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Bisquaternary Ammonium Compounds as Potential Tumor Imaging Agents

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Reports of rapid accumulation of ^{3}H- or ^{14}C-labelled hexamethonium in cartilaginous tissues after intravenous administration to rats and mice and of binding of hexamethonium to chondroitin sulfate *in vitro* prompted synthesis of radioiodinated analogs of hexamethonium. Tissue distribution studies in rats performed at 0.5 and 2 h after intravenous administration of ^{14}C-hexamethonium confirmed the propensity of this drug to accumulate in cartilaginous tissues. Tissue distribution studies were performed in both normal and chondrosarcoma tumor-bearing rats at 0.5 and 2 h after intravenous administration of the radioiodinated analogs. The radioiodinated analogs showed profiles of distribution of radioactivity comparable to that of hexamethonium. Levels of uptake were highest in kidney and urine at all time periods studied. High levels of activity were seen in trachea, intervertebral discs and, when present, tumor. A rapid dispersal of a relatively low level of radioactivity was seen in most other tissues and was followed by rapid clearance in all tissues except meninges which continued to accumulate radioactivity for as long as 2 h. A chondrosarcoma tumor was successfully imaged in a rat 15 min following intravenous administration of a radioiodinated analog of hexamethonium.

Introduction

GLYCOSAMINOGLYCANS (mucopolysaccharides) are an ill-defined group of compounds which form the amorphous ground substances of connective tissue, the blood group specificity substances, and the protective mucous secretions of the digestive and respiratory passages. They are nearly always associated with proteins in some way. A characteristic feature of these macromolecules is the presence of many closely packed, negatively charged groups associated with repeating disaccharide units. As a result of this anionic nature, they show a great affinity for quaternary and especially bisquaternary drugs. These macromolecules represent sites of loss for the quaternary drugs as interaction results in no physiological effect.

Hexamethonium is a bisquaternary, anti-hypertensive agent which acts at the ganglionic synapse by antagonizing acetylcholine.[1] This agent has been shown to accumulate in poorly vascularized tissues such as cartilage within minutes after administration. Autoradiographic studies in mice and rats have shown selective concentration of ^{3}H- or ^{14}C-hexamethonium in articular cartilage and intervertebral disc as compared to surrounding tissues.[2,3] The high levels of radioactivity in cartilaginous tissues of mice following administration of ^{3}H-hexamethonium has been shown to be associated with areas presumably containing glycosaminoglycans.[3] Quaternary ammonium compounds have also been shown to bind *in vitro* to glycosaminoglycans such as chondroitin sulfate.[2,4]

Agents such as these when appropriately radiolabelled could be useful tools in diagnosis of diseases of cartilage and of diseases characterized by localized increases in levels of glycosaminoglycans such as regions of inflammation, arthritis and cystic fibrosis. Hexamethonium has been radiolabelled with ^{11}C and preliminary evaluations have been encouraging.[5] However, the short half-life of ^{11}C (20 min) may limit its availability for clinical use.

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Thus, a goal in our laboratory has been the structural modification of the hexamethonium molecule to allow incorporation of a foreign γ-emitting nuclide (radioiodine) without the loss of characteristics which are essential for accumulation in cartilage. Substitution of the C_6 polymethylene chain of hexamethonium with a xylylene group was an attractive modification as it allowed introduction of an aromatic radiohalogen which would be more stable than an aliphatic halogen. Moreover, the *p*-xylylene substituted hexamethonium analog has been shown to have no significant ganglionic blocking activity in the cat.[6] It was hoped that the lower ganglionic blocking activity of these analogs would not decrease their ability to bind at anionic sites of loss present in cartilaginous tissues.

Thus, two radioiodinated analogs of hexamethonium have been synthesized and evaluated in normal and tumored rats for their ability to concentrate in tissues known to contain high concentrations of glycosaminoglycans.

