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Development and validation of a liquid chromatography–tandem mass spectrometry method for the determination of pyridostigmine bromide from guinea pig plasma

Shane R. Needham [a,*], Binying Ye [a], J. Richard Smith [b], William D. Korte [b]

[a] *Alturas Analytics, Inc., 1282 Alturas Drive, Moscow, ID 83843, USA*
[b] *Pharmacology Division, US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010, USA*

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Abstract

An HPLC/MS/MS method was validated for the low level analysis of pyridostigmine bromide (PB) from guinea pig plasma. An advantage of this strong-cation exchange HPLC/MS/MS method was the enhancement of the ESI-MS signal by providing good retention and good peak shape of PB with a mobile phase of 70% acetonitrile. In addition, the use of 70% acetonitrile in the mobile phase allowed the direct injection of the supernant from the protein precipitated extracted sample. The assay was linear from the range of 0.1 to 50 ng/ml using only 25 μl of sample. The precision and accuracy of the assay was better than 9.1 and 113%, respectively.

Keywords: Pyridostigmine bromide

1. Introduction

Pyridostigmine bromide (PB) is part of a pretreatment regimen utilized by the US military to protect the warfighter against organophosphorus (OP) chemical warfare nerve agents [1]. Significant decreases in lethality have been achieved in animal models utilizing a regimen consisting of pretreatment with a carbamate cholinesterase inhibitor such as pyridostigmine, followed by administration of atropine and oxime treatment upon exposure [2,3]. The role of pyridostigmine is to reversibly inhibit the active site of acetylcholinesterase (AChE), thereby protecting it from irreversible phosphorylation by the OP. Protection of AChE is based upon the spontaneous decarbamylation of the pyridostigmine-inhibited enzyme. PB received recent FDA approval for use by the US military for prophylaxis against the lethal effects of Soman nerve agent poisoning [4]. Other uses for PB include the treatment of myasthenia gravis [5], obesity [6], post polio syndrome [7], dementia [8], cardiovascular parameters [9] and epilepsy [10].

Fig. 1 shows the structure of PB. The analysis of PB is a challenge since PB has a low molecular weight (181 amu) and is thus subject to interferences

* Corresponding author. Tel.: +1-208-883-3400; fax: +1-208-882-9246.
E-mail address: sneedham@alturasanalytics.com (S.R. Needham).

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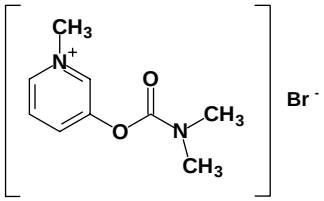

Fig. 1. Structure of PB.

from small molecules present in biological fluids. Also, PB is a polar quaternary amine compound that is difficult to obtain good retention and good peak shape on typical hydrophobic HPLC stationary phases such as C8 or C18. Although methods exist for the analysis of PB from biological fluids including GC/MS methods [11,12], enzymatic or immunoassay methods [13–15], HPLC methods [16–19] and a thermospray LC/MS method [20], these methods are not as selective, as rapid or as sensitive as HPLC/MS/MS methods. In addition, the GC methods require laborious derivitizations that require more than 15 min per sample, whereas the enzymatic methods are often not selective enough to distinguish between drugs and metabolites in biological fluids. More recently, a HPLC/ESI/MS method was developed, however, the assay suffered from interferences near low levels of quantitation without the use of more selective MS/MS [21]. In addition to long analysis times (>15 min), the majority of the HPLC methods use ion-pairing or ion-suppressing agents in the mobile phase to obtain adequate peak shape and retention on C8 or C18 stationary phases. Although, ion-pairing agents at times may be necessary with HPLC/ESI/MS, ion-pairing and ion-suppressing agents should be avoided in ESI/MS since these agents have been shown to decrease the ESI/MS signal for the analysis of basic drugs [22]. In this study, we report on the development and validation of an HPLC/ESI/MS/MS assay for the determination of PB from guinea pig plasma.

2. Experimental

2.1. Reagents and standards

Pyridostigmine bromide (>99% purity) was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA USA). Standard stock solutions (1.0 mg/ml) were prepared by dissolving a weighed amount of the compound in water. Standards of PB were prepared fresh daily to circumvent the need to perform stock solution stability experiments. Acetonitrile (J.T. Baker, Phillipsburg, NJ, USA) was of HPLC grade or better. Water was obtained in-house from a Milli-Q Water System (Millipore Corporation, Bedford, MA, USA). Ammonium formate was obtained from J.T. Baker and was of >96% purity. Formic acid was obtained from Sigma (St. Louis, MO, USA) and was of >95% purity. All reagents were used without further purification.

