Ethyl Chloroformate as Derivatizing Reagent for Gas Chromatographic Determination of α-Keto acids and Amino acids from Pharmaceutical Preparation

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Summary: α-keto acids pyruvic acid (PYR), 2-ketobutyric acid (KBA), 3-methyl-2-oxobutyric acid (MKBA), 3- methyl-2-oxovalaric acid M3KVA), 4- methyl-2-oxovalaric acid (M4KVA), 2-ketohexanoic acid (KHA) and phenylpyruvic acid (PPYR) together with amino acids as derivatives of ethyl chloroformate (ECF) eluted and separated from GC column HP-5(30m x 0.32 mm id). The detection was by flame ionization ((FID). Linear calibration curves were obtained within 18-127.8 µg/mL and detection limits were within 6-8.6 µg/mL of each α-keto acid. The separation was repeatable with inter and intraday variation with relative standard deviation (RSD) within 0.2-3.5%. A pharmaceutical preparation Ketosteril was analyzed and amounts of MKBA, M3KVA, M4KVA, and PPYR correlated with labeled values and their amount extracted from pharmaceutical preparation by standard addition were calculated to 93.92 -96.1 % with RSD 0.3-0.7%. The amino acids present in Ketosteril tablet separated completely and were determined simultaneously together with α-keto acids with recovery of 95.8 to 98.65% with RSD 0.2-0.9%.

Introduction

2-Keto acids are important compounds in human body and they take part in several metabolic process including gluconeogenesis, amino acids metabolism, fatty acid oxidation and maintenance of acid – base balance [1, 2]. Pyruvic acid (PYR) is involved in biosynthesis of alanine and a change in the concentration of PYR is reported in hypercatabolic states [3, 4]. Phenylpyruvic acid (PPYR) exhibits greatly increased level in plasma from the patients with hereditary metabolic disease [5, 6]. Branch chain keto acids 3- methyl-2-oxovalaric acid (M3KVA) and 4- methyl-2-oxovalaric acid (M4KVA) can regulate protein turn over [7, 8].

A number of methods have been developed for the determination of α- keto acids because of their importance in biological system. α- keto acids have been determined by spectrophotometry [9], spectrofluorimetry [10], liquid chromatography [7] and gas chromatography (GC) [11]. Derivatization with suitable reagent is generally carried out prior the detection. The quatitation of α-keto acids with fluorescence HPLC has been reviewed [12]. GC mainly involved the formation of O- (trimethylsilyl) quinoxalinols [13, 14], silylated oximes [15], N- and O- alkylated quinoxalinols [16], pentafluorobenzyl ester [17], oxime-methylated with diazomethane [18], tetramethylsilane (TMS) [11] and methyl ester of 2, 4-dinitrophenylhydrazone [19]. The procedures involved one of two reagents to bind carboxyl groups, followed by silylsation or estrification of acid residues. However some problems are reported due to decomposition or tautomeric transformation from o-trimethylsilyl-N- trimethylsilyl- quinoxalinol after approximately 30 min [20], making a serial investigation of repeated samples impossible [12].

Ethyl chloroformate (ECF) is reported as general purpose derivatizing reagent for acids, phenol, amino acids and amines using GC [21-23] or GC-MS [24-26]. The use ECF as GC reagent has been reviewed [27, 28]. The work examines the use of ECF as derivatizing reagent for GC elution and separation of α -keto acids PYR, KBA, MKBA, M3KVA, M4KVA, KHA, PPYR and amino acids and the determination of M3KVA, M4KVA, MKBA, PPYR, L- threonine, L- lysine, L-histadine, L-tyrosine, and L- trytophan from a pharmaceutical preparation of α-keto acids and amino acids. Analytical conditions are optimized for derivatization, GC elution, separation and determination of α-keto acids and amino acids.

