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Gas Chromatographic Determination of α-Keto Acids in Pharmaceutical Preparation Using 1,2-Propylenediamine as Derivatizing Reagent

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Analytical procedure has been developed for the gas chromatographic separation and determination of α-keto acids: pyruvic acid, 2-ketobutyric acid, 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid, 2-ketohexanoic acid and phenylpyruvic acid after derivatization with 1,2-propylenediamine from the column HP-5 (30 × 0.32 mm i.d). The detection was made by flame ionization. Linear calibration curves were obtained within 9.0-84 μg/mL and detection limits within 3.0-5.6 μg/mL of each α-keto acids. A pharmaceutical preparation ketosteril was analyzed for phenylpyruvic acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid and 3-methyl-2-oxobutyric acid and the found amounts correlated with the labeled values. The extraction recovery from pharmaceutical preparation tested by standard addition of these compounds was calculated to be 94.0-96.8 % with RSD 0.1-0.8 %. A number of pharmaceutical additives and amino acids added did not affect the determination of α-keto acids with relative error within ± 3 %.

Key Words: Gas Chromatography, α-Keto acids, 1,2-Propylenediamine, Drug analysis.

INTRODUCTION

α-Keto acids are key intermediate in a number of major biochemical pathways including glycolysis, amino acids and carbohydrate metabolism[1,2]. Each of the α-keto acids has specific function. Pyruvic acid is involved in biosynthesis of alanine[3,4], while the concentration of phenylpyruvic acid is increased in plasma of patients with heriditery metabolic desease[5,6] and it inhibits renal gluconeogenesis[7]. The branched chain α-keto acids 3-methyl-2- oxovaleric acid and 4-methyl-2-oxovaleric acid are involved in the regulation of protein turn over and are of interest for pathological processes such as sepsis and burns[8,9]. The pharmaceutical preparation containing mixture of essential amino acids and α-keto acids are used for therapy of condition associated with deficient protein metabolism. A number of analytical procedures are reported for their determination such as spectrophotometry[10], spectrofluorimetry[11,12], paper[13] and thin layer chromatography[14], high performance liquid chromatography[15,16] and gas chromatography[17-20]. The chromatography of α-keto acids involves derivatization before their detection. The quantitation of α-keto acids by fluorescence HPLC has been reviewed[21]. The GC procedures mainly involved the formation of O-(trimethylsilyl) quinoxalinols[22,23], silylated oximes[24], *N*- and *O*-alkylated quinoxalinols[25], pentafluorobenzyl ester[26], oxime-methylated with diazomethane[19] or tetramethylsilane (TMS)[17] and methyl ester of 2,4-dinitrophenylhydrazone[27]. Two reagents are used, one to bind carbonyl groups and other carboxylic acid residue. Frigerio *et al.*[28] have reported decomposition or tautomeric transformation from *o*-trimethylsilyl-*N*-trimethylsilyl-quinoxalinol after *ca.* 0.5 h, making a serial investigation of repeated samples impossible[21].

1,2-Propylenediamine has been employed for the first time as a reagent for the derivatization of α-keto acids and their determination from pharmaceutical formulation by GC. 1,2-Propylenediamine has been examined for the ease of elution and separation at lower temperature. The experimental conditions for derivatization and GC separations are optimized and tested in the terms of repeatability, linearity of response, limits of detection, limit of quantitation and inter and intraday variations.

EXPERIMENTAL

1,2-Propylenediamine (PDA) (Fluka, Switzerland), phenylpyruvic acid (PPYR) (Sigma, USA), pyruvic acid (PYR), 2-oxobutyric acid (KBA), 3-methyl-2-oxobutyric acid (MKBA), 3-methyl-2-oxovaleric acid (M3KVA), 4-methyl-2-oxovaleric acid (M4KVA), 2-ketohexanoic acid (KHA) sodium salt (Fluka, Switzerland), methanol and chloroform (E. Merck, Germany) and sodium chloride (Fluka, Switzerland)

