Article

6- ^{18}F] Fluoro-Dehydroascorbic Acid : Synthesis and Tissue Distribution in Mice

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6-Deoxy-6- ^{18}F]fluoro-L-threo-2,3-hexodiulosonic acid γ -lactone (6- ^{18}F]fluoro-dehydroascorbic acid, ^{18}F -FDHA) was synthesized by oxidation of 6-deoxy-6- ^{18}F]fluoro-L-ascorbic acid (^{18}F -DFA) with Br_2 , a route that is analogous to the original synthesis of FDHA, in order to develop a radiotracer for nuclear medical imaging. Tissue biodistribution of ^{18}F -FDHA in fibrosarcoma-bearing mice showed a low accumulation of radioactivity in the brain, with both substantial in vivo defluorination and low tumoral uptake levels. Several organs of mice unfed for 24 h showed a more significant increase in radioactivity postinjection of ^{18}F -FDHA than did fed mice. Co-injection with 2-deoxyglucose of ^{18}F -FDHA did not significantly alter brain and tumor uptake, as confirmed by tissue-to-blood ratios, and as compared to control experiments. Thus, ^{18}F -FDHA does not exhibit desirable properties as a brain-targeting imaging agent for ascorbic acid bioactivity.

Key Words : 6-deoxy-6- ^{18}F]fluoro-L-threo-2,3-hexodiulosonic acid γ -lactone,
radiosynthesis, tissue distribution, brain, fibrosarcoma, mouse

1. Introduction

Oxidative stress in the living body can induce various pathological conditions such as cancer, neurological disorders and aging¹⁾. Dehydroascorbic acid (DHA) is an oxidized form of L-ascorbic acid (AsA) that plays crucial roles in tissue redox homeostatics as a biologic antioxidant molecule. DHA is either not detectable or found only at low levels in blood circulation and tissues under normal physiological conditions, but it can be used to regenerate AsA in

many types of cells²⁾⁻⁴⁾. Recent studies on transport mechanisms demonstrated that the facilitative glucose transporters (GLUTs) are efficient transporters for DHA, whereas AsA is transported through the sodium-dependent transporters⁵⁾⁻⁷⁾. It was demonstrated more than 50 years ago that, by whole autoradiography of a mouse injected with ^{14}C -labeled DHA, considerable radioactivity uptake was observed in the central nervous system at early time points after injection⁸⁾. Recently, rapid transport of DHA across the blood-brain barrier (BBB) through the GLUTs and its retention in the brain of AsA as a result of reduction of transported DHA have been proven in rats and mice using ^{14}C -labeled tracers⁹⁾. This DHA-

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GLUTs transport mechanism is also a means by which tumor cells accumulate and retain AsA *in vivo*¹⁰. These findings suggest that the transport and reduction of DHA in cells may be important steps regulating tissue uptake and accumulation of AsA.

We anticipated that radiolabeled analogs of AsA with selective transport characteristics would provide a new probe for *in vivo* assessment of tissue redox status resulting from oxidative stress by a nuclear imaging technique. In our previous studies, we reported on the synthesis and characterization of 6-deoxy-6-[¹⁸F]fluoro-L-ascorbic acid (¹⁸F-DFA) as a positron-labeled analog of AsA for the evaluation of ascorbate bioactivity in the brain^{11)–13)}. Experiments on rodent distribution of ¹⁸F-DFA demonstrated that uptake in the brain was low, but with a progressive increase in brain radioactivity with time, similar to that observed for ¹⁴C-labeled AsA. In view of the uptake behavior of the prodrug type of DHA *in vivo*, in spite of its limited stability in aqueous solution, we became interested in the use of the oxidized form of ¹⁸F-DFA as a potential molecule to improve the bioavailability of ¹⁸F-DFA to the brain. The purpose of this study is to prepare 6-deoxy-6-[¹⁸F]fluoro-L-threo-2,3-hexodiulosonic acid γ -lactone (6-[¹⁸F]fluoro-dehydroascorbic acid, ¹⁸F-FDHA), and evaluate its biodistribution properties in tumor-bearing mice in comparison with that of ¹⁸F-DFA.