2.2. Synthesis of hexadeuteropyridostigmine

The hexadeuteropyridostigmine bromide (3-[[(dimethylamino)-carbonyl]oxy1]-1,1,1-trideuteromethyl)-2,4,6-trideuteropyridinium bromide) was synthesized in four steps from 3-hydroxypyridine. 3-Hydoxypyridine was heated at 160 °C in a sealed high-pressure bottle with basic D_2O for 10 days [23,24]. After neutralization, an 85% yield of a mixture of 2,4,6-trideutero-3-hydroxypyridine and dideutero-3-hydroxypyridines with a melting point of 124–127 °C was obtained. GC/MS analysis indicated that 85–89% of the product was trideuterated ($m/z = 98$). The mixture was then converted to the carbamates with dimethylcarbamyl chloride. The *m/z* value for the trideuterated carbamate was 169. The carbamate was then treated with 1,1,1-trideuteroiodomethane to give an oil that was primarily hexadeuteropyridostigmine iodide. Ion exchange over a Dowex 1-X4 (bromide form) column followed by high vacuum drying of the hygroscopic products gave a solid. Analysis by NMR, MALDI-TOF/MS, and LC/MS all indicated that the major product (85–89%) was the hexadeuteropyridostigmine bromide ($m/z = 187$).

2.3. HPLC

A Shimadzu series 10Advp HPLC system equipped with a controller, two pumps and a degasser delivered the mobile phase at 0.6 ml/min. The mobile phase consisted of 70% acetonitrile/30% 100 mM ammonium formate. A CTC LEAP Technologies HTC PAL autoinjector (Carrboro, NC, USA) injected 30 μl onto the HPLC column. The use of 100 mM ammonium formate gave the best peak shapes for the analysis. In ad-

dition, the MS signal was not diminished with the use of 100 mM ammonium formate mobile phase when compared to 10 mM ammonium formate. The HPLC column was a 2 mm i.d. × 35 mm length strong-cation exchange SCX poly(2-sulfoethyl aspartamide) available from PolyLC (Columbia, MD, USA). A 10-port switching valve from Rheodyne (Rohnert Park, CA, USA) was used to divert the void volume of the column to waste for the first 0.35 min of the analysis.

2.4. Mass spectrometry

A Sciex API 3000 triple quadrupole mass spectrometer (MDS-Sciex, Toronto, Ontario, Canada) equipped with a turbo ion spray interface was used for the detection of analytes. Data was acquired in the positive ion mode with an ESI probe voltage of 1500 V. Nebulizer gas and curtain gas settings were set to 15 and 12 units, respectively. The turbo ion spray was operated at a temperature of 400 °C and a drying gas setting of 7000 ml/min. Analyst software version 1.2 was used for all data acquisition and analysis. Microsoft (Redmond, WA, USA) Excel 2000 was used for all other calculations.

Multiple reaction monitoring (MRM) mode was used for data collection. Based on collision induced dissociation (CID) product ion scan spectra the following MRM transitions were used for detection of analytes; *m/z* 180.8 → 124.0 (pyridostigmine) and *m/z* 186.8 → 130.0 (D6-pyridostigmine, internal standard). Collision pressure with nitrogen was set to 2.9 mbar. The collision energy for pyridostigmine and internal standard was 23 eV. The mass spectrometer was operated at unit mass resolution. Dwell times were approximately 0.2 s per ion.

2.5. Calibration, quantitation and sample preparation

Control guinea pig plasma was obtained from Cocalico Biologicals (Reamstown, PA, USA) and stored at −20 °C until use. Calibration and quality control (QC) samples were prepared from stock solutions prepared fresh daily to cover the range of 0.1 to 50 ng/ml. The standards were fortified to give standard curve points of 0.1, 0.25, 0.5, 1.0, 5.0 and 50 ng/ml. Quality control samples were prepared at the upper limit of quantitation (ULOQ) and lower limit of quantitation (LLOQ) of 50 and 0.1 ng/ml, respectively. Additional QC samples were prepared at 0.3, 10 and 40 ng/ml. Calibration was performed by plotting peak area ratios of drug to internal standard against drug concentration. A linear $1/x^2$ fit was employed for all calibration curves.

