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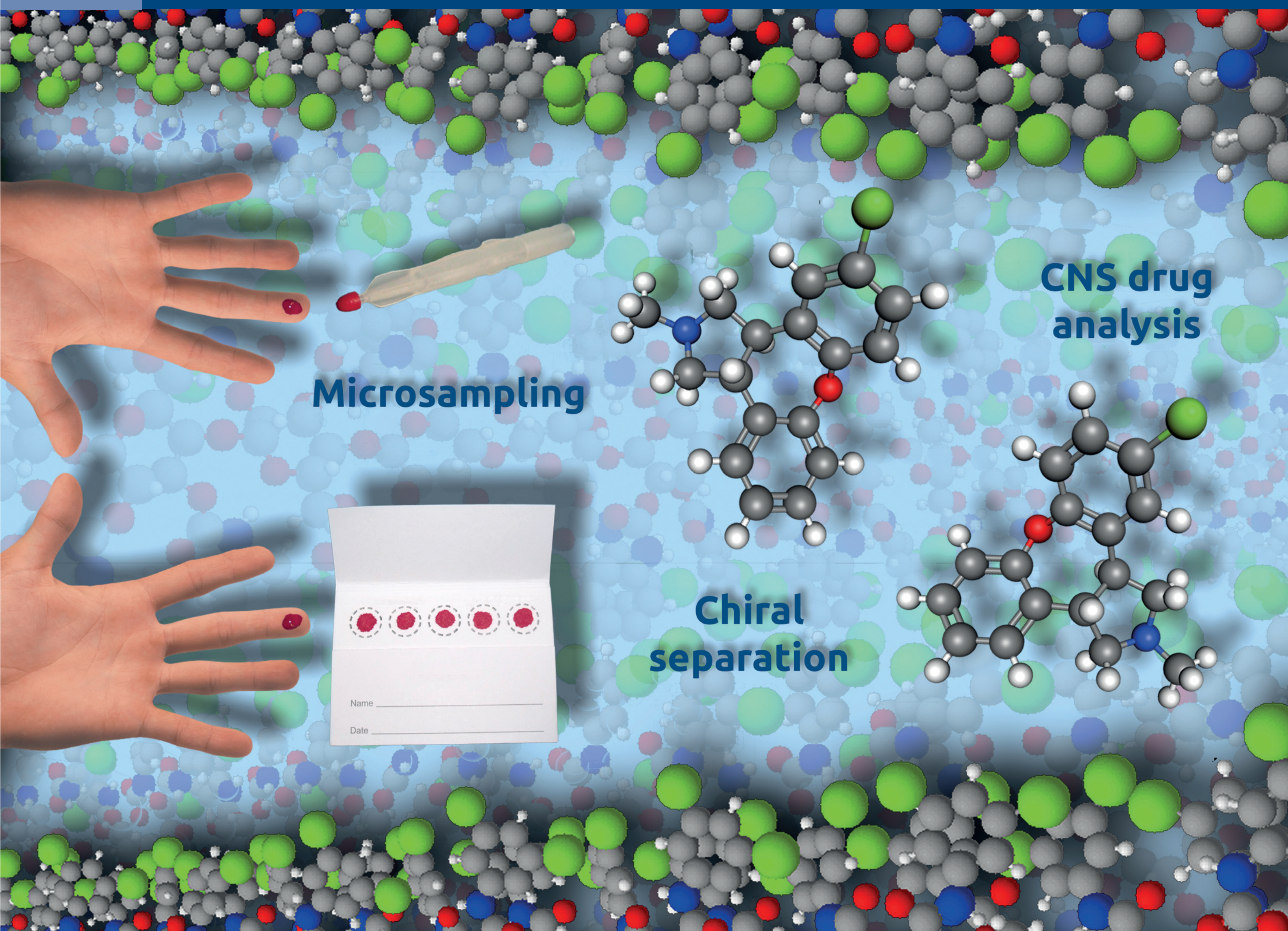

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RESEARCH ARTICLE

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Development of a high-performance liquid chromatography method for the simultaneous determination of chiral impurities and assay of (*S*)-clopidogrel using a cellulose-based chiral stationary phase in methanol/water mode

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A simple reversed-phase high-performance liquid chromatography method for the chiral separation of the active pharmaceutical ingredient (*S*)-clopidogrel has been developed on the cellulose-based Chiralcel OJ-RH chiral stationary phase. The *S* enantiomer was baseline resolved from its *R* impurity (impurity C) with a mobile phase consisting of methanol/water (100:15) without any interference coming from the other two potential chiral impurities A and B. The enantio- and chemoselective method was partially validated and compared with that reported in the United States Pharmacopoeia for the drug product. The versatility of the Chiralcel OJ-RH allowed separating the enantiomers of the impurity B also under normal phase and setting up an efficient strategy to convert the racemic sample into the enantiomeric *S* form on a semipreparative scale.

