

A LCMS Compatible Stability-Indicating HPLC Assay Method for Clopidogrel bisulphate

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Abstract: Clopidogrel bisulphate is an inhibitor of adenosine diphosphate (ADP)-induced platelet aggregation which makes it an effective drug for reducing the incidence ischemic strokes, heart attacks or claudication due to vascular diseases such as atherosclerosis. A stability-indicating HPLC method for the quantitative determination of Clopidogrel bisulphate is described.

Separation was achieved on a Inertsil C8 HPLC column using a mobile phase which consists of a mixture of 0.1 % trifluoroacetic acid (Solvent A) and acetonitrile (Solvent B). Degradation studies were performed on bulk samples of Clopidogrel bisulphate using acidic (0.5 N hydrochloric acid), basic (0.1 N sodium hydroxide), neutral (water : acetonitrile mixture 1:1), oxidative (6 % v/v hydrogen peroxide), thermal (105 °C) and photolytic (UV light -254 nm) conditions.

Degradation was observed under acidic, basic and neutral hydrolysis conditions to give Clopidogrel carboxylic acid. Two additional degradation products were observed under the conditions of oxidative degradation. The degradation products observed during forced degradation studies were monitored using the HPLC method developed. Method developed was LCMS compatible and the same was used to identify the degradation products. The mass spectrum provides the identity of degradation products formed and proves the specificity of the method unambiguously. The method was validated with respect to specificity, linearity, accuracy, precision and robustness.

Keywords: Clopidogrel bisulphate • HPLC • LCMS • Forced degradation studies

Introduction

Clopidogrel bisulfate is an inhibitor of adenosine diphosphate (ADP)-induced platelet aggregation acting by direct inhibition of ADP binding to its receptor and of the subsequent ADP-mediated activation of the glycoprotein GPIIb/IIIa complex. Clopidogrel's platelet inhibiting activity makes it an effective drug for reducing the incidence ischemic strokes, heart attacks or claudication due to vascular diseases such as atherosclerosis. By inhibiting platelet aggregation, Clopidogrel reduces the chance of arterial blockage, thus preventing strokes and heart attacks¹.

Chemically it is methyl (+)-(S)- α -(2-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)-acetate sulfate

(1:1). The empirical formula of Clopidogrel bisulphate is $C_{16}H_{16}ClNO_2S \cdot H_2SO_4$ and its molecular weight is 419.9. So far to our present knowledge no LCMS compatible stability-indicating analytical method for Clopidogrel bisulphate has been published in the literature²⁻¹², although a method for determination of Clopidogrel in Pharmaceutical Preparations has been published¹². However the method is not LCMS compatible and does not discuss the identity of the degradation products. The present work deals with method development, conducting forced degradation studies and validation of the method.

Experimental

Chemicals and Reagents

Clopidogrel bisulphate sample was received through the kind courtesy of Aarti Drugs limited. The drug substance was used as received. HPLC (gradient) grade acetonitrile was procured from Merck, Germany. Trifluoroacetic acid was procured from Spectrochem India whereas sodium hydroxide, hydrochloric acid and hydrogen peroxide were procured from Qualigens India. High purity water was prepared with a Millipore Milli Q plus system.

Chromatographic Conditions

A Waters Alliance 2695 Separations model LC system equipped with a photodiode array detector (PDA) was used. The output signal was monitored and processed using Empower software (Waters) on a Pentium computer (HP). The column used was a Inertsil C8 (250 X 4.6 mm, 5 micron) and the mobile phase consisted of solvent A (1.0 ml of trifluoroacetic acid in 1,000 ml of HPLC grade water) and solvent B (acetonitrile). The flow rate was 1.0 ml/ min. The HPLC gradient was T/B (where T is time in minutes and B is % concentration of solvent B in terms of volume by volume i.e. v/v) : 0 min / 20%, 6 min / 20% , 40 min / 80% , 45 min /80 % , 48 min / 20%, and 50 min / 20% v/v. The column temperature was maintained at 25°C and the analysis was carried out at wavelength $\lambda = 225$ nm. The injection volume was 20 μ L. A mixture of water and Acetonitrile (1:1) was used as diluent.