To investigate the stability of PB throughout the analysis and storage procedures, short- and long-term stability experiments were performed. Stability was investigated by the preparation of QC samples at time 0 (T0) and comparison to a different set of QC samples taken through a series of conditions. The conditions investigated for short-term stability were freeze–thaw stability (three cycles 12 h freeze minimum), benchtop stability and stability of samples awaiting analysis in the autosampler. Long-term stability was investigated for 6 months at −80 °C.

Twenty-five microliter aliquots of samples were placed in a 96-well plate, where 50 μl of internal standard in 95% acetonitrile was added and the mixture was shaken for 30 s. The plate was centrifuged for 10 min. at approximately 2400 × *g*. Approximately 70 μl of supernant was transferred to a clean well of a 96 well plate. Then, the sample was injected onto the HPLC/MS/MS system. To reduce carry-over after each extracted plasma sample injection, the column was rinsed by injecting 100 μl of 1 M ammonium formate and running the method with the column eluent directed to waste.

Precision was expressed as percent relative standard deviation (R.S.D.). Accuracy was calculated as percent by ((mean calculated concentration × 100)/intended concentration). The ULOQ and LLOQ points were validated by the analysis of a replicate of six replicate QC samples each prepared at 50 and 0.1 ng/ml, respectively. Intra-assay variability studies were performed by analysis of six replicates of QC samples at 0.3, 10 and 40 ng/ml. Inter-assay variability studies were performed by analysis of at least three replicates of QC samples at each QC concentration (0.3, 10 and 40 ng/ml) on three different days.

3. Results and discussion

Fig. 1 displays the structure of PB. The drug and deuterated internal standard formed predominant cations (M^+) in the ESI source. The ions formed

in the ESI/MS source are typical of those found in previous research with quaternary amines [25].

Initially we developed an HPLC/MS/MS method for the analysis of PB from guinea pig plasma with the use of a poly-styrene-divinyl benzene column [26]. However, this assay needed an ion-pairing agent and 98% aqueous mobile phase to achieve good retention and peak shape. In addition, the protein precipitated (by acetonitrile) sample had to be evaporated and reconstituted in aqueous mobile phase to obtain adequate results. Since the limits of quantitation near 0.1 ng/ml were necessary for upcoming PB guinea pig studies, we investigated other stationary phases that would allow the use of a 70% acetonitrile mobile phase without ion-pairing agents. The use of a 70% acetonitrile mobile phase would allow the direct injection of the precipitated sample and would increase the MS signal with the use of a higher percentage of acetonitrile in the mobile phase [27]. Fig. 2 shows results from the flow injection analysis of PB onto the HPLC/MS/MS system with a variance of the amount of organic modifier in the mobile phase. As shown in Fig. 2, the increase in MS signal is two times higher at 85% acetonitrile compared to 10% acetonitrile. Thus, a column that gives good retention and peak shape with >70% acetonitrile would be optimum. After investigation of several different columns and conditions a strong-cation exchange (SCX) column with 70% acetonitrile/30% 100 mM ammonium formate was found to give good retention, peak shape and MS signal. These and other ion-exchange columns have been shown previously to work well for the analysis of polar basic drugs, metabolites and peptides [28,29]. When compared to the previous reversed-phase method, we developed for the HPLC/MS/MS analysis of PB from plasma this SCX assay had a lower LLOQ by five times greater due to better sample preparation methods and the increased amount of organic modifier in the mobile phase [26]. Since Fig. 2 demonstrates a factor of at least two times greater signal from the use of 70% acetonitrile compared to <10% acetonitrile, the remaining gain in assay sensitivity is most likely to due better sample preparation. Since the current assay is a precipitation with a direct injection losses of sample are minimized compared to the previous assay which required evaporation of extraction solvent and reconstitution in mobile phase. Losses of PB during evaporation and reconstitution could be significant. After thorough method development, the HPLC/MS/MS method was fully validated in accordance with the latest FDA guidelines for bioanalytical validation [30,31].

3.1. Linearity and dynamic range

Calibration curves were constructed in the range of 0.1 to 50 ng/ml with six points in duplicate for PB in guinea pig plasma. Correlation coefficients were >0.99 on three different days of validation. The limits of quantitation were validated by the preparation of QC samples ($n = 6$) at the ULOQ, 50 ng/ml and LLOQ, 0.1 ng/ml. For the ULOQ an accuracy and precision of 93.5 and 6.82% were obtained, respectively.