Materials and Methods

Radiolabelled compounds

The synthesis of ^{14}C-hexamethonium (Fig. 1, Scheme I) was accomplished by treatment of *N,N,N',N'*-tetramethyl-1,6-hexanediamine with $^{14}CH_3I$. An excess of the tertiary amine was used to ensure complete utilization of $^{14}CH_3I$. Subsequent addition of unlabelled CH_3I assured complete quaternization. Purity of the product was confirmed by thin-layer chromatography (TLC) using silica gel plates developed in $CHCl_3$:MeOH (1:2).

Iodinated analogs of hexamethonium (NM-194 and NM-202) were synthesized using the general method (Scheme II) in Fig. 1. Results of NMR (Varian A-60A), i.r. (Perkin–Elmer 281) and elemental analyses (Spang Microanalytical Laboratory, Ann Arbor, Michigan, or Midwest Microlab, Inc., Indianapolis, Indiana) on the final product and on all intermediates were all consistent with the proposed structures. Melting points (Thomas–Hoover Apparatus, uncorrected) of the two analogs were *para*-243°C dec, and *meta*-250°C dec. TLC analyses of the compounds were performed using either silica gel or cellulose plates (Eastman Kodak) developed in methyl cellosolve:propionic acid:water (15:15:70) saturated with NaCl. Iodine vapor or u.v. light were used to visualize the spots. R_f values for the *para* analog were 0.30 and 0.93 for silica gel and cellulose, respectively. The *meta* analog had R_f values of 0.27 and 0.95 for silica gel and cellulose, respectively.

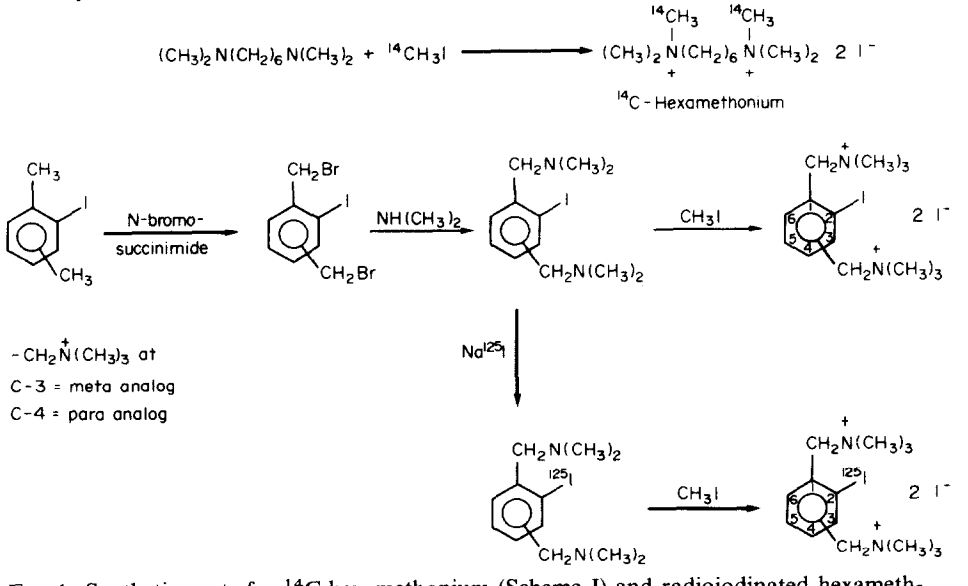

FIG. 1. Synthetic route for ^{14}C-hexamethonium (Scheme I) and radioiodinated hexamethonium analogs (Scheme II).

Incorporation of radioiodide into the analogs was achieved by exchange reaction with $Na^{125}I$ performed on the tertiary amines followed by quaternization with CH_3I. Radiochemical purity was confirmed by TLC analysis using the systems described above. A sample of each radiolabelled analog and the corresponding authentic non-radiolabelled compound were co-chromatographed. The location of the radioactive peak (determined by a Baird Atomic Radiochromatogram Scanner) was coincident with the location of the spot from the unlabelled material. No other radioactive peaks or spots were detectable.