Result and Discussion

Derivatization and Separation

The carboxylic group of α- keto acids could react with ECF to form ethyl carbonate derivatives (Fig. 1). PYR, KBA, MKBA, M3KVA, M4KVA, KHA and PPYR were treated with ECF separately and the derivatives formed were extracted in chloroform, and examined for elution from GC column. Each of the derivatives eluted separately as a

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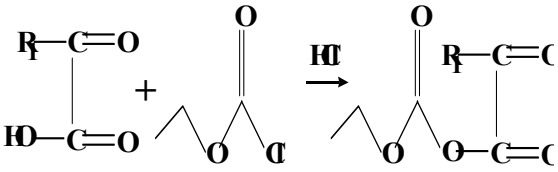
single peak and separated from the derivatizing reagent. It was therefore the derivatization conditions and separation of PYR, KBA, MKBA, M3KVA, M4KVA, KHA and PPYR were optimized. The effect of reaction medium, pH, amount of the reagent ECF added per analysis, heating time and temperature were examined on the derivatization. Each time constant volume (1 µl) was injected and the condition which gave maximum response (average peak height /peak area) was considered as optimum. The derivatization reaction was examined in aqueous, aqueous-methanol, aqueous – acetonitrile and aqueous phase containing pyridine base. A similar reaction was observed in each case, but aqueous – acetonitrile phase gave better results and was selected. The pH was varied from 1-10 at unit interval and maximum derivatization was observed at pH 9 using sodium carbonate buffer as has been reported with related compounds [19-21] (Fig. 2). Pure ECF was added within 0.2-2.0 at an interval of 0.4 ml and the addition of ECF was not observed critical as long excess of ECF was present the reaction mixture. The maximum derivatization was observed with 0.5ml of ECF and above. The heating time was examined within 10-40 min at an intervale of 10 min and temperature within 40-100° C at an interval of 10°C. Warming temperature at 90 to100 °C for 10 minute gave reproducible results and was selected. The GC separation was next examined. The separation between PYR, KBA, MKBA, M3KVA, M4KVA, KHA and PPYR was easily 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') for PYR, KBA, MKBA, KHA and PPYR were obtained 0.673, 1.171, 1.366, 2.457, 2.623, 3.528 and 6.544 respectively. The separation was repeatable (n = 6) with relative standard deviation (RSD) of 0.2 – 1.2 % and 0.6- 1.4 % in terms of retention time and peak height. The 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 at the optimized conditions for α- keto acids also reacted with ECF to form volatile derivatives and eluted and separated from GC column [21, 25], after the elution of α-keto acids the elution and separation of α-keto acids and amino acids was repeatable with RSD within 1.7% in terms of retention time and peak height.

Quantization and Validation

The relationships between peak height / peak area and concentrations of the individual α- keto acids were linear from 18 – 127.8 µg/mL, corresponding to1.8 – 12.78 ng injected on the GC column. The coefficients of determination from five calibrators were calculated to 0.9983, 0.9978, 0.9978, 0.9979, 0.9986, 0.9982 and 0.9978 for PYR, KBA, MKBA, M3KVA, M4KVA, KHA and PPYR 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 acids.



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 $R_1 = C_6H_5CH_2$
M_3KVA $R_1 = CH(CH_3)CH_2CH_3$
M_4KVA $R_1 = CH_2CH(CH_3)_2$

Fig 1: Schematic diagram reactions of (1) PYR (2) KBA (3) MKBA (4) M3KVA (5) M4KVA (6) KHA (7) PPYR with ECF.

