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Purity of antidotal oxime HI-6 DMS as an active pharmaceutical ingredient for auto-injectors and infusions

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As reactivators of inhibited acetylcholinesterase, oximes are essential antidotes in poisoning by organophosphorus compounds. Due to its superior efficacy in cases of soman, cyclosarin, and sarin poisoning, the oxime HI-6 represents a promising option for an active pharmaceutical ingredient (API) in the further development of antidote therapy for nerve agent poisoning. Developmental lots of HI-6 DMS (dimethanesulfonate) provided by different manufacturers were examined with respect to their content and purity with a view to their future use as an API. There are distinct differences in the HI-6 content from three manufacturers. With respect to purity, gradual differences arise with the known synthetic by-products as well as with unknown accompanying compounds. It became apparent that in the case of a modified synthesis using protective groups, the proportion of some synthesis by-products decreases considerably. With one exception, they are thus below the reporting threshold for API in accordance with pertinent regulatory guidelines. In HI-6, an unknown impurity always occurs, whose percentage necessitates identification due to regulations. This unknown impurity, which has not been described so far, could be identified as an isomer. These findings supply data required for the description of pharmaceutical quality in accordance with module 3 of a Common Technical Document (CTD). They thus contribute to the marketing authorization of this substance as an API for auto-injectors and infusions. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: antidote; HI-6; oxime; regulatory affairs; spectrometry

Introduction

As reactivators of inhibited acetylcholinesterase, oximes are essential antidotes in poisoning by organophosphorus compounds.[1–3] In the therapy of nerve agent poisoning, the oxime HI-6 DMS represents a promising option for an active pharmaceutical ingredient in the further development of antidote therapy due to its superior efficacy in soman, cyclosarin, and sarin intoxication.[4–7]

HI-6 DMS, 1-({[4′-(aminocarbonyl)pyridinium]methoxy}methyl)-2-((hydroxyimino)methyl)pyridinium dimethanesulfonate is an asymmetric bis-pyridinium aldoxime (Figure 1). The coupling of the two pyridine rings can be effected by bis(chloromethyl)ether (BCME), which is highly reactive and carcinogenic. As an alternative avoiding the carcinogenic intermediate BCME, a synthesis has been described using bis(methylsulfonoxymethyl)ether (BSME) as a coupling reagent.[8,9] The percentage of synthesis by-products was reduced through the use of protective groups.[10] Essential synthesis steps are depicted in Figure 1. Detailed information about protection is not given because of proprietary reasons.

Knowing the synthetic pathway is necessary in order to take the specific steps required for substance characterization with a view to identifying the synthetic by-products to be expected. In this case, the following synthetic by-products are to be expected: Isonicotinamide (INA), pyridine-2-aldoxime (P2A), isonicotinamide-dimer (INA-dimer) and pyridine-2-aldoxime-dimer (P2A-dimer) (Figure 1).

With a view to its future use as an active pharmaceutical ingredient (API), four developmental lots of HI-6 DMS (dimethanesulfonate) and HI-6 dichloride respectively provided by different manufacturers were examined with respect to their content and purity. By comparing two developmental lots of the same manufacturer, which were synthesized with or without protective groups, the success of synthesis development could be verified analytically.

Even if the synthetic pathway uses protective groups, an unknown impurity appears. Unknown impurities in a new API are problematic from an ethical and legal point of view. For this purpose, internationally acknowledged threshold values have been established under pharmaceutical law.[11] With an area proportion of 0.11%, the content of this impurity lies above the value requiring identification. Therefore, investigations were carried out in order to elucidate the structure of this so far unknown impurity.

Experimental

Test substances

HI-6 DMS, 1-({[4′-(aminocarbonyl)pyridinium]methoxy}methyl)-2-((hydroxyimino)methyl)pyridinium dimethanesulfonate, CAS 144252-71-1, was provided by Phoenix Ltd (Bromborough, UK) in two different lots. A HI-6 DMS reference sample had been produced by Minakem SAS (Beuvry la Foret, France), and a HI-6 dichloride reference sample came from Pharmsynthez JSC (St Petersburg, Russia).

