Pharm Methods, 2017; 8(1): 22-30
A multifaceted peer reviewed journal in the field of Pharm Analysis and Pharmaceutics
www.phmethods.net | www.journalonweb.com/phm

Original Article

Stability-indicating UFLC method for uncoupling and estimation of impurities in clopidogrel, aspirin and omeprazole in their tablet dosage form using PDA detection

Jinesh Bahubali Nagavi[1*], Bannimath Gurupadayya[1]

[1]Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS University, Mysuru, Karnataka, INDIA.

ABSTRACT

Introduction: In this paper a fast and novel stability-indicating ultra fast LC method for separation and estimation of impurities in clopidogrel and aspirin in their combined tablet dosage form and omeprazole was developed and validated according to ICH guidelines. **Methodology:** The separation of USP related substances of clopidogrel (A, B and C), aspirin (D), omeprazole (A, B and C) and few other unknown impurities was detected by using ultra fast liquid chromatography with PDA detection. The maximum detection was set as follows: 237 nm for aspirin, its impurities and for the impurity C of clopidogrel and 254 nm for Clopidogrel and its impurities except for impurity C and 280 nm for omeprazole and its impurities. Phenomenex C8 (250 mm × 4.6 mm, 5 µ) was used as a stationary column to separate and analyze the mixture within 11 min with a programmed gradient elution of 0.01 M phosphate buffer pH 2.0 and acetonitrile. The tablets were exposed to acid, alkaline, thermal, higher humidity, oxidative and photolytic stress conditions. Samples undergone stressed conditions were analyzed by the novel proposed method. **Results:** The method was successfully validated in accordance to the International Conference of Harmonization (ICH) guidelines for clopidogrel and its impurities, aspirin and its impurity D and omeprazole and its impurities A, B and C. Separation was satisfactory for all the significant degradation products from the principal peaks of drug substances and the impurities from each other. **Conclusion:** The method complies for the peak purity test for clopidogrel, aspirin and omeprazole in all the samples under stress and showed no co-elution of degradation products. The method was found to be stable, precise, linear, accurate, sensitive, specific and robust. The method can be used routinely to test the adulteration in the pharmaceutical formulations of clopidogrel, aspirin, and omeprazole.

Key words: Clopidogrel, Aspirin, Omeprazole, LC, ICH, PDA.

Correspondence:
Jinesh Bahubali Nagavi, Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS University, Mysuru, Karnataka, INDIA.
Phone no: +919738516415

E-mail: nagavi.jinesh@gmail.com
DOI : 10.5530/phm.2017.8.4

INTRODUCTION

Registration authorities compulsorily require the purity testing method, which is a chief constituent of the method development for a pharmaceutical molecule. A validated analytical method for assuring the maximum safety of drug therapy is necessary.[1,2] Liquid chromatography is one of the most widely applied tools for pharmaceutical analysis and its latest technical tackle called "ultra fast liquid chromatography" has a significant advantage like short analysis time, better resolution, higher peak capacity and sensitivity, minimum solvent utilization.[3] Monographs depicting the HPLC method for related substances are devoted to clopidogrel and aspirin in European Pharmacopoeia[4] as well as in United State Pharmacopoiea.[5] Stability-indicating[6] and non stability-indicating[7,8] assay methods for simultaneous estimation of aspirin and clopidogrel bisulphate have been published. Determination of aspirin Ph. Eur. impurity D[4] and omeprazole Ph. Eur. impurity B[4] in combination was published under reversed phase HPLC conditions.[9] Omeprazole impurities A, B and C named as per European Pharmacopoiea[4] were determined by a RP- HPLC method suitable for both assay of drug substances and their purity test. UPLC methods for impurities of aspirin[10] and clopidogrel were determined. Alternate chromatographic methods in combination have been focused majorly on the estimation of the active molecules. Clopidogrel and omeprazole were analyzed simultaneously by stability-indicating assay methods. Non-stability-indicating assay methods for a combination of clopidogrel, omeprazole and aspirin are also reported. Clopidogrel Ph. Eur. impurity D[4] as its main acidic degradation product was analyzed by stability-indicating purity testing methods for combinations of Clopidogrel with atorvastatin calcium. As mentioned, combined pharmaceutical dosage forms containing aspirin or clopidogrel have been analyzed mostly by assay methods and purity test methods have been published mainly for combination with omeprazole. The triple combination of aspirin, clopidogrel and omeprazole has so far been analyzed only by assay methods. The retention ability of the drug substances in reversed phase HPLC methods depends on the pH of mobile phase. If a low pH mobile phase is used, the analytes elute in the order omeprazole, Clopidogrel and aspirin. If weakly acidic or neutral conditions are used, the elution order of Clopidogrel and aspirin is reversed and, if neutral or basic buffer is used, aspirin is eluted first, followed by omeprazole and Clopidogrel leaves the column. Few stability-indicating methods have been published for this triple combination. However, no purity data for the peaks obtained and identification of degradation products were provided. Spectrophotometric methods for determination of aspirin, clopidogrel and omeprazole were also performed The aim of this work was to develop and validate a stability-indicating UFLC analytical method for quantitative purity testing of aspirin, clopidogrel and omeprazole, as no method of assurance for their foreign particles in such a combination was found in the literature. The still unofficial USP describes only an HPLC column for the determination of organic impurities of this triple combination. According to authors learning, no UFLC method for analysis of this combination of drug substances and impurities has been published till date.

MATERIAL AND METHODOLOGY

Chemicals

Standard molecules of aspirin, clopidogrel, omeprazole, tablets and placebo were supplied by Deviz Enterprises (Navi Mumbai, Maharashtra, India).

