

## RESEARCH ARTICLE

# To Synthesize and Characterize Metabolite Enalapril: PKPD and Toxicity Predictions by Computational Approaches

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## ABSTRACT

Enalapril is a prodrug that facilitates the transformation of angiotensin I to angiotensin II, an ingredient that brings constriction of blood vessels by action of the enzyme angiotensin-converting enzyme (ACE). Reversed-phase high-performance liquid chromatography (RP-HPLC) method is the focus of this work, which also includes its characterization, optimization, and synthesis. The objective is to correctly, precisely, and sensitively determine both enalapril and its synthesized metabolite, enalaprilat, when present as an impurity. The paper also forecasts the ADME and toxicity characteristics of ENLP by utilizing several ADME databases, including SWISS ADME and molesoft. The laboratory synthesized enalaprilat, the metabolite derived from enalapril, and characterized. The Kinetex C18 stationary phase was employed in the analytical approach. Flow rate was 1.0 mL/min, and the injection volume was 20 µL. With a wavelength of 244 nm, the run lasted for 10 minutes. The mobile phase used was an 80% acetonitrile: 20% pH 3 phosphate buffer. The HPLC technique validation, which involved the identification of the enalapril metabolite, followed the parameters outlined in ICH Q2B (R1). The analysis exhibited a range of accuracy and precision between 98.75 and 102.5% for all substances being tested. This approach is appropriate for identifying and measuring the metabolite in enalapril bulk or formulations. The method for assessing enalaprilat in biological fluids has the potential to be further refined and applied in clinical and bioequivalence research. The study furthermore presents an evaluation of toxicity utilizing computational methods such as the Swiss tool, with the objective of aiming to mitigate potential risks.

**Keywords:** Enalapril, Enalaprilat, RP-HPLC, ADME database.

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**Conflict of interest:** None

## INTRODUCTION

Currently, cardiovascular disease (CVD) stands as the primary contributor to mortality worldwide. In 2019, non-communicable diseases led to 17 million untimely deaths (under the age of 70), accounting for 38% of the total.<sup>1</sup>

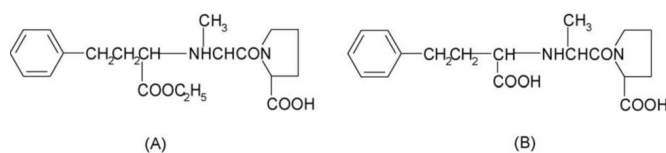
Enalapril, chemically denoted as N-[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl]-L-proline (Figure 1A), is classified as a substituted N-carboxymethyl dipeptide. It acts as a prodrug, which means that it is converted into an active angiotensin-converting enzyme (ACE) inhibitor once it is absorbed and undergoes hydrolysis. Enzyme ACE facilitates the transformation of angiotensin I to angiotensin II, a vascular-constricting chemical. Furthermore, the adrenal cortex is stimulated to secrete aldosterone by angiotensin II. Inhibiting the renin-angiotensin-aldosterone pathway is primarily responsible for the observed advantages in hypertension and heart failure. Reducing the activity of vasopressors and the

generation of aldosterone is the result of ACE inhibition, which lowers blood levels of angiotensin II.<sup>2</sup>

Enalaprilat (Figure 1B) is the prime metabolite of enalapril and has demonstrated efficacy in treating hypertension and congestive heart failure while avoiding notable adverse effects.

Detecting enalapril and enalaprilat concurrently in biological fluids is crucial, particularly for pharmacokinetic studies in human plasma. Various analytical methods for these compounds in biological samples have been documented, encompassing GC-MS<sup>3</sup> radioimmunoassay<sup>4</sup> and enzyme kinetics.<sup>5</sup> Enalapril maleate and similar chemicals can now be officially measured using HPLC technology, according to the European Pharmacopoeia (EP).<sup>6</sup> To evaluate enalapril and enalaprilat, researchers have recently turned to LC-MS/MS<sup>7,8</sup> and LC-MS.<sup>9</sup> Yet, when it comes to quantitative biosample analysis, long analysis time (>3.5 minutes), bulky plasma sample volume (>0.5 mL), or poor extraction retrieval could

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**Figure 1:** A: Structure of enalapril; B: Structure of enalaprilat

not be enough to meet the needs for speed, sensitivity, and high throughput.

