Purification of Cyclopiazonic Acid by Liquid Chromatography

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A purification procedure for cyclopiazonic acid has been developed, using sequential preparative and semi-preparative liquid chromatography. Crude cyclopiazonic acid (324 mg) was extracted from a 1 L fermentation medium with chloroform-methanol (80 + 20), dried, dissolved in chloroform, and chromatographed on an oxalic acid/silica preparative column with chloroform-methanol (99 + 1) as the eluant. A semi-preparative oxalic acid/silica column and chloroform-methanol (99.5 + 0.5) were then used for rechromatography of the partially purified cyclopiazonic acid. This second chromatographic treatment yielded fractions from which cyclopiazonic acid was readily crystallized (106.7 mg; 33% recovery). Analytical chromatography was developed using an amino column in an ion-exchange mode, with a methanol-phosphate buffer eluant. Response was linear from 10 to 800 \( \mu \)g/injection of standard solutions. Cyclopiazonic acid chemically binds sodium from soda-lime vials.

Cyclopiazonic acid (CPA) is a neurotoxic mycotoxin, acutely toxic in rats and ducklings (1, 2). CPA is produced by Penicillium cyclopium (3), P. camemberti, and a number of other species of Penicillium as well as Aspergillus, including A. flaus (4). Fungi producing CPA have been isolated from meats, cheese, corn, and peanuts. The mycotoxin occurs naturally in corn (5), cheese (6), peanuts (7), and millet (8). Although there is evidence that CPA may be a potential problem in feeds and foods, there have been no definitive studies of the extent of its occurrence in agricultural commodities, possibly because of the lack of a convenient, reliable method of analysis.

Methods of analysis of CPA proposed most recently involve quantitation by colorimetric measurements, thin-layer chromatography (TLC), and liquid chromatography (LC). A colorimetric method developed for CPA was applied successfully to rice, corn, and wheat inoculated and incubated with P. cyclopium (9). Extracts of the fermented grains were subjected to preparative TLC to separate CPA. CPA extracted from TLC zones was measured using the color reagent p-dimethylaminobenzaldehyde. Concentrations of 25 \( \mu \)g/g of CPA in grains could be determined. Addition of sulfuric acid and ferric chloride to solutions of p-dimethylaminobenzaldehyde resulted in limits of detection of 5 \( \mu \)g/g for CPA (10). However, preparative TLC is very time consuming, and the colorimetric analysis did not have the sensitivity required for detecting CPA as a natural contaminant.

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In a method developed for determining CPA in corn and peanuts, extracts were purified by partitioning CPA into aqueous sodium bicarbonate, followed by acidification and transfer into chloroform (11). CPA was measured on TLC plates sprayed with dimethylaminobenzaldehyde–hydrochloric acid by reflection densitometry at 450 nm. The value of ng CPA for the sample extract was calculated from the linear regression line of log (ng CPA standard spot) vs log (area counts). If peanuts were not defatted before analysis, difficulties with emulsions were encountered during partition. The detection limit for CPA in peanuts or corn was 125 ng/g.

Two methods have been reported recently for the LC quantitative analysis of CPA. In the first, CPA was measured using a UV detector (280 nm) by ligand-exchange chromatography on a C$_8$ or C$_{18}$ column loaded with 4-dodecyldiethylenetriamine and an aqueous mobile phase containing 4-dodecyldiethylenetriamine, zinc acetate, ammonium acetate, 2-propanol, and acetonitrile (12). The limit of detection for standard CPA was approximately 4 ng. Extracts for LC analysis were purified by partition into sodium bicarbonate, acidification, and repartition into dichloromethane. The second LC method measured CPA by normal-phase chromatography using a mobile phase of ethyl acetate–2-propanol–25% aqueous ammonia (55 + 20 + 5) (13). The detection limit for pure CPA in this system was 0.2 ng. Extracts of corn, defatted peanut meal, and rice were purified on SepPak silica cartridge columns for measurement of CPA by the normal-phase LC method. The lower limit for quantitation of CPA in corn was about 100 ng/g.

