Review Article

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Analytical methods for the determination of aspirin in its combinations with clopidogrel and some statins in medications for treatment of acute coronary syndromes (ACS)

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Abstract

The combination of aspirin (ASP) and clopidogrel (CLD) has demonstrated efficacy in managing coronary syndromes, including unstable angina and myocardial infarction. This regimen prevents clotting and effectively reduces the risk of vascular events by inhibiting both adenosine diphosphate and the cyclooxygenase pathway. Furthermore, cholesterol-lowering agents such as statins are also employed as a preventive measure for patients with acute coronary syndromes (ACS). This article reviews the current analytical methods for the quantitative determination of aspirin in combination with clopidogrel, or the combination of the two drugs with one of the statins, in various marketed formulations in the period of 2005 -2024. The most commonly used methods for determining these combinations include chromatographic techniques such as high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC), as well as spectrophotometric methods. Recent trends in the analysis of these combination samples show a preference for HPLC (60%), thin-layer chromatography (TLC) (22.5%) and spectrophotometric methods (17.5%) reflecting a general shift towards more sensitive methods with higher resolution capabilities. These methods also offer the advantages of requiring smaller quantities of samples and reagents and shorter analysis times.

Keywords: Aspirin, Clopidogrel, Statins, Dosage forms, Combinations.

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1. Introduction

While monotherapy is traditionally the preferred option, initial treatment for chronic conditions like cardiovascular diseases and diabetes, require polytherapy from the outset. Effective treatment of these chronic conditions can be sometimes hindered by patient non-adherence to polytherapy secondary to different reasons such as forgetfulness, financial problems, and unavailability of medications (1). Polytherapy options include the sequential addition of individual drug products to create an effective multi-drug regimen or the use of a single fixed-dose combination (FDC) therapy product. FDCs are becoming increasingly popular due to their synergistic effects, improved patient adherence, simplified dosing schedules, prevention of dose dividing/sharing, and reduction of drug resistance (2).

Despite these advantages, a significant drawback of fixed-dose combination formulations is the need to develop complex analytical techniques or new methods for the simultaneous determination of multiple active pharmaceutical ingredients (APIs). This process can be costly and time-consuming, requiring highly specialized equipment and expertise (3).

Acute coronary syndromes (ACS), including clinical conditions such as ST-elevation myocardial infarction (STEMI), non-ST-elevation

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myocardial infarction (NSTEMI), and unstable angina, significantly contribute to cardiovascular mortality and morbidity worldwide (4). The combination of aspirin (ASP) and clopidogrel (CLD) has been proven effective in managing coronary syndromes like unstable angina and myocardial infarction. This combination inhibits platelet aggregation as well as clotting and effectively prevents vascular events through dual blockade of adenosine diphosphate and cyclooxygenase pathway inhibition (5, 6). Additionally, therapeutic interventions with cholesterol-lowering agents, such as statins (e.g. atorvastatin (ATR) and rosuvastatin (ROS)), are another therapeutic/preventive measure for patients with ACS (7).

Various analytical techniques are employed in pharmaceutical analysis, each with its own strengths and applications. In the context of aspirin combined with clopidogrel and statins, these analytical methods are utilized to determine the content of active ingredients, detect impurities, study degradation pathways, assess formulation stability, and evaluate pharmacokinetic parameters. Overall, the selection of analytical techniques depends on the specific requirements of the analysis and the properties of the compounds being studied. Proper validation of these methods is essential to ensure accurate and reliable results, which are critical for regulatory submissions and ensuring patient safety and efficacy of the medications.

This scientific literature review aims to provide an in-depth overview of advancements in evaluating the combination of aspirin and clopidogrel with or without statins from 2005 to 2024. The review categorizes the data according to the analytical methods used. This can assist readers in quickly understanding the different approaches employed to binary mixtures of aspirin with clopidogrel or ternary mixtures containing the two drugs plus a statin, offering detailed insights from various published studies. Such a structured presentation enables pharmaceutical researchers and professionals to easily find pertinent information about the analytical techniques relevant to their research or work.

2. Methodology

A systematic review was conducted on previously published research in the fields of chemistry and biomedicine using the most comprehensive scientific databases: Science Direct, Taylor and Francis, Springer Link, Pubmed, Scopus, Google Scholar and Wiley. The search terms included "determination of aspirin, clopidogrel, and statins" and "analysis of combination therapy for ACS." This review resulted in a list of research papers focused on the analysis of aspirin and clopidogrel combination, as well as their combination with statins in various matrices. The first phase of the review involved applying specific filters to each database, restricting the search to: 1) original articles in English, and 2) papers published between 2005 and 2024. Then, two independent reviewers manually analyzed the selected articles. The selected articles were then manually analyzed. In the first round of selection, articles were excluded if they: 1) were duplicates, 2) were reviews, book chapters, or abstracts, or 3) had titles, abstracts, or keywords that did not match the search criteria. In the second round, full articles were reviewed, and the exclusion criteria were: 1) studies not conducted in matrices other than bulk and dosage forms, 2) articles that did not present details of the analytical method, and 3) studies whose results and analysis did not align with the objectives of this review.

3. Results

A flow diagram depicting the flow of information through the different phases of a systematic review is given in figure 1. It depicts the number of records identified, included and excluded, and the reasons for exclusions (Figure 1).

We summarized the general characteristics of spectrophotometric methods included in this review are in Table 1. We found that six of the eight studies were conducted in India and that one was conducted in Egypt and the other in Palestine. It was also found that derivative and chemometric techniques are predominant (Table 1).

