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ESTIMATION OF SOME COMPOUNDS IN BIOLOGICAL AND PHARMACEUTICAL SAMPLES USING HPLC TECHNOLOGY

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Abstract

The enantiomers of Leucine and Tryptophan in human plasma samples were monitored using the validated SPE and HPLC methods. TSPE and Chiral HPLC procedures were established for the chiral separations of DD-, DL-, LD-, and LL-enantiomers in human plasma, and they are environmentally friendly, repeatable, accurate, cheap, and effective. At a neutral pH (i.e. 9.0), the highest percentage binding of the DD-, DL-, LD-, and LL- enantiomers to plasma was 56.79 (DD-), 63.39 (DL-), 73.11 (LD-), and 84.11 (LL-), respectively. Both separation and resolution factors were more than 1.0, showing chiral resolution above the norm. These 115 LOQ values varied from 5.0 to 10.0 g mL-1, whereas the LOD values for DD-, DL-, LD-, and LL- enantiomers were 1.0 to 2.7 g mL-1. Good agreement was found between the experimental findings and the modeling results. The procedures described here were successfully used on plasma samples taken from the real world. As a result, the disclosed approach may be used to any patient to track the chiral ratio of these dipeptide stereomers. Additionally, the modeling approach is helpful for determining the structure of the isolated enantiomers.

single paper from the lab as of late. High-performance liquid chromatography (HPLC), say Aboul-Enein and Ali, is the optimum method for this purpose because of its dependability, simplicity, efficiency, and selectivity.

The four leucine-tryptophan stereoisomers are shown in Figure 1. Separation efforts have been made between them. The HPLC technique used an AmyCoat-RP column, which contains a stationary phase made of amylose polysaccharides. The results of the investigation are presented here. D-leucine, L-leucine, D-tryptophan, and L-tryptophan have all been iden- tified as possible stereoisomers of leucine. The enantiomer elution order and recognition procedure were modeled nu- merically. Both the theoretical foundation and experimental evidence are provided here.

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I. INTRODUCTION

It is well known that one stereoisomer may have pharma- cological action whereas the other may be inert, poisonous, or used just as a filler 1]. Academics, researchers, medical profes- sionals, and government agencies all worry about the presence of optically active stereoisomers in medications with major medical applications. Different stereoisomers have different biological functions because they undergo different metabolic processes, are excreted in different ways, are cleared from the body at different rates, and are distributed in different ways [1]. Certain principles established by the Racemic pharmaceuticals destined for commercial distribution are regulated by table 3he . Dipeptides are critical in a wide variety of activities, including protein synthesis, neurotransmission, fertilisation, key functions of pathogenic microorganisms, and regulation of inflammatory processes. This has led to the usage of dipep- tides in the search for novel drugs and the identification of biomarkers. [2] . It is well established that dipeptides are com- pounds with two asymmetric centres and four stereoisomers, all of which include amino acids in the DL-configuration. Therefore, it is possible for a single dipeptide to manifest in the human body as if it were four different dipeptides. Important roles in protein and nucleic acid recognition are played by dipeptides containing aromatic amino acids. Several studies on the separation of derivatized dipeptides are found upon a thorough literature review. This procedure is notorious for being time-consuming, difficult, and solvent-intensive, all of which contribute to its hefty price tag [3]. Separation of aromatic underivatized dipeptides has only resulted in a

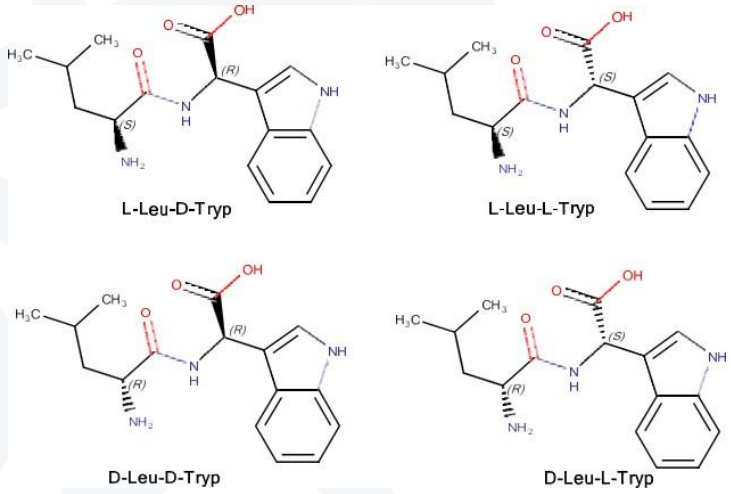


Fig. 1. Four Stereomers of Leucine-Tryptophan

II. LITERATURE REVIEW

A comprehensive and meticulous review of the literatu- rAnalytical and Chemical Abstracts, PubMed, Science Di- rect, SciFinder, and a number of publications published by the American Chemical Society, Royal Society of Chemists, Springer link, and Wiley Online Library were used in the research that was carried out. Based on the results of the examination of the relevant literature, it seems that there are only a select few analytical methods available for the separa- tion of racemic substances that include numerous centres. The literature section is broken down into two basic parts, namely:

(i) the method of sample preparation by solid-phase extraction, and (ii) the analysis of medicines using high-performance liquid chromatography. Both of these parts are discussed in the following paragraphs [7].

