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Brief communication (Original)

Validation of a high-performance liquid chromatography method for the determination of pyridostigmine in plasma

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Background: Pyridostigmine is a reversible acetylcholinesterase inhibitor that is used in military medicine as a prophylactic agent against intoxication by nerve agents and in the treatment of myasthenia.

Objective: We developed and validated a high-performance liquid chromatography (HPLC) method for the quantification of pyridostigmine in plasma samples.

Methods: Pyridostigmine was isolated from plasma using one-step protein precipitation by chloroform. The separation was performed on an analytical C18 column (250 × 4.6 mm; 4 µm particle size). The detector wavelength was set at 270 nm. The mobile phase was a mixture of acetonitrile and aqueous solution (15:85 v/v) of sodium-1-hepatene sulfonate and triethylamine, adjusted to pH 3.0 at a flow rate of 1.5 ml min^{-1}. Hydrochlorothiazide was used as internal standard.

Results: The recovery of drug from plasma samples was above 90%. Using the current method, pyridostigmine (r_t = 7.3) and hydrochlorothiazide (r_t = 6.4) peaks were well resolved. The calibration curve was linear over the concentration range of 15–60 ng ml^{-1}. The inter- and intraday assay coefficients of variation were found to be less than 8%.

Conclusion: The currently described procedure could be used as a simple, rapid and sensitive tool in bioavailability and bioequivalency investigations for the quantification of pyridostigmine in human plasma samples.

Keywords: Drug, high-performance liquid chromatography, Mestinon, method validation

Pyridostigmine is an anticholinergic agent that acts through inhibition of enzyme cholinesterase. This drug is indicated for the treatment of myasthenia gravis (a progressive autoimmune neuromuscular disorder characterized by fluctuating muscle weakness and fatigue), along with gastrointestinal motility disorders, postoperative urinary retention, and prophylaxis of muscular paralysis as a result of nerve agents. Pyridostigmine has low oral absorption and undergoes extensive metabolism in the liver and plasma. The primary elimination route of this drug is renal, with a half-life around 3 to 4 hours [1].

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In 2002, the US-FDA approved the indication of pyridostigmine for the prophylaxis of respiratory muscles paralysis as a result of the nerve agent soman. This has been the first approval of a drug without passing necessary clinical trials and was based only on preclinical findings on guinea pigs and apes (the animal efficacy rule) [1, 2]. Soman is a potent nerve agent that acts through irreversible inhibition of acetylcholinesterase, thereby causing flaccid paralysis and respiratory failure, which eventually leads to death. Cholinergic compounds such as atropine and pralidoxime are the antidotes for nerve agents and should be used intravenously immediately after exposure [3]. On the other hand, pyridostigmine and related medications prevent the interaction of cholinesterase with nerve agents through reversible

binding to the enzyme. Therefore, these drugs should be used prior to the exposure and only for prophylaxis purposes. The suggested dose for pyridostigmine is 30 mg every 8 hours [4]. Because this dose of pyridostigmine is lower than that used for myasthenia gravis, the side effects are predictable and there is no need to conduct time-consuming toxicology studies [5]. With respect to the short half-life of pyridostigmine on the one hand, and the importance of producing constant plasma concentrations together with the ease of use during harsh war conditions on the other hand, a sustained release form of this drug is the preferred dosage form. Recently, sustained release pyridostigmine has been presented as 180 mg tablets that could be used once daily. Such a dosage form of pyridostigmine is only available in a limited number of countries including Germany (MEDA Pharma, Bad Homburg) and the United States (Valeant Pharmaceuticals International, CA). It has been recently prepared in Iran by the School of Pharmacy at the Shaheed Beheshti University of Medical Sciences (Tehran, Iran). Like every new dosage form, this sustained release formulation requires investigation in terms of bioavailability and bioequivalency. The prerequisite for performing such investigations is development of a validated method for the quantification of drug in plasma samples. In the current research, a high-performance liquid chromatography (HPLC)-based method with high sensitivity and reproducibility was designed and applied for the extraction and determination of pyridostigmine in plasma.

Materials and methods

Chemicals

Standard pyridostigmine powder, hydrochlorothiazide (employed as internal standard), sodium-1-hepatene sulfonate, triethylamine, and HPLC grade acetonitrile were obtained from Merck, Darmstadt, Germany. Frozen human plasma was supplied by the blood bank.

Instruments and chromatographic conditions

A Knauer HPLC system (Germany) was employed and consisted of a Wellchrom K-1001 pump, a Rheodyne 7125 injector, and a K 2501 UV detector connected to a Eurochrom 2000 integrator. The separation was performed on an analytical C18 column (Knauer; 250 × 4.6 mm; 4 μm particle size). The wavelength was set at 270 nm. The mobile phase was a mixture of acetonitrile and aqueous solution (15:85 v/v) of sodium-1-hepatene sulfonate (0.2% w/v) and triethylamine (5% v/v) (5 mL) adjusted to pH 3.0 at a flow rate of 1.5 mL min^{-1}.

