Trade Science Inc.

January 2008 Volume 6 Issue 2

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ 6(2) 2008 [000-000]

HPLC method development for the determination of substances related to ampicillin in bulk drug

Ch.B.V.N.Raju*[1], Hemant K.Sharma[1], Ch.Sreenivas Rao[1], G.Nageswara Rao[2]

[1]Aurobindo Pharma Limited, Survey No. 66-69, Pydibhimavaram, Ranastali Mandal, Srikakulam-532409, (INDIA)

[2]Department of Inorganic and Analytical Chemistry, Andhra University, Visakhapatnam-530003, (INDIA)

Tel: +91-8942-288292, 330, 333; Fax: +91-8942-288293

E-mail : chbvn_5@yahoo.co.in

Received: 28th March, 2008 ; Accepted: 2nd April, 2008

ABSTRACT

A gradient, reverse phase liquid chromatographic (RPLC) method was developed for the quantitative determination of related substances in ampicillin drug substance. The chromatographic separation was achieved on Hypersil BDS C8 (150mm×4.6 mm, 5µm) column using 0.025M sodium dihydrogen orthophosphate dihydrate buffer, adjusted to a pH of 5.00 ± 0.05 with 1M sodium hydroxide as mobile phase A and acetonitrile as mobile phase B. Flow rate was 1.5ml min^{-1} and eluents were monitored at 220nm. Column oven temperature was maintained at 40°C. Forced degradation studies were performed for ampicillin drug substance using acid, base, oxidation, temperature, humidity and photo light. The limit of detection and limit of quantification of all related substances were found to be below 0.043 and 0.070 (%w/w), respectively. The percentage recovery for each related substance in bulk drug sample was in the range of 95 to 105. The ampicillin sample solution in mobile phase was found to be stable for at least 2 hours at 25°C and for 5 hours at 6°C. The developed method was validated with respect to specificity, linearity, accuracy, precision, ruggedness, and robustness and forced degrading studies to prove the stability indicating nature of the method. This method was applied to three quality control samples and results are reported.

KEYWORDS

Ampicillin;
HPLC;
Forced degradation;
Validation;
Quality control.

INTRODUCTION

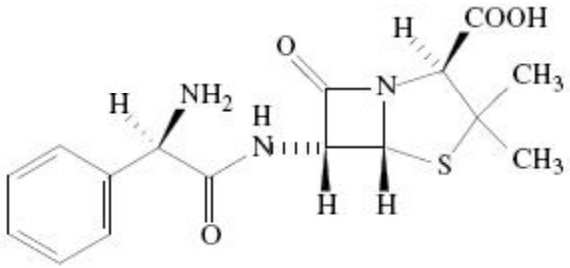
Ampicillin (Figure 1) is a β-lactam antibiotic. It is bactericidal and an aminopenicillin with amino group side-chain attached to the basic penicillin structure. Ampicillin is able to penetrate the outer membrane of some gram-negative bacteria and has a broader spectrum of activities. Ampicillin is more active than benzyl penicillin against some gram-negative organisms. Minimum inhibitory concentrations for gram-positive and gram-negative organisms have been reported to range from 0.02 to 1.5µg ml^{-1} and 0.03 to 3µgml^{-1}[1] respectively.

A number of HPLC methods have been reported in literature for the determination of ampicillin in biological samples[2-9]. Some methods were reported for the separation of ampicillin from other penicillin's or other drugs[10-13]. A number of methods has been reported

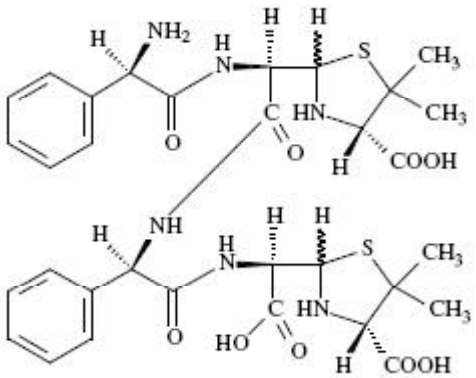
Full Paper

for the separation of ampicillin[14-16] and its related substances[14,15,17,18]. However no validated LC method was reported for the better separation and quantitative analysis for 18 individual related substances including isomers in ampicillin drug substance. In the literature, reported analytical method[13,15] selectivity towards related substances was not discussed. Zhu et al proposed an analytical method[18], but the selectivity towards related substances was poor. This method was not stability indicating and was not validated.