We also summarized the general characteristics of the high performance liquid chromatographic methods included in the review in table 2. The global distribution of the studies; shown that twenty-four studies were conducted in India, three in Egypt, two in Pakistan and one in Romania. We found that reverse-phase high-performance liquid chromatography (RP-HPLC) on C18 column is the most frequently used method for both the binary and ternary mixture of the analytes studied (Table

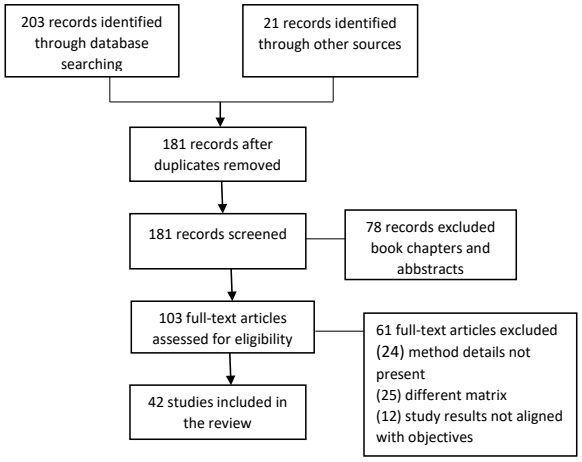

Figure 1. PRISMA Flow Diagram for the literature review process.

2).

3.1. Spectrophotometric methods

The analysis and quality control of such combinations pose significant challenges to analytical chemists, especially with direct spectrophotometry due to spectral bands overlapping. Various approaches involving mathematical manipulation of spectral data have been developed to address this issue, with their effectiveness depending on the degree of overlapping and the number of components involved (8, 9).

Several methods have been reported for the quantitative determination of aspirin when combined with clopidogrel and certain statins in pharmaceutical dosage forms. Most cases employ derivative spectrophotometry by using various derivative orders, the ratio derivative method, and the double divisor ratio approach (10, 11, 13-16). Additionally, chemometric techniques such as inverse least squares (ILS), classical least squares (CLS), principal component regression (PCR), and partial least squares (PLS) are used to resolve overlapping spectral bands and allow for the quantitative determination of the analytes involved (12, 16). Other methods include the H-point standard addition method (17), simultaneous equation approach enhanced by Cramer's rule (17), and the area under the curve technique (10). Below are the details of the UV spectrophotometric methods used.

Two spectrophotometric methods have been developed for the simultaneous determination of CLD and ASP in a combined tablet dosage form. The first method was a ratio derivative method, which involves dividing the spectrum of the mixture by the standardized spectra of each

Table 1. The general characteristics of the recruited spectrophotometric methods.

Study Number	First Author	Publication Year	Country	Analytes	Technique (s)
1	Kale (10)	2011	India	CLD and ASP	Ratio derivative and simultaneous equations
2	Game (11)	2010	India	CLD and ASP	Simultaneous estimation
3	Rajput (12)	2008	India	CLD and ASP	ILS and CLS
4	Singh (13)	2017	India	ASP, CLD and ROS	Third order derivative
5	Singh (14)	2010	India	ASP, CLD, and ATR	First order derivative
6	Pandey (15)	2021	India	ASP, CLD and ROS	Double divisor ratio-spectra derivative
7	Al Sawy (16)	2023	Egypt	ASP, CLD, and either ATR or ROS	PCR, PLS, and CLS
8	Issa (17)	2013	Palestine	ASP, ATR, and CLD	H-point and Cramer's rule

Table 2. The general characteristics of the recruited high performance liquid chromatography methods.

Study Number	First Author	Publication Year	Country	Analytes	Technique, column	Elution
1	Patel (19)	2008	India	ASP and CLD	RPHPLC, C8 column	Isocratic
2	Anandakumar (25)	2007	India	ASP and CLD	RPHPLC, C18 column	Isocratic
3	Dos (26)	2016	India	ASP and CLD	RPHPLC, C18 column	Isocratic
4	Gandhimathi (27)	2007	India	ASP and CLD	RPHPLC, C18 column	Isocratic
5	Kahsay (28)	2013	India	ASP and CLD + impurities	Chiral separation	Gradient
6	Kahsay (29)	2012	India	ASP and CLD+ impurities	RPHPLC, C18 column	Gradient
7	Kachhadia (30)	2009	India	ASP and CLD	RPHPLC, C8 column	Isocratic
8	Mahesh (31)	2015	India	ASP and CLD	RPHPLC, Phenylhexyl Column, Ion pair	Gradient
9	Sreekanth (32)	2020	India	ASP and CLD	RPHPLC, C18 column	Isocratic
10	Chatrabhuji (33)	2014	India	ASP and CLD	RPHPLC, C18 column	Isocratic
11	Usman (34)	2023	Pakistan	ASP and CLD	RPHPLC, C18 column	Isocratic
12	Rashad (35)	2023	Egypt	ASP and CLD	RPHPLC, C18 column	Isocratic
13	Gandla (36)	2015	India	ASP and ROS	RPHPLC, C18 column	Isocratic
14	Panda (37)	2010	India	ASP and CLD	RPHPLC, C18 column	Isocratic
15	Shrivastava (38)	2008	India	ASP and CLD	RPHPLC, C18 column	Isocratic
16	Shah (39)	2007	India	ATR and ASP	RPHPLC, C18 column	Isocratic
17	Panchal (40)	2014	India	ASP and ROS	RPHPLC, C18 column	Isocratic
18	Dumasiya (41)	2012	India	ROS and ASP	RPHPLC, C8 column	Isocratic
19	Solanki (42)	2012	India	ROS and ASP	RPHPLC, C18 column	Isocratic
20	Sultana (43)	2011	Pakistan	ASP and CLD	RPHPLC, C18 column	Isocratic
21	Spiridon (44)	2015	Romania	CLD, ASP, and ATR	RPHPLC, C18 column	Isocratic
22	Pisal (45)	2018	India	CLD, ASP, and ROS	RPHPLC, C18 column	Isocratic
23	Tiwari (46)	2019	India	CLD, ASP, and ROS	RPHPLC, C18 column	Isocratic
24	Sundar (47)	2014	India	CLD, ASP, and ATR	RPHPLC, C18 column	Isocratic
25	Londhe (48)	2011	India	CLD, ASP, and ATR	RPHPLC, C18 column	Isocratic
26	Mostafa (49)	2023	Egypt	ASP, CLD, ATR, and ROS	RPHPLC, C18 column	Gradient
27	Devika (50)	2011	India	ASP, ATR, and CLD	RPHPLC, C18 column	Isocratic
28	Elkady (51)	2017	Egypt	ASP, ATR, and CLD	RPHPLC, C18 column	Gradient
29	Dubey (52)	2013	India	ASP, ATR, and CLD	RPHPLC, C18 column	Isocratic
30	Sathiyasundar (53)	2014	India	ASP, ATR, and CLD	RPHPLC, C18 column	Isocratic

