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HPLC Determination of α -keto Acids in Human Serum and Urine after Derivatization with 4-Nitro-1,2-phenylenediamine

Khalida Parveen Mahar^{*1}, Kulsoom Ubedullah Abbasi², Muhammad Yar Khuhawar², Gulam Murtaza Mastoi², Abdul Hameed Channer², Shamroz Bano Sahito¹ and Abdul Jabbar Kandhro²

¹Shah Abdul Latif University Khairpur Pakistan

²Institute of Advanced Research Studies in Chemical Sciences, University of Sindh, Jamshoro, Pakistan

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Abstract

The determination of α -keto acids has clinical importance, because these are intermediates in a number of biochemical processes. This work reports the development of an HPLC procedure for the analysis α -keto acids in blood and urine samples after derivatization with 4-nitro-1,2-phenylenediamine (NPD). Nine α -keto acids: glyoxylic acid (GA), pyruvic acid (PYR), 2-oxobutyric acid (KB), 3-methyl-2-oxobutyric acid (MKBA), 3-methyl-2-oxovaleric acid (K₃MVA), 2-oxoglutaric acid (KG), 4-methyl-2-oxovaleric acid (K₄MVA), 2-oxohexanoic acid (KHA) and phenylpyruvic acid (PPY) were derivatized with (NPD) at pH 3 and separated on a Zorbax 300 SB-C18 HPLC column (4.6x150mm id) and photodiode array detection at 255 nm. The isocratic elution was performed with methanol: water: acetonitrile (42: 56:2, v/ v/ v) with a flow rate 0.9 mL/min. The keto acids separated within 14 min. The method was repeatable with a relative standard deviation (RSD) of 0.1-2.9% for each of the α -keto acids. The limits of detection and quantitation were obtained within the range 0.05-0.26 μ g/ mL and 0.15-0.8 μ g/ mL respectively. The method was applied for determination of α -keto acids from a pharmaceutical preparation, human serum and urine samples of healthy volunteers and diabetic patients. The results were further confirmed by standard addition technique. The method is rapid and simple and is suitable for the separation and determination of α -keto acids from clinical samples.

Keywords: α -Keto acids; 4-Nitro-1,2-phenylenediamine; HPLC; Serum; Urine.

Introduction

α -Keto acids are key intermediates in a number of major biochemical pathways including glycolysis, amino acids and carbohydrate metabolism [1]. Each of the keto acid has its specific function. Pyruvic acid (PYR) is involved in the biosynthesis of alanine [2], and branched chain keto acids: 3-methyl-2-oxovaleric acid (K₃MVA) and 4-methyl-2-oxovaleric acid (K₄MVA) can regulate protein turn over [3, 5]. The determination of the α -keto acids is of interest for pathological conditions such

as sepsis [6], burns [7] and hepatic disorders [8]. The concentration of phenylpyruvic acid increases in the serum of patients with hereditary metabolic diseases [9]. A higher risk of infectious diseases have been reported in elderly people, because of protein malnutrition and decline in the immune system [10]. The subjects may improve on supplementation with α -keto acids. Therefore, the determination of α -keto acids in biological fluids is of considerable interest and relevance.

*Corresponding Author Email: khalidamahar@yahoo.com

A number of analytical methods for the determination of α -keto acids are based on gas chromatography (GC-FID, GC-MSD) [11, 19], high performance liquid chromatography (HPLC) [20, 27] and capillary zone electrophoresis (CZE) [28]. Several derivatizing reagents have been reported for the HPLC determination of α -keto acids with spectrophotometric and spectrofluorimetric detection. Among these frequently used reagents are 2,4-dinitrophenylhydrazine [29-30], 4-hydrazine-2-stilbanzole [31], o-phenylenediamine [32, 33], 1,2-diamino-4,5-dimethoxybenzene [34], 1,2-diamino-4,5-methylenedioxybenzene [35] and 4,5-diaminophthalhydrazide [36]. 4-nitro-1,2-phenylenediamine (NPD) is related to o-phenylenediamine and has been used for the determination of α -keto acids by paper chromatography [37, 39]. However, many of these techniques are time-consuming and involving extensive sample preparation. The present work was aimed at developing a rapid HPLC method for the analysis of α -keto acids, using 4-nitro-1,2-phenylenediamine (NPD) as derivatizing reagent from a pharmaceutical preparation, human serum and urine of healthy volunteers and diabetic patients.

Materials and Methods

Chemicals and Reagents

GA monohydrate, PYR, KB, MKBA, KHA, K₃MVA, K₄MVA, sodium salt, KG monosodium salt, NPD (Fluka, Switzerland), and PPY (sigma, USA) were used. The standard solutions of α -Keto acids (1.0 mg/mL) were prepared in 10 % acetic acid (w/v). Further solutions were prepared by appropriate dilution.

