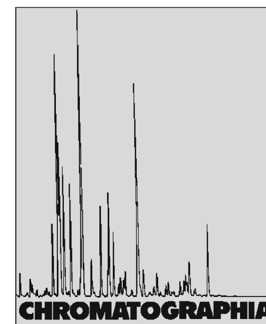
RP-LC Simultaneous Determination of Nebivolol Hydrochloride and Amlodipine Besilate in Bi-Layer Tablets



2009, *70*, 557–561

Sneha J. Joshi[1,2,✉], Pradnya A. Karbhari[1,2], Suvarna I. Bhoir[2]

[1] Ipca Laboratories Ltd, 48, International House, Kandivali Industrial Estate, Kandivali-West, Mumbai 400 067, India;
E-Mail: sneha_joshi@vsnl.net
[2] C.B. Patel Research Centre for Chemistry and Biological Sciences, 3rd Floor, Bhaidas Hall, Vile Parle West, Mumbai 400 056, India

Received: 2 December 2008 / Revised: 21 April 2009 / Accepted: 22 April 2009
Online publication: 18 June 2009

Abstract

A simple and rapid LC method was developed and validated for simultaneous estimation of nebivolol and amlodipine in a bi-layer tablet formulation. Efficient chromatographic separation was achieved on (USP L10) Hypersil BDS cyano, 5 μm, 250 mm × 4.6 mm column with simple mobile phase composition delivered in isocratic mode. The method had requisite accuracy, selectivity, sensitivity, robustness and precision to assay nebivolol and amlodipine in pharmaceutical dosage form. Degradation products resulting from the stress studies did not interfere with the detection of nebivolol and amlodipine, these peaks remained pure and thus proved to be stability indicating. The mass balance of the stressed sample was in the range 99.0–100.2% for amlodipine and 99.3–100.3% for nebivolol.

Keywords

Column liquid chromatography
Stability indicating study
Nebivolol hydrochloride
S-Amlodipine besilate

Introduction

Nebivolol hydrochloride (NH) is a third generation antihypertensive drug, a very specific β_1-blocker which can be characterized by having nitric oxide (NO) potentiating vasodilatory effect [1–3].

Amlodipine besilate is prescribed for the management of angina and hypertension. It is used therapeutically as a racemic mixture composed of *S* and *R* enantiomers but its Ca channel blocking effect is confined to *S*-amlodipine besilate (AB) which has a 1,000 times higher activity than the *R* form [4].

A combination containing racemate amlodipine besilate and nebivolol hydrochloride is available in the market in strength of 5 mg each. For the present study bi-layer tablet dosage form was chosen which contained *S*-amlodipine besilate and nebivolol hydrochloride with a strength of 2.5 and 5 mg respectively.

Tablet dosage forms containing AB, NH as single or in combination with other drugs are available in the local market for effective therapy. NH is not official in pharmacopeia. Literature survey revealed several analytical methods such as spectrophotometery, simple and stability indicating TLC, LC, HPTLC, LC-MS that have been reported for determination of NH [7–12] and AB [13–16] in pharmaceutical dosage forms and biological samples alone or in combination with other drugs. However no report is available on a simultaneous assay, forced degradation study of AB and NH. The method [8] reported a broad peak shape of NH, but did not give the necessary separation among NH and AB. Also, in the reported LC method for AB, NH eluted as poor shaped peak. Hence, objective of the present study is to develop and validate a stability indicating assay method for quantification of the drugs in combination. Stability indicating assay method [17] was conducted under the conditions of hydrolysis, oxidation, thermal and photolysis in accordance with ICH guidelines.

