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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 186–195
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Characterization and quantitative determination of impurities in piperaquine phosphate by HPLC and LC/MS/MS

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Received 27 April 2006; received in revised form 26 June 2006; accepted 1 July 2006
Available online 17 August 2006

Abstract

Four impurities in piperaquine phosphate bulk drug substance were detected by a newly developed gradient reverse phase high performance liquid chromatographic (HPLC) method. These impurities were identified by LC/MS/MS. The structures of impurities were confirmed by spectroscopic studies (NMR and IR) conducted using synthesized authentic compounds. The synthesized reference samples of the impurity compounds were used for the quantitative HPLC determination. The system suitability of HPLC analysis established the validity of the separation. The method was validated according to ICH guidelines with respect to specificity, precision, accuracy and linearity. Forced degradation studies were also performed for piperaquine phosphate bulk drug samples to demonstrate the stability indicating power of the newly developed HPLC method.

Keywords: Piperaquine phosphate; Impurities; HPLC; LC/MS/MS; Characterization; Validation; Forced degradation

1. Introduction

Piperaquine phosphate—1,3-bis-[4-(7-chloroquinolyl-4)-piperazinyl-1]-propane phosphate, is an established drug for the treatment and suppuration of falciparum malaria [1,2]. A combination therapy (piperaquine—dihydroartimisinin) has been developed and found effective against malarial parasites that are resistant to the commonly used drug, chloroquine. [3]. This simple, safe, and relatively inexpensive combination could become the treatment of choice for falciparum malaria [4].

A few bioanalytical methods are reported in the literature for the quantitative determination of piperaquine [5–7]. The Chinese pharmacopeia [8] describes a thin layer chromatographic method for the qualitative assessment of the related substances. However there are no reports available on the identification, characterization and quantitative determination of related substances in piperaquine active pharmaceutical ingredient (API).

Since the impurity profile study of any pharmaceutical substance is a crucial part of process development, it was felt necessary to develop a reliable method for quantitative determination of impurities in piperaquine phosphate.

During process development studies [9], four impurities were detected in both crude and pure samples of piperaquine using a newly developed gradient reversed phase HPLC method. A comprehensive study was undertaken for the identification of these impurities using LC/MS/MS followed by their synthesis and further characterization by various spectroscopic techniques. This paper also deals with the validation of a new HPLC method for quantitative determination of these impurities.

2. Experimental

2.1. Materials and reagents

Samples of piperaquine phosphate API (batch no. PPQ-Pure and PPQ Crude) were obtained from Ipca Laboratories Ltd., Chemical Research Division, Mumbai, India. HPLC grade acetonitrile and ammonium acetate were purchased from Merck India Limited. Chloroform—d_3 and dimethyl sulphoxide—d_6 (for NMR) were purchased from Aldrich Chemical Co., USA.

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doi:10.1016/j.jpba.2006.07.005

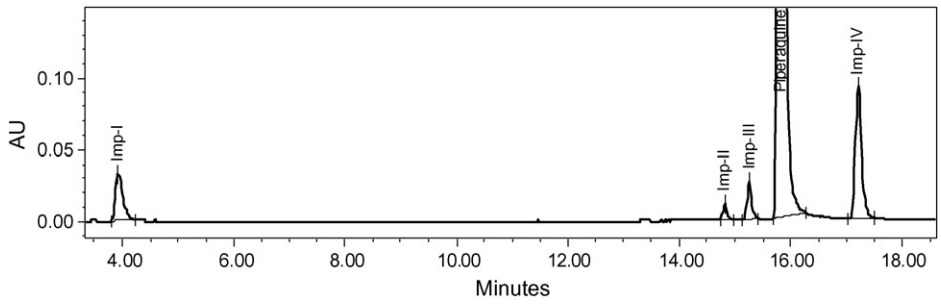

Fig. 1. A typical chromatogram of piperaquine crude sample.

2.2. High performance liquid chromatography

Samples were analysed on a Waters Alliance 2690 separation module equipped with 2487 UV detector. A C18 column (Waters XTerra 150 cm × 4.6 cm i.d., 3 μ particles) was used for chromatographic separations. The mobile phase consisting of A: 0.02 M disodium hydrogenphosphate, 0.1% triethylamine (pH 7 adjusted with phosphoric acid) and B: acetonitrile, with a timed gradient programme *T* (min)/%B: 0/30, 5/30, 12/70, 18/70, 22/30, 30/30, and a flow rate of 0.7 mL per min was used. The injection volume was 20 μL and the detector wavelength was fixed at 320 nm. The column was maintained at 40 °C throughout the analysis.

2.3. Liquid chromatography–tandem mass spectrometry (LC/MS/MS)

The MS and MS/MS studies were performed on LCQ-Advantage (Thermo Electron, San Jose, CA) ion trap mass spectrometer. The source voltage was maintained at 3.0 kV and capillary temperature at 250 °C. Nitrogen was used as both sheath and auxiliary gas. The mass to charge ratio was scanned across the range of *m*/*z* 150–500. MS/MS studies were carried out by keeping normalized collision energy at 35% and an isolation width of 6 amu. The HPLC consisted of an Agilent-1100 series quaternary gradient pump with a degasser, an auto sampler and column oven. A C18 column (Waters XTerra 150 cm × 4.6 cm i.d., 3 μ particles) was used for separation. The mobile phase consisting of A: 0.01 M ammonium acetate (pH 7 adjusted with ammonium hydroxide) and B: acetonitrile, with timed gradient programme of *T* (min)/%B: 0/20, 7/20, 15/50, 23/50, 27/70, 35/70, 40/20, with the flow rate of 0.7 mL per min was used.

2.4. NMR spectroscopy

^{1}H and ^{13}C NMR spectra of the synthesized impurities were recorded on Bruker 400 MHz instrument. The ^{1}H and ^{13}C chemical shift values were reported on the δ scale (ppm) relative to $CDCl_3$ (7.26 ppm).