Based on these observations, it was felt necessary to develop stability indicating LC method for the determination of Ampicillin (1) and its related substances D-Phenylglycine (2), 6-Amini penicillanic acid (3), Pencilloic acids of ampicillin-1(4), Pencilloic acids of ampicillin-2 (5), L-Ampicillin (6), Penilloic acids of ampicillin-1 (7), Ampicillin diketopiperazine-1 (8), Penilloic acids of ampicillin-2 (9), Ampicillin diketopiperazine-2 (10), 3-Phenyl pyrazine 2-ol (11), N-Pivaloyl Phenylglycine (12), (3R,6R)-3,6-Diphenyl pirazine-2,5-dione (13), Dimer of ampicilloic acids (14), D-Phenylglycyl ampicillin (15), N-Pivaloyl 6-APA (16), Dimer of ampicillin and ampicilloic acids (17), Ampicillinyl D-Phenylglycine (18) and Trimer of ampicillin and ampicilloic acids (19) in drug substance. All the related substances except substance 14 (Figure 1) are listed in European Pharmacopoeia. This paper deals with the development of a stability indicating LC method, separating all related substances from each other and from the main peak. The developed method was subjected to stress study as per ICH guidelines[19] like acid hydrolysis, base hydrolysis, oxidation, heat, photolytic and humidity. This paper also deals with the analytical method validation for accurate quantification of all individual related substances in ampicillin drug substances as per ICH guidelines[20]. The developed method was applied to three real quality control samples.



Ampicillin



Dimer of Ampicilloic acids

Figure 1: Molecular structure of Ampicillin and dimer of ampicilloic acids

EXPERIMENTAL

Chemicals

Samples of ampicillin and related substances 2-19 were received from Process Research Department of Aurobindo Pharma Ltd, Hyderabad, India. HPLC grade acetonitrile (Merck, Germany) and A.R. grade potassium dihydrogen orthophosphate (Rankem, Mumbai, India) were used. High pure water was prepared by using Millipore Milli-Q plus purification system.

Equipment

The LC system used for the method development and forced degradation studies was Waters Alliance 2695 separation module with 2996 Photo Diode Array (PDA) detector. The output signal was monitored and processed with Waters Empower software. The LC system used for the validation and intermediate precision study was Waters Alliance consisting of 2695 separation module with thermostatic compartment and a 2487 dual wavelength detector. The mass (m/z) of impurities was recorded on Perkin Elmer triple quadrupole mass spectrometer (API 2000, PESCIEX). Analyst software was used for data acquisition and data processing. 1H experiments were performed on Bruker Avance DPX-300 M[Faellanden, Switzerland] NMR spectrometer.

Chromatographic conditions

The chromatographic column used was Hypersil BDS-C8 150 mm×4.6 mm with 5µm particles (Thermo

TABLE 1: Chemical shift value of TDI

Electron Corporation, USA). The mobile phase A was aqueous sodium dihydrogen orthophosphate dihydrate, adjusted to a pH of 5.00±0.05 with 1M sodium hydroxide and acetonitrile used as mobile phase B. Finally a good separation was achieved between all individual substances 1-19 using mobile phase A:B linear gradient time program mentioned in Table1. The flow rate of the mobile phase was 1.5ml min^{-1}. The column temperature was maintained at 40°C and the absorbance was monitored at a wavelength of 220nm. The injection volume was 20µl. A mixture of pH 5.00 buffer and acetonitrile 98:02 was used as diluent.

LC and mass spectrometer (MS) conditions for determination of mass (m/z) values

The substances 2-19 isolated by preparative HPLC, were used for mass determination. For mass determination on LC-MS, where 0.01M ammonium acetate buffer (adjusted to a pH of 5.0 with diluted AcOH) was used as mobile phase A and acetonitrile was used as mobile phase B. The ratios of A and B were changed in linear gradient program from 100:0 to 50:50, in a duration of 50 minutes. For LC separation Hypersil C8 column was used and eluents monitored at 230 nm. The mass spectrometer detector turbo ion spray voltage was maintained at 5.5 kV and temperature was set at 375°C. The auxiliary gas and sheath gas used was high pure Nitrogen. Zero air was used as Nebular gas. Mass spectra were recorded from m/z 100 to 1500 in 0.1 amu steps with 2.0 sec dwell time.

