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Quantification of pralidoxime methylsulfate (Contrathion®) in human urine by capillary zone electrophoresis

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Abstract

Pralidoxime methylsulfate (Contrathion®) is widely used to treat organophosphate poisoning. For the first time, we developed a specific assay for urinary pralidoxime using capillary zone electrophoresis (CZE) in the following conditions: fused-silica capillary (length: 47 cm, internal diameter: 75 μm), electrolyte solution: 25 mM sodium borate (pH 9.1), voltage: 15 kV, temperature: 25 °C, injection time: 1 or 2 s, on-line UV detection: 280 nm. Sample preparation did not require a deproteinization step (1:5 dilution in water). The method was linear between 0.125 and 2 mg mL^{-1} of pralidoxime (quantification limit: 0.10 mg mL^{-1}). Coefficients of variation for intra- and inter-assay precision were below 10% for all three control levels (0.15–1.15 mg mL^{-1}). This assay was successfully applied to urine specimens from organophosphate poisoned patients treated by Contrathion® ($n = 10$). This CZE method allows the measure of pralidoxime in urine within 15 min with excellent precision, selectivity, and sensitivity. It is simple (no pretreatment) and convenient, thus suitable for the monitoring of Contrathion® therapy in organophosphate poisoned patients.

Keywords: Pralidoxime; Capillary zone electrophoresis; Urine

1. Introduction

According to the World Health Organization, organophosphates are annually involved in about 3,000,000 of poisoning cases with about 10% of mortality [1]. Organophosphate poisonings result mostly from accidental causes and suicidal attempts, especially in developing countries. Standardized therapeutic scheme includes supportive treatment, antidote infusion (atropine and/or oximes) and decontamination [2]. Oximes are nucleophilic agents able to re-activate phosphorylated receptors by binding to organophosphorus compounds [3]. Among approved oximes [4,5], pralidoxime is used as a methylsulfate (Contrathion®) (Fig. 1a) or chloride salts (Protopam®) and obidoxime as dichloride salts (Toxogonine®) (Fig. 1b). Oxime pharmacokinetic is characterized by a rapid plasma decrease secondary to renal excretion [4]. Non-renal elimination only accounts for 5% of pralidoxime total clearance (versus 15% for obidoxime) [6,7]. Supportive treatments, including artificial ventilation and cardiovascular support, might significantly modify oxime pharmacokinetic [8]. It would be, therefore, particularly helpful to obtain additional information about oxime urinary excretion to optimize their administration, especially in severely poisoned patients [8].

Several techniques have been described to measure oxime concentration in plasma [9–11] and in automatic injection device [12]. Two high liquid performance chromatography (HPLC) methods have been published for urine analysis [9,13]. They require 1 mL urine volume and a preliminary deproteinization step. Capillary electrophoresis (CZE) has never been used for oxime determination in urine, although CZE is often a method of choice to determine pharmaceutical compounds [14,15] in preparations and biological samples [16]. We developed a rapid CZE method to measure pralidoxime concentration in a small volume of urine (200 μL) and without sample deproteiniza-

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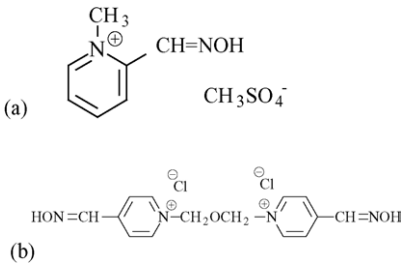

Fig. 1. Chemical structures of pralidoxime and obidoxime. (a) Pralidoxime methylsulfate (Contrathion®, MW = 137 as free base) and (b) obidoxime chloride (Toxogonine®, MW = 287 as free base).

tion. Daily urinary pralidoxime excretion was determined in ten patients treated with a continuous infusion of Contrathion® for organophosphate poisoning.

2. Experimental

2.1. Reagents

SERB laboratories (Paris, France) kindly provided pralidoxime methylsulfate. Sodium decahydrate tetrahydroborate was obtained from Merck (Nogent-sur-Marne, France). Normal Lyphochek Quantitative Urine Control® was purchased from Bio-Rad laboratories (Marnes-la-Coquette, France). Obidoxime, hypoxanthine, xanthine, cytosine, guanosine, adenosine, 5-fluorouracile, cytarabine, 6-*O*-methylguanine, and 7-methylguanine were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France). Distilled water, used to prepare reagents and standards, was from Frésénius (France Pharma, Louviers, France).

