A novel and sensitive analytical method for identification and quantification of organic acids in pharmaceutical products by HPLC with UV-Visible detector

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Abstract: A novel, sensitive, specific and reliable gradient HPLC (High Performance Liquid Chromatography) method has been developed and validated for the identification and quantification of acids namely, Glyoxalic acid, Glycolic acid, Formic acid, Acetic acid, Diglycolic acid, Chloro acetic acid, Dichloro acetic acid and Trichloro acetic acid. A novel, suitable, rapid and sensitive analytical method was developed using HPLC technique for the above acids quantification and identification. The chromatographic separation of the acids were achieved on Atlantis T3 (250*4.6) mm, 5.0 μ column using 0.15 % TFA (Tri Fluoro Acetic acid) in water (v/v): Solvent mixture (98.5:1.5 v/v) as a mobile phase-A and Acetonitrile: Methanol (90:10%v/v). The solvent mixture was prepared by mixing the acetonitrile and methanol solvents in the ratio of 90:10 volumes. The HPLC method was validated with respect to limit of detection, limit of quantitation, specificity, linearity, accuracy and precision.
Keywords: Organic residual Acids / Development / HPLC / Validation.

INTRODUCTION

Various types of organic acids (OAs), such as Acetic acid, Glyoxalic acid, Glycolic acid, Formic acid, Diglycolic acid, Chloro acetic acid, Dichloro acetic acid, Trichloro acetic acid oxalic acid, succinic acid, fumaric acid, maleic acid, tartaric acid, citric acid and quinolic acid etc., are considered to be the residual impurities or byproducts during the synthesis process of the raw materials, intermediates and finished products.

These organic acids are to be quantified to avoid the adverse effect in further process, but have been little studied using different analytical techniques such as high performance liquid chromatography (HPLC) with electrochemical detection (ED) for determining organic acids in fruit wines [1-3]. Various types of organic acids (OAs), such as Oxalic, succinic, fumaric, malic, tartaric, citric and quinolic acids. The Gas chromatography (GC) method for the analysis of OAs would appear to offer significant advantages compared to HPLC since the former could be readily interfaced with MS permitting unequivocal identification of known acids and the detection of new compounds [4].

Residual organic acids in pharmaceuticals are volatile organic chemicals that are used in and are produced during the synthesis of drug substances or can be in excipients used in the production of drug formulations. These residual volatiles are remains from processing agents. Many of these volatile organic chemicals generally cannot be completely removed by standard manufacturing processes or techniques and are left behind, preferably at low levels. It is important to quantify these organic acids in bulk drug substance and finished pharmaceutic products for a number of reasons. High levels of residual organic acids represent a risk to human health because of their adverse effect. Finally, residual organic acids can create odor problems and color changes in the finished product and, thus, can lead to issues.

In the recent past, guidelines for residual organic acids have generally been vague and not up to date. The USP set official limits in USP [5] & ICH, Trichloro acetic acid, Formic acid, Acetic acid and

Chloro acetic acid and has stated limits ranging from 3000 to 5000 parts per million (ppm). The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [4] has made much progress in recent years with residual solvent guidelines and limits [6, 7].

As a part of our research programme in this area, a novel analytical method for the quantification of OAs was developed. The literature procedures described for the analysis of these compounds using GC [8, 9], ion chromatography [10, 11], ELSD [12] and HPLC techniques were not satisfactory with respect to

time and complexity of the detectors used as part of the techniques. The GC method using tri methyl silyl derivatives [13] could be applied to the analysis of only a few Organic acids, whilst the HPLC methods previously described have been employed mainly for the chlorogenic acids.

