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Nature of the main contaminant in the anti malaria drug primaquine diphosphate: a qualitative isomer analysis☆

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Abstract

The main contaminant of primaquine (CAS 90-34-6) has been tentatively identified, by using two liquid chromatography (LC) methods and liquid chromatography–mass spectrometry (LC–MS), as the positional isomer quinocide (CAS 525-61-1). The first LC system was equipped with a chiral Chirex (S)-VAL and (R)-NEA column and the second system was equipped with an Adsorbosphere Nucleotide-Nucleoside 7 μ column. Comparison of the main contaminant of primaquine with an authentic quinocide standard by using co-chromatography in both LC systems and LC–MS (mass fragmentation) supported the hypothesis. The toxicity of quinocide batch 17172, primaquine batch 16039, and the drug primaquine diphosphate batch 20107 used in pharmaceutical industry, and the effect of the substances on respiratory and electron transport chain were compared in the eucaryotic unicellular fresh water green alga *Chlamydomonas reinhardtii* as a model system. These studies suggest that minor amount of other related substances can contribute more to the toxicity of the drug primaquine diphosphate than the positional isomer quinocide.

Keywords: Primaquine; Quinocide

1. Introduction

Malaria is a widely spread protozoan disease with a death rate of 1.5 million humans per year. The micro organisms that cause malaria in humans are *Plasmodium falciparum*, *Plasmodium vivax*, *P. ovale* and *P. malariae*. Primaquine diphosphate, 8-[(4-amino-1-methylbutylamino)]-6-methoxyquinoline di-(dihydrogen phosphate) and quinocide 8-[(4-amino-4-methyl-butylamino)]-6-methoxyquinoline (CAS 525-61-1) are the only anti malarial drugs, which are gametocidal and effective against the hypnozoites, and against the tissue schizonts. Primaquine (CAS 90-34-6) is not effective against blood schizonts [1]. Synthetic anti malarials have been developed in Germany and France before the World War II and in Great Britain, the USA, and the USSR during and since the World War II [2]. Out of the thousands of compounds investigated, only a few have achieved a significant place in the chemotherapy of malaria. The most important of the synthetic anti malarials being currently used are chloroquine, amodiaquine, primaquine, proguanil, and pyrimethamine. The plasmocide and quinocide have been widely used in the USSR [2]. Synthesis of primaquine has been proposed by Elderfield et al. [3]. An improved procedure was also proposed by Elderfield et al. [4]. The latter procedure is still in use today. Synthesis of quinocide was described by Braude and Stavrovskaya [5,6].

Primaquine is primarily used for the causative (radical) treatment of *P. vivax* infections in people returning from malarious areas. The drug is anti malarial, and killing the exo-erythrocytic stages of *P. vivax* and the pre-erythrocytic form of *P. falciparum*. It also kills gametocytes, or renders them incapable of developing in the mosquito, but it has little effect on other erythrocytic stages of Plasmodium [2].

An analysis of related substances of primaquine is not described in the British Pharmacopoeia [7]. But in the British

☆ The article is dedicated to I.B.'s teacher, Prof. Tyge Greibrokk.
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Pharmacopoeia 1988 addendum 1990 a liquid chromatography (LC) method for analysis of related substances is presented [8]. Related substances are allowed to be present in the drug at a maximum of 6%. In the British Pharmacopoeia 1993, vol. I, demands to the purity requirements are the same as in British Pharmacopoeia 1988 addendum 1990. The drug primaquine phosphate is listed as "primaquine phosphate and enantiomer" for the first time in the British Pharmacopoeia [9]. In the British Pharmacopoeia 1993 addendum 1997 [10] purity requirements are the same as in the third edition of the European Pharmacopoeia (EP), i.e. the related substances are allowed to be present in the drug at a maximum of 3% [11]. The limits were still valid in the British Pharmacopoeia [12] and in European Pharmacopoeia third, supplement [13].

It has been assumed that the main contaminant to primaquine is the enantiomer [3], which was proposed to have the same toxicity as the main product. A possible separation of the racemate has been reported [3].

In the present paper, it is shown by LC using two different systems that the main contaminant of primaquine is not its enantiomer but the positional isomer.

Methemoglobinemia [14–18] and neurotoxicity of the 8-aminoquinolines are well known [19,20]. Polyamines may be involved in the development of a hepatic encephalopathy and cerebral oedema [21]. Neuropsychiatric manifestations after therapy with the quinoline derivative *mefloquine* for *P. falciparum* malaria and the carcinogenicity of primaquine have also been reported [22,23]. Separation of the enantiomers of primaquine has been described [3,24], and the different metabolisms of the enantiomers of primaquine have been discussed [25]. Even the evidence of the effect of aliphatic side-chain substituents on the anti malarial activity and on the metabolism of primaquine was questioned [26].

In vitro toxicity assays with the unicellular green alga *Chlamydomonas reinhardtii* were used to evaluate to what extent primaquine and the contaminating positional isomer quinocide contribute to the overall toxic effect of the drug primaquine diphosphate.

Toxicity was assessed by monitoring growth of *C. reinhardtii* cells [27] at increasing concentrations of quinocide standard, primaquine standard, and the drug primaquine diphosphate used in pharmaceutical industry, and by determining the maximal concentration of quinocide and primaquine that the cells could tolerate. The effect of primaquine and quinocide on respiratory and electron transport chain was measured with a Clark-type oxygen electrode [28].

2. Materials and methods

2.1. Primaquine and quinocide

Primaquine diphosphate from different producers was analysed: drug primaquine diphosphate used in pharmaceutical industry (sample is resaved from one of the authors, J. Røe) batch 20107 (Shanghai Zhong XI Pharmaceutical Co., Shanghai, China), standard primaquine diphosphate p.a. quality from (Aldrich-Chemie, Steinheim, Germany) batch 16039 and p.a. quality standard quinocide dihydrochloride batch 17172 from the Martsinovsky Institute of Medical Parasitology and Tropical Medicine (Moscow, Russia).

2.2. Preparation of substances for LC and LC–MS

Primaquine diphosphate batch 20107 and batch 16039 were used. 2.5 mg of primaquine diphosphate were dissolved in 10 ml of purified water and analysed by LC and LC–MS. The solution was protected from light. The same procedure was used for the quinocide batch 17172.

2.3. Liquid chromatography

For LC analysis a HP 1100 instrument (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode array detector, and ChemStation software was used. Analysis was done in the first system on the chiral Chirex (S)-VAL and (R)-NEA column 250 mm long and 4.6 mm i.d., from Phenomenex (Torrance, CA, USA) with a flow of 0.5 ml/min. The mobile phase was hexane (Merck, Darmstadt, Germany)/1,2-dichloroethane (Merck)/ethanol (Arcus, Oslo, Norway), trifluoroacetic acid (TFA) (Sigma, St. Louis, MO, USA) (55:44:1, with ethanol–TFA premixed 20:1). The injection volume was 20 μl. Detection was done at 268 nm, and a simultaneous UV scan was obtained in the range 190–400 nm.

