Research article

Drug Testing and Analysis

Received: 31 May 2011 Revised: 1 July 2011 Accepted: 3 July 2011 Published online in Wiley Online Library: 18 November 2011

(wileyonlinelibrary.com) DOI 10.1002/dta.340

Quantification of pralidoxime (2-PAM) in urine by ion pair chromatography-diode array detection: application to *in vivo* samples from minipig

Harald John,[a]* Michael Eddleston,[b] R. Eddie Clutton,[c] Franz Worek[a] and Horst Thiermann[a]

Pralidoxime (2-PAM) is a monopyridinium oxime used as an antidote for the treatment of poisoning with organophosphorus (OP) compounds, for example, pesticides and nerve agents, reactivating OP-inhibited acetylcholinesterase. However, appropriate dosing and efficacy remains a matter of discussion requiring experimental data.

Therefore, we developed and validated an ion pair chromatography-diode array detection (IPC-DAD) method suitable for quantitative analysis of 2-PAM in human and porcine urine. Before injection of 20 μl, urine was acidified with trichloroacetic acid, mixed with internal standard (pyridine-4-aldoxime, 4-PAO), and diluted with IPC solvent yielding a total dilution of 1:49.5 and a 100% recovery. Isocratic separation was carried out at 25 °C on a LiChrospher 60 RP-select B column (125 x 4.0 mm I.D.) using phosphate buffer (7.5 mM Na_2HPO_4, 7.5 mM KH_2PO_4, pH 2.6) mixed with octanesulfonate (2.5 mM) as ion pair reagent and acetonitrile (6% *v/v*) as organic modifier (1 ml/min). 2-PAM was detected at 293 nm and 4-PAO at 275 nm. The method is rugged, selective, and characterized by good intra-day and inter-day precision (RSD, 1.3–6.0%) and accuracy (88–100%) with a limit of detection at 4.9 μg/ml, a limit of quantification at 9.8 μg/ml, and a broad calibration range from 4.9–2500 μg/ml.

The procedure was applied to urine samples obtained from dimethoate poisoned minipigs receiving 2-PAM therapy (intravenous bolus injection and infusion). Results indicate that 60–80% of infused 2-PAM is rapidly (within 1–2 h) excreted in the urine.

Keywords: chromatography; nerve agents; oxime; pesticides; pharmacokinetics

Introduction

Pralidoxime (2-PAM, N-methyl-2-aldoximinopyridinium, Figure 1A) is a monopyridinium oxime administered for the causal therapy of poisoning with organophosphorus (OP) compounds; for example, pesticides and nerve agents. The abbreviation 2-PAM is used to address the pure oxime structure, not specifying any of the counterions typically present in pharmaceutical formulations; for example, chloride, iodide, methanesulfonate.

OP poisons react (phosphylation) with the serine residue of the active site of acetylcholinesterase (AChE) thus inhibiting the enzyme, sometimes irreversibly, and preventing regulatory ester-cleavage of the neurotransmitter acetylcholine (ACh). Consequently, ACh accumulates in the synaptic cleft and overstimulates effector cells, leading initially to miosis, fasciculation, and increased secretion of body fluids (saliva, tears, sweat) and ultimately to death by respiratory paralysis.[1]

To reactivate the enzyme and thus restore its physiological function, 2-PAM is administered intravenously (i.v.) or intramuscularly (i.m.) typically as solutions of its completely dissociated chloride, iodide or methanesulfonate salts. [2] *In vivo* 2-PAM is partly deprotonated and forms the active zwitterionic oximate that is capable of cleaving the OP-moiety from the enzyme by nucleophilic substitution. [3] Free active AChE and the unstable intermediate phosphylated oxime (POX) are produced.[4–6]

Even though 2-PAM was introduced as the first oxime antidote against OP poisoning in 1955,[3] there remains ongoing debate over its optimum use and poison-specific efficacy. [2,7–13] Although human *in vivo* data have been obtained from OP pesticide poisoned patients [8–10,12,13] controlled animal studies are required to elaborate indications and limitations of therapy. Present *in vitro* approaches are helpful and promising but cannot replace *in vivo* studies entirely. [14,15]

Therefore, studies were performed using the Göttingen minipig, which is a good model for biomedical research having many physiological, anatomical, nutritional, and metabolic similarities to humans. [16–18]

Therapy optimization necessitates analytical approaches for quantification of 2-PAM in body fluids and distinct compartments.

* *Correspondence to: Harald John, Bundeswehr Institute of Pharmacology and Toxicology; Neuherbergstr. 11, 80937 Munich, Germany. E-mail: HaraldJohn@bundeswehr.org*

a *Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany*

b *Clinical Pharmacology Unit, Centre for Cardiovascular Science, University of Edinburgh, and National Poisons Information Service - Edinburgh, Royal Infirmary of Edinburgh, UK*

c *Royal (Dick) School of Veterinary Sciences, University of Edinburgh, UK*

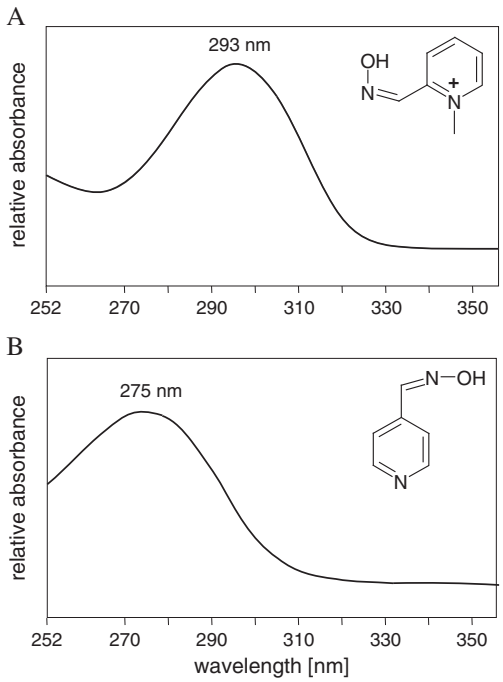

Figure 1. UV-spectra extracted from IPC-DAD runs (A) 2-PAM (pralidoxime) (B) 4-PAO (pyridine-4-aldoxime, internal standard).

Several methods have been introduced including paper chromatography, [6] ion metal affinity chromatography (IMAC), [19] capillary zone electrophoresis (CZE), [20,21] cation exchange chromatography,[13,22] and reversed-phase chromatography (RPC). [23,24] But most frequently ion pair chromatography (IPC) coupled to electrochemical detection (ECD), [25,26] radio detection (RD), [27] and UV detection [14,28,29] has been applied. For a detailed overview, see the review on 2-PAM quantification in this issue. [30] Although more modern RPC-tandem mass spectrometric (RPC-MS/MS) methods provide better sensitivity and selectivity, [23,24] UV-detection appears to be sufficient for the quantification of high micromolar 2-PAM concentrations present in plasma and urine samples. [12–14,20,23–27] In general, reports of quantitative urine analysis are quite rare, covering only 2-PAM concentrations in rats by IMAC-UV [19] and IPC-RD [27] and in humans by CZE-UV. [20] However, no procedure applied to porcine urine has been published so far. Therefore, we developed and validated a quantitative IPC-diode array detection (DAD) procedure that allows for selective, precise and accurate analysis of 2-PAM in human and porcine urine. This procedure was applied to samples from minipigs undergoing 2-PAM therapy after poisoning with the OP pesticide dimethoate, which is commonly used in suicide attempts.

