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Isolation and identification of impurities in chlorin e6

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Abstract

The tetrapyrrolic compound chlorin e6 is currently used as a pharmaceutical substance for Photolon formulation, which is utilized in photodynamic therapy of various diseases. It was found that chlorin e6 could contain both process- and degradation-related impurities. In order to understand their origin, the most abundant impurities were prepared by liquid extraction, preparative chromatography or chemical synthesis. By means of HPLC-PDA–MS, 1D and 2D NMR spectroscopy, these impurities were identified as chlorin e6 17^4-ethyl ester, chlorin e4, 15-hydroxyphyllochlorin, rhodochlorin, 15^1-hydroxymethylrhodochlorin δ-lactone, rhodochlorin-15-oxymethyl δ-lactone, rhodochlorin-15-oxymethyl δ-lactone 17^4-ethyl ester, 15^1-hydroxymethylrhodoporphyrin δ-lactone, rhodoporphyrin-15-oxymethyl δ-lactone and purpurin 18. The possible routes of formation of the chlorin derivatives upon the production and storage of chlorin e6 are discussed.

Keywords: Chlorin e6; Photolon; Impurities identification; Chlorin derivatives

1. Introduction

Presently chlorin e6 and related structures are of special pharmacological interest thanks to their high photosensitizing efficacy. This feature makes chlorins attractive molecules for photodynamic therapy (PDT) of pathological tissues—treatment modality that includes combined action of an "exited-to-a-triplet-state" photosensitizer and molecular oxygen dissolved in tissues [1]. The main advantages of chlorin e6 in PDT are the low dark toxicity, fast and sufficiently selective accumulation in target tissue, and a well-balanced combination of diagnostic properties and tumoricidal ability [2]. The use of chlorin e6 bound to a polyvinylpyrrolidone carrier allowed us to develop the new promising photosensitizer (PS) Photolon [3,4]. The formulation is efficient both in photodynamic diagnostics and in therapy of oncological lesions of skin, bladder, as well as in PDT of age-related macular degeneration and microbial infections [5–10].

Several synthetic methods of chlorin e6 preparation from available precursors (chlorophyll "a", pheophytin "a", pheophorbide "a") have been proposed to produce the compound in various yields and of different purity degrees [11–13]. Also, the studies associated with different degradation approaches and synthetic modifications of chlorin e6 have been performed in order to obtain certain porphyrin and chlorin derivatives [11,12,14–16]. In particular, boiling of chlorin e6 in pyridine is known to result in its decarboxylation, yielding chlorin e4. When degraded by oxidizing agents, chlorin e6 can be transformed to tetrapyrroles such as purpurin 5, rodochlorin-15-oxymethyl lactone and other similar compounds [11,17,18]. Moreover, chlorin e6 readily decomposes when exposed to light with formation of the compounds, whose structure has not been elucidated so far [19].

With ever increasing regulatory concerns on the quality and safety of pharmaceuticals, a systematic investigation of impurities and degradation products in chlorin e6 as an active pharmaceutical ingredient is of paramount importance. Such studies may help to understand how the source of raw materials, manufacturing process and storage conditions can affect the impurity profile of chlorin e6, which would eventually lead to a development of pharmaceutical forms of chlorin e6 with a long shelf life which would contain less process-related impurities and degradation products.

This work describes separation and identification of impurities and degradation products in chlorin e6 using high-performance liquid chromatography/photodiode array UV detector (HPLC-PDA), HPLC–MS and NMR methods. Special attention has been paid to the development of procedure for iso-

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lation and/or synthesis of related substances which can be used as external standards in routine analysis.

2. Experimental

2.1. Samples and reagents

Chlorin e6 (**1**) samples were produced by the modified method of Loetjoenen et al. [13] using chlorophyll "a" extracted from *Spirulina platensis* at RUE Belmedpreparaty, Minsk, Belarus. Trifluoroacetic acid (TFA), sodium hydroxide, and hydrochloric acid of analytical purity were purchased from Sigma–Aldrich. Acetonitrile for HPLC, deuterated pyridine (99.8%), methanol, chloroform and other solvents were obtained from Merck, Darmstadt, Germany.

2.2. Synthesis of the impurities

Impurity **2** was synthesised by partial esterification of chlorin e6. Chlorin e6 was stirred in 4% sulfuric acid/ethanol medium (2 h) with subsequent fractionalization by preparative HPLC of the resultant mixture of chlorin e6, mono (17^4) and diethyl (15^3, 17^4) esters. Due to different reactivity of the carboxyl groups of chlorin e6, the formation of other possible (mono substituted) isomeric esters was negligible, allowing one to isolate pure (according to ^{1}H NMR) impurity **2**.

In order to prepare impurity **8**, impurity **2** was degraded under the action of air at 30 °C (storage for 14 days in a dark place) with subsequent purification by means of preparative chromatography.

Impurity **10** was synthesised by dehydrogenation of the impurity **7** by dichlorodicyanobenzoquinone in chloroform as described for other chlorins [20] and purified by preparative chromatography. To avoid possible side reactions, impurity **7** was initially methylated by 5% (v/v) sulfuric acid in methanol.

2.3. Equipment

HPLC analysis was performed on a Waters chromatography system consisting of a Waters 600E HPLC pump, a gradient controller, a type 996 photodiode array detector (PDA) (all from Waters, Milford, MA). HPLC separations were monitored at 407 nm (analytical HPLC) and 515 nm (preparative separation). The following chromatography columns were used: an analytical 150 mm × 4.6 mm column and a semipreparative 150 mm × 10 mm column packed with XTerra RP-18 (Waters, Milford, MA) with 3.5 and 5 μm particles, respectively. The mobile phase flow rate was 1 and 4.5 ml/min for analytical and preparative separations, respectively. During the analytical run, the gradient elution was carried out using mobile phases A (0.1% TFA in water) and B (acetonitrile). Chlorin e6 impurities were separated under the conditions determined earlier using a 20-min gradient of B from 45 to 100% [21]. During the preparative run, mobile phase B contained also 0.1% TFA. To achieve efficient elution of porphyrin-like impurities, phase B was additionally modified with dimethylformamide (DMF) (up to 30% of DMF to isolate impurity **9** and up to 50% of DMF to recover impurity **10**). Samples were injected in dimethylsulfoxide (chlorin type structures) or dimethylformamide (porphyrin type) in 10 μl and 100–500 μl aliquotes for analytical and preparative separations, respectively.

