Quantitative analysis of imetelstat in plasma with LC–MS/MS using solid-phase or hybridization extraction

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Aim: Imetelstat, a 13-mer oligonucleotide with a lipid tail is being evaluated for treating hematologic myeloid malignancies. This report describes the development of extraction and quantification methods for imetelstat. **Methodology & results:** Imetelstat was extracted using SPE (rat plasma) or by hybridization using a biotinylated capture probe (human plasma) and was quantified by LC–MS/MS. Calibration curves were established (0.1–50 µg/ml). Stability of imetelstat in plasma was demonstrated. Concentrations of imetelstat extracted using either of the methods and quantified with LC–MS/MS were comparable with a validated ELISA. **Conclusion:** Two extraction methods (solid phase and hybridization) were developed for quantifying imetelstat in plasma using LC–MS/MS. The hybridization extraction in combination with LC–MS/MS is a novel extraction approach.

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In recent years, oligonucleotide drugs (ODNs) have gained popularity owing to their ability to target specific genes and provide specificity. ODNs comprise an oligonucleotide sequence, containing 10–50 nucleotides [1,2]. Numerous ODNs have been investigated so far [1–4]. But only six ODNs (fomivirsen, pegaptanib, mipomersen, eteplirsen, nusinersen and defibrotide sodium) have been approved by the US FDA since 1998 [5].

Pharmaceutical development of ODNs has several technical challenges such as maintaining *in vivo* stability, ensuring delivery and bioavailability, and minimizing off-target effects. These challenges can be minimized by chemical modifications of ODNs to enhance their pharmacokinetic and pharmacodynamic properties [4,6]. These chemical modifications include: sulfuration of the phosphodiester bond to avert degradation by endogenous exonucleases, addition of methoxy or methoxyethyl groups to sugar moieties [7,8], locked and unlocked nucleic acids, and alterations in the internucleotide linkage (e.g., amide linkage) resulting in peptide nucleic acids [1]. Sulfuration results in phosphorothioate analogs that are more hydrophobic, exhibit more complex secondary structures with higher protein binding and greater accumulation in organs than phosphodiester analogs [9–11].

Imetelstat is a novel, first-in-class telomerase inhibitor ODN [12], being investigated for the treatment of hematologic myeloid malignancies [13,14] and certain solid tumors [12,15,16]. Imetelstat is a 13-mer oligonucleotide N3′–P5′ thio-phosphoramidate (NPS) with a covalently linked C16 (palmitoyl) lipid moiety at the 5′-end (Figure 1; Supplementary Table 1). Addition of a lipid chain and the modified oligonucleotide backbone enables imetelstat to penetrate cells and tissues, with high biodistribution into normal and malignant cells [12].

Imetelstat differs from antisense oligonucleotides in its mechanism of action. Imetelstat is complementary to the template region of the RNA component of telomerase, to which it binds with high affinity and specificity, and directly competes with telomere binding, thereby inhibiting telomerase activity [17]. Thus, imetelstat acts as a classical active-site enzyme inhibitor, rather than an antisense oligonucleotide.

Preclinical and clinical pharmacokinetic and toxicokinetic studies for imetelstat are supported with a validated competitive nucleic acid hybridization ELISA [15]. Though hybridization ELISA is highly sensitive [18], it has a narrow dynamic range, is labor intensive and can lack selectivity toward truncated metabolites, potentially leading

Figure 1. Partial oligonucleotide structures showing (A) imetelstat with NPS linkages and (B) internal standard with OPS linkages between subsequent nucleotides.

The palmitoyl lipid tail at the 5' or 3' position for both molecules is presented as well.

NPS: Thio-phosphoramidate linkage; OPS: Two bridging oxygens and one nonbridging sulfur.

to overestimation of parent ODN levels and does not provide insight into potential imetelstat metabolites. LC-MS is a potential alternative method to increase assay selectivity [19]. MS has become a method of choice to study structural properties [19–21], as well as for quantitative analyses of ODNs. Other chromatographic strategies (utilizing capillary electrophoresis or ion exchange in combination with fluorescence or UV detection [22–25]) are also reported, although these methods are often incompatible with MS.

There are several technical issues associated with LC-MS analysis of ODNs as they are large acidic molecules with limited ionization efficiency in ESI-MS. High polarity of ODNs leads to limited chromatographic retention. Ion-pair reversed-phase HPLC increases the retention and the nature and concentration of the ion-pairing reagent impacts the MS response. Applying the ion-pair reagent with acidic modifiers enables the right balance between improved MS sensitivity and LC separation [26,27]. Hydrophilic interaction LC has been proposed in combination with MS-friendly mobile phases for the analysis of synthetic and chemically modified oligonucleotides [28].

Sample cleanup is also a critical part of ODN analysis and displacement of ODNs bound to protein needs to be ensured. Various approaches for ODN extraction from the biological matrix have been used including protein precipitation, liquid-liquid extraction (LLE) and SPE, though each method has its own shortcomings and compound-dependent success rates [27,29–33].

