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Short communication

# Development and validation of a liquid chromatographic method for purity control of clopidogrel–acetylsalicylic acid in combined oral dosage forms

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ABSTRACT

A reversed phase liquid chromatographic method with UV detection for the simultaneous determination of clopidogrel and acetylsalicylic acid and their related substances in combined oral formulations was developed and validated. Good separation was achieved on a Luna C18 column (150 mm × 4.6 mm, 3 μm) using gradient elution at a flow rate of 1 mL/min and a column temperature of 35 °C. UV detection was performed at 220 nm. The validation was performed according to the ICH guidelines. The method proved to be specific, sensitive (LOQ = 0.975 μg/mL and 0.0384 μg/mL for clopidogrel and acetylsalicylic acid, respectively), linear in the concentration range from LOQ to 325 μg/mL for clopidogrel and from LOQ to 650 μg/mL for acetylsalicylic acid, precise (RSD values for intermediate precision <1%) and accurate with mean recovery values of 100.7% and 100.2% for clopidogrel and acetylsalicylic acid, respectively. Moreover, the solution stability and method robustness were examined. The method gives satisfactory separation of impurities of clopidogrel and acetylsalicylic acid and so it is suitable for quantification of the related substances as well as for the assay of the actives.



## 1. Introduction

Clopidogrel (CLP) and acetylsalicylic acid (ASA) (Fig. 1) are two widely used antithrombogenic agents and coadministration produces an enhanced therapeutic effect in many clinical conditions, particularly in high-risk patients with acute coronary syndromes [1]. Thus, their mechanisms are complementary and may decrease clot formation over either agent alone.

The synthesis of an active pharmaceutical ingredient (API) normally consists of several synthetic steps. The process related impurities can be formed at any step and could ultimately appear in the final drug substance. Impurities may also develop upon aging of both API and formulated APIs to medicines [2]. In addition, for an optically active single isomer drug there could be enantiomeric impurities present in the API. So, to guarantee the quality, safety and efficacy of drugs, sensitive and selective control methods are advisable. The chemical structures for impurities of CLP (usually used in its hydrogen sulfate form) and ASA are depicted in Figs. 2 and 3 [3,4].

A number of analytical methods have been reported in the literature for the individual determination of CLP or ASA. These methods include: spectrofluorimetry [5], reversed phase liquid chromatography (LC) [6–9], thin-layer chromatography [10], and spectrophotometry [11,12]. The simultaneous assay determination of CLP and ASA in tablets and capsules was also reported [13,14]. Moreover, a chemometric approach for the simultaneous estimation of CLP and ASA from combined dosage forms has been described by Rajput et al. [15]. For the determination of related substances in pharmaceutical dosage forms, several analytical methods [16,17] based on LC were reported for ASA. Concerning CLP, LC methods have been described for purity control of bulk drugs and pharmaceutical dosage forms including an enantiospecific LC method [18,19]. Both the European Pharmacopoeia (only drug substance) and United States Pharmacopeia (also drug products) describe separate LC methods for the determination of CLP and ASA and their respective impurities [3,4]. However, no LC method for the simultaneous analysis of CLP, ASA and their related substances in combined dosage forms has been reported so far. In this study, a reversed phase LC method for this purpose was developed and validated.

## 2. Experimental

### 2.1. Reagents and samples

Reference substances for CLP, ASA and their impurities, as well as Coplavix® 75/100 (combination tablets containing 75 mg of CLP/tablet and 100 mg of ASA/tablet) were kindly provided by Sanofi-Aventis Groupe (Paris, France). Two other tablet formulations containing 75 mg of CLP/tablet and 75 mg of ASA/tablet

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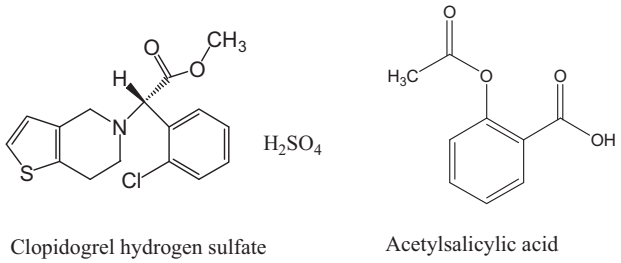



