Food Chemistry 142 (2014) 114–120

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Fast and reliable artemisinin determination from different *Artemisia annua* leaves based alimentary products by high performance liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 7 June 2012
Received in revised form 12 June 2013
Accepted 6 July 2013
Available online 18 July 2013

Keywords:
Artemisia annua
Artemisinin
Malaria
Medicinal plant
Liquid chromatography
Mass spectrometry

ABSTRACT

In many tropical countries malaria is endemic, causing acute illness and killing people, especially children. The availability of recommended malaria medicines is scant, even though these medicines are based on artemisinin, a compound extracted from the *Artemisia annua* plant that grows in many of these countries. New sources of treatment drawn from traditional medicine are therefore used, such as the tea infusion. An analytical method based on high-performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) was developed to quantify the artemisinin content of foods prepared with *Artemisia annua* leaves. A fast and reliable analytical method is described. The technique does not require any derivatisation prior to injection and offers excellent analytical intermediate precision. Robust qualitative and quantitative results were obtained using tea, biscuit or porridge specimens.

Although further research is needed to define the potential therapeutic benefits of these alimentary formulations, the analytical method described can be employed in developing more convenient and appropriate foods for administering artemisinin to those infected with malaria.

1. Introduction

Malaria is a parasitic infection of the blood caused by any of 5 *Plasmodium* species. The life cycle of plasmodia is complex and involves human and mosquito hosts. The Anopheline mosquito transmits infectious forms of the parasite from infected patients to uninfected individuals. The parasite damages erythrocytes and cellular debris engorges the sinusoids of the spleen and liver, causing splenomegaly, hepatomegaly, and other nonspecific features (Sturm et al., 2006). Malaria is endemic in most tropical regions, and each year affects approximately 225 million people worldwide. Of these people, 781,000, most of whom are African children, die from the disease (World Health Organization, 2012). Progress in shrinking the malaria map (the geographical range of endemic malaria) has been remarkable over the past 100 years, even though malaria continues to pose a major public health threat (Feachem et al., 2010). Malaria still represents a major health burden, particularly in Africa. The fragility of many health systems, the rise of insecticide and drug resistance, and particularly the expected decline both in funding and in the coverage of key interventions are a few of the challenges that urgently need to be addressed (Alonso & Tanner, 2013). Artemisinin and active derivative (dihydro-artemisinin, artesunate, artemether, and arteether) based combination therapies (ACTs) are effective in reducing malaria transmissibility and are currently recommended by WHO as the first-line treatment for malaria caused by *P. falciparum*, the most dangerous of the *Plasmodium* parasites that infect humans (World Health Organization, 2012). In Africa, however, only 27 of 44 countries are using these medicines in the public sector (Feachem et al., 2010). In particular, in some countries, the proportion of febrile children given antimalarials who receive ACTs remains low, which implies that a proportion of patients with malaria does not receive appropriate treatment (World Health Organization, 2012).

Pure artemisinin has a low solubility in water and oil, but can be administered orally (when possible, in patients without severe malaria and vomiting), rectally, and intramuscularly. Artemisinin and related compounds are extracted from the *Artemisia annua* plant, where it is present mainly not only in the leaves, but also in the small green stems, buds, flowers, and seeds. *Artemisia annua* is a vigorously growing annual weedy herb, usually single-stemmed, reaching up to 2–3 m in height. Cultivation of this plant requires a minimum of 6 months, and extraction, processing and manufacturing of the final product requires at least 2–5 months (World Health Organization, 2006). Artemisinin is a sesquiterpenoid

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http://dx.doi.org/10.1016/j.foodchem.2013.07.052

compound, the content of which in dry leaf mass of *Artemisia annua* is between 0.1% and 1.5% (Barbacka & Baer-Dubowska, 2011). The plant also contains other bioactive compounds, such as flavonoids, coumarines, steroids, phenolics, and purines (de Vries & Dien, 1996).

The cultivation of *Artemisia annua* has improved in many tropical regions (Ellman, 2010), in particular in African countries where malaria morbidity and mortality of children under five years are the highest (UNICEF, 2007; World Health Organization, 2006). In these countries, where needs and rights are frequently denied to majority of the population at risk of malaria (especially children) and where access to ACTs is limited, a tea preparation with *Artemisia annua* (5 g dried leaves in 1 litre of water every day for 7 days) has been suggested as a remedy, and is now widely used in the treatment of uncomplicated *Plasmodium falciparum* malaria (de Ridder, van der Kooy, & Verpoorte, 2008) and to prevent the disease and related fevers (Ogwang et al., 2011). Moreover, it has been proven that artemisinin is absorbed faster from herbal tea preparations than the pure compound (Mueller et al., 2004). However, even though malaria symptoms diminished rapidly, the cure rates in controls at follow up were lower than in those with recommended therapies (de Ridder et al., 2008). This could be due to the low dosage of artemisinin (40.2 mg/day taken in 1 litre of tea preparation versus at 500 mg/day in the adult as the reported effective clinical cure) (Willcox, 2009). There has been much debate, following comparisons with traditional remedies, as to whether the 500-mg artemisinin daily dose is unnecessarily high when taking into account results from *in vitro* tests of infected erythrocytes, and the fact that the Artemisia plant contains many compounds with potential antimalarial activity that synergize the activity of artemisinin (de Vries & Dien, 1996). However, more research is needed to, amongst other things, determine the most suitable species and genetic variety of Artemisia as well as the optimal preparation method and dosage of traditional preparations. It is true that considering the chemical–physical–therapeutic characteristics of artemisinin alone, and at the suggested therapeutic dose, a "tea formulation" is not feasible for the moment, given it would require consumption of 12.5 L/day for adults (500 mg/day: 40.2 mg/L) and 2.5 L/day for children (100 mg/day: 40.2 mg/L). Instead, other traditional sources must be used such as common foods (biscuits, porridge) enriched with *Artemisia annua* (Bonati, Severino, Bagnati, Carrà, & Fanelli, 2011).

