Reversed-phase HPLC enantioseparation of pantoprazole using a teicoplanin aglycone stationary phase—Determination of the enantiomer elution order using HPLC-CD analyses

Lajos Attila Papp | Mohammadhassan Foroughbakhshfasaei | Béla Fiser | Péter Horváth | Eszter Kiss | Khaled Sekkoum | Árpád Gyéresi | Gabriel Hancu | Béla Noszál | Zoltán-István Szabó | Gergő Tóth

1 Department of Pharmaceutical Chemistry, University of Medicine, Pharmacy, Sciences and Tehnology, Târgu Mureș, Romania
2 Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary
3 Institute of Chemistry, Faculty of Materials Science and Engineering, University of Miskolc, Miskolc, Hungary
4 Ferenc Rákóczi II. Transcarpathian Hungarian Institute, Beregszász, Transcarpathia, Ukraine
5 Bioactive Molecules and Chiral Separation Laboratory, Faculty of Science and Technology, University of Béchar, Béchar, Algeria
6 Department of Drugs Industry and Pharmaceutical Management, University of Medicine, Pharmacy, Sciences and Technology, Târgu Mure, Romania

Correspondence
Gergő Tóth, Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary. Email: toth.gergo@pharma.semmelweis-univ.hu

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1 | INTRODUCTION

Chiral synthesis, isolation of chiral compounds from natural sources, or simply HPLC enantioseparation are all common situations when the absolute configuration of the investigated compounds may be questionable, and the isolation is not possible for various reasons, such as the small sample quantity or due to the fast decomposition of the analyte. In this case, the hyphenation of circular dichroism (CD) measurements with HPLC, supported by molecular modeling can help to determine the absolute configuration and the enantiomer elution order in chiral separations. The aim of this work was to develop a method, which could further be applied for the determination of absolute configuration of enantiomers in chiral separations without any time- and labor-intensive isolation. As a chiral model drug of choice, pantoprazole was selected.

Pantoprazole, (5-(difluoromethoxy)-2-[(3,4-dimethoxy-2-pyridyl) methylsulfinyl]-1H-benzimidazole), is a proton pump inhibitor (PPI), frequently prescribed in gastric hyperacidity-related disorders (Figure 1). The molecule has a chiral sulfoxide moiety, and it is used as a racemate in therapy. However, it has been proved that its S-enantiomer has some therapeutic advantages over the racemate, because of its stereoselective pharmacokinetics. Moreover, one of the best-known examples of chiral switch is the structurally similar PPI, esomeprazole, the S-enantiomer of omeprazole (Figure 1).

Several chromatographic and electrophoretic techniques, such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), capillary electrochromatography (CEC), thin layer chromatography (TLC), supercritical fluid chromatography (SFC), and gas chromatography (GC) are frequently used for enantioseparation of pharmaceutical substances. Among these techniques, direct HPLC enantioseparations using chiral stationary phases (CSPs) is the golden standard in this field.

Macrocyclic antibiotics have been introduced as chiral selectors for HPLC in 1994 by Armstrong et al. Since then, it became clear that the macrocyclic glycopeptides, such as teicoplanin, vancomycin, ristocetin A, and its analogues, are among the most useful chiral selectors for the enantioseparation of many biologically active compounds. The variety and combination of their chiral interaction capabilities, including electrostatic, hydrophobic, H-bonding, steric repulsion, dipole stacking, and π-π-interactions, are the factors that make these chiral selectors useful in chiral separations. Another advantage of these CSPs includes their great versatility, which makes them suitable in several combinations of mobile phases, such as normal-phase, reversed-phase, and polar organic mobile phase modes.

Several HPLC chiral separation methods have been published for the chiral discrimination of pantoprazole enantiomers. The most frequently used CSPs for this purpose are polysaccharide-type CSPs such as Chiralcel OJ-R, Chiralpak IA, Chiralpak ID-3, and Chiralpak IE-3. However, studies using other CSP classes such as protein-based or ligand-exchange have also been published in the past two decades.

