for her willingness to help us with the assessment of an unskilled operator, and Dr. L. Goldstein for her technical assistance.

References
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Detection of Interference by Cyclobenzaprine in Liquid-Chromatographic Assays of Tricyclic Antidepressants
Patricia R. Puopolo and James G. Flood

We evaluated a technique for detecting cyclobenzaprine interference with liquid-chromatographic assays for tricyclic antidepressants. The technique involves dual-wavelength absorbance monitoring of the column effluent at 214 and 254 nm. Ratios of analyte peak heights at each wavelength are used to check for the presence of co-eluting interferences. With this technique, one can detect interference with an amitriptyline assay caused by 10 μg of cyclobenzaprine per liter.

The muscle-relaxing drug cyclobenzaprine interferes with assays for tricyclic antidepressants. Schneider and Giardina (1) reported that cyclobenzaprine interfered with the reversed-phase liquid-chromatographic determination of imipramine. Tasset et al. (2) reported a case indicating that cyclobenzaprine interfered in each of seven different analytical techniques for detecting amitriptyline, including enzyme-mediated immunoassay, thin-layer chromatography, gas chromatography, liquid chromatography, and gas chromatography/mass spectrometry. We find that cyclobenzaprine and amitriptyline have nearly identical retention times on popularly used commercial liquid-chromatographic columns.

Evidently cyclobenzaprine may interfere with many procedures for quantifying tricyclic antidepressant drugs. We describe here a dual-wavelength absorbance-ratio technique for detecting such interference. Variations of this technique have been used to check the homogeneity of liquid-chromatographic peaks and help identify drugs and nucleosides in complex mixtures (3-5). In this technique, the column effluent is monitored at two different wavelengths. If Beer’s Law is adhered to, the ratio of the absorbances at the two wavelengths will be constant and independent of analyte concentration during elution of a “pure” peak. Deviations from this constant ratio may be used to warn the chromatographer that an interfering compound(s) may be co-eluting with the compound of interest.

Materials and Methods
Apparatus
We used a QA-1 liquid chromatograph (Waters, Division of Millipore, Inc., Milford, MA 01757) equipped with 214-

and 254-nm fixed-wavelength detectors and a 4.6 × 150 mm LC-PCN column (Supelco, Inc., Bellefonte, PA 16823). We injected 50-μL samples. The flow rate of the mobile phase was 2 mL/min. Column temperature was maintained at 30 °C. For peak-height measurements we used HP 3390A electronic integrators (Hewlett Packard, Inc., Avondale, PA 19311).

Chemicals and Solutions
All chemicals were reagent grade or better, and only de-ionized water was used. The chromatographic mobile phase consisted of acetonitrile/methanol/phosphate buffer (10 mmol/L, pH 7.1), 60:15:25 by vol. Amitriptyline HCl and cyclobenzaprine HCl were from Merck, Sharp & Dohme, Rahway, NJ 07065. Stock (1 mg/mL) solutions of these compounds were prepared by drying each compound under reduced pressure at 23 °C for 2 h, then dissolving a weighed amount of the solid in methanol. Standard solutions and serum calibrators were prepared by appropriately diluting the stock solutions with mobile phase or drug-free plasma.

Results
Figure 1 shows the ultraviolet-visible absorbance spectra of amitriptyline and cyclobenzaprine. The differences in the spectra indicate that wavelength “ratioing” can be used to detect co-elution of cyclobenzaprine with amitriptyline.

We chromatographed various amitriptyline standards re-
peatedly over a 10-h period to determine if the 214/254 nm peak-height ratio was independent of amitriptyline concentration. We found (Table 1) the ratio to be relatively constant over the 50–500 μg/L range. Predictably, the imprecision of the ratio estimate increased as smaller amounts of amitriptyline were chromatographed. We have no explanation for the slightly lower ratio observed in the case of the most dilute amitriptyline standard. We estimated the between-run imprecision of the amitriptyline ratio by analyzing a 200 μg/L serum-based calibrator by our routine tricyclic antidepressant drug assay, which involves an organic-solvent extraction procedure that effectively concentrates the sample by twofold (85–90% absolute recovery of amitriptyline and 97–100% absolute recovery of cyclobenzaprine are typical). The peak-height ratios for 20 different runs made during four weeks averaged 4.24 (SD 0.04).

