Development and validation of a copper ligand-exchange chromatographic method for the estimation of D-lactic acid in Ringer-lactate solution

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Abstract

In this study, the chromatographic conditions for separation and determination of L- and D-lactic acid enantiomers by copper ligand exchange chromatography have been examined and optimized statistically using a response surface methodology (RSM). The chromatographic variables: copper sulfate, acetic acid and organic modifier were screened by operating a 2-level full factorial design (FFD). The significant effect of independent chromatographic variables was analyzed using the analysis of variance (ANOVA). Variables proved significant (p < 0.05) were cautiously tuned using RSM with a face-centered central composite design. Moreover, a D-optimality design was employed to minimize the variation in the regression coefficients of the fitted model. The proposed model represented an excellent example of fulfilling the efficiency of factorial designs in optimizing the chromatographic conditions and maximizing the output. The chromatographic separation was achieved on Supelco Astec CLC-D chiral bidentate ligand (5.0 µm, 150.0 × 4.6 mm). The isotropic mobile phase composition was 7 mM anhydrous copper sulfate in 1.0 mM acetic acid containing 4% methanol. A photodiode array detector was used to determine the optimal detection wavelength, which was at 236 nm. A linear calibration curve was obtained in the range of 30.0–3600 µg mL⁻¹ with a high value for the coefficient of determination (R² ≥ 0.999). The optimized method has been successfully applied to the determination of lactate in the commercial Ringer-lactate solution for injection. The results obtained were in excellent agreement with the label claim with no interference from other additives commonly co-formulated with the drug. Intra- and inter-day precision, detection and quantification limits, as well as percent coefficient of variation, have been estimated according to ICH guidelines for assessment of analytical procedures.

1. Introduction

Design of experiments (DOE) is a process in which reliable information can be systematically extracted by well-designed study plans. Recently, several researchers have proven that application of DOE in chromatographic separation [1] has many advantages in optimization and development of a robust and rugged method [2]. The attractiveness of DOE generates from the fact that it requires considerably less investment, effort and resources for optimization than those of the univariate procedures [3]. DOE experiments can be differentiated according to the objective of the experiment into screening and optimization designs. The number of executed experiments in the screening designs is much lower than that in the optimization one. Response surface methodology is a powerful and efficient tool for the development and optimization of analytical methods and many articles have demonstrated the potential of these chemometric methodologies in enantiomeric separation of pharmaceutical compounds by capillary electrophoresis (CE) [4] and high-performance liquid chromatography (HPLC) [5]. Central composite design (CCD) is one of the fundamental response surface methodologies that are used in the experimental design and in the optimization process studies [6].

In the late 1960s to early 1970s, Davankov et al. invented the first reversed phase liquid chromatographic technique based on chiral ligand-exchange model [7]. This technique can separate the enantiomers of the most important classes of underivatized natural and synthetic compounds with chelating moieties (diamines, amino acids, amino
alcohols, small peptides, diols, etc.). The principles of chiral ligand-exchange chromatography (CLEC) have been interpreted in the literature, and the technique has been found to be successful for direct enantiomeric resolution of amino acids, beta-blockers and hydroxy acids by thin-layer chromatography (TLC) [8]. Chiral resolution of lactic acid has been conducted by HPLC with chiral ligand-exchange phases [9]. It has been reported that direct chiral resolution of lactic acid was possible by CE using 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) [10]. A monoclonal anti-d-hydroxy acid antibody was used as chiral selector for chromatographic enantiomer separation and quantification of lactic acid contained in human urine samples [11]. The teicoplanin based stationary phase has been used for chiral separation of lactic acid enantiomers in urine by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [12]. Recently, the capacity of quinine- and quinidine-derived chiral stationary phases to perform the enantioseparation of eight chiral hydroxy carboxylic acid was evaluated [13]. Diseases due to metabolic disorders may lead to accumulation of short chain organic acids in different body fluids. The produced D-lactic acid from human metabolism is minimal. Under certain pathological conditions, the bacterial flora of the gut liberates D-lactic acid that is absorbed into the blood and transported to different fluids as urine. D-lactic acid cannot be metabolized by mammals and at high concentration, can cause encephalopathy [14]. Therefore, the presence of D-lactic acid in fermented foods is not recommended as it is not readily metabolized and under specific circumstances it may lead to accumulation in the blood [15]. It has also been reported that D-lactic acidosis may lead to neurological impairment and alterations in cardiac rhythm [16]. Since its creation, lactated Ringer's solution has ended up one of the most famous fluids that are used for the treatment of various human and veterinary clinical disorders, including states of hypo-perfusion [17]. The by-products of lactate metabolism counteract acidosis generated from a chemical imbalance that occurs with acute fluid loss or renal failure. However, some evidence has indicated that it is the D-isomer of lactate, which is predominantly responsible for the pro-inflammatory and toxic properties of racemic lactated Ringer’s solution [18]. The clinical magnitude of administering D-lactate in racemic lactated Ringer’s solution continues to remain argued; however, some authors recommend the manufacturers to stop adding D-lactate to fluid formulas [19]. Because of the above findings lactated Ringer’s solution containing only 28 mmol/L of the L-isomer of sodium lactate has become readily available and more widely used as a resuscitation fluid [19]. Sodium lactate in Ringer-lactate solution was assayed in British Pharmacopeia (BP) and in United State Pharmacopeia (USP) by liquid chromatographic methods. However, none of these methods is capable of determining the amount of D-isomer. In this work, the optimization of the separation conditions of the two optical isomers of lactic acid by a factorial design has been studied. An experimental design with three quantitative factors (copper sulfate concentration, the percentage of methanol as an organic modifier, and acetic acid concentration) was evaluated. The method was applied to determine both isomers in Ringer-lactate solution.