Mass spectra (*m/z* 400–800) were obtained by online HPLC–MS using a Waters ZQ2000 mass detector (Waters, Milford, MA) working with ESI interface in positive ionization mode. The MS detector parameters were varied to obtain preferentially either molecular ion or fragmentation spectra (capillary voltage 2–4 kV, cone voltage 30–100 V). The spectra of the impurities in the UV–vis region (300–700 nm) were recorded in the mobile phase using Waters PDA detector.

NMR experiments were performed using Bruker Avance 500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C). All spectra were recorded in deuterated pyridine with reference to the solvent signal (71,808 ppm (meta-proton) for ^{1}H and 135.9 ppm (C_4) for ^{13}C) [22]. Two-dimensional [^{1}H–^{1}H] COSY, [^{1}H–^{1}H] NOESY (mixing time 500 ms), [^{1}H–^{13}C] HMBC (J = 8.0 Hz) and [^{1}H–^{13}C] HSQC spectra were recorded using standard impulse sequences provided by Bruker.

3. Results and discussion

A typical HPLC chromatogram of a batch of chlorin e6 (**1**) is shown in Fig. 1. Characteristics of chlorin e6 and main impurities obtained using HPLC–MS and HPLC-PDA are shown in Table 1. One of the chlorin e6 impurities was identified earlier during development of the HPLC assay method as monoethyl ester of chlorin e6 (impurity **2**) [21]. In this work, by means of HPLC we isolated and characterised other nine most significant related substances in chlorin e6 API. The structures of the identified impurities, as well as their trivial names are shown in Table 2. In order to give unambiguous identification of isolated and/or separated molecules, proton-magnetic resonance spectra were recorded together, if considered necessary, with two-dimensional [^{1}H–^{1}H] and [^{1}H–^{13}C] correlation spectra (Tables 3 and 4). Description of the preparation of the impurities and the main steps of structural elucidation are presented below.

3.1. Isolation and purification of impurities

From HPLC-PDA analysis of chlorin e6 API, the main peak of chlorin e6 (~7.5 min) and the peaks of several more hydrophobic impurities were detected in the chromatogram (Fig. 1). Because of low content of impurities in the initial sample, an induced degradation process was used to increase their content. The most suitable sample for the isolation of impurities appeared to be the one oxidized by the action of air (ageing for 1 year in the dark at room temperature) shown in the chromatogram in Fig. 2 (referred to thereafter as "degraded chlorin"). The attempts to prepare degraded chlorin within a shorter term proved unsuccessful, since acceleration of the degradation process under the action of different reagents lead to a lower selectivity of the reaction, which entailed generation of very complex mixtures of tetrapyrrols with lower content of the com-

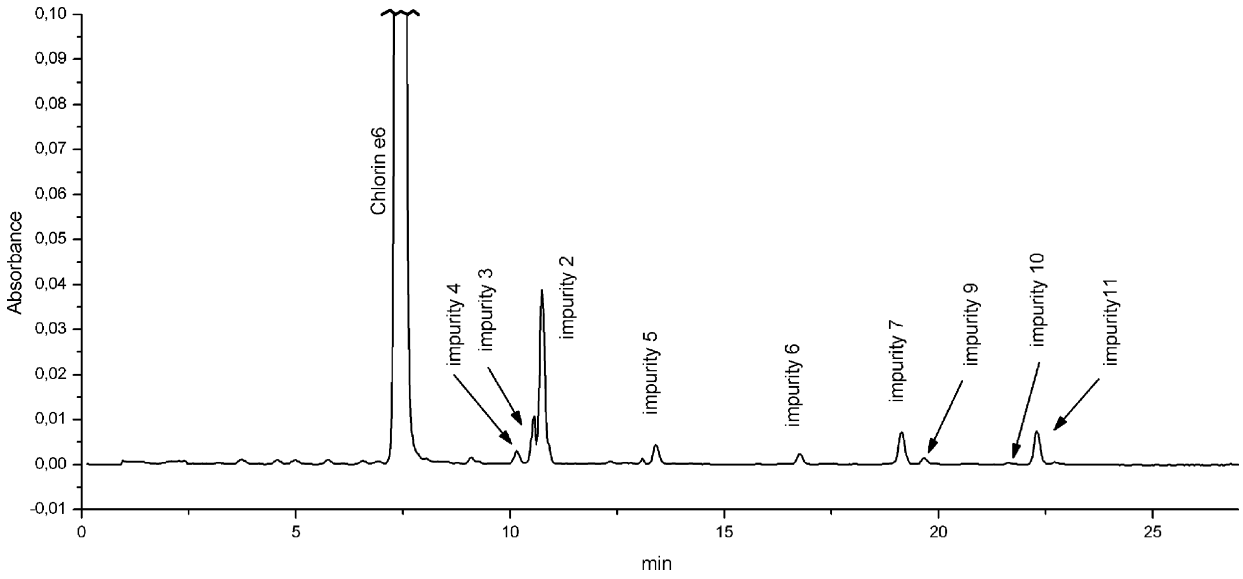


Fig. 1. Chromatogram of impurities in chlorin e6 pharmaceutical substance.

pounds of interest or predominant formation of relatively pure but undesired chlorins.

Using HPLC fractionation of the degraded chlorin, impurities **4**–**7** were obtained. Individual impurities **6** and **7** were isolated by preparative chromatography using linear gradient elution with 0.1% TFA/acetonitrile mixtures. To facilitate separation of **4** and **5**, these impurities were enriched using hydrochloric acid/ether fractionation [17]. At first, polar porphyrins were washed out from degraded chlorin solution in ether using 3% HCl, followed by recovery of the fraction containing **4**, **5** and **9** as the major compounds in 8% HCl. Further, pure **4** and **5** were prepared by means of preparative HPLC.

