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High Performance Liquid Chromatographic Determination Of α-keto Acids From A Pharmaceutical Preparation (Tablet) Using 4-Nitro-1, 2-Phenylene Diamine As A Derivatizing Reagent

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ABSTRACT
A rapid, sensitive and selective HPLC separation with photodiode array detector was developed for the determination of the α-keto acids from pharmaceutical tablet. α-keto acids (1) 3-methyl 2-oxobutyric acid (MKBA), (2) 3-methyl 2-oxovaleric acid (K3MVA), (3) 4-methyl-2-oxovaleric acid (K4MVA) and (4) Phenyl pyruvic acid (PPY) as derivatives with 4-dinitro 1,2-phenylenediamine (NPD) eluted and separated from HPLC column Zorbax C–18 and the UV detection was carried out by photodiode array at 255 nm at pH 3. The isocratic elution was with methanol-water-acetonitrile (42:56:2V/V/V) with flow rate 0.9 ml/min. The standard curves were linear from 0.45-100 µg/ml and detection limits were within 0.1-0.266 µg/ml of each acid. The elution and separation of four keto acids was rapid within 10 min.The method was repeatable with relative standard deviation (RSD) within 0.1-2.9% for each of the α-keto acids. The method was applied for determination of α-keto acids from a pharmaceutical preparation. The result was further confirmed by standard addition technique.

Keywords: HPLC, α-Keto acids, 4-nitro1, 2-phenylenediamine, Ketostril tablet

1. INTRODUCTION
α-Keto acids are important intermediates in the biosynthesis of amino acids and carboxylic acids, glycolysis cabohydrates metabolism[1-2]. Each of the keto acid has its specific function, Pyruvic acid (PYR) is involved in biosynthesis of alanine and a change in the concentration of phenylpyruvic acid is reported in hypercatobolic states[3-4] , while the concentration of phenylpyruvic acid is increased in plasma from patients with hereditary metabolic diseases[5-6]. Branched chain keto acids, 3-methyl 2-oxovaleric acid (K3MVA) and 4-methyl-2-oxovaleric acid (K4MVA) are involved in protein turn over, hence their determination is of interest of pathological situation such as sepsis burns and heptic disorders[7-9].

The common methods for the determination of α-keto acids are based on spectrophotometric and spectroflurimetric, High Performance Liquid Chromatography HPLC[10-15] and gas chromatography[16-19]. The chromatography of α-keto acids involves derivatization before their detection. The derivatizing reagents used for the sensitive HPLC determination are mainly 2,4-dinitro-phenylhydrazine[20], 4-hydrazine-2-stilbanzole[21], 1, 2- diamino-4, 5-dimethoxybenzene[22] , 1, 2- diamino-4, 5-methylenedioxybenzene[23] and 4, 5-diaminophthalhydrazide[24]. o-phenylenediamine[25-27]. 4-nitro1, 2-phenylenediamine (NPD) is related to o-phenylenediamine and has been used for the determination of α-keto acids by paper chromatography. However these techniques are required time consuming and procedure of sample preparation involving complications.

The present work examines the use of 4-nitro1, 2-phenylenediamine (NPD) as a derivatizing reagent for HPLC separation and determination of α-keto acids from a pharmaceutical preparation.

2. MATERIAL AND METHODS

2.1 *Reagents*
MKBA, K3MVA, K4MVA, sodium salt, (Fluka, Switzerland), PPY (sigma,USA) and NPD (Fluka, Switzerland) 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 recrystalized 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.

The Buffer solutions within pH 1-10 at unit interval were prepared from the following: Hydrochloric acid (0.1 M) and potassium chloride (1 M)), acetic acid (1 M) and sodium acetate (1 M), ammonium acetate (1 M) boric acid (1 M) and sodium tetraborate (1 M), sodium bicarbonate (1 M), sodium carbonate (saturated) and ammonium chloride (1 M) and ammonia solution (1 M).

2.2 *Equipment*
pH measurement was made with an 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

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Spectrophotometer (Hitachi (Pvt) Ltd Tokyo, Japan) with dual 1 cm silica cuvettes. High Performance Liquid Chromatography was carried out on Agilent model 1100–network HPLC System (Agilent Technology Inc, USA) 1100 series with diode array detection system. The computer with Chemstation software controlled the HPLC. The column ZORBAX 300 SB-C18 (4.6x150mm id) (Agilent Technology Inc, USA) was used throughout the study.

2.3 *Analytical Procedures*

2.3.1 *Spectrophotometric Procedure*

The solution (1-2 ml) containing (5-100 µg) MKBA, K3MVA, K4MVA and PPY was transferred to 10 ml volumetric flask separately. Each of the solution was added NPD solution (1.5 mL, 1%, w/v in methanol), acetic-acid sodium acetate buffer (pH 3) (1 ml) and contents were warmed on water bath at 80 ^{0}C for 30 minutes. The volume was adjusted with methanol and absorption spectrum was recorded within 400-250 nm against reagent blank. The reagent blank was prepared following the same procedure, without the addition of α- keto acids.