HPLC-PDA-ESI-MS (LC-MS)

The analyses were conducted using a HPLC coupled with LCQ Advantage (ThermoElectron, USA) The output signal was monitored and processed using Xcalibur software (Thermo) on a Pentium computer (HP).

The mass spectrometer operating in the positive and negative ion modes with electrospray ionisation (ESI) source. The mass spectra were obtained as an average of 50 scans.

The chromatographic conditions were maintained the same as described above, however the flow of mobile phase from HPLC was directed into the ESI source at a flow rate of 0.2 ml /min by using a flow splitter.

ESI source conditions were as follows: heated capillary temperature 250 °C; sheath gas (Nitrogen gas) flow rate 40 units (ca. 0.60 L/min); spray voltage 4.5 kV; capillary voltage 25 V; tube lens off set voltage 25 V. For ESI-MS/MS, the precursor ions were first isolated by applying an appropriate waveform across the end cap electrodes of the ion trap to resonantly eject all trapped ions except those ions of

the m/z ratio of interest. The isolated ions were then subjected to a supplementary ac signal to resonantly excite them and so cause collision-induced dissociation (CID). The relative collision energy was set to a value at which product ions were produced in measurable abundance and varying from 18 to 40 %. The isolation width used in the MS/MS experiments was 3unit.

Forced degradation studies

Stress studies were carried out under ICH prescribed stress conditions Degradation studies were performed on the bulk drug to provide an indication of the stability-indicating property and specificity of the proposed method. Forced degradation was carried out under stress conditions of photolysis (UV light - 254 nm), thermal (105°C), acidic (0.5 N hydrochloric acid), basic (0.1N sodium hydroxide), neutral (diluent) and oxidative condition (6.0 % H₂O₂) to evaluate the ability of the proposed method to separate Clopidogrel bisulphate from its degradation products. For thermal and photolytic studies, the study period was 10 days whereas treatment with acid, base and neutral media was carried out for 12 hours. Degradation studies in acidic and neutral media were carried out at an elevated temperature of 80°C. Degradation under oxidative stress conditions was studied for a period of 5 days.

Preparation of samples for HPLC and LCMS analyses

A sample concentration of 1000 μ g/ml was used to conduct degradation studies. The degradation samples were neutralised (in case of acid and base hydrolysis) and diluted suitably to a concentration of 400 μ g/ml for analysis by HPLC and LCMS. The solid samples were with diluted with diluent. All the solutions were filtered using 0.22 micron membrane filter before HPLC and LCMS injections.

Method Validation

Preparation of Standard Solutions

Standard solution of 400 μ g/ ml was prepared for assay determination and to carry out degradation studies analysis.

Specificity

The specificity of the method for Clopidogrel was carried out in the presence of its degradation products i.e. degradant I, II and III. Degradant I is product of hydrolysis of Clopidogrel observed under acidic, basic and neutral conditions [figure I and 2]. Degradants II and III were observed under oxidative stress conditions. [figure I and 2]

Peak purity assessment was carried out on the stressed samples of Clopidogrel bisulphate by using PDA

detector. The specificity was also demonstrated by subjecting the degraded samples to LCMS analysis using the same method.

Precision

Precision of the method was evaluated by carrying out six independent assays of a test sample of Clopidogrel bisulphate against a reference standard and calculating the percent RSD. The reproducibility of the method was also evaluated using a different analyst and a different instrument in the same laboratory.

Linearity

Linearity test solutions were prepared from stock solution at seven concentration levels of analyte (50, 100, 200, 300, 400, 500 and 600 µg/ml). The peak area versus concentration data was performed by least-squares linear regression analysis. The calibration curve was drawn by plotting Clopidogrel bisulphate average area for triplicate injections and the concentration expressed as a percentage.

Accuracy

The accuracy of the method was evaluated in triplicate at five concentration levels i.e., 200, 300, 400, 500 and 600 µg/ml in bulk drug sample. The % recoveries were calculated.

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

The LOD and LOQ for Clopidogrel bisulphate was estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively by injecting a series of diluted solutions with known concentration.

