SEPARATION OF (R)-LIPOIC ACID AND (S)-LIPOIC ACID BY HPLC	ID #	AM-1000
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	Auth.	ARS
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I. Purpose and Scope:

This analytical method provides instructions for the separation of (R)-Lipoic Acid and (S)-Lipoic Acid by normal phase HPLC.

II. Summary of Methodology:

This is a chiral assay for quantitatively determining Lipoic Acid enantiomers in purified preparations or racemic mixtures. The sample is dissolved in the mobile phase and diluted to the appropriate volume. This normal phase separation is performed with an isocratic mobile phase comprised of Hexanes and Isopropanol with 0.5% TFA. A detection wavelength of 215 nm is used.

III. Instrumentation and Supplies:

a. Analytical balance; capable of weighing to ± 0.01 mg
b. HPLC system equipped with programmable variable wavelength detector (VWD) or diode array detector (DAD) and data acquisition system.
c. Ultrasonic bath
d. Class A volumetric glassware
e. Pipettes
f. HPLC system sample vials

IV. Reagents, Solutions and Standards:

a. (R)-Lipoic Acid reference standard
b. (S)-Lipoic Acid reference standard
c. Hexanes: HPLC grade
d. Isopropanol: HPLC grade
e. Trifluoroacetic acid (TFA), ≥99%
f. Isopropanol with 0.5% TFA

Note: Proportionally larger amounts may be prepared.
In a 500 mL volumetric flask, add 2.5 mL of TFA in approximately 100mL of Isoproponal and dilute to volume with Isopropanol. Mix well.

g. Mobile Phase (Hexanes : Isopropanol with 0.5% TFA) (95 : 5) (premixed)

Note: Proportionally larger amounts may be prepared. Prepare sufficient mobile phase for both sample preparation and HPLC use.
Add 50 mL Isopropanol with 0.5% TFA to a 1000 mL volumetric flask and dilute to volume with Hexanes. Mix well and transfer into a suitable HPLC reservoir.

h. Sample diluent (mobile phase)

V. Standard and Sample Solutions Preparation:

Proportionally larger amounts of each of these solutions may be prepared. For analyzing (S)-Lipoic acid samples, replace (R)-Lipoic acid with (S)-Lipoic acid.

a. (R)-Lipoic Acid Standard Solution (nominal 1000 µg/mL):
Accurately weigh and quantitatively transfer about 25 mg of a validated (R)-Lipoic Acid standard material into a 25 mL volumetric flask. Dissolve (sonicate if necessary) and dilute to volume in mobile phase. Mix well and transfer a portion into an HPLC sample vial.

b. Resolution Stock (nominal 250 µg/mL of (S)-Lipoic Acid):
Accurately weigh and quantitatively transfer about 25 mg of (S)-Lipoic Acid into a 100 mL volumetric flask. Dissolve (sonicate if necessary) and dilute to volume in mobile phase. Mix well.

c. Resolution Solution:
Using a volumetric pipette, transfer 2 mL of Resolution Stock Solution to a 10 mL volumetric flask and dilute to volume with (R)-Lipoic Acid Standard Solution. Mix well and transfer a portion into a HPLC sample vial.

d. Sample Solution:
Accurately weigh and transfer about 25 mg of (R)-Lipoic Acid sample into a 25 mL volumetric flask. Dissolve (sonicate if necessary) and dilute to volume with mobile phase. Transfer a portion into a HPLC sample vial.

Note:
For analysis of the water soluble Lipoic Acid salts (Na^+, K^+, Mg^{2+}, etc):
Accurately weigh and transfer about 50 mg of Lipoic Acid salt into a 25 mL volumetric flask. Dissolve (sonicate if necessary) the sample in approximately 20 mL pure water (distilled or R.O.; ≥18MΩ). Slowly adjust solution to pH 2.0 (± 0.25) with phosphoric acid. Dilute to final volume with water. Extract the Lipoic Acid from the aqueous sample by mixing 2 mL of the sample with 2 mL of Hexanes. Shake vigorously then allow the phases to completely settle; Transfer a portion of the (Hexanes) top layer containing the extracted 'free acid' LA into a HPLC sample vial.

