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Nature of the main contaminant in the drug primaquine diphosphate: SFC and SFC–MS methods of analysis

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Dedicated to the memory of the former Editor of the Journal of Chromatography B, Prof. Zdenek Deyl.

Abstract

The drug primaquine diphosphate is used for causative treatment of malaria. Using HPLC–MS and GC–MS, this research group was previously able to show that the main contaminant of primaquine is the positional isomer quinocide [I. Brondz, D. Mantzilas, U. Klein, D. Ekeberg, E. Hvattum, M.N. Lebedeva, F.S. Mikhailitsyn, G.D. Soulimanov, J. Roe, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 800 (2004) 211–223; I. Brondz, U. Klein, D. Ekeberg, D. Mantzilas, E. Hvattum, H. Schultz, F. S. Mikhailitsyn, Asian J. Chem. 17 (2005) 1678–1688]. Primaquine and quinocide are highly toxic substances which can have a number of side effects upon use in medical treatment. A standard for quinocide is not typically commercially available. In the present work, supercritical fluid chromatography–mass spectrometry (SFC–MS) with two different columns was used to achieve a shorter analysis time for the separation between the positional isomers quinocide and primaquine in primaquine diphosphate and to elucidate additional information about differences in their MS fragmentation. Unlike using HPLC–MS, it was possible to achieve the differential fragmentation of positional isomers at branching points using the SFC–MS technique.

The desired short analysis time was achieved using SFC equipped with a Discovery HS F5 column and the differential fragmentation of positional isomers during SFC–MS provides information on the differences in the structure of these substances. Using a Chiralpak AD-H chiral column, it was possible to resolve the enantiomers in primaquine and separate quinocide from those enantiomers.

Keywords: Primaquine; Quinocide; Positional isomers; Chiral chromatography; SFC–MS; Anti-malaria drug

1. Introduction

Malaria is one of the most widely spread diseases on the globe with an estimated case rate of about 500 million and a death rate of about 2.7 million humans per year. Children are the most vulnerable victims of this disease. In Africa, the childhood death rate from malaria is about 90% of the total human death rate from this disease. The drug primaquine diphosphate is used for causative treatment of malaria. Specifications regarding the purity of primaquine diphosphate are less strictly indicated than for most drugs used in the treatment of other diseases.

Primaquine and quinocide are highly toxic substances which can have a number of negative side effects upon treatment; methemoglobinemia [1–5] and neurotoxcity of the 8-aminoquinolines [6,7] are well known. Polyamines may be involved in the development of a hepatic encephalopathy and

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cerebral oedema [8]. Neuropsychiatric manifestations after therapy with the quinoline derivative mefloquine for *Plasmodium falciparum* malaria and the carcinogenicity of primaquine have also been reported [9,10].

Due to the time consumption, uncertainties and difficulties of synthesis, it is preferable that an analyst should not synthesize standards for detection of a target drug substance. The identity of toxins in drugs should be established without any possible source of error. There is an urgent need for the development of a precise analytical procedure for the determination of toxic contaminants in drugs especially in cases where there is an absence of a commercially available authentic standard.

Using HPLC–MS and GC–MS, this team previously showed that the main contaminant of primaquine is the positional isomer quinocide [11,12]. In a prior publication [12], detailed schemes were presented for MS fragmentations for primaquine and quinocide and the differences in fragmentation were noted. However, GC analysis could result in thermal degradation of these substances. As quinocide is less thermally stable than primaquine, thermal degradation can interfere with the correct quantification of the contaminant in the drug. The separation of contaminants in the drug primaquine by HPLC or even GC is considerably more time-consuming than SFC [13]; the latter is more suited to the needs of a high throughput analytical laboratory where time-consuming analyses need to be avoided. A major objective of this study was to develop a more efficient separation and verification analysis. In SFC separations, a column produced for general HPLC use was used as well as a column produced specifically for SFC.

