Biodistribution of covalent antithrombin-heparin complexes

Paul A. Chindemi¹, Petr Klement¹,², Filip Konecny¹, Leslie R. Berry¹,³, Anthony K. C. Chan¹,³

¹Henderson Research Centre, Hamilton, Canada
²University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic
³McMaster University, Department of Pediatrics, Hamilton, Canada

Summary
We have developed a covalent antithrombin-heparin (ATH) complex with advantages compared to non-covalent antithrombin-heparin (ATH:H) mixtures. In addition to increased activity, ATH has a longer intravenous half-life that is partly due to reduced plasma protein binding. Given ATH’s altered clearance, we investigated biodistribution of ATH in vivo. ATH made from either human plasma-derived AT (pATH) or recombinant human (produced in goats) AT (rhATH) was studied. [¹²⁵I-ATH + unlabeled carrier was injected into rabbits at different doses. [¹³¹I]-labeled albumin was administered just before sacrifice as a marker for trapped blood in tissues. Immediately after sacrifice, animal components were removed, weighed, and subsamples were counted for gamma-radioactivity. Percent recoveries of ATH in various organs/compartments at different time points were calculated, and kinetic distribution plots generated. At saturating doses, early disappearance of rhATH from the circulation was much faster than pATH. Co-incident with clearance, 26 ± 3% of dose for rhATH was liver-associated compared to only 3.7 ± 0.5% for pATH by 20 min. Also, at early time periods, >60% of all extravascular ATH was liver-associated. Analysis of the vena cava and aorta suggested that vessel wall binding might also account for initial plasma loss of rhATH. By 24 h, most of pATH and rhATH were present as urinary degradation products (51 ± 3% and 63 ± 8%, respectively). In summary, systemic elimination of ATH is greatly influenced by the form of AT in the complex, with liver uptake and degradation playing a major role.

Keywords
Antithrombin, heparin, pharmacodynamics

Introduction
Covalent antithrombin-heparin complex (ATH) has emerged as a model compound for investigation of heparin mechanisms and as a potential therapeutic agent. Previous experiments employing ATH have indicated that antithrombin (AT) can wrest heparin pentasaccharide from interaction with other AT molecules in which the heparin remains covalently tethered at the aldose terminus (1). Results from these studies suggested interesting potential transition states in the catalysis by unfractionated heparin (UFH) of thrombin or factor Xa reaction with AT. Furthermore, ATH has played an important role in understanding the resistance of fibrin-bound thrombin to inhibition by AT + UFH (2). As an anticoagulant, ATH has shown promise in both treatment and prophylaxis. ATH possesses a rapid rate of direct thrombin inhibition as well as a high specific activity to catalyze AT reaction with coagulation factors (3–5). On cell surfaces, the conjugate was vastly superior to non-covalent AT + UFH for preventing tissue factor-associated thrombin formation (6, 7). Moreover, while non-covalent AT-heparin is ineffective at inhibiting fibrin-bound thrombin, ATH rapidly neutralizes thrombin regardless of the presence of fibrin (8, 9). In vivo, tests in rabbit venous (10) and arterial (9) thrombosis models showed that ATH caused reduced clot mass in conjunction with diminished hemorrhagic side effects compared to UFH. Studies of intra-tracheally instilled ATH illustrated that, unlike heparin, covalent AT-heparin complexes can be retained in the airspace for long periods as a potential treatment for prevention of coagulation that is a major factor in respiratory distress syndrome leading to bronchopulmonary dysplasia (3, 7).

A critical property responsible, in part, for increased efficacy of ATH is its increased intravenous half-life in the circulation compared to uncomplexed heparin. In vivo investigations showed that ATH remained in the circulation 6- to 8-fold longer...
than UFH (3) with a β-phase half-life that was 3-fold more than the time of peak plasma levels for subcutaneous LMWH (4). There are a number of potential factors that may play a role in lengthening bioavailability of the conjugate. Due to the vast increase in size, covalent linkage of heparin chains to AT would prevent intravenous loss via glomerular filtration (3). Additional studies have intimated that a major reason for ATH’s slower removal from the vascular space is due to reduced binding to plasma and endothelial cell surface proteins (11), which is a significant factor in UFH elimination (12, 13). However, once ATH leaves the circulation, it can become sequestered within other compartments. Early experiments demonstrated that subcutaneous ATH is greatly restricted but does act as a depot for release of small quantities back into the bloodstream (3). Furthermore, although ATH captured inside alveoli had no measurable secretion into the lumen (3, 7), increase in stress may alter the pulmonary permeability (14–16). Thus, the altered metabolism of heparin due to covalent AT-linkage may depend on still unexplored features within the ATH complex.

