Research article

Drug Testing and Analysis

Received: 26 August 2011 Revised: 16 September 2011 Accepted: 21 September 2011 Published online in Wiley Online Library: 22 February 2012

(wileyonlinelibrary.com) DOI 10.1002/dta.376

Photostability of antidotal oxime HI-6, impact on drug development

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HI-6 exhibits superior efficacy in the therapy of intoxication by different highly toxic organophosphorus nerve agents. Therefore HI-6 is a promising candidate for the development of new antidotes against nerve agents. For ethical and safety reasons antidotes containing HI-6 should get marketing authorization. Active pharmaceutical ingredients of medicinal products have to fulfil regulatory conditions in terms of purity and stability. Photostability is an essential parameter in this testing strategy. HI-6 was tested under conditions of ICH Q1B 'Photostability testing of new drug substances and products'. The data showed a marked degradation of HI-6 after exposure to daylight. The mechanism of degradation could be detected as photoisomerism. The light burden dependent rate of photoisomerism was followed quantitatively. Based on these quantitative results on the amount of light induced isomeric product a pharmacological qualification was made. A standardized *in vitro* test showed a decreased ability of light exposed HI-6 to reactivate sarin- and paraoxon-inhibited human acetylcholinesterase. These results have an impact on the further development of antidotes containing HI-6, as light protection will probably be necessary during handling, packaging, storage and application. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: antidote; Hi-6; photostability; reactivation constants acetylcholinesterase; regulatory affairs

Introduction

HI-6 is an asymmetric bis-pyridinium aldoxime. Numerous *in vitro* and *in vivo* studies demonstrated a superior therapeutic efficacy of HI-6 in poisoning by different organophosphorus nerve agents. Hence, this oxime represents a promising option for an active pharmaceutical ingredient (API) in the further development of antidote therapy of nerve agent poisoning. [1–4]

Data on photostability are state of the art for new API and represent an essential condition in marketing authorization of new medicinal products. Technical requirements for marketing authorization are harmonized within Europe, the USA, and Japan by a series of guidelines published by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Photostability has to be tested under standardized conditions as described in ICH Q1B '*Photostability Testing of New Drug Substances and Products*'.[5] The background of these studies is to demonstrate that light exposure does not result in unacceptable change of the API. If significant changes occur, a qualification of the degradation product in terms of its pharmacological and toxicological characteristics can be required. This is to support the decision about necessary changes in packaging or handling of the API and dosage form to avoid light exposure.

Experimental

Chemicals

HI-6 DMS [HI-6; (1-({[4′-(aminocarbonyl)pyridinium]methoxy} methyl)-2-((hydroxyimino)methyl)pyridinium dimethanesulfonate, CAS 144252-71-1] was provided by Phoenix Ltd. (Bromborough, UK). Acetylthiocholine iodide (ATCh) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were supplied by Sigma (Deisenhofen, Germany). Sarin (Isopropylmethylphosphonofluoridate, >98% by GC–MS, ^{1}H- and ^{31}P-NMR) was made available by the German Ministry of Defence. Paraoxon (O,O′-diethyl-p-nitrophenyl phosphate) was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) and was cleaned of disturbing *p*-nitrophenol as desribed by Worek *et al.*[6] Pure water (Millipore water) was produced with a Milli-Q Gradient system (Millipore Corporation, Billerica, MA, USA).

Analytics on photodegradation

For the analysis of the HI-6 samples, a high performance liquid chromatography (HPLC) method compatible to mass spectrometry detection with the following chromatographic conditions was used: For the separation of HI-6 DMS and its isomer we used an Atlantis HILIC Silica, 150 x 4.6 mm I.D. column with 3 μm particle size (Waters, Eschborn, Germany). The HPLC pump delivered a gradient of 9:1 (*v/v*) acteonitrile /100 mM ammonium formate (eluent A) and 4.5:4.5:1 (*v/v*) acteonitrile / water /100 mM ammonium formate (eluent B) with the following conditions: 10% B at a flow rate of 1 ml/min, linear increase to 100% B from 4 to 16 min, and return to initial conditions from 16 to 19 min. The total run time was 35 min. The injection volume was 5 μl and the auto sampler was cooled to 8 °C. For the detection an Agilent (Agilent Technologies, Waldbronn, Germany) diode array detector (DAD) was used (λ=254 nm).

