An Enzyme-Linked Immunosorbent Assay (ELISA) for Methylphenidate (Ritalin®) in Urine

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Abstract

A direct enzyme-linked immunosorbent assay (ELISA) for urinary immunoreactive methylphenidate (Ritalin), in which a standard 96-well microtiter plate is used, is described. For this ELISA, a methylphenidate-thyroglobulin conjugate is immobilized to the microtiter plate and competes with methylphenidate in the standard or urine sample for antibody-binding sites. After washing, the sheep methylphenidate antibody bound to immobilized methylphenidate is detected with peroxidase-labelled goat antishep IgG. Following a further wash, tetramethylbenzidine is added, color is developed, and the plate is read at 450 nm on an ELISA plate reader. This method is unaffected by drugs of abuse and is suitable for routine use in the toxicology laboratory.

Introduction

Methylphenidate (Ritalin) is commonly used for the treatment of attention deficit hyperactivity disorder (ADHD). More than 2 million children are treated annually in the United States (1). Unfortunately, as therapeutic use increases, so does the risk of misuse, which can include diversion from legitimate use to abuse in preteens and adolescents (2). Monitoring the use and abuse of the drug has proved difficult and time consuming. Previous methods have used extraction and derivatizing steps, including thin-layer chromatography (TLC) and gas chromatography (GC) procedures (3,4), but these methods do not lend themselves to the expected rapid growth in analytical requests. More recently, a commercial enzyme-linked immunosorbent assay (ELISA) for methylphenidate and ritalinic acid in urine has been developed by Diagnostix Ltd. (Mississauga, ON, Canada) presumably in response to anticipated growth. However, this ELISA uses immobilized ritalinic acid and competition with methylphenidate in the samples for antibody-binding sites. Following washing, bound sheep ritalin antibody is detected using antishep peroxidase, and substrate is added after final washing.

Experimental

Isolation of methylphenidate

Although we had a small amount of pure methylphenidate for preparation of the standards, we required a larger amount for conversion to ritalinic acid for conjugation to bovine thyroglobulin as a carrier protein in the ELISA. To expedite this, a larger amount of methylphenidate was extracted from Rubifen® (10-mg tablets, Lab. RUBI6, S.A., Spain) using a biphasic extraction. Rubifen tablets containing 10 mg methylphenidate.HCl were dissolved in water, and an equimolar amount of NaOH was added, converting the methylphenidate into its organic soluble form. The solution was extracted with diethyl ether (3 x 10 mL), and the aqueous phase was discarded. The organic phase was dried down under nitrogen to yield the methylphenidate, which was confirmed by gas chromatography-mass spectrometry (GC-MS), in which the major peak was eluted cleanly at 12.8 min, consistent with authentic methylphenidate. This was converted to ritalinic acid as described.

Isolated methylphenidate was dissolved in methanol and the pH adjusted to 12–14 by the addition of LiOH (0.1M). The solution was then stirred at 50–60°C for 1–2 h until there was no migration by TLC (chloroform/methanol/ammonia, 90:9:1). The solution was neutralized with the addition of H2SO4 (1.25M) and taken to dryness. The product ritalinic acid was then dissolved in methanol, leaving unwanted Li2SO4 as a precipitate. The ritalinic acid was analyzed by GC–MS and found to have an elution time of 7.48 min.
Coupling of ritalinic acid to bovine thyroglobulin

Ritalinic acid was coupled to bovine thyroglobulin by the mixed anhydride method (5). Briefly, ritalinic acid (~ 6 mg) was dissolved in dimethylformamide (400 µL), and to this were added tri-n-butylamine (10 µL) and iso-butylchloroformate (4 µL). The solution was then stirred for 20 min to allow activation to occur, and following this the mixed anhydride was added drop-wise to aqueous bovine thyroglobulin (10 mg in 1 mL) on ice. The pH was maintained at 8–8.5 by the addition of NaOH (1M) with stirring, and, after the addition of H₂O₂ (4 mL), left stirring at 4°C for 24 h. The conjugate was then dialyzed against water, sodium azide added, and stored frozen in aliquots.

