Differences in the Intracellular Processing of the Radiolabel Following the Uptake of Iodine-125- and Technetium-99m-Neogalactosyl Albumin by the Isolated Perfused Rat Liver

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Neogalactosyl albumin (NGA) is a synthetic ligand to the asialoglycoprotein receptor (hepatic binding protein), which has been proposed as a useful receptor binding radiopharmaceutical for the noninvasive assessment of liver function. We have compared the uptake and intracellular processing of iodine-125- (^{125}I) and technetium-99m- (^{99m}Tc) NGA following its administration as a 1-min pulse (147 pmol) to the isolated perfused rat liver. Approximately 40% of a pulse of either ^{125}I- or ^{99m}Tc-NGA were taken up first pass by the liver. Of the ^{125}I taken up by the liver, 82% was released after 15–20 min at the sinusoidal pole of the hepatocyte, predominantly as small molecular weight metabolites. A further 8% of the ^{125}I-associated radioactivity was secreted as intact NGA into bile by the non-lysosomal (direct) pathway while 6% remained in the liver 1 hr after the pulse. In contrast, of the ^{99m}Tc taken up by the liver, only 4% reappeared in the perfusate while 40% was secreted into bile by the lysosomal (indirect) pathway and 55% remained in the liver 1 hr after the pulse. Since labeled metabolites of ^{99m}Tc-NGA do not appear in plasma, this permits kinetic modeling with ^{99m}Tc-NGA without correction for labeled metabolites. Thus, ^{99m}Tc-NGA is an excellent candidate as a receptor-binding radiopharmaceutical.

J Nucl Med 1991; 32:506–512

Among the more interesting recent developments in nuclear medicine are the receptor-binding radiopharmaceuticals. These are radionuclide-labeled ligand molecules which become bound by receptor molecules contained in target tissues. One example of a potential receptor-binding radiopharmaceutical is neogalactosyl albumin (NGA). This is a synthetic ligand to the asialoglycoprotein receptor (also known as hepatic binding protein) in which sugar molecules are covalently linked to human serum albumin via primary amino groups (*1,2*). The asialoglycoprotein receptor resides exclusively in the sinusoidal membrane of hepatocytes (*3*). The receptor recognizes and binds glycoproteins with galactose or N-acetyl-galactosamine residues exposed due to removal of terminal sialic acid residues. In vitro studies using purified rat (*4*), rabbit (*5*), and human (*6*) liver plasma membranes preparations have shown that receptor binding of NGA correlates well with the binding characteristics of other ligands to the asialoglycoprotein receptor.

NGA, labeled with technetium-99m (^{99m}Tc), has been proposed as a useful agent for assessing liver function (*7,8*). Indeed, ^{99m}Tc-NGA imaging has been shown to provide a valuable means of evaluating ischaemia and rejection following liver transplantation (*9*). In addition, estimation of hepatic asialoglycoprotein receptor concentration by ^{99m}Tc-NGA functional imaging correlates well with Child-Turcotte scores in patients with alcoholic cirrhosis, hepatoma and acute viral hepatitis (*8*).

However, most experimental studies on the hepatic handling of asialoglycoproteins have used ^{125}I-labeled ligands. The most commonly used ligands have been orosomucoid (α_1-acid glycoprotein) or fetuin that first have to be chemically desialated before they will serve as ligands to the asialoglycoprotein receptor. After binding, these ligands are internalized into the endosome compartment by a process known as receptor mediated endocytosis (*10*). The majority of the ligand is delivered to the lysosomes for degradation by proteases and glycosidases (*11*) while some is returned intact to the sinusoidal membrane by a process known as diacytosis (*12*) and some is secreted into bile as a result of missorting in the endosome compartment (*13*). We have recently shown that ^{125}I-NGA is internalized and proc-

Received May 15, 1990; revision accepted Aug. 21, 1990.
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essed by the isolated perfused rat liver in an essentially similar manner (*14,15*).

Technetium-99m is the preferred isotope for use in diagnostic nuclear medicine, principally because of its short half-life and ease of imaging. However, the hepatic handling of ^{99m}Tc-NGA has not been previously investigated and in this study we have compared the uptake and intracellular processing of ^{99m}Tc- and ^{125}I-labeled NGA. Pulse-chase experiments in the isolated perfused rat liver were used since this allows the protein to be presented to the liver as a short pulse under single-pass conditions.