A validated HPLC-MS method capable for enantiomeric determination of pantoprazole in dog plasma was reported recently. The authors used both macrocyclic antibiotic-based (Chirobiotic T and Chirobiotic V2) and protein-based (AGP and Ultron ES-OVM) columns in their work, but successful chiral separation was only achieved on protein-based columns.

To the best of our knowledge, no report appeared so far on the enantioseparation of pantoprazole, using teicoplanin aglycone-based CSP. The aim of this work was to develop, optimize, and validate an HPLC method for the chiral separation of pantoprazole enantiomers, using macrocyclic-type CSPs. In-depth analysis of different chromatographic parameters using face-centered composite design (FCCD) was used to evaluate their influence on the enantioseparation. Another objective of this work was to predict the enantiomer elution order without the time-consuming collection of pure enantiomers. CD spectroscopy, the dedicated technique to distinguish between enantiomers and the measured online

![FIGURE 1](image-url) Constitutional formulas of pantoprazole (left) and esomeprazole (right). Asterisk denotes the chiral center

**KEYWORDS**

chiral separation, enantiorecognition, molecular modelling, omeprazole, online circular dichroism, pantoprazole, proton pump inhibitor, teicoplanine-aglycone
HPLC-CD signal or spectral information supported by computational method, was used for the elucidation of enantiomer elution order. Molecular docking was applied to simulate the interaction processes between teicoplanin aglycone and pantoprazole enantiomers at the molecular level, as the calculated binding energy difference between the enantiomers could also predict the enantiomer elution order.

2 | MATERIALS AND METHODS

2.1 | Materials

Macro cyclic glycopeptides-type chiral columns with identical dimensions (100 × 4.6 mm, 5-μm particle size)—ristocetin A-based Chirobiotic R, teicoplanin-based Chirobiotic T, teicoplanin aglycone-based Chirobiotic TAG, and vancomycin-based Chirobiotic V were purchased from Supelco/Astec, (Milwaukee, Wisconsin). Pantoprazole sodium hydrate (≥98%), esomeprazole magnesium hydrate (≥98%), and triethylamine (≥99%) were ordered from Sigma-Aldrich Hungary (Budapest). Gradient grade methanol (MeOH), acetonitrile (ACN), ethanol (EtOH), glacial acetic acid, ammonium acetate (≥98%), ammonium formate (≥99%), and formic acid (≥98%) were purchased from Merck (Darmstadt, Germany). Controloc 20-mg tablets were obtained from Central Pharmacy of Semmelweis University (Budapest, Hungary). Ultrapure, deionized water was prepared by a Milli-Q Direct 8 Millipore system (Milford, Massachusetts).

2.2 | LC-UV analysis

LC-UV analysis was carried out on a JASCO HPLC system (JASCO PU-2089 Plus binary gradient pump, AS-4050 autosampler, MD-2010 Plus diode array detector and column oven (CO2065 plus)). The software used for the operation of the equipment and data processing was ChromNAV. UV detection was performed at 288 nm. Stock solutions of 1-mg/mL pantoprazole were prepared in MeOH and were further diluted with the same solvent. An injection volume of 10 μL was used, and three parallel measurements were performed in all cases. Other chromatographic conditions including validation processes are given in Section 3.

2.3 | LC-CD method

The inline CD spectra of the separated enantiomers were measured by an LC system composed of a JASCO intelligent pump (PU-980), hyphenated with a JASCO spectropolarimeter (J720) equipped with a flow through cell (path length: 0.5 cm), suitable for the simultaneous detection of CD and UV spectra (JASCO, Tokyo, Japan). During this method, 1-mg/mL pantoprazole solutions were prepared in methanol, and 20 μL of these solutions were injected on the Chirobiotic TAG column using a Rheodyne injector. The signs of CD signal of pantoprazole enantiomers at 273 and 229 nm were compared with those of esomeprazole. The esomeprazole (c = 0.5 mg/100 mL in methanol) CD spectrum was recorded on the same JASCO J720 spectropolarimeter in a 1-cm quartz cuvette at 25°C. The spectrum was collected from three times accumulation with a bandwidth of 1 nm and a scanning step of 0.2 nm at a scan speed of 50 nm/min. Besides, high-speed, multiscan CD spectra were also registered continuously between 240 and 330 nm with 1000-nm/min scanning speed during the separation process.