Robustness

To determine the robustness of the method, experimental conditions were purposely altered. The flow rate of the mobile phase was 1.0 ml/ min. To study the effect of flow rate on the resolution, it was changed from 0.9 to 1.1 ml/ min while the other mobile phase components were held constant. The effect of column temperature on resolution was studied at 20 and 30°C instead of 25°C while the other mobile phase components were held constant.

Results and Discussion

Optimisation of Chromatographic Conditions

The objective was to separate Clopidogrel from its degradation products. Different types of column chemistry like C-18, C8, phenyl, lengths of column (100, 150 and 250 mm) and brands were evaluated. An Inertsil C8 column (250 X 4.6 mm, 5 micron) was found suitable. Different mobile phases were considered but trifluoroacetic acid was found suitable for the analysis. Also different concentrations of the

acid were considered but at higher concentration ion suppression in mass spectrometer was observed and at lower concentration separation of degradation products was not optimum. A concentration of 0.1 % trifluoroacetic acid as mobile phase was found suitable. The mobile phase consisted of a mixture of solvent A and B given above in a gradient elution program. The flow rate of mobile phase was 1.0 ml/min. The HPLC gradient was kept as T/B (where T is time in minutes and B is % concentration of solvent B in terms of volume by volume i.e. v/v) : 0 min / 20%, 6 min / 20% , 40 min / 80% , 45 min / 80 % , 48 min / 20%, and 50 min / 20% v/v.. The column was maintained at 25°C. Analysis was carried out at different wavelengths; however a wavelength of 225 nm was found most suitable to monitor Clopidogrel and its degradation products. Hence the samples were analysed at 225 nm. Under the optimised conditions, Clopidogrel bisulphate and its degradation products were well separated and the method was found to be specific for Clopidogrel bisulphate and its degradation products.

The mass spectrometer conditions were optimised for obtaining a good signal and high sensitivity for Clopidogrel and its degradation products. The conditions like capillary voltage and spray voltage along with tube lens voltage were varied to maximise the response for Clopidogrel and its degradation products, even at a very low concentration. The above mentioned conditions were found suitable for the purpose.

Method Validation

Specificity

The peaks due to degradation products and Clopidogrel bisulphate are distinct and well separated [figure 2]. Peak purity test results obtained from PDA confirm that the Clopidogrel peak was homogeneous and pure in all the analysed stressed samples. The mass balance of Clopidogrel in stressed samples was above 98.0 %, which confirms the stability indicating power of the method. [Table :I]. The stressed samples were further analysed using LCMS. The mass detector showed excellent mass purity for each of the degradation product and the Clopidogrel drug substance which unambiguously proves the method specificity [figure 3].

Precision

The percent RSD of assay of Clopidogrel bisulphate during assay method precision studies was well within 1% thus confirming good precision of the method.

Table I : Mass balance for Clopidogrel bisulphate drug substance in presence of degradation products formed during forced degradation studies.

Degradation Studies	Time	Assay (by % area)	Assay (% w/w)	Remarks
Control		99.5%	100 %	
Thermal at 105°C	10 days	100 %	99.2 %	No significant degradation observed
Base hydrolysis (at RT)	12 h	54.5 %	54.2 %	Degradation product I observed
Acid hydrolysis (at 80°C)	12 h	85.2 %	84.5 %	Degradation product I observed
Neutral hydrolysis (at 80°C)	12 h	93.1 %	92.4 %	Degradation product I observed
Oxidation by H ₂ O ₂	5 days	65.9 %	63.8 %	Degradation products I and II observed
Photolytic Condition	10 days	99.0 %	98.2 %	No significant degradation observed

Linearity

A linear calibration plot for the method was obtained over the calibration ranges 50–600 µg/ml with a correlation coefficient equal to 0.9995. Linearity was checked over the same concentration range for three consecutive days. The results showed that an excellent correlation exists between the peak area and concentration of the analyte.

Accuracy

The percentage recovery of Clopidogrel bisulphate in bulk drug samples was ranged from 98.0 to 102.0 % w/w.