Hence, in the present study a new method has been re-investigated for the identification, quantification of eight organic acids (Acetic acid, Glyoxalic acid, Glycolic acid, Formic acid, Diglycolic acid, Chloro acetic acid, Dichloro acetic acid, Trichloro acetic acid) in representative pharmaceutical test samples by using a simple HPLC connected with UV detector.
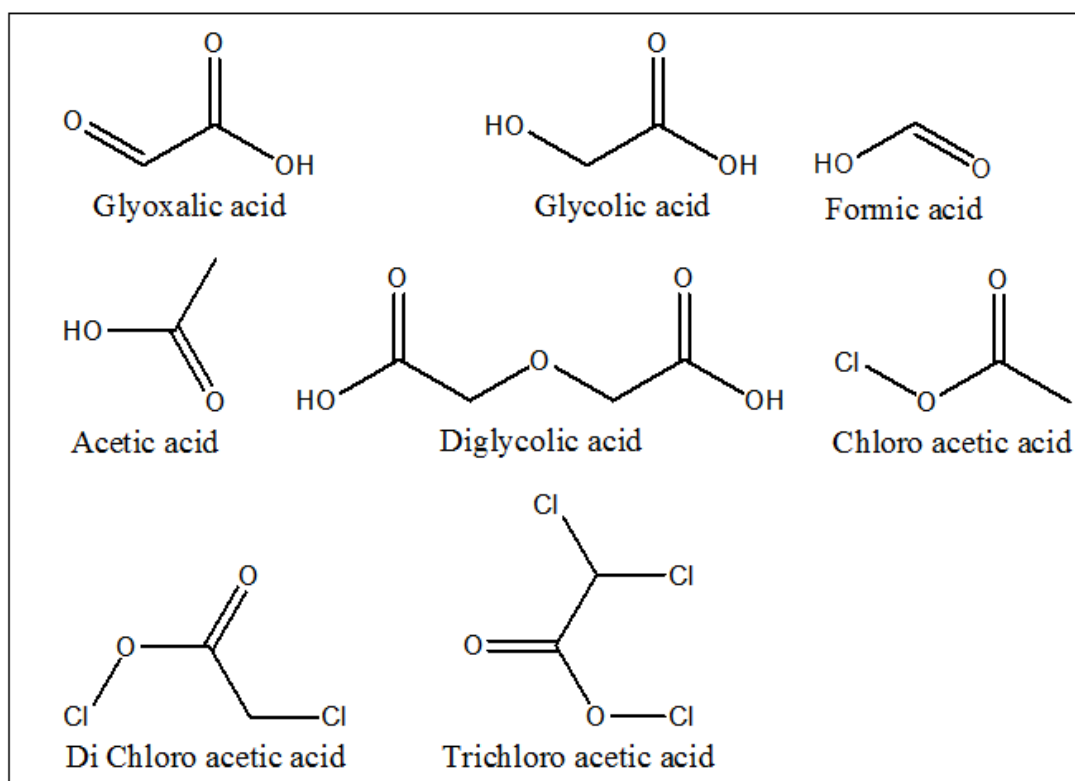

Fig-1: Chemical structures of all the acid compounds with decoding numbers used to identify on chromatograms

Experimental
Chemicals and reagents
HPLC grade acetonitrile and potassium dihydrogen phosphate were purchased from Rankem (Mumbai, India). Acetic acid, Glyoxalic acid, Glycolic acid, Formic acid, Diglycolic acid, Chloro acetic acid, Dichloro acetic acid and Trichloro acetic acid were purchased from S.D. Fine Chemicals (Mumbai, India). Mill-Q water (Barnstead, USA) was used.

Acetonitrile (make of Rankem and HPLC grade solvent), Methanol (make of Rankem and HPLC grade solvent), Tri Fluoro Acetic acid (make of Rankem and HPLC grade) and (make of Rankem and HPLC grade reagent) were used for the method development.

Instrumentation
All experiments were carried out on Agilent liquid chromatographic system (Agilent Technologies, 1100 series, USA), equipped with both PDA (Photo diode array) and VWD detector. The system consisted of a quaternary gradient pump (GP40) with automated membrane eluent degassing capability, a chromatographic column oven (LC20), UV-visible VWD detector (ED40) and an auto sampler with temperature controller (AS40). A personal computer equipped with Empower 3 software was used for instrument control. Data collection and data processing were performed using an HP-Vectra (Hewlett Packed, Waldron, Germany) computer system with empower version 3 (Waters Corporation, Build 2154, USA) data acquiring software.

Chromatographic conditions

Reversed phase HPLC analysis was performed using an Agilent 1100 series HPLC system (USA) which consists of vacuum degasser, quaternary pump, column compartment with thermostat, UV/Visible detector and auto sampler with temperature controller. The initial analytical methods were tried on X-bridge C8, SB Phenyl and Atlantis T3 analytical columns. Different mobile phases like e.g. TFA (Tri Fluoro Acetic acid), Phosphate buffer at different pH by mixing in the different ratios of organic modifiers and without organic modifiers were tried. But final trials were carried out on Atlantis T3, 250 mm×4.6 mm and with particle size 5 μm column.

