JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Absorption, Uptake and Tissue Affinity of High-Molecular-Weight Hyaluronan after Oral Administration in Rats and Dogs

LAJOS BALOGH,[†] ANDRAS POLYAK,[†] DOMOKOS MATHE,[†] REKA KIRALY,[†] JULIANA THUROCZY,[†] MARIAN TEREZ,[†] GYOZO JANOKI,[†] YAOTING TING,[‡] LUKE R. BUCCI,[§] AND ALEXANDER G. SCHAUSS*,[||]

Department of Applied Radioisotopes, National "F.J.C." Research Institute for Radiobiology and Radiohygiene, "Fodor József" National Center of Health, Budapest, Hungary, FenChem Biotek LTD, Nanjing, China, Schiff Nutrition Group, Salt Lake City, Utah, and Life Sciences Division, American Institute for Biosocial and Medical Research, Inc., Puyallup, Washington

The purpose of this study was to determine the absorption, distribution and excretion of ^{99m}technetium-labeled, high-molecular-weight hyaluronan ((^{99m}Tc-HA) and ^{99m}technetium pertechnetate (^{99m}Tc-P) after single dose, oral administration to Wistar rats and Beagle dogs. A pilot study utilized ^{99m}Tc-HA alone, and a second confirmatory study compared uptake of labeled ^{99m}Tc-HA with ^{99m}Tc-P. Urinary and fecal excretion after ^{99m}Tc-HA ingestion by rats showed 86.7−95.6% of radioactivity was recovered, almost all in feces. All tissues examined showed incorporation of radioactivity from ^{99m}Tc-HA starting at 15 min and persisting for 48 h, in a pattern significantly different from ^{99m}Tc-P. Whole-body scintigraphs and close-ups of the ventral chest region showed nonalimentary radioactivity from ^{99m}Tc-HA concentrated in joints, vertebrae and salivary glands four hours after administration. Autoradiography of skin, bone and joint tissue pieces after 24 h showed incorporation of radioactivity from ^{99m}Tc-HA, but not from ^{99m}Tc-P. Conversely, absorption, distribution and excretion of ^{99m}Tc was completely different from ^{99m}Tc-HA, showing an expected pattern of rapid absorption and excretion in urine, with accumulation in thyroid glands, stomach, kidney and bladder. This report presents the first evidence for uptake and distribution to connective tissues of orally administered, high-molecular-weight HA.

KEYWORDS: Hyaluronan; oral absorption; ^{99m}technetium; scintigraphy; dietary supplements; joint tissues

INTRODUCTION

Hyaluronan (HA) is a high-molecular-weight glycosaminoglycan found in animals consisting of repeating disaccharide units of *N*-acetylglucosamine and glucuronate (*1*−*3*). Ubiquitous in animal tissues and fluids, HA is found in high concentrations in synovial fluid, vitreous humor and skin (*2*). HA is listed as an ingredient in an ever-increasing number of dietary supplements targeted to joint and skin health for animals and humans (*1*). In the United States, dietary supplements are regulated as a subset of foods under the Dietary Supplement Health & Education Act of 1994 (*4*). Estimates of hundreds of thousands of doses per year from dietary supplements containing HA in the United States alone show that HA is widely consumed, in addition to amounts normally present in animal-derived foodstuffs. HA in dietary supplements is derived from extraction of chicken combs or by microbial fermentation, although some sources are simple cartilage powders or hydrolyzed cartilage, which are not well characterized with respect to HA content and molecular weight (*1*). Thus, most HA being ingested by consumers is in a high-molecular-weight form around 1 million daltons (1 MDa). Obviously, if biological effects are expected from oral consumption of HA, then it is logical to assess the oral uptake into tissues, especially connective tissues and skin. However, we could not find any reports in the literature concerning absorption or uptake of high-molecular-weight HA after oral administration.

HA is typically used as a medical device for treatment of degenerative joint conditions, and is used in eye surgery and wound healing (*1*−*3, 5, 6*). HA is administered by injectable or topical routes of administration for these uses. Because of the large molecular weight and size of individual HA molecules (usually 1 MDa or more), and rapid clearance from the

* Corresponding author. Mailing address: Life Sciences Division, American Institute for Biosocial and Medical Research, Inc, 4117 South Meridian, Puyallup, WA 98373. E-mail: alex@aibmr.com. Phone: 253-286-2888. Fax: 253-286-2451.

[†] "Fodor József" National Center of Health.

[‡] FenChem Biotek LTD.

[§] Schiff Nutrition Group.

[||] American Institute for Biosocial and Medical Research, Inc.

10.1021/jf8017029 CCC: $40.75
Published on Web 10/30/2008

bloodstream by the liver (*2*), it has been assumed that oral HA would exhibit poor systemic uptake and/or clinical utility. However, this assumption has not been tested or reported in the scientific literature using modern techniques. This study investigated the uptake into bloodstream and tissues in two animal models after oral ingestion of radioactively labeled, high-molecular-weight HA, similar to that used in some dietary supplements. We found evidence for uptake of radioactively labeled HA into tissues, especially connective tissues, after oral administration to animals.

MATERIALS AND METHODS

Test Materials. Food grade sodium hyaluronate (trade name Nutrihyl, manufactured by Bioconti spol.sr.o, Tvardkova, 1191, Usti nad Orlici, Czech Republic), containing 93.6% sodium hyaluronate, was used for the pilot study. Molecular weight was approximately 1 MDa. Food grade sodium hyaluronate (trade name HyaMax, manufactured by FenChem Biotek Ltd., Nanjing, China) was used for the controlled experiment. Molecular weight was approximately 1.1−1.5 MDa for each HA.

^{99m}Technetium Labeling of Hyaluronan. ^{99m}Tc-pertechnetate (^{99m}Tc-P) was collected from a ^{99m}Mo−^{99m}Tc generator (Drygen, Sorin Biomedica) (*7*). Purity of the eluted TcO_4^- (pertechnetate) solution was over 99%. The labeling process for HA that yielded the highest efficiency was as follows: 9 mg of HA in 900 μL of distilled water, 30 μL of 10^{-3} M calcium glucoheptonate, 1.1 mL of 2.2 GBq ^{99m}Tc-pertechnetate solution, and 100 μL of 1 mg/mL $SnCl_2$ in 1.0 M HCl, pH 4.0. This mixture was incubated at 50 °C for 90 min with gentle stirring.

Two thin layer chromatograph (TLC) methods were used to separate the ^{99m}Tc-labeled HA (^{99m}Tc-HA) from free pertechnetate and labeled tin colloid and deduce labeling efficiency: TLC (0.2 mm silica gel 60 on aluminum sheets, Merck Inc.) developed in acetone for determining the free pertechnetate impurity and ITLC-SG (silica gel impregnated glass fiber sheets, Gelman Sciences Inc.) developed in 0.9% NaCl solution for determining tin colloid impurity. In vitro stability of ^{99m}Tc-HA was checked by the same methods after 6, 24, and 48 h incubation in physiological saline, canine blood sera and canine synovial fluids as mediums at room temperature.

Urinary and Fecal Excretion in Rats. A single dose of ^{99m}Tc-HA (100 MBq/0.2 mg/0.2 mL) or ^{99m}Tc-P (100 MBq/0.2 mL) was administered per os via a Teflon feeding tube to five Wistar rats weighing between 150 and 200 g in each study. ^{99m}Tc agents were administered within one hour after finishing the labeling process. Rats were held in individual metabolism cages for separate collection of urine and feces at 12, 24, 48 and 72 h post administration. Radioactivity collected from urine and feces was reported as the percentage of applied dose (mean ± SD from five animals). Animals were maintained and handled according to the Declaration of Helsinki guidelines.

Blood and Urinary Clearance in Dogs. A single dose of ^{99m}Tc-HA (370 MBq/10 mg/1.5 mL) or ^{99m}Tc-P (370 MBq/1.5 mL) was administered per os via a Teflon feeding tube to two Beagle dogs weighing 10−15 kg in each study. Blood and urine samples were collected at multiple time points after administration, including 2, 5, 15, 30 min, 1, 2, 4, 6, 12, 24, 48 and 72 h after administration. Results were reported as the percent of administered dose/gram of whole blood or urine. Fitted time activity curves were plotted to assess blood and urine clearance over time.

Tissue Distribution Assay in Rats. A single dose of ^{99m}Tc-HA (100 MBq/0.2 mg/0.2 mL) or ^{99m}Tc-P (100 MBq/0.2 mL) was administered via feeding tube per os to Wistar rats weighing 150−200 g in both studies. Three animals per time point were euthanized at each of the following times after administration of the labeled compound: 5, 15, 30 min, 1, 2, 4, 6, 12, 24 h. For time points of 48 and 72 h, 2000 MBq/0.2 mL was administered.

Radioactivity in the following organs and tissues was counted in both studies: blood, bone, heart, large intestine (two sections), liver (two pieces), lungs, kidneys, muscle, small intestines (two sections), spleen, stomach and urinary bladder in both studies and additionally, brain, complete knee joint, skin and thyroids in the controlled study. Percentages of applied dose/whole organ and applied dose/gram tissue were calculated as mean ± SD. After removal of other organs and tissues, carcass radioactivity was measured, and this data was used to calculate radioactivity, assuming the following percentages of body weight in blood (6.5%), bone (10.8%) and muscles (45%).

Scintigraphic Examinations of Animals. In the pilot study, early, middle and late static pictures (whole body and zoomed pictures from different regions of the body) were taken 30 min, 1, 2, 4, 6, 12, 24, 48 and 72 h after oral administration of ^{99m}Tc-HA to assess its absorption, distribution and excretion in two Wistar rats and a Beagle dog with a Nucline x-ring gamma camera (*8*). In the controlled study, early, middle and late static pictures were taken 30 min, 1, 2, 4, 6, 12, 24, and 48 h after oral application of labeled compounds in four Wistar rats. Dorso-ventral and lateral whole body views were taken at each time point for each agent for each study.

SPECT/CT Scans of Rats. In the controlled study, supersensitive nanoSPECT/CT scans were taken in rats to image and localize biodistribution of radioactivity in the whole body, and to determine the presence or absence of radioactivity in ex vivo samples of bone, joints and skin (Nano SPECT/CT, Bioscan Ltd.). Hybrid images of whole body, three-dimensional lateral views and sagittal, coronal and tranverse slices (through areas of high radioactivity as shown by the three-dimensional image) were imaged from 0.5−48 h, showing the skeleton and ghost images of the rest of the body with radioactivity superimposed in color. In addition, transverse slice images of joints were also imaged at 48 h. Semiquantitative biodistribution analysis via ROI-data calculations was performed to measure bioutilization of ^{99m}Tc-HA in rats.

Autoradiography of Rat Tissue Samples. In the controlled study, a single dose of ^{99m}Tc-HA (100 MBq/0.2 mg/0.2 mL) or ^{99m}Tc-P (100 MBq/0.2 mL) was administered per os via a Teflon feeding tube to four Wistar rats weighing 150−200 g. Animals were sacrificed 24 and 48 h after administration and parallel samples of complete femurs, forelegs, hind legs, tails and skin were removed and contact microautoradiography and scintigraphy performed.

