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Short communication

ICH guidance in practice: Validated stability-indicating HPLC method for simultaneous determination of ampicillin and cloxacillin in combination drug products

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Abstract

Ampicillin and cloxacillin were degraded together under different stress test conditions prescribed by International Conference on Harmonization. The samples so generated were used to develop a stability-indicating high performance liquid chromatographic (HPLC) method for the two drugs. The drugs were well separated from degradation products using a reversed-phase (C-18) column and a mobile phase comprising of acetonitrile:phosphate buffer (pH 5.0), which was delivered initially in the ratio of 15:85 (v/v) for 1 min, then changed to 30:70 (v/v) for next 14 min, and finally equilibrated back to 15:85 (v/v) from 15 to 20 min. Other HPLC parameters were: flow rate, 1 ml/min; detection wavelength, 225 nm; and injection volume, 5 μl. The method was validated for linearity, precision, accuracy, specificity and selectivity. It was also compared with the assay procedures given in British Pharmacopoeia for individual drugs. Similar results were obtained, indicating that the proposed single method allowed selective analysis of both ampicillin and cloxacillin, in the presence of their degradation products formed under a variety of stress conditions. The developed procedure was also applicable to the determination of instability of the drugs in commercial products.

Keywords: Ampicillin; Cloxacillin; Stability; HPLC; Validation

1. Introduction

In recent times, there is an increased tendency towards the development of stability-indicating assays [1–3], using the approach of stress testing as enshrined in the International Conference on Harmonization (ICH) guideline Q1AR(2) [4]. Even this approach is being extended to drug combinations [5,6], to allow accurate and precise quantitation of multiple drugs, their degradation products, and interaction products, if any.

The combination containing ampicillin and cloxacillin shows synergistic effect [7] and is extensively used for the treatment of infections in premature infants and newborns [8]. It is also employed for therapy of resistant infections, and for veterinary purposes [9]. There is no stability-indicating assay method reported yet for this combination, developed using the ICH approach of stress testing. Otherwise, there are several HPLC procedures known for the analysis of ampicillin [10–12] and cloxacillin [13–15] individually, and some methods even exist for simultaneous analysis of the two drugs, either in a veterinary combination [16] or biological fluids [17,18]. Recently, we also developed a derivative spectrophotometric method targeted to selective analysis of ampicillin and cloxacillin in drug combination [19]. The procedure gave acceptable results with fresh products, but gave overestimation during analysis of stability samples and aged products.

Therefore, the focus in the present study was to develop an HPLC stability-indicating method for the combination, by degrading the drugs together under various stress conditions according to ICH. The drugs were separated from degradation products on a reversed-phase HPLC column. The method was compared with the procedures given in British Pharmacopoeia for individual drugs [20,21], and also extended to marketed products. The results are discussed in this paper.

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2. Experimental

2.1. Materials

Pure ampicillin and cloxacillin were obtained as gift samples from Arbro Research and Development Center, New Delhi, India. Combination products containing the two drugs were purchased from local shops. HPLC grade acetonitrile was purchased from J.T. Baker (Mexico City, Mexico). Ultra pure water was obtained from a water purification unit (Elga Ltd., Bucks, England). Buffer materials and all other chemicals were of analytical-reagent grade.

2.2. Equipment

The HPLC system consisted of an on-line degasser (DGU-14A), low-pressure gradient flow control valve (FCV-10AL$_{VP}$), solvent delivery module (LC-10AT$_{VP}$), auto injector (SIL-10AD$_{VP}$), column oven (CTO-10AS$_{VP}$), UV–vis dual-wavelength detector (SPD-10A$_{VP}$), photo-diode array (PDA) detector (SPD-M10A$_{VP}$), system controller (SCL-10A$_{VP}$) and CLASS-VP software, ver. 6.13 (all from Shimadzu, Kyoto, Japan). The separations were achieved on a inertsil ODS2 column (250 mm × 4.6 mm, 5 μm) from Flexit Jour, Pune, India and grace vydac C-18 (250 mm × 4.6 mm, 5 μm) column from Hesperia, CA, USA. The latter was used for intermediate precision studies. A precision water bath equipped with MV controller (Julabo, Seelbach, Germany) was used to carry out selected reactions in solution. Stability studies were carried out in humidity (KBF720, Binder, Germany) and photostability (KBWF240, WTC Binder, Germany) chambers both set at 40 °C ± 1 °C/75% RH ± 3% RH. The photostability chamber was equipped with an illumination bank on inside top consisting of a combination of two black light UV lamps (OSRAM L18W/73) and four white fluorescent lamps (OSRAML 18W/20) in accordance with option two of International Conference on Harmonisation (ICH) guideline [22]. The samples were placed at a distance of 9 in. from the light bank. Both fluorescent and UV lamps were put on simultaneously. Thermal stability study was carried out in dry air oven (NSW Limited, New Delhi, India). Other equipments used were sonicator (Branson Ultra-sonic Corporation, Danbury, CT, USA), analytical balance (Mettler Toledo, Schwerzenbach, Switzerland) and auto pipettes (Eppendorf, Hamburg, Germany).