As per the rules set by the International Conference on Harmonisation (ICH), it is obligatory to identify and characterize any impurities present in medicinal substances or products. ICH defines impurities as all substances that aren't excipients or active ingredients in medicinal products. Rules impose stringent restrictions on the permissible levels of impurities in pharmaceutical goods, mandating the identification and qualification of impurities above 0.1%. Regardless of the amount, if the pollutants are thought to be very harmful, detection and identification are necessary.<sup>10</sup>

In order for pharmaceutical goods to ensure safety and efficacy, it is crucial to accurately identify and characterize impurities. The main objectives of a medical researcher conducting this type of study are to achieve sufficient separation, selectivity, detection sensitivity, and accurate quantification.

In order to address off-target effects and metabolic pathways, it is critical to conduct an important evaluation of ADME during drug discovery. Early ADME assessment in drug development helps identify potential pharmacokinetic issues, and in silico models, such as the SwissADME database, provide a robust alternative to experimental methods for predicting ADMET properties. The work also anticipated attributes like ADMET for the synthesized metabolite of enalapril, enalaprilat, through the publicly accessible SwissADME database and molesoft at <http://www.swissadme.ch>.<sup>11</sup>

This study aims to synthesize and characterize the metabolite of enalapril (ENL), namely enalaprilat (ENLP). In order to quantify the synthesized metabolite using chromatographic methods like HPLC, this message further stresses the need to use non-compendial reference standards. In silico models are investigated in the second part of the study as a possible substitute for experimental methods in ADME prediction. To forecast characteristics like ADME and toxicity for the produced metabolite ENLP, the study used a number of ADME databases, including Molesoft and SWISS ADME.

## MATERIAL AND METHODS

### Chemicals and Reagents

Cadila Pharmaceuticals, India, graciously offered gift samples of functioning quality and pure ENL/vastatin. The Envas TAB, manufactured by Cadila Pharmaceuticals, was obtained from a pharmacy located nearby. The analysis utilized HPLC-grade chemicals, all of which were of analytical grade. Triple distilled water used.

## Synthesis and Characterization of ENLM

### Procedure

A carefully measured amount of 3.48 g (equivalent to 0.01 moles) of the chemical was placed into a flask with a circular bottom. About 5 mL of ethanol and 10 mL of a 0.1 N solution of sodium hydroxide were added one after the other to. Subsequently, a reflux condenser was connected to the flask, and the resulting solution was subjected to reflux for a duration of three hours using a water bath. The resultant solution was further cooled using an ice salt solution. Glacial acetic acid was employed to lower pH of the combination to a neutral value of 7. Raw ENLP was subjected to filtration using a pump and then recrystallized using ethanol. Analytical thin-layer chromatography was employed to monitor the progression of the reaction.

### Characterization

Comprehensive characterization of the synthesized molecule was carried out using a range of spectroscopy methods, encompassing UV, fourier-transform infrared (FTIR), mass spectroscopy (MS), and nuclear magnetic resonance (NMR). The compound's ultraviolet (UV) spectra were captured by means of a UV-vis spectrophotometer (JASCO V-730 Double Beam). The FTIR, which displayed absorption bands associated with various vibrational modes, were recorded using a JASCO FTIR-4600 analyzer.

To further characterize the synthesized metabolite, <sup>1</sup>H-NMR spectroscopy was employed. Solubility testing of the compound was conducted in CDCl<sub>3</sub>, DMSO-d<sub>6</sub>, and D<sub>2</sub>O, subsequently, for additional NMR analysis to confirm the molecular structure.

