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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE MYCOTOXIN CITRININ AND ITS APPLICATION TO BIOLOGICAL FLUIDS

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SUMMARY

Citrinin is a toxic metabolite produced by several species of *Penicillium* and *Aspergillus*. Citrinin is nephrotoxic and has been implicated in disease outbreaks in animals and humans. Citrinin was resolved as a sharp peak by reversed-phase high-performance liquid chromatography on a small-particle (10 μm) column by elution in 4.25 min with a phosphoric acid (0.25 *N*)–acetonitrile–2-propanol solvent (55:35:10). Detection was by ultraviolet absorbance at 340 nm. The relationship between peak height and area and quantity injected was linear over a range of 2–50 ng at 340 nm and 5–200 ng at 365 nm. Retention time and peak area were highly reproducible. As little as 2–5 ng citrinin was detectable. Complete recovery of citrinin from plasma samples containing known quantities of [^{14}C]citrinin was obtained over a range of 5–40 μg/ml by treatment of the plasma with 1 *N* hydrochloric acid followed by extraction with ethyl acetate. The method provides for the direct analysis of citrinin in urine and bile without prior extraction.

INTRODUCTION

Citrinin (Fig. 1), a secondary metabolite of several *Penicillia* and a few *Aspergilli*, was discovered initially in 1931 by Hetherington and Raistrick[1]. Citrinin, however, proved too toxic for clinical use because of its nephrotoxic action[2]. Later studies have shown an action at the proximal tubules as evidenced by alterations in transport parameters[3] and necrosis[4]. As an environmental health hazard, citrinin was implicated first in Japan in disease outbreaks associated with molded rice shortly after World War II[5]. More recently citrinin has been suggested as one of the causal

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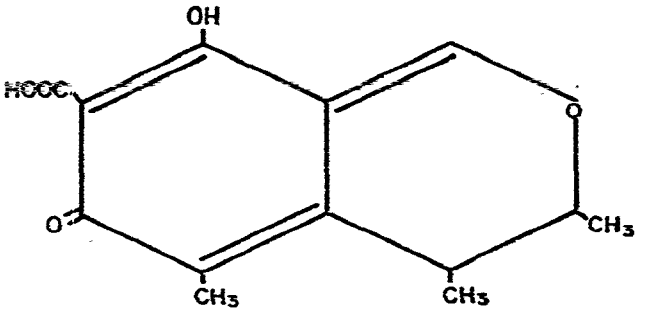


Fig. 1. Structure of citrinin, $C_{13}H_{14}O_5$.

factors in porcine[6–9] and avian[10,11] nephropathy and possibly in a fatal renal disease in humans known as Balkan nephropathy[12].

Procedures for the extraction and estimation of citrinin from natural products and synthetic growth medium have been published[13–16]. A method for citrinin extraction from grains and its estimation using high-performance liquid chromatography (HPLC) also has been described[17]. However, except for the attempt by Wang and Ting[18,19] to estimate citrinin by a colorimetric method, no procedure exist for the extraction and quantitation of citrinin from mammalian tissues and fluids. This paper describes a modification of the method of Marti *et al.*[17] to quantitate citrinin in urine, bile and plasma, and a procedure for the extraction of citrinin from plasma.

EXPERIMENTAL

Citrinin

Highly purified citrinin was produced by a strain of *Penicillium citrinum* and purified according to the method of Davis *et al.*[16]. [^{14}C]Citrinin (sp. act. 31.0 μCi/mmol) was produced by adding labeled acetate (2.0 μCi) to the growth medium on day 7 and harvesting on day 21. The purity of citrinin was confirmed by thin-layer chromatography (TLC), HPLC and melting point determination. Citrinin was dissolved in appropriate volumes of acetonitrile to yield standards containing 0.5, 0.1, 0.05, 0.01 and 0.005 mg/ml.

