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A validated RP-HPLC method for the determination of piperidone analogue of curcumin

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Abstract: Curcumin (Diferuloylmethane) is a natural product extracted from the root of *Curcuma longa*. 5-Bis (4-hydroxy-3-methoxybenzylidene)-N-methyl-4-piperidone, the piperidone analogue of curcumin (PAC), was one of the analogues that, demonstrated potential anticancer effects against breast and colon cancers compared with native curcumin. A simple, accurate, and rapid isocratic reverse phase high performance liquid chromatography (HPLC) analytical method utilizing UV detection was developed and validated for the determination of PAC utilizing C_{18} column with run time was 7 min. Chromatogram showed a peak of PAC at retention time of 5.8±0.92 min. The method was validated for linearity, accuracy, precision, limit of detection, limit of quantitation and robustness. Linear relationship ($r > 0.99$) was observed between AUP of PAC and the corresponding concentrations over 100-10000µg/mL. The LOQ of this assay was 3.9ng/mL with a corresponding relative standard deviation of 4.8 and 4.0%. The LOD was 13.1ng/mL at a signal-to-noise ratio of >3.

Keywords: Piperidone analogue of curcumin, reverse phase high performance liquid chromatography.

INTRODUCTION

The dried powdered rhizome of *Curcuma longa* L. is commonly known as turmeric and used worldwide as a food-coloring agent (Martins *et al*., 2009). Turmeric extract contains curcuminoids, which are phenolic compounds composed mainly of three bioactive substances, curcumin, demethoxy curcumin, and bis-demethoxy curcumin (PAC) (Martins *et al*., 2009).

Curcuminoids are well-known in traditional medicine because of their ant inflammatory (Jurenka, 2009), antibacterial (Martins *et al*., 2009), antidepressant (Kulkarni *et al*., 2009), antidiabetic (Wickenberg *et al*., 2010), antitumor (Wilken *et al*., 2011), immunomodulatory (Rogers *et al*., 2010) and gastro protective (Kim *et al*., 2005) properties. In addition, curcuminoids haves been successfully used in the treatment of Alzheimer's disease (Ahmed *et al*., 2010) and cardiac disorders (Morimoto *et al*., 2010). Turmeric is widely accepted as a spices with the highest antioxidant activity (Wojdylo *et al*., 2007). Moreover anticancer effects of curcumin have been elucidated in many *in vitro* and *in vivo* studies against various cancer types (Dhillon *et al*., 2008, Sharma *et al*., 2001, Anand *et al*., 2008).

Curcumin is characterized by poor aqueous solubility, low gastrointestinal absorption, high rate metabolism, low of bioavailability and low stability. In neutral pH conditions, contributing to their limited clinical uses (Anand *et al*., 2007a, Nimiya *et al*., 2016). In addition, these molecules have poor bioactivity and are unstable in neutral and alkaline aqueous solutions (Yallapu *et al*., 2012, Tønnesen and Karlsen, 1985).

Khairia M. Youssefa *et al*. (Youssef *et al*., 2004) synthesized various curcumin analogues in 2004. 5-Bis (4-hydroxy-3-methoxybenzylidene)-N-methyl-4 piperidone, piperidone analogue of curcumin (PAC) (fig. 1), was one of the curcumin analogues that, showed promising anticancer effects against breast and colon cancers (Al-Hujaily *et al*., 2011, Al-Qasem *et al*., 2016). PAC is 27-fold more soluble than curcumin in water(Al-Hujaily *et al*., 2011).

Various analytical techniques for the quantification of total and isolated curcumin in different matrices have been reported, particularly spectrophotometric methods for the determination of total curcuminoids (Kadam *et al*., 2013, Silva-Buzanello *et al*., 2015, Ahmed *et al*., 2012). However, this approach cannot be used to quantify individual curcuminoids. Methods such as high performance thin layer chromatography (HPTLC) have been used for analysis of curcumin (Rasmussen *et al*., 2000, Ansari *et al*., 2005). High-pressure liquid chromatography detection (HPLC) is the most common used method for the determination of curcuminoids and curcumin in turmeric samples, biological samples, or dosage forms (Jadhav *et al*., 2007, Li *et al*., 2009, Jangle and Thorat, 2013, do Nascimento *et al*., 2012, Buadonpri *et al*., 2009, Jayaprakasha *et al*., 2002, Syed *et al*., 2015, Koop *et al*., 2013). Table 1, summarizes the studies in which curcumin was determined using HPLC assay, published in the last two decades.