Statistical Analysis. Comparisons of the percentage of ingested dose in tissues between the ^{99m}Tc-P and ^{99m}Tc-HA (HyaMax) groups were determined by ANOVA (SPSS ver 12.0). Other statistical analyses were performed with GraphPad Prism Version 5.01 or GraphPad InStat 3 software.

RESULTS

Labeling Purity, Efficiency and Stability. Purity of eluted $^{99m}TcO_4^-$ (pertechnetate) solution was always over 99%. Results of chromatographic analyses showed that labeling efficiency was greater than 85% in both studies. Free pertechnetate in ^{99m}Tc-HA preparations was less than 3% of radioactivity, and labeled tin colloid was less than 7% of radioactivity in ^{99m}Tc-HA (**Figure 1**). Label proved to be stable within 48 h after labeling when stored in saline, canine serum and canine synovial fluid (data not shown).

Urinary and Fecal Excretion in Rats. In the pilot study, average total excretions of Nutrihyl ^{99m}Tc-HA over the 72 h period were 92.3 ± 1.7% of ingested dose in feces and 3.2 ± 0.42% of ingested dose in urine, for a total urine + feces excretion of 95.6 ± 2.0% ingested dose (**Table 1**). In the confirmatory study, average total excretions of HyaMax ^{99m}Tc-HA over the 72 h period were 84.6 ± 7.8% of ingested dose in feces and 2.0 ± 0.63% of ingested dose in urine, for a total urine + feces excretion of 86.7 ± 8.0% of ingested dose. Average total excretions of ^{99m}Tc over the 72 h period were 1.2 ± 0.09% of ingested dose in feces and 93.2 ± 4.5% of ingested dose in urine, for a total urine + feces excretion of 94.4 ± 4.5% of ingested dose. The similarity of excretion pattern for the two ^{99m}Tc-HA groups is in marked contrast to the pattern for ^{99m}Tc-P. Both ^{99m}Tc-HA groups exhibited excretion of almost all radioactivity in feces after 24 h, coinciding with gut transit time

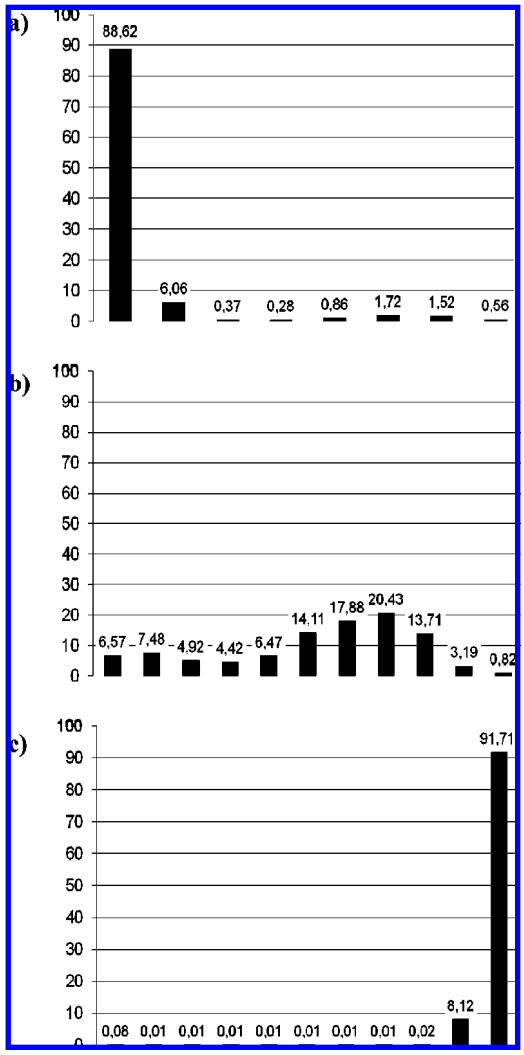


Figure 1. Chromatographic separation of ^{99m}technetium-labeled HyaMax hyaluronate (^{99m}Tc-HA) and ^{99m}Tc-pertechnetate label (^{99m}Tc). (**a**) TLC of ^{99m}Tc-HA developed in acetone; (**b**) ITLC of ^{99m}Tc-HA developed in physiological saline; and (**c**) TLC of ^{99m}Tc developed in acetone. *X*-axis: arbitrary fraction number. *Y*-axis: % of applied radioactivity.

Table 1. Urinary and Fecal Excretion of ^{99m}Tc-Hyaluronan and ^{99m}Tc-Pertechnetate in Rats (as % of Ingested Dose ± SD)

group	hours	feces	urine	total
Nutrihyl ^{99m}Tc-HA	0–12	2.1 ± 1.3	0.40 ± 0.17	2.5 ± 1.3
	0–24	39.5 ± 11.7	1.3 ± 0.38	40.7 ± 13.2
	0–48	74.7 ± 7.4	2.2 ± 0.29	76.9 ± 8.6
	0–72	92.3 ± 1.7	3.2 ± 0.42	95.6 ± 2.3
HyaMax ^{99m}Tc-HA	0–12	1.8 ± 1.6	0.34 ± 0.11	2.2 ± 1.6
	0–24	42.2 ± 9.5	0.78 ± 0.18	42.9 ± 9.4
	0–48	69.4 ± 9.7	1.4 ± 0.38	70.8 ± 9.8
	0–72	84.6 ± 7.8	2.0 ± 0.63	86.7 ± 8.0
^{99m}Tc-pertechnetate	0–12	0.44 ± 0.09	65.8 ± 3.7	66.2 ± 3.7
	0–24	0.64 ± 0.09	82.4 ± 4.7	83.1 ± 4.7
	0–48	0.88 ± 0.08	89.0 ± 4.2	89.8 ± 4.2
	0–72	1.2 ± 0.09	93.2 ± 4.4	94.4 ± 4.5

and verified by whole-body scintigraphy. However, the ^{99m}Tc-P group excreted almost all radioactivity in urine, not feces. This pattern of excretion for ^{99m}Tc-P is typical and well-known.

Blood and Urinary Clearance in Dogs. In the pilot study, administration of a single dose of ^{99m}Tc-HA to Beagle dogs showed a peak blood clearance of 0.05%/g of blood between 2−4 h postadministration (**Table 2**). The blood clearance curve showed a sharp rise in the first two hours, with a steady decrease over the ensuing 72 h time period. In the controlled study, blood clearance rose immediately, and returned to background by 6 h. Blood clearance of ^{99m}Tc started at 15 min, reached a peak between 2 and 4 h, and then returned to background after 48 h.

In the pilot study, urinary clearance in Beagle dogs peaked at 0.70%/g of urine 48 h after administration of ^{99m}Tc-HA (**Table 2**). The urinary clearance curve showed a progressive increase in clearance toward a peak at 48 h, followed by a decline over the next 24 h period to 0.20%/g of urine at the 72 h time point. In the controlled study, urinary clearance of ^{99m}Tc-HA also appeared after 0.5 h, but returned to background after 12 h. Urinary clearance of ^{99m}Tc-P started at 0.5 h and continued to 72 h.

Comparing clearance patterns of ^{99m}Tc-P to ^{99m}Tc-HA, there were obvious differences between the two in the confirmatory study. The data from ^{99m}Tc-HA in the pilot study looked similar to the ^{99m}Tc-P control pattern. However, the difference between urine and fecal excretion was similar for both HA groups and diametrically opposed to the ^{99m}Tc-P control pattern.

Tissue Biodistribution of Radioactivity. Three Wistar rats were sacrificed at each of 5, 15, 30 min, 1, 2, 4, 6, 12, 24, 48 and 72 h after administration of agents, and radioactivity in blood, bone, brain, heart, kidneys, knee joints, two sections of large intestine, two sections of liver, lung, muscle, two sections of small intestine, skin, spleen, stomach, thyroids and urinary bladder was counted and expressed as percentage of ingested dose per organ (**Table 3**). Brain, knee joint, skin and thyroid tissues were not measured in the pilot study (Nutrihyl group). Patterns of tissue distribution of radioactivity between ^{99m}Tc-P and ^{99m}Tc-HA groups (characterized by ratios between tissues at each time point) were immediately different, and remained different throughout the time course of the studies. ^{99m}Tc-P administration showed a characteristic pattern of rapid removal of radioactivity from the gastrointestinal tract (less than two hours), rapid uptake into and removal from most tissues at low levels (within two hours), with later residual radioactivity in thyroids, kidneys and urinary bladder. Loss of radioactivity from the body after ^{99m}Tc-P administration was more rapid than both ^{99m}Tc-HA groups, as shown by the total radioactivity in tissues per time point. After 72 h, only 0.5% of the ingested dose for ^{99m}Tc-P was recovered from tissues, consistent with the recovery in urine and feces in **Table 1**. ANOVA analysis of total radioactivity (fecal and urinary) excreted for each time period found significant differences among groups ($P < 0.0001$ for 0−12 and 0−24 h, $P = 0.0099$ for 0−48 h, $P = 0.0487$ for 0−72 h).

In contrast, the pattern of distribution of radioactivity exhibited by both ^{99m}Tc-HA groups was remarkably different from the ^{99m}Tc-P group, and similar to each other. The time interval between the pilot study and controlled study was four years, suggesting reproducible biological effects after oral administration of ^{99m}Tc-HA. It was clear that the majority of orally administered ^{99m}Tc-HA remained in the gastrointestinal tract, as evidenced by the localization of radioactivity in first stomach, then small intestine segments, then large intestine segments in keeping with normal gut transit times for rats. Radioactivity after ^{99m}Tc-HA administration showed accumulation in blood, bone, knee joint, muscle and skin tissues, but not other organs. Interestingly, a very rapid uptake of radioactivity in bone, muscle and small and large intestine tissues at the 5 and 15 min time points was seen, even though the ^{99m}Tc-HA bolus was clearly not in intestinal segments yet (as shown by scintigraphic data). Rapid uptake of radioactivity into these tissues was not seen for the ^{99m}Tc-P group at these time points,

Table 2. Blood and Urinary Clearance after Oral Administration of ^{99m}Tc-Hyaluronan (^{99m}Tc-HA) or ^{99m}Tc-Pertechnetate in Beagle Dogs (% Ingested Dose/Gram of Fluid)

		time (h)										
group	fluid	0.08	0.25	0.5	1	2	4	6	12	24	48	72
Nutrihyl ^{99m}Tc-HA	blood	0.00	0.01	0.01	0.02	0.05	0.05	0.01	0.01	0.03	0.02	0.00
	urine	0.00	0.00	0.07	0.05	0.03	0.02	0.05	0.10	0.30	0.70	0.20
HyaMax ^{99m}Tc-HA	blood	0.06	0.06	0.02	0.04	0.02	0.01	0.01	0.00	0.00	0.00	0.00
	urine	0.00	0.00	0.30	0.07	0.11	0.09	0.12	0.02	0.00	0.00	0.00
^{99m}Tc-pertechnetate	blood	0.00	0.01	0.02	0.03	0.06	0.05	0.03	0.02	0.03	0.01	0.00
	urine	0.00	0.00	0.06	0.06	0.04	0.03	0.05	0.03	0.02	0.08	0.03

indicating uptake was not due to possible contaminating free ^{99m}Tc-P. Afterward, tissue uptake of radioactivity into blood and peripheral tissues coincided with presence of ^{99m}Tc-HA in absorptive sections of the gastrointestinal tract. After 72 h, 10.0% of ingested radioactivity remained in tissues, corresponding closely with the figure of 13.3% excreted from **Table 1**.