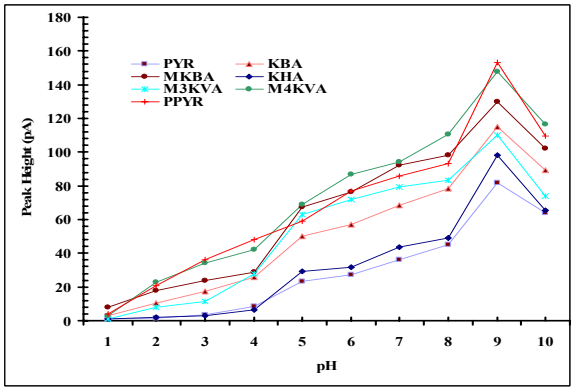


Fig 2: GC-FID study of effect of pH on α-keto acids as derivatives of ECF from the column HP-5 (30m x 0.32 mm i.d.) with film thickness 0.25 µm at initial column temperature 75 °C for 10 min, followed by heating rate 10°C/min up to 175 ^{O}C with heating 15 ^{O}C/min up to 250 ^{O}C. The nitrogen flow rate was 1.5 ml/min with split ratio 10:1. Detection was by FID. Injection port and detector temperatures were 250 and 280 ^{0}C, respectively. Nitrogen make up flow rate was 45 mL/min. FID air and hydrogen flow rate were 450 and 40 mL/min, respectively.

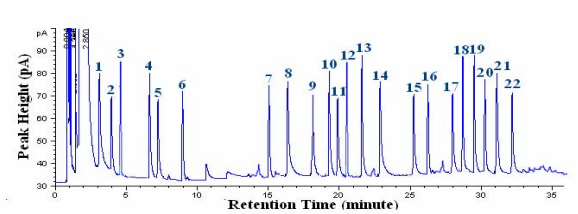


Fig 3: GC separation of (1) PYR (2) KBA (3) MKBA (4) M3KVA (5) M4KVA (6) KHA (7) PPYR (8) Glycine (9) L-valine (10) L-leucein (11) Isoleucein (12) L-threonine (13) L-aspartic acid (14) L-serine (15) Alanine (16) L-glutamine (17) L-cystein (18) L-methionine (19) L- lysine (20) L-Histadine (21) L-tyrosine and (22) L-Tryptophane as derivatives of ECF. Conditions as Fig 2.

The limit of detection (LOD) calculated as signal-noise ratio (3:1) for PYR, KBA, MKBA, M3KVA, M4KVA, KHA and PPYR were 6.0, 7.0, 7.5, 8.0, 8.6, 8.3 and 7.2 µg/mL corresponding to 0.6 ng, 0.7 ng, 0.75 ng, 0.8 ng, 0.6 ng, 0.83 ng and 0.72 ng respectively injected on the column with split ratio 10:1. The limit of quantitation measured as signal-noise ratio (10:1) were 18 µg/mL PYR, 21 µg/mL KBA, 22.5 µg/mL MKBA, 24 µg/mL M3KVA, 25.8 µg/mL M4KVA, 24.9 µg/mL KHA and 21.6 µg/mL PPYR (Table-1). The amino acids L- threonine, L-lysine, L-histadine, L-tyrosine, and L- trytophan are present in the pharmaceutical preparation of α-keto acids and amino acids, thus quantitation of the amino acids was considered. The linear calibration curve for each amino acid was observed within 10.4-331.4 µg/mL with r^2 within 0.9973-0.9988 (Table-1). LOD's were observed within 3.5-6.9 µg/mL. The pharmaceutical additives methylparabin, propylparabin, gum acacia, manitol, lactose, fructose, glucose, galactose, sodium chloride, sodium lacuryl sulphate and methyl hydroxypropyl cellulose was added at least twice the amount of the *α*-keto acids and analysis was carried out following the analytical procedure 2.3. The results obtained were compared with *α*-keto acids standards. The additions of additives did not interface the determination of the *α*-keto acids with relative error ±3.5.