MATERIALS AND METHODS

Materials

NGA, synthesized by the reductive amination of human serum albumin with lactose (*16*), and a ^{99}Mo/^{99m}Tc generator were supplied by Medgenix Diagnostics, Fleurus, Belgium. Analysis for galactose (*17*) and for protein (*18*) showed it to contain 28 mol of sugar per mol of protein and its molecular weight was therefore assumed to be 72,000. Nembutal (60 mg/ml pentobarbitone) was from May and Baker, Dagenham, Essex, U.K., PP10 cannulation tubing was made by Portex Ltd., Hythe, Kent, U.K., and heparin (5,000 U/ml) was from C.P. Pharmaceuticals, Wrexham, U.K. All other fine chemicals were from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Human erythrocytes were obtained through the courtesy of the Merseyside Blood Transfusion Service and were washed free of plasma in Krebs-Ringer bicarbonate buffer, pH 7.4 containing 1 m*M* $CaCl_2$ and 5m*M* glucose.

Radiolabeling of NGA

Iodination. Iodine-125 iodide (185 MBq, 50 μl) and 40 μl of a 50 mg/ml NGA solution were diluted into 0.5 ml of 0.2 *M* borate buffer, pH 8.5. The mixture was transferred to an Iodogen coated vial and after 10 min, 10 μl of freshly prepared chloramine T (1 mg/ml) was added. After 2 min, the reaction was stopped by addition of 10 μl of sodium metabisulphite (2 mg/ml). Iodine-125-NGA was purified by gel exclusion chromatography on Biorad 10DG Econo-pac columns eluted with saline and sterilized by filtration through a 0.22-μm Millex-GV filter. The specific activity of the final preparation was 10 MBq/mg of protein.

Technectium-99m Labeling. Reaction vials contained 15 mg NGA, 0.1 mg of $SnCl_2 \cdot 2H_2O$), 12 mg of NaCl adjusted to pH 2.7 with HCl before freeze drying. Vials were reconstituted with 400 MBq of $^{99m}TcO_4^-$ in a volume of 10 ml of saline. This was further diluted with saline to a specific activity of 0.3 MBq per 10.6 μg of NGA. Radiochemical purity was assessed by ascending chromatography on Whatman No. 1 paper using methanol:water (85:15) as solvent. The labeling yield was routinely greater than 99%. In addition, the possible formation of labeled tin colloid in Tc-NGA kits was assessed by Sephadex G200 chromatography; <1% of colloidal material (eluting at the void volume) was detected.

Isolated Perfused Rat Livers

Male Wistar rats weighing 250–300 g bred within the Liverpool Polytechnic Animals Unit were used throughout. These had been maintained on a standard laboratory diet in a constant temperature environment (22°C) and under a constant 12-hr light-dark cycle.

Livers were perfused in situ essentially as described by Gores et al. (*19*). Briefly, the common bile duct was cannulated while the rats were under Nembutal anaesthesia; bile was collected on ice throughout the experiment into preweighed tubes. Bile flow was determined gravimetrically. Approximately 10 min after cannulation of the common bile duct, heparin (2,500 U in 0.5 ml) was administered via the inferior vena cava and after a further 1 min a 17.5-gauge Wallace cannula was inserted into the hepatic protal vein. To minimize anoxia, perfusion was started immediately. The thoracic vena cava was then cannulated with a 16-gauge cannula and recycling perfusion established.

The perfusion medium was Krebs-Ringer bicarbonate buffer, pH 7.4 containing 1 m*M* $CaCl_2$, 5 m*M* glucose, 1% (w/v) bovine serum albumin, a physiologic amino acid mixture (*20*) and 20% (v/v) washed human erythrocytes. Flow rate was 17 ml/min (~1.2–1.6 ml/min/g of liver). The perfusion medium was gassed with O_2:CO_2 (19:1) and maintained at 37°C. All perfusions were carried out in a thermostatically controlled cabinet. The viability of the liver was assessed macroscopically by a marked color change in the afferent and efferent perfusate, its homogeneity and a bile flow of at least 0.8 μl/min/g of liver. Livers were allowed to approach steady-state for 30 min with recycling perfusion, after which they were converted to a one-pass perfusion using fresh medium and maintained in this mode until the end of the experiment. Radiolabeled (^{125}I or ^{99m}Tc) NGA was infused from a syringe pump via a side arm port into the perfusion line just prior to the portal cannula and was administered as a 1-min pulse (147 pmol, 10.6 μg) dissolved in erythrocyte-free perfusion medium. Hepatic venous outflow was collected in 0.6-min aliquots for a total of 60 min after the pulse of radiolabeled NGA. Bile was collected in 2-min samples for 20 min and then 5-min samples for the rest of the experiment. At the end of the experiment, the liver was removed, weighed, and cut up into several pieces for determination of radioactivity.

