DRUG FORMULATIONS AND CLINICAL METHODS

Simultaneous Estimation of Acetylsalicylic Acid and Clopidogrel Bisulfate in Pure Powder and Tablet Formulations by High-Performance Column Liquid Chromatography and High-Performance Thin-Layer Chromatography

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This paper describes validated high-performance column liquid chromatographic (HPLC) and high-performance thin-layer chromatographic (HPTLC) methods for simultaneous estimation of acetylsalicylic acid (ASA) and clopidogrel bisulfate (CLP) in pure powder and formulations. The HPLC separation was achieved on a Nucleosil C8 column (150 mm length × 4.6 mm id, 5 μm particle size) using acetonitrile–phosphate buffer, pH 3.0 (55 + 45, v/v) mobile phase at a flow rate of 1.0 mL/min at ambient temperature. The HPTLC separation was achieved on an aluminum-backed layer of silica gel 60F_{254} using ethyl acetate–methanol–toluene–glacial acetic acid (5.0 + 1.0 + 4.0 + 0.1, v/v/v/v) mobile phase. Quantitation was achieved with UV detection at 235 nm over the concentration range 4–24 μg/mL for both drugs, with mean recoveries of 99.98 ± 0.28 and 100.16 ± 0.66% for ASA and CLP, respectively, using the HPLC method. Quantitation was achieved with UV detection at 235 nm over the concentration range of 400–1400 ng/spot for both drugs, with mean recoveries of 99.93 ± 0.55 and 100.21 ± 0.83% for ASA and CLP, respectively, using the HPTLC method. These methods are simple, precise, and sensitive, and they are applicable for the simultaneous determination of ASA and CLP in pure powder and formulations.

Acetylsalicylic acid (ASA) is a nonsteroidal drug that exhibits anti-inflammatory, analgesic, antipyretic, and platelet aggregation inhibitory activity (1, 2). Clopidogrel bisulfate (CLP), (αS)-α-(2-chlorophenyl)-6, 7-dihydrothieno [3, 2-c] pyridine-5-(4H)-acetic acid methyl ester, is an antiplatelet agent that belongs to the class of thienopyridines (3, 4).

The combination of ASA and CLP has been shown to be effective in the management of coronary syndrome such as unstable angina and myocardial infarction. The combination of ASA and CLP inhibits clotting and completely prevents vascular events with dual blockade of adenosine diphosphate inhibition and cyclo-oxygenase pathway inhibition (5, 6).

A literature survey revealed that different analytical methods involving column high-performance liquid chromatography (HPLC) for determination of ASA in biological fluids (7–14) and in pharmaceutical preparations (15–31) have been developed. Literature reports concerning HPLC and high-performance thin-layer chromatographic (HPTLC) determination of CLP in pharmaceutical dosage forms (32–34) as well as an enantiospecific HPLC method to determine the impurities and to perform the assay (33) have been published. Also, thermal differential scanning calorimetry, thermogravimetric analysis, hot stage microscopy, X-ray diffraction, and Fourier transform infrared methods for characterization and quantitation of 2 enantiomers (R and S) of CLP have been reported (35). Because of the absence of an official pharmacopoeial method for the simultaneous determination of ASA and CLP in pharmaceutical formulations, efforts were made to develop an analytical method for the estimation of ASA and CLP in their combined dosage form using HPLC and HPTLC methods.

Experimental

Apparatus

A Series 200 HPLC system (PerkinElmer, Shelton, CT) equipped with a Series 200 diode array detector, Series 200 quaternary gradient pump, Series 200 column oven, manual injector (Rheodyne valve) with 20 μL fixed loop, Turbochrom navigator software (Version 6.1.1.0.0:K20), and Nucleosil (SGE, Austin, TX) C8 SS column (150 mm × 4.6 mm id, 5 μm particle size) was used. For HPTLC, a Linomat V autosprayer, TLC Scanner III with winCATS-4 software, twin-trough

Received May 22, 2007. Accepted by SW October 7, 2007.
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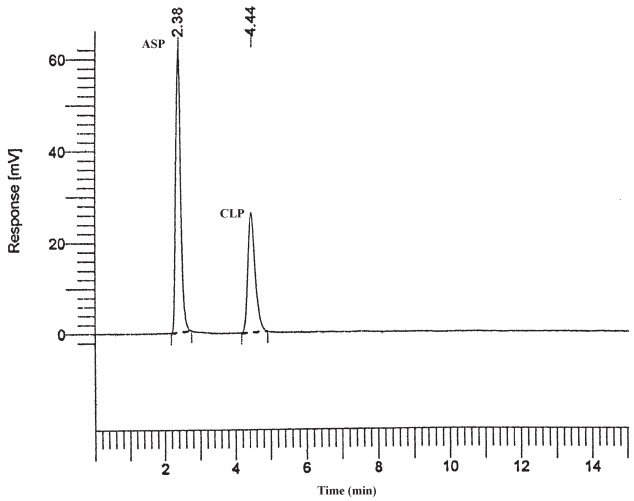

