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Development and validation of UPLC method for determination of primaquine phosphate and its impurities

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Abstract

With the objective of reducing analysis time and maintaining good efficiency, there has been substantial focus on high-speed chromatographic separations. Recently, commercially available ultra-performance liquid chromatography (UPLC) has proven to be one of the most promising developments in the area of fast chromatographic separations. In this work, a new isocratic reverse phase chromatographic method was developed using UPLC for primaquine phosphate bulk drug. The newly developed method is applicable for assay and related substance determination of the active pharmaceutical ingredient. The chromatographic separation of primaquine and impurities was achieved on a Waters Acquity BEH C18, 50 × 2.1 mm, 1.7 μm column within a short runtime of 5 min. The method was validated according to the regulatory guidelines with respect to specificity, precision, accuracy, linearity and robustness. Forced degradation studies were also performed for primaquine phosphate bulk drug samples to demonstrate the stability indicating power of the UPLC method. Comparison of system performance with conventional HPLC was made with respect to analysis time, efficiency and sensitivity.

Keywords: HPLC; UPLC; Method development; Validation; Stability indicating

1. Introduction

Though high-performance liquid chromatography (HPLC) is a well-established reliable technique used in controlling the quality and consistency of active pharmaceutical ingredients (API's) and dosage forms, it is often a slow technique because of the complexity of some of the samples, it could still be improved.

Ultra-performance liquid chromatography (UPLC) is a new category of separation technique based upon well-established principles of liquid chromatography, which utilizes sub-2 μm particles for stationary phase. These particles operate at elevated mobile phase linear velocities to affect dramatic increase in resolution, sensitivity and speed of analysis [1]. Because of its speed and sensitivity, this technique is gaining considerable attention in recent years for pharmaceutical and biomedical analysis [2–5]. In the present work, this technology has been applied to the method development and validation study of related substance and assay determination of primaquine phosphate bulk drug.

Primaquine phosphate is used for causative treatment of malaria [6]. It is highly effective against the gametocytes of all plasmodia, and thereby prevents spread of the disease to the mosquito from the patient. It is also effective against the dormant tissue forms of *P. vivax* and *P. ovale* malaria, and thereby offers radical cure and prevents relapses [7].

Reports on the bioanalytical methods for primaquine are available in the literature [8–11]. Few methods are available for assay determination of primaquine phosphate [12,13]. European Pharmacopeia [14] describes the HPLC method for the impurity analysis, while few reports are also available on the isomeric impurity of primaquine [15,16]. However, there are no

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reports available on stability indicating analytical method for primaquine phosphate API. It is, therefore, felt necessary to develop a new stability indicating method for the related substance determination and quantitative estimation of primaquine phosphate. We intend to opt for a faster chromatographic technique, UPLC, for the said study.

In this work, we show how the HPLC method for primaquine and its impurities has been transformed to UPLC. An attempt was made on determining whether UPLC can reduce analysis times without compromising the resolution and sensitivity.

2. Experimental

2.1. Materials and reagents

Samples of primaquine phosphate and its two impurities were received from Chemical Research Division, Ipca laboratories Ltd. Mumbai, India. The chemical structure of primaquine phosphate and impurities are shown in Fig. 1. HPLC-grade acetonitrile and trifluroacetic acid (TFA) were purchased from Merck India Ltd. High-purity water was obtained by Millipore Milli Q water purification system.

2.2. High-performance liquid chromatography

The HPLC system used for initial chromatographic development was Waters Alliance separation module with a 2487 UV detector. A Kromasil C18, 250 × 4.6 mm, 5 μm column was used for separation. Chromatographic separation was achieved in both the modes (isocratic and gradient). Mobile phase consisting of a mixture of A: 0.01% aqueous trifluroacetic acid and B: acetonitrile in the ratio 75:25 (v/v) for isocratic mode while a timed gradient programme T (min)/%B: 0/25, 15/25, 25/80, 30/80, 35/25, with the flow rate of 1 ml/min was employed. The injection volume was 10 μl while detector was set at 265 nm. The column was maintained at 35 °C.