Equipment

Reversed-phase chromatography was performed using a Waters Associates HPLC system including a U6K septumless injector and a fixed-wavelength absorbance detector, Model 440 (Waters Assoc., Milford, Mass., U.S.A.) and an Omniscribe dual-pen recorder with an electronic integrator (Houston Instruments, Austin, Texas, U.S.A.). Separations were achieved with a μBondapak C_{18} (particle size, 10 μm) column (30 cm × 4 mm I.D.) at an elution rate of 2.0 ml/min at a nominal pressure of 1300 p.s.i. A pre-column (Whatman, Clifton, N.J., U.S.A.), connected just before the μBondapak column, increased the retention time of citrinin only 1–2 sec but allowed bile and urine to be applied directly without prior extraction. Citrinin was detected at 340 nm, with the absorbance detector at sensitivities of 0.005–0.5 absorbance units full scale (AUFS).

Elution solvent system

The elution system consisted of distilled-in-glass acetonitrile and 2-propanol (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.), and 0.25 *N* phosphoric acid distilled-in-glass. The elution solvent was prepared by mixing acetonitrile and 2-propanol and then adding 0.25 *N* phosphoric acid to 0.25 *N* H_3PO_4–acetonitrile–2-propanol (55:35:10). The solvent was degassed by stirring under vacuum.

RESULTS AND DISCUSSION

Optimization of chromatography

Solvent programming (Model 660, Waters Assoc.) was used to establish optimum solvent ratios. Marti *et al.*[17] reported that 0.25 *N* phosphoric acid and acetonitrile or methanol were acceptable as an elution system for citrinin extracted from corn. The addition of 2-propanol or ethyl acetate, increased both resolution and sensitivity. Also, with acetonitrile, the sensitivity (ε) was doubled at 340 nm compared to methanol (Table I). With an elution solvent of 0.25 *N* phosphoric acid–acetonitrile–2-propanol (55:35:10), citrinin eluted as a sharp peak (Fig. 2) within 4.5 min. Increasing the proportion of acetonitrile to phosphoric acid and increasing the 2-propanol concentration decreased the retention time. No decrease in column efficiency was noted over two weeks of daily injections. The first two peaks appearing on the chromatogram are due to acetonitrile which was the vehicle for injection of citrinin onto the column. If the mobile phase was used to inject the standard citrinin, the early solvent peaks are absent.

TABLE I

ABSORBANCE CHARACTERISTICS OF CITRININ IN HPLC ELUTION SOLVENTS

Solvent A: methanol–0.25 *N* phosphoric acid–2-propanol (55:35:10); solvent B: 0.25 *N* phosphoric acid–acetonitrile–2-propanol (55:35:10).

Parameter	*Solvent*	
	A	*B*
λ_{max}	216	335
ε_{max}	22,350	15,250
ε, 340 nm	8000	14,750
ε, 254 nm	9150	9500

A list of various mobile phases tested for citrinin with their respective capacity ratios (k') and t' values are presented in Table II. The t' value is a reflection of peak tailing and was calculated by dividing the total peak width minus the width of the tail by the total peak width. A peak with no tailing would yield a t' value of 1.0. The capacity ratio $[k' = (V_1 - V_0)/V_0]$ is indicative of the retention and resolution of a compound. Usually the k' is changed by varying the solvent strength. A k' value in the range of 2–6 tends to optimize resolution.

In analysis of biological samples such as urine, bile and plasma which may contain polar metabolites, reversed-phase chromatography is indicated for separation of these more polar metabolites from the lipophilic parent compound, citrinin. The