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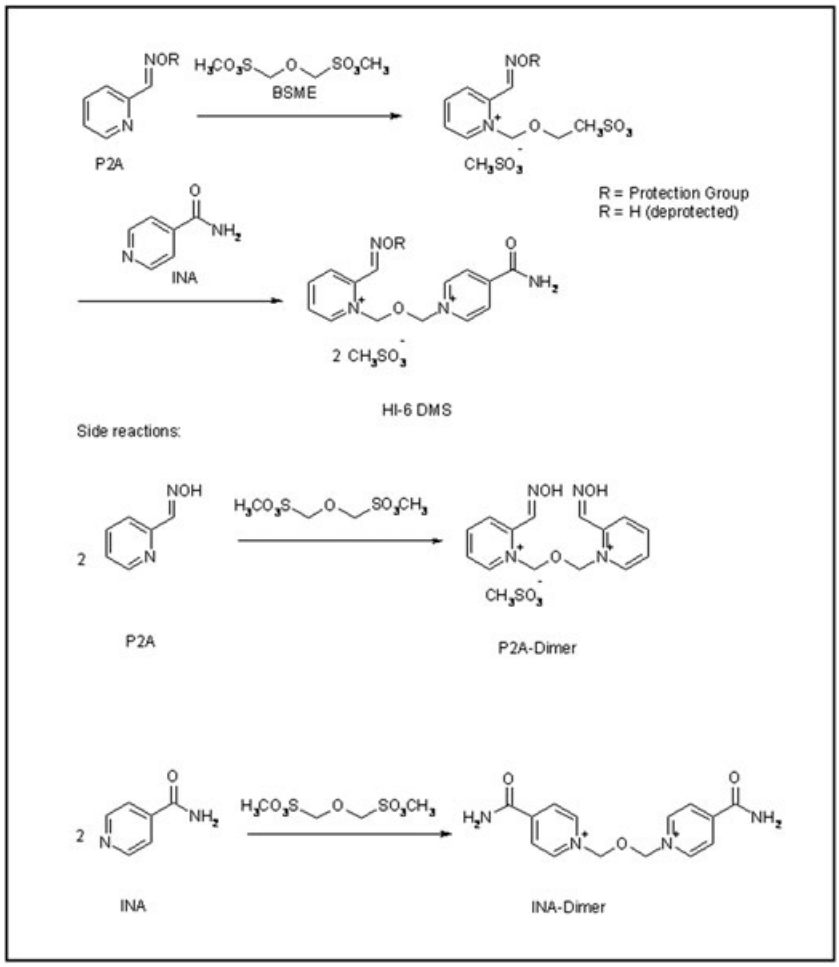

Figure 1. Synthetic pathway of HI-6 DMS using BSME.

HPLC – DAD examinations

HPLC – DAD was conducted on an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany). For reasons of confirmation two HPLC methods, suitable for further MS detection were developed and validated.

HPLC method with stationary phase Atlantis dC18

A column Atlantis dC18 (Waters, Eschborn, Germany), 150 x 4.6 mm I.D. (particle size 5 μm), was used, which is a reversed phase column enabling separation without ion pair reagent thus being suitable for MS-detection. The high performance liquid chromatography (HPLC) pump delivered a gradient of 100 mM ammonium formate in water (solvent A_1) and methanol with 100 mM ammonium formate (solvent B_1) with the following conditions: 0% B_1 at a flow of 1 ml/min, linear increase to 40% B_1 from 0 to 6 min, linear increase to 100% B_1 from 6 to 9 min and return to initial conditions from 12 to 14 min. Total run time was 20 min. The injection volume was 5 μl and the auto sampler was cooled to 8 °C. UV detection was performed by DAD at 254 nm. This method was used for quantification of HI-6 DMS.

HPLC with stationary phase HILIC Silica

A column Atlantis HILIC Silica (Waters, Eschborn, Germany), 150 x 4.6 mm I.D. (particle size 3 μm), was used. HILIC represents hydrophilic lipophilic interaction chromatography with orthogonal separation suitable for MS-detection. The HPLC pump delivered a gradient of 9:1 (*v/v*) acetonitrile 100 mM ammonium formate (solvent A_2) and 4.5:4.5:1 (*v/v*) ACN/ water/100 mM ammonium formate (solvent B_2) with the following conditions: 10% B_2 at a flow of 1 ml/min, linear increase to 100% B_2 from 4 to 16 min, and return to initial conditions from 16 to 19 min. Total run time was 35 min. The injection volume was 5 μl and the auto sampler was cooled to 8 °C. UV detection was performed by DAD at 254 nm. This method was used for the quantification of samples prepared for nuclear magnetic resonance (NMR) studies.

HPLC-MS examinations

For identification of the unknown impurity, an Agilent 1100 HPLC system was coupled to an Agilent XCT ultra ion trap mass spectrometer (Agilent Technologies, Waldbronn, Germany). The HPLC method was the same as described above using an Atlantis dC18 stationary phase. MS and 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).

NMR examinations

Preliminary tests had shown that under light irradiation, the percentage of the unknown impurity increased. In order to cause the unknown impurity to accumulate, the samples were put in aqueous solution (2 mg/ml) and placed in a climatic chamber equipped with an irradiation device (HCL 4057/S, Heraeus Vötsch, Reiskirchen-Lindenstruth, Germany; light source: SOL 2000 lamp with H1 filter, Dr Hönle UV-Technologie, Gräfelfing, Germany). There, they were exposed to artificial daylight wavelength 320 nm – 820 nm, dose rate10^5 lux, temperature of 25 °C, time up to 20 h. The samples thus produced were lyophilized. Upon quantification of the proportion of the unknown impurity by means of HPLC, the samples were examined by NMR. For the NMR study, approximately 2–3 mg HI-6 DMS were dissolved in 600 µL DMSO-d6; ^{1}H-NMR spectra, ^{13}C-Attached Proton Test (APT)-NMR spectra and nuclear Overhauser enhancement spectroscopy (NOESY)-NMR spectra were recorded with a Avance III 400 MHz Microbay Ultrashield with 5 mm broadband PABBO BB-1H/D probe head (Bruker Biospin, Rheinstetten, Germany) at a measuring temperature of 30 °C.

ATR-IR examinations

Attenuated total reflectance - infrared (ATR-IR) spectra of HI-6 DMS and/or HI-6 dichloride of the different manufacturers were recorded directly on an Excalibur FTS 3000 IR-spectrometer (Bio Rad, Munich, Germany) between 600 and 4000 [cm^{-1}].