2. Experimental

2.1 General

Pure samples of 6-deoxy-6-fluoro-L-ascorbic acid (DFA) and 6-deoxy-6-fluoro-L-threo-2,3-hexodiulosonic acid γ -lactone (6-fluoro-dehydroascorbic acid) (FDHA) were prepared as described previously^{11), 12)}. The chemical reagents

and solvents were of commercial quality and were used without further purification unless otherwise noted. Thin layer chromatography (TLC) was performed on silica gel 60F₂₅₄ plate (Merck), and the spots were visualized with UV light or by spraying with 5% alcoholic molybdophosphoric acid. Analysis of the radioactivity of the TLC plates was performed with an ALOKA radio chromatogram scanner. HPLC was done using a TOSOH CCP & 8020 series HPLC system by monitoring the radioactivity as well as the UV absorption (at 254 nm). The radioactivity was also quantified with a Capintec radioisotope calibrator (CRC-30). Identity of the labeled compounds was confirmed by co-injection with authentic samples into HPLC and TLC. Radiochemical yields were expressed at the end of synthesis (not corrected for decay), relative to the amount of [¹⁸F]fluorinating agent measured as total radioactivity present in the reaction vessel.

All animal experiments were carried out in accordance with the regulations on animal experiments of the Faculty of Pharmaceutical Sciences, Kyushu University.

2.2 Preparation of 6-deoxy-6-[¹⁸F]fluoro-L-threo-2,3-hexodiulosonic acid γ -lactone (6-[¹⁸F]fluoro-dehydroascorbic acid, ¹⁸F-FDHA, Scheme 1)

Fluorine-18 was produced from 8 or 16% enriched [¹⁸O]H₂O by the ¹⁸O(p, n)¹⁸F reaction, as described previously¹⁴⁾. Tetramethylammonium [¹⁸F]fluoride (Me₄N¹⁸F) was prepared by the addition of 10% tetramethylammonium hydroxide in methanol (3 μ L) to the irradiated water in a TPX (polymethylpentene) vessel and subsequent removal of the water under a stream of argon by co-evaporation with dry acetonitrile. 6-Deoxy-6-[¹⁸F]fluoro-L-ascorbic acid (¹⁸F-

Scheme 1 Synthesis of 6- ^{18}F fluoro-dehydroascorbic acid (^{18}F -FDHA)

DFA) was prepared by a slight modification of the method described previously^{11), 12)}. Briefly, methyl 2,3-*o*-isopropylidene-2-keto-L-gulonate 4,6-cyclic sulfate (1–2 mg) dissolved in dry acetonitrile (300 μL) was added to a TPX vessel containing the $\text{Me}_4\text{N}^{18}\text{F}$ (37–74 MBq). The mixture was refluxed at 120 $^\circ\text{C}$ for 15 min and then the solvent was removed under a stream of argon. The residue was dissolved in 40% aqueous H_2SO_4 (300 μL) and the mixture was heated in a closed vessel at 80 $^\circ\text{C}$ for an additional 15 min. After briefly cooling, aliquots of the reaction mixture were analyzed by radio-HPLC (mobile phase : 0.04 M Na_2HPO_4 with 0.1% triethylamine, pH = 5.65, column : COS-

mixture was then extracted with ethyl acetate. After the organic layer was evaporated in vacuo to remove the solvent, the crude radioactive product, ^{18}F -DFA, was used for the next step without further purification, although the original method that we developed for the synthesis of ^{18}F -DFA involved a HPLC purification step¹¹⁾.

