Brain Delivery of Biotin Bound to a Conjugate of Neutral Avidin and Cationized Human Albumin

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The delivery of pharmaceuticals through the brain capillary endothelial wall, which makes up the blood-brain barrier (BBB) in vivo, may be facilitated by conjugation of therapeutics to brain drug delivery vectors. Since cationized albumin has been shown to undergo absorptive-mediated transcytosis through the BBB in vivo, cationized human serum albumin (cHSA) is a potential brain drug delivery vector in humans. Conjugation of biotinylated therapeutics to brain drug delivery vectors is facilitated by the preparation of vector/avidin conjugates. Therefore, the present studies describe the preparation of a cHSA-avidin conjugate and the delivery of ^{3}H-biotin bound to this conjugate through the BBB in vivo in anesthetized rats. Since the cationic nature of avidin (AV) accelerates the removal of avidin-based conjugates from blood in vivo, the present studies also describe the preparation and the pharmacokinetics of ^{3}H-biotin bound to a conjugate of cHSA and neutral avidin (NLA). The bifunctional nature of the conjugate was retained: the cHSA/NLA conjugate contained 2.8 to 6.8 biotin binding sites per conjugate, and the BBB permeability-surface area (PS) product for ^{3}H-biotin bound to cHSA/NLA was at least 7-fold greater than the BBB PS product for ^{3}H-biotin bound to a conjugate of NLA and native HSA (nHSA). The systemic clearance of the cHSA conjugate was reduced 10-fold by the use of NLA as opposed to AV. The increased area under the plasma concentration curve (AUC) of the cHSA-NLA conjugate correlated with an increase in brain delivery of ^{3}H-biotin as compared to the brain delivery achieved with the cHSA/AV conjugate. In conclusion, these studies demonstrate that cHSA serves as a brain drug delivery vector and that the use of neutral forms of avidin increases the plasma AUC of the conjugate and enhances the brain delivery of biotin.

KEY WORDS: blood-brain barrier; pharmacokinetics; drug delivery; avidin; biotin.

INTRODUCTION

Brain drug delivery is limited by the poor transport of hydrophilic therapeutics through the brain capillary endothelial wall, which makes up the blood-brain barrier (BBB) in vivo (1). One strategy for BBB drug delivery is the use of chimeric peptides (2), which may be considered a form of pro-drug (3). Chimeric peptides are comprised of a transportable brain delivery vector, which is conjugated to a non-transportable therapeutic compound generally via a cleavable disulfide linkage (2). Peptides and proteins are proposed to undergo receptor-mediated endocytosis into cells in peripheral tissues (4), and receptor-mediated or absorptive-mediated transcytosis through the BBB (2). The model vector which undergoes receptor-mediated transcytosis through the BBB is the OX26 monoclonal antibody to the transferrin receptor (5,6), which is highly enriched on brain microvascular endothelium (7,8). Cationized albumin (9) and some cationic peptides (10,11) undergo absorptive-mediated transcytosis through the BBB. The coupling of drugs to brain transport vectors is facilitated by the use of avidin-biotin technology (12). In this approach, vector/avidin conjugates are formed, which potentially allows for the transport through the BBB of many different biotinylated compounds. Previous studies have described the preparation of an OX26/avidin conjugate and the use of this conjugate to mediate an in vivo pharmacologic response in brain following the systemic delivery of relatively low doses (12 μg/kg) of peptide therapeutic, a vasoactive intestinal peptide (VIP) analogue (13).

The use of vector/avidin conjugates in humans may be limited by the immunogenicity of these compounds. However, the immunogenicity of the avidin moiety may be minimal owing to human tolerance to avidin induced by antigen feeding of egg whites (14–16). The immunogenicity of murine monoclonal antibodies may be minimized by "humanization" of the monoclonal antibody using recombinant DNA methods (17). While the immunogenicity in humans of antibody-based brain drug delivery vectors is at present only conjectural, it may be useful to consider the development of brain drug delivery vectors that are comprised of proteins that are not antibodies. Previous studies have shown that cationized bovine albumin (cBSA) may be used as a brain drug delivery vector (9). However, the administration of cBSA to rabbits induces an immunogenic response (18). This immunogenicity arises when heterologous proteins are administered. Different results are obtained when cationized proteins are administered in a homologous system. No significant immune response or measurable tissue toxicity is detected following chronic administration of cationized rat albumin (cRSA) to rats (19). These findings suggest that cationized human albumin (cHSA) may be used as a brain drug delivery vector in humans (2).

The present studies were designed to prepare a conjugate of cHSA and avidin (AV) and to measure the efficacy of cHSA as a brain drug delivery vector by determining the brain uptake of ^{3}H-biotin bound to the cHSA/AV conjugate. However, recent studies show that the systemic clearance of avidin-based vectors is very rapid (20). Owing to the cationic nature of AV, there is marked peripheral tissue uptake of avidin-based vectors in vivo. This results in a reduction in the area under the plasma concentration curve (AUC) and a proportionate reduction in brain delivery of the therapeutic. Conversely, the AUC of avidin-based vectors is greatly increased by the use of neutral forms of avidin (21). Neutral avidin (NLA), like its bacterial homologue, streptavidin (22,23), is cleared from the plasma compartment at a much slower rate as compared to AV (21). The present studies describe the preparation of cHSA/NLA or cHSA/AV conjugates, quantitate the number of biotin binding sites per conjugate, and demonstrate the higher plasma AUC and brain delivery of ^{3}H-biotin bound to the cHSA/NLA conjugate as compared to cHSA/AV.

