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Article

Separation and determination of D-malic acid enantiomer by reversed-phase liquid chromatography after derivatization with (*R*)-1-(1-naphthyl) ethylamine

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L-Malic acid is the Active Pharmaceutical Ingredient of the latest generation of compound electrolyte injection (STEROFUNDIN ISO, Germany) and plays a very important role in the rescue of critically ill patients. The optical purity of L-malic acid is a Critical Quality Attributes. A new reversed-phase high performance liquid chromatography (RP-HPLC) method for pre-column derivatization of D-malic acid enantiomer impurity in L-malic acid bulk drug was established. The derivatization reaction was carried out using (*R*)- 1-(1-naphthyl)ethylamine ((*R*)-NEA) as a chiral derivatization reagent. The Kromasil C_{18} column was used with a detection wavelength of 225 nm, a flow rate of 1.0 mL·min^{-1}, and a column temperature of 30 °C. The mobile phase was acetonitrile-0.01 mol·L^{-1} potassium dihydrogen phosphate solution (containing 20 mmol·L^{-1} sodium heptanesulfonate, adjusted to pH 2.80 with phosphoric acid) (at a ratio of 45:55) and the resolution of D-malic acid and L-malic acid derivatization products reached 1.7. The proposed method possesses the advantages of simple operation, mild conditions, stable derivatization products and low cost. Also it gave better separation and was more accurate than previous methods.

Keywords: L-Malic acid. (*R*)-NEA. Chiral purity. Pre-column derivatization.

INTRODUCTION

Since December 2013, the Ebola virus (ebov) has spread in West Africa, primarily in Guinea, Sierra Leone, and Liberia, on an unprecedented scale, with an actual mortality rate of up to 70%. However, in October 2014, successful treatment of a critical Ebola patient from West Africa was achieved in Germany using Sterofundin ISO (B. Braun Medical Supplies) (Kreuels et al. 2014). "Sterofundin ISO" by Braun was approved in Germany in 2003. It is the latest generation of crystal balance fluid. The first generation of electrolyte injection is physiological saline, and normal saline is the basic preparation for infusion treatment. The content of sodium in physiological saline is similar to that in plasma, but the content of chlorine is significantly higher than the content of chlorine in plasma. When supplemented with normal saline, it can cause high chloride metabolic acidosis (Soussi et al. 2017). The second generation of electrolyte injection is Ringer's solution and sodium lactate Ringer's solution. The Ringer's solution increases the electrolytes such as Ca^{2+} and K^+ on the basis of physiological saline, and the chloride ion concentration is higher than the normal concentration range of plasma chloride ions. It can cause loss of bicarbonate and is limited in clinical application. Sodium lactate Ringer's solution is based on Ringer's solution. Because of the slow metabolism of lactic acid, it is mainly metabolized in the liver. Therefore, when the amount of infusion is large and liver dysfunction, it may cause lactic acid accumulation and cause acidosis (Kohnle et al. 1977). The third generation of electrolyte injection is sodium acetate Ringer's solution. The osmotic pressure of sodium acetate Ringer's solution is close to normal plasma, and it does not contain lactate. Therefore, it can avoid

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high-chlorine metabolic acidosis and high lactatemia when a large amount of supplementation occurs (Doucet, Hall, 1999). The new generation of electrolyte injection is L-malic acid crystal balance solution (Hafizah, Liu, Ooi, 2017). In this paper, we use the Fischer (*D*/*L*) notation for malic acid enantiomers, while retaining the Hughes-Ingold-Kahn (*R*/*S*) notation for the chiral reagent (*R*)-NEA. The malic acid in the formula acts as a free radical scavenger and plays an important role in the body. Malic acid is known for its ability to increase energy and tolerance to exercise, and has a strong antioxidant effect, which is an important factor in life activities and organ protection. Malic acid can also directly reduce the production of succinic acid by fumarate as a precursor, and simultaneously produce adenosine triphosphate (ATP), which can protect the integrity of myocardial cell membrane. Besides, it rapidly replenished energy and improved the efficiency of mitochondrial energy metabolism, thereby shortening the rescue time for critically ill patients. With increase of the success rate of critical fluid recovery in these patients, the survival rate will also increase. Structurally, malic acid contains a stereogenic centre, therefore a pair of enantiomers. Because D-malic acid affects the metabolism of L-malic acid (Buser-Suter, Wiemken, Matile, 1982), L-malic acid has high requirements for optical purity in food and medicine (Eisele, 1996; Horiba et al. 1982; Kamencev, Komarova, Morozova, 2016; Martinez-Luque, Castillo, Blasco, 2001; Mazzei, Botrè, Favero, 2007; Nakajima et al. 1993; Zotou, Loukou, Karava, 2004). Several reports on D-malic acid enantiomer detection methods have been published, such as direct determination using chiral high-performance liquid chromatography (HPLC) columns (Calderon, Santi, Lammerhofer. 2018), separation by biological enzyme reaction (Mori, Shiraki, 2008; Shapiro, Silanikove, 2011; Trojanowicz, Kaniewska, 2013), chiral mobile phases (CMP) (Doner, Cavender, 1988), and pre-column derivatization with chiral reagents (Fransson, Ragnarsson, 1998; Miwa, 2000; Miwa, Yamamoto, 1996). Due to the high separation cost and poor reproducibility of the chiral column, the consumption of additives in the CMP additive (CMPA) method is very high, and the bio-enzyme analysis requires a specific kit for each specific organic acid. Although the enzymatic method is simple to use, the enzyme of the catalytic reaction is not highly specific. The pre-column derivatization reagent method can overcome the shortcomings of the weak chromaticity for the carboxyl group, since it does not require expensive reagents and complicated detectors, and has improved sensitivity and selectivity. In the previously reported method for pre-column derivatization of DL-malic acid (Fransson, Ragnarsson, 1998; Miwa, 2000; Miwa, Yamamoto, 1996), complete separation of DL-malic acid derivatives was not achieved. Therefore, the purpose of this study was to develop a liquid chromatography method for the separation and determination of DL-malic acid for the quality assessment of L-malic acid bulk drugs.