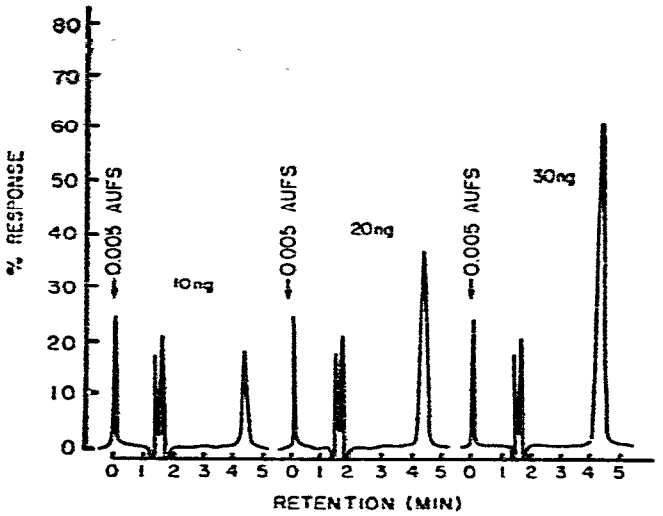


Fig. 2. HPLC resolution of citrinin on μBondapak C_{18}, elution system, 0.25 *N* phosphoric acid–acetonitrile–2-propanol (55:35:10); flow-rate, 2.0 ml/min.

TABLE II

PARAMETERS OF CITRININ IN VARIOUS SOLVENTS ON μBONDAPAK C_{18} COLUMN

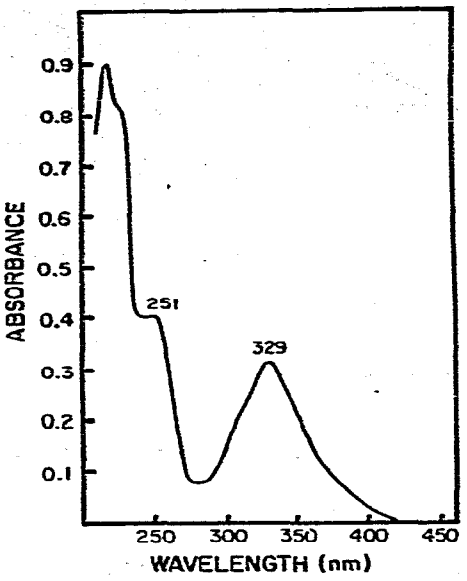
t' = (total peak width − tail width)/total peak width; $k' = (V_t - V_0)/V_0$ (V_0 = void volume; V_t = volume required for peak elution); R_t = retention time.

Mobile phase	*Ratio (v/v)*	*Flow-rate (ml/min)*	*R_t (sec)*	*t'*	*k'*
Methanol–water	70:30	2	138.8 ± 0.8	0.586	1.875
Methanol–0.25 *N* phosphoric acid	55:50	2	416.0 ± 0.0	0.766	7.667
Acetonitrile–0.25 *N* phosphoric acid	70:30	1.5	176.0 ± 0.0	0.616	2.667
Acetonitrile–0.25 *N* phosphoric acid	90:10	1	198.0 ± 0.0	0.733	3.125
Methanol–ethyl acetate–0.25 *N* phosphoric acid	55:10:35	2	140.6 ± 0.7	0.852	1.929
Acetone–ethyl acetate–0.25 *N* phosphoric acid	5:1.5:4.5	2	183.6 ± 1.3	0.726	2.825
Acetonitrile–acetone–0.25 *N* phosphoric acid	40:25:35	2	140.0 ± 0.0	0.083	1.917
Acetonitrile–acetone–0.25 *N* phosphoric acid	50:20:30	2	125.7 ± 1.3	0.049	1.619
Methanol–2-propanol–0.25 *N* phosphoric acid	5.5:2.0:2.5	2	99.0 ± 0.7	0.702	1.062
Methanol–2-propanol–0.25 *N* phosphoric acid	5.5:1.0:3.5	2	136.0 ± 0.0	0.818	1.833
0.25 *N* phosphoric acid–acetonitrile–2-propanol	55:35:10	2	256.0 ± 0.3	0.817	4.333

more polar compounds elute prior to citrinin. A retention time of at least 3 min but not longer than 5 or 6 min then is desirable for speed in analysis. The solvent system using 0.25 *N* phosphoric acid–acetonitrile–2-propanol (55:35:10) produced an acceptable t', k' and retention time (R_t).