An electron impact source was used to interface a Shimadzu QP 3010 mass spectrometer with gas chromatography for mass analysis and detection. The sample analysis was carried out using helium mobile phase at flow rate 1-mL/minutes keeping electron impact source temperature at 280 nm and using a gas chromatographic real analyzer as the detector were the two primary components. A 300 m long column with a 0.2 mm diameter inside diameter was used.

### HPLC Method Development

#### Chromatographic conditions

The investigative method was established and validated using an RP-HPLC system equipped by UV detector, a Kinetex C18 column and a particle size of 5 μm. The optimization procedure took into consideration a 20 μL injection volume, a 1.0 mL/min flow, a 10-minute run time, and a 230 nm wavelength. An 80% acetonitrile and 20% 20 mM phosphate buffer solution with a pH of 3 was used as the mobile phase in a gradient mode.

Various instruments used in validation process, such as the electronic balance from Shimadzu, ultrasonicator from Wensar, and pH meter from Equip-tronics.

#### Buffer solution preparation

Accurate measurements were taken, and then 5.299 g of disodium hydrogen phosphate and 1.625 g of potassium

dihydrogen phosphate were combined with 550 mL of distilled water. The compounds were dissolved by continuously spinning the mixture. The buffer solution was passed through a Millipore membrane filter with a pore size of 0.45  $\mu\text{m}$ .

#### *Mobile phase*

An 80:20 volumetric ratio of methanol to phosphate buffer solution made an updiluent, mobile phase, and blank solution. We filtered and degassed this solution. A pH of 3 was achieved in the mobile phase *via* addition of ortho-phosphoric acid.

#### *Stock and standard solutions*

Weighing precisely 10 mg each of ENL and ENLP, they were individually dissolved in an adequate volume of the mobile phase within 100 mL calibrated volumetric flasks. After that, the solutions were sonicated for 10 minutes to help the medication dissolve, and then the mobile phase was added to bring the total volume to 100 mL. The solutions that were produced were named stock solutions and had a concentration of 100  $\mu\text{g/mL}$ . Then, from the ENL and ENLP stock solutions, aliquots of 0.5 to 3 mL were taken and placed into separate 10 mL volumetric flasks.

This step was performed to obtain final concentrations spanning from 0.5 to 30  $\mu\text{g/mL}$  for both ENL and ENLP, correspondingly.

#### *Sample solution preparation*

Almost 20 enalapril tablets (Envas) were measured, crushed, and combined. A 100 mL volumetric flask was used to hold the precisely measured 10 mg of enalapril powder, which was then mixed with an appropriate quantity of diluent. After 20 minutes of sonication, the volume was brought down to 100 mL by adding more of the same diluent. We used this produced solution as our stock solution and diluted it further as needed to get the concentration of ENL.

#### **Method Validation**

Analytical method validation followed ICH standards, which included checking system appropriateness, specificity, accuracy, linearity, ruggedness, precision (system and method), and establishing limit of detection (LoD) and limit of quantitation (LoQ).<sup>12,13</sup>

#### *System suitability study*

The investigation of system suitability parameters was conducted using freshly generated standard stock solutions of ENL and ENLP. The chromatographic system was optimized for the injection of the medication and its metabolite. For the purpose of determining if the system was appropriate, several parameters were investigated.

#### *Specificity*

Injecting ENL standards and test solutions allowed us to evaluate the analytical method's specificity. In order to identify any possible interference peaks, the sample chromatograms were carefully examined. Most importantly, the chromatograms of reference and test solutions had to show that they were comparable to one another, particularly with regard to retention times.

#### *Linearity*

In order to reach final concentrations ranging from 5 to 30  $\mu\text{g/mL}$  for ENL and ENLP, respectively, 0.5, 1, 1.5, 2, 2.5, and 3-mL volumes of ENL and ENLP stock solutions were put into individual 10 mL volumetric flasks. To make the solutions more water-soluble, the mobile phase was next added. Afterwards, 20  $\mu\text{L}$  of each solution that had been made was injected for a period of 10 minutes at a flow rate 1.0 mL/min. Analysis of samples was conducted at a wavelength of 230 nm. Calibration curves were constructed by correlating the concentrations with the corresponding peak areas for both ENL and ENLP.

