***In Vivo* Multitracer Analysis Technique: Screening of Radioactive Probes for Noninvasive Measurement of Physiological Functions in Experimental Animals**

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A novel screening experiment, to find radioactive probes for non-invasive measurements of physiological functions in experimental animals, was tested using the *in vivo* multitracer analysis technique. The details of the efficiency of the detector settings used in the *in vivo* multitracer analysis technique were examined by both computer simulations and practical measurements. Multiple radioactive isotopes, *i.e.* multitracer, were prepared by irradiating a silver foil target with a heavy ion beam at the RIKEN ring cyclotron. After chemical separation of the silver target, the multitracer was finally dissolved in isotonic citrate buffer. The multitracer solution was intravenously injected into rats. Using a γ -ray detector equipped with a well-defined slit, the collimated γ -rays from the upper abdomen of living rats were measured. After correction of detection efficiencies, it was possible to compare the distribution of radioactive elements between two groups of rats different in body weight. The *in vivo* measurement showed that the tissue substantial volume of the selenium-deficient (SeD) rat liver increased compared to normal rats. The possibility of a functional estimation of tissue/blood volume for living rats was proposed based on the characteristic *in vivo* distribution of ^{74}As , ^{83}Rb and ^{103}Ru .

Key words blood volume; extracellular volume; intracellular volume; liver; rat; bio-trace element

The multitracer technique was developed at the RIKEN ring cyclotron (Wako, Japan) and supplied to several Japanese research groups. The multitracer technique has been applied to many disciplines, such as chemistry,²⁾ agricultural chemistry,^{3,4)} environmental chemistry,⁵⁾ and biology and medical sciences.^{6,7)} The wide variety of applications can be attributed to the high resolution of detectors for γ -ray energies, and allows for the simultaneous use of many tracers under identical conditions. We have applied this technique to study 1) the uptake of trace elements in rat organs,^{8–10)} 2) the affinity of metal ions to blood components,^{11,12)} and 3) the affinity of metal ions to sub-cellular components.^{13–15)} The multitracer technique itself has some limitations at the present time because of its low quantitative nature among the produced radioactive isotopes and the presence of long lived radioisotopes. However, the technique is still quite useful as a screening tool for biological and/or biochemical kinetics of radioisotopes in *in vivo* and *in vitro* experiments.

The multitracer is produced when a metal foil target, such as silver and/or gold, is irradiated by a highly-accelerated heavy ion beam (^{16}O , ^{14}N , ^{12}C , *etc.*). The various nuclides are produced in the target by nuclear spallation reactions. After chemical separation of the target elements, the various nuclides are obtained as the multitracer. Combining the multitracer with γ -ray spectrometry, simultaneous analysis of various elements is achievable.^{16,17)}

From past experiments each nuclide in the multitracer has been calculated to be on the order of 10^{-6} – 10^{-8} mol/g target. However, the carrier content in the multitracer is quite low after the chemical separation of the target element. The molar doses of each element when injected into an animal were on the order of 10^{-14} – 10^{-16} mol/g b.w. This concentration level is significantly lower compared to doses of commonly used medicines (10^{-6} – 10^{-8} mol/g b.w.) or the natural contents of most bio-trace elements (10^{-8} – 10^{-12} mol/g

b.w.). Such a low dose of multitracer is suitable for the estimation of the physiological behavior of its elements. In addition, even though a relatively toxic element such as arsenic is contained within the multitracer, no toxicity would be expected at the levels injected.

Animals were given intravenous (i.v.) injections of the multitracer. Using a γ -ray detector equipped with a well-defined slit, the collimated γ -rays from a particular region of a living animal can be measured. Time courses of the radioactivity provide information on the *in vivo* dynamics of bio-trace elements for the specific region of a living animal. Using this method, the noninvasive and simultaneous assessment of the dynamics of several elements in a specific region within an hour was reported.^{18–20)} The method was suitable in analyzing the tracer distributions in the upper abdomen (liver region) of a rat.¹⁸⁾ The results of *in vivo* and *in vitro* measurements were comparable based on the specific tissue distributions of ^{74}As and ^{83}Rb as the internal standard.^{19,20)}

The tissue blood volume is quite an important function when analyzing data obtained from noninvasive imaging techniques, such as CT, PET, SPECT, and MRI. In addition, the previous knowledge about intra and extra cellular volume ratio in the region analyzed should be useful information when assessing the biological, physiological and/or pathological functions using either CT, PET, SPECT, or MRI. However, individual assessments of tissue blood volumes, extra cellular volumes, and/or intra cellular volumes of intact tissue in a living animal, in our opinion, have not been successful.