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Although HPLC techniques are characterized by a short run time, to the best of our knowledge, no study has reported a validated assay for PAC with simple and efficient extraction techniques. The retention time for curcumin in literature review varied from 26 to 1.2 min. Our study aimed to develop a rapid, robust, selective, sensitive, and precise HPLC method for the determination of PAC. The assay method was validated for linearity, accuracy, precision, specificity, limit of detection (LOD), and limit of quantification (LOQ) and used for in determination of drug content of the PAC-liposome formulation (under publication).

MATERIALS AND METHODS

Materials

PAC was prepared by the Medicinal Chemistry Department, College of Pharmacy, King Saud University, KSA (Youssef *et al*., 2004). All other reagents and chemicals were of HPLC analytical grade, and were used as received. Water was deionized and purified using a Milli-Q Reagent Grade water system (Millipore Corporation, Bedford, MX 01730, USA).

Liquid chromatography conditions

The HPLC system consisted of Waters 1525 binary pump Separation module (Waters, USA) fitted with C_{18} column (300 mm × 4.6 mm). The autosampler injection system (Waters 2707) used was a 10µL sample loop. A Millipore Swinnex type filter (pore size =0.45µm) was obtained from Millipore (Bangalore, India). A Waters HPLC system equipped with a Waters 484 variable UV absorbance detector and a Waters 2707 plus auto sampler was used. Waters 515 solvent delivery system was used to operate the gradient flow through a C_{18} column (4.6mm × 150 mm, 3µm spherical particles). Acetonitrile: 5% acetic acid (50:50, v/v) was used as the mobile phase at a flow rate of 1 mL/min and the run time was 7.0 min. A Waters 2489 UV/Visible detector used at a wavelength of 392 nm was used for detection. Degassing was achieved via filtration through a 0.45µm Millipore membrane filter and sonication for 10 min. The injection volume was 10µl and detection was at 392 nm. The HPLC system was operated at 25°C. Data were collected with a Breeze Chromatography Manager Data Collection System. A daily standard calibration curve (6 standards ranging from 100 ng/mL to 10000ng/mL) was prepared to determine the unknown PAC concentration.

Preparation of stock solutions

The stock standard solution of PAC was prepared in acetonitrile at a concentration of 10µg/mL and stored in 4.0mL glass vials in a refrigerator at 4°C. Different working standard solutions of PAC (100-10000ng/ml) were prepared by diluting of the above mentioned stock solution in pure acetonitrile and were stored at 4°C.

Validation of PAC HPLC assay

The RP-HPLC method for PAC assay was validated in term of accuracy, reproducibility, linearity, specificity, LOD, LOQ and robustness according to ICH Harmonized Tripartite Guidelines (Guideline, 2005). Three standard calibration curves were prepared at different times (at least three months) to evaluate the linearity, precision, accuracy and stability.

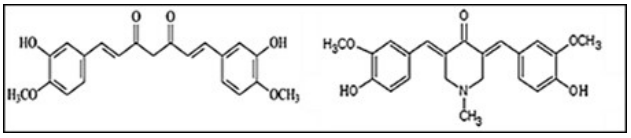

Fig. 1: Chemical structure of curcumin (left) and the novel analouge (PAC) (right) (Youssef *et al*., 2004)

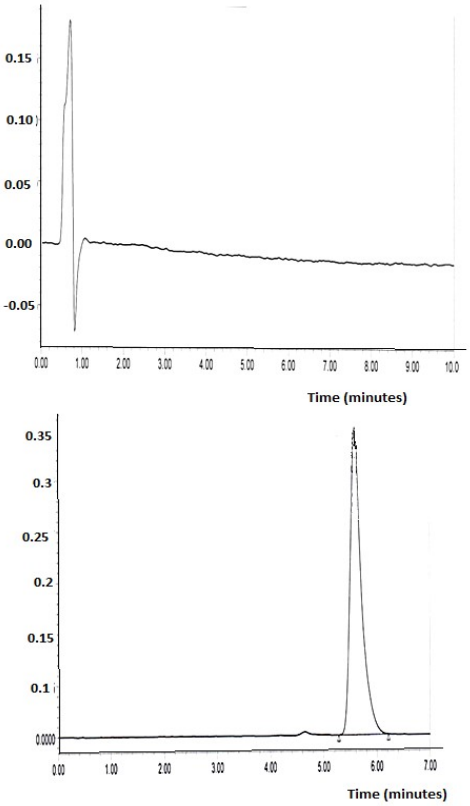

Fig. 2: HPLC chromatograms of mobile phase (chromatogram A) and HPLC chromatograms of mobile phase containing 1000ng/ml PAC and 100µg/ml (chromatogram B).