The optically pure chiral reagent (*R*)-NEA has strong ultraviolet (UV) absorption, and (*R*)-NEA is also used as a derivatization reagent to enhance the UV absorption of malic acid-derived products, which is inexpensive and mild. In the present study, 1-hydroxybenzotriazole (HOBT) and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC-HCl) were used to activate the carboxyl group of malic acid, and then the activated DL-malic acid was pre-column derivatized with (*R*)-NEA. It is separated by C_{18} column and has the advantages of simple operation, stable derivatization products and low cost. In this study, the effect of different chromatographic conditions on the resolution of DL-malic acid derivatives was investigated by means of pre-column derivatization, and a series of optimizations were performed on the derivatization method. This method achieved the separation and quantitative analysis between D-malic acid and its L-malic acid enantiomer by a common C_{18} column with a good resolution (more than 1.7).

MATERIAL AND METHODS

Chemicals

The following chemicals were used. L-malic acid (Changmao Biochemical Engineering Company Limited, Changzhou, China; ≥99.5%), D-malic acid (Aladdin, China; ≥99%), 1-Hydroxybenzotriazole (HOBT) (Adamas-Beta, Shanghai,China; 99%), 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC-HCl) (Acros Organics, NJ, USA; ≥98%), (*R*)-1-(1-naphthyl) ethylamine ((*R*)-NEA) (Alfa Aesar, Haverhill,

MA, USA; 99%), Acetonitrile (chromatographic grade; Honeywell, Morris Plains, NY, USA), phosphoric acid (chromatographic grade; TEDIA, Fairfield, OH, USA), sodium heptanesulfonate (chromatographic grade; Merck), and Milli-Qultrapure water. All other reagents were of analytical grade.

Instrumentation

Chromatographic analysis was performed using the following instruments: HPLC, Shimadzu LC-2010A; LC-solution chromatography workstation; Sartorius BT25S electronic analytical balance; GL-88B vortex mixer (Haimen Kylin-Bell Lab Instruments Co., Ltd., Qilin, China); mass spectrometer, The Waters Quattro Micro API (Waters, Milford, MA, USA); Workstation: Masslynx4.0 data analysis system (Waters, Milford, MA, USA).

Mass spectrometry conditions

Electrospray ionization (ESI) was conducted as follows: scanning mode: positive ion; positive ionization spray voltage: 3.5 kV; cone voltage: 20 V; secondary cone extraction voltage: 1 V; radio frequency lens: 0.5 V; source temperature: 120 °C; desolvation gas temperature: 350 °C; atomizing gas flow rate: 400 L/hr; air curtain air flow: 50 L/hr; collision energy: 10 V; collision chamber vacuum: $3.5e^{-3}$ mbar.

Standard stock and sample preparation

5 mg each of L-malic acid and D-malic acid were added to a 100 mL volumetric flask, and dissolved in acetonitrile to prepare a 0.1 mg/mL racemic DL-malic acid control solution. 10 mg of HOBT was added to a 10 mL volumetric flask and dissolved in acetonitrile to prepare a 1 mg/mL HOBT solution. 15 mg of EDC-HCl was added to a 10 mL volumetric flask and dissolved in acetonitrile to prepare a 1.5 mg/mL EDC-HCl solution. 100 mg of (*R*)-NEA was added to a 10 mL volumetric flask and dissolved in acetonitrile to prepare a 10 mg/mL derivatization reagent solution.

Derivatization procedure

100 µL of DL control solution and 200 µL of HOBT solution were vortexed and mixed for 20 s. Then, 200 µL of EDC-HCl solution was added and was vortexed for 20 s. The solution was left undisturbed at room temperature for 2 min to fully activate the carboxyl group, and then 20 µL of (*R*)-NEA solution was added. The mixture was diluted with 180 µL of acetonitrile, heated at 40 °C for 2 h, and 20 µL was injected into the liquid chromatography column.

