Project:

Measuring Lipid Composition – LC-MS/MS

Verification of expected lipid composition in nanomedical controlled release systems by liquid chromatography–tandem mass spectrometry

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Version	Approval Date	Description of the Change		Author / Changed by
1.0	12/05/2016	All	Initial Document	Sven Even Borgos

Version	Approval Date	Description of the Change		Author / Changed by
1.1	31/03/2017	7 4	Specified preparation of QC samples by a separate person. Added reference to two other SOPs in Chapter 4.	Sven Even Borgos

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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.

For lipid based delivery systems (e.g. liposomes), the presence and proportion of lipids should be verified in the final formulation. These are generally major components present in significant concentrations. It should be noted, however, that the lipid components might not be stoichiometrically pure, e.g. they could be PEGylated with polydisperse PEG chain lengths.

2 Principle of the Method

In order to measure the lipid composition, the delivery vehicle (nanoparticle) should be dissolved and non-covalent interactions should be broken. That is done in this SOP by dissolving the nanoparticles in a suitable solvent, commonly an organic solvent like methanol, acetonitrile, acetone, DMSO or halogenated solvents.

Quantification of the lipids in the solubilized state is performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). This ensures precise quantification with very high specificity as the compound of interest has to match all of the following criteria:

- Retention time on a specific LC stationary phase with a specific mobile phase composition, i.e. highly defined chemical molecular interactions of the compound with the two-phase separation system
- Molecular mass
- Mass and relative intensity of molecular fragments generated when the intact molecular ion is fragmented in the gas phase in the mass spectrometer

In principle, the extraction, separation and detection parameters have to be specifically optimized for each lipid-containing delivery system, even though small structural variations in lipids can usually be run within the same chromatography methods. MS/MS fragmentation is always fully compound specific. This will generally imply a certain method development work, or adaptation of previously developed methods.

3 Applicability and Limitations (Scope)

The methodology described here is applicable to all controlled release drug formulations where the lipids can be fully extracted from the release formulation with a suitable solvent. This implies that it should be ensured the solvent could solubilize all lipids to be measured in the relevant concentrations.

In the quantification step, it is required that the lipids can be sufficiently well separated from other components of the controlled release formulation so that interferences during the ionization process are negligible. This also implies that the lipid 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.

3.1 Unknown lipid components

The current method only applies to quantification of known components. If possible, however, it could be considered to complement this with an LC-MS scan for unknown major components. This could be lipid contaminants or altered (e.g. oxidized) lipids. This would not fully exclude the possible presence of unknowns, since no LC-MS method can be optimal for all types of compounds simultaneously. It could, however, give an indication of whether contaminants are present. Please note that in the case of e.g. PEGylated lipids, the desired lipid components might already constitute a highly complex mixture, which would complicate the observation of unknowns.

4 Related Documents

Table 1:

Document ID	Document Title
EUNCL_PCC_30	Measuring Total Drug Loading – LC-MS/MS
EUNCL_PCC_32	Free/Bound Drug Ratio – LC-MS/MS

5 Equipment and Reagents

5.1 Equipment

For extraction of lipid, pipettes and vortex mixers are generally sufficient.

For analysis of the extracted lipids, 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

For *extraction of lipids* from nanocarriers, standard organic solvents are used. The exact choice of solvent depends on the solubility of the lipid in question.

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 lipid*. Pure lipid 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 controls in the experiments to exclude the possibility that addition of the payload drug has altered the carrier significantly.

Where available and not prohibitively expensive, a *standard of the lipid labeled* with stable atomic isotopes (e.g. ^{2}H, ^{13}C, ^{15}N) can 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 lipid 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 lipid, both for lipid extraction and LC-MS/MS analysis. New formulations including lipids for which a method for quantification already exists, might require only minor modifications of the existing method.

Stability data on both the nanoformulation and the free lipid in different aqueous and organic solvents should be requested from the sponsor. The same applies to solubility data, as this will make the initial choice of extraction protocol as effective and rational as possible.

6.2 Flow chart

A flow chart outlining the overall method is shown in Figure 1. A separate flowchart describing the general principles for LC-MS/MS method development is shown in Figure 2.

