PHARMACOKINETIC STUDIES ON THE HEPATOTOXICITY OF LUTEOSKYRIN. (III)

QUANTITATIVE DETERMINATION OF LUTEOSKYRIN IN THE LIVER

Ikuko UENO

Institute of Medical Science, University of Tokyo, Tokyo 108, Japan

Accepted September 12, 1975

Abstract—A method for the quantitative determination of luteoskyrin in mouse liver is described. Luteoskyrin was extracted with ether from the liver homogenate after acidification with HCl, purified by thin-layer chromatography and determined by spectrophotometry. Five μg of luteoskyrin added to the liver homogenate which was equivalent to one gram tissue was determined with a recovery rate of 97% and 1 μg , under the same conditions, with a recovery rate of 70%. Luteoskyrin in the amount of 0.5 μg could be detected on the chromatogram, but could not be determined quantitatively. A radioactivity corresponding to 0.92 $\mu\text{g/g}$ tissue of ^3H -luteoskyrin in the livers from the mice injected intravenously with this compound was extracted into ether with a recovery rate of 93% and separated on the thin-layer chromatogram with a recovery rate of 81%. The lower limit of quantitative determination of luteoskyrin in one gram of liver was 1 μg . Amounts of luteoskyrin in the livers of mice poisoned with this mycotoxin were estimated. Luteoskyrin was recovered in a large quantity in livers from male mice while detection in the liver of females was nil.

Luteoskyrin is a hepatotoxic and hepatocarcinogenic metabolite of *Penicillium islandicum* Sopp (1~4).

In previous studies on the distribution of ^3H -luteoskyrin in mice, it was demonstrated that radioactive compounds were selectively concentrated in the liver and these high contents in the liver were closely associated with the disturbance of liver functions (5). In addition, there was a larger amount of the radioactive compounds in the livers of animals which showed a higher susceptibility to this toxin.

Experiments were carried out to identify the radioactive compounds in the liver sub-cellular fractions and it was revealed that they consisted mainly of unmetabolized ^3H -luteoskyrin (7).

These findings indicate that the accumulation of luteoskyrin in the liver cells plays an important role in the occurrence of acute liver injury caused by this mycotoxin.

In the present work, isolation techniques developed in the preceding work (7) were applied to a quantitative determination of luteoskyrin contents in the liver to give satisfactory results with high recovery rates.

MATERIALS AND METHODS

Luteoskyrin (LS) and ^3H -LS were furnished as described previously(6) and ^3H -LS was

re-purified by thin-layer chromatography just before use. The purity of ^3H -LS was 96.5%. DDD strain mice were used in all the experiments.

Recovery test with the liver homogenate to which LS has been added

LS was dissolved in a drop of 1 N NaOH and diluted with water and then the alkaline solution was neutralized with diluted HCl. A constant small volume (0.1 ml) of this aqueous solution of LS was pipetted into a tube, acidified by adding a drop of diluted HCl and then diluted with 0.9 ml of acetone. A visible absorption spectrum from 400 $\text{m}\mu$ to 500 $\text{m}\mu$ of this acetone solution was measured using a Shimazu Multipurpose spectrophotometer. The concentration of LS was estimated from the difference in extinction between 448 $\text{m}\mu$ and 500 $\text{m}\mu$ of the above spectrum. The following steps were carried out under cooling in a semidark room. Five ml each of aqueous LS solutions of 2.0, 0.2, 0.1 and 0.04 $\mu\text{g/ml}$ was transferred into a glass-stoppered centrifuge tube (50 ml) and to this was added five ml of 20% mouse liver homogenate in water. After thorough mixing, two ml of concentrated HCl was added and the mixture stirred. To this acidified liver homogenate was added thirty ml of ether and the tube was shaken vigorously for one minute several times at short intervals. The ether layer separated by centrifugation was saved and the ether extraction was repeated again twice in the same way. Three ether extracts were pooled in a flask and rinsed with water. The solvent was evaporated at 40°C under light vacuum. The residue was dried and stored in a desiccator. The residue dissolved in a small volume of ether or acetone was applied in a line 4 cm from the bottom of a thin-layer plate spread with 0.1 M oxalic acid-treated Kiesel gel G (20 cm $\times$ 20 cm) and developed with the upper layer of a mixture of acetone-*n*-hexane-water (4:2:1) until the yellow band of LS was clearly separated from the other brown pigments (usually 10 to 12 cm from the origin). The yellow band of LS was scraped into a glass-stoppered centrifuge tube and LS was extracted into one ml of acetone with vigorous shaking, followed by centrifugation to precipitate the gel. The concentration of LS in the acetone extract was estimated by the procedure described above.

