In Vitro Stabilization of a Low-Tin Bone-Imaging Agent (^{99m}Tc-Sn-HEDP) by Ascorbic Acid

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The presence of oxidants in the ^{99m}Tc-pertechnetate and of oxygen in diagnostic kits containing low concentrations of Sn(II) has a detrimental effect upon in vitro and in vivo stability. Maintaining a nitrogen atmosphere or increasing the Sn(II) concentration inhibits the formation of $^{99m}TcO_4^-$. However, the latter remedy is likely to cause uptake in the reticuloendothelial system and has been associated with false positive or negative brain scans. We used ascorbic acid (an antioxidant) to ensure the in vitro stability with the low-Sn(II) bone agent disodium etidronate. In vitro stability studies by instant thin-layer chromatography, using high-activity generators and "instant pertechnetate," yielded less than 2% free pertechnetate at 24 hr after preparation. Distribution studies in guinea pigs show neither altered distribution of the bone agent nor abnormal distribution of ascorbic acid, suggesting its sole function as a noncomplexing stabilizer.

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Oxidants added during the processing of ^{99}Mo for ^{99m}Tc-pertechnetate sources, and also the presence of oxygen, have been indicted as major causes of in vitro instability in diagnostic kits containing Sn(II) as the reducing agent. Clinically the instability manifests itself by visualization of the gut and thyroid, indicating the presence of free pertechnetate. The use of higher-activity ^{99}Mo–^{99m}Tc generators, the introduction of fission-produced molybdenum generators of high specific activity, and the increasing popularity of instant pertechnetate from high-specific-activity sources all involve radiation doses capable of reducing the pertechnetate. Consequently, they require increased oxidants to maintain high ^{99m}Tc extraction or elution yields. Thus, diagnostic kits of low Sn(II) content, originally designed to be used with pertechnetate sources of relatively low activity, are becoming even more susceptible to high levels of oxidants present in the pertechnetate. The diagnostic kit investigated here is stannous etidronate (Sn·HEDP), a bone-imaging agent whose formulation contains a very low level of Sn(II). Although there is sufficient stannous ion for the initial reduction of pertechnetate and subsequent formation of the complex, the level is not sufficient to prevent slow oxidation with time.

In vitro instability of low-Sn(II) chelates can be minimized by nitrogen purging of the saline eluant and the diagnostic kit vials, in order to reduce the oxygen content before adding the pertechnetate (*1,2*). Another method of minimizing the stability problem is to increase the level of Sn(II) to counteract the oxidants (*2*). However, recent papers (*3–6*) report altered activity distributions in clinical brain scans for up to 2 weeks after the administration of bone-seeking agents containing high levels of tin. Accordingly, the use of an antioxidant, ascorbic acid (H_2Asc), and its sodium salt (NaHAsc) was investigated as a means of eliminating the interfering oxidants without abandoning the low levels of Sn(II) needed to maintain the high selectivity of the ^{99m}Tc-Sn·HEDP bone-seeking agent.

MATERIALS AND METHODS

The pertechnetate sources for this study were commercially available ^{99m}Tc generators (Squibb or Mal-

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linckrodt) containing 300–400 mCi of ^{99}Mo derived from (n,γ) reactions or from fission, and "instant" pertechnetate (CISR, Medi-Physics) extracted by methyl ethyl ketone. Each vial contained 0.10 mg of tin and 5.9 mg of sodium etidronate (ethane-1-hydroxy-1,1-diphosphonate) and was prepared according to the manufacturer's instructions (Procter & Gamble, Cincinnati, Ohio). Our stabilized bone-imaging agent contained this material with the addition of 0.1 or 0.6 mg of the sodium ascorbate (Merck & Co.) per vial of the bone agent. In some cases 0.1 mg of ^{14}C-labeled H_2Asc was added (3.8 μCi of ^{14}C-1-L-ascorbic acid, New England Nuclear Corp., North Billerica, Mass.). Before adding the $^{99m}TcO_4^-$, the vials were purged with nitrogen by repeated evacuation of air followed by nitrogen sweeping in a vacuum chamber. The effect of oxygen, with and without NaHAsc, was determined from nitrogen-purged vials that were vented directly to the air. Oxygen analysis (Beckman Model 778) of the head space in ten air-vented vials yielded a mean value of 12.6 ± 5.2% oxygen, against less than 0.5% oxygen for the nitrogen-purged vials.