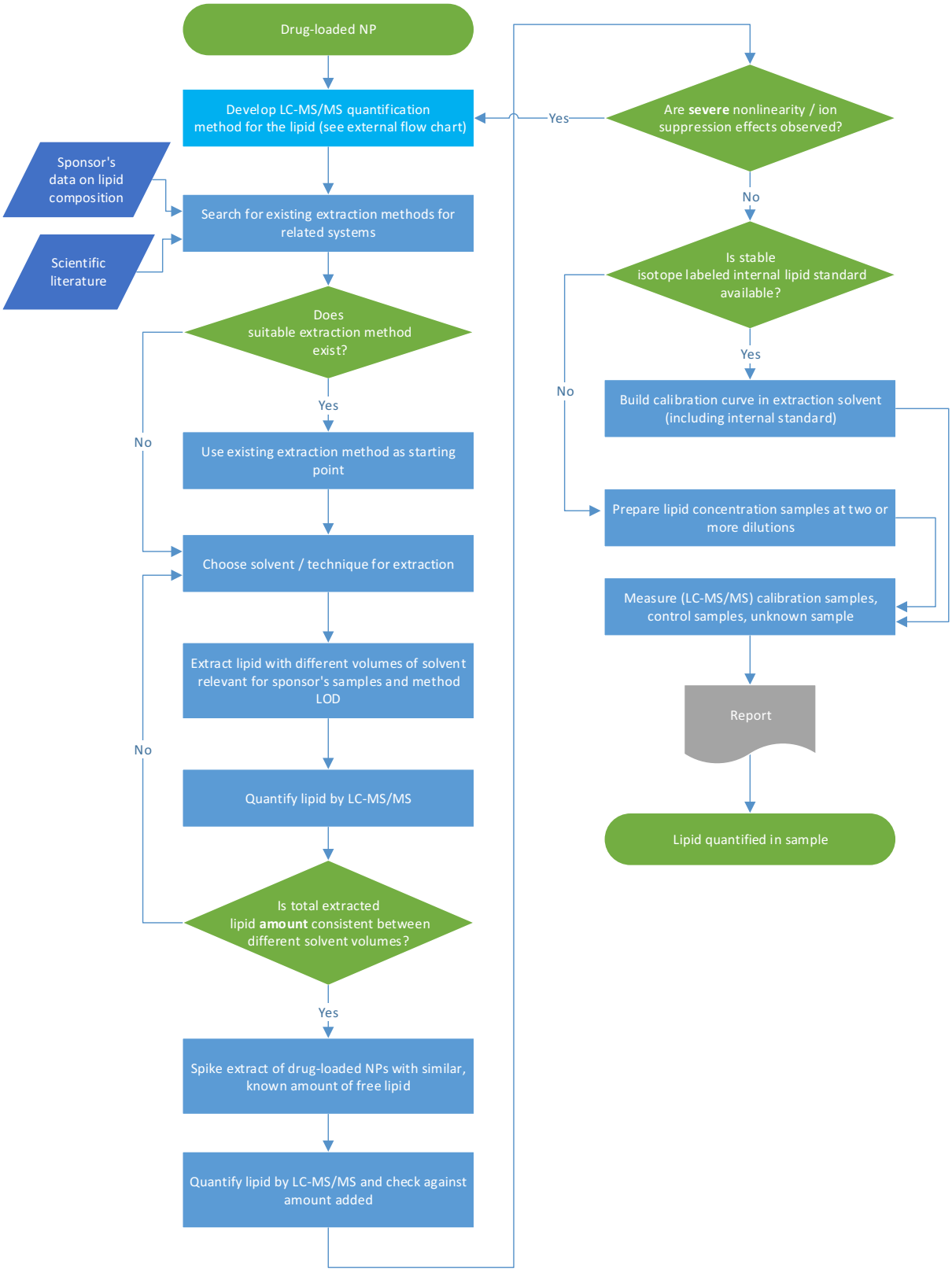

Figure 1. Brief outline of work flow for complete extraction and analysis.

