# Estimation of Some Amino-Based Drugs in Human Blood Using HPLC Technique

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*Enantiomers in human plasma were analyzed using Solid Phase Extraction (SPE) and Chiral High-Performance Liquid Chromatography (HPLC). The chiral HPLC strategy allowed fractionation of DL, DD, LL, and LD varieties in human blood plasma with Targeted Solid Phase Extraction (TSPE). These methods are precise, economical, eco-friendly, and reproducible. Maximum plasma binding percentages for DL-, DD-, LL-, and LD-enantiomers were 63.39%, 56.79%, 84.11%, and 73.11%, respectively, at pH 9. Chiral resolution was strong, with separation factors over 1.0. Limits of Detection (LOD) ranged from 1 to 2.7 µg/mL, and Limits of Quantitation (LOQ) ranged from 5 to 10 µg/mL. The experimental data aligned well with the model, successfully applied to real plasma samples, enabling chiral monitoring of dipeptide stereoisomers in patients. HPLC, using an AmyCoat-RP column with an amylose polysaccharide stationary phase, proved effective for consistent and robust analysis, confirming the suitability of D-tryptophan, L-leucine, D-leucine, and L-tryptophan stereoisomers for clinical and research applications.*

**Keywords:** enantiomers, HPLC, chiral separation modeling plasma

## Introduction

The significance of stereoisomers in pharmacology cannot be overstated, as one stereoisomer can be therapeutic while its counterpart may be inactive or even harmful. This creates considerable interest among researchers, scholars, medical professionals, and regulatory agencies in understanding how optically active stereoisomers influence medications with strong therapeutic effects. A given stereoisomer's variations can differ in metabolic pathways, distribution profiles, and elimination rates, resulting in distinct biological roles [1].

Dipeptides play a critical role in various biological processes, such as neurotransmission, protein synthesis, microbial activity, fertilization, and the regulation of inflammatory responses. Research on dipeptides has thus contributed significantly to biomarkers and drug discovery. Dipeptides, characterized by two asymmetric centers, can exist in four stereoisomeric forms, each influencing the behavior of the dipeptide in the human body [2]. Dipeptides with aromatic amino acids are especially important in protein interactions and nucleic acid recognition.

A comprehensive literature review highlights the substantial work done on dipeptide separation, noting that traditional methods, though effective, tend to be tedious, complex, and solvent-intensive, making them costly [3]. The review [4-6] also reveals the limited availability of analytical methods for separating chiral compounds with multiple asymmetric centers. Key topics in this review include sample preparation for solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) in pharmaceutical analysis, which are explored in subsequent sections.

In 2020, Nora et al. developed a new method for analyzing the optical purity of the DL-alanine-DL-phenylalanine complex. This method determines the enantiomeric composition of DL-Ala-DL-Phe using a mobile phase mixture of $CH_3COONH_4$ (10 mM), methanol (MeOH), and formic acid (HCOOH) in a 70:30:0.05 (v/v) ratio at a flow rate of 0.8 mL/min. An AmyCoat-RP column was employed as the stationary phase to separate the enantiomers, specifically designed to analyze enantiomeric profiles of two distinct dipeptides [7]. A simulation further validated the accuracy of these chromatographic analysis results.

Additional studies explored the enantiomers of DL-tyrosine and DL-alanine using novel methods. For DL-alanine-DL-tyrosine, a mobile phase of CAN (10 mM, pH 6.0) [50:50 v/v] was utilized, and for DL-alanine-DL-tryptophan, the mobile phase comprised methanol, acetonitrile (50:40:10), and a 10 mM ammonium acetate solution, with a flow rate of 0.8 mL/min. An AmyCoat-RP column was used as the stationary phase to achieve improved enantioseparation, as described by Ali et al. (2018) [8].

In a method suggested by Aydogan et al. (2018) for amino acid enantiomer detection, a solvent system of 85% acetonitrile, 10% methanol, and 5% water with 0.10% trifluoroacetic acid was employed, maintaining a flow rate of 0.8 mL/min. This setup, combined with a porous-layer stationary phase, facilitated

enantioseparation [9].

Pucciarini et al. (2019) conducted validation research on a novel approach for detecting carnosine enantiomers, utilizing a water-based mobile phase containing 0.10% formic acid with 20–40% acetonitrile at a flow rate of 1 mL/min [10]. Using a chirobiotic T column as the stationary phase created an environment conducive to enantioseparation [11].

