PHARMACOKINETIC AND PHARMACODYNAMIC STUDY OF PYRIDOSTIGMINE IN CONGESTIVE HEART FAILURE

By

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PHARMACOKINETIC AND PHARMACODYNAMIC STUDY OF PYRIDOSTIGMINE IN CONGESTIVE HEART FAILURE

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Abstract: Sympathetic overactivity and parasympathetic withdrawal indicate profound dysregulation of autonomic control in patients with congestive heart failure. Pyridostigmine binds to the acetylcholinesterase enzyme and inhibits its action on acetylcholine. Thus, pyridostigmine may contribute to restoring the balance between the sympathetic and parasympathetic arms of the autonomic system in the heart. We hypothesized that pyridostigmine increases parasympathetic tone and thus improves autonomic balance in the heart. In the first aim, a rodent model of heart rate recovery (HRR) was developed to test the hypothesis that subacute pyridostigmine administration enhances HRR in rats. Rapid heart rate deceleration after exercise or HRR is associated with the activation of parasympathetic tone. Male Sprague-Dawley rats treated with pyridostigmine (0.14 mg/ml/day in the drinking water) showed a significant decrease in acetylcholinesterase activity in plasma and red blood cells (RBCs) ($P<0.001$), whereas plasma butyrylcholinesterase activity did not significantly change ($P=0.99$). HRR recorded 1 min after the end of exercise was higher in the pyridostigmine-treated group as compared with the control group ($P=0.002$). The parasympathetic tone was higher in the pyridostigmine-treated rats as compared with control ($P<0.001$) indicating that pyridostigmine enhanced parasympathetic tone and associated HRR in rats. Under the second aim, analytical methods were developed to quantify pyridostigmine and its metabolite 3-hydroxy-N-methylpyridinium in human plasma using sensitive hydrophilic interaction liquid chromatography–electrospray ionization–tandem mass spectrometry assays. Accuracy and precision values for each assay were within the acceptable limits described in FDA guidelines. In the final aim of the study, a population-based pharmacokinetic model was used to compare two types of structural base models after oral pyridostigmine administration for ten weeks. The two-compartment model was determined to best fit the pyridostigmine plasma data and parameter estimates for pyridostigmine were reported. In conclusion, subacute pyridostigmine administration to rats enhanced HRR by increasing cardiac parasympathetic tone, making this rodent model an appropriate tool for further testing of the effects of pyridostigmine of autonomic tone. A sensitive method was developed to quantify pyridostigmine and its metabolite 3-hyroxy-N-methylpyridinium in human plasma. Lastly, a population-based pharmacokinetic model estimated the variability in the pharmacokinetic parameters of pyridostigmine in heart failure patient population.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

CHAPTER V

LIST OF FIGURES

Figure Page

CHAPTER II

CHAPTER III

CHAPTER I

INTRODUCTION

The prevalence of heart failure has increased over the years, from 4.9 million people in the United States suffering from heart failure in 2001 [1] to 5.7 million in 2013 [2], and it continues to be one of the leading causes of sudden death in cardiovascular disease patients. Sudden cardiac death is a major concern in these patients with an estimated annual incidence ranging from 184,000 to more than 400,000 per year [3]. Neuroendocrine dysregulation is considered to be an important determinant of mortality and morbidity in patients with congestive heart failure. Sympathetic overactivity concurrent with parasympathetic withdrawal indicates profound dysregulation of the autonomic control of the heart. Heart failure is treated pharmacologically, with one goal being to decrease mortality by improving sympathovagal balance. Several conditions such as coronary artery disease, myocardial infarction, abnormal heart valves, diabetes, severe lung disease, obesity, and high blood pressure can lead to heart failure [4]. Heart failure occurs due to the irreversible damage caused to the heart over time. However, successful treatment strategies can be applied to manage this condition. Treatment plans include changes in lifestyle, medications and, if required, devices and surgical procedures. These treatment procedures target specific pathophysiological alterations that occur during heart failure. For example, one recommended lifestyle change for heart failure patients is to decrease sodium intake, so that less water retention occurs in the body, thereby decreasing circulatory volume and the resulting load on the heart and vessels. A second common therapeutic goal is the administration of beta blockers to counter excessive, chronic sympathetic stimulation of the heart. The administration of angiotensin converting enzyme inhibitors reduces blood pressure and circulating volume, thus decreasing preload and afterload on the heart. However, current therapeutic approaches do not offset the withdrawal of parasympathetic activity that is also an important event in heart failure. Acetylcholinesterase inhibitors such as pyridostigmine may contribute in enhancing the parasympathetic tone and normalize autonomic balance in heart. Acetylcholinesterase inhibitors may be used to augment parasympathetic neurotransmission by blocking the enzymatic breakdown of acetylcholine at cholinergic receptor sites.

In this dissertation, we focus on the administration of an indirect parasympathomimetic drug, pyridostigmine. Pyridostigmine is a carbamate, short-acting acetylcholinesterase inhibitor approved by the FDA for the treatment of myasthenia gravis. We studied the effects of pyridostigmine on heart rate recovery in exercising rats. We also determined the pharmacokinetic behavior of pyridostigmine in human congestive heart failure patients. Population pharmacokinetic modeling was performed to study the pharmacokinetic effects of the drug in a congestive heart failure patient population.

The overall hypothesis of this dissertation was that pyridostigmine administration improves autonomic regulation during congestive heart failure, as predicted by pharmacokinetic parameters and their associated covariates. Our objectives were to determine the effects of subacute exposure of pyridostigmine on heart rate recovery in rats and to develop a population pharmacokinetic model to describe the variability in the pharmacokinetic behavior of pyridostigmine in human congestive heart failure patients.

Heart rate recovery is a measure of reactivation of the parasympathetic nervous system and is considered to be an index of cardiovascular fitness in people. Acetylcholinesterase inhibitors, such as pyridostigmine, can enhance parasympathetic tone in the heart and possibly HRR in CHF patients. To our knowledge, a study validating the use of HRR to assess autonomic tone in rats has not been reported previously. We hypothesize that HRR can be used to assess autonomic tone in rats and that subacute administration of pyridostigmine in rats will enhance parasympathetic tone and HRR after exercise. To test this hypothesis, our objectives were arrived at as follows:

The validation of pyridostigmine as a modulator of HRR in rats will be useful in future studies that will benefit from the availability of a rodent model to assess pharmacological effects on parasympathetic tone. To that end, our first objective was:

AIM 1: To validate the use of HRR to assess autonomic tone in rats and to determine the subacute effects of pyridostigmine on HRR in rats

In order to examine the disposition of pyridostigmine in heart failure patients, an analytical method was required to quantify the levels of pyridostigmine and its metabolite, 3-OH NMP, in human plasma. Therefore, our second objective was:

AIM 2: To quantify the plasma concentrations of pyridostigmine and its metabolite, 3-OH NMP, by developing a highly sensitive and specific assay

The pyridostigmine plasma data generated under the second aim was used to develop a population pharmacokinetic model. We hypothesized that population pharmacokinetic modeling would explain the variability in the dose-response effect amongst the CHF patient population and could be used to estimate pharmacokinetic parameters of pyridostigmine in patients with CH. This led us to our third objective:

AIM 3: To develop a population-based pharmacokinetic model characterizing the pharmacokinetic (PK) properties of repeated oral dosing of pyridostigmine in patients with CHF and estimate variability in the resulting pharmacokinetic parameters.

Our overall hypotheses and specific aims to test that hypotheses integrated several diverse fields of science, such as pharmacology, exercise physiology, analytical chemistry, and pharmacokinetic modeling. By utilizing and integrating the knowledge from these different areas of investigation, we were able to develop a heart rate recovery exercise model in rats. Moreover, we developed analytical methods to quantify pyridostigmine and its metabolite 3 hydroxy N-methyl pyridinium in human plasma. We also developed a population-based pharmacokinetic model of pyridostigmine in CHF patients.

In this dissertation, the studies were organized into six chapters. Chapter I introduces the topic of the dissertation and presents the specific aims and hypotheses of the study. In chapter II the current knowledge and findings, as well as theoretical and methodological contributions to the dissertation topic are discussed as 'Literature Review.' 'Subacute pyridostigmine exposure increases heart rate recovery and cardiac parasympathetic tone in rats' was presented in chapter III. In chapter IV, the analytical methods were described, 'Use of hydrophilic interaction liquid chromatography–tandem mass spectrometry for the quantification of pyridostigmine and its metabolite, 3 hydroxy N-methylpyridinium in human plasma'. In Chapter V, 'Population pharmacokinetic analysis of pyridostigmine in congestive heart failure patients' is discussed. Finally, in chapter VI, the topic is summarized and concluded by presenting the impactful findings of the study.

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CHAPTER II

REVIEW OF LITERATURE

1. Congestive Heart Failure

The 2016 Heart Disease and Stroke Statistical Update by the American Heart Association (AHA), in conjunction with the Centers for Disease Control and Prevention, the National Institutes of Health, and other government agencies [1] reported that in 2013, 1 in 9 death certificates in the United States mentioned heart failure. Data from the Framingham Heart Study [2] and the Olmsted County Study [3] shows that survival after diagnosis of heart failure has improved over time. However, the death rate is still high with approximately 50% of people dying within five years after diagnosis with CHF [3]. Data from NHANES 2009 to 2012 in the United States show an estimated 5.7 million people of more than 20 years of age have CHF (Figure 1). Projections show that the prevalence of CHF will increase from 2012 to 2030 by 46% [4] (Figure 2). According to the census from 2005, in the US, approximately 5 million cases of CHF per year are reported. Elderly patients above the age of 65 present the majority of cases of CHF [5].

Figure 1: Prevalence of heart failure by sex and age as reported by National health and nutritional examination survey: 2009-2012. Sources: National Center for Health Statistics and National Heart, Lung, and Blood Institute. This figure is adapted from 2015 Heart Disease and Stroke Statistical Update by American Heart Association (AHA) [6].

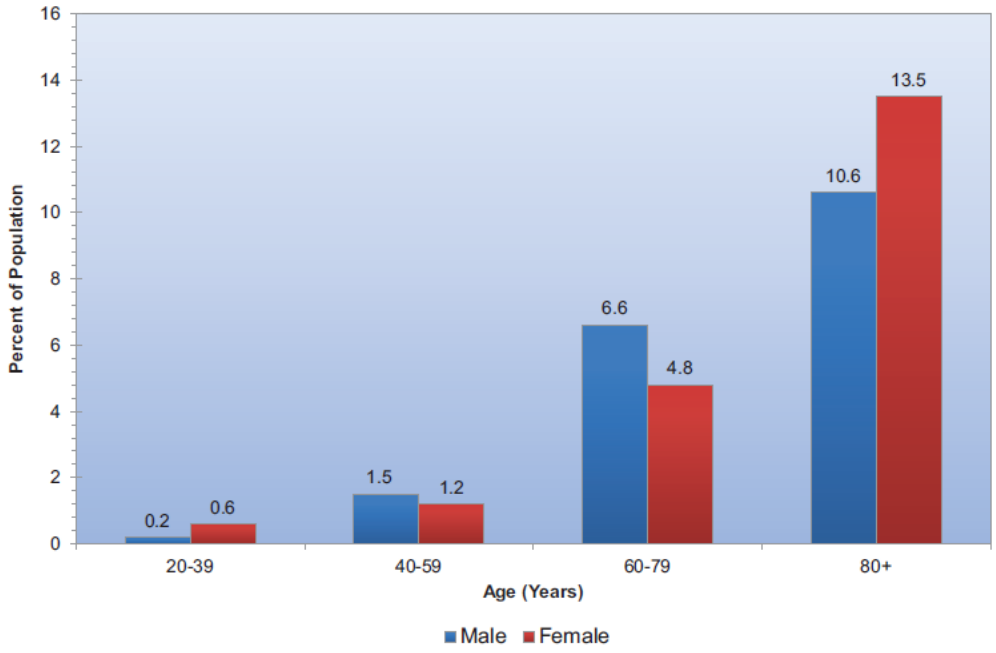
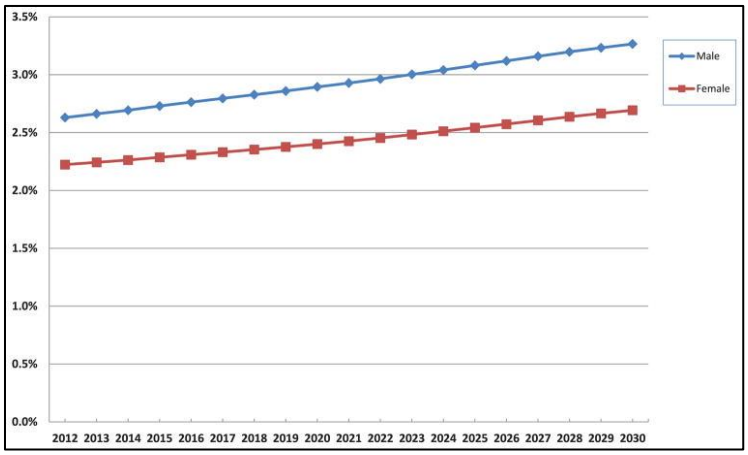


Figure 2: Projected prevalence of HF from 2012 to 2030 is shown for men and women in the United States. The prevalence of HF remains highest among men throughout the period, although it increases among both groups over time. HF indicates heart failure. This figure is adapted from a manuscript by Paul A. Heidenreich, entitled 'Forecasting the Impact of Heart Failure in the United States: A Policy Statement from the American Heart Association' [4].



Congestive heart failure (CHF) can be defined as "the pathophysiologic state in which, because of an abnormality of cardiac function, the heart fails to pump the blood at a rate commensurate with the requirements of the metabolizing tissues, or else does so only with an elevated filling pressure" [7]. Heart failure is often a long-term (chronic) condition, but it may also occur suddenly.

There are two major categories of heart failure: systolic and diastolic heart failure. In systolic heart failure, the heart muscle cannot pump the blood effectively, whereas, in diastolic heart failure, the heart muscle becomes stiff and does not relax sufficiently to fill with blood, resulting in a decreased ejection volume.

Abnormalities such as pressure and volume overload, loss of cardiac muscle, excessive peripheral demands, etc. may cause CHF [8]. In developed countries, ventricular dysfunction is more prevalent and occurs mainly due to myocardial infarction and hypertension. Other causes of heart failure include degenerative valve disease, idiopathic cardiomyopathy, and alcoholic cardiomyopathy [9].

Clinical signs and symptoms

Symptoms of CHF can be of slow or sudden onset. Initially, symptoms may occur only during maximum activity. Eventually, patients may notice breathing problems even when resting. Common symptoms of CHF are cough, tiredness, weakness, fainting, loss of appetite, the urge to urinate during the night, irregular pulse, short breath, bloating, swelling of extremities and internal organs and weight gain [10].

Pathophysiology

Disturbance in myocardial contraction leads to decreased cardiac output. To improve the cardiac output, several compensatory mechanisms including Frank-Starling relationship, sympathetic nervous system and the renin-angiotensin-aldosterone system (RAAS) activation, secretion of arginine vasopressin, and the development of myocardial hypertrophy are utilized by the cardiovascular system. These mechanisms are effective in preserving cardiac output, supporting systemic blood pressure, and ensuring adequate cerebral perfusion in the short term. However, the persistence of such pathophysiologic alterations has long-term deleterious effects on cardiac function [11]. In the usual form of heart failure, contractility of the heart muscle is decreased which produces a reduction in cardiac output, and then the heart becomes unable to

meet the peripheral demands of the body. Disturbances in cardiac output alter the following four primary determinants of left ventricular (LV) performance:

(1) Decrease in cardiac muscle contractility.

(2) Increase in preload or left atrial filling pressure causes congestion and dyspnea.

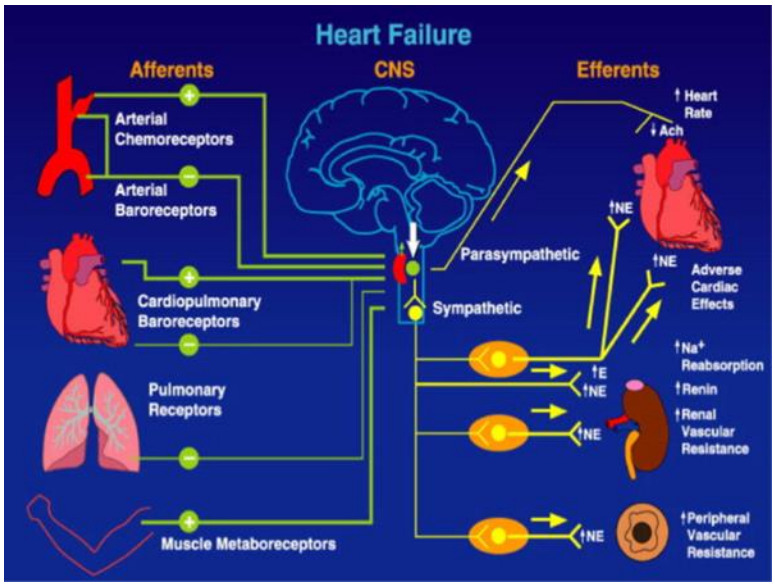
(3) The increase in afterload or systemic vascular resistance causes hypertensive conditions.

(4) The increase in heart rate as part of the compensatory mechanism produces an increase in sympathetic tone and circulating catecholamines [8].

Neurohormonal activation

CHF associates with alterations in the nervous and endocrine systems. Under normal physiological conditions, these neurohormonal compensatory mechanisms provide valuable support for the heart. However, in CHF conditions, they play a role in the development and subsequent progression of the disease. These neurohormonal alterations include stimulation of the RAAS, increase in the activity of the sympathetic nervous system, release of natriuretic peptides, increase in the levels of antidiuretic hormone vasopressin, the release of endothelin, and withdrawal of cardiac parasympathetic activity (Figure 3) [12]. In our research, we focus on pharmacological management of the changes that occur in the parasympathetic control of the heart during CHF.

Figure 3: Neurohormonal alterations occurring during CHF. This figure was adapted from a published article by Floras, J.S., 'Sympathetic nervous system activation in human heart failure: clinical implications of an updated model.' J Am Coll Cardiol, 2009. Ach = acetylcholine; CNS = central nervous system; E = epinephrine [18, 19].



Management

The therapy of CHF is mostly directed at restoring normal cardiopulmonary physiology and reducing the hyper-adrenergic state. Dietary management of CHF includes less salt intake to control fluid retention [13, 14]. Most patients also need diuretic therapy. Vasodilators like nitrates have also been used to reduce the vasoconstriction caused by the over-activation of the sympathetic nervous system [15]. These management approaches improve preload. Decreasing the activity of the RAAS by administering angiotensin-converting-enzyme (ACE) inhibitors reduces afterload. Another approach in the management of heart failure is to improve the

contractility of cardiac muscle with a positive inotrope, like digoxin, and controlling the activity of beta-receptors with beta blockers like atenolol and propranolol. Phosphodiesterase inhibitors also increase contractility of the heart and cause peripheral vasodilation [11]. In some cases, life-supporting devices such as a defibrillator or pacemaker may be implanted.

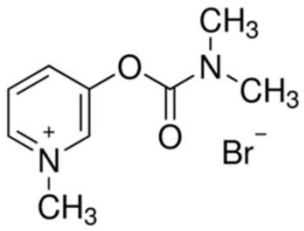
Recent efforts to treat CHF have focused on specific treatment modalities, such as spinal cord stimulation, electrical vagus nerve stimulation [16], activation of baroreceptors, and denervation renal sympathetic nerve [17]. One such emerging treatment modality is the reactivation of the parasympathetic nervous control of the heart, which is withdrawn in CHF.

2. Pyridostigmine Bromide

Chemical Structure and Mechanism of Action

Pyridostigmine (PYR) is a positively charged quaternary ammonium carbamate compound (Figure 4). PYR carbamylates the active site serine residue of acetylcholinesterase (AChE) which prevents acetylcholine (ACh) from binding to these same sites and being hydrolyzed by the enzyme. AChE inhibition thus leads to a buildup of Ach in the neuromuscular junction (NMJ) or cholinergic synapse, resulting in an increased/prolonged action of ACh at cholinergic receptors. Pyridostigmine also has additional actions, such as direct binding and stimulation of ACh receptors [20]. Administration of pyridostigmine (25 mg/kg/day) for a prolonged period can downregulate acetylcholine receptors and lead to neuromuscular dysfunction, with or without receptor changes [21].

Figure 4: Structure of pyridostigmine bromide (This figure was adapted from sigmaldrich.com, http://www.sigmaaldrich.com/catalog/substance/pyridostigminebromide2611210126811?lang=en &region=US)



Effects of pyridostigmine occur via the action of ACh at the nicotinic and muscarinic cholinergic receptors in the skeletal NMJ, autonomic end-organs of the sympathetic and parasympathetic nervous systems, autonomic ganglia, and the central nervous system. Effects of enhanced nicotinic receptor stimulation can include changes in muscle tone, altered cognitive functions, modulation of reward, arousal and motor control, and analgesia [22]. Similarly, the action of ACh at muscarinic receptors in smooth muscles, glands, and the heart is affected by pyridostigmine. These muscarinic effects can include spasm of smooth muscles, increased glandular secretions, and slow of the heart rate [23]. ACh activity is mostly regulated by the enzyme acetylcholinesterase (also called "red blood cell cholinesterase" or "RBC cholinesterase" or "true cholinesterase" and abbreviated as "AChE"). ACh binds and is rapidly inactivated by AChE in cholinergic synapses. This hydrolytic breakdown of ACh prevents inappropriate, excessive signaling at the postsynaptic cholinergic terminal. Butyrylcholinesterase (also called "plasma cholinesterase" or "pseudocholinesterase," and abbreviated as "BChE") plays a less critical role. BChE is made in the liver and secreted into the circulation, but is found in most tissues throughout the body [24].

