Project:

Free/Bound Drug ratio – LC-MS/MS

Measurement of free versus encapsulated /conjugated drug ratio

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1 Introduction

Controlled release systems for delivery of drugs have a number of properties that can be very advantageous to the treatment of medical conditions. Targeting of the delivery system to the desired site of therapeutic action will strongly reduce undesired systemic side effects. In addition, a sustained release could help maintain therapeutically optimal concentrations of the drug with significantly longer intervals between administrations than what is the case with free drugs. Finally, the encapsulation of drugs might help them escape first-pass and other undesired metabolism and thus bring a much larger proportion of the injected drug to the site of action.

To verify that the drug is actually encapsulated rather than just co-delivered in solution together with nanoparticles, it is necessary to quantitate the amount of free drug vs. the amount encapsulated. This is important for the interpretation of observed biological toxicity data *in vitro* and *in vivo*, and for assessment of pharmacodynamic/pharmacokinetic observations *in vivo.*

2 Principle of the Method

Ideally, drug concentration should be measured separately for the loaded nanoparticles (bound drug) and the surrounding liquid medium (free drug). For many nanoparticles, however, the complete separation from the medium is very challenging, as they are not easily separated by centrifugation. The practical approach will then commonly be to quantify the drug in the complete sample (including medium and nanoparticles) and then subtract the amount of drug measured in the medium not containing nanoparticles (free drug). It then becomes critical to separate the medium from the particles by a method that does not disturb their integrity or induce leakage of drug by other means. It should be noted that any kind of separation of the particles from the surrounding medium might influence the concentration equilibrium of the drug between the encapsulated and the free state. This will be particularly important in cases where a significant fraction of the drug is found dissolved in the medium, e.g. when solubility is relatively high.

The method for separation preferred here is the use of ultrafiltration, specifically centrifugal filters with specific molecular weight cut-off. After separation, drug concentrations in the particle-free medium is measured by LC-MS/MS, referenced against quantification standards of the same drug. Total drug is measured as described in SOP 'EUNCL-PCC-30 total drug loading'. In general, the same LC-MS/MS methodology can be used also for the free drug measurements, although the sample used for analysis (injected in the instrument) should have approximately the same solvent composition, i.e. the same proportion of the organic solvent as used in the total drug measurements. This is to avoid possible matrix effects during analysis.

3 Applicability and Limitations (Scope)

The same limitations apply here as to the measurements of total drug loading, i.e. that the methodology described here is applicable to all controlled release drug formulations where the drug can be fully extracted from the release formulation with a suitable solvent. This implies that it should be ensured the solvent could solubilize both the nanocarrier and the encapsulated drug in the relevant concentrations.

In the quantification step, it is required that the drug can be sufficiently well separated from components of the controlled release formulation so that interferences during the ionization process are negligible. This also implies that the drug can be ionized with one or several of the available ionization methods to provide sufficient detection sensitivity, and that it can generate suitable molecular fragments for MS/MS verification. This should be obtainable for virtually all organic compounds by careful selection of separation and ionization parameters.

4 Related Documents

Table 1:

EUNCL_PCC_30	**Measuring Total Drug Loading – LC-MS/MS**
EUNCL_PCC_31	**Measuring Lipid Composition – LC-MS/MS**

5 Equipment and Reagents

Equipment and reagents used for measuring the total drug loading (used to calculate the encapsulated drug) is described in the SOP EUNCL_PCC_30 'Measuring Total Drug Loading – LC-MS/MS'.

5.1 Equipment

For separation of the medium from the nanoparticles, Amicon Ultra Centrifugal Filters (Millipore) with regenerated cellulose (RC) membrane is the preferred starting point. These are available in a range of volumes; generally, the smaller sizes (0.5 ml or 2 ml) volume will be sufficient. In terms of molecular weight cut-off (MWCO), the 10 kDa or 30 kDa should be suited for most small molecule drugs; MWCO up to 100 kDa are available. As a rule of thumb, the nanoparticle (or other compound) to be retained above the membrane should be at least 5 times the MWCO. Also, alternative ultrafiltration systems exist with other membrane materials, *e.g.* polyethersulfone; these could be applicable if unacceptable binding is found to occur with the RC membranes.

A standard centrifuge with adjustable speed should be used for the filtration step.