Impact of the column oven temperature and different ratios of the solvent mixture on the resolution between the different acids has been studied for the optimization of the method. Chromatographic separation was carried out at the column oven temperature 25ºC ± 2ºC with a flow rate 0.8 mL/min of gradient elution using two mobile phases: MP-A – (0.15 % TFA in water (v/v) : Solvent mixture (98.5 :1.5)) and B – Acetonitrile : Methanol (90:10%v/v). Solvent mixture has been prepared by mixing the acetonitrile and methanol in the ratios of 90:10(v/v). Wavelength of 210 nm was used for the analysis. 20 μL of injection volume used to inject the standard and test sample solutions. All the solutions were filtered using 0.22 μm membrane filter before HPLC analysis.

Sample preparation

The test samples (API-A) were prepared at 40 mg/mL concentration with mobile phase-A. All the organic acids (Acetic acid, Glyoxalic acid, Glycolic acid, Formic acid, Diglycolic acid, Chloro acetic acid, Dichloro acetic acid and Trichloro acetic acid) were prepared at concentration of 0.04 mg/mL to get 0.10% w/w with respect to test sample concentration. All the test samples and acid standard solutions were kept in refrigerator at 2-8°C and all the solutions were filtered through 0.22 μm membrane filters before HPLC analysis.

RESULTS AND DISCUSSION
Optimization of chromatographic conditions

During the method development initial analytical methods were tried on Inertsil C8, X-bridge C8, SB Phenyl and Atlantis T3 stationary phases to optimize the peak shape of the acid peaks and to improve the resolution between the acid peaks. Different mobile phases like e.g. TFA (Tri Fluoro

Acetic acid), Phosphate buffer at different pH by mixing in the different ratios of organic modifiers and without organic modifiers were tried. Inertsil C8 stationary phases was used for initial trials as this is stable stationary phase at lower pH 20mM of Potassium dihydrogen phosphate buffer, adjusted the pH with diluted phosphoric acid to 2.5. Buffer and Acetonitrile have mixed at the ratio of 95:5%v/v to prepare the mobile phase. Flow rate was adjusted to 0.7 mL/Min with isocratic conditions and diluent was used is acetonitrile and water mixture in the ratio of 90:10 v/v. But the separation of acids and retention time of compound peak (API) is not good and observed column to column variation. Same chromatographic conditions were used and stationary phase was changed to X-bridge C8. But observed there is no proper resolution between acid peaks and compound peak. Buffer has been replaced with 0.15% v/v TFA in water as mobile phase. This column has given good separation when 0.15% v/v TFA modifier in water was used for initial trials.

Then trials were attempted by mixing with the different composition of organic solvents with buffer, but separation was lost when more ratio of organic modifier was used. Similar conditions were used by changing the stationary phase to SB Phenyl column and observed there is no separation between all the acids more than 1.5 resolution. Therefore it is concluded that the solvent mixture ratio at lower level and more aqueous will improve the resolution between the acid peaks. To use more aqueous mobile phase and at lower pH of mobile phase Atlantis T3 column will be more compatible and further trials were carried out on Atlantis T3 column (250mm, 4.6mm & 5.0μm). Column oven temperature maintained at 40°C± 2ºC and with a flow rate of 0.8 mL/Min gradient elution. The resolution between Acetic acid and Diglycolic acid was lost when column temperature was increased to 40°C± 2ºC.

