Pharmacokinetics and Pharmacodynamics of Hyaluronan Infused into Healthy Human Volunteers

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Abstract: The pharmacodynamics and elimination kinetics of escalating doses (1.5-12 mg/kg) of hyaluronan (HA) infusions were studied in healthy human volunteers. Metabolic breakdown of serum HA and associated adverse events were monitored throughout the study. The HA-binding capacities of circulating CD4+ and CD8+ T lymphocytes, CD19+ B-lymphocytes and CD14+ peripheral blood monocytes (PBMC) were also quantified. Breakdown of infused HA into small fragments (<37 kDa) were not detected and adverse events related to HA infusions were infrequent and non-serious in nature. Binding of FITC-HA was greatest to CD14+ monocytes and the binding capacity of these cells for FITC-HA was significantly increased by the final HA infusion. At that time, binding to CD14+ monocytes was related to serum HA levels suggesting a close relationship between PK and PD of serum HA. Drug level analysis demonstrated a disproportional increase in the area under the serum concentration vs. time curve with increasing HA dose. The observed non-linear HA kinetics appears to result from a saturable elimination process as revealed by pharmacokinetic modeling. These results have implications for the use of injected HA for drug delivery or in imaging applications.

INTRODUCTION

HA is an ubiquitous glycosaminoglycan produced by three distinct but homologous HA synthases (HAS1-3) and is degraded by five known hyaluronidases (HYAL1-5) most of which are lysosomal. HA is retained in tissues as a result of specific interactions with extracellular and cellular HA-binding proteins defined as hyaladherins and amounts are regulated during morphogenesis, wound repair, chronic inflammatory disorders, and neoplasia [1, 2]. Altered tissue HA results from changes in the activity and expression of HAS and hyaluronidases as well as rate of HA uptake into the cell [3-6]. HA modifies the physico-chemical nature of the extracellular matrix within tissues and contributes to both homeostasis and response-to-injury processes [1, 4, 5, 7-10]. For example, interactions with cell receptors activates signaling cascades that promote migration and proliferation, which consequently influence both differentiation and immune trafficking/function during tissue homeostasis and response-to-injury [2, 11]. HA is metabolized in multiple compartments including tissues, lymphatics and vasculature [6] and alteration of this metabolism can have serious consequences to homeostasis. For example, modification of tissue HA metabolism during response-to-injury promotes unremitting, non-resolving inflammation [4, 12]. A proportion of tissue HA escapes into the lymphatics, is processed in lymph nodes and from there gains entry into the blood vasculature [13].

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Uptake and metabolism of HA occurs in each of these compartments as well. Endocytosis of HA by liver, and to a lesser extent kidney endothelium, removes most serum HA [14]. Ultimately HA is degraded in lysosomes by hyaluronidases [15, 16].

In spite of this limited knowledge of both the metabolic fate and functions of HA, it is currently extensively used in humans (e.g. for joint supplementation and tissue replacement, surgical adhesion prevention, regulation of inflammatory diseases, tissue healing/regeneration and tissue engineering) [17-20]. Future products of HA or modified HA are also being developed to enhance targeted drug delivery into traditionally inaccessible compartments such as tumors, peripheral lymph nodes, and bone marrow [21-23]. HA is also being investigated as an imaging agent. For example, increased HA accumulation at the periphery of invading breast tumors enhances ultrasound detection of the margins of the invading tumor [24]. HA can be readily modified with non-specific contrast agents such as metals, and these complexes are being studied for their ability to detect areas of altered HA metabolism [25].

The current clinical uses of HA often result in repeated exposure of patients to high levels of exogenous HA in serum, far exceeding normal serum concentrations, which are in the order of ug/L [26]. Surprisingly few studies have addressed the pharmacokinetics or dynamics of the exogenous HA polymer itself or in combination with other drugs [27, 28] and to our knowledge there are no reports of the pharmacodynamics of chronically administered, high dose (mg/kg) exogenous HA under conditions that patients might experience when HA is used as a delivery vehicle for therapeutic reagents. Since quantifying the pharmacokinetics of administered HA is an essential first step to characterize its pharmacodynamics for identifying potential deleterious side effects of exogenous HA, and for further refinement of HA as a drug delivery and imaging agent, we assessed the elimination kinetics and toxicity of multiple, escalating doses of HA from 1.5 mg/kg-12 mg/kg administered to healthy male and female human volunteers over a 4 week period.