#### *Precision*

The execution of a precision study verified the method's repeatability. The accuracy of the system and approach was assessed.

- *System precision*

The system was dosed with ENL standard solution five times in accordance with the normal procedure. When calculating peak area, make sure the RSD cannot exceed over 2%.

- *Method precision*

Standard solution of ENL was individually injected 6 times following the specified test method. The assay of ENL should fall within the range of not less than 90.0% and not more than 110.0%.

#### *LoD and LoQ*

The system's minimum detectable concentration was determined as LoD, while the lowest accurate and precise quantifiable amount was defined as LoQ.

#### *Accuracy*

The experiment was performed three times using the prescribed procedure, with ENL concentrations that were 80, 100, and 120% of the specified quantity. The accuracy was evaluated by comparing it to the standard solution at equivalent concentrations, and the average percentage of ENL recovered was calculated. The predicted range for the mean % recovery for each spike level is 90.0 to 110.0%.

#### *Robustness*

Deliberately altering the flow rate proved the effectiveness of the devised strategy. The HPLC system was fed a standard solution at flow rates of 0.9, 1, and 1.1 mL/min in accordance with the specified test protocol. The system's appropriateness under these changed settings was then determined by evaluating system suitability factors.

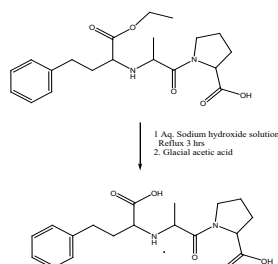
#### **PKPD Predictions**

In a direction to minimize expenses, exertion, and the potential for delayed malfunctions in compound analysis, it is crucial to get preliminary assessments of favorable results at an early stage. We conducted ADME analysis using computational techniques. ENLP's work involved utilizing internet servers such as SWISS ADME and molesoft to analyze ADME, toxicity, and drug similarity. The chemical ENLP was analyzed

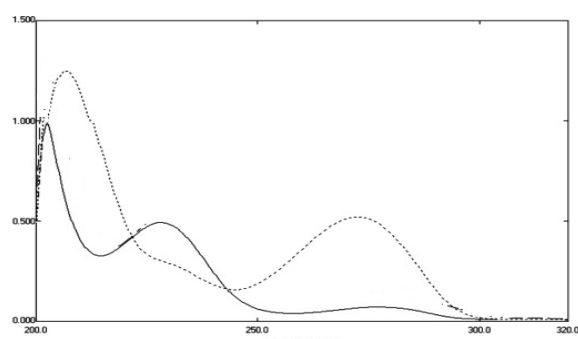
using the Swiss-ADME web server to evaluate its drug-like characteristics and forecast probable toxicity.<sup>14,15</sup>

## RESULT AND DISCUSSION

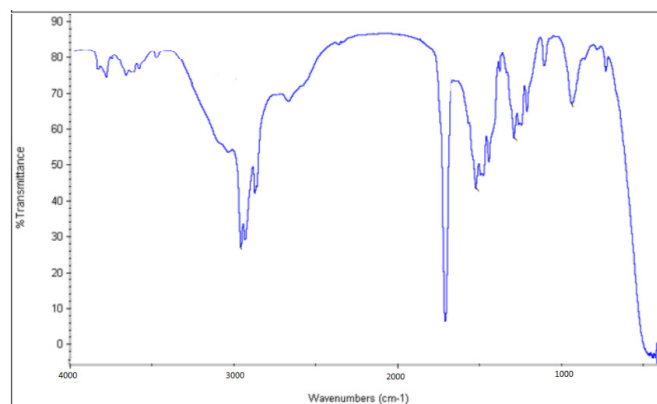
Following a comprehensive review of the literature, the synthesis scheme for ENLP from ENL is depicted in Figure 2. In order to characterize ENLP, spectroscopic methods such as