Preparation of standard and sample solutions

Standard solution was prepared by dissolving ampicillin qualified standard (0.015mg ml^{-1}) in diluent. Sample solution was prepared by dissolving ampicillin drug substance (1.5mg ml^{-1}) in diluent.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its related substances. The specificity of the developed LC method was evaluated for ampicillin in the presence of the substances 2-19. The sample (0.75mg ml^{-1}) solution was also subjected to stress conditions like heat (105°C/30 min), acid (1M HCl/85°C/20 min), base (0.5M NaOH/ initial), oxidation (10%H_2O_2/85°C/10 min), photolytic (10,000 lux/432 hrs) and humidity (92% RH/25°C/ 432 hrs) to evaluate the ability of the proposed method to separate ampicillin from its degradation products. Peak purity of ampicillin peak was evaluated by using PDA detector in stress samples and control sample. The peak purity test was also performed for the peaks of the substances 1-19 in the test solution spiked with all related substances.

Method validation

Precision

System precision of the method was evaluated by injecting ampicillin standard solution six times and by calculating %RSD for area count of ampicillin peak. The method precision was evaluated by injecting six individual preparations of ampicillin drug substance spiked with all related substances 2-19 at 1%w/w concentration. The % RSD for content of individual and total related substances in six determinations were calculated. The intermediate precision of the method was evaluated also by using different column, different instrument and different analyst in the same laboratory. The %RSD for results obtained by two analysts for each individual and total related substances were calculated.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ values of each related substance were predicted from a separate linearity experiment performed at lower concentration of the impurities. Each predicted concentration was verified, by preparing solutions at about predicted concentration and injecting each solution six times into the HPLC by following test method and conditions as specified in section 2.3. The %RSD for each related substance at LOD and LOQ levels were calculated.

Linearity

A series of solutions were prepared using ampicillin qualified standard and its related substances at six concentration levels from the LOQ to 1.5% w/w. The calibration curves were plotted for peak areas of ampicillin and the related substances 2-19 versus their corresponding concentrations. The slope, Y-intercept, residual sum of squares and correlation coefficient were calculated from linear calibration curves. Response factor

Full Paper

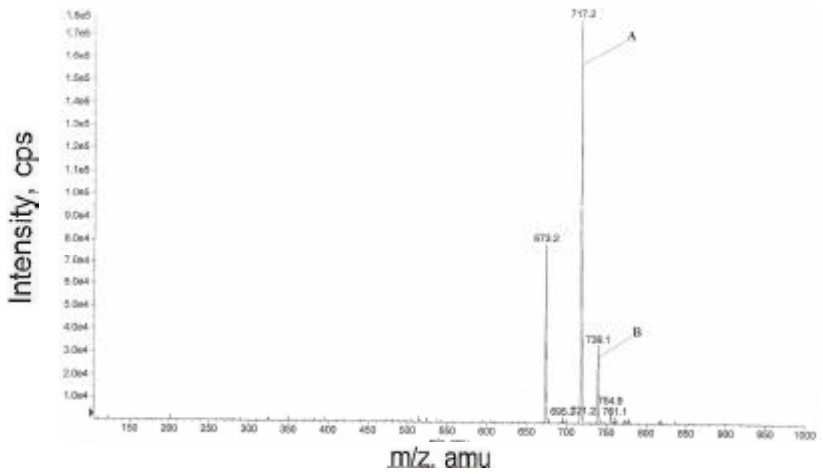


Figure 2 : The mass spectrum for dimer of ampicilloic acids where A is molecular ion peak[(MH)$^+$] and B is sodium adduct of molecular ion peak[(MH)$^+$]+Na