2.3. Degradation studies

In general, degradation studies were carried out at a concentration of 1 mg/ml of each drug in the solution. For hydrolysis in water, the solution was refluxed for 6 h. The corresponding reactions in 0.1 M HCl, 0.1 M NaOH and 3% H_2O_2 were carried out for 1 h at room temperature. Degradation was also carried out in solid state by exposing pure drugs to dry heat at 80 °C for 24 h, and in dark and photostability chambers for 5 days. Samples were withdrawn periodically and subjected to analysis after suitable dilution.

2.4. Development of method

HPLC studies were carried out on all the reaction solutions individually, and on a mixture of the solutions in which decomposition was observed. The separations were achieved by gradient elution using acetonitrile:phosphate buffer (10 mM, potassium dihydrogen orthophosphate, pH 5.0) as the mobile phase. It was filtered through 0.45 μm nylon filter and degassed before use. The injection volume was 5 μl and mobile phase flow rate was 1 ml/min. The detection was carried out at 225 nm.

2.5. Validation of the method

The method was validated for linearity, precision (inter-day, intra-day and intermediate precision), accuracy, specificity and selectivity. Standard plots were constructed for both ampicillin and cloxacillin in the range of 50–1000 μg/ml. The experiment was repeated thrice on the same day and additionally on two consecutive days to determine intra- and inter-day precision, respectively. The intermediate precision of the method was determined by repeating the experiment on two different columns. Accuracy was determined by fortifying the mixture of degraded solutions with three known concentrations of the drugs. Further, specificity of the method was assessed by study of the resolution factor of the drug peaks from nearest resolving peaks. The selectivity was determined by checking peak purity of all the peaks, including those of degradation products, using a PDA detector.

2.6. Comparison of developed method with the procedures given in British Pharmacopoeia for individual drugs

The developed method was compared with the individual HPLC assay procedures given in British Pharmacopoeia for ampicillin and cloxacillin [20,21]. This was done by analyzing the degraded samples both by the developed method and through compendial procedures, after preparing calibration curves individually for the two drugs.

2.7. Application of the developed method to the marketed FDC formulations containing ampicillin and cloxacillin

The developed method was extended to the analysis of five marketed capsule formulations containing ampicillin and cloxacillin together. The contents of the capsules were dissolved in 250 ml water. The resultant solution was filtered through 0.22 μm nylon and analysed by the developed method.

3. Results and discussion

3.1. Degradation behaviour

HPLC studies on the combination under different stress conditions indicated the following degradation behaviour.

3.1.1. Acidic condition

The combination showed sufficient degradation within 1 h at room temperature in 0.1 M HCl. Cloxacillin showed higher

degradation as compared to ampicillin. The major degradation products formed were at retention times (RTs) 9.3, 9.6 and 9.9 min.

3.1.2. Neutral (water) degradation

Sufficient degradation was observed upon refluxing the combination for 6 h. Similar to acid, cloxacillin showed higher degradation as compared to ampicillin. The degradation products appeared at RTs 3.1, 9.6, 10.6 and 11.3 min.

3.1.3. Degradation in alkali

Both the drugs were found to be highly labile to alkaline hydrolysis in 0.1 M NaOH at room temperature and most of the drug decomposed within 1 h. The major products appeared at RTs 3.1, 9.6 and 9.9 min.

3.1.4. Oxidative degradation

The drugs showed sufficient degradation when the combination was degraded in 3% H_2O_2 for 1 h. The three major degradation products appeared at 3.1, 9.9 and 10.6 min.

3.1.5. Solid state studies

Solid state studies showed that the combination was unstable in dark, light as well as thermal conditions. Sufficient degradation was observed in accelerated conditions in dark and light after 5 days. The degradation in light was more as compared to dark conditions. On the other hand, enough degradation was observed when the combination was exposed to dry heat at 80 °C for 24 h. Cloxacillin showed higher degradation in all the cases. The major degradation products resolved at 9.3, 10.6 and 11.0 min.

