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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF A CANDIDATE 8-AMINOQUINOLINE ANTILEISHMANIAL DRUG USING OXIDATIVE ELECTROCHEMICAL DETECTION

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SUMMARY

An analytical method was developed for the quantitation of a candidate antileishmanial drug, 6-methoxy-8-(6-diethylaminohexylamino)-4-methylquinoline, dihydrochloride, in canine plasma. The assay utilized internal standard technique with a structurally similar 8-aminoquinoline, 6-methoxy-8-(7-diethylaminoheptylamino)-4-methylquinoline, dihydrochloride, as the internal standard. The method employs a liquid—solid extraction procedure with prepackaged silica gel columns upon which the drug and internal standard are adsorbed, then selectively washed and eluted. Reversed-phase chromatography was then employed to analyze the extracted sample by means of oxidative electrochemical detection at + 0.75 V. Good accuracy and precision were obtained over the range of concentration tested (10—1500 ng/ml plasma). Analyses of plasma samples from human volunteers given the drug demonstrate the method is also suitable for analysis of human plasma samples. The entire procedure is relatively simple and requires only 1 ml of plasma.

INTRODUCTION

The leishmaniases are parasitic diseases affecting perhaps 100,000,000 people in the tropical and semitropical world [1]. The major forms of human disease are papular ulcerative cutaneous disease (cutaneous disease), erosive oral, nasal or pharyngeal disease (mucocutaneous disease), or hepatosplenomegaly (visceral disease). The leishmaniases result from infection of the macrophages of the respective organs by the amastigote form of the parasite. The leishmaniases are presently treated with pentavalent antimonials such as sodium stibogluconate (Pentostam®, Burroughs Wellcome) or N-methylglucamine (Glucantime®, Rhodia) [2—4]. The 10—25% of cases that are antimony-resistant [4] are treated with more or higher doses of antimony, with

pentamidine or with amphotericin B [2—4]. These compounds are parenterally administered and have relatively low therapeutic indices.

The Walter Reed Army Institute of Research has screened more than 3000 compounds in an effort to find an orally administered effective antileishmanial compound. 6-Methoxy-8-(6-diethylaminohexylamino)-4-methylquinoline, dihydrochloride, is presently the most active drug discovered thus far in a screening program for antileishmanial activity in the hamster model of visceral leishmaniasis [5]. In this model, the above compound has consistently shown suppressive activity 400—700 times greater than the reference compound, Glucantime [5]. This compound is being developed for human use and has been tested in a double blind study of safety and tolerance in humans and was well tolerated up to and including a 60-mg single oral dose [6].

To support the development of this drug, the estimation of pharmacokinetic parameters in animals and humans must be determined. Therefore a sensitive and specific assay for this candidate 8-aminoquinoline antileishmanial drug in plasma was needed and is described in this paper.

EXPERIMENTAL

Chemicals

6-Methoxy-8-(6-diethylaminohexylamino)-4-methylquinoline, dihydrochloride* (I) and 6-methoxy-8-(7-diethylaminoheptylamino)-4-methylquinoline dihydrochloride** (II), the internal standard, were synthesized on contract from Starks Assoc. (Buffalo, NY, U.S.A.) and Ash Stevens (Detroit, MI, U.S.A.), respectively (Fig. 1). Compound I and the internal standard were found to be 99.7% [7] and > 99% pure, respectively. 6-Methoxy-8-(6-ethylaminohexylamino)-4-methylquinoline, dihydrochloride hemihydrate***, the salt form of a metabolite of compound I found in both rat and hamster microsomal preparations [8], was synthesized under contract by Starks Assoc. (purity > 99.0%, Fig. 1). Acetonitrile was of HPLC grade and obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All chemicals used in this study were of reagent grade. Trizma buffer was purchased from Sigma (St. Louis, MO, U.S.A.). Ammonium formate was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

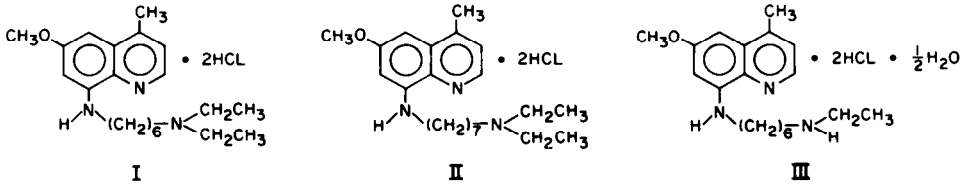

Fig. 1. Chemical structures of compound I (I), the internal standard (II) and the N-deethylated metabolite of compound I (III).

