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Journal of Pharmaceutical and Biomedical Analysis
27 (2002) 1–7

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Dual-mode gradient HPLC procedure for the simultaneous determination of chloroquine and proguanil

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Received 20 December 1999; received in revised form 22 March 2001; accepted 27 March 2001

Abstract

In order to assay the antipaludic capsule of the Service de Santé des Armées (SSA), that contains two antimalarial drugs, i.e. chloroquine sulfate (CQS, cp1) and proguanil hydrochloride (PGH, cp5), a HPLC procedure was developed. A reversed-phase ion-pair high-performance liquid chromatography (HPLC) method with an ultraviolet detection at 254 nm was set up and validated. Elution system includes programming of both organic concentration and flow-rate known as 'dual-mode gradient'. This method allows the simultaneous determination of both active compounds and separation of four process related substances. The method is simple, rapid, selective and accurate, and the precision is good with an inter- and intra-assay of $< 2\%$. The sensitivity is particularly suitable for pharmaceutical quality control.

Keywords: Chloroquine sulfate; Proguanil hydrochloride; HPLC-UV; Quality-control; Validation; Elution and flow-rate gradient

1. Introduction

Chloroquine has been the most prescribed drug for the treatment of malaria for more than 50 years and proguanil (also termed chlorguanide) has been used since 1946. The pharmaceutical form of SSA includes the two major active compounds whose chemical synthesis requires several steps leading to different related substances, either synthetic intermediate products or degradation products (i.e. 7-chloro-4-(2-methyl-pyrrolidin-1-yl)quinoline (cp2), 5-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline (cp3), 7-chloro-4-(4-ethylamino-1-methylbutylamino)quinoline (cp4)) and (isopropylamine (cp6), 4-chloroaniline (cp7), isopropyldicyandiamide (cp8)). Some of them could be found in the raw material or in the pharmaceutical form.

For the pharmaceutical control of CQS and PGH contained capsule, an improved HPLC procedure was developed to carry out the quality control of the antimalarial capsule compounded by the SSA quantifying these two active substances. In order to increase the specificity, we looked for a method able to discriminate both process-related substances and potential degradation products from parent drugs.

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0731-7085/02/$ - see front matter
PII: S0731-7085(01)00555-6

In the literature, there was no report of assays allowing simultaneous determination of all these compounds. Some papers [1–3] only described the separation of either CQS and PGH. In contrast, the assay reported by Chaulet et al. [4] was of major interest as it allowed the separation of CQS, PGH and some metabolites in blood using an RP-HPLC method. However, this HPLC method was not adapted to routine determination of PGH regarding the long retention time (> 35 min). Moreover, the elution conditions did not make possible the separation of CQS, PGH and the four related substances. Thus, the present work deals with the simultaneous determination of both active compounds, for the assessment of the stability of the bulk drug and of pharmaceutical dosage forms, using a polarity (i.e. organic concentration) and flow-rate gradient RP-HPLC.

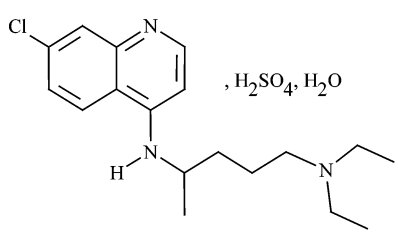
2. Experimental

2.1. Materials

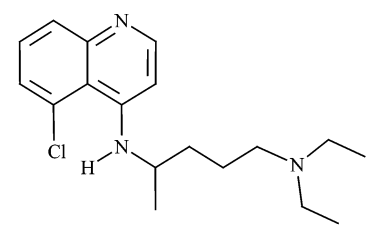
All chemicals were of analytical grade. CQS and PGH were purchased from Rhône Poulenc Rorer (Antony, France) and Panchim (Lisses, France), respectively. Ammonium formate (Fluka, St Quentin, France), phosphoric acid 85% (Merck, Darmstadt, Germany) and methanol (Baker, Noisy, France) were used for the mobile phase preparation. All aqueous solutions were prepared using high-purity water obtained from the water purification system Milli Q® (Millipore, St Quentin, France). Stock and working solutions were made in 0.01 M hydrochloric acid (Merck, Darmstadt, Germany); final concentrations were 0.68 and 1 mg/ml for CQS and PGH, respectively. Working solutions contained 34 and 50 μg/ml, for CQS and PGH, respectively, and were diluted with 0.01 M hydrochloric acid prior to use.