Studies in vitro. The degree of instability of the HEDP complex was determined by measuring the percentage of free pertechnetate with two different thin-layer chromatographic systems: (A) precoated cellulose acetate plastic sheets (Brinkman Polygram Gel MN 300) with methanol–water (85:15); or (B) instant thin-layer chromatography (ITLC) strips (Gelman SG) with 100% methyl ethyl ketone. Strips were spotted with instant pertechnetate at the time of preparation and at 3, 6, or 24 hr after preparation; and at 3 or 6 hr using pertechnetate obtained from generators.

Studies in vivo. Biologic distribution studies were conducted in Heartly female albino guinea pigs (210–370 gm) to determine whether adding H_2Asc to the skeletal imaging agent altered the distribution of either the ^{99m}Tc-Sn·HEDP or the ^{14}C-H_2Asc. The agents used were ^{99m}Tc-Sn·HEDP alone and with 0.1 mg of ^{14}C-H_2Asc (^{99m}Tc-Sn·HEDP [^{14}C-H_2Asc]), and ^{14}C-H_2Asc alone. Guinea pigs were used because they are known to be similar to man in their H_2Asc requirements (*7*). After an overnight fast, these animals were injected intravenously with 0.5 ml of the radioactive complex as previously described (*8*). Water was offered ad libitum and the animals were killed by decapitation at 3 and 24 hr after administration. Whole organs were removed, weighed, and placed in scintillation vials for immediate radioassay of ^{99m}Tc; these included femur, tibia, liver, kidneys, spleen, heart, brain, pancreas, adrenals, uterus (including ovaries), eyes, submandibular gland and parotid gland, urine (0–24 hr), and samples of bone marrow, skeletal soft tissue, and blood. To assay the ^{14}C-ascorbic acid, the ^{99m}Tc was allowed to decay before complete combustion of the samples in a high-temperature oxygen combustion train with trapping of $^{14}CO_2$ in an ethanolamine–methyl cellosolve mixture (1:7), followed by analysis with a liquid-scintillation beta spectrometer (Packard Model 2450). The effects of oxygen on the tissue retention of the bone-seeking agent was studied in femur, blood, and muscle. The tracer was allowed to stand in vials for 3 hr before administration, and the animals were killed 3 hr after the dose. The effects were examined with the NaHAsc at concentration levels of 0.1 and 0.6 mg per vial and the results were compared with those obtained in the absence of NaHAsc, under oxygen, and after oxygen deprivation by nitrogen sweeping.

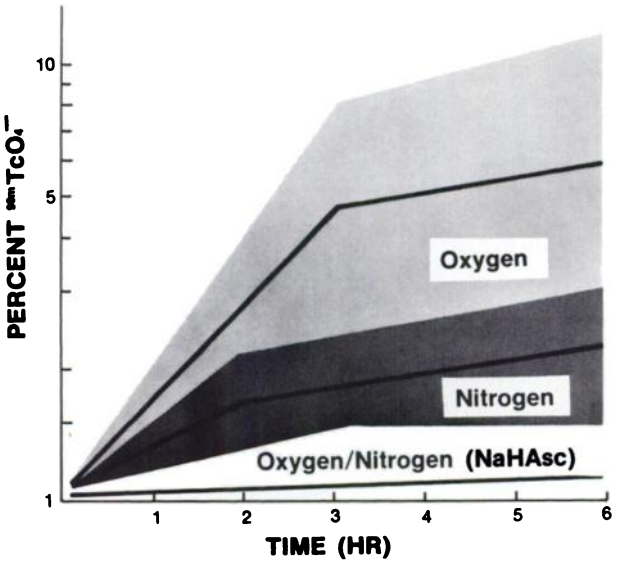


FIG. 1. In vitro stability of ^{99m}Tc-Sn·HEDP without (top two curves) and with (bottom curve) sodium ascorbate stabilizer at either 0.1 or 0.6 mg per vial. High-activity generators were used, with vials under oxygen (air) and nitrogen atmospheres.

To avoid decay corrections, the percent of dose per organ, or percent dose per gram of sample, was determined using standards prepared from the administered ^{99m}Tc and ^{14}C and counted with the tissue samples.