In order to determine the enantiomeric makeup of DL- alanine-DL-phenylalanine complexes, Nora and her colleagues (2020) used an innovative method. The mobile phase that was used was a combination that had a flow rate of 0.8 mL per minute and included MeOH, CH_3COONH_4 (10 mM), and HCOOH in the proportions of 70: 30: 0.05 (v/v). In order to promote enantioseparation by creating a environment, the stationary phase was an AmyCoat-RP column, which was used in the separation process. came up with a fresh method in order to determine the enantiomeric makeup of two different dipeptides. A quality was present in the mobile phase that was used. In order to promote enantioseparation by creating a environment, the stationary phase was an AmyCoat-RP column, which was used in the separation process. In addition, a simulation study was carried out in order to verify the reliability of the results obtained from the chromatographic analysis. After that, the same group of researchers from 2015 came up with an innovative method for determining the enantiomers of two more dipeptides, namely DL-alanine-DL- tyrosine. For the DL-alanine-DL-tyrosine analysis, the mobile phase that was used was composed of ACN:CH_3COONH_4 (10 mM, pH 6.0) [50:50, v/v], and for the same purpose, the mobile phase was composed of ACN: MeOH:CH_3COONH_4 (10 mM; pH:4.5) [8]. With a flow rate of 0.8 mL/minute, a concentration of 50% leucine and 20% phenylalanine in a DL form was utilised. In order to promote enantioseparation by creating a environment, the stationary phase was an AmyCoat- RP column, which was used in the separation process. Ali and colleagues (2018) proposed an innovative method for de- termining whether enantiomers of DL-alanine-DL-tryptophan exist. The mobile phase that was used was a combination that included methanol, ammonium acetate (10 mM), and methyl cyanide (50:40:10), and it was provided at a flow rate of 0.8 mL

per minute. In order to promote enantioseparation by cre- ating a environment, the stationary phase was an AmyCoat-RP column, which was used in the separation process. A unique strategy for determining the enantiomers of amino acids has been developed by Aydogan and colleagues (2018) [9]. The mobile phase that was used had a flow rate of 0.8 mL/minute and was composed of 85% acetonitrile, 10% methanol, and 5% water with 0.1% volume/volume trifluoroacetic acid. It was provided in this configuration. The porous-layer stationary phase was used as the stationary phase, which contributed to the creation of a environment, which in turn enabled the enantioseparation. A validation study on a new method for detecting the enantiomers of carnosine was carried out in 2019 by Pucciarini and colleagues together with other researchers. It made use of the mobile phase that was used [10].

At a flow rate of one millilitre per minute, a solution that was used was one that was made up of water containing 0.1% formic acid and 20-40% ensures. A Chirobiotic T column was used in the research as the stationary phase in order to promote enantioseparation by producing a environment [11].

A. Solid Phase Extraction

Undoubtedly, there exist numerous techniques for sample preparation, however, solid phase extraction holds significant prominence among them. Initially, a solvent capable of dis- solving all enantiomers was prepared through the combination of methanol and water in a 50:50 (v/v) ratio. The concen- trations of all enantiomers subsequent to dissolution in the solvent were recorded as 1 mg/mL. Subsequently, a volume of 1 milliliter was extracted from the solution of each enantiomer and combined with 5.0 milliliters of recently frozen human plasma [12]. One milliliter of enantiomeric solutions was mixed with plasma and subjected to vortexing for a duration of 2-3 minutes, followed by a resting period of 25-35 minutes. In each instance, a volume of 15 mL of acetone was combined with the sample, subjected to vortexing, and subsequently incubated for a duration of 30 minutes to facilitate deproteina- tion. Subsequently, the samples underwent centrifugation for a duration of 10 minutes at a specific velocity of 10,000 revo- lutions per minute to facilitate the isolation of the supernatant. Following the process of evaporation and subsequent drying of the supernatant, the resulting residue was reconstituted in a 10 mL solution of phosphate buffer with a concentration of 5mM and a pH of 7. The Sep-Pac C18 cartridges underwent preconditioning using 1.5-2.5 mL of MeOH and 5.0 mL of Millipore H2O. Enantiomers were introduced into a 10 mL phosphate buffer solution with a concentration of 5mM and a pH of 7. The solution was then passed through cartridges at a flow rate of 0.2 mL/minute, followed by a washing solvent consisting