Experimental

Chemicals

Acetonitrile (ACN, gradient grade), water (LiChrosolv), KH_2PO_4, Na_2HPO_4*$2H_2O$, trichloroacetic acid (TCA) and phosphoric acid (all guaranteed reagent) were purchased from Merck (Darmstadt, Germany). Pralidoxime (2-PAM) chloride (CAS No. 51-15-0), pyridine-4-aldoxime (4-PAO used as internal standard, CAS No. 696-54-8), N-methyl-2-pyridone (CAS No. 694-85-9) and sodium 1-octanesulfonate were delivered by Sigma-Aldrich (St Louis, MO, USA) in purities of 98–99.6%, respectively. Human urine was randomly collected from male and female volunteers not controlling diet or medications. Porcine urine used for blank samples and standards was taken from study pigs before poisoning and therapy. Dimethoate (DIM, CAS-No. 60-51-5) and omethoate (OM, CAS-No. 1113-02-6) were provided by Dr Ehrenstorfer (Augsburg, Germany) in a purity of 99% and 97%, respectively.

HPLC equipment

The HPLC system consisted of two pumps (P580), an autosampler (Gina 50), column oven (STH 585), and degasser (DG 2410) from Dionex, Germering, Germany that was coupled to a diode array detector (DAD) (UVD 320S, Gynkotek, Germering, Germany). The HPLC system was controlled by the Chromeleon software (vers 6.01 Build 447, Dionex) also allowing DAD data acquisition and processing.

IPC-DAD analysis

Chromatography was performed at 25 °C with a flow of 1 ml/min on a LiChrocart 125–4 LiChrospher 60 RP-select B column, 5 μm, 125 x 4.0 mm I.D. (Merck, Darmstadt, Germany) protected by a replacement filter disc (0.5 μm, Rheodyne, Chromatographie-Handel Müller, Fridolfing, Germany). Solvent A (7.5 mM Na_2HPO_4, 7.5 mM KH_2PO_4, 2.5 mM sodium octanesulfonate in water, adjusted to pH 2.6 with H_3PO_4) and solvent B (7.5 mM Na_2HPO_4, 7.5 mM KH_2PO_4, 2.5 mM sodium octanesulfonate in ACN/water 50:50 *v/v*, adjusted to pH 2.6 with H_3PO_4) were used as mobile phase. The prepared sample solution (20 μl) was injected and separated isocratically at 25 °C (solvent A/solvent B 88:12 *v/v*) monitoring UV-absorbance at 293 nm (2-PAM, $\varepsilon = 12.40\ mM^{-1}\ cm^{-1}$, Figure 1A) and 275 nm (4-PAO as internal standard, IS, $\varepsilon = 15.77\ mM^{-1}\ cm^{-1}$, Figure 1B). In addition the DAD detected absorptions at wavelengths between 253–353 nm thus allowing us to analyze the corresponding UV-spectra of analyte and IS, and any interference. When analyzing pesticide-containing samples from the minipig study, a subsequent washing step was performed to elute DIM prior to injection of the next sample. This washing step was carried out in the gradient mode starting with 60% (*v/v*) solvent B held for 7 min and proceeding with 12% solvent B for 13 min to re-equilibrate the column for oxime analysis. All samples were measured in duplicate.

Preparation of samples and standards

If not otherwise stated, 2-PAM concentrations given in this report refer to the pure oxime; the the counter ion (chloride) being ignored.

Pralidoxime chloride stock solutions

2-PAM chloride stock solution was prepared by dissolving weighed material (about 568 mg) in water resulting in 365 mM, corresponding to 50 mg pure 2-PAM/ml. Concentration was controlled by determination of UV-absorption at 293 nm in a cuvette photometer (UV/VIS photometer Cary 3/300, Varian, Darmstadt, Germany) after dilution to 0.1 mM in 0.01 M HCl. Stock solution was stored at −80 °C prior to use.

Pyridine-4-aldoxime (IS) stock and working solution

4-PAO (Figure 1B) was used as internal standard. The stock solution was prepared by dissolving weighed material (about 75 mg) in ethanol resulting in a concentration of 41 mM (5 mg/ml). The working solution was produced by diluting the stock solution 1:5

with solvent A (1 mg/ml). Stock and working solution were stored at −80 °C before use.

Urine samples and blank

Urine (200 µl) was acidified with 60 µl 1 M TCA and mixed either with 30 µl solvent A (samples and blank without IS: $sample_{noIS}$ and BL_{noIS}) or with 30 µl IS working solution (samples and blank with IS: sample and BL) followed by vortex mixing and centrifugation (16 000 g for 4 min). Subsequently, 25 µl of supernatant was added to 825 µl solvent A thus causing a total urine dilution of 1:49.5. These solutions were analyzed by IPC-DAD following the standard protocol.

Urine standards and quality control samples

Ten urine standards covering a concentration range from 5000 µg/ml (Std A) to 9.76 µg/ml (Std J) were generated by serial dilution (1:2; 250 µl + 250 µl) of urine spiked with 2-PAM chloride. Std A was prepared by adding 100 µl of the 2-PAM stock solution to 900 µl urine. Preparation was carried out by the standard procedure described above (total dilution 1:49.5).

Quality control samples (QCs) containing 2-PAM were produced from the stock solution diluted in urine to three concentration levels (QC_{low}: 40 µg/ml, QC_{med}: 200 µg/ml and QC_{high}: 1000 µg/ml), prepared with IS as described above and stored as aliquots of 100 µl at −20 °C prior to use.

Characteristics of IPC-DAD performance

Performance characteristics of the IPC-DAD procedure were elaborated for validation. Below we describe the principle procedures to determine the quality criteria.

Effect of column temperature on chromatographic retention

Urine was spiked with 2-PAM (100 µg/ml) and prepared as described above. HPLC analysis was performed at different column temperatures controlled by the column oven (10, 15, 20, 25, 30, 35, and 40 °C). Resulting retention times (t_R) of 2-PAM and 4-PAO were plotted against the corresponding temperature. Capacity factors k′ of 2-PAM and IS were calculated by $(t_R\text{-}t_0)/t_0$, where t_0 is the dead time. Temperature dependent chromatographic resolution (α) was calculated as the ratio of k′(4-PAO)/k′(2-PAM).

Linear range, lower limit of quantification and detection, precision, and accuracy

Urine standards additionally including lower concentrated standards (down to 1.22 µg/ml, Std M) were prepared (n = 4) and analyzed as described above to determine the linear range, lower limit of quantification (LLOQ) and lower limit of detection (LLOD).

LLOQ was defined as the lowest concentration not exceeding either 20% relative standard deviation (RSD) or 80–120% accuracy. LLOD was set to be the lowest concentrated standard allowing unambiguous qualitative analyte detection in all replicates.

Precision and accuracy of the method were assessed by intra- and inter-day validation using QC_{low}, QC_{med} and QC_{high}. Intra-day accuracy and precision were evaluated by processing QCs in six replicates (n = 6) at one day. Inter-day accuracy and precision were determined by analyzing QCs in duplicate at six non-consecutive days. Corresponding concentrations were calculated from daily calibrations curves. Accuracy was calculated as the relative ratio between the determined concentration and the nominal value. RSD (SD/mean * 100) was used as a measure of precision.

