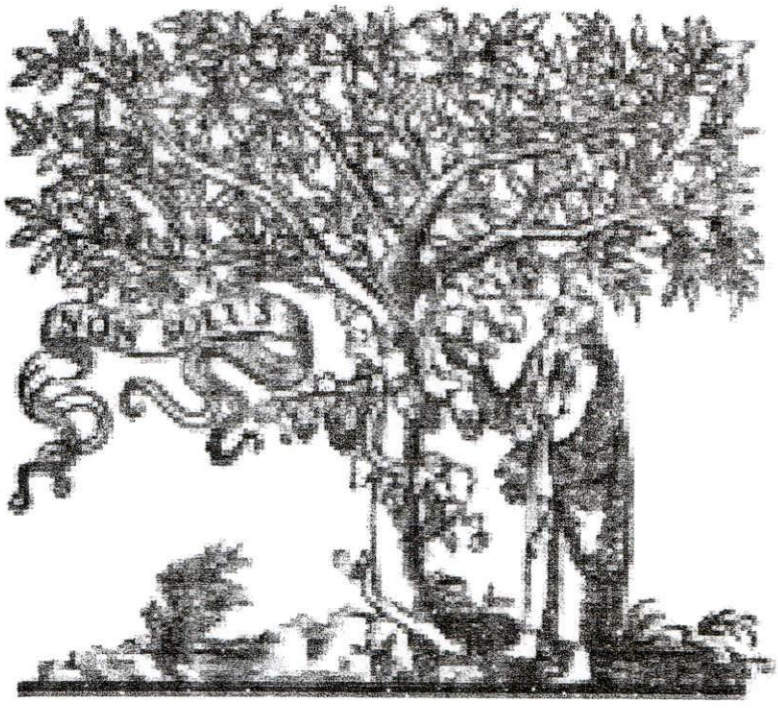

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2

Clopidogrel Bisulfate

Maria L.A.D. Lestari,* Suciati,*
Gunawan Indrayanto,* and **Harry G. Brittain†**

Contents

1. Description 72
1.1. Nomenclature [1] 72
1.2. Formulae [2] 72
1.2.1. Empirical formula, molecular weight, CAS number 72
1.2.2. Structural formula 73
1.3. Appearance 73
1.4. Elemental analysis [1] 73
1.5. Uses and applications 73
2. Physical Characteristics 74
2.1. Ionization constant 74
2.2. Solubility characteristics and partition coefficient 74
2.3. Crystallographic properties 74
2.4. Thermal properties 75
2.4.1. Melting range 75
2.4.2. Differential scanning calorimetry 75
2.5. Spectroscopic properties 78
2.5.1. Ultraviolet absorption spectroscopy 78
2.5.2. Optical activity 78
2.5.3. Infrared absorption spectroscopy 79
2.5.4. Raman spectroscopy 79
3. Methods of Analysis 82
3.1. Known impurities of clopidogrel 82
3.2. Compendial methods of analysis 84
3.2.1. Identification 84
3.2.2. Impurity analysis 85

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Profiles of Drug Substances, Excipients, and Related Methodology, Volume 35
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3.2.3. Assay methods 86
3.3. Spectroscopic methods of analysis 86
3.3.1. Ultraviolet absorption spectroscopy 86
3.3.2. Vibrational spectroscopy 88
3.3.3. Nuclear magnetic resonance 89
3.4. Potentiometric method of analysis 90
3.5. X-Ray powder diffraction methods of analysis 90
3.6. Thermal methods of analysis 92
4. Chromatographic Methods of Analysis 93
4.1. Thin-layer chromatography 93
4.2. Gas chromatography 93
4.3. Liquid chromatography 93
4.4. Capillary electrophoresis 100
5. Determination in Biological Samples 101
5.1. Liquid chromatography 101
5.2. Gas chromatography 113
References 113

1. DESCRIPTION

1.1. Nomenclature [1]

Clopidogrel bisulfate is the hydrogen sulfate salt of clopidogrel, for which the free compound is known by the following systematic names:
Systematic chemical name

- (α*S*)-α-(2-Chlorophenyl)-6,7-dihydrothieno[3,2-*c*]pyridine-5(4*H*)-acetic acid methyl ester

Alternate systematic chemical names

- Methyl-(+)-(*S*)-α-(*o*-chlorophenyl)-6,7-dihydrothieno[3,2-*c*]pyridine-5(4*H*)-acetate
- (+)-Methyl-α-5-[4,5,6,7-tetrahydro[3,2-*c*]thienopyridyl]-(2-chlorophenyl)acetate

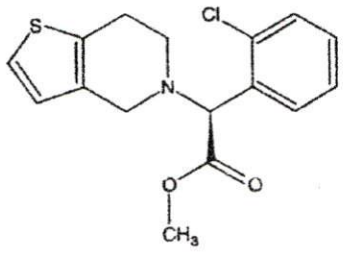
1.2. Formulae [2]

1.2.1. Empirical formula, molecular weight, CAS number

Clopidogrel	$C_{16}H_{16}ClNO_2S$	321.82	[113665-84-2]
Clopidogrel bisulfate	$C_{16}H_{18}ClNO_6S_2$	419.90	[135046-48-9]

1.2.2. Structural formula

The structural formula of clopidogrel is:



The site of interaction for salt formation is at the pyridine nitrogen, which is capable of forming salts only with extremely strong acids.

1.3. Appearance

Clopidogrel is a colorless oil under ambient conditions, while the bisulfate sale is obtained in the form of white crystals [1].

1.4. Elemental analysis [1]

	C (%)	HX (%)	Cl (%)	N (%)	O (%)	S (%)
Clopidogrel	59.71	5.01	11.02	4.35	9.94	9.96
Clopidogrel bisulfate	45.77	4.32	8.44	3.34	22.86	15.27

1.5. Uses and applications

Clopidogrel contains a center of dissymmetry, and hence is capable of being resolved into its two mirror image compounds. It has been found that only the (*S*)-enantiomer (which corresponds to the dextrorotatory form) has antithrombotic activity and that the (*R*)-enantiomer (which corresponds to the levorotatory form) does not exhibit antithrombotic activity. Moreover, in animal studies the (*R*)-enantiomer triggered convulsions at high doses [4, 5]. Consequently, (*R*)-clopidogrel bisulfate is considered to be one of the impurities in (*S*)-clopidogrel bisulfate bulk drug substance.

2. PHYSICAL CHARACTERISTICS

2.1. Ionization constant

Using the Advanced Chemistry Development Physical Chemistry program, the pK_a of clopidogrel has been calculated as 4.56 ± 0.20.

2.2. Solubility characteristics and partition coefficient

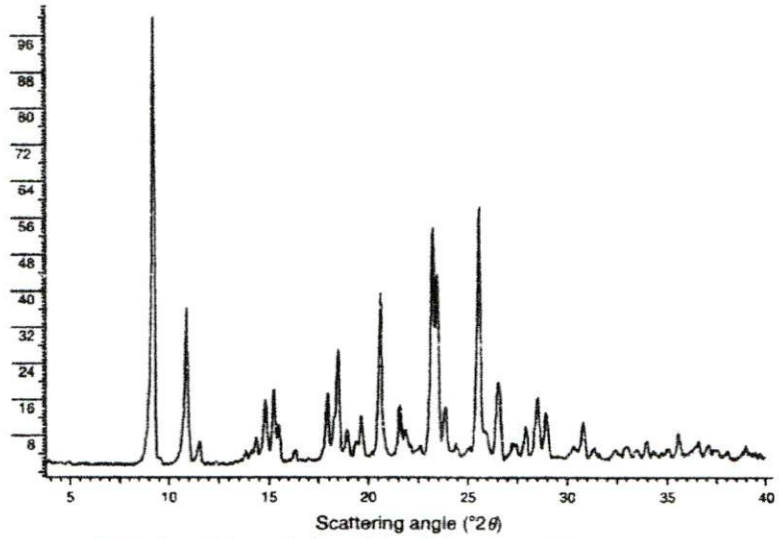
Clopidogrel bisulfate has been reported to be freely soluble in methanol, sparingly soluble in methylene chloride, and practically insoluble in ethyl ether and in water at neutral pH. At pH 1, the compound is freely soluble in water [3], which is one of the reasons why the hydrogen sulfate salt is the preferred form of the drug substance.

The partition coefficient in octanol/water of clopidogrel bisulfate has been reported to be about 3.9 at pH 7.4 [3].

2.3. Crystallographic properties

Although clopidogrel bisulfate has been found to crystallize in six different polymorphic forms [9, 10], only Form-I and Form-II are used in pharmaceutical products. Although the density of orthorhombic Form-II is less dense (1.462 g/cm^3) than is the monoclinic Form-I (1.505 g/cm^3), the two crystal forms appear to be fairly equivalent in their relative stability. However, Form-II is more compact and much less electrostatic than is Form-I, making it more useful for secondary processing [9].

The methods for the preparation of clopidogrel bisulfate Form-I and Form-II, as described in United States patent 6,504,030 [9], have been reproduced. X-ray powder diffraction (XRPD) patterns of these products were obtained using a Rigaku MiniFlex powder diffraction system, equipped with a horizontal goniometer operating in the $\theta/2\theta$ mode. The X-ray source was nickel-filtered Kα emission of copper (1.54184 Å). Samples were packed into the sample holder using a back-fill procedure, and were scanned over the range of 3.5–40° 2θ at a scan rate of 0.5° 2θ/min. Using a data acquisition rate of 1 point per second, the scanning parameters equate to a step size of 0.0084° 2θ. Calibration of the diffractometer system pattern was effected by scanning an aluminum plate, and using the aluminum scattering peaks having *d*-spacings of 2.338 and 2.024 Å to verify both the angle and scan rate. The intensity scale for all diffraction patterns was normalized so that the relative intensity of the most intense peak in the pattern equaled 100%. The patterns obtained for the two polymorphic forms of clopidogrel bisulfate are shown in Figs. 2.1 and 2.2, along with tables containing the angles and calculated *d*-spacings of the most intense scattering peaks.



Scattering angles and *d*-spacings of the 10 most intense peaks

Scattering angle (°2θ)	*d*-Spacing (Å)	Scattering angle (°2θ)	*d*-Spacing (Å)
9.22	9.580	20.63	4.302
10.89	8.117	23.23	3.825
15.27	5.798	23.47	3.788
17.95	4.937	25.59	3.479
18.50	4.793	26.54	3.356

FIGURE 2.1 X-ray powder diffraction pattern of clopidogrel bisulfate, Form-I.