analyte and deriving the ratio to obtain a spectrum dependent on the concentration of the analyte used as a divisor. The amplitudes at 218.11 nm and 247.002 nm in the second derivative of the

ratio spectra were selected for ASP and CLD, respectively. In area under curve method the sample solutions were scanned in the range of 200-350 nm and area under curve between 215.66-218.78 nm and 235.15-238.27 nm were integrated, by using integrated areas. Two simultaneous equations were created and solved to determine concentrations of analytes. The coefficients of the simultaneous equations were obtained from the area under the curve between 215.66-218.78 nm and 235.15-238.27 nm after scanning suitable dilutions of the standard solutions of the drugs in the range of 200-350 nm. Both drugs exhibited linearity within the concentration range of 5.0 - 25.0 µg/mL (10).

Simultaneous estimation of clopidogrel bisulphate and aspirin was performed using the first-order derivative zero-crossing method. The first-order derivative absorption at 232.5 nm (the zero-crossing point of aspirin) was utilized for measuring clopidogrel bisulphate, while the absorption at 211.3 nm (the zero-crossing point of clopidogrel bisulphate) was used for measuring aspirin. Both drugs exhibited linearity within the concentration range of 5.0 - 25.0 µg/mL (11).

Two chemometric methods, ILS and CLS, were applied to simultaneously assay clopidogrel bisulphate and aspirin in their combined tablet formulation. Twelve mixed solutions were prepared as a training set for chemometric calibration, and ten mixed solutions were prepared as a validation set. The absorbance data matrix was obtained by measuring absorbance at 16 wavelength points, from 220 to 250 nm at 2 nm intervals. Calibration or regression models were created using the concentration and absorbance data matrices to predict unknown concentrations (12).

Simultaneous determination of ASP, CLD and ROS was carried out by using the third order derivative spectroscopy at 290.36 nm, 243.84 nm and 256.16 nm for ASP, CLD and ROS, respectively. A linear calibration curve was obtained in the concentration range of 20-100 µg/mL for ASP; 6-20 µg/mL for ROS calcium, and 10-100 µg/mL for CLD (13).

First derivative and multicomponent spectrophotometry were used for the simultaneous estimation of ASP, CLD, and ATR combination in pharmaceutical formulations. In the first derivative the amplitudes at 276, 226 and 222 nm were utilized for the estimation, while the absorbance at 247, 220 and 235 nm at 247, 220 and 235 nm were utilized in the multicomponent mode. The linearity of responses was observed over the concentration ranges of 2-6, 15-52.5, and 15-52.5 µg/ml of ATR, CLD and ASP, respectively (14).

A double divisor ratio-spectra derivative method was developed and validated for the determination of ASP, CLD and ROS in tablets. The measurement was carried out at 240.2 nm, 231.8 nm and 263.2 nm for ASP, CLD and ROS, respectively. The linearity range for all three drugs was 2-20 µg/mL (15).

Three chemometric techniques —PCR, PLS, and CLS — were employed for the simultaneous determination of ASP, CLD, and either ATR or ROS in their FDC formulations. To apply these techniques, an absorbance data matrix corresponding to the concentration data matrix was generated using absorbance measurements in the 250-280 nm range at 0.2 nm intervals from their zero-order spectra. Calibration or regression models were then developed using these concentration and absorbance data matrices to predict the unknown concentrations of the components (16).

Two chemometrics-assisted UV spectrophotometric methods were proposed for resolving ternary mixtures without requiring any chemical pretreatment. The first method involves a modified H-point standard addition method, allowing for the simultaneous analysis of three species using a single calibration set by adding all three analytes simultaneously. In this method, the absorbance of a ternary mixture (*Am*) containing ASP, ATOR, and CLOP at wavelengths 317 nm and 231 nm. They are the sum of the absorbance of the three analytes at these wavelengths. The selection of these wavelengths was based on the equality of the absorbance ratio of ATR at 317 nm and 231 nm to that of CLD at the same wavelengths. Thus, when ASP, ATR, and CLD are added, the slope resulting from the addition of the species maintains a constant ratio at 317 and 231 nm for CLD and ATR, respectively. The necessary data for this method includes the absorbance of the mixture and the absorbance of the mixture spiked with known amounts of ASP, ATR, and CLD at 317 nm and 231 nm, which is then mathematically processed to determine the concentrations of the three analytes. The second method combines the first derivative spectra with Cramer's matrix rule. Molar absorptivity (ε) val-

