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Determination and validation of low molecular mass organic acids in Pharmaceutical drug substances by capillary electrophoresis

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ABSTRACT

A sensitive capillary electrophoresis method has been developed and optimized for the determination of various organic acid present in the pharmaceutical drug substances. The organic acids under study are Formic acid, Succinic acid, Methanesulfonic acid, Acetic acid, Trifluoro acetic acid and Pivalic acid. These organic acids are potential impurities in various drug substances and causes undesirable by product. The method was developed in such a way that to enhance the detection by employing an indirect UV-mode, and minimizing acquisition time by using suitable electrolyte of potassium phthalate and flow modifier Cetylammonium bromide in water. To prove the performance characteristic of the optimized method, the study was extended toward validation parameters as per the ICH guideline requirement for selectivity, sensitivity (limit of detection and quantification), linearity, precision, robustness, and accuracy. The limit of detection and limit of quantification for the six organic acids were found to be in between 1.6 and 2.6µg/mL and 4.9 and 7.8µg/mL respectively.

Keywords: Capillary electrophoresis, Formic acid, Acetic acid, Methanesulfonic acid, Succinic acid, Pivalic acid and Trifluoroacetic acid, Pharmaceutical matrices

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INTRODUCTION

A great number of organic acids are present in drug substances, which are used during the manufacturing process of the pharmaceutical drug substances. Generally the organic acids are used for pH-controlled reaction, or introduced in to side chain of the drug molecules. Organic acids may be by-product during the synthesis of drug substances, which make important contributions to residual impurity in the drug substances. These residual organic acids tends to react with the basic drug molecules or basic group in the drug substances and form a undesirable product which will be hammering the quality of the drug product or which act as catalyst to facilitate the decomposition of the drug substances during the storage.

Simple organic acids have been quantified by capillary electrophoresis [CE] in a variety of sample types including fruit juices and wines [1-3]. These organic acids generally have limited UV activity and are often detected in CE using indirect UV detection. However, use of short UV wavelengths such as 190 nm can allow direct UV detection of selected simple organic acids [4, 5]. The requirements of a method to routinely quantify the organic acids are

speed, and simplicity. Traditionally ion chromatography (IC) has been used for this type of analysis. However it was expected that CE might offer a simple and inexpensive alternative to IC for determination of organic acid drug counter-ion and residual organic acids. CE is gradually gaining acceptance as an alternative and complementary technique to high performance liquid chromatography (HPLC) for the pharmaceutical analysis. The principle advantages of CE include, among others, high separation efficiency, improved selectivity, low operational cost and speed of analysis. The CE method has been recognized as a promising technique for the separation and quantization of organic acids in various matrices [6-8]. Complementary to established chromatographic methods, like ion-exclusion chromatography (IEC) [9-10], CE provides advantageous features, including short analysis time, low buffer quantity, sample consumption and low detection limits [11]. In this work, it was proposed to assess the use of CE with indirect UV detection as a suitable method for stable, repeatable content of organic acids. The in-direct UV detection has been used with various background electrolytes such as chromate [12], 3,5-dinitrobenzoic acid [13], salicylic acid [14] and 2,6- pyridinedicarboxylic acid (PDC) [15].

In this paper we describe the simple CE method used for the determination of some short chain organic acids in pharmaceutical drug substances, which are used in the manufacturing process of the drug substances. It is very important for the manufacturer to monitor the level of anticipated process related and degradation impurities present in the drug substance before commercial release to prove the consistency of the manufacturing process employed, by using appropriate analytical techniques. In addition, reaction of organic acid with basic drug substances or a basic group present in the drug substances is also quite possible during the storage which leads to undesirable by product. Therefore, short chain organic acids are observed to be a potential impurity of drug substances, and to be monitored during stability storage as well. The ICH guideline on impurities describes that any impurity other than active moiety is to be controlled with suitable limits in the drug substance irrespective of its harmful nature [16].