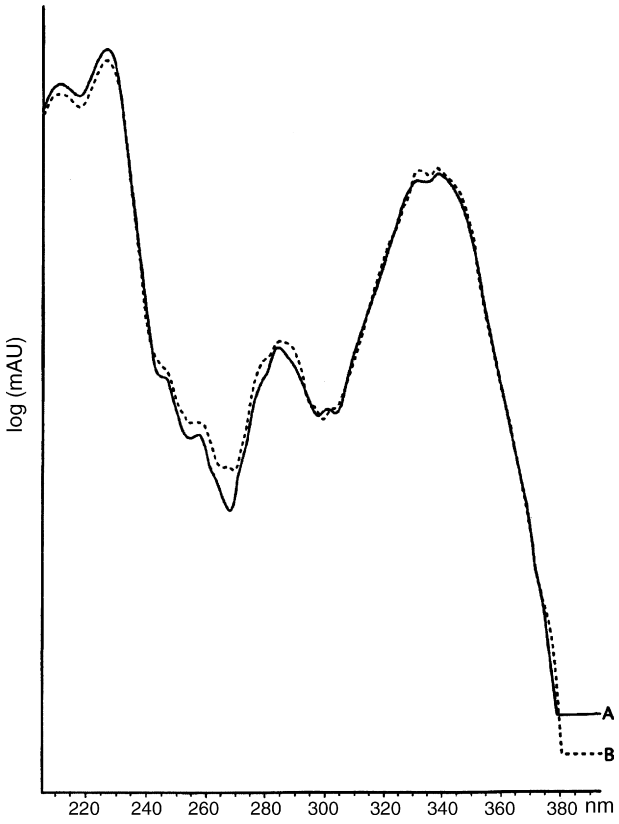

Fig. 1. UV spectra of quinocide (A) and primaquine (B) taken dynamically by using a diode array detector (DAD). Experiments were performed using the conditions stated above in Section 2.1.

2. Materials and methods

2.1. UV spectroscopy

The HPLC instrument used was an Agilent 1100 chromatograph with a diode array detector (DAD) and ChemStation software (Hewlett–Packard, Palo Alto, CA, USA). A Discovery HS F5 column, 250 mm × 4.6 mm i.d., 5 μm particle size (Supelco, Bellefonte, PA, USA) was utilized to enhance the separation. The injection volume was 20 μL. The mobile phase composition was acetonitrile p.a. quality (Merck, Darmstadt, Germany)/20 mM ammonium acetate p.a. quality (Merck) in deionised water (pH 7.0), 50/50 (v/v). The flow rate was 1.0 mL min^{-1}. Baseline separation was achieved isocratically [13,16]. The analytes were detected at 268 nm with reference at 300 nm. The separation was done at room temperature. The UV spectra were obtained dynamically in the mobile phase solvent, normalized and overlaid as Fig. 1.

2.2. Supercritical fluid chromatography (SFC) and supercritical fluid chromatography–electrospray ionisation–mass spectroscopy (SFC–ESI–MS)

The following equipment was used for all the SFC experiments: Berger SFC MiniGram (Berger Instruments Inc., Newark, DE, USA) equipped with an UV K-2501 detector (Advanced Scientific Instruments GmbH Ing., Dr. Knauer, Berlin, Germany) and ProNTo software (Berger Instruments Inc.). Samples were injected with an automated liquid sampler (Berger Instruments Inc.) with an injection volume of 5 μL. A part of the flow stream exiting the UV detector was diverted by a fixed splitter and used to feed a Micromass PLCZ 4190 Mass Spectrometer equipped with ESI running under MassLynx (Waters-Micromass, Manchester, UK). MS conditions were: cone voltage, 60 V, ion energy 1.0 V, multiplier 650 V, analyzer vacuum 2.6 kPa, dissolvation gas flow 495 L h^{-1}, function type Scan, mass range *m/z* 50–400. UV detection was recorded at 268 nm. Samples were prepared as described in Brondz et al. [11].

2.2.1. SFC on a 2-ethyl-pyridine column

The first stationary phase used was a 2-ethyl-pyridine SFC column, 250 mm × 4.6 mm i.d., 5 μm particle size (Berger Instruments Inc.). The mobile phase was CO_2 (AGA, Oslo, Norway) in the supercritical state modified with ethanol (Arcus) containing 0.4% (v/v) diethylamine p.a. quality (Merck, Darmstadt, Germany). The gradient program of the mobile phase was: 5% modifier held for 2 min, increased by 10% min^{-1} to 25% and held at this level for 10 min, then lowered by 10% min^{-1} to 5% modifier. The flow rate was 5 mL min^{-1}. The oven temperature was 35 °C. UV and ESI–MS experiments were performed using the conditions stated above. The results are shown in Fig. 2.

