



## Research Article

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## Validation of UV-HPLC method for simultaneous quantification of organic acids in disinfectants for haemodialysis machines

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### Abstract

A UV-HPLC method was validated for quantification of citric, lactic and malic acids in disinfectants. The chromatographic separation was performed at 40°C by using mobile phase comprised a mixture (90:10) of potassium phosphate monobasic (50mM) and acetonitrile and pH was adjusted to 2.8 with ortho phosphoric acid, on C<sub>18</sub>-5µm and 100<sup>0</sup>A pore size reverse phase column (250mm×4.6mm) at λ=210 nm using UV-Vis detector with 1.25ml/min flow rate. ICH guidelines were adopted to perform the validation studies that the analytical method's characteristics are reliable throughout the time during analysis. Validation parameters showed efficiency and adequate linearity with correlation coefficient (r<sup>2</sup>) 0.9997, 0.9998 and 0.9997 for 20-80 µg/ml citric acid, 2-8µg/ml lactic acid and malic acid respectively. The repeatability and intermediate precision (%RSD<2) indicated the method reproducibility. The limits of detection (LOD) 1.93µg/ml, 0.11µg/ml, 0.21 µg/ml and the limit of quantification (LOQ) were 6.46µg/ml, 0.34µg/ml and 0.68µg/ml for citric acid, lactic acid and malic acid respectively. % Recovery ± SD for citric, lactic and malic acids was 98.22±0.24 - 100.3±0.03, 98.26±0.03 - 101.26±0.03 and 97.42±0.03 - 100.5±0.07 respectively for three spikes levels.

**Key words:** Organic acids, HPLC, Disinfectants, Haemodialysis

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### 1. Introduction

The substances which are employed for prevention, control and destruction of microbes for example fungi, bacteria and viruses are called disinfectants [1]. Uremic patients are at high risk of acquiring a biofilm related illness. During chronic haemodialysis infections with related inflammatory events activation takes place not only from vascular access but also from dialysis apparatus [2]. Biofilm has been detected in hydraulic circuit of haemodialysis machines by which dialysis monitors are at risk of contamination by bacteria and endotoxins [3,4]. Broad spectrums of bacteria are killed by the rapid action of organic acids and work effectively over a wide range of temperature [5]. The acidic

pH of environment destroyed the nucleic acid bond and proteins become precipitated which is detrimental to many microorganisms [6]. Reverse phase HPLC method has become popular, developed and gained importance for determination of organic acids in various type of mixtures like wine, root exudates, soils, fruits, juices, fast foods and acid based biodegradable polymers using  $\text{KH}_2\text{PO}_4$  phosphate buffer (5-50mM) combination separately with methanol (0-10%v/v) and acetonitrile (1%v/v) mobile phase at pH 2.4-3.3 and detection wavelength 200-240nm at room temperature. The detection limits for citric, lactic and malic acids were reported 3-330  $\mu\text{g/ml}$ , 2.5-419  $\mu\text{g/ml}$  and 2-155  $\mu\text{g/ml}$  respectively [7-13]. Method validations ensure the accuracy, precision, reproducibility and robustness of analytical methodology over the specified concentration of analyte present during normal use and provide the document evidence of its intended purpose [14]. The aim of this study was to validate a simple and expeditious UV-HPLC method for the quantification of organic acids (citric acid, lactic acid and malic acid) in disinfectant formulations used for disinfection of haemodialysis machines under the framework provision by International Conference on Harmonization (ICH).

## 2. Materials and Methods

### Reagents and Chemicals

Citric acid (Merck, Germany), Malic acid (BDH, UK), Lactic acid (Thermo Fischer, UK), Phosphoric acid (Merck, Germany), Potassium phosphate monobasic (Merck, Germany) Acetonitrile (Sigma Aldrich USA). Deionized water with a specific resistivity of 18 M $\Omega$  cm was prepared in house using Milli-Q water purification system (Millipore, USA).