Clopidogrel impurities A, (+)-(S)-(2-Chlorophenyl)[6,7-dihydrothieno (3,2-c) pyridine-5(4H)-yl) acetic acid hydrochloride, Clopidogrel impurities B, (RS)-Methyl (2-chlorophenyl)-4,5-dihydrothieno (2,3-c) pyridine-6 (7H)-acetate hydrochloride and). Clopidogrel impurities C, Methyl (2R)-(2-Chlorophenyl) (6, 7-dihydrothieno (3, 2-c) pyridin-5(4H)-yl) acetate Hydrogen (according to Ph. Eur. (8)) were purchased from Sai Traders (Ahmedabad, Gujarat, India).

Aspirin impurity D (according to Ph. Eur. (8)) was supplied by TLC Pharma Labs (Hyderabad, Telangana, India).

Omeprazole impurities A, B and C were supplied by Veeprho Pharmaceuticals (Pune, Maharashtra, India).

Ultra gradient HPLC grade acetonitrile and HPLC gradient grade methanol were purchased from Arihant Enterprise (Maharashtra, India). The eluent and other solvents were prepared using potassium dihydrogen ortho phosphate (98.0%), ortho-phosphoric acid (90%), hydrochloric acid (37.5%), sodium hydroxide (99.0%) and hydrogen peroxide (30%); all purchased from Merck (Darmstadt, Germany) and water was processed using Milli-Q system from Merck Millipore (Billerica, USA).

Chromatographic parameters

The study was carried out using Shimadzu prominence ultra fast liquid chromatography having a column oven and a UV-PDA detector from Shimadzu (SPD-M20A). obtained chromatograms were integrated with the help of LC Solutions software from Shimadzu. Phenomenex C8 250 mm×4.6 mm, 5µ particle size, from Shimadzu (Santa Clara, CA), thermostatted at 28°C was optimized to separate and identify the molecules. The eluent was a gradient mixture of component A (1.15 g/l solution of potassium dihydrogen ortho phosphate adjusted to pH 2.0 with ortho-phosphoric acid) and component B (acetonitrile). The flow rate of the mobile phase was 1.2 mL/min. The final gradient program ((min)/% B) was 0/5, 5/25, 7.5/50, 10/75, 12.5/50 and 15/5. The temperature of the sample was set to 25°C and the volume of injection was 10 µl. Chromatograms obtained for the impurities of aspirin and impurity C of clopidogrel were evaluated at a wavelength of 237 nm. Other Clopidogrel impurities were detected and evaluated at a wavelength of 254 nm. Based on the UV spectra of Omeprazole and it's the impurities, 280 nm was selected as the wavelength for this method. The PDA detector operated at sampling rate 20 points per second. A Mark ultrasonic bath from RC Systems (Bangalore, Karnataka, India) was used for sample sonication. Samples were centrifuged with an eppendorf centrifuge 5810R from Eppendorf AG (Hamburg, Germany).

Preparation of solvent systems
Sample preparation

Ten milliliter of ortho-phosphoric acid (90%) was pipetted into a 1000 mL volumetric flask and diluted upto 1000 mL with water. This solution was mixed with acetonitrile and methanol in a ratio of 50/30/20 (ortho-phosphoric acid solution/acetonitrile/methanol; v/v/v).

Preparation of sample solution

Twenty tablets of clopidogrel, aspirin and omeprazole were thoroughly homogenized. An amount of 750 mg of the homogenized sample was transfered into a 50 mL amber volumetric flask and 40 mL of sample was added. The sample was sonicated for 30 min. During the sonication, the sample was occasionally shaken and the temperature of the bath was controlled not to exceed 26°C. After the sonication, the sample was made up to the mark with the sample solvent. Then the sample was stirred for 15 min using a magnetic stirring plate. After this, the sample was centrifuged for 15 min at 1.0×10^4 rotations per minute and 10°C. The supernatant was carefully transferred into a vial using a pipette tip and crimped. The final concentrations of the drug substances were 2.5 mg/mL of aspirin, 13.5 mg/mL of Clopidogrel and 0.2 mg/mL of omeprazole calculated as per the label claim in the formulated tablets (Table 1).

Preparation of standard solution

Aspirin, clopidogrel and omeprazole standard molecules were solubilized in the sample solution corresponding to the concentration level to 0.5% concentration of the sample solution prepared for all the three standard substances.

Preparation of placebo solution

An amount of 400 mg of homogenized placebo (mainly composed from microcrystalline cellulose, stearate and anhydrous colloidal silica) was transferred into a 50 mL amber colored measuring flask. The placebo solution was further prepared as the sample solution.

Method validation

As per the ICH Q2 (R1) guideline, the developed method was validated[12] for clopidogrel impurities, aspirin impurity D and omeprazole impurities. The method was found to be selective for all the impurities in addition to various available clopidogrel impurities E, F, G and aspirin impurities B. These impurities of clopidogrel and aspirin were quantified and labeled as unknown impurities in the sample solution because they did not exceeded the limits during preliminary stability studies of the tablets and therefore it was not fully validated.

Precision

The method was confirmed for the repeatability by analyzing the replicates of six samples of the tablets and the % RSD were calculated for the contents of impurities. Six replicates of the samples of same batch of formulated tablets were analyzed to determine the intermediate precision injected by a different analyst on a different day in a different laboratory by using a different column (same specification but of different batch). The % RSD values for the content of impurities were calculated for all the replicates (two analysts together).