Specificity

The specificity of the HPLC method was evaluated to ensure that there was no interference from the excipients present in the formulations. The specificity was studied by injecting the excipients.

Linearity and range

Linearity is the ability to obtain test results that are directly proportional to the concentration of the analyte. Linearity was determined by three injections of seven different PAC concentrations (100, 250, 500, 1000, 2500, 5000 and 10000ng/ml). The average peak areas were plotted against concentrations. Then linearity was evaluated using the calibration curve to calculate coefficient of correlation, slope and intercept. In general, a value of correlation coefficient (r^2) > 0.998 is considered as the evidence of an acceptable fit for the data to the regression line.

Table 1: High performance chromatographic techniques used during last two decays (2000-2017) for quantitative determination of curcumin

	Sample origin	Column temperature °C	Stationary phase	Mobile phase	Detector	RT* (min)	Reference
1	-Curcumin and PAC	25	C18	Acetonitrile: Acetic acid 5% (50:50, v/v)	UV at 392 nm	5.8	Current study
2	-Plasma	33	Luna® C18	Acetonitrile: water (50:50, v/v)	UV at 425 nm	13.6	(Fonseca-Santos *et al.*, 2016)
3	-Herbal extract -Pharmaceutical dosage form	-	Lichrosorb® CN column	acetonitrile: citric acid 1.0% 50:50,v/v)	PDA at 425 nm	ND	(Paulucci *et al.*, 2013, Zamarioli *et al.*, 2015, Martins *et al.*, 2013)
4	-Herbal extract	40	Wondasil™ C18	Acetonitrile: 10 mM phosphate buffer pH 5 (50:50 ,v/v)	electrochemical detector	14	(Long *et al.*, 2014)
5	-Herbal extract -Pharmaceutical dosage form	33	ACE C18	Acetic acid 2%: acetonitrile (40:60, v/v)	PDA at 425 nm	7.4	(Monton *et al.*, 2016)
6	-Plasma	40	Capcell Pak C8	acetonitrile : water (40:60, v/v)	VWD at 230 nm	10.56	(Kim *et al.*, 2017)
7	-Pharmaceutical dosage form	30	Waters X-bridge® C18	Gradient elution of mobile of 0.05 mM phosphate buffer in Methanol to acetonitrile 1:1 ratio	PDA at 288 nm	36.71	(Korany *et al.*, 2013)
8	-Plasma	40	Reprosil-Pur C18	Acetonitrile: water: methanol (34 :52:14, v/v/v)	fluorescence detector at 426 nm	ND	(Kocher *et al.*, 2015)
9	-Plasma	-	Symmetry RP C18	citric acid 1%: tetrahydro-furan (50:50, v/v)	UV/VIS at 430 nm	5.17	(Pak *et al.*, 2003)
10	-Herbal extract	-	C18	acetonitrile: methanol: water (40:20:40, v/v/v)	UV/VIS at 370 nm	7	(Syed *et al.*, 2015)
11	-Pharmaceutical dosage form	35	Luna C18	0.1% ortho phosphoric acid : acetonitrile (45:55, v/v)	PDA at 262 nm	8.6	(Moorthi *et al.*, 2013)
12	-Synthetic curcumin	-	C 18	Gradient elution of 0.4% acetic acid in acetonitrile	PDA at 422 nm	4.5	(Scotter, 2009)
13	-Pharmaceutical dosage form	35	Waters® X Terra MS C18	The gradient program 2% acetic acid in acetonitrile	PDA at 420 nm	8.9	(Lee and Choung, 2011, Perko *et al.*, 2015)
14	-Herbal extract	55	Phenomenex® fused-core C18	Gradient elusion of 0.1% acetic acid in acetonitrile	PDA at 425 nm	1.2	(Osorio-Tobón *et al.*, 2016)
15	-Plasma	-	Phenomenex ODS C 8	Gradient elution of acetonitrile in 0.1% phosphoric acid, pH 3.5	PDA at 420 nm	44.7	(Xie *et al.*, 2007)
16	-Herbal extract -Pharmaceutical dosage form	30	YMC ODS-A C18	Gradient elusion of acetonitrile in 0.1% formic acid	UV at380 nm	26.5	(Li *et al.*, 2011)
17	-Herbal extract	25	Kromasil C18	0.05% acetate acid: methanol (15:85, v/v)	PDA at 420 nm	15.8	(Cheng *et al.*, 2010)
18	-Herbal extract	35	Merck Lichrosphery C18	Gradient elusion of methanol in 0.1% phosphoric acid	PDA at 425 nm	15.2	(Li *et al.*, 2014)
19	-Pharmaceutical dosage form	-	Zobrax Eclipse Plus C18	Ethanol: water (90:10, v/v)	UV at 423 nm	2.5	(Yu *et al.*, 2017, Yu *et al.*, 2016, Nguyen *et al.*, 2016, Nguyen *et al.*, 2015)
20	-Plasma -Food samples	-	Macherey Nagel Nucleosil-C18	Water: methanol (80:20,v/v)	UV at 420 nm	ND	(Bahrani *et al.*, 2017)
21	-Pharmaceutical dosage form	25	C18	2% glacial acetic acid : acetonitrile (50:50, v/v)	MWD at 425 nm	13.28 ±0.25	(Shinde *et al.*, 2015)