2.3. Ultra-performance liquid chromatography

UPLC was performed using a Waters Acquity system equipped with binary solvent delivery pump, an auto sampler and tunable UV detector. The chromatographic separation was performed using a Waters Acquity BEH 50 × 2.1 mm, 1.7 μm, C18 column. The mobile phase containing a mixture of 0.01% aqueous trifluroacetic acid and acetonitrile in the ratio of 75:25 (v/v) at a flow rate of 500 μl/min was used. The detection was obtained at a wavelength of 265 nm. The injection volume was 0.8 μl; mobile phase was used as a diluent while the column was maintained at 35 °C.

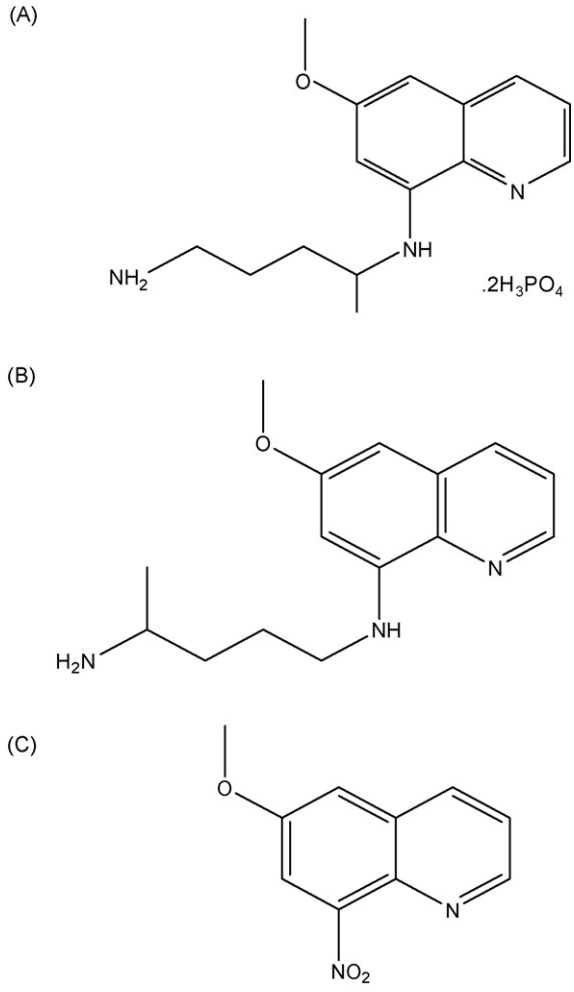

Fig. 1. Chemical structures of primaquine phosphate and impurities. (A) primaquine phosphate (B) impurity I: 8-(4-amino-4-methylbutyl amino)-6 methoxyquinoline (C) impurity II: 8-nitro-6- methoxyquinoline.

Forced degradation studies were carried out with a 2996 photo diode array detector.

2.4. Preparation of solution

A standard and test solutions of primaquine phosphate (200 μg/ml) were prepared using diluent for assay determination. System suitability solution was prepared by dissolving 5 mg of impurity, I and II each, and 20 mg of primaquine phosphate in 100 ml of diluent.

Table 1
A Comparison of system performance of HPLC and UPLC

Component	Elution time (min)		Resolution		Tailing factor		USP plate count	
	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC
Impurity I	8.580	0.87	–	–	1.83	1.20	3242	3915
Primaquine	9.739	0.99	1.24	1.57	2.43	1.71	973	1724
Impurity II	25.17 (46.02)[a]	1.80	6.12	8.47	1.62	1.01	14443 (3642)[a]	6125

[a] In an isocratic HPLC mode.

