HINNAO™

HINNAO™ HIGH STABILITY LIPOSOME METHYLCOBALAMIN WITH HPLC ANALYSIS

Analysis Conducted

For

HINNAO™

20 White Church Lane, London, E1 7QR

DIFFUSION RESULTS OF HIGH STABILITY LIPOSOMAL METHYLCOBALAMIN

Diffusion: Movement of a fluid from an area of higher concentration to an area of lower concentration. Diffusion is a result of the kinetic properties of particles of matter. The particles will mix until they are evenly distributed.

Materials used:

- PBS buffer solution
- Franz diffusion apparatus
- Sheep mucosal membrane
- Syringe
- HPLCInstrument

Franz's diffusion cell:

Franz showed an excellent correlation between in vitro and in vivo studies. Majority of In-vitro experiments are conducted in animal skin i.e. hairless mouse, guinea pig, rabbit etc. Although these exist a number of similarities there is as yet no animal skin that complete mimics the penetration characterization of human skin.

The receptor chamber of the cell is placed in circulation water in a water bath with a temperature of 37 ºC keeping the temperature at the skin surface at 37° to imitate a real life skin condition as much as possible. The receptor fluid is kept homogenous in concentration and in temperature by a magnetic stirring bar. The fluid in the receptor chamber is manually sampled at predefined time intervals. Any type and any amount of vehicle (that will fit into the donor chamber) may be applied to the skin.

Preparation of PBS buffer solution:

Measure a volume of 800 ml of ddH2O with a graduated cylinder and transfer to an Erhlenmyer flask. Add a magnetic stir bar to the Erhlenmyer flask and place the flask on a magnetic stir plate Adjust the speed of the magnetic stir bar so that oxygen is not introduced into the solution while it is rapidly mixed.

Transfer to the flask:

- 8g of NaCl, 0.2 g KCl
- 1.44 g of Na_2HPO_4
- 0.25 g of KH_2PO_4

Ensure that the pH meter has been properly calibrated and rinse the pH probe with double distilled H2O. Remove the excess water from the probe tip (without touching the probe tip) with a clean paper towel. Place the pH probe into the solution. Slowly add 1 M HCl dropwise with a transfer pipette and allow the HCl to fully dissolve into the solution. Stop stirring the solution.

Measure the pH with the pH meter. Repeat until the pH of the solution is 7.4. Pour the solution into a fresh graduated cylinder and adjust the final volume to 1 liter with double distilled H2O. Store the PBS solution at room temperature. The PBS Solution is sterile; when using the PBS Solution, ensure that sterile techniques are employed

Drug Diffusion Study:

The drug diffusion studies through membrane experiments were conducted by using vertical type diffusion cell (Franz type) having receptor compartment 60ml volume with 10.18cm2 area. The receptor compartment was filled 60ml of phosphate buffer pH 7.4; the activated mucosal membrane was mounted on the flange of the diffusion cell receptor compartment. The whole assembly was kept on a magnetic stirrer and solution in the receptor compartment was constantly and continuously stirred using a magnetic bead and at 37 1oC maintained. This temperature and rpm was maintained by magnetic stirrer. The tissue was stored

in Krebs buffer at 4°C upon collection. After the buccal membrane was equilibrated for 30 min with the buffer solution between both the chambers, the receiver chamber was filled with fresh buffer solution (pH 7.4), and the donor chamber was charged with 5mL (1mg/mL) of drug solution. Aliquots (5mL) were collected at predetermined time inter wells up to 45min and the amount of drug permeated through the mucosa was then determined by measuring the values at 215 nm using HPLC method. The medium of the same volume (5mL), which was pre-warmed at 37°C, was then replaced into the receiver chamber.

The experiments were performed and values were used to calculate flux (J) and permeability coefficient (P).

$$J = \frac{dQ}{A\,t}$$

$$P = \frac{dQ}{\Delta C A}$$

Where, **J** is Flux (mg.hrs-1cm-2)
P is permeability coefficient (cm/h)
dQ/dt is the slope obtained from the steady state portion of the curve
ΔC is the concentration difference across the mucosa and
A the area of diffusion (cm2)

METHOD DEVELOPMENT FOR METHYLCOBALAMIN (VITAMIN B12) BY USIN HPLC

CHROMOTOGRAPHIC CONDITIONS

Column	:	INERTSIL ODS C 18 150*4.6mm,5 µ particle Size.
Mobile Phase	:	Acetonitrile: water (50:50 v/v).
Flow rate	:	0.8 ml/min
Injection Volume	:	10 µl
Wavelength	:	215 nm
Temperature	:	5o C ± 2
Runtime	:	6 mins
Rt	:	2.215 mins.

Preparation of Linearity Solutions:

Weigh Accurately 10 mg of Methylcobalamin and take it into a 10 ml of volumetric flask to this add 3 ml of diluent and make up the solution up to the mark with same solution.(1000 µg/ml)

From the above stock solution take 0.1 ml into a 10 ml of volumetric flask make up the solution with diluent up to the mark.(10 µg/ml).

From the Above solution take a series of solutions 0.3ml,0.6ml,0.9ml,1.2ml,1.5ml,1.8 ml into different 10 ml volumetric flasks and make up the solution with diluent to get a concentration range of 0.3 µg/ml to 1.8 µg/ml.

(*mobile phase is used as a Diluent)

The diffused samples are injected into HPLC by maintaining above chromatographic conditions, from the data obtained area of the peak were calculated and the % drug release.

Samples were tested as per the Client requirement. Franz Tests were retested up to 45 min to determine % drug diffuse and Flux rate.

METHOD DEVELOPMENT FOR PARTICLE SIZE ANALYSIS

SIZE DISTRIBUTION BY INTENSITY WAS ANALYZED USING MALVERN

LINEARITY AND RANGE METHOD

Preparation of stock solution

Weigh accurately 10mg Vitamin B12 Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

HINNAO™ Vitamin B12:

Preparation of linearity solution (20%)

0.2ml of stock solution was taken in 10ml of volumetric flask dilute up to the mark with diluent. The solution mixed well and used for chromatographic injection.

Preparation of linearity solution (30%)

0.3ml of stock solution was taken in 10ml of volumetric flask dilute up to the mark with diluent. The solution mixed well and used for chromatographic injection

Preparation of linearity solution (40%)

0.4ml of stock solution was taken in 10ml of volumetric flask dilute up to the mark with diluent. The solution mixed well and used for chromatographic injection.

Preparation of linearity solution (50%)

0.5ml of stock solution was taken in 10ml of volumetric flask dilute up to the mark with diluent. The solution mixed well and used for chromatographic injection.

Preparation of linearity solution (60%)

0.6ml of stock solution was taken in 10ml of volumetric flask dilute up to the mark with diluent The solution mixed well and used for chromatographic injection.

