Review

Drug Testing and Analysis

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Review of UV spectroscopic, chromatographic, and electrophoretic methods for the cholinesterase reactivating antidote pralidoxime (2-PAM)

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Pralidoxime (2-PAM) belongs to the class of monopyridinium oximes with reactivating potency on cholinesterases inhibited by phosphylating organophosphorus compounds (OPC), for example, pesticides and nerve agents. 2-PAM represents an established antidote for the therapy of anticholinesterase poisoning since the late 1950s. Quite high therapeutic concentrations in human plasma (about 13 μg/ml) lead to concentrations in urine being about 100 times higher allowing the use of less sensitive analytical techniques that were used especially in the early years after 2-PAM was introduced. In this time (mid-1950s until the end of the 1970s) 2-PAM was most often analyzed by either paper chromatography or simple UV spectroscopic techniques omitting any sample separation step. These methods were displaced completely after the establishment of column liquid chromatography in the early 1980s. Since then, diverse techniques including cation exchange, size-exclusion, reversed-phase, and ligand-exchange chromatography have been introduced. Today, the most popular method for 2-PAM quantification is ion pair chromatography often combined with UV detection representing more than 50% of all column chromatographic procedures published. Furthermore, electrophoretic approaches by paper and capillary zone electrophoresis have been successfully used but are seldom applied.

This review provides a commentary and exhaustive summary of analytical techniques applied to detect 2-PAM in pharmaceutical formulations and biological samples to characterize stability and pharmacokinetics as well as decomposition and biotransformation products. Separation techniques as well as diverse detectors are discussed in appropriate detail allowing comparison of individual preferences and limitations. In addition, novel data on mass spectrometric fragmentation of 2-PAM are provided.

Keywords: 2-PAM; antidote; column chromatography; mass spectrometry; paper chromatography; oxime

Introduction

Introduced in 1955,[1] pralidoxime (2-PAM, 2-hydroxyiminomethyl-1-methylpyridinium or 1-methyl-2-aldoximinopyridinium or N-methyl-2-aldoximinopyridinium, $C_7H_8N_2O^+$, CAS-No. 51-15-0 as chloride) (Figure 1, **I**) became a well established small molecule antidote used for therapy of poisoning with organophosphorus compounds (OPC), for example, pesticides and nerve agents. It is administered clinically especially in the USA, the UK, Canada, France, and Japan as well as in several developing countries.

Diverse analytical techniques have been developed to quantify 2-PAM in pharmaceutical formulations and body fluids to elaborate stability and pharmacokinetic parameters. This review focuses on those analytical approaches.

The first part of the review provides basic data on important pharmacological and toxicological characteristics to point out resulting analytical requirements. Subsequently, chemical properties of 2-PAM determining the adequate choice of separation and detection techniques are addressed.

The second part summarizes diverse methods applied for 2-PAM analysis. We cover both the phase of development and early clinical trials (mid-1950s until the end of the 1970s), that was distinguished by the primary use of spectroscopic methods and paper chromatography, as well as the subsequent column chromatographic era (early 1980s until today), which is presently headed by hyphenated highly selective mass spectrometric methods.

The third part addresses diverse detectors that were applied for 2-PAM analysis in chromatography and electrophoresis.

Pharmacological and toxicological aspects

The following sections give a brief summary on the pharmacological, toxicological and clinical background of 2-PAM as antidote.

Mode of action. 2-PAM is clinically used as an antidote for the therapy of poisoning with OPC that inhibit cholinesterases by covalent binding to the active site serine residue (phosphylation of enzymes). Phosphylation of serine denominates both phosphorylation (reaction with organophosphates) and phosphonylation (reaction with organophosphonates). 2-PAM-induced enzyme reactivation occurs as a two-step mechanism including the

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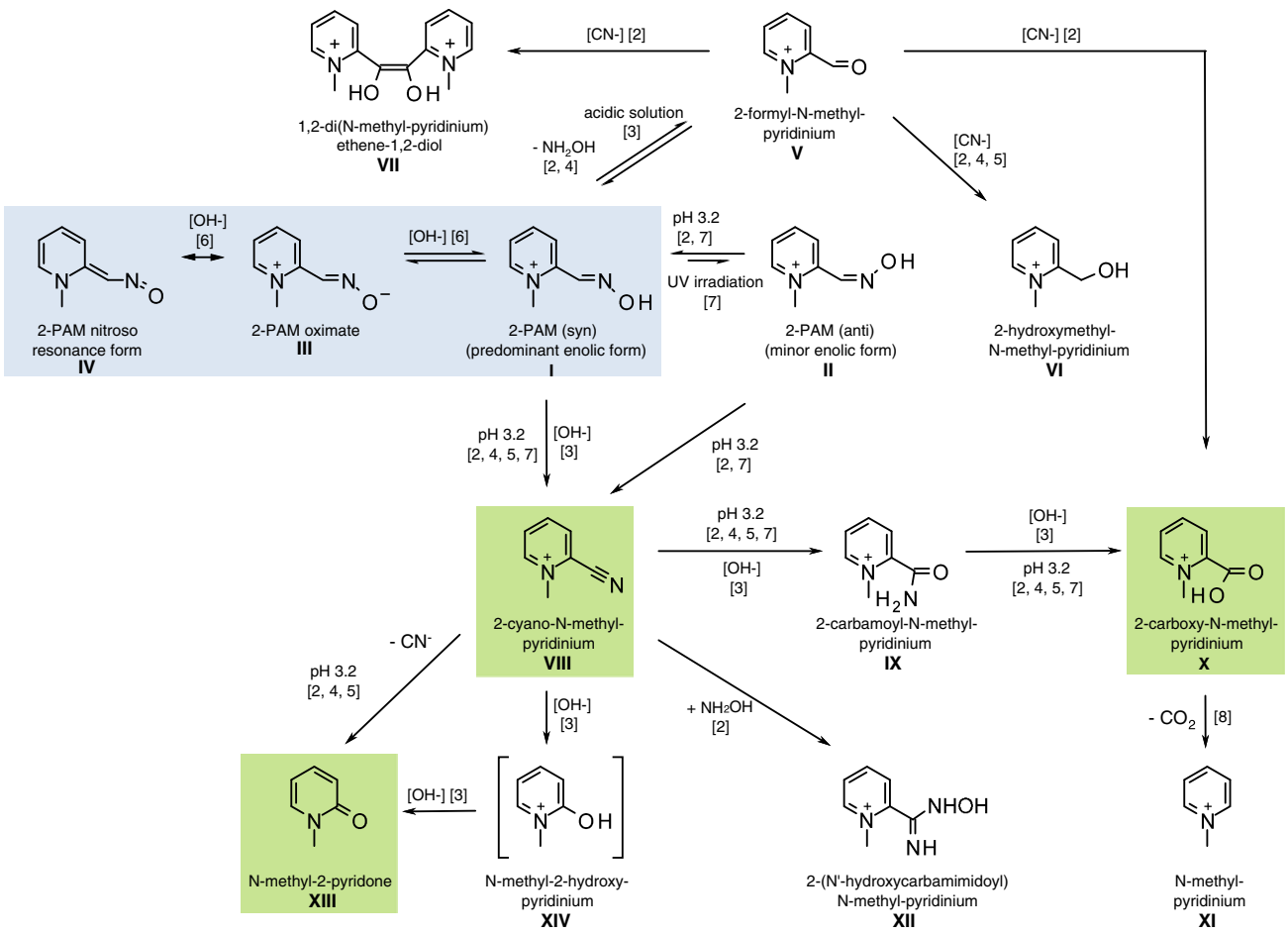

Figure 1. Decomposition and biotransformation products of 2-PAM. Structures highlighted in blue illustrate the dissociation equilibrium that determines the choice of analytical methods. Structures highlighted in green represent biotransformation products found to a less extent in plasma and urine.

formation of a Michaelis complex characterized by the association of the oxime and the phosphylated-enzyme followed by a nucleophilic attack of the oximate anion on the phosphorus atom. Subsequent diffusion of the phosphylated oxime (POX) liberates the reactivated enzyme thus supporting reconstitution of health[9–11] (Figure 2). Despite this common mechanism, 2-PAM cannot be regarded as a universally suitable enzyme reactivator since it lacks sufficient reactivating potency with different OPC.[9]

Clinical use. Typically 2-PAM is administered intravenously (i.v.) or intramuscularly (i.m.) as solutions of its iodide, chloride, methylsulfate, or methanesulfonate (mesylate) salts.[10] In addition, oral administration to man also caused concentrations in plasma representing 20–30% of the oral dose (2–3 h after intake).[12] However, this route of administration is inadequate for immediate response in acute intoxication scenarios.

In general, 2-PAM should be administered by intermittent doses (1 g every 6 h), bolus or short time infusion (1 g within 2 min) followed by long-term infusion (0.5 g/h).[9,10] Best results with respect to minimum adverse effects and maximum reactivating potency are expected for a short (30 min) infusion of 1 g 2-PAM chloride causing concentrations in plasma of 13 μg/ml at steady-state.[10] In contrast, Pawar *et al.* conclude that optimum benefit is achieved after a 2 g loading dose prior to a high-dose regimen of

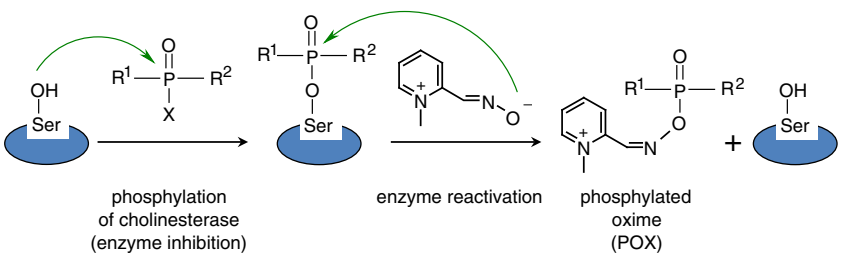

Figure 2. Inhibition of cholinesterases and reactivation by 2-PAM. The blue ellipse symbolizes a cholinesterase enzyme (e.g. acetylcholinesterase or butyrylcholinesterase) exhibiting an active site serine residue that is phosphylated by organophosphorus poisons (e.g. nerve agent or pesticide) thus causing inhibition. After nucleophilic attack of the oximate antidote 2-PAM a phosphylated oxime (POX) is generated and the enzyme is reactivated.

long term infusion (48 h) with 1 g/h.[13] However, the clinical benefit to the treatment of OPC poisoning with 2-PAM under intensive-care-unit conditions is sometimes challenged, for example by the need for artificial ventilation.[10]

In emergency scenarios, 2-PAM autoinjectors (500–600 mg in 2 ml) are used for immediate i.m. administration of the antidote. Therefore, autoinjectors belong to the personal medical countermeasure equipment of soldiers, and are also provided to civilian first responder, for example, firefighters and paramedics.