*This compound is an investigational drug and has no generic name. An institutional identification of WR 6026 ◦ 2HCl (Lot AF) is used to identify this compound.

**The internal standard was also obtained from the Walter Reed Inventory and has no generic name. An institutional identification of WR 223,658 ◦ 2HCl (Lot AA) is used.

***This metabolite was synthesized and also obtained from the Walter Reed inventory. The institutional identification for the chemical is WR 211,789 ◦ 2HCl ◦ ½H_2O.

Dehydrated ethanol was purchased from U.S. Industrial Chemical (Tuscola, IL, U.S.A.). Silica gel Bond-Elut® columns (3 ml capacity) were purchased from Analytichem International (Harbor City, CA, U.S.A.). Plasma was prepared from freshly drawn heparinized (20 I.U./ml) canine blood. All water was demineralized, double glass-distilled and stored in glass.

Liquid—solid extraction of compound I from plasma

Canine plasma samples (1 ml) that had been previously spiked with various levels of compound I were thawed at room temperature. The internal standard was dissolved in 0.01 *M* hydrochloric acid and appropriate amounts were added to the thawed samples and vortexed for 10 sec. Acetonitrile (2 ml) was added to the sample and immediately vortexed vigorously for 30 sec. Samples were then centrifuged for 10 min at 500 *g* and 5°C in a Sorvall refrigerated RC-2 centrifuge (DuPont, Newtown, CT, U.S.A.). The supernatant was transferred to another precleaned (tubes rinsed with acetonitrile then methanol) disposable glass test tube and acetonitrile was evaporated to dryness under nitrogen gas (< 35°C) using a N-Evap® analytical evaporator manufactured by Organomation (South Berlin, MA, U.S.A.). The sample was reconstituted with 2.0 ml of water and vortexed vigorously for 15 sec.

The sample was placed onto a wetted silica gel Bond-Elut column previously equilibrated by the passage of 4 ml of ethanol followed by 6 ml of water. The test tube was rinsed with 2.0 ml water and the rinse solution was also put on the column. The sample and test tube rinse were drawn through the column in this and subsequent wash steps by use of a vacuum (200 mmHg) with a Vac-Elute® manifold obtained from Analytichem International. After the sample had been drawn through the column, the volume was washed with 6 ml of water followed by 6 ml of water—ethanol (20:80). Finally, the drug plus internal standard were eluted off the silica gel Bond-Elut column with 8 ml of 0.1 *M* trizma, pH 7.4—ethanol (20:80) into a precleaned glass test tube and evaporated to dryness under nitrogen gas at < 35°C. The dry residue was dissolved in mobile phase for HPLC analysis.

HPLC analysis

Chromatographic system consisted of a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Model U6K universal injector (Waters Assoc.), an electrochemical detector with a single glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a Model 585 strip-chart recorder (Linear Instruments, Irvine, CA, U.S.A.) and a 250 mm × 4.6 mm I.D. 5-μm particle size Spherisorb-CN® column (Thomson Instrument, Newark, DE, U.S.A.) Column was at ambient temperature. Mobile phase consisted of 0.1 *M* ammonium formate, pH 4.5—acetonitrile (67:33) and the flow-rate was 2.0 ml/min. The pH of the ammonium formate was titrated with concentrated formic acid to pH 4.5 prior to the addition of acetonitrile. Samples were reconstituted in 250 μl of mobile phase and 5—20% of the samples were injected. The electrochemical detector was set in the oxidative mode at an applied potential of +0.75 V using a single glassy carbon electrode at a sensitivity of 2—10 nA full scale. The electrochemical detector signal was filtered at a maximum cut-off frequency of 0.05 Hertz by means of a Model 1021A filter (Spectrum Scientific, Newark, DE, U.S.A.).

