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Enantiomeric purity test of R-(+)-alpha lipoic acid by HPLC using immobilized amylose-based chiral stationary phase

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Abstract: Alpha lipoic acid, an antioxidant, is widely used for treatment of various diseases. It is a racemic mixture, with R-(+)-α lipoic acid exhibiting greater potency, bioavailability, and effectiveness than those of the S-form. Thus, selective R-(+)-α lipoic acid has been recently used in various applications, necessitating the development of a method to test the enantiomeric impurity in R-(+)-α lipoic acid. We developed a simple and fast high-performance liquid chromatography method using a new immobilized amylose-based chiral column (Chiralpak IA-3). Design of experiment was applied to accurately predict the effects and interactions among various factors affecting the analytical parameters and to optimize the chromatographic conditions. This optimized method could completely separate the two enantiomer peaks with a resolution > 1.8 within a short running time (9 min). Then, the optimized method was validated according to the guidelines of the International Conference on Harmonization and applied for quantification of S-(−)-α lipoic acid in some commercial R-(+)-α lipoic acid tromethamine raw material. Our results suggested that the developed method could be used for routine quality control of R-(+)-α lipoic acid products.

Key words: R-(+)-α lipoic acid, High-performance liquid chromatography (HPLC), Enantiomeric purity test, Immobilized chiral stationary phase

1. Introduction

Alpha lipoic acid (ALA) is an antioxidant derived from both plants and animals. The R-enantiomer of ALA is naturally present in prokaryotic and eukaryotic cells,[1] where it plays an important role in the antioxidant defense system of the organisms.[2] Owing to its vital antioxidant properties, the anti-inflammatory effects of ALA have been widely studied;[3] in addition, its potential as a treatment for cardiovascular diseases, diabetes, and hypertension has been investigated.[2,4] Another research suggested that ALA might be beneficial as an anti-obesity and lipid-lowering agent.[5] Therefore, ALA has been added to various dietary supplements.

For chemical structure, alpha lipoic acid has two different enantiomeric forms, the S-(-)-ALA and R-(+)-ALA (*Fig*. 1). R-(+)-ALA is the biologically

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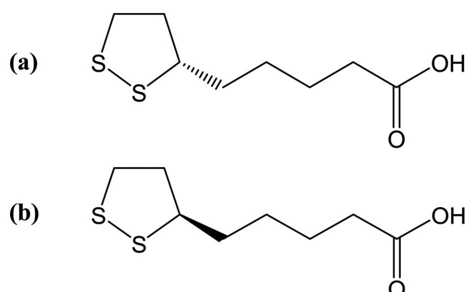

Fig. 1. Chemical structure of alpha-lipoic acid enantiomers. (a) S-(-)-α Lipoic acid; (b) R-(+)-α Lipoic acid

active enantiomer, which has higher bioavailability, potency, and therapeutic efficiency than the S enantiomer.[2,6-9] In some cases, the S-enantiomer was reported to be inactive[10,11] and was even shown to cause mortality.[12] Therefore, the use of enantioselective R-(+)-ALA in supplements and drugs is preferable. The development of a method to determine the enantiomeric purity and quantify S-(-)-ALA impurity in the R-(+)-ALA material is important to ensure the quality of pharmaceutical products. No enantiomeric purity test of R-(+)-ALA is reported in the current pharmacopoeias. Some chiral separation methods have been established using different techniques, such as capillary electrophoresis (CE),[13] liquid chromatography-mass spectrometry (LC-MS),[6,14,15] and high-performance liquid chromatography (HPLC).[16] Using CE, the two enantiomers in the racemic mixture could be partially resolved (resolution [R_s] = 1.2) within 18 min. Therefore, CE is not suitable for optical purity tests. The combination of HPLC and tandem MS (MS/MS) detector brought several advantages, including the possibility of sensitive determination of ALA enantiomers in urine,[14] rat plasma,[6] and racemate of dietary supplements.[15] However, no enantiomeric purity test has been developed for R-(+)-ALA tromethamine raw material. HPLC, a well-known as the most popular analytical method used on the industrial scale, was also used to quantify R-(+)-ALA and S-(-)-ALA in plasma in a previous study. However, the sample preparation involved many complex steps, including liquid-liquid extraction of plasma samples, chemical reduction, and precolumn derivatization with *O*-phthalaldehyde in the presence of *D*-phenylalanine.[16] Besides, the target enantiomer, S-(-)-ALA, was eluted after the main peak, R-(+)-ALA. Thus, if this method was applied to determine the enantiomeric purity of R-(+)-ALA, the small peak of S-(-)-ALA impurity could overlap with and might be masked by the large and broad R-(+)-ALA peak, thus affecting the analysis results.