Under the conditions developed initially for preparative HPLC, impurity **9** eluted as a wide and asymmetric peak, leading to a low recovery and significant contamination by other compounds. However, double re-crystallization of the degraded chlorin from tetrahydrofuran and chloroform enabled us to obtain a mixture that contained **9** as a major compound along with minor amounts of some chlorins and porphyrins. Furthermore, the HPLC conditions were optimized to facilitate the elution of impurity **9**. This was achieved using a mobile phase B (0.1% in acetonitrile) containing 30% (v/v) of DMF.

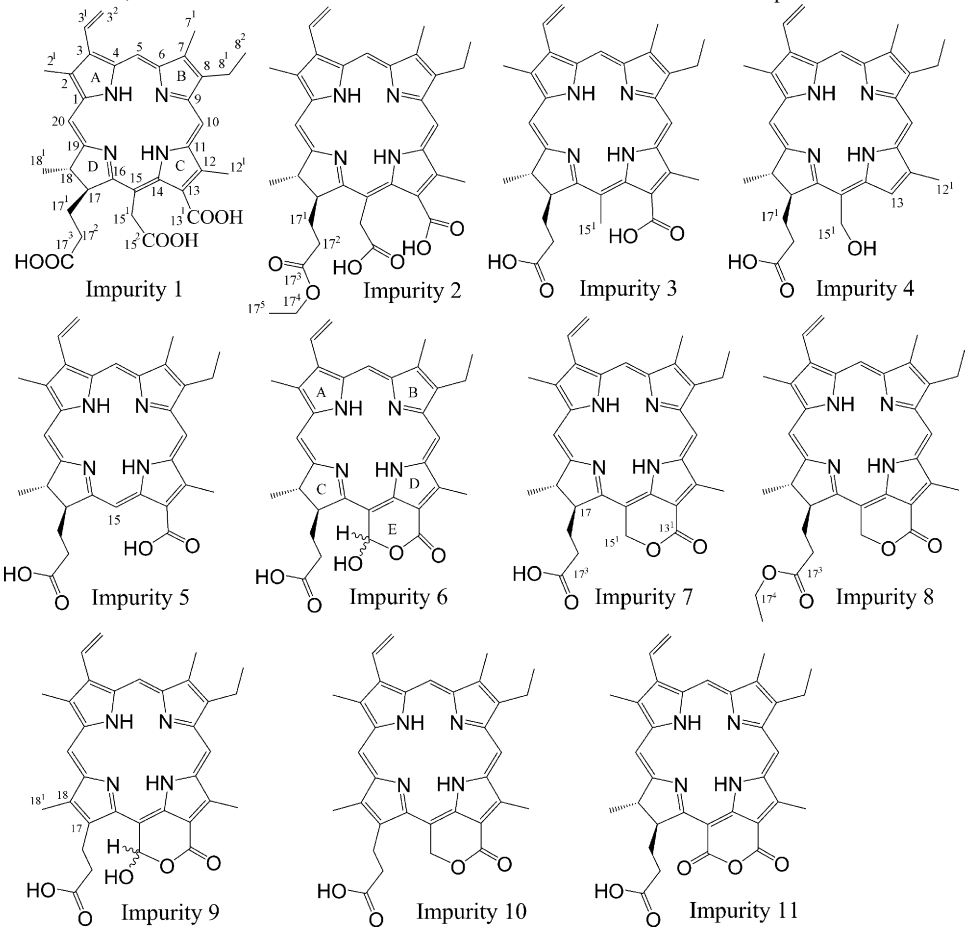
Impurities **2**, **3**, **8**, **10** and **11** were hard to isolate from the degraded chlorin because of their low content and/or insufficient resolution under the HPLC conditions used. However, online HPLC-PDA–MS investigation allowed us to establish the most probable structures and synthesise them. To prepare impurities **3** and **11** in the amounts sufficient for NMR investigation, data available from the literature were used [11]. The synthesis of impurities **2**, **8** and **10** was carried out using common reactions of

Table 1
UV–vis and mass-spectral characteristics of chlorin e6 and related impurities

Compound	Vis (Q_1, Q_2, Q_3, Q_4, Q_5, Soret bands)	MS (ESI, +ve)
Chlorin e6	643, 592, –, 528, –, 406	597.7 (100, MH$^+$), 553.8 (40, –CO_2), 538.7 (38, –CH_2CO_2H), 509.8 (5, –2CO_2), 494.8 (5, –CH_2CO_2H, –CO_2), 465.7 (5, –3CO_2)
Impurity **2**	644, 592, –, 528, –, 408	625.8 (100, MH$^+$), 581.8 (29, –CO_2), 566.7 (23, –CH_2CO_2H)
Impurity **3**	644, 591, –, 532, –, 409	553.7 (100, MH$^+$)
Impurity **4**	634, 582, –, 526, –, 402	525.8 (100, MH$^+$), 507.8 (30, –H_2O), 495.8 (10, –CH_2OH), 435.8 (10, –CH_2OH, –CH_3, –CO_2)
Impurity **5**	641, 591, –, 523, –, 403	539.8 (100, MH$^+$), 495.9 (5, –CO_2)
Impurity **6**	665, 608, (558)[a], 530, 500, 405	567.8 (100, MH$^+$), 549.7 (42, –H_2O), 539.8 (9, –CH_2O), 523.8 (10, –CO_2)
Impurity **7**	661, 604, (558), 530, 500, 404	551.8 (100, MH$^+$), 507.8 (5, –CO_2)
Impurity 8	661, 604, (560), 530, 500, 404	579.8 (100, MH$^+$), 551.8 (23, –C_2H_4), 535.8 (10, –CO_2)
Impurity **9**	(631), 580, 561, 520, –, 409	565.7 (100, MH$^+$), 547.8 (18, –H_2O), 537.8 (21, –CH_2O), 521.8 (21, –CO_2)
Impurity **10**	(630), 577, 554, 515, –, 408	549.7 (100, MH$^+$), 505.3 (7, –CO_2)
Impurity **11**	700, 644, 548, 509, –, 407	565.7 (100, MH$^+$), 521.6 (11, –CO_2), 503.6 (15)

[a] Bands of low intensity are shown in parentheses.