## Experimental part

*Reagents*

A methanol–water solution (50:50, v/v) was prepared using HPLC-grade methanol obtained from Sigma-Aldrich (St. Louis, MO, USA) and ultrapure water (Millipore, Billerica, MA, USA). Freshly frozen human plasma was sourced from a certified supplier. Acetone (for protein precipitation, 99.8%) was purchased from Merck (Darmstadt, Germany). The phosphate-buffered solution (5 mM, pH 7) was prepared using analytical-grade reagents from Sigma-Aldrich. Methanol (for cartridge preconditioning) and ultrapure water (Millipore) were used as preconditioning solvents. The elution solvent consisted of methanol with 0.10% (v/v) trifluoroacetic acid (TFA) supplied by Sigma-Aldrich.

*Apparatus*

Sep-Pak C18 cartridges (Waters, Milford, MA, USA) were used for solid-phase extraction. A vortex mixer (IKA, Staufen, Germany) was employed to ensure thorough mixing of solutions. Samples were centrifuged at 10,000 rpm using a high-speed centrifuge (Eppendorf 5430, Hamburg, Germany). A hot air blower (VWR, Radnor, PA, USA) was utilized to dry the cartridges post-wash. Chromatographic analyses were performed using a high-performance liquid chromatography (HPLC) system (Agilent 1260 Infinity, Santa Clara, CA, USA).

*Solid-phase extraction (SPE) procedure*

A 50:50 (v/v) methanol-water mixture was initially used to dissolve the various enantiomers, with concentrations standardized to 1 mg/mL. Next, one milliliter of each enantiomer solution was added to five milliliters of freshly frozen human plasma. The mixture was vortexed for two to three minutes and allowed to stand for twenty to thirty minutes, depending on the ratio used. Acetone (15 mL) was added to facilitate protein precipitation, followed by further vortexing and a 30-minute incubation period.

Samples were centrifuged for 10 minutes at 10,000 rpm to separate the supernatant more effectively. After centrifugation, the supernatant was dried, and 10 mL of a 5 mM phosphate-buffered solution (pH 7) were added to reconstitute the residue.

Preconditioning of Sep-Pak C18 cartridges was performed with 1.5-2.5 mL of methanol and 5.0 mL of Millipore water. The enantiomer solutions were then passed through the cartridges at a flow rate of 0.20 mL per minute, followed by a wash with 2.0 mL of Millipore water at the same rate. The cartridges were then dried with hot air. Analytes were extracted using a solvent containing 10 mL of methanol with 0.10% trifluoroacetic acid at a flow rate of 0.5 mL per minute. Elution and subsequent evaporation concentrated the drug solutions to a final concentration of quantity/0.5 mL. HPLC analysis was conducted after this process, confirming the presence of illicit substances.

Several experimental parameters were optimized to improve the SPE conditions.

## Results and discussion

### Solid phase extraction

Optimizing solid-phase extraction (SPE) involves adjusting several critical factors, including the pH and concentration of the phosphate buffer and the elution flow rates for both the solvent and the buffer. Additionally, various solvents have been employed in sample elution, such as acetone, methanol, ethyl acetate, and ethanol [11-13]. Table S1 summarizes the binding and recovery percentages observed for the drug under investigation on a C18 cartridge when exposed to human plasma. Recovery ratios for the enantiomers of specific chiral pharmaceuticals were calculated to evaluate the effectiveness of the developed method. The percentage recoveries for leucine-tryptophan at pH 9.0 were 36.61% (DL-), 43.21% (DD-), 15.90% (LL-), and 26.89% (LD-). The corresponding plasma binding percentages for leucine-tryptophan enantiomers at pH 9.0 were 63.39% (DL-), 56.79% (DD-), 84.11% (LL-), and 73.11% (LD-).

Accounting for potential experimental errors was essential for accurately estimating drug-protein binding percentages in human plasma. The elution solutions included 0.10% to 1% acetic acid or trifluoroacetic acid to maximize recovery values. This report meticulously documents the most significant experimental conditions. The enantiomers were extracted with optimal recovery rates from a C18 cartridge using a phosphate buffer (50 mM, pH levels 5, 7, 9, and 10) at a 0.1 mL/min flow rate. Methanol (MeOH) with 0.1% trifluoroacetic acid, delivered at a flow rate of 0.1 mL/min, proved to be the most effective solvent for eluting all drug enantiomers.

