HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD DEVELOPMENT AND VALIDATION FOR DETECTION AND QUANTIFICATION OF SUBSTANCES RELATED TO ALPHA KETO VALINE CALCIUM SALT

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ABSTRACT

A High-Performance Liquid Chromatography method was developed for identification, quantification and control of possible impurities related to α -ketovaline calcium salt. A stainless-steel column (YMC Triart-C18: 250 $\times$ 4.6 mm, 5 μ m, made: Japan) and two different mobile phases [Mobile phase A containing 0.1% of orthophosphoric acid aqueous solution and B containing acetonitrile and methanol in a ratio 90:10 (v/v)] as an eluent at flow rate 1.0 mL/min were used to achieve the separation and determination of impurities related to α -ketovaline calcium salt. The column temperature was kept 35 $^{\circ}$ C. The injection volume was 20 μ L. UV detection was performed at 210nm. The method was validated for Specificity, Limit of Detection (LOD), Limit of Quantification (LOQ), Linearity, Range and Recovery (Accuracy).

Keywords: Keto Analogs, Anticonvulsant, Impurities, HPLC, Renal Failure, Validation

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INTRODUCTION

Ketoacid is one type of organic acid that has a vital role *in vivo*, and it plays a central role in amino acid metabolism and the process of keeping redox state. α -keto valine calcium salt [chemical name: ($\pm$)-3-Methyl-2-oxobutanoate calcium salt] is one of the main raw material of compound α -ketoacid formulation preparation, which is useful to slow down the chronic renal failure (chronic uremia) deterioration and help to cure the uremia symptom; can provide a shield to protein malnutrition.¹⁻⁵ Therefore, these types of salts must be used with fewer impurities as much as possible. Therefore α -keto valine calcium salt has importance as a substance for pharmaceutical applications and has been monographed in Pharmacopoeias accordingly (e.g., European Pharmacopoeia). For the sake of convenience α -ketovaline calcium salt was abbreviated as " α -kvc" in this text later. Hydantoin is used as starting material in the manufacturing process of α -kvc. 5-(Propan-2-Ylidene) imidazolidine-2, 4-dione is the intermediate stage of the process. While DL-2-Hydroxy-3-Methylbutyric acid is the possible impurity of α -kvc. The chemical structures of compounds analyzed here are given in Fig.-1.

The maternal use of phenytoin drugs during pregnancy can cause fetal hydantoin syndrome.⁶ Phenytoin is a hydantoin derivative. Phenytoin is extensively metabolized and arene oxide intermediate is generated from the metabolism of phenytoin.⁷ It is thought that this reactive intermediate is responsible for many adverse effects of phenytoin such as damaging the liver and other unpredictable adverse effects which cannot be explained by pharmacologic properties (Idiosyncratic reactions).⁷ So, the presence of hydantoin or its derivatives is harmful to health. 5-(Propan-2-Ylidene) imidazolidine-2, 4-dione is also a hydantoin derivative. So, the amount of hydantoin must be in control.

DL 2-Hydroxy-3-methylbutyric acid is found in urine patients with acidosis (a condition in which a high level of acid is observed in body fluids) and diabetic ketoacidosis (a serious complication of diabetes). The concentration of this metabolite was also abnormally high in the urine of the severely asphyxiated group. In the case of the maple syrup urine disease, when also the amount of the DL 2-Hydroxy-3-methylbutyric was increased in the urine, at that time the early proper diagnosis and treatment can save the brain to damage of the patient and improve prognosis.^{8,9} This method can quantify the compound.

Fig.-1

Considering Hydantoin, 5-(Propan-2-Ylidene) imidazolidine-2, 4-dione and DL-2-Hydroxy-3-Methylbutyric acid (later abbreviated as Imp-1, Imp-2, and Imp-3 respectively in the text) are the possible impurities and the limit of these impurities are not more than 0.50 mg per 100 mg of α -kvc individually. The literature reviews related calcium salts keto analogs essential amino acids, disinterment that few analytical methods have been developed for quantification of this type of salts from different biological fluids such as blood, plasma, and serum¹⁰⁻¹² and also a method of simultaneous quantification of keto analogous acid by UHPLC (latest and fastest technology in liquid chromatography).¹³ But there is no method reported for the quantification of possible impurities present in α -kvc. This method outlines the separation of different process-related impurities and the developed method has been validated as per ICH guideline.¹⁴

EXPERIMENTAL

Chemicals and Solvents

The working standard of α -kvc, Imp-1, Imp-2, Imp-3 and other samples, HPLC Grade Water, ortho-phosphoric acid (AR grade), Acetonitrile (HPLC grade), Methanol (HPLC grade) was used to develop the method.