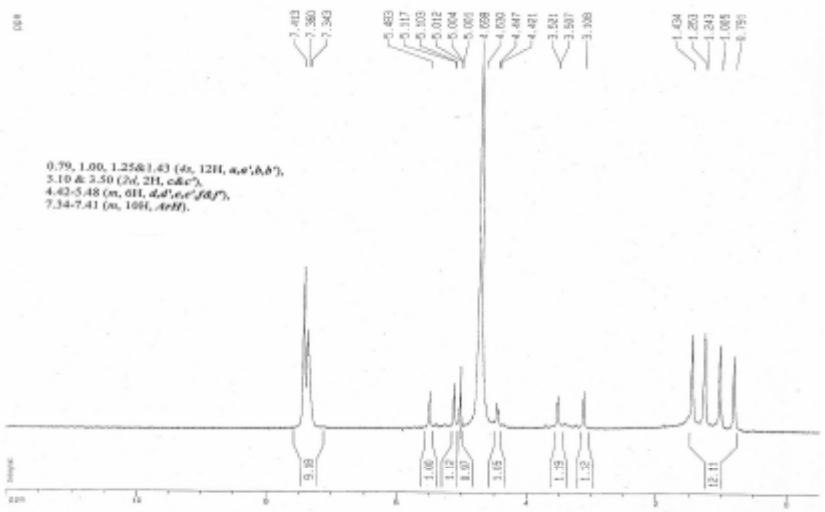


Figure 3 : The proton NMR spectrum for dimer of ampicilloic acids

for each impurity was calculated by comparing slope of the linear calibration curve of each impurity with that of ampicillin curve.

Accuracy

Prepared solutions in triplicate using ampicillin drug substance spiked with substances 2-19 substances at 50%, 100% and 150% of specification level (1% w/w) prepared were injected as mentioned in Section 2.3. The % recovery was calculated for each level of concentration.

Robustness

To determine the robustness of the developed method, the following parameters of the experimental conditions were purposely altered: the flow rate of the mobile phase was altered by ±10% (1.35ml min^{-1} and 1.65ml min^{-1}); the percent organic strength by ±2% absolute; the detector wavelength by ±5nm; the column oven temperature by ±5°C; and pH of the mobile phase was altered by ±0.2 unit. The rest of the chromatographic conditions for each alteration study were held constant as per the Section 2.3. The effect of these deliberate changes on USP resolution, USP tailing and USP plate count for system suitability and on selectivity of each impurity comparing ampicillin peak as reference peak in test solution spiked with all related substances at 1.0% level were checked.

Solution and mobile phase stability

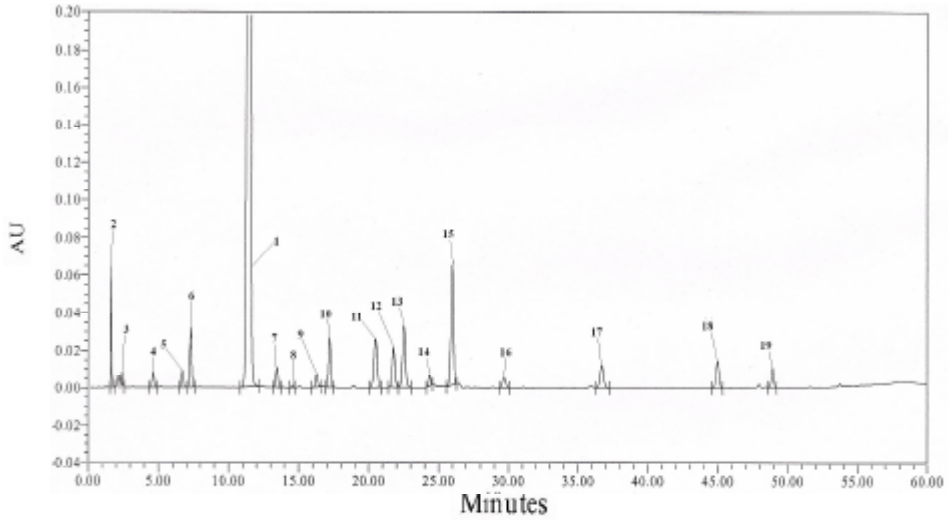


Figure 4: Chromatogram of ampicillin drug substance spiked with substances 2-19 using mobile phase A (pH 5.00) as diluent, linear gradient program of A and B specified in Table1 and proposed method conditions specified

The solution stability of ampicillin in test solution was tested by analyzing sample solution (prepared using ampicillin drug substance spiking with known related substances at 1.0%w/w level) initially and at different time intervals (1hr) by keeping sample solution at room temperature (25°C) and at refrigerated (6°C) conditions separately. The % difference between the responses obtained at initial and different time intervals were calculated.