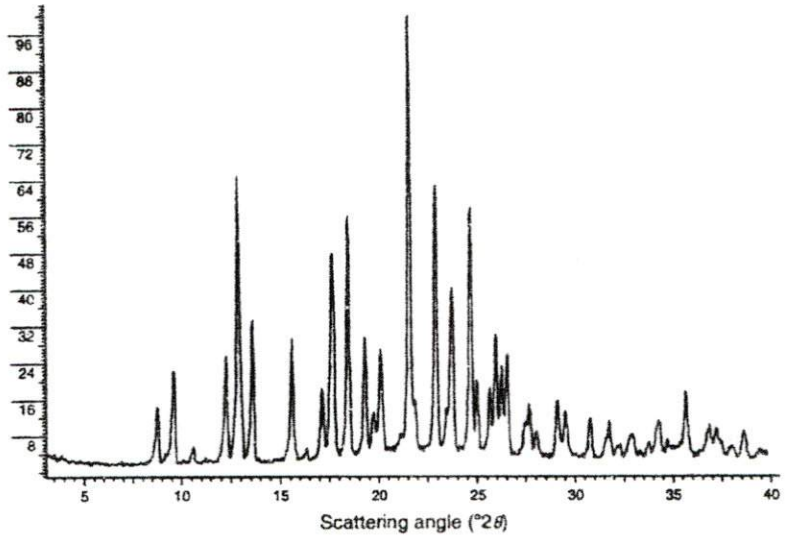
2.4. Thermal properties

2.4.1. Melting range

Polymorphic Form-I has been reported to exhibit a melting point range spanning 198–200 °C, while Form-II exhibits a melting point range spanning 176–178 °C [11].

2.4.2. Differential scanning calorimetry

Measurements of differential scanning calorimetry (DSC) were obtained on clopidogrel bisulfate Form-I and Form-II using a TA Instruments 2910 thermal analysis system. Samples of approximately 1–2 mg were accurately weighed into an aluminum DSC pan, and then covered with an aluminum lid that was inverted and pressed down so as to tightly contain



Scattering angles and *d*-spacings of the 12 most intense peaks

Scattering angle (°2θ)	*d*-Spacing (Å)	Scattering angle (°2θ)	*d*-Spacing (Å)
9.61	9.199	18.47	4.800
12.28	7.204	19.29	4.597
12.89	6.864	21.61	4.109
13.62	6.497	22.95	3.872
15.62	5.670	23.76	3.742
17.66	5.018	24.71	3.601

FIGURE 2.2 X-ray powder diffraction pattern of clopidogrel bisulfate, Form-II.

the powder between the top and bottom aluminum faces of the lid and pan. The samples were then heated over the temperature range of 20–200 °C, at a heating rate of 10 °C/min.

The thermograms obtained for the two clopidogrel bisulfate polymorphic forms are shown in Figs. 2.3 and 2.4. While the temperature maximum associated with the melting endotherm of Form-II (180.7 °C) agreed reasonably well with the melting point value, the temperature maximum associated with the melting endotherm of Form-I (184.1 °C) was significantly less than the melting point value.

The endothermic melting transitions were integrated, enabling a determination of the enthalpies of fusion for the two forms. For Form-I it was found that $\Delta H_f = 70.6$ J/g, while for Form-II it was found that

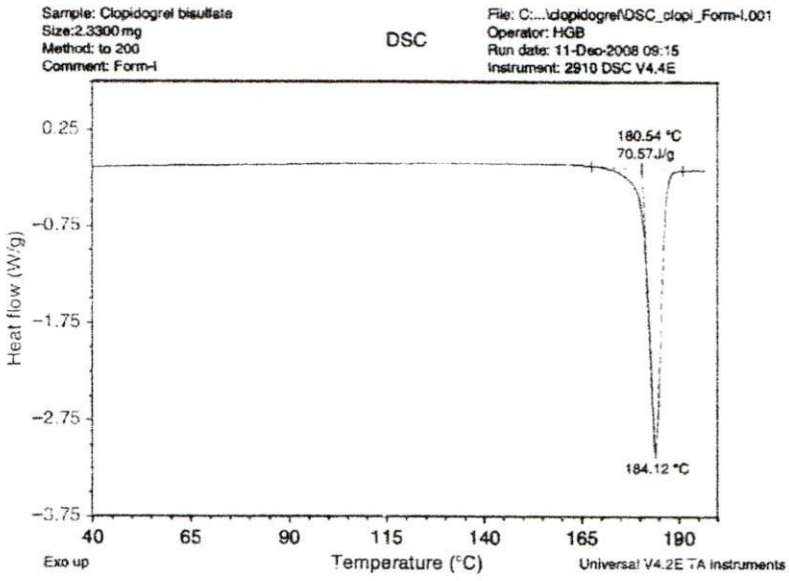


FIGURE 2.3 Differential scanning calorimetry thermogram of clopidogrel bisulfate, Form-I.

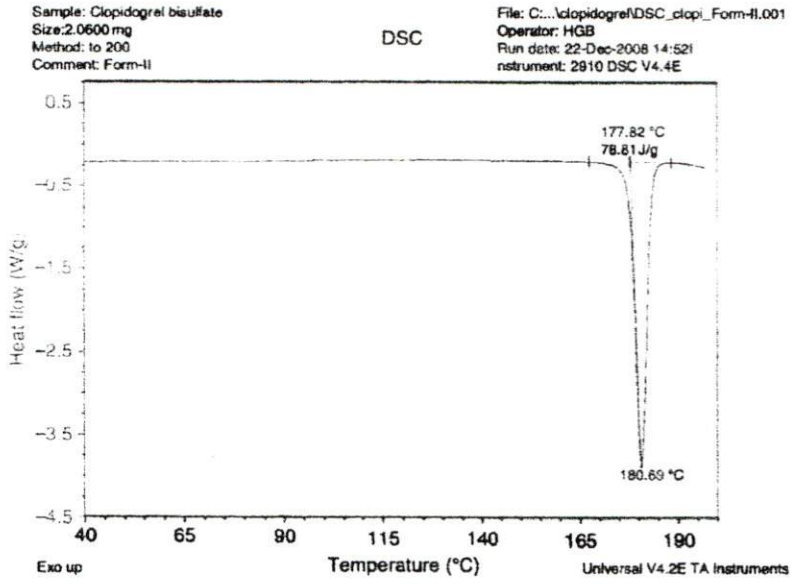


FIGURE 2.4 Differential scanning calorimetry thermogram of clopidogrel bisulfate, Form-II.

$\Delta H_f = 78.8$ J/g. Since the higher melting polymorphic form exhibited the lower heat of fusion, it is concluded that the two polymorphs are enantiotropically related.

2.5. Spectroscopic properties

2.5.1. Ultraviolet absorption spectroscopy

Clopidogrel bisulfate was dissolved at a concentration of 5.5 μg/ml in methanol that had been acidified with sulfuric acid. The UV absorption spectrum of this solution was obtained using a Beckman model Lambda 3B spectrophotometer, and the resulting spectrum is found in Fig. 2.5. The most intense absorption band was observed at a wavelength of 220 nm, while (as the inset shows) two very weak peaks were also noted at 270 and 278 nm.

2.5.2. Optical activity

The Merck Index reports that the specific rotation ($[\alpha]_D^{20}$) of clopidogrel as +51.52°, when the measurement is made at a concentration of 1.61 g/dl in methanol. It was also reported that for the bisulfate salt, $[\alpha]_D^{20} = +55.10°$ ($c = 1.891$ in methanol). The latter value is consistent with the data

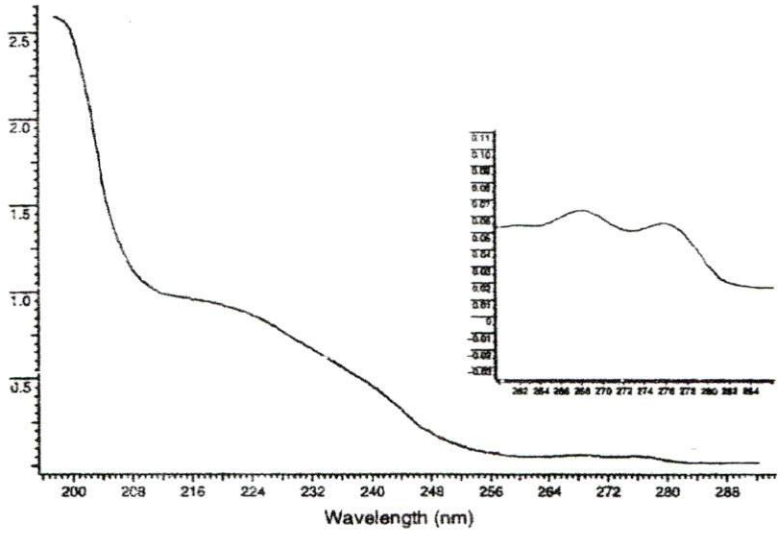


FIGURE 2.5 Ultraviolet absorption spectrum obtained clopidogrel bisulfate, dissolved at a concentration of 5.5 μg/ml in methanol that had been acidified with sulfuric acid. The inset spectrum represents an expansion of the absorbance scale.

published in the patent literature, namely $[\alpha]_D^{20} = +55.16°$ ($c = 1.68$ in methanol) for Form-I and $[\alpha]_D^{20} = +55.10°$ ($c = 1.68$ in methanol) for Form-II [11].

2.5.3. Infrared absorption spectroscopy

The infrared absorption spectra of clopidogrel bisulfate Form-I and Form-II were obtained at a resolution of 4 cm^{-1} using a Shimadzu model 8400S Fourier-transform infrared spectrometer, with each spectrum being obtained as the average of 40 individual spectra. The data were acquired using the attenuated total reflectance sampling mode, where the samples were clamped against the ZnSe crystal of a Pike MIRacle™ single reflection horizontal ATR sampling accessory.

As shown in Figs. 2.6–2.9, the infrared absorption spectra of the two polymorphic forms of clopidogrel bisulfate are quite different. These differences are summarized in Table 2.1, which lists the energies of the main observed peaks and which contrasts the energies of peaks assigned to the same vibrational mode.

2.5.4. Raman spectroscopy

The Raman spectra of clopidogrel bisulfate Form-I and Form-II were obtained in the fingerprint region using a Raman Systems model R-3000HR spectrometer, operated at a resolution of 5 cm^{-1} and a laser

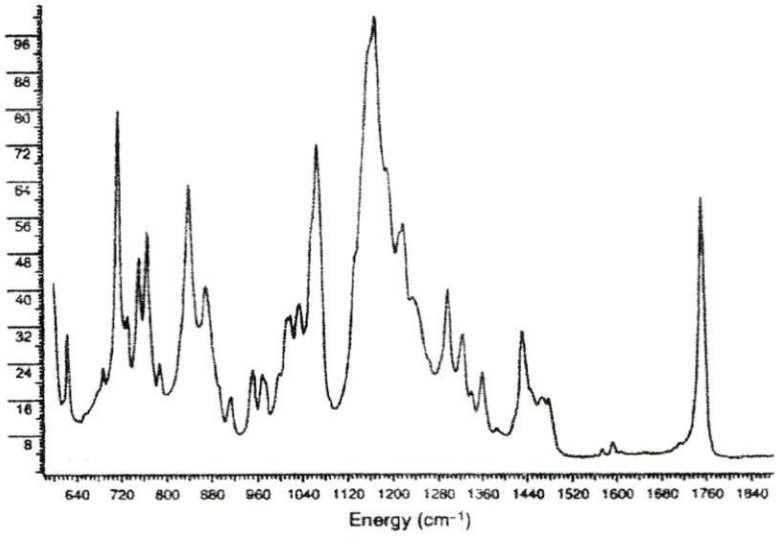


FIGURE 2.6 Infrared absorption spectrum obtained in the fingerprint region for clopidogrel bisulfate, Form-I.