KEYWORDS
(*S*)-clopidogrel, Chiralcel OJ-RH, deracemization, reversed-phase enantioseparation, semipreparative enantioseparation

1 | INTRODUCTION

Clopidogrel (CLOP), methyl (1)-(*S*)-α-(2-chlorophenyl)-6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-acetate) (Fig. 1), is a block-buster antiplatelet drug used for the management and prophylaxis of cardiovascular and cerebrovascular thromboembolic events. About 85% of the drug is in vivo hydrolyzed to an inactive carboxylic acid derivative. The remainder is activated by a two steps process to a thiol metabolite, which binds to a free cysteine on the $P2Y_{12}$ receptors and irreversibly blocks the adenosine diphosphate binding [1].

The antiplatelet drug is currently available as (*S*)-clopidogrel hydrogen sulfate ((*S*)-CLOP) under the trade name Plavix and (*S*)-clopidogrel besylate in generic drugs. The *R* enantiomer of clopidogrel ((*R*)-CLOP), which is inactive, is considered as an impurity (IMP-C).

According to the monograph reported in the United States Pharmacopeia (USP), the enantiomeric excess (ee) of (*S*)-CLOP in the drug product is checked by an enantioselective HPLC method based on the use of the ovomucoid-based Ultron ES-OVM-C (150 × 4.6 mm, 5 μm) column with a mobile phase containing a mixture of acetonitrile–phosphate buffer (25:75) [2]. The same method is able to determine the content of the other two chiral impurities (IMP-A and IMP-B). The production of (*S*)-CLOP on an industrial scale is carried out by fractional crystallization using camphorsulfonic acid as a resolving agent. Since IMP-B is a constitutional isomer of CLOP, the fraction precipitated could contain an enantiopure *S* form of impurity or an enantiomerically enriched mixture.

Abbreviations: (*R*)-CLOP, *R* enantiomer of clopidogrel; (*S*)-CLOP, (*S*)-clopidogrel hydrogen sulfate; API, active pharmaceutical ingredient; CLOP, clopidogrel; CSP, chiral stationary phase; ee, enantiomeric excess; IMP-A, impurity A; IMP-B, impurity B; IMP-C, impurity C; MeOH, methanol; MeONa, sodium methoxide; USP, United States Pharmacopeia; vol, volume

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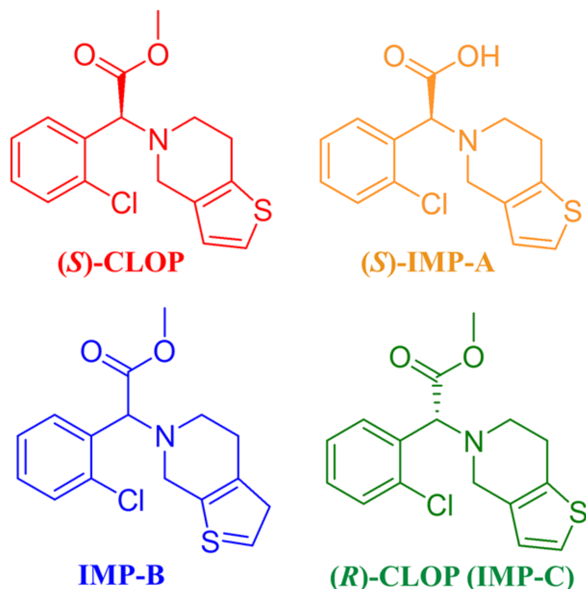

FIGURE 1 Structures of (*S*)-clopidogrel and its chiral impurities A–C

It is interesting to highlight that in the USP system suitability testing the reference standard of IMP-B is used in the racemic form and the stereochemistry of the enantiomeric peaks is not reported. Therefore, it is not possible to identify and, consequently, integrate in the chromatogram resulting from the pharmaceutical analysis of related substances the chromatographic peak corresponding to the (*S*)-IMP-B form. So, the enantioselective HPLC analysis of IMP-B enantiomers requires standards of known absolute configuration.

The unavailability of standards of the enantiopure forms of IMP-B on a commercial scale reflects the limits of the protein selector ovomucoid for preparative applications. In fact, although it shows a good enantiodiscrimination ability towards the chiral compounds structurally related to CLOP, due to its low capacity of loading [3] and limited chemical and biochemical stabilities with aqueous mobile phases containing an organic modifier content greater than 50% [4], it cannot be employed for isolating mg/g amounts of enantiomers of IMP-B.