Adverse effects. Adverse effects in man include blurred vision, diplopia and dizziness occurring at 2-PAM concentrations in plasma of at least 80 μM (14 μg/ml).[14] After rapid i.v. injection causing high systemic concentrations vomiting, transient impairment of respiration, tachycardia, muscle rigidity and transient neuromuscular blockade were also observed.[10]

Pharmacokinetic aspects. 2-PAM is rapidly distributed and exhibits terminal elimination half-lives ($t_{1/2\beta}$) of 70–80 min in healthy man (i.v.[15,16]) and prolonged times in OPC-poisoned patients (i.m. and i.v. 170–206 min[17,18]). Parameters are species-dependent as shown for example, for man, rat, and sheep.[19–22] Elimination of 2-PAM is best described by a two-compartment open model not showing significant binding to plasma proteins.[10]

Therapeutic doses result in quite high levels of 2-PAM in body fluids (1–15 μg/ml plasma and 100–2000 μg/ml urine[17,19,23–30]).

These drug concentrations are quite comfortable to analyze as they do not require modern high-end detectors of extraordinary sensitivity as may be provided by mass spectrometry.

2-PAM is almost quantitatively excreted via urine as the unchanged compound (80–90%,[31] 84%[32]), thus biotransformation processes do only occur to a minor extent producing N-methyl-2-pyridone (Figure 1, **XIII**), 2-cyano-N-methyl-pyridinium (Figure 1, **VIII**),[33,34] 2-carboxy-N-methyl-pyridinium (Figure 1, **X**),[34] and SCN^-.[35]

Structural analogues of 2-PAM are currently tested to optimize pharmacokinetic (PK) behaviour (passage of blood-brain-barrier) and reactivating potency.[36,37]

Use of 2-PAM for catalytic decontamination. Even though the following aspect is not of relevance for therapeutic treatment *in vivo* it represents a highly interesting application of 2-PAM for the production of protective barriers and reactive coatings enabling direct decontamination while contact to OPC.[38] Bromberg *et al*. very recently introduced a clay forming phyllosilicate (Montmorillonite K-10, $(Si_{7.8}Al_{0.2})^{IV}(Al_{3.4}Mg_{0.6})^{VI}O_{20}(OH)_4$) that intercalates 2-PAM oximate by ionic interaction thus providing active centres for OPC hydrolysis (parathion, diisopropylfluorophosphate).[38] Initially OPC phosphylates the oximate to produce POX followed by aqueous hydrolysis liberating regenerated 2-PAM and the hydrolyzed non-toxic OPC. The clay component and 2-PAM act synergistically providing chemisorbtion and catalyzed degradation of OPC. Although general feasibility and practical benefit still have to be demonstrated, this concept represents a promising innovation in OPC countermeasures.

Chemical properties

2-PAM belongs to the class of monopyridinium oximes. The abbreviation 2-PAM used in this review refers to the pure pyridinium structure (MW 137.16 g/mol) thus not including the counterion that may be chloride, iodide or methanesulfonate in pharmaceutical formulations.[10] 2-PAM chloride is a white odourless, crystalline powder of good water solubility (650 g/L; 3.8 M) that dissociates as a weak acid.[39]

Structural characteristics and impact on analysis. With respect to the geometry of the N-substitution of the oxime-group, 2-PAM is found exclusively as its syn-isomer in fresh preparations[40] (Figure 1, **I**) whereas its anti-form (Figure 1, **II**) is merely produced under UV irradiation and thermal stress.[7] Therefore, we refer to the syn-isomer when using the abbreviation 2-PAM if not stated otherwise.

2-PAM possesses a quaternary methylated nitrogen atom that provides a permanent positive charge of the molecule (Figure 1, **I**). Its aromatic pyridinium structure causes a UV absorption maximum at λ_{max} 293 nm under acidic conditions (molar absorptivity in 0.1 M HCl, ε_{293} $1.25*10^4 M^{-1}cm^{-1}$).[39] This maximum is shifted to 336 nm (bathochromic shift) after dissociation of the oximino group (deprotonation) thus generating a zwitterionic betaine structure with a modified Π-electron system resulting in yellow coloured solutions (ε_{336} in 0.1 M NaOH $1.84*10^4 M^{-1}cm^{-1}$)[39,41] (Figure 1, structures highlighted in blue). The pK_a of this group was found to be 7.94 for the syn-isomer[39] and 8.7 for the anti-isomer.[7] Therefore, at pH > 8.0 2-PAM is predominantly present as a zwitterion (Figure 1, **III**) and the chargeless nitroso resonance form (Figure 1, **IV**).

The pH-dependent shift of λ_{max} was used to quantify 2-PAM in early UV spectroscopic approaches without any chromatographic separation step (see section *UV spectroscopic procedures*). Furthermore, strong light absorption is a valuable property for online UV detection in column chromatography and electrophoresis.

In addition, net charge is a critical issue for chromatographic separation affecting retention properties and elution behaviour, especially for ion exchange and ion pair chromatography (IPC). Accordingly, the pH of solvents used as a mobile phase in chromatography exerts an essential influence on charge and UV absorption relevant for separation and detection. Therefore, strict control of this parameter is required for reproducible and reliable analysis.

Stability in aqueous buffers. Characterization of 2-PAM stability in pharmaceutical formulations and buffers is relevant for long-time storage especially in auto-injectors stockpiled for emergency scenarios of acute threat or attack by OPC poisons.

The decomposition rate of 2-PAM increases with higher pH and elevated oxime concentrations (1–50% *w/v*).[8] In citrate injection solutions, maximum stability was found at pH 3.2 and several products were found especially after exposure to thermal stress (80 °C).[2,7] As depicted in Figure 1, the proposed consecutive decomposition reaction of 2-PAM (Figure 1, **I**) is an initial OH^--catalyzed dehydration to 2-cyano-N-methyl-pyridinium (Figure 1, **VIII**), which is subsequently hydrolyzed to either N-methyl-2-pyridone (Figure 1, **XIII**) and CN^- or to 2-carbamoyl-N-methyl-pyridinium (Figure 1, **IX**). This product is further hydrolyzed to 2-carboxy-N-methyl-pyridinium (Figure 1, **X**), which is decarboxylated to N-methyl-pyridinium (Figure 1, **XI**).[2,5,8] The limited stability under basic conditions restricts sample preparation and analysis thus favouring neutral or acidic pH.

However, under acidic conditions 2-PAM also undergoes decomposition (Figure 1). 2-formyl-N-methyl-pyridinium (Figure 1, **V**) is formed by the release of hydroxylamine (NH_2OH)[3] followed by the production of 2-hydroxymethyl-N-methyl-pyridinium (Figure 1, **VI**).[2,4,5] **V** can also be transformed into 2-carboxy-N-methyl-pyridinium (Figure 1, **X**) in the presence of CN^-.[2]

Utley has shown that decomposition is initiated by an isomerization step converting the predominant syn-form of 2-PAM (Figure 1, **I**) into its anti-form (Figure 1, **II**).[7] Decomposition products as listed above for basic conditions were shown to be derived from the anti-isomer establishing an equilibrium with syn-2-PAM. UV spectra of the corresponding syn- and anti-oximates varied markedly in terms of λ_{max} (anti: 338 nm, syn: 335 nm) and molar absorptivity ($\varepsilon_{anti}=0.3\,\varepsilon_{syn}$).[7] Therefore, early UV spectroscopic methods applied to investigate the stability of 2-PAM did not differentiate between both structures thus causing erroneous calculations of stability. In contrast, the chromatographic method applied by Utley allowed separation of both forms.[7]

Figure 1 depicts the relevant structures that were detected by diverse chromatographic techniques thus reinforcing the requirement for sufficient resolution and appropriate detection properties. The second part of this review addresses these methods.

Furthermore, the liberation of NH_2OH in acidic solutions is the key step for a UV spectroscopic quantification procedure based on the production of an azo-dye after oxidation of NH_2OH to nitrous acid (Figure 3).[42–44] This procedure will also be discussed *vide infra*.

Analytical techniques for 2-PAM quantification

2-PAM was introduced as antidote in the mid-1950s,[1] an era in which liquid column chromatographic (LC) techniques were rarely used and the theory of separation mechanisms was unexplored. Therefore, LC procedures were missing for 2-PAM analysis until 1967.[6] In contrast, gas chromatography (GC) represented an established and robust technique but it is unsuitable for 2-PAM determination due to the polar and charged oxime structure. Therefore, no reports on GC analysis could be found to date. Paper (PC) and thin layer chromatography (TLC) were also well elaborated in general but were of limited resolution and especially less appropriate for quantification. Therefore, PC was used for early studies of biotransformation and no reports on TLC were found that time.[34,45–48] Only in 2010 was a rare example presented using TLC for neat aqueous solutions of 2-PAM and numerous additional oximes.[49] As this procedure did not provide any application for stability analysis or quantification, it will not be addressed in more detail.

