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Mass Fragmentographic Determination of Ferulic Acid in Plasma after Oral Administration of γ -Oryzanol¹⁾

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A mass fragmentographic method for the quantitative determination of ferulic acid, a main metabolite of γ -oryzanol in rabbit and dog plasma, was developed. After its isolation by Amberlite XAD-2 chromatography and by solvent extraction, ferulic acid was converted to the heptafluorobutyryl ethyl ester derivative and analyzed by using 4-hydroxy-3-trideuteromethoxycinnamic acid (ferulic acid- d_3) as an internal standard. The recovery of ferulic acid from plasma samples was $100.5 \pm 4.5\%$ (mean \pm standard deviation, $n=20$) in the range of 5.0–150.0 ng/ml plasma. γ -Oryzanol was administered orally to rabbits and dogs at doses of 25, 50 and 100 mg/kg body weight as a solution of sesame oil, and then the plasma concentrations of ferulic acid were determined by mass fragmentography. A good correlation between the administered dose of γ -oryzanol and the area under the concentration curve (AUC) of ferulic acid was obtained in both animals, so the AUC of ferulic acid may be used as an index for estimating the extent of gastrointestinal absorption of γ -oryzanol.

Keywords— γ -oryzanol; ferulic acid; ferulic acid- d_3 ; AUC; dog; rabbit; plasma; mass fragmentography

In the previous paper²⁾ we reported the metabolic fate of γ -oryzanol in rabbits following oral administration of the ¹⁴C-labeled compound. Only a little radioactivity derived from γ -oryzanol was detected in the blood at the early stage after the administration. On the other hand, the radioactivity derived from the metabolites reached the maximum 3 h after oral administration, then decreased gradually with time to a negligible level at 24 h. A main metabolite in the blood was elucidated to be ferulic acid, but its maximum level was calculated to be only about 100 ng/ml plasma even at the dose of 40 mg/kg.

Although there are some reports³⁾ concerning the absorption, metabolism, distribution and excretion of γ -oryzanol in rabbits and rats by using the ¹⁴C-labeled compound, no studies with unlabeled γ -oryzanol have been reported. The determination of ferulic acid in plasma is of great interest for the evaluation of the gastrointestinal absorption and the metabolic fate of γ -oryzanol in experimental animals and humans. This paper deals with a mass fragmentographic method for the quantitative determination of ferulic acid in plasma, and with the relationship between the area under the concentration *versus* time curve (AUC) of ferulic acid and the dose of γ -oryzanol.

Experimental

Materials— γ -Oryzanol was recrystallized from benzene-methanol (mp 130–135°C). Heptafluorobutyric anhydride (HFBA) was obtained from Wako Pure Chemical Industries, Ltd. and sesame oil was obtained from Nakarai Chemicals Co., Ltd. Ferulic acid was obtained from Tokyo Kasei Kogyo Co., Ltd. and used after recrystallization from water. 4N ethanolic HCl was prepared by blowing hydrogen chloride into anhydrous ethanol under cooling. 4-Hydroxy-3-trideuteromethoxycinnamic acid (ferulic acid- d_3), used as an internal standard, was synthesized from 3,4-dihydroxybenzaldehyde according to the method of Markey *et al.*⁴⁾ and Pearl and Beyer⁵⁾ shown in Chart 1, and purified by recrystallization from water to give light yellow needles, mp 169–171°C. The mass spectrum showed the molecular ion at m/z 197, and the isotopic purity was above 98%. Its structure was also confirmed by NMR spectral analysis. NMR (in DMSO- d_6) δ ppm: 6.34 and 7.47 (each 1H, d, $J=15$ Hz, $-\text{CH}=\text{CH}-$), 6.77, 7.08 and 7.24 (each 1H, d, $J=8$ Hz,

