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Metabolite analysis of [^{11}C]flumazenil in human plasma: Assessment as the standardized value for quantitative PET studies

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Analysis of carbon-11 labeled metabolites in plasma was carried out during positron emission tomography (PET) studies with a central benzodiazepine receptor ligand [^{11}C]flumazenil ([^{11}C]FMZ) in 24 human subjects (14–76 y.o.) including five normal volunteers and 19 patients with neurological disorders. Arterial plasma samples were obtained at 3, 5, 10, 15, 20, 30 and 60 min after i.v. injection of the tracer, and were analyzed by high-performance liquid chromatography. The rate of plasma [^{11}C]FMZ degradation was associated with a large individual variation, but no significant difference was found in the degradation of [^{11}C]FMZ either between male and female, young and old, or between normal subjects and patient groups. When the mean fraction of unchanged [^{11}C]FMZ at each time point was used instead of individually measured metabolite data for the arterial input function, as much as a 30% error occurred in the distribution volume of the [^{11}C]FMZ binding in the brain. These results indicate that the mean percentage of unchanged [^{11}C]FMZ fraction in subjects cannot be used as the standardized value, and that the analysis of metabolites in plasma is necessary to determine the exact arterial input function for quantitative PET measurement.

Key words: [^{11}C]flumazenil, metabolism, benzodiazepine receptor, human, PET

INTRODUCTION

FOR QUANTITATIVE POSITRON EMISSION TOMOGRAPHY (PET), the radioactive tracer concentration in arterial plasma is used as an input function based on a kinetic model. Because only a limited number of positron-emitting radiopharmaceuticals are free of metabolic alteration, on most occasions analysis of labeled metabolites in the plasma should be performed to provide the exact input function for quantitative PET measurement.[1,2]

[^{11}C]Flumazenil ([^{11}C]FMZ) has a high and selective affinity for benzodiazepine receptors in the central nervous system and has been used for research and diagnosis of psychiatric and neurological disorders by PET.[3,4] The metabolism of this tracer in human plasma has been investigated with thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), or other simplified methods.[3] As labeled metabolites, an acidic metabolite Ro 15-3890 and a lipophilic metabolite (probably the hydroxy-ethyl ester Ro 15-7965) have been separated from the unchanged [^{11}C]FMZ,[5–7] although another investigator group has not detected the latter metabolite.[8,9] For rapid separation of the unchanged [^{11}C]FMZ from metabolites, an ion-exchange column[10] or a C-18 cartridge[6,7] allows many samples to be analyzed, but [^{11}C]FMZ was not completely separated from metabolites with these columns. The HPLC-method obviously gives a better resolution of the metabolites.[3] To eliminate the troublesome work of metabolite analysis in routine PET

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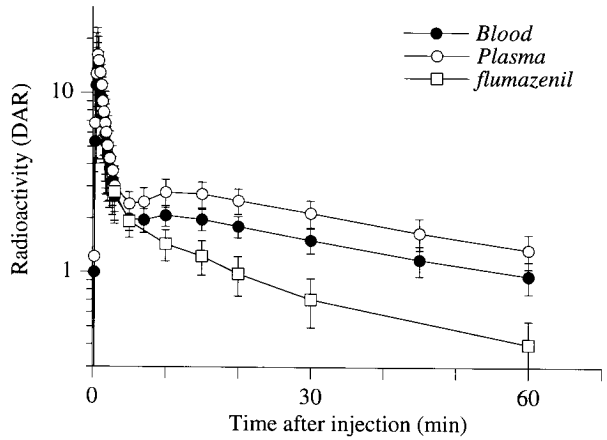

Fig. 1 Time-radioactivity curves of blood and plasma after i.v. injection of [^{11}C]flumazenil in human subjects. The level of radioactivity was expressed as the DAR.

