Chem. Pharm. Bull.
28(10)3093—3097(1980)

Quantitative Determination of Ergothioneine in Plasma and Tissues by TLC-Densitometry[1]

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(Received April 23, 1980)

A rapid and reliable thin-layer chromatographic (TLC) method for the quantitative analysis of ergothioneine in plasma and in various organs is presented. After extraction from biological materials with acetone/methanol (1: 1), ergothioneine was separated by TLC and the spot corresponding to the compound with *Rf* 0.29 was quantified by scanning with a densitometer at 256 nm. The sensitivity of the assay was 0.05 μg/20 μl of liver extract. This procedure should be useful in pharmacokinetic studies of ergothioneine in relation to its physiological role.

Keywords——ergothioneine; TLC-densitometry; rat liver; distribution in rat organs; intravenous injection

Ergothioneine is a sulfur-containing substance whose chemical structure is 2-mercaptohistidine trimethylbetaine. Because of its wide distribution in the organs of animals, several physiological roles have been suggested for ergothioneine. Numerous attempts[3] have been made to confirm a physiological role of ergothioneine, but so far unsuccessfully. However, the development of assay methods has made it possible to determine precisely the endogenous levels of ergothioneine in biological materials. Analytical procedures for ergothioneine in several organs were reviewed by Melville.[3a] The diazo method[4] was preferred because of its precision. A *p*-chloromercuribenzoate (PCMB) method, based on the reaction of the sulfhydryl group of ergothioneine with PCMB, was reported by Hama *et al.*[5] These assay methods are not sensitive enough to measure low levels of endogenous ergothioneine in the organs of animals. Recently, higher sensitivity was attained with a procedure based on the use of high pressure liquid chromatography (HPLC).[6] However, this method is rather complicated due to the need for lyophilization of biological samples. Therefore, we have developed a thin-layer chromatography (TLC-densitometry) method which is rapid while retaining the specificity, sensitivity and accuracy of the HPLC method[6] for determining ergothioneine in biological samples. This procedure can be used to determine the pharmacokinetic profile of ergothioneine and should be useful in studies of the physiological role of ergothioneine in animals in relation to the levels in specific organs.

Experimental

Reagent——L-Ergothioneine was obtained from Sigma. Methanol and acetone used in this experiment were of reagent grade for high pressure liquid chromatography and were used as received. A 0.02% stock

1) Presented at the 29th Meeting of the Kinki Branch of the Pharmaceutical Society of Japan, Kyoto, Nov. 1979.
2) Location: *Arise, Igawadani-cho, Tarumi-ku, Kobe, 673, Japan.*
3) *a*) D.B. Melville, *Vitamins and Hormones*, **17**, 155 (1959); *b*) T. Konishi, H. Okumura, N. Tamaki, and T. Hama, *Vitamins*, **46**, 131 (1972); *c*) T. Hama, H. Okumura, N. Tamaki, and T. Konishi, *Yakugaku Zasshi*, **93**, 369 (1973).
4) D.B. Melville, W.H. Horner, and R. Lubschez, *J. Biol. Chem.*, **206**, 221 (1954).
5) T. Hama, T. Konishi, N. Tamaki, F. Tunemori, and H. Okumura, *Vitamins*, **46**, 121 (1972).
6) T. Mayumi, H. Kawano, Y. Sakamoto, E. Suehisa, Y. Kawai, and T. Hama, *Chem. Pharm. Bull.*, **26**, 3772 (1978).

solution of ergothioneine was prepared as follows; 40 ml of methanol was added to ergothioneine in a 50 ml volumetric flask and sonicated for five minutes to dissolve the ergothioneine. Sonication was repeated in the same manner to dissolve any remaining ergothioneine and methanol was added to give 50 ml at room temperature.

Preparation of Biological Samples——Seven- or eight-week-old rats (Sprague-Dawley strain) weighing 200—250 g were used to obtain plasma and tissue samples. During blood sampling the animals were unanesthetized and restrained. The plasma was obtained by centrifugation after taking blood samples from a jugular vein. The plasma was then shaken with an equal volume of acetone/methanol (1 : 1) for ten minutes and the supernatant was obtained by centrifugation for five minutes ($1000 \times g$). After sacrifice by exsanguination, the liver, the kidneys, the testicles and the brain were removed and weighed. Acetone/methanol (1 : 1) was added to each of the tissue samples (three times the tissue weight) and the samples were homogenized in a Potter homogenizer. The homogenate was shaken for ten minutes and then centrifuged for five minutes. The supernatant was used for the assay.