For analysis of the extracted drug, a liquid chromatography system coupled to a tandem mass spectrometer should be used. The chromatography system will typically consist of an automated liquid sampling unit, a binary or quaternary mobile phase pump, a vacuum degasser for the mobile phases, and a thermostatted column compartment, in addition to the suitable chromatography column. The mass spectrometer should be either a quadrupole-time-of-flight (QTOF) or – preferably – a triple quadrupole instrument, equipped with the suitable ionization interfaces. For most applications, an electrospray (ESI) ion source will be suitable; for some specific analytes, however, chemical (APCI) or photon-induced (APPI) ionization might be necessary.

5.2 Reagents

Mobile phases for LC-MS/MS are prepared from standard reagents (organic solvents, volatile buffers, water) with sufficient purity, typically HPLC or – preferably – LCMS grade.

For quantification, standard curves will be built using solutions with known concentrations of the *free drug*. Pure drug standards are preferably obtained from the sponsor, but can also be purchased as analytical standards from commercial sources.

When *empty nanocarriers* can be obtained from the sponsor, it is highly advantageous to run these as negative controls in the experiments to exclude the possibility of any matrix interferences.

Where available and not prohibitively expensive, a *standard of the drug labeled* with stable atomic isotopes (e.g. ^{2}H, ^{13}C, ^{15}N) should be used to ensure exact quantification. Such labeled compounds can often be delivered by the sponsor, otherwise they have to be purchased commercially or synthesized on demand. The cost of the latter option can potentially be very high.

5.3 Reagent Preparation

All drug calibration series and LC-MS/MS mobile phases are prepared according to normal procedures. Calibration samples are prepared in the same solvent matrix as the samples to be measured.

6 Procedure

6.1 General remarks

The method described here involves specific method development for each new drug, both for drug extraction and LC-MS/MS analysis. New formulations of drugs for which a method for quantification already exists, might require only minor modifications of the existing method. The method development will normally be done through the measurement of total drug loading (SOP EUNCL_PCC_30), and the same LC-MS/MS method should be applicable.

Stability data on both the nanoformulation and the free drug in different aqueous and organic solvents should be requested from the sponsor. The same applies to solubility data, and is of particular interest with regards to the (predicted or measured) solubility of the drug in the surrounding medium, which will generally be aqueous.

The separation method and equipment should be chosen based on the physico-chemical characteristics of the free drug and the nanoformulation. For example, the selection of pore size of the centrifugal filters should be verified depending on particle size (determined in the batch mode DLS measurement, EUNCL_PCC_001) and the choice of filter material should be based on the expected charge of the drug molecule and the nanoparticles in the given buffer (Zeta potential, EUNCL_PCC_002).

Specifically, for the separation of free from bound drug, the compatibility of the filtration with the specific drug/nanoparticle system has to be verified. Non-specific binding of drug to the filter device has to be tested at relevant concentrations. Also, integrity of the nanoparticles during separation should be tested. This can be tested by performing dynamic light scattering (DLS) on the filtrate; the presence of nanoparticles indicates that not only the free drug has crossed the membrane. DLS can also be performed on the retentate and compared with the original sample to check for

agglomeration/aggregation. Centrifugation should be performed at the lowest practical g-force (speed) to minimize the shear and compression forces on the nanoparticles.

Centrifugal filter separation of the free drug from the particles should be performed in the original reconstituted nanoparticles, i.e. in the clinically relevant suspension. The solution should not be diluted before filtration, as this could easily shift the bound vs. free drug equilibrium, especially if the free drug is at or near saturation concentration in the buffer.

6.2 Workflow

It is assumed that total drug loading has already been measured, according to SOP EUNCL_PCC_30.

If possible, a preliminary LC-MS/MS quantification of the free drug could be performed in order to choose optimal concentrations for the subsequent standard curve. This could be done by running a filtered sample (cfr. below) against one or two free drug standards to estimate the drug concentration (order of magnitude). Under no circumstance will the highest free drug concentration exceed the measured total drug loading, so if no pre-quantification is performed as described above, a reasonable highest standard concentration will be equal to the total drug concentration measured.

Standard samples of the free drug should be prepared from stock solution with known concentration, diluted in the same buffer as used to reconstitute the nanoparticles (the medium). These standards will be used to assess binding of the drug to the filter membrane, and concentrations should be chosen to be both above (or at) and below the sample target concentration of free drug.

The following suspension/solutions are loaded in separate ultrafiltration devices, and centrifuged at the lowest practical speed (g force):

1. [Sample NSD]: The nanoparticle suspension (i.e. drug-loaded nanoparticles suspended in the recommended buffer).
2. [Samples STFD]: Standard samples of the free drug, diluted from stock solution with known concentration in the same buffer as used to reconstitute the nanoparticles (the medium).
3. [Sample BM]: One blank medium (no drug added) to check for possible interferences from the filter
4. [Sample NSDhigh]: One nanoparticle suspension identical to 1. above, but *centrifuged at significantly higher speed* (g-force).

