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High-performance liquid chromatography coupled with electrospray tandem mass spectrometry (LC/MS/MS) method for the simultaneous determination of diazepam, atropine and pralidoxime in human plasma

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ABSTRACT

A high-performance liquid chromatography coupled with electrospray tandem mass spectrometry (LC/MS/MS) procedure for the simultaneous determination of diazepam from avizafone, atropine and pralidoxime in human plasma is described. Sample pretreatment consisted of protein precipitation from 100 μl of plasma using acetonitrile containing the internal standard (diazepam D5). Chromatographic separation was performed on a X-Terra® MS C_8 column (100 mm × 2.1 mm, i.d. 3.5 μm), with a quick stepwise gradient using a formate buffer (pH 3, 2 mM) and acetonitrile at a flow rate of 0.2 ml/min. The triple quadrupole mass spectrometer was operated in positive ion mode and multiple reaction monitoring was used for drug quantification. The method was validated over the concentration ranges of 1–500 ng/ml for diazepam, 0.25–50 ng/ml for atropine and 5–1000 ng/ml for pralidoxime. The coefficients of variation were always <15% for both intra-day and inter-day precision for each analyte. Mean accuracies were also within ±15%. This method has been successfully applied to a pharmacokinetic study of the three compounds after intramuscular injection of an avizafone–atropine–pralidoxime combination, in healthy subjects.

1. Introduction

Organophosphate "OP" nerve agents (sarin, soman and tabun) are extremely toxic chemicals that were developed by the German chemist, Gerhard Scharder, before and during World War II. They pose potential neurotoxic effects to both military and civilian population, as evidenced by armed conflicts or terrorist attacks.

Exposure to organophosphorous cholinesterase inhibitors causes a progression of toxic signs and symptoms, including hypersecretion, fasciculation, tremors, convulsions, coma and respiratory distress, which can lead to death. These toxic effects are due to hyperactivity of the cholinergic system because cholinesterase is inhibited and acetylcholine increases at central and peripheral sites.

Benzodiazepines are effective against OP-induced symptoms, with strong synergistic effects when combined with cholinesterase deactivators and anticholinergic drugs [1]. Different benzodiazepines have been tested and enhanced activity of diazepam was observed [2]. Thus, diazepam has been recommended for standard treatment therapy of convulsions caused by nerve agents, along with cholinesterase reactivators (pralidoxime, HI-6) and a muscarinic antagonist such as atropine [3,4].

Three-drug regimens are currently packaged in a single autoinjector which must be used intramuscularly. However, compared to cholinesterase reactivators and atropine, diazepam is not water soluble. This chemical property limits the pharmacological potency of diazepam for intramuscular (i.m.) injection and requires incorporation of an organic solvent in the triple injectable formulation.

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Table 1
Retention times and monitored transitions of the analytes and the internal standard

	Retention time (min)	Precursor (*m*/*z*)	Product (*m*/*z*)
Pralidoxime	1.8–2.1	137	119
Atropine	6–6.2	290	124
Diazepam	7–7.5	285	193
Diazepam D5	7–7.5	290	198

Therefore, a water-soluble prodrug of diazepam, avizafone, was developed as a component of an aqueous drug mixture with atropine and a cholinesterase reactivator. Avizafone is effective in arresting soman-induced seizures [5].

The three-drug regimen is currently packaged in a single auto-injector device containing a combination of avizafone hydrochloride, atropine sulfate and pralidoxime methyl sulfate. The formulation is a lyophilized powder to be diluted with water for injection before i.m. administration.

Several methods have been reported in the literature for the quantification of benzodiazepines. Several analytical methods, such as thin-layer chromatography (TLC) [6], column-switch high-performance liquid chromatography [7–9] and immunoassay [10], as well as different sensitive and selective LC/MS/MS [11,12], and CE/MS/MS [13,14] are currently used for the quantification of diazepam and 1–4 benzodiazepines.

In fact, several LC methods have already been reported for the quantification of pralidoxime in plasma. These assays involved UV detection [15,16] and electrochemical detection [17].

Additionally, quantification of atropine in the biological matrix has been accomplished by different techniques including HPLC with UV detector [18] or tandem mass spectrometry [19,20] and gas chromatography with mass spectrometry [21]. In addition, atropine was quantified as dl-hyoscyamine equivalents with a radioimmuno assay [22] and a radio receptor assay [23]. Nevertheless, no method is yet published describing the simultaneous quantification of pralidoxime, atropine and diazepam in a biological matrix.

In this paper, we describe the development and validation of a rapid, sensitive and specific method for the quantification of three molecules used in the treatment of OP intoxication in human plasma, using HPLC coupled with electrospray ionization tandem mass spectrometry.

This method was combined with a simple sample pretreatment, and the validation of the method was performed based on the most recent international guidelines for bioanalytical validation.