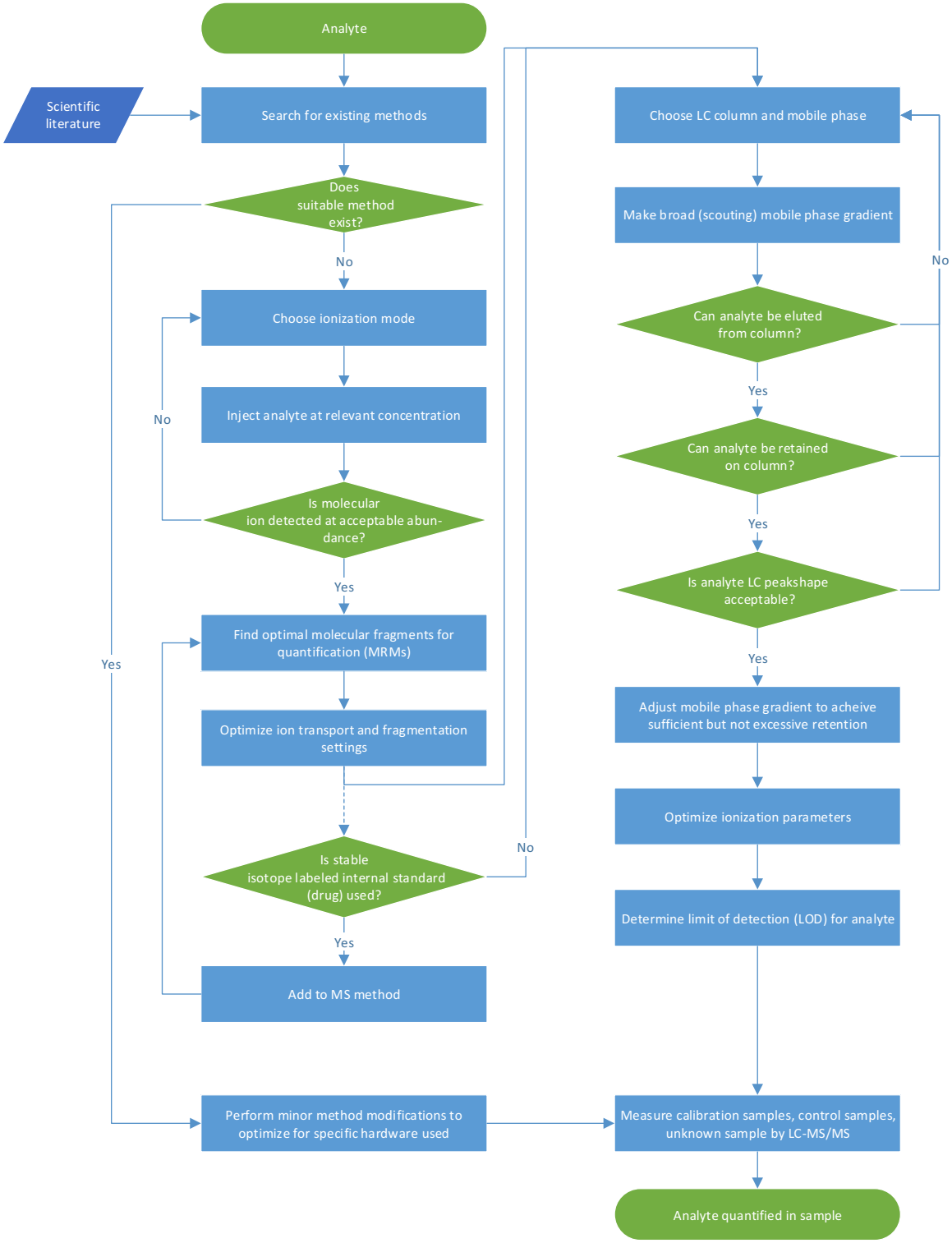

Figure 2. Brief outline of work for LC-MS/MS method development.

6.3 Reporting

The measurements report should include the following:

- Product number, CAS number and batch number of lipid standard(s) used, including isotope labeled compounds if applicable
- Choice of solvent for extraction of lipid from nanocarriers, and volumes used (solvent and nanomedicine sample)
- Predicted lipid concentration (from sponsor)
- Any observations made during lipid extraction (precipitation, color changes, other phenomena)
- The lipid 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 lipid sample, 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.

7 Quality Control, Quality Assurance, Acceptance Criteria

Calibration curves for lipid quantification should encompass (at least) 5 concentration points. The curves should be measured in triplicate throughout the run sequence, distributed as follows; one complete, single calibration curve (no replicates) at beginning of sequence, one at the end of the sequence, and one curve distributed as single calibration points interlaced during the sample 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.

If available, stable isotope labeled internal lipid 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 (lipid) 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 do not 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 lipids and the desired end concentrations; all calculations should be performed separately by the QC preparing person. At least two QC samples with different lipid 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 lipid in the chosen solvent, QC samples could be prepared on 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 contamination and to minimize exposure to personnel; 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 LC mobile phase containing test analytes should be discharged as appropriate for the active ingredient.

9 Abbreviations

SOP	Standard Operating Procedure
LC	Liquid Chromatography
MS/MS	Tandem mass spectrometry
DMSO	Dimethyl sulfoxide