Liquid-Chromatographic Analysis for Methylphenidate (Ritalin) in Serum

Steven J. Soldin, Ying-Pui M. Chan, Barbara M. Hill, and James M. Swanson

We describe a “high performance” liquid chromatographic method for quantitating methylphenidate in serum. The internal standard, 4,5-diphenylimidazole, and a serum or plasma sample are extracted in chloroform, evaporated, and redissolved in 20 mmol/L potassium phosphate (pH 3.5)/high-purity acetonitrile, 80/20 by vol. A centrifuged aliquot is chromatographed on a Bondapak C-18 with the phosphate/acetonitrile solvent as mobile phase, a flow rate of 1.6 mL/min, and a column temperature of 40 °C. Absorbances are read at 192 nm. This method reliably measures concentrations >20 μg/L and has analytical recoveries of 74%.

Additional Keyphrases: hyperkinesis • ritalinic acid • microprocedure • pediatric chemistry • HPLC • 4,5-diphenylimidazole internal standard

Methylphenidate [methyl α-phenyl-α-(2-piperidyl)acetate, (ritalin)] has pharmacological properties similar to those of dextroamphetamine (1) and has been used to treat children with hyperkinesis (2) as well as patients with depression. According to recent estimates from the United States, 1–2% of the children labeled as hyperactive are currently receiving this medication (3). Following oral administration of methylphenidate, about 75% of the original dose is recovered as ritalinic acid [α-phenylα-(2-piperidyl)acetic acid] in urine (4, 5) (see Figure 1). Minor metabolic pathways for both these compounds include parahydroxylation of the aromatic ring, oxidation to the 6-oxo-derivatives, and glucuronide formation (5–11).

Gas-chromatographic and gas-chromatographic-mass-spectrometric procedures recently described for quantitation of methylphenidate and ritalinic acid (11–14) require relatively large sample volumes (2–5 mL of plasma or serum). Because the hyperactive syndrome is a childhood disorder, a microprocedure for analysis of both these compounds would be a distinct improvement over current methodologies. We have recently described a reliable microprocedure for the analysis for ritalinic acid in serum (15), and we now present a rapid, reliable, and sensitive “high performance” liquid chromatographic procedure for the analysis for methylphenidate in serum.

Materials and Methods

Apparatus

Throughout these studies, we used a “high performance” liquid chromatograph, series 2/2 (Perkin-Elmer Corp., Norwalk, CT 06856), and a 4 mm × 30 cm μ-Bondapak C-18 column mounted in a temperature-control block (both from Waters Associates, Inc., Milford, MA 07157). The detector was a Perkin-Elmer Model LC-55 variable wavelength spectrophotometer attached to a Perkin-Elmer/Coleman 560 recorder. The methylphenidate and 4,5-diphenylimidazole spectra were obtained with a Zeiss spectrophotometer PMQ II (Carl Zeiss, Oberkochen, P.R.G.).

Reagents

We obtained acetonitrile and methanol from Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442, and 4,5-diphenylimidazole from the Aldrich Chemical Co., Inc., Milwaukee, WI 53233. Methylphenidate hydrochloride, ritalinic acid 6-oxo-methylphenidate, and 6-oxo-ritalinic acid were obtained from Ciba-Geigy Corp., Ardsley, NY 10502. Reagent A consists of 20 mmol/L potassium phosphate buffer, pH 3.5. Reagent B is acetonitrile.

Extraction Procedure and Condition's of Analysis

Add 3.0 mL of chloroform containing 50 μg of 4,5-diphenylimidazole per liter to 300 μL of serum or plasma in a 13 × 100 mm glass test tube. Vortex-mix for 60 s. Centrifuge at 2000 rpm for 5 min. Place 2.5 mL of the organic phase into another test tube and evaporate under nitrogen at 60 °C. Dissolve the residue in 50 μL of mobile phase (an 80/20 by vol. mixture of reagent A/reagent B), vortex-mix, centrifuge, and inject 40 μL into the chromatograph. The conditions of analysis are shown in Table 1.

Results and Discussion

Analytical Variables

Detector wavelength. Figure 2 shows an absorbance scan for methylphenidate and 4,5-diphenylimidazole. Clearly, monitoring the absorbance at 192 nm will give a more sensitive assay for methylphenidate than will a higher wavelength. However, using the 192-nm wavelength necessitates use of high-purity acetonitrile. Many commercial brands could not be used, but the Burdick and Jackson acetonitrile, distilled in glass (UV), is adequate. A wavelength below 192 nm was not selected because of interference from acetonitrile.

Column temperature. Column temperature is not too critical in this assay. We chose 40 °C because the viscosity of the mobile phase increases significantly at lower temperatures, which results in undesirably high column pressures.

Composition of the mobile phase. Increasing the pH from 3.5 to 4.5 has little effect on the retention time of methylphenidate, but considerably increases the retention time of 4,5-diphenylimidazole. Because this would result in a longer
Methylphenidate (Ritalin)

**Methylα-phenylα-(2-piperidyl) acetate**

**Ritalinic acid**

α-phenylα-(2-piperidyl) acetic acid

Analysis time, the lower pH was selected. Ratios of reagent A/reagent B greater than 80/20 (by vol) increase retention times for both methylphenidate and 4,5-diphenylimidazole. Hence, for extractions from serum, the optimum pH of 3.5 and the reagent ratio of 80/20 (reagent A/reagent B) will separate methylphenidate and 4,5-diphenylimidazole from interfering peaks.