Procedure

Each level of the above solutions was injected into the chromatographic system for five replicate and the peak area was measured. A graph was plotted (peak area versus concentration) and the correlation coefficient (R^2) was calculated.

Linearity

Fig. 1 : Chromatogram Of HINNAO™ Vitamin B12 Linearity-1

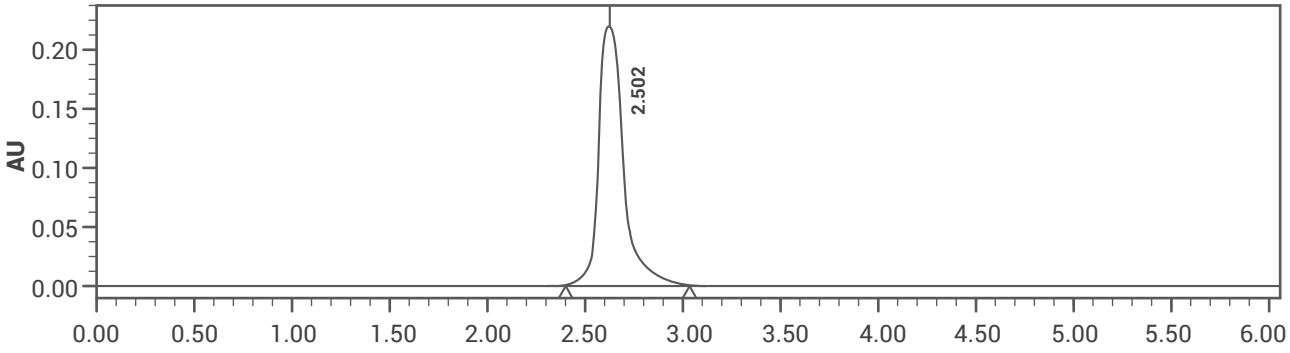


Fig. 2 : Chromatogram of HINNAO™ Vitamin B12 Linearity-2

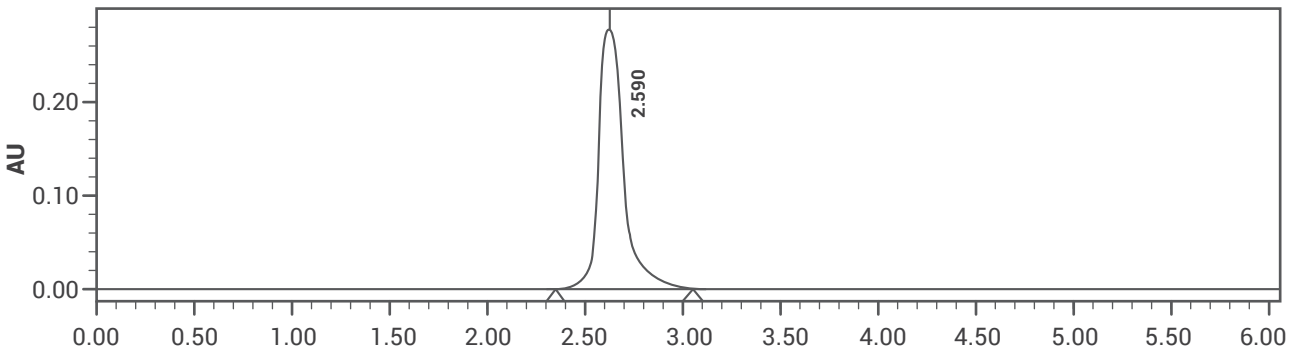


Fig. 3 : Chromatogram of HINNAO™ Vitamin B12 for Linearity-3

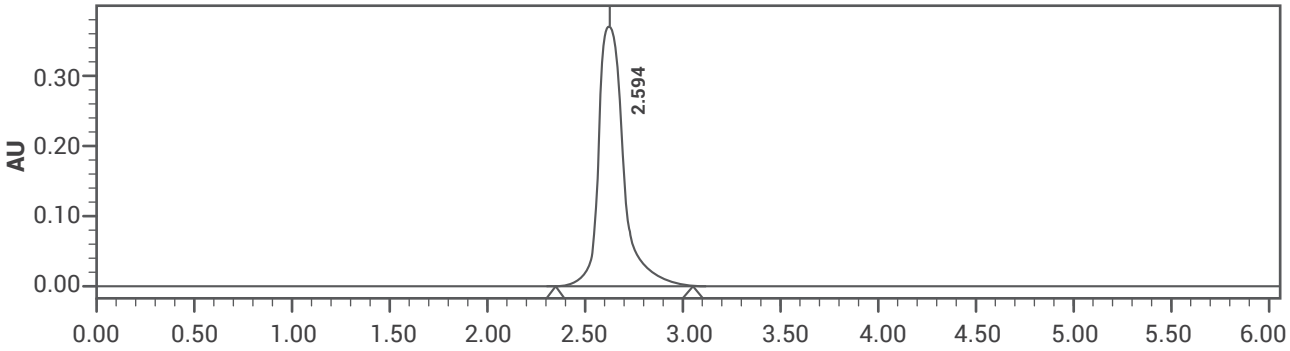