This paper describes the development of an LC-MS/MS assay for imetelstat as an orthogonal assay to the hybridization ELISA. The assay presented is risk-based and fit-for-purpose to support limited analysis for explorative

work and a lean validation approach was taken [34]. In the event, the LC–MS assay would substitute the validated hybridization ELISA, a complete validation as per regulatory guidance would be required [35,36].

Materials & methods

Chemicals & reagents

Imetelstat, a 13-mer oligonucleotide (5′-TAGGGTTAGACAA-3′) N3′–P5′ NPS with a palmitoyl tail at the 5′-position (5′-Palm-TAGGGTTAGACAA-NH₂-3′) and the internal standard (IS), a 12-mer phosphorothioate (OPS; ACAGATTGGGAT) with a palmitoyl group linked at 3′-position (Figure 1; Supplementary Table 1) were provided by Geron Corporation (CA, USA). Two biotinylated capture probes having biotin label at the 3′- or 5′-position, in other words, TTGTCTAACCCTA-3′ TEG (tetraethylene glycol)-biotin and 5′ biotin-TEG-TTGTCTAACCCTA capture probes (Supplementary Table 1) were synthesized by Integrated DNA Technologies (Leuven, Belgium). The 16-mer reference phosphodiester (OPO) oligonucleotide (ATCTATACAAGCTGTC) was also custom made by Integrated DNA Technologies.

For SPE, Clarity® OTX™ 96-well plates (100 mg/well) were obtained from Phenomenex (Utrecht, The Netherlands). Pierce™ streptavidin magnetic beads (bead concentration: 10 mg/ml; binding capacity: 3500 pmol biotinylated fluorescein/mg of beads) were purchased from Thermo Scientific (Gent, Belgium). Human and rat blank plasma (K₂EDTA) were obtained from Bioreclamation (West Sussex, UK). LoBind DNA tubes, LoBind 96-well plates, all solvents (HPLC grade or equivalent) and all chemicals (AR or HPLC grade) were purchased from Sigma-Aldrich or Merck (Overijse, Belgium). Multivette® 600 K3E tubes were procured from Sarstedt (Nümbrecht, Germany).

The HPLC column (X-bridge C₁₈ 2.1 × 50 mm, 3.5 μm) was procured from Waters (Zellik, Belgium).

Animals

Sprague Dawley rats were obtained from Charles River Laboratories (Sulzfeld, Germany).

Preparation of imetelstat standard, IS & capture probe solutions

A 2 mg/ml stock solution of imetelstat in water (HPLC grade) was prepared. Calibration standards of imetelstat were prepared by adding 5, 10 and 25 μl of the stock solution to a total volume 1 ml of plasma (rat or human K₂EDTA plasma), resulting in 10, 20 and 50 μg/ml imetelstat, respectively. Two further serial dilutions of each of these three dilutions were prepared in the appropriate plasma (rat or human; 100 + 900 μl) to obtain standard imetelstat concentrations of 0.1, 0.2, 0.5, 1, 2 and 5 μg/ml. Quality control (QC) samples were prepared by diluting an independently prepared stock solution of imetelstat to achieve concentrations of 0.8, 1.6, 8 and 40 μg/ml in human or rat plasma.

IS stock solution (2 mg/ml) and working dilutions (5 or 10 μg/ml) were prepared in water.

For both capture probes, stock solutions (1 mg/ml) were prepared in water. Working dilutions (1–10 nmol/ml) were prepared from the stock solution using buffer (15 mM sodium citrate, 150 mM sodium chloride).

LC–MS/MS system

The LC–MS/MS system consisted of a Shimadzu LC20AD HPLC with an SIL-HTC autosampler (Shimadzu Scientific Instruments, MD, USA), coupled to an API4000™ triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada) equipped with Turbo Ionspray source. Additional details of LC and MS/MS parameters are provided in Supplementary Tables 2 and 3, respectively. Data acquisition and processing were performed using Analyst® software Version 1.6.2 (AB Sciex, Toronto, Canada).

For quantitation, linear regression was executed on log-transformed peak-area ratios (imetelstat-IS) against the log-transformed imetelstat concentrations. The concentrations in the incurred samples were calculated by interpolation from the calibration curve.

Imetelstat extraction from plasma

SPE from rat & human plasma

SPE of imetelstat from rat or human plasma was conducted using Clarity OTX extraction kits according to the manufacturer's protocol (Phenomenex) with certain modifications to optimize the extraction. The wash steps were modified to improve the matrix cleanup and to avoid contamination due to overfilling of wells. The final method is detailed in Supplementary Table 4.

Hybridization extraction for human plasma

The hybridization extraction method is based on the annealing of sense and antisense DNA probes (Supplementary Figure 1A). The antisense probe, also named the capture probe, includes a biotin label for affinity capture of the sense–antisense complex from the biological matrix (plasma), using streptavidin-coated magnetic beads. The method is summarized in Supplementary Table 5.