Solutions

Saline solution (NaCl, 8 g/L) containing 0.05% Tween 20 (v/v) was used for washing the microtiter plates. Phosphate-buffered saline (PBS) containing 0.05% Tween 20 (v/v) and 0.1% gelatin (w/v) was used as the assay buffer.

Tetramethylbenzidine (TMB) was used as the substrate for horseradish peroxidase and was made up as follows. Sodium acetate anhydrous (8.2 g) and citric acid (3.6 g) were dissolved in H₂O (600 mL), and TMB (270 mg) dissolved in methanol (400 mL) was added. H₂O₂ (30%, 0.5 mL) was added, and the solution was stored in the dark at room temperature.

Antisera

Sheep antiserum to methylphenidate (Ritalin) was purchased from Cortex Biochem, Inc. (San Leandro, CA, CR 6084SP). Lyophilized rabbit antisheep IgG (Fab')2 conjugated to peroxidase (CB 1348) was also obtained from Cortex Biochem and was reconstituted to the manufacturer’s specifications.

ELISA method

ELISA plates (Falcon 3912 Microtest III, Becton Dickinson, Franklin Lakes, NJ) were coated by adding 100 µL of conjugate solution to each well and leaving the covered plate overnight at 4°C. The conjugate solution was prepared by adding ritalinic acid/thyroglobulin (5 µL) to aqueous guanidine hydrochloride (6M, 10 mL). Unbound conjugate was removed by washing, and the remaining binding sites were “blocked” by the addition of assay buffer (150 µL) for 30 min at room temperature.

Following blocking, the plates were shaken dry and the methylphenidate standards (50 µL) added in duplicate down the left-hand side of the plate to create the standard curve. Methylphenidate standards (0–200 ng/mL) were prepared in assay buffer from a stock solution of methylphenidate (1 mg/mL) in methanol (Diagnostix Ltd.). Urine samples and controls were diluted 1:20 with assay buffer in a dilution block and then added (50 µL) in duplicate to their relative position on the plate.

Ritalin antibody (1:2000 in assay buffer, 50 µL) was then added to each well and the plate covered and incubated for 1 h at room temperature. The plate was then washed and shaken dry, and peroxidase-labelled rabbit antisheep IgG (1:1000 in assay buffer, 100 µL) was added to each well and incubated for an additional hour. Plates were again washed and shaken dry prior to the addition of TMB substrate solution (100 µL).

Color developed over 15 min at room temperature and was stopped by the addition of H₂SO₄ (1.25M, 100 µL), and the absorbance was read on an ELISA plate reader at 450 nm. Results were interpolated on the standard curve and then corrected for the urine dilution. The results are expressed in methylphenidate equivalents, as urine will contain predominantly ritalinic acid (6) and only minor amounts of methylphenidate, which will be equally recognized by the antibody because it has 100% cross-reactivity to both methylphenidate and ritalinic acid.

For comparative studies, the commercial methylphenidate ELISA kit was purchased from Diagnostix Ltd. and used according to the manufacturer’s instructions.

Measurement of creatinine

The urine dilutions were also used for the measurement of creatinine by a microtiter plate adaptation of the Jaffé picric acid method (7). The optical density of complex formation was read at 492 nm on an ELISA plate reader following a 15-min incubation.

Validation

Cross-reactivity studies were performed to determine the specificity of the assay towards methylphenidate. The antibody was raised against ritalinic acid following conjugation to KLH and was equally specific for both methylphenidate and ritalinic acid at 100%. Patient urine samples known to contain drugs other than methylphenidate were assayed to determine any interference. In addition, a blank urine sample was spiked with a mixture of various drugs each at 2000 ng/mL to determine relative cross-reactivity.

The precision was evaluated using 4 control urine samples, and each was measured 8 times in duplicate for within-assay variation and between-assay variation was calculated from 15 consecutive assays.