Instrumentation

Shimadzu HPLC model LC 2010C_{HT} system equipped with binary gradient capability, UV detector, an auto sampler and column oven connected to a data acquisition system and processing system LabSolution version 5.2 was used to perform development and validation. A stainless-steel column filled with porous silica particles chemically bonded with Octadecyl silane {YMC Triart C18 (250 x 4.6) mm, 5 μ m, made: Japan} was used to achieve the determination of the content of imp-1, imp-2 and imp-3 in α -kvc. An analytical weight balance (made by Shimadzu, Japan) was used for weighing purposes.

Preparation of Mobile phase A

Accurately 1.0 mL of ortho-Phosphoric acid was pipetted and transferred into a 1000 mL measuring cylinder and diluted to 1000 mL with distilled water, mixed thoroughly, filtered through 0.45 μ filter and sonicated to degas it.

Preparation of Mobile phase B

Acetonitrile and Methanol in the ratio of 9:1 were mixed and sonicated to degas it.

Chromatographic Condition

YMC Triart C18 (250 x 4.6) mm, 5 μ m, made: Japan was used to analyze targeted molecules at column oven temperature 35 $^{\circ}$ C and the flow rate was maintained 1.0 mL/min. The sample injection volume was

20 µl and the sample temperature was kept at 25 °C. The UV detector was set to a wavelength of 210 nm to monitor the presence of targeted molecules. The chromatographic Gradient System Runtime was 40 minutes. A degassed mixture of Water: Acetonitrile in the ratio of 1:1 was used for rinse purpose.

Method Development

The analytical method developed here has consisted of mobile phase selection, detection wavelength selection and stationary phase selection. For this purpose, a literature survey has been optimized. The physical and chemical properties of chemicals and reagents used here have been studied.

Mobile Phase Selection

After some trial and error better chromatographic compatibility, resolution and selectivity have been achieved by using an aqueous orthophosphoric acid solution prepared as above as mobile phase-A and the mixture of Acetonitrile and Methanol in the ratio of 9:1 as mobile phase-B in the gradient form given in Table-1.

Column Selection

Among the column chemistry available in the lab a stainless-steel column filled with porous silica particles chemically bonded with Octadecyl silane C18 having specifications (250 x 4.6 mm, 5 µm) was found to generate better results.

Table-1: Gradient Composition

Time in Minutes	Mobile Phase A (%v/v)	Mobile Phase B (%v/v)
Initial	98	2
5	98	2
25	50	50
27	98	2
40	98	2

Detection Wavelength Selection

On the base of the method studied given in reference¹¹ for the first wavelength of 215 nm was used to detect the targeted molecules. But maximum chromatographic compatibility was observed with the wavelength of 210 nm, so it has been selected to optimize the targeted molecules in the proposed method.

Preparation of Diluent

A mixture of Water and Acetonitrile in 9:1 ratio was prepared and sonicated to degas it for 5 minutes.

Preparation of Standard Dilution Solution of α -kvc

A solution of α -kvc having was prepared by accurately weighed 50.050 mg of working standard of α -kvc and transferred into a 50 mL volumetric flask, added about 35 mL of diluent into it and sonicated with occasional swirling to dissolve the solid. Diluted to the volume with diluent and mixed thoroughly. This solution was used for dilution purposes also.

Preparation of Sample Solution

The sample solution of α -kvc was prepared by accurately weighed 40.05 mg of α -kvc (samples, different batch) and was transferred into a 10 mL volumetric flask. Added about 8 mL of diluent into it. Sonicated with occasional swirling to dissolve the solid. Diluted to the volume with diluent and mixed thoroughly.

A stock solution of Imp- 1, 2 and 3

Accurately weighed and transferred 10.010 mg of Imp-1, 10.030 mg of Imp-2 and 10.080 mg of Imp-3 into a 10 mL volumetric flask separately and added 7 mL of diluent in each flask, sonicated to dissolve and made the volume with further adding diluent into each volumetric flask.

Peak Identification Solution

Accurately pipetted 1 mL of each of stock solution of Imp- 1, 2 and 3 into a 10 mL volumetric flask and added 10 mg accurately weighed of standard α -kvc and added 5 mL of the diluent, sonicated for 5 minutes and made the volume up to the mark with the diluent.

RESULTS AND DISCUSSION

Concerning the method development, the chromatographic parameters given in the Table-2 were found suitable for the validation given here.

