3.2.P.5.3 Validation Report for Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Abbreviations
LEF – Laboratório de Estudos Farmacêuticos

References
LEF Study no. PEI/2009/00190, Revision 1: «Relatório de Validação Analítica: Metodologia Analítica de Cromatografia Líquida de Alta Pressão para Doseamento de Aminoácidos no Medicamento Ketosteril® comprimidos»
P.6.00013_00_i: «Ketosteril Tablets 100/300»

Materials and Methods

Equipments
Chromatographic Systems
System 1 and 2
Separations Module Waters 2695 Alliance, Photodiode Array Detector Waters 2996, Column Waters AccQ-Tag, 150mmx3.9mm, 4µm

Other Equipments
Analytical Scale Sartorius R200D, Scale Recorder Sartorius YDP03 OCE, Mettler Scale AB204-S, Mettler Scale Recorder RS-P42, Mettler Scale AE24OS, Eppendorf Centrifugation Equipment 5702R, Ultrasonic Bath Sonorex RK51OS, Water Bath GFL 1013, Magnetic stirrer Variomag Telesystem HP 15, Transferpettor Micropipette 100µL Brand, VWR Micropipette 10-100µL, Finnpipette Micropipette 20-200µL Thermolabsystems

Reagents and Others
AccQ.Tag Eluent A Waters, AccQ-Fluor Reagent Kit Waters, Acetonitrile FISHER, 37% Hydrochloric Acid Merck, PALL ACRODISC 0.45µm GHP Filters, Ultra-purified water obtained by an Ultra Purelab Enkrott system

Reference Substances
The reference substances were sent by the applicant.

Table 3.2.P.5.3- 1 Reference Substances

Name	Batch	Content [%] [(a)]	Expiry date
Lysine acetate	80702535	99.80	May 2011
Tyrosine	80501838	99.40	Mar. 2012
Histidine	80602251	99.40	Mar. 2010
Threonine	80501850	100.60	Jan. 2014
Tryptophan	80501789	100.10	Oct. 2010

[(a)] content “as is”

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Samples

Table 3.2.P.5.3- 2 Identification of Samples

LEF Code	Identification	Batch	Exp. Date
Finished Products			
PEI/2009/00190	Ketosteril Tablets	D24061/D24062	July 2011
Placebo			
ASS-PEI/2009/00190-01	Placebo with Ketoacids and Hydroxyacids	-	-

Table 3.2.P.5.3- 3 Finished Product Composition

Component	Amount per tablet
Keto and Hydroxy Acids	
α-Ketovaline, Calcium Salt	86.0 mg
α-Ketoisoleucine, Calcium Salt	67.0 mg
α-Ketoleucine, Calcium Salt	101.0 mg
α-hydroxymethionine, Calcium Salt	59.0 mg
α-Ketophenylalanine, Calcium Salt	68.0 mg
Amino Acids	
Lysine acetate	105.0 mg
Threonine	53.0 mg
Tryptophan	23.0 mg
Histidine	38.0 mg
Tyrosine	30.0 mg
Excipients	
Excipients	170.0 mg
Total	**800.0 mg**

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Analytical Conditions

Column: AccQ-Tag, 150 mm x 3.9mm, 4µm
Mobile Phase A: 100 mL concentrated solution of AccQ-Tag eluent A to 1000 mL with water
Mobile Phase B: Acetonitrile
Mobile Phase C: Water
Gradient: see following table

Table 3.2.P.5.3- 4 Gradient Conditions

Time [min]	A [%]	B [%]	C [%]	Curve
0	100	0	0	-
0.5	99	1	0	11
18	95	5	0	6
19	91	9	0	6
29.5	83	17	0	6
35	0	60	40	11
38	100	0	0	11
60	0	0	100	11
90	0	60	40	11
120	0	60	40	6
121	100	0	0	6
125	100	0	0	-
135	100	0	0	- *

* Running time was extended to 135min to guarantee the column stabilization prior to a new injection

Flow: 1.0 mL/min
Detection: Ultra-violet – 248nm
Injection Volume: 10 µL
Column Temperature: Room Temp.
Injector Temperature: 20 ºC
Solvent: HCl 0.02 M

Derivatization Reagent: Waters AccQ-Fluor Reagent Kit consisting of AccQ-Fluor Borate Buffer (vial 1), AccQ-Fluor Reagent Powder (vial 2A) and AccQ-Fluor Reagent Diluent (vial 2B).
Reconstitution of Derivatization Reagent: Pipette 1.0 mL of vial 2B (after discarding the 1st mL) into vial 2A – that should be previously tapped – cap and vortex during 10sec. Finally heat at 55 ºC with occasional agitation until powder complete dissolution. This reconstituted reagent has a shelf life of 1 week of and should be kept in a desiccator at room temperature. (The derivatization reagent will be henceforward designated as Derivatizing I and the AccQ-Fluor Borate Buffer (vial 1) as Derivatizing II).

Table 3.2.P.5.3- 5 Working Concentrations

Substance	C [µg/ mL]
Lysine acetate	21.0
Threonine	10.6
Tryptophan	4.6
Histidine	7.6
Tyrosine	6.0

Solutions Preparation
Selectivity
Solvent: HCl 0.02 M (dilute 1.67 mL HCl 37 % to 1000 mL with H_2O). Derivatize the solvent – see table "Derivatization reaction". The solvent was analyzed prior and after filtration (filter GHP 0.45 µm).

Table 3.2.P.5.3- 6 Individual Solutions

Substance	Stock Solution			Intermediate Solution			Final Solution[(b)]	
	Sample drawn [mg]	V [mL] [(a)]	C [µg/ mL]	Aliquot [mL]	V [mL]	C [µg/mL]	Derivatization Rx	C [µg/mL]
Lysine acetate	52.61	50	1050	5	25	210		21.0
Threonine	26.34	50	530	5	25	106		10.6
Tryptophan	11.49	50	230	5	25	46		4.6
Histidine	19.12	50	380	5	25	76		7.6
Tyrosine	15.09	50	300	5	25	60		6.0

a) Solvent was added and submitted to ultrasonic bath at room temperature for approx. 5min, the volume was completed with the same solvent
b) The intermediate solution was submitted to derivatization reaction

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Table 3.2.P.5.3- 7 Reference Solution

Substance	Stock Solution			Intermediate Solution			Final Solution[(b)]	
	Sample drawn [mg]	V [mL] [(a)]	C [µg/ mL]	Aliquot [mL]	V [mL]	C [µg/mL]	Derivatization Rx	C (µg/mL)
Lysine acetate	52.61	50	1050	5	25	210		21.0
Threonine	26.34		530			106		10.6
Tryptophan	11.49		230			46		4.6
Histidine	19.12		380			76		7.6
Tyrosine	15.09		300			60		6.0

a) Solvent was added and submitted to ultrasonic bath at room temperature for approx. 5min, the volume was completed with the same solvent
b) The intermediate solution was submitted to derivatization reaction.

Table 3.2.P.5.3- 8 Sample Solution

Substance	Stock Solution			Intermediate Solution			Final Solution [(d)]	
	Sample drawn [mg] [(a)]	V [mL] [(b,c)]	C [µg/mL]	Aliquot [mL]	V [mL]	C [µg/mL]	Derivatization Rx	C [µg/mL]
Lysine acetate	≈800	100	1050	5	25	210		21.0
Threonine			530			106		10.6
Tryptophan			230			46		4.6
Histidine			380			76		7.6
Tyrosine			300			60		6.0

a) Tablets sample reduced to powder (≈800mg, equivalent to 1 tablet) b) Solvent was added and submitted to magnetic agitation during 60min, afterwards submitted to ultrasonic bath at room temperature for approx. 10 min, and finally the volume was completed with the same solvent
c) Filtration by GHP 0.45µm filter d) The intermediate solution was submitted to derivatization reaction.

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Table 3.2.P.5.3- 9 Placebo Solution

Stock Solution			Intermediate Solution			Final Solution [d]	
Sample drawn [mg] [a]	V [mL] [b,c]	C [µg/mL]	Aliquot [mL]	V [mL] [d]	C [µg/mL]	Derivatization Rx	C [µg/mL]
≈551	100	-	5	25	-		-

a) The placebo contains the Keto acids, Hydroxid acids and Excipients (≈551mg, equivalent to 1 tablet). b) Solvent was added and submitted to magnetic agitation during 60min, afterwards submitted to ultrasonic bath at room temperature for approx. 10min, and finally the volume was completed with the same solvent
c)Filtration by GHP 0.45µm filter
d)The intermediate solution was submitted to derivatization reaction.