Optimization is a fundamental process in the development of analytical methods. The conventional method used for optimization is the trial with one-factor-at-a-time (OFAT) concept, which is time-consuming, tedious, and costly. In addition, it might not be able to predict interactions between various factors. Recently, design of experiment (DoE) has been extensively applied for the optimization of analytical methods.[17-19] DoE has the advantages of not only reducing the number of experiments, work, and reagent consumption but also fixing critical and unpredictable errors.[20] However, DoE has not been used for the development of analytical methods for ALA.

In this study, we aimed to develop a new, simple, and convenient analytical method for determination of the enantiomeric purity of R-(+)-ALA tromethamine raw material. A new-generation chiral stationary phase, amylose tris (3,5-dimethylphenylcarbamate) immobilized on 3-μm silica gel, was used. To optimize the chromatographic conditions, DoE was applied Design-Expert 11 software, giving a reliable and robust result.

2. Experimental

2.1. Chemicals and reagents

S-(-)-ALA and R-(+)-ALA standards were purchased from Sigma-Aldrich (Saint Louis, MO, USA). R-(+)-lipoic tromethamine raw material were obtained from Bukwang Pharm. Co., Ltd. and Korea Biochem Pharm. Inc. Glacial acetic acid and formic acid (≥ 99.5 %) were purchased from Daejung (Siheung, Korea). HPLC grade methanol was obtained from Honeywell Burdick & Jackson (B&J – Ulsan, Korea).

2.2. Chromatographic conditions

The developed method was performed using

Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan), including a DGU–20A5R degasser, two LC-20AD pumps, SIL–20A autosampler, CBM-20A communication bus module, SPD-M20A 230V photodiode array (PDA) detector, and CTO-20AC column oven. Agilent 1100 series HPLC system was also used for determination of the intermediate precision.

A Chiralpak IA3 HPLC column (100 × 4.6 mm ID, 3 μm), Chiralpak IA guard column (10 × 4 mm ID, 5 μm; Daicel Corporation), and another Phenomenex C18 guard column (3 × 4 mm ID) were used.

2.3. Sample Preparation

Stock standard solutions of S-(-)-ALA and R-(+)-ALA (1000 μg/mL) were prepared in methanol. Diluted S-(-)-ALA standard solution (50 μg/mL) was prepared using methanol:water mixture (1:1). Standard S-(-)-ALA (10 μg/mL) and sample R-(+)-ALA (500 μg/mL) solutions were also prepared.

2.4. Method development

We performed some preliminary experiments to select a suitable mobile phase composition (type and concentration of organic solvents [methanol, acetonitrile, etc.] and additives [acetic acid, formic acid, etc.]) and guard column for optimization of the chromatographic conditions. Then, various factors, including methanol and acetic acid concentrations, temperature, and flow rate, were optimized easily reliance on DoE software.

2.5. Method validation

The optimized method was validated according to the guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). The validation procedure included:

Specificity: Injection of blank, S-(-)-ALA (10 ppm), R-(+)-ALA (500 ppm), standard mixture, and sample solution to assess the effects of other components, such as the mobile phase and excipients, on the responses of analytes.

System suitability: Repetitive injection of mix standard solution to ensure the stability of the system for the proposed method.

Linearity and limit of detection (LOD)/ limit of quantification (LOQ): Standard solutions of S-(-)-ALA with concentrations ranging from 0.5 to 40 ppm were prepared and analyzed to construct the calibration curve. The LOD and LOQ were determined from the ratio of signal and noise in the chromatograms of the diluted solution.

Precision and accuracy: Intraday, interday, and intermediate precision (different HPLC system) were determined. The accuracy was evaluated using spiked S-(-)-ALA (5, 10, and 15 ppm) into R-ALA tromethamine raw material (500 ppm).

Robustness: The robustness of the proposed method was examined by changing the concentration of methanol (± 2 %), percentage of acetic acid (± 0.05 %), temperature (± 2 °C), and flow rate (± 0.1 mL/min).

