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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

were examined to ensure that the absorbance response was directly proportional to the concentration of the analyte. The HI-6 to 2-PAM area ratio was calculated for each replicate and plotted against the sample concentration. The relationship was analyzed by least squares linear regression to confirm a minimum coefficient of determination, $R^2 \geq 0.95$. The standard curve generated from the linearity samples was used to produce the standard curve for the analysis of the remainder of the validation tests.

2.5.2. Range

The LLOQ, upper and lower working range concentration samples for the determination of linearity of the method were examined to comply with limits for precision and accuracy. The LLOQ was defined as the concentration that produced at least five times the response compared to the blank [15]. Five replicate injections of five separately prepared plasma samples were examined for precision and accuracy within ±15% at the upper and lower working concentration range, and ±20% at the LLOQ

2.5.3. Accuracy

Accuracy was determined by comparison of the observed (calculated) concentration values with the expected values. Five replicate injections of five separately prepared plasma samples of three different concentrations (low, 4 μg/mL; medium, 200 μg/mL; and high, 800 μg/mL concentrations of HI-6) were examined to ensure that the mean of the concentration calculated based on the peak area ratio of HI-6 to 2-PAM was within ±15% of the expected value (relative error, %RE).

2.5.4. Precision

Precision of intrabatch and interrun samples was examined to ensure elution times and peak areas of both HI-6 and 2-PAM were reproducible within ±15%. Five separate injections (of five separately spiked plasma preparations) of three different concentrations (low, 4 μg/mL; medium, 200 μg/mL; and high, 800 μg/mL concentrations of HI-6) were analyzed. Intrabatch precision was asessed by the determination of the relative standard deviation (RSD, RSD = standard deviation/mean) of concentrations within the five separate injections. The interrun precision was analyzed by determining the RSD of the 2-PAM peak area and retention time and the HI-6 retention time across the three concentrations of HI-6 tested.

2.5.5. Selectivity

Selectivity was determined by the confirming that the presence of major sample components did not interfere greatly with the 2-PAM and HI-6 peaks. Five replicate injections of five separately prepared plasma samples of four selectivity samples (naïve plasma, plasma + 2-PAM, plasma + HI-6, plasma + AS) and two base samples (plasma + 2-PAM + HI-6 and plasma + 2-PAM + HI-6 + AS) at one concentration of HI-6 (medium working range concentration, 400 μg/mL) were examined. The series was also tested in a saline matrix. The average time of each component peak was compared between each selectivity sample and the base samples to confirm accuracy within ±15%. The HI-6 and 2-PAM peak areas for samples with and without AS were compared to confirm accuracy within ±15%. Chromatograms were also visually inspected to ensure that there was not any evidence of undue interference on the HI-6 and 2-PAM peaks by other components present in the sample.

2.5.6. Ruggedness

Evaluation of ruggedness was carried out to assess the reproducibility of the test system from the point of sample collection through to analysis. Low and high concentration incurred samples were collected from animals in the pharmacokinetic studies of HI-6 in guinea pigs. Each incurred sample was analyzed at least 2 weeks post-primary sample analysis. The incurred samples were analyzed for evidence of changes from primary study samples (samples first analyzed). As suggested by Rocci et al. [13] the upper and lower ratio limits (acceptable ratio limit range of 0.83–1.20) and limits of agreement (acceptable limits of agreement range of 0.83–1.20) were determined as well as the RSD. Acceptable relative standard deviation was set for ±20%.

2.5.7. Stability and intra-day variation

For stability low (8 μg/mL), medium (128 μg/mL) and high (2048 μg/mL) concentrations of HI-6 (DMS and 2Cl) in plasma were prepared and stored at −20 and −80 °C. Samples were analyzed biweekly over a period of 4 months. Relative error was calculated for stability. Acceptable relative error was set for ±15%.

For intra-day variation 13 different concentrations of HI-6 (DMS and 2Cl) were tested with 10 replicates. Relative error was calculated for intra-day variation with acceptable relative error set for ±15%.

