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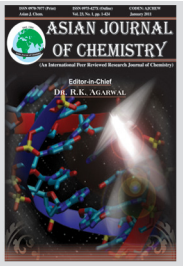

HPLC Determination of α-Oxoaldehydes and α-Oxoacids from Human Serum of Diabeties and Uremic Patients Using 4-Nitro-1,2-phenylenediamine as Derivetizing Reagent

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Reversed phase HPLC method has been developed for the determination of α-oxoaldehydes: glyoxal (Go) and methylglyoxal (MGo) and α-oxoacids: 3-methyl-2-oxovaleric acid (K3MVA), 2-oxoglutaric acid (KG), 4-methyl-2-oxovaleric acid (K4MVA) and phenylpyruvic acid (PPY) using 4-nitro-1,2-phenylenediamine (NPD) as derivatizing reagent. HPLC was carried out on a Zorbax C-18 column with isocratic elution using methanol-water-acetonitrile (46:52:2 v/v/v) as mobile phase at a flow rate of 0.9 mL/min. UV detection was carried out by photodiode array at 255 nm. Calibration curves were linear in the rage of 0.2-100 µg/mL and limit of detections were from 0.045-2.5 µg/mL. The method was applied for the determination of α-oxoaldehydes and α-oxo acids from the serum of diabetic and uremic patients. The results obtained were compared with healthy volunteers. Higher concentration of oxoaldehydes and oxoacids were observed in the uremic patients. The result obtained indicated relative standard deviation (RSD) within 1.1-2.2 %.

Key Words: α-Oxoaldehydes, α-Oxoacids, 4-Nitro-1,2-phenylenediamine, Dervatization, HPLC.

INTRODUCTION

α-Oxoaldehyde and α-Oxoacids are biological active compounds and are playing an active role in the pathogenesis of many chronic age related diseases[1]. α-Oxoaldehyde may accumulate in body fluids, due to accelerated oxidative stress and modify proteins and phospholipids to form biological active adducts such as advanced glycation end products and lipid oxidation end products[2]. α-Oxo-acids are intermediates in the metabolism of glycolysis, amino acid and carbohydrate. These can regulate protein turnover[3,4]. Among oxoaldehydes, glyoxal (Go) and methylglyoxal (MGo) have attracted attention because they are highly reactive and may exert various biological effects[5-7]. The concentration of oxo-acids 3-methyl-2-oxovaleric acid (K3MVA) and 4-methyl-2-oxovaleric acid (K4MVA) may change significantly in diabetic mellitus[8] and uremic patients[9]. Phenylpyruvic acid (PPY) exhibits greatly increased level in plasma from patients with hereditary metabolic diseases[10,11] and it inhibits renal gluconeogenesis[12]. 2-Oxoglutarate (KG) can also provide information about the cellular energy supply in metabolically super active cells as neutrophils[13].

Oxoaldehyde and oxoacids may be present in the serum of diabetic and uremic patients simultaneously. An analytical method for separation and determination of oxoaldehydes and oxoacids could be of value for the simultaneous determination of oxoaldehydes and oxoacids.

Analytical procedures for the determination of oxoaldehydes and oxoacids are mainly based on high performance liquid chromatography (HPLC)[14-24], gas chromatography (GC)[25-31] and capillary electrophoresis (CE)[32]. The most commonly used derivatizing reagents for the labeling of oxoaldehydes and oxoacids are 2,4-dimitrophenylhydrazine[31,32]. *o*-Phenylenediamine and 1,2-diamino-4,5-dimethoxybenzene. The derivatives formed are monitored by spectrophotometric or spectrofluorimetric method[14,17,23,33,34]. Da Silva Ferreira determined Go, MGo, DMGo, 2,3-pentandioxe, α-keto-γ-(methylthio)butyric acid and β-phenyl pyruvic acid in wine by HPLC using diaminobenzene as derivatizing reagent[35]. 4-Nitro-1,2-phenylenediamine (NPD) is similar to *o*-phenylenediamine and has been used for the determination of oxo-acids by paper chromatography[35,36] and Go, MGo and DMGo from foods, bervarages and wines[37]. The present work is an attempt to examine the simultaneous HPLC separation of oxo-aldehydes and oxo-acids from the serum in diabetic and uremic patients with spectrophotometric detection. The derivatization and separation condition are optimized in term of linearity, limit of detection (LOD), limit of quantitation (LOQ) and repeatability (inter and intraday precision).