### pH effects of phosphate buffer

The highest percentage recoveries of dipeptide stereoisomers onto the sorbent after acetone deproteinization is critically dependent on buffer pH. Consequently, pH plays a key role in the desorption of dipeptide stereoisomers from C18 SPE (solid-phase extraction) cartridges. Different pH values yielded varying percentage recoveries for each analyte. For the C18 sorbent, retention variations were observed across different pH levels due to the presence of acidic regions on the C18 surface, which consists of unbound silanols. These acidic regions enhance dipeptide stereoisomer binding through hydrogen bonding and cation-exchange interactions by interacting with the imine (-NH-) group.

The buffer pH influences the ionization of dipeptide stereoisomers' enantiomers. Since each enantiomer

contains amino and amide groups, dipeptide stereoisomers are positively charged in acidic environments. Due to their distinct stereochemical configurations, these dipeptides exhibit different recovery rates. As mentioned, the varying hydrogen bonding interactions between dipeptide stereoisomer molecules and the sorbent may contribute to the diverse recovery percentages observed at different buffer pH levels. The ionization capacity of dipeptide stereoisomers significantly impacts their recovery percentages. The effect of pH on the percentage recoveries of DL-, DD-, LL-, and LD- dipeptide stereoisomers was investigated using solid-phase extraction techniques (see Figure 1).

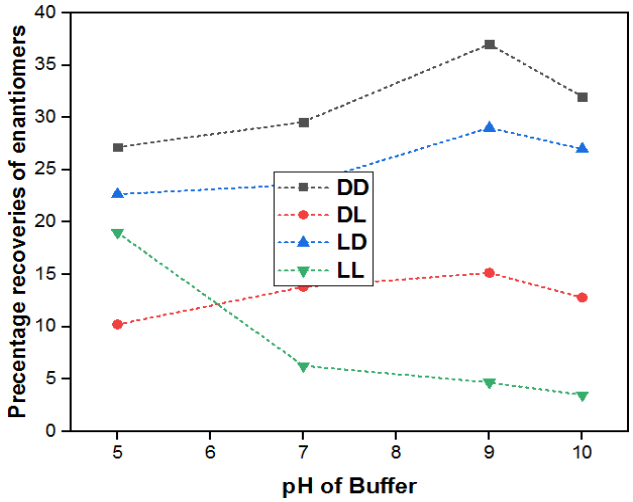


**Figure 1.** Effect of buffer pH on percentage recoveries of dipeptide stereoisomers.

### Phosphate buffer flow rate Effects

To enhance the current SPE technique, various eluting solvents were tested. Selecting the appropriate solvent is crucial to achieving maximum recoveries of DL-, DD-, LL-, and LD- enantiomers. The right eluting solvent is essential for effectively removing interfering compounds and maximizing analyte recovery from human plasma samples. Four solvents—acetone, ethanol, methanol, and ethyl acetate—were used to optimize DD, DL, LD, and LL enantiomers' percentage recoveries. Additionally, varying acetic and trifluoroacetic acid concentrations were added to previously described solvents to increase dipeptide stereoisomer recoveries further. The highest and lowest recoveries for DD-, LD-, DL-, and LL-enantiomers were achieved with methanol containing 0.10% trifluoroacetic acid (TFA) and with acetone, respectively, as indicated by the percentage recovery plots of these enantiomers (see Figure 2).

### Effect of other eluting solvents

For the percentage recoveries of these dipeptide stereoisomers, methanol with 0.10% TFA (v/v) demonstrated the highest efficacy, surpassing acetone, which in turn was more effective than ethyl acetate, followed again by methanol. Methanol (0.10% TFA, v/v) proved to be the optimal eluting solvent, yielding the maximum recovery percentages of the specified dipeptide stereoisomers from the C18 cartridge. This can be attributed to the significant differences in polarity and dielectric constants among the solvents, which influence the recovery rates observed for each. The high polarity and low dielectric constant of methanol and ethyl acetate enable effective cleavage of C18 bonds with DL-, DD-, LL-, and LD- enantiomers, leading to substantial recovery percentages for the studied dipeptide stereoisomers. The best outcomes were achieved with methanol and 0.10% TFA, as this combination produced the highest recovery values for DL-, DD-, LD-, and LL- enantiomers. Methanol's high polarity facilitated the disruption of analyte and C18 phase bindings in the presence of 0.10% TFA (v/v) [14,15]. The impact of various solvents on percentage recovery values of dipeptide enantiomers are shown in Figure 3.