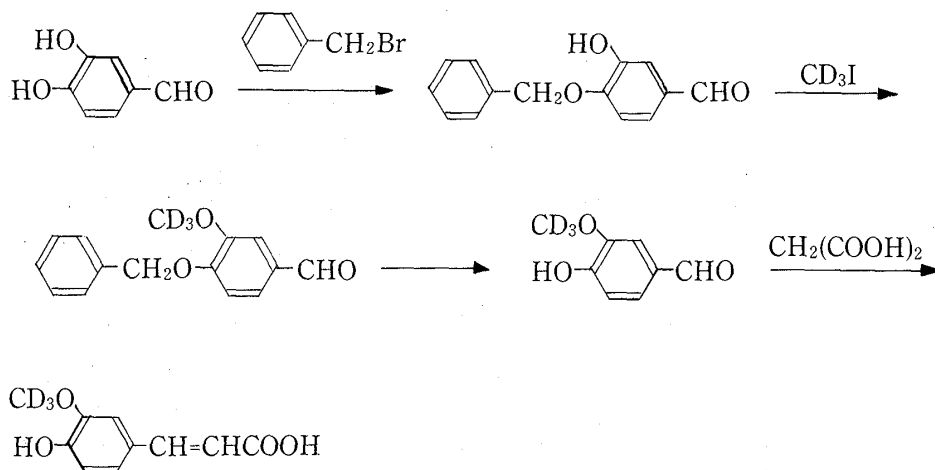


Chart 1

dd, $J=2.8$ Hz and d, $J=2$ Hz, aromatic protons). All other chemicals used were of analytical-reagent grade and were obtained commercially.

Mass Spectrometry, Mass Fragmentography and Mass Chromatography—A Hitachi model-60 gas chromatograph-mass spectrometer was used under the following conditions. A glass tube (2 m \times 3 mm i.d.) was packed with 2% OV-17 on Chromosorb W 80—100 mesh (AW-DMCS). The flow rate of the carrier gas (helium) was 20 ml/min. The temperatures of the column, injection port and ion source (chamber) were 210°C, 240°C and 180°C, respectively. The ionization potential was 25 eV. Mass chromatography was carried out by attaching a Hitachi model-002B data processing system to the gas chromatograph-mass spectrometer.

Animal Experiments—Five male rabbits (2.5—3.2 kg body weight) and three male beagle dogs (9.5—10.8 kg body weight) were fasted for 20—24 h prior to and during the experiment, but they were allowed free access to water throughout the experiment. γ -Oryzanol was administered orally to the animals as a solution of sesame oil (25—100 mg/ml) at dosage levels of 25, 50 and 100 mg/kg body weight. The dosing volume employed was 1 ml/kg. Approximately 3 ml of blood was collected into a heparinized syringe prior to administration and at 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 24 h postadministration. The blood samples were then centrifuged, and 1.0 ml of each plasma sample was used for the assay of ferulic acid by mass fragmentography.

Clean-up, Derivatization and Calibration Curve—To 1.0 ml of the plasma were added 1 ml of 0.2 N HCl and the internal standard (ferulic acid- d_3), and then this sample was applied to a column of Amberlite XAD-2 (10 cm \times 7 mm i.d.). The preparation of an Amberlite XAD-2 column and the clean-up procedure were carried out according to the method of Takahashi *et al.*⁹⁾ The column was washed with 10 ml of 0.1 N formic acid and 30 ml of water, then eluted with 10 ml of methanol. The eluate was evaporated to dryness under a stream of N_2 at 50°C. The residue was dissolved in 1 ml of 0.2 N NaOH and extracted with 3 ml of ethyl acetate. The organic layer was discarded after centrifugation, and the aqueous layer was acidified by adding 1 ml of 1 N HCl, then re-extracted with 5 ml of ethyl acetate. The organic layer was evaporated to dryness under a stream of N_2 at 50°C, and the residue was heated at 70°C with 1 ml of 4 N ethanolic HCl for 30 min. After the removal of alcoholic HCl under N_2 , the dried residue was derivatized in 100 μl of a mixture of ethyl acetate and HFBA (1:1, v/v) at room temperature for 30 min. An aliquot of this solution was injected into a gas chromatograph-mass spectrometer. The calibration curve for ferulic acid was obtained as follows. To 1 ml of dog or rabbit blank plasma sample were added 10, 25, 50, 100, 150 or 200 ng of ferulic acid and 100 ng of the internal standard. These samples were extracted, derivatized and analyzed by mass fragmentography as described above, and then the ratios of the peak height of ferulic acid to the internal standard were plotted against the amounts of ferulic acid added to plasma. In order to check the accuracy of this method, 5.0 to 150.0 ng of ferulic acid and the internal standard were added to 1 ml of dog blank plasma, and the plasma concentrations of ferulic acid were determined in the same manner as described above.