ADME

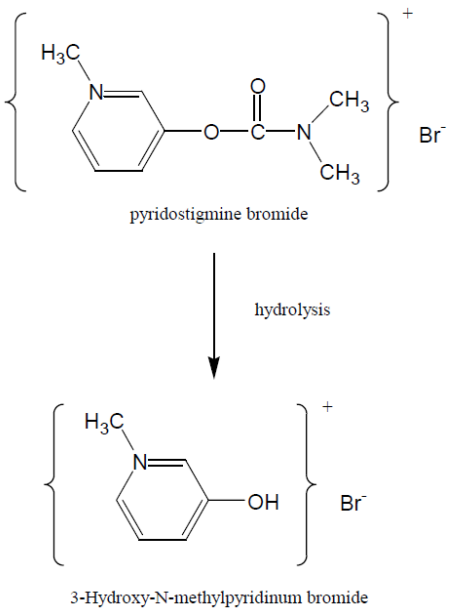
Oral bioavailability of pyridostigmine was reported to be 7.6 ± 2.4%, indicating that pyridostigmine is poorly absorbed from the GI tract [25]. The charged quaternary ammonium leads to high water solubility, and it is mostly absorbed in the duodenum, but overall it is poorly absorbed from the gastrointestinal tract [26]. PYR is rapidly excreted by glomerular filtration and active tubular secretion by the kidneys. In a study performed in healthy controls and myasthenia gravis patients, peak pyridostigmine concentrations were reached between 1 ½ to 2 hr after oral administration of a 60 mg dose and the time of peak concentration was similar between the groups [27].

In healthy subjects in which 2.5 mg of pyridostigmine was administered IV, the plasma elimination half-life was 1.52 hr, the volume of distribution (Vd) was 1.43 L/kg, and plasma clearance was 0.65 L/kg*hr. The pharmacokinetic parameters obtained after oral administration of 120 mg tablet of pyridostigmine were slightly different from that reported after IV dose; elimination half-life was 1.78 ± 0.24 hr, the Vd was 1.64 ± 0.29 L/kg, and the plasma clearance was 0.66 ± 0.22 L/kg*hr. A greater than the seven-fold difference in steady state pyridostigmine plasma concentrations was found between myasthenia gravis patients taking similar daily doses. However, the pharmacokinetic data from the healthy subjects was not able to explain these higher differences in the steady state pyridostigmine plasma concentrations [25].

In humans and other animal species, such as dogs and rats, pyridostigmine is mainly metabolized to 3-hydroxy-N-methylpyridinium (3-OH NMP) (Figure 5), which is excreted in urine along with the unchanged drug [29]. This metabolite does not contribute to antagonism of

neuromuscular blockade in dogs [30]. The drug and the metabolite are not bound to protein. Both blood cholinesterases and microsomal enzymes in liver play an important role in the metabolism of pyridostigmine [31]. In another study, the elimination of unchanged drug in rats remained constant for different levels of doses when administered via the portal vein. However, the percentage of the metabolite eliminated decreased with the increase in the level of doses suggesting a zero-order elimination for pyridostigmine and a dose-dependent process (first order kinetics) for the elimination of 3-OH NMP [29].

Figure 5: Metabolic pathway of pyridostigmine bromide. This figure was adapted from a review article by Zhao B et al., 2006, 'Determination of pyridostigmine bromide and its metabolites in biological samples' [28].



Indications

Pyridostigmine bromide is one of the main drugs for the treatment of myasthenia gravis. [32]. In military personnel, pyridostigmine is used as a prophylactic agent against the nerve agents such as sarin and soman, which lead to very long-term inhibition of AChE [34]. The protective property of pyridostigmine is due to its ability to shield the active site of AChE for a short time, to prevent the much longer inhibition elicited by the nerve agents. Pyridostigmine may, therefore, be useful as a pre-treatment to minimize the effect of organophosphate nerve agent intoxication, especially if used in conjunction with the standard antidotes, atropine and pralidoxime chloride (2-PAM) [35, 36]. The quaternary nature of pyridostigmine impedes its passage across the blood–brain barrier to limit central nervous system effects [37].

3. Reactivation of parasympathetic function

Pyridostigmine is an indirect parasympathomimetic drug which enhances parasympathetic tone by covalently binding to AChE, thus inhibiting the hydrolysis of ACh molecules in the synapse. This concept is supported by many human and animal studies indicating that pyridostigmine enhances parasympathetic tone [38-43]. Recently, Lataro et al. administered pyridostigmine in the drinking water (four weeks at 0.14 mg/mL) to rats with experimental heart failure and reported that PYR enhanced cardiac vagal tone and reduced sympathetic tone [42]. Similarly, Sabino et al. administered pyridostigmine to heart failure rats at this same dose rate. Pyridostigmine was efficient at preventing the attenuation of baroreflex sensitivity, which is a typical outcome of heart failure. Besides, pyridostigmine treatment avoided changes in iHR (intrinsic heart rate) and autonomic cardiovascular control, as examined by

arterial pressure variability and cardiac parasympathetic tone [44]. The same group also found that pyridostigmine prevented increased peripheral chemoreflex sensitivity and partially blunted central chemoreflex sensitivity, but did not affect basal pulmonary ventilation in HF rats [45].

It has been previously reported that pyridostigmine improved tolerance to peak exercise and inhibited the chronotropic response to submaximal exercise in human patients with exercise-induced angina [46]. These results suggest that pyridostigmine can provide protection against exercise-induced myocardial ischemia. In another study in human coronary artery disease patients, pyridostigmine reduced QTc interval [39]. These results indicate that oral pyridostigmine produces tonic cardiac cholinergic stimulation while exerting no effect on its reflex changes. Further studies are needed to understand the potential role of pyridostigmine on the prognosis of patients with acute myocardial infarction [47].

4. Heart rate recovery

Cardiac autonomic function can be assessed in healthy subjects or CHF patients by using various tools, including the chronotropic response of the heart to physiological stress and pharmacological blockade [48,49], heart rate variability (HRV) [50,51], quantification of baroreflex sensitivity [52], plasma or coronary sinus catecholamine levels [53], HR turbulence [53], and others. Heart rate recovery (HRR) is the rate at which heart rate decelerates after moderate to heavy exercise and is dependent on the relative changes between the parasympathetic and sympathetic nervous activities of the autonomic nervous system [54]. The analysis of beat by beat heart rate decay after exercise may have the potential to deduce an indicator of vagally mediated heart rate recovery, providing fine time resolution [55].

Parasympathetic activation and HRR

Intrinsic activity of the sinus node, in conjunction with the parasympathetic and sympathetic system, determines the heart rate. At the beginning of the exercise, the increase in heart rate is mediated by the withdrawal of the parasympathetic system. Activation of sympathetic system is responsible for maintaining exercise tachycardia with the vigorous levels of exercise. When exercise is stopped, reactivation of parasympathetic system and withdrawal of sympathetic system contributes to the recovery of heart rate. However, the relative contribution of these components of the autonomic system during recovery phase was not completely elucidated.

Savin et al. postulated that in healthy subject sympathetic withdrawal contributes more to the HRR soon after peak exercise, while parasympathetic reactivation plays a greater role later in recovery [56]. In a subsequent similar study, Imai et al. found that HRR was accelerated in trained athletes but blunted in patients with heart failure [55]. They concluded that immediately after exercise the decrease in heart rate is primarily due to parasympathetic reactivation. The increase in plasma norepinephrine levels at about 1 minute after high-level of exercise indicates that sympathetic activity remains high during the early recovery phases [57]. A higher level of sympathetic activity during early phases of recovery implies that the fall in heart rate that occurs during this period is highly dependent on parasympathetic reactivation.

Savin et al. attempted to assess the contribution of the autonomic nervous system to heart rate recovery following exertion by parasympathetic blockade with atropine sulfate, sympathetic blockade with propranolol hydrochloride, double blockade with both drugs, all compared to responses with no drug administration [56]. Results suggested that heart rate recovery occurs in

an exponential manner. In the same study, the authors suggested that deceleration of heart rate could be an intrinsic property of the heart because it occurred under each experimental condition. These authors proposed that the contribution of sympathetic withdrawal to heart rate recovery soon after peak exercise is higher and parasympathetic activation plays a major role later in recovery [56].

However, the above results were contradicted by Perini et al. when he studied the time course of heart rate and venous blood epinephrine concentration as an expression of sympathetic nervous system activity during recovery from periodic exercise cycles [58]. His results suggested that in the first minute of recovery, independent of exercise intensity, the recovery was mainly due to the restoration of vagal tone. The further decrease in heart rate was attributed to the return of sympathetic nervous activity to pre-exercise level [58].

Congestive heart failure and HRR

On comparing the heart rate recovery between two different physiological groups, it was found that HRR is blunted in chronic heart failure patients and accelerated in trained athletes. This difference could exist because the parasympathetic activity is altered in CHF patients [48, 59].

Heart rate recovery as a predictor of mortality

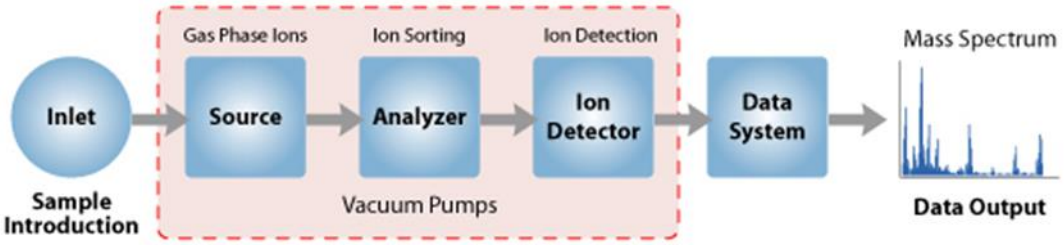
Delayed heart rate recovery can be an important prognostic marker in patients with CHF. Cole et al. performed a cohort study in patients without evidence of cardiovascular disease [60]. HRR, measured one minute after termination of exercise, of 12 bpm or less after peak exercise was defined as abnormal (26% of patients in the study) and a delayed decrease in HR after graded

exercise was regarded as a powerful predictor of overall mortality [61]. Nishime EO et al. recommended HRR as a better technique than treadmill exercise scoring for routine incorporation into exercise test interpretation [62]. In the cohort study performed by Watanabe J, an abnormal HRR was defined as ≤18 bpm [63]. An abnormal heart rate recovery was able to predict mortality whether or not left ventricular systolic dysfunction was present [63]. Dimkpa U has also discussed the physiological implications and clinical and practical applications of HRR [64]. Dimkpa U associated HRR to maximum oxygen uptake, endurance capacity and central hemodynamic variables like resting HR and resting blood pressure [64].

5. Tandem mass spectrometry

Mass spectrometry generates ions from either inorganic or organic compounds by a suitable method. Then the mass spectrometer separates these ions according to their mass-to-charge ratio (m/z) to identify and quantify their abundance (Figure 6). Different methods of ionization can be used such as thermal, by electric fields, or by impacting energetic electrons, ions, or photons on the analytes. Ion separation is effected by dynamic electric or magnetic fields [65]. There are different types of mass analyzers available such as time of flight (TOF), quadruple, ion traps, and Fourier transform (FT) resonance detectors. The ion sources can be matrix-assisted laser desorption/ionization (MALDI), electrospray, and plasma fields (ICP, APCI).

Figure 6: Components of a mass spectrometer. This figure was adapted from the webpage of Premier Biosoft (http://www.premierbiosoft.com/tech_notes/mass-spectrometry.html).



Principle of tandem mass spectrometry

As described above, a mass spectrometer works on the principle of ionization and detection. However, in a tandem mass spectrometer, there are two mass spectrometers linked end to end. In the first mass spectrometer, the parent ion is selected, and then it is fragmented into several daughter ions by bombardment of electrons on the molecules in the ionization chamber. These daughter ions can then be detected or measured in the second mass spectrometer.

Uses and advantages

Liquid chromatography-tandem mass spectrometry (LC/MS/MS) provides superior sensitivity and selectivity of analytes. The liquid chromatography, in most cases, eliminates the need for derivatization. It is efficient in separating and identifying similar compounds. When operating in multiple reaction monitoring (MRM) mode, quantification of low levels of compounds in biological matrices becomes feasible.

Analytical methods available in literature

Several analytical methods for the quantification of pyridostigmine have been developed and published. There are different analytical techniques involved in those methods such as radioactive [28, 66, 67], chromatographic, capillary electrophoresis [68, 69] techniques, and other biological techniques e.g. RIA [70]. Assays reported in the published materials for quantifying pyridostigmine and 3-OH NMP are listed in Table 1. There is only one mass spectrometry (MS) method published so far for the quantitation of pyridostigmine [71]. However, this MS method is developed to analyze pyridostigmine in guinea pig plasma. The lower limit of quantitation (LLOQ) of the method is 0.1 ng/mL. To our knowledge, there is no MS method for the determination of the metabolite 3-OH NMP.

Table 1: Reported procedures for the determination of pyridostigmine and its metabolite (Adapted from Zhao B et al., 2006 [28])

Compound	Sample	Extraction method	Stationary Phase	Detection Mode	Validation Data	Refs.
PYR and 3-OH NMP	Plasma& urine (rat)	SPE	µBondapak C18	UV, 280	LIN: 100 – 1000ng/ml LOD: 50ng/ml (for both)	[72]
PYR	Plasma (guinea pig)	LLE	SCX poly(2-sulfoethyl aspartamide) column	MS, *m/z* 180.8 → 124.0	LIN: 0.1 – 50ng/ml LOQ: 0.1ng/ml	[71]
PYR	Plasma (human)	SPE	Axiom Silica column	UV, 208	LIN: 1.53 – 76.3ng/ml LOQ: 1.53ng/ml	[36]
PYR and 3-OH NMP	Serum (human)	LLE	LiChrosorb RP-8	UV, 214	LIN: 0 – 1000ng/ml (PYR) LOQ: 5ng/ml (PYR)	[73]

PYR	Plasma (human)	SPE	µBondapak C18	UV. 254	LOD: 1ng/ml	[32]
PYR and 3-OH NMP	Plasma & urine (human & rat)	SPE	µBondapak C18	UV, 270	LIN: 49 – 4900ng/ml (PYR)	[74]
PYR	Plasma (human)	SPE	CPS Hypersil NC-04 column	UV, 272	LIN: 5 – 200ng/ml LOD: 1ng/ml	[75]
PYR	Plasma (Human)	LLE	µBondapak C18	UV, 269	LIN: 20.8 – 104.1ng/m	[76]
PYR	Plasma (human)	SPE	Radial-Pak CN column	UV, 270	LIN: 10 – 200ng/g LOD: 2ng/g	[77]
PYR	Plasma (human)	SPE	Altex Ultra-sphere Octyl column	UV, 208	LIN: 0 – 68.3ng/ml LOD: 1.37ng/ml	[78]
PYR and 3-OH NMP	Plasma& urine (rat)	SPE	µBondapak C18	UV, 280	LIN: 100 – 1000ng/ml LOD: 50ng/ml (for both)	[79]
PYR	Plasma (human)	SPE	Cation exchange column	UV	LIN: 5 – 100ng/ml LOQ: 5ng/ml	[80]

6. Population pharmacokinetic modeling

Modeling plays a critical role during drug development. It has been very well said by Atkinson and Lalonde that "dose selection and dose regimen design are essential for converting drugs from poisons to therapeutically useful agents [81]." Modeling and simulation serve as important tools during drug development and its usage. In general, pharmacokinetic and

pharmacodynamic modeling is useful in predicting the time course of exposure and response Moreover, population modeling, in particular, is useful for quantitating and explaining variability in drug exposure and response.

Population modeling was first introduced in 1972 by Sheiner et al. [82]. The earlier methods such as naïve pooled average and standard two-stage have some problems which may lead to biased parameter estimates. These problems become worse when samples in the data are missing, sampling is sparse, or poor dosing compliance exists [83]. The population approach developed by Sheiner et al. addressed these problems. Moreover, the population modeling approach allows pooling of sparse data from many different sources to estimate mean population parameters, between subject variability (BSV). Population approach also helps in estimating the covariate effects that quantitate and explain variability in drug effect and exposure. Measuring parameter precision was also feasible with this approach by the generation of standard errors.

The components of population model

A population model is comprised of three main components:

a. The structural model consists of algebraic or differential equations which describe the time course of effect and exposure of the drug.

b. The statistical model describes the variability (such as interindividual, interoccasion) or random effects variability in the population.

c. The covariate model describes the influence of factors on the variability and parameter estimates of the population.

In population modeling approach, all data from all individuals are analyzed simultaneously using a nonlinear mixed effects model. The term “nonlinear” indicates that dependent variable (e.g., concentration) is nonlinearly related to the model parameters and independent variables. Parameters that do not vary across individuals are called “fixed effects,” and the parameters that vary across individuals are called “random effects” When both, fixed and random effects parameters are estimated simultaneously in a model, it indicates a mixed effect model. [84].

Population model development

Data consideration

While developing a population model, several different considerations are involved including database formation, selection of software and estimation method, comparing models, structural model development, statistical model selection, covariate model building and finally performing model evaluations. All these steps when performed aid efficiently in an accurate, appropriate and reliable model.

The first step of generating database for population analysis is one of the most critical and time-consuming part of the evaluation [85]. Data need to be thoroughly scrutinized for accuracy. Data can be graphed initially to identify potential errors and outlier. Early diagnostic analysis can also be performed to understand the data and its content. The right section of the software and estimation method is very important in modeling. Many population modeling software packages are available and so choosing a package requires careful consideration including familiarity with the software package, available online support for the package, cost,

and how well-establishes the package is. The software packages available today are mostly based on the maximum likelihood approach where parameter estimations are performed by minimizing an objective function value (OFV) [85]. The OFV is the negative twice the log of the likelihood, and this single number tells us how closely the model predictions match to the observed data. Considering the influence of fixed and random effects on the model, an approximate marginal likelihood is calculated by employing different estimation methods such as FO, FOCE, LAPLACE and SAEM [86]. All estimation methods have their advantages and disadvantages which are mostly related to the speed, robustness, precision and stability [87, 88].

While developing population models, different approaches are tried, and the resulting models are compared. As mentioned above, the minimum OFV is important for comparing and ranking models. However, Akaike information criterion (AIC) and Bayesian information criterion (BIC) can also be used to compare models in case of increased complexity regarding a number of parameters and number of data observations.

$$AIC = OFV + 2*np$$

$$BIC = OFV + np * Ln(N)$$

Where, np is the total number of parameters in the model and N is the number of data observations.

The careful evaluation of structural model is important because it has implications in selecting a covariate model [89]. The structural model describes the kinetics of the drug after IV (systemic model) and extravascular (absorption model) administration.

Similar to the structural base model, an appropriate statistical model is also important for the evaluations of covariates. The statistical model helps in describing the variabilities such as between subject and random variability in the population. Statistical models also have implications in determining the amount of remaining unexplained variability in the data and performing simulations [85].

Identification of covariates that explain the variability in the pharmacokinetic response of the drug in population is important. Building a covariate model is a stepwise approach in which the potentially important covariates are selected first, and then they are screened based on regression, correlation, etc. Covariates can also be evaluated graphically for visible and significant relationships. Thorough initial evaluation to limit the number of important covariates in the model can considerably reduce runtime. In the last step of covariate model development, all relevant covariates which meet inclusion criteria during screening are included in the model. Significance levels are pre-specified, and the covariates are dropped one after the other and changes in the goodness-of-fit of the model are tested. This process is continued until the simplest possible model is left by testing and reducing not significant covariates.

Model evaluation is the final step of population analysis. In initial stages, models are compared based on their OFV. However, in later stages, simulations are performed, and methods such as the visual predictive check (VPC) may be more useful for effective model evaluation. Some important evaluation methods are listed below:

a. Graphical evaluation: Graphical representation of residuals to evaluate model fitting is fundamental in pharmacometrics.

b. Parameters, SE and confidence intervals: Assessment of precision of the parameter estimates and their plausibility is an important method of model evaluation.
c. Visual predictive checks: New database is simulated using the final model and prediction intervals are constructed from simulated concentration-time profiles and compared with observed data.

There are two population studies performed on pyridostigmine. The first study, by Marino MT et al. [36] was conducted on healthy volunteers to assess the use of pyridostigmine bromide for prophylaxis against nerve agent poisoning. In this multiple doses, placebo-controlled and randomized double-blind study, pyridostigmine was administered orally at a dose rate of one 30 mg tablet every eight hours for 21 days. Samples were collected once before and at multiple later time points until 8 hours after the first dose. During the 21 day treatment period, samples were collected once every three days. After the last dose had been administered, samples were collect until 72 hours afterward. These samples were analyzed for plasma concentrations of pyridostigmine and red blood cell cholinesterase levels. The red blood cell cholinesterase activity had shown correlation with survival in nerve agent exposure in some animal models and therefore, RBC ChE activity was selected as the pharmacodynamic endpoint [90].

The methods adopted in this article report that standard two-stage procedure was followed using NONMEM IV version 2.1. A two-compartment model with first-order absorption (with lag time) and elimination was used to fit the pyridostigmine plasma concentrations.

The results of this study suggested that the PK parameters of pyridostigmine are dependent on both gender and weight. The pharmacodynamic (PD) effects were directly

proportional to the plasma concentration and returned to near baseline conditions within 8 hours after pyridostigmine administration.

In another retrospective pop PKPD study in Chinese subjects [91], pyridostigmine was administered at a similar 30 mg dose every 8 hours for three days, and the plasma concentrations of pyridostigmine and RBC AChE activity were determined at various time points. Unlike the previous study by Marino et al., a one-compartmental open model with first order absorption (with a lag term) and elimination fitted the PK data best. First-order conditional estimate (FOCE) with interaction and inhibitory Emax model within NONMEM were used to develop the PK and PD model respectively.

Unlike the previous study, the tested covariates were not correlated with any of the model parameters. Simulations performed by the researchers suggested a dose of 30 mg every 6 hours to maintain a steady state trough percentage above 10% in healthy Chinese males.

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CHAPTER III

SUBACUTE PYRIDOSTIGMINE EXPOSURE INCREASES HEART RATE RECOVERY AND CARDIAC PARASYMPATHETIC TONE IN RATS

Abstract

The rapid deceleration of heart rate after strenuous exercise, heart rate recovery (HRR), is an indicator of parasympathetic tone. A reduction in parasympathetic tone occurs in patients with congestive heart failure resulting in delayed HRR. Acetylcholinesterase inhibitors, such as pyridostigmine, can enhance parasympathetic tone by increasing cholinergic input to the heart. The objective of this study was to develop a rodent model of HRR to test the hypothesis that subacute pyridostigmine administration decreases cholinesterase activity and enhances HRR in rats. Ten days after implantation of radiotelemetry transmitters, male Sprague-Dawley rats were randomized to control (CTL) or treated (PYR) (0.14 mg/ml pyridostigmine in the drinking water, 29 days) groups. Rats were exercised on a treadmill to record HRR, and blood samples were collected on days 0, 7, 14, and 28 of pyridostigmine administration. Total cholinesterase and acetylcholinesterase (AChE) activity in plasma was decreased by 32-43% and 57-80%, respectively, in PYR rats on days 7-28, while plasma butyrylcholinesterase activity did not significantly change. AChE activity in RBCs was markedly reduced by 64-66%. HRR recorded 1 min after exercise was higher in the PYR group on days 7, 14 and 28, and on day 7 with HRR recorded at 3 and 5 min. The autonomic tone was evaluated pharmacologically using sequential administration of muscarinic (atropine) and adrenergic (propranolol) blockers. The parasympathetic tone was increased in PYR rats, but no significant difference was noted in the CTL group. These data support the study hypothesis that subacute pyridostigmine administration enhances HRR by increasing cardiac parasympathetic tone.