Results

Validation parameters of the HPLC methods

The chromatographic parameters of the MS-compatible HPLC methods are summarized in Table 1. The still unknown impurity, which will be identified in the following, is already referred as an isomer at that point.

A mixture of HI-6 DMS (Phoenix) with the synthetic by-products to be expected, which is dissolved in solvent A (concentration 0.5 mg/ml each for column Atlantis dC 18, 0.05 mg/ml each for column HILIC Silica) resulted in the chromatograms represented in Figure 2A and 2B.

The isomer can be recognized clearly in Figure 3. It elutes directly before the main peak and exhibits almost a base line separation.

Comparison of manufacturers

Identity of HI-6

HI-6 DMS or HI-6 dichloride samples by three manufacturers (Phoenix, Minakem, and Pharmsynthez) were tested for HI-6 identity by means of infrared and mass spectrometry. For this purpose, the Phoenix product, which had been specified with respect to the HI-6 DMS identity by NMR, was used as a reference.

The ATR-IR spectra from HI-6 lots from Phoenix and Minakem are almost identical both in the wavenumber range of valence vibrations (4000–1600 cm^{-1}) and in the range of molecular vibrations (1600–1000 cm^{-1}, fingerprint). The Pharmsynthez sample shows a very similar spectrum in the valence vibration range, whereas the spectrum in the fingerprint range deviates (Figure 4A–4C).

This is in line with the fact that the substance concerned is HI-6 dichloride instead of HI-6 DMS. However, all three

Table 1. Chromatographic parameters of the MS-compatible HPLC methods with detection by DAD 254 nm.

a) Method with stationary phase Atlantis dC18

	INA- Dimer	HI-6 DMS Isomer	HI-6 DMS	P2A-Dimer	INA	P2A
t_R [min]	2.35	3.40	3.74	4.84	7.34	10.75
Resolution [1)]	-	3.67	1.29	6.48	15.82	26.01
Selectivity [2)]	-	1.31	1.10	1.30	1.52	1.47
LOD [3)] [µg/mL]	0.7	n.c.	2.5	1.9	0.5	0.7
LOQ [4)] [µg/mL]	2.5	n.c.	8.3	6.3	1.5	2.2

b) Method with stationary phase HILIC Silica

	P2A	INA	P2A-Dimer	HI-6 DMS	HI-6 DMS Isomer	INA-Dimer
t_R [min]	3.88	5.82	12.72	13.91	14.24	15.19
Resolution [1)]	-	7.28	25.26	7.65	2.38	1.07
Selectivity [2)]	-	1.50	2.19	1.09	1.02	7.43
LOD[3)] [µg/mL]	0.7	1.0	1.3	0.9	n.c.	1.5
LOQ[4)] [µg/mL]	2.3	3.2	4.4	3.2	n.c.	5.0

[1)] Reported in relation to preceding peak. Calculation according Pharmacopoea Europea Ph.Eur. 6.4/2.02.46.00.[12]

[2)] Defined as quotient of capacity factors of vicinal peaks.

capacity factor $k = \frac{t_R - t_o}{t_o}$

t_R absolute retention time

t_0 hold-up time.

[3)] Limit of detection based on a signal/ noise ratio of 3/1 according ICH Q2(R1).[13]

[4)] Limit of quantification based on a signal/ noise ratio of 10/1according ICH Q2(R1).[13]

n.c. not calculated as no defined weight available.

