Determination of Physostigmine in Plasma and Cerebrospinal Fluid by Liquid Chromatography with Electrochemical Detection

L. K. Unni, M. E. Hannant, R. E. Becker, and E. Giacobini

Physostigmine (Phy) was determined in plasma and cerebrospinal fluid (CSF) by HPLC with electrochemical detection, with use of a normal-phase column and methanolic sodium acetate buffer, pH 4.6. The detection limit of the method was 0.5 μg/L for a 2-mL sample of plasma or 0.5 mL of CSF. Analytical recovery of Phy in the range from 0.5 to 40 μg/L was 80% (SD 5%) for plasma and 78% (SD 8%) for CSF. Excellent chromatographic separation of Phy without column deterioration during extended usage and constant recovery for a wide range of Phy concentrations makes the routine monitoring of plasma from patients with Alzheimer's disease economically feasible. Using our method, we measured Phy in 13 such patients' plasmas at 105 and 135 min after a 135-min intravenous infusion of 300, 600, and 900 μg of Phy per square meter of body surface. Mean values significantly increased with dose (P = 0.001), but differences between 108 and 135 min (P = 0.229) or between dose and time (P = 0.849) were not significant.

Additional Keyphrases: Alzheimer's disease · pharmacokinetics · cholinesterase inhibitor

Physostigmine (Phy), an alkaloid from the calabar bean, is a reversible inhibitor of cholinesterase (EC 3.1.1.8) and was first used in 1883 in the treatment of glaucoma (1). A tertiary amine, it can cross the blood-brain barrier; thus, unlike other reversible cholinesterase inhibitors such as neostigmine and pyridostigmine, Phy has gained prominence as an antidote for anticholinergic drugs and also for managing side effects from overdose of tricyclic antidepressants (2-4). Recently, owing to its effect on the central nervous system, Phy has been used in the treatment of Alzheimer's disease. Phy decomposes in alkaline medium to the hydrolysis products, eseroline and rubreserine. It is rapidly metabolized in plasma and liver, resulting in a short half life after intravenous or oral administration (5-7).

Only in the last few years have sensitive analytical methods become available for measuring Phy in biological samples. These have lacked either a linear recovery for low Phy concentrations or chromatographic conditions that would permit both a good separation of Phy from metabolites and plasma contaminants and extended usage of the column. A high-performance liquid chromatographic (HPLC) method with electrochemical detection (EC), performed with a Spherisorb column and basic buffer (pH 8.9), and having a detection limit of 0.1 μg/L, has been reported for determining Phy concentrations in plasma (8). The HPLC method of Brodie et al. (9), involving a fluorescence detector, also gave a detection limit of 0.1 μg/L; here, also, a Spherisorb column and basic buffer (pH 9.9) were used for the chromatographic separation. However, silica-based columns should be used under acidic conditions because alka-line media disrupt the packing and drastically shorten column life and reproducibility. Moreover, Phy deteriorates under basic chromatographic conditions. Isaksson and Kissing (10) reported an HPLC-EC method involving a Biopack octyl column, an acidic buffer, and a solid-phase extraction step. Because recovery of Phy decreased from 53% for 10 μg/L to 35% for 1 μg/L, they recommended the use of an internal standard or liquid-liquid extraction method to circumvent this problem. Unfortunately, the two types of extraction methods are not necessarily interchangeable within a given methodology, because the contaminants remaining in the sample after one separation step may respond differently during subsequent steps of the separation. Moreover, because the concentrations of Phy in plasma after its intravenous, intramuscular, or subcutaneous administration to humans are so low [expected range: 0.5-10 μg/L (7)], it is critical that the extraction efficiency be linear in this range.

Here, we report a sensitive HPLC-EC method for determination of Phy in plasma and cerebrospinal fluid (CSF). Determination of Phy in CSF has not been reported earlier. The advantages of our method over the previously reported HPLC-EC methods for plasma samples are: excellent separation of Phy under chromatographic conditions that do not cause column deterioration during extended usage; constant recovery for a wide range of Phy concentrations; and sensitivity to detect clinically significant concentrations of Phy, enabling routine and economical monitoring of patients' plasmas. We also report the use of our method to determine concentrations of Phy in plasma of patients with Alzheimer's disease who received intravenous infusions of three different doses of Phy.

Materials and Methods

Drugs and Chemicals

Physostigmine salicylate was obtained from Sigma Chemical Co., St. Louis, MO. Eseroline salicylate was a gift from Dr. A. Galli, Universita Degli Studi Di Firenze, Italy. Sodium acetate, methanol, diethyl ether, and chloroform, all of "HPLC" grade, were from Fisher Scientific Co., St. Louis,
MO. Reagent-grade trifluoroacetic acid was obtained from J. T. Baker and Co., Phillipsburg, NJ, and ammonium hydroxide from Fisher Scientific Co.