The streptavidin-coated magnetic beads were prepared before use. For each experiment, an appropriate volume of the bead suspension (based on 500–3000 pmol beads/sample depending on the experiment, with an extra allowance) was centrifuged ($2430 \times g$, 22°C , 3 min). The beads were precipitated using a magnet and the supernatant fraction was discarded. The beads were washed three-times by suspending in wash buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 2 M NaCl) using three-times the volume of the original bead suspension. After the final wash step, the beads were resuspended in wash buffer containing 2% Tween 80 to achieve the required pmol beads/200 μl . During method development, the pmol of beads in 200 μl of buffer was varied to optimize the procedure.

The annealing process was initiated by the addition of 100 μl of the capture probe (100 pmol of the 5' probe) and 200 μl of prepared beads per well of a LoBind 96-well plate, followed by the addition of 100- μl phosphate-buffered saline with 0.01% Tween 80. The plate was subsequently incubated for 10 min at 22°C in a thermomixer. Annealing was initiated by the addition of 20- μl aliquots of samples (study samples, QC samples and calibrator human K₂EDTA plasma) to the beads-capture probe solution. The plate was incubated in the thermomixer for 90–120 min at 22°C , followed by centrifugation ($2000 \times g$, room temperature [RT], 2 min) and precipitation of the beads using a magnet. The plate was washed twice by discarding the supernatant fraction, adding 200 μl of ice-cold water, vortexing, cooling the plate on ice-water for 20 min, followed by centrifugation ($2000 \times g$, 4°C , 2 min) and precipitation of the beads using a magnet.

To denature the antisense–sense oligonucleotide complex, 200 μl of water (80°C) was then added to each well. The plate was incubated in a thermomixer (1100 rpm, 80°C , 20 min), followed by centrifugation ($2000 \times g$, RT, 2 min) and precipitation of the beads using a magnet. The supernatant fractions were then transferred to a new 96-well LoBind plate and 25- μl IS was added to each well and mixed before injection onto the LC–MS system.

hybridization ELISA

Quantitative analyses of preclinical (rat plasma) and clinical (human plasma) samples using the described extraction method, coupled with LC–MS/MS method were compared with the results from the validated hybridization ELISA for imetelstat [15]. The hybridization ELISA is based on the competitive binding between imetelstat and a detection probe (i.e., a DNA probe with a digoxigenin label at the 3'-position) to hybridize to the complementary DNA strand (Supplementary Figure 1B). An antidigoxigenin antibody conjugated to alkaline phosphatase allows fluorescent detection of the digoxigenin-labeled probe. Consequently, a low signal intensity in the ELISA signifies high imetelstat concentrations.

Clinical samples

Blood samples were obtained from five patients from the Baerlocher *et al.*'s study [14] in which patients with polycythemia vera or essential thrombocytopenia had received imetelstat infusion of either 7.5 or 9.4 mg/kg for 2 h. Blood samples were collected on K₂EDTA before the start of infusion (from two patients), at the end of the infusion and at 1 and 2 h after the end of the infusion. Plasma was isolated and stored in Nunc[®] vials at -20°C . Samples with concentrations above the ULOQ were diluted five-times in human plasma for the final analysis.

Rat pharmacokinetic study

A pharmacokinetic study of imetelstat was conducted in male Sprague-Dawley rats ($N = 16$; 250–280 g). An intravenous bolus of imetelstat was administered in the saphenous vein at either 1 or 5 mg/kg ($n = 8$ each dose). The selected doses were the same as used in the preclinical studies carried out to support clinical development of imetelstat (data on file). Blood (0.4 ml) was sampled from the tail vein at 7 and 20 min, and at 1, 2, 4, 7, 24, 48, 72 and 96 h postdose ($n = 3$ per time point) and collected into Multivette[®] 600 K3E tubes (containing K₂EDTA). Plasma was separated by centrifugation ($1900 \times g$, 5°C , 10 min). Each plasma sample was divided into two aliquots and stored in micronic vials at -20°C . One aliquot was used for quantification of imetelstat with the SPE–LC–MS/MS method and the other aliquot was used for quantification with the hybridization ELISA method (assay range: 0.7–3.5 $\mu\text{g/ml}$). The plasma samples of the first two time points of both groups, 5 mg/kg dose group and 1 mg/kg dose group were analyzed following five-times dilution in rat plasma.

Table 1. Intrabatch precision and accuracy of quality control data (n = 6) for imetelstat in rat plasma (the SPE–LC–MS/MS method) and in human plasma (the hybridization–LC–MS/MS method).