In this report, the efficiency of the detector settings for the *in vivo* multitracer analysis technique was examined in detail to verify the experimental conditions. The dynamics of bio-trace elements 3 h after administration were analyzed using the *in vivo* multitracer analysis technique. The results between the normal and the selenium-deficient (SeD) rats were

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compared. The possible advantages of the technique as a screening tool for examining physiological and/or pathological functions, such as tissue blood volume and/or ischemia, in a living experimental animal are discussed.

MATERIALS AND METHODS

Multitracer A silver foil target was irradiated with a ^{14}N beam of 135 MeV/nucleon at the RIKEN ring cyclotron. After the chemical separation of Ag^+ , a hydrochloric acid solution containing the multitracer was prepared.^{16,17} The solution was evaporated to dryness and then the multitracer was dissolved in 2 ml of isotonic citrate buffer (pH 6.2) for administration to rats. The elements in our multitracer solution are probably present in ion form as Be^{2+} , Sc^{3+} , VO^+ , Cr^{3+} , Mn^{2+} , Fe^{3+} , Co^{2+} , Zn^{2+} , AsO_4^{3-} , SeO_4^{2-} , Rb^+ , Sr^{2+} , Y^{3+} , and ZrO^{2+} , although no chemical analyses have been carried out. These elements may weakly bind with the citrate molecules in the multitracer solution.

Instrumentation The rat holder (acrylic plate), lead slit, and arrangement of detectors are shown in Fig. 1. A slit (7.5 mm width, 50 mm thickness, and 70 mm length) was cut in the base of the apparatus between the rat holder and a γ -ray detector. A high-purity Ge semiconducting detector (ORTEC, Model No. GEM-15190, Oak Ridge, TN, U.S.A.) equipped with a multichannel analyzer (Seiko EG & G Co., Ltd., Matsudo, Japan) was placed 10 mm under the slit.

Simulation of Geometrical Detection Efficiency The detection efficiency on a central sagittal-axial plane (dotted square in Fig. 1B) was computer-simulated using a program designed in our laboratory with only the geometrical factors for input. At first, an angle range α , which is made by a position of a point source and the edges of the slit, was calculated (Fig. 2). One end of α was defined as an absolute angle α_s and other end as α_r . Second, an angle range β , which is made by the position of the point source and the edges of the detector, was calculated by every 0.001 radian increment from α_s to α_r . The integration of β from α_s to α_r gives an approximate solid angle S , in which γ -rays from the point source can be detected. The geometrical detection efficiency was given by $S/4\pi$. The geometrical detection efficiency for each pixel coordinate was calculated, and then the relative detection efficiency was obtained by comparing the geometrical detection efficiency of each pixel with that of the center point of the slit entrance as 100%.

Measurement of Detection Efficiency The detection efficiency of the present set-up was examined using a ^{152}Eu standard point source ($\phi 3$ mm) embedded in an aluminum holder ($\phi 15$ mm). The ^{152}Eu point source was measured at 0, 2, 10, 20, and 30 mm distance from the slit. An anesthetized rat was mounted on the rat holder (2 mm thick acrylic plate), and γ -rays passing through the rat upper abdomen (31 mm thick) or the head (23 mm thick) were measured. The detection efficiency was identified and quantified based on the γ -ray counts of 122, 245, 344, 411, 444, 779, 867, 964, and 1408 keV emitting from the ^{152}Eu . When the ^{152}Eu point source was directly placed on the slit entrance, the relative detection efficiency was standardized as 100%.

Noninvasive Measurement in Living Rats Normal healthy Wistar rats were purchased from Japan Laboratory Animals, Inc. (Tokyo, Japan). SeD rats were prepared as de-

Fig. 1. Schematic Description of Lead Slit and Detector Setting

(A) Arrangement of the rat, rat holder, lead slit, and detector. (B) Detail of the arrangement and position of the ^{152}Eu γ -ray point source used for estimating the relative detection efficiency (RDE) of this setting.

