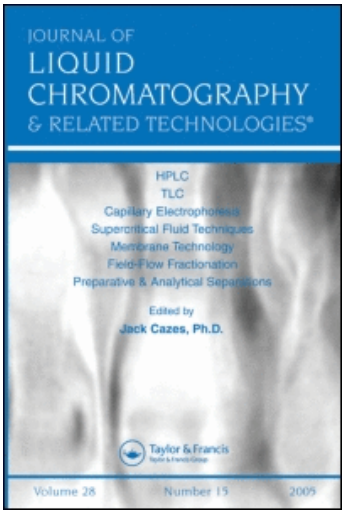

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Analysis of Pyridostigmine Bromide in Human Plasma and its Application in Bioequivalence Studies

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Abstract: A high performance liquid chromatographic (HPLC) method was developed using liquid-liquid extraction and ultra-violet detection for quantifying pyridostigmine bromide in human plasma. Liquid chromatography was performed on a BDS Hypersil C18 coulmn (5 μm, 250 × 4.6 mm i.d.) using an isocratic mobile phase comprised of acetonitrile-5 mM potassium dihydrogen phosphate (40:60, v/v) and the eluent was detected at 269 nm by UV. Clonazepam was used as the internal standard. The limit of quantification was 20 ng/mL using 1 mL plasma. The intra and inter-day precision expressed as the relative standard deviation was less than 15%. The assay was applied to the analysis of samples from a pharmacokinetic study. Following the oral administration of 60 mg pyridostigmine bromide (test) to volunteers, the maximum plasma concentration (Cmax), area under the curve (AUC) and elimination half-life ($t_{1/2}$) were 176.03 ± 9.176 ng/mL, 819.999 ± 109.64 ng hr/mL and 3.787 ± 0.08 hr, respectively. The method was demonstrated to be highly feasible and reproducible for pharmacokinetic studies of pyridostigmine bromide in humans.

Keywords: Pyridostigmine bromide, Pharmacokinetics, HPLC

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INTRODUCTION

Pyridostigmine bromide (PB) belongs to a class of neuroactive compounds called carbamates. It is chemically 3-dimethylaminocarbonyloxy-*N*-methylpyridinum bromide and structurally-similar to physostigmine.[1] It is a powerful and reversible acetylcholinesterase (AChE) inhibitor, which effectively increases the concentration of acetylcholine at the sites of cholinergic transmission.[2]

PB is one of the main drugs currently used to treat myasthenia gravis,[3] a progressive neuromuscular disease characterized by an apparent insufficient stimulation of peripheral muscarinic receptors. PB competes with acetylcholine (ACh) for binding to AChE, and like ACh, is hydrolyzed by the enzyme. However, hydrolysis of PB proceeds much more slowly than that of ACh, resulting in effective inhibition of ACh hydrolysis and enhancement of muscarinic stimulation.[2]

In military medicine, PB is used as a prophylactic agent against intoxication with irreversible organophosphorus AChE inhibitors, such as the nerve agents sarin and soman[4,5] and is useful as a pretreatment to minimize the effect of nerve gas poisoning if used in conjunction with the standard treatment of atropine and pralidoximine chloride (2-PAM).[6,7]

There is little evidence of adverse effects of PB treatment in humans.[8–10] PB undergoes hydrolysis by cholinesterases. It is also metabolized by microsomal enzymes in the liver. The main metabolite of PB is 3-hydroxy-*N*-methylpyridinum (3-OH NMP) to which few references of a biological action has been made.[11] To help understand the full consequences of the biological activity of PB and its metabolites, various analytical techniques, mainly (HPLC) high performance liquid chromatography[12–15] and (GC) gas chromatography[16] have been utilised for their measurement in biological samples. Several methods have been used in the last 25 years for the analytical determination of PB and its metabolites. Biological techniques, e.g., immunoassay,[17] are among the most sensitive analytical methods, but are limited by the availability of the specific antisera and are subjected to cross reactivity from biological samples. HPLC technique, on the other hand, although not as sensitive as biological techniques, enables simultaneous screening of both PB and its metabolites. The objective of this study was the identification and quantification of PB by HPLC and its subsequent utilization in bioequivalence study on human healthy volunteers. Basic information about the type of sample used for analysis, sample preparation, chromatographic column, mobile phase, detection mode, and validation data have been extensively studied, however very little literature is available on the application of a validated and robust HPLC method to a pharmacokinetic study in humans.