Results

Mass Fragmentography

The heptafluorobutyryl ethyl ester (HFB-Et) derivatives of ferulic acid and ferulic acid- d_3 showed one peak whose retention time was 3.4 min as determined by total ion monitoring (TIM). Both mass spectra, shown in Fig. 1 (A) and (B), gave the molecular ions (base peak)

at m/z 418 and 421 with other characteristic fragment ions at m/z 373 and 221, and 376 and 224 ($M^+ - OC_2H_5$, $M^+ - COC_3F_7$), respectively. Mass fragmentography was carried out by monitoring m/z 418 and 421. To determine the ratio of ferulic acid to ferulic acid- d_3 accurately, the HFB-Et derivative of ferulic acid- d_3 was injected into the gas chromatograph-mass spectrometer and the ion intensities at m/z 418 and 421 were measured. It was found from the mass fragmentogram that the ratio of ferulic acid to ferulic acid- d_3 was less than 0.1%.

Typical mass fragmentograms of the blank plasma and the sample containing ferulic acid and ferulic acid- d_3 (50 ng each) are shown in Fig. 2. A small peak was observed at the same retention time as the derivative of ferulic acid in blank plasma samples, and the concentrations calculated as ferulic acid were 1.2 ± 0.2 ng/ml in dogs and 2.6 ± 0.3 ng/ml in rabbits (four animals each, mean \pm S.D.). Though we used 2% OV-1 and OV-101 as a stationary phase instead of OV-17 to separate this peak from the HFB-Et derivative of ferulic acid, this peak could not be separated and further, the concentrations determined by monitoring at m/z 418 and 421 were the same as those mentioned above. The same results were also obtained when mass fragmentography was carried out after derivatizing the extract from blank plasma to the HFB methyl ester. It seems likely from these results that this peak is due to endogenous ferulic acid, although this remains to be confirmed.