The ^{14}C-hexamethonium (15 mg, 310 μCi) was dissolved in saline (5 ml) for use in tissue distribution studies. The *meta* analog (12.5 mg, 1.3 mCi) was dissolved in saline (5 ml). The *para* analog was synthesized twice for tissue distribution studies. For the studies in normal animals, 26 mg of compound (250 μCi) was dissolved in saline (2 ml). For the studies in tumored rats, 8 mg of compound (280 μCi) was dissolved in saline (2 ml). The compound dose for tissue distribution studies was 0.8–1.4 mg/kg except for studies in normal rats using the *para* analog when 5.0–6.0 mg/kg was given. A third synthesis of the *para* analog, necessary for the scan attempt, resulted in 34 mg of compound (1.7 mCi) which was dissolved in saline (1 ml).

Tissue distribution studies in normal rats

Adult male Sprague–Dawley rats (Spartan Research Animals, Haslett, Michigan) weighing 200–300 g were used as experimental animals. The rats were housed in temperature and light-controlled quarters and had access to food (Teklad 4% Rat and Mouse Diet) and water *ad libitum*. The rats received intravenous injections of the test materials via the tail vein in 0.1 ml of saline, while under ether anesthesia. The rats were killed by cardiac exsanguination under ether anesthesia.

Each of 15 rats received 6.3 μCi of ^{14}C-hexamethonium and was killed at either 0.5 h (2 groups of 5 rats) or 2 h (5 rats) post-injection. Twenty rats were divided into 2 groups and the rats each received either 11.7–12.3 μCi of the *para* analog (10 rats) or 23.8–25.1 μCi of the *meta* analog (10 rats). Half of the rats in each group were killed at 0.5 h and half were killed at 2 h post-injection. Following the killing of all rats, samples of blood were taken and the major organs removed, dissected free of fat and connective tissue, blotted dry and weighed. Large organs were minced with scissors. Samples of tissues from rats that received ^{14}C-hexamethonium were placed in tared liquid scintillation vials and weighed. To each vial was added 0.3 ml of 2 N NaOH solution and the vials were allowed to digest overnight. The vials were heated on a hot plate at 40°C for 5–10 min to complete digestion and then allowed to cool whereupon 50 μl of H_2O_2 (30% solution) was added to each vial. The vials were reheated at 40°C for 1–3 min and allowed to cool. Enough 0.5 M acetic acid to neutralize the base and 13.5 ml of PCS (Amersham Corp.) were added and the vials shaken on a vortex mixer. The samples were assayed for radioactivity in a liquid scintillation counter (Beckman LS-150) for 10 min or until enough counts had accumulated to reduce counting error to 2% at 95% confidence level, whichever was shorter. For samples which counted the full 10 min, maximum counting error at 95% confidence level was 10%. The observed cpm were corrected for efficiency using the external standard channels ratio method. Samples from rats that received radioiodinated analogs were placed in tared cellulose acetate capsules and weighed. The capsules were then placed in counting tubes and assayed for radioactivity in a well scintillation counter (Searle Automatic Gamma System) for 10 min or until enough counts had accumulated to reduce counting error to 2% at 95% confidence level, whichever was shorter. For samples which counted the full 10 min, maximum counting error at 95% confidence level was 6%. Counting efficiency for ^{125}I was 87%.

Chondrosarcoma tumor model

Two rats inoculated 10 weeks previously with the Swarm transplantable chondrosarcoma tumor first described by CHOI *et al.*[7] and characterized by OEGEMA *et al.*[8]

were received from the laboratory of Dr Dominic Dziewiatkowski of The University of Michigan, Ann Arbor. These rats were used to start the tumor line carried in this laboratory, and the transplantation procedure routinely used was as follows: donor rats were killed with ether, and the tumors removed and placed on ice. Samples of these tumors were preserved in 10% formalin solution for histopathological evaluation. The remaining portions of the tumors were finely minced with scissors, and 0.1 ml of the tumor mince was injected via syringe subcutaneously into the left flank of male Sprague–Dawley rats weighing 180–200 g. At 6–8 weeks following tumor inoculation these rats had tumors of adequate size for experimental use. Rats weighed 350–450 g at this time.