2. Experimental

2.1. Chemicals and reagents

Drug standards pralidoxime, atropine and diazepam were purchased from Sigma–Aldrich (St. Quentin Fallavier, France). The

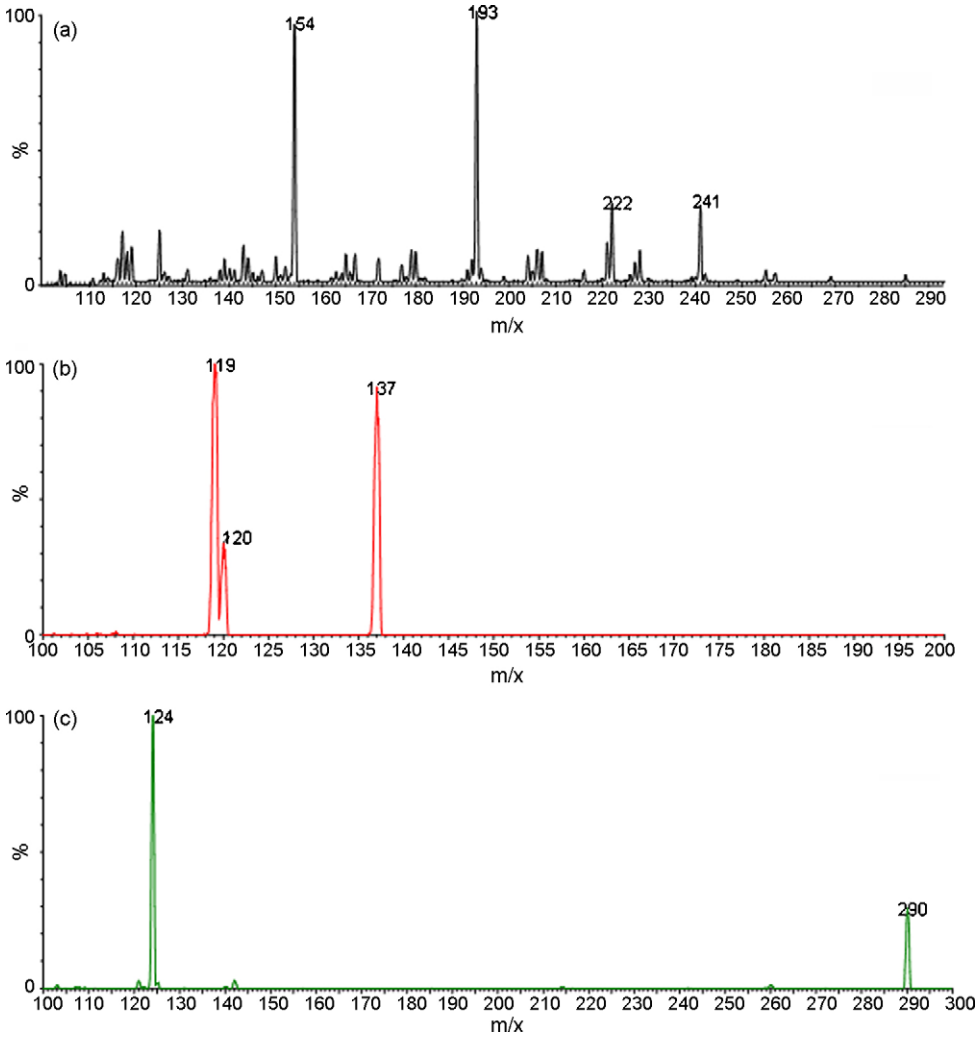

Fig. 1. Full scan mass spectra of pralidoxime (a), diazepam (b) and atropine (c).

Table 2
Interday and intraday precision (CV%) and accuracy (deviation%) for the three analytes in spiked human plasma samples

Analyte	Inter-day reproducibility ($n=6$)				Intra-day reproducibility ($n=6$)		
	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy%	CV%	Measured concentration (ng/ml)	Accuracy%	CV%
Pralidoxime	15	15.9	5.89	6.67	16	6.67	6.74
	300	330	9.9	4.25	311	3.77	6.33
	800	785	−1.83	6.64	799	−0.17	5.88
Atropine	0.75	0.78	3.65	7.48	0.77	3.00	3.24
	15	15.6	3.72	5.92	16	6.67	6.94
	40	39.5	−1.19	6.07	44.8	7.33	7.33
Diazepam	3	3.12	3.65	7.48	3.08	2.78	12.5
	150	156	3.72	5.92	165	10.2	2.11
	400	395	−1.19	6.07	384	−4.01	4.82

Table 3
Lower limit of quantification precision (CV%) and accuracy (deviation%) for the three analytes in spiked human plasma samples

Analyte	Lower limit of quantification ($n=6$)			
	Nominal concentration	Measured concentration	Accuracy%	CV%
Pralidoxime	5	5.68	13.5	3.49
Atropine	0.25	0.238	−4.80	18.9
Diazepam	1	1.12	11.6	3.66

internal standard (I.S.) pentadeuterated diazepam was obtained from Promochem (Molsheim, France). HPLC grade acetonitrile and methanol, and analytical grade formic acid and ammonium formate were purchased from Merck (Fontenay sous Bois, France). Milli-Q water was used throughout the analysis. Drug-free human plasma was obtained from the hospital blood bank (Angers Hospital, France).