Fig. 1 : Chromatogram of HINNAO™ Vitamin B12 for Linearity-4

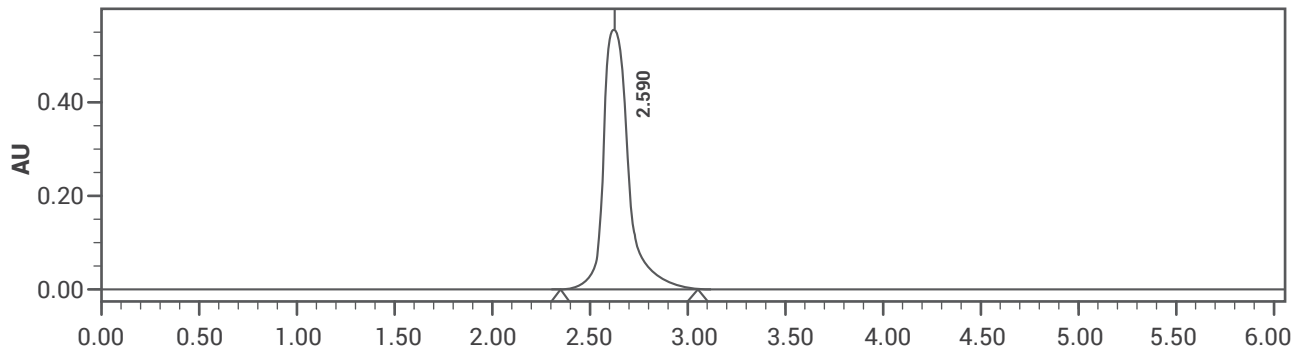


Fig. 1 : Chromatogram of Vitamin B12 for Linearity-5

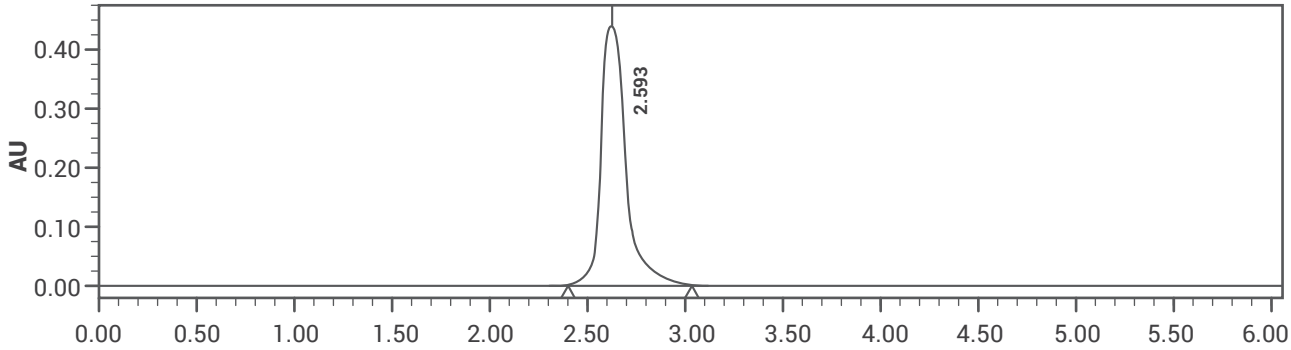
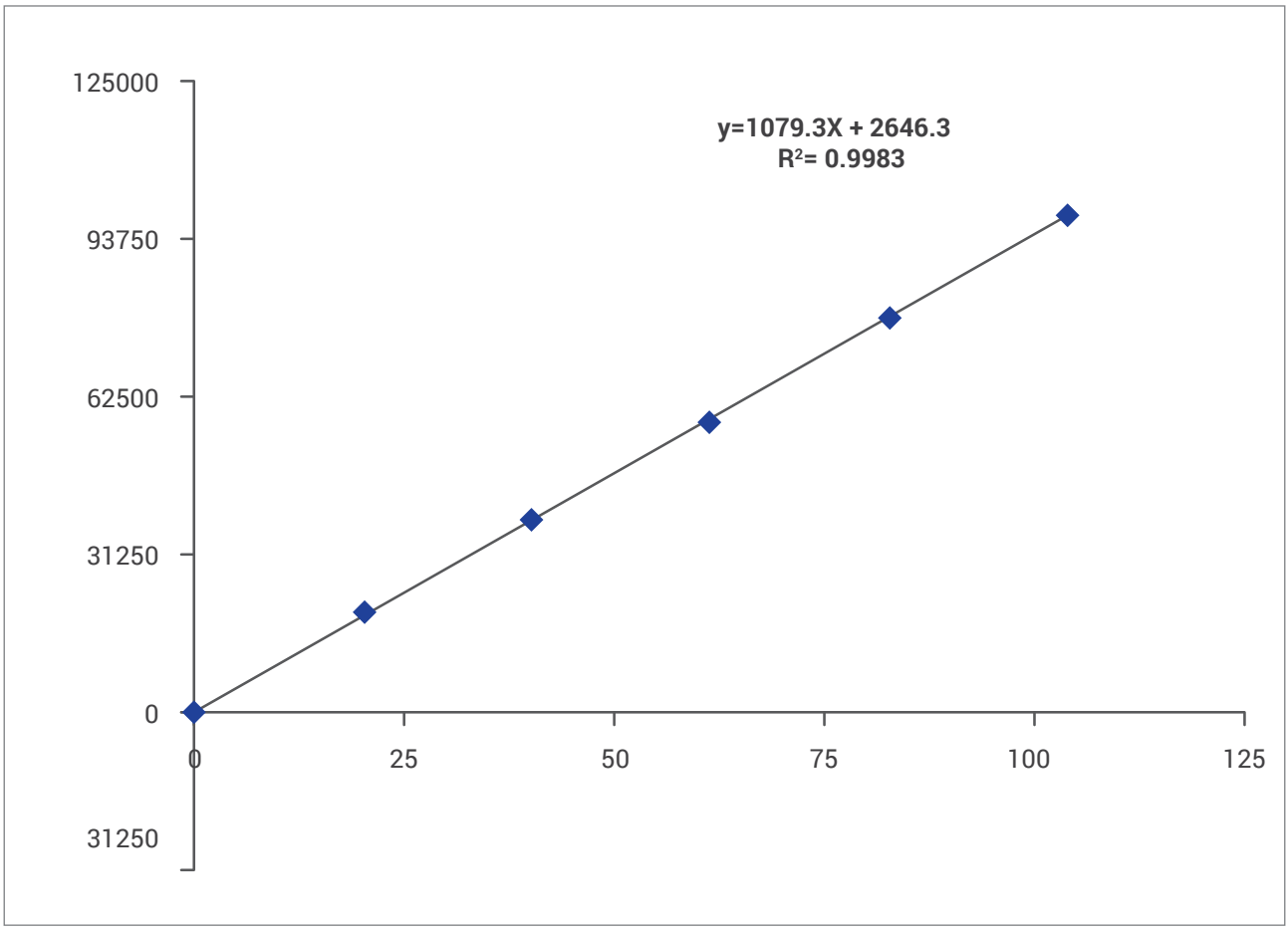


TABLE 1: Linearity of HINNAO™ Vitamin B12

Sample ID	HINNAO™ Vitamin B12	
	Concentration	Area
20% of operating concentration	20	924140
40% of operating concentration	30	1395681
60% of operating concentration	40*	1792966
80% of operating concentration	50	2256546
100% of operating concentration	60	2697214
Correlation Coefficient 0.998		

