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A High-Performance Liquid Chromatography Method for the Determination of Artemisinin in *Artemisia annua* L. Leaf Extracts.

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A simple, sensitive, accurate and precise high-performance liquid chromatography (HPLC) method for determination of artemisinin in crude plant material was developed and validated. Optimal separation of artemisinin from matrix components in the plant extracts was achieved using a Waters XTerra® RP18, 5 μm, 250 × 4.6 mm column, maintained at 40 °C, a mobile phase consisting of 0.05 M potassium phosphate buffer, pH 6.0 - acetonitrile (60:40) containing 5 mM hexane sulfonate in isocratic flow. The mobile phase flow rate was 1.0 ml/min while elution was monitored at 216 nm. The method satisfied the International Conference on Harmonization (ICH) validation criteria for linearity, accuracy, precision and sensitivity. The developed method is applicable in routine quality control of *Artemisia annua* crude extracts.

Key words: *Artemisia annua*, artemisinin content, crude extract

INTRODUCTION

Malaria is a life-threatening parasitic disease that occurs in many tropical and subtropical areas especially the Africa region where about 92 % of the global malaria cases were recorded in 2017. The 2018 World Malaria Report estimated that 435,000 deaths occurred globally in the year 2017, of which an estimated 93 % were recorded in the World Health Organization (WHO) Africa Region [1].

In Kenya, malaria is still a major cause of morbidity and mortality with more 70 % of the population at risk of the disease [2]. There are various methods used in prevention of malaria for the vulnerable groups, particularly children aged under 5 years and pregnant women. Use of long-lasting insecticide-treated nets (LLITNs) is recommended for populations living in malaria endemic areas [2]. Intermittent preventive treatment in pregnancy (IPTp) via administration of sulfadoxine-pyrimethamine (SP) in the 2nd - 3rd trimester is stipulated in the Kenyan malaria treatment guidelines. Chemoprophylaxis in non-immune persons specifies use of mefloquine, atovaquone-proguanil or doxycycline [2,3].

Malaria case management is guided by the infecting Plasmodium species, clinical status of the patient and drug susceptibility of the infecting parasite [4]. Current WHO guidelines for treatment of falciparum malaria employs artemisinin-based combination therapy (ACT). In Kenya, the first line ACT is artemether-lumefantrine (AL) while the second line products comprise of dihydroartemisinin-piperaquine [3].

The reliance of ACT on artemisinin (Figure 1) and its derivatives has led to increased demand for the compound worldwide [1]. Artemisinin is obtained from cultivation of *Artemisia annua* since chemical synthesis is an expensive multistep process with low yields. Briefly, the dried aerial parts are extracted using apolar

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organic solvents followed by evaporation of solvent to obtain the crude extracts. The pure compound is achieved through clean-up of the extracts and crystallization to the desired quality [4].

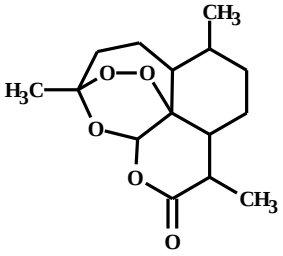

Figure 1: Chemical structure of artemisinin

Artemisinin (ART) is mainly found in the aerial part of the *A. annua* plant, where its concentration peaks just before or during flowering corresponding to plant age of 12 - 13 weeks. However, variation in artemisinin content (0.01 - 1.4 % w/w) has been observed in *A. annua* plants grown in different regions of the world. This is attributable to climatic conditions, plant variety, or other undetermined factors [5-7].

To maximize on yield, it is critical that the ART content of the crude plant material is monitored to establish the optimum growth conditions and harvesting time. Hence, the need for suitable high-performance liquid chromatography (HPLC) methods with the desired efficiency, specificity and precision to permit determination of ART in crude plant extract matrix.

The WHO monograph for ART assay specifies reversed phase HPLC - UV detection (216 nm) while the crude extracts are analyzed by semi-quantitative thin layer chromatography (TLC) [8]. Other workers have reported TLC and HPLC methods for *A. annua* extracts based on UV detection with prior derivatization using NaOH, which complicates the procedures and renders them unreliable in routine analyses [9,10].

Lapkin et al. developed and validated two HPLC methods for pure ART and extracts, utilizing refractive index and evaporative light scattering detectors. They further compared these two methods with UV detection, the workhorse of routine HPLC work. No marked differences in performance were observed although the authors asserted that UV detection was unsuitable for analysis of extracts [11]. This paper reports the development and validation of a simple, sensitive, accurate and precise reversed phase HPLC method for the quantification of ART in *A. annua* extracts.

