International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 3, Issue 1, 2011

Research Article

DEVELOPMENT AND VALIDATION OF A NEW STABILITY INDICATING ANALYTICAL METHOD FOR THE DETERMINATION OF RELATED COMPONENTS OF BRIMONIDINE TARTRATE IN DRUG SUBSTANCES AND DRUG PRODUCT USING UPLC

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Received: 11 Oct 2010, Revised and Accepted: 11 Nov 2010

ABSTRACT

A novel stability-indicating Ultra high-performance liquid chromatography (UPLC) method has been developed and validated for the quantitative determination of potential impurities in Brimonidine tartrate drug substance and drug product.

A simple reverse phase MS compatible assay method has a unique advantage over the other methods. The output from UPLC system is directly applied to hyphenate mass spectrometry (MS). The elution pattern of impurities in UV detector is correlated with the TIC (Total ion current) of Mass spectrometry detector, which enables rapidly identification of impurities. Chromatographic separation of impurities achieved on the C18 column. The variable mixture of aqueous buffer containing a mixture of 20mm ammonium acetate pH 6.0 ± 0.05 (adjusted with acetic acid) and Methanol with Acetonitrile as a mobile phase delivered at a flow rate of 0.3 ml/min and the detection was carried out using UV detector at wavelength 247 nm. The chromatographic resolution between Brimonidine and its potential impurities was found to be greater than 2. The responses was determined and (correlation coefficient) regression r values were obtained greater than 0.998 for all known related components and Brimonidine.The method was capable of detecting all known impurities at a level of 0.003 % with respect to 0.400 mg/ml sample concentration with injection volume of 1.5µL. The Brimonidine tartrate samples were treated for thermal, acid, base and oxidative stress conditions as per ICH and peak purity was checked at each conditions. The drug was subjected to stress conditions as prescribed by the ICH. Degradation was found to occur slightly under oxidative stress conditions but the drug was stable to aqueous, acidic, and basic hydrolysis, and photolytic and thermal stress conditions. The inter and intraday precision for all known impurities were found to be within acceptable limits. The test solution was found to be stable upto 24 hrs.

Keywords: Brimonidine tartrate; Degradation products; Stability indicating; UPLC

INTRODUCTION

Brimonidine tartrate (5-bromo-6-(2-imidazolidinylideneamino) quinoxaline L-tartrate) Fig.1 is a α_2-adrenoreceptor agonist. It is used to lower intra-ocular pressure in patients with open-angle glaucoma or ocular hypertension Brimonidine tartrate ophthalmic solution is indicated for lowering intraocular pressure in patients with open-angle glaucoma or ocular hypertension. The IOP lowering efficacy of Brimonidine tartrate ophthalmic solution diminishes over time in some patients. This loss of effect appears with a variable time of onset each patient and should be closely monitored[1-2].

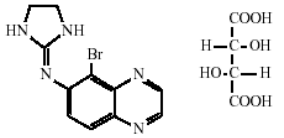


Fig. 1 Chemical structure of 5-bromo-6-(2-imidazolidinylideneamino) quinoxaline L-tartrate

All analytical methods found in literature are described with complex buffers. Separation of three related impurities of Brimonidine was reported with ion pair buffer by reverse phase HPLC[3]. A gas chromatographic-mass spectrometric assay method was reported for analysis of Brimonidine tartrate in human plasma[4]. This report describes the development and validation of related impurities and major degradation products in bulk drug and drug product with volatile buffer and MS compatible method.

MATERIALS AND METHODS

Instrumentation

An Ultra High performance liquid chromatography (UPLC) system consisted of Waters Acquity with PDA detector and data-handling system Empower Pro, Version 6.10.00.00, and HPLC Data acquisition was performed using Waters alliance chromatography 2695 separation module software package. Version 1.2., TQD Tandem Quadrupole UPLC/MS/MS instrument with Empower 2 Software from waters corporation Milford, USA, All pH measurements were performed on a pH meter (Metrohm, model 654 Herisau)

Materials and reagents

Brimonidine tartrate and all related impurities (Fig. 2) were synthesized in Chemical Research department of Wockhardt Research Center, Aurangabad, India. Obtained as a gift HPLC grade solvents Acetonitrile, Methanol was purchased from Merck-Specialties private Ltd., India. Gradient grade Ammonium acetate was purchased from Merck KgaA Germany. Acetic Acid GR grade were purchased from Merck-Specialties private Ltd India. Water was deionised and further purified by means of a Milli-Q Plus water purification system, Millipore Ltd (U.S.A)