TABLE-1
QUANTITATIVE GC/FID DATA OF α-KETO ACIDS AS DERIVATIVES OF ECF

S. NO	Name of compound	Calibration range	Coefficient R^2	Least square or regression	LOD	LOQ
1	PYR	(9-45 µg/mL)	0.9974	Y = 0.3727x + 0.0033	3.0	9
2	KBA	(12-60 µg/ mL)	0.9966	Y = 0.1913x + 0.0185	4.0	12
3	MKBA	(17.2-51.6 µg/ mL)	0.9987	Y = 0.1803x + 0.01	4.5	13.5
4	M3KVA	(15-75 µg/mL)	0.9979	Y = 0.1122x + 0.0105	5.0	15
5	M4KVA	(16.8-84 µg/ mL)	0.9949	Y = 0.1001x + 0.066	5.6	16.8
6	KHA	(15.9-79.5 µg/ mL)	0.9961	Y = 0.1048x + 0.0067	5.3	15.9
7	PPYR	(12.6-63 µg/ mL)	0.9986	Y = 0.2825x + 0.0614	4.2	12.6

were used. The buffer solutions at the concentration of (0.1 M) within pH 1-12 at unit interval were prepared from the following: Hydrochloric acid and potassium chloride (pH 1-2), acetic acid and sodium acetate (pH 3-6), ammonium acetate (pH 7), sodium bicarbonate and sodium carbonate (pH 8-9), ammonium chloride and ammonia (pH 10-11) and sodium chloride and sodium hydroxide (pH 12).

pH Measurements were made with Orion 420 A pH meter (Orion Research Inc. Boston, USA) with glass electrode and internal reference electrode.

The gas chromatographic studies were carried out on Agilent model 6890 Network GC system gas chromatograph (Agilent Technologies Inc. USA) coupled with flame ionization detection (FID), split injector with split ratio (10: 1), hydrogen generator Parker Balson Model H2-90, analytical gas system (Parker Hannifin Haverhill, M.A. USA) and pure nitrogen (British Oxygen Company (BOC) Karachi). The gas chromatograph was controlled by Chemstation software (Agilent Technologies). Capillary GC column HP-5 (5 % phenyl methyl siloxane) (30 m × 0.32 mm i.d.) with film thickness 0.25 µm (J and W Scientific GC column, USA) was used throughout the study.

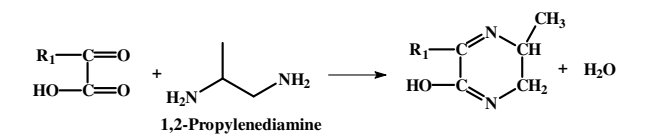
GC analytical procedure: To the aqueous solution (1 mL) containing 9 to 84 µg of each keto acid: pyruvic acid, 2-oxobutyric acid, 3-methyl-2-oxobutyric acid, 3- methyl-2-oxovalaric acid, 4-methyl-2-oxovalaric acid, 2-oxohexanoic acid, phenylpyruvic acid was added 1 mL of 5 % 1,2-propylenediamine and 1 mL of acetic acid-sodium acetate buffer pH 3.2. The contents were heated on water bath at 95 ºC for 30 min and allowed to cool at room temperature. Chloroform 1 mL was then added and contents were mixed well. The layers were allowed to separate and an aliquot of organic layer (200 µL) was transferred to screw capped sample vial. The solution (1 µL) was injected on the column at column temperature 75 ºC for 10 min with heating rate 10 ºC/min up to 175 ºC with total run time 20 min. Nitrogen flow rate was 1.0 mL/min. Detection was by FID. Injection port and detector temperatures were 250 and 280 ºC, respectively. Nitrogen make up flow rate was 45 mL/min. FID air and hydrogen flow rates were 450 and 40 mL/min, respectively.

Analysis of pharmaceutical preparation: Five tablets Ketostril (Fresenius Kabi Bad Homburg, Germany) were ground to fine powder and amount (0.802 g) corresponding to one tablet was dissolved in methanol: water (1:1 v/v). The solution was filtered and volume adjusted to 100 mL. Aliquots of 0.2 mL and 0.4 mL from well mixed solution were taken and GC analytical procedure was followed. The quantitation was carried out using external calibration curves, prepared from at least five standard solutions within the range indicated in Table-1. A blank determination was also carried out with an aliquot of 0.4 mL of the prepared solution of ketosteril following the same procedure, but without addition of the derivatizing reagent 1,2-propylenediamine.