Analysis was done in the second system using an Adsorbosphere Nucleotide-Nucleoside 7 μ column, 250 mm × 4.6 mm i.d., from Alltech (Deerfield, IL, USA) with a flow of 1 ml/min. The mobile phase was 75% methanol (Merck), and 25% of 0.2 M ammonium carbonate p.a. quality (Merck) in purified water. The injection volume was 20 μl. Detection was done at 268 nm, and a simultaneous UV scan was obtained in the range 190–400 nm.

2.4. Liquid chromatography–mass spectrometry

The chromatographic system consisted of a Waters 2690 mobile phase pump, equipped with an auto-sampler (Waters, Milford, MA, USA). Analysis was done using an Adsorbosphere Nucleotide-Nucleoside 7 μ column 250 mm × 4.6 mm i.d., from Alltech with a flow of 0.5 ml/min. The mobile phase was 75% methanol (Merck), and 25% of 0.2 M ammonium carbonate p.a. quality (Merck) in purified water. The injection volume was 2 μl. The LC system was coupled on-line to a Quattro HPLC–MS/MS triple-quadrupole mass spectrometer (Micromass, Altricham, UK) equipped with a pneumatically assisted electrospray ionisation source. The eluent was split 1:4 prior to introduction into the mass spectrometer. Data acquisition and processing were performed using a Masslynx NT 3.1 data system.

The LC effluent entered the mass spectrometer through an electrospray capillary set at 3.0 kV at a source block temperature of 80 °C and a desolvation gas temperature of 150 °C. Nitrogen was used both as drying gas and nebulizing gas at flow rates of approximately 610 and 80 l/h, respectively. The ion-source parameters were optimised with respect to the positive molecular ions of primaquine and quinocide, and the cone voltage was set to 30 V. The mass spectra, between *m*/*z* 20 and *m*/*z* 400, were obtained at a scan speed of 200 *m*/*z* unit scan/s with a mass resolution corresponding to 1 unit at half peak height. The instrument was previously calibrated with sodium iodide.

2.5. Toxicological test

2.5.1. Algae

C. reinhardtii, strain CC124, from the *Chlamydomonas* Genetics Centre at Duke University, NC, USA, was maintained on high salt medium (HS) [29] in 100 ml liquid cultures in 250 ml Erlenmeyer flasks or on agar plates (1.5% agar). Algae were transferred to fresh medium at regular intervals.

2.5.2. Determination of cell number

The number of cells per ml was determined by counting in a Thoma hemocytometer (Brand, Germany) after im-

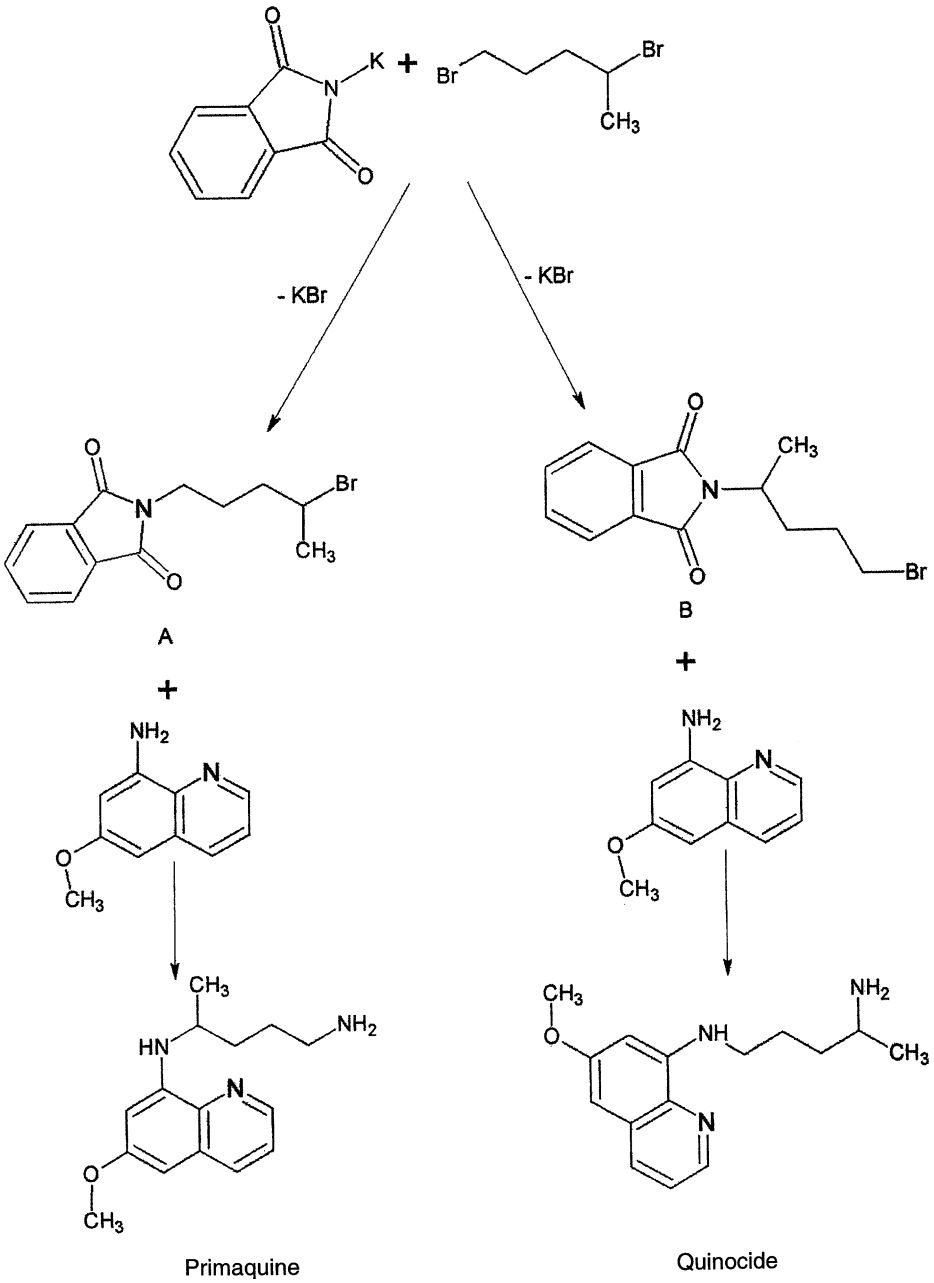

Fig. 1. Main routes for synthesis of primaquine and the related substance quinocide. There are two possible positional isomers. One isomer has the methyl group in position C_1 of the chain, the other in position C_4. Details are described in the text.

mobilization of cells by a small amount of phenol. Samples were diluted to approximately 1×10^6 cells/ml before counting.