Chromatographic conditions and measurement procedure

Chromatographic separation was achieved on a reversed phase hypersil Gold column (100×2.1 mm i.d., 1.9µm particle size.) Thermo scientific. USA. A flow rate of 0.30 ml/min of UPLC analysis was conducted at 40°C column oven temperature and sample cooler temperature was 10°C. The mobile phase consists of about 1.54g of ammonium acetate dissolved in 1000mL of water. Adjust pH 6.0 ± 0.05 with acetic acid. Filter through 0.45µ (or finer porosity) membrane filter.

Gradient system

Time (Min.)	Mobile phase A %	Mobile phase B%
0.0	98	2
2.0	95	5
6.0	85	15
9.0	70	30
10.0	65	35
11.0	98	2
13.0	98	2

Preparation of Mobile Phase-A: Prepare a mixed and degassed solution of buffer and methanol in the ratio of 90: 10 (v/v). Preparation of Mobile Phase-B: Acetonitrile.

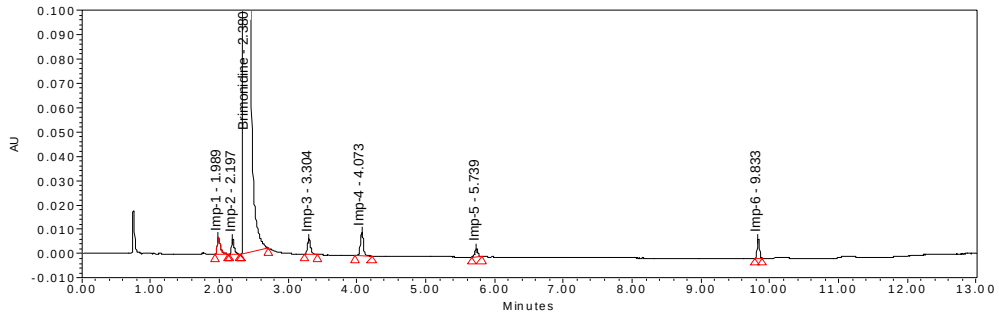


Fig. 2: Typical chromatogram obtained from: brimonidine tartrate spiked with the known related compound

Imp-1 N-(4,5-dihydro-1H-imidazol-2-yl)quinoxalin-6-amine, Imp-2 5-chloro-6-(2-imidazolin-2-ylamino)quinoxaline, Imp-3 1-(2-aminoethyl)-3-(5-bromoquinoxalin-6-yl)thiourea, Imp-4 1-(5-bromoquinoxalin-6-yl)thiourea, Imp-5 5-bromoquinoxalin-6-amine, Imp-6 1,1'-(ethane-1,2-diyl)bis(3-(5-bromoquinoxaline-6-yl)thiourea

Preparation of solutions

Standard solution

A stock of Brimonidine tartrate working standard solution was prepared in diluent (Mixture of methanol and 20mM ammonium acetate pH 7.5 with ammonia solution, 60:40 v/v) and diluted stepwise to get a solution concentration of 4.0 µg/ml filtered through 0.45 µm PTFE membrane filter.

System suitability solution

Brimonidine tartrate working standard spiked with Imp-1 a closely eluting peak was prepared at 0.15% level and injected throughout the study to check the system performance. The resolution between Imp-1 and Brimonidine peak should be not less than 2.0

Sample preparation solution

Contents of not less than three sample bottles of Brimonidine tartrate ophthalmic solution was mixed and transferred accurately 4.0 ml of pooled sample into 25 ml volumetric flask add 5ml of 3% aqueous boric acid solution and mixed well to form a precipitate add 1ml of 1N sodium hydroxide mixed and allow to settled down the precipitates, dilute 5.0 ml of supernatant clear solution to 10 ml volumetric flask and volume made up to the mark with diluent (Mixture of methanol and 20mM ammonium acetate pH 7.5 with ammonia solution , 60:40 v/v). The resultant solution obtained was 400 µg/ml filtered through 0.45 µm PTFE membrane filter.

Method validation

The method was validated for system suitability specificity, linearity, range, precision, accuracy, sensitivity and robustness asper ICH guidance[5].