Fig. 2. Geometrical Arrangements Used for the Simulation

The integration of β from α_s to α_r gives an approximate solid angle S , in which γ -rays from the point source can be detected. The geometrical detection efficiency was given by $S/4\pi$.

scribed elsewhere.¹⁴ The rats used for experimentation were at 8 weeks of age, with a body weight of 225.1 ± 1.4 g for normal and 142.7 ± 8.7 g for SeD groups (mean $\pm$ S.D., $n=3$). Individual rats were anesthetized with Nembutal (0.05 mg/g b.w.; i.p. injection) (Dinabot, Osaka, Japan), and then the multitracer solution (200 μl i.v.) was administered. The rats were re-anesthetized 3 h after the administration of the multitracer solution. The γ -ray spectrum of the upper abdomen was collected for 15 min. The multitracer solution (10 μl)

was collected in a plastic tube as a standard sample. The animal experiments were carried out in compliance with the Guideline for Animal Care and Use at Showa Pharmaceutical University (2001), and approved by the Ethical Committee for Animal Care and Use of Showa Pharmaceutical University.

Measurement of Removed Organs After the final non-invasive measurement, rats were sacrificed by bleeding from the abdominal aorta under anesthesia. The blood was collected into a test tube. The plasma was separated with centrifugation ($1000 \times g$ for 10 min). Then, the whole body was perfused with ice-cold physiological saline (0.9% NaCl) until blood was sufficiently removed. The liver, kidney, spleen, heart, lung, colon, brain, femoral muscle, femoral bone, back skin and testis were isolated, cut into pieces, and weighed. Aliquots of the organs, blood, and plasma were collected in a plastic tube and sealed. γ -ray spectra of the samples were measured with the high-purity Ge semiconducting detector using a normal setting. A dilute (1/200) multitracer solution (2 ml) was collected in a plastic tube as a standard sample.

Data Analysis The radioactivities of ^7Be , ^{46}Sc , ^{48}V , ^{54}Mn , ^{59}Fe , ^{58}Co , ^{65}Zn , ^{74}As , ^{75}Se , ^{83}Rb , ^{85}Sr , ^{88}Y , ^{88}Zr , and ^{103}Ru were determined by the photopeaks at 477.6, 889.3, 983.5, 834.8, 1099.2, 810.8, 1115.6, 595.8, 264.7, 520.4, 514.0, 898.0, 392.9, and 497.1 keV, respectively. The radioactivity of a tracer nuclide in a removed organ was estimated as the normalized uptake rate (%):^{10,14)}

$$\text{normalized uptake rate (\%)} = (R_O/W_O)/(R_A/W_B) \times 100 \quad (1)$$

where R_O is the radioactivity of the tracer in the organ (cps), R_A is the total radioactivity of the tracer administered (cps), W_O is weight of the organ (g), and W_B is body weight of the rat (g).

The *in vivo* distribution of an element in a liver region of a rat was estimated as the *in vivo* relative distribution (%).

$$\text{in vivo relative distribution (\%)} = (R_M/W_M)/(R_A/W_B) \times 100 \quad (2)$$

where R_M is the radioactivity of measured section (cps) and W_M is weight of the measured section (g). This concept is proposed according with the normalized uptake rate. Since the W_M is an indeterminate value, this is given as 1 g for all measurements to simplify calculation.

To compare the *in vivo* relative distributions and the normalized uptake rates between normal and SeD rats, values were standardized with those of ^{74}As or ^{83}Rb , respectively. The mean value of the *in vivo* relative distribution of ^{74}As was standardized to 1.0 of the ^{74}As -standardized *in vivo* distribution. Similarly, the mean value of the normalized uptake rate and the *in vivo* relative distribution of ^{83}Rb were standardized to 1.0 of the ^{83}Rb -standardized uptake rate and the ^{83}Rb -standardized *in vivo* distribution, respectively.