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MATERIALS AND METHODS

Materials

^{3}H-biotin (60 Ci/mmol) was purchased from Dupont-NEN (Boston, MA). The 2-iminothiolane (Traut's reagent), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), and the bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce Chemical Corporation (Rockford, IL). Human serum albumin (HSA) (globulin-free fraction V, catalog #A8763), avidin, N-ethyl maleimide (NEM), and N-ethyl-N'-3-(dimethylaminopropyl) carbodiimide (EDAC) were purchased from Sigma Chemical Company (St. Louis, MO). Neutral avidin (24) was obtained from Accurate Chemical and Scientific Company (Westbury, NY). Sephacryl S300 HR and pI standards were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Hexamethylenediamine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) supplies were obtained from Bio-Rad Inc. (Richmond, CA). Male Sprague-Dawley rats (220–250 g) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN).

Cationized HSA preparation

2 M hexamethylenediamine (13.4 ml) was slowly added to 200 mg of HSA while stirring and the pH was adjusted to 7.8 (19). Following stirring for 30 minutes at room temperature, 200 mg of fresh EDAC was added, the pH was readjusted to 7.8 and the mixture was stirred at room temperature for 4 hours, followed by dialysis overnight at 4°C against 20 L of water followed by dialysis against another 20 L of water at 4°C for 8 additional hours, using a 12 kDa molecular weight cutoff dialysis tubing. An aliquot was removed for measurement of protein using the BCA assay and the cHSA was then evaporated to dryness and stored at −20°C. The isoelectric point (pI) of the native HSA (nHSA) or cHSA was determined with polyacrylamide gel isoelectric focusing (IEF) as described previously (19,21). The proteins were desalted with a G25 spin column in 0.05 M Na_2HPO_4, pH = 7.4 (PBW) containing 2% NP40. Approximately 8 μg of either nHSA or cHSA was applied per well in parallel with pI standard proteins. The pI of HSA was raised following cationization from approximately 5.3 to approximately 8.1 (Figure 1). The molecular size of the HSA following cationization was unchanged since the mobility through SDS-PAGE or either native or cationized HSA was equivalent (data not shown).

Preparation of HSA-avidin conjugates

Three different HSA-avidin conjugates were prepared: (a) cHSA/AV, (b) cHSA/NLA and (c) nHSA/AV. The avidin was thiolated by solubilizing 20 mg of AV or NLA in 2 ml of 0.16 M sodium borate (pH = 8.0) containing 0.1 mM EDTA; to this mixture was added 125 μL of Traut's reagent (4 mg/ml), which represented a molar excess of Traut's reagent over the avidin tetramer of 12 to 1 (12). Following stirring at room temperature for 60 minutes, the mixture was dialyzed at room temperature for 90 minutes against 2 L of 0.01 M TBS (0.01 M TRIS, 0.15 M NaCl, pH = 7.4) containing 0.1 mM EDTA using a 12 kDa molecular weight cutoff dialysis tubing. The cHSA or nHSA (20 mg) was dissolved in 2 ml of 0.05 M PBW and 36 μL of MBS (20 mg/ml, dissolved in dimethyl formamide) was added while vortexing, and the solution was stirred at room temperature for 60 minutes. The molar ratio of the MBS to HSA was 7.8 to 1. The MBS reaction was quenched by the addition of 100 μL of 5 mM NEM dissolved in water to block any free sulfhydryls on the cHSA. The solution was then dialyzed for 45 minutes at room temperature against 4 L of 0.05 M PBW followed by an additional 60 minute dialysis against 4 L fresh PBW using a 12 kDa molecular weight cutoff. Following conjugation of MBS to HSA, this protein and the thiolated avidin were then mixed for 90 minutes at room temperature; 0.5 μCi of ^{3}H-biotin was added and the mixture was then applied to a 1.6 × 94 cm column of Sephacryl S300 HR that had been pre-equilibrated in 0.01 M TBS containing 0.05% Tween-20. The column was eluted at room temperature at 40 ml/hr and 2.0 ml fractions were collected and measured for both the A_{280}