Introduction

The autonomic nervous system is important in controlling cardiovascular functions. Neuroendocrine dysregulation, indicated by sympathetic over-activity and parasympathetic withdrawal, is an important determinant of mortality and morbidity in patients with congestive heart failure (CHF) (1). The cardiac autonomic function can be assessed in healthy subjects or CHF patients by using various tools. These assessment methods include chronotropic response of the heart to both physiological stress and pharmacological blockade (2, 3), heart rate variability (HRV) analysis (4, 5), quantification of baroreflex sensitivity (6), plasma or coronary sinus catecholamine levels (7), HR turbulence (7), and others. Heart rate can be recorded noninvasively and inexpensively with the aid of commercially-available and automated equipment. Heart rate recovery (HRR) and HRV can also be easily derived (8), and so more focus has been directed to analysis of HRR and HRV. Recently, HRR, preferably measured after submaximal exercise to avoid possible sympathetic activity interference (9, 10), has been used to approximate parasympathetic tone in human subjects (11). HRR is an index of cardiac parasympathetic tone and has clinical utility in predicting cardiovascular fitness, disease prognosis, and mortality in heart failure patients (12-16).

Some of the earliest clinical evaluations of heart rate recovery were performed by Perini and Imai and coworkers (9, 10). They observed that the early (within 1 min) recovery of heart rate after exercise is mediated by parasympathetic reactivation. Later, Pierpont and coworkers quantitatively linked HRR to parasympathetic function in healthy volunteers (17, 18). Shelter and Watanabe validated the use of HRR as a predictor of mortality in human subjects (13, 19). However, there are very few studies which support the use of HRR to evaluate parasympathetic tone in rats. Barnard (1974) discussed the effect of training rats to near maximal heart rates and changes in HRR during this period but did not correlate these with changes in cardiac parasympathetic tone (20).

Acetylcholinesterase inhibitors, such as pyridostigmine, can increase parasympathetic activity by increasing acetylcholine levels at the vagal cholinergic nerve terminals innervating cardiac tissue. Previous studies by Androne and co-workers demonstrated that short-term pyridostigmine treatment augmented parasympathetic tone and increased HRR in stable CHF patients (21). Similarly, Serra et al. found that the chronotropic response to exercise in heart failure patients was reduced by pyridostigmine (22). The objective of our study was to develop a rodent exercise model of HRR and to test our hypothesis that subacute administration of pyridostigmine enhances HRR after submaximal exercise in normal rats by increasing cardiac parasympathetic tone.

Material and methods

Optimization of experimental methods

A pilot study was performed to estimate the number of animals needed per group. From the pilot study, it was determined that intraabdominal implantation of the radiotelemetry transmitter device with an intradermal suture pattern led to a faster recovery as compared to the subcutaneous implantation and simple interrupted sutures. The exercise performance of rats with increasing treadmill speed was tested and accordingly, the highest running speed was set at 20 meters per minute (m/min). From the pilot study (data not shown), a sample size of eight rats per treatment group was calculated to be sufficient to detect a difference of 44 beats/min in HRR at 1 minute between each treatment group with a power of 80% and an alpha of 0.05. As per the findings of the pilot study, we designed the following experiment.

Experimental animals

Sixteen male, 8-9 week old, Sprague-Dawley rats weighing 275-300 g at onset were purchased from Charles River (Wilmington, MA). The rats were housed individually and maintained on a 12-hour light-dark cycle in an Association for Assessment and Accreditation of

Laboratory Animal Care International (AAALAC International)-accredited facility. Rats were given *ad libitum* access to water and standard laboratory rodent chow. All animal experiment protocols were reviewed and approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Surgical implantation of radiotelemetry device

After one week of acclimatization, the CTA-F40 small animal radiotelemetry transmitter device from Data Sciences International (DSI, St. Paul, MN) was surgically implanted under isoflurane general inhalation anesthesia. The surgical protocol followed was adapted by the recommendations of DSI (ETA, CTA, EA, or CA Device Surgical Manual). A single dose of morphine (5 mg/kg, IM) was administered before surgery. Meloxicam (2 mg/kg, bid, SQ) was administered to all rats for three days after surgery and enrofloxacin (Baytril®, 5 mg/kg, bid, SQ) for five days after the surgery. The rats recovered uneventfully.

Measuring heart rate (ECG) by telemetry

Data were collected using a TA11CTA-F40 implant, PhysioTel Receiver Model RPC-1, and DataQuest ART (Advanced Research Technology) Version 4.0 software (DSI). This allowed the continuous collection of electrocardiograms from conscious rats.

Exercise training and exercise protocol

After recovering from surgery, each rat was trained for three days to run on the treadmill. During this training period, rats were allowed to run continuously under increasing speeds to determine their submaximal level of exercising. Rats initially ran at a speed of 5 m/min at a 10% grade for 10 min. After that, the treadmill was held at a constant grade while the speed was increased by 5 m/min every 5 min until the rat was unable or unwilling to maintain pace with the treadmill belt (modified from [3]). The heart rate at this stage was considered as their maximum

exercise heart rate, and the speed was recorded. The submaximal exercise level was considered as 80% of the maximal HR and the speed, 20 m/min, at which it was achieved, was noted for future experiments. According to the final exercise protocol, the treadmill was inclined at 10% grade and the speed of treadmill was increased by 5 m/min every 3 min until reaching 20 m/min. At the 20 m/min speed, the rats were run for 6 min, after which the treadmill was stopped and the rats were allowed to rest on the treadmill for 5 min.

Pyridostigmine treatment

After recovery from the surgery, rats were randomly divided into pyridostigmine treated (PYR) and control (CTL) groups (n=8 per group). The PYR group received 0.14 mg/mL [4-6] of pyridostigmine dissolved in drinking water, whereas the CTL rats received only water for 29 days. Stability of pyridostigmine in water was confirmed by mass spectrometry (MS). Pyridostigmine in water was found to be stable in both ambient and cold (4° C.) temperatures for up to 8 days.

Assessment of heart rate recovery

Heart rate recovery (beats/min) was determined by subtracting the heart rate at one, three and five minutes after exercise from the maximum heart rate achieved at 20 m/min speed at the end of the exercise period. These were designated as HRR1, HRR3, and HRR5 (e.g., HRR1 = beats/min at 0 minutes after exercise – beats/min at 1 min after exercise). Heart rate recovery data were collected on days 0 (pre-treatment), 7, 14 and 28 after initiating PYR administration.

Acetylcholinesterase and butyrylcholinesterase activity

Blood samples were collected on days 0, 7, 14 and 28 for determining blood cholinesterase (ChE) activities. An aliquot of whole blood was collected, and plasma and red blood cells (RBCs) were separated by centrifugation (5000 x g, 2 min). The RBCs were washed

by resuspension in phosphate buffered saline solution, centrifuged as before and the supernatant was discarded. The washing step was repeated three times. Plasma and washed RBCs were then stored at -80°C until analysis. Acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and total ChE activity were measured in the plasma, whereas only AChE activity was measured in RBCs, using a radiometric method with [^{3}H] acetylcholine iodide (1 mM final concentration) as the substrate and incubation at 26°C [7]. To determine total cholinesterase activity, plasma samples with no selective inhibitor were assayed in the enzyme reaction. To estimate BChE activity, the specific acetylcholinesterase inhibitor BW284c51 (1, 5-bis[allyldimethylammoniumphenyl] pentane-3-dibromide, 2 µM, Sigma-Aldrich (St. Louis, MO, USA) was added 1 minute prior to adding substrate. AChE activity was defined as the difference in acetylcholine hydrolysis between total ChE and BChE. Preliminary assays were carried out to determine incubation times that resulted in linear rates of substrate hydrolysis from 30 sec to 30 min incubation.

To determine the *in vitro* sensitivity of cholinesterases to PYR, blood was collected from a different set of three naïve rats, and plasma and RBCs were separated as before and stored at -80°C. To determine the *in vitro* sensitivity of plasma BChE to pyridostigmine, all reactions contained 284c51 (2 µM) and either vehicle (50 mM potassium phosphate buffer, pH 7) or one of a range of concentrations of pyridostigmine (0.0003 to 30 µM) in the vehicle. To determine *in vitro* sensitivity of plasma AChE to pyridostigmine, all samples contained the butyrylcholinesterase-selective inhibitor ethopropazine (Sigma-Aldrich) (10 µM dissolved in 50 mM potassium phosphate buffer, pH 7 containing 1% ethanol and either vehicle or one of a range of concentrations of pyridostigmine. In all cases, reactions were pre-incubated at 26°C for 30 minutes before adding substrate and measuring residual activity.

Pharmacological determination of autonomic tone

The autonomic tone was assessed in freely moving, conscious rats. On day 27, basal heart rate (HR_{basal}) was recorded for 10 minutes. Atropine sulfate (Henry Schein, Dublin, Ohio, USA) was injected intraperitoneally, at a dose rate of 2 mg/kg [8-10] to block parasympathetic input to the heart. The HR recorded 15 min after atropine injection was defined as $HR_{atropine}$, i.e., HR primarily under the influence of the sympathetic and the intrinsic cardiac nervous systems. Propranolol (4 mg/kg, IP, West-Ward Pharmaceutical Corp., Eatontown, NJ, USA) was then administered and HR was again recorded 15 min later and defined as $HR_{intrinsic}$, i.e., HR with both parasympathetic and sympathetic innervation blocked. The sympathetic tone was estimated using $HR_{atropine}$ and $HR_{intrinsic}$ as below in equation 1.

Equation 1:

$$Sympathetic\ tone = \frac{HR_{atropine} - HR_{intrinsic}}{HR_{intrinsic}} * 100$$

Where, $HR_{atropine} = HR_{sympathetic} + HR_{intrinsic}$

Similarly, on day 29, after recording HR_{basal} for 10 min, propranolol was administered as described above [8-10]. HR recorded 15 min after propranolol injection was termed the $HR_{propranolol,}$ under the influence of the parasympathetic and intrinsic cardiac nervous systems. Atropine was administered at a dose rate of 2 mg/kg and the HR was recorded after 15 min of injection for determination of $HR_{intrinsic}$. The parasympathetic tone was calculated using $HR_{propranolol}$ and $HR_{intrinsic}$ as shown below in equation 2.

Equation 2:

$$Parasympathetic\ tone = \frac{HR_{propranolol} - HR_{intrinsic}}{HR_{intrinsic}} * 100$$

Where, $HR_{propranolol} = HR_{parasymapathetic} + HR_{intrinsic}$

Statistical analysis

All data are presented as the mean ± SEM. Comparison of the basal HR and maximum HR were performed using two-way repeated measures ANOVA followed by Dunnett's test for multiple comparisons. Similarly, HRR and ChE enzyme activity were analyzed using repeated measures two-way ANOVA followed by Sidak's (to compare between treatment groups) and Dunnett's (to compare day 7, 14 and 28 with day 0) test for multiple comparisons. For measurements that were obtained only once (i.e., sympathetic tone, parasympathetic tone, intrinsic HR, weight gain, and water intake), a student's t-test was used to assess differences. The level of significance was set at $\alpha < 0.05$. *In vitro* inhibition data were fitted to a nonlinear curve to calculate the IC_{50} by using nonlinear curve fitting of the inhibitor dose-response function in GraphPad Prism 6® (GraphPad Software, Inc., La Jolla, CA).

Results

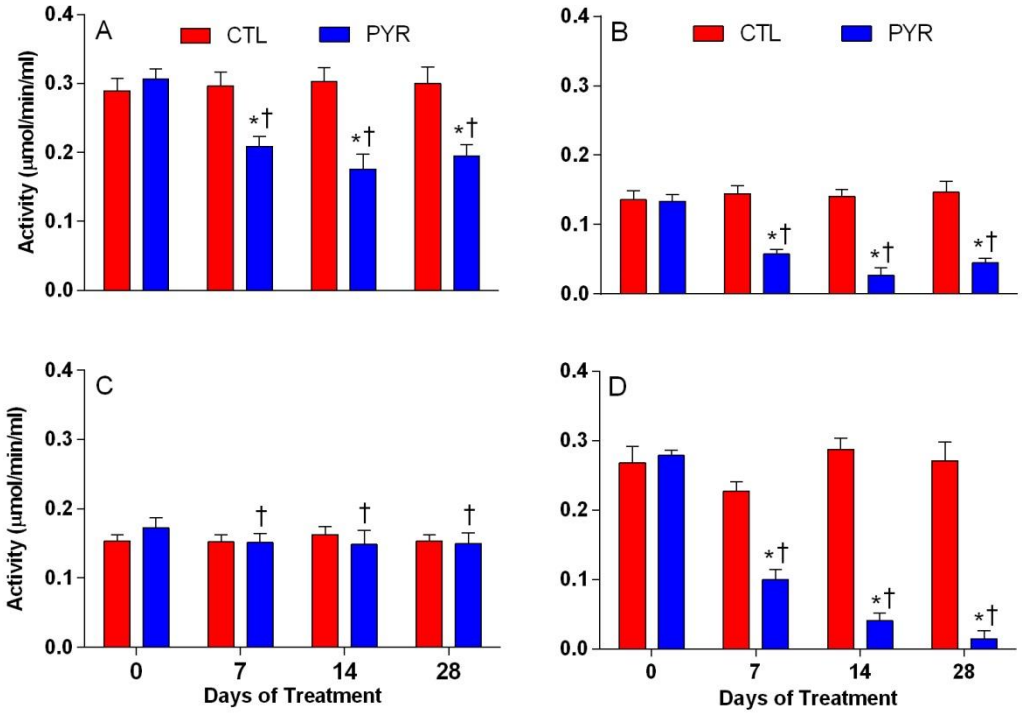
Pyridostigmine intake and *in vivo* and *in-vitro* inhibition of cholinesterase activity

Pyridostigmine in tap water was stable in the dark at both room temperature and 4° C for up to 8 days. During the experimental period, water bottles containing either fresh water or water containing freshly-prepared pyridostigmine solution were replaced after every three days. There were no significant differences in body weight gain (CTL, 55 ± 4 g; PYR, 51 ± 4 g, P=0.53)) or water consumption (CTL, 100 ± 5 ml/kg/day; PYR, 106 ± 6 mL/kg/day, P=0.47).Pyridostigmine intake was estimated at 15 ± 1 mg/kg/day in the PYR group.

Rat blood plasma contains relatively similar levels of both AChE and BChE while rat RBCs contain primarily only AChE [11]. Fig. 1A shows that total ChE activity in plasma was decreased by 32%, 43% and 36% in the PYR group relative to controls on days 7, 14 and 28,

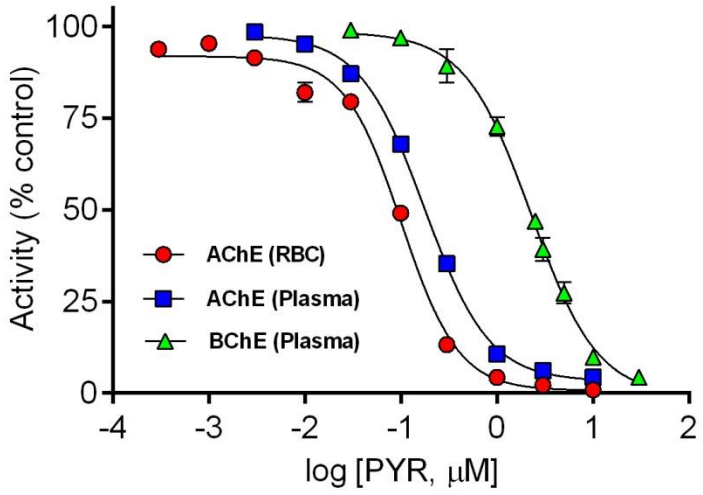
respectively ($P<0.001$ for interaction term). Fig. 1B shows the effects of PYR on plasma AChE activity, which was reduced by 57%, 80%, and 66% of control on days 7, 14 and 28, respectively ($P<0.001$ for interaction term). In contrast, there was no significant reduction in plasma BChE activity following PYR exposure at any time-point seen in Fig. 1C. Similar to reductions in plasma AChE, AChE activity in RBCs was also markedly lower (64%, 66%, and 66%, Fig. 1D) on days 7, 14, and 28 following PYR administration ($P<0.001$ for interaction term). When compared to day 0, all the aforementioned enzyme activities were significantly lower on all subsequent days in the PYR group ($P<0.001$ for total ChE, plasma AChE, and RBC AChE, and $P<0.01$ for plasma BChE). These *in vivo* data suggested that AChE was more sensitive to inhibition by PYR than BChE.

Figure 1: Cholinesterase activity in CTL and PYR rats. The total plasma cholinesterase (A), plasma acetylcholinesterase (B) and plasma butyrylcholinesterase (C) and red blood cell acetylcholinesterase activity (D) were analyzed on days 0, 7, 14 and 28 of pyridostigmine administration. Data are presented as the mean ± SEM. *P<0.05 compared to CTL group, †P<0.05 compared with day 0.



We also evaluated *in vitro* sensitivity of AChE and BChE to inhibition by PYR. Figure 2 shows that the IC_{50} (95% confidence interval) for PYR was relatively similar against AChE in both RBC (0.1020 µM; 0.088 - 0.119) and plasma (0.1775 µM; 0.139 - 0.227). In contrast, BChE in plasma was markedly (more than an order of magnitude) less sensitive to *in vitro* inhibition by PYR, with a mean IC_{50} of 2.272 µM (2.071 - 2.493).

Figure 2: In vitro inhibition of red blood cell AChE, plasma AChE and BChE activity by pyridostigmine. Tissues were pre-incubated with pyridostigmine for 30 minutes at 26°C prior to adding substrate and measuring residual activity. Enzyme activity was expressed as mean percent of control activity in the absence of inhibitor.



Heart rate recovery

As shown in Table 1 and Fig. 3A, basal HR was similar between groups (P=0.325). However, Table 2 and Fig. 3B shows that the maximum or peak HR at the maximum running speed of 20 m/min was significantly lower in PYR rats as compared to pretreatment values on days 7, 14 and 28 (P=<0.001, for days term).

Table 1: Effect of pyridostigmine on the basal heart rate of rats on day 0, 7, 14, and 28 of pyridostigmine administration.

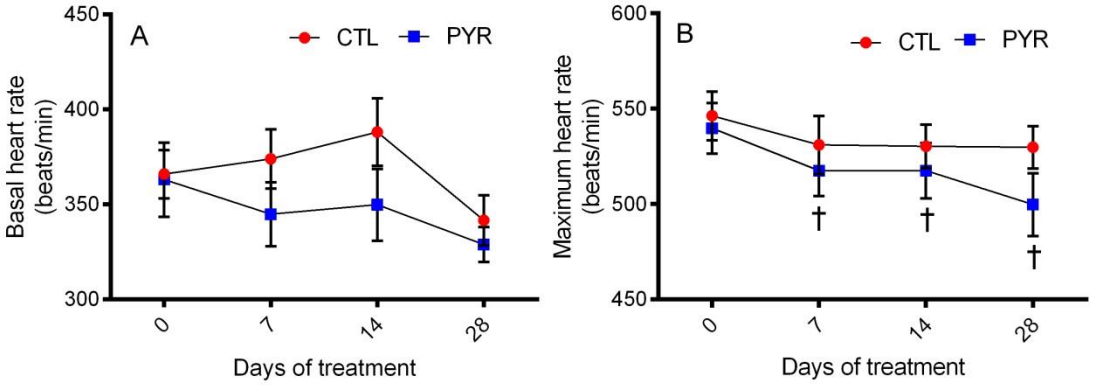
Day	Basal HR of CTL (beats/min)	Basal HR of PYR (beats/min)
0	366±13	363±20
7	374±16	344±17
14	388±18	350±19
28	342±13	329±9

Data are presented as the mean±SEM. HR=heart rate, CTL=control group, PYR=pyridostigmine treated group.

Table 2: Maximum heart rate of rats at 20 m/min running speed recorded during exercise on day 0, 7, 14, and 28 of pyridostigmine administration. Data are presented as the mean±SEM. HR=heart rate, CTL=control group, PYR=pyridostigmine treated group.* $P<0.05$ compared with day 0.

Day	Max HR of CTL (beats/min)	Max HR of PYR (beats/min)
0	546±13	540±13
7	531±15	518±13*
14	530±11	518±14*
28	530±11	500±16*

Figure 3: Basal and maximum heart rate. The basal heart rate (A) before exercise and maximum heart rate (B) at the maximum running speed of 20 m/min was measured on days 0, 7, 14 and 28 during pyridostigmine administration. Data are presented as the mean ± SEM. †P<0.05 compared with day 0.

