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Chemiluminescent detection of artemisinin Novel endoperoxide analysis using luminol without hydrogen peroxide

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Abstract

A novel method for artemisinin quantitation employing high-performance liquid chromatography (HPLC) with chemiluminescence (CL) detection in the absence of hydrogen peroxide (H_2O_2), is reported. After elution from the HPLC column, artemisinin is combined with an alkaline solution of hematin and luminol. The resulting CL signal is detected by use of a spectrofluorometer with the excitation lamp disabled, and is proportional to artemisinin concentration. The CL method was optimized and applied to the analysis of artemisinin in spiked human serum.

CL in the absence of H_2O_2 or other known oxidizing species is remarkable since such oxidizers are usually required to produce CL from luminol under alkaline conditions. Artemisinin, a naturally occurring sesquiterpene, is one of several natural products that contain an endoperoxide functional group. Since H_2O_2 is not needed in the analysis, the endoperoxide moiety on artemisinin is implicated as a contributing source of superoxide radicals required for the light-producing reaction with luminol.

1. Introduction

Artemisinin or qinghaosu is a naturally occurring sesquiterpene endoperoxide (Fig. 1) that is receiving considerable attention in the treatment of malaria because of growing resistance of *Plasmodium falciparum* strains to traditional quinoline-based antimalarial drugs [1,2].

Detection of artemisinin has proven to be a challenge as it does not possess any sensitive or

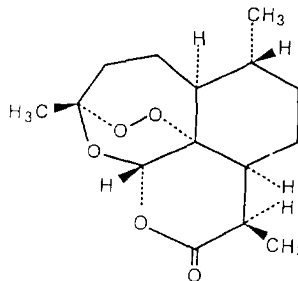

Fig. 1. Structure of artemisinin.

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specific spectrophotometric characteristics. Although artemisinin does absorb ultraviolet radiation between 210 and 220 nm, the extinction coefficient is relatively small and detection at the lower wavelengths usually results in poor selectivity and increased baseline noise when high-performance liquid chromatographic (HPLC) methods are used. Precolumn and postcolumn derivatization methods have been developed to convert artemisinin into a compound that absorbs UV radiation at longer wavelengths [3–5]. These methods lack adequate sensitivity and specificity and can be very time-consuming. An HPLC method that uses reductive electrochemical methods has been reported for artemisinin detection [6]. A disadvantage to this method is that detection interferences due to oxygen in the mobile phase require rigorous deoxygenation prior to and during analysis.

We now report a novel detection method for the analysis of artemisinin involving a chemiluminescent (CL) reaction with luminol, utilizing hematin as a catalyst. Luminol, with either cytochrome *c* or a heme compound as catalysts, has been used for CL detection of lipid hydroperoxides [7–10]. The CL response produced by the oxidized luminol in alkaline solution catalyzed by iron(III) from hematin, cytochrome *c* and ferric ions normally requires H_2O_2 or some other oxidizing species [11]. The iron-catalyzed reduction of peroxides involves the formation of free radicals which react with luminol to produce the CL.

Meshnick et al. [12] have reported evidence of iron-dependent free radical formation from the endoperoxide moiety of artemisinin. Free radicals ($A^{\cdot}$) formed in this manner could react with both the luminol anion (LH^-) and O_2 to form the luminol radical anion ($L^{\cdot -}$) and superoxide ($O_2^{\cdot -}$), respectively. The latter two species, which are requisite for CL in this system, react to form the luminol hydroperoxide (LOO^-) anion. It forms a covalent bond with the paracarbon atom to give the transient luminol endoperoxide (LOOL). The latter decomposes to give an aminophthalate (AP) in the lowest excited singlet state (^1AP) and N_2. The excited aminophthalate then gives off CL as it returns to the ground state as shown in the scheme below [8,9,11]. If $O_2^{\cdot -}$ is cleaved off as indicated by the results of Meshnick et al. [12], then step (a) in the following scheme would not be needed.

(a) $A^{\cdot} + O_2 \rightarrow O_2^{\cdot -} + A$

(b) $A^{\cdot} + LH^- \rightarrow L^{\cdot -} + A$

(c) $L^{\cdot -} + O_2^{\cdot -} \rightarrow LOO^-$

(d) $LOO^- \rightarrow LOOL$

(e) $LOOL \rightarrow {}^1AP + N_2$

(f) ${}^1AP \rightarrow h\nu + AP$

The CL detection method described in this paper is optimized with respect to sensitivity (optimum response for artemisinin) and the linearity and precision of the CL response evaluated using a flow-injection technique prior to its application in an HPLC system for the analysis of artemisinin in serum.

