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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF THE MYCOTOXINS, RUBRATOXINS A AND B, AND ITS APPLICATION TO THE ANALYSIS OF URINE AND PLASMA FOR RUBRATOXIN B

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SUMMARY

The rubratoxins are toxic metabolites produced by *Penicillium rubrum* and *P. purpurogenum* on food and feedstuffs. Rubratoxin B is hepatotoxic, mutagenic and teratogenic. Rubratoxins A and B were resolved as sharp peaks in the order A–B by reversed-phase high-pressure liquid chromatography on a small-particle (10 μm) column in 3 min by an acetonitrile–water–ethyl acetate elution solvent (11:9.9:3), with detection by ultraviolet absorbance at 254 nm. The relationship between peak height and quantity injected was linear over a range of 0.25–5 μg for rubratoxin A and 0.05–5 μg for rubratoxin B. Retention time and peak height and peak area were highly reproducible for both toxins. Detection was very sensitive, allowing detection of 3–5 ng rubratoxin B, and 15–20 ng rubratoxin A. Quantitative recovery of rubratoxin B from spiked urine samples was obtained over a range of 5–40 μg/ml, with maximum recovery from urine samples adjusted to pH 2 before extraction. Good recovery of rubratoxin B was also obtained from spiked plasma samples subjected to treatment with 3 *N* hydrochloric acid followed by extraction with ethyl acetate.

INTRODUCTION

Rubratoxin B, a metabolite of *Penicillium rubrum* and *P. purpurogenum*, was brought first to the attention of researchers in 1952, following a disease outbreak among pigs and cattle that had consumed moldy corn[1]. Other similar field intoxications involving these fungi also have been reported[2,3]. Rubratoxin B (Fig. 1) is hepatotoxic[4], mutagenic[5], embryocidal and teratogenic[5,6]. Umeda[7] described cytoplasmic and nuclear pleomorphism in cultured cells from rat liver, kidney and lung treated with rubratoxin B. Numerous reports have described procedures for the extraction and estimation of rubratoxin from natural and semisynthetic growth media[8–12]. However, aside from radioisotope and bioassay techniques, no procedures exist for the extraction and estimation of rubratoxin from biological fluids. This paper describes a high-pressure liquid chromatography (HPLC) system for the quantitation of rubratoxins A and B and procedures for the isolation of rubratoxin B from plasma and urine.

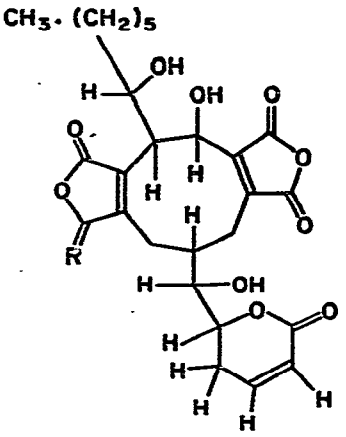

Fig. 1. Structure of rubratoxins. Rubratoxin A, $C_{26}H_{32}O_{11}$; R = H, OH. Rubratoxin B, $C_{26}H_{30}O_{11}$; R = O.

EXPERIMENTAL

Standards

Highly purified rubratoxin A was donated by M. O. Moss, University of Surrey, Great Britain. Highly purified rubratoxin B was prepared according to the procedure of Hayes and Wilson[10]. The purity of both rubratoxins was confirmed by HPLC and mass spectral analysis. Crystalline rubratoxins A and B were accurately weighed and dissolved in acetonitrile to prepare individual stock standards containing 1 mg/ml. Aliquots of the stock standards of rubratoxins A and B were dissolved in the appropriate volume of acetonitrile to yield standards containing 0.5, 0.25, 0.1 and 0.05 mg/ml and 0.5, 0.25, 0.1 and 0.01 mg/ml, respectively.

Equipment

Reversed-phase chromatography was performed using a Waters Assoc. 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 equipped with an electronic integrator (Houston Instrument, 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 1.7 ml/min at a nominal pressure of 1500 p.s.i. The rubratoxins were detected at 254 nm, with the absorbance detector at sensitivities of 0.005–0.5 absorbance units full scale (AUFS).