Table 2
Nomenclature, HPLC retention times and molecular masses of chlorin e6 and relative compounds



No.	Trivial name	RT (HPLC)	MW
Compound 1	Chlorin e6	7.5	596.7
Impurity **2**	Chlorin e6 17^4-ethyl ester	10.7	624.7
Impurity **3**	Chlorin e4	10.5	552.7
Impurity **4**	15^1-Hydroxyphyllochlorin	10.0	524.7
Impurity **5**	Rhodochlorin	13.4	538.6
Impurity **6**	15^1-Hydroxymethylrhodochlorin δ-lactone	16.9	566.6
Impurity **7**	Rhodochlorin-15-oxymethyl δ-lactone	19.2	550.6
Impurity **8**	Rhodochlorin-15-oxymethyl δ-lactone 17^4-ethyl ester	22.1	578.7
Impurity **9**	15^1-Hydroxymethylrhodoporphyrin δ-lactone	19.7	564.6
Impurity **10**	Rhodoporphyrin-15-oxymethyl δ-lactone	21.7	548.6
Impurity **11**	Purpurin 18	22.3	564.6

the porphyrin chemistry as described in the Section 2.2. Identity of the prepared compounds to the corresponding impurities in chlorin e6 was confirmed by comparison of the HPLC retention times, HPLC-PDA profiles and HPLC–MS data.

3.2. Identification of impurities

UV–vis spectral characteristics of the chlorin e6 impurities in the mobile phase (Table 1) suggest that the impurities **2–8** possess chlorin-type spectra with intensive absorption bands at 402–408 (Soret band) and at 634–664 nm. As it follows from the absence of an intensive long wave adsorption band along with presence of the Soret band in the visible spectra of **9** and **10**, these impurities are the products of dehydrogenation of the chlorin cycle at C17-18 position (porphyrins).

Typical mass fragmentation of chlorin e6 and related chlorins is well-documented in the literature [22–24]. In the majority of cases, formation of fragment ions is accompanied by chlorin decarboxylation, and loss of carboxyalkyl and hydroxyl groups. Accordingly, in the MS-ESI spectra of chlorin e6, intensive peaks belonging to $[MH - CO_2]^+$ and $[MH - CH_2CO_2H]^+$ fragments due to fission of the most active C15 side chain group were observed. Under relatively mild electrospray ionization conditions, two remaining carboxylic groups (at C17 and C13)

Table 3
^{1}H NMR signal assignments for chlorin e6 and impurities **2**–**8** (chemical sifts in ppm, coupling constants in Hz)

Position	Chlorin e6	Compound **2**	Compound **3**	Compound **4**	Compound **5**	Compound **6** (a mixture of 15^1-*R*/*S* isomers)		Compound **7**	Compound 8
10-H	9.96/s	9.99/s	10.02/s	10.08/s	10.71/s	9.99/s	9.93/s	10.01/s	10.05/s
5-H	9.96/s	9.97/s	9.94/s	9.98/s	10.15/s	9.83/s	9.74/s	9.85s/	10.87/s
15-H	–	–	–	–	10.02/s				
20-H	9.08/s	9.09/s	9.06/s	9.13/s	9.00/s	8.98/s	8.91/s	8.97/s	8.97/s
13-H	–	–	–	9.25/s	–				
3^1-C*H*═CH_2	8.27/dd 17.8, 11.6	8.27/dd 17.8, 11.5	8.27/dd 17.8, 11.5	8.35/dd 17.8, 11.5	8.30/dd 17.8, 1.5		2H 8.16/m	8.23/dd 17.8, 11.6	8.25/dd 17.8, 11.6
3^2-CH═C*H*$_2$ (trans)	6.42/dd 18.0, 1.2[a]	6.42/dd 17.9, 0.7	6.42/d 17.9	6.43/dd 17.8, 1.1	6.43/dd 17.9, 1.2		4H 6.37/d 18.0	6.40/d 17.9	6.41/d 17.8
3^2-CH═C*H*$_2$ (cis)	6.11/dd 11.6, 1.2	6.12/dd 11.6, 0.9	6.12/d 11.5	6.14/dd 11.5, 1.2	6.15/dd 11.5, 1.3		4H 6.13/d 11.5	6.16/d 11.6	6.17/d 11.7
15^1-CH_2	6.43/d 18.6[a], 6.18/d 18.8	6.35/d 18.9, 6.03/d 18.4	CH_3 4.44/s	C*H*$_2$OH 5.94/s	–	C*H*OH 9.02/s	C*H*OH 8.7/s[a]	7.15/dd 14.5. 9.6	7.05/'t' 15.4
17-H	5.08/d 10.1	4.95/dd 10.4, 1.8	4.82/dd 10.2, 1.8	5.18/dd 10.1, 2.0	2H 4.61/m	5.19/d 9.8	5.05/d 8.2	4.66/d 10.3	4.55/m
18-H	4.68/q 7.2	4.62/q 7.1	4.63/q 7.2	4.72/q 7.2			2H 4.73/m	4.61/q 7.2	
17^4-CH_2	–	4.12/q 7.0	–	–	–	–	–		4.07/m
8^1-CH_2	3.81/q 7.4	3.81/q 7.7	3.83/q 7.6	3.85/q 7.5	3.88/q 7.7		4H 3.76/m	3.80q 7.7	3.82/q 7.2
12^1-CH_3	3.76/s	3.78/s	3.81, 3.43, 3.31/s	3.47, 3.46, 3.35/s	4.08, 3.43, 3.34/s	3.96/s	3.92/s	3.97/s	3.98, 3.39, 3.28/s
2^1-CH_3	3.42/s	3.42/s				3.37/s	3.35/s	3.39/s	
7^1-CH_3	3.30/s	3.31/s				3.25/s	3.22/s	3.27/s	
17^2-CH_2	3.02, 2.66/m	2.83, 2.48/m	2.71, 2.13, 3.01,	3.08, 2.87, 2.73,	2.82, 2.71, 3.11,		3.50–2.35/m	2.74, 2.17, 3.00,	2.60, 2.11, 2.81,
17^1-CH_2	2.74, 2.04/m	2.61, 2.00/m	2.78/m	2.28/m	2.97/m			2.68/m	2.49/m
18^1-CH_3	1.84/d 7.0	1.84/d 7.2	1.78/d 7.2	1.82/d 7.2	1.88/d 7.1	1.80/d 7.0	1.74/d 6.7	1.77/d 7.3	1.77/d 7.1
8^2-CH_3	1.72/t 7.6	1.73/t 7.6	1.74/t 7.6	1.73/t 7.6	1.77/t 7.7		6H 1.70/t 7.2	1.72/t 7.6	1.73/t 7.5
17^5-CH_3	–	1.07/t 7.1				–	–	–	1.04/t 7.0

