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Development and validation of a sensitive HPLC method for the quantification of HI-6 in guinea pig plasma and evaluated in domestic swine☆,☆☆

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ABSTRACT

A rapid and small volume assay to quantify HI-6 in plasma was developed to further the development and licensing of an intravenous formulation of HI-6. The objective of this method was to develop a sensitive and rapid assay that clearly resolved HI-6 and an internal standard in saline and plasma matrices. A fully validated method using ion-pair HPLC and 2-PAM as the internal standard fulfilled these requirements. Small plasma samples of 35 μL were extracted using acidification, filtration and neutralization. Linearity was shown for over 4 μg/mL to 1 mg/mL with accuracy and precision within 6% relative error at the lower limit of detection. This method was utilized in the pharmacokinetic analysis HI-6 dichloride (2Cl) and HI-6 dimethane sulfonate (DMS) in anaesthetized guinea pigs and domestic swine following an intravenous bolus administration. From the resultant pharmacokinetic parameters a target plasma concentration of 100 μM was established and maintained in guinea pigs receiving an intravenous infusion. This validated method allows for the analysis of low volume samples, increased sample numbers and is applicable to the determination of pharmacokinetic profiles and parameters.

1. Introduction

Organophosphorous nerve agents (NA) inhibit acetylcholinesterase (AChE), an enzyme central to the metabolism of acetylcholine (ACh). AChE inhibition results in ACh accumulation within the central and peripheral nervous systems, causing overstimulation that manifests as a cholinergic crisis [1]. Antidotal regimens used to treat OP poisoning generally include an antimuscarinic agent (such as atropine) [2] that competitively binds to muscarinic receptors and antagonizes the actions of ACh and an oxime [2] that reactivates AChE by breaking the AChE-nerve agent bond. This treatment is supplemented with an anticonvulsant [3]. The standard practice is to distribute atropine/oxime autoinjector syringes to first responder personnel for the acute treatment of NA exposure. While the use of autoinjectors is convenient for immediate field treatment, casualties should have access to improved treatment options upon arrival at an appropriate medical facility. Improved treatment options may include intravenous (iv) administration of atropine/oxime to allow for controlled therapy.

The current treatment regimen used by the Canadian Forces for NA poisoning involves administration of three atropine/oxime autoinjectors and the anticonvulsant diazepam. The oxime currently preferred for use by the Canadian Forces is HI-6 ([(Z)-[1-[(4-carbamoylpyridin-1-yl)methoxymethyl]pyridin-2-ylidene]methyl]-oxo-azanium). For NA exposures in which a casualty may require more than the standard treatment of three autoinjectors, such as a topical exposure, a continuous intravenous infusion of atropine/HI-6 should improve the clinical management. While much data has been collected on the pharmacokinetics of HI-6 following intramuscular (im) administration, little data exists on the pharmacokinetics of HI-6 administered by the intravenous (iv) route. Previous studies in guinea pigs and male human volunteers suggest that repeated intramuscular administration of HI-6 does not affect the pharmacokinetic profile relative to a single im dose. However, no studies have been published on the effect of continuous intravenous infusion.

Defence Research & Development Canada Suffield (DRDC Suffield) is conducting pharmacokinetic studies of iv administered HI-6 in guinea pig and swine animal models using two salts of HI-6: HI-6 dichloride (HI-6 2Cl) and HI-6 dimethane sulfonate (HI-6 DMS). To identify and quantify HI-6 in plasma samples, a rapid high performance liquid chromatography (HPLC) method was required. HPLC techniques for the quantitative analysis of HI-6 have been reported that generally require relatively tedious extractions [4], lengthy chromatographic separations [4–7] and large sample vol-

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umes [5]. The method reported here was designed to improve on previous methods and also be in compliance with Good Laboratory Practices, so as to enable the data to be used for regulatory submission. The present study outlines the development and validation of a method using ion-pair chromatography to quantify plasma HI-6 in both anaesthetized guinea pigs and domestic swine. Derived pharmacokinetic parameters were then used to achieve a target plasma concentration over an 8-h infusion period in anaesthetized guinea pigs.

2. Materials and methods

2.1. Materials

The HI-6 salts tested were as follows: HI-6 2Cl ([(Z)-[1-[(4-carbamoylpyridin-1-yl)methoxymethyl]pyridin-2-ylidene]methyl]-oxo-azanium dichloride) 359.21 g/mol and HI-6 DMS ([(Z)-[1-[(4-carbamoylpyridin-1-yl)methoxymethyl]pyridin-2-ylidene] methyl]-oxo-azanium dimethane sulfonate) 478.50 g/mol. HI-6 2Cl was obtained from Pharmsynthez ZAO (Saint-Petersburg, Russia) and HI-6 DMS from BioQuadrant (Laval, QC, Canada). Pralidoxime chloride (2-PAM) (2-[(Hydroxyimino) methyl]-1-methylpyridinium chloride) (Sigma–Aldrich Ltd., Oakville, ON, Canada). The oximes were stored at 2–6 °C. Naïve pooled male guinea pig plasma for *in vitro* spiked samples and standards were obtained from Charles-River Laboratories (Saint-Constant, QC, Canada). Naïve pooled domestic swine plasma was obtained from ~10-week old, castrated, Yorkshire-Landrace cross animals housed at DRDC Suffield. Chemicals were of American Chemical Society Grade or better and solvents were of HPLC grade; both were obtained from Sigma–Aldrich. HPLC Reagent PIC B7 (acetic acid, methanol, sulfonate salts and water) was obtained from Waters Limited (Mississauga, ON, Canada). Water was purified using a Barnstead Nanopure ultrapure water system (Fisher Scientific, Nepean, ON, Canada) to a resistivity of at least 18 MΩ cm.

