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

SOP No. and Version: TDM05/02

Date first effective : 1st January 2025

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

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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 $\mu\text{g/ml}$ 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

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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 (5-ethyl-5-para-tolybarbituric acid-S.I.S) 1mg/ml:

5-ethyl-5-para-tolybarbituric acid-(I.S) 1mg/ml:	Add 10 mg of S.I.S in 10 ml of Methanol,	Shake it well.
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v. Preparation of Working (7ml stock) Internal Standard (5-ethyl-5-para-tolybarbituric acid-S.I.S) 1mg/ml:

Add 6.5 ml of distilled water in 10 ml stopper tube	Add 0.5 ml S.I.S(5-ethyl-5-para-tolybarbituric acid-S.I.S) 1mg/ml:	7 ml of stock.
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vi. Preparation of Internal Standard (I.S):

Dilution by Acetonitrile (ACN) in the ratio of 1:9

1ml of 7ml stock	9 ml of ACN.	10 ml
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1.2. Preparation of Plasma Standards: 2ml Stock

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

1.3. Preparation of Plasma Standards:

Std I	0.9 ml blank plasma	0.1 ml of Stock
Std 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.

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2.0 Extraction procedure

Add 1 mL of plasma (blank/std/quality control/sample)



Add 1 mL of working internal standard in a clean glass test tube



Mix it well, white precipitate will be formed



Centrifuge at 3000 to 3500 rpm for 20 mins



Take 0.1mL of glacial acetic acid with the help of 1 mL glass pipette in a clean dry 'U' shaped stoppered test tube. To that add supernatant solution of centrifuged samples



Add it to 5 mL of chloroform (organic phase).



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



Centrifuge it again for 20mins at 3000 to 3500 rpm



Discard the middle precipitate (**protein layer**) and centrifuge the U-tube for 5-10 minutes at 2500rpm



Aspirate 4.5 mL (Bottom layer) of the organic solution in 'V' shaped tube without disturbing the upper layer



Evaporate the organic solvent under the continuous stream of nitrogen at **approx.**

45°C till it is fully dried.



During analysis by HPLC method reconstitute the sample with 100µL of methanol.

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2. 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.

3. HPLC Conditions

- a. Injecting volume : 10 μ L
- 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.

4. Abbreviations:

- a. **CBZ** = Carbamazepine
- b. **HPLC** = High Performance Liquid Chromatography
- c. **I.S.** = Internal Standard
- d. **PBT** = Phenobarbital
- e. **PHT** = Phenytoin
- f. **Std** = Standard