Method/parameter	Nominal imetelstat concentration (μg/ml) in QC samples			
	0.8	1.6	8	40
Observed imetelstat concentration (μg/ml) in rat plasma (SPE–LC–MS/MS)				
Intrabatch mean (SD)	0.746 (0.0605)	NA	8.4 (0.1)	40.0 (6.0)
Intrabatch precision (CV%)	8.1	NA	1.7	14.9
Intrabatch accuracy (%)	93.2	NA	105	100
Observed imetelstat concentration (μg/ml) in human plasma (hybridization–LC–MS/MS)				
Intrabatch mean (SD)	NA	1.68 (0.159)	8.8 (0.726)	35.9 (2.7)
Intrabatch precision (CV%)	NA	9.5	8.4	7.6
Intrabatch accuracy (%)	NA	105	108	89.8

NA: Not applicable; QC: Quality control.

Acceptance criteria for assay qualification & execution

A calibration curve covering the concentration range of interest needs to have at least five calibration points with relative errors (RE) less than 20% (<25% at the LLOQ), following application of an appropriate linear regression model. The QC samples were evaluated at three concentration levels for accuracy and precision (n = 6, Table 1) [34]. The average back-calculated value of the QCs at each concentration level should have an accuracy between 80 and 120% with a CV of less than 20%. For the analysis of study samples, calibration standards and QC samples at three concentration levels in duplicates were included in each analytical run. Results were accepted if calibration standards (at least five concentration levels) as well as four of six QC samples (with each concentration level represented) had accuracies between 80 and 120%. Selectivity was evaluated by analysis of LLOQ QCs prepared in six different lots of rat or human plasma. The average back-calculated value of the QCs should have an accuracy between 75 and 125% with a CV of less than 25%.

Ethical approval

The study was approved by appropriate institutional review board and followed the principles outlined in the Declaration of Helsinki for all clinical and preclinical experimental investigations. For clinical investigations, informed consent was obtained from the participants involved. Preclinical investigations in animals were carried out in accordance with the guidance for the care and use of laboratory animals.

Results & discussion

In this paper, we describe the development of a qualified assay to quantify imetelstat in plasma (rat and human) using LC–MS/MS assay along with two alternative extraction strategies to allow appropriate sample preparation.

LC–MS/MS method development

LC solvents

ODNs are detected in MS as multiply charged negative ions. The abundance of these different charge states is highly dependent on the choice of LC solvent. For imetelstat, the 6-charge state was the most abundant ion when hexafluoroisopropanol (HFIP; as modifier) and triethylamine (as ion-pairing reagent) were used in LC analysis while the 4-charge state was the most abundant ion with other ion-pairing reagents, such as hexylamine. The 6-charge state was selected for further analysis. A spectrum with the charge state distribution and the product ion spectrum was obtained on an API4000 instrument and presented at an European Bioanalysis Forum meeting [37]. The findings are consistent with previous results for the HPLC/MS–MS analysis of a 24-mer phosphorothioate oligonucleotide in rat plasma [38]. Using alkylamines with higher Henry's law constant (hexylamine, 1.57 vs triethylamine, 0.35) as ion-pairing agents reduces the charge distribution by forming complexes with the oligonucleotide in gaseous phase while the ion-pairing reagents with a lower Henry's law constant value are depleted in the ion spray droplets [39]. For imetelstat, increasing the concentration of the ion-pairing reagent (i.e., triethylamine) in the LC mobile phase while improving the dynamic range of the analysis resulted in deterioration of the peak shape and sensitivity (data not shown). The optimal combination for the LC mobile phase was 0.2% HFIP and 0.2% triethylamine.

The findings with LC solvents used in our analysis are consistent with previous findings [26,27] in which introducing the solvent combination HFIP/triethylamine enhanced the LC separation and increased ESI-MS efficiency. These findings have been extended by the systematic investigation of modifiers for the quantification of phosphorothioate DNA and recently other fluorinated alcohols alongside HFIP have been proposed [22,38]. Furthermore, the impact of different ion-pairing reagents has also been reported for the LC–MS/MS analysis of specific ODNs [39–41].

Attaining a robust signal

The LC–MS/MS conditions were carefully controlled and optimized. Initially, LC with new, unused silica-based C₁₈ column required multiple injections (>10) of matrix-containing samples to obtain a stable signal for imetelstat. This stabilization behavior was less pronounced for the IS. During the initial phase of method development, a reference compound (16-mer natural oligonucleotide without a lipid tail; Supplementary Table 1) which was assessed in the LC–MS/MS system demonstrated a more stable signal than what was seen for both imetelstat and the IS (i.e., oligonucleotides with lipid tails).

For both imetelstat and the IS, a decline in response was observed over a typical analytical run of approximately 60–100 injections. Various modifications were evaluated to optimize the LC method. Since HFIP was considered to have a potential role in the response decline owing to its volatility, additional precautions were taken to avoid evaporation, and solvents were replenished daily. However, this resulted in only a minimal improvement. An alternative strategy of decreasing HFIP concentrations from the LC mobile phase and introducing post-LC column (to enhance ionization of the oligonucleotides) was also assessed. Decreasing the HFIP concentration from 2 to 0.1% resulted in a substantial increase in response (around 350-fold). However, this strategy proved unviable since the peak shape and column performance deteriorated rapidly. Decreasing HFIP concentration significantly increased mobile phase pH to 11–12, which reduced the life time of the LC column. Complete removal of HFIP from the mobile phase and performing LC at a higher pH with ammonium carbonate resulted in a 50–100-fold decrease in response. Hence, this method was not pursued.