Analysis of pharmaceutical preparation by standard addition: Five tablets of ketostril were taken and processed as described under analysis of pharmaceutical preparation and aliquot of the solutions of 0.2 mL, 0.4 mL and 0.6 mL were taken in duplicate. An aliquot from each was added 3-methyl-2-oxovaleric acid (25 µg), 4-methyl-2-oxovaleric acid (26 µg), phenylpyruvic acid (22 µg) and 3-methyl-2-oxobutyric acid (23 µg) and all the solutions were analyzed following the GC analytical procedure. The quantitation was made from external calibration curve and an increase in response with added standards.

RESULTS AND DISCUSSION

Derivatization and separation: The carboxyl group is present on adjacent carbon atom to carboxylic acid in pyruvic acid, 2-oxobutyric acid, 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid, 2-ketohexanoic acid and phenylpyruvic acid and reacted with 1,2-propylenediamine to form cyclic ring structure dihydropyrazinol (Fig. 1). The derivatives formed were extracted in chloroform and examined for elution from GC column separately. Each of the compounds eluted and separated from the derivatizing reagent. It was therefore the derivatization conditions and separation of pyruvic acid, 2-oxobutyric acid, 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid, 2-ketohexanoic acid and phenylpyruvic acid were optimized. The effect of pH, amount of the reagent 1,2-propylenediamine added and warming time and temperatures were optimized for derivatization. Each time constant volume (1 µL) with split ratio 10: 1 was injected and the condition which gave maximum response (average peak height/peak area) was considered as optimum. The pH was varied from 1-10 at unit interval and the derivatization reactions were observed in acidic medium within pH 2-5 and maximum at pH 3.2 using acetic acid and sodium acetate buffer (Fig. 2). Heating temperature between 40-100 ºC at an interval of 10 ºC and heating time between 10-50 min at an interval of 10 min were varied. Maximum derivatization was observed by warming in boiling water (95 ºC) for 0.5 h and was selected. The derivatives once formed were stable and did not show any change in response (average peak height) upto 24 h. GC separation between pyruvic acid, 2-oxobutyric acid, 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid, 2-ketohexanoic acid and phenylpyruvic acid was examined and was easily



PYR–R_1 = CH_3; KBA–R_1 = C_2H_5; MKBA–R_1 = $CH(CH_3)_2$; KHA–R_1 = C_4H_9; PPYR–R_1 = $C_6H_5CH_2$; M3KVA–R_1 = $CH(CH_3)CH_2CH_3$; M4KVA–R_1 = $CH_2CH(CH_3)_2$

Fig. 1. Schematic diagram of reaction of α-keto acids with 1,2-propylenediamine

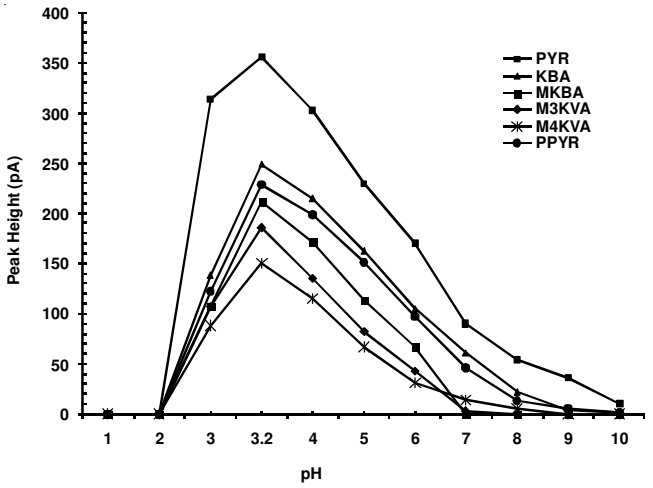


Fig. 2. Effect of pH on derivatization on GC response of PYR, KBA MKBA, M3KVA, M4KVA, KHA and PPYR as derivatives of PDA from the column HP-5 (30 m × 0.32 mm i.d.) with film thickness 0.25 μm at column temperature 75 ºC for 10 min with heating rate 10 ºC/min up to 175 ºC with total run time 20 min. Nitrogen flow rate was 1.0 mL/min with split ratio 10:1, v/v. Detection was by FID. Injection port and detector temperatures were 250 and 280 ºC, respectively. Nitrogen make up flow rate was 45 mL/min. FID air and hydrogen flow rates were 450 and 40 mL/min, respectively

achieved each time, but a better separation was obtained using temperature elution program (experimental) (Fig. 3) with resolution factor (Rs) > 1.5. The capacity factors (K') were calculated for pyruvic acid, 2-oxobutyric acid, 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid, 2-ketohexanoic acid and phenylpyruvic acid and were obtained 0.844, 1.415, 1.637, 2.673, 2.829, 3.734 and 6.648 respectively. The separation was repeatable (n = 6) with relative standard deviation of 0.1-0.7 % and 0.6-1.4 % in terms of retention time and peak height/peak area respectively.

