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Simultaneous separation of atovaquone, proguanil and its metabolites on a mixed mode high-performance liquid chromatographic column

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Abstract

An isocratic high-performance liquid chromatographic (HPLC) method for simultaneous separation of the components in the antimalarial combination drug Malarone® with UV detection is described. An HPLC system using a mixed mode column composed of 50% C_{18} phase and 50% strong cation-exchanger has been optimised for the simultaneous separation of atovaquone, proguanil and its two main metabolites. The mobile phase was optimised for factors such as pH, counter ion concentration and acetonitrile. Elimination of interferences from other antimalarial drugs was achieved by adding sodium perchlorate to the mobile phase. With a mobile phase of acetonitrile-phosphate buffer (60:40, v/v) pH 6.8, 50.7 mmol l^{-1} K^+ and 10 mmol l^{-1} $Na{\cdot}ClO_4$, separation was achieved within a run time shorter than 17 min.

Keywords: Atovaquone; Proguanil; Malarone

1. Introduction

Atovaquone (ATQ), a hydroxynaphthoquinone, and proguanil (PG), a biguanide, have shown synergistic activity against *P. falciparum* in vitro [1]. These substances were combined in the new antimalarial drug Malarone®, developed by Glaxo Wellcome (UK). ATQ is thought to inhibit the mitochondrial respiratory process of the malaria parasite by blocking electron transfer mechanism at the cytochrome complex [2,3].

ATQ is a lipophilic compound and difficult to handle because of its physiochemical properties. It is poorly soluble in a variety of organic solvents and its solubility in aqueous solvents is very limited (<100 ng ml^{-1}) [4]. PG and its metabolites, cycloguanil (CG) and 4-chlorphenylbiguanid (4-cpb), are more hydrophilic and show basic properties.

The major chromatographic difficulty was to establish chromatographic conditions for simultaneous separation of ATQ, PG and its two metabolites with a suitable internal standard by a common hydrophobic stationary C-18 phase, due to the great differences in the chemical properties of the analytes. One method for a reversed-phase liquid chromato-

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graphic separation of atovaquone, proguanil and related substances was optimised [5] but the method presented no separation for the metabolites of PG. Methods for analysis of PG and its main metabolites CG and 4-cpb have been developed earlier [6–9].

A recently commercially available mixed mode column with a stationary phase composed of 50% hydrophobic C-18 phase and 50% strong cation-exchanger allows simultaneous analysis of hydrophobic and ionic analytes [10]. By using this column a method for the separation of the components of Malarone® was developed. ATQ and the internal standard interact with the hydrophobic phase and their retention times could be changed by the amount of acetonitrile and the pH of the mobile phase. PG and its metabolites interact mainly with the strong cation-exchanger and thus their retention times could be changed by the kind and concentration of counter ions in the mobile phase. By a combination of these mobile phase components a baseline separation was achieved for these five analytes.

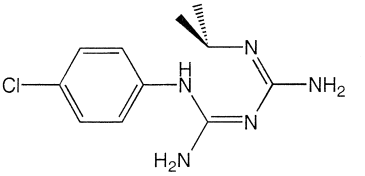
2. Experimental

2.1. Chemicals and materials

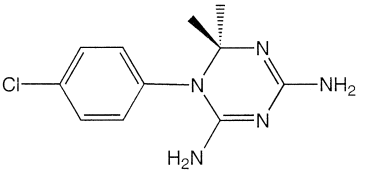
Atovaquone (566C80) and the internal standard (IS), *trans*-2-hydroxy-3-(4-phenylcyclohexyl)-1,4-naphthalenedione, (59C80) were obtained from Glaxo Wellcome (UK). PG, CG and 4-cpb were obtained from Zeneca (Cheshire, UK). The structures are shown in Fig. 1. Acetonitrile (HPLC-grade), methanol (p.a.) and dimethylformamide (p.a.) were obtained from Merck (Darmstadt, Germany). All other reagents and chemicals used were of HPLC or analytical grade from Merck (Darmstadt, Germany). The aqueous solutions were prepared using high purity water obtained from a Milli-Q deionized water system (Millipore, Bedford, MA, USA).