2.4 | Pharmaceutical sample preparation

Ten Controloc tablets were weighted and afterwards ground and mixed in a mortar. Then an amount of powder equivalent to 20 mg of racemic compound was dissolved in 100-mL methanol. The suspension was sonicated for 10 minutes and then centrifuged for 10 minutes applying 4000 rpm (Sartorius 2-16P benchtop centrifuge, Goettingen, Germany). The clear supernatant was filtered through a 0.22-μm pore size syringe filter and diluted with MeOH to the appropriate concentrations before injection. Determination of the pantoprazole content in pharmaceutical formulation was performed in triplicate.

2.5 | HPLC-ESI-QqQ-MS methods

LC–ESI–MS/MS analysis was carried out on an Agilent 1260 Infinity HPLC system (G1312B binary gradient pump, G1367E autosampler) hyphenated with an Agilent 6460 triple quadrupole system equipped with a JetStream (ESI) ion source (Agilent Technologies, Waldbroon, Germany). The ESI was operated in positive ion mode. High purity N₂ was used as collision gas. Applied ion source parameters for pantoprazole were the following: flow and temperature of the drying gas (N₂): 10 L/min and 250°C, pressure of the nebulizer gas (N₂): 45 psi, capillary voltage: 3000 V, nozzle voltage: 500 V, sheath gas flow and temperature: 10 L/min and 300°C, respectively. Multiple reaction monitoring (MRM) transitions for the target analytes, associated fragmentor voltages, and collision energies were automatically determined in positive ion mode using Agilent MassHunter Optimizer.
Software. Two transitions were used to identify and quantify pantoprazole 384.1 200.0 MRM transition with 80-V fragmentor voltage and 8-eV collision energy were the parameters of quantifier ion and 384.1 138.1 transition with 80-V fragmentor voltage and 36-eV collision energy were the parameters of qualifier ion. Mass spectra were processed by Agilent MassHunter B.04.00 software.

HPLC-MS/MS measurements were used to detect the pantoprazole enantiomers in 5-ng/mL concentration in spiked mouse serum sample. Therefore, an appropriate amount of pantoprazole stock solution was added to 200-µL mouse serum sample. After 1 minute on vortex, samples were centrifuged at 8000 rpm for 10 minutes. The supernatant was transferred to a glass vial and evaporated under nitrogen gas stream to complete dryness. The residue was dissolved in 100-µL purified water and filtered through 0.22-µm syringe filter. The resulting solution was injected into the HPLC/ESI-MS system. The injection volume was 10 µL.

2.6 Docking simulations

The structure of teicoplanin aglycone (TAG) was obtained from the Protein Data Bank (rcsb.org), by downloading the 3mgb entry. The 3mgb is a molecular complex containing TAG and other molecules. Only one teicoplanin aglycone molecule was kept, while the other parts of the complex (protein, water, etc) were deleted. Hydrogens were added, then, partial charges were computed, and the resulting TAG structure was minimized by using the MMFF94x force field. This minimized structure has been used as host in docking simulations. Pantoprazole enantiomers were docked by using AutoDock Vina, and their affinity towards the teicoplanin aglycone model was determined. The grid box in the calculations was 30 × 30 × 30 Å³, the maximum number of binding modes was set to 9, while the exhaustiveness was 8. Binding affinities (ΔE_A) of the best pantoprazole binding modes were compared.

2.7 Quantum chemical calculations

Geometry and frequency calculations were carried out at the M06-2X/6-31G(d)/CPCM (methanol) level of theory. The optimized structures of the pantoprazole enantiomers were used to compute their CD spectra at M06-2X/6-311 + G(2d,p)/CPCM (methanol) level of theory. Overlapping Gaussian functions with σ = 0.20 eV fitting parameter were applied to simulate the CD spectra in the SpecDis program. All quantum chemical calculations were carried out by using the Gaussian 09 program package.