The ^{18}F -DFA obtained was redissolved in methanol (2 mL) and to this solution was added one gt. of bromine using a syringe. Immediately after the addition of bromine, the mixture was evaporated in vacuo to remove excess amount of bromine and solvent. The resulting mixture was injected into reverse-phase HPLC

Fig. 1 (A) HPLC radiochromatogram of purified ^{18}F -DFA. (Column : COSMOSIL 5C18 AR-II 4.6×250 mm, Mobile phase : 0.04 M Na_2HPO_4 with 0.1% triethylamine (pH=5.65), Flow rate : 0.7 mL/min) (B) HPLC radiochromatogram of the reaction mixture obtained by oxidation of ^{18}F -DFA with Br_2 , prior to purification. (Column : COSMOSIL 5C18 AR-II 4.6×250 mm, Mobile phase : 0.04 M Na_2HPO_4 with 0.1% triethylamine (pH=5.65), Flow rate : 0.7 mL/min) (C) TLC radiochromatogram of the reaction mixture obtained by oxidation of ^{18}F -DFA with Br_2 , with the addition of non-radioactive reference substances to the mixture for comparison. (silica gel 60F₂₅₄ plates (Merck), EtOAc : MeOH : AcOH = 5 : 1 : 1)

(mobile phase : 0.04 M Na_2HPO_4 with 0.1% triethylamine, pH = 5.65, column : COSMOSIL 5C18 AR-II 4.6×250 mm, flow rate = 0.7 mL/min). The radioactive fraction corresponding to FDHA ($R_t = 7.6$ min) was collected. The final product was obtained as a solution of the HPLC eluate, ready for animal studies. Total synthesis time was 70 min and the radiochemical yields were 3.5–4.0% in several experiments. The specific activity of ^{18}F -FDHA was estimated to be around 350 GBq/mmol from that of an ^{18}F -DFA preparation in the same synthetic runs. Neither radiochemical nor chemical contaminations were detected by HPLC analysis performed immediately after the end of the synthesis.

2·3 Biodistribution in tumor-bearing mice

NFSa-fibrosarcoma was inoculated s.c. into the right hind leg muscle of female C3H/He mice (5 weeks old, 18–20 g). These mice which developed tumors with a diameter of about 1 cm at 9–14 days after inoculation were used for in vivo biodistribution studies.

Two groups of mice were studied. The first was given access freely to food and water during the investigation (fed group). The second group was prevented from eating for 24 h but allowed access to water before the investigation. The mice unfed for 24 h were also used in 2-deoxyglucose co-injection experiments.

The fibrosarcoma-bearing fed and unfed mice were intravenously injected through the tail vein with ^{18}F -FDHA (185–370 kBq) dissolved in a buffer solution (0.3 mL, pH = 5.65) obtained by HPLC purification. ^{18}F -FDHA was also administered intravenously in the unfed mice together with 2-deoxyglucose (5.3 mg/mouse). This dose of 2-deoxyglucose has been reported to have a significant effect leading to

uptake inhibition of ^{14}C -labeled DHA into the mouse brain^{9),10)}. In a separate set of experiments, the same tumor-bearing fed mice were injected with ^{18}F -DFA (250 kBq) dissolved in the same buffer solution in order to determine their tissue uptake at an early time point. The mice were sacrificed by exsanguinations under anesthesia at the predetermined time after injection of ^{18}F -FDHA or ^{18}F -DFA. A sample of 0.2–0.5 mL of blood was collected at the time of euthanasia. Samples of the tissues and tumors were excised immediately and weighed. The tissue radioactivity was counted by gamma counter (ALOKA, ARC-400) and corrected for decay. The tissue radioactivity levels were calculated as a percentage of the injected dose per gram of tissue (%ID/g tissue). The uptake ratio of tissue-to-blood was also used as an indicator of specificity and accumulation of radioactivity in tissues.

2·4 Statistical Analysis

All values are expressed as the mean $\pm$ S.D. and a lack of a significant difference using the Student's *t*-test was considered to be $p > 0.05$.

3. Results and Discussion

On the basis of the previously reported oxidation of DFA which gives FDHA, we reasoned that the preparation of ^{18}F -DFA and its subsequent oxidation should provide the target-labeled compound, as illustrated in Scheme 1. Thus, intermediate ^{18}F -DFA was prepared by a slight modification of the method described previously¹¹⁾ and isolated from the reaction mixture by extraction with ethyl acetate to give high radiochemical purity, as evidenced by HPLC analysis (Fig. 1A). The subsequent conversion of ^{18}F -DFA into ^{18}F -FDHA by treatment with Br_2 was completed almost instantaneously.

