Technetium-99m-MRP20, a Potential Brain Perfusion Agent: In Vivo Biodistribution and SPECT Studies in Non-Primate Animals

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MRP20 (N-(2(1H pyrolylmethyl))N′-(4-pentene-3-one-2)) ethane-1,2-diamine) complexes with technetium-99m, yielding a neutral, lipophilic species. This compound has been characterized as [TcO(MRP20)]. Biologic investigation of [^{99m}Tc][TcO(MRP20)] in female rats showed 2.35% ID in the brain 30 min p.i. with no significant wash-out over 3 hr. A single-photon emission computed tomography (SPECT) study in a dog demonstrated rapid tracer uptake in the brain, reaching a maximum within 1 min, with 2.24% i.d. 15 min p.i., decreasing to 1.7% after 4 hr. The complex undergoes hydrolysis in vitro forming a cationic species. This is possibly the trapping mechanism in the brain in vivo. The main excretory route of [^{99m}Tc][TcO(MRP20)] is via the hepatobiliary tract. There is evidence of some "in vivo" cell labeling and soft-tissue uptake.

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There is considerable interest in the development of a new radiopharmaceutical for cerebral imaging that is capable of accurately reflecting regional cerebral blood flow (rCBF). Neurologic nuclear medicine has potential applications in the diagnosis of stroke patients, dementias including Alzheimer's disease patients, epilepsy and psychiatric disorders, e.g., schizophrenia (*1–6*). The radiopharmaceuticals currently employed for these studies, either commercially or in research include iodine-123-N-isopropyl-p-iodoamphetamine (Spectamine®, I-AMP); [^{123}I] N,N,N′-trimethyl-N′-[2-hydroxy-3-methyl-5-iodobenzyl]-1,3-propanediamine (I-HIPDM); technetium-99m-hexamethylene propylamine oxime (Ceretec®, Tc-d,l HMPAO); ^{99m}Tc-ethyl cysteinate dimer (Neurolite®, Tc-ECD) (*7*), and [^{99m}Tc][TcCl(DMG)$_2$2MP] (*8*). The high cost and limited availability of ^{123}I makes a technetium agent the radiopharmaceutical of choice in many clinics and there is presently only one such technetium compound suitable for these studies licensed for sale in most parts of the world. A prerequisite of these radiopharmaceuticals is their ability to cross the blood-brain barrier (BBB) and be retained within the brain matter for a length of time suitable for the required study. Retention occurs because of a trapping mechanism which will prevent the tracer from crossing the BBB in the opposite direction. The mechanism may be enzymatic, causing a fundamental change in the dependent molecule or pH due to the different pH values in blood and brain matter (*9–11*) or a simple chemical reaction that renders the complex non-diffusable. Certain factors, such as charge (*9–11*), molecular weight (*12*), and lipid solubility (*13*) are known to affect a compound's ability to pass the BBB. The ideal molecule has an overall neutral charge, <500 Daltons and is within a window of Log P values 0.9–2.5. In this study, we report a ligand MRP20 (N-(2(1H pyrolylmethyl))N′-(4-pentene-3-one-2)ethane-1,2-diamine), which, by the loss of three protons, is designed to form a neutral technetium complex containing a Tc = O^{3+} core, with technetium in the +5 oxidation state (Fig. 1). This complex has a molecular weight of 336 Daltons and lies well within the accepted limit for molecular mass. The synthesis of the no-carrier added (NCA) ^{99m}Tc complex and its in vivo behavior in non-primate animals are reported in this paper.

MATERIALS AND METHODS

All chemicals were of standard laboratory grade except for high-performance liquid chromatography (HPLC) grade solvents that were obtained from Romil Chemicals Ltd., Loughborough, United Kingdom. Technetium-99m-HMPAO was prepared from a commercially available kit (Ceretec®, Amersham International). Distilled water was used at all times and nitrogen gas (Air Products, Wavre, Belgium) was used as found without further drying or removal of oxygen. Pertechnetate was obtained from a ^{99}Mo/^{99m}Tc generator (Elutec®, Medgenix) in isotonic saline.