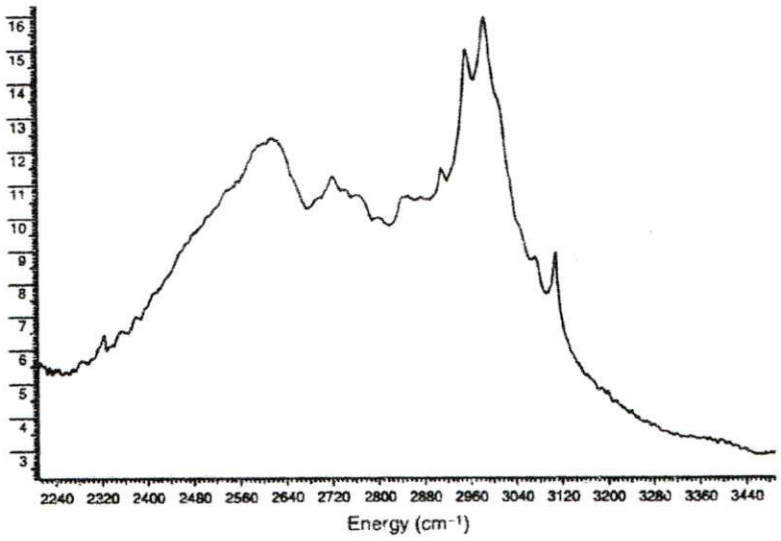


FIGURE 2.7 Infrared absorption spectrum obtained in the high-frequency region for clopidogrel bisulfate, Form-I.

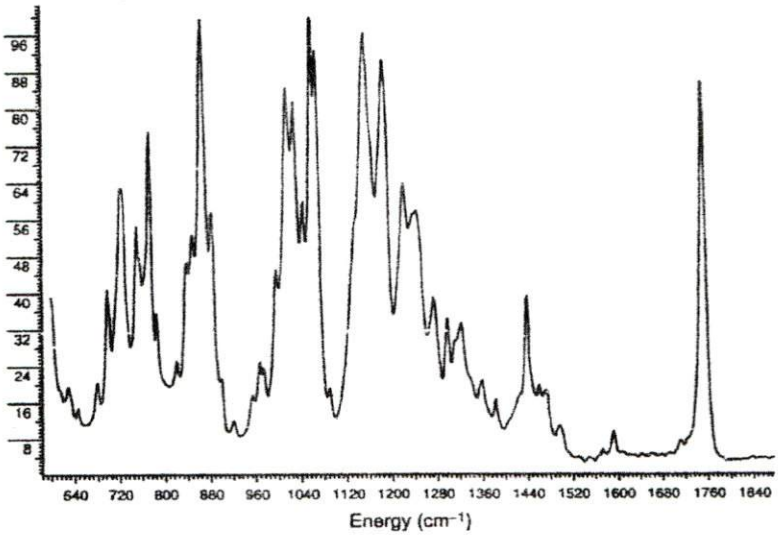


FIGURE 2.8 Infrared absorption spectrum obtained in the fingerprint region for clopidogrel bisulfate, Form-II.

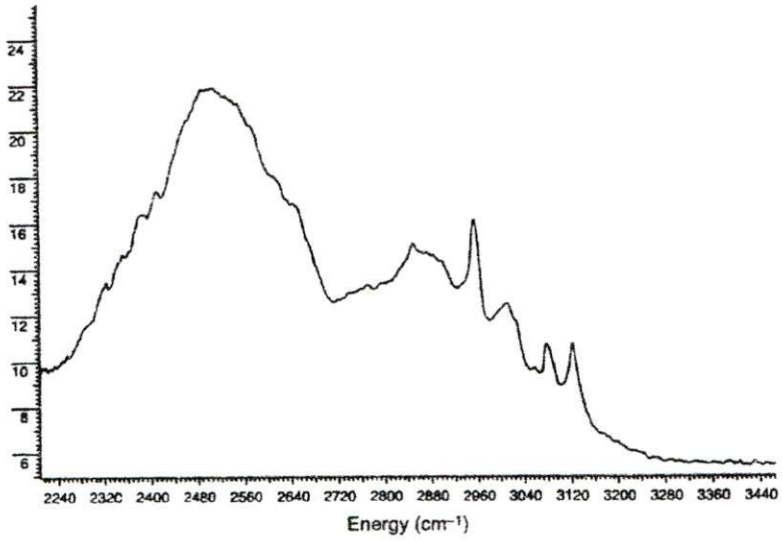


FIGURE 2.9 Infrared absorption spectrum obtained in the high-frequency region for clopidogrel bisulfate, Form-II.

TABLE 2.1 Energies of corresponding bands in the infrared absorption spectra of clopidogrel bisulfate, Forms I and II

Energy, Form-I band (cm^{-1})	Energy, Form-II band (cm^{-1})	Energy, Form-I band (cm^{-1})	Energy, Form-II band (cm^{-1})
624.9			1151.4
	698.2	1170.7	
715.5	721.3		1184.2
752.2	750.3	1218.9	1220.9
765.7	773.4		1244.0
839.0			1272.9
869.8	866.0	1298.0	1298.0
912.3		1325.0	1321.1
950.8		1359.7	
968.2		1431.1	1438.8
1018.3	1014.5	1477.4	
1033.8	1028.0		1591.2
	1056.9	1751.2	1749.3
1066.6	1066.6		

wavelength of 785 nm. The data were acquired using front-face scattering from a thick powder bed contained in an aluminum sample holder.

As shown in Figs. 2.10 and 2.11, the Raman spectra of the two polymorphic forms of clopidogrel bisulfate are substantially different. These differences are summarized in Table 2.2, which lists the energies of the main observed peaks and which contrasts the energies of peaks assigned to the same vibrational mode.

3. METHODS OF ANALYSIS

3.1. Known impurities of clopidogrel

Clopidogrel bisulfate has several related compounds that are to be impurity species [2, 6, 7]. The molecular structures of these impurities are shown in Fig. 2.12.

Related compound (**A**) has the systematic name (+)-(*S*)-(*o*-chlorophenyl)-6,7-dihydrothieno[3,2-*c*]pyridine-5-(4*H*)-acetic acid [2], and is a hydrolysis product of the ester group of clopidogrel. This impurity is formed as a result of the combined effects of moisture and temperature [2, 6, 7].

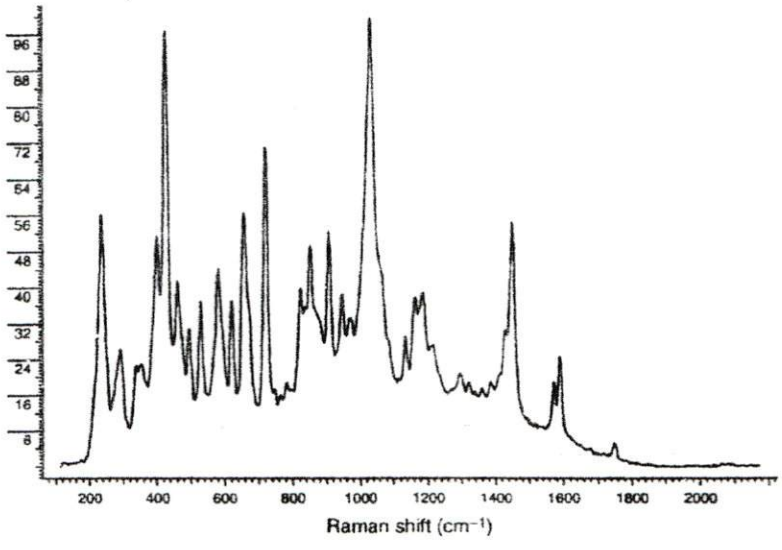


FIGURE 2.10 Raman spectrum obtained in the fingerprint region for clopidogrel bisulfate, Form-I.

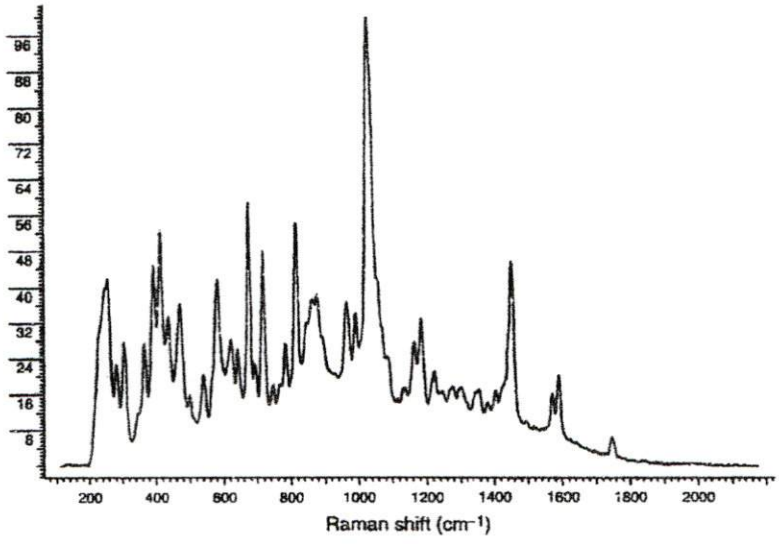


FIGURE 2.11 Raman spectrum obtained in the fingerprint region for clopidogrel bisulfate, Form-II.

TABLE 2.2 Energies of corresponding bands in the Raman spectra of clopidogrel bisulfate, Forms I and II

Raman shift, Form-I band (cm^{-1})	Raman shift, Form-II band (cm^{-1})	Raman shift, Form-I band (cm^{-1})	Raman shift, Form-II band (cm^{-1})
235.4	254.4		781.9
	278.9	822.0	814.2
292.3	303.5	852.2	
351.4	364.8		875.6
	390.4	907.9	
399.4		947.0	964.8
	412.8		989.3
426.1	435.1	1032.8	1025.0
460.7	469.6	1133.2	
495.3		1162.2	1163.3
528.7	538.8	1185.6	1183.4
580.0	581.2		1221.3
620.2	621.3	1449.9	1449.9
657.0	641.4	1571.5	1570.4
	673.7	1589.3	1588.2
720.6	716.1		1746.6

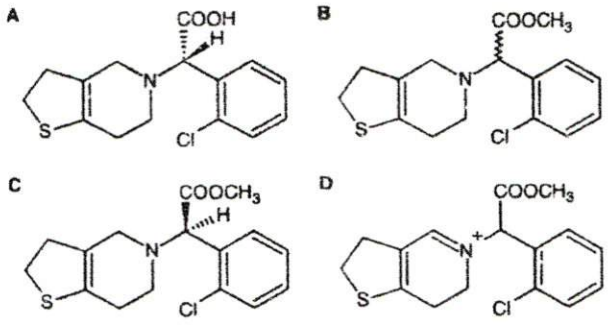


FIGURE 2.12 Structures of four known impurities of clopidogrel bisulfate.