EXPERIMENTAL

Go and DMGo (Across, New Jersey, USA), MGo (Fluka, Switzerland), K3MVA, K4MVA sodium salt, KG monosodium

salt (Fluka Switzerland) and PPY (Sigma, USA) and NPD (Fluka Switzerland) were used. The standard solutions of oxoaldehyde and oxo acids (1 mg/mL) were prepared in 10 % acetic acid and working solutions were then prepared by appropriate dilution with the same solvent compostion. 4-Nitro-1,2-phenylenediamine was recrystallized from *n*-heptane prior to use. Methanol (HPLC grade), acetic acid (RDH, Germany), hydrochloric acid (37 %), potassium chloride, sodium acetate, ammonium acetate, boric acid, sodium tetra borate, sodium bicarbonate, sodium carbonate, ammonium chloride and ammonia (25 %) (E-Merk, Germany) were used.

The buffer solution within pH 1-10 at unit interval were prepared from the following: hydrochloric acid (0.1 M), potassium chloride (1 M) (pH 1-2), acetic acid (1 M), sodium acetate (1 M), (pH 3-6), ammonium acetate (1 M)) (pH 7), boric acid (1 M), sodium tetraborate (1 M) (pH 8), sodium bicarbonate (1 M), sodium carbonate (saturated) (pH 9), ammonium chloride (1 M) and ammonia (1 M) (pH 10).

pH measurement were made with an Orion 420 A pH meter (Orion (Pvt) Ltd., Boston, USA) with combined glass electrode and internal reference electrode. Spectrophotometric study was carried out with double beam Hitachi 220 Spectrophotometer (Hitachi (Pvt) Ltd, Tokyo Japan) with duel 1 cm silica cuvettes. HPLC was carried out on Agilent model 1100-network HPLC system (Agilent Technology Inc.-Palo Alto, CA, USA), a syring loading sample injector (Rhedyne 7725) containing a 20 µL loop, two quart pump G1311A, Degasser G1379A and diode array DAD G1315B detection system The computer with Chemstation software controlled the HPLC. The column Zorbax 300 SB C-18 (4.6 × 150 mm id) (Agilent Technology Inc, USA) was used throughout the study.

Analytical procedures

Spectrophotometric procedure: To the solution (0.1-1.0 mL) containing Go, MGo, DMGo, K3MVA, KG, K4MVA and PPY separately in seven 10 mL volumetric flask was added 1.5 mL of NPD solution (1 % w/v in methanol) and 1 mL acetic acid-sodium acetate buffer (pH 3). The final concentration of each compound was controlled in the range of 5-100 µg/mL. The contents were then warmed at 80 ºC for 0.5 h. The volume was adjusted with methanol and absorption spectrum was recorded within 500-250 nm against reagent blank. The reagent blank was prepared following the same procedure, without the addition of analyte.

HPLC procedure: The solution (0.1-1.0 mL) containg a mixture of Go, MGo, DMGo, K3MVA, KG, K4MVA and PPY was transferred in 10 mL volumetric flask. The final concentrations of each component were adjusted as in Table-1. The contents were treated as spectrophotometric procedure. The solution (20 µL) was injected on the column Zorbax C-18 and eluted with methanol-water-acetonitrile (42:56:2 v/v/v) with a flow rate 0.9 mL/min. Detection was carried out by the photodiode array detector at 255 nm.