VI. Procedure:

1. The HPLC conditions are as follows:

Column	Diacel, Chiralpak AD-H, 5 μm, 25 cm (L) X 4.6 mm (ID)
Guard Column	Diacel, Chiralpak AD-H, 0.4 cm (ID) X 1 cm
Mobile Phase	Hexanes : Isopropanol with 0.5% TFA (premixed); (95:5)
Column Temperature	25°C
Detection Wavelength	215 nm
Flow Rate	1 mL/min
Injection Volume	10 μL
Run Time	25 min

2. System Suitability:

a. Blank Determination:
Inject the mobile phase used to prepare the standards and samples. Determine if there are any peaks in the chromatogram present at the expected retention times of the Lipoic Acid stereoisomers. If a peak is observed, continue to inject the blank until an interference-free baseline is established.

b. Resolution Determination:
Inject the Resolution Solution. The resolution between (R)-Lipoic acid and (S)-Lipoic acid peaks from the Resolution Solition should be ≥ 1.5.

c. Replicate Injection Precision:
Make five (5) replicate injections of (R)-Lipoic Acid Standard Solution. The RSD of the peak area responses for the (R)-Lipoic Acid peaks must be ≤ 2.0%.

d. Assay Sequence:
   1) Following a successful system suitability evaluation, inject one (1) 10 μL portion of each Sample Solution preparation and record the peak areas of (R)-Lipoic Acid and (S)-Lipoic Acid.
   2) Bracket each set of six (6) samples with (R)-Lipoic Acid Standard Solution until all the samples have been analyzed.
   3) Assure that the final injection of the sequence is from (R)-Lipoic Acid Standard Solution.

3. Reporting Requirements:
Report chiral assay results of (R)-Lipoic Acid as a percentage to two decimal places.

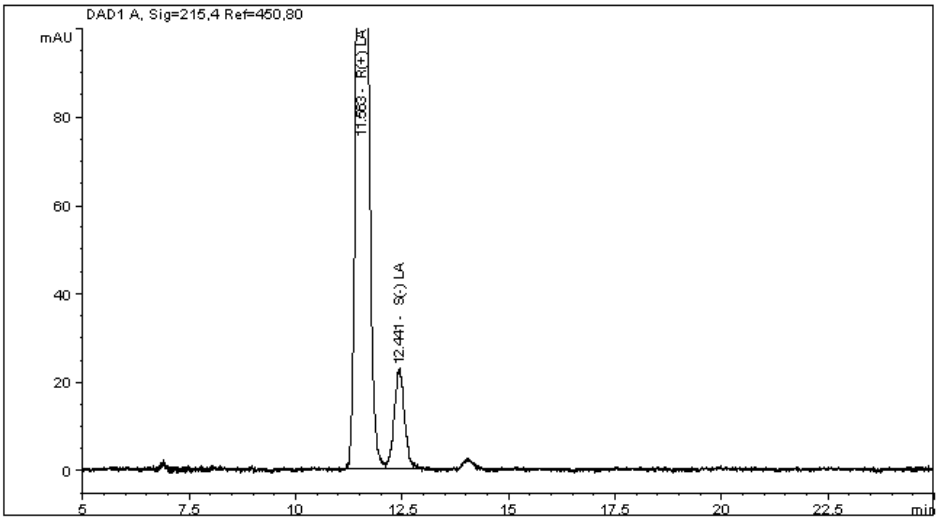
VII. Calculation:

1. Chiral Assay:
   Calculate the chiral assay of (R)-Lipoic Acid by peak area % as follows:

$$\%\,(R)\text{ - Lipoic Acid} = \frac{\text{Peak Area (R) - Lipoic Acid}}{\text{(R) - Lipoic Acid} + \text{(S) - Lipoic Acid}} \times 100$$

VIII. Sample Chromatography:

1. Resolution Solution



2. *Racemic*-Lipoic Acid test sample