



Stability-Indicating UFLC Method For Uncoupling And Estimation Of Impurities In Clopidogrel, Aspirin And Omeprazole In Their Tablet Dosage Form Using PDA Detection

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Abstract

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. 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 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. 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. Separation was satisfactory for all the significant degradation products from the principal peaks of drug substances and the impurities from each other. 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.

Keywords: Clopidogrel, aspirin, omeprazole, LC, ICH, PDA.

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1. Introduction

Registration authorities compulsorily require the purity testing method, which is an important part of the method development for a drug 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 Pharmacopoeia⁵. 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 D⁴ and omeprazole Ph. Eur. impurity B⁴ in combination was published under reversed phase HPLC conditions [9]. Omeprazole impurities A, B and C named according to Ph. Eur.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 D⁴ 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 for determination of their impurities 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.

2. Material and Methods

2.1. Chemicals

Reference materials of aspirin, clopidogrel, omeprazole and samples of 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 mobile phase and 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 for chromatography was treated by the Milli-Q system from Merck Millipore (Billerica, USA).

2.2. Chromatographic Conditions

The experiments were performed on the Shimadzu prominence UFLC system with column thermostat and PDA detector from Shimadzu (SPD-M20A). Data were collected and evaluated by LC Solutions software from Shimadzu. UFLC column Phenomenex C8 250 mm × 4.6 mm, 5μ particle size, from Shimadzu (Santa Clara, CA), thermostatted at 28°C was used for the separation. The mobile phase 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 sample temperature was set at 25°C and the injection volume was 10μl. Data 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 its the impurities, 280nm was selected as the wavelength for this method. The PDA detector operated at sampling rate 20 points per second. A Mark ultrasonic bath from RCSsystems (Bangalore, Karnataka, India) was used for sample sonication. Samples were centrifuged with an eppendorf centrifuge 5810R from Eppendorf AG (Hamburg, Germany).

2.3. Preparation of solutions

2.3.1. Sample Solvent 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).

2.3.2. Sample Solution Preparation

Twenty tablets of clopidogrel, aspirin and omeprazole were thoroughly homogenized. An amount of 750 mg of the homogenized sample was weighed into a 50mL amber volumetric flask and 40mL of sample solvent were added. The sample was put in an ultrasonic bath and 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 with respect to their contents in the formulated tablets (Table 1).

2.3.3. Standard Solution Preparation

Aspirin, clopidogrel and omeprazole reference materials were dissolved in the sample solvent corresponding to the concentration level to 0.5% of the concentration of the sample solution for all the drug substances.

2.3.4. Placebo Solution Preparation

An amount of 400 mg of homogenized placebo (mainly composed from microcrystalline

Table 1. Composition of the formulated triple tablets.

Compound	Amount (mg)	Concentration		Impurity	Limit (%)
		in sample	solvent (mg/ml)		
lopido grel	75	13.5		Impurity A	0.3
				Impurity B	0.2
				Impurity C	0.2
				Unidentified Impurity	0.2
Omepra zole	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

cellulose, stearate and anhydrous colloidal silica) was weighed into a 50 mL amber volumetric flask. Then the placebo solution was prepared in the same way as the sample solution.

2.4. Method Validation

The method was validated according to the ICH Q2 (R1) guideline [12] for clopidogrel impurities, aspirin impurity D and omeprazole impurities. The selectivity of the method was confirmed for all the above mentioned impurities with addition of the other 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 exceed the limits during preliminary stability studies of the tablets and therefore it was not fully validated.

2.4.1. Precision

The repeatability of the method was verified by analyzing the six replicate samples of the tablets and the % RSD values of the contents of the detected impurities were calculated. The intermediate precision was analyzed by injecting six replicate samples of the same batch of formulated tablets by a different analyst in a different laboratory on a different day using a different column (same type, different batch). The % RSD values of the impurities content were calculated for all the replicates (two analysts together).

2.4.2. 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 dissolved in the sample solvent and then spiked into the weighed sample of tablets at five concentration levels, three samples per 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 also prepared to rectify the amount of impurities which were originally identified. The linearity and accuracy for determination of drug substances were also evaluated for quantification of unknown impurities. Reference materials of aspirin, omeprazole and clopidogrel were dissolved in the sample solvent and spiked into the weighed sample of placebo at five concentration levels, three samples per level. These samples were treated according to the procedure of the placebo solution preparation (Section 2.3.4). The volume of sample solvent was reduced by the volume of spikes of impurities. The linearity was calculated for each impurity and each drug from the whole range of concentrations. Samples for evaluation of the linearity and accuracy were prepared corresponding to the concentrations of the related drugs in the sample solution (Section 2.3.2) 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 each impurity and each drug as the percent recovery of the sum of the impurity/drug that was added to the sample at three selected levels across the range of concentrations.