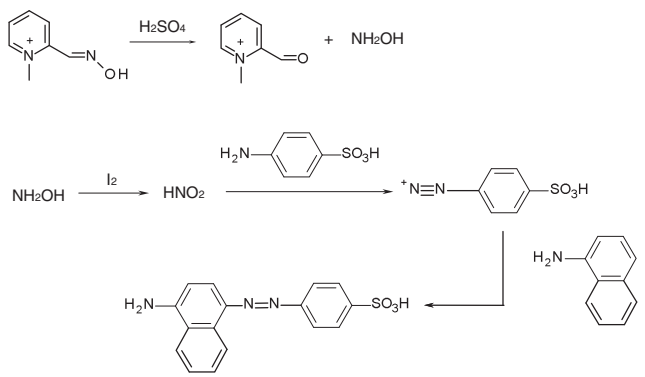

Figure 3. Reaction scheme for quantification of 2-PAM by production of an azo-dye. Under acid conditions 2-PAM is converted into its formyl-derivative liberating hydroxylamine in stoichiometric amounts. NH_2OH is oxidized by I_2 and transferred into an azo-dye photometrically measured at 604 nm.[44]

Due to these technical restrictions in analytical performance it appears plausible that 2-PAM quantification was most often performed by non-chromatographic UV spectroscopic procedures until the end of 1970s. These simple but effective approaches are addressed in the following section.

UV spectroscopic procedures

Numerous early publications are focused on the characterization of 2-PAM in terms of *in vitro* stability[15,39,42] and pharmacokinetics in diverse species including man,[16,32,44,50–56] cat,[43] dog,[57] rabbit,[58,59] and guinea pig.[60] These studies were conducted with related variants of spectroscopic procedures to quantify specimens of whole blood, plasma, urine, aqueous humour, tissue, and faeces.

Quantification by conversion into oximate anion. In principle, most procedures were based on deprotonization of 2-PAM under alkaline conditions producing a yellow-coloured solution of the 2-PAM oximate whose absorption was measured spectroscopically (333–336 nm). As depicted in Figure 4, this wavelength does neither interfere with undissociated 2-PAM nor with its primary degradation product 2-formyl-N-methyl-pyridinium (Figure 1, **V**) produced under acidic conditions (Figure 1).[42] Ellin and Kondritzer were the first who introduced this procedure for 2-PAM in neat aqueous solution,[42] that was subsequently adopted by Creasey and Green for analysis of biological samples.[15] The common basic work flow is comprised of initial dilution of the fluid (e.g. whole blood) with water, followed by hemolysis induced with 0.3 M $Ba(OH)_2$ and subsequent precipitation of blood proteins by addition of $ZnSO_4$ (0.33 M) and NaCl (3.4 M). After centrifugation, the protein-free supernatant was mixed with NaOH (5 M) and analyzed at 335 nm.[15,39] Satisfying linearity was obtained by external calibration up to 120 µg/ml plasma.[59]

However, blank samples showed noticeable absorption and interference requiring provision (subtraction from sample values) for quantification as elaborated by Creasey and Green[15] and May *et al.*[39]

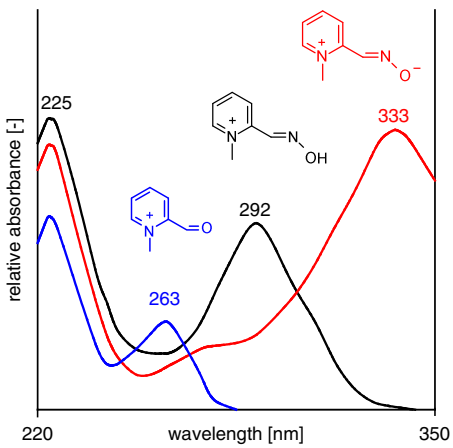

Figure 4. UV spectra of 2-PAM, its oximate and formyl-derivative. UV spectra of 2-PAM were monitored under basic (oximate, red curve) and acidic conditions (black curve). 2-formyl-N-methyl-pyridinium was also measured in acidic solution (blue curve). Data were obtained from Ellin and Kondritzer.[42] UV absorption at 333–336 nm allows to differentiate the oximate (λ_{max} 333 nm) and undissociated 2-PAM (λ_{max} 292 nm) without interference of the primary formyl-decomposition product (λ_{max} 263 nm). Based on this selectivity 2-PAM has been quantified in diverse studies.[15,39,42,59]

Unspecific absorbance of matrix ingredients is the general major disadvantage of the UV spectroscopic method. Due to insufficient sample preparation (simple precipitation omitting any subsequent separation step) and limited selectivity of UV detection, significant interferences with matrix compounds (e.g. endogenous compounds, metabolites from diet, drugs, and biotransformation products) might falsify results and hamper exact quantification.

Additional drawbacks were based on the laborious and time consuming steps of manual sample preparation and single spectroscopic measurements in cuvettes.

These limitations were overcome by the establishment of an automated approach presented by Groff and Ellin in 1969 that provided improved accuracy and a 40-sample/h throughput.[41] The apparatus comprised of an autosampler (providing the body fluid samples), mixing coils (dilution of samples with saline and surfactant), a double membrane dialyzer (dialysis of 2-PAM into an alkaline recipient stream), an on-line flow cell UV spectrophotometer (detection of the oximate at 335 nm under alkaline conditions) and a recorder (monitoring the absorption data). In contrast to earlier approaches, which required manual precipitation and centrifugation steps that were almost impossible to automate, this procedure used the principle of membrane dialysis in helical channels to remove matrix proteins. High molecular weight matrix components were separated from 2-PAM that is able to pass the membrane into the alkaline recipient (0.01 M NaOH). The concentration of 2-PAM in the receptor fluid was proportional to the concentration in the original sample. Sensitivity (0.5 μg/ml), linear range (0.5–24 μg/ml) and excellent inter-day coefficients of variation (1.5%) were highly appropriate for *in vivo* samples. Therefore, this analytical system was successfully applied to numerous *in vivo* studies in the following years.[16,32,52,53,55,56,60]

Quantification after derivatization into an azo-dye. A quantitative UV spectroscopic approach more selective than the oximate variant was presented by Sundwall converting the 2-PAM degradation product hydroxylamine into a purple coloured azo-dye (Figure 3).[44]

The procedure was based on the initial quantitative degradation of 2-PAM under harsh acidic conditions (1 M H_2SO_4) producing 2-formyl-N-methyl-pyridinium (Figure 1, **V**) and hydroxylamine (NH_2OH) (Figures 1 and 3). Subsequent addition of iodine in acetic acid oxidized NH_2OH to nitrous acid (HNO_2). Mixing with sulfanilic acid (4-aminobenzene sulfonic acid) as diazotizable produced its corresponding diazonium salt. Addition of 1-naphthylamine as the coupling reagent yielded a purple coloured dye that was analyzed spectroscopically using a 604 nm filter (λ_{max} 553 nm[61]). A schematic overview of the chemical reactions is shown in Figure 3. The general procedure had been introduced before by Csaky[62] and adopted by Askew *et al.*[63] for quantification of non-pyridinium oxime reactivators like MINA (monoisonitrosoacetone) and DAM (diacetyl monoxime). Unfortunately, a detailed description of analytical performance characteristics for 2-PAM was not provided.[44] Nevertheless, it was successfully applied to *in vivo* studies in human[44] and cat.[43] Apart from oxime analysis, such azo-dyes generated from nitrous acid are also used nowadays for nitrite quantification in water samples.[61]

Chromatographic procedures

The resolving power of chromatographic procedures is indispensable when analyzing complex mixtures of 2-PAM derivatives from non-enzymatic degradation in buffered solutions (stability studies) or from biotransformation *in vivo* (pharmacokinetic studies). Prior to the establishment of reliable column chromatographic methods, paper chromatography was used to analyze such samples.

Paper chromatography (PC). In 1962, Enander *et al.* performed a metabolic study with ^{14}C-2-PAM (^{14}C-labelled N-methyl group) in rats after oral and i.m. administration. Urine samples were separated by PC using a paper chromatograph (Frieseke and Höpfner, FH 52) equipped with two Geiger-Müller tubes (FH 215a) to detect radioactivity.[34] In addition, autoradiography (1–4 week exposure of an X-ray film placed on the chromatographic paper) was also performed to confirm results. Prior to PC urine was simply diluted with water and spotted onto a cellulose paper (Whatman No. 1) of 0.18 mm thickness allowing flows of 130 mm/min. A mobile phase consisting of n-butanol:acetic acid:water (4:1:1) was used.

Quantification of metabolites documented that 80–90% of 2-PAM (R_f 0.53) was directly excreted into urine within 24 h without structural changes.[34] In addition, at least three spots of biotransformation products were found assigned to N-methyl-2-pyridone (R_f 0.70) (Figure 1, **XIII**), 2-cyano-N-methyl-pyridinium (R_f 0.44) (Figure 1, **VIII**) and 2-carboxy-N-methyl-pyridinium (homarine, R_f 0.35) (Figure 1, **X**) (Figure 1, highlighted in green).

A similar ^{14}C-2-PAM approach was used by the group of Way investigating biotransformation in isolated perfused rat liver.[46,47,64,65]

However, quantitative assessment by PC relied on the use of a radio-labelled drug thus restricting its general use for *in vivo* studies especially in man.

PC separation combined with a spray reagent (iodoplatinic acid) for spot visualization was applied to *in vitro* stability studies of diverse 2-PAM preparations differing in their counterions (e.g. iodide, chloride, phosphate, perchlorate and trichloroacetate).[45] Counterions or sample matrix-derived anions associated with pyridinium-compounds and were shown to alter chromatographic retention causing either a shift of the retention factor or spot splitting that impaired spot assignment and quantitative interpretation. Therefore, more reliable techniques providing better chromatographic resolution were required.