A) Chromatographic separation was carried out at the column oven temperature 25ºC ± 2ºC with a flow rate 0.8 mL/min of gradient elution using two mobile phases: MP-A – (0.15 % TFA in water (v/v) : Solvent mixture (98.5 :1.5)) and B – Acetonitrile : Methanol (90:10%v/v). Solvent mixture has been prepared by mixing the acetonitrile and methanol in the ratios of 90:10(v/v). (A) Impact of Tri Fluoro Aceticacid on the separation of organic acid peaks in standard.
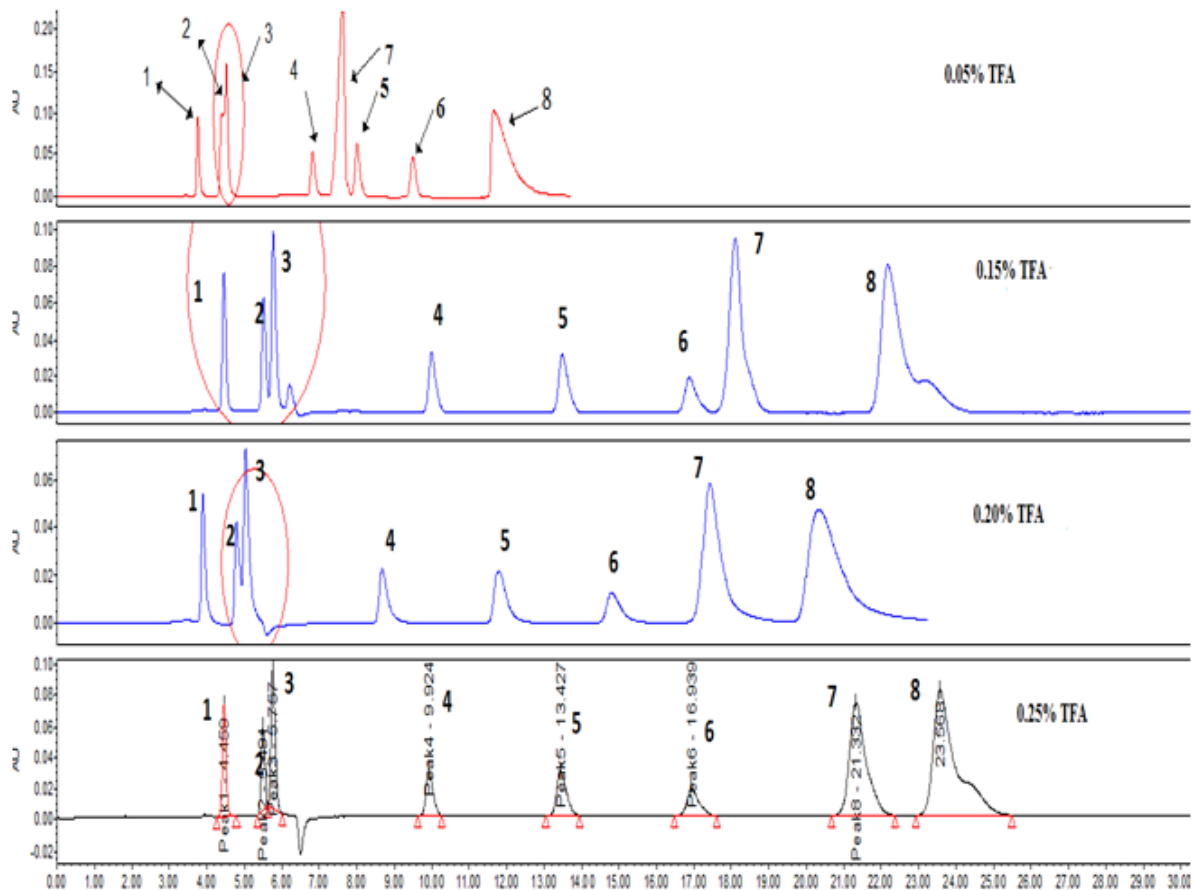
Fig-2: Typical HPLC chromatograms of (A) Impact of Tri Fluoro Aceticacid on the separation, (B) Impact of organic modifier (C) Impact of column temperature, (D) Impact of mobile phase composition (E) System suitability chromatogram