Linearity and accuracy

Spiked samples of tablet powder were examined for the linearity and accuracy. Reference materials of Clopidogrel impurity A, B, and C, omeprazole impurities A, B and C and aspirin impurity D were solubilized in the sample solution, further spiked into the sample of tablets, weighed previously at five different concentration levels, triplicate samples for each level. Sample solution preparation procedure was followed to prepare the solutions. The volume of sample solvent was reduced according to the volume of spikes of impurities. Unspiked samples of formulated tablets were prepared to rectify the impurities which were identified previously by amount. The accuracy and linearity for determination of drug substances were also evaluated for quantification of unknown impurities. Reference materials of aspirin, omeprazole and clopidogrel were solubilized in the sample solution and spiked into the placebo solution at five different concentration levels, triplicate of samples in each level. Samples were prepared as per the procedure of the placebo solution preparation. The volume of sample solvent was reduced by the volume of spikes of impurities. The linearity was calculated for all the drug and impurities for all the concentration range. According to the concentration of drugs in sample solution, linearity and accuracy samples were prepared at the concentration levels: 0.10-0.50% for Clopidogrel, 0.10-0.30% for omeprazole and aspirin, 0.10-0.50% for Clopidogrel impurity C, 0.05-1.20% for omeprazole impurities A and B, 0.10-0.20% for omeprazole impurity C and 0.15-0.30% for impurity D of aspirin. The accuracy was computed for all the impurities and each drug as percentage recovery of the sum of the impurity/drug that was spiked into the sample solution at three different levels across the concentration range.

Table 1: Composition of the formulated triple tablets

Compound	Amount (mg)	Concentration in sample solvent (mg/ml)	Impurity	Limit (%)
Clopidogrel	75	13.5	Impurity A	0.3
			Impurity B	0.2
			Impurity C	0.2
			Unidentified Impurity	0.2
Omeprazole	10	0.2	Impurity A	1.0
			Impurity B	0.5
			Impurity C	0.2
Aspirin	100	2.5	Impurity D	0.2
			Unidentified Impurity	0.2

Selectivity

The developed method was found to be selective by evaluating the impurities in the spiked sample solution. Concentration of the drugs in the sample was used relatively to spike the impurities. Based on the limit concentrations for each impurity, the concentration levels were set (Table 1). To demonstrate the absence of interferences with the peaks for the spiked sample, chromatograms of the sample solution and the placebo solution were evaluated. The stability-indicating property of the method was tested by performing the forced degradation study. In order to confirm that there was no interference of any unknown impurity with the drugs peak, peak purity test was done.

Robustness

Chromatographic parameters were altered accordingly to confirm that the developed method was robust, conditions such as the column temperature (±5°C), flow rate (±0.3 mL/min), pH of the buffer (±0.5), percentage of acetonitrile at different gradient levels (±3%), concentration of potassium dihydrogen ortho phosphate in the buffer (±15%) and the column. The spiked samples were prepared exactly as the sample for evaluation of the selectivity, the retention times of principle peaks and impurities were analyzed.

Stability of the sample and standard solutions

Sample solutions from the linearity estimation (Section 2.4.2) were used for a stability study. The samples were stored in an autosampler at 10°C in the dark as well as at room temperature exposed to daylight. Solution were evaluated after 12, 24, 36 and 48 h. The standard solution was also stored under the same conditions as the sample solution and was analyzed after 12, 24, 36, 48 and 72 h. The difference in the contents of the drug and the impurities at the initial time and at the end of the study were calculated.

Forced degradation study

The potential of the method to separate the drug and their known and unknown degradation products were examined by the forced degradation study. The samples of homogenized tablets were treated under acidic, alkaline, oxidative, thermal, hydrolytic and photolytic stress conditions. Then they were prepared according to the procedure mentioned in Linearity. An unstressed sample solution was also prepared as a blank in this study. Evaluation of the peak purity for clopidogrel, omeprazole and aspirin was done.

Stress conditions

For dry thermal stress conditions, the weighed sample was placed in an oven at 65°C for 18 h. For hydrolytic stress conditions, one milliliter of water was added to the weighed sample and then the sample was kept at 65°C for 18 h. For acidic and alkaline stress conditions, three milliliter of 0.5 M HCl and two milliliter of 0.2 M NaOH were added to the weighed sample and then the sample was treated at 50°C for 2 h. To simulate oxidative stress conditions, three milliliter of 30% H_2O_2 was added to the weighed sample and then the sample was kept at 50°C for 2 h. For photolytic stress conditions, the sample was prepared according to the procedure described in Section 2.3.2 but without using an amber colored volumetric flask. The sample was exposed to daylight for 18 h before centrifugation.

RESULTS AND DISCUSSION

Optimization and method development

Chromatographic conditions

The UFLC method with reversed phase and gradient elution of the mobile phase consisting of acetonitrile and low pH phosphate buffer was chosen as chromatographic conditions because of its promising and good peak shapes provided in the assay methods. The sample for optimization of the chromatographic conditions was prepared with 50% methanol as a solvent and the sample was spiked with all the available impurities at concentration levels corresponding to their limits (Table 1). Low content of acetonitrile was used to start the gradient elution to achieve the appropriate retention of highly polar omeprazole. A successful separation was achieved on an Phenomenex C8 (250×4.6 mm, 5µ) column at 23°C with a linear gradient of 90% of 0.01 M potassium dihydrogen ortho phosphate buffer pH 2.0 and 10% acetonitrile at the beginning to 30% of the buffer and 70% acetonitrile after 10 min (flow rate 0.6 mL/min) with injection volume of 10 µL. Satisfactory sensitivity for Clopidogrel and its impurities was achieved with this injection volume. Many critical pairs of compounds (i.e., omeprazole impurity A and omeprazole; omeprazole impurities B and A; unknown omeprazole impurity and aspirin impurity D; omeprazole impurity C and unknown impurity of aspirin; aspirin and Clopidogrel impurity B; and Clopidogrel impurities B and C) were carefully observed to achieve acceptable resolution. A larger injection volume (20 µL) was used to obtain maximum sensitivity for clopidogrel and its impurities. But, the column was seemed to be overloaded by omeprazole and thus its peaks were distorted when 20 µL were injected. 25% or 30% of acetonitrile in gradient elution resulted in better peak shapes for aspirin impurity D. A higher content of acetonitrile at the start of the gradient program led to co elution of omeprazole impurities B and A. Other columns with similar dimensions, such as phenomenex C8 RRHD 1.8mm and phenomenex C8 2.7 mm were also analysed in the same conditions and both gave a similar elution profile to the Phenomenex C8 4.6 mm column but underwent distortion of the omeprazole peaks. Finally, the column Phenomenex C8 (250×4.6 mm, 5 µ)