Selectivity

Urine blank samples from 13 human volunteer donors and 6 minipig individuals were analyzed in the presence (BL) and absence of IS (BL_{noIS}) as well as after spiking with 2-PAM to the LLOQ concentration (9.76 µg/ml). BL, BL_{noIS} and spiked samples were measured in duplicate by IPC-DAD following the standard procedure allowing elaboration of selectivity.

Furthermore, N-methyl-2-pyridone, the potential biotransformation product of 2-PAM, was chromatographed by the standard protocol to identify potential interferences with the analyte or IS.

Effects in the presence of pesticides

DIM and OM were dissolved separately in solvent A resulting in concentrations of 150 µg/ml and 50 µg/ml, respectively, corresponding to values previously determined in urine of the same animal study.[31] Pesticides were chromatographed under standard conditions except the run time was prolonged to 40 min and the DAD was set to monitor absorbance between 200 and 356 nm. Chromatograms obtained at 205 nm were extracted from DAD data to detect pesticides.

Recovery

Recovery was elaborated by comparing the slope of a calibration curve obtained from urine following the standard protocol to the slope of a calibration curve that was simply prepared in water. Standards of the latter curve contained 2-PAM in similar concentrations to those theoretically present in prepared urine samples.

Stability of 2-PAM: thaw-and-freeze cycles

Blank urine was spiked with 2-PAM to 40 and 200 µg/ml and aliquots of 200 µl were generated. Immediately after spiking each concentration was prepared and analyzed in triplicate following the standard protocol. Additional samples were stored for 24 h at −20 °C prior to complete thawing and storage at room temperature for 1 h. Subsequently, samples were re-frozen for 24 h and completely thawed again. This procedure was carried out four times in total. After each thawing step, samples of both concentrations were analyzed in triplicate by duplicate IPC-DAD measurement. Resulting concentrations were compared to those obtained without thawing.

Stability of 2-PAM in TCA acidified urine

Urine was spiked with 2-PAM yielding concentrations of 50 and 500 µg/ml. Two aliquots each (3 ml) were mixed with 900 µl 1 M TCA and 450 µl solvent A and stored at room temperature as typical for sample preparation. Samples (290 µl) were taken immediately after mixing (0) and after 3, 7, 11, 17, and 35 min followed by a 4 min centrifugation step (16 000 g for 4 min) except for sample 0. The supernatant of each sample (25 µl) was mixed with 825 µL solvent A and analyzed by IPC-DAD in duplicate. Mean of measured 2-PAM peak areas were plotted versus total storage time in TCA acidified urine (1, 8, 12, 16, 22, 40 min).

Stability of 4-PAO in TCA acidified urine

Urine was spiked with 4-PAO (130 µg/ml) and two aliquots (3500 µl) were acidified with 913 µl 1 M TCA each yielding a 4-PAO concentration as present under standard protocol conditions. Samples (290 µl) were taken immediately after mixing (0) and after 1, 5, 15, and 35 min followed by a 4 min centrifugation

step (16 000 g for 4 min) except for sample 0. The supernatant of each sample (25 µl) was mixed with 825 µl solvent A and analyzed by IPC-DAD in duplicate. Mean of measured 4-PAO peak areas were plotted versus total storage time in TCA acidified urine (1, 5, 10, 20, 40 min).

Stability of 2-PAM and 4-PAO in the autosampler

Stability of prepared urine samples stored in the autosampler for 24 h at room temperature was investigated using QC_{med} (200 µg/ml) and QC_{high} (1000 µg/ml), that were chromatographed hourly in duplicate. Resulting concentrations were plotted against the storage time.

Ruggedness

Ruggedness of the IPC-DAD method describes how analytical results were influenced by slightly varying parameters during analysis. Selected parameters of the standard protocol (variant A) were changed to slightly lower (variant B) and slightly higher settings (variant C). Detection wavelength for 2-PAM was changed from 293 nm to 291 nm and 295 nm, wavelength for 4-PAO from 275 nm to 273 nm and 277 nm, LC flow from 1.0 ml/min to 0.95 ml/min and 1.05 ml/min, isocratic solvent composition (solvent A/solvent B, *v/v*) from 88:12 to 86:14 and 90:10, and solvent pH from 2.6 to 2.4 and 2.8.

Urine standards (Std H, 39.1 µg/ml and Std D, 625 µg/ml) were analyzed using the IPC-DAD parameter conditions of variants A, B, and C in triplicate each and were quantified by a calibration curve measured under standard conditions (variant A). Resulting precision and accuracy were evaluated.

Animal study

The study was performed under UK Home Office Licence in male Göttingen minipigs (Ellegaard Göttingen Minipigs ApS, Dalmose,