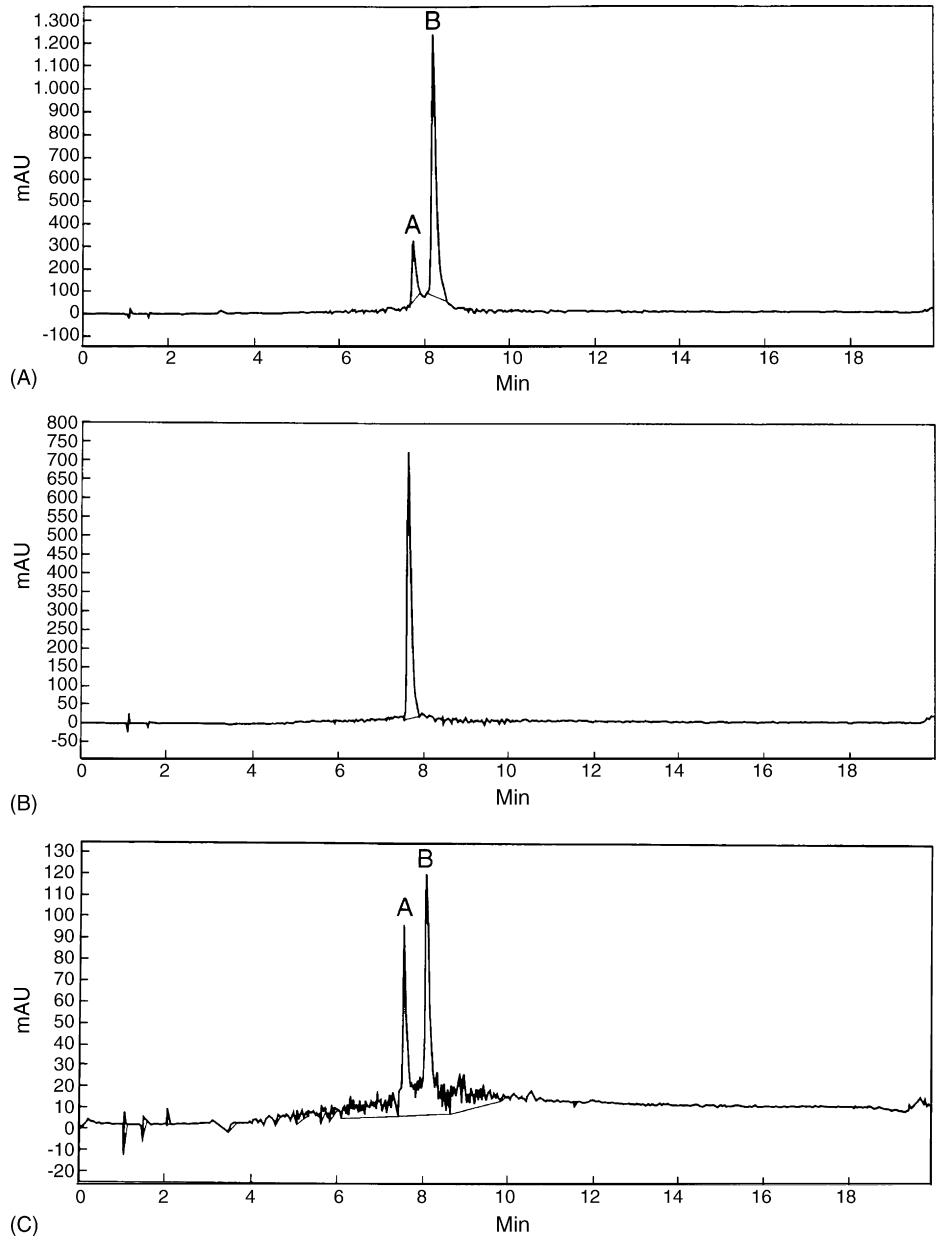

Fig. 2. Separation of the contaminant quinocide from the primaquine in the drug primaquine diphosphate done on a 2-ethyl-pyridine SFC column. UV detection was recorded at 268 nm. Experiments were performed using the conditions stated above in Section 2.2.1. (A) There are two peaks in the chromatogram of primaquine diphosphate: peak A is the contaminant quinocide and peak B is primaquine. (B) This figure shows a chromatogram of quinocide. (C) Co-chromatography of the drug primaquine diphosphate with a quinocide standard is shown. There are two peaks in the chromatogram, peak A and B; these have retention times of quinocide (peak A) and primaquine (peak B).

2.2.2. SFC on Chiralpak AD-H column—chiral separation

Separation of the enantiomers of primaquine in the drug primaquine diphosphate was performed by SFC–UV and SFC–MS on a Chiralpak AD-H column (amylase tris-3,5-dimethylphenylcarbamate coated on 5 μm silica–gel substrate), 250 mm × 4.6 mm i.d., 5 μm particle size (Daicel Chemical Industries, LTD. Exton, PA, USA). The isocratic mobile phase was CO_2 (AGA) in the supercritical state modified with methanol (Merck) (20%) containing 0.4% (v/v) diethylamine p.a. quality (Merck). The flow rate was 4 mL min^{-1} and the oven temperature was 35 °C. UV and ESI–MS experiments were performed using conditions stated above. The results are shown in Fig. 3.