2.4.3. Selectivity

The selectivity of the method was confirmed by analysis of the sample spiked with all the available impurities. Impurities were spiked relative to the concentrations of the related drugs in the sample solution (Section 2.3.2). 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 solvent and the placebo solution were obtained. The stability-indicating property of the method was tested by performing the forced degradation study (see Section 2.5). In order to confirm that there was no interference of any unknown impurity with the principal peaks of the drugs, peak purity test was done.

2.4.4. Robustness

The robustness of the method was examined by varying the chromatographic conditions, such as the column temperature ($\pm 5^\circ\text{C}$), flow rate (± 0.3 mL/min), pH of the buffer (± 0.5), volume of acetonitrile at all points of the gradient ($\pm 3\%$), concentration of salt in the buffer ($\pm 15\%$) and particular column used. The spiked sample prepared in the same way as the sample for evaluation of the selectivity (Section 2.4.3) was analyzed and the retention times of all the impurities and principal peaks were monitored.

2.4.5. Stability of the Sample and Standard Solutions

One of the sample solutions from the linearity evaluation (Section 2.4.2) was used for a stability study of the sample solution. The sample was stored in an autosampler at 10°C in the dark

as well as at ambient temperature not protected from the light. It was analyzed 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 absolute differences in the contents of the drug and the impurities at the beginning and at the end of the stability study were evaluated.

2.5. 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 Section 2.3.2. 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.

2.5.1. 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.

3. Results And Discussion

3.1. Method development and optimization

3.1.1. 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 mm × 4.6 mm, 5 μ) 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 μ L. 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 μ L) 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 μ L 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 tested under these conditions and both of them yielded similar elution profile to the Phenomenex C8 4.6mm column but underwent distortion of the omeprazole peaks. Finally, the column Phenomenex C8 (250 mm × 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 μ L 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 ($R \geq 2.0$). 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.

3.1.2. 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.

3.2. Method Validation

3.2.1. Precision

The intermediate precision and repeatability were determined as mentioned in Section 2.4.1. Impurities present in the sample were determined as a percent of the amount of related drug in one tablet against the calibration obtained by injection of a standard solution. The % RSD of the content was calculated for one unknown impurity of aspirin, Clopidogrel impurity C and omeprazole impurities A, B and C, which were present in the samples. The % RSD values for five injections peak area of standard solution were also calculated. The intermediate precision was evaluated as the % RSD values for all the results obtained by the first and second analysts. The values of RSD \leq 0.5% for five injections of the standard solution and RSDs 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 the contents 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).

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.

3.2.2. Linearity, Accuracy, LOD and LOQ

The linearity and accuracy of the method were verified by analyzing the spiked samples of tablets and placebo as described in Section 2.4.2. Recovery was calculated based on the relative response factor for each impurity. Based on the peak areas of the impurities the relative response factor was calculated as the ratio of the slopes of the linearity regression lines of the drug substance and its particular 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, as well as the fact that points in the residual plots were randomly distributed around the horizontal axis. The LOD and LOQ values were determined as the ratios $3.3 \times \sigma / S$ and $10 \times$

σ / S , respectively, where σ is the mean value of the baseline noise obtained from six chromatograms of the placebo and S is the slope of the regression line (based on peak heights) obtained from the linearity data. The LOD and the LOQ data are reported in Table 4 and showed satisfactory sensitivity of the method. The relative response factors and recovery data are reported in Table 5 and indicated that the method is accurate, since the recovery values were in the range 92–104%. The RSD value of the recovery $\leq 4\%$ demonstrated the repeatability of the determination for all the spiked 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.

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	$y = 1.9347 \times 10^4 x - 131$	211	0.51	89
Imp. A (Clopidogrel)	0.05–1.00	1.000	$y = 9.4960 \times 10^3 x + 34$	46	1.5	911
Imp. B (Clopidogrel)	0.05–1.00	1.000	$y = 1.9135 \times 10^4 x - 213$	102	0.9	132
Imp. C (Clopidogrel)	0.05–1.14	0.999	$y = 6.9387 \times 10^4 x - 511$	209	3.2	315
Aspirin	0.05–0.30	1.000	$y = 1.0349 \times 10^6 x + 1723$	412	0.9	295
Imp. D (Aspirin)	0.05–0.41	1.000	$y = 4.8862 \times 10^4 x - 197$	296	1.1	396
Omeprazole	0.05–0.30	0.999	$y = 7.1284 \times 10^4 x - 304$	34	0.93	217
Imp. A (Omeprazole)	0.10–0.60	0.999	$y = 1.9448 \times 10^4 x + 36$	37	2.0	166
Imp. B (Omeprazole)	0.10–0.30	0.999	$y = 1.1037 \times 10^6 x + 649$	24	2.8	931
Imp. C (Omeprazole)	0.10–0.50	1.0000	$y = 9.3291 \times 10^4 x + 46$	47	1.7	159

Regression equation – relationship between concentration and peak area.