Sample Analysis

A pharmaceutical preparation Ketosteril of α- keto acids and amino acids is used for prevention and therapy of damages due to deficient protein metabolism in chronic renal insufficiency in connection with limited protein food. The drug was analysed after extraction of the active ingredients in methanol-water. ECF derivatives formed were eluted from GC column. The GC responses corresponding to MKBA, M3KVA, M4KVA and PPYR were noted. The peak identification was made by comparing retention times with standards. Quantitation was from external calibrations based on least square linear regression equation Y = ax + b. The amounts of M3KVA, M4KVA, MKBA and PPYR found were 64.43, 96.8, 81.03 and 64.81 mg/ tablet as calcium salt with RSD of 0.41%, 0.38%, 0.68% and 1.0 % (n = 4). The observed value agreed with labeled values 67, 101, 86 and 68 mg/tablet respectively. Aqueous – methanolic solution of the tablet Ketosteril was spiked with standard solutions of MKBA, M3KVA, M4KVA and PPYR 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 recovery rates of MKBA, M3KVA, M4KVA and PPYR were calculated to 94.22%, 96.16% 95.84% and 95.3 % with RSD 0.4 %, 1.0%, 0.7 % and 0.1 % (n = 3). The Ketosteril tablet also contained amino acids as L-threonine, L-lysine acetate, L-histadine, L-tyrosine and L-tryptophane. The amino acids eluted and separated completely from each other and from α-keto acids, thus their amount in the tablet were also estimated using external calibration curve. Their amount found agreed with expected value with RSD within 0.2-0.9% (Table-2) with recovery of 95.8%, 98.65%, 97.73%, 95.4% and 94.6% respectively (Fig. 4).

Table-1: Quantitative GC-FID data of α-keto acids as derivatives of ECF.

S. No	Name of compound	Calibration range	Coeffici R^2	Least square or Regression	LOD µg/mL	LOQ µg/mL
1	PYR	(18-90 µg/mL)	0.9983	Y= 0.2212x + 0.0288	6	18
2	KBA	(21-105 µg/mL)	0.9978	Y=0.1299x + 0.001	7	21
3	MKBA	(22.5-112.5 µg/mL)	0.9978	Y= 0.1103x + 0.0467	7.5	22.5
4	M3KVA	(24-120 µg/mL)	0.9979	Y= 0.0924x + 0.0662	8.0	24
5	M4KVA	(25.8-127.8 µg/mL)	0.9986	Y= 0.0851x + 0.0789	8.6	25.8
6	KHA	(24.9-124.5 µg/mL)	0.9982	Y=0.0887x + 0.0076	8.3	24.9
7	PPYR	(21.6-108 µg/mL)	0.9978	Y= 0.2177x + 0.0476	7.2	21.6
9	L-Threonine	(15.3-244.8 µg/mL)	0.9973	Y= 0.0712x + 0.0268	5.1	15.3
10	L-lysine acetate	(20.7-331.7 µg/mL)	0.9988	Y=0.1149x + 0.051	6.9	20.7
11	L-histadine	(12.6-201.6 µg/mL)	0.9974	Y= 0.0603x + 0.0567	4.2	12.6
12	L-tyrosine	(14.6-233.3 µg/mL)	0.9980	Y= 0.0924x + 0.0862	4.9	14.6
13	L-tryptophane	(10.4-166.1µg/mL)	0.9982	Y= 0.1151x + 0.0919	3.5	10.4

Table-2: Analysis of M3KVA, M4KVA, MKBA and PPYR from Ketosteril tablet using ECF as derivatizing reagent.

S. No	Name of Standard	Amount Labeled mg/tablet	Amount Found mg/tablet (RSD %)
1	M3KVA	67	64.5(0.5)
2	M4KVA	101	95.87(0.2)
3	MKBA	86	80.98 (0.9)
4	PPYR	68	64.91 (0.6)
5	L-Threonine	53 mg	50.78 (0.5)
6	L-lysine acetate	105 mg	103.58 (0.2)
7	L-histadine	38 mg	36.91 (0.8)
8	L-tyrosine	30 mg	28.63 (0.2)
9	L-tryptophane	23 mg	21.77 (0.3)