**Figure 2:** Synthesis scheme for metabolite enalaprilat (ENLP)



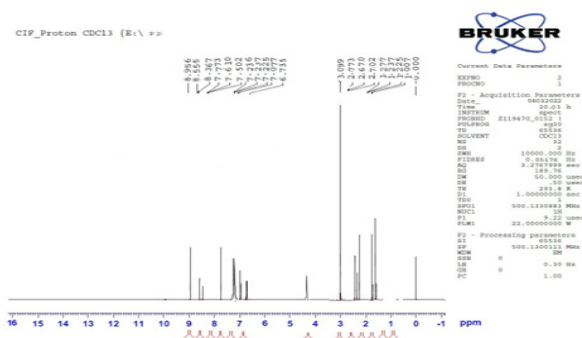
**Figure 3:** UV spectrum overlain of ENL and ENLP



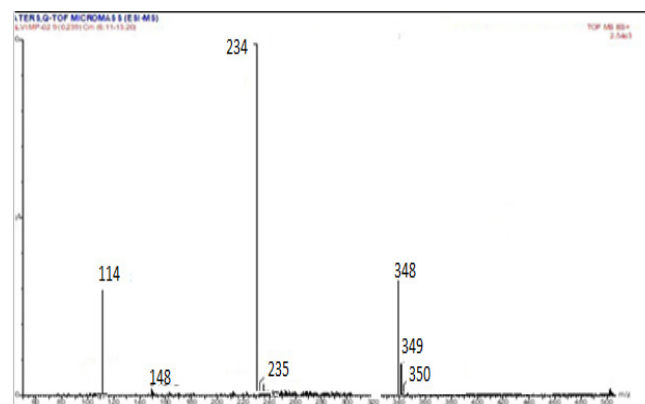
**Figure 4:** IR of ENLP

NMR, IR, UV and GC/MS were employed. FTIR, mass, UV, and NMR spectra are shown in Figures 3-6. Spectral analysis provided irrefutable proof that the product was synthesized to the specified purity and quality standards.

The development of analytical methods relies heavily on the selection of the stationary phase, which is mostly dictated by the molecule's solubility and molecular weight. Based on what is already known about the ENL and how it should be examined in RP-HPLC, C18 column was selected for this exploration. By adjusting the concentrations of acetonitrile and buffer, we were able to reduce the run time and still reach the drug's symmetric peak. Acetonitrile (80%): Phosphate buffer (20 mM) pH 3 (20%) produced excellent symmetrical peaks and a well-resolved separation. At 2.90 minutes, the ENL retention time was noted, while the ENLP retention time was 5.99 minutes. The several mobility phases that were observed



**Figure 5:** NMR of ENLP



**Figure 6:** MS of ENLP

**Table 1:** ENL and ENLP Chromatographic performance

Mobile Phase	Concentration (% v/v)	Retention time (min)	
		ENL	ENLM
Methanol:Water	90:10	No peak obtained	4.5
Acetonitrile: Water	90:20	2.6	Peak Not obtained up to 15 min
Acetonitrile: Phosphate Buffer 20 mM pH 3	80:20	2.90	5.99

**Table 2:** Optimized chromatographic conditions

Parameters	Details
Column	Kinetex C18, 4.6 x 250 mm, 5 µm column
Injection volume	20 µL
Flow rate	1.0 mL/min
Detector wavelength	230 nm
Retention time	2.90 and 5.99 minutes
Run time	10 minutes

are listed in Table 1. Table 2 displays the chromatographic conditions that were optimized. In Figure 7, you can see chromatograms of both standard and test solutions. The procedure has been verified in accordance with the ICH Q2R1 (Q2B) standards.

