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1. Table of Contents

1. **Purpose:**

This SOP describes the technique for qualitative and quantitative estimation of isoniazid in human plasma by Liquid Chromatography Tandem Mass Spectrometry (LCMS/MS).

2. **Scope:**

This SOP is limited to find out the concentration in µg/ml of isoniazid in human plasma by Liquid Chromatography Tandem Mass Spectrometry (LCMS/MS).

3. **Responsibilities:**

The head of the department is responsible for the medical care and welfare of all patients under her/his care. The task of performing estimation of isoniazid will be delegated to trained personnel who will perform this function.

4. **Applicable rules, regulations and guidelines**

- ICMR Good Clinical Laboratory Practices Guidelines 2021 (http://icmr.nic.in/guidelines/GCLP.pdf)

5. **Reference to other applicable SOPs**

- SOP No.24/01: Biomedical waste management.

6. Detailed instructions:

A. Chemicals and Materials:

i. Pure Powder of Isoniazid
ii. Pure powder of Phenacetin (IS)
iii. Miscellaneous: : Methanol and Acetonitrile (HPLC Grade-Merck make), Formic Acid (HPLC Grade-Merck make), Ammonium acetate buffer (GR grade-Merck), Purified water.
iv. LC column: C18, 150 mm x 4.6 mm, 5-micron particle size
v. Glassware: Eppendorf vials (2.0mL), Glass Test Tubes (10ml), Conical Flasks (100, 250, 500, 1000 mL), Measuring Cylinders (100ml, 500ml), falcon tubes, volumetric flasks (10ml).

B. Equipments: High Performance Liquid Chromatography (Shimadzu), Mass Spectrometry (API 2000), Cooling Centrifuge 15,000 X g (Biofuge), Ultra sonicator machine (Imeco), Vortex machine (Spinage), Auto pipettes-eppendorf (10-100µL, 100-1000µL), Centrifuge (Remi-R23).

C. Preparation of solutions, mobile phase and calibration standards:

a. Solutions:

i. **Preparation of stock 5mM ammonium acetate buffer, pH 3.00: -** 0.3854 gm of ammonium acetate dissolved and diluted up to 1000 mL of purified water and adjusted pH 3.0 with formic acid solution.
ii. **Preparation of aqueous methanol solution: -** 90 mL of methanol diluted up to 100 mL with 5mM ammonium acetate buffer solution and 0.1 mL of formic acid solution.
iii. **. Preparation of 1: 1 Methanol: Purified water solution**: - 50 ml of Methanol diluted up to 100 mL with purified water.
iv. **Preparation of 90% methanol solution (0.1 % formic acid solution):** 90 mL of methanol diluted up to 100 mL with purified water and add 100 µL formic acid solution.

v. **Preparation of system suitability solution**: 975 uL of mobile phase + 25 uL of 1mg/mL of stock (1mg/mL)

vi. **Mobile phase:** 5mM ammonium acetate buffer: Methanol: Acetonitrile (30:50:20)

b. **Calibration standards (Isoniazid):**

i. **Preparation of aqueous stock standard solution (1 mg/ml) of isoniazid:** 10 mg of isoniazid hydrochloride dissolve and diluted up to 10 mL with 1:1 methanol: purified water solution and vortexes for 2 mins.

ii. **Preparation of aqueous stock standard solution (100 µg/ml) of isoniazid:** 1 mL of stock solution (1 mg/mL) diluted up to 10 mL with 90% methanol solution (0.1% formic acid)

iii. **Preparation of aqueous standard solution (75 µg/mL) of isoniazid:** 750 µL of stock solution (1 mg/mL) diluted up to 10 mL with 90% methanol solution (0.1% formic acid)

iv. **Preparation of aqueous standard solution (50 µg/mL) of isoniazid:** 5 mL of stock solution (100 µg/mL) diluted up to 10 mL with 90% methanol solution (0.1% formic acid)

v. **Preparation of aqueous standard solution (25 µg/ml) of isoniazid:** 5 mL of stock solution (50 µg/mL) diluted up to 10 mL with 90% methanol solution (0.1% formic acid)

vi. **Preparation of aqueous standard solution (10 µg/ml) of isoniazid:** 1 mL of stock solution (100 µg/mL) diluted up to 10 mL with 90% methanol solution (0.1% formic acid)

vii. **Preparation of aqueous standard solution of isoniazid (Tuning solution):** 1 mL of stock solution (10 µg/mL) diluted up to 10 mL with 90% methanol solution (0.1% formic acid)

viii. **Preparation of aqueous standard solution (0.5 µg/mL) of isoniazid:** 5 mL of stock solution (1 µg/mL) diluted up to 10 mL with 90% methanol solution (0.1% formic acid)

ix. **Preparation of aqueous standard solution (0.1 µg/mL) of isoniazid:** 1 mL of stock solution (10 µg/mL) diluted up to 10 mL with 90% methanol solution (0.1% formic acid)

x. **Preparation of aqueous standard solution (0.05 µg/mL) of isoniazid:** 5 mL of standard solution (0.1 µg/mL) diluted up to 10 mL with 90% methanol solution (0.1% formic acid)

c. Preparation of Internal Standard (Phenacetin):

i. **Preparation of stock aqueous internal standard (1 mg/mL):** 10 mg of phenacetin dissolve and diluted up to 10 mL of methanol (100%) and vortexes for 2 mins.