Analysis

Radioactivity in 1-ml samples of efferent perfusate, bile samples, and the liver was determined in a LKB 1275 Minigamma counter. Technetium-99m counts were time-corrected to allow for its short half-life (6 hr). Intact ^{125}I-NGA was determined as acid precipitable radioactivity after addition of 1 ml of 15% (w/v) trichloroacetic acid to 1 ml of efferent perfusate or bile samples. Using this technique, the ^{125}I-NGA preparation was >95% acid precipitable. However, acid precipitation could not be used on ^{99m}Tc-NGA, since this treatment released large amounts (30%–60%) of the ^{99m}Tc from the denatured protein (Gore and Billington, unpublished observations).

RESULTS

Figure 1a shows a typical time course of ^{125}I-associated radioactivity in the venous effluent following a 1-min pulse of ^{125}I-NGA to the isolated perfused rat liver operating under single-pass conditions. A peak of radioactivity occurred over the first 3 min; this was >90% trichloroacetic acid precipitable showing it to be the

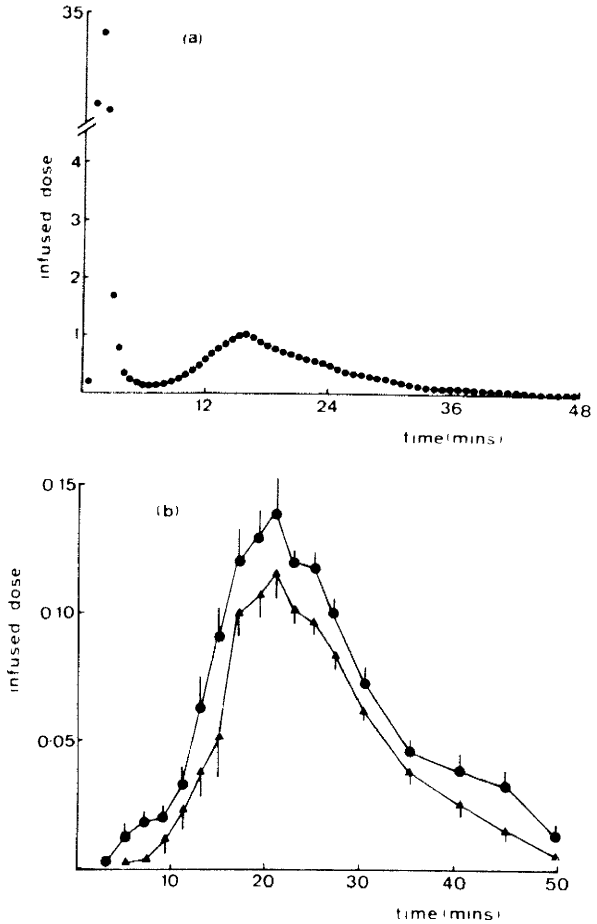

FIGURE 1
Fate of the radioactivity following a pulse of ^{125}I-NGA to the isolated perfused rat liver operating under single-pass conditions. Iodine-125-NGA (147 pmole, 10.6 μg, 5×10^6 cpm) was infused into the perfusion line via a side arm port just prior to the portal cannula at t = 0–1 min. (a) A typical experiment showing the time course of appearance of ^{125}I associated radioactivity in the venous effluent. (b) Biliary output of ^{125}I associated radioactivity; values are means ± s.e.m. of five observations where (●) are total counts and (▲) are acid precipitable counts.

intact, native protein and therefore ^{125}I-NGA not taken up by the liver. Subtracting the counts recovered in this peak from that infused in the original pulse showed that approximately 37% of the pulse was taken up and this was equivalent to an initial rate of uptake of 4.9 pmole/min/g of liver (Table 1). Following a similar pulse of ^{99m}Tc-NGA to the isolated perfused rat liver (Fig. 2a), 44% of the pulse was taken up and this was equivalent to an initial rate of uptake of 5.0 pmole/min/g of liver (Table 1). When assessed by Student's t-test, these values are not significantly different from those for ^{125}I-NGA.