2.5.3. Chlorophyll determination

The concentration of chlorophyll (a + b) was determined according to Arnon [30]. Chlorophyll was extracted from the cells with 80% acetone (Merck). After pelleting cell debris in a centrifuge, the absorption of the supernatant was measured at 645 and 663 nm in a Shimadzu UV-1601 spectrophotometer (Shimadzu, Tokyo, Japan). The concentration of chlorophyll was calculated using the formula $20.2 \times A_{645} + 8.02 \times A_{663}$ = chlorophyll (a + b) mg/ml.

2.5.4. Measuring growth and viability of C. reinhardtii

To assess the toxicity of p.a. quality primaquine, of the drug primaquine diphosphate used in pharmaceutical industry and of quinocide, algae were grown in plastic Petri dishes (5 cm in diameter) on HS medium in the presence and absence of primaquine, of the drug primaquine diphosphate and of quinocide. The Petri dishes were continuously

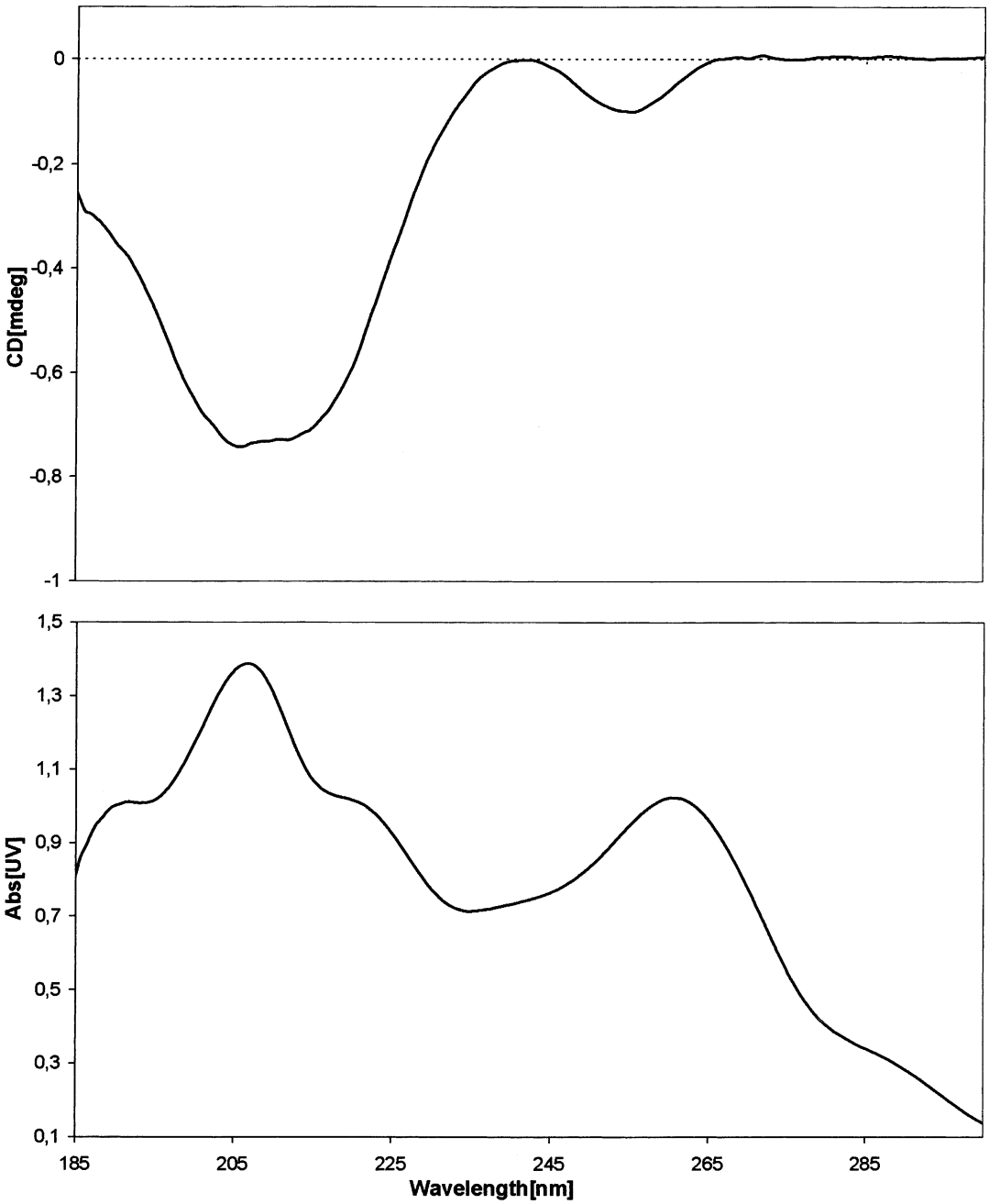

Fig. 2. CD (upper) and UV spectra of primaquine (batch 16039). The spectra in purified water were all characteristic of optically active samples, and not of a racemate. Details are described in the text.

illuminated with four fluorescent lamps Osram L18W/20 Cool white, yielding a combined light intensity of the 71.5 μmol/(m^2 s) at the top of the Petri dishes. The starting cell density for all samples was 0.15×10^6 cells/ml. Cell number and chlorophyll content were determined after 3 days of incubation as described above. Cells were also inspected under the microscope for visual damage and for their ability to move.