22	-Pharmaceutical dosage form	-	RP-C18	acetonitrile: 0.1% trifluor-acetic acid (50:50, v/v)	UV at 427 nm	ND	(Coradini *et al.*, 2014)
23	-Herbal extract	-	Eurospher I 100-5 C18	acetonitrile: water (90:10.v/v)	UV at 420 nm	ND	(Sahne *et al.*, 2017)
24	-Pharmaceutical dosage	35	Waters spherisorb column C18	acetonitrile: water(60:40, v/v).	UV at 454 nm	11.44	(Biswas *et al.*, 2016, Moorthi and Kathiresan, 2013)
25	-Pharmaceutical dosage form -Plasma	-	Waters uBondapak C18	acetonitrile : citric buffer, pH 3.0 (55:45, v/v)	UV at 428 nm	8.44	(Ma *et al.*, 2007, Kheradpezhouh *et al.*, 2010, Molina-Jijón *et al.*, 2011)
26	-Pharmaceutical dosage form	-	Thermo BDS C18	Gradient elution of 0.2% formic acid in acetonitrile	PDA at 422 nm	ND	(Zhu *et al.*, 2016)
27	-Plasma	-	C18	Gradient ekusion of acetonitrile in 0.1% trifluoroacetic acid.	UV at 570 nm	12.8	(Purkayastha *et al.*, 2009, Anand *et al.*, 2007b)
28	-Herbal extract	-	Inertsil® ODS-3 C8	Acetonitrile :water: acetic acid (45:55:1.), flow rate 1 ml/min.	UV- 410 nm	ND	(Hou *et al.*, 2007)
29	-Herbal extract	-	A Tosoh TSK-C18	acetonitrile: methanol: water: acetic acid (41:23:36:1, v/v/v/v)	UV at 422 nm	10.8	(Jang *et al.*, 2007)
30	-Pharmaceutical dosage form	25	Phenomenex C18	Gradient elution of acetonitrile in 50 mM Phosphate buffer pH 5.5	PDA at 420 nm	11.15	(Xu *et al.*, 2014)
31	-Pharmaceutical dosage form	35	Inertsil1C18	acetonitrile: tetrahydrofuran, (4:1) : citric acid 1% (65:35. v/v)	PDA at 430 nm	4.4	(Jokerst *et al.*, 2011, Yadav and Kumar, 2014)
32	-Pharmaceutical dosage form	-	C18	Acetonitrile: methanol:1.0% acetic acid (30:40:30, v/v/v)	UV at 420 nm	3.3	(Kamalasanan *et al.*, 2014)
33	-Pharmaceutical dosage form	40	C18	acetonitrile: water: acetic acid (45L55:1, v/v/v)	LED at 420 nm	20	(Maniglia *et al.*, 2014)
34	-Plasma	-	C18	methanol :water (80:20, v/v)	UV at 420 nm	2.57	(Subhashini *et al.*, 2013)
35	-Plasma	-	Merck Lichro-C18	50 mM tetrahydrofuran (THF): citrate buffer (pH 6) (66:34, v/v)	UV at 419 nm		(Derochette *et al.*, 2014)
36	-Pharmaceutical dosage form	-	C18	Gradient elution of acetonitrile in 5% acetonitrile, 1%TFA).	UV at 328 nm	5.3	(Wang *et al.*, 2013)
37	-Plasma	-	Eclipse XDB C18	Acetonitrile : 10 mM monosodium phosphate (pH 3.5 (40:60, v/v)	PDA at 425 nm	22	(Tsai *et al.*, 2011)
38	-Plasam	35	C18	Acetonitrile: tetrahydrofuran (4:1) : 1% (w/v) citric acid monohydrate (pH 3.0) (65 : 35%)	PDA at 435 nm	3.5	(Wahlang *et al.*, 2011)
39	-Plasma	40	TSKgel- ODS C8	Acetonitrile: 50 mM phosphoric acid (48:52, v/v)	UV at 425 nm	17	(Asai and Miyazawa, 2000, Suresh *et al.*, 2007)
40	-Pharmaceutical dosage form	-	Spherisorb S3 ODS2-C8	gradient elution of 0.1% TFA in acetonitrile	PDA at 427 nm	14.8	(Awasthi *et al.*, 2000)
41	-Pharmaceutical dosage form	-	C18	acetonitrile :citric buffer 1% (w/v) (3:2, v/v)	UV at 430 nm	8.8	(Mohanty *et al.*, 2010, Khan *et al.*, 2016)
42	-Plasma	-	Diamonsil C18	Acetonitrile:5% acetic acid (75:25, v/v)	UV at 420 nm		(Li *et al.*, 2009)
43	-Herbal extract	30	Gemini C18	gradient elution of 3 mM phosphoric acid in acetonitrile	PDA at 425 nm	13.22	(Jayaprakasha *et al.*, 2013)
44	-Herbal extract -Pharmaceutical dosage form	-	Supelco Exil-Amino C8	Propanol: water (95:05, v/v)	PDA at 425 nm	11.09	(Naidu *et al.*, 2009, Bellary *et al.*, 2011)