Results and Discussion

Mass spectrometry analysis

The reaction scheme of DL-malic acid with HOBT (as an activator), EDC-HCl (as a coupling agent) and (*R*)-NEA (as a derivatization reagent), is shown in Figure 1. The derivatization product of DL-malic acid was analyzed by tandem mass spectrometry (MS/MS) using a triple quadrupole mass spectrometer. The MS/MS for the derivatives of DL-malic acid is shown in Figure 2.

The derivative of DL-malic acid was subjected to ESI ionization to form $[M+H]^+$ (m/z 441), and the secondary mass spectrum with a collision energy of 10 V is shown in Figure 3. Among the fragment ions generated under electrospray ionization tandem mass spectrometry (ESI-MS/MS) conditions, the fragment ions of m/z 155 and m/z 287 are product ions generated by the carbon-nitrogen bond cleavage. The possible secondary cleavage pathways for mass spectrometry are shown in Figure 4.

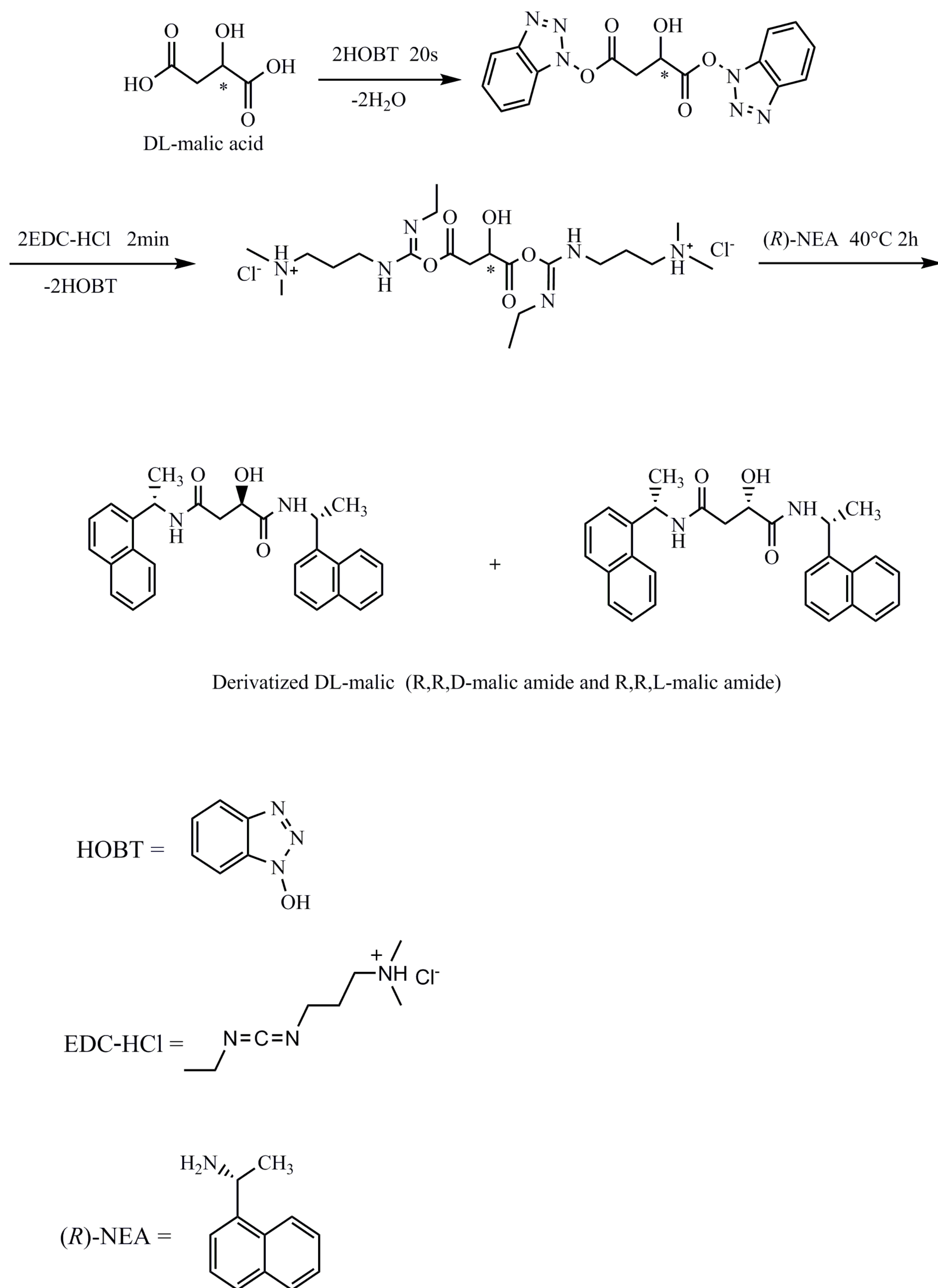

FIGURE 1 - The reaction scheme of (*R*)-NEA with the carboxyl group of DL-malic acid molecule, HOBT is an activator, and EDC-HCl is a coupling agent.