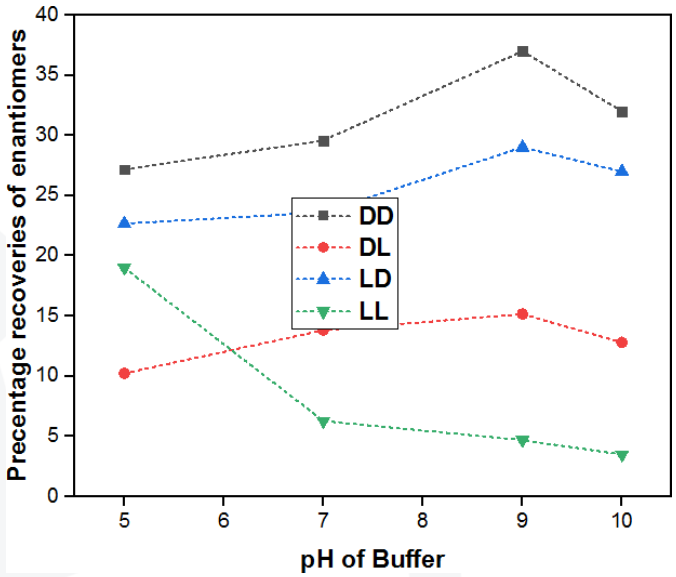
of 2mL Millipore H2O at the same flow rate. Subsequently, heated air was utilized to desiccate the cartridges. The reported drugs were eluted using a flow rate of 0.5 mL/minute with a solvent consisting of 10 mL of methanol containing 0.1% trifluoroacetic acid. The drug solutions that were reported were subjected to elution and subsequently evaporated to increase their concentration, resulting in a final concentration of amount/0.5 mL. Upon completion of this task, a -HPLC analysis was conducted. The use of illicit substances was reported. In order to enhance the conditions of solid phase extraction, multiple experimental factors were optimized. To assess the efficacy of the devised approach, the percentage recuperation values of the enantiomers of the aforementioned pair of -centered pharmaceuticals were computed. At a pH of 9.0, the Leucine-Tryptophan exhibited percentage recoveries of 43.21 (DD-), 36.61 (DL-), 26.89 (LD-), and 15.90 (LL-). In contrast, the plasma binding percentage of the enantiomers of Leucine-Tryptophan reached a maximum of 56.79% (DD-), 63.39% (DL-), 73.11% (LD-), and 84.11% (LL-) at a pH of 9.0. The determination of the drug-protein binding percentage in human plasma necessitated the inclusion of potential investigative errors.

In order to attain optimal recoveries, the eluting sol- vents mentioned above were supplemented with 0.1 to 1% CH3COOH or CF3COOH. The investigative conditions that were most appropriate have been duly observed and docu- mented in this report. The drug's enantiomers were extracted from a C 18 cartridge with the aid of phosphate buffer (50.0 mM, pH 5.0, 7.0, 9.0, and 10.0) at a flow rate of 0.1 mL min- 1, resulting in the highest percentage recoveries. The optimal solvent for eluting all enantiomers of the drug in question was found to be methanol (MeOH) with a trifluoroacetic acid concentration of 0.1% and a flow rate of 0.1 mL/min. Table 1 displays the proportions of bindings and recoveries observed for the drug under investigation when subjected to human plasma on a C18 cartridge [11].

TABLE I PERCENTAGE RECOVERIES ANDBINDING AFFINITIES OF DIPEPTIDES ENANTIOMERS WITH HUMAN PLASMA

		% Recovery by SPE with Plasma Protein on C18	
S.NO.	Enantiomceorlsumn		
		Percentage recoveries	Human plasma bindings
1	DD	43.21	56.79
2	DL	36.61	63.39
3	LD	26.89	73.11
4	LL	15.90	84.11

conducted at pH levels of 5, 7, 9, and 10. The outcomes of the aforementioned experiments pertaining to DD-, DL-, LD-, and LL- have been illustrated in Figure 4.2. The percentage recoveries of DD-, DL-, and LD-enantiomers of a dipeptide were observed to be low at pH 5.0, with the exception of the LL-stereomer. At a pH of 7.0, the recoveries of DD-, DL-, and LD- increased, while that of LL- decreased. At a pH of 9.0, the recoveries of DD- and LD- exhibited an increase, whereas a decrease was observed in the case of DL- and LL-. The observed percentage recoveries of the dipeptide stereomers decreased at a high pH of 10.0. The observed phenomenon could potentially be attributed to a reduced level of desorption of the stereoisomers of the dipeptide in question under conditions of elevated pH. Thus, it can be inferred that a pH of 9.0 yielded the highest recoveries for the dipeptide stereomers [13].



1) Optimization of SPE: There were many conditions whose change was involved in the optimization of SPE such as pHs and concentration of phosphate buffer, flow rates of eluting solvents and phosphate buffer. Besides, many solvents such as methanol, C_2H_5OH, CH_3COCH_3 and ethyl acetate, were used in the elution of the sample. The effects of various parameters on SPE are discussed below [12].

III. EFFECT OF PHOSPHATE BUFFER PH

The pH of the buffer plays a crucial role in achieving the highest possible percentage recoveries of dipeptide stereomers onto the sorbent following deproteinization with acetone. Therefore, the pH value plays a crucial role in the desorption process of dipeptide stereoisomers from the C18 cartridge of solid-phase extraction (SPE).

Various pH levels resulted in varying percentage recoveries for a specific analyte. A varia- tion in retention was observed at varying pH levels for the C18 sorbent. The C18 sorbent is composed of silanols that are not bound, resulting in acidic regions on the surface of the sorbent. These regions facilitate the binding of dipeptide stereomers through hydrogen bonding and cation-exchange mechanisms by interacting with the imine (–NH-) group. The ionization of dipeptide stereoisomers' enantiomers is contingent upon the pH level of the buffer. Under acidic conditions, dipeptides consisting of stereoisomers exhibit a positive charge as a result of the amino and amide groups present in each enantiomer. The distinct arrangements of stereoisomers in these dipeptides are accountable for their varying rates of recuperation. Furthermore, the preceding discourse has indicated that the varying percentage recoveries observed at distinct buffer pH levels can be attributed to the existence of diverse hydrogen bonding interactions between the molecules of dipeptide stereoisomers and the sorbent. The percentage recoveries are significantly in- fluenced by the ionization capabilities of dipeptide stereomers. In order to determine the impact or influence. The impact of pH on the percentage recoveries of DD-, DL-, LD-, and LL- was investigated through solid phase extraction procedures