EXPERIMENTAL

Plant material

The aerial parts of *Artemisia annua* L. were collected from six locations in diverse counties in Kenya, namely; Malakisi (Bungoma), Emusaga (Kakamega), Kehancha (Migori), Kitengela (Kajiado) and Kenyatta University (Nairobi) in 2014 - 2015. The plants were harvested prior to flowering. They were identified in the field and specimens submitted to Department of Botany, University of Nairobi for preservation. The aerial parts of the plant were air dried, ground into powder and stored in separate labeled plastic containers until use.

Chemicals, solvents and reagents

Analytical grade hexane (Sigma Aldrich Chemie, St Louis, MO, USA) was used for extraction of plant material. Analytical grade hexanesulfonic acid sodium salt (Fisher Scientific, Loughborough, UK), KH_2PO_4 (Merck PTY Ltd, Gauteng, South Africa), K_2HPO_4 (RFCL Ltd., New Delhi, India) were employed as buffer reagents. HPLC grade acetonitrile (Rankem, Avantor Performance materials Ltd, India) was used as solvent and organic modifier in HPLC experiments. Purified water was prepared in the laboratory using an Arium RO and Arium VF water system (Satorius AG, Göttingen, Germany). Artemisinin working standard (99.9% w/w) was a kind donation of East African Botanicals Ltd (Nairobi, Kenya).

Instrumentation

Hexane extracts were evaporated using a rotary vacuum evaporator equipped with a WB2000 water bath (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) and cooled by means of an ethanol based PolyScience chiller (Niles, IL, USA). Plant samples were stored in a refrigerator maintained at 2 - 8 ℃ till analysis.

All mobile phases were degassed on a MRC DC 200H ultrasonic bath (MRC Ltd., Holon, Israel).

HPLC system

An integrated D–7000 Merck-Hitachi HPLC system (Hitachi High Technologies Corporation, Tokyo, Japan), supported by a HSM 7000 version 2.0 software was used for all chromatographic experiments. It consisted of a D-7000 interface with L-7400 UV detector, L-7200 auto sampler and L-7100 low pressure gradient pump. The temperature of the column was controlled using L-7350 column oven. A Waters XTerra® RP18, 5 µm chromatography column, of dimensions 250 × 4.6 mm ID (Waters Corp., Wexford, Ireland) was used as the stationary phase. The injection volume was 10 µl while the flow rate was maintained at 1.0 ml/min. The eluents were monitored by means of UV detection at a wavelength of 216 nm.

Mobile phases

Mobile phases were prepared as mixtures of acetonitrile and phosphate buffer/ion pairing agent. Buffers were prepared by mixing equimolar (0.05 M) solutions of KH_2PO_4 and K_2HPO_4 to the desired pH under magnetic stirring. Where applicable, the desired weight of hexane sulfonic acid (HSA) was dissolved in buffer solutions prior to pH adjustment. During preparation of mobile phases, appropriate volumes of buffer/HSA were mixed with acetonitrile. Mobile phases were degassed using an ultra-sonic water bath for about 15 minutes immediately before use.

Sample and standard solutions

About 100 mg of the crude plant extract was also dissolved in 100 ml acetonitrile (1 mg/ml), filtered and injected in the HPLC system. Standard solutions were prepared by dissolving 20 mg of artemisinin working standard in 20 ml acetonitrile with aid of sonication. These solutions were used during method development and validation.

Method validation

Method validation was carried out according to International Conference on Harmonization (ICH) guidelines [12]. For this purpose, 1.0 mg/ml artemisinin in acetonitrile was taken as 100 % solution. The validation parameters applied were, linearity, accuracy, precision and sensitivity.

Linearity: Solutions of artemisinin working standard were prepared at five concentration levels corresponding to 120, 100, 75, 50 and 25 %. These were run in triplicate and used to determine the linearity of the method.

Accuracy: Accuracy was determined by spiking the plant extract with artemisinin working standard and determining recovery. This was tested at the 80, 100, and 120 percent levels, whereof the percentage recovery of artemisinin was used as a measure of accuracy.

Precision: Repeatability was determined by making six injections of 100% solution on the same day. For inter-day precision, six replicate injections of a freshly prepared artemisinin solution (100%) were run on three consecutive days. The peak areas of the analytes were normalized and the coefficient of variation (CV) of the corrected areas computed.