A previous investigation [5] indicated that the use of the chromatographic supports containing tris(4-methylbenzoate) of cellulose provided the interesting opportunity to direct the separation of the enantiomers of CLOP with normal-phase eluents. An important attribute of the cellulose-derived chiral stationary phase (CSP) is that it can operate enantiodiscrimination in multimodal conditions on both analytical and semipreparative scale [6–11].

In this light, with the aim of obtaining more efficient enantioselective systems for the resolution of racemic CLOP and IMP-B, the present research was addressed in the study of the influence of different mobile phases containing *n*-hexane or water as cosolvents on the performance of the cellulose tris(4-methylbenzoate)-based Chiralcel OJ-RH CSP.

Special attention was dedicated to the evaluation of the influence of water content in reversed-phase eluents on enantio- and chemoselectivity exhibited by Chiralcel OJ-RH CSP in the analysis of samples containing a mixture of (*S*)-CLOP and its chiral related substances.

2 | MATERIALS AND METHODS

2.1 | Reagents, samples, and instruments

(*S*)-CLOP and its chiral impurities A, B, and C were obtained by Reference Standards Unit of the USP (Maryland, USA). Tablets labeled to contain 75 mg of CLOP, manufactured by Sanofi Aventis (Plavix®), were obtained from Italian market, and were used within their shelf life. HPLC enantioseparations were performed by using stainless-steel Chiralcel OJ-RH (150 × 4.6 mm, 5 μm), Chiralcel OJ (250 × 10 mm, 10 μm) columns (Daicel, Tokyo, Japan), and Ultron ES-OVM-C (150 × 4.6 mm, 5 μm) (Shinwa Chemical Industries, Kyoto, Japan) columns. HPLC-grade solvents were supplied by Sigma–Aldrich. The HPLC system consisted of a Dionex P580 LPG pump (Dionex, Sunnyvale, CA, USA), an ASI-100 T autosampler, a STH 585 column oven, and a PDA-100 UV detector. The data were acquired and processed by a Chromeleon Datasystem (Dionex).

For semipreparative separation of IMP-B a Perkin-Elmer (Norwalk, CT, USA) 200 LC pump equipped with a Rheodyne (Cotati, CA, USA) injector, a 2000 μL sample loop, a Perkin-Elmer LC 101 oven, and Waters 484 detector (Waters, Milford, MA, USA) were used. The signal was acquired and processed by Clarity software (DataApex, Prague, The Czech Republic). Standard solutions of IMP-B were prepared by dissolving the racemic sample in the mixture *n*-hexane/ethanol/diethylamine (50:50:0.1). After semipreparative separation, the collected fractions were pooled, evaporated, and analyzed by a chiral analytical column to determine their ee.

The circular dichroism spectra of enantiomers of CLOP and IMP-B, dissolved in ethanol (concentration about 0.3 mg/mL), in a quartz cell (0.1 cm path length) at 25°C, were measured by using a Jasco Model J-700 spectropolarimeter. The spectra were average, computed over three instrumental scans and the intensities were presented in terms of ellipticity values (mdeg).

In analytical enantioseparations, standard solutions were prepared by dissolving 1–3 mg of each sample, into 10 mL of ethanol or methanol (MeOH). The injection vol was 10–20 μL.

In off-column racemization studies, solutions of the enantiomers of IMP-B (concentration about 0.5 mg/mL) were held

at constant temperature in a reaction solvent composed of MeOH containing variable concentrations of sodium methoxide (MeONa). The temperature was set and monitored by a Julabo (Julabo Labortechnik, Seelbach, Germany) Model HP-4 thermostat. Samples were withdrawn at fixed time intervals and analyzed by HPLC on the Chiralcel OJ-RH CSP using pure ethanol as a mobile phase. The ee was estimated from the integrated areas in the chromatograms.

For the partial validation of the reversed-phase HPLC method based on OJ-RH CSP, standard stock solutions of CLOP and single impurity were prepared separately by transferring 100 mg of CLOP into a 100 mL volumetric flask and 10 mg of each impurity into a 200 mL volumetric flask, to which small amounts of diluents were added. Mixtures were sonicated to dissolution and made up to vol with the mobile phase. Final concentrations of 0.8–0.01 mg/mL (160–2%) of CLOP and 0.0125–0.0005 mg/mL (2.5–0.1%) of each impurity were prepared for calibration standard curves.

Sample solutions were prepared from marketed formulation tablets, which were accurately weighed and crushed to a fine powder in a mortar. A portion of the ground tablet powder, corresponding to the average weight of four tablets, was transferred into a 150 mL volumetric flask, to which 100 mL of mobile phase were added. The mixture was sonicated to dissolve the excipients and then made up to vol with mobile phase. After 15 min of mechanical shaking, the solution was maintained in an ultrasonic bath for 15 min and then filtered through 0.45 μm filter. Aliquots of 2.5 mL of the filtered solution were transferred to a 10 mL volumetric flask and made up to vol with mobile phase to yield a final concentration of 0.5 mg/mL in CLOP.

