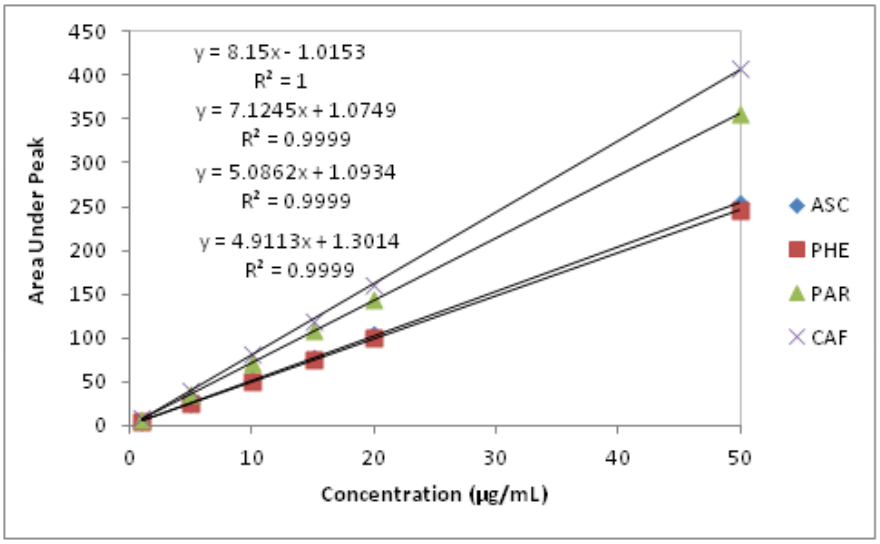

Figure3. *Calibration curves of ASC, PHE, PAR, and CAF using the proposed HPLC method*

Table3. *Results of analysis for the four drugs in pure form using the proposed method*

ASC			PHE			PAR			CAF		
Taken µg/mL	**Found µg/mL**	**Recovery %**	**Taken µg/mL**	**Found µg/mL**	**Recovery %**	**Taken µg/mL**	**Found µg/mL**	**Recovery %**	**Taken µg/mL**	**Found µg/mL**	**Recovery %**
1	0.97	97.34	**1**	1.01	101.12	**1**	0.98	97.93	**1**	0.98	98.01
5	4.98	99.61	**5**	4.89	97.86	**5**	4.98	99.61	**5**	4.91	98.13
10	10.05	100.52	**10**	9.87	98.72	**10**	9.92	99.16	**10**	9.80	98.02
15	15.06	100.37	**15**	15.13	100.87	**15**	15.11	100.77	**15**	14.71	98.07
20	19.94	99.69	**20**	20.18	100.88	**20**	20.19	100.98	**20**	19.62	98.12
50	49.02	98.04	**50**	49.93	99.85	**50**	49.91	99.81	**50**	49.83	99.67
Mean		**99.26**			**99.88**			**99.71**			**98.33**
±SD		1.28			1.33			1.11			0.65
±RSD		1.29			1.34			1.12			0.66
±SE		0.57			0.60			0.50			0.29
Variance		1.66			1.79			1.25			0.42
LOD (µg/mL)		0.76			0.82			0.47			0.24
LOQ (µg/mL)		2.53			2.73			1.57			0.80

3.2.3. Limits of Detection and Quantification

Limit of detection (LOD) of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Limit of quantification (LOQ) is the l owest amount of analyte in a sample which can be quantitatively determined with suitable precision an d accuracy. LOD = 3.3 S/K and LOQ = 10 S/K, were used for the values calculation where S is the sta ndard deviation of three replicate determination values under the same conditions and K is the slope of calibration graph. LODs were reported to be 0.76, 0.82, 0.47 and 0.24 µg/mL, while LOQs were calcu lated to be 2.53, 2.73, 1.57 and 0.80 µg/mL for ASC, PHE, PAR, and CAF respectively (Table 3). The se results show that the proposed method is highly sensitive in comparison with reported methods [13] and applicable not only for pharmaceutical analysis but also for pharmacokinetic studies.

3.2.4. Precision

The precision of the method was calculated in terms of repeatability and intermediate precision (intra-day and inter-day precision). Standard deviation (SD) of five replicate determinations using the same s olution containing pure drug during the same day and five consecutive days were calculated as shown in Table 4. The SD values (1.30 to 1.78) for intra-day and those (0.10 to 3.35) of inter-day prescision were in the acceptable range and showed that the proposed method has an adequate precision in respec t of the simultaneous determination of the 4 cited drugs in their pharmaceutical formulation.

Table4. *Results of Intra-and inter-day precision of the four drugs*

Drugs	Conc. µg/mL	Intra-day		Inter-day	
		Mean ± SD	RSD%	Mean ± SD	RSD%
ASC	5	96.54 ± 1.57	1.63	96.10 ± 1.26	1.13
	10	97.43 ± 1.58	1.62	95.90 ± 2.10	2.12
	15	97.29 ± 1.57	1.62	96.00 ± 1.88	1.96
PHE	5	96.19 ± 1.78	1.86	96.30 ± 0.10	0.94
	10	97.09 ± 1.73	1.79	99.69 ± 3.35	3.37
	15	99.23 ± 1.75	1.26	101.50 ± 2.83	2.79
PAR	5	98.90 ± 1.33	1.34	96.90 ± 2.10	2.18
	10	98.46 ± 1.30	1.32	97.10 ± 0.61	0.63
	15	100.06 ± 1.32	1.32	99.34 ± 0.94	0.95
CAF	5	99.58 ± 1.33	1.34	98.37 ± 0.50	0.54
	10	97.15 ± 1.67	1.67	100.07 ± 0.91	0.91
	15	100.63 ± 1.32	1.31	99.13 ± 0.20	0.21

3.2.5. Accuracy and recovery

Accuracy was assessed using 9 determinations over 3 concentration levels of 5, 10, and 15 µg/mL cov ering the specified ranges. The results showed excellent recoveries with lower SD values as seen in table 5.

