**Phenytonin - FREE**

1. **PURPOSE**

Phenytonin (diphenylhydantoin) has been used extensively for seizure control in patients having both grand mal epilepsy (major motor) and cortical focal seizures and temporal lobe epilepsy. Serum level monitoring of the drug is essential in order to achieve maximal seizure control while maintaining minimal blood levels through a routine daily dosage.

Although total phenytonin is traditionally monitored, phenytonin is approximately 90% protein bound in most patients. It is the unbound or free fraction of the drug that possesses the ability to cross membranes or bind receptors. Therefore, the pharmacological effects of phenytonin are related to the concentrations of the unbound or free drug.

1. **TEST PRINCIPLE**

Fluorescence polarization

COBAS INTEGRA systems therapeutic drug monitoring measurements are made on the COBAS INTEGRA systems using the principle of fluorescence polarization. When a fluorescent molecule, or fluorophore, is irradiated with light of the proper wavelength (the excitation wavelength) some of the light is absorbed. Within a few nanoseconds the absorbed light is emitted, although at a longer wavelength (the emission wavelength). Whether or not the emitted light is polarized depends on the freedom of the fluorophore to rotate in solution. A small molecule, such as fluorescein, can rotate rapidly before light emission occurs, results in depolarization of the emitted light. In contract, a fluorescent macromolecule, such as a fluorescein-labeled protein, will rotate much more slowly. Thus, in the time frame between excitation and emission, the macromolecule will have rotated only very slightly and the emitted light will be polarized. Fluorescence polarization is a reproducible function of the drug concentration, and is suitable for the quantitative determination of drug concentrations in serum for the purpose of therapeutic drug monitoring.

Surface active agents are sued to ensure dissociation of the drug from serum proteins and to prevent nonspecific binding of the tracer.

1. **SCOPE**

In vitro diagnostic test for the quantitative determination of free phenytonin in serum or heparinized plasma on COBAS INTEGRA systems.

1. **RESPONSIBILITIES**

| **Group/Person** | **Responsibility** |
| --- | --- |
| Quality Assurance | * Provides Document Control System. |
| Medical Director | * Ensures that the procedure is followed. * Review and approval of this document. |
| Supervisor | * Ensures that the procedure is followed. * Review and approval of this document. |
| End User | * Follows the procedure |

1. **ACRONYMS/DEFINITIONS**

| **Abbreviation** | **Definition** |
| --- | --- |
| VALP | Valproic Acid |
| SW | Strong West |
| URMC | University of Rochester Medical Center |

1. **SPECIMENS**

Un-hemolyzed serum

Sample preparation for the COBAS FP Free Phenytonin assay requires the separation of the free phenytonin fraction from the protein-bound fraction prior to analysis. The COBAS FP Free Phenytonin controls and calibrators do not require this separation step because all of the drug is in the free state. Methods to isolate the free fraction include equilibrium dialysis and ultrafiltration.

Samples should be processed by ultrafiltration upon receipt in the lab, if possible. Alternately, samples may be stored refrigerated at 2-8°C for up to 7 days prior to ultrafiltration, if necessary. Frozen samples should be warmed to room temperature andmixed completely before ultrafiltration. Freezing samples for less than 16 weeks has been reported to have little effect on free phenytonin concentrations. Samples should be securely capped prior to ultrafiltration to prevent pH changes.

Procedure for filtration:

1. Add sample (~1 mL) to the sample reservoir of the assembled ultrafiltration device.

2. Place the filter in a fixed-angle rotor of a centrifuge, capable of maintaining the temperature a 25°C ±3°C. The rotor and centrifuge must be equilibrated to 25°C before adding the filters.

3. Centrifuge at 1000-2000xg for 30 minutes at 25°C ±3°C. AT least 220 uL of ultrafiltrate should be obtained to ensure accurate recovery. Smaller volumes may not yield recovery of the free drug.

Ultrafiltrates containing free drug should be assayed immediately.

Refer to SW.CP.GL.jad.0101 for sample stability.

1. **QUALITY CONTROL**

**QC Materials**

Analyze quality control material as indicated on the Roche Integra analyzer set up form (SW.CP.GL.frm.0101).

**QC Frequency**

Analyze quality control level and frequency as indicated on the Roche Integra analyzer set up form (SW.CP.GL.frm.0101). Analyze all levels of quality control material each time calibration is performed, following any major troubleshooting/service/preventative maintenance or a change to a new lot of reagent.

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Follow the laboratory’s QC procedure for the Roche systems to evaluate QC acceptability and troubleshooting actions. Refer to QC policy SW.CP.GL.adm.0002 and the BioRad Unity Real Time QC procedures.

1. **SPECIAL SAFETY PRECAUTIONS**

Exercise the normal precautions required for handling all laboratory reagents and biohazardous patient samples. Refer to Safety data sheets.

Disposal of all waste material should be in accordance with local guidelines.