2. Experimental

2.1. Materials and reagents

Sodium acetate, acetic acid, sodium hydroxide, ammonium hydroxide, hydrochloric acid (37%) and anhydrous copper sulfate (99.0%) were of analytical grade and were purchased from Sigma–Aldrich (Steinheim, Germany). Methanol and acetonitrile were of HPLC grade and were purchased from Fisher Scientific (Waltham, MA, USA). Glass distilled water was further purified using Milli-Q water purification system (Millipore, Bedford, MA, USA). D-(-)-lactic acid (98.0%) and L- (+)-lactic (99.0) were purchased from Sigma–Aldrich (Steinheim, Germany). Ringer-lactate intra-venous sterile solution, (labeled to contain 0.32% w/v of sodium lactate), was purchased from the Egyptian market.

2.2. Instrumentation and software

The HPLC system consists of a low-pressure gradient preparative system (LC-20A Prominence Liquid Chromatography). FCV-200 AL: a low-pressure gradient unit, a system controller (CBM-20A), gradient mixer 4.5 mL for analytical applications. SPD-M20A: Prominence photo diode array detector. DGU-10B: Helium degassing unit, SIL-10AP autosampler (injection volume: 1–5000 µL). FRC-10A: fraction collector and automatic rinsing pump, all from Shimadzu, (Japan). The analytical-preparative HPLC was controlled remotely using LabSolution LC Workstation Ver. 5.51 Multi LC-PDA (Japan). Minitab® 17.1.0 was used to establish the mathematical models for optimization.

2.3. Preparation of stock and standard solutions

Individual stock solutions of D-(-)-lactic acid and L- (+)-lactic acid (10 mg/mL) were prepared by dissolving 100 mg of D-lactic acid and L- (+)-lactic acid in water and diluting to 10 mL in a volumetric flask. The stock solutions were prepared once a month, kept at 2 – 8 °C in a refrigerator and brought to room temperature before use. The D- and L-lactic acid working solutions were prepared by adding 1 mL of the stock solutions to a 100 mL volumetric flask and diluting to volume with water to obtain the working solution 100 µg mL⁻¹ of each enantiomer. The combined working solutions of D- and L-lactic acid enantiomers in the desired concentration range (30–3600 µg mL⁻¹) were prepared by appropriate dilution of the stock and working solutions with water into 10 mL volumetric flasks. The quality control (QC) samples were prepared from other prepared enantiomers stock solutions.

A stock solution of 0.1 M anhydrous copper sulfate was prepared by dissolving 15.96 g anhydrous CuSO4 in 1 L of de-ionized water. The working solutions of a copper sulfate in the desired concentrations were prepared by appropriate dilution of standard stock solutions with water.