Tetrahydrofuran was chosen as the ternary mobile phase to wash out the LC system at the end of the chromatographic run since it reduced the carryover for the analyte.

LC–MS/MS method performance

The LC–MS/MS method for imetelstat in plasma (Supplementary Tables 2 & 3) was optimized to balance sensitivity requirements (lowest expected concentrations in preclinical and clinical samples, 0.1 and 0.5 µg/ml, respectively) while providing a stable response across an analytical batch.

Representative chromatograms of imetelstat and of the IS are presented in Figure 2. Although there were some methodological challenges owing to the lipid tail on these oligonucleotides, the LC and MS profiles were in-line with published reports.

Imetelstat extraction from plasma

Mixed-mode anion exchange SPE method development

Initially, a similar extraction approach as described by the authors of the 18-mer Trabedersen assay was assessed [33]. This method combined an LLE method using chloroform-phenol-isoamylalcohol with a hydrophilic-lipophilic-balanced SPE. However, for imetelstat, the recovery in the LLE step was very low (<20%), despite good recovery (>70%) of the experimental reference 16-mer oligonucleotide that was tested alongside. Combinations of protein precipitation (with acetonitrile or ethanol) and SPE to extract imetelstat were also ineffective due to losses in the initial protein precipitation step. Hence, mixed-mode anion-exchange extraction on SPE was explored as a potential solution to obtain good recovery.

Initial experiments with the commercially available kit (Clarity OTX extraction kit; 100 mg/3-ml mg/3 ml cartridges) also yielded a very low (<10%) recovery for imetelstat, though recovery for the reference 16-mer oligonucleotide was more than 50% (Supplementary Table 1). Imetelstat recovery improved to ≥65%, following incubation of 20 µl plasma with 200 µl lysis buffer at 50°C for 10 min before loading on the SPE cartridge. While imetelstat recovery was acceptable under these conditions, drift in the LC–MS/MS response still occurred in larger analytical runs, most likely due to residual matrix or buffer components. The wash and elution steps were further optimized (buffer composition, solvents and pH; data not shown). Finally, the fractionated elution (Supplementary Table 4) with 500 µl fractions of 100 mM ammonium bicarbonate pH 8.8/acetonitrile (50/50 v/v) revealed no

Figure 2. LC-MS/MS chromatograms of (A) imetelstat 0.1 µg/ml and (B) imetelstat 10 µg/ml, and (C) IS 20 µg/ml. Chromatographic and MS conditions are described in Supplementary Tables 2 & 3. cps: Counts per second; IS: Internal standard.

elution of imetelstat in the first 500 µl. Using 500 µl elution fractions (Supplementary Table 4), imetelstat recovery was 54% in the second elution and 10% in the third 500 µl elution fraction (first 500 µl removed). The IS used in our analysis contains the 12-mer 5'-nucleotide sequence of imetelstat including the lipid tail (the sequence is identical but linkage opposite from 3' to >5') and the NPS linkage is replaced by the OPS linkage (Figure 1; Supplementary Table 1). Other oligonucleotide structures such as the 13-mer OPO of imetelstat without a lipid tail were evaluated as well as IS but behaved more different than the 12-mer with the lipid tail. Nevertheless, this IS had a different elution pattern with approximately 40, 30 and 10% recovery in the first, second and the last 500 µl elution fractions, respectively. Combining all eluted fractions had a negative impact on signal robustness in the LC-MS/MS analysis. Since the IS showed a different elution profile compared with imetelstat, the IS was spiked after SPE elution, so that the IS only compensated for the signal drift in the LC-MS/MS but not for SPE recovery. Although addition of the IS following extraction is less optimal, all analytical runs are controlled by inclusion of QC samples prepared in the corresponding blank matrix as means of in-study validation.

Hybridization extraction method development

For imetelstat hybridization extraction, two capture probes were synthesized. The biotin labels were introduced via a TEG linker. This linker differs slightly when included at the 3'- or 5'-position as reflected in the molecular weight (Supplementary Table 1). In theory, the 3' probe is preferred as the TEG linker is slightly longer, thereby

Figure 3. Imetelstat recovery by different concentrations of beads, capture probe and imetelstat.

potentially minimizing steric hindrance. However, at this position steric hindrance could have more pronounced spatial hindrance as both the lipid tail and the biotin label are in close spatial proximity when the complementary strands hybridize (Supplementary Figure 1A), thereby limiting the recovery. Therefore, both options were explored, and recovery was assessed for 3'- and 5'-biotin probes. Following incubation of 100 pmol imetelstat with 100 pmol of capture probe and elution for 30 min at 80°C, imetelstat recovery was 97.8 and 35.2% with the 5'- and 3'-capture probes, respectively.