Related compound (**B**) has the systematic name methyl-(±)-(*o*-chlorophenyl)-4,5-dihydrothieno[2,3-*c*]pyridine-6-(7*H*)-acetate, hydrogen sulfate salt [2], and is a racemic residue formed during the manufacturing process. This compound may appear as a racemic mixture in samples of bulk drug substance as impurities (**1a**) and (**1b**) [6, 7].

Related compound (**C**) is the (*R*)-enantiomer of clopidogrel bisulfate, and therefore has the systematic name methyl-(−)-(*R*)-(*o*-chlorophenyl)-6,7-dihydrothieno[3,2-*c*]pyridine-5(4*H*)-acetate, hydrogen sulfate salt [2, 6, 7].

Related compound (**D**) has been reported to result from an oxidation process, and has the systematic name 5-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-6,7-dihydrothieno[3,2-*c*]pyridine-5-ium [8].

3.2. Compendial methods of analysis

3.2.1. Identification

USP32-NF27 [2] sets forth the use of infrared absorption spectroscopy (method ⟨197K⟩), a chromatographic method, and the sulfate test (method ⟨191K⟩) for identification of clopidogrel bisulfate bulk drug substance. The IR absorption method requires mixing the assayed substance with potassium bromide, and then recording the spectra of the test specimen and the USP reference standard over the range of 2.6–15 μm (3800–650 cm^{-1}). The chromatographic method uses the liquid chromatography (LC) method of the assay section, with the monograph specifying that the retention time of the major peak be similar with that of the reference standard. Furthermore, treatment of a sample with barium chloride TS should result in the formation of a white $BaSO_4$ precipitate which is

insoluble in hydrochloride acid and nitric acid. As a confirmatory test, addition of lead acetate TS to a sample will result in the formation of a white $PbSO_4$ precipitate that is insoluble in ammonium acetate TS.

For identification of clopidogrel bisulfate in pharmaceutical preparations, USP32-NF27 [2] recommends use of a UV absorption and a chromatographic method. The test solution required for performance of the UV absorption method is obtained as for the dosage form uniformity test, where one tablet containing clopidogrel bisulfate is dissolved in 0.1 N HCl. The spectrum is recorded over 250–300 nm, where clopidogrel bisulfate exhibits a wavelength maximum at 270 nm. The chromatographic method to be used for identification of clopidogrel bisulfate in pharmaceutical preparations is the same as used for assay of the bulk drug substance.

3.2.2. Impurity analysis

USP32-NF27 [2] requires use of a liquid chromatographic method to determine three clopidogrel bisulfate-related compounds, and this method is the same as the chromatographic method used to determine clopidogrel in bulk substances. Stock solutions of clopidogrel bisulfate and each of its related compounds are prepared by dissolution in methanol, and these are subsequently diluted with methanol to obtain concentrations of 20 μg/ml (for clopidogrel bisulfate RS), 40 μg/ml (for related compound **A**), 120 μg/ml (for related compound **B**), and 200 μg/ml (for related compound C). Five milliliters of each of these solutions is then diluted with mobile phase to obtain final concentrations of 0.5, 1, 3, and 5 μg/ml, respectively, for clopidogrel and the three impurities.

Test solutions of analytes are prepared by dissolving 100 mg of sample with 5 ml methanol, and then diluting to volume with mobile phase. With the flow rate set adjusted to 1.0 ml/min, the relative retention times will be approximately 0.5 for related compound **A**, 0.8 and 1.2 for the two enantiomers of related compound **B**, 1.0 for clopidogrel, and 2.0 for related compound C. The resolution between clopidogrel and the first enantiomer of related compound **B** must be greater than 2.5. The monograph specification of USP32-NF27 is that not more than 0.2% of related compound **A** is found, not more than 0.3% of the first enantiomer of related compound **B** is found, and not more than 1.0% of related compound **C** is found. In addition, the monograph requires that not more than 0.1% of any other impurity is found, and that the total impurity content be less than 1.5%. The concentrations of all clopidogrel-related compounds are to be expressed as bisulfate salts, obtained by using bisulfate salt equivalents as stated on the USP reference standard labels.

3.2.3. Assay methods

A liquid chromatographic method is utilized for the determination of clopidogrel bisulfate in samples of the bulk drug substance. The method uses a column (L57 column size 15 cm × 4.6 mm) packed with ovomucoid (a chiral-recognition protein) that is chemically bonded to silica particles of 5 μm diameter and a pore size of 120 Å. Both the reference standard and the sample to be analyzed are dissolved in methanol, and then diluted with mobile phase. The mobile phase is 75:25 0.01 M phosphate buffer/acetonitrile, and the flow rate is adjusted to 1.0 ml/min. Observation is made on the basis of the UV absorbance at 220 nm, and the clopidogrel peak has a relative retention time about 1.0 min.

The assay method for determination of clopidogrel bisulfate content in tablets uses the same LC method as used for the bulk drug substance. Here, not less than 20 tablets are finely powdered, and a quantity of the powder (equivalent to about 75 mg of clopidogrel base) is dissolved in and diluted with methanol. The quantity of clopidogrel base (in mg units) is calculated using a gravimetric factor based on the molecular weight of clopidogrel base and the molecular weight of clopidogrel bisulfate. The calculation also takes into account the peak responses of the standard and the sample:

$$1000 \times \left(\frac{321.82}{419.90}\right) C \left(\frac{r_u}{r_s}\right) \tag{1}$$

where C is concentration of clopidogrel bisulfate USP reference standard, while r_u and r_s are the respective peak responses obtained from the sample and the standard.

3.3. Spectroscopic methods of analysis

3.3.1. Ultraviolet absorption spectroscopy

Sankar *et al.* [12] reported a spectrophotometric method for the analysis of clopidogrel and repaglinide in their pure forms and in their combination tablet. In this method, water was used as the solvent, and clopidogrel was determined on the basis of its absorbance at a wavelength of 225 nm. The regression curve was linear over the range of 10–60 μg/ml.

Another spectrophotometric method for determining clopidogrel bisulfate in aspirin combination tablets was reported by Mishra and Dolly[13]. In this method, the absorbance additivity technique and the graphical determination of absorbance ratios methods were applied. Stock solutions of clopidogrel bisulfate and aspirin were prepared in methanol, and working solutions were then prepared by mixing 1 ml of the stock solutions with 1 ml of H_2SO_4 and heating in a water bath for

30 min. Linearity in the calibration curve was obtained over the range of 4–18 μg/ml for both methods, and ranged from 97.0% to 101.4%.

Rajput *et al.* reported another method to determine of clopidogrel bisulfate and aspirin in combination tablets, developing and validating a spectrophotometric assay based on chemometric methods [14]. Methanol was used to prepare standard solutions of clopidogrel bisulfate and aspirin, as well as to extract analytes from the tablets. In this method, chemometric methods based on inverse least square (ILS) and classical least square (CLS) were applied. For the purpose of establishing a calibration set, 12 mixture compositions of aspirin (0–20 μg/ml) and clopidogrel bisulfate (0–30 μg/ml) were employed, and the UV spectra of each mixture was recorded over the spectral region of 200–310 nm (16 wavelength point absorbance measurements were obtained with a 2-nm interval spanning 220–250 nm). In order to validate the method, calibration models developed based on ILS and CLS methodologies were used to determine clopidogrel bisulfate and aspirin in mixtures. Ten combination mixtures were used as a validation set and subjected to recovery studies. It was found that the recovery based on the ILS method was 97.33–103.33%, while the recovery obtained using the CLS method was 95.55–103.50%.

A spectrophotometric method was developed by Zaazaa *et al.* [15] to analyze clopidogrel bisulfate and its alkaline degradation, where the method used two spectrophotometric derivatization methods and a spectrophotometric method based on bivariate calibration. The first method used second derivative spectrophotometry, in which the derivative curves were recorded at $\Delta\lambda = 4$ with a scaling factor of 100. The wavelength values used for the calibration curve were 219.6, 270.6, 274.2, and 278.4 nm, which corresponds to the zero-crossing points of the degradant. This second derivative spectrophotometric method was able to determine clopidogrel bisulfate in the presence of 65% degradant, with a recovery ranging from 99.32% to 100.51% and linearity over the 4–37 μg/ml concentration range. In addition, this method was also employed to study the kinetics of the alkaline degradation of clopidogrel bisulfate at 278.4 nm.

The second spectrophotometric method developed by Zaazaa *et al.* [15] used the first derivative of ratio spectra, with peak amplitudes being recorded at 217.6 and 229.4 nm ($\Delta\lambda = 4$ nm, scaling factor = 10). The absorption spectrum of clopidogrel bisulfate was then divided by the absorption spectrum of a 15 μg/ml solution of the degradant over the range of 5–38 μg/ml. By using this latter method, clopidogrel bisulfate could be determined in the presence of up to 70% of its degradant.

The third spectrophotometric developed by Zaazaa *et al.* [15] used a bivariate calibration technique, which is simple and does not require performance of a derivatization procedure. In addition, no full spectrum information is required, eliminating the need for extensive data

processing. In this method, each substance (clopidogrel bisulfate and an alkaline analyte) are observed at two different wavelengths (210 and 225 nm), and calibration curves were established for each wavelength. The bivariate calibration technique could enable the determination of clopidogrel bisulfate in the presence of up to 70% of its alkaline degradant with recoveries ranging from 98.0% to 101.10%. Linearity in the clopidogrel bisulfate calibration curve was obtained over the range of 5–38 μg/ml, while linearity for its alkaline degradant extended over the range of 5–25 μg/ml.

3.3.2. Vibrational spectroscopy

Two infrared absorption methods (i.e., FTIR) and a Raman spectroscopic method were used to quantify polymorphic clopidogrel bisulfate Form-I and Form-II [16, 17]. In addition, qualitative analysis of these polymorphs was also conducted using FTIR [16], where each sample was scanned over in the spectral region of 450–4000 cm^{-1} at a resolution of 4 cm^{-1}. The sampling procedure used KBr pellets, loaded to contain approximately 3% of analyte. It was found that absorption bands associated with C–H and C–O bonds were stronger for Form-II relative to Form-I, and that unique absorption bands for Form-I and Form-II were observed at 841 and 1029 cm^{-1}, respectively. These absorption bands were reported to be useful in the quantitative or qualitative analysis of clopidogrel polymorphs.

For quantitative analysis, it was necessary to sieve both polymorph forms prior to analysis in order to obtain some degree of uniformity in particle size. The sieving method was chosen owing to the possibility of polymorphic interconversion during grinding [16]. The quantitative analysis of Form-I was performed on the basis of absorption intensities at 2987, 1175, and 841 cm^{-1}, and at 1497, 1187, and 1029 cm^{-1} for Form-II. Quantification of Form-I in a binary polymorph mixture was performed at 841 cm^{-1} due to the sensitivity of this band, and the readily interpretable spectral difference. Moreover, this band was not affected by pressure effects associated with preparation of the KBr pellet. The range of the method was evaluated between 10% and 90% of Form-I in samples of Form-II. However, the method was found to be limited to low levels of Form-I in Form-II since the characteristic Form-II bands were not visible for mixtures containing less than 30% of Form-I. The recovery of the method ranged from 100.0% to 101.0% for Form-I and in Form-II percentages of 20%, 50%, 60%, and 80% [16].