Sample 'NSDhigh' above will serve as a potential positive control; if Samples 'NSD' and 'NSDhigh' give different results by either DLS measurements and/or drug quantification, this is an indication that breakage/leakage/aggregation might be occurring in one *or both* samples. If no differences are seen, this is an indication that the centrifuge filtration is sound.

Drug in the samples are then quantified by LC-MS/MS, referenced against calibration standards. The following samples should be measured:

- Calibration samples
- QC samples

- Filtrates from the samples NSD, NSDhigh, STFD and BM as described above, diluted in the same solvent (matrix) as the calibration samples and QC samples
- Original (non-filtered) samples NSD, NSDhigh, STFD and BM as described above, diluted in the same solvent (matrix) as the calibration samples and QC samples

The measured free drug is then calculated as a fraction of measured total drug, and the encapsulated drug is then calculated by subtraction of the free drug from the total drug.

LC-MS/MS method development has generally been performed previously during measurements of drug loading. A flow chart describing the process is shown below in Figure 1 for clarity.

DLS measurements should be performed and compared for the following sample sets:

- Filtrates NSD, NSDhigh, BM and the STFD standard with the drug concentration found to be closest to the NDS sample
- Retentates NSD and NSD high, as well as the original unfiltered NSD sample

Exact measured DLS values are not critical; the comparison within the groups is the key measurand. Ideally, no differences in DLS sizes should be observed within each of the above two groups. Note that particles concentrations will generally be different in the retentate samples, and that chromogenic drugs might interfere with the DLS measurements.

6.2 Reporting

The measurements report should include the following:

- Product number, CAS number and batch number of drug standard(s) used, including isotope labeled compounds if applicable
- Choice of solvent for extraction of drug from nanocarriers, and volumes used (solvent and nanomedicine sample)
- Predicted drug concentration (from sponsor)
- Any observations made during drug extraction (precipitation, color changes, other phenomena)
- The drug concentrations of the calibration samples, and the linearity of the calibration curve reported as the R^2 value
- The measured concentration of the QC samples
- The measured concentration of the drug samples, including variability between the replicates. Also, if dilution parallels of the sample are analyzed,
- Analytical hardware used, and instrument settings including LC gradient and ionization parameters. This can take the form of a standard instrument-generated run report.