We now report purification of CPA produced by fermentation using preparative LC separation. The purified CPA is being used in a study of an analytical method for CPA.

**Experimental**

**Reagents and Organism**

(a) **Solvents.**—Chloroform, methanol, acetonitrile, acetone, water.

(b) **Extraction solvent.**—Chloroform–methanol (8 + 2).

(c) **Potassium dihydrogen phosphate solution.**—25mM. Contains 3.4 g ACS grade KH$_2$PO$_4$ dissolved in 1 L water.

(d) **Analytical LC eluting solvent.**—Methanol–25mM potassium dihydrogen phosphate solution (8 + 2).

(e) **Preparative and semi-preparative LC conditioning solvent.**—500mM oxalic acid in acetone. Contains 126 g ACS grade H$_2$C$_2$O$_4$·2H$_2$O dissolved in 2 L acetone.

(f) **Preparative and semi-preparative LC eluting solvents.**—Chloroform–methanol (99 + 1, and 99.5 + 0.5, respectively).

(g) **CPA standards.**—Crystalline CPA (by this method) dissolved in methanol to contain 10, 50, 100, 200, 400, 600, 800 µg/mL. Protected from light and stored at 4°C in Pyrex (or equivalent) containers.

(h) **Penicillium griseofulvum.**—Obtained from L. Leistner, Federal Centre for Meat Research, 8650 Kulmbach, Federal Republic of Germany, and designated as strain Sp368 (NRRRL 13458, ARS Culture Collection, Peoria, IL). Grown on glucose–mannitol–peptone (GMP) medium composed of (g/L): glucose (30), mannitol (20), peptone (10), NaNO$_3$ (4.5), MgSO$_4$·7H$_2$O (0.5), KCl (0.5), and K$_2$HPO$_4$ (1).

**Apparatus**

(a) **Wrist-action shaker.**—Burrell Corp. (Pittsburgh, PA).

(b) **Rotary evaporator.**—Buchi Model RE-121 Rotavapor (Brinkmann Instruments, Inc., Westbury, NY).

(c) **Preparative LC apparatus.**—Waters Associates (Milpore Corp., Milford, MA) Prep LC/System 500 chromatograph.

(d) **Semi-preparative LC apparatus.**—DuPont Instruments (Wilmington, DE) chromatographic pump; Rheodyne Inc. (Cotati, CA) 7125 injector; Isco (Lincoln, NE) V$^4$ variable-wavelength detector; Houston Instruments (Austin, TX) Omniscrife recorder.

(e) **Analytical LC apparatus.**—Spectra-Physics, Autolab Division (San Jose, CA) SP8700 solvent delivery system; Rheodyne 7125 injection valve; LKB Instruments, Inc. (Gaithersburg, MD) column oven; LDC/Milton Roy (Riviera Beach, FL) spectroMonitor D variable wavelength detector; and ModComp (Modular Computer Systems, Inc., Fort Lauderdale, FL) 32/85 mainframe computer.

(f) **LC columns.**—Preparative: Waters Associates PrepPak 500 silica (300 mm × 57 mm id). Semi-preparative: Rainin Instrument Co., Inc. (Woburn, MA) Dynamax-60A silica (250 mm × 21.4 mm id). Analytical: Alltech Associates (Deerfield, IL) Econosphere Amino (250 mm × 4.6 mm id).

**Production and Extraction of CPA**

Ten flasks of sterile GMP medium (100 mL in 500 mL Erlenmeyer flask) were prepared and inoculated with *P. griseofulvum*, as described by Hermansen et al. (4), and then harvested after 14 days of incubation at 24°C on a rotary shaker (200 rpm). Solids were filtered and washed with water. The combined filtrate and washings (1100 mL total volume) was adjusted to pH 2 with 2N HCl and divided into 8 equal portions. Each portion was then shaken with 300 mL CHCl$_3$–methanol (8 + 2) for 30 min on a wrist-action shaker. The organic layers were pooled and evaporated to near dryness on a rotary evaporator under partial vacuum. This procedure was repeated 2 more times to ensure maximum extraction of CPA. The original solids were lyophilized (13.4 g) and extracted sequentially with three 300 mL portions of CHCl$_3$–methanol (8 + 2). These extracts were combined, assayed, and evaporated to dryness with those from the culture filtrate. The dried extract was then redissolved in 50 mL extraction solvent and placed in a freezer at −15°C overnight. The resulting precipitate was filtered and washed with cold extraction solvent. Remaining solids were insoluble in methanol, CHCl$_3$, or water and were discarded. The combination of filtrate and washings was evaporated to dryness (2.17 g).