Derivatization Reaction

In a glass tube, of approx. 1 cm of diameter and 10 cm of height, introduce:

Table 3.2.P.5.3- 10 Derivatization Reaction

Solution	Aliquot [µL][a]	Derivatizing I [µL]	Derivatizing II [µL] [b]
Reference	40	280	80
Sample	40	280	80
Placebo	40	280	80

Derivatizing I – derivatizing reagent reconstituted (vial 2A+ vial 2B)
Derivatizing II – AccQ-Fluor Borate Buffer (vial 1)

a) Aliquot from respective intermediate solutions
b) After adding derivatizing II it was submitted to vortex for approx. 15sec, it was left at rest for 1 min at room temperature and incubated at 55 ºC for 10 min.

Linearity

Table 3.2.P.5.3- 11 Stock solution

Substance	Stock Solution		
	Sample drawn [mg]	V [mL] [a]	C [µg/mL]
Lysine acetate	105.22	100	1050
Threonine	52.68		530
Tryptophan	22.98		230
Histidine	38.23		380
Tyrosine	30.18		300

a) Solvent was added and submitted to ultrasonic bath at room temperature for approx. 5min, the volume was completed with the same solvent

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

The following solutions were prepared from the stock solution:

Table 3.2.P.5.3- 12 Preparation of Solutions

Solution [a]	Intermediate Solution		Final Solution[b]
	Aliquot [mL]	V [mL]	
50.0%	2.5	25	Derivatization Rx
80.0%	4	25	
100.0%	5	25	
120.0%	6	25	
150.0%	6	20	

a) Percentage related to working concentration b) Intermediate solution was submitted to derivatization reaction

The respective concentrations are:

Table 3.2.P.5.3- 13 Concentration of the solutions

Substance	Solution[a]				
	50.0%	80.0%	100.0%	120.0%	150.0%
	C [µg/mL]				
Lysine acetate	10.50	16.80	21.00	25.20	31.50
Threonine	5.30	8.48	10.60	12.72	15.90
Tryptophan	2.30	3.68	4.60	5.52	6.90
Histidine	3.80	6.08	7.60	9.12	11.40
Tyrosine	3.00	4.80	6.00	7.20	9.00

(a) Percentage related to working concentration

Accuracy

Sample solutions

Placebo samples spiked with lysine acetate, threonine, tryptophan, histidine and tyrosine were prepared at 3 levels of concentration, corresponding to 80 %, 100 % and 120 % of working concentration, 3 replicates of each level, in a total of 9 determinations. The solutions were prepared according to the following table:

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Table 3.2.P.5.3- 14 Accuracy Stock Solutions

Level [(a)]	Substance	Stock Solution			
		Sample drawn [mg]	Placebo [mg] [(b)]	V [mL][(c,d)]	C [µg/ mL]
80 %	Lysine acetate	84.17	≈551	100	840.0
	Threonine	42.15			424.0
	Tryptophan	18.39			184.1
	Histidine	30.59			304.1
	Tyrosine	24.15			240.1
100 %	Lysine acetate	52.61	≈361	50	1050.0
	Threonine	26.34			530.0
	Tryptophan	11.49			230.0
	Histidine	19.12			380.0
	Tyrosine	15.09			300.0
120 %	Lysine acetate	63.13	≈361	50	1260.0
	Threonine	31.61			636.0
	Tryptophan	13.79			276.0
	Histidine	22.94			456.0
	Tyrosine	18.11			360.0

a) Percentage related to working concentration b) The placebo contains the Keto acids, Hydroxid acids and Excipients. c) Solvent was added and submitted to magnetic agitation during 60min, afterwards submitted to ultrasonic bath at room temperature for approx. 10min, and finally the volume was completed with the same solvent

d) Filtration by GHP 0.45µm filter

The following solutions were prepared from the respective stock solutions:

Table 3.2.P.5.3- 15 Accuracy Sample Solutions

Level[a]	Substance	Intermediate Solution			Final Solution[b]	
		Aliquot [mL]	V [mL]	C [µg/mL]		C [µg/mL]
80 %	Lysine acetate	5	25	168.0	Derivatization Rx	16.80
	Threonine			84.8		8.48
	Tryptophan			36.8		3.68
	Histidine			60.8		6.08
	Tyrosine			48.0		4.80
100 %	Lysine acetate	5	25	210.0	Derivatization Rx	21.00
	Threonine			106.0		10.60
	Tryptophan			46.0		4.60
	Histidine			76.0		7.60
	Tyrosine			60.0		6.00
120 %	Lysine acetate	5	25	252.0	Derivatization Rx	25.20
	Threonine			127.2		12.72
	Tryptophan			55.2		5.52
	Histidine			91.2		9.12
	Tyrosine			72.0		7.20

a) Percentage related to working concentration b) Intermediate solution was submitted to derivatization reaction

Reference Solution
A solution of the reference substances at working concentration was prepared as described in Selectivity.

Precision

System Repeatability
A solution of reference substances at working concentration was prepared as described in Selectivity.

Analysis Repeatability
Sample Solutions
6 sample replicates were prepared at working concentration as described in Selectivity.

Reference Solution
A solution of reference substances at working concentration was prepared as described in Selectivity.

Intermediate Precision
2 operators prepared independently, in different days, 6 sample replicates and 1 reference solution, as described in Analysis Repeatability.

Solutions Stability
Sample solution prepared as described in Selectivity.
Solution of reference substances at working concentration prepared as described in Selectivity.
These solutions were stored at room temperature, protected from light during 18h.

Chromatographic System Suitability
Solution of reference substances at working concentration prepared as described in Selectivity.

Results
Selectivity
The method selectivity was evaluated by the analysis of:
- Mobile phase
- Filtered and non-filtered solvent
- Individual solutions of Amino acids at working concentration
- Reference solution at working concentration
- Placebo solution at a concentration equivalent to the sample solution
- Sample solution at working concentration

Acceptance criteria
The peaks corresponding to the substances of interest should be separated between themselves, that is, the resolution factors should be ≥1.5.
See the results of method selectivity in the following table.

Table 3.2.P.5.3-16 Selectivity

Solution	Identification	Rt [min]	RRt	RF
Mobile phase	-	-	-	-
Solvent (HCl 0.02M + Derivatization Reag.) non-filtered	- -	11.187 92.087	- -	- -
Solvent (HCl 0.02M + Derivatization Reag.) filtered	- -	11.742 92.601	- -	- -
Placebo	Solvent Solvent	11.026 92.071	- -	- -

Rt: Retention time RRt: Tryptophan relative retention time RF: Resolution factor in relation to the previous analyte peak

Table 3.2.P.5.3-16 Selectivity (cont.)

Solution	Identification	Rt (min)	RRt	RF
Histidine individual solution	Solvent Histidine Solvent	11.055 18.027 92.084	- - -	- - -
Threonine individual solution	Solvent Threonine Solvent	11.102 21.337 92.097	- - -	- - -
Tyrosine individual solution	Solvent Tyrosine Solvent	11.088 26.600 92.083	- - -	- - -
Lysine individual solution	Solvent Lysine Solvent	11.233 29.661 92.083	- - -	- - -
Tryptophan individual solution	Solvent Tryptophan Solvent	11.100 35.289 92.084	- - -	- - -
Reference solution	Solvent Histidine Threonine Tyrosine Lysine acetate Tryptophan Solvent	11.072 18.020 21.227 26.589 29.550 35.063 92.083	0.32 0.51 0.60 0.76 0.84 1.00 2.63	- - 14.05 32.91 15.48 20.83 -
Sample solution (D24062 batch)	Solvent Histidine Threonine Tyrosine Lysine acetate Tryptophan Solvent	11.147 18.312 21.305 26.731 29.940 35.997 92.071	0.31 0.51 0.59 0.74 0.83 1.00 2.56	- - 13.38 33.15 16.31 21.01 -

Rt: Retention time RRt: Tryptophan relative retention time RF: Resolution factor in relation to the previous analyte peak

