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Original Article

Simultaneous Estimation of Cal DL-2-Hydroxy,4-methyl thio-butyrate and Ca-2-oxo3-phenyl Propionate in Alpha-Ketoanalogues Tablets by Validated RP-HPLC Method

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ABSTRACT

Introduction: Alpha-Ketoanalogues is a dietary supplement drug containing Calcium-DL-2-hydroxy-4-(methylthio) butyrate (CHMTB) and Calcium-2-oxo-3-phenyl propionate (COPP) for the management of chronic renal disease. **Objectives:** In this study, a simple RP-HPLC method has been developed to simultaneously determine Calcium-DL-2-hydroxy-4-(methylthio) butyrate (CHMTB) and Calcium-2-oxo-3-phenyl propionate (COPP) in Alpha-Ketoanalogues Tablets. **Materials and Methods:** The compounds were separated in a single run using a Waters XBridge 250 mm x 4.6 mm C18 column with 5µm particle size and 130 Å pore size. Elution was carried out via gradient mode with the UV detector wavelength set at 210 nm. The mobile phase selected was a binary mixture of ammonium hydrogen sulphate buffer (pH 7.0) and acetonitrile at a 1.0 mL/min flow rate. **Results:** The retention time of CHMB and COPP was found to be about 7 min and 20 min, respectively. The chromatographic method shows detector linearity in 50-150% of the operating range with the square of correlation coefficient at 0.9998 and 0.9999 for these two compounds, respectively. The parentage recovery of both CHMB and COPP was within 98-102% range. The method validation was carried out per the USP (chapter 1225) and ICH guidelines (Q2-R2). The developed method was found to be specific, precise, linear, robust, and accurate. **Conclusion:** This method could be utilized to assay tablet formulation of CHMB and COPP during the in-process and finished product quality control.

Keywords: Alpha-Ketoanalogues, Chronic kidney disease, Chromatography, Method Validation, Simultaneous estimation, Formulation.

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INTRODUCTION

Alpha-Ketoanalogues is a dietary supplement drug containing Calcium-DL-2-hydroxy-4-(methylthio) butyrate (CHMTB) and Calcium-2-oxo-3-phenyl propionate (COPP) for the management of chronic renal disease. Ketoanalogues supplementation decreases dialysis and mortality risk in patients with anemic advanced chronic kidney disease.[1] CHMTB is a α-hydroxy analog of methionine calcium salt[2] (Figure 1), and COPP (Figure 2) is the alpha-keto analogs of phenylalanine calcium salt.[3] In the 1980s, Mitch and colleagues found that Alpha-Keto Analogs (KA) supplemented Low-Protein Diet (LPD) slowed or halted the progression of renal insufficiency in CKD patients.[4] LPD supplemented with KA also alleviated the decrease in Glomerular Filtration Rate (GFR) and maintained body mass index.[5] It was hypothecated that the KA of essential amino acids, converted into essential amino acids in the body via transamination, could improve nutritional deficiencies caused by protein-restricted diets in Chronic Kidney Disease (CKD) patients. Several marketed formulations like Ketogia are currently available as nutritional supplements containing CHMTB and COPP.[6] However, an accurate analytical method is required to quantify these two Alpha-Ketoanalogues (KA) of essential amino acids in formulations. An HPLC method of CHMTB estimation is reported by Rinda R. Ontiveros *et al.*[7] However, no method is available for simultaneous estimation for CHMTB and COPP by HPLC. In this article, a specific, precise, linear, accurate, stable,

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and robust RP HPLC method has been developed to estimate both drugs in tablet formulation simultaneously.

MATERIALS AND METHODS

Chemicals and reagents

CHMTB and COPP salt was gifted by RPG life sciences Ltd. Water (HPLC grade), Acetonitrile (HPLC grade), and Orthophosphoric acid (HPLC grade) were procured from the Fisher Scientific India Pvt. Ltd. Tetra-n-butyl ammonium hydrogen sulfate (For synthesis) was procured from SRL chemicals, Sodium Hydroxide (Analytical Reagent grade) and Concentrated Hydrochloric acid (Analytical Reagent grade) was purchased from LOBA Chaime Pvt. Ltd.,

Chromatographic Analysis

The chromatographic system consisted of a Waters HPLC system (Model: 2489) with a UV detector, degasser, pump, column heater and auto-injector. These optimized chromatographic conditions are mentioned in Table 1.