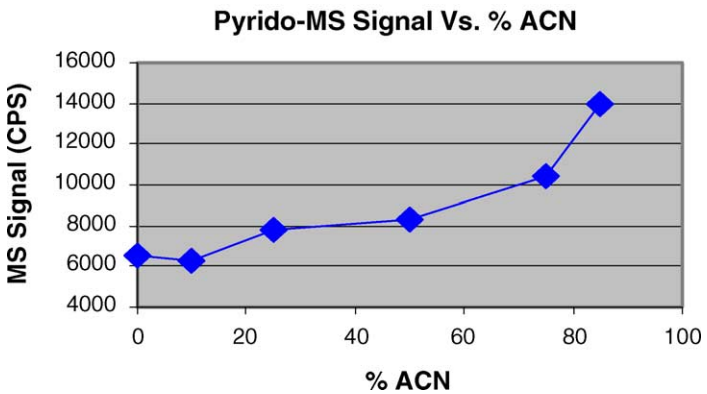

Fig. 2. Flow Injection analysis of PB onto the HPLC/MS/MS system demonstrating the affect of the amount of organic modifier in the mobile phase to the ESI/MS signal.

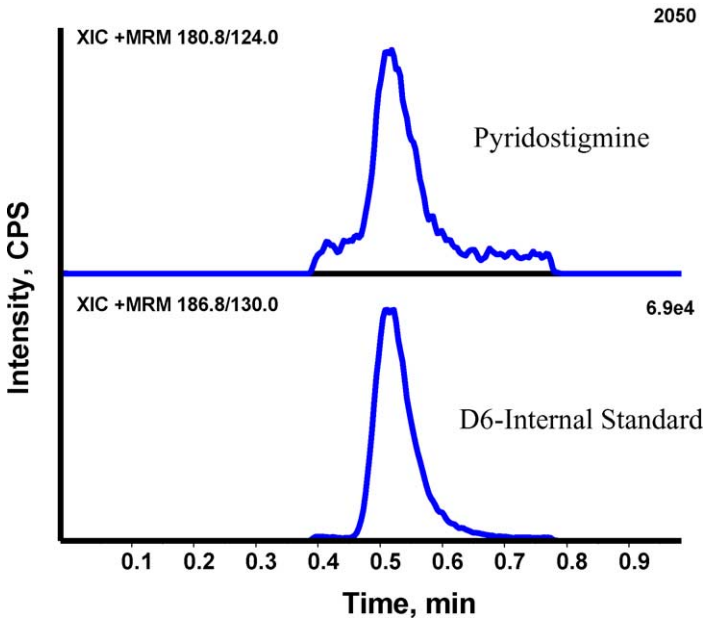

Fig. 3. HPLC/MS/MS chromatogram from the analysis of an extracted standard in guinea pig plasma fortified at 0.1 ng/ml.

For the LLOQ an accuracy and precision of 101 and 6.28% were attained, respectively. Fig. 3 shows a chromatogram from the HPLC/MS/MS analysis of a standard fortified at the LLOQ of 0.1 ng/ml in guinea pig plasma.

3.2. Accuracy and precision

Table 1 displays the data on the performance of the HPLC/MS/MS assay of PB from guinea pig plasma. For each QC sample at 0.3, 10 and 40 ng/ml, the interday and intraday accuracy and precision was better than 113 and 11%, respectively. The accuracy and precision of all the standard curve points was better than 113 and 13% on three different days of validation. In addition, at least five different lots of plasma were successfully tested with standards and QC samples for good accuracy and precision.

Table 1
Quality control (QC) data for the HPLC/MS/MS analysis of PB from guinea pig plasma

Nominal concentration (ng/ml)	Accuracy (%)[a]	Intraassay precision (%)[b]	Interassay precision (%)[a]
0.3	92.4	5.3	9.1
10	101	10.8	9.1
40	113	4.8	6.6

[a] Mean of three different days of validation of $n = 15$.
[b] Mean of 1 day of validation of $n = 6$.

3.3. Specificity and selectivity

Assay selectivity is demonstrated by the absence of interfering peaks at the retention times of PB and PB-D6 in an extracted blank guinea pig plasma sample. Figs. 4 and 5 depict extracted-ion chromatograms from the HPLC/MS/MS analysis of a blank sample (Fig. 4) and a blank sample fortified with internal standard (Fig. 5). At least five different lots of guinea pig plasma were tested to demonstrate assay specificity and selectivity. This data shows that the assay is selective and specific for the analysis of PB from guinea pig plasma.