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Chromatograms

Figure 3.2.P.5.3- 1: Mobile Phase

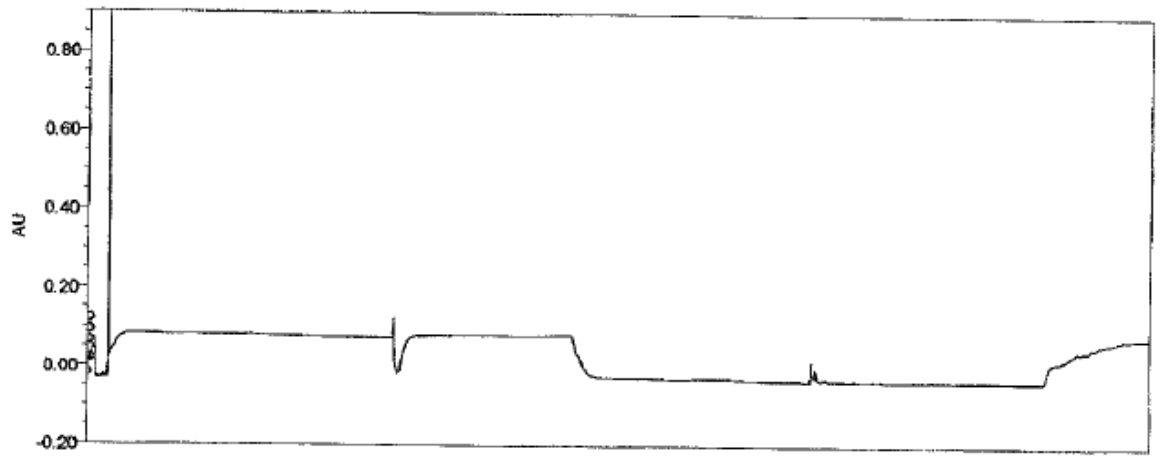


Figure 3.2.P.5.3- 2 Unfiltered Solvent

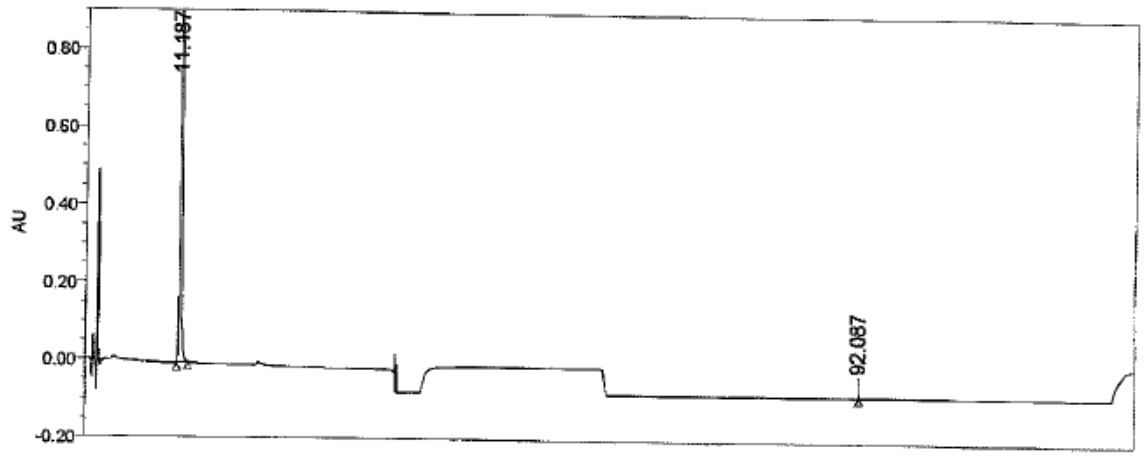


Figure 3.2.P.5.3- 3 Filtered Solvent

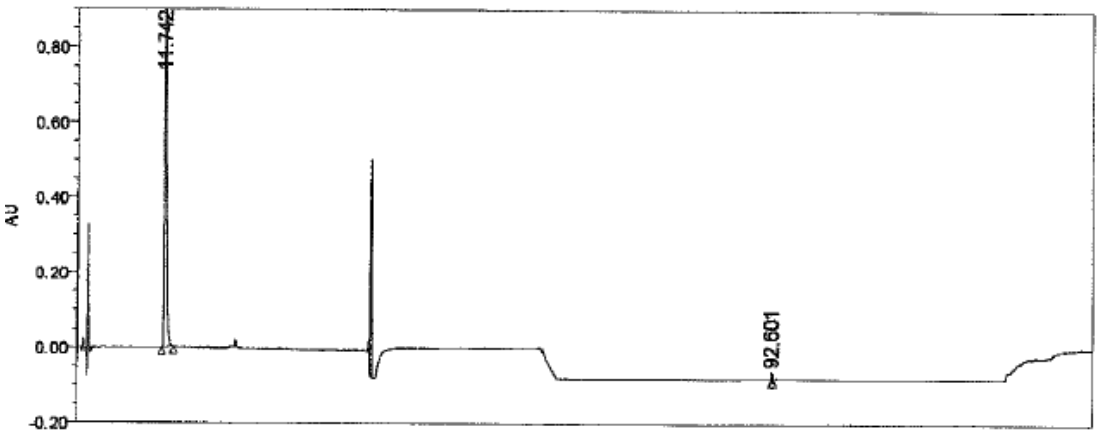


Figure 3.2.P.5.3- 4 Histidine individual reference solution at Working Concentration

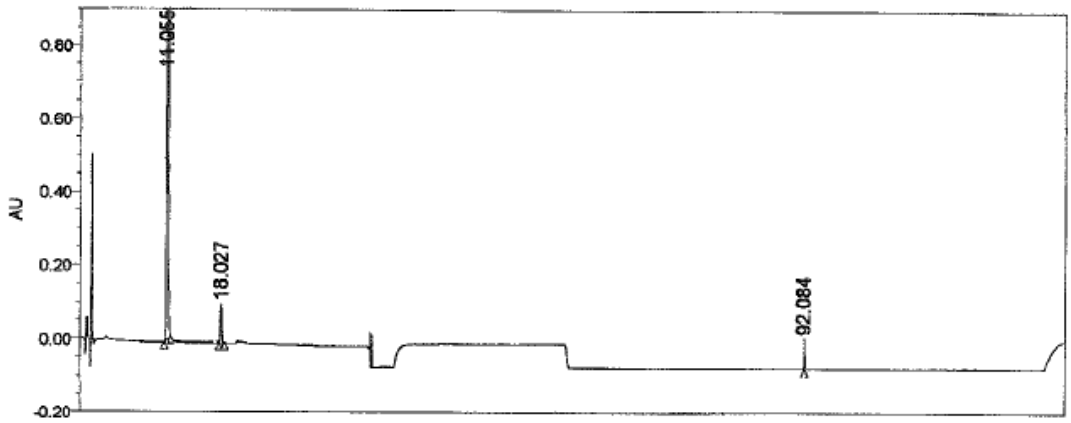


Figure 3.2.P.5.3- 5 Threonine individual reference solution at Working Concentration

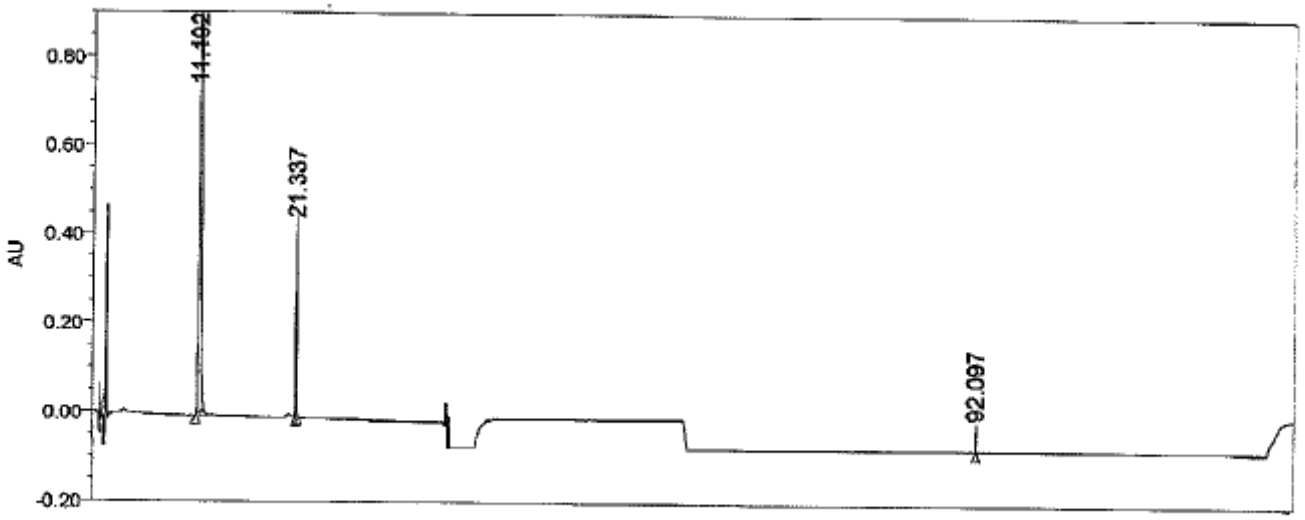


Figure 3.2.P.5.3- 6 Tyrosine individual reference solution at Working Concentration