### Table I. Recovery from Urine

<table>
<thead>
<tr>
<th>Amount added (ng)</th>
<th>Amount recovered (ng)</th>
<th>%Recovered</th>
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<tbody>
<tr>
<td>48</td>
<td>59</td>
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<td>64</td>
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<tr>
<td>1033</td>
<td>1178</td>
<td>114</td>
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</tbody>
</table>

### Table II. Linearity

<table>
<thead>
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<th>Dilution factor</th>
<th>Sample 1 (ng/mL)</th>
<th>Sample 2 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:160</td>
<td>53</td>
<td>57</td>
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<td>1:80</td>
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<td>282</td>
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<tr>
<td>1:20</td>
<td>420</td>
<td>509</td>
</tr>
<tr>
<td>1:10</td>
<td>1030</td>
<td>1297</td>
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Recovery was carried out using urine spiked with either 1033, 533, 283, 158, 95.5, 64, or 48 ng/mL of methylphenidate. Recovery was calculated by expressing the total methylphenidate determined by ELISA as a percentage of added methylphenidate. Results are shown in Table I.

To show the assay was linear, two high urine samples were taken and serially diluted from 1:10 to 1:160. Results are shown in Table II.

Clearance studies
Following local informed consent procedures, one of the authors took one 10-mg Rubifen tablet on two separate occasions, and urine samples were collected for 48 h following the dose. The samples were assayed for methylphenidate equivalents, as well as creatinine and the results plotted to obtain a clearance curve. Methylphenidate equivalents are plotted as previously described.

Results and Discussion
A typical standard curve is shown in Figure 1. Specificity studies showed negligible cross-reactivity to other drugs of abuse. These included patient urines containing paracetamol, cetirizine, amitriptyline, nortriptyline, amphetamine, ephedrine, paroxetine, lignocaine, carbamazepine, caffeine, methadone, cannabinoids, nicotine, benzodiazepines, chlorpromazine, citalopram, cyclizine, codeine, morphine, cocaine, pethidine, quinine, sulfapyridine, quinalbarbitone, amylobarbitone, nitrazepam, and clozapine. In addition, the spiked urine sample (2000 ng/mL each of all of these drugs) returned values less than 25 ng/mL, similar to the levels measured in normal drug-free volunteers. The calculated cross-reactivity of these drugs is less than 1%. The minimal detectable dose of ritalin is 15 ng/mL. The precision studies on the four urine controls gave mean results of 27, 47, 382, and 399 ng/mL with within-assay variation of 8.0, 3.8, 7.4, and 4.0%, respectively. The between-assay variations for 15 consecutive assays gave mean results of 30, 47, 373, and 390 ng/mL with variations of 18, 6, 17, and 15%, respectively.

Patients taking the prescribed dose of methylphenidate had urine concentrations between 40 and 500 ng/mL of methylphenidate equivalents and a methylphenidate/creatinine ratio greater than 5 µg/mmol creatinine with a proviso that the creatinine is above 5. This is in contrast with methylphenidate-free volunteers, for whom the methylphenidate equivalent was less than 25 ng/mL and the methylphenidate/creatinine ratio always less than 3 µg/mmol creatinine.

Data from the clearance study in Figure 2 (10 mg methylphenidate) show peak values of methylphenidate equivalents 1.5–2 h after ingestion. These values then rapidly decline to low but significant levels, even 9 h (540 min) after that dose. The normal prescribed dose of 20–60 mg per day in 2 or 3 divided doses should be permanently detectable. Methylphenidate assays should take into account the creatinine concentration of the urine and, therefore, patient results are expressed as micrograms-per-millimole creatinine. Patients on methylphenidate with a high fluid intake may return a very low urine creatinine and, hence, partially circumvent the test. In these cases, a fluid restricted repeat sample should be requested.

We also used the commercial kit to test urine from a variety of patients who were known ritalin users (n = 15), nonritalin users (n = 15), as well as the kit controls. In all cases the results were concordant and for the “in house” ELISA, quantitative, providing the creatinine was greater than 5.0 mmol/L. Both ELISAs could be circumvented by substantial urine dilution, but this is a common problem with drug screening.

Although a commercial ELISA test kit is available for ritalin, it is expensive and provides only a positive or negative result and, hence, does not allow for creatinine correction. The ELISA described in this paper is inexpensive, reliable, and easy to perform. It is quantitative, thus allowing creatinine correction for excretion, which provides a more robust indicator of methylphenidate compliance. Apart from compliance issues, this simple quantitative ELISA could also prove useful for pharmacological studies.
References


Manuscript received September 17, 2002; revision received March 5, 2003.