Determination of oxoaldehyde and oxo-acids from serum: The blood samples (5 mL) collected from healthy volunteers, diabetic and uremic patients were allowed for 1 h at room temperature (30 ºC) and centrifuged at 3000 g for 0.5 h. The supernatant layer (2.5 mL) was collected and methanol (5 mL) was added. The contents were mixed well and centrifuged at 3000 g for 20 min. The supernatant layer was collected and was added DMGo (1.0 µg/mL) and HPLC procedure was followed. Final volume was adjusted to 5 mL. The quantitation was carried out by external calibration curve and ratio of the peaks with internal standard (DMGo).

Determination of oxoaldehyde and oxoacids from serum using linear calibration curve and spiked sample: Blood sample (5 mL) collected from diabetic or uremic patients was treated as determination of oxoaldehyde and oxo-acids from serum. The serum after deproteinization with methanol was divided in to two equal parts. A part was treated as HPLC procedure and other was added Go (10 µg), MGo (5 µg), DMGo (1.0 µg/mL), k3MVA (90 µg), KG (50 µg), K4MVA (60 µg), PPY (70 µg) and HPLC procedure was again followed. Final volume was adjusted to 5 mL. The quantitation was made by linear calibration and an increase in the response with added standards.

The blood samples of diabetic and uremic patients were obtained (with verbal consent) from Liaquat University of Medicines and Health Sciences Hospital, Jamshoro. The blood samples were collected by vein puncture with hypodermic syringe. The samples were analyzed as received. The blood glucose level of the patients was collected from the records of hospital on the day of sample collection. The data was collected with permission of the duty doctor and the patients. The blood samples of healthy volunteers with the verbal consent were collected from Dr. M.A. Kazi Institute of Chemistry, University of Sindh and their glucose level was determined by Micro Lab (Merck). The patients and healthy volunteers were informed about the objectives of research project.

RESULTS AND DISCUSSION

Derivatization: 2-Oxoaldehyde (Go, MGo and DMGo) and 2-oxo acids (K3MVA, KG, K4MVA and PPY) react with NPD to form derivatives (Fig. 1). The reaction was initially monitored by spectrophotometer for each of the compound

TABLE-1
QUANTITATIVE HPLC DATA FOR Go, MGo DMGo AND α-KETO ACIDS USING NPD AS A DERIVATIZATION REAGENT

Name of compound	Calibration range (µg/mL)	Coefficient of determination (R^2)	Least square or regression	Limit of detection (µg/mL)	Limit of quantification (µg/mL)
Go	(0.2-2.0)	0.9991	Y = 5.2468x + 0.0354	0.045	0.135
MGo	(0.2-1.0)	0.9983	Y = 0.2122x + 0.2436	0.071	0.213
DMGo	(0.2-1.0)	0.9995	Y = 0.5066x – 0.0229	0.075	0.225
K3MVA	(0.6-100)	0.9976	Y = 0.2584x + 0.0286	0.266	0.800
KG	(0.5-100)	0.9988	Y = 0.1137x + 0.0333	0.160	0.500
K4MVA	(0.6-100)	0.9983	Y = 0.1480x + 0.0333	0.200	0.600
PPY	(0.4-100)	0.9957	Y = 0.1196x – 0.0519	0.100	0.300

