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APPLICATION OF A NEW RADIOMETRIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY TO DEFINE PHYSOSTIGMINE PHARMACOKINETICS IN GUINEA PIGS[a]

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SUMMARY

A sensitive high-performance liquid chromatographic method was developed to determine pharmacokinetic parameters of [^{3}H]physostigmine from serial plasma samples from guinea pigs. Physostigmine was totally resolved from its metabolite, eseroline. The limit of sensitivity was 0.05 ng/ml from 0.2 ml plasma. Extraction efficiency was 99.6%. Within-run and among-run coefficients of variation ($n=6$) for 0.2, 0.75, 1.5 and 2.5 ng/ml [^{3}H]physostigmine ranged from 0.7 to 20% and 16 to 32%, respectively. Physostigmine (5 μg/kg) intramuscularly administered to the guinea pig ($n=6$) reached maximum serum concentration (1.5 ng/ml) in 26 min. The apparent volume of distribution and systemic clearance were 1.4 l/kg and 26 ml/min/kg, respectively. This method was successful in defining physostigmine pharmacokinetic parameters in guinea pigs and can be employed for other small animal pharmacokinetic studies.

INTRODUCTION

Physostigmine, an alkaloid derived from the calabar bean, was the first carbamate identified. It combines reversibly with acetylcholinesterase at the es-

[a]The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army of the Department of Defense. In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, revised 1985). The use of trade names does not constitute an official endorsement or approval of the use of such commercial hardware or software. This document may not be cited for purposes of advertisement.

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teratic site to produce an inactive methylcarbamoyl enzyme. As a tertiary amine, physostigmine penetrates the blood–brain barrier to elicit central nervous system effects. Clinical uses include treatment of open-angle glaucoma, acute-angle closure glaucoma and overdoses of drugs with antimuscarinic activity (antimuscarinic drugs, tricyclic antidepressants, H_1-antihistamines). Physostigmine has also been shown to be a valuable treatment for Alzheimer's patients [1].

Small animal studies have recently revealed physostigmine's effectiveness as a pretreatment against organophosphate poisoning [2] but since behavioral toxicity appears at low doses [2], dosing must be carefully administered for maximal efficacy and minimal toxicity. Pharmacokinetic studies are of interest in order to define further the dose–response relationship and to extrapolate results to man. Until recently, pharmacokinetic analyses of physostigmine have been limited by lack of an appropriate analytical technique. Effective doses of physostigmine produce low concentrations (ng/ml) in plasma, requiring extremely sensitive analytical methods.

A high-performance liquid chromatographic (HPLC) technique has been used to study the pharmacokinetics of [^{3}H]physostigmine in rats [3]. However, because analysis of each sample requires a large volume of blood, the method is unsuitable for serial sampling in small animals and is excessively time-consuming to analyze.

In this paper we describe a method to study physostigmine pharmacokinetics in small animals that are serially sampled for blood. The concentration–time profile can be determined in each animal, minimizing variability and animal requirements. The method uses flow-through scintillation counting, thus providing decreased labor and increased speed.

EXPERIMENTAL

Materials

Physostigmine (benzene ring-^{3}H) with 16.1 Ci/mmol specific activity was purchased from Amersham International (Arlington Heights, IL, U.S.A.). Physostigmine (free base) and neostigmine bromide were obtained from Aldrich (Milwaukee, WI, U.S.A.) and Hoffman-LaRoche (Nutley, NJ, U.S.A.), respectively. Eseroline was obtained from Walter Reed Army Institute of Research under Contract No. DAMD17-83-C-3207.

Reagents

Hydrochloric acid, 1-octanesulfonic acid (sodium salt) and monobasic sodium phosphate were analytical grade. Glacial acetic acid was reagent grade. Methanol, water and Ultrafluor scintillation fluid (National Diagnostics, Somerville, NJ, U.S.A.) were HPLC grade. All reagents and chemicals were obtained from commercial sources.

Equipment and HPLC conditions

A Beckman 341 HPLC system was equipped with a 100-μl sample loop, a Waters disposable C_{18} guard column and a stainless-steel Waters μBondapak C_{18} separation column (30 cm$\times$3.9 mm I.D., 10 μm pore size). A Radiomatic Instruments Flo-One B radioactive flow detector with a 2.5-ml sample chamber was coupled to the separation column.