Detection

Absorbance spectra for citrinin were determined in two solvent systems: solvent A, methanol–0.25 *N* phosphoric acid–ethyl acetate (55:35:10) (Fig. 3) and solvent B, 0.25 *N* phosphoric acid–acetonitrile–2-propanol (55:35:10) (Fig. 4). The



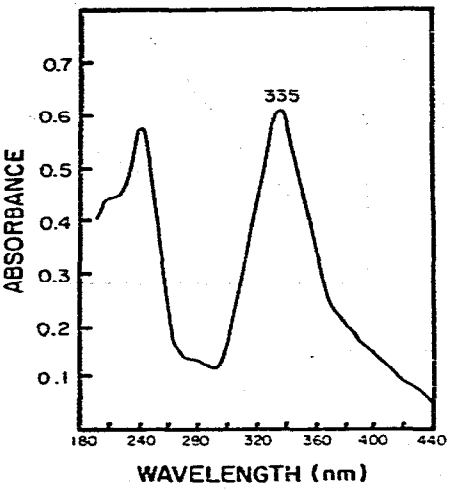


Fig. 3. UV absorbance spectra of citrinin (10 ng/ml) in methanol–0.25 *N* phosphoric acid–ethyl acetate (55:35:10).

Fig. 4. UV absorbance spectra of citrinin (10 ng/ml) in 0.25 *N* phosphoric acid–acetonitrile–2-propanol (55:35:10).

detection wavelength of 340 nm was chosen as the closest to the absorption maxima of 335 nm for citrinin in elution solvent B (Table I). The molar extinction coefficient (ε) of citrinin in solvent system B at 340 nm was approximately twice that of citrinin in solvent system A. Although Marti *et al.*[17] earlier reported detection of citrinin at 254 nm, the inherent peaks in plasma extract, and in urine and bile were much lower at 340 nm than at 254 nm. In solvent system B, the molar extinction coefficient of citrinin also was greater at 340 nm, than at 254 nm regardless of the solvent system (Table I). Therefore detection of citrinin in this procedure was performed at 340 nm.

Linearity

The relationship between peak heights and areas and amount of citrinin injected was linear over 2–50 ng at 340 nm and 5–200 ng at 365 nm (Fig. 5). Over a three-day period, 48 injections (5 μl each) of citrinin standard were made, using microliter syringes (Hamilton, Reno, Nev., U.S.A.).

Precision and sensitivity

Precision was evaluated by injecting ten 5-μl aliquots of citrinin standard containing 25 ng of citrinin. Reproducibility of both peak height and peak area measurements were good, with coefficients of variation of 0.95 for peak height and 1.23 for peak area, representing the combined errors of HPLC resolution, injection and detection (Table III).

Mean sensitivity of detection, mm peak height/ng, of citrinin (5.24) shown in Table III, and the chromatogram shown in Fig. 2 indicate that 10–30 ng of citrinin can easily be detected.

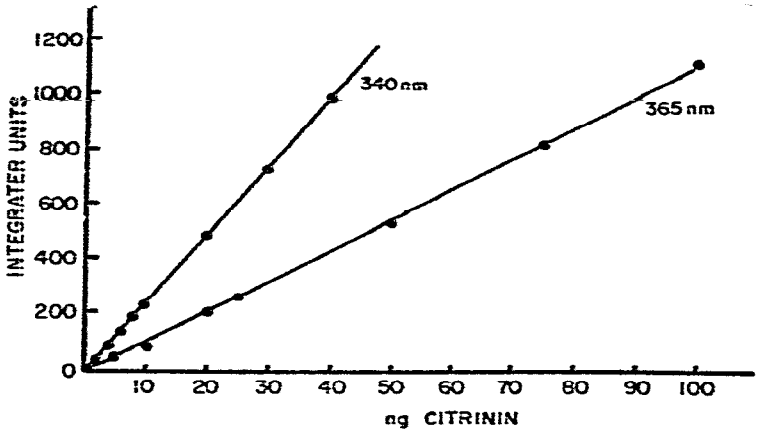


Fig. 5. Linearity of absorbance *vs.* quantity of citrinin at 340 nm (2–50 ng) and 365 nm (5–200 ng).