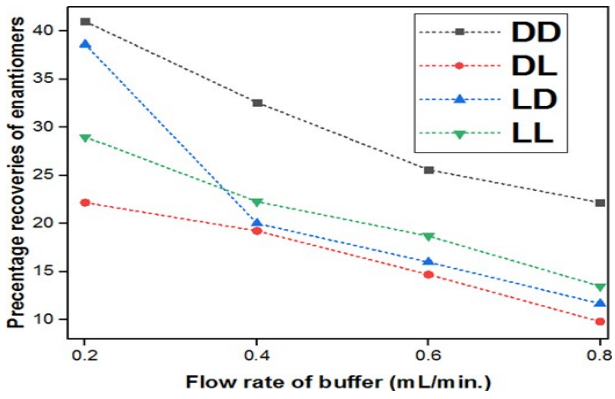


**Figure 2.** Impact of buffer flow rate on percentage recoveries of dipeptide enantiomers.

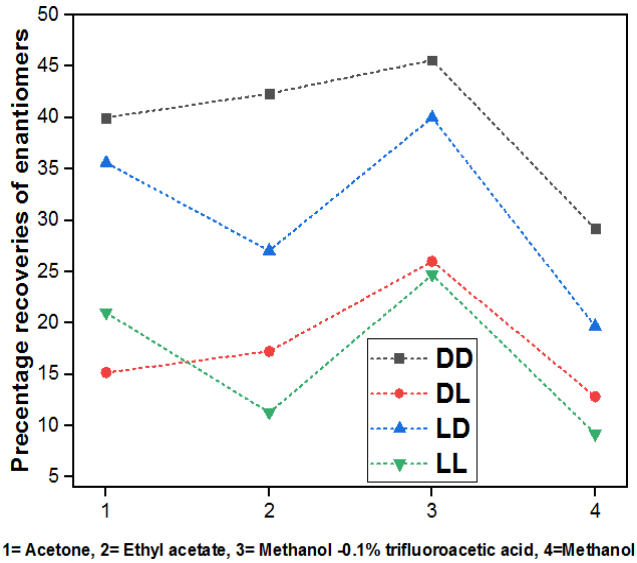


**Figure 3.** Impact of various solvents on percentage recovery values of dipeptide enantiomers.

**Effects of the flow rates of the elution solvent**

The flow rate of the eluting solvent (methanol with 0.10% TFA, v/v) through the C18 cartridge also influenced the percentage recoveries of DL-, DD-, LL-, and LD- enantiomers from human plasma. Depending on the flow rate, varying amounts of each dipeptide stereoisomer enantiomer were recovered. To optimize the extraction of DL-, DD-, LD-, and LL-enantiomers from human plasma, the elution solvent flow rate through the C18 cartridge was adjusted. Results demonstrated that significant recoveries were achieved at lower flow rates compared to higher ones. Based on this finding, we tested four distinct flow rates: 0.20, 0.40, 0.60, and 0.80 mL/min.

To determine which conditions yield the highest percentage recovery of dipeptide stereoisomers, the maximum and minimum recovery values at a 0.1 mL/min flow rate are displayed in Figure 4 [17], showing the experimental results. Recovery for enantiomers DL-, DD-, LD-, and LL- increased progressively with flow rates of 0.2, 0.4, 0.6, and 0.8 mL/min. At a 0.2 mL/min flow rate, all described dipeptide stereoisomers were effectively recovered from the C18 cartridge. Consequently, at 0.1 mL/min, a portion of DL-, DD-, LD-, and LL-enantiomers remained bound to the C18 material in the SPE cartridge. Elution was ultimately achieved through the gradual disintegration or desorption of these bonds at lower flow rates. In contrast, higher flow rates led to lower recovery percentages, as they did not allow sufficient time for the C18 material in the SPE cartridge to break (desorb) the DL-, DD-, LD-, and LL- enantiomer bonds.

***Enantioselective Binding of Dipeptide Stereoisomers to Human Plasma Proteins:*** Various levels of interaction between the enantiomers of the dipeptide stereoisomers and plasma proteins were observed throughout the solid-phase extraction and chromatographic analysis. These interactions showed no specific pattern, likely due to the stereo-specific properties of plasma proteins, which provide an enantioselective environment due to their surface loops. The dipeptide stereoisomers bind stereoselectively to the protein surfaces, resulting in distinct enantiomeric interactions. Furthermore, pH influenced these enantiomer-protein interactions, as differing functional groups on enantiomers and plasma proteins affect binding capacities at various pH levels. The recovery values for C18 cartridge-bound enantiomers at pH levels of 5.0, 7.0, 9.0, and 10.0 were as follows: DD- (18.60%, 23.31%, 26.89%, 24.61%), DL- (14.28%, 17.31%, 36.61%, 14.11%), and LL- (21.51%, 18.60%, 15.90%, 14.20%). These findings highlight pH 9.0 as the optimal condition for maximum recovery. Accordingly, percentage recoveries at pH 9.0 identified enantiomer binding in human plasma, with bindings recorded at 56.79% (DD-), 63.39% (DL-), 73.11% (LD-), and 84.11% (LL-), as shown in Table S2 [18].

