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Journal of Chromatography B, 666 (1995) 183–187

JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

Short communication

Packed-column supercritical fluid chromatography of artemisinin (qinghaosu) with electron-capture detection

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First received 5 July 1994; revised manuscript received 22 November 1994; accepted 5 December 1994

Abstract

In this preliminary report, a supercritical fluid chromatographic method is described for the determination of artemisinin in whole blood. The chromatography is carried out on a 20 cm × 1 mm I.D. Deltabond cyano supercritical fluid chromatographic column with detection of the artemisinin via an electron-capture detector. The sample work-up uses a liquid–liquid extraction with hexane, giving a recovery of 82%. The current limit of detection using 1 ml of blood is 20 ng/ml. We speculate that the endoperoxide moiety accounts for the response to the electron-capture detector and thus provides a new approach by which this class of compounds may be analyzed.

1. Introduction

Artemisinin, also called qinghaosu (QHS), is a naturally occurring, sesquiterpene lactone endoperoxide (Fig. 1) isolated from *Artemesia annua L.* and chemically characterized by Chinese scientists during the 1970s and early 1980s [1–4]. QHS and several synthetic derivative analogs constitute a new class of antimalarial drugs that have been shown to be effective against the erythrocytic stages of the plasmodial parasite, even against strains that have developed resistance to other currently available drugs such as chloroquine [5–7]. This is particularly important since malaria is still a major health problem in many areas of the world [8,9]. Artemisinin

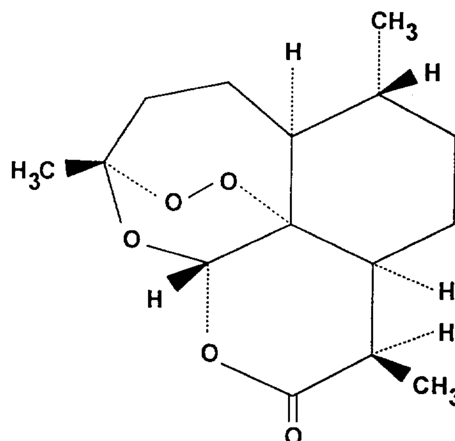

Fig. 1. Structure of artemisinin (qinghaosu, QHS).

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SSDI 0378-4347(94)00560-5

antimalarial activity and history have been reviewed by Klayman [10].

Specific and sensitive methods for determining QHS, metabolites or synthetic analogs in biological fluids are needed in conducting therapeutic drug monitoring, pharmacokinetic and metabolic studies. For metabolic studies, thermospray high-performance liquid chromatography (HPLC)–mass spectrometry is currently being used [11,12]. Because of the expense and complexity of operation and maintenance of these instruments, they are not practical for routine use required for therapeutic drug monitoring and pharmacokinetic studies. Other analytical approaches for determining QHS and analogs in biological fluids have been developed and tried, such as gas chromatography (GC) and HPLC. Neither approach has been acceptable because this class of compounds is thermally labile [13–15] and does not contain an ultraviolet, visible or fluorescent chromophore. Acid or base hydrolysis of QHS producing an ultraviolet-chromophore prior to HPLC analysis has been employed [16–18], but this approach lacks specificity. (Both parent and metabolite compounds produce the same derivative in many instances.) Base hydrolysis, after HPLC separation of the analytes [19,20], solves this problem, but this technique is experimentally cumbersome and may not have the sensitivity required for therapeutic drug monitoring. The technique that has best met the sensitivity and specificity requirements so far has been HPLC with reductive electrochemical detection [21–24]. The major drawback of this technique is the experimental rigor required to maintain a low level of oxygen in the HPLC system and the sample solutions which is essential for the sensitive and stable operation of the electrochemical detector in the reductive mode.

We report a promising new approach for determining QHS in biological fluids by using supercritical fluid chromatography (SFC) with electron-capture detection (ECD). Speculations that the endoperoxide moiety of the QHS molecule might effect electron-capture detectability and that supercritical carbon dioxide might have sufficient solvating power to elute QHS compounds below the temperature of degradation, led us to try the SFC-ECD analysis of QHS. Use of the SFC-ECD technique has been previously reported for the analysis of chlorinated pesticides [25–27], polycyclic aromatic hydrocarbons [26], polychlorinated biphenyls [26], nitro and other chlorinated compounds [27] and certain fungicides [26,28,29]. A SFC-ECD method has been reported for the analysis of the antimalarial drug, mefloquine, in blood [30]. Mefloquine was eluted from a packed column using modified supercritical pentane.