The beginning era of column chromatography brought novel and useful applications based on diverse principles including, for example, cation exchange, reversed-phase and most of all ion pair chromatography.

Cation exchange chromatography (CIEC). The principle of CIEC relies on the interaction of positively charged molecules (analytes) with a stationary phase providing ionic functional ligands of negative charge. Attractive ionic forces bind the analyte and are eliminated or at least minimized for elution by changing the composition of the mobile phase. Usually gradients providing increasing portions of metal ions (e.g. Na^+) or hydroxonium ions (H_3O^+) are used to displace cationic analytes of lower attraction.

Under neutral and acidic conditions 2-PAM exists as single charged cation with its charge located at the quaternary nitrogen atom. When raising the pH, the oximino group is deprotonated and the molecule loses its net charge thus not binding to the stationary phase. These properties make the oxime an ideal candidate for CIEC.

Gibbon and Way introduced a CIEC approach for separating 2-PAM from its biotransformation products monitored by UV absorption at 280 nm (interference filter).[6] To separate small

amounts of transformation products from huge amounts of 2-PAM the sample was buffered in 0.5 M sodium borate (pH8.9) allowing the zwitterionic 2-PAM oximate molecule to pass the column without retention whereas cationic products were bound. Limited 2-PAM stability under alkaline conditions required rapid performance at 0–2 °C using a short column packed with Amberlite CG-50-Na^+ material. Metabolite elution was carried out using a 1 mM sodium phosphate buffer (pH6.7). More chromatographic details are summarized in Table 1. However, analysis of real samples from *in vitro* or *in vivo* studies was not reported.

About 15 years later, Kientz *et al*. presented a methodical publication systematically investigating the influence on diverse chromatographic parameters (type of ion exchange resin, pH, temperature, composition of mobile phase) on retention in CIEC of different mono- and bispyridinium oximes including 2-PAM (Table 1).[66] In principle, retention was caused by different mechanisms including ionic interaction, van-der-Waals forces and normal-phase partitioning. The latter one was favoured by a higher content of an organic modifier (e.g. methanol) in the mobile phase whereas ionic interaction was favoured by low ratios of methanol. Application to real life samples with more complex matrices was not part of that work.

Application to human plasma samples was presented by Willems *et al*. in 1992 using CIEC on R-Sil Cat as well as Ionospher-C material to analyze pharmacokinetics of 2-PAM in OPC-poisoned patients under 2-PAM (contrathion) therapy (Table 1).[17] This procedure made use of pyridostigmine as internal standard (IS) for antidote quantification by UV detection (270 nm for IS and 300 nm for 2-PAM). Plasma samples were mixed with trichloroacetic acid for deproteinization prior to CIEC analysis. Methods provided a sufficient linear range (covering 2 orders of magnitude) and good lower limits of detection (LLOD 0.01 μg/ml for R-Sil Cat and 0.2 μg/ml for Ionospher-C) thus underlining the applicability of CIEC for quantitative approaches.

Accordingly, Singh *et al*. also presented a CIEC method with UV detection (296 nm) for guinea pig plasma analysis quite recently (Table 1).[25] Plasma samples (100 μl) were prepared by acidification with phosphoric acid, centrifugation and subsequent ultra-filtration (10,000 MW cut-off) to remove particulate and protein matter prior to chromatography (recovery 80–100%).

Even though CIEC was the first column chromatographic method introduced for 2-PAM analysis, this technique did not achieve the broad acceptance and popularity as observed for ion pair chromatography (Figure 5). Most likely, this was due to the limited quality of stationary phase material and lack of commercially available, reproducibly filled columns causing inappropriate chromatographic resolution during the early years of practical use of high pressure (performance) liquid chromatography. In addition, reliable pump systems allowing high pressure applications were not wide spread. In contrast, columns of diverse dimensions packed with optimized ion exchange material of small particle and pore size are commercially available today, providing satisfying resolution, reproducibility and stability.

However, prior to the triumph of IPC a very rare example of size-exclusion chromatography was published in 1971.[68]

Size-exclusion chromatography (SEC). Size-exclusion chromatography is used for separation of molecules depending on their size and shape. Porous particles of the stationary phase enable small molecules to enter the pores whereas larger molecules are hindered. Therefore, small analytes cover a longer distance and thus exhibit longer retention times than bigger analytes for leaving the column in the mobile phase buffer.

A self-packed glass column containing Sephadex G-10, a cross-linked dextran gel (particle size 40–120 μm), or carboxymethyl-cellulose (CM) was applied as stationary phase for SEC to analyze 2-PAM from neat aqueous solutions (Table 2).[68] Fractionated eluates were alkalized to measure UV absorption at 336 nm off-line. Crone investigated diverse chromatographic parameters (e.g. flow rate, buffer salt composition) to characterize SEC properties to address more methodical and mechanistic aspects than providing a practical approach for real life sample analysis.[68]

In general, inappropriate resolution made SEC unfeasible for 2-PAM analysis when degradation and biotransformation products should be separated. A more powerful and adequate method was established in the early 1980s with the introduction of IPC.

Ion pair chromatography (IPC). Charged small molecules like 2-PAM are hardly retained on reversed-phase columns (C8, C18) thus hampering chromatographic separation and reliable analysis. Adding ion pair reagents to the mobile phase allows this restriction to be overcome. Salt additives like alkyl-sulfonates (e.g. sodium heptane-, octane- or dodecyl sulfonate) interact with the charged analyte and form an ion pair complex that exhibits a modulated hydrophilicity. Strong interaction between the RP-phase and the associated alkyl-chain of the reagent enhance peak shape and retention. This kind of chromatography is called IPC.

In 1981, Brown *et al*. were the first who introduced IPC to analyze the degradation of 2-PAM in acidic and basic aqueous solutions.[3] After subjecting the antidote to thermal stress (95 °C up to 1 h) diverse decomposition products were isocratically baseline separated on a μBondapak C18 column using 1-heptane sulfonic acid as ion pair reagent supplied under the trade name PIC B7® (Waters) (Table 3). Monitoring at 280 nm two major products (N-methyl-2-pyridone (Figure 1, **XIII**, 61.4%) and 2-carboxy-N-methyl-pyridinium (Figure 1, **X**, 37.8%) and a small byproduct (2-carbamoyl-N-methyl-pyridinium, Figure 1, **IX**) were found under alkaline conditions (Figure 1). Under acidic conditions only 2-formyl-N-methyl-pyridinium (Figure 1, **V**) (10%) was detected alongside unchanged 2-PAM.

As summarized in Table 3, diverse IPC modifications were introduced in the following years varying the stationary phase material and mobile phase composition. IPC was applied to qualitative and quantitative analysis of neat solutions,[3,8,70,71] pharmaceutical formulations[2,7,72] and biological specimen from different species including plasma and serum,[20–22,29–31,69–75] urine,[20,27,31,73] and brain tissue[76] (Table 3).

Despite this widespread use, a certain drawback of isocratic methods is due to lipophilic matrix components (e.g. remaining proteins, phospholipids, exogenous poisons, and drugs) that bind to the hydrophobic stationary phase but are not eluted because of insufficient ratio of organic modifier in the mobile phase. Therefore, successive column pollution may result in altering retention properties and interferences thus perturbing chromatographic analysis.[27]

An additional constraint of IPC is the requirement for high salt concentrations of the mobile phase leading to incompatibility with selective mass spectrometric detection. Therefore, most methods use UV detection at diverse wavelengths between 260 nm and 290 nm providing sufficient sensitivity and selectivity for reliable analysis (Table 3).

Table 1. 2-PAM analysis by cation exchange and reversed-phase chromatography.

sample matrix	stationary phase	dimension L. × I.D. [mm]	solvent	flow [ml/min]	detection	linearity [μg/ml]	LLOQ [μg/ml]	IS	ref.
cation exchange chromatography (CIEC)									
neat solution	Amberlite CG-50	102 × 4.5	conditioning: 0.5 M Na-borate, pH8.9; gradient elution: 1–5 mM Na-phosphate buffer, pH6.7	2.0	280 nm	3–1000 [mg/ml]	n.s.	none	6
neat solution	Nucleosil-SA	250 × 5	0.5 M KFA in MeOH/ACN/H_2O or EtOH/EE/H_2O, pH6.3, different ratios, different temperatures	1.0	RID or 290 nm	n.s.	n.s.	none	66
neat solution	Partisil-SCX	250 × 5	0.5 M KFA in MeOH/H_2O 80:20v/v, pH6.3	1.0	290 nm	n.s.	n.s.	none	66
neat solution	R-Sil Cat	250 × 5	0.5 M KFA in MeOH/H_2O 80:20v/v, pH6.3	1.0	290 nm	n.s.	n.s.	none	66
guinea pig plasma	PolyCATA	100 × 2.1	0.03 M NH_4OAc, 44% (v/v) ACN, pH4.5	0.3	296 nm	n.s.	n.s.	none	25
hum plasma	R-Sil Cat	250 × 5	0.02 M FA, 0.35 M KOH in 83% MeOH, pH5.0	1.0	300 nm	0.01–20	0.01[a]	pyridostigmine	17
hum plasma	Ionospher-C	100 × 3	0.003 M FA, 0.06 M KOH in 97% MeOH, pH5.0	0.6	300 nm	0.2–20	0.2[a]	pyridostigmine	17
reversed-phase chromatography (RPC)									
formulation[b]	Spherisorb-ODS	250 × 3.2	5 mM H_3PO_4, 1 mM Et_4NCl/ACN 48:52v/v	0.8	270 nm	125–375 [mg/ml]	n.s.	none	5, 67
hum plasma	X-Terra C8	100 × 2.1	solvent A: 2 mM NH_4FA, pH3, solvent B: ACN, gradient mode	0.2	ESI-MS/MS	0.005–1	0.005	d5-diazepam	26, 19

ACN, acetonitrile; d5-diazepam, five-fold deuterated diazepam; EE, ethyl acetate; ESI-MS/MS, positive electrospray ionization-tandem mass spectrometry; EtOH, ethanol; FA, formic acid; hum, human; I.D., inner diameter of column; IS, internal standard; KFA, potassium formate; L., column length; LLOQ, lower limit of quantification; MeOH, methanol; n.s., not specified; OAc, acetate; RID, refractive-index detection; ref., reference.