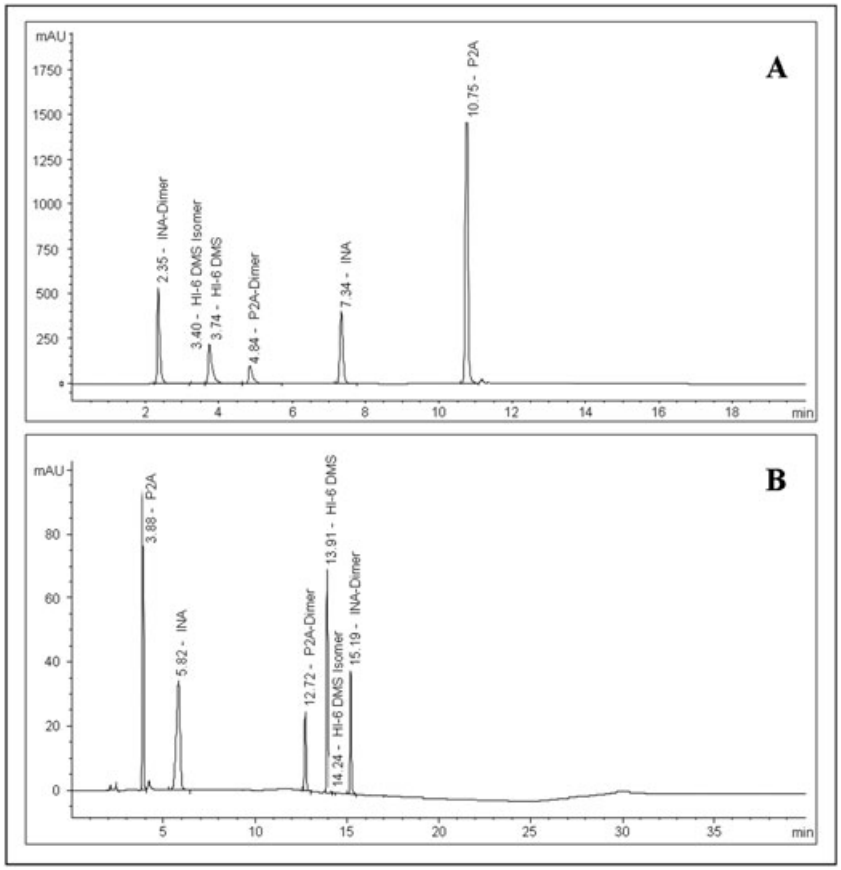

Figure 2. HPLC chromatogram of HI-6 DMS and synthetic by-products; column Atlantis dC18 150 x 4.6 mm I.D. using ammonium formate water – methanol gradient (A), and column HILIC Silica 150 x 4.6 mm ID using ammonium formate water – acetonitrile gradient (B); flow rate 1 ml/min, UV detection at 254 nm.

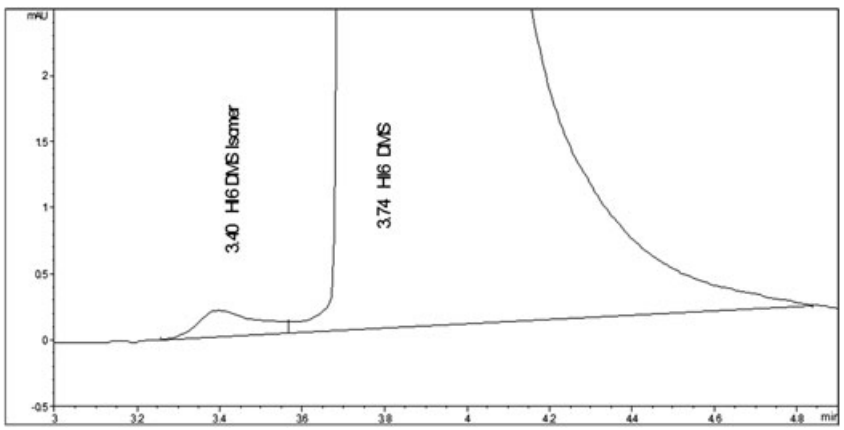

Figure 3. Chromatographic separation of the HI-6 isomer on column Atlantis dC18 150 x 4.6 mm I.D. using ammonium formate water – methanol gradient, flow rate 1 ml/min, UV detection at 254 nm.

compounds uniformly exhibit characteristic bands at 3400 cm^{-1} (OH stretching vibration), 2900 cm^{-1} (CH stretching vibration), 1700 cm^{-1} (CO stretching vibration), 1650 cm^{-1} and 1600 cm^{-1} (NH and CN stretching vibration), 1100 cm^{-1} (NO stretching vibration) as well as in the range from 1600 cm^{-1} to 1450 cm^{-1} (molecular vibrations of aromatic rings). With respect to the reference sample used, the ATR-IR spectra confirm the identity of HI-6.

Also the MS and MS/MS spectra of all three HI-6 samples are identical.

Conformity with the certified reference Phoenix sample was verified and proven by means of ATR-IR and MS/ MS/MS. Thus, the identity of the product HI-6 is correct for all three manufacturers.

Purity of HI-6

The three substances were examined for their content and for by-products by means of HPLC. A comparative summary of the quantitative composition is provided in Table 2.