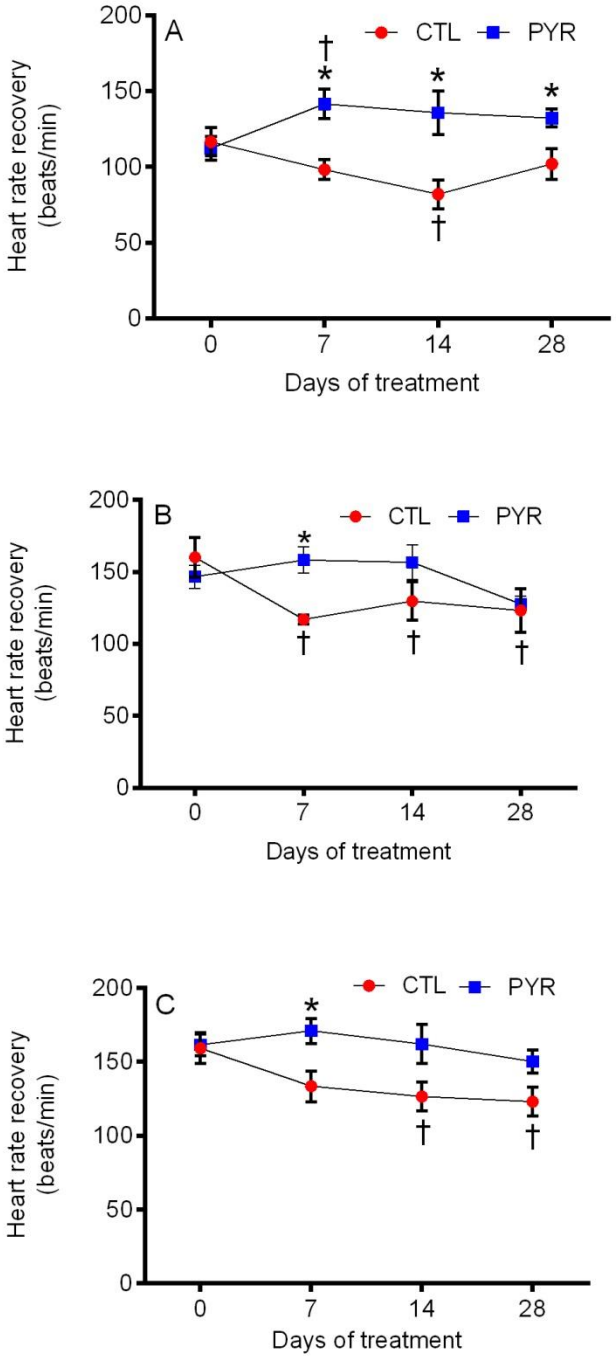


Pyridostigmine administration increased HRR as compared with CTL rats, at 1, 3 and 5 minutes after the end of the exercise, i.e., HRR1, HRR3 and HRR5 (Table 3 and Figs. 4A, B, and C respectively). The HRR1 in PYR rats was significantly higher than in the CTL group on days 7, 14 and 28 (P=0.002 for interaction term, Fig. 4A). The HRR3 was significantly higher in PYR rats on day 7, but not on day 14 or 28 (P=0.015 for interaction term, Fig. 4B). Similarly, HRR5 was significantly higher on days 7 but not on day 14 and 28 (P = 0.036 and 0.019 for days and treatment term, Fig. 4C).

Table 3: Heart rate recovery of rats recorded before the treatment on day 0 and days 7, 14, and 28 during the pyridostigmine administration. Data are presented as the mean±SEM. HR: heart rate, HRR: heart rate recovery, CTL: control group, PYR: Pyridostigmine group. *P<0.05 compared to CTL group, †P<0.05 compared with day 0.

Days	CTL (beats/min)	PYR (beats/min)
	HRR1	
0	117±9	112.3±8
7	98.3±7	142±10*†
14	82±10†	136±14*
28	102±10	132.3±6*
	HRR3	
0	160.3±14	147±8
7	117±3†	158.3±9*
14	130±13	157±12
28	124±15†	128±5
	HRR5	
0	159.3±10	162±7
7	134±10	171±8*
14	127±10†	162±13*
28	123.1±10†	150.3±8

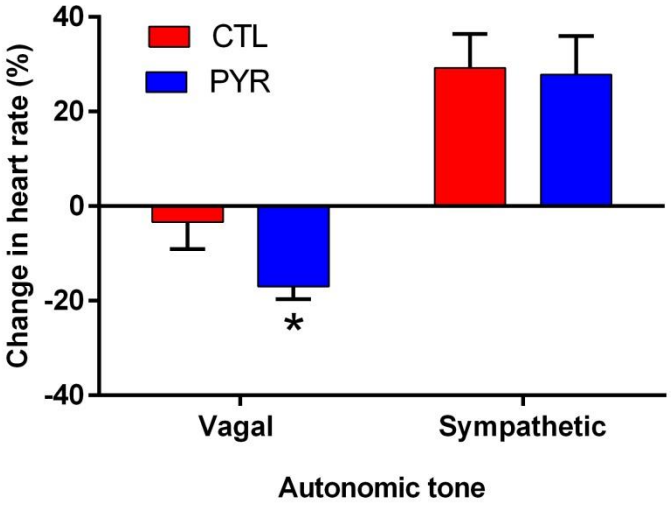
Figure 4: Heart rate recovery after exercise. The 1, 3 and 5 min (A, B, and C) HRR was measured on days 0, 7, 14 and 28 during pyridostigmine administration. Data are presented as the mean ± SEM. *P < 0.05 compared to CTL group, †P<0.05 compared with day 0.



Autonomic tone and intrinsic heart rate

Fig. 5 shows that parasympathetic tone was increased in the PYR rats as compared to the CTL group, as indicated by a greater bradycardic response (3% vs 17% decrease in HR, $P<0.001$) after propranolol administration. In contrast, there was no difference in sympathetic tone (28% vs. 29% increase in HR, $P=0.99$) between the treatment groups after atropine administration. Intrinsic HR was 374 ± 9 vs. 368 ± 8 beats/min ($P=0.4$) and 349 ± 10 vs. 352 ± 11 beats/min ($P=0.53$) in CTL and PYR groups on days 27 and 29, respectively (data not shown). Basal HR was also similar between the groups on day 27 (338 ± 12 and 324 ± 10 beats/min in CTL and PYR groups, respectively, $P=0.4$); however, on day 29, basal HR was significantly lower in the PYR group (350 ± 7 vs. 321 ± 9 beats/min in CTL and PYR groups, respectively $P=0.02$; data not shown).

Figure 5: Modulation of autonomic tone represented by the alteration in heart rate following pharmacological intervention in PYR and CTL groups. The HR response associated with atropine sulfate administration is represented by the negative change in HR, whereas the bradycardic response caused by propranolol administration is represented by a positive change in HR. Data are presented as the mean ± SEM. *P<0.05 compared to CTL group.



Discussion

Heart rate recovery is measured as the reduction in heart rate at specified times after moderate to heavy exercise. In other words, it is the rate at which the heart decelerates after moderate to heavy exercise and is dependent on the dynamic relationship between parasympathetic and sympathetic innervation [12] of the cardiac tissues. During and after exercise, parasympathetic and sympathetic control of the heart adjusts according to metabolic demands. Savin and colleagues [2] studied cardiac function in normal patients with and without selective autonomic blockers and proposed that sympathetic withdrawal contributes more to the decrease in heart rate soon after exercise, while the parasympathetic system is predominant later in the recovery phase. However, recent studies have proposed that parasympathetic reactivation

occurs faster than sympathetic withdrawal in heart rate recovery, thus playing a major role in the early deceleration of the heart [13-17]. Our study was designed to explore the relative contributions of the sympathetic and parasympathetic systems on heart rate recovery following forced running exercise in rats.

Barnard and coworkers reported relatively few differences in HRR among different ages of rats exercised until exhaustion [18]. To the best of our knowledge, there is no other published literature on HRR in rats for comparison. To develop an exercise model in rats to study HRR and the cardiac neuroendocrine system, we modified an exercise protocol from a previous study by Copp and coworkers [3]. Using this exercise model, we determined that subacute administration of pyridostigmine in rats enhanced heart rate recovery (Fig. 4). Pyridostigmine can modify parasympathetic control of cardiac function by blocking the enzymatic breakdown of acetylcholine at vagal nerve terminals. This can lead to enhanced activation of muscarinic receptors regulating cardiac muscle contraction. A single dose of pyridostigmine in CHF patients led to an increase in heart rate recovery recorded 1 min after exercise [19]. In 2007, Dewland et al. showed that in sedentary adults, pyridostigmine decreased resting heart and increased post-exercise heart rate recovery at 1 min, but had no significant effect on trained athletes [20]. The study by Dewland and colleagues explained post-exercise heart rate recovery as an index of cholinergic signaling in the sinoatrial nodal junction. Serra et al. reported that repeated pyridostigmine dosing increased HRR and other hemodynamic parameters in human CHF patients [21]. Our study demonstrated the augmented effect of subacute pyridostigmine treatment (28 days in the drinking water) on heart rate recovery in rats (Fig. 4).The results from our study thus generally agree with and model those reported previously in humans. However, in this study, basal heart rate did not differ significantly between treatment groups (Fig. 3), contrasting with results obtained from a study performed in rats with induced heart failure rats [22]. This discrepancy may be due to physiological differences between normal rats used in the current

study as compared to those with heart failure. In heart failure rats, a positive chronotropic condition occurs as a consequence of the withdrawal of the parasympathetic system [22]. The indirect parasympathomimetic drug, pyridostigmine, enhances cardiac vagal tone. Thus a greater negative chronotropic effect would be expected in the rats with heart failure. A smaller drug effect would, therefore, occur with pyridostigmine administration to normal rats. This smaller magnitude effect, along with day to day variability, may also explain why mean basal HR was lower in PYR rats as compared to CTL rats before exercise and on day 27 before pharmacological estimation of autonomic tone, but was only significantly lower on day 29 before pharmacological estimations.

Soares et al. reported that 0.14 mg/mL pyridostigmine in the drinking water of rats resulted in an average pyridostigmine intake of 31 mg/kg/day [5]. These investigators reported that this concentration of pyridostigmine led to approximately 40% inhibition of plasma acetylcholinesterase activity after a seven day treatment period [5]. Although the average pyridostigmine intake in our study was estimated to be only 15 mg/kg/day, AChE inhibition after seven days of drug administration was 57% and 64% in rat plasma and RBCs, respectively (Fig. 1). Other studies used the same pyridostigmine dosing conditions in rats as Soares et al. and the resulting intake of the drug was also higher compared to our estimate of intake [4, 6, 22, 23]. The relatively lower intake of pyridostigmine by our rats may be attributed to evidence of higher water intake by young rats used in the above-listed studies as compared to the older rats used in our study [24].

Moreover, when compared to 30% cholinesterases inhibition by pyridostigmine in HRR studies performed in human subjects, the level of inhibition of acetylcholinesterases was generally higher in our study (Fig. 1). This may contribute to the substantial enhancement of HRR noted in our study (Fig. 4).

We noted substantial differences in the level of AChE and BChE inhibition following *in vivo* PYR exposure (Fig. 1). We, therefore, evaluated the relative *in vitro* sensitivity of AChE and BChE to inhibition by pyridostigmine. In both the RBC and plasma, pyridostigmine was a more potent *in vitro* inhibitor of AChE compared to BChE, confirming that pyridostigmine has a higher inhibitory effect on AChE than BChE (Fig. 2). The lower IC_{50} of AChE compared to BChE agrees with the lesser BChE inhibition noted in our *in vivo* studies. A previous report indicated that *in vitro* measurements of human and rat AChE may also show differences in sensitivity towards pyridostigmine [25]. The slight difference in *in vitro* sensitivity of AChE in plasma vs. AChE in RBCs noted in our study may be due to differences in PYR detoxification in the plasma vs. RBC fractions. Pyridostigmine is a carbamate acetylcholinesterase enzyme inhibitor, and some carbamate anticholinesterases are metabolized by plasma carboxylesterases [26]. Thus, there may be more stoichiometric binding sites for pyridostigmine in the plasma vs. the RBC fraction, and thus less inhibition of AChE in plasma with similar concentrations of pyridostigmine. Pyridostigmine may also bind to plasma albumin [27], decreasing its relative *in vitro* inhibition of the enzyme in plasma.

In the present study, subacute administration of pyridostigmine increased parasympathetic tone but had no apparent effect on sympathetic tone (Fig. 5). In contrast, in a previous study on heart failure in rats, both parasympathetic and sympathetic tones were decreased by pyridostigmine administration [22]. However, in both heart failure rats from this previous study and in the normal rats used in our current study, intrinsic heart rate did not differ between the PYR and CTL groups. According to Lataro et al., in rats with heart failure, improvement of cardiac sympathetic tone could be due to effects of pyridostigmine on cholinergic transmission in the sinoatrial node [22]. Pharmacological blockade of the sympathetic and parasympathetic nervous systems by the administration of selective blocking agents are useful for assessing autonomic tone [15, 28-30]. Lataro et al. subtracted HR_{basal} from $HR_{atropine}$ to

estimate parasympathetic tone, and then subtracted that value from $HR_{propranolol}$ to estimate sympathetic tone [22]. However, after subtracting the basal HR from the $HR_{atropine}$ or $HR_{propranolol}$, the intrinsic HR still influences HR and confounds the interpretation of relative effects of the autonomic system. We proposed that changes in autonomic tone could be better evaluated by subtracting the intrinsic HR from $HR_{atropine}$ to get sympathetic tone, and then subtracting that value from $HR_{propranolol}$ to estimate parasympathetic tone (Equations 1 and 2). This approach may exclude the influence of intrinsic activity while individually isolating the parasympathetic and sympathetic contributions.

Conclusion

The data support our hypothesis that subacute PYR dosing enhances HRR by increasing cardiac parasympathetic tone in rats. Post-exercise HRR can be used to assess autonomic tone and thus can serve as an indicator of cardiovascular fitness. The present model of HRR after submaximal exercise should be suitable for future studies in rats with heart failure.

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CHAPTER IV

USE OF HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY FOR THE QUANTIFICATION OF PYRIDOSTIGMINE AND ITS METABOLITE, 3 HYDROXY N-METHYLPYRIDINIUM IN HUMAN PLASMA

Abstract

Analytical methods were developed to quantify pyridostigmine (PYR) and its metabolite 3-hyroxy-N-methylpyridinium (3-OH NMP) in human plasma using a sensitive liquid chromatography–electrospray ionization–tandem mass spectrometry assay. For PYR sample preparation, plasma was processed by a solid phase extraction procedure, using weak cation exchange (WCX) cartridges and 3-OH NMP was precipitated by acetonitrile. Hydrophilic interaction liquid chromatography separation was performed with a Restek® Ultra PFPP (3µm, 150 X 4.6mm) column. The mobile phase consisted of 30% of 50 mM ammonium formate (pH 3.25) and 70% of 1:1 acetonitrile:methanol for PYR and 2mM ammonium formate in 1% formic acid solution in water and methanol for 3-OH NMP. Two calibration curves were utilized for PYR to achieve linearity over the range of concentrations, low and a high calibration curves spanned over 0.25–10 ng/mL and 5–100 ng/mL, respectively. The calibration curve for 3-OH NMP ranged from 1 to 200 ng/mL. The intra and inter-day accuracy of PYR were above 95%, and coefficient of variation was at or below 15%. Similarly, intra and inter-day accuracy of 3-OH NMP were above 95%, and coefficient of variation was at or below 9%. The lower limit of quantitation (LLOQ) of PYR and 3-OH NMP assays was determined to be 0.25 and 1 ng/mL, respectively. At the LLOQ, intra-day accuracy was above 80%, and coefficient of variation was below 14% for PYR and 94% and below 10% respectively for 3-OH NMP. The average recovery of PYR from plasma was 93%, 97% and 86% at 0.8, 8 and 80 ng/mL, respectively. The average recovery for the internal standard, neostigmine was similar to that of PYR at 92%.

Introduction

Pyridostigmine (PYR) is a positively charged quaternary ammonium carbamate compound, which binds to the acetylcholinesterase (AChE) enzyme on a negatively charged site and an esteric site. Thus PYR prevents acetylcholine (ACh) from binding to these same sites and being broken down by the enzyme. This inhibition leads to the buildup of ACh, which is not being hydrolyzed, resulting in an increased action of ACh on its receptor. Pyridostigmine inhibits acetylcholinesterase enzyme for a longer duration of time than neostigmine (1). Pyridostigmine bromide was first approved by the FDA in 1955 to treat a neuromuscular disease, myasthenia gravis. The FDA has given pyridostigmine "orphan drug" status. FDA designates orphan drug status to drugs and biological products which are intended for use in treatment, diagnosis or prevention of rare diseases. Pyridostigmine is the first drug to be approved under the "animal efficacy rule of FDA", in Feb 2003 as a prophylactic agent at 30 mg dose taken three times a day. Use of PYR in military personnel was intended to increase survival after exposure to Soman nerve gas poisoning (2).

Pyridostigmine is being studied for its beneficial cardiovascular effects during heart failure. Recent studies suggest that pyridostigmine attenuates the development of hypertension and inflammation in spontaneously hypertensive rats (3). Pyridostigmine also improves the cardio-circulatory function in rats with chronic heart failure (4) and prevents ventricular dysfunction during the onset of the heart failure in rats (5). In human congestive heart failure patients, a 45 mg pyridostigmine tablet administered orally three times a day resulted in improved post-exercise hemodynamic profile such as inhibition of chronotropic response, enhancement of heart rate reserve and heart rate recovery at 1 min after the exercise (6).

There are several published methods for the identification and quantification of pyridostigmine from biological fluids using gas chromatography-mass spectrometry (GC/MS) (1,

7), enzymatic or immunoassay methods (8, 9), HPLC methods (10-12) and a thermospray LC/MS method (13). These methods are less selective, rapid or sensitive. As compared to liquid chromatography, due to the derivatization steps, the time required for sample processing is longer in gas chromatography. Moreover, the enzymatic methods are not as selective in distinguishing between drugs and the metabolites which resemble chemically in biological fluids. Additionally, HPLC methods followed by UV or fluorescence detectors have long analysis time (run time) and may require ion-pairing agents in the mobile phase to obtain proper peak shape and for the retention on the C8 and C18 stationary phases (14). These ion-pairing agents may interact with the analytes of interest and interfere in quantification. A mass spectrometry method with the low runtime, high selectivity, and enhanced sensitivity is required to address these limitations. In this study, we report two separate tandem mass spectrometry methods for the determination of pyridostigmine and its metabolite, 3-hydroxy-N-methylpyridinium, in human plasma. This method was applied for the quantification of these analytes in the plasma collected from human congestive heart failure patients.

Materials and methods

Chemicals and reagents

Pyridostigmine bromide (Pyridinium, 3-[[(dimethylamino)carbonyl]oxy]-1-methyl-, bromide; 99.8% purity) (PYR) was obtained from U.S. Pharmacopeial Convention (USP, Rockville, MD USA). 3-hydroxy-N-methylpyridinium bromide (3-OH NMP) was obtained from TLC PharmaChem Inc. (Vaughan, Ontario, Canada). The internal standards, Neostigmine bromide (NEO) (≥98% purity) for PYR method and Eserine (ESE) (≥98% purity) for the 3-OH NMP method, were obtained from Sigma-Aldrich (St. Louis, MO USA). Standard stock solutions were prepared 1% formic acid in methanol solution. HPLC grade ammonium formate, formic acid, citric acid, sodium phosphate, methanol, and other reagents were purchased from Fisher

Scientific Company (New Jersey, NJ). Oasis® WCX 3mL solid phase extraction cartridges were purchased from Waters ® Corporation, (Milford, MA).

Instrumentation

An Applied Biosystems 4000 Q-Trap MS/MS system (Applied Biosystems, Foster City, CA) and a NitroGen N300DR nitrogen generator (Peak Scientific Instruments Ltd., Paisley, United Kingdom) were used with an electrospray ionization source for the fragmentation and detection of the analytes. A Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) was used to separate the analyte for detection and quantification. This liquid chromatography system consisted of a system controller (CBM-20A), binary solvent delivery unit (LC-20AD), in-line degasser (DGU-20A5), an auto-sampler (SIL-20AC) with thermostatic injector set at 4 °C and a column oven set at 40 °C (CTO-20AC). The separation column used was a Restek® Ultra PFPP (3µm, 150 X 4.6mm) column (Restek, Bellefonte, PA). Data was acquired and analyzed by the Multiquant and Analyst® 1.5 software. For the measurement of both PYR and 3-OH NMP, 25 µL were injected, and the auto-sampler was set at 4°C temperature.

Data was collected in a Multiple reaction monitoring (MRM) mode. Based on collision-induced dissociation (CID) product ion scan spectra, MRM transitions ions were selected for detection of analytes. The method was run under positive ion mode. The values of curtain gas pressure (CUR), ion spray voltage (IS), temperature (TEM), pressure of ion source gas 1 (GS1), ion source gas 2 (GS2), declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) for both methods are shown in Table 1.

Table 1: Optimal mass spectrometry instrument parameter settings for pyridostigmine and 3-OH NMP methods.

Parameters	Value for Pyridostigmine assay	Value for 3-OH NMP assay
CUR	20	20
IS	1000	1000
TEM	700	550
GS1	40	80
GS2	30	60
DP	40	40
EP	5	5
CE (eV)	20	20
CXP	10	10

Abbreviations: Curtain gas pressure (CUR), ion spray voltage (IS), temperature (TEM) , pressure of ion source gas 1 (GS1), ion source gas 2 (GS2), declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP)

a. Mobile phase

The mobile phase for the PYR method consisted of 30% of 50 mM ammonium formate (pH 3.25) and 70% of 1:1 acetonitrile: methanol. Mobile phase for 3-OH NMP method consisted of 2mM ammonium formate in 1% formic acid solution in water and methanol. Isocratic elution achieved separation at a flow rate of 0.5 mL/min.

b. Standard solutions

Stock solutions of PYR and 3-OH NMP were made in 1% formic acid in methanol solutions. Then the stock solutions were diluted make the working solutions in mobile phase B.

The working solutions were used to fortify pooled unmedicated human plasma (Innovative Research®, Novi, MI) to get the final concentrations of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 75, and 100 ng/mL for PYR and 1, 2.5, 5, 10, 25, 50, 100, and 200 for 3-OH NMP standard curves. Working solutions of 10ug/mL NEO and ESE were prepared similarly.

c. Sample preparation

Different separation techniques were employed for PYR and 3-OH NMP. After thawing, the pooled human plasma was fortified by PYR or 3-OH NMP separately at different concentrations to get a total volume of 500 uL of fortified plasma as required for calibration curves and quality control samples. For sample preparation of PYR, 500 uL of fortified plasma or patients' plasma sample were diluted with 500 uL of 50 mM citrate phosphate (CP) buffer, pH=5.8, containing 50 ng/mL of NEO. Solid phase extraction technique was used to separate PYR through weak cation exchange (WCX) cartridges from Oasis®. The cartridges were conditioned with 2mL of methanol and CP buffer and then the diluted sample was loaded as passed slowly through using a positive pressure manifold. The cartridge was then rinsed with one mL of CP buffer followed by one mL methanol and dried under maximum pressure for ten mins. Lastly, the retained analytes were eluted with 20% formic acid solution of pH 2 in 80% methanol and collected in glass tubes. The samples were then dried under a gentle nitrogen gas stream for 30 min at 35°C. Samples were reconstituted with 100 µL of a mixture of 30% mobile phase A and 70% mobile phase B solution and transferred to injection vials.