Figure 2 illustrates uptake of radioactivity in selected tissues as a function of time, allowing a direct visual comparison between ^{99m}Tc-P and ^{99m}Tc-HA groups. Radioactivity (*y*-axis) scales were adjusted and plotted as a function of time (*x*-axis) on a logarithmic scale for better visualization. In general, a common pattern for blood, bone, knee joint, liver, muscle and skin is apparent. These tissues showed uptake of radioactivity from ^{99m}Tc-P between 0 and 2 h, and then little or no uptake afterward. However, these tissues showed uptake of radioactivity after ^{99m}Tc-HA from 2 h onward, usually with accumulation at later time points (24−72 h). Kidney, thyroid and urinary bladder tissues exhibited a much different pattern, with uptake of radioactivity from ^{99m}Tc-P persisting for 0−12 h and longer, whereas uptake after ^{99m}Tc-HA was nil or in trace amounts only. Small and large intestines (not pictured) showed little or no appreciable radioactivity after ^{99m}Tc-P left the stomach. After ^{99m}Tc-HA, these tissues exhibited the majority of ingested radioactivity in a pattern consistent with normal gut transit time. In fact, at the appropriate time points, radioactivity was seen in formed feces in the terminal large intestine and rectum, as shown by scintigraphs (not pictured). The remaining tissues (brain,

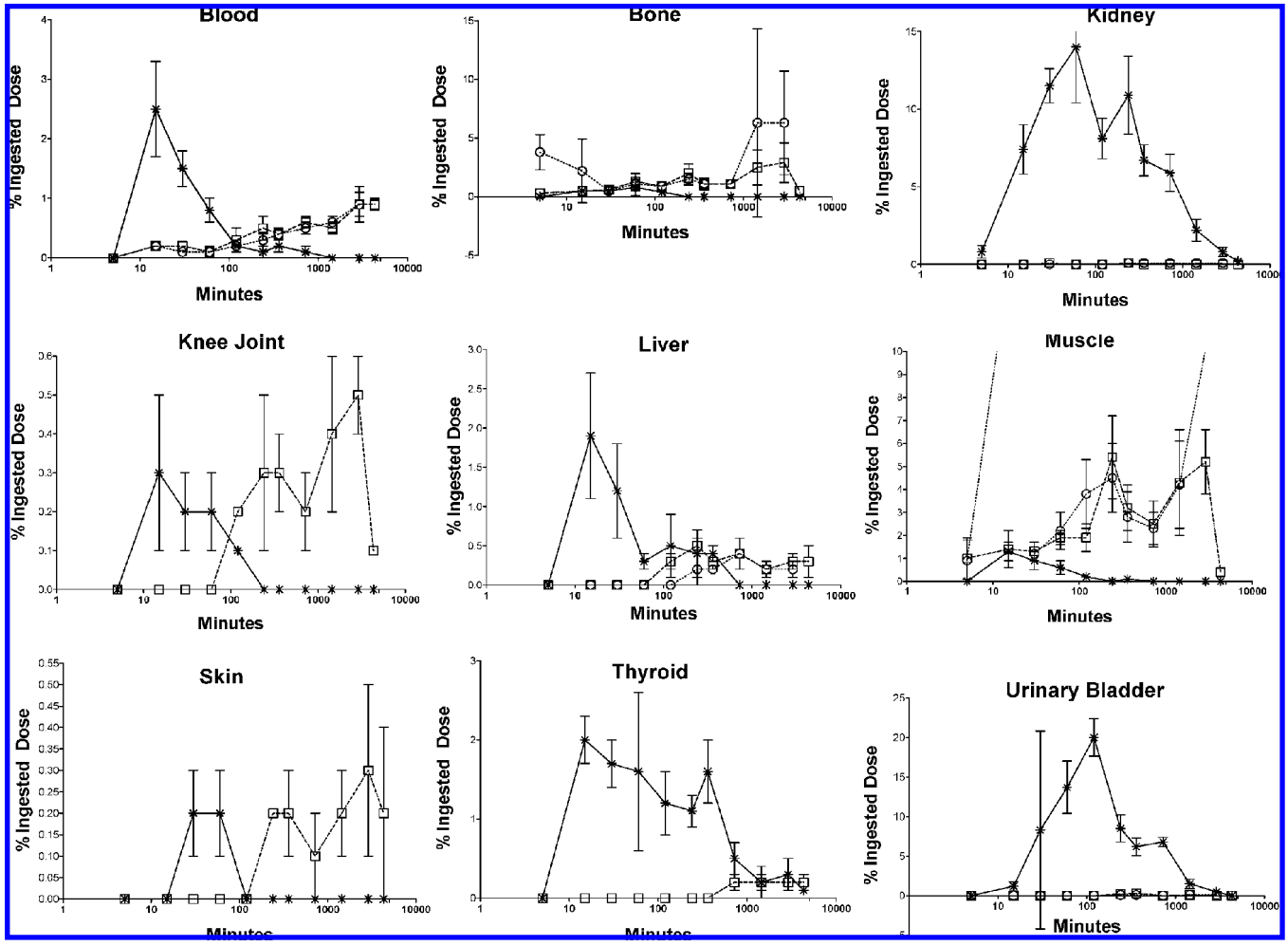


Figure 2. Radioactivity (% of ingested dose ± SD) in selected tissues of rats, comparing uptake between ^{99m}Tc-pertechnetate (*) and ^{99m}Tc-hyaluronans (□ for HyaMax, ○ for NutriHyl) over a 72 h period. There were three rats per time point. *Y*-axis scales have been adjusted, and time plotted with a logarithmic scale to more easily visualize data, especially 0−12 h. Time points were 5, 15, 30, 60, 120, 240, 360, 720, 1440, 2880 and 4320 min after administration.

Table 3. Tissue Biodistribution of Radioactivity as % of Ingested Dose after Oral Administration of ^{99m}Tc-Pertechnetate (^{99m}Tc) and ^{99m}Tc-Hyaluronan (^{99m}Tc-HA) from Each Study (Mean ± SD) to Wistar Rats ($n = 3$ per time point)[a]

		^{99m}Tc-HA				^{99m}Tc-HA	
tissue	^{99m}Tc	HyaMax	Nutrihyl	tissue	^{99m}Tc	HyaMax	Nutrihyl
	5 min				60 min		
blood	ND	ND	ND	blood	0.8 ± 0.2	0.1 ± 0.0	0.1 ± 0.1
bone	ND	0.3 ± 0.2	3.8 ± 1.5	bone	0.8 ± 0.7	1.3 ± 0.7	1.1 ± 0.6
brain	ND	ND	NA	brain	0.1 ± 0.0	ND	NA
heart	ND	ND	ND	heart	0.2 ± 0.1	ND	ND
kidneys	0.8 ± 0.4	ND	ND	kidneys	14.0 ± 3.6	ND	ND
knee joint	ND	ND	NA	knee joint	0.2 ± 0.1	ND	NA
large intestine (1)	ND	0.2 ± 0.1	0.1 ± 0.1	large intestine (1)	0.7 ± 0.2	ND	ND
large intestine (2)	ND	0.1	0.1 ± 0.0	large intestine (2)	0.1 ± 0.0	ND	ND
liver (1)	ND	ND	ND	liver (1)	0.2 ± 0.1	ND	ND
liver (2)	ND	ND	ND	liver (2)	0.1 ± 0.0	ND	ND
lung	ND	ND	0.1 ± 0.0	lung	0.2 ± 0.1	ND	ND
muscle	ND	1.0 ± 0.9	0.9 ± 0.9	muscle	0.6 ± 0.3	1.9 ± 0.3	2.2 ± 0.8
skin	ND	ND	NA	skin	0.2 ± 0.1	ND	NA
small intestine (1)	ND	6.3 ± 2.6	11.5 ± 12.0	small intestine (1)	3.3 ± 0.9	23.3 ± 20.1	18.9 ± 16.4
small intestine (2)	ND	0.7 ± 0.2	0.5 ± 0.5	small intestine (2)	1.3 ± 0.3	23.3 ± 19.2	29.7 ± 32.0
spleen	0.5 ± 0.2	ND	ND	spleen	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
stomach	91.9 ± 5.0	82.6 ± 2.4	80.8 ± 12.0	stomach	28.4 ± 4.8	45.0 ± 4.2	32.6 ± 28.3
thyroids	ND	ND	NA	thyroids	1.6 ± 1.0	ND	NA
urinary bladder	ND	ND	ND	urinary bladder	13.7 ± 3.3	ND	ND
total	93.2 ± 5.5	90.8 ± 0.6	97.9 ± 1.8	total	65.4 ± 6.7	94.9 ± 4.0	85.0 ± 13.3
	15 min				120 min		
blood	2.5 ± 0.8	0.2 ± 0.0	0.2 ± 0.0	blood	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.0
bone	0.5 ± 0.1	0.5 ± 0.1	2.2 ± 2.7	bone	0.4 ± 0.3	0.9 ± 0.4	0.9 ± 0.1
brain	ND	ND	NA	brain	0.2 ± 0.1	ND	NA
heart	0.3 ± 0.2	ND	ND	heart	0.1 ± 0.0	ND	ND
kidneys	7.4 ± 1.6	ND	ND	kidneys	8.1 ± 1.3	ND	ND
knee joint	0.3 ± 0.2	ND	NA	knee joint	0.1 ± 0.0	0.2 ± 0.0	NA
large intestine (1)	0.5 ± 0.2	0.7	0.3 ± 0.4	large intestine (1)	0.2 ± 0.1	0.1 ± 0.0	ND
large intestine (2)	ND	0.3	0.1 ± 0.1	large intestine (2)	0.1 ± 0.0	2.9 ± 3.6	2.0 ± 1.9
liver (1)	0.8 ± 0.4	ND	ND	liver (1)	0.3 ± 0.3	0.2 ± 0.1	ND
liver (2)	1.1 ± 0.4	ND	ND	liver (2)	0.2 ± 0.1	0.1 ± 0.0	ND
lung	1.2 ± 0.4	ND	0.1 ± 0.0	lung	0.1 ± 0.1	ND	ND
muscle	1.3 ± 0.4	1.4 ± 0.8	13.5 ± 20.2	muscle	0.2 ± 0.1	1.9 ± 0.6	3.8 ± 1.5
skin	ND	ND	NA	skin	ND	ND	NA
small intestine (1)	6.2 ± 0.4	20.7 ± 8.7	26.3 ± 12.3	small intestine (1)	0.9 ± 0.4	35.3 ± 21.7	36.7 ± 25.8
small intestine (2)	2.2 ± 0.5	7.1 ± 0.8	7.9 ± 1.4	small intestine (2)	0.3 ± 0.2	17.3 ± 21.8	12.9 ± 11.0
spleen	0.3 ± 0.1	ND	ND	spleen	ND	ND	ND
stomach	62.0 ± 1.6	57.8 ± 5.7	51.1 ± 15.1	stomach	23.4 ± 3.4	42.1 ± 12.3	39.0 ± 12.5
thyroids	2.0 ± 0.3	ND	NA	thyroids	1.2 ± 0.4	ND	NA
urinary bladder	1.2 ± 0.6	ND	ND	urinary bladder	20.0 ± 2.4	ND	ND
total	89.3 ± 0.7	88.1 ± 9.3	98.9 ± 0.5	total	55.5 ± 4.1	94.3 ± 3.5	95.7 ± 3.3
	30 min						
blood	1.5 ± 0.3	0.2 ± 0.0	0.1 ± 0.0				
bone	0.5 ± 0.3	0.6 ± 0.3	0.5 ± 0.2				
brain	0.1 ± 0.0	ND	NA				
heart	0.3 ± 0.3	ND	ND				
kidneys	11.5 ± 1.1	0.1	ND				
knee joint	0.2 ± 0.1	ND	NA				
large intestine (1)	0.9 ± 0.3	0.2	0.1 ± 0.1				
large intestine (2)	1.1 ± 0.1	ND	ND				
liver (1)	0.7 ± 0.3	ND	ND				
liver (2)	0.5 ± 0.3	ND	ND				
lung	ND	ND	ND				
muscle	0.9 ± 0.4	1.3 ± 0.4	1.2 ± 0.3				
skin	0.2 ± 0.1	ND	NA				
small intestine (1)	5.4 ± 0.6	18.4 ± 11.7	28.3 ± 20.9				
small intestine (2)	4.3 ± 0.3	10.4 ± 12.0	9.1 ± 7.9				
spleen	0.2 ± 0.1	ND	ND				
stomach	42.8 ± 4.9	67.5 ± 4.2	58.4 ± 16.8				
thyroids	1.7 ± 0.3	ND	NA				
urinary bladder	8.3 ± 12.5	ND	ND				
total	80.5 ± 15.4	98.4 ± 0.5	97.9 ± 1.9				