A second peak of ^{125}I associated radioactivity was seen in the venous effluent at 15 min after the pulse of ^{125}I-NGA (Fig. 1a). This radioactivity was only 20%–40% trichloroacetic acid precipitable and represented

TABLE 1
Uptake of NGA Following a 1-min Pulse of Either ^{125}I- or ^{99m}Tc-NGA (147 pmole) to the Isolated Perfused Rat Liver Operating Under Single-Pass Conditions

Radiolabel	Percent taken up	Rate of uptake (pmole/min/g of liver)
^{125}I	36.9 ± 5.9	4.91 ± 0.29
^{99m}Tc	44.4 ± 14.4	5.04 ± 0.73

Values are means ± s.d. of five observations.

82% of that taken up by the liver (Table 2). A further 7.7% of the ^{125}I associated radioactivity taken up by the liver appeared in bile (Table 2). Peak biliary secretion occurred after 20 min and this biliary radioactivity was

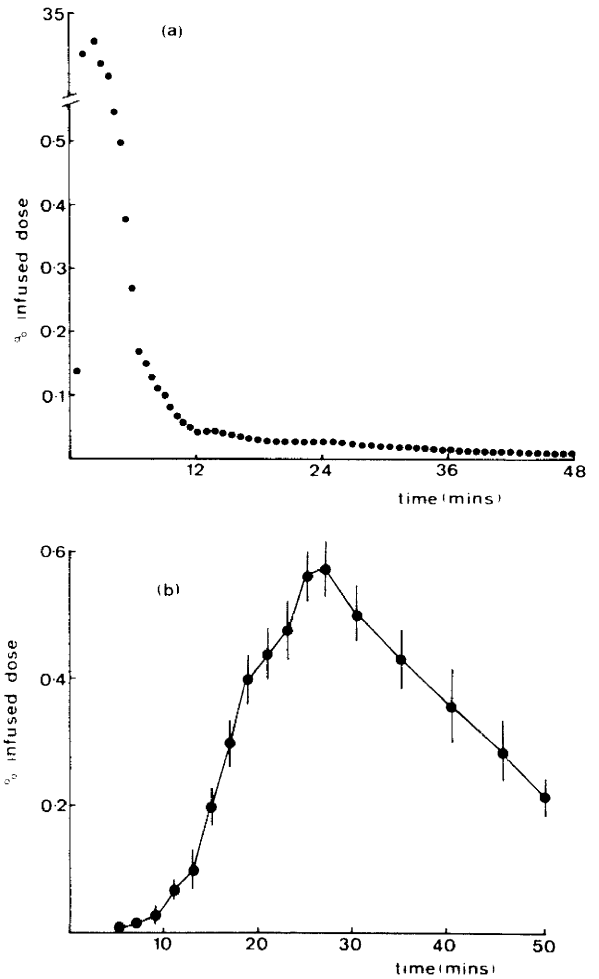

FIGURE 2
Fate of the radioactivity following a pulse of ^{99m}Tc-NGA to the isolated perfused rat liver operating under single-pass conditions. Technetium-99m-NGA (147 pmole, 10.6 μg, 2×10^7 cpm) was infused into the perfusion line via a side arm port just prior to the portal cannula at t = 0–1 min. (a) A typical experiment showing the time course of appearance of ^{99m}Tc associated radioactivity in the venous effluent. (b) Total biliary output of ^{99m}Tc associated radioactivity; values are means ± s.e.m. of five observations.

TABLE 2
Fate of Radioactivity Taken up by the Isolated Perfused Rat Liver After a 1-min Pulse of Either ^{125}I- or ^{99m}Tc-NGA (147 pmole)

	Percent recovery of	
	^{125}I	^{99m}Tc
Venous outflow	82.1 ± 5.9	4.4 ± 0.8*
Bile	7.7 ± 1.6	40.4 ± 12.9*
Remaining in liver 1 hr after pulse	6.4 ± 1.8	55.2 ± 22.8*
Total recovery	96.2 ± 1.2	100.0 ± 0.4

Values are means ± s.d. of five observations. Statistical differences were assessed by Student's t-test and are indicated by * ($p < 0.05$).