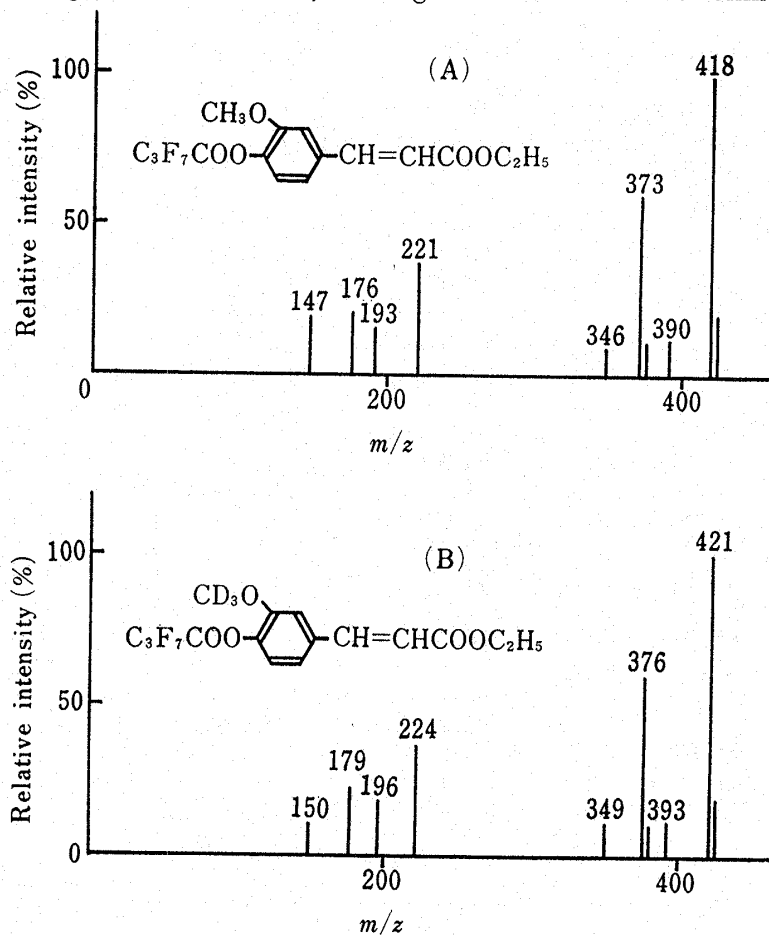


Fig. 1. Mass Spectra of the HFB-Et Derivatives of Ferulic Acid (A) and the Internal Standard (B)

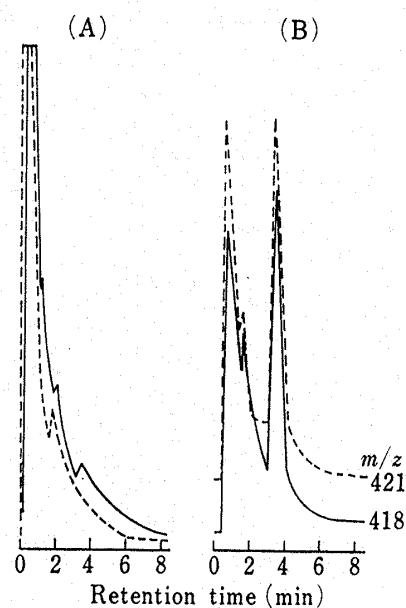


Fig. 2. Mass Fragmentograms of Blank Plasma (A) and a Sample with added Ferulic Acid and Internal Standard (B)

The amounts of ferulic acid and the internal standard were 50 ng each.

Calibration Curve

The calibration curve for ferulic acid is shown in Fig. 3. A good linear relationship was obtained in the range of 10 to 200 ng/ml ($r=0.99$).

Accuracy

The results of the recovery tests are summarized in Table I. The recoveries were in the

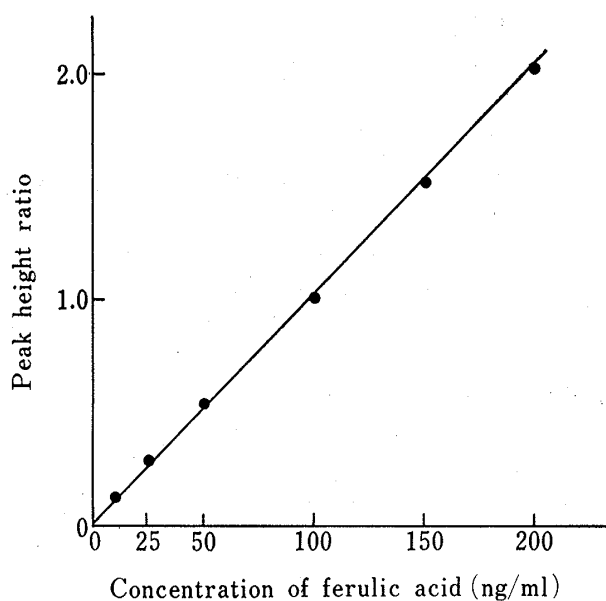


Fig. 3. Calibration Curve of Ferulic Acid

The amount of internal standard added to plasma was 100 ng.

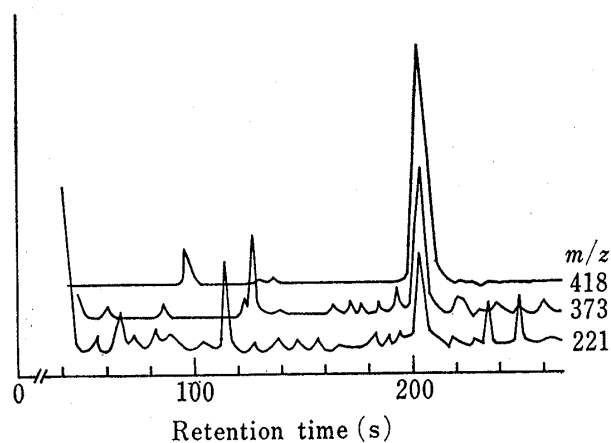


Fig. 4. Mass Chromatogram of Dog Plasma Extract following Oral Administration of γ -Oryzanol