In our work, indirect detection with potassium hydrogen phthalate as background electrolyte and Cetyltrimethylammonium bromide (CTAB) as electro osmotic flow (EOF) modifier was chosen. The optimized CE method has been validated according to ICH guidelines [17] to prove its suitability and reliability for the determination of short chain organic acids such as Formic acid (FA), Succinic acid (SA), Methanesulfonic acid (MSA), Acetic acid (AA), Trifluoroacetic acid (TFA) and Pivalic acid (PIA), in various drug substances such as Cefazolin sodium, Lamivudine, Atorvastatin calcium, and Lopinavir during routine as well as during the stability storage analysis.

EXPERIMENTAL SECTION

Chemicals and Reagents

All the reagents and standards used in the study were of analytical reagent grade, unless otherwise specified were purchased from E. Merck, India, Pivalic acid procured form Fluka, USA, Methanesulfonic acid was purchased from CDH, India, Propionic acid was supplied by Loba Chime, India and water is double distilled and purified by using Milli Q purification system (Millipore, Billerica, MA). Drug substance and their related known compounds were prepared at Aurobindo Pharma Research Centre, Hyderabad India.

Preparation of Internal standard solution

Dissolve 50mg of Propionic acid **(**PRA**)** in a mixture of 2000ml water and Acetonitrile in the ratio of 1:1v/v.

Preparation of standard solution

Stock solutions of organic acids were prepared individually by accurately weighed quantity of 100mg each of FA, AA, MSA, SA, PA and TFA in separate 100mL volumetric flask and each of the organic acids were in dissolve in internal standard solution and made into 100mL with the internal standard solution. A blend of organic acid solution is prepared at the concentration of 25µg/ml with appropriate dilution with internal standard solution.

Preparation of Sample solution

Sample solution is prepared by accurately weighed 250mg of the each drug substances in 10ml of internal standard solution (25µg/ml) of PRA solution except Atorvastatin calcium drug substance. Due to the poor solubility of Atorvastatin calcium, the concentration of the sample solution was reduced to 10mg/mL in internal standard solution.

Instrumentation and Procedure

An Agilent instrument CE system equipped with a diode array detector along with Chemstation software for data acquisition and processing was used. Separation was carried out in fused silica capillary with extended light path length (Agilent, Germany) of effective length of 56cm and i.d. of 50μm.

The Background electrolyte used was 6-mmole potassium hydrogen phthalate buffer, and 1.2mmole of CTAB adjusted to pH 6.5 with 0.1M molar sodium hydroxide solution. The sample and standard solutions were introduced by hydrodynamic pressure of 50 mbar for 5 sec, and the separation was carried out with constant applied voltage of (-) 20kV at ambient temperature (~20°C). Before introducing the sample solution, the capillary was conditioned with background electrolyte for 3 min at the inlet pressure of 3 bars for 3min.The analyte signal was detected by indirect UV photometric method, the wavelength was set at 330 nm against reference signal at 220 nm. New capillaries were rinsed with water for 5min and then 5min with 0.1M sodium hydroxide solution, followed by background electrolyte for 10 min.

RESULTS AND DISCUSSION

Optimization of experimental variable for effective separation of organic acids

Short chain organic acids are available as anionic species in aqueous solution and are inert to UV photometric absorption. Therefore, it is difficult to determine the content of short chain organic acid by using conventional analytical techniques like HPLC, where UV photometric detection is employed. However, this kind of anionic species can be determined either by Ion chromatography or by CE. In-direct UV photometric detection is greatly employed in zone electrophoresis technique for UV inactive substances. The organic acids are easily ionizable in aqueous solution and acquire a negative charge in the moiety. The negative species are analyzed easily in CE by reversing the EOF (electro osmotic flow) by means of introducing a long chain cationic surfactant.

The long chain cationic surfactant, CTAB is added to the electrolyte to form a positively charged surface coating. The addition of the cationic surfactants CTAB, to the carrier electrolyte, at concentrations below the critical micelle concentration (CMC), reverses the EOF by dynamically coating the inner capillary wall. The positively charged surface generates an EOF in the same direction as the migration of the organic acids, which significantly improves peak shapes and reduces analysis time. The reversing the EOF can reduce the analysis time for anionic species. Under such conditions, a negative power supply causes anionic compounds to migrate in the same direction as the electro osmotic flow.