EXPERIMENTAL

Materials and Reagents

Pyridostigmine bromide and clonazepam standards were gifted by Central Drugs Laboratory, Kolkata. Pyrido 60 mg tablets and Mestinon (ICN Canada Ltd.) were gifted by Shree Ganesh Pharmaceuticals Ltd., Mumbai; acetonitrile and water (both HPLC grade) were purchased from Merck India. All other reagents were of analytical grade.

Instrumentation

The HPLC system used was a Knauer, Germany, HPLC system equipped with a solvent delivery pump (K-1001), a Rheodyne injector, and an UV-visible detector (K-2501). Integration was done using Eurochrom 2000 software. Chromatographic separation was achieved on a BDS Hypersil C18, (250 $\times$ 4.6 mm, 5 μ particle size) column. Compounds were eluted up to a total retention time of 20 min, using an isocratic mobile phase consisting of acetonitrile-potassium dihydrogen phosphate (5 mM) (40:60 (v/v) at a flow rate of 1 mL/min.

Preparation of Stock Solution and Calibration Standards

Primary stock solutions of pyridostigmine bromide (1 μg/mL) were prepared in acetonitrile, and spiking standard solutions of 20, 50, 75, 100, 125, 150, 200 ng/mL were prepared by diluting the stock solution with acetonitrile. The clonazepam (IS) working stock solution was made up to 200 ng/mL in acetonitrile. All the stock solutions were stored at 4°C when not in use. Calibration standards of pyridostigmine bromide and clonazepam were prepared by spiking the appropriate amount of the stock solutions into the blank plasma obtained from healthy and non-smoking volunteers. Prepared calibration curves covered the range 20–200 ng/mL plasma.

Sample Preparation and Extraction Procedure

A 100 μL aliquot of clonazepam (200 ng/mL) stock solution was added to 1 mL of each plasma sample and vortex mixed. Acetonitrile (2 mL) was added and vortex mixed for 1 min, and centrifuged at 3000 $\times$ g for 10 min. The upper supernatant layer was transferred to another tube to which 4 mL dichloro methane was added and vortex mixed for 10 min. The organic layer was then transferred to another tube and evaporated under a stream of nitrogen gas at 55°C until completely dry. The residue was reconstituted in 500 μL of mobile phase and injected into the HPLC.

Assay Validation

Assay Specificity

Specificity was assessed by extracting samples of six different batches of blank plasma, blank plasma sample (spiked with clonazepam only), and then comparing the results for plasma spiked with clonazepam (IS) and pyridostigmine bromide that were the lowest (20 ng/mL) or the highest concentration (200 ng/mL) of pyridostigmine bromide in the calibration standard. The chromatograms were also inspected visually for interfering chromatographic peaks from endogenous substances.

Linearity

Calibration standards at seven pyridostigmine bromide concentrations (range, 20–200 ng/mL) were extracted and assayed. Least squares linear regression was used to determine the plasma concentration from peak area ratios (pyridostigmine bromide versus clonazepam).

Precision and Accuracy

The precision of the assay was determined from plasma samples of seven concentrations of pyridostigmine bromide (20–200 ng/mL). Intra-day precision was determined by repeating the analysis of standards five times in a single day, and inter-day precision and accuracy were determined by repeating analysis on five consecutive days. Sample concentrations were determined using calibration standards prepared on the same day. Assay precision was defined as the relative standard deviation (S.D.) from the mean (M), as calculated using R.S.D., % = (S.D./M) × 100. Accuracy was defined as the ratio of the mean computed value (E) to the true value (T) expressed as a percentage accuracy (%).

Recovery

The recovery of pyridostigmine bromide and clonazepam by extraction was determined by comparing peak areas in plasma sample and in acetonitrile solution samples, spiked with pyridostigmine bromide at 20 ng/mL and clonazepam at 50 ng/mL (n = 5). Recovery was defined as the ratio of the peak areas in plasma samples to those in acetonitrile solution sample for pyridostigmine bromide and clonazepam, respectively, and expressed as recovery %.