Mass spectrometry (MS) examinations of the degradation product were performed on an Agilent 1100 HPLC system

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coupled to an Agilent XCT ultra ion trap mass spectrometer. The HPLC method was the same as described above. MS and tandem mass spectrometry (MS/MS) detection was done by electrospray ionization (ESI) and ion trap mass spectrometry (positive mode). The mass spectrometer was calibrated with an ESI tuning mix (Agilent) from *m/z* 200–2200 and a scan speed of 26 000 *m/z* per second (ultra scan mode). The ionization parameters were set to the following values: drying gas (N_2), 12.0 L/min; dry temperature, 350 °C; capillary voltage, 3500 V; skimmer voltage, 40 V; capillary exit voltage, 109.8 V. The mass analyzer parameters were: octopole 1 DC, 12.0 V; octopole 2 DC, 1.7 V; octopole RF 142.5 Vpp; lens 1, -5.0 V; lens 2, -60.0 V; trap drive, 31.9 V; ion charge control (ICC) on, smart target 100 000; maximum accumulation time, 200 ms; number of averaged scans 2; target mass, *m/z* 300. MS/MS experiments were performed with He as collision gas and auto MS/MS acquisition cycle, respectively multiple reaction mode (MRM).

Confirmatory studies about the structure of the degradation product were made by ^{1}H-NMR. Therefore the light exposed aqueous samples were lyophilized. HPLC measurements before and after lyophilization showed no influence of lyophilization on the sample. For the NMR study, approximately 2–3 mg of light exposed HI-6 were dissolved in 600 µl DMSO-d_6; ^{1}H-NMR spectra were recorded with an Avance III 400 MHz Microbay Ultrashield with 5 mm broadband PABBO BB-1H/D (BBO) probe head (Bruker Biospin, Rheinstetten, Germany) at a measuring temperature of 30 °C.

Light exposure

Light exposure was conducted in a climate chamber equipped with a light source and appropriate filter (HCL 4057/S, Heraeus Vötsch, Reiskirchen-Lindenstruth, Germany; light source: SOL 2000 lamp with H1 filter, Dr. Hönle UV-Technologie, Gräfelfing, Germany) delivering a spectral output (λ = 320 nm – 820 nm) as described in ISO 18909. [7] The measured dose rate was 1 x 10^5 lux. The temperature within the light chamber was stabilized at 25 °C. HI-6 was exposed to light under the following test conditions:

1. HI-6 solution in pure water pH 7, concentration 0.5 mg/ml, total volume 20 ml in a clear glass flask.
2. HI-6 solution in 0.1 % (*v/v*) formic acid pH 2, concentration 0.5 mg/ml, total volume 20 ml in a clear glass flask.
3. Solid HI-6 powder 10 mg, in a clear glass dish.

Exposure times were up to 33 h (pH 7), 57 h (pH 2), and 72 h (powder) corresponding to doses from 3.3 x 10^6 lux*h to 7.2 x 10^6 lux*h. For the analytical procedure 1 ml sample from each solution was taken during light exposure and analyzed by HPLC-DAD and LC-MS, respectively. For the solid HI-6 powder one 10 mg specimen was prepared and exposed for each sampling time. For HPLC measurement the powder was dissolved in 0.1 % (*v/v*) formic acid (pH 2) to a final concentration of 0.5 mg/ml.

The samples for pharmacological qualification of the isomer were produced as described above at pH 7.

In order to check the influence of temperature comparative studies at 60 °C for up to 47 h without light exposure were conducted.

Determination of reactivation constants

Influence of light exposure on pharmacological activity was examined by determination of reactivation constants of untreated and light exposed HI-6. Therefore sarin stock solutions (0.1 % *v/v*) were prepared in acetonitrile (ACN) and were stored at 20 °C. Paraoxon stock solutions in 2-propanol (10 mM) were stored at 4 °C. HI-6 preparations in Millipore water (1 mM) were used which were either non treated or light exposed.

Sarin, paraoxon, and HI-6 were diluted as required in distilled water immediately before use.