Elution solvent

The elution solvent consisted of distilled-in-glass acetonitrile and ethyl acetate (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.), and distilled-in-glass water. The distilled water was degassed by heating. The elution solvent was prepared by mixing the acetonitrile and water (11:9.9), allowing the mixture to warm to room temperature, and then adding ethyl acetate (12.5%, v/v).

RESULTS AND DISCUSSION

Optimization of chromatography

Solvent programming (Model 660, Waters Assoc.) was used to establish optimum solvent ratios. Acetonitrile–water (11:9.9) gave adequate separation of rubratoxin A from the low retention time peak found in all rubratoxin samples[10]. The addition of ethyl acetate (12.5%, v/v) produced sharper peaks and increased the sensitivity to both rubratoxins. With this elution solvent, the two rubratoxins eluted as sharp peaks (Fig. 2) in three min. Increasing the proportion of acetonitrile to distilled water and increasing the ethyl acetate concentration decreased the retention times of both rubratoxins. No decrease in column efficiency was noted over 1–2 weeks of daily injections. Some decrease in sensitivity was noted if standards were injected from the elution solvent rather than acetonitrile.

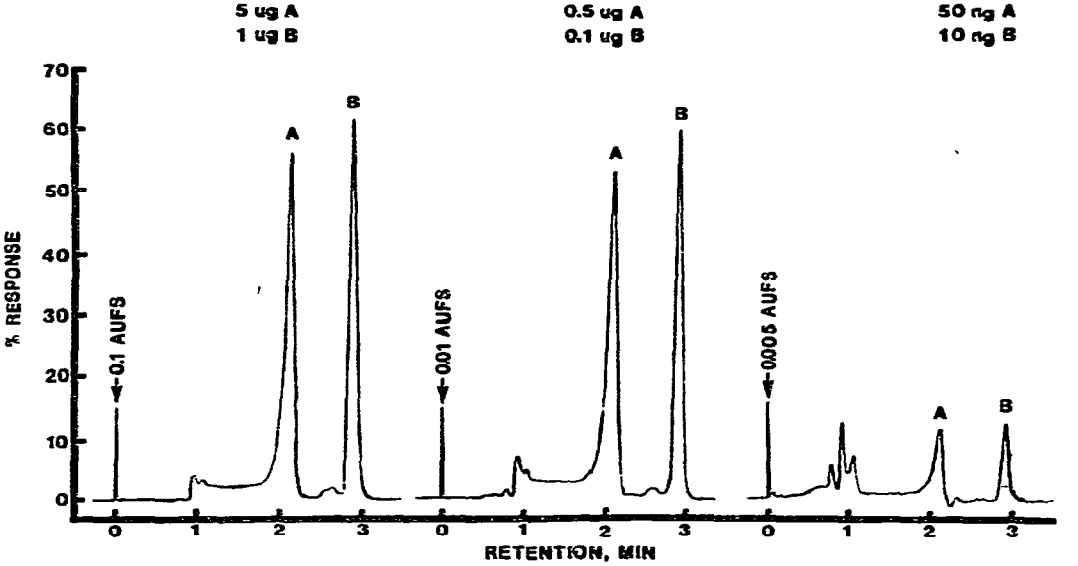

Fig. 2. HPLC resolution of rubratoxins A and B on μBondapak C_{18}, elution solvent acetonitrile–water–ethyl acetate (11:9.9:3); flow-rate 1.7 ml/min.

The elution pattern A–B was, as expected, the reverse of the elution pattern observed for silica gel thin-layer chromatography[10,13]. The second peak appearing on the chromatogram (retention time, t_R = 58 sec) was present in all rubratoxin samples exposed to water. The first and third peaks were found to be due to the acetonitrile in which the standards were dissolved.

The capacity ratios (k') and t' values of rubratoxin B in various HPLC systems are listed in Table I. 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. As indicated in the table, the k' values for rubratoxin B on μPorasil in all the solvent systems tested were poor, and peak tailing was a problem. The k' values for rubratoxin B using reversed-phase

TABLE I

PARAMETERS OF RUBRATOXIN B IN VARIOUS HPLC SYSTEMS

$k' = (V_1 - V_0)/V_0$; t' = (total peak width − tail width)/total peak width.