was chosen. In the column with diameter of 4.6 mm, it was significant to use a flow rate of 0.8 mL/min. An injection volume of 20 µL of the sample solution could be used with this column as the omeprazole peaks were no longer distorted, the column was not overloaded and, despite the higher column volume, the sensitivity was maintained for Clopidogrel impurity C. A smoother baseline compared to that observed with the narrower columns was also achieved. The gradient program (slower compared to the original gradient program) and column temperature were gradually adjusted to their final values as described in Section 2.2 and consequently satisfactory resolution of all the critical pairs of peaks was achieved (R≥2.0). The detection wavelengths were set as a compromise between the sensitivity and selectivity for each drug substance based on their absorption spectra. Clopidogrel exhibited strong absorption at 238 nm and 254 nm. The latter was chosen as an optimal wavelength because of the better signal to noise ratio, except for impurity C, which did not absorb at this wavelength. This impurity was finally evaluated at 237 nm, which was also the optimal wavelength for aspirin and its impurities.

Sample preparation

To achieve satisfactory sensitivity for all the drug substances and impurities, an amount of 652 mg of homogenized tablets was dissolved in a volume of 50 mL. 50% methanol was used as a solvent in the beginning. It was observed that omeprazole is quite unstable in this solvent, as the area of omeprazole impurity B increased rapidly (80% of area after 18 h at room temperature). Also, the recovery of omeprazole impurity C in 50% methanol was not satisfactory. Degradation of omeprazole to its impurity B was stopped by using a 2.0% solution of ortho-phosphoric acid instead of water in 50% methanol. The recovery of impurity C improved when 75% methanol or 50% acetonitrile were used as solvents. Thus, the peak areas of omeprazole and its impurities were disturbed. Therefore, a final sample solvent consisting of a 2.0% solution of ortho-phosphoric acid, 50% acetonitrile and methanol in a volume ratio of 40:30:30 (v/v/v).

Omeprazole was found to be stable in this sample solvent for up to 36 h at 10°C (Section 3.2.5) and satisfactory recovery of omeprazole impurity B was achieved (Section 3.2.2). Instability of omeprazole may also have happened because of not maintaining a constant temperature during sonication (26°C) and centrifugation (10°C). When the temperature was not maintained constant, a significant increase (up to 60%) in the peak area of omeprazole was found when compared with the sample prepared under controlled conditions.

Method validation

Precision

The intermediate precision and repeatability were determined as mentioned in Section 2.4.1. Impurities in the sample solution were identified as a percentage of the amount of related drug in one tablet compared to the calibration obtained from a standard solution. The % RSD was calculated for Clopidogrel impurity C, omeprazole impurities A, B and C and one unknown impurity of aspirin, present in the sample solution. The % RSD values for five injections peak area of standard solution also was analysed. The intermediate precision results were calculated as the % RSD values for all the results obtained by two different analysts. The values of % RSD was ≤0.5% for five different injections of the standard solution and RSDs were in the range 0.44-1.8% for the contents of impurities met the acceptance criteria[12] and expressed good repeatability of the method. The % RSD values of impurities calculated from all the replicates were in the range 1.5-10% met the acceptance criteria[12] and the intermediate precision of the method was expressed satisfactorily (Table 2).

Linearity, accuracy, LOD and LOQ

The linearity and accuracy of the method were analyzed by the spiked samples of tablets and placebo as mentioned in Section 2.4.2. Recovery was calculated based on the relative response factor for each impurity. From the obtained peak areas of the impurities the relative response factor was calculated for the drug substance and its impurity. Data such as RSD of the area/concentration ratio, regression equation, correlation coefficient and standard deviation values of the slope and the intercept are reported in Table 3 and showed good linearity between the peak area and the concentration for each compound. The LOD and LOQ values were determined as the ratios 3.3 and 10 respectively, The LOD and the LOQ data are reported in Table 4 indicating the method to be sensitive. The recovery and relative response factor data are reported in Table 5 and indicated that the method is accurate, since the percentage recovery values were in the range 92-104%. The % RSD value for the recovery ≤4% confirmed the repeatability of all the analytes. LOD, LOQ, Baseline noise and recovery of Clopidogrel were evaluated at a detection wavelength of 254 nm because only impurity C from among the Clopidogrel impurities was detected at 237 nm.