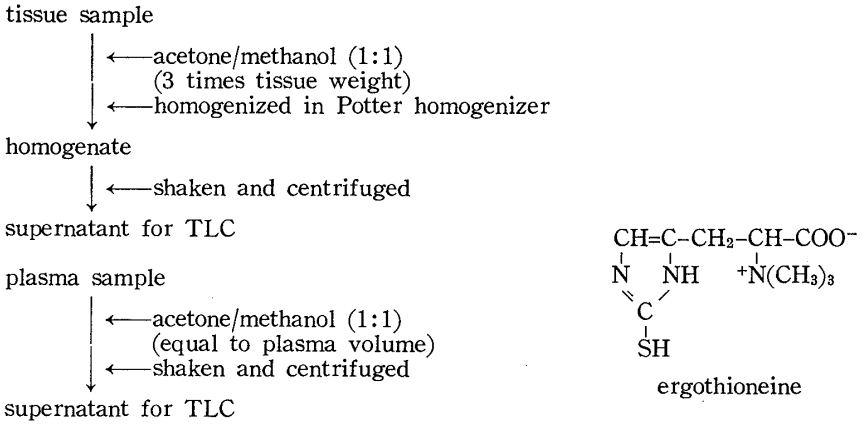

Fig. 1. Analytical Procedure for Ergothioneine in Biological Materials

Analytical Procedure——A schematic representation of the analytical procedure is shown in Fig. 1. A 2 ml portion of plasma or tissue extract was evaporated to dryness in a tapered tube by passing N_2 gas. The residue was dissolved in 0.25 ml of methanol by mixing well, and 20 μl of this sample solution was spotted on a TLC plate (Merck, TLC plates Silica gel 60 F_{254}, 20×20 cm) and developed with methanol/H_2O (3 : 1). Thirteen samples were spotted on each TLC plate. After development, the plate was allowed to dry for five minutes at room temperature and each spot of ergothioneine was detected and analyzed at 256 nm by scanning the TLC plate with a spectrodensitometer (Shimadzu, Dual-Wavelength TLC Scanner CS-910). The slit width was 10.0×1.0 mm. The scan speed was 40 mm/min, and the chart speed of the recorder was 20 mm/min. For the determination of endogenous ergothioneine in the liver, 20 μl of liver extract was spotted on the TLC plate without dilution or concentration, and treated in the same manner as other tissue samples.

Intravenous Administration——Ergothioneine (100 mg/kg) was administered *i.v.* to rats *via* the jugular vein. Blood samples were taken from another jugular vein just before administration and 0.25, 0.5, 0.75, 1, 1.25, 1.5 and 2 hour after administration and were then centrifuged to obtain plasma samples for the determination of ergothioneine.

Results and Discussion

Typical densitometric tracings of assay samples are shown in Fig. 2. On scanning a pure ergothioneine standard (A), the compound appeared at *Rf* 0.29 (peak 1). When an extracted liver sample containing added ergothioneine was scanned, peak 2 with the same *Rf* value was observed on the densitogram (B). In the case of the extracted liver sample without added ergothioneine, a similar peak (peak 3) with *Rf* 0.29 was also detected on the densitogram (C). By using the HPLC method[6] presented for the assay of ergothioneine in biological materials, peak 3 obtained from the extracted liver sample was found to consist of endogenous ergothioneine (typical HPLC assay chromatograms are shown in Fig. 3). Peak 2 was also confirmed to be a single peak corresponding to the total amount of added and endogenous ergothioneine in the sample. Accordingly, the liver sample contained no interfering materials in the region of ergothioneine on the TLC plate. Thus, it is possible to quantify the endogenous level of ergothioneine by TLC-densitometry. This was also the case in other biological samples tested.

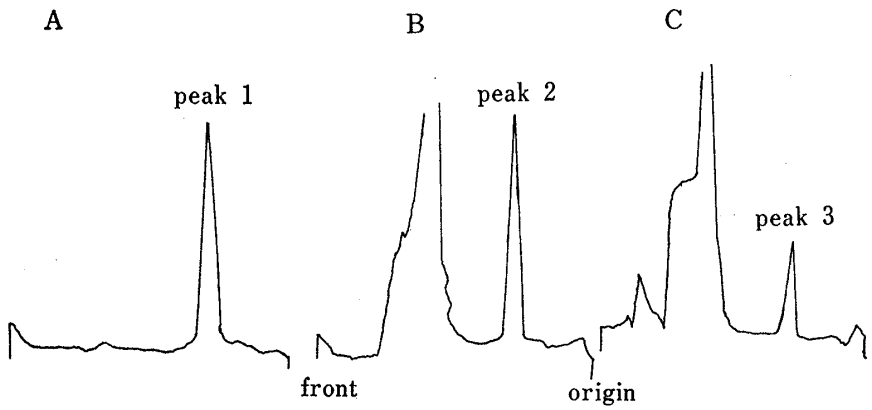

Fig. 2. Typical Densitograms of Extracted Liver Samples Containing Ergothioneine

A: pure ergothioneine;
B: liver extract with added ergothioneine;
C: liver extract.