***High-Performance Liquid Chromatography:*** Chromatographic analysis revealed a single peak for chromatographically pure leucine-tryptophan, with distinct signals for each of the four possible enantiomers. Retention factor (k), resolution (Rs), and separation (Sep) factors were calculated for the resolved leucine-tryptophan stereoisomers. Four unique retention factor values were recorded for each separable stereoisomer. These parameter values are listed in Table S3. Close examination of the chromatographic parameters and chromatograms confirmed the resolution for all four stereoisomers. Simulation analysis facilitated the identification of isolated stereoisomers, following the extraction order of LL>DD>DL>LD. In accordance with standard FDA protocol (2000), retention times and peak areas were used for quantitative and qualitative analysis of the dipeptide stereoisomers [19].

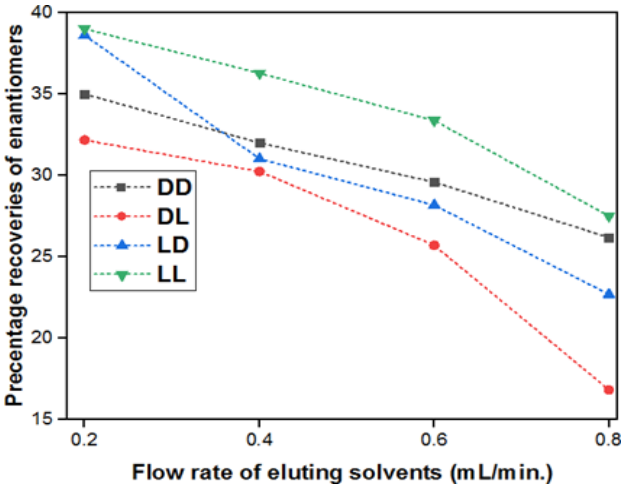


**Figure 4.** The effect of the elution solvent (methanol with 0.10% TFA) on the recovery percentages of dipeptide enantiomers in the drug samples.

The accuracy of the chromatographic process was validated using several metrics, including linearity, limit of quantification (LOQ, S/N 1:10), and limit of detection (LOD, S/N 1:5). For the HPLC suitability test, six separate injections (n = 6) of a reference solution were utilized. Additionally, we calculated the relative standard deviation (RSD) for retention times, the tailing factor, and the RSD of peak area. The RSDs for peak area and retention time were less than 2% and 1%, respectively.

***Optimization of the HPLC Method:*** Various mobile phase compositions were tested to optimize HPLC experimental conditions. Ratios of acetonitrile to ammonium acetate included 10:90, 20:80, 30:70, 60:40, and 50:50. Additional combinations of methanol, acetonitrile, and ammonium acetate were tested, such as 50:10:40, 40:30:30, 50:10:40, and 50:20:30, with ammonium acetate concentrations ranging from 5 to 50 mM. Solvent flow rates varied between 0.2 and 2.0 mL/min. Broad peaks were observed at 0.20 and 0.50

mL/min flow rates, while well-resolved peaks were achieved at 1.0 and 1.5 mL/min. At a 2.0 mL/min flow rate, no separation was observed. Detection covered a broad wavelength range from 210 to 350 nm.

The performance of detection and quantification limits was shown to be suboptimal at wavelengths above 20 nm. Injection volumes varied significantly, ranging from 5 µL to 25 µL, and optimization was conducted at temperatures between 10 and 50°C. This study details how comprehensive analyses were used to determine optimal parameters for HPLC.

*System Suitability Test.* The HPLC system suitability test was conducted using six injections of a standard solution (n = 6). The tailing factors, peak areas, and retention time deviations were calculated, with RSD values for retention time and peak area being 1% and 2%, respectively. The tailing factors for DL-tyrosine -DL-alanine stereoisomers (DD, LD, DL, and LL) were recorded as 1, 1, 1, and 1.11, respectively.