2.2 | Validation of the method

The parameters assessed for the validation of HPLC method were linearity, accuracy, precision (as repeatability), robustness, LOD, and LOQ.

Linearity was evaluated for (*S*)-CLOP in the range 0.01–0.8 mg/mL and in the range 0.5–12.5 μg/mL for (*S*)-IMP-A, for (*S*)-IMP-B, and for (*R*)-IMP-C.

The accuracy was determined by standard addition method. Samples of CLOP previously analyzed for API (active pharmaceutical ingredient) were added with standard drug solutions and the recovery (%) and RSD (%) were calculated.

The repeatability of sample injection was determined by analyzing six times a test solution containing 0.5 mg/mL (100%) of CLOP.

The robustness of the method was studied purposely altering experimental conditions, such as flow rate, column temperature, and water content in the mobile phase.

The LOD values were determined at S/N = 3, and the LOQ values at S/N = 10.

3 | RESULTS AND DISCUSSION

3.1 | Normal-phase analytical and semipreparative enantioseparation of IMP-B

Rao et al. reported in a recent study an effective HPLC method to separate the enantiomers of CLOP using the coated-type Chiralcel OJ-H CSP containing the tris(4-methlybenzoate) of cellulose as a polysaccharide selector [5]. The enantioselective analytical method was developed with a normal phase eluent constituted of *n*-hexane/ethanol/diethylamine (70:30: 0.1, v/v/v).

As an extension and deepening of that investigation, in the first step of current work ethanol was probed as alcohol modifier at different concentrations in mixture with *n*-hexane, in the enantioseparation of IMP-B, which is the constitutional isomer of CLOP (Fig. 1), on the cellulose-derived Chiralcel OJ-RH CSP.

Figure 2 shows the curves obtained by plotting the retention and enantioselectivity factors versus the percentage of alcohol in mobile phase.

It is interesting to note how the retentive behavior of the two enantiomers depends strictly on the composition of the mobile phase. In fact, a crossover alcohol% (60%) defines

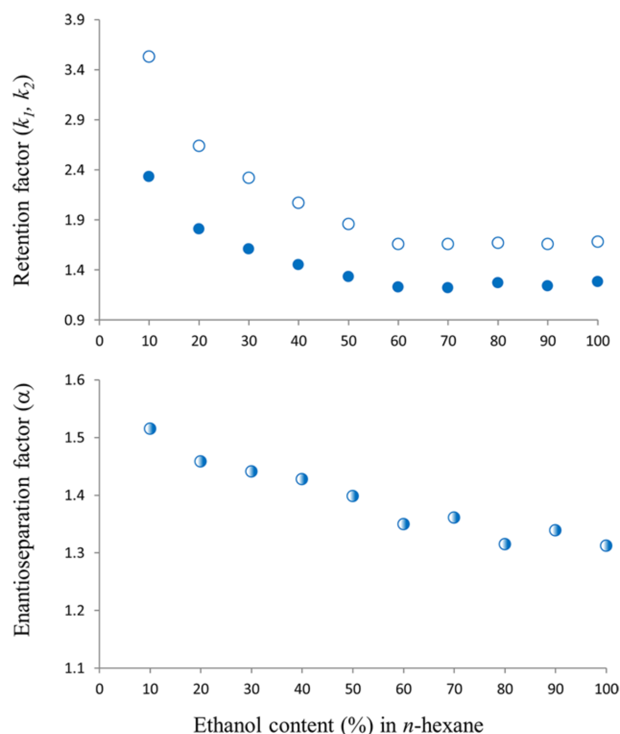

FIGURE 2 Plots of the retention and enantioselectivity factors of the impurity B as a function of the ethanol content in *n*-hexane/ethanol mixtures. Chromatographic conditions: column, Chiralcel OJ-RH 150 × 4.6 mm id; eluent, *n*-hexane/ethanol mixtures; flow rate, 1.0 mL/min; temperature, 25°C; detection, UV at 226 nm

two distinct elution regions. Within the left branch of the graph k versus alcohol%, retention decreases as the content of ethanol increases. In this elution mode, ethanol acts as a typical normal phase organic modifier, weakening complementary interactions established by polar selector/selector fragments.

Beyond the critical value of ethanol concentration no appreciable retention variation was recorded and the graphs were flat. The retention balancing could be produced by the intervention of non-specific solvophobic interactions. This assumption appears to be confirmed by the fact that using different *n*-pentane/MeOH mixtures as eluents the competitive interaction is strengthened and shape of the retention graphs becomes concave (Fig. 3) [12].