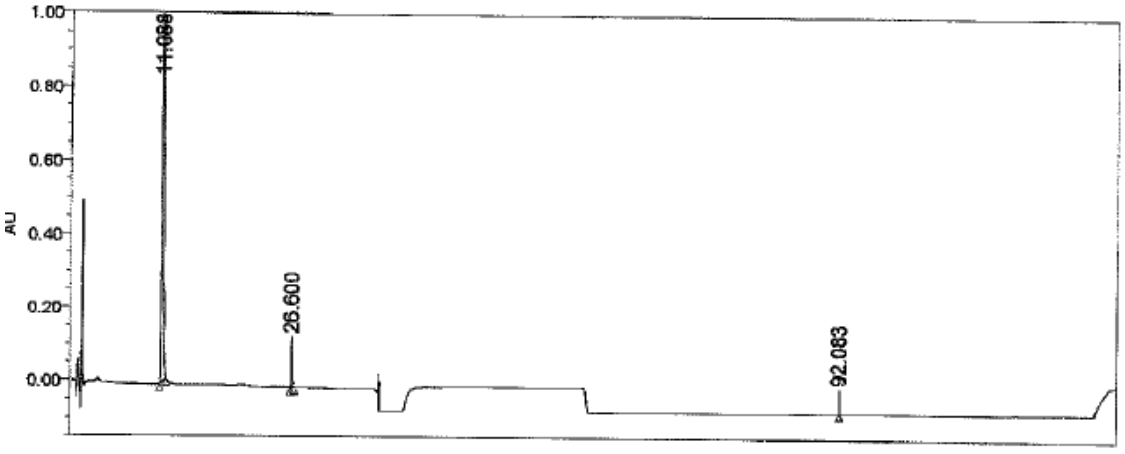


Figure 3.2.P.5.3- 7 Lysine individual reference solution at Working Concentration

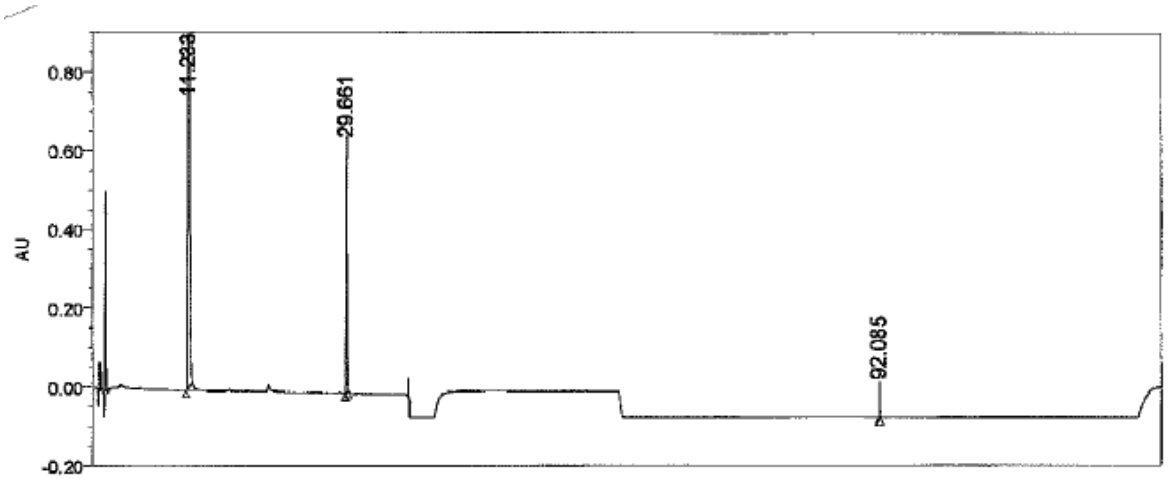


Figure 3.2.P.5.3- 8 Tryptophan individual reference solution at Working Concentration

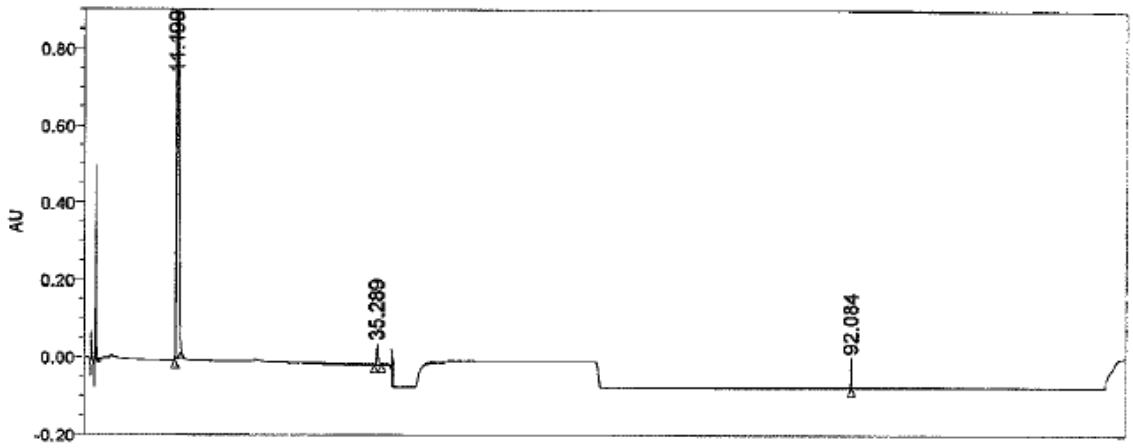


Figure 3.2.P.5.3- 9 Reference Solution at Working Concentration

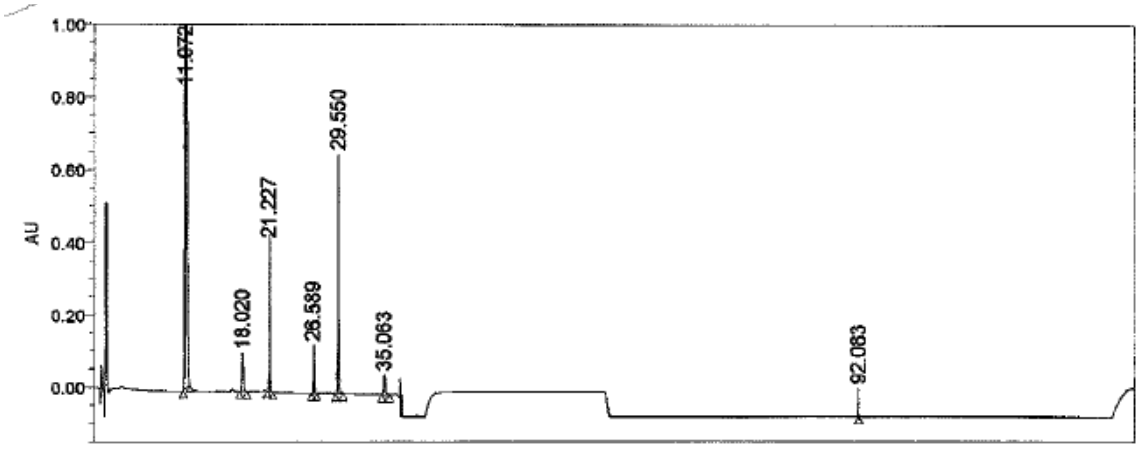


Figure 3.2.P.5.3- 10 Placebo

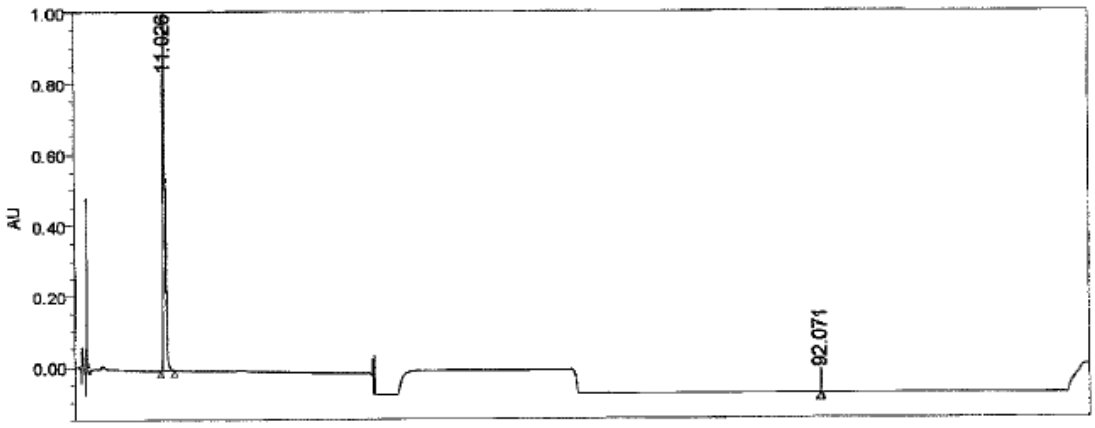
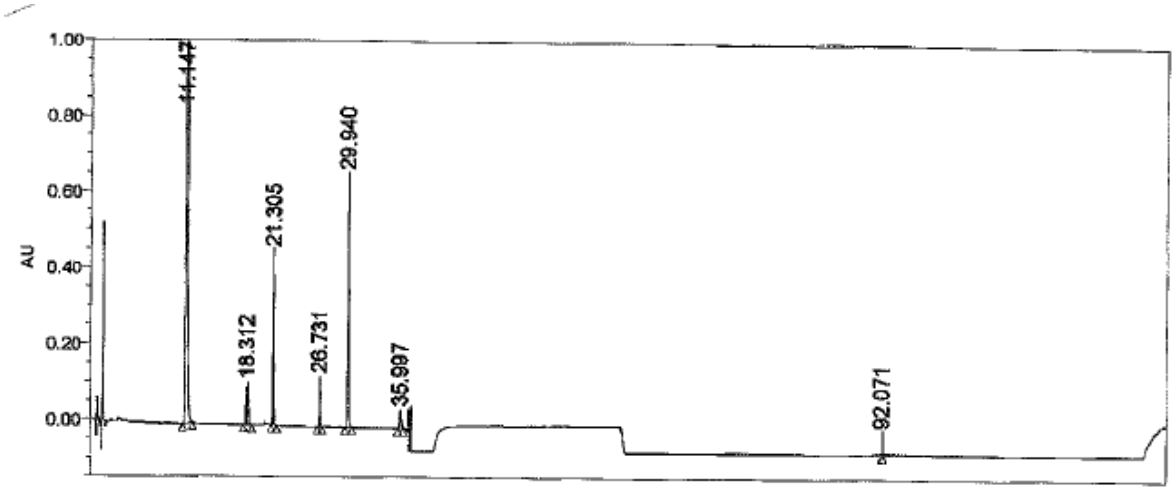