Tissue distribution studies in tumor-bearing rats

Twenty tumor-bearing rats were divided into two groups and the rats each received either 22.8–23.1 μCi of the *para* analog (10 rats) or 24.7–25.0 μCi of the *meta* analog (10 rats). Half of the rats in each group were killed at 0.5 h and half of the rats were killed at 2 h post-injection. Tissue handling was as described for tissues from normal rats receiving radioidinated compounds. In addition, samples of each tumor were preserved in 10% formalin solution for histopathological evaluation. Urine samples obtained from some of these rats were also analyzed using the TLC system described for the radioiodinated hexamethonium analogs. An aliquot of the solution injected was chromatographed concurrently with the urine samples for comparison. The TLC plates were scanned to locate the radioactivity on the plates (Berthold Radio Scanner Model 6000).

Scan of chondrosarcoma-tumored rat

Five tumored rats received intravenous injections of the *para* analog at doses ranging from 83–178 μCi (1.7–6.5 mg). Higher radioactive doses were required for detection of activity with the small animal scanner. Animals were killed by ether overdose at 5–30 min post-injection and scanned using a small animal scanner (Berthold Radio Scanner Model 6000). Following completion of one scan, the tumors were removed and the animals rescanned. After the second scan was complete, samples of tumor and other tissues were assayed for radioactivity as described previously.

Results of tissue distribution studies are expressed as % administered dose/gram of tissue and were calculated as follows:

$$\%\ \text{dose/g} = \frac{\text{Sample dpm} \times 100\ \text{mg/g}}{\text{sample wt (mg)} \times 22.2 \times 10^5\ \text{dpm}/\mu\text{Ci} \times \text{dose}\ (\mu\text{Ci})} \times 100$$

Results

The distribution of radioactivity following intravenous injection of ^{14}C-hexamethonium or its radioiodinated analogs to normal and/or tumored rats is summarized in Table 1.

The distribution of radioactivity following injection of ^{14}C-hexamethonium to normal rats is comparable to that reported for autoradiographic studies in rats and mice by WASSERMAN[3] and ASGHAR and ROTH.[2] The highest concentration of radioactivity was seen in the kidney at all time periods studied. There was a rapid dispersal of a relatively low level of activity in most tissues followed by a rapid clearance. The clearance of radioactivity from the blood was especially rapid with % dose/g values of 0.310 $\pm$ 0.028 and 0.020 $\pm$ 0.006 at 0.5 and 2 h, respectively. High levels of uptake were seen in trachea and intervertebral discs initially which were also cleared rapidly, although not as rapidly as blood. Trachea-to-blood ratios were 3.7 $\pm$ 0.8 and 6.9 $\pm$ 1.0 at 0.5 and 2 h, respectively. The tendons and articular cartilage showed higher levels of uptake than surrounding muscle. While there was very little concentration of radioactivity in the brain, the meninges showed initially moderate levels of uptake at 0.5 h which increased at 2 h.

TABLE 1. Distribution of radioactivity following intravenous administration of ^{14}C-hexamethonium or its radioiodinated analogs to rats