SUBJECTS, MATERIALS AND METHODS

Patient Eligibility

Twelve non-smoking male and 12 non-smoking female volunteers, 30-50 years of age, weighing at least 60 kg (males) or 45 kg (females), and who were within 15% of their optimum weight [29] were enrolled in this study by

Table 1. Patient Demographics and Exclusion Criteria

A. Patient Demographics	12 males, 12 females
	[a]Non-Smokers
	Age (30 – 50 yrs)
	[b]Weight (avg. 60 kg for males, 45 kg for females)
	Height (avg. 178 cm for males, 160 cm for females)
B. Medical Histories (for exclusion from study)	Significant Cardiovascular disease
	Significant hepatic disease
	Significant renal disease
	Significant CNS disease
	Significant hematological disease
	Significant gastrointestinal disease
	Clinically significant illness 4 weeks prior to study entry
	Alcoholism or drug abuse in previous year before study entry
	Hypersensitivity or idiosyncratic reaction to HA or other GAG
	Pregnancy
	Nursing
	Inadequate contraception
	[c]Personality disorders
	Conditions interfering with drug absorption, distribution, metabolism or excretion
	Use of enzyme inducing drugs within 30 days of study entry
	Treatment drugs toxic to major organs within 3 months of study entry
	Abnormal diet during 4 weeks preceding study
	Through completion of study, donation of >900 mL of blood over 20 weeks
	Participation a drug study 4 weeks prior to study entry
C. Prohibited (for duration of study)	[d]Medication (including over-the-counter products)
	Use of alcohol-containing or xanthine-containing beverages through sample collection

See Methods and Results sections for descriptions of procedures.
[a]Subjects could not have used nicotine within 3 months of study entry.
[b]Patients were within 15% of their ideal weight ("Table of Desirable Weights of Adults", Metropolitan Life Insurance Company, 1983).
[c]Includes disorders that would preclude informed consent or compliance with protocol requirements.
[d]If medication other than that specified in the protocol was required, decision to continue subject was made by L.A.B. Medical Personnel.

L.A.B. Pharmacological Research International Clinical Research Center (Montreal, Canada). Subjects were further screened for medical histories/demographic data (Table **1**). All underwent a complete physical examination and standard laboratory tests to detect medical abnormalities (Table **2**). Participants were restricted to non-smoking, medically healthy subjects with clinically acceptable laboratory profiles. Exclusion criteria are listed in Table **1**.

Ethical Approval

The protocol was reviewed and approved (IND#BV5200-02) by TPD (formerly HPB, Regulatory Body of Canada). The protocol was also internally reviewed by the L.A.B. Pharmacological International Institution Review Board and was carried out in accordance with established clinical research guidelines [30, 31]; and the principles enunciated in the Declaration of Helsinki [32]. The purpose of the study, the procedures that were carried out, and the potential hazards were described to the subjects in non-technical terms in conformity with regulatory requirements.

Study Design

This was a single-dose, dose escalation, pharmacokinetic study. The primary objective was to evaluate the pharmacokinetic profile of HA after 120 min intravenous infusion of a sterile 1% HA solution at escalating doses of 1.5 mg/kg,

Table 2. Clinical Laboratory Tests Performed on Subjects

A. Hematology[a]	hemoglobin[d] hematocrit[d] total and differential leukocyte count red blood cell count platelet count calculated indices sedimentation rate PT/PTT
B. Serum Chemistry[a]	BUN creatine total bilirubin alkaline phosphatase[e] SGOT[e] SGPT[e] LDH sodium potassium calcium phosphorus glucose B-HCG (for female subjects only)
C. Urinalysis[a]	pH specific gravity protein glucose ketones bilirubin blood nitrate urobilinogen-microscopic examination B-HCG (for female subjects only)[a]
D. Urine drugs-of-abuse screen[c]	cocaine cannabis
E. Other[c]	HIV