2.2. Structures and physico-chemical characteristics of compounds

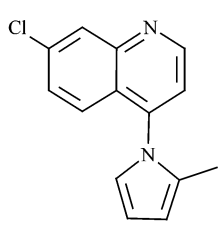
The structures of chloroquine sulfate (CQS, cp1) or (*RS*)-7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline sulfate and their related substances (i.e. 7-chloro-4(2-methylpyrrolidin-1-yl)quinoline (cp2), 5-chloro-4(4-diethylamino-1-methylbutylamino)quinoline (cp3), 7-chloro-4(4-ethylamino-1-methylbutylamino)-



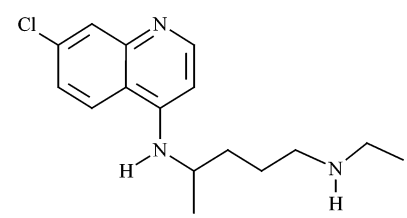
Chloroquine sulphate
(Compound 1)



Compound 3



Compound 2



Compound 4

Fig. 1. Chemical structures of chloroquine sulfate and their synthesis intermediates.

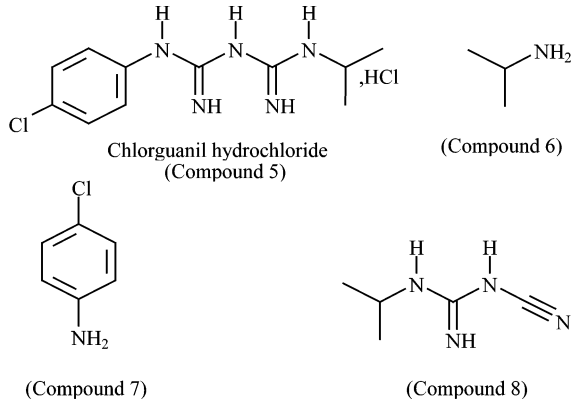


Fig. 2. Chemical structures of proguanil hydrochloride and their synthesis intermediates.

quinoline (cp4)) are depicted in Fig. 1. The latter compounds were supplied by the manufacturer of the parent drug.

The structures of proguanil hydrochloride (PGH, cp5) or 1-(4-chlorophenyl)-5-isopropyl biguanide hydrochloride and their synthetic intermediate products (isopropylamine (cp6), 4-chloroaniline (cp7), isopropyldicyandiamide (cp8)) are shown in Fig. 2. The synthesis of PGH requires isopropylamine and the sodium salt of dicyandiamide to form isopropyldicyandiamide. The condensation of the latter with 4-chloroaniline leads to proguanil. The intermediates were supplied by the same manufacturer as for PGH.

The UV absorbance spectra of CQS, PGH, compounds **2**, **3**, **4** and **7** exhibit local maxima in the 254-nm region but compounds **6** and **8** could not be determined because they do not absorb at this wavelength.

2.3. HPLC instrumentation and chromatography

The LC material was a HP serie 1100 system (Hewlett Packard, Waldbronn, Germany) driven by ChemStation® software. This system was composed of a quaternary pump, an autoinjector, a mobile phase degazer and an ultra-violet detector equipped with a 14-μl flow cell which was set at 254 nm. Chromatographic separations were performed on a 250 × 4 mm i.d. column packed with 5-μm C_{18} Lichrospher® 60 RP-select B (Merck) specially designed to separate alkaline compounds. A 5-μm C_{18} Lichrospher® guard column (10 × 4 mm i.d.) was set between the injector and the analytical column. Injection volumes were 5 or 10 μl. The column was thermostated at 25° C during analysis. The mobile phase consisted of 0.5 M ammonium formate, 0.075 M phosphoric acid and methanol. The pH of the aqueous phase was adjusted to 4 with phosphoric acid and filtered through a 0.45-μm filter and the mobile phase degassed by sonication. Initial proportion of the mobile phase was water/methanol (60:40, v/v) and an elution gradient was used during the analysis (60:40, to 45:55, v/v). The flow-rate was increased during the run from 0.7 to 1 ml/min after 7 min.

