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Measurement of serum pralidoxime methylsulfate (Contrathion®) by high-performance liquid chromatography with electrochemical detection

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Abstract

Pralidoxime methylsulfate (Contrathion®) is widely used to treat organophosphate poisoning. Despite animal and human studies, the usefulness of Contrathion® therapy remains a matter of debate. Therapeutic dosage regimens need to be clarified and availability of a reliable method for plasma pralidoxime quantification would be helpful in this process. We here describe a high-performance liquid chromatography technique with electrochemical detection to measure pralidoxime concentrations in human serum using guanosine as an internal standard. The assay was linear between 0.25 and 50 $\mu g\,mL^{-1}$ with a quantification limit of 0.2 $\mu g\,mL^{-1}$. The analytical precision was satisfactory, with variation coefficients lower 10%. This assay was applied to the analysis of a serum from an organophosphorate poisoned patient and treated by Contrathion® infusions (100 and 200 $mg\,h^{-1}$) after a loading dose (400 mg).
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1. Introduction

According to the World Health Organisation, organophosphates (OP) are implied in about 3,000,000 of poisoning cases with over 220,000 deaths [1]. Organophosphate poisonings mostly result from accidental causes and suicidal attempts, especially in developing countries. Fortunately, they have been involved only in a few cases of bioterrorism, such as the attack in Tokyo. These compounds act by inhibition of the enzyme acetylcholinesterase, which results in an excess of acetylcholine and an overstimulation of both muscarinic and nicotonic receptors of the parasympathetic nervous system [2]. The standard therapeutic scheme includes supportive treatment, antidote infusions (atropine and/or oximes), and decontamination [3]. Atropine counteracts muscarinic effects of organophosphates, but is ineffective on nicotonic effects [4]. Oximes are nucleophilic agents which can re-activate all phosphorylated receptors by binding to the organophosphorus compound [5]. Among oximes, pralidoxime is used as a methylsulfate (Contrathion®) (Fig. 1) or as chloride salts (Protopam®) [6]. Despite human [7–9] and animal studies [10,11], the usefulness of pralidoxime therapy is still questioned. Treatment by oximes is not always successful due to differing toxicological activity among OP compounds, delays between poisoning and treatment, "ageing" of the compound at the receptor, lack of central action and absence of standardized therapeutic infusion schemes [12]. Furthermore, the relationship between oximes plasma concentration and protection against organophosphate poisoning is not well defined [10]. According to some studies [7,13], a plasma concentration at or above 4 $mg\,L^{-1}$ is required to observe a therapeutic efficacy. Due to its rapid renal excretion, various administration protocols have been tested in man to achieve this therapeutic range [7,8,14]. In these studies, pralidoxime plasma concentrations were determined, but pharmacokinetic param-

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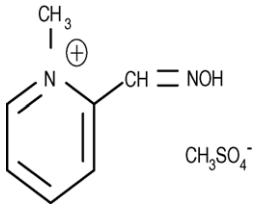

Fig. 1. Chemical structure of pralidoxime methylsulfate (Contrathion®).

eters were calculated only in few cases of human poisoning [7].

Different techniques have been reported for pralidoxime measurement in pharmaceutical formulations [15,16] and in biological material [7,17,18]. In the later, the employed methods were often high-performance liquid chromatography (HPLC) with UV detection, all derived from the original method of Benschop et al. [17]. The detection limit of this technique is about 0.5 μg mL^{-1}, with a 100 μL plasma. Due to pralidoxime short half-life [19], repeated sampling must be carried out to calculate pharmacokinetic parameters. It is therefore necessary to reduce the volume of blood sampled, especially in animal studies, thus requiring an increase in method sensitivity.

In the present study, we describe a chromatographic method with electrochemical (EC) detection to determine pralidoxime concentrations in biological samples. This method is 10-fold more sensitive than UV detection and pralidoxime concentrations are easily measurable with 25 μL of plasma. This method is therefore applicable to pharmacological studies both in man and in animals. Given its degree of sensitivity, this method will also permit the exploration of oxime diffusion in other biological fluids, such as cerebrospinal fluid.