Figure 3.2.P.5.3- 11 Sample solution



Conclusion

The results are according to the acceptance criteria, showing that the analytical method is selective for the assay of amino acids.

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

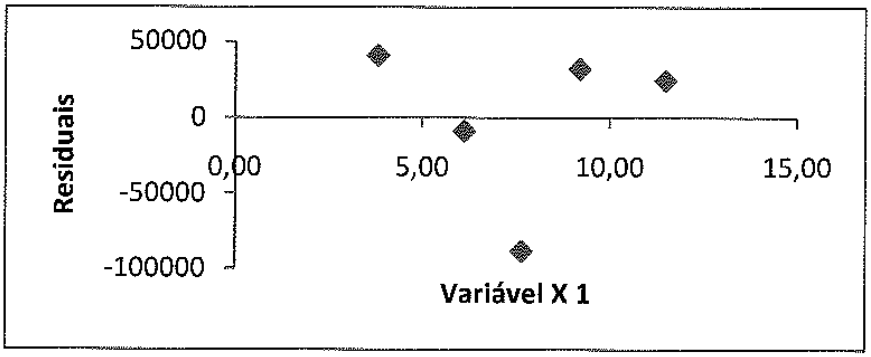
Linearity

The method linearity was evaluated with reference solutions of lysine acetate, threonine, tryptophan, histidine and tyrosine at a concentration range corresponding to 50 % to 150 % of working concentration, using a minimum of 5 standard solutions, 3 injections per solution. Using the least squares method, the linear regression of the analytical results against the concentration of solutions was calculated, and the correlation coefficient (r^2), the slope, the intercept and the respective 95 % confidence limits, and the graphic representation of the regression line were reported. The distribution of residuals was also analysed.

Table 3.2.P.5.3-17 Linearity Parameters for Histidine

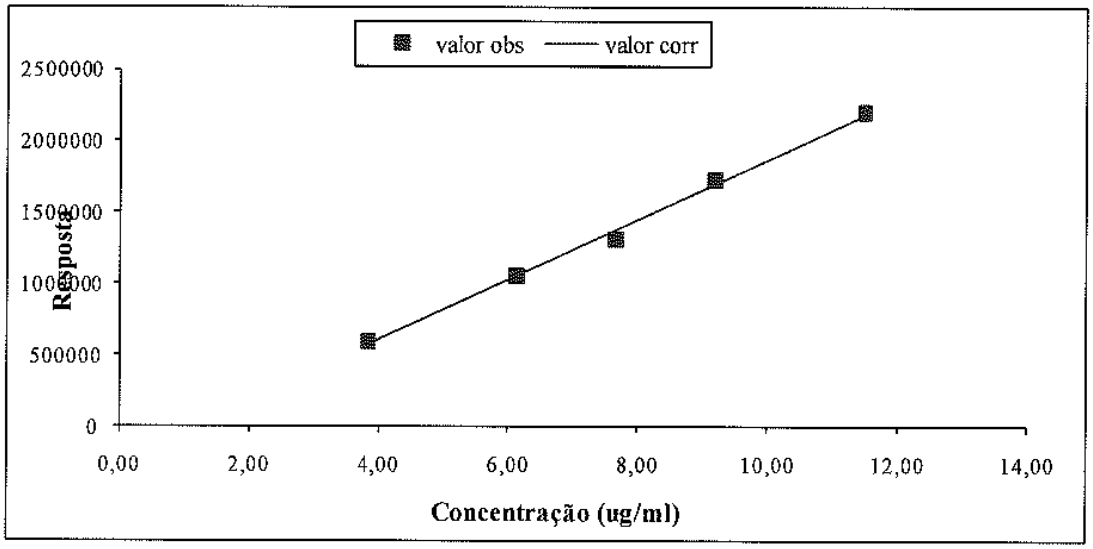
Parameters	Results	Acceptance Criteria
Linearity Range [µg/mL]	3.83; 6.13; 7.67; 9.20; 11.50	-
r^2	0.995731	≥ 0.99
Line equation		
Slope	210512.042	-
Intercept	-237004.418	-
95 % intercept limits	-442151.202/-31857.635	-

Figure 3.2.P.5.3- 12 Graphic Representation of the Distribution of Residuals for Histidine



Residuais = Residuals
Variável X 1= Variable X 1

Figure 3.2.P.5.3- 13 Graphic Representation of the Regression Line for Histidine

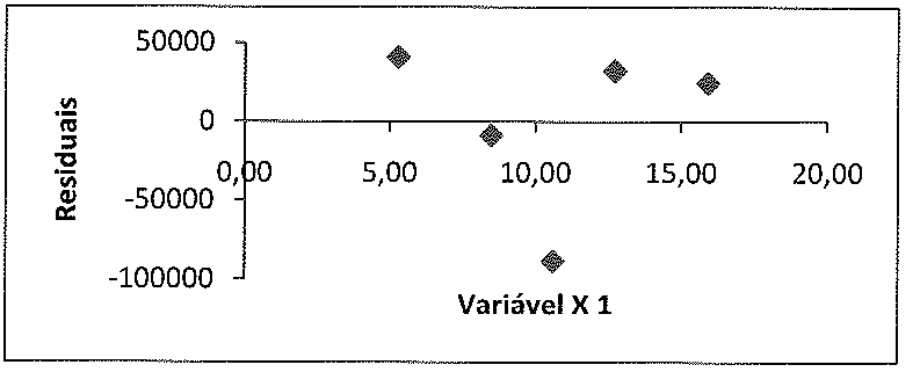


Respostas = Results
Concentração= Concentration

Table 3.2.P.5.3- 18 Linearity Parameters for Threonine

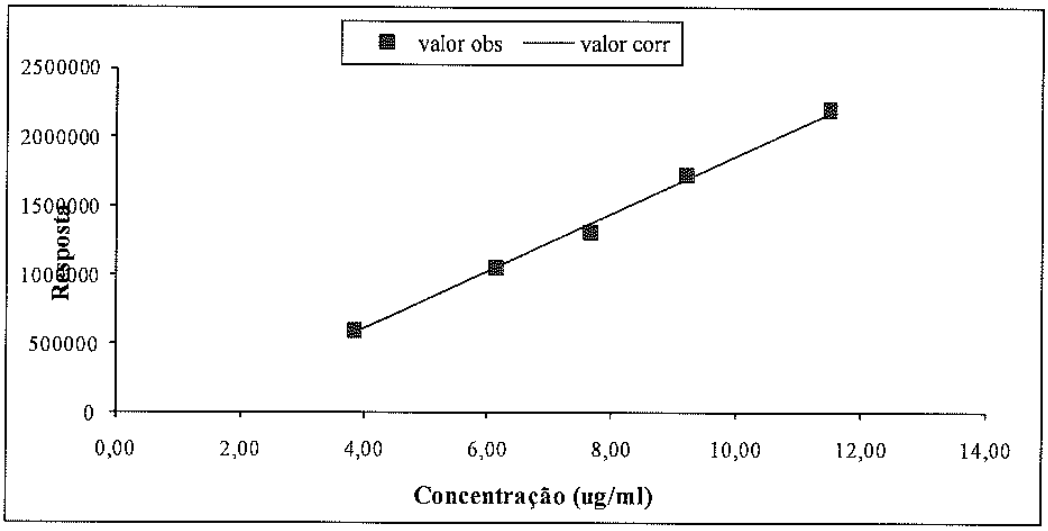
Parameters	**Results**	**Acceptance Criteria**
Linearity Range [µg/mL]	5.31; 8.49; 10.61; 12.73; 15.92	-
R^2	0.995330	≥0.99
Line equation		
Slope	275531.720	-
Intercept	-379634.037	-
95% intercept limits	-768375.982/9107.908	