The NPD was recrystallized from n-heptane before use. Methanol (Fisher Scientific, HPLC grade, Leicestershire, UK), acetic acid, (Riedel-dehaen, Germany), hydrochloric acid (37%), potassium chloride, sodium acetate, ammonium acetate, boric acid, sodium tetraborate, sodium bicarbonate, sodium carbonate, ammonium chloride and ammonia (25%) (E. Merck, Germany) were used. Freshly prepared double distilled water was used throughout the study.

The Buffer solutions within pH 1-10 at unit interval were prepared from Hydrochloric acid (0.1 M) and potassium chloride (1 M) (pH 1-2), acetic acid (1 M) and sodium acetate (1 M) (pH 3-6), ammonium acetate (1 M) (pH7), boric acid (1 M) and sodium tetra borate (1 M) (pH-8), sodium bicarbonate (1 M), sodium carbonate (saturated) (pH-9) and ammonium chloride (1 M) and ammonia solution (1 M) (pH-10).

All pH measurements were made with Orion 420 A pH meter (Orion (Pvt.), Ltd, Boston, U.S.A) with combined glass electrode and internal reference electrode. Spectrophotometric study was carried out with a double-beam Hitachi 220 Spectrophotometer (Hitachi (Pvt) Ltd Tokyo, Japan) with dual 1 cm silica cuvettes.

Analytical Procedures

Spectrophotometric Procedure

To the solution (1-2 mL) containing (5-100 μ g) GA, PYR, KB, MKBA, K₃MVA, KG, K₄MVA, KHA, and PPY was transferred to 10 mL volumetric flask separately. Each of the solution was added NPD solution (1.5 mL, 1.0 %, w/v in methanol), acetic acid sodium acetate buffer (pH 3) (1 mL) and contents were warmed on water bath at 80 °C for 30 min. The volume was adjusted with methanol and absorption spectrum was recorded in the spectral range of 400-250 nm against reagent blank. The reagent blank was prepared following the same procedure, without the addition of α - keto acids.

HPLC Procedure

To an aqueous solution (1mL) containing GA, PYR, MKBA, KB, K₃MVA, KG, K₄MVA, KHA, and PPY of each within the concentration range as indicated in (Table 1), was added NPD reagent solution (1 mL, 1%, w/ v in methanol) and acetic acid-sodium acetate buffer pH 3 (1 mL). The contents were warmed at 80 °C for 30 min and volume was adjusted with methanol to 10 mL. The solution (20 μ l) was injected on the column and eluted with methanol: water: acetonitrile (42: 56: 2 v/ v/ v) with a flow rate 0.9 mL/ min. The detection was set at 255 nm.

Table 1. HPLC parameter for α -Keto acids using NPD as a derivatization reagent

Sr. No.	Name of Compound	Calibration Range ($\mu\text{g/mL}$)	Coefficient of determination (R^2)	Least Square Or Regression	Limit of Detection N (LOD) ($\mu\text{g/mL}$)	Limit of Quantitation (LOQ)
1	GA	(0.2-5)	0.9968	$Y=5.0029x+0.0095$	0.06	0.2
2	PYR	(0.2-20)	0.9978	$Y=3.1505x+0.0286$	0.05	0.15
3	KB	(0.3-100)	0.9986	$Y=0.3676x+0.0381$	0.13	0.39
4	MKBA	(0.45-100)	0.998	$Y=0.4897x+0.0119$	0.15	0.45
5	K3MVA	(0.6-100)	0.9976	$Y=0.2584x+0.0286$	0.266	0.8
6	KG	(0.5-100)	0.9988	$Y=0.1137x+0.0333$	0.16	0.5
7	K4MVA	(0.6-100)	0.9983	$Y=0.148x+0.0333$	0.2	0.6
8	KHA	(0.7-100)	0.9953	$Y=0.1265x+0.001$	0.25	0.75
9	PPY	(0.4-100)	0.9957	$Y=0.1196x-0.0519$	0.1	0.3

Chromatographic Conditions

HPLC was carried out on Agilent model 1100-network HPLC System (Agilent Technology Inc, USA) 1100 series a syringe loading sample injector containing a 20 μL loop, two quart pump G1311A, Degasser G1379A with DAD G1315B detection system. The computer with Chem Station software controlled the HPLC. The column ZORBAX 300 SB-C18 (4.6 x 150 mm i.d.) (Agilent Technology Inc, USA) was used throughout the study. Retention times and peak areas were determined by enhanced integrator.