2.6. Animal studies

This research was conducted in accordance with the Canadian Council on Animal Care guidelines, under protocols approved by DRDC Suffield Animal Care Committee.

2.6.1. Guinea pig studies

Male Hartley guinea pigs weighing 400–600 g were obtained from Charles-River (Saint-Constant, QC). Animals were acclimatized for at least 2 weeks prior to use and were housed in pairs in clear shoebox cages using a 12-h light/dark cycle. Standard guinea pig chow and tap water were provided *ad libitum* with carrot slices provided daily. Small shelters were also placed in each cage for environmental enrichment.

An indwelling catheter was placed in the left jugular vein using isoflurane (ABBOTT Laboratories Ltd., Montreal, QC) as an anaesthetic, buprenorphine (0.05 mg/kg) (McGill University, Montreal, QC) as a pre and post analgesic and glycopyrrolate (0.04 mg/kg) (CDMV Western Distribution Centre, Calgary, AB) as an anticholinergic. Animals were allowed to recover from surgery for a minimum of 48 h prior to administration of HI-6.

On the day of HI-6 administration animals were administered glycopyrrolate and anaesthetized using isoflurane to establish intravenous access in the medial saphenous vein of a pelvic limb.

A single intravenous (iv) bolus dose of 1.1×10^{-4} mol/kg of HI-6 salt (41.5 mg/kg 2Cl; 52.6 mg/kg DMS) in 0.2 mL of saline was administered to guinea pigs to determine the pharmacokinetics of iv HI-6 (2Cl $n = 6$, and DMS $n = 6$). Dosing solutions were prepared in 0.9% NaCl and sterile filtered prior to administration. Prior to HI-6 administration, control blood samples (150 μL) were collected in EDTA tubes from an indwelling jugular catheter. Following oxime administration samples were collected at 5, 30, 45, 75, 90, 120, 150, 180, 210, 240, 300, 360, 420, 480 and 510 min and every 24 h thereafter up to 48 h post-dosing. Samples were centrifuged at $3000 \times g$ for 10 min at 4 °C. Plasma was collected and divided into two aliquots, snap frozen in liquid nitrogen, and stored at −80 °C.

The pharmacokinetic parameters were determined from the HPLC analysis of HI-6 (2Cl and DMS) in plasma samples. These parameters were used to determine an iv maintenance infusion dose that would maintain a target plasma HI-6 concentration of 100 μM. This molar concentration equates to 37.7 and 47.9 μg/mL

for 2Cl and DMS salts of HI-6 respectively. The following equation was used for calculation of infusion dose,

Infusion dose = clearance × target plasma concentration

Eighteen guinea pigs ($n = 6$ per treatment) were administered a continuous iv infusion of HI-6 (2Cl, DMS or saline control) over 8 h. HI-6 2Cl was administered at a rate of 149 μg/min kg and HI-6 DMS was administered at a rate of 229 μg/min kg. Blood samples were collected at 5, 30, 60, 120, 180, 240, 300, 360, 420, 480 and 510 min during oxime infusion and every 24 h thereafter for 7 days. Blood samples were handled in the same manner as described for iv bolus administration of HI-6.

2.6.2. Domestic swine studies

Male castrated York-Landrace cross pigs weighing approximately 15 kg were purchased from a local supplier. The animals were housed in groups of 6 per pen in a temperature controlled area with a 12-h light/dark cycle. The animals had free access to water and were fed twice per day. Swine were allowed to acclimatize for at least 1 week prior to experimental use. Animals weighed 20.0 ± 1.5 kg at the time of treatment.

