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Journal of Pharmaceutical and Biomedical Analysis 42 (2006) 132–135
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

Development and validation of a reversed-phase LC method for analysing potentially counterfeit antimalarial medicines

Maria Cristina Gaudiano *, Eleonora Antoniella, Paola Bertocchi, Luisa Valvo

Dipartimento del Farmaco, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Roma, Italy

Received 24 October 2005; received in revised form 30 January 2006; accepted 31 January 2006
Available online 6 March 2006

Abstract

Pharmaceutical counterfeiting is more and more a public health problem, especially in developing countries where the most counterfeit drugs are antibiotics, antimalarials and other life-saving drugs. The evaluation of the phenomenon extent is of great concern to the World Health Organization for carrying out a global strategy to combat the phenomenon. To this purpose, a reversed-phase liquid chromatographic method to perform the separation and simultaneous determination of three different kinds of antimalarial drugs (chloroquine, quinine and mefloquine) was developed. The method was validated by using both commercial and in-laboratory produced tablets and was then verified on various in-laboratory produced formulations differing in excipient composition. Finally, the method was successfully applied to the analysis of medicinal samples purchased from the informal market in Congo, Burundi and Angola.
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Keywords: Antimalarials; Counterfeit drugs; Sub-standard drugs; Reversed-phase liquid chromatography; Screening analysis

1. Introduction

Pharmaceutical counterfeiting is a world-wide emerging health problem, but it is more and more diffused in developing countries where drug-regulatory systems are weak and controls on production, distribution and import are insufficient. In developing countries 20–60% of marketed medicines are counterfeit, mainly in the informal market (open-air market, stalls) [1–3].

The World Health Organization (WHO) defines a counterfeit medicine as that one which is deliberately and fraudulently mislabelled with respect to identity and/or source. Counterfeiting can apply to both branded and generic products and counterfeit products may include products with correct ingredients, wrong ingredients, without active ingredients, with incorrect quantity of active ingredients or with fake packaging. In developed countries the most frequently counterfeit medicines are new, expensive lifestyle medicines, such as hormones, steroids and antihistamines, while in developing countries most counterfeit medicines are those used to treat life-threatening conditions, such as antibiotics, antimalarials, anti-tuberculosis and antiretroviral drugs [4].

In the last years many articles reported examples of counterfeit and sub-standard medicines in developing countries. Counterfeit antimalarial drugs were found in Africa, Asia and South America. In particular, chloroquine counterfeit preparations with lower or no active substance were found in Tanzania, Sudan, Sierra Leone [5], artesunate and other antimalarial counterfeit drugs were purchased in the South-East Asia [6,7]. Considering the importance of the antimalarial treatment in the tropical developing countries, where malaria is endemic and is the first cause of death for children, the presence of counterfeit and sub-standard drugs in the pharmaceutical market is a risk for the life of million peoples. Moreover, the diffusion of sub-standard antimalarial medicines, i.e. medicines with a lower amount of active substance, induces drug resistance as well as it occurs with antibiotics [8,9].

The evaluation of pharmaceutical counterfeiting extent is of great concern to WHO for carrying out a global strategy to combat the phenomenon. According to WHO recommendations on the development of simple screening methods, a single liquid chromatographic method for determining the three most employed antimalarial drugs (chloroquine, quinine and mefloquine) in tablets and capsules of different formulations was

* Corresponding author. Tel.: +39 06 49902160; fax: +39 06 49902830.
E-mail address: gaudiano@iss.it (M.C. Gaudiano).

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doi:10.1016/j.jpba.2006.01.059

developed and validated. The method was successfully applied to the analysis of tablets from African market.

2. Experimental

2.1. Chemicals

Chloroquine diphosphate salt (purity > 98%) and quinine sulphate salt USP (purity = 99.8%) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Mefloquine hydrochloride reference standard was obtained from *European Pharmacopoeia* (EDQM, Strasbourg, France). Chloroquine 250 mg tablets (Bayer AG Leverkusen, Germany), mefloquine 250 mg tablets (Roche S.p.A., Milano, Italy) and quinine 250 mg tablets (Nova Argentia, Milano, Italy) were purchased from the Italian national market.

All other reagents were of analytical grade.