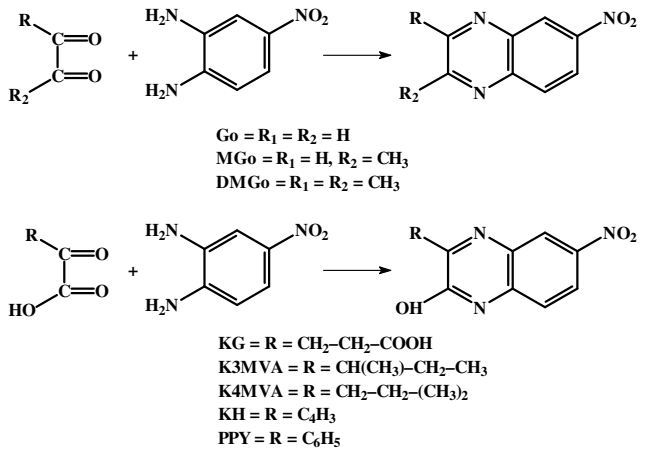

Fig. 1. Reaction of oxoaldehyde and oxo acids with NPD

separately to optimize the reaction conditions for the maximum formation of the derivatives. The effect of pH, addition of the reagent NPD, heating time and temperature were examined. The absorbance was measured against reagent blank prepared under the same condition except the addition of analyte. The maximum absorbance of α-oxoacids and oxoaldehydes derivatives was measured within 340-300 nm. The effect of pH was examined within pH 1-10 at unit interval, reagent NPD 1 % (w/v) in methanol was added 0.5-3.0 mL at an interval of 0.5 mL and warmed at 80 ºC for 10-50 min at an interval of 10 min. The reaction was observed in acidic medium within pH 1-5 and maximum was observed at pH 3 (Fig. 2). A similar response was observed after the addition of 1 mL and higher amount of reagent solution and addition of 1.5 mL was selected. The reactions of oxoaldehydes were rapid and reached to maximum by warming at 10-20 min, but the reaction of oxo-acids were somewhat slow and reached at the maximum within 0.5 h and was selected. The derivatives once formed were highly stable and did not show any change in absorbance up to 48 h. Each of the derivatives obeyed the Beers law at their λ_{max} within 5-100 µg/mL.

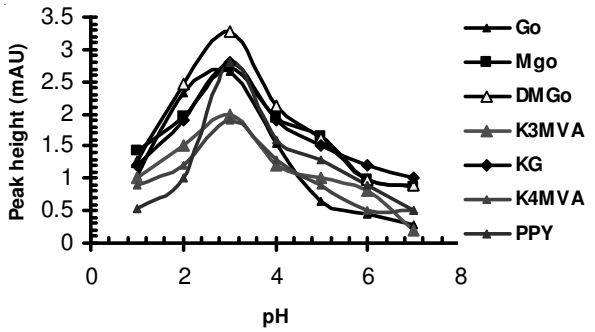

Fig. 2. Effect of pH on the absorbance of α-oxo aldehyde and α-oxoacids derivatives

Chromatography: The derivatization reactions with oxoaldehydes and oxo-acids yield different nitro quinoxaline and nitro quinoxalanol derivatives (Fig. 1) and could be separated by HPLC. The derivatives eluted from reverse phase C-18 column with methanol-water and separated from derivatizing reagent. The derivatives absorbed within UV range and wavelength for maximum absorbance was scanned with photodiode array detector during elution within 300-200 nm. For the simultaneous detection wavelength of 255 nm was selected for peak maximum.

Chromatographic separation was examined from Zorbex C-18 column using isocratic elution. Different mixtures of methanol-water including the addition of different buffers comprising of sodium acetate, sodium phosphate, sodium carbonate and sodium tetraborate were examined. Ease of the separation was obtained with methanol-water-acetonitrile (46:52:2 v/v/v) with a flow rate of 0.9 mL/min. Identification of each peak was based on the comparison of retention time with that of standard and by spiking each of compound in sequence with standard. Oxoaldehydes eluted before oxoacids. Complete separation was obtained between seven component mixture of oxoaldehydes and oxo acids plus derivatizing reagent NPD, except some overlapping of the peaks of NPD and Go was observed (Fig. 3). However for the separation of Go from NPD separately, the concentration of derivatizing reagent NPD was reduced to 1 mL (1 % w/v) and a complete separation was again observed. The separation was repeatable (n = 6) with relative standard deviation (RSD) of (0.2-0.8 %) for retention time and 0.4-1.2 %, for peak height/peak area.