The mobile phase flowed through the column at a flow-rate of 1.5 ml/min. Prior to reaching the detector's sample compartment, the column effluent was mixed with Ultrafluor scintillation fluid in a 4:1 (v/v) ratio, the resulting mixture achieving a flow-rate of 5.5 ml/min through the Flo-One's cell.

The mobile phase was made according to the method of Somani and Khalique [3]. Briefly, an aqueous buffer (0.5 m*M* 1-octanesulfonic acid–5 m*M* monobasic sodium phosphate–1% acetic acid) was mixed with methanol in a 60:40 (v/v) ratio and the pH adjusted to 3.1. The mixture was then filtered through a 0.45-μm Nylon 66 membrane (Alltech Assoc.) under vacuum.

Standards

Plasma spiked with 50 μg neostigmine per ml was used in preparing stock solution of 29.3 mCi [^{3}H]physostigmine per ml plasma. All working solutions were prepared from this stock. Standard [^{3}H]physostigmine concentrations were 0.1, 0.5, 1.0, 2.5 and 5.0 ng/ml.

Animal preparation

Six guinea pigs (250–350 g, Hartley Albino) were anesthetized with ketamine (30 mg/kg) and rompun (6 mg/kg). Hair was clipped from the incision site and the skin surgically scrubbed. A ventral midcervical incision was made and the area dissected to expose the right internal carotid artery. A polyethylene 90 catheter was inserted into the artery and anchored over the shoulder. While placing the catheter, 1–3 ml of heparin (4 U/ml) was used to keep the artery patent. The incision was closed with 3-0 and 4-0 silk ligatures. A three-way stopcock was attached to the end of the catheter. After surgery, animals were housed in custom-designed acrylic cages that prevented them from chewing and pulling on the catheters as they awakened. They were allowed to completely recover from surgical anesthesia before proceeding with the rest of the experiment, usually about 24 h post-surgery.

Dosing

[^{3}H]Physostigmine (5 μg/kg body weight) was intramuscularly administered in the right hind limb, and blood samples were collected from the indwelling carotid catheter at the following times: 0, 5, 10, 15, 20, 30, 40, 60, 120, 180, 240 and 300 min. Blood was transferred into heparinized tubes and placed immediately on ice until centrifugation. After all blood was drawn, the tubes were centrifuged on a Beckman tabletop refrigerated centrifuge at 1200 *g* for 5

min at 4°C. Plasma (200 μl) from individual samples was then transferred into microcentrifuge tubes containing 50 μl of aqueous neostigmine bromide solution (50 μg/ml).

Sample preparation

For each analysis, 200 μl of plasma were placed into a Centricon microconcentrator tube (Amicon, Danvers, MA, U.S.A.). For unknown samples, 50 μl of a 50 μg/ml solution of neostigmine bromide in distilled water were added to the tubes. Standards already had neostigmine added. The microconcentrator tubes contained a membrane filter with a 10 000 molecular mass cutoff. Samples were centrifuged at 7000 *g* for 70 min at 4°C in a Dupont Sorvall RC-5B centrifuge. Approximately 150 μl of protein-free filtrate resulted (per sample). The filtrate was kept refrigerated until HPLC analysis. Except where noted, all samples were analyzed within several hours of preparation.

Quantitation

Physostigmine concentrations were determined from weighted standard curves derived each study day from analyses of standard physostigmine concentrations (0.1, 0.5, 1.0, 2.5 and 5.0 ng/ml) in neostigmine-spiked plasma. The area under the curve (AUC) values (radioactive counts) for physostigmine from the Flo-One detector were plotted versus the corresponding standard concentrations. Data were analyzed by weighted least-squares regression analysis, with weight based upon the reciprocal of the variance of six samples at each concentration. For samples obtained from animals, a multiplication factor (1.25) was applied in the data analysis to adjust for the increased volume resulting from the addition of neostigmine bromide solution to plasma.

Sensitivity

The sensitivity of the analytical method was defined as the lowest physostigmine concentration producing a peak with an AUC value greater than three times the standard deviation above background.

