DISPOSITION OF FRACTIONATED $^3$H-HEPARIN IN RATS*

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To evaluate the disposition of fractionated $^3$H-heparin, high affinity fraction (fraction A) and low affinity fraction (fraction NA) to protamine were prepared in this study from commercial $^3$H-heparin by affinity chromatography on protamine-Sepharose and gel filtration chromatography.

Following intravenous administration of fraction A to rats, it seemed that the radioactivity in plasma declined one-exponentially with time to a pseudo-steady-state level, and 20.5% of dose was excreted in urine in 5 h. However, following the administration of fraction NA to rats, the plasma radioactivity curve was different from that of fraction A, and 69.8% of dose was excreted in urine in 5 h. It was found that fraction A and fraction NA had the distinctly different disposition characteristics in the body. Additionally, whole-body autoradiography with fraction A showed that the liver, bone marrow, kidney cortex, salivary gland and intestine played an important role in the distribution of it. It was suggested by gel filtration chromatography and affinity chromatography that the main material of the radioactivity excreted in urine in 5 h has the same properties as that of the administered heparin fraction.

**Keywords**—heparin; fractionated $^3$H-heparin; affinity chromatography; gel filtration chromatography; disposition; distribution; metabolism; pharmacokinetics; whole-body autoradiography; rat

INTRODUCTION

The disposition of $^{35}$S-heparin or $^3$H-heparin in rats was studied by many investigators. But it could not not be considered plausible that sufficient pharmacokinetic studies have been carried out by using radioactive heparin.

To evaluate the distribution of water-soluble macromolecular compounds in the body, heparin has been selected as one of the model compounds in our laboratory. In our previous report, urinary and fecal excretion and plasma levels following intravenous administration of commercial $^3$H-heparin were investigated at three dose levels. Whole-body autoradiography was also carried out periodically following intravenous administration of commercial $^3$H-heparin at single dose level. However, all the results obtained in these experiments were not in agreement with those of the other reports.

The commercial heparin is polydisperse with respect to molecular weight and also heterogeneous with respect to the biological and chemical properties. Since “heparin” is not a homogeneous substance, it is difficult to study the mechanism of heparin disposition by using the commercial heparin. Therefore, fractionation of heparin was required to diminish heterogeneity and polydispersity of heparin then to study the disposition of the fractionated heparin in detail.

Shanberge and coworkers have reported the disposition of $^3$H-heparin which was fractionated in regard only to the polydispersity.

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However, there is no report concerning the heparin disposition in rats by using fractionated
³H-heparin in regard to both polydispersity and heterogeneity of it.

In this study, the commercial ³H-heparin was fractionated by affinity chromatography on pro-
tamine-Sepharose in regard to heterogeneity and then fractionated by gel filtration chromatog-
raphy in regard to polydispersity of it, and two separated fractions, which were of different
affinity to protamine, were temporarily named fraction A and fraction NA. Investigations of the
periodical urinary excretion and whole-body autoradiography following intravenous admin-
istration to rats were made only for fraction A because enough amount of fraction NA was not
obtained to perform the similar experiments, while plasma levels and cumulative urinary excre-
tion were investigated for both fractions. Radioac-
tive material in the urine and plasma were fraction-
ated by gel filtration chromatography and then fractionation by affinity chromatography. The
remarkable difference in the disposition characteristics of fraction A from those of fraction NA
was observed.

MATERIALS AND METHODS

Materials — The radioactive heparin was
[PH(G)] heparin sodium salt of porcine mucosal
origin (#1396-171; specific anticoagulant activity
was 1443 units/mg and specific radioactivity was
0.27 mCi/mg; M.W.; 6000-20000, New Eng-
land Nuclear, Boston, Mass.). ³H-Water (the
specific radioactivity was 1 mCi/ml; New Eng-
land Nuclear, Boston, Mass.) of 0.7 mCi/ml was
prepared by dilution with distilled water. Pro-
tamine sulfate (from salmon roe) was obtained
from Wako Pure Chemical Industries, Ltd., Japan.
Cyanogen bromide-activated Sepharose 4B
(CNBr-Sepharose 4B) and Sephadex G-25 (fine)
were purchased from Pharmacia, Uppsala,
Sweden. Protamine-Sepharose was prepared by
coupling protamine to CNBr-Sepharose 4B
according to the procedure of Piepkorn et al.
All other chemicals were of analytical grade and used
without further purification.