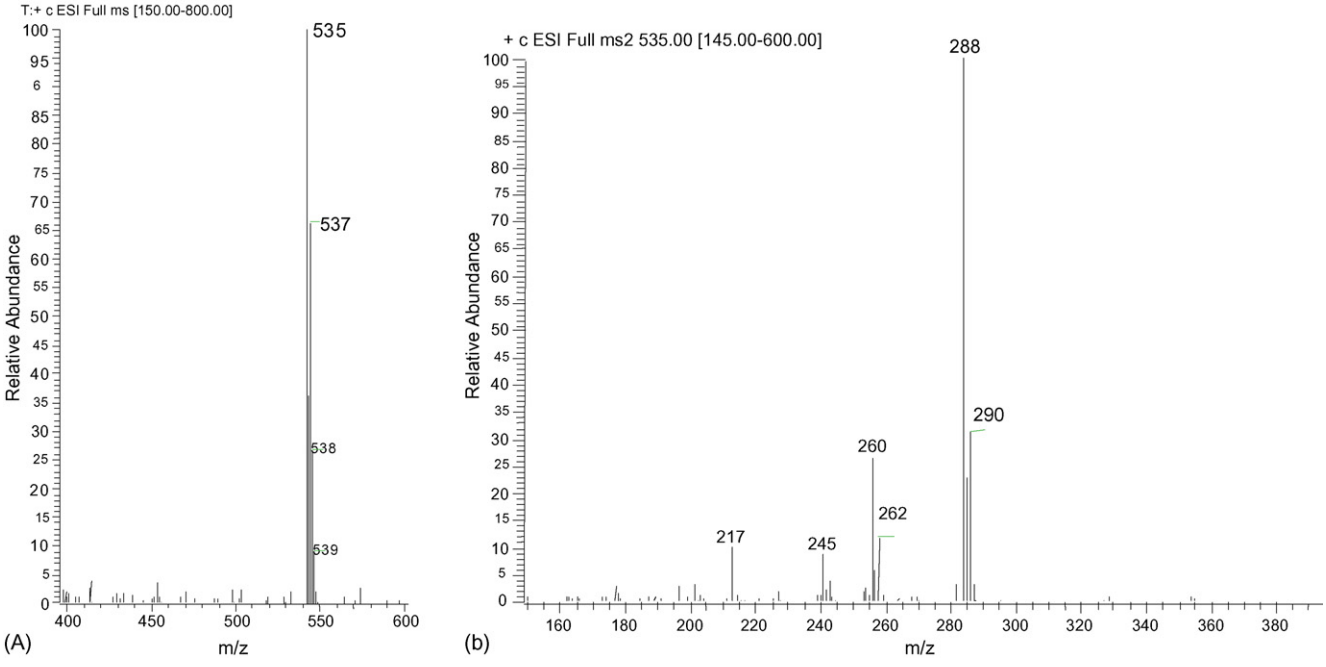

Fig. 2. (A) Mass spectrum of piperaquine and (B) MS/MS spectrum of piperaquine.

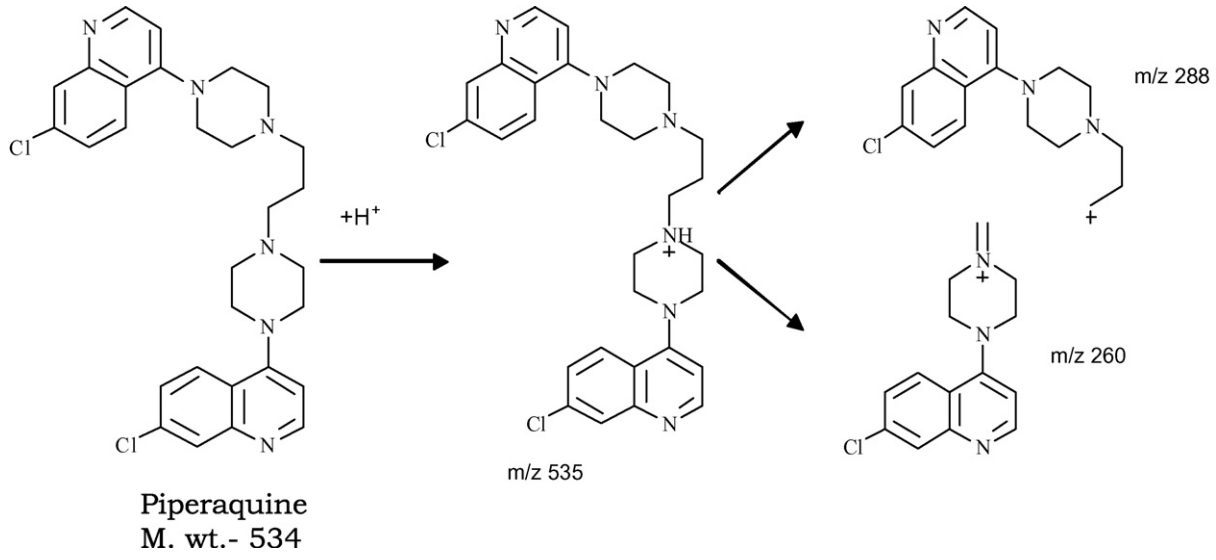

Fig. 3. Fragmentation mechanism for formation of ions *m/z* 288 and *m/z* 260 from M + H peak of piperaquine.

2.5. IR spectroscopy

The IR spectra for isolated impurities were recorded in the solid state as KBr powder dispersion using Perkin-Elmer spectrum one FT-IR spectrometer.

2.6. Preparation of solutions for validation of HPLC method

A test preparation of 200 μg/mL of piperaquine phosphate bulk drug sample was prepared using the diluent (mixture of water and acetonitrile in the ratio of 60:40). A stock solution of mixture of impurities was prepared by dissolving 6 mg of Imp-I, 4 mg of Imp-III, 10 mg of Imp-IV and 2 mg of piperaquine phosphate in 100 mL of diluent. From this stock solution a standard solution containing 0.6 μg/mL of Imp-I, 0.4 μg/mL of Imp-III, 1 μg/mL of Imp-IV and 0.2 μg/mL of piperaquine phosphate was prepared. This standard solution was also used for checking system suitability parameters.

3. Result and discussion

3.1. Detection of impurities by HPLC

HPLC analysis using the method described in Section 2.2 revealed the presence of four impurities at RRTs 0.20, 0.85, 0.9 and 1.10 with respect to principle peak. The target impurities under study are marked as Imp-I, Imp-II, Imp-III and Imp-IV, respectively. The typical chromatogram highlighting the retention times of these impurities is shown in Fig. 1.

3.2. Identification of impurities by LC/MS/MS

A mass spectrometry compatible HPLC method, as described in Section 2.3 is used to detect impurities. The mass spectrum obtained for piperaquine showed a protonated molecular ion $(M+H)^+$ at *m/z* 535. The characteristic ^{37}Cl isotopic peak, at *m/z* 537 with approximately 65% relative abundance, is obtained due to the presence of two chlorine atoms in piperaquine molecule (Fig. 2A). The spectral data obtained from MS/MS studies showed two product ion peaks (*m/z* 288 and *m/z* 260) with a characteristic single chorine isotopic pattern (Fig. 2B). The formation of these product ions can be explained by the dissociation mechanism shown in Fig. 3.

The mass spectrum of Imp-I showed protonated molecular ion peak at *m/z* 248, which was identified as 7-chloro-4-piperazinyl quinoline. This impurity was appeared due to traces of unreacted intermediate (Ia) during the synthesis of piperaquine (Fig. 6A). The identity of this impurity was further confirmed by co-elution of the said intermediate with the reference sample in the HPLC chromatogram.