2.2. Standard and sample solutions preparation

Standard working solutions at the concentrations in the calibration range were prepared by dissolving the appropriate amount of chloroquine diphosphate in 50 mM potassium phosphate buffer, pH 2.9, and quinine sulphate and mefloquine hydrochloride in methanol. For System Suitability Test, a standard mixture of chloroquine, quinine and mefloquine at 0.1 mg/ml was prepared in a phosphate buffer/methanol mixture (1:2, v/v).

Chloroquine, quinine and mefloquine sample solutions were prepared by dissolving in buffer (for chloroquine) or in methanol (for quinine and mefloquine) the suitable amount of each powder obtained from six tablets to obtain a 1 mg/ml concentration in active substance. Solutions were then sonicated, centrifuged and the clarified solutions were diluted 1:10 with the same solvent.

2.3. Chromatographic analysis

The chromatographic apparatus consisted of an HPLC system Series 1100 equipped with an automatic injector and a photo-diode array detector (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). The chromatographic column was a Symmetry C18, 75 mm × 4.6 mm i.d., 3.5 μm particle size (Waters Corporation, MA, USA) thermostated at 30 °C. The detection wavelength was 230 nm and the injection volume was 10 μl.

Mobile phase A was prepared by dissolving 6.80 g of anhydrous potassium dihydrogen phosphate and 1.22 g of 1-pentanesulphonic acid sodium salt in about 900 ml of bi-distilled water and adding 1 ml of triethylamine. The pH was then adjusted to 2.9 ± 0.1 with phosphoric acid and the volume was brought to 1 l with water.

Mobile phase B consisted of acetonitrile.

The elution was a gradient delivered at 1 ml/min as follows: 0–5 min, from 90 to 70% A; 5–8 min, from 70 to 50% A; 8–10 min, 50% A. The system was then re-equilibrated to 90% A. Quantification of medicinal samples was obtained by calibration straight line at three points with standard solutions at a concentration of 0.08, 0.1 and 0.12 mg/ml.

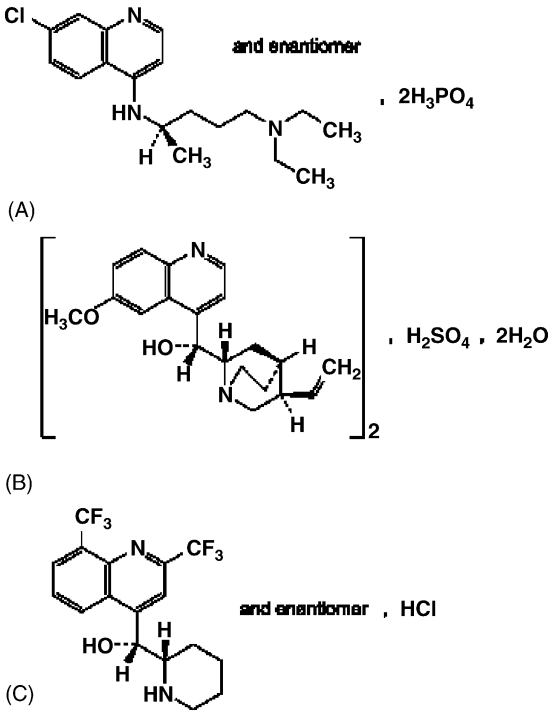

Fig. 1. Structure of chloroquine diphosphate (A), quinine sulphate (B) and mefloquine hydrochloride (C).

3. Results and discussion

3.1. Method validation

The method allows the separation and simultaneous determination of three different antimalarial drugs (chloroquine, quinine and mefloquine in different tablet formulations). The structure of the three analytes and the standard mixture chromatogram are reported in Figs. 1 and 2, respectively.