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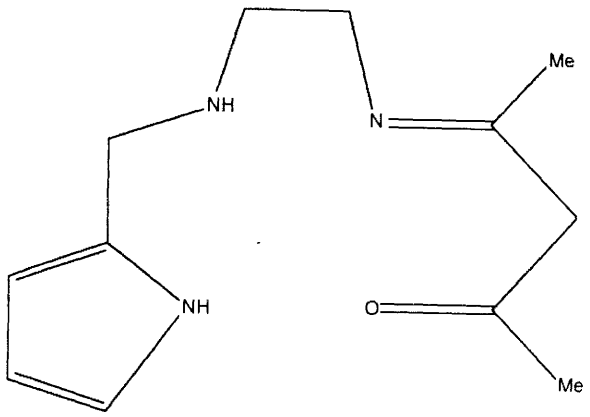

FIGURE 1
MRP20, N-(2(1H pyrolylmethyl))N'-(4-pentene-3-one-2) ethane-1,2,-diamine.

Ligand Synthesis

MRP20 was synthesized and fully characterized by a single crystal X-ray structure determination. The details of the synthetic procedure will be reported elsewhere.

Formulation of the NCA ^{99m}Tc Complex

Five milligrams (2.26×10^{-5} mole) of MRP20 ligand in ethanol was mixed with a pertechnetate and saline mixture and 20 μg (8.86×10^{-8} mole) of stannous chloride (Janssen Chimica, Beerse, Belgium) to a final concentration of 50% v/v EtOH:NaCl. The reaction was left at room temperature for 15 min followed by a separation step on a C-18 reverse-phase chromatography column (Seppak® Millipore, Waters). The column was prepared in the recommended way by pre-wetting with ethanol followed by a water rinse before loading the sample. The lipophilic complex was retained on the column and all impurities (e.g., excess stannous ions, free ligand, and unreacted pertechnetate) were removed by washing the column with water. The complex was then eluted in a pure form in ethanol and conserved until required.

Complex Analysis

Standard analysis was performed using instant thin-layer chromatography silica gel support (ITLC-SG®, Gelman, St. Louis, MO) with 20% saline and methyl ethyl ketone (MEK) as mobile phases. The strips were analyzed on a Berthold automatic TLC linear analyzer with a Berthold chromatography data system and an Epson LX 86 printer. In this chromatography system, the complex and pertechnetate migrate to the solvent front in MEK, while only pertechnetate migrates to the solvent front in saline. By subtraction therefore, the overall yields of TcO_4- and TcO_2 can be determined as well as the yield of complex.

Analysis of the yield of lipophilic complex was also made using an octanol/saline extraction system. To a tube containing 1 ml of saline-saturated octanol and 1 ml of octanol-saturated saline a small aliquot (10–20 μl) of the reaction solution was added. This was mixed vigorously on a vortex mixer for several minutes and then centrifuged for 3 min at 1500 rpm. The two phases were separated and 0.8 ml of each phase was counted in a Capintec CRC120 gamma counter. The percent of activity found in the organic phase, normalized to 1 ml, was taken as the percent yield of lipophilic complex.

To assess the charge on the technetium complex, electrophoresis was performed using electrophoresis chamber (Gelman Sciences), a veronal buffer at pH 8.5, and a stationary phase with Whatman's No. 1 chromatography paper. Routinely, a voltage of 10 V/cm was applied and the experiment ran for 30 min.

Reverse-phase HPLC was carried out routinely to check reaction reproducibility. A Spectra-Physics system (Darmstadt-Kranickstein, Germany), comprised of a SP8800 ternary HPLC pump for gradient elution, a chromjet integrator connected to a Spectra 100 variable wavelength u.v./visible detector, and a Canberra gamma detector, was used with a Hamilton PRP-1 (10 μm reverse phase) column fitted with a rheodyne injection valve. A gradient elution profile of 5% tetrahydrofuran (THF):95% phosphate buffer reaching 45% THF:55% buffer after 5 min was employed, and under these conditions the ligand R_v was calculated to be 6.99 ml and that of the technetium complex, 10.7 ml. The dead volume of the system was calculated as 1.39 ml using NaI detected by absorption at 254 nm.