2. Experimental

2.1. Reagents[1] and standards

The CL reagent, consisting of a solution of 15 μg/ml luminol (Sigma, St. Louis, MO, USA) and 30 μg/ml hematin (Sigma) in 0.1 *M* sodium hydroxide, was allowed to stand for about 30 min before use. This reagent was prepared daily although weekly preparations may be used if stored away from light. Hemin (Sigma) and cytochrome *c* (Sigma) were also investigated for use as a catalyst for the CL reaction. Artemisinin was obtained from Sigma. The artemisinin derivatives: artemether, arteether and their main metabolite, dihydroartemisinin were provided as a generous gift from Dr. P. Olliaro, World Health Organization.

A stock solution of artemisinin was prepared

[1] Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the US Department of Health and Human Services.

by adding the compound to a 10-ml glass volumetric flask that had been previously tared on a Mettler AE163 balance. The weight of the compound was 0.0020 g. The flask was then filled to the mark with methanol and sonicated for about 5 min. The final stock concentration was 200 μg/ml. Concentrations of 100, 50, 10, 5, 2.5, 1, 0.5, and 0.25 μg/ml were then prepared from the stock. Standard samples were stored at 4°C to reduce evaporation and equilibrated to room temperature before analysis.

2.2. Apparatus and conditions

In the flow-injection technique, the CL reagent was combined with the sample carrier solvent in a PCRS 500 reaction module (Kratos, Ramsey, NJ, USA) by means of a μLC-500 Isco syringe pump (Isco, Lincoln, NE, USA). The PCRS 500 reaction module consists of a mixing device designed to induce a flowing vortex in the inlet fluid streams and a reaction coil where convoluted tubing is used to minimize band broadening. A Rheodyne sample injector valve Model £7125 (Rheodyne, Cotati, CA, USA) with a 10-μl sample loop was used to deliver the sample into the carrier stream. The carrier stream, consisting of methanol, was delivered to the reaction module by a SP8700XR LC reciprocating pump (Spectra-Physics Analytical, Fremont, CA, USA). The subsequent CL reaction produced by the combined sample and CL reagent was detected by a photomultiplier tube (PMT) from a Perkin-Elmer (Norwalk, CT, USA) 650-S fluorescence spectrophotometer fitted with a flow cell with a path length of 8 mm and volume of 20 μl. The emission wavelength and slit width were set at 425 nm and 20 nm, respectively. The flow-rate for the sample carrier solvent and CL reagent was 0.5 ml/min for both streams with a dead volume of about 1.1 ml from the point of mixing to the detector. The CL signal was recorded by using an HP 3390A integrator (Hewlett-Packard, Palo Alto, CA, USA).

Artemisinin was chromatographically separated from dihydroartemisinin and interfering endogenous components of extracted serum by the addition of a 15 × 0.2 cm Ultrasphere octadecylsilyl (ODS) bonded silica column with a particle size of 5 μm (Beckman, Fullerton, CA, USA) to the flow-injection system described above.

Further modifications of the detection system were made to maximize the signal response for the HPLC analysis of artemisinin in spiked serum. A section of PTFE tubing (0.51 mm I.D.) was placed directly over the opening of the PMT. The opening was covered with aluminum foil to prevent light leak and to reflect the light generated by the sample back to the PMT.

Artemisinin and dihydroartemisinin were eluted from the column at about 7 and 4 min, respectively, with an acetonitrile–water (50:50, v/v) mobile phase pumped at a flow-rate of 0.3 ml/min. The CL reagent flow-rate was 0.5 ml/min.

2.3. Extraction of artemisinin from serum

Human serum was spiked with artemisinin to yield a final concentration of 25, 50, 250 and 500 ng/ml. Dihydroartemisinin was added to each sample and used as an internal standard. The samples were allowed to equilibrate at room temperature for 7 h prior to storage at 0°C.