DOI: 10.1365/s10337-009-1165-1
0009-5893/09/08
© 2009 Vieweg + Teubner | GWV Fachverlag GmbH

Experimental

Materials and Reagents

Working standard of AB and NH were supplied by Ipca Laboratories, Mumbai, India. All reagents sodium hydroxide pellets, hydrochloric acid, potassium dihydrogen phosphate, hydrogen peroxide, acetonitrile and methanol were procured from Merck (Darmstadt Schuchardt, Germany). Ultra pure water obtained from a Millipore purification unit was used. NEBICARD SM manufactured by Torrent Pharmaceuticals (Ahmedabad, India) bi-layer tablets for oral administration (2.5 and 5 mg per tablet AB equivalent to *S*-amlodipine and NH equivalent to Nebivolol respectively) were used throughout the experiment.

Instrument and Equipment

Water bath (thermo constant temperature) was used for solution degradation. Dry oven used for solid state thermal stress studies and stability walk in chamber for stability studies were from Newtronic (Mumbai, India). Photo stability studies were performed in a photostability chamber (Newtronic, Tuttlingen, Germany) equipped with an illumination of two UV and four fluorescent lamps providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 W h m^{-2} in accordance to Q1B Option 2 [18].

The LC system comprised of Waters Alliance LC System with precision pump (Waters 2695 separation module) a photodiode array detector (Waters 2998), auto injector (Waters 2695 separation module) and Waters Empower software Version 2.6. The chromatographic columns used were: (1) Hypersil BDS cyano (250 mm × 4.6 mm, 5 µm) Thermo Electron Corporation, Runcorn, UK, and ii) Zorbax cyano (250 mm × 4.6 mm, 5 µm) Agilent, USA.

Chromatographic Conditions

Mobile phase used was methanol, acetonitrile and 50 mM potassium dihydrogen phosphate buffer (pH adjusted to 6.0 with triethylamine) in a ratio of 10: 45: 45 v/v. The flow rate was 1 mL min^{-1} and wavelength 220 nm. The mobile phase was filtered through a 0.45 µm membrane filter, degassed by vacuum and ultrasonication.

Preparation of Standard Solution

A working standard solution (100 µg mL^{-1} nebivolol and 50 µg mL^{-1} amlodipine) was prepared in a degassed 60 : 40 (v/v) mixture of water and acetonitrile (this solvent is subsequently referred to as 'diluent') for assay determination.

Preparation of Sample Solution

Ten whole bi-layer tablets were transferred to a 500 mL volumetric flask, 100 mL of diluent was added and tablets were dispersed by shaking and dissolved by sonication for 10 min. The sample was then diluted to 500 mL with diluent. The resulting solution was filtered by Whatmann filter paper GFC and used for analysis.

Method Development

A variety of mobile phases were investigated in the development of an RP-LC suitable for simultaneous analysis of AB and NH. The suitability of mobile phase was decided on the basis of selectivity and sensitivity for the assay, suitability for stability studies, separation among impurities formed during forced degradation studies on tablet, individual and mixture of drug substance.

Validation of Developed Method

Validation was as per ICH Q2 (R1) [19] for parameters specificity, linearity and range, accuracy, precision, filter paper evaluation, stability of the drug in solution, LOD and LOQ and robustness.

Results and Discussion

Development and Optimization of Method

Literature survey revealed pK_a values of AB and NH to be 8.6 and 8.22 respectively [20]. Considering the fact that the pK_a value is on the basic side, method development attempts were focused using acidic mobile phase.

Initial attempts with C8, C18 stationary phases using acidic mobile phase showed long and broad eluting NH peaks. Peak modifier such as triethyl amine was therefore introduced in the mobile phase to minimize tailing. Among the different mobile phases investigated the mobile phase comprising of acetonitrile : 50 mM phosphate buffer (pH adjusted to 6.0 with triethylamine) in ratio 50:50 v/v using 250 mm × 4.6 mm, 5 µm C18 column gave the necessary separation but showed high retention and asymmetry of 2.1 for the NH peak. To decrease the retention time and to improve peak shape a cyano column was introduced, as it had less hydrophobic and reduced silanol analyte interactions with basic compounds in RP-LC.