To determine linearity, calibration curves were created for ENL and ENLP, covering concentrations ranging from 5 to 30 µg/mL. The ENL and ENLP linearity graphs are shown in Figures 8 and 9, correspondingly. Correlation coefficients of 0.9998 and 0.9996 for ENL and ENLP, correspondingly, were obtained from the least square lines that were plotted against

concentrations (Table 3). This confirms that the approach is linear.

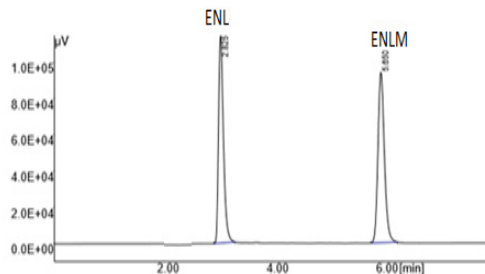
The results of the ENLP recovery experiments, which demonstrate that the approach was accurate (Table 4), range from 98.57 to 101.86% and have a %RSD of 1.97 or higher. The method's repeatability and reproducibility have been demonstrated by intra- and inter-day precision studies (Table 5).

**Specificity**

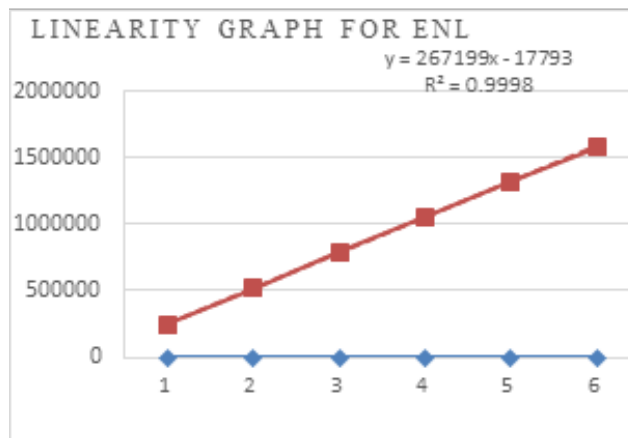
Retention times of ENL and ENLP in control and experimental solutions were unaffected by one another. The specificity

**Table 3:** Linearity of ENL and ENLP

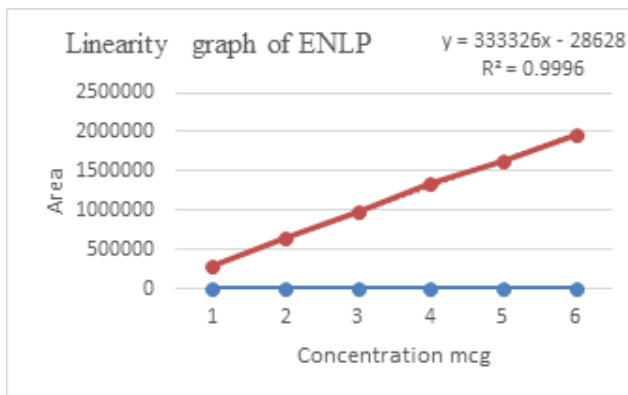
S. No	Sample			
	ENL		ENLM	
	Conc <sup>n</sup> (µgmL <sup>-1</sup> )	Area (N = 3)	Conc <sup>n</sup> (µgmL <sup>-1</sup> )	Area (N = 3)
1	5	243106.1	5	295871.2
2	10	516123.7	10	6372620.2
3	15	789563.1	15	972239.2
4	20	1057862.2	20	1329139.1
5	25	1320619.1	25	1633582.2
6	30	1577139.1	30	1959978.9
Straight line equation	Y = 267199x - 17793		Y = 333326x - 28628	
R <sup>2</sup>	0.9998		0.9996	
SD	456363.9		569382.9	
Slope	267199		333326	