Analysis of Pharmaceutical preparation

Five tablets Ketostril (Fresenius Kabi Bad Homburg, Germany) were ground to powder and amount (0.802 g) corresponding to one tablet was dissolved in 10% acetic acid (20 mL). The solution was filtered and volume adjusted to 100 mL. Well mixed solution 0.4 mL and 0.6 mL were

transferred to 10 mL volumetric flasks and HPLC analytical procedure was followed. The quantitation was carried out using external calibration curve.

Analysis of Pharmaceutical Preparation by Standard Addition

Five tablets of Ketostril were treated as Analysis of Pharmaceutical preparation. The solutions 0.4 mL and 0.6 mL were taken in duplicate. A solution was processed as HPLC analytical procedure and others were added MKBA 50 μg , K₃MVA 60 μg , and K₄MVA 100 μg , PPY 40 μg , and again processed as HPLC analytical procedure. The quantitation was carried out from external calibration curve and an increase in the response with added standards.

Sample Collection and Pretreatment

The blood and urine samples of diabetic patients with verbal contents were obtained from LUMHS Hospital, Jamshoro. The blood samples were collected by vein puncture with hypodermic

syringe. Urine samples were collected in the morning in clean plastic bottle. The blood glucose level of the patient was collected from the record of the hospital of the patient on the day of sample collection. The data was collected with permission of duty doctor and the patients. The blood samples of healthy volunteers with verbal consent were collected from Dr. M. A. Kazi Institute of Chemistry and their blood glucose level were determined by Micro-lab (E-Merck, Germany).

Determination of α -Keto Acids from Serum

The blood samples (5 mL) collected from healthy volunteers and diabetic patients were allowed at room temperature for 1 h and were centrifuged at 3000 rpm for 20 min. The supernatant layer was separated and added methanol twice in volume (5 ml). The contents were mixed well and again centrifuged at 3000 g for 20 min. The supernatant layer was collected and procedure HPLC analytical procedure was followed. The final volume was adjusted to 5 mL. The quantitation was carried out using external calibration curve.

Determination of α -Keto Acids from Serum using Linear Calibration Curve with Spiked Sample

Blood sample (5 ml) collected from the diabetic patient was treated as determination of α -Keto acids from Serum. The serum after deproteinization with methanol was divided in two equal parts. A part was treated as HPLC analytical procedure and other was added PYR (3 μ g), KB (8 μ g), MKBA (6 μ g), K₃MVA (30 μ g), KG (30 μ g), K₄MVA (60 μ g), PPY (40 μ g), and procedure HPLC was again followed the final volume was adjusted to 5 mL. Quantitation was carried out using linear calibration and increase in the response (average peak height / area) with added standard.

Determination of α -Keto acids from Urine

The urine (2 mL) collected from the diabetic patients in Stoppard test tube (Quick fit) was diluted with methanol (1 mL) and was centrifuged at 3000 rpm for 20 min. The clear solution was transferred to volumetric flask and procedure HPLC was followed. The final volume

was adjusted to 5 mL. The quantification was carried out by calibration curve.

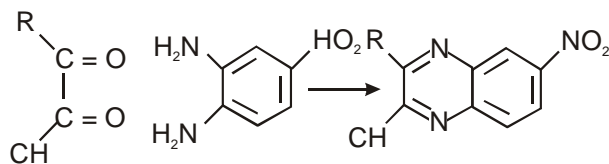
Determination of α -Keto acids from Urine by Standard Addition

The urine sample (2 mL) in duplicate from diabetic patient was treated as Determination of α -Keto acids from Urine. A sample was processed as HPLC procedure and other was added PYR (2 μ g), KB (6 μ g), MKBA (8 μ g), K₃MVA (40 μ g), KG (30 μ g), K₄MVA (70 μ g) and PPY (60 μ g), again processed as HPLC procedure. The final volume was adjusted to 5 mL. The quantitation was made by linear calibration and an increase in the response with added standard.

Results and Discussion

Derivatization and Separation

α -keto acids react with NPD to form nitroquinoxanol derivatives (Fig 1). Nine α -keto acids: GA, PYR, KB, MKBA, K₃MVA, KG, K₄MVA, KHA, and PPY were treated with NPD separately and the derivatives formed were extracted in chloroform, and examined for elution from HPLC column. The effect of reaction conditions such as pH, amount of reagent NPD added per analysis, warming time and temperature were investigated. The reactions were initially monitored by a UV-Vis spectrophotometer. The derivatives showed absorbance maxima in the range of 299 to 340 nm against the reagent blank. The λ_{max} obtained were considered for monitoring the reactions. The effect of pH within 1 to 10, at a unit interval was examined and the reaction was observed maximum in acidic medium and acetate buffer (pH 3) was selected (Fig 2a). The reagent solution (1% w/v in methanol) was varied from 0.5-4.0 mL and at an interval of 0.5 mL. A similar response was observed with the addition of 1 mL and above and addition of 1.5 mL was selected. The warming time at 80 °C was varied from 10-40 min at an interval 5 min. Maximum absorbance was observed with warming time of 15 min and above and warming time of 30 min was selected (Fig 2b). Each of the derivatives at optimized conditions obeyed the Beer's law within 5-100 μ g/mL and did not show any change in absorbance up to 24 h.