2. Experimental[1]

2.1. Standards

QHS was a gift from the Walter Reed Army Institute of Research (Washington, DC, USA). A stock solution was prepared in absolute ethanol containing 480 μg/ml QHS. An aliquot of this solution was further diluted with ethanol to give a working solution of 48 ng/μl.

2.2. Reagents and solvents

Spectroscopic-grade hexane was from Burdick and Jackson Laboratories (Muskegon, MI, USA). Carbon dioxide, SFC grade, was obtained from Matheson Gas Products (East Rutherford, NJ, USA). Ethanol, dehydrated, USP absolute, was obtained from Midwest Grain Products Company (Weston, MO, USA).

2.3. Apparatus

We constructed the SFC-ECD system from a Model μLC-500 high-pressure syringe pump (Isco, Lincoln, NE, USA), a Series 210A HPLC injector with 20-μl loop (Beckman Instruments, San Ramon, CA, USA), a Model 3700 gas

[1] Use of trade names and commercial sources is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

chromatograph with a ^{63}Ni electron-capture detector (Varian, Walnut Creek, CA, USA) and a 200 × 1 mm I.D. stainless steel, Deltabond cyano 5 μm SFC column (Keystone Scientific, Bellfonte, PA, USA).

The HPLC injector was mounted above the GC injector port. The injector was first connected to an in-line solvent HPLC filter (with a 2-μm frit) above the GC injector port with 0.178-mm I.D. stainless steel tubing. This same tubing material was then used to complete the connection from the in-line filter through the GC injection port orifice to the column inside the GC oven. The exit end of the column was joined to the detector with 30 cm × 20 μm I.D. (0.375-mm O.D.) fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA). The column and capillary tubing were connected with a 1.6 × 0.4 mm I.D. vespel/graphite-reducing ferrule (Alltech, Deerfield, IL, USA). A 1.6 × 0.5 mm I.D. graphite-reducing ferrule (Scientific Glass Engineering, Ringwood, Australia) was used to connect that capillary tubing to a 6.35-mm capillary "T" conversion adapter (Scientific Glass Engineering) which was then interfaced directly to the detector. Nitrogen gas was supplied at a flow-rate of 200–300 ml/min through the arm of the "T" adapter. PTFE tubing forced over the exit nozzle of the detector carried the effluent to a fume hood.

2.4. Chromatographic procedure

A new column was installed and a flow of carbon dioxide was applied at a pressure of 8.2 MPa at ambient temperature while leaving the detector end of the fused-silica capillary transfer line unconnected to expel any solvent from the column. The transfer line was then connected to the detector and the pressure was raised to 17.2 MPa at a column temperature of 80°C, the operational parameters used for analysis. About one hour was required for the baseline response of the detector to stabilize. After this initial conditioning sequence, the time for equilibration at the beginning of each day was ca. 10 min. A minimum amount of nitrogen makeup gas was applied until the electrometer could be adjusted in establishing a baseline. The detector temperature was set at 350°C. The volume of sample injected was 3 μl. The retention time for QHS at these conditions was 8.2 min. Chromatographic responses were determined by peak height.

2.5. Extraction of QHS from whole blood

A 1-ml volume of blank whole blood was fortified with 10 μl of a 48 ng/μl ethanolic solution of QHS in a 15-ml screw-capped glass centrifuge tube. A 2-ml volume of deionized water and 7 ml of hexane were added to the tube. The tube was capped, shaken vigorously for about 15 s by hand and centrifuged at 600 *g* for about 1 min. The hexane layer was transferred, by using a Pasteur pipet, to a clean centrifuge tube and evaporated to dryness with a gentle flow of nitrogen in a 60°C water bath. The residue in the tube was reconstituted in 35 μl of hexane with vortex-mixing.