Regarding the optimum temperature required for adequate denaturation and elution of the double-stranded molecule, it was observed that 50°C was insufficient while elution for 30 min at 80°C resulted in lower recovery compared with the elution for 30 min at 50°C, followed by 10 min at 80°C. Therefore, elution for 20 min at 80°C was finalized as the elution condition (Supplementary Table 5) and proved to be robust as the assay acceptance criteria were met, as demonstrated by the performance of the QC samples.

The combination of the amount of beads and the concentration of the capture probe was also investigated. In theory, streptavidin has four biotin-binding sites but, in practice, steric hindrance limits binding efficiency. The streptavidin magnetic beads used in this analysis had a binding capacity of 3500 pmol/mg at a concentration of 10 mg/ml. Preliminary experiments demonstrated the loss of capture probe in the supernatant fraction at equimolar binding capacity of beads and capture probe (data not shown). Thus, a relative excess of binding capacity (i.e., amount of beads) was selected. Figure 3 shows imetelstat recovery at different concentrations of streptavidin beads and 5' biotin-capture probe. We found that at least equal amounts of capture probe and imetelstat were a prerequisite for the sufficient recovery (>90%) of imetelstat. Excess capture probe was considered, but increased capture probe levels, in turn, required increased concentrations of streptavidin beads, thereby increasing the assay costs. The ULOQ of the assay determines the amount of capture probe needed in the assay; a slight excess ($\times 1.5$) was considered to be able to evaluate study samples with concentrations above the ULOQ.

Technologies such as polymerase chain reaction and southern blots rely on the hybridization principle, in other words, single-stranded DNA or RNA annealing to the complementary DNA or RNA strand. In a validated hybridization ELISA for imetelstat [15] (Supplementary Figure 1B), a complementary oligonucleotide sequence containing a biotin label at the 3'-position provided the scaffold for competition of imetelstat and a 3'-labeled digoxigenin analog. Application of this 'affinity' to extract oligonucleotides from a biological matrix for qualitative

Table 2. Stability of imetelstat in rat and human plasma spiked at 20 µg/ml.

Storage condition	Rat plasma		Human plasma	
	Measured concentration (µg/ml)	% vs reference (time = 0 h)	Measured concentration (µg/ml)	% vs reference (time = 0 h)
Reference control (time = 0 h)	20.0 (0.3)	100.0	21.7 (1.2)	100.0
2 h at RT (22°C)	18.6 (0.2)	92.8	21.7 (0.3)	100.0
2 h at 4°C	18.5 (0.6)	92.5	21.9 (0.7)	100.8
2 h at 37°C	17.5 (0.1)	87.4	14.9 (1.5)	68.7
24 h at RT (22°C)	15.6 (0.4)	77.9	19.8 (2.0)	91.0
5x freezing and thawing cycles	16.7 (3.3)	83.4	20.8 (1.2)	95.6

Extraction performed using SPE-Clarity approach. Results are mean (SD) of three independent samples.
RT: Room temperature.

and quantitative analysis in combination with LC–MS/MS is newly explored and has only been briefly mentioned previously [42,43].

SPE–LC–MS/MS performance

Precision & accuracy

Using the final SPE method presented in Supplementary Table 4, the method was qualified in rat plasma in a single LC–MS/MS run (Supplementary Tables 2 & 3). This qualification included nine calibration levels of imetelstat. Using log–log linear regression back-calculated values with %RE ≤ 12% at all concentration levels of the calibration curve were obtained. The precision and accuracy of the method were determined at three QC levels – low, medium and high imetelstat concentrations (0.8, 8 and 40 µg/ml, respectively).

In rat plasma, the calibration curve for imetelstat was established in the range of 0.1–50 µg/ml. The calibration curve for imetelstat in rat plasma exhibited good linearity. Table 1 shows the intrabatch precision and accuracy of the analysis of imetelstat in rat plasma. The intrabatch precision was acceptable, with the CV being less than 15%. The intrabatch accuracy ranged between 93.2 and 100%. Selectivity was evaluated in six different rat plasma batches at the LLOQ level and showed an average accuracy of 94.7% and a CV of 8.7%.

Since the SPE–LC–MS/MS assay in human plasma was identical apart from the plasma source, no formal qualification was carried out for human plasma, but all analytical runs included QC samples prepared in human plasma as means of in-study validation.

Imetelstat stability

The stability of imetelstat in rat and human plasma was assessed (Table 2). Imetelstat was stable in plasma stored for 2 h at either 4°C or at RT, and during repeated freezing and thawing as the measured values were more than 80% of reference control. Stability of imetelstat at RT for 24 h and after freezing and thawing in rat plasma showed more degradation compared with the human plasma, probably explained by the enhanced esterase and hydrolase activity observed in rodent plasma [44].