Stability

The (1) freeze/thaw; (2) short term room temperature; (3) long-term storage; (4) stock solution and (5) post-preparative stabilities were tested. To test the

stability of pyridostigmine bromide in plasma, two sets of samples with low and high concentration (20 and 200 ng/mL) were stored under different conditions. The freeze/thaw stability test was performed by freezing at −20°C for 24 h and thawing at room temperature. During each cycle, triplicate 10 μL aliquots were processed, analyzed, and the results averaged. Short term stability testing was performed at room temperature over 6 h, and long term stability was examined at −20°C over 2 weeks. The results of the freeze/thaw, and short and long term stability were compared to the average of intra-day results. To test the stock solution stability of pyridostigmine bromide and IS, stock standard (pyridostigmine, 1000 ng/mL) and IS (clonazepam, 200 ng/mL) solutions were left at room temperature for 6 h. Post operative stability was performed by comparing after intra-day analysis to the first intra-day analysis.

Bioavailability Study Design

Subjects

The Drugs Controller General of India (DCGI) and the Institutional Ethics Committee (IEC) on human studies at Jadavpur University, approved the present bioavailability study. Twelve healthy volunteers aged 19–30 years and weight 50–81 kg were enrolled in this study. All volunteers gave written informed consent before entering in the study.

Based on medical history, clinical examinations, and laboratory tests, which included hematology, blood biochemistry, and urine analysis, no subject had a history or evidence of hepatic, renal, gastrointestinal, or hematological deviations, or any acute or chronic disease or drug allergy. None of the volunteers were taking any prescribed or investigational medication during the 4 weeks preceding the enrollment. None of the volunteers was a smoker or had a history of alcohol consumption.

Study Design

This was a balanced, randomized, double-blind, single oral dose, crossover study. After the screening visit, each eligible volunteer entered into a randomized schedule to either receive 60 mg of reference formulation (ICN Canada Ltd., Canada) or pyridostigmine bromide 60 mg (Shree Ganesh Pharmaceuticals, Mumbai, India) after an overnight fast. All subjects were admitted in the clinical research ward in the evening prior to the dosing day, and remained in the ward until 24 hours post dosing.

Treatment and Dosage

Our analytical method was applied to a pharmacokinetic study in which the pyridostigmine bromide concentration was measured in 12 human volunteers. After

an overnight fast (more than 10 h) each subject received a single dose of 60 mg pyridostigmine bromide, either reference or test formulation, according to the prior randomization schedule, orally with 240 mL of drinking water. A standard breakfast, lunch, and dinner was served to all subjects at 3, 6, and 12 hours, respectively, after drug administration. Subjects were not permitted to smoke, consume alcohol or caffeine containing beverages 12 hours before dosing and throughout their stay in the unit. Vital signs like pulse rate, blood pressure, respiratory rate, and temperature were recorded before drug administration.

Blood Sampling and Analytical Method

Approximately, 10 mL blood samples were drawn into Vacutainer™ tubes containing EDTA from a forearm vein, using an indwelling catheter or by direct veinpuncture before dosing (0 h), and then at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 6.0, 8.0, 10.0, 12.0, and 24.0 after dosing. Collected blood samples were centrifuged immediately, plasma was separated and stored frozen at $-20^{\circ}C$ with appropriate labeling of volunteer code no., study date, and collection time, till the date of analysis.

Abnormal signs/symptoms were monitored, during the study period and for one week after the study period and, if noticed, their details were entered in the case report sheets and tabulated at the end of the study. On the study days volunteers were permitted normal activities, excluding strenuous exercise.

The instrumentation and chromatographic conditions employed for analysis are described above. The pharmacokinetic parameters of pyridostigmine bromide were performed using non-compartmental pharmacokinetic methods with the WinNolin software package (Pharsight Coporation, California). The non-compartmental analysis was performed using standard methods for each subject. The area under the plasma concentration time curve (AUC) was calculated using the trapezoidal rule and extrapolated to infinity. Total clearance (CL_{total}) was divided by AUC_{∞}. The elimination half-life ($t_{1/2}$) was calculated using Equation (1).

$$t_{1/2} = \frac{0.693}{k_e} \tag{1}$$

All data were expressed as means $\pm$ standard deviation.