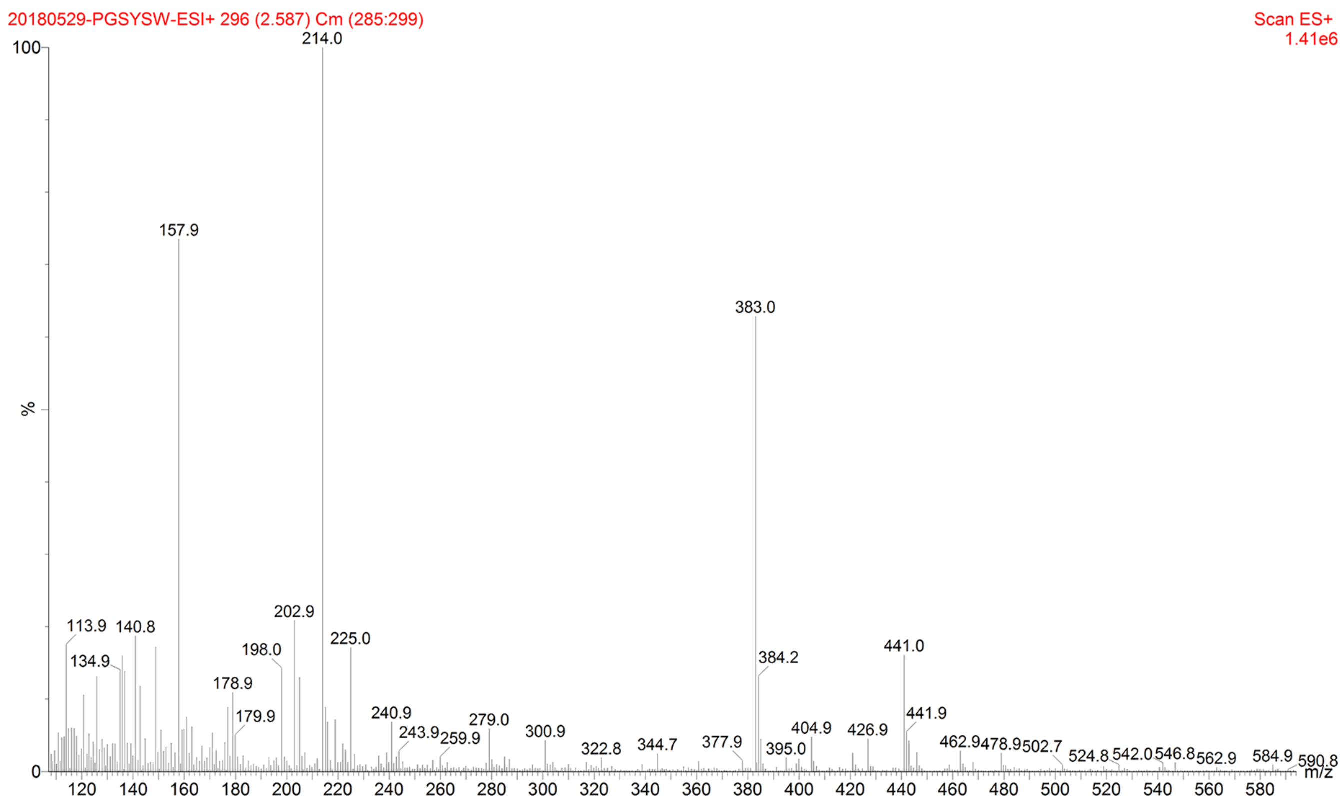

FIGURE 2 - The mass spectrum for derivatives of DL-malic acid.