All solutions were kept on ice until the experiment. If not otherwise stated, the buffer consisted of 0.1 M sodium phosphate, pH 7.4.

Human hemoglobin-free erythrocyte ghosts served as acetylcholine esterase (AChE) source and were prepared as described before. [8] Aliquots of the erythrocyte ghosts with an AChE activity adjusted to that found in whole blood were stored at −80 °C. Prior to use, aliquots were homogenized on ice with a Sonoplus HD 2070 ultrasonic homogenator (Bandelin Electronic, Berlin, Germany), three times for 5 s with 30-s intervals, to achieve a homogeneous matrix for the kinetic studies.

Determination of AChE activity

AChE activities were measured with a modified Ellman assay [9,10] on a Cary 3Bio spectrophotometer (Varian, Darmstadt, Germany) at 436 nm using polystyrol cuvettes and 0.45 mM ATCh as substrate and 0.3 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.4).

All experiments were performed at 37 °C and pH 7.4. All concentrations refer to final concentrations.

Preparation of organophosphate (OP)-inhibited AChE

Erythrocyte ghosts were incubated with a small volume (≤1 %, *v/v*) of appropriate sarin and paraoxon concentrations in distilled water for 15 min at 37 °C to achieve an AChE inhibition by >95 %. Then, the treated ghosts were dialyzed against 0.1 M phosphate buffer, pH 7.4, overnight at 4 °C to remove residual inhibitor. Finally, the absence of inhibitory activity was tested by incubation of treated and control ghosts (30 min, 37 °C), and aliquots were stored at −80 °C until use.

Reactivation of OP-inhibited AChE

Sarin- and paraoxon-inhibited human AChE was incubated with a small aliquot (<3 % *v/v*) of the different HI-6 preparations resulting in final HI-6 concentrations of 10 and 30 µM. At various times (1–30 min) a sample was taken and the AChE activity was determined. The data were referred to control AChE and the % reactivation was calculated according to Worek *et al.*[11]

Inhibition of human AChE by HI-6

Various HI-6 concentrations, 10, 30, 60, and 100 µM, were added to human AChE and the enzyme activity was determined. Data were referred to control AChE and are given as % of control.

Data analysis

Processing of experimental data for the determination of the kinetic constants was performed by non-linear regression analysis using curve fitting programs provided by Prism™ Vers. 4.03 (GraphPad Software, San Diego, CA, USA). Differences in the reactivation kinetics of inhibited AChE were analysed with the two-tailed Mann-Whitney U-test. A $p < 0.05$ was considered to be significant.

Results

Analytics on photodegradation

The HI-6 samples were analyzed by HPLC before and during light exposure. Figure 1 shows an overlay HPLC chromatogram of an aqueous HI-6 solution at pH 7 during light exposure.

The peak at t_R 14.6 min corresponds to HI-6. During light exposure the HI-6 peak decreased marked with exposure time, while a second peak at t_R 14.8 min increased. Another small peak arising at t_R 15.6 min was not examined. The additional peak at t_R 14.8 was further investigated by LC-MS/MS giving the MS/MS spectra shown in Figures 2A and 2B.

The MS/MS spectrum of the base peak at *m/z* 257.1 is identical in both spectra at t_R 14.6 and 14.8 min and there are more than two identical mass transfers of this main peak for HI-6 and the degradation product. As recently shown by Bogan *et al.* [12] HI-6 could be degraded in its isomer at the aldoxime group. The MS/MS spectra at 14.6 and 14.8 min are in good agreement with our results during the study of the purity of HI-6. So we suppose that the degradation product is again a constitutional isomeric compound, presumably the Z-isomer. Light burden increased the amount of this compound.

HPLC quantification of the irradiated batch for NMR experiments showed a content of about 25% of the degradation product. To demonstrate that the degradation product which was generated during light exposure is the Z-isomer, ^{1}H-NMR experiments were used.