Figure 1. High-performance liquid chromatogram of ASA and CLP and corresponding retention times with detection at 235 nm.

flat-bottom TLC developing chambers, and viewing cabinet with UV lamps (Camag, Muttenz, Switzerland) were used. HPTLC plates used were 10 × 10 cm silica gel with an indicator fluorescing at 254 nm, layer thickness 0.2 mm, aluminum-backing (E. Merck KGaA, Darmstadt, Germany). An Orion pH meter 420A (Allometric Ltd., Baton Rouge, LA) was used for pH measurements.

Reagents and Materials

ASA and CLP pure powder were procured as gratis samples from Sun Pharmaceuticals (Baroda, India). HPLC grade acetonitrile, methanol, and water were purchased from E. Merck (Mumbai, India). Disodium hydrogen phosphate, orthophosphoric acid, triethylamine, ethyl acetate, toluene, and glacial acetic acid were procured from SDfine Chemical Ltd. (Ahmedabad, India) and were of analytical grade. Membrane filters (nylon 0.45 μm, 47 mm) were purchased from Gelman Laboratory (Mumbai, India). Tablets containing ASA (75 mg) and CLP (75 mg) of 2 brands, Torrent Pharmaceutical Ltd. (Gujarat, India) and Ajanta Pharma Ltd. (Mumbai, India), were purchased from the local market.

Table 1. System suitability test parameters for ASA and CLP for the proposed HPLC method

Parameter	ASA ± RSD[a] (n^b = 6)	CLP ± RSD (n = 6)
Retention time, min	2.38 ± 0.02	4.44 ± 0.01
Tailing factor	1.22 ± 0.01	1.21 ± 0.03
Resolution	—	6.24 ± 0.04
Selectivity	—	1.98 ± 0.03
Theoretical plate No.	7096 ± 0.35	11151 ± 0.50

[a] RSD = Relative standard deviation, %.
[b] n = Number of determinations.

Chromatographic Conditions

(**a**) *HPLC method*.—The Nucleosil C8 column (18) was used at ambient temperature. The mobile phase consisted of acetonitrile–phosphate buffer (55 + 45, v/v) with the final pH adjusted to 3.0 ± 0.02 with orthophosphoric acid–triethylamine; it was pumped at a flow rate of 1 mL/min. The mobile phase was passed through a nylon 0.45 μm membrane

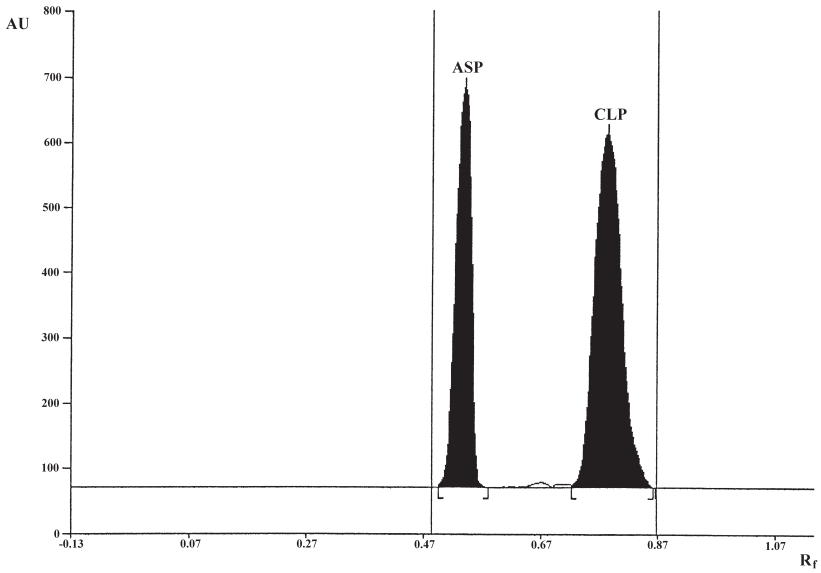

Figure 2. HPTLC densitogram of ASA and CLP with scanning at 235 nm.