The mass spectrum of Imp-II showed a protonated molecular ion peak ($[M+H]^+$, *m/z* 324) with the characteristic isotopic pattern for two chlorine atoms (Fig. 4A). In the MS/MS studies the parent ion (*m/z* 324) gave three prominent product ion peaks at *m/z* 288, *m/z* 205 and *m/z* 164 (Fig. 4B). All the product ion peaks showed a single chlorine isotopic pattern. The fragment at *m/z* 288 was presumed to be identical with the product ion peak of piperaquine, showing difference of 36 amu from the mass of the parent ion peak ($[M+H]^+$) of impurity II. This diagnostic difference was ascribed to the loss of neutral HCl from the protonated molecular ion. It is evident from reaction scheme of piperaquine that intermediate (Ia) is converted to piperaquine via intermediate (Ib), 1-chloro-3-(7-chloro-4-quinolyl-4-piperazinyl) propane (molecular mass 323) (Fig. 6C). Taken together, the mass spectral data and reaction scheme of piperaquine support the appearance of Imp-II in the final product in that it can be attributed to traces of unreacted intermediate (Ib). The formation of other product ions (e.g. *m/z* 205 and *m/z* 164) can be rationalized by considering the proposed structure of the impurity shown in Fig. 5.

The mass spectrum of Imp-III showed a protonated molecular ion at *m/z* 535, isobaric with the protonated molecular ion of piperaquine. Since the MS/MS spectrum obtained from this

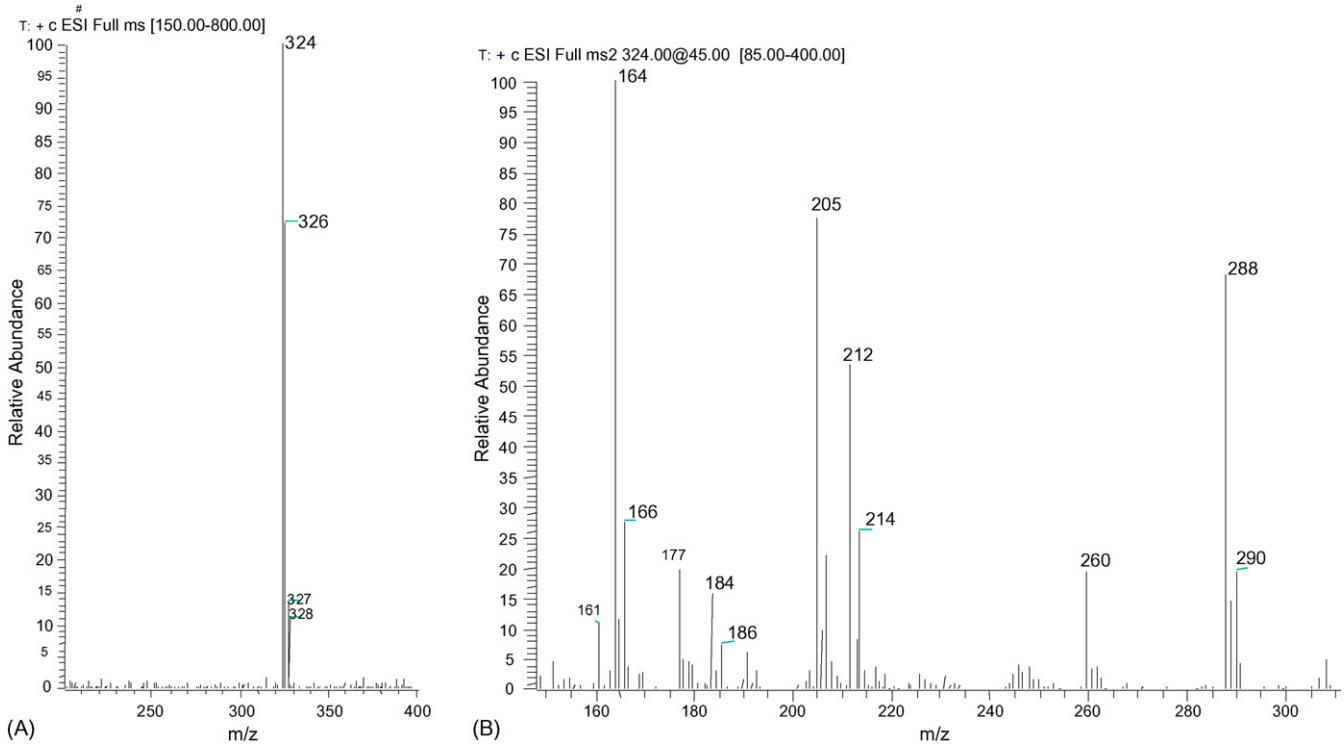

Fig. 4. (A) Mass spectrum of Imp-II and (B) MS/MS spectrum of Imp-II.

parent ion also resembled with that of MS/MS spectrum of piperaquine, this impurity was suspected to be a regioisomer of piperaquine. The most plausible structure for this regioisomer can be assigned as 1-(1-5-chloro-4-quinolyl-4-piperazinyl)-3-(1-7-chloro-4-quinolyl-4-piperazinyl) propane. The presence of 4,5-dichloroquinoline (isomeric impurity) in the starting material (4,7-dichloroquinoline) was found to contribute the formation of Imp-III (Fig. 6D).

The mass spectrum of Imp-IV exhibited a protonated molecular ion peak at *m/z* 409. The characteristic chlorine isotopic peak at *m/z* 411 with approximately 65% relative abundance (Fig. 7A), indicated the presence of two chlorine atoms in the molecule, possibly due to the presence of two chloroquinoline moieties in the molecule. The difference of 161 amu between protonated molecular ion of this impurity and intermediate Ia (Imp-I) corresponds to the monochloroquinoline moiety. The molecular mass (408 amu) obtained for Imp-IV differs by 126 amu from piperaquine (534 amu), which can be accounted for by *N*-propyl piperazine moiety. During the synthesis of intermediate Ia, there is a possibility of formation of a by-product (Fig. 6B),