### Instrumentation

The chromatographic system used for assay validation was Shimadzu HPLC system (Kyoto, Japan) consisting of a Shimadzu DGU-4A degasser, LC-20AD pump. A Rheodyne manual syringe-loading valve injector fitted with 20 $\mu\text{L}$  loop, SPD20A UV-Visible detector and CTO-20A column oven maintained at 40  $^\circ\text{C}$  were used. All the chromatograms were analyzed at a single wavelength of 210nm and data acquisition was performed using a chromatography software package LC solution.

### Chromatographic Conditions

Chromatography was performed at 40 $^\circ\text{C}$  temperature on Promosil and Purospher Star column (for robustness studies) C<sub>18</sub>-5 $\mu\text{m}$  and 100 $^\circ\text{A}$  pore size reversed phase column (250 mm x 4.6 mm). The mobile phase was 50mM  $\text{KH}_2\text{PO}_4$  with pH= 2.8 made by addition of ortho-phosphoric acid. Elution was carried out isocratically of  $\text{KH}_2\text{PO}_4$  and aceto nitrile (90:10) at a flow rate of 1.25ml/min and prior to use ultra-sonication for mobile phase degassing was done. The UV detection wavelength was set at 210nm and the injection volume was 20 $\mu\text{l}$ .

**Standards preparation:** Citric acid (1.0 g), Malic acid (1.0 g), Lactic acid (1.0 g) was diluted to 1000 ml of deionized water to form stock solution. Standard working solution (20-100 $\mu\text{g/ml}$  for citric acid and 2-10 $\mu\text{g/ml}$  for lactic acid and malic acid) were prepared by serial dilution of stock standard solution with mobile phase.

### Sample preparation

Each disinfectant was diluted with mobile phase (50mM  $\text{KH}_2\text{PO}_4$  and acetonitrile, 90:10) prior to HPLC analysis final dilution was filtered using 0.45 $\mu\text{m}$  nylon filter.

### Optimization of Method

Optimization of HPLC system was done for short analysis time, good separation (high peak resolution) and high sensitivity. For quantification of three organic acids (lactic, citric and malic acids) in disinfectant solution with high sensitivity, a suitable wavelength was investigated by using spectrophotometric system. The standard solution of each acid at pH 2.8 was prepared in the mobile phase. An absorption spectrum in range of 190–380 nm of each solution was recorded. The detection wavelength of 210 nm was chosen because the maximum absorption of all the studied acids was obtained. For reproducible results in separation of mixture of organic acids by UV-HPLC, use of mobile phase with appropriate pH has prime importance. 25 to 50mM phosphate buffer in 2.5–2.8 pH range was most appropriate for the separation of organic acids in reverse phase applications because organic acids above this pH are in un-dissociated form. We performed the separation of organic acids in above mentioned buffer pH range but the good separation was achieved at pH=2.8 due to their lower pKa values. Mobile phase with high concentration of phosphate (50mM) was used because acidic compounds may deposit on the silica packing due to low pH. So for method robustness the separation of organic acids in a mixture should be done at mobile phase pH which did not affect the retention of analyte in reverse phase column.

### Method validation

The validation of optimized method was carried out according to procedures methodology by ICH (International Conference on Harmonization). The UV-HPLC method was validated for specificity, precision, accuracy and recovery, linearity and calibration curve, range, limit of detection and limit of quantitation and robustness. Before the validation of UV-HPLC method for quantification of organic acids, system suitability tests were performed.

### System suitability

Capacity factor ( $k' > 2$ ), tailing factor ( $T \leq 2$ ), resolution ( $R_s > 2$ ), injection precision by peak area measurements ( $\% \text{RSD} \leq 1$  for  $n \geq 5$ ) and theoretical plates ( $N > 2000$ ) determined the suitability of chromatographic system for quantification of organic acids in disinfectants as per ICH guidelines.

**Specificity**

Unequivocally assessment of analyte in the presence of matrix components such as impurities and degradation products determine specificity for analytical method. Reputable authorities like IUPAC, AOAC and ISO/IEC define the term selectivity rather than specificity. The specificity of optimized UV-HPLC method was assessed by comparing the chromatographic peaks of mixture of organic acids with individual acid whether the peaks are pure or not within the chromatogram of sample.