Sensitivity: The limit of detection (LOD) and limit of quantitation (LOQ) were determined by analysis of dilutions of artemisinin standard solution (1 mg/ml). The LOD was derived from the lowest concentration that yielded a signal to noise ratio (S/N) of 3:1, while the LOQ corresponded to S/N, 10:1.

Artemisinin content of plant material

Dried ground plant material (100 g) were subjected to cold maceration in 1 liter (1000 ml) of cold hexane for 24 hours. The resulting extract was rotary evaporated at 40 °C to dryness. The residue was re-dissolved in 1000 ml of acetonitrile and analyzed using the developed method against a standard solution of concentration 1 mg/ml. The percentage content of artemisinin in each sample was calculated based on the dry weight of plant material.

RESULTS AND DISCUSSION

Chromatographic optimization

During method development, the stationary phase (XTerra® RP18, 5 µm, 250 x 4.6 mm ID), detector wavelength (216 nm), flow rate (1.0

ml/min) and the injection volume (10 µl) were kept constant. In addition, the organic modifier (acetonitrile), buffer system (potassium phosphate) and ion pairing agent (sodium hexane sulfonate) were maintained.

Optimization was therefore applied for the following chromatographic factors; buffer concentration, pH, sodium hexane sulfonate (HSA) concentration, acetonitrile proportion and column temperature. Evaluation was based on resolution of the artemisinin (ART) peak from matrix peaks and attainment of acceptable peak parameters. Unknown peaks adjacent to ART were designated PK1 and PK2 for that eluting before and after the ART peak, respectively. Optimum values for chromatographic factors were established by plotting charts of the capacity factors (k') for PK1, ART and PK2 vs quantities to demonstrate separation trends of the three peaks.

Based on the work carried out by Lapkin et al. [11], a modified mobile phase consisting of water-acetonitrile (60:40 v/v) and column temperature of 40 °C was used as the starting point in method development. Under these conditions there was poor separation between artemisinin and adjacent unknown peaks (Figure 2).

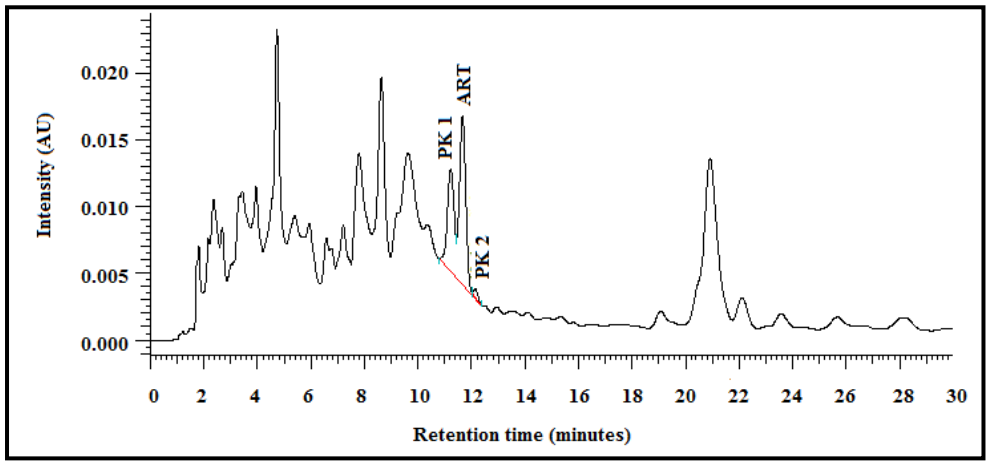

Figure 2: Typical chromatogram of *A. annua* leaf extract obtained using un-buffered mobile phase.
Column: XTerra® RP18, 5 µm, 250 × 4.6 mm ID. Temperature: 40 °C. Mobile phase: water - acetonitrile (60:40, % v/v). ART-artemisinin, PK1 and PK2-unknown peaks.

Using un-buffered mobile phase, acetonitrile concentration was investigated at 40, 50, 60 and 70 % v/v. Acetonitrile concentration of 40% v/v was taken as optimum and used in all subsequent experiments. The proportion of buffer relative to acetonitrile in the mobile phase was investigated within the range 40-60%, with 60% buffer yielding the best results. Buffer pH was run at 4.5, 6.0 and 7.5, whereof pH 6.0 was found to be optimal. To achieve baseline separation of the ART peak, hexane sulfonic acid sodium salt (5-15 mM) was employed as ion pairing agent. The optimum HSA concentration was 5 mM expressed as final concentration in the mobile phase.