Work to date involving this new anticoagulant has employed complexes of plasma-derived AT bound to heparin (pATH). Recently, we have prepared conjugates of recombinant human AT (rhAT) and heparin (rhATH) that have shown superior results for clinical applications, such as that demonstrated in a porcine cardiopulmonary bypass model (17). RhAT may have enhanced potential to prepare ATH for a pharmaceutical grade product (18). With the availability of ATH complexes that contain different AT moieties, it would be possible to determine the extent to which features of the glycoprotein portion direct the fate of heparin conjugates in vivo. Therefore, we studied the biodistribution of ATH preparations to determine key target organs, study metabolism and elucidate important structural components responsible for its pharmacodynamics.

Materials and methods

Chemicals
Human AT was obtained from Affinity Biologicals (human plasma derived AT, Ancaster, ON, Canada) or GTC Biotherapeutics (recombinant human AT in goats, Framingham, MA, USA). Heparin was obtained from Sigma (Sodium Salt, grade I-A, from porcine intestinal mucosa, Catalogue # H-3393; Mississauga, ON, Canada). Na125I and Na131I were from Perkin Elmer (Boston, MA, USA). IODO-BEAD was from Pierce Biotechnology Inc. (Rockland, IL, USA) and bovine serum albumin (BSA) was purchased from Sigma (99% purity, Catalogue # A-7638).

ATH preparation
Production of ATH has been described previously (3). Briefly, AT (1,052 µg) and heparin (64 µg) were separately dialyzed against 2M NaCl followed by PBS, then mixed together in a total volume of approximately 900 ml and incubated for 14 days in a water bath set at 40°C. Subsequently, 0.5 M NaBH₄CN was added to the mixture (final concentration of 0.1M) and incubation was continued for another 5 hours at 37°C. ATH was purified from the reaction mixture by a two-step procedure involving hydrophobic chromatography (Butyl-Sepharose; Amersham Biosciences, Baie d’Urfé, QU, Canada) followed by anion exchange chromatography (DEAE-Sepharose; Amersham Biosciences) (3, 11).

Physical and activity analyses
Protein concentrations of the ATH products were determined by absorbance at 280 nm, as described previously (1). Heparin content in ATH preparations was determined by a protamine sulfate turbidimetric assay, according to a published method (1, 19). Anti-Xa activity assay of pATH and rhATH employed the Stachrom Heparin Kit and was determined as the ability of the heparin moiety to catalyze inhibition of factor Xa by exogenous AT (3). In all molar calculations, the molecular weight of AT was taken to be 59,000 and the molecular weight of ATH heparin chains was taken as 18,000 (1).

Isotopic labeling
ATH or BSA (in PBS) was radiolabeled using a single IODO-BEAD. The IODO-BEAD was pre-washed with 1.0 ml of PBS and then incubated for 5 min at 23°C with 10 ml of PBS and 0.5 ml (0.5 mCi) of the appropriate isotope. One hundred µl of either protein (200 µg) was then added to the bead suspension and incubated for another 5 min at 23°C. For the labeling of ATH, the labeled product was transferred to an eppendorf tube containing enough 1 M NaI to yield a final concentration of 0.1 M in terms of NaI (addition of NaI was done to displace any non-covalently bound radioactive iodine). The labeled sample was allowed to incubate for 20 min before being applied to a previously equilibrated (PBS containing 0.1M NaI) PD-10 column (Amersham Biosciences). Fractions (0.5 ml) were collected and sampled for radioactivity measurement using a 1272 Clinigamma counter (Amersham Biosciences). Peak fractions were pooled (typically fractions 6 and 7) and stored at −70°C until needed. Specific activities were determined by assaying AT protein using a human AT ELISA kit (Affinity Biologicals). Free radioactive I was measured by counting soluble supernatant after protein precipitation with 5% phosphotungstic acid in 2 M HCl (PTA). For BSA labeling, non-radioactive NaI was not required.