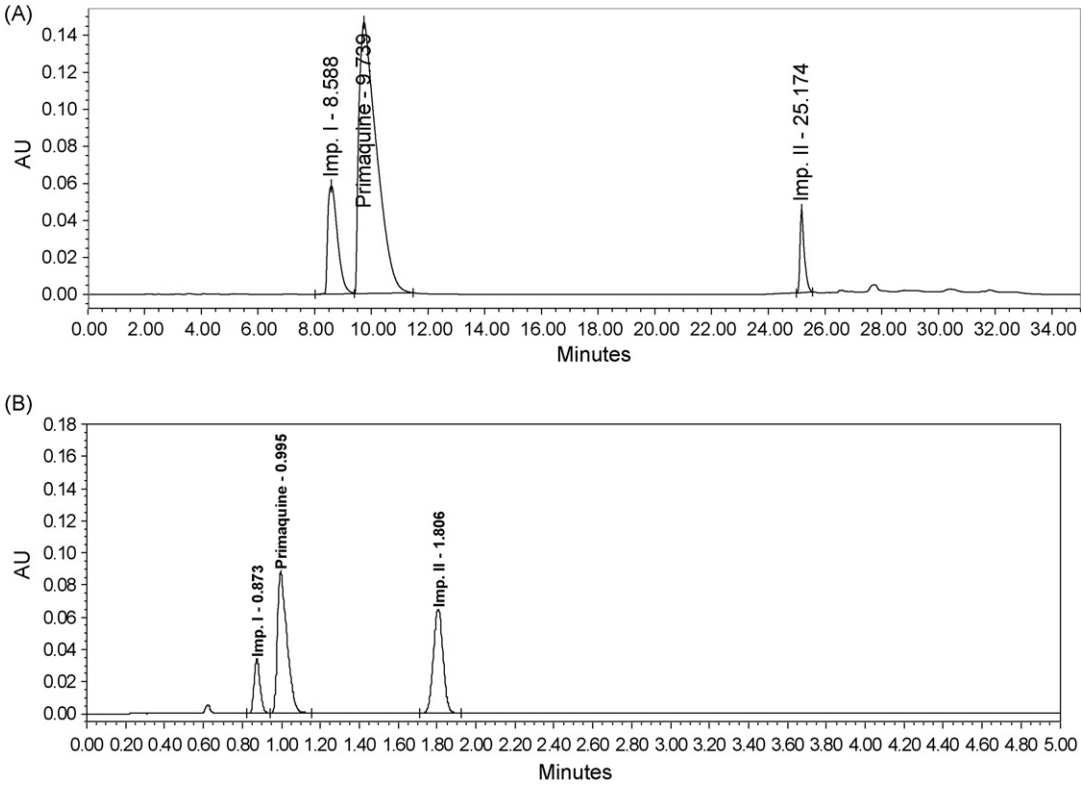

Fig. 2. Comparison of chromatograms of primaquine phosphate and impurities obtained from (A) HPLC and (B) UPLC.