Stationary phase	*Mobile phase*	*Ratio (v/v)*	*Flow-rate (ml/min)*	t_R *(sec)*	k'	t'
μPorasil	Acetonitrile–chloroform	1:1	2.0	175	1.3	0.394
	Acetonitrile–chloroform	2:1	2.0	130	0.71	0.258
	Acetonitrile–chloroform–methylene chloride	63.3:31.7:5	2.0	140	0.84	0.397
	Acetonitrile–chlorcform–methylene chloride	56.7:28.3:15	2.0	165	1.17	0.258
	Acetonitrile–chloroform–ethyl acetate	63.3:31.7:5	2.0	133	0.75	0.424
	Acetonitrile–chloroform–ethyl acetate	9:9:2	2.0	160	1.10	0.316
	Acetonitrile–chloroform–ethyl acetate	2:2:1	2.0	152	1.00	0.306
	Acetonitrile–chloroform-ethyl acetate	1:1:1	2.0	145	0.91	0.345
μBondapak C_{18}	Acetcnitrile–chloroform	1:1	2.0	82	3.31	0.5
	Acetonitrile–chloroform	7:3	1.5	110	3.40	0.2
	Methylene chloride–acetonitrile	4:1	2.0	110	4.79	0.361
	2-Propanol–acetonitrile	1:1	1.5	105	3.20	0.450
	Acetonitrile–water–ethyl acetate	11:9.9:3	1.7	175	6.95	0.6

chromatography were more acceptable. The best k' and t' values were obtained with acetonitrile–water–ethyl acetate (11:9.9:3) and the μBondapak C_{18} column. Analysis of ethyl acetate extracts of spiked plasma samples using the more promising HPLC systems (based on k' and t' values) revealed that only the acetonitrile–water–ethyl acetate solvent with the μBondapak C_{18} column yielded adequate separation from interfering peaks.

It was discovered, during the course of these experiments, that upon exposure to water, rubratoxin B was converted to a low-retention-time compound (t_R = 58 sec) using the reversed-phase HPLC system (Fig. 3). A 50-μl volume of acetonitrile containing 50 μg of rubratoxin B was mixed with 100 μl of glass-distilled deionized water

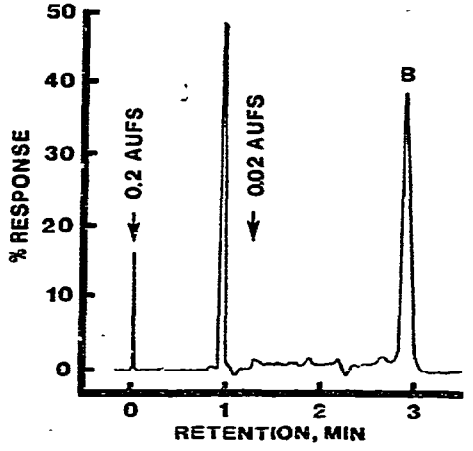

Fig. 3. Chromatogram tracing of 3-μl aliquot of mixture of 50 μg rubratoxin B in 50 μl acetonitrile and 100 μl water after 32 min incubation.

to give a ratio of 1:2 (v/v). Then, 3-μl aliquots of the mixture were injected in rapid succession. As shown in Fig. 4, the loss in absorbance of the rubratoxin B peak was biexponential (●) and concomitant with the increase in absorbance of the 58-sec peak (○). A summation of the area of the 58-sec peaks and the extrapolated values for the area of the rubratoxin B peaks at the same time points indicates that 40% of the original UV absorbance is lost by 55 min of exposure to water (▲).