RESULTS AND DISCUSSION

Specificity

The method selectivity was demonstrated on twelve blank plasma samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks. Pyridostigmine and the internal standard were well

resolved, with retention times of 3.668 and 8.453 min, respectively. A typical chromatogram of blank plasma and the chromatogram of a plasma sample are shown in Figures 1A and 1B.

Linearity and Limit of Quantitation

The calibration curves were linear in the range studied. The calibration curve equation took the form $y = bx + c$, where y is the ratio of pyridostigmine bromide to clonazepam peak areas and x concentration of the pyridostigmine bromide. The proposed assay is linear in the range of 20.0 ng/mL to 200 ng/mL. The mean equation (curve coefficients $\pm$ S.D.) for calibration curve ($n = 6$), obtained from seven points, was $y = 0.0045\ (\pm 0.0004)x + 0.0172(\pm 0.007)$ (correlation coefficient, $r = 0.9975$). The limit of quantitation was 20 ng/mL ($n = 6$).

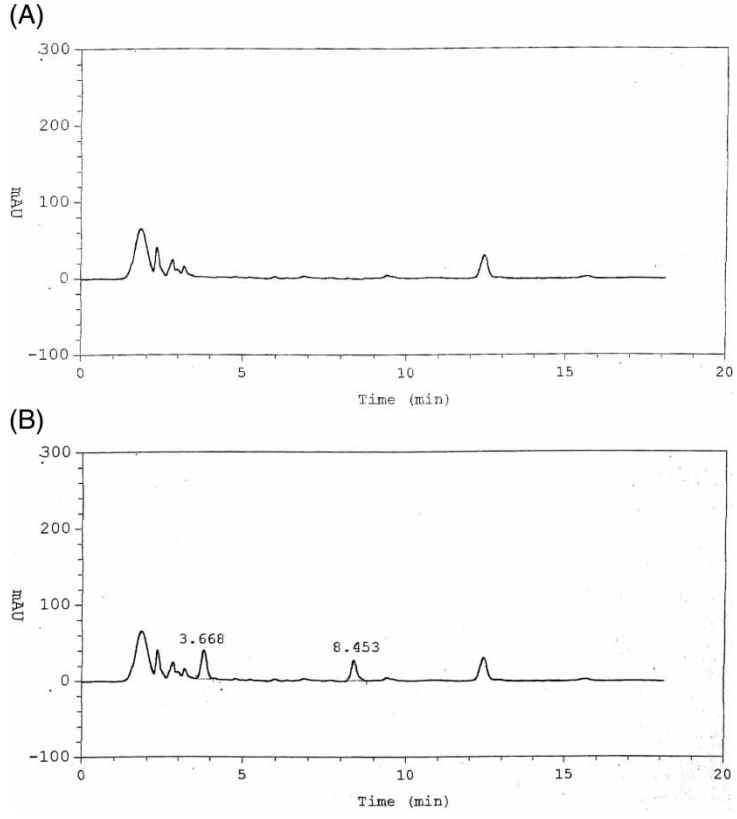

Figure 1. Chromatograms of (A) Blank plasma; (B) Plasma from healthy volunteers after administration of pyridostigmine bromide, 60 mg.

Accuracy and Precision

The intra-assay accuracy and precision of the method are illustrated in Table 1 and were found to be satisfactory for our purposes. Intra-day precision (R.S.D.%) was less than 15% with results ranging from 0.543 to 0.818%. Inter day precision was also less than 15% with results ranging from 0.789 to 3.937%. Accuracy was within 85–115% at all concentrations investigated. These results show that the developed method has good precision and accuracy.