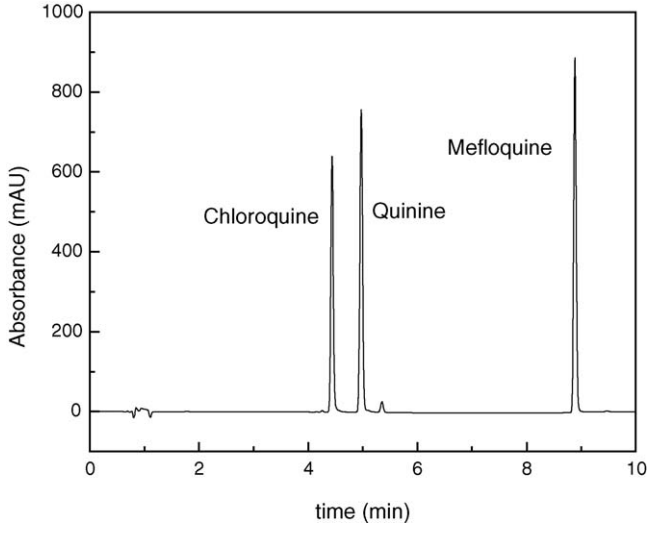

Fig. 2. Chromatogram of chloroquine, quinine and mefloquine standard mixture.

Table 1
Method linearity data

	Range (%)	Equation	r^2
Chloroquine	10–200	$Y = 20787.5X + 8$	0.99998
Quinine	10–200	$Y = 26453.1X + 10$	0.99996
Mefloquine	10–200	$Y = 26146.7X + 178$	0.998
Mefloquine	10–120	$Y = 28264X + 70$	0.9995

The method was validated following the EMEA 3AQ13a Guideline [10] by means of either Italian market branded drugs (chloroquine by Bayer and mefloquine by Roche) or an in-laboratory produced tablet lot composed of quinine sulphate, silica, sucrose, maize starch, talc and magnesium stearate.

Specificity was investigated by using tablets containing different excipients without active substance and verifying the absence of interferences.

Considering the large variability of counterfeit and substandard medicines marketed in developing countries, the linearity was evaluated in the range 10–200% of the test concentration on nine different concentrations. A good linear relationship was observed for chloroquine and quinine, while for mefloquine a slight deviation from linearity was observed at a concentration higher than 120%. In Table 1 the calibration curve equations and their correlation coefficient (r^2) are reported. For each calibration standard the concentration value from the linear equation was back calculated to confirm the linearity of the calibration curve. The back calculated values are in good agreement with the nominal values, thus confirming the linear calibration model. Only for mefloquine very high (150–200%) and very low (10–20%) values of the test concentration show a deviation from linearity.

Accuracy was determined on samples to which 5, 10 and 15% of active substance was added and calculated as difference between the recovery and the theoretical quantity. Precision was evaluated as intra-day (six determinations) and inter-day (three determinations) repeatability. In Table 2 the results of accuracy and repeatability are reported.

A System Suitability Test was performed by six replicate injections of the standard mixture verifying the following parameters: chloroquine/quinine resolution ≥2; %R.S.D. of each peak area <0.7%; %R.S.D. of each peak retention time <0.4%.

Standard and sample solutions were stable at least for one week at 2–8 °C, protected from light.

3.2. Application of the method to different tablet formulations and to potentially counterfeit tablets from African market

The performance of the method and its applicability to samples of unknown formulation were verified on in-laboratory prepared tablets containing different excipients. More than 10 different formulations with and without active substance were prepared by using different excipients mixed in different proportions: silica, sucrose, maize starch, talc, magnesium stearate, microcrystalline cellulose, lactose, mannitol, sugar and flour (to mimic a possible "hand-made" production in developing countries).

Table 2
Accuracy and intra-day (six determinations) and inter-day (three determinations) repeatability of the method

	Chloroquine	Quinine	Mefloquine
Intra-day precision			
Mean ($n = 6$) (%)	99.9	97.5	101.6
%R.S.D.	0.80	0.29	0.80
Mean ($n = 6$) (%)	102.7	98.0	100.9
%R.S.D.	0.46	0.66	0.30
Mean ($n = 6$) (%)	100.4	97.9	101.3
%R.S.D.	0.76	0.86	0.61
Inter-day precision			
Mean ($n = 3$) (%)	101.0	97.8	101.3
%R.S.D.	1.40	0.30	0.37
Accuracy (%)	−0.08	−2.00	−0.16
Linear equation (measured (%) vs. added (%))	$Y = 1.06095X + 99.92$	$Y = 0.9889X + 98.00$	$Y = 0.9571X + 99.84$
r^2	0.998	0.992	0.9995

In all cases the active substance content, measured in triplicate analysis, was found in the 98–102% range.