Precision

Variability was determined using within-day and between-day coefficients of variation (C.V.) values. For both determinations, data were used from four concentrations (0.2, 0.75, 1.5 and 2.5 ng/ml) of physostigmine in plasma. To estimate within-day variability, three samples of each of the four concentrations were assayed the same day. C.V. values were determined from these three samples for concentrations derived from a daily standard curve. The mean of six sets (one each day) of these C.V. values represented within-day variability. C.V. values were also calculated from eighteen samples of each of the four concentrations assayed over the six days. These values represented total variability attributed to within-day and between-day differences. The difference

of these values from the within-day C.V. values represents day-to-day (between-day) variability.

Efficiency

Four samples each of filtered and unfiltered [^{3}H]physostigmine solutions (1 ng/ml of mobile phase) were analyzed. The average AUC of filtered solutions was divided by that of the unfiltered to determine efficiency. The standard deviation was computed by the method of estimating the error of a computed result from the errors of component factors [4].

Protein binding

The efficiency study was replicated with exception of filtered [^{3}H]physostigmine-containing plasma (1 ng/ml) replacing the unfiltered mobile phase solution. Concentrations determined from filtered plasma were divided by those from unfiltered mobile phase to determine plasma protein binding.

Pharmacokinetic analysis

Time and plasma concentration data from individual guinea pigs were fitted to a standard pharmacokinetic model using the PCNONLIN computer program [5]. The following pharmacokinetic parameters were estimated by the program: apparent volume of distribution (V_d), maximal plasma concentration (C_{max}), time to reach C_{max} (T_{max}), absorption rate constant (k_{01}), elimination rate constant (k_{10}) and AUC. The apparent total systemic clearance of physostigmine was calculated as the dose/AUC. For graphic representation, average concentrations and standard error of the means for each time point were plotted as a function of time. The best-fit curve of those means weighted against the inverse of the variance was also displayed.

RESULTS AND DISCUSSION

Pharmacokinetic parameters are best determined by serial blood sampling. Because of the limited blood volume in small animals, their physostigmine pharmacokinetics must be determined with minimal sample size. Too much blood removed may profoundly alter the hemodynamics and consequently produce inappropriate pharmacokinetics. Our method is currently the only one sensitive enough to measure physostigmine concentrations in the low volumes required for serially blood sampling small animals.

Because physostigmine is oxidized to eseroline in vitro [6] and in vivo [7], we first assure resolution of the two for our method development. The mobile phase and HPLC column in our study was the same as that of Somani and Khalique [3] and therefore the resolution we found was expected (Fig. 1). Due to unavailability of radioactive eseroline, the observed separation required ul-

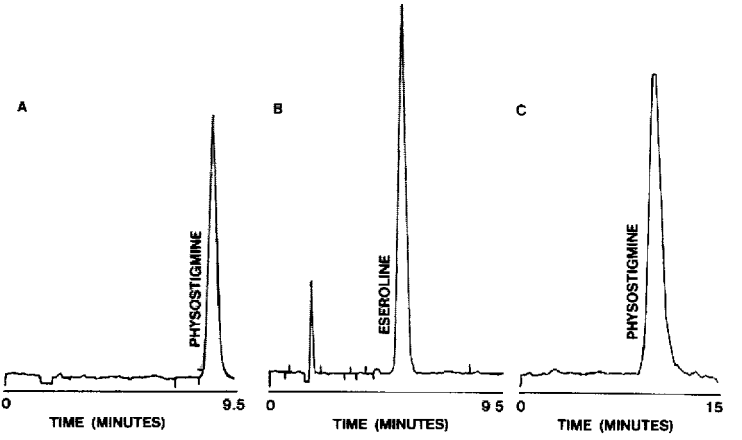

Fig. 1. Chromatograms of (A) physostigmine (retention time 8.1 min) and (B) eseroline (retention time 5.8 min) detected by an ultraviolet spectrophotometer and of (C) [^{3}H]physostigmine (retention time 10.1 min) detected by a flow-through scintillation counter.

TABLE I

PRECISION EXPRESSED AS WITHIN-DAY AND BETWEEN-DAY COEFFICIENTS OF VARIATION

Concentration (ng/ml)	Coefficient of variation (%)	
	Between-day[a]	Within-day[b]
0.2	12.1	20.2
0.75	11.7	4.7
1.5	9.1	7.5
2.5	10.7	9.7

[a]Eighteen samples of each concentration assayed over six working days were used to calculate between-day C.V. values.
[b]Means of six working day C.V. values (each determined from triplicate samples) represented within-day C.V. values.

traviolet detection. The mobile phase flow path was slightly shorter to the ultraviolet detector than to the radioactive detectors, causing the retention times of physostigmine to vary for the two detectors. HPLC effluent in all other parts of this study went directly to the radioactive flow detector.