3 RESULTS AND DISCUSSION

3.1 Preliminary screening

The enantiomeric resolution capabilities of the four macrocyclic antibiotic-type CSPs (Chirobiotic TAG, Chirobiotic T, Chirobiotic R, and Chirobiotic V2) were evaluated, in polar organic, polar ionic, and reversed-phase chromatographic mode. In polar organic mode, pure MeOH and ACN were used. In polar ionic mode, 0.1% ammonium formate or acetic acid/triethylamine mixture in different ratios was added to MeOH or ACN in order to facilitate ionization of the analyte. In reversed-phase mode, eluents containing mixtures of ACN/water, MeOH/water, and EtOH/water in different proportions were used to establish the most suitable organic modifier. In all preliminary experiments, a constant 0.5-mL/min flow rate and 20°C column temperature was used.

Preliminary chromatographic runs revealed that under the applied conditions, only the teicoplanin aglycone-based Chirobiotic TAG column has enantiorecognition ability for pantoprazole enantiomers. The highest enantiorecognition ability was achieved in reversed-phase mode when using a 60/40 (v/v%) MeOH/water mobile phase. Further experiments were carried out to evaluate the pH dependence of the chiral separation; 0.1% acetic acid (pH 3.30), 20-mM ammonium acetate/acetic acid (pH 5.00), and 20-mM ammonium acetate (pH 6.90) were used as aqueous component of the mobile phase, while the MeOH/water ratio was kept constant 60/40 (v/v%). No significant differences were obtained in enantiomeric resolution or retention times using the above-mentioned mobile phase compositions, compared with pure water. However, 20-mM ammonium acetate buffers resulted the best peak shape. Taking also into account the relative instability of pantoprazole in acidic conditions, 20mM ammonium acetate in water (pH 6.90) was chosen for further method optimization. The influence of column temperature (10-40°C) and flow rate (0.3-0.8 mL/min) were also investigated. Applying higher temperature, the resolution value decreased. Based on these findings, the range of 5°C to 15°C was chosen for the following optimization design, while 0.5 to 0.7 mL/min flow rate range was selected in accordance with resolution and analysis time.

3.2 Method optimization using FCCD

To optimize the analytical conditions and to investigate the effect of the different chromatographic parameters on the separation a face-centered central composite design (FCCD) for three factors with 20 experiments
was applied. The variable analytical parameters and their applied ranges were as follows: MeOH percentage in the mobile phase (50%-70% v/v) (factor A) column temperature (5°C-15°C) (factor C) and flow rate (0.5-0.7 mL/min) (factor B). As a result, two experimental responses were screened: resolution value between pantoprazole enantiomers and retention time of the second eluting enantiomer.

The experimental plan and the results are summarized in Table 1.

A second-order polynomial quadratic model was applied, and analysis of variance (ANOVA) was carried out to estimate the significance of the model. The insignificant model terms were deleted one by one, while the model was re-evaluated after each deleted term. The following final regression models were obtained for the resolution and analysis time, respectively:

\[
\text{Resolution} = +1.90 - 0.16A - 0.13B + 0.017C - 0.078A^2 + 0.042B^2 - 0.19C^2
\]  

\[
\text{Analysis time} = +7.80 - 3.75A - 1.66B + 0.79A^2 + 1.17B^2
\]

where \(A\) is the methanol content in % (v/v), \(B\) is the flow rate in milliliters per minute, and \(C\) is the temperature in degree celcius.

By increasing the methanol content of the mobile phase, a shortened analysis time was observed, while the resolution optimum was around 60%. Upon enhancing the flow rate, both resolution and analysis time decreased. The temperature influenced the resolution to a great extent, which reached a maximum value around 10°C, while it had no significant effect on the analysis time in the studied range (5-15°C).

Both models showed good performance indicators: \(R^2 = 0.9866, R^2_{adj} = 0.9804\) for resolution and \(R^2 = 0.9292, R^2_{adj} = 0.9104\) for analysis time, respectively. The values obtained verify that both estimated models fit the experimental results.

Three-dimensional response surface plots obtained by the regression models are presented in Figure S1.