Table-2: Chromatographic Conditions

Parameters	Optimum Condition
Mobile Phase-A	0.1% orthophosphoric acid in water
Mobile Phase-B	Acetonitrile: Methanol (90:10)
Column	YMC Triart C18(250 x 4.6 mm, 5 μ m particle size)
Flow Rate	1.0 mL/min
Gradient Elution at	As per table 1
Detection Wavelength	210 nm
Diluent	Water: Acetonitrile (90:10)
Injection Volume	20 μ L

Method Validation

The method for the determination and quantification of the α -kvc and related known impurities in bulk drug was further validated as ICH Q2 (R1) guidelines.¹²

Specificity

The Specificity of the method was evaluated by 1.0 mL from each stock solution- 1, 2 and 3 were pipetted and transferred into 10 mL of volumetric flask individually and diluted up to mark and these solutions were injected into the system (Fig.-2 to 4). The sample solution α -kvc prepared as above was also injected to examine. (See Fig.-5). The chromatogram of the sample solution showed the peaks of some unknown impurities present in the sample of α -kvc. A solution having the concentration at the 100% level of specification of impurities spiked studied here was injected with the sample preparation to examine for if any interference could be observed with Imp-1, Imp-2 and Imp-3 peaks (see Fig.-6). The Specificity of the method was further evaluated by injecting the blank solution and observing that any interference with the peaks of interest in the chromatograms obtained from the Standard Solution. To identify the retention time of each component the peak identification solution prepared as above was injected (see Fig.-7). The retention time for α -kvc was observed at 14.85 minutes. Peak shapes were found satisfactory for each known impurity. It has been confirmed that no interference was observed in the elution zone of the blank/diluent. Specificity data also revealed that the impurities do not elute in the elution zone of the α -kvc retention time. The results of specificity Data for individual Impurity and spiked Sample summarized in Table-3 and 4 respectively.

Table-3: Specificity Results

Compound Name	Retention Time (minutes)
Imp-1	3.80
α -kvc	14.74
Imp-2	16.58
Imp-3	19.30
Blank	-

Table-4: Specificity Results, Spiked Sample Individual Impurities

Compound Name	Retention Time (minutes)	Relative Retention Time(about)
Imp-1	3.81	0.26
α -kvc	14.74	1.00
Imp-2	16.59	1.13
Imp-3	19.31	1.31

Fig.-2: Chromatogram of Individual Imp-1 (Specificity)

Fig.-3: Chromatogram of Individual Imp-2 (Specificity) (5-(Propan-2-Ylidene) imidazolidine-2,4-dione)

Fig.-4: Chromatogram of Individual Imp-3

Fig.-5: Chromatogram of α-kvc as such (Specificity)(DL-2-Hydroxy-3-Methylbutyric acid)

System Suitability

For the study of system suitability, a solution having a concentration of 20 µg/mL (specification level) of each component studied here was prepared. For these purposes, different solutions were made as described below:

- 2 mL solution was pipetted from the standard dilution solution into 10 ml volumetric flask and made the volume up to mark with the diluent.
- 2 mL of each solution of the stock solution of Imp-1, 2 and 3 were pipetted and transferred into a 10 mL volumetric flask and made the volume with the diluent.
- Further took 1 mL of each the solution prepared as above into a 10 mL volumetric flask and made the volume to the mark with the diluent. This solution was used to evaluate the system suitability of the method developed by injecting the replicate injections at the specification level. The chromatogram obtained here was represented by Fig.-8.

The theoretical plates are obtained greater than 2000 by the method developed for all substances studied here. Moreover, the chromatogram of the peak identification solution indicates the system suitability of the test. The system suitability data were obtained according to Table-5. The Relative Standard Deviation (%RSD) found 0.0, 0.3, 0.7 and 0.0 for Imp-1, α-kvc, Imp-2 and Imp-3 respectively.

Table-5: System Suitability Data

Description	Imp-1	α-kvc	Imp-3	Imp-2
Conc. Of Solution (µg/ml)	20.00	20.00	20.00	20.00
Conc. With respect to Test in Percentage (%)	0.50	0.50	0.50	0.50
Average (n=6)	633221	162154	19320	927079
Standard Deviation	107	549	127	204
%RSD	0.02	0.34	0.66	0.02

Fig.-6: Chromatogram of Spiked Solution at 100% level of specification (Specificity)

Fig.-7: chromatogram of peak identification solution

LOD and LOQ

Limit of Detection (LOD) and Limit of Quantification (LOQ) were derived based on signal to noise ratio. Each component was injected at the concentration level giving a minimum detectable peak. Here minimum detectable peak area was observed for the concentration of 0.15 $\mu\text{g/mL}$ for Imp-1, 0.3 $\mu\text{g/mL}$ for $\alpha\text{-kvc}$, 0.15 $\mu\text{g/mL}$ for Imp-2 and 0.3 $\mu\text{g/mL}$ for Imp-3.