2.2. Electrophoresis

Capillary zone electrophoresis (CZE) was performed using the P/ACE 5500 system (Beckman, Gagny, France) equipped with a variable wavelength UV detector. A fused-silica capillary tube [total length (*L*), 57 cm; injector-detector length (*l*), 50 cm; internal diameter (I.D.), 75 μm] was selected. The part of the capillary ensuring electrophoretic separation is maintained at a constant temperature by immersion in a coolant circulating in a cartridge with a rectangular detection window (800 μm × 100 μm).

Electrophoretic separation was performed in normal polarity (input: anode, output: cathode), the capillary being thermostated at 25 °C. Samples were hydrodynamically injected for 1 or 2 s under a pressure of 20 psi. The CZE electrolyte solution consisted of a 25 mM sodium borate solution (pH 9.1) filtered through a 0.45 μm membrane (Polylabo, Strasbourg, France). A constant voltage of 15 kV was applied and direct UV detection was obtained at 280 nm. Data were collected and analyzed by the Gold® System (Beckman).

The capillary was conditioned before each series of analysis with 1N sodium hydroxide (10 min), followed by distilled water (10 min) and a final 5 min equilibrium with CZE electrolyte solution. Between each sample, the capillary was washed with 1N sodium hydroxide (2 min), followed by distilled water (2 min) and a final 2 min equilibrium with CZE electrolyte solution. These washings were intended to improve the reproducibility of the electroosmotic flow and, thus, that of migration time.

2.3. Preparation of calibration standards and controls

Pralidoxime methylsulfate stock solution (4 mg mL^{-1} as pralidoxime base) was prepared in distilled water and stored at −80 °C for up to 6 months. Pralidoxime calibration standards (0.125–2 mg mL^{-1}) were obtained by spiking 200 μL of normal free urine (Bio-Rad) by increasing volumes of pralidoxime stock solution (31.5–500 μL); all standards were completed to 1 mL with distilled water. Point 0 consisted of distilled water (800 μL) and free urine (200 μL). Hypoxanthine stock solution (10 mg mL^{-1}) was prepared in distilled water with 10 μL of sodium hydroxide 1 M and stored at −80 °C. In these conditions, the solution is stable for 6 months.

Three urine samples from patients treated by Contrathion® infusion were used as controls for the precision study: low (C1 = 0.15 mg mL^{-1}), medium (C2 = 0.45 mg mL^{-1}) and high levels (C3 = 1.15 mg mL^{-1}); controls were stored at −80 °C.

2.4. Samples preparations

No extraction procedure was necessary to perform the assay. Urinary controls and samples (200 μL) were first diluted in 700 μL of distilled water. A volume of 100 μL of hypoxanthine stock solution was added to urine standard, controls and samples, as internal standard (IS). Calibration standards and controls were hydrodynamically injected in the capillary for 1 or 2 s (depending on their pralidoxime concentration); patient urine samples were injected for 1 s.

2.5. Correlation to reference technique

Results were compared with a reference HPLC method first developed to measure obidoxime in urine [13] and slightly modified for pralidoxime analysis [11]. Pralidoxime concentration was measured by the developed CZE assay and HPLC in 30 human urine samples.

2.6. Clinical application

Pralidoxime methylsulfate concentration was measured in 30 urine samples from 10 patients treated in the ICU of Tunis Hospital for a severe organophosphate poisoning. After a loading dose of 5 mg kg^{-1}, Contrathion® was infused at the dose of 50 mg kg^{-1} using a perfusion (n = 5) or an electric syringe (n = 5). Urine was collected before therapy, then on a daily basis and for 3 consecutive days following the beginning of Contrathion® therapy. Urine samples were stored at −20 °C until assayed. Pralidoxime urinary excretion was expressed as mean area under curve (AUC) ± standard error of the mean (S.E.M.).

The Mann–Whitney test was used to test correlation with reference technique and compare AUC values. A p value <0.05 was chosen as a statistically significant value.

3. Results and discussion

3.1. Choice of CZE method to measure urinary pralidoxime

CZE is an attractive method for separation and quantification of drugs in biological fluids because of a small sample/reagent volume, short analysis time and high resolution [17,18].