Table 2. System suitability test parameters for ASA and CLP for the proposed HPTLC method

Parameter	ASA ± RSD[a] (n[b] = 6)	CLP ± RSD (n = 6)
R_f[c]	0.55	0.79
Area (average)	9332.01 ± 161.37	13707.44 ± 416.44
Peak purity	>0.9995	>0.9999

[a] RSD = Relative standard deviation, %.
[b] n = Number of determinations.
[c] R_f = Retardation factor.

filter and degassed before use. The elution was monitored at 235 nm, and the injection volume was 20 μL.

(**b**) *HPTLC method*.—Solutions of ASA and CLP were applied to silica gel $60F_{254}$ HPTLC plates (10 × 10 cm) by means of a Linomat V automatic spotter equipped with a 100 μL syringe and operated with settings of band length, 6 mm; distance between bands, 5 mm; distance from the plate edge, 10 mm; and distance from the bottom of the plate, 10 mm. The plate was developed in a twin-trough chamber previously saturated for 30 min with the mobile phase, ethyl acetate–methanol–toluene–glacial acetic acid (5.0 + 1.0 + 4.0 + 0.1, v/v/v/v), for a distance of 8 cm. The spots on the air-dried plate were scanned with the Scanner III at 235 nm using the deuterium source.

Preparation of ASA and CLP Mixed Standard Stock Solutions

For both the HPLC and HPTLC methods, a stock solution was prepared by weighing ASA (10 mg) and CLP (10 mg). Weighed powder of both drugs was accurately transferred to the same 100 mL volumetric flask and dissolved in, and then diluted to the mark with, methanol to obtain a mixed standard stock solution of ASA (100 μg/mL) and CLP (100 μg/mL).

Preparation of Sample Solutions

Twenty tablets were weighed and finely powdered. A mass equivalent to 75 mg of both ASA and CLP was weighed and transferred in a 100 mL volumetric flask, and methanol (80 mL) was added. The solution was sonicated for 15 min, and the final volume was diluted to the mark with methanol to obtain a solution containing 750 μg/mL each of ASA and CLP. An aliquot of this solution (0.4 mL) was further diluted to 25 mL with methanol to obtain a solution containing 12 μg/mL each of ASA and CLP. The mixture was then passed through a nylon 0.45 μm membrane filter.

Method Validation

(**a**) *Calibration graph (linearity of the HPLC method)*.—Calibration graphs were constructed by plotting peak areas vs concentrations of ASA and CLP, and the regression equations were calculated. The calibration graphs were plotted over 6 different concentrations in the range of 4–24 μg/mL for both drugs. Accurately measured mixed standard solution aliquots of ASA and CLP (0.4, 0.8, 1.2, 1.6, 2.0, and 2.4 mL) were transferred to a series of 10 mL volumetric flasks and diluted to the mark with mobile phase. Aliquots (20 μL) of each solution were injected under the operating chromatographic condition described above [number of replicates (n = 6)].

(**b**) *Calibration graph (linearity of the HPTLC method)*.—Calibration graphs were plotted over the concentration range of 400–1400 ng/spot for both the drugs. Accurately prepared mixed standard solutions of ASA and CLP (4.0, 6.0, 8.0, 10.0, 12.0, and 14.0 μL) were applied to the plate. The calibration graphs were developed by plotting peak area vs concentrations (n = 6) with the help of the winCATS software.

(**c**) *Accuracy (recovery)*.—The accuracy of the methods was determined by calculating recoveries of ASA and CLP by the standard addition method. Known amounts of mixed standard solution of ASA and CLP (6.0, 12.0, and 18.0 μg/mL for the HPLC method and 300, 600, and 900 ng/spot for the HPTLC method) were added to prequantitated sample solutions of tablet dosage forms. The amounts of ASA and CLP were estimated by applying values of peak area to the regression equations of the calibration graph.

Table 3. Regression analysis of calibration graphs for ASA and CLP for the proposed HPLC and HPTLC methods

	HPLC		HPTLC	
Parameter	ASA	CLP	ASA	CLP
Concn range	4–24 μg/mL	4–24 μg/mL	400–1400 ng/spot	400–1400 ng/spot
Slope	50466.22	32355.47	7354.12	11346.08
SD[a] of the slope	13.67	16.48	345.32	388.81
Intercept	829.09	–2369.02	6492.18	9573.21
SD of the intercept	230.65	507.56	203.13	426.11
Correlation coefficient	0.9998	0.9999	0.9985	0.9993

[a] SD = Standard deviation.