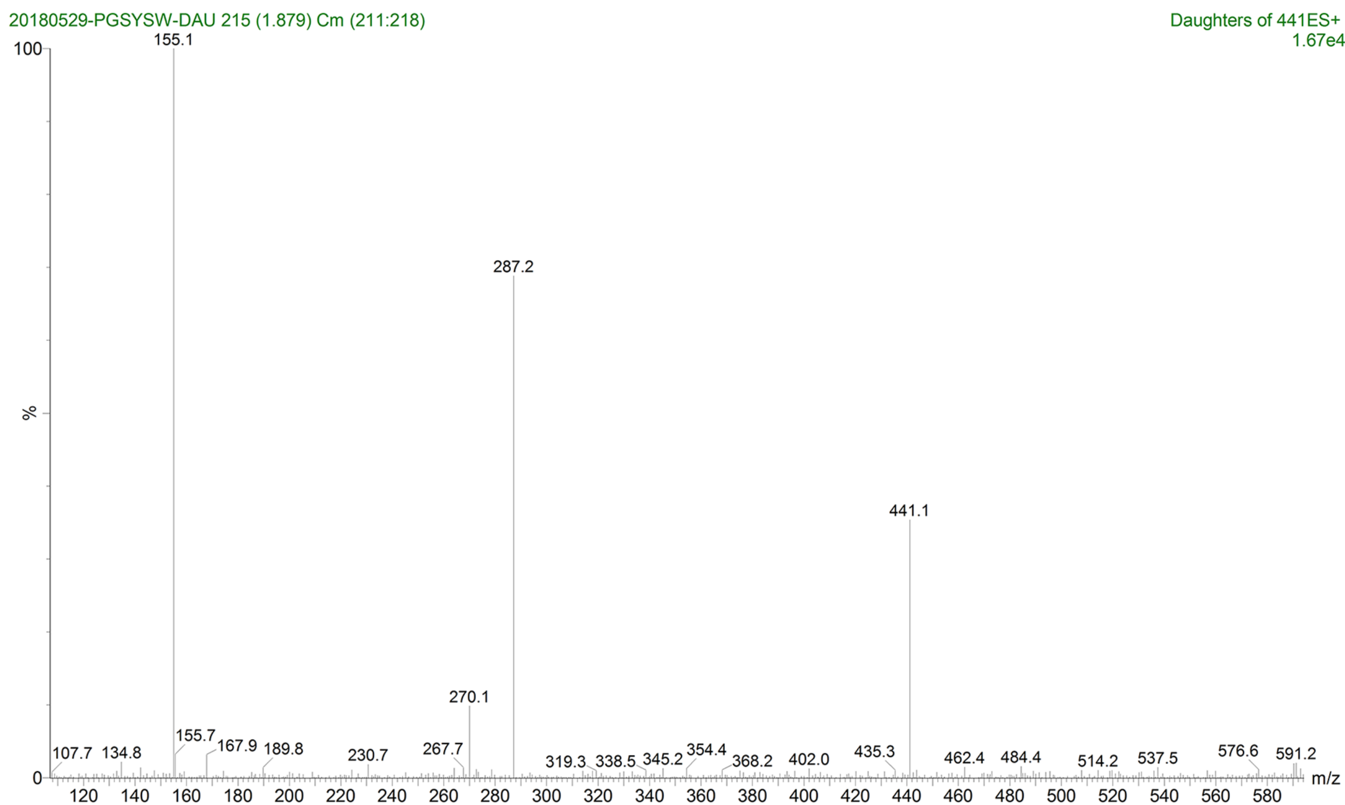

FIGURE 3 - The tandem mass spectrum (MS/MS) of DL-malic acid derivatives.