Clopidogrel hydrogen sulfate Acetylsalicylic acid

**Fig. 1.** Chemical structures of clopidogrel hydrogen sulfate and acetylsalicylic acid.

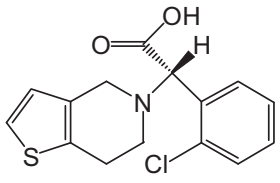
(Closprin® 75/75) or 150 mg of ASA/tablet (Noklot Plus® 75/150) were from Venus international (Mumbai, India) and Hetero Labs Ltd. (Kalyanpur, India), respectively. Sodium octane sulfonate monohydrate was purchased from Acros Organics (Geel, Belgium), HPLC gradient grade acetonitrile was from Fisher Scientific (Leicestershire, UK), HPLC grade methanol from Biosolve Ltd. (Valkenswaard, The Netherlands) and phosphoric acid, 98% purity from BDH (Briare, France).

### 2.2. Apparatus

LC analyses were performed on a Dionex Ultimate 3000 system from Dionex Softron GmbH (Germering, Germany) equipped with a high pressure pump (LPG-3400A), autosampler (WPS-3000T SL) and UV/VIS detector (MWD-3000). For data processing and acquisition, Chromeleon software version 6.80 from Dionex was used. An ultrasonicator from Branson Ultrasonics Corporation (Danbury, CT, USA) and a pH meter from Metrohm (Herisau, Switzerland) were used.

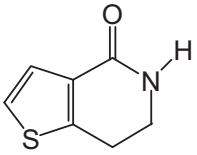
### 2.3. Chromatographic conditions

Chromatographic separations were achieved on a Luna C-18 (150 mm × 4.6 mm, 3 μm) column (Phenomenex, CA, USA). A Julabo

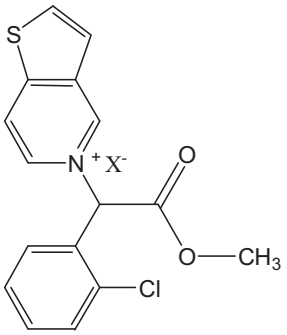



Imp. C1 (SR 26334)
(acid derivative)

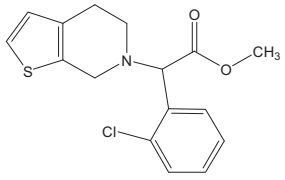
Ph. Eur. : Impurity A
USP : Related compound A




Imp. C4 (PCR 2482)

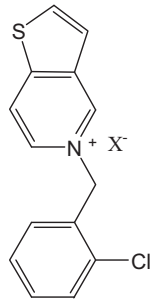



Imp. C7 (SR 26577)

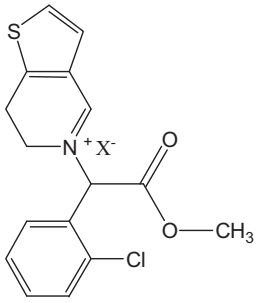



Imp. C2 (SR 24726)
(regio-isomer)

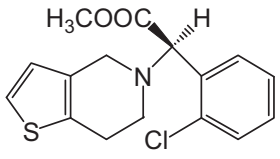
Ph. Eur. : Impurity B
USP : Related compound B




Imp. C5 (PCR 0625)

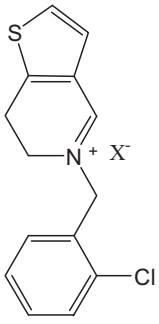



Imp. C8 (SR 120867)



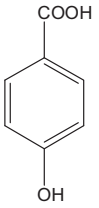

Imp. C3 (SR 25989)
(enantiomer)

Ph. Eur. : Impurity C
USP : Related compound C

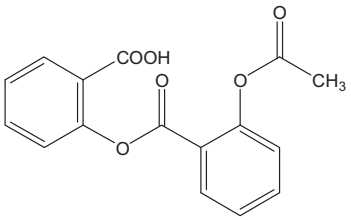



Imp. C6 (PCR 2549)

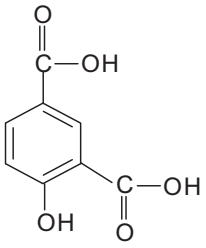
**Fig. 2.** Chemical structures of impurities of clopidogrel.