Refer to Safety procedure SW.CP.GL.adm.0005

1. **MATERIALS**
2. Equipment

Roche Integra 400 PLUS analyzer

Data Innovations Middleware

BioRad Unity Real Time QC Application

Hettich EBA 21 centrifuge

1. Supplies

Roche Sample cups

Falcon tubes

Pipets

Pipet tips

Serum Filters for ultrafiltration (Cat. No. 21986643 122)

1. Reagents

PHNY reagent (order # 20737879 322)-ready for use

**R1** Antibogy reagent

Anti-phenytonin monoclonal antibody (mouse) in buffer, pH 6.5

**SR** Tracer reagent

Fluorescein-labeled valproic acid derivative in buffer, pH 7.5.

R1 is in position B and SR is in position C.

Shelf life at 2-8 °C

See expiration date on cobas c pack label

On-board in use at 10-15°C-stable for 12 weeks

The on-board in use stability period begins at the time of cobas c pack puncture.

1. Calibrator

COBAS FP Free Phenytonin Calibrator (order # 20759015 322)-A (1 x 3.5 mL) B-F (5 x 1 x 1.5mL)

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| --- | --- |
| Calibration mode | Exponential 5 |
| Calibration replicate | Duplicate recommended |
| Calibration interval | Each lot, every 20 weeks, and as required following quality control procedures |

A calibration curve must be prepared using the COBAS FP Free Phenytonin Calibrators. Calibrators must be placed from the higher concentration (F) first, to the lower (A) last, on the CAL/QC rack. This curve is retained in memory by the COBAS INTEGRA systems and recalled for later use.

Traceability: The COBAS FP Free Phenytonin calibrators are prepared to contain known quantities of valproic acid in normal human serum and are traceable to USP reference materials.

Calibrators should be assayed within 2 hours after placing on-board the instrument.

1. **PROCEDURE**

Refer to general Integra 400 PLUS analyzer operating procedure SW.CP.GL.lab.0101

Test Definition:

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| Measuring mode | FP |
| Reaction mode | R1-SDR2/S-SR |
| Reaction direction | Increase |
| Wavelength A | 485nm (excitation)/515 nm (emission) |
| Reading cycle blank/test | 29/45 |
| Unit | µg/mL |

Pipetting Parameters:

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| --- | --- | --- |
|  |  | Diluent (H2O) |
| R1 | 120 µL | 10 µL |
| Sample | 9 µL | 5 µL |
| Special diluent (SDR II) | 6 µL |  |
| SR | 15 µL | 10µL |
| Total volume | 175 µL |  |

1. **LIMITATIONS**

Refer to SW.CP.GL.jad.0102 for the chart indicating at what Roche H, I, L indice level the test is affected if any.

1. **CALCULATIONS**

COBAS INTEGRA analyzers automatically calculate the analyte concentration of each sample.

1. **MEASURING RANGE**

Refer to the Roche Range Chart for the measuring range guidelines (SW.CP.GL.jad.0104).

1. **INTERPRETATION**

Refer to Reference Range guide for age appropriate reference ranges and critical value levels (SW.CP.GL.jad.0103).

***Critical value(s)***

Age range Critical Low Critical High

n/a n/a ≥ 3.0 µg/mL

The critical value limits and appropriate reporting process for this assay can be referenced by reviewing the URMC Clinical Laboratory Policy Manual, Policy VI-14 Clinical Laboratory Critical Value Immediate Notification Policy (**SH.CP.AA.lgp.0049).**

1. **RESULT REPORTING**

Results are generally reported via the DI Middleware-refer to procedure SW.CP.GL.lab.0103.

1. **TRAINING**

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| **Role** | **Training Needed** |
| Management | Read procedure |
| Employees | Read procedure |

1. **ANALYTICAL SPECIFICITY**

The following cross-reactive, structurally related and/or co-administered substances were evaluated on the COBAS INTEGRA systems in filtered normal human serum spiked with phenytonin at 1.9 µg/mL (7.5 µmol/L). Each substance was tested at 10 times the highest concentration for its therapeutic or normal range, as per the protocol described by NCCLS.12 The imprecision of the assay was taken into account when determining cross-reactivity. Cross-reactivity was designated as “not detectable” (ND) if the obtained value was less than the sensitivity of the assay.

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| Cross-reactivity (%) = | 100 × (analytical result - analyte concentration) | | |
| concentration of interferent | | |
| Drug | | Level tested  µg/mL | Cross-reactivity  % |
| 5-p-(Hydroxyphenyl)-5phenylhydantoin (HPPH) | | 100 | 1.8 |

ND = Not Detectable

1. **REFERENCES**

|  |  |
| --- | --- |
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| 11 | National Committee for Clinical Laboratory Standards. User Evaluation of Precision Performance of Clinical Chemistry Devices; Tentative Guideline. Villanova, PA.: NCCLS;1992;4(12). NCCLS Publication EP5-T2. |
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**Training or Read/Review Signature Log**

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| Trainer(s):  (if applicable, or NA) | | | | |

***Your signature below indicates that you have read/been trained and understood the information.***

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**Preparation of samples for Testing of Free Phenytoin and Free Valproic Acid on the Integra 400 - Skills Assessment**

Trainee Name: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Trainer Name: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Review Date: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

| **Skill** | **Trainee**  **Initial and date** | **Trainer**  **Initial and date** |
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**Employee name (print)**

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**Employee signature (Date)**

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**Supervisor name (print)**

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**Supervisor signature (Date)**