Description of the numbers and related organic acid peak names

1. Glyoxalic acid, 2. Glycolic acid, 3. Formic acid, 4. Acetic acid, 5. Diglycolic acid, 6. Chloro acetic acid, 7. Dichloro acetic acid and 8. Trichloro acetic acid.
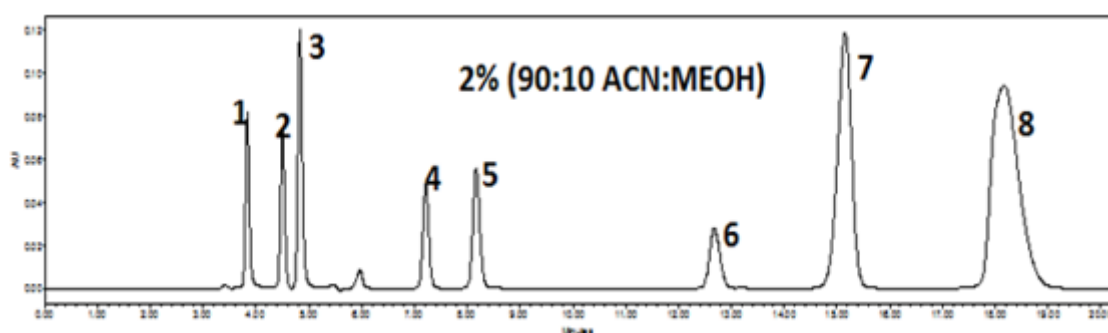

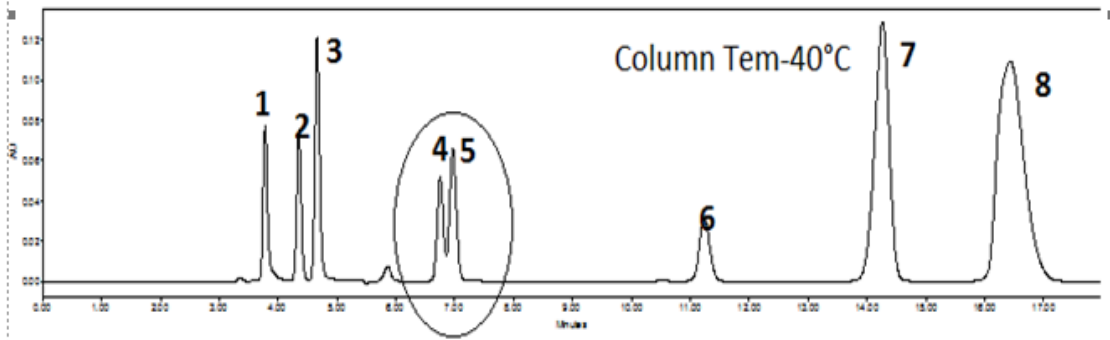**Fig-3: Typical HPLC chromatograms of Impact of organic modifier (B)**

Fig-4: Typical HPLC chromatograms (C) Impact of column oven temperature on the separation of organic acid peaks

Fig-5: (D) Impact of mobile phase composition on the separation of organic acid peaks
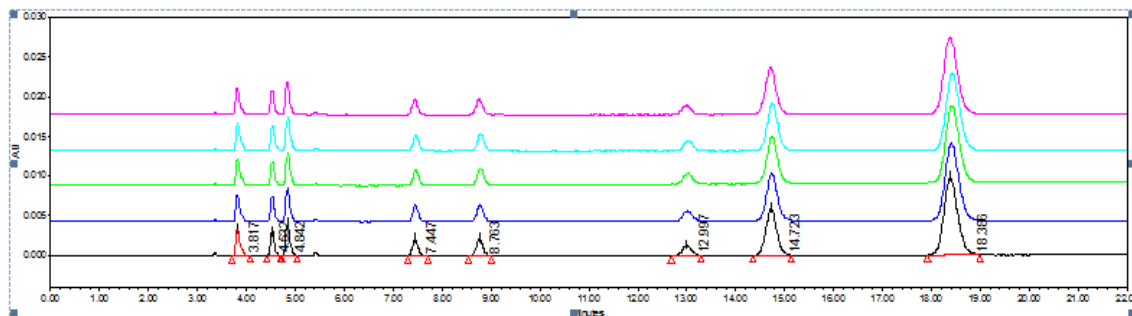

Fig-6: System suitability chromatograms overlaid

Table-1: %RSD of organic acid peak areas at 0.1% level from system suitability standards