2.4. Standard solution and sample preparation

Stock solution of CQS and PGH (1 mg/ml and 0.68 mg/ml) were prepared in 0.01 N hydrochloric acid.

To determine the assay of the SSA capsules, samples were prepared by dissolving one capsule in 200 ml of HCl 0.01 N to afford a 1 mg/ml solution of CQS and a 0.68 mg/ml solution of PGH. These solutions were filtered through 0.45-μm membrane filter and directly injected.

By means of different placebo mixtures it was demonstrated that the following excipients contained in the SSA capsule do not adversely affect the results—pregelatinized starch, magnesium stearate, aerosil 200 and the capsule container.

2.5. Validation protocol

According to 'ICH topic Q2A et Q2B' [1,2], the validation of an analytical technique requires specificity, linearity, accuracy, precision and the measurement range. Experiments regarding the analytical validation were carried out according to SFSTP guidelines [3]. According to the 'note for guidance on validation of analytical procedures: methodology' of ICH (CPMP/ICH/281/95) topic Q2B [2], the range for the assay of an active substance or a finished product is normally from 80 to 120% of the test concentration.

On 3 different days, two series of calibration samples corresponding to 80, 90, 100, 110 and

120% of nominal concentration of drug were prepared. One with the active substances in solution and one with the active substances from the finished product. Calibration curves were performed in triplicate for five concentration levels. Accuracy was daily assessed with five calibration points made with drug contained capsules. The precision was done over 3 days. Six measurements of sample prepared from the capsule were daily performed corresponding to 18 independent results. Statistical analysis was carried out using AVA software (SFSTP-QUALILAB, Orléans, France).

3. Results and discussion

3.1. Optimisation of the LC procedure

The LC procedure was optimised with a view to develop a quantitative and stability indicating method in a convenient time analysis according to previous papers measuring either CQS and PGH [4–6] or both [7]. At the first attempt, an isocratic mobile phase (methanol 40%) was used to separate the six compounds which absorb at 254 nm. Unfortunately, CQS and compound **2** were not well resolved, and time analysis (i.e. 35 min) was far too long.

We decided to investigate the use of an elution gradient to improve resolution and time analysis. At the beginning, the organic proportion of the mobile phase was set at 40% until 4 min to allow a sufficient resolution, and subsequently increased to 60% to obtain a time analysis suitable for routine use. To avoid elution condition problems on the next chromatogram, we chose to combine a flow-rate gradient with an elution gradient also termed as dual-mode gradient. The first one was used to separate the CQS and its pyrrolidinyl derivative and to reduce the time of analysis. Then, the flow-rate's gradient was set to reduce the time analysis. Fig. 3 represents the diagram of the gradient used. The initial proportion of methanol was kept at 40% (v/v) until 4 min. The methanol was successively increased up to 50% (v/v) from 4 to 7 min and to 55% (v/v) from 7 to 8 min, allowing fine resolution of the last compounds. The methanol proportion was kept at 55% (v/v) for 7 min (15 min) in order to appropriately separate compounds **3**, **7** and **4**. After this time, both methanol proportion and flow-rate gradient were applied, increasing the flow-rate from 0.7 to 1 ml/min in 1 min (7–8 min). A 1-min gap was scheduled to return to the initial conditions. These parameters were held constant in order to shorten time analysis. This analytical method allows the simultaneous determination of the six compounds we had to chromatography as shown on Fig. 4 and can be used for routine control of the pharmaceutical form.

3.2. Validation of the method

3.2.1. Specificity

Separation of CQS and PGH from their related substances is achieved. Fig. 4 shows HPLC profiles of a working solution of both parent drug (CQS and PGH) and related compounds prepared in 0.01 N HCl spiked with 0.68, 1.0 and 0.2 mg/ml, respectively. The resolution factors (R_s) obtained between the different compounds are

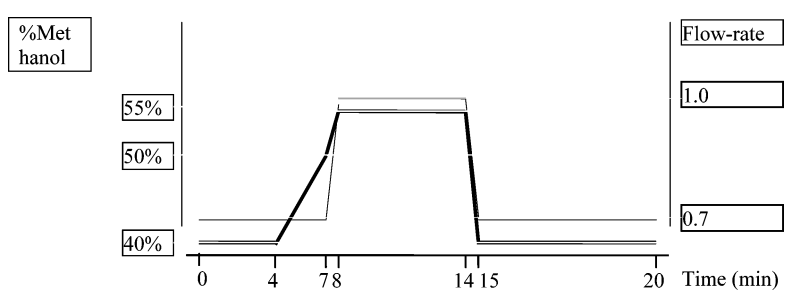


Fig. 3. Detail of elution and flow-rate gradient.