Forced degradation studies conducted using these chromatographic conditions showed poor resolution between oxidative degradant of AB and AB peak. To achieve the necessary resolution between oxidative degradant and AB peak supplementary polar solvent methanol was added. Optimum mobile phase thus developed was methanol : acetonitrile : 50 mM phosphate buffer (pH adjusted to 6.0 with triethylamine) 10 : 45 : 45 v/v. The phase gave good separation of AB ($t_R = 8.9$ min) and NH ($t_R = 13.4$ min), with clear baseline separation in presence of their degradation products at a flow rate of 1 mL min^{-1} and wavelength set at 220 nm. Injection volume 20 µL for column and LC system was found to be the best for analysis. Mixture of water : acetonitrile, 60: 40 (v/v) was confirmed for use as diluent as it could easily extract both the analyte from the whole bilayer tablets with shaking and sonication for 10 min.

The final decision of chromatographic conditions was based on suitability of mobile phase, sensitivity to assay, suitability for stability studies, time required for analysis, ease of preparation and readily available cost effective solvents.

Validation of Proposed Method

Specificity

Specificity of a method is the extent to which it can be used for analysis of a particular analyte in a mixture or matrix without interference from other components. In this method specificity was tested by analysis of solutions containing degradation products produced in forced degradation studies. A mixture of AB and NH and separate pure samples of the drugs were subjected to different stress conditions acidic and basic, oxidation, thermal, photo induced degradation. The resulting solutions were appropriately diluted with diluent and injected for LC analysis. Acidic and basic hydrolysis was performed at 75 °C for 60 min on tablets, active pharmaceutical ingredient individual and in combination. The sample showed nominal degradation of 3–10% in acid hydrolysis using 0.5 M hydrochloric acid and showed degradation of 10–50% within 20 min in basic hydrolysis using 0.5 M sodium hydroxide solution. These samples showed formation of degradant impurities of AB at RRT = 0.25, 0.36, 0.61 and that of NH at RRT = 0.43, 0.45, 0.49, 0.84, (all RRT calculated with respect to NH peak) (Fig. 1g). Photolytic degradation and dry heat induced degradation showed decrease in the assay of AB and NH. These samples resulted in formation of degradant impurities of AB at RRT = 0.62 and those of NH at RRT = 0.44, 0.48. The peaks due to analyte however confirmed the peak purity test. Oxidative stress was carried out by treating the sample with 3.0% (*v*/*v*) hydrogen peroxide for 3 h with heating at 60 °C. This resulted in unknown degradation products of AB at RRT = 0.38, 0.42, 0.44, 0.48, 0.62 and those of NH at RRT = 0.44, 0.48