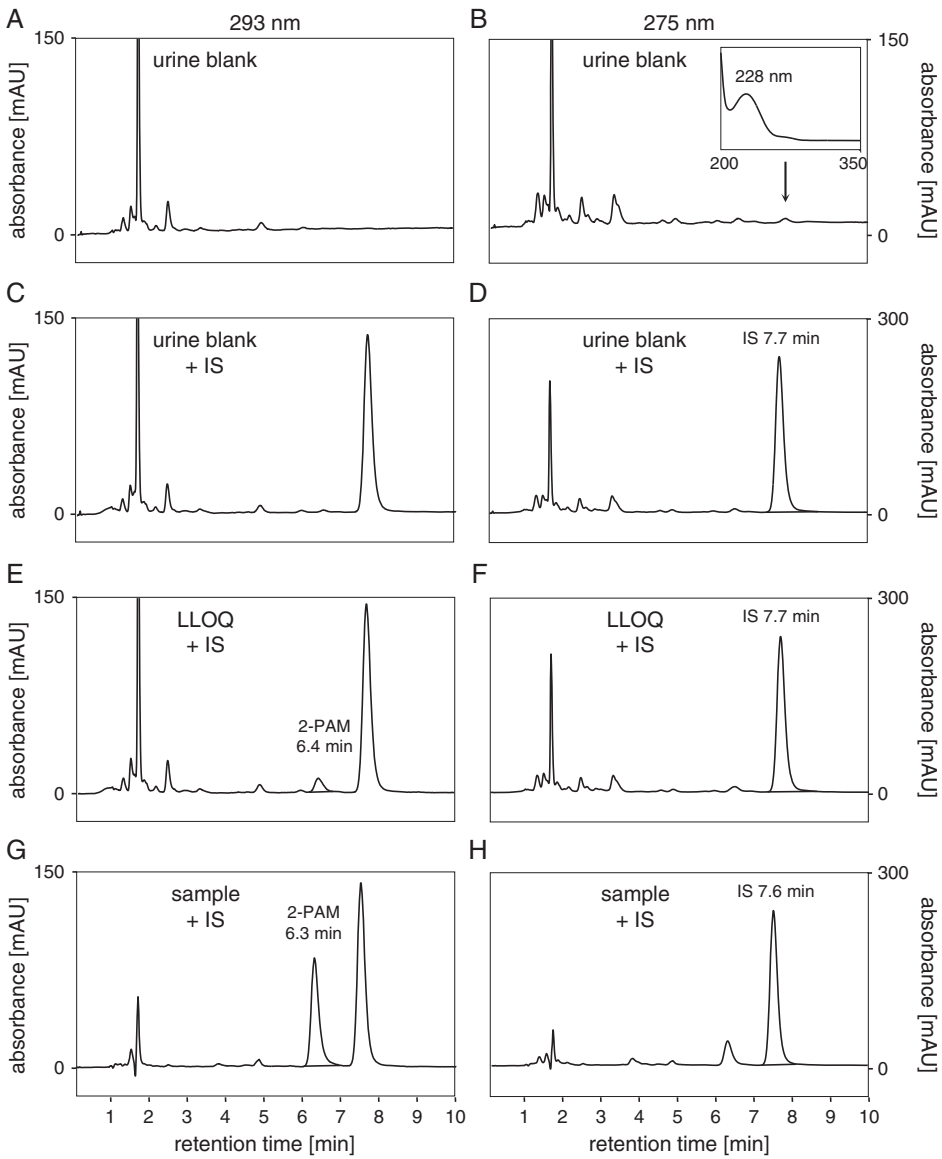

Figure 2. Representative chromatograms of urine analysis by IPC-DAD. (A,B) urine blank; insert: UV-spectrum of an unknown peak interfering with internal standard (C,D) urine blank with internal standard (E,F) urine standard spiked with 2-PAM at LLOQ level (9.8 µg/ml) (G,H) sample from minipig study taken 52 min after initial intravenous bolus injection of 2-PAM chloride corresponding to 22 min after start of 2-PAM infusion. Isocratic ion pair chromatography-diode array detection (IPC-DAD) was carried out at 25 °C on a LiChrospher 60 RP-select B column (125 x 4.0 mm I.D.) using phosphate buffer (7.5 mM Na_2HPO_4, 7.5 mM KH_2PO_4, pH 2.6) mixed with octanesulfonate (2.5 mM) as ion pair reagent and acetonitrile (6% *v/v*) as organic modifier (1 ml/min). Optimum detection was performed at 293 nm (2-PAM) and 275 nm (4-PAO) as indicated. IS, internal standard; LLOQ, lower limit of quantification.

Denmark) after institutional ethics review. The animals were treated in accordance with the Animals (Scientific Procedures) Act 1986. The study was funded by the Wellcome Trust (grants 063560 and 085979)

Anaesthetized (isoflurane) and mechanically ventilated minipigs (approximately 20 kg body weight) were poisoned by gavage with a commercial emulsifiable concentrate EC40 (BASF plc, Cheadle Hume, UK) of the organophosphorus pesticide DIM (400 g/l) in a dose of 2.5 ml/kg body weight. Two hours later, animals were treated intravenously (i.v.) with 2-PAM chloride given by venous cannula as an initial bolus (8 mg/kg body weight) and subsequent infusion (3.5 mg/kg/h). When calculating the corresponding doses for pure 2-PAM (only the oxime, not considering the chloride ion) the bolus and infusion delivered 6.4 mg/kg and 2.8 mg/kg/h, respectively. Therapy was based on standard human treatment protocols. [32] During a 12 h-period, blood samples (taken from a central arterial cannula) and urine samples (drawn by an indwelling bladder catheter) were collected every hour. Urine and EDTA plasma was stored at −22 °C before shipment on dry ice to Munich, Germany, for analysis.

Results and discussion

It has been shown that therapeutic concentrations of 2-PAM are quite high in rat plasma and serum (1.4–5.5 μg/ml; [27] up to 100 μg/ml; [26] 0.7–3.3 μg/ml [28]), in sheep plasma (up to 7 μg/ml, [33] and in human plasma and serum (0.5–3 μg/ml; [23,24] 3–6 μg/ml; [25] 3–15 μg/ml [12,13]). Concentrations in human urine were more than 100-times higher (250–2000 μg/ml [20]). Due to these extraordinarily high values, we developed a more conservative IPC method coupled to UV detection. Whilst promising less sensitivity than more modern hyphenated mass spectrometric procedures [23,24] simple sample preparation by acidification and dilution followed by conventional IPC separation and diode array detection proved adequate for the quantitative analysis of clinical urine samples.

Photometric characterization of 2-PAM and 4-PAO

To assure that both 2-PAM and 4-PAO (IS) were detected at maximum absorptivity under chromatographic conditions, their UV-spectra were extracted from DAD data (Figure 1). Both spectra revealed one maximum each at wavelengths (λ_{max}) of 293 nm for 2-PAM ($\varepsilon = 12.40\ mM^{-1}\ cm^{-1}$) and 275 nm for 4-PAO ($\varepsilon = 15.77\ mM^{-1}\ cm^{-1}$). Therefore, the DAD was set to these values for quantitative measurement under standard conditions. However, earlier reports applied a broad variety of different wavelengths, for example, 262, 270, 280, 290, 294, and 300 nm [13,28,29,34–36] to detect 2-PAM quantitatively. These values may be due to different HPLC solvent components, the pH used, or simply to non-optimum settings.

Characteristics of IPC-DAD analysis

The presented procedure was validated for the analysis of human urine in a total dilution of 1:49.5. Representative chromatograms of blank urine without IS (Figures 2A and 2B), blank urine with IS (Figures 2C and 2D), standard at the LLOQ level (Figures 2E and 2F), and a sample from the animal study (Figures 2G and 2H) are illustrated including both wavelength used for optimum detection of 2-PAM (293 nm, t_R 6.4 ± 0.1 min) and 4-PAO (275 nm, t_R 7.7 ± 0.1 min).

Effect of column temperature on chromatographic retention

Retention times of 2-PAM and IS were markedly influenced by the column temperature. Longest retention times (7.9 min for 2-PAM and 10.2 min for IS) resulted at 10 °C and were permanently reduced with elevated temperature. At 40 °C shortest t_R were detected (5.1 min for 2-PAM and 5.7 min for IS) not providing sufficient chromatographic resolution anymore ($\alpha = 1.17$). Figure 3 depicts the course of retention times depending on the column temperature. We decided to set 25 °C as standard protocol condition allowing the best compromise of short run times and satisfying chromatographic resolution ($\alpha = 1.25$).

Sample preparation and recovery

Due to the high 2-PAM concentrations that can be expected in clinical samples (up to several hundred μg/ml), [20] a simple dilution of urine (1:49.5) in combination with acidification (1 M TCA) was sufficient to prepare samples. Subsequent centrifugation was performed to remove any potential particulate matter that could deteriorate robust analysis.

2-PAM calibration curves (2-PAM concentrations plotted versus area ratio of 2-PAM/IS) obtained in pure aqueous solution and in urine after preparation showed identical regression data for slope ($m = 0.00448 \pm 0.00002$ ml/μg) and y-intercept (0.0190 ± 0.0006) with excellent coefficients of variation ($r^2 = 0.99997$ for urine and 0.9996 for water) indicating both optimum linearity in the calibration range (4.9-2500 μg/ml) and excellent recovery (100.7%, calculated by the ratio of the slopes: 100 x m_{urine}/m_{water}).

Linear range, LLOQ and LLOD

The linear range was found to be excellent from 4.9–2500 μg/ml ($r^2 \geq 0.9990$) corresponding to absolute on-column-amounts of 2.0–1010 ng. Following Food and Drug Administration (FDA) guidelines for bioanalytical method validation [37] the calibration range was fixed considering that precision should not exceed ± 20% and accuracy should be between 80–120%. Table 1 summarizes relevant data for the lowest concentration standards tested in quadruplicate (Std I-Std L). Std L and K did not meet the quality criteria mentioned above and therefore, 9.8 μg/ml (Std J) was defined as LLOQ and 4.9 μg/ml (Std K) as LLOD.