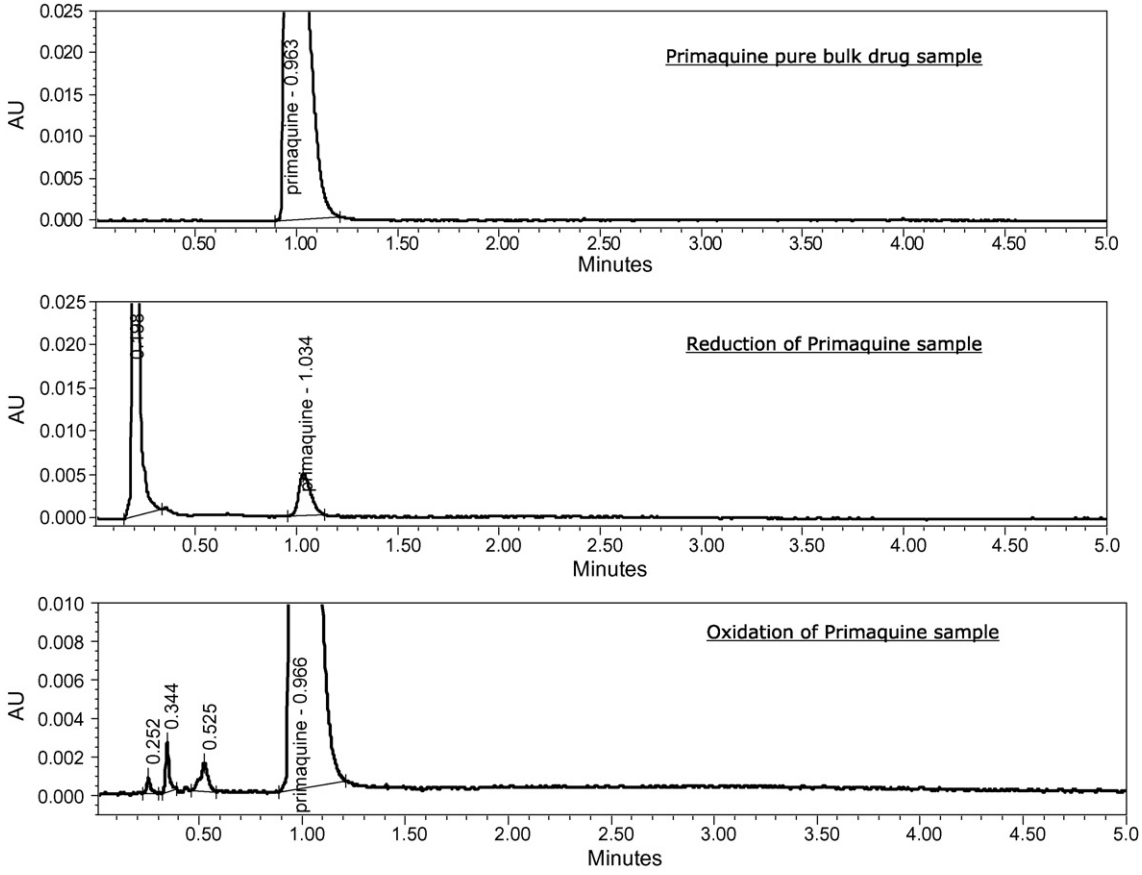

Fig. 3. UPLC chromatograms of primaquine phosphate bulk drug sample and stressed samples.