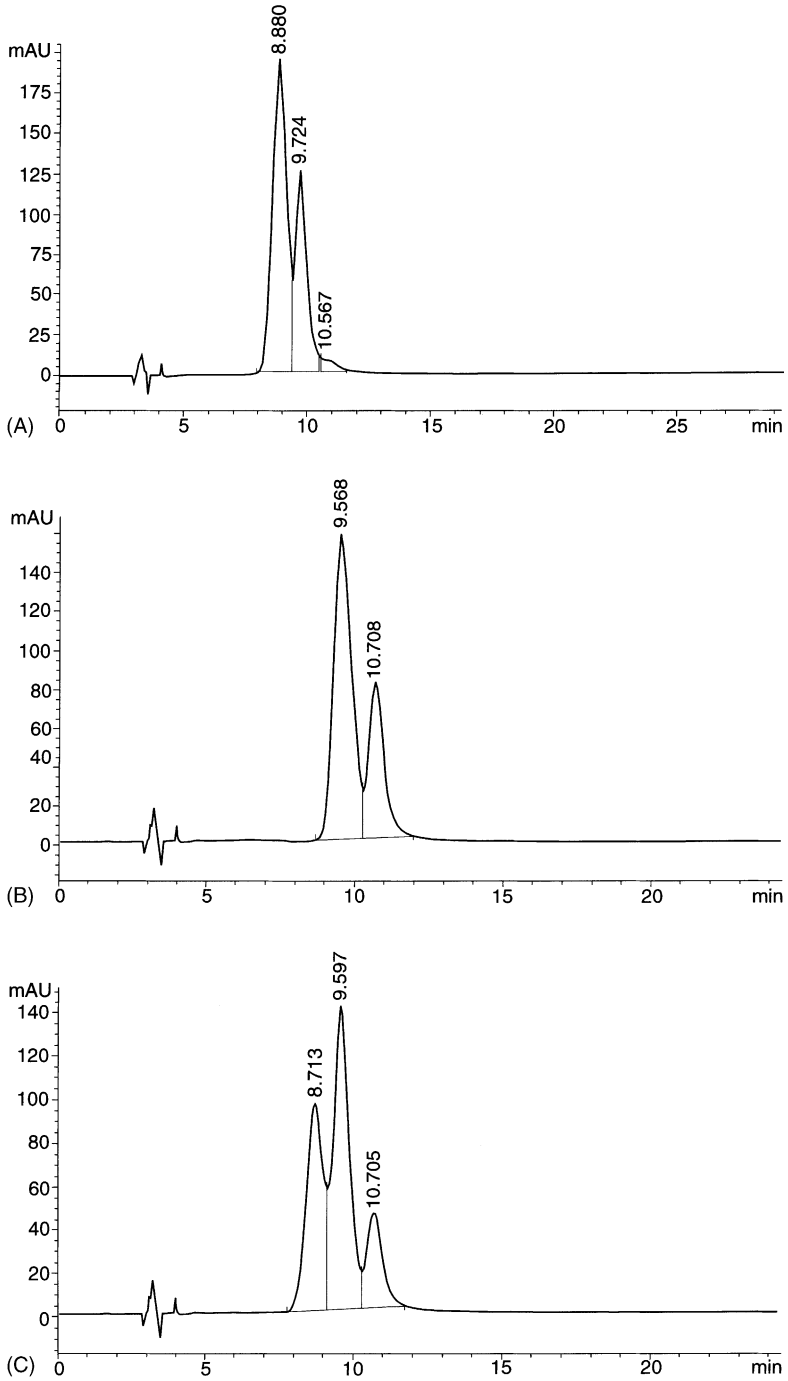

Fig. 3. LC chromatograms of primaquine and quinocide. Samples were analyzed on the chiral Chirex (S)-VAL and (R)-NEA column 250 mm long and 4.6 mm i.d., from Phenomenex with a flow of 0.5 ml/min. The mobile phase was hexane/1,2-dichloroethane/ethanol, trifluoroacetic acid (TFA) (55:44:1, with ethanol–TFA premixed 20:1). The injection volume was 20 μl. Detection was done at 268 nm. A simultaneous UV scan was obtained in the range 190–400 nm. (A) Typical LC chromatogram of primaquine batch 20107. Three peaks with retention times of 8.880, 9.724 and 10.567 min are detected. (B) Typical LC chromatogram of quinocide. Two peaks with retention times of 9.568 and 10.708 min are detected. (C) A co-chromatography of primaquine batch 20107 and quinocide batch 17172. Three peaks with retention times of 8.713, 9.597 and 10.705 min are detected. Details are described in the text.

2.5.5. Determination of oxygen uptake (respiration) and oxygen production (photosynthesis)

Uptake and production of oxygen were measured in a 1 ml plastic chamber equipped with a Clark-type oxygen electrode (Hansatech Instruments, Kings Lynn, UK). Cells were suspended in HS medium at a cell density of 0.5×10^6 cells/ml in the absence and presence of primaquine, of the drug primaquine diphosphate and of quinocide (1.54 mM/ml each). Oxygen evolution (photosynthesis) was measured in the presence of 10 mM $NAHCO_3$. Light intensity was 2.400 μmol/(m^2 s) at the side of the plastic chamber in those experiments. Recorder readings were calibrated using values of 0.253 mM oxygen for air-saturated water at 25 °C and 0 mM oxygen for water depleted of oxygen by addition of sodium dithionite.

2.6. Optical purity analysis by circular dichroism (CD) spectroscopy

CD spectra of primaquine diphosphate (batch 16039 and 20107) were recorded by using a Jasco J-810 spectropolarimeter (Jasco International Co., Tokyo, Japan) calibrated with ammonium D-camphor-10-sulfonate (Icatayama Chemicals, Tokyo, Japan). Measurements were performed at 23 °C by using a quartz cuvette with a path length of 0.1 cm. All the measurements were performed with a sample concentration of 0.01 M in purified water. Samples were scanned five times at 50 nm/min with a bandwidth of 1 nm and a response time of 1 s, over the wavelength range 185–300 nm. The data were averaged and the spectrum of a sample-free control sample (purified water) was subtracted. The resul-

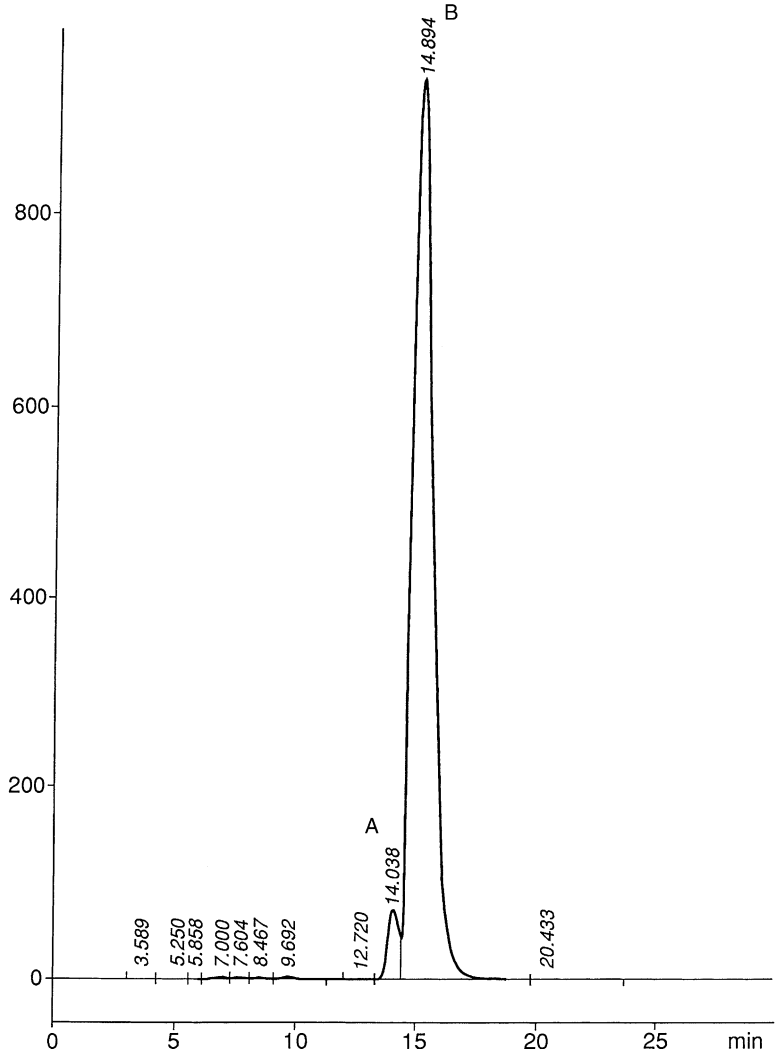

Fig. 4. Typical LC chromatogram of primaquine batch 20107. There are two major peaks detected. Peak A is eluted at a retention time of 14.038 min, and peak B primaquine at 14.894 min. Samples were analyzed on an Adsorbosphere Nucleotide-Nucleoside 7 μ column 250 mm long and 4.6 mm i.d., from Alltech with a flow rate of 1 ml/min. The mobile phase was 75% methanol and 25% of 0.2 M ammonium carbonate p.a. quality in purified water. The injection volume was 20 μl. Detection was done at 268 nm, and a simultaneously UV scan was obtained in the range 190–400 nm. Further details are described in the text.

tant spectra were then smoothed with the means-movement method. All measurements were conducted at least twice.