Fig. 6 : Showing calibration graph for HINNAO™ Vitamin B12

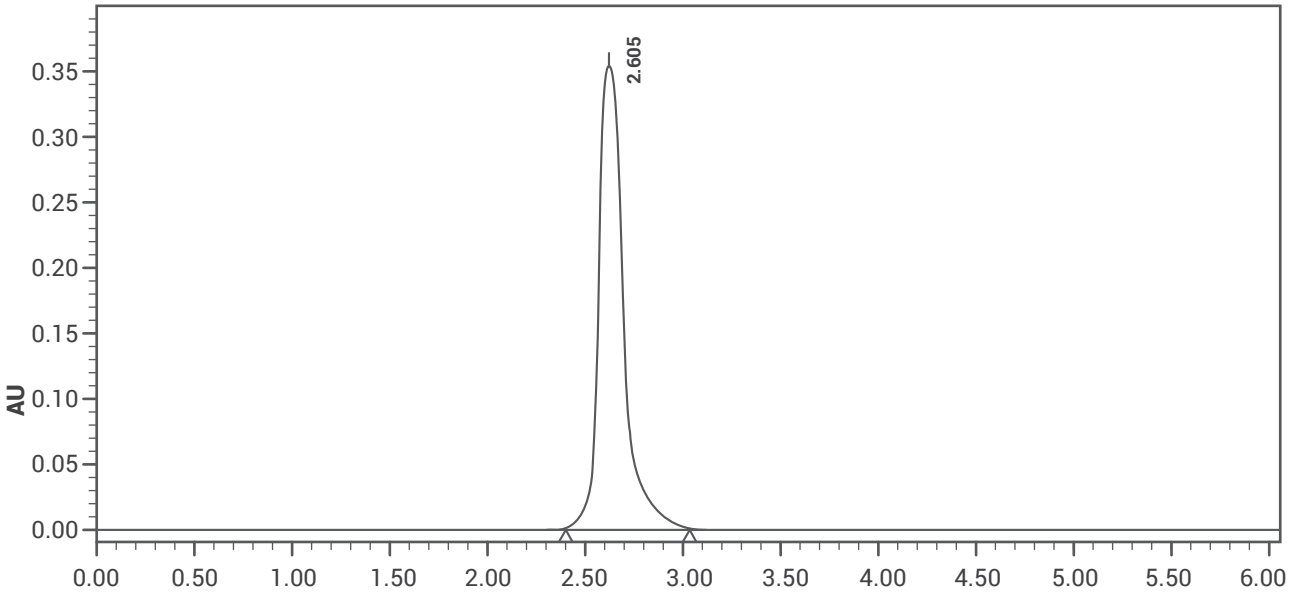


Acceptance criteria: Correlation Coefficient should be NLT 0.998

Discussion: The relationship between the concentration of HINNAO™ Vitamin B12 was linear in the specific range and the correlation coefficient was found to be within limit only. The correlation coefficient of HINNAO™ Vitamin B12 was found to be 0.9999 .

Optimized method

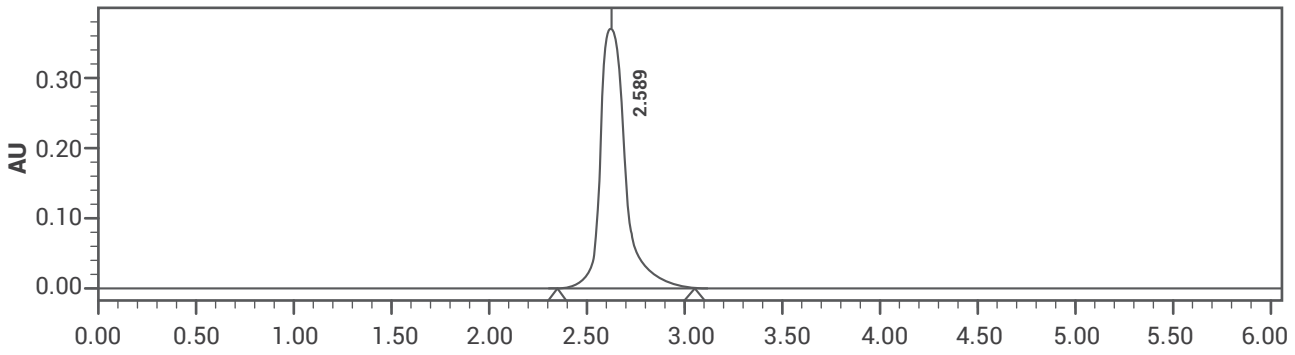
Fig. 7 : Chromatogram of Optimized method



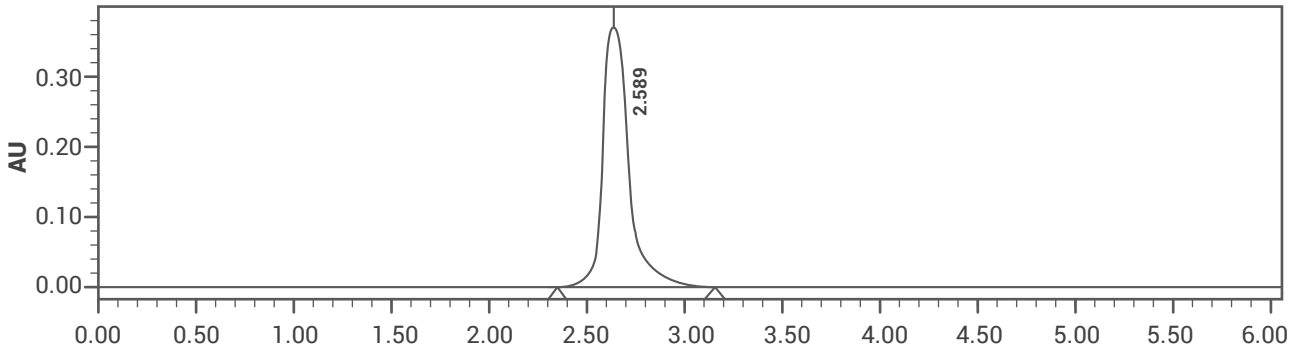
S. No	Peak Name	Rt	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Vitamin b12	2.605	2233704	365596	4456	1.4	

Discussion: The separation of two analytical peaks was good. The plate count also above 2000, tailing factor below 2, and the resolution is above 2. The condition is taken as optimized method.