ues are calculated by using absorbance measurements at 212 nm, 250 nm, and 316.8 nm from the first-order spectra of each compound in the ternary mixture. These wavelengths were chosen because CLD has zero-crossing points at 316.8 nm and 212 nm, and ATR has a zero-crossing point at 250 nm, simplifying the matrix and making it easier to solve. By using these ε values, a system of equations with three unknowns can be created for the compounds in the ternary mixture. Matrix calculations based on Cramer's rule can then be employed to simultaneously determine the concentrations of these compounds in the ternary mixture and pharmaceutical formulations. The linearity range was 2.5-20 µg/mL for ASP aspirin, 2.5-17.5 µg/mL for ATR, and 2.5-20 µg/mL for CLD regarding both methods (17).

3.2. Chromatographic methods

Analyzing multicomponent mixtures through volumetric or spectrophotometric methods is challenging due to the numerous independent procedures required for determining individual components, making the process labor-intensive and time-consuming. Contemporary chromatographic techniques such as gas chromatography (GC), chromatography–mass spectrometry (GC/MS and HPLC/MS), high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC) are extensively utilized to assess the purity, uniformity, and stability of industrial products. These methods are also employed in analyzing biological fluids and tissues to determine appropriate dosages, study metabolic pathways and pharmacokinetics, and also in forensic medicine. Following are the different chromatographic methods reported for the determination of the combinations:

3.2.1 Thin layer chromatography

Recent advancements in instrumentation for TLC in term of the stationary phase or the detection system; have significantly enhanced its accuracy and precision, making it increasingly popular for the quantitative determination of drug combinations.

All reported methods in this review, utilized normal phase chromatography on silica gel plates for separating the combinations, employing various mobile phases. Spot detection was performed in the UV region, although most methods did not clearly explain the rationale for selecting specific detection wavelengths.

Sinha and colleagues (18) reported stability indicating high performance thin layer chromatographic method of analysis for ASP and CLD. TLC aluminum plates precoated with silica gel 60 F254 were used as the stationary phase and carbon tetrachloride-acetone (6: 2.4 v/v) as the mobile phase. The analytes were detected densitometrically at 220 nm. The calibration plots showed good linear relationship in the concentration range of 200-600 ng/spot and 300-600 ng/spot for ASP and CLD, respectively. In this method, it has been found that to achieve the reproducibility of peak shapes and areas, it is necessary to prewash the TLC plates with methanol followed by drying, activation, and pre-saturation of TLC chamber with mobile phase for 20 min.

The HPTLC separation of ASP and CLD was achieved on an aluminum-backed layer of silica gel 60F254 and a mobile phase composed of ethyl acetate: methanol: toluene: glacial acetic acid (5.0+1.0+4.0+0.1, v/v/v/v). Quantifying was achieved with UV detection at 235 nm over the concentration range of 400-1400 ng/spot for both drugs (19).

A thin-layer chromatographic method has been developed for the analysis of rosuvastatin calcium and aspirin in bulk and capsule dosage forms. Chromatography was performed on silica gel 60 F254 plates by using a mobile phase of toluene: butanol (9:1:0.2 v/v/v). Densitometry analysis of ROS and ASP was conducted in absorbance mode at 234 nm. The linear regression analysis data for the calibration plots indicated a good linear relationship with respect to peak area in the concentration range of 200-1200 ng/spot for ROS and 1500-9000 ng/spot for ASP (20).

Beside this, ROS and ASP were determined by using TLC. The method involved separating the components on precoated silica gel 60 F254 plates with a mobile phase consisting of n-Hexane: Acetone: Ethyl acetate: Formic acid (6:3:1:0.2 v/v). Detection of the spots was performed at 240 nm for both ROS and ASP. The linearity range was 500-1000 ng/spot for ROS and 3750-7500 ng/spot for ASP (21).

An optimized HPTLC method, developed using experimental design, was employed for the

simultaneous determination of ASP, CLD, and ROS in capsules. The central composite experimental design optimization assessed the effects of chamber saturation time, ionic modifier volumes, and mobile phase ratio on the Rf value and band shape. This led to the establishment of optimized HPTLC conditions: a 20-minute saturation time and a mobile phase ratio of 7:3:0.2 v/v/v (ethyl acetate, chloroform, and glacial acetic acid as the pH modifier). These conditions were then applied for separation (22).

3.3. High performance liquid chromatographic methods

HPLC is particularly well-suited for examining the purity and quality of pharmaceutical preparations, especially when gas-liquid chromatography (GLC) is unsuitable due to the insufficient thermal stability or low volatility of components. Consequently, HPLC is preferred over GLC for product quality control in most pharmaceutical companies and is included in most international pharmacopeias (23, 24). The development of highly selective adsorbents and advancements in the sensitivity of flow-through spectrophotometric, fluorometric, and electrochemical detectors have further enhanced the use of HPLC in pharmaceutical analysis.

This review revealed that RP-HPLC has been extensively used for quantifying ASP and CLD combination, as well as their combination with statins in bulk and pharmaceutical dosage forms. Most of the reported methods utilized isocratic elution with a reversed-phase column (C8 or C18) and mobile phases consisting of organic solvent and buffer mixtures adjusted to specific pH levels.