Table 3. Continued

tissue	^{99m}Tc	^{99m}Tc-HA		tissue	^{99m}Tc	^{99m}Tc-HA	
		HyaMax	Nutrihyl			HyaMax	Nutrihyl
	4 h				24 h		
blood	0.1 ± 0.0	0.5 ± 0.2	0.3 ± 0.1	blood	ND	0.5 ± 0.1	0.6 ± 0.1
bone	ND	2.0 ± 0.8	1.5 ± 0.5	bone	ND	2.5 ± 1.5	6.3 ± 8.0
brain	ND	ND	NA	brain	ND	ND	NA
heart	ND	ND	ND	heart	ND	ND	ND
kidneys	10.9 ± 2.5	0.1 ± 0.0	0.1 ± 0.0	kidneys	2.2 ± 0.7	ND	0.1 ± 0.0
knee joint	ND	0.3 ± 0.2	NA	knee joint	ND	0.4 ± 0.2	NA
large intestine (1)	0.2 ± 0.2	10.0 ± 9.8	8.5 ± 8.8	large intestine (1)	ND	21.3 ± 10.4	21.8 ± 11.1
large intestine (2)	ND	26.3 ± 13.6	25.9 ± 11.5	large intestine (2)	ND	15.4 ± 7.2	19.8 ± 9.1
liver (1)	0.2 ± 0.1	0.3 ± 0.0	0.1 ± 0.1	liver (1)	ND	0.1 ± 0.1	0.1 ± 0.0
liver (2)	0.2 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	liver (2)	ND	0.1 ± 0.0	0.1 ± 0.0
lung	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	lung	ND	ND	0.1 ± 0.0
muscle	ND	5.4 ± 1.8	4.5 ± 1.5	muscle	ND	4.3 ± 2.3	4.2 ± 1.9
skin	ND	0.2 ± 0.0	NA	skin	ND	0.2 ± 0.1	NA
small intestine (1)	1.1 ± 0.2	25.0 ± 10.5	25.3 ± 9.5	small intestine (1)	0.4 ± 0.2	3.2 ± 1.5	3.7 ± 1.6
small intestine (2)	0.6 ± 0.3	6.6 ± 4.5	7.2 ± 6.3	small intestine (2)	ND	2.3 ± 1.9	1.9 ± 1.5
spleen	ND	ND	ND	spleen	ND	ND	ND
stomach	23.1 ± 5.4	22.4 ± 3.9	18.5 ± 1.6	stomach	6.1 ± 1.0	4.0 ± 3.5	4.2 ± 3.9
thyroids	1.1 ± 0.2	ND	NA	thyroids	0.2 ± 0.2	0.2 ± 0.1	NA
urinary bladder	8.5 ± 1.7	0.2 ± 0.1	0.1 ± 0.1	urinary bladder	1.5 ± 0.6	0.1 ± 0.0	0.1 ± 0.0
total	46.1 ± 2.1	98.9 ± 0.6	92.3 ± 3.3	total	10.4 ± 1.4	54.4 ± 22.1	62.9 ± 25.6
	6 h				48 h		
blood	0.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	blood	ND	0.9 ± 0.3	0.9 ± 0.2
bone	ND	1.1 ± 0.5	1.1 ± 0.5	bone	ND	2.9 ± 1.7	6.3 ± 4.4
brain	ND	ND	NA	brain	ND	ND	NA
heart	ND	ND	ND	heart	ND	ND	0.1 ± 0.0
kidneys	0.8 ± 0.4	ND	0.1 ± 0.0	kidneys	0.8 ± 0.3	ND	0.1 ± 0.0
knee joint	ND	0.3 ± 0.1	NA	knee joint	ND	0.5 ± 0.1	NA
large intestine (1)	ND	29.5 ± 11.3	27.7 ± 9.5	large intestine (1)	ND	8.6 ± 5.6	5.3 ± 4.8
large intestine (2)	ND	41.7 ± 13.7	41.7 ± 9.0	large intestine (2)	ND	4.3 ± 4.2	3.9 ± 3.6
liver (1)	ND	0.1 ± 0.0	0.1 ± 0.0	liver (1)	ND	0.2 ± 0.1	0.1 ± 0.1
liver (2)	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	liver (2)	ND	0.1 ± 0.0	0.1 ± 0.0
lung	ND	ND	ND	lung	ND	ND	0.1 ± 0.0
muscle	0.1 ± 0.0	3.2 ± 1.0	2.8 ± 1.1	muscle	ND	5.2 ± 1.4	10.1 ± 9.2
skin	ND	0.2 ± 0.1	NA	skin	ND	0.3 ± 0.2	NA
small intestine (1)	0.2 ± 0.1	12.4 ± 9.6	12.4 ± 9.2	small intestine (1)	ND	0.3 ± 0.1	0.3 ± 0.1
small intestine (2)	0.3 ± 0.1	4.7 ± 7.5	4.7 ± 7.6	small intestine (2)	ND	0.3 ± 0.2	0.3 ± 0.2
spleen	ND	ND	ND	spleen	ND	ND	0.1 ± 0.0
stomach	15.1 ± 1.1	1.2 ± 1.4	1.0 ± 1.5	stomach	2.1 ± 0.3	0.7 ± 0.5	0.7 ± 0.5
thyroids	1.6 ± 0.4	ND	NA	thyroids	0.3 ± 0.2	0.2 ± 0.1	NA
urinary bladder	6.2 ± 1.1	0.3 ± 0.0	0.1 ± 0.1	urinary bladder	0.5 ± 0.1	ND	0.1 ± 0.0
total	30.6 ± 2.2	94.6 ± 6.2	92.1 ± 3.4	total	3.7 ± 0.3	23.9 ± 8.3	28.5 ± 9.2
	12 h				72 h		
blood	0.1 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	blood	ND	0.9 ± 0.1	NA
bone	ND	1.1 ± 0.3	1.1 ± 0.1	bone	ND	0.5 ± 0.3	NA
brain	ND	ND	NA	brain	ND	ND	NA
heart	ND	ND	ND	heart	ND	ND	NA
kidneys	5.9 ± 1.2	ND	0.1 ± 0.0	kidneys	0.2 ± 0.1	ND	NA
knee joint	ND	0.2 ± 0.1	NA	knee joint	ND	0.1	NA
large intestine (1)	ND	43.1 ± 6.0	37.3 ± 19.4	large intestine (1)	ND	2.1 ± 0.7	NA
large intestine (2)	ND	46.6 ± 6.8	39.4 ± 15.0	large intestine (2)	ND	4.9 ± 2.6	NA
liver (1)	ND	0.2 ± 0.0	0.2 ± 0.0	liver (1)	ND	0.2 ± 0.1	NA
liver (2)	ND	0.2 ± 0.1	0.2 ± 0.1	liver (2)	ND	0.1 ± 0.1	NA
lung	ND	ND	ND	lung	ND	ND	NA
muscle	ND	2.5 ± 1.0	2.3 ± 0.7	muscle	ND	0.4 ± 0.1	NA
skin	ND	0.1 ± 0.1	NA	skin	ND	0.2 ± 0.2	NA
small intestine (1)	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.2	small intestine (1)	ND	0.3 ± 0.1	NA
small intestine (2)	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	small intestine (2)	ND	0.3 ± 0.2	NA
spleen	ND	ND	ND	spleen	ND	ND	NA
stomach	8.7 ± 1.2	ND	0.1 ± 0.0	stomach	0.2 ± 0.2	0.3 ± 0.2	NA
thyroids	0.5 ± 0.2	0.2 ± 0.1	NA	thyroids	0.1 ± 0.0	0.2 ± 0.1	NA
urinary bladder	6.8 ± 0.6	ND	ND	urinary bladder	ND	ND	NA
total	22.5 ± 1.8	94.9 ± 6.5	81.6 ± 26.5	total	0.5 ± 0.3	10.0 ± 2.5	NA

[a] NA = not analyzed; ND = not detected (radioactivity was below detection limits).

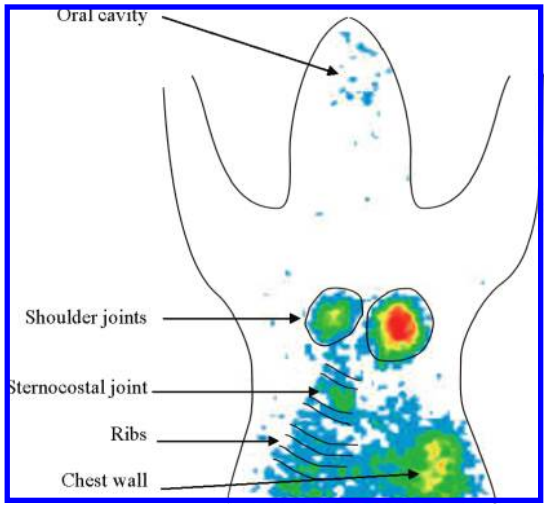


Figure 3. Ventral view of a scintigraphic scan of rat chest from the pilot study showing biodistribution of radioactivity four hours after administration of ^{99m}Tc-hyaluronan (NutriHyl) in joints, bones, muscles and salivary glands.

heart, lungs and spleen) showed only minor amounts of radioactivity after ^{99m}Tc-P from 0 to 2 h, and no radioactivity after ^{99m}Tc-HA.