Here the LOD and LOQ value derived using the equations below,

$\text{LOD} = 3 \times \text{The concentration at minimum detectable peak observed for the solution}$

$\text{LOQ} = 3 \times \text{LOD}$

The LOD values derived here are 0.45 $\mu\text{g/mL}$, 0.9 $\mu\text{g/mL}$, 0.45 $\mu\text{g/mL}$ and 0.9 $\mu\text{g/mL}$ for Imp-1, $\alpha\text{-kvc}$, Imp-2 and Imp-3 respectively.

The LOQ values derived here are 1.35 $\mu\text{g/mL}$, 2.7 $\mu\text{g/mL}$, 1.35 $\mu\text{g/mL}$ and 2.7 $\mu\text{g/mL}$ for Imp-1, $\alpha\text{-kvc}$, Imp-2 and Imp-3 respectively.

Linearity Study

Linearity of the detector response for the working standard of $\alpha\text{-kvc}$ and the imp-1, imp-2 and imp-3 were determined across the range of 5% to 120% of the specification limit.

The proper concentration needed here had been prepared from stock solution of Imp-1, 2 and 3 by pipetting 2 mL of each solution and transferred into 10 mL volumetric flask separately and made the volume up to mark for each flask. These solutions were used for the dilution purpose for the Imp-1, 2 and 3 described in the Table-6. For the linearity study of $\alpha\text{-kvc}$ standard dilution, solution had been used for dilution purpose. 2 mL solution was pipetted and transferred into a 10 mL volumetric flask and diluted with the diluent up to the mark. This solution was used to get the proper concentration required for the study of linearity of $\alpha\text{-kvc}$ which was shown by the Table-6.

On the base of the linearity study, the linearity graph was obtained for each component study here. Different components of the regression equation were obtained as given in Table-6. The predicted value LOD of $\alpha\text{-kvc}$, imp-1, imp-2 and imp-3 were found 0.54 $\mu\text{g/mL}$, 0.21 $\mu\text{g/mL}$, 0.24 $\mu\text{g/mL}$ and 0.31 $\mu\text{g/mL}$ respectively. The predicted LOQ value of $\alpha\text{-kvc}$, imp-1, imp-2 and imp-3 were found 1.65 $\mu\text{g/mL}$, 0.64 $\mu\text{g/mL}$, 0.72 $\mu\text{g/mL}$ and 0.94 $\mu\text{g/mL}$ respectively. The predicted value of LOQ and LOD value was calculated using the equations given below:

$$\text{LOQ} = \frac{10 \times \text{Residual Standard Deviation}}{\text{Slope}}$$

$$\text{LOD} = \frac{3.3 \times \text{Residual Standard Deviation}}{\text{Slope}}$$

The results found in the linearity study were summarized in Table-6.

Table-6: Linearity Study

S. No.	% level of linearity conc.	Pipetted volume (mL)	Made up of vol. (mL)	Imp-1		α -kvc		Imp-2		Imp-3	
				Conc. ($\mu\text{g/mL}$)	Area	Conc. ($\mu\text{g/mL}$)	Area	Conc. ($\mu\text{g/mL}$)	Area	Conc. ($\mu\text{g/mL}$)	Area
1	120	1.20	10	24.00	763964	24.00	197536	24.00	1116787	24.00	23427
2	100	1.00	10	20.00	633017	20.00	161666	20.00	926560	20.00	19290
3	80	0.80	10	16.00	507445	16.00	129315	16.00	739528	16.00	15510
4	30	0.30	10	6.01	188208	6.01	46983	6.02	271144	6.05	5897
5	10	0.10	10	2.00	64206	2.00	15856	2.01	90746	2.02	1961
6	5	0.50	100	1.00	32893	1.00	7519	1.00	44823	1.01	981
Slope				31751.7		8212.2		46608.8		971.4	
Intercept				-242.02		-1307.7		-4636.5		-6.4	
Coefficient of determination(r^2)				1.0000		0.9998		1.0000		0.9999	
STEYX				2025.7		1354.0		3370.8		91.0	
LOQ($\mu\text{g/mL}$)				0.64		1.65		0.72		0.94	
LOD($\mu\text{g/mL}$)				0.21		0.54		0.24		0.31	

STEYX = Residual Standard Deviation

Study of Precision at LOD and LOQ Concentration Level

This work has been tested the precision of the validation process for the solution prepared of known concentrations.