Equipment

The LC system consisted of a PM-30 pump, two LC-4B amperometric controllers, a dual glassy-carbon working electrode, and an Ag/AgCl reference electrode (all from Bioanalytical Systems, West Lafayette, IN). Samples were injected via a Rheodyne Model 7125 injector valve with a 200-μL loop. The chromatograms were plotted with a Bioanalytical Systems Model RYT recorder.

Chromatographic conditions: We used a 5-μm particle diameter Spherisorb silica column, 150 mm × 4.6 mm, i.d., a guard column packed with pellicular silica (both from Alltech Associates, Chicago, IL), and a mobile phase consisting of sodium acetate buffer (10 mmol/L, pH 4.6) in methanol (1/10 by vol). The mobile phase was filtered under reduced pressure through a 0.45-μm pore-size Nylon 66 filter (Rainin Instrument Co., Woburn, MA) and sonicated to remove the air bubbles. The flow rate was 2 mL/min. The dual glassy-carbon electrode was used in series and operated at oxidation potentials of +0.25 V (W1) and +0.95 V (W2), respectively.

Extraction Procedures

For plasma: We combined human plasma (2 mL), ammonium hydroxide (0.5 mL of a 35 mL/L solution), and diethyl ether (5 mL) in a screw-cap test tube, vortex-mixed for about 10 s, then centrifuged the mixture at 900 × g for 10 min at 0–5 °C in an IEC Centra-TR centrifuge. After centrifugation, we transferred 3 mL of the ether (upper) layer to a 5-mL conical-bottom glass vial (Microfuge Products, Wheaton, IL) and evaporated the solvent under a gentle stream of nitrogen at room temperature. The residue was re-extracted with a mixture of 200 μL of ether and 225 μL of water and again centrifuged at 900 × g for 15 min at 0–5 °C. The aqueous (lower) layer (about 200 μL) was pipetted into a microcentrifuge tube and centrifuged (11 000 × g, 5 min, 5 °C) in a microcentrifuge. We then injected an aliquot of the supernate into the chromatographic column. During all extraction steps, the tubes were shielded from light as much as possible with aluminum foil.

For CSF: We pipetted human CSF (0.5 mL) and trifluoroacetic acid (50 μL of a 1 g/L solution) into a screw-cap glass test tube and vortex-mixed for 10 s. Chloroform (2.5 mL) was added, and the mixture was vortex-mixed again. After centrifugation at 800 × g for 15 min at 0–5 °C, we transferred 2 mL of the chloroform (lower) layer to a conical glass vial and evaporated the solvent under nitrogen. We reconstituted the residue with 200 μL of methanol, centrifuged this (11 000 × g, 5 min, 5 °C) in a microcentrifuge, and injected an aliquot (about 190 μL) of the supernate into the chromatographic column.

Calibration Curves

Standard Phy salicylate solutions in water: Phy concentrations ranging from 0.5 to 10 μg/L were injected into the chromatographic column, and the peak heights for Phy were measured.

Plasma supplemented with Phy salicylate solution: To 2-mL plasma samples we added various concentrations of Phy, ranging from 0.5 to 20 μg/L, and extracted as described above.

CSF supplemented with Phy salicylate solution: We supplemented 0.5-mL samples of CSF with Phy in concentrations ranging from 0.5 to 40 μg/L and extracted as described above.

Other Studies

In vitro stability of Phy salicylate in water at 5 °C. Phy salicylate was dissolved in sterile water to give final concentrations of 10 mg/L and 100 μg/L, then kept refrigerated, in the dark. Known volumes of solution were injected into the chromatograph and measured at various times during the next six weeks.

Alkaline hydrolysis of Phy solution. Mix 1 mL of 1 μg/mL Phy solution in water with 1 mL of an ammonium hydroxide solution (100 mL/L) in a glass test tube (not light protected) and leave at room temperature.

Phy dosing in patients with Alzheimer's disease. Phy (300, 600, or 900 μg per square meter of body surface) was administered to 13 patients by intravenous infusion over 135 min (one-third of the total dosage was given during the first 15 min and the remainder over the subsequent 2 h). Details of drug administration and blood collection are discussed elsewhere (11). Blood, collected in heparinized tubes, was centrifuged (900 × g, 15 min, 0–5 °C). Plasma was separated and stored at −70 °C until analysis. Phy was extracted from the plasma as described above.

Results

Phy (retention time, 4.33 min) was well separated from its metabolite eseroline (retention time, 3.33 min). Peak heights were used as a measure of Phy concentration; they varied linearly for Phy standards in the range from 0.5 to 10 μg/L. The standard curve for pure Phy had a slope of 17.472 mm·L/μg and a y-intercept of 0.68 mm (r = 0.999).