Scintigraphic Biodistribution Studies in Rats and Dogs. Scintigraphic, whole body scans of rats from both studies were set at low gain and confirmed the tissue distribution data, with less sensitivity (data not presented). After ^{99m}Tc-P administration, radioactivity was quickly found throughout the entire body, as evidenced by a low, diffuse opacity from 30 min to six hours. Thyroid, kidneys and urinary bladder showed clear uptake from 0.5 to 6 h, but most radioactivity was associated with gastrointestinal tissues. At later time points (12, 24, 48 h), radioactivity was seen only in gastrointestinal regions.

After ^{99m}Tc-HA administration, radioactivity quickly entered the proximal small intestine, and was seen to traverse the gastrointestinal tract over time, ending as formed feces 12−24 h later (data not presented). Radioactivity entered the large intestine between 6 and 12 h. There was no visualization of diffuse radioactivity throughout the entire body at any time point with the low gain setting. There was no appearance of radioactivity in thyroid or bladder at any time point. Ventral scans of rat chests taken at high gain in the pilot study four hours after administration of ^{99m}Tc-HA showed that measurable amounts of radioactivity were seen in shoulder joints, vertebrae and sternocostal joints, but not thyroid (**Figure 3**). Ventral images taken at high gain in the pilot study from dogs two and four hours after administration of ^{99m}Tc-HA also showed that measurable amounts of radioactivity were localized in bones, joints and salivary glands, but not thyroid, in dogs (**Figure 4**). Thus, the pattern of tissue distribution of radioactivity differed between ^{99m}Tc-P and ^{99m}Tc-HA groups, mirroring and confirming the pattern seen in the tissue distribution data.

Ex vivo contact autoradiography of connective tissue samples from the controlled study showed a clear-cut, marked difference in uptake of radioactivity between ^{99m}Tc-P and ^{99m}Tc-HA groups 24 h after administration (**Figure 5**). Each sample examined (femur, foreleg, hindleg, skin and tail section) showed greater incorporation of radioactivity from the ^{99m}Tc-HA group, ranging from 113−342 times greater than incorporation from ^{99m}Tc-P. Uptake in cleaned femurs (including cartilage on both ends) showed that radioactivity was taken up in connective tissues other than skin. Color-enhanced scintigraphy of skin

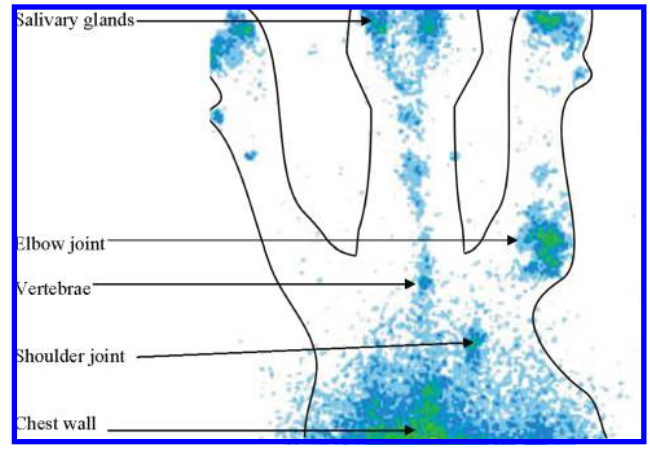


Figure 4. Ventral view of a scintigraphic scan of dog chest from the pilot study showing biodistribution of radioactivity two hours after administration of ^{99m}Tc-hyaluronan (NutriHyl) in joints, bones and salivary glands.

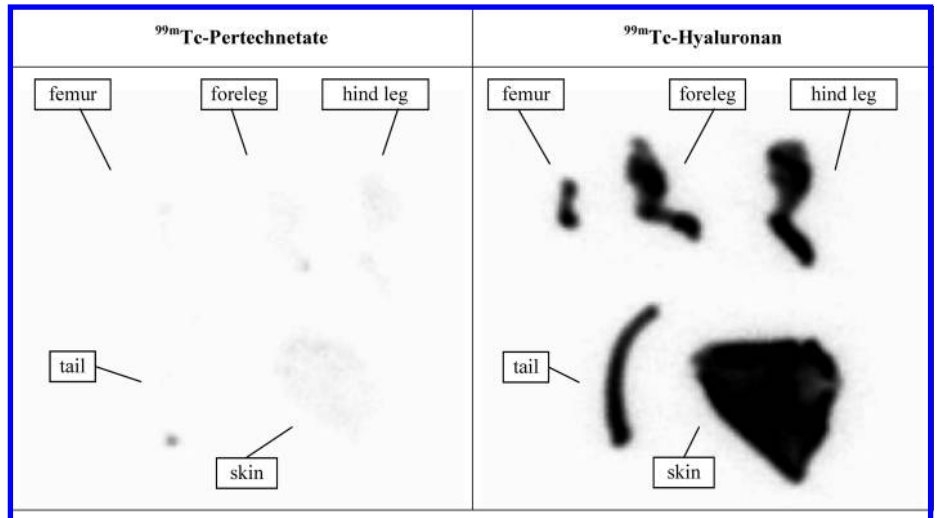
samples from rats 24 and 48 h after administration of ^{99m}Tc-P and ^{99m}Tc-HA showed identical results: virtually no radioactivity from ^{99m}Tc-P skin samples, but marked uptake of radioactivity from ^{99m}Tc-HA skin samples (data not presented). Thus, it is obvious that the ^{99m}Tc-HA group exhibited significantly increased connective tissue uptake of radioactivity compared to the ^{99m}Tc-P control group.

NanoSPECT/CT Scans. Three-dimensional visualization of radioactivity in whole rat bodies allowed precise localization of ingested radioactivity at a relatively low gain setting. Whole-body scans allowed areas of interest to be selected, and sagittal and coronal sections were then illustrated based on position of the crosshair. Transverse sections were selected from the intersection of sagittal and coronal scans. Thus, precise localization of tissue radioactivity was able to rule out overlapping tissues as a location of uptake, which was not possible with scintigraphs.

From 30 min to six hours after ^{99m}Tc-P administration, most radioactivity was present in the stomach, but radioactivity was unequivocally found in thyroid, salivary glands, saliva and urinary bladder, along with some uptake by intestines and soft tissues throughout the body. However, transverse images conclusively determined that radioactivity was not visualized in bones and joints. At 12 h, most radioactivity was still in the stomach, with more uptake in intestines, including formed feces. By 24 h, most radioactivity was gone, although thyroid still showed uptake, but radioactivity was not seen at 48 h.

At the gain setting used, radioactivity after ^{99m}Tc-HA administration appeared to remain in the gastrointestinal tract, following normal gut transit. The diffuse, soft tissue uptake as seen in the ^{99m}Tc-P group was not visualized after ^{99m}Tc-HA. Likewise, there was no observable radioactive uptake in thyroid, salivary glands, kidneys or urinary bladder after ^{99m}Tc-HA. Representative nanoSPECT/CT scans from the six hour time point are presented in **Figure 6**.

NanoSPECT/CT scans at a higher gain setting were also conducted on connective tissue pieces to ascertain whether radioactivity was incorporated into joints, and which compartments of joints, 48 h after administration (**Figure 7**). Radioactivity was seen in cartilage on the end of the femur, over bones and joints, and in skin. Radioactivity was also seen in surrounding muscle tissue. Transverse images through synovial spaces of six different joints (tarsal, knee, hip, carpal, elbow and shoulder) showed that radioactivity was seen in synovial fluid, cartilage and bone. Uptake was not seen in the ^{99m}Tc-P group



	Radioactivity (cpm)	
Organ	^{99m}Tc-Pertechnetate	^{99m}Tc-Hyaluronan
Femur	214	24082
Foreleg	862	141591
Hind leg	1214	197215
Tail	316	102492
Skin	3033	1036615
Total	7571	1501862

Figure 5. Ex vivo scintigrams of removed target organs from two rats 24 h after administration of ^{99m}Tc-pertechnetate or ^{99m}Tc-hyaluronan (HyaMax). Both rats received the same dose of radioactivity (100 MBq).

at this time point. Thus, three-dimensional imaging of radioactivity showed uptake into joint tissues after oral ingestion of ^{99m}Tc-HA.

DISCUSSION

This report presents the first evidence for uptake and distribution to tissues of orally administered, high-molecular-weight HA. The authors were unable to find other reports in the peer-reviewed literature concerning oral uptake of HA when these experiments were conceived and conducted in 2002−2003 and when the pilot study was published in abstract form in 2004 (*9*). Based on the possibility of oral uptake of hyaluronan found in the pilot study, and need for confirmation of a previously unexplored finding, a controlled study comparing the uptake of radioactivity from unbound and bound radioactive isotope (^{99m}Tc) was done. In the controlled experiment, 13.3% of radioactivity from ingested HA remained in animals after 72 h, compared to 5.6% of radioactivity remaining in animals after free pertechnetate administration. The pattern of uptake and removal from the body differed significantly between the two forms of radioactivity, indicating that uptake was different between the two forms, and suggesting tissue uptake of HA after oral ingestion. Several lines of reasoning suggest that the ingested radioactivity from HA represented uptake of some form of HA into circulation and tissues.

There are three major possibilities that explain the observed results: (1) trace amounts of contaminating radioactivity not bound to HA accounted for observed results; (2) ^{99m}Tc label was removed from HA by metabolism in cells (animal or gut microbial); (3) intact or partially depolymerized HA was absorbed into systemic circulation and taken up by peripheral tissues; and (4) a combination of these possibilities. Considering the first possibility, there was approximately 10% of radioactivity in ^{99m}Tc-HA preparations used in this investigation that was from starting materials. ^{99m}Tc-P was up to 3% and ^{99m}Tc-tin colloid was the remainder. ^{99m}Tc-P in ^{99m}Tc-HA would behave similarly to the control group, with far less radioactivity measurable. This pattern of uptake was not seen in the ^{99m}Tc-HA results; therefore, the contribution of contaminating ^{99m}Tc-P to the results is unlikely. Even the rapid tissue uptake of radioactivity seen in the first 30 min for the ^{99m}Tc-HA group did not resemble that seen for ^{99m}Tc-P, based on tissue uptake pattern dissimilarity with the ^{99m}Tc-P control group. Labeled tin colloid uptake is not well-known, but likely has poor absorption, as seen for similar metals (*10*). Even if all of the tin colloid were absorbed, the amount of radioactivity not recovered (13.3%) exceeded the amount of tin colloid radioactivity (<10%). Tin is not known for affinity to connective tissues. However, the very rapid appearance of radioactivity in bone tissue at 5 and 15 min after oral administration of ^{99m}Tc-HA (see **Table 3**) may be due to small amounts of non-^{99m}Tc-P and/or non-^{99m}Tc-HA radioactivity. Thus, with the possible exception of the first two time points in bone tissue, it is unlikely that contamination by starting materials could account for the observed results over the course of study.