Using a normal polarity, cationic species are eluted first, thus reducing their analysis time. Pralidoxime, a *N*-methylpryridium derivative, was eluted in 6.60 min (apparent electrophoretic mobility, 0.003069 $m^2\ V^{-1}\ s^{-1}$) before neutral compounds such as urea and creatinine, or dimethylsulfoxide, a marker of electroosmotic flow (apparent electrophoretic mobility: 0.002960 $m^2\ V^{-1}\ s^{-1}$, elution time: 6.90 min). Using an HPLC method [13], obidoxime was eluted in 9 min using a 1.2 mL min^{-1} flow rate and pralidoxime was eluted in 13 min using a 1.0 mL min^{-1} flow rate [11]. The major drawback of CZE with direct UV detection is low sensibility due to limited optical length (in our conditions, capillary I.D.: 75 μm) [17]. Molecular absorption coefficient of pralidoxime in 1N NaOH is 4130 $M^{-1}\ cm^{-1}$ at 280 nm (personal data) is low. However, more than 90% of the injected dose of pralidoxime is found in urine, with resulting high concentrations (0.5–2 mg mL^{-1}) [11]. Overall, pralidoxime cationic structure and high urinary concentrations are two major arguments to select a CZE method for a urinary assay. The absence of an preliminary extraction procedure is also attractive. HPLC assays require a deproteinization of urinary samples [11,13]. By CZE, anionic proteins are eluted after neutral compounds, thus not interfering with pralidoxime.

Using 25 mM borate buffer and a voltage 15 kV (the maximum allowed by Ohm's law), pralidoxime was eluted with an excellent repeatability (CV: 0.3%, $n = 10$) and efficiency (290,000 plates/m, following a 1 s injection). Increasing the injection time from 1 to 3 s did not substantially reduce the method efficiency (2 s: 275,000 plates/m, 3 s: 250,000 plates/m).

3.2. Choice of internal standard

The ideal internal standard must absorb at 280 nm, be separated from pralidoxime and not physiologically found in human urine. Obidoxime (Fig. 1b) was first tested, but it displayed a similar elution time (6.62 min). CZE separation is based on the ratio z/M [19], which is 0.00729 (1/137) for pralidoxime and 0.00696 (2/287) for obidoxime. These z/M ratios are too close to allow an optimum separation (selectivity coefficient $r = \mu$ pralidoxime/μ obidoxime = 1).

Some natural (hypoxanthine, xanthine, cytosine, guanosine, adenosine, 7-methylguanine) or synthetic (5-fluorouracile, cytarabine, 6-*O*-methylguanine) nucleotides, which all absorb at 280 nm, were tested as potential internal standards. Their selectivity coefficient was determined with the pralidoxime peak as a reference. Some of them showed a too low selectivity coefficient: cytarabine ($r = 2$), cytosine ($r = 2$), and 6-*O*-methylguanine ($r = 3$). Guanosine, 5-fluorouracil, and adenosine displayed excellent selectivity coefficients ($r > 30$), but retention times were over 15 min. Selectivity coefficients were 12, 18, and 22, respectively, for 7-methylguanine (retention time: 9.51 min), hypoxanthine (10.83 min), and xanthine (13.44 min). The former was discarded as an interfering peak was often found in urine samples. Hypoxanthine was selected IS, as it possessed the shortest elution time and an excellent separation from pralidoxime ($r = 18$). In physiologic state [20,21] and malignancies, hypoxanthine is always excreted in small amounts (<0.1 mg mL^{-1}) by kidneys. Concentration of hypoxanthine used for spiking (10 mg mL^{-1}) is also much higher than that found in urine. Overall, physiologic concentration of hypoxanthine cannot interfere with our pralidoxime assay as illustrated by an electrophoregram from a blank sample urine (Fig. 2a) [21,22].

3.3. Validation of the method

The CZE method was validated for routine use with criteria generally employed for HPLC [23]: specificity, repeatability of migration time and of pralidoxime concentration, linearity, sensitivity, percentage of recovery, as well as intra- and inter-day accuracy and precision.

3.4. Specificity

Fig. 2 shows electrophoretic profiles of a blank urine sample without IS (a), a blank urine sample (b), the 0.50 mg mL^{-1} calibration standard (c), and a urine sample from a patient treated with Contrathion® infusion (d). After preparation of blank urine (Fig. 2a and b), no additional peaks interfered with the measured pralidoxime methylsulfate and hypoxanthine. Compounds were well separated with a migration time of 6.60 min for pralidoxime methylsulfate and 10.83 min for hypoxanthine. For pralidoxime, the peak shape showed no evidence of tailing (symmetry factor: 1.01).