In the ^{1}H-NMR spectrum of the light exposed material an additional aldoxime peak with an area proportion of 26 % at

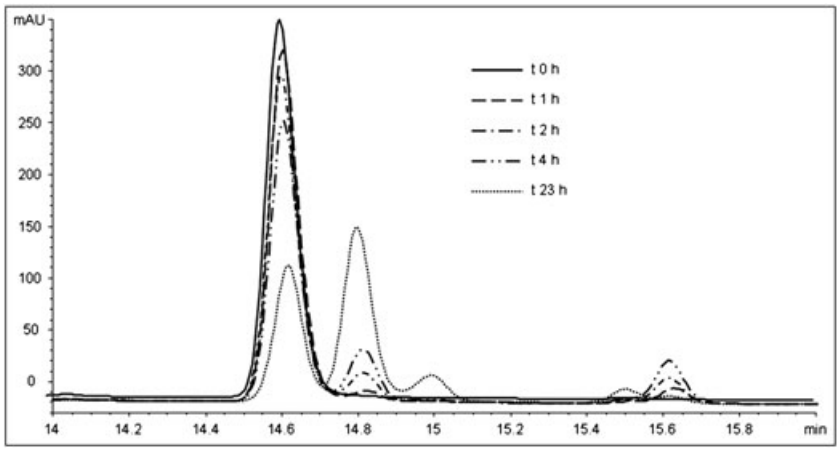

Figure 1. Overlay HPLC chromatogram of an aqueous HI-6 solution at pH 7 during light exposure. HPLC was performed on an Atlantis HILIC Silica column 150 x 4.6 mm I.D. using an ammonium formate/water/acetonitrile gradient with a flow rate of 1 ml/min and UV detection at λ=254 nm.

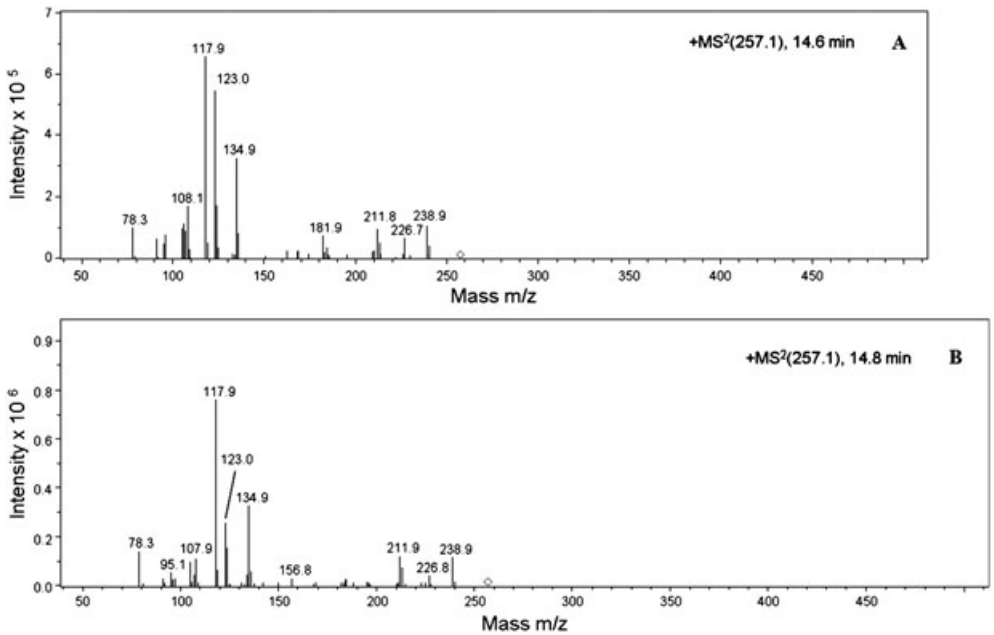

Figure 2. MS/MS spectra of HI-6 (A) and light-induced degradation product (B). Spectra were recorded with an Agilent XCT ultra Ion Trap Mass Spectrometer in ESI-MS mode (positive mode) from *m/z* 200–2200, with a scan speed of 26 000 *m/z* per second in ultra scan mode and He as collision gas and auto MS/MS acquisition cycle respectively MRM; separation of the compounds was performed on a HILIC Silica column 150 x 4.6 mm I.D. using an ammonium formate/water/acetonitrile gradient (flow rate 1 ml/min).