Table5. *Results of accuracy study (recovery)*

Drugs		ASC		PHE		PAR		CAF	
		Mean ± SD	RSD%	Mean ± SD	RSD%	Mean ± SD	RSD%	Mean ± SD	RSD%
Conc. µg/mL	5	96.69 ± 2.57	2.66	97.50 ± 0.86	0.88	99.47 ± 0.63	0.64	98.20 ± 2.05	2.09
	10	99.79 ± 1.25	1.25	97.93 ± 1.69	1.73	98.83 ± 0.94	0.95	99.80 ± 2.07	2.08
	15	96.79 ± 3.13	3.22	101.90 ± 1.61	1.61	100.79 ± 0.39	0.39	99.30 ± 2.04	2.05

3.2.6. Robustness

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variat ions in method parameters. In the proposed method, a small variation in the flow rate and mobile phas e composition showed a negliegble effect on the on the results as revealed by small SD values (SD ≤ 3 .97) for all applied changes (Table 6).

Table6. *Results of robustness*

Drugs	ASC	PHE	PAR	CAF
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
pH 3	93.12 ± 0.49	96.17 ± 2.63	99.60 ± 0.81	99.00 ± 0.78
pH 4	93.19 ± 0.58	97.49 ± 3.92	100.11 ± 2.29	99.20 ± 0.33
Mobile phase (68:22:10)	94.90 ± 0.54	99.75 ± 2.62	96.60 ± 0.81	100.58 ± 0.78
Mobile phase (72:18:10)	95.10 ± 0.59	101.2 ± 3.97	97.14 ± 2.25	100.70 ± 0.33

3.2.7. Application on Pharmaceutical Preparation

The proposed method was successfully applied on pharmaceutical preparation containing ASC, PHE, PAR, and CAF. Results obtained were established in Table 7, showing a high degree of accuracy and p recision where excipients and impurities did not show interference on the selected values. Also, results

obtained were compared to those obtained by reference methods [11,26,37,47] where Student's t-test a nd F-test were performed for comparison. Results shown in Table 8 indicated that calculated t and F v alues were less than tabulated ones for the 4 drugs which in turn indicate that there is no significant dif ference between proposed method and reference ones relative to precision and accuracy.

Table7. *Results of analysis of ASC, PHE, PAR and CAF in pharmaceutical formulations*

ASC			PHE			PAR			CAF		
Taken µg/mL	**Found µg/mL**	**Recovery %**	**Taken µg/mL**	**Found µg/mL**	**Recovery %**	**Taken µg/mL**	**Found µg/mL**	**Recovery %**	**Taken µg/mL**	**Found µg/mL**	**Recovery %**
3	2.97	99.02	**0.25**	0.24	99.28	**20**	19.29	96.49	**1.75**	1.73	99.27
6	5.99	99.99	**0.50**	0.48	96.70	**40**	39.40	98.51	**3.5**	3.47	99.32
9	9.02	100.29	**0.75**	0.74	98.90	**60**	59.09	98.49	**5.25**	5.19	99.01
12	12.09	100.81	**1**	0.98	98.09	**80**	78.31	97.88	**7**	7.02	100.3
18	17.96	99.81	**1.5**	1.47	98.52	**120**	119.6	99.67	**10.5**	10.46	99.70
Mean	**99.99**			**98.24**			**98.21**			**99.53**	
±SD	0.66			1.14			1.15			0.52	
±RSD	0.65			1.16			1.18			0.52	
±SE	0.29			0.51			0.51			0.23	
Variance	0.43			1.31			1.34			0.27	

Table8. *Statistical analysis of results obtained by the proposed HPLC method applied on pharmaceutical formu lations compared with reference methods*

DRUG	Recovery ± SD				Reference method number	Student t- values	F- Values
	Proposed Method	N	Reference Method	N			
ASC	100.00 ± 0.37	4	100.30 ± 0.17	4	[11]	**0.65** (1.94)[a]	**4.71** (6.39)[b]
PHE	100.60 ± 0.29	3	100.40 ± 0.10	3	[26]	**0.64** (2.13)[a]	**7.93** (9.28)[b]
PAR	100.50 ± 0.36	3	100.00 ± 0.13	3	[37]	**1.24** (2.13)[a]	**7.77** (9.28)[b]
CAF	99.43 ± 0.13	3	99.73 ± 0.05	3	[47]	**2.07** (2.13)[a]	**6.48** (9.28)[b]

4. CONCLUSION

A simple, precise, accurate, valid, robust, highly sensitive and reliable HPLC method was established f or determination of ascorbic acid. phenylephrine, paracetamol and caffeine in bulk and pharmaceutica l preparation. In the proposed method, the chromatographic resolution was achieved within 6 minutes f or the four drugs. Linearity was observed over a concentration range of 1-50 µg/mL for all drugs. The method has been successfully applied for the analysis of tablet formulation in respect of quality control in addition to performing statistical comparison with reference methods showing no significant differences.

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Citation: *Mahmoud M. Sebaiy, et.al, "HPLC Method for Simultaneous Determination of Ascorbic acid, Phenylephrine, Paracetamol, Caffeine in Their Pure and Dosage Forms", International Journal of Advanced Research in Chemical Science, 7(6), pp. 7-16. DOI: https:// doi.org/10.20431/2349-0403.0706002*