Gas-Chromatographic Quantification of Methylphenidate in Plasma with Use of Solid-Phase Extraction and Nitrogen-Sensitive Detection

Brian D. Potts,1 Catherine A. Martin,2 and Mary Vore3

The gas-chromatographic assay for methylphenidate described here involves isolation by solid-phase extraction and quantification by thermionic nitrogen-phosphorus detection. Methylphenidate and the internal standard, ethylphenidate, are extracted from plasma by partition onto C2 reversed-phase packing. Methylphenidate and ethylphenidate are eluted, dried, derivatized with trifluoroacetic anhydride, and gas-chromatographed, with nitrogen-sensitive detection. The standard curve for the assay is linear in the range 5–100 μg/L. The within-run CV is <4%, the between-run CV <6%. Mean analytical recovery of methylphenidate was >90%. The smallest measurable concentration of methylphenidate is 2 μg/L. The sensitivity, reproducibility, and economy of this assay make it suitable for clinical monitoring and pharmacokinetic studies.

Additional Keyphrase: sympathomimetic drugs

Methylphenidate (MEPH; Figure 1), a sympathomimetic drug with pharmacologic properties similar to those of amphetamine, is generally considered to be the drug of choice for treatment of children with hyperactivity (attention-deficit disorder with hyperactivity) (7), a condition seen in 5 to 20% of all children in North America (2–4). Reportedly, about 80% of such hyperactive children respond favorably to treatment with MEPH; about 20% are nonresponders (5).

In humans, MEPH is metabolized primarily by de-esterification to therapeutically inactive ritalinic acid (alphaphenyl-2-piperidine acetic acid, RA; Figure 1), which accounts for roughly 80% of its metabolites (6, 7). In rats, MEPH concentration in the brain declines in parallel with its concentration in the plasma after an intravenous dose (8). Because of the rapid and extensive tissue distribution and organic-aqueous partitioning characteristics of MEPH, the brain apparently is part of a central, rapidly equilibrating compartment, along with the plasma (8). If this parallel relationship holds true in humans, the concentration of MEPH in plasma would be a good index to the concentration of active drug in the brain.

Several recent studies (9–11) have explored the pharmacokinetics of MEPH in children. All note considerable interpatient differences in several of the pharmacokinetic variables. The oral dose required for optimal clinical response also varies widely among individuals (5, 10). Accordingly, effective doses of MEPH are typically determined by starting the hyperactive child on a small dose of MEPH and gradually increasing it until a "response" is seen at school and (or) at home (12, 13). However, this procedure presents difficulties, because the child's response may be different at home, at school, or in the physician's office, or confounding environmental factors may affect behavior independently of drug response. If a significant correlation between MEPH concentration in plasma and clinical response was observed, assays for it could be used as a guide in determining the clinically effective dose of MEPH. Thus, a clinically applicable method for quantifying MEPH in plasma is needed.

Numerous methods have been described for measuring MEPH and RA in plasma and urine. RA has been measured in biological fluids (14–16) because much more of it than MEPH is present after administration of MEPH (9). However, quantification of this inactive metabolite as an indicator of MEPH concentration in the plasma of children is inappropriate because the ratio of RA to MEPH in plasma concentration varies considerably among individuals (9).

Methods for measuring MEPH by gas chromatography with flame ionization detection (16, 17) and liquid chromatography (18) lack the sensitivity required to detect MEPH at the concentrations of 5 to 20 μg/L (9–11) that are present in plasma after oral administration of therapeutic dosages. Recently published gas-chromatography/mass-spectroscopic (GC/MS) methods are much more sensitive. Milberg et al. (19) developed a method for MEPH in blood and urine that involves solvent extraction and quantification, by GC/MS, of underivatized MEPH—which, however, reportedly undergoes extensive and variable decomposition in the injection port of the gas chromatograph (20). A GC/MS procedure described by Gal et al. (8) involves solvent extraction, derivatization with trifluoroacetic anhydride, and quantification by comparison with an internal standard. Although this procedure works well for MEPH concentrations of 1–5 μg/L in plasma, higher concentrations are difficult to measure because the signal from the MEPH overlaps the signal from the internal standard in the mass chromatogram (21). Although GC/MS selected-ion monitoring assays (8, 10, 19) are adequately sensitive to measure low concentrations of MEPH, difficulties are encountered in the analysis of patients with high concentrations of RA.
MEPH in plasma, they are currently impractical for use in the routine clinical laboratory because the cost of the instrumentation is prohibitive. Hungund et al. (21) developed a method based on gas chromatography with nitrogen-sensitive detection that has a sensitivity of 1 μg/L, but it is not suitable for routine clinical use because the extensive preparation steps take 4 to 6 h.