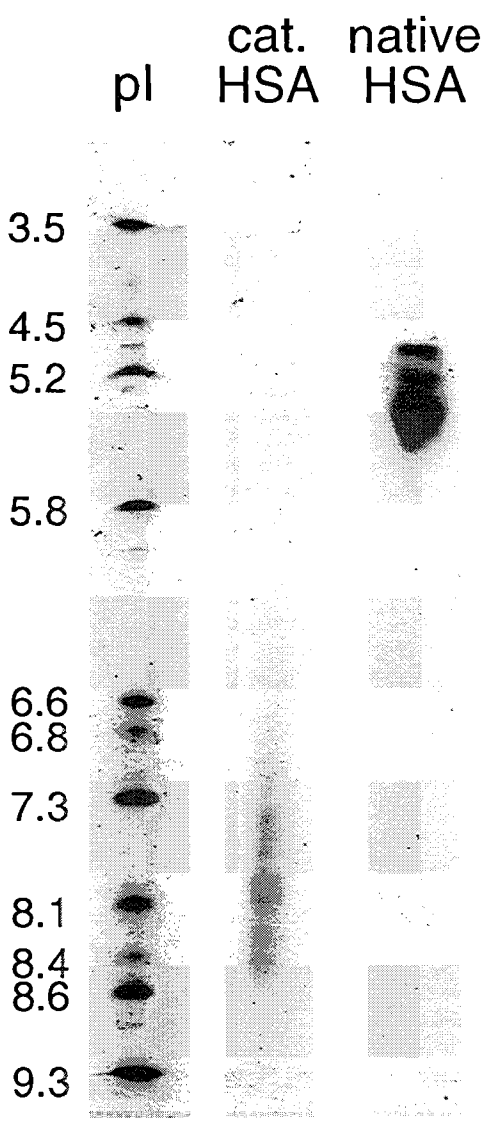

Figure 1. Isoelectric focusing of cationized (cat.) human serum albumin (HSA), native HSA, and isoelectric standards (pI).

and ^{3}H-radioactivity. The column was standardized by determining the elution of known molecular weight standards: blue dextran 2000 (molecular weight = 2 million), ferritin (molecular weight = 440 kDa), aldolase (molecular weight = 158 kDa), bovine serum albumin (BSA, molecular weight = 67 kDa), and chymotrypsinogen (molecular weight = 25 kDa). Four peaks of ^{3}H-biotin binding proteins were identified on the column: (a) fractions 38–43, which represented the void volume of the column and high molecular weight aggregates, (b) fractions 44–49 (peak 1), which contained the cHSA/AV conjugate with a stoichiometry greater than 1:1, (c) fractions 50–58 (peak 2), which contained the cHSA/AV conjugate in an approximate molar ratio of 1:1, and (d) fractions 59–66, which contained unconjugated avidin. The cHSA/AV conjugate eluting at fractions 44–49 and 50–58 were designated as peak 1 and peak 2, respectively.

The peak 2 cHSA/AV conjugate was analyzed further by re-chromatography on Sephacryl S300 HR and by elution through SDS-PAGE, and was utilized in subsequent pharmacokinetic studies (see below); 12 μg of peak 2 cHSA/AV conjugate was applied to a 1.5 mm SDS-PAGE gel and was eluted in parallel with SDS-PAGE high and low molecular weight standards (Bio-Rad) and the gel was stained with Coomassie Blue and photographed. A 500 μg aliquot was enriched with 2.5 μCi of ^{3}H-biotin and reapplied to the Sephacryl S300 HR column followed by elution with the same conditions as used to isolate the conjugate. The fractions were then measured for ^{3}H-radioactivity.

Biotin binding assay

The maximal capacity (B_{max}) of biotin binding to the peak 1 cHSA/NLA conjugate, to the peak 2 cHSA/AV conjugate, or to unconjugated NLA was measured with a centrifugal filtration assay exactly as described previously (12). In this assay, varying concentrations up to 1000 nM of unlabeled biotin are incubated with ^{3}H-biotin (2 nM), and the binding assay is initiated by the addition of either the cHSA/NLA conjugate or the unconjugated NLA. The number (n) of biotin binding sites per monomer was calculated from the B_{max}/conc. ratio, where conc. = the molar concentration of biotin binding protein, e.g., cHSA/AV, NLA, or cHSA/NLA.

Pharmacokinetics and brain delivery

The pharmacokinetics and brain delivery of ^{3}H-biotin bound to either cHSA/AV, cHSA/NLA, or nHSA/NLA were determined following single intravenous injection in rats anesthetized with ketamine (100 mg/kg) and xylazine (2 mg/kg) intraperitoneally. The femoral vein injection solution contained 0.2 ml of Ringer-Hepes buffer (pH = 7.4), 0.1% native rat serum albumin (nRSA), 10 μCi of ^{3}H-biotin (0.2 nmol), and 20 μg of HSA-avidin conjugate (0.15 nmol). Blood samples were collected from a femoral artery cannulated with heparinized PE50 tubing at 0.25, 1, 2, 5, 15, 30, 60, 90, 120, 360, and 1440 minutes after injection. The animals were anesthetized for up to 360 minutes; in the 24 hour measurements, the rats regained consciousness after one hour of anesthesia. After each blood sampling, the blood volume was replaced with the same volume of normal saline, and the plasma was separated by centrifugation. At 1, 2, 6, or 24 hours after intravenous injection, the animals were decapitated and the brains removed, weighed, and solubilized in 3 ml Soluene-350 (Packard Instrument Company, Downer's Grove, IL) for liquid scintillation counting of brain radioactivity.