Table 2: Inter- and Intra-day statistics

PAC		Measured concentration (ng/mL)		
		Nominal concentration (ng/mL)		
	Day-1	500	1000	5000
		485	1190	5126
		515	981	4874
		495	1240	5240
		490	955	4760
	Day-2	525	1110	5148
		505	984	4874
		522	1080	5100
		487	992	4910
		492	909	4864
	Day-3	519	1113	5123
		523	1235	5210
		506	1185	4850
		488	1100	5020
		491	1021	5150
Inter-day statistics	n	6	6	6
	Mean	501.9286	1094.571	5001.143
	SD	16.24896	112.677	153.7644
	Accuracy (%RSD)	3.237304	10.29416	3.074586
Intra-day (on day 3), n = 6	n	18	18	18
	Mean	502.5	1076.667	5003.667
	SD	15.41104	120.9755	192.1725
	Accuracy (%RSD)	3.066873	4.23611	3.840633

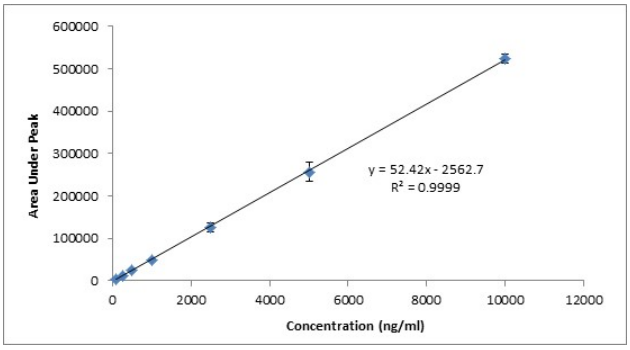

Fig. 3: Mean drug concentration ranging from 1000-10000 ng/ml ± SD. Y denotes dependent variable and X is the in dependent variable while R is the regression coffefficient

Accuracy

The accuracy of an analytical method expresses the nearness between the expected value and the value found. It is obtained by calculating the percent recovery (R%) of the analyte recovered. In this case, to evaluate the accuracy of the developed method, successive analysis (n =3) for three different concentrations (500ng/ml, 250 ng/ml and 100ng/ml) of standard PAC solution were performed using the developed method. The data of the experiment were statistically analyzed using the formula [% Recovery = (Recovered conc. /Injected conc.)×100] to study the recovery and validity of the developed method. The mean recovery should be within 90-110% to be accepted.