Figure 3.2.P.5.3- 14 Graphic Representation of the Distribution of Residuals for Threonine



Residuais = Residuals
Variável X 1= Variable X 1

Figure 3.2.P.5.3- 15 Graphic Representation of the Regression Line for Threonine



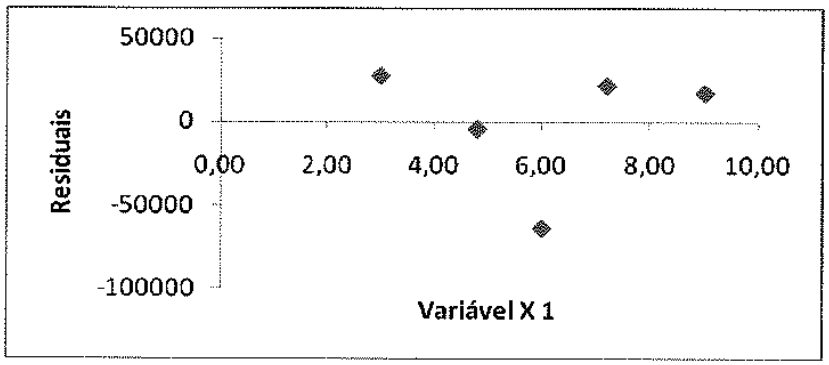
Respostas = Results
Concentração= Concentration

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Table 3.2.P.5.3 – 19 Linearity Parameters for Tyrosine

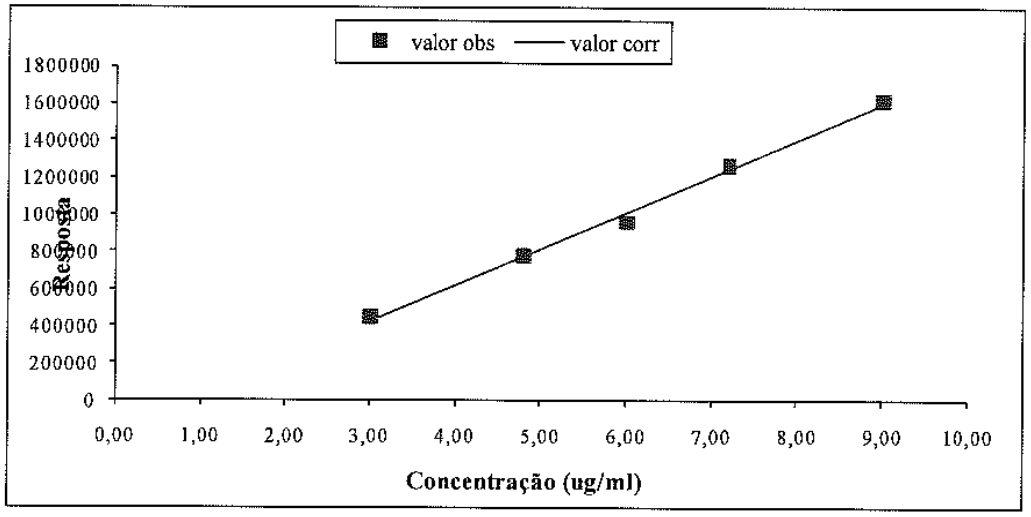
Parameters	Results	Acceptance Criteria
Linearity Range [µg/mL]	3.00; 4.81; 6.01; 7.21; 9.01	-
R^2	0.995058	≥ 0.99
Line equation		
Slope	195549.229	-
Intercept	-162411.897	-
95 % intercept limits	-323161.298/-1662.495	

Figure 3.2.P.5.3- 16 Graphic Representation of the Distribution of Residuals for Tyrosine



Residuais = Residuals
Variável X 1= Variable X 1

Figure 3.2.P.5.3- 17 Graphic Representation of the Regression Line for Tyrosine



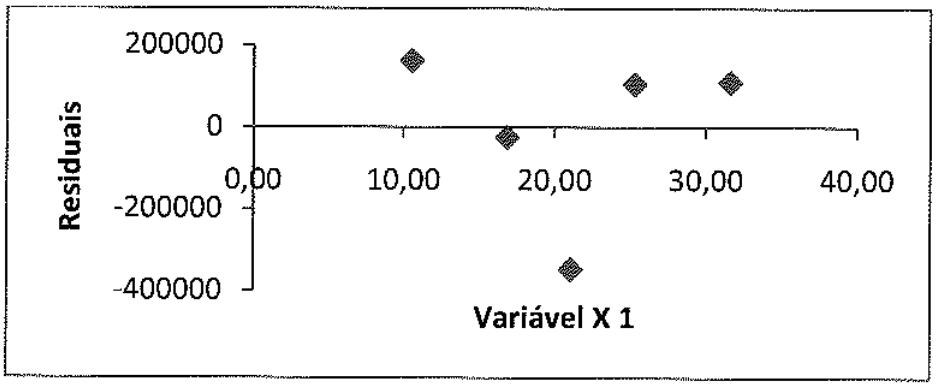
3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Respostas = Results
Concentração= Concentration

Table 3.2.P.5.3- 20 Linearity Parameters for Lysine

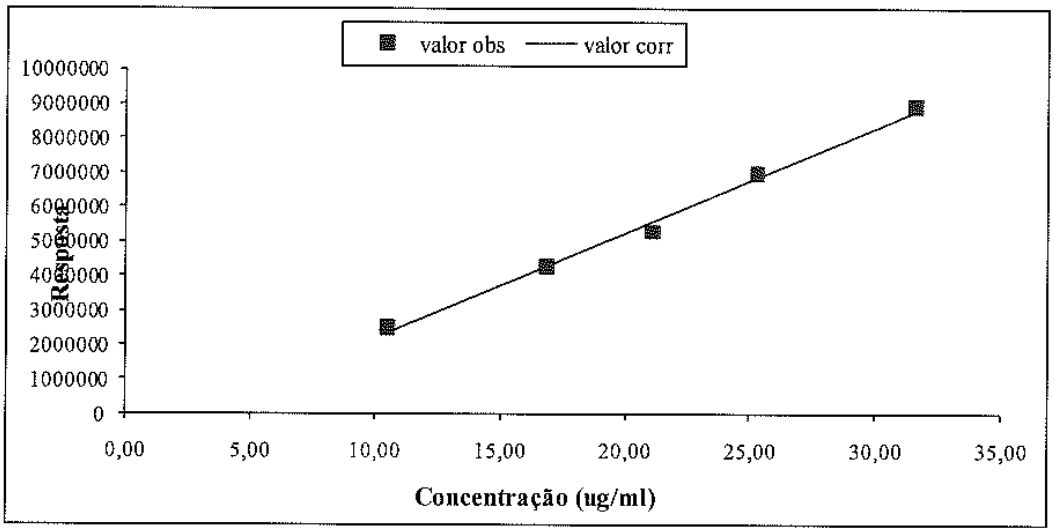
Parameters	Results	Acceptance Criteria
Linearity Range [µg{ml]	10.54; 16.86; 21.08; 25.29; 31.61	-
R^2	0.994470	≥ 0.99
Line equation		
Slope	305938.817	-
Intercept	-875397.177	-
95 % intercept limits	-1808705.96/57911.605	

Figure 3.2.P.5.3- 18: Graphic Representation of the Distribution of Residuals for Lysine