Pharmacokinetic parameters were determined by fitting plasma radioactivity data to the following biexpoential equation,

$$A(t) = A_1e^{-K_1t} + A_2e^{-K_2t}$$

where A(t) = %ID/ml of plasma ^{3}H-radioactivity; ID = injected dose. The plasma data were fit to the biexponential equation using a derivative free nonlinear regression analysis (PARBMDP, Biomedical Computer P-Series developed at the UCLA Health Sciences Computing Facilities), and the data were weighted using weight = 1/(concentration)^2, where concentration = either DPM/μL plasma or %ID/ml. The area under the plasma concentration curve (AUC), the steady state volume of distribution (V_{ss}), the steady state plasma clearance (Cl_{ss}) and the mean residence time (MRT) were calculated from A_1, A_2, K_1, and K_2, as described by Gibaldi and Perrier (25). The brain volume of distribution (V_D) of ^{3}H-biotin bound to the conjugates was determined from the ratio of DPM/g brain divided by DPM/μl of the corresponding terminal plasma at 1, 2, 6, and 24 hours.

The BBB permeability-surface area (PS) product of the ^{3}H-biotin bound to the conjugate was calculated as follows:

$$PS = \frac{[V_D - V_O]\, C_p(T)}{\int_0^t C_p(t)\, dt}$$

where Cp(T) = the terminal plasma conventration, V_o = the brain volume of distribution of a plasma marker (10 μL/g) such as nRSA, which has been measured previously (20). The brain delivery of ^{3}H-biotin was determined as follows:

$$\frac{\%\ ID}{g}(t) = PS \times AUC(t)$$

where PS and AUC correspond to the respective time after injection.

The BBB transcytosis of ^{3}H-biotin bound to cHSA/NLA conjugate was assayed with the capillary depletion technique described previously (26). In these experiments, 30 μCi of ^{3}H-biotin and 20 μg of cHSA/NLA were administered per rat.

Chromatography of Plasma and Brain

The metabolic stability in plasma of the ^{3}H-biotin bound to the cHSA/NLA conjugate was examined by high performance liquid chromatography (HPLC) analysis of plasma radioactivity using a 7.8 × 300 mm TSK-GEL G2000SW$_{XL}$ gel filtration column (Tosohaas, Montgomeryville, PA). In these studies, the doses of ^{3}H-biotin and cHSA/NLA were increased to 25 μCi and 150 μg per rat, respectively. At 6 and 24 hours after injection, blood were removed from the anesthetized rats; 20 μL of serum from 2 rats was pooled, diluted to a total volume of 250 μL with 0.01 M Na_2HPO_4/0.15 M NaCl, pH = 7.4 (PBS), and injected on to the HPLC column followed by isocratic elution in PBS/0.05% Tween-20 at a

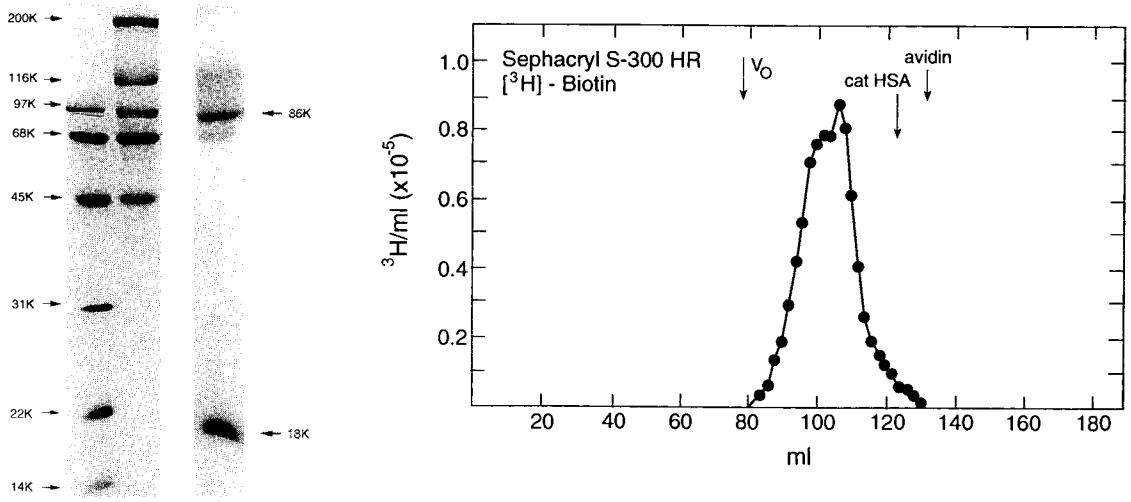

Figure 2. (Right) Sephacryl S300-HR gel filtration chromatography of ^{3}H-biotin bound to the cHSA/AV conjugate. The elution volumes of blue dextran 2000 (V_0), unconjugated cationized HSA (cat HSA), and unconjugated avidin are shown in the Figure. **(Left)** SDS-PAGE of low molecular weight markers (lane 1), high molecular weight markers (lane 2), and cHSA/AV. The calculated size of the heavy chain (86 kDa) and light chain (18 kDa) of the cHSA/AV conjugates are shown.

flow rate of 0.5 ml/min for 30 minutes. Column fractions (0.5 ml) were counted for ^{3}H radioactivity. The void and salt volumes of the column are 6 and 11 ml, respectively (21).

