A Liquid-Chromatographic Analysis for Ritalinic Acid [α-Phenyl-α-(2-piperidyl) Acetic Acid] in Serum

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

We describe a "high-performance" liquid-chromatographic assay for measuring ritalinic acid in 300 μL of serum. The procedure includes adsorption of ritalinic acid and internal standard (α,α-dimethyl-β-methylsuccinimide) from serum onto charcoal, from which both compounds are eluted with methanol. The methanol extract is evaporated, the residue dissolved in a 93/7 (by volume) mixture of potassium phosphate buffer (20 mmol/L, pH 3.8) and acetonitrile, and an aliquot of this solution is chromatographed. We use a μ-Bondapak C-18 column with the above-mentioned buffer system as mobile phase, a flow rate of 2.0 mL/min, and a column temperature of 40 °C. Ritalinic acid and internal standard are detected by their absorbance at 192 nm and quantitated by measuring peak heights. The procedure allows for the reliable analysis for ritalinic acid in serum at concentrations >50 μg/L. Analytical recoveries were >73% and the method affords good day-to-day precision (CV, <12%).

Additional Keyphrases: methylphenidate • Ritalin (Ciba) • HPLC • α,α-dimethyl-β-methylsuccinimide internal standard • pediatric chemistry

About 5% of children in North America are referred for medical treatment with symptoms suggesting a disorder called "hyperactivity" (1), characterized by extreme impulsivity, distractability, and an inability to attend to simple tasks in school and at home. The most common medical treatment for this disorder is therapy with stimulants. Bradley (2) initially demonstrated that amphetamines, given in small doses, benefit about 70% of all children who display these symptoms. Recently another stimulant, methylphenidate [methyl-α-phenyl-α-(2-piperidyl) acetate], has become the most commonly used stimulant for treating hyperactivity, presumably because it produces fewer and less-serious side effects than does amphetamine (3).

After a favorable response to methylphenidate has been documented by psychological testing, the optimal dosage regimen must be ascertained. The oral dose required for optimal response varies widely among individuals (4). The obvious shortcoming of current practice is that behavioral response has been correlated with the amount of drug administered, despite the knowledge that inter-individual differences in drug absorption, metabolism, and excretion make unpredictable the relationship between drug dose and the concentration in serum of the parent drug and its metabolites. Correlation of concentrations in serum with behavioral response would eliminate the semi-empiricism of current procedures and enable more rational diagnosis and management.

Methylphenidate, synthesized in 1944 by Panizzon from benzyl cyanide and 2-chloropyridine (5), is marketed as the hydrochloride (Ritalin, Ciba). Its pharmacological properties are similar to those of amphetamine and other phenylisopropylamines. In man, it is rapidly metabolized to ritalinic acid [α-phenyl-α-(2-piperidyl) acetic acid] (Figure 1), which is then eliminated via the kidney. After oral administration of methylphenidate, about 75% of the original dose is present as ritalinic acid in urine. Minor metabolic pathways for both these compounds include para-hydroxylation of the aromatic ring, oxidation to the 6-oxoderivatives, and glucuronide formation (6-12). After oral administration of 20 mg of [14C]-methylphenidate hydrochloride, Faraj et al. (13) found its concentration in plasma to be less than 20 μg/L, while radioactivity representing its metabolites was maximal at 2 h. Furthermore, Bartlett and Egger (10) indicated that they could find no measurable methylphenidate in plasma after oral administration of the drug, even though their lower limit of detection was 2 μg/L. In sharp contrast, we can detect methylphenidate in the serum of patients who are receiving the drug orally. This work, details of which will be reported shortly, enables us to assess whether the therapeutic effect of methylphenidate is due to the parent drug, its major metabolite, or both, by correlating psychological performance with the concentration of these compounds in serum.