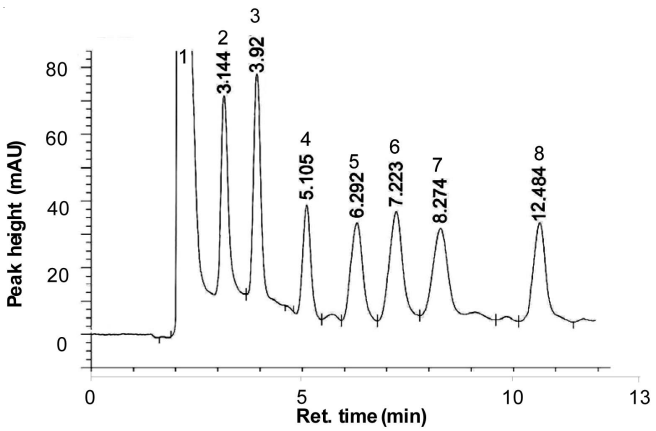

Fig. 3. HPLC separation of (1) reagent, (2) Go, (3) MGo, (4) DMGo (5) K3MVA, (6) KG, (7) K4MVA and (8) PP; Conditions: column Zorbax C-18 (4.6 × 150 mm id) isocratic elution with methanol-water-acetonitrile (46:52:2 v/v/v) with a flow rate of 0.9 mL/min. UV detection by photodiode array at 255 nm

Quantitation: In order to develop analytical procedure for the determination of oxoaldehydes and oxo acids linearity, repeatability, limit of detection (LOD), limit of quantitation (LOQ) and accuracy were verified. A linear relationship was observed between peak height/peak area and analyte concentration for each of the compound. The calibration parameters are shown in Table-1. Repeatability (precision within runs) covering derivatization and chromatographic separation was satisfactory. Inter (n = 5) and intra (n = 6) day variation was observed with RSD within 0.1and 1.4 % in retention times and 1.1 and 2.0 % peak height/peak area. Limit of detections measured as three times signal to noise ratio were within 0.045-2.5 µg/mL. Limit of quantifications measured as ten times signal to noise ratio were calculated within 0.35-7.5 µg/mL. Test solutions (n = 4) containing different amounts of oxoaldehyde and oxo acids within calibration range were analyzed and relative error was obtained within ±1.1-2.9 %.

The additives methylparabin, propylparabin, gum acacia, manitol, lactose, fructose, glucose, sodium chloride, sodium lauryl sulphate and methyl hydroxypropyl cellulose were added atleast twice the concentration of α-keto acids and their effects were examined and they did not affect the determination of α-keto acids or oxoaldehyde.

Analysis of biological samples (serum): The serum samples of healthy volunteers, diabetic and uremic patients were analyzed after deproteinization with methanol and the analytes were identified by comparison of chromatographic retention times with those of standards. Representative separation for analyses of serum from uremic patients is shown in Fig. 4a. The chromatographs obtained from serum are similar to that of standard (Fig. 3) and indicate that sample matrix did not interfere with the compounds analyses. The results of analyses are reported in Tables 2-4 for healthy volunteers, diabetic and uremic patients respectively. The accuracy of the method was verified by analyzing serum samples spiked with known amounts of oxoaldehyde and oxo-acids within the calibration range (Table-1), (Fig. 4b). The recovery of the compounds from the serum was calculated within 96-98 % with RSD 2.1 %. The amount of oxoaldehyde and oxo acids observed in serum of diabetic and uremic patients were higher than healthy volunteers.