Recovery test with the liver homogenate from mice injected with ^3H -LS

An aqueous solution of ^3H -LS (sp. act. 1.12×10^4 dpm/ μg /0.2 ml saline/animal) prepared by the above procedure was injected i.v. into mice ten minutes before sacrifice. With the animals under ether anesthesia, the livers were perfused with saline *in situ* via the portal vein and then removed. Three livers (4.5 g) were combined and homogenized in water to make a total of 15 ml. A small volume (0.1 ml) of the 30% liver homogenate containing ^3H -LS was put on a small piece of filter paper and burnt in a Packard Tri-Carb 306 sample oxidizer. The radioactivity of $^3\text{H}_2\text{O}$ trapped in 10 ml of Monophase-40 scintillator (Packard) was counted using a Beckman LS 250 liquid scintillation counter. The remaining 30% homogenate was diluted first with water to make a 10% liver homogenate containing ^3H -LS, then it was again diluted with a 10% normal liver homogenate obtained from untreated mice to make five samples containing radioactivities of respectively 9.2, 4.6, 0.92, 0.46 and 0.18 μg of ^3H -LS in 10 ml of the homogenate (equivalent to one gram wet liver). ^3H -LS in each 10 ml of the homogenate was extracted with ether by the procedure described in I. Before

thin-layer chromatography, a small portion of the pooled ether extracts was put into a vial to determine the radioactivity. To the remaining part was added a drop of ethanol solution of pure LS and after evaporation of the solvent, the residue was applied on a thin-layer plate. After development of the plate, the gel from the starting line to the solvent front was divided into ten equal portions and each portion except the one which contained the yellow band of LS was put directly into a vial and 10 ml of the ANPO solution (6) was added. The gel containing LS was scraped into a glass-stoppered centrifuge tube and the yellow pigment was extracted with acetone. After the acetone extract was diluted to an appropriate volume, a portion of the diluted acetone solution was transferred into a vial. The solvent was evaporated and the ANPO solution was added for counting.

Measurement of LS contents in the livers of male and female mice orally administered LS

Four male and three female mice (16 weeks-old) were given orally 250 $\mu\text{g}/10\text{ g}$ of LS dissolved in olive oil two days before sacrifice. The amount of LS in the livers was determined by the method described in I.

RESULTS

Recovery of LS added to the liver homogenate in vitro

The amount and percent of recovery of LS from the liver homogenate to which this mycotoxin had been externally added are shown in Table 1. When 5 or 10 μg of LS was added to 10 ml of the 10% liver homogenate, LS was recovered with a rate of 97%, while with 1 μg of LS added to 10 ml of the homogenate, the recovery rate was 70%. However, when the amount of LS added was reduced to 0.5 μg no observable peak at 448 $\text{m}\mu$ of the visible absorption spectrum was obtained, although a faint yellow band of LS was observed

TABLE 1. Recovery of luteoskyrin in liver homogenate

Amount of luteoskyrin added recovered		Recovery
($\mu\text{g}/10\text{ g}$)		(%)
10.0	9.9	97
	9.5	
5.0	4.7	97
	5.0	
1.0	0.8	70
	0.6	
0.5	slightly*	
0.2	not detectable**	

Luteoskyrin added to the 10% homogenate of the mouse liver was extracted and determined as described in Materials and Methods.

* A faint yellow band of luteoskyrin was observed on the thin-layer chromatogram.

** A yellow band of luteoskyrin was not observed on the chromatogram.

on the thin-layer plate. 0.2 μg of LS could not be detected either chromatographically or spectro-photometrically.

Recovery of ^3H -LS from the liver homogenate of the mice i.v. injected with ^3H -LS

Table 2 shows radioactivities transferred into ether from the liver homogenate containing different amounts of ^3H -LS and those identified as ^3H -LS on the thin-layer plate. When the homogenate equivalent to one gram of the liver contained a radioactivity of 0.92 μg or more of ^3H -LS, more than 93% of it was extracted with ether. When the level of ^3H -LS was lowered to 0.18 μg , the recovery rate was still around 80%. In all the doses tested more than 90% of the ether-extractable radioactivity was recognized chromatographically as ^3H -LS.

TABLE 2. Recovery of ^3H -luteoskyrin from liver homogenate of the mice injected intravenously with ^3H -luteoskyrin

^3H -luteoskyrin in one gram liver		Recovered radioactivity			
μg	dpm $\times 10^2$	Ether extract $\times 10^2$	(%)*	^3H -luteoskyrin on TLC $\times 10^2$	(%)*
9.2	1030	924	90	844	82
		1001	97	882	86
4.6	515	497	97	435	84
		483	94	442	86
0.92	103	96	93	83	81
		96	93	83	81
0.46	51.5	43	84	40	78
		43	84	40	78
0.18	20.6	16	78	14	69
		17	83	16	78

^3H -luteoskyrin present in the livers of mice i.v. injected with ^3H -luteoskyrin was extracted and determined by the method described in Materials and Methods.

* Percent of total radioactivity in one gram of liver.