Acceptance criteria¹⁴. 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	λ (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.

3.2.3. Selectivity

The selectivity of the method was examined by analyzing a sample spiked considering all the available impurities at their limit levels (Table 1), as mentioned in Section 2.4.3. The chromatograms of the placebo solution and sample solvent were also obtained to examine the possible interferences. All the peaks of the sample solvent and placebo solution were separated from the peaks of the impurities and drugs in the sample solution and are designated in the chromatogram of the spiked sample (Figure 1). Stability-indicating ability of the method was confirmed by carrying out the forced degradation study (Section 3.3). 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

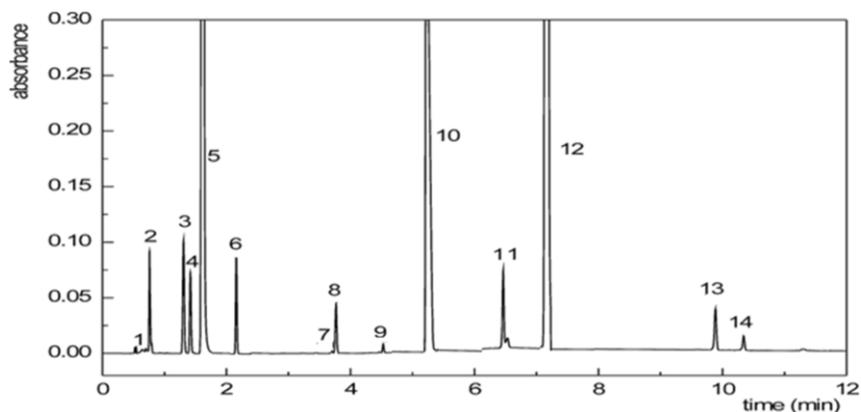


Figure. 1. Chromatogram of the sample solution of formulated tablets spiked with impurities at limit concentration levels (Table 1). Evaluated at 254 nm.

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.

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.

3.2.4. Robustness

The robustness of the method was tested by changing the variable chromatographic conditions as described in Section 2.4.4. 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 showed that the method is 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 ($R \geq 1.9$).

3.2.5. Stability of reference and sample solutions

The reference and sample solutions, the same solutions as those used for evaluation of the accuracy (Section 3.2.2), were stored and analyzed as described under Section 2.4.5. The reference solution (Section 2.3.2) was found to be stable for up to 72 h stored in the autosampler at 10°C and also at ambient temperature not protected from the light, as the maximum difference in the concentrations of the drugs was 1.5% relative (0.003% absolute) at a concentration level of 0.200% and therefore the difference was within the acceptance criteria (change $\leq 10\%$ relative over the specified time)⁵⁹. The sample solution was

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].

$0.05 \leq c < 0.1\%$; recovery: 50.0–150.0%.

$0.1 \leq c < 0.5\%$; recovery: 70.0–130.0%.

$0.5 \leq c < 1.0\%$; recovery: 80.0–120.0%. $c \geq 1.0\%$; recovery: 90.0–110.0%.

found to be stable for up to 48 h when stored in

the autosampler at 10°C, as the difference in the contents of impurities was in the range 0.01–2.86% relative and therefore within the acceptance criteria (change $\leq 10\%$ over the specified time)⁵⁹. 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 \geq reporting limit)[12]. In

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)	
							1.04 g/l	1.27 g/l	0.7 mL/min	0.9 mL/min	25 °C	35 °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.

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.

3.3. Forced Degradation Study

Degraded samples were analyzed to confirm the stability-indicating property of the method. The 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

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 ₂ O) 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 ₂ O 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.

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 satisfactorily separated from each other ($R \geq 1.2$). The peak purity test successfully passed 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.

3.3.1. 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 exposure 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 (Section 3.3.2). Other degradation products including several unknown impurities were detected and data including peak purities are reported in Table 7.

3.3.2. 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.

3.3.3. 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.

4. 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.

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