Next, another possibility is removal of ^{99m}Tc label from HA, in which case ^{99m}Tc-P and other similar compounds would be formed. Label was found to be stable in presence of plasma and synovial fluid, so it is highly unlikely that there was

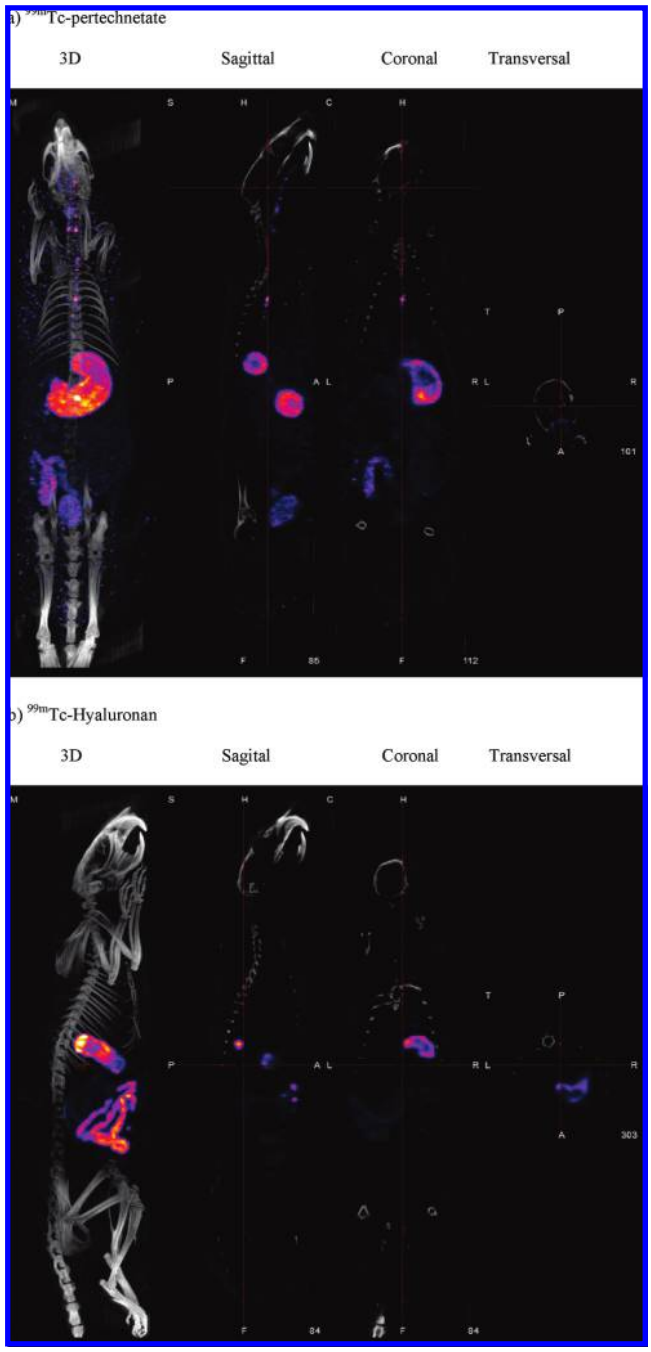


Figure 6. Representative NanoSPECT/CT scans from two rats 6 h after administration of (**a**) ^{99m}Tc-pertechnetate or (**b**) ^{99m}Tc-hyaluronan (HyaMax). Both rats received the same dose of radioactivity (100 MBq). Whole body (3D), sagittal, coronal and transverse images are presented. Radioactivity is clearly visible in thyroid, salivary glands, stomach, urinary bladder and soft tissues after ^{99m}Tc-pertechnetate, but after ^{99m}Tc-hyaluronan, only alimentary tract shows radioactivity at this gain setting.

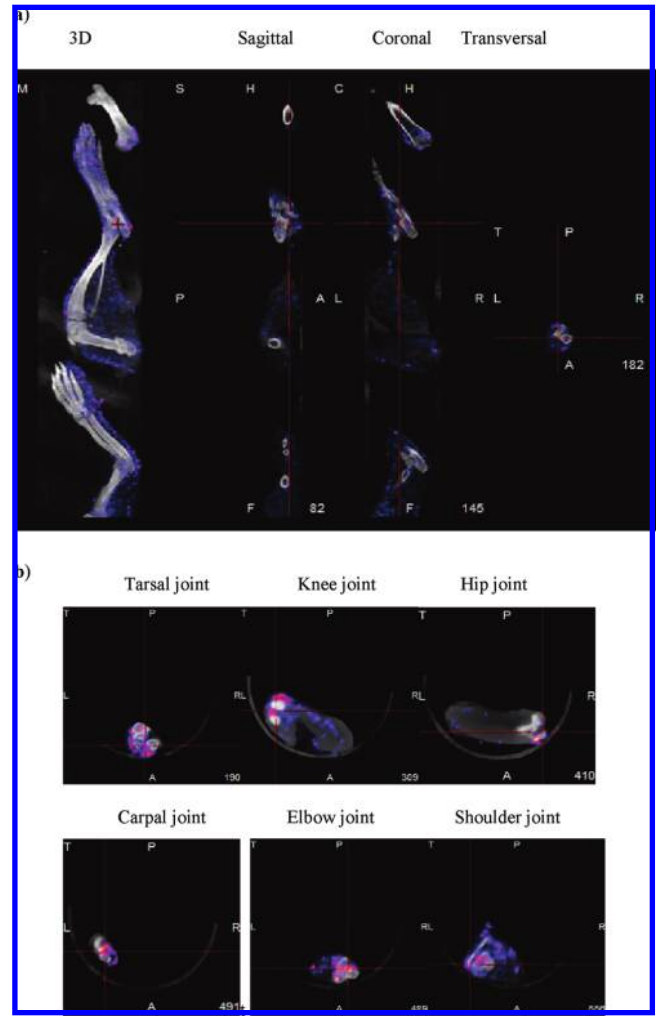


Figure 7. NanoSPECT/CT scans of rat connective tissue pieces ex vivo with higher gain setting 48 h after ingestion of 2000 MBq of ^{99m}Tc-hyaluronan (HyaMax) in the controlled study. (**a**) Ex vivo whole tissue (3D), sagittal, coronal and transverse images of femur, hindleg and foreleg (top to bottom). Radioactivity is apparent in cartilage at end of femur and inside the tarsal joint, as well as skin and soft tissues around bones. (**b**) Transverse images through synovial spaces of tarsal, knee, hip, carpal, elbow and shoulder joints showing radioactivity associated with joint structures (synovial fluid, cartilage, bone) as well as some uptake in muscle.

nonspecific removal of ^{99m}Tc from ^{99m}Tc-HA from bodily fluids. That leaves the possibility that HA would need to be metabolized to its smallest components by cellular actions in order to release free ^{99m}Tc. Metabolism of HA to its smallest components does occur in cells (*2*), which would require internalization and enzymatic degradation. This could occur in gastrointestinal microbes or the animals' own cells or both. If the animals' own cells degraded HA to release ^{99m}Tc, then a pattern similar to the control group would be seen, albeit delayed and to a lesser extent. The pattern of tissue uptake does not support this possibility: the ratio of radioactive counts between tissues is very different between ^{99m}Tc-P and ^{99m}Tc-HA groups (see **Figure 2** and **Table 3**). However, it was obvious from the observed results that orally administered ^{99m}Tc-HA remained in the lumen of the entire gastrointestinal tract for up to 48 h and was present in feces, meaning it was exposed to gut microbes. Passage of the ^{99m}Tc-HA bolus into the large intestine was seen after six hours, and this is also when greatest accumulation of radioactivity in tissues was seen. Some microbes are known to possess hyaluronidases, which have the capability to degrade ^{99m}Tc-HA into smaller monosaccharide components. This would potentially release free ^{99m}Tc-P available for absorption and excretion into urine. However, the pattern of tissue uptake and urinary excretion of radioactivity for ^{99m}Tc-HA did not resemble that for ^{99m}Tc-P, meaning that gut microbes in rats and dogs either did not degrade ^{99m}Tc-HA to any appreciable extent, or any radioactive ^{99m}Tc released from ^{99m}Tc-HA remained inside gut microbe cells, unavailable for absorption or further dispersal. In fact most of the radioactivity from ^{99m}Tc-HA was excreted with feces, as shown in **Table 1**

and scintigraphic and nanoSPECT/CT images (data not presented). These findings indicate that radioactivity seen outside the intestinal lumen after six hours was not free ^{99m}Tc label, as would be expected for microbial or cellular degradation of ^{99m}Tc-HA to free ^{99m}Tc. There is still a possibility that radioactivity in tissues could have been due to degraded monomer components of ^{99m}Tc-HA, such as *N*-acetylglucosamine or glucuronate, or even HA oligosaccharides, but these compounds are rapidly metabolized by liver and other tissues (*1, 2*). The pattern of radioactivity uptake observed does not support this possibility either. Thus, the third possibility, that tissue radioactivity was due to uptake of high-molecular-weight HA remains as the most likely explanation for the observed results.

Further support for the explanation that tissue uptake of radioactivity was due to intact HA comes from the pattern of tissue uptake. HA is known to have an affinity for connective tissues, and these tissues exhibited accumulation of radioactivity throughout the study period from ^{99m}Tc-HA, but not after ^{99m}Tc-P. Overall, the findings suggest that a small amount of orally administered HA was absorbed into systemic circulation and taken up by connective tissues.

Other evidence supports our findings of oral uptake of HA into connective tissues. First, oral uptake of other high-molecular-weight glycosaminoglycans (10−40 kDa) into circulation and tissues is not unprecedented, and has been repeatedly found by many investigators after feeding chondroitin, dermatan, heparan and/or unfractionated heparin sulfates to humans and other mammals (*11*−*41*). Other reports did not find evidence of absorption of chondroitin sulfates, but these reports each had issues that would explain a lack of ability to find absorption (*42*−*45*). Of interest to the present investigation is that the percent of ingested dose of other glycosaminoglycans (between 5−20%) entering circulation was similar to that reported here (*11*−*41*). Thus, there is a clear precedent for partial uptake of large glycosaminoglycans after oral ingestion.

Second, reports of biological effects after oral administration of high-molecular-weight HA consistent with known properties of HA are found in the scientific literature (*1, 46*−*51*). Animal studies have found significant improvements in lameness in horses (*46*), reversal of bone loss in ovariectomized mice (*47*), and reductions in joint effusions in horses (*48, 49*), including an increase in synovial HA levels (*49*). Human studies using HA alone (*50*) or in combination with other nutrients with HA presence the major variable (*51*) found improvements in joint pain and function in subjects with osteoarthritis. Importantly, oral administration studies with high-molecular-weight HA did not show proinflammatory effects, as would be expected if HA was converted or metabolized into small HA oligosaccharides. Although biological effects after oral administration of an agent are not proof of absorption, a plausible reason for effects is related to uptake into systemic circulation and tissues. Thus, other studies have found that oral HA has evidence of biological activity, similar to the situation reported for other glycosaminoglycans, lending further support for findings of oral uptake in this investigation.