RESULTS

Figure 3A shows the mapping of relative detection efficiency (RDE) on the central sagittal-axial plane (the area indicated in Fig. 1B). The simulation considered only the geometrical factor, otherwise other factors characteristic to the radiations, such as absorption, coherent scattering, back-scattering, and *etc.* were ignored. The geometrical efficiency was recalculated as a normalized value, *i.e.* the relative detection

Fig. 3. Geometrical Arrangement of the Simulated RDE and Rat Liver

(A) Simulated mapping of the RDE. (B) NMR image of sagittal section of the upper abdomen of an eight week old normal Wistar male rat.

efficiency, taking into account the data collected from the ^{152}Eu phantom. Therefore, the highest geometrical efficiency, obtained at the center point of the slit entrance, is normalized to be 100%. As shown in Fig. 3A, the RDE gradually decreases with distance from the slit. The expansion of detectable area along the axial direction was relatively small. Figure 3B illustrates the position of the liver of an 8 week old normal male Wistar rat, which was fixed on the rat holder stomach side down. The liver occupies most of the area under the diaphragm. This position covers most of the detectable area shown in Fig. 3A.

The simulated values of RDE with geometrical effects on the central line indicated in Fig. 3A are re-plotted in Fig. 4A as a function of distance from the slit (crosses). The RDE gradually decreased such that at the 35 mm point it was 40%. The average loss of γ -rays through the slit was 36.3% when calculated from the range of 0 to 35 mm (which was the range occupied by the rat holder and the upper abdomen).

The triangles and circles in Fig. 4 show the RDE of γ -rays for the various positions of the ^{152}Eu source measured with or without rat holder on the slit. The RDE simulated with geometrical effects was in good agreement with the measured ones. The RDE measured with the 2 mm rat holder (triangle) tended to decrease compared to the RDE without rat holder. Although, the losses of γ -rays by the rat holder were dependent on the γ -ray energies and distance from the slit, this loss amounted to no more than 4% of the total. When the γ -rays were detected through the rat body (squares), the RDE was smaller than the simulated values, especially in the case of the low-energy γ -rays. The RDE through the rat head decreased to 72.3–83.8% of the geometrically simulated values for 122–1408 keV γ -rays. The RDE measured through

Fig. 4. The Comparison of Simulated and Measured Values of RDE and the Corresponding Correction Considering the γ -Ray Energy

(A) The plot of simulated and measured values of RDE as a function of the distance from the slit. The crosses indicate the simulated RDE. Circles are the measured RDE using the ^{152}Eu point source. Triangles are the measured RDE through the rat holder positioned on the slit. Squares indicate the measured RDE through the rat head (25 mm) and upper abdomen (33 mm). Gray color scales of the marks are dependent on the energy of the γ -rays. (B) Energy of γ -rays and the averaged losses of the RDE in a rat body. Ratio of measured RDE to the simulated value (%) were averaged between the 0–35 mm range and plotted with γ -ray energies. The differential absorbance of γ -rays by the rat body is due to the different energies of the γ -rays and was corrected based on the data shown in the figure.

the rat upper abdomen were 63.4–78.6% of the geometrically simulated values. The ratio of measured RDE to the simulated value (%) were averaged within the 0–35 mm range and plotted against the γ -ray energies (Fig. 4B). From this loss of data the RDE in the rat upper abdomen was estimated to be 10.7–18.3% for 122–1408 keV. The *in vivo* relative distribution was corrected according to the γ -ray energy using the equation in Fig. 4B. The correction was done depending on the γ -ray energies. Since the averaged loss of RDE was dependent on the sample volume, the correction was recalculated for every animal group having different body weights (thickness of upper abdomen).

Figure 5A shows the normalized uptake rates of ^{74}As in various organs of normal and SeD rats 3 h after administration of multitracer solution. The blood shows markedly higher concentrations of the ^{74}As . The plasma, however, showed very low concentrations of the ^{74}As . This suggests that ^{74}As is mainly taken into red blood cells. The kidney, spleen, and bone also showed a relatively high uptake of ^{74}As . The relatively high ^{74}As in the kidney may reflect its concentration during the urinary excretion. The spleen and bone marrow are the hemopoietic organ/tissue.