13.08 ppm and additional signals between 6 and 9.5 ppm could be recognized. Figure 3 demonstrates the ^{1}H-NMR spectrum of the mixture of HI-6 and the degradation product. The important peaks at 13.08 ppm, 8.12 ppm and the shifted protons of the aldoxime group between 6 and 9.5 ppm are highlighted. The ^{1}H-NMR data are identical to the NMR data from our recent study about the purity of HI-6.[12]

All the chromatographical and MS data as well as the NMR findings are in accordance with previous findings to an unknown impurity of HI-6. This was proven as an E/Z isomer. [12] Therefore the mechanism of light induced degradation is photo isomerism. It leads to an isomeric form of HI-6 whereas the Z-isomer increases by light exposure (Figure 4).

The amount of genuine HI-6 and its arising isomer was determined and thus the rate of isomerism was followed up to 72 h. As we saw no shift in λ_{max} for the isomeric form, we assumed the same molar absorptivity for both isomeres. The resulting concentration-time curves are shown in Figures 5A–5C.

At all tested conditions, corresponding curves of HI-6 isomerization could be recorded. This effect was more pronounced at pH 7. In the case of HI-6 powder only a very limited effect was determined. All curves approximate to a stable maximum rate of isomerization. These maximum rates of isomerization are given in Table 1.

Longer lasting duration of light exposure up to 7 days at all test conditions resulted in an almost quantitative degradation with many unspecified degradation products.

Temperature of 60 °C for 47 h without light exposure did not yield in formation of the isomer.

Pharmacological qualification of the degradation product

Pharmacological qualification was made *in vitro* by determination of the reactivation kinetics of OP inhibited AChE. For this purposes HI-6 samples with a rate of isomerization of 59% isomeric form, prepared as described above, were used.

The reactivation of sarin-inhibited human AChE by non- and light exposed HI-6 resulted in significantly lower increase of AChE activity with light exposed oxime (10 μM and 30 μM; Figures 6A and 6B). A comparable result was recorded with paraoxon-inhibited AChE and use of 100 μM oxime (Figure 7). With both inhibitors mono-exponential reactivation curves were obtained and the pseudo-first order reactivation rate constants k_{obs} could be determined (Table 2). In all cases k_{obs} values of non exposed HI-6 were significantly higher compared to those of light exposed HI-6.

The pseudo-first order reactivation rate constant k_{obs} was determined by non-linear regression analysis according to Worek *et al.* [13] Data are given as means ± SD.

Incubation of native AChE with non- and light exposed HI-6 at different concentrations resulted in a comparable, concentration dependent inhibition of the enzyme (Figure 8). This proves that there is no influence of isomerism on the intrinsic inhibitory activity of HI-6. [14]

Discussion

Though HI-6 is an important candidate for antidotal treatment of poisoning by nerve agents, studies on the photostability of this

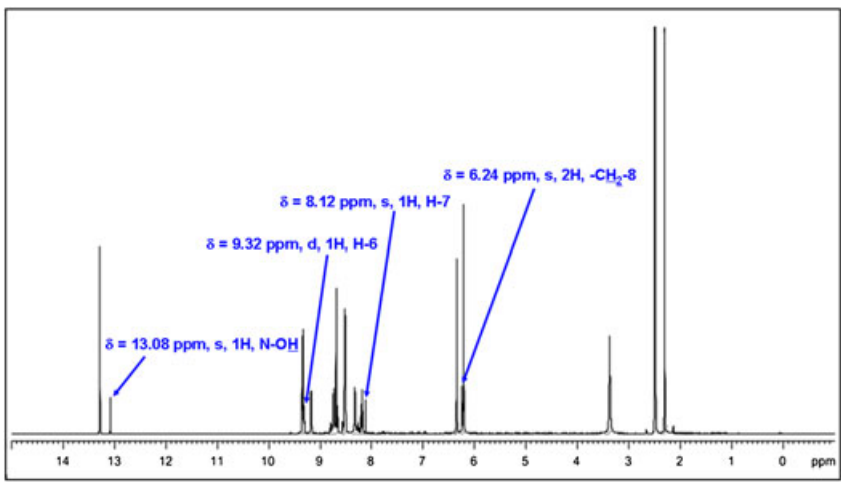

Figure 3. ^{1}H-NMR spectrum of light exposed HI-6. The spectrum was recorded with a Bruker Biospin 400 MHz microbay including BBO probe, in DMSO-d_6, at a measuring temperature of 30 °C.