Characterization of the NCA Neutral Complex

The ^{99}Tc complex of MRP20 was prepared from [^{99}Tc][TcO(ethyleneglycolate)$_2$]$^{2-}$ and characterized by standard analytical techniques as the [MOL] complex, where M = Tc(V) and L = $(MRP20)^{3-}$. The technetium complex structure was identified by mass spectrometry, FAB$^+$MS, infra-red spectrometry, microanalysis, and additionally by ^{13}C NMR spectroscopy as [^{99}Tc][TcO(MRP20)]. Additionally, a crystal of [^{99}Tc][TcO(MRP20)] suitable for X-ray analysis was obtained and its structure recently solved. Details of the synthesis and characterization of these complexes will be the subject of a separate report. Equivalence of the ^{99}Tc and [^{99m}Tc][TcO (MRP20)] compounds was shown by coinjection of the two complexes onto the HPLC.

Calculation of Log P Values for the Ligand and the NCA Complex

A method for determining Log P was developed with an HPLC system based on published examples (*14–16*). A series of ligands of similar structure to MRP20 were analyzed by octanol:saline extraction and the partition coefficient was calculated using HPLC with O.D. measurements at 311 nm. Calculation of the capacity factors on the HPLC column of the ligands was made according to:

$$k' = \frac{V_r - V_o}{V_o}$$

where V_r is retention volume of the ligand and V_o is the dead volume of the column.

Animal Studies: Biodistribution in Rats

Biodistributions were performed using a formulation of the MRP20 complex that had been purified on a C-18 reverse-phase column and eluted with ethanol:saline (45:55 v/v). Approximately 5–10 μCi of MRP20 were injected into the femoral vein of female rats while under ether anesthesia. The rats were allowed to recover from the anesthetic and placed in individual cages where no attempt was made to collect urine and feces. Three rats were killed by cardiac puncture at five time points: 5, 15, 30, 60, and 180 min postinjection. The organs of interest were excised, washed with saline to remove

surface blood, weighed on an analytical balance, and counted in an Intertechnique automatic gamma counter (CG 400, attached to a teletype printer). Suitable standards, representing 1/1000 of the injected dose, were prepared from the injection material. Blood and muscle values were taken as 6% and 43% of the total body weight, respectively.

In order to study the biologic fate of the hydrolysis product of MRP20, a preparation of MRP20 of >95% purity was allowed to degrade for over 4 hr. The remaining lipophilic complex was fully extracted with CH_2Cl_2 and an assay on the HPLC confirmed the absence of both the neutral MRP20 and pertechnetate. A biodistribution study in rats was performed following the same protocol as above, with three animals killed at 5, 15, and 30 min postinjection. A subsequent whole-body study on a beagle dog was performed by taking a number of views to cover the whole body with a large field of view camera. The hydrolysis compound was prepared using the technique previously described.

Canine Biodistribution

An adult beagle dog, weighing 19 kg, was anesthetized with i.v. pentobarbital (1500 MBq per study) which was injected in a vein in the foreleg. In order to study initial kinetics of brain uptake, serial dynamic images of the head in a left lateral position were obtained with a frame rate of 1/sec for the first 2 min after injection, and 10 sec/frame for the next 13 min. To generate time-activity curves, a region of interest including the whole brain, was selected. At the end of the 15 min and again 4 hr after injection, a number of views covering the whole body were obtained with a large field of view camera. Blood samples were withdrawn into heparinized tubes from a site other than the injection site at 2, 5, 10, 20, 45, 60, 120, and 180 min after injection for blood clearance analysis. An aliquot from each sample was pipetted and counted as whole blood and the remaining sample was centrifuged at 3000 rpm for 10 min and an aliquot of plasma was taken. Both the whole blood and plasma were counted in a gamma counter with suitable standards prepared from the injection material.