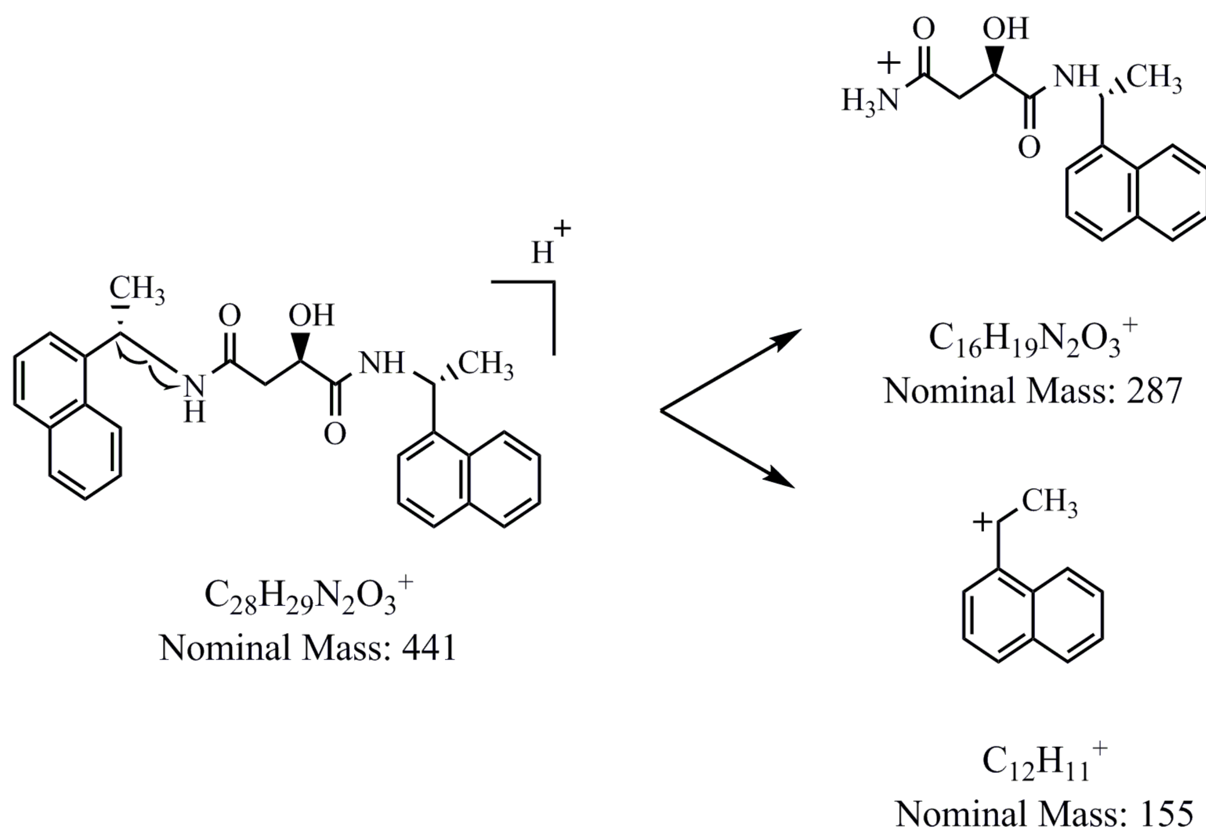

FIGURE 4 - Proposed fragment pathway for derivatives of malic acid.

Method development

By optimizing the derivatization reaction conditions, including adjusting the ratio of derivatization reagent and DL-malic acid, derivatization reaction time, reaction temperature and activation time, the optimal conditions for reaction of DL-malic acid derivatization could be eventually obtained.

Effect of activation time on derivatization efficiency

If there is no activator in the derivatization of malic acid, the reaction rate will slow down and the reaction will be difficult to complete. Therefore, HOBT and EDC-HCl were added to activate malic acid before derivatization. The activation of HOBT and EDC-HCl with carboxyl groups is mild and rapid, and an excess activator does not affect the derivatization reaction.In order to determine the effect of activator activation time on the derivatization reaction, we mixed 100 μL of DL-malic acid control solution and 200 μL of HOBT solution and vortexed for 20 s, 200 μL of EDC-HCl solution was added and vortexed for 20 s. Allow the solution to mix well. Then, the solution was left undisturbed at room temperature for 0, 20, 120, 300 and 600 seconds, respectively, and then 20 μL of the (*R*)-NEA solution was added. The mixture was diluted with 180 μL of acetonitrile, reacted at 40 °C for 2 h, and 20 μL was injected into the liquid chromatography. The results showed that the peak area of the product did not significantly change. To ensure full activation, the duration of optimal test activation was chosen to be 2 min.

Effect of ratio of (R)-NEA to malic acid on derivatization efficiency

The amount of derivatization reagent in the derivatization reaction was changed in order to investigate the effect of the reaction molar ratio on the derivatization efficiency, and the excess (*R*)-NEA ensured that the reaction was completed. Afterwards, the DL-malic acid

was fully activated at room temperature. The reaction was carried out at 40° C according to the ratios of (*R*)-NEA to malic acid at 4:1, 8:1, 16:1, 40:1, 80:1, and 160:1. The results showed that the peak area of the reaction product reached the maximum when the ratio of (*R*)-NEA to DL-malic acid was 16:1. In order to avoid wastage of (*R*)-NEA, the optimum ratio of (*R*)-NEA to DL-malic acid was maintained at 16:1.

Effect of temperature and duration of reaction on derivatization efficiency

After activation of DL-malic acid, the derivatization time was optimized at room temperature i.e. at 20 ° C at time intervals of 0, 0.25, 0.5, 1,2,3 and 4 h by keeping the ratio of (*R*)-NEA to DL-malic acid was 16:1. It was found that the minimum time required for derivatization was 3 h at 20 ° C. To evaluate which temperature is optimal in terms of yield of derivatization reaction for analysis, the effect of a wide range of temperatures on the yield of derivatization was tested. The effect of increase in temperature on the rate of formation of the derivative was checked by keeping the time constant (3 h). The reaction was carried out at a temperature of 20, 30, 40, 50 and 60 °C, respectively, and it was observed that the peak area of the derivative product remained unchanged from 40 °C. Finally using all the optimized conditions.100 µL of DL-malic solution and 200 µL of HOBT solution were vortexed and mixed for 20 s. 200 µL of EDC-HCl solution was added and vortexed for 20 s. Then the solution was left undisturbed at room temperature for 2 min and 20 µL of (*R*)-NEA solution was added. The mixture was diluted with 180 µL of acetonitrile, reacted at 40 °C . The peak area of derivatives was observed every half hour. As a result, it was found that the derivative formation was constant from 2 h to 4 h. The derivatization time optimized to 2 h at 40 °C.