TABLE I. Recovery Tests for Ferulic Acid

Amount added (ng)	Amount found (ng)	Recovery (%)	Mean \pm S.D.
5.0	5.2	104.0	99.0 \pm 4.8
	4.8	96.0	
	4.7	94.0	
	5.1	102.0	
25.0	27.1	108.4	103.9 \pm 4.3
	26.6	106.4	
	25.5	102.0	
	24.7	98.8	
50.0	49.7	99.4	102.3 \pm 4.6
	48.8	97.6	
	53.8	107.6	
	52.2	104.4	
100.0	104.5	104.5	100.7 \pm 3.9
	95.3	95.3	
	102.3	102.3	
	100.5	100.5	
150.0	140.9	93.9	96.9 \pm 3.1
	142.8	95.2	
	146.1	97.4	
	151.4	100.9	
			100.5 \pm 4.5 (n=20)

range of 93.9 to 108.4% and the mean value was 100.5%. The standard deviation of these recoveries was below 5%. It was found that the accurate determination of ferulic acid in plasma at concentrations as low as 5 ng/ml was possible by this method.

Identification of the Main Metabolite of γ -Oryzanol in Dog Plasma

Dogs are convenient experimentally for comparing the bioavailability of the drug preparations from the viewpoint of the ease of administration, but no studies on the metabolism of γ -oryzanol in dogs have yet been done. To confirm that dogs also give ferulic acid as a metabolite of γ -oryzanol, as do rabbits, 1 g of γ -oryzanol dissolved in 10 ml of sesame oil was

administered orally to a beagle dog. Plasma (2 ml each) was collected at 1, 2, 3 and 4 h after dosing. These samples were treated according to the extraction and derivatization procedures described in the experimental section, and then mass chromatography was performed.

Mass chromatograms at m/z 418, 373 and 221 are shown in Fig. 4. One peak which had the same retention time (3.4 min) as the HFB-Et derivative of authentic ferulic acid was observed in every plasma sample, and the peak height ratios of m/z 221 and 373 to m/z 418 were the same as those of the derivative of authentic sample shown in Fig. 1 (A). These results suggest that the peak obtained in mass chromatography is the HFB-Et derivative of ferulic acid.

Plasma Concentration of Ferulic Acid in Rabbits and Dogs

γ -Oryzanol was administered orally to three beagle dogs and five rabbits as a solution of sesame oil at doses of 25, 50 and 100 mg/kg. Plasma levels of ferulic acid for 24 h after oral

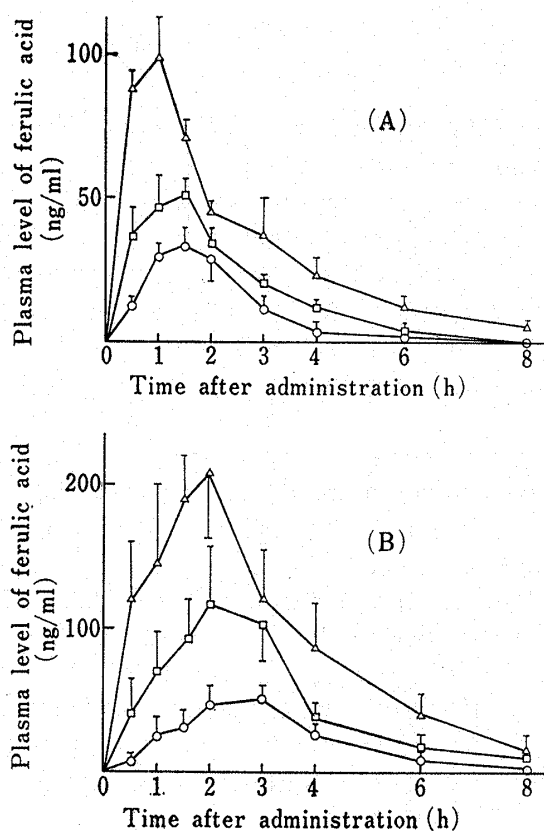


Fig. 5. Plasma Concentrations of Ferulic Acid in Beagle Dogs (A) and Rabbits (B) after Oral Administration of γ -Oryzanol at Different Doses

—○—: 25 mg/kg, —□—: 50 mg/kg, —△—: 100 mg/kg.
Each point represents the mean \pm S.E.