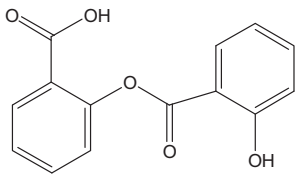

Imp. A1 = 4-hydroxybenzoic acid



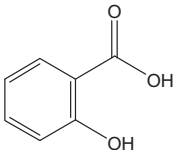

Imp. A4 = acetylsalicylsalicylic acid



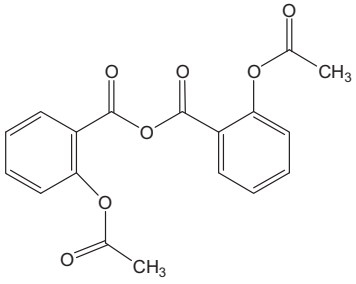

Imp. A2 = 4-hydroxyisophthalic acid




Imp. A5 = salicylsalicylic acid




Imp. A3 = salicylic acid




Imp. A6 = acetylsalicylic anhydride

**Fig. 3.** Chemical structures of impurities of acetylsalicylic acid.

EM immersion thermostat (Seelbach, Germany) was used to keep the water bath of the column at 35 °C. The mobile phase was a gradient mixture of mobile phase A (5:95 (v/v) mixture of methanol and a 1.0 g/L solution of sodium octane sulfonate monohydrate adjusted to pH 2.5 with dilute phosphoric acid) and mobile phase B (5:95 (v/v) mixture of methanol and acetonitrile) pumped at a flow rate of 1 mL/min. The gradient programme [time (min)/%B] was set as 0/10.5, 3/10.5 to 48/68.5, 68/68.5 to 80/10.5. The injection volume was set at 10 μL and the UV detection was made at 220 nm.

### 2.4. Assay determination of CLP and ASA

#### 2.4.1. Preparation of sample and reference solutions

*Sample solution*: Twenty tablets from each Coplavix®, Closprin® and Noklot Plus® products were weighed and finely powdered with a mortar and pestle. A quantity of the powder equivalent to 20 mg of CLP and 26.67 mg (Coplavix®), 20 mg (Closprin®) or 40 mg (Noklot Plus®) of ASA was transferred into a 20.0 mL volumetric flask, sonicated with solvent mixture (mobile phase A – acetonitrile (40:60, v/v)) for ten minutes and then the solution was completed to volume with the same solvent. This solution was filtered through a 0.2 μm nylon filter (Whatman, Dassel, Germany) and 1.0 mL of the filtered solution was diluted to 10.0 mL with the solvent mixture. An aliquot of this solution was used for analysis.

*Reference solutions*: A stock reference solution of CLP was prepared by dissolving 26.10 mg of clopidogrel hydrogen sulfate (equivalent to 20 mg of CLP) in 20.0 mL of solvent mixture. A similar stock reference solution of ASA (20 mg/20 mL) was made. Since the content of ASA is different for the three products, 2 different dilutions were prepared: 1.0 mL of the stock reference solution of CLP and 1.0 mL of the stock reference solution of ASA were diluted to 10.0 mL (reference solution used for Coplavix® and Closprin®) while 1.0 mL of the stock reference solution of CLP and 2.0 mL of the stock reference solution of ASA were diluted to 10.0 mL with the solvent mixture (reference solution used for Noklot Plus®). For Coplavix®, peak areas of ASA reference were corrected with a factor 1.33 to compensate for the higher amount of ASA compared to Closprin® (100 mg vs. 75 mg).

### 2.5. Determination of related substances

#### 2.5.1. Preparation of test and reference solutions

*Test solutions*: From the tablet powder used for assay, a quantity equivalent to 65 mg of CLP and 86.67 mg, 65 mg and 130 mg of ASA for Coplavix®, Closprin® and Noklot Plus®, respectively, was dissolved in a 10.0 mL volumetric flask and sonicated with the solvent mixture for 10 min. The solutions were cooled to room temperature and completed to volume with the same solvent. The solution was filtered through a 0.2 μm nylon filter to ensure the absence of particulate matter before analysis.

*Reference solution*: 1.0 mL of the test solution was diluted to 100.0 mL with the solvent mixture. 1.0 mL of this solution was further diluted to 10.0 mL (=0.1%) with the same solvent.

### 2.6. Method validation

The proposed method was validated according to the ICH guidelines [20] for its specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, solution stability and robustness. Moreover, relative response factors for the impurities of CLP and ASA were also determined.

#### 2.6.1. Specificity

The specificity of the developed method was examined for the presence of possible interference from excipients or sample matrix by overlaying chromatograms of (1) APIs spiked with impurities, (2) the drug products and (3) the blank.