[a]values represent the lower limit of detection.

[b]injectable formulation (not specified).

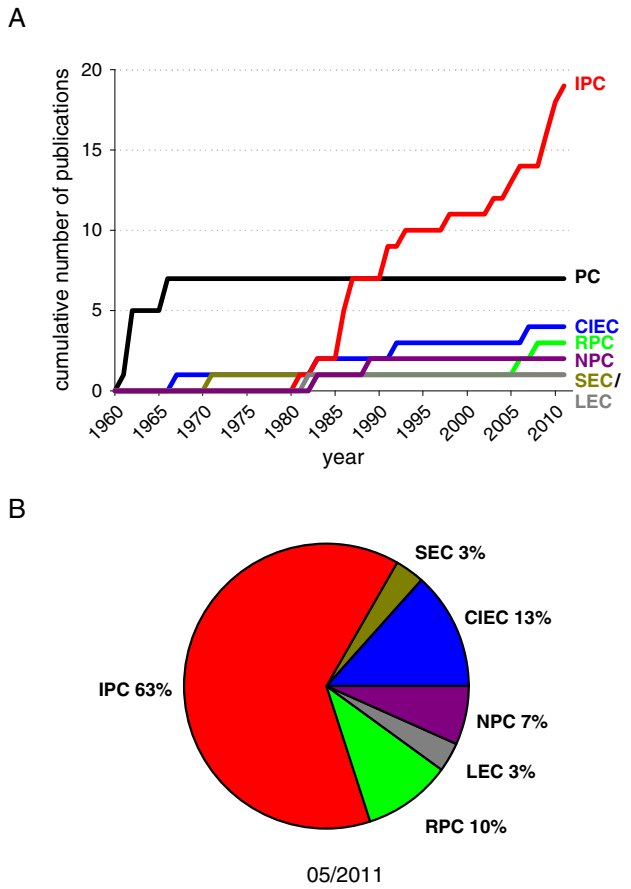

Figure 5. Trends in chromatographic analysis of 2-PAM. (A) Cumulative number of published reports from the early development phase of 2-PAM until today. CIEC, cation exchange chromatography; IPC, ion pair chromatography; LEC, ligand-exchange chromatography; NPC, normal-phase chromatography; PC, paper chromatography; RPC, reversed-phase chromatography; SEC, size-exclusion chromatography. Considered reports were found by PubMed database search and references cited therein. (B) Relative distribution (status from May 2011) of column liquid chromatographic methods applied for 2-PAM analysis counted by the number of published reports. For abbreviation see above.

Independent of the kind of detection IPC became the most frequent and popular chromatographic technique for 2-PAM analysis covering more than 50% of all column chromatographic approaches (Figure 5B).

Nevertheless, a few additional liquid chromatographic methods were introduced that will be addressed below.

Reversed-phase chromatography (RPC). The principle mechanism of RPC is based on the adsorption of the analyte to the stationary phase by hydrophobic interaction. Chromatographic resin that contains non-polar n-alkyl chains immobilized on its surface is best suited for retention of hydrophobic compounds. Desorption of analytes is achieved with a mobile phase containing appropriate ratios of organic modifier causing decreasing polarity of the solvent. Due to this mechanism, polar and charged analytes may be barely retarded.

Therefore, only two methods were found that applied RPC for 2-PAM analysis (Table 1).[5,19,26,67] In 1983, Prue *et al.* presented a quantitative method that was used for the determination of decomposition products of 2-PAM in injectable pharmaceutical formulations.[5] Isocratic separation of highly concentrated samples was performed on a Spherisorb-ODS (C_{18}) column at ambient temperature using a solvent mixture of 5 mM phosphorus acid, 1 mM NEt_4Cl and ACN (48:52 v/v) monitored at 270 nm (Table 1). NEt_4Cl is a typical ion pair reagent for IPC but attractive ionic interaction between the positively charged pyridinium compounds and the positive ammonium ion appears less plausible. Therefore, we included this report in the group of RPC and not IPC methods.

Decomposition products were characterized by monitoring the UV profile (200–340 nm) applying stop-flow spectroscopy. This procedure was successfully used before more modern diode-array detectors (DAD) were established that detect an entire range of wavelengths simultaneously.[27] Identification of degradation products was done by comparison to reference standards. Products found included the 2-carboxy (Figure 1, **X**), 2-carbamoyl (Figure 1, **IX**), 2-hydroxymethyl (Figure 1, **VI**) and 2-cyano (Figure 1, **VIII**) derivatives of N-methyl-pyridinium as well as N-methyl-2-pyridone (Figure 1, **XIII**) (Figure 1). The referred method was also used recently to investigate the

Table 2. 2-PAM analysis by ligand-exchange, size-exclusion and normal-phase chromatography.

sample matrix	stationary phase	dimension L. × I.D. [mm]	solvent	flow [ml/min]	detection	linearity [µg/ml]	LLOQ [µg/ml]	IS	ref.
ligand-exchange chromatography (LEC)									
rat plasma, rat urine	Partisil 5 silica, Cu^{2+}-loaded	150 × 4.8	0.5 M NH_3 in ACN/water 75:25 v/v	1.5	340 nm	0.21–42,000	0.21[a]	4-PAM	69
size-exclusion chromatography (SEC)									
neat solution	Sephadex G-10	360 × 9	0.01 M Tris/HCl, 0.15 M NaCl or KCl, pH 7.4	0.083	336 nm off-line	n.s.	n.s.	none	68
neat solution	CM	165 × 8.1	0.01 M Tris/HCl, 0.15 M NaCl or KCl, pH 7.4	0.083	336 nm off-line	n.s.	n.s.	none	68
normal-phase chromatography (NPC)									
neat solution	LiChrosorb	250 × 5	0.5 M KFA in MeOH/H_2O 80:20 v/v, pH 6.3	1.0	290 nm	n.s.	n.s.	none	66
formulation[b]	µ-Porasil	300 × 3.9	ACN/8.36 mM Et_4NCl, 52.5 mM HOAc, pH 2.9, 86:14 v/v	1.0 1.5	295 nm	65–85	0.1	none	4

4-PAM, N-methyl-pyridinium-4-aldoxime; ACN, acetonitrile; CM, carboxymethylcellulose; HOAc, acetic acid; I.D., inner diameter of column; IS, internal standard; KFA, potassium formate; L., column length; LLOQ, lower limit of quantification; MeOH, methanol; n.s., not specified; ref., reference.

[a]value represents the lower limit of detection.

[b]formulation from autoinjector ComboPen®.

Table 3. 2-PAM analysis by isocratic ion pair chromatography.

sample matrix	stationary phase	dimension L. × I.D. [mm]	solvent	flow [ml/min]	detection	linearity [µg/ml]	LLOQ [µg/ml]	IS	ref.
neat solution	µBondapak C_{18}	300 × 3.9	0.01 M PIC-B7®/ACN 80:20 v/v, pH 3.4	1.5	280 nm	100–1500	n.s.	none	3
neat solution	Hibar-Lichrosorb RP-C18	125 × 4.0	2 mM Na-OSA, 50 mM Me_4NCl, 20 mM HOAc, 10% v/v MeOH, pH n.s.	0.8	200–300 nm	n.s.	n.s.	none	8
neat solution	Zorbax RX-C„	250 × 4.6	1 mM Me_4NCl, 1 mM Na-OSA, 3% v/v ACN, pH 3.5	1.0	203 nm	n.s.	n.s.	none	70
neat solution	Spherisorb ODS	250 × 4.6	0.05 M Et_2NH phosphate, 0.01 M Na-laurylsulfate, pH 3.0/MeOH 75:25 v/v	n.s.	265 and 293 nm	n.s.	n.s.	n.s.	72
formulation[a]	Hypersil ODS	125 × 4.6	0.1 M TMAP, 0.01 M Na-laurylsulfate, pH 3.0 in MeOH/H_2O 10:90 v/v	1.0	262 nm	2000–15,000	n.s.	nicotine-amide	7
formulation[b]	Hypersil ODS	150 × 4.6	0.1 M phosphate, 9.2 mM Na-laurylsulfate, 10% v/v ACN, pH 3.0	1.5	262 nm	n.s.	n.s.	none	2
Tris/HCl buffer	Puresil C18	250 × 4.6	0.2 M H_3PO_4, 2 mM Na-OSA, 0.1 M Et_2NH, pH n.s.	1.0	230 nm	n.s.	n.s.	none	71
hum serum and urine, rat serum	Inertsil ODS	250 × 4.6	13.8 mM Na_2HPO_4, 13.8 mM KH_2PO_4, 0.92 µM Na-OSA, 8% v/v MeOH, pH 2.6	1.0	ECD and 280 nm	0.25–50	0.2	guanosine	73, 20
hum plasma	Purosphere Star RP18	55 × 4.0	5 mM PIC-B7 and MeOH (gradient mode)	1.4	293 nm	0.15–20	0.15	none	30, 28
hum serum	Bondapak C_{18}	n.s.	PIC-8 buffer, pH 2.6/ACN 80:20 v/v	1.1	294 nm	1–50	n.s.	pyridostigmine	29
mnipig plasma	LiChrospher 60 RP-select B	125 × 4.0	7.5 mM Na_2HPO_4, 7.5 mM KH_2PO_4, 2.5 mM Na-OSA, 5% v/v ACN, pH 2.6	1.0	293 nm	0.39–400	0.39	none	74
minipig urine	LiChrospher 60 RP-select B	125 × 4.0	7.5 mM Na_2HPO_4, 7.5 mM KH_2PO_4, 2.5 mM Na-OSA, 6% v/v ACN, pH 2.6	1.0	293 nm	4.9–2500	9.8	4-PAO	27
rat plasma, sheep plasma	Radial-Pak Resolve C^{18}	100 × 2.5	0.01 M HOAc, 0.05 M Et_4NCl, 0.005 M OSA/MeOH 60:40 v/v, pH 4.0	0.2	295 nm	0.5–100	n.s.	none	21, 22
rat serum	µBondapak C18	n.s.	PIC-8 buffer, pH 2.6/ACN 78:22 v/v	0.8	294 nm	1–25	n.s.	pyridostigmine	75
rat urine, rat plasma	Lichrosorb RP-18	300 × 4	0.05 M PIC-B7®/ACN 50:50 v/v	1.5	RD	n.s.	n.s	none	31
rat brain[c]	Puresil C18	250 × 4.6	0.2 M H_3PO_4, 2 mM Na-OSA, 0.1 M Et_2NH, pH n.s.	1.0	290 nm	n.s.	n.s.	none	76