The animals underwent an inhalation induction with 5% isoflurane in a carrier gas of 100% oxygen at a flow rate of 8 L/min. Post-induction, the animals were placed in the dorsal recumbent position on a heated operating table. Once the animals were intubated, the isoflurane concentration was reduced to 3% in 100% O_2 at a flow rate of 1 L/min. Core body temperature was maintained at 38.5 ± 1.2 °C. Once the instrumentation was completed, isoflurane was maintained at a rate of ~2% in room air supplemented with oxygen to a fraction of inspired oxygen of 30% to achieve a non-responsive plane of anaesthesia. The animals received 0.9% normal saline via an iv line for fluid replacement. A cystostomy was performed in order to monitor urinary output. Animals were allowed to stabilize at the low isoflurane level for at least 30 min, during which time steady-state anaesthesia (SSA) was established. Continuous physiological parameters were monitored while the animals were under anaesthesia.

Swine were administered a single iv bolus of HI-6 DMS (1899 mg) as previously determined by Lundy et al. [16] or HI-6 2Cl (1509 mg) in 3 mL of saline. Blood samples were collected from a catheter placed in a branch of the saphenous artery into EDTA tubes at 1, 3, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min following oxime administration. Blood samples were handled in the same manner as described for guinea pigs with the exception of larger sample volumes being retained for blood and plasma analysis.

2.7. Sample preparation

Aliquots of all dosing solutions used in the pharmacokinetic studies of HI-6 in guinea pigs were collected for HPLC analysis to confirm dosing levels. Dosing solution samples were obtained daily as prepared, snap frozen in liquid nitrogen and stored at −80 °C until analysis. Samples were allowed to thaw on ice. Equal volumes of dosing solution and 2-PAM (0.1 mg/mL) were combined, vortexed and filtration centrifuged prior to the filtrate being analyzed.

Guinea pig and swine plasma samples were obtained as blood samples were collected from treatment animals. Blood samples were centrifuged for 10 min (3000 × *g*, 4 °C), the plasma was collected, divided into aliquots, snap frozen in liquid nitrogen and stored at −80 °C until analysis. Samples were allowed to thaw on ice. Plasma (35 μL), 2-PAM and TCA were combined in the following proportions respectively, 1:1:2 (v:v:v), vortexed and clarified with centrifugation. Supernatant was removed and the remainder of sample preparation was completed as described for the plasma standards.

2.8. Quantification

All analyses were performed on an individual assay basis. HI-6 concentrations were determined from standard curves derived from the analysis of the solution and plasma standards peak area ratios between HI-6 and the internal standard 2-PAM. Each assay included two sets of standards spanning the working range of concentrations for that particular assay as well as three sets of low, medium and high concentrations of HI-6/2-PAM to act as quality control (QC) samples. Integration of chromatograms was set as part of the method in ChemStation Software and was the same for all assays.

2.9. Pharmacokinetics

Pharmacokinetic parameters were determined for plasma HI-6 concentrations of an iv bolus in both guinea pigs and swine. The $t_{1/2}$ (elimination half-life), V_d (volume of distribution) and CL (systemic clearance) were determined using PK Solutions software (Summit Research Services, Montrose, CO) assuming a one-compartment model and first order rate of elimination. Parameters were based on the exponential expression of 1st order kinetics;

$$C_{(t)} = C_{(0)} \exp\left[-\left(\frac{\mathrm{Cl}}{V_d}\right) t\right] \quad (1)$$

where $C_{(t)}$ is the concentration, $C_{(0)}$ the initial concentration, Cl the clearance, V_d the volume of distribution, and t is the time.

Pharmacokinetic parameters were used to determine maintenance dosing to achieve 100 μM plasma concentration over an 8-h infusion period in guinea pigs. Infusions were performed with both salts without AS and with DMS co-administered with AS.

3. Results and discussion

3.1. Method development

To conduct HI-6 intravenous infusion studies, the development of a rapid and validated method was necessary for the determination of HI-6 plasma concentrations and pharmacokinetic parameters. Previous methods have been used to determine HI-6 plasma concentrations in mice [11], guinea pigs [17], rats [5,6,9], rabbits [10], canines [8,9], swine [7,16,18], non-human primates [17–19] and man [4].

