

SEPARATION OF LIPOIC ACID AND DIHYDROLIPOIC ACID BY HPLC	ID #	AM-1010
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	Auth.	ARS
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I. Purpose and Scope:

This analytical method provides instructions for the separation of Lipoic Acid by reverse-phase HPLC.

II. Summary of Methodology:

This is an assay for quantitatively determining Total Lipoic Acid in purified preparations or racemic mixtures. This reverse-phase HPLC separation is adapted from the USP-NF official method for Lipoic Acid determination. The sample is dissolved in 50% Ethanol and diluted to the appropriate volume. The separation is performed with an isocratic mobile phase comprised of 50mM Phosphate Buffer, pH 2.7: Acetonitrile: Methanol 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. K_2HPO_4
- c. Ethanol (95%): HPLC grade
- d. Acetonitrile: HPLC grade
- e. Methanol: HPLC grade
- f. Water: HPLC grade (double distilled or reverse osmosis treated, ≥ 18 M Ω)
- g. Phosphoric acid: HPLC grade
- h. 50 mM K_2HPO_4 , pH 2.7

Note: Proportionally larger amounts may be prepared.

In a 2 L volumetric flask, add 13.78 g K_2HPO_4 in approximately 1800 mL of water. Mix well and adjust pH to 2.7 with phosphoric acid. Dilute to volume with water and filter if necessary (0.2 μ m pore size).

- i. Mobile Phase (50 mM K_2HPO_4 , pH 2.7: Acetonitrile: Methanol) (50:30:20) (premixed)

Note: Proportionally larger amounts may be prepared.

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Add 300 mL Acetonitrile and 200 mL Methanol to a 1000 mL volumetric flask and dilute to volume with 50 mM K_2HPO_4 , pH 2.7. Mix well and transfer into a suitable HPLC reservoir. Degas with 20 min sonication if necessary.

j. Sample diluent

Note: Proportionally larger amounts may be prepared. Prepare sufficient volume for all standard and sample preparations.

Add 500 mL Ethanol (95%) to a 1L volumetric flask. Dilute to volume with water and mix well.

V. Standard and Sample Solutions Preparation:

Proportionally larger amounts of each of these solutions may be prepared.

- a. (R)-Lipoic Acid Standard Stock (nominal 5000 $\mu\text{g/mL}$):
Accurately weigh and quantitatively transfer about 125 mg of a validated (R)-Lipoic Acid standard material into a 25 mL volumetric flask. Dissolve (sonicate if necessary) and dilute to volume in sample diluent. Mix well.
- b. (R)-Lipoic Acid Standard Solution (nominal 500 $\mu\text{g/mL}$): Accurately measure and transfer 1 mL of (R)-Lipoic Acid Standard Stock solution to a 10 mL volumetric flask and dilute to volume with sample diluent. Mix well. Transfer a portion into an HPLC sample vial.
- c. Sample Solution (nominal 500 $\mu\text{g/mL}$):
Accurately weigh and transfer about 125 mg of Lipoic Acid sample into a 25 mL volumetric flask. Dissolve (sonicate if necessary) and dilute to volume with sample diluent. Dilute 1/10 by transferring 1 mL of this solution to a 10 mL volumetric flask and diluting to volume with sample diluent. Mix well. Filter if necessary and transfer a portion into a HPLC sample vial.

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VI. Procedure:

1. The HPLC conditions are as follows:

Column	Phenomenex Gemini C18, 5 μ m, 25 cm (L) X 4.6 mm (ID)
Guard Column	Phenomenex Security Guard, 4 x 2 mm; C18
Mobile Phase	50 mM K ₂ HPO ₄ , pH 2.7: Acetonitrile: Methanol (50:30:20)
Column Temperature	25°C
Detection Wavelength	215 nm
Flow Rate	1 mL/min
Injection Volume	20 μ L
Run Time	15 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 time of Lipoic Acid. If a peak is observed, continue to inject the blank until an interference-free baseline is established.

- b. 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 $\leq 2.0\%$.

- c. Assay Sequence:

- 1) Following a successful system suitability evaluation, inject one (1) 20 μ L portion of each Sample Solution preparation and record the peak areas of 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 assay results of Total Lipoic Acid as a percentage to two decimal places. Though (R)-Lipoic Acid is used as the standard, this assay does not resolve R/S Lipoic acid enantiomers. No chiral results are determined in this assay.

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VII. Calculation:

1. Total Lipoic Acid Assay:

Calculate Total Lipoic Acid by peak area % as follows:

$$\% \text{ Lipoic Acid} = \frac{\text{Sample Lipoic Acid Peak Area}}{\text{(R) - Lipoic Acid Standard Peak Area}} \times 100$$

VIII. Sample Chromatography:

1. (R)-Lipoic Acid Standard Solution