Third, evidence exists for uptake into portal circulation and lymphatics of orally ingested, high-molecular-weight microparticles. A well-developed literature has shown that in normal animals and humans, about 5−20% of orally administered microparticles reach portal blood and thoracic lymph (*52*). Note the similarity in percent of ingested dose of microparticles absorbed into circulation to the percent of ingested HA dose remaining in animals after 72 h from this investigation. HA molecular size in solution is close to 1 μm in diameter (*2*), which is in the range of lymphatic uptake of microparticles (*52*). Interestingly, absorption of microparticles was also seen for colonic mucosa (*52*), which corresponds with timing of accumulation of connective tissue radioactivity in this investigation (see **Table 3** and **Figures 2**, **5**, **7**). Bioadhesiveness was shown to increase microparticle binding to mucosal enterocytes and facilitate penetration (*53*). HA exhibits bioadhesiveness at least via several types of specific cell surface receptors (*2, 3, 54, 55*). Thus, in addition to evidence for uptake after oral administration of related glycosaminoglycans, there is substantial evidence that microparticles similar in size and bioadhesiveness to HA are absorbed through gastrointestinal mucosa and delivered to portal circulation and lymphatics. However, results from this investigation do not support a portal route of uptake of radioactivity from ^{99m}Tc-HA, since no appreciable radioactivity was seen in liver tissue at any time point. Since liver is the major site of disposal for HA in the blood, a portal route of entry for oral HA is unlikely, leaving lymphatic uptake as the remaining likelihood. The observed results could be explained by lymphatic entry and transport of ^{99m}Tc-HA, since the timing of tissue uptake (after 6 h) and ability of lymphatics to reach connective tissues is coincident with appearance and accumulation of radioactivity.

Fourth, lymphatic transport of HA to and from tissues is a normal physiologic event (*2, 56, 57*). Transport of high-molecular-weight HA via lymphatics, known transport into and out of synovial spaces via lymphatics (*2, 56, 57*) and normal presence in blood and other fluids (*2*) lend mechanistic support for appearance of orally ingested HA in connective tissues. Also, unlabeled chondroitin sulfates were measured in human knee synovial fluid and plasma after oral administration, showing that ingested glycosaminoglycans can transit intact to synovial fluid (*30*). Thus, means for orally ingested HA to enter systemic circulation and to transit to connective tissues are apparently normal physiological events.

Fifth, another line of evidence supporting uptake of oral HA into systemic circulation is the use of HA in peritoneal dialysis fluids. Breborowicz and colleagues reported that a peritoneal dialysis fluid containing 10 mg/dl of high-molecular-weight HA (1.2−2.4 MDa) led to 25% of the HA being absorbed over an eight hour period in rats, with measurable increases in HA content of the peritoneal interstitium and blood (*58*). These results suggest that high-molecular-weight HA can cross peritoneal membranes and enter circulation. Intraperitoneal HA was found to enter circulation via specialized end lymphatic openings (stomata) normally present in subdiaphragmatic peritoneum, and also by visceral lymphatic vessel uptake (*58*), which would place HA into the lymphatic system. Thus, lymphatic vessels have the ability to take up and transport high-molecular-weight HA.

Sixth, of interest to a possible route of uptake is the finding in both ^{99m}Tc-HA groups that appearance of radioactivity in tissues preceded appearance in blood. This finding suggests that lymphatic uptake and transport of ^{99m}Tc-HA to peripheral tissues may have occurred initially, before any uptake into the bloodstream either via the thoracic duct or direct entry. Also, it is well-known that HA in plasma is rapidly taken up and metabolized by the liver (*2*). However, levels of radioactivity in liver tissue were always negligible or absent in both HA groups. This finding, along with the appearance of radioactivity in tissues before blood, suggests that HA was delivered to tissues via a nonblood transport system. Lymphatic uptake and transport could explain these findings.

This investigation did not characterize the molecular form of radioactivity that reached tissues. Attempts to analyze size

distribution of radioactivity in blood and tissues were not successful. Thus, it is possible that some or all of radioactivity observed in tissues was no longer attached to HA. Several lines of evidence other than those previously discussed support that radioactivity was attached to some form of HA. First, ^{99m}Tc-labeled chondroitin sulfate fed to animals was associated with high-molecular-weight chondroitin in blood and tissues (*14*). Second, control experiments demonstrated that the label on HA was stable and covalently bound. Third, the tissue distribution pattern for ^{99m}Tc-HA did not fit the pattern exhibited by free pertechnetate, which is well-known from use in bone scan and other diagnostic imaging procedures. Free pertechnetate tends to concentrate in kidneys and urinary bladder more than seen in the tissue distribution for ^{99m}Tc-HA in **Table 3** and **Figure 2**.

Reproducible results from two separate experiments suggest that orally administered, high-molecular-weight HA may reach peripheral tissues, including joints and skin, in small amounts. These findings support reports of biological actions seen after oral administration of high-molecular-weight HA in animal and human studies (*1, 46*−*51*). Thus, a rationale for inclusion of HA in dietary supplement products designed for joint and skin health exists. Further research is needed to identify the composition of radioactivity in tissues after feeding ^{99m}Tc-HA, and to confirm biological effects from potentially absorbed HA.

ABBREVIATIONS USED

^{99m}Tc, ^{99m}technetium; ^{99m}Tc-HA, ^{99m}technetium-labeled hyaluronan; ^{99m}Tc-P, ^{99m}technetium as pertechnetate; GBq, gigabecquerels; HA, hyaluronan/hyaluronic acid/sodium hyaluronate; ITLC-SG, silica gel-impregnated thin-layer chromatography; kDa, kilodaltons; MBq, megabecquerels; MDa, megadaltons; TLC, thin-layer chromatography; WOMAC, Western Ontario and McMaster University osteoarthritis scale.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical skills of the staff at the Research Institute for Radiobiology and Radiohygiene, "Fodor József" National Center of Health, Budapest, Hungary. Portions of this research were presented as posters at FASEB meetings in 2004 (pilot study) and 2008 (controlled study).

LITERATURE CITED

(1) Bucci, L. R.; Turpin, A. A. Will the real hyaluronan please stand up? *J. Appl. Nutr.* **2004**, *54*, 10–33.

(2) Laurent, T. C.; Fraser, J. R. E. Hyaluronan. *FASEB J.* **1992**, *6*, 2397–2404.

(3) Kogan, G.; Soltes, L.; Stern, R.; Gemeiner, P. Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnol. Lett.* **2007**, *29*, 17–25.

(4) Dietary Supplement Health and Education Act of 1994 (DSHEA), Public Law 103-417. *Fed. Regist.* **1994**, *59*, 4325−4329.

(5) Samson, D. J.; Grant, M. D.; Ratko, T. A.; Bonnell, C. J.; Ziegler, K. M.; Aronson, N. Treatment of primary and secondary osteoarthritis of the knee. *Evidence Report/Technology Assessment No. 157* (Prepared by Blue Cross and Blue Shield Association Technology Evaluation Center Evidence based Practice Center under Contract No. 290-02-0026). AHRQ Publication No. 07-E012. Rockville, MD: Agency for Healthcare Research and Quality. September 2007.

(6) Campbell, J.; Bellamy, N.; Gee, T. Differences between systematic reviews/meta-analyses of hyaluronic acid/hyaluronan/Hylan in osteoarthritis of the knee. *Osteoarthritis Cartilage* **2007**, *15*, 1424–1436.

(7) Dilworth, J. R.; Parrott, S. J. The biomedical chemistry of technetium and rhenium. *Chem. Soc. Rev.* **1998**, *27*, 43–55.

(8) Balogh, L.; ocs, G.; Thuroczy, J.; Nemeth, T.; Lang, J.; Bodo, K.; Janoki, G. A. Veterinary nuclear medicine. Scintigraphical examinations—a review. *Acta Vet. Brno* **1999**, *68*, 231–239.

(9) Schauss, A. G.; Balogh, L.; Polyak, A.; Mathe, D.; Kiraly, R.; Janoki, G. Absorption, distribution and excretion of ^{99m}technetium labeled hyaluronan after single oral doses in rats and beagle dogs. *Fed. Am. Soc. Exp. Biol. J.* **2004**, *18*, A150−A151[abstract 129.4]

(10) Balogh, L.; Kerekes, A.; Bodó, K.; Körösi, L.; Jánoki, G. A. [Evaluation of a complex trace element composition and bioutilization using isotope technics and total body measurement]. *Orv. Hetil.* **1998**, *139*, 1297–1302.

(11) Yamaguchi, T.; Horie, K.; Miyoshi, S.; Kobayashi, Y.; Nagatuka, M.; Okuyama, T. Studies on absorption, distribution and excretion of ^{35}S-sodium chondroitin polysulfate in rats. *Oyo Yakuri* **1972**, (2), 405–413.

(12) Morrison, L. M.; Schjeide, O. A.; Murata, K. Absorption, distribution, metabolism and excretion of acid mucopolysaccharides administered to animals and patients. In *Coronary Heart Disease and the Mucopolysaccharides (Glycosaminoglycans)*; Morrison, L. M., Schjeide, O. A. Eds.; Charles C. Thomas: Springfield, 1974; pp 109−127.

(13) Konador, A.; Kawiak, J. Distribution of radioactivity in the mouse organism after administration of 35S-chondroitin sulphate. *Folia Biol. (Krakow)* **1976**, *24*, 177–190.

(14) Thilo, G. Etude de 35 cas d'arthrose traités par l'acide chondroïtine sulfurique. [A study of 35 cases of arthrosis treated with Chondroitin Sulfuric Acid]. *Schweiz. Rundsch. Med./Prax.* **1977**, *66*, 1696–1699.

(15) Pescador, R.; Diamantini, G.; Mantovani, M.; Malandrino, S.; Riva, A.; Casu, B.; Oreste, P. Absorption by the rat intestinal tract of fluorescein-labelled pig duodenal glycosaminoglycans. *Arzneim. Forsch.* **1980**, *30*, 1893–1896.

(16) Pescador, R.; Madonna, M. Pharmacokinetics of fluorescein-labelled glycosaminoglycans and of their lipoprotein lipase-inducing activity in the rat. *Arzneim. Forsch.* **1982**, *32*, 819–824.

(17) Clevidence, B. A.; Failla, M. L.; Vercellotti, J. R.; Pescador, R. Pharmacokinetics of catalytically tritiated glycosaminoglycans in the rat. *Arzneim. Forsch.* **1983**, *33*, 228–230.

(18) Gross, D. Orale chondroitin-Sulfatmedikation zur behandlung von arthrosen. [Oral chondroitin sulfate medication for the treatment of osteoarthritis]. *Therapiewoche* **1983**, *33*, 4238–4244.