3.2. Development and optimization of the stability-indicating HPLC method

A gradient method was found necessary to optimize the separation of major degradation products formed under various stress conditions. The best resolution was achieved with initial run of acetonitrile: phosphate buffer (pH 5.0) in the ratio of 15:85 (v/v) for 1 min, which was then changed to 30:70 (v/v) for next 14 min and finally equilibrated back to the same ratio of 15:85 (v/v) from 15 to 20 min. The method worked well with the mixture of degradation solutions and was even applicable to degraded formulations. Fig. 1a–c shows the chromatographic resolution of the blank, mixture of stressed samples, and a degraded formulation, respectively.

3.3. Validation of the developed stability-indicating method

The linearity could be established for both the drugs in the concentration range of 50–1000 μg/ml (see Table 1). Table 2 lists the relative standard deviation (R.S.D.) data obtained on analysis of the samples on the same day ($n = 3$) and on consecutive days ($n = 3$). As evident, the R.S.D. values were <1% and <2% for intra- and inter-day studies, respectively, demonstrating that the method was sufficiently precise. Even intermediate precision was established for the method, as almost similar resolution was

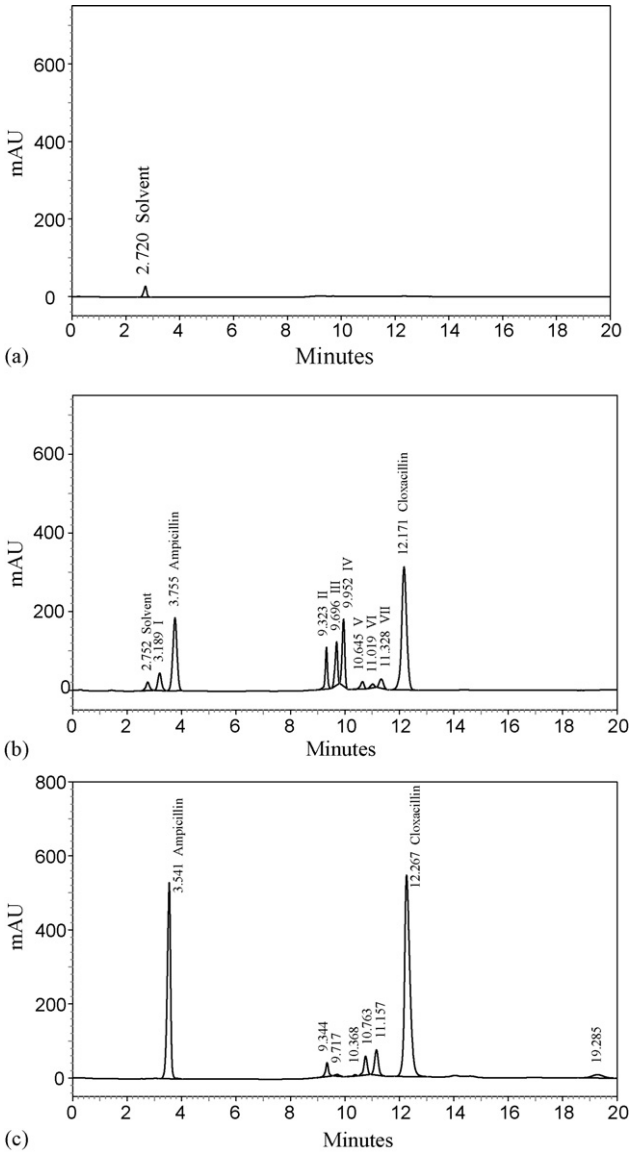

Fig. 1. Chromatograms showing resolution of components in blank (a), mixture of stress samples (b), and degraded formulation (c). Key—I: formed in H_2O_2, H_2O and NaOH; II: formed in light, dark, thermal conditions and HCl; III: formed in NaOH, HCl and H_2O; IV: formed in NaOH, HCl, H_2O_2; V: formed in light, dark, thermal conditions, H_2O_2 and H_2O; VI: formed in light, dark and thermal conditions; VII: formed in H_2O only.

observed on repeating the experiment on two different reversed-phase HPLC columns (Table 3). Table 4 shows that recovery of the added drug, obtained from the difference between peak areas of unfortified samples and fortified samples, was satisfactory at all the tested concentrations. As shown in Fig. 1b, the method had sufficient specificity and selectivity as the two drugs and

Table 1
Linearity data for ampicillin and cloxacillin ($n = 3$)

Drug	Range (μg/ml)	Regression parameters	
		Equation of regression line	R^2 value
Ampicillin	50–1000	$y = 3722.1x + 11076$	0.9999
Cloxacillin	50–1000	$y = 10849x + 13715$	0.9999