In another study, samples for polymorphic analysis were processed using gentle grinding after mixing [17]. Here, a combination of FTIR and Raman spectroscopy with multivariate analysis was utilized to quantify Form-II in mixtures with Form-I. Multivariate analysis using the PLS

method gave the best results when compared to other methods such as CLS and PCR. For the Raman spectroscopic method, the respective LOD and LOQ values were determined to be 1.0% and 3.0% when using PLS multivariate analysis, while for the FTIR method the respective LOD and LOQ values were found to be 0.7% and 0.2%. Both methods were able to quantify down to 3% of clopidogrel bisulfate Form-II in mixtures with Form-I. The accuracy of the method was characterized by RSD values smaller than 10% over the analysis range of 3–15% (w/w). It was concluded that by employing PLS multivariate analysis for both FTIR and Raman spectroscopy, quantification of lower amounts of clopidogrel bisulfate Form-II polymorph was possible [17].

3.3.3. Nuclear magnetic resonance

An NMR spectroscopic method was utilized by Badorc and Fréhel [5] to determine the enantiomeric purity of the dextrorotatory and levorotatory enantiomers of clopidogrel bisulfate. The chiral lanthanide shift reagent, $Eu(tfc)_3$ [tfc = 3-(trifluoromethyl-hydroxymethylene)-*d*-camphorato], was added to a $CDCl_3$ solution containing the racemic drug substance, whereupon the enantiomeric purity could be determined using 60 MHz ^1H NMR spectroscopy. In the absence of the chiral shift reagent, NMR analysis of the racemate showed that the hydrogen attached to the asymmetric center in the α position to the ester function appeared as a singlet, characterized by a chemical shift $\delta = 4.87$ ppm in $CDCl_3$. After the addition of the shift reagent, the singlet became separated into two well-resolved singlets (separation of about 6 Hz) which corresponded to the protons of the two enantiomers. It was determined that the band having the smaller chemical shift corresponded to the proton of the dextrorotatory enantiomer, while the band having the larger chemical shift was due to the levorotatory enantiomer. It was reported that this method was able to detect more than 5% (w/w) of one enantiomer in the presence of the other.

Mohan *et al.* [8] used an NMR spectroscopic method to characterize impurity D in samples of clopidogrel bisulfate. The method entailed ^1H NMR (at 400.13 MHz) and ^{13}C NMR (at 100.62 MHz), with a sample concentration of 1 mg/ml in DMSO-d_6 (this solvent also served as an internal chemical shift standard). It was found that the ^1H NMR spectrum of impurity D exhibited one hydrogen band than did that of clopidogrel, while the ^{13}C NMR and DEPT135 NMR spectra indicated the presence of one methyl carbon, two methylene carbons, eight methine carbons, and five quaternary carbons. This corresponded to a similar structure as for clopidogrel, but with one less methylene carbon and one more methine carbon.

3.4. Potentiometric method of analysis

Saber *et al.* [19] reported the development and validation of a potentiometric method to determine clopidogrel bisulfate in pharmaceutical preparations. In this method, a polyvinyl chloride membrane reference electrode was developed by using two plasticizers having different dielectric constants. The reference electrode consisted of a 7:63:30 (w/w/w) mixture of tetrakis (*p*-chlorophenyl) borate, *o*-nitrophenyl octyl ether or dioctyl phthalate as plasticizers, and PVC. This reference electrode was soaked in 0.1 M clopidogrel solution for a minimum of 2 days prior to use, and maintained in the same solution when not in use. An EIL-type RJ 23 calomel reference was used as working electrode, while an Ag/AgCl combination electrode was used for pH measurement, with the internal solution being silver–silver chloride in a 0.1 M clopidogrel solution.

It was found that the limit of detection was 1.0×10^{-5} M for both plasticizers and that linearity and Nernstian response was obtained over the range of 1.0×10^{-5}–1.0×10^{-2} mol/l (pH range of 1.5–4.0). This potentiometric method was validated for the determination of clopidogrel over the concentration range of 4.2 μg/ml to 4.2 mg/ml with an average recovery of 100.65%. Both sensors showed stable potential readings and calibration slopes for extensive time periods.

3.5. X-Ray powder diffraction methods of analysis

Quantitative and/or qualitative XRPD methods have been reported to determine the polymorphic content of clopidogrel bisulfate samples, and these have been summarized in Table 2.3. Koradia *et al.* [16] reported the qualitative analysis of clopidogrel bisulfate in both active pharmaceutical ingredients and tablet dosage forms. Based on the interplanar distances (*d*-spacing) associated with each polymorph, it was concluded that the molecular packing in Form-I was more dense than that of Form-II, indicating that Form-II would be less stable relative to Form-I. This result was similar with that reported by Bousquet [9].

Uvarov and Popov [20] reported the quantification of polymorphic forms I and II using two different XRPD methods. One method was based on the single peak intensity or direct method, and the second used whole-powder-pattern decomposition (WPPD) method. The Form-I nonoverlapping peaks suitable for analysis work were found at *d*-spacings 8.13, 5.98, and 4.32 Å, while the useful peaks for Form-II had *d*-spacings of 7.24 and 6.88 Å. It was shown that use of the WPPD method yielded better

TABLE 2.3 Summary of X-ray powder diffraction methods used to analyze clopidogrel bisulfate

Radiation source	Scan range (° 2θ)	Step size (° 2θ)	Tube voltage (kV)	Current (mA)	Type of analysis	References
Cu Kα	2–40	Not reported	Not reported	Not reported	Qualitative	[9]
Cu Kα	5–40	0.02	35	20	Qualitative	[16]
Cu Ni	1–40	0.02	60	300	Qualitative	[54]
Cu Kα	6–36	0.02	40	40	Quantitative	[20]
Not reported	8.99–9.30	Not reported	Not reported	Not reported	Quantitative	[21]
Cu Kα	2–40	0.013	40	40	Quantitative	[17]

reproducibility as compared to the direct method, although the limits of detection were comparable for both methods.

Alam *et al.* [21] reported an XRPD method for quantification of the two polymorphic forms of clopidogrel bisulfate. In this method, particle size uniformity of both polymorphs was obtained by ball milling samples to obtain mean particle sizes of 9–17 μm. The highest peak intensity of clopidogrel bisulfate using the highest intensity scattering peak of Form-I (9.14° 2θ), linearity was characterized by an R^2 value of 0.9885, LOD and LOQ values of 0.29% and 0.91%, respectively, and recoveries in the range of 97–102%. This method was shown to be able to detect low concentration of Form-I (down to levels of 1%) in mixtures of Form-I and Form-II. It was also noted that the presence of excipients changed the peak intensities and caused baseline shifting. These problems must be considered in any quantitative analysis of polymorph of clopidogrel bisulfate in pharmaceutical dosage forms by means of XRPD [16].

3.6. Thermal methods of analysis

Koradia *et al.* [16] have used hot stage microscopy (HSM), DSC, and Thermogravimetric analysis (TGA) to study Form-I and Form-II of clopidogrel bisulfate. For the HSM method, samples were heated on the hot stage and simultaneously observed with both normal and polarized illumination. It was found that Form-I exhibited an irregular plate morphology and that agglomeration was characteristic for Form-II. Under polarized light examination, Form-I appeared as alternately dark and bright when rotated by 45°, while Form-II appeared as fully bright and turned to dark at a rotation of 90°. In addition, half extinction was exhibited for Form-II at rotation angles of 30° and 60°.

As detailed by Koradia *et al.* [16], the DSC and TGA studies were run at a heating rate of 10 °C/min, and a nitrogen purge was set at 80 ml/min for the DSC work and at 20 ml/min for the TGA work. The DSC thermogram of Form-I consisted of three endothermic transitions, with temperature maxima at 181, 186, and 190 °C. The DSC thermogram of Form-II also consisted of three endotherms, characterized by temperature maxima at 177, 179, and 182 °C. During the TGA analysis, no temperature-induced loss of mass was observed for either polymorph, indicating that the two polymorphic crystal forms were anhydrous and nonsolvated.

Alam *et al.* [21] reported that the endothermic transition of Form-I was observed at 179.78 °C, and that the endothermic transition of Form-II was observed at 177.34 °C.

4. CHROMATOGRAPHIC METHODS OF ANALYSIS

4.1. Thin-layer chromatography

Table 2.4 contains a summary of some of the thin-layer chromatography (TLC) methods that have been developed and validated for the analysis of clopidogrel bisulfate. High-performance TLC methods have been shown to be able to separate clopidogrel from its degradants in accelerated degradation studies that used stressing by acid–base, hydrogen peroxide, heat degradation, and photochemical means [15, 22]. In the acid–base degradation study (performed using both 1.0 N HCl and 1.0 N NaOH), two additional peaks appeared in front of the main clopidogrel peak [22].

Similar results were obtained in another study of the alkaline degradation of clopidogrel that used either 0.5 N NaOH or 1.0 N NaOH, with one degradant peak appearing before the elution of the main peak [15]. Infrared spectroscopic and mass spectrometric structural elucidation of the alkaline degradants using showed the presence of the carbonyl group and the hydroxyl group of carboxylic acids [15]. An additional peak was also found eluting after the main peak for both the peroxide and dry heat (100 °C) degradation studies. In this work, no degradant peaks of photochemical origin were noted when clopidogrel bisulfate was exposed to direct sunlight for 24 h, indicating that clopidogrel bisulfate was photochemically stable under these conditions [22].

4.2. Gas chromatography

Kamble and Venkatachalam [23] reported a gas chromatography (GC) method for the determination of clopidogrel in tablet dosage forms. This method used a DB-17 capillary column of 30 m length and 0.25 mm internal diameter, equilibrated at an oven temperature of maintained at 250 °C. Dioctyl phthalate was used as an internal standard, and clopidogrel was detected at 4.1 min by means of a flame ionization detector. Linearity in the method ranged from 0.5 to 5.0 mg/ml, with a recovery of 99.89%.