HPLC separation and detection

Ion pairing agents are commonly used as additives in mobile phases to adjust selectivity and achieve improved peak shape with reliable separation (Wannerberg, Persson, 1988). In this experiment, ion-pair chromatography was used to develop a pre-column derivatization method in order to achieve more sensitive and selective detection by reversed phase HPLC (RP-HPLC) and improve the resolution. The Kromasil C_{18} column was used with a detection wavelength of 225 nm, a flow rate of 1.0 $mL·min^{-1}$, and a column temperature of 30 °C. The mobile phase was acetonitrile-0.01 $mol·L^{-1}$ potassium dihydrogen phosphate solution (containing 20 $mmol·L^{-1}$ sodium heptanesulfonate, adjusted to pH 2.80 with phosphoric acid). The injection volume was 20 µL. The method has the advantages of simple operation, mild conditions, stable derivatization products, as well as being cost-effective, and can more accurately realize the separation and detection of the DL-malic acid enantiomer.

Acetonitrile and methanol are commonly used as organic solvents mobile phases in reverse chromatography. When methanol and water (at a ratio of 45:55) are used as mobile phases, D-malic acid and L-malic acid cannot be separated, and the baseline drift is high, affecting qualitative and quantitative analyses. The organic solvent for mobile phase was selected as acetonitrile. Conventional detection methods cannot be easily used to completely separate L-malic acid from D-malic acid on a common C_{18} column, and the resolution is difficult to reach 1.5 or more, thus attempts should be made to adjust the organic phase for mobile phase and pH. Phosphate buffer saline (PBS) and sodium heptane sulfonate (as an ion-pair reagent) were added to the mobile phase to improve the peak shape and increase its retention on the column, so that the duration of retention would change, thereby improving the resolution. The retention behavior of the solute on the column is typically represented by the retention factor (k'). When the k' was increased from 2.5 to 25 $mmol·L^{-1}$, the k' of the derivatization product of DL-malic acid was observed to decrease from 12 to 6. The ion-pair reagent was varied between 2.5–25 $mmol·L^{-1}$ at a constant ratio of acetonitrile to 0.01 M phosphate buffer (45:55). Within this range, the resolution of D- and L-malic acid increased from 1.3 to 1.7. It was higher than 1.15 reported in the literature (Fransson, Ragnarsson, 1998; Miwa, 2000; Miwa, Yamamoto, 1996). However, the acceptability of the column should be considered, therefore, the concentration of ion pair reagent was chosen as 20 $mmol·L^{-1}$. The effect of the amount of acetonitrile in the mobile phase and the pH

of the aqueous phase on the resolution and duration of retention was investigated in the range of 35% to 55%. The results showed that the lower the acetonitrile ratio and pH, the better the separation effect. However, lower organic ratio may prolong duration of retention, and very low pH may damage the column. The mobile phase was eventually determined to be an acetonitrile-0.01 mol·L^{-1} potassium dihydrogen phosphate solution, containing 20 mmol·L^{-1} sodium 1-heptanesulfonate, adjusted to pH 2.80 with phosphoric acid. The typical chromatograms of (*R*)-NEA derivative of DL-malic acid standard solution and L-malic acid standard solution were shown in Figure 5.