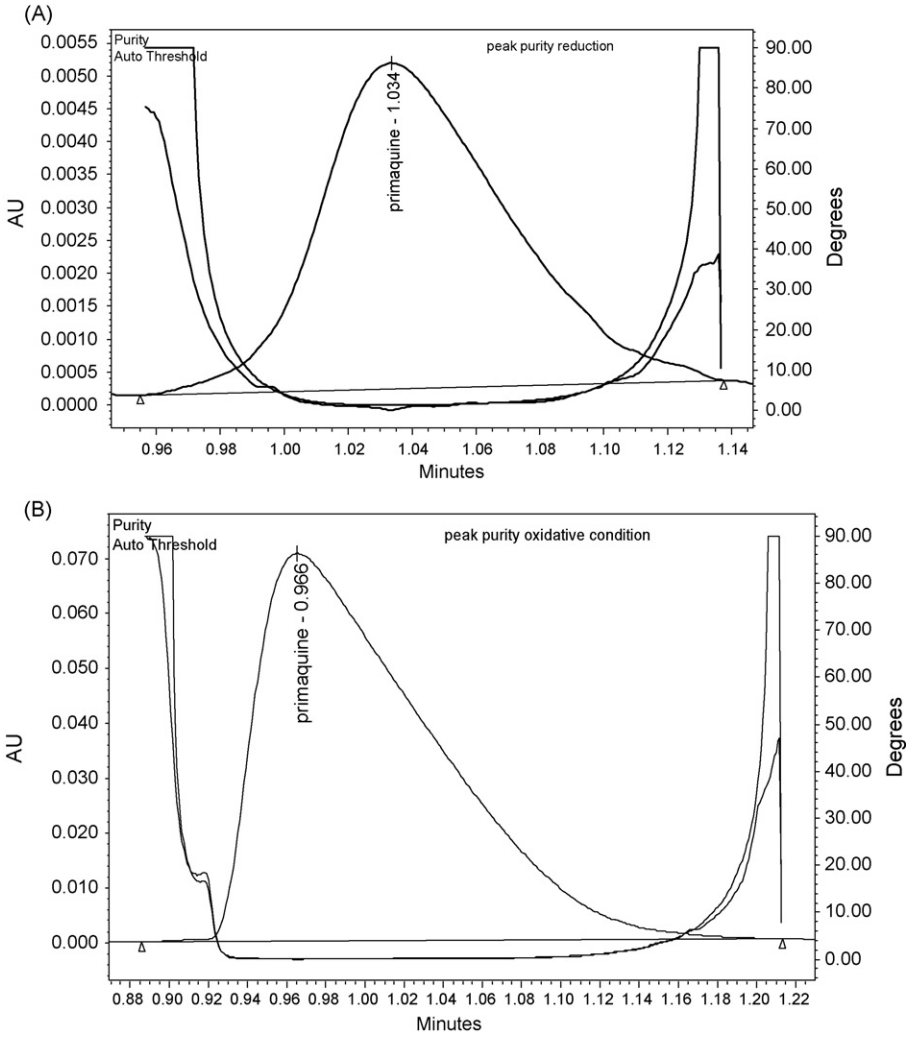

Fig. 4. PDA peak purity (A) reductive degradation (B) oxidative degradation.

The test solution (500 μg/ml) was prepared by dissolving appropriate amount of primaquine phosphate in diluent for related substance analysis. A stock solution of mixture of impurities was prepared by dissolving 25 mg each of impurity I and II in 50 ml of diluent. The standard impurity solution containing 0.5 μg/ml each of impurity I, II and primaquine phosphate was prepared using the stock and test preparation. This standard solution was also used as system suitability solution for related substance.

2.5. Validation procedure

The newly developed UPLC method was validated in terms of precision accuracy and linearity according to International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines [17]. Assay method precision was carried out using six independent test solutions and a standard preparation. The standard impurity solution was used for determination of precision for related substance method. The intermediate precision of the assay and related substance method was also evaluated using different analyst on three different days. The accuracy of the assay method was evaluated in triplicate using three concentration levels 160, 200 and 240 μg/ml. Standard addition and recovery experiments were conducted to determine accuracy of the related substance method. The study was carried out in triplicate using three concentration levels 0.75, 0.50 and 0.25 μg/ml of impurity I and II. Linearity test solutions for related substance method were prepared by diluting the impurity stock solution. The limit of detection (LOD) and limit of quantification (LOQ) for Impurity I and II were estimated by injecting a series of dilute solutions with known concentration. The limit of quantification was estimated from standard deviation values of replicate response of impurities (signal to noise ratio 10:1). Precision study was also carried at the LOQ level. To determine the robustness of the method experimental conditions were purposely altered and the resolution between primaquine and impurities was examined by injecting system suitability solution for related substance. The flow rate was changed to 475 and 525 μl/min. Column temperature was varied by (±)2 °C. The organic strength was varied by (±)1%.

Forced degradation studies of the bulk drug sample were also performed using the following conditions: acid hydrolysis (0.1N hydrochloric acid), base hydrolysis (0.1N sodium hydroxide),