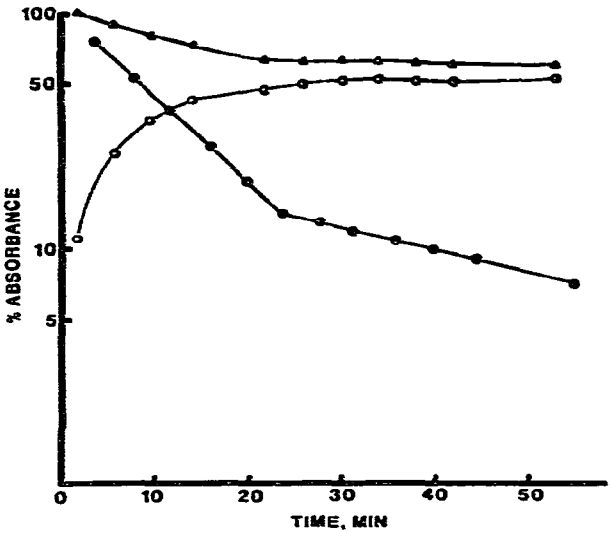

Fig. 4. Semi-log plot of rubratoxin B peak absorbance (●), 58-sec peak absorbance (○) and total absorbance (▲) at 254 nm, *vs.* time after exposire to water. See text for experimental protocol. Sum of absorbance of 58-sec peak and rubratoxin B peak of 1 μg rubratoxin B standard was taken as 100%. Column and conditions listed in text. The points in the rubratoxin B absorbance curve are mean of values fitted to $y = ae^{bx}$ ($0.96 \leqslant r^2 \leqslant 0.99$); the points in the 58-sec peak absorbance curve are mean of observed values. Rubratoxin B absorbance was extrapolated to the 58-sec peak times and the absorbance of both peaks summed to give total absorbance.

Approximately 3.8% of the peak area of rubratoxin B was lost in the 3-min exposure to the elution solvent. Because of the conversion of rubratoxin B upon exposure to water, the concentration of water in the elution solvent must be carefully controlled and exposure of rubratoxin B to the elution solvent prior to injection must be kept to a minimum.

Detection

The detection wavelength of 254 nm was chosen as being closest to the absorption maxima of 242–246 nm for rubratoxins A and B in the elution solvent (Table II). The molar extinction coefficient, ε, at 254 nm of rubratoxin B was approximately 1.9 times that of A. However, the $\varepsilon_{max.}$ of both toxins in this solvent system was approximately half the $\varepsilon_{max.}$ reported for the rubratoxins in acetonitrile[14].

TABLE II

ADSORPTION CHARACTERISTICS OF RUBRATOXINS A AND B IN HPLC ELUTION SOLVENT

Solvent: acetonitrile–water–ethyl acetate (11:9.9:3).

Characteristic	*Rubratoxin*	
	A	*B*
$\lambda_{max.}$	242	246
$\varepsilon_{max.}$, 242–246 nm	5333	5440
ε, 254 nm	2813	5233

Retention time

Retention times were highly reproducible with the HPLC solvent and column used; 46 injections over a 4-day period gave mean retention times of 127 (A) and 176 (B) sec with coefficients of variation [(σ/mean)·100] of 0.53% and 0.77%, respectively (Table III).

TABLE III

REPRODUCIBILITY OF RETENTION TIME FOR THE RUBRATOXINS BY HPLC

Statistic	*Rubratoxin*	
	A	*B*
Injections, N*	46	46
Retention time (sec)		
Range	126–130	174–180
Mean	127	176
Mean retention time (min)	2.1	2.9
Standard deviation (sec)	0.67	1.35
Coefficient of variation (%)**	0.53	0.77

* Successive injections of rubratoxin A standard, 0.25–5 μg, or rubratoxin B standard, 0.05–5μg, over 4 days.

** $\frac{\text{Standard deviation}}{\text{mean}} \times 100.$

Linearity

The relationship between peak heights and areas and amount of rubratoxin injected was linear over 0.25–5 μg range for rubratoxin A and 0.05–5 ug range for rubratoxin B (Fig. 5). Over a 4-day period, 46 injections, 2.5–10 μl, of each rubratoxin standard were made, using microliter syringes (Precision Sampling, Baton Rouge, La., U.S.A.).

Precision and sensitivity

Precision was evaluated by injecting ten 5-μl aliquots of each rubratoxin standard containing 0.5 μg of rubratoxin. Reproducibility of both peak height and peak area measurements were good, with coefficients of variation of 0.98–1.03% for