System suitability

A system suitability test was defined based on the result obtained in several representative chromatograms. The column efficiency determined from the analyte peak more than 7000,the tailing factor not more than 2.0 and the R.S.D for six replicate injection of standard solution not more than 2.0%. The results are recorded in Table-5.

Specificity

The Specificity[5,7] is the ability for the method to measure the analyte response in presence of all potential impurities. Retention time of individual components and diluent were injected and the result are recorded in Table 1. PDA detector was used to evaluate the homogeneity of the peak in the chromatogram. Chromatographic peak purity was determined for Brimoniodine tartrate spiked with known related substances **(Fig. 2)**.

Table 1: Specificity data

Component name	Retention time (in minute)	RRT*	Purity angle**	Purity threshold**
Impurity-1	1.989	0.84	0.81	1.77
Impurity-2	2.197	0.92	0.85	1.82
Brimonidine	2.380	1.00	0.34	1.00
Impurity-3	3.304	1.39	0.79	1.67
Impurity-4	4.073	1.71	0.55	1.46
Impurity-5	5.739	2.41	0.72	1.76
Impurity-6	9.833	4.13	0.61	1.47

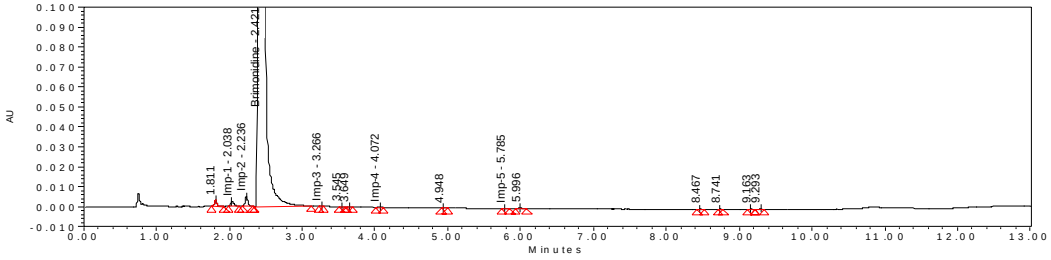
* RRTs are calculated with respect to Brimonidine peak

** Purity angle and Purity Threshold of the related substances in the sample spiked with known related substances

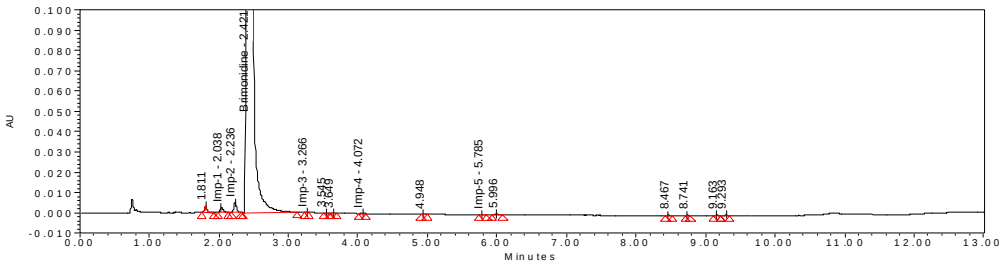
Degradation study

In order to assure the selectivity and provide an indication of the stability-indicating properties of the proposed method, forced degradation studies were performed under various stress conditions[6,8,9,10]. Thus, Brimonidine tartrate sample, were stressed with 1.0 N HCl (Heated at 70ºC for 30 minutes), 1.0 N NaOH solution (heated on water bath at about 70°C for 30min) and 1 ml of 30%w/v H_2O_2 (heated on water bath at about 70°C for 30min) before injection the solution were allow to cool at room temperature. Samples were kept for thermal degradation at 105°C for 24hr and for Photolytic degradation Sample was exposed to UV

degradation in sun test apparatus at 55Klux for 22 hours (exposure ≃1.2 million Lux hours). After the degradation treated samples were neutralized with acid / base as required and analyzed asper the proposed method. Brimonidine was shown a significant stability in all conditions and no major degradation were observed. In peroxide stress degradation briminidine showed about 2 % degradation and the impurity peaks were well-resolved .The mass balance was achieved in all conditions. Typical chromatograms of stress conditions were shown in **Fig. 3** and Forced Degradation data presented in **Table 2**.