Table 1 Biodistribution of ^{18}F -FDHA and ^{18}F -DFA in tumor-bearing mice

Organ	Uptake (%dose/g) ^{a)}					
	^{18}F -FDHA				^{18}F -DFA	
	2 min	10 min	30 min	60 min	2 min	60 min ^{b)}
Blood	9.36 $\pm$ 0.68	5.25 $\pm$ 0.35	1.62 $\pm$ 0.29	0.87 $\pm$ 0.12	4.01 $\pm$ 0.44	1.90 $\pm$ 1.11
Lung	6.44 $\pm$ 1.02	6.34 $\pm$ 0.64	2.94 $\pm$ 0.80	2.26 $\pm$ 0.09	30.10 $\pm$ 2.69	7.27 $\pm$ 2.49
Liver	7.10 $\pm$ 0.72	6.20 $\pm$ 0.83	2.12 $\pm$ 0.41	1.29 $\pm$ 0.18	21.21 $\pm$ 1.82	6.23 $\pm$ 2.20
Small intestine	5.47 $\pm$ 0.46	6.46 $\pm$ 1.39	2.30 $\pm$ 0.36	2.25 $\pm$ 0.73	29.88 $\pm$ 2.53	9.71 $\pm$ 4.34
Adrenals	4.11 $\pm$ 1.98	5.39 $\pm$ 1.84	4.24 $\pm$ 1.37	2.51 $\pm$ 0.98	23.43 $\pm$ 8.20	12.90 $\pm$ 7.83
Kidneys	14.66 $\pm$ 1.73	11.28 $\pm$ 0.93	3.56 $\pm$ 0.43	2.06 $\pm$ 0.24	41.42 $\pm$ 6.64	8.31 $\pm$ 2.55
Heart	6.35 $\pm$ 0.75	4.37 $\pm$ 0.47	2.04 $\pm$ 0.65	1.10 $\pm$ 0.02	4.78 $\pm$ 0.17	1.73 $\pm$ 0.39
Muscle	2.94 $\pm$ 0.37	4.47 $\pm$ 3.70	1.16 $\pm$ 0.27	0.65 $\pm$ 0.14	1.86 $\pm$ 0.41	0.76 $\pm$ 0.17
Bone	11.76 $\pm$ 0.45	6.42 $\pm$ 1.94	12.54 $\pm$ 4.80	16.65 $\pm$ 1.93	2.04 $\pm$ 0.18	1.62 $\pm$ 1.06
Brain	0.41 $\pm$ 0.12	0.32 $\pm$ 0.03	0.34 $\pm$ 0.07	0.41 $\pm$ 0.05	0.73 $\pm$ 0.15	0.49 $\pm$ 0.08
Tumor	4.05 $\pm$ 0.12	3.85 $\pm$ 0.52	2.23 $\pm$ 0.17	1.54 $\pm$ 0.23	4.31 $\pm$ 0.49	3.21 $\pm$ 0.70

a) All data were shown as means $\pm$ S.D. (n=3)

b) Data from previous report (ref. 11)

neously, as shown in Fig. 1B and 1C. The required ^{18}F -FDHA was obtained, following preparative HPLC, with an isolated radiochemical yield (referring to the starting activity of ^{18}F fluoride) of 3.5–5.0% in a total preparation time of 70 min. Since FDHA displays no special absorption in the accessible ultraviolet region, the specific activity was estimated to be around 350 GBq/mmol from that of the precursor ^{18}F -DFA in the same synthetic runs. The overall radiochemical yield was low, but was in keeping with multistep radiofluorination reactions. As shown by TLC on silica gel and HPLC, the final product in the HPLC eluate solution was found to be >95% chemically and radiochemically pure. However, when the injection solution was allowed to stand at ambient temperature for 30 min after the end of preparation, HPLC analysis showed that one radioactive species ($R_t = 4.7$ min), except the parent compound ($R_t = 7.6$ min), appeared with a radioactivity ratio of about 30%, thus indicative of the limited radiochemical stability of ^{18}F -FDHA. This chemical species was not identified in this study. We considered at

this stage that this should not affect the suitability of the radiotracer for in vivo use if favorable pharmacokinetic behavior, such as a target-blood ratio of >1, towards the target brain could still be achieved.