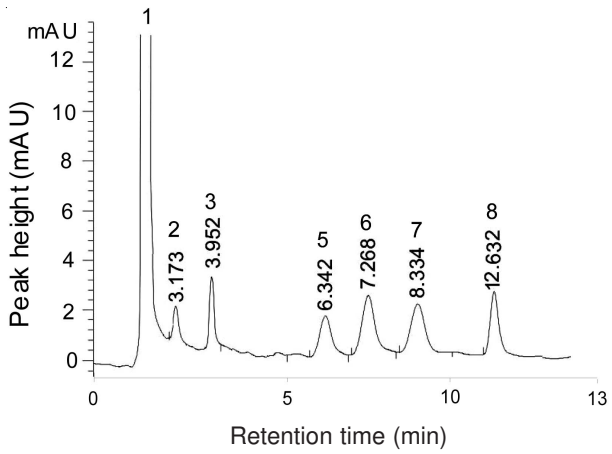

Fig. 4a. HPLC responses of (1) reagent (2) Go, (3) MGo, (5) K3MVA, (6) KG, (7) K4MVA and (8) PP from blood samples of uremic patients; Condition as Fig. 2

TABLE-2
QUANTITATIVE ANALYSIS OF α-ALDEHYDES AND α-KETO ACIDS FROM DIABETIC BLOOD AND URINE PATIENTS

Age M = Male F = Female	Blood glucose level at time collection of blood samples (mg/dl)	Go (μg/mL) (n = 6) (RSD	MGo (μg/mL) (n = 6) (RSD	K3MVA (μg/mL) (n = 6) (RSD	KG (μg/mL) (n = 6) (RSD	K4MVA (μg/mL) (n = 6) (RSD	PPY (μg/mL) (n = 6) (RSD
M/60	478	0.576 (1.2)	0.71 (1.9)	7.43 (2.2)	9.53 (1.6)	8.42 (2.1)	14.22 (2.2)
		0.578 (1.4)*	0.72 (1.2)*	7.44 (2.8)*	9.54 (1.1)*	8.43 (1.9)*	14.24 (2.0)*
M/57	469	0.556 (1.6)	1.58 (1.9)	7.72 (2.3)	9.20 (1.2)	8.59 (2.6)	14.69 (2.4)
M/55	451	0.552 (1.3)	1.33 (1.6)	7.52 (2.6)	9.48 (1.9)	8.28 (2.4)	14.03 (1.4)
F/51	430	0.548 (2.0)	1.55 (1.4)	7.26 (2.4)	9.92 (2.0)	8.16 (2.6)	14.98 (2.4)
F/48	405	0.532 (1.4)	1.12 (2.0)	6.62 (1.2)	9.68 (2.2)	8.13 (2.9)	14.86 (2.6)
		0.533 (1.6)*	1.13 (1.9)*	6.63 (3.1)*	9.67 (1.3)*	8.14 (2.5)*	14.87 (1.9)*

*Spiked sample

TABLE-3
QUANTITATIVE ANALYSIS OF Go, MGo AND α-KETO ACIDS FROM BLOOD SAMPLES OF URINE AND DIABETIC PATIENTS

Age M = Male F = Female	Blood glucose level at time collection of blood samples (mg/dl)	Go (μg/mL) (n = 6) (RSD	MGo (μg/mL) (n = 6) (RSD	K3MVA (μg/mL) (n = 6) (RSD	KG (μg/mL) (n = 6) (RSD	K4MVA (μg/mL) (n = 6) (RSD	PPY (μg/mL) (n = 6) (RSD
M/58	480	1.92 (0.9)	2.32 (2.1)	8.92 (2.0)	10.83 (1.1)	6.96 (1.2)	19.77 (1.4)
		1.93 (1.6)	2.32 (1.1)	8.93 (1.3)	10.85 (1.6)	6.97 (1.8)	19.76 (1.2)
M/56	478	1.90 (1.6)	2.26 (2.2)	8.90 (2.1)	10.80 (1.6)	6.91 (2.2)	19.76 (2.4)
M/51	460	1.88 (2.8)	2.24 (2.6)	8.82 (2.2)	9.78 (2.0)	6.83 (1.4)	18.64 (2.6)
F/48	455	1.84 (1.9)	2.15 (2.4)	7.66 (1.9)	8.66 (2.4)	6.82 (1.6)	16.61 (2.8)
F/46	420	1.74 (2.6)	2.06 (2.2)	7.52 (1.6)	8.60 (2.6)	6.75 (1.2)	15.52 (1.9)
		1.75 (2.4)*	2.07 (2.6)+	7.53 (1.1)*	8.62 (1.6)*	6.75 (0.9)*	15.52 (1.2)*