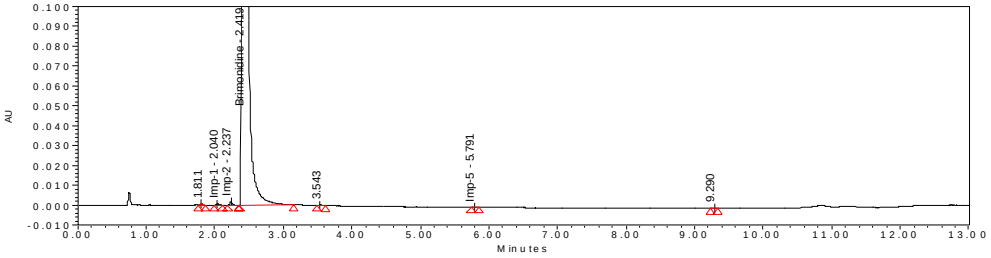


a. Brimonidine tartrate under reflux with 1 N HCl for 70°C, 30min

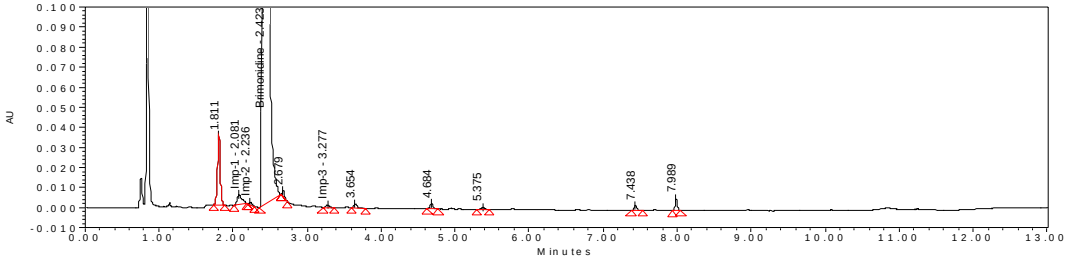


b. Photolytic degradation sample was exposed in sun test apparatus at 55Klux for 22 hours

.(exposure ≃ 1.2 million Lux hour)



c. Thermal degradation sample, 105°C, 24hr



d. Sample treated with H_2O_2, 70°C for 30min

Fig. 3: Typical chromatograms of stress conditions

Precision

The precision of the method was studied by injecting six individual preparations of Brimonidine tartrate samples and calculated for the percentage levels of impurities present in the sample. Also intermediate precision was evaluated by use of a different column (same packing but from a different batch), by a different analyst, using a different instrument, in the same laboratory. These results from both the experiments were compared and confirmed the ruggedness of the method.

Table 2: Degradation data

Stress condition	Total impurities (%)	% Difference (Initial Purity Angle and Stress)		Purity threshold	Purity flag
Acid hydrolysis, 1N HCl, 70°C, 30min.	0.19	0.027	0.821	1.034	No
Base hydrolysis, 1N NaOH, 70°C, 30min.	0.18	0.026	0.820	1.032	No
Thermal, 105°C, 24hr	0.17	0.006	0.333	1.06	No
Peroxide 1 ml 30% H_2O_2, 70°C, 30min	2.30	2.135	0.21	1.025	No
Photolytic, 55Klux, 22 hours (exposure ~1.2 million Lux hours)	0.42	0.258	0.29	1.006	No

Purity angle and Purity threshold data shows that the analyte chromatographic peaks are not attributed to more than one component, i.e. Method is specific and stability indicating.

Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) for Imp-1 to Imp-6 were established as 0.013, 0.009, 0.02, 0.02, 0.012, 0.020 µg/ml and are 0.102, 0.069, 0.158, 0.159, 0.096, 0.162 µg/ml respectively with sample concentration of 400 µg/ml at 1.5µL injection volume. The LOD and LOQ were determined by plotting graph of linearity solutions and the concentrations were confirmed for precision by preparing standards and injecting a series of diluted solutions of known concentration six times. The % RSD values of peak areas for each impurity for LOD and LOQ was obtained were less than 33 % and 10 % respectively.