Below are the details of the chromatographic methods, used for the analysis of Dual antiplatelet therapy (aspirin with clopidogrel±a statin):

ASP and CLD have been quantified by using HPLC. Separation was performed on a Nucleosols C8 column (150 mm length, 4.6 mm id, 5 µm particle size) with a mobile phase of acetonitrile and phosphate buffer (pH 3.0) in a 55:45 (v/v) ratio, at a flow rate of 1.0 mL/min at ambient temperature. Quantification was carried out with UV detection at 235 nm, covering a concentration range of 4–24 µg/mL for both drugs (19).

Simultaneous estimation of ASP and CLD in capsules was achieved by using a reversed-phase high-performance liquid chromatography (HPLC) method. Separation was performed on a C18 octadecyl column with an eluent consisting of acetonitrile, methanol, and 20 mM phosphate buffer at pH 3 (50:7:43 v/v), at a flow rate of 1 mL/min. Detection was conducted at 240 nm, with linearity for both ASP and CLD in the range of 10-50 µg/mL (25).

A stability-indicating HPLC method was developed for the simultaneous determination of ASP and CLD. The method utilized a reversed-phase C18 column (5 µm, 250 mm x 4.6 mm i.d.) in isocratic mode with a mobile phase of 0.3% orthophosphoric acid buffer solution and acetonitrile (65:35, v/v). The flow rate was set at 1 mL/min, and detection was carried out at 266 nm. Linearity was demonstrated with a seven-point calibration curve, covering the concentration ranges of 30–120 µg/mL for ASP and 15–60 µg/mL for CLD. The stability-indicating capability of the method was confirmed through forced degradation studies on both drugs (26).

A reverse-phase HPLC method was also used to determine ASP and CLD in the combined tablet dosage form. Chromatographic separation was achieved on a C18 column (5 µm, 150 mm x 4.6 mm i.d.) with an isocratic elution using a mobile phase of 0.1% v/v triethylamine (pH 4.0) and acetonitrile in a 25:75 (v/v) ratio. The detection wavelength was set at 225 nm, and the flow rate was 1 mL/min. Nimesulide (20 µg/mL) was used as an internal standard. The method demonstrated linearity in the range of 1-5 µg/mL for both drugs (27).

CLD and ASP combination dosage forms was evaluated for their content, impurities, and drug release. An enantiospecific (chiral) method was used for the quantification of the R-enantiomer of CLD, while an achiral method was employed for determining the content of CLD, ASP, and their impurities. The following stationary phases were utilized: A CHIRALCEL® OJ column (250 mm x 4.6 mm, 10 µm) was used for the quantification of the R-enantiomer of CLD. The mobile phase consisted of anhydrous ethanol and heptane (15:85 v/v) at a flow rate of 0.8 mL/min. UV detection was performed at 220 nm, with the column maintained at room temperature. A C18 column

(150 mm x 4.6 mm, 3 µm) was used for the determination of the content of CLD, ASP, and their impurities, as well as for monitoring drug release. The column temperature was maintained at 35°C by using a water bath. 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) at a flow rate of 1 mL/min. The gradient program [time (min) / %B] was as follows: 0/10, 3/10 to 48/68 to 50/10, 55/10. Ten commercial batches of different marketed drugs containing CLD and ASP combined dosage forms (tablets and capsules) were analyzed. Although no specific monograph exists for combined dosage forms of CLD and ASP, most samples complied with the United States Pharmacopeia (USP) specifications for content and related substances of the individual monographs. However, there were notable differences in the in vitro drug release among the examined samples (28).

A reversed-phase liquid chromatographic method with UV detection was developed and validated for the simultaneous determination of clopidogrel, acetylsalicylic acid, and their related substances in combined oral formulations. Effective separation was achieved by using a C18 column (150 mm × 4.6 mm, 3 µm). The mobile phase consisted of 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 program [time (min)/%B] was as follows: 0/10.5, 3/10.5 to 48/68.5, 68/68.5 to 80/10.5. The column temperature was maintained at 35°C, and UV detection was performed at 220 nm (29).

A HPLC assay method for determining CLD and ASP in tablet formulations was described. Separation of the drugs from degradation products formed under stress conditions was achieved by using an octylsilane (C8) column with a mobile phase consisting of 0.3% orthophosphoric acid and acetonitrile (65:35, v/v). The mobile phase flow rate was set at 1.0 mL/min, and analytes were detected at 226 nm. The method demonstrated linear response ranges of 30.0-120.0 µg/mL for ASP and 15.0–60.0 µg/mL for CLD (30).

A stability-indicating RPLC method has been developed and validated for the simultaneous estimation of CLD and ASP in tablet dosage form. Chromatographic separation was achieved on a 100 × 4.6 mm, 2.6 µm phenylhexyl stationary phase column, with the column oven temperature maintained at 35 °C. The mobile phase A consisted of diammonium hydrogen phosphate (1.32 g/L) and octane-1-sulphonic sodium salt (2 g/L), with the pH adjusted to 2.3±0.05. Mobile phase B was a mixture of acetonitrile and methanol in a 50:50 ratio. The flow rate was adjusted to 1.0 mL/min in gradient elution mode. The gradient program was set as follows: T/%B: 0/30, 2/45, 4/55, 10/55, 11/30, and 15/30. Detection was performed at a wavelength of 220 nm (31).

A liquid chromatography (LC) method was outlined for quantifying CLD and ASP in tablet dosage forms. Separation was conducted using a C18 column (250×4.6mm, 5µm), with a mobile phase composed of phosphate buffer and acetonitrile in a ratio of 55:45 (v/v). Detection occurred at a wavelength of 235 nm (32).