Affinity Chromatography — For the fractiona-
tion of ³H-heparin in regard to the affinity to pro-
tamine, polypropylene mini column packed with
2.5 ml of protamine-Sepharose gel was used.
About 2 mCi (8 mg) of ³H-heparin in 1 ml of buffer
(0.6 M NaCl - 0.02 M imidazole, pH 7.35)
was applied to the protamine-Sepharose column
equilibrated with the same buffer at room tem-
perature. The column was washed with 20 ml of
the same buffer as above and then eluted with 20
ml of 2.5 M NaCl - 0.02 M imidazole buffer (pH
7.35) at a mean flow rate of 17 ml/h. Each 50-
drop fraction of the eluate was collected into a
glass tube (about 1.6 ml/tube) using an automatic
fraction collector (PC-80, Gilson, France). For the
radioactivity measurement, 3 µl of each fraction
was transferred to the counting vial containing
0.5 ml of distilled water and then 10 ml of scin-
tillator (ACS II, Amersham Searle Corp., U.S.A.)
was added to it.

Gel Filtration Chromatography — Each fraction
in tube number 3, 4, 5, 15, 16 and 17 on affinity
chromatography was placed on a 1.5 × 40 cm col-
umn of Sephadex G-25. Then elution was per-
formed with distilled water at a mean flow rate of
38 ml/h. The elute was collected into 50-drop
fraction. Three µl of each fraction was transferred
to the counting vial for radioactivity measure-
ment.

Preparation of the Solutions for Injection — The
solution of fraction NA for injection was pre-
pared by pooling the fractions in number 16
through 20 on gel filtration chromatography of
the tube number 3, 4 and 5 on affinity chroma-
tography, by lyophilization and then by dissolving in physiological saline. In the same
way, the solution of fraction A was prepared by
pooling the fractions in number 16 through 20 on
gel filtration chromatography of the fractions of
the number 15, 16 and 17 on affinity chro-
matography.

Animals — Male Wistar rats weighing 170 to
240 g were used. All rats were cannulated into
the right external jugular vein with silicone polymer
tubing (i.d. 1.0 mm; o.d. 1.5 mm, Dow Corning,
Tokyo, Japan) under light anesthesia with ether
about 2 d before the start of the experiments. In the surgery, 10% sodium citrate was used to fill the cannula instead of heparinized saline.

Plasma Levels — Rats were injected with 100 μCi/kg of fraction NA or fraction A through the cannula. The dose of each fraction was 0.37 mg/kg body weight, which was calculated on the assumption that fraction NA and fraction A had the original specific radioactivity. The administered volume was about 5 ml/kg body weight. Blood samples of 0.25 ml were drawn through cannula periodically at 1, 3, 5, 10, 20, 35, 60, 120, 180, 240 and 300 min and were then centrifuged at 3000 rpm for 15 min to obtain plasma samples. For radioactivity measurement, 100 μl of each plasma sample was transferred to the counting vial containing 1 ml of Soluene-350 (Packard Instrument Co., Downers Grove, III., U.S.A). Then 10 ml of toluene scintillator (PPO 6 g, POPOP 75 mg, toluene 1000 ml) was added to it.

Urinary Excretion — Urine samples were also collected from the above rats over 5 h in order to measure the cumulative urinary excretion and to obtain the samples for fractionation. Following the injection of fraction NA or fraction A, rats were housed individually in glass metabolic cage and the urination was induced by making them sniff at a cotton pad wetted with ether. Urine was collected in a volumetric flask (50 ml). Some aliquots of urine samples were frozen (−20°C) and stored until fractionation was carried out.

To obtain periodical urinary excretion profiles, another group of rats received the intravenous injection of fraction A (100 μCi/kg) through the cannula. Urine was collected over 0—0.5, 0.5—1.0, 1.0—1.5, 1.5—2.0, 2.0—3.0, 3.0—4.0, 4.0—6.0, 6.0—8.0 and 8.0—24.0 h in the same way as described previously.3)

One ml of urine sample was transferred to the counting vial for radioactivity measurement and then 10 ml of scintillator (ACS II) was added to it.