4.3. Liquid chromatography

High-performance liquid chromatography (HPLC) methods represent the most commonly used analytical technology for the determination of clopidogrel bisulfate in either its bulk drug substance form or its pharmaceutical dosage forms. HPLC methods are most preferred in pharmaceutical compendia [2]. A summary of HPLC methods for the analysis of clopidogrel bisulfate is provided in Table 2.5. Since clopidogrel bisulfate contains a center of dissymmetry, some HPLC methods are

TABLE 2.4 Summary of TLC methods used to analyze clopidogrel bisulfate

Stationary phase	Mobile phase (v/v)	Solvent	Wavelength	TLC preparation	Sample	Limit of detection (LOD), limit of quantitation (LOQ), and recovery (Rec)	References
Silica gel 60F-254 HPTLC plate	Carbon tetrachloride: chloroform: acetone = 6:4:0.15	MeOH	230 nm	• Prewashing plate with MeOH and heating at 60 °C for 5 min • Saturating chamber with mobile phase for 30 min	Drug powder and tablet	LOD: 40 ng/spot LOQ_2: 120 ng/spot Rec: 99.13–101.30%	[22]
Silica gel 60F-254	*n*-Heptane: tetrahydrofuran = 1:1	MeOH	• Spot detected at 254 nm (UV light) • Quantitative analysis at 230 nm	• Saturating chamber with mobile phase for 15 min	Drug powder and clopidogrel impurity (SR 25334)	Clopidogrel bisulfate (impurity level of SR 26334: 0.028%) LOD: 0.024 mg/ml LOQ: 0.014 mg/ml Rec: 98.5–100.3% SR 25334 (impurity level of SR 0.095%) LOD: 0.079 LOQ: 0.047 Rec: 94.5–107.1%	[55]

Silica gel 60F-254 HPTLC plate	Acetone:MeOH:toluene: glacial acetic acid = 5.0:1.0:4.0:0.1	MeOH	235 nm	• Saturating chamber with mobile phase for 30 min	Drug powder and tablet	LOD: 112.66 ng/ml LOQ: 375.55 ng/ml Rec: 99.42–100.86%	[12]
Silica gel plate	Hexane:MeOH:ethyl acetate = 8.7:1:0.3	MeOH	248 nm	n/a	Tablet	LOD: 0.04 μg/band LOQ: 0.4 μg/band Rec: 98.90–102.17%	[15]

n/a: not available.

TABLE 2.5 Summary of HPLC methods used to analyze clopidogrel bisulfate in bulk drug substance and pharmaceutical dosage forms

Column	Mobile phase	Column temperature (°C)	Detection (nm)	Solvent	Limit of detection (LOD), limit of quantitation (LOQ), and recovery (Rec)	References
BDS C_8 semimicro column (250 mm × 2.1 mm i.d.)	0.010 M disodium hydrogen phosphate (pH 3):ACN = 35:65	Ambient	235	Standard: stock solution in MeOH then diluted with mobile phase Sample: dissolved in ACN then with water then with mobile phase	LOD: 0.12 μg/ml LOQ: 0.39 g/ml Rec: 99.62%	[31]
Chromolith Performance 18e (100 mm × 4.6 mm i.d.)	ACN:50 mM phosphate buffer = 50:50 (pH 3)	n/a	235	MeOH	LOD: 0.97 μg/ml LOQ: 3.53 μg/ml Rec: 99.1%	[32]
Phenomenax C_{18} (250 mm × 4.6 mm i.d.)	ACN:MeOH:20 mM phosphate buffer (pH 3) = 50:7:43	20	240	Mobile phase	LOD: n/a LOQ: n/a Rec: 100.20%	[26]
Phenomenax Gemini C_{18} column	0.1% (v/v) triethylamine (pH 4.0):ACN = 25:75	n/a	225	MeOH	LOD: 0.03 μg/ml LOQ: 1.0 μg/ml Rec: 100.34%	[29]
Phenomenex Luna 5 m C_{18} 100A column	ACN:50 mM potassium dihydrogen phosphate buffer:MeOH = 50:30:20 (solution adjusted to pH 3.0)	Ambient	240	Mobile phase		[30]
Nucleosil C_8 SS column (150 mm × 4.6 mm i.d.)	ACN:phosphate buffer = 55:45 (solution adjusted to pH 3.0)		235	MeOH	LOD: 0.047 μg/ml LOQ: 0.157 μg/ml Rec: 99.34–100.94%	[28]
Waters C_{18} (150 mm × 3.9 mm)	0.05 M ammonium formate (pH 4.0):ACN = 40:60	Ambient (+25 °C)	225	Stock solution dissolved in MeOH	LOD: 0.3 μg/ml LOQ: 1.0 μg/ml	[19]

				then standard series/samples diluted with mobile phase	Rec: 99.20–99.88%	
Hypersil BDS C_8 column	Solvent A: ACN:potassium phosphate buffer (pH 2.3; 10 mM) = 20:80 (v/v) Solvent B: ACN:potassium phosphate buffer (pH 2.3; 10 mM) = 80:20 (v/v) Time gradient program of T (min)/%B (v/v): 0.01/0, 5/0, 15/15, 40/30, 45/060/0	n/a	220	n/a	Qualitative analysis of degradation sample	[8]
Hypersil BDS C_8 column	Solvent A: ACN:potassium phosphate buffer (pH 2.3; 10 mM) = 20:80 (v/v) Solvent B: ACN:potassium phosphate buffer (pH 2.3; 10 mM) = 80:20 (v/v) Time gradient program of T (min)/%B (v/v): 0.01/0, 5/0, 15/15, 40/30, 45/060/0	n/a	LC–MS/MS operated in electrospray ionization positive and negative mode Fragmentation pattern: *m/z*: 321/323 *m/z*: 212/214 *m/z*: 183/185 *m/z*: 125/127 *m/z*: 155/157	n/a	Characterization of clopidogrel bisulfate degradation products	[8]

(*continued*)

ChiraDex® column (4 mm × 250 mm)	ACN:MeOH:0.01 potassium dihydrogen phosphate = 15:5:80	220	Mobile phase	Impurity 2: LOD: 1.4 ng/ml LOQ: 4.2 ng/ml Rec: 98.9–100.9% *R*-enantiomer LOD: 1.04 ng/ml LOQ: 3.17 ng/ml Rec: 99.9–101.0% *S*-Clopidogrel: LOD: n/a LOQ: n/a Rec: 99.9–101.2% *R*-enantiomer: LOD: 0.25 μg/ml (impurity level 0.07%) LOQ: 0.75 μg/ml (impurity level 0.2%) Rec: 104.6–105.2% *S*-acid: LOD: 0.031 μg/ml (impurity level 0.008%) LOQ: 0.09 μg/ml (impurity level 0.024%) Rec: 104.0–105. 0%	[25]
Chiral-AGP (100 mm × 4.0 mm)	ACN:1 mM DMOA in 10 mM ammonium acetate (pH 5.5) = 16:84	230	n/a	n/a	[59]

TABLE 2.5 *(continued)*

Column	Mobile phase	Column temperature (°C)	Detection (nm)	Solvent	Limit of detection (LOD), limit of quantitation (LOQ), and recovery (Rec)	References
ULTRON ES-OVM column (4.6 mm × 150 mm i.d.)	ACN:0.01 M potassium dihydrogen phosphate = 25:75	17	220	Tablets were dissolved with 5.0 ml and further diluted with mobile phase	Hydrolysis product: LOD: 0.5 ng LOQ: 0.15 ng Rec: n/a *R*-enantiomer: LOD: 1.5 ng LOQ: 4.9 ng Rec: n/a	[24]
ULTRON ES-OVM column (4.6 mm × 150 mm i.d.)	ACN:0.01 M potassium dihydrogen phosphate = 22:78	25	220	Tablets were dissolved with 5.0 ml and further diluted with mobile phase	Hydrolysis product: LOD: 0.5 ng LOQ: 0.15 ng Rec: n/a *R*-enantiomer: LOD: 1.5 ng LOQ: 4.9 ng Rec: n/a	[6]
CHIRAL-AGP (4.0 mm × 100 mm)	ACN:0.015 M di-potassium hydrogen phosphate (pH 6.5) = 40:60	30	220	MeOH	Clopidogrel: LOD: 194.6 ng/ml LOQ: 589.7 ng/ml Rec: 99.8–101.2% Impurity 1a: LOD: 1.9 ng/ml LOQ: 5.7 ng/ml Rec: 98.9–101.6% Impurity 1b: LOD: 1.79 ng/ml LOQ: 5.44 ng/ml Rec: 99.6–101.2%	[7]

conducted using chiral chromatography in the determination of clopidogrel bisulfate and its impurities [6, 7, 24, 25], since the use of chiral columns enables separation of the (*S*)- and (*R*)-enantiomers, as well as separation from other impurities and degradants.

However, most of the HPLC methods use C_{18} column or C_8 column technology [8, 19, 26–28], including the determination of clopidogrel bisulfate in an aspirin combination tablet [29, 30]. Sippel *et al.* [27] also reported an HPLC method using a C_{18} column to determine clopidogrel bisulfate in coated tablets, and evaluation of its degradation under acid, alkaline, and peroxide conditions, as well as its photostability. Alkaline conditions induce rapid degradation of clopidogrel bisulfate, as has been shown by HPLC analysis of the alkaline degradation products of clopidogrel bisulfate [27, 31].

Mohan *et al.* [8] reported a successful separation of clopidogrel bisulfate and its degradants using a C_8 HPLC column, followed by preparative-HPLC collection of the degradants for their characterization by LC–MS/MS. The preparative-HPLC isolation of the impurities was conducted by using an Xterra MS C_{18} ODB HPLC column. Degradation studies were conducted in acid, base, and peroxide conditions by moistening clopidogrel bisulfate with water and maintenance of the samples in an oven at 120 °C for 24 h. In this study, the presence of related compound D was found when the drug substance was exposed to oxidative condition, while this compound was not found for the other degradation conditions.

The use of semimicro column and monolithic columns for the HPLC determination of clopidogrel bisulfate have also been reported [31, 32]. The use of semimicro or small-bore columns is advantageous when compared to conventional columns, since the reduction in diameter enables a reduction in solvent usage owing to the lower flow rates and a concomitant reduction in run times. Analysts must consider, however, the possibility that higher back pressures might be encountered when using semimicro columns [33]. Monolithic columns have also been used to obtain shorter run times, but here higher flow rates (i.e., 4.0 ml/min) are used [32]. Due to their internal structure, monolithic silica columns provide higher separation efficiencies, as well as higher permeability. These qualities enable running the columns at higher flow rates with little effect on sample separation or interfering back pressures [34, 35]. In these works, the retention times for clopidogrel bisulfate were 6.28 min (semimicro column) and 1.13 min (monolithic column).

4.4. Capillary electrophoresis

One capillary zone electrophoresis method was reported by Fayed *et al.* [18] for the separation and determination of clopidogrel and its impurities. In this method, an uncoated fused silica capillary was used at 20 °C,

with an applied voltage of −12 kV and sample detection at 195 nm. Optimum separation of clopidogrel and its impurities (related compounds **A**, **B**, and **C**) was achieved by using 5% (m/v) of the sodium salt of sulfated β-cyclodextrin in 10 mM (pH 2.3) buffer to achieve chiral selection. The buffer composition consisted of equal volumes of triethylamine/phosphoric acid, and was pH adjusted with 1.0 M HCl. The reported limits of detection were 0.40, 1.00, 0.50, and 0.25 μg/ml for clopidogrel and related compounds **A**, **B**, and **C**, respectively. The reported limits of quantification for clopidogrel, and related compounds **A**, **B**, and **C** were 0.13, 0.33, 0.16, and 0.08 μg/ml, respectively. The average recovery for clopidogrel in this method was 99.45%, and it was successfully used to analyze clopidogrel and its impurities in two commercial bulk samples.