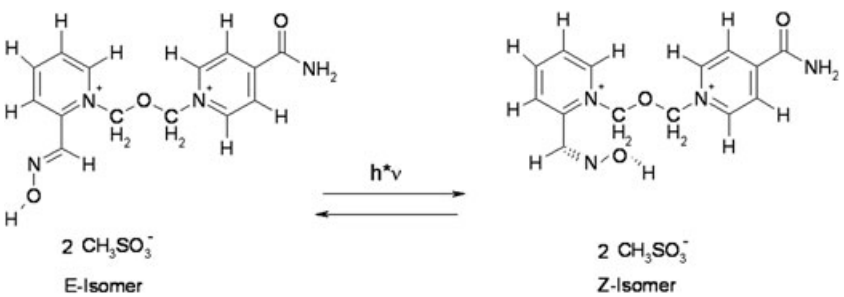

Figure 4. Photo-isomerism of HI-6.

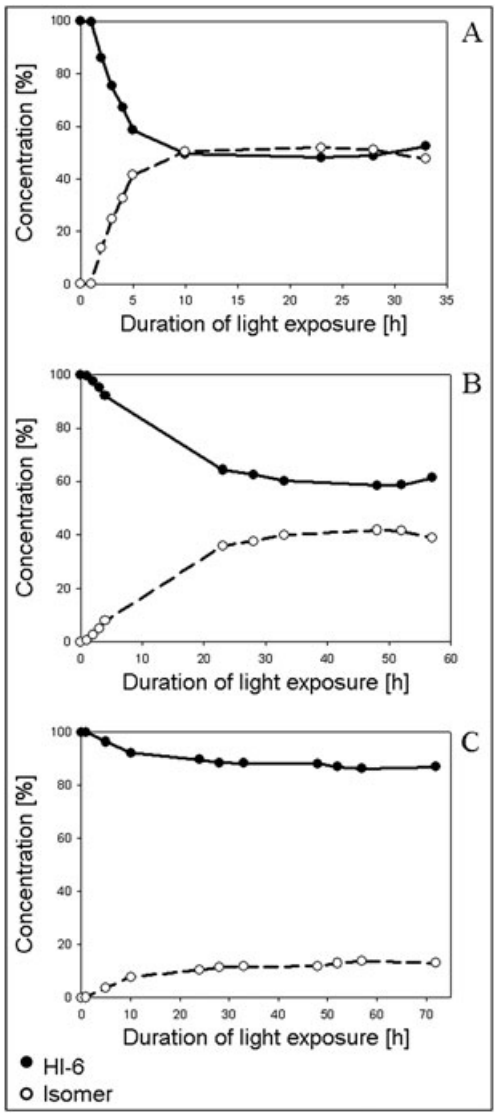

Figure 5. Concentration-time curves of HI-6 during light exposure. Test conditions were: HI-6 solution in pure water pH 7, concentration 0.5 mg/ml, total volume 20 ml in clear glass flask (A); HI-6 solution in 0.1 % (*v/v*) formic acid pH 2, concentration 0.5 mg/ml, total volume 20 ml in clear glass flask (B), and solid HI-6 powder 10 mg, clear glass dish (C). Light exposure was accomplished in a climate chamber equipped with a light source delivering a spectral output (λ = 320 nm – 820 nm) as described in ISO 18909, measured dose rate was 1 x 10^5 lux, temperature was 25 °C. Analytics of HI-6 was performed by HPLC, using an Atlantis HILIC Silica column 150 x 4.6 mm I.D. with an ammonium formate/water/acetonitrile gradient at a flow rate of 1 ml/min and UV detection at λ=254 nm.