Figure 5B shows the normalized uptake rates of ^{83}Rb in various organs of rats 3 h after administration. The ^{83}Rb was present in the blood but not in the plasma; more precisely, ^{83}Rb is only in the red blood cells. Since the hematocrit of rats is about 50, ^{83}Rb level in the red blood cells should be double as much as the blood. The brain showed low levels of

Fig. 5. The Normalized Uptake Rates of ^{74}As and ^{83}Rb in Various Organs of Normal and SeD Rats Isolated 3 h after Administration of the Multitracer Solution

The normalized uptake rates of (A) ^{74}As , (B) ^{83}Rb , and (C) ^{103}Ru .

^{83}Rb presumably because brain is protected by the blood-brain-barrier. The range of the normalized uptake rates of the ^{83}Rb among the organs except for the brain and plasma was about 70–250%, which was the lowest variability compared with the other elements. For example, the normalized uptake rates of the ^{59}Fe among various organs ranged about 10–2500% (data not shown).

Figure 5C shows the normalized uptake rates of ^{103}Ru in various organs of rats 3 h after administration. The ^{103}Ru was present in blood at half the level as in plasma. Very little of the ^{103}Ru was detected in brain. Relatively high levels of ^{103}Ru were measured in kidney 3 h after administration. The range of distribution of ^{103}Ru was quite variable but the amounts were relatively low 3 h after administration. These results suggest that the ^{103}Ru exists as a membrane impermeable form. Therefore, ^{103}Ru exists in the blood plasma and extracellular fluid, and was excreted into the urine by the kidney with a relatively fast rate.

For the normalized uptake rates of ^{74}As , ^{83}Rb , and ^{103}Ru , no significant difference was obtained between the normal and SeD rats, except that the ^{103}Ru in kidney was higher in normal rat than SeD rat.

Figure 6 shows the ^{83}Rb -standardized uptake rate of several elements in the removed blood and the excised liver 3 h after injection of the multitracer. The ^{83}Rb -standardized uptake rate of ^{74}As and ^{103}Ru , i.e. $^{74}\text{As}/^{83}\text{Rb}$ and $^{103}\text{Ru}/^{83}\text{Rb}$ ratio, in the corrected blood were 17.27 ± 3.28 and 2.07 ± 0.16 for normal rat, and 22.05 ± 4.82 and 2.50 ± 0.47

Fig. 6. The ^{83}Rb -Standardized Uptake Rates of Several Elements in Normal and SeD Rat Liver and Blood

(A) The blood and (B) the liver were isolated 3 h after injection of the multitracer.

for SeD rat respectively. ^{83}Rb -standardized uptake rates of ^{74}As and ^{103}Ru in the isolated liver were below 1.

Figure 7A shows the ^{74}As -standardized *in vivo* distributions of various trace elements in the upper abdomen of rats 3 h after administration of the multitracer. Figure 7B shows the ^{83}Rb -standardized *in vivo* distribution in the upper abdomen of rats 3 h after administration of the multitracer. The ^{74}As - and ^{83}Rb -standardized *in vivo* distributions indicate standardized values depending on the contributions from the blood (red blood cells) volume, and intracellular volume (blood cells and tissues). The *in vivo* distributions of elements, now, can be comparable between normal and SeD rat groups using those factors, even though rat groups have markedly different body weights.

If it is assumed that *in vivo* distribution of ^{74}As came entirely from the blood, the blood- ^{83}Rb can be estimated by the $^{74}\text{As}/^{83}\text{Rb}$ ratio. The tissue- ^{83}Rb can be obtained by subtraction of the blood- ^{83}Rb from total *in vivo* ^{83}Rb . The ratio between the tissue- ^{83}Rb and blood- ^{83}Rb , *i.e.* tissue/blood volume ratio, was 41.92 ± 6.52 for normal rats ($n=3$) and 55.06 ± 6.16 for SeD rats ($n=3$). Since the measured tissue- ^{83}Rb is mainly from the liver in these experiments, it is likely that the liver/blood ratio of the SeD rats were larger than the normal rats, but not significantly.

Similarly, tissue- $^{83}\text{Rb}/\text{tissue-}^{103}\text{Ru}$ can also be related to the intracellular/extracellular volume ratio. Calculated values were 2.48 ± 1.10 for normal rats ($n=2$) and 3.46 ± 0.23 for SeD rats ($n=3$). This result also shows the trend that the intracellular volume of SeD rats liver may be larger than that of normal rats, although these values are not significant.