Linearity

Different Concentrations from LOQ to 0.7 % level of the test concentration were prepared from series of dilutions from known amount of stock solution (i.e. LOQ, 10, 20, 40, 60, 80, 100, 120, 140 and 150 %) and injected. From the peak area responses and concentration data plots for all known related components and Brimonidine the correlation coefficient values were obtained greater than 0.998 .The best–fit linear equation obtained for Imp-1 was y=15360x-469, Imp-2 was y=13178x-137, Imp-3 was y=11526x+490, Imp-4 was y=15194x+1469, Imp-5 was y=9644x+121 and Imp-6 was y=16858x-594.

Accuracy

Accuracy of the method was studied in triplicate (n = 3) by analyzing known concentrations of all components adding at different levels of 50 %, 100%, and 150% in Brimonidine samples. The recovery (%) was calculated for Imp-1, imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 and were found within the acceptance criteria of 80-120 % as per ICH. The result of mean values of recovery were recorded in Table 3.

Table 3: Result of accuracy

Spike level (%, n=3))	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6
50	106.47	100.00	93.18	104.17	101.53	82.89
100	100.21	107.89	90.98	99.73	97.96	100.00
150	98.83	101.72	89.89	97.40	96.21	108.86

Table 4: Result of robustness

Experiment	Resolution between Imp-1 and Brimonidine peak	Theoretical plates of Brimonidine peak	Tailing factor for Brimonidine peak
Precision	4.5	17928	1.5
Robustness (-Wavelength)	4.5	17929	1.5
Robustness (+Wavelength)	4.5	17963	1.5
Robustness (-Temperature)	5.2	19500	1.5
Robustness (+Temperature)	6.3	19790	1.4
Robustness (-Flow rate)	5.9	21005	1.4
Robustness (+Flow rate)	5.7	18180	1.4
Robustness (-Organic)	4.7	31661	1.6
Robustness (+Organic)	5.9	16531	1.4
Robustness (-pH)	5.6	16979	1.4
Robustness (+pH)	5.6	19776	1.4

Robustness

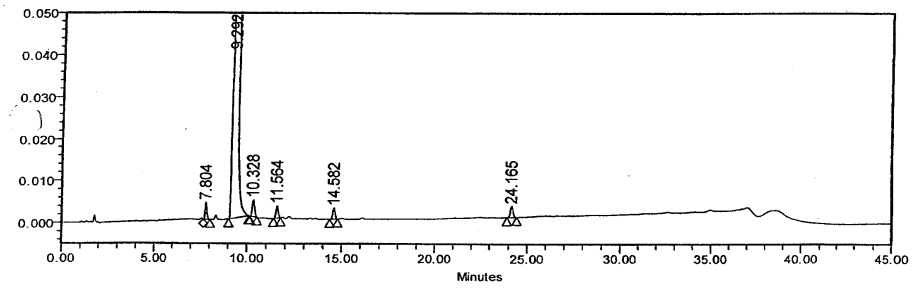
Robustness of the method was studied by making small but deliberate changes in the optimized conditions. The effects of deliberate changes were confirmed by injecting system suitability solution and monitored the resolution between brimonidine and Imp-1, Imp-2, Imp-3. Imp-4. Imp-5 and Imp-6.

Mobile phase flow rate was changed by ±10 % from 0.27 to 0.33, pH of buffer solution was varied by ±0.1 units from 5.9 and 6.1. The column oven temperature was changed from ±5°C from 35 to 45 °C. The deliberate organic phase change ±2% composition in methanol and buffer. When one parameters are changed rest of all the other conditions were kept constant. The result are recorded in **Table 4.**

