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Simultaneous Estimation of Tryptophan and Valine by RP- HPLC Method

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Abstract: **A simple, rapid, specific and precise HPLC method has been developed for the determination of amino acid (Tryptophan) and ketoanalogue (Calcium-3-methyl-2-oxo-butyrate) in solid dosage form. Separation was achieved with Acclaim, OA, C-18 column having 250 × 4.6 mm i.d, 5 µM particle size analytical column using mixture of acetonitrile, and Methane sulfonic acid solution with isocratic mode. The instrumental settings are at a flow rate of 1.0mL/min, column temperature at 40°C and UV detection at 235 nm. The retention time was found to be about 4 and 9 min respectively. RSD and tailing factor were found to be well within the limit for both the compounds. The percentage contents found by the proposed method were more than 98 % for both the compound. Precision were found 0.10% and 0.30 % respectively. This method was validated and meets the regulatory requirements for precision, specificity and linearity. The proposed method can be used for the quantification of Tryptophan and Ca-3-methyl -2-oxo-butyrate in solid dosage forms.**

Keywords: Tryptophan, Valine, Calcium salts, Ketoanalogue, RP-HPLC Method.

I. INTRODUCTION

Tryptophan (symbol Trp or W)[1] is a α-amino acid that is used in the biosynthesis of proteins. It contains a α-amino group, an α-carboxylic acid group, and a side chain indole, making it a non-polar aromatic amino acid. It is essential in humans, meaning the body cannot synthesize it: it must be obtained from the diet. Tryptophan is also a precursor to the neurotransmitter serotonin and the hormone melatonin. [2] It is encoded by the codon UGG.

Like other amino acids, tryptophan is a zwitterion at physiological pH where the amino group is protonated (–NH3+; pKa = 9.39) and the carboxylic acid is deprotonated (–COO−; pKa = 2.38). [3]

Because tryptophan is converted into 5-hydroxytryptophan (5-HTP) which is then converted into the neurotransmitter serotonin, it has been proposed that consumption of tryptophan or 5-HTP may improve depression symptoms by increasing the level of serotonin in the brain. Tryptophan is sold over the counter in the United States (after being banned to varying extents between 1989 and 2005) and the United Kingdom as a dietary supplement for use as an antidepressant, anxiolytic, and sleep aid. It is also marketed as a prescription drug in some European countries for the treatment of major depression. There is evidence that blood tryptophan levels are unlikely to be altered by changing the diet,[4][5] but consuming purified tryptophan increases the serotonin level in the brain, whereas eating foods containing tryptophan does not.[6] This is because the transport system that brings tryptophan across the blood–brain barrier also transports other amino acids which are contained in protein food sources.[7] High blood plasma levels of other large neutral amino acids prevent the plasma concentration of tryptophan from increasing brain concentration levels.

Ca-3-methyl-2-oxo-butyrate is also known as ketoanalogues of valine and calcium salt of 3-methyl -2 butyric acid. [8]

The literature survey revealed that there was no method available for quantitative determination of Tryptophan and Calcium-3-methyl-2-oxo-butyrate**.** We hereby report a simple and reliable RP-HPLC [11][12] method for estimation of both the compound with single method.

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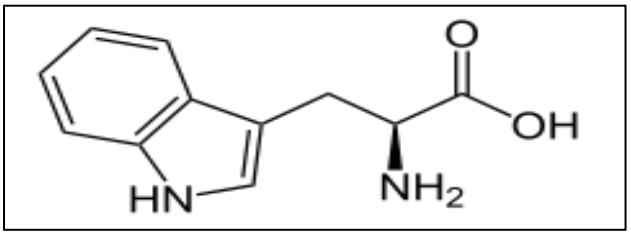