Whole-Body Autoradiography — Rats were injected with 100 μCi/kg of fraction A through the cannula. At 5 min and 5 h following the injection, rats were sacrificed and freeze-dried sections were prepared from them according to the similar method described in our previous paper.3) The freeze-dried sections were contacted with Sakura 3H-type X-ray films (MARG, Konishiroku Photo Ind. Co. Ltd., Tokyo, Japan) for 4 weeks at 4°C. The films were developed at 20°C for 5 min.

Fractionation of Excreted Radioactivity in Urine — To fractionate the excreted radioactivity in the urine with respect to its molecular weight, 1 ml of each urine sample was placed on a 1.5 × 40 cm column of Sephadex G-25 and eluted with distilled water. Fifty-drop fractions were collected and 100 μl of each fraction was used for

![Affinity Chromatogram on Protamine-Sepharose of Commercial 3H-Heparin Used in This Experiment](image-url)

- a) Tube number 15, 16 and 17 were gel filtrated to obtain fraction A.
- b) Tube number 3, 4 and 5 were gel filtrated to obtain fraction NA.
the radioactivity measurement.

The fractions in tube number 16 through 20, which were the same fractions as injected, were pooled and lyophilized to fractionate the radioactive material with respect to its affinity to protamine-Sepharose. Then, 1.5 ml of buffer (0.6 M NaCl - 0.02 M imidazole, pH 7.35) was added to it. By using 1 ml of this solution, affinity chromatography was carried out in the same way as described above. One hundred μl of each fraction on affinity chromatography was used for the radioactivity measurement.

**Fractionation of Radioactivity in Plasma** — At 300 min following the injection of fraction A (100 μCi/kg), about 7.3 ml of blood was drawn from the cannula and mixed with 10 mg of disodium edetate dihydrate. The mixture was centrifuged at 3000 rpm for 15 min. To obtain defibrinated plasma, about 4.3 ml of plasma was transferred to another centrifuge tube. Then it was incubated at 56°C for 5 min and centrifuged at 3000 rpm for 15 min. One ml of the defibrinated plasma was fractionated by gel filtration chromatography followed by affinity chromatography and then the radioactivity of the eluates was measured.

To compare the gel filtration chromatograms of defibrinated plasma and urine with that of [H]-water, gel filtration chromatography of [H]-water was performed under the same condition.

**Radioactivity Measurement** — The radioactivity was determined in a Mark II liquid scintilla-

**FIG. 2(a).** Gel Filtration Chromatogram of Tube Number 15 (High affinity fraction) in Fig. 1 (b). Gel Filtration Chromatography of Tube Number 3 (Low affinity fraction) in Fig. 1

a) The fractions in tube number 16 through 20 were pooled to obtain fraction A.
b) The fractions in tube number 16 through 20 were pooled to obtain fraction NA.
tion spectrometer (Nuclear-Chicago corporation, Des Plaines, Ill., U.S.A.). The counting efficiencies were determined by $^{133}$Ba external standard channels method and cpm values were converted to dpm.

RESULTS AND DISCUSSION

Fractionation of Commercial $^3$H-Heparin

Affinity Chromatography — The elution pattern of radioactivity following the affinity chromatography on protamine-Sepharose is shown in Fig. 1. The commercial $^3$H-heparin which was used in this experiment was fractionated into low and high affinity fractions on protamine-Sepharose, comprising approximately 14 and 81% of low and high affinity fraction, respectively.

Gel Filtration Chromatography — To obtain the same fraction with respect to its molecular weight, both the low and high affinity fractions on protamine-Sepharose were fractionated on Sephadex G-25. The gel filtration chromatogram of tube number 15 (high affinity fraction) in Fig. 1, and that of tube number 3 (low affinity fraction) in Fig. 1 are shown in Fig. 2(a) and Fig. 2(b), respectively.

In the gel filtration chromatogram of the high affinity fraction, single peak of radioactivity was observed in void volume (Fig. 2(a)). However, as for low affinity fraction of commercial $^3$H-heparin, two peaks of radioactivity existed primarily in void volume and smaller molecular weight portion (Fig. 2(b)).