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

The limit of detection (LOD) of Clopidogrel bisulphate was 0.25 µg/ml for 20 µl injection volume. Limit of

quantification (LOQ) for Clopidogrel bisulphate was 0.75 µg/ml for 20 µl injection volume.

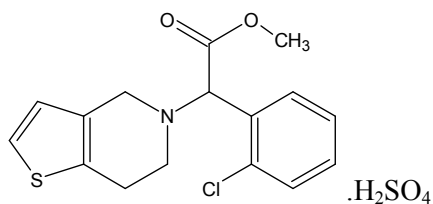
Robustness

Deliberate changes made in chromatographic conditions (flow rate and column temperature) have no significant change in assay value was observed, which confirms the robustness of the method.

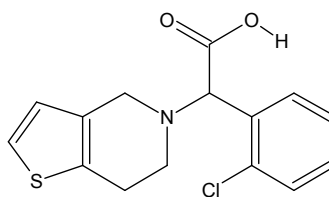
System suitability

The system suitability was established by evaluating parameters like asymmetry factor and plate count. The results were established by six replicates of Clopidogrel solution (400µg/ml), which prove the method suitability for intended purpose. The mean results for six replicates is given below,

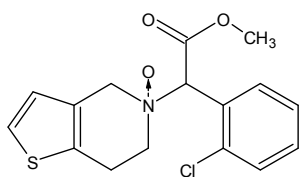
Parameters	Observed	Preferred
% RSD (for area)	0.957	less than 2.0
% RSD(Retention time)	0.20	less than 1.0
Peak tailing factor (as per USP)	1.30	less than 1.5
Plate count (N) (as per USP)	82535	more than 15000

Figure 1: Structures of Clopidogrel bisulphate and its degradation products

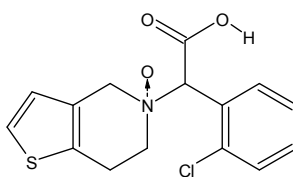
Clopidogrel bisulphate
Molecular weight : 419.9
Clopidogrel (base) :
Molecular weight : 321



Degradation Product I
Clopidogrel Acid
Molecular weight : 307



Degradation Product III
Clopidogrel N-oxide
Molecular weight : 337



Degradation Product II
Clopidogrel Acid N-oxide
Molecular weight : 323

Results of Degradation Studies

Clopidogrel bisulphate degrades under basic conditions at ambient temperature and under acidic to neutral conditions at an elevated temperature forming a single major degradation product i.e. carboxylic acid of Clopidogrel via hydrolysis of the ester group. [figure 1 and 3].

Clopidogrel bisulphate also degrades under oxidative stress conditions forming two major degradation products (II and III). Degradation product II is N-oxide [figure 1 and 3] of Clopidogrel carboxylic acid formed by oxidation of Clopidogrel carboxylic acid formed by hydrolysis of Clopidogrel in solution. Degradation product III is the N-oxide [figure 2] of Clopidogrel formed by oxidation of Clopidogrel. These products are not stable and hence could not be isolated for further characterisation. However the mass spectrum observed during the LCMS analysis confirms the formation of N-oxides. [figure 1 and 3].

Peak purity test and the mass purity confirmed that the Clopidogrel bisulphate peak was homogeneous and

pure in all the analysed stress samples. The mass balance of stressed samples was greater than 98.0 % (Table I). The assay of Clopidogrel bisulphate is unaffected in the presence of degradation products confirming the stability- indicating ability of the method.

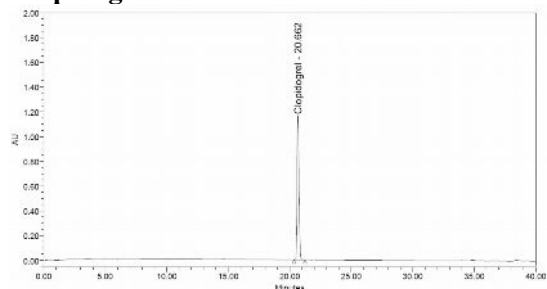
Conclusions

This paper describes a novel stability indicating method for Clopidogrel bisulphate by HPLC. The results of forced degradation studies undertaken according to the ICH guidelines reveal that the method is selective and stability-indicating. The method is LCMS compatible, the results of which further establishes method specificity and helps in confirming the identity of degradation products formed.