2.2. Preparation of drug standards (stock solutions, working solutions and plasma standards)

Stock solutions of each analyte were prepared in methanol at a concentration of 1 mg/ml.

During analysis, two stock solutions were used for each analyte: one to spike the plasma calibration standards, the other to prepare the quality control "QC" samples.

Stock solutions were diluted further in methanol:water (50:50, v/v) to obtain two working solutions. The first solution contained pralidoxime and atropine at 20 and 1 μg/ml, respectively. The second solution contained diazepam at 10 μg/ml.

These working solutions were then diluted in human drug-free plasma in order to obtain calibration curve standards and QC samples.

The stock solution of the I.S. (diazepam D5) was prepared in methanol at a concentration of 1 mg/ml. This solution was diluted to a concentration of 100 ng/ml in acetonitrile. The final solution

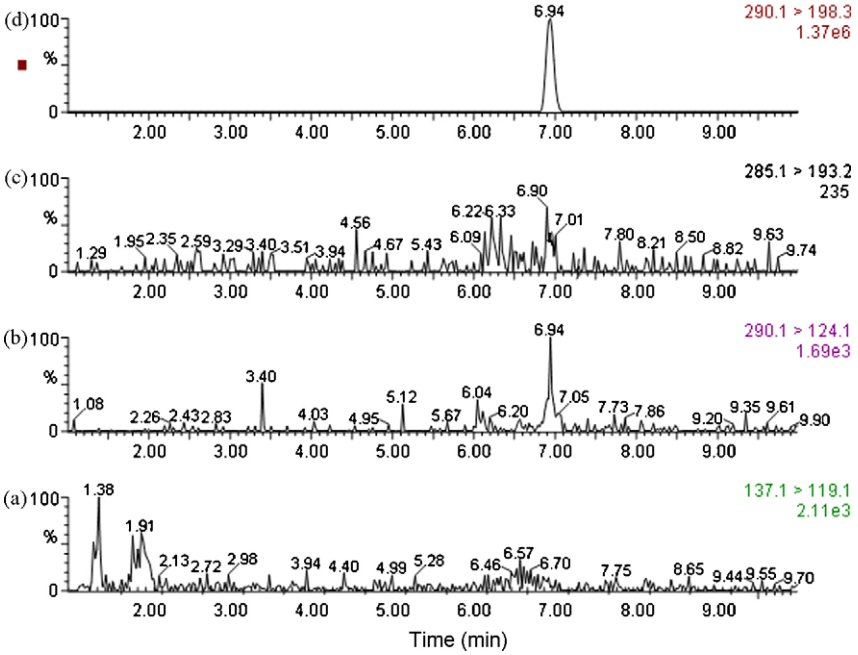

Fig. 2. MRM chromatograms (a–d) of a processed zero point sample: pralidoxime transition (a), atropine transition (b), diazepam transition (c) and diazepam D5 transition (d).

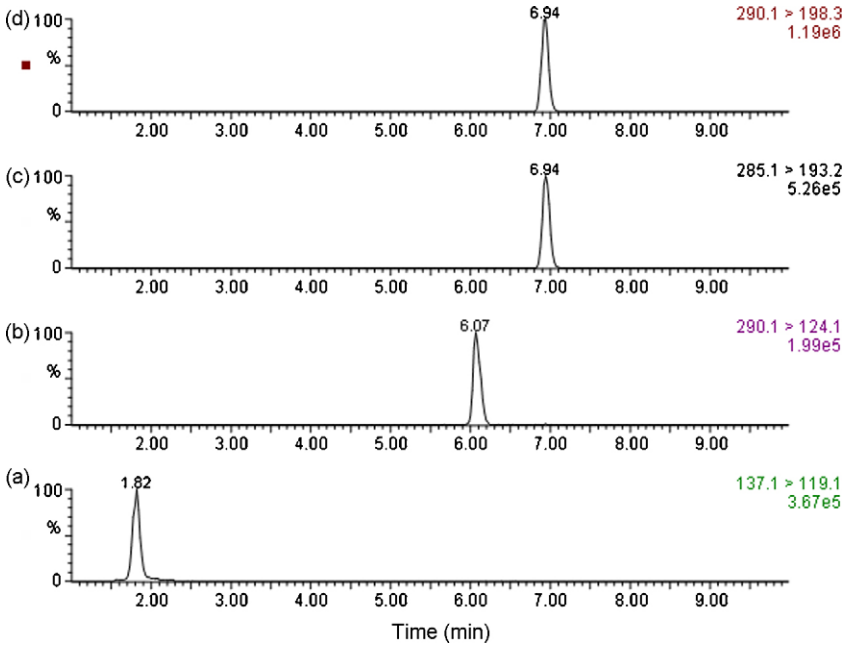

Fig. 3. MRM chromatograms (a–d) of a processed quality control sample at high concentration level: pralidoxime transition (a, 790 ng/ml), atropine transition (b, 38 ng/ml), diazepam transition (c, 391 ng/ml) and diazepam D5 transition (d).

was used as a protein precipitation reagent. All stock solutions were stored at −20 °C.