Mobile Phase A Preparation

In 1000 mL of water, 3.0 g of Tetra-n-butyl ammonium hydrogen sulfate were dissolved. Then, 1.0 mL of Orthophosphoric acid was added, and the pH was adjusted to 7.0 (±0.05) with a diluted sodium hydroxide solution. Afterward, the mixture was filtered through a 0.45 µ nylon filter and degassed.

Standard solution

About 59.0 mg of CHMTB and 68.0 mg of COPP were weighed accurately and transferred in a 250 mL volumetric flask. Then, about 60 mL of 0.1 N HCl was added to a volumetric flask. Then the mixture was sonicated for 20 min to dissolve, and the volume was made up with diluent (25 mM Sodium Hydroxide) to get concentrations of 236 µg/mL and 272 µg/mL, respectively, for CHMTB and COPP. The final standard solution was prepared by transferring 2.5 mL of this stock solution in a 20 mL volumetric flask and diluting up to the mark with diluent to get final concentrations of sample 29.5 µg/mL and 34 µg/mL.

Test Solution

Alfalog tablets (RPG Life Sciences) were collected from the market, and 20 tables were crushed to make fine powder and then transferred equivalent to one tablet into a 250 mL volumetric flask. 60 mL of 0.1 N HCl was added and kept for sonication for 20 min with occasional shaking. After that, 150mL of diluent (25 mM Sodium Hydroxide) were added. Then the mixture was kept for 15 min sonication with occasional shaking. A portion of the sample solution was filtered through 0.45 µ Nylon membrane filter paper after discarding the initial 3.0 mL. Then 2.5 mL of the filtrate was diluted with diluent up to 20 mL to get the final expected concentrations of CHMTB and COPP to be 29.5µg/mL and 34µg/mL respectively.

System Suitability

The analytical system's suitability for CHMTB and COPP was ensured by meeting specific performance criteria during the analysis. These criteria included achieving a theoretical plate count greater than 5000, maintaining a tailing factor of less than 2.0, and ensuring that the relative standard deviation for five replicate injections did not exceed 2.0%.

Method Validation

After successfully optimizing the developed method, it was validated using different validation parameters as the acceptance criteria of USP (1225) guidelines and ICH (Q2R2) guidelines. A detail of validation outcomes is described below with proper data and results.

Specificity

The Specificity of the method was evaluated by injecting the blank, mobile phase, and placebo solution to observe for interference within the retention time of CHMTB and COPP. The blank, mobile phase, Sample, and Placebo were prepared according to the test method and injected into the HPLC system.

Precision

System Precision: The standard was prepared per the test method, injected six times, and the percent relative standard deviation for CHMTB and COPP peak areas was calculated.

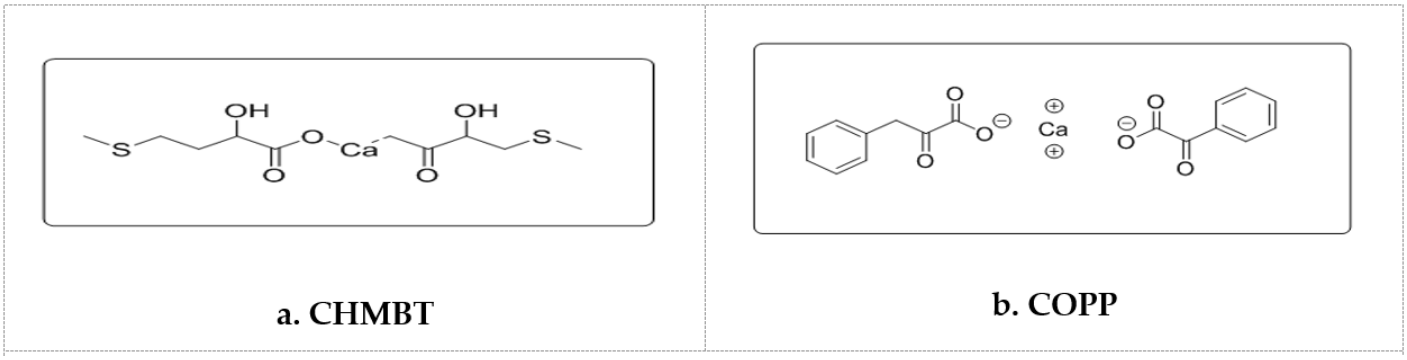

Figure 1: Structure of CHMBT and COPP.