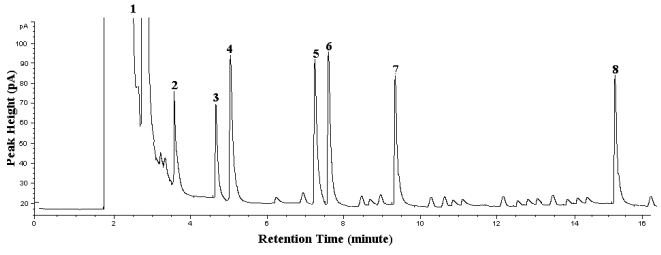


Fig. 3. GC separation of (1) Solvent and PDA (2) PYR (3) KBA (4) MKBA (5) M3KVA (6) M4KVA (7) KHA (8) PPYR as derivatives of PDA. Conditions as Fig. 2

Quantitation and validation: The relationships between peak height/peak area and amounts of the individual α-keto acids were linear from 9.0-84.0 μg/mL, corresponding to 0.9-8.5 ng injected on the column. The coefficients of determination from five calibrators were calculated to 0.9974, 0.9966, 0.9987, 0.9979, 0.9949, 0.9961 and 0.9986 for pyruvic acid, 2-oxobutyric acid, 3-methyl-2-oxobutyric acid, 3-methyl-2- oxovaleric acid, 4-methyl-2-oxovaleric acid, 2-ketohexanoic acid and phenylpyruvic acid respectively. The precision was established by repeated determination (n = 6) using a mixture of the seven α-keto acids (50 μg/mL each). The RSD did not exceed 2.5 % for any acid.

The limit of detection calculated as signal-noise ratio (3:1) for pyruvic acid, 2-oxobutyric acid, 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid, 2-ketohexanoic acid and phenylpyruvic acid were 3.0, 4.0, 4.5, 5.0, 5.6, 5.3 and 4.2 μg/mL corresponding to 0.3 ng, 0.4 ng, 0.45 ng, 0.5 ng, 0.56 ng, 0.53 ng and 0.42 ng respectively injected on the column. The limit of quantitation measured as signal-noise ratio (10: 1) were 9.0 μg/mL pyruvic acid, 12.0 μg/mL 2-oxobutyric acid, 13.5 μg/mL 3-methyl-2-oxobutyric acid, 15.0 μg/mL 3-methyl-2- oxovaleric acid, 16.8 μg/mL 4-methyl-2- oxovaleric acid, 15.9 μg/mL 2-ketohexanoic acid and 12.6 μg/mL phenylpyruvic acid (Table-1).

The pharmaceutical additives and amino acids methylparabin, propylparabin, gum acacia, manitol, lactose, fructose, glucose, galactose, sodium chloride, sodium lauryl sulphate, methyl hydroxypropyl cellulose and amino acids: Glycine, L-valine, L-leucein, isoleucein, L-threonine, L-aspartic acid, L-serine, alanine, L-glutamine, L-cystein, L-methionine L-lysine, L-histadine, L-tyrosine and L-tryptophane were added at least twice the concentration of the α-keto acids and analysis was carried out following the analytical procedure. The results obtained were compared with α-keto acids standard. The additions of additives and amino acids did not interfere with the determination of the pyruvic acid, 2-oxobutyric acid, 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid, 2-ketohexanoic acid and phenylpyruvic acid with relative error ± 3.5 %. The inter (n = 4) and intra (n = 4) day variation was examined with 50 μg/mL of each α-keto acid in terms retention time and peak height and RSD did not exceed 3.0 %.