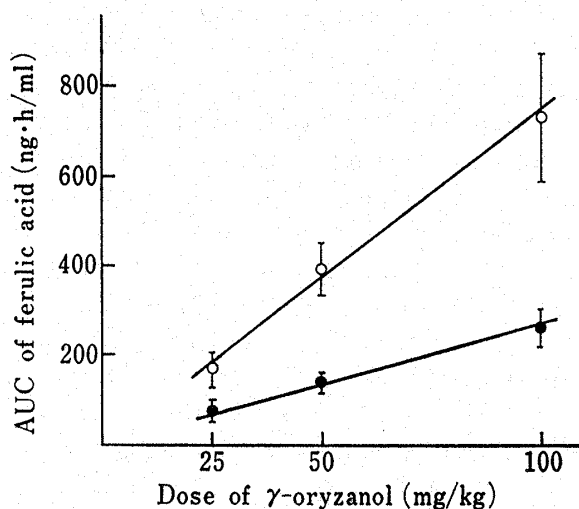


Fig. 6. Relationship between the Administered Dose of γ -Oryzanol and the AUC_{0-8h} of Ferulic Acid

—○—: rabbit, —●—: dog.
Each point represents the mean \pm S.E.

TABLE II. AUC, C_{max} , T_{max} , K_{el} and $t_{1/2}$ Values of Ferulic Acid

Animal	Dose (mg/kg)	AUC (ng·h/ml)	C_{max} (ng/ml)	T_{max} (h)	K_{el} (1/h)	$t_{1/2}$ (h)
Dog	25	77.8	32	1.5	0.533	1.3
	50	138.9	51	1.5	0.566	1.2
	100	263.8	99	1.0	0.438	1.6
Rabbit	25	169.9	51	3.0	0.528	1.3
	50	393.8	116	2.0	0.434	1.6
	100	729.7	209	2.0	0.415	1.7

administration were followed by mass fragmentography, and the results are shown in Fig. 5. At 24 h after the administration, the plasma level of ferulic acid was the same as that before dosing in both animals. Plasma concentration reached the maximum faster in dogs (1—1.5 h) than in rabbits (2—3 h) at every dose, but no difference of the elimination rate constant (K_{el}) was observed between the two animals. The pattern of the plasma concentration curve in rabbits (Fig. 5 (B)) was very similar to that of γ -oryzanol metabolites reported previously.²⁾ The plasma concentration increased with increase of the amount of γ -oryzanol administered. Table II summarizes T_{max} (time required to reach maximum), C_{max} (concentration at maximum), K_{el} , $t_{1/2}$ (half life) and the AUC_{0-8h} of ferulic acid obtained from the mean plasma concentration curve. The T_{max} and K_{el} were essentially consistent with different doses, while C_{max} and AUC increased proportionally with the dose of γ -oryzanol. The AUC in rabbits was 2.2 to 2.8 times higher than that in dogs, and the coefficient of variation was greater in rabbits. As shown in Fig. 6, a good correlation was observed between the administered dose of γ -oryzanol and the AUC_{0-8h} of ferulic acid in both animals.

Discussion

Generally in order to estimate the bioavailability of drugs, it is necessary to measure the plasma concentration or the amount of urinary excretion of the administered drug.

