# Pharmacokinetic Analysis and Cellular Distribution of the Anti-HIV Compound Succinylated Human Serum Albumin (Suc-HSA) *in Vivo* and in the Isolated Perfused Rat Liver

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*Received May 18, 1992; accepted May 17, 1993*

After intravenous injection of a low dose (25 μg/kg) in rats, the anti HIV-1 compound succinylated human serum albumin (Suc-HSA) is taken up mainly in the liver and spleen and is proteolytically degraded. Ten minutes after injection of $^{125}$I-Suc-HSA, 72 and 14% of the dose were found in the liver and spleen, respectively. With immunohistochemistry we demonstrated that in both organs, Suc-HSA was specifically endocytosed in endothelial cells. In the isolated perfused rat liver preparation, liver uptake was shown to be saturable, with a $K_m$ of 2.9 $10^{-8}$ *M* and a $V_{max}$ of 2.4 μg/min/100 g body weight. The apparent $K_m$ and $V_{max}$ *in vivo* were 2.2 $10^{-7}$ *M* and 10.3 μg/min/100 g, respectively. Uptake in liver and spleen was inhibited by preadministration of an excess of formaldehyde-treated albumin and with polyinosinic acid, indicating the involvement of the scavenger receptor, as anticipated for such polyanionic compounds. Suc-HSA is not absorbed intact from the colon and the ileum. After injecting (i.v.) rats with a high dose of Suc-HSA (10 mg/kg), the elimination $t_{1/2}$ was 3 hr, and therefore, sustained plasma levels above the concentration needed for *in vitro* anti-HIV-1 activity can be achieved.

KEY WORDS: succinylated human serum albumin; anti-HIV compound; pharmacokinetics; perfused rat liver.

## INTRODUCTION

We recently described that succinic anhydride- and *cis*-aconitic anhydride-modified human serum albumins (Suc-HSA and Aco-HSA) have potent *in vitro* anti-HIV activity (1,2). The 50% HIV inhibitory concentrations ($IC_{50}$) of Suc-HSA and Aco-HSA are 0.9 μg/mL (5 n*M*) and 0.023 μg/mL (270 p*M*) respectively, which renders Aco-HSA one of the most potent *in vitro* anti-HIV-1 compounds described to date. Aco-HSA and Suc-HSA are not toxic to a variety of cells, at concentrations up to 1 mg/mL. Therefore, the *in vitro* selectivity index ($IC_{50}/CC_{50}$) of these compounds is more than 4 orders of magnitude.

To date, several compounds have been described with a promising anti-HIV activity *in vitro*, e.g., dextran sulfate, a sulfated polysaccharide, and recombinant soluble CD4 (3–5). *In vivo*, however, these compounds were dramatically less effective (6,7). This large discrepancy between *in vitro* and *in vivo* anti-HIV activity is probably caused by unfavorable pharmacokinetic profiles (8). Therefore, it is important to perform early pharmacokinetic screening of compounds with potent *in vitro* pharmacologic activity.

We report here on the kinetics in rats of the recently developed anti-HIV compound, Suc-HSA (1,2). With $^{125}$I-labeled material, we studied the plasma disappearance and organ distribution. We determined the apparent $K_m$ and $V_{max}$ in intact rats and in the isolated perfused rat liver and performed competition studies with other polyanions. With immunohistochemistry, we show which cell type is responsible for the clearance of Suc-HSA. Finally, we investigated whether Suc-HSA is absorbed intact from the colon, from the ileum, or after subcutaneous injection.

## MATERIALS AND METHODS

### Preparation of Succinic Anhydride-Treated Albumin (Suc-HSA)

Derivatization of HSA with succinic anhydride was performed as described previously (1). Briefly HSA (500 mg) was dissolved in 50 mL 0.2 *M* $K_2HPO_4$, pH 8.0. Solid succinic (or *cis*-aconitic) anhydride (500 mg) was added and the solution was stirred until all anhydride was dissolved. The pH was kept between 8.0 and 8.5 with 6 *M* sodium hydroxide. For purification, the solution was filtered over a 0.2-μm filter to remove insoluble material, purified on a Sephadex G25 column, washed with distilled water on a PM10 membrane in an Amicon stirred cell concentrator, and finally, lyophilized.