Figure 2 also shows the enantioselectivity did not show dramatic fluctuations in the concentration range of the ethanol employed.

An effective chromatographic method able to resolve the racemic sample of IMP-B into its enantiomers at semipreparative scale was developed using the mixtures *n*-hexane/ethanol (70:30 v/v) in combination with a 1-cm i.d. OJ column.

In Supporting Information Figure 1S is shown the typical chromatograms pertinent to the baseline enantioseparation of 2, 5, and 10 mg of IMP-B at the column temperature of 25°C. The good performance of the chiral support in the normal-phase conditions made possible the production of about 4.9 mg for each enantiomer with ee > 99% within 12 min.

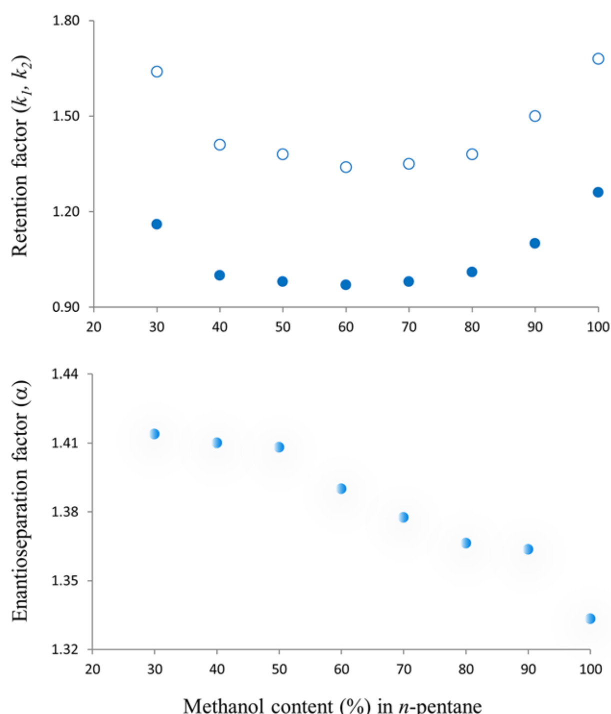

FIGURE 3 Plots of the retention and enantioselectivity factors of the impurity B as a function of the methanol content in *n*-pentane/methanol mixtures. Chromatographic conditions: column, Chiralcel OJ-RH 150 × 4.6 mm id; eluent, *n*-pentane/methanol mixtures; flow rate, 1.0 mL/min; temperature, 25°C; detection, UV at 226 nm

3.2 | Conversion of rac-IMP-B into *S*-IMP-B

A significant limitation of the semipreparative production of (*S*)-IMP-B by enantioselective HPLC, is the collection of the undesired *R* enantiomer as waste. For this reason, the yield for the target *S* enantiomer could not ever exceed 50%.

To overcome the intrinsic disadvantage of the resolution method, a simple strategy, which is potentially applicable at industrial scale, was set up to convert the undesirable *R* enantiomer into (*S*)-IMP-B.

Observing the structure of IMP-B, the stereogenic center bears a proton and acidity-enhancing substituents such as a carbonyl group, a nitrogen atom, and an aromatic moiety [13]. Thus, the base-induced deprotonation at chiral carbon of the *R* enantiomer can lead to a carbanion↔enolate intermediate and the following random readdition of the removed proton to the racemic form. The unwanted *R* enantiomer isolated by semipreparative HPLC may be therefore off-column converted into its racemic form, and the resulting racemate resubmitted to enantioselective HPLC.

To obtain a direct confirm of the theoretical prediction on stereolability, (*R*)-IMP-B was submitted to off-column racemization HPLC experiments. The base selected for inducing racemization was MeONa in MeOH solution. The progress of the racemization reaction of the enantiopure sample, which was incubated into a thermostatted reactor at 45, 50, 55, and 60°C in presence of three different concentrations of MeONa (0.023, 0.035, and 0.046 M), was monitored by enantioselective HPLC on the OJ-RH CSP using pure ethanol as a mobile phase.

The plots of the natural logarithm of the decreasing of ee as a function of time were highly linear (R^2 greater than 0.995) and allowed determining the pseudo-first order rate constants for the base-catalyzed racemization process (k_{obs}).

In Fig. 4 is shown the chromatogram pertinent to the repetitive HPLC check of the ee during the fast racemization ($\tau_1/_2$ was about 11 min) induced by a [MeONa] = 0.046 M at 60°C and the corresponding plot ln(ee) versus time.