After ^{18}F -FDHA was injected intravenously into the fibrosarcoma-bearing mice which were allowed free access to food and water, the tissue biodistribution of the tracer was determined at a function of time after injection. The results are shown in Table 1 and also included for comparison are data for ^{18}F -DFA. ^{18}F -FDHA showed the highest concentration of radioactivity in the kidney at early time points. The concentration of radioactivity in the brain at 2 min after injection was $0.41 \pm 0.12\%$ ID/g, representing about 60% of that observed for ^{18}F -DFA at the same time, and then remained at this level for up to 60 min after injection, thus demonstrating the poor targeting of radioactivity in the brain. The fibrosarcoma exhibited higher levels of radioactivity ($4.05 \pm 0.12\%$ ID/g) than the muscle ($2.92 \pm 0.37\%$ ID/g) at 2 min after injection, but with fast elimination of radioactivity from the tumor at later time

Table 2 Effect of co-injection with 2-deoxyglucose (2-DG) and lack of food on the accumulation of radioactivity at 2 min after injection of ^{18}F -FDHA in mice

Tissue	Tissue uptake (%dose/g) ^{a)}			Tissue-to-blood ratio ^{a)}		
	Free ^{b)}	Fasted ^{c)}	2-DG injection ^{d)}	Free ^{b)}	Fasted ^{c)}	2-DG injection ^{d)}
Blood	9.36 ± 0.68	19.03 ± 3.44	15.10 ± 2.02	-	-	-
Brain	0.41 ± 0.12	0.68 ± 0.26**	0.53 ± 0.06	0.043 ± 0.012	0.035 ± 0.007	0.035 ± 0.002
Bone	11.76 ± 0.45	9.68 ± 5.02	6.89 ± 1.46	1.263 ± 0.144	0.490 ± 0.161**	0.453 ± 0.036
Kidneys	14.66 ± 1.73	50.28 ± 6.66**	36.78 ± 7.68	1.564 ± 0.114	2.659 ± 0.171**	2.421 ± 0.252
Adrenals	4.11 ± 1.98	12.62 ± 3.96*	13.58 ± 6.98	0.429 ± 0.188	0.653 ± 0.088	0.899 ± 0.460
Tumor	4.05 ± 0.12	5.89 ± 0.41*	4.60 ± 0.67	0.435 ± 0.047	0.315 ± 0.049*	0.307 ± 0.048

a) means ± S.D. (n=3)

b) Free access for food group

c) Fasted for 24hr group

d) Co-injection of 2-DG with ^{18}F -FDHA into fasted for 24hr group mice

* $p < 0.05$, ** $p < 0.01$ compared with free access for food group (Student's t-test)

points. Considerable high bone radioactivity was observed at early time points, which reached about 35% ID/organ even at 2 min after injection, clearly indicating that ^{18}F -FDHA is susceptible to in vivo defluorination.

In tracer studies by Agus et al., approximately 4% ID/g of radioactivity was found in the mouse brain at 1 min after injection of ^{14}C -labeled DHA^{9),10)}. It was also reported that xenograft tumors accumulated 1 – 2% ID/g of radioactivity at 1 min after injection of ^{14}C -labeled DHA in mice¹⁰⁾. The results have proved that injected ^{18}F -FDHA yielded only low radioactivity in the mouse brain, being less than blood radioactivity with a moderate affinity for fibrosarcoma, unlike ^{14}C -labeled DHA. We did not attempt to identify the chemical species of radioactivity accumulated in the tissues due to our methodological limitation.