Tomographic images were obtained at 30 min, 1, 2, and 4 hr after injection using a rotating gamma camera equipped with a low-energy high-resolution collimator. Data were collected in 64 × 64 matrices with a zoomed 30 × 30 cm field of view using 64 angular increments over 360° and an acquisition time of 30 sec/view. Before reconstruction, a 50% scatter correction was performed by subtracting 50% of the image acquired in the scatter window (100 keV, 20%) from the image acquired in the ^{99m}Tc peak window (140 keV, 15%). Transaxial, sagittal, and frontal slices were generated by filtered backprojection using a Hamming-Hann filter.

RESULTS

Chemistry of the Carrier and NCA Complexes

The reduction of pertechnetate by stannous ions in the presence of a solution of MRP20 at ambient pH (pH 8–8.5) yields a neutral and lipophilic technetium complex that does not migrate under electrophoresis conditions and has an R_f of 1 in MEK. Octanol extraction shows that it is quantitatively extracted into the organic phase and its behavior on the HPLC column confirms its lipophilic nature. Figure 2 shows an HPLC

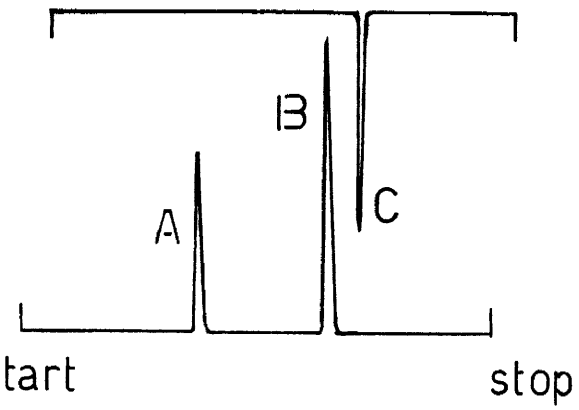

FIGURE 2
HPLC chromatograms shows the equivalence of ^{99m}Tc complexes of MRP20 following coinjection of the two isotopes onto the Hamilton PRP-1 reverse-phase column. Details of the elution gradient are given in the Methods and Materials section. **A** is the free, uncomplexed ligand, MRP20, which is retained on the column and elutes in 6.57 ml. Detection was by uv/visible spectrometry at 311 nm. **B** is the ^{99m}Tc complex, retained for a longer time on the column and eluted in 10.78 ml. Detection was by a NaI crystal gamma scintillation well. **C** is the ^{99m}Tc complex (10.7 ml) which is shown inverted and offset to emphasize the identical retention volume of the two technetium complexes. Detection was by uv/visible spectrometry at 360 nm.

trace of both the uncomplexed ligand and [^{99m}Tc] Tc complex, with the latter retained on the column significantly longer; R_v [ligand] 6.99 ml; R_v [complex] 10.68 ml. The chromatogram obtained with MRP20 is also included to show the equivalence of the two compounds. Log P of the ligand was calculated as 1.13 and that of the technetium complex as 1.93. The Log P of the complex is within the range of values quoted for lipophilicity (0.9–2.5) that are suitable for crossing the BBB. The same system was used to calculate a Log P for ^{99m}Tc-HMPAO, reported as 1.2 by Neirinckx et al. (*17*). We calculated experimentally by HPLC and achieved a Log P of 1.81. This is in reasonable agreement with the Log P value quoted by Anderson et al. (*18*) of 1.9 which was determined by octanol:saline extraction.

MRP20 Biodistribution in Rats

The results of the rat biodistributions on the neutral technetium complex are given in Table 1. The data represents the % ID/organ and shows significant brain activity after 30 min and retention in the brain of the injected material up to 3 hr. Tracer excretion was through the hepatobiliary system. There was considerable soft-tissue and muscular uptake, and the blood clearance was slow with a half-life of 3.8 hr over the first 30 min and a half-life of 11.5 hr from 30 to 180 min.