2.4. Liquid chromatographic method

Aliquots of 20.0 µL of enantiomers standard working solution at seven different concentrations (30.0–3600.0 µg mL⁻¹) were injected into the HPLC system. The procedure was carried out in triplicate for each concentration. The enantiomers peak areas obtained were plotted against the corresponding concentrations of each enantiomer (expressed as µg mL⁻¹). Chromatographic separation was achieved on Supelco Astec CLC-D chiral bidentate ligand (5.0 µm, 150.0 × 4.6 mm). The isocratic mobile phase composition was 7 mM anhydrous copper sulfate in 1.0 mM acetic acid containing 4% methanol. Mobile phase was filtered through a 0.45 µm nylon membrane filter, degassed and pumped at a flow rate 1.0 mL min⁻¹. Column equilibrium of at least 1 h was required to get a reproducible data. The detector was set at 200–350 nm and the quantitative measurement was calculated at 236.0 nm.

2.5. Analysis of sodium lactate in Ringer-lactate solution for injection

To 100 mL Ringer lactate solution for injection, add 25 µL hydrochloric acid (37%) to bring the pH to 2.5 ± 0.20. Aliquots of 20.0 µL of this solution were injected into the HPLC system. The procedure was carried out as mentioned under “Section: 2.4.”

2.6. Experimental design

All chromatographic conditions that might affect the anticipated responses (resolution, area under the peak (AUP), tailing at 10%,
number of theoretical plates (NTP), and retention time) are illustrated in the Electronic supplementary data (Table S1). The main goal of experimental designs was to decrease the number of experiments needed while examining the maximum number of factors. The experimental designs, 2-level FFD and face-centered central composite design (FCCD) were generated using Minitab*17 software for screening and optimization phases, respectively. A 2³-FFD (8 runs in the base design, 3 replicates, and 3 centre points in total added to the matrix of design) was developed keeping all factors free from aliasing. The factors, their levels, and the 2³-FFD are presented in Supplementary data (Table S2).

For optimization, FCCD was implemented. A full factorial design with an additional design that has 24 cube points, 18 axial points and 12 centre points in cube was used to generate the full face-centered design. Experiments were run in three replicates and in three base blocks. The factors, their levels, and full face-centered design are presented in Supplementary data (Table S3). The polynomial function was applied to obtain the best response taking into account the linear, 2-way, as well as the quadratic interactions. ANOVA at 95.0% confidence limits was used to validate each model structure.

3. Results and discussion

3.1. Copper ligand exchange chromatography

Astecl CLC columns depend on the copper ligand theory that is illustrated by Davankov and Rogozhin [7] to achieve enantomer separation. The method depends on the formation of transient diastereomeric complexes via the coordination of the copper ions with a chiral bidentate ligand (D-form) on the stationary phase and functional groups on the analytes (Fig. 1). One of the most important advantages of CLEC is its ability to give small acids with no UV chromophore a strong signal at 254 nm [20].

3.2. Experimental design

It is essential to run some preliminary chromatographic experiments before carrying out a design to verify the feasibility of the experimental design. This can be achieved by running two experiments – one with all variables at their lowest settings and another with them all at their highest settings. If the results from the two experiments are close, this suggests that the domain is too narrow and the difference between variables needs to be increased. However, there is another possibility, namely the curvature in the response. This can be verified by running some experiments with all variables set at their mean values. This not only investigates for curvature but also provides information about the reproducibility of responses. Cube plots for the 2-level FFD experiments showing the process variables with their mean corresponding chromatographic responses are shown in Supplementary data. The experiments may have to be blocked. Blocking is necessary for chromatographic applications when many experiments are required to be carried out on more than one day.

3.2.1. Inspection of significant variables (screening experiment – 2³-FFD)

The main target is to execute a minimum number of experiments on a maximum number of factors. Furthermore, the purpose of the screening phase is to determine which one of the chromatographic variables significantly affects the chromatographic responses. The data resulting from the investigation of the effect of three chromatographic variables on the chromatographic responses are presented in Supplementary data (Table S2). The analysis and the interpretation of the results generated from 2³-FFD are illustrated in Supplementary data.

3.2.2. Optimization of significant variables (RSM) - FCCD

In this stage, crucial factors were inserted to Minitab*17 with the purpose of finding the "optimal" chromatographic conditions. Levels of these factors were cautiously chosen to obtain an experimental domain that satisfies the experimental specifications. The FCCD data resulting from the investigation of the effect of three variables on the chromatographic responses are presented in Supplementary data (Table S3).