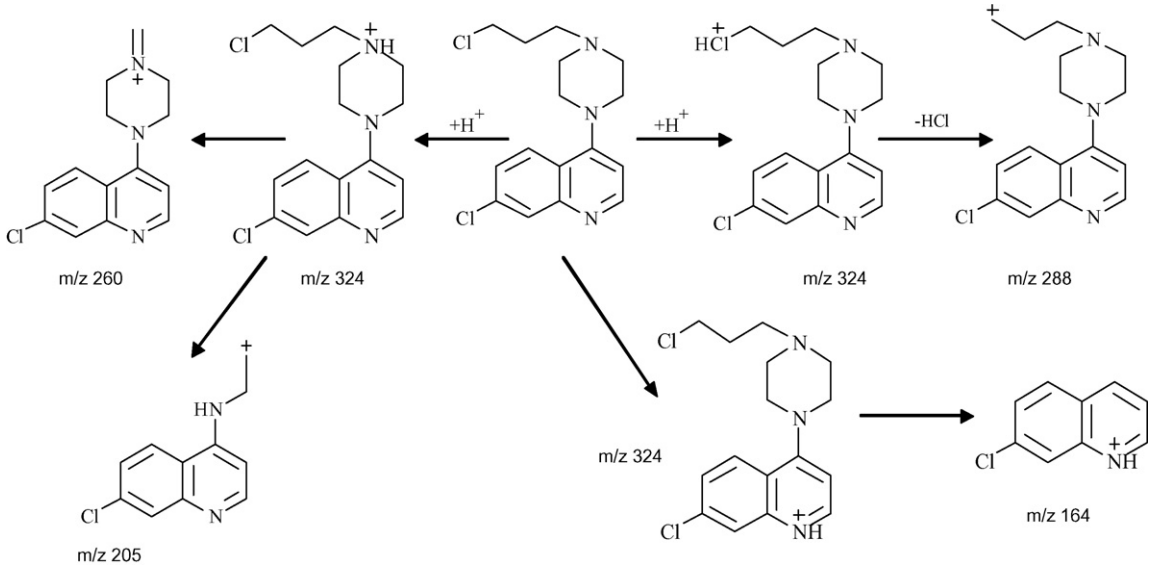

Fig. 5. Plausible scheme for fragmentations of Imp-II.

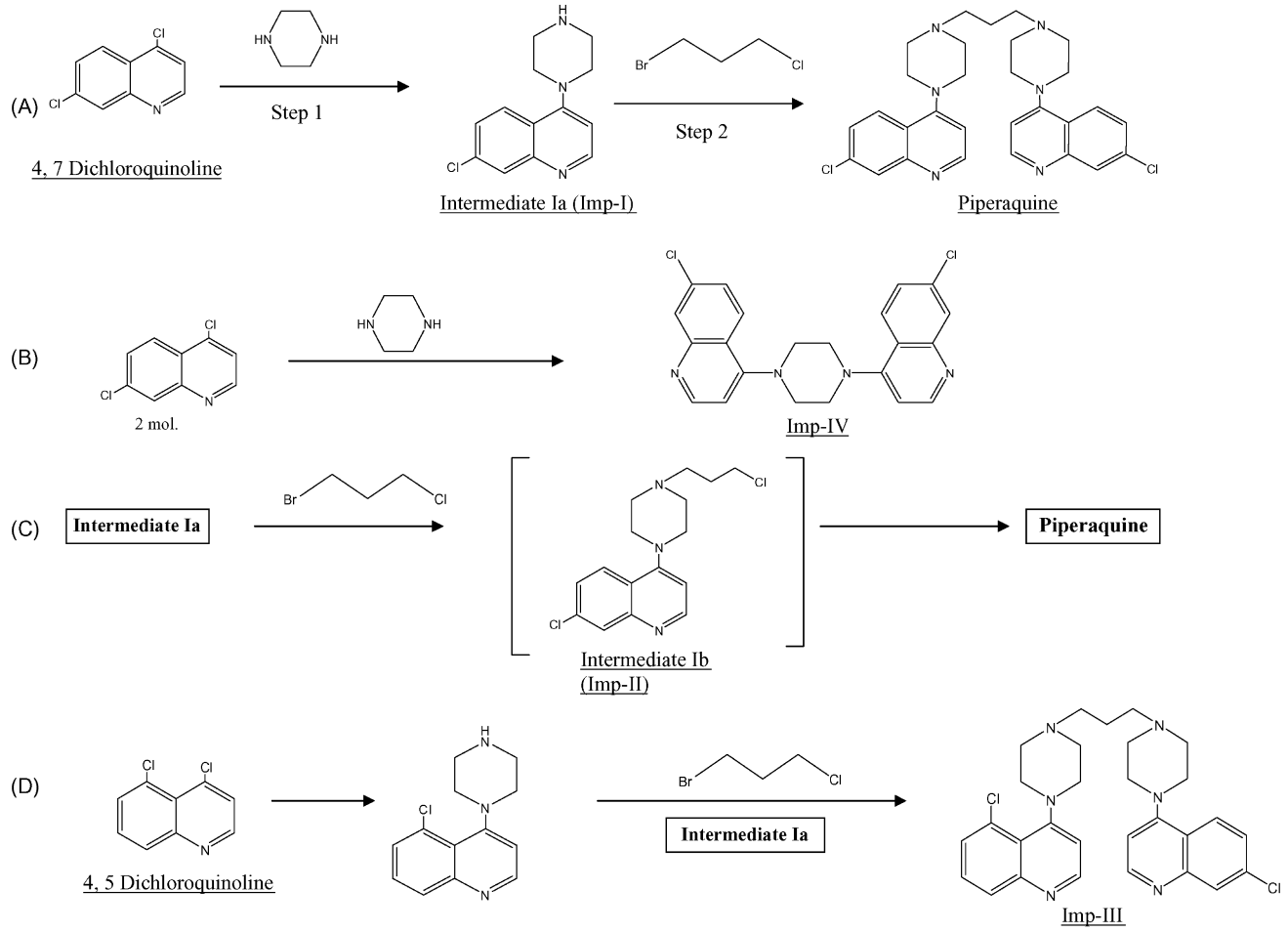

Fig. 6. (A) Scheme for synthesis of piperaquine, and formation of Imp-I, (B) formation of Imp-IV, (C) formation of Imp-II and (D) formation of Imp-III.