Five hundred uL of fortified human plasma or a patient plasma sample was precipitated with one mL of acetonitrile containing 25 ng/mL of the internal standard ESE to clean the samples for 3-OH NMP. The tubes were then centrifuged for 10 min at 4000 g speed. One mL of supernatant was transferred to a new glass tube and dried under nitrogen gas in the Turbovap for

30 min at 35°C. Samples were reconstituted with 100 µL of a mixture of 50% mobile phase A and 50% mobile phase B solution.

d. Calibration curves

For the quantification of pyridostigmine and 3-OH NMP, the ratio of peak area of the analyte to the peak area of the respective internal standards was used. For PYR, the calibration curve split into low and high curves, with the high curve ranging from 0.25 to 10 ng/mL and the low curve from 5 to 100 ng/mL, to obtain acceptable linearity. For quantification of 3-OH NMP, a single calibration curve ranging from 1 to 200ng/mL was used. To plot a calibration curve, at least six calibrators were included. In the analysis of PYR, $1/y^2$ weighting was used for high and low calibration curves whereas, for 3-OH NMP curve, weighting was not required. At the lower limit of quantitation, the acceptable limit of accuracy was set to 20% of nominal concentrations and coefficient of variation within 20%. Acceptability criteria for all runs included accuracy within 15% of at least five retained calibrants and an R^2 of 0.99 or above. Quality control (QC) samples of three different concentrations (0.8, 8 and 80 ng/mL for PYR and 8, 40 and 160 ng/mL for 3-OH NMP) in six replicates were also included in the validation experiments.

e. Method Validation

Method was evaluated by determining the intraday and interday accuracy and CV% of the six replicates of the of the quality control samples. Coefficient variation (CV), or standard deviation divided by the mean predicted concentration was expressed as a percentage, and this represented the precision of the method. The accuracy of within 20% of the nominal concentration at the lower limit of quantitation (LLOQ) and 15% of the nominal concentration at all (at least five out of six) higher concentrations was set as the acceptable limits. Similarly, a CV of less than 20% at the LLOQ and less than 15% at higher concentrations were set as the acceptable parameters for a successful run.

In mass spectrometry methods, plasma components that are extracted with the analytes may alter the signal response, causing either ion suppression or enhancement. This may result in poor analytical accuracy and linearity. Thus, matrix effects were determined in the method by preparing samples in triplicates at nominal concentrations of 0.8, 8 and 80 ng/mL of pyridostigmine in plasma. Peak areas of analytes in resuspending buffer were defined as A; analytes fortified before extraction as B and analytes fortified after extraction from plasma as C. The determination of the matrix effect, recovery efficiency and process efficiency were determined by the following formulae:

$$\text{Matrix effect (\%)} = C/A \times 100$$

$$\text{Recovery efficiency (\%)} = B/C \times 100$$

$$\text{Process efficiency (\%)} = B/A \times 100$$

f. Specificity

Samples were assayed along with internal standard and in the presence of metabolites and drugs in the plasma. It was important to determine the effect of the presence of an internal standard, metabolite (in case if pyridostigmine method) and drug (in the case of 3-OH NMP method) on the quantification. Thus to assess this effect, the peak areas of the quality control samples of each analyte were compared with and without the present of other analytes.

g. Stability

To determine the stability of the analytes during storage (-80°C), test were performed on samples of the drug and the metabolite. A reversed-phase high-performance liquid chromatography method was originally developed with a maximum sensitivity of 10ng/mL. This method was used to test the stability of PYR and 3-OH NMP in the 1% formic acid in methanol

solution. This solution was used to prepare the stock and working solutions to store until use and thus it was necessary to test the stability of the drug in this solution.

h. Application to human pharmacokinetic study of pyridostigmine and its metabolite in congestive heart failure patients

This novel highly sensitive analytical method was altered to address the issues of impaired specificity and then successfully applied to quantify the concentrations of pyridostigmine and 3-OH NMP in plasma collected from16 adult CHF patients involved in a clinical trial at New York University. These patients orally self-administered pyridostigmine initially at 15 mg every 8 hours for two weeks with subsequent planned uptitration to 30 mg every 8 hours for two weeks, and 60 mg every 8 hours for four weeks. The dosing regimen then followed a downtitration to 30 and 15 mg every 8 hours for one week each. Plasma samples were collected at different times across the study period.

Results and Discussion

Sample pretreatment optimization

Several different sample processing methods were investigated to optimize sample recovery and chromatography. Previously published solid phase extraction (SPE) by Octadecyl (C18)-bonded silica has been the most widely employed SPE adsorbent in the cartridge for both PYR and 3-OH NMP (17, 18). Abu-Qare et al. used the C18 SEP-PAK cartridge and reported recovery rates of 77% for PYR and 79% for 3-OH NMP, whereas Ellin et al. (19) claimed recovery rates of >90% for PYR with similar SPE material. Other previously reported SPE cartridges, such as HLB (20), C8 Bond Elute (12), Bond Elut C2 (21), and CBA (22), were also tested for recovery. In our experimental settings with human plasma, the recoveries of PYR with HLB, CBA, and WCX cartridges were promising (Figure 2). However, the recovery of the internal standard NEO was lower with HLB as compared to PYR, and there was interference in

chromatography which may be due to insufficient cleaning of the samples when CBA cartridges were used. Hence, WCX cartridges were ultimately used to extract PYR and NEO from human plasma with approximately 90% recovery as shown in Table 2.

Figure 1: Recovery of Pyridostigmine and Neostigmine through different SPE cartridges

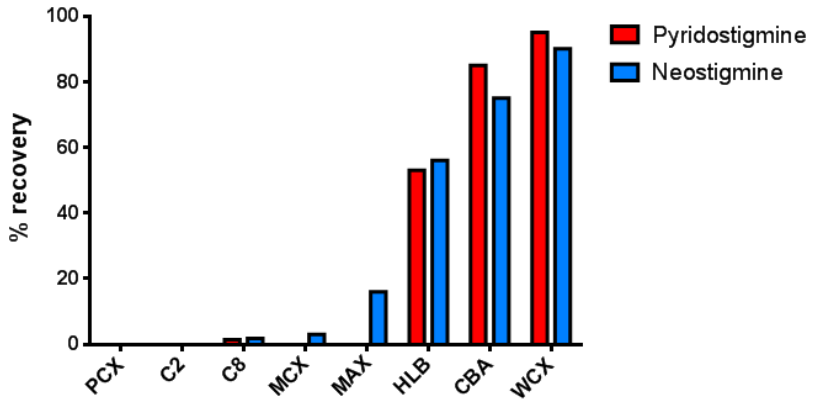


Table 2: Mean percentages of matrix effects, recovery efficiency, and process efficiency of extraction of plasma samples fortified with pyridostigmine and neostigmine in triplicate; fortified at the concentrations of 0.8, 8 and 80 ng/mL pyridostigmine and 100 ng/mL neostigmine.

PYR conc (ng/mL)	**Matrix Effect (%)**	**Recovery Efficiency (%)**	**Process Efficiency (%)**
0.8	17	93	78
8	2	97	99
80	4	86	89
NEO 100	5	92	97

To extract 3-OH NMP from human plasma, HLB, Trace B, Trace N and MCX cartridges were tested. Trace N and HLB columns had poor recoveries, whereas Trace B and MCX columns did not remove the interfering substance. Hence, a protein precipitation method was

successfully employed. Protein in the plasma samples was precipitated with acetonitrile, and the supernatant was collected for drying. The sensitivity of 3-OH NMP method was poorer than that of the PYR method because more drug was lost during protein precipitation and sample preparation whereas, most of the drug is recovered in the SPE method.

Chromatographic optimization

Few obstacles made the analyses of PYR and 3-OH NMP difficult. Low molecular weights of PB (181.21) and 3-OH NMP (79.9) predispose interference from other small molecules present in biological matrices (23, 24). The molecule is highly polar, and thus it is difficult to obtain ideal chromatographic results in terms of peak shape and retention on the typical hydrophobic stationary columns such as C8 or C18 (25).

In previous studies, columns used for the determination of PYR and its metabolites are almost exclusively reverse-phase (RP) (10, 11, 14, 17, 19, 26-28). These RP columns ranged from 150 to 300 mm in length and 3.2 to 4.6 mm in internal diameter. The particle size in these columns was either 5 or 10 µm. Needham et al. compared previous reversed-phase methods to their LC/MS/MS method for the analysis of PYR in guinea pig plasma using SCX columns and reported five folds lower LLOQ (25). We explored the RP-18, Atlantis HILIC, and Restek Ultra PFPP columns. Restek Ultra PFPP column using hydrophilic interaction chromatography (HILIC) method for separation gave the best results for our analytes. Polar compounds are retained well in HILIC through preferential partitioning between a more hydrophobic mobile phase and an immobilized liquid layer enriched in water (29, 30). Pyridostigmine and its metabolite 3-OH NMP are highly polar in nature, and so the HILIC method of separation allowed the analytes to retain at the stationary phase.

Different mobile phases were also tried to improve the separation of analytes. Isocratic elution with 30% of 50 mM ammonium formate (pH 3.25) and 70% of 1:1 acetonitrile:methanol

for PYR and 2mM ammonium formate in 1% formic acid solution in water and methanol for 3-OH NMP at a flow rate of 0.5 mL/min efficiently resulted in pertinent separation and Gaussian peak shape of the analytes. Resuspending buffer was injected after every highest calibration run, and it was found that there are no subsequent carryovers of the analytes from the previous runs.

Mass Spectrometry Instrument setting and optimization

Analytes were dissolved in methanol and directly injected to determine the optimal instrument settings. The mass spectrometry setting such as curtain gas pressure (CUR), ion spray voltage (IS), temperature (TEM) , pressure of ion source gas 1 (GS1), ion source gas 2 (GS2), declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were optimized. The aim of the optimization was to remove or decrease interfering molecules, increase the sensitivity of the method and gain optimum separation of the analytes. We also compared the chromatograms in ESI vs. APCI mode and found that there was more background noise in APCI mode, and it had low sensitivity. To overcome these problems, ESI with positive mode ionization was used to get satisfactory sensitivity.

Multiple reaction monitoring (MRM) in which a typical fragment ion is selected amongst several ions from the complex matrix and quantified was used in this method (31). MRM plots are very simple, usually containing only a single peak. These characteristics make the MRM plot ideal for sensitive and specific quantitation (31).

Specificity

The identification (ID) ratios of qualifier ions for each analyte was determined to assess that specificity of the method. Based on FDA guidelines for method development ID ratios within ±20% of mean of ID ratios were considered as acceptable (32). The mass to charge ratios (m/z) of precursor ions were 180.7, 223.3, 109.98, and 276.1 for pyridostigmine, neostigmine, 3-OH NMP, and eserine, respectively. For quantitation of analytes, major transitions were chosen, such

as 180.7/72.95, 223.3/151.0, 109.98/66.9 and 276.1/219 for pyridostigmine, neostigmine, 3-OH NMP, and eserine, respectively. For identification of analytes, qualifier transitions were 180.7/125.1, 223.3/208.9, 109.98/38.9 and 276.1/94.4 for pyridostigmine, neostigmine, 3-OH NMP, and eserine respectively. Figure 3 and 4 shows chromatograms of PYR and NEO and 3-OH NMP and ESE respectively.

Figure 2: Representative chromatogram of fortified human plasma with 100ng/mL pyridostigmine (PYR) and 100 ng/mL neostigmine (NEO, internal standard).

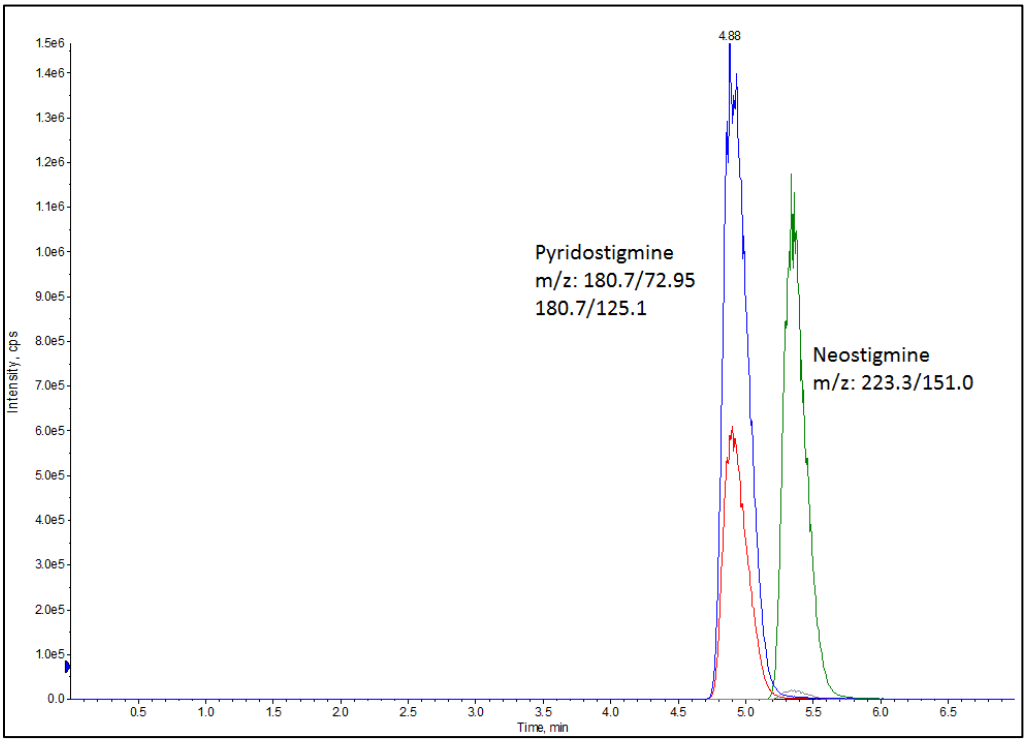
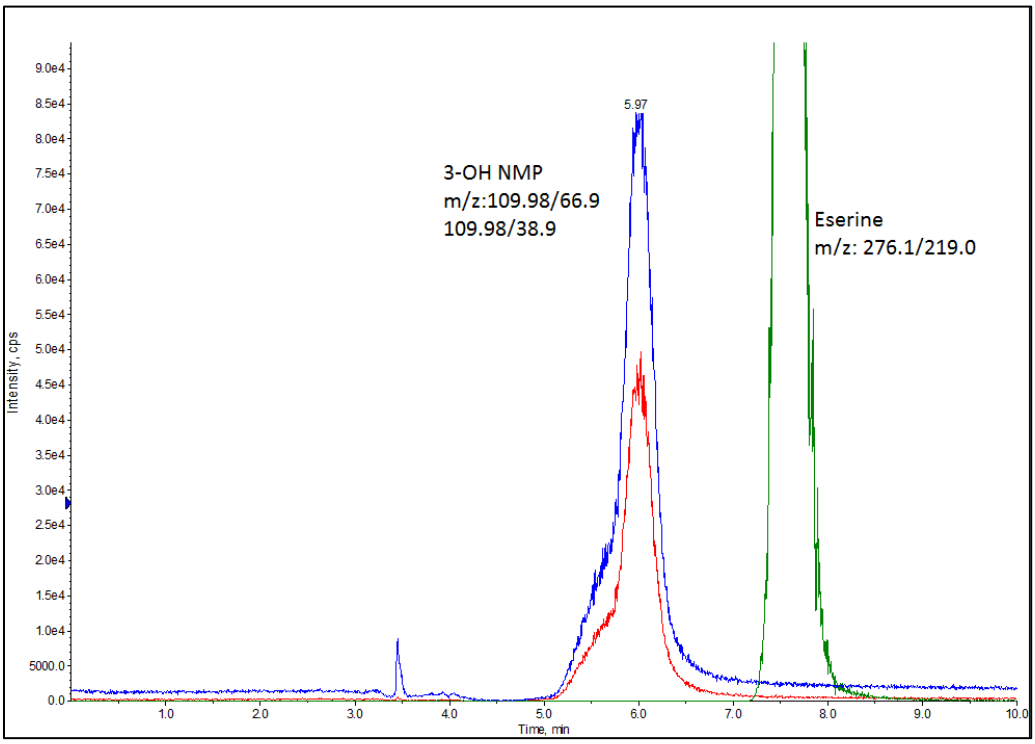


Figure 3: Representative chromatogram of fortified human plasma with 100ng/mL N-methyl pyridinium bromide (3-OH NMP) and 25 ng/mL eserine (ESE, internal standard).



Stability

Both PYR and 3-OH NMP, at 10 and 100 ng/mL, were stable in 1% formic acid in methanol and mobile phase solution for up to 30 days. The potency of 10 and 100 ng/mL PYR samples over a 30 day period remained > 98% and the coefficient of variation < 3%. Similarly, the potency of 10 and 100 ng/mL 3-OH NMP samples over a 30 day period was maintained at > 95%, and CV was <5%.

Calibration curve

A wide range of pyridostigmine calibrants was included (0.25–100 ng/mL) in plasma to quantify the expected concentrations of pyridostigmine in human plasma. Peak areas of PYR were not linear over the range of anticipated plasma concentrations, so the calibration curve was split into two, 0.25–10 ng/mL and 5–100 ng/mL for the low and a high calibration curves,

respectively (16). Both of the curves were linear across the range with an R2 value of >0.997. A 1/(concentration)2 weighting scheme was applied to both low and high curves. The 3-OH NMP curve was linear across the 1 to 200ng/mL range, and the R^2 value of >0.999 was obtained without weighting. The PYR calibration curve reported in a previously published method for guinea pig plasma samples was narrow and ranged from 0.1 to 50 ng/mL (25). The calibration curves reported in this paper were broad to cover the expected higher plasma concentrations of pyridostigmine in human samples.

Method validation

Matrix effects, recovery efficiency, and processing efficiency were each calculated for pyridostigmine and neostigmine in plasma (15). Table 2 shows that recovery at all QC concentrations of PYR was higher in this reported method as compared to a previously published method by Needham et al. in Guinea pig plasma (25). The higher recovery could be attributed to the use of an SPE method for sample preparation as compared to the liquid-liquid extraction method reported by Needham et al. (25). In the current method for developed for PYR, the intra-day accuracy for pyridostigmine was above 97%, and coefficient of variation (CV) was at or below 5%, whereas, the inter-day accuracy was 95% and CV was below 15% respectively (Table 3 and 4). The lowest value of the intraday and inter-day accuracy was 95%, indicating a good accuracy of the developed method. The LLOQ of pyridostigmine was 0.25ng/mL with an accuracy and CV of 80.2 and 13.3%, respectively (Table 4). For 3-OH NMP, the calibration curve ranged from 1 to 200 ng/mL, and the LLOQ was one ng/mL with an accuracy and CV of 94 and 9.5% respectively (Table 4). The intra-day accuracy for 3-OH NMP was above 96%, and coefficient of variation (CV) was at or below 9%, whereas the inter-day accuracy was 95% and CV was below 9%, respectively (Table 3 and 4).

Table 3: Intra-day accuracy and precision of analytes in plasma were presented as a mean ± standard deviation. The intraday accuracy and precision of both analytes were within acceptable limits.

Analyte	Intra-day precision and accuracy (n=6)			
	Nominal conc. (ng/mL)	Observed conc. (ng/mL) (mean ± SD)	CV (%)	Accuracy (%)
PYR	0.8	0.8±0.04	5	98.6
	8	7.8±0.13	1.7	97.8
	80	77.8±1.5	1.9	97.2
3-OH NMP	8	8.3±0.3	4	96.7
	40	40.7±2.9	7	98
	160	160.3±13.7	8.6	99.8

Table 4: The values of inter-day accuracy and precision of analytes in plasma in eight runs of PYR and three runs of 3-OH NMP were presented as a mean ± standard deviation. The values of inter-day accuracy and precision of both analytes were within acceptable limits.

Analyte	Inter-day precision and accuracy (n=6)			
	Nominal conc. (ng/mL)	Observed conc. (ng/mL) (mean ± SD)	CV (%)	Accuracy (%)
PYR	0.8	0.8±0.13	15	95
	8	7.8±0.24	3.2	97.2
	80	79.1±1.7	2.1	98.9
	0.25 (LLOQ)	0.23±0.03	13.3	80.2
3-OH NMP	8	7.7±0.4	5.5	97.2
	40	41.7±0.97	2.3	95.5
	160	160.3±13.7	8.6	99.8
	1 (LLOQ)	1.06±0.1	9.5	94

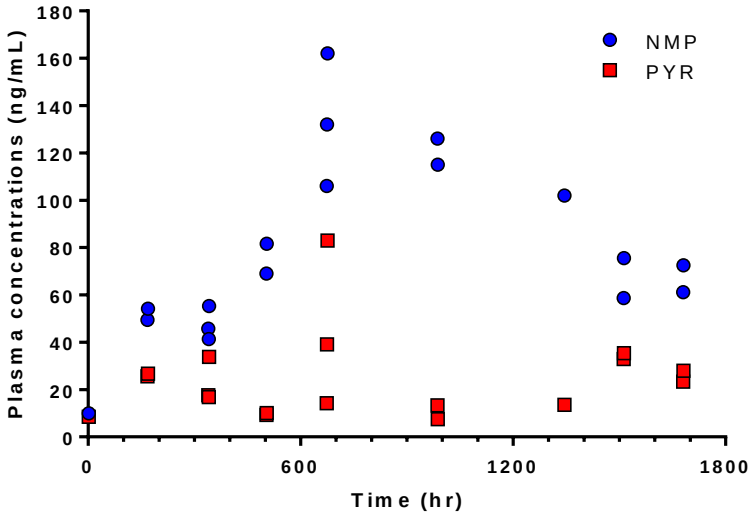
Application to pyridostigmine in human plasma samples

The PYR and 3-OH NMP concentrations determined in human plasma ranged from 1.15 to 88.09ng/mL and from 1.07 to162 ng/mL, respectively. Figure 5 represents a pharmacokinetic profile of pyridostigmine and its metabolite, 3-OH NMP, in a human congestive heart failure patient. As seen in figure 5, the assays were adequately able to describe the plasma disposition of orally administered pyridostigmine in this human CHF patient. However, the observed plasma concentrations of the metabolite 3-OH NMP were higher than the plasma concentrations of PYR at all time points. Previous studies indicate that orally administered pyridostigmine undergoes high first pass metabolism, which reduces the fraction of unchanged drug eliminated in urine, and the fraction of the metabolite is increased as compared with intravenous administration (33).