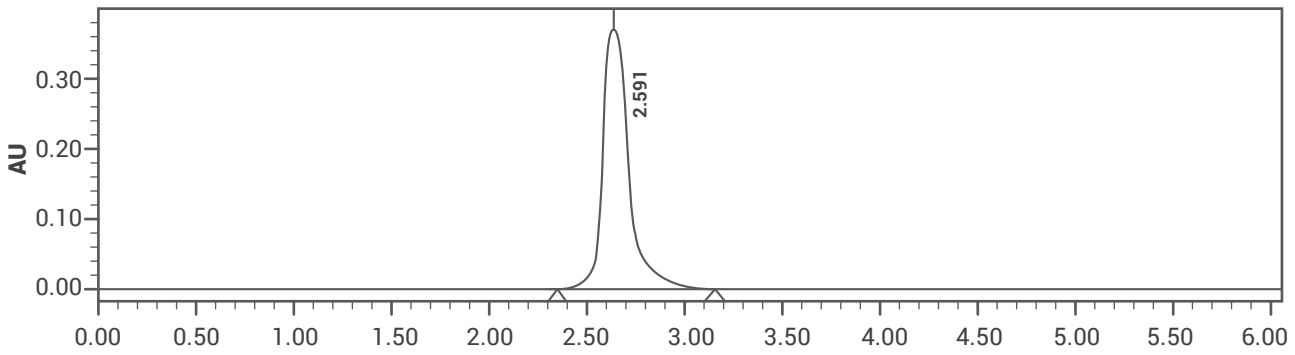
HINNAO™ Vitamin B12



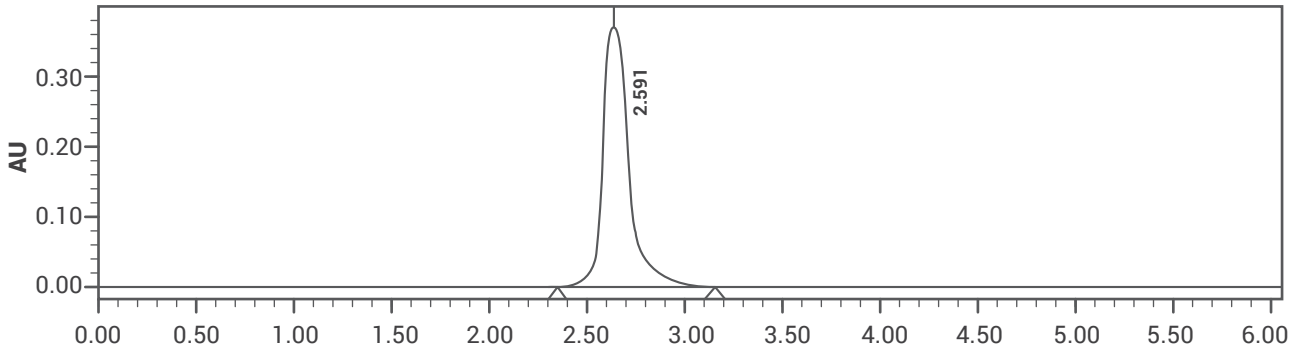
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.588	4742422	218734	1	Vit b12 10 sec



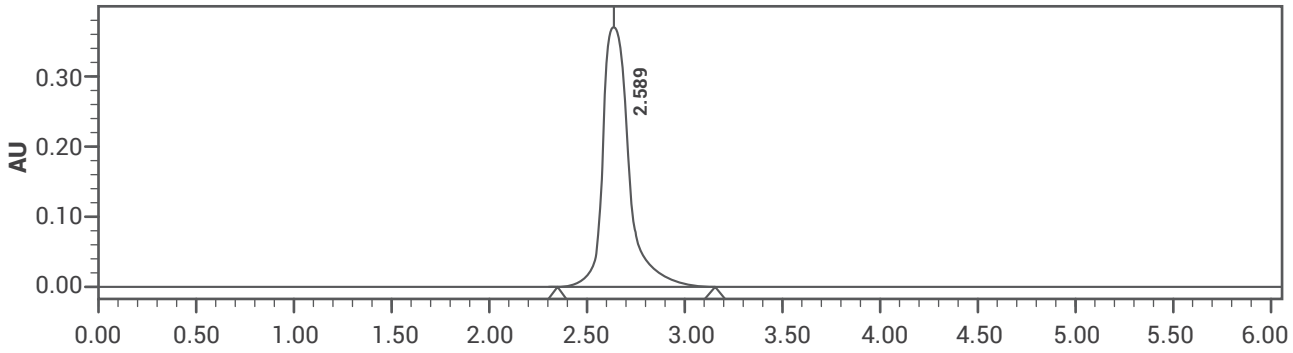
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.589	5842428	229874	1	Vit b12 20 sec



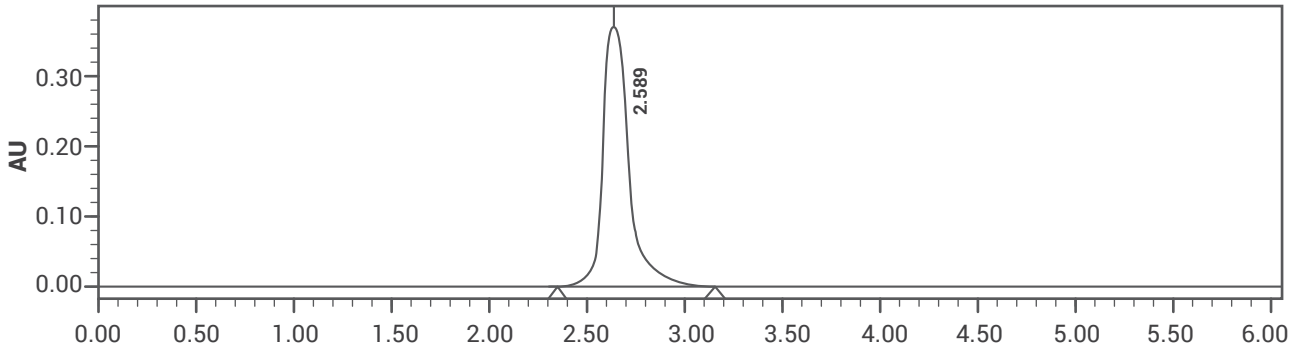
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.591	6852430	218784	1	Vit b12 30 sec



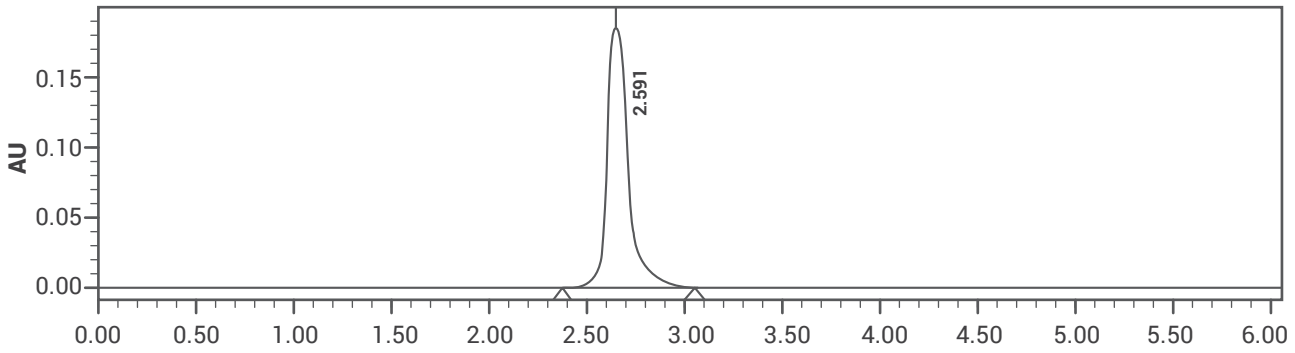
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.590	6862435	216764	1	Vit b12 40 sec