All previously reported methods used an isocratic flow of slightly varying mobile phase compositions. The use of a gradient of two different mobile phases allowed for a reduced flow rate (0.8 mL/min) compared to that reported by McCluskey et al. [10] (1.5 mL/min) and thus a reduction in the amount of solvent required for analysis. The mobile phase component composition was based on Klimmek and Eyer's [8] method. Triethylamine (TEA) was added to component A of the mobile phase to counteract tailing [17] of the HI-6 peak caused by the interaction of the silanol (from the column stationary phase) with the amine groups of HI-6. The TEA (an amine) interacts more strongly with the silanols of the stationary phase than amino groups of the test and standard compounds in the sample thus inhibiting the silanol-sample amine interaction, improving the retention time and peak shape of HI-6. The addition of a second mobile phase (component B) aided in increased resolution and decreased retention times through the establishment of a chemical gradient. The use of a binary pump and gradient flow allowed for quicker equilibration of the column between samples

and thus establishment of a very consistent and low level baseline that never exceeded a height greater than 0.5 mAU and a rapid sample turnaround time of 10.5 min.

The use of an internal standard enhances the precision and reliability of the method by providing an internal check on the extraction efficiency and by reducing technical artifacts such as potential variations in injection volumes. The internal standard 2-PAM was eluted around 2.4 min (±0.1 min) with HI-6 eluting at approximately 6.0 min (±0.15 min depending on the salt being tested, and the testing matrix). Fig. 1 represents typical chromatograms of 2-PAM and HI-6 in saline (dosing solution sample) and guinea pig plasma. Previously published methods all reported HI-6 elution times greater than 7 min [5,8,10]. Busker et al. [17] reported an HI-6 elution time of 4 min in guinea pig plasma, however an internal standard was not included in this method. 2-PAM was selected for use as the internal standard because it elutes prior to HI-6 and the baseline returns to normal prior to HI-6 elution. Obidoxime, which has been previously used [4,7,18] and pyridoxine were also evaluated for use as internal standards. However their elution times were too close to those of HI-6, leading to reduced resolution and poor HI-6 peak shape.

Previously reported methods for rats have required sample volumes of up to 5 mL of whole blood [5], while methods for quantification in swine required sample volumes up to 50 μL of plasma [7]. The method used here requires a maximum of 35 μL of plasma or a minimum of 20 μL for analysis, the lowest volume to date reported for the analysis of HI-6 by HPLC. The low sample volume requirement is particularly important for the quantification and detection of HI-6 over time in small animals such as guinea pigs, as it allows for an increased number of serial samples to be obtained from a single small animal. The method used requires a 5 μL injection, the lowest reported injection volume to date. Total sample extraction was rapid and produced a clean sample for injection thus extending the life of the column and aiding in the prevention of carry over between samples within a run.

Lundy et al. [16] used a column temperature of 30 °C, the only previously reported method examined that noted using a heated column. The current method was optimized to use a column compartment of 40 °C. A temperature of 40 °C resulted in the quickest possible retention times of peak elution with minimal effect on resolution.

Sodium hydroxide (NaOH) was added in the final step of sample preparation to neutralize the acidified sample. Plasma samples that were not neutralized with NaOH resulted in broad non-symmetrical HI-6 peaks with an elongated front slope (fronting) and a plateau at their highest point rather than a sharp apex. The extraction procedure also offers the advantage of providing a clean solution for injection and thus prolongs the life of the column, which aids method reproducibility. The cause of the significant peak following 2-PAM (as shown in Fig. 1) has yet to be identified. It is unknown whether this peak is the result of acidification of 2-PAM resulting in ionized and neutral entities. However, the separation distance between the 2-PAM peak and the unknown peak is not consistent with typical ionization products of chemicals in general. The unknown peak may also be the result of acidification of a constituent within the plasma. Determination of the unknown peak using mass spectrometry has not been carried out due to interference of mobile phase solvents.

The method has been successfully used with whole blood swine samples. However a modification was necessary for sample extraction to ensure clean samples. A larger volume of TCA was found to be required for extraction of whole blood samples.