Tissues	% Dose/g*					
	^{14}C-Hexamethonium		*Para* analog		*Meta* analog	
	1/2 h	2 h	1/2 h	2 h	1/2 h	2 h
Normal rats						
Blood	0.310 ± 0.028†	0.020 ± 0.006	0.277 ± 0.029	0.015 ± 0.002	0.274 ± 0.013	0.021 ± 0.007
Brain	0.018 ± 0.004	0.018 ± 0.003	0.044 ± 0.008	0.038 ± 0.005	0.072 ± 0.009	0.059 ± 0.004
Cartilage	0.290 ± 0.088	0.043 ± 0.011	—	—	—	—
Inter-vertebral disc	1.094 ± 0.531	0.144 ± 0.021	0.607 ± 0.068	0.087 ± 0.022	0.913 ± 0.130	0.113 ± 0.040
Kidney	2.616 ± 0.302	1.384 ± 0.236	3.305 ± 0.651	1.834 ± 0.269	4.292 ± 0.488	3.647 ± 0.191
Liver	0.112 ± 0.012†	0.049 ± 0.006	0.129 ± 0.016	0.085 ± 0.011	0.210 ± 0.018	0.175 ± 0.008
Lung—normal	0.386 ± 0.046‡	0.137 ± 0.018	0.257 ± 0.029	0.087 ± 0.011	0.268 ± 0.011	0.078 ± 0.005
Lung—abnormal	0.466	0.497	—	—	—	—
Meninges	0.301 ± 0.063	0.413 ± 0.020	0.467 ± 0.066	0.658 ± 0.138	0.764 ± 0.081	0.869 ± 0.070
Muscle	0.094 ± 0.021	0.014 ± 0.002	0.080 ± 0.014	0.022 ± 0.002	0.073 ± 0.004	0.021 ± 0.004
Tendon	0.297 ± 0.091	0.039 ± 0.012	—	—	—	—
Thyroid	0.206 ± 0.046	0.047 ± 0.015	0.095 ± 0.010	0.060 ± 0.008	0.189 ± 0.028	0.245 ± 0.016
Trachea	1.350 ± 0.476	0.127 ± 0.035	0.789 ± 0.085	0.089 ± 0.018	1.116 ± 0.158	0.144 ± 0.061
Tumored rats						
Blood			0.217 ± 0.015	0.039 ± 0.017	0.185 ± 0.011	0.022 ± 0.002
Kidney			3.179 ± 0.250	1.340 ± 0.171	3.533 ± 0.332	3.269 ± 0.217
Liver			0.085 ± 0.009	0.053 ± 0.005	0.163 ± 0.005	0.149 ± 0.006
Trachea			0.814 ± 0.051	0.160 ± 0.036	0.598 ± 0.041	0.102 ± 0.013
Tumor			0.639 ± 0.062	0.181 ± 0.038	0.707 ± 0.039	0.161 ± 0.019

* Values represent mean ± SEM for 5 rats unless noted; † 10 rats; ‡ 6 rats.

TABLE 2. Radioactivity in tissues of rats scanned after injection of the radioiodinated *para* analog of hexamethonium

Time (min)	Dose (μCi)	Scan results	% Dose/g		
			Kidney	Liver	Tumor
5	104	–	2.538	0.323	0.175
15	83	–	2.700		0.598
15	178	+	2.020	0.144	0.647
25	83	–	3.958		0.477
30	83	–	3.084	0.142	0.481

Meninges-to-brain ratios were 17.7 $\pm$ 1.8 and 25.3 $\pm$ 7.3 at 0.5 and 2 h, respectively.

Intravenous administration of either radioiodinated analog of hexamethonium also resulted in highest levels of radioactivity being present in the kidneys at both time periods studied. There was also a rapid dispersal of relatively low levels of radioactivity throughout the tissues followed by rapid clearance. Trachea and intervertebral discs had high levels of activity, although not as high as when ^{14}C-hexamethonium was administered. Trachea-to-blood ratios were 3.0 $\pm$ 0.4 and 6.2 $\pm$ 0.9 for the *para* analog and 4.0 $\pm$ 0.5 and 6.2 $\pm$ 0.6 for the *meta* analog at 0.5 and 2 h, respectively, and similar ratios were noted for intervertebral discs. As with ^{14}C-hexamethonium, the levels of radioactivity increased with time in meninges. The meninges-to-brain ratios were 11.5 $\pm$ 1.9 and 17.4 $\pm$ 3.1 for the *para* analog and 10.8 $\pm$ 0.6 and 15.1 $\pm$ 2.0 for the *meta* analog at 0.5 and 2 h, respectively.

Tumors developed in all rats injected with tumor mince. Histopathological evaluation of the tumors* confirmed that these tumors were chondrosarcomas. Measurements taken of the tumors at their greatest lengths in two perpendicular directions varied from 1.0 $\times$ 2.0 to 2.7 $\times$ 4.0 cm and weights varied from 1.04 to 7.66 g.