Calibration standards (at seven concentrations) and QC samples (at low, medium and high levels) containing the three studied molecules were prepared in plasma by diluting various volumes of working solutions in human drug-free plasma. The following concentration ranges were validated: 5–1000 ng/ml for pralidoxime, 0.25–50 ng/ml for atropine and 1–500 ng/ml for diazepam.

2.3. Sample preparation

To 100 μl of plasma (QC, calibration standard and subject), 150 μl of protein precipitation reagent (including I.S.) was added. After vortex mixing for 30 s, the samples were ultra centrifuged at 10,000 rpm for 5 min. The supernatant was then transferred into autosampler vials, and 10 μl aliquot was then analyzed by the chromatographic system.

2.4. Chromatographic and mass spectrometric conditions

The LC separation was performed using a Waters Alliance® 2695 separation module system. Chromatography was carried out at 40 °C in a reversed phase system using a X-Terra® MS C_8 column (100 mm × 2.1 mm, i.d. 3.5 μm) protected with X-Terra® MS C_8 (10 mm × 2.1 mm, i.d. 3.5 μm) precolumn (Waters, Saint Quentin en Yvelines, France).

A quick stepwise gradient was used to elute the compounds from the column. At time zero, a mixture of 99% of ammonium formate buffer (pH 3, 2 mM) and 1% of acetonitrile was flushed through the column. From 2 to 3 min, the percentage of acetonitrile was increased to 2% linearly. Then, from 3 to 3.1 min, acetonitrile percentage was augmented to 95%. This solution of 95% acetonitrile was held till 7.5 min. From 7.5 to 8 min, the solution was changed to 1% of acetonitrile. From 8 to 11 min the HPLC column was re-equilibrated before the next injection. The flow rate was maintained at 0.2 ml/min. The column outlet was connected to the electrospray sample inlet.

The separated compounds were detected with a Waters Micromass Quattro Premier® triple quadrupole mass spectrometer with an electrospray source operating in positive ionization mode. The ionization source conditions were as follows: capillary voltage of 3.0 kV, source temperature of 120 °C and desolvation temperature of 80 °C. The cone and desolvation gas flows were 60 l/h and 651 l/h, respectively, and were obtained from a nitrogen generator (Peak Scientific). Argon was used as the collision gas and regulated at 0.25 ml/min. The multiplier was set to 650 V.

Mass spectrometer conditions (cone and collision energy) were optimized by direct infusion of the compound into the source (solutions at 0.5 mg/ml in water:methanol (50:50, v/v)). Multiple reaction monitoring (MRM) was used for data collection and the precursor/product ion transitions were listed in Table 1. ESI mass spectra of pralidoxime, atropine and diazepam are shown in Fig. 1. Data were processed by MassLynx® NT software.

2.5. Validation procedure

Validation of the analytical method was based on the Food and Drug Administration (FDA) guidelines for bioanalytical method validation published on-line [24].

2.5.1. Linearity

Calibration standards were prepared and analyzed in triplicate in three independent runs. Calibration curves (area ratio with I.S. vs. nominal analyte concentration) were fitted by least square linear regression without weighting and using $1/X$ and $1/X^2$ (X = concentration) as weighting factors. In order to establish the best weighting factor the goodness of the fit graphs were examined. To assess linearity, deviation of the mean calculated concentration over three runs should be within ±15% of nominal concentration with a coefficient of variation (CV) <15%. At the lower limit of quantification (LLOQ) level, a deviation of ±20% and a CV up to 20% was permitted.

Table 4
Stability study results

Analyte	Nominal concentration	Three freeze/thaw cycles		−20 °C		25 °C		Extracted at 25 °C	
		Recovery%	CV%	Recovery%	CV%	Recovery%	CV%	Recovery%	CV%
Pralidoxime	15	104.67	14.00	101.33	13.40	102.67	9.61	110.33	3.84
	300	100.33	5.30	96.13	4.26	91.07	4.55	102.25	10.49
	800	96.90	9.20	96.63	4.60	99.80	5.30	91.41	5.51
Atropine	0.75	95.56	12.00	96.24	10.20	104.32	9.75	105.20	6.80
	15	104.23	9.00	97.50	6.80	98.64	4.21	104.52	7.52
	40	106.32	8.00	101.20	5.80	102.30	7.80	96.30	5.23
Diazepam	3	107.87	8.93	105.56	7.29	105.67	10.15	110.00	4.29
	150	105.00	9.59	97.73	5.50	103.53	3.77	101.33	5.12
	400	106.50	6.31	104.70	4.30	105.88	6.02	97.2	2.98

2.5.2. Accuracy and precision

The QC samples already prepared were used for precision and accuracy determination, the three QC levels were chosen to cover the calibration curve range.

Precision was calculated as the CV% with a single run (Intra-assay) and between different runs (Inter-assay). Accuracy was determined as the percentage of deviation between measured and nominal concentration.