2. Experimental

2.1. Reagents

Guanosine, 1-octane sulfonate sodium salt (OSS), dihydrated disodium phosphate and monopotassium phosphate were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). Pralidoxime methylsulfate was kindly provided by SERB Laboratories (Paris, France). Obidoxime dichloride and orthophosphoric acid, perchloric acid, hydroxide natrium and methanol, all of analytical quality, were purchased from Merck (Nogent-sur-Marne, France). Water for injectable preparations (Frésénius France Pharma, Louviers, France) was used for preparation of different reagents.

2.2. Chromatographic conditions

The chromatographic system consisted of an isocratic pump (Waters 515, St-Quentin-en Yvelines, France) and an automatic injector (Autosampler 712, Waters). Separation was performed at room temperature on an Inertsil ODS 3250 × 4.6 mm column (i.d.) (Chrompack, Les Ulis, France). The mobile phase (1 mL min^{-1}) was composed of 8% methanol and 92% buffer containing disodium phosphate (15 mM), monopotassium phosphate (15 mM), and 1-octanesulfonate sodium salt (1 μM) adjusted to pH 2.6 with orthophosphoric acid.

For parlidoxime detection in serum, the oxidation of oxime function was ensured by an electrochemical detector composed of a Coulochem 5100 A model, a Coulochem 5011 analytic cell and a cell guard 5020 (Eurosep, Cergy-pontoise, France). Measurement cells had respective potential of +0.30 V (E1) and +0.95 V (E2), while the potential of the guard cell was +1.20 V. The detector gain was set at 20 μA. Data were collected on an integrator (Data Module 746) with attenuation set at 32.

In urine, parlidoxime was detected at 280 nm using an UV–vis detector (Waters 2487).

2.3. Preparation of the calibration range and controls

The calibration range and various controls (low, medium and high) were prepared from pooled serum obtained from healthy subjects. The pool was stirred on activated charcoal for 24 h and then filtered (Nalgène 0.45 μm filter, Merck) before being fractionated and frozen at −80 °C.

Pralidoxime methylsulfate stock solutions (1 mg mL^{-1} expressed as pralidoxime base) was prepared in water and stored to −80 °C for up to 6 months.

Contrathion® serum standards (calibration standards) were prepared at concentrations of 0.25–50 μg mL^{-1}. The first serum standard concentration (50 μg mL^{-1}), obtained by dilution (1:20) of stock solution in pooled serum, was then fractionated and frozen at −80 °C. The calibration range was extemporaneously prepared by diluting 50 μg mL^{-1} serum standard in free pooled serum; point 0 consisted of pooled serum only.

For the internal standard, mother guanosine solution (1 mg mL^{-1}) was prepared in water (with 10 μL of hydroxide natrium 1 M), fractionated and frozen at −80 °C. In these conditions, this solution is stable for 6 months. Working solutions of internal standard (IS) in water were extemporaneously prepared by diluting 1:100 the stock solution.

Low (C1 = 0.75 μg mL^{-1}), medium (C2 = 7.5 μg mL^{-1}) and high (C3 = 40 μg mL^{-1}) controls were obtained by diluting 50 μg mL^{-1} serum standard in free pooled serum. They were fractionated and frozen at −80 °C.

Urine standards were prepared at concentrations ranging from 1 to 0.25 mg mL^{-1}. The first urine standard concentration (1 mg mL^{-1}) corresponded to the pralidoxime stock solution. The calibration range was extemporaneously prepared by diluting the stock solution with distilled water; point 0 consisted of distilled water only.