Artemisinin and dihydroartemisinin were extracted from spiked serum using a quick and simple solid-phase extraction technique. An Empore, 4 mm diameter C_{18} extraction membrane was prepared by passing 0.5 ml of methanol followed by 0.5 ml of water. The membrane was not allowed to dry before sample application. One ml of serum was centrifuged to eliminate particulates, passed directly through the membrane and subsequently washed by passing 0.3 ml of water. Elution of artemisinin and dihydroartemisin was accomplished by passing 0.1 ml of 65% acetonitrile through the membrane. Extraction recovery ranged from 65 to 91% at serum artemisinin concentrations between 25 and 500 ng/ml ($n = 3$ for each concentration). An aliquot of 50 μl of the eluate was injected directly into the HPLC system.

3. Results and discussion

3.1. Chemiluminescent method optimization and evaluation

Using the flow-injection technique, we optimized artemisinin detection by adjusting the hematin and luminol concentrations of the CL reagent (Fig. 2). The flow-rate ratio of the carrier stream to the CL reagent stream was also optimized while maintaining a total flow of 1 ml/min (Fig. 3).

Using hematin as the catalyst is desired because it is relatively inexpensive and it facilitates a quicker reaction, resulting in higher sensitivity. Less sensitivity was observed when cytochrome *c* was substituted for the hematin. The use of freshly prepared hemin in the CL reagent exhibited a very weak CL response from luminol and artemisinin. Exposure of hemin to 0.1 *M* NaOH for about 6 h resulted in increased CL response, comparable to that observed with hematin. Conversion of hemin to hematin occurs under alkaline conditions and accounts for this. Very alkaline conditions (0.1 *M* NaOH) afforded the greatest sensitivity.

The flow-injection technique was used in determining the linearity and variability of re-

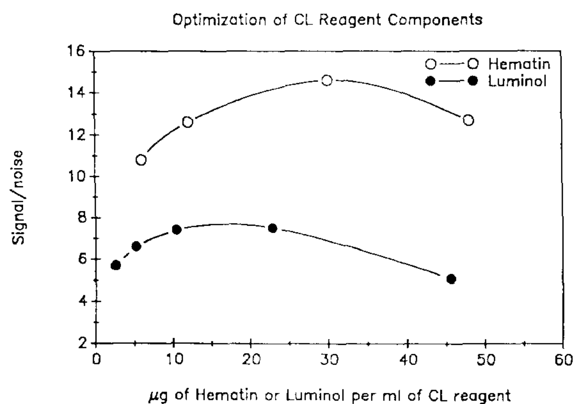

Fig. 2. Effects of hematin or luminol concentrations on the chemiluminescent response of artemisinin. Concentrations of luminol were varied while the concentration of hematin was kept constant at 27 µg/ml. Concentrations of hematin were varied while luminol concentration was constant at 15 µg/ml. The optimal concentration (maxima) of each component were used in subsequent analyses.

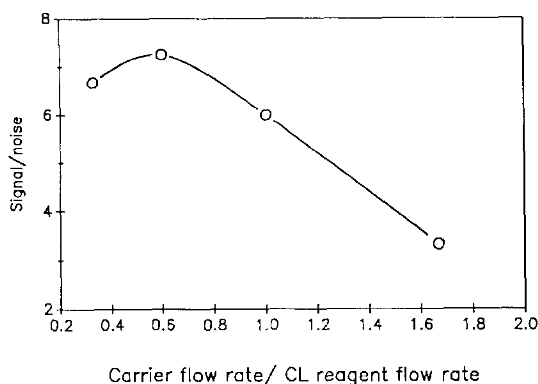

Fig. 3. Adjustment of the flow-rate ratio between the carrier stream and the chemiluminescence (CL) reagent. The total flow-rate was maintained at 1.0 ml/min.

sponse for the CL detection method before the detection method was applied to an HPLC system. A log–log transformation of artemisinin mass versus CL response curve was linear over the range of 2.5 to 2000 ng ($y = 1.17x - 0.29$, $r = 0.999$, $n = 9$ data points). Within-day response variability was assessed by determining the coefficient of variation (standard deviation of the mean divided by the mean multiplied by 100) from the signal peak heights produced by five replicate injections of 10 µl from each artemisinin concentration. The coefficient of variation for day-to-day response variability was determined from the mean signal ($n = 5$) from each of five days. The coefficient of variation for within day and day-to-day variability was $\leq 5\%$ from 25 to 2000 ng of artemisinin and $< 20\%$ from 2.5 to 10 ng of artemisinin. The limit of detection for artemisinin under these conditions is 2.5 ng and is defined as the minimum mass that gave a peak height (signal response) of three times the baseline noise. The relatively high coefficient of variation at the lower amounts of artemisinin is a result of the small perturbation of the carrier stream caused by sample injection.