Animal model and dosing
Animal experiments were approved by the Animal Research Ethics Board and performed according to their guidelines. Animals were housed in an isolated room, designated for radioactive work, and experiments were monitored by the McMaster University Health Physics department. Male, New Zealand White rabbits, weighing between 2.8 and 3.5 kg were purchased from Charles River (ON, Canada) and given free access to autoclaved Rabbit Chow (Charles River) and water. In the experiments, one catheter was placed in the artery of one ear and one catheter was placed in a vein of the other ear. Dose solutions were infused in the arterial line, while blood samples were drawn from the venous line. Rabbits were administered with ATH (containing 125I-ATH as a radioactive tracer) at one of 2 dose levels (3 mg/kg or 3 µg/kg). For those rabbits receiving the higher dosage, labeled ATH was mixed with unlabeled ATH in order to obtain the required load. For those rabbits receiving the lower ATH dosage, the ATH load was supplemented with unlabeled BSA (1mg/ml in PBS) as a carrier protein. Concentrations of either ATH dose sol-
ution were prepared such that each rabbit received an approximate volume of 1.0 ml. Thus, for a 3 kg rabbit, either 1.0 ml of a 9 mg/ml load was injected to give 3 mg/kg or 1.0 ml of a 9 µg/ml load was injected to give 3 µg/ml. Confirmation that the prepared dose was received by each rabbit was determined by measuring the weights of the syringes pre and post administration. The clock was started (T₀) as soon as the dose was injected. This was followed by a 2.0 ml saline flush. At a time 5 min prior to sacrificing the animal, 1 ml of the ¹³¹I-labeled BSA solution was administered (used as a marker for the amount of injected protein contained in blood trapped within tissue samples taken postmortem). The rabbits were sacrificed by injection of euthanyl, followed by blood sampling (2–3 ml) into heparin-coated syringes. In order to determine the amount of metabolized ATH in the blood samples, PTA precipitation tests were done. Subsamples (0.4 ml) of blood from different time points after injection were added to 1.6 ml of H₂O₂, followed by mixture with 2.0 ml of 5% PTA in 2 M HCl and centrifugation. Radioactivity detected in the resultant supernatant was calculated as a percent of total radioactivity in the starting blood subsample to give the proportion of circulating ATH-derived material present as acid-soluble degradation products. The fraction of radiolabel present as ATH degradation products was subtracted from the total to give the amount of intact ATH in blood at time of sacrifice.

Tissue sample preparation
At specific time intervals (20 min, 4 h or 24 h) the rabbits (n = 5 per group) were sacrificed and exsanguinated, followed by extraction of the liver, heart, lung, spleen, kidneys, and segments of aorta and vena cava. Weights of individual organs, as sub-samples in duplicate (approximately 1 gm), were recorded. Samples were counted in 75 x 12 mm plastic tubes, with water added to a total pre-determined volume mark of 2.0 ml to maintain a consistent counting geometry. All urine excreted into the bottom cage trays was collected and pooled with that remaining in the bladder. The total volume was recorded and duplicate 2 ml sub-samples were counted. To check that urinary radioactivity was from ATH metabolites, a small volume of urine was mixed with BSA (1 mg/ml), and the resultant solution was combined with 2 volumes of 5% PTA in 2 M HCl, followed (after 10 min) by centrifugation and γ-counting to determine the percentage of radiolabel as acid-soluble degradation products. Feces were also collected from the trays and pooled with feces removed from the colon. Water was added to the pooled feces in order to prepare homogeneous slurry, from which sub-samples (approximately 1 gm) were taken. Radioactivity was measured in an LKB Wallac model 1272 Clinigamma counter. To correct for radioactive decay (especially in the case of ¹³¹I at 8 days) and to allow for accurate calculations of the accumulated radioactivity in each organ, injection standards from each dose type were measured simultaneously with the samples.

Biodistribution calculations
Following correction for isotopic crossover (¹³¹I to ¹²⁵I channel window of the gamma counter), the net (tissue associated) content of ¹²⁵I-labelled material in each organ was calculated by multiplying the ¹³¹I-protein content by the ¹²⁵I/¹³¹I ratio in the minimal blood sample, followed by subtraction of this value from the total ¹²⁵I content. Amounts of ATH in each organ or tissue sample were then expressed as percentages of the ATH dose injected. Anatomical measurements reported in the literature (20) were used to estimate amounts of total ATH bound to venous or arterial surfaces.