The amount of protein was determined, according to Lowry *et al.* (9) and using the protein assay kit of Bio-Rad according to Bradford (10). Estimation of the free ε-lysine groups of the derivatized HSA's performed according to the method of Habeeb (11) showed that more than 95% of the amino groups were derivatized.

### Fast Protein Liquid Chromatography (FPLC)

*Charge Estimation.* The relative net negative charge of the modified albumins was determined on a FPLC system (Pharmacia, Woerden, The Netherlands) equipped with a Mono-Q anion-exchange column (Pharmacia) as described previously (12). Buffer A was a Tris–HCl buffer (0.02 *M*), pH 7.4, and buffer B consisted of buffer A plus 2 *M* NaCl. Elution was performed at a flow rate of 0.25 mL/min, with a gradient from 100% A to 100% B in 30 min. Samples were dissolved to 1 mg/mL in buffer A and 100 μL was injected into the FPLC system. The retention times of HSA and Suc-HSA were 20.3 and 30.8 min, respectively.

*Molecular Weight Estimation.* The percentages monomers, dimers, and polymers were determined on a FPLC system, using a Superose-12 column (Farmacia) as described previously (13), and were found to be 91.1, 8.1, and 0.8, respectively. The eluant was PBS pH 7.4 and the flow was 0.5 mL/min.

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0724-8741/93/1100-1611$07.00/0 

### Radioiodination

Proteins were labeled with $^{125}I$ to a specific activity of 2 μCi/μg using a chloramine-T method (14). Unattached $^{125}I$ was removed by gel filtration on a Sephadex G25 column. Immediately prior to experiments noncovalently bound $^{125}I$ was removed on a PD-10 column and radioactivity recovered in the void volume was more than 98% precipitable with trichloroacetic acid (TCA) at a final concentration of 10%.

### Isolated Perfused Rat Liver Experiments

Experiments with isolated perfused livers were performed as previously described (15). Briefly, rats were anesthetized with pentobarbital (Nembutal, 60 mg/kg i.p.). The bile duct, the portal vein, and the superior vena cava were cannulated. The liver was excised and placed in the perfusion apparatus. Temperature was kept at 37°C, and perfusate flow was maintained at 35 mL/min at a hydrostatic pressure of 10–12 cm. The recirculating perfusion medium (100 mL) consisted of a Krebs–bicarbonate buffer supplemented with 0.1% glucose and 1% BSA and was constantly gassed with 95% oxygen and 5% carbon dioxide. To replace bile salts, a 15 μmol/hr infusion of sodium taurocholate was given. All inhibitors were given 5 min prior to injection of Suc-HSA. Samples of 300 μL were mixed with 300 μL ice-cold 20% TCA and centrifuged at 2500 rpm for 10 min. The pellet was washed with 600 μL 20% TCA. The radioactivity of the combined supernatants and the pellet was counted in a LKB-Multichannel gamma counter.

### Immunohistochemical Staining of Liver Sections

One hundred fifty micrograms of Suc-HSA was injected in the vena penis dorsalis, and after 10 min pieces of the liver and spleen were frozen in liquid freon (−80°C). Liver sections of 4 μm were cut in a cryostat (20°C). Staining of the endocytosed material was performed as described by Harms *et al.* (16). Briefly, sections were fixed in acetone for 10 min and incubated with a rabbit anti-human serum albumin antibody (1:250 diluted in a buffer containing 0.02 *M* Tris, 0.15 *M* NaCl, 0.5 *M* $CaCl_2$, pH 7.8) for 30 min, and subsequently the sections were incubated with a peroxidase conjugated swine anti-rabbit antibody (diluted 1:20 in the same buffer) for 15 min. Finally, the peroxidase activity was visualized with the aminoethylcarbazole (AEC) reaction for 10 min and at last sections were counterstained with Mayer's hematoxylin for 10 sec.