3.4. Short-term and long-term stability

Table 2 displays the short-term and long-term stability of PB. As per FDA guidelines, the compound is deemed “stable” if the concentration of the compound

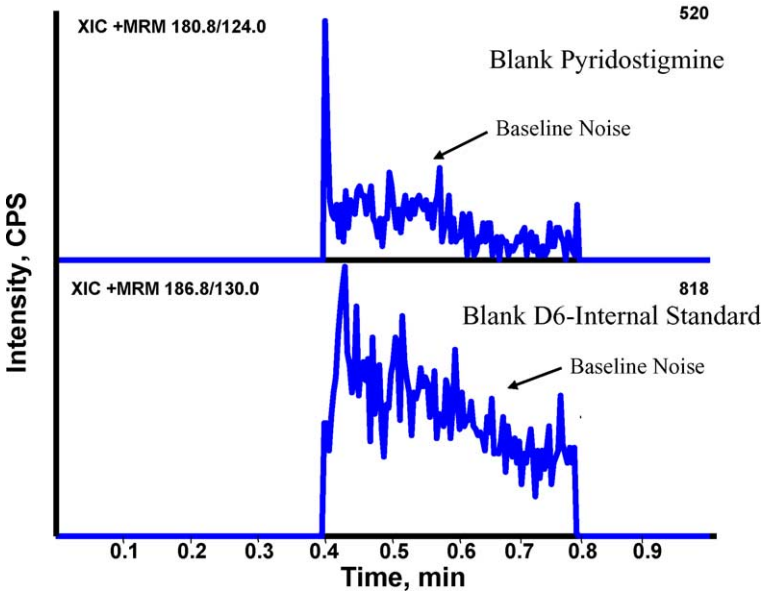

Fig. 4. HPLC/MS/MS chromatogram from the analysis of an extracted guinea pig plasma blank sample.

through the stability experiment does not deviate by more than 15% compared to a control (time 0) concentration. In summary, stability for PB under the following conditions has been shown; (1) stability of PB in guinea pig plasma for at least 4 h on the benchtop at room temperature, (2) stability of PB in guinea pig plasma through at least three freeze–thaw cycles, (3) stability of PB sitting in the autosampler for at least

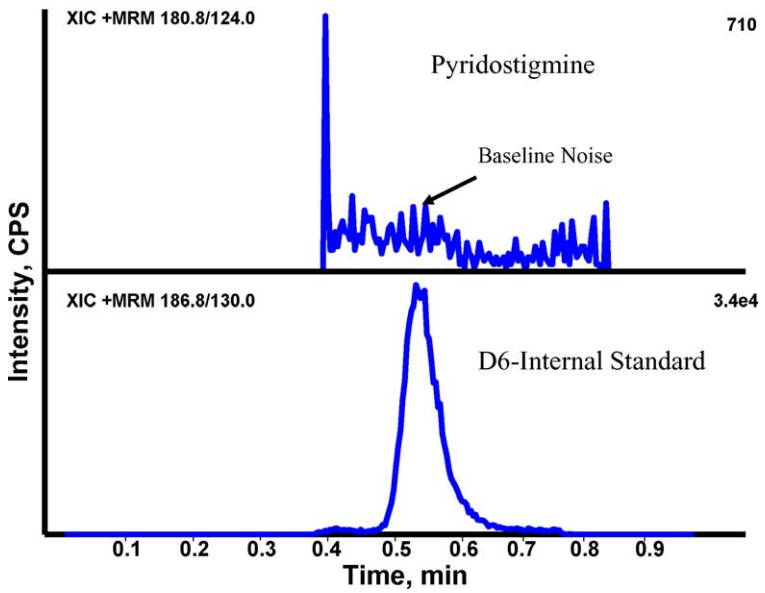

Fig. 5. HPLC/MS/MS chromatogram from the analysis of an extracted guinea pig plasma sample fortified with PB-D6, internal standard.

8 h and (4) stability of PB for at least 6 months frozen at −80 °C.