[a] Partially unresolved signals (approximate resonance position was derived by deconvolution).

Table 4
^{1}H NMR signal assignments for impurities **9**–**10** (chemical sifts in ppm, coupling constants in Hz)

Position	Compound **9**	Compound **10**
10-H	10.16/s	10.11/s
5-H	10.07/s	10.02/s
20-H	10.00/s	9.98/s
3^1 -C*H*=CH_2	8.35/dd 17.8, 11.4	8.32/dd 16.7, 11.4
3^2 -CH=C*H*$_2$ (trans)	6.38/d 18.0	6.37/d 17.8
3^2 -CH=C*H*$_2$ (cis)	6.18/d 11.5	6.18/d 11.1
15^1-CH_2	C*H*OH 9.48/s	8.78/s
17-H	–	–
18-H	–	–
8^1-CH_2	3.99/q 7.6	3.95/m
12^1-CH_3	4.11/s	4.08/s
2^1-CH_3 18^1-CH_3	3.58, 3.59/s	6H 3.58/s
7^1-CH_3	3.44/s	3.41/s
17^1-CH_2 17^2-CH_2	5.00, 4.63/m 3.71, 3.52/m	4.85–3.35/m
8^2-CH_3	1.72/t 7.7	1.69/m

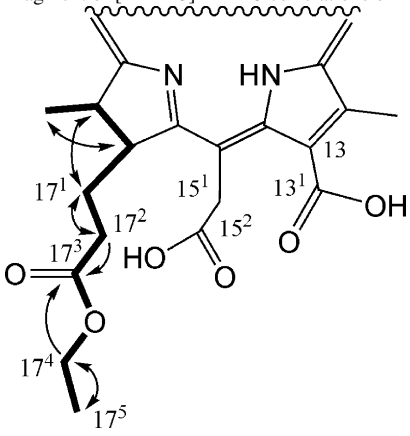
were more stable and, therefore, only low intensity signals of $[MH - 2CO_2]^+$ and $[MH - 3CO_2]^+$ were produced in the spectrum (Table 1).

The ^{1}H NMR spectrum of chlorin e6 was used as a reference to elucidate the structures of unknown impurities. Chemical shifts of chlorin e6 in deuterated pyridine (Table 3) correlate with those published elsewhere with one exception, namely, the low field proton resonances are atypically deshielded (especially 15^1-CH_2 group for ~1 ppm) compared to chlorin e6 spectra in $CdCl_3$ or acetone-d_6 [25,26].

3.2.1. Structural elucidation of impurity 2

Impurity **2** showed UV–vis spectrum of a typical chlorin and differed from chlorin e6 only by the presence of ethyl ester group, as it was established earlier with the use of HPLC–MS [21]. Treatment of chlorin e6 with acidic ethanol during purification stage was supposed to be responsible for the formation and accumulation of this impurity. After several optimization steps treatment of chlorin e6 in EtOH with 4% H_2SO_4 allowed receiving the largest quantity of the ester with the same HPLC–MS characteristics as detected for **2**. The ^{1}H NMR spectrum of impurity **2** showed characteristic system of ethyl ester group, comprising signals at $\delta_H = 4.12$ ppm (q) and 1.07 ppm (t). The 2D HMBC spectrum presented intensive over three bond correlations between 17^4 methylene protons and 17^3 carbon ($\delta_C = 173.2$ ppm), which, in turn, showed correlations with both 17^2 and 17^1 protons (Table 5). These data allows one to

Table 5
Fragment of [^{1}H–^{13}C] HMBC correlations of impurity **2**



Carbon atom	Signal position δ_C (ppm)	Interacting protons in HMBC spectrum
17^5	14.1	17^4 (++)
17^1	30.2	18 (+), 17^1 (+)
17^2	31.6	–
15^1	39.5	–
17^4	60.4	17^5 (+++)
13	128.6	12^1 (+++)
13^1	172.1	12^1 (++)
17^3	173.2	17^2 (++), 17^4 (++)
15^2	175.5	15^1 (+)