Distribution of radioactivity following intravenous administration of radioiodinated hexamethonium analogs to chondrosarcoma tumor-bearing rats was not significantly different from results in normal rats. Levels of uptake in the tumors were similar to that in the trachea. Tumor-to-blood ratios were 3.0 $\pm$ 0.3 and 6.3 $\pm$ 1.1 for the *para* analog and 3.9 $\pm$ 0.4 and 7.1 $\pm$ 0.5 for the *meta* analog at 0.5 and 2 h, respectively. Urine obtained from the bladder of 17 of the 20 tumored rats had levels of radioactivity ranging from 18–93% dose/g at 0.5 h and 6–46% dose/g at 2 h post-injection. As no attempt was made to collect all urine excreted between injection and killing, calculations of total amounts of radioactivity present in the urine cannot be made. If enough urine remained after counting samples had been taken, TLC analyses were performed. TLC analysis of urine from five of the rats (*para* analog–3, *meta* analog–2) killed at 0.5 h post-injection showed only one detectable radioactive peak which had the same R_f value as the injected material which was cochromatographed with the urine samples. In some, but not all, 2 h urine samples, a shoulder was apparent in the radioactive peak which may be indicative of another metabolite. Further characterization of urinary radioactivity was not attempted.

Initial attempts to visualize the chondrosarcoma tumor were unsuccessful. The radioiodinated *para* analog was used for imaging studies with the small animal scanner. A dose of 83 μCi (4.2–5.0 mg/kg) was given to each of the 3 rats which were killed at 15, 25 or 30 min after injection. Although results of these scans were encouraging, close proximity of the tumor to the kidneys prevented positive indentification. A fourth rat was injected with 104 μCi (8.2 mg/kg) and killed 5 min post-injection. The tumor was not visualized. Subsequent analysis of the tissues showed that levels of activity in the tumor at 5 min were only 0.175% dose/g. A final attempt to visualize the tumor

* Performed by Drs D. A. Repola and R. H. Nishiyama of the Department of Pathology at The University of Michigan.

FIG. 2. Scan of chondrosarcoma tumored rat using the *para* analog at 15 min after i.v. injection. Urine from the bladder was withdrawn with a syringe.

was made after preparation of a fifth rat with the tumor mince injected subcutaneously over the left rear leg instead of the left flank. A 15-min interval was selected because this time period gave the highest radioactivity in the tumors of the other 4 rats studied (Table 2). The dose injected was 178 μCi (15.8 mg/kg). Following injection, the rat exhibited tremors and decreased motor activity. No visible signs of intoxication were seen with the other rats in this study. The initial scan of the fifth tumored rat showed four areas of increased radioactivity. Two of these corresponded to the position of the kidneys and the third was thought to represent urinary activity in the bladder. The fourth area corresponded to the location of the tumor. A syringe was used to remove the urine from the bladder, and the animal was rescanned. Only three areas of increased radioactivity remained (Fig. 2). When the animal was rescanned following removal of the tumor, the corresponding area of increased radioactivity was no longer evident (Fig. 3).