4-PAO, pyridinium-4-aldoxime; ACN, acetonitrile; ECD, electrochemical detection; HOAc, acetic acid; I.D., inner diameter of column; IS, internal standard; L., column length; LLOQ, lower limit of quantification; n.s., not specified; OSA, octanesulfonic acid; PIC-B7®, ion-pairing reagent heptanesulfonic acid (Waters); PIC-8, 0.01 M Na-octanesulfonate, 0.002 M Me_4NCl, 0.005 M NaOAc, pH 2.6; RD, radio detection for ^{14}C; ref., reference; TMAP, trimethylamine phosphate.

[a] 250 mg/ml pralidoxime mesylate, 1 mg/ml atropine sulfate in 0.1 M citrate buffer, pH 3.2.

[b] 250 mg/ml pralidoxime mesylate in 0.1 M citrate buffer, pH 3.2

[c] as rat brain microdialysate.

stability and purity of 2-PAM in autoinjectors stock-piled in hospitals.[67]

The next example of RPC we would like to discuss belongs to the smartest and most modern methods applied for 2-PAM analysis. In 2008, Abbara *et al*. presented a validated, selective, sensitive, precise, and accurate method for the simultaneous quantification of most relevant antidotes in human plasma including atropine, diazepam and 2-PAM itself (Table 1).[19,26] Plasma sample volumes as small as 100 µl obtained from a pharmacokinetic study were simply precipitated by the addition of ACN prior to centrifugation and analysis of supernatant (10 µL). Interestingly, a less hydrophobic C_8 column (X-Terra MS C_8, 100 mm x 2.1 mm I.D., Waters, Saint Quentin, France) was used in combination with 2 mM ammonium formate (pH 3) and ACN as solvents (200 µl/min) causing slight but sufficient retention of 2-PAM ($t_R \approx 2$ min) (Table 1). Unfortunately, the chromatographic dead time was not given thus not allowing the extent of retention to be conclusively addressed. Other analytes were eluted by a step-wise ACN gradient. Optimum selectivity was achieved by using tandem mass spectrometric detection (MS/MS) in the multiple reaction monitoring mode (MRM) after electrospray ionization (ESI). Deuterated diazepam was used as IS for all analytes. To the best of our knowledge, this is the only report applying MS detection for 2-PAM. Detector properties in general and mass spectrometric behaviour of 2-PAM in particular will be addressed below in the section *Detectors for chromatography and electrophoresis*.

The linear range for 2-PAM was from 5 up to 1000 ng/ml. However, plasma samples of patients under 2-PAM therapy are highly concentrated (1–14 µg/ml)[17,19,23,25,26] thus not necessarily requiring such sensitive detection. Nevertheless, the convincing charm of this method lies in the simultaneous detection of relevant compounds from a small plasma volume thus saving sample material, time for analysis and therefore costs in general.

Even though RPC appears to be less favourable for separating small charged pyridinium compounds due to minor hydrophobic interaction, its counter part normal-phase chromatography (NPC) has also not been used more frequently. For the sake of completeness NPC will be addressed next.

Normal-phase chromatography (NPC). The principle mechanism of NPC is based on hydrophilic interaction between the polar analyte and a polar stationary phase (e.g. silica). A non-polar mobile phase (e.g. hexane, ethylacetate) allows analyte adsorption while resorption is enabled by increased portions of aqueous solvent causing increasing polarity.

Investigating chromatographic mechanism Kientz *et al*. tested the retention behaviour of diverse pyridinium aldoximes including 2-PAM on bare silica material (Lichrosorb, Merck, Darmstadt, Germany) (Table 2).[66] Analyte separation was discussed as a consequence of dynamic partition equilibria between the less polar (methanol) and highly polar (water) mobile phases enabling rapid mass transfer of the solutes. The silica resin was considered as a kind of aqueous phase and to a small extent as a weak cation exchanger. Elevated temperature (80 °C) resulted in high chromatographic efficiencies and resolutions.

Schroeder *et al*. applied NPC to analyze 2-PAM decomposition products on a silica gel column (µPorasil, Waters, Milford, MA, USA) with a high ratio of ACN (86% *v/v*) in the acidic aqueous mobile phase (Table 2).[4] Despite validation for quantification and satisfying data for accuracy and precision, chromatographic separation and non-selective UV detection at 295 nm (Table 4) were not optimum causing numerous overlapping peaks.

Table 4. Wavelengths at maximum UV absorption of 2-PAM and some decomposition products.

compound	λ_{max} [nm]
2-carbamoyl-N-methyl-pyridinium (Figure 1 **IX**)	266[a]
2-carboxy-N-methyl-pyridinium (Figure 1 **X**)	276[a]
2-cyano-N-methyl-pyridinium (Figure 1 **VIII**)	224/269/276 (sh)[a]
2-formyl-N-methyl-pyridinium (Figure 1 **V**)	226/263[a,b]
2-hydroxymethyl-N-methyl-pyridinium (Figure 1 **VI**)	206/263[a]
N-methyl-2-pyridone (Figure 1 **XIII**)	227/301[a]
pralidoxime (2-PAM, not dissociated) (Figure 1 **I**)	225/293[b](295) [a]
pralidoxime (2-PAM, dissociated) (Figure 1 **III**)	225/333[b](336)[c]

[a]measured at pH 2.9[4]
[b]measured in neutral and acidic solution[42]
[c]measured in 0.1 M HCl[39]
[d]measured in 0.1 M NaOH[39]
sh, shoulder.
Roman numbers refer to structures depicted in Figure 1.

Even though columns packed with classical normal-phase material were the first to be provided commercially, NPC never played a major role in 2-PAM analysis (Figure 5).

Finally, a quite special and unique method should be presented to complete our review on column chromatographic techniques.

Ligand-exchange chromatography (LEC). LEC requires a stationary phase that contains immobilized (transition) metal ions capable for coordination of compounds or ligands (analytes), which are adsorbed forming labile complexes. Resorption and elution are achieved by displacement (exchange) of analytes with other ligands of higher metal/ligand stability provided in the mobile phase.[77]

In 1982, Guyon *et al*. introduced LEC on copper(II)-modified silica gel to quantify 2-PAM in rat plasma and urine for determination of PK parameters.[69] The stationary phase was prepared from Partisil 5 silica gel (Whatman, Clifton, NJ, USA) that was flushed with a solution of 0.01 M $CuSO_4$, 1 M NH_3 to strongly immobilize Cu^{2+}-cations on the silicate resin prior to column equilibration with the mobile phase (0.5 M NH_3 in ACN/water 75:25 *v/v*) (Table 2). A 20 µl aliquot of supernatant obtained from plasma precipitation with ACN, was injected onto the column allowing 2-PAM to be bound by copper ions. Whether binding to Cu^{2+} appears predominately by ionic interaction of the negatively charged oximate group or by coordination to the aromatic pyridinium system was not discussed. However, the same procedure was also applied to separate a series of amino acids and peptides not necessarily requiring aromatic systems for retention.[77–79]

Isocratic elution of the antidote was performed within 6 min with NH_3 in the mobile phase desorbing the solute from the coordination site. Therefore, the chromatographic mechanism appears to be related to immobilized-metal affinity chromatography (IMAC), CIEC and NPC.

The use of a structural 2-PAM analogue as IS (4-PAM, N-methyl-4-aldoximino-pyridinium) allowed precise and sensitive determination of 2-PAM. When compared to IPC, LEC provided improved selectivity and better sensitivity due to UV detection of the oximate anion under alkaline conditions (340 nm)

instead of the undissociated oxime under acidic conditions (295 nm).[69]

Even though this method sounds quite exotic, performance characteristics demonstrated its reliability and capability for 2-PAM quantification in biological fluids. The more complex and laborious column preparation, its limited stability as well as the sensitivity towards mobile phase composition (ratio of water and concentration of NH_3) might be reasons for the lack of further propagation (Figure 5).[79]

Besides the chromatographic methods referred to, a small number of electrophoretic approaches have been described in the literature, which in principle represent complementary techniques and thus will be discussed in the next section.

Electrophoretic methods

Electrophoresis denotes separation techniques that use a spatially uniform electric field causing electrokinetic effects that make dispersed charged analytes migrate within a conductive liquid medium (buffer) from one electrode to the opposite one. Separation (electrophoretic mobility) is influenced by, for example field strength, viscosity and dielectric constant of the dispersant as well as size (frictional forces and hydrodynamic radius) and charge of the analyte. Depending on the kind of conductive medium carrier, electrophoresis is classified as for example (polyacrylamide) gel electrophoresis (GE), paper electrophoresis (PE) and capillary zone electrophoresis (CZE). Whereas GE is most appropriate for quite large analytes (e.g. proteins), PE and CZE are well suited for small molecules, including 2-PAM. These applications are now addressed.