See Methods and Results sections for descriptions of procedures.
[a]Conducted at screening and post study.
[b]Conducted prior to entry into each section.
[c]Conducted at screening only.
[d]Conducted prior to and following period 4.
[e]Conducted at 72 hrs. post-dose in periods 1, 2, and 3.

3.0 mg/kg, 6.0 mg/kg and 12 mg/kg. Each dose subsequent to the 1.5 mg/kg dose was administered following a 7-day washout period. In each case a total volume of 250 ml of stock solution of HA, diluted appropriately with 0.9% sodium chloride, was administered over a period of 120 min. Blood was sampled from all volunteers at 2, 1.5, 1, and 0.5 h before administration and at 2, 4, 6, 10, 14, 18, 22, 32, 38, 50 and 74 h after administration of the HA solution. Blood samples were collected and serum HA levels were measured on site by L.A.B., Inc.. Blood collected from a subset of six subjects (3 females and 3 males) at 0, 1, 4, 12, 24 and 72 h post-infusion of each HA dose were analyzed to determine the molecular weight profile of serum HA and to assess HA binding by T cells, B cells and blood monocytes. Measurement of these parameters was the first step in assessing pharmacodynamic disposition of HA and as described below. Samples were not segregated according to gender.

Antibodies and Reagents

FMC63 (CD19) antibody was conjugated to PE. PE conjugated CD4 and CD8 antibodies were purchased from Becton Dickinson (San Jose, CA). Isotype matched control monoclonal antibodies (mAb) were from Southern Biotech (Birmingham, AL). For infusions, lyophilized HA (medical grade, lyophilized HA form prepared by Hyal Pharma, Mississauga ON from original fermented HA product purchased from Kyowa Hakkos, Japan) was dissolved in phosphate buffered saline (PBS) and autoclaved to sterilize and reduce molecular weight. HA samples from this commercial source were analyzed with respect to MW using HPLC/SEC multi-angle laser light scattering (LifeCore Biomedical, MS). For cell binding assays, HA (Healon, medical grade) was obtained from Pharmacia (Dorval ZB) and conjugated to fluorescein (FITC) as described previously [33].

Assessment of HA Purity

HA preparations were tested for the presence of protein, DNA and endotoxin. Protein content was assayed by absorption at 280 nm. DNA content was determined by electrophoresis of 10-100 µg HA in agarose gels containing 0.7% agarose and 0.5 mg/ml ethidium bromide at 100 V for 3 h. DNA was visualized using a UV transilluminator (wavelength: 302nm) [34]. Endotoxin was detected using a colorimetric Limulus ameobocyte lysate assay with a sensitivity of 0.01 endotoxin units/ml [35].

Injections of HA, Sampling and Pharmacokinetic Assessment

Each subject received four escalating doses of 1% HA (1.5, 3.0, 6.0, and 12 mg/kg) one week apart as a 2 h infusion. Between 8-10 a.m. on day one of the study, blood was drawn from volunteers 2 h prior to HA infusion, to determine baseline serum HA. Blood was collected in 1 X 1.8 ml citrated vacu-containers (BD Canada, Oakville, On). On the first day, 24 volunteers were infused i.v. with 1.5 mg/kg HA (1%) over a 2 h period (period 1). Blood was sampled at the times listed above in *"Study Design"*. Subsequently, at 7-day intervals, volunteers were infused with 3.0 mg/kg (period 2), 6.0 mg/kg (period 3) and 12 mg/kg (period 4) and blood samples taken as described for period 1.