In order to establish an optimal combination of the studied analytical conditions for both responses, the Derringer desirability function was applied. In this approach, experimental results are transformed in desirability values on a scale between 0 and 1, 0 representing the most undesirable and 1 the most desired outcome of each response of interest. In our case, resolution had to be enhanced, and analysis time had to be minimized. Global desirability was calculated as geometric mean of the individual desirability values, and then the overall optimum was searched in the experimental space.

Optimal analytical conditions obtained, based on the desirability function, were as follows: Chirobiotic TAG stationary phase, thermostated at 10°C, mobile phase consisting of methanol/20mM ammonium acetate 60:40 v/v, and 0.6-smL/min flow rate.

Using these optimal analytical conditions, baseline resolution \((R_s = 1.91 \pm 0.03)\) within 10 minutes was achieved between pantoprazole enantiomers.

### 3.3 Method validation and application

To prove that our method is appropriate and reliable, a validation study was carried out according to ICH guidelines, based on precision, linearity, accuracy, limit of detection (LOD), and limit of quantification (LOQ). Method precision was evaluated based on intraday and interday precisions. Six consecutive injections \((n = 6)\) from a standard solution \((c = 20 \, \mu g \, mL^{-1})\) were performed in order to estimate the intraday precision by calculating the RSD values for peak areas for each

| Experiments | Standard Order | Run Order | Factors | Response | \(t_r\) (min) |
|-------------|----------------|-----------|---------|----------|-------------|
| 3           | 15             | 50        | 0.7     | 5        | 1.68        |
| 15          | 2              | 60        | 0.6     | 10       | 1.86        |
| 5           | 3              | 50        | 0.5     | 15       | 2.01        |
| 13          | 4              | 60        | 0.6     | 5        | 1.69        |
| 8           | 5              | 70        | 0.7     | 15       | 1.42        |
| 11          | 6              | 60        | 0.5     | 10       | 2.07        |
| 10          | 7              | 70        | 0.6     | 10       | 1.68        |
| 12          | 8              | 60        | 0.7     | 10       | 1.82        |
| 4           | 9              | 70        | 0.7     | 5        | 1.36        |
| 6           | 10             | 70        | 0.5     | 15       | 1.61        |
| 14          | 11             | 60        | 0.6     | 15       | 1.74        |
| 7           | 12             | 50        | 0.7     | 15       | 1.71        |
| 17          | 13             | 60        | 0.6     | 10       | 1.90        |
| 18          | 14             | 60        | 0.6     | 10       | 1.86        |
| 20          | 15             | 60        | 0.6     | 10       | 1.91        |
| 19          | 16             | 60        | 0.6     | 10       | 1.93        |
| 2           | 17             | 70        | 0.5     | 5        | 1.64        |
| 9           | 18             | 50        | 0.6     | 10       | 1.97        |
| 1           | 19             | 50        | 0.5     | 5        | 1.95        |
| 16          | 20             | 60        | 0.6     | 10       | 1.90        |

Note. Factor A—methanol content (% v/v); Factor B—flow rate (mL/min); Factor C—temperature (°C); \(t_r\)—retention time of the second eluting enantiomer.

\(\text{Analysis time} = +7.80 - 3.75A - 1.66B + 0.79A^2 + 1.17B^2\)
enantiomer. Interday precision was determined by performing six injections of a standard solution \((c = 20 \mu g \text{ mL}^{-1})\) over three consecutive days \((n = 18)\), and calculating RSD values for peak areas. Linearity of the models was investigated over the concentration range 1 to 50 \(\mu g \text{ mL}^{-1}\), at six concentrations, using three replicates at each concentration level. LOD and LOQ were calculated from signal/noise ratio 3:1 and 10:1, respectively. Method accuracy was investigated by recovery experiments at three concentration levels for both enantiomers, covering the linearity range, each solution injected in triplicate. The validation data including precision, accuracy, and linearity, LOD, and LOQ values are summarized in Table 2.

The validated method was applied for the chiral separation and quantitative determination of pantoprazole from a commercially available dosage form. Results of the quantitative evaluation are presented in Table 3. Recoveries were in good agreement with the declared drug content. Moreover, no interference was observed on the chromatograms from drug formulation excipients (Figure 2A). Using our optimized HPLC method supplemented with optimized MS/MS parameters, pantoprazole enantiomers in 5-ng/mL concentration were detected in spiked mouse serum, indicating that the developed method could be a good starting point for bioanalytical purpose as well (Figure 2B).