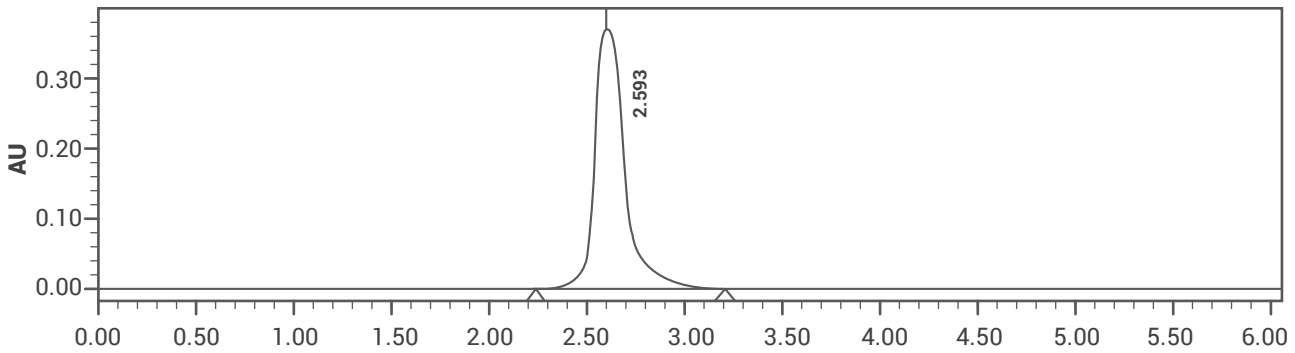
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.580	7863440	318768	1	Vit b12 50 sec



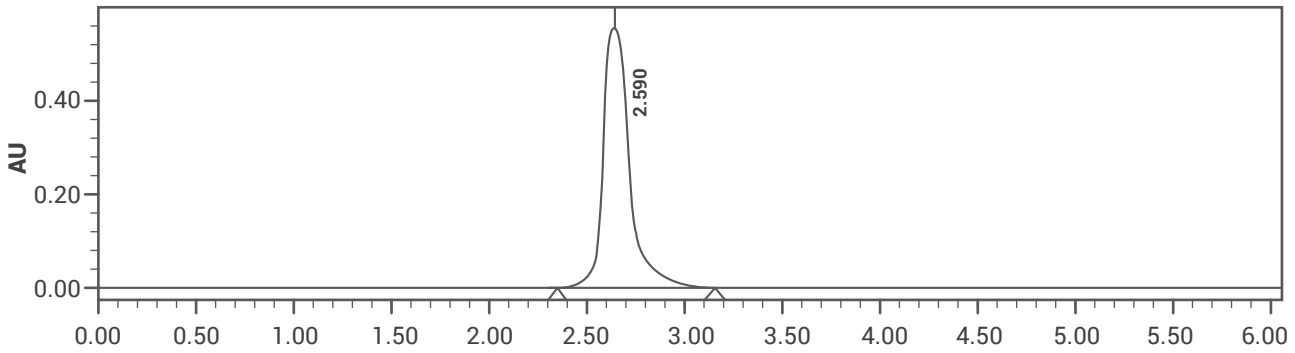
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.588	7872450	328468	1	Vit b12 60 sec



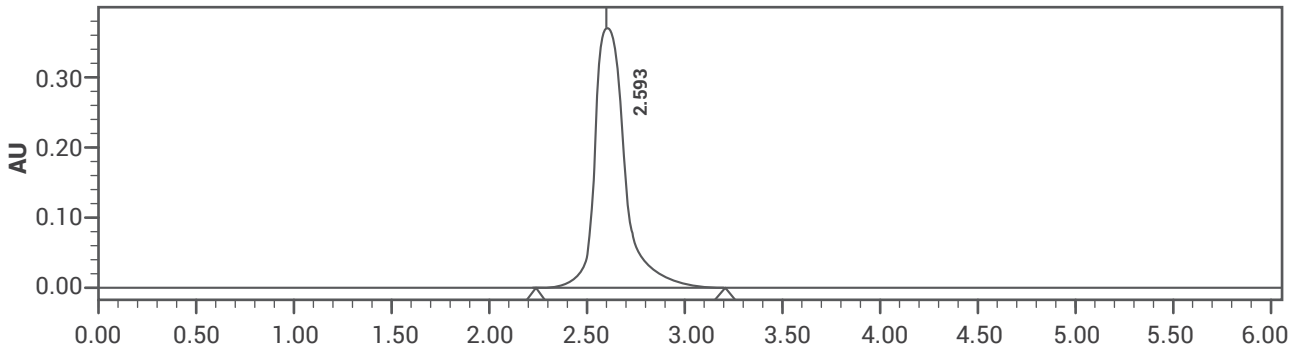
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.591	7982560	328479	1	Vit b12 70 sec



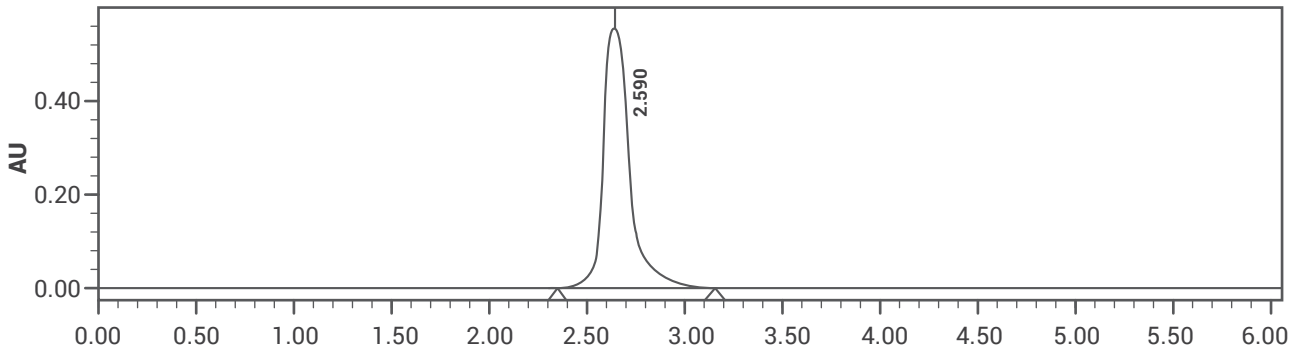
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.593	7983565	328479	1	Vit b12 80 sec