3. Results and discussion

Fig. 1 illustrates the main stages in the synthesis of primaquine as described [3].

Appearance of a positional isomer is possible, but other isomers or even polyamines could also be produced. There are two possible positional isomers.

One isomer has the methyl group in position C_1 of the chain, the other in position C_4 (Fig. 1).

In the synthesis of primaquine described by Elderfield et al. [3] it was mentioned that the rearrangement may occur during the attachment of the side chain to the ring system. The same article proposes the existence of racemates. In the literature there are several publications mentioning the separation of enantiomers of primaquine [3,31]. In the synthesis [3,4] the possibility of appearance of 8-(4-amino-4-methylbutylamino)-6-methoxyquinoline exists.

When 1,4-dibromopentane is refluxed with potassium phthalimide in acetone solution, 1-phthalimido-4-bromopentane (**A**) is formed, then condensation of **A** with 6-methoxy-8-aminoquinoline leads to the end product primaquine. When the product 1-phthalimido-1-methyl-4-bromobutane (**B**) is formed, then condensation of **B** with 6-methoxy-8-aminoquinoline leads to the positional isomer of primaquine, the quinocide (Fig. 1).

Each positional isomer may also have a sterio-isomer. Consequently, the production of four substances is possible.

Even sterio-isomers of primaquine have different toxicity [31], however, positional isomers as a rule have quite different toxicity. The differential metabolism of the enantiomers of primaquine was discussed by Baker and McChesney [25]. The effect of aliphatic side-chain substituents on the anti malarial activity and on the metabolism of primaquine was

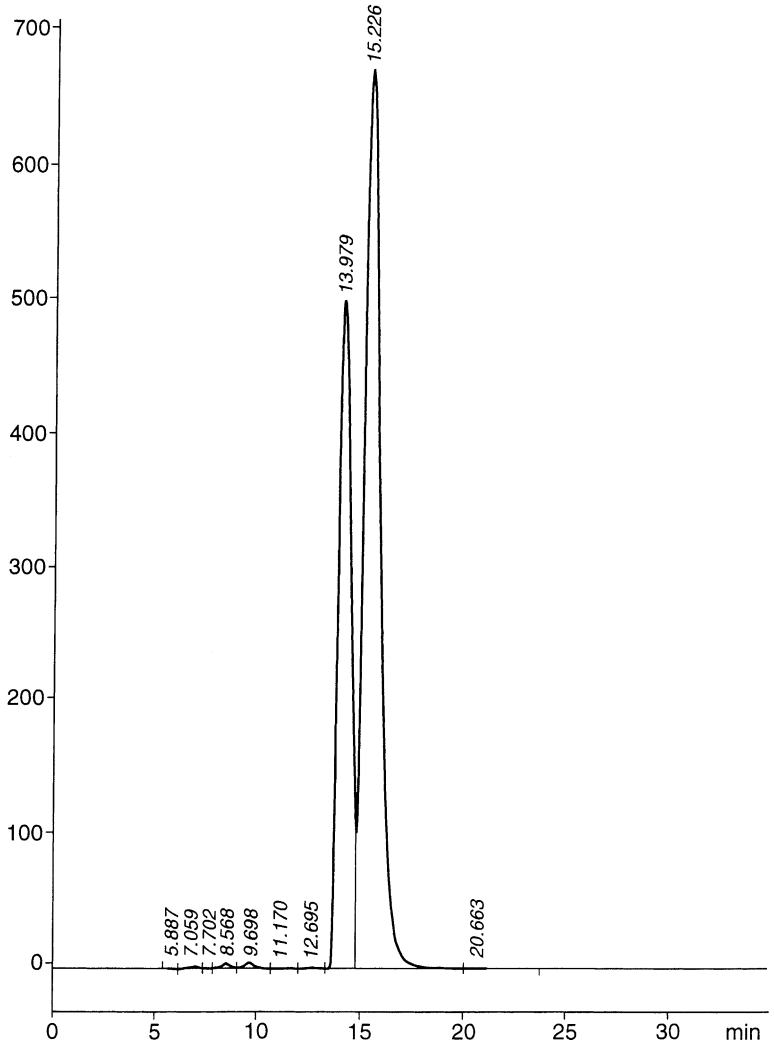

Fig. 5. Co-chromatography of primaquine batch 20107 and quinocide batch 17172. Two peaks with retention times of 13.979 and 15.226 min are detected. Samples were analyzed on an Adsorbosphere Nucleotide-Nucleoside 7 μ column. Details are described in Fig. 4 and in the text.

presented in [26]. Quite different metabolites were formed from primaquine and the positional isomer quinocide; they are carboxyprimaquine and quinocide ketone metabolite, respectively [26].

The drug primaquine diphosphate exhibited the CD spectrum which could not be the spectrum of the racemic mixture (Fig. 2).

3.1. LC analysis

Two LC systems were used to insure the separation of stereo-, and geometrical isomers.

The fist system utilised a chiral Chirex (S)-VAL and (R)-NEA column.

In Fig. 3A the characteristic chromatogram for the drug primaquine diphosphate is shown. Three peaks are detected. In Fig. 3B the characteristic chromatogram for the quinocide is shown. Two peaks are detected.

The result of co-chromatography of primaquine diphosphate and a standard of quinocide is shown in Fig. 3C. One of the stereo isomers of quinocide is co-detected with one of the stereo isomers of primaquine.

The UV spectra of primaquine and quinocide are very similar to each other (UV spectra of primaquine is shown in Fig. 2).

The co-chromatography of the primaquine batch 20107 with quinocide batch 17172, demonstrated that one (or both) of sterio-isomer(s) of quinocide might be contaminants of primaquine Fig. 3C.

In the second system the reverse D-phase column Adsorbosphere Nucleotide-Nucleoside 7 μ was utilised.

In Fig. 4 a characteristic chromatogram for the drug primaquine diphosphate is shown. There are two major peaks detected. Peak A is eluted at a retention time of 14.038 min, and peak B primaquine at 14.894 min.

The result of co-chromatography of primaquine diphosphate and a standard quinocide standard is shown in Fig. 5.