Sample preparation

To 300 μL of plasma, 20 μL of hydrochlorothiazide solution (180 ng mL^{-1}; used as internal standard) and 450 μL of acetonitrile and saturated sodium chloride solution were added. The resulting mixture was vortexed for 60 seconds and then centrifuged at 8000 rpm for 5 minutes. At the next step, 600 μL of the supernatant was mixed with 1000 μL chloroform and again vortexed (60 seconds) and centrifuged (8000 rpm; 5 minutes). After discarding the upper aqueous layer, the organic layer was dried under nitrogen. Dry residue was then dissolved in water (60 μL), sonicated (2 minutes), and centrifuged (2 minutes). This final solution (50 μL) was injected into the HPLC column.

Plasma calibration curves and quantification

Blank plasma was prepared from heparinized whole-blood samples collected from healthy volunteers and stored at –20°C. After thawing, final concentrations of 5, 10, 20, 30, 60, 80, and 100 ng mL^{-1} were prepared from pyridostigmine. Internal standard solution was added and the samples were then prepared for analysis as described above. Calibration curves were constructed by plotting peak area ratio (y) of pyridostigmine to the internal standard versus pyridostigmine concentrations (x). A linear regression method was used for quantification.

Precision, accuracy, and recovery

The precision and accuracy of the method were examined by adding known amounts of pyridostigmine to drug-free plasma (quality control samples) in order to achieve 15, 45, and 60 ng mL^{-1} concentrations. For intraday precision and accuracy, three replicate quality control samples at each concentration were assayed on the same day. The interday precision and accuracy were evaluated on three different days. The analytical recovery for plasma at three different concentrations of pyridostigmine (15, 45, and 60 ng mL^{-1}) was also determined. Known amounts of pyridostigmine were added to drug-free plasma and the internal standard was then added. The relative recovery of pyridostigmine was calculated by comparing the peak areas for extracted pyridostigmine from spiked plasma and a standard aqueous solution of pyridostigmine

containing internal standard with the same initial concentration, using the following formula:

% recovery = peak area in plasma sample/peak area in aqueous sample × 100

Results

Under the chromatographic conditions described above, pyridostigmine and the internal standard peaks were well resolved. **Figure1** shows typical chromatograms of blank plasma in comparison to spiked samples analyzed. The average retention times of pyridostigmine and hydrochlorothiazide were 7.3 and 6.4 minutes, respectively. The regression coefficient of the calibration curve for the determination of pyridostigmine in plasma was 0.998 (**Figure 2**).

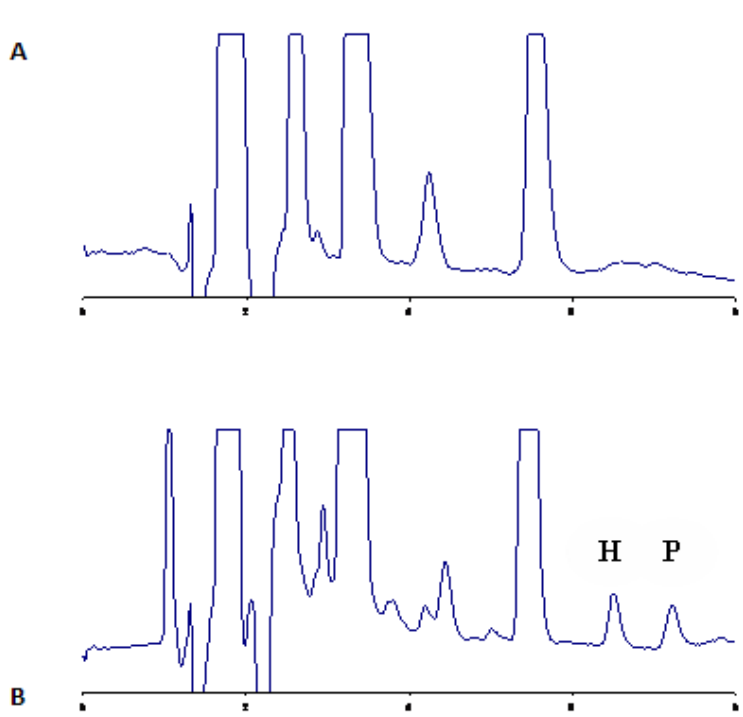

Figure 1. Chromatograms of (**A**) blank plasma and (**B**) blank plasma spiked with pyridostigmine (P) and hydrochlorothiazide (H) as internal standard