Statistics
Data were expressed as mean ± SEM (n = 5). Analysis of significant differences between time courses of different groups was performed by general linear model ANOVA. Investigation of differences between types or dose of ATH for material present in each organ at each time point was done using student’s t-test. Values of p < 0.05 were considered significant.

Table 1: Physicochemical and activity analyses of ATH compounds.
The catalytic anti-Xa activity measured (using a standard kit) in test samples of product was divided by the mg of heparin present to give the specific activity. Heparin mass (assessed by a previously described turbidimetric method) and AT mass (protein determined by absorbance at 280 nm) were used to calculate the mole ratio of heparin to AT (molecular weight of heparin in ATH preparations is 18,000 and AT molecular weight is 59,000).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Synthetic yield (in terms of starting AT)</th>
<th>Anti-Xa units per mg heparin</th>
<th>heparin:AT mol ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATH</td>
<td>39 %</td>
<td>471</td>
<td>1.04</td>
</tr>
<tr>
<td>RhATH</td>
<td>35 %</td>
<td>508</td>
<td>1.03</td>
</tr>
</tbody>
</table>

Figure 1: Clearance of covalent antithrombin-heparin (ATH) from plasma. Radiolabeled ATH, prepared from either plasma-derived antithrombin (pATH, administered at 3 mg/kg) or human recombinant antithrombin (rhATH, administered at either 3 mg/kg or 3 µg/kg), was measured in blood samples taken at various time samples after injection. The fraction of radioactivity due to acid-soluble degradation products was subtracted, and the amount of intact ATH in the total blood volume was calculated as a percentage of the starting dose at time 0. Values are expressed as mean ± SEM.
Results

Chemical and activity assays

Results from analyses of mass and activity recovered during pATH and rhATH preparations are reported in Table 1. Overall, yields of ATH during synthesis were similar, regardless of whether pAT or rhAT was used as the starting material. Likewise, anti-Xa specific activities of pATH and rhATH were comparable (difference between pATH and rhATH in units/mg heparin was less than 8%, as shown in Table 1). Further analyses of protein and heparin content were performed to assess the substitution of heparin molecules on AT in the conjugates used in this study. Results showed that the heparin:AT mole ratio was close to 1.0 for both pATH and rhATH (Table 1), in agreement with all previous characterization of ATH complexes (1, 3).

Plasma clearance

Time course elimination of injected ATH is given in Figure 1. Circulating ATH (plasma- or recombinant-derived) dropped significantly over early time periods, whereby more than 40% of the dose could not be accounted for by 20 min after injection. However, at dosages that would theoretically give starting plasma levels of conjugate (= 0.9 µM or = 53 µg/ml for a 3 mg/kg dose) approaching those of endogenous AT (= 3 µM or = 200 µg/ml), while the majority of pATH was recovered in the blood, only one third of rhATH could be detected (p = 0.002). Since plasma samples were heparinized to prevent coagulation prior to γ-counting, no direct measurement of systemic anticoagulant activity could be done. However, if ATH detected in plasma represents intact complex [calculated plasma values were corrected for acid-soluble (metabolically degraded) material], maximal activities present in the circulation might be estimated from the pATH and rhATH specific activities given in Table 1. Thus, given the units/mg heparin for the conjugates, the disappearance curve data (Fig. 1) and the plasma volume (57 ml/kg), it was calculated that up to 4.3 units/ml of pATH and 2.6 units/ml of rhATH remained at 20 minutes after injection. Similarly, by 4 hours, a theoretical maximum anticoagulant activity of 0.76 units/ml of pATH and 0.62 units/ml of rhATH would have been in the plasma phase. An attempt was made to assess if the significant loss in vascular rhATH might be receptor-mediated (dose-dependent). When rhATH was administered at loads of 3 µg/kg, the recovery of labeled drug at 20 min suffered a further decrease (3-fold, p < 0.001) compared to experiments with the larger dose (Fig. 1). Following initial plasma loss, the clearance from 20 min to 24 h proceeded at similar rates (p > 0.05) for high dose pATH and rhATH (half-lives were 3.5 h and 4.0 h, respectively).