It has been well known that "heparin" is polydisperse with respect to molecular weight and also heterogeneous with respect to the biological and chemical properties. In the present experiment, commercial $^3$H-heparin was fractionated into 14% of low affinity fraction and 81% of high affinity fraction by affinity chromatography on protamine-Sepharose. By affinity chromatography on antithrombin III-Sepharose, Höök et al.\(^{50}\) have reported that the percentages of high affinity fraction are 38, 64 and 50% using three types of heparin, and Bhargava et al.\(^{52}\) have reported that the percentages of high

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**FIG. 3. Plasma Levels of Radioactivity following Intravenous Administration of Fraction A (a) and Fraction NA (b) to Rats**

Each point represents the mean ± S.D. of 3 rats. Dose: 100 μCi/kg. •: fraction A, ○: fraction NA. The broken lines show the eye-fitted curves.

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**FIG. 4. Cumulative Excretion of Radioactivity in Urine following Intravenous Administration of Fraction A to Rats**

Each point represents the mean ± S.D. of 3 rats. Dose: 100 μCi/kg.
affinity fraction are 40 and 83% and the percentages of low affinity fraction are 60 and 17% from two types of heparin, respectively. From comparison with these reported values, it was found that the commercial \(^{3}H\)-heparin used in this experiment consisted of relatively large amount of high affinity fraction.

_Elimination from Plasma_

Fig. 3 shows the time course of total radioactivity in plasma following intravenous administration of fraction A or fraction NA.

It seems that following the administration of fraction A, plasma radioactivity declined one-exponentially to a pseudo-steady-state level. If the plasma levels of radioactivity in early period (0–60 min) following the administration of fraction A are analyzed according to the classical one-compartment open model, \(46.5 \pm 1.7 \, \text{ml/kg of } V_d \) and \(7.85 \pm 0.65 \, \text{min of } t_{1/2} \) (mean \(\pm\) S.E., \(n=21\)) would be estimated. But the plasma radioactivity following the administration of fraction NA showed a different curve from that obtained after the administration of fraction A.

These declining patterns of plasma radioac-

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**FIG. 5.** Autoradiograms showing the Distribution of Radioactivity (Dark Area) in Rats at 5 min (A) and 5 h (B) following Intravenous Administration of Fraction A (100 \(\mu\)Ci/kg)
tivity following the administration of fraction A and fraction NA (Fig. 3) could not be analyzed according to the classical two-compartment open model although the plasma radioactivity following the administration of unfractionated commercial $^3$H-heparin could be analyzed by the model in our previous paper. The observation suggested that the different elimination curves from different commercial $^3$H-heparin might have been due to the different ratios of the fraction A to NA in the commercial heparin.

**Urinary Excretion**

The excreted radioactivities in urine (mean ± S.D.) within 5 h following intravenous administration of fraction A and fraction NA were 20.5±3.1% ($n=3$) and 69.8±9.6% ($n=3$) of the administered dose, respectively. A significant difference was observed in these values ($p < 0.01$). This observation also indicated that fraction A and fraction NA have distinctly different disposition characteristics in the body. In our previous paper, the cumulative urinary excretion of radioactivity following intravenous administration of unfractionated commercial $^3$H-heparin was approximately 70% at 4 h. On the basis of the present results of urinary excretion, it may be considered that the commercial $^3$H-heparin used in the previous experiment was composed of a relatively large amount of fraction NA and small amount of fraction A.

The periodical urinary excretion profiles following the administration of fraction A is shown in Fig. 4. The excreted percentage of radioactivity in urine was 41.0% of dose 24 h after administration of fraction A and most of it was excreted in early period (Fig. 4). Besides, it is interesting to note that the approximate zero-order excretion in urine (Fig. 4) seemed to correspond to the pseudo-steady state of the plasma radioactivity (Fig. 3).

**Whole-Body Autoradiography**

Whole-body autoradiograms following

![Graphs showing radioactivity over tube number]

**FIG. 6. Gel Filtration Chromatogram of Urine Sample in 5 h following Intravenous Administration of Fraction A (a) or Fraction NA (b)**

$V_o$: void volume. a) The fractions in tube number 16 through 20 were pooled to obtain the sample for affinity chromatography.
intravenous injection of fraction A are shown in Fig. 5. A remarkable blackening in blood and lung was observed at 5 min but not at 5 h (Fig. 5). In contrast, a relatively strong blackening was observed in the liver, bone marrow, kidney cortex, salivary gland and intestine at both 5 min and 5 h (Fig. 5).