A phthalate buffer of 5mmole was initially used, as background electrolyte was adjusted pH to 8.0 with sodium hydroxide. A constant level of EOF in this method is essential and relies upon a consistent positive charge on the capillary wall, a 0.25mmole of CTAB were selected as flow modifier for the separation of the organic acid in the pharmaceutical drug substances and temperature of 25°C and voltage applied was (-) 25kV. In this condition the peaks of TFA and MSA were co-eluted with each other. To improve the resolution of peaks of organic acid, the critical parameters such as pH and concentrations of electrolyte were optimized.

The optimized method utilizes a phthalate additive in the buffer in the concentration of 6mmole to permit indirect UV detection, as the mobility of phthalate is well matched with that of the organic acids. This is essential in order to ensure acceptable peak shapes and resolution. A CTAB concentration of 0.5mmole is added to the electrolyte to form a positively charged surface coating. A pH of 6.0 is selected for the separation of each acid. However a system peak was observed near the migration time of PIA. As increase in concentration of CTAB from 0.25mmole to 1.25mmole the system peak near the PIA was gradually diminished, while the peak of TFA and AA were interchanged. As increase in pH the resolution between TFA and AA was increased, while the resolution between FA and SA was decreased. At the pH 6.5, a maximum separation of organic acids with the optimum concentration of 1.2mmole of CTAB was achieved. A short capillary length, in combination with a relatively low voltage of 20kV, was selected to give a low current while still maintaining a fast separation time. The production of a low current helps to flatten the baseline and reduces the buffer depletion effects. This optimized CE method to determine the content of organic acids in various pharmaceutical drug substances has been validated according to ICH guidelines [17].

Validation of the method

The experiments that have been demonstrated during validation studies were selectivity, sensitivity by means of limit of detection and quantification, linearity, precision (system precision, method precision and intermediate precision), stability of sample solution and accuracy, and the results obtained from the experiments were briefly summarized below.

Selectivity

The solution of blank, FA, SA, MSA, TFA, AA and PIA were prepared separately, and injected as per procedure to identify the migration time of each component in the sample matrix. The sample solution was spiked with process related impurity of short chain organic acid with other known impurities of each drug substances separately and were prepared, injected as per procedure to confirm any co-elution of any peaks from the sample matrix. The chromatograms obtained from the analyses show that, all the organic acids peaks were well resolved from that of blank, sample and other related components of sample matrix as well, indicating the selectivity of the method to determine the content of organic acid in pharmaceutical drug substances. An overlay electropherograms of blank solution with internal standard and, standard solution containing all organic acid mixture at target level are shown in Fig. 1. The alphabetic "a, b, c, d, e, f and g" in the electropherograms denotes the peaks corresponding to organic acids FA, SA, AA, MSA, TFA, PRA and PIA respectively.

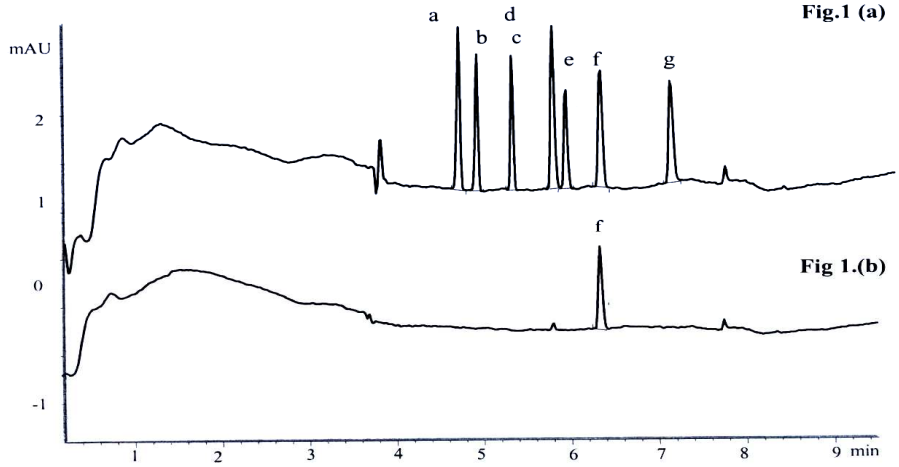

Fig 1: Representative Electropherogram of (a) Standard solution of all organic acids and (b) Diluent containing internal standard

Table 1: Experimental data obtained from linearity and sensitivity experiment.