3.2.3. FCCD for the chromatographic resolution between the two enantiomers

Supplementary data in Table S3 were run through RSM to construct an empirical model for the representation of the resolution of the two enantiomers in terms of copper sulfate concentration, percentage of methanol and acetic acid concentration. Based on a regression analysis at 95% of confidence interval, the lack of fit error and p-values of parameter estimations were found to be significant. This indicates that a model except linear would fit the data better [21]. The quadratic model was used to fit the observed data by least squares analysis and the following empirical model was obtained:

\[
\text{Resolution(USP)} = 3.890 + 0.0389 \text{ Copper Sulfate (mM)} - 0.1888 \text{ Methanol(\%)} - 0.5665 \text{ Acetic Acid (mM)} + 0.09180 \text{ Copper Sulfate (mM)} \times \text{Copper Sulfate(mM)} + 0.01078 \text{ Methanol (\%)} \times \text{Methanol(\%)} + 0.0356 \text{ Acetic Acid (mM)} \times \text{Acetic Acid (mM)} - 0.01162 \text{ Copper Sulfate (mM)} \times \text{Methanol (\%)} - 0.00010 \text{ Copper Sulfate (mM)} \times \text{Acetic Acid (mM)} + 0.00785 \text{ Methanol(\%)} \times \text{Acetic Acid (mM)}
\]

(1)

ANOVA results of the quadratic model presented in Table 1 indicate that the model equation sufficiently describes the response surface of the chromatographic resolution of the two enantiomers in the interval of the investigation. The average chromatographic resolution of the two enantiomers at the design centre, 5 mM copper sulfate, 6% methanol and 3 mM acetic acid was 1.74 ± 0.04 calculated from Eq. (1). The fit of the model was expressed by the determination coefficient R², which was found to be 96.65%. The adjusted R² value is 95.77% indicated that this model did not explain only 4.23% of the total variations. The high value of R² indicates that the quadratic equation is capable of representing the system under the given experimental domain. Predicted R² is a measure of how good the model predicts a response value. The predicted R² of 94.46% is in reasonable agreement with the adjusted R² of 95.77%.
The limit of detection and the limit of quantitation-resolution).

Table 1: Analysis of variance (ANOVA) at 95% confidence level for FCCD (for chromatographic response-resolution).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F-Value</th>
<th>P-Value</th>
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<td>Copper Sulfate (mM)</td>
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<td>0.0465</td>
<td>2.21</td>
<td>0.145</td>
</tr>
<tr>
<td>Methanol (%)</td>
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<td>2.3823</td>
<td>2.3823</td>
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<td>0.000</td>
</tr>
<tr>
<td>Acetic Acid (mM)</td>
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<td>12.7766</td>
<td>12.7766</td>
<td>607.44</td>
<td>0.000</td>
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<tr>
<td>Square</td>
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<td>0.1906</td>
<td>9.06</td>
<td>0.000</td>
</tr>
<tr>
<td>Copper Sulfate (mM) Copper</td>
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<td>0.0021</td>
<td>0.0021</td>
<td>0.10</td>
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<tr>
<td>Sulfate (mM)</td>
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<td>0.0753</td>
<td>0.0753</td>
<td>3.58</td>
<td>0.065</td>
</tr>
<tr>
<td>Methanol (%) Methanol (%)</td>
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<td>0.0753</td>
<td>0.0753</td>
<td>3.58</td>
<td>0.065</td>
</tr>
<tr>
<td>Acetic Acid (mM) Acetic Acid</td>
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<td>0.1622</td>
<td>7.71</td>
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<tr>
<td>(mM)</td>
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<td>0.0000</td>
<td>0.00</td>
<td>0.983</td>
</tr>
<tr>
<td>2-Way Interactions</td>
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<td>0.1053</td>
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<td>0.005</td>
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<tr>
<td>Copper Sulfate (mM) Methanol</td>
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<td>0.2627</td>
<td>0.2627</td>
<td>12.49</td>
<td>0.001</td>
</tr>
<tr>
<td>(%)</td>
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<td>0.0533</td>
<td>0.0533</td>
<td>2.53</td>
<td>0.119</td>
</tr>
<tr>
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<td>0.8834</td>
<td>0.0210</td>
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<td>0.7904</td>
<td>0.1976</td>
<td>80.76</td>
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<tr>
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<td>38</td>
<td>0.0930</td>
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<tr>
<td>Total</td>
<td>53</td>
<td>26.3370</td>
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</tr>
</tbody>
</table>

\(\text{Adj SS} = \text{sum of squares and MS is mean of squares. Significant factors (2-way interactions)} (P-value ≤ 0.05) appear in bold italic.\)