	SampleName	Vial	Injection	GOA (µV*sec)	GA (µV*sec)	FA (µV*sec)	AA (µV*sec)	DGA (µV*sec)	MCA (µV*sec)	DCA (µV*sec)	TCA (µV*sec)
1	ACID MIX 0.1%	2	1	19634	16145	23823	16704	20324	17181	91678	189480
2	ACID MIX 0.1%	2	2	19416	16093	23902	17211	20319	17027	92038	189011
3	ACID MIX 0.1%	2	3	19563	16127	24004	16844	20540	16837	91216	188368
4	ACID MIX 0.1%	2	4	19228	16114	23762	17141	20563	17969	90665	188394
5	ACID MIX 0.1%	2	5	19762	16241	24047	16689	20821	16971	91082	187615
Mean				19520.63	16143.83	23907.68	16917.86	20513.42	17196.78	91335.75	188573.74
Std. Dev.				205.719	57.482	119.53E	244.612	206.920	449.008	533.827	708.049
% RSD				1.05	0.36	0.50	1.45	1.01	2.61	0.58	0.38

Description of the numbers and related organic acid peak names

GOA: Glyoxalic acid, GA: Glycolic acid, FA: Formic acid, AA: Acetic acid, DGA: Diglycolic acid, MCA: Chloro acetic acid, DCA: Dichloro acetic acid and TCA: Trichloro acetic acid.

Validation

Specificity, selectivity and system suitability (SST)

Specificity is the ability of the analytical method to measure the analyte concentration accurately in presence of all the individual components. To establish specificity for all the acid peaks, all the individual organic acid solutions (Acetic acid, Glyoxalic acid, Glycolic acid, Formic acid, Diglycolic acid, Chloro acetic acid, Dichloro acetic acid and Trichloro acetic acid) were prepared and injected at 0.10% w/w level. Specificity and selectivity were determined by comparing the chromatograms of the standard solution and the blank samples. System suitability is commonly used to verify resolution, column efficiency, and repeatability of the chromatographic system to ensure its adequacy for the particular analysis. System suitability is performed by injecting the six standard injections of acid solutions. The percentage relative standard deviation (RSD) of the area and retention time of each acid from six replicate injections were below 1.5%. Low values of RSD for replicated injections indicate that the system is precise. The results of other system suitability parameters such as peak asymmetry and theoretical plates are presented in Table 1. As seen from these data, the acceptable system suitability parameters would be as follows: the relative standard deviation of replicate injections is not more than 5.0 %. Method is found to be specific for all the acid peaks.

Linearity

The linearity was analyzed by calculating the correlation coefficient for the (straight line) analytical curve of Acetic acid, Glyoxalic acid, Glycolic acid, Formic acid, Diglycolic acid, Chloro acetic acid, Dichloro acetic acid and Trichloro acetic acid individually. A calibration curve for each component was constructed by linear regression of the observed average peak area versus concentration. The coefficients of the regression curves (the slope and the intercept on the y axis) and the squares of the correlation coefficients ($r2$) were calculated by the least squares method. Linearity was evaluated using standard samples over five calibration points with six measurements for each calibration point. The calibration curve at all concentration ranges was better described by a quadratic equation with the correlation coefficient > 0.99. Calibration curves were linear for all analytes investigated. Table 2 presents the equation of the regression line, correlation coefficients ($r2$), relative standard deviation (RSD), values of the slope and intercept for each compound, the results are shown in Table-2.

The results have indicated a good linearity. The limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield a signal-to-noise ratio in-between 2.0 & 3.4 for LOD and 9.5 & 11.0 for LOQ respectively. Established LOD and LOQ values were found to be in between 0.01 to 0.03 %w/w respectively. This sensitivity of the method can be further improved by using the suitable concentrations of the test sample based on the solubility.

Table-2: Linearity results

%level	Glyoxalic acid	Glycolic acid	Formic acid	Acetic acid	Diglycolic acid
LOQ	6510.1	5232.42	7898.25	5558.3	6765.8
50%	9821	8125.2	11925.3	8455.6	10258.5
75%	14820.1	12112.5	17925.3	12584.5	15452
100%	19545.6	16254.3	23895.5	16915.8	20512.5
125%	24562.5	20175	29885.8	21145.8	25635.2

