Determination of citrinin in red yeast (mold) rice and dietary supplement by immunoassay

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Abstract

Citrinin is a mycotoxin produced by *Aspergillus* and *Penicillium* species and is found in various foods and feeds such as cheese, sake, red yeast rice, wheat, barley and corn. During the fermentation processes of Red Yeast (Mold) Rice (*Monascus purpures*) to produce blood cholesterol lowing compound, Monacolin K, the mycotoxin citrinin is also produced by *Monascus* spp. Citrinin is a potent renal and hepatic toxins, which causes kidney damage and alter liver metabolism. Citrinin has been analyzed by liquid and gas chromatography. However, they require clean-up procedures for sample extract before analysis such as solid-phase extraction (SPE) or liquid-liquid extraction (LLE). A competitive immunoassay was developed for rapid and sensitive detection of citrinin in foods without using extensive clean-up procedures. Rabbit polyclonal antibodies against citrinin were produced, and showed a good sensitivity (IC 50 = 25 ppb). Detection limit of the immunoassay was 2 µg/kg (ppb). The assay range was 2 to 300 ppb in buffer solution. Total assay time was 20 minutes. Red yeast rice and dietary supplements were extracted with 70% methanol and analyzed by the immunoassay after centrifugation and filtration. Citrinin from dietary supplements was determined by the immunoassay and compared with HPLC (High Performance Liquid Chromatography). One dietary supplement contained high levels of citrinin, 45 ppm by immunoassay and 46 ppm by HPLC).

Introduction

Citrinin (4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid) is a toxic secondary metabolite from *Penicillium citrinum*. There are other species of *Penicillium*, *Aspergillus sp*. and *Monascus sp*. that produce citrinin as well. Citrinin is known as a nephrotoxin causing renal disease in poultry, pigs, dogs, and rats. Contaminations of citrinin in various commodities such as fermented maize, cheese, wheat, barley, red yeast rice, apples, brewed beer, and cereal products have been reported. Red yeast rice is a product of yeast (and fungus) grown on rice and used to color various food products in China and other Asian countries. It has been used in China as a traditional medicine for gastric illnesses (indigestion and diarrhea), blood circulation, and spleen and stomach health. It was found that red yeast rice contains a group of compounds known as monacolins. One of these compounds, monacolin K inhibits cholesterol synthesis and lowers total and LDL-cholesterol. Therefore, red yeast rice extract has been sold in the market as a natural cholesterol-lowering agent. However, it was found that some red yeast rice dietary supplements were contaminated with citrinin (Gordon et al., www.consumerlab.com). Citrinin is an undesirable by-product produced by *Monascus* sp. during red yeast rice fermentation. Many analytical methods have been developed to detect citrinin with using a clean-up procedure such as solid-phase extraction (SPE; C18) and liquid-liquid extraction. However, a rapid and sensitive method to detect citrinin is important to ensure food safety. In this study, a competitive immunoassay was developed and used to determine citrinin content in red yeast rice supplements.

Figure 1. Red yeast rice and dietary supplement made from its extract.

Methodology

Polyclonal antibody production:
In the development of this assay, citrinin was coupled to a modified protein carrier, which served as an immunogen, and horse radish peroxidase (HRP) which served as the enzyme conjugate. The antibody was produced through the immunization of New Zealand white rabbits. These rabbits were shaved and injections were administered intradermally on the back. The serum was collected monthly.

Sample preparation:
Weigh 10 g of red yeast rice in a 50 mL centrifuge tube and add 20 mL of 70% MeOH in water.
(For supplement sample, weigh 1 g of sample in a 20 mL scintillation vial and add 10 mL of 70% MeOH in water.)
Vortex for 3 min and filter with paper filter (Fisher Scientific, P5).
Transfer 1 mL into a microcentrifuge tube and centrifuge for 2 minutes at 10,000 rpm.
Dilute with deionized water as needed.

Assay protocol:
50 µl of HRP conjugate is added to each well followed by 50 µl of calibrator or sample extract. Lastly 50 µl of antibody solution is added to the wells and mixed gently for 30 seconds and incubated for 10 minutes. The well contents are then decanted and washed 4 times with deionized water. Substrate (100 µl) is added to the wells and incubated for 10 minutes. The reaction is stopped with 100 µl of Stop solution and the contents of the wells are read at 450nm-650nm.