3.5. Linearity

The linearity of the technique (corrected peak area ratio of the drug/IS versus drug concentration) was evaluated over a concentration range of 0.125–2 mg mL^{-1}. Regression analysis, performed by the least-squares method, gave the following results: $y = 1.715x - 0.009$, $r^2 > 0.998$ (equation determined by five calibrations obtained on different days). The determination coefficient ($r^2 > 0.998$) confirms the excellent linearity of this method.

3.6. Precision

The three controls (C1–C3) were injected 15 times; results are indicated in Table 1. For C1 and C2, within-run CV was below 10% and for C3 below 4%. For between-run precision, CV was 10% for C1 and below 10% for C2 and C3.

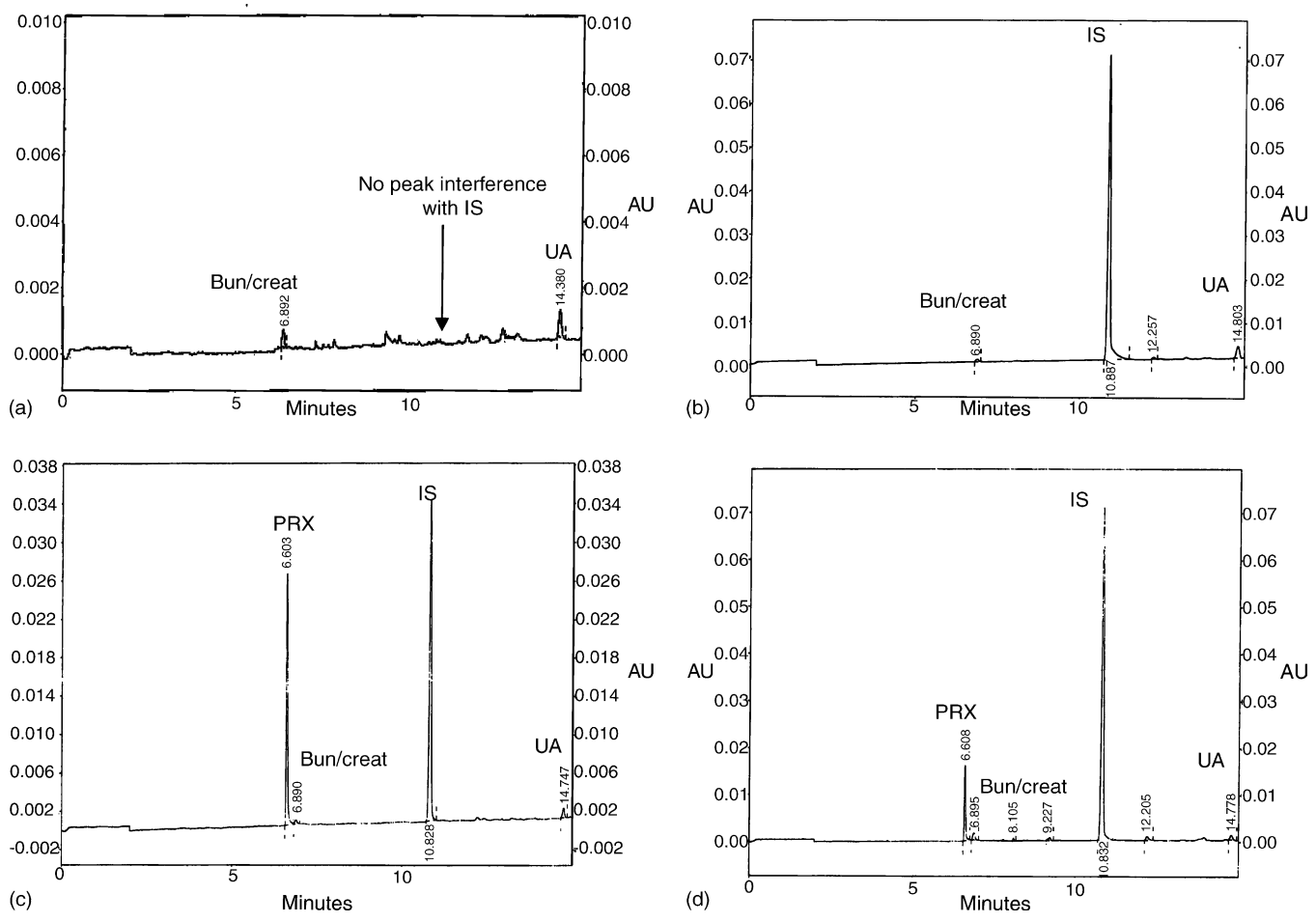

Fig. 2. Selected CZE profiles. (a) Sample blank urine without IS, (b) sample blank urine, (c) urine containing 0.50 mg mL^{-1} pralidoxime, (d) human urine (concentration = 0.14 mg mL^{-1}) spiked with IS (1 mg). Elution times were 6.60 and 10.83 min for pralidoxime (PRX) and IS, respectively. Bun/creat, bun/creatinine; UA, uric acid. Electrophoretic conditions are as described in Table 1 with an injection time of 2 s for (a and b), and 1 s for (c and d).

3.7. Detection and quantification limits

Detection limit, defined as the smallest quantity of pralidoxime distinguishable from baseline, was calculated as three times the signal-to-noise ratio (S/N = 3) estimated by the baseline thickness. Solutions with decreasing concentrations of pralidoxime were analyzed (injection time: 2 s). In our conditions, the pralidoxime detection limit was 0.033 mg mL^{-1} corresponding to 0.10 mg mL^{-1} as quantification limit.

Table 1
Precision of the CZE pralidoxime method

Pralidoxime concentration		Within-run precision (n = 15)	Between-run precision (n = 15)
C1 (0.15 mg mL^{-1})	Mean concentration (mg mL^{-1})	0.16	0.14
	R.S.D. (mg mL^{-1})	0.008	0.015
	CV (%)	5.5	10.2