FIG.1: STRUCTURE OF TRYPTOPHAN

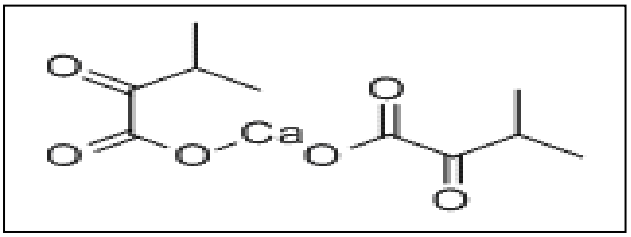

FIG.2: STRUCTURE OF Ca-3-METHYL-2-OXO-BUTYRATE (Valine)

II. MATERIAL AND METHODS

A. Chemicals and Reagents:

All reagents were of analytical-reagent grade unless stated otherwise. HPLC grade water, Methane sulfonic acid, acetonitrile HPLC grade (Rankem) Sodium hydroxide AR grade (Merck) Hydrochloric acid AR grade (Spectrochem). The analysed samples are obtained from local market.

B. Instrument:

The HPLC system Waters e2695 separation module was composed of UV detector (Waters 2489), degasser, pump, column heater and auto injector.

An Acclaim OA,[10] C-18 HPLC column with dimension of 250 cm length with 4.6 mm internal diameter and 5-µm particle size (Make- Thermo) was used for the separation. The chromatograms and integrated data obtained from analysis were recorded by using a computer system with Empower software. All the glass wares used were 'A' grade.

C. Chromatographic Conditions:

The analysis was carried out on Thermo, Acclaim OA, C-18 column using a isocratic method, which consist of buffer and Acetonitrile as organic phase. For Preparation of buffer transfer 3.0 mL of Methane sulfonic acid to 1000 mL in HPLC grade water. Further diluted above solution from 50 mL to 2000 mL with HPLC grade water. Thoroughly mix the buffer, filter through 0.45 µm nylon filter, and degassed. Mixed buffer and Acetonitrile in the ration of 95:5. The detection was carried out on 235 nm wavelength with a flow rate of 1.0 mL and with 40°C HPLC column temperature. Peak shape, asymmetry was found satisfactory which were shown in **Fig.3**

D. Standard Preparation:

Prepared Stock solution of Tryptophan and Calcium-3-methyl -2-oxo-Butyrate (96 µg/mL and 340 µg/mL) respectively in 0.1 N hydrochloric acid with sonication up to 15 minutes and dilute stock solution with diluted sodium hydroxide solution finally to get (12 µg/mL and 42.5 µg/mL) respectively.

E. Sample Preparation:

Taken Tablet sample from Market, crushed it into fine powder, transferred equivalent to 1 tablet powder into 250 mL volumetric flask, Added 0.1 N hydrochloric acid, and kept on sonication about 30 minutes. Remove the flask from sonicator and kept on bench top for cooling. Filtered the sample from 0.45µm nylon filter paper and further diluted with 0.1 N sodium hydroxide solution to get final concentration (12 µg/mL and 42.5 µg/mL) respectively.

III. METHOD VALIDAITON

The RP-HPLC method was validated according to ICH guidelines [10] for validation of analytical procedures for different validation parameters. The method was validated for its specificity, linearity, accuracy, precision, and robustness.

F. System Suitability:

System suitability test was carried out to verify that the analytical system was working properly and can give accurate and precise results. The overall system suitability was evaluated for the system suitability of the proposed method. Data from six injections of Tryptophan 12 µg/mL and Ca-3-methyl-2-oxo-butyrate 42.5 µg/mL were utilized for calculating parameters like theoretical plates, resolution, tailing factor and %RSD. The results were shown in **Table 1.**

G. Specificity:

The specificity studies were carried out by varying specific conditions, *i.e*., placebo study. A study conducted to demonstrate that diluent and Placebo were not interfering with the analyte peak in the proposed method. Solutions of sample, placebo and blank were prepared individually and chromatograms were obtained. The results were shown in **Fig. 4, 5 and 6**

H. Precision:

Precision of an analytical procedure as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The results were shown in **Table 2**