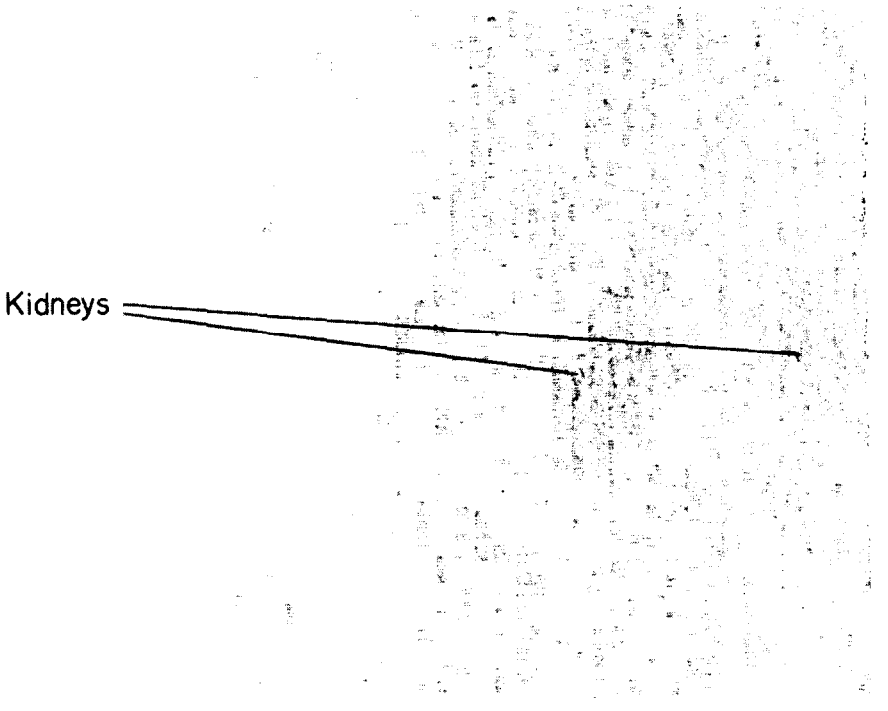

FIG. 3. Rescan of rat in Fig. 2 following removal of the chondrosarcoma tumor.

Discussion

As reported in the literature,[2] and confirmed in this study, hexamethonium accumulates rapidly in avascular tissues such as cartilage following injection to rats. Radioactivity in such tissues after administration of radiolabelled hexamethonium is related to the tissue concentration of negatively-charged glycosaminoglycans,[3] and such tissues represent sites of loss for bisquaternary drugs. The structural alterations present in the analogs of hexamethonium evaluated in this study allow for retention of the ability to accumulate in cartilaginous tissues as well as introduction of a γ-emitting nuclide.

The rapid urinary excretion reported for hexamethonium[9,10] is apparently responsible for the high levels of radioactivity in the kidneys after injection of the ^{14}C-labelled compound. High renal activity was also seen following administration of either radioiodinated analog suggesting that the kidneys are important in clearance of these compounds.

Hexamethonium is not metabolized *in vivo* and has been isolated unchanged from the urine in several species.[11] TLC analysis of urine from rats 0.5 h after administration of the radioiodinated hexamethonium analogs showed only one radiolabelled product with R_f value the same as the injected material suggesting no detectable *in vivo* metabolism. By 2 h after administration, however, a second radioactive substance seen as a shoulder on the radioactive peak was detectable in some urine samples. Further work is necessary to ascertain the exact structures of the radioactive product(s) present in the urine. The low levels of radioactivity present in the thyroid indicate a minimum amount of *in vivo* deiodination.

The meninges showed a delayed pattern of accumulation of radioactivity as compared to other cartilaginous tissues. While these tissues showed highest levels of radioactivity at 0.5 h, it was at the 2-h interval when radioactivity in other tissues had decreased that meninges showed highest uptake. This delayed uptake resulted in favorable meninges-to-blood and brain ratios suggesting that agents of this type may also have value in the diagnosis of meningiomas.

Visible toxic effects were only seen when high dose (15 mg/kg) of low specific activity material were administered for imaging purposes. Doses as high as 8 mg/kg resulted in no apparent toxic effects. Nonetheless, future studies should strive for the use of agents with a specific activity in the order of at least 1 mCi/mg to minimize possible pharmacological effects.

The feasibility of using these hexamethonium analogs for imaging areas of glycosaminoglycan concentration has been shown by successful visualization of a rat chondrosarcoma tumor. The clinical use of these hexamethonium analogs as scanning agents would require the substitution of ^{125}I with the more energetic isotopes of iodine such as ^{131}I and ^{123}I. The short time required for synthesis of these analogs as well as the ability of the compounds to accumulate in target tissues at early time periods makes labelling with ^{123}I feasible.

The encouraging results obtained in these preliminary studies with two radioiodinated hexamethonium analogs suggest that further studies be carried out in larger animals to confirm accumulation of these compounds in cartilage of other species and to evaluate their toxicity at doses necessary for scanning.

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