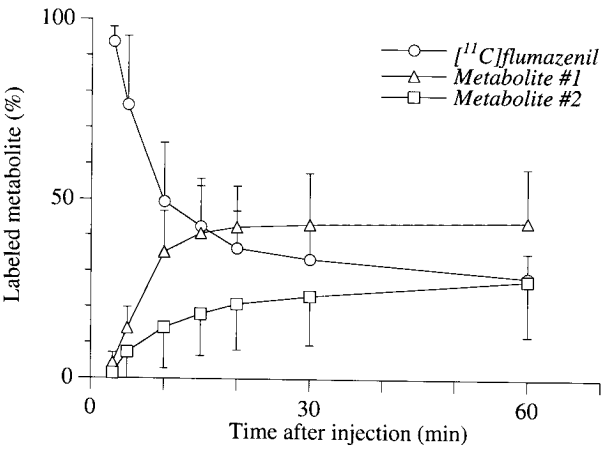

Fig. 2 Percentage of ^{11}C-labeled metabolites in the plasma after i.v. injection of [^{11}C]flumazenil into human subjects.

studies, Delforge et al. approximated the unchanged plasma [^{11}C]FMZ time-concentration curve with a biexponential function in baboon studies.[11] When there is a large individual variation in metabolism, however, this assumption is suspected of resulting in a significant error in PET measurement.

In the present work, we performed the metabolite analysis of [^{11}C]FMZ in the plasma of 24 human subjects by HPLC, and examined whether the average unmetabolized [^{11}C]FMZ fraction can be used as a standardized value for the quantitative PET measurement of the benzodiazepine receptors in the human brain.

MATERIALS AND METHODS

Synthesis of [^{11}C]flumazenil

FMZ and its desmethyl FMZ were generously supplied by Hoffman-La Roche (Basel, Switzerland). [^{11}C]FMZ was prepared by ^{11}C-methylation of desmethyl FMZ as described.[12] [^{11}C]Methyl iodide prepared as described[13] was trapped in 0.25 mL of dimethylformamide solution containing 0.25–0.5 mg desmethyl FMZ and 1 mg of sodium hydride. The solution was heated at 120°C for 1 min. After adding 1.3 m*l* of 0.05 M HCl, the solution was applied to HPLC. The column used was a YMC-Pack ODS-A (S-5 μm, 120 Å, 10 mm i.d. × 250 mm) and the mobile phase was a mixture of acetonitrile and 10 mM phosphoric acid (4/6, v/v) with a flow rate of 7 m*l*/min. The [^{11}C]FMZ fraction was eluted at 4.0–4.5 min and evaporated to dryness. The residue was dissolved in a mixture of physiological saline and 0.7% $NaHCO_3$ (1 1/1, v/v) and filtered through a 0.22 μm membrane filter. The specific activity was 55 ± 32 GBq/μmol (range, 16 to 182 GBq/μmol).

Measurement of labeled metabolites in human plasma

Metabolic analysis was carried out in 24 human subjects (9 men and 15 women; 49.8 ± 20.3 y.o.; range 14–76 y.o.) including five normal subjects (61.4 ± 6.1 y.o.; range 57–72 y.o.), five patients with Alzheimer's disease (70.4 ± 4.6 y.o.; range 65–76 y.o.), four patients with cerebral infarction (65.0 ± 7.5 y.o.; range 55–72 y.o.), nine epileptic patients (27.0 ± 9.8 y.o.; range 14–45 y.o.), and a patient with brain tumor (41 y.o.). The dose of [^{11}C]FMZ administered was 9.1 ± 2.6 MBq/0.33 ± 0.12 nmol/kg body weight.

After i.v. injection of the tracer into the subjects, the arterial blood was sampled at 0.17, 0.33, 0.5, 0.67, 0.83, 1.0, 1.17, 1.33, 1.5, 1.67, 1.83, 2, 2.25, 2.5, 3, 5, 7, 10, 15, 20, 30, 45 and 60 min. The sampled blood volumes were 1 m*l*. To analyze the labeled metabolites, 3 m*l* of the additional blood was taken at 3, 5, 10, 15, 20, 30 and 60 min. The blood and plasma were weighed and measured for radioactivity with an NaI(Tl) auto-gamma scintillation counter. The level of radioactivity was expressed as the differential absorption ratio {DAR, [sample radioactivity/total injected radioactivity]/[sample weight (g)/body weight (g)]}.