An RP-HPLC method was developed and validated for the simultaneous estimation of CLD and ASP from bulk drug samples. Chromatographic separation was achieved isocratically on a C18 column (250×4.6 mm, 5 µ particle size) by using a mobile phase consisting of 0.3% ortho-phosphoric acid (v/v) and acetonitrile (40:60 v/v). The flow rate was maintained at 1 ml/min, and effluent was detected at 226 nm. Linearity was observed in the concentration range of 30-120 µg/mL for ASP and 15-60 µg/mL for CLD (33).

Quantitative estimation of CLD and ASP, along with the degradation products generated under various stress conditions as recommended by the ICH guideline, was accomplished via HPLC. The separation of the two drugs was conducted on a C18 column (4.6 x 150 mm), utilizing a mobile phase composed of acetonitrile and buffer in a ratio of 350:650 v/v. The flow rate was set at 1.3 mL/min, and detection was performed at 220 nm (34).

A factorial design comprising 23 runs was employed to refine and enhance a green HPLC method for the concurrent quantification of CLD and ASP in tablet formulations. The chromatographic separation utilized a C18 analytical column (150×4.6 mm; 5 µm) and a mobile phase consisting of a blend of ethanol and 0.08 M so-

dium dihydrogen phosphate in a 70:30 v/v ratio, adjusted to pH 6.0 with o-phosphoric acid. Detection was carried out at 230 nm, maintaining a flow rate of 1 mL/min (35).

An isocratic separation of ROS and ASP was conducted by utilizing an X-Terra column (4.6 × 150 mm, 5 µm particle size) with a mobile phase comprising Methanol: Buffer (45:55% v/v). Quantification was performed at a wavelength of 215 nm, and the flow rate was maintained at 1 mL/min. Linear regression analysis demonstrated a favorable correlation for ASP and ROS across the concentration ranges of 5-25 µg/mL and 6.25-31.25 µg/mL, respectively (36).

The simultaneous determination of ASP and CLD in a commercial formulation was accomplished by using an RP-HPLC method with a C18 column (250 mm × 4 mm i.d., 5 µm). The mobile phase consisted of acetonitrile and 0.01 M tetrabutyl ammonium hydrogen sulphate (ion-pair reagent) in a 50:50% v/v ratio, with a flow rate of 1.0 mL/min. Quantification was carried out by using UV detection at 240 nm. The method exhibited linearity within the ranges of 1-250 µg/mL for ASP and 0.5-125 µg/mL for CLD (37).

An RP-HPLC method was developed for the simultaneous estimation of ASP and CLD in tablet dosage form. To achieve optimal resolution, a mobile phase consisting of acetonitrile, 50 mM potassium dihydrogen phosphate buffer, and methanol in a 50:30:20 %v/v ratio with the solution pH adjusted to 3 was selected. This mixture provided effective separation of both components at a flow rate of 1.5 mL/min and a detection wavelength of 240 nm. The method demonstrated linearity in the concentration ranges of 1.5-7.5 µg/mL for CLD and 3.5-15.0 µg/mL for ASP (38).

RP-HPLC method was developed for the simultaneous determination of ATR and ASP in capsule dosage forms. Separation was achieved using a C18 column (250×4.6 mm i.d., 5 µm) in isocratic mode, with a mobile phase consisting of 0.02 M potassium dihydrogen phosphate and methanol (20:80% v/v ratio), adjusted to pH 4 with orthophosphoric acid. The flow rate was set at 1.0 mL/min, and analytes were detected at 240 nm. The method exhibited linearity for ATR in the range of 0.5-4 µg/mL and for ASP in the range of 5-25 µg/mL (39).

The simultaneous determination of ASP and ROS in bulk and pharmaceutical formulations was performed using RPLC on an RP18 column (150 mm x 3.0 mm, 5 µm). The mobile phase comprised 20 mM KH2PO4 and methanol in a 30:70 % v/v ratio, with a flow rate of 1.0 mL/min. Detection was carried out by using a photodiode array (PDA) detector. Linearity was established for ASP in the concentration range of 15.0-90.0 µg/mL and for ROS within the range of 2.0-12.0 µg/mL (40).

An isocratic, stability-indicating RP-HPLC method was developed for the quantitative determination of ROS and ASP in a combined dosage form. The separation was achieved using a C8 column (125 mm × 4.6 mm i.d., 5 µm) with a mobile phase consisting of acetonitrile, methanol, and water (25:30:45, v/v/v) adjusted to pH of 3. The flow rate was 1.0 mL/min, and detection was performed at 243 nm. Linearity was established for ROS within the range of 0.2-10 µg/mL and for ASP within the range of 1.5-75µg/mL. The method demonstrated stability-indicating capability by effectively resolving forced degradation products from the parent compounds (41).

A reversed-phase HPLC method was developed and validated for the simultaneous estimation of ROS and ASP in capsule dosage forms. Separation was achieved using a C18 column (200 mm × 4.6 mm, 5.0 µm) with a mobile phase of acetonitrile and 0.050 M potassium dihydrogen phosphate buffer (adjusted to pH 3.0 with orthophosphoric acid) in a 55:45% v/v ratio, at a flow rate of 1 mL/min. Detection was performed at a wavelength of 241 nm. Linearity was established over the concentration ranges of 0.5-6 µg/mL for ROS and 2.5-30 µg/mL for ASP (42).