Paper electrophoresis (PE). Horizontal PE enables separation of charged small molecule analytes that are spotted onto a cellulose paper strip moistened with an aqueous buffer to make it electrically conductive. The ends of the strip are dipped into separated buffer reservoirs also containing electrodes. Under cooling analyte ions migrate by the driving force of a high voltage electric field. After electrophoresis separated compounds can be detected by a variety of staining techniques.

PE was applied to investigate the biotransformation of ^{14}C-2-PAM in isolated perfused rat liver as a complimentary technique to PC.[47,64,65] Separation of cationic biotransformation products was achieved with 0.05 M sodium acetate buffer (pH 4.5) and 0.05 M sodium phosphate (pH 7.2) at 2 °C for 4 h applying 400 V and 7 mA.[65] Detection was performed by UV absorption and measurement of radioactivity. Unfortunately, no further details on performance characteristics were provided.

However, inappropriate resolution and limited robustness presumably prevent more frequent use of this technique. In contrast, capillary (zone) electrophoresis has become a useful and robust analytical tool for reliable separation of a large variety of diverse molecules within the last two decades.

Capillary zone electrophoresis (CZE). In CZE, charged analytes are separated according their charge-to-size ratio within a cooled fused-silica capillary connecting two buffer reservoirs which are equipped with electrodes for application of high voltage. Migrating analytes are typically detected online by UV absorption.

In 2005, Houze *et al.* introduced the first CZE method applied to the quantification of 2-PAM in human urine without a need for any sample preparation other than simple dilution (1:5) (Table 5).[24] Urine was separated from anode to cathode under constant voltage (15 kV) at 25 °C in a 50 cm capillary (75 μm I.D.) with 25 mM sodium borate buffer (pH 9.1). 2-PAM was detected at 280 nm (t_M 6.6 min) and quantified by an IS (hypoxanthine) covering a linear range from 125–2000 μg/ml. This selective, precise and accurate method was used to analyze urine from OPC-poisoned patients under 2-PAM therapy within 15 min per run. However, the selected wavelength of 280 nm appears to be less adequate for 2-PAM with respect to the alkaline buffer used in the separation, leading to the formation of the oximate anion which shows much stronger UV absorption at 336 nm as discussed above (Figure 4).

A major limitation of CZE is due to the small sample volume (nl range) that can be analyzed after hydrodynamic or electrokinetic injection. High concentrations of the analyte are required that are typically higher than for LC analysis. Therefore, the LLOQ of the method was 100 μg 2-PAM/ml urine. Considering the 1:5 dilution of urine, the corresponding injection solution had a concentration of 20 μg/ml.

Kalasz *et al.* also used CZE for 2-PAM quantification in rat tissue and body fluids.[80] A surprising difference is noted with respect to the LLOQ (Table 5). Kalasz *et al.* presented linear ranges of 0.3–10 μg/ml for serum, 0.1–7 μg/ml for cerebrospinal fluid (CSF) and 0.3–7 μg/ml brain tissue thus being about 1000 times lower than elaborated by Houze *et al.*[24] Unfortunately, it remained unclear how μg/ml-concentrations given for brain tissue correlate to concentrations in wet or dry tissue (μg/mg).

Similar to Houze *et al.*,[24] sample preparation for serum and CSF simply comprised a 1:5 dilution step with distilled water without control of pH or ionic strength. Separation was also carried out in fused-silica capillaries of comparable length (30 cm) and identical I.D. (75 μm) with detection at 280 nm. In contrast to Houze *et al.*,[24] an acidic buffer at pH 4.0 was used (0.05 M β-alanine-acetic acid) (Table 5) at constant current (22 μA) thus favouring 2-PAM detection at 293 nm instead of the chosen 280 nm (Figure 4). Furthermore, sample injection differed as Kalasz *et al.* used isotachophoretic concentration allowing hydrodynamic injection of 5-times larger volumes.[80] However, considering similarities (sample dilution, detection wavelength) and

Table 5. 2-PAM analysis by capillary zone electrophoresis.

sample matrix	capillary	dimension L. × I.D.	buffer	voltage [kV]	detection	linearity [μg/ml]	LLOQ [μg/ml]	IS	ref.
hum urine	fused-silica	47 cm × 75 μm	25 mM Na-borate, pH 9.1	15	280 nm	125–2000	100	hypoxanthine	24
rat brain,	fused-silica	30 cm × 75 μm	0.05 M β-alanine-HOAc, pH 4.0	20	280 nm	0.3–7	0.1[a]	none	80
rat CSF,						0.1–7	0.05[a]		
rat serum						0.3–200	0.1[a]		

CSF, cerebrospinal fluid; HOAc, acetic acid; hum, human; I.D., inner diameter of capillary; IS, internal standard; L., capillary length to detector; LLOQ, lower limit of quantification; ref., reference.

[a]values represent the lower limit of detection.

differences (buffer composition, pH and injection) reasons for the enormous difference in LLOQ remain surprising.

Despite the above mentioned ambiguity with respect to certain experimental details, CZE surely represents an alternative technique to LC methods to quantify 2-PAM in biological samples allowing simple dilution for sample preparation prior to analysis. In addition, modern instruments for CZE and LC are of comparable robustness and validity.

Detectors for chromatography and electrophoresis

In principle, most chromatographic and electrophoretic methods use UV detectors for analysis of 2-PAM and its decomposition or biotransformation products. Nevertheless, a few exceptions were found that will also be addressed in the next sections.

UV detector (UVD). UV detectors are cheap, robust, and provide sufficient sensitivity for high 2-PAM concentrations in *in vivo* samples. Furthermore, selectivity is appropriate despite simple sample preparation procedures when combined with any separation step. Whereas older simple UV detectors were able to analyze just one wavelength at time, modern diode array detectors can monitor several wavelengths and an entire UV spectrum simultaneously.[27]

Even though optimum absorptivity of 2-PAM is well known to be at 293 nm under acidic and at 333–336 nm under alkaline conditions (Figure 4) only a minority of methods applied these wavelengths (Tables 1, 2, 3 and 5). Instead, a broad variety of different wavelengths, for example, 262, 270, 280, 290, 294, and 300 nm[2,3,5,17,75,76] were used. These settings might be due to (i) instrumental restrictions of older detectors in terms of light sources and monochromators (e.g. filters for fixed wavelengths, especially for 280 nm), (ii) a compromise for multicomponent detection at fixed wavelength (Figure 4, Table 4), (iii) influence of LC solvent components and pH, or (iv) simply non-optimum choice.

By far the most selective detection is realized by mass spectrometers especially when operating in the multiple reaction monitoring mode.

Mass spectrometric detector (MSD). The permanent positive charge of 2-PAM under acidic conditions makes this compound an ideal candidate for MS detection not requiring any proton transfer in electrospray ionization. In principle, the use of mass spectrometric detectors in LC is restricted by the need for minimal salt concentrations in the solvent. Otherwise pollution and clogging of the MS entrance (e.g. curtain plate and skimmer) may impair ion transfer into the mass analyzer and thus deteriorate qualitative and quantitative analysis. Therefore, IPC and CIEC for 2-PAM have never been coupled to MS detection (Tables 1, 3). Instead, low concentrations of volatile buffer components, for example, NH_4FA are compatible with ESI-MS and were therefore applied to RPC (Table 1).[19,26]

Figure 6 depicts the MS/MS spectra in the range from *m/z*10 to *m/z*200 of the 2-PAM precursor ion (*m/z*137) generated by collision-induced dissociation (CID) at rising collision energies after positive electrospray ionization using an API 4000 QTrap (Applied Biosystems, Darmstadt, Germany). In agreement with the findings of Abbara *et al.*[26] (Table 1) we detected predominant product ions at *m/z*119 (Figures 6, Figure 7F2) and *m/z*120 (Figure 6, Figure 7F1), whereas D´Agostino *et al.* found a single product at *m/z*119 exclusively.[81] In addition smaller fragments (*m/z* < 100, Figure 6, Figure 7F3–F8) were also detected especially when using higher energies. These fragments were not presented by Abbara *et al.*, who published their

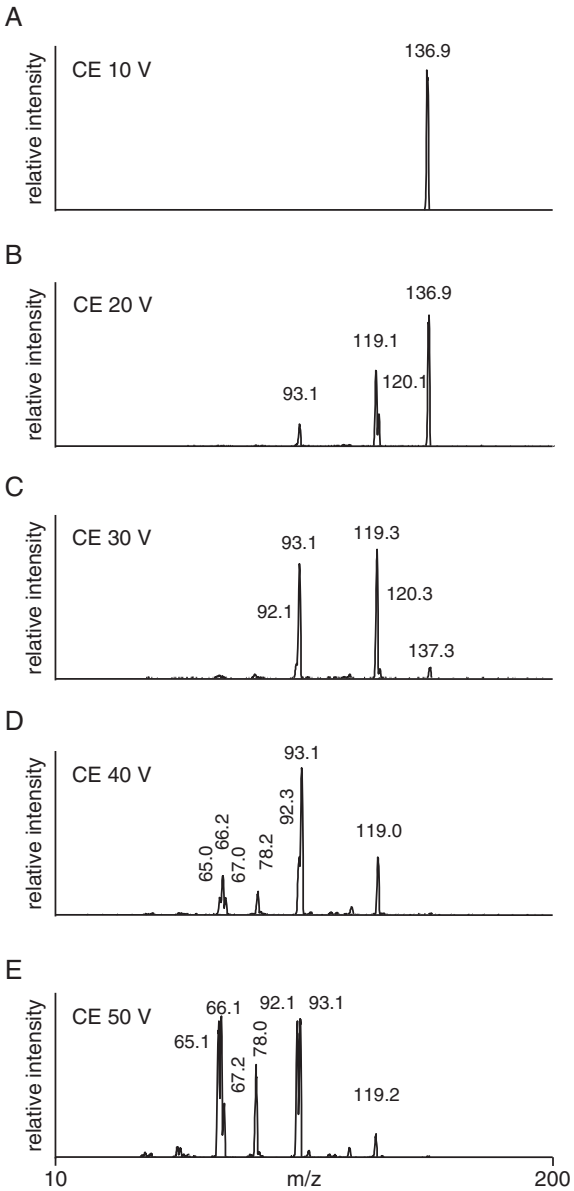

Figure 6. Collision energy-dependent ESI-MS/MS spectra of 2-PAM. 2-PAM chloride was dissolved in 0.1% (*v/v*) formic acid/acetonitrile (80:20 *v/v*) (30 ng/ml) and infused into a QTrap 4000 linear ion trap mass spectrometer (Applied Biosystems) by positive electrospray ionization. MS conditions were: ionization spray voltage 2 kV, declustering potential 80 V, entrance potential 10 V, temperature 300 °C, curtain gas and gas spray 20 psi ($1.38*10^5$ Pa), rising collision energy (CE) as indicated, mass range *m/z*10–200. The precursor ion of 2-PAM (*m/z*136.9) fragmented into diverse product ions to a collision energy-dependent extent. Structural assignments for product ions are proposed in Figure 7.