2.6. Method application

The developed method was applied to quantify S-(-)-ALA impurity in R-(+)-α lipoic tromethamine raw material from Bukwang Pharm. Co., Ltd. and Korea Biochem Pharm. Inc. The following equation was used to calculate the percentage of S (-) enantiomer:

$$\text{Percentage of S-enantiomer} = (r_U/r_S) \times (C_S/C_U) \times 100$$

Where:

$\mathbf{r_U}$ is the peak response of S-(-)-ALA in the sample solution,

$\mathbf{r_S}$ is the peak area of S-(-)-ALA in the standard solution,

$\mathbf{C_S}$ is the concentration of S-(-)-ALA in the standard solution, and

$\mathbf{C_U}$ is the concentration of R-(+)-ALA in the sample solution.

3. Results and Discussion

3.1. Method development

One of the outstanding points in preliminary experiments is the influence of the guard column on the eluted peak. The original method involved the use of Chiralpak IA guard column (4 mm ID × 10

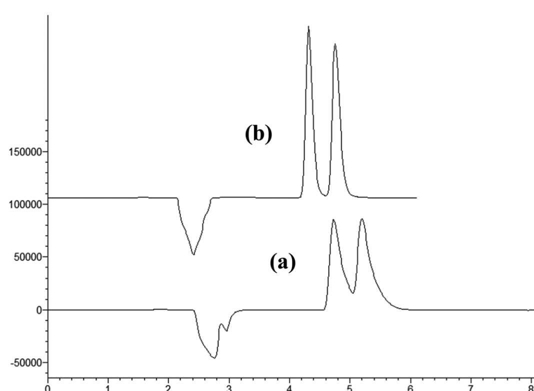

Fig. 2. The difference between using two types of guard column (a) Chiralpak IA (4 mm ID × 10 mmL; 5 μm); (b) Phenomenex C18 (3 × 4 mm ID)

mmL; 5 μm) for protection; however, this resulted in poor sensitivity and peak shape. Therefore, we manipulated the type and pH of the buffer, ratio of organic solvents, additives, and ionic liquids. However, these manipulations did not result in satisfactory results without changing the guard column. The use of Phenomenex C18 guard column (3 × 4 mm ID) significantly improved the technical parameters of chromatogram peaks, such as peak area, peak height, tailing factor, and number of theoretical plates (*Fig.* 2).

Then, screening and optimization of the chromatographic conditions were performed using DoE concept. Five factors, including methanol concentration, additive type (acetic acid and formic acid), additive concentration in the mobile phase, column temperature, and flow rate were selected as screening parameters. The subtype chosen in Design-Expert version 11.1.2.0 was split-plot, in which the additive type and concentration were hard-to-change factors. The responses selected for assessment were the peak height, capacity, tailing factor, number of theoretical plates of the S-(-)-ALA peak, and resolution between the two enantiomer peaks. Analysis of variance (ANOVA) was used to analyze the results of 32 runs (*Table* 1). Additive type was excluded from the vital factors because it did not significantly affect any response ($p > 0.1$ for all responses).

The range of remained factors also was shrunk for optimization step. The design domain for optimization included methanol concentration (75–90 %), additive concentration (0.05–0.2 %), temperature (25–35 °C), and flow rate (0.6–0.8 mL/min). Response surface methodology using I-optimal design with split-plot subtype was used for optimization, including 22 runs divided into 2 blocks. The detailed design and results of each run are shown in *Table* 2. From Box-Cox transformation, the peak height and capacity responses were transformed to the square root and natural log transformations, respectively. The equations for each response based on factors (A: additive concentration, B: methanol percentage, C: Temperature and D: Flow rate) were as follows:

Response 1 – Peak height:

$$\mathrm{Sqrt}\,(H) = 77.986 + 12.934B + 3.996C - 1.295D - 0.407AB - 1.176BC + 0.520CD - 1.038B^2 - 0.669C^2.$$

Response 2 – Capacity:

$$\ln(k') = 0.930 - 0.022A - 0.511B - 0.140C + 0.003D - 0.003AB + 0.036BC + 0.003BD + 0.033B^2.$$