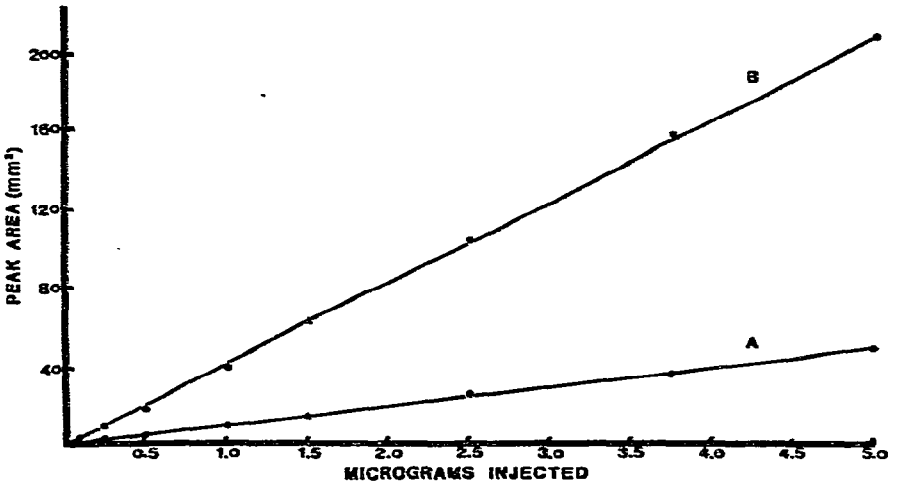

Fig. 5. Linearity, absorbance *vs.* amount of rubratoxin A (0.25–5 μg) and B (0.05–5 μg) injected.

peak height and 1.45–1.65% for peak area, representing the combined errors of HPLC resolution, injection and detection (Table IV).

Mean sensitivity of detection, mm peak height/ng, of A (0.37) and B (1.97), shown in Table IV, and the chromatogram shown in Fig. 2 indicate that 15–20 ng of rubratoxin A and 3–5 ng of rubratoxin B can easily be detected.

TABLE IV

PEAK HEIGHT AND PEAK AREA REPRODUCIBILITY IN RUBRATOXIN SEPARATION BY HPLC

Statistic	*Rubratoxin*	
	A	*B*
Injections, *N**	10	10
Peak height (mm)		
Range	90–93	98–100
Mean	92.3	98.5
Standard deviation (mm)	0.95	0.97
Coefficient of variation (%)**	1.03	0.98
Sensitivity (mm peak height/ng)***	0.37	1.97
Peak area (mm^2)		
Range	484–505	363–381
Mean	493	374
Standard deviation (mm^2)	7.2	6.2
Coefficient of variation (%)**	1.45	1.65
Sensitivity (mm^2 peak area/ng)***	1.97	7.49

* Successive 5 μl injections of 0.5μg rubratoxin A standard, 0.01 AUFS or 0.5 μg rubratoxin B standard, 0.05 AUFS.

** $\frac{\text{Standard deviation}}{\text{mean}} \times 100.$

*** Calculated to maximum sensitivity, 0.005 AUFS.

BIOLOGICAL APPLICATIONS

Extraction of rubratoxin B from human urine

Recovery of rubratoxin from spiked urine samples was dependent upon urine pH with maximum recovery obtained at pH 2 or below.

A volume of 25 ml of urine was divided into 5-ml aliquots and 100 μg rubratoxin B added to each aliquot. The pH of the spiked urine samples then was adjusted with 1 *N* HCl to 1.0, 2.0, 3.0, 4.0 or 5.0, and 1-ml aliquots of each sample were freeze-dried. The freeze-dried material was extracted with 0.5 ml acetonitrile by heating to 73° for 2 h, with constant shaking, in a water bath. The results (Fig. 6)

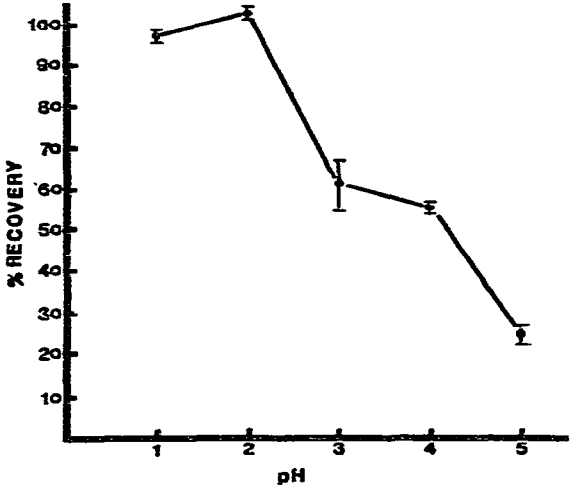

Fig. 6. Recovery of rubratoxin B from urine. Values are mean ± S.E. of three individual extractions.

indicated that maximum recovery was achieved at pH 2 or below with a gradual diminution to about 24% recovery at pH 5. To determine the linearity of rubratoxin B recovery from urine, 200, 100, 50 and 25 μg of toxin were added to 5-ml urine samples and extracted as above. A typical chromatogram is shown in Fig. 7a. Data in Table V indicated good recovery over the toxin concentration range investigated.