2.2.3. SFC–ESI–MS on Discovery HS F5 column

For these SFC–UV and SFC–ESI–MS experiments the mobile phase consisted of CO_2 in the supercritical state; the modifier was chromatography grade MeOH (Merck). The gradient program was from 5 to 55% of modifier with a rate 10% min^{-1} at a flow of 3 mL min^{-1}. Oven temperature was 35 °C. A Discovery HS F5 column, 250 mm × 4.6 mm i.d. 5 μm particle size (Supelco) conditioned specifically for SFC [13,16] was

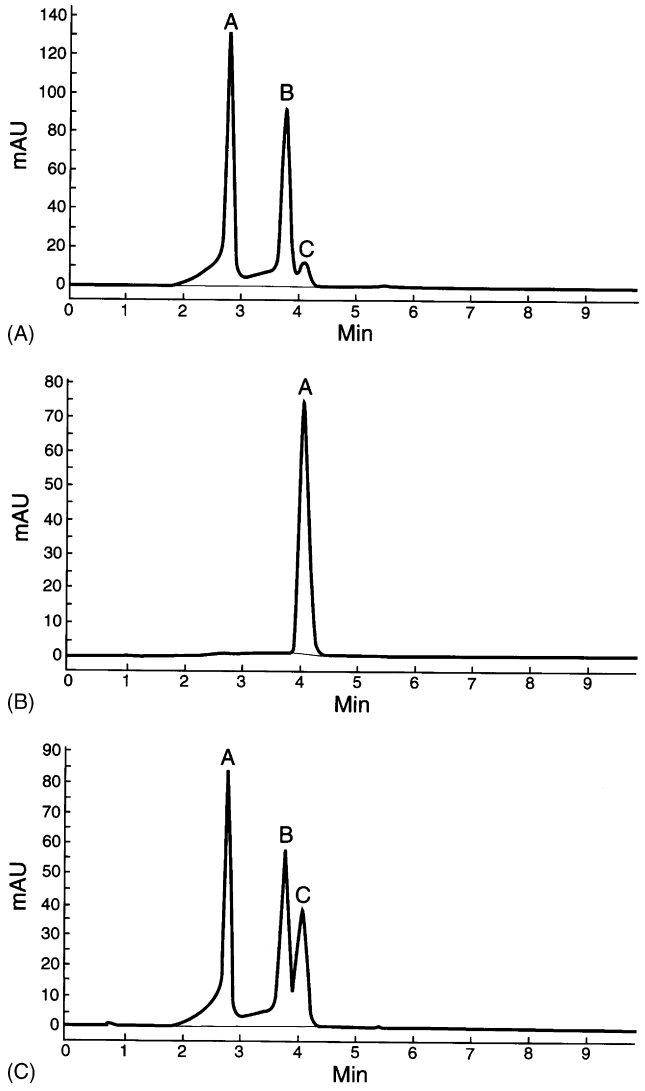

Fig. 3. Separation of the enantiomers of primaquine in the drug primaquine diphosphate was performed by SFC on a Chiralpak AD-H column (amylase tris-3,5-dimethylphenylcarbamate coated on 5 μm silica–gel substrate), 250 mm × 4.6 mm i.d., 5 μm particle size. UV detection was recorded at 268 nm. Experiments were performed using the conditions stated above in Section 2.2.2. (A) There are three peaks in this chromatogram: peaks A and B, which are enantiomers of primaquine, and peak C which is the contaminant quinocide. (B) The only peak in this chromatogram is the quinocide (peak A). (C) The co-chromatogram of the drug primaquine diphosphate and a quinocide standard shows three peaks: A–C. Peak C does exhibit increased height and area.