GA = R = H
 PYR = R = CH₃
 KB = R = C₂H₅
 MKBA = R = (CH₃)₂ CH
 KG = R = CH₂ . CH₂ . COOH
 KHA = R = C₄H₉
 PPY = R = C₆H₅ . CH₂
 K₃MVA = R = CH₃ . CH₂ . CH - CH₃
 K₃ MVA = R = (CH₃)₂ CH . CH₂

Figure 1. Reaction of α -Keto acids with NPD.

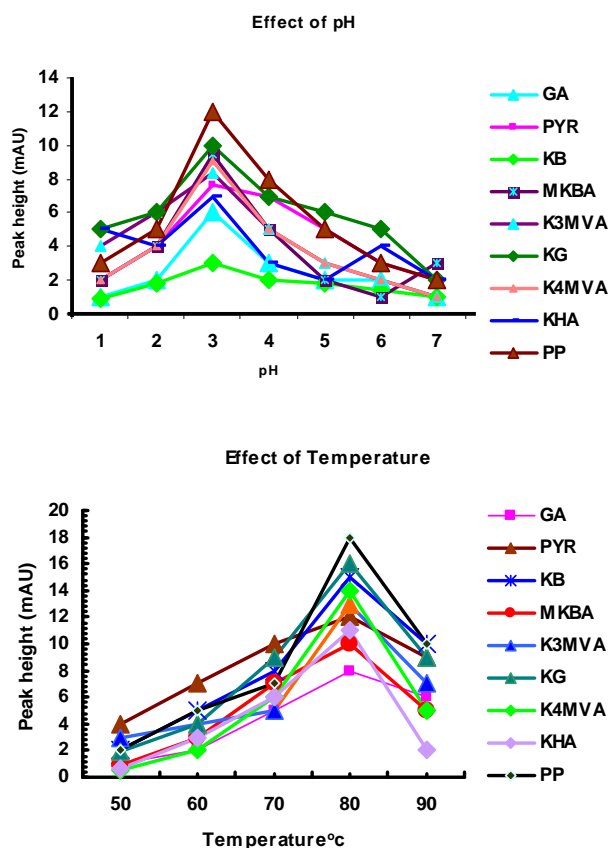


Figure 2. (a) Effect of pH (b) Effect of Temperature, Amount of reagent (1%, w/v) for the derivative of 2-oxo acids with NPD. Conditions: column Zorbax C-18 (4.6 x 150 mm id) isocratic elution with methanol - water - acetonitrile (42:56:2 v/v/v) with a flow rate of 0.9 ml/min. UV detection by photodiode array at 255 nm

For the separation and simultaneous determination of the α -keto acids, a Zorbax C-18 HPLC column was used. Each of the derivatives,

eluted as a single peak and separated from the derivatizing reagent. For the separation of the α -keto acids, composition of the mobile phase solvents was changed. An isocratic elution with methanol: water: acetonitrile (42: 56: 2, v/ v/ v) with a flow rate of 0.9 mL/ min resulted a better resolution of GA PYR, KB, MKBA, K₃MVA, KG, K₄MVA, KHA, and PPY peaks. Although a baseline separation was achieved but excess of the derivatizing reagent affected the elution of GA derivative. In order to improve the separation of GA concentration of the derivatizing reagent was decreased to 1 mL (Fig 3). The peak identification was based on the retention time and by spiking each of the α -keto acids in sequence. Repeatability of the separation (precision) in terms of retention time and peak height was examined (n=5) and relative standard deviations (RSD) were observed within 0.1-2.0% and 1.5-2.9%, respectively. The derivatives absorb in the UV region due to π - π^* transition within quinoxalinols rings. The wavelength at peak maximum was examined with the diode-array detection at 255 nm.