Extraction of rubratoxin B from human plasma

A 0.5-ml volume of 3 *N* hydrochloric acid was added to small test tubes containing 0.5 ml of spiked plasma samples. The tubes were incubated for 15 min at 80° with constant shaking. The mixture was extracted twice with 1.0 ml of cyclohexane by vortexing for 2 min each time. After centrifugation at high speed for 15 min in a table-top clinical centrifuge, the upper cyclohexane layer was discarded. The lipid-extracted aqueous phase then was extracted twice with 1.0 ml of ethyl acetate by vortexing for 2 min each time. After centrifugation (<800 *g*) the upper ethyl acetate layers were pooled, evaporated to dryness and taken up into acetonitrile for HPLC analysis. Denaturation of plasma protein by acid treatment followed by centrifugation eliminated the need for filtration of the plasma samples, thus allowing analysis of

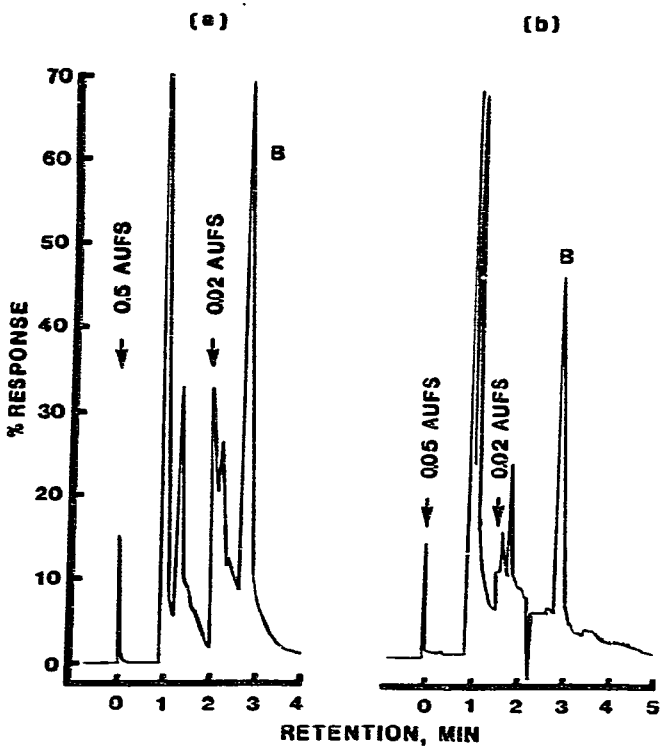

Fig. 7 (a) Chromatogram tracing of extract of urine spiked to 40 μg/ml. (b) Chromatogram tracing of extract of plasma spiked to 20 μg/ml. See text for column and conditions.

TABLE V

RECOVERY OF RUBRATOXIN B FROM SPIKED URINE SAMPLES

Rubratoxin B added (μg/ml)	*Recovery (%), mean ± S.E.*
0	—
5	103.8 ± 2.1
10	98.7 ± 2.0
20	95.2 ± 5.2
40	91.7 ± 4.1

samples of small volume. No increase in system back-pressure was observed despite many days of repeated injections, indicating adequate sample preparation.

As indicated in Table VI, the recovery of rubratoxin B from plasma was linear over a range of 2–20 μg/ml of plasma. A typical chromatogram is shown in Fig. 7b. The inclusion of the lipid-extraction step in the extraction procedure eliminated much of the interference seen previously in the latter part of the elution profile.