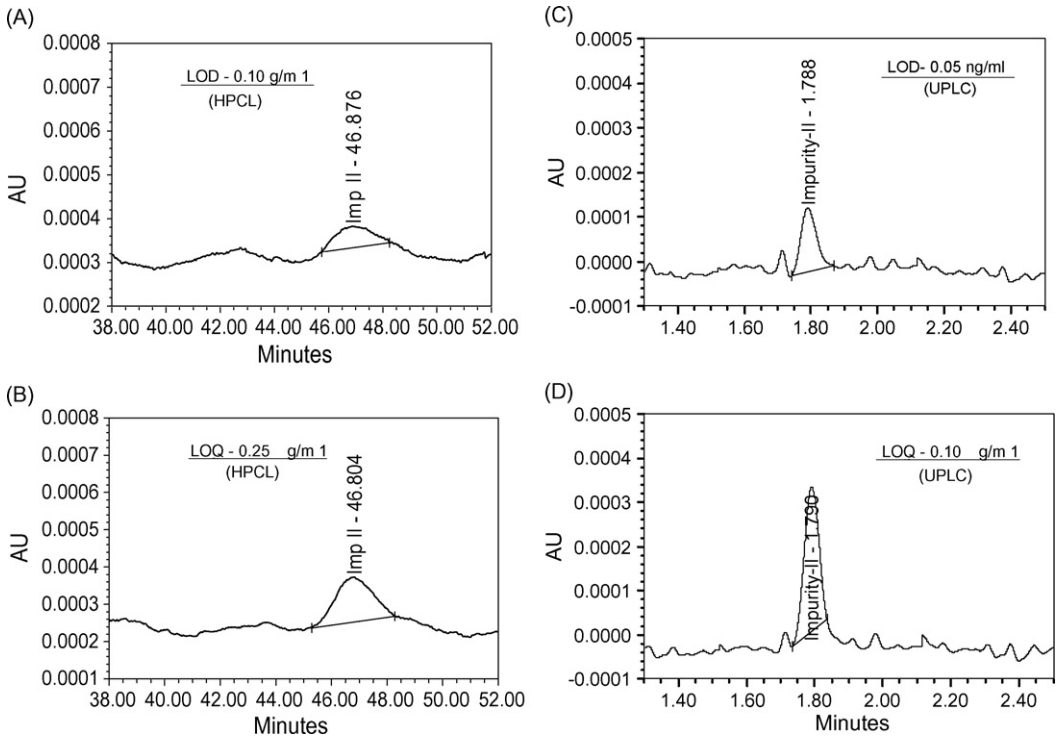

Fig. 5. The LOD and LOQ demonstrated for HPLC (isocratic mode) and UPLC.

heat (105 °C for 48 h), photolytic (UV and sunlight for 48 h), oxidation (30% hydrogen peroxide) and reduction (10% sodium metabisulphite). Peak purity test was carried out for primaquine peak by using photodiode array (PDA) detector in stress samples.

3. Results and discussions

3.1. LC method development and transfer to UPLC

Initially, the isocratic and gradient HPLC conditions were optimized for primaquine phosphate and impurities in API sample. The main target of the chromatographic method was to achieve separation of impurities and main component primaquine. Impurity I is a regioisomer of primaquine and a potential impurity in primaquine phosphate bulk drug sample. The impurity II is an intermediate of primaquine, which may carry forward during the synthesis of API. During LC development study, it was observed that impurity I was closely eluting with primaquine, while impurity II was eluting at higher retention time. The response of impurities and primaquine was found to be adequate at 265 nm.

The chromatographic separation was achieved on a Kromasil C18, 250 × 4.6 mm, 5 μm column maintained at 35 °C. In an isocratic mode using a mobile phase consisting of buffer (0.01% aqueous TFA and acetonitrile in the ratio (75:25, v/v), there was a good separation between impurity I and primaquine, while impurity II was found to be eluting at higher retention time (around 50 min) with broadening of peak. To reduce the run time and achieve better peak shape, it was decided to switchover to gradient HPLC mode. A gradient programme for mobile phase was optimized as mentioned in Section 2.2.

The basic chromatographic conditions like stationary phase, solvents and UV detection, employed in HPLC were taken into account while developing the new UPLC method. The detection wavelength, column temperature, buffer and solvent used in HPLC were kept constant. The stationary phase C18 was chosen in order to have similar chemistry as that used in the HPLC. A BEH C18, 50 × 2.1 mm, 1.7 μm column was employed for the separation. The injection volume was scaled to 0.8 μl from 10 μl as used in HPLC. At the beginning, an isocratic mode was chosen with the same ratio of buffer to acetonitrile as used in isocratic HPLC mode (75:25, v/v). The flow rate was scaled to 200 μl/min. Using these conditions, a satisfactory separation was achieved between primaquine and impurity I, while impurity II was eluting around 4–5 min giving a total run time of 7–8 min. A backpressure of 6000 psi was observed. Taking in to account the capability of high operating pressure of UPLC, flow rate was increased further to 500 μl/min with a backpressure of 13,500 psi. At this flow rate, the runtime was decreased to 4–5 min without affecting the separation of primaquine and impurity I.

An attempt was made by switching over to gradient mode after 1 min, wherein elution time of impurity II was observed to be marginally decreased with interference of gradient peak. Hence, the isocratic mode was preferred for UPLC analysis.