#### 2.6.2. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for CLP and ASA were determined based on a signal-to-noise ratio of 3 and 10, respectively. Precision at the LOQ level was determined as % RSD for both APIs.

#### 2.6.3. Linearity

Linearity was examined for the assay of the APIs and for the related substances. ASA: For the assay method, five concentrations (50 μg/mL, 100 μg/mL, 150 μg/mL, 200 μg/mL and 250 μg/mL) which cover the different concentrations of ASA in the tablets (75, 100, 150 mg/tablet) were injected. For related substances, concentrations ranging from LOQ to 650 μg/mL of the test solution were examined. CLP: Similarly, for the assay method, five concentrations ranging from 25 to 125% of the assay analyte concentration (25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL and 125 μg/mL) and for the related substances concentrations ranging from LOQ to 5% of the test solution (100% = 6.5 mg/mL) were examined.

#### 2.6.4. Precision and accuracy

Repeatability was examined by three fold analyses of two preparations of 100 μg/mL of CLP and 200 μg/mL of ASA in one day. Between days variation (intermediate precision) was examined on five consecutive days. The % RSD on the peak areas was evaluated. Accuracy of the proposed method was determined by the standard addition method on the dosage form to which known amounts of CLP and ASA standards have been added at different concentrations (CLP: 7.8 μg/mL, 15.6 μg/mL, 23.4 μg/mL; ASA: 20 μg/mL, 40 μg/mL, 60 μg/mL). The determination was carried out using three replicates at each concentration level. The accuracy was determined as percent recovery of amount of analyte added to the sample.

#### 2.6.5. Robustness

To evaluate the robustness of the method, experimental factors that might cause variability in the method responses were examined. 4 factors (column temperature, pH, the time at which percent v/v of mobile phase B reaches its maximum and the percent v/v of mobile phase B at its plateau) were investigated. A two-level full factorial experimental design with $2^k + n$ number of runs, where $k$ is the number of factors and $n$ is the number of center points was applied. Thus, 19 experiments including three center points were performed in duplicate. The design was generated by using Modde 5.0 Umetrics software (Umeå, Sweden). For this test, one lower value and one higher value of the factors were used. The different values used for each factor in the design are given in Table 1. In every experiment, as response, the amount of impurities of CLP and ASA were calculated against a reference solution of 0.1%.

#### 2.6.6. Response factors

The response factors of the impurities were determined from the ratio of the slopes of the impurities and the APIs. Solutions were prepared at concentrations from LOQ to 65 μg/mL of the test concentration for all impurities and APIs.

**Table 1**
Chromatographic parameter settings applied in the robustness investigation, corresponding to low (−), central (0) and high (+) levels.

| Parameter | Low value (−) | Central value (0) | High value (+) |
|---|---|---|---|
| Temperature (°C) | 33 | 35 | 37 |
| pH | 2.3 | 2.5 | 2.7 |
| MP B (%, v/v) | 67 | 68.5 | 70 |
| MP B (time, min) | 46 | 48 | 50 |

MP: mobile phase.

## 3. Results and discussion

### 3.1. Optimization of the chromatographic conditions

The starting conditions were taken from the European Pharmacopoeia monograph for clopidogrel hydrogen sulfate [3]. Three different columns have been tested for the separation of the specified and unspecified impurities of both APIs from the principal peaks: Luna C18 column (150 mm × 4.6 mm, 3 μm), Kromasil C18 column (150 mm × 4.6 mm, 5 μm) and Symmetry C18 column (150 mm × 4.6 mm, 3.9 μm). The specified impurities were better separated from the main peaks of CLP and ASA using the Luna column. Using the Kromasil column, impurity C1 and ASA were not well separated. Similarly, impurity C1 and ASA were coeluted using the Symmetry column. As a consequence, the Luna column was used for further method development and optimization. To optimize the chromatographic conditions for satisfactory separation of the APIs and their impurities, the effect of different chromatographic parameters was examined. The percentage of the organic modifier (acetonitrile) was changed from 37.5% to 80% in different gradient runs. The temperature of the column was varied from 25 °C to 40 °C (25 °C, 30 °C, 35 °C and 40 °C). Similarly the effect of pH was examined at 2.0, 2.5, 3.0 and 3.5. The concentration of the ion pairing reagent (IPR) (sodium pentane sulfonate monohydrate) was changed from 4.8 mM, 5.0 mM, 5.5 mM, 5.9 mM, 7.5 mM till 10.0 mM. Sodium pentane sulfonate monohydrate is the IPR used in the European Pharmacopoeia monograph for clopidogrel hydrogen sulfate in mobile phase A. However, not all impurities of CLP and ASA could be well separated. Changing the IPR to sodium octane sulfonate (SOS) monohydrate (investigated in the range 3.9–4.9 mM) did substantially improve the selectivity. It was found that a column temperature of 35 °C and an IPR concentration of 4.3 mM SOS in mobile phase A at pH 2.5 were the optimum conditions for the separation of the APIs and their impurities (Fig. 4).