The co-chromatography of the primaquine batch 20107 with quinocide standard batch 17172 demonstrated that the main contaminant in the drug primaquine diphosphate was co-detected with quinocide Fig. 5. Separation of stereoisomers on the reverse D-phase column is unlikely, however, the geometrical isomers could be separated.

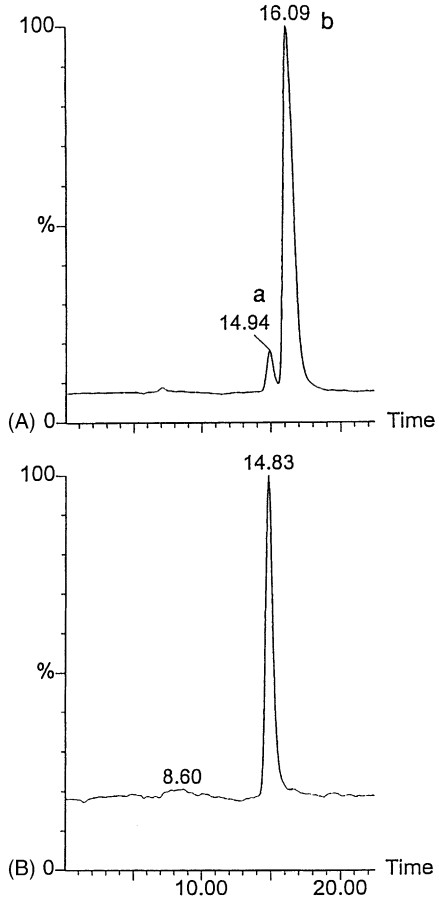

Fig. 6. Reconstructed positive ion chromatogram after LC/ESI–MS analysis with MS1 set to the scanning mode of; (A) primaquine batch 20107; (B) quinocide batch 17172. Conditions are described in the experimental section. Samples were analyzed on an Adsorbosphere Nucleotide-Nucleoside 7 μ column 250 mm long and 4.6 mm i.d., from Alltech with a flow rate of 1 ml/min. The mobile phase was 75% methanol and 25% of 0.2 M ammonium carbonate p.a. quality in purified water. The LC Waters 2690 mobile phase pump, equipped with an auto-sampler system was coupled on-line to a Quattro HPLC-MS/MS triple-quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ionisation source.

3.2. LC–MS

Because of the importance of a correct decision in the case of a human drug, additional evidence of the identity of the main contaminant with quinocide is provided by LC–MS.

Table 1
Threshold concentrations of primaquine and quinocide for inhibition of growth of *C. reinhardtii* cells

Control	Primaquine batch 16039			Primaquine batch 20107			Quinocide batch 17172	
Concentration (mM)	0.00	0.0001	0.0002	0.0004	0.0001	0.0002	0.0001	0.0002
Cells (ml^{-1})	7.0×10^6	4.7×10^6	1.3×10^6	0.7×10^6	2.4×10^6	0.7×10^6	2.2×10^6	0.3×10^6
Chlorophyll[a] (a + b) (mg/ml)	2.4	2.0	0.6	0.1	1.1	0.3	1.0	0.1

Cell numbers were 0.15×10^6 per ml at the start of the experiment. Number of cells and concentrations of chlorophyll (a + b) given in the table were determined after 3 days of growth in the present of primaquine and quinocide at the indicated concentrations.

[a] $n = 5$, R.S.D. = 3%.

The drug primaquine diphosphate batch 20107 and the authentic standard quinocide batch 17172 were analysed by LC/ESI–MS. MS was set to the scanning mode. Fig. 6A and B show the reconstructed ion chromatograms (RIC) of the drug primaquine and the authentic standard quinocide, respectively. Fig. 6A shows are two peaks, where peak a is the geometrical isomer and peak b primaquine. In Fig. 6B there is one peak corresponding to the retention time of geometrical isomer, which is the quinocide standard.

All three substances were detected as the protonated molecules with *m/z* 260, and fragmented by losing NH_3 to *m/z* 243 (Fig. 7). The main contaminant of primaquine has $[M+H]^+$ *m/z* 260 as the major ion. Fig. 7A and B show the mass spectra of the two peaks found in the drug primaquine diphosphate, and Fig. 7C shows the mass spectrum of the peak found in quinocide standard. The ion at *m/z* 260 is the protonated molecule of primaquine and quinocide, i.e. the $[M+H]^+$ ion. The other ions found in the mass spectra are cone-voltage induced fragments of the protonated molecule. The proposed fragmentation of the protonated molecules of primaquine and quinocide are shown in Figs. 8 and 9, respectively.

The comparison of the relative signal intensities of the ions in the three mass spectra from the Fig. 7A and C, show that the relative signal intensities of the ions in the mass spectra of the minor peak for the drug primaquine diphosphate and in the authentic standard quinocide are similar, and that they are different from the relative signal intensities of the ions in a mass spectrum of the major peak for the drug primaquine diphosphate, primaquine Fig. 7B. This indicates that quinocide is identical to the minor peak a in Fig. 6A.

3.3. Toxicity

Multiple toxic effects of primaquine such as neurotoxicity and methemoglobinemia in the not Caucasian race, has been described since it was in use [31]. The biological activity of enantiomers of primaquine is in general well established [32]. Schmidt et al. [33] found that the capacities of the isomers and the racemate to cure infections with *P. cynomolgi* in rhesus monkeys were essentially identical. On the other hand, in the same investigation, conflicting evidence was also presented concerning the toxicity of D- and