2.5.3. Limit of detection (LOD) and lower limit of quantification (LLOQ)

The limit of detection was set to the lowest concentration where the signal of the compound was threefold higher than background noise.

The LLOQ was experimentally chosen as the minimal concentration in plasma samples that could be confidently determined. FDA guidelines recommend that the deviation between measured and nominal concentration at LLOQ should not deviate more than ±20% with a precision <20%.

2.5.4. Specificity

Specificity refers to the ability of analytical method to differentiate and quantify the analyte in the presence of other components. Specificity was examined by applying the pretreatment procedure to drug-free human plasma samples ($n = 5$) as previously described by Shah et al. [25].

2.5.5. Stability

The stability of analytes was investigated at various concentrations during all steps of analysis.

Therefore, freshly prepared QC samples at three concentration levels were stored for 24 h at room temperature, 3 months at −20 °C and underwent three freeze/thaw cycles.

The stability of extracted samples was assessed by reanalyzing a calibration curve and duplicate QC samples at each level after storage for approximately 20 h at room temperature.

2.5.6. Assessment of matrix effects

To assess any possible suppression or enhancement of ionization due to sample matrix, three types of experiments were performed.

In the first experiment, blank plasma samples of subjects at time 0 (before administration of any drug), used as negative samples were analyzed.

The second experiment included the evaluation of the matrix effect as described by Matuszewski et al. [26]. For this test two sets of samples are necessary. Set A consists of standard solutions. For set B blank samples are supplemented with the same amount of standards as used for set A.

Absolute matrix effects were calculated with the formula: $ME\% = B/A \times 100$.

Relative matrix effect was based on direct comparison of the MS/MS responses of extracts originating from different batches (sources) of biological fluid (set B) with analytes spiked into. The variability of the responses, expressed as CVs (%), can be considered as a measure of the relative matrix effect for a given analyte.

The final procedure was based on the post-column infusion of an analyte in a chromatographic run of an extract or a blank matrix [27]. The signal was compared to the signal obtained with the post-column infusion of the same model analyte in a chromatographic run with eluent only.

2.6. Clinical application

Twenty healthy adult male volunteers between the ages of 18 and 45 years (29.7 ± 6.3 years, mean ± SD) were selected for the clinical study. All subjects provided written informed consent and the Ethics Committee has approved the clinical protocol. All volunteers were assessed as healthy based on medical history, clinical examination, blood pressure, ECG and laboratory investigation (hematology, blood biochemistry and urine). No subject had a history or showed evidence of hepatic, renal, gastrointestinal, or hematological deviations, or any acute/chronic disease or drug allergy.

The study was conducted in an open, randomized, single-dose, three-way, cross-over design with a 3 weeks washout period between the treatments. Each subject received the following treatments by i.m. injection: 20 mg of avizafone chlorhydrate, 11.3 mg of diazepam and 20 mg of avizafone chlorhydrate combined with 2 mg of atropine sulfate and 350 mg of pralidoxime methyl sulfate using the bi-compartmental auto-injector under development (AIBC).

Blood samples were collected before i.m. administration, and 0.0833, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120 and 168 h after treatment administration. The blood samples were centrifuged and plasma was separated and stored at −80 °C until drug assay.

3. Results and discussion

3.1. Chromatographic conditions

The method permitted the separation and the simultaneous quantification of molecules used in the treatment of intoxication by organophosphorous compounds. These molecules vary greatly with respect to their hydrophilic nature. Hence, application of the stepwise gradient method was necessary in order to obtain an acceptable run time with accurate and precise quantification.

Upon injection, a small percentage of acetonitrile was applied in order to elute pralidoxime with enhanced resolution. Once the elution of pralidoxime and atropine was completed, the percentage of acetonitrile was increased to 95% to elute diazepam. Use of a dramatic gradient permitted simultaneous quantification of the three compounds in the treatment. It was necessary to increase the

Table 5
Matrix effect study ($n = 6$) results

Analyte	Nominal concentration (ng/ml)	Recovery%	CV%
Pralidoxime	15	37.4	2.69
	300	39.2	1.33
	800	45.2	1.17
Atropine	0.75	40.5	1.94
	15	37.7	0.63
	40	38.7	1.35
Diazepam	3	31.2	2.71
	150	31.1	3.16
	400	30.6	5.89

acetonitrile percentage quickly because of the differences in the hydrophilic nature of the molecule (atropine and pralidoxime are highly hydrophilic while diazepam is highly lipophilic).

Varying pretreatment procedures, including a liquid–liquid or a solid phase extraction were not applicable due to the variability in molecules nature. Also, the pretreatment procedure permitting the extraction of three molecules would be labor intensive and time consuming. To expedite the sample processing, a protein precipitation using acetonitrile was applied. This sample pretreatment procedure allowed the quantification of pralidoxime atropine and diazepam with a LLOQ that favorably compared to methods already published [9,17].