Selectivity

The method was confirmed to be selective by analyzing a sample spiked considering the impurities at their limit levels (Table 1). The chromatograms obtained from the placebo solution and sample solvent were examined for any possible interferences. Peaks of the impurities and analyte molecules in the sample solution were clearly segregated from the sample solution peaks and the placebo solution peaks and are represented in the chromatogram of the spiked sample solution (Figure 1). Stability-indicating ability of the method was confirmed by carrying out the forced degradation study. The chromatogram of the spiked sample solution evaluated at 254 nm (all the impurities were detected) is shown in (Figure 1). It demonstrates satisfactory selectivity of the method as the resolution of all the peaks of interest was not less than 2.2 with the exception of partial co-elution of aspirin impurity C with aspirin unknown D (resolution R=1.02) and partial co-elution of Clopidogrel impurity C with an unknown impurity of aspirin (R=0.75). The selectivity of the method was enhanced by using different detection wavelengths for each drug substance and its impurities. As a result, the partial co-elution of Clopidogrel impurity C and unknown impurity of aspirin visible at 237 nm was resolved by detection of Clopidogrel impurities at 254 nm. Aspirin and its impurities did not absorb at 280 nm and impurity D was evaluated as a single peak. Despite the partial co-elution, Clopidogrel impurity C was quantified successfully and accurately (Table 5). Due to the resolution, aspirin impurity D was cut off from the unknown impurity. In addition, both impurities had similar aspirin's absorption spectra and the unknown impurity never exceeded the reporting limit for aspirin (0.05%) during the preliminary stability studies of tablets and the forced degradation study.

Robustness

The method was found to be robust when subjected to variable conditions. The retention times of the drugs, all spiked impurities and several unknown impurities were monitored and resolution values for all peaks were calculated. The data are reported in Table 6 and confirmed that the method was robust since resolution values did not change significantly with an exception when a buffer with higher pH was used. The pair of compounds omeprazole impurity C and the unknown impurity of aspirin RRT 0.54 was co-eluted when the buffer with pH 2.8 was used. The separation of these compounds was robust at pH values up to 2.7 (R ≥ 1.9).

Table 2: Precision data

Compound	Content range (%)	Precision RSD (%)	Intermediate precision RSD (%)
Omeprazole	–	0.66	–
Clopidogrel	–	0.44	–
Aspirin	–	0.57	–
Unknown imp. of aspirin	≤0.05	0.57	2.0
Imp. C (Clopidogrel)	≤0.05	0.89	10
Imp. A (omeprazole)	0.05–0.20	0.45	3.0
Imp. B (omeprazole)	≤0.05	0.71	2.8
Imp. C (omeprazole)	0.20–0.50	1.8	1.5

Content range – the content of the impurity in percent of the related drug amount in one tablet.

Precision RSD – for omeprazole, clopidogrel and aspirin calculated from the peak areas of five injections of the standard solution; for impurities calculated from the content determined from six replicate samples.

Intermediate precision RSD – for impurities calculated from the content determined from twelve replicate samples (combined from two analysts). Acceptance criteria.

Table 3: Linearity data

Compound	Concentration range (%)	Correlation coefficient	Regression equation	SD of the intercept	RSD of area/ concentration ratio (%)	SD of the slope
Clopidogrel at 237 nm	0.05–0.30	1.000	$y = 1.9347*10^4 x - 131$	211	0.51	89
Imp. A (Clopidogrel)	0.05–1.00	1.000	$y = 9.4960*10^3 x + 34$	46	1.5	911
Imp. B (Clopidogrel)	0.05–1.00	1.000	$y = 1.9135*10^4 x - 213$	102	0.9	132
Imp. C (Clopidogrel)	0.05–1.14	0.999	$y = 6.9387*10^4 x - 511$	209	3.2	315
Aspirin	0.05–0.30	1.000	$y = 1.0349*10^6 x + 1723$	412	0.9	295
Imp. D (Aspirin)	0.05–0.41	1.000	$y = 4.8862*10^4 x - 197$	296	1.1	396
Omeprazole	0.05–0.30	0.999	$y = 7.1284*10^4 x - 304$	34	0.93	217
Imp. A (Omeprazole)	0.10–0.60	0.999	$y = 1.9448*10^4 x + 36$	37	2.0	166
Imp. B (Omeprazole)	0.10–0.30	0.999	$y = 1.1037*10^6 x + 649$	24	2.8	931
Imp. C (Omeprazole)	0.10–0.50	1.0000	$y = 9.3291*10^4 x + 46$	47	1.7	159

Regression equation – relationship between concentration and peak area.

Acceptance criteria (14). Correlation coefficient >0.98 for impurities and >0.99 for drug substances. RSD of area/concentration ratio ≤ 10.0% for impurities and ≤ 3.0% for drug substances.

Table 4: LOD and LOQ data

Compound	_(V)	LOD (g/mL)	LOQ (g/mL)	LOD (%)	LOQ (%)
Omeprazole	51	0.30	0.102	0.004	0.008
Clopidogrel at 237 nm	–	–	–	–	–
Clopidogrel at 254 nm	55	0.311	0.097	0.003	0.008
Aspirin	32	0.90	0.114	0.026	0.048
Imp. A (omeprazole)	45	0.238	0.109	0.006	0.021
Imp. B (omeprazole)	107	0.054	0.172	0.004	0.041
Imp. C (omeprazole)	61	0.061	0.119	0.0003	0.011
Imp. C (Clopidogrel)	65	0.087	0.092	0.021	0.017
Imp. D (aspirin)	49	0.91	0.162	0.044	0.012

– Baseline noise obtained from the chromatogram of the placebo solution at the retention time of the analyte, calculated as the mean of six injections.

LOD - limit of detection, LOQ - limit of quantification.