Intra- and inter-day precision and accuracy

Intra-day and inter-day precision (RSD, 1.3–5.5%) and accuracy (88–100%) were determined from replicate measurements of

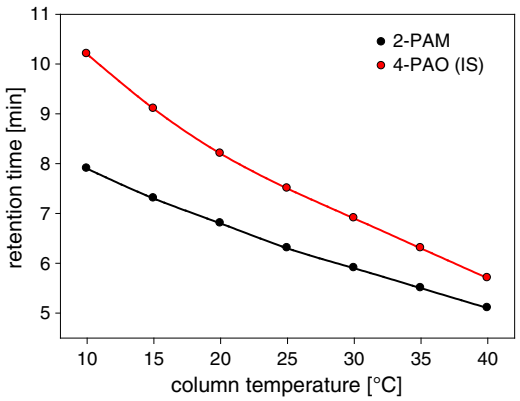

Figure 3. Retention times in dependence on column temperature. Data points (red: 4-PAO; black: 2-PAM) represent mean of retention times determined in duplicate (SD ≤ 0.05 min).

Table 1. Intra- and inter-day precision (RSD) and accuracy of 2-PAM quantification

Standard code	Theoretical concentration [μg/ml]	Mean of measured concentration [μg/ml]	Precision RSD [%]	Accuracy [%]
		Intraday		
L	2.44	4.3 [a]	97.5	175.4
K	4.88	6.2 [a]	17.0	127.6
J	9.76	10.8 [a]	14.4	110.5
I	19.5	18.4 [a]	12.9	94.3
QC_{low}	40.0	35.3 [b]	2.5	88.3
QC_{med}	200	185.2 [b]	1.3	92.6
QC_{high}	1000	971.7 [b]	2.8	97.2
		Inter-day		
QC_{low}	40.0	38.1 [b]	5.5	95.3
QC_{med}	200	196.0 [b]	3.2	98.0
QC_{high}	1000	999.8 [b]	2.2	100.0

Lower limit of quantification (LLOQ) was determined at 9.8 μg/ml and lower limit of detection (LLOD) at 4.9 μg/ml after 1:49.5 dilution of urine.
[a], measured with n = 4; [b], measured with n = 6
QC, quality control sample; inter-day data were obtained on six non-consecutive days

QCs covering the lower, middle and higher concentration range and indicated good and appropriate quality for *in vivo* sample analysis (Table 1).

Selectivity

Within the first 4 min the peak profiles of the 13 individual urine blanks were quite variable for both wavelength (293 and 275 nm) with respect to peak number and height (Figures 2A and 2B). This might be due to various polar urine matrix ingredients depending on, for example, individual diet, hormonal status or potential medication. In contrast, few peaks were found between 4 and 10 min representing the time frame for analyte and IS elution (Figures 2A and 2B). Example chromatograms of human urine blank samples with and without IS are shown in Figures 2A–D indicating the absence of any interfering compound at the retention time of 2-PAM (t_R 6.4 ± 0.1 min) but showing a small co-eluting interference for the IS (t_R 7.7 ± 0.1 min) marked by an arrow (Figure 2B). The relevant compound was not identified, but its UV-spectrum showed an absorption maximum at λ = 228 nm which can be found for carbon double-bonds, for example, or sulfonate esters and amides (Figure 2B). However, reasonable changes of chromatographic conditions did not prevent from partial coelution.

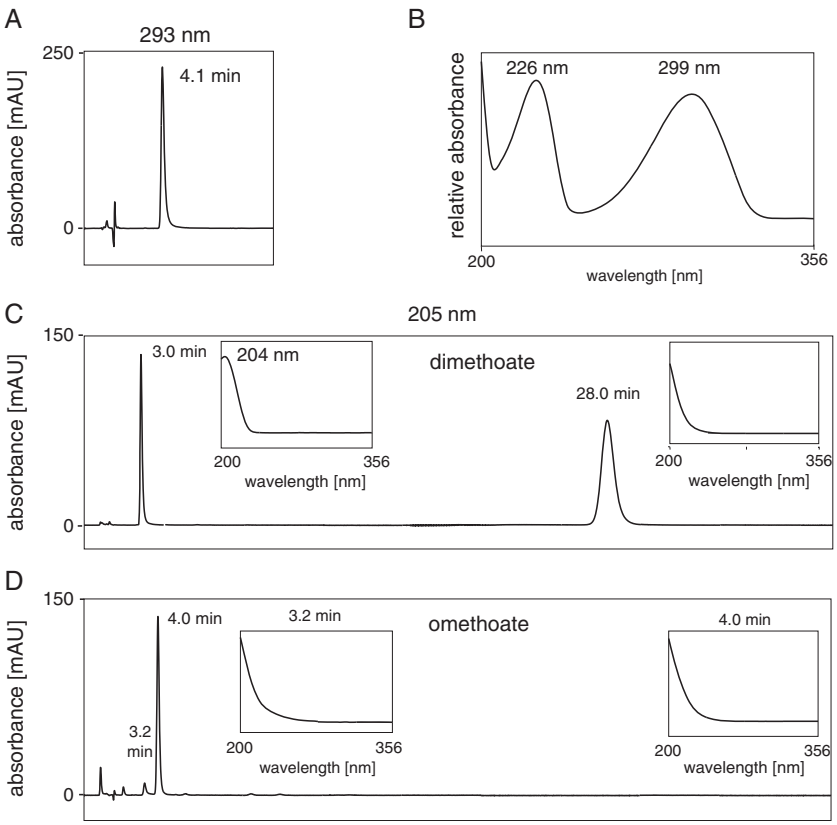

Figure 4. Chromatography of a potential biotransformation product and the organophosphorus pesticides dimethoate and omethoate. (A) N-methyl-2-pyridone (B) UV-spectra of N-methyl-2-pyridone obtained from the peak at 4.1 min (Figure 4A) (C) Dimethoate (DIM); inserts: UV-spectra of peaks occurring after DIM injection (D) Omethoate (OM); inserts: UV-spectra of peaks occurring after OM injection. Isocratic ion pair chromatography-diode array detection (IPC-DAD) was carried out at 25 °C on a LiChrospher 60 RP-select B column (125 x 4.0 mm I.D.) using phosphate buffer (7.5 mM Na_2HPO_4, 7.5 mM KH_2PO_4, pH 2.6) mixed with octanesulfonate (2.5 mM) as ion pair reagent and acetonitrile (6% *v/v*) as organic modifier (1 ml/min). Detection was performed at 293 nm for N-methyl-2-pyridone and at 205 nm for pesticides.

To elaborate whether this interference was of relevance for quantification, the 13 human urine blanks were chromatographed in the presence and absence of IS and after spiking with 2-PAM to the LLOQ level. Blank samples without IS allowed us to integrate the individual interfering peak exclusively. In contrast, urine blanks mixed with IS only allowed us to determine the total peak area of IS and coeluting interference. To obtain a corrected IS value, the peak area of the interference was subtracted from the total area (IS + interference). Peak area of the interference represented 2–16% (mean 9%, n = 13) of the mean pure IS area indicating only slight impact on quantitative analysis.