Table 4. Summary of validation parameters for the proposed HPLC and HPTLC methods

	HPLC		HPTLC	
Parameter	ASA	CLP	ASA	CLP
LOD[a]	0.014 μg/mL	0.047 μg/mL	82.86 ng/mL	112.66 ng/mL
LOQ[b]	0.046 μg/mL	0.157 μg/mL	276.21 ng/mL	375.55 ng/mL
Accuracy, %	99.73–100.30	99.34–100.94	99.20–100.49	99.42–100.86
Repeatability (RSD[c], %, *n* = 6)	0.102	0.189	0.279	0.311
Precision (RSD, %)				
Interday (*n* = 3)	0.375–1.371	0.241–0.473	1.28–2.02	1.56–2.61
Intraday (*n* = 3)	0.310–1.316	0.297–0.526	1.36–1-95	1.32–2.15

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantitation.
[c] RSD = Relative standard deviation.

(**d**) *Method precision (repeatability).*—The precision of the instruments was checked by repeatedly injecting (*n* = 6) mixed standard solutions of ASA and CLP (12 μg/mL) for the HPLC method. Repeatability of HPTLC instruments was assessed by applying the same sample solution 6 times on a plate with the automatic spotter using the same syringe and by taking 6 scans of the sample spot for both ASA and CLP (600 ng/spot) without changing the position of the plate.

(**e**) *Intermediate precision (reproducibility).*—The intraday and interday precisions of the proposed methods were determined by analyzing mixed standard solution of ASA and CLP at 3 different concentrations (4.0, 12.0, and 24.0 μg/mL for the HPLC method and 400, 800, and 1200 ng/spot for the HPTLC method) 3 times on the same day and on 3 different days. The results are reported in terms of relative standard deviation (RSD).

(**f**) *Limit of detection (LOD) and limit of quantitation (LOQ).*—The LOD with signal-to-noise (S/N) ratio of 3:1 and the LOQ with S/N ratio of 10:1 were calculated for both drugs using the following equations according to International Conference on Harmonization guidelines (36):

$$\text{LOD} = 3.3 \times \sigma/S$$

$$\text{LOQ} = 10 \times \sigma/S$$

where σ = the standard deviation (SD) of the response and S = the SD of the *y*-intercept of the regression line.

(**g**) *Specificity.*—The excipients hydroxypropylcellulose, mannitol, microcrystalline cellulose, polyethylene glycol 6000, and lactose monohydrate (Signet Ltd., Mumbai, India), and Methocel E5 Premium LV EP (Colorcon Asia Pvt. Ltd., Goa, India) were spiked into a preweighed quantity of drugs to assess the specificity of the methods. The peak area was measured to determine the quantity of the drugs.

(**h**) *Robustness.*—Robustness of the methods was studied by changing the composition and the pH of mobile phase and determining the stability of the drugs in methanol for 24 h at ambient temperature. Spot stability was observed by performing 2-dimensional HPTLC development using the same mobile phase (37).

Analysis of ASA and CLP in Tablet Dosage Forms

The responses of sample solutions were measured at 235 nm for quantitation of ASA and CLP by using the HPLC and HPTLC methods as described above. The amounts of ASA and CLP present in sample solution were determined by applying values of peak area to the regression equations of the calibration graph.

Results and Discussion

HPLC Method

To optimize the HPLC parameters, several mobile phase compositions were tried. A satisfactory separation of ASA and CLP with good peak symmetry and steady baseline was obtained with the mobile phase acetonitrile–phosphate buffer (55 + 45, v/v) adjusted to pH 3.0. Quantitation was achieved with UV detection at 235 nm based on peak area. Complete resolution of the peaks with clear baseline separation was obtained (Figure 1). The system suitability test parameters are shown in Table 1.

HPTLC Method

Several mobile phases were tried to accomplish good separation of ASA and CLP. Using the mobile phase ethyl acetate–methanol–toluene–glacial acetic acid (5.0 + 1.0 + 4.0 + 0.1, v/v/v/v) and 10 × 10 cm HPTLC silica gel $60F_{254}$ aluminum-backed plates, good separation was attained with retardation factor (R_f) values of 0.55 for ASA and 0.79 for CLP. A wavelength of 235 nm was used for the quantitation of the drugs. Resolution of the peaks with clear baseline separation was found (Figure 2). The system suitability test parameters are shown in Table 2.