Range

Range of analytical method was inferred from the linearity and accuracy data.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The main target of the chromatographic method is to get the separation of ampicillin and its related substances in ampicillin drug substance. All the related substances except the substance 16 were reported earlier in the official monograph of the European Pharmacopoeia[14]. The new substance found in process was isolated using preparative HPLC and characterized using LC-MS and NMR spectrometer. The Mass spectrum and NMR spectrum mentioned in figures 2 and 3 respectively. As per the analytical method reported by Zhu[18] with Hypersil ODS C18 column, the following impurities are merging with each other: ampicilloic acid (5S) and ampicillin (5S), piperazine-2,5-dione; ampilloic acid (5R) and ampicillin (5R) piperazine-2,5-dione; 2-hydroxy-3-phenylpyrazine, diphenylpiperazine-2,5-dione and D-phenylglycylampicillin. Poor selectivity was observed, using other two columns mentioned in the method (Bio-sil C18 and Spherisorb ODS-1) and some of the peaks were found to be merging. The substances 2-19 were spiked with ampicillin drug substance and analysed using European pharmacopoeia method[14]. It was observed that as per the method[14] all the substances were found not to be separated well and some of the impurities were eluted at the same retention times or separated with less resolution. For example substances 4,5 are merging with 6; substances 11 and 13 are merging; substance 12 is merging with 14; 6-APA peak has poor UV response at 254nm. Our efforts towards modification of this method could not achieve better separation of the individual substances (including isomers). But a chromatographic separation was achieved using 0.05M sodium dihydrogen orthophosphate dihydrate (pH 5.0±0.05) solution as mobile phase A and acetonitrile as mobile phase B. Different trials were made with stationary phase C18, Phenyl and C8 to achieve good selectivity towards individual related substances. Finally good selectivity towards all related substances was achieved (Figure 4) by different trials made by changing flow rate (finally fixed at 1.5 ml/min), composition of mobile phase A and B at different time intervals (Gradient program) and C8 stationary phase

Full Paper

(Hypersil BDS-C8). Column oven temperature was maintained at 40°C for good separation and peak shape. At 254 nm 6-APA has low UV response. Ampicillin and all related substances response was good at 220 nm. Gradient composition of mobile phase A and B is mentioned in TABLE 1. The resolution between substances 12 and 13 not less than 1.5, and USP plate count for ampicillin peak not less than 7500 was fixed as system suitability. Peak purity angle is a measure of spectral homogeneity determined from the weighted average of all spectral contrast angles across a peak. Spectral contrast angles are calculated by comparing the spectra from each data point in an integrated peak against the peak apex spectrum. Peak purity threshold is the largest spectral contrast angle that can be due to noise or solvent contribution alone, and not due to an actual component absorbance difference in spectral shape. The purity angle value less than the purity threshold value indicates spectral homogeneity. Peak purity values grater than purity threshold indicate spectral inhomogeneity. The peak purity angle and peak purity threshold were collected from Empower software, Copyright@2002 Waters Corporation, Service pack A-D and ICH guidelines[20]. The retention times and peak purity data for each related substance mentioned in TABLE 2, confirm good selectivity towards the 19 substances. The purity and mass (m/z) values for all related substances were given in TABLE 2.

TABLE 1: Mobile phase A and B linear gradient program at different time intervals

Time (min)	Buffer (pH 5.0) (Mobile phase A)	Acetonitrile (Mobile phase B)
0.01	98%	02%
20.00	90%	10%
40.00	85%	15%
50.00	80%	20%
55.00	75%	25%
60.00	75%	25%
62.00	98%	02%

Results of forced degradation experiments

Degradation was observed for ampicillin drug substance during stress conditions like temperature, oxidation, acid and base degradation and no degradation was observed for photolytic and humidity degradation. The peak purity data of ampicillin in control sample and the sample spiked with all related substances, and the peak purity data of ampicillin in all stress conditions, indicated that the peak was homogeneous and had no

TABLE 2: Summary of experiments (purity, mass, retention times) for each related substance, method validation (Linearity, LOD, LOQ, response factor, peak purity and specificity) and the results of real samples (samples 1,2&3) using chromatographic conditions specified