*Spiked sample

TABLE-4
QUANTITATIVE ANALYSIS OF Go, MGo AND α-KETO ACIDS FROM BLOOD OF HEALTHY VOLUNTEERS

Age M = Male F = Female	Blood glucose level at time collection of blood samples (mg/dl)	Go (μg/mL) (n = 6) (RSD	MGo (μg/mL) (n = 6) (RSD	K3MVA (μg/mL) (n = 6) (RSD	KG (μg/mL) (n = 6) (RSD	K4MVA (μg/mL) (n = 6) (RSD	PPY (μg/mL) (n = 6) (RSD
F/42	140	0.059 (1.1)	0.092 (1.4)	0.303 (1.2)	0.240 (2.0)	0.541 (2.6)	0.548 (2.6)
		0.058 (1.6)*	0.093 (1.2)*	0.303 (1.1)*	0.240 (1.6)*	0.541 (1.4)*	0.470 (1.6)*
F/38	138	0.055 (1.2)	0.900 (1.6)	0.414 (1.9)	0.212 (26)	0.535 (0.8)	0.569 (2.4)
M/40	130	0.052 (1.6)	0.086 (1.6)	0.419 (2.0)	0.306 (2.2)	0.512 (2.4)	0.574 (1.4)
M/44	120	0.050 (1.9)	0.082 (1.2)	0.406 (1.6)	0.334 (2.4)	0.538 (2.6)	0.567 (1.1)
M/51	110	0.053 (2.0)	0.083 (2.3)	0.412 (1.2)	0.322 (1.9)	0.583 (1.9)	0.595 (1.6)
		0.054 (2.1)*	0.082 (2.0)*	0.414 (1.4)	0.324 (1.3)	0.584 (1.1)	0.596 (1.9)

*Spiked sample

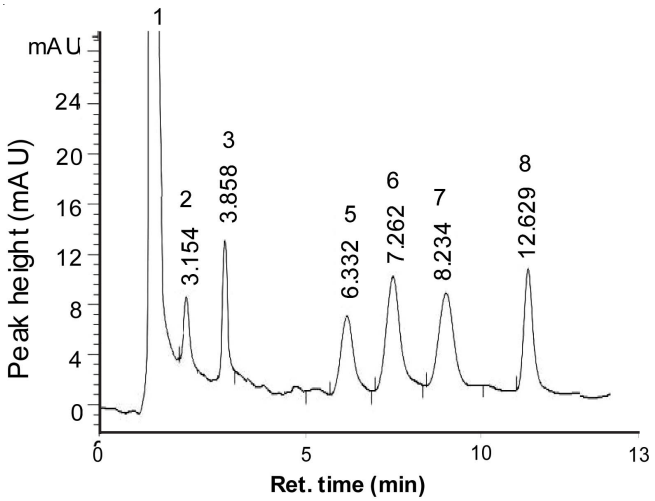

Fig. 4b. HPLC separation of (1) reagent (2) Go, (3) MGo, (4) DMGo (5) K3MVA (6) KG, (7) K4MVA and (8) PP after spiking as Fig 2

Conclusion

4-Nitro-1,2-phenylenediamine (NPD) has been examined as a useful precolumn derivatizing reagent for the analysis of oxoaldehyde and oxo-acids in biological samples such as serum of diabetic and uremic patients. The compounds indicated high sensitivity in UV region and separated easily with isocratic elution with a simple mixture of methanol-water-acetonitrile. The selectivity and reproducibility of the method can allow determination of oxo- aldehyde and oxo- acids for diagnostic purposes in metabolic diseases.

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