(19) Ronca, G.; Ronca-Testoni, S.; Lualdi, P. Pharmacocinetique du chondroitin sulfate−3H après administration orale chez l'animal [abstract]. *Z. Rheumatol.* **1988**, *47*, 325–326.

(20) Dawes, J.; Hodson, B. A.; Pepper, D. S. The absorption, clearance and metabolic fate of dermatan sulphate administered to man--studies using a radioiodinated derivative. *Thromb. Haemostasis* **1989**, *62*, 945–949.

(21) Dawes, J.; Hodson, B. A.; MacGregor, I. R.; Pepper, D. S.; Prowse, C. V. Pharmacokinetic and biological activities of dermatan sulfate (Mediolanum MF701) in healthy human volunteers. *Ann. N.Y. Acad. Sci.* **1989**, *556*, 292–303.

(22) Palmieri, L.; Conte, A.; Giovannini, L.; Lualdi, P.; Ronca, G. Metabolic fate of exogenous chondroitin sulfate in the experimental animal. *Arzneim. Forsch.* **1990**, *40*, 319–323.

(23) Conte, A.; Palmieri, L.; Segnini, D.; Ronca, G. Metabolic fate of partially depolymerized chondroitin sulfate administered to the rat. *Drugs Exp. Clin. Res.* **1991**, *17*, 27–33.

(24) Conte, A.; de Bernardi, M.; Palmieri, L.; Lualdi, P.; Mautone, G.; Ronca, G. Metabolic Fate of Exogenous Chondroitin Sulfate in Man. *Arzneim. Forsch.* **1991**, *41*, 768–772.

(25) Dawes, J.; McLaren, M.; Forbes, C.; Belch, J. J.; Lane, D. A.; Bray, B.; McEwen, J.; Houin, G.; Gianese, F. The pharmacokinetics of dermatan sulphate MF701 in healthy human volunteers. *Br. J. Clin. Pharmacol.* **1991**, *32*, 361–366.

(26) Ronca, G.; Conte, A. Metabolic fate of partially depolymerized shark chondroitin sulfate in man. *Int. J. Clin. Pharmacol. Res.* **1993**, *13*, 27–34.

(27) Silvestro, L.; Lanzarotti, E.; Marchi, E.; Gori, M.; Pescador, R.; Ferro, L.; Nilani, M. R.; Da Col, R.; Coppini, A. Human pharmacokinetics of glycosaminoglycans using deuterium-labeled and unlabeled substances: evidence for oral absorption. *Semin. Thromb. Hemostasis* **1994**, *20*, 281–292.
(28) Conte, A.; Volpi, N.; Palmieri, L.; Bahous, I.; Ronca, G. Biochemical and pharmacokinetic aspects of oral treatment with chondroitin sulfate. *Arzneim. Forsch.* **1995**, *45*, 918–925.
(29) Volpi, N. Physico-chemical properties and the structure of dermatan sulfate fractions purified from plasma after oral administration in healthy human volunteers. *Thromb. Haemostasis* **1996**, *75*, 491–496.
(30) Ronca, F.; Palmieri, L.; Panicucci, P.; Ronca, G. Anti-inflammatory activity of chondroitin sulfate. *Osteoarthritis Cartilage* **1998**, *6*, 14–21.
(31) Imanari, T.; Washio, Y.; Huang, Y.; Toyoda, H.; Suzuki, A.; Toida, T. Oral absorption and clearance of partially depolymerized fucosyl chondroitin sulfate from sea cucumber. *Thromb. Res.* **1999**, *93*, 129–135.
(32) Eddington, N. D.; Du, J.; White, N. Evidence of the Oral Absorption of Chondroitin Sulfate as Determined by Total Disaccharide Content After Oral and Intravenous Administration to Horses. *A.A.E.P. Proc.* **2001**, *47*, 326–328.
(33) Adebowale, A.; Du, L.; Liang, Z.; Leslie, J. L.; Eddington, N. D. The bioavailability and pharmacokinetics of glucosamine hydrochloride and low molecular weight chondroitin sulfate after single and multiple doses to beagle dogs. *Biopharm. Drug Dispos.* **2002**, *23*, 217–225.
(34) Volpi, N. Oral bioavailability of chondroitin sulfate (Condrosulf) and its constituents in healthy male volunteers. *Osteoarthritis Cartilage* **2002**, *10*, 768–777.
(35) Volpi, N. Oral absorption and bioavailability of ichthyic origin chondroitin sulfate in healthy male volunteers. *Osteoarthritis Cartilage* **2003**, *11*, 433–441.
(36) Barthe, L.; Woodley, J.; Lavit, M.; Przybylski, C.; Philibert, C.; Houin, G. In vitro intestinal degradation and absorption of chondroitin sulfate, a glycosaminoglycan drug. *Arzneim. Forsch.* **2004**, *54*, 286–292.
(37) Du, J.; White, N.; Eddington, N. D. The bioavailability and pharmacokinetics of glucosamine hydrochloride and chondroitin sulfate after oral and intravenous single dose administration in the horse. *Biopharm. Drug Dispos.* **2004**, *25*, 109–116.
(38) Lamari, F. N.; Theocharis, A. D.; Asimakopoulou, A. P.; Malakavi, C. J.; Karamanos, N. K. Metabolism and biochemical/physiological roles of chondroitin sulfates: analysis of endogenous and supplemental chondroitin sulfates in blood circulation. *Biomed. Chromatogr.* **2006**, *20*, 539–550.
(39) Hiebert, L. M. Oral heparins. *Clin. Lab.* **2002**, *48*, 111–116.
(40) Pineo, G; Hull, R; Marder, V. Oral delivery of heparin: SNAC and related formulations. *Best Pract. Res., Clin. Haematol.* **2004**, *17*, 153–160.
(41) Arbit, E.; Goldberg, M.; Gomez-Orellana, I.; Majuru, S. Oral heparin: status review. *Thromb. J.* **2006**, *4*, 6.
(42) Konador, A.; Kawiak, J. Changes in chondroitin sulfate concentration in rabbit blood plasma depending on the method of its administration. *Arch. Immunol. Ther. Exp.* **1977**, *25*, 895–903.
(43) Andermann, G.; Dietz, M. The influence of the route of administration on the bioavailability of an endogenous macromolecule: chondroitin sulphate (CSA). *Eur. J. Drug Metab. Pharmacokinet.* **1982**, *7*, 11–16.
(44) Baici, A.; Horler, D.; Moser, B.; Hofer, H. O.; Fehr, K.; Wagenhauser, F. J. Analysis of glycosaminoglycans in human serum after oral administration of chondroitin sulfate. *Rheumatol. Int.* **1992**, *12*, 81–88.
(45) Jackson, C. G.; Plaas, A. H.; Barnhill, J. G.; Harris, C. L.; Clegg, D. O. The pharmacokinetics of oral glucosamine and chondroitin sulfate in humans [abstract 488 L13]. In *American College of Rheumatology Annual Scientific Meeting: 13−17 November 2005; San Diego*; American College of Rheumatology: Atlanta, GA, 2005; p 15.
(46) Pierce, S. W. Efficacy of orally administered sodium hyaluronate gel in the racing thoroughbred. In *Hyaluronan 2003 Proceedings. Chapter 6 Musculoskelatal System*: Balazs, E. A., Hascall, V. C., Eds.; Matrix Biology Institute: Cleveland, OH, 2004; pp 1−4.
(47) Stancikova, M.; Svik, K.; Istok, R.; Rovensky, J.; Velebny, V. The effects of hyaluronan on bone resorption and bone mineral density in a rat model of estrogen deficiency-induced osteopenia. *Int. J. Tissue React.* **2004**, *26*, 9–16.
(48) Bergin, B. J.; Pierce, S. W.; Bramlage, L. R.; Stromberg, A. Oral hyaluronan gel reduces post operative tarsocrural effusion in the yearling Thoroughbred. *Equine Vet. J.* **2006**, *38*, 375–378.
(49) Martinez-Puig, D.; Carmona, J. U.; Arguelles, D.; Deulofeu, R.; Ubia, A.; Prades, M. Oral hyaluronic acid administration improves osteochondrosis clinical symptoms and slightly increses intra-articular concentration of hyaluronic acid in a horse model: a pilot survey. *Osteoarthritis Cartilage* **2007**, *15*, C62−C63(abstract 96).
(50) Kalman, D. S.; Heimer, M.; Valdeon, A.; Schwartz, H.; Sheldon, E. Effect of a natural extract of chickencombs with a high content of hyaluronic acid (Hyal-Joint®) on pain relief and quality of life in subjects with knee osteoarthritis: a pilot randomized double-blind placebo-controlled trial. *Nutr. J.* **2008**, *7* (3), 1–9.
(51) Bucci, L. R.; Sheldon, E.; Schwartz, H.; Pachon, J.; Kalman, D.; Mederos, M.; Pezzullo, J. C.; Beer, C. Comparison between glucosamine with chondroitin sulfate and glucosamine with chondroitin sulfate and hyaluronate for symptoms of knee osteoarthritis. *Osteoarthritis Cartilage* **2005**, *13*, S99.
(52) Florence, A. T.; Jani, P. U. Oral uptake of microparticles across the gastrointestinal mucosa. In *Drug Targeting and Delivery: Concepts in Dosage Form Design*; Junginger, H. E., Ed.; Ellis Horwood: New York, NY, 1992; pp 113−128.
(53) Thanos, C. G.; Yip, K. P.; Mathiowitz, E. Intestinal uptake of polymer microspheres in the rabbit studied with confocal microscopy. *J. Bioact. Compat. Polym.* **2004**, *19*, 247–266.
(54) Knudson, C. B.; Knudson, W. Hyaluronan and CD44: modulators of chondrocyte metabolism. *Clin. Orthop. Relat. Res.* **2004**(Suppl. 427), 152−162.
(55) Jiang, D.; Liang, J.; Noble, P. W. Hyaluronan in tissue injury and repair. *Annu. Rev. Cell Dev. Biol.* **2007**, *23*, 435–461.
(56) Liu, N. Metabolism of macromolecules in tissue. *Lymphat. Res. Biol.* **2003**, *1* (1), 67–70.
(57) Liu, N. Trafficking of hyaluronan in the interstitium and its possible implications. *Lymphology* **2004**, *37* (1), 6–14.
(58) Breborowicz, A.; Moberly, J. B.; Pawlaczyk, K.; Polubinska, A.; Kuzlan-Pawlaczyk, M.; Wieczorowska-Tobis, K.; Ogle, K.; Martos, L.; Oreopoulos, D. G. Effects of hyaluronan used as a supplement in peritoneal dialysis solutions. In *Hyaluronan. Volume 2. Biomedical, Medical and Clinical Aspects*; Kennedy, J. F., Phillips, G. O., Williams, P. A., Hascall, V. C., Eds.; Woodhead Publishing Unlimited: Cambridge, England, 2002; Vol. 2, pp 453−460.

Received for review June 3, 2008. Revised manuscript received August 22, 2008. Accepted September 8, 2008. This work was supported by a grant from Schiff Nutrition Group, Salt Lake City, UT, and from FenChem BioTek LTD, Nanjing, China.

JF8017029