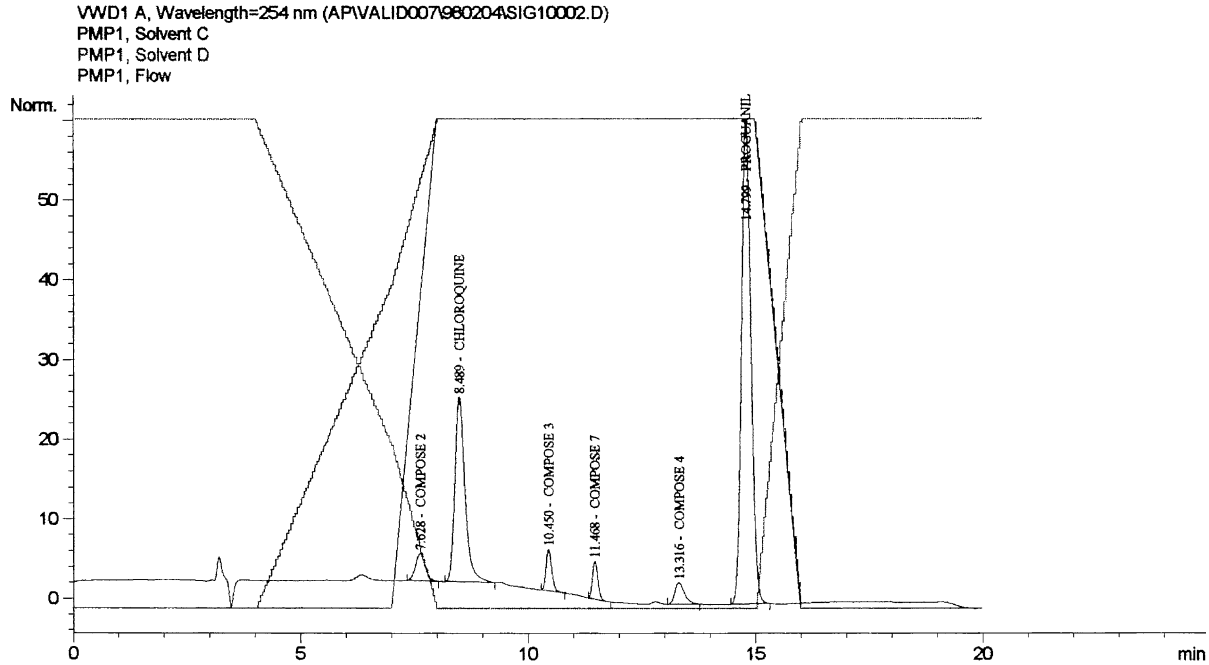


Fig. 4. Chromatogram of a solution containing active substances and intermediate compounds with the dual-mode elution gradient system. NB: proguanil is the other name of proguanil.

shown in Table 1. In any case, R_s values were greater than 2 highlighting the good specificity of this HPLC method. None of the auxiliary substances absorb at 254 nm.

3.2.2. Linearity

Over the concentration range 80–120% of the nominal concentrations of active compounds, the areas exhibited a linear responses with $r^2 = 0.993$ for CQS and $r^2 = 0.998$ for PGH, in both solutions of active substance alone (AS) and solutions of active substance of the pharmaceutical form (PF). Least square linear regression curves obtained for CQS in solution and drug contained capsules led to the following equations, $y = 29.02x + 12.00$ and $y = 30.08x - 6.37$, respectively, and for PGH, $y = 35.02x + 11.44$ and $y = 35.25 - 16.66$, respectively. These curves demonstrate the perfect linearity of the method in the range 80–120% of nominal concentration.