150%	29325.4	24258.2	35855.2	25438.5	30871.2
Correlation:	**0.99997**	**0.99996**	**1.00000**	**0.99998**	**0.99999**
slope:	**195225.5**	**162122.0**	**239098.6**	**169842.8**	**205648.8**
%level	**Chloro acetic acid**	**Chloro acetic acid**	**Trichloro acetic acid.**		
LOQ	5675.5	30155.2	62532.5		
50%	8601.2	45701.2	94892.5		
75%	12598.6	68498.2	141625.3		
100%	17201.2	91201.5	188923.5		
125%	21525.6	113999.2	231215.2		
150%	25801.2	136992.5	282456.2		
Correlatio	**0.99987**	**1.00000**	**0.99980**		
Y intercept:	-91.35984	47.12556	1441.07178		
slope:	**172516.8**	**912317.2**	**1863037.1**		

Accuracy, precision and recovery

Accuracy of the method was determined for both intra-day and inter-day variations using the six times analysis of the samples. Precision was determined by repeatability (intra-day) and intermediate (inter-day). Assay precision of the method was evaluated by repeatability (intra-day) and intermediate (inter-day) by analysis of six replicates of the standard solution for two concentrations in 2 days. The mean of percentage recoveries and the relative standard deviation were also calculated. The results are shown in Table 3. The RSD values for intra-day precision was ≤2.6% and for intra-day ≤2.5%. The relative error values for intra-day accuracy were ≤3.5% and for inter-day accuracy was ≤3.5%. The results show good reproducibility and precision of the developed method.

Table-3: Method Precision

Glyoxalic acid	Glycolic acid	Formic acid	Acetic acid	Diglycolic acid	Chloro acetic acid	Chloro acetic acid	Trichloro acetic acid
Area	Area	Area	Area	Area	Area	Area	Area
19634	16145	23823	16704	20324	17181	91678	189480
19416	16093	23902	17211	20319	17027	92038	189011
19563	16127	24004	16844	20540	16837	91216	188368
19228	16114	23762	17141	20563	17969	90665	188394
19762	16241	24047	16689	20821	16971	91082	187615
Avg:19520.6	16144	23907.6	16917.8	20513.4	17197	91335.8	188573.6
SD: 205.8	57.4	119.5	244.6	207.0	448.8	533.6	708.2
%RSD: 1.05	0.36	0.50	1.45	1.01	2.61	0.58	0.38

Table-4: Accuracy at 100% level

Glyoxalic acid	Glycolic acid	Formic acid	Acetic acid	Diglycolic acid	Chloro acetic acid	Chloro acetic acid	Trichloro acetic acid
99.8	99.4	100.8	101.7	99.5	100.1	99.9	100.0
99.8	99.2	100.6	101.7	99.4	100.0	99.9	100.5
99.9	**99.7**	**100.4**	101.6	99.7	100.0	99.9	100.0
99.8	**99.6**	**100.6**	100.5	99.4	100.0	100.2	100.3
100.2	99.7	100.8	**101.7**	**99.6**	**100.0**	100.0	100.3
99.9	99.7	100.4	**101.7**	99.0	**100.0**	100.1	100.7
Avg. : 99.9	**99.6**	**100.6**	**101.5**	**99.5**	**00.0**	**00.0**	**100.3**

Method accuracy at LOQ level has shown very good recovery and this further can be improved to achieved more sensitive depending upon the solubility of the analyte compounds.

CONCLUSIONS

A novel analytical method has been developed, validated and applied in routine for screening, identification and quantitation of Acetic acid, Glyoxalic acid, Glycolic acid, Formic acid, Diglycolic acid, Chloro acetic acid, Dichloro acetic acid and Trichloro acetic acid in the testing samples. Using this single analysis by HPLC, we can quantify all the 8 organic acids in a short span of time. There are so many analytical methods are available to quantify the residual acids by using Ion chromatography, conventional Gas

chromatography and Liquid chromatography, but this method enables to identify and quantify the residual acids using simple HPLC coupled with UV detector. Morover single method can be applied to identify and quantify Acetic acid, Glyoxalic acid, Glycolic acid, Formic acid, Diglycolic acid, Chloro acetic acid, Dichloro acetic acid and Trichloro acetic acid in any of the pharmaceutical materials.

This method was found to be satisfactory in terms of linearity of response, system precision, accuracy and quantification in the range of LOQ to 0.10%w/w. Recovery studies have shown good results for all solutes (99.5–101.5%). The method is linear for all compounds over the concentration range tested.

Acknowledgment

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