RESULTS

Studies in vitro. Figure 1 shows the average values and range of the percent of unbound pertechnetate over a period of three consecutive weeks, Monday through Thursday, with 300- and 400-mCi generators. Pertechnetate levels for unstabilized ^{99m}Tc-Sn·HEDP ranged over 2.5–8% at 3 hr and 3–11% at 6 hr for vials vented to the air; they ranged over 1.5–3% at 6 hr for vials purged with nitrogen. Vials of the bone-imaging agent containing 0.1 and 0.6 mg

of sodium ascorbate as the antioxidant, used with high-activity generators, consistently had free pertechnetate values of approximately 1% in the presence of either oxygen or nitrogen over a 6-hr period in vitro.

The oxygen–oxidant susceptibility of the ^{99m}Tc-Sn·HEDP with MEK-extracted pertechnetate is given in Fig. 2. The percent of free pertechnetate is an average of ITLC values from three air-vented and three nitrogen-purged vials, with NaHAsc (0.1 and 0.6 mg) and without NaHAsc at 0, 3, 6, and 24 hr after preparation.

Studies in vivo. The biologic distribution of the intravenously administered ^{99m}Tc-Sn·HEDP containing the ^{14}C-H_2Asc is compared to the bone agent without the ^{14}C-H_2Asc and the ^{14}C-H_2Asc alone at 3 hr (Table 1) and 24 hr (Table 2) after administration. As expected (*8*), the diphosphonate bone agent was specifically localized on osseous tissue with less than 2% of the dose in soft tissue at 3 and 24 hr. The uptake of ^{14}C-H_2Asc alone in the liver, kidney, spleen, heart, brain, pancreas, adrenals, uterus, eyes, submandibular gland, and parotid gland is consistent with the ^{14}C autoradiographic distribution studies of Hornig et al. (*7*). Comparisons of ^{99m}Tc and ^{14}C uptake for the individual components with the ^{99m}Tc-Sn·HEDP (^{14}C-H_2Asc) show very good agreement in organ distribution, indicating that H_2Asc does not complex with the ^{99m}Tc-Sn·HEDP nor does it alter the distribution of the ^{99m}Tc-labeled diphosphonate bone agent.

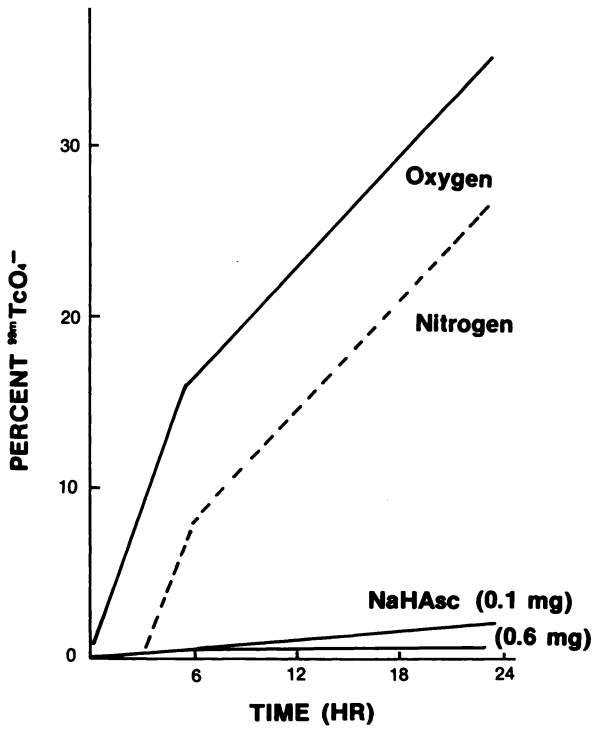


FIG. 2. In vitro stability of ^{99m}Tc-Sn·HEDP without stabilizer (top two curves), with vials under oxygen (air) and nitrogen atmosphere; and with stabilizer (0.1 mg and 0.6 mg sodium ascorbate—bottom two curves) under either oxygen (air) or nitrogen atmosphere. Instant pertechnetate was used.

The effects of oxygen in the vial upon the biologic distribution and the role of the NaHAsc antioxidant are given in Table 3. The significant increase in ^{99m}Tc retention in muscle, as compared with retention in the presence of two levels of NaHAsc or under nitrogen atmosphere, illustrates the detrimental effect of oxygen upon soft-tissue background. The higher blood values in the absence of NaHAsc probably reflect the effect of oxygen, even at the very low oxygen levels in the nitrogen-purged vials.