Subst. number	Purity (%)	Mass (m/z)	R.T (min)	LOD (%w/w)	LOQ (%w/w)	(R.F) Response Factor #	Purity angle	Purity threshold	Slope	y-intercept	Residual Sum of squares	Sample-1 (%w/w)	Sample-2 (%w/w)	Sample-3 (%w/w)
1	99.50	349	11.541	--	--	1.00	0.133	0.320	15665	-139	2056	--	--	--
2	100.0	151	1.636	0.010	0.020	1.00	0.300	5.146	15708	809	2462	BLQ**	0.02	BLQ**
3	99.90	216	2.427	0.022	0.040	1.95	0.381	5.606	8020	1597	2029	ND	BLQ**	ND
4	96.74*	367	4.646	0.026	0.038	1.61	0.540	5.735	9701	-186	3368	0.04	0.04	0.04
5	96.74*	367	6.719	0.026	0.038	1.61	0.431	5.550	9701	-186	3368	0.18	0.18	0.17
6	99.47	349	7.326	0.017	0.024	1.00	0.223	5.191	15741	1948	3993	0.06	0.15	0.06
7	96.75*	323	13.457	0.026	0.035	1.54	0.413	5.519	10166	-1255	3129	0.04	0.05	0.04
8	99.16*	349	14.540	0.027	0.049	1.03	5.619	14.433	15173	-249	2415	ND	ND	ND
9	96.75*	323	16.273	0.026	0.035	1.54	0.679	5.846	10166	-1255	3129	0.06	0.05	0.06
10	99.16*	349	17.208	0.027	0.049	1.03	0.138	5.201	15173	-249	2415	ND	ND	ND
11	97.83	172	20.479	0.013	0.025	0.66	1.229	5.149	23709	-1572	4920	ND	ND	ND
12	99.67	235	21.785	0.019	0.033	1.07	0.251	5.273	14632	1476	4225	0.04	0.06	0.04
13	95.84	266	22.523	0.014	0.024	0.57	0.178	5.172	27439	4212	10785	ND	ND	ND
14	78.58	716	24.374	0.035	0.067	2.10	0.546	5.836	7475	2192	1654	ND	ND	ND
15	96.23	482	25.978	0.010	0.017	0.83	0.074	5.079	18876	-3920	7092	0.17	0.30	0.18
16	94.20	300	29.690	0.043	0.070	2.42	0.618	5.849	6462	618	1510	ND	ND	ND
17	83.22	698	36.688	0.026	0.038	1.17	0.789	5.430	13403	-2765	3337	0.41	0.45	0.39
18	79.74	482	44.959	0.022	0.034	1.00	0.282	5.366	15643	-697	5035	ND	ND	ND
19	79.30	1047	48.898	0.035	0.056	1.95	0.363	5.509	8029	85	1396	ND	ND	ND

Regression equation y=mx+b ('x' is concentration and 'y' is peak response of the substance); # Multiply the R.F of each impurity with the same impurity obtained by direct quantification against ampicillin standard (0.15mg ml $^{-1}$ solution) area to get the result (%w/w); *Total purity of isomers 1& 2. ** - Detected. BLQ- Below Limit of Quantification. ND - Not Detected

TABLE 3: Forced degradation studies conducted for specificity using control sample (0.75mg ml $^{-1}$) solution

Condition	Time	Ampicillin	Degradation (% Area)	Purity angle	Purity threshold
Un-degraded	-	99.45	-	0.106	0.275
Acid degradation (1M HCl/ 85^{0}C)	20min	86.86	12.70	0.066	0.264
Base degradation (0.5M NaOH)	Initial	91.70	7.80	0.058	0.266
Peroxide Pegradation (10% H_2O_2/85^{0}C)	10 min	80.35	19.20	0.049	0.259
Thermal degradation (105^{0}C)	3 hr	81.05	18.50	0.056	0.261
Photolytic degradation (10,000 Lux)	432 hr	99.42	0.00	0.089	0.278
Humidity degradation (92%RH/25^{0}C)	432 hr	99.54	0.00	0.087	0.273

co-eluting peaks, confirming the specificity and stability indication nature of the method. The summary of forced degradation is given in TABLE 3.

Precision

The % RSD for area count of ampicillin standard solution (system precision) was within 2.0%. The % RSD for concentration of each related substance and total related substances in six determinations (Method precision) was within 3.0% and the % RSD for the results obtained by two analysts (intermediate precision) was within 5.0%, confirming good precision of the method.

LOD and LOQ

The % RSD for peak area of each related substance at LOQ and LOD levels were less than 10% and 26% respectively. The LOD and LOQ values of each impurity are given in TABLE 2.