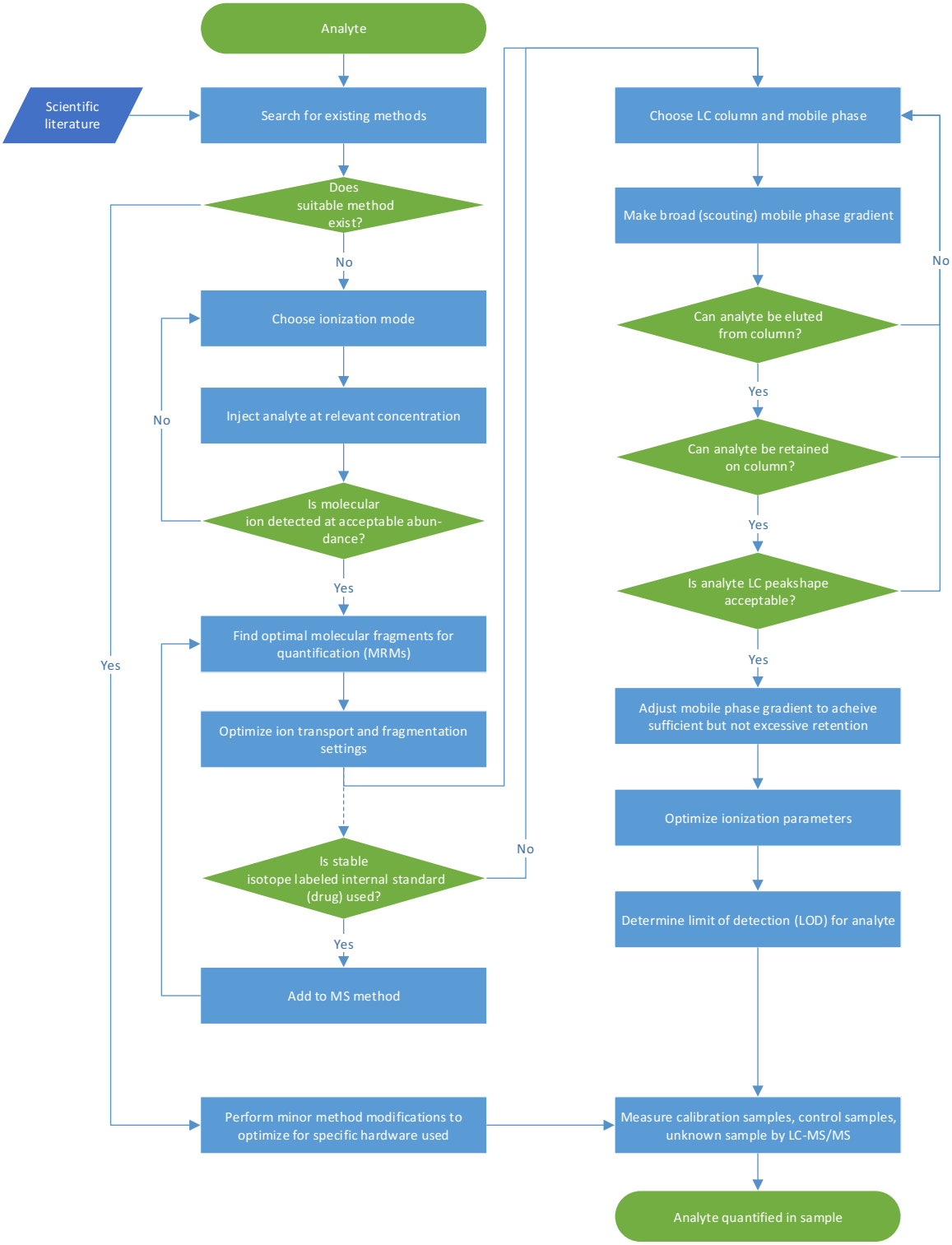

Figure 1. Brief outline of work for LC-MS/MS method development.

7 Quality Control, Quality Assurance, Acceptance Criteria

DLS analysis of the separated fractions should verify NP integrity during the process. Separation methods with more than 10% change in the zeta-average value of the concentrate compared to the

starting material are not acceptable. Presence of NPs in the filtrate suggests non-suitable separation method/equipment.

Spiking experiments should be performed in order to exclude matrix effect or interference caused by the NPs in the chosen analytical method.

Calibration curves for drug quantification should encompass (at least) 5 concentration points. The curves should be measured in triplicate throughout the run sequence, including one complete calibration curve at beginning of sequence, one at the end of the sequence. The analyzed sample concentration should fall between two calibration points. Samples should be extracted and measured in triplicate. Both the calculated mean concentrations and corresponding standard deviations, retention times and peak area values have to be reported both for the calibration points and sample measurements. For the calibration curves, the curve fit linearity (R^2 value) should be given.

It is strongly advisable that stable isotope labeled internal drug standards is used during the quantification to correct for matrix effects and sample preparation losses. The labeled standard should be added in the same concentration to samples and calibration curves, and should follow the analyte (drug) during the sample workup. If an internal standard is *not available*, it is necessary to analyze at least two dilution parallels of the sample and check that measured concentration is in concordance with the dilution factor. This reveals possible matrix effects, as matrix interferences in LC-MS/MS don't scale linearly with dilution.

QC samples should be prepared the same way as the calibration samples, but in a separate preparation (weighing, dissolution). Specifically, the QC samples should be prepared by a different person, who should only get as input the solvent, the non-dissolved drug and the desired end concentrations; all calculations should be performed separately by the QC preparing person. At least two QC samples with different drug concentrations should be run. These should be analyzed at least two times during the sampling sequence to reveal instrument drift. If no preexisting stability data is available (from literature or sponsor) for the drug in the chosen solvent, QC samples should be prepared at least 2 time points and run in the same LC-MS/MS sequence to assess stability of the analyte.

8 Health and Safety Warnings, Cautions and Waste Treatment

Samples should be prepared with care to protect the sample from particulate and to minimize exposure; appropriate safety precautions and protective gear such as gloves, lab coat and goggles must be worn. After the measurement, the carrier containing samples should be discharged as appropriate for nanomaterials. Eluted HPLC mobile phase containing test drug should be discharged as appropriate for the active ingredient.

9 Abbreviations

DLS dynamic light scattering

LC liquid chromatography

MS mass spectrometry

MWCO molecular weight cutoff