**Precision**

The precision of analytical method was assessed by three different concentration of citric acid, lactic acid and malic acid covered the complete specified range. Repeatability (intra-assay precision) was performed over short interval of time under same operating conditions on same day. Intermediate precision (inter-assay) was determined within the laboratory on three different days for verification of optimized method results. Intra-assay and inter-assay was statistically analyzed set  $\alpha=0.05$  by applying two-way analysis of variance (ANOVA). The acceptable precision for inter and intra-assay is  $\leq 2\%$ .

**Accuracy and Recovery**

The accuracy of analytical method was evaluated by analyzing the known concentration of citric acid, lactic acid and malic acid and compared the true value with the measured value because it determined the effectiveness of sample preparation. The results of accuracy were explained by recovery studies and it can be used for correction of final results for different concentration. The acceptable recovery is 95-105%.

**Linearity and Calibration curve**

The test results either directly or well-defined mathematical transformation within a range determined the linearity of analytical procedure. Six injections each of five standards in 80-120 percent concentration span of target concentration range was applied for determination of linearity. Calibration curve was obtained by plotting the peak area of individual acid against its concentration. For accuracy purpose linearity curve's parameters like correlation coefficient ( $r^2$ ), y-intercept, slope of the regression line and residual sum of squares was reported.

**Range**

20-100 $\mu\text{g/ml}$  for citric acid, 2-10  $\mu\text{g/ml}$  for lactic acid and malic acid was demonstrated for accuracy, precision and linearity of analytical procedure. The range was in 80-120 percent of specified test concentration of citric acid, lactic acid and malic acid in disinfectants samples.

**Limit of Detection (LOD) and Limit of Quantitation (LOQ)**

LOD and LOQ were determined by standard deviation of the response based on the slope of the calibration curve by six injections of five samples each of citric acid, lactic acid and malic acid under the optimized chromatographic conditions.

Limit of detection (LOD) =  $yB + 3sB$

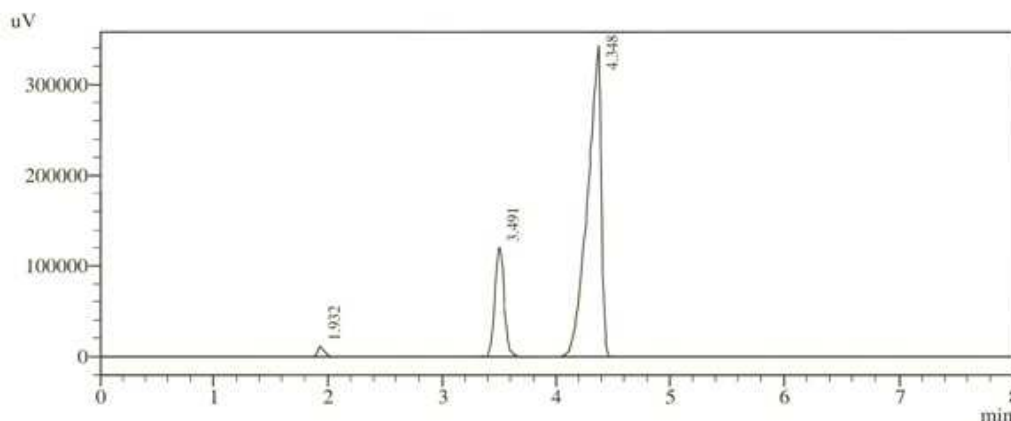
Limit of quantitation (LOQ) =  $yB + 10sB$

Where  $yB$  is intercepts of regression line and  $sB$  is standard deviation of intercepts of regression line [15].

**Robustness**

The study of robustness was carried out to evaluate the influence by slight changes in chromatographic conditions. The factors chosen for this study were the flow rate (ml/ min), pH of mobile phase, mobile phase concentration (mM), wavelength (nm), column temperature ( $^{\circ}\text{C}$ ) and different column, by giving five replicate injections of standards of citric acid, lactic acid and malic acid.