Table 5: Relative response factors and accuracy data

Parameter	Clopidogrel				Aspirin		Omeprazole			
	Substance	Imp. A	Imp. B	Imp. C	Substance	Imp. D	Substance	Imp. A	Imp. B	Imp. C
RRF	–	1.91	1.31	1.34	–	1.01	–	0.94	1.31	1.34
Level 1 (%)	0.05	0.05	0.05	0.10	0.10	0.10	0.05	0.05	0.05	0.10
Recovery (%)	97.1	92.1	95.4	99.0	98.3	103.4	102.4	101.4	95.3	99.5
RSD (%)	1.3	0.6	1.3	1.5	2.7	0.8	2.3	0.9	1.0	2.1
95% confidence interval	99.5–101.7	97.1–109.4	91.7–97.1	96.6–103.5	91.7–97.1	96.6–103.5	91.4–92.2	94.9–112.5	93.4–95.4	95.1–98.3
Level 2 (%)	0.31	2.12	1.04	1.31	0.41	0.73	1.11	0.41	1.42	0.63
Recovery (%)	100.3	99.2	95.4	100.5	95.7	95.6	95.5	95.0	95.4	95.0
RSD (%)	0.5	0.6	0.4	1.3	1.4	1.7	0.9	1.1	0.7	1.3
95% confidence interval	94.6–96.3	94.9–96.3	94.5–96.3	94.3–95.7	98.6–102.3	94.6–96.9	99.5–101.2	99.0–99.4	94.5–96.3	94.3–95.7
Level 3 (%)	0.30	1.20	1.20	1.24	0.30	0.66	0.3	0.41	1.20	1.24
Recovery (%)	97.1	99.5	96.4	98.6	99.0	97.1	98.9	99.6	96.9	96.5
RSD (%)	1.0	1.2	1.3	0.7	0.4	1.0	1.2	1.4	0.9	0.3
95% confidence interval	93.9–99.0	94.7–95.8	99.5–100.3	98.9–100.1	94.8–96.7	94.7–96.3	97.6–100.9	94.7–96.4	94.8–96.7	94.7–95.8

RRF – relative response factor, calculated as a ratio of slopes of regression lines of the drug substance and its particular impurity.

Acceptance criteria[14].

0.05 ≤ c < 0.1%; recovery: 50.0–150.0%.

0.1 ≤ c < 0.5%; recovery: 70.0–130.0%.

0.5 ≤ c < 1.0%; recovery: 80.0–120.0%. c ≥ 1.0%; recovery: 90.0–110.0%.

Table 6: Robustness data expressed as resolution between adjacent peaks

Parameter	Standard conditions	Second column	% acetonitrile		pH of the buffer (2.5)		Salt concentration in the buffer (1.15 g/l)		Flow rate (0.8 mL/min)		Column temperature (30°C)	
Compound			--2%	+2%	2.2	2.8	1.04 g/l	1.27 g/l	0.7 mL/min	0.9 mL/min	25° C	35° C
Clopidogrel	7.9	8.1	7.8	7.9	7.7	8.0	8.0	8.0	8.0	7.6	8.3	7.4
Imp. A (Clopi)	13.9	12.0	14.1	13.2	14.2	14.3	14.1	14.0	14.5	13.3	14.9	13.1
Imp. B (Clopi)	2.4	2.4	3.1	1.9	2.4	2.4	2.6	2.5	2.6	2.4	2.7	2.4
Imp. C (Clopi)	6.2	6.1	6.2	6.2	6.2	6.1	6.3	6.4	6.2	6.2	6.4	6.2
Omeprazole	14.4	14.1	14.3	14.5	15.4	13.7	15.4	15.4	15.7	12.0	15.5	14.1
Imp. A (Omp)	3.3	4.1	3.5	3.0	3.3	3.2	3.3	3.3	3.3	3.2	3.4	3.2
Imp. B (Omp)	5.9	5.7	5.5	5.5	5.5	5.8	5.7	5.6	5.8	5.4	5.9	5.2
Imp. C (Omp)	26.9	25.7	27.5	24.9	26.4	26.2	26.9	26.7	25.9	26.8	26.9	25.3
Aspirin	0.76	0.84	0.84	0.71	0.77	0.76	0.77	0.77	0.87	0.74	0.81	0.67
Imp. D (Asp)	2.1	2.4	3.2	4.1	4.2	1.9	1.8	1.2	2.6	3.5	2.7	3.4

The first integrated peak in the chromatogram. The retention times in minutes are reported. Omp – omeprazole, Asp – aspirin, Clopi – Clopidogrel.

Stability of reference and sample solutions

The sample and standard solutions, used for the estimation of accuracy (Section 3.2.2), were utilized and estimated for stability as described under Section 2.4.5. The reference solution (Section 2.3.2) was identified to be stable upto 72 h stored at 10°C in the autosampler and also at room temperature exposed to sunlight, as the concentrations of the drugs showed maximum difference of 1.5% relative (0.0041% absolute) at a concentration level of 0.200% and therefore the difference was found to well within the limit (change ≤10% relative over the specified time). Upto 48 h at 10°C, the sample was stable, as the amount of impurities were in the range 0.01–2.86% relative and therefore within the acceptance criteria (change ≤10% over the specified time). The partial instability of omeprazole and its impurity C resulted in stability of the sample solution only for up to 36 h at ambient temperature. After that, the contents of two unknown omeprazole impurities RRT 1.25 and RRT 1.57 increased over the reporting limit (0.05%) and thus did not meet the acceptance criteria (no new impurity ≥ reporting limit) (12). In addition, the content of omeprazole impurity C decreased by 7.5% relative after 36 h at ambient temperature.

That value met the acceptance criteria (12) but the degradation was significant compared to the value of the solution stored in the autosampler at 10°C.

Table 7: Forced degradation study data

Stress condition	Omeprazole			Clopidogrel			Aspirin		
	Degradation products	Purity		Degradation products	Purity		Degradation products	Purity	
		Angle	Threshold		Angle	Threshold		Angle	Threshold
Thermal 65°C, 18 h	Imp. B	0.094	0.243	Imp. C	0.043	0.244	Imp. D	0.056	0.379
Hydrolytic (1mL H_2O) 65°C, 18 h	Imp. B, C, A	0.009	0.361	Imp. A	0.081	0.240	-	0.053	0.281
Acidic (3 mL 0.5 M HCl) 50°C, 2 h	Imp. B, C, A	0.062	0.269	Imp. C	0.034	0.290	Imp. D	0.021	1.304
Alkaline (2 mL 0.2 M NaOH) 50°C, 2 h	Imp. B, C	0.278	0.517	Imp. C	0.067	0.221	–	0.477	1.011
Oxidative (3 mL 3% H_2O_2) 50°C, 2 h	Imp. B	0.07	0.317	Imp. D	0.031	0.217	Imp D	0.060	0.402
Photolytic (daylight) 18 h	–	0.030	0.402	Imp. D	0.025	0.418	–	0.038	1.320

The peak is spectrally clear if the purity angle < the purity threshold.