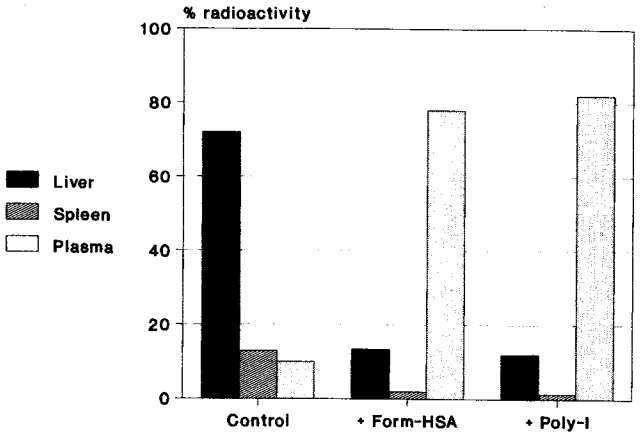


Fig. 2. Organ distribution of a tracer dose of $^{125}I$-Suc-HSA 10 min after i.v. injection. Data are the mean of three separate experiments.

### *In vivo* Clearance of Suc-HSA

Briefly, rats (200–220 g) were anesthetized with pentobarbital (Nembutal, 60 mg/kg i.p.). The temperature was kept at 37°C using an electrically heated table. The arteria carotis was cannulated and $^{125}I$-labeled Suc-HSA was injected into the vena penis dorsalis. At the indicated times blood samples were taken from the arteria carotis. In the organ distribution experiment, rats were sacrificed and the liver and spleen were removed. Inhibitors of the scavenger receptor (5 mg) were given 5 min prior to the addition of $^{125}I$-Suc-HSA. Radioactivity was counted in a LKB multichannel gamma counter.

## RESULTS

After intravenous injection of a relatively small dose (10 μg) of $^{125}I$-labeled Suc-HSA, TCA-precipitable radioactivity, representing intact Suc-HSA, disappeared rapidly from

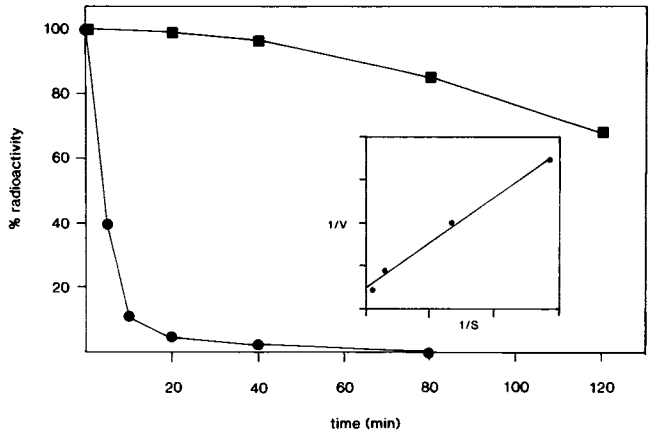


Fig. 1. Plasma disappearance of $^{125}I$-Suc-HSA after i.v. injection of 10 μg (circles) and 2.5 mg (squares). The inset shows a Lineweaver–Burk plot of different concentrations. Data are the mean of three separate experiments.

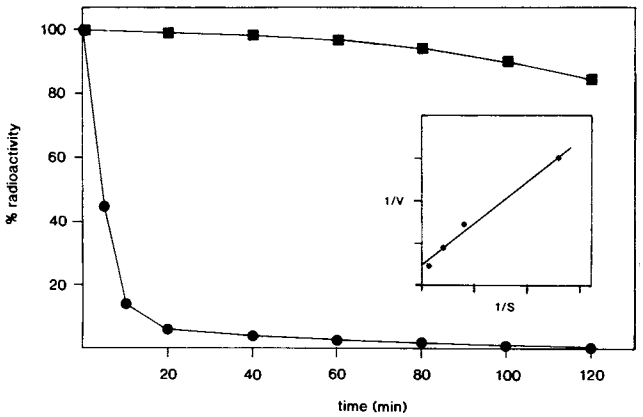


Fig. 3. Perfusate disappearance of $^{125}I$-Suc-HSA after injection of 10 μg (circles) and 2.5 mg (squares). The inset shows a Lineweaver–Burk plot of different concentrations. Data are the mean of three separate experiments.

plasma (Fig. 1). The initial plasma disappearance $t_{1/2}$ for this 10-μg dose was 3.6 ± 0.3 min. For a larger dose of 1 mg the corresponding $t_{1/2}$ was several hours, indicating a saturable clearance process.