A chromatographic method for the simultaneous determination of clopidogrel and aspirin from bulk material and dosage formulations, using meloxicam as an internal standard, has been developed. Separation was performed on a Purospher Star C18 column (5 µm, 250 × 4.6 mm) at ambient temperature. The mobile phase consisted of methanol and water (80:20, v/v), with the pH adjusted to 3.4 using orthophosphoric acid, and was pumped at a flow rate of 1 mL/min using an isocratic pump system. The multivariate chromatographic calibration technique was applied to high-performance liquid chromatography (HPLC) data for the simultaneous quantitative analysis of binary mixtures of clopidogrel and aspirin. HPLC

data based on analyte peak areas were obtained at five wavelengths (225, 230, 235, 240, and 245 nm). The mathematical algorithm of the multivariate chromatographic calibration technique relies on linear regression equations. Calibration plots for clopidogrel and aspirin were constructed at each wavelength using the peak areas corresponding to the concentrations of each active compound (43).

Besides ASP and CLD, the chromatographic methods; used for the analysis of ASP and CLD plus a statin combination (Triple therapy) were described as follows:

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed for the simultaneous estimation of CLD, ASP, and ATR in tablet dosage form. The chromatographic analysis was conducted on a C18 column using a mobile phase composed of 0.01 M KH_2PO_4 buffer (pH adjusted to 2.6 with phosphoric acid), acetonitrile, and methanol in a 20:40:40 v/v/v ratio, pumped at a flow rate of 0.8 mL/min. UV detection was performed at 220 nm for clopidogrel and acetylsalicylic acid, and at 244 nm for atorvastatin. The method demonstrated a good linear relationship between peak area and concentration over the ranges of 0.03-2 µg/mL for ASP, 0.03-5 µg/mL for CLD, and 0.04-1.25 µg/mL for ATR (44).

A stability-indicating assay method was developed for the simultaneous qualitative and quantitative estimation of aspirin, rosuvastatin, and clopidogrel in bulk and pharmaceutical dosage forms. Separation was achieved isocratically using a C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase consisting of water (pH 2.51 adjusted with 0.1% v/v orthophosphoric acid) and acetonitrile in a 50:50 ratio, at a flow rate of 1 mL/min. The effluents were monitored at 237 nm. Linearity was confirmed using seven-point calibration curves within the concentration range of 1-7 µg/mL for all three drugs. The method's stability-indicating capability was validated through forced degradation studies on the drugs (45).

The simultaneous estimation of ROS, CLD, ASP in bulk and pharmaceutical dosage forms was performed by using RP-HPLC. Chromatographic separation was achieved on a C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase consisting of KH_2PO_4 buffer (pH 6.0) and acetonitrile in a 60:40 ratio, at a flow rate of 1 mL/min. The effluents were monitored at 242 nm. The calibration curves were linear over the concentration ranges of 7.5-22.5 µg/mL for CLOP and 1-3 µg/mL for ASP and ROS (46).

A central composite rotatable design and Derringer's desirability function were utilized to optimize an RP-HPLC method for the simultaneous estimation of Aspirin, Atorvastatin, and Clopidogrel in pharmaceutical dosage forms. The optimized chromatographic conditions included a C18 column (150 mm × 4.6 mm i.d., 5 µm) and a mobile phase consisting of acetonitrile, methanol, and 0.1% triethylamine (52:05:43, v/v/v), with the pH of the aqueous phase adjusted to 3.0 using 10% orthophosphoric acid. Analyte detection was performed at 220 nm. Method validation studies confirmed the suitability of these chromatographic conditions for the analysis of the drugs (47).

A RP-HPLC method was developed and validated for the simultaneous determination of ASP, ATR, and CLD in capsules. Chromatographic separation was achieved using an ODS analytical column (150 × 4.6 mm, 5 µm) with a mobile phase of acetonitrile and phosphate buffer (pH 3.0 adjusted with orthophosphoric acid) in a 50:50 v/v ratio, at a flow rate of 1.2 mL/min. UV detection was carried out at 235 nm. The calibration plots were linear over the concentration range of 5-30 µg/mL for atorvastatin calcium and 30-105 µg/mL for ASP and CLD (48).

A Box-Behnken design with three center points was employed to optimize the key parameters impacting HPLC separation. The effects of pH, the organic ratio in the mobile phase during the first stage of gradient elution, and the organic ratio in the mobile phase during the second stage of gradient elution on the theoretical plates of the first peak and the tailing factor of the last peak were studied. The number of theoretical plates for the ASP peak and the tailing factor for the CLD peak were identified as the most critical responses for chromatographic separation, guiding the optimization of the developed method. ASP, CLD, ATR, and ROS were then estimated in bulk and pharmaceutical dosage forms using the optimized method. The separation was conducted on a C18 column (100 mm x 4.6 mm, 5 µm) using an RP-HPLC system with a diode array detector. The mobile phase consisted of acetonitrile and KH2PO4 buffer (pH

3.2), operated in gradient mode at a flow rate of 1 mL/min. The gradient program began with 40% acetonitrile for 5 minutes, increased to 95% over 1 minute, maintained at 95% acetonitrile for 2 minutes, then reverted to 40% acetonitrile within 0.3 minutes, and held at initial conditions until 10 minutes. The total run time was 10 minutes at room temperature. The four drugs exhibited linearity over the concentration range of 0.05–50 µg/mL (49).

ASP, ATR, and CLD in pharmaceutical dosage forms were analyzed using a Hypersil BDS C18 column (250 × 4.6 mm, 5 µm). The mobile phase consisted of acetonitrile, water (pH 3), and methanol in a 50:40:10 (v/v) ratio. Detection of the eluents was performed by UV absorbance at 248 nm. The analytes exhibited linear responses over the concentration ranges of 120-489 µg/mL for ASP, 8-32 µg/mL for ATR, and 60-240 µg/mL for CLD (50).