3.2. Method validation

Standard curves were linear and correlation coefficients for all matrices tested with each HI-6 salt with or without AS were all greater than 0.9990 over the entire concentration range tested. The relative error and relative standard deviation for 2Cl and DMS in plasma and saline are shown in Table 1. Validation samples tested

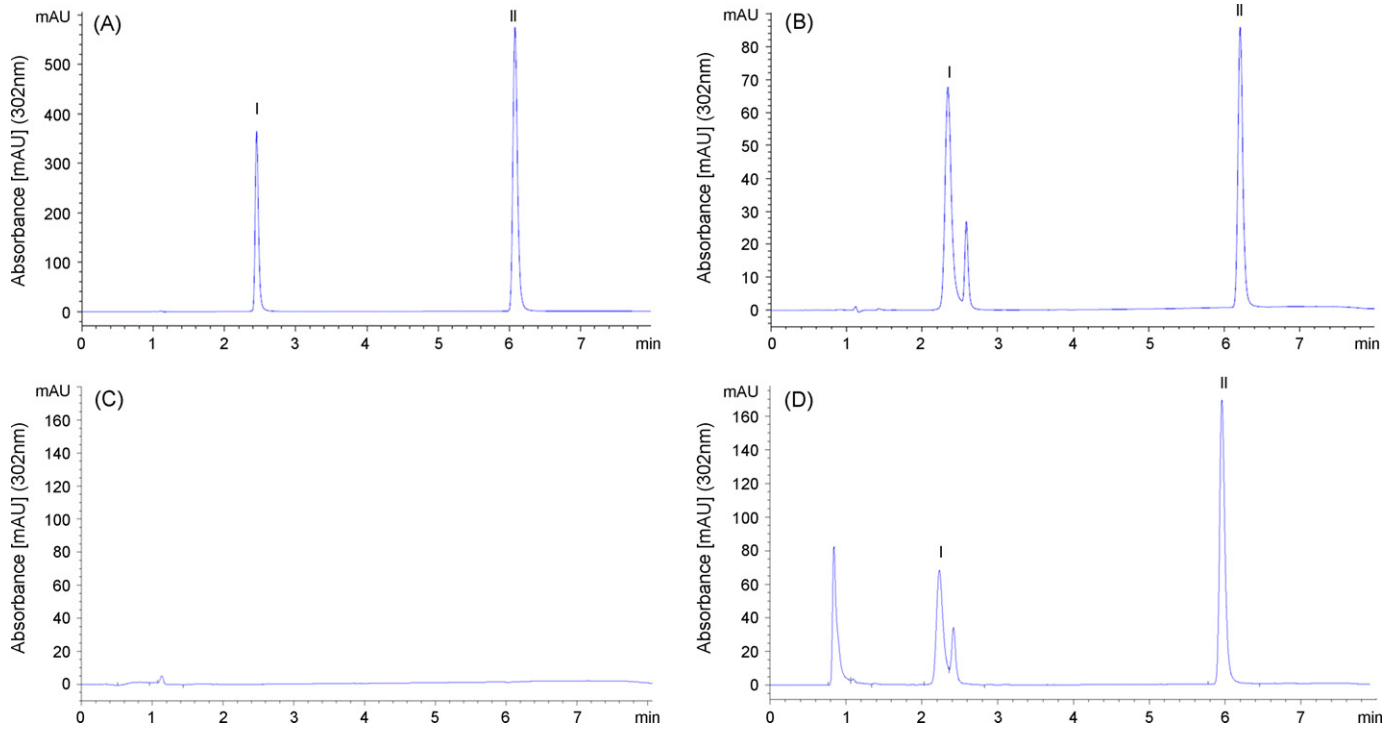

Fig. 1. Representative chromatogram of 200 μg/mL 2-PAM and 200 μg/mL HI-6 DMS added to saline (A). Chromatogram of HI-6 DMS (30 μg/mL of HI-6 and 25 μg/mL of 2-PAM final concentration) in guinea pig plasma (B). A representative chromatogram of guinea pig plasma is shown without the addition of oxime (C). Chromatogram of HI-6 DMS (67 μg/mL of HI-6 and 25 μg/mL of HI-6 final concentration) in swine plasma (D). The elution peaks are labeled, 2-PAM (I) and HI-6 DMS (II). All samples were separated using ion-pair chromatography HPLC. HPLC parameters and sample processing were as described in Section 2.