5. DETERMINATION IN BIOLOGICAL SAMPLES

Clopidogrel is extensively metabolized *in vivo* by carboxylesterase hydrolysis on the ester function, resulting the formation of clopidogrel carboxylic acid (CCA) as the inactive metabolite of clopidogrel. In addition, small amounts of clopidogrel are converted to a pharmacologically active metabolite (AM) via the intermediate metabolite inactive 2-oxoclopidogrel which is then converted to an AM by a two-step cytochrome P450 oxidation process [36, 37].

Due to the instability of clopidogrel AM and the abundant availability of the more stable CCA in human plasma (±85%), CCA is used to indirectly determine the pharmacokinetics of clopidogrel [36–38]. Furthermore, there is also possibility that (*S*)-clopidogrel undergoes an *in vivo* chiral inversion into the other clopidogrel enantiomer, which becomes hydrolyzed to (*R*)-CCA [36]. Metabolic pathways and potential *in vivo* chiral inversions of clopidogrel are described in Fig. 2.13. Until recently, only chromatographic methods were used to determine clopidogrel in biological samples.

5.1. Liquid chromatography

Most of the liquid chromatographic methods use CCA as a standard in the analysis of clopidogrel metabolites, facilitating the acquisition of pharmacokinetic information for clopidogrel [31, 39–43]. Although the analysis of clopidogrel AM in plasma is difficult due to its instability [44], some workers have reported the successful development of LC methods to determine AMs of clopidogrel [37, 38, 44–46]. Despite previous reports of very small levels of unchanged clopidogrel being detected in plasma [47, 48], a number of successful determinations of unchanged clopidogrel

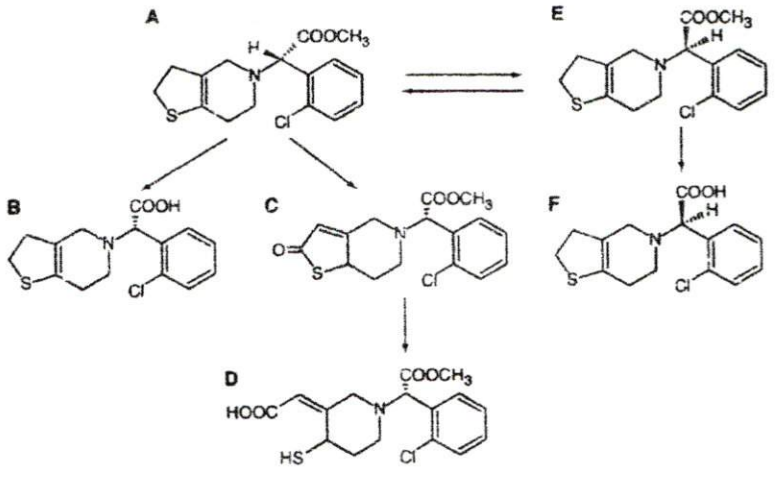


FIGURE 2.13 Metabolic pathway and possible *in vivo* chiral inversion of clopidogrel, modified from references [37, 38]. Clopidogrel (**A**) is metabolized into inactive metabolite (**B**) (clopidogrel carboxylic acid), and an inactive 2-oxoclopidogrel (**C**). The latter compound can undergo a two-step cytochrome P450 process, and become converted to the active metabolite of clopidogrel (**D**). The *in vivo* process results in a chiral inversion, changing clopidogrel into its other enantiomer (**E**), which then is metabolized into the clopidogrel (*R*)-acid (**F**).

in biological samples have been reported that use LC methods [38, 46, 49–51]. Summaries of the LC methods for analysis in biological samples of clopidogrel, its active metabolite, and its inactive metabolite are detailed in Table 2.6.

For the determination of CCA in biological samples, methods not based on LC–MS/MS technology [39, 41–43] and methods that used LC–MS/MS [40, 52] have been reported. Most of the sample extraction methods used liquid–liquid extraction (LLE) technology, since this extraction method is simpler and able to minimize matrix effects. Consequently, LLE methods are considered to provide cleaner samples as compared to solid phase extraction (SPE) methods. Since LC–MS/MS methodology uses nonvolatile solvents or a combination of nonvolatile and volatile solvents, difficulties in the evaporation process and associated interferences when samples are injected onto the system can arise [51]. However, Bahrami as well as Souri [42, 43] applied a combination of nonvolatile and volatile solvents in which the nonvolatile solvents were acidic buffers (pH 5 or less). Analytes eluted from SPE prepared samples did not undergo evaporation as applied commonly encountered in extraction procedures [37, 45].

TABLE 2.6 Summary of HPLC methods used to analyze clopidogrel bisulfate and its metabolites in biological samples

Analyte(s)	HPLC conditions	Sample	Preparation of standard, sample extraction and clean up	Limit of detection (LOD), limit of quantitation (LOQ), and recovery (Rec)	References
CCA and sulfafurazole (i.s.)	Column: Hypercarb PGC (50.0 mm × 3.0 mm) at ambient temperature Detector: LC–MS operated in electrospray ionization positive mode SIM monitoring: Carboxylic acid metabolite: m/z 308 Clopidogrel: m/z 322 Sulfafurazole: m/z 268 Mobile phase: 70% methanol in water containing 0.1% (v/v) trifluoroacetic acid	Human plasma	Standard: MeOH Sample: SPE: Hypercarb cartridge Precondition: 1.0 ml MeOH and 1.0 ml water Column wash: 0.5 ml of 10% MeOH in water Elution: 0.8 ml mixture of 70% ACN in water containing 0.1% TFA	Carboxylic acid metabolite LOD: 28 ng/ml LOQ: 93 ng/ml Rec: 73.0–75.2%	[52]
CCA and atorvastatin (i.s.)	Column: Kromasil ODS (250 mm × 4.6 mm i.d.) at 30 °C Detector: UV/Vis 220 nm Mobile phase: Solvent A (0.05% TFA in water)/solvent B (ACN) run at gradient program started from 90% solvent A and 10% solvent B then decreased to	Rat plasma	Standard: Water:MeOH:ACN = 40:40:20 Sample: LLE: ethyl acetate: dichloromethane = 80:20 Reconstitution of dry residue with similar solvent as for standard	Carboxylic acid metabolite LOD: 75 ng/ml LOQ: 125 ng/ml Rec: 85.8–88.5%	[41]

(continued)

TABLE 2.6 *(continued)*

Analyte(s)	HPLC conditions	Sample	Preparation of standard, sample extraction and clean up	Limit of detection (LOD), limit of quantitation (LOQ), and recovery (Rec)	References
	10% solvent A and 90% solvent B which was held constant for 3 min then increased to 60% solvent A and 40% solvent B at time 16 min held constant for 2. At time 18 min increased to 90% solvent A and 10%, held for 2 min until time 20 min, then baseline				
CCA and repaglinide (i.s.)	Column: Luna 3μ C_{18} (75 mm × 4.6 mm i.d.) at 35 °C	Human plasma	Standard: SR25990C: MeOH	Carboxylic acid metabolite LOD: n/a LOQ: 20 ng/ml Rec: 85–90%	[40]
	Detector: LC–MS operated in electrospray ionization SIM monitoring: SR25990C: m/z 308.00 Repaglinide: m/z 453.55 Mobile phase: ACN:water: formic acid = 60:40:0.1		Repaglinide: stock solution in MeOH then diluted with water Sample: SPE continued with LLE: Sample added with 250 ml water and 500 ml 0.1 ammonium acetate, then loaded on 1 ml Chem Elut extraction cartridge. LLE performed with 4 ml dicholorometane, dried, and reconstituted with 200 μl of mobile phase		

CCA and ticlopidine (i.s.)	Column: Nova-pack® C_8 4 μm (250 mm × 4.6 mm) Detector: UV/Vis 220 nm Mobile phase: 30 mM K_2HPO_4: THF:ACN = 79:2:19 (pH mobile phase = 3)	Human plasma	Standard: MeOH Sample: LLE: Sample added with 200 μl of 300 mM K_2HPO_4 (pH 5) and 4 ml chloroform, then extracted. Dry residue obtained from the organic layer was reconstituted with 100 μl mobile phase	Carboxylic acid metabolite LOD: 0.02 μg/ml LOQ: 0.2 μg/ml Rec: 77.4–83.2%	[42]
CCA, aspirin, and salicylic acid	Column: semimicro column ODS (250 mm × 1.5 mm) Detector: n/a Mobile phase: 10 mM phosphate buffer (pH 2.5) and ACN. Composition of mobile phase was not reported	Human plasma	Standard: n/a Sample: LLE using 10% *n*-hexane/ethyl acetate	Carboxylic acid metabolite LOD: n/a LOQ: 80.7 ng/ml Rec: n/a	[39]
CCA and phenytoin (i.s.)	Column: Shimpack CLC-ODS (150 mm × 4.6 mm i.d.) at 50 °C Detector: UV/Vis 220 nm Mobile phase: 0.05 M sodium phosphate buffer (pH 5.7): ACN = 56:44	Human serum	Standard: MeOH Sample: LLE using 2.0 N HCl and extracted with ethyl acetate. Residue reconstituted in mobile phase	LOD: 0.02 μg/ml LOQ: 0.05 μg/ml Rec: 96.1–101.8%	[43]

(*continued*)

TABLE 2.6 *(continued)*

Analyte(s)	HPLC conditions	Sample	Preparation of standard, sample extraction and clean up	Limit of detection (LOD), limit of quantitation (LOQ), and recovery (Rec)	References
CCA and clopidogrel	Column: C_{18}-ether HPLC analytical column at 50 °C Detector: LC–MS/MS operated in electrospray ionization (ESI) interface at positive ion mode MRM monitoring: Clopidogrel: *m/z* 322.0663 → 212.0478 CCA: *m/z* 308.0506 → 198.0322 Mobile phase: Gradient from 10% to 100% of ACN:0.1% formic acid	Human plasma	Standard: n/a SPE	Clopidogrel: LOD: 0.25 μg/l LOQ: n/a Rec: n/a CCA: LOD: 25 μg/l LOQ: n/a Rec: n/a	[58]
Purified analyte of clopidogrel AM	Column: Lichrocart 60RP8E (125 mm × 4 mm) Detector: LC–MS operated in electrospray ionization positive mode SIM monitoring: *m/z* 356.5 *m/z* 358.5 Mobile phase: MeOH:water: ACN:diethylamine = 40:60:0.2:0.1	Fraction H obtained from incubation of (7S)-2-oxoclopidogrel with human microsomes	HPLC preparative at UV detection 234 nm. Elution was done using ACN/10 mM ammonium acetate (pH 6.5) gradient (10–24%) using Ultrabase UB225 column	LOD: n/a LOQ: n/a Rec: n/a	[44]