For measurement of plasma metabolites, the plasma sample was treated with a third volume of CH_3CN containing 20% trichloroacetic acid in an ice-water bath. Two min later the suspension was centrifuged at 6,000 *g* for 2 min at 2°C and divided into the acid-soluble and acid-precipitable fractions. The precipitate was washed twice with 0.5 m*l* of CH_3CN containing 10% trichloroacetic acid. The supernatant was combined with the acid-soluble fraction. After adding 10 μL of 0.1 mM authentic FMZ, the concentration of CH_3CN in the combined acid-soluble supernatant was made 30% by adding water. Then the solution was analyzed by HPLC: column, Nova-pak C8 equipped with an RCM 8 × 10 compression module (Waters); eluent, CH_3CN: 50 mM sodium acetate, pH 4.5 (3 : 7, v/v); flow rate, 2 m*l*/min. The elution profile was detected with a radioactivity monitor (FLO-ONE/Beta A200, Packard). A portion of the applied sample was also measured for radioactivity to calculate the recovery yield of the radioactivity in the HPLC. As the radioactivity was

corrected for the physical decay of ^{11}C, the recovery of the radioactivity in the HPLC analysis was essentially quantitative. The percentage of the unchanged [^{11}C]FMZ fraction in the total plasma radioactivity was calculated as well as its plasma concentration in DAR units.

PET measurement of [^{11}C]flumazenil binding to the human brain

For each subject, a serial dynamic scan was performed for 60 min starting at the time of [^{11}C]FMZ injection with a PET scanner Headtome-IV[14] which provided 14 slices of images with center-to-center intervals of 6.5 mm. The image spatial resolution was 7.5 mm FWHM and the axial resolution was 9.5 mm FWHM. A kinetic analysis was carried out with a single tissue-compartment model to estimate the rate constant of tracer incorporation (K_1) and washout (k_2), and the K_1/k_2 ratio was computed as the distribution volume (DV) reflecting the binding capacity of benzodiazepine receptors.[15] As an input function for the analysis, we used the plasma concentration of unchanged [^{11}C]FMZ that was computed from the fraction of unchanged [^{11}C]FMZ in the plasma interpolated through the scanning period. To assess whether the mean percentage of unchanged [^{11}C]FMZ fraction averaged across the subjects can be used as a standardized metabolite data for the input function instead of the individual metabolite data, regions of interest were placed over the cortical area and cerebellum in five patients and the DV values were calculated by using the mean values for the unchanged [^{11}C]FMZ fraction and the individual plasma radioactivity curve.

Table 1 Percentage of unchanged form in the plasma after i.v. injection of [^{11}C]flumazenil into human subjects

		10 min	30 min
Male	(53.9 ± 18.4 y.o., n = 9)	52.1 ± 15.1	33.5 ± 9.8
Female	(47.4 ± 21.5 y.o., n = 15)	51.0 ± 12.8	33.9 ± 9.9
Young	(24.8 ± 7.6 y.o., n = 8)	50.1 ± 17.3	34.7 ± 12.5
Old	(65.1 ± 6.9 y.o., n = 14)	51.9 ± 11.2	32.8 ± 8.4
Normal	(61.4 ± 6.1 y.o., n = 5)	52.3 ± 9.4	35.2 ± 3.2
Epilepsy	(27.0 ± 9.8 y.o., n = 9)	47.7 ± 15.7	34.3 ± 11.7
Alzheimer's disease	(70.4 ± 4.6 y.o., n = 5)	48.7 ± 15.8	29.9 ± 12.5
Cerebral infarction	(65.0 ± 7.5 y.o., n = 4)	54.8 ± 8.6	33.5 ± 7.5

No significant difference between sex or age or diseases ($p > 0.05$)

RESULTS AND DISCUSSION

Time-radioactivity curves of the whole blood and plasma after injection of [^{11}C]FMZ are summarized in Figure 1. After i.v. injection of the tracer, the level of radioactivity in the blood and plasma both decreased rapidly for the first 5 min. The radioactivity increased for the next 5 min, and then decreased. The level of radioactivity was higher in the plasma than in the whole blood over 60 min.