2.3.2 *HPLC Procedure*

The solution (1ml) containing MKBA, K3MVA, K4MVA, and PPY of each within the concentration range as indicated in Table-1, was added NPD reagent solution (1.5 ml, 1%, w/v in methanol) and acetic acid-sodium acetate buffer pH 3 (1 ml). The contents were warmed at 80 ^{0}C for 30 minutes and volume was adjusted with methanol to 10 ml. The solution (20 µl) was injected on the column Zorbax C-18 (4.6x150 mm-id) and eluted with methanol-water-acetonitrile (42:56:2V/V/V) with a flow rate 0.9 ml/min. The detection was at 255 nm.

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

S.no	Name of Compound	Calibration Range (µg/mL)	Coefficient of determination (R^2)	Least Square Or Regression	Limit of Detection N (LOD) (µg/mL)	Limit of Quantitation (LOQ)
1	MKBA	(0.45-100)	0.998	Y=0.4897x+0.0119	0.15	0.45
2	K3MVA	(0.6-100)	0.9976	Y=0.2584x+0.0286	0.266	0.8
3	K4MVA	(0.6-100)	0.9983	Y=0.148x+0.0333	0.2	0.6
4	PPY	(0.4-100)	0.9957	Y=0.1196x -0.0519	0.1	0.3

2.3.3 *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 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 analytical procedure 2.3.2 was followed. The quantitation was carried out using linear calibration curves based on

$$Y=ax=+b.$$

2.3.4 *Analysis of Pharmaceutical Preparation by Standard Addition*

Five tablets of Ketostril were treated as 2.3.3. The solutions 0.4 ml and 0.6 ml were taken in duplicate. A solution was processed as 2.3.2 and others were added MKBA 50 µg, K3MVA 60 µg, and K4MVA 100 µg, PPY 40 µg, and again processed as 2.3.2. The quantitation was carried out from external calibration curve and an increase in the response with added standards.

3. RESULTS AND DISCUSSION

α-keto acids react with NPD to form nitroquinoxanol. The effect of reaction conditions in terms of pH, amount of reagent NPD added per analysis, warming time and temperature were examined. The reactions were initially monitored by spectrophotometer. The derivatives indicated maximum absorbance within 299-340 nm against reagent blank and were used for monitoring of each reaction. The condition which gave maximum absorbance was considered optimum. The effect of pH within 1 to 10 at unit interval was examined and the reaction was observed maximum in acidic medium and acetate buffer pH 3 was selected. 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 ^{0}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. Each of the derivatives at optimized conditions obeyed the Beer's law within 10-100 µg/ml and did not show any change in absorbance up to 24 hours. For the simultaneous determination of the α-keto acids HPLC was examined from Zorbax C-18 column. Each of the derivatives eluted as single peak and separated from the derivatizing reagent. The separation of the α-keto acids was next examined using different compositions of eluting solvent system. An optimal separation between MKBA, K3MVA, K4MVA and PPY was obtained when eluted isocratically with methanol-water-

acetonitrile (42:56:2 v/v/v) with a flow rate of 0.9 ml/min (Fig-1). Complete and baseline separation was obtained. The peak identification was made on the basis of 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 within UV region due to π-π* transition within quinoxalinols rings and wave length at peak maximum was examined with the diode array detector and wave length of the UV detector was fixed at 255 nm.

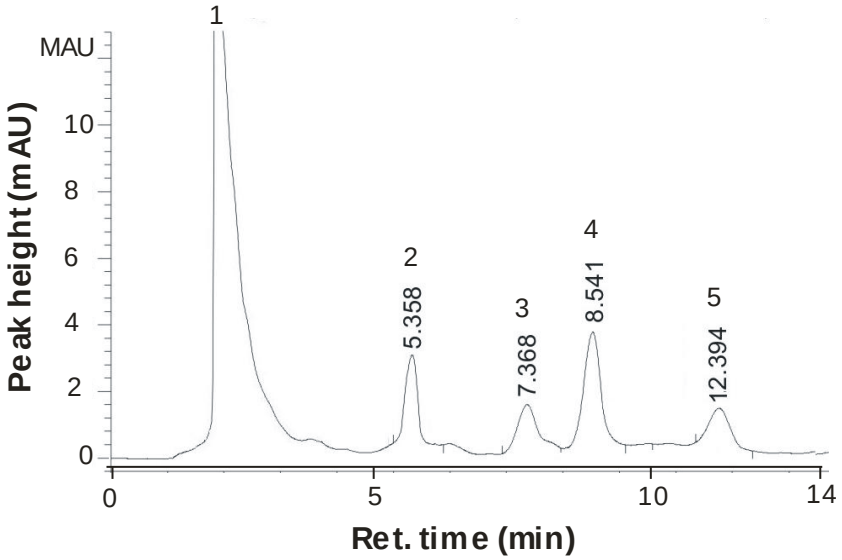

Fig-1: HPLC separation of (1) Reagent, (2) MKBA, (3) K3MVA, (4) K4MVA, (5)PPY from Tablet sample.