### 3.4 Determination of enantiomer elution order by online HPLC-CD

Since pantoprazole enantiomer standard was not available, two approaches were applied for the elucidation of elution order.

I. The online registered CD spectra of pantoprazole enantiomers were compared with CD spectrum of enantiopure esomeprazole, a chemically related compound.

#### TABLE 2 Method validation data

| Pantoprazole | S-pantoprazole | R-pantoprazole |
|--------------|----------------|----------------|
| Precision    |                |                |
| Intraday     | RSD% of peak area | 1.17 | 1.32 |
| Interday     | RSD% of peak area | 1.59 | 1.82 |
| Accuracy     | (Recovery %)     | 101.22 102.4  |
| 5 \(\mu g \text{ mL}^{-1}\) (n = 3) | 100.33 99.94 |
| 10 \(\mu g \text{ mL}^{-1}\) (n = 3) | 99.11 98.36 |
| Linearity    | Regression equation |
| (1-50 \(\mu g \text{ mL}^{-1}\)) | \(y = 0.245x + 1.065\) | \(y = 0.240x + 0.896\) |
| \(R^2\)     | 0.999 0.998     |
| LOD (\(\mu g \text{ mL}^{-1}/l\)) | 0.65 0.59 |
| LOQ (\(\mu g \text{ mL}^{-1}/l\)) | 1.96 1.78 |

#### TABLE 3 Results obtained during quantification of pantoprazole enantiomers from commercially available pharmaceutical product

| Pharmaceutical product | Declared Enantiomer Quantity (mg) | Found Enantiomer Quantity (mg) (n = 3) |
|-------------------------|-----------------------------------|---------------------------------------|
|                         | 1st enantiomer                    | 2nd enantiomer                       |
|                         | 1st enantiomer                    | 2nd enantiomer                       |
| Controloc 20 mg         | 10                                | 10                                    |

FIGURE 2 Representative chromatogram for the chiral separation of pantoprazole enantiomers under optimized analytical conditions (Chirobiotic TAG stationary phase, thermostated at 10°C, mobile phase: methanol/20mM ammonium acetate 60:40 v/v, 0.6 mL/min flow rate) (A) from commercially available dosage forms (UV detection at 288 nm), (B) from spiked mouse serum (MS detection: 384.1 200.0 MRM transition with 80 V fragmentor voltage and 8 eV collision energy)
II. The inline CD signal of pantoprazole enantiomers was compared with the quantum chemically calculated CD spectra.

HPLC coupled with a CD spectrometer can be used as chiroptical detector, and it can be useful for determination of enantiomer elution order of racemic drugs; if structurally similar, pure enantiomer is available. Comparing the character of CD spectra of the reference and the investigated molecule, the configuration can be assumed.24 This so-called empirical approach implies that a minor structural difference does not alter the CD spectrum of the investigated molecule.24-26 In our study, the detected CD sign of pantoprazole enantiomers was compared with the structurally related esomeprazole at two wavelengths (229 and 273 nm), where the signs of the CD spectrum of esomeprazole are opposite. As it can be seen in Figure 3, both esomeprazole and the foremost eluting pantoprazole enantiomer show positive CD signal at 229 nm as well as similarly negative CD signal at 273 nm; thus, elution order can be empirically established as S-pantoprazole, followed by R-pantoprazole.

Rapid multiscan chiroptical spectral recording method is unique and useful method to characterize peak purity27 and determine enantiomer elution order.28 Using 1000-nm/min scanning speed, we were able to register two appropriate ECD spectra between 330 and 240 nm. The two spectra collected from the pantoprazole chromatogram show mirror images of each other. The first eluted isomer shows negative band, whereas the second eluted isomer shows positive band (Figure 4A). To further verify the identity of the isomers, their spectra were calculated by using M06-2X/6-311 + G(2d,p)/CPCM (methanol) level of theory and compared with the experimental results (Figure 4B). The calculated and measured spectra can be paired easily and allow assigning the S-pantoprazole absolute configuration to the first eluted peak, so this approach also confirmed the elution order as being: R isomer follows S.

