e-ISSN: 2320-1215
p-ISSN: 2322-0112

RESEARCH AND REVIEWS: JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES

A Liquid Chromatography – Mass Spectrometry Method for the Determination of Alpha-Ketoglutaric Acid in Human Plasma.

Gundoju Suresh*, PE Thirugnanam, and Atmakuru Ramesh.

Department of Analytical Chemistry, International Institute of Bio-technology and Toxicology (IIBAT), Padappai, Chennai 601 301, Tamil Nadu, India.

Research Article

Received: 25/11/2013
Revised : 26/12/2013
Accepted: 30/12/2013

***For Correspondence**

Department of Analytical Chemistry, International Institute of Bio-technology and Toxicology (IIBAT), Padappai, Chennai 601 301, Tamil Nadu, India.

Keywords: Alpha-ketoglutaric acid (α-KG), LC-MS/MS, Plasma, Cyanide antidote,

ABSTRACT

Alpha-ketoglutaric acid (A-KG) is currently under investigation as promising cancer cell damaging agent and cyanide antidote. A simple, specific liquid chromatography-mass spectrometry (LC-MS/MS) method was developed for the quantitative determination of A-KG in human plasma. Derivatization of A-KG was achieved by using N-methyl imidazole in presence of trifluroacetic anhydrate. Several parameters, which affected the yield of the derivatization reaction, such as reaction temperature and time were optimized. An imidazole derivative of A-KG was extracted using methyl tertiary butyl ether and quantified in positive electron spry ionization mode (mass-to-charge ratio (m/z):197). The method was fully validated using the parameters such as specificity, linearity, limit of detection and quantification, precision, accuracy, recovery, robustness and ruggedness. Linearity was demonstrated over the concentration rage of 100-2500 ng mL^{-1} with a correlation coefficient (R^2) 0.9991. The limit of detection and quantification were found to be 40 and 100 ng mL^{-1} respectively. Intra- and inter-day accuracy and precision were within the acceptable limit (<8%).The robustness and ruggedness was evaluated using Plackett-Burman experimental design.

INTRODUCTION

Alpha-ketoglutaric acid (A-KG) is a derivative of glutaric acid. It is a key compound for the proper metabolism of all essential amino acids and the transfer of cellular energy in the citric acid cycle. In combination of L-glutamate, alpha-ketoglutaric acid can reduce levels of ammonia formed in the brain, muscles and kidneys, as well as help balance the body's nitrogen chemistry and prevent nitrogen excess in body tissues and fluids. Oral and parenteral administration of A-KG leads to reduced oxidative stress and improvement of preoperative exercise capacity. This, in turn, leads to shorter recovery time and reduced risk of complications in fast rack surgery programs and lung surgery with resultant lower hospitalization and morbidity. In addition, A-KG is said to help treat or prevent health problems such as bacterial infections, cataracts, kidney disease and yeast infections [1, 2]. A-KG is currently being pursued widely as a cyanide antidote, A-KG react with cyanide to form nontoxic cyanohydrins derivative [3,4,5,6].

Several methods have been developed for the quantification of alpha-ketoglutaric acid in biological samples as its corresponding phenyl-hydrazone, dinitrophenylhydrazone or quinoxalone coupled to photometric detection [8,9,10,11] or as its dipyrene, 2-quinoxalinol, quinoxalinone or zinc-pyridoxamine derivatives coupled to fluorimeric detection [12,13,14,15,16].

The aim of this present study is to develop a LC-MS/MS method for the determination of A-KG in human plasma. In the present study A-KG was derivatized using n-methyl imidazole and derivatization reaction was optimized with respect to the reaction temperature and time. The present proposed method can be useful for the routine analysis of A-KG in human plasma samples.

e-ISSN: 2320-1215
p-ISSN: 2322-0112

EXPERIMENTAL

Chemicals and reagents

Alpha-ketoglutaric acid disodium salt (purity 96%) was purchased from Sigma-Aldrich, USA. N-methyl imidazole (NMI), trifluroacetic anhydride (TFAA) and methyl tertiary butyl ether (MTBE) were purchased from Sigma-Aldrich, USA. All other solvents and reagents of analytical grade were from Merck (Darmstadt, Germany).

Instrumentation and LC-MS/MS conditions

The quantification of alpha-ketoglutaric acid was performed by an Agilent 1200 HPLC system (Germany) consisting of a binary pump, an autosampler, a diode array detector and temperature controlled column oven, coupled to Bruker MS detector with Electron Spry Ionization (ESI) interface. The separations were performed on a Zorbax SB C8 (4.6 x 75 mm, 3.5 µ) using isocratic mixture 65:35 v/v (acetonitrile: water 0.1% formic acid).