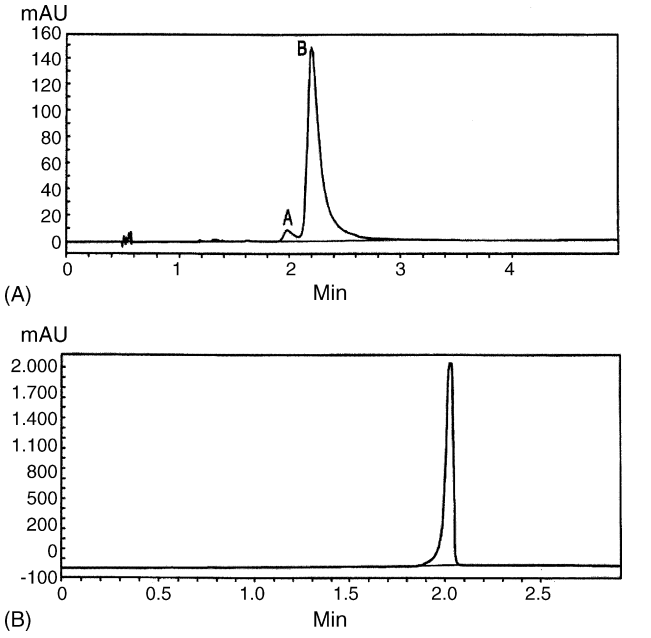

Fig. 4. Chromatograms obtained using SFC–ESI–MS equipped with a Discovery HS F5, are shown in A and B. UV detection was recorded at 268 nm. Experiments were performed using the conditions stated above in Section 2.2.3. (A) There are two peaks in the chromatogram of the drug primaquine; peak A is the contaminant quinocide and peak B is the primaquine. (B) There is one peak in the chromatogram of quinocide.

used. UV and ESI–MS experiments were performed using conditions stated above. The results are shown in Figs. 4 and 5.

3. Results and discussion

In order to distinguish between the two positional isomers of primaquine diphosphate and to identify the quinocide contaminant, an informative mass spectrum is necessary. Although primaquine and quinocide can be partially degraded at high temperature, informative GC–MS analysis was achieved in a previous study [12]. Detailed knowledge about the differences in MS fragmentation between primaquine and the main contaminant was integral to the development of detailed schemes for the MS fragmentation of primaquine and quinocide as well as the formation of an explanation for the differences in the fragmentation pathways [12].

Under HPLC–MS conditions it was possible, in previous experiments, to obtain important information about the nature of the main contaminant in the drug primaquine diphosphate [11]. As per the results from that study, it was clear that the main contaminant in the drug is quinocide. However, a requirement in *Pharmacopoeias* [14,15] is baseline separation of the contaminant from the main substance. In the current study, it is evident that the UV spectra of primaquine and quinocide are very similar, though some differences can be observed in the strong field (see Fig. 1A and B).

Separation of the contaminant quinocide from primaquine (in the drug primaquine diphosphate) using SFC–UV and SFC–MS with a 2-ethyl-pyridine column is shown in Fig. 2. There are two peaks in the chromatogram Fig. 2A: peak A is the contaminant quinocide and peak B is primaquine. A chromatogram of a quinocide is shown in Fig. 2B. There is only one peak in this chromatogram. Co-chromatography of the quinocide with the drug primaquine diphosphate is shown in Fig. 2C. There are two peaks in this chromatogram: peak A, which has the same retention time of quinocide and B, which has the same retention time as primaquine. Peak A does exhibit increased height and area.

A Discovery HS F5 column [13,16] has been used to achieve the desired baseline resolution of the main contaminant from primaquine, however, the time of analysis was quite long in HPLC analyses. SFC equipped with a Discovery HS F5 column [13]