Clopidogrel (*S*)-acid and (*R*)-acid	Column: Hypersil ODS (250 mm × 4 mm) Detector: spectrofluorometric with excitation wavelength = 280 nm and emission wavelength = 330 nm Mobile phase:ACN: triethylammonium acetate buffer 0.01 M (pH 3.3) = 55:45	Rat plasma	SPE and LLE: First elution with hexane (discarded) then analytes Plasma sample was then derivatized with (*S*)-(−)-α-(1-naphtyl)ethylamine with the aid of HOBT and EDAC. Extraction was performed with water and hexane. Organic phase was then evaporated and reconstituted with mobile phase	LOD: n/a LOQ: n/a Rec: n/a Linearity of (*S*)-acid: 0.60–40 mg/l Linearity of (*R*)-acid: 0.60–4.0 mg/l	[36]
Clopidogrel AM, MPB derivative of clopidogrel AM (MP-AM) (i.s.)	Column: Inertsil ODS-3 column (2.1 mm × 50 mm) at 40 °C Detector: LC–MS/MS operated in ESI interface at positive ion mode MRM monitoring: Clopidogrel AM: m/z 356 → 155 MP-AM: m/z 504 → 354 Mobile phase: MeOH:1% formic acid = 70:30	Human plasma	Standard solution: derivatization of clopidogrel AM in ACN with 3′-methoxyphenacyl bromide (MPB) in ACN and ammonium chloride buffer (pH 9). Volume was then made with ACN Sample: Derivatization as for standard solution followed by extraction Extraction: SPE prewashed with MeOH and water in equal. After sample application, SPE then washed with 1% formic acid followed by 50 mM ammonium acetate equivolume. Analyte eluted with MeOH followed by 50 mM aqueous ammonium acetate. Both eluates then mixed and injected	LOD: n/a LOQ: n/a Rec: 88.9–98.6%	[37, 45]

(continued)

TABLE 2.6 (*continued*)

Analyte(s)	HPLC conditions	Sample	Preparation of standard, sample extraction and clean up	Limit of detection (LOD), limit of quantitation (LOQ), and recovery (Rec)	References
Free clopidogrel base, CCA, clopidogrel AM, and diltiazem (i.s.)	Column: Aquasil C_{18} column (100 mm × 3 mm) Detector: LC–MS/MS operated in electrospray ionization positive mode SRM monitoring: Unchanged clopidogrel: m/z 322 → 212 Carboxyl metabolite: m/z 308 → 198 Active metabolite: m/z 356 → 212 Diltiazem: 415 → 178 Mobile phase: gradient of ACN/0.1% formic acid (10–90%, v/v)	Human plasma	Standard: n/a Sample: dissolved in ACN	n/a	[46]
Free clopidogrel base, CCA, clopidogrel AM, and 1-methyl-4-phenylpyridinium bromide (i.s.)	Column: Kromasil C_8 column (100 mm × 3 mm) Detector: LC–MS/MS operated in electrospray ionization positive mode SRM monitoring: Unchanged clopidogrel: m/z 322 → 212	Human plasma	Standard: n/a Sample: n/a	n/a	[56]

	Carboxyl metabolite: m/z 308 → 198 Active metabolite: m/z 356 → 212 1-Methyl-4-phenylpyridinium bromide: 170 → 127 Mobile phase: ACN/0.1% formic acid:water/0.1% formic acid = 90:10				
Clopidogrel and CCA	Column: Varian Monochrom silica 3u (100 mm × 4.6 mm) Detector: positive MRM Other details were not available Mobile phase: ACN:ammonium acetate 2 nM = 500:140	Human plasma	Standard: Sample: LLE using ethyl acetate: pentane = 1:1 (pH 4). Dry extract was reconstituted in ACN	LOD: n/a LOQ: n/a Rec: n/a	[57]
Clopidogrel bisulfate and ticlopidine HCl (i.s.)	Column: Waters symmetry C_8® (150 mm × 4.6 mm i.d.) at 30 °C Detector: LC–MS/MS operated in electrospray ionization MRM monitoring: Clopidogrel: m/z 322.2 → 212.1 Ticlopidine: m/z 264.1 → 154.2 Mobile phase: 5 mM ammonium formate:MeOH = 5:95	Human plasma	Standard: stock standard in MeOH then diluted with MeOH:water = 50:50 Sample: LLE: sample extracted with extraction mixture diethyl ether/n-hexane (8:2, v/v) then dried organic residue was reconstituted with 250 µl mixture of 5 mM ammonium formate/MeOH (20:80)	LOD: not reported LOQ: 5 pg/ml Rec: 88.2–99.5%	[51]

(continued)

TABLE 2.6 *(continued)*

Analyte(s)	HPLC conditions	Sample	Preparation of standard, sample extraction and clean up	Limit of detection (LOD), limit of quantitation (LOQ), and recovery (Rec)	References
Clopidogrel and $^{2}H_{3}$-clopidogrel (i.s.)	Column: Luna C_8 (50 mm × 2.0 mm) at 50 °C Detector: LC–MS/MS operated in atmospheric pressure ionization positive mode MRM monitoring: Clopidogrel: *m/z* 322.07 → 212.15 $^{2}H_{3}$-clopidogrel: *m/z* 327.00 → 217.10 Mobile phase: Solvent A (ACN containing 0.1% (v/v) formic acid) and solvent B (water containing 0.1%, v/v formic acid) was run in gradient mode. 35% solvent A and 65% solvent B were held for 0.8 min. At 1.40 min, solvent A% was 70% and solvent B 30%, held until 1.60 min. At 1.70 min, composition was 35% solvent A and 65% solvent B, held until time 3.0 min then baseline	Human plasma	Standard: stock standard in MeOH then diluted with MeOH:water = 50:50 Sample: LLE: sample added with 500 μl of 50 mM ammonium acetate buffer (pH 6.8), then with 2 ml diethyl ether. Dried organic residue was reconstituted in 50 μl of 0.1% (v/v) formic acid in ACN then with 50 μl of 0.1% formic acid in water	LOD: not reported LOQ: 10 pg/ml Rec: 61.5–68.6%	[50]

Clopidogrel, ticlopidine HCl (i.s.)	Column: Hypersil GOLD C_{18} column (2.1 mm × 150 mm) Detector: LC–MS/MS operated in turbo ion spray ionization MRM monitoring: Clopidogrel: m/z 322.2 → 211.9 Ticlopidine: m/z 264.1 → 125.10 Mobile phase: ACN:10 mM ammonium acetate in water = 85:15	Human plasma	Standard: MeOH Sample: LLE using pentane. Dried organic residue was dissolved in mobile phase		[49]
Clopidogrel	Column: Zorbax SB-C_8 Detector: LC–MS/MS operated in electrospray ionization Mobile phase: MeOH: ammonium formate (5 mM/l, pH 6.0)	Human plasma	LLE	LOD: n/a LOQ:n/a Rec: 103.1–109.3%	[47]

According to Ksycinska *et al.* [40], buffering plasma samples with 0.1 M ammonium acetate at pH 4.0 helped to weaken drug that had become bound to plasma, therefore making easier the partitioning of drug from an aqueous environment into the organic solvent. In contrast with the common preference for extracting CCA from biological sample, Mitakos and Panderi [52] reported an SPE method for extracting CCA from human plasma samples that did not require acidification. The extraction efficiency obtained using this SPE method ranged from 73.0% to 75.2%. However, this value is seen to be lower relative to other values obtained using the LLE method, which uses either buffer/volatile solvent mixtures or volatile solvents only [40–42].

In the biological samples (blood or plasma), AMs of clopidogrel contain a thiol group, which is reactive and causes degradation of the AM. To overcome the instability of clopidogrel AM, a derivatization method was used, where an alkylating reagent (3-methoxyphenacyl bromide) served to block reactivity of the thiol group. Since this method enabled a successful stabilization of the thiol group, higher amounts of clopidogrel AM in human plasma samples were reported when compared to samples which did not undergo the derivatization reaction [37, 45].

Due to the instability of unchanged clopidogrel, Taubert *et al.* [46] obtained standards of clopidogrel free base by extracting the substance from crushed clopidogrel bisulfate tablets with methanol, followed by extraction using potassium carbonate/cyclohexane, and finally purified over Celite™ 545. However, other workers have reported [49–51] obtaining the free base of clopidogrel by dissolving clopidogrel bisulfate in methanol without the use of potassium carbonate/cyclohexane extraction.

In the analysis of biological samples, the use of an internal standard is needed to ensure that the method employed is specific and selective for the analyte. Isotopically labeling of the analyte is preferable. However, this type of internal standard is not commercially available, and an alternative is to choose an internal standard that has a relevant structure and similar retention behavior to the target analyte [51]. Several drugs have been used as internal standards, with ticlopidine being commonly used due to its close similarity with clopidogrel [42, 49, 51]. However, it should be noted that ticlopidine was observed to have a similar retention time to CCA, so high percentages of aqueous content in the mobile phase and lower flow rates should be used to increase the resolution of ticlopidine and clopidogrel. However, this can result in longer run times and in the reduction of sensitivity [42, 43].

Stability of samples prior to and during analysis is an important consideration when developing and validating an analytical method. For analysis of CCA, Souri *et al.* [42] reported that the stability of CCA in rat plasma samples was up to 48 days, or 3 cycles of freeze–thaw, when stored at -70 ± 5 °C. When stored at ambient temperature (20–25 °C),

samples can withstand up to 24 h of exposure, and after reconstitution, samples were stable up to 35 h in an autosampler maintained at 15 °C.

Ksycinska as well as Bahrami [40, 43] reported that clopidogrel in human plasma was stable for 1 month storage at −20 °C, and 60 days for human serum stored at −40 °C. Rapid degradation of clopidogrel AM can be overcome by derivatization, with it being reported that clopidogrel AM in plasma was stable for up to 4 months [45]. For the analysis of unchanged clopidogrel in plasma, Robinson *et al.* [50] reported that clopidogrel was only stable for 4 h at room temperature. In contrast, Shin as well as Nirogi [49, 51] reported that clopidogrel in human plasma samples did not show significant loss when stored at room temperature for as long as 24 h.

5.2. Gas chromatography

Only one gas chromatographic method has been reported to determine CCA in human plasma samples [53]. The standard metabolite used was the hydrochloride salt of CCA, while an analogous hydrochloride salt of the carboxylic acid was used as internal standard. Extraction by LLE method followed by SPE, and subsequent derivatization, was used to extract the metabolite of clopidogrel from human plasma and serum since this procedure could minimize matrix effects. In the LLE procedure, formic acid and diethyl ether were used to extract the analyte, followed by an SPE extraction of the residue in methanol using a C_{18} SPE column.

Derivatization was conducted by the addition of a 10% *n*-ethyl-diisopropylethylamine solution and α-bromo-2,3,4,5,6-pentafluorotoluene. Sample obtained from the derivatization procedure were dissolved in ethyl acetate prior to injection in splitless mode using a DB-1 capillary column. Helium was used as the mobile phase, and the injector temperature was set at 290 °C with a transfer line temperature of 270 °C. Sample detection used ion trap MS for detection, with the detector being set at negative chemical ionization with $m/z = 262$ (for CCA) and $m/z = 286$ (for the internal standard). The limit of quantitation was 5 ng/ml, and the average recovery ranged from 92.0% to 114%. In addition, the extraction efficiency ranged from 48.2% to 55.6% for concentrations of 5, 50, and 250 ng/ml. Samples were reported to be stable for up to 6 months when stored at −18 °C.

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