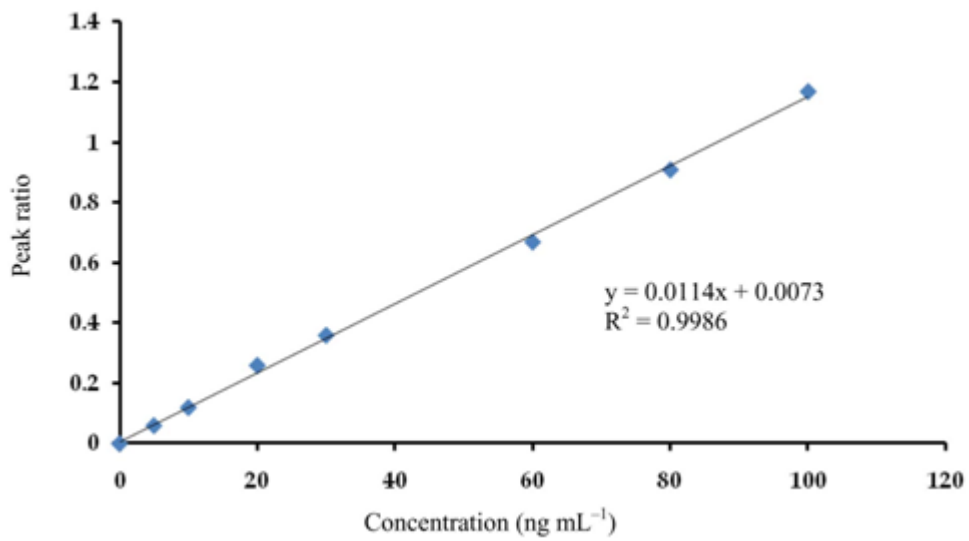

Figure 2. Calibration curve for the determination of pyridostigmine in plasma. Peak ratio represents the ratio between pyridostigmine peak area to internal standard (hydrochlorothiazide) peak area

The relative analytical recovery for plasma at three different concentrations of pyridostigmine was determined. In the concentration range of 15–60 ng mL^{-1}, the average recovery was 90.12 ± 1.04% (**Table 1**). The limit of quantification (LOQ) was defined as the pyridostigmine concentration that produced a signal-to-noise ratio greater than 10. The LOQ in plasma was 5 ng mL^{-1}. The precision of the method was assessed using repeated analysis of plasma specimens containing known concentrations of pyridostigmine. As shown in **Table 1**, coefficients of variation were less than 8%, which is acceptable for the routine measurement of pyridostigmine.

Discussion

Immediate release tablets of pyridostigmine have a short duration of action and hence need to be used every 8 hours. Therefore, modification of pyridostigmine formulation into a sustained release dosage form would be highly desirable for military medicine in terms of producing long lasting effects and ease of use in the harsh conditions of war. Such an improvement in the formulation would influence the release pattern and plasma concentration of drug. These changes necessitate bioavailability and bioequivalency studies to be conducted. On the other hand, an important prerequisite for such studies is to set a validated method for the quantification of drug in plasma in a simple, rapid, accurate, and reproducible fashion. To this end, a validated HPLC-based method was designed in the present study, capable of measuring drug concentrations with high accuracy and precision. The extraction method that was introduced here is a simple procedure that can yield recovery rates greater than 90%. In addition, this method has a favorable reproducibility with %CV rates between 3% and 7% for both interday and intraday variations. The high sensitivity (LOQ of 5 ng mL^{-1}) and linearity over a wide concentration range (15–60 ng mL^{-1}) are other advantages of the introduced method. Hitherto, different methods have been applied for the determination of pyridostigmine in human plasma. Among these methods, HPLC has been among the most reliable [6, 7]. Because UV absorption of pyridostigmine is low, specific detectors and extraction procedures are required for quantification. Terry and Teitelbaum could detect as low as 5 ng mL^{-1} concentrations of pyridostigmine in plasma using ion-pair extraction and CN-propyl column chromatography [8]. In spite of its efficacy, this method requires several consecutive and time-consuming extraction steps that limit its application in routine practice. Some other researchers have applied solid phase extraction for the determination of pyridostigmine. However, this method has a lower sensitivity compared to that described here, and is not applicable in all laboratories because of its high cost [9-12]. With liquid–liquid extraction, problems such as low LOQ have been raised which make this method unsuitable for using at terminal time points of bioavailability studies [9]. Recently, LC–MS approaches have been employed for the detection of pyridostigmine, but such methods are costly and require proficient operators [13, 14]. Taken into account the problems of the aforementioned methods, the procedure described here can provide a simple, rapid, inexpensive, reproducible, and sensitive method to be used for reliable determination of plasma concentrations of pyridostigmine in bioavailability and bioequivalency studies.

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Table 1. Reproducibility and recovery of pyridostigmine in plasma

Spiked concentration of pyridostigmine (ng mL^{-1})	Concentration measured (ng mL^{-1})				% recovery
	Interday		Intraday		
	Mean ± SD	%CV	Mean ± SD	%CV	
15	0.15 □ 0.01	6.88	0.15 □ 0.01	7.00	90.03 □ 1.44
45	0.47 □ 0.02	3.32	0.47 □ 0.04	7.58	91.20 □ 1.10
60	0.70 □ 0.03	4.52	0.72 □ 0.04	5.80	89.13 □ 1.19

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