Baseline, endogenous serum HA was 35-40 ng/ml, consistent with that reported in healthy persons 18-65 years old [26]. HA serum levels had dropped to these baseline levels in all subjects during the washout period between dose periods. These values were remarkably consistent and were 0.1-0.4% of serum HA at the earliest time point (0.5 h) after injection of the lowest amount of HA (1.5 mg/kg). For simplicity of analysis, baseline serum HA levels were subtracted prior to analysis of the data.

Maximum serum concentrations (C_{max}) were the values obtained 2 h after HA infusion and AUC ("area-under-curve", in a plot of HA concentration *vs* time infusion [0-75]) was determined using a linear trapezoid method. Model-dependent analysis of the serum concentration-time profiles was used to estimate zero-order endogenous HA synthesis rate (k_{syn}), saturable elimination process (K_m, serum concentration at half-maximal elimination rate; V_{max}, maximum elimination rate) and one-compartment volume of distribution (V_d) (Fig. **1**). Hence, the rate of change in HA mass in serum as a function of time was described as:

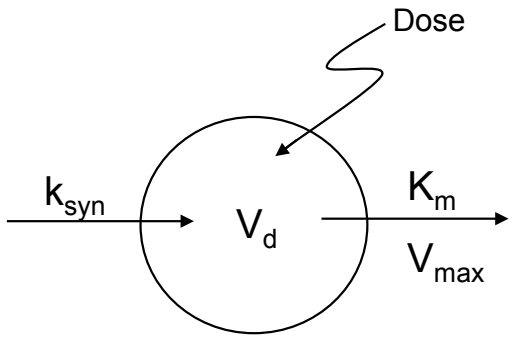

Fig. (1). Hyaluronan Pharmacokinetics Model. The pharmacokinetics of HA was modeled with a one-compartment distribution scheme to which the dose is delivered as a 2-hour infusion. Endogenous HA synthesis is described as a zero-order process (k_{syn}) while the rate of saturable HA elimination is governed by the parameters K_m and V_{max}.

$$\frac{dHA}{dT} = k_{syn} - \frac{V_{max} \times [HA]}{K_m + [HA]} \qquad \text{Eq. I}$$

where the concentration of HA is:

$$[HA] = \frac{HA}{V_d} \qquad \text{Eq. II}$$

The initial HA concentration was set as the pre-infusion level. It was assumed that the rate of HA appearance in serum (k_{syn}) remained constant over the sampling period. The entire mean data set over time 0 to 75 h for each dose regimen (1.5, 3, 6, and 12 mg/kg administered over 2 h) was fitted to the model to estimate parameter values using a least-squares minimization procedure with optimized weighting determined by visual inspection of the fitted result (SCIENTIST, MicroMath Scientific Software, St. Louis, MO). The present model was employed because preliminary kinetic analyses determined that simpler models describing elimination as a single linear function, or more complex models such as those including a combination of saturable and linear clearance processes, did not yield improved fits of the data after comparisons of sums of squared deviation, residual plots, standard deviation of estimates and values for a modified Akaike criterion.

Clinical Tests Performed to Identify Serious Adverse Reactions

Clinical laboratory tests (Table **2**) were conducted at screening and post-study. *B*-HCG urine levels were assessed in female subjects at screening, prior to entry into each dosing period and post-study (Table **1**). Hemoglobin and hematocrit were both tested on screening, prior to entry into the study, prior to and following period 4 dosing, and at the end of the study. Serum alkaline phosphatase, SGOT, and SGPT levels were determined at screening, prior to entry into the study and 72h post-dose in periods 1, 2 and 3, and at the end of the study.

Measurement of Serum HA

The analysis for HA levels in serum samples from 23 healthy volunteers for each time point described above was performed using a Pharmacia HA Test (Kabi Pharma, Upsala, Sweden) based upon the same principles as the presently available HA Test Kit (Echelon). The HA of serum samples reacts with a specific HA binding protein labeled with ^{125}I. The limit of quantification (LOQ) of the assay was 10 ng/ml, and the limit of detection (LOD) was 4.73 ng/ml. Inter and intra-day variation of the assay was controlled through incorporation of an analysis of HA analysis standards, provided with the kit.