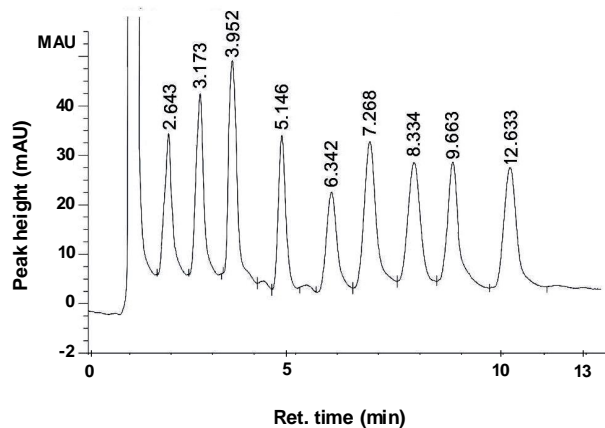


Figure 3. HPLC separation of (1) Reagent (2) GA (3) PYR (4) KB (5) MKBA, (6) K₃MVA (7) KG (8) K₄MVA (9) KHA and (10) PPY. Condition as Fig 2.

Quantitation and Validation

Linearity and limit of detection

Linear calibration curves were obtained by recording average peak height/ peak area (n = 4) versus concentration of GA (0.2-5.0 μ g/ mL), PYR (0.2-20 μ g /mL), KB (0.3-100 μ g/ mL), MKBA (0.45-100 μ g/mL), KG (0.5-100 μ g/ mL), K₃MVA (0.6-100 μ g/ mL), K₄MVA (0.6-100 μ g/ mL),

KHA (0.7-100 $\mu\text{g}/\text{mL}$), PPY (0.7-100 $\mu\text{g}/\text{mL}$) with coefficient of determination (r^2) ranging 0.9953-0.9988. The limits of detection (LOD) measured as three times the signal to noise ratio (3:1) were obtained within the range 0.05-0.26 $\mu\text{g}/\text{mL}$ and limit of quantitation (LOQ) measured as signal to noise ratio (10: 1) were within (0.15-0.8) $\mu\text{g}/\text{mL}$ (Table1). The analysis of test solutions ($n = 6$) of the mixture of the α -keto acids indicated relative error within ± 0.1 - 2.9%.

Precision Recovery

The derivatization reaction and separation was repeatable and reproducible and variation in the response (peak height/ peak area) of each of the α -keto acid was examined intra and inter day by the same operator under same conditions on the same day and different days ($n = 5$) and RSDs were observed within (0.3-2.8%) and (0.5-3.2%) respectively. The derivatives were found to be highly stable and did not show any change in response up to 48 h.

The pharmaceutical preparations additives methylparabin, propylparabin, gum acacia, manitol, lactose, fructose, glucose, galactose, sodium chloride, sodium lacuryl sulphate and methyl hydroxypropyl cellulose were added at least twice the concentration of α -keto acids and analysis was carried out following analytical procedure. The results obtained were compared with α -keto acids standards. The addition of additives did not affect the determination as the relative error was $\pm 2.9\%$.

Sample Analysis

A pharmaceutical preparation, Ketostril tablet was analyzed for the contents of the α -keto acids. The amounts of MKBA, K_3MVA , K_4MVA , and PPY found per tablet were 82.5 mg, 64.8 mg, 98 mg, and 65.8 mg, with a RSD of 2.1%, 2.6%, 2.2% and 1.9%, respectively. The values found were in correlation with the labeled values (per tablet) of 86 mg, 67 mg, 101 mg, and 68 mg, respectively. The analysis was also carried out by standard addition method by spiking the solutions of the drug with MKBA, K_3MVA , K_4MVA and PPY. The amounts found correlated with the labeled values and % recovery was in the range of

95.9-97% with RSD between 0.91-2.02% for MKBA, K_3MVA , K_4MVA and PPY.

The blood samples of healthy volunteers and diabetic patients were analyzed for the contents of α -Keto acids. Five healthy volunteers of 35-42 years age were subjected to test for blood glucose levels. The blood glucose levels measured at the day of sample collection were within 120-140 mg/dL. The GA, PYR, KB, KG and K_4MVA were detected in the concentration range of 0.234-0.246 $\mu\text{g}/\text{mL}$, 0.166-0.175 $\mu\text{g}/\text{mL}$, 0.45-1.81 $\mu\text{g}/\text{mL}$, 0.58-1.20 $\mu\text{g}/\text{mL}$ and 0.712-0.73 $\mu\text{g}/\text{mL}$ RSD ranging between 0.3-2.6%.