DISCUSSION

The subject-slit-detector arrangement in this paper looks

Fig. 7. *In Vivo* Distributions of Various Elements in the Upper Abdomen Measured 3 h after Administration of the Multitracer Solution and Standardized Uptakes of Various Elements in the Liver Isolated 3 h after Administration of the Multitracer Solution into Rats

(A) ^{74}As -Standardized *in vivo* distributions of various elements in the upper abdomen. (B) ^{83}Rb -Standardized *in vivo* distributions of various elements in the upper abdomen.

well suited for *in Vivo* measurements of rat liver. When rats were set stomach side down on the slit, approximately 73–74% of the information was from the liver. Therefore, the elemental pattern obtained in the isolated liver (Fig. 6B) and obtained by the *in vivo* measurement of upper abdomen (Fig. 7) showed similar patterns.

The performance of the technique at the present time is limited due to slit shape and subject-slit-detector geometry. The geometrical factor mainly affects the accuracy due to the single detector measurement. Therefore, the slit shape and the subject-slit-detector geometry should be carefully optimized to a subject animal species and for a region of interest. The coherent scattering of radiation from a subject body and the slit materials should also be corrected in the data acquisition to achieve more accurate quantitative measurements of γ -rays in general. However, the fundamentally low quantitative ability of the multitracer itself does not require such an accurate measurement in this time. The error from individual biological systems of subject animals was quite large, while the distinct advantages of the *in vivo* measurement are that the time course^{18–20} of the relative amount of tracers could be compared to each other in an individual animal, and that the information relating to the intact tissues and/or organs are available.

The dose of multitracer was difficult to standardize, since the amounts and ratios of radioactive isotopes synthesized in the target were changeable by the condition of the heavy ion beam irradiation. Therefore, we proposed the use of a normalized uptake rate in order to diminish the effect of different doses.^{10,14} Even if the normalized uptake rate is used, comparison is difficult among animal groups with different

age and pathological conditions. In addition, we cannot know the actual volume of a tested animal in *in vivo* experiments. Therefore, an internal standard is required for comparing elemental distributions among different animal conditions. The use of either ^{83}Rb or ^{74}As was as an internal standard of the *in vivo* relative distribution in this paper made it possible to compare the elemental distribution between rat groups with markedly different body weights.

^{83}Rb belongs to the Ia group in the periodic table of the elements and acts similarly to potassium which is main intracellular location. The distribution of ^{74}As indicates the distribution of blood alone, although the accumulation of arsenic into red blood cells is a characteristic phenomenon for rats.^{21–23} In addition, ^{103}Ru was also proposed as another probe reflecting extracellular volume.

In this paper, the different tissue/blood volume ratios and intracellular/extracellular volume ratios between normal and SeD rats were also estimated by ^{74}As , ^{83}Rb , and ^{103}Ru . Although the difference was not significant, the trend agreed well with the fact that the hepatocytes of SeD rat were swollen.¹⁴ The SeD rat liver may have a larger cytoplasmic volume than the normal rat liver. Therefore, the combination of ^{74}As , ^{83}Rb , and ^{103}Ru is applicable as indices of the tissue/blood and intracellular/extracellular volume ratios. The levels of ^{74}As and ^{83}Rb can principally indicate the blood and tissue volume in a measured region of rat at a particular time point. Therefore, a decrease in the $^{74}\text{As}/^{83}\text{Rb}$ and/or $^{103}\text{Ru}/^{83}\text{Rb}$ ratio may also indicate ischemia. The ratios measured will be more accurate when the required radioactive isotopes are extracted from the multitracer, or suitably tailored according to an experimental purpose. In addition, the method will be more powerful when it is combined with imaging techniques such as SPECT (single photon emission computed tomography),^{24,25} MTGEI (multitracer gamma-ray emission imaging),^{26,27} or a highly sensitive direct conversion detector gamma camera imaging.²⁸

Although the shape of the slit and the subject-slit-detector arrangement should be examined in future experiments to obtain information from other animals and/or other regions, the possibility of functional estimation of tissue blood volume and/or ischemia was proposed using ^{74}As and ^{83}Rb and ^{103}Ru in the multitracer. Our results show the potential of combining radioactive tracers as a tool that provides useful functional information in living experimental animals.

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