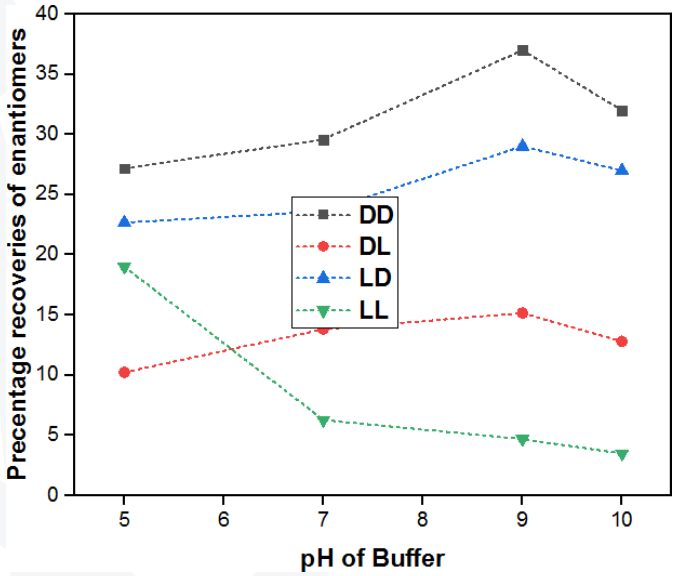


Fig. 2. Effect of pH of buffer on the percentage recoveries of dipeptide stereomers.

A. Effect of Phosphate Buffer Flow Rates

Various eluting solvents were also used to fine-tune the developed SPE technique. If you want to get the highest possible recoveries of DD-, DL-, LD-, and LL- enantiomers, you need to use the right solvent. For efficient elimination of interference chemicals and optimal analyte recovery from human plasma samples, the right eluting solvent must be chosen.

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The highest % recoveries of the DD-, DL-, LD-, and LL- enantiomers were achieved by using four differ- ent solvents: acetone, methanol, ethanol, and ethyl acetate. Furthermore, acetic acid and trifluoroacetic acid were added to the aforementioned solvent at varying concentrations to achieve the highest recoveries of these dipeptide stereomers. Plotting the percentage recoveries of DD-, DL-, LD-, and LL-enantiomers (Fig.4) makes it evident that the highest and lowest recoveries of DD-, DL-, LD-, and LL- enantiomers were achieved using methanol containing 0.1% trifluoroacetic

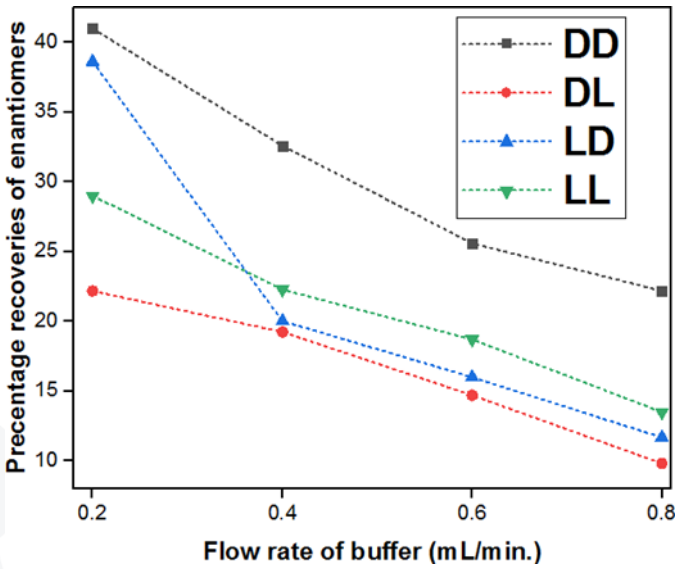

Fig. 3. Effect of buffer flow rate on the percentage recoveries of enantiomers of a dipeptide. Effect of Other Eluting Solvents

acid (TFA) and acetone, respectively. Methanol (0.1% TFA, v/v) > acetone > ethyl acetate > methanol for the percent- age recoveries of these dipeptide stereomers. Since methanol (0.1% TFA, v/v) resulted in the highest percentage recoveries of the reported dipeptide stereomers from C18 cartridge, it was determined to be the best solvent for the elution. This is due to the fact that polarity and dielectric constant vary greatly amongst various solvents. These factors accounted for the varying degrees of recovery shown by each. Both methanol and ethyl acetate are capable of cleaving C18 bonds with DD-, DL-, LD-, and LL- enantiomers due to their high polarity and low dielectric constant. As a consequence, the settings have led to high recoveries of the investigated dipeptide stereomers as a percentage. In addition, methanol with 0.1% TFA produced the greatest results. Using methanol with 0.1% TFA resulted in the highest percentage recoveries of DD-, DL-, LD-, and LL- enantiomers, as shown above. Methanol's strong polarity allowed it to dissolve bindings between the analytes and the C18 phase (0.1% TFA, v/v) [15]

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B. Effect of Eluting Solvent Flow Rates

The percentage recoveries of DD-, DL-, LD-, and LL- enantiomers from human plasma were also impacted by the flow rate of the eluting solvent (methanol containing 0.1% TFA, v/v) through the C18 cartridge. Both enantiomers of these dipeptide stereomers were recovered at high and low percentages depending on the flow rate. Maximum recoveries of DD-, DL-, LD-, and LL- enantiomers from human plasma have been sought by adjusting the elution solvent flow rate through the C18 cartridge. Low eluting solvent flow rates were shown to result in considerable recoveries, as compared to high flow rates. With this information in mind, we experimented with four different flow rates (0.2, 0.4, 0.6, and 0.4 Ml min-1)