The apparent $K_m$ and $V_{max}$ of this process, calculated from the nonlinear decay curves (50-, 100-, 500-, and 1000-μg doses), are $2.2 \times 10^{-7}$ $M$ and 10.3 μg/min/100 g rat, respectively (Fig. 1, inset).

Figure 2 shows that, 10 min after injection of a tracer dose of $^{125}$I-Suc-HSA, most of the radioactivity (73 ± 3.4%) was found in the liver, 13 ± 1.8% was recovered from the spleen, and 8.1% remained in the plasma compartment. Suc-HSA was not excreted intact in urine or in bile. Preadministration of an excess of formaldehyde-treated albumin (form-HSA) or polyinosinic acid significantly decreased the amount of Suc-HSA in both liver and spleen. This indicates that the scavenger receptor, responsible for the uptake of polyanions, is involved in the uptake of Suc-HSA.

The perfusate disappearance of Suc-HSA in the isolated perfused rat liver (IPRL) is shown in Fig. 3. A saturable uptake process was observed with an apparent $K_m$ and $V_{max}$ of $2.9 \pm 0.3 \times 10^{-8}$ $M$ and 2.4 ± 0.3 μg/min/100 g rat, respectively (Fig. 3, inset).

With immunohistochemistry we visualized the endocytosed Suc-HSA in liver and spleen (Fig. 4). Suc-HSA was endocytosed in the sinusoidal liver cells, predominantly in the endothelial cells (Fig. 4A). Also in the spleen (Fig. 4B), the endothelial cells (lining the sinuses) were the main cell type responsible for the uptake of Suc-HSA.