C2 (0.45 mg mL^{-1})	Mean concentration (mg mL^{-1})	0.46	0.48
	R.S.D. (mg mL^{-1})	0.036	0.039
	CV (%)	7.9	8.2
C3 (1.15 mg mL^{-1})	Mean concentration (mg mL^{-1})	1.2	1.12
	R.S.D. (mg mL^{-1})	0.044	0.077
	CV (%)	3.7	6.9

Three control levels of pralidoxime (C1 = 0.15 mg mL^{-1}, C2 = 0.46 mg mL^{-1} and C3 = mg mL^{-1}) were used. CZE conditions: fused-silica capillary (L = 47 cm, l = 40 cm, I.D. = 75 μm); electrolyte: 25 mM solution of sodium borate, pH 9.1; voltage applied: 15 kV; temperature: 25 °C; injection time: 1 or 2 s; detection: UV direct (280 nm). R.S.D., relative standard deviation; CV, coefficient of variation.

Table 2
Pralidoxime recovery study

Overloaded 4 mg mL^{-1} pralidoxime solution (μL)	Expected values (mg mL^{-1})	Measured values (mg mL^{-1})	Recovery (%)
C1 (0.15 mg mL^{-1})			
	0.15	–	
25	0.22	0.21	95
50	0.28	0.28	100
100	0.37	0.37	101
125	0.40	0.38	95
150	0.43	0.42	98
C1 (0.45 mg mL^{-1})			
	0.46	–	
25	0.51	0.48	95
50	0.54	0.57	106
100	0.58	0.60	103
125	0.60	0.62	104
150	0.61	0.63	103

Electrophoretic conditions are the same as described in Table 1.

3.8. Recovery tests

Two human urine controls (C1 and C2) were overloaded with increasing volumes (25, 50, 100, 125 and 150 μL) of a 4 mg mL^{-1} pralidoxime stock solution. The percentages of recovery were within the limits of recovery tests (generally 80–120%) (Table 2).

3.9. Correlation with a reference technique

Our modification of the Grasshoff et al. [13] urinary obidoxime HPLC assay for pralidoxime [11] was selected as the reference technique. In 30 human urine samples, the correlation between the two methods was excellent (slope: 0.972, intercept: −0.02, correlation coefficient: 0.99). Using a Bland–Altmann analysis [24], the mean difference was −0.051 mg mL^{-1}; the 95% confidence interval for the difference of means was −0.24 to 0.025 mg mL^{-1}, without significant difference between two techniques ($p = 0.215$, Mann–Whitney test).