Figure 1 shows the chromatograms obtained after extraction of ordinary human plasma and human plasma with added Phy, 20 μg/L. The standard curves obtained by supplementing plasma (2 mL) or CSF (0.5 mL) with Phy (0.5–40 μg/L) gave correlation coefficients of 0.999 for both
kinds of specimens. The slope and y-intercept values for the standard curves were 17.030 mm · L/μg and -4.343 mm for plasma and 14.368 mm · L/μg and -4.649 mm for CSF, respectively.

Analytical recovery of Phy from plasma and CSF was 60% (SD 5%) and 78% (SD 8%), respectively, with a detection limit of 0.5 μg/L in 2-mL samples of plasma or 0.5-mL samples of CSF. The within- (n = 6) and between-day (n = 10) coefficients of variation were less than 7.9% and 8.7% for plasma, and 9.3% and 10.1% for CSF, respectively.

The stability of Phy salicylate dissolved in sterile water and refrigerated at 5 °C in the dark was unchanged for six weeks. Alkaline hydrolysis of Phy solution (1 μg/mL) by addition of an equal volume of ammonium hydroxide (100 mL/L) at room temperature resulted in a gradual loss of Phy: from 28% by 30 min, 39% by 50 min, 43% by 105 min, 73% by 21.5 h, 82% by 26.25 h, 88% by 46 h, 92% by 70 h, and 100% by 84 h.

Phy samples after extraction from plasma can be stored at -70 °C for at least one month before injection into HPLC without causing any degradation of Phy. Similarly, human plasma containing Phy can be stored under those conditions for at least six months without loss.

Phy concentrations in plasma from patients with Alzheimer's disease, 105 and 135 min after intravenous infusion of three different doses of Phy, are shown in Figure 2. Statistical analysis of variance (ANOVA) showed a significant increase in mean plasma Phy concentration with increase in dose (P = 0.001). Differences between the mean plasma Phy concentrations at 105 and 135 min were not significant (P = 0.229), nor was there a significant interaction between dose and time (P = 0.949).

Discussion

The liquid chromatographic method with electrochemical detection permitted excellent separation of Phy with no column deterioration for at least 500 injections each of extracts from plasma and CSF. Constant recovery proportions and sensitivity for clinically significant concentrations of the drug make the method economical for routine monitoring of drug concentrations in patients' plasma.

Phy metabolizes to eseroline and rubreserine under alkaline conditions. Absence of any peak at the retention time of Phy after alkaline hydrolysis indicates that rubreserinone does not co-elute with Phy. By applying a potential of +0.25 V to the upstream electrode, we oxidize plasma and CSF contaminants before Phy oxidation, which occurs at the downstream electrode (0.95 V).

We used a liquid–liquid extraction method to isolate Phy from plasma and CSF. Alkaline precipitation of plasma proteins was preferred because acid treatment yielded an extraneous peak that co-eluted with Phy. The solubility of Phy salicylate in water at 5 °C is about 3.33 times higher than in an equal volume of ether. Substituting water during the re-extraction step with hydrochloric acid (10 mmol/L) or with trifluoroacetic acid solution (a 1 mL/L solution) decreased Phy recovery to <40%. A re-extraction step was essential to remove plasma contaminants, which otherwise co-eluted with Phy. Brodie et al. (9), reported a recovery >90% for Phy after alkaline precipitation of plasma, extraction with tert-butyl ether, and re-extraction from the ether layer into hydrochloric acid (10 mmol/L). Therefore, alkaline precipitation apparently does not produce a loss of Phy recovery through hydrolysis to the metabolites. The extraction procedures for plasma and CSF were not interchangeable. The extraction of Phy from CSF was different from the plasma extraction procedure because contaminant peaks co-eluted with Phy. Lower Phy concentrations in plasma and CSF can be detected by using larger sample volumes than 2 mL plasma or 0.5 mL CSF without affecting the recovery.

Analysis of plasmas from patients with Alzheimer's disease after administration of Phy by intravenous infusion over 135 min showed a significant increase in mean plasma Phy concentration with dose, and that the concentration of plasma Phy was in a steady state from 105 to 135 min. These findings are in agreement with the observed inhibition of plasma cholinesterase observed in these patients, which increased with drug dosage but was constant from 105 to 135 min (11). We have also used this method to measure Phy concentrations in CSF after administering Phy intracerebroventricularly to patients with Alzheimer's disease (12).