Previously it was suggested by periodical whole-body autoradiography that the reticuloendothelial system might be involved in $^3$H-heparin distribution. In the present experiment, it was also found by whole-body autoradiography that there was the same tendency, and these organs and tissues played an important role in the distribution of fraction A.

**Metabolism**

**Fractionation of Excreted Radioactivity in Urine**—Gel filtration chromatograms of urine

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**FIG. 8(a).** Gel Filtration Chromatogram of Defibrinated Plasma at 5 h following Intravenous Administration of Fraction A (100 μCi/kg)

(b). Affinity Chromatogram of the Sample from Pooled Fraction of Tube Number 16 through 20 in Fig. 8(a)

a) The fractions in tube number 16 through 20 were pooled to obtain the sample for affinity chromatography. The broken line shows the gel filtration chromatogram of $^3$H-water. $V_0$: Void volume.
Disposition of Fractionated $^3$H-Heparin

sample following the administration of fraction A and fraction NA are shown in Fig. 6(a) and (b), respectively. Although some radioactivity existed at smaller molecular weight portion in both gel filtration chromatograms, the main peak of radioactivity existed at void volume (Fig. 6(a) and (b)).

Affinity chromatograms were then obtained from pooled samples of tube number 16 through 20 on gel filtration chromatography (Fig. 6(a) and (b)) and are shown in Fig. 7.

The sample (Fig. 6(a)) obtained following the administration of fraction A showed that the main peak existed at high affinity portion (Fig. 7). On the other hand, the main peak of radioactivity was observed at low affinity portion of the affinity chromatogram for the sample which was obtained following the administration of fraction NA (Fig. 7).

**Fractionation of Radioactivity in Plasma** — Gel filtration chromatogram of defibrinated plasma at 5 h following the administration of fraction A is shown in Fig. 8(a) along with that of $^3$H-water. Gel filtration chromatogram of the defibrinated plasma showed that the main peak existed at higher molecular weight portion than that of $^3$H-water. However, the radioactivity existed not only at void volume but also spreaded over other portions (Fig. 8(a)).

Affinity chromatogram of the sample which was prepared from the pooled tubes of number 16 through 20 on gel filtration chromatogram of the defibrinated plasma indicated that the main peak existed at high affinity portion as shown in Fig. 8(b).

By fractionation of radioactivity in the urine and plasma, it was verified that the radioactivity in the urine and plasma was not due to $^3$H-water. Additionally, it may be suggested that most of the radioactivity excreted in urine within 5 h had the same properties as those of the administered fraction. This result is similar to that of Losito et al.\(^2\)\(^b\)

They have observed by microelectrophoresis that the peak of Sepharose 4B chromatogram of excreted radioactivity in urine from the rat which was given $^3$H-heparin was indistinguishable from the original heparin.\(^2\)\(^b\)

**CONCLUSION**

By employing the fractionation technique on commercial $^3$H-heparin, the disposition kinetics for the fraction A or NA was investigated following intravenous administration in rats.

The plasma level of radioactivity for fraction A declined one-exponentially with time to a pseudo-steady-state. However, the plasma level for fraction NA showed the different pattern from that for the fraction A.

The urinary excretion for the fraction A was considerably slower than that for the fraction NA, and showed an approximate zero-order kinetics after about 60 min following the administration. This seemed to correspond to the pseudo-steady-state of the plasma radioactivity.

Whole-body autoradiography with the fraction A showed that liver, bone marrow, kidney cortex, salivary gland and intestine played an important role in the distribution of it. In this respect the result was rather similar to that reported in the previous paper.\(^3\)

As for metabolism of the fraction A or NA in rats, much of the fraction seemed to be excreted in unchanged form in urine.

In this study it was suggested that the different disposition kinetics from different commercial $^3$H-heparin might be mainly dependent on the different ratios of the fraction A to NA in the commercial heparin. Therefore, further investigation on the fractionated heparin should be necessary to reveal the mechanism of disposition of commercially available heparin, and the study on the dose-dependency of disposition kinetics for fractionated heparin is in progress in our laboratory.

**REFERENCES**