LS contents in the livers of the male and female mice two days after oral administration of LS

Two days after oral administration of LS (250 $\mu\text{g}/10\text{ g}$), LS was detected in all the livers of the four male mice with their mean concentration of $1.8 \pm 0.5\text{ }\mu\text{g}/\text{g}$ liver, while LS was not detected in the livers of the three females.

DISCUSSION

In the present experiment, it was demonstrated that when one gram of wet liver contained 5 μg of luteoskyrin it could be determined with a recovery rate of 97% and the recovery rate from 1 μg under the same conditions was 70%. However, 0.5 μg of luteoskyrin was detectable only on the thin-layer chromatogram (Table 1).

As stated in the preceding report (7), luteoskyrin is hardly obtainable from the liver of animals when organic solvents are used as extractors. This is attributable to a weak binding to the cellular components of the liver. Therefore, to extract this mycotoxin with a high recovery rate, it is necessary to liberate the bound-luteoskyrin from the cellular components

of the liver by acidification with HCl.

To determine whether or not the present method was actually applicable to determination of luteoskyrin present in the liver, extraction and determination of the ^3H -luteoskyrin which entered the liver through the blood flow were undertaken. As shown in Table 2, when the radioactivity corresponded to $0.92\ \mu\text{g}$ of ^3H -luteoskyrin, in one gram of the liver, 93% of the luteoskyrin was recovered into the ether-extract. Taking into account the high purity of ^3H -luteoskyrin it may be concluded that when $1\ \mu\text{g}$ of luteoskyrin is contained in one gram of the liver it is almost completely extractable with ether under acidic conditions. Even when the level of radioactivity was equivalent to $0.18\ \mu\text{g}$ of ^3H -luteoskyrin in one gram of the liver, luteoskyrin was extracted with a recovery rate of about 80%. Therefore, luteoskyrin of at least 0.2 ppm in the liver is quantitatively extractable.

In the present experiment in which one gram of the liver was used for analysis, the minimum amount of luteoskyrin that could be assayed remained at $1\ \mu\text{g}$ per gram (1 ppm). However, so far as luteoskyrin at a level of 0.2 ppm in the liver is easily extractable as mentioned above, use of larger amounts of liver should make it possible to determine even minute amounts of luteoskyrin. Actually, 0.1 ppm of luteoskyrin could be determined by using 10 g of the liver as will be described elsewhere.

The assay method developed in the present experiment was applied to estimation of the luteoskyrin contents in the livers of male and female mice which had been poisoned with this mycotoxin. The results showed that when the same dose of luteoskyrin was given to both male and female mice, luteoskyrin was detected in large quantities in the livers of the males but was not detectable in the livers of the females. Differences in the liver radioactivity according to the sex were indeed observed in the mice which had been injected s.c. with ^3H -luteoskyrin (6). These findings may correlate with differences according to sex observed in susceptibility of mice to hepatotoxicity (8~10) and hepatocarcinogenicity (3) as related to this mycotoxin. More detailed information regarding the sex related phenomenon will be described in a succeeding report.

REFERENCES

- 1) URAGUCHI, K., TATSUNO, T., SAKAI, F., TSUKIOKA, M., SAKAI, Y., YONEMITSU, O., ITO, H., MIYAKE, M., SAITO, M., ENOMOTO, M., SHIKATA, T. AND ISHIKO, T.: *Japan. J. exp. Med.*, **31**, 19 (1961)
- 2) URAGUCHI, K.: *International Encyclopedia of Pharmacology and Therapeutics*, Section 71. Edited by H. RASKOVA, Part V, p. 143, Pergamon Press, Oxford & New York (1971)
- 3) URAGUCHI, K., SAITO, M., NOGUCHI, Y., TAKAHASHI, K., ENOMOTO, M. AND TATSUNO, T.: *Fd. Cosmet. Toxicol.*, **10**, 193 (1972)
- 4) UENO, I., SAITO, M., ENOMOTO, M. AND URAGUCHI, K.: *Proc. Japan. J. Canad. Assoc.* the 32nd Annual Meeting, p. 187 (1973)
- 5) URAGUCHI, K., UENO, I., UENO, Y. AND KOMAI, T.: *Toxicol. appl. Pharmacol.*, **21**, 335 (1972)
- 6) UENO, I., HAYASHI, T. AND UENO, Y.: *Japan. J. Pharmacol.*, **24**, 535 (1974)
- 7) UENO, I.: *Japan. J. Pharmacol.*, **25**, 171 (1975)
- 8) URAGUCHI, K.: *Proc. of the 15th General Assembly of the Japan Medical Congress*, p. 313 (1959) (in Japanese)
- 9) NOGUCHI, Y.: *Folia pharmacol. japon.*, **56**, 126 (1960) (in Japanese)
- 10) URAGUCHI, K., UENO, I., AKABORI, A. AND ENOMOTO, M.: *Folia pharmacol. japon.* **64**, 1 (1968) (in Japanese)