The method was then applied to the analysis of antimalarial tablets purchased from the informal market in Congo, Burundi and Angola. The aim of this study was to evaluate the quality of antimalarials sold in open-air markets and stalls, where the drug price is low in comparison with pharmacies. A form containing information on the site where the sample was sold, the price, the declared sample concentration and the storing conditions was written out for each sample. In the majority of the cases samples were without both primary and secondary packaging and tablets were packaged in a little plastic bag or enveloped with paper and the expiry date and strength of the active substance was written on a piece of paper. All samples were within the period of validity on the base of the declared expiry date. Only for some samples the Company name and the lot number were available.

The comparison between the chromatogram of a quinine tablets sample from Congo and an in-laboratory produced quinine tablets sample is reported in Fig. 3A and B. The sample from Congo was purchased from the informal market in Goma city, without primary packaging and at a cost of US$ 2.5 for 20 tablets. Only 88.6% (%R.S.D. = 0.6) of declared active substance (300 mg) was found. Moreover, a high quantity of impurities was observed with respect to the reference preparation, indicating some degradation probably due to the bad storing conditions. In particular, impurities at 4.44 and 5.30 min were found to be 4.8% ($\sigma_{n-1} = 0.1$) and 7.27% ($\sigma_{n-1} = 0.02$) of the quinine peak area, respectively. In the reference preparation the same impurities, on six determinations, were found at a concentration of 1.46% ($\sigma_{n-1} = 0.05$) and 3.66% ($\sigma_{n-1} = 0.03$), respectively. In all drugs from African market, the percentages of these impurities were variable and in many cases higher than those found both in the reference preparation and in the tablets from Italian market.

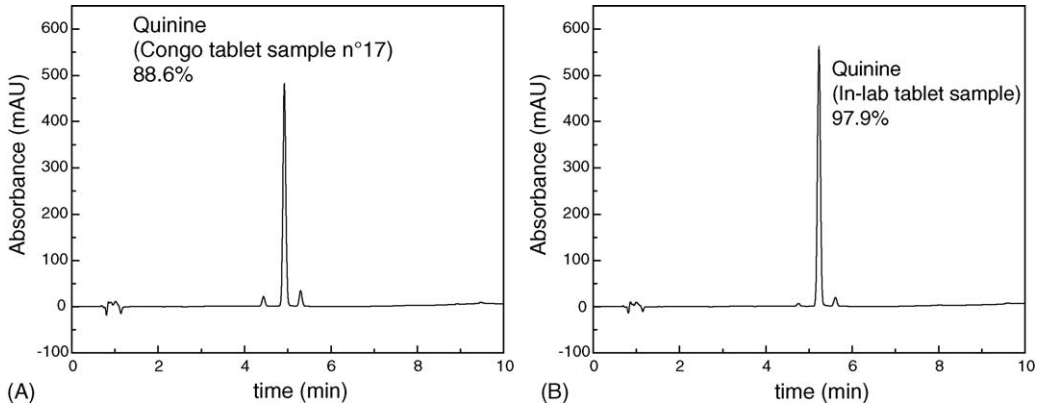

Fig. 3. Chromatogram of a sample of quinine sulphate tablets from Congo informal market (A) and of in-laboratory produced quinine sulphate tablets (B).

4. Conclusions

The method here described is suitable for determination of chloroquine, quinine and mefloquine in different formulations. The method is characterised by good linearity, precision and accuracy.

The use of a single method for the three most employed antimalarial drugs allows to perform screening analysis on different potentially counterfeit drugs to check their quality and to show a possible substitution of the more expensive active substance with the cheaper one (for example, mefloquine substitution by chloroquine or quinine).

Pharmaceutical counterfeiting of life-saving medicines should be considered a crime towards the humanity and the access to good quality medicines a right of all people in the world. Considering the world-wide diffusion of pharmaceutical counterfeiting, and in particular in developing countries, the development and validation of suitable methods for analysing medicines of unknown formulation can play key role in the strategy against pharmaceutical counterfeiting.

The developed method could be utilised by the national quality control laboratories in developing countries for the analysis of medicines of dubious origin. A future development of this work will be the extension of the method to other antimalarial dugs and the statistical evaluation of drugs quality in a number of developing countries.

Acknowledgements

The authors thank the Italian NGO VIS, Dr. Mariangela Agus, Dr. Giuseppe Guglielmi and Dr. Eugenio Marchetti for their help in obtaining the antimalarial drugs from African market.

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