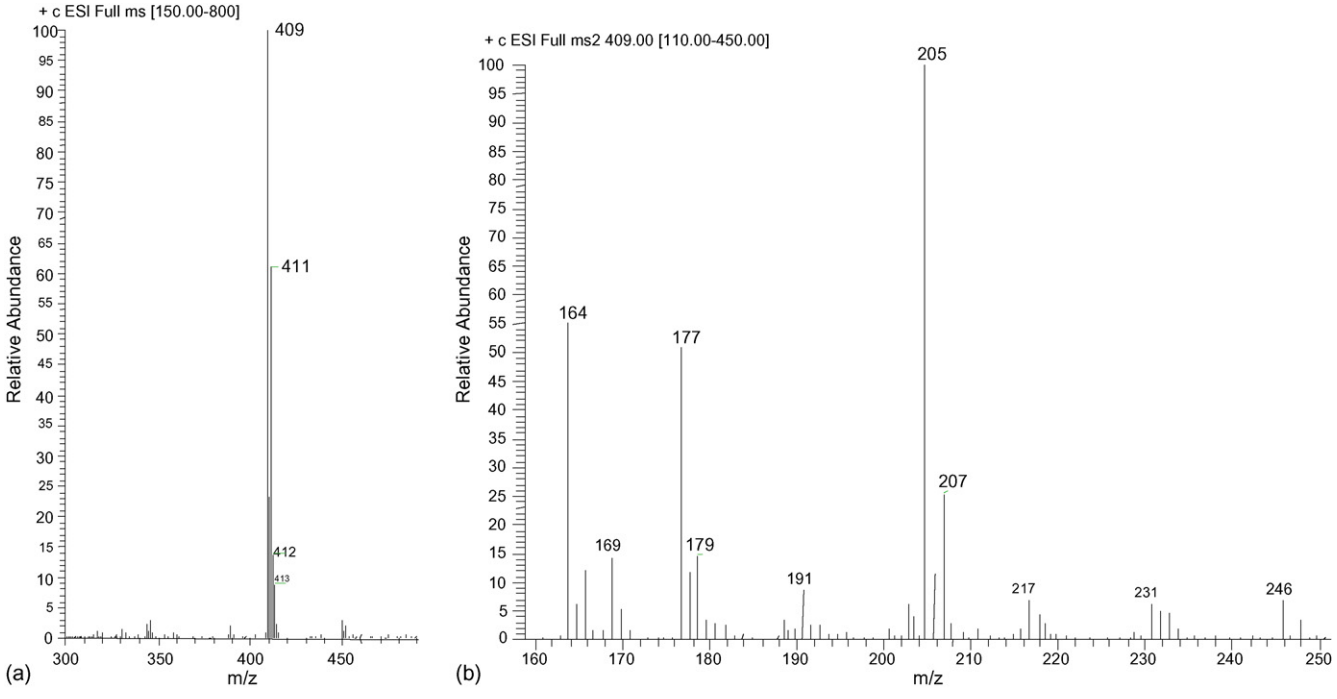

Fig. 7. (A) Mass spectrum of Imp-IV and (B) MS/MS spectrum of Imp-IV.

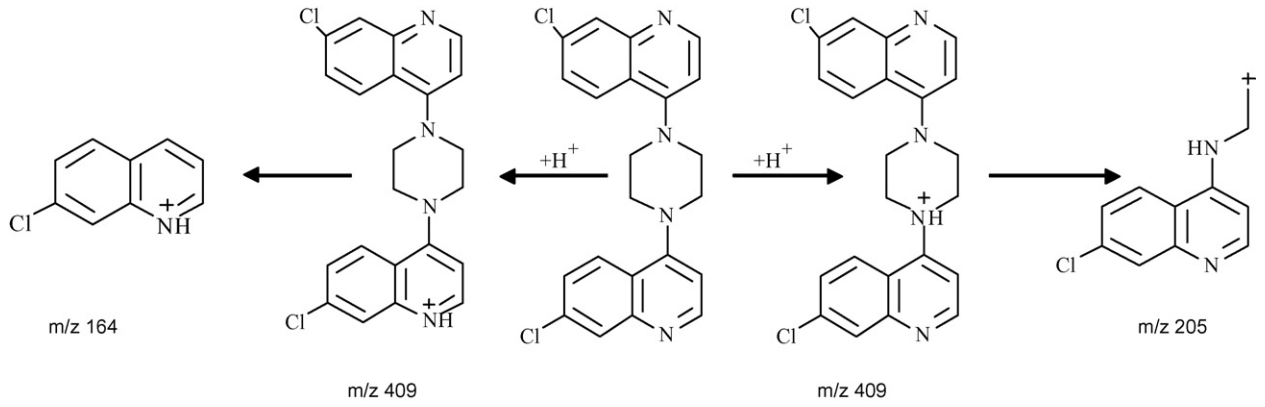

Fig. 8. Plausible scheme for fragmentations Imp-IV.

which carry forwards into the final product. The mass spectral data is in agreement with this proposed by-product, i.e. 1,4-bis-(4,7-dichloroquinoline) piperazine (molecular mass 408), which appeared as Imp-IV in the piperaquine bulk drug substance. Further MS/MS studies of Imp-IV showed two product ions at *m/z* 205 and *m/z* 164 (Fig. 7B). The formation of these fragments is depicted in Fig. 8.

3.3. Synthesis and structural elucidation of Imp-II

Since Imp-II is not isolable from the reaction mixture of the piperaquine synthesis, it was independently synthesized.

7-Chloro-4-piperazinyl quinoline was treated with 1,3-bromochloropropane at room temperature (28 °C) using *N*,*N*-dimethylformamide as a solvent. The reaction mixture showed an enrichment of Imp-II by HPLC analysis. It was purified by column chromatography using silica gel (60–120 mesh) and an eluent mixture of chloroform and ethyl acetate (80:20). The chromatographic purity was found to be 95%. ^{1}H NMR spectral data confirmed the proposed structure. The MS/MS spectrum obtained for synthesized authentic compound of impurity using direct infusion mode was exactly same as MS/MS spectrum of Imp-II obtained from on-line LC/MS/MS analysis.

3.4. Synthesis and structural elucidation of Imp-III

Synthesis of Imp-III was carried out in two steps. The first step involved condensation of piperazine with 4,5-dichloroquinoline

Table 1
Comparative NMR assignment of Imp-III and piperaquine

Positions[a]	Number of protons (H)	Imp-III			Piperaquine	
		Proton chemical shift, δ (ppm)	J[b] (Hz)	^{13}C chemical shift	Proton chemical shift, δ (ppm)	^{13}C chemical shift
1	1H	7.96		129.56	7.88	121.904
1′	–				–	
2	1H	7.42	dd	126.25	7.35	125.256
2′	1H	7.51		128.79	–	
3	–				–	
3′	1H	7.50	7.58	128.5	–	
4	1H	8.03	2.02 d		7.98	128.870
4′	1H	7.94	2.78	125.31	–	
5	–				–	
6	–				–	
7	1H	8.67	5.05 d	150.79	8.65	156.918
7′	1H	8.71	5.05 d	152.05	–	
8	1H	6.83	5.05 d	109.08	6.77	108.965
8′	1H	6.92	5.05 d	109.65	–	
9	–				–	
10	1H	3.27		52.26–56.69	3.20	52.178
10′	1H			52.26–56.69	–	
10″	1H			52.26–56.69	–	
11	1H	2.93		52.26–56.69	2.71	53.154
11′	1H	2.77		52.26–56.69	–	
12 and 12′	2H	2.56	7.83, 7.33 t	56.68	2.51	56.567
13	2H	1.86	7.58 q	24.41	1.78	24.418

[a] Refer the structural formula in Fig. 9A and C for numbering.
[b] ^{1}H–^{1}H coupling constants.