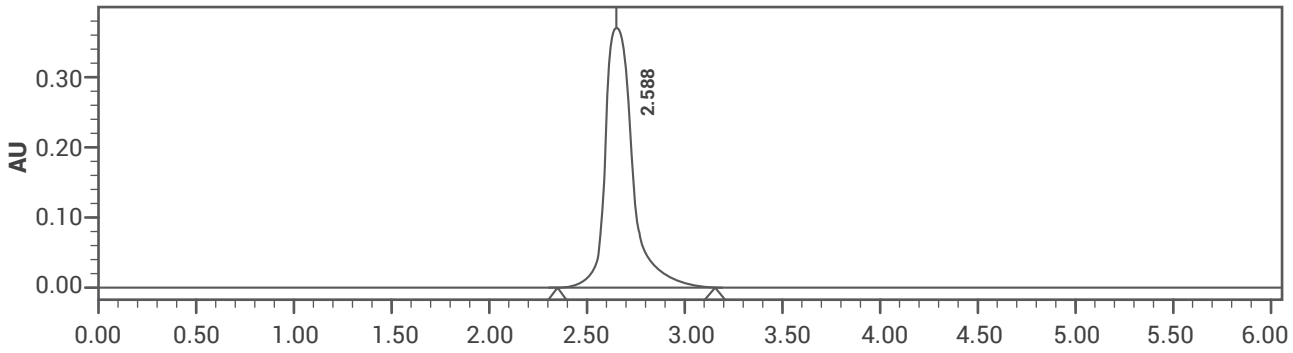
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.590	8984664	228478	1	Vit b12 90 sec



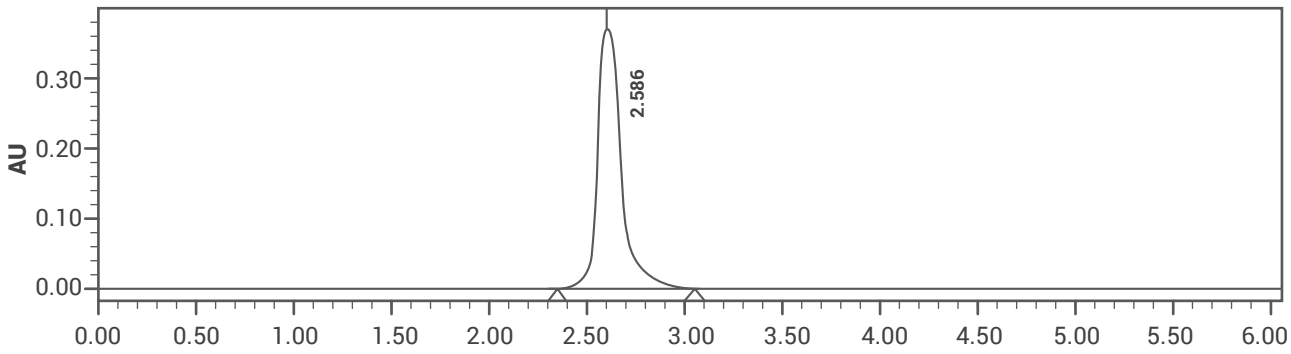
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.588	8985670	238577	1	Vit b12 120 sec



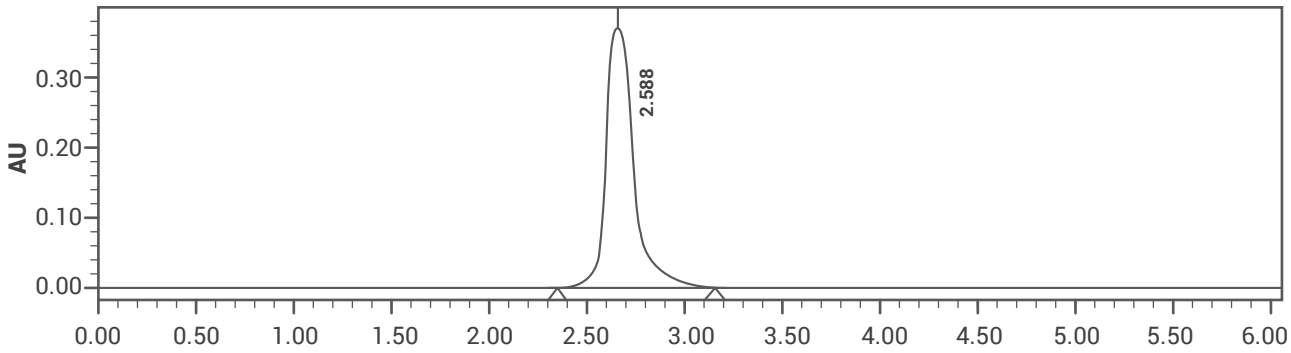
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.586	8986675	437587	1	Vit b12 150 sec



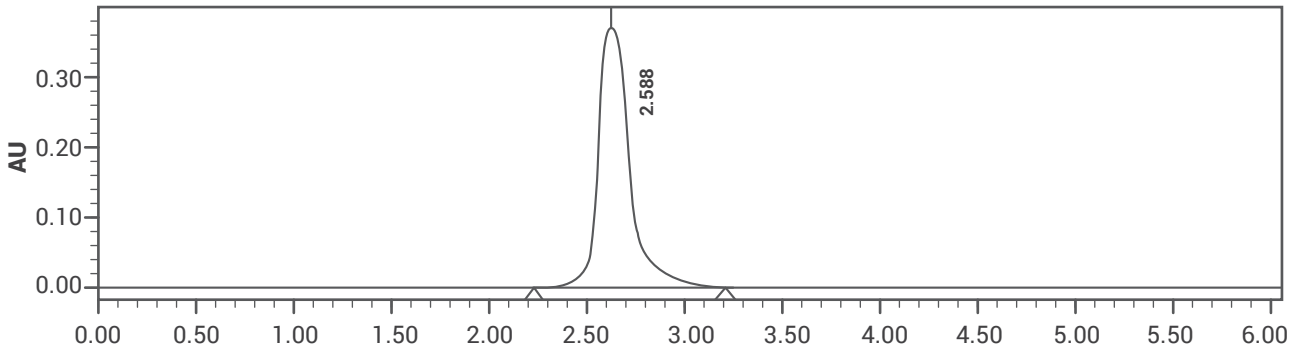
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.589	9987685	448588	1	Vit b12 180 sec



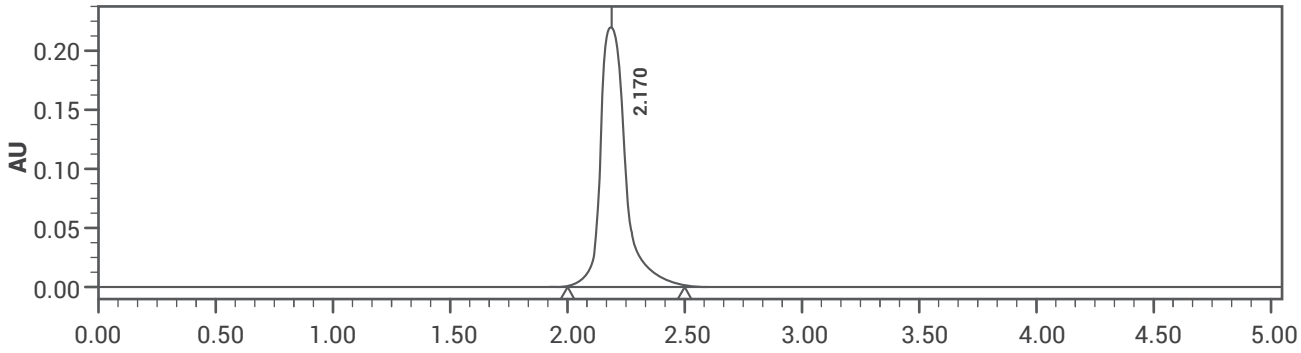
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.586	8986675	437587	1	Vit b12 150 sec