### 3. Results and Discussion



**Figure 1: Chromatogram for citric acid, lactic acid and malic acid**

System suitability test was performed by injecting five times of standard solution (5 $\mu\text{g/mL}$ ) each of citric acid, malic acid and lactic acid into the HPLC system. The values of system suitability parameters were compliance with ICH guidelines which indicated that chromatographic system was suitable for determination of organic acids (citric acid,

lactic acid and malic acid) in disinfectants in aqueous phase. The identity of the peak corresponding in retention time to that of citric acid, lactic acid and malic acid in samples were matched to reference standards which indicated the purity of chromatographic peak shown in figure 01.

The peak correlation of reference standard with sample indicated the specificity of method. In addition, the retention times for the different concentrations of three acids (citric acid 1.93 min, lactic acid 3.49 min. and malic acid 4.35 min.) remain the same throughout used for the calibration. The results of system suitability tests and specificity are represented in table 1.

**Table 1: System Suitability test results and Specificity**

Components	Retention time ( $t_R$ ) Mean $\pm$ SD	Peak Area %RSD for n=5	Capacity factor (k')	Tailing factor (T)	Resolution (Rs)	Theoretical plates (N)
Citric acid	1.931 $\pm$ 0.003	0.27	2.39	0.98	--	6730
Lactic acid	3.491 $\pm$ 0.003	0.16	5.12	0.92	4.27	7265
Malic acid	4.348 $\pm$ 0.026	0.14	6.65	0.89	5.44	5739

The repeatability and intermediate precision of analytical method was evaluated by nine replication of which covered complete specified range for citric acid (20-100 $\mu$ g/ml), lactic acid and malic acid (2-10 $\mu$ g/ml) three times during the day and for three consecutive days respectively. The RSDs for repeatability and intermediate precision were in the range of 0.06 to .15 % and 0.15 to 0.55% for citric acid, 0.21 to 1.25 % and 0.44 to 1.28 % for lactic acid, 0.31 to 1.05 and 0.15 to 1.28 for malic acid respectively which indicated that assay was precise. The accuracy of the method was evaluated by the recovery studies of citric acid, lactic acid and malic acid sample solutions spiked at concentration levels of 80%, 100% and 120% in five replicates. The accuracy in terms of recovery for three different spike concentrations of citric acid, lactic acid and malic acid into sample was 98.22 $\pm$ 0.24 - 100.3 $\pm$ 0.03, 98.26 $\pm$ 0.03 - 101.26 $\pm$ 0.03 and 97.42 $\pm$ 0.03 - 100.5 $\pm$ 0.07 respectively for three consecutive days which were in required range for compliance of assay as per ICH guidelines. The repeatability (intra-assay precision), intermediate precision (inter-assay precision) and recovery data obtained for each level are summarized in table 2.

**Table 2: Repeatability, Intermediate precision and Recovery for citric acid, lactic acid and malic acid**

Repeatability Standard concentration	Intermediate precision		Spiked level of Concentration (%)	%Recovery $\pm$ SD			
	Mean(n=3)	%RSD					
Citric acid ( $\mu$ g/ml )	20	19.9767	0.15	19.8700	0.55	80	100.3 $\pm$ 0.03
	60	59.9533	0.14	59.8533	0.16	100	98.22 $\pm$ 0.24
	100	100.0733	0.06	99.7033	0.14	120	99.80 $\pm$ 0.04
Lactic acid ( $\mu$ g/ml )	2	2.0067	1.25	1.96	1.28	80	98.26 $\pm$ 0.03
	6	5.9667	1.21	6.000	0.44	100	101.26 $\pm$ 0.03
	10	9.9967	0.21	9.943	0.46	120	99.13 $\pm$ 0.06
Malic acid ( $\mu$ g/ml )	2	1.986	1.05	1.96	1.28	80	97.42 $\pm$ 0.03
	6	5.9867	0.35	5.9467	0.48	100	100.5 $\pm$ 0.07
	10	10.0033	0.31	9.923	0.15	120	99.50 $\pm$ 0.04

The linearity of the method was performed by a series of six injections of five standards of citric acid (20-100 $\mu$ g/ml), lactic acid and malic acid (2-10 $\mu$ g/ml) to verify the reproducibility of the detector response at each concentration level. The concentrations of analytes were calculated from the simple linear equation using regression analysis of calibration standard. The plots of peak area of each sample against respective concentration of citric acid, lactic acid and malic acid were found to be linear. The range suitable for linearity, accurate and precise analysis of citric acid, lactic acid and malic acid in disinfectants for haemodialysis machines which covered the concentration span of 80-120 percent, calibration curve's parameters, limit of detection and limit of quantitation are summarized in table 3.