The metabolic stability in brain of the ^{3}H-biotin bound to the cHSA/NLA conjugate was also examined by HPLC analysis of brain homogenate obtained 6 hours after intravenous administration of 30 μCi of ^{3}H-biotin and 20 μg of cHSA/NLA per rat. After decapitation, the brain was homogenized in 3 volumes of PBS 0.05% Tween-20 in a Polytron homogenizer and centrifuged at 20,000 RPM for 30 minutes at 4°C. The supernatants from 3 rats were pooled and 250 μL (containing 1.8 mg brain protein) was injected on to the HPLC column followed by elution as described above.

RESULTS

The cHSA was conjugated to AV and was purified by Sephacryl S300-HR gel filtration column chromatography. The peak 2 conjugate was then reapplied to the Sephacryl S300 column, and eluted as a single peak as shown in Figure 2 (right-hand panel). This cHSA/AV conjugate represented a 1:1 conjugate based on the findings with SDS-PAGE. Following denaturation of the cHSA/AV conjugate in SDS sample buffer containing 5% β-mercaptoethanol, and boiling, the cHSA/AV conjugate eluted as two principal proteins: an 86 kDa heavy chain, and an 18 kDa light chain (Figure 2, left-hand panel). The 18 kDa light chain approximates the molecular weight of the avidin monomer (27), and the 86 kDa heavy chain corresponds to the sum of molecular weights of cHSA and an AV monomer, which are covalently jointed through a stable thiol ether linkage.

The number of biotin binding sites (n) per conjugate is shown in Table 1. The observed number of sites (n = 4) for NLA corresponds to the expected number for the avidin homotetramer (27). The number of biotin binding sites for the peak 1 cHSA/NLA conjugate exceeds the number for the peak 2 cHSA/AV conjugate (Table 1), consistent with the higher molar ratio of avidin to cHSA in peak 1 relative to peak 2 (Methods).

The pharmacokinetics of ^{3}H-biotin bound to the cHSA/AV conjugate was initially determined, and the plasma radioactivity for up to two hours is shown in Figure 3. These data show that the cHSA/AV conjugate was rapidly cleared from the plasma compartment, as 99% of the injected dose had been removed from plasma within 60 minutes following a single intravenous injection (Figure 3, left-hand panel). The pharmacokinetic parameters for ^{3}H-biotin bound to cHSA/AV are given in Tables 2–3, and the brain delivery data is shown in Figure 4. Owing to the relatively rapid plasma clearance of ^{3}H-biotin bound to cHSA/AV, the steady state clearance value was high at 6.0 ± 1.0 ml/min/kg. Accordingly, the plasma AUC of this conjugate was reduced (Table 3) and the brain delivery of the conjugate was reduced in parallel (Figure 4). Only 0.02 %ID/g brain delivery of ^{3}H-biotin bound to cHSA/AV was achieved with this vector (Figure 4).

The brain delivery of cationic AV-based vectors is re-

TABLE 1. [^{3}H]-BIOTIN BINDING B_{max} AND NUMBER (n) OF BIOTIN BINDING SITES PER CONJUGATE.

protein (concentration)	B_{max} (nM)	n
cHSA/AV (11.0 μg/ml), peak 2	234 ± 4	2.8
NLA (4.3 μg/ml)	261 ± 16	4.0
cHSA/NLA (6.5 μg/ml), peak 1	334 ± 9	6.8

The molecular weights of the cHSA/AV or cHSA/NLA used to calculate n is 132,000, and the molecular weight of neutral avidin (NLA) is 64,000 Daltons.

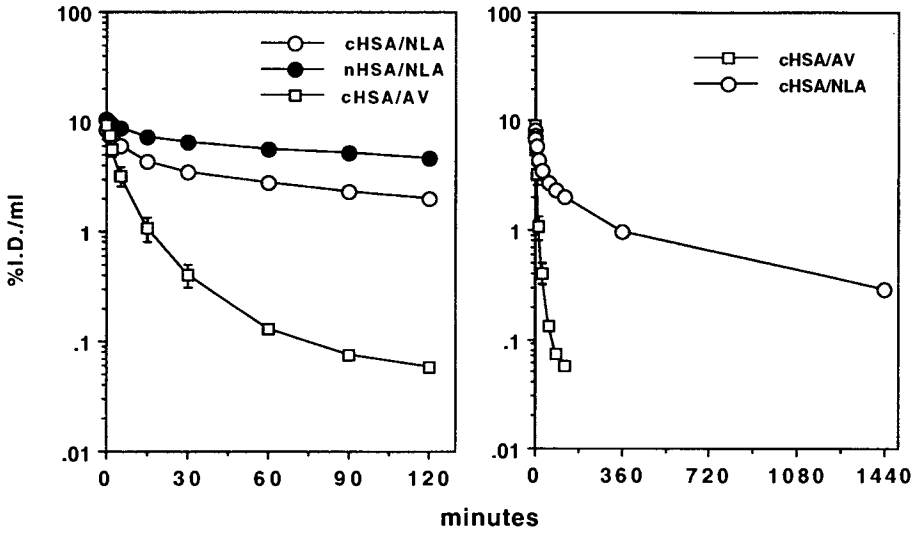

Figure 3. The clearance from plasma of ^{3}H-biotin bound to cHSA/NLA, nHSA/NLA, or cHSA/AV is shown for up to 2 hours **(left-hand panel)** or 24 hours **(right-hand panel)**.

