Bioanalysis ZONE

Alturas Analytics
Bioanalytical Experts

Method development for small molecules: a step-by-step guide

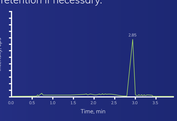
Method development begins with selecting a solvent in which the target analyte is both **soluble** and **stable**. Once this is established, the molecule should be tuned on the most appropriate platform based on the sensitivity requirements of the assay.

If a low pg/mL detection limit is required:
Many newer instruments have the capability to reach these very low limits of detection. The mass spectrometer — typically a triple quad — conditions should be optimized to find the most abundant fragment and a potential backup fragment in case the most abundant has selectivity interferences when extracted from matrix.

If a selective fragment cannot be found:
High Resolution Mass Spectrometry (HRMS) can be used. Some HRMS instruments are not capable of accurate quantitation, however advanced technology has been shown to provide acceptable accuracy and precision for small molecules.

Once the MS method has been established, the liquid chromatography (LC) method should be developed

- The column selection should be based on the lipophilicity of the analyte, which is sometimes unknown for novel compounds where a chemical structure is not provided.
- A C18 column with 0.1% formic acid in water (mobile phase A) and Acetonitrile 0.1% formic acid (mobile phase B) should be tested first.
  - This column and mobile phase are used in many small molecule methods, allowing the platform to be used for multiple methods without switching the column or mobile phases.
- A good starting gradient at T=0 is 5% mobile phase B, ramping up to 95% at T=3 minutes.
- The retention factor for the analyte should be >5 with adequate peak shape. The gradient should be altered to increase the retention if necessary.



- If the analyte elutes at 95% organic, change the column to a C8. If retention can't be retained with a shallow gradient, change the column to a more polar phase and/or add buffer or ion pairing reagent.
  - The aqueous/organic solvent ratio should be tested to verify good peak shape at an injection volume of up to 40 µL.

Extraction method development criteria

Extraction method development should start with simple protein precipitation methods, advancing to liquid/liquid extractions or solid phase extraction if necessary.

Sample volume
The assay should be developed keeping in mind the sample volume available.

Samples for analysis
Enough sample should be available for three analysis batches.

Precipitation volume
The precipitation volume should be no less than 5X the sample volume.

Internal standard
Should be added in a small volume immediately after the sample is aliquoted for extraction.

Once the extraction method is developed, **selectivity**, **matrix stability** and **matrix effects** should be tested.

If enzymatic instability is observed:
the plasma should be stabilized with an enzyme inhibitor, i.e., sodium fluoride, dichlorvos or potassium oxalate anticoagulant.

If chemical instability is observed:
the plasma should be stabilized with the addition of acid.

The developed method may then be transferred to an **automated sample extraction system**.

Automated sample extraction systems

Automated sample extraction systems are capable of tracking entire batch processing with an integrated barcode reader. Many systems are installed with a tube decapper, shaker, evaporator, positive pressure manifold and centrifuge, and can process multiple plates to completion. Additionally, systems may aliquot samples (clot detection enabled), dilute samples and add the internal standard.

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