Response 3 – Tailing factor:

$$T_f = 1.324 + 0.046B + 0.029C - 0.030C^2.$$

Table 1. *p*-value obtained by ANOVA analysis in screening step

Responses	Whole-plot			Subplot											
	A	B	AB	C	D	E	AC	AD	AE	BC	BD	BE	CD	CE	DE
H	0.5736	<0.0001	0.0163	<0.0001	<0.0001	0.0002	0.0201	0.1019	0.0004	<0.0001	0.0033	0.0028	0.0391	0.116	0.0013
k'	0.5479	<0.0001	0.1435	<0.0001	<0.0001	0.0206	0.0611	0.1143	0.1885	0.0038	0.1739	0.8914	<0.0001	0.8575	0.8596
T_f	0.5707	0.7206	0.5956	0.0297	0.0137	0.0608	0.4094	0.1182	0.0349	0.0031	0.3388	0.5813	0.0394	0.6029	0.2428
N	0.7252	0.0006	0.965	0.5771	0.2408	0.0077	0.0152	0.8299	0.0665	0.0239	0.1353	0.0901	0.6573	0.4201	0.0109
R_s	0.3596	<0.0001	0.498	<0.0001	<0.0001	0.0020	0.0054	0.4180	0.1547	0.4095	0.0073	0.3995	0.0056	0.1355	0.0026

Responses: H (Peak height), k' (Capacity), T_f (Tailing factor), N (Number of Theoretical Plate), R_s (Resolution). Factors: A (Additive type), B (Additive concentration), C (Methanol ratio), D (Temperature), E (Flow rate).

Table 2. Optimization design and results

Block	Run	Factors				Responses				
		Additive conc. (%)	MeOH (%)	Temperature (°C)	Flow rate (mL/min)	Peak height (uV)	Capacity	Tailing factor	Theoreotical Plate	Resolution
1	1	0.2	80	35	0.7	5499	2.56	1.361	7658	1.803
	2	0.2	75	25	0.6	3369	5.14	1.25	9077	2.128
	3	0.2	90	25	0.7	6907	1.72	1.321	6716	1.494
	4	0.05	85	25	0.8	5864	2.54	1.332	6851	1.661
	5	0.05	75	30	0.7	4028	4.52	1.278	8564	2.018
	6	0.05	85	35	0.6	7410	1.95	1.357	7661	1.512
	7	0.1	90	25	0.6	7647	1.76	1.324	7407	1.508
	8	0.1	85	35	0.7	7153	1.92	1.336	6990	1.501
	9	0.1	85	30	0.7	6689	2.18	1.317	7411	1.649
	10	0.2	90	35	0.6	7936	1.40	1.350	6972	1.227
	11	0.2	85	30	0.8	6023	2.15	1.303	6908	1.607
	12	0.2	75	35	0.8	4211	3.65	1.250	7808	1.940
2	13	0.1	75	35	0.6	4837	3.66	1.264	9264	1.972
	14	0.1	80	30	0.8	5215	3.02	1.336	7138	1.875
	15	0.1	80	25	0.7	4651	3.52	1.237	8217	1.996
	16	0.15	75	25	0.8	3452	5.19	1.257	7507	2.013
	17	0.15	85	30	0.6	7418	2.12	1.374	8005	1.681
	18	0.15	80	30	0.7	5727	3.00	1.324	8102	1.833
	19	0.15	90	35	0.8	8830	1.41	1.385	6029	1.126
	20	0.05	90	30	0.7	8679	1.63	1.338	6698	1.225
	21	0.05	75	35	0.8	4942	3.79	1.261	7817	1.823
	22	0.05	80	25	0.6	5393	3.62	1.243	9045	1.945