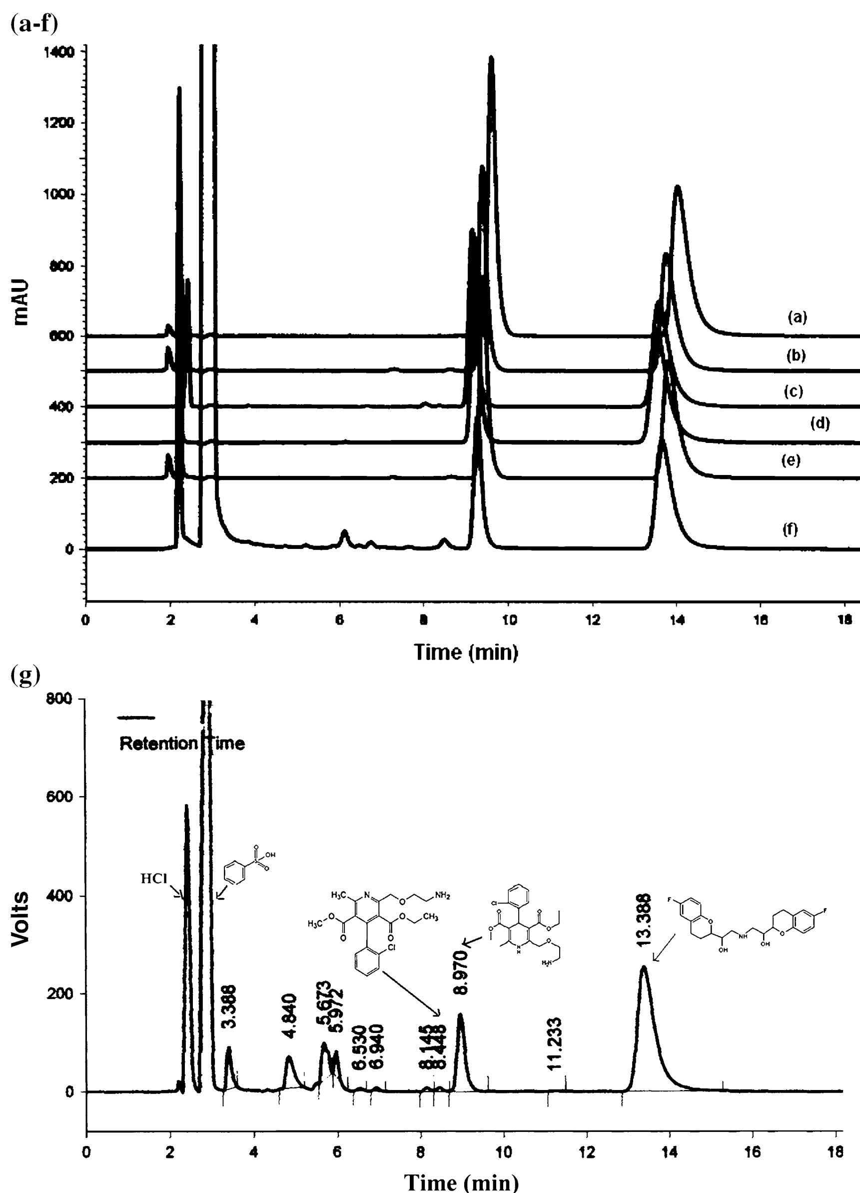


Fig. 1. Chromatograms of (**a**) standard (**b**) marketed sample (**c**) sample exposed to photostability (**d**) sample exposed to thermal degradation (**e**) sample exposed to acid hydrolysis (**f**) sample exposed to oxidation (**g**) base degraded sample at 20 min

(Fig. 1f). Oxidative stressing concluded that AB was sensitive to oxidation. PDA data showed the purity factors were within the threshold limit 1.000 in the range of 200–800 nm for all the stressed samples, hence peak purity test results confirmed that the AB and NH peaks from all the stressed samples were homogenous and pure.

The mass balance of the stressed sample was in the range 99.0–100.2% for AB and 99.3–100.3% for NH (Table 1). Assay of AB and NH were unaffected in presence of degradant products, confirming stability indicating nature of the method. The mass balance is a process of adding the assay values and the amounts of degradation products to see how closely the total matches 100% of the initial value, with due consideration of marginal error [21].

Calibration Plots

A linear calibration plot was obtained for the assay method over the calibration range tested, by suitably diluting a stock solution to concentration range of amlo-

Table 1. Results from forced degradation study

Stress conditions	Time	(%) Assay		% Total impurities		Mass balance (% assay + % total impurities)	
		AB	NH	AB	NH	AB	NH
Base/0.5 N NaOH/75 °C	20 min	51.6	91.7	49.4	9.5	100.0	100.2
Acid/0.5 N HCl/75 °C	90 min	88.0	97.3	11.9	3.0	99.9	100.3
Oxidation/3% H_2O_2/60 °C	60 min	67.4	97.7	32.8	1.6	100.2	99.3
Oxidation/3% H_2O_2/60 °C	180 min	45.1	94.1	54.1	5.8	99.2	99.9
Thermal degradation/100 °C	1 week	99.1	100.2	0.5	–	99.6	100.2
Photolytic degradation	12 days	98.5	96.0	0.5	3.4	99.0	99.4

Table 2. Summarized validation parameters and study of commercial tablets using proposed RP-LC method

