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A Quantitative Method for Determining Hyaluronan Content in Plasma by LC/MS/MS and Plasma HA as a PharmacodynamicMarker for PEGylated-Hyaluronidase PH20 (PEGPH20) in a Phase 1b Trial for Pancreatic Ductal Adenocarcinoma

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ABSTRACT

Purpose:
Hyaluronan (HA) is a non-sulfated linear glycosaminoglycan comprised of repeating disaccharide units of n-acetylglucosamineand glucuronic acid. HA is abundant in the extracellular matrix of tissues (e.g. skin, those undergoing rapid growth and development) and has been associated with wound repair and aggressive malignancies. In solid tumors, abundant HA has been implicated in abnormallyhigh interstitial fluid pressures (IFP), poor vascular perfusion, hypoxia, and is associated with poorer prognosis. PEGPH20, a systemically administered investigational hyaluronidase, degrades tumor-associated HA leading to an increase in tumor vascular perfusion, a reduction in IFP/hypoxia, and more efficient delivery of co-administered anti-cancer agents. Quantitation of plasma HA may provide a useful tool to characterize tumors that accumulate HA and may also be used as a pharmacodynamic(PD) marker for PEGPH20.

Methods:
Human plasma samples containing K3-EDTA as the anticoagulant were assayed for total HA content by enzymatic digestion with chondroitinaseABC to liberate HA disaccharide. HA disaccharide was recovered by precipitation with ethanol and subsequently derivatizedwith 4-nitrobenzylhydroxylamine. A deuterium labeled 4-nitrobenzylhydroxylamine derivative of HA disaccharide was incorporated as an internal standard. Excess derivatizingreagent was removed by a solvent wash step and then the extracts were separated by HPLC using a PhenomenexSynergiMAX-RP column. The mobile phase was nebulized using heated nitrogen in a Z-spray source/interface set to electrospray negative ionization mode. The ionized compounds were detected using MS/MS.

Results:
Derivatization of HA disaccharide with 4-nitrobenzylhydroxylamine imparted greater hydrophobic retention and allowed chromatographic separation from chondroitin disaccharide, which has identical mass to HA. Moreover, derivatization allowed for the incorporation of a deuterium labeled 4-nitrobenzylhydroxylamine derivative of HA disaccharide as an internal standard. This feature of the assay was key to successful validation. Method validation studies demonstrated acceptable performance characteristics with intraday and interdayprecision and accuracies <15%, LLOQ intraday and interdayprecision and accuracies <20%, enzymatic digestion robustness precision and accuracies of <10%, appropriate selectivity/matrix interference characteristics and analytecarry-over <5%.

Conclusions:
A novel LC/MS/MS method for the quantitation of total HA content in plasma samples was developed and validated, which provides accurate quantitation of HA independent of chain length. This allows for determination of intact HA as well as oligosaccharide catabolites in human plasma samples.

Repeating polymers of HA can vary in size from small oligosaccharide chains to high molecular weight forms (1-4 MDa). Ligand-binding assays (LBA) and histochemical assays for HA tend to be semi-quantitative in nature due to the heterogeneity of the glycosaminoglycan, variable efficiencies of detection based on size, and the ability of small molecular weight HA to interfere in detection of the larger species. Early HPLC-based methods allowed for quantitative determination of HA content by total disaccharide content but were limited in sensitivity, making measurements of low endogenous serum/plasma concentrations difficult. We sought to improve the sensitivity of the HA disaccharide HPLC methods by coupling with MS/MS. The goal of the improved assay is to provide ng/mL sensitivity and the precision, accuracy,and dynamic range necessary for use as a PD marker and in PK/PD modeling for oncology trials investigating PEGPH20 in the treatment of solid tumors.

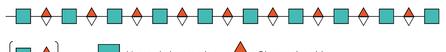
Quantitation of HA content in unknowns was determined by interpolation from calibration curves of known disaccharide standard quantities. The method was validated for use in human plasma for concentrations ranging from basal endogenous levels (~40 ng/mL) to approximately 400 µg/mL and allows for the accurate quantitation of HA independent of fragment size. Plasma samples from stage IV pancreatic ductal adenocarcinoma (PDA) patientsenrolled in a Phase 1b clinical trial of PEGPH20 in combination with gemcitabine were evaluated for HA content at baseline and varyingtime points post-PEGPH20 administration to evaluate impact of systemic exposure to hyaluronidase on circulating concentrations of HA.

PDA patients in 3 cohorts were administered twice weekly doses of PEGPH20 at 1.0, 1.6, and 3.0µg/kg in combination with gemcitabine.Plasma samples evaluated for HA catabolites by LC/MS/MS revealed a dose and time-dependent increase over the first 3 days. In general, HA concentrations continued to increase with time, reaching a steady-state after approximately 1 week of dosing, although inter-individual variation was observed.
A dose-dependent increase in circulating plasma HA concentrations post-PEGPH20 administration provided evidence for systemic exposure to active hyaluronidase and supports the use of HA as a PD marker. The performance characteristics of the method allow for further use of data in subsequent PK/PD modeling. The measure of endogenous plasma HA is being investigated as an alternative prognostic markerfor PDA.

This method has utility for measuring HA catabolites as a PD marker for PEGPH20-based treatment in patients with PDA.