Imaging Characteristics in the Beagle

In the dog brain, uptake is fast; maximum brain activity was seen within 1 min postinjection with 2.24%

TABLE 1
Biodistribution in Rats of MRP20

Organ	5 min	15 min	30 min	60 min	180 min
Brain	1.20	1.40	2.35	2.08	1.68
Heart	1.20	1.06	1.43	1.31	0.88
Liver	9.70	9.78	13.20	8.98	4.27
Kidneys	4.00	4.59	5.84	6.43	7.95
Intestine	5.60	6.66	9.32	9.89	15.27
Spleen	0.30	0.32	0.48	0.49	0.32
Lung	2.10	1.97	2.21	2.05	1.31
Stomach	1.50	1.82	2.85	2.51	1.73
Muscle	28.82	37.97	—	43.25	—
Blood	13.11	12.43	11.80	11.10	8.65

The values are expressed as % dose/organ and represent the mean of three animals.

ID in the brain at 15 min postinjection. This level of activity remained fairly constant throughout the 4-hr study (1.74% after 4 hr). A comparison of the rate of uptake into the brain of MRP20 and HMPAO made in the same dog on two different occasions shows that the rate of uptake for both compounds was very similar (Fig. 3). In spite of the small size of the dog's brain when considering the spatial resolution of a rotating camera system, SPECT studies showed a selective cerebral uptake with a high contrast between the brain and the skull (Fig. 4). No apparent redistribution was observed during the successive SPECT studies. As previously observed with Ceretec®, MRP20 also showed uptake in the olfactory region (*19*) with approximately the same kinetic pattern as the brain. The blood clearance of MRP20 was slow with a whole blood activity of 14% at 2 min decreasing to 6% by 2 hr. The corresponding plasma activities were 3% and 1.1%, indicating a strong affinity with the cellular fraction of the blood (Fig. 5). The major excretory route was through the hepatobiliary system, although the whole-body images at 4 hr showed considerable activity in the urinary bladder as well.

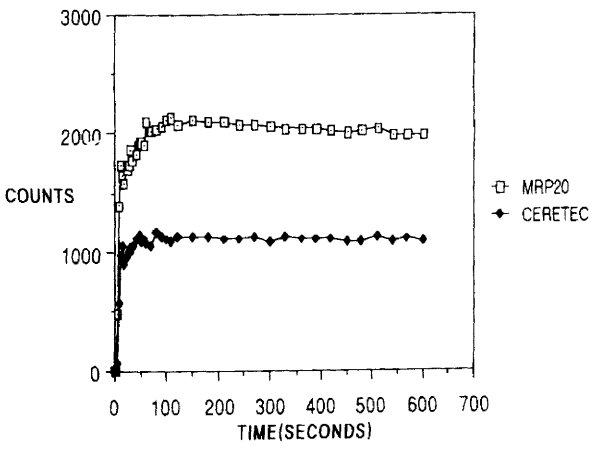

FIGURE 3
A comparison of the rate of uptake into the brain of a dog of 25 mCi [^{99m}Tc][TcO(MRP20)] and 15 mCi of [^{99m}Tc][Tc-HMPAO]. The graph shows the uptake and retention of both compounds over a period of 10 min. The same dog was used for the studies on two separate occasions.

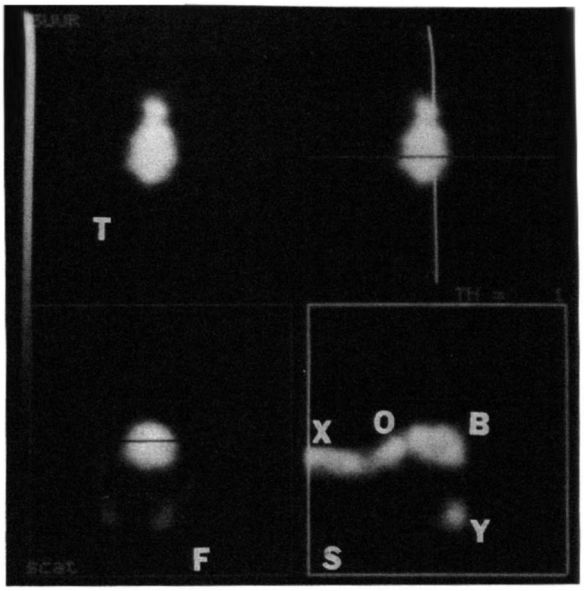

FIGURE 4
The SPECT images of the dog were acquired 3 hr after injection. Three different views are shown; transverse (T), frontal (F), and sagittal (S) slices were reconstructed. The sagittal view clearly shows the brain (B), olfactory (O), and snout (X) activity. There is also evidence of some salivary (Y) gland activity.