Biodistribution in major organs and extravascular spaces

Measurements of 125I-labeled material in the vascularized organ systems at different times post-injection revealed the course of metabolic processing for ATH complexes. Tissue content of pATH and its metabolites shortly after injection was minimal in the extravascular spaces that were examined. At 20 min, only the liver contained a significant amount of the dose (3.7%) with < 0.2% of injected material having accumulated in the lung, spleen, kidney and heart (Fig. 2A). Analyses of urine and feces showed that only 0.5% of pATH appeared as excreted degradation products early in the experiment. As the majority of pATH started to disappear from the vascular compartment (Fig. 1), increasing amounts of labeled material accumulated in the liver and urine (6.5% and 15.5% at 4 h, respectively) along with small quantities in other organs (Fig. 2B). Finally, by 24 h, the majority of pATH had been degraded and excreted into the urine, combined with another 5.0% of the load recovered as fecal waste (Fig. 2C). Supplementation of small volumes of urine with BSA (1 mg/ml) and mixture with 2 volumes of PTA confirmed that

Figure 2: Organ and extravascular distribution of covalent antithrombin-heparin prepared from plasma-derived antithrombin (pATH) or recombinant human antithrombin (rhATH). The amount of radiolabel in various organs and extravascular spaces at 20 min (A), 4 h (B) and 24 h (C) after injection of 3 mg/kg 125I-pATH, 3 mg/kg 125I-rhATH or 3 µg/kg 125I-rhATH was determined by γ-counting of subsamples and corrected for blood trapped in the tissue, as revealed by the presence of 131I-BSA injected just prior to sacrifice. Total amount in each organ, urine or feces was calculated as a percentage of the dose and expressed as mean ± SEM.
\(^{125}\)I-label was attached to soluble fragments derived from the original ATH. Thus, from 20 min to 24 h, a steady state process was observed involving removal of pATH to the liver followed by ultimate deposition of degraded species into the excretory compartments.

Determination of the pharmacodynamics for rhATH disclosed a very different metabolic course. By the initial 20 min time point, a large proportion of \(^{125}\)I-rhATH had already been taken up by the liver (26.1%, Fig. 2A), corresponding to a vast loss from the vascular compartment (Fig. 1). Over the next 3.7 h, presence of label shifted from the liver (6.1%) to appearance as degradation products (17.1%) in urine (Fig. 2B). The vast majority of rhATH-derived material was contained in the urine at the 24 h end point, somewhat similar to pATH. In comparison, however, rhATH progressed much more rapidly to the liver for processing than pATH \((p < 0.001)\). In order to assess whether the significant hepatic uptake of rhATH involved a limited number of membrane receptors, experiments were conducted using trace doses of the labeled conjugate. At 1/1,000 of the previous experimental dosage, the majority (51.2%) of rhATH was now liver-associated by 20 min (Fig. 2A). This quantity of label represented a significant \((p < 0.001)\) 2-fold increase in liver adsorption compared to that with 3 mg/kg injections of rhATH. Interestingly, low dose rhATH also resulted in significant accumulations of label in lung (1.5%), spleen (1.3%) and kidney (3.1%) during this early timeframe (Fig. 2A). The fact that a large majority of the 3 µg \(^{125}\)I-rhATH/kg injected was contained in tissues after 20 min was reflected by the extremely low amount of label remaining in blood (12.9%, Fig. 1). By 4 h, although vascular amounts of low dose rhATH remained essentially unchanged (Fig. 1), there was a highly significant reduction \((p < 0.0001)\) in label within the liver and a corresponding appearance of products in urine (Fig. 2B). Other organs, particularly the kidney (1.4%), also possessed significant quantities of \(^{125}\)I at 4 h (Fig. 2B). Finally, by the end of experiments with low dose rhATH, most of the radiolabel was recovered in urine (53.8%) and feces (4.2%, Fig. 2C).