The blood samples of two volunteers were spiked with GA, PYR, KB, KG and K_4MVA and amounts were found to correspond with the values evaluated by calibration curve (Table 2). The % recovery from the blood was 90 - 93% with RSD 2.5%. The urine samples of five healthy volunteers with blood glucose level 110-190 mg/ dL were analyzed and the amounts of GA, PYR, KB, KG, K_3MVA , and K_4MVA found were within the range 0.383-0.389 $\mu\text{g}/\text{mL}$, 1.91- 2.93 $\mu\text{g}/\text{mL}$, 1.51-1.82 $\mu\text{g}/\text{mL}$, 0.223-1.62 $\mu\text{g}/\text{mL}$, 0.60-0.93 $\mu\text{g}/\text{mL}$, and 0.778-0.868 $\mu\text{g}/\text{mL}$, respectively, with RSD ranging 1.0-2.6 % (Table 2). Two urine samples were spiked with α -keto acids and analyses were again carried out. The results obtained correlated with that of direct calibration with recovery of α -keto acids from the urine around 95%.

Similarly 10 diabetic patients with blood and urine glucose level within 310- 450 mg/ L on the day of sample collection were analyzed and PYR, KB, MKBA, K_3MVA , KG, K_4MVA , and PPY were found within 9.28-11.65 $\mu\text{g}/\text{mL}$, 1.65-4.83 $\mu\text{g}/\text{mL}$, 6.08-7.86 $\mu\text{g}/\text{mL}$, 6.15-8.25 $\mu\text{g}/\text{mL}$, 5.19-7.81 $\mu\text{g}/\text{mL}$, 7.34-7.84 $\mu\text{g}/\text{mL}$ and 7.72-11.54 $\mu\text{g}/\text{mL}$ respectively with RSD ranging 0.19-3.2% (Fig 4a) (Table 3). Among these, 2 blood samples were also analyzed by standard addition. The results obtained correlated and indicated % recovery of 80%, 83%, 85%, 88%, 78%, 82%, and 90% for PYR, KB, MKBA, K_3MVA , KG, K_4MVA , KHA, and PPY with RSD within 1.0-2.8, respectively. The urine samples of 10 diabetic patients with blood glucose levels within 310-480 mg/ dL indicated the α -keto acids PYR, KB, MKBA, K_3MVA , KG, K_4MVA and PPY at the

concentration levels 9.32-14.36 $\mu\text{g/ml}$, 6.08-6.75 $\mu\text{g/ml}$, 7.22-8.52 $\mu\text{g/ml}$, 5.58-8.34 $\mu\text{g/ml}$, 6.09-10.98 $\mu\text{g/ml}$, 12.06-12.56 $\mu\text{g/ml}$ and 13.39-15.86 $\mu\text{g/ml}$, respectively with RSD within 1.0-3.1% (Fig 4b). Two samples were also analyzed by standard addition and the amounts of α -keto acids found agreed with calibration procedure and indicated recovery of PYR, KB, MKBA, K₃MVA, KG, K₄MVA and PPY 90.2%, 90%, 95%,

88.4%, 82.6%, 85.4%, 87%, respectively with RSD of 0.9-1.9%, respectively. The amount of α -keto acids observed were higher in diabetic patients as compared healthy volunteers. The amount of α -keto acids in diabetic patients correlated positively with blood glucose level with coefficient of determination and may be considered as markers for diabetic patients.

Table 2. Quantitative analysis of α -Keto acids from Healthy blood and Urine person

Sr. No.	Age M= F=	Glucose level mg/dl Random/ fasting	Blood and Urine Samples	GA $\mu\text{g/ml}$ (n=6) (RSD)	PYR $\mu\text{g/ml}$ (n=6) (RSD)	KB $\mu\text{g/ml}$ (n=6) (RSD)	KG $\mu\text{g/ml}$ (n=6) (RSD)	K ₃ MVA $\mu\text{g/ml}$ (n=6) (RSD)	K ₄ MVA $\mu\text{g/ml}$ (n=6) (RSD)
1	M/42	140	Blood	0.246 (1.3) 0.246 (1.4)*	0.233 (1.6) 0.233 (1.1)*	0.45 (1.3) 0.46 (1.6)*	0.88 (1.3) 0.89 (1.1)	Nil	0.725 (1.1) 0.7269 (2.0)*
2	M/40	130	Blood	0.234 (1.4) 0.234 (1.3)*	0.175 (1.4) 0.175 (1.8)*	0.61 (0.1) 0.61 (2.0)*	0.60 (1.2) 0.61 (1.4)	Nil	0.629 (1.4) 0.628 (1.8)*
3	M/35	120	Blood	0.242 (1.0)	0.173 (1.6)	0.94 (0.3)	0.58 (2.6)	Nil	0.73 (2.6)
4	F/40	135	Blood	0.238 (2.0)	0.178 (1.3)	0.96 (0.3)	0.89 (2.1)	Nil	0.724 (1.2)
5	F/35	120	Blood	0.243 (2.6)	0.166 (1.1)	1.81 (0.6)	1.20 (2.3)	Nil	0.712 (1.2)
6	F/30	190	Urine	0.389 (1.6) 0.390 (1.8)*	2.93 (1.2) 2.94 (2.4)*	1.51 (1.3) 1.52 (1.2)	0.223 (1.1) 0.223 (1.6)	Nil (1.1) 0.78 (1.4)*	0.761 (1.6) 0.762 (1.2)*
7	F/35	170	Urine	0.385 (1.3)	2.88 (1.1)	1.55 (1.8)	0.62 (0.9)	0.60 (1.3)	0.802 (1.0)
8	M/40	160	Urine	0.383 (1.2) 0.384 (1.6)*	2.12 (2.2) 2.13 (2.0)*	1.82 (1.4) 1.82 (1.3)*	1.62 (1.2) 1.62 (1.8)*	0.85 (1.0) 0.85 (1.3)*	0.778 (1.9) 0.778 (1.7)*
9	M/35	130	Urine	0.384 (2.0)	2.44 (1.3)	1.66 (1.1)	1.60 (2.0)	0.93 (1.8)	0.863 (2.2)
10	M/38	110	Urine	Nil	1.91 (1.9)	1.58 (1.6)	1.20 (2.3)	0.68 (2.0)	0.868 (2.1)