TABLE III

PEAK HEIGHT AND PEAK AREA REPRODUCIBILITY IN CITRININ SEPARATION BY HPLC

Results were obtained by ten successive 5-μl injections of 25 ng citrinin standard at full scale sensitivity. Sensitivity for peak height is (mm peak height/ng) and for peak area (mm^2 peak area/ng).

	Peak height (mm)	*Peak area (mm^2)*
Range	129.5–133.5	690–718
Mean	131.1	700.6
Standard deviation	1.25	8.6
Coefficient of variation (%)	0.95	1.23
Sensitivity	5.24	28.02

Retention time

Retention times were highly reproducible with the HPLC solvent and column used. Using solvent system B, 48 injections over a three-day period gave a mean retention time of 257 sec with a coefficient of variation [(σ/mean) × 100] of 0.56% (Table IV). As expected, the retention time was shorter than that for the less polar solvent system A. Twenty-four injections over a three-day period gave a mean retention time of 148.4 sec with a coefficient of variation of 1.64%.

Extraction of citrinin from plasma

Rat plasma (1.0 ml) containing 5, 10, 25 or 50 μg/ml of citrinin was acidified by adding an equal volume of 1.0 *N* hydrochloric acid. The mixture was extracted three times with equal volumes of ethyl acetate. The ethyl acetate was evaporated to dryness under nitrogen. Acetonitrile (1.0 ml) was added to the residue and 5 μl aliquots were analyzed by HPLC. The recovery of citrinin from plasma was 95.5–100% over a range of 5–50 μg/ml of citrinin (Table V).

Rat plasma (1.0 ml) containing 100 μg of [^{14}C]citrinin also was extracted as outlined above. The dried extracts were dissolved in 100 μl of acetonitrile and 10 μl aliquots were analyzed by HPLC. Eluate fractions were collected at 10-sec intervals.

TABLE IV

REPRODUCIBILITY OF RETENTION TIME FOR CITRININ BY HPLC

Solvent A: methanol–0.25 *N* phosphoric acid–ethyl acetate (55:35:10); solvent B: 0.25 *N* phosphoric acid–acetonitrile–2-propanol (55:35:10).

Parameter	*Solvent system*	
	A	*B*
Injections*	24	48
Retention time (sec)		
Range	144–150	254–260
Mean	148.4	257.0
Mean retention time (min)	2.47	4.28
Standard error (sec)	0.51	0.22
Coefficient of variation (%)	1.64	0.56

* Successive injections of citrinin standard, 0.002–0.500 μg, over 4 days.

TABLE V

RECOVERY OF CITRININ FROM PLASMA, URINE AND BILE SAMPLES

Citrinin added (μg/ml)	*Recovery (%) ($\bar{X} \pm$ S.D.)*		
	Plasma	*Urine*	*Bile*
5	100.0 ± 2.52	96.43 ± 2.02	107.14 ± 2.52
10	97.4 ± 2.93	100.88 ± 2.35	102.78 ± 3.37
25	97.2 ± 1.16	98.85 ± 2.46	100.22 ± 0.96
50	95.5 ± 0.38	104.95 ± 0.18	103.03 ± 0.93

To each of these, 15 ml of PCS® (Amersham, Arlington Heights, Ill., U.S.A.) were added and the samples subjected to liquid scintillation spectrometry. More than 96% of the ^{14}C activity eluted with an R_f of 254 sec corresponding with the citrinin peak. The remaining radioactivity eluted with a peak at 164 sec, normally seen from plasma extracts (Fig. 6). This may indicate that a fraction of the citrinin originally added to plasma is associated with an extractable component of plasma or is altered during the extraction process.

Analysis of citrinin in urine and bile

Urine and bile samples were analyzed before and after the addition of known quantities of citrinin. A chromatogram of urine (5 μl) and urine (5 μl) plus 30 ng of citrinin is presented in Fig. 7. There were no interferring peaks at 340 nm and resolution of the citrinin was good. Fig. 8 presents a chromatogram of bile (5 μl) and bile (5 μl) plus 30 ng of citrinin. Separation from inherent substances in the bile was good and comparable to that seen with urine. The presence of the guard column described earlier allowed the direct injection of urine or bile without sample clean-up.