Linearity

Linear calibration plot for related substances was obtained over the calibration ranges tested, i.e. LOQ to 150% of specification level (1.0% w/w). The correlation coefficient was greater than 0.999. The slope, Y-intercept, residual sum of squares and response factor are listed in TABLE 2. The results show that an excellent correlation exists between the peak area and concentration for all the related substances.

Accuracy

The percentage recovery for the substances 2-19 was found to be in the range 92.0 to 108.0. The recovery results at 0.5%, 1.0% and 1.5% w/w concentrations indicate that the test method has an acceptable level of accuracy for the determination of all related substances in ampicillin drug substance.

Robustness

In all the deliberately varied chromatographic conditions, viz., flow, percentage organic strength, column temperature, wavelength, mobile phase pH etc., the resolution between substances 12 and 13 is not less than 1.5, and USP plate count for ampicillin peak is not less than 7500. Selectivity of each substance is also not affected by varied chromatographic conditions. This shows robustness of the method.

Solution stability

The test solution was found to be stable for 2 hours at 25°C, and at least 5 hours at 6°C.

CONCLUSIONS

The RPLC method developed for the determination of ampicillin and its related substance is rapid, precise, accurate and selective. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for assessing the stability of ampicillin as drug substance.

ACKNOWLEDGMENTS

The authors thank the management of APL group for supporting the work and the process research group for providing reference samples. The authors also thank their colleagues in APL R&D Separation Science Division of Analytical Research of Custom Chemical Services for their co-operation in carrying out this work.

REFERENCES

[1] Martindale; 'The complete drug reference', 33rd Ed., 151 **(2002)**.

[2] T.B.Vree, Y.A.Hekster, A.M.Baars, E.Van Der

Full Paper

Kleijn; J.Chromatogr., **145**, 496 **(1978)**.
[3] D.Westerlund, J.Carlqvist, A.Theodorsen; Acta. Pharm.Suecica., **16**,187 **(1979)**.
[4] T.Uno, M.Masada, K.Yamaoka, T.Nakagawa; Chem.Pharm.Bull., **29**, 957 **(1981)**.
[5] K.Miyazaki, K.Ohtani, K.Sunada, T.Arita; J. Chromatogr., **276**, 478 **(1983)**.
[6] M.T.Rosseel, M.G.Bogaert, Y.J.Valcke; Chromato graphia., **27**, 243 **(1989)**.
[7] W.Luo, C.Y.W.Ang, H.C.Thompson Jr; J. Chromatogr., **694**, 401 **(1996)**.
[8] J.Lal, J.K.Paliwal, P.K.Grover, R.C.Gupta; J. Chromatogr., **655**, 142 **(1994)**.
[9] M.Jamil Akhtar, S.Khan, M.A.Salam Khan; J. Pharm.Biomed.Anal., **11**, 375 **(1993)**.
[10] W.A.Moats; J.Chromatogr., **366**, 69 **(1986)**.
[11] K.L.Tyczkowska, R.D.Voyksner, R.F.Straub, A. Aronson; J.Assoc.Off.Anal.Chem., **77**, 1122 **(1994)**.
[12] L.K.Sorensen, L.K.Snor, T.Elkær, H.Hansen; J.Chromatogr., **734**, 307 **(1997)**.
[13] F.Nachtmann, K.Gstrein; Int.J.Pharm., **7**, 55 **(1980)**.
[14] European Pharmacopoeia, 5th Ed., **(2005)**, Conseil de Europe, Strasbourg, 998 **(2004)**.
[15] British Pharmacopoeia, The Stationary office, London, 138 **(2005)**.
[16] USP 28NF 23, 'The United States Pharmacopeia', 28th rev., the National Formulary, 23rd Ed., United States Pharmacopoeial Convention, Inc., Rockville, 152 **(2005)**.
[17] F.Salto; J.Chromatogr., **161**, 379 **(1978)**.
[18] Y.Zhu, E.Roets, Z.Ni, M.L.Moreno, E.Porqueras, J.Hoogmartens; J.Pharm.Biomed.Anal., **14**, 631 **(1996)**.
[19] ICH stability testing of new drug substances and products (Q1AR), International Conference on Harmonization, IFPMA, Geneva, **(2000)**.
[20] ICH Draft Guidelines on Validation of Analytical Procedures: Definitions and Terminology, Federal Register, IFPMA, Switzerland, **60**, **(1995)**.