Therefore, enterohepatic recycling of the metabolite may be another possible reason for the increased metabolite concentration in plasma as compared to the parent drug.

It is also possible that during storage and thawing steps, pyridostigmine underwent hydrolysis by the cholinesterases enzymes present in the plasma leading to higher concentrations of NMP in plasma. However, this contradicts with the previous findings where pyridostigmine was slowly metabolized and so the metabolite concentrations in urine and liver did not exceed more than 25% of the excreted radioactivity (34). A freeze-thaw stability of pyridostigmine and metabolite in plasma would be helpful in determining the effect of storage and thawing on the analytes. It is also possible that the analytical method developed for NMP produced erroneous results, since this assay was subsequently found to be difficult to replicate due to the presence of contaminants that co-eluted with NMP. Given that interfering substances were detected in subsequent attempts to use theses assays to quantify both PYR and NMP in plasma, further modification and validation of these assays will be required before they can be applied to patient samples.

Figure 4: A representative time course disposition of pyridostigmine bromide (PYR) and 3-hydroxy N-methyl pyridinium bromide (3-OH NMP) in a human congestive heart failure patient (104.3 kg body weight), that received oral doses of 15, 30 and 60 mg (initially escalating and then descending) tablets three times a day.



Conclusion

In conclusion, a sensitive and selective method was validated for the routine LC/MS/MS analysis of PYR and 3-OH NMP from human plasma. The method used WCX columns to retain PYR and separate the analytes from the plasma, whereas a protein precipitation method was employed to separate 3-OH NMP from plasma. The HILIC mode of LC separation was used, and eluted drugs were quantified via MRM positive ion mode ESI in a tandem mass spectrometer. The method, with modifications to improve specificity, was applied to a population pharmacokinetic and study of pyridostigmine bromide in congestive heart failure patients.

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CHAPTER V

POPULATION PHARMACOKINETIC ANALYSIS OF PYRIDOSTIGMINE IN CONGESTIVE HEART FAILURE PATIENTS

Abstract

This study was conducted to characterize the population pharmacokinetics of pyridostigmine given as pyridostigmine bromide. For eight weeks, 16 congestive heart failure patients received oral escalating doses of 15, 30 and 60 mg pyridostigmine bromide every eight hours. Plasma concentrations of pyridostigmine were determined at various times within visit 1 to 12 conducted on days -14, -7, 0, 7, 14, 21, 28, 42, 56, 63, 70 and 77 with a ±4 day window. One and two compartment models with first-order absorption and elimination was fitted to the resulting pharmacokinetic data. Nonlinear mixed effects modeling was performed using NONMEM, interfaced with PDx- Pop. Model-to-data fitting was performed using the first-order conditional estimation (FOCE) method with interaction. The performance of each model was evaluated by computing the objective function values (OFV), Akaike Information Criterion (AIC), Schwarz Bayesian criterion (SIC) and diagnostic plots. As a result, in a one-compartment model, the typical values for CL/F, Vd/F, and Ka were 187 ± 14.6 L/h, 101 ± 16 L and 0.06 ± 0.004/h, respectively. The inter-individual variability for CL/F, Vd/F, and Ka was 29.0%, 1.39%, and 21.3%, respectively and residual variability was 52.4% for the model. The terminal half-life (t½) was 0.37 h. However, in a two compartment model, typical values for CL/F, Vd/F, Q/F, Vss/F and Ka were, 208 L/h, 1110 L, 509 L/h, 9290 L, and 0.748 L/h, respectively. The interindividual variability in CL/F, Vd/F, Q/F, Vss/F and Ka was 31.4%, 36.5%, 25.9%, 35.6% and 5.32% respectively and residual variability was 46.7% for the model. The terminal phase half-life (t½) was 3.7 h. In conclusion, a two compartment model showed a lower OFV, AIC, and SIC and had better diagnostic plots suggesting a better representation of the data.

Introduction

Heart failure is a complex clinical syndrome that suggests impairment of the heart as a pump supporting physiological circulation. Heart failure is caused by structural or functional abnormalities of the heart (1). Abnormalities in neuroendocrine regulation are considered to be an important determinant of disease progression in patients with chronic heart failure (2, 3). Abnormal cardiac autonomic control, characterized by sympathetic activation and parasympathetic withdrawal, is one of the major manifestations of neuroendocrine dysregulation in heart failure (4). Pharmacological inhibition of sympathetic activation by the systemic administration of adrenergic receptor blockers has been shown to improve survival in clinical trials (5). Therefore, traditional heart failure therapy mainly includes β-blockers, angiotensin converting enzyme (ACE) inhibitors, positive inotropic agents and aldosterone antagonists (5).

In recent studies, attention has focused on the management of heart failure with the administration of parasympathetic system activators, such as pyridostigmine. Pyridostigmine is an acetylcholinesterase inhibitor, which activates the parasympathetic system by reversibly binding to acetylcholinesterase enzyme (6-8). Studies suggest that pyridostigmine administration benefits rats with heart failure during its progression by reducing sympathetic tone and decreasing cardiac remodeling and attenuating left ventricular dysfunction (9). Pyridostigmine also reduced baroreflex sensitivity, power in the low-frequency (LF) band of the systolic atrial pressure spectrum, cardiac parasympathetic tone, and intrinsic HR (10) which had beneficial effects on heart failure rats. In human subjects, repeated pyridostigmine administration increased heart rate recovery (HRR) and had beneficial effects on other hemodynamic parameters in human CHF patients (11, 12).

Initially, pyridostigmine bromide was indicated for the symptomatic treatment of myasthenia gravis, a chronic muscle disease, by the US Food and Drug Administration (FDA) in

1955. Later, pyridostigmine bromide was approved by the FDA for use during the 1990–1991 Gulf War as a prophylactic agent against nerve gas poisoning by Soman (13).

However, very limited human data is available for the pharmacokinetic and pharmacodynamic of pyridostigmine bromide (14-20). Available studies were performed on small subject sizes or did not incorporate all the important covariates in the model. Two population pharmacokinetic and pharmacodynamic (PKPD) studies were previously reported for pyridostigmine bromide (21, 22). In the first study by Marino et al., the data were obtained from Caucasian subjects, and this analysis incorporated only demographic covariate information during model development (22). In the second population PKPD study by Seng et al., relevant dosing regimens were identified for Chinese subjects and other potential covariate relationships were determined (21). However, both the population PKPD studies were conducted with regards to nerve agent exposure. Hence, our population pharmacokinetic study was designed to characterize the pharmacokinetic parameters of pyridostigmine and identify the variability of these parameters in a population of patients with heart failure.

The overall aim of this population analysis was to develop a model characterizing the steady state pharmacokinetics of repeated oral administration of pyridostigmine bromide in patients with congestive heart failure. Also, in this study, we tested the hypothesis that population pharmacokinetic modeling will determine the variability in the dose-response amongst the CHF patient population and estimate pharmacokinetic parameters of pyridostigmine in patients with CHF.

Material and methods

Study design, study population and pyridostigmine regimen

A prospective, randomized, double-blind, parallel group, forced titration, ascending dose study was designed. Thirty-three eligible subjects with chronic heart failure associated with left

ventricular systolic dysfunction were enrolled in the study. All participants were provided written consent and necessary HIPAA authorization in accord with institutional and Federal guidelines. Eligible subjects were selected from the heart failure population at NYU Langone Medical Center. Inclusion criteria; age 21-75 years, symptomatic NYHA Class II-III heart failure >6 months, left ventricular ejection fraction <35%, etc. and exclusion criteria; e.g. contraindications to cholinergic stimulation, sick sinus syndrome, resting heart rate <60 or >100 min-1, pregnant or breastfeeding women, etc., were used to determine the eligibility of the subjects to participate in the study. Various demographics such as race, ethnicity, age and gender, as well as clinical and laboratory parameters of liver and renal function, etc. were documented during each study visit for all of the subjects (Table 1). Creatinine clearance (CLCR) was calculated according to Schwartz's formula (23). Glomerular filtration rate (GFR) was calculated by the Cockcroft-Gault formula (24).

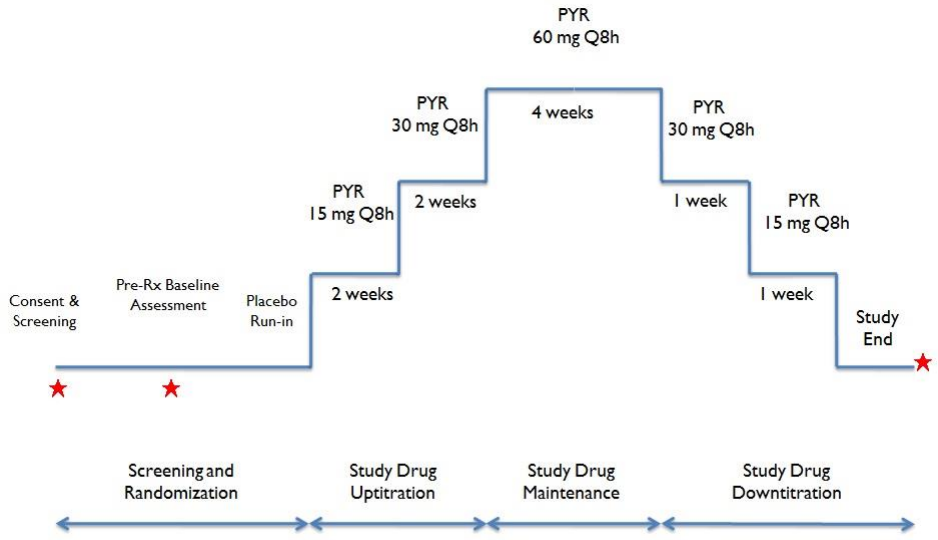
Table 1: Variables for the study population in the pyridostigmine pharmacokinetic analysis

Characteristics	**Units**	**Mean ± SD (n=16)**	**Range**
Hemoglobin	g/dL	13.11 ± 1.33	6.5 to 15.5
Sodium	mmol/L	139.35 ± 2.36	132 to 147
Serum Creatinine	mg/dL	1.11 ± 0.34	0.6 to 2.2
Glomerular filtration rate	mL/min	92.02 ± 23.86	42.29 to 169.95
Alanine Aminotransferase	units/L	28.56 ± 11.17	10 to 59
Aspartate aminotransferase	units/L	27.66 ± 8.49	14 to 61
Weight	kg	82.27 ± 16.81	51.6 to 116.6
Height	cm	166.91 ± 6.28	156 to 176
Age	years	54.31 ± 9.58	27 to 70
Gender	N/A	N/A	14 Male, 16 Female
Race	N/A	N/A	9 White, 4 Black, 3 Asian
Ethnicity	N/A	N/A	8 Hispanic, 8 non-Hispanic
NYHA Functional Classification	N/A	N/A	14 Class II, 2 Class III

The study drug, pyridostigmine bromide was self-administered by the subjects at approximately 8-hour intervals. The study drug was initiated at 15 mg every 8 hours for two weeks with subsequent planned uptitration to 30 mg every 8 hours for two weeks, and then maintained at 60 mg every 8 hours for four weeks. Study drug was then downtitrated to 30 and 15 mg every 8 hours for one week each (Figure 1). If a subject did not tolerate a dose of study drug due to excess cholinergic effects such as, sitting or standing systolic blood pressure <90 mmHg,

increase in QTC by >50 msec, cholinergic symptoms score > 16, or increased >8 from placebo run-in period, resting HR<50 beats/min, etc. the dose was downward adjusted.

Figure 1: Study Schematic representing the study design and dosing regimen



Profiling of Plasma pyridostigmine and 3 hydroxy N-methylpyridinium, red blood cell acetylcholinesterase and plasma butyrylcholinesterase activity levels

The study was conducted in 4 stages: 1) Screening and randomization (Visits 1-3); 2) Study drug uptitration (Visits 4-7); 3) Study drug maintenance and primary endpoint assessment (Visits 8-9); and 4) Study drug downtitration (Visits 10-12). Visits 1 to 12 were conducted on days -14, -7, 0, 7, 14, 21, 28, 42, 56, 63, 70 and 77 with a ±4 day window. Patients were examined and screened, and the first dose of the study drug was administered during visit 3. Blood was collected in heparinized tubes at visits 3 to 11. The samples were immediately centrifuged at 4°C, and plasma was separated for determination of plasma levels of pyridostigmine (PYR) and its major metabolite 3 hydroxy-N-methylpyridinium (3-OH NMP) and butyrylcholinesterase (BChE) levels. Erythrocytes were washed in isotonic saline solution and

centrifuged to discard the supernatant. RBC was stored for determination of acetylcholinesterase (AChE) activity. In order to obtain representative blood samples over a wide range of times, post-administration, the timing of the study visits were staggered to occur at 2-4 hours post study drug at one visit (visits 4, 6, and 9) and 4-6 hours post–administration at one visit (visits 5, 7, and 8) for each dose level of study drug. At these visits, one blood sample was drawn upon arrival, and another sample was drawn approximately one hour later. In addition, at visits 3, 5, and 7, blood samples were drawn immediately before and at 2 hours after administration of study drug in the office. The compliance of study drug administration was recorded at each visit.

Pharmacokinetic assay

The analytical method which was developed in the previous chapter to quantify pyridostigmine (PYR) in human plasma using a sensitive liquid chromatography–electrospray ionization–tandem mass spectrometry assay was modified to address the impaired specificity by the OSU Forensic Toxicology and Trace Laboratory at Center for Health Sciences, Tulsa, OK. Plasma samples were sonicated for approximately 20 minutes to separate the plasma from the proteins and clotting factors that could clog the extraction column. The sonicated plasma samples are then centrifuged and the supernatant filtered by syringe filters (Thermo Scientific, Waltham, Massachusetts, USA) used for extraction of pyridostigmine by solid phase extraction technique using weak cation exchange (WCX) cartridges. Secondly, the pumps were set at a binary gradient mode to prevent the elution of the contaminant at the retention time of pyridostigmine and thus improved the specificity of the method. The MRM ions for pyridostigmine and neostigmine were also re-optimized, and results suggest slight changes in the mass to charge ratios (m/z) of precursor ions as 182.2 and 223.2 for pyridostigmine and neostigmine, respectively. For quantitation of analytes, major transitions were chosen, such as 182.2/71.7 and 223.2/150.0 for pyridostigmine and neostigmine, respectively. For identification of analytes, qualifier transitions were 182.2/125.1 and 223.2/56.0 for pyridostigmine and neostigmine, respectively. Hydrophilic

interaction liquid chromatography separation was performed with a Restek® Ultra PFPP (3µm, 150 X 4.6mm) column (Restek Corporation, U.S., Bellefonte, PA). The mobile phase consisted of 30% of 50 mM ammonium formate (pH 3.25) and 70% of 1:1 acetonitrile: methanol and the pumps were set in binary gradient mode.

Calibration curve for PYR to achieve linearity spanned over 1–50 ng/mL with a quadratic fit and 1/x weighting. The intra and inter-day accuracy of PYR were above 95%, and coefficient of variation was at or below 15%. The lower limit of quantitation (LLOQ) of PYR assays was determined to be 1 ng/mL. At the LLOQ, intra-day accuracy was above 80%, and coefficient of variation was below 20%.

Acetylcholinesterase activity inhibition assay

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were measured in the RBC and plasma, respectively. A radiometric method was used with [3H]acetylcholine (1 mM final concentration) as the substrate, with a reaction volume of 100 µL, and incubation at 26°C (25). Butyrylcholinesterase activity in plasma samples was assayed in 50 mM potassium phosphate buffer, pH 7.0, without Triton X-100. AChE activity in RBC was assayed in the presence of Triton X-100 (0.1% in 50 mM potassium phosphate buffer, pH 7.0). Either 20 µL of plasma or 20 µL washed RBC, and 20 µL of radiolabeled substrate ([3H] acetylcholine iodide) in 50 mM potassium phosphate buffer, pH 7.0 was added in each case. Preliminary assays were carried out with 30 s to 30 min incubation times to determine incubation times that produced linear rates of substrate hydrolysis.

To determine non-enzymatic hydrolysis of the substrate, sample reactions with only buffer (no tissue) were included. The radiolabeled substrate was added to the reaction vials at staggered (10 seconds) intervals to start the incubation and reactions were terminated by staggered addition of 100 µl of ChE “stop” solution. The cholinesterase stop solution was

prepared by adding 9.45 grams of chloroacetic acid, 2 grams of sodium hydroxide and 11.6 grams of sodium chloride to 100 ml of deionized water. Once the reactions had been stopped, 5 ml of organic scintillation cocktail was added to each vial. Organic scintillation cocktail for the cholinesterase assay consisted of 0.5% (w/v) 2, 5-diphenyloxazole (PPO), 0.03% (w/v) 1, 4-bis [5-phenyl-2-oxazolyl] benzene (POPOP) and 10% (v/v) isoamyl alcohol in toluene. The vials were capped and vortexed for 5-10 seconds and were then counted in a Tri-Car 2810 TR (PerkinElmer, Waltham, MA).

Population pharmacokinetic model

The plasma pyridostigmine concentration and time data were simultaneously fitted using the nonlinear regression program NONMEM (NONMEM 7.3.0, ICON Development Solution, Hanover, MD), interfaced with PDx- Pop (v5.2, GloboMax LLC, Hanover, MD, USA), in conjunction with a GFORTRAN compiler and R (R Foundation for Statistical Computing, Vienna, Austria) for statistical computing and graphics. Structural base, i.e. covariate-free, models were developed to compare model fitting in one and two compartment models. The pharmacokinetic models were developed under assumptions of first-order absorption and elimination (21, 22). This model was implemented using the ADVAN2 and TRANS2 PREDPP subroutines for one compartment and ADVAN4 and TRANS3 for a two compartment model. ADVAN2 and ADVAN4 are routines in PREDPP's library that implement the kinetic equations for the one and two compartment linear models with first-order absorption, respectively. TRANS2 is a routine in PREDPP's library which performs a re-parameterization of the basic pharmacokinetic (PK) parameters to the internal parameters, elimination constant (K) and absorption constant (Ka). Similarly, TRANS3 is a routine in PREDPP's library that performs a re-parameterization of the basic PK parameters to the internal parameters K, K12, K21 (intercompartmental elimination constants), and Ka.

The model was fitted to the data by using the first-order conditional estimation (FOCE) method with interaction. The pharmacokinetic parameters that were estimated by the one compartment analysis were absorption rate constant (Ka), apparent plasma oral clearance (CL /F) and apparent volume of distribution after oral administration (Vd/F).The structural pharmacokinetic parameters that were estimated in the two compartment analysis were absorption rate constant (Ka), apparent plasma oral clearance (CL/F), intercompartmental clearance (Q), apparent volume of distribution in the central compartment after oral administration divided by bioavailability (V/F), and the volume of distribution at steady state divided by bioavailability (Vss/F). The interindividual variability, or between subject variability (BSV), in pharmacokinetic parameters was modeled using exponential error models:

$$\theta i = \theta * \text{expo}(\eta i)$$

Where, θij is the ith individual value of the parameter, θ the typical value in the population and ηi, is the random effect or interindividual variability. A mean of zero was assumed for ηi normally distributed variable with standard deviation ω. The covariance of the parameters was studied during the modeling process. Residual variability was described by a standard proportional error model.

$$Yij = Fij*(1+\varepsilon 1ij)$$

Where, Yij is the observed concentration of ith individual on the jth occasion, Fij is the population predicted the concentration of ith individual on the jth occasion and $\varepsilon_1 ij$ is the residual error. It was assumed that ε had a mean zero and was normally distributed with standard deviation σ.

The performance of each model was evaluated by computing the objective function values (OFV), Akaike Information Criterion (AIC), Schwarz Bayesian criterion (SIC) (26), checking standard diagnostic plots of observed concentrations vs. model-predicted

concentrations, and plots of conditional weighted residuals vs. population model-predicted concentrations, subject identification, etc.

Results

Alterations in cholinesterase activities

Fig. 2 shows that the BChE activity in plasma was not affected when compared between the groups, however, it was lower on visits six to nine ($P<0.001$) when compared with pretreatment values. As shown in Fig. 3, AChE activity was progressively decreased with time and dosing and then returned to normal during down-titration of the pyridostigmine dose, until it was discontinued completed on visit 11 (approximately 70 days after beginning pyridostigmine therapy). When compared with the pretreatment value, the AChE activity decreased by approximately 10, 20 and 30% on visits 4-8. The activities of AChE were significantly lower on visits six to nine when compared to the pretreatment value ($P<0.001$) as well as between groups ($P<0.013$).

Figure 2: Plasma butyrylcholinesterase activity in congestive heart failure patients were analyzed on visit 3 to 12 of pyridostigmine administration. Data are presented as the mean (bars) and SEM (error bars).