Radioactive metabolites in the acid-soluble fraction were analyzed by radio-HPLC. In the deproteinized plasma, in which protein bound [^{11}C]FMZ was recovered in the acid-soluble fraction, at least two radioactive metabolites (retention time: peak #1, 2.0 min; and peak #2, 3.3 min) were detected besides unchanged [^{11}C]FMZ (retention time: 5.4 min) that was identified by the retention time of the co-injected authentic sample. Contrasted with previous results,[5–7] the metabolites appearing as

Table 2 Percentage of unchanged plasma [^{11}C]flumazenil in human subjects as arbitrarily classified into three groups based on the degradation rate

3 min	5 min	10 min	15 min	20 min	30 min	60 min
Group 1, slow degraders (53.3 ± 20.1 y.o., n = 7)						
97.4 ± 1.7**	90.6 ± 2.0***	67.1 ± 9.2***	58.2 ± 10.5***	45.6 ± 12.4*	44.5 ± 9.0***	34.1 ± 8.2
Group 2, medium degraders (48.9 ± 21.7 y.o., n = 15)						
92.9 ± 4.1	76.6 ± 8.2	47.1 ± 5.6	38.0 ± 5.7	34.9 ± 4.1	32.1 ± 5.2	28.0 ± 4.3
Group 3, fast degraders (46 and 67 y.o., n = 2)						
88.4	66.2	27.4	20.1	19.8	15.8	12.8

Mean ± s.d. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ (Student's t-test between group 1 and group 2)

Table 3 The coefficients in the equation $A + (100 - A)e^{-Bt}$ to assess percentages of unchanged plasma [^{11}C]flumazenil in all human subjects and in the arbitrarily classified three groups

	A	B
All subjects (49.8 ± 20.3 y.o., n = 24)	25.9 ± 7.3	0.103 ± 0.036
Group 1, slow degraders (53.3 ± 20.1 y.o., n = 7)	29.0 ± 10.4	0.060 ± 0.021
Group 2, medium degraders (48.9 ± 21.7 y.o., n = 15)	26.6 ± 4.9	0.113 ± 0.029***
Group 3, fast degraders (46 and 67 y.o., n = 2)	13.2	0.138

Mean ± s.d. ***$p < 0.001$ (Student's t-test between group 1 and group 2)

Table 4 Error in the distribution volume of [^{11}C]flumazenil in the human brain using the mean percentage of unchanged [^{11}C]flumazenil in the plasma as an input function

	Percent error in the distribution volume*				
	Subjects in slow degraders		Subjects in medium degraders		
Metabolite data	ID 946	ID 997	ID 729	ID 845	ID 879
Mean percentage					
of total subjects	28.3 ± 0.6	26.7 ± 6.3	13.5 ± 0.3	3.3 ± 0.1	5.4 ± 2.9
of slow degraders	3.2 ± 1.0	0.6 ± 0.4			
of medium degraders	6.9 ± 0.1	11.9 ± 0.4	13.6 ± 4.5		

*The percent error in the distribution volume calculated from the mean metabolite values as compared with those calculated from the individually measured values. Mean ± s.d. of ten regions of interest: left and right regions of frontal cortex, temporal cortex, occipital cortex, parietal cortex, and cerebellum

peaks #1 and #2 were considered to be an acidic compound Ro 15-3890 and a lipophilic metabolite Ro 15-4965 (the hydroxy-ethyl ester), respectively. In analysis by TLC, these metabolites could not be separated.[8,9]

Figure 2 shows the time course of the fraction of unmetabolized [^{11}C]FMZ and the two radioactive metabolites within the plasma. The percentage of unchanged [^{11}C]FMZ rapidly decreased for the first 15 min and then gradually decreased over time. Metabolite #1 rapidly increased for the first 15 min and remained constant, whereas the metabolite #2 gradually increased over 60 min. The time course of unchanged [^{11}C]FMZ concentration in the plasma, as shown in Figure 1, rapidly decreased over 60 min.