After injecting $^{125}$I-Suc-HSA in the colon or the ileum,

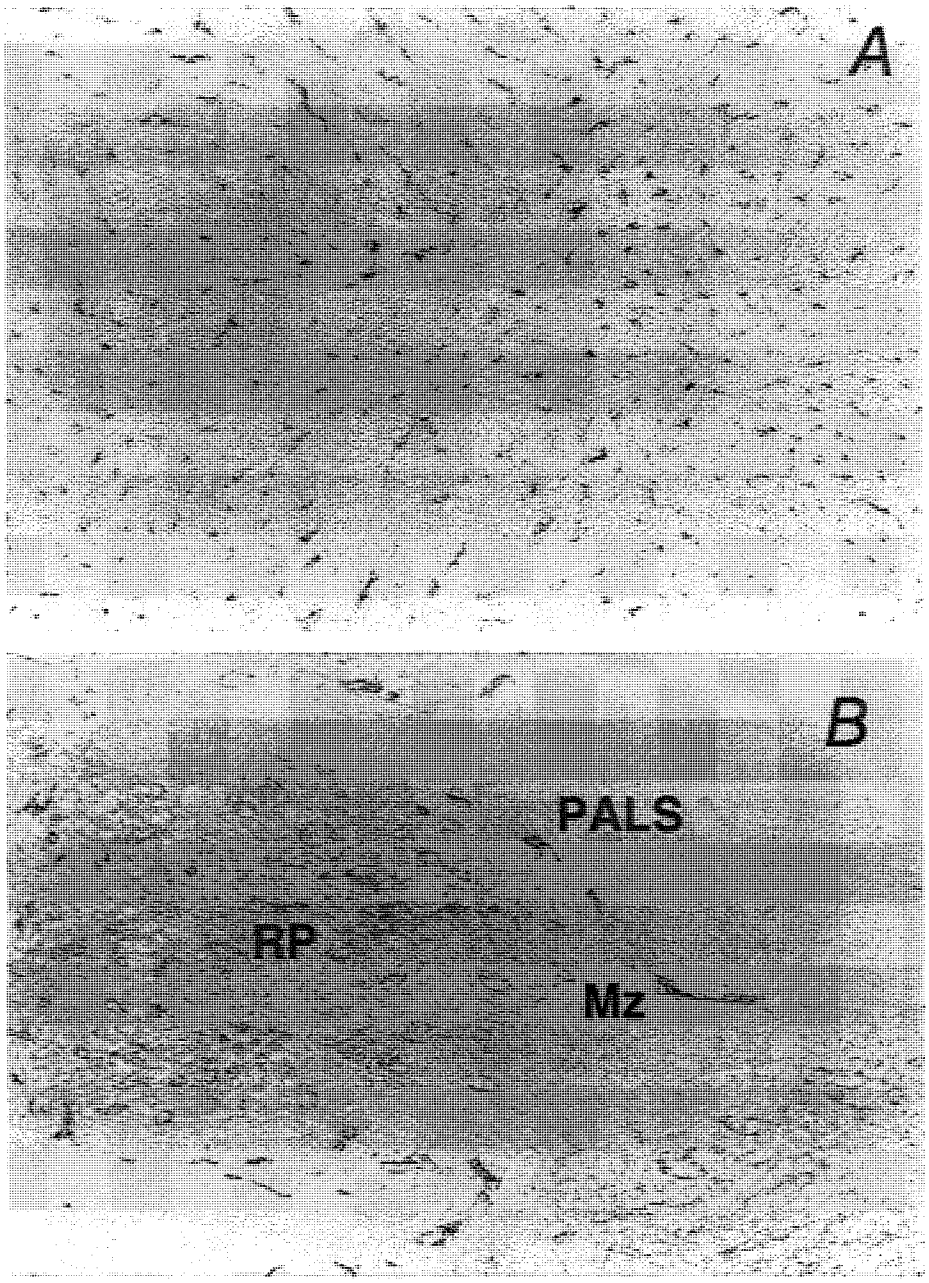


Fig. 4. Immunohistochemical staining of endocytosed Suc-HSA in liver (A) and in spleen (B). Mz, marginal zone; RP, red pulp; PALS, periarterial lymphocyte sheet.

Table I. Cumulative Percentage of the Dose in the Circulation, 2 hr After Injection of a Tracer Amount of $^{125}$I-Suc-HSA

| | |
|---|---|
| Colon | 0.025 |
| Ileum | 0.02 |
| Subcutaneous | 0.025 |

or after subcutaneous injection, less than 0.025% of the dose reached the central compartment (Table I). TCA precipitation of the blood samples revealed that none of the radioactivity found in blood represented intact Suc-HSA.

## DISCUSSION AND CONCLUSIONS

We recently described the potent *in vitro* anti-HIV activity of negatively charged human serum albumins (1,2). They inhibit the virus entry and the syncytia formation probably because of interference with the viral fusion protein gp41. Prior to the *in vivo* anti-HIV screening we investigated whether their kinetic profile permits prolonged contact time with gp41. Suc-HSA is rapidly removed from the circulation after i.v. injection of a tracer amount (Fig. 1). Increasing the dose of Suc-HSA corresponded with a longer plasma disappearance, $t_{1/2}$, indicating a saturable uptake process. When a relatively high dose was injected, in relation to the $V_{max}$ of the uptake process (10.3 μg/min/100 g rat), plasma levels well above the concentration needed for anti-HIV activity ($IC_{50}$ *in vitro* is 1 μg/mL) were obtained for several hours.

Most of the compound was taken up by the liver and, to a lesser extent, by the spleen. In both organs, Suc-HSA was found in the endothelial cells. The inhibition of uptake in liver and spleen by coadministration of other polyanions such as form-HSA and polyinosinic acid indicates that scavenger receptor systems are involved. We recently described the existence of two different scavenger receptors, present in sinusoidal liver cells (13), one on endothelial liver cells, recognizing monomeric formaldehyde-treated HSA, and one on Kupffer cells, which endocytoses predominantly the polymeric form of this denatured protein (13). The Suc-HSA used in the present study consisted predominantly of monomers (>91%), and the endothelial liver cell localization agrees with these previous results. Thus far, a scavenger receptor had not been reported to be present on endothelial cells in the spleen. The fact that the apparent $K_m$ ($2.9 \times 10^{-8}$ *M*) in the isolated perfused rat liver is one order of magnitude lower than the $K_m$ ($2.2 \times 10^{-7}$ *M*) determined from the *in vivo* experiments suggests that, although the receptors in liver and spleen have some characteristics in common, they are not identical.

Finally, we showed that Suc-HSA is not absorbed from the colon and the ileum as expected from its strongly polyanionic character. Therefore, for *in vivo* anti-HIV testing this compound should be injected intravenously or intramuscularly.

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