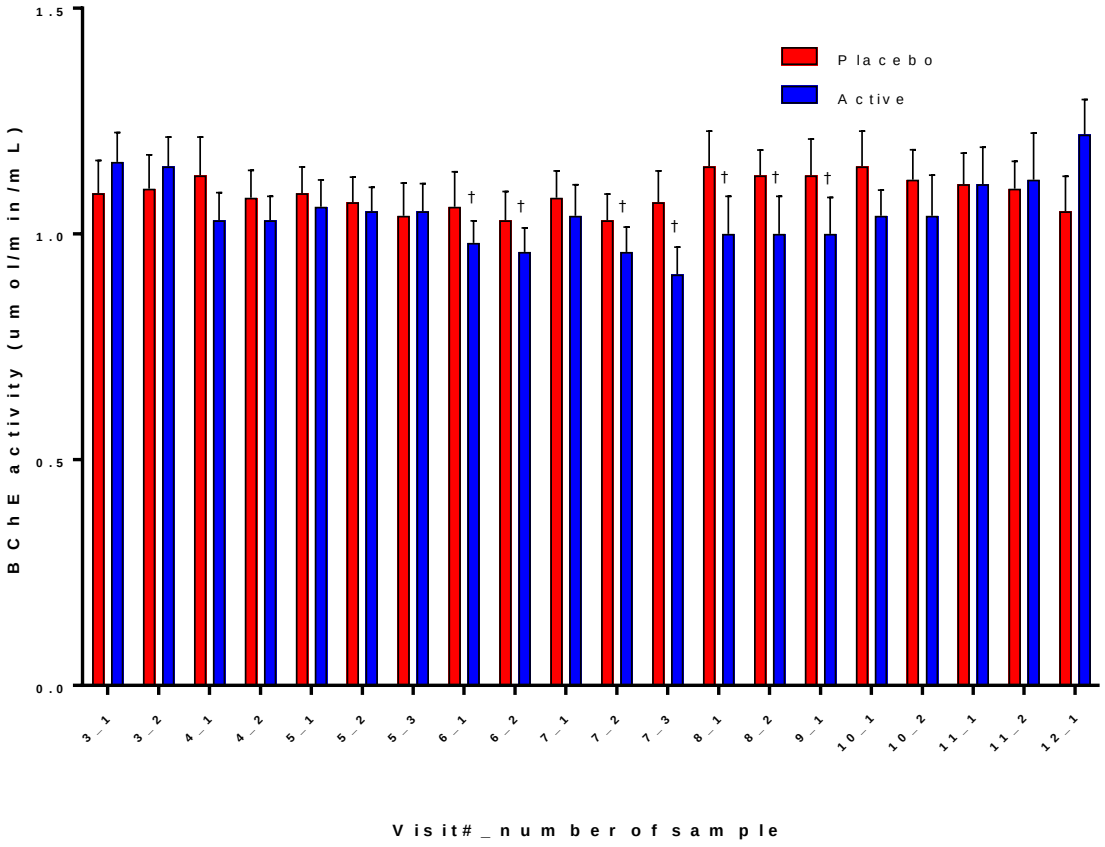
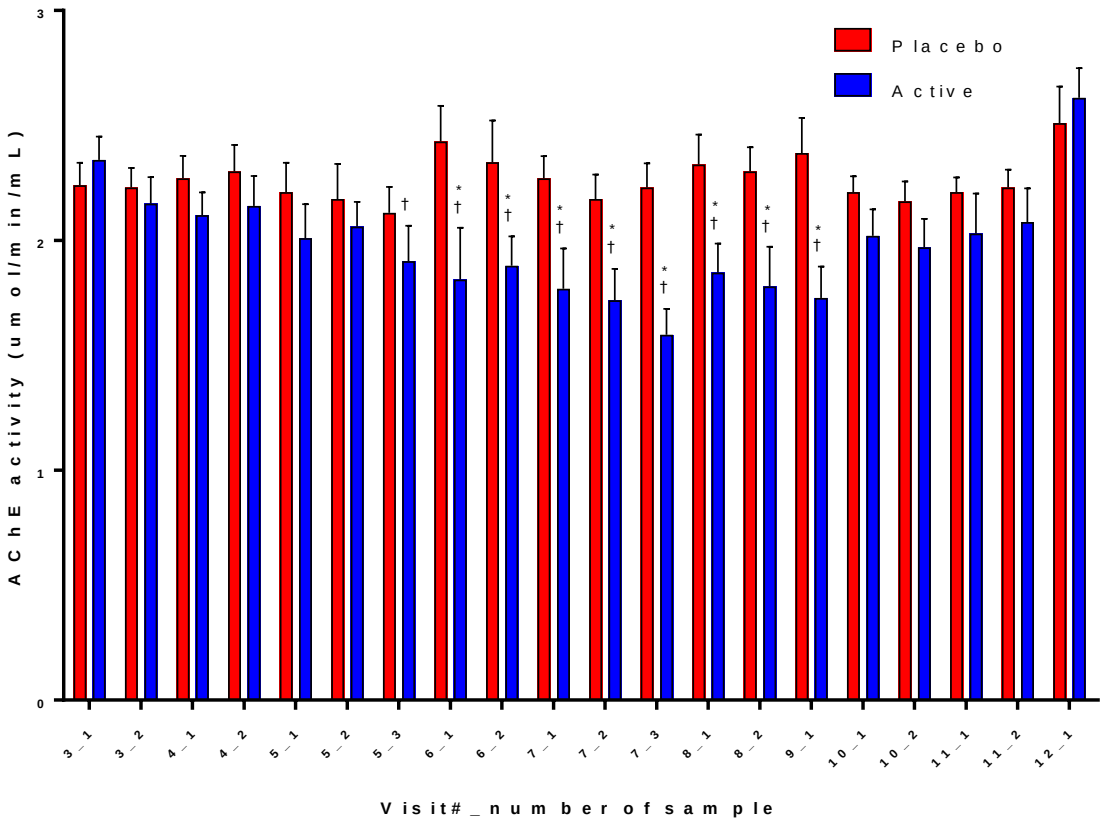


Figure 3: RBC Acetylcholinesterase activity in congestive heart failure patients were analyzed on visit 3 to 12 of pyridostigmine administration. Data are presented as the mean (bars) and SEM (error bars).



Population pharmacokinetic analysis

In total, 217 plasma pyridostigmine concentration–time points were available from the subjects for analysis. Based on the minimal change in the objective function value (OFV) and similarity in the goodness of fit plots with and without incorporation of a lag time, it was concluded that the data did not support the inclusion of an absorption lag time in the population

pharmacokinetic model. Parameter values for the one and two compartment population pharmacokinetic model are shown in Table 2.

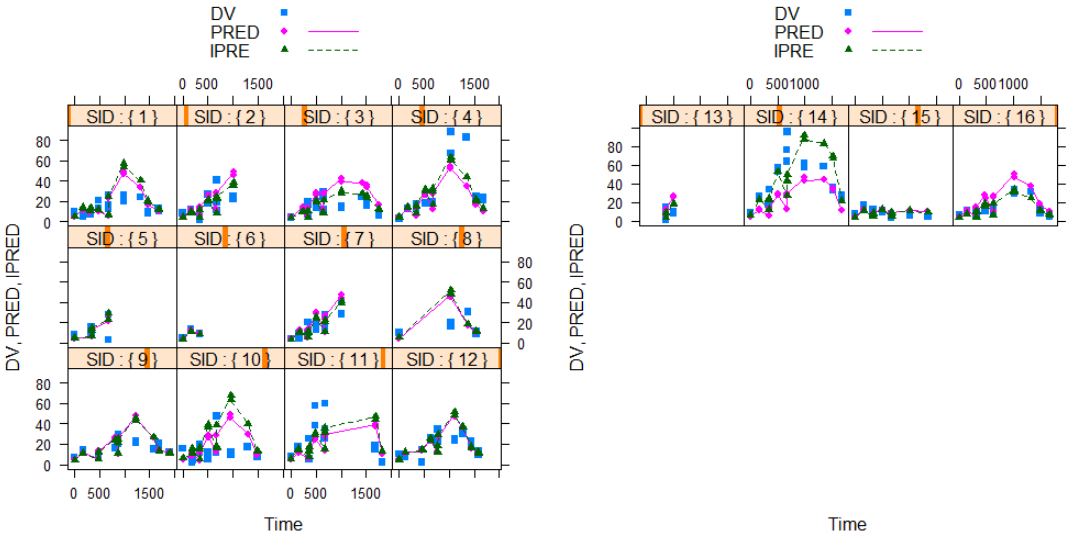
Table 2: Parameter estimations of the one and two compartment models for pyridostigmine

Parameter estimates	**One compartment**	**Two compartment**
CL/F (l/h)	187	208
Vd/F (l)	101	1110
Q/F (l/h)	-	509
V_{ss}/F (l)	-	9290
K_a (1/h)	0.0624	0.748
K_e (1/h)	1.85	0.19
T_{max} (h)	1.89	2.47
Half-life (h)	0.37	3.70
Interindividual variability (%)		
CL/F	29.0	31.4
Vd/F	1.39	36.5
Q/F	-	25.9
V_{ss}/F	-	35.6
K_a	21.3	5.32
Residual variability (%)		
	52.4	46.7

One compartment model: Fig. 4 represents the individual plots showing time vs. observed concentrations (DV), population predictions (PRED), and individual predictions (IPRE) for each

individual. It was noted that at many time point for most of the individuals the PRED and IPRE we overestimated indicating a bad fit of the model.

Figure 4: Individual plots showing time vs. observed concentrations (DV), population predictions (PRED), and individual predictions (IPRE) fitted in a one compartmental PK model.



The observed vs population-predicted and individual predicted concentrations are presented in Fig. 5 and 6, respectively.

Figure 5: Observed concentration (DV) vs. population prediction (PRED) in one compartmental PK model

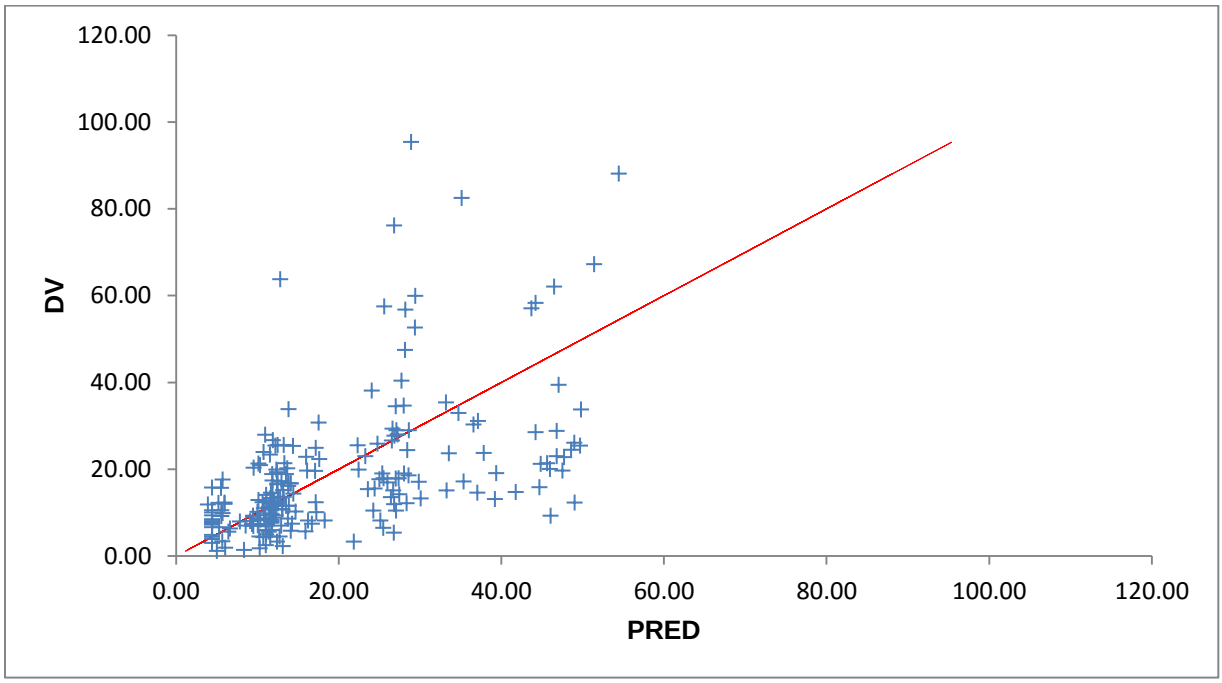
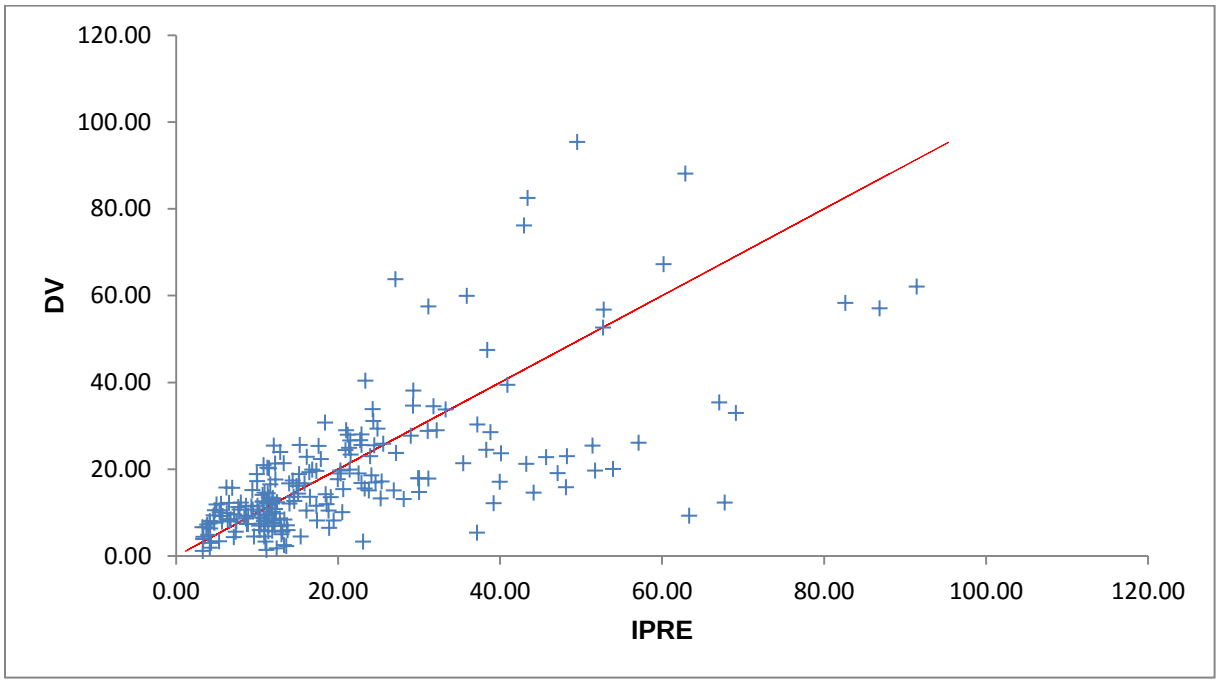


Figure 6: Observed concentration (DV) vs. individual prediction (IPRE) in one compartmental PK model



Overall the fit was judged to be good. However, in the distributions of weighted residuals shown in figures 7 and 8 as a function of sampling time and population-predicted values were homogenous indicating a good fit of the model to the data but had a non-significant slight negative slope.

Figure 7: Weighted residual (WRES) vs. time plot for one compartmental PK model

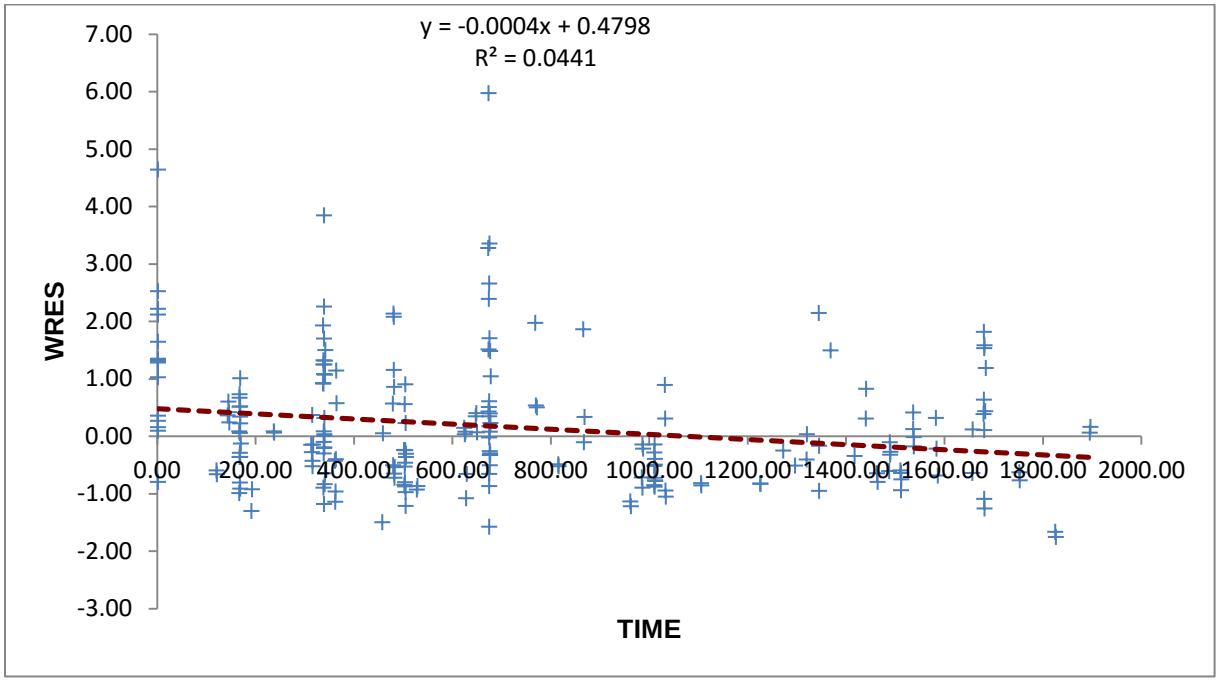
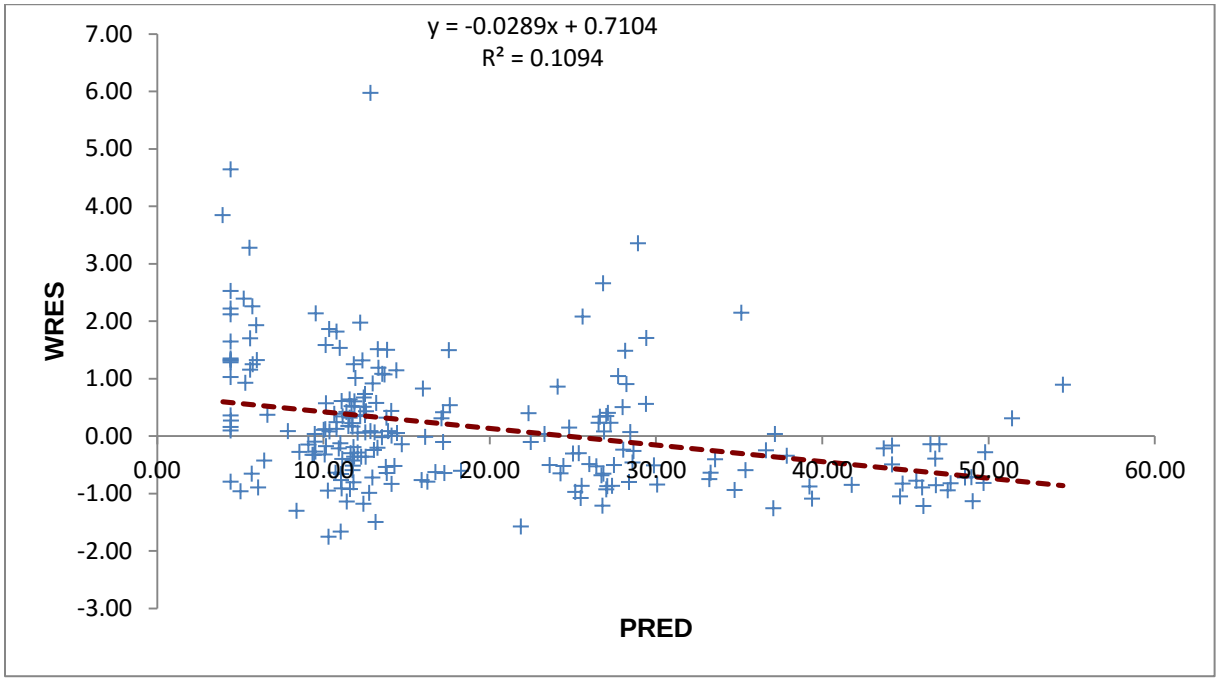


Figure 8: Weighted residual (WRES) vs. population prediction (PRED) plot for one compartmental PK model



The OFV, AIC, and SIC values for the models are shown in Table 3. It was seen that all the parameter values were lower for the two compartment model than the one compartment.

Table 3: Comparison OFV, AIC and SIC values of one and two compartment model for pyridostigmine

Parameters	One compartment	Two compartment
OFV	1175.4	1120.3
AIC	1189.4	1142.3
SIC	1213.1	1179.5

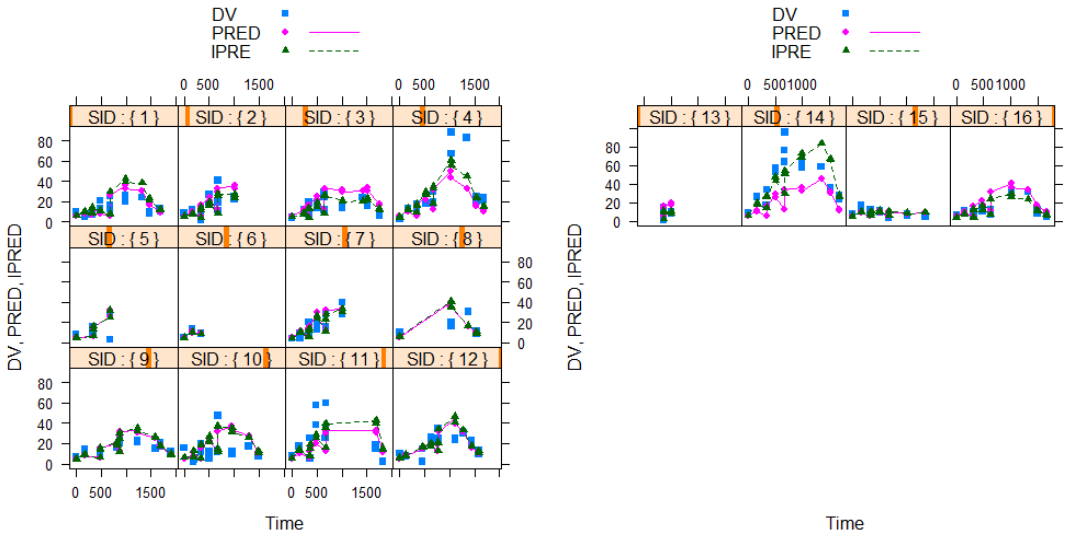
The estimated times for maximum concentration after a dose (T_{max}) was 1.89 h, calculated from each subject's conditional estimates of K_a and K by the standard formula: $T_{max} = \ln(K_a/K)/(K_a–K)$ for a one compartment extravascular administration model (27). Typical values for CL/F calculated for the studied population were 187 ± 14.6 l/h. The typical values of Vd/F and Ka for all subjects were 101 ± 16 and 0.06 ± 0.004/h, respectively. The inter-individual variability about CL/F, Vd/F, and Ka was 29.0%, 1.39%, and 21.3%, respectively. The residual variability was 52.4% for the model. The terminal half-life (t½), derived from the expression t½ = (0.693* Vd/F)/(CL/F), with individual estimates of CL/F and Vd/F from each patient, for pyridostigmine plasma concentrations was 0.37 h (Table 2).