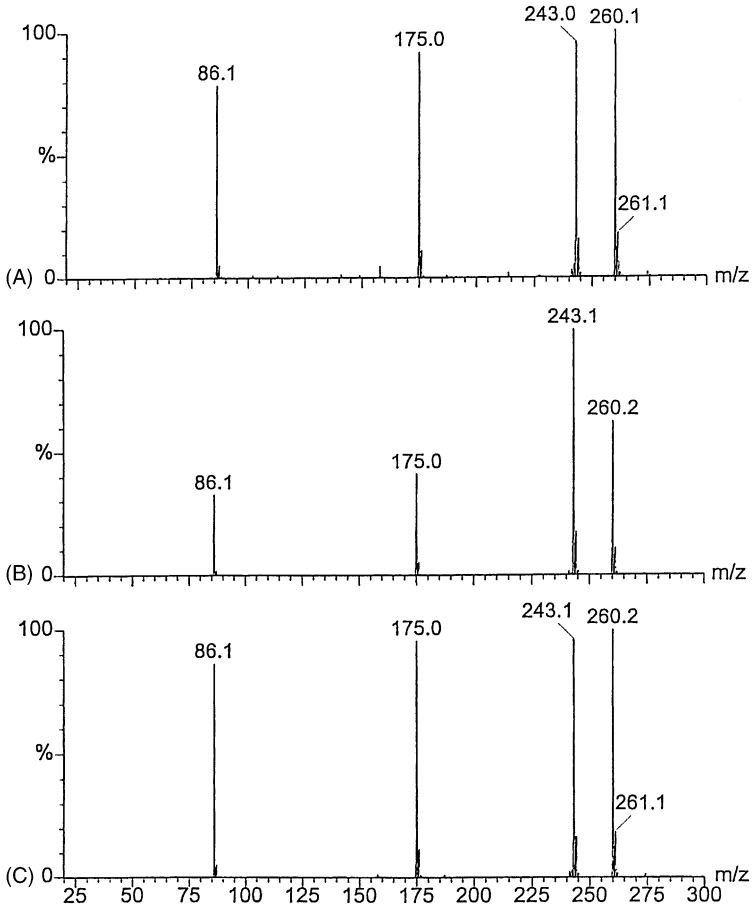

Fig. 7. Positive ion LC/ESI–MS mass spectra with cone voltage at 30 V obtained from the reconstruction ion chromatogram (Fig. 6). The main contaminant of primaquine has $[M+H]^+$ *m/z* 260 as the major ion. (A and B) Show the mass spectra of the two peaks found in the drug primaquine diphosphate, and (C) shows the mass spectrum of the peak found in the quinocide standard. (A and C) Show that the relative signal intensities of the ions in the mass spectra of the minor peak for the drug primaquine diphosphate and in the authentic standard quinocide are similar.

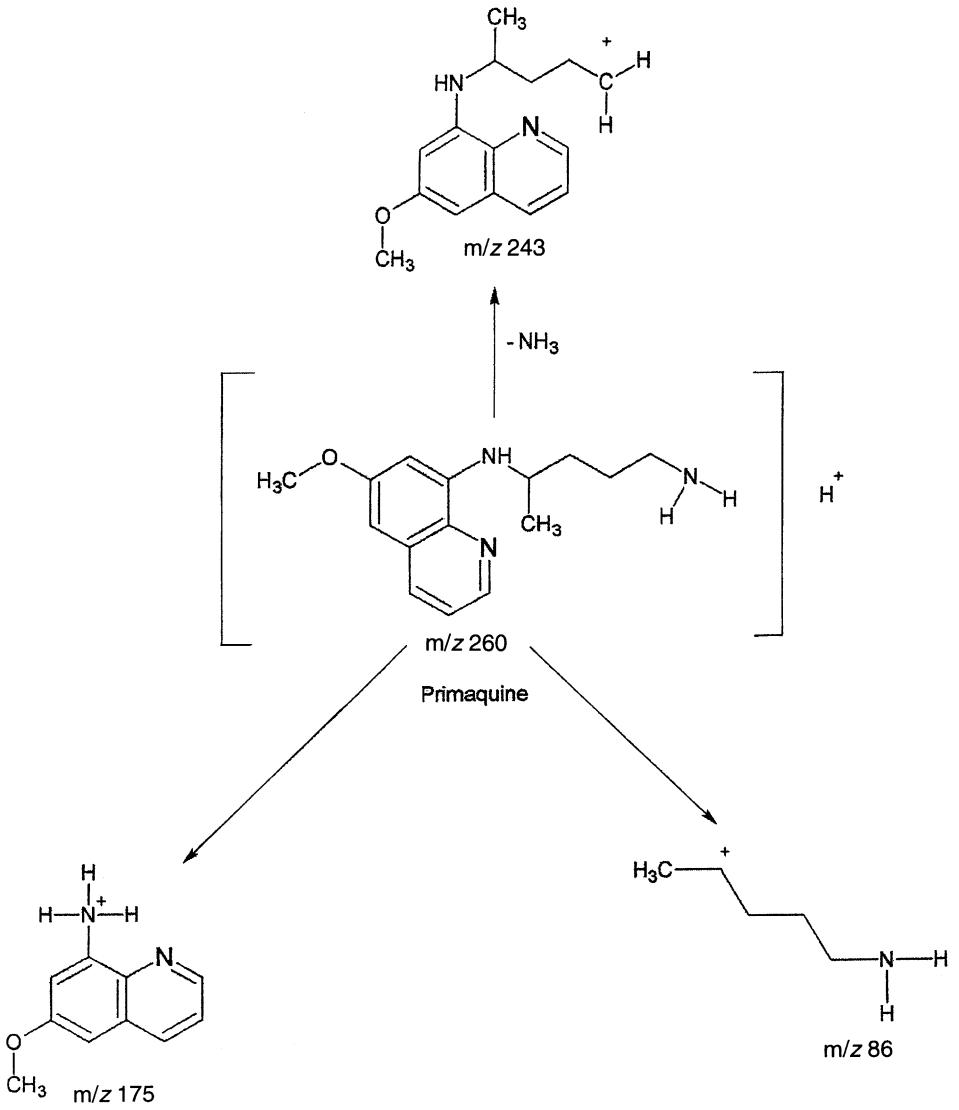

Fig. 8. The main routes of fragmentation for primaquine, as suggested by LC/ESI–MS spectrometry.

L-isomer in rhesus monkeys versus mice. However, the question about the nature of the related substances and they toxicity in terms of content of the actual related substances are never discussed in the Pharmacopoeias.

The unifying hypothesis of the biochemical toxicology of primaquine was suggested in [34]. From the hypothesis it is clear that primaquine can participate in oxygen metabolism. 8-Aminoquinolines interfere with redox processes, primaquine can also induce superoxides production [35]. Oxygen production/consumption was therefore chosen as the indication of toxicity. The alga *C. reinhardtii* was used to assess the toxicity of the primaquine batch 16036 (containing 0.3% of quinocide), the drug primaquine diphosphate batch 20107 (containing 5.8% quinocide), and the quinocide batch 17172.

The substances were toxic (algaecidal) at concentrations as low as 1.5×10^{-4} mg/ml for primaquine batch 16039, 1.0×10^{-4} mg/ml for the drug primaquine diphosphate batch 20107, and 1.0×10^{-4} mg/ml for the quinocide batch 17172. No growth was observed at these concentrations. Cells exhibiting no growth after 2 weeks looked severely damaged, swollen, and were immobile indicating that they were killed by these substances. Growth inhibition increased in the order primaquine batch 16039, drug primaquine diphosphate batch 20107 and quinocide batch 17172, as determined by the number of cells and the concentration of chlorophyll (a + b) mg/ml after 3 days of growth (Table 1).