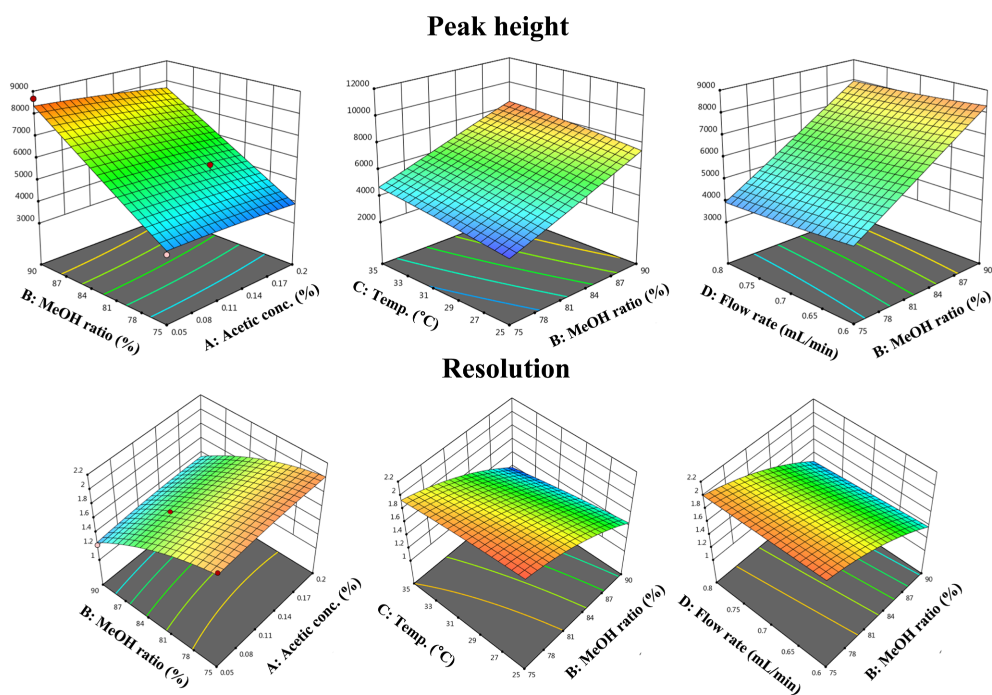

Fig. 3. 3D plots for the interaction influence of vital factors on important responses

Response 4 – Number of theoretical plate:

$N = 7685.28 - 928.4B - 125.296C - 660.995D + 150.516BD.$

Response 5 – Resolution:

$R_s = 1.779 - 0.348B - 0.107C - 0.040D - 0.031BC - 0.097B^2.$

The 3D plots in model graph clearly displayed the interactions among the factors influencing on achieved responses. Among the five selected responses, the height of S-(-)-ALA peak and the resolution between the two enantiomer peaks were the most important responses determining the reliability of the method. The interactions between among the factors affecting on these responses were demonstrated in *Fig*. 3. The conditions of high methanol concentration, low

Table 3. Confirmation method of peak S-enantiomer

Response	Predicted mean	95 % PI low	Data Mean	95 % PI high
Peak height	6458.30	4758.78	6236.15	8405.28
Capacity	2.49	2.46	2.49	2.52
Tailing factor	1.32	1.25	1.31	1.40
Number of theoretical Plate	8319.63	8044.05	8180.67	8595.20
Resolution	1.81	1.73	1.84	1.89