Preparation of standard solutions

Standard stock solution containing 100 mg/L of A-KG was prepared in distilled water and stored at 4°C. Working standards were freshly prepared by diluting stock solution with distilled water.

Optimization of the derivatization reaction

The derivatization reaction was optimized by comparing the peak areas of the derivatized A-KG. The ion *m/z* 179 for A-KG was monitored to ensure that the derivatization was as efficient as possible. Derivatization reactions were carried out at different temperatures (60, 65 and 70°C) and times (30, 60 and 90 min) and five-replicate analyses were done for each condition. The reaction was performed by evaporating the standard solution of A-KG to dryness in a test tube under a gentle stream of nitrogen. Then, 40 µL of derivatization reagent was added. The test tubes were allowed to stand for 60 and 80°C for 30, 60 and 90 min. The resulting solutions were injected into the LC-MS/MS.

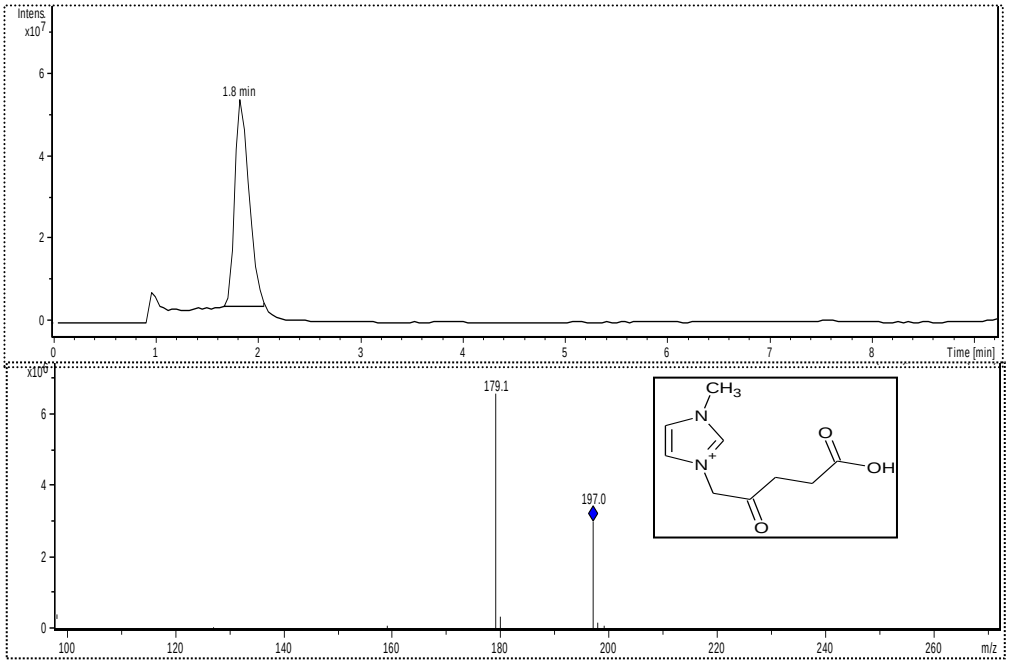


Figure 1: Chromatogram obtained from spiked plasma: (a) Total ion chromatogram, (b) mass spectra

Sample preparation, extraction and derivatization

One hundred microliters plasma or aqueous sample solution of A-KG (100µg/mL) was added to a glass vial and mixed with 20µL N-methyl imidazole reagent. Twenty microliters trifluroacetic anhydride was added to the mixture. The reaction was allowed to proceed for 15minutes at 65°C in a water bath. Added 3mL of methyl tertiary butyl ether (MTBE) to the mixture and the vial was closed after short vigorous vortex and extracted for 10 minutes at a wrist action shaker. After centrifugation at 4000 rpm for 5min, the organic layer was collected, the solvent evaporated under a steam of nitrogen, and the dry residue reconstituted in methanol, samples were transferred into auto sampler vials. The vial were closed with crimp-top caps and stored at -20°C until LC-MS/MS analysis.

e-ISSN: 2320-1215
p-ISSN: 2322-0112

Method Validation

Specificity

Specificity of the method was evaluated by analyzing different blank samples. This was done to investigate whether there had been any interference from the endogenous substance. The chromatograms obtained were examined for any interference at the retention time by monitoring the ions 179 m/z and 197 m/z. Carryover effect of the method was assessed by injecting high concentrations of A-KG into spiked plasma followed by blank samples.