2.6. Determination of extraction recovery

Four 1-ml blank whole blood samples were each fortified with 10 μl of the 48 ng/μl ethanolic solution of QHS in 15-ml screw-capped glass centrifuge tubes and carried through the extraction procedure. A reference solution was prepared by transferring 10 μl of the QHS solution to a 4-ml screw-capped glass vial, evaporating the ethanol under a stream of nitrogen, and then reconstituting with the same volume of hexane as added to the blood extracts. The four blood extracts, along with the reference solution, were injected in duplicate onto the chromatographic system. All of the injection samples were stored in the freezer during the injection sequence to reduce the volatility of hexane. Without this, the concentration of QHS in the hexane solution can be observed to increase with time.

3. Results and discussion

The chromatograms of the blank and QHS-fortified whole blood extracts shown in Fig. 2

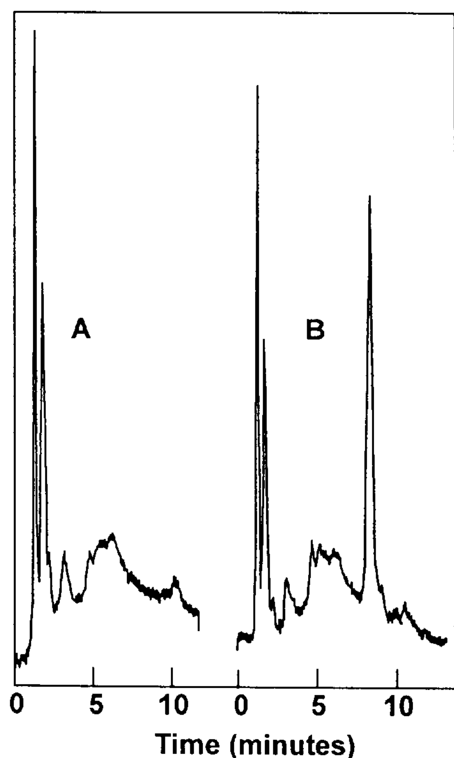

Fig. 2. Chromatograms from (A) an extract of 1.0 ml of drug-free whole blood and (B) an extract of 1.0 ml of drug-free whole blood fortified with standard artemisinin at a level of 480 ng/ml.

demonstrate that QHS can be extracted from fortified whole blood and chromatographed on a packed micro-bore column with minimal interference from endogenous components extracted from whole blood. From these chromatograms, a detection limit of approximately 20 ng/ml was calculated, which corresponds to a peak height of three times the baseline noise (2 mm). A detection limit of 5 ng/ml was reported for arteether (a synthetically produced ethyl ether derivative of QHS) in plasma using HPLC with reductive electrochemical detection [24]. QHS was used as an internal standard compound in this assay.

The limit of detection for QHS using SFC-ECD can possibly be further lowered by reducing the baseline response of the detector by improving the quality of the supercritical carbon dioxide with respect to the oxygen and moisture content. This could be accomplished by using a grade of carbon dioxide that was manufactured with a lower oxygen and moisture content or by using a high-pressure filtering system between the carbon dioxide tank and the syringe pump to remove oxygen and moisture while filling the pump.

The calculated percent recovery for the extraction method was found to be 81.6 ± 8.4% (R.S.D. 10.3%). The calculated values ranged from 71.3 to 91.0%. From four simultaneous injections of the reference solution, the R.S.D. of the injection-to-injection reproducibility was calculated to be 13.0%. Thus, it appears that injection reproducibly effects most of the scatter observed in the recovery experiment. Injection reproducibility can be improved by incorporating an appropriate internal standard into the analysis scheme.

In summary, these preliminary results demonstrate that QHS can be extracted from fortified whole blood with good recovery, can be chromatographed on a packed micro-bore column with supercritical carbon dioxide with reasonable separation from endogenous blood components, and is electron-capture detectable. This SFC-ECD technique may prove to be sensitive enough to measure the drug levels of QHS encountered in therapeutic drug monitoring and pharmacokinetic studies. This provides a promising new approach for determining QHS, and perhaps other QHS analogs and metabolites, in body fluids. However, further work is needed in the development of a rigorous analytical method, such as finding an appropriate internal standard to improve the precision of the analysis and improving the limit of detection by lowering oxygen and moisture content in the carbon dioxide mobile phase.

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