Method Precision: Six individual test preparations were prepared per the test method, and the percent Related Standard Deviation of six Assay results was calculated.

Intermediate Precision: Six individual test preparations were prepared as per the test method by different HPLC and columns on different days by different analysts, and the percentage Related Standard Deviation of six Assay results were calculated. Also, the percent absolute difference between the Assay result of the method precision sample and the intermediate sample was calculated.

Linearity and Range

Linearity and Range study was carried out within the 50%-150% range of the target standard concentration, and correlation coefficient (r^2) value was calculated. The standard solutions were prepared at 50%, 80%, 100%, 120%, and 150% of the target standard concentration of CHMTB (236 µg/mL) and COPP (272 µg/mL), which also covered the method's lower and higher level linearity ranges.

Accuracy

Three target standard/sample concentration levels were selected for the percentage recovery or accuracy study. A homogenous tablet blend (triplicate) was prepared by mixing the drug substances and placebo at 80%, 100%, and 120% of the target initial concentration and analyzed as per the test method.

Robustness

The robustness was estimated to observe whether this analytical method is stable to small changes in experimental conditions. The standard and sample solutions were prepared per the test method and analyzed by changing the chromatographic conditions. The variation in Flow rate was ±0.1 mL of 1.0 mL (0.9 mL/min and 1.1 mL/min); the variation in the Column oven was ±5°C (35°C-45°C), and the variation in pH of the buffer of Mobile Phase were ±0.2 (6.8-7.2).

Table 1: **Optimized Chromatographic Condition.**

Mobile Phase A: Ammonium hydrogen sulphate buffer. MobilePhase-B: Acetonitrile. Gradient programme			
Time (min)	**Flow (mL)**	**% A (Buffer)**	**%B (Acetonitrile)**
0.00	1.0	87.0	13.0
9.00	1.0	87.0	13.0
15.00	1.0	75.0	25.0
25.00	1.0	75.0	25.0
30.00	1.0	87.0	13.0
35.00	1.0	87.0	13.0
Column: Xbridge 250 x 4.6 x 5 µm (Make: Waters)			
Injection volume: 50 µL			
Column temperature: 40°C			
Auto Sampler temperature: 15°C			

Table 2: **Accuracy measurement for CHMTB assay.**

Level / set	Wt. of API spiked/added (mg)	Wt. Recovered (mg)	% Recovery	Average	% RSD
80% set-1	23.83	23.89	100.2	101.0	1.07
80% set-2	23.84	24.27	101.8		
80% set-3	23.55	24.11	102.4		
100% set-1	29.66	29.97	101.1	101.2	0.21
100% set-2	29.59	29.99	101.4		
100% set-3	29.63	30.14	101.7		
120% set-1	36.05	36.35	100.8	101.3	1.4
120% set-2	35.96	35.98	100.1		
120% set-3	36.07	37.12	102.9		

Ruggedness

This validation parameter was assessed to check whether changes in the analyst or the day of testing can impact the assay result. This intermediate precision was estimated with different analysts on different days. Percentage relative standard deviation value was observed at 0.8 and 1.2 on different days by different analysts, which was within the limits. The result is given in Table 4.

RESULTS AND DISCUSSION

Specificity

From blank, mobile phase, and Placebo, no interference was observed at the retention time of main analyte peaks (Figure 2).

Precision

In System Precision, the Percentage Standard Deviation (RSD) value was obtained as 0.1 and 0.9, which was well within the 2.0% RSD limit. Method precision was carried out using six different sample preparations, and the %RSD values of CHMTB and COPP were respectively obtained as 0.8 and 1.1, well within the 2.0% RSD limit of six replicate preparations. The intermediate precision was performed using six different samples preparation, and the %RSD value were respectively obtained as 0.8 and 1 for two drugs. The absolute difference between method precision and intermediate precision was within acceptable range (0.1 and 0.0, respectively). The percentage relative standard deviation and absolute difference obtained were also within the limit. Hence the