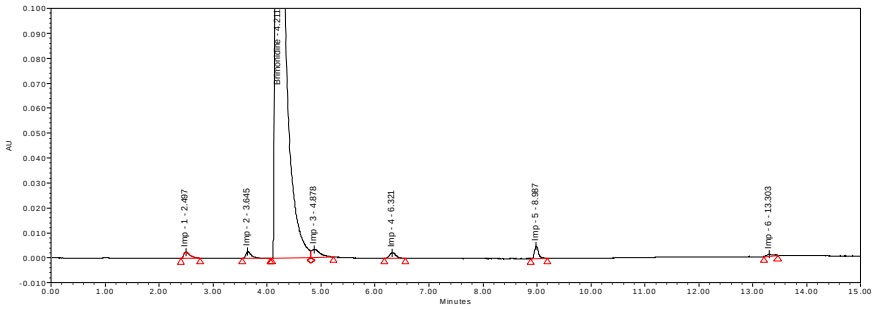
RESULTS AND DISCUSSION

Analytical method development

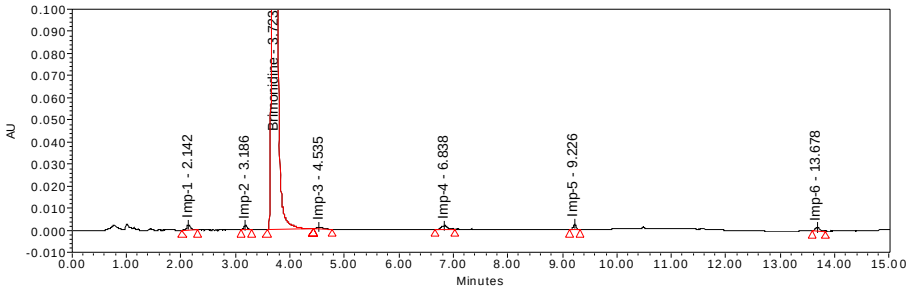
Primarily the objective was to develop stability indicating, cost effective, and rugged method which can be used for faster samples testing routinely in QC lab.



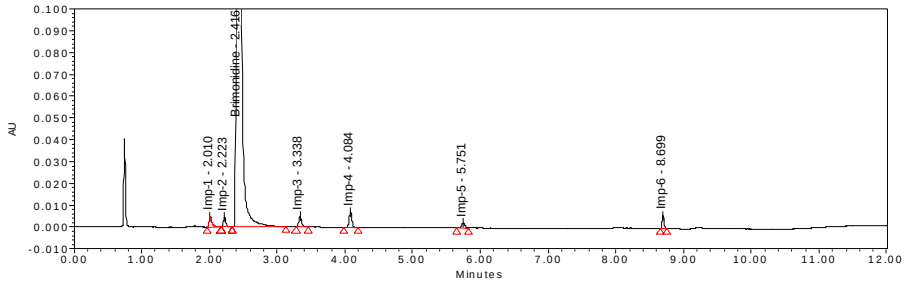
(a) Kromasil C-18,150 × 4.6mm, 5µm, aqueous buffer n-pentane sulphonate and potassium phosphate, triethyl amine pH 7.5 gradient with methanol.



(b) BEH C-18,100 × 2.1mm, 1.7µm column, Ammonium format Buffer pH 7.5 Imp-3 is merged to brimonidine peak (at PH-7.5)



(c) BEH C-18,100 × 2.1mm, 1.7µm column, Ammonium acetate Buffer pH 7.4, Imp-3 elutes closed to brimonidine peak and Imp-6 was highly retained on column.



(d) Hypersil Gold 100 × 2.1mm, 1.9µm column impurity peaks asymmetric

Fig. 4: Typical chromatograms obtained from Development

Several trials has been performed on HPLC to separate all six impurities and Brimonidine tartarate, but only satisfactory separation was achieved with gradient elution mode having a variable mixtures of a) Aqueous buffer n-pentane sulphonate and potassium phosphate, pH 7.5 with triethyl amine and b) Organic phase methanol : THF, 90:10 v/v The column was Kromasil C-18,150*4.6mm,5µm particle size. The limitation of this method was that run time and total analysis time per sample was about 45 to 50 minutes and it could not dropped down further on HPLC. **Fig.** 4 (a)

To achieve the targeted separation further development were tried on UPLC. To begin with knowledge gained from optimized HPLC condition were utilized and experiments planed accordingly.

Resolution between Imp-1 and Imp-2 was very much critical and pH sensitive. Brimonodine tart rate sample solution spiked with all 6 impurities prepared in a diluent (Mixture of methanol and 20mM ammonium acetate pH 7.5 with ammonia solution, 60:40 v/v) and injected throughout development studies.

Solution of aqueous volatile buffers (20mM) of ammonium acetate and ammonium format with varying pH range from 3 to 7 were prepared and gradient elution with organic phase as acetonitrile, methanol or in combination of both studied. Column used a waters BEH 100 mm x 2.1mm; having C18 stationary phase and 1.7µm particle size, data acquired on PDA detector in the range of 200 to 400 nm. The peaks Imp-1 and Imp-2 were resolved well but Imp-3 peak was eluted at the tailing edge of brimonidine peak and peaks shapes were asymmetric.