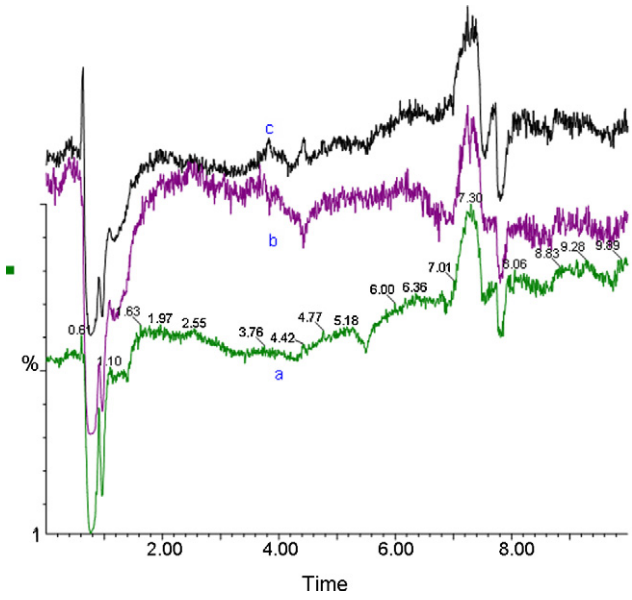

Fig. 4. MRM chromatograms for pralidoxime (a), atropine (b) and diazepam (c) during post-column infusion and subsequent injection of blank human plasma extracted according to the assay. Retention times for pralidoxime, for atropine and diazepam are, respectively, 1.8, 6.0 and 6.9 min.

3.2. Validation procedure

The assay was linear over the validated concentration ranges of 1–500 ng/ml for diazepam, 5–1000 ng/ml for pralidoxime and 0.25–50 ng/ml for atropine, with determination coefficients $R^2 \geq 0.99$. The best calibration curves fitting was obtained using a weighting factor of $1/(\text{concentration})^2$ for all analytes. Deviations from the nominal concentration ranged from −5 to 15% for all analytes at all concentrations. The overall precision of the back-calculated standard concentrations was <14.3% for all analytes and at all concentration levels.

Intra- and inter-assay validation results are presented in Table 2.

The intra-assay precision as measured by the CV% of mean of six analyses of three QC sample concentrations in one run. It was <12% for all tested concentrations for all compounds.

The mean inter-assay precision did not exceed 9.6% for all analytes.

Intra-assay accuracies were within ±13.5% for the limit of quantification and within ±11.1% for the other concentrations.

Samples above the upper limit of quantification could be reanalyzed and quantified with acceptable accuracy after dilution with drug-free human plasma.

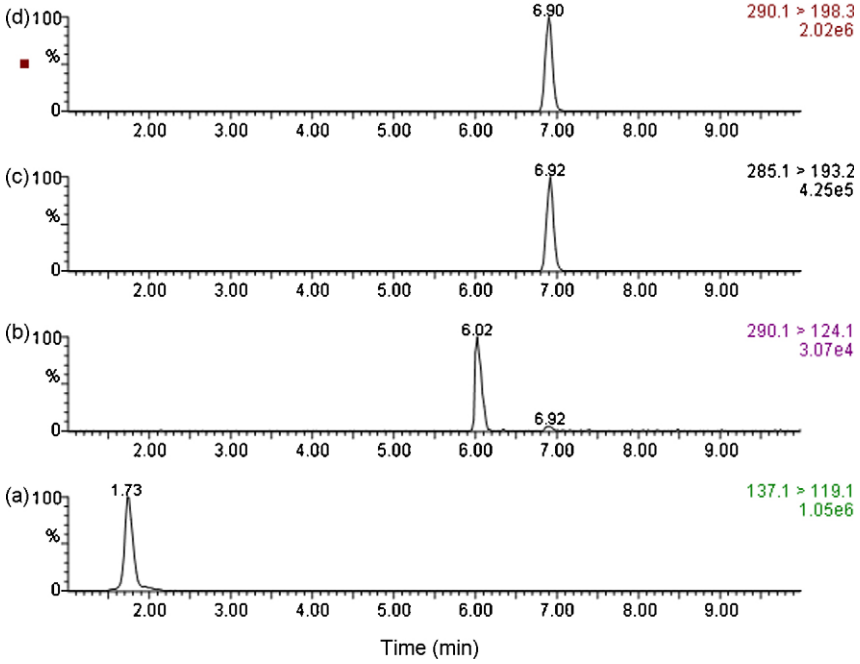

Fig. 5. MRM chromatograms (a–d) of a processed subject sample 30 min after the intramuscular administration of the three analytes: pralidoxime transition (a, 1998 ng/ml), atropine transition (b, 3.8 ng/ml), diazepam transition (c, 207 ng/ml) and diazepam D5 transition (d).