**Vessel wall binding**

It was apparent from early portions of the experimental time course that, particularly in the case of rhATH, significant amounts of \(^{125}\)I-label were rapidly lost from the circulation (Fig. 1). Although, the liver was a major target for this removal (Fig. 2), it was likely that the most rapid mechanism for clearance would be through adsorption onto vessel wall surfaces. Thus, the entire vena cava and aorta were excised and analyzed for \(\gamma\)-radioactivity. Results appear in Figure 3. Analysis of venae cavae, 20 min post injection, showed small but measurable amounts of \(^{125}\)I-binding for all ATH experiments (Fig. 3A). However, quantities (as percent dose) of rhATH bound to the vena cava tended to be greater than that for pATH, and the decreased dose further shifted the proportion of injected rhATH taken up onto the vessel surface (Fig. 3A). At longer time points, the amounts of both pATH and rhATH on the vena cava surface approached baseline (Fig. 3A). In an attempt to project the amount of ATH that may be associated with venous surfaces throughout the animal, a calculation was done using vena cava binding as a model for the rest of the vasculature. Prior reports have shown detailed geometrical relationships between large and small blood vessels (21). Using measurements of the number and surface area for all the veins in a small mammal, as determined by other workers (20), we calculated the ratio of surface area for the vena cava to the total surface area for all veins to be 1:1,005. In order to get a rough estimate of the degree of vascular binding, we assumed that the distribution of ATH bound on the vena cava may be similar to that on other venous surfaces and used the vena cava total vein surface area ratio to calculate the potential % dose of radiolabel taken up onto the venous system. The right-hand axis in Figure 3A indicates that total \(^{125}\)I-rhATH taken up onto veins, shortly after injection, was likely a significant proportion of the dose. Turning attention to measurements of \(^{125}\)I-ATH binding to the aorta revealed a somewhat similar pattern to that of the vena cava. Although binding at 20 min of pATH was low, significantly greater
amounts (p < 0.05) of bound rhATH could be observed (Fig. 3B). However, while rhATH was lost from the aorta surface after 4 h in experiments with high doses of rhATH, arterial binding of rhATH persisted when trace amounts of the complex were administered. This dose dependence of elevated and long-term arterial binding by rhATH mirrored that found on the vena cava (compare Figs. 3A and B). Similarly, estimation of potential quantities of ATH present on all arterial vessels (aorta:total arterial surface area = 1:424) suggested that these surfaces were a major site responsible for initial removal from the circulation. All told, both veins and arteries could account for much of the rapid drop in rhATH from the blood.

One important aspect of vessel wall uptake concerns the impact of the surface-bound anticoagulants on local haemostatic effects. In an attempt to obtain some appreciation of the potential alteration in vessel surface character by the adsorbed ATH compounds, a calculation was performed based on the amount of labeled conjugate bound and the anti-Xa activities reported in Table 1. If the starting specific activities can be used as an estimate for bound drug, then 0.014 and 0.057 units of pATH and rhATH, respectively, was associated with the vena cava after 20 minutes in high dose experiments (approximately 500 units/kg were injected). By a similar token, 0.0036 and 0.44 units of pATH and rhATH, respectively, would be aorta-bound after 20 minutes in high dose experiments.

Soluble fragments
A final confirmation of the metabolic breakdown of injected drug over time was made by assessment of the levels of PTA-soluble $^{125}$I-label in some of the fluids that were collected. Addition of PTA to diluted blood samples showed that change in non PTA-precipitable fragments was minor up to 4 h (Table 2). However, by 24 h, much of the minute % of label remaining in the circulation was comprised of degradation products (Table 2). Coinciding with the loss in blood-borne drug was an increase in urinary ATH derivatives over time. As expected, essentially all $^{125}$I in the urine was from degraded conjugate (radiolabel in all urine samples tested was > 90% PTA soluble).

Discussion
Although heparin is the major anticoagulant used clinically, it suffers from a number of limitations. UFH has a short dose-dependent intravenous half-life which gives rise, in part, to an unpredictable anticoagulant effect (22). LMWH has been prepared that has a prolonged half-life and more controlled anticoagulant response due to lower binding to surfaces (11, 23). However, both UFH and LMWH are ineffective at catalyzing the inhibition of fibrin-bound thrombin (24–26). These issues have been addressed by development of a covalent ATH product that can neutralize clot procoagulant function (8, 10) and persists in the circulation with high anticoagulant activity (3). Recent studies have confirmed that one explanation for the increased intravenous half-life is that ATH has reduced binding to plasma and endothelial surface proteins compared to UFH (11). To more fully determine the factors involved in the performance of ATH in vivo, detailed analysis of the conjugate’s pharmacodynamics was necessary.

Radiolabeled ATH prepared from either plasma-derived AT (pATH) or recombinant human AT produced in goats (rhATH) was intravenously injected into rabbits, and the presence in various organs or compartments measured. Disappearance of a saturating pATH bolus from the circulation (Fig. 1) displayed a 2-phase profile with a clearance rate in the range of that found previously (3, 27). When similar doses of rhATH were tested, a much more significant initial loss of compound was observed than with pATH (Fig. 1). However, after this early time period, decreases in blood levels of pATH and rhATH over time were comparable. As suspected, examination of vascularized organs showed that a major site responsible for increased quick removal of rhATH relative to pATH was the liver (Fig. 2A). Further experiments with trace amounts of rhATH (Fig. 2) lead to even greater proportions of the load being deposited in the liver, suggestive that this rapid hepatic uptake was receptor-limited (28).