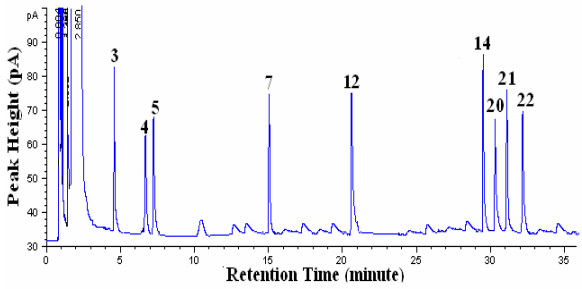


Fig 4: GC elution of (3) MKBA (4) M3KVA (5) MKBA (7) PPYR (12) L-threonine (14) L-lysine acetate (20) L-Histadine (21) L-tyrosine and (22) L-Tryptophane from Ketosteril tablet after precolumn derivatization with ECF. Conditions as Fig 2.

Now comparing the results with reported GC procedures for analysis of α- keto acids, Fu *et al.*, [11] have reported separation of nine α- keto acids by GC-MS with oxime-trimethylsilyl derivatization with total run time of 50 min, but the present method reports the separation of seven α- keto acids within 20 min and simultaneous analysis of amino acids is an added advantage.

Experimental

Chemicals and Reagents

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

Equipment

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/splitless injector operated in split mode, 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 the computer with Chemstation software (Agilent Technologies), HP 1300 Laser jet was used throughout the study. Capillary GC column HP-5 (5% phenyl methyl siloxane) (30m x 0.32 mm i.d.) with film thickness 0.25 µm (J & W Scientific GC column, USA) was used throughout the study.

GC Analytical Procedure

The solution (1ml) containing pyruvic acid (PYR), 2-oxobutyric acid (KBA), 3-methyl 2-oxobutyric acid (MKBA), 3- methyl-2-oxovalaric acid (M3KVA), 4- methyl-2-oxovalaric acid (M4KVA), 2-oxohexanoic acid (KHA), phenylpyruvic acid (PPYR) (18-127.8 µg each) and a mixture of amino acids was added 0.5 ml of ethyl chloroformate, 0.5 ml acetonitrile and sodium carbonate buffer pH 9 (1 ml). The contents were heated in boiling water for 10 minutes and allowed to cool at room temperature and chloroform 1 ml was added. The contents were mixed well and layers were allowed to separate. An aliquot of organic layer was transferred to screw capped sample vial. The solution (1µl) was injected on the column HP-5 (30 m x 0.32 mm i.d.) with film thickness 0.25 µm at column temperature 75 ^{0}C for 10 min followed by heating rate 10^{0}C/min up to 175 ^{0}C and heating rate 15°C/min up to 250°C. Nitrogen flow rate was 1.5 mL/min with split ratio 10:1, v/v. Detection was by FID. Injection port and detector temperatures were 250 and 280 ^{0}C, respectively. Nitrogen as make up flow rate was 45 mL/min. FID air and hydrogen flow rate 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 twice in 20 ml portions of methanol: water (1:1). The solution was filtered and volume adjusted

to 100 ml. Well mixed solutions 0.1, 0.3 ml, 0.4 ml and 0.5 ml were taken and GC Analytical Procedure was followed. The quantitation was carried out using linear external calibration curves based on y = ax + b.

Analysis of Pharmaceutical Preparation by Standard Addition

Five tablets of Ketostril were taken and processed as Analysis of Pharmaceutical preparation. The well mixed solutions 0.2 ml, 0.3 ml and 0.4 ml were taken in duplicate. A solution from each was processed as analytical procedure and other was added M3KVA 25 µg, M4KVA 26 µg, PPYR 22 µg and MKBA 23 µg and again GC Analytical Procedure was followed. The quantitation was carried out from external calibration curve and an increase in response with added standards.

Conclusion

A GC procedure has been developed for the determination of MKBA, M3KVA, M4KVA PPYR and amino acids from a pharmaceutical preparation after precolumn derivatization with ECF. A number of pharmaceutical additives present together with the α- keto acids did not affect the determination. Their extraction α-keto acids from pharmaceutical preparation was calculated within 94.22% to 96.16%. The derivatization, GC elution and separation were repeatable with RSD 0.1- 1.0 %.

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