3.2. Comparison study of chromatographic performance

A comparative data on chromatographic performance of HPLC (gradient and isocratic) and UPLC (isocratic) has been obtained by injecting a solution of primaquine and impurities (impurity I and II 50 μg/ml each, and primaquine phosphate

Table 2
System suitability report for UPLC related substance method

Component	Resolution (USP)	USP Tailing Factor	USP Plate count
Impurity I	–	1.13	4900
Primaquine	2.80	1.06	5308
Impurity II	10.99	1.02	7202

200 μg/ml). The performance parameters of both the systems are shown in Table 1. It is observed that the elution time of impurity II in UPLC was reduced by 10-fold to that of gradient mode HPLC and 20-fold to that of isocratic mode HPLC. The resolution and theoretical plates obtained for primaquine and impurity I in UPLC showed comparatively better separation efficiency than HPLC. Theoretical plates obtained for impurity II in gradient mode HPLC was obviously higher, but in case of isocratic HPLC mode (eluting at 46 min), it is lesser than UPLC. The typical chromatograms obtained from final HPLC and UPLC conditions are depicted in Fig. 2.

3.3. UPLC method validation

The validation study allowed the evaluation of the method for its suitability for routine analysis. The API samples of primaquine phosphate showed presence of impurity I (regioisomer of primaquine) up to level of 0.2% while impurity II was not detected. The assay values of different bulk drug samples were found to be in the range of 98–100%. The system suitability parameters obtained for related substance method are given in Table 2.

Forced degradation studies were also performed for primaquine phosphate bulk drug sample to demonstrate the stability indicating power of the newly developed UPLC method.

3.3.1. Specificity

It is the ability of analytical method to measure the analyte response in the presence of its potential impurities and degradents. The specificity of the UPLC method was determined by injecting individual impurity samples, wherein no interference was observed for any of the components. During the forced degradation study, a considerable degradation of drug substance was observed in oxidative and reductive conditions (Fig. 3).

The chromatograms were checked for the appearance of any extra peak. Peak purity of these samples under stressed conditions was verified using a PDA detector (Fig. 4). The purity of the principle and other chromatographic peaks was found to be satisfactory. This study confirmed the stability indicating power of the UPLC method.

3.3.2. Precision

The precision of the assay method was evaluated by carrying out six independent assays. The %R.S.D. of assay of primaquine phosphate determination was within the acceptable limit of 2%. The precision of related substance method was examined using six replicate injections of standard impurity solution. The R.S.D. for impurity I and II were found to be 5.52% and 3.78%, respectively. These values are well within the generally acceptable limit of 10%.

Table 3
Accuracy of impurities

Level	Amount added (μg/ml)	Amount recovered (μg/ml)	Recovery (%)
At 80% ($n=3$)			
Impurity I	0.201	0.210	104.26
Impurity II	0.200	0.215	107.48
At 100% ($n=3$)			
Impurity I	0.251	0.252	100.24
Impurity II	0.250	0.264	105.79
At 120% ($n=3$)			
Impurity I	0.301	0.311	103.22
Impurity II	0.300	0.320	106.74

The R.S.D. of assay results obtained in intermediate precision study was within 2% and the R.S.D. of responses for impurity I and II were well within 10%, confirming good precision of the assay and related substance method.

3.3.3. Accuracy

The accuracy of the method was determined for the related substance by spiking known amount of impurities in primaquine bulk sample (test preparation) in triplicate at levels 80%, 100% and 120% of the specified limit. The recoveries of impurities were calculated and given in Table 3. The accuracy of the assay method was evaluated in triplicate at three concentration levels, 160, 200 and 240 μg/ml in bulk drug sample. The percentage recovery of primaquine phosphate in bulk drug samples ranged from 98.0 to 99.0% (Table 4).

3.3.4. Limit of quantification and limit of detection

LOQ values for impurity I and II were found to be 0.02% each of analyte concentration (500 μg/ml). The LOD values for impurity I and II were 0.01% each of analyte concentration (500 μg/ml).