Two compartment model:

Fig. 9 represents the individual plots showing time vs. observed concentrations (DV), population predictions (PRED), and individual predictions (IPRE) for each individual in a two compartment model. When compared to the one compartment model, for most of the individuals

the PRED and IPRE values were close to the observed concentrations indicating better fitting of the two compartment model.

Figure 9: Individual plots showing time vs. observed concentration (DV), population prediction (PRED), and individual prediction (IPRE) fitted in a two compartmental PK model.



The observed vs. population-predicted and individual predicted concentrations are presented in Fig. 10 and 11, respectively.

Figure 10: Observed concentrations (DV) vs. population predictions (PRED) in two compartmental PK model

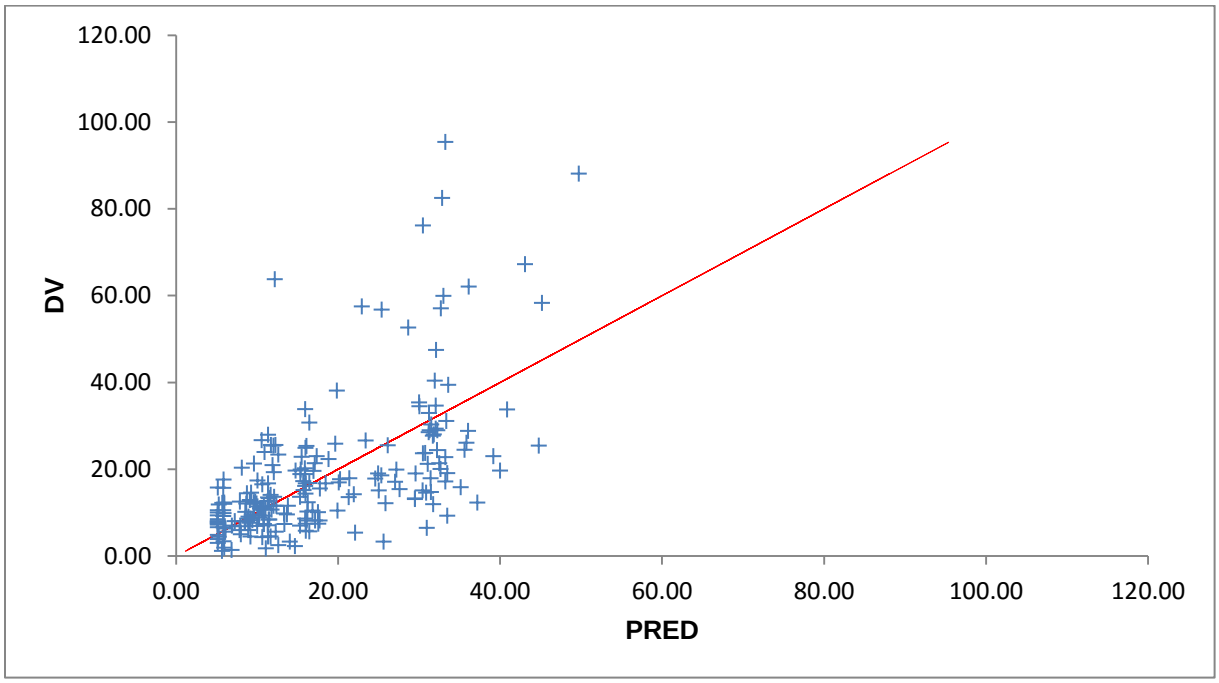


Figure 11: Observed concentrations (DV) vs. individual predictions (IPRE) in two compartmental PK model

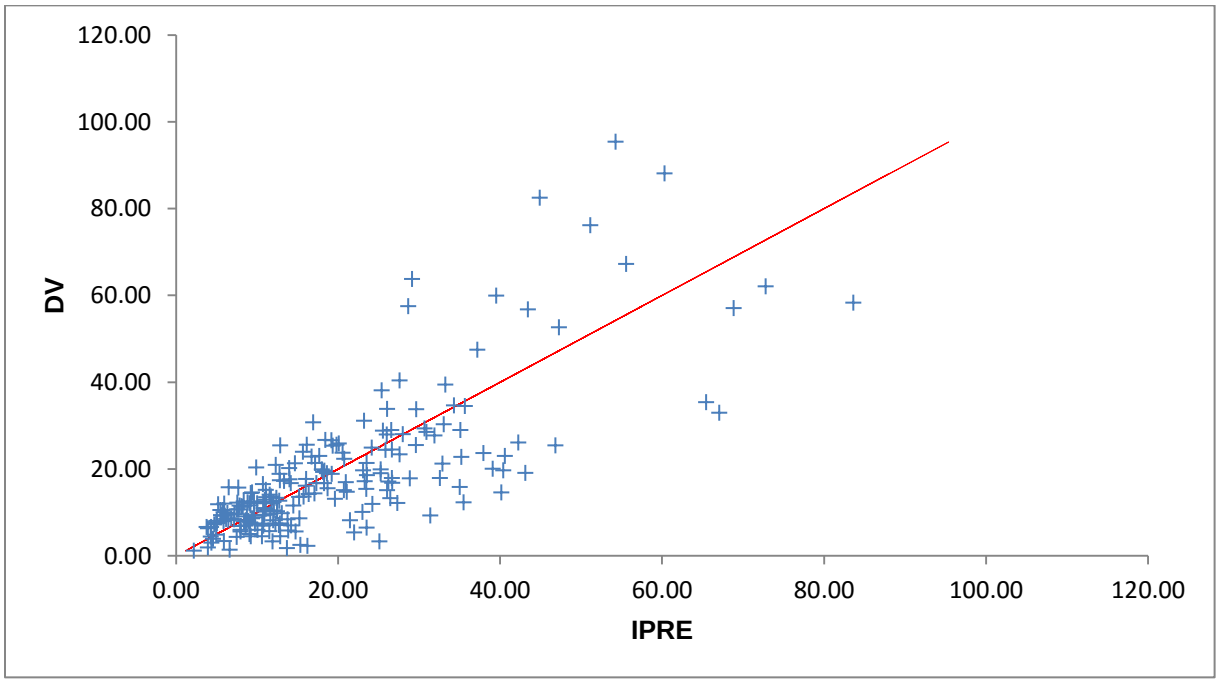


Figure 12 and 13 represent the distribution of the weighted residuals as a function of sampling time and population-predicted values. The distribution was homogenous indicating a good fit of the model to the data.

Figure 12: Weighted residual (WRES) vs. time plot for two compartmental PK model

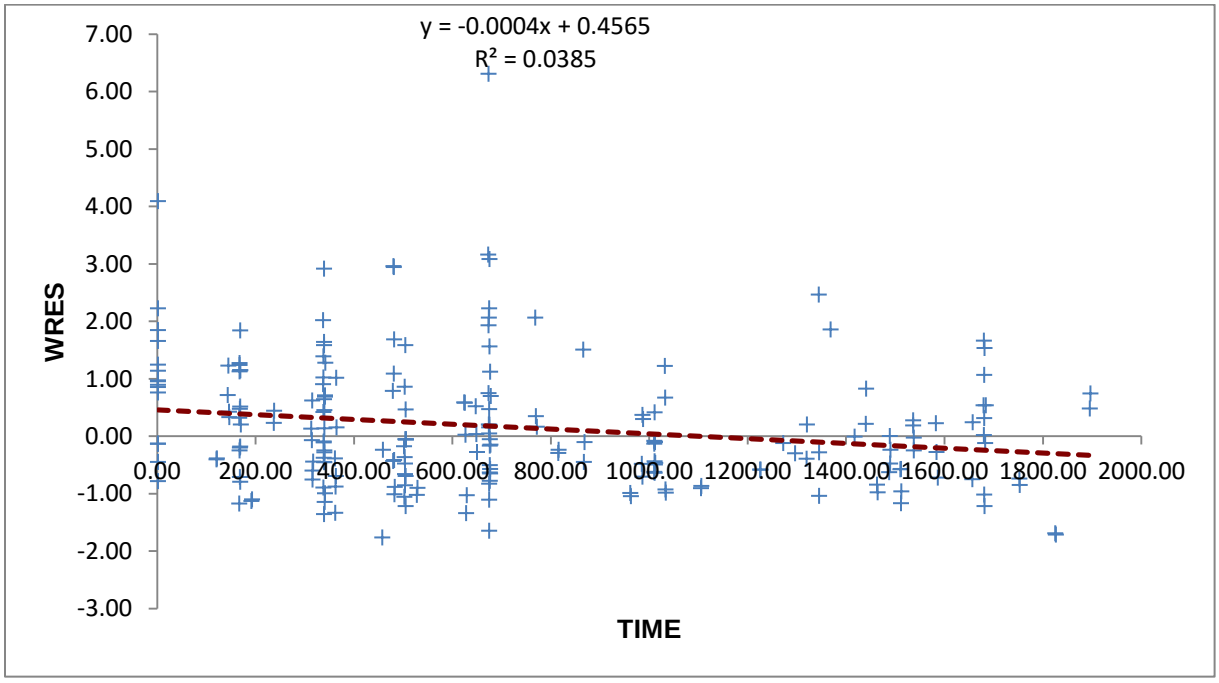
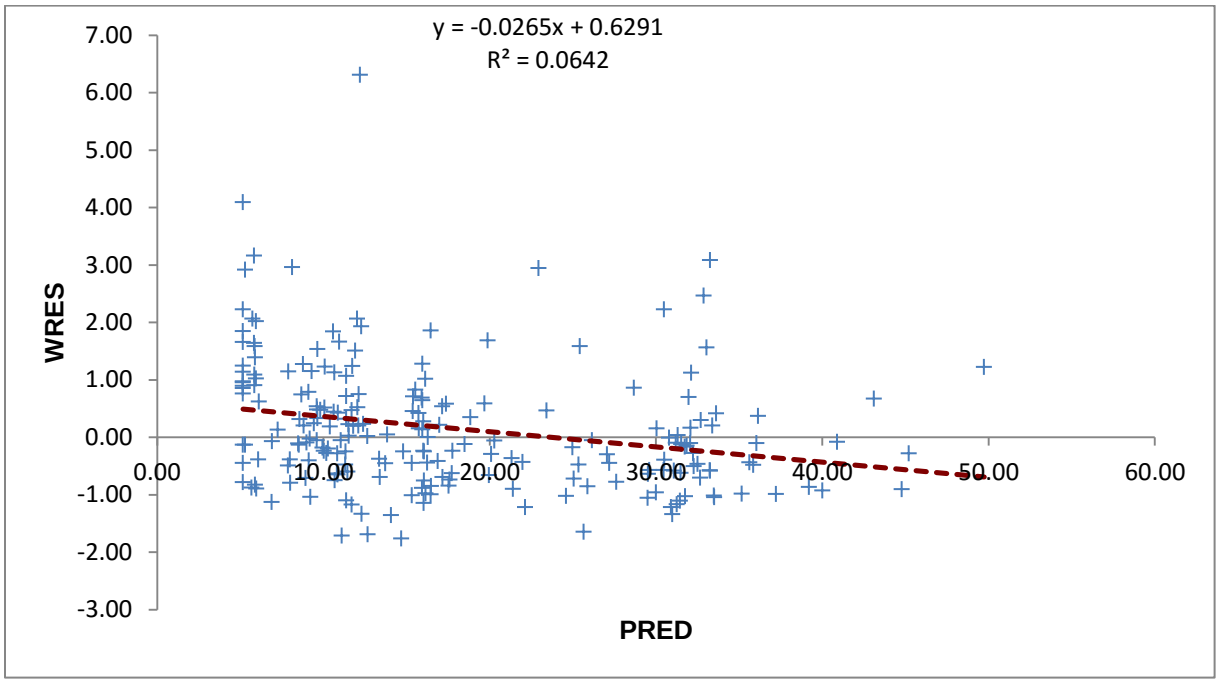


Figure 13: Weighted residual (WRES) vs. population prediction (PRED) plot for two compartmental PK model



The estimated time (T_{max}) of maximum plasma pyridostigmine concentrations after oral pyridostigmine administration was 2.47 h, calculated by the standard formula $T_{max} = \ln(K_a/K)/(K_a – K)$ for a one-compartment extravascular model. The mean value of CL/F calculated for the studied population was 208 L/h. The mean values of Vd/F, Q, V_{ss} and K_a were, 1110 L, 509 L/h, 9290 L, 0.748, respectively. The interindividual variabilities in CL/F, Vd/F, Q, V_{ss} and K_a were 31.4%, 36.5%, 25.9%, 35.6% and 5.32%, respectively. The residual variability was 46.7% for the model. The terminal phase half-life (t½), determined by the formula, t½ = (0.693* Vd/F)/(CL/F) using individual estimates of CL/F and Vd/F, for pyridostigmine plasma concentrations, was 3.7 h. The observed vs. population-predicted and individual predicted concentrations are presented in Figures 10 and 11. Overall the fit was judged to be good, as the distributions of weighted residuals as a function of sampling time, population-predicted values, or subject ID were homogenous without a significant slope to the best fit linear regression line of the residual values (Figures 12 and 13; Table 2).

Discussion

The progressive decrease in the AChE activity corresponds to the drug uptitration from visits 4-7 (15 and 30 mg) and drug maintenance (60 mg) from visits 8-9. As expected, during the down-titration of the drug, AChE activity gradually increased back to approximate pretreatment values during visits 10 to 12. Previous studies in humans reported approximately 10% inhibition of the RBC AChE activity after administration of 30 mg tablet three times a day which was true for animal studies also (22, 28). The dosing simulations performed by Seng et al. suggested that the oral administration of 30 mg every six hours would be required to achieve steady-state trough percentage inhibition above the recommended 10% in healthy Chinese males. However, in

another study by Marino et al. in the Caucasian population, a similar dose every 8 hours was recommended (22).

To our knowledge, this is the first investigation in which nonlinear mixed effect modeling has been applied to investigate oral pyridostigmine pharmacokinetics in human congestive heart failure patients. The current study developed a population pharmacokinetic model and estimated the associated population pharmacokinetic parameters and their associated variability. One and two compartment models were compared in this study. For both models, there were no trends or patterns in the goodness-of-fit plots, and other model evaluation criteria were also indicated that the models described the data well.

Although we did not estimate the interoccasion variability, the high interindividual variability values estimated in this study suggest a low interoccasion variability. In a previous study by Seng et al., the authors did not include the interoccasion variability in the model because it was found to be greater than the interindividual variability. In such cases, each individual is different from each other individual as well as being different from himself on other occasions. In that case, it is reasonable to treat the individuals as different subjects on each unique occasion.

In this study, the residual variability was similar between one and two compartment models but was much higher than that estimated previously (21). Residual variability arises from multiple sources, including assay variability, errors in sample time collection, and model misspecification. In our case, it is important to note that the time of sample collection was not identical for all subjects. A wide window of ±4 days around each visit was designed for the convenience of the patients. This may have contributed to the higher values of residual variability in our models. Secondly, the quantitation limits of the pyridostigmine assay ranged from 1 to 50 ng/mL. The concentrations that were higher than 50 ng/mL were extrapolated using a quadratic

equation and 1/x weighting scheme. This extrapolation may also have added to the increased residual variability in the model.

Our estimates of oral Cl/F, determined from either compartmental model were comparable to that reported in a one-compartmental model study by Marino et al. and supports the idea of poor absorption of pyridostigmine from the GIT or high metabolism of the drug due to the first pass effect (29). The K_a of the two compartment model estimated in this study was compatible with the K_a reported in the two compartment population study by Seng et al. (21). However, K_a measured by using the one-compartment model in our study was substantially lower than the K_a reported by Marino et al., supporting the inadequacy of the one-compartment model in our study (22).

In our current study, the two-compartment model showed lower values of OFV, AIC and SIC (Table 3) as compared with the use of the one-compartment model. The minimum OFV determined by parameter estimation during model development is important for comparing models and ranking them. Similarly, AIC and SIC values aid in comparing complex models by compensating for improvements of fit due to increased model complexity (e.g. increased number of compartments) (30). Individual plots illustrate how well the model describes the data for any given subject. The individual plots of observed, population predicted, and individual predicted concentration plotted against time in fig. 4 showed that for most of the individuals and most time points, the PRED and IPRE were overestimated by the one-compartment model, again indicating a poor fit of the model. In contrast, the two compartment model gave a better fit for the individual plasma pyridostigmine concentration data and prediction values (Fig. 9). Other diagnostic plots, such as observed concentration vs. population and individual predictions (Figure 5, 6, 10 and 11), did not show as much difference between the compartmental models. Similarly, when weighted residuals were plotted against time and population predictions (Figure 7, 8, 12 and 13), the resulting scatter remained similar for both of the models, without any significant trend.

The diagnostic plots give a similar idea of the goodness of the models, but the OFV value and parameter estimates indicated that the two-compartment model was a better fit for this data set.

In our study population, the subjects' renal functions exceeded the upper limits of the normal values and body weight, age and liver functions showed a relatively broad range as compared with other covariates (Table 1). These abnormal function values of kidneys and liver were expected in congestive heart failure patients. However, studies suggest that GFR has an inverse graded association with heart failure severity (31), but biochemical parameters of liver function, such as AST and ALT, are moderately elevated to two to three times the upper normal reference level (32). It would be interesting to include these covariates in the mixed effect model to study their relationships to the parameter estimates and test whether such covariates can partially explain the variability in the pharmacokinetic model. In the study by Marino et al. (22), body weight and gender were significantly correlated to Vd/F and Cl/F but such relationships were not observed in the Chinese population study by Seng et al. (21). This discrepancy between the two studies was attributed to the narrow range of covariates present in the study by Seng et al. and a wider range of covariates in the study by Marino et al. (21, 22).

However, to support these study findings it is required to study the relationship of covariates with the parameter estimates and variability terms. We also recommended performing more efficient analysis by procuring larger sample sizes, extensive sampling after the last dose and incorporating a higher number of potentially important covariate information.

Conclusion

The pharmacokinetics of pyridostigmine was described using a nonlinear nixed effect model and the effects of pyridostigmine on red blood cell AChE and BChE activities were determined. A one-compartment and two-compartment model was successfully fitted to the plasma pyridostigmine concentration–time data.

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CHAPTER VI

SUMMARY AND CONCLUSION

Neuroendocrine dysregulation is considered to be an important determinant of mortality and morbidity in patients with congestive heart failure (1). Sympathetic overactivity and parasympathetic withdrawal indicate profound dysregulation of autonomic control. It is possible that pharmacological intervention may play a role in decreasing mortality by improving sympathovagal balance.

Pyridostigmine is a cholinergic stimulant that binds to the acetylcholinesterase enzyme and inhibits its action on acetylcholine. Studies suggest that pyridostigmine can be effective in returning the balance between sympathetic and parasympathetic control of the heart (2-5). As a consequence, we hypothesized that pyridostigmine administration to congestive heart failure (CHF) patients would restore the balance between the sympathetic and parasympathetic arms of the autonomic system, improve heart rate recovery, and enhance the rate of survival associated with this disease.

Determination of the effects of pyridostigmine on heart rate recovery in rats

Rapid heart rate deceleration within the first minute after maximum exercise is regulated primarily by reactivation of the parasympathetic nervous system (6, 7). A blunted decrease in heart rate during the first minute after maximal exercise has been associated with an increase in the subsequent risk of mortality in subjects with and without cardiovascular disease (8-12). To the best of our knowledge, there is no other published literature on the use of heart rate recovery (HRR) in rats. We developed a rodent model of HRR to assess parasympathetic tone. Using HRR to assess parasympathetic tone is non-invasive, easy to perform and less technical than heart rate variability and other such complex assessment methods. Using this exercise model, we determined that subacute administration of pyridostigmine in rats enhanced HRR. The results of our study were generally in agreement with current knowledge regarding the effects of pyridostigmine on heart rate recovery. Pyridostigmine enhanced heart rate recovery by enhancing

the parasympathetic tone of the heart. The model could be used in future studies that can answer emerging questions regarding the best use of pyridostigmine in CHF patients.

Analytical method to quantify pyridostigmine and its metabolite in human plasma

A sensitive analytical method was developed to quantify pyridostigmine (PYR) and its metabolite 3-hyroxy-N-methylpyridinium (3-OH NMP) in human plasma using a sensitive liquid chromatography–electrospray ionization–tandem mass spectrometry assay. For PYR sample preparation, plasma was processed by a solid phase extraction procedure, using weak cation exchange (WCX) cartridges, whereas 3-OH NMP was precipitated by acetonitrile. The HILIC mode of LC separation was used, and eluted drugs were quantified via MRM positive ion mode ESI in a tandem mass spectrometer. The lower limit of quantitation (LLOQ) of PYR and 3-OH NMP assays were determined to be 0.25 and one ng/mL, respectively. To our knowledge, the 3-OH NMP assay developed in our study was the first method to use mass spectrometry to quantify 3-OH NMP in human plasma.

Overall, a sensitive and selective method was validated for the routine LC/MS/MS analysis of PYR and 3-OH NMP from human plasma. After incorporating modifications to improve specificity, the method was applied to a population pharmacokinetic and study of pyridostigmine bromide in congestive heart failure patients. This method has higher sensitivity as compared to other methods available to quantify pyridostigmine in human samples.

Population pharmacokinetic model of pyridostigmine in congestive heart failure

Pyridostigmine bromide was orally administered to human CHF patients for several months, and blood samples were collected during the study period to determine the plasma concentrations of pyridostigmine and its metabolite, as well of the activity of blood and plasma esterases. The plasma concentrations of pyridostigmine were analyzed using NONMEM software and two structural base models; one and two compartment models were compared using

a population-based pharmacokinetic approach. A two-compartment model was best fitted to the data obtained from repeated oral dosing of pyridostigmine in these CHF patients. The two compartment model showed a lower OFV, AIC, and SIC and had better diagnostic plots than did the one-compartment model. The typical values for CL/F, Vd/F, Q/F, Vss/F and Ka for the two compartment model were, 208 L/h, 1110 L, 509 L/h, 9290L, and 0.748 L/h, respectively. The interindividual variability in CL/F, Vd/F, Q/F, Vss/F and Ka was 31.4%, 36.5%, 25.9%, 35.6% and 5.32%, respectively, and residual variability was 46.7% for the model. The terminal phase half-life (t½) was 3.7 h for the same model. In the future, this population PK base model will aid in the development of a model that can assess associations between pyridostigmine dose, efficacy, safety, and patient covariates.

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