2-PAM MS/MS spectrum with a narrower range from *m/z*100 to *m/z* 200.[26]

Interestingly, product ions appear as a pair of signals differing by one hydrogen mass (*m/z*120/119, 93/92, 65/66/67) (Figure 6). As illustrated in Figure 7, we propose structural assignments to these signals not considering potential mesomeric structures.[82]

Whereas ESI is known to produce mainly even electron (EE) product ions from EE precursors[83] we herein suggest also the generation of radical odd electron (OE) cations. The occurrence of *m/z* 119 and 120 is most probably due to the loss of H_2O and

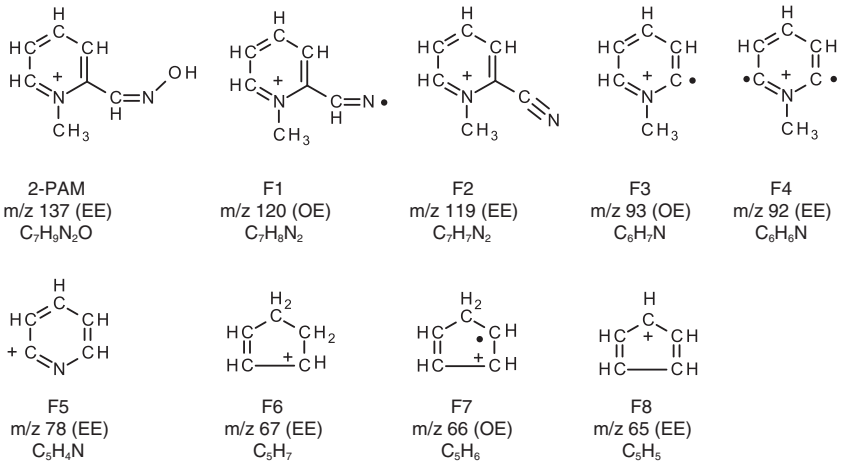

Figure 7. Proposed structural assignment to MS/MS fragments of 2-PAM. Signal pairs of fragments differing by 1–2 Da were found (*m/z* 120/119, 93/92, 67/66/65) presumably reflecting the presence of suggested even electron cations (EE) and odd electron radical cations (OE). Potential mesomeric structures are not depicted. Fragments were obtained under MS conditions presented in Figure 6.

an OH-radical producing the EE structure F2 and OE structure F1 (Figure 7). A more detailed discussion of detected fragments would go beyond the focus of this review and will thus not be provided.

Besides excellent selectivity MS detection also provides superior sensitivity allowing trace analysis. However, high 2-PAM concentrations in urine and plasma of treated mammals do not require that sensitivity. This argument in combination with high investments costs for MS systems may be an important reason why MS detection has rarely been used so far. Furthermore, commercially available, reliable and robust LC-ESI MS and CZE-ESI MS systems became available only in the late 1990s and could therefore not have been applied before.

Radioactivity detector (RD). Early approaches to detect decomposition and biotransformation of 2-PAM by paper chromatography were carried out with the radioactive labelled parent drug containing ^{14}C-methyl.[34,46,47,64,65] Radioactive products fixed on the paper were monitored at the end of chromatography either by autoradiography[34] or by a scanning technique using Geiger-Müller tubes (radiochromatograph).[34] Both methods allowed continuous (non-fractionated) monitoring of separated compounds. In contrast, other radio assays were based on elution from chromatographic paper (1 mM phosphate buffer[47]) after slitting the paper (fractionation) followed by quantification of radioactivity (windowless gas flow proportional counter).[47,64,65]

More modern approaches would have used liquid or solid scintillation techniques to detect the β^--emitters ^{14}C or tritium (^{3}H).[84] This equipment also allows online detection for column chromatography and is nearly independent of solvent composition. Potential quenching effects influencing the amount of light output can be determined experimentally. Accordingly, Garrigue *et al.* analyzed *in vivo* samples from rats treated with ^{14}C-labelled 2-PAM by IPC on-line coupled to RD (Table 3).[31]

In principle, the use of radio-labelled analytes mixed with a large excess of non-labelled molecules enables sensitive analysis of all products that still contain the labelled moiety. But any transformation product that results from the loss of that label cannot be detected anymore. Nevertheless, due to the lack of analytical methods providing sufficient and satisfying sensitivity this strategy was also applied in early studies on, for example, atropine,[85–88] prostaglandins,[89–91] and many other compounds. However, restricted use of radioactive compounds in animal models and especially in humans, the need for specialized laboratories and high disposal fees for radioactive waste as well as the missing possibility for structural elucidation make these procedures unattractive compared to the alternative methods available today.

UVD, MSD, and RD represent the most common, most selective and sensitive detectors used for 2-PAM analysis. Nevertheless, for the sake of completeness two additional detectors should be mentioned that were also applied even though in unique examples.

Refractive-index detector (RID). The RID belongs to the least sensitive LC detectors inappropriate for gradient elution but well suited for non-ionic, non-UV-absorbing and non-fluorescent analytes. Compounds exhibiting a different refractive-index (RI) than the mobile phase are monitored by positive or negative changes of the RI in comparison to the pure solvent.

A RID was used by Kientz *et al.* in CIEC for detection of 2-PAM and other quarternary pyridinium aldoximes (Table 1).[66] These analyses were performed to elucidate chromatographic mechanisms and not to quantify the antidote in real life samples. Therefore, a demand for high sensitivity did not restrict the capability of this detector.

In contrast, appropriate sensitivity for *in vivo* samples was provided by electrochemical detection.[20,73]

Electrochemical detector (ECD). The ECD is capable to detect any compound that is oxidizable or reducible by a chemical reaction at the surface of electrodes. In principle, composed of a working electrode for analyte oxidation or reduction, an auxiliary electrode and a reference electrode, the ECD operates either in a coulometric or ampiometric mode. The resulting electron flow (electric current) is proportional to the analyte concentration thus allowing robust, sensitive and selective detection. For a more detailed description we refer to respective textbooks.

Houze *et al.* applied an ECD for quantitative measurement of 2-PAM in human urine and serum after IPC separation (Table 3).[20,73] For sample preparation urine and serum were precipitated by the addition of perchloric acid prior to dilution of the supernatant with water. This validated method using guanosine as IS was shown to be precise, sensitive (LLOQ 0.2 μg/ml) and linear over two orders of magnitude (0.25–50 μg/ml).[73] Very small sample

volumes of 25 µl were sufficient for reliable coulometric analysis using 30 mM phosphate buffer. Optimization of electrode potentials (electrode 1, +0.3 V for elimination of interfering substances; electrode 2, +0.95 V for oxidation of 2-PAM and IS; guard cell +1.2V) allowed selective determination in body fluids of an OPC-poisoned patient under 2-PAM therapy[73] and of paraoxon-poisoned rats treated for a PK study.[20]

Therefore, the ECD was proven to be a reliable and useful tool for drug detection being 10 times more sensitive than UV detection.[73]

Nuclear magnetic resonance and infrared spectroscopy (NMR and IR)

Fourier-transform infrared (FTIR) and infrared spectroscopy after embedding in a potassium bromide disc or in a Nujol mull have been used to characterize 2-PAM synthesized for clinical treatment.[38,39,92] Furthermore, ^{13}C-, ^{1}H-, and ^{15}N-nuclear magnetic resonance (NMR) spectroscopy as well as X-ray crystal structure determination were also primarily applied to identify purity and integrity of synthetic lots allowing for discrimination between syn- and anti-configuration of 2-PAM.[40,92–94]

However, no quantitative application to biological samples was reported for any of these techniques. Therefore, we will only refer to these spectroscopic methods without detailed discussion.

Conclusions

As discussed, numerous analytical methods were developed that allow quantification and characterization of 2-PAM to evaluate stability and *in vivo* behaviour. By far the most frequent methods today are liquid chromatographic approaches with UV detection (Figure 5). Especially IPC has been shown to be a highly valuable tool for rapid, robust, and reliable analysis. Determination of therapeutic concentrations in body fluids like plasma and urine are established for small sample volumes with sufficient selectivity and sensitivity.

With respect to the rising propagation of mass spectrometers that enable on-line coupling to LC, an increasing number of applications for 2-PAM quantification can be expected for the future.

However, 2-PAM is an established reactivating antidote that has been extensively characterized in the past. Therefore, corresponding novel analytical approaches can rather be anticipated for additional antidotal oximes that are currently under development (e.g. HI6, MMB-4) requiring clinical studies.

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