PI: Prediction Interval

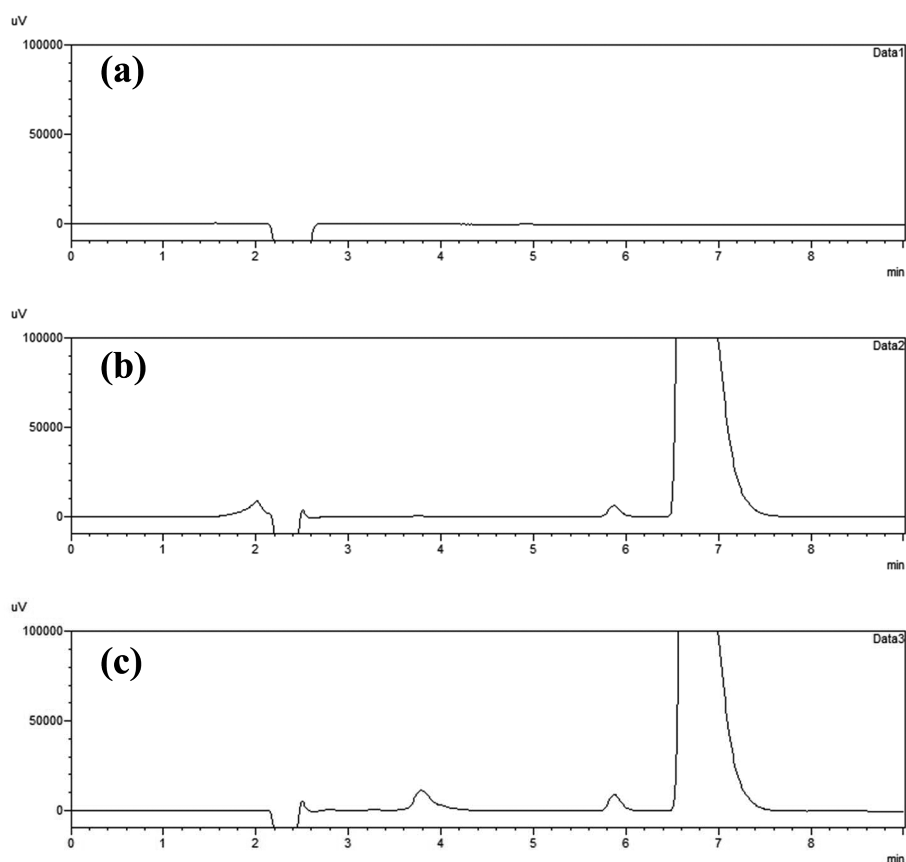

Fig. 4. Chromatogram showing the specificity. (a) Blank: Methanol-Water (1:1), (b) Mix standard solution, (c) Sample solution from material

acetic acid concentration in the mobile phase, high temperature, and low flow rate provided the highest peak height. To achieve a good resolution, lower methanol concentration and temperature would be required.

Finally, the conditions recommended by the software (desirability = 0.752) were applied for a confirmation test (n = 6). A comparison between the predicted and actual values (*Table* 3) verified that the results were close to the predicted values and reached to the goal of experiment with the satisfied parameters.

The final HPLC condition used Chiralpak IA3 column (100 × 4.6 mm ID, 3 µm); and Phenomenex C18 guard column (3 × 4 mm ID) which were put in column oven at temperature 27 °C. Mobile phase contained methanol, water and acetic acid in a ratio of 84 %, 16 %, and 0.1 %, respectively. Flow rate was 0.6 mL/min; injection volume 20 µL. The detection wavelength of the PDA detector to detect lipoic acid was 215 nm.

3.2. Validation

3.2.1. Specificity and system suitability

As illustrated in *Fig.* 4, the biologically inactive or less active enantiomer, S-(-)-ALA, was eluted prior to R-(+)-ALA. This elution order could avoid the overlapping between the small S-(-)-ALA peak and the tail of the large R-(+)-ALA peak. The S-(-)-ALA peak was eluted at 5.758 min and was completely separated from the R-(+)-ALA peak ($R_s > 1.8$).

The system suitability for the developed method was assessed based on the value and relative standard deviation (RSD) of the retention time, peak area, peak height, tailing factor, number of theoretical plates of the S-(-)-ALA peak, and resolution between the 2 enantiomer peaks (*Table* 4).

Table 5. Linearity results

Parameter	S-(-)-α-lipoic acid
Regression equation	y = 13986x + 999.05
Range (µg/mL)	0.5 − 20
Correlation coefficient (R^2)	0.9998
Number of data points	6
Slope ± SD	13986 ± 491.93
Intercept ± SD	999.05 ± 1573.64
LOD (µg/mL)	0.1
LOQ (µg/mL)	0.3

SD: Standard deviation

3.2.2. Linearity and LOD/LOQ

The sensitivity of the developed method was confirmed by calculating the ratio of the S-(-)-ALA signal/noise (LOD = 0.1 µL/mL and LOQ = 0.3 µg/mL [n = 6]).

The linearity of method was evaluated using S-(-)-ALA concentrations ranging from 0.5 to 20 µg/mL. The method showed good linearity along this range with a correlation coefficient (R^2) > 0.9997 for all 6 sets. Statistical parameters of ANOVA ($p = 0.05$) showed the regression linearity of the method (*Table* 5).

3.2.3. Precision and accuracy

For the intraday precision, the recovery of each sample was 98−102 %, and the RSD% of each concentration was < 1 %. The method also exhibited good interday precision with a recovery of 100-102 % and RSD% ≤ 0.64 %. The obtained results of intraday and interday precision are shown in *Table* 6. For intermediate precision, the method was validated using another system (AGILENT 1100 series).

Moreover, the accuracy of the developed method was good with a recovery ranging from 98.77 to 100.64 % and RSD% ≤ 1.33 % (*Table* 6).