Stability of the NCA Complex

The stability of the NCA complex in ethanolic solution was studied by HPLC, electrophoresis, and by octanol:saline extraction. Both the ligand and complex are susceptible to hydrolysis under certain conditions with the cleavage occurring at the >C = N-linkage which is formally present in the molecule (Figure 6). Hydrolysis of MRP20 results in a technetium complex (or complexes) which has been identified by electrophoresis to be cationic. A charged and more hydrophilic character is reflected by the lack of extraction of this hydrolysis product into octanol. Good separation on HPLC has not been achieved and this is attributed to the presence of a vacant coordination site arising from the cleavage of the >C = N bond, which may be loosely coordinating solvent molecules easily lost or exchanged under HPLC conditions. It was observed that the neutral MRP20 decomposed preferentially into the cationic species rather than to pertechnetate over the first 3 hr of the study. It is possible to arrest the hydrolysis of both the ligand and complex by paying careful attention to the reaction conditions. This work is under extensive investigation in our laboratories.

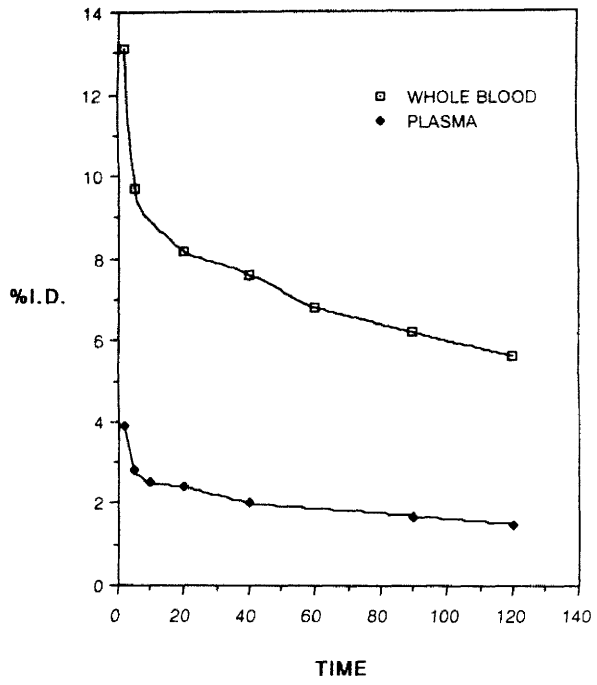

FIGURE 5
Graph plotting the blood clearance in the beagle of MRP20 over a 2 hr. Points represent % ID in the whole blood or plasma as a function of time.

Biodistribution of the Hydrolysis Product

Under dilute, aerobic conditions where no pH adjustments have been made, a NCA preparation of >90% radiochemical purity will degrade in 4 hr to less than 20% neutral, lipophilic MRP20. The results of the biodistribution of the hydrolysis product, after separation of the neutral compound from the reaction mixture, are given in Table 2. This study shows a lack of brain uptake, rapid clearance from the blood with a half-life of 24 min, and mixed hepatobiliary/renal elimination in rats. These observations were subsequently confirmed with a canine whole-body study (unpublished experimental results).