Gas-chromatographic methods involving flame-ionization detection of methylphenidate and ritalinic acid in blood and urine were described by Schubert (12) and Wells et al. (15). These approaches lack the required sensitivity and require relatively large (10 mL) volumes of blood (12). More recently, gas-chromatographic–mass-spectrometric procedures with improved sensitivity, but still requiring large volumes of plasma (2-5 mL), have been described (14, 15).

We present here a reliable, and sensitive high-performance liquid-chromatographic procedure for analysis for ritalinic acid which requires only 300 μL of serum or plasma. Analysis of such micro-scale samples should allow the concentration of ritalinic acid in serum and behavioral response in children to be correlated. Because the hyperactive syndrome is a childhood disorder, the availability of a micro-scale procedure for the analysis for ritalinic acid in serum represents a considerable improvement over existing techniques for monitoring ritalinic acid concentrations.

Materials and Methods

Apparatus. We used a high-performance liquid chromatograph, series 2/2 (Perkin Elmer Corp., Norwalk, CT 06856)
**Results and Discussion**

**Analytical Variables**

*Detector wavelength.* Figure 2 shows an absorbance scan for ritalinic acid and \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide. Clearly, monitoring the absorbance at 192 nm will provide for a more sensitive assay for ritalinic acid than would use of a higher wavelength.

Ideally, a bichromatic approach would be optimal, with the column eluate being continuously monitored at 192 nm and, say, 198 nm. This could afford increased specificity, as ritalinic acid has a known absorbance ratio at these wavelengths and the presence of an interfering compound would be likely to skew the ratio, thereby alerting the operator to the problem. Using a wavelength of 192 nm necessitates the use of high-purity acetonitrile. Many commercial brands could not be used, but the Burdick and Jackson distilled-in-glass (ultra-violet grade) acetonitrile is adequate.

*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, resulting in undesirably high column pressures.

**Table 1. Conditions of Analysis**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Assay conditions required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>93/7 mixture of reagent A</td>
</tr>
<tr>
<td></td>
<td>/ reagent B</td>
</tr>
<tr>
<td>Temperature</td>
<td>40 °C</td>
</tr>
<tr>
<td>Pressure</td>
<td>13.8 MPa (2000 psi)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>2.0 mL/min</td>
</tr>
<tr>
<td>Absorbance units</td>
<td>0.02</td>
</tr>
<tr>
<td>Wavelength</td>
<td>192 nm</td>
</tr>
<tr>
<td>Internal standard</td>
<td>(\alpha,\alpha)-Dimethyl-(\beta)-methylsuccinimide</td>
</tr>
<tr>
<td>Chromatography time</td>
<td>15 min</td>
</tr>
</tbody>
</table>

![Graph](image)
Recovery, I
2
Mean

Fig. 3. Determination of the relative response factor for ritalinic acid (O•O) and \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide (O–O) at 192 nm

Fig. 4. The elution pattern obtained by the procedure outlined for a serum sample containing 400 \(\mu\)g of ritalinic acid per liter
Peaks 1 and 2 correspond to \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide and ritalinic acid, respectively

**Composition of mobile phase.** Higher pH’s shorten the retention time of ritalinic acid, but have little effect on the retention time of \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide. Reagent A/reagent B ratios of less than 93/7 shortened the retention times of both ritalinic acid and \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide. In the case of serum samples, the optimum pH for reagent A for separating ritalinic acid and \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide from interfering peaks is 3.8 at a reagent A/reagent B ratio of 93/7.

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

\[
\text{Concentration of ritalinic acid} = \frac{A}{B} \times \frac{C}{D} \times \frac{E}{F}
\]

where

- \(A\) = peak height for ritalinic acid
- \(B\) = peak height for \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide
- \(C\) = recovery of \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide
- \(D\) = recovery of ritalinic acid
- \(E\) = concentration, in \(\mu\)g/L, of \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide
- \(F\) = relative response factor (peak height of ritalinic acid compared to that for an equal amount of \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide)