Linearity and limits of the method (LOD and LOQ)

The linearity of the method was assessed by making calibration curves (in blank matrix and solvent) over the concentration rage 100-2500 ng/mL. The calibration curves were constructed by plotting the peak area versus concentration of A-KG. The coefficient of determination was evaluated.

The limit of detection (LOD) and limit of quantification (LOQ) were established based on the following expressions.

$$LOD = Xb + 3S$$
$$LOQ = Xb + 10S$$

Where, Xb is the mean peak area of blank and S is the standard deviation of the peak area.

Limit of quantification (LOQ) is the least concentration that can be detected and quantified with an acceptable precision and accuracy. The acceptable precision and accuracy at the LOQ point are 20% and 80-120%, respectively.

Precision and accuracy

Precision and accuracy of the method was evaluated by performing intra-day and inter-day precision and accuracy tests. To perform the intra-day test, 100 µL plasma samples were spiked with the appropriate amount of A-KG standard solution in order to obtain samples at three concentration levels (100, 500 and 1500 ng/mL). The sample were processed for LC-MS/MS analysis and analyzed on the same day. To evaluate the inter-day precision and accuracy, the spiked plasma samples at three concentration levels (100, 500 and 1500 ng/mL) were prepared over five days and analyzed on each of the five days. At each concentration level five replicate samples (n=5) were prepared and analyzed in duplicate. The concentration of A-KG in each sample was calculated using slope and intercept obtained from calibration curves, which were constructed daily. Precision was represented by percent relative standard deviation (%RSD) and accuracy was expressed by bias.

Recovery

The recovery studies were conducted at three different concentration levels (100, 500 and 1500 ng/mL). Appropriate volumes of working standards were spiked into 100 µL of blank plasma and the samples were processed and analyzed by LC-MS/MS.

Robustness and ruggedness

The robustness and ruggedness of the method were simultaneously carried out using the Plackett-Burman experimental design. Five variables were selected, which were significant in the performance of the method twelve experimental runs were performed (Table 1 and 2). Experiments were performed in a random order to minimize the effect of unexplained variability on the response. The results were assessed using the STATISTICA 10 statistical software.

Table 1: The variable tested for the robustness and ruggedness

Variables	Low level (-)	High level (+)
Analyst	Analyst I	Analyst II
Column	Column I	Column II
NMIZ	NMIZ I	NMIZ II
Derivatization time (min)	25	35
Derivatization temperature (°C)	63	67

e-ISSN: 2320-1215
p-ISSN: 2322-0112

Table 2: Plackett-Burman experimental design matrix for five variables

	Variables				
Exp.no	Analyst	Column	NMIZ	Derivatization time (min)	Derivatization temperature (°C)
1	+	-	+	-	-
2	+	+	-	+	-
3	-	+	+	-	+
4	+	-	+	+	-
5	+	+	-	+	+
6	+	+	+	-	+
7	-	+	+	+	-
8	-	-	+	+	+
9	-	-	-	+	+
10	+	-	-	-	+
11	-	+	-	-	-
12	-	-	-	-	-

Table 3: Intra- and inter-day precision and accuracy results for α-KG in spiked plasma samples (n=5)

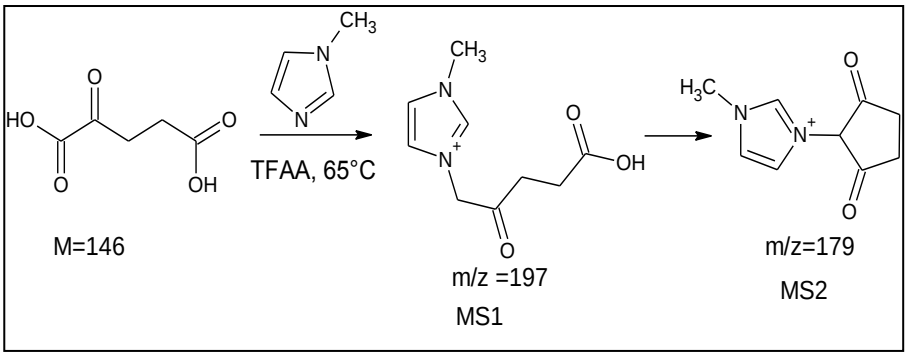
	Inter-day			Intra-day		
Nominal amount (ng mL^{-1})	Mean Recovered (%)	Precision[a] (%)	Accuracy[b] (%)	Mean Recovered (%)	Precision[a] (%)	Accuracy[b] (%)
100	98.02	7.58	-1.98	98.53	6.95	-1.47
500	494.56	5.94	-1.088	498.75	5.63	-0.25
1500	1492.68	4.86	-0.488	1502	4.73	0.13

[a] = percent relative standard deviation [b] = [(found – added)/added] x 100.