I. Accuracy:

Accuracy was carried out by % recovery studies of Tryptophan and Ca-3-methyl-2-oxo-butyrate at three different concentration levels (50 %, 100 %, and 150 %). Percentage recovery was calculated from the amount added and the amount recovered. The percentage recovery was within the acceptance criteria which indicate the accuracy of the method at different concentrations. (Acceptance criteria: % recovery between 98.0 % to 102%). The results were shown in **Table 3 & 4**

J. Linearity:

Linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the samples. The linearity of the method was determined by preparing serial dilutions of minimum 5 concentrations of working stock solutions for Tryptophan in the range of 6 to 18 µg/ml and for Ca-3-methyl-2-oxo-butyrate was 21.2 to 63.8 µg/ml. The area of each injection was obtained and the peak area was plotted against actual concentration. The regression coefficient r^2, y-intercept and slope of the regression were calculated. The results were shown in **Fig. 7 and 8**

K. Robustness:

The robustness of an analytical variation in method parameters such as flow rate, column temperature were varied within a realistic range and the quantitative influence of the variables were determined. The results were shown in **Table** 5**.**

IV. RESULTS AND DISCUSSION

Optimization of the method was carried out by performing various trials by change in mobile phase composition, column and flow rate etc. The chromatographic conditions were achieved by Methane sulfonic acid buffer and acetonitrile in a ratio of 95:5 v/v. Acclaim OA, C-18 HPLC column used as stationary phase because of better resolution, number of Theoretical plates and symmetric peaks. Tryptophan and Ca-3-methyl-2-oxo-butyrate were found to show appreciable absorbance at 235 nm determined spectro-photometrically and hence it was selected as the detection wavelength. Represents chromatogram of mixture of standard solutions are below in **Fig. 3**

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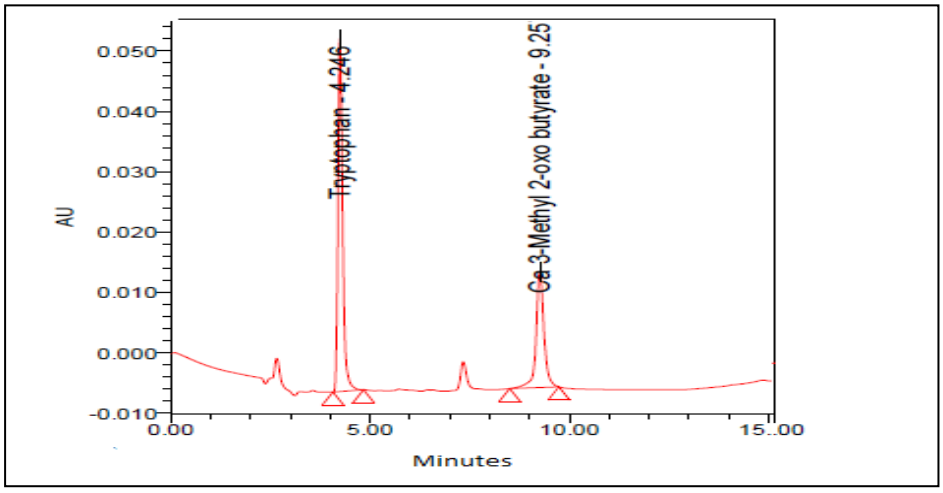

Fig.3: REFERNCE CHROMATOGRAM

Table-1.1: Optimised Chromatographic Conditions

Optimised Chromatographic Conditions	
Mobile Phase	Buffer : ACN 95:5
Column	Acclaim OA, C-1O 250 x 4.6 x 5µm
Flow	1.0 mL/min.
Column Temperature	40°C
Wave Length	235 nm
Run Time	15 min.