As shown in Table 2, the chromatographic retention times for cyclobenzaprine and amitriptyline are nearly the same—but the 214/254 nm peak-height ratios of these compounds differ markedly. To see if their co-elution could be detected by wavelength ratioing, we chromatographed mixtures of these compounds. Using the within-run precision data from Table 1, we define an "interference-free" peak of amitriptyline at 100 μg/L as one exhibiting a mean ratio of 4.18 (2 SD = 0.10). The ratio technique correctly alerts the chromatographer to as little as 10 μg of cyclobenzaprine interference per liter in a sample containing 100 μg of amitriptyline per liter.

Serum samples from a subject on a therapeutic cyclobenzaprine regimen were processed through our tricyclic antidepressant drug assay. The subject was not taking amitriptyline or nortriptyline. The chromatogram of the subject's serum exhibited two distinct peaks, one almost identical in retention time to amitriptyline, the other near that of nortriptyline. The wavelength ratios of both peaks were not those of a "pure" amitriptyline or nortriptyline peak. The ratio technique thus warned us of the expected interference with amitriptyline, but also to an unexpected one with nortriptyline. As expected, the wavelength ratio of the interference with amitriptyline matched that of cyclobenzaprine. Interestingly, the wavelength ratio of the peak near nortriptyline was also similar to that of cyclobenzaprine. This peak perhaps represents a metabolite of cyclobenzaprine.

Table 1. Within-Run Precision of the Ratio

<table>
<thead>
<tr>
<th>Amitriptyline* concn, μg/L</th>
<th>Ratiob (mean, n, SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>4.22, 11, 0.01</td>
</tr>
<tr>
<td>200</td>
<td>4.22, 10, 0.02</td>
</tr>
<tr>
<td>100</td>
<td>4.18, 10, 0.05</td>
</tr>
<tr>
<td>50</td>
<td>4.18, 9, 0.09</td>
</tr>
<tr>
<td>25</td>
<td>4.10, 10, 0.10</td>
</tr>
</tbody>
</table>

*"Simulated" concentration in mobile phase to reflect a twofold concentration step in the pre-chromatographic part of assay; 100% recovery assumed. aRatio of peak heights as measured with the 214- and 254-nm detectors.

Table 2. Effect of Cyclobenzaprine on the Amitriptyline Ratio

<table>
<thead>
<tr>
<th>Test solutions,μg/L</th>
<th>Ratiob</th>
<th>Retention time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (100)</td>
<td>1.85</td>
<td>2.74</td>
</tr>
<tr>
<td>A (100)</td>
<td>4.18</td>
<td>2.78</td>
</tr>
<tr>
<td>A (100) + C (5)</td>
<td>4.11</td>
<td>2.78</td>
</tr>
<tr>
<td>A (100) + C (10)</td>
<td>3.98</td>
<td>2.79</td>
</tr>
<tr>
<td>A (100) + C (15)</td>
<td>3.84</td>
<td>2.78</td>
</tr>
<tr>
<td>A (100) + C (20)</td>
<td>3.73</td>
<td>2.78</td>
</tr>
</tbody>
</table>

*"Simulated" concentration in mobile phase of cyclobenzaprine (C) and amitriptyline (A) to reflect a twofold concentration in the pre-chromatographic part of the assay; 100% recovery assumed. aRatio of peak heights as measured with the 214- and 254-nm detectors (mean of three measurements). b214-nm detector.

Discussion

We believe this technique is a powerful tool for detecting interferences. Wavelength selection for such "ratioing" techniques has been discussed by Fell et al. (6). The wavelengths we used here are not necessarily the optimum ones for detecting cyclobenzaprine interference with amitriptyline; rather, we used 214 and 254 nm because of the sensitivity of these wavelengths for measuring all tricyclic drugs and the ability to detect other potential interferences such as the phenothiazines.

Cyclobenzaprine concentrations of 5–25 μg/L (7) are to be expected in plasma after therapeutic use of this drug. If proper ratioing techniques are used, even such low concentrations should not result in clinically significant interference with amitriptyline going undetected. Cyclobenzaprine masquerades as amitriptyline in many procedures (2), but liquid chromatography with dual-wavelength ratioing can detect such interference.

References