The percentage of unchanged [^{11}C]FMZ fraction in the plasma radioactivity presented a large individual variation. An even larger variation was observed in the unchanged [^{11}C]FMZ plasma concentration in DAR units. As shown in Table 1, no significant difference was observed when the percentage at 10 min or 30 min was compared in male versus female, young versus old, or between any diseases. A previous study suggested that the degradation of a histamine receptor ligand [^{11}C]doxepin was significantly enhanced in epileptic patients with medication compared with age-matched normal volunteers.[2] In the present study no enhanced degradation of [^{11}C]FMZ was found in the epileptic patients compared with normals or any other patient groups, although we did not examine the metabolism in age-matched young adults. Therefore, the large variation found in our subjects is attributed to the individual variation in metabolism.

Based on the rate of degradation of [^{11}C]FMZ, we tentatively divided subjects into three groups. Ten min after the tracer injection, more than 60% of the total plasma radioactivity was detected as unchanged [^{11}C]FMZ in seven subjects (group 1, slow degraders), and less than 30% of the plasma radioactivity was detected as unchanged [^{11}C]FMZ in two subjects (group 3, fast degraders). The other 15 subjects were classified as group 2, medium degraders. The mean percentage of unchanged [^{11}C]FMZ in slow and medium degraders was significantly different (Table 2). Any known factors such as age, sex, disease or injected dose did not affect the classification.

To easily assess unchanged [^{11}C]FMZ as an input function for the quantitative PET measurement, Delforge et al. applied a biexponential function to the plasma time-concentration curve of the unchanged [^{11}C]FMZ concentration without metabolite analysis in baboon studies,[11] but our data did not fit the biexponential law, but rather a monoexponential law:

$$\% = A + (100 - A)e^{-Bt},$$

as previously described.[16] Again a large individual variation was found for coefficients A and B (Table 3).

We assessed whether the mean percentage of unchanged [^{11}C]FMZ can be used as the standardized metabolite values for the quantitative PET measurement of the benzodiazepine receptors in the human brain. We arbitrarily selected five subjects (two slow degraders and three medium degraders), and measured the distribution volume of the [^{11}C]FMZ binding by using an input function derived from individually measured plasma radioativity curve and three kinds of metabolite data: the individual data, the mean values for total subjects, and the mean values for each of two groups. Table 3 shows the error in the distribution volume calculated from the mean metabolite values as compared with those calculated from the individual values. In two slow degraders, approximately a 30% error was observed when the mean values for total subjects were used, whereas only a 3% error was found when the mean values for the slow degraders were used. In the three subjects belonging to group 2, the error was 3% to 14%, which depended on the mean values used. Such errors are not tolerable because they are comparable with the amount of reduction (approximately 20%) observed in the cortex and cerebellum in patients with Alzheimer's disease as compared with age-matched normal subjects (data will be reported elsewhere).

To enable rapid separation of the unchanged [^{11}C]FMZ in many samples an ion-exchange column or a C-18 cartridge was used, but [^{11}C]FMZ was not completely separated from metabolites.[3] In our experience, we also

could not separate unchanged [^{11}C]FMZ from metabolite #2 with a C-18 cartridge (data not shown).

In conclusion, the mean percentage of unchanged [^{11}C]FMZ cannot be used as the standardized metabolite values for the quantitative PET analysis because of large individual variation in the metabolism of [^{11}C]FMZ in human subjects, and the metabolite analysis is necessary in individual subjects to accurately determine the input function for the quantitative PET studies.