L. System Suitability:

An RP -HPLC method was developed by monitoring the system suitability parameters, *i.e*. Tailing factor (T), the number of theoretical plates (N), the runtime and the cost effectiveness. System suitability method acceptance criteria set in each validation run were: tailing factor ≤ 2.0 and theoretical plates > 2000. In all cases, the relative standard deviation (R.S.D) for the analytic peak area for six consecutive injections was < 2.0%. A chromatogram obtained from reference substance solution was presented. System suitability parameters were shown in **Table 1.2**. All the system suitability parameters are found to be satisfactory. The peak is reasonably symmetrical. High numbers of theoretical plates indicate the efficient performance of the column with reasonable retention times.

Table-1.2: Summary of System Suitability Parameters

Optimised Chromatographic Conditions		
	Tryptophan	Ca-3-Methyl-2-Oxo-Butyrate
% RSD	0.35	0.71
Theoretical Plates	9325	16147
Tailing Factor	1.3	1.1
Resolution	-	6.2

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M. Specificity:

The blank chromatogram showed no interference at the retention time of Tryptophan and Ca-3-Methyl-2-Oxo-Butyrate . This indicates that diluent solution used in sample preparation do not interfere in the estimation of both the compounds. Similarly the placebo sample chromatogram also showed no interference peaks at the retention time of both the compounds respectively, which indicates the specificity of the proposed method. The results were given in **Fig. 4, 5** and **6**.