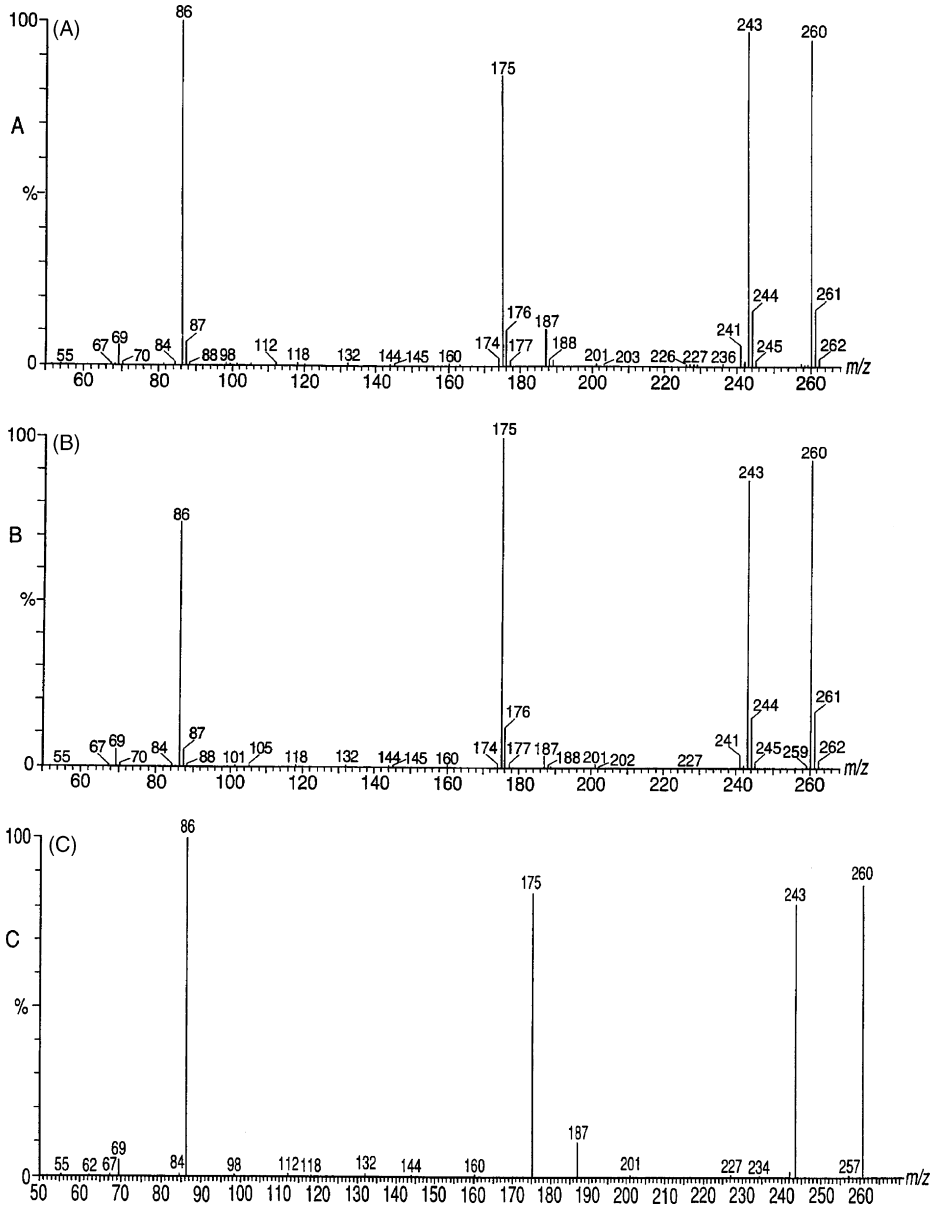

Fig. 5. Fragmentations of quinocide versus primaquine were done by using SFC–ESI–MS. ESI–MS experiments were performed using conditions stated above in Section 2.2. (A) Fragmentation of the contaminant peak A, in Fig. 4A. (B) Fragmentation of the primaquine peak B, in Fig. 4A. (C) Fragmentation of the quinocide from the single peak in Fig. 4B.

or a 2-ethyl-pyridine column significantly reduced the analysis time. In gradient mode both columns gave good separation of the main contaminant from the drug primaquine.

Separation of quinocide from primaquine in primaquine diphosphate on the Discovery HS F5 column by SFC–ESI–MS is shown in Fig. 4. In SFC–ESI–MS analysis, it can be difficult to use the mobile phase additive diethylamine without compromising the fragmentation and contaminating the MS instrumentation. Therefore, in this experiment only methanol was used as the mobile phase modifier, i.e. the additive was omitted. Without the addition of diethylamine there was some loss in resolution and it was difficult to achieve baseline separation between quinocide and primaquine, however, this is compensated for by the use of MS detection.

There are two peaks in the chromatogram of the drug primaquine diphosphate in Fig. 4A: peak A is the contaminant quinocide and peak B is primaquine. There is one peak in the chromatogram of quinocide (Fig. 4B); the retention time of this peak corresponds to retention time of the quinocide peak (peak A) in Fig. 4A.

SFC–ESI–MS using a Discovery HS F5 column results in separations of quinocide from primaquine with extremely short run times and also yields informative differences in the fragmentation of quinocide versus primaquine. In Fig. 5A, the fragmentation of the contaminant (peak A in Fig. 4A) is shown. The fragmentation of primaquine (peak B in Fig. 4A) is shown in Fig. 5B. The fragmentation of quinocide from the single peak in Fig. 4B is shown in Fig. 5C. The fragmentation patterns showed