To improve the peak shape the pH was reduced to 3 which improved the peak symmetry of the brimonidine peak but, because of ionizable amine groups in brimonidine and its impurities, their interaction with the column was reduced, and all seven compounds were eluted rapidly separation was improved but not satisfactory hence neutral buffers may be better choice for the separation. The chromatographic data retention factor (k) and number of theoretical plates (N) were also recorded and observed during these studies. Typical chromatograms of method development shown in Fig. 4.

The UV absorption maxima of brimonidine, Imp-1, Imp-2, Imp-3, Imp-4 and Imp-6 were at approximately 247 nm whereas that for Imp-5 was at 263 nm. at 247 nm the relative responses of all peaks and Brimodine was approximately same, so detection wavelength was chosen at 247 nm, where as during method development all data monitored and acquired on PDA detector at 200 to 400 nm.

To improve the peak shapes tried another column Hypersil Gold 100 x 2.1mm I.d with 1.9µm, The flow rate was 0.3 ml/min, a gradient elution was planned with 20mM ammonium acetate buffer, pH adjusted to 6.0 by addition of acetic acid and acetonitirile the retention time of brimonidine and resolution between the impurities were satisfactory but column performance decreased over the injections and loss of resolution and peak asymmetry was noted. Buffer solution was found tobe hazy after 24 hrs of storage.

To overcome with this problem buffer solution was mixed with the methanol. Finally method optimized with variable mixture of Mobile phase-A and Mobile phase-B. Which consist of 20mM ammonium acetate buffer, pH adjusted to 6.0 with acetic acid:methanol (90:10 v/v) (Mobile Phase-A) and Acetonitrile (Mobile phase-B) the retention time of brimonidine tartrate was about 2.4 minute and resolution between the all the impurities impurities >2.

The system suitability results are given in **Table 5**. Buffer pH, and amount of methanol and acetonitrile all played a major role in achieving a symmetric brimonidine peak and separation of all six impurities. When different batches of the bulk drug (n = 3) were analyzed results were within accepted limits. results from study of the stability of brimonidine tartrate in accordance with ICH Q1A (R2)[6] were also well within accepted limit.

Table 5: Result from solution of Brimonidine spiked with all six impurities

Compound name	RRT*	USP resolution(Rs)	USP tailing factor	No of theoretical plates N
Imputity-1	0.84	-	1.63	10700
Imputity-2	0.92	2.8	1.32	16766
Brimonidine	1.0	-	1.58	14225
Imputity-3	1.39	11.0	1.29	26648
Imputity-4	1.71	9.3	1.06	42731
Imputity-5	2.41	20.2	1.02	75744
Imputity-6	4.13	57.0	1.06	465440

*RRT's are calculated with respect to Brimonidine peak

UPLC / MS of Brimonidine tartrate

The output from Acquity UPLC system was directly applied to hyphenated mass spectrometry (MS) a TQD Tandem Quadrupole UPLC/MS/MS instrument. The TIC (Total ion current) spectra recorded at atmospheric pressure ionization in both +ve and – ve ion mode. The elution pattern of impurities in UV detector were correlated with the impurities in Mass Spectrometry detector. Brimonidine and all other impurities were ionized in $(M+H)^+$ and Imp-6 were ionisesd in $(M-H)^-$ ion. The MS and MSMS spectra of impurities enable rapidly identification of impurities.

CONCLUSION

A new, sensitive, and stability-indicating UPLC method has been successfully developed for quantitative analysis of related substances in brimonidine tartrate drug substance and drug product the proposed method has been successfully validated for specificity, linearity, range, precision, accuracy, sensitivity and robustness.

The added advantage of the volatile buffers used in the method has a unique advantage over the other methods .The output from UPLC system is directly applied to hyphenated Mass Spectrometry detector, which enables rapidly identification of impurities. The method was accurate and precise with good consistent recovery at all the levels. The method enables high resolution of degradation products and impurities both from brimonidine tartrate and from each other.

Conventional reported HPLC methods to be replaced by the proposed UPLC method because of cost effective, analysis time saving per sample and better detection. For faster samples testing routinely in QC lab the validated method may be used for brimonidine tartrate in the bulk drug and drug product.

ACKNOWLEDGMENT

The authors wish to thank the management of Wockhardt Research center for supporting this work.

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