**Preparative and Semi-Preparative LC Purification of CPA Extract**

The crude, dried extract (2.17 g) was dissolved in 100 mL CHCl$_3$ and pumped onto the pre-equilibrated preparative column at 250 mL/min. Elution was contained with CHCl$_3$–methanol (99 + 1) and monitored with the built-in refractive index detector of the Prep LC/System 500 chromatograph. CPA-containing fractions were combined and evaporated to 5 mL on a rotary evaporator. This concentrate was further purified in 1 mL portions, on the pre-equilibrated semi-preparative system at 6 mL/min with CHCl$_3$–methanol (99.5 + 0.5). Elution was monitored at 282 nm (2.0 AUFS) with the variable wavelength detector. Fractions 2–4 (Figure 1) from each injection were collected, and similar fractions were combined, evaporated to dryness, redissolved in 10 mL CHCl$_3$, and washed twice with 20 mL water to remove resid-
Figure 1. LC chromatograms of (a) cyclopiazonic acid standard (5 μL, 101 μg/mL), (b) 5 μL concentrate from preparative LC, and (c) 1 mL concentrate from preparative LC with fractions collected.

A Dynamax 25 cm × 21.4 mm id silica column, pre-equilibrated with oxalic acid, was eluted at 6 mL/min with chloroform-methanol (99.5 + 0.5). Elution was monitored at 282 nm with absorbance settings (AUFS) of 0.02, 0.2, and 2, respectively.

**LC Quantitation of Cyclopiazonic Acid**

A series of standards containing 10–800 μg/mL crystalline CPA in methanol was prepared. The analytical chromatography was conducted on an amino bonded-phase column, used in a weak anion-exchange mode with the methanol-phosphate buffer eluant to minimize "tailing" or irreversible binding to the column packing material. Temperature was maintained at 60°C by the column oven. Samples (5 μL/injection) of various concentrations were eluted at a flow rate of 0.5 mL/min and monitored at 225 nm (0.2 AUFS) with a UV detector connected to the ModComp computer system for integration and subsequent response calculations. A response factor was determined with the formula:

\[
\text{Response factor} = \frac{[\text{concentration (ng/μL)}] \times [\text{injection volume (μL)}]}{\text{peak area}}
\]

which yields values numerically equivalent to μg/mL or mg/L.

**Results and Discussion**

*P. griseofulvum* Sp638, grown under the conditions cited by Hermansen et al. (4), is reported to yield 1000 mg/L of CPA, as determined by TLC assay. In this experiment, a yield (as determined by analytical LC) of 291 mg CPA was obtained in the culture filtrate (Table 1). The filtrate extract contained only 180 mg (62% extraction efficiency), while the dried solids extract provided an additional 144 mg of CPA for an apparent yield of 324 mg/L. However, assuming a 62% efficiency also in the solids extraction procedure, our total estimated yield would be 523 mg/L. The final crystalline CPA (106.7 mg) from fraction 3 of the semi-preparative separation represents a recovery of 20%, or approximately 33% of the apparent yield. A second crystallization from the mother liquor of fraction 3 gave additional crystalline material (23 mg) contaminated with an impurity. CPA in LC fractions was then quantitated by solving the expression:

\[
\text{Concentration (ng/μL)} = \text{response factor} \times \frac{\text{peak area}}{\text{injection volume (μL)}}
\]

which yields values numerically equivalent to μg/mL or mg/L.