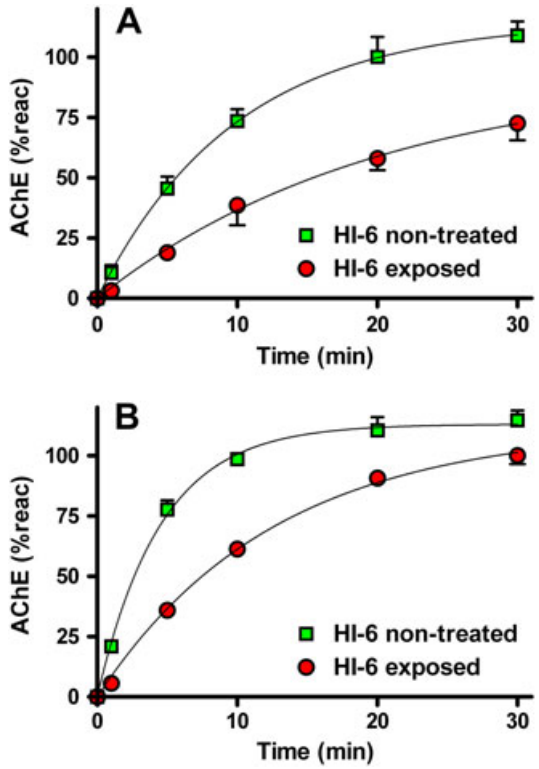

Figure 6. Reactivation of sarin-inhibited human AChE by light-exposed and non-treated HI-6. Sarin-inhibited AChE was incubated with 10 μM (A) and 30 μM (B) HI-6 and AChE activity was determined after various times. Data (n = 4; means ± SD) were analyzed by non-linear regression analysis.

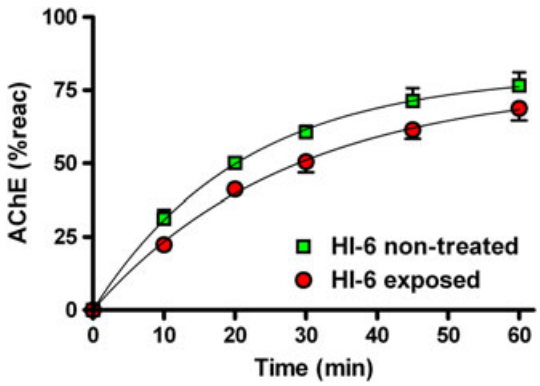

Figure 7. Reactivation of paraoxon-inhibited human AChE by light-exposed and non-treated HI-6. Paraoxon-inhibited AChE was incubated with 100 μM HI-6 and AChE activity was determined after various times. Data (n = 4; means ± SD) were analyzed by non-linear regression analysis.

bispyridinium oxime are still lacking. Drug development consists of stability studies of drug substances and products towards certain stress conditions i.e. temperature, humidity, oxidation, hydrolysis at different pH values as well as photolysis.[15] Photostability testing is a special issue of this testing strategy to elucidate whether and to which degree photodegradation will occur after light exposure.[16] Light irradiation has very complex effects on molecules, which can result in a pattern of irradiation

Table 1. Maximum rates of isomerization after light exposure.

Test conditions	Dose rate [lux*h]	Rate of isomerization [%] mean ± SD, n = 3
HI-6 solution conc. 0.5 mg/ml in Millipore water, pH 7	2.3 x 10^6	52 ± 7
HI-6 solution conc. 0.5 mg/ml in formic acid 0.1 % (*v/v*), pH 2	4.8 x 10^6	42 ± 3
HI-6 dry powder 10 mg	5.2 x 10^6	14 ± 1

Table 2. Reactivation constants of HI-6 preparations with sarin- and paraoxon-inhibited human AChE.

	Sarin-inhibited AChE				Paraoxon-inhibited AChE	
	10 μM HI-6		30 μM HI-6		100 μM HI-6	
	Non exposed	Light exposed	Non exposed	Light exposed	Non exposed	Light exposed
k_{obs} (min^{-1})	0.102 ± 0.006	0.051 ± 0.023	0.221 ± 0.013	0.079 ± 0.013	0.049 ± 0.006	0.036 ± 0.002
	$p < 0.05$		$p < 0.05$		$p < 0.05$	