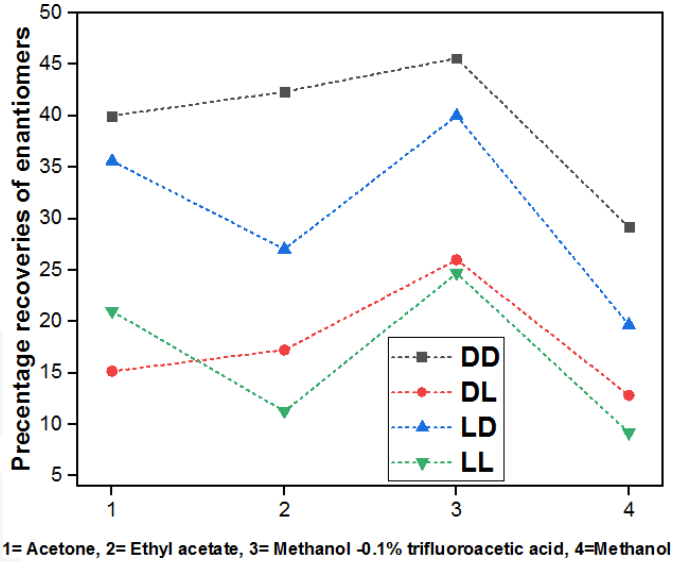

Fig. 4. Effect of other solvents on percentage recoveries of enantiomers of the dipeptide.

To see which would result in the highest percentage recoveries of dipeptide stereomers. The experimental findings are shown in (Fig.5), where the greatest and lowest % recoveries of the dipeptide stereomers in question at the 0.1 mL/min flow rate are presented [17].,

respectively. Recoveries for DD-, DL-, LD-, and LL- enan- tiomers increased in order from 0.2 to 0.4 to 0.6 to 0.8 millilitres.All of the described dipeptide stereomers were suc- cessfully recovered from a C18 cartridge with a flow rate of 0.2 mL. Because of this, 0.1 mL of DD-, DL-, LD-, and L-L enantiomers remain attached to the C18 cartridge material used in SPE. It was the equilibrium dissolution of these bonds (i.e. desorption) that ultimately led to the elution of the dipeptide stereomers. Slowly breaking these compounds at low flow rates allowed for their entire elution as DD-, DL-, LD-, and LL- enantiomers. However, high flow rates did not allow enough time

for the C18 material of the SPE cartridge to break (desorbs) the bonds of the DD-, D-L-, LD-, and LL- enantiomers, leading to low percentage recoveries of these isomers.

1) Enantioselective Bindings of Dipeptide Stereomers with Human Plasma Proteins: During solid-phase extraction and chromatographic analysis, it was observed that enantiomers of the reported dipeptide stereomers interacted with plasma proteins at different extents. It was observed that enantiomers interacted with plasma proteins at different magnitudes. There was no specific trend of interactions of these enantiomers. The different extents of interactions of the enantiomers of these dipeptide stereomers were because of the stereo specificities of plasma proteins. The plasma proteins contain surfaces including loops, which provide an enantioselective environ- ment to the enantiomers of the reported dipeptide stereomers. Therefore, the enantiomers of these dipeptide stereomers fit

TABLE II PERCENTAGE BINDINGS OF DD, DL, LD AND LL WITH HUMAN PLASMA PROTEIN.

S.No.	pH of 0.1%TFA in MeOH	DD % Recovery	% Binding with plasma protein	DL % Recovery	% Binding with plasma protein	LD % Recovery	% Binding with plasma protein	LL % Recovery	% Binding with plasma protein
1	5	21.78	78.22	14.28	85.72	18.6	81.4	21.51	78.49
2	7	37.51	62.49	17.31	82.69	23.31	76.69	18.6	81.4
3	9	43.21	56.79	36.61	63.39	26.89	73.11	15.90	84.11
4	10	41.21	58.79	14.11	85.89	24.61	75.39	14.19	85.81

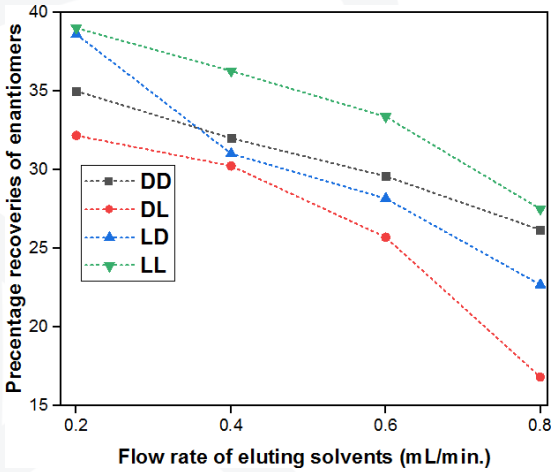

Fig. 5. Effect of eluting solvents (methanol containing 0.1%TFA) on the percentage recoveries of enantiomers of a dipeptide of drugs.