Table 1
HPLC method validation results for HI-6 2Cl and DMS spiked saline and guinea pig plasma samples.

		2Cl		DMS	
		Plasma	Saline	Plasma	Saline
Linearity (4–1000 μg/mL)	(R^2)	0.9991	0.9991	0.9995	0.9996
Accuracy	(%RE)	4	5	3	3
	(%RE at LLOQ)	5	4	5	4
Precision	(RSD)	4.5	1.5	2.8	1.5
	(RSD at LLOQ)	6	2	2	4
Selectivity		Pass	Pass	Pass	Pass
Ruggedness	(RL)	1.01–1.07	–	1.01–1.07	–
	(LA)	0.95–1.13	–	0.95–1.13	–
	(RSD)	7.5	–	8.9	–
Lower limit of detection (μg/mL)		1	1	1	1

All validation terms are defined in Section 2.5. RSD: relative standard deviation; %RE: relative error; LLOQ: lower limit of quantification (4 μg/mL); RL: ratio limit; LA: limit of agreement; R^2: coefficient of determination; (–) indicates not tested.

that included AS produced similar results to samples that did not include AS. The addition of AS did not affect method selectivity of HI-6 or quantification. All validation parameters at LLOQ were less than 7% for all matrices tested. The method was found to be selective for HI-6 (2Cl and DMS) and 2-PAM with minimal relative error noted. All values reported were well within the limitations set indicating a precise and accurate method.

The limit of detection for all matrices and salts was determined to be 1 μg/mL plasma concentration and the lower limit of quantification was determined to be 4 μg/mL. The upper limit of quantification determined was the highest concentration utilized in the aqueous solution standard curves (4.1 mg/mL).

Preliminary validation has been completed to examine the utility of this assay for the use with swine samples. Results attained to date do not show any differences between swine and guinea pig plasma sample analysis.

3.3. Pharmacokinetic application of method

The assay was used to determine the pharmacokinetic parameters for both HI-6 2Cl and DMS in guinea pig plasma following a single iv bolus dose. Fig. 2 shows the plasma HI-6 concentrations following administration of an iv bolus of 41.5 and 52.6 mg/kg (1.1×10^{-4} mol/kg) of 2Cl or DMS respectively. The pharmacokinetic constants calculated are presented in Table 2. At equimolar doses the two salts displayed similar pharmacokinetic profiles, as found previously in other species such as swine [16]. The apparent volume of distribution for HI-6 2Cl and DMS were 0.266 and 0.355 L/kg. These values correspond with the volume of extracellular fluid which is expected for a charged compound and the rate of elimination follows first order kinetics.

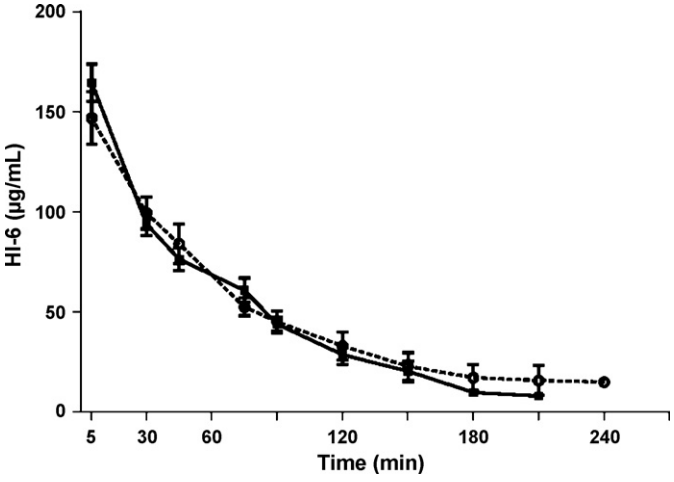

Fig. 2. Comparison of HI-6 2Cl (■) and HI-6 DMS (○) plasma concentrations for various time points following intravenous bolus administration in anaesthetized guinea pig. The dose of each salt administered at 0 min was 41.5 mg/kg body weight and 52.6 mg/kg weight for 2Cl and DMS salts respectively. Each data point represents the mean plasma concentration ± SEM ($n = 6$).