### 3.2. Specificity

The specificity of the developed method was determined by examining the presence of possible interference from excipients or sample matrix. The chromatogram overlay of the spiked mixture of impurities and APIs, the drug product and the blank showed that the proposed method is specific for both APIs and their related substances as there was no interference observed. Absence of interference was verified for all forms. As the method is not enantiospecific, impurity C3 (=R-enantiomer of CLP) cannot be determined.

### 3.3. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for CLP and ASA were determined by injecting a series of dilutions of known concentrations of the analytes. It was found that for CLP, the LOD and LOQ were 0.3 μg/mL and

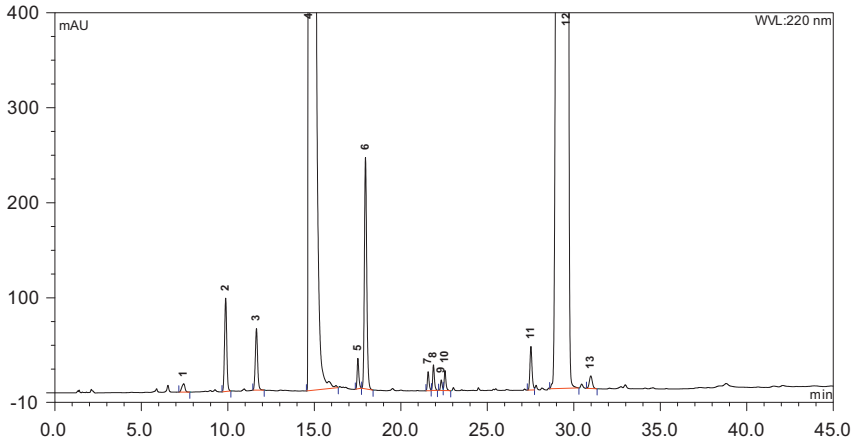



**Fig. 4.** Typical chromatogram of a solution of APIs spiked with all available impurities. Chromatographic conditions: see Section 2.3. **1**. Impurity A1; **2**. Impurity A2; **3**. Impurity C4; **4**. Aspirin; **5**. Impurity C1; **6**. Impurity A3; **7**. Impurity C5; **8**. Impurity C6; **9**. Impurity C7; **10**. Impurity C8; **11**. Impurity A4; **12**. Clopidogrel; **13**. Impurity C2.

1.0 μg/mL (RSD = 0.6%), respectively and for ASA, the LOD and LOQ were 0.04 μg/mL and 0.13 μg/mL (RSD = 0.6%), respectively.

### 3.4. Linearity

In the examined concentration ranges described under Section 2.6.3, linear responses were observed between the peak areas and the concentration of the analytes. The points in the residual plots were randomly distributed around the horizontal axis. The random dispersion of the residuals suggests that the linear model gives a good fit of the data. The coefficients of determination of the regression lines, the standard error of estimate and the linear regression equations are shown in Table 2.

### 3.5. Precision and accuracy

The precision of the method was evaluated as repeatability and intermediate precision. Repeatability was examined by three fold analyses of two preparations of 100 μg/mL of CLP and 200 μg/mL of ASA in one day. The RSD on the peak areas of these six determinations was not more than 0.5%. Intermediate precision was also determined for five consecutive days. The RSD on the peak areas ($n$ = 30, 5 days) was not more than 1.0% (Table 2) suggesting that the proposed method is suitable for simultaneous analysis of CLP and ASA in combined dosage forms. In addition, the between days (intermediate) precision suggests that the developed method gave repeatable results for five consecutive days. Accuracy of the method was determined as percent recovery of a known added amount of analyte to the sample. The proposed method was found to give a mean recovery of 100.7% for CLP and 100.2% for ASA in the examined dosage form. So, the developed method gave satisfactory recoveries for both CLP and ASA.