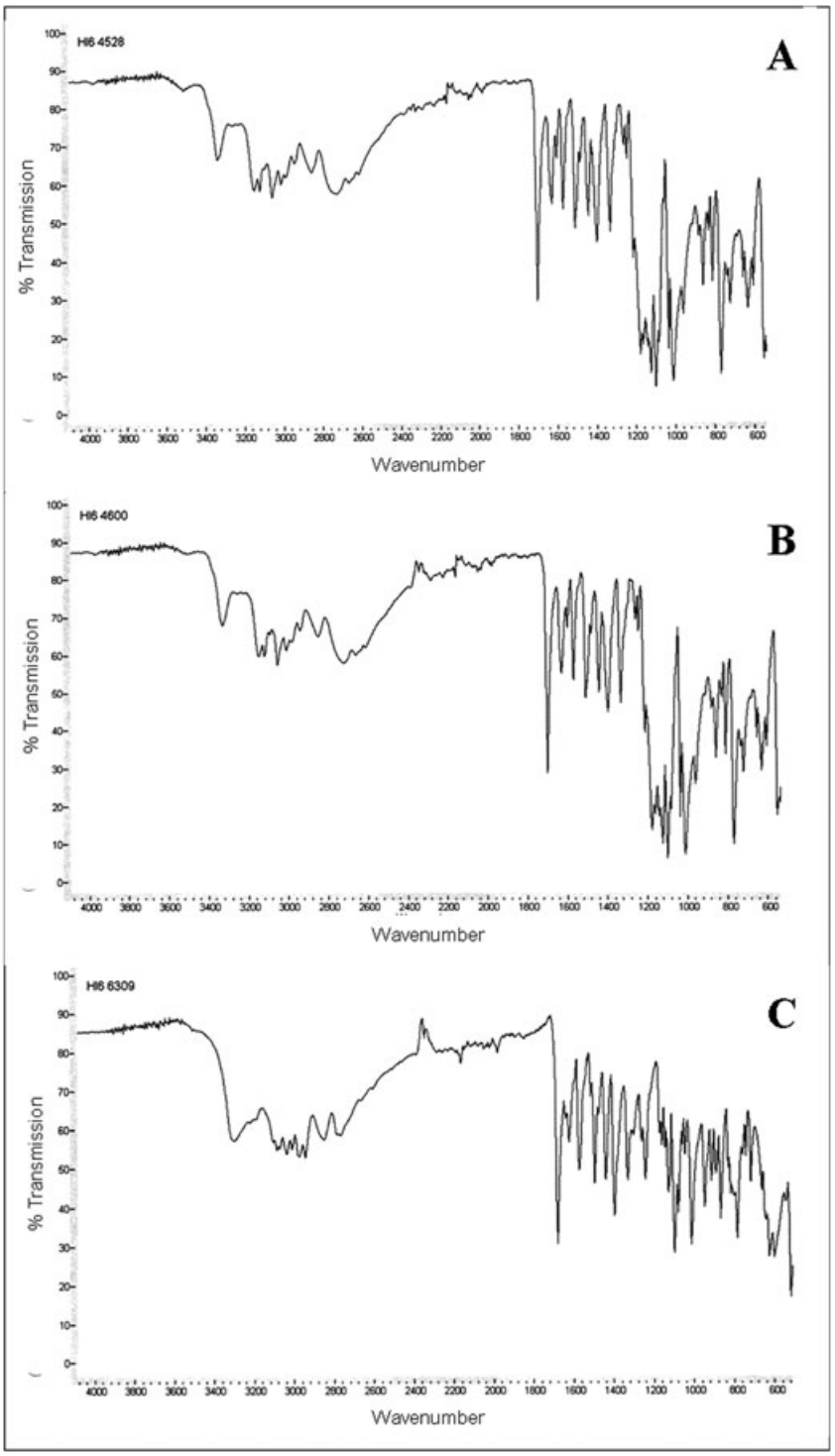

Figure 4. ATR-IR spectra of HI-6 lots from Phoenix (A), Minakem (B), and Pharmsynthez (C) directly recorded on an Excalibur FTS 3000 IR-spectrometer between 600 and 4000 [cm^{-1}].

With values of 97.8% (Minakem), 99.3% (Phoenix Lot II) and 99.7% (Pharmsynthez), there are distinct differences in the HI-6 content by the three manufacturers. With respect to purity, gradual differences occur with the known synthetic by-products as well as with unknown impurities not to be reported according regulatory provision.[14] It is remarkable that P2A and P2A dimer is lacking in the Pharmsynthez product.

Analytics accompanying the synthesis of the API during the development phase

Synthesis development by Phoenix was accompanied analytically. The result was the quantitative composition of two different developmental lots given in Table 2. Lot I was synthesized without protective groups whereas for the synthesis of lot II,

Table 2. Quantitative composition of HI-6 DMS/HI-6 dichloride for three manufacturers.

	Phoenix (HI-6 DMS)		Minakem (HI-6 DMS) [%]	Pharmsynthez (HI-6 dichloride) [%]
	Lot I synthesis without protective group [%]	Lot II synthesis with protective group [%]		
INA-Dimer	0.06	0.04	0.03	0.04
HI-6 isomer	0.21	0.11	1.89	0.16
HI-6	98.32	99.26	97.80	99.74
P2A-Dimer	0.49	0.54	0.06	0.00
INA	0.75	0.02	0.10	0.06
P2A	0.17	0.03	0.12	0.00

Areas [%] are given in percent of all reported peaks which is a normalized content according Ph.Eur. 6.0/2.02.46.00.[12]

protective groups were used. The new synthetic pathway with protective groups resulted in a considerable reduction of the synthetic by-products INA, P2A and INA-dimer. INA was reduced in the final product by 97%, P2A by 83% and INA-dimer by 33 % compared to the former values. P2A-dimer, on the other hand, is present in almost the same concentration. The proportion of the hitherto unknown impurity (HI-6 isomer) was reduced by 48%. Its value amounts to 0.11%, which is still within a range relevant to pharmaceutical law.

Identification of the unknown impurity (HI-6 isomer)

With the MS-compatible HPLC method (Column Atlantis dC18) developed for this purpose, HI-6 DMS and the so far unknown