For comparison, 2-PAM concentrations at the LLOQ level of the 13 samples were quantified using both the corrected and the non-corrected IS area. Resulting concentrations revealed that precision ($RSD_{non\text{-}corrected}$ 7%, $RSD_{corrected}$ 9%) and accuracy (non-corrected: 101%, corrected 110%) were of similar quality as expected for the LLOQ level. Therefore, the more complex procedure of IS correction did not provide more reliable results and was thus neither necessary nor favourable for 2-PAM quantification. However, IS correction may be useful for untypical urine samples presenting interferences of exceedingly high peak areas (≥ 20% of IS area).

To elaborate such phenomena for minipig urine, six individual urine blanks were analyzed accordingly but did not demonstrate any interference for analyte and IS.

In addition, the potential biotransformation product N-methyl-2-pyridone eluted at 4.1 min did not interfere with either 2-PAM or the IS (Figure 4A). Therefore, the IPC-DAD method is of sufficient selectivity to allow 2-PAM quantification in urine for clinical purposes in humans and minipigs.

Stability of 2-PAM: thaw-and-freeze cycles

2-PAM in urine was found to be stable after four thaw-and-freeze cycles. All concentrations measured were identical within the range of precision (RSD ± 6%) and did not show any trend. Urine samples were stable at −20 °C for at least nine months.

Stability of 2-PAM and 4-PAO in TCA acidified urine

Neither 2-PAM nor 4-PAO showed any degradation within 40 min storage in TCA acidified urine (pH 1) at room temperature as depicted in Figure 5. Therefore, duration of typical sample preparation including TCA addition, vortex mixing, centrifugation and subsequent dilution in solvent A will not deteriorate analyte and IS stability and thus analytical quality.

Stability of 2-PAM and 4-PAO in the autosampler

2-PAM and 4-PAO remained stable in both concentrations during storage at room temperature in the autosampler. Concentrations showed a RSD of ± 4% without any trend. Therefore, 2-PAM concentrations in prepared urine samples will not change during the time needed for the analysis of a set of samples. These findings are in accordance with Utley's demonstration of maximum stability of 2-PAM in acidic solutions (pH 3.2).[34]

Ruggedness

The IPC-DAD procedure was demonstrated to be rugged since slightly altered parameter settings (flow rate, wavelengths, solvent composition and solvent pH) impaired neither precision (0.1–2.4%) nor accuracy (95-105%) for 2-PAM (Table 2). Only retention times were markedly influenced.

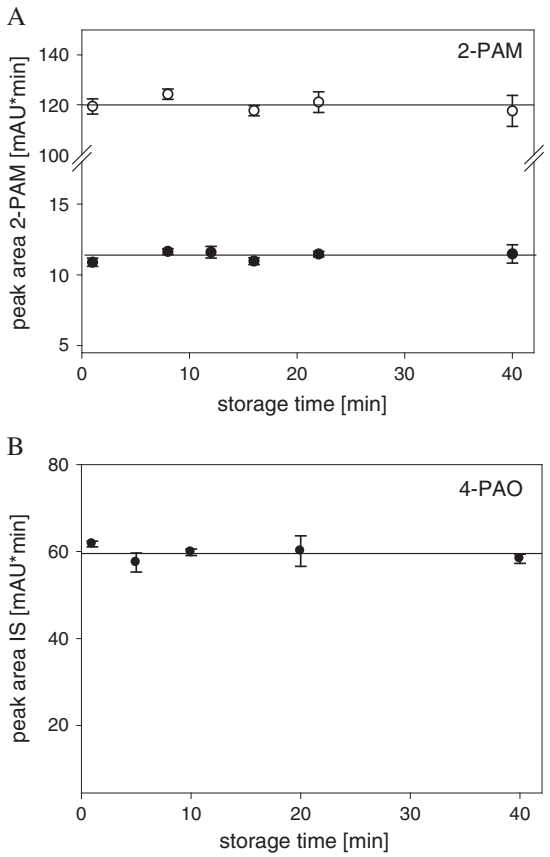

Figure 5. Stability of analyte and IS in urine acidified with TCA. (A) Stability of 2-PAM. Analyte was stored in urine acidified with TCA at 50 (open circles) and 500 µg/ml (black circles) under standard protocol conditions with respect to acid and temperature. 2-PAM was analyzed by IPC-DAD and peak areas are given as mean and SD (n = 2, measured in duplicate). (B) Stability of 4-PAO. IS was stored in urine acidified with TCA under standard protocol conditions with respect to concentration and temperature. 4-PAO was analyzed by IPC-DAD and peak areas are given as mean and SD (n = 2, measured in duplicate).

Under standard conditions 2-PAM and 4-PAO were detected at t_R 6.4 ± 0.1 min and 7.7 ± 0.1 min, respectively (Figure 2). As expected, retention times of both compounds were prolonged by decreased flow (6.7 min and 8.1 min) and decreased ratio of organic modifier (solvent B) (7.5 min and 9.4 min). Accordingly, shorter retention times were observed with increased flow rate (6.0 min and 7.4 min), elevated ratio of solvent B (5.5 min and 6.5 min), and to a lesser extent by lower pH (6.2 min and 7.5 min). Nevertheless, QC samples and standards that are analyzed just before or immediately after unknown samples allow unambiguous peak identification by comparing retention times, thus allowing unambiguous peak assignment and reliable analysis.

Application to animal study

2-PAM was administered intravenously to minipigs for causal therapy of oral poisoning with DIM. The validated method for 2-PAM quantification presented herein was applied to analyze urine samples collected in this study.

We had tested linear range, precision and accuracy and found no differences between human and porcine urine matrix. In addition selectivity had been investigated by analyzing six blank urine samples from individual minipigs not showing any interference with 2-PAM or IS as described above. The

Table 2. Ruggedness of 2-PAM quantification in urine by IPC-DAD

	Std H 39.1 μg/ml		Std D 625 μg/ml	
	RSD [%]	Accuracy [%]	RSD [%]	Accuracy [%]
Standard conditions[a]	2.4	95.4	0.2	101.9
Flow rate [ml/min]				
0.95	1.1	94.5	0.1	100.8
1.05	0.1	94.5	0.6	102.5
Wavelength 2-PAM [nm]				
291	2.3	90.8	0.6	97.1
295	1.5	95.5	0.2	103.7
Wavelength 4-PAO [nm]				
273	1.4	93.5	0.5	101.3
277	0.5	96.3	0.3	102.7
Solvent composition [A:B v/v]				
86:14	1.8	104.9	0.3	101.8
90:10	3.2	95.1	0.2	102.5
Solvent pH				
2.4	0.3	97.5	0.0	101.6
2.8	0.8	92.3	0.1	99.9

[a], standard conditions: flow rate: 1.0 ml/min; wavelength 2-PAM: 293 nm, wavelength 4-PAO: 275 nm, solvent composition: 88:12 *v/v*; solvent pH: 2.6.
RSD, relative standard deviation (precision); Std, standard
Analyses were performed in triplicate for each variation.

main difference in matrix composition between standards and samples arose from the presence of the highly concentrated organophosphorus pesticides DIM and OM. Therefore, we paid special attention to this fact.

Matrix effects in the presence of pesticides

After incorporation of DIM and *in vivo* biotransformation into OM both pesticides are excreted via the kidney resulting in high micromolar concentrations in urine as demonstrated earlier for the presented animal study (up to 150 μg/ml for DIM and up to 50 μg/ml for OM).[31] Therefore, the influence of pesticides on retention behaviour or detection of 2-PAM and its IS had to be evaluated.