We describe here a gas-chromatographic assay for MEPH in which the ethyl ester homolog of the drug, ethylenediamine (ETPH; Figure 1), is used as the internal standard. MEPH and ETPH are isolated from plasma samples by using minicolumns for solid-phase extraction, and are quantified as their N-trifluoroacetyl derivatives in a gas chromatograph equipped with a nitrogen-sensitive detector. This procedure is sensitive (2 μg/L), reproducible (CV <6%), and rapid for determining MEPH in blood.

Materials and Methods

Materials

Instrumentation. We used a Model 3700 gas chromatograph equipped with a thermionic nitrogen–phosphorus-sensitive detector (Varian Associates, Palo Alto, CA 94303). This instrument was fitted with a 3.9 m × 2 mm (i.d.) coiled-glass column packed with 3% SP-2250-DB on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA 16823). The carrier gas was helium, the flow rate 30 mL/min. Injection temperature was 240 °C, oven temperature 170 °C, and detector temperature 270 °C. Bias voltage was set at −4 V and bead current at 5.5 A. Flow rates of hydrogen and air to the alkali-ceramic bead were 4.5 and 180 mL/min, respectively. We used a reporting integrator (Model 3390A; Hewlett-Packard Co., Avondale, PA 19311) with this gas chromatograph to quantify peak areas.

Reagents. MEPH-HCl and RA were generous gifts of CIBA-Geigy Corp., Ardelay, NY 10502. The internal standard, ETPH-HCl, was prepared from RA as described by Gal et al. (6). The purity of MEPH-HCl and ETPH-HCl was assessed from their melting points and GC/MS analysis. A stock solution of ETPH was prepared by dissolving 4.0 mg of ETPH-HCl in 10 mL of water and diluting to a final concentration of 2000 μg/L. This stock solution, divided into 1-mL aliquots, was stored at −70 °C until use.

All solvents (methanol, diethyl ether, cyclohexane, acetone) were “nagrador” and glass distilled (Burdick & Jackson, Muskegon, MI 49442). Trifluoroacetic anhydride was obtained from Pierce Chemical Co., Rockford, IL 61105. Water was doubly distilled, de-ionized, and stored in glass. All glassware was acid washed; successively rinsed with water, alkali (0.1 mol/L sodium hydroxide), water, and acetone; and oven dried.

C2 mini-columns were prepared by packing 0.2 g of “preparative” grade C2 Sepaplyte (Analytichem International, Harbor City, CA 90710) in a 2-mL glass syringe (Micromate; American Scientific Products, Mcgraw Park, IL 60085), with glass wool as frits.

Samples. Plasma was obtained from behaviorally disordered children who participated in a methylphenidate dose-escalation study conducted by one of us (C. A. M.). Blood was collected in a 5-mL tube containing EDTA (Vacutainer Tube; Becton-Dickinson, Rutherford, NJ 07070) to inhibit the plasma esterases that metabolize MEPH to RA (11). It was promptly centrifuged (3000 × g, 10 min), after which we separated the plasma, and stored it at −70 °C until analysis.

Standards. For preparing standard curves we added MEPH to drug-free plasma and serially diluted with plasma to give final concentrations of 5 to 100 μg/L. These samples were taken through the assay procedure and we constructed a standard curve by plotting MEPH concentration vs MEPH/ETPH peak-area ratio.