Components	FA	SA	MSA	AA	TFA	PIA
Correlation coefficient (r^2)	0.9987	0.9982	0.9985	0.9993	0.9987	0.9990
Concentration range (µg/ml)	5 - 40	5 - 40	5 - 40	5 - 40	5 - 40	5 - 40
Intercept	0.047	0.045	0.027	0.014	-0.005	0.035
Slope	0.0537	0.0423	0.0360	0.0271	0.0327	0.0327
STEYX (Standard error)	0.0356	0.0209	0.0282	0.0175	0.0230	0.0183
CC	0.9987	0.99926	0.99822	0.99873	0.99851	0.99904
RSQ	0.9974	0.9985	0.9964	0.9975	0.9970	0.9981
Limit of detection (µg/ml)	2.2	2.6	2.3	1.6	2.1	1.9
Precision for limit of detection, %RSD*	3.9	3.5	6.8	9.8	6.3	11.9
Limit of quantification (µg/ml)	6.6	7.8	7.0	4.9	6.5	5.6
Precision for limit of quantification, %RSD*	2.3	3.6	4.1	2.8	3.2	3.6

**Average of n=6 experimental determinations*

Linearity

The linear relationship of analyte response against concentration was verified in the working concentration range by analyzing different level of solutions containing each organic acid from about 5µg/ml to 40µg/ml. The linear regression line was plotted against analyte response versus concentration. The correlation coefficient of the regression line was found to be more than 0.99. The statistical analysis of linear regression line was evaluated and is summarized in Table 1and linearity plot of concentration of each organic acid Vs area response is shown in the fig 2.

Fig 2: Linearity plot of concentration of Organic acids Vs Area response of organic acids normalized with propionic acid.

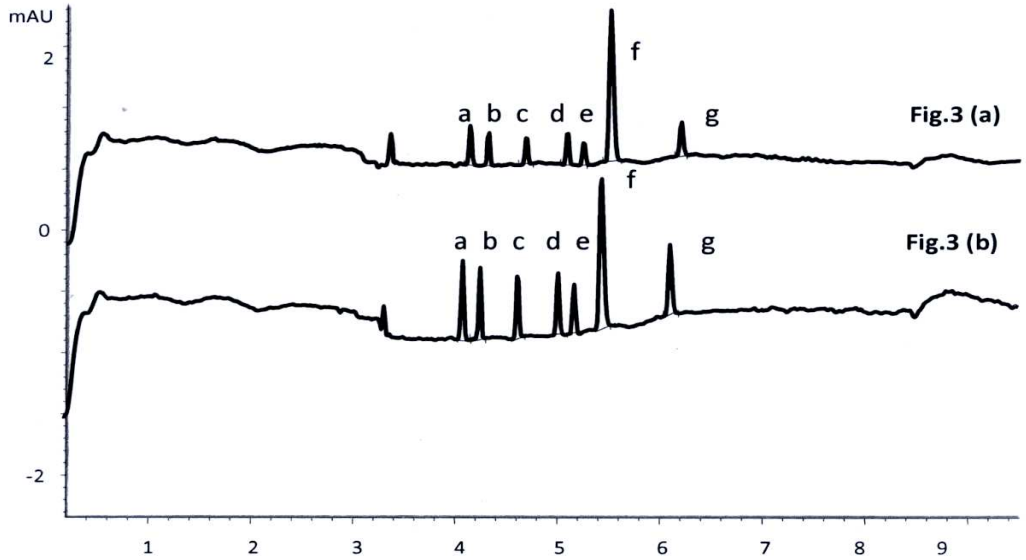

Fig 3: Electropherogram obtained from (a) LOD level concentrations of organic acids and (b) LOQ level concentrations of organic acids

Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) were predicted using slope (S) and residual standard deviation (SD) that obtained from a linear regression line performed using organic acid solution prepared at lower

concentration levels between 5µg/mL and 40µg/mL, is being one of the three approaches described in ICH guidelines [17] for the prediction of LOD and LOQ. The formula used for the prediction of LOD and LOQ were 3.3× STEYX/ S and 10× STEYX/S respectively. The solutions were prepared at the predicted concentration of LOD and LOQ levels, and analyzed for six times, and the percentage relative standard deviations were tabulated in Table.1. The representative electropherogram obtained from the LOQ and LOQ concentrations are shown in the Fig 3.

Precision (system precision, method precision and intermediate precision)

System precision/repeatability was demonstrated by analyzing six replicate injections of organic acid standard solution of formic acid, succinic acid, trifluoroacetic acid, methanesulfonic acid, acetic acid and pivalic acid as per procedure. The percentage relative standard deviation of six replicate injections of each acid performed on six different days was found to be less than 3.7.

Repeatability of the test method (method precision) was demonstrated by analyzing six separate sample solution prepared using single batch of Cefazolin sodium, which was spiked with each of 0.05%w/w of AA and PIA and Lopinavir drug substance was spiked with FA, TFA and MSA at about 0.05%w/w. The lamivudine drug substances and Atorvastatin calcium was spiked with MSA at about 0.1%w/w. since these organic acids are not detected in the samples and SA and AA were detected in the sample well above the LOQ level in Lamivudine and Atorvastatin calcium drug substances respectively. The percentage relative standard deviation of FA, SA, TFA, MSA, AA and PIA content in six sample preparations were found to be less than 6.1. The electropherogram obtained with Atorvastatin Calcium, Cefazolin, Lamivudine and Lopinavir drug substance with appropriate organic acids are shown in the Fig 4a, 4b, 4c and 4d respectively.

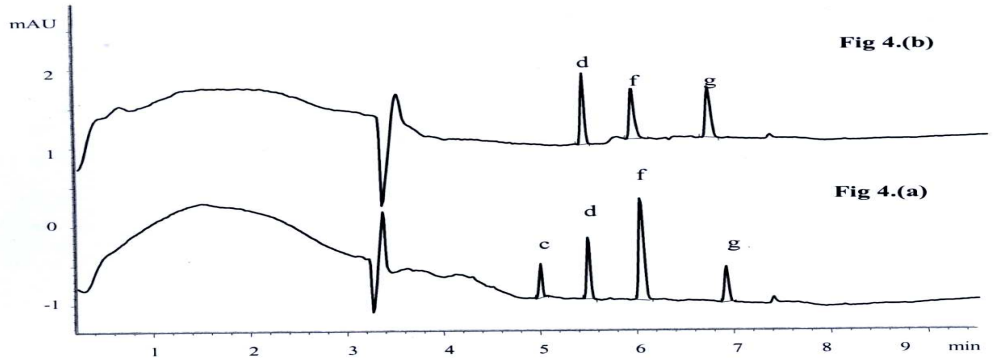

Fig 4a&4b: Electropherogram obtained from spiking corresponding organic acids at target level to (a) Atorvastatin Calcium (b) Cefazolin drug substance