2.1.1. Preparation of reference substances

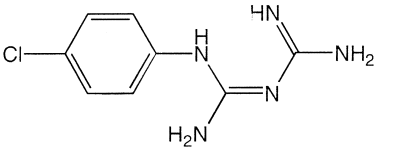
Stock solutions of ATQ (500 μmol l^{-1}) and I.S. (250 μmol l^{-1}) were prepared in methanol-dimethylformamide (99:1 v/v). Stock solutions (500 μmol l^{-1}) of PG, CG and 4-cpb were prepared in water. Various working solutions were obtained by diluting the stock solutions with the mobile phase



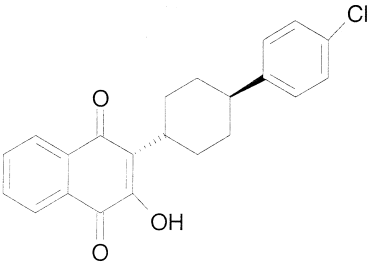
Proguanil



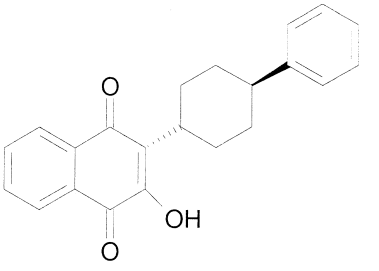
Cycloguanil



4-chlorphenylbiguanid



Atovaquone



Internal standard

Fig. 1. Structural formulas.

(acetonitrile-phosphate buffer 60:40 v/v, pH 6.8). The solutions were stored at +4°C.

2.1.2. Preparation of buffer solutions

The buffer solutions used in the mobile phases were prepared by mixing the right amount of potassiumhydroxide and potassium-di-hydrogen-phosphate with water and thus achieving the desirable pH and K^+ concentration.

2.1.3. Instrumentation

The HPLC system consisted of a ConstaMetric III HPLC pump (LDC/Milton Roy, Riviera Beach, FL, USA) and a WISP 710B autoinjector (Waters Assoc., Milford, MA, USA) with an Absorbance detector 757 set at 254 nm (Applied Biosystems, USA). The column was Hypersil Cation Duet 5 μ, 250×4.6 mm (Hypersil, Cheshire, UK). The peaks were evaluated with Chromatography Station for Windows 1.6 (Data Apex Ltd. Prague, Czech Rep.). The flow-rate through the column at ambient temperature, was 1.0 ml min^{-1}.

2.2. Optimisation

The effects on the capacity factors for the analytes caused by variations of pH, acetonitrile and K^+ concentration in the mobile phase were investigated. The pH was changed between pH 3 and 7, measured on the aqueous part of the mobile phase, in 60% v/v acetonitrile (ACN) and 60 mmol l^{-1} K^+. The concentration of acetonitrile in the mobile phase was changed between 50 and 60 vol% at pH 5.5 and 60 mmol l^{-1} K^+. The concentration of K^+ was changed between 20.9 mmol l^{-1} and 80.8 mmol l^{-1} at constant pH of 6.8 and 60% v/v acetonitrile.

2.3. Selectivity

To evaluate the separation selectivity several antimalarial drugs were tested. Water solutions of chloroquine, desethylchloroquine, mefloquine, carboxymefloquine, pyrimethamine, sulfadoxine and amodiaquine with a concentration of 10 μmol l^{-1} were injected. The mobile phase consisted of acetonitrile-phosphate buffer 60:40 v/v, pH 6.8, 65 mmol l^{-1} K^+. Mefloquine and amodiaquine interfered with CG and 4-cpb, but this was eliminated by adding sodium perchlorate to a concentration of 10 mmol l^{-1} to the mobile phase ACN–phosphate buffer 60:40 v/v, pH 6.8, 50.7 mmol l^{-1} K^+.