3.10. Interfering substances

No endogenous substances were found to interfere with pralidoxime by CZE (Fig. 3). Two peaks were observed, one at 6.89 min corresponding to urea/creatinine and one at 14.80 min for uric acid. We tested obidoxime, an antidote approved in several European countries [5,13]. In our conditions, it was eluted in 6.65 min and could not be separated from pralidoxime. To our knowledge, these oximes are never co-infused and our assay could be used to measure urinary pralidoxime or obidoxime according the therapy advised in each country. Atropine is the main antidote in organophosphate poisoning and is usually administered at high dose [25]; it is eliminated partially unchanged in urine. In our conditions, atropine was eluted at

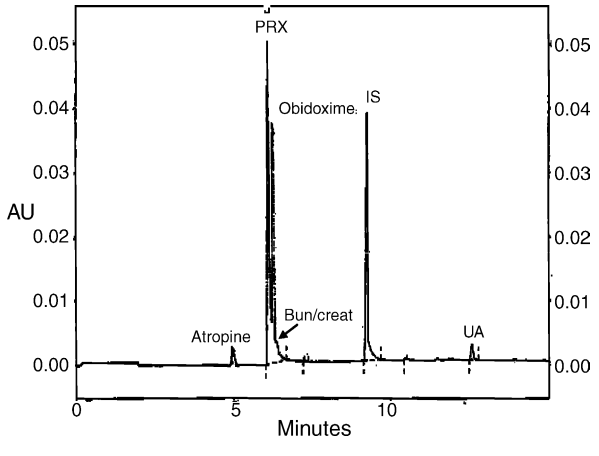

Fig. 3. CZE profile of potential interfering substances (endogenous and chemical) Urine spiked with atropine (0.5 mg mL^{-1}), pralidoxime (PRX) (0.75 mg mL^{-1}), obidoxime (0.75 mg mL^{-1}), IS (1 mg). Bun/creat, bun/creatinine; UA, uric acid. Electrophoretic conditions are as described in Table 1 with an injection time of 1 s.

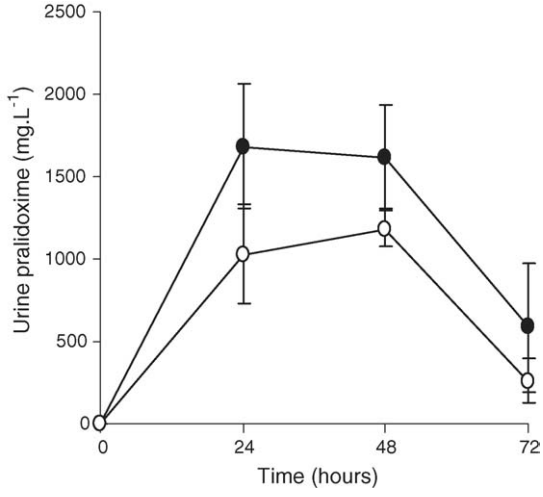

Fig. 4. Urinary pralidoxime excretion profile during Contrathion® therapy. Pralidoxime was administered by an electric syringe (dark circle, $n = 5$) or by a perfusion (open circle, $n = 5$) in organophosphate poisoned patients. Results are expressed as mean ± S.E.M. (standard error of the mean).

6.20 min, but could not be detected at 280 nm (optimum detection: 214 nm), even at high concentration, such as 0.5 mg mL^{-1} (Fig. 3).

3.11. Clinical application

Daily urinary pralidoxime concentrations were determined in 10 patients admitted to the ICU of the Tunis hospital for an acute organophosphate poisoning. In samples obtained before the beginning of Contrathion® therapy, no pralidoxime peak was found. Fig. 4 shows the mean profile of urinary pralidoxime excretion during treatment. Pralidoxime concentrations ranged from 0.2 to 1.8 mg mL^{-1}, as previously reported [11]. The two infusion modes showed a similar excretion profile (Fig. 4) and AUC: syringe (73.271 ± 44.409 mg mL^{-1} h^{-1}), and perfusion (44.653 ± 21.882 mg mL^{-1} h^{-1}) ($p = 0.0616$, NS).

4. Conclusion

In conclusion, the proposed CZE assay allows the determination of urinary pralidoxime in 15 min, with excellent precision, selectivity, and sensitivity. This procedure has the advantage of being simple (no pretreatment) and convenient, thus suitable for the therapeutic monitoring of Contrathion® therapy during organophosphate poisoning.

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