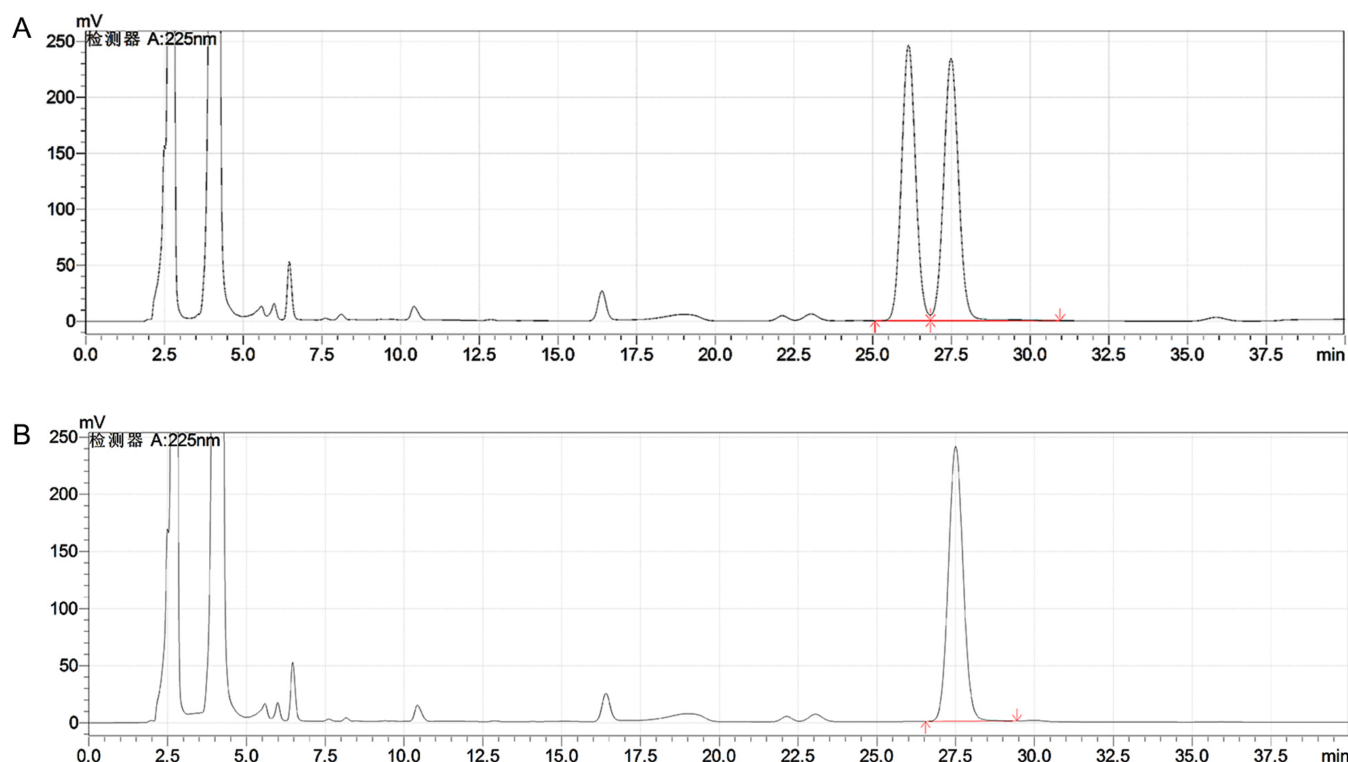

FIGURE 5a - (a) HPLC chromatogram of (*R*)-NEA derivative of DL-malic acid (standard solution). The duration of retention for the chromatographic peaks of D-malic derivative and L-malic acid derivative was 26.1 and 27.5 min, respectively. (b) HPLC chromatogram of (*R*)-NEA derivative of L-malic acid (standard solution).

Validation of the method

In order to facilitate accurate detection of the content of D-malic acid, the linear response of D-malic acid at low concentrations was specifically investigated according to the ICH guidelines.

Thus, it was attempted to precisely measure the appropriate amount of the reference stock solution and prepare a linear solution containing D-malic acid concentrations of 0.05, 0.10, 0.15, 0.20, 0.25μg·mL^{-1}, respectively. Derivatization was carried out under optimized conditions, and 20 μL was injected for HPLC analysis. The peak area and concentration of derivative product of D-malic acid showed a linear relationship. The regression equation of D-malic acid was $Y = 1.151\times10^6 X - 2.521\times10^4$, $R = 0.9999$. The limit of detection of D-malic acid was 0.5 ng (S/N=10), and the minimum detection limit was 0.1 ng as well (S/N=3).

The test solution containing 0.05%, 0.10%, and 0.15% of D-malic acid was separately prepared in triplicate, and measured under the above mentioned chromatographic conditions. Then, the peak area of D-malic acid was recorded and the rate of recovery was calculated. The average rates of recovery for three concentrations were

99.52%, 101.2%, and 101.5%, respectively, indicating that the method was very accurate. The precision expressed in relative standard deviation (RSD, %) was determined by analyzing the samples on the same day and seven subsequent dates (n = 6). Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) and reported in RSD (%), which were 0.90% and 0.72%, respectively. Consequently, the analytical measurements obtained from this method were reliable.

The separation between D-malic acid derivative and L-malic acid derivative was more than 1.7 under different chromatographic conditions, such as flow rate, flow ratio, injection volume, UV wavelength, and column temperature, indicating the robustness of the method.

Assay of bulk drug materials

The method was applied to determine the D-malic acid enantiomer impurity in three batches of L-malic acid bulk drug, and the product was also analyzed in triplicate (n = 3) for derivatization. The RSD rates of the measurement results were 1.27%, 1.43%, and 0.53%, respectively (Table I). These results indicated that the developed method was very simple, fast, and reliable, and could be used to determine the D-malic acid enantiomer impurity in L-malic acid bulk drugs.

TABLE I - Results of bulk drugs determination

Lot No.	D-malic acid%	RSD% *
20180201	1.02	1.27
	1.09	
	1.07	
20180202	0.89	1.43
	0.85	
	0.84	
20180203	1.53	0.53
	1.52	
	1.56	

*Relative standard deviation

CONCLUSIONS

In this study, the effects of different chromatographic conditions on the separation of derivatives of DL-malic acid were assessed by pre-column derivatization, and a series of optimizations were also carried out on the derivatization method. This method achieved separation and quantitative analysis between D-malic acid and its L-enantiomer using a conventional C_{18} column. The reaction conditions were mild, the reproducibility was satisfactory, the derivatized product was stable, use of a chiral column was unnecessary. All in all, the method for the quality control of L-malic acid shows a number of advantages over previous methods.

ACKNOWLEDGMENTS

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest in relation to this research.

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