Table 2
Intra-day and inter-day precision studies ($n = 3$)

Drug	Added (μg/ml)	Intra-day precision	Inter-day precision
		Found ± S.D. (μg/ml), R.S.D. (%)	Found ± S.D. (μg/ml), R.S.D. (%)
Ampicillin	200	204.64 ± 0.24, 0.11	207.79 ± 2.01, 0.96
	400	402.37 ± 0.15, 0.03	405.29 ± 0.32, 0.08
	600	602.11 ± 3.52, 0.58	600.54 ± 2.37, 0.39
	800	791.79 ± 7.17, 0.90	792.31 ± 2.08, 0.26
	1000	1003.09 ± 2.45, 0.24	1002.58 ± 8.76, 0.87
Cloxacillin	200	202.35 ± 0.37, 0.18	202.57 ± 2.76, 1.36
	400	400.15 ± 1.25, 0.31	403.64 ± 4.85, 1.20
	600	602.42 ± 3.51, 0.58	600.09 ± 5.03, 0.83
	800	792.91 ± 7.38, 0.93	786.60 ± 5.03, 0.63
	1000	1003.56 ± 2.56, 0.25	994.57 ± 1.86, 0.18

Table 3
Intermediate precision studies

Column	Retention time (min)	
	Ampicillin	Cloxacillin
Inertsil-C18	~3.60	~12.20
Grace-vydac-C18	~4.00	~12.50

Table 4
Recovery studies of ampicillin and cloxacillin

Drug	Added concentration (μg/ml)	Measured concentration (μg/ml)	% recovery	Mean % recovery
Ampicillin	50	50.25	100.51	100.78 ± 0.42%
	100	100.56	100.56	
	200	202.54	101.27	
Cloxacillin	50	49.97	99.95	100.40 ± 0.39%
	100	100.52	100.52	
	200	201.45	100.72	

even degradation products were well separated from each other, with the resolution factor of >2 in all cases. All the peaks were pure, which was proved through PDA purity studies. Data of peak purity index and single point threshold values are listed in Table 5.

Table 5
PDA peak purity parameters for ampicillin, cloxacillin and the degradation products formed under various stress conditions

Drug/degradation products	Peak purity index	Single point threshold
I	0.999986	0.999115
Ampicillin	0.999999	0.999897
II	0.999991	0.999289
III	0.999987	0.999123
IV	1.000000	0.999883
V	0.999671	0.979359
VI	0.999578	0.958321
VII	0.999851	0.988022
Cloxacillin	1.000000	0.999940

Key: I–VII, same as that of Fig. 1.

Table 6
Analysis of a degraded sample by the developed method, and the individual procedures given in British Pharmacopoeia [19,20] for the two drugs

Drugs	Quantity of drug (mg) ± S.D.	
	Developed method	BP method
Ampicillin	99.28 ± 1.33	100.22 ± 0.48
Cloxacillin	91.22 ± 0.91	90.80 ± 0.46

Table 7
Analysis of formulations containing ampicillin and cloxacillin in combination ($n = 3$)

Formulation	% label claim	
	Ampicillin	Cloxacillin
BL-1	90.52	86.21
BL-2	99.22	86.87
ST-1	102.43	98.03
ST-2	103.31	95.88
ST-3	103.08	100.26

Key: BL: blister pack containing 250 mg each of ampicillin and cloxacillin, ST: strip pack containing 250 mg each of ampicillin and cloxacillin.

3.4. Comparison of the developed method with procedures given in BP for individual drugs

Table 6 gives the comparison of the results obtained on analysis of a degraded sample containing ampicillin and cloxacillin by the developed and pharmacopoeial methods. As evident, the results were comparable.

3.5. Application of the developed method to marketed FDC formulations containing ampicillin and cloxacillin

The developed method was used to analyze marketed formulations containing the two drugs. A clear resolution of the drugs and degradation products was achieved even for all formulations tested, with no interference from excipients. In almost all the cases, chromatographic pattern was similar to the one shown in Fig. 1c. This indicated that the method could be extended for the study of available drug content in commercial products. The data in Table 7 indicates that overall cloxacillin was more prone to degradation, as the assay was less than the label claim in four out of five formulations. In comparison, ampicillin was less affected.

4. Conclusion

This study presents a simple and validated stability-indicating HPLC method for simultaneous estimation of ampicillin and cloxacillin in the presence of degradation products. The method yielded results similar to those determined by application of compendial procedures on individual drugs. The method could be applied with success even to the analysis of marketed products, as no interference was observed due to excipients or other components present.

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