2.4. Determination of the limit of detection

The limit of detection (LOD) was defined as the analyte concentration resulting in a peak height three times the peak-to-peak noise level of the baseline when a sample of 100 μl was injected. Following mobile phase was used: acetonitrile–phosphate buffer 60:40 v/v, pH 6.8, 65 mmol l^{-1} K^+.

2.5. Column degradation

The mobile phase acetonitrile–phosphate buffer 60:40 v/v, pH 6.8, 65 mmol l^{-1} K^+ was used for the comparison of separations made on a new and a former used Hypersil Cation Duet column. The same test solution was injected on both columns.

3. Results and discussion

3.1. pH variation

Fig. 2 shows the influence of mobile phase pH on the capacity factors of the compounds. PG and its metabolites show basic properties with pKa values above the pH interval suitable for an HPLC column and thus they are positively charged and interact with the strong cation-exchanger and are not affected by changes in pH. At pH 7 one can see a small increase in their capacity factors as the pH gets closer to their

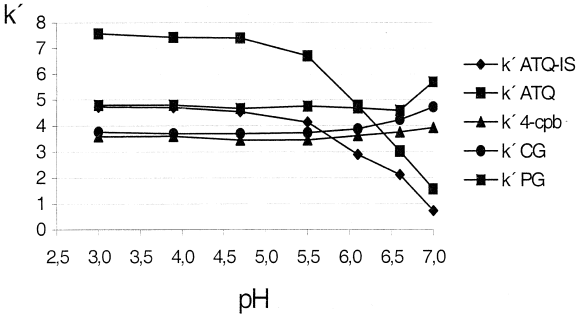


Fig. 2. Effect of pH variation on the capacity factor for the five analytes.

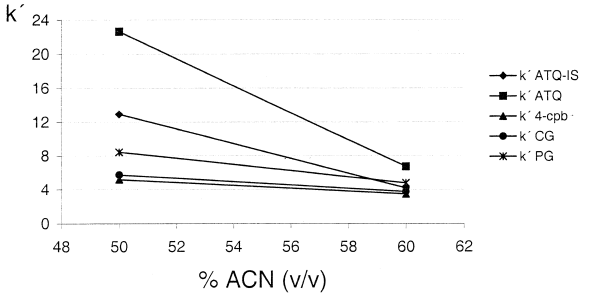


Fig. 3. Effect of acetonitrile on the capacity factor for the five analytes.

pKa values. ATQ and I.S. have acidic properties and their capacity factors decrease rapidly as the pH increases. This is an effect of the weaker interactions with the hydrophobic part of the stationary phase as these analytes get negatively charged.

3.2. Acetonitrile variation

Fig. 3 shows that the concentration of acetonitrile has a strong effect on the retention of ATQ and I.S. Their lipophilic character demand very high concentrations of organic modifiers. The capacity factors of PG, CG and 4-cpb are less sensitive to changes in the acetonitrile concentration since they interact mainly with the ion-exchanger.

3.3. K^+ variation

Fig. 4 clearly shows that the concentration of K^+ in the mobile phase greatly influences the capacity factors for PG and its metabolites and therefore is the main tool in changing their retention times. PG and metabolites are basic analytes with positive charges at pH below 7, resulting in an ion-exchange interaction with the stationary phase. The decrease in retention time for the basic analytes with increased concentration of K^+ seems to be an ion-exchange mechanism were K^+ function as counter ions and compete with the positively charged analytes about the negatively charged sites on the stationary phase.