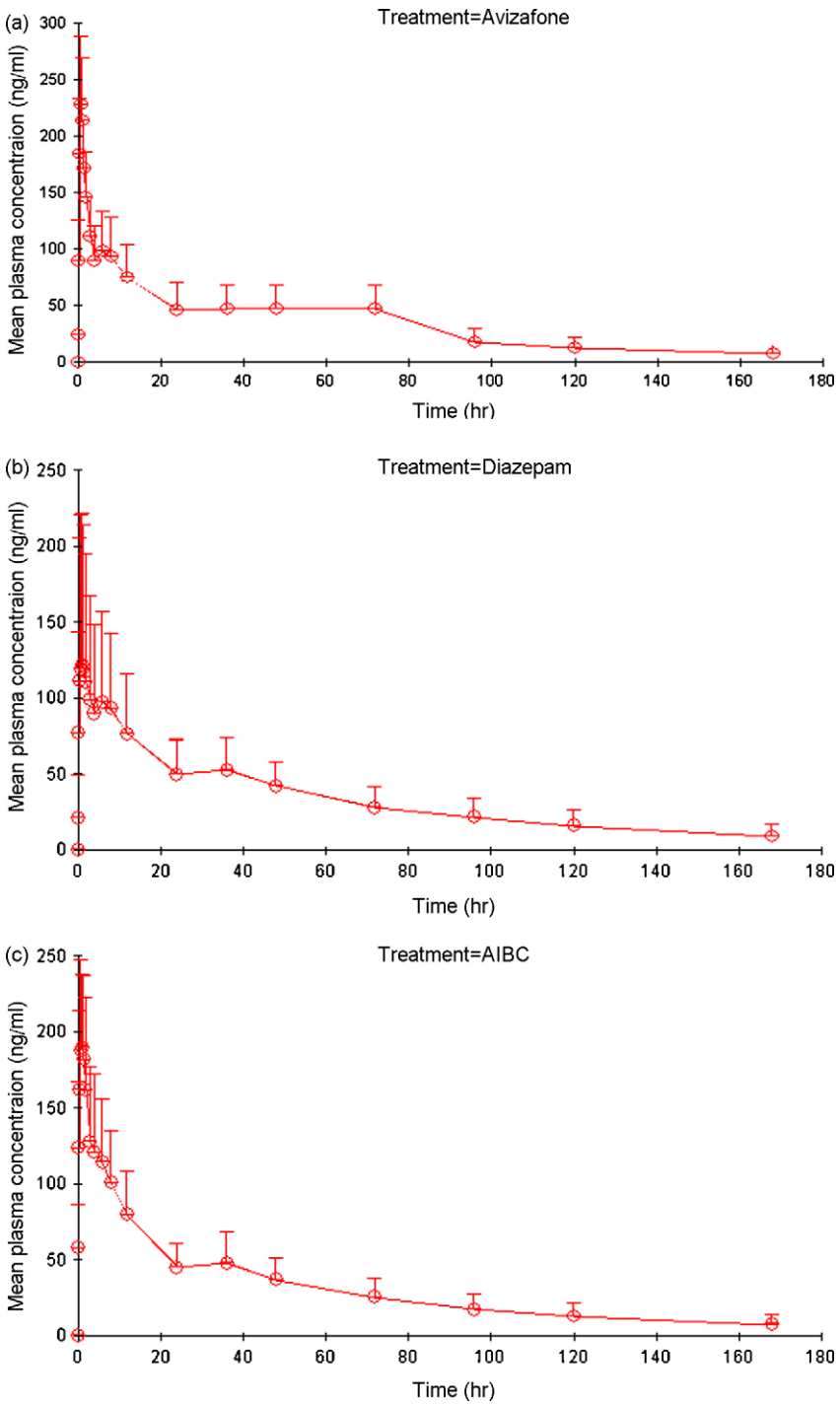

Fig. 6. Diazepam mean pharmacokinetic profile after the intramuscular administration of avizafone alone (a), diazepam alone (b) and avizafone with atropine and pralidoxime using the bi-compartmental auto-injector "AIBC" (c).

The LOD was set to 0.1 ng/ml for diazepam, 1 ng/ml for pralidoxime and 0.075 ng/ml for atropine.

The LLOQ was validated at 1 ng/ml for diazepam, 5 ng/ml for pralidoxime and 0.25 ng/ml for atropine in human plasma.

For LLOQ, the CV ($n = 6$) of the quantified concentrations ranged from 3.49 to 18.9%. The accuracies for LLOQs were within ±13.5% for all analytes (Table 3).

3.2.1. Selectivity and specificity

Drug-free human plasma ($n = 5$) was analyzed using the chromatographic conditions used to quantify the studied molecules. Fig. 2 shows the extracted single ion chromatograms of drug-free human plasma spiked with internal standard. Fig. 3 shows the extracted single ion chromatograms of high-level quality control sample. No significant endogenous interfering peaks were noticed at the retention time of the studied analytes.