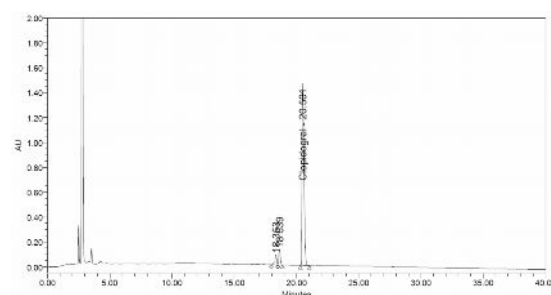
The method is specific, accurate, precise, stability-indicating and validated for the routine analysis of Clopidogrel bisulphate in bulk drug form. The method may also be extended to evaluate active drug substance in the finished dosage form.

Figure 2 : HPLC chromatograms of Clopidogrel and its degradation products under stress conditions

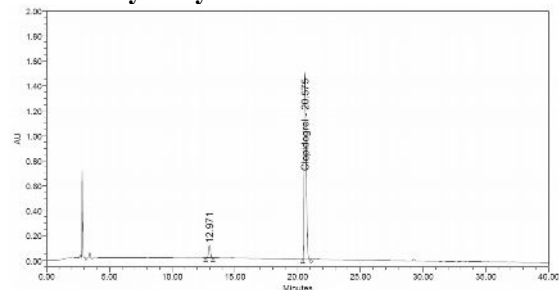
Clopidogrel standard



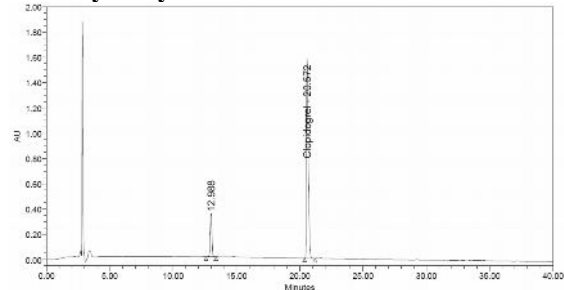
Oxidative Degradation



Neutral hydrolysis



Acid hydrolysis



: Base hydrolysis

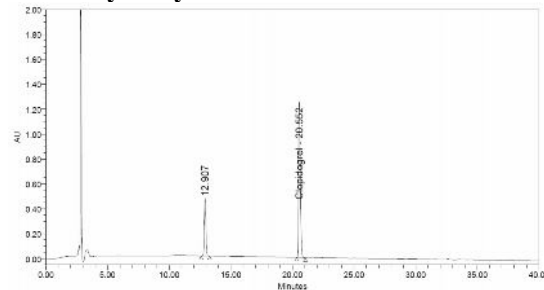
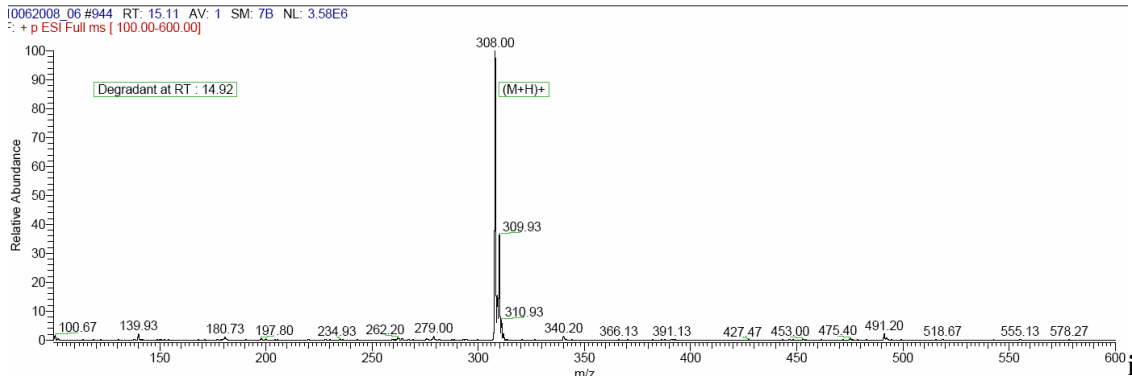
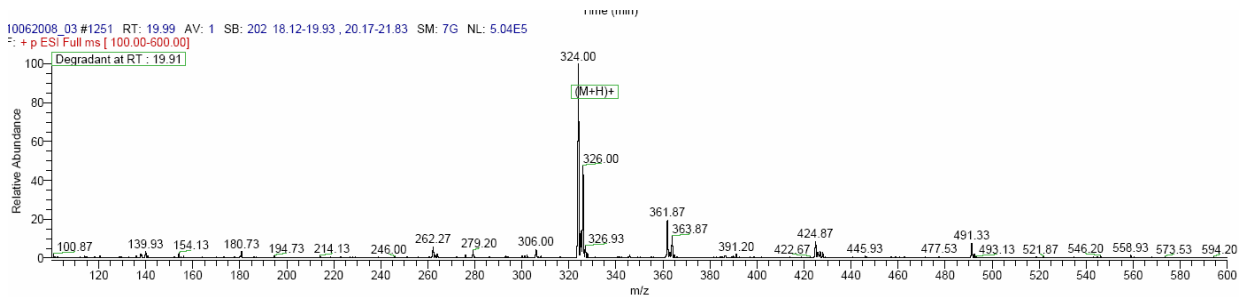