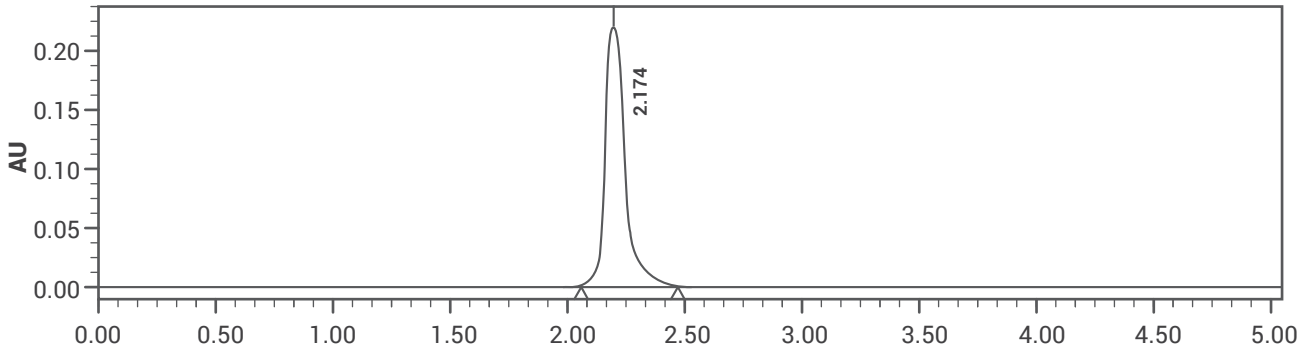
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.589	9987685	448588	1	Vit b12 180 sec



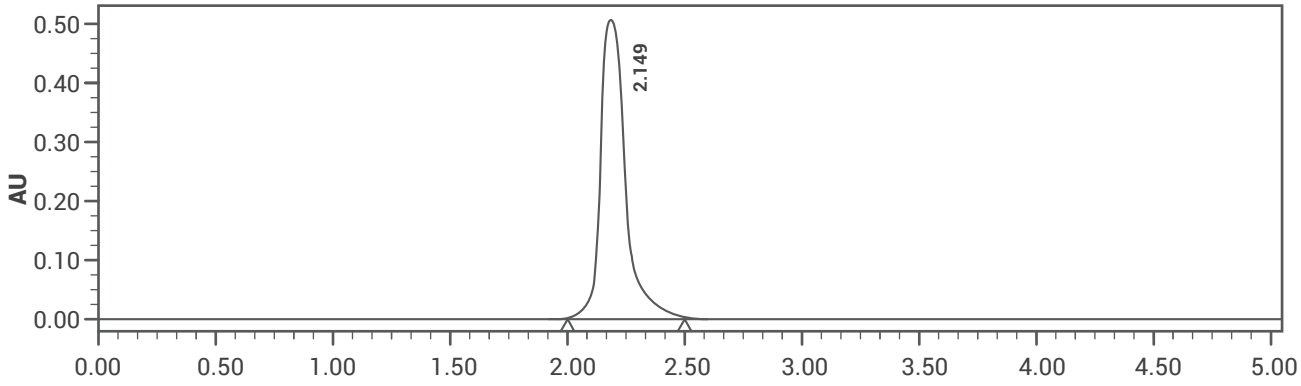
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.586	9988765	448588	1	Vit b12 210 sec



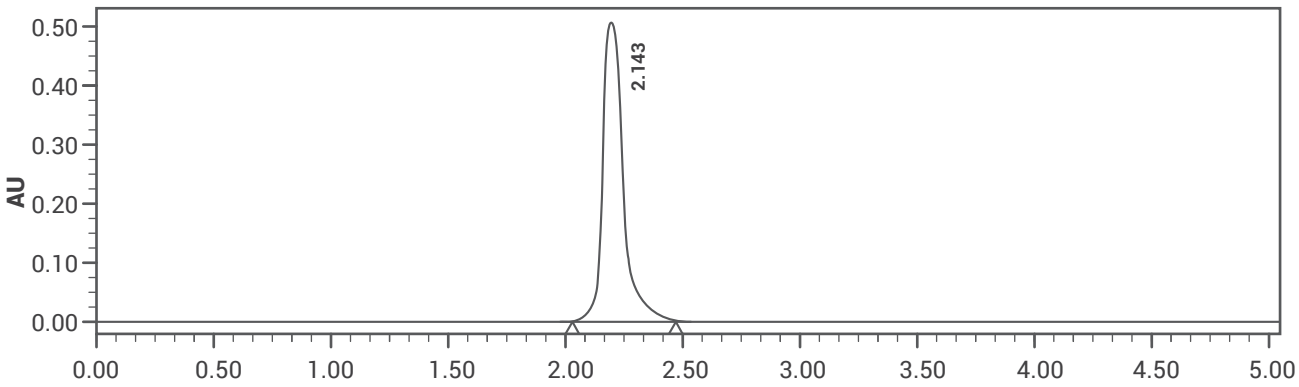
	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.170	885752	864288	1	Vit b12 10 min



	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.174	7834732	753250	1	Vit b12 15 min



	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.149	7632621	743140	1	Vit b12 25 min



	Peak Name	RT	Area	Height	Injection	SampleName
1	vitamin b12	2.143	5322411	713240	1	Vit b12 45 min

FLUX VALUES

Time (sec)	Dose (mg/ml)	Slope	Area (f1)	Y INER-CEPT	CONCNETRA-TION	AMT DRUG RELEASED	% Drug Diffused	Flux
10	1	1007	4742422	2146	4707.324727	117.6831182	17.6831182	62.59740328
20	1	1007	5842428	2146	5799.684211	144.9921053	44.9921053	154.2469205
30	1	1007	6852430	2146	6802.665343	170.0666336	70.0666336	271.3829259
40	1	1007	6862435	2146	6812.600794	170.3150199	70.3150199	362.3723827
50	1	1007	7863440	2146	7806.647468	195.1661867	95.1661867	519.0590072
60	1	1007	7872450	2146	7815.594836	195.3898709	95.3898709	623.5846944
70	1	1007	7982560	2146	7924.939424	198.1234856	98.1234856	737.6938294
80	1	1007	7983565	2146	7925.937438	198.1484359	98.1484359	843.1848338

*Amount drug released=Amount of drug diffused
%drug released=%drug diffused

The flux value for 3 minutes was 2373.540826 for 45 minutes it was 18969.23068 the decrease in the values was observed due to super saturation