The observed pseudo-first order rate constants, $k^{r'}_{obs}$, were linearly dependent (Supporting Information Figure 3S) on the increasing concentration of the basic catalyst (MeONa), according to the following equation:

$$k_{obs} = k_{MeONa}\,[MeONa] + k_0 \quad (1)$$

in which k_{MeONa} is the second-order rate constant for MeONa and k_0 is the pseudo-first order rate constant which includes

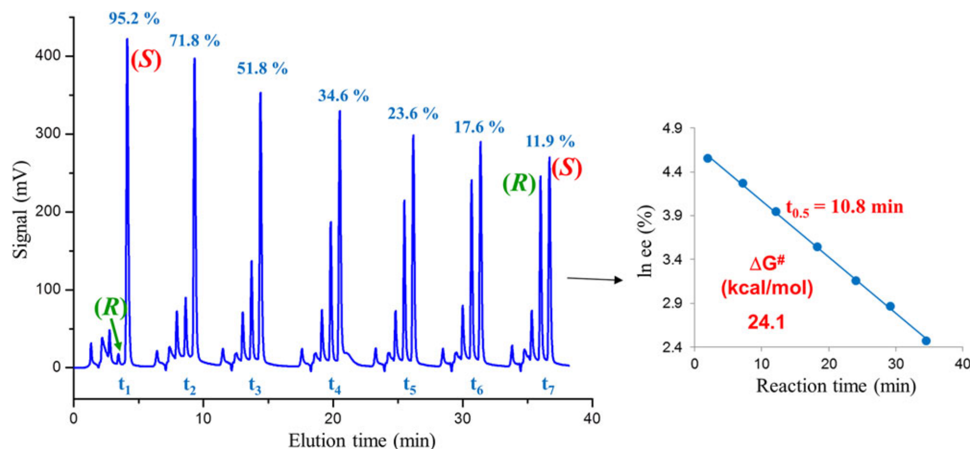

FIGURE 4 Off-column racemization of the impurity B monitored by enantioselective HPLC (reaction solvent: methanol/MeONa (0.046 M); temperature: 60°C; ee (enantiomeric excess) changed from 95.2 to 11.9%) and pertinent plot of ln(ee) versus time

contributions to racemization by potential impurities present in the reaction media.

By taking into account the correlation between racemization (k^r) and enantiomerization (k^e) rate constants ($k^r = 2k^e$), the activation barriers $\Delta G^{\#}_{2°(T)}$, corresponding to the second order enantiomerization process at each considered temperature were calculated by Eyring equation and are reported in Supporting Information Table 1S.

Subsequent Eyring $\Delta G^{\#}_{2°(T)}$ versus 1/T plot analyses (Supporting Information Figure 4S) show good correlation coefficient values (R^2 greater than 0.996) and afforded the thermodynamic terms $\Delta H^{\#}_{2°}$ and $\Delta S^{\#}_{2°}$ listed in Supporting Information Table 1S. By inspection of the calculated transition state parameters, it is possible to highlight as the negative values for $\Delta S^{\#}_{2°}$ ($\Delta S^{\#}_{2°}$ ranging from −22.18 to −24.64 entropy units) were compatible with a dissociative two-step base-catalyzed racemization mechanism involving a transition state with more charge separation and restricted degree of freedom than the ground state.

As suggested by kinetic and HPLC experiments, it is therefore theoretically possible to convert the racemic IMP-B into the single desired enantiomer through repetitive cycles of resolution/racemization steps.

In Fig. 5 is schematized a single cycle of the deracemization process. Starting from 10.0 mg of racemic sample it was possible to isolate about 7.0 mg of the enantiopure *S* form under very simple operational conditions.

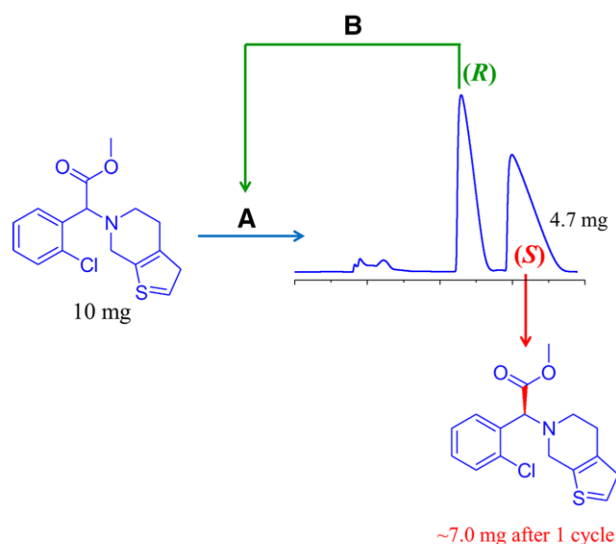

FIGURE 5 Optimized semipreparative conversion of the racemic impurity B into the *S* enantiomer. A, Chromatographic resolution. Column, Chiralcel OJ (250 × 10 mm id); eluent, *n*-hexane/ethanol (70:30, v/v); flow rate, 4.5 mL/min; temperature, 25°C; detection, UV at 280 nm. B, Off-column racemization. Solvent, methanol/MeONa (0.046 M); temperature: 60°C