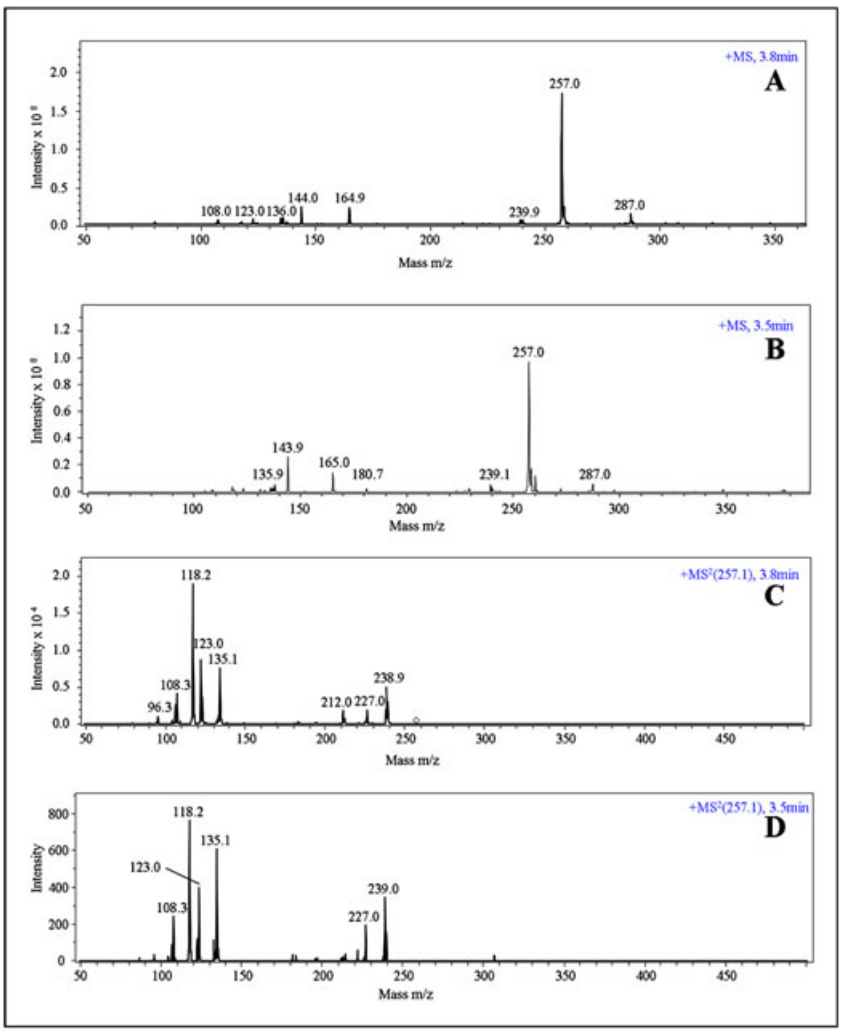

Figure 5. MS and MS/MS spectra of HI-6 DMS and the unknown impurity. (A) and (B) demonstrate the MS spectra of HI-6 DMS at $t_R = 3.8$ min and the unknown impurity at $t_R = 3.5$ min. (C) and (D) show the corresponding MS/MS spectra of HI-6 DMS at $t_R = 3.8$ min and the unknown impurity at $t_R = 3.5$ min. The spectra were recorded with an Agilent XCT ultra ion trap mass spectrometer in ESI-MS mode (positive mode) from *m/z* 200–2200, scan speed of 26 000 *m/z* per second in ultra scan mode; MS/MS experiments with He as collision gas and auto MS/MS acquisition cycle respectively MRM; separation of the compounds on column Atlantis dC18 150 x 4.6 mm ID using ammonium formate water – methanol gradient (flow rate 1 ml/min).

impurity were separated and characterized mass spectrometrically. Figure 5A–5D show the corresponding MS and MS/MS spectra of HI-6 at t_R 3.8 min and the unknown impurity at t_R 3.5 min.

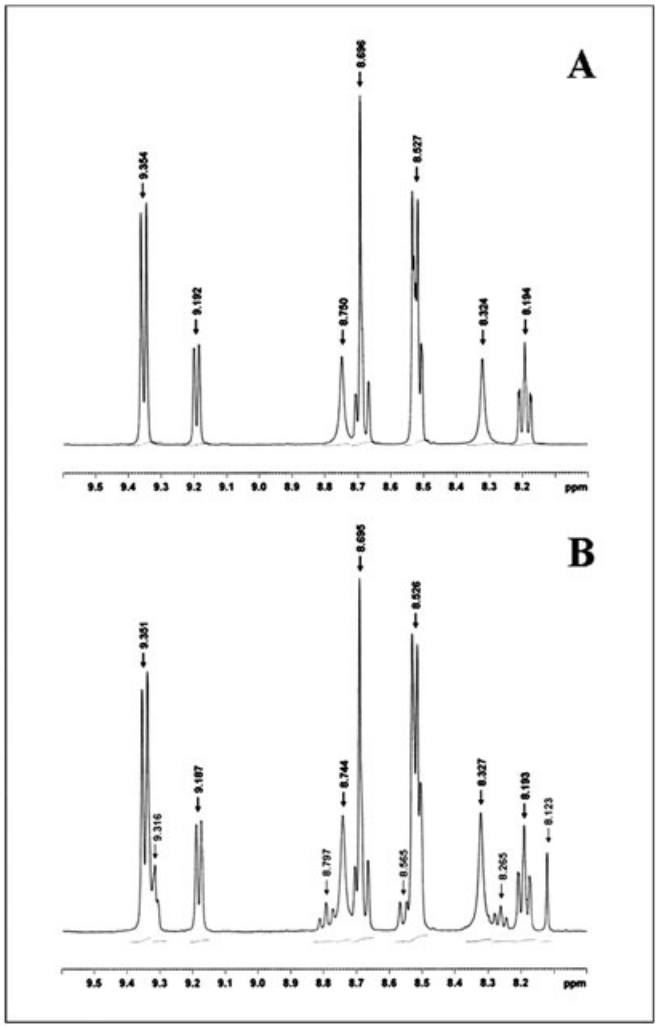

Figure 6. Extracted ^{1}H-NMR spectra between 8 and 9.5 ppm of HI-6 DMS from Phoenix (A) and of HI-6 DMS enriched with approximately 25% of the unknown compound (B). The essential signals of the unknown compound are highlighted in grey (B). The spectra were recorded with a Bruker Biospin 400 MHz microbay including BBO probe, in DMSO, at measuring temperature of 30 °C.