>90% trichloroacetic acid precipitable suggesting it to be the intact, native protein (Fig. 1b). Approximately 6.4% of the radioactivity taken up remained in the liver 1 hr after the ^{125}I-NGA pulse giving a total recovery of ^{125}I taken up by the liver of 96% (Table 2).

Following a pulse of ^{99m}Tc-NGA to the isolated perfused rat liver, a second peak of ^{99m}Tc associated radioactivity was only just evident in the venous effluent at 15–20 min (Fig. 2a). This represented only 4.4% of the radioactivity taken up by the liver (Table 1) and was much smaller than that seen following a pulse of ^{125}I-NGA. Biliary output of ^{99m}Tc associated radioactivity was slower than that of ^{125}I and did not peak until approximately 25–30 min (Fig. 2b). In addition, biliary output of ^{99m}Tc associated radioactivity was substantial and accounted for 40% of the radioactivity taken up (Table 2). Approximately 55% of the radioactivity taken up remained in the liver 1 hr after the pulse of ^{99m}Tc-NGA giving a total recovery of 100% (Table 2).

DISCUSSION

The NGA used in this study was synthesized by the reductive amination of human serum albumin with lactose and thus the covalently attached sugar group is a disaccharide with a terminal galactose. NGA used by other workers (*4–9*) was synthesized by reductive amination using 2-imino-2-methoxyethyl-1-thio-β-D-galactopyranoside and results in single galactose residues covalently linked to the protein backbone. While it is possible that these two different forms of NGA have different liver metabolism, this possibility is extremely remote.

This study has shown that NGA, whether labeled with ^{125}I or ^{99m}Tc, is avidly taken up by the isolated perfused rat liver and is therefore an excellent ligand for the asialoglycoprotein receptor. Approximately 37% of a 147-pmole pulse of ^{125}I-NGA and 44% of a similar pulse of ^{99m}Tc-NGA were taken up first pass by the liver; this corresponds to a rate of uptake of approximately 5 pmole/min/g of liver (Table 1). These values compare favorably with those reported by other workers for endogenous asialoglycoproteins; for example Perez et al. (*21*) have reported that 34% of asialofetuin is extracted first pass by the isolated perfused rat liver. In addition, these results show that radiolabeling NGA with either ^{125}I or ^{99m}Tc does not affect its affinity for the asialoglycoprotein receptor and its subsequent uptake into hepatocytes.

Although both ^{125}I- and ^{99m}Tc-NGA were taken up by the isolated perfused rat liver to the same extent, the intracellular processing of the two radiolabels was markedly different. In the case of ^{125}I-NGA, the majority of radioactivity (82%) reappeared in the perfusate with smaller amounts secreted into bile (8%) or remaining in the liver 1 hr after the pulse (6%). In contrast, following ^{99m}Tc-NGA pulsing, only small amounts of radioactivity (4%) reappeared in the perfusate while much larger amounts were secreted into bile (40%) or remained in the liver (55%) (Table 2).

The differences in the fate of the radiolabel can be explained by the different chemistries for introducing the radiolabel into NGA. Iodination of proteins by the chloramine-T method results in ^{125}I atoms becoming covalently substituted into one or both ortho positions of the phenolic ring of tyrosine residues in the protein (*22*). In contrast, ^{99m}Tc-labeling of proteins is nonspecific and the TcO_4^- ion is held within the three-dimensional structure of the protein either by electrostatic bonding to positively charged amino acids or by forming complexes with basic amino acids; so called "low affinity bonding" (*23*). Further bonding occurs with free thiol groups of cysteine residues; so called "high affinity bonding" (*24*). Indeed, following acid precipitation of iodinated NGA, ^{125}I remains attached to the denatured protein while acid precipitation of ^{99m}Tc-NGA releases much of the ^{99m}Tc, presumably due to disruption of electrostatic bonds and complex formation.