<table>
<thead>
<tr>
<th>Material analyzed</th>
<th>Total mg of CPA</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation culture (1 L)</td>
<td>523* (est.)</td>
<td>100</td>
</tr>
<tr>
<td>Culture filtrate</td>
<td>281</td>
<td>—</td>
</tr>
<tr>
<td>Total extractable CPA</td>
<td>324</td>
<td>62</td>
</tr>
<tr>
<td>(Culture filtrate extract)</td>
<td>(180)</td>
<td>—</td>
</tr>
<tr>
<td>(Solids extract)</td>
<td>(144)</td>
<td>—</td>
</tr>
<tr>
<td>Concentrate after preparative LC</td>
<td>217</td>
<td>41</td>
</tr>
<tr>
<td>Concentrate after semi-preparative LC</td>
<td>184</td>
<td>35</td>
</tr>
<tr>
<td>Crystalline CPA</td>
<td>106.7</td>
<td>20</td>
</tr>
</tbody>
</table>

*Based on calculation of the efficiency of filtrate extraction and applying this value to solids extract (i.e., 1.6 × 144 = 232 mg, and 232 + 291 = 523 mg).
The authors acknowledge Gordon L. Adams, Ronald D. Plattner, and David Weisleder (U.S. Department of Agriculture, Peoria, IL) for their technical expertise in the fermentation, mass spectral, and NMR analysis procedures.

Acknowledgments

The 2-stage (preparative and semi-preparative) LC separation on oxalic acid/silica columns provides a rapid, large-capacity alternative to purification methods that use thin-layer chromatography (TLC) plates, solvent partitioning, or gravity-fed ion-exchange resin columns (3, 9, 14, 15). Although this study describes a 2.17 g/100 mL injection onto the preparative column, loading of 10 g or more is possible.

The CPA response curve from the amino column analytical system exhibited good linearity over the 10–800 µg/mL range, with a calculated response factor of 8.8 × 10⁻⁴. A lower detection limit of 50 ng/injection was estimated with this system; however, this could reasonably be reduced to 5 ng/injection if the detector sensitivity were increased to 0.02 AUFS. In addition, injection volumes of 10–50 µL would provide 2–to 10-fold more sensitivity for dilute samples. The detector was nearing saturation at the 600 µg/mL concentration level; values above this may require dilution of the sample for greater accuracy.

Either of the 2 described adsorbent/solvent systems can routinely quantitate CPA in standards or relatively simple mixtures. The oxalic acid/silica-based support provides better resolution of CPA from interfering compounds, but is susceptible to leaching of oxalic acid by the elution solvent, causing a slow drift of retention times. A detection wavelength of 282 nm is necessary because of the higher UV cutoff value of CHCl₃–methanol; this reduces detector sensitivity by almost 50%. The NH₄⁺/silica-based support system gives higher detector sensitivity with its methanol–buffer solvent by allowing monitoring at the maximum coefficient of extinction (225 nm).

An interesting phenomenon was noted during mass spectrometry (MS) analysis of CPA. All procedures were routinely performed in borosilicate glass vessels, including preparation of standards for MS, NMR, and spectrophotometric studies. When an aliquot of standard solution, stored in an inexpensive soda-lime glass vial, was analyzed by MS, the usual CPA spectrum was not observed. Only a weak signal at (M + Na) was recorded, which suggests that CPA may form a complex with Na ions from the soda-lime vial. This reaction proceeded rapidly (< 10 min), as later monitored by intermittent sampling and MS determinations, and produced a CI parent ion of 359 atomic mass units (336 + 23). Evidently, Na-containing compounds (glassware, buffers, drying agents, etc.) can have a detrimental effect on recovery of CPA during production, purification, or analysis, and should be avoided.

CPA quantitation is difficult because of the acidic structure of the molecule, which may cause its tenacious affinity for Na ions; caution must be observed in all phases of handling to preserve molecular integrity of CPA. Potassium salts (as buffers) appear to afford a mediating effect on stability. Further studies of analytical methods for determination of naturally occurring CPA in grains are currently being conducted under the above-mentioned constraints.

References


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Figure 4. Absorbance spectrum of CPA in methanol.