Residuais = Residuals
Variável X 1= Variable X 1

Figure 3.2.P.5.3- 19 Graphic Representation of the Regression Line for Lysine

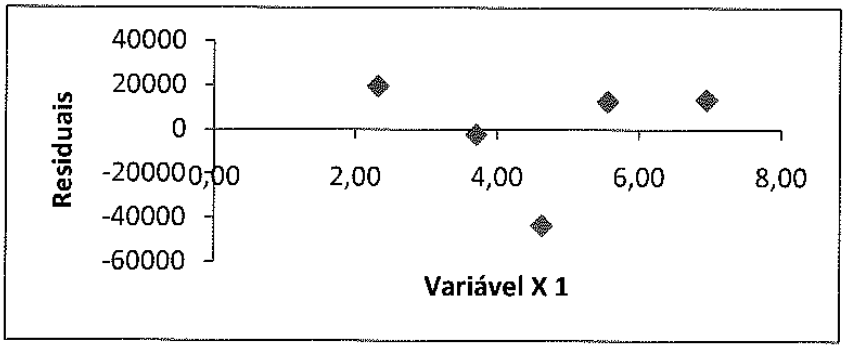


Respostas = Results
Concentração= Concentration

Table 3.2.P.5.3- 21 Linearity Parameters for Tryptophan

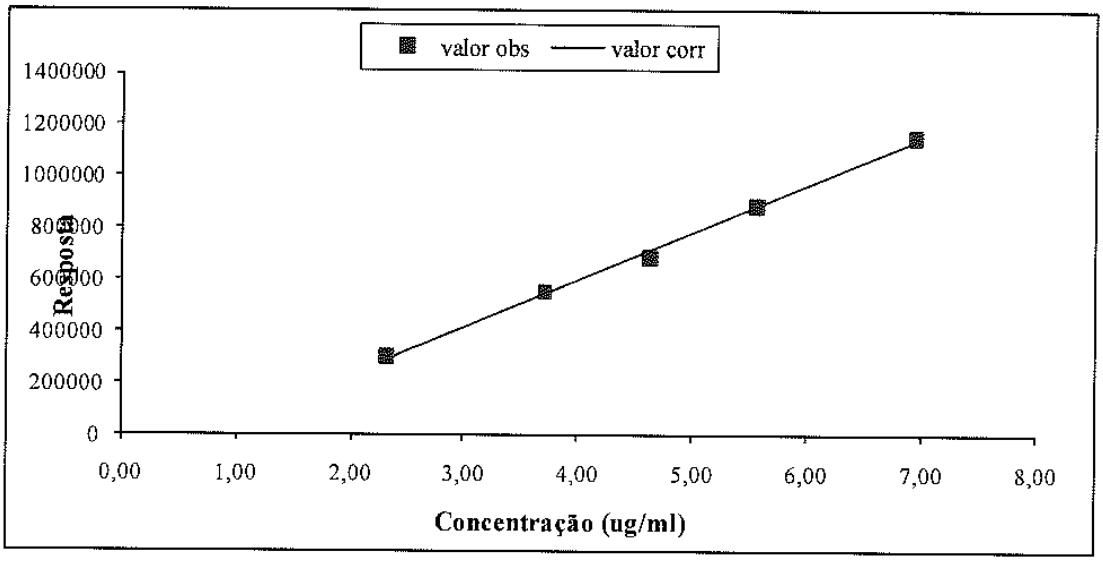
Parameters	Results	Acceptance Criteria
Linearity Range [µg/mL]	2.32; 3.71; 4.63; 5.56; 6.95	-
R^2	0.997333	≥ 0.99
Line equation		
Slope	182915.307	-
Intercept	-132423.237	-
95 % intercept limits	-217521.823 / -47324.650	

Figure 3.2.P.5.3- 20 Graphic Representation of the Distribution of Residuals for Tryptophan



Residuais = Residuals
Variável X 1= Variable X 1

Figure 3.2.P.5.3- 21 Graphic Representation of the Regression Line for Tryptophan



Respostas = Results
Concentração= Concentration

Conclusion

The results obtained are according to the established acceptance criteria, showing that the method is linear in the range of 50% to 150%.

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Accuracy

The method accuracy was evaluated through the analysis of placebo spiked with lysine acetate, threonine, tryptophan, histidine and tyrosine at 3 levels of concentration, corresponding to 80 %, 100 % and 120 % of working concentration, 3 replicates of each level, in a total of 9 determinations, and was expressed as recovery percentage:

% Recovery= Calculated concentration / Nominal concentration x 100

For each level of concentration, the individual recovery %, the average recovery %, as well as the 95 % confidence interval, were reported. The results can be analysed in the following table.

Table 3.2.P.5.3 – 22 Accuracy

Level	Concentration [µg/mL]		Recuperation [%]				Acceptance criteria*
	Nominal	Calculated	Individual	Average	CV [%]*	CI 95%	Individual R %
Histidine							
80 %	6.087 5.976 5.966	6.309 6.160 6.119	103.65 103.09 102.57	103.10	0.53	0.61	95.0-105.0
100 %	7.813 7.634 7.785	7.601 7.807 7.838	97.29 102.27 100.69	100.08	2.54	2.88	95.0-105.0
120 %	9.491 9.280 9.137	9.306 9.066 9.356	98.06 97.69 102.39	99.38	2.63	2.96	95.0-105.0
Threonine							
80 %	8.382 8.456 8.561	8.527 8.771 8.618	101.73 103.72 100.66	102.04	1.52	1.76	95.0-105.0
100 %	10.684 10.792 10.792	10.372 11.087 10.736	97.08 102.73 99.47	99.76	2.84	3.21	95.0-105.0
120 %	12.808 12.925 12.708	12.862 12.889 13.289	100.42 99.72 104.57	101.57	2.58	2.97	95.0-105.0
Tyrosine							
80 %	4.853 4.926 4.853	5.065 5.129 4.970	104.37 104.12 102.41	103.63	1.03	1.21	95.0-105.0
100 %	6.024 6.059 6.079	5.943 6.309 6.095	98.66 104.12 100.26	101.02	2.78	2.18	95.0-105.0
120 %	7.201 7.288 7.411	7.208 6.950 7.097	100.10 95.37 95.76	97.07	2.71	2.97	95.0-105.0

Table 3.2.P.5.3 – 22 Accuracy (continued)

Level	Concentration [µg/mL]		Recuperation [%]				Acceptance criteria*
	Nominal	Calculated	Individual	Average	CV [%]*	CI 95%	Individual R %
Lysine							
80 %	16.689 16.730 16.671	17.230 17.303 16.565	103.25 103.42 99.37	102.01	2.25	2.59	95.0-105.0
100 %	21.006 21.102 20.970	20.567 21.709 20.880	97.91 102.88 99.57	100.12	2.52	2.86	95.0-105.0
120 %	25.297 25.221 25.289	25.022 24.718 25.894	98.91 98.01 102.39	99.77	2.32	2.62	95.0-105.0
Tryptophan							
80 %	3.672 3.660 3.886	3.710 3.726 3.898	101.04 101.82 100.32	101.06	0.74	0.85	95.0-105.0
100 %	4.641 4.625 4.669	4.492 4.652 4.476	96.79 100.59 95.87	97.75	2.56	2.83	95.0-105.0
120 %	5.493 5.582 5.546	5.374 5.646 5.642	97.82 101.15 101.74	100.24	2.11	2.39	95.0-105.0

* The acceptance criteria were changed to CV ≤ 3.0 % and Individual R %: 95.0 – 105.0 due to the fact that the preparation involves a derivatization reaction, the work volumes are very low - µL - and due to the fact that the solvent used is very volatile. These factors, when conjugated, introduce a high error contribution associated with the volume to the final results.

Conclusion

The results obtained are according to the established acceptance criteria, showing that the method is accurate in the evaluated concentration range.

Precision

System repeatability

The system repeatability was evaluated through the analysis of the analytical response variability, expressed by the coefficient of variation (CV), of 6 consecutive injections of a standard solution at working concentration; see results on the following table.

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Table 3.2.P.5.3- 23 System Repeatability

Parameters	Results	Acceptance criteria*
Histidine		
Concentration [µg/ mL]	7.67	-
Mean Area, n=6	1306916.8	-
CV [%]	0.84	≤ 3.0
Mean Rt	18.31	-
CV [%]	0.33	-
Threonine		
Concentration [µg/ mL]	10.61	-
Mean Area, n=6	2419699.7	-
CV [%]	0.91	≤ 3.0
Mean Rt	21.34	-
CV [%]	0.07	-
Tyrosine		
Concentration [µg/ mL]	6.01	-
Mean Area, n=6	956972.3	-
CV [%]	0.82	≤ 3.0
Mean Rt	26.79	-
CV [%]	0.10	-
Lysine		
Concentration [µg/ mL]	21.08	-
Mean Area, n=6	5255068.2	-
CV [%]	0.94	≤ 3.0
Mean Rt	29.82	-
CV [%]	0.07	-
Tryptophan		
Concentration [µg/ mL]	4.63	-
Mean Area, n=6	685768.2	-
CV [%]	0.50	≤ 3.0
Mean Rt	35.49	-
CV [%]	0.16	-

* The acceptance criteria were changed to CV ≤ 3.0 % due to the fact that the preparation involves a derivatization reaction, the work volumes are very low - µL - and due to the fact that the solvent used is very volatile. These factors, when conjugated, introduce a high error contribution associated with the volume to the final results.

Repeatability of Analysis

The repeatability of analysis was evaluated through the study of the variability, expressed by the coefficient of variation (VC), of the assay of 6 sample replicates (batch D24062), prepared at working concentration; see results on the following table.