Sample analysis: A pharmaceutical preparation ketosteril of α-keto acids and amino acids (Table-2) was analyzed after extraction of the active ingredients in methanol-water. 1,2-propylenediamine derivatives formed were eluted from GC column. The GC responses corresponding to 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid and phenylpyruvic acid were noted. The peak identification was made by comparing retention times with standards. The quantitation was from external calibrations based on least square linear regression equation Y = ax + b. The amounts of 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid and phenylpyruvic acid found were 81.5, 63.8, 96.9 and 65.8 mg/tablet as calcium salts with RSD of 0.2 % and 0.9 % (n = 4) (Fig. 4). The observed value agreed with labeled values 86, 67, 101 and 68 mg/tablet respectively. The blank determination of ketosteril tablet was carried out without the addition of the derivatizing reagent 1,2-propylenediamine and chromatogram did not indicate response corresponding to α-keto acids or

TABLE-2
KETOSTERIL TABLET IS MIXTURE OF α -KETO ACIDS AND ESSENTIAL AMINO ACIDS

S.NO	Name of compound	Amount (mg/tablet)	Amount Found mg/tablet (RSD %) (n = 4)
1	(RS) 3-methyl-2-oxovalaric acid calcium salt	67 mg	63.9 (0.2)
2	4-methyl-2-oxovalaric acid calcium salt	101 mg	97.64(0.4)
3	2-oxo-3-phenylpropanoic acid calcium salt	68 mg	65.78 (0.7)
4	3-methyl-2-oxobutyric acid calcium salt	86 mg	80.83 (0.1)
5	(RS)-2-hydroxy-4-methylthiobutyric acid calcium salt	59 mg	-
6	L-lysine acetate	105 mg	-
7	L-threonine	53 mg	-
8	L-tryptophan	23 mg	-
9	L-histadine	38 mg	-
10	L-tyrosine	30 mg	-

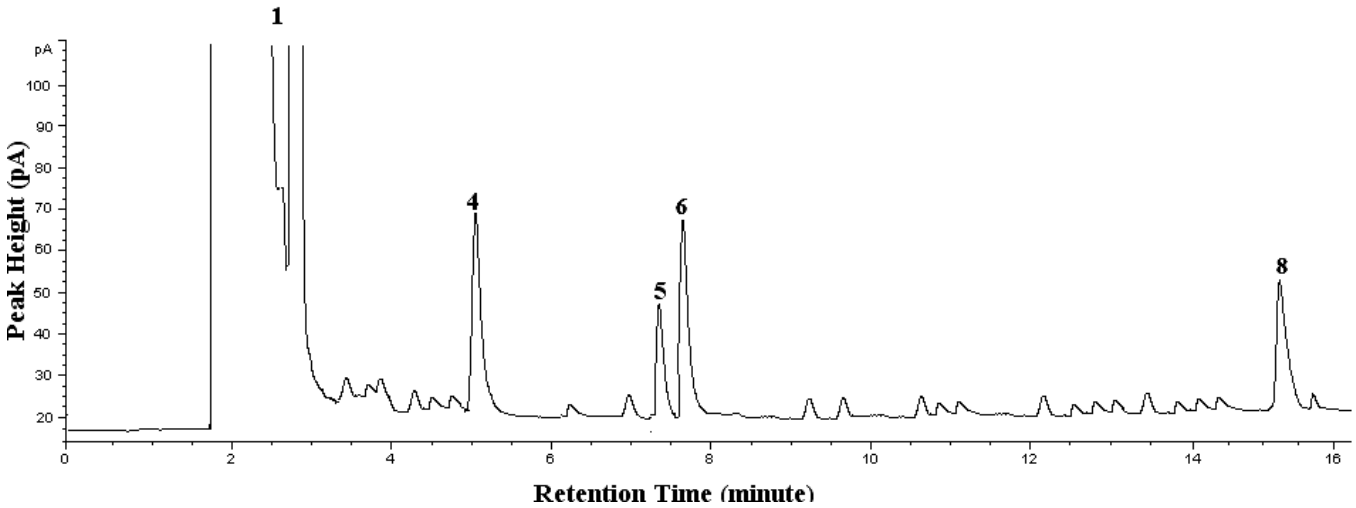


Fig. 4. GC elution of (4) MKBA (5) M3KVA (6) M4KVA (8) PPYR from Ketosteril tablet. Conditions as Fig. 2

amino acids. Aqueous-methanolic solution of the tablet ketosteril was spiked with standard solutions of 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid and phenylpyruvic acid and derivatization procedure was carried out. GC indicated an increase in the response corresponding to the added standards, without any change in peak shapes. The recoveries of 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid and phenylpyruvic acid were calculated 94.8, 95.0, 95.9 and 96.8 % respectively, while the corresponding RSDs were 0.3, 0.8, 0.3 and 0.1 % (n = 3). GC elution of α-keto acids with derivatizing reagents 1,2-propylenediamine and phenylenediamine were compared under same operating conditions (Fig. 5) and it was observed that derivatives of 1,2-