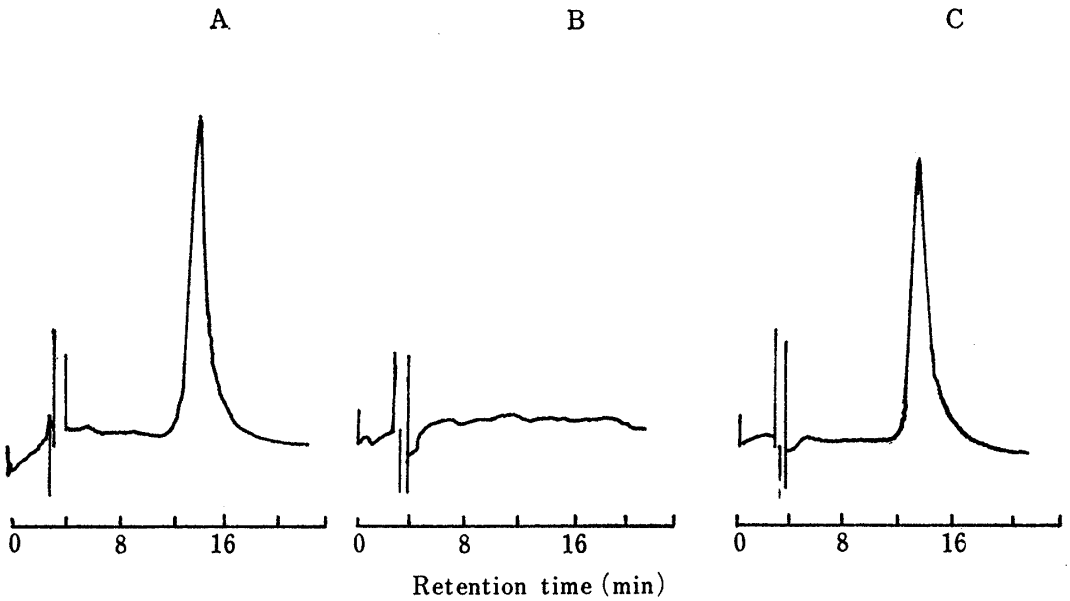

Fig. 3. Sample Chromatograms of Ergothioneine from Liver Extract and Pure Ergothioneine using the HPLC Method[6]

A: standard solution of ergothioneine; B: sample obtained from the spot in the region of ergothionine without loading the extracted liver sample on the TLC plate; C: sample obtained from the spot corresponding to peak 3 in Fig. 2 after loading liver extract on the TLC plate. Column, μBondapak CH; eluent, CH_3CN/H_2O (80: 20); flow rate, 1.0 ml/min; sample volume, 10 μl.

Further, the results for samples with added ergothioneine indicated that accurate determination was possible by this method.

The reproducibility of the assay was tested by assaying extracted liver samples containing ergothioneine at concentration of 0.4 and 0.8 μg/20 μl. For this determination, 20 μl samples were spotted on the TLC plate. Coefficients of variation in a triplicate study on a single plate were 1.1 and 0.2%, respectively. Recoveries were 99.2 ±1.1% for triplicate samples at 0.4 μg/20 μl and 99.6±2.0% at 0.8 μg/20 μl, as shown in Table I. The content of added ergothioneine in this experiment was calculated as described above, *i.e.*, the peak height of B minus the peak height of C, in comparison with peak heights for pure ergothioneine samples. The sensitivity limit of the assay was about 0.05 μg/20 μl ($CV\% = 23.6$). Above 0.05 μg/20 μl, a linear relation was obtained between the peak height and the

amount of ergothioneine added to the liver samples, as shown in Fig. 4. The correlation coefficient of the calibration curve was 0.9991, indicating good accuracy. In the application of this TLC-densitometry method to plasma and other tissues, it was found that this method could be successfully used to quantify endogenous ergothioneine in several organs. The data for ergothioneine content in rat organs determined by the present and the conventional meth-

Table I. Recovery of Ergothioneine

Sample			
Pure ergothioneine: A Liver extract with added ergothioneine: B Liver extract: C			
Data			
Ergothioneine (μg/20 μl)		0.4	0.8
Peak height of A	(cm)	3.68±0.06	7.35±0.70
Peak height of B	(cm)	5.43±0.06	9.10±0.02
Peak height of C	(cm)	1.78±0.04	1.78±0.04
Recovery (%)		99.2±1.10	99.6±2.00

Detection limit: 0.05 μg/20 μl.
Values are the means ± S.D. of three determinations.