The MS spectrum of HI-6 is in good agreement with the data from D'Agostino *et al.*[15] The molecular peak of HI-6 was observed as $[HI\text{-}6\text{-}H^+]^+$-ion at m/z 287.0 and significant product ions were found at *m/z* 257.0, 164.9, 144.0 and 136.0. In contrast to D'Agostino *et al.*, we obtained the base peak at *m/z* 257.1, which results from loss of formaldehyde ($M^+\text{-}CH_2O$).[15] This phenomenon is the result of the chosen MS parameters. Figure 5A and 5B demonstrate the MS spectra of HI-6 DMS at 3.8 min and the unknown compound at 3.5 min after HPLC separation. Both spectra show the base peak at *m/z* 257.0 ($[HI\text{-}6\text{-}H^+\text{-}CH_2O]^+$), identical product ions and a mass peak at *m/z* 287.0, which is significant for the $[HI\text{-}6\text{-}H^+]^+$-ion of HI-6. Beyond that, Figures 5C and 5D show the MS/MS spectra of the base peak at *m/z* 257.1 which are in agreement with the known MS data of HI-6.[15] Product ions at *m/z* 238.9 (loss of H_2O from *m/z* 257.1), 227.0 (loss of neutral CH_2O from *m/z* 257.1), 135.1, 123.0, 118.2 (loss of H_20 from *m/z* 136 $C_7H_8N_2O$) and 108.3 with similar intensities were recorded in the MS/MS spectra of HI-6 and the unknown compound. Both spectra provide identical fragmentation patterns with more than two identical mass transfers for the base peak of HI-6 and the unknown impurity. This proves that the unknown impurity should be a constitutional isomeric compound. In this context, possible E-Z isomerism at the aldoxime group of HI-6, whose rotation is restricted, must be considered.[16,17]

For further investigation of the possible isomeric form, ^{1}H- and ^{13}C-NMR spectra were recorded in dimethyl sulfoxide (DMSO) of pure HI-6 from Phoenix and a batch with the unknown impurity enriched by irradiation. For the ^{13}C-NMR studies the APT experiment was used to separate carbons unattached to protons from -CH and -CH_2 groups. The APT experiment is a simple multiplet decoupled ^{13}C-NMR experiment, which allows the separation of quaternary carbons, CH, CH_2, and CH_3 carbons. In an APT spectrum, CH and CH_3 carbons give a negative signal, whereas the CH_2 and quaternary carbons give positive signals.

In the ^{1}H-NMR spectrum of the irradiated batch, containing about 25% of the unknown substance, an additional aldoxime band whose area proportion amounts in accordance to our preliminary HPLC quantification to 20–25 % at 13.08 ppm and additional signals between 6 and 9.5 ppm occur. The very low field resonance of the aldoxime proton at 13.28 ppm and 13.08 ppm indicates a hydrogen bond to the pyridinium ring system in both compounds. For the determination of the structure of the unknown compound, the additional doublet at 9.32 ppm (1H, H-6) as well as the singlets at 6.24 ppm (2 H, CH_2-8) and 8.12 ppm (1 H, H-7) provide important information for structure elucidation. The concerning peaks of the

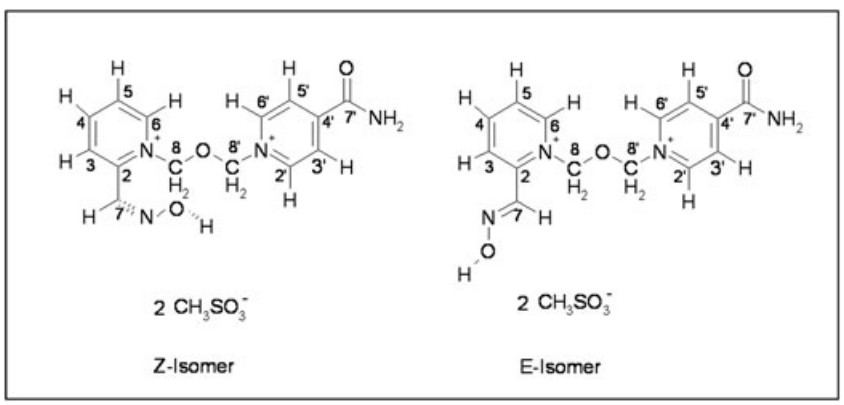

Figure 7. Isomeric forms of HI-6 DMS.

unknown show a significant shift to the higher field: 13.28 to 13.08 ppm (aldoxime proton), 8.70 to 8.12 ppm (olefinic proton), and 6.35 to 6.24 ppm (methylene protons neighboured to the aldoxime group). Within the aromatic ring system the protons of the aldoxime bearing group show a down-field shift while the protons of the amide bearing ring are not affected at all by this isomerization (Figures 6A and 6B).