**Table 3: Linearity, Calibration curve parameters, LOD and LOQ of Citric acid, Lactic acid and Malic acid**

Components	Concentration $\mu$ g/ml	R <sup>2</sup>	Slop	y-intercept	Regression equation	Residual sum of squares	LOD $\mu$ g/ml	LOQ $\mu$ g/ml
Citric acid	20-100	0.9997	1910.3	956.3	y = 1910.3x + 956.3	2891000	1.93	6.45
Lactic acid	2-10	0.9998	301769	33300	y = 301769x + 33300	3.2E + 008	0.11	0.37
Malic acid	2-10	0.9997	489290	29348	y = 489290x + 29348	3.3E + 009	0.21	0.68

The lower limit of detection 1.93, 0.11, 0.21 and limit of quantitation 6.45, 0.37, 0.68 for citric acid, lactic acid and malic acid respectively indicated a good sensitivity of the method. Experimental design matrixes for the robustness

study with chromatographic conditions like flow rate ( $\pm 10\%$ ), pH of mobile phase ( $\pm 0.05$ ), mobile phase concentration ( $\pm 2\%$ ), detection wavelength ( $\pm 2\text{nm}$ ), temperature ( $\pm 5^\circ\text{C}$ ) and with different columns (Promosil and Purospher Star) were performed for citric acid, lactic acid and malic acid in both disinfectants. Five samples of each disinfectant were analyzed and expressed as the percentage amount of assay value. By varying the above mentioned parameters, no significant change in results of both the disinfectants was observed which indicated the method was robust. The results of assay are presented in table 4.

**Table 4: Assay results for citric acid, lactic acid and malic acid in disinfectants**

Column	Disinfectant	Contents	Mean $\pm$ SD (n=5)	%RSD
Promosil	Citrosteril FMC, Germany	Citric acid	21.10 $\pm$ 0.023	0.09
		Lactic acid	2.34 $\pm$ 0.028	0.97
		Malic acid	2.09 $\pm$ 0.030	0.75
	RenaX Nephro-aid, Pakistan	Citric acid	22.08 $\pm$ 0.023	0.08
		Lactic acid	3.08 $\pm$ 0.032	0.84
		Malic acid	2.09 $\pm$ 0.029	1.10
Purospher Star	Citrosteril FMC, Germany	Citric acid	21.08 $\pm$ 0.021	0.08
		Lactic acid	2.32 $\pm$ 0.029	0.99
		Malic acid	2.11 $\pm$ 0.032	0.78
	RenaX Nephro-aid, Pakistan	Citric acid	22.12 $\pm$ 0.022	0.08
		Lactic acid	3.06 $\pm$ 0.032	0.82
		Malic acid	2.06 $\pm$ 0.028	1.09

#### 4. Conclusions

A relatively simple reverse phase UV-HPLC method was optimized and validated with system suitability for the simultaneous determination of the three organic acids in a disinfectant in aqueous form. The method validation according to the ICH guidelines was specific for quantification of disinfectant formulation containing organic acids (citric acid, lactic acid and malic acid). The method was linear over the proposed range (20-100 $\mu\text{g/ml}$  for citric acid, 2-10 $\mu\text{g/ml}$  for lactic acid and malic acid) with a correlation coefficient 0.9997, 0.9998 and 0.9997 for citric acid, lactic acid and malic acid respectively. The data validation showed that the UV-HPLC method was accurate and precise from P-value > 0.05 indicate no significant differences in the results between days and robust regarding the flow rate, mobile phase concentration, mobile phase pH, detection wavelength, column temperature and columns of different brands. The major advantage of the validated method is that three different organic acids can be determined on a single chromatographic run with the same detection wavelength simultaneously.

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