SOP Title: TnBP Analysis Using LCMS
SOP Number: 23016
Revision: 00

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1. PURPOSE

The determination of Tri(n-butyl)phosphate (TnBP) residue in biopharmaceutical products using liquid chromatography with mass spec detection. Biopharmaceutical products are extracted with acetonitrile, centrifuged and supernatant is analyzed. TnBP is analyzed by liquid chromatography coupled with electrospray ionization MS/MS monitoring selected positive ion transition of 267 m/z to 99 m/z. Peak areas are measured and quantified against a standard curve that is generated.

2. SCOPE

This Procedure applies to Pa/QC personnel who performed the analysis.

3. RESPONSIBILITIES

3.1 The Director, Technical Operations, Process Analytics/Quality Control (PA/QC)

- Defines this procedure.

3.2 Process Analytics/Quality Control (PA/QC)

- Trains Laboratory personnel.
- Performs this procedure.
- Reviews data

3.3 BQ

- Provides quality oversight.

4. MATERIALS AND REAGENTS

Part Number	Description	BDP Approved Substitution Permitted?
21471	1ml pipette tips	☒ YES ☐ NO
21470	200 µL pipette tips	☒ YES ☐ NO
21472	20 µL pipette tips	☒ YES ☐ NO
20006	15 mL Centrifuge Tube	☒ YES ☐ NO
31065	Acetonitrile, HPLC grade	☐ YES ☒ NO
30881	0.1% FA (formic acid) in water	☐ YES ☒ NO
30880	0.1% FA (formic acid) in Acetonitrile	☐ YES ☒ NO
10060	Tri(n-butyl)phosphate	☐ YES ☒ NO
20595	1.5mL Microcentrifuge tubes	☒ YES ☐ NO
22127	Waters Acquity UPLC peptide BEH C-18 column	☐ YES ☒ NO
N/A	High Quality water (which is deionized, reverse-osmosis, Milli-Q, WFI or other purified water)	☒ YES ☐ NO

5. EQUIPMENT

- Waters UPLC system.
- Waters Xevo-G2-XS QTOF system.
- Select HEATBlock, VWR scientific products.
- Pipettes 1ml, 200ul, 20ul.
- Tabletop Centrifuge

6. PROCEDURE

6.1 Sample Handling and Storge

6.1.1 Received frozen samples are stored at ≤ -70°C in PA/QC and thawed at ambient temperature prior to use.

6.1.2 Any remaining samples are stored at ≤ -70°C after use.

6.2 Sample Preparation

6.2.1 Preparation of Biopharmaceutical Sample

6.2.1.1 A 2x100 µL aliquot of sample is mixed with 900 µL of acetonitrile in a 1.5mL centrifuge tube to produce a protein precipitate of the sample.

6.2.1.2 Allow mixture to sit at ambient temperature for 15 minutes.

6.2.1.3 Centrifuge tube of protein precipitate at 12,000 x g for 20 minutes.

6.2.1.4 Transfer approximately 900 µL of the supernatant from each tube to a 15 ml tube.

6.2.1.5 Preparation of samples will be documented on **Form 23016-02 TnBP testing form.**

6.3 Instrument Operating Conditions

6.3.1 Waters Acquity UPLC System (Following **SOP 22964**)

6.3.1.1 Column: Waters Acquity UPLC peptide BEH C-18 column, BDP PN 22127 or equivalent.

6.3.1.2 Mobile phase composition: A= 0.1% FA (formic acid) in Water and B = 0.1% FA (formic acid) in Acetonitrile

6.3.1.3 Document outlining the conditions that were used for the operation of LCMS system will be printed and included in the test report.

6.4 Calibration Curve Samples

6.4.1 Dilution 1: prepare fresh 1mg/mL solution of TnBP in Acetonitrile by diluting 10.3 µL of TnBP (Density: 0.9729 g/mL) with 9989.7 µL of Acetonitrile to make 10mL of Dilution 1.

6.4.1.1 Dilution 2: prepare a 100 µg/mL of TnBP in Acetonitrile by diluting 1mL of Dilution 1 with 9mL of Acetonitrile.

6.4.1.2 Dilution 3: prepare a 10 µg/mL stock solution of TnBP in Acetonitrile by diluting 1mL of Dilution 2 in 9mL of Acetonitrile.

6.4.1.3 Preparation of calibration standards will be documented on **Form 23016-02 TnBP testing form.**

6.4.1.4 Prepare Calibration Standards in duplicate from Dilution 3.

Standard ID	Volume Of Dilution 3 (mL)	Volume Of Acetonitrile (mL)	Concentration Of TnBP (µg/mL)
Blank	0	10.0	0
1	1.0	9.0	1.0
2	0.5	9.5	0.5
3	0.25	9.75	0.25
4	0.1	9.9	0.1
5	0.05	9. 95	0.05
6	0.01	9. 99	0.01

6.4.2 Preparation of Spike Sample in duplicate

Sample ID	Volume Of Dilution 3 (µL)	Volume Of Acetonitrile (µL)	Sample From Step 6.2.1.4	Concentration Of Tnbp (µg/mL)
Sample A	0	900	100	0
Sample A + Spike 1	5	895	100	0.05
Sample A + Spike 2	10	890	100	0.1

6.4.3 Analyze calibration standards in duplicate by LCMS. Remove the peak area value of blank solution from the standard samples. Prepare a calibration curve of concentration in µg/mL TnBP versus peak area in Excel. Use Excel to fit the points and determine the equation of the fit and R^2 value. If the R^2 value is ≥ 0.95 it would be accepted for the validity of the test. If R^2 value is not within the limits, then the highest and/or lowest concentrations may be removed, and remaining points may be fit using Excel. If removing of some data points provide an acceptable R^2 value, then the equation from those points may be used. If removal of some points does not provide a R^2 value within limits the assay needs to be reperformed. This will be documented on **Form 23016-03** to specify if some points are not used in the analysis.

6.4.4 Analyze biopharmaceutical sample solution plus spikes from section 6.4.2 in duplicate by LCMS. Calculate the concentration of Tnbp in sample and sample plus spikes using the equation generated in 6.4.3 to determine sample concentration of TnBP. If the spike recovery is within 100 ± 30% of spiked amount, then the assay is accepted as working, if not it needs to be reperformed.

6.4.4.1 Peak areas will be documented on ***Form 23016-03 – Peak Readings for the determination of TnBP concentration.***

6.4.4.2 TnBP in undiluted sample can be calculated as following.

TnBP (µg/mL) in undiluted sample = Calculated concentration * 10 (Dilution factor)

6.4.5 Results for the determination of TnBP will be documented on ***Form 23016-04 Determination of TnBP Concentration***.

7. DOCUMENTATION AND RECORDS

Document on Form 23016-01, 23016-02, 23016-03 and 23016-04. Mass spec operating conditions will be printed and included as part of the documentation.

8. REFERENCES AND RELATED DOCUMENTS

Document Number	Title
22702	Solutions Used in Process Analytics
22955	Routine Calibration of the Waters Xevo G2-XS QToF (QToF) Mass Spectrometer
22964	Operating the Waters Acquity UPLC Using MassLynx
22971	Guidelines for Analyses Conducted on the UPLC/QTOF Mass Spectrometer
23016-01	TnBP testing – Reagents, Materials, and equipment.
23016-02	TnBP testing form
23016-03	TnBP testing – Peak area readings for determination of TnBP concentration
23016-04	Determination of TnBP Concentration