HPLC analysis:
System: Agilent 1200 series
Column: Columbus C18 (150 X 4.6 mm, 5 µm, Phenomenex)
Mobile phase: Acetonitrile/Water/Trifluoroacetic acid (50/50/0.03)
Detector: Fluorescence detector; Ext. 330 nm and Em. 500 nm

Sample preparation procedure is the same as the immunoassay except for the extraction solvent. The mobile phase is used to extract citrinin instead of 70% MeOH.

Table 1. Citrinin calibrators and inhibition ratios.

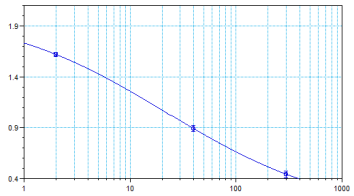
Calibrator (ppb)	%B0
0	100
2	79
40	43
300	22

Results

Table 2. Determination of citrinin by Immunoassay and HPLC

No.	Sample*	Immunoassay (µg/g, ppm)	HPLC (µg/g, ppm)
1	S	45	46
2	S	17.9	< 1
3	S	6.6	< 1
4	S	1.3	< 1
5	S	0	< 1
6	S	22.8	< 1
7	S	19.3	< 1
8	R	0.4	-**
9	G	0	-
10	S	2	-
11	R	0.05	-

*S: Supplement, R: Red yeast rice, G: Gluten added with red yeast rice.
**Not tested.



4-parameter curve fit: Y = (A – D)/(1 + (X/C)^B) + D

A	B	C	D	R^2
2.05	0.544	22.8	0.0554	1

IC 50: 24 ppb

Figure. 2. Standard curve.

Discussion

A competitive immunoassay was developed for the detection of citrinin from red yeast (mold) rice and dietary supplements. For determination of citrinin with instrumental analysis such as HPLC (high-performance liquid chromatography), sample extraction (clean-up) procedure is very time consuming and laborious due to use of high temperature, acidic and organic solvents. Therefore, a simple and easy method to determine citrinin is necessary. Sensitivity of the developed immunoassay was high enough to detect low levels of citrinin in samples. This high sensitivity of the assay makes sample preparation easy. Dilution of sample extracts with deionized water could be easily conducted by a user until the citrinin concentration is within the detection range (2 – 300 ppb). The detection limit of the assay is 2 ppb in buffer solution. The recovery was 70% when 10 ppm of citrinin was spiked in dietary supplements. Eleven samples (8 dietary supplements and 3 red yeast rice added products) were tested by immunoassay. Citrinin contents in the samples determined by immunoassay ranged from 0 to 45 ppm. However, only one high concentration of citrinin was determined by HPLC (sample 1) and matched with the immunoassay result. There could be several reasons for this discrepancy between immunoassay and HPLC. First, since detection limit of HPLC is too poor to detect low levels of citrinin, it could be difficult to detect low levels of citrinin from some samples (4, 8, 10, 11) with HPLC. Second, there could be a cross reaction with citrinin related compounds formed during fermentation and processing. Citrinin can be degraded at high temperature forming degraded compounds (Xu et al., 2006). Some of the degraded compounds increase or decrease cytotoxicity depending on the heating temperatures (Trivedi et al., 1993). During drying process to make dietary supplement from red yeast rice extract, citrinin in the red yeast rice can be degraded to form several compounds including citrinin H_1 and H_2. Then, these compounds may react to the anti-citrinin polyclonal antibody. This could be the reason why the immunoassay showed higher concentration of citrinin than HPLC due to the cross reaction of the antibody to these compounds. However, since most of the degraded compounds are unknown except citrinin H_1 and H_2, it is impossible to test cross reactivity with them. Citrinin H_1 and H_2 are not commercially available at this time. It is not uncommon that immunoassay for the determination of citrinin has the higher result than HPLC due to cross reactions (Xu et al., 2006). Since the clean-up procedure for instrumental analysis is laborious and time consuming, an immunoassay as a screening tool to estimate the level of citrinin in samples could be attractive to have a fast and less expensive screening method especially when testing a lot of samples.

References

Gordon R.Y., Cooperman T., Obermeyer W. and Becker D.J. 2010. Marked variability of monacolin levels in commercial red yeast rice products: buyer beware! Arch. Intern. Med., 170:19, 1722-1727.

Trivedi A.B., Doi E. and Kitabatake N. 1993. Toxic compounds formed on prolonged heating of citrinin under watery conditions. J. Food. Sci., 58 (1), 229-231.

www.cosumerlab.com, Red Yeast Rice Supplement Review.

Xu B., Jia X., Gu L. and Sung C. 2006. Review on the qualitative and quantitative analysis of the mycotoxin citrinin. Food Control, 17, 271-285.