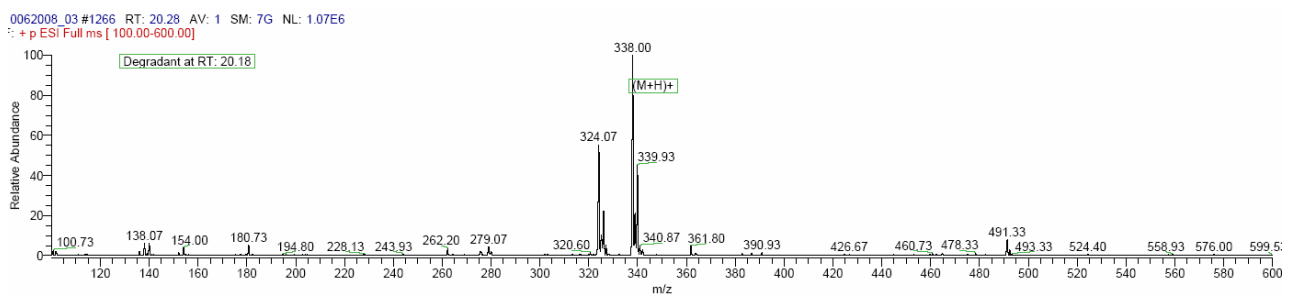
Figure 3: LCMS Analysis : Mass spectrum of Clopidogrel and its degradation products
Mass spectrum of Degradant I (M+H)⁺ = 308



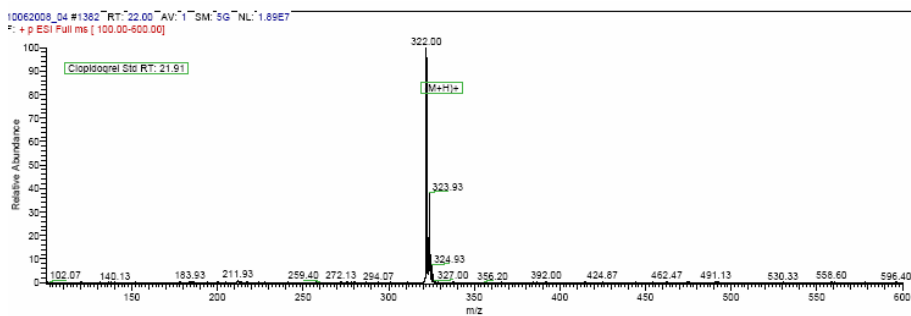
Mass spectrum of Degradant II (M+H)⁺ = 324



Mass spectrum of Degradant III (M+H)⁺ = 338



Mass spectrum of Clopidogrel bisulphate (M+H)⁺ = 322



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