2.4. Sample preparations

Serum standards, controls or samples (25 μL), and diluted internal standard solution (50 μL) were mixed in 1 ml Eppendorff tubes. One hundred microliters of perchloric acid (0.6 M) was added to precipitate proteins. Eppendorff tubes were shaken for 1 min and centrifuged at 15 000 rpm for 5 min. Supernatants were further diluted with distilled water (1:5) to reduce acidity. A 50 μL volume of this mixture was then injected in the chromatographic system.

Urine samples were prepared according to the method described by Grasshoff et al. [24]. Urine standards, prediluted samples (1:20 and 1:100 in distilled water) (500 μL), and diluted internal standard solution (50 μL) were mixed in 1 ml Eppendorff tubes with 300 μL of perchloric acid (0.6 M). Then, urinary samples preparation was similar to serum samples.

2.5. Choice of potential measurement cells

To select appropriate potentials, a serum standard containing pralidoxime (5 $\mu g\,mL^{-1}$) and IS (10 $\mu g\,mL^{-1}$) was used. In a first step, E1 potential was adjusted to 0 V. A 50 μL serum standard was injected into the chromatographic system for different values of E2 potential, from 0 to +1 V. For each potential, signal intensities were recorded for pralidoxime and IS. The same experiment was then performed with E1 potentials varying between 0 and +1 V. E2 was set at the potential determined during the first step procedure and giving the best signal intensity.

2.6. Clinical application

To validate our technique, pralidoxime methylsulfate concentrations were measured in a case of acute organophosphorus poisoning treated with Contrathion®. A 52-year-old man was admitted in Intensive Care Unit (ICU) in Algeria suspected of organophosphorus poisoning with abdominal pains, hypersalivation, nausea, dyspnea and cyanosis. Ingested quantity and name of product were unknown, but cholinesterase activity was decreased in serum (1.3 $UI\,L^{-1}$; normal range: 2–8 $UI\,L^{-1}$) and erythrocytes (860 $UI\,L^{-1}$: normal range: 2800–5200 $UI\,L^{-1}$). Initially, the patient was treated by atropine (1.5 $mg\,kg^{-1}$) before transferred to the MedTox ICU at Lariboisière Hospital (Paris, France). Atropine therapy was continued and an oxime (Contrathion®) treatment was initiated. After a loading dose of 400 mg in slow perfusion over 30 min, Contrathion® was infused at the dose of 100 $mg\,kg^{-1}$ over 18 h, then 200 $mg\,kg^{-1}$ over 3 days, followed by of 100 $mg\,kg^{-1}$ over 60 h. Blood and urine specimens were obtained during pralidoxime methylsulfate treatment. Five milliliters of blood were collected in glass tubes without anticoagulant and immediately centrifuged (at 2000 × *g* during 15 min). Samples not immediately assayed were stored at −20 °C; an aliquot of urine was frozen at −20 °C.

3. Results and discussion

3.1. Choice of mobile phase buffer and optimization of detection potentials

Coulometric detection requires salt in the mobile phase. Among different salts, phosphate is frequently employed. We selected a 30 mM phosphate buffer since an optimum response has been previously reported for 5–45 mM phosphate buffer molarity [20].

The Coulochem 5011 system is equipped with two electrodes of measure (E1 and E2) and a guard cell. The voltage applied to the guard cell allows mobile phase oxidation before sample injection without modifying the response of measurement electrodes. E1 and E2 electrodes permit simultaneous determination of two oxido-reductive substances. In our study, the second electrode (E2) was used to measure pralidoxime, whereas the first electrode (E1) eliminated interfering substances.

To optimize the detection, we tested a serum standard containing pralidoxime and guanosine (IS) according to the procedure previously described (Section 2.5). The Fig. 2 shows the intensity/potential curves obtained with the two electrodes. E2 response was undetectable between 0 and +0.7 V, then increased to become maximal at +0.95 V. No difference was observed between +0.95 and +1 V. For E1 potential optimization, E2 value was set at +0.95 V. Fig. 2 shows that the signal intensity was maximal between 0 and +0.3 V and then decreased because of the beginning of pralidoxime oxidation. E1 voltage was thus set at +0.3 V to eliminate interfering substances without reducing the signal intensity. For guanosine (IS), intensity/potential curves were determined in the same manner (Fig. 2). Potentials were similar (E1 = +0.40 V, E2 = +0.90 V) to those determined for pralidoxime.