3.5 Docking study of pantoprazole and teicoplanin aglycone complexes

Using molecular docking, the interactions between pantoprazole enantiomers and teicoplanin aglycone can be investigated at the molecular level. Furthermore, by using molecular docking, the strength of complexation can be compared and studied. The TAG model from a molecular complex (PDB ID: 3MGB) was prepared and used as host in docking simulations. AutoDock Vina was used to dock the enantiomers and study the interactions and the corresponding affinities between them and the TAG model. The best R- and S-pantoprazole binding modes have been selected based on their binding affinities towards the TAG model. The three-dimensional (3-D) structures of the selected diastereomeric complexes between pantoprazole enantiomers and TAG are presented in Figure 5 (for additional views, see Figures S2 and S3), together with the calculated relative binding

FIGURE 3 Comparison of circular dichroism (CD) spectrum of esomeprazole (A) with CD (B) and UV (C) signals of the separated pantoprazole enantiomers at 229 (red) and 273 (black) nm. Chromatographic conditions are the same as in Figure 2, except for column temperature (ambient)
Within the formed complexes, three different interaction types can be identified: π-stacking, –CH–Ph and –NH–O. The number of interactions is four (two π-stacking, one –CH–Ph, and one –NH–O) and five (three π-stacking, one –CH–Ph, and one –NH–O) in case of the S- and R-pantoprazole-TAG complex, respectively. More interactions can be associated with stronger binding, which is confirmed by the complexation energies of pantoprazole enantiomers. Thus, the R-enantiomer binds stronger to the chiral selector than its antipode (ΔR–SSEA = −0.3 kcal/mol), in accordance with the elution order on the column. Our results also show that molecular docking can efficiently contribute to predict elution order of enantiomers.

4 | CONCLUSION

Pantoprazole enantiomers were separated with a novel, reversed-phased HPLC method on a Chirobiotic TAG column. Our method was optimized using a multivariate approach, leading to baseline separation of enantiomers within 10 minutes, which is one of the fastest methods in the literature. Further benefits of our method are that it is environmentally friendly cheap especially compared with the often used normal-phased system in chiral HPLC, and it is MS-compatible. Applicability of the method was checked by analyzing commercial pharmaceutical preparations, and after MS-hyphenation, it was also proved that it could be a good starting point for bioanalytical purposes as well. The study further underlines the ease of use of reversed-phase mode using macrocyclic glycopeptide-based CSPs. Since individual enantiomers were not available, the method was used as a prerequisite for the identification of the separated antipodes without the time-consuming sample collection and prediction of the elution order. Thus, methods were elaborated to determine the absolute configuration of the eluted pantoprazole enantiomer peaks. Three separate approaches were used for the determination of elution order without isolation. At first enantiomer, elution order was determined using HPLC coupled CD spectroscopy as detection method, by two approaches: The online registered CD spectra of pantoprazole enantiomers were compared with CD spectrum of enantiopure esomeprazole, and the inline CD signal of pantoprazole enantiomers was compared with the calculated CD spectra. Furthermore, molecular docking was also applied to determine the binding strength between the chiral selector and the enantiomers. The docking calculations showed that the R-pantoprazole binds stronger the chiral selector than its antipode, in accordance with the elution order on

FIGURE 4  Online registered (A) and calculated (B) circular dichroism (CD) spectra of pantoprazole enantiomers

FIGURE 5  3-D structures of the diastereomeric complexes between pantoprazole enantiomers and teicoplanin aglycone (TAG) and difference between their calculated relative binding affinities (ΔR–SSEA)
the column. Our results demonstrate the efficiency of combined methods, HPLC-CD and theoretical calculations, to determine enantiomer elution order.

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**ORCID**

Gabriel Hancu  
[https://orcid.org/0000-0003-2564-9271](https://orcid.org/0000-0003-2564-9271)

Gergő Tóth  
[https://orcid.org/0000-0001-5341-319X](https://orcid.org/0000-0001-5341-319X)

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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