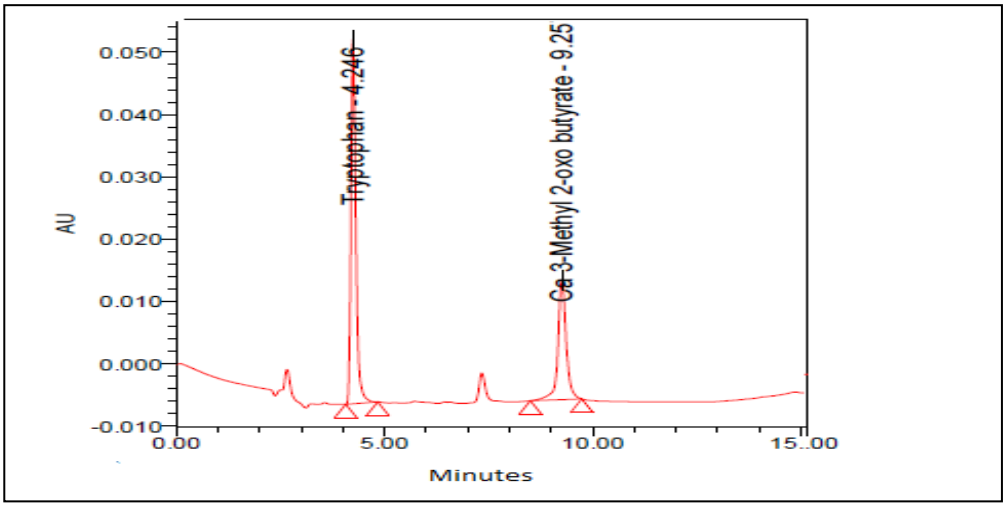

Fig.4: Standard Chromatogram

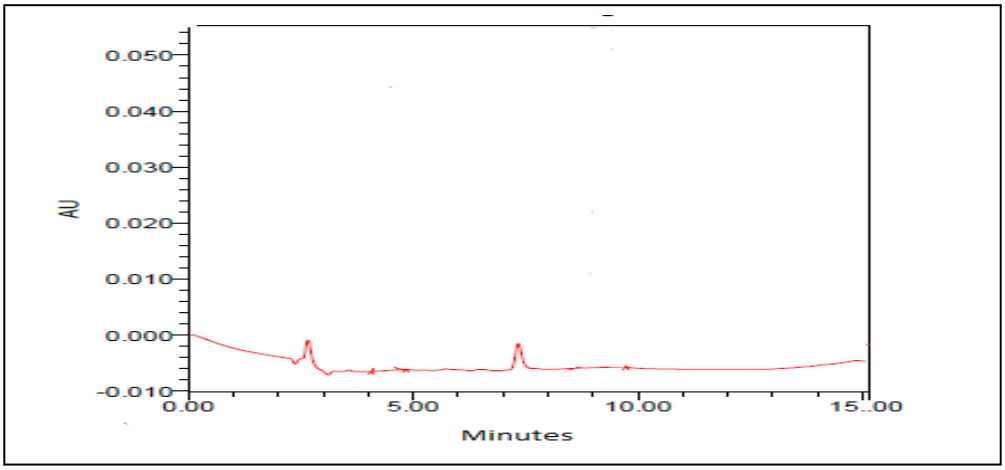

Fig.5: Blank Chromatogram

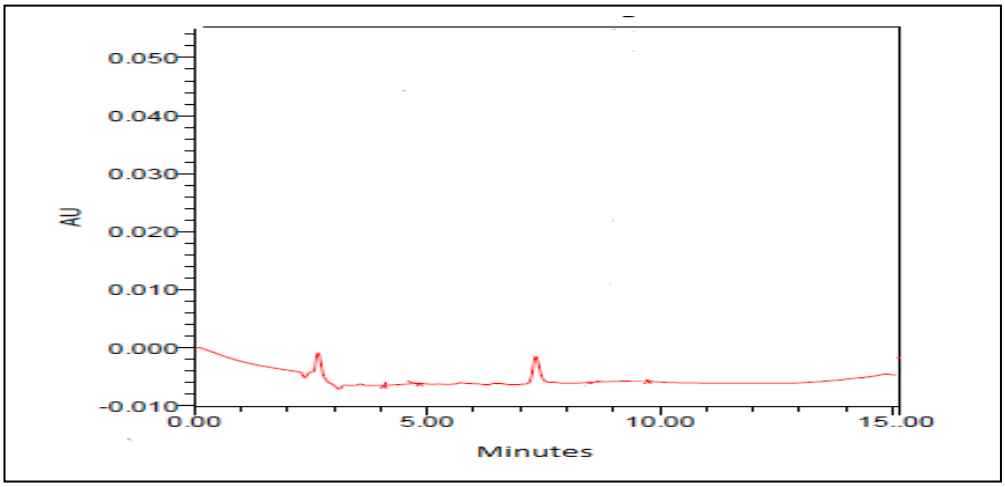

Fig.6: Placebo Chromatogram

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N. Precision:

The percentage relative standard deviation was calculated for the peak areas of both the drugs and it was found to be 0.71 % and 0.57 % for Tryptophan and Ca-3 methyl-2-oxo butyrate respectively. The % RSD for the area of six standard injections was should not be more than 2 % and the method was found to be précised. The results were shown in **Table 2**

Table 2 Method Precision data

Sr. No.	Tryptophan	Ca-3 methyl-2-oxo butyrate
1	100.1	99.0
2	98.6	98.5
3	98.2	98.2
4	99.7	99.5
5	99.5	98.9
6	99.4	99.7
Average	**99.3**	**99.0**
%RSD	**0.71**	**0.57**

O. Accuracy:

The accuracy of an analytical method is the closeness of that results obtained by that method to the true value. Accuracy may often be expressed as percent recovery by the assay of known added amount of analyte. The data shown below in **Table 3 and 4**

Table 3: Accuracy Data of Tryptophan

level	Tryptophan	Average	SD	% RSD
50%-1	98.7	98.9	0.252	0.25
50%-2	98.9			
50%-3	99.2			
100%-1	99.5	99.6	0.100	0.10
100%-2	99.7			
100%-3	99.6			
150%1	100.2	100.5	0.306	0.30
150%2	100.8			
150%3	100.4			

Table 4: Accuracy Data of Ca-3methyl-2-oxo-butyrate

level	Ca-3methyl-2-oxo-butyrate	Average	SD	% RSD
50%-1	99.6	99.7	0.153	0.15
50%-2	99.7			
50%-3	99.9			
100%-1	100.2	100 .5	0.666	0.66
100%-2	101.3			
100%-3	100.1			
150%1	99.5	100.1	0.513	0.51
150%2	100.2			
150%3	100.5			