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.

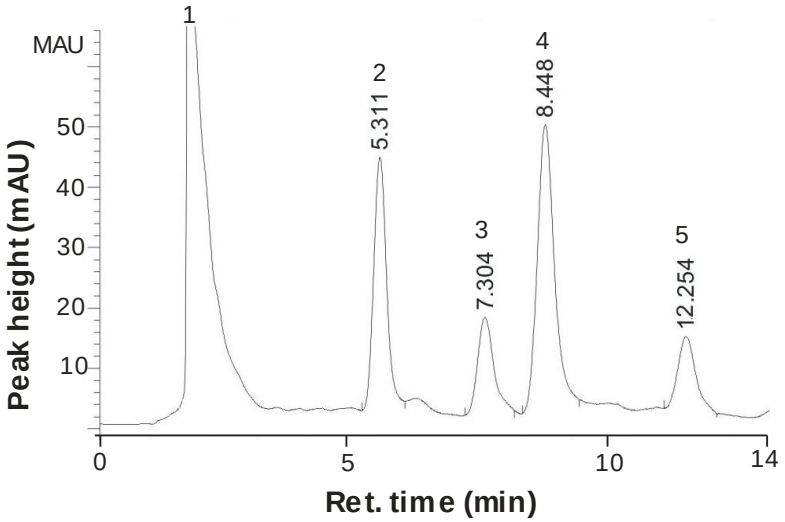

Fig-2: HPLC separation of (1) Reagent, (2) MKBA, (3) K3MVA, (4) K4MVA, (5)PPY by standard addition method Conditions as Fig-1

4. QUANTITATION AND VALIDATION

Linear calibration curves were obtained by recording average peak height / peak area (n=4) versus concentration with MKBA (2.5-100 µg/ml), K3MVA (8-100 µg/ml), K4MVA (6-100 µg/ml), and PPY (3-100 µg/ml) with coefficient of determination (r^2) 0.999, 0.9943, 0.9961 and 0.9983 respectively. The standard deviation (SD) and 95% confidence limit of slope (b) and intercept (a) for the linear regression equation Y=bx+a were calculated (Table-2). The limits of detection (LOD) measured as three times the signal to noise ratio (3:1) were obtained within the range 0.05-2.5 µg/ml and limit of quantitation (LOQ) measured as signal to noise ratio (10:1) were within (0.15-2.7) µg/ml (Table-1). The analysis of test solutions (n=6) of the mixture of the α-keto acids indicated relative error within ±0.1-2.9%. 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 formed were highly stable and did not show any change in response up to 48 h.

Table-2: HPLC analytical results of α-keto acids from Pharmaceutical preparation using 4-nitro-1, 2, Phenylenediamine as a derivatization reagent

Product	Name of α-keto	Amount reported mg/tab	Amount found mg/tab	Relative deviation (RSD)	% recovery
Tablet Ketosteril Bad Homburg, V.D.H.Germany	MKBA	86	82.5	2.1	95.9
	K3MVA	67	64.8	2.6	96.7
	K4MVA	101	98	2.9	97.0
	PPY	68	65.8	0.9	97.0

5. SAMPLE ANALYSIS

A pharmaceutical preparation Ketostril tablet was analyzed for the contents of the α-keto acids and amount of MKBA, K3MVA, K4MVA, and PPY found were 82.5 mg/tab, 64.8 mg/tab, 98 mg/tab 65.8 mg/tab with RSD 2.1%, 2.6%, 2.2% and 1.9% and correlated with the labeled values of 86 mg/tab, 67 mg/tab 101 mg/tab and 68 mg/tab respectively. The analysis was also carried out by standard addition by spiking the solutions of the drug with MKBA, K3MVA K4MVA and PPY. The amounts found again correlated with labeled value and % recovery was observed 95.9%, 96.7%, 97.0% and 97% with RSD 0.91%, 1.2%, 1.8% 2.0% for MKBA, K3MVA, K4MVA and PPY respectively. Kieber and Mopper[1] have reported the separation of nine α-keto acids within 24 min. using o-phenylenediamineas dervatizing reagent, spectrophotometric detection and gradient elution for sample analysis, but the present method reports the separation of nine α-keto acids within 14 min and more commonly available spectrofluorometric detection and isocratic elution.

Table-3: By standard addition method, 20, 80, 30 &60 ppm

Product	Name of α-keto	Amount reported mg/tab	Amount found mg/tab	Relative deviation (RSD)	% recovery
Tablet Ketosteril Bad Homburg, V.D.H.Germany	MKBA	86	82.0	2.2	94.6
	K3MVA	67	64.9	1.9	96.0
	K4MVA	101	98.6	2.0	95.0
	PPY	68	65.2	1.2	98.2

6. CONCLUSION

An analytical procedure has been developed for the determination of α-keto acids using NPD as derivatizing reagent. The Results of the analysis using the developed method for three α-keto acids agreed with the labeled values in pharmaceutical preparation. The method reports quick and simple isocratic HPLC elution method for the separation and determination of α-keto acids.

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