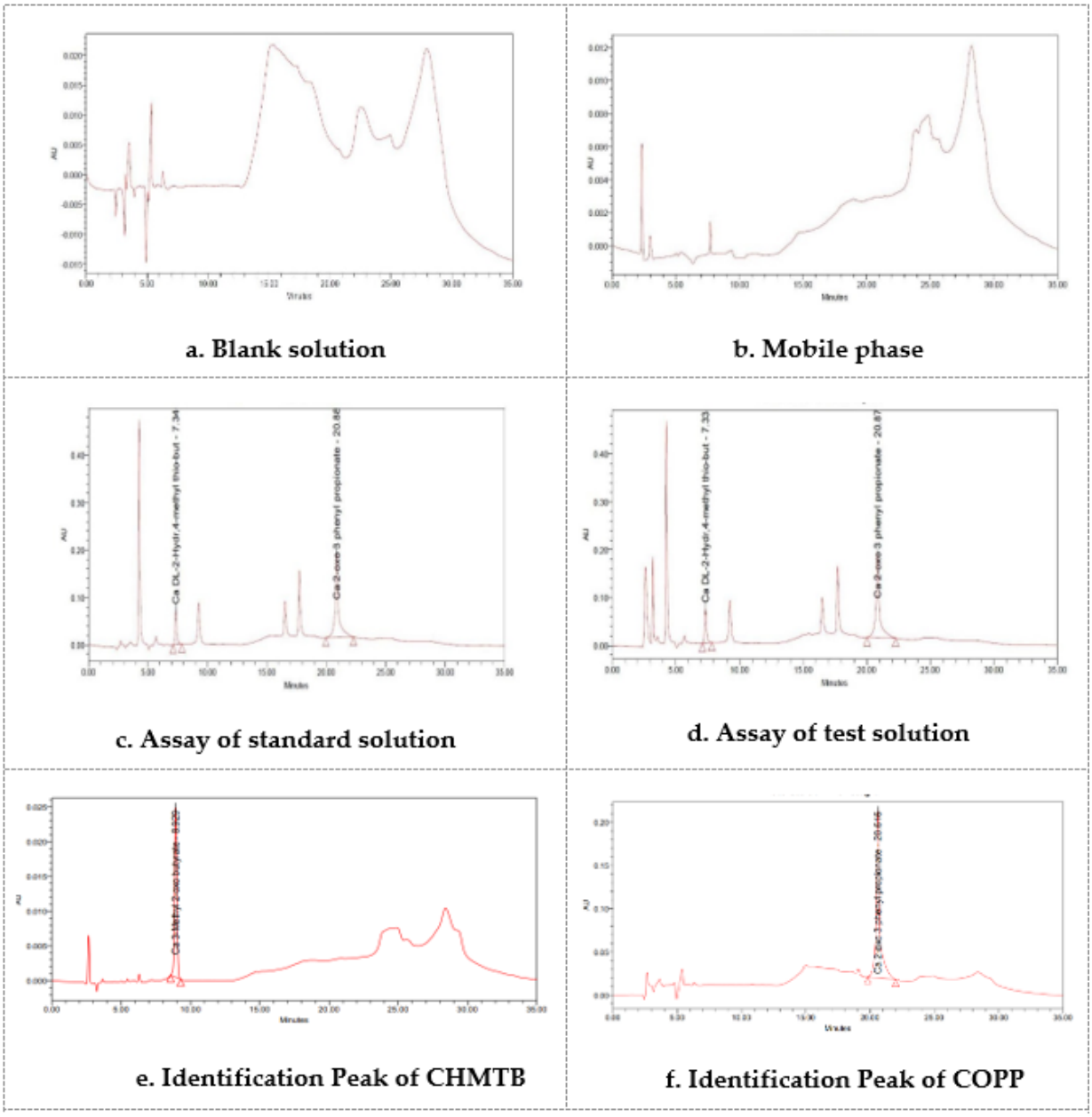

Figure 2: Specificity of the chromatogram at 210 nm.

Table 3: Accuracy measurement for COPP assay.

Level/set	Wt. of API spiked/added (mg)	Wt. Recovered (mg)	% Recovery	Average	% RSD
80% set-1	27.37	27.14	100.7	100.9	0.6
80% set-2	28.08	28.50	101.6		
80% set-3	27.17	27.28	100.4		
100% set-1	34.20	33.95	99.3	99.5	0.36
100% set-2	34.35	34.28	99.8		
100% set-3	34.06	34.10	100.1		
120% set-1	41.33	41.93	101.5	100.8	0.96
120% set-2	41.50	41.53	100.1		
120% set-3	41.22	41.65	101.1		

Table 4: Evaluation of method robustness.