Standard curves were linear ($r^2 = 0.997$) over the entire concentration range (0.1–5.0 ng/ml). The limit of sensitivity for physostigmine as assessed by a detected peak three standard deviations (S.D.) above background was 0.05 ng/ml.

Inter- and intra-day variabilities, expressed as C.V. values, are listed in Table I. The highest variability (C.V. = 20%) was found within a day at the 0.2

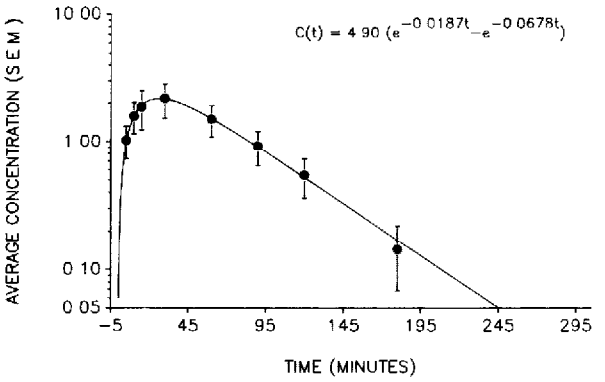

Fig. 2. Physostigmine plasma concentration (mean ± S.E.M.)-time profile in the guinea pig ($n=6$) given 5 μg/kg [^{3}H]physostigmine intramuscularly.

TABLE II

PHARMACOKINETIC ESTIMATES OF PHYSOSTIGMINE INTRAMUSCULARLY ADMINISTERED TO GUINEA PIGS

Pharmacokinetic parameters were determined by model fitting the mean concentration-time profile of six animals intramuscularly administered 5 μg/kg physostigmine.

Parameter[a]	Value (estimate ± S.D)
V_d/F (l/kg)	1.41 ± 0.56
k_{01} (min)	0.068 ± 0.004
k_{10} (min)	0.019 ± 0.001
AUC (ng·min/ml)	190 ± 3.7
Cl_s/F (ml/min/kg)	26.3 ± 0.4
$t_{1/2}$ abs (min)	10.2 ± 0.6
$t_{1/2}$ elim (min)	37.1 ± 1.9
T_{max} (min)	26.2 ± 0.5
C_{max} (ng/ml)	2.17 ± 0.25

[a]abs, absorption; AUC, area under the concentration-time curve; C, concentration; Cl_s, systemic clearance; elim, elimination; F, fraction of drug absorbed; k_{01}, rate constant for absorption; k_{10}, rate constant for elimination; max, maximum; T, time; $t_{1/2}$, half-life; V_d, apparent volume of distribution.

ng/ml concentration. Considering the magnitude of this concentration, a 20% C.V. value is small and indicates a very precise analytical procedure within the concentration range studied.

Efficiency of filtering physostigmine through the microconcentrator tubes was 99.6–3.1% (mean ± S.D.), indicating no significant absorption to the filtering tubes. With a 10 000 molecular mass cutoff, the membrane filters provided a clean filtrate to extent HPLC column life and to measure physostigmine plasma protein binding. The percentage of plasma protein-bound

physostigmine was 37 ± 4.3% (mean ± S.D.), and therefore the major concentration of physostigmine in plasma is unbound.

Physostigmine pharmacokinetics in the guinea pigs were described using a one-compartment open model with first-order absorption and elimination (Fig. 2). Maximum plasma concentration from intramuscular absorption was reached at 26 min (Table II). Assuming 100% bioavailability from the intramuscular route, the apparent volume of distribution, 1.4 l/kg, was smaller than that found in rats (2.3 l/kg) [8]. However, in both species the values were larger than total body water (0.6 l/kg), indicating tissue sequestering of physostigmine from plasma. Clearance was 26 ml/min/kg, much quicker than liver blood flow and therefore physostigmine elimination must be attributed to other processes in addition to hepatic metabolism.

In conclusion, a precise and sensitive method has been developed to measure radiolabelled physostigmine concentrations in small volumes of plasma. The method allows for serial sampling, thereby affording better results with less animals in generating pharmacokinetic data. Future work will apply this method to more thoroughly define pharmacokinetic parameters of physostigmine in small animals.

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