Procedure

Add 100 μL (200 ng) of the ETPH stock solution to 2 mL of plasma sample. Add 1 mL of saturated sodium borate to the sample, which increases its pH to 9.3. Condition a C2 mini-column by passing two column volumes of methanol through the column, then two column volumes of water. This can be done quickly by using a commercially available reduced pressure-evolution apparatus (Analytichem International) or by centrifuging the columns, supported in centrifuge tubes, at 30 × g for 30 s. Pass the plasma sample through the column. The drug and internal standard are retained on the column. Wash the column with three column volumes of distilled water, then dry it for 5 min by applying negative pressure to the bottom of the column. Elute the MEPH and ETPH with three 1.5-mL portions of diethyl ether, collecting these fractions in one 7-mL conical centrifuge tube. Evaporate the diethyl ether at 35 °C in a water bath, under a gentle stream of nitrogen. Add 100 μL of cyclohexane to the residue and mix to redissolve. Add 20 μL of a 50 nmol/L solution of triethyamine in cyclohexane and 20 μL of trifluoroacetic anhydride. Cap, mix, and incubate the mixture for 15 min in a water bath set at 50 °C, then wash the mixture with 2 mL of 1 mol/L sodium hydroxide and aspirate the aqueous (bottom) layer. For this we used a drawn-out Pasteur pipet attached to a 2-mL Bel-Art pipetter pump (American Scientific Products). Inject 2 μL of the cyclohexane layer into the gas chromatograph.

To determine the concentration of MEPH in clinical samples, calculate the peak-area ratio (MEPH/ETPH) of the unknown and interpolate the MEPH concentration from the standard curve.

Results and Discussion

A standard curve was constructed as described above, by plotting the concentration of MEPH in μg/L (x) vs the peak-area ratio of MEPH/ETPH (y). The data for this standard curve were obtained on three different days for a series of five concentrations of standard. For these 15 points, the regression equation (±1 SD) was x = −122.83 (± 6.09) + 0.088 (± 0.13), with a standard error of estimate of 9.28 and a correlation coefficient of 0.99.

Stability and between-run precision were evaluated in a series of 10 experiments done during two weeks. We prepared two solutions of MEPH (20 and 100 μg/L) in drug-free plasma, apportioned them into vials, and stored them at −70 °C until analysis. These two solutions yielded mean values of 19.8 (SD 1.17) and 104.2 (SD 5.89) μg/L and CVs of 5.9 and 5.7%, respectively. We noted no trend in the observed values during this 14-day period, an indication that the samples were stable under these conditions.

Within-run precision was determined by preparing two solutions of MEPH (20 and 90 μg/L of plasma) as outlined above and analyzing them in replications of 10. For these solutions, the mean values were 23.83 (SD 0.907) and 92.59 (SD 2.84) μg/L and the CVs were 3.8 and 3.1%, respectively.

We assessed the variability of the gas-chromatographic determination, excluding the sample-extraction steps, by multiple injections of the same sample extract. For a concentration of 20 μg/L the CV was 0.6% for 10 replicate injections.

We measured the analytical recovery of MEPH and ETPH from plasma by adding known amounts of MEPH and ETPH to drug-free plasma to obtain the concentrations shown in Table 1. The extraction and analysis procedures were performed as outlined above, except that we did not
add the internal standard, ETPH, to the plasma samples. We injected precisely measured aliquots of the final solutions into the gas chromatograph and determined the peak areas of the N-trifluoroacetyl derivatives of MEPH and ETPH. The peak areas of the recovery samples were compared with the peak areas obtained by injection of known amounts of MEPH and ETPH. As shown in Table 1, the analytical recoveries of MEPH and ETPH were 89–92% and 93%, respectively.

The minimum detection limit is based on the peak height relative to the baseline noise (22) and on the reproducibility of the peak height for a given concentration of MEPH. In the procedure described, a signal-to-baseline noise ratio of 2 corresponds to a minimum detection limit of 1 µg/L. This, in combination with the precision data, establishes 2.0 µg/L as a lower limit of sensitivity above which MEPH concentrations can be accurately measured.