RESULTS AND DISCUSSION

N-methyl imidazole was selected as the derivatization reagent in order to introduce imidazole group to A-KG. The possible reaction of A-KG with methyl imidazole is provided in Fig.2. In the A-KG - methyl imidazole reaction, the influence of the reaction time on the reaction yield at 60, 65 and 70°C was evaluated. Each temperature was tested for 20, 30 and 50 minutes and yields of the reaction under different conditions are given in Fig.3. It was found that the reaction was most effective at 65°C. The yield of the reaction was increased from 60 to 65°C in 60 min time. Therefore, it was concluded that the reaction is optimally conducted at 65°C for 30 min.

Figure 2: The possible reaction of α-KG with methyl imidazole - The imidazole adduct was formed with α-KG at optimized temperature in presence of TFAA further the MS/MS was shows the cyclization of the glutaric acid.



e-ISSN: 2320-1215
p-ISSN: 2322-0112

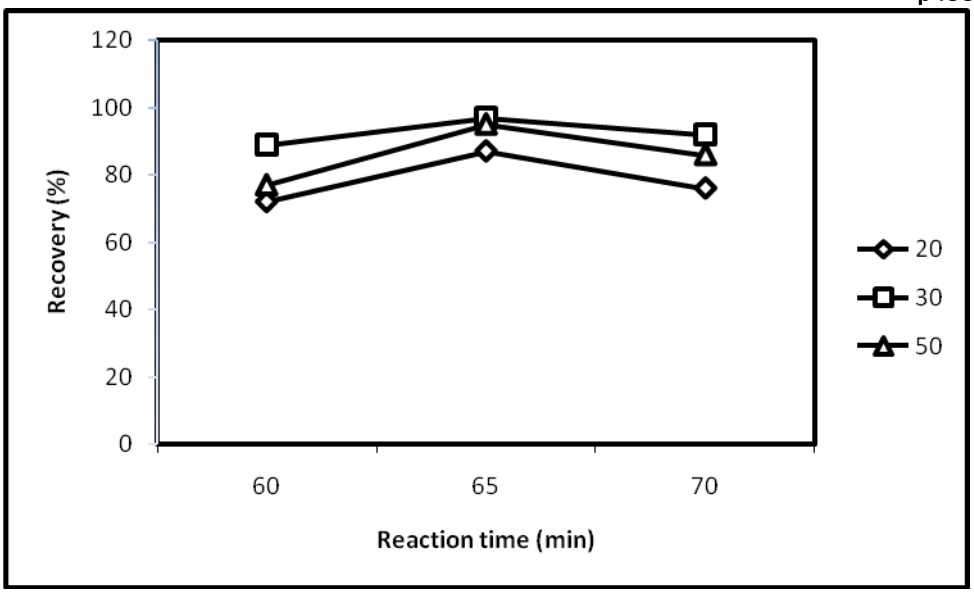


Figure 3: Yield of the derivatization reaction under different reaction conditions

Method validation

Specificity

Chromatograms obtained for specificity tests were examined for any interference at the retention time of A-KG. No interference was detected. Therefore the developed method is considered to be specific.

In the LC-MS/MS assay, no carryover problem was observed after the repeated injections of high concentrations of A-KG.

Linearity and limits of the method (LOD and LOQ)

The LOD and LOQ were found to be 40 ng/mL and 100 ng/mL (RSD 4.9%) respectively. The method was linear over the concentration range of 100-2500 ng/mL with a coefficient of determination (R^2) greater than 0.999.

Precision and accuracy

The results for precision and accuracy are summarized in Table 3. The intra- and inter-day precision did not exceed 7.58%, while the values for accuracy varied between -1.98% and 0.13%. The method developed was thus considered to be precise and accurate.

Recovery

The recovery values for each concentration point was determined and they fell in the range of 71.36-89.65%, the precision values calculated at each point remained within 3.01-1.12%, which means the recovery of A-KG is consistent and precise.

Robustness and ruggedness

The ANOVA test with the Plackett-Burman experimental design was used to identify the effects of each factor on the response. The results showed that the effects of selected variable on the response were not significant ($p > 0.05$). In other words, the variable for this method did not have significant effects on the response. Therefore, the method can be considered to be robust and rugged with regard to the variations tested in this study.