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References

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Fig. 2. Plasma Phy concentration in 13 patients with Alzheimer's disease after intravenous infusion of 300, 600, and 900 μg of Phy per square meter of body surface, over a 135-min infusion period. a and b denote blood samples drawn at 105 and 135 min, respectively. The solid and dashed lines connect the points representing the mean (+ SD) Phy concentration (μg/L) in plasma after 105 and 135 min, respectively, for the three Phy doses.
Detection of Poisoning by Plant-Origin Cardiac Glycoside with the Abbott TDx Analyzer

Kee Cheung, John A. Hinds,1 and Patrick Duffy2

Cardiac glycoside poisoning caused by ingestion of plant material is common in tropical and subtropical areas. In evaluating the use of the Abbott TDx Digoxin II assay to detect such cases of poisoning, we found it a rapid and convenient method for confirming the ingestion of glycosides from the plants Nerium oleander, Thevetia peruviana, and Adonis microcarpa, and from the toad Bufo marinus. Here we report some clinical cases illustrating our experience with the use of this assay, and describe results of cross-reactivity studies with compounds structurally similar to digoxin. Because of the competitive nature of the immunoassay as well as the complexity of the mixture of cross-reacting cardiac glycosides present in the plant material, the measured apparent digoxin concentration is not linearly related to the cardiac glycoside concentration.

Additional Keyphrases: toxicology · veterinary chemistry · "kit" methods

A wide variety of tropical and subtropical plants contain cardiac glycosides, and ingestion of these plants is a relatively frequent cause of poisoning in humans and animals (1). Children are at particular risk, being attracted to the bright flowers, the glossy green leaves, and the fruit-like seeds of these plants, which are commonly grown as ornamental garden shrubs. Recent cases of fatal human poisoning have been reported in Australia (2), Melanesia (3), Thailand (4), India (5-7), and the United States (8, 9). In veterinary medicine, cardiac glycosides in weeds contaminating grazing areas are an important cause of mortality, and cases of animal poisoning have been reported (10, 11).

Use of commercial digoxin immunoassay kits to detect poisoning by such plants has been limited by the highly specific antibodies in these kits. For years we have been using an in-house radioimmunoassay to detect plant cardiac glycosides involving antibodies tailored to have a substantial cross-reactivity against many common plant glycosides (12). Use of this assay reportedly confirms the presence of cardiac glycosides in fatal cases of Thevetia peruviana poisoning (2, 3).

Recently, we observed that the Abbott TDx analyzer, used with Digoxin II reagents, gave an apparent digoxin concentration result similar to our own in-house immunoassay for serum from swine experimentally fed Adonis microcarpa seeds (which reportedly contain cardiac glycosides (13, 14)). We detail here case reports and other experimental data to show that the current TDx Digoxin II assay is a simple, rapid assay of serum for detecting many cases of plant cardiac-glycoside poisoning.

Materials and Methods

Swine serum samples were referred from a veterinary research institute investigating an occurrence of Adonis microcarpa poisoning in pigs. Four swine had been given Adonis microcarpa seeds as part of their food over a four-day period, and four control animals had been fed the same diet without the adonis additive (15).

We also obtained, from another hospital, serum and urine samples from a 17-year-old mentally retarded boy who had been admitted for observation after suspected ingestion of a leaf from an oleander tree. After observation showed the patient to be clinically normal and to have a normal electrocardiogram, he was discharged.

Fresh leaves from Nerium oleander (Mediterranean oleander; 2 g) and Thevetia peruviana (yellow oleander; 2 g), seeds from Adonis microcarpa (pheasant’s eye; 0.5 g), and secretion (0.5 g) and skin (0.5 g) from the cane toad (Bufo marinus) were crushed and extracted with 10 mL of phosphate-buffered saline (sodium potassium phosphate buffer, 10 mmol/L, pH 7.4, containing 150 mmol of sodium chloride per liter) by inversion for 30 min. Aliquots were diluted into pooled, drug-free human serum for assay. Solutions of lanatoside C (Sandoz AG, Basle, Switzerland), digitoxin (Sigma Chemical Co., St Louis, MO), procislaridin (Knoll AG, Ludwigshafen, F.R.G.), and ouabain (BDH Chemicals Ltd., Melbourne, Australia) were prepared in ethanol/water (80/20, by vol) and diluted with pooled, drug-free human serum for assay.

For the in-house radioimmunoassay, we used an 125I-labeled digoxin derivative (DuPont, Billerica, MA) as tracer and polyethylene glycol 6000 (BDH Chemicals Ltd.) for separation of bound and free fractions. Antibody was prepared as described previously (12). We carried out the assay at room temperature by mixing 25 µL of specimen, 100 µL of 125I-labeled digoxin, and 300 µL of antibody in phosphate-buffered saline containing 10 g of bovine gamma globulin (Cohn Fractions II, III), 1 g of bovine serum albumin (Cohn Fraction V), and 0.8 g of 8-anilinonaphthalene-1-sulfonic acid in saline. The assay was performed with serum Aliquots were diluted into pooled, drug-free human serum for assay.

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