The optimized chromatographic conditions were established thus: a mobile phase consisting of 0.05 M KH_2PO_4, pH 6.0 - acetonitrile (60:40 % v/v), enriched with 0.005 M hexanesulfonate delivered at a flow rate of 1.0 ml/min; as stationary phase, Waters XTerra® RP18, 5 µm, 250 × 4.6 mm ID, maintained at 40 °C; UV detector set at 216 nm; with a run time of 40 min. Figure 3 is a typical chromatogram obtained under the optimum conditions.

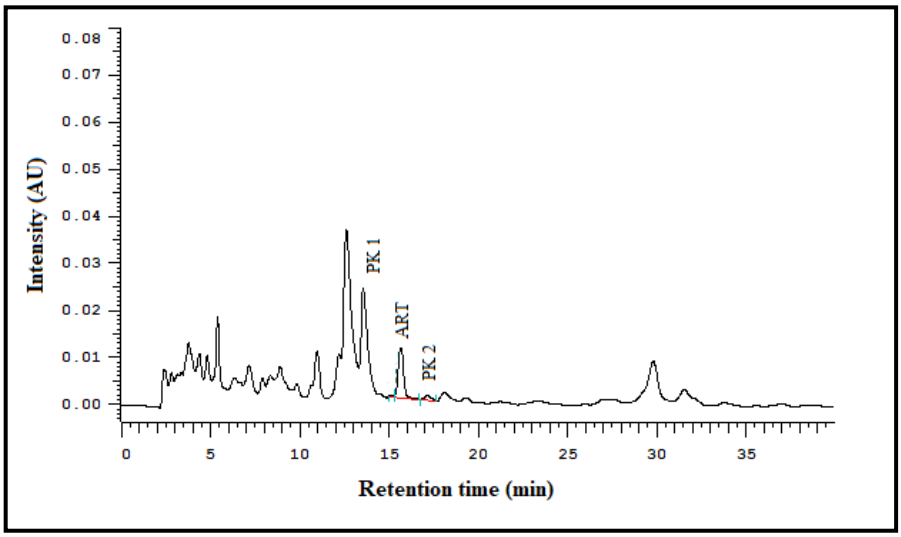

Figure 3: Typical chromatogram of *A. annua* leaf extract obtained under optimum chromatographic conditions.

Column: XTerra® RP18, 5 µm, 250 × 4.6 mm; Temperature: 40 °C; Mobile phase: 0.05 M KH_2PO_4, pH 6.0 buffer - acetonitrile (60:40 % v/v) with 0.005 M sodium hexane sulfonate. ART-artemisinin, PK1 and PK2-unknown peaks.

Method validation

Validation parameters were evaluated with respect to the ICH acceptance criteria for pharmaceuticals [12,13]. The method was found to be linear over the range of 25-120 % with r^2 of 0.9976. The method demonstrated good accuracy as evidenced by a recovery range of 98.5 - 100.2 % at the three concentration levels investigated. The CV for repeatability and inter-day precision were 0.7 and 2 respectively. The sensitivity test yielded LOD and LOQ values of 20 ng and 30 ng respectively. The low LOQ attained permits the determination of artemisinin in the plant at low concentrations.

Artemisinin content of plant material

Table 1 shows artemisinin content of plant samples collected from five regions in Kenya. The highest content was found in plants grown in Emusaga, followed by Malakisi and Kehancha.

Table 1: **Artemisinin content of *Artemisia annua* samples**

Collection Site (County)	Artemisinin Content (% w/w)
Malakisi (Bungoma county)	0.87
Emusaga (Kakamega county)	0.89
Kehancha (Migori county)	0.85
Kitengela (Kajiado county)	0.75
Kenyatta University (Nairobi county)	0.68

Previous studies have demonstrated that artemisinin content of *A. annua* varies between 0.01 to 2 % w/w of the dried leaves of varieties

grown in different geographical regions depending on farming practices, harvesting time and environmental factors such as temperature and availability of nutrients [6, 14].

CONCLUSION

A simple, accurate and precise HPLC method was developed and validated for the analysis of crude plant extracts of *Artemisia annua*. The method achieved separation of the artemisinin from other compounds also present in the plant extract. The developed method offers the advantage of artemisinin assay in crude plant extracts of *A. annua* directly without sample clean up procedures. The method can be used for routine quality control of plant material.

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