In conclusion, pralidoxime optimal voltage values were +0.95 and +0.30 V for E2 and E1, respectively. These values were applied during method validation and clinical application.

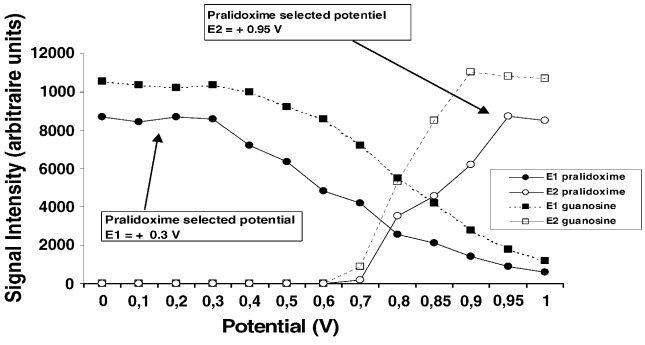

Fig. 2. Intensity/potential curves of pralidoxime and guanosine (IS) to determine optimal potentials for E1 and E2. Curves were obtained after injection of 50 μL of serum standard containing pralidoxime (5 $\mu g\,mL^{-1}$) and IS (10 $\mu g\,mL^{-1}$) in the chromatographic system.

3.2. Choice of internal standard

In most HPLC methods with UV detection, pyridostigmine is selected as an internal standard [7,17]. With our electrochemical detection method, the response observed with this compound was limited, even at high concentration (up to 10 mg L^{-1}). We choose guanosine as an internal standard (IS) since it contains an amine function easily oxidized at similar potentials as pralidoxime (Fig. 2). Guanosine is a natural compound not detected in serum during physiologic conditions because of immediate transformation in guanine by purine nucleoside phosphorylase [21].

3.3. Validation of the method

The method was validated for routine pralidoxime methylsulfate assays in human serum. Criteria retained for this optimization are those generally used for high-performance liquid chromatography: specificity, reproducibility of migration time, linearity, sensitivity, and percentage of recovery as well as within-run and between-run precision.

3.3.1. Specificity

Fig. 3 shows the separation and determination of methylsulfate of pralidoxime in human serum using guanosine as internal standard. In the chromatogram obtained after preparation of blank serum, no additional peaks interfered with the measured Contrathion® and IS (Fig. 3a). The compounds were well separated with a migration time of 14.65 min for pralidoxime and 21.70 min for IS (Fig. 3b and c). For pralidoxime, the peak shape had no evidence of tailing (symmetry factor 1.03). A chromatogram obtained by analyzing serum from a patient treated with Contrathion® had a similar appearance (Fig. 3d).

3.3.2. Linearity

The linearity of the method (peak height ratio of the drug/IS versus drug concentration) was evaluated over a range of concentration of 0.25–50 μg mL^{-1}. Regression analysis performed by the least-squares method gave the following formula: $y = 0.0569x + 0.0324$. This equation was determined by 5 calibrations obtained on different days.