3.2.4. Response optimizer

Many experimental designs include finding out the optimal conditions that will yield the best value for the response. In this study, we want to maximize the resolution between the two enantiomers, AUP, and NTP by varying three input variables: copper sulfate, methanol, and acetic acid. At the same time, we want to minimize the tailing factor and the retention time, which are also affected by the same three variables. Using response optimizer, we defined target goals for resolution, AUP, NTP, tailing factor and retention time. Minitab calculates optimal setting for the input variables along with desirability values to indicate how well those settings achieve the response targets. Response optimization is a method that allows for compromise among the various responses. As shown in Fig. 2, composite desirability evaluates how well a set of input variables fulfills the goals you have defined for the responses. Desirability has a range of zero to one where one represents the ideal case; zero indicates that one or more responses are outside their acceptable limits.

The optimization plot shows the effect of each factor (columns) on the responses or composite desirability (rows). The vertical red lines on the graph represent the current factor settings. The numbers displayed at the top of a column show the current factor level settings (in red). The horizontal blue lines and numbers represent the responses for the current factor level.

The optimization plot, as revealed in Fig. 2, provides the “optimum” solution for the contributing variable combinations. The conditions shown on the Fig. 2 (7 mM copper sulfate, 4% methanol and 1 mM acetic acid) produce the best chromatographic responses.

3.3. Method validation

3.3.1. Linearity

Linear correlation coefficients \((r ≥ 0.999)\) were individually achieved for each lactic acid enantiomer over the concentration range 30.0–3600.0 µg mL\(^{-1}\). Quantitative measurement was calculated at 236 nm rather than at 254 nm; where the chromatographic response area under the peak at 236 nm is larger than that at 254 nm (see Fig. 3). The limit of detection and the limit of quantification of L-lactic acid were 4.78 and 15.94 µg mL\(^{-1}\), respectively. The estimated limits were verified by analyzing a suitable number of samples containing the analyte at the corresponding concentrations.

3.3.2. Accuracy and precision

Table 2 summarizes the intra- and inter-day accuracy and precision for L- and D-lactic acid at four different concentration levels (450, 900, 1500 and 1800 µg mL\(^{-1}\)) on the same day \((n = 3)\) and on consecutive days \((n = 3)\). The percentage CV values of the intra-day precision ranged from 0.49% to 1.85% and from 0.46% to 1.53% for L-lactic acid and D-lactic acid, respectively. While the percentage CV values of the inter-day precision, ranged from 0.75% to 1.15% and from 0.54% to 1.32% for L-lactic acid and D-lactic acid, respectively, proved that the method was sufficiently precise. The intra- and inter-day recoveries ranged from 97.28% to 103.3% for both enantiomers, these results demonstrated that both intra- and inter-day accuracy values were within the acceptance variability limits. The proposed method was successfully applied to the determination of lactate in the commercial Ringer-lactate solution for injection (see Fig. 4d). The detected amount of D-lactic acid was found 6.67%, if it is compared to the estimated L-lactic acid.

3.3.3. Specificity

Specificity was determined by comparing the HPLC-PDA chromatogram results from an analysis of samples lactate containing placebo ingredient (NaCl [0.6% w/v], KCl [0.04% w/v] and CaCl\(_2\).2H\(_2\)O
with those obtained from analysis of samples without placebo ingredients. As shown in Fig. 4a and b, the injected placebo ingredients are well resolved from the peaks of interest. Fig. 4c represents spiked lactic acid enantiomers in the placebo ingredients. By comparing the area under the peaks for each enantiomer in Fig. 4a to that corresponding to it in Fig. 4c, it was found that the area under the peaks was not affected by spiked placebo ingredients, which indicates that the method was specific.

4. Conclusion

A simple, reliable and sensitive HPLC-PDA method was developed and successfully applied for the determination of sodium lactate in Ringer-lactate solution. The proposed method explained the urgent need to estimate the amount of D-lactic acid in Ringer-lactate solution. Factorial design experiments were used to both investigate and optimize all the variables affecting the chromatographic responses. For screening, a 2³-FFD was used. For optimization, a RSM based on a face-centered central composite design was utilized. The results from
optimization procedures, ANOVA testing and desirability functions revealed that the model is adequate and precise. Chromatographic method validation was performed following the ICH guidelines. Results obtained in terms of linearity, sensitivity, accuracy, and precision, further support the validity of the optimized procedure.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2018.06.068.

References