CURRENT

Category : Patient Care
Title : Procedure for estimation of Phenobarbitone (PBT), Phenytoin (PHT) and Carbamazepine (CBZ) in human plasma by High Performance Liquid Chromatography (HPLC) Eliminate of Nitrogen Gas

SOP No. and Version: TDM 17/02

Date first effective : 1st January 2025 **Review date: 31st December 2025**

Department of Clinical Pharmacology, 1st Floor, New MS Building, Seth GS Medical College & KEM Hospital, Parel, Mumbai 400012.

Category: Patient Care

Title : Procedure for estimation of Phenobarbitone (PBT), Phenytoin (PHT) and Carbamazepine (CBZ) in human blood plasma by High Performance Liquid Chromatography (HPLC). Eliminate Nitrogen Gas

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Confidential

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1. Table of Contents

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1. Purpose:

This SOP describes the technique for qualitative and quantitative estimation of Phenobarbitone (PBT), Phenytoin (PHT) and Carbamazepine (CBZ) in human plasma by reverse phase High Performance Liquid Chromatography (HPLC).

2. Scope:

This SOP is limited to the estimation of PBT, PHT and CBZ in µg/ml in human plasma by reverse phase High Performance Liquid Chromatography (HPLC).

3. Responsibilities:

The Head of the department is responsible for the medical care and welfare of all patients pertaining to TDM of PBT, PHT and CBZ. The task of performing estimation of PBT, PHT and CBZ will be delegated to trained personnel who will perform this function.

4. Applicable rules, regulations and guidelines

- ICMR Good Clinical Laboratory Practices Guidelines 2008 (http://icmr.nic.in/guidelines/GCLP.pdf)

5. Reference to other applicable SOPs

- SOP No.24/02: Biomedical waste management.
- SOP No.TDM 01/02: Collection and separation of blood sample for TDM
- SOP No. TDM 05/02: Operation of High-Performance Liquid Chromatography

6. Detailed instructions

1. Preparation of standards and calibrators

1.1 . Preparation of Stock Standards

i. Preparation of Stock Standard (Phenobarbital-PBT, Phenytoin-PHT and Carbamazepine-CBZ) 1mg/ml:

(Phenobarbital-PBT) 1mg/ml	Add 10 mg of PBT in 10 ml of Methanol	Shake it well.
(Phenytoin-PHT) 1mg/ml	Add 10 mg of PHT in 10 ml of Methanol	Shake it well.
(Carbamazepine-CBZ) 1mg/ml	Add 10 mg of CBZ in 10 ml of Methanol	Shake it well.

iv. Preparation of Stock Internal Standard (10- Methoxy Carbamazepine I.S) 1mg/ml:

10-Methoxy-Carbamazepine I.S) 1mg/ml:	Add 10 mg of S.I.S in 10 ml of Methanol,	Shake it well.

V. Preparation of Working Internal Standard (5-ethyl-5-para-tolybarbituric acid-S.I.S) 1mg/ml:

Working standard (IS) I (100µg/ml)	10-Methoxy-Carbamazepine **1mg/ml 1mL**	**9 mL of Acetonitrile.**	**10ml**
Working standard (IS) II (10µg/ml)	**IS (I) 1mL**	**9 mL of Acetonitrile.**	**10ml**

1.2. Preparation of Plasma Standards: 2ml Stock

Blank plasma	**1750 µl**
(Phenobarbital-PBT)1mg/ml Stock	**100 µl**
(Phenytoin-PHT) 1mg/ml	**100 µl**
(Carbamazepine-CBZ) 1mg/ml	**50 µl**

1.3. Preparation of Plasma Standards:

Standard I	0.9 ml blank plasma	0.1 ml of Stock
Standard II	0.8 ml blank plasma	0.2ml of Stock

1.4. Preparation of buffer for Mobile Phase:

- 0.2 M of KH_2PO_4 buffer at pH = 6.0
- Weigh 27.2 g of Potassium dihydrogen phosphate (KH_2PO_4) in 1000 mL of distilled water.
- Adjust pH = 6 with 1 M NaOH.

2. Extraction procedure

Add 100µl of plasma (blank/std/quality control/sample) in 2ml Plastic Eppendorf tube

⇩

Add 100µl of internal standard (10µg/ml) to it Vortex Mix thoroughly for 30 sec

⇩

Add 100µl of Methanol and Vortex Mix thoroughly for 2 min

⇩

Mix it well, white precipitate will be formed

⇩

Extract the drug in organic phase by shaking it well (approx. 100 times)

⇩

Centrifuge at 8000 rpm for 10 min

⇩

Remove 100µl of supernatant and add transfer into HPLC vials.

Inject into HPLC for analysis.

3. Preparation of Mobile phase

a. Take 8.2 mL of buffer + 216.4 mL of Distilled Water + 185 mL of methanol + 90.6 mL of acetonitrile = 500 mL approximately for 40 samples.

b. Filter the mobile phase through **0.22-micron** filter and sonicate for 15 minutes.

4. HPLC Conditions

a. Injecting volume : 10µL

b. Flow rate: 1.3 mL/min.

c. Wavelength: 255 nm

d. HPLC Column: C18 column

e. Run Time: 6.5 min (approximately)

f. Retention times for PBT- 3.4-4.0 min, I.S – 4.20 -4.50 min PHT- 4.6 – 5.0 and CBZ – 5.3 – 6.0 min approximately.

5. Abbreviations:

a. **CBZ** = Carbamazepine

b. **HPLC** = High Performance Liquid Chromatography

c. **I.S.** = Internal Standard

d. **PBT** = Phenobarbital

e. **PHT** = Phenytoin

f. **Std** = Standard