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REFERENCES

1. Ishiwata K, Hatazawa J, Kubota K, Kameyama M, Itoh M, Matsuzawa T, et al. Metabolic fate of L-[methyl-^{11}C]methionine in human plasma. *Eur J Nucl Med* 15: 665–669, 1989.
2. Ishiwata K, Yanai K, Iwata R, Takahashi T, Hatazawa J, Itoh M, et al. Analysis of plasma metabolites during human PET studies with three receptor ligands, [^{11}C]YM-09151-2, [^{11}C]doxepin and [^{11}C]pyrilamine. *Tohoku J Exp Med* 178: 129–136, 1996.
3. Pike VW, Halldin C, Crouzel C, Barre L, Nutt DJ, Osman S, et al. Radioligands for PET studies of central benzodiazepine receptors and PK (peripheral benzodiazepine) binding sites—current status. *Nucl Med Biol* 20: 503–525, 1993.
4. Malizia AL, Richardson MP. Benzodiazepine receptors and positron emission tomography: ten years of experience. A new beginning? *J Psychopharmacol* 9: 355–368, 1995.
5. Swahn C-G, Persson A, Pauli S. Metabolism of the benzodiazepine antagonist ^{11}C-Ro 15-1788 after intravenous administration in man. *Human Psychopharmacol* 4: 297–301, 1989.
6. Loc'h C, Hantraye Ph, Khalili-Varasteh M, Mazière B, Delforge J, Brouillet E, et al. [^{11}C]-Flumazenil metabolites: measurements of unchanged ligand in plasma using thin layer chromatography and rapid liquid chromatography. *J Label Compds Radiopharm* 30: 234, 1990.
7. Frey KA, Holthoff VA, Koeppe RA, Jewett DM, Kilbourn MR, Kuhl DE. Parametric *in vivo* imaging of benzodiazepine receptor distribution in human brain. *Ann Neurol* 30: 663–672, 1991.
8. Barre L, Debruyne D, Abadie P, Moulin M, Baron JC. A comparison of methods for the separation of [^{11}C]Ro 15-1788 (flumazenil) from its metabolites in the blood of rabbits, baboons and humans. *Appl Radiat Isot* 42: 435–439, 1991.
9. Debruyne D, Abadie P, Barre L, Albessard F, Moulin M, Zarifian E, et al. Plasma pharmacokinetics and metabolism of the benzodiazepine antagonist [^{11}C]Ro 15-1788 (flumazenil) in baboon and human during positron emission tomography studies. *Eur J Drug Metab Pharmacokinet* 16: 141–152, 1991.
10. Pauli S, Liljequist S, Farde L, Swahn C-G, Halldin C, Litton J-E, et al. PET analysis of alcohol interaction with the brain disposition of [^{11}C]flumazenil. *Psychopharmacology* 107: 180–185, 1992.
11. Delforge J, Syrota A, Bottlaender M, Varastet M, Loc'h C, Bendriem B, et al. Modeling analysis of [^{11}C]flumazenil kinetics studied by PET: application to a critical study of the equilibrium approaches. *J Cereb Blood Flow Metab* 13: 454–468, 1993.
12. Suzuki K, Inoue O, Hashimoto K, Yamasaki T, Kuchiki M, Tamate K. Computer-controlled large scale production of high specific activity [^{11}C]RO 15-1788 for PET studies of benzodiazepine receptors. *Appl Radiat Isot* 36: 971–976, 1985.
13. Ishiwata K, Seki H, Ishii K, Ishii S, Nozaki T, Senda M. Synthesis and *in vivo* evaluation of [^{11}C]semotiadil, a benzothiazine calcium antagonist. *Appl Radiat Isot* 45: 439–443, 1994.
14. Iida H, Miura S, Kanno I, Murakami M, Yamamoto S, Amano M. Design and evaluation of Headtome-IV, a whole-body positron emission tomography. *IEEE Trans Nucl Sci* NS-37: 1006–1010, 1989.
15. Koeppe RA, Holthoff VA, Frey KA, Kilbourn MR, Kuhl DE. Compartmental analysis of [^{11}C]flumazenil kinetics for the estimation of ligand transport rate and receptor distribution using positron emission tomography. *J Cereb Blood Flow Metab* 11: 735–744, 1991.
16. Millet P, Delforge J, Mauguiere F, Pappata S, Cinotti L, Frouin V, et al. Parameter and index images of benzodiazepine receptor concentration in the brain. *J Nucl Med* 36: 1462–1472, 1995.