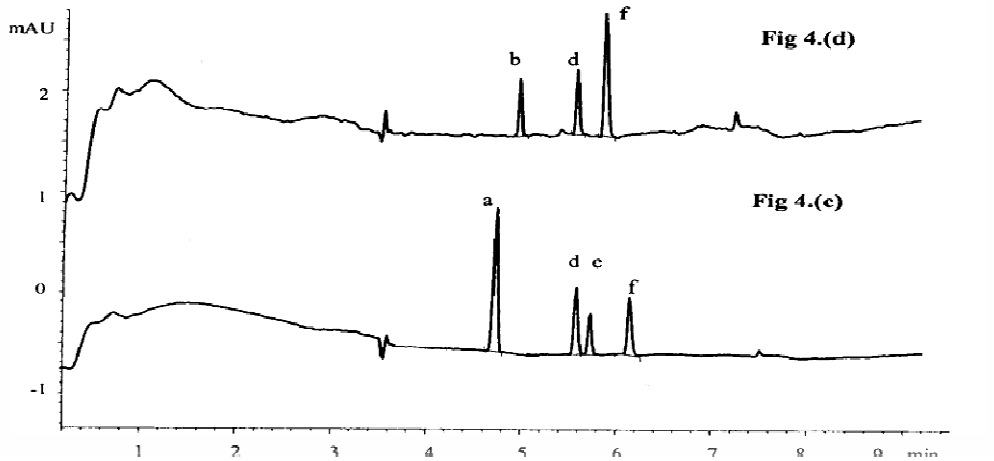

Fig 4c&4d: Electropherogram obtained from spiking corresponding organic acids at target level to (c) Lopinavir (d) Lamivudine drug substance.

Intermediate precision/reproducibility of the test method was demonstrated by analyzing six separate sample solution prepared using single batch of Cefazolin, Lopinavir, Lamivudine and Atorvastatin calcium drug substances and same amount of respective organic acids were spiked in the drug substances (that used for method precision), and the sample analysis were employed by different analyst, on different day with another lot of column. The percentage relative standard deviations were found to less than 5.5, and the results are summarized in Table 2.

Table 2 : Experimental summary data from precision

SYSTEM PRECISION (Repeatability) *Area ratio of PRA with*					
S.No/Statistics	**FA**	**AA**	**SA**	**TFA**	**PIA**
1	1.234	0.827	1.113	0.758	0.579
2	1.210	0.825	1.092	0.724	0.603
3	1.208	0.802	1.133	0.779	0.584
4	1.220	0.828	1.043	0.814	0.627
5	1.185	0.805	1.008	0.753	0.609
6	1.181	0.845	1.134	0.793	0.594
Average	1.206	0.822	1.087	0.770	0.599
SD	0.02	0.02	0.05	0.03	0.02
%RSD	1.7	1.9	4.7	4.2	3.0
METHOD PRECISION (Reproducibility) *in %w/w*					
Drug substance	**Org. acid**	**Average**	**SD**	**%RSD**	**95%CI (±)**
Atorvastatin Calcium	AA	0.17	0.009	5.3	0.007
	MSA	0.198	0.007	3.5	0.009
	PIA	0.100	0.004	4.0	0.004
Cefazolin	AA	0.05	0.001	3.3	0.001
	PIA	0.047	0.001	5.9	0.001
Lamivudine	SA	0.319	0.011	3.5	0.013
	MSA	0.096	0.002	2.1	0.002
Lopinavir	FA	0.056	0.002	3.6	0.002
	MSA	0.049	0.003	6.1	0.003
	SA	0.051	0.001	2.0	0.001
INTERMEDIATE PRECISION (Reproducibility) *in %w/w*					
Drug substance	**Org. acid**	**Average**	**SD**	**%RSD**	**95%CI (±)**
Atorvastatin Calcium	AA	0.168	0.006	3.6	0.006
	MSA	0.186	0.003	1.6	0.003
	PIA	0.101	0.003	3.0	0.003
Cefazolin	AA	0.053	0.001	3.0	0.001
	PIA	0.049	0.001	5.3	0.001
Lamivudine	SA	0.311	0.014	4.5	0.016
	MSA	0.091	0.003	3.3	0.003
Lopinavir	FA	0.055	0.003	5.5	0.003
	MSA	0.056	0.001	1.8	0.001
	SA	0.047	0.001	2.2	0.001

SD stands for stanadard deviation, 95%CI stands for 95% confidence interval
MR, R and SD represent Mean % recovery, % recovery and Standard deviation respectively.

Accuracy

The accuracy of the method was verified by preparing sample solution spiked with known amount of organic acid at three different levels. The PIA and AA in Cefazolin sodium and TFA, MSA and FA in Lopinavir were spiked at about LOQ, 0.05%w/w and 0.075%w/w level. Similarly in Lamivudine sample was spiked with SA and MSA with about 0.05% 0.1% and 0.15%. In case of Atorvastatin Calcium, sample was spiked with AA at about 0.15%, 0.3% and 0.45% and MSA was a spiked with about 0.1%, 0.2% and 0.3%. Each concentration levels were prepared in triplicate and analyzed as per the method. The percentage recoveries of the individual organic acids were evaluated and it lies between 85% and 113% and results are summarized in the Table 3.