Several analytical methods have been reported in the literature for extraction and detection of artemisinin. Solvent extraction of dried plant material is the most commonly applied extraction technique because it is simple and supported by considerable solubility data (Liu, Lu, & Pang, 2009). According to the literature, chloroform (Van Nieuwerburgh et al., 2006), toluene (Mannan et al., 2010) and hexane (Erdemoğlu, Orhan, Kartal, Adıgüzel, & Bani, 2007) are the most frequently used solvents. Other techniques, such as super critical fluid extraction (Kohler, Haerdi, Christen, & Veuthey, 1997) or microwave assisted extraction, have also been reported (Hao, Han, Huang, Xue, & Deng, 2002). The analytical techniques that have been developed for the detection and quantification of artemisinin are: thin layer chromatography with visible light densitometric detection (Koobkokkruad, Chochai, Kerdmanee, & De Eknamkul, 2007), high-performance liquid chromatography with UV detection (ElSohly, Croom, & ElSohly, 1987), HPLC with electrochemical detection (Acton, Klayman, & Rollman, 1985), HPLC with evaporative light-scattering detection (Avery, Venkatesh, & Avery,1999), HPLC with peroxyoxalate chemiluminescence detection (Karikari, Kishikawa, Ohba, Nakashima, & Kuroda, 2006), HPLC/tandem mass spectrometry (HPLC–MS/MS) (Xing, Yan, Zhang, Ren, & Gao, 2006), high performance capillary electrophoresis using a self-designed conductivity detection system (Huang & Yao, 2006), gas chromatography/mass spectrometry (GC/MS) (Woerdenbag et al., 1991), and enzyme-linked immunosorbent assay (ELISA) (Ferreir & Janick, 1996).

In the present work, HPLC–MS/MS was applied to analyse artemisinin from three different food preparations (tea, biscuits, and porridge) enriched with dried leaves of *Artemisia annua*.

2. Materials and methods

2.1. Materials and reagents

Artemisia Annua leaves from a hybrid plant cultivated in Brazil were used (de Magalhães, Delabays, & Sartoratto, 1997). New hybrid lines of antimalarial species *Artemisa annua* L. guarantee its growth in Brazil. The dried leaves were sieved (sieve size = 5 mm) and packed up, according to national drug regulatory indications, in 1.25 g preparations by Farmanguinhos (Rio de Janeiro, RJ, Brazil). These bags were used to prepare tea, biscuits, and porridge. Artemisinin analytical standard was purchased from Sigma–Aldrich (Milwaukee, WI, USA); artemether, in the form of Coartem tablets, was obtained from Novartis (Basel, Switzerland) and was used as internal standard (IS). Acetonitrile (HPLC grade) and ethanol (analytical grade) were purchased from Carlo Erba (Milano, Italy). HPLC grade Milli-Q water was obtained with a Millipore Milli-Q RO Plus 90 apparatus (Molsheim, France). Ammonium acetate (HPLC–MS grade) was purchased from Fluka (Buchs, Switzerland).

2.2. Tea preparation

Two different procedures, reported by Räth, Taxis, Walz, Gleiter, Li, & Heide in 2004, were used to prepare tea samples, which were called Tea-A and Tea-B, respectively.

Tea-A: 1 L of tap water was heated to the boiling point, then, after removing the heat source, 5 g of dried *Artemisia annua* leaves were added. After 5 min and gentle mixing, the water was filtered and allowed to cool at room temperature. An aliquot of 0.2 mL of tea was spiked with the internal standard, at the concentration of 50 μg/mL, and then diluted 1:100 with MilliQ water. Tea-B: 1 L of tap water was heated to the boiling point and 5 g of dried *Artemisia annua* leaves were added. After 30 min of continuous boiling, the water was filtered and allowed to cool at room temperature. An aliquot of 0.2 mL of tea was spiked with the internal standard, at the concentration of 50 μg/mL, and then diluted 1:100 with MilliQ water. These procedures were repeated three times and all samples were directly analysed by LC/MS.

2.3. Biscuit preparation

In Kenya, a malaria endemic country, millet (a cereal with high nutritional benefits) is cultivated and used to prepare, amongst other foods, unrefined biscuits. To reproduce this local product, 20 g or 40 g of millet flour were combined with 1.5 g (Biscuit-A) or 5 g (Biscuit-B) of dried *Artemisia annua* leaves, respectively, in a bowl. The dry ingredients were mixed together and 60–120 ml of milk or water was added until the dough pulled away from the sides of the bowl. A blank sample was also prepared without *Artemisia annua* leaves. Salt, butter and sugar or honey can be added at discretion. The dough was dropped into moulds and then weighed. The three unbaked biscuits were called Bis-A, Bis-B and Bis-blank respectively. They were baked at 180 °C for 20 min and then weighed again to assess their weight loss. All samples were stored frozen at −20 °C. Before analysis, the frozen samples were ground in a cold mortar and three aliquots of 0.05 g of each sample were weighed and extracted with 0.5 mL of ethanol to which 60 μg of internal standard was added. Extraction was performed by vortex mixing and ultrasonication in a water bath for 40 min. Samples