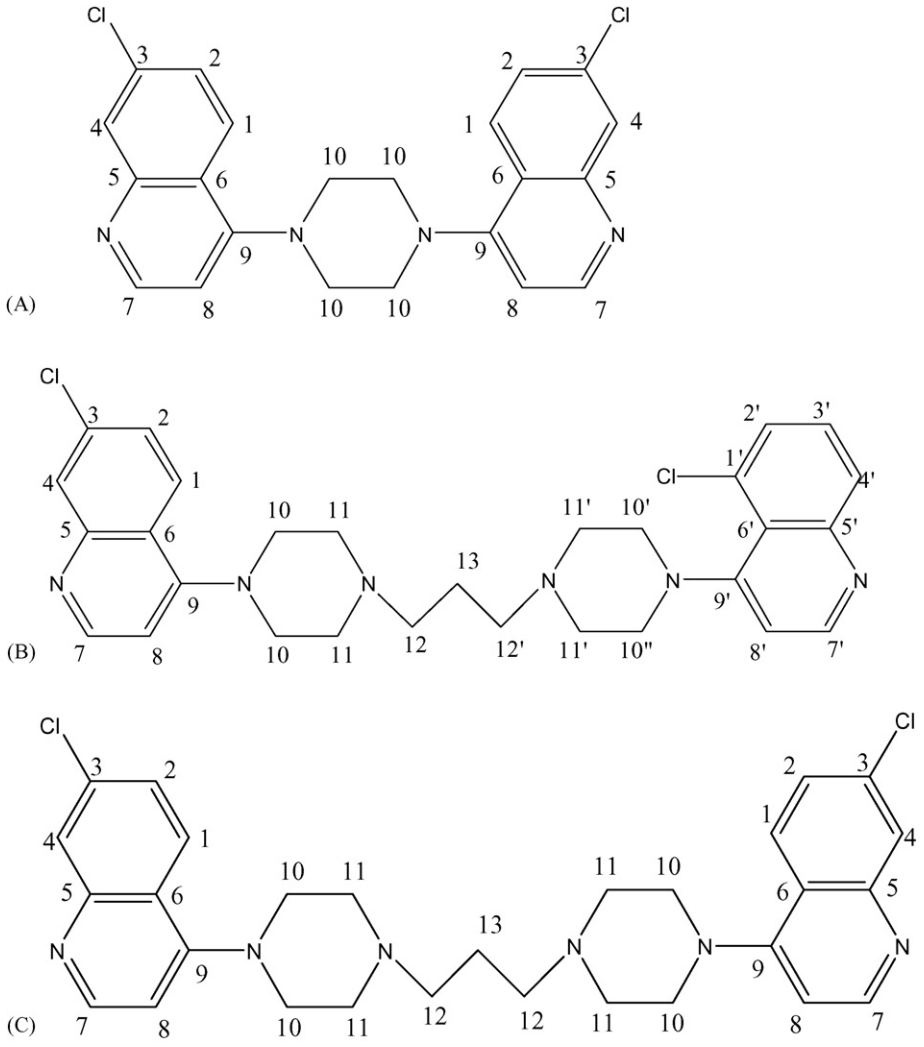

Fig. 9. NMR assignment of (A) Imp-IV, (B) Imp-III and (C) piperaquine.

under reflux in methanol to get 5-chloro-4-piperazinyl quinoline. The reaction mass was dried under vacuum and washed with water. The product obtained was treated with 1-chloro-3-(7-chloro-4-quinolyl-4-piperazinyl) propane at 90 °C using *N*,*N*-dimethylformamide as solvent. The reaction mixture was filtered and washed with water. The product obtained was recrystallised with ethanol. The chromatographic purity was found to be 99%.

The ^{1}H NMR spectrum of Imp-III was compared with ^{1}H NMR spectrum of piperaquine. The number of protons and chemical shift values in the aliphatic region were found to be similar for both the molecules. The aromatic region of the piperaquine spectrum showed five signals, each corresponding to two protons (due to the presence of two qinoline rings with same chemical environment), while in the case of Imp-III, 10 signals were obtained with each corresponding to one proton. This behavior might be due to the existence of two slightly different chemical environments for the two quinoline rings (for complete NMR details refer to Table 1 and Fig. 9B). These data support the structure of Imp-III that was presumed on the basis of the MS/MS data and synthetic scheme of piperaquine.

3.5. Synthesis and structural elucidation of Imp-IV

Synthesis of Imp-IV was carried by the reaction of 4,7-dichloroquinoline (3 mol) with piperazine (1 mol) using triethylamine as a base under reflux in methanol. The reaction mass was dried under vacuum and washed with water. The product obtained was checked by HPLC and found to be 99% pure.

In the ^{1}H NMR spectrum of Imp-IV, there were no signals corresponding to any residual propyl group. The singlet at δ 4.29, integrating for eight protons, indicated that only one piperazine ring is present in the structure. The aromatic region showed the existence of two identical qinoline rings in the molecule. These data were found to be in agreement with the structure assigned on the basis of MS and MS/MS spectral data. The NMR and IR assignments are shown in Tables 2 and 3, respectively.

4. Validation of HPLC method

The newly developed method for piperaquine and its related impurities was validated according to ICH guidelines [10,11].

Table 2
NMR assignment of Imp-IV

Positions[a]	Number of protons	Proton chemical shift, δ (ppm)	J^b (Hz)	^{13}C chemical shift
1	1H	8.32	9.6 dd	119
2	1H	7.74	9.2 dd	126
3	–	–	–	137
4	1H	8.08	2	129
5	–	–	–	140
6	–	–	–	116
7	1H	8.69	7.1 d	142
8	1H	7.09	7.6 d	103
9	–	–	–	158
10	1H			49

[a] Refer the structural formula in Fig. 9B for numbering.
[b] ^1H–^1H coupling constants.

Table 3
IR assignment

Functional group	Wave numbers (cm^{-1})		
	Imp-III	Imp-IV	Piperaquine
Aromatic C–H bending	721, 771	722, 775	719, 775
Aliphatic C–N stretching	1250, 1268	1232, 1247, 1264	1257, 1274
Aromatic C–N stretching	1323, 1360	1364, 1380	1324, 1367
Aliphatic C–H stretching	2822, 2870	2838, 2877, 2977	2820, 2874
Aromatic C–H stretching	3010	3014	3009
Aromatic C–Cl stretching	1129, 1149	1136, 1160	1126, 1148

Table 4
System suitability report

Component	Resolution	USP tailing factor	USP plate count
Imp-I	–	1.3	3851
Imp-III	57	1.1	157744
Piperaquine	3.3	1.1	134920
Imp-IV	7.2	1.0	129624

The validation study was carried out for the analysis of Imp-I, III and IV. The Imp-II was found unstable under the present experimental condition and hence was not included in the validation study. However, the response factor of Imp-II was determined with respect to a diluted solution of piperaquine, which can be used for quantitative determination of this impurity. The system suitability was established to verify the chromatographic separation (Fig. 10). The results are shown in Table 4.