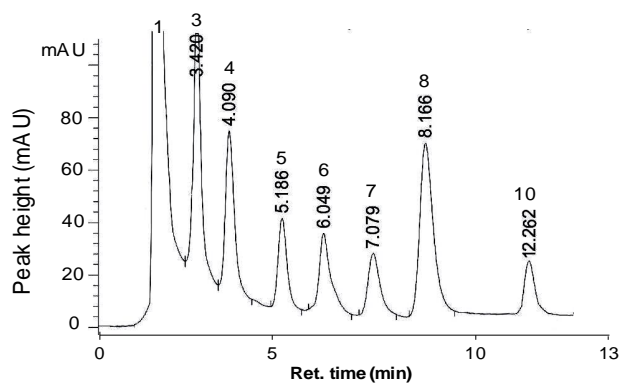
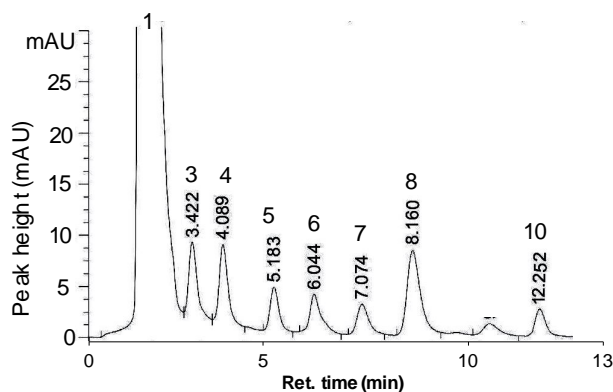


Figure 4(a). HPLC responses of (1) Reagent (3) PYR (4) KB (5) MKBA, (6) K₃MVA (7) KG (8) K₄MVA (9) KHA and (10) PPY, From blood samples of diabetic patients Condition as Fig 2.

Figure 4(b). HPLC separation of (1) Reagent (3) PYR (4) KB (5) MKBA (6) K₃MVA (7) KG (8) K₄MVA (9) KHA and (10) PPY, after spiking HPLC Condition as Fig 2.

Table 3. Quantitative analysis of α -Keto acids from Diabetic blood and Urine patients