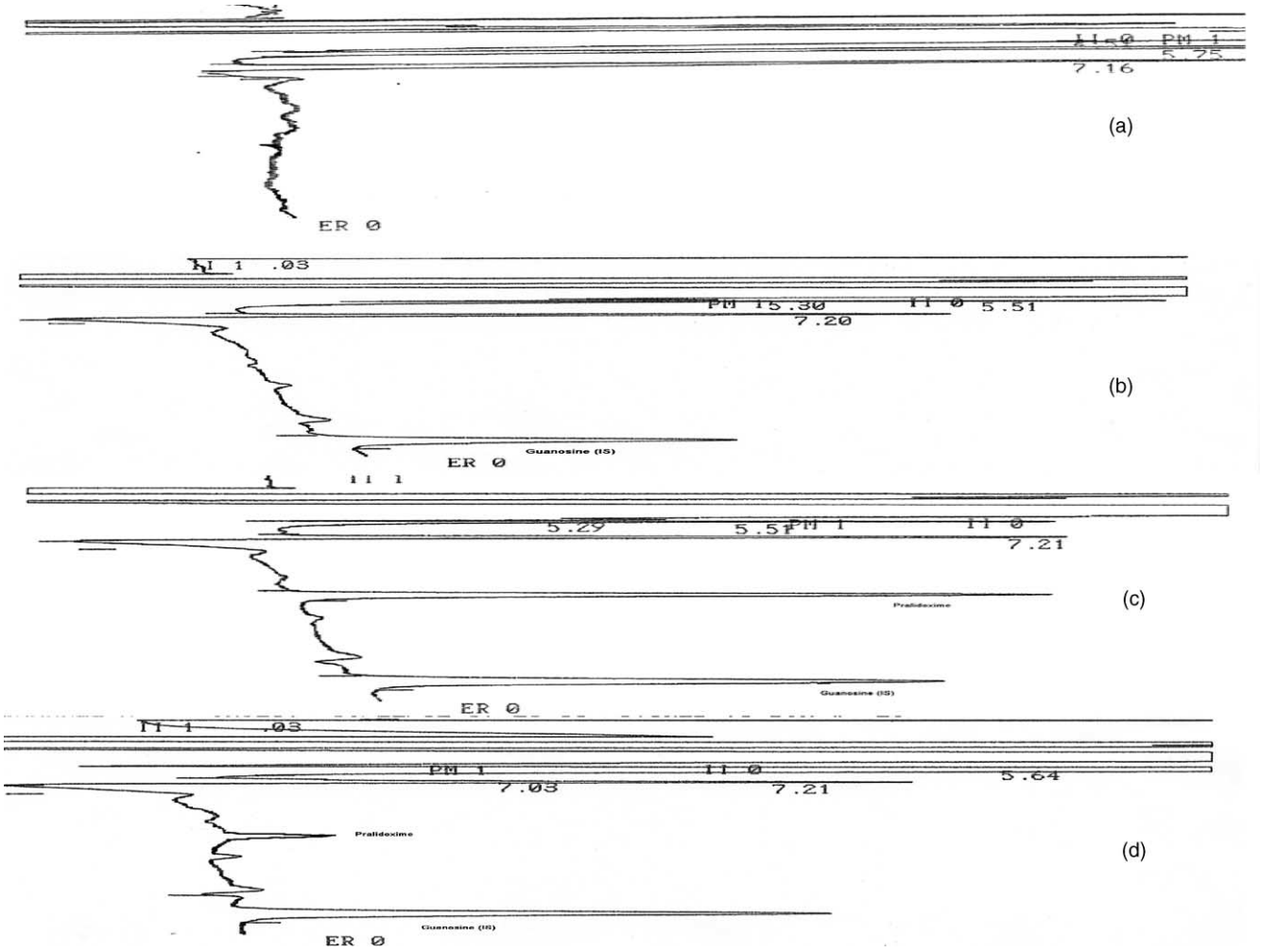

Fig. 3. HPLC profiles of extracted blank serum without IS (a), extracted blank serum with IS (b), spiked human serum containing 20 μg mL^{-1} pralidoxime and IS (c) and human serum (concentration = 3.1 μg mL^{-1}) with IS (d). Retention time were 14.65 and 21.70 min for pralidoxime and IS, respectively.