stereo-specifically onto the surface of the proteins, leading to the different enantiomeric interactions. Furthermore, it was observed that enantiomeric interaction was pH control. This is because enantiomers and the plasma proteins have different functional groups, which have different binding capacities at different pHs. C18 cartridge enantiomer recoveries for DD- were 21.78, 37.51, 43.21, and 41.21 at 5.0, 7.0, 9.0, and 10.0 pHs; for DL-, they were 14.28, 17.31, 36.61, and 14.11; for LD-, they were 18.60, 23.31, 26.89, and 24.61; and for LL-, they were 21.51,18.60, 15.90, and 14.2. At a pH of 9.0, these numbers clearly demonstrated optimal recovery. Therefore, percentage recoveries at pH 9.0 were used to determine enantiomer binders in human plasma. Therefore, the bindings of the DD-, DL-, LD-, and LL- enantiomers in human plasma at pH 9.0 were 56.79 (DD-), 63.39 (DL-), 73.11 (LD-), and 84.11 (LL-), respectively. Table2 displays these values. [18].

2)High-Performance Liquid Chromatography: . Chromatographically pure Leucine-Tryptophan was revealed by a single peak in an a analysis. Each of the four potential enantiomers was represented by a separate signal in the study. Resolution (Rs), retention (k), and separation (Sep) factors of high-performance liquid chromatography were calculated for the resolved Leucine-Tryptophan stereoisomers. There were four different retention factor values for the four stereomers that might be separated. Each isomer's parameter values are tabulated in Table.3. shown in Fig. 6. Resolution of all four stereoisomers of the reported compound was val- idated by carefully examining the chromatographic parameters and chromatograms. The simulation investigation pinpointed the isolated stereoisomers. Stereomers were eluted from the system as follows: LL > DD > DL > LD. Retention periods and peak areas of the reported dipeptide stereomers were used for the qualitative and quantitative analysis as per the standard protocol (FDA, 2000) [19].

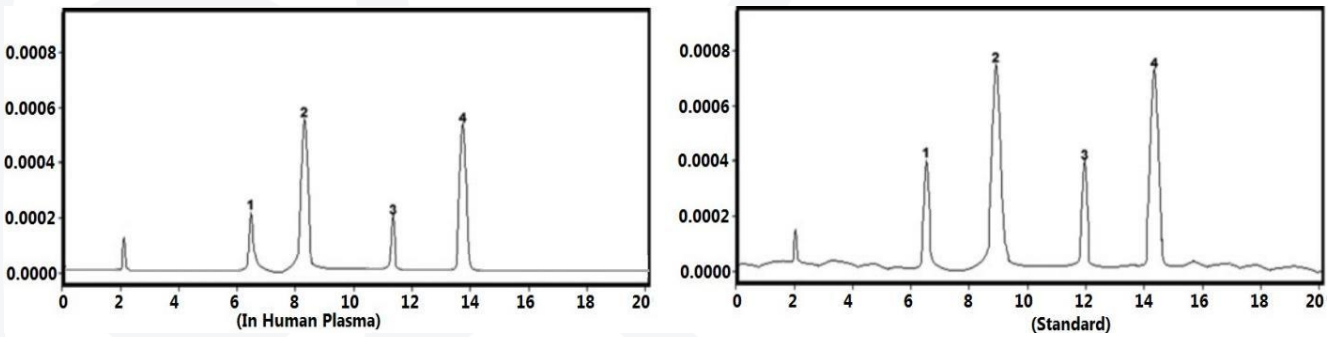

Fig. 6. HPLC chromatograms of Enantiomers of DL-Leucine-DL- Trypto- phan dipeptide. (1): LL- (2): DD- (3): DL- and (4): LD- stereomers in (a): human plasma sample and (b): standard solution.

Multiple parameters, including linearity, the limit of detec- tion (LOD, S/N 1:5), and the limit of quantification (LOQ, S/N 1:10), were used to verify the accuracy of the chromatographic procedure. Six independent injections (n = 6) of a reference solution were used to conduct the HPLC suitability test. We also determined the tailing factor, the relative standard deviation (RSD) of peak area, and the RSD of retention times. The relative standard deviations (RSDs) for peak area and retention duration were both below 2% and 1%, respectively.

3) HPLC method optimization: Various mobile phases were utilised to optimise the experimental conditions of HPLC. Several ratios of ammonium acetate and acetonitrile were utilised, comprising 10:90, 20:80, 30:70, 60:40, and 50:50. The study utilised different proportions of ammonium ac- etate, methanol, and acetonitrile, including but not limited to 50:10:40, 40:30:30, 50:10:40, and 50:20:30. The ammonium acetate concentrations exhibited a range of 5 to 50 millimolar. The mobile phase flow rates were varied from 0.2 mL/min to 2.0 mL/min. At 0.2 and 0.5 mL/min, considerable breadth peaks were seen. In contrast, results show that peaks were somewhat resolved at flow rates of 1.0 and 1.5 mL/min.In addition, at a flow rate of 2.0 mL/min, no segregation was observed. Detection was possible throughout a broad spectrum,

TABLE III THE RETENTION (K), SEPARATION (α) AND RESOLUTION (RS) FACTORS FOR DD-, DL-, LD- AND LL- DIPEPTIDE STEREOMERS ON AMYCOAT-RP COLUMNS.