DISCUSSION

Radiation effects upon elution of ^{99m}Tc from ^{99}Mo–^{99m}Tc alumina-column generators were studied by Afka and Veseley (*9*). They reported a 50% elution yield of ^{99m}Tc at doses of (0.1–1.5) × 10^{19} eV/ml, which is approximately equivalent to the ionizing radiation of a 100-mCi generator for 2–3 hr on 1 ml of saline aqueous solution on a column. The poor ^{99m}Tc yield with this relatively low-activity generator, attributable to formation of lower oxidation states, illustrated the effect of photon radiation upon water on the generator column. More recently, Boyd (*10*) reported on generator elution efficiencies and found a rapid fall in efficiency at approximately 250 mCi, which was preventable by addition of an oxidant, potassium dichromate. For instant pertechnetate, a situation exists that is even more sensitive to radiation effects. Organic molecules (the methyl ethyl ketone and trace organic impurities) have a much higher tendency to form radiolysis byproducts than has water (*11*). Both sources of pertechnetate necessitate the use of oxidants to maintain the correct oxidation state for elution or extraction.

In commercially available high-activity generators, oxidant analysis by iodine thiosulfate titration (*12*) has yielded values up to 38 ppm and 76 ppm in the saline eluant and first eluate, respectively. Since a large number of substances (e.g., HClO, $Cr_2O_7^{-2}$, O_2, H_2O_2, NO_2^-, and organic peroxides) yield positive results, the iodometric analysis does not define the oxidant, but it does indicate variable levels of oxidants, either from the processing and manufacturing of the pertechnetate sources, or possibly radiolysis products that complex with oxygen. The antioxidant effectiveness of ascorbate and the role of oxygen in accelerating in vitro instability suggest two oxidants whose role as oxidation intermediates is explainable by the antioxidant mechanism of sodium

TABLE 1. TISSUE DISTRIBUTION OF ^{99m}Tc-Sn · HEDP, ^{99m}Tc-Sn · HEDP (^{14}C-ASCORBIC ACID), AND ^{14}C-ASCORBIC ACID ALONE IN GUINEA PIGS 3 HR AFTER INTRAVENOUS DOSE*

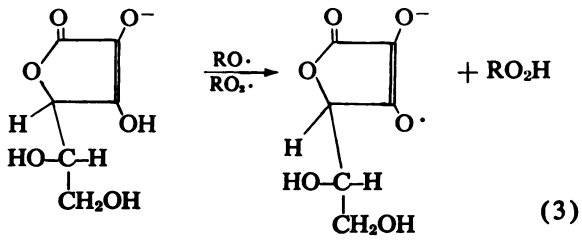
Organ	^{99m}Tc-Sn · HEDP alone	Sodium etidronate complexed with ascorbic acid		
		^{99m}Tc-Sn · HEDP	^{14}C-H_2Asc	^{14}C-H_2Asc alone
PERCENT OF DOSE PER WHOLE ORGAN				
Femur	2.1 (2.0–2.2)	2.0 (1.8–2.1)	0.30 (0.28–0.31)	0.36 (0.29–0.45)
Tibia	1.7 (1.6–1.9)	1.6 (1.5–1.7)	0.20 (0.19–0.21)	0.23 (0.20–0.29)
Liver	0.42 (0.33–0.49)	0.31 (0.30–0.34)	11.3 (10.8–11.9)	18.3 (14.8–21.6)
Kidney	0.67 (0.64–0.72)	0.46 (0.45–0.48)	1.02 (0.98–1.05)	1.97 (1.59–2.22)
Spleen	0.010 (0.010–0.012)	0.008 (0.008–0.009)	0.53 (0.42–0.64)	0.77 (0.57–0.95)
Heart	0.013 (0.012–0.014)	0.012 (0.012–0.013)	0.19 (0.17–0.23)	0.35 (0.32–0.37)
Brain	0.018 (0.013–0.023)	0.015 (0.005–0.024)	0.38 (0.32–0.46)	0.47 (0.40–0.52)
Pancreas	0.011 (0.007–0.014)	0.007 (0.006–0.008)	0.23 (0.18–0.30)	0.58 (0.46–0.67)
Adrenals	0.009 (0.005–0.016)	0.003 (0.003–0.005)	0.33 (0.26–0.38)	0.64 (0.58–0.69)
Uterus (ovary)	0.026 (0.021–0.036)	0.017 (0.011–0.024)	0.49 (0.29–0.65)	0.69 (0.59–0.82)
Eyes	0.006 (0.0059–0.0062)	0.006 (0.005–0.006)	0.35 (0.28–0.40)	0.38 (0.29–0.49)
Submandibular gland	0.010 (0.009–0.012)	0.010 (0.009–0.011)	0.70 (0.57–0.94)	0.68 (0.33–1.07)
Parotid gland	0.003 (0.0024–0.0041)	0.003 (0.0019–0.0039)	0.03 (0.02–0.04)	0.03 (0.03–0.03)
PERCENT OF DOSE PER GRAM				
Marrow (femur)	0.12 (0.023–0.26)	0.11 (0.05–0.18)	0.60 (0.58–0.61)	0.67 (0.43–1.01)
Blood	0.034 (0.030–0.038)	0.022 (0.019–0.026)	0.052 (0.040–0.062)	0.069 (0.062–0.082)
Muscle (skeletal)	0.0047 (0.0044–0.0049)	0.0044 (0.0033–0.0052)	0.039 (0.034–0.044)	0.051 (0.042–0.057)