The mouse is an animal capable of synthesizing AsA from glucose in the liver. It is known that variations in diet can affect the mechanism for biosynthesis, metabolism and/or elimination of this vitamin^{15), 16)}, and a typical diet contains both AsA and DHA. We compared

the tissue distribution at 2 min after injection between in the unfed and fed mice to investigate the influence of the nutritional state on tissue accumulation of radioactivity after injection of ^{18}F -FDHA. We also evaluated the effect of co-injection of ^{18}F -FDHA with 2-deoxyglucose (2 mg/kg) in the unfed mice to test whether the accumulation of radioactivity observed was due to a specific uptake mechanism, because it has been reported that transport of ^{14}C -DHA into brain and tumor tissue is inhibited by 2-deoxyglucose⁹⁾. In these experiments we used two parameters, tissue uptake and the tissue-to-blood ratio of radioactivity, as the index to correct the delivery of the tracer. As is evident from Table 2, the unfed mice caused a statically significant increase of radioactivity in the brain, tumor, kidney and adrenal glands, in spite of much slower clearance of radioactivity from the blood compared to the fed mice. The tissue-to-blood ratios of radioactivity in the tumor, bone and kidney, except for the brain and adrenal glands, also varied significantly due to no food intake for 24 h. Thus, the results with the unfed mice indicate increased bioavailabil-

ity of radioactivity in some organs postinjection of ¹⁸F-FDHA, although the uptake mechanism is unclear.

On the other hand, co-injection with 2-deoxyglucose in the unfed group did not produce any significant change in either parameter compared to the control (fed group) except for the tumor uptake. Insensitivity to 2-deoxyglucose blocking in this study suggests that the GLUTs mechanism may not be involved in the delivery of radioactivity from ¹⁸F-FDHA into the brain. However, this notion might be premature because of the investigation using a single dose of 2-deoxyglucose at a single time point after injection. Recent studies on ascorbate transport in vitro have found that the oxidized forms of 6-chloro-6-deoxy-⁶⁾ and 6-bromo-deoxy-L-ascorbic acid¹⁷⁾ are not transported or bound by the GLUT-1 and -3, unlike DHA transport. It was further pointed out by Nishikawa et al.¹⁸⁾ that FDHA can not cyclize as a bicyclic hemiketal structure like DHA in aqueous solution, which is thought to be a structural requisite for interaction with the GLUTs, because the C₆-hydroxy group of the DHA structure is substituted by the fluorine atom (Scheme 2). This consideration may explain the observed low uptake of radioactivity in the brain after injection of ¹⁸F-FDHA, but it is largely speculation in the absence of detailed studies. In addition to defluorination, tracer degradation of ¹⁸F-FDHA, possibly in a manner similar to DHA it-

self¹⁹⁾, would also have contributed to the effect of reducing the influx of radioactivity into the brain.

4. Conclusion

In this study we have described a three-step synthesis of ¹⁸F-FDHA as a positron-labeled analog of DHA and its tissue biodistribution in mice by the simple means of uptake in vivo. The in vivo instability against defluorination and low cerebral uptake of radioactivity preclude its use as a positron-labeled analog of DHA for imaging. We are currently conducting further studies for new analogs of AsA both with good metabolic stability and better BBB permeability, mainly aiming at the structural modifications at the C₅-side chain of ascorbate molecule.

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Scheme 2 Predicted structure of 6-fluoro-dehydroascorbic acid (FDHA) in aqueous solution

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6- ^{18}F フルオロ-デヒドロアスコルビン酸：合成とマウス生体内分布

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核医学イメージング剤として標識アスコルビン酸の開発展開を目的として, 6-デオキシ-6- ^{18}F フルオロ-L-スレオ-2,3-ヘキソジウロソン酸 γ -ラクトン (6- ^{18}F フルオロ-デヒドロアスコルビン酸, ^{18}F -FDHA) の標識合成を, 6-デオキシ-6- ^{18}F フルオロ-L-アスコルビン酸に臭素分子を作作用させることにより達成した。線維筋腫 fibrosarcoma を移植したマウスにおける ^{18}F -FDHA の生体内分布を検討した。脳及び腫瘍組織への放射能集積は低く, 一方, 骨には非常に高い放射能の集積が観察された。24 時間絶食マウスでは, いくつかの臓器に放射能集積の増加が認められた。2-デオキシグルコースとの同時投与では, 脳及び腫瘍部位への放射能分布に影響を与えなかった。 ^{18}F -FDHA は, 脳や腫瘍を標的とするデヒドロアスコルビン酸のポジトロン標識プローブとして有用性を持っていないことがわかった。