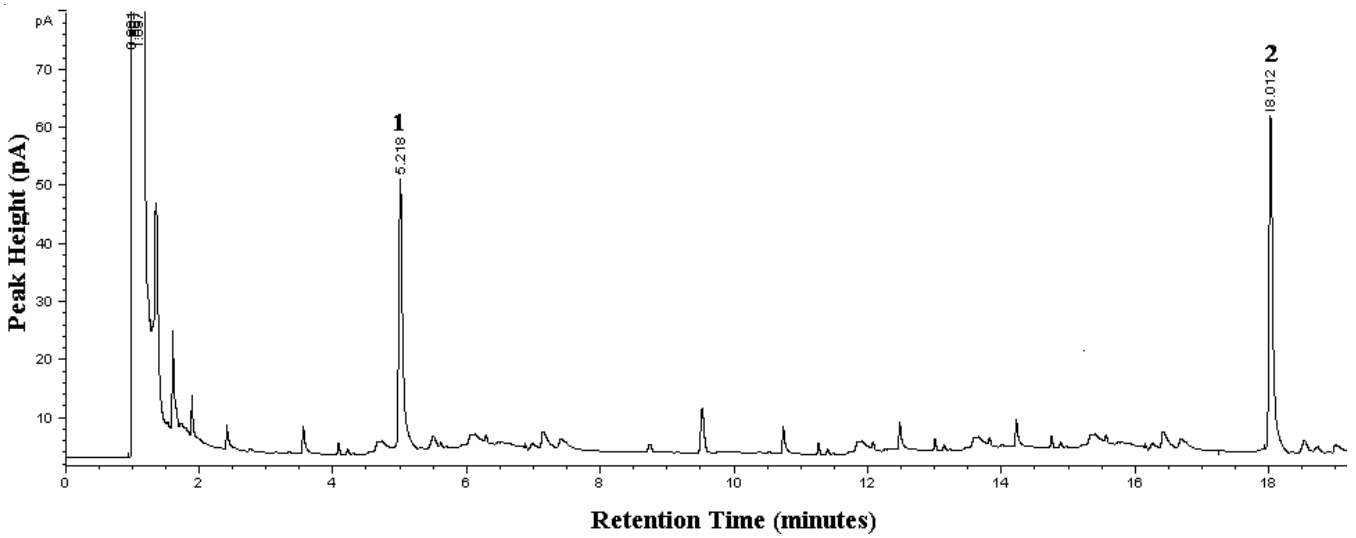


Fig. 5. GC elution of M4KVA as derivatives (1) PDA and (2) Phenylenediamine at a concentration of 50 µg/mL from the column HP-5 (30 m × 0.32 mm i.d.) with film thickness 0.25 µm at initial column temperature 100 ºC for 5 min, followed by heating rate 10 ºC /min up to 200 ºC with total run time 20 min. Nitrogen flow rate was 1.0 mL/min with split ratio 10:1, v/v. Detection was by FID. Injection port and detector temperatures were 250 and 290 ºC, respectively. Nitrogen make up flow rate was 45 mL/min. FID air and hydrogen flow rates were 450 and 40 mL/min, respectively

propylenediamine eluted quickly at lower operating temperatures than phenylenediamine.

Conclusion

A GC procedure has been developed for the determination of 3-methyl-2-oxobutyric acid, 3-methyl-2- oxovaleric acid, 4-methyl-2- oxovaleric acid and phenylpyruvic acid from a pharmaceutical preparation after precolumn derivatization with 1,2-propylenediamine. A number of pharmaceutical ingredients present in the formulation along with 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid and phenylpyruvic acid did not react to form volatile derivatives with 1,2-propylenediamine and did not affect the determination. Extraction of 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, 4-methyl-2-oxovaleric acid and phenylpyruvic acid from pharmaceutical preparation was calculated 94.8-96.8 %. The overall reproducibility of the method was calculated with RSD within ± 3 %.

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