4.1. Specificity

The ability of analytical method to unequivocally assess the analyte in the presence of other components (impurities and degradents) can be demonstrated by evaluating specificity. The specificity of the HPLC method was determined by injecting individual impurity samples, wherein no interference was observed for any of the components. Forced degradation studies of the bulk drug sample using the following conditions were also performed: acid hydrolysis (0.1 N hydrochloric acid), base hydrolysis (0.1 N sodium hydroxide), heat (105 °C for 48 h), photolytic (UV and sunlight for 48 h), oxidation (30% hydrogen peroxide) and reduction (10% sodium metabisulphite). The considerable degradation of drug substance was observed in oxidative and reductive conditions (Fig. 11).

The chromatograms were checked for the appearance of any extra peaks. Peak purity of these samples was verified using a photodiode array (PDA) detector. The purity of the principle and other chromatographic peaks was found to be satisfactory. This study confirmed the stability indicating power of the HPLC method.

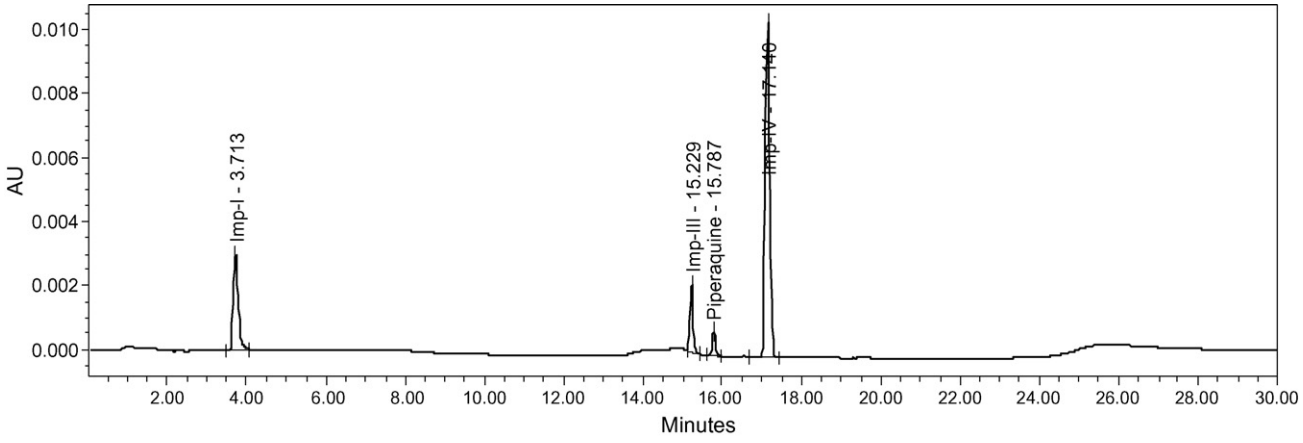

Fig. 10. Chromatogram of system suitability solution.

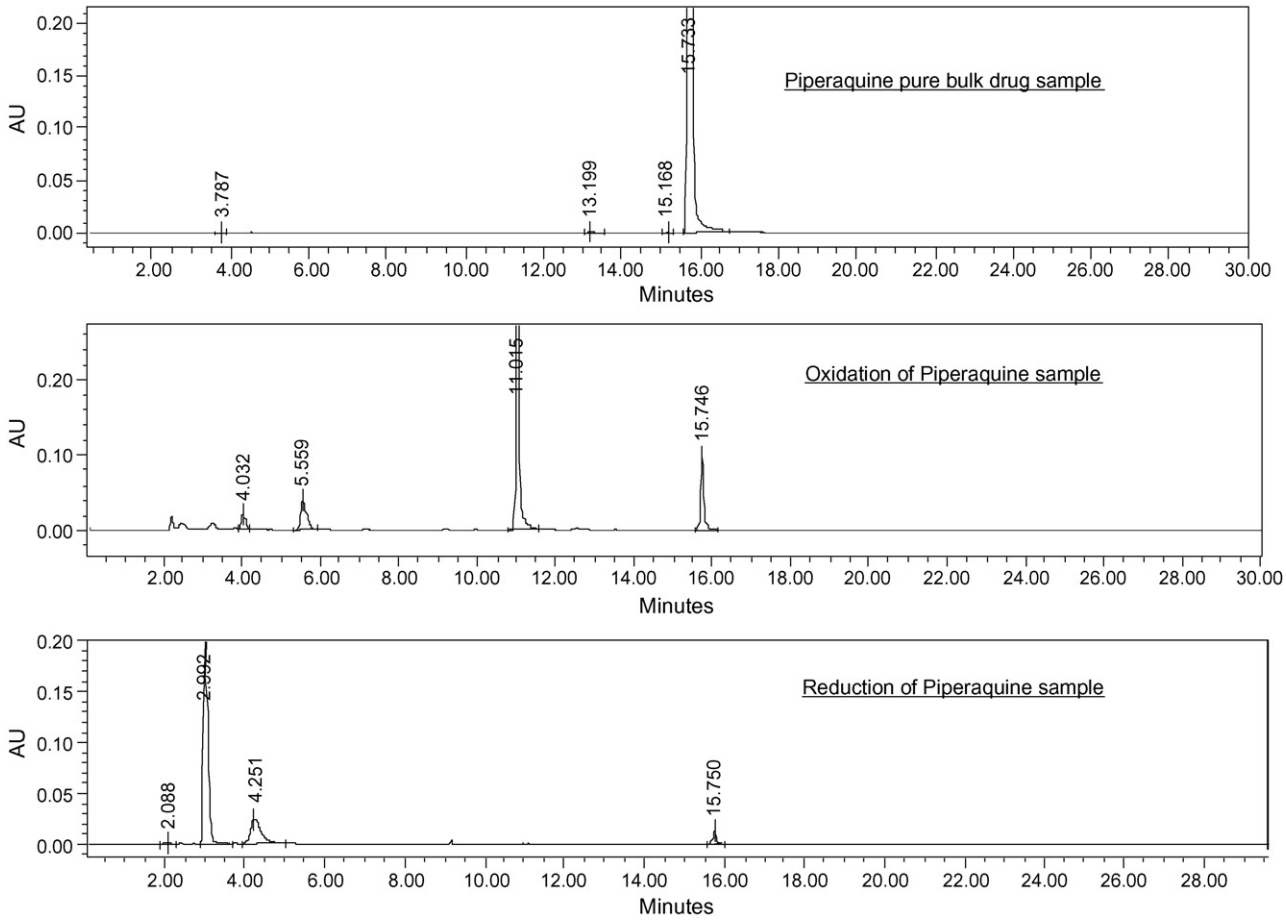

Fig. 11. Chromatograms of piperaquine phosphate bulk drug sample and stressed sample showing degradation.