Table 1
Precision of the HPLC pralidoxime method

Pralidoxime concentration		Within-run precision ($n = 20$)	Between-run precision ($n = 20$)
C1 (0.75 μg mL^{-1})	Mean concentration (μg mL^{-1})	0.76	0.72
	R.S.D. (μg mL^{-1})	0.07	0.09
	CV (%)	9.2	12.5
C2 (7.5 μg mL^{-1})	Mean concentration (μg mL^{-1})	7.37	7.7
	R.S.D. (μg mL^{-1})	0.28	0.51
	CV (%)	3.7	6.6
C3 (40 μg mL^{-1})	Mean concentration (μg mL^{-1})	38.7	38.4
	R.S.D. (μg mL^{-1})	1.2	2.3
	CV (%)	3.4	5.9

Three control levels (C1 = 0.75 μg mL^{-1}, C2 = 7.5 μg mL^{-1} and C3 = 40 μg mL^{-1}) were used. HPLC conditions were similar those described in Section 2. R.S.D.: relative standard deviation; CV: coefficient of variation.

Correlation coefficient ($r^2 > 0.999$) confirm the excellent linearity of the method.

3.3.3. Precision

To determine between run and within run precision, injections were realized 20 times for three serum control levels C1 (0.75 μg mL^{-1}), C2 (7.5 μg mL^{-1}) and C3 (40 μg mL^{-1}). Table 1 indicates mean values, relative standard deviations (R.S.D.) and coefficients of variation (CV). For the C1 concentration, within run CV was below 10%, but higher (12%) in between-runs. In other controls (C2 and C3), precision was excellent, with CVs below 5% for within run and below 10% for between-runs.

3.3.4. Detection (DL) and quantification limits (QL)

Detection limit, defined as the smallest quantities of pralidoxime clearly distinguishable from baseline, was calculated to be three times the signal-to-noise ratio (S/N = 3) estimated by the baseline thickness. DL was determined by analysis of solutions with decreasing concentrations of pralidoxime. For this compound, the detection limit was 0.05 μg mL^{-1} corresponding to 0.2 μg mL^{-1} as quantification limit. The observed DL was 10-fold more sensitive than HPLC procedures with UV detection [17].

3.3.5. Recovery tests

These tests were performed by overloading two sera containing 7.2 and 18 μg mL^{-1} of pralidoxime with increasing volumes (10, 20, 40, 80 and 100 μl) of a 50 μg mL^{-1} pralidoxime aqueous solution. The percentages of recovery indicated in Table 2 are within the limits of the recovery test (generally between 80 and 120%).

3.3.6. Correlation with a reference technique

For pralidoxime measurement in biological samples, HPLC with UV detection is considered as the reference method [17]. Serum pralidoxime concentrations were measured once with the both techniques (developed and reference) in 15 samples. We found an excellent correlation between UV and electrochemical detection. Slope of the regression curve was 0.93 with an intercept of 0.56 and a correlation coefficient 0.99. Using a Bland–Altman analysis [22], the mean difference was −0.206 μg mL^{-1}, with 95% confidence interval for difference of means included from 0.524 to 0.112 μg mL^{-1} without significant difference between two techniques ($p = 0.1869$).

3.3.7. Interfering substances

We tested physiologic and pharmacologic substances for potential interference. Among physiologic compounds, thiols compounds (cysteine, glutathione, homocysteine) may be oxidized at our working potential (E2 = +0.9 V), thus potentially interfering with our pralidoxime assay. Under our laboratory conditions, these compounds did not induce any interference. Cysteine and glutathione are rapidly eluted (RT = 6.2 and 8.6 min, respectively). Free homocysteine is present in serum at very small concentrations and cannot be thus detected. Total homocyteine could interfere with our method (RT is close to that of pralidoxime), but only after reduction of oxidized forms by a reducing agent, such as sodium borohydride [23]. In our method, reducing agents are not used and no interference was observed. Products with a pharmacological action, such as *N*-acetylcysteine, atropine and DL-penicillamine, were eluted 6.2, 19.5 and 20.5 min after injection, respectively. Atropine injection resulted in a very small peak and, therefore, cannot interfere even at high concentration (0.5 mg mL^{-1}). Among others oximes, we tested obidoxime which is the antidote approved in Germany and several other European Countries [24]. In our conditions, obidoxime is eluted after the internal standard