Recovery and Stability

The recovery (%) (mean ± S.D.) of pyridostigmine and clonazepam was 88.6 ± 1.01% and 85.2 ± 0.66%, respectively. The stability experiments were aimed at testing all possible conditions that the samples might be exposed to during sample shipping and handling. No significant degradation of pyridostigmine bromide and clonazepam were observed. Three freeze/thaw cycles and 6 h room temperature storage had no substantial effect on the results. The samples were stable at room temperature for at least 2 weeks. Standard solutions of pyridostigmine bromide and clonazepam were analyzed using HPLC and then stored at room temperature for 6 h; the samples were analyzed to compare with assayed sample before 6 h and the difference (%) in values in stock solution stability were 1.06% and −0.49% for pyridostigmine bromide and clonazepam (IS), respectively. The post-preparative samples were stable at room temperature for at least 5 days.

Application to Pharmacokinetic Study

Based on our validation results, we used this method to determine the plasma concentrations of pyridostigmine bromide in an open, balanced,

Table 1. Intra-day and inter-day precision and accuracy for pyridostigmine bromide analyzed using HPLC-UV method

	Precision (R.S.D., %)		Accuracy (%)	
Concentration (ng/mL)	Intra-day (n = 5)	Inter-day (n = 5)	Intra-day (n = 5)	Inter-day (n = 5)
QCL 50	0.818	3.937	91.364	87.953
QCM 100	0.539	1.825	94.852	93.158
QCH 180	0.543	0.789	96.324	95.50

Table 2. Pharmacokinetic parameters of pyridostigmine bromide after oral administration of 60 mg test and reference tablet

Pharmacokinetic parameters	Reference preparation	Test preparation
C_{max} (ng/mL)	181.998 ± 11.211	176.03 ± 9.176
t_{max} (hr)	1.458 ± 0.0396	1.542 ± 0.396
AUC_{0-24} (ng · hr/mL)	834.513 ± 90.175	819.999 ± 109.64
$AUC_{0-\infty}$ (ng · hr/mL)	933.697 ± 91.862	909.86 ± 110.84
k_{el} (hr^{-1})	0.184 ± 0.006	0.183 ± 0.004
$t_{1/2}$ (hr)	3.764 ± 0.117	3.787 ± 0.08
Relative bioavailability (%)	100%	98.26%

randomized, pharmacokinetic study of 24 healthy volunteers to assess the bioavailability of 60 mg of pyridostigmine bromide after a single oral dose. The limit of quantitation of pyridostigmine bromide allowed the plasma concentration to be followed for up to 24 hr after drug administration. The pharmacokinetic parameters of pyridostigmine bromide were calculated using WinNolin software package (Pharsight Inc.). The calculated parameters are given in Table 2. Figure 2 shows the mean of the plasma concentration time curve following oral administration of 60 mg of pyridostigmine bromide.

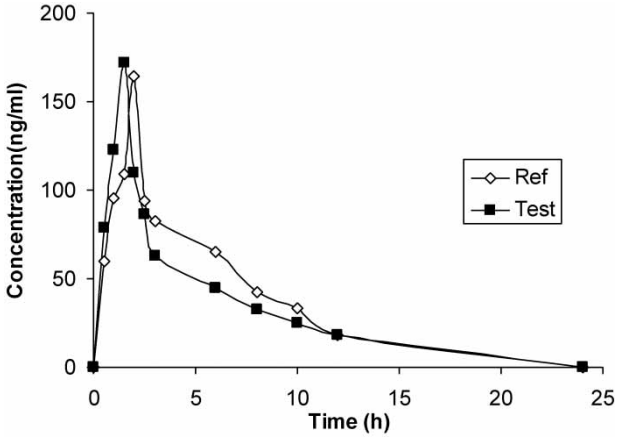

Figure 2. Plasma concentration – time curves after administration of test and reference formulations of pyridostigmine bromide, 60 mg. ◇ = Reference formulation (Mestinon) of ICN Canada Ltd., Canada and ■ = Test formulation (Pyrido) manufactured by Shree Ganesh Pharmaceuticals, India.

CONCLUSIONS

A simple, easy, and sensitive HPLC method was developed to determine pyridostigmine bromide in human plasma. The method was validated as per the standard guidelines and was found to be reproducible. This method was successfully used to determine the pharmacokinetics of pyridostigmine bromide after a single oral dose administration in human volunteers.

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