Sl. No.	Change Parameter	%RSD	CHMTB	COPP
1	Column 35°C		0.6	0.7
2	Column 45°C		0.2	0.5
3	pH (6.8)		0.3	0.9
4	pH (7.2)		0.3	1.4
5	Flow rate (0.9 mL/min)		1.0	4.4
6	Flow rate (1.1 mL/min)		0.7	1.4

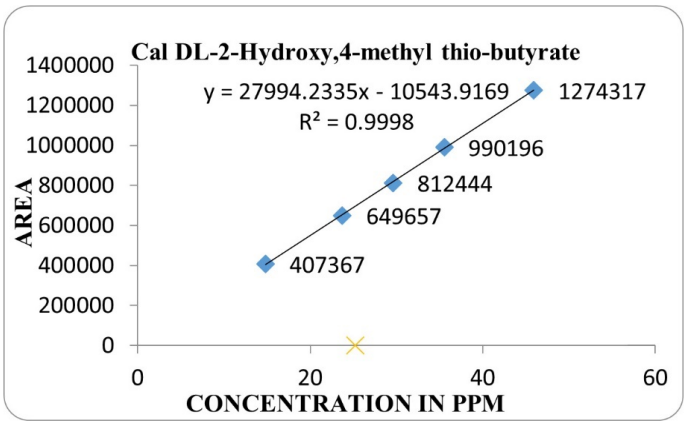

Figure 3: Linearity graph of CHMTB.

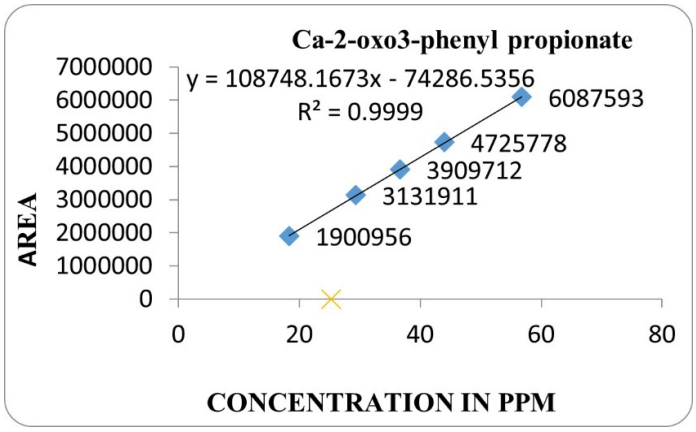

Figure 4: Linearity graph of COPP.

method was precise with respect system, method, and different analysts.

Linearity and Range

The square of the correlation coefficient was obtained at 0.9998 and 0.9999 for CHMTB and COPP, which was within the acceptance limit as per the guideline (not less than 0.995). The linearity graphs are given in Figures 3 and 4.

Accuracy

The results obtained for percentage recovery are within 98-102 % (Tables 2 and 3), respectively.

Robustness

Flow rate (±0.1 mL), pH of buffer (±0.2), and column oven temperature (±5°C) were changed, and the percentage RSD was calculated (Table 4). The results showed no significant change in the actual and change conditions parameters. So, the method was robust as per ICH guidelines.

CONCLUSION

RP-HPLC method was successfully developed for CHMTB and COPP and validated in terms of validation parameters per ICH guidelines (Q2R2) and USP 1225. The method was specific, precise, linear, robust, and accurate. Hence, the method was used for the percentage Assay determination of CHMTB and COPP in the in-process and finished product Tablet formulation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ICH: International Conference on Harmonisation; **USP:** United States Pharmacopoeia**; RSD:** Relative Standard Deviation; **HPLC:** High Performance Liquid Chromatography.

SUMMARY

- A New Analytical method has been developed for estimation of CHMBT & COPP in Alpha-Ketoanalogues Tablets.
- RP-HPLC gradient chromatography method has been used for the separation of two components.
- Tetra-n-butyl ammonium hydrogen sulfate (pH 7.0) which is an ion pairing agent has been used as a buffer for mobile phase A & Acetonitrile as a Mobile Phase B.
- The retention time of CHMBT & COPP has been found about 7minutes & 20 minutes with well resolve peaks.
- The % Assay method for CHMBT & COPP has been validated as per ICHQ2 (R2) & USP 1225.
- All the validation parameter is well within the acceptance limit & the method can be used for quantification purpose.

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