duced in parallel to the reduction in plasma AUC (20). Therefore, cHSA conjugates were then prepared with NLA (21), and the pharmacokinetics and brain delivery was determined. As shown in Figure 3, the rate of plasma clearance of ^{3}H-biotin bound to cHSA/;NLA is markedly reduced relative to the rate of plasma clearance of ^{3}H-biotin bound cHSA/AV. The steady state clearance of ^{3}H-biotin bound to cHSA/NLA was reduced 10-fold to 0.60 ± 0.03 ml/min/kg, and this steady state clearance value is approximately 3-fold greater than that for ^{3}H-biotin bound to nHSA/NLA (Table 2). The V_{ss} of the ^{3}H-biotin bound nHSA/NLA, 58 ± 3 ml/kg (Table 2), is not significantly different from the steady state volume of distribution of nRSA, which is confined to the plasma volume (33). Extension of the pharmacokinetic analysis to 24 hours allows for a computation of the steady state volume of distribution of the ^{3}H-biotin bound to cHSA/NLA conjugate, and this volume, 181 ± 16 ml/kg (Table 2), was 3-fold greater than the plasma volume; the MRT of this conjugate was 11.6 ± 0.3 hours (Table 2).

The BBB PS product of ^{3}H-biotin bound to cHSA/NLA was not significantly different from the BBB PS product of ^{3}H-biotin bound to cHSA/AV (Table 3). In parallel to the increase in plasma AUC of the cHSA/NLA conjugate, the brain delivery of ^{3}H-biotin bound to cHSA/NLA was increased relative to the brain delivery of ^{3}H-biotin bound to cHSA/AV (Figure 4). The maximal brain delivery was reached at 6 hours following injection and approximated the previously reported brain delivery of a ^{3}H-biotin bound to the OX26/NLA conjugate (21).

The transcytosis through the BBB of ^{3}H-biotin bound to the cHSA/NLA conjugate was examined with capillary depletion analysis of brain homogenate obtained 6 hours after injection (26); 88 ± 5% (mean ± S.E., n = 3 rats) of the total brain homogenate radioactivity was recovered in the postvascular supernatant.

The metabolic stability of the ^{3}H-biotin bound to cHSA/NLA conjugate was examined by HPLC gel filtration analysis of plasma obtained 6 and 24 hours after injection (Figure

Table 2. PHARMACOKINETIC PARAMETERS

	2 hrs.		24 hrs.
Parameter	nHSA/NLA	cHSA/AV	cHSA/NLA
K1 (min^{-1})	0.12 ± 0.02	0.16 ± 0.02	0.028 ± 0.006
K2 (min^{-1})	0.0033 ± 0.0002	0.017 ± 0.002	0.0013 ± 0.0001
distribution t1/2 (min)	6.1 ± 0.9	4.5 ± 0.7	27 ± 5
elimination t1/2 (min)	214 ± 13	42 ± 5	550 ± 26
A1 (%ID/ml)	3.2 ± 0.6	7.5 ± 0.5	5.0 ± 0.3
A2 (%ID/ml)	6.9 ± 0.6	0.40 ± 0.08	1.8 ± 0.3
Vss (ml/kg)	58 ± 3	151 ± 44	181 ± 16
CLss (ml/min/kg)	0.19 ± 0.01	6.0 ± 1.0	0.26 ± 0.02
MRT (min)	305 ± 19	24 ± 3	699 ± 19

Abbreviations: cHSA/NLA, neutral avidin-cationized HSA; nHSA/NLA, neutral avidin-native HSA; cHSA/AV, avidin-cationized HSA. ^{3}H-biotin was injected as a complex with 1 of the 3 conjugates with a single intravenous injection in rats and plasma was collected for up to 24 hours after injection. The pharmacokinetic parameters for 2 or 24 hours were computed from the plasma ^{3}H-radioactivity data shown in Figure 3.

Table 3. PLASMA AUC, BBB PERMEABILITY (PS), AND BRAIN VOLUME OF DISTRIBUTION (V_D)

parameter	Time (hrs.) 1	2	6	24
cHSA/NLA				
AUC (%ID · min/ml)	300 ± 14	376 ± 24	688 ± 56	1346 ± 118
PS (μL/min · g)	0.20 ± 0.04	0.24 ± 0.01	0.23 ± 0.04	0.13 ± 0.02
V_D (μL/g)	27 ± 3	54 ± 1	179 ± 27	598 ± 115
cHSA/AV				
AUC (%ID · min/ml)	64 ± 7	69 ± 12	—	—
PS (μL/min · g)	0.26 ± 0.13	0.19 ± 0.03	—	—
V_D (μL/g)	126 ± 51	226 ± 16	—	—
nHSA/NLA				
AUC (%ID · min/ml)	—	714 ± 55	—	—
PS (μL/min · g)	—	<0.04	—	—
V_D (μL/g)	—	15 ± 1	—	—

Abbreviations defined in Table 2. The PS product was computed from the AUC and V_D (see Methods) for each of the time points. These AUC and PS values were used to compute the %ID/g shown in Figure 4.