Table 2
Pharmacokinetic parameters of HI-6 salts in anaesthetized guinea pig following an intravenous bolus administration.

Parameter	HI-6 2Cl	HI-6 DMS
Elimination half-life (min)	47.36 ± 9.70	53.74 ± 14.68
V_d (L/kg)	0.260 ± 0.036	0.355 ± 0.054
Cl (mL/min kg)	3.96 ± 1.06	4.77 ± 1.22

Parameters were determined assuming first order elimination and a one-compartment model using PK Solutions Software (Summit Research Services, Montrose, CO). All values are the mean ± SD ($n = 6$). V_d: volume of distribution; Cl: clearance.

The pharmacokinetic parameters were used to determine the required iv HI-6 concentration for an 8 h infusion of HI-6 to achieve

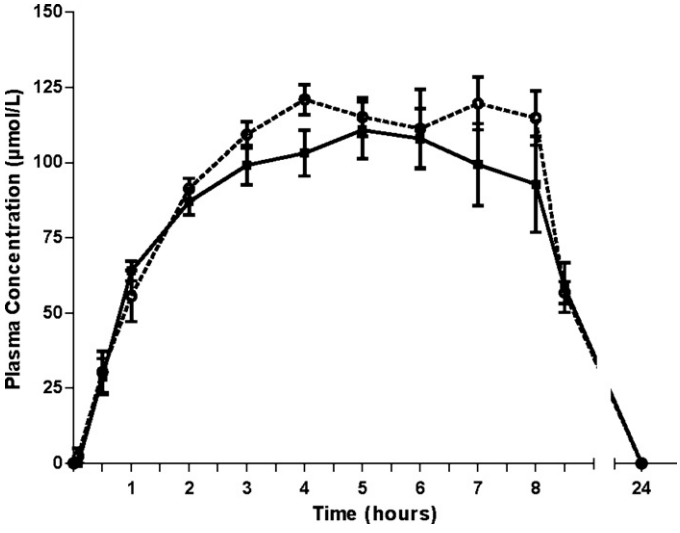

Fig. 3. Plasma HI-6 2Cl and HI-6 DMS concentrations in anaesthetized guinea pigs at various time points during a continuous 8 h infusion. Infusion rates were based on determined pharmacokinetic parameters (Table 2) to achieve a target plasma concentration of 100 μM, 37.7 and 47.9 μg/mL for the 2Cl and DMS salts respectively. The dosing rates for HI-6 2Cl and HI-6 DMS were 149 μg/min kg body weight (■) and 229 μg/min kg body weight (○) respectively. Each data point represents the mean plasma concentration ± SEM ($n = 6$).

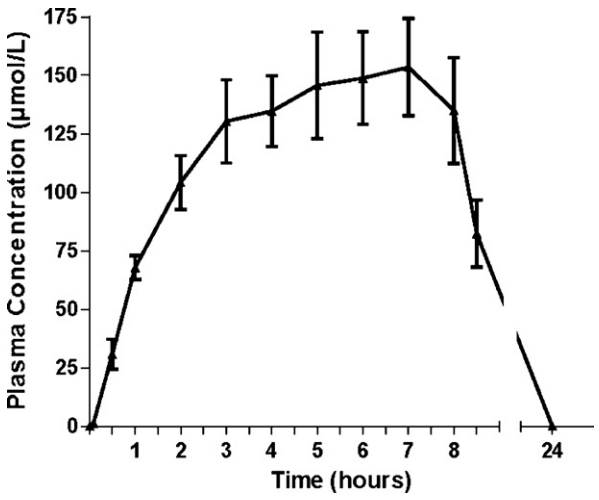

Fig. 4. Plasma HI-6 DMS concentrations in anaesthetized guinea pigs at various time points during a continuous 8 h infusion of HI-6 DMS and atropine sulfate. Infusion rates were based on determined pharmacokinetic parameters (Table 2) to achieve a target plasma concentration of 100 μM (47.9 μg/mL). The dosing rates for HI-6 DMS and atropine were 229 μg/min kg body weight and 4.4 μg/min kg body weight respectively. Each data point represents the mean plasma concentration ± SEM ($n = 6$).