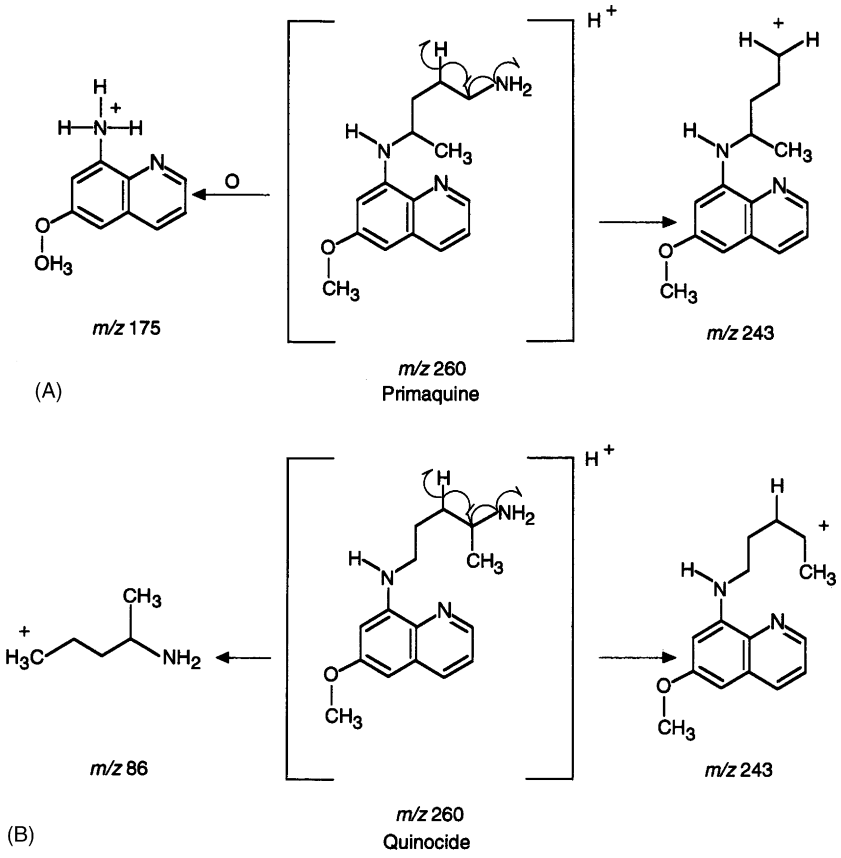

Fig. 6. The main routes of fragmentation for the quinocide and primaquine, as suggested by SFC–ESI–MS. ESI–MS experiments were performed using conditions stated above in Section 2.2. (A) The schema of the main routes of fragmentation for primaquine, as suggested by SFC–ESI–MS. (B) The schema of the main routes of fragmentation for quinocide, as suggested by SFC–ESI–MS.

in Fig. 5A matches that of Fig. 5C and the retention times of both match the retention time for quinocide.

Both primaquine and quinocide gave $[M+H]^+$ ion *m/z* 260. Elimination of NH_3 is observed from the protonated molecules of both primaquine and quinocide with the production of an ion with *m/z* 243. The ion with *m/z* 243 in the spectrum of primaquine is isomeric to the ion with *m/z* 243 in the spectrum of quinocide as is shown in Fig. 6A and B.

In-source CID fragmentation patterns for SFC–ESI–MS are similar to the fragmentation patterns observed for HPLC–ESI–MS and also GC–MS. However, in this particular case, fragmentation utilizing SFC–ESI–MS is more informative compared to HPLC–ESI–MS fragmentation. The characteristic base peak ion for quinocide is *m/z* 86 with SFC–ESI–MS. Statistically, there is more charge localized on the aliphatic fragment (Fig. 6B). In contrast, the characteristic base peak ion for primaquine is *m/z* 175, in this case statistically there is more charge localized on the aromatic fragment (Fig. 6A). The proposed fragmentation schemes are presented in Fig. 6A and B. The main fragmentations are the cleavages of the molecules into aromatic and aliphatic fragments. Thus, for quinocide, the preferable charge localization is on the aliphatic fragment, while the preferable charge localization for primaquine is on the aromatic fragment. It appears that the difference in the preferable localization of charge in fragmentation using SFC–ESI–MS depends on the position of branching. The branching points of the aliphatic chain in these molecules constitute a major difference between the substances.

Both substances display ions with *m/z* 243; however, the ion produced from quinocide is isomeric to the ion produced from primaquine, as is shown in Fig. 6A and B. The significant differences in the fragmentation pattern provide a means to distinguish the contaminant even in the absence of an authentic standard. The differential fragmentation using SFC–ESI–MS provides necessary and important information about the branching point and the position of the chiral carbon in the chain.