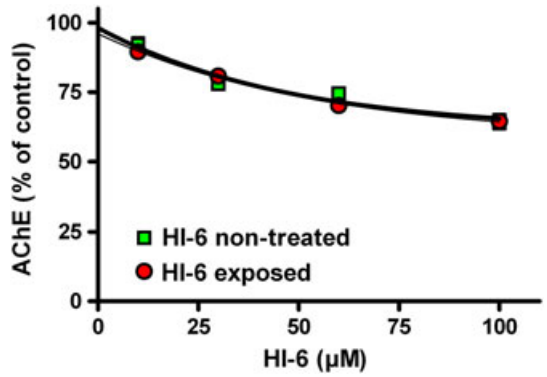

Figure 8. Inhibition of human AChE by HI-6. Human AChE was incubated with 10, 30, 60, and 100 μM light-exposed and non-treated HI-6 and AChE activity was determined after various times. Data (n = 2; means ± SD) were analyzed by non-linear regression analysis.

products.[17] If photodegradation occurs, knowledge about the putative mechanisms is essential to estimate effects such as formation of toxic degradation products and loss of efficacy. In order to meet regulatory requirements and due to the very distinct effects on further drug development especially formulation, package and storage conditions, several attempts for standardization and validation of these studies are reported. [18–20] The results reported here were gathered under the conditions of ICH Q1B [5] whereas the spectral distribution according ISO 18909 [7] was assured by suitable filter and dose rate was measured during all experiments. Thus, the results can be used to a statement of photostability as required by regulatory authorities.

Detection and quantification of degradation products are based on a suitable stability-indicating analysis. In this case the emerging of an isomeric compound of the API had to be followed. In a previous work we showed that a HPLC method based on stationary phase HILIC Silica has advantages compared to conventional reversed phase HPLC for this issue in terms of resolution between API and its isomer. [12] Therefore, this method was used here for quantification of the isomer.

The measured photodegradation is based on photoisomerism as proven by means of MS/MS and NMR. This effect of light irradiation on chemical structures with oxime groups is also reported in the literature.[21–24] Thus our results are in accordance with cited findings.

Our data on photostability show that HI-6 is unstable to daylight. This applies for solutions and to a lesser extent for dry powder. Reports on photodegradation showed that several parameters influence the kinetics of degradation, for example, excipients, pH and concentration of drug. [25–29] It can be expected that higher concentrations will lead to lower isomerization rates. The concentrations used here for explorative studies on photostability are much lower than the ones used for clinical treatment. Therefore the data presented here should be supplemented by additional, confirmatory studies with the final dosage form as stipulated by regulatory legislation.[5]

The observed light-induced changes have consequences on quality, safety, and efficacy of a further formulation of HI-6 as an antidote. These consequences depend to a great extent on the pharmacological characteristics of the isomer. Efficacy of oximes is based on binding to the inhibited acetylcholinesterase, which is in part sterically determined. [11,30,31] Therefore, it may be expected that isomerism has an influence on reactivation efficacy.

Compared to non-treated HI-6, light-exposed HI-6 resulted in a moderate but significant reduction of the reactivation kinetics with sarin- and paraoxon-inhibited human AChE. An impact of isomeric configuration of oximes has been shown before, for example, the syn isomer of 4-PAM had a higher reactivity towards sarin-inhibited electric eel AChE than the anti isomer.[32] Also for obidoxime the ZZ isomer is in accordance to our findings reported to be less effective. [33] In view of the fact that reactivation of inhibited AChE is the major mechanism of action of oximes, any reduction of the reactivation potency will probably lead to a reduced therapeutic efficacy of an oxime. Hence, the observed impact of light exposure on the ability of HI-6 for reactivation has to be taken into account in the process of drug development.

The regulatory guideline appropriate for degradation products [34] gives several algorithms for threshold values of degradation products dependent on the maximum daily dose of drug substance. The marked photodegradation observed in our studies makes data on the mechanism and the identity of the degradation product mandatory. The degradation product was identified as an isomeric form of HI-6. This way our results on photostability contribute to meet regulatory requirements for marketing authorization of HI-6.

Our results on photostability and on subsequent *in vitro* efficacy testing have an impact on the further development of this antidote. As a conclusion of our work, light protection will probably be necessary for handling of the API, packaging of the dosage form, storage, and application of antidotes containing HI-6.

Acknowledgements

The authors are grateful to Christiane Hochreiter, Nada Vujtovic-Ockenga and Tamara Hannig for skilful technical assistance.

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