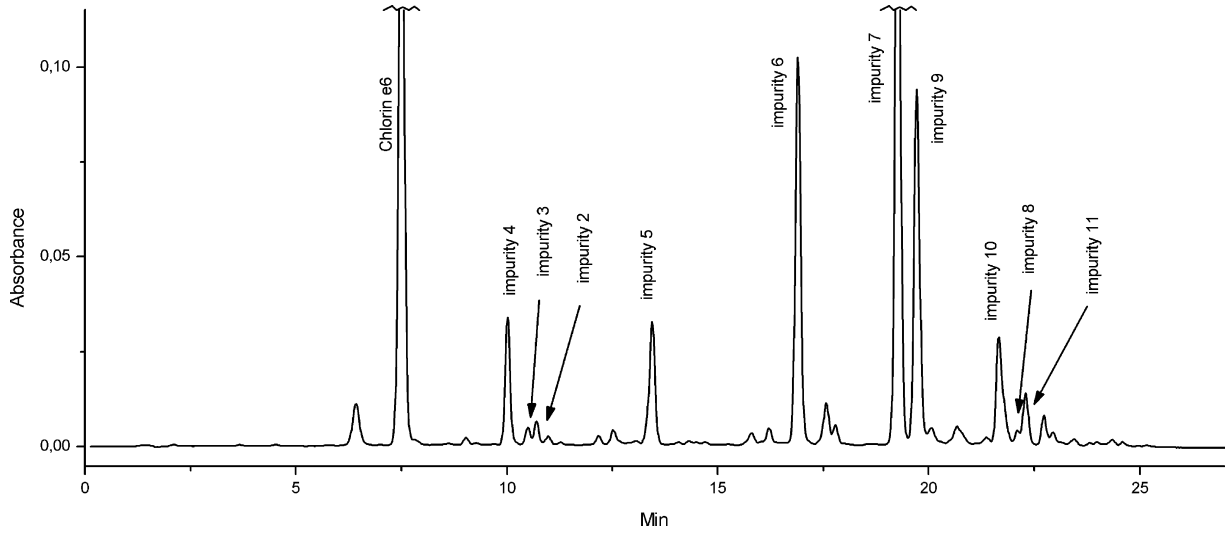


Fig. 2. HPLC chromatogram of degraded chlorin e6 (ageing for 1 year in the dark at the room temperature).

deduce localization of the esterified group to propionic acid (17^4) residue, revealing the structure of **2** as chlorin e6 17^4-ethyl ester.

3.2.2. Structural elucidation of impurity 3

Impurity **3** was characterised by UV–vis spectra very similar to that of chlorin e6 and appeared to be slightly more hydrophobic than RP-HPLC. The mass spectrum of **3** showed only a molecular ion with *m/z* 553.7 (Δ 44 in comparison with initial **1**) suggesting loss of the carboxyl group from the parent molecule. Potentially, any of three acidic residues could be altered in the chlorin e6 molecule. However, it is known that the decarboxylation of chlorin e6 begins at the least stable 15-acetic chain leading to the formation of chlorin e4 known from the works of Fischer [11]. The initial loss of 13-COOH group is possible if the others are protected by esterification and demands significantly more harsh conditions (>200 °C) than the ones used in the synthesis of chlorin e6. HPLC-PDA–MS analysis of synthesised chlorin e4 showed its equivalence to the characteristics of impurity **3**. ^{1}H NMR spectrum of impurity **3** confirmed the disappearance of a doublet of doublets of the 15^1-CH_2 group of **1** and appearance of the methyl proton resonance (δ_H = 4.44 ppm) corresponding to the appropriate signal of its dimethyl-esterified derivative [14]. These findings prove decarboxylation of the 15-acetic acid residue of chlorin e6 and allow characterising impurity **3** as chlorin e4.

3.2.3. Structural elucidation of impurity 4

The mass spectrum of **4** showed molecular ion at *m/z* 525.8 and the main product ion at *m/z* 507.8. The mass loss difference of 18 units allowed us to conjecture the presence of hydroxyl group in the structure of **4**. An analysis of the integral intensity and resonance positions of proton-bearing structural elements in comparison to **1** revealed that possible oxidation of vinyl group ($C3^1$-3^2) or reduced pyrrole ring (at C17-18 positions) did not occur [15,22]. Although almost all of the chlorin e6 substituents survived, the transformation of 15^1-CH_2 group was found due to absence of its signals. Additionally, two new singlet signals appeared in the low field at δ = 9.25 ppm (1H) and 5.94 ppm (2H). In comparison to chlorin e6, the signals of 17^x-methylene protons remained essentially the same, while the singlet belonging to 12-CH_3 protons was noticeably deshielded (∼0.3 ppm). These data implied that the electron withdrawing (carboxylic) group was removed at position 13 of the macrocycle, producing a resonance in the low field (δ_H = 9.25 ppm) [22]. The remaining two-proton singlet at 5.94 ppm was assigned to the 15-CH_2OH group. This suggestion was further supported by the presence of the fragment ion at *m/z* 495.8 in the MS spectrum corresponding to $[MH - CH_2OH]^+$. General examination of the experimental results allowed assigning the structure of **4** to 15^1-hydroxyphyllochlorin.

3.2.4. Structural elucidation of impurity 5

The MS-ESI spectrum of impurity **5** showed an ion at *m/z* 539.8 which formally corresponded to simultaneous removal of carboxyl (Δ 44) and methylene fragments (Δ 14) from parent molecule. A straightforward investigation of ^{1}H NMR data revealed only elimination of the AB system of 15^1-methylene group while the other characteristic multiplets remained in the spectrum. Therefore, we conjectured a loss of the acetic acid chain at C15 of chlorin e6 with the formation of the known substance rhodochlorin [11]. In this case the resonance of 15-CH had to be formed in the low field. The relevant signal was found in the expected region at δ_H = 10.02 (δ_H ($CdCl_3$) = 9.81 for rhodochlorin dimethyl ester [14]) and confirmed the structure of **5** as rhodochlorin.

3.2.5. Structural elucidation of impurity 6

In the mass spectrum of **6**, the molecular ion peak (*m/z* 567.8) and several fragment peaks which could be assigned to dehydration (*m/z* 549.7), deformylation (*m/z* 539.8) and decarboxylation (*m/z* 523.8) were observed. In spite of the homogeneity of the chromatographic peak of **6** (according to PDA and MS spectral purity), ^{1}H NMR spectrum of this impurity revealed a set of duplicated lines of all resolved resonances. Correlations in 2D [^{1}H–^{1}H] COSY and [^{1}H–^{1}H] NOESY spectra indicated the presence of two compounds with the same set of scalar and spatial couplings (Fig. 3, [^{1}H–^{1}H] COSY data not shown). Furthermore, the ^{1}H NMR spectrum of **6** differed from that of chlorin e6 by (i) the presence of strongly deshielded singlets (1H each) at δ_H = 9.0 and 8.7 ppm for both impurities, respectively, and (ii) by the absence of characteristic system of 15^1-CH_2 group. By assuming the oxidation at 15^1-position (with one proton loss) and taking into account the MS fragmentation pattern the structure of **6** can be rationalized as a hydroxylactone (which is a known substance, chlorin 5 [27]) in the form of 15^1-stereoisomers mixture.