2.2. HPLC apparatus

Gradient ion-pair chromatography was carried out using HPLC equipment and columns supplied by Agilent Technologies Canada Ltd. (Mississauga, ON, Canada). The columns used included an Agilent Zorbax Analytical Guard Column (C_{18}, 4.6 mm × 12.5 mm, 5 μm) and an Agilent Zorbax Rapid Resolution SB-C_{18} (4.6 mm × 75 mm, 3.5 μm). The HPLC equipment included an 1100 Vacuum Degasser, 1200 Binary Pump, 1100 Autosampler, 1100 Thermostatted Column Compartment and 1100 Variable Wavelength Detector controlled by Agilent ChemStation for LC 3D Systems©(Rev. B.03.01).

2.3. HPLC conditions

HI-6 2Cl and DMS (in all three matrices: guinea pig plasma, swine plasma and aqueous solutions) were resolved under gradient conditions at a flow of 0.8 mL/min. The mobile phase gradient used was an initial ratio of 60:40 (Component A (A):Component B (B)), ending with 0:100 (A:B). Component A consisted of water:PIC B7:triethylamine (TEA) – 974:25:1 (v:v:v). Component B consisted of A:methanol (MeOH) – 1:1 (v:v). Mobile phase solvents A and B were filtered through a 0.22 μm filter (Fisher Scientific, Nepean, ON, Canada) prior to use. The injection volume was set to 5.0 μL and the absorbance for both salts of HI-6 was optimally set for 302 nm. Column compartment temperature was set at 40 °C. Total run time including re-equilibration to gradient starting point was 10.5 min.

2.4. Preparation of plasma and aqueous solution standards

Standards for all three matrices included the internal standard of 2-PAM. 2-PAM (0.10 mg/mL, initial concentration) was prepared in 0.9% sodium chloride (NaCl) solution. All standard solutions were prepared on ice on the day they were to be analyzed. 2-PAM standard solutions were found to be stable for at least 4 months (relative error calculated to be 1.25%) in saline stored at 4 °C. Relative error is calculated as the absolute difference between the expected and observed values divided by the expected value and then presented as a percentage.

Aqueous solution standards for both HI-6 salts were prepared in the same manner and in the same range of concentrations. HI-6 2Cl and DMS solutions in saline were found to be stable for at least 4 months (relative error calculated to be 4.5%) stored at −20 °C. The HI-6 was initially prepared to a concentration of 10 mg/mL in 0.9% NaCl; all solutions used for the administration of HI-6 in the pharmacokinetic studies were prepared in the same manner. Serial dilutions from the stock solution were performed to attain a final sample HI-6 (DMS and 2Cl) concentration range of 4–4100 μg/mL (the highest concentration tested in aqueous solutions). A 1:1 (v:v) ratio of HI-6:2-PAM was used for all concentrations of HI-6 to establish a standard curve to determine the concentration of study samples. All prepared samples were filter centrifuged (10 min, 3000 × *g*, 4 °C) and collected in HPLC vials for analysis.

Plasma standards (purchased pooled guinea pig and swine) were prepared in the same manner. HI-6 was initially prepared to a concentration of 10 mg/mL in 0.9% NaCl. Dilutions from the stock solution were performed to attain a final sample concentration range of 4–1000 μg/mL (the highest concentration tested in plasma). Naïve plasma (35 μL), HI-6, 2-PAM and trichloroacetic acid (TCA) were combined in the following proportions respectively, 9:1:10:20 (v:v:v:v), vortexed and clarified with centrifugation. Supernatant was removed and combined with 0.2 M sodium hydroxide (NaOH) in a 1:12 (v:v) ratio, vortexed and filtration centrifuged using a 0.45 μm filter. Filtrate was collected in an HPLC vial for analysis. Plasma samples for standard curve values were prepared in the same manner as described for all other standards. Sample preparation was slightly changed to account for atropine sulfate (AS) levels with the addition of AS to the HI-6 stock solution in an equivalent concentration to animal plasma samples.

2.5. Method validation

The method has been tested and validated using the prepared plasma and aqueous solutions to determine assay linearity over a range of concentrations, accuracy, precision, selectivity and robustness to effects caused by metabolized test substances through incurred samples. In this study incurred samples were samples taken to evaluate the effects of *in situ* metabolism as well as sample preparation and storage. These samples were frozen and retained for analysis following initial sample analysis. All validation parameters described have been completed for HI-6 DMS and HI-6 2Cl. Validation of HI-6 DMS, HI-6 2Cl solutions (with and without AS), HI-6 2Cl/AS and HI-6 DMS/AS has been completed for all parameters excluding determination of ruggedness through incurred samples. Validation parameters and acceptable limits were based upon recommendations laid out by Viswanathan et al. [12], Rocci et al. [13] and Fast et al. [14].

2.5.1. Linearity

Linearity was determined by a series of five replicate injections of five separately prepared plasma samples spanning a concentration range from the lower limit of quantification (LLOQ) to 120% of the upper working range (0, 4, 10, 40, 80, 100, 160, 200, 280, 350, 400, 600, 800, and 1000 μg/mL). Samples