It became obvious from the previous study²⁾ on the metabolism of γ -oryzanol in rabbits that the blood level of γ -oryzanol following oral administration was extremely low, and that γ -oryzanol disappeared very rapidly and could not be detected in the blood at 2 h after administration owing to its poor absorbability, rapid distribution to tissues and extensive metabolism, probably in the liver. Although the bioavailability of γ -oryzanol should be estimated from the AUC of γ -oryzanol itself, the quantitative analysis of γ -oryzanol in blood by high performance liquid chromatography and/or gas chromatography-mass spectrometry is very difficult at the present time because of its low concentration in blood. Also we reported in the previous paper that the radioactivity excreted in the 48-h urine was only 6.4% of the administered dose. Unchanged γ -oryzanol was not detected in the urine at all, and the six metabolites identified, such as ferulic acid and vanillic acid, were excreted in nearly equal proportions when γ -oryzanol-¹⁴C was administered orally to rabbits. These difficulties may account for the absence of studies on the bioavailability of γ -oryzanol using the unlabeled compound.

Based on the results reported in the previous paper that the main metabolite in blood was ferulic acid (more than 80% of the total radioactivity at the maximum concentration) following oral administration of γ -oryzanol-¹⁴C to rabbits, we considered that the AUC of ferulic acid might be a good indicator for estimating the extent of gastrointestinal absorption of γ -oryzanol.

First, we investigated the quantitative determination of ferulic acid in plasma. As mentioned previously, the plasma concentration of ferulic acid was extremely low. Thus, we used the mass fragmentographic method because of its high selectivity and sensitivity, and we also used the stable isotope labeled compound as an internal standard. Such an internal standard for quantitative mass fragmentographic analysis should be multi-labeled and moreover should have a high isotopic purity. We investigated two methods to incorporate deuterium atoms into ferulic acid. One was to replace three hydrogen atoms of the aromatic ring with deuterium atoms, and the other was to introduce deuterium atoms into the methoxy group. We tried to prepare [aromatic- d_3] ferulic acid by dissolving ferulic acid in 9% DCl in D₂O and heating for 6 h at 80°C according to the method of Muskiet *et al.*,⁷⁾ but deuterium atoms were not incorporated at all, presumably due to the presence of the double bond. On the other hand, 4-hydroxy-3-trideuteromethoxycinnamic acid (ferulic acid- d_3) which had a high isotopic purity was successfully synthesized by the use of methyl iodide- d_3 (its isotopic purity was above 99.0%), and it was found that the obtained ferulic acid- d_3 could be used as an internal standard.

In the case of mass fragmentographic analysis, it is very important to extract a very small amount of drug in body fluids (such as plasma, spinal fluids and urine) quantitatively and to remove many other constituents in body fluids which interfere with the analysis. For this purpose, we used a column of Amberlite XAD-2, because the removal of the interfering substances and deproteinization were carried out simultaneously. It was confirmed by an experiment with the ^{14}C -labeled compound that ferulic acid was adsorbed quantitatively and that its elution from this column was accomplished with 10 ml of methanol.

Though there are many reports concerning methods for preparing derivatives of phenolic acids, such as trimethylsilyl (TMS),⁸⁾ ethyl ester-TMS ether,⁹⁾ HFB methyl ester,¹⁰⁾ HFB ethyl ester¹¹⁾ and pentafluoropropionyl methyl ester¹²⁾ derivatives, the HFB ethyl ester (HFB-Et) derivative gave excellent reproducibility and stability among the derivatives investigated.

As mentioned above, after isolating ferulic acid from plasma samples by Amberlite XAD-2 chromatography and by solvent extraction followed by the formation of the HFB-Et derivative, plasma levels of ferulic acid were quantitatively determined with good accuracy by mass fragmentography using ferulic acid- d_3 as an internal standard. The lower limit of detection was about 5 ng/ml. Furthermore, it was confirmed by mass chromatography that ferulic acid appeared in dog plasma as a metabolite of γ -oryzanol, as was the case in rabbits, when γ -oryzanol was administered orally to dogs.

Second, γ -oryzanol was administered orally to dogs and rabbits at different doses and the plasma concentrations of ferulic acid were determined by mass fragmentography, then the $\text{AUC}_{0-8\text{ h}}$ of ferulic acid was calculated. The results indicated a good correlation between the dose of γ -oryzanol and the AUC of ferulic acid in both animals. Thus it is considered from these results that the AUC of ferulic acid may be used as an index for estimating the extent of gastrointestinal absorption of γ -oryzanol preparations in experimental animals and humans.

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References and Notes

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