4.2. Precision

The precision of the method was examined using six replicate injections of a standard solution (mixture of impurities). The relative standard deviation (R.S.D.) was calculated for response (area) of each impurity. The R.S.D.'s for Imp-I, Imp-III and Imp-IV were found to be 0.28%, 3.29% and 0.29%, respectively. These values are well within the generally acceptable limit of 10%. The RSD for diluted standard solution was also evaluated and found to be 3.79%. The method precision was established by analyzing samples of piperaquine phosphate using six different test preparations. The calculated R.S.D. of these results was found to be 2.67%.

4.3. Accuracy

The accuracy of the method was determined for the related substances by spiking of known amounts of an impurity in piperaquine bulk sample (test preparation) at levels, 80%, 100% and 120% of the specified limit. The recoveries of impurities were calculated and are given in Table 5.

4.4. Limit of quantification and limit of detection

The limit of quantification values for Imp-I, Imp-III and Imp-IV were found to be 0.06%, 0.06%, and 0.025% of analyte concentration (200 μg/mL), respectively. The limit of detection

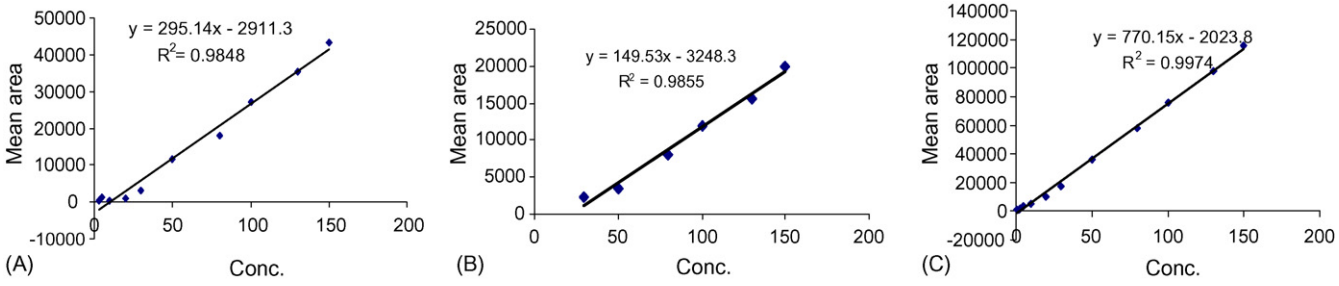

Fig. 12. Linearity plots of (A) Imp-I, (B) Imp-III and (C) Imp-IV.

Table 5
Accuracy of impurities

	Amount added (μg/mL)	Amount recovered (μg/mL)	Recovery (%)	Mean
At 80% level				
	0.493	0.501	101.62	
Imp-I	0.493	0.502	101.83	102.7
	0.493	0.504	102.23	
	0.330	0.332	100.61	
Imp-III	0.330	0.331	100.30	100.4
	0.330	0.334	100.21	
	0.821	0.942	114.74	
Imp-IV	0.821	0.947	115.35	115.5
	0.821	0.956	116.44	
At 100% level				
	0.613	0.619	100.98	
Imp-I	0.613	0.618	100.82	100.7
	0.613	0.615	100.33	
	0.410	0.401	97.80	
Imp-III	0.410	0.405	98.78	98.5
	0.410	0.406	99.02	
	1.123	1.231	109.62	
Imp-IV	1.123	1.225	109.08	110.8
	1.123	1.256	111.84	
At 120% level				
	0.714	0.732	102.52	
Imp-I	0.714	0.735	102.94	102.9
	0.714	0.738	103.36	
	0.490	0.500	100.20	
Imp-III	0.490	0.501	102.24	101.8
	0.490	0.504	102.89	
	1.214	1.325	109.14	
Imp-IV	1.214	1.361	112.11	110.3
	1.214	1.332	109.72	

values for Imp-I, Imp-III and Imp-IV were 0.009%, 0.01%, and 0.005% of analyte concentration (200 μg/mL), respectively.

4.5. Linearity

Linear calibration plots for the related substances method were obtained over the range (100–150% of standard solutions of impurities and diluted standard). The method showed linear response for all the impurities and the diluted standard. The linearity plots are shown in Fig. 12.

5. Conclusion

A new HPLC method was developed for separation of impurities in piperaquine phosphate bulk drug sample. These impurities were identified by LC/MS analysis. Characterization of the impurities was carried by synthesis followed by spectroscopic analysis. The newly developed HPLC method has been validated as per regulatory guidelines; it can be conveniently used for the quantitative determination of related substances in piperaquine phosphate bulk drug sample. The method was found to be specific, accurate and precise, and can be used for the routine analysis as well as to monitor the stability studies.

Acknowledgements

The authors wish to thank the management of Ipca Laboratories and Head, Department of Chemistry, University of Mumbai for providing necessary facilities. We would also like to thank Mr. P.P. Rao, Mr. Kailash Mungase, Mr. Prakash Sangle, Mr. R.K. Singh and Mr. Ch. Balaraju for providing technical assistance.

References

[1] L. Chen, Chin. Med. J. 104 (1991) 161–163.
[2] J.L. Vennerstrom, W.Y. Ellis, A.J. Ager, S.L. Andersen, L. Gerena, W.K. Milhous, J. Med. Chem. 35 (1992) 2129–2134.
[3] T.T. Hien, C. Dolecek, P.P. Mai, N.T. Dung, N.T. Truong, L.H. Thai, D.T.H. An, T.T. Thanh, K. Stepniewska, N.J. White, J. Farrar, Lancet 9402 (2004) 18–22.
[4] E.A. Ashley, R. McGready, R. Hutagalung, L. Phaiphun, T. Slight, S. Proux, K.L. Thwai, M. Barends, S. Looareesuwan, N.J. White, F. Nosten, Clin. Infect. Dis. 41 (2005) 425–432.
[5] N. Lindegårdh, N.J. White, N.P.J. Day, J. Pharm. Biomed. Anal. 39 (2005) 601–605.
[6] M. Malm, N. Lindegårdh, Y. Bergqvist, J. Chromatogr. B 809 (2004) 43–49.
[7] T.Y. Hung, T.M.E. Davis, K.F. Ilett, J. Chromatogr. B 791 (2003) 93–101.
[8] Chinese Pharmacopeia 2 (2000) 530.
[9] A. Kumar, B.N. Nerurkar, C.H. Shaha, R.K. Singh, Indian Patent application 639/MUM/2005.
[10] ICH topic Q2B, Validation of analytical methods: procedure and methodology.
[11] United States Pharmacopeia 26 (2006) 3050–3053.