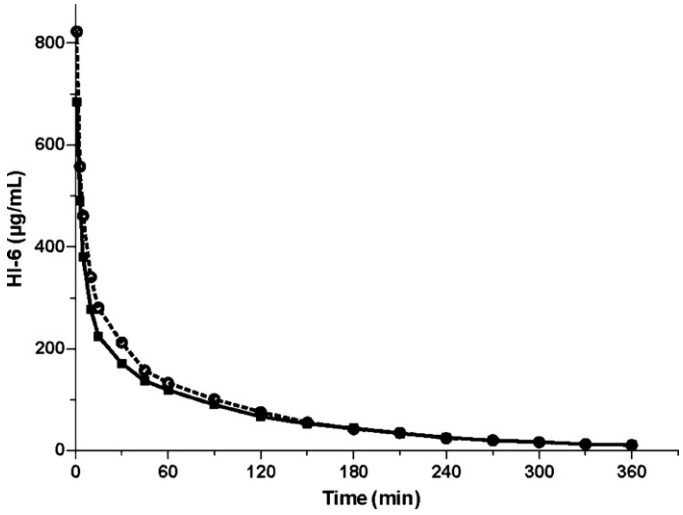

Fig. 5. Plasma HI-6 2Cl (■) and HI-6 DMS (○) concentrations in anaesthetized domestic swine following an iv bolus administration. The dose of HI-6 2Cl and HI-6 DMS administered at 0 min was 1509 and 1899 mg respectively.

a target plasma concentration of 100 μM. Fig. 3 represents the profile of each HI-6 salt over the course of an 8-h infusion period in guinea pigs. For both HI-6 2Cl and DMS the target plasma concentration was achieved as expected within four to five half-lives. Once steady state was achieved the mean ± standard deviation of the plasma concentration was 102.6 ± 6.9 and 116 ± 4.0 μM for 2Cl and DMS salts respectively. When the infusion was stopped (8 h) a decline in plasma HI-6 concentration was observed but due to sample number limitations the rate of elimination could not be determined. When HI-6 DMS was co-administered with AS the steady-state plasma concentration was higher (140 ± 8.1 μM) than the targeted 100 μM which may be due to blood flow affects of AS (Fig. 4). All animals tolerated the infusion both in the presence and absence of AS and survived with no observable signs of toxicity over the 7-day test period.

The pharmacokinetic profile of HI-6 following iv bolus administration was also determined in swine and is represented in Fig. 5. In this figure the average maximal HI-6 plasma peak level following iv injections for 2Cl was 685 μg/mL and for DMS 823 μg/mL. The profile observed was similar to that reported by Lundy et al. [16].

4. Conclusions

A rapid, sensitive and validated method for the detection and characterization of HI-6 in guinea pig plasma as well as in solution (HI-6 in saline) has been developed. The method has been validated to be linear over a large range of concentrations (0.4–4100 μg/mL), precise, accurate, selective and rugged. The use of gradient flow in conjunction with the sample extraction described allows for the rapid detection of HI-6, rapid equilibration of the column and thus a longer lifespan for the column. 2-PAM and HI-6 peaks are clearly resolved with the signal returning to baseline following 2-PAM elution prior to HI-6 elution. The method allows for serial blood sampling due to the low sample volume requirement and thus affords the generation of very accurate pharmacokinetic profiles. Future studies will validate this method for use in other species, as well as continue to define the pharmacokinetic profile of HI-6 in guinea pigs and swine.

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