Antibody-drug conjugates (ADCs) are a novel class of therapeutic agents, in which one or more small cytotoxic molecules (payload) are linked covalently to a monoclonal antibody (mAb). The exposure of unconjugated payload and/or its metabolites in circulation is important for the evaluation of the safety and efficacy of ADCs. In this work, we describe the development and validation of an LC-MS/MS method for the determination of unconjugated DM4 and DM4-Me in human plasma. DM4, a derivative of maytansine, is the cytotoxic payload of an ADC developed at Covance Laboratories Inc., Madison, WI; 2Novartis Institutes for BioMedical Research, East Hanover, NJ.

**Introduction**

Antibody-drug conjugates (ADCs) are a novel class of therapeutic agents, in which one or more small cytotoxic molecules (payload) are linked covalently to a monoclonal antibody (mAb). The exposure of unconjugated payload and/or its metabolites in circulation is important for the evaluation of the safety and efficacy of ADCs. In this work, we describe the development and validation of an LC-MS/MS method for the determination of unconjugated DM4 and DM4-Me in human plasma. DM4, a derivative of maytansine, is the cytotoxic payload of an ADC developed at Novartis Institutes for BioMedical Research, East Hanover, NJ. DM4 contains a free sulfhydryl group, which reacts with other thiol-containing molecules in biological matrices to form conjugates, leading to underestimation of the free payload concentration. This renders a technical challenge in developing a robust bioanalytical assay in support of clinical development.

**Method**

Antibody Drug Conjugate (ADC) and DM4

![Image](Image1.png)

**Figure 1. Representation of ADC and structures of DM4 and DM4-Me. Isotope labeled DM4 and DM4-Me were used as Internal Standards (structures not shown).**

**Sample Preparation Procedure**

1. **50.5 µL aliquot of Human Matrix**
2. **25.0 µL ISTD mix**
3. **Protein precipitation w/ ACN:MeOH:FA (90:10:0.1, v:v:v)**
4. **Reduction using TCEP for 90 min at 60°C**
5. **Transfer supernatant**
6. **Reduction using TCEP recovers the endogenous conjugated DM4**
7. **Whole Blood Stability on wet ice for up to 2h**
8. **Stability in plasma on wet ice for up to 24h, after 5 freeze-thaw cycles, 9 days in freezer set at <-60°C**
9. **Matrix effect in hemolysis or hyperlipidemia plasma**
10. **Matrix effect in hemolysis or hyperlipidemia plasma**
11. **The stability of the linker was evaluated by stressing the ADC spiked QCs prepared in human plasma**
12. **The linker was stable for the sample preparation steps, on wet ice up to 24h, after 5 freeze-thaw cycles, 9 days in freezer set at <-60°C**

**Conclusion**

- A sensitive and robust method for the quantitation of DM4 and DM4-Me was developed and it is currently being validated according to FDA guidance for bioanalytical method validation.
- Reduction step in the sample preparation aids deconjugation of DM4 from the matrix components to facilitate reliable quantitation of unconjugated DM4 in human plasma.

**References**


**Summary**

- Quantitative method for unconjugated DM4 and DM4-Me in clinical study.
- Choice of matrix (Plasma vs Serum) driven by analyte properties.
- Extraction: Protein precipitation followed by chemical reduction and SPE.
- Calibration range for DM4 and DM4-Me: 0.100-50.0 ng/mL.

**LC-MS/MS**

**Parameter**

- **Compound Name**
- **Transition**
- **Retention Time (min)**

**Accuracy and Precision**

**Bias**

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<th>Compound Name</th>
<th>Nominal Concentration (ng/mL)</th>
<th>LLOQ QC (n=6)</th>
<th>LQC (n=6)</th>
<th>LMQC (n=6)</th>
<th>MQC (n=6)</th>
<th>HQC (n=6)</th>
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<td>DM4</td>
<td>0.103 ± 0.315</td>
<td>2.48 ± 1.03</td>
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<td>DM4-Me</td>
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<td>2.33 ± 0.7</td>
<td>19.7 ± 3.4</td>
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<tr>
<td>ISTD2</td>
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**Significance of Reduction Step: Choice of Matrix**

- **Protein precipitation using ACN:MeOH:FA (90:10:0.1, v:v:v)** provided the best ADC precipitation efficiency (data not shown). This is a crucial step to remove the ADC and prevent it from undergoing reduction.
- **Conjugation is more predominant at room temperature compared to wet-ice conditions in blood or plasma (Data not shown).**
- **Sample thawing and processing is on wet ice.**
- **TCEP, DTT and 2-Mercaptoethanol were evaluated for reduction step.**
- **Reduction using TCEP recovers the endogenous conjugated DM4 more efficiently in human plasma vs. in human serum, driving the choice of matrix as human plasma for this clinical study.**

**Talking point:** Why does human serum fail to show similar DM4 recovery and matrix factor?