**Figure 7:** ENL and ENLP chromatogram



**Figure 8:** Linearity of ENL



**Figure 9:** Linearity of ENLP

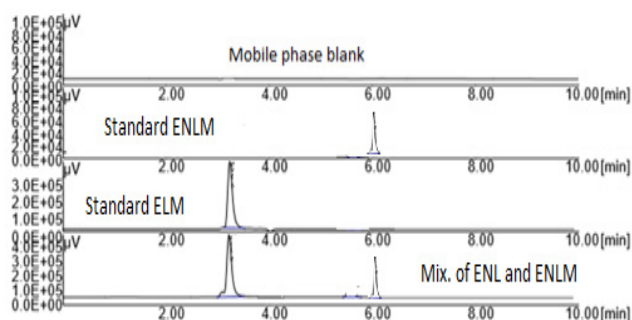
**Table 4:** ENL and ENLP accuracy studies

Sample	Drug in formulation (mg)	Spiked level (%)	Conc <sup>n</sup> added (mg)	Area (N = 3)	Conc <sup>n</sup> found (mg)	%Recovery	SD	%RSD
ENL	10	80	08	1058878	7.9	98.75	1411.92	0.34
	10	100	10	1239273	10.01	100.1	1908.01	0.42
	10	120	12	1529906	12.1	100.8	16282.29	0.92
ENLM	10	80	08	1060192	8.2	102.5	1256.51	0.82
	10	100	10	1240252	10.1	101.0	1637.08	0.51
	10	120	12	1528892	11.9	99.16	1467.19	0.86

\*Acceptance standards < 2.0.

**Table 5:** ENL and ENLP intra and interday variations

Details of Parameter	Sample		
	ENL	ENLP	ENLP
Day 1	Mean (N = 3)	1059153	1326729.2
	SD	1528.2	1432.2
	%RSD	0.65	1.2
Day 2	Mean (N = 3)	1062546.3	13219289.2
	SD	1409.2	1393.2
	%RSD	0.89	1.7
Day 3	Mean (N = 3)	1061889.3	1328292.2
	SD	1502.3	1414.8
	%RSD	0.14	0.67


**Figure 10:** Specificity

test was successful since the retention times of control and experimental solutions were determined to be equal (Figure 10).

### LoD and LoQ

It was found that the LoD for ENLP was 0.012 µg/mL and for ENL it was 0.37 µg/mL. The LoQ for ENLP was determined to 0.32 µg/mL, but for ENL it was 0.56 µg/mL. Using this method, the sensitivity to LoD and LoQ was excellent.

### Robustness

By varying the flow rate from 0.9 to 1.1 mL, a study tested the method's resilience. There was no discernible effect on the percentage of drugs recovered, and the procedure was found to stay within acceptable ranges. The devised approach is resilient, according to the robustness study's results, showing stability even when subjected to small changes in chromatographic conditions. Table 6 provides a summary of the effects observed as a result of parameter changes.

### PKPD Predictions

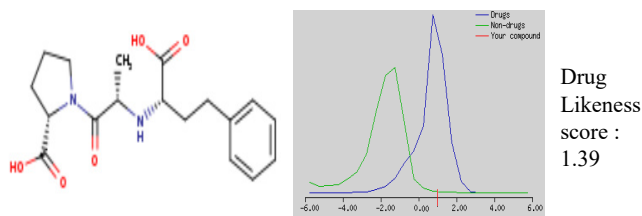
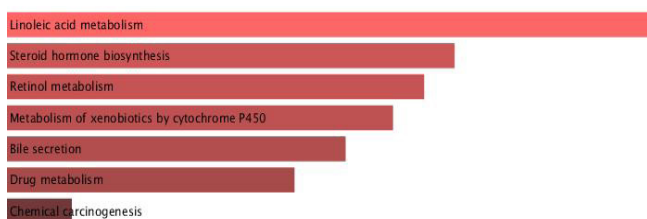
To evaluate the ADME properties, harmfulness, and drug similarity of ENLP, the online servers SWISS ADME and molesoft were employed. The drug-likeness assessment for the compound CDA was conducted using the molsoft online server package, as illustrated in Figure 11.