3.3 | Chemo- and enantioselective HPLC analysis of (*S*)-CLOP and its impurities under MeOH/water mode

3.3.1 | Specificity

Worldwide regulatory authorities require effective analytical tools to identify, characterize, and control the levels of the impurities in the APIs as well as pharmaceutical preparations. Usually, organic impurities in an API may arise from unreacted starting materials, products of side reactions, or degradation products. In the case of an enantiopure drug there is an additional type of impurity constituted by the therapeutically unwanted enantiomer. Thus, to assure quality and safety of chiral medicines it is necessary to develop sensitive, accurate, and precise chemo- and enantioselective analytical methods capable of monitoring trace amounts of any impurity.

As anticipated in the introduction section, a monograph dedicated to the analysis of the enantiopure API CLOP

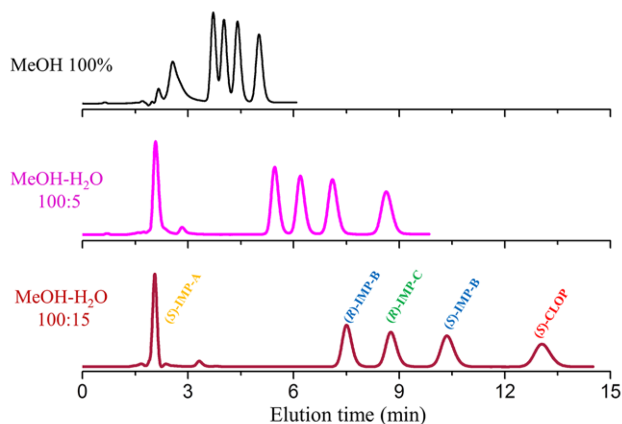

FIGURE 6 Typical HPLC chromatograms of a mixture of (*S*)-clopidogrel and its impurities A–C on the OJ-RH CSP under methanol/water mode. Chromatographic conditions: column, Chiralcel OJ-RH 150 × 4.6 mm id; eluent, from top to bottom: methanol, methanol/water (100:5, v/v) and methanol/water (100:15, v/v); flow rate, 1.0 mL/min; temperature, 25°C; detection, UV at 226 nm

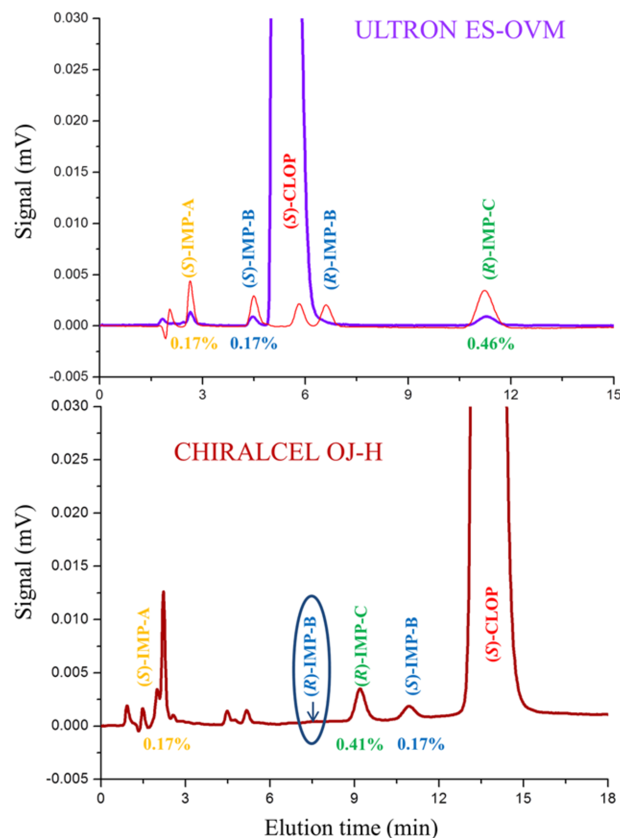

FIGURE 7 HPLC determination of the impurities A–C of (*S*)-clopidogrel in the finished product Plavix Chromatographic conditions: columns, Ultron ES-OVM-C (150 × 4.6 mm, 5 μm) (top) and Chiralcel OJ-RH (150 × 4.6 mm id) (bottom); eluent, acetonitrile–phosphate buffer (25:75) (top) and methanol/water (100:15) (bottom); flow rate, 1.0 mL/min; temperature, 25°C; detection, UV at 220 nm

bisulfate was published in the US Pharmacopoeia. Here, an alternative HPLC system to that authorized by regulatory agencies was developed using the OJ-RH CSP in reversed-phase mode.