METHODS

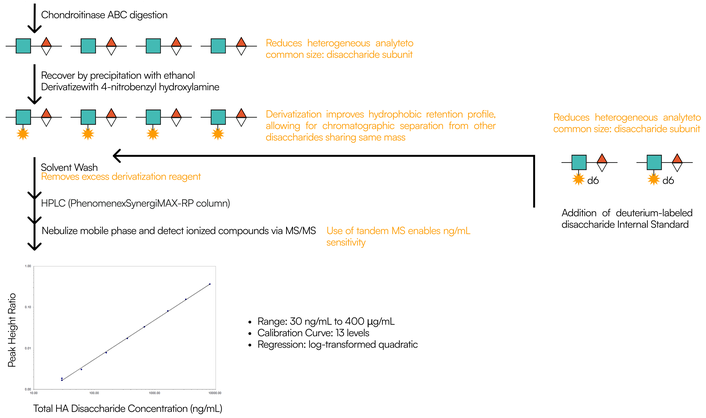
Hyaluronan Structure



HA can be up to 25,000 disaccharide repeats in length
HA size can range from <5 kDa to 10,000 kDa

Method Workflow

Samplematrix: Human K3-EDTA plasma



- Range: 30 ng/mL to 400 µg/mL
- Calibration Curve: 13 levels
- Regression: log-transformed quadratic

RESULTS

Method Validation Studies Demonstrate Suitable Performance Characteristics

Validation Parameter		Acceptance Criteria	N	Results
Results	Intraday	≤15.0% CV	6	1.48% to 5.44%
	Intraday	≤15.0% CV	30	3.41% to 4.23%
Accuracy	Intraday	≤±15% DEV	6	-7.62% to +5.85%
	Intraday	≤15.0% CV	30	-5.83% to +3.35%
LLOQ QC Interday	Intraday	≤±15% DEV	6	6.39%
	Accuracy	≤±20% DEV	30	+6.85%
LLOQ QC Intraday	Precision	≤20.0% CV	6	2.25% to 7.68%
	Accuracy	≤±20% DEV	6	2.74% to 14.4%
Specificity	Analyte	80% of lots tested	10	100%
	Internal Standard	80% of lots tested	10	100%
MatrixEffect / Selectivity		80% of lots tested	10	80%
Hemolysis Effect	Precision	≤15.0% CV	3	0.45% to 6.94%
	Accuracy	≤±15% DEV	3	-13.1% to 4.99%
Lipemic Effect	Precision	≤15.0% CV	3	2.54% to 4.40%
	Accuracy	≤±15% DEV	3	10.9% to 11.0%

RESULTS (continued)

Method Validation Studies Demonstrate Suitable Performance Characteristics

Validation Parameter	Acceptance Criteria	N	Results
MatrixEffect / Selectivity	≤15.0% CV	6	1.48% to 5.44%

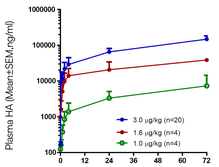
Stability	Conditions	N	Duration	Pass/Fail
Processed Sample	5°C	6	74 hours	Pass
Whole Blood	Room Temp	3	30 & 60 minutes	Pass
	On Ice	6	30 & 60 minutes	Pass

Successful Application of Method as Pharmacodynamic Marker in Phase 1b Study of PEGPH20 in Stage IV Pancreatic Ductal Adenocarcinoma

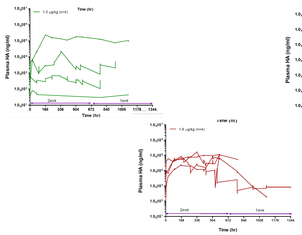
Study Design and Treatment

- Multicenter, international, open-label, dose-escalation, phase 1b study to evaluate the safety and tolerability of PEGPH20 plus gemcitabine in patients with advanced pancreatic cancer.
- Patients were treated with PEGPH20 at 1 of 3 dose levels (1.0, 1.6, and 3.0 µg/kg twice weekly for 4 consecutive weeks, then once weekly for the next 3 weeks during Cycle 1; for subsequent cycles.
- PEGPH20 was administered for 3 consecutive weeks) in combination with intravenous gemcitabine 1,000 mg/m2. Gemcitabine was administered once weekly throughout the study

Figure 1. Dose-Dependent Changes in Plasma HA Concentration After PEGPH20 Administration



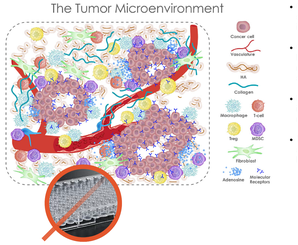
Individual Patient Profiles



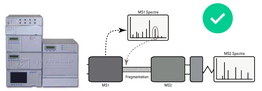
- Increase in circulating plasma HA levels provide evidence for systemic exposure to active hyaluronidase
- Plasma HA responses were on average dose-dependent

BACKGROUND

The Tumor Microenvironment



- Glycosaminoglycans (GAGs) such as HA are components of the extracellular matrix implicated in a number of disease processes.
- GAGs are difficult analytes, due to their heterogeneity in size, varying structural modifications (e.g., sulfation) and their association with binding proteins.
- Ligand binding assays for HA suffer inaccuracies in measuring heterogeneous sizes of the repeating polymer.
- The presence of small HA fragments can also directly interfere with the detection of high molecular weight species.



- HPLC provides the advantage of size-independent quantitation, however, lacks sufficient sensitivity and selectivity to measure endogenous hyaluronan levels in plasma.
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- Coupling HPLC with tandem-MS overcomes a number of constraints suffered by both LBAs and HPLC alone.

SUMMARY

- A novel LC/MS/MS method for the quantitation of total HA content in plasma samples was developed and validated, which provides accurate HA quantitation independent of chain length. This allows for determination of a heterogeneous population of HA in human plasma samples which can be present after systemic hyaluronidase exposure.
- This method has utility for measuring HA catabolites as a PD marker for PEGPH20-based treatment in patients with PDA.
- LC MS/MS may be a viable approach to successful bioanalysis of other gylcosaminoglycans.

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