Table 4. System suitability data (n=6)

	Retention time	Peak area	Tailing factor	N	Resolution
AVERAGE	5.770	193239	1.351	8098	1.804
RSD%	0.15	0.67	1.75	0.45	0.06

N: Number of Theoretical plate

Table 6. Results of intra-day/inter-day precision and accuracy validation

Conc. (μg/mL)	Intra-day Precision (n=5)		Inter-day Precision (n=3)		Accuracy (n=3)	
	Recovery	RSD%	Recovery	RSD%	Recovery	RSD%
5	99.19	0.99	101.1	0.64	98.77	0.80
10	100.83	0.53	100.34	0.36	100.64	0.49
15	101.87	0.16	101.55	0.27	100.60	1.33

3.2.4. Robustness

The robustness of the method was proven with slight variations in the ratio of methanol (± 2 %), concentration of acetic acid (± 0.05 %), temperature (± 2 °C), and flow rate (± 0.1 mL/min).

Table 7. Content of S-(−)-lipoic acid in R-(+)-lipoic tromethamine raw materials

Manufacturer	Content	RSD%
Korea Biochem Pharm (n=6)	0.901	1.26
Bukwang Company (n=6)	0.972	1.65

3.3. Application

The proposed method was successfully applied on R-(+)-ALA tromethamine raw material. The amount of S-(-)-ALA present in R-(+) lipoic tromethamine raw material obtained from Korea Biochem Pharm. Inc. (*n* = 6) and Bukwang Pharm. Co., Ltd. (*n* = 6) was 0.901 and 0.972 %, respectively (*Table* 7).

Table 8. Comparison of proposed method and conventional methods

Method	Column	Condition	Elution order	Runtime (min)	Rs	Reference
HPLC	Chiralpak IA3 (100 × 4.6 mm; 3 μm)	Mobile phase: MeOH-Water-Acetic acid (84 : 16 : 0.1) Temperature: 27 °C Flow rate: 0.6 mL/min Detector: PDA 215 nm	S-R	9	>1.8	Current study
HPLC	LiChrospher 60 RP-Select B (250 × 4 mm; 5 μm)	Derivatization with o-phthalaldehyde in presence of D-phenylalanine. Mobile phase: 55 % K_2HPO_4 20 m pH 5.8 and 45 % acetonitrile/methanol (1:1) Flow rate: 1.7 mL/min Detector: Fluorescence 230 nm	R-S	20	N/A	(16)
LC-MS	Chiralpak AD-3R	Mobile phase: Acetonitrile-Methanol-Formic acid (10mM) (25:25:50, v/v/v) Temperature: 30°C Flow rate: 0.2 mL/min	S-R	30	1.8	(14)
LC-MS	Chiralpak AD-RH (150 × 2.1 mm; 5 μm)	Mobile phase: 0.1 % (v/v) formic acid/water (A) and 0.1 % (v/v) formic acid/methanol (B) - gradient Temperature: 30 °C Flow rate: 0.3 mL/min	S-R	10	>1.5	(6)
LC-MS	Chiralpak AD-RH	Mobile phase: 10 mM Formic acid (A) and Acetonitrile : Methanol (50 : 50) (B). (A : B = 60 : 40). Temperature: 40 °C Flow rate: 0.2 mL/min	S-R	30	N/A	(15)
CE	FunCap-CE Type S (80.5 cm× 50 μm)	Phosphate buffer (100 mM; pH 7.0) containing TM-β-CD (8 mM) Temperature: 20 °C. Supply voltage: +18 kV	S-R	18	1.2	(13)

N/A : Not applicable

4. Conclusions

For the first time, a simple, fast and effective methods for analysis the enantiomeric impurity in the R-(+) lipoic tromethamine raw material was developed. The application of DoE tool supported to predict important factors affecting the analytical parameters, as well as the interactions among them to optimize the developed method. Compared to other enantioseparation methods, this method could completely separate the two enantiomers in the shortest running time via the use of new-generation stationary phase column (*Table* 8). The elution order (S-enantiomer prior to R-enantiomer) could avoid the masking of the small peak. The developed method exhibited a high sensitivity since it could detect 0.06 % S-(-)-ALA in R-(+)-ALA. The proposed method was validated and successfully applied on R-(+)-ALA raw material. These results showed that the developed method might be appropriate for the quality control and testing the enantiomeric purity of R-(+)-ALA.

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