The specificity of the HPLC based method was investigated by evaluating the effect of progressive additions of vol of water (through 5 vol increments) to 100 vol of ethanol, MeOH, or acetonitrile on retention of API and its correlated substances [14]. The column temperature and the flow rate were fixed at 25°C and 1 mL/min, respectively.

As shown in Fig. 6, the objective to baseline separating the peak pertinent to (*S*)-CLOP from those of its related substances was achieved when the mixture MeOH/water (100:15) was used as a mobile phase. It is interesting to emphasize three aspects of the chromatographic analysis: (i) the OJ-RH CSP preserved in the aqueous conditions the good enantioselectivity showed in normal phase mode (the enantioseparation factor values for CLOP and IMP-B were 1.62 and 1.50, respectively); (ii) the enantiomer elution order of CLOP was opposite than that observed using the Ultron ES-OVM CSP ($S < R$, see Fig. 7); the (*S*)-CLOP was the more retained analyte and no overlapping of other components of the mixture test was observed.

The successive step was to analyze the finished product Plavix by the HPLC methods based on OJ-RH and Ultron ES-OVM CSPs. From the comparison of the obtained chromatograms shown in Fig. 7, it is possible to note that the peak pertinent to the API (*S*)-CLOP obtained under the experimental conditions reported in the USP masks the second eluting (*R*)-IMP-B enantiomer. Therefore, it is not possible to evaluate the potential presence of (*R*)-CLOP-B in the drug and finished product using the Ultron ES-OVM CSP.

The chromatogram of the same sample of Plavix obtained with the chiral method developed with OJ-RH highlights instead how the peak pertinent to API does not interfere with the integration of the peaks of the related substances. It was so demonstrated that the impurity CLOP-B is present in the finished product only in the *S* form.

Therefore, for its specificity, the proposed HPLC method based on the OJ-RH CSP was partially validated and applied for the ee determination of API and impurity content in the finished product Plavix.

3.3.2 | Validation

Calibration curves showed good linearity in the range examined, with a target concentration of 0.5 mg/mL (100%) for CLOP, with a correlation coefficient (r^2) ≥ 0.999 and regression equation $y = 2E \times 10^{-8} \times -0.0037$. For (*S*)-IMP-A,

(*S*)-IMP-B, and (*R*)-IMP-C good linearity was obtained in the range 0.5–12.5 μg/mL (0.1–2.5%) with correlation coefficient of (r^2) ≥ 0.999 and regression equations $y = 2E \times 10^{-8} \times -3E \times 10^{-5}$, $y = 2E \times 10^{-8} \times -4E \times 10^{-5}$ and $y = 2E \times 10^{-8} \times -6E \times 10^{-5}$, respectively.

The accuracy, determined by adding a previously analyzed test solution with drug standard solution at three concentrations (50, 100, and 160%) was 99.80–101.30% with an RSD value of 0.96%.

The repeatability as RSD (%) regarding the peak area CLOP was 0.2% (<2%).

Robustness of the method was valuated varying the chromatographic parameters governing the separation. The flow rate varied from 0.8 to 1.2 mL/min, column temperature varied from 23 to 27°C, and the vol of water added to 100 vol of MeOH varied from 113 to 117. No appreciable change in resolution was observed.

The LOD values were determined at S/N = 3, and the LOQ values at S/N = 10 with values of 0.15 and 0.5 ng/mL, respectively.

4 | CONCLUDING REMARKS

A new RP-HPLC method based on the Chiralcel OJ-RH CSP for the simultaneous separation of (*S*)-CLOP and its chiral impurities is reported. The method is simple, reliable, linear, accurate, and reproducible for quantitative analysis of CLOP in bulk and in tablet formulations.

The proposed HPLC assay presents some advantages respect to that reported in the USP monography. First, the enantiomeric elution order of CLOP is opposite to that observed by using the Ultron ES-OVM CSP. So, the chromatographic peak pertinent to active ingredient does not interfere with the integration of second eluted peak of the IMP-B. Second, unlike reported in the dedicated monography, the absolute configuration of the enantiomers of IMP-B is known. The knowledge of their stereochemistry allows a clear identification and integration of the pertinent chromatographic peaks.

Finally, the versatility and good chiral ability discrimination of the OJ-RH CSP towards compounds structurally related to CLOP have permitted to separate the enantiomers of IMP-B in normal phase conditions on a semipreparative scale and to determine its configurational stability through off-column kinetic determinations.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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