Retention factors (k)				Separation factors (α)			Resolution factors (Rs)		
LL (k1)	DD (k2)	DL (k3)	LD (k4)	α 1	α 2	α 3	Rs1	Rs2	Rs3
2.25	3.60	5.00	6.50	1.60	1.39	1.30	7.76	8.05	7.19
Number of theoretical plates				Tailing factors					
LL	DD	DL	LD	LL	DD	DL	LD		
1156	2540	5112	9216	1.00	1.21	1.15	1.11		

from 210 to 350 nanometers in wavelength. When tested at wavelengths exceeding 20 nm, detection and quantification limits were shown to perform poorly. There was a wide range, from 5 L to 25 L, for the injection quantities. Temperatures between 10 and 50 degrees Celsius were maintained in order to complete the optimisation procedure. This article details how the optimal settings for high-performance liquid chro- matography (HPLC) were determined via a series of in-depth analyses.

C. System suitability test

Six independent injections of the standard solution were used to conduct the HPLC suitability test (n = 6). The standard deviations of the peak areas and retention periods as well as the tailing factor were computed. The relative standard deviation (RSD) was just 1% for retention time and 2% for peak area. The tailing factors for the DL-alanine-DL-tyrosine LL-, DD-, DL-, and LD- stereomers were 1, 1, 1, and 1.11, respectively.

D. Linearity

To determine the system's linearity, a graph was drawn to show how the peak area changed when the concentration of a dipeptide was changed. Coefficients of determination (r, r2, and the slope and y-intercepts) were calculated. Within the concentration range of 50.0-400 g/mL, the dipeptides showed excellent linearity. T

E. Detection and quantitation limits

A signal-to-noise ratio of 10 was used to determine both the LOD and LOQ, with the LOD being 5 times less stringent than the LOQ. The research indicates that 2.3, 1.0, 2.0, and

2.7 g/mL are the analytical sensitivities for identifying LL-, DD-, DL-, and LD-stereomers, respectively. The research established quantification limits of 11.6 g/mL for the LL stereoisomer, 5.8 g/mL for the DD stereoisomer, 10.8 g/mL for the DL stereoisomer, and 14.0 g/mL for the LD stereoisomer. The acquired data was sensitive enough for detection and quantitative enough for quantification.

F. Precision

The HPLC technique's accuracy was assessed by comparing results from different days. Accuracy throughout the course of a day was evaluated intraday. Contrast evaluation of inter-day accuracy was performed on a different date. The stereoisomers LL, DD, DL, and LD were tested in the intraday precision assay. The researchers looked at outcomes at 0.08, 0.10, and 0.12 mg. A dosage of 0.08 mg yielded values of 98.9 for the LL-, 99.0 for the DD-, 99.7 for the DL-, and 99.5 for the LD-stereoisomers. The results for 0.1 mg were 100.1, 100.6, 100.3, and 99.9, whereas the results for 0.12 mg were 101.4, 101.2, 100.2, and 101.0. Six samples were tested over the course of two days to determine the percentage of accuracy for LL-, DD-, DL-, and LD-stereomers. The observed values fell within the ranges of 99.4-100.9, 99.2-99.9, 99.9-100.6, and 98.2-99.5. Precision tests performed on the same day yielded

% RSD values of 0.62 for the LL-stereomer, 0.25 for the DD- stereomer, 0.26 for the DL-stereomer, and 0.54 for the LD- stereomer.

G. Accuracy

The precision of the HPLC technique was verified by employing varying concentrations of the stereoisomers as re- ported. Three concentrations were

employed, specifically 0.1, 0.05, and 0.025 mg mL-1. The experiment was conducted with six replicates (n=6) of chromatographic runs. The determina- tion of accuracies was carried out through the interpolation of peak areas of six replicates of these stereomers.The study reports that the RSD and confidence levels fell within the range of 0.54-0.70% and 98.3-98.8%, respectively.

H. Robustness

The study evaluated the resilience of high-performance liquid chromatography (HPLC) by systematically altering in- dividual parameters while maintaining the stability of the remaining factors, and subsequently monitoring the resultant chromatograms for any potential impact on the efficacy of HPLC. The flow rate and temperature were altered. The HPLC method was confirmed to be robust as the changes in retention time (Rt) and peak area were found to be less than 1.5.

1) Mechanism of Separation: By combining the docking results and the sequence of chromatographic elution, the mechanism of separation was clarified. As can be seen in Figure 4.7, the base of the AmyCoat-RP column is a deriva- tized amylose polysaccharide. Amylose, unlike cellulose and other polysaccharides, displays a greater degree of helicity, as shown by Streel et al. (2002). grooves on an AmyCoat-RP column were shown to be responsible for the successful separation of dipeptide stereoisomers. The stereoisomers of the dipeptides display a wide range of stereo-specificity. Multiple interactions control the stereoisomer configuration. Hydrogen bonding, van der Waal's forces, and steric effects are also among the most important types of interactions (ápka and Xu, 2001; Miller-Stein and Fernandez-Metzler, 2002). This dipeptide's aromatic properties make it especially well suited for - interactions with selectors. This means that the forces in question are accountable for the stereoisomer separation reaching its ideal state. However, it is important to remember that the aforementioned interactions also contribute to the stereoisomer separation process.