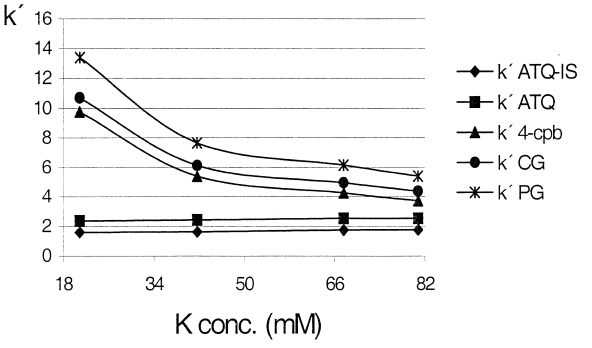


Fig. 4. Effect on the capacity factor for the five analytes caused by changes in the K^+ concentration of the mobile phase.

3.4. Optimising separation conditions

The optimisation of a mobile phase that gave a good separation ($R_s \geq 2$) was performed from the results given in Fig. 2 to 4. The mobile phase that gave the best separation consisted of acetonitrile–phosphate buffer (60:40 v/v) pH 6.8, K^+ concentration 65 mmol l^{-1}. Fig. 5 shows the chromatogram.

3.5. Selectivity

Amodiaquine and mefloquine interfered with CG and 4-cpb and it was not possible to eliminate this interference by changing the pH, ACN or potassium ion concentration of the mobile phase. But separation was achieved when sodium perchlorate was added to the mobile phase as an ion pairing reagent. Adding of the sodium perchlorate also resulted in shorter retention times for PG, CG and 4-cpb.

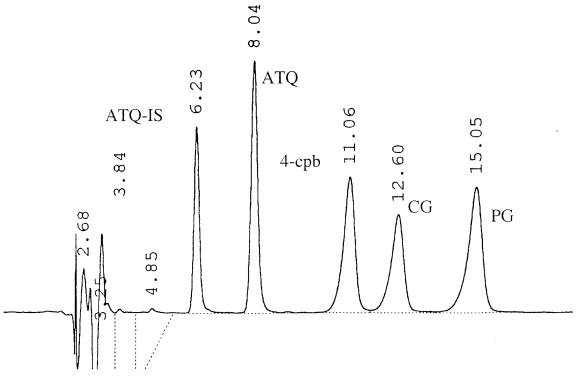


Fig. 5. HPLC profiles for the separation of the analytes. Column: C 18/SCX, Hypersil Cation Duet. Mobile phase: acetonitrile–phosphate buffer pH 6.8 (60:40 v/v), 65 mmol l^{-1} K^+. Figures on the top of the peaks are retention times in min.

3.6. Limit of detection

The limit of detection was 3 pmol for ATQ and 5 pmol for PG, CG and 4-cpb.

3.7. Column degradation

More than 25 000 column volumes have been pumped through the first column. It seems to be the ion-exchange part of the stationary phase that is degraded over time, see Fig. 6. Separation is achieved with both columns but the retention times of the bases seem to become shorter as the column is used. The difference in retention times for ATQ and I.S. is so small that it's more likely caused by batch variation or just differences between two different separation runs. The retention of the bases could preferable be changed by adjusting the concentration of K^+.

A validation of an analytical HPLC method with SPE mixed mode technique with the presented separation of the Malarone® components will be presented.

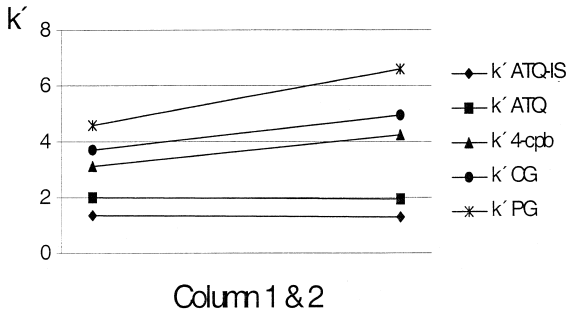


Fig. 6. Differences in the capacity factor for the five analytes with separation made on a former used (1) and a new (2) column C 18/SCX Hypersil Cation Duet.

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