* Figures in parenthesis indicate range (n = 3).

ascorbate. The first is peroxide, in whose absence atmospheric oxidation is not normally possible (*13*). The oxygen–oxygen bond is weakest in peroxides and their scission gives use to powerful oxidizing alkoxy or hydroxy radicals (Eq. 1, below), which play a major role in autoinitiated oxidation by molecular oxygen. Peroxy radicals, the other intermediate from radiolysis products and oxygen (Eq. 2), are also explainable since their removal leads to interruption of radical-chain-propagated reactions. Both of these intermediates are stabilized by the ascorbate through transfer of H atom to the intermediate (Eq. 3), yielding a resonance-stabilized and nonreactive molecule, RO_2H (*14*).

$$RO\text{–}OR' \rightarrow RO\cdot + \cdot OR' \qquad (1)$$

$$R\cdot + O_2 \rightarrow RO_2 \qquad (2)$$



(3)

The elimination of the intermediate by the ascorbate is believed to provide in vitro stability by inhibition of the slow oxidation of TcO_2 to Tc(VII). This slow oxidation of the TcO_2 is responsible for the production of Tc(VII) (*1*). The mechanism of the HEDP bone agent's instability is suggested by Hambright et al. (*15*) to be dissociation and hydrolysis of stable chelated Tc(IV) into TcO_2 rather than direct interaction of the chelate with oxygen.

TABLE 2. TISSUE DISTRIBUTION OF ^{99m}Tc-Sn · HEDP, ^{99m}Tc-Sn · HEDP (^{14}C-ASCORBIC ACID), AND ^{14}C-ASCORBIC ACID ALONE IN GUINEA PIGS 24 HR AFTER INTRAVENOUS DOSE*

Organ	^{99m}Tc-Sn · HEDP alone	Sodium etidronate complexed with ascorbic acid		^{14}C-H_2Asc alone
		^{99m}Tc-Sn · HEDP	^{14}C-H_2Asc	
PERCENT OF DOSE PER WHOLE ORGAN				
Femur	1.8 (1.6–1.9)	1.7 (1.6–1.8)	0.25 (0.18–0.35)	0.31 (0.25–0.34)
Tibia	1.4 (1.3–1.5)	1.3 (1.2–1.4)	0.35 (0.29–0.43)	0.22 (0.21–0.24)
Liver	0.73 (0.48–1.1)	0.60 (0.42–0.73)	1.66 (1.59–1.76)	2.59 (1.38–4.53)
Kidney	0.36 (0.34–0.40)	0.30 (0.29–0.32)	0.67 (0.53–0.77)	0.87 (0.60–1.14)
Spleen	0.009 (0.008–0.012)	0.007 (0.006–0.008)	0.86 (0.80–0.96)	0.73 (0.47–0.90)
Heart	0.005 (0.004–0.006)	0.004 (0.003–0.004)	0.23 (0.21–0.25)	0.22 (0.15–0.30)
Brain	0.026 (0.002–0.062)	0.010 (0.007–0.012)	0.94 (0.91–0.97)	0.90 (0.84–0.95)
Pancreas	0.002 (0.002–0.003)	0.002 (0.002–0.003)	0.18 (0.15–0.20)	0.30 (0.16–0.52)
Adrenals	0.005 (0.004–0.007)	0.001 (0.001–0.002)	0.24 (0.20–0.32)	0.34 (0.22–0.54)
Uterus (ovary)	0.005 (0.004–0.006)	0.006 (0.004–0.008)	0.41 (0.33–0.47)	0.39 (0.20–0.62)
Eyes	0.002 (0.001–0.003)	0.001 (0.001–0.002)	0.39 (0.26–0.47)	0.32 (0.30–0.35)
Submandibular gland	0.005 (0.004–0.005)	0.005 (0.005–0.006)	0.87 (0.84–0.90)	0.66 (0.54–0.72)
Parotid gland	0.002 (0.0010–0.0026)	0.001 (0.0005–0.0015)	0.028 (0.010–0.056)	0.024 (0.015–0.041)
Urine	45.4 (42.0–51.0)	41.6 (37.7–45.9)	6.7 (5.8–7.4)	8.4 (7.2–10.8)
PERCENT OF DOSE PER GRAM				
Marrow (femur)	0.026 (0.018–0.034)	0.042 (0.007–0.062)	0.62 (0.38–1.10)	0.56 (0.54–0.59)
Blood	0.006 (0.0060–0.0063)	0.005 (0.0043–0.0056)	0.018 (0.015–0.021)	0.017 (0.015–0.018)
Muscle (skeletal)	0.0011 (0.0011–0.0012)	0.0010 (0.0009–0.0012)	0.059 (0.056–0.063)	0.073 (0.069–0.076)