Precision

Precision of a measurable technique is the degree of agreement among individual tests, when the technique is applied repetitively to analyze multiple replicates in three different occasions. The intraday precision was assessed by analyzing the calibration curves of six replicates of different concentrations of PAC within the same day. The inter-day precision was determined by analyzing of six replicates of different concentrations of PAC on three different days. The total precision of the method was expressed as the relative standard deviation (%RSD). In the current method development and validation protocol, precision was determined by six replicate analyses at a concentration of 5000ng/mL of standard PAC solution using the developed method and % RSD ≤2% was accepted.

Limit of detection and Limit of quantification

LOD is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. LOQ is the lowest concentration of analyte that can be determined with acceptable precision and accuracy. These two parameters were calculated using the formula LOD =3.3×SD/S and LOQ =10×SD/S, where SD =standard deviation of response (peak area) and S = slope of the calibration curve.

Robustness
The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions.

STATISTICAL ANALYSIS

In vitro results were expressed as mean ± SD of at least three replicates. The HPLC results of PAC were calculated using linear regression without weighting, according to the equation: Y = 0.0403×-0.0119, where Y is the area under the peak (AUP) ratio of the drug and X is the concentration of PAC. The % RSD was calculated for all values. Student's t-test was used to inspect the concentration difference at each day and one-way analysis of variance (ANOVA) was used to assess the reproducibility of the assay using IBMSPSS Statistics 21. The level of confidence was 95%.

RESULTS

Development of HPLC method
In fig. 2, chromatogram A represents the blank mobile phase and chromatogram B represents PAC with an average retention time of 5.8±0.92 min and with no interfering peaks. This is an indication of the specificity of the developed HPLC method. The retention time was comparable with the shorter published data for curcumin (table 1).

Specificity
The specificity of the method was monitored by analyzing the placebo and standard solution. No peak was detected close to the retention time of PAC, which proved the high degree of specificity of the method (fig. 2).

Linearity, limit of quantification, limit of detection
Linear relationship (r>0.99) was observed between AUP of PAC and the corresponding concentrations over 100-10000µg/mL (fig. 3). The mean linear regression equation of the peak area ratios (Y) versus drug concentrations (X) of PAC was typically of the form Y = (b ± S.D.)X ± (a ± S.D.) and it was Y=52.42 X-2562.7 for PAC. The LOQ of this assay was 3.9ng/mL with a corresponding relative standard deviation of 4.8 and 4.0%. The LOD was 13.1ng/mL at a signal-to-noise ratio of >3.

Recovery, accuracy and precision
Within-day precision and accuracy of the method were determined from replicate analysis (n=6) of PAC test standards at concentrations within the linear range of the assay for each drug (table 2). The reproducibility of the assay was evaluated by comparing the linear regressions of three standard plots prepared on three different days over a 3-week period. The mean correlation coefficient was >0.999 with % R.S.D. of the slopes of the three lines beign 8.4%. ANOVA of the data indicated no significant difference (p>0.05) in the slopes, intra- and inter-day, of the calibration curves. The results confirmed the reproducibility of the assay method. The mean percentage recovery of 100-10000ng/mL PAC was 95.2±4.9%.

DISCUSSION

As curcuminoids hydrophobic compounds which is practically insoluble in water (Anand *et al*., 2007a, Nimiya *et al*., 2016), it is freely soluble in acetonitrile, so the C_{18} columns were preferred for HPLC analysis (Jadhav *et al*., 2007). many studies reviewed the use of C_{18} for separation of the drug using acetonitrile as the main solvent. These HPLC methods reported in (table 1), particularly older studies, have several disadvantages, including unsatisfactory separation times, poor resolution, complicated solvent mixtures with gradient elution, and long analysis times. The aim of this study was to develop and validate a new simple and rapid analytical method for PAC.

This article describes fast and specific HPLC method for PAC quantification, the drug eluted within 5.8 minutes. A significant reduction in the analysis time is achieved utilizing this method, which also indicate a significant reduction in the solvent consumption

CONCLUSIONS

A simple, rapid and sensitive analytical method was developed and validated for the analysis for piperidone analogue of curcumin (PAC). The chromatographic runtime was also short. The developed analytical method can be reliably used for further *in vitro* pharmaceutical and pharmacokinetic study of any dosage form containing piperidone analogue of curcumin.

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