Recovery of citrinin added to urine and bile samples was complete as indicated in Table V. Recovery was independent of the quantity of citrinin added due to elimination of an extraction step by the direct analysis of urine or bile.

The described method for quantitative determination of citrinin in plasma, urine and bile offers several advantages over the colorimetric methods of Wang and

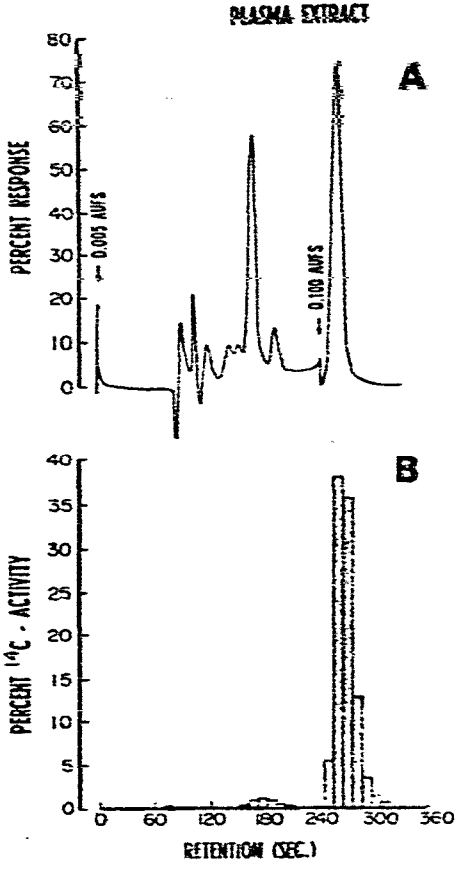


Fig. 6. Chromatogram of plasma extract of [^{14}C]citrinin (A) and radioactivity collected from the column at 10-min intervals (B).

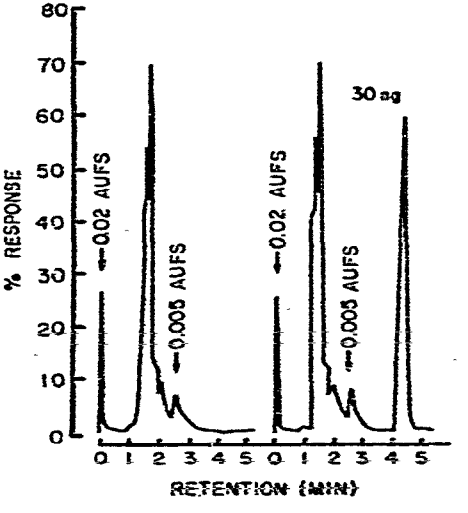


Fig. 7. Chromatogram of urine (5 μl) and urine (5 μl) plus 30 ng of citrinin.

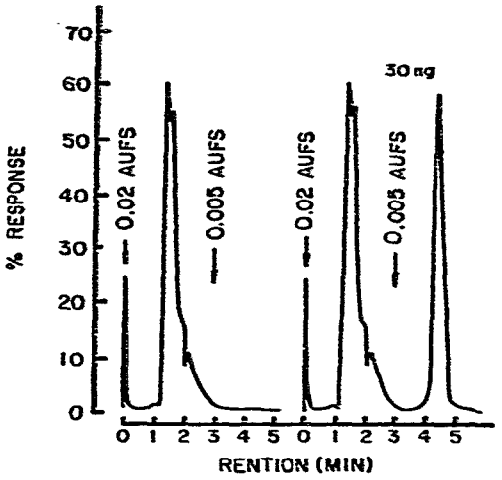


Fig. 8. Chromatogram of bile (5 μl) and bile (5 μl) plus 30 ng of citrinin.

Ting[18,19] and previous TLC methods[20,21]. The HPLC procedure is simple and rapid, with a time for analysis of 5–6 min. The procedure allows direct analysis of urine and bile and a simple one-step extraction from plasma. The method is well-suited for field monitoring of plasma levels of citrinin in farm animals, humans or for pharmacokinetic studies.

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