Artemether, arteether and dihydroartemisinin are derivatives of artemisinin that also possess the endoperoxide moiety. Their ability to produce a chemiluminescent reaction under the above conditions was tested. Although the chemiluminescent response for dihydroar-

temisinin is comparable to artemisinin, the response produced by the other artemisinin derivatives, arteether and artemether were about 1000 fold less. This observation is likely to be related to the stability of these endoperoxides when exposed to an alkaline environment. For example, Idowu et al. [13] has reported that unlike artemisinin, arteether is stable in alkaline solution.

3.2. HPLC of spiked serum

The chromatogram shown in Fig. 4 demonstrates that artemisinin can be extracted from serum, separated from dihydroartemisinin and endogenous compounds by HPLC and detected by the CL reaction with luminol. From this chromatogram, a detection limit of 10 ng/ml of artemisinin in 1 ml of serum was determined, which corresponds to a peak height of three times the baseline noise. The sensitivity of this method is comparable to currently available methods and may be improved by further instrumental modifications.

The analysis of artemisinin in spiked serum was evaluated in terms of day-to-day variability (Table 1). Although a log–log conversion produced a linear relationship over the wide artemisinin mass range (2.5–2000 ng) in the flow-injection evaluation, a direct linear relationship between artemisinin concentration and signal response was observed over the on-column artemisinin mass range of 12.5 to 250 ng and used to determine the calculated serum artemisinin concentration. Even though dihydroartemisinin was used as an internal standard in the chromatographic analysis, another suitable internal standard must be used, as dihydroartemisinin is a metabolite of artemisinin and will be present in actual biological samples.

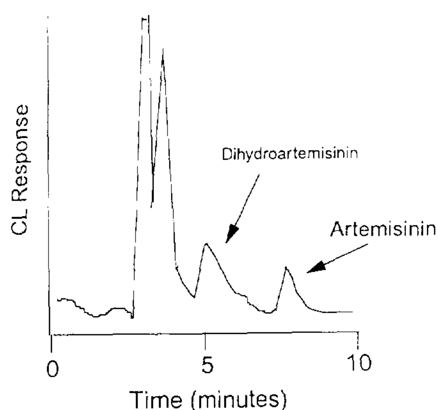

Fig. 4. HPLC chromatogram of artemisinin in spiked serum. An aliquot of 50 μl (50%) of total extract from spiked serum (25 ng/ml) was injected into the HPLC system described in the Experimental section. CL response is equivalent to 12.5 ng of artemisinin on-column.

Table 1
Day-to-day variability of artemisinin concentration in spiked serum samples

Amount added (ng/ml)	Calculated amount (mean ± S.D.) $n = 5$	R.S.D. (%)
25	24 ± 3	13
50	50 ± 6	12
250	254 ± 18	7
500	493 ± 12	3

Peak height ratio of artemisinin to dihydroartemisinin was used to quantitate in all samples. Standard curve ranged from 25 to 500 ng/ml and the correlation coefficient of concentration vs. response was 0.998 ± 0.002, $n = 5$ determinations.

4. Conclusions

The discovery that artemisinin and dihydroartemisinin can be detected by a light producing reaction with luminol provides a new and promising detection method for HPLC analysis of these compounds in body fluids. The many advantages of HPLC with CL detection include selectivity, high sensitivity, wide dynamic range and use of inexpensive and simple equipment [14].

The CL response is thought to be derived from the endoperoxide moiety of artemisinin and dihydroartemisinin yet the response is greatly diminished for the artemisinin derivatives, artemether and arteether. These compounds, which also contain an endoperoxide, have an ether group substituted for the keto group of

artemisinin. Therefore the diminished CL response is attributed to the greater stability of these compounds in alkaline solution due to the ether arrangements. Although hematin is thought to act as a catalyst under the described conditions, it may also be a source of oxygen free-radicals contributing to the chemiluminescent response.

The assay has good potential use for the analysis of dihydroartemisinin. Dihydroartemisinin, which is biologically active against malaria [2] has recently become very important due to reports of its suggested neurotoxicity [15]. Therefore, a sensitive and precise analysis method using chemiluminescent detection for dihydroartemisinin in biological fluids is currently being optimized and evaluated.

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