Parameters	AB	NH
Retention time (min)	8.9	13.4
Tailing factor	1.1	1.6
Resolution (USP)	–	8.0
Theoretical plates	10,765	5,119
Linearity range (μg mL^{-1})	5–100	10–200
LOD (μg mL^{-1})	0.025	0.01
LOQ (μg mL^{-1})	0.06	0.05
Regression equation ($y^* = a + bc$)		
Slope (b)	103,013.8	38,084.6
Intercept (a)	−1,025.9	+6,448.9
Correlation coefficient (r)	0.99999	0.99998
Residual sum of squares (r^2)	0.99998	0.99996
Method precision		
Mean % assay	100.1	99.9
(CV, %) ($n = 6$)	0.6	1.1
Ruggedness mean % assay (CV, %) ($n = 12$)	0.9	1.3
Accuracy/recovery		
at 80% level	100.6	99.6
at 100% level	99.9	99.4
at 120% level	99.0	98.9
Robustness parameters		
Change in pH		
(a) 5.8	(a) 99.8	(a) 99.2
(b) 6.2	(b) 99.4	(b) 99.9
Change in column (Zorbax Cyano)	99.9	100.1
Change in methanol composition		
(a) 0. 8% *v*/*v*	(a) 100.4	(a) 99.6
(b) 0.12% *v*/*v*	(b) 100.8	(b) 99.4

$y^* = a + bc$, where c is the concentration

dipine (5–100 μg $mL^{-1)}$ and nebivolol (10–200 μg mL^{-1}) in triplicate. High values of correlation coefficients (r^2) 0.9999 for AB and 0.9998 for NH, confirmed excellent correlation between peak area and concentrations of the analyte.

Accuracy

Accuracy as recovery was determined by spiking the marketed sample with standard at 80, 100, 120% and analyzed by the proposed method. The experiment was performed in triplicate and values of recovery (%) are listed in Table 2.

Precision

The drug product was assayed by the proposed method six times on the same day and on different days by different analysts on different systems. % RSD values for the intra- and inter-day were less than 2, confirming high precision of method (Table 2).

Filter Paper Evaluation

Sample after assay preparation was filtered through Whatmann filter paper 41, 42, 1, GFC and 0.45 μm PTFE syringe filter to check compatibility with the sample solution. % assay results conclude that these filters were suitable for filtration of sample.

Drug Stability in Solution

Sample and standard solution were stored at room temperature for 24 h. These solutions were assessed for stability by injecting at specific time intervals. Results showed an increase in AB impurity at RRT = 0.62 almost after 22 h of preparation, indicating solutions to be stable for 20 h.

The Limit of Detection and Quantitation

LOD and LOQ represent the concentration of the analyte that would yield S/N of 3 and 10 respectively, by injecting series of dilute solutions of known concentration. The LOD for amlodipine was achieved at 0.025 μg mL^{-1} and for nebivolol at 0.01 μg mL^{-1}. The LOQ for amlodipine was achieved at 0.06 μg mL^{-1} and for nebivolol at 0.05 μg mL^{-1}.

Robustness

Robustness of the proposed method was assessed with respect to the effect of small but deliberate variation in chromatographic conditions such as column

Table 3. Result of market bilayer formulation kept for stability study

Duration	Market formulation					
	40 °C/75% RH		30 °C/65% RH		25 °C/60% RH	
	NH	AB	NH	AB	NH	AB
Initial control	100.1	99.9	100.1	99.9	100.1	99.9
1 month	99.7	98.9	99.4	99.2	99.9	99.4
2 month	99.1	98.2	99.7	98.8	98.9	99.1
3 month	98.5	97.9	99.2	98.1	98.3	98.7

change (different supplier), pH of buffer (±0.2), percentage of methanol (8 and 12 *v*/*v*) and flow rate (±0.2 mL min^{-1}). No significant change was seen in % assay of AB and NH in tablets with these changes, results under these variations are listed in Table 2. The results confirm robustness of the method.