The existence of two epimers of impurity **6** was confirmed by RP-HPLC separation of its methylated form. After esterification by methanol with catalytic H_2SO_4, two nearly baseline resolved peaks corresponding to dimethyl esters (*m/z* 595.7) of **6** appeared in the chromatogram at 10.0 and 10.9 min. The MS fragmentation spectra of these two peaks were found to be identical to the main product ion at *m/z* 563.7, which is apparently formed due to elimination of the methanol molecule. Considering molecular ions and the fragmentation pattern, the structure of methylated **6** can be deduced as the mixture of 15^1-(*S*)-**6b** and 15^1-(*R*)-**6b**.

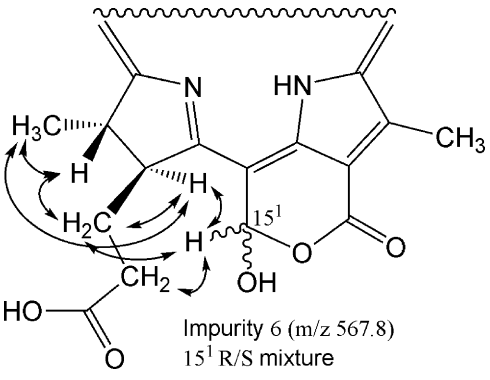


Fig. 3. [^{1}H–^{1}H] NOESY correlations of structure **6**.

By presenting correlation between 15^1 proton and a carbon with a $\delta_C = 98.7$ ppm, heteronuclear single quantum coherence (HSQC) spectrum unambiguously justified the above deduced skeletal structure of **6**, since in 15-formylrhodochlorin (the only other feasible structure) similar correlation should have been appeared in the typical aldehyde region (purpurin **5**, $\delta^1_{C15} =$ 191.6 ppm [14]).

*3.2.6. Structural elucidation of impurities **7** and **8***

^{1}H NMR spectra of impurity **7** and chlorin e6 were almost identical with the exception of a significant low field shift of the signals of the 15^1-CH_2 group (Δ0.85 ppm) and an up-field shift of the 17-CH group (Δ0.42 ppm). Consequently, the saturation degree of CH_x-type groups compared to the parent compound most likely remained the same. The molecular ion of **7** was found at *m/z* 551 without noticeable fragmentation. Therefore, it was suggested that the most probable structure of **7** was a chlorin lactone formed due to the loss of COOH at 15^1-position and the ring fusion of the residue with the adjacent carboxylic group. Due to several possibilities of the ring closure (with 13 and 17 side chains), the atom connectivities in the HMBC spectra were studied with regard to long-range *CH* couplings of 13^1 and 17^3 acidic carbons. The presence of intensive correlations between 13^1-C (164.3 ppm) and 15^2-CH_2 and 13-CH_3 protons were detected, while 17^1-C shows correlation only with 17^x-CH_2 side chain protons. These data proved cyclisation of 13–15 chains and allowed us to determine the structure of **7** as a rhodochlorin δ-lactone described earlier by Conant [28]. The 2D spectral pattern of **7** was found to be in a good agreement with the one observed for 17^4-methylated derivative of this molecule, which provides an additional support of its correct interpretation [20].

Impurity **8** showed the visible spectrum identical to that of **7**, appeared to be slightly more hydrophobic according to RP-HPLC and displayed characteristic MS ions at *m/z* 579.8 $[MH]^+$ and 551.8 $[MH - C_2H_4]$. The main fragment ion detected in MS spectra of **8** was identical to the molecular ion of the impurity **7**. This fact taken together with the increase in RP-HPLC retention and similarity of visible spectra allowed us to suppose **8** as esterifed derivative of impurity **7**. Further investigations clarified that **8** was not detected among the degradation products of purified chlorin e6. However, the main product obtained by mild oxidation of chlorin e6 17^4-ethyl ester (which is present chlorin e6 as impurity **2**) matched to **8** in its HPLC–MS characteristics. Based on these findings and ^{1}H NMR spectra of the impurity **8**, one can identify its structure as rhodochlorin δ-lactone 17^4-ethyl ester.

*3.2.7. Structural elucidation of impurities **9** and **10***

Impurities **9** and **10** have been considered as porphyrins according to their visible spectra. This assumption was confirmed due to the absence of 17 and 18 proton resonances of the reduced pyrrole ring and, at the same time, an additional singlet of the 18-CH_3 group ($\delta_H = 3.58$ ppm) was detected in ^{1}H NMR spectrum. The molecular ions of 9 (*m/z* 565.7) and 10 (*m/z* 549.7) were two units lower than those of the impurities **6** and **7**, respectively, and showed similar fragmentation patterns (considering incremental mass difference). These findings allowed us to conjecture that **9** and **10** are porphyrin derivatives of **6** and **7**. To obtain a solid confirmation of their structures, impurity **9** was collected from degraded chlorin, whereas impurity **10** was prepared synthetically. Finally, the scalar and spatial relationships in the 2D [^{1}H–^{1}H] correlation spectra (data not shown) proved the structures of **9** and **10** as 17,18-didehydrogenated analogs of hydroxylactone **6** and lactone **7**, respectively.

*3.2.8. Structural elucidation of impurity **11***

Impurity **11** could be quite consistently identified solely on the basis of the specificity of its visible spectrum (700, 644, 548, 509, 407 and 360 nm) which is characteristic of purpurin-type compounds. Impurity **11** prepared by Fischer's method [11] was identical to its analog in chlorin e6 substance according to its HPLC-PDA–MS characteristics. ^{1}H NMR spectrum of **11** was found to be in agreement with the one available in the literature [25].

3.3. The scheme of the formation of impurities

The majority of synthetic routes of the preparation of chlorin e6 (including the one used at RUE Belmedpreparaty) include alkaline hydrolysis of exocyclic E-ring of the parent chlorin in an inert atmosphere. It is known that the composition of impurities formed depends on the concentration of alkali and the presence of residual oxygen [11,29].