Since pATH and rhATH both contain heparin chains from the same source, it is apparent that biodistribution differences would result from the type of AT used. Coincident with our findings here, the reported half-life of rhAT (29, 30) is reduced compared to plasma AT (3, 31–33). Constituently, plasma-derived AT has a 432 amino acid chain (34) containing either 4 (35) or 3 (36) N-linked glycans. Since it was produced in goats, rhAT was found to be structurally identical to plasma-derived AT (37), except for major differences in N-glycosylation (38). The main change in glycosylation is the presence on rhAT of oligomannose structures at Asn 155 compared to plasma AT that only contains complex type glycans (37). Loss of native plasma-derived AT occurs primarily through redistribution into the extravascular space (39, 40), as well as slow conversion to a latent form with similar pharmacokinetics (41). Alternatively, the presence of oligomannose chains on rhAT would direct it to the well-characterized hepatic mannose receptor (42–44), consistent with the rapid liver uptake of our heparin-conjugated rhAT (Fig. 2).

Further analyses revealed detectable venous and arterial binding of ATH during the early phase after administration (Fig. 3). Literature reports for plasma ATH show clearance to be biphasic (39) with initial rapid binding of a small fraction to heparan sulfate proteoglycans on vessel wall endothelium (36, 45). In our experiments, swift uptake onto the vena cava and aorta was greatly enhanced for rhATH over pATH (Fig. 3). Given that the preponderance of endothelial mannose receptors (46) make this lectin the principal mode for vessel binding of heteroglycosyl-
ated proteins (47), glycan differences on rhATH may play a role similar to that found with the liver.

When the assumption was made that vena cava and aorta binding may be representative of binding throughout veins and arteries, the amount of rhATH rapidly associating with the vessel wall was a sizable proportion, even at high doses (Fig. 3). Any uptake by the capillary bed would further augment clearance. However, one caveat about estimation of total venous and arterial uptake is the potential variation in binding capacity within the vasculature. Indeed, variation in expression by cells such as smooth muscle cells can occur from segment to segment within the same vessel (48). Nevertheless, consideration of the calculation for potential total arterial/venous surface-bound ATH does have merit. For example, it has recently been shown that the mannose receptor is widely distributed on endothelium (49) from large vessels on down to the microvasculature (50). Furthermore, murine studies indicated that the sinusoidal endothelium has consistent expression of mannose receptor from embryonic stages through to adulthood (51).

Selective targeting of rhATH to the vessel lumen could be an elegant modality for treatment. Current theories postulate that inhibition of coagulant enzymes in vivo principally occurs by reaction with activated AT bound to endothelial heparan sulfate (52–55). Thus, immediate placement of rhATH on the vessel wall would efficiently position this drug for neutralization of thrombotic activity at the site of generation and in a way that mimics the patient’s natural anticoagulant system. Calculations that used specific activities of the injected materials as an estimate for bound ATH gave values of up to 0.4 anti-Xa units on the rabbit vena cava and aorta, which would be appreciable given the fact that surface areas of these vessels are in the order of 1–3 cm² (56–58). Of course, it must be cautioned that estimates of systemic and local surface activities reported here were indirect since no ex vivo assays were possible. Nevertheless, there is a rational basis for these projected values since plasma disappearance curves of the AT protein and heparin activity for pATH are virtually identical (3), and there is no more than a 25% difference in anti-Xa activity across the entire molecular weight spectrum of ATH heparin chains (1). To confirm the activity of vessel-bound ATH, future experiments involving sophisticated new surface activity assays (59) are being developed.

With this basic pharmacokinetic data at hand, studies can be devised to refine potential treatment protocols and verify mechanistic aspects. For example, the different distribution sites for pATH and rhATH might be probed by administration of agents that block binding to the known receptors in these organs. Furthermore, effects of various pathological states on biodistribution could also be studied to determine the impact of local injury on ATH utilization in the organism. From these types of investigations, more detailed understanding of ATH metabolism may be achieved.

References