It was found by chromatographic research that the enan- tiomers of the described medicines interacted with plasma proteins to varying degrees during solid phase extraction. It was found that the interactions between R- and S-enantiomers and plasma proteins varied in intensity. The enantiomers interacted in no discernible pattern. The stereo specificities of plasma proteins accounted for the varying degrees to which the enantiomers of these medicines interacted. Chiral surfaces, such as chiral loops, present on plasma proteins provide an enantioselective environment for the enantiomers of the described medicines. Different enantiomeric interactions result from the stereospecific conformational fit of the enantiomers of these medicines to

the surfaces of the proteins. It was also shown that the pH had an effect on the enantiomeric interaction. Reasons include 0 to 5 to 10 to 15 to 20 to 25
to 30 to 35 to 40 to 45 Percentage Enantiomer (S) recovery Eluting Solvent Flow Rate (mL/min) Oxybutynin Pheniramine Cetirizine The binding capabilities of enantiomers and plasma proteins are pH-dependent, since their functional groups are distinct. 25.18(R) and 20.67(S) enantiomers were recovered at a pH of 5.0, 32.59(R) and 36.40(S) at a pH of 7.0, 39.00(R)
and 42.10(S) at a pH of 9.0, and 35.00(R) and 40.10(S) at a pH of 10.0 from a C18 cartridge. At a pH of 9.0, these numbers clearly demonstrated optimal recovery. Therefore, percentage recoveries at pH 9.0 were used to determine enantiomer binders in human plasma. In a similar vein, the percentage recoveries of R- and S-enantiomers of oxybutynin on a C18 cartridge were 11.24 and 13.17 at pH 5.0, 7.0, and 9.0, and 11 and 13.0% at pH 10.0. These numbers strongly suggested that the optimal pH for recovery was 9.0. Therefore, percentage re- coveries at pH 9.0 were used to determine enantiomer binders in human plasma. Cetirizine enantiomers were recovered at percentages of 20.68(R) and 17.59(S) on C18 cartridge at pH 5.0, 7.0, 9.0, and 10.0, and 28.61(R) and 22.20(S), 27.02(R)
and 25.91(S), and 26.00(R) and 23.50(S), respectively. These numbers strongly suggested that the optimal pH for recovery was 9.0. Therefore, percentage recoveries at pH 9.0 were used to determine enantiomer binders in human plasma. 19.00(R) and 20.40(S), 6.28(R) and 17.59(S), 4.70(R) and 15.90(S),
and 3.50(R) and 14.2(S) were the brinzolamide enantiomer recoveries on C18 cartridge at pH 5.0, 7.0, 9.0, and 10.0, respectively. At a pH of 9.0, these values obviously rebounded the most. The % recoveries at pH 9.0 were used to deter- mine the enantiomers' binding affinities in human plasma. The enantiomers of 154 pheniramine, oxybutynin, cetirizine, and brinzolamide all bound to human plasma with different strengths, with 11.00(R) and 7.91(S), 36.83(R) and 36.11(S), 20.98(R) and 26.09(S), and 42.80(R) and 36.60(S) at pH 9.0.

Table 4 provides these numerical values.

S. No.	pH of buffer	Pheniramine % Recovery 0.1%TFA in MeOH	% Binding with plasma protein	Oxybutynin % Recovery 0.1%TFA in MeOH	% Binding with plasma protein	Cetirizine % Recovery 0.1%TFA in MeOH	% Binding with plasma protein	Brinzolamide % Recovery 0.1%TFA in MeOH	% Binding with plasma protein
1	5.0	25.18	24.82	11.24	37.76	20.68	27.32	19.00	28.50
		(20.67)	(29.33)	(13.17)	(37.83)	(17.59)	(34.41)	(20.40)	(32.10)
2	7.0	32.59	17.41	10.82	38.18	28.61	19.39	6.28	41.22
		(36.40)	(13.60)	(16.20)	(34.80)	(22.20)	(29.80)	(17.59)	(34.91)
3	9.0	39.00	11.00	12.17	36.83	27.02	20.98	4.70	42.80
		(42.10)	(7.90)	(14.89)	(36.11)	(25.91)	(26.09)	(15.90)	(36.60)
4	10.0	35.00	15.00	11.00	38.00	26.00	22.00	3.50	44.00
		(40.10)	(9.90)	(13.00)	(38.00)	(23.5)	(28.50)	(14.2)	(38.30)

IV. CONCLUSION

The research describes the development of environmentally friendly, repeatable, accurate, low-cost, and efficient SPE and HPLC techniques for the separation of DD-, DL-, LD-, and LL-enantiomers found in human plasma. These methods use HPLC. At a pH of 9.0, the LL-enantiomer was found to have the highest percentage of binding to plasma, which was 84.11%. This was followed by the LD-enantiomer, which had 73.11%, the DL-enantiomer, which had 63.39%, and the DD-enantiomer, which had 56.79%. Both the separation and resolution factors had values that were larger than 1.0, which indicated that resolution was achieved overall. The measured values for the limit of detection (LOD) for the enantiomers DD-, DL-, LD-, and LL- ranged from 1.0 to 2.7 g mL

1. This was the range that was observed. It was discovered that the limit of quantification (LOQ) values for the identical enantiomers ranged from 5.0 to 10.0 g mL-1. The results of the experiment were compared to the outcomes predicted by the model, and a degree of concordance that was deemed sufficient was found. The approaches that were disclosed had a positive outcome when they were applied to plasma samples taken from real-world circumstances. As a result, the procedure that has been described as being useful for monitoring the ratio of dipeptide stereomers in patients may be put into practise. In addition, the use of the modelling approach shows to be beneficial in the process of figuring out how the various enantiomers are laid up in their respective configurations.

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