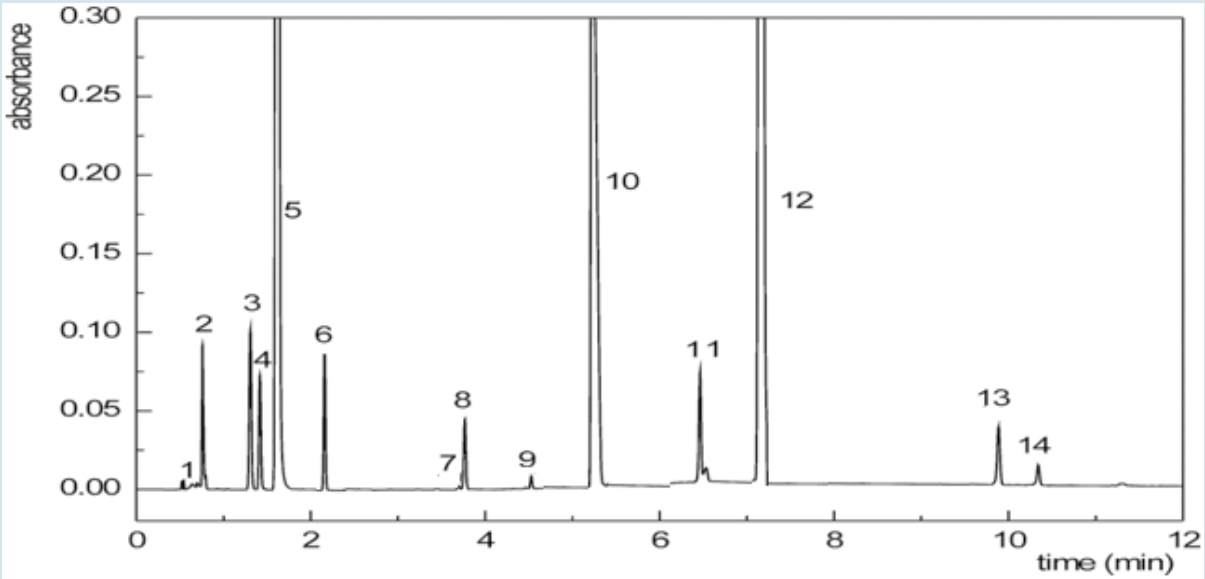


Figure 1: Chromatogram of the sample solution of formulated tablets spiked with impurities at limit concentration levels (Table 1). Evaluated at 254 nm.

Peaks:

(1) solvent peaks; (2) bisulphate; (3) impurity B (CLOPIDOGREL); (4) impurity A (CLOPIDOGREL);

(5) omeprazole; (6) impurity A (OMEPRAZOLE); (7) minor unknown impurity of omeprazole; (8) impurity C (CLOPIDOGREL); (9) impurity D (ASPIRIN); (10) Clopidogrel; (11) impurity C (OMEPRAZOLE); (12) aspirin; (13) impurity B (OMEPRAZOLE); (14) solvent peaks.

CLOPIDOGREL – omeprazole, ASPIRIN – Clopidogrel, OMEPRAZOLE – aspirin.

Forced degradation study

Degraded samples were estimated to justify the stability-indicating property of the method. Samples were stressed under the acidic, alkaline, oxidative, thermal, hydrolytic and photolytic conditions as described in the Section 2.5 and analyzed. Data of analyzed samples were evaluated. Degradation products were assigned to the proper drug substance on the basis of the UV spectra and preliminary experiments with separately stressed active pharmaceutical ingredients. All the impurities and the detected degradation products were satisfactory separated from each other (R≥1.2). The peak purity test passed successfully for the peaks of clopidogrel, omeprazole and aspirin in analysis of all the stressed samples and thus confirmed the spectral clearness of the principal peaks. Forced degradation studies data are summarized in Table 7 and represent the stability-indicating ability of the method. The method was found to be acceptable for the analysis of stability samples.

Clopidogrel

Clopidogrel degraded mainly to its impurity C. The content of impurity C increased under all the stress conditions including day-light. After 24 h of expose to day-light, the amount of impurity C increased twice and this resulted in the necessity of using amber glass for preparation of the sample solution. Similar to omeprazole, the main degradation was observed under hydrolytic stress conditions. Other degradation products including several unknown impurities were detected and data including peak purities are reported in Table 7.

Omeprazole

As mentioned in Sections 3.1.2 and 3.2.5, omeprazole was found to be a relatively unstable molecule. Impurity B of omeprazole was found to be the main degradation product as its content increased significantly under all the stress conditions with the exception of daylight conditions. The maximum degradation was observed under the hydrolytic stress condition as the content of impurity B increased 150 times in comparison with an unstressed sample. The impurities A and C were also found as degradation products under the hydrolytic stress conditions, as their contents increased two-fold and three-fold, respectively. Degradation of omeprazole under different conditions and peak purity data are reported in Table 7.