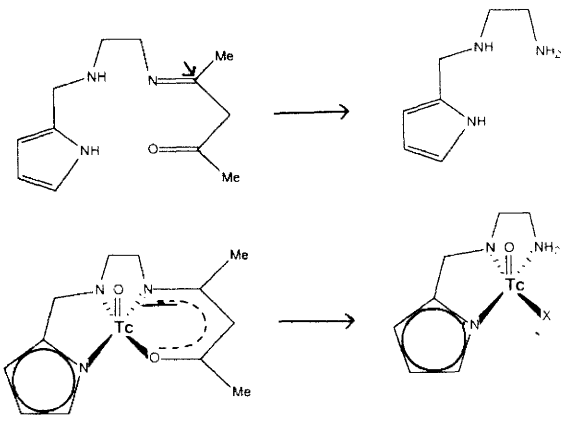

FIGURE 6
Schematic diagrams shows the probable sites of hydrolysis in the ligand MRP20 (**A**) and the technetium complex (**B**). The hydrolysis of the ligand, **A**, results in the formation of a tridentate ligand containing a primary amine group. The hydrolysis of the complex, **B**, results in a compound of uncertain structure, with a vacant coordination site **X**, which may loosely coordinate solvent molecules.

TABLE 2
Biodistribution Data of Hydrolyzed MRP20 in Rats

Organ	5 min	15 min	30 min
Brain	0.15	0.08	0.09
Heart	0.31	0.11	0.11
Liver	16.53	22.47	21.76
Kidneys	10.83	11.70	9.40
Intestine	3.62	8.64	12.09
Spleen	0.21	0.24	0.20
Lungs	0.93	0.31	0.25
Stomach	0.53	0.84	0.78
Muscle	15.20	4.60	4.03
Blood	10.71	3.07	2.01

The values are expressed as % dose/organ and represent the mean of three animals.

DISCUSSION

The formulation of MRP20 presently requires more than a simple reconstitution step, but a freeze-dried kit has been prepared that has a proven shelf-life of more than 6 mo which is currently undergoing stability tests. Following our protocol, we can prepare this compound with a radiopharmaceutical purity of more than 93%, where the impurities contain <5% TcO_2 and TcO_4^-. The stability of the complex can be guaranteed for more than 2 hr at greater than 80% of the desired lipophilic complex with the remaining 15% comprising the cationic hydrolysis product which is cleared through the hepatobiliary system. We are currently exploring several alternative methods to improve the shelf-life and post-reconstitution of this compound.

The Log P (or degree of lipophilicity) of our complex is comparable to that of ^{99m}Tc-HMPAO, however, we have shown that the mechanism of uptake (even considering passive diffusion) is not completely controlled by calculation of molecular weight, charge, and lipophilicity (*14*).

The neutral, lipophilic complex, [^{99m}Tc][TcO-(MRP20)], is readily taken up into non-primate brains and is retained over a prolonged period. The uptake in rat brain reached a maximum between 15 and 30 min postinjection and the absolute amount of radioactivity in the brain is comparable to that reported in rats for ^{99m}Tc-HMPAO (*17*).

In the dog, brain activity peaked within 1 min, followed by a small decrease before reaching a plateau at 2.24% ID, calculated at 15 min postinjection. The blood clearance in the beagle of MRP20 is slow compared to the values quoted in animals for ^{99m}Tc-ECD

20) but is comparable to those of ^{99m}Tc-HMPAO (*19*). There was significant soft-tissue uptake, however, the activity clears fairly rapidly from the soft tissues while remaining trapped within the brain matter with no apparent washout or redistribution of the radiopharmaceutical.

In common with other technetium complexes scanned for rCBF imaging, the olfactory gland of the dog was in evidence, probably due to the neural tissue that comprises part of that gland. Approximately 1% of the injected dose was found in the olfactory gland at 15 min postinjection, which is significantly less than the 2.24% ID found in the brain.

In conclusion, [^{99m}Tc][TcO(MRP20)] has been shown to cross the BBB in non-primate animals and is retained within the cerebral matter for a sufficient time to allow good quality SPECT images to be acquired for up to 4 hr after injection. The distribution of the tracer within the cerebral tissues appears qualitatively to be in accordance with CBF with no redistribution of the radioactivity with time. We are currently designing the experiments necessary to quantify these observations. The results obtained in animals suggest that this complex may be useful in cerebral imaging and warrants further study in normal human volunteers (*21*).

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