**Relative response factor.** By injecting known amounts of ritalinic acid and internal standard into the chromatograph we demonstrated a linear relationship between peak height and drug concentration over a concentration range far in excess of that required for clinical purposes (Figure 3). From Figure 3 a relative response factor of 1.00 was calculated. The absorbance of 100 ng of ritalinic acid is 0.01 and absorbance of 100 ng of \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide is 0.01. Therefore the relative response factor is 0.01/0.01 = 1.00. Figure 4 shows the type of elution pattern obtained for a serum specimen containing 400 \(\mu\)g of ritalinic acid per liter. Peaks 1 and 2 correspond to \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide and ritalinic acid. All the other peaks appear to be unrelated to methylphenidate administration.

**Precision.** We assessed the between-day precision of the method \((n = 12)\) by the repeated analyses of sera containing various concentrations of ritalinic acid. At concentrations of 94 and 524 \(\mu\)g/L the method yielded coefficients of variation of 11.7 and 6.5%, respectively. Over a longer period \((n = 25)\), the coefficient of variation at 247 \(\mu\)g/L was 8.3%.

**Sensitivity.** The minimum detection limit is based on the peak height relative to baseline noise (17) and also on the reproducibility of that peak height for a serum sample containing a given concentration of ritalinic acid. In the above analysis a signal to baseline noise ratio of 2 corresponds to a minimum detection limit of 25 \(\mu\)g/L. This, together with the precision data, suggests that ritalinic acid concentrations below 50 \(\mu\)g/L cannot be reliably estimated by the above procedure.

**Recovery.** We measured the absolute recovery from plasma of ritalinic acid and \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide as follows. The two compounds were added to drug-free plasma to achieve the concentration shown in Table 2. The analysis procedure was then carried out as described except that the 1 mL of water added to the 300 \(\mu\)L of serum did not contain any \(\alpha,\alpha\)-dimethyl-\(\beta\)-methylsuccinimide. Carefully measured aliquots of the final solution were chromatographed and the peak heights determined. Percentage recovery was calculated by comparing these peak heights with the peak heights obtained by the direct injection of the pure compounds. As

<table>
<thead>
<tr>
<th>Added to plasma pool, (\mu)g/L</th>
<th>Mean recovery, % ((n = 12))</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ritalinic acid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>450</td>
<td>80</td>
<td>6.2</td>
</tr>
<tr>
<td>225</td>
<td>73</td>
<td>4.9</td>
</tr>
<tr>
<td>(\alpha,\alpha)-Dimethyl-(\beta)-methylsuccinimide</td>
<td>505</td>
<td>83</td>
</tr>
<tr>
<td>232</td>
<td>80</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Table 2. Recovery Studies
shown in Table 2, the percentage recovery of ritalinic acid and α,α-dimethyl-β-methylsuccinimide at various serum concentrations was 73–80% and 80–83%, respectively.

Interference studies. Twenty sera from individuals known not to be receiving methylphenidate were analyzed by the described procedure. No peaks with retention times similar to ritalinic acid or α,α-dimethyl-β-methylsuccinimide were found. With the procedure described, the following compounds on extraction from serum were found not to interfere in the analysis: phenobarbital, phenytoin, primidone, ethosuximide, carbamazepine, theophylline, salicylate, amphetamine, and dextroamphetamine. The retention times of methylphenidate and the 6-oxo metabolites of both methylphenidate and ritalinic acid differed markedly from those of α,α-dimethyl-β-methylsuccinimide and ritalinic acid.

Patient Studies

Figure 5 shows the concentrations of ritalinic acid in the serum of a 14-year-old male patient who requires a higher than usual dose of methylphenidate to elicit a beneficial response. Methylphenidate, 35 mg, was administered orally at 0730, 1130, and 1530 h. Figure 6 shows the serum concentrations of ritalinic acid in a 9-year-old male patient. Methylphenidate, 10 mg, was administered orally at 0730, 1130, and 1530 h.

Correlations between psychological performance and serum concentrations of ritalinic acid are currently being undertaken.

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References