**Method of calculation.** Peaks on the chromatogram are identified on the basis of their retention times relative to the internal standard. Methylphenidate concentration is calculated from the peak heights according to the formula:

\[
\text{Methylphenidate concn} = \frac{(A/B) \times (C/D) \times (E/F)}{
\text{where:}}
\]

- \(A\) = peak height for methylphenidate
- \(B\) = peak height for 4,5-diphenylimidazole
- \(C\) = recovery of 4,5-diphenylimidazole
- \(D\) = recovery of methylphenidate

**Table 1. Assay Conditions**

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>80/20 mixture of reagent A/reagent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>40 ºC</td>
</tr>
<tr>
<td>Pressure</td>
<td>1000 psi</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.6 mL/min</td>
</tr>
<tr>
<td>Absorbance with full scale</td>
<td>0.016</td>
</tr>
<tr>
<td>Wavelength</td>
<td>192 nm</td>
</tr>
<tr>
<td>Internal standard</td>
<td>4,5-diphenylimidazole</td>
</tr>
<tr>
<td>Chromatography time</td>
<td>18 min</td>
</tr>
</tbody>
</table>

**Fig. 2.** Absorbance scan for 5 mg of methylphenidate (●–●) and 5 mg of 4,5-diphenylimidazole (○–○) dissolved in 1 L of mobile phase

**Fig. 3.** Determination of the relative response factor for methylphenidate (●–●) and 4,5-diphenylimidazole (○–○) at 192 nm

**Fig. 4.** Type of elution pattern obtained for a serum specimen containing 125 μg of methylphenidate per liter.
Precision. The between-day precision of the method (n = 20) was assessed by the repeated analyses of serum specimens containing various concentrations of methylphenidate. At concentrations of 135, 252, and 497 µg/L, the method yielded coefficients of variation of 12.2, 7.5, and 9.4%, respectively.

Sensitivity. The minimum detection limit is based on the peak height relative to the baseline noise (16) and on the reproducibility of that peak height for a serum sample with a given concentration of methylphenidate. In the analysis for linearity a signal-to-baseline-noise ratio of 2 corresponds to a minimum detection limit of 10 µg/L. This, together with the precision data, suggests that methylphenidate cannot be reliably estimated by our procedure at concentrations of less than 20 µg/L.

Recovery. We measured absolute recovery from plasma of methylphenidate and 4,5-diphenylimidazole as follows. To three different samples of drug-free plasma we added the two compounds to the concentrations shown in Table 2. We performed the analysis as described, except that the 3.0 mL of chloroform added to the 300 µL of serum contained no 4,5-diphenylimidazole. We carefully measured and chromatographed aliquots of the final solutions and determined the peak heights. We calculated percentage recovery by comparing these peak heights with the peak heights obtained by direct injection of the pure compounds. As shown in Table 2, the percentage recovery of methylphenidate and 4,5-diphenylimidazole at various serum concentrations was 74 and 68%, respectively.

Interference studies. We also analyzed twenty sera obtained from individuals known not to be receiving methylphenidate. No peaks with retention times similar to methylphenidate or 4,5-diphenylimidazole were found in any of the samples. Furthermore, the following compounds, added to and extracted from serum, did not interfere in the analysis: phenobarbital, phenytoin, primidone, ethosuximide, carbamazepine, theophylline, salicylate, amphetamine, dextroamphetamine, 6-oxo-methylphenidate, and 6-oxo-ritalinic acid.

Patient studies. Figure 5 shows the serum concentrations

Table 2. Recovery Studies

<table>
<thead>
<tr>
<th>Added to plasma pool, µg/L</th>
<th>Recovery, % (mean; n = 12)</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylphenidate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>74</td>
<td>10.5</td>
</tr>
<tr>
<td>125</td>
<td>74</td>
<td>9.9</td>
</tr>
<tr>
<td>4,5-Diphenylimidazole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>68</td>
<td>9.2</td>
</tr>
<tr>
<td>250</td>
<td>68</td>
<td>8.8</td>
</tr>
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</table>

Fig. 4. Elution pattern obtained using the procedure outlined for a serum sample containing 125 µg of methylphenidate per liter. Peak 1 is methylphenidate and peak 2 is 4,5-diphenylimidazole.

Fig. 5. Variation of the serum concentrations of methylphenidate (○-○) and ritalinic acid (O-O) with time in a 12-year-old boy. The subject received 15 mg methylphenidate at 7:30.
of methylphenidate and ritalinic acid in a 12-year-old boy. From this graph we calculated the half-life for methylphenidate and ritalinic acid to be 2.6 and 3.2 h, respectively. Correlations between psychological performance and serum concentrations of both methylphenidate and its major metabolite ritalinic acid are currently being undertaken. The availability of procedures for the analysis of these compounds will enable us to assess whether the therapeutic effect of methylphenidate is due to the parent drug, its major metabolite, or both, by correlating psychological performance (17) with serum concentration of these compounds.

The relationship between drug dose and the concentration of parent drug and its metabolites in serum is unpredictable because of inter-individual differences in drug absorption, metabolism, and excretion. Correlation of concentrations in serum with behavioral response would eliminate the semi-empiricism of current practice and make possible more rational diagnosis and management.

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References