Application of the Method to Stability Testing

Accelerated stability studies are performed to establish the effect of storage and to show stability indicating nature of the analytical method. The tablet samples stored under long term conditions of 30 ± 2 °C and relative humidity of 65 ± 5% and under accelerated storage conditions of 55 ± 2 °C, 40 ± 2 °C and relative humidity of 75 ± 5% were analyzed by the method at intervals of 1, 2 and 3 months. The results clearly indicated that the drugs were stable under these conditions confirming the method was stability indicating (Table 3).

Conclusion

A simple, economical, accurate, specific, sensitive, precise, robust and validated RP-LC has been described in this paper. The combination of drugs AB and NH under various stress conditions were studied and the results presented. All degradation products formed during the application of stress conditions are well separated indicating the method to be stability indicating and suitable for quality control of pharmaceutical preparations containing NH and AB.

Acknowledgment

The authors wish to thank Dr. Jadhav, Head of the Analytical Development Laboratory and entire team at Ipca Laboratories Ltd and C. B. Patel Research centre for their encouragement and support during the work.

References

1. Bowman AJ, Chen CP, Ford GA (1994) Br J Clin Pharmacol 38(3):199–204
2. Agabiti Rosei E, Rizzoni D (2007) Drugs 67(8):1097–1107. doi:10.2165/00003495-200767080-00001
3. Weiss R (2006) Vasc Health Risk Manag 2(3):303–308. doi:10.2147/vhrm.2006.2.3.303
4. Ph MD, Ji-Young D, Ph P, Kyoung-Ah Kim D, Ph MD, Pil-Whan Park D, Ock-Je Lee BS, Jong Hyeon Ryu MS, Geun Hyeog Lee MS, Mun Choun Ha MS, Jin Sun Kim MS, Seoung Woo Kang MS, Ph MD, Kyung Ryul Lee D (2006) Clin Ther 11(28):1837–1847
5. British Pharmacopeia 2009
6. United States Pharmacopeia (2009) 32 NF27, Rockville, MD
7. Shah DA, Bhatt KK, Mehta RS, Baldania SL, Gandhi TR (2008) J AOAC Int 5(91):1075–1082
8. Kachhadia PK, Doshi AS, Joshi HS (2008) J AOAC Int 91(3):557–561
9. Kamila MM, Mondal N, Ghosh LK, Gupta BK (2007) Pharmazie 62(7):486–487
10. Raja RK, Sankar GG, Rao AL, Raju DB, Rao JV, Seshagiri LN (2005) Asian J Chem 17:1259–1263
11. Reddy TS, Devi PS (2007) J Planar Chromatogr Mod TLC 20:149–152
12. Ramakrishna NVS, Vishwottam KN, Koteshwara M, Manoj S, Santosh M, Varma DP (2005) J Pharm Biomed Anal 39:1006–1013. doi:10.1016/j.jpba.2005.05.021
13. Gohil K, Trivedi P, Molvi KI (2005) Indian J Pharm Sci 67:376–378
14. Rao JR, Kadam SS, Mahadik KR (2002) Indian Drugs 39:378
15. Gowri N, Vaidhyalingam V, Shantha A (2002) Indian Drugs 39:532
16. Avadhanulu AB, Srinivas JS, Anjaneyulu A (1996) Indian Drugs 33:36
17. Trissel LA (1983) Am J Hosp Pharm 40:1159–1160
18. ICH (1996) In: International conference on harmonization of technical requirements for registration of pharmaceuticals for human use, stability testing: photostability testing of new drug substances and products Q1 B
19. ICH (2005) In: International conference on harmonization of technical requirements for registration of pharmaceuticals for human use, validation of analytical procedures: text and methodology Q2 (R1), Switzerland
20. Moffat AC, Osselton MD, Widdop B (2004) Clarke's analysis of drugs and poisons, 3rd edn. Pharmaceutical Press, London
21. Bakshi M, Singh S (2002) J Pharm Biomed Anal 28:1011–1040. doi:10.1016/S0731-7085(02)00047-X