For determination of retention times, DIM and OM were dissolved in solvent A (150 and 50 μg/ml, respectively) followed by acidification and further dilution according to the standard protocol. Isocratic chromatography was continued for 40 min instead of 10 min used for 2-PAM analysis allowing detection of the more lipophilic DIM. As depicted in Figure 4C, two peaks were found after DIM injection at t_R 3.0 min and the major component at 28.0 min, the latter one most reasonably representing original DIM. Following injection of the more polar OM one major peak was detected at 4.0 min accompanied by a much smaller one at 3.2 min presumably representing a form of impurity or hydrolysis product (Figure 4D). Corresponding UV-spectra of DIM and OM showed quite non-specific absorption profiles as expected for both pesticides not containing chromophoric groups (Figures 4C and 4D). Nevertheless, when monitoring at 275 nm DIM was also detectable as a broad peak of weak intensity (data not shown). This phenomenon caused obvious interferences with the IS after injection of DIM-containing minipig samples when analyses were performed simply by consecutive 10 min-standard runs: DIM, that had been injected with the first sample, eluted not earlier than during the third run, where it appeared with a retention time of about 7–8 min (2 x 10 min separation time + 2 x 0.3 min for sample aspiration and injection + 7–8 min additional separation time ≈ 28 min). Therefore, partial coelution with the IS of the third injection and deterioration of quantitative analysis were found. Consequently, we took account of this fact by inserting a washing step with a higher ratio of organic modifier (60% B *v/v*) subsequent to each standard analytical IPC run. DIM was rapidly eluted and the column re-equilibrated before next sample injection. This procedure prevented pesticide-derived interferences and enabled reliable and accurate analysis thus underlining the method's suitability for 2-PAM analysis of samples from DIM poisoned animals.

Urinary excretion of 2-PAM in minipig

DIM solution (EC40) was given to minipigs by gavage allowing poison uptake from the gastrointestinal tract and mimicking intentional or accidental pesticide poisoning in humans. [8–10] The primary toxic biotransformation product of DIM is OM. It is produced in the liver by desulfuration, inhibits acetylcholinesterase (AChE) by phosphylation, and induces an ultimately fatal cholinergic crisis.

2-PAM chloride was administered as antidote to reactivate phosphylated AChE by an initial i.v. bolus injection (8 mg/kg) followed by i.v. infusion (3.5 mg/kg/h). Resulting oxime concentrations in plasma were quantified by an in-house IPC-DAD procedure similar to the one presented herein [14] proving rapid establishment of 2-PAM concentrations in the expected range of 4–6 μg/ml. [14,32]

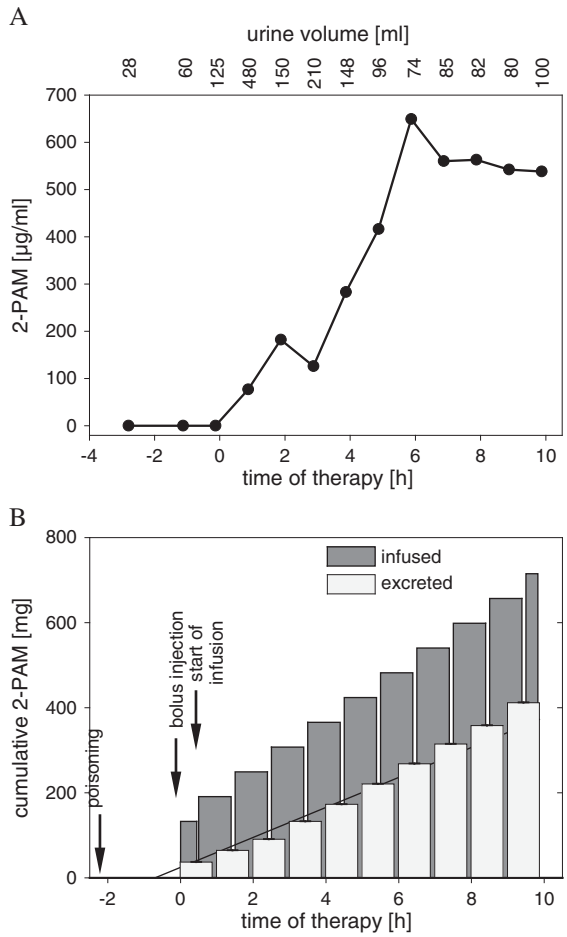

Figure 6. Urinary excretion of 2-PAM in minipig after intravenous administration. (A) Concentration-time profile of 2-PAM also indicating urine volumes obtained during collection periods. 2-PAM chloride therapy was initiated at t = 0 h (2.13 h after oral poisoning with dimethoate) by bolus injection (8 mg/kg) followed by infusion (3.5 mg/kg/h) starting at t = 0.5 h. (B) Cumulative amounts of 2-PAM infused (dark grey bars) and excreted (light grey bars). Mean infusion rate was 58.2 mg/h and mean excretion rate over the entire experimental phase was 35 mg/h (first order regression line) increasing to 46 ± 5 mg/h during the last 5 h. Data indicate that 60-80% of infused 2-PAM was excreted immediately within 1–2 h.

To monitor renal excretion of 2-PAM, urine was sampled from minipigs by an indwelling catheter allowing the determination of both the volume excreted within the sampling period (1 h) and the corresponding 2-PAM concentration. Figures 2G and 2H show the chromatographic analysis of a urine sample taken 52 min after initial 2-PAM chloride bolus injection (and 22 min after start of infusion) monitored at 293 nm and 275 nm. Significant 2-PAM concentrations of about 77 µg/ml were detected.

An example (from 1 minipig) of the time course of 2-PAM concentration in urine is illustrated in Figure 6A. This shows a rising concentration during the experimental phase with a maximum at 650 µg/ml. Nevertheless, when considering the different volumes of urine collected between two time points (Figure 6A, labelling at the top) a quite constant rate of 2-PAM excretion of 35 mg/h was found for the entire experimental phase as illustrated by the constantly rising cumulative amount of excreted pralidoxime over the time (Figure 6B, light-grey bars and covering first order regression line). The mean value for excretion during the last 5 h exclusively was 46 ± 5 mg/h. Because of constant infusion during therapy, 2-PAM uptake also increased constantly with a slope of 58.2 mg/h as illustrated by the dark-grey bars in Figure 6B.

When comparing infusion and excretion rate, it was found that 60% of administered 2-PAM were excreted almost immediately (within 1–2 h) rising up to 80% during the last 5 h of the experiment. Therefore, it appears that large amounts of this reactivating oxime are required to establish full therapeutic benefit.

These data are in accordance with a limited number of reports dealing with renal excretion of 2-PAM. Garrigue *et al*. and Enander *et al*. have shown that 2-PAM was excreted nearly quantitatively via urine as unchanged compound. [6,27] Six hours after intramuscular administration of 2-PAM iodide to healthy male Wistar rats already 73% of 2-PAM has been excreted by urine and 90% were recovered after 24 h. [27] Accordingly, plasma elimination half-lives ($t_{1/2\beta}$) of 2-PAM in rats after i.v. and i.m. administration were determined to be as short as 27–31 min and 38–62 min, respectively, indicating quite rapid elimination and clearance. [26] In healthy man $t_{1/2\beta}$ was found to be 70–80 min after i.v. application. Until now, data for minipig were unavailable.