CONCLUSIONS

The proposed LC-MS/MS method can be readily applied for the identification and quantification of α-KG in human plasma samples. Prior to LC-MS/MS analysis, a simple dericvatization method with methyl imidazole was successfully applied and imidazole derivative of α-KG was formed. The obtained derivative was detected with the characteristic fragments (m/z 179 and 197) with mass spectral analysis. Linearity was achieved over the

concentration range of 100-300 ng/mL. The validation data showed that the developed method is specific, precise, accurate, robust ad rugged.

ACKNOWLEDGEMENT

The authors are thankful to the Management, IIBAT, for providing necessary facility to conduct the experiment.

REFERENCES

1. Jeppsson A, Ekroth R, Friberg P, Kirno K, Milocco I, Nilsson FN, et al. Renal effects of alpha-ketoglutarate early after coronary operations. Ann Thorac Surg. 1998;65(3):684-690.
2. Riedel E, Nundel M, Hampl H. Alpha-ketoglutarate application in hemodialysis patients improves amino acid metabolism. Nephron, 1996;74 (2):261-265.
3. Bhattacharya R, Tulsawani RK. *In Vitro* and *In Vivo* evaluation of various carbonyl compounds against cyanide toxicity with particular reference to alph-ketoglutaric acid. Drug Chem Toxicol. 2008; 31(1):149-161.
4. Tulsawani RK, Debnath M, Pant SC, Kumar Om, Prakash AO, Vijayaraghavan R, et al. Effect of sub-acute oral cyanide administration in rats: protective efficacy of alpha-ketoglutarate and sodium thiosulfate. Chemico-Biol. Interact. 2005;156(1):1-12.
5. Mittal G, Singh T, Kumar N, Bhatnagar A, Tripathi RP, Tulsawani R, et al. Radiolabeling and dose fixation study of oral alpha-ketoglutarate as a cyanide antidote in healthy human volunteers. Clin Toxicol (Phila), 2010;48(6):509-515.
6. Bhattacharya R, Vijyaraghavan R. Promising role of alpha-ketoglutarate in protecting the lethal effects of cyanide. Hum Exp Toxicol. 2002; 21(6):297-303.
7. Dulanev MD, Brumely M, Willis JT, Hume AS. Protection against cyanide toxicity by oral alpha-ketoglutaric acid. Vet Human Toxicol. 1991, 33 (6):571-575.
8. Liao JC, Hoffman NE, Barboriak JJ, Roth DA. High-performance liquid chromatography of pyruvic acid and α-ketoglutaric acids and its application to urine samples. Clin Chem.1977; 23(5):802-805.
9. Lang M, Malyusz M. Fast method for the simultaneous determination of 2-oxo acids in biological fluids by high-performance liquid chromatography. J Chromatogr B Biomed Appl.1994; 662(1); 97-102.
10. Qureshi AA, Elson CE, Lebeck LA. Application of high-performance liquid chromatography to the determination of glyoxylate synthesis in chick embryo liver. J.Chromatogr A. 1982; 249(2):333-345.
11. Hemming BC, Gubler CJ. High-pressure liquid chromatography of alph-keto acid 2,4-dinitropheylhydrazones. Anal Biochem. 1979; 92(1):31-40.
12. Nakamura H, Tamura Z. Fluorometric determination of pyruvic acid and alpha-ketoglutaric acid by high performance liquid chromatography. Anal Chem. 1979; 51(11):1679-1683.
13. Nohta H, Sonoda J, Yoshida H, Satonozo H, Ishida J, Yamaguchi M. Liquid chromatographic determination of dicarboxylic acids based on intramolecular excimer-forming fluorescence derivatization. J Chromatogr A. 2003; 1010(1):37-44.
14. Darmaun D, D'Amore D, Haymond MW. Determination of glutamine and alph-ketoglutarate concentration and specific activity in plasma using high-performance liquid chromatography. J Chromatogr B Biomed Appl. 1993; 620(1):33-38.
15. Hayashi T, Tsuchiya H, Todoriki H, Naruse H. High-performance liquid chromatographic determination of alpha-keto acids in human urine and plasma. Anal Biochem. 1982; 122(1):173-179.
16. Muhling J, Fuchs M, Campos ME, Gonter J, Engel JM, Sablotzki A,et al. Quantitative determination of free intracellular alpha-keto acids in neutrophils. J Chromatogr B. 2003; 789(2):383-392.