5). At 6 hours after injection, more than 90% of the serum ^{3}H-radioactivity co-migrated on the column with the cHSA/NLA conjugate, whereas <10% migrated in the low molecular weight salt volume, representing ^{3}H-biotin metabolites. At 24 hours after injection, approximately 75% of the plasma ^{3}H-radioactivity co-migrated with the cHSA/NLA conjugate (Figure 5B). The metabolic stability of ^{3}H-biotin bound to the cHSA/NLA conjugate in brain at 6 hours after injection was also assessed by HPLC analysis of brain homogenate. Approximately 60% of the ^{3}H-radioactivity co-migrated with the cHSA/NLA conjugate and the remainder eluted at the salt volume of the column (Figure 5C).

DISCUSSION

The present studies describe the preparation of cHSA/NLA and cHSA/AV conjugates and demonstrate the higher plasma AUC and brain delivery achieved with the NLA-based conjugate. Cationized HSA is a model brain drug delivery vector that crosses the BBB via absorptive-mediated transcytosis, and the OX26 monoclonal antibody is a model brain drug transport vector which traverses the BBB via receptor-mediated transcytosis (2). The OX26 monoclonal antibody is transported through the BBB by virtue of its binding to an extracellular projecting epitope on the transferrin receptor (28), which participates in the receptor-mediated transcytosis of transferrin through the BBB (29). The cationized albumin binds to negatively charged residues at the BBB, which on the lumenal side of the brain capillary endothelium are predominantly sialic acid residues (30). The electrostatic interaction between these negative charges and the positively-charged moieties on the cationized albumin trigger absorptive-mediated endocytosis, which is succeeded by exocytosis of the cationized albumin into the brain interstitial fluid (26). Previous studies have shown that either AV- or NLA-based OX26 conjugates are transcytosed through the BBB with PS values that approximate those for the unconjugated vector (12,21). Therefore, differences in brain delivery of avidin-based conjugates are caused by altered plasma AUC.

The OX26/NLA conjugate is transported through the rat BBB approximately 4-fold faster than is the cHSA/NLA conjugate. For example, the BBB PS product of OX26/NLA is 0.88 ± 0.11 μL/min/g at 2 hours following intravenous injection (21), in comparison to the corresponding BBB PS value of 0.24 ± 0.01 μL/min/g for cHSA/NLA (Table 3). Despite the 3- to 4-fold difference in BBB permeability between the OX26/NLA and cHSA/NLA, the brain delivery of ^{3}H-biotin at 6 hours following injection is nearly comparable for the two vectors. The near equivalence of the two vectors in terms of brain delivery at 6 hours after injection is due to the efflux from brain of ^{3}H-biotin or metabolites between 2 and

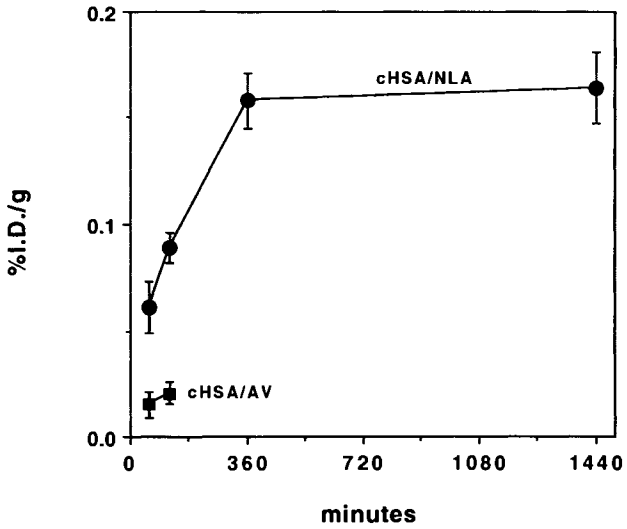

Figure 4. The % injected dose (ID) per gram brain of ^{3}H-biotin bound to either cHSA/NLA or cHSA/AV for up to 24 hours after administration is shown. Data are mean ± S.E. (n = 3 rats per point).