*Linearity.* To assess linearity, a graph was generated to illustrate changes in peak area in response to dipeptide concentration. Slope, y-intercept, and determination coefficients (r, $r^2$) were calculated. The dipeptides demonstrated strong linearity in the 50–400 µg/mL concentration range.

*Limits of Detection and Quantification.* The LOQ and LOD were identified with signal-to-noise ratios of 10 and 5, respectively. LOD was approximately five times lower than LOQ. Analytical sensitivities for detecting stereoisomers LL-, DD-, DL-, and LD- were found to be 2.3, 1, 2, and 2.7 µg/mL, respectively, with quantification limits of 10.8 µg/mL for DL-, 14.0 µg/mL for LD-, 5.8 µg/mL for DD-, and 11.6 µg/mL for LL-, demonstrating sufficient sensitivity for quantification.

*Precision.* The method's precision was evaluated through intraday and interday tests. Intraday assessments of DD-, DL-, LD-, and LL- stereoisomers were conducted at concentrations of 0.08, 0.10, and 0.12 mg. Results at 0.1 mg were 100.1, 100.6, 100.3, and 99.9, while 0.12 mg yielded 101.4, 101.2, 100.2, and 101.0 values, respectively. Interday tests over two days showed accuracy between 99.4–100.9%, 99.2–99.9%, 99.9–100.6%, and 98.2–99.5% for LL-, DD-, DL-, and LD-, respectively, with RSDs of 0.62, 0.25, 0.26, and 0.54.

*Accuracy.* The HPLC method's accuracy was confirmed using three concentrations (0.1, 0.05, and 0.025 mg/mL) across six replicates (n = 6). Peak area interpolation indicated accuracy levels with confidence intervals and RSDs between 98.3–98.8% and 0.54–0.70%, respectively.

*Robustness.* The robustness of HPLC was validated by altering parameters such as temperature and flow rate while keeping other conditions stable. Chromatograms showed variations in peak area and retention time of less than 1.5%, affirming method robustness.

**Mechanism of separation**

The separation mechanism was elucidated by combining chromatographic elution data with docking results. The AmyCoat-RP column, based on derivatized amylose polysaccharides, features grooves that facilitate dipeptide stereoisomer separation, as illustrated in Figure 4.7 Streel et al. (2002). Amylose, with a higher helicity than other polysaccharides, enables effective stereoisomer separation through interactions involving van der Waals forces, hydrogen bonding, and steric effects. Due to the aromatic nature of the dipeptides, these forces support optimal stereoisomer separation.

Chromatographic analysis revealed varying interactions between enantiomers and plasma proteins during solid-phase extraction. The interactions did not follow a specific pattern, likely due to the stereospecific environment provided by chiral loops on the protein surfaces. This structural fit leads to distinct enantiomeric interactions, which are also influenced by pH. For example, R- and S-enantiomer recoveries from a C18 cartridge were 25.18% and 20.67% at pH 5, 32.59% and 36.40% at pH 7, 39% and 42.10% at pH 9, and 35% and 40.10% at pH 10, with pH 9.0 showing optimal recovery. Similar patterns were observed for oxybutynin, cetirizine, pheniramine, and brinzolamide, with the highest enantiomer recoveries consistently achieved at pH 9.0.

The binding affinities of cetirizine, pheniramine, brinzolamide, and oxybutynin enantiomers to plasma at pH 9.0 showed distinct differences: 11R and 7.91S for cetirizine, 36.83R and 36.11S for pheniramine, 20.98R and 26.09S for brinzolamide, and 42.80R and 36.60S for oxybutynin.

## Conclusion

The study presents the development of SPE and HPLC methods that are cost-effective, accurate, reproducible, and environmentally friendly for the separation of LD, DL, LL, and DD enantiomers in human plasma, with HPLC being utilized in these procedures. Among the enantiomers, the LL-enantiomer showed the highest plasma binding percentage (84.11%) at a pH of 9.0, followed by the LD-enantiomer (73.11%), DL-enantiomer (63.39%), and DD-enantiomer (56.79%). Complete resolution was achieved, as separation and resolution factors exceeded 1. The LOD values for DD-, DL-, LD-, and LL-enantiomers were measured within a range of 1 to 2.7 µg/mL, while the LOQ values for the same enantiomers ranged from 5 to 10 µg/mL. The experimental results showed strong concordance with model predictions. When applied to plasma samples from real-world scenarios, the methods produced favorable results, suggesting that they can effectively monitor the chiral composition of dipeptide stereoisomers in patients. These findings demonstrate a promising approach for clinical applications and further pharmacological research.

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