3.3.5. Linearity

Linear calibration plots for the related substance method were obtained over the calibration range (LOQ to150%) at six concentration levels in triplicate. For impurity I corre-

Table 4
Assay recovery of Primaquine phosphate

Level (%)	Amount added (μg/ml)	Amount recovered (μg/ml)	Recovery (%)	Mean
80	160.08 ($n=3$)	161.03	100.59	99.92
		159.43	99.59	
		159.38	99.56	
100	200.1 ($n=3$)	197.43	98.66	98.53
		196.83	98.36	
		197.29	98.59	
120	240.12 ($n=3$)	236.87	98.65	98.70
		237.19	98.78	
		236.97	98.69	

sponding regression equation was $y = 2397.5x - 22$, with the correlation coefficient (R^2) greater than 0.998. For impurity II, corresponding regression equation was $y = 9406.4x - 12$, with the correlation coefficient (R^2) greater than 0.999. The results showed excellent correlation between the peak area and concentration of impurities.

3.3.6. Robustness

In all the deliberately varied chromatographic conditions, the chromatogram for system suitability solution for related substance showed satisfactory resolution between primaquine and impurity I (Rs > 2).

3.4. Sensitivity of UPLC

Sensitivity of UPLC over HPLC was evaluated by comparison of LOQ values of impurity II obtained in isocratic mode for both systems (Fig. 5). The LOQ concentration for UPLC was found to be 0.1 μg/ml, with R.S.D. 2.9% at injection volume 0.8 μl. The LOQ concentration for HPLC was found to be 0.25 μg/ml, with R.S.D. 6.97% at injection volume 10 μl. The lower LOQ values with higher precision are attributed towards better sensitivity of UPLC method.

4. Conclusion

The newly developed UPLC method for related substance and assay determination of primaquine phosphate was found to be capable of giving faster retention times maintaining good resolution than that achieved with conventional HPLC. The method was completely validated showing satisfactory data for all the parameters tested. This method exhibited an excellent performance in terms of sensitivity and speed. It is a stability indicating method suitable for rapid analysis of primaquine bulk drug and its impurities.

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References

[1] A.D. Jerkovich, J.S. Mellors, J.W. Jorgenson, LC–GC North America 21 (2003) 600–610.
[2] R. Plumb, J.C. Perez, J. Granger, I. Beattie, K. Joncour, A. Wright, J. Rapid Commun. Mass Spectrom. 18 (2004) 2331–2337.
[3] S.A.C. Wren, P. Tchlitcheff, J. Chromatogr. A 1119 (2006) 140–146.
[4] S.A.C. Wren, P. Tchlitcheff, J. Pharm. Biomed. Anal. 40 (2006) 571–580.
[5] R. Li, L. Dong, J. Huang, Anal. Chim. Acta 546 (2005) 167–173.
[6] Dee Ann Casteel, Burger's medicinal chemistry and drug discovery, in: Donald J. Absa (Ed.), Chemotherapeutic Agents, vol. 5, sixth ed., Johnwieth and Sons Inc., 2003, p. 949.
[7] http://www.malariasite.com/malaria/primaquine.htm.
[8] Y. Bergqvist, F.C. Churchill, J. Chromatogr. 434 (1988) 1–20.
[9] J.K. Baker, J.D. McChesney, C.D. Hufford, A.M. Clark, J. Chromatogr. 230 (1982) 69–77.
[10] J. Lal, N. Mehrotra, R.C. Gupta, J. Pharm. Biomed. Anal. 32 (2003) 141–150.
[11] V.K. Dua, P.K. Kar, R. Sarin, V.P. Sharma, J. Chromatogr. B 675 (1996) 93–98.
[12] A.K. Dwivedi, D. Saxena, S. Singh, J. Pharm. Biomed. Anal. 33 (2003) 851–858.
[13] M.V. Nora, G.W. Parkhurst, R.W. Thomas, P.E. Carson, J. Chromatogr. 307 (1984) 451–456.
[14] European Pharmacopoeia 5.0 vol. 2, 2004, pp. 2308–2309.
[15] I. Brondz, D. Mantzials, U. Klein, D. Ekeberg, E. Hvattum, M.N. Lebedeva, F.S. Mikhailitsyn, G.D. Souleimanov, J. Roe, J. Chromatogr. B 800 (2004) 211–223.
[16] V.G. Dongre, P.P. Karmuse, M.M. Nimbalkar, D. Singh, A. Kumar, J. Pharm. Biomed. Anal. 39 (2005) 111–116.
[17] ICH topic Q2(R1), Validation of analytical procedures: text and methodology version 4 (2005).