Hybridization extraction–LC–MS/MS performance

Precision & accuracy

Using the final hybridization method (Supplementary Table 5), the method was qualified in human plasma in a single LC–MS/MS (Supplementary Tables 2 & 3) run. This qualification included seven calibration levels of imetelstat. The back-calculated concentrations levels obtained following log–log linear regression showed RE less than 5%. The precision and accuracy of the method were determined at three QC levels – low, medium and high imetelstat concentrations (1.6, 8 and 40 µg/ml, respectively). Selectivity was evaluated in six different plasma batches at the LLOQ level and showed an average accuracy of 79.8% and a CV of 13.3%.

In human plasma, the calibration curve for imetelstat was established in the range of 0.5–50 µg/ml (Supplementary Figure 2B). The LLOQ of the method is defined as the lowest calibration point with S/N ratio more than 5 and %RE less than 25%, and can be adapted as a function of the lowest concentrations of imetelstat expected in (pre)clinical studies.

Table 3. Comparison of imetelstat concentrations in clinical plasma samples as analyzed by three independent assays on three different occasions.

Patient and sampling times	Imetelstat concentration (μg/ml) hybridization ELISA	Imetelstat concentration (μg/ml) SPE–LC–MS/MS [†]	Accuracy % of ELISA result [‡]	Imetelstat concentration (μg/ml) hybridization–LC–MS/MS	Accuracy % of ELISA result [‡]
Patient #1					
Before infusion	<LLOQ	<LLOQ	–	<LLOQ	–
End of infusion	105	113	108	96.8	92.3
1 h after infusion	80.7	86.0	107	75.9	94.0
2 h after infusion	102	86.0	84.3	86.8	85.1
Patient #2					
Before infusion	<LLOQ	<LLOQ	–	<LLOQ	–
End of infusion	158	181	114	127	80.7
1 h after infusion	111	153	138	119	107
2 h after infusion	124	129	103	98.6	79.3
Patient #3					
End of infusion	98.6	108	110	98.6	100
1 h after infusion	83.6	104	124	97.3	116
2 h after infusion	81.8	85.5	105	77.3	94.5
Patient #4					
Before infusion	0.6	0.588	98.8	<LLOQ	–
End of infusion	120	119	99.2	108	89.6
1 h after infusion	99.2	93.5	94.2	88.6	89.3
2 h after infusion	85.3	78.5	92.0	78.2	91.6
Patient #5					
Before infusion	<LLOQ	<LLOQ	–	<LLOQ	–
End of infusion	133	112	83.7	100	75.1
1 h after infusion	140	133	94.9	117	83.7
2 h after infusion	116	113	96.9	99.5	85.8

[†]Samples > ULOQ were analyzed after a 1:5 dilution.
[‡]SPE–LC–MS/MS versus hybridization ELISA.
[§]Hybridization–LC–MS/MS (LLOQ LC–MS/MS assay = 0.500 μg/ml) versus hybridization ELISA.
LLOQ hELISA = 0.35 μg/ml.

Table 1 shows the intrabatch precision and accuracy of the analysis of imetelstat in human plasma. The intrabatch precision was acceptable, with the CV being less than 8%. The intrabatch accuracy ranged between 105 and 112%.

Comparison of the SPE–LC–MS/MS & hybridization extraction–LC–MS/MS methods with the validated hybridization ELISA to quantify imetelstat in study samples

Human plasma

For comparison of the SPE–LC–MS/MS and hybridization extraction–LC–MS/MS methods with the validated hybridization ELISA, 19 clinical samples from five patients who had received imetelstat infusion of either 7.5 or 9.4 mg/kg for 2 h were evaluated. Imetelstat concentrations were determined with the validated hybridization ELISA [15].

Prior to LC–MS/MS analysis, plasma samples were stored at –80°C for about 2 years. No 2-year stability data are available for this assay but in the validated ELISA 113 days stability in the freezer was proven. The study samples were analyzed at risk with the goal to encipher whether ELISA results in higher concentration levels compared with the LC–MS assay.

Table 3 presents imetelstat concentrations from the SPE–LC–MS/MS and hybridization–LC–MS/MS methods compared with those from the hybridization ELISA. With both methods, imetelstat concentrations were in agreement with the values obtained with hybridization ELISA.

Since the hybridization ELISA also measures truncated oligonucleotides, our data that show comparable imetelstat levels measured with SPE–LC–MS/MS and hybridization ELISA may suggest that imetelstat metabolite

Figure 4. Concentration versus time profiles for imetelstat in rat plasma after intravenous bolus dosing at 1 and 5 mg/kg after analysis by orthogonal methods: SPE–LC–MS/MS and hybridization ELISA.

concentrations are low (though no formal data are available to prove this). Moreover, the specificity of the LC–MS/MS system for imetelstat and its metabolites has not been established. The LC–MS/MS assay separates the truncated forms (n-1) of imetelstat; however, the NPO metabolite (one S exchanged for an O) coelutes with imetelstat but very low signals were observed in the incurred samples. Nonetheless, whether other Na^+ adducts of multiple-charged ions can contribute to the imetelstat transition is unclear due to the unavailability of reference materials. It should be noted though that LC conditions with HFIP are believed to minimize adduct formation [38].