ii. **Preparation of stock aqueous internal standard (100 µg/mL):** 1 mL of stock solution (1 mg/mL) diluted up to 10 mL with methanol (100%)

iii. **Preparation of working internal standard (10 µg/mL):** 1 mL of stock solution (100 µg/mL) diluted up to 10 mL with methanol (100%)

iv. **Preparation of tuning solution (1 µg/mL):** 1 mL of stock solution (10 µg/mL) diluted up to 10 mL with 90% methanol (0.1% formic acid).

d. Preparation of plasma Standard (Isoniazid):

i. **Preparation of stock plasma standard (100 µg/mL) of isoniazid:** 200 µL ml of aqueous stock solution (1 mg/mL) of isoniazid diluted with 1800 µL of blank plasma

ii. **Preparation of plasma standard (75 µg/mL):** 750 µL of stock plasma standard (100 µg/mL) of isoniazid diluted with 250 µL of blank plasma.

iii. **Preparation of plasma standard (50 µg/mL):** 500 µL of stock plasma standard (100 µg/mL) of isoniazid diluted with 500 µL mL of blank plasma.

iv. **Preparation of plasma standard (25µg/mL):** 0.5 mL of stock plasma standard (50 µg/mL) of isoniazid diluted with 0.5 mL of blank plasma.

v. **Preparation of plasma standard (10µg/ml):** 200 µL of stock plasma standard (100 µg/mL) of isoniazid diluted with 1800 µL of blank plasma.

vi. **Preparation of plasma standard (5µg/ml):** 500 µL of plasma standard (10µg/mL) of isoniazid diluted with 500 µL of blank plasma.

vii. **Preparation of plasma standard (1 µg/mL):** 200 µL of stock plasma standard (10µg/mL) of isoniazid diluted with 1800 µL of blank plasma.

viii. **Preparation of plasma standard (0.5µg/mL):** 500 µL of plasma standard (1 µg/mL) of isoniazid diluted with 500 µL of blank plasma.

ix. **Preparation of plasma standard (0.1µg/mL):** 200 µL of plasma standard (1.0 µg/mL) of isoniazid diluted with 1800 µL of blank plasma.

x. **Preparation of plasma standard (0.05µg/mL):** 500 µL of plasma standard (0.1 µg/mL) of isoniazid diluted with 500 µL of blank plasma

e. **Preparation of quality control samples:**

i. **Preparation of lower limit of quantification (LLOQ) sample (0.5µg/mL):** 500 µL of plasma standard (1 µg/mL) of isoniazid diluted with 500 µL of blank plasma.

ii. **Preparation of Middle Quality Control (MQC) sample (10 µg/ml):** 100 µL of stock plasma standard (100 µg/mL) of isoniazid diluted with 900 µL of blank plasma.

iii. **Preparation of High Quality Control (HQC) sample (40 µg/mL):):** 400 µL of stock plasma standard (100 µg/mL) of isoniazid diluted with 600 µL of blank plasma.

f. Extraction procedure

Simple protein precipitation method

100 µL of BP/ plasma standard/QC + 50 µL of (10 µg/mL) working internal standard. Vortex for 1 minute.

↓

Add 300 µL of aqueous Methanol solution

↓

Vortex for 2 mins

↓

Centrifuge at 15,000 rpm for 10 mins

↓

Transfer clear supernatant in another vial and again centrifuge at 15,000 rpm for 5mins

↓

Inject 5 uL of supernatant on LCMS instrument

g. LCMS condition:

LC parameters

i. Injecting volume: 5µL
ii. Flow rate: 0.350 mL/min
iii. Column: C18, 150 mm x 4.6 mm, 5-micron particle size
iv. Auto sampler temperature: 25° C
v. Column oven temperature: 30° C
vi. Total run time: 8 mins

Comp name	Q1	Q3	Time (msec)	ID	DP	FP	EP	CE	CXP
INH	138.1	121.1	200	INH	20	310	5	21	5
Phenacetin	180.1	110.1	200	IS	23	250	8	30	5

MS parameters:

Scan type: MRM
Polarity: Positive
Curtain gas: 30 PSI
CAD: 10 PSI
Ion Spray voltage: 5500 v
Heater temperature: 425 °C
GS1: 50
GS2: 60

1. Abbreviations:

i. **LCMS** = Liquid Chromatography Mass Spectrometry
ii. **QC** = Quality Control
iii. **LLOQ :** Lower Limit of Quantification
iv. **MQC :** Middle Quality Control
v. **HQC :** High Quality Control
vi. **I.S.** = Internal Standard
vii. **DP** = Declustering Potential
viii. **FP** = Focusing Potential
ix. **EP** = Entrance Potential
x. **CE** = Collision Energy
xi. **CXP** = Collision Exit Potential