3.2.2. Stability

The results of stability study are presented in Table 4. The stability of extracted samples was tested by reinjecting a calibration standards and duplicate QC samples at each concentration after storage for approximately 20 h stored at room temperature. Stan-

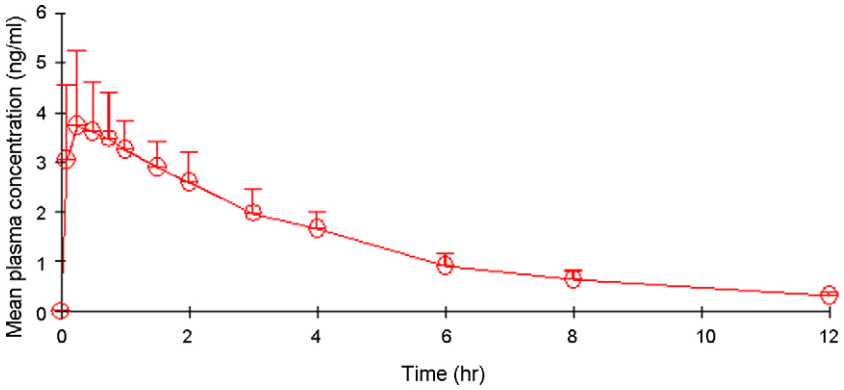

Fig. 7. Atropine mean pharmacokinetic profile after the intramuscular administration of atropine with avizafone and pralidoxime using the bi-compartmental auto-injector "AIBC".

dards and QC samples deviation remained within ±13.4% for all analytes.

The stability of plasma samples exposed to three cycles of freezing and thawing prior to analysis was assessed by analyzing triplicate QC samples at each concentration level after three freeze/thaw cycles. The mean plasma concentrations for QC samples remained within ±7.87% of nominal levels and that for all molecules at the three levels indicated acceptable stability for samples thawed up to three times before analysis.

The stability of plasma samples exposed to thaw at room temperature storage conditions was tested by analyzing triplicate QC samples at each concentration level after storage at room temperature during 24 h. Mean plasma concentrations for these QC samples were within ±9.93% of nominal levels, indicating acceptable stability of analytes in samples thawed 24 h prior to analysis.

The stability of frozen samples was tested by analyzing QC samples in triplicate at each concentration after storage for approximately 3 months at −20 °C. Mean plasma concentrations for QC samples remained within ±5.56% of nominal levels, indicating acceptable stability for samples stored at least 3 months at −20 °C.

3.2.3. Assessment of matrix effects

Plasma samples were obtained before treatments administration. These samples were used as negative controls to compare the baseline chromatograms with those obtained after drug administration.

The matrix effect evaluation procedure showed a low influence on the qualitative and quantitative determinations, and this was further confirmed by the post-column infusion test. As far as an absolute matrix effect is concerned, the percentages of recovery were <100% indicating an ionization suppression. Meanwhile, the assessment of the relative matrix effect showed that the precision of the determination of set B at three concentration levels varied very slightly from 0.63 to 5.89%, for all analytes (Table 5). These data showed that the relative matrix effect for the three analytes was nearly absent.

Post-column infusion (10 μl/min) of the three analytes into the mobile phase while injecting extracted blank matrix is a very useful tool to determine the location of interference peaks that cause ion suppression. No critical area around the retention times of the three analytes was detected (Fig. 4).

3.3. Clinical application

For pharmacokinetic purpose, over 1200 plasma samples from 20 subjects have been assayed using the bioanalytical method described above. Fig. 5 shows the extracted single ion chromatograms of a subject's plasma, spiked with internal standard, 30 min after i.m. administration of the three compounds. Fig. 6 shows the mean pharmacokinetic profile of diazepam after i.m. administration of avizafone alone, diazepam alone and avizafone with atropine and pralidoxime using the bi-compartmental auto-injector "AIBC".

Fig. 7 shows the mean pharmacokinetic profile of atropine after i.m. administration of atropine with avizafone and pralidoxime using the bi-compartmental auto-injector "AIBC".

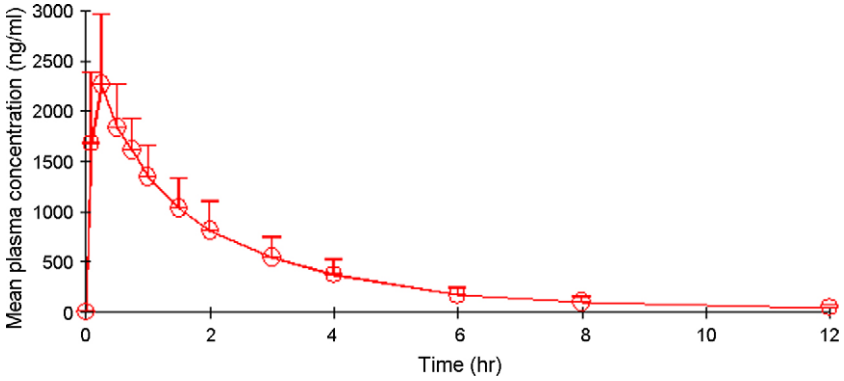

Fig. 8. Pralidoxime mean pharmacokinetic profile after the intramuscular administration of atropine with avizafone and pralidoxime using the bi-compartmental auto-injector "AIBC".

Fig. 8 shows the mean pharmacokinetic profile of pralidoxime after i.m. administration of pralidoxime with atropine and avizafone using the bi-compartmental auto-injector "AIBC".

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