Table 2
Recovery study

50 μg mL^{-1} pralidoxime solution (μL)	Expected values (μg mL^{-1})	Measured values (μg mL^{-1})	Recovery values (%)	Expected values (μg mL^{-1})	Measured values (μg mL^{-1})	Recovery (%)
0	7.2	–		18.0	–	
10	7.7	7.4	96	18.3	18.5	101
20	8.1	8.1	100	18.7	18.1	97
40	9.0	9.2	102	19.4	19.6	101
80	10.7	9.9	92	20.6	20.5	99
100	11.5	11.6	101	21.2	20.8	98

HPLC conditions were similar those described in Section 2.

Table 3
Pralidoxime serum and urine concentrations and pharmacokinetic parameters in a patient treated with pralidoxime (Contrathion®) infusion after loading dose

Day after admission (Days)	Time after admission in French ICU (Hours)	Pralidoxime infusion rate / day infused amount ($mg.h^{-1}$ / g)	Atropine infusion rate ($mg.h^{-1}$)	Pralidoxime concentrations Serum ($\mu g.mL^{-1}$)	Pralidoxime concentrations Urines ($\mu g.mL^{-1}$)	Urine Output (mL)	Pralidoxime excreted / metabolised amount (g)	Renal Clearance ($L.kg^{-1}.h^{-1}$)
D0	T0		1.5	< 0.2	<1			
D0	T+3			< 0.2				
D1	**T4h-T4.5**	**Loading dose (400 mg) then 100 / 2.4**						
D1	T+10			3.1	810	1100	0.9/ 0.5	
D1	T+16			3.1				
D1	T+19			3.0	2727			
D1	**T+21**	**200 / 4.8**						
D3	T+35			5.6	2385	2600	6.2/ NC	0.75
D4	T+59				2051	2100	4.3/ 0.1	
D4	**T+69**	**100 / 2.4**	**Stop**					
D5	T+83			2.1	1651	2600	4.3/ NC	
D6	T+107				1382	1750	2.4 / 0	
D7	T+131			2.9	1987	1100	2.2 / 0.2	0.50
D8	**T+132**	**Stop**						
D9	T+155				841	1100	0.92/NC	

NC: not calculated.

(RT = 25.2 min) and cannot interfere with pralidoxime measurement.

3.4. Clinical application

In a patient treated with Contrathion®, we measured serum and urine concentrations of pralidoxime (Table 3). No detectable serum pralidoxime concentration was observed before the beginning of treatment showing the specificity of our method. In this patient, the therapeutic scheme (loading dose followed by continuous infusion) allowed serum concentrations of about 4 $\mu g\,mL^{-1}$, the presumed therapeutic concentration [14]. Daily urine pralidoxime concentrations were determined according the method of Grasshoff et al. [24] and high concentrations ranging from 810 to 2720 $\mu g\,mL^{-1}$ were obtained. Daily, metabolized amounts of pralidoxime are very small ranging from 0 to 0.5 g/24 h. The amount of metabolized pralidoxime (drug infused − drug excreted) was here calculated (Table 3). The excreted amount in urine accounted for 88–100% of the administered dose. These values are higher than those reported after intravenous [25] or intramuscular injections [26]. In two samples and after modifications of pralidoxime infusion regimen, excreted amounts in urine were greater than infused quantities. Renal clearance was 0.50 and 0.75 $L\,h^{-1}\,kg^{-1}$ for pralidoxime infusion to 100 and 200 $mg\,h^{-1}$, respectively (Table 3). These values are similar to these of Willems et al. [7] in poisoned patient.

In conclusion, the HPLC assay described allows the determination of serum pralidoxime with a good precision, selectivity and a sensitivity 10-fold higher than UV detection. The procedure has the advantage of being a relatively simple and convenient, thus suitable for therapeutic monitoring during organophosphate poisonings.

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