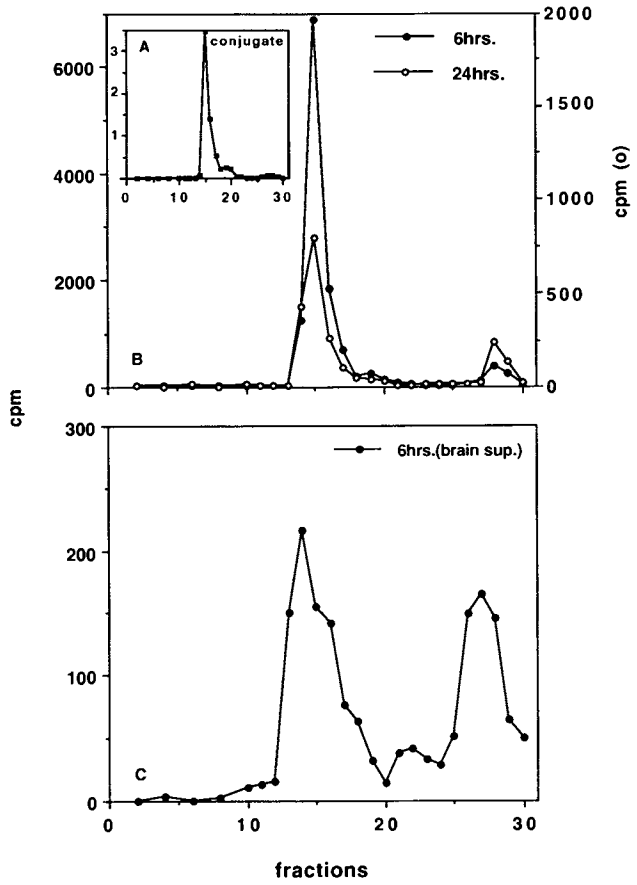

Figure 5. (A) Elution through the HPLC gel filtration column of ^{3}H-biotin bound to the purified cHSA/NLA conjugate before injection into rats; CPM $\times 10^{-5}$, (B) Elution of plasma obtained 6 and 24 hours after a single intravenous injection of ^{3}H-biotin bound to the cHSA/NLA conjugate, (C) Elution of brain obtained 6 hours after a single intravenous injection of ^{3}H-biotin bound to the cHSA/NLA conjugate.

6 hours after injection of the OX26/NLA conjugate (21), whereas there is no efflux of ^{3}H-biotin or metabolites bound to cHSA/NLA during this time period. For example, the PS product for ^{3}H-biotin bound to cHSA/NLA at 1, 2, and 6 hours is not significantly different, although there is approximate 50% reduction in the PS product at 24 hours after administration (Table 3). The decrease in calculated PS product at 24 hours after injection represents efflux from brain of ^{3}H-biotin or metabolites. The HPLC analysis of brain and plasma obtained 6 hours after administration demonstrates a greater amount of free ^{3}H-biotin or metabolites in brain relative to plasma (Figure 5). These data provide evidence that brain is able to degrade the cHSA/NLA conjugate subsequent to its transport across the BBB.

The present studies with cHSA corroborate previous investigations with the OX26 monoclonal antibody, showing the higher plasma AUC and brain delivery achieved with NLA as compared to cationic AV (21). The pI of NLA is approximately 5–6 (21), as opposed to the pI of AV, which is greater than 9.5 (31). The cationic nature of AV results in a marked increase in systemic clearance of the protein by organs such as liver and kidney (22). This rapid systemic clearance causes reduced plasma AUC and a parallel reduction in brain delivery of avidin-based vectors (Figure 4). Conversely, NLA is removed from the plasma compartment slowly (21), and NLA-based vectors have higher plasma AUC and enhanced brain delivery (Figure 4). Neutral avidin has a decreased pI (21), yet the biotin binding of NLA is unimpaired; the expected four biotin binding sites are present on NLA (Table 1). The use of NLA demonstrates that the biologic activity of a protein, e.g., biotin binding, may be retained following a change in the charge of the protein. However, the conversion of a cationic AV to the neutral NLA results in a marked increase in the plasma AUC and brain delivery of ^{3}H-biotin bound to cHSA/NLA (Figure 4).

Finally, the measurements of brain delivery of ^{3}H-biotin bound to cHSA/NLA (Figure 4) or OX26/NLA (21) shows that 0.15–0.25% ID/g is delivered to brain with existing conjugates of NLA and BBB drug delivery vectors. This level of brain delivery may be compared to quantitative measurements of brain delivery of sucrose (32), which has a molecular weight >500-fold lower than the cHSA/NLA or OX26/NLA vectors. The BBB PS product for sucrose, 0.39 ± 0.05 μL/min/g (32), is about 2-fold greater than the PS product for cHSA/NLA (Table 3) and is more than 2-fold lower than the PS product for OX26/NLA (21). However, the plasma AUC for sucrose, 24 ± 1%ID · min/ml, is more than 10-fold lower than the plasma AUC for cHSA/NLA (Table 3) or OX26/NLA (21). Therefore, the brain delivery (%ID/g) of ^{3}H-biotin bound to either cHSA/NLA (Figure 4) or OX26/NLA (21) is 15- to 30-fold greater than the brain delivery of sucrose, or a metabolically stable opioid peptide (32). These considerations underscore the general principle that brain drug delivery is a dual function of the BBB PS product and the plasma AUC. The conjugate of biotinylated peptide therapeutics to a brain drug delivery system takes advantage of the dual properties of the vector to both increase the BBB PS product and the plasma AUC of the peptide therapeutic (33).

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