Conclusions

We herein present the development and validation of an IPC-DAD procedure that allows for precise, accurate and reliable quantification of 2-PAM in urine. Due to high concentrations in clinical samples simple acidification and dilution (1:49.5) was sufficient to reduce the potential deteriorating impact of matrix ingredients and to adjust analyte concentrations within the broad linear range of the method. This procedure was validated for human urine but porcine urine did not show relevant differences thus enabling the quantification of samples from a minipig study. Therefore, our chromatographic procedure provides a useful tool for future pharmacokinetic studies, especially allowing elucidation of renal clearance and excretion. As the ideal dose of 2-PAM is still unknown, this method will provide valuable support to improve medical care of patients poisoned with OP compounds.

Acknowledgments

We thank Steffen Krüger and Johann Baur (both Bundeswehr Institute of Pharmacology andToxicology) and Jonathan Street for technical assistance, Dick Dyason and Keith Lambert (BASF) for the dimethoate EC40, and Professor P. Blain for the pralidoxime.

References

[1] H. John, F. Balszuweit, K. Kehe, F. Worek, H. Thiermann, in *Handbook of Toxicology of Chemical Warfare Agents*, (Ed: R. Gupta). Academic Press/Elsevier: Amsterdam, **2009**, pp. 755–790.
[2] P. Eyer, *Toxicol. Rev.* **2003**, *22*, 165.
[3] I. B. Wilson, S. Ginsburg, *Biochim. Biophys. Acta* **1955**, *18*, 168.
[4] C. Becker, H. John, F. Worek, *Drug Test. Analysis* **2010**, *2*, 460.
[5] I. Enander, A. Sundwall, B. Sörbo, *Biochem. Pharmacol.* **1961**, *7*, 232.
[6] I. Enander, A. Sundwall, B. Sörbo, *Biochem. Pharmacol.* **1962**, *11*, 377.
[7] F. Worek, M. Bäcker, H. Thiermann, L. Szinicz, U. Mast, R. Klimmek, P. Eyer, *Hum. Exp. Toxicol.* **1997**, *16*, 466.
[8] M. Eddleston, N. A. Buckley, P. Eyer, A. H. Dawson, *Lancet* **2008**, *371*, 597.
[9] M. Eddleston, L. Karalliedde, N. Buckley *et al. Lancet* **2002**, *360*, 1163.

[10] F. Konradsen, A. H. Dawson, M. Eddleston, D. Gunnell, *Lancet* **2007**, *369*, 169.
[11] M. Jokanovic, M. Maksimovic, *Arch. Toxicol.* **1995**, *70*, 119.
[12] J. L. Willems, H. C. De Bisschop, A. G. Verstraete, C. Declerck, Y. Christiaens, P. Vanscheeuwyck, W. A. Buylaert, D. Vogelaers, F. Colardyn, *Arch. Toxicol.* **1993**, *67*, 79.
[13] J. L. Willems, J. P. Langenberg, A. G. Verstraete, M. de Loose, B. Vanhaesebroeck, G. Goethals, F. M. Belpaire, W. A. Buylaert, D. Vogelaers, F. Colardyn, *Arch. Toxicol.* **1992**, *66*, 260.
[14] F. Worek, N. Aurbek, N. M. Herkert, H. John, M. Eddleston, P. Eyer, H. Thiermann, *Chem-Biol. Interact.* **2010**, *187*, 259.
[15] F. Worek, H. Thiermann, L. Szinicz, P. Eyer, *Biochem. Pharmacol.* **2004**, *68*, 2237.
[16] P. Vodicka, K. Smetana Jr, B. Dvorankova, T. Emerick, Y. Z. Xu, J. Ourednik, V. Ourednik, J. Motlik, *Ann. N. Y. Acad. Sci.* **2005**, *1049*, 161.
[17] W. J. Dodds, *Fed. Proc.* **1982**, *41*, 247.
[18] J. P. Hannon, C. A. Bossone, C. E. Wade, *Lab. Anim. Sci.* **1990**, *40*, 293.
[19] F. Guyon, A. Tambute, M. Caude, R. Rosset, *J. Chromatogr.* **1982**, *229*, 475.
[20] P. Houze, H. Thabet, A. Delfour, L. Larrouy, T. Le Bricon, F. J. Baud, *J. Chromatogr. B* **2005**, *826*, 63.
[21] H. Kalasz, E. Szökö, T. Tabi, G. A. Petroianu, D. E. Lorke, A. Omar, S. Alafifi, A. Jasem, K. Tekes, *Med. Chem.* **2009**, *5*, 237.
[22] H. Singh, D. Moorad-Doctor, R. H. Ratcliffe, K. Wachtel, A. Castillo, G. E. Garcia, *J. Anal. Toxicol.* **2007**, *31*, 69.
[23] C. Abbara, I. Bardot, A. Cailleux, G. Lallement, A. Le Bouil, A. Turcant, P. Clair, B. Diquet, *J. Chromatogr. B* **2008**, *874*, 42.
[24] C. Abbara, J. M. Rousseau, B. Lelievre, A. Turcant, G. Lallement, S. Ferec, I. Bardot, B. Diquet, *Brit. J. Pharmacol.* **2010**, *161*, 1857.
[25] P. Houze, S. W. Borron, F. Scherninski, B. Bousquet, B. Gourmel, F. Baud, *J. Chromatogr. B* **2005**, *814*, 149.
[26] P. Houze, D. E. Mager, P. Risede, F. J. Baud, *Toxicol. Sci.* **2010**, *116*, 660.
[27] H. Garrigue, J. C. Maurizis, J. C. Madelmont, C. Nicolas, J. M. Meyniel, A. Louvel, P. Demerseman, H. Sentenac-Roumanou, A. Veyre, *Xenobiotica* **1991**, *21*, 583.
[28] J. D. Shiloff, J. G. Clement, *Toxicol. Appl. Pharmacol.* **1987**, *89*, 278.
[29] K. Sakurada, K. Matsubara, K. Shimizu, H. Shiono, Y. Seto, K. Tsuge, M. Yoshino, I. Sakai, H. Mukoyama, T. Takatori, *Neurochem. Res.* **2003**, *28*, 1401.
[30] H. John, M. M. Blum *Drug Test. Analysis*. DOI: 10.1002/dta.327.
[31] H. John, M. Eddleston, R. E. Clutton, F. Worek, H. Thiermann, *J. Chromatogr. B* **2010**, *878*, 1234.
[32] M. Eddleston, A. Dawson, L. Karalliedde, W. Dissanayake, A. Hittarage, S. Azher, N.A. Buckley, *Crit. Care* **2004**, *8*, R391.
[33] D. H. Moore, I. J. Hayward. F. S. Tucker, B. Lukey, *Biopharm. Drug Dispos.* **1991**, *12*, 223.
[34] D. Utley, *J. Chromatogr.* **1987**, *396*, 237.
[35] D. G. Prue, R. N. Johnson, B. T. Kho, *J. Pharm. Sci.* **1983**, *72*, 751.
[36] N. D. Brown, M. P. Strickler, H. K. Sleeman, B. P. Doctor, *J. Chromatogr.* **1981**, *212*, 361.
[37] *Guidance for Industry: Bioanalytical Method Validation,* US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine, May **2001**.