In the oxygen-free solution, E-ring hydrolysis yields exclusively chlorin e6 and its esters. As far as one of the stages of the chlorin e6 synthesis is performed in an acidic ethanol medium, the main impurity **2** is formed upon partial esterification of the target compound (Fig. 4). Further, impurity **3** is formed as a result of chlorin e6 partial decarboxylation upon hydrolysis. Therefore, the impurities **2** and **3** are considered to be process-related.

In the presence of oxygen during E-ring hydrolysis, several so-called "unstable chlorins" are typically formed, leading after a series of transformations to the stable impurity **11** (Fig. 4).

The remaining impurities **4**–**10** readily form upon induced degradation of chlorin e6 even under relatively mild conditions (e.g. under exposure to atmospheric oxygen) and, therefore, are related to degradation of API. The study of chlorin e6 synthesis revealed that accumulation of these impurities starts even at the late stages of preparation and purification, and can hardly be avoided (though several improvements to the technology have been made). This oxidative destruction of chlorin e6 is, most likely induced in the C15 (acetic acid) side chain and involves action of atmospheric oxygen or oxygen containing particles. Excessive spatial hindrance of the acetic acid radical is an additional promoter of such reactions [27]. Thus, the oxidative cleavage of the C15 side chain leads to decarboxylation and formation of impurities **6** and **7** (analogous transformation of **2** leads to **8**). Furthermore, impurities as **4** and **5** can be formed if transformation of the C15 chain is accompanied with the loss of the C13 carboxylic acid group. Afterwards, under the action of air, chlorin-like impurities are gradually converted to more

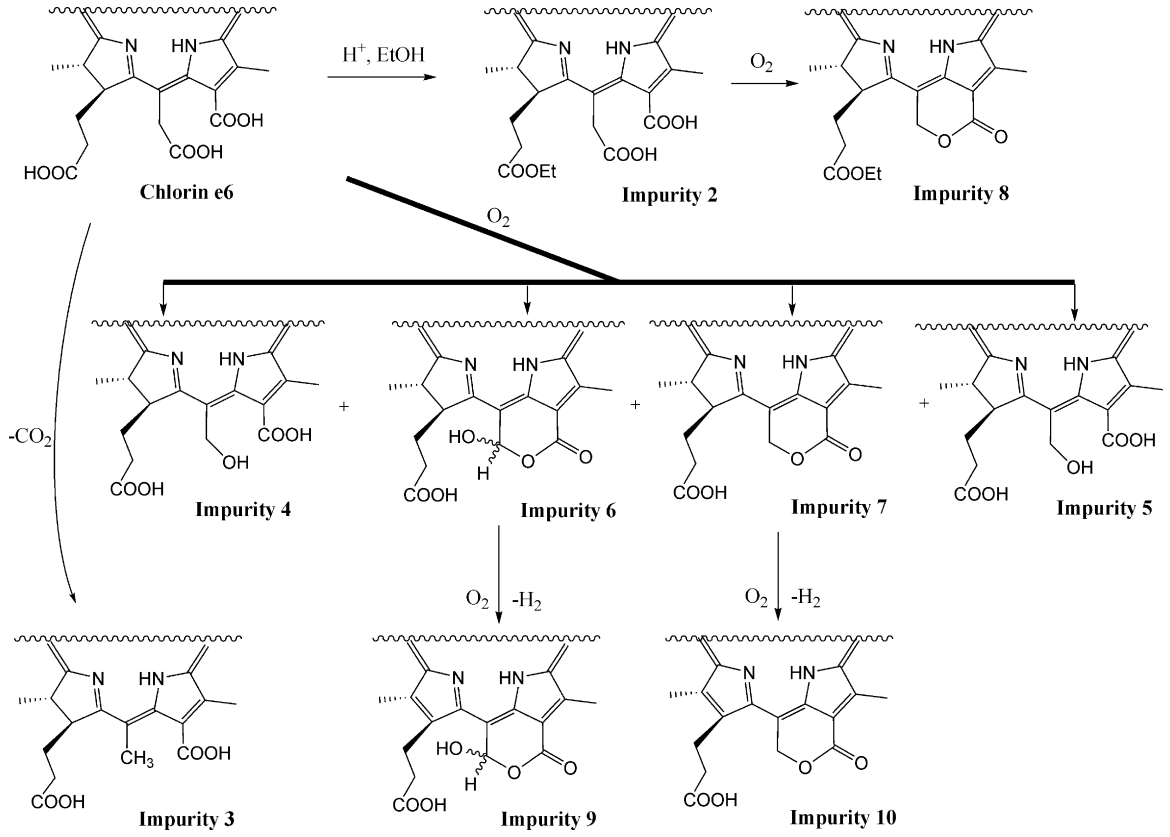


Fig. 4. Suggested scheme of the formation of impurities **2**–**10**.

stable 17,18-didehydrochlorin derivatives yielding porphyrins **9** and **10** [30].

4. Conclusions

It was established that both process and degradation related impurities can be detected in the pharmaceutical substance of chlorin e6. The main impurities detected in the samples included: chlorin e6 17^4-ethyl ester, chlorin e4, 15^1-hydroxyphyllochlorin, rhodochlorin, 15^1-hydroxymethylrhodochlorin δ-lactone, rhodochlorin-15-oxymethyl δ-lactone, rhodochlorin-15-oxymethyl δ-lactone 17^4-ethyl ester, 15^1-hydroxymethylrhodoporphyrin δ-lactone, rhodoporphyrin-15-oxymethyl δ-lactone and purpurin 18. The chemical structures of these impurities have been unambiguously proved by HPLC-PDA, HPLC–MS and NMR data. Syntheses and isolation procedures have been developed to prepare impurities from chlorin e6 or pheophytin *a* using the methods of induced destruction, extraction and chromatographic fractionation. On the basis of the identified structures of the impurities and the data on their development in chlorin e6, a scheme of impurities formation was suggested.

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