Plasma samples from individuals known not to be receiving MEPH were analyzed by the described procedure, except that ETPH was not added. No peaks eluted that would interfere with quantification of MEPH or ETPH (Figure 2a). We then added MEPH to the plasma samples and analyzed these by the described procedure; retention times for the trifluoroacetyl derivatives of MEPH and ETPH were 3.84 and 4.42 min, respectively (Figure 2b). We also tested the following compounds for interference by adding them to plasma and extracting by the described procedure: diphenhydramine, chlorpromazine, desipramine, haloperidol, amphetamine, ethosuximide, carbamazepine, phenytoin, theophylline, chlorpheniramine, imipramine, primidone, and acetaminophen. None gave interfering peaks with retention times similar to the trifluoroacetyl derivatives of MEPH or ETPH.

The only difficulty observed in the development of the solid-phase extraction procedure was interference from plasticizers. A plasticizer was leached from the polyethylene frits in commercially prepacked solid-phase extraction columns (Analyticem International) upon elution with diethyl ether, which interfered with the gas-chromatographic quantification of MEPH. We solved this problem by obtaining the packing material in bulk and preparing the minicolumns as outlined under Reagents. One must also be careful to avoid contact of samples and reagents with soft plastic containers.

The solid-phase extraction procedure requires approximately 1.5 h to prepare eight to 12 samples for analysis. Sample purity is similar to that of samples prepared by more extensive and time-consuming liquid extractions (21), and substantial economies are realized through use of less solvent and glassware. The assay outlined here, because of its sensitivity, reproducibility, and economy, can be used routinely in the clinical laboratory to quantitate MEPH concentrations in plasma.

Figure 3 shows the concentrations of MEPH in the plasma of three behaviorally disordered children who received a 10-mg oral therapeutic dose of MEPH. Maximum MEPH concentration in plasma ranged from 10 to 20 µg/L. Our initial data support the earlier observations (9–11) of considerable interpatient variation in absorption, metabolism, and excretion of MEPH. Studies attempting to correlate plasma concentrations of MEPH to therapeutic effect and subjective, physiological, and behavioral responses are currently in progress.

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References
Carboxyhemoglobin, Cotinine, and Thiocyanate Assay Compared for Distinguishing Smokers from Non-Smokers

R. Pojer, J. B. Whitfield, V. Poulos, I. F. Eckhard, R. Richmond, and W. J. Hensley

We compared cotinine, carboxyhemoglobin, and thiocyanate concentrations in blood sampled from 187 cigarette smokers and 181 non-smokers. All three differed significantly between smokers and non-smokers. Cotinine performed best as a test for assessing smoking status, with a sensitivity of 98% as compared with 94% for carboxyhemoglobin and 80% for thiocyanate, all at a specificity of 95%. These differences were statistically significant. Results by none of these three methods correlated well with number of cigarettes smoked per day.

Additional Keyphrases: validating smoking claims · cutoff value

Several biochemical methods are used to distinguish smokers from non-smokers in health surveys, anti-smoking programs, and heart-disease prevention studies (1–3). These methods—which include plasma cotinine, blood carboxyhemoglobin, plasma thiocyanate, and expired-air carbon monoxide—are a valuable aid to interpretation of the questionnaires used in smoking-cessation programs. Information supplied solely by the volunteers in these programs is often unreliable (5, 6), and to validate results independent measures of cigarette smoking are necessary. Thus it seems important to document and inter-compare the performance of these tests, but there are few such studies adequately comparing the discriminating power of the various biochemical methods (1, 2). Here, we examine the performance of three of these tests: cotinine, carboxyhemoglobin, and thiocyanate, and compare them, both with each other and with the number of cigarettes reportedly smoked per day. We attempted to fulfill the criteria suggested by Zweig and Robertson (7): subject selection, independent classification, performance of all tests on all subjects, and comparison of tests at the same specificities. We have also performed statistical tests to validate impressions of difference in performance.

Subjects and Methods

The study population consisted of 187 smokers and 181 non-smokers, the former being participants in a voluntary smoking-reduction campaign in an suburban general practice, the latter being unsolicited patients attending the clinic.