Table 3.2.P.5.3 – 24 Repeatability of Analysis

Parameters	Results	Acceptance criteria*
Histidine		
Content [% related to labelled content]	100.82 95.12 99.55 96.91 96.42 100.86	-
Average	98.28	-
CV [%]	2.5	≤ 3.0
Threonine		
Content [% related to labelled content]	102.00 95.18 100.86 98.73 97.77 103.28	-
Average	99.64	-
CV [%]	3.0	≤ 3.0
Tyrosine		
Content [% related to labelled content]	101.82 95.03 100.59 97.95 97.58 102.78	-
Average	99.29	-
CV [%]	3.0	≤ 3.0

Table 3.2.P.5.3 – 24 Repeatability of Analysis (continued)

Parameters	Results	Acceptance criteria*
Lysine		
Content [% related to labelled content]	101.68 95.09 100.48 98.21 98.66 103.82	-
Average	99.66	-
CV [%]	3.0	≤ 3.0
Tryptophan		
Content [% related to labelled content]	102.94 96.00 100.71 98.78 98.46 102.05	-
Average	99.82	-
CV [%]	2.6	≤ 3.0

* The acceptance criteria were changed to CV ≤ 3.0% due to the fact that the preparation involves a derivatization reaction, the work volumes are very low - µL - and due to the fact that the solvent used is very volatile. These factors, when conjugated, introduce a high error contribution associated with the volume to the final results.

Intermediate Precision

The intermediate precison was evaluated through the study of variability of the assay results of the same sample (batch D24062), obtained by 2 analysts, in different days, according to the procedure used for the repeatability of analysis.

The relative difference (D %) between the 2 tests and the variability of the 2 tests, expressed by the combined coefficient of variation, were determined by the following expressions:

D (%) = |Average test 1 – Average test 2|

$CV_{combined}$(%) = (s / average of the 2 tests)x100

S is the combined standard deviation of the 2 tests, calculated by the following expression.

$$S^2=\frac{(n1-1)s_1^2+(n2-1)\,s_2^2}{(n1+n2-2)}$$

n_x = number of determinations of test x s_x^2 = variance of data of test x

It was also evaluated the t-test of the obtained results. See results on the following table.

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Table 3.2.P.5.3- 25 Intermediate Precision

Parameters	Results		Acceptance criteria*
	Test I	Test II	
Histidine			
Content [% related to labelled content]	100.82 95.12 99.55 96.91 96.42 100.86	100.80 97.32 96.44 95.57 98.26 98.72	-
Average	98.28	97.85	-
SD	2.45	1.85	-
CV [%]	2.5	1.9	≤ 3.0
Difference between tests [%]	0.43		≤ 3.0
Combined CV [%]	2.2		≤ 3.0
T Stat	0.48		-
Threonine			
Content [% related to labelled content]	102.00 95.18 100.86 98.73 97.77 103.28	100.57 98.88 96.68 95.76 99.19 99.68	-
Average	99.64	98.46	-
SD	2.98	1.85	-
CV [%]	3.0	1.9	≤ 3.0
Difference between tests [%]	1.18		≤ 3.0
Combined CV [%]	2.5		≤ 3.0
T Stat	0.92		-
Tyrosine			
Content [% related to labelled content]	101.82 95.03 100.59 97.95 97.58 102.78	100.51 98.24 96.75 95.06 97.92 97.19	-

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Table 3.2.P.5.3- 25 Intermediate Precision (continued)

Parameters	Results		Acceptance criteria*
	Test I	Test II	
Average	99.29	97.61	-
SD	2.94	1.80	-
CV [%]	3.0	1.9	≤ 3.0
Difference between tests [%]	1.68		≤ 3.0
Combined CV [%]	2.5		≤ 3.0
T Stat	1.31		-
Lysine			
Content [% related to labelled content]	101.68 95.09 100.48 98.21 98.66 103.82	99.96 97.72 97.35 95.52 98.34 99.15	-
Average	99.66	98.01	-
SD	3.04	1.55	-
CV [%]	3.0	1.6	≤ 3.0
Difference between tests [%]	1.65		≤ 3.0
Combined CV [%]	2.4		≤ 3.0
T Stat	1.58		-
Tryptophan			
Content [% related to labelled content]	102.94 96.00 100.71 98.78 98.46 102.5	101.06 99.17 98.47 96.26 99.80 97.11	-
Average	99.82	98.65	-
SD	2.57	1.76	-
CV [%]	2.6	1.8	≤ 3.0
Difference between tests [%]	1.18		≤ 3.0
Combined CV [%]	2.2		≤ 3.0
T Stat	0.98		-

* The acceptance criteria were changed to CV ≤ 3.0% due to the fact that the preparation involves a derivatization reaction, the work volumes are very low - µL - and due to the fact that the solvent used is very volatile. These factors, when conjugated, introduce a high error contribution associated with the volume to the final results.

Conclusion

The results obtained are according to the established acceptance criteria, showing that the method is precise in the evaluated concentration range.

Stability of Solutions

The standard and sample solutions stability, stored at room temperature (20-24ºC) and protected from light during 18 h, was evaluated through the evaluation of the analytical response variability, expressed by the coefficient of variation (CV), of solution injection immediately after preparation and after a defined period.

See result on the following table.

Table 3.2.P.5.3 – 26 Stability of Solutions During 18 h

Substance	Areas (n=6)	CV [%]	Acceptance criteria*
Standard solution			
Histidine	t = 0 h 1172689 1189257	1.41	≤ 3.0
	t = 18 h 1200703 1212181		
Threonine	t = 0 h 2154320 2170684	0.77	
	t = 18 h 2182350 2193243		
Tyrosine	t = 0 h 869560 875537	0.57	
	t = 18 h 880843 879105		
Lysine	t = 0 h 4656328 4713749	0.96	
	t = 18 h 4741993 4759892		
Tryptophan	t = 0 h 613427 617488	0.27	
	t = 18h 616026 615631		

Table 3.2.P.5.3 – 27 Solutions Stability During 18 h (continued)

Substance	Areas (n=6)	CV [%]	Acceptance criteria*
Sample solution			
Histidine	t = 0 h 1174326 1195560	2.20	≤ 3.0
	t = 18h 1225181 1231056		
Threonine	t = 0 h 2253923 2287900	2.40	
	t = 18 h 2352213 2373586		
Tyrosine	t = 0 h 884352 899614	2.45	
	t = 18 h 930025 928147		
Lysine	t = 0 h 4895076 4942517	2.49	
	t = 18 h 5127425 5138106		
Tryptophan	t = 0 h 629053 643003	1.95	
	t = 18 h 642975 659733		

* The acceptance criteria were changed to CV≤3.0% due to the fact that the preparation involves a derivatization reaction, the work volumes are very low - µL - and due to the fact that the solvent used is very volatile. These factors, when conjugated, introduce a high error contribution associated with the volume to the final results.

Conclusion

The results obtained show that the standard and sample solutions are stable for 18 h, when stored at room temperature (20-24 ºC), protected from light.

System Suitability Tests

The system suitability tests are established to ensure that the method and the equipment present the expected performance previously to the analysis of the test samples.

The following table shows the results of the system suitability test, determined according to the Ph. Eur.

3.2.P.5.3 Validation Report for the Determination of Amino Acids in Ketosteril Film-Coated Tablets by High Pressure Liquid Chromatography (HPLC)

Table 3.2.P.5.3- 27 System Suitability cy

Parameters [(a)]	Results	Recommended values
Theoretical plates number Histidine (≈ 18.3 min) Threonine(≈ 21.3 min) Tyrosine (≈ 26.8 min) Lysine (≈ 29.8 min) Tryptophan (≈ 35.5 min)	 52082 386075 332387 363661 165133	≥ 2000 [(b)]
Symmetry factor Histidine (≈ 18.3 min) Threonine(≈ 21.3 min) Tyrosine (≈ 26.8 min) Lysine (≈ 29.8 min) Tryptophan (≈ 35.5 min)	 1.03 1.12 1.08 1.10 1.06	0.8 – 1.5 [(b)]
Resolution factor Histidine (≈ 18.3 min) Threonine(≈ 21.3 min) Tyrosine (≈ 26.8 min) Lysine (≈ 29.8 min) Tryptophan (≈ 35.5 min)	 - - 33.79 15.84 20.75	≥ 1.0 [(d)]
Injection repeatability, CV (%), n≥6 [(c)] Histidine (≈ 18.3 min) Threonine(≈ 21.3 min) Tyrosine (≈ 26.8 min) Lysine (≈ 29.8 min) Tryptophan (≈ 35.5 min)	 0.84 0.91 0.82 0.70 0.50	≤ 2.0

(a) according to European Pharmacopoeia (b) Recommendations of European Pharmacopoeia and CDER (FDA), Validation of Chromatographic Methods (c) Data collected in System repeatability
(d) according to customer recommendation

Conclusion
The results obtained show compliance with the recommended values.

Conclusions

The HPLC analytical methodology, with UV detection, for the assay of amino acids in the product Ketosteril® film coated tablets, was validated.