**Smile:** O=C(O)CC(O)CC(O)CCC2=C(C)C=CC1=CC(C)CC(OC(=O)C(C)C)CC1C12

Based on pharmacokinetic considerations, the chemical ENLP is a viable therapeutic candidate, as shown by the graphic data, which clearly displays a drug-likeness score of 1.39.

**Table 6:** Flow rate variations

Flow rate (mL/min)	ENL		ENLP	
	RT (min)	Peak area	RT (min)	Peak area
0.9	3.08	223923	6.05	296373
1.0	3.01	230722	5.99	289363
1.1	2.99	2293736	5.92	289933


**Figure 11:** Graphical data drug-likeness score of ENLP

**Figure 12:** The following route could be impacted by the inhibition of the CYP450 (CYP3A4) class of enzymes

The compound ENLP displays a lipophilicity (XlogP3) of -0.74, a molecular weight of 348.39, a total polar surface area (TPSA) of 106.94, and an ESOL (Log S) value of -1.12. Additionally, the compound exhibits nine flexible obligations, six hydrogen bond receptors, and three hydrogen bond donors, indicating structural adaptability that suggests favorable gastrointestinal (GI) absorption but limited permeability through the blood-brain Barrier, suggesting a low likelihood of crossing the central nervous system (CNS). Predictions indicate that compound ENLP is likely to be a substrate for P-glycoprotein (Pgp), suggesting a high probability of efflux shown in Table 7.

Upon further examination of the metabolic factor, it was observed that compound ENLP is anticipated to function as a substrate for the CYP250 class of enzymes (specifically, CYP1A2, CYP2C19, CYP2C9, and CYP3A4) while also being predicted as an inhibitor. This suggests that the selected compound may pose mild challenges in terms of metabolism, potentially resulting in delayed excretion from the system. Several metabolic pathways can be impacted by inhibiting these particular CYP450 enzymes. This class of pathways includes cytochrome P450 xenobiotic metabolism, biliary release, biochemical cancer development, steroid hormone synthesis, retinol metabolic processes, linoleic acid metabolism and xenobiotic oxidative stress (Figure 12).

Here are the results of the ADMET SAR version 2 analysis of chemical ENLP's toxicity:

There are findings from both regression and classification in

**Table 7:** PKPD investigation from Swiss-ADME server

<i>Parameter</i>	<i>Predication</i>
Pgp substrate	Yes
BBB permeant	No
GI absorption	High
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
Ghose #violations	0
CYP2D6 inhibitor	No
Lipinski #violations	0
CYP3A4 inhibitor	No
CYP1A2 inhibitor	No

**Table 8:** ADMET TOX estimate by means of ADMETSAR tool box

<i>Parameter</i>	<i>Prediction</i>	<i>Probability</i>
Carcinogenicity	-	0.3733
EYE corrosion	-	0.4700
Hepatotoxicity	+	0.25
Eye irritation	-	0.034
Respiratory toxicity	-	0.66
Acute oral toxicity	+	1.49
Nephrotoxicity	-	0.56
Ames Mutagenicity	-	0.69

the ADMET property Table 8. Endpoint, value, and likelihood are the columns in the table that are used for categorization. Value is in the labels that are predicted. For toxicity endpoints, for instance, a “+” indicates a positive or poisonous result, whereas a “-” indicates a negative or nontoxic result. The value is associated with the probability, which is often greater than 50%, since the alternative outcome would have been foreseen if the probability was lower. There are three columns in the regression models: endpoint, value, and unit.

## CONCLUSION

Notable features of the developed approach for ENL are its sensitivity, selectivity, accuracy, and precision; the same qualities also apply to the estimation of ENLP, the metabolite that ENL is derived from. This technique is useful for measuring ENLP, an impurity that is present in many ENL and bulk medication formulations. Additional optimization could make the approach useful for measuring ENLP in biological fluids, which would increase its potential use in clinical and bioequivalence research. Toxicological evaluations employing computational tools, such as the Swiss tool, have been documented in an effort to lessen possible dangers.

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