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P. Linearity:

The concentration range of 6 to 12 µg/ml for Tryptophan and 21.2 to 63.7 µg/ml of Ca-3-methyl-2-oxo-butyrate were found to be linear with correlation coefficients 0.999 and 1.000 for Tryptophan and Ca-3-methyl-2-oxo-butyrate respectively. The results were given in **Fig.7** and **8**.

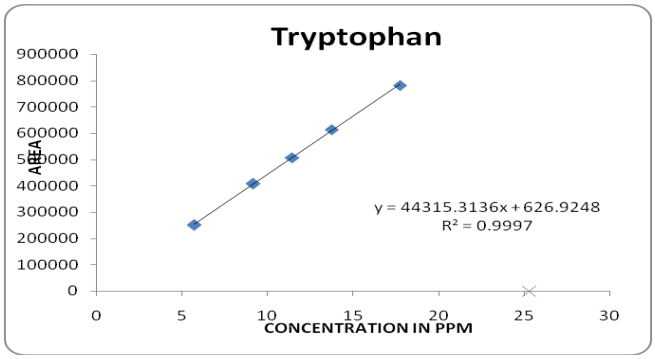

Fig.7 Linearity Plot of Tryptophan

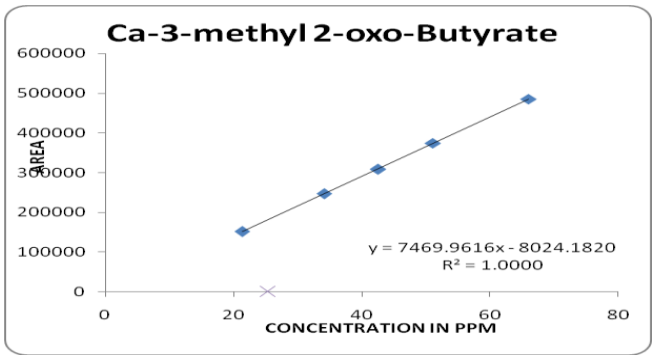

Fig.8 Linearity Plot of Ca-3-Methyl-2-Oxo-Butyrate (Valine)

Q. Robustness:

Robustness of the method tested with different flow rates such as 1.1 ml/min, 0.9 ml/min and different column temperatures such as 35 °C, 45 °C. The peak was observed as sharp with good resolution and passes all system suitability parameters indicating the method was robust. The results were reported in **Table 5**

Table 5: Result of Robustness Studies

Analyte	Robustness Condition	System Suitability Parameter		
		Tailing Factor	Theoretical Plates	Resolution
Tryptophan	(+ Flow) 1.1mL/min.	1.3	15532	-
Ca-3-methyl-2-oxo-butyrate		1.2	8632	5.3
Tryptophan	(- Flow) 0.9 mL/min	1.2	11230	-
Ca-3-methyl-2-oxo-butyrate		1.2	7800	6.7
Tryptophan	(+ Temp.) 45°C	1.1	17014	-
Ca-3-methyl-2-oxo-butyrate		1.2	9385	4.9
Tryptophan	(- Temp.) 35°C	1.1	12365	-
Ca-3-methyl-2-oxo-butyrate		1.2	6900	5.8

V. CONCLUSION

The study was undertaken in order to develop and validate the RP-HPLC method for estimation of Tryptophan and Ca-3-methyl-2-oxo-butyrate in pharmaceutical formulations. The method was developed and validated by means of specificity, accuracy, precision, linearity, and robustness as per ICH guidelines. The results of the study indicate that the proposed RP-HPLC method can be used with respect to routine analysis for the assay of the tablets containing Tryptophan and Ca-3-methyl-2-oxo-butyrate.

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