Finally NOESY spectra were recorded of both variants of HI-6. The irradiated form instead shows the same couplings as Phoenix HI-6 but an additional coupling between the new up-field shifted methylene protons and the new up-field shifted olefinic proton and demonstrates the steric modification at the aldoxime group. In contrast to the ^{1}H-NMR spectrum of the HI-6 reference standard from Phoenix with equal interactions and cross signals at 8.70 ppm (s, 1H) and 6.35 ppm (s, 2H), we observed a shift to higher field of the olefinic proton H-7 ($\Delta = 0.58$ ppm) and $C\underline{H_2}$-8 ($\Delta = 0.11$ ppm). The additional bands developing due to the accumulation of the isomer are also clearly discernible in the ^{13}C-APT-NMR spectrum. In the ^{13}C-NMR spectrum, additional CH bands and a supplementary $-CH_2$ signal at 85.03 manifest themselves, which reflect the steric proximity to the changed aldoxime group. Thus, the NMR spectra described, prove that the so far unknown impurity is an E/Z-isomer at the aldoxime group with restricted rotation in HI-6 DMS. These data are in good agreement with the findings of Silva *et al*. who reported about three energy minima conformers for the E configuration and one for the Z configuration of HI-6.[18] Figure 7 depicts the isomeric forms of HI-6 DMS identified this way.

Discussion

Two MS-compatible HPLC methods have been developed which have made it possible to separate HI-6 from its isomer. As expected, HILIC exhibited a clearly changed elution behavior.[19] Atlantis dC 18 and HILIC Silica revealed comparable LOD and LOQ values. Although for a pure routine analysis of oximes, HILIC also seems suitable, but it does not have any advantages compared with a conventional RP18-phase. This is in accordance with literature describing RP-phase in this case using ion-pairing reagent as suitable for analysis of HI-6 and by-products.[20] The resolution between HI-6 and HI-6 isomer is much better in the case of HILIC silica, where it amounts to 2.38 as opposed to 1.29 for Atlantis dC18. For investigations on quantitative changes of the isomer, stationary phase HILIC silica should thus be preferred.

The identity of HI-6 was verified by means of ESI mass spectrometry. Due to the thermolability and low volatility of oximes, this mild ionization method is particularly suited for this substance class. The spectra obtained correspond to the spectra reported for HI-6 in literature if this method is applied.[15]

Available samples of three manufacturers (Phoenix, UK: HI-6 DMS; Minakem, FRA: HI-6 DMS; Pharmsynthez, RUS: HI-6 dichloride) were subjected to a comparative study which revealed distinct differences in the HI-6 content. This data may thus support finding a suitable manufacturer for this substance. The synthesis of HI-6 DMS can be carried out by two different methods. A method that has been known and used for a relatively long time is to employ the highly reactive, electrophilic but as a result also highly carcinogenic BCME as a coupling reagent. Thus, the resulting dichloride is converted into better water soluble dimethanesulfonate[21] via ion exchange.[20,22] An alternative method uses the less reactive BSME and in this way produces HI-6 DMS directly, but with smaller yields.[8,9] Phoenix and Minakem use this method; the synthetic pathway of Pharmsynthez is not known. If the latter use the synthetic pathway using BCME, this would correspond to the 99.7% and thus maximum yield in the final product, which would also be in line with the fact that the product present in this case is the dichloride.

A suitable analysis for an API is always based on knowing the synthetic pathway. This is laid down in documents pertaining to regulatory affairs (e.g. Certificate of Suitability CEP, Drug Master File DMF). The synthetic pathway for HI-6 DMS by Phoenix is known. It suggests a defined spectrum of by-products. With a MS-compatible HPLC method specific to this spectrum of by-products, synthesis development of the potential API was accompanied analytically. This has made it possible to confirm analytically the success of this synthesis described according to Eddols *et al*.[10] It becomes evident that in the last developmental lot from Phoenix, which was produced according to a modified synthetic pathway introducing protective groups, the synthetic by-products INA, P2A and INA-dimer decrease considerably while P2A-dimer remains almost constant. Based on a maximum daily dose (MDD) < 2 g, INA, P2A and INA-dimer, whose percentages amount to 0.02%, 0.03% and 0.04%, are now below the reporting threshold.[11] With respect to API licensing, these by-products do not necessitate further investigations. The analytical results presented here thus support marketing authorization of HI-6 DMS as an API.

In the products of all manufacturers, including the current Phoenix product, an unknown impurity appears, which almost coelutes with HI-6 DMS. Unknown side products in new API are problematic from an ethical and legal point of view. For this purpose, internationally acknowledged threshold values have been established.[11] With an area proportion of 0.11%, the impurity occurring here is above the threshold value and thus must be identified for regulatory reasons. Due to structural and chromatographic analogies with other oximes, possible E/Z-isomerism at the aldoxime group with restricted rotation has been considered.[16,17] Literature discusses the occurrence of an isomeric impurity in HI-6.[22] In this context, the paper quoted interprets an additional spot appearing in HI-6 during thin-layer chromatography as an isomer. It does not report about more extensive studies. With the results presented here, a proof of the structure of this so far unknown impurity always occurring in HI-6 has successfully been furnished. The MS and NMR findings prove that the unknown impurity is an E/Z-isomer of HI-6 DMS and confirm the findings of Silva *et al*.[18] Isomers are excluded in the ICH Q3A guideline, which focuses on toxicological aspects. Structure elucidation of this impurity thus represents an essential contribution to the pharmaceutical characterization of this potential API, and a further requirement for its marketing authorization as an antidote has been met with this work.

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