3.5. Extraction recovery and matrix effects

Extraction recovery was determined by comparing peak areas of extracted samples against those of non-extracted standards prepared in blank extracted guinea pig plasma to account for matrix affects. These standards were prepared at three QC concentrations spanning the calibration curve. Theaverage recovery ($n = 6$) of PB was 82.1% at 0.300 ng/ml, 74.3% at 10.0 ng/ml, and 87.3% at 40.0 ng/ml. The average recovery ($n = 6$) for the internal standard, PB-D6 was 82.8%. The precision across all concentrations of QC samples for extraction recovery was 11.9% or better. Fig. 6 shows an extracted ion chromatogram comparing the extracted to the non-extracted standards for the HPLC/MS/MS analysis of PB at 0.3 ng/ml. As shown in Fig. 6, the only noticeable difference in the chromatograms is the slightly lower peak height. An average recovery >80% in five different lots of plasma along with good precision and a good chromatographic comparison to a fortified non-extracted standard suggests a low occurrence of matrix effects with this assay. Furthermore, the good accuracy and precision obtained when standards and QC were prepared in at least five different lots of plasma demonstrates the minimal matrix effects in this assay.

Table 2
Short-term and long-term stability data from the HPLC/MS/MS analysis of PB from guinea pig plasma

Stability parameter	Deviation from control[a] (%)
Freeze–thaw	−6.9
Samples awaiting analysis in autosampler	−15
Benchtop	−13
6 months at −80 °C	−3.1

For short-term stability, the conditions were three freeze–thaw cycles, 8 h and 4 h used for freeze–thaw, samples in autosampler and benchtop stability, respectively.

[a] The deviation is calculated as an average from three QC levels (0.3, 10 and 40 ng/ml).

Although, the analysis time is <1 min per sample under the HPLC conditions shown above, the retention factor for PB is >2.2 which is well outside the solvent front of the column. In addition to the enhanced selectivity of strong-cation exchange compared to reverse

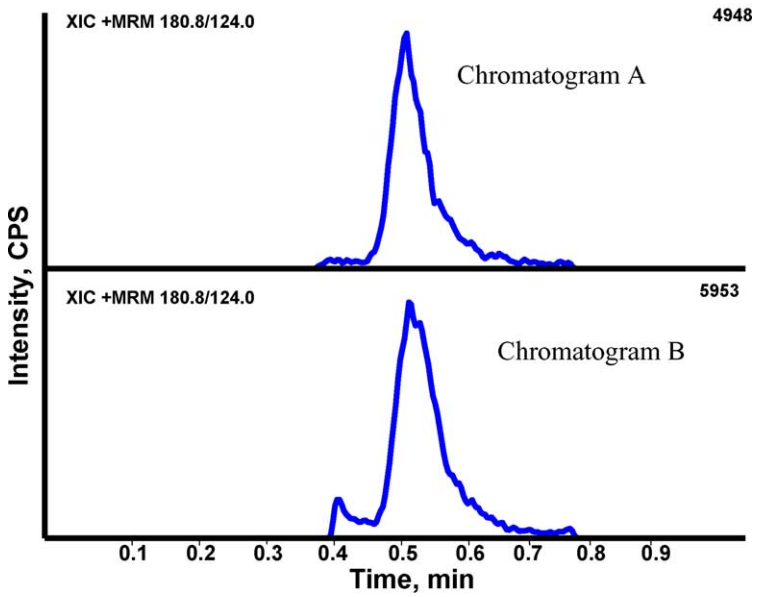

Fig. 6. Chromatograms from the HPLC/MS/MS analysis of a guinea pig plasma standard fortified with PB at 0.3 ng/ml (Chromatogram A) and from a guinea pig plasma sample extracted and fortified with PB at 0.3 ng/ml, equivalent (Chromatogram B) to calculate extraction recovery.

phase HPLC to typical endogenous plasma components such as salts, this retention factor along with isotopically labeled internal standards have been shown to be adequate for LC/MS/MS bioanalysis [32,33].

4. Conclusion

In conclusion, a sensitive and selective method was validated for the routine HPLC/MS/MS analysis of PB from guinea pig plasma. The method uses SCX to retain PB with 70% acetonitrile mobile phase whereas previous reversed-phase methods required only 2% acetonitrile to obtain similar retention. The high concentration of acetonitrile improved the desolvation process in the HPLC/ESI/MS/MS method and thus produced detection limits five times better than previous HPLC/MS/MS methods. In addition, the protein precipitated sample can be directly injected onto the system, which is not possible in typical reversed-phase methods without evaporation and reconstitution. Possible additions to this method include the investigation of the simultaneous determination of other nerve agent pretreatment compounds such as atropine and pralidoxime in various matrices.

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