* Figures in parenthesis indicate range (n = 3).

The ascorbate's sole function as an in vitro stabilizer is evident from the unaltered bone specificity of the ^{99m}Tc-Sn·HEDP imaging agent in the presence of ascorbic acid. Although ascorbic acid is itself a weak reducing agent, under slightly acid conditions the reduction of pertechnetate proceeds very slowly (*16*). The lack of any increased renal uptake, at 3 and 24 hr, when the bone agent contains the H_2Asc, clearly negates formation of any Tc(IV)–ascorbate complex. Consequently, the Sn(II) is responsible for the rapid reduction of ^{99m}Tc(VII) and the subsequent complexing of the ^{99m}Tc label with the diphosphonate.

Ascorbic acid is nontoxic when administered in moderate doses to humans (*17*). While most toxicity studies were focused upon oral administration, a number of studies have been conducted with intravenously administered ascorbic acid. Demole (*18*) administered intravenously an aqueous solution of sodium ascorbate at 1 gm/kg to mice, rats, and rabbits and at 0.2 gm/kg to dogs. The doses were well tolerated in every case and no symptoms of hypervitaminosis were observed. In addition, many reviews of ascorbic acid levels in serum, plasma, and whole blood have appeared (*19–21*). A study with normal volunteers yielded a value of 1.5 ± 0.6 mg of ascorbic acid per 100 ml of whole blood (*22*). Comparing this value to the level of ascorbate in a vial of ^{99m}Tc-Sn·HEDP (0.1–0.6 mg), the stabilized bone agent contributes only 0.0018–0.011 mg/100 ml of

TABLE 3. EFFECTS OF OXYGEN, OXYGEN LACK, AND SODIUM ASCORBATE (3 HR IN VITRO) UPON BIOLOGIC DISTRIBUTION OF ^{99m}Tc-Sn · HEDP*

	Oxygen			Nitrogen
Organ	no ascorbate	0.1 mg ascorbate	0.6 mg ascorbate	no ascorbate
Bone	2.01 (1.96–2.10)	2.42 (2.26–2.57)	2.36 (2.16–2.56)	2.30 (2.15–2.47)
Blood	0.033 (0.031–0.037)	0.020 (0.017–0.022)	0.017 (0.015–0.018)	0.026 (0.022–0.028)
Muscle	0.0051 (0.0044–0.0055)	0.0028 (0.0027–0.0030)	0.0030 (0.0026–0.0035)	0.0033 (0.0030–0.0038)

* Figures in parentheses indicate range ($n = 3$). Data are expressed as percent dose per gram of tissue.

whole blood to a standard man, an insignificant increase in blood concentration to the above "normal" values.

Thus, the addition of ascorbic acid or its salt is a safe and effective means of inhibiting the effect of oxygen and oxidants and permits the use of diagnostic kits containing low levels of Sn(II), a consideration of growing importance in nuclear medicine.

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