Pharmacopoeias [14,15] describe the drug primaquine as primaquine and an enantiomer; however, neither the separation nor quantification method for enantiomers is described in the *Pharmacopoeias*. In a previous publication [11], the author described the separation of enantiomers of primaquine by HPLC and HPLC–MS on a chiral column; however, the enantiomers were not resolved to the baseline. Several publications describe the separation of enantiomers of primaquine by HPLC [17], SFC [18] and even by capillary zone electrophoresis (CZE) [19]. In two papers [17,18], the authors failed to produce reliable documentation in the form of chromatograms supporting the claim of the separation of enantiomers of primaquine. In the CZE study [19], the authors claimed that primaquine is racemic. However, the definition for racemic is reserved for a mixture of equal quan-

tities of pure dextrorotatory and levorotatory isomers of the same compound; therefore, the racemic mixture is optically inactive. There is evidence that the drug primaquine diphosphate is not racemic. In previous related work [11] it was shown by CD that the solution of primaquine is optically active and by HPLC–MS it was shown that solution of primaquine is a mixture of several optic and positional isomers. Other evidence pointing to unequal quantities of *l*- and *d*- isomers involves toxicity studies [11,20].

Schmidt et al. [20], described differences in toxicity of *l*- and *d*-isomers of primaquine. In their experiments, the *l*-isomer was found to be three to five times as toxic as the *d*-isomer. The conclusion was that the *d*-isomer had a therapeutic index at least twice that of primaquine.

Fig. 3A shows the separation of the enantiomers of primaquine on a Chiralpak AD-H column using SFC. There are two major peaks, A and B, which are primaquine enantiomers, and a minor peak (peak C) which is the contaminant quinocide. The chromatogram of quinocide is shown in Fig. 3B; there is only one peak in this chromatogram. The co-chromatography of primaquine with quinocide is shown in Fig. 3C; here peak C is expected to be the contaminant co-eluting with quinocide, and indeed this peak does exhibit increased height and area. Peaks A and B, primaquine enantiomers, are also seen in Fig. 3C. The chiral separation of the enantiomers of primaquine (peaks A and B) is accomplished nearly to the baseline. Thus, the drug primaquine diphosphate is not a racemate as was claimed in [19]; rather it is a mixture of enantiomers of primaquine and the positional isomer quinocide. These results support the previous CD [11] and toxicity [11,20] evidence.

In the present work, the aim was mainly qualitative as opposed to quantitative. Also, the minimum amount of time required for resolution of the contaminant from primaquine was tested. Baseline separation using HPLC [13,16] required over 30 min, whereas it was possible to achieve nearly baseline resolution in less than 3 min on the same Discovery HS F5 column using SFC–MS. This is half the time of the fastest run, 6–7 min, published in ref. [13]. Of course some reduction in the quality of resolution is a result of the shortened analysis time. The minimum run time limit was tested because the consumption of time in analytical performance is typically of great importance in industrial laboratories.

The pairing of separation equipment to a mass spectrometer can be essential in resolution and verification of enantiomers from other isomers. As it was shown in ref. [11] the resolution of enantiomers from other isomers on a chiral phase (Chirex (S)-VAL and (R)-NEA was not complete. MS detection, especially in the case of toxic contaminants in drugs, should be used.

The instrumentation was optimally tuned to receive informative fragmentation around the branching point in the aliphatic chain. In the present experiment using the Chiralpak AD-H column, resolution between the optical *l*- and *d*-isomers, and between optical isomers versus positional isomers was monitored by MS. Differences in fragmentation between primaquine and quinocide were taken as criteria for verification. The SFC–ESI–MS combination was more suitable for this task than SFC–APCI–MS despite better sensitivity in the latter.

4. Conclusion

SFC–ESI–MS analysis supports earlier findings [11–13,16] that the main contaminant in the anti-malaria drug primaquine diphosphate is quinocide, the positional isomer of primaquine. SFC gave good resolution of these positional isomers using both a 2-ethyl-pyridine column and a Discovery HS F5 column. The analysis time was shorter than with HPLC and GC yet still provided adequate resolution; thus, SFC provides a means for efficient and rapid analyses.

Fragmentation of isomers during SFC–ESI–MS identified branching points in the aliphatic chains of these substances. It was shown that fragmentation can be used to distinguish between primaquine and quinocide, making it an important tool especially for cases such as this, where a standard for one of the positional isomers (quinocide) is not commercially available.

These experiments with SFC–ESI–MS could be useful in the branching point analysis for molecules other than those described in this work. Establishment of the branching point in a molecule often requires the preparation of a derivative or time-consuming experiments, but it may be possible to eliminate these steps by using SFC–ESI–MS.

Using SFC equipped with a Chiralpak AD-H column, nearly baseline chiral separation of the enantiomers of primaquine was accomplished, and quinocide was separated from the primaquine *l*- and *d*- isomers.

SFC is an efficient time-saving technique for analytical procedures as well as a powerful tool for enantiomeric separations.

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