Robustness

A systematic variation of the method parameter was employed for robustness studies. The investigated parameters are temperature, pH and applied voltage, buffer concentration, and flow modifier concentration (about 2-10% as per the method parameter) and evaluate the resolution between organic acid peaks. The variation of pH is shows remarkable decrease and increase in the resolution of the peaks for trifluoroacetic acid and acetic acid. The concentration of the CTAB is also playing an important role in the separation of the trifluoroacetic acid and acetic acid.

Table 3: Accuracy experimental data for organic acids with different drug substances

Drug substance	Organic acid	Level^	Amount %w/w		Statistical data^^			Overall statistical data		
			Added	Found	MR	SD	%RSD	R	SD	%RSD
Atorvastatin Calcium	AA	L1	0.149	0.152	102.4	3.25	3.2	101.7	2.08	2.0
		L2	0.298	0.312	102.9	1.94	1.9			
		L3	0.446	0.446	99.8	1.04	1.0			
	MSA	L1	0.104	0.098	94.0	6.06	6.4	92.7	2.87	3.1
		L2	0.209	0.197	92.9	1.20	1.3			
		L3	0.313	0.285	91.3	1.30	1.5			
	PIA	L1	0.052	0.048	92.9	2.17	2.3	96.7	2.09	2.2
		L2	0.102	0.101	99.0	2.00	2.0			
		L3	0.151	0.149	98.3	2.10	2.2			
Cefazolin	AA	L1	0.029	0.030	103.5	0.23	0.2	102.1	1.97	1.9
		L2	0.050	0.051	101.3	3.01	3.0			
		L3	0.071	0.072	101.4	1.40	1.4			
	PIA	L1	0.027	0.027	101.2	4.33	4.3	99.8	4.56	4.6
		L2	0.046	0.045	97.1	6.21	6.4			
		L3	0.076	0.076	101.0	3.18	3.1			
Lamivudine	MSA	L1	0.050	0.048	96.7	3.06	3.2	96.8	6.32	6.5
		L2	0.100	0.093	93.3	1.53	1.6			
		L3	0.150	0.143	94.7	3.06	3.2			
	SA	L1	0.250	0.224	90.9	2.37	2.6	94.9	2.71	2.9
		L2	0.499	0.511	104.0	3.78	3.6			
		L3	0.747	0.704	95.5	2.71	2.8			
Lopinavir	FA	L1	0.016	0.018	110.4	3.58	3.2	107.3	5.17	4.8
		L2	0.051	0.055	108.5	4.50	4.1			
		L3	0.076	0.078	103.1	5.47	5.3			
	MSA	L1	0.020	0.018	88.3	5.77	6.5	93.6	6.46	6.9
		L2	0.050	0.040	89.3	4.16	4.7			
		L3	0.080	0.070	96.9	5.41	5.6			
	TFA	L1	0.029	0.027	94.3	9.08	9.2	91.5	6.04	6.6
		L2	0.052	0.048	91.7	5.54	6.0			
		L3	0.078	0.074	94.9	2.19	2.3			

^ L1,L2 and L3 represent target level concentration of 50%, 100% and 150% respectively.

^^ Average experimental result from n=3 determination at each level

MR, R and SD represent Mean % recovery, % recovery and Standard deviation respectively.

CONCLUSION

The level of organic acid in drug substance is to be controlled/ monitored during routine as well as during stability storage analysis to conform the desired purity of active moiety. This optimized CE is simple and uses lesser reagents, shorter acquisition time, very sensitive and accurate. The results obtained from validation experiments prove that the CE method used to determine the content of organic acid in drug substance is selective, sensitive, linear, precise and accurate. Hence, this optimized CE method is suitable and reliable to determine the content of short chain organic acid in various drug substances during routine as well as during stability storage analysis.

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