### 3.6. Robustness test

A two-level full factorial experimental design was used to check the robustness of the LC method by introducing intentional variation of the experimental factors as described in Section 2.6.5. From the regression coefficient plots of the impurities it can be concluded that the percentage of impurities A1, A4, C2, C6 and C8 was not significantly ($\alpha$ = 0.05) affected when the chromatographic parameters were varied as shown in Table 1. Temperature and pH variations affect somewhat the percentage of impurities A2, C1, C4, C5 and C7 in the range examined. Although the effects are significant, their absolute value is small. The assay values of CLP and ASA were not affected by these small variations of the chromatographic factors investigated. Nevertheless, it is advisable to control these effects rather strictly.

### 3.7. Relative response factor

The results for the relative response factors of each impurity versus the APIs are shown in Table 3. The purity of the reference impurities, as given by the supplier, was taken into account.

### 3.8. Application to real samples

The proposed method was applied for the determination of CLP and ASA in three tablet products. The assay results are shown in Table 3. According to the USP specification, CLP or ASA tablets contain not less than 90.0% and not more than 110.0% of the labeled amount of CLP or ASA [4]. Hence, the content of ASA in the two generics (Closprin® and Noklot Plus®) does not comply with the prescribed limits stipulated by the USP. Next, impurities of CLP and ASA were quantified in the three products. The disregard limits for CLP and ASA impurities were 0.05% and 0.03%, respectively. Impurities of CLP were calculated versus the diluted CLP reference (0.1% dilution of the test solution) while impurities of ASA were calculated versus the diluted ASA reference (0.1% dilution of the test solution). The results are shown in Table 3. The level of impurity C1 in Closprin® was above the 1.2% limit of the USP monograph of CLP tablets [8]. In Closprin® and Noklot Plus®, the amount of impurity

**Table 2**
Results of linearity and intermediate precision determinations.

| Method | APIs | Concentration range (μg/mL) | Regression equation | $R^2$ | $S_{y,x}$ | Intermediate precision (RSD, $n$ = 30) |
|---|---|---|---|---|---|---|
| Assay | CLP | 25–125 | $y = 0.3933x - 2.1044$ | 0.9995 | 0.8 | 0.4 |
| | ASA | 50–250 | $y = 0.3903x - 0.6833$ | 0.9994 | 0.4 | 0.6 |
| Related substances | CLP | 0.975–325 | $y = 0.4059x + 0.1299$ | 1.0000 | 0.1 | |
| | ASA | 0.128–650 | $y = 0.3000x - 0.1047$ | 0.9999 | 0.3 | |

Where $R^2$ = coefficient of determination; $S_{y,x}$ = standard error of estimate; $y$ = mean ($n$ = 3) peak area; $x$ = concentration (μg/mL).

**Table 3**
Results for relative response factor, content and impurities determinations.

| | CLP and ASA impurities (%, m/m) | | | | | | | | | | | Content (RSD, $n = 6$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A1 | A2 | A3 | A4 | C1 | C2 | C4 | C5 | C6 | C7 | C8 | CLP | ASA |
| Coplavix® | <0.03 | <0.03 | 0.64 | 0.10 | 0.05 | 0.10 | 0.11 | <0.05 | 0.05 | <0.05 | 0.08 | 99.9 (0.5) | 98.2 (1.7) |
| Closprin® | 0.53 | <0.04 | 20.94 | <0.03 | 2.81 | 0.14 | <0.05 | <0.05 | 0.42 | <0.05 | 0.06 | 96.2 (0.7) | 75.5 (1.4) |
| Noklot Plus® | 0.19 | 0.03 | 9.31 | 0.27 | 1.03 | 0.15 | 0.24 | 0.08 | 0.33 | 0.15 | 0.20 | 94.4 (0.3) | 89.2 (0.3) |
| RRF | 0.66 | 4.72 | 0.86 | 1.29 | 0.99 | 0.89 | 3.11 | 1.23 | 1.95 | 1.38 | 1.14 | (1.00) | (1.00) |

RRF: relative response factor; Ax: aspirin impurities; Cx: clopidogrel impurities.

A3 was far above the 3.0% limit prescribed in the USP monograph for coated ASA tablets.

## 4. Conclusion

The developed reversed phase LC method is specific, linear, sensitive, precise and accurate for the separation and determination of CLP, ASA and their impurities. As there is no official method, the LC method can be applied for routine quality control of the APIs and their related substances in combined oral dosage forms.

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