The precision of α -kvc related impurities at about Limit of detection and Limit of quantification was conducted. Six test preparations at LOD concentration level and six test preparations at LOQ concentration level for each impurity along with α -kvc were made and injected into the system.

The Relative Standard Deviation (RSD) values observed at LOQ level of concentration were 0.4%, 3.8%, 0.3% and 1.7% for the Imp-1, α -kvc, Imp-2 and Imp-3 respectively. The Relative Standard Deviation (RSD) values observed at LOD concentration level were 0.4%, 3.1%, 0.7% and 5.5 for the Imp-1, α -kvc, Imp-2 and Imp-3 respectively. These data are summarized in Table-7 and 8 and graphical representation is given by Fig.-9 and 10.

Table-7: Precision Study at LOQ Concentration Level

Description	Imp-1	α -kvc	Imp-2	Imp-3
Conc. of Solution ($\mu\text{g/mL}$)	1.00	2.00	1.00	2.00
Conc. of Solution in Percentage (%) with respect test	0.025	0.05	0.025	0.05
Average area (n=6)	32893	15298	44823	1961
Standard Deviation	139	580	145	33
%RSD	0.4	3.8	0.3	1.7

Table-8: Precision Study at LOD Concentration Level

Description	Imp-1	α -kvc	Imp-2	Imp-3
Conc. of Solution ($\mu\text{g/mL}$)	0.50	1.00	0.50	1.00
Conc. of Solution in Percentage (%) with respect to test	0.013	0.025	0.013	0.025
Average area (n=6)	16336	7088	22031	942
Standard Deviation	68	218	162	52
%RSD	0.4	3.1	0.7	5.5

Fig.-8: Chromatogram of System Suitability at Specification Level Solution

Fig.-9: Chromatogram of LOQ Level Concentration Solution

Fig.-10: Chromatogram of LOD Level Concentration Solution

The Relative Response Factor

The Relative Response Factor (RRF) of each known impurity and Alpha Keto Valine Calcium was calculated using the slope value obtained from the linearity data mentioned above. The Relative Response Factor (RRF) of each known impurity was calculated by using the following formula:

$$\text{RRF} = \frac{\text{The slope of } \alpha\text{-kvc (A)}}{\text{The slope of each individual known impurity linearity curve (B)}}$$

The RRF value founded was 0.26 for Imp-1, 0.18 for Imp-2 and 8.45 for Imp-3.

Accuracy (Recovery)

A study of recovery of α -kvc related to known impurities in a spiked sample of α -kvc test preparation was conducted. Samples were prepared by spiking of all known impurities in test preparation at LOQ level, 80%, 100% and 120% of the specification level concentration.

When a control sample of α -kvc was injected at test concentration, with some peaks of unknown impurities, a peak of Imp-3 was also observed (see Fig.-5). The amount of Imp-3 presented in the sample was 0.54 concerning test. %recoveries of α -kvc related known impurities were calculated by established RRF as described in Table- 9.

Table-9: Recovery Study

Level	Amount Present	Amount Added	Amount Found	%Recovery
Hydantoin (Conc. with respect to test in percentage (%))				
120%	00	0.60	0.61	101.7
100%		0.50	0.51	102.0
80%		0.40	0.41	102.5
LOQ Level		0.025	0.026	104.0
DL-2-Hydroxy-3-Methylbutyric acid (Conc. with respect to test in percentage (%))				

120%	0.54	0.60	1.2	108.3
100%		0.50	0.99	102.0
80%		0.40	0.88	97.5
LOQ Level		0.050	0.56	100.0
5-(Propan-2-Ylidene) imidazolidine-2,4-dione (Conc. with respect to test in percentage (%))				
120%	00	0.60	0.61	103.3
100%		0.50	0.50	104.0
80%		0.40	0.40	102.5
LOQ Level		0.025	0.030	116.0

CONCLUSION

This method validated for the α -kvc and related known impurities by using the column described as above as per the ICH guidelines. This method can be adopted in bulk and formulation industries for α -kvc and its impurities. Good results were observed by this method and the validation parameters likewise linearity, specificity, method precision and assay accuracy had been generated. By this method, this work concluded that the results obtained through the column are accurate and precise and the method can quantify and detect known all impurities summarized here with a good precise level. Hence this method can be used for the determination of α -kvc and its impurities for formulation purposes. The specificity data given here showed the ability of the method to correctly identify and quantify the analyte in the presence of all potential sample components.

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