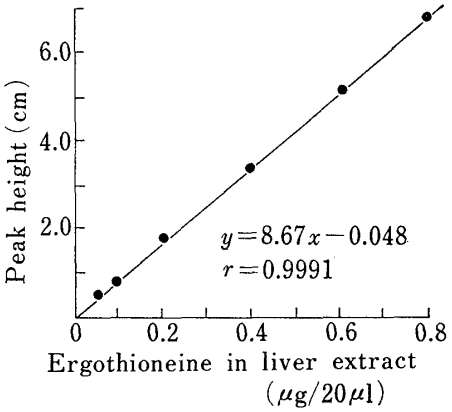

Fig. 4. Calibration Curve for Ergothioneine

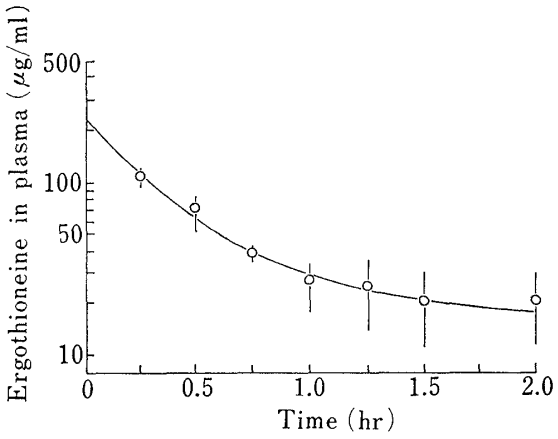

Fig. 5. Elimination Pattern of Ergothioneine in Rat Plasma Following *i. v.* Administration of 100 mg/kg

$C_p = 207.7\, e^{-3.37t} + 27.0\, e^{-0.222t}$

Table II. Ergothioneine Contents in Rat Organs

	Ergothioneine (mg/100 g of wet tissue)			
	Present method	HPLC method	PCMB method	Diazo method
Liver	7.82±0.70	4.83±0.45	4.74	13.3
Kidney	1.58±0.65	1.87±0.07	6.73	4.3
Testicle	0.50±0.03	0.24±0.01	—	—
Brain	0.41±0.04	0.21±0.02	—	—
Plasma	0.21±0.08	—	—	—

Mean ± standard error.

ods, including the HPLC method,[6] are summarized in Table II. The values of ergothioneine content determined by TLC-densitometry were in reasonably good agreement with those obtained by the HPLC method,[6] *i.e.*, both methods gave the same order of magnitude for the content of ergothioneine in the samples of rat organs used. It is known, however, that blood levels vary among individuals within a species,[3a] and the differences between the two methods shown in Table II are presumably due largely to intersubject variation in the content of ergothioneine in the rat organs.

The TLC-densitometry method was further used to determine plasma ergothioneine levels in rats after the intravenous administration of 100 mg/kg of ergothioneine (Fig. 5). Each point is the average of eight experiments, with the standard deviation. In each animal, the level of ergothioneine just before administration was subtracted from the total value determined at each point. The plasma concentration of ergothioneine (C_p) declined with time (t) in a bi-exponential manner ($C_p = Ae^{-\alpha t} + Be^{-\beta t}$), corresponding to a two-compartment pharmacokinetic system; the curve depicted as a solid line in Fig. 5 was obtained by iterative least-squares regression analysis. The calculated parameters, A, B, α, β, are also shown in Fig. 5. The mean rate constant β obtained from the slope, $-\beta/2.303$, of the terminal exponential phase was 0.222 hr^{-1}, corresponding to a half-life of 3.12 hr. From the data obtained in this experiment, it appears that the TLC-densitometry method described here is suitable for practical use in studying the pharmacokinetic behavior of ergothioneine in relation to its physiological role in animals.

In conclusion, the results of this study indicate that the TLC-densitometry developed here can be employed to quantify endogenous and exogenous ergothioneine levels in biological materials. Furthermore, this assay method is simple, requiring only one extraction of ergothioneine from biological samples with acetone/methanol (1: 1).