Sr. No.	Age M= F=	Blood Glucose level mg/dl	Blood & Urine Sample	PYR µg/ml (n=6) (RSD)	KB µg/ml (n=6) (RSD)	MKBA µg/ml (n=6) (RSD)	K ₃ MVA µg/ml (n=6) (RSD)	KG µg/ml (n=6) (RSD)	K ₄ MVA µg/ml (n=6) (RSD)	PPY µg/ml (n=6) (RSD)
1	M/50	450	Blood	13.24 (1.9)	4.62 (2.2)	6.35 (1.1)	9.08 (2.6)	8.16 (2.4)	7.42 (3.0)	12.06 (2.2)
2	M/45	440	Blood	13.09 (1.2)	4.44 (1.6)	6.28 (2.6)	9.19 (2.2)	8.05 (2.6)	10.18 (2.5)	12.32 (2.6)
3	M/44	410	Blood	12.65 (1.9)	4.52 (2.2)	5.98 (2.8)	8.42 (2.5)	7.35 (1.9)	7.32 (2.2)	12.05 (2.5)
4	M/44	360	Blood	12.48 (2.0)	4.19 (2.5)	5.65 (2.2)	8.66 (1.9)	6.26 (3.0)	7.22 (2.4)	11.26 (2.9)
5	M/43	360	Blood	12.39 (1.6)	4.46 (2.6)	5.48 (2.4)	7.79 (1.1)	6.86 (2.2)	7.38 (3.0)	11.84 (2.9)
6	M/43	360	Blood	12.39 (1.2)*	4.45 (1.9)*	5.48 (1.2)*	7.79 (1.6)*	6.85 (2.9)*	7.38 (1.6)*	11.84 (1.4)*
7	M/40	310	Blood	12.81 (1.6)	3.55 (1.9)	5.88 (2.2)	7.22 (1.3)	6.09 (2.9)	7.48 (1.4)	11.16 (2.5)
8	F/40	430	Blood	11.42 (1.9)	3.22 (1.1)	7.24 (2.6)	6.38 (1.5)	6.67 (2.6)	7.54 (3.0)	10.888 (2.4)
9	F/38	400	Blood	10.42 (1.3)	2.96 (1.9)	6.62 (2.2)	5.94 (2.5)	6.34 (2.2)	7.72 (1.1)	9.16 (2.4)
10	F/35	360	Blood	10.19 (3.0)	3.61 (1.1)	5.58 (1.1)	5.74 (2.6)	6.05 (2.9)	5.33 (2.5)	9.22 (2.0)
11	M/48	440	Blood	10.08 (2.6)	3.32 (1.8)	5.26 (2.2)	5.27 (3.0)	6.16 (2.4)	5.38 (2.1)	8.45 (2.5)
12	M/45	440	Urine	16.42 (1.1)	6.82 (1.9)	8.65 (2.0)	9.28 (2.1)	10.62 (2.5)	10.09 (2.5)	19.22 (2.6)
13	M/44	410	Urine	16.26 (2.8)	6.85 (1.9)	8.19 (3.0)	9.46 (2.1)	10.24 (1.6)	10.72 (2.9)	18.64 (3.0)
14	M/44	360	Urine	15.35 (2.2)	6.84 (1.6)	8.28 (2.4)	9.02 (1.2)	10.14 (2.4)	10.44 (2.8)	16.38 (2.2)
15	M/44	360	Urine	15.62 (2.4)	5.95 (1.9)	7.92 (2.8)	9.36 (2.4)	10.82 (1.1)	10.28 (2.6)	18.44 (2.82)
16	M/43	360	Urine	15.12 (2.2)	5.35 (1.1)	7.26 (1.5)	8.24 (2.4)	10.36 (2.6)	10.46 (2.5)	16.72 (2.9)
17	M/43	360	Urine	15.12 (2.6)	5.45 (1.6)	7.75 (1.1)	8.18 (2.6)	11.29 (1.1)	10.04 (2.9)	16.28 (1.2)
18	M/40	410	Urine	15.53 (1.9)	5.25 (2.4)	7.67 (3.0)	8.35 (2.4)	11.08 (1.1)	10.16 (2.2)	16.36 (2.6)
19	M/40	330	Urine	15.58 (2.6)	5.72 (2.2)	7.95 (2.9)	8.72 (2.8)	11.42 (2.6)	10.82 (2.8)	16.44 (2.2)
20	M/38	400	Urine	14.72 (2.2)	5.48 (1.9)	8.16 (2.9)	6.42 (2.9)	10.36 (2.6)	10.72 (1.9)	16.22 (2.4)
21	M/35	360	Urine	14.54 (2.5)	5.75 (1.9)	8.28 (2.2)	6.36 (2.5)	10.22 (2.2)	10.96 (1.9)	16.48 (3.0)

Kieber and Mopper [1] have reported the separation of nine α -keto acids within 24 min. using o-phenylenediamine as derivatizing reagent, with spectrofluorometric detection and gradient elution. The present method enabled separation of nine α -keto acids within 14 min with more commonly available spectrophotometric detection and isocratic elution.

Conclusion

In conclusion we have developed a highly sensitive and specific method, for the determination of α -keto acids using NPD as derivatizing reagent. Chromatographic separation of nine α -keto acids is performed at a flow rate 0.9 mL/ min at 255 nm photodiode array detection, affording good separation of the peaks, good recoveries and shorter retention times than other techniques. The results for the analysis using the developed method for four α -keto acids agreed with the labeled values in pharmaceutical preparation. α -keto acids were detected and determined quantitatively from blood serum and urine samples of diabetic patients (with metabolic disorders or suffering from various diseases) with RSD within 2.9 %. The blood and urine samples were also analyzed by standard addition. Both the results agreed with each other and the amounts of α -keto acids found were higher in diabetic than healthy volunteers.

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