HA Molecular Weight Analysis

HA and other glycosaminoglycans were precipitated using cetylpyridinium chloride treatment of serum samples from 6 subjects using the 0.5hrs and 48 hrs sampling times [35]. Equal amounts of precipitated glycosaminoglycans, detected with a modified anthrone assay, which detects uronic acid, were used for molecular weight analyses [36]. The heterogeneity of HA size range was determined using non-denaturing gel filtration. Precipitates were dissolved in PBS and then passed through a 1.6 x 61.5 cm Sephacryl 500 column, previously calibrated using dextran standards (MW 11.3-2,000 kDa). One ml fractions from a total 120 ml volume were analyzed for uronic acid. Inter- and intra-day variations in amounts of HA detected with the modified anthrone assay were determined and adjusted for by using HA standards to generate concentration curves for each assay. Elution profile curves were plotted and molecular weight categories of $>2 \times 10^6$, 1.9×10^5-2.0×10^6; and $<1.9 \times 10^5$ were quantified by measuring AUC using Adobe Photoshop to obtain pixel number/unit area.

Measurement of HA Binding by Subsets of Peripheral Blood Mononuclear Cells (PBMC)

PBMC were purified by centrifugation over Ficoll PAQUE (Pharmacia, Dorval, QB), washed and stained for surface marker expression as well as for the ability to bind FITC-HA as previously described [33]. To determine the ability of PBMC subsets to bind HA, PBMC were stained in two-color immunofluorescence with PE-coupled mAb to a defined surface marker and HA-FITC. The optimum amount of mAb-PE and of FITC-HA was determined by titration using PBMC from untreated normal donors. HA binding by T cells (CD4+ or CD8+), B cells (CD19+) or monocytes (CD14+) was analyzed in replicate aliquots of each PBMC sample. Isotype-matched Ab binding was also assessed as a control for background staining.

PBMC stained with mAb-PE and FITC-HA were analyzed by FACS (Becton Dickinson). Data from 10,000 cells (after exclusion of erythrocytes and dead cells) were analyzed using Lysis II software. Cells expressing CD4+, CD8+, CD19+ and CD14+, (depending on the mAb used) were assessed for staining with FITC-HA in each PBMC subset. Auto-fluorescence of PBMC and PBMC stained with IgG-FITC or avidin-FITC were used as negative controls in evaluation of staining by FITC-HA. Staining was moderately bright for FITC-HA for 13 untreated normal donors, with mean fluorescence intensity reported in arbitrary units. HA binding by subsets of PBMC after infusion of HA was compared to the normal values. Assay variability was compensated through assessment of HA binding to CD14+ monocytes (collected at the 20 hr time point from each period) in each FACS analysis.

RESULTS

Characterization of HA Used for Study

The average MW of HA used in this study was 276.6 (hereafter referred to as 280) kDa after heat sterilization (which fragments HA slightly), and 519.7 kDa prior to heat exposure. Protein contamination of less than 0.001 µg/mg was detected (although values obtained were at the lower limit of detection of the assays). DNA contamination in commercial samples of HA, which has the capacity to induce cytokines in monocytes *in vitro* has been reported [34]. Thus, we assessed samples for DNA contamination but none was detected. Endotoxin was also not detected. These results indicate that contaminants common to commercial HA batches were below the detection limits of our assays.

Table 3. Frequency of Adverse Effects

Adverse Effects	Frequency	Related to Study	Severity	Subject Continued or Discontinued in Study
Influenza	1	Unrelated	Mild	Discontinued
Lower Arm Rash	1	Possibly related	Mild	Continued
Headache	4	Possibly related	Mild	Continued
Pro-thrombin Time	2	[a]Unrelated	Mild	Continued
Practical Thromboplastic Time	2	[a]Unrelated	Mild	Continued
Others	7	Possibly related	Mild	Continued

See Methods and Results sections for descriptions of procedures.
[a]Levels were altered at normal, endogenous HA levels.

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