Reversed-phase HPLC-UV and UPLC-DAD methods were developed, validated, and utilized for the determination of ASP, CLP, ATR in bulk and capsule forms, along with their primary synthesis impurities and degradation products: salicylic acid (SAL), clopidogrel carboxylic acid metabolite related impurity A (CLD-RA), and clopidogrel inactive R-enantiomer related impurity C (CLD-RC). The HPLC method employed an Agilent Eclipse Plus C18 column (250 mm × 4.6 mm, 5 µm), with a gradient mobile phase consisting of 20 mM anhydrous KH2PO4 buffer solution (containing 0.2% TEA, v/v), pH adjusted to 2.7 using orthophosphoric acid (solution A), and acetonitrile (solution B). The gradient program was set as follows: 0 min/45% B, 5 min/45% B, 18.2 min/35% B, 18.3 min/45% B, and 20 min/45% B. The mobile phase was pumped through the column at a flow rate of 1 mL/min.

For the UPLC method, an Agilent Eclipse Plus C18 column (100 mm × 2.1 mm, 1.7 µm) was used, with an isocratic mobile phase consisting of 20 mM anhydrous KH2PO4 buffer solution (containing 0.2% TEA, v/v), pH adjusted to 2.7 using orthophosphoric acid, and acetonitrile (55:45, v/v). The flow rate was set at 0.3 mL/min. Detection was performed for both methods at 220 nm for ASP, CLP, SAL, CLP-RA, and CLP-RC, and at 240 nm for ATR (51).

A RP-HPLC method has been developed and validated for the simultaneous estimation of ASP, ATR, and CLD in combined capsule dosage forms. The RP-HPLC method utilized a C18 column and a mobile phase consisting of acetonitrile, methanol, and water (adjusted to pH 3.5 with orthophosphoric acid) in a 50:30:20 (v/v/v) ratio. Detection was performed by using a diode array detector set at 250 nm. The chromatographic responses were linear over the concentration ranges of 5-100 µg/mL for ASP, 2-24 µg/mL for ATR, and 5-100 µg/mL for CLD (52).

A stability-indicating assay method has been developed and validated for the simultaneous estimation of aspirin, atorvastatin, and clopidogrel in active pharmaceutical ingredients and commercial dosage forms. This reverse-phase liquid chromatographic method utilized a C18 column (150 x 4.6 mm i.d., 5 µm) with a mobile phase composed of acetonitrile, methanol, and 0.1% TEA (pH adjusted to 3.0 with orthophosphoric acid) in a 52:05:43% (v/v/v) ratio, at a flow rate of 1.4 mL/min. Detection was conducted by using a photodiode array detector at 220 nm, based on peak area. Linear calibration curves were established in the concentration range of 2-10 µg/ml for ASP and CLD and 1-5 µg/ml for ATR. The method was successfully applied to commercially marketed formulations, showing no interference from excipients. ASP, ATR, and CLD, as well as their combination products, were subjected to acid, alkali, and neutral hydrolysis, oxidation, dry heat, and photolytic stress conditions. The stressed samples were analyzed using the proposed method. This method effectively separated the drugs from their degradation products, making it suitable as a stability-indicating approach for determining the stability of these drugs in bulk and pharmaceutical dosage forms (53).

4. Conclusion

Detailed scrutiny of the published work revealed that, only three out of the reported methods were optimized using an experimental design approach (35, 47, 49), while the others relied on the famous one-factor-at-a-time (OFAT) approach or the developer's experience and instinct to determine optimal separation conditions. Although these methods are fully validated, in accordance with global guidance on method validation (ICH Q2 (R1)) (54), some are still found to be non-ro-

bust during routine QC testing, indicating they are not fit for purpose (55). Given the stringent regulatory requirements and the shift towards applying quality by design (QbD) principles in the analytical field, it is essential to establish more rigorous standards for publishing analytical methods. This ensures the development of robust analytical methods suitable for use in quality control laboratories.

This literature review did not provide specific details on the best mobile phase, column, detector, and other conditions for determining aspirin in combination with clopidogrel or statins using HPLC. However, it did highlight the prevalence of HPLC as the most commonly used method for analyzing these combinations due to its versatility and sensitivity.

In general, when using HPLC methods to determine drugs like aspirin, clopidogrel, and statins in combination: Mobile Phase): A suitable mobile phase is typically a mixture of solvents that ensures good separation of the compounds. The choice of mobile phase depends on the chemical properties of the analytes and may include combinations such as acetonitrile-water or methanol-water with additives like acids or buffers to adjust pH; Column): The selection of an appropriate column is crucial for achieving good separation and resolution. C18 columns are commonly used for pharmaceutical analysis due to their versatility and compatibility with a wide range of compounds; Detector): UV-Vis detectors are often used for quantification due to their sensitivity and ability to detect compounds at low concentrations. For more specific detection needs, fluorescence or mass spectrometry detectors may be employed; Other Conditions): In addition to mobile phase composition, column type, and detector choice, other conditions such as flow rate, injection volume, temperature control (if necessary), and gradient elution programs can significantly impact method performance.

To determine the best parameters for quantifying these combinations using HPLC methods specifically tailored to aspirin-clopidogrel-statin analysis would require further detailed investigation into recently published studies focusing on these exact combinations or conducting empirical research in a laboratory setting based on sample characteristics available locally.

All in all, review of the current analytical methods for the quantitative determination of aspirin in combination with clopidogrel, or the combination of the two drugs with one of the statins revealed that the most commonly used methods for determining these combinations is high-pressure liquid chromatography (60%), thin-layer chromatography (TLC) (22.5%) and spectrophotometric methods (17.5%), reflecting a general shift towards more sensitive methods with higher resolution capabilities.

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Conflict of Interest

The authors declare no conflict of interest.

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