To determine the optimum time for the acid treatment, 0.5-ml plasma samples spiked with 2.5 μg rubratoxin B were incubated with 0.5 ml 3 *N* hydrochloric acid for 0, 5, 10, 15, 20, 30, 45 and 60 min at 80° with constant shaking and extracted as above. As shown in Fig. 8 (●), maximum recovery was obtained at 15 min, with a

TABLE VI

RECOVERY OF RUBRATOXIN B FROM SPIKED PLASMA SAMPLES

Rubratoxin B added (μg/ml)	*Recovery (%), mean ± S.E.*
2	92.99 ± 3.61
5	92.99 ± 2.22
10	90.22 ± 2.21
20	90.01 ± 5.76

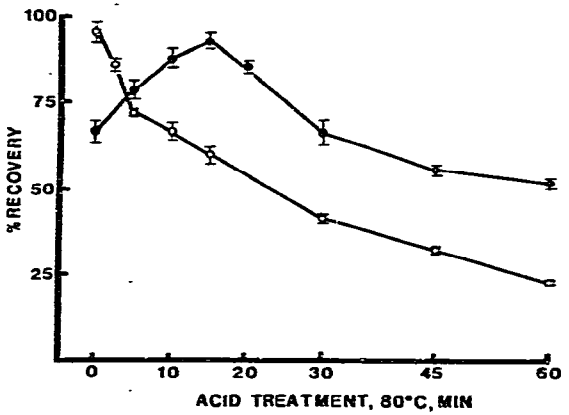

Fig. 8. Recovery of rubratoxin B from plasma (●) and Ringer's solution (○) *vs.* duration of acid treatment at 80°. Values are mean ± S.E. of three individual extractions.

decrease in recovery to about 52% by 60 min of incubation. Ethyl acetate extraction of plasma samples (with no acid treatment) yielded only 2.2% recovery of rubratoxin B.

In an attempt to determine the cause for the decrease in recovery seen after 15 min of hydrolysis, 2.5 μg rubratoxin B was added to aliquots of 0.5 ml of Ringer's solution with 0.5 ml 3 *N* hydrochloric acid and incubated at 80° with constant shaking for 0, 2.5, 5, 10, 15, 30, 45 and 60 min. Ethyl acetate extracts of these samples were evaporated to dryness and analyzed. Maximum recovery was obtained with no heating of the acidified Ringer's solution (Fig. 8, ○). The recovery of rubratoxin decreased exponentially after 5 min of hydrolysis, with the observed data points nicely fitting the equation $y = ae^{bx}$ ($r^2 > 0.999$). Ethyl acetate extraction of Ringer's solution (without addition of acid) yielded no recovery of rubratoxin B.

It seems likely, in view of the recovery data, that rubratoxin B was converted to the low retention-time compound by exposure to water in the aqueous solution, a reaction that was reversed by the addition of hydrochloric acid. Numerous reports have indicated that adjustment of the pH of liquid fungal growth media to about 1.5 before ethyl–ether extraction yields the greatest recovery of rubratoxin B[9,10,15]. In addition, complex formation of rubratoxin B with some plasma component was likely since simple acid treatment was not sufficient to give full recovery of the rubratoxin from plasma. Since rubratoxin B recovery from acidified plasma actually increased

for 15 min following the application of heat, it appeared that some plasma component offered protection to rubratoxin B from the immediate degradation by heat and acid seen in Ringer's solution (Fig. 8, ○).

The form to which rubratoxin B was converted in aqueous solution is apparently readily converted back to the original compound. It is thought that the maleic anhydride rings are converted to the open form(s) by scission of the two anhydride rings in the presence of hydroxylating solvents[16,17]. We feel that the low-retention-time compound to which rubratoxin B was converted is this carboxylated form. Toxicity studies have shown that the opening of the maleic anhydride rings on the rubratoxin B molecule by formation of the tetrasodium salt of rubratoxin B resulted only in a quadrupling of the LD_{50} to mice from 3 mg/kg to 12 mg/kg body weight[18].

According to the results contained in the present report, rubratoxin B exists in three phases in plasma. Approximately 2% remains in the parent form, 65% is present in the carboxylated form, with the remaining 33% reversibly associated with some plasma component, probably albumin. There is no evidence to indicate that the rubratoxin B is bound to the plasma albumin fraction in the carboxylated form (may be bound in the parent form), nor is there any evidence that the carboxylated form cannot be taken up by body tissues. Indeed, recent studies performed in this laboratory indicate that rubratoxin B (as quantitated using the procedure described here) is removed from the plasma of isolated perfused rat liver preparations with a half-life of about 200 min (unpublished data). This would not be possible if the carboxylated rubratoxin B were inert or biologically inactive.

The extraction procedure outlined in this report converted the carboxylated form back to the parent form and also released any rubratoxin B which may have been bound to plasma components. The result, therefore, is that the total rubratoxin content of the plasma can be determined.

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