The combination of selective hybridization extraction with LC–MS/MS detection potentially offers selectivity and specificity for imetelstat evaluation. However, the selectivity of the hybridization has not yet been fully explored. It is unknown if shorter oligonucleotide fragments (n-1; n-2 metabolites) can also hybridize with the capture probe; the cutoff fragment length is unknown. If only unchanged imetelstat is present and quantified, the evaluation using hybridization extraction and LC–MS/MS is not expected to have a substantial impact. However, if metabolite identification is required, then the selectivity of this method will require further exploration.

Rat plasma

The mean plasma exposures of imetelstat after receiving an intravenous bolus dose at either 1 or 5 mg/kg as determined from both the SPE–LC–MS/MS method and the hybridization ELISA are shown in Figure 4; the data are virtually superimposable. As shown, the SPE–LC–MS/MS assay is slightly more sensitive than the hybridization ELISA and is capable of detecting plasma exposure of imetelstat above the LLOQ at one additional time point.

Conclusion

LC–MS/MS assay was developed for the quantitative analysis of imetelstat in preclinical and clinical plasma samples. The assay performance was fit for purpose and in line with typical bioanalytical acceptance criteria. Furthermore, the analysis of human and rat plasma samples using both methods, SPE–LC–MS/MS and hybridization extraction–LC–MS/MS yielded results that were comparable to those obtained using a validated hybridization ELISA assay.

The presence of the lipid tail in imetelstat alters the overall physicochemical properties of the oligonucleotide such that classical ODN extraction approaches are not applicable. Two alternative extraction strategies in combination with LC–MS/MS were explored and qualified. The SPE approach using Clarity OTX anion exchange was optimized for recovery and to minimize matrix interferences, achieved through a fractionated extraction procedure. The hybridization extraction of an oligonucleotide in combination with LC–MS/MS is a novel approach and offers a more selective extraction, with a dynamic range dependent on the amount of capture probe. The specificity of this extraction toward truncated forms of imetelstat is currently unknown, although evidence suggests that this hybridization method is comparable to the validated hybridization ELISA. However, the mass spectrometric detection offers additional selectivity.

Future perspective

Development of quantitative LC–MS/MS assays for oligonucleotides continues to encounter some methodological challenges. Nevertheless, such methods are invaluable to support results obtained with ELISA or polymerase chain reaction (PCR) technology.

Cleanup using 2D chromatography, as well as trap/elute approaches or full orthogonal 2D strategies may enhance the signal stability. MicroLC with low flow rates could enhance ionization efficiency and reduce signal variability as less matrix is introduced. Strategies to avoid using HFIP and ion-pairing reagents as constituents in the mobile phase, such as by hydrophilic interaction LC before introduction to the MS, may strengthen the MS response.

High-resolution MS provides the benefit of improved selectivity and can eliminate the fragmentation step used in the multiple reaction monitoring approach, which results in nonselective product ions in case of oligonucleotides.

Oligonucleotide extraction from a biological matrix is a critical step, especially for oligonucleotides such as imetelstat, which combine a lipid tail at the 5'-position and a hydrophilic nucleotide strand. These characteristics alter the physicochemical properties, and the more traditional liquid extractions using chloroform–phenol approaches do not result in sufficient recovery. Disrupting the strong protein binding by use of organic solvents, detergents or proteinase K treatment can also be examined. With SPE, pretreatment with lysis buffer is essential; however for the hybridization approach, currently no special treatment for dissociation of the protein binding is included. The high hybridization affinity will probably displace the oligonucleotide from the proteins, to which it is bound. For hybridization extraction, understanding the functional specificity of assay conditions in relation to the truncated variants, as well as endogenous oligonucleotides present in the sample requires further exploration to apply and adapt this extraction method to new oligonucleotide sequences.

Finally, extraction of oligonucleotides from other biological matrices, such as tissue homogenates, is often a requirement in development programs. Whether the proposed approaches can successfully be applied to these matrices requires further elaboration.

Summary points

- Imetelstat is a novel, first-in-class telomerase inhibitor oligonucleotide, being developed for the treatment of hematologic myeloid malignancies. This report describes a quantitative LC–MS/MS method using solid phase or hybridization extractions for the quantitation of imetelstat as an orthogonal method for the validated hybridization ELISA.
- Two independent extraction approaches were developed to capture imetelstat from plasma: an anion exchange SPE method with fractionated elution, and a hybridization extraction approach using a complementary biotinylated DNA probe.
- The developed method was qualified and was sufficiently sensitive to detect the range of imetelstat concentrations expected in preclinical (rat plasma) and clinical (human plasma) samples.
- Imetelstat concentrations in incurred human and rat plasma samples with the qualified LC–MS/MS assay with solid phase or hybridization extraction were in agreement with the concentration values obtained with the validated ELISA.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.future-science.com/doi/full/10.4155/bio-2017-0145

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved. Animal experiments were carried out in accordance with the guide for the care and use of laboratory animals.

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