The effect on uptake of oxygen in the dark (respiration) and production of oxygen in light (photosynthesis) was determined for the substance primaquine batch 16039 and quinocide batch 17172, which had the weakest and strongest toxic effects, respectively, in the growth and viability assay. Both substances had measurable inhibitory effects on

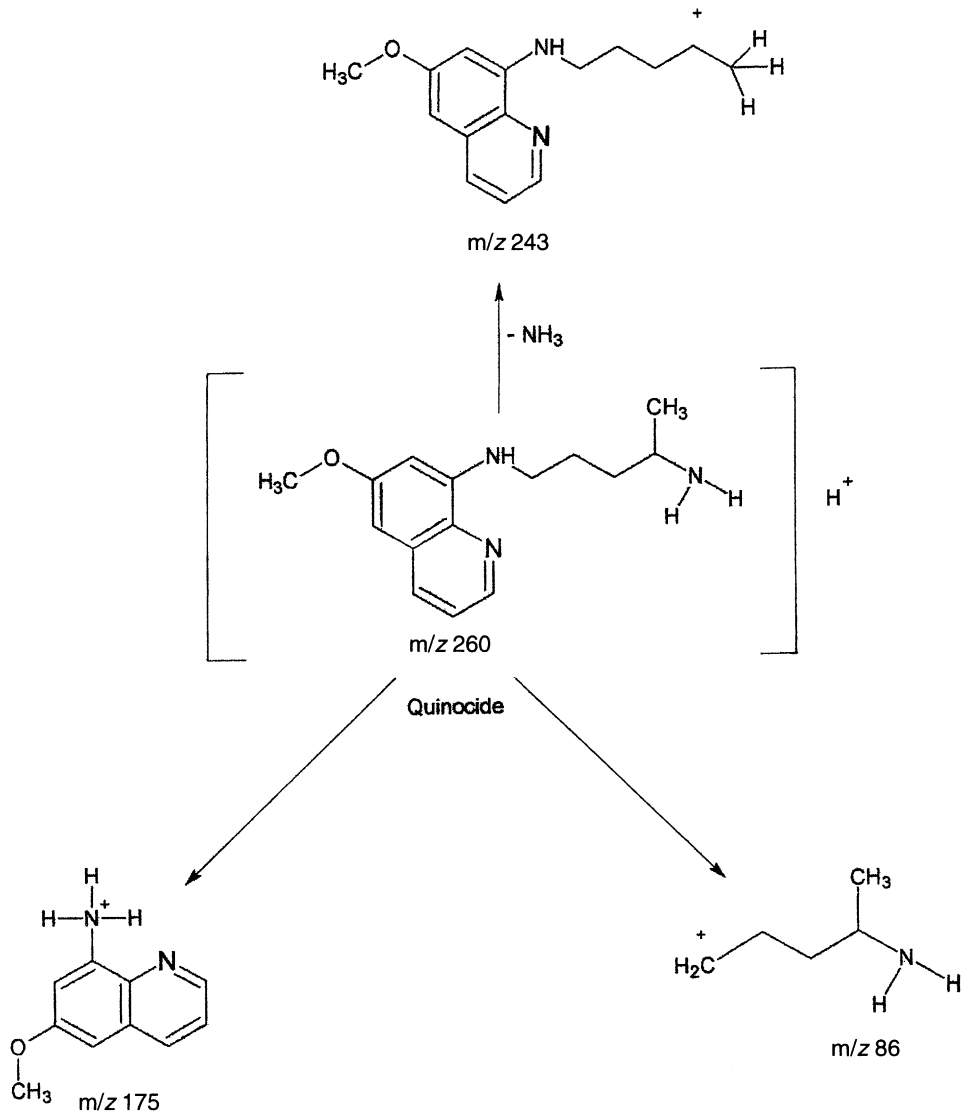

Fig. 9. The main routes of fragmentation for quinocide, as suggested by LC/ESI–MS spectrometry.

oxygen consumption in the dark (Table 2), but, more conspicuously, they abolished evolution of oxygen in the light. Moreover, both substances induced a net uptake of oxygen in the light, at rates that were two to three times higher than oxygen uptake of the control cells in the dark. The effect resembles the enhanced uptake of oxygen that is observed with uncouplers of respiration.

Because primaquine and quinocide reverse oxygen production in the light to oxygen consumption, these substances seem to interfere with the photosynthetic electron transport chain in the chloroplast of the algae.

The toxic effect of the quinocide batch 17172, primaquine batch 16039 and primaquine batch 20107 against algae show that the quinocide is about twice as toxic as primaquine, and that the primaquine batch 20107 is more toxic than primaquine batch 16039.

The toxicity of the primaquine batch 20107 is higher than can be expected to be for a mixture of approximately 6% quinocide in primaquine. This could be explained by synergistic effects of primaquine and quinocide or by other minor related contaminants in the drug that are very toxic. The latter explanation is more likely, and will be investigated further.

Table 2
The effect of primaquine and quinocide on oxygen metabolism of *C. reinhardtii* in the dark and in light

	Dark	Δ[a] (%)	Light	Δ (%)
O_2 uptake (−) or evolution (+) (nmol O_2/min mg *Chl*)				
Control	−7.2		+14.0	
Primaquine 1.5 mM	−6.2	−14	−23.8	−263
Quinocide 1.5 mM	−5.3	−26	−16.0	−214

Negative and positive numbers refer to uptake and evolution of oxygen, respectively. $n = 9$, R.S.D. = 5%.

[a] Δ: oxygen production/consumption percentage difference between control and sample with 1.5 mM primaquine or quinocide.

3.4. Optical purity

In the literature primaquine diphosphate has been described as a racemate [36,37]. The is point is very important in case of toxicological studies [32,33], however, the optical purity of primaquine diphosphate used was not analytically supported. Physico-chemical data are inconsistent [33] (D-primaquine has a $[\alpha]_D = +28.7^\circ$, however, L-primaquine has a $[\alpha]_D = -26.6^\circ$ and -27.8°). Each enantiomers should rotate the plane of polarization of plane-polarized light in opposite direction, however, at the same angle. In addition different lots vary in melting points from 176 to 183 °C, showing that the primaquine used was contaminated.

We measured the optical purity in solution of primaquine diphosphate from different supplies. Representative CD and UV spectra of primaquine diphosphate batch 16039 are shown in Fig. 2 and batch 20107 (results not shown). The spectra in purified water were all characteristic of optically active samples [38], and not of a racemate.

Judged from the data described in [33] and measurements done in this report, the primaquine diphosphate is not a racemate but a mixture of D- and L-isomers, with L-primaquine being predominant.

4. Conclusion

It is concluded that the main contaminant in the drug primaquine diphosphate used in pharmaceutical production is not an enantiomer as believed previously, but the positional isomer quinocide.

The primaquine diphosphate is not a racemate, but a mixture of stereo- and positional isomers in the D- and L-forms.

These conclusions are based on two different LC systems, LC–MS and CD analysis.

It is not possible to separate the enantiomers by the analytical methods described in the Pharmacopoeias [7–13]. *Related substances* separated by Pharmacopoeias' analytical methods are not enantiomers to primaquine, although, the presence of enantiomers cannot be excluded [3,24,36,37]. This is supported by the measurements presented, in this paper.

Because of the relative high percentage of main related substance allowed to be present in the drug, and the present knowledge of the nature of the main contaminant, the total toxicity of the human drug primaquine diphosphate used in pharmaceutical production should be re-evaluated.

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