Once internalized into the hepatic endosome compartment, the majority of asialoglycoproteins are delivered to the lysosomes for proteolytic degradation (*11*). Thus, following proteolysis of NGA, it would be expected that ^{125}I would remain covalently bound to tyrosine while much of the ^{99m}Tc would be released as the TcO_4^- ion. The majority (60%–80%) of the ^{125}I associated radioactivity released back into the perfusate after pulsing with ^{125}I-NGA was acid soluble, suggesting it to be iodotyrosine or small peptides. Several transport systems for amino acids are known to exist in the sinusoidal membrane (*25*) and it is possible that ^{125}I-iodotyrosine exits the hepatocyte in this manner accounting for the large amount of ^{125}I associated radioactivity reappearing in the perfusate. This conclusion is supported by Galli et al. (*26*) who report that following

administration of ^{131}I-asialoorosomucoid to rabbits, blood radioactivity declines to approximately 1% of the dose 10 min after injection with a subsequent increase to a plateau of 10% of the dose 40 min after injection. The remaining 20%–40% of ^{125}I associated radioactivity released into the perfusate was acid precipitable, suggesting it to represent intact NGA which has escaped lysosomal degradation and returned to the sinusoidal membrane by diacytosis. Similarly, up to 40% of internalised asialoorosomucoid is returned to the cell surface of cultured hepatocytes (*12*). In contrast, TcO_4^- would not be expected to either diffuse or be transported by a specific process across the sinusoidal membrane, accounting for only small amounts of ^{99m}Tc reappearing in the perfusate.

Close examination of the time course of the appearance of radioactivity in bile provides an explanation for the large differences in the biliary output of ^{125}I and ^{99m}Tc. Peak biliary output of ^{125}I-associated radioactivity occurred 20 min after a pulse of ^{125}I-NGA (Fig. 1b) and accounted for 8% of the radioactivity taken up while peak biliary output of ^{99m}Tc associated radioactivity occurred after 25–30 min (Fig. 2b) and accounted for 40% of the radioactivity taken up. In a recent review, Coleman (*27*) points out that internalized protein can reach bile by one of two routes. The non-lysosomal (direct) route takes about 20 min and delivers intact proteins to the canaliculus while the lysosomal (indirect) route takes approximately 30 min and invariably delivers fragments of the original molecules (*27*). The ^{125}I associated radioactivity secreted into bile following a pulse of ^{125}I-NGA was >90% acid precipitable showing it to be present as the intact protein and its time course of appearance suggests it arises via the direct, non-lysosomal pathway. Similar amounts of ^{125}I associated radioactivity are secreted into bile following the administration of either ^{125}I-asialoorosomucoid or ^{125}I-asialofetuin to whole animals or isolated perfused rat livers (*15,21*). Biliary secretion of intact asialoglycoproteins has been attributed to the mis-sorting of ligands in the endosome compartment (*13,28*).

While some ^{99m}Tc secretion into bile can be accounted for by missorting of ligands, this does not explain the 5-fold greater biliary output of ^{99m}Tc compared to ^{125}I. The time course of ^{99m}Tc appearance in bile suggests the majority arises via the lysosomal (indirect) pathway (*27*). Lysosomal degradation of ^{99m}Tc-NGA would be expected to liberate the ^{99m}Tc as TcO_4^-. It is only possible to speculate on how this TcO_4^- could enter bile. Organic anions such as bilirubin diglucuronide are transferred to bile via an organic anion transport protein in the canalicular membrane (*29*). TcO_4^- is a large inorganic anion and it is possible that TcO_4^- itself, or complexes of TcO_4^- with other molecules, can enter bile via this transporter. Indeed, the majority of injected ^{99m}Tc-associated radioactivity is present in the small intestine of rabbits 60 min after administration o ^{99m}Tc-NGA (*2*) and presumably arises there via the bile

In summary, the results presented here show tha NGA, whether labeled with ^{125}I or ^{99m}Tc, is an excellen potential receptor-binding radiopharmaceutical in tha it is avidly taken up by the liver. The fact that ^{99m}Tc remains within the liver for longer than ^{125}I, and is eventually secreted mainly in the bile, represents an artifact of radiolabel handling. Nevertheless, this is beneficial to diagnostic nuclear medicine in that ^{99m}Tc-NGA will persist in the liver in steady-state conditions for sufficient time to enable planar and SPECT imaging as well as estimation of hepatic asialoglycoprotein receptor concentration. In addition, the fact that labeled metabolites of ^{99m}Tc-NGA do not appear in plasma permits kinetic modeling with this radiopharmaceutical without time-consuming and expensive correction for labeled metabolites.

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