3.2.3. Accuracy

Accuracy was calculated as the relative difference (%) between the amount of drug added to a 0.01 M HCl solution and the amount of drug measured in the finished product. Results obtained for both drugs are displayed in Table 2. One could expect that the confidence interval of mean recovery should include the 100% value. However, this relative inaccuracy is acceptable as the confidence interval is low and the average

Table 1
Resolution factor between the different compounds

Compounds	Resolution factor (R_s)
Compound **2**	2.0/CQ
Chloroquine (CQS)	2.0/Cp2;17.5/PG; 7.6/Cp3
Compound **3**	7.6/CQ; 5.1/Cp 7
Compound **7**	5.1/Cp 3; 5.7/Cp 4
Compound **4**	5.7/Cp 7; 4.8/PG
Proguanil (PGH)	4.8/Cp4; 17.5/CQ

Table 2
Statistical results of the accuracy study of each active substance

	Confidence interval (0.95)	Mean of recovery
Chloroquine	[101.07; 102.39]	101.74
Proguanil	[98.24; 99.87]	99.06

value of recovery is close to 100%. Moreover, acceptance for values were defined as $\pm 5\%$.

3.2.4. Precision

The coefficient of variation for repeatability (CVr) and for reproducibility (CVR), for both active substances, were 0.79, 1.05, respectively for CQS and 1.65, and 1.74, respectively, for PGH. For the intermediate precision as well as the repeatability all CV values were less than 2%.

In addition to the validation parameters above, the robustness of the method was evaluated. The effect of the pH of the buffer used to prepare the mobile phase, the effect of the temperature, the effect of the methanol concentration and column-to-column reproducibility were investigated. As the pH of the buffer was increased up to 5, the resolution between compound **2** and CQS decreased while other compounds were not affected. No temperature effect was observed when the column oven was removed: from 18 to 35 °C we did not observed any significant differences regarding the separation of the six compounds. Minor increases in methanol concentration caused a decrease in the resolution of the compounds cited previously while there were no significant change in the relative retention times of the other compounds. Reproducibility of the column was considered acceptable. In fact, after more than 5000 injections done in the last 2 years, the mean $t_R = 7.85 \pm 0.18$ min for CQS and 14.32 ± 0.09 for PGH. The validation data for this procedure substantiates the use of this method.

4. Conclusion

There are a few reports of reversed-phase dual-mode gradient elution (RP-dmGE) in the literature [8–12]. The RP-dmGE process is used to improve resolution and shorten the time analysis. However, it seems that one could be reluctant to develop an RP-GE method because it is acknowledged to be a less reproducible, more complex and less transferable technique.

Benefits of RP-GE have been clearly demonstrated. Among many papers, some [10–12] pointed out the improvement of resolution using a gradient elution system. Dappen et al. [10] clearly showed that an elution gradient improves separation and that a segmented gradient leads to further improvement. Allan et al. [13] showed that this system provides better detection. Others authors [8,9] demonstrated that this type of elution mode allows shorten retention times.

In contrast, we found only one reference in the literature describing both elution and flow-rate gradient. These authors used a dual-mode gradient ion-pair chromatograph to measure amino acids and bioactive amines in biological fluids [9].

To our knowledge this paper is the first report of a validation of a HPLC method using both a elution and flow-rate gradients for the pharmaceutical quality control. All validation parameters meet the requirements of ICH and SFSTP guidelines.

In comparison to the method used by Chaulet et al. [4], elution and flow-rate gradient allows identification of four compounds known as intermediates during organic synthesis while the time analysis was dramatically decreased by approximately 42%. Even though the retention mechanism of different compounds is not totally elucidated, changes in the mobile phase composition did not alter the validation parameters of the method.

This work clearly demonstrated that the official requirements in terms of analytical method complied with a sophisticated use of HPLC technique using both type of gradients (elution and flow-rate). In that way, the proposed technique illustrated the usefulness of the ICH and SFSTP validation requirements which was conveniently fulfilled in this case.

During the pharmaceutical control analysis none of intermediate compounds were found in any samples assayed. This method is perfectly suitable for measurement of drug (CQS and PGH) into capsules and in solution after dissolution tests.

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