Aspirin

Aspirin was found to be relatively stable in comparison with clopidogrel and omeprazole. It degraded significantly only to impurity D under thermal, hydrolytic, acidic and oxidative stress conditions (Table 7). The content of impurity D increased over the reporting limit (0.05%) only under the thermal and hydrolytic stress conditions. Despite the degradation pathway, the limit for unknown impurities (0.2%) was found to be suitable for impurity D and thus it did not need to be validated as described in Section 3.2. No degradation of aspirin was observed under the alkaline and photolytic stress conditions. An increase in the contents of several unknown impurities was observed under oxidative and hydrolytic stress conditions. The list of degradation products and peak purity data are reported in Table 7.

CONCLUSION

A novel, fast, gradient-reversed phase UFLC method was developed and validated for separation of clopidogrel, aspirin, omeprazole and their impurities in tablet dosage form. The method successfully separated clopidogrel and related substances A, B and C, omeprazole and related substances A, B and C and aspirin Ph. Eur. related substance D and several unknown impurities of all the drug substances. The method is precise, linear, accurate, sensitive, specific, robust and stability-indicating. The method can be used as a routine quality control method for triple combined dosage form and also for stability studies.

ACKNOWLEDGEMENT

The authors express their sincere thanks to the Head of the Department, Dept. of Pharmaceutical Chemistry and The Principal, JSS College of Pharmacy, JSS University, Mysore for providing the necessary facilities to carry out the research work.

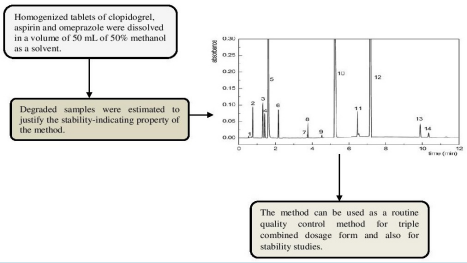
CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Ahuja S. Assuring quality of drugs by monitoring impurities. Adv. Drug Deliv. 2007;59(1):3-11. http://dx.doi.org/10.1016/j.addr.2006.10.003 ; PMid:17218038.
2. Gorog S. Drug safety, drug quality, drug analysis. J Pharm Biomed Anal. 2008;48(2):247-53. http://dx.doi.org/10.1016/j.jpba.2007.10.038; PMid:18082992.
3. Gumustas M, Kurbanoglu S, Uslu B, Oskan SA. UPLC versus HPLC on drug analysis: advantageous, Appl. Valid. Parameters Chromatogr. 2013;76 (21-22):1365-427.
4. European Pharmacopoeia. European Directorate for the Quality of Medicines & HealthCare. 2014;8.2.
5. USP 37, The United States Pharmacopeial Convention, 2014.
6. Krishnaiah C, Reddy AR, Kumar R, Mukkanti K. Stability -indicating UPLC method for determination of Aspirin and their degradation products in active pharmaceutical ingredient and pharmaceutical dosage forms. J Pharm Biomed Anal. 2010;53:483-9. http://dx.doi.org/10.1016/j.jpba.2010.05.022 ; PMid:20646890.
7. Ramadan NK, Mohamed HM, Moustafa AA. Rapid and highly sensitive HPLC and TLC methods for quantitation of Clopidogrel besilate and aspirin in bulk powder and in pharmaceutical dosage forms and in human plasma, Anal. Lett. 2010;43(4):570-81. http://dx.doi.org/10.1080/00032710903406953.
8. Ivanovic D, Malenovic A, Jancic B, Madenica M, Maskovic M. Monitoring of impurity level of aspirin and omeprazole employing RP-HPLC gradient mode. J Liq Chromatogr Relat Technol. 2007;30(19):2879-90. http://dx.doi.org/10.1080/10826070701588638.
9. USP 37 Second supplement, The United States Pharmacopeial Convention, 20.10.2014. ICH Q2 (R1) Validation of Analytical Procedures: Text and Methodology, 2005.
10. Lo Brutto R, Patel T. Method validation, in: Y. Kazakevich, R. LoBrutto (Eds.), HPLC for Pharmaceutical Scientists. John Wiley & Sons, Hoboken; 2007:455-502. http://dx.doi.org/10.1002/9780470087954.ch9.
11. Crowther JB, Validation of pharmaceutical test method, in: S. Ahuja, S. Scypin- sky (Eds.), Handbook of Modern Pharmaceutical Analysis, Academic Press. San Diego; 2001:415-43. http://dx.doi.org/10.1016/s0149-6395(01)80014-3.
12. ICH Q2 (R1) Validation of Analytical Procedures: Text and Methodology, 2005

PICTORIAL ABSTRACT



SUMMARY

- A novel, sensitive and robust stability indicating UFLC method was developed and validated for estimation of clopidogrel, aspirin, omeprazole, related substances and impurities in tablet dosage form.
- The pharmaceutical tablets were exposed to acid, alkaline, thermal, higher humidity, oxidative and photolytic stress conditions. Samples undergone stressed conditions were analyzed by the novel validated method.
- The method can be used routinely to test the adulteration in the pharmaceutical formulations of clopidogrel, aspirin, and omeprazole.

ABOUT AUTHORS

Jinesh Bahubali Nagavi: Is a Research Scholar at JSS College of Pharmacy, JSS University, Mysuru. He has work experience as an executive in Neopharma, UAE and as a Lecturer in RAKMHSU, UAE for 4 years. His main research interests are in the area of Bioanalytical method validation, drug-drug interaction and pharmacokinetic studies. He has published and presented his research widely in various international journals and conferences.

Gurupadayya Bannimath: Currently working as a Professor in the Department of Pharmaceutical chemistry at the JSS College of Pharmacy, JSS University, Mysuru. He has obtained his Ph.D. Degree in 1996 from Kuvempu University, Shimoga. His main research interests are in the areas of drug/herb interaction, chiral and herbal compound analysis.