Validation of the Proposed Methods

Linearity.—Linear correlation was obtained between peak areas and concentrations of ASA and CLP in the range of

Table 5. Assay results for the combined dosage form using the proposed HPLC and HPTLC methods

	ASA ± SD[a] (n^b = 5), %		CLP ± SD (n = 5), %	
Tablet	HPLC	HPTLC	HPLC	HPTLC
A	99.77 ± 0.28	100.49 ± 0.55	100.07 ± 0.66	100.86 ± 0.71
B	100.04 ± 0.63	99.76 ± 0.79	100.16 ± 0.77	101.32 ± 0.83

[a] SD = Standard deviation.
[b] n = Number of determinations.

4–24 μg/mL for both the drugs, respectively, for the HPLC method and 400–1400 ng/spot for both the drugs, respectively, for HPTLC. Data of the regression analysis are summarized in Table 3.

Accuracy.—The recovery experiments were performed by the standard addition method. The recoveries obtained were 99.98 ± 0.28 and 100.16 ± 0.66% for ASA and CLP, respectively, by the HPLC method and 99.93 ± 0.55 and 100.21 ± 0.83% for ASA and CLP, respectively, by the HPTLC method (Table 4). The high values indicate that both methods are accurate.

Method precision.—The RSD values for ASA and CLP were found to be 0.102 and 0.189%, respectively, using HPLC and 0.279 and 0.311%, respectively, for HPTLC (Table 4). The RSD values were found to be <1%, which indicates that the proposed methods are repeatable.

Intermediate precision.—The RSD values were found to be <2%, which indicates that the proposed methods are reproducible (Table 4).

LOD and LOQ.—LOD values for ASA and CLP were found to be 0.014 and 0.047 μg/mL, respectively, for HPLC and 82.86 and 112.66 ng/mL, respectively, for HPTLC. LOQ values for ASA and CLP were found to be 0.046 and 0.157 μg/mL, respectively, for HPLC and 276.21 and 375.55 ng/mL, respectively, for HPTLC (Table 4). These data show that nanogram quantity of both drugs can be accurately determined.

Specificity.—Excipients used in the specificity studies did not interfere with the estimation of either of the drugs by the proposed methods. Hence, the methods were found to be specific for estimation of ASA and CLP.

Robustness.—Peak area and retention time variation were found to be <1%. Also, no significant change in peak area was observed during 24 h. No decomposition was observed in either the first or second direction of the 2-dimensional analysis for both drugs on the HPTLC plate. Hence, the methods were found to be robust for estimation of ASA and CLP.

Assay of the Tablet Dosage Form (ASA and CLP 75 mg/Tablet)

The proposed validated methods were successfully applied to determine ASA and CLP in their tablet dosage forms (tablets A and B). The results obtained for ASA and CLP were comparable with the corresponding labeled amounts (Table 5).

Comparison of the Proposed Methods

The assay results for ASA and CLP in their combined dosage form obtained using the HPLC and HPTLC methods were compared by applying the paired t-test. The calculated t-values of 0.72 for ASA and 1.04 for CLP were less than the tabulated t-value (2.13) at the 95% (P = 0.05) confidence level. Therefore, there was no significant difference in the determined content of ASA and CLP by the HPLC and HPTLC methods.

The literature describes an HPLC method (38) for determination of ASA, and HPLC (32) and HPTLC (34) methods for determination of CLP in tablet dosage forms. The assay results obtained by these methods were used for statistical comparison to evaluate the validity of developed HPLC and HPTLC methods. For ASA, the calculated F-value was found to be 1.58 (for HPLC), which is less than the tabulated F-value (5.05) at the 95% (P = 0.05) confidence level. For CLP, the calculated F-values were found to be 1.35 (for HPLC) and 2.84 (for HPTLC), which were less than the tabulated F-value (3.48) at the 95% (P = 0.05) confidence level. Therefore, there were no significant differences among the methods.

Conclusions

The proposed methods have the advantages of simplicity and convenience for the separation and quantitation of ASA and CLP in combination and can be used for the assay of their dosage form. Also, the low solvent consumption and short analytical run time lead to environmentally friendly chromatographic procedures.

The results were compared statistically, and both methods were found to be precise and accurate. The additives usually present in the pharmaceutical formulations of the assayed analytes did not interfere with determination of ASA and CLP. The methods can be used for the routine simultaneous analysis of ASA and CLP in pharmaceutical preparations.

Acknowledgments

We are grateful to Sun Pharmaceuticals for the gratis samples of pure ASA and CLP.

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