Quantitation

Quantitation was achieved using peak height ratios of compound I to internal standard (II). For each analysis, a standard curve was generated by adding known and varying amounts of compound I and a known and constant amount of the internal standard to canine plasma. The ranges of standard curves (8—120 ng or 100—1500 ng) were designed to encompass the range of expected experimental values. Spiked samples were treated as unknowns to evaluate the precision and accuracy of the method. Additionally, blind plasma samples spiked with low concentrations of compound I were prepared by another laboratory and were analyzed to further validate the procedure. Human plasma samples were obtained from volunteers, participating in a safety and tolerance study, 24 h following a single 60-mg oral dose of placebo. The human plasma samples were then frozen (—20° C) for approximately 30 days and then analyzed by this procedure.

Percent recovery was determined by comparison of peak height of compound I for each unknown sample to an external standard curve of ng of compound I on column versus peak height (cm). An external standard curve was constructed for every analysis.

RESULTS

The method was validated by means of spiked canine plasma samples treated as unknowns and blind spiked canine plasma samples prepared in another laboratory. The results are shown in Tables I—III and they demonstrate good precision and accuracy over the concentration range studied. The overall accuracy and precision from 10 to 1000 ng of compound I per ml of plasma were ± 7.2% and 7.2% coefficient of variation (C.V.), respectively. In addition, the overall (10—1000 ng/ml) mean percentage recovery of compound I as determined by external standard technique was 55 ± 13.2% (n = 103).

In order to accurately quantitate plasma levels of compound I over a large concentration range (10—1500 ng/ml) two separate but overlapping standard curves were run. Both low- and high-range standard curves consisted of seven

TABLE I

PRECISION AND ACCURACY DATA FOR ANALYSIS OF LOW CONCENTRATIONS OF COMPOUND I IN PLASMA (n = 16)

Data are a summarization of data of four separate experiments. A standard curve of 8 to 130 ng of compound I per ml of plasma bracketed spiked unknowns. For additional information, see Experimental.

Amount added (ng)	Amount measured (ng, mean ± S.D.)	C.V. (%)	Accuracy $\lvert\%\Delta\rvert$*
10.0	9.5 ± 1.32	13.9	11.2
20.0	20.4 ± 1.74	8.5	7.1
50.0	53.5 ± 7.45	13.9	11.7
100.0	99.5 ± 10.38	10.4	8.8

*Represents the mean of individual determinations for the absolute percent difference of amount of drug added to sample versus amount of drug assayed.

TABLE II

PRECISION AND ACCURACY DATA FOR ANALYSIS OF HIGHER CONCENTRATIONS OF COMPOUND I IN PLASMA (n = 12)

Data are a summarization of data of two separate experiments. A standard curve of 100—1500 ng of compound I per ml of plasma bracketed spiked unknowns. For additional information, see Experimental.

Amount added (ng)	Amount measured (ng, mean ± S.D.)	C.V. (%)	Accuracy $\lvert\%\Delta\rvert$*
500.0	520.3 ± 13.96	2.7	4.7
1000.0	1036.6 ± 17.90	1.7	3.6

*Refer to footnote to Table I.

TABLE III

PRECISION AND ACCURACY DATA FOR ANALYSIS OF BLIND SAMPLES (n = 5)

Data represent a single experiment for the analysis of plasma samples spiked with compound I that were supplied by another laboratory. The actual levels of compound I were made known to the analyst only after the blind samples were analyzed. The unknowns were bracketed by a standard curve from 8 to 120 ng of compound I per ml of plasma. For additional information, see Experimental.

Amount added (ng)	Amount measured (ng, mean ± S.D.)	C.V. (%)	Accuracy $\lvert\%\Delta\rvert$*
17.2	16.7 ± 0.69	4.1	3.6
34.3	35.3 ± 1.95	5.5	4.0
85.1	76.9 ± 2.82	3.7	9.7

*Refer to footnote to Table I.

points bracketing the anticipated unknown sample concentrations. Good linearity and a negligible Y-intercept were routinely found. Least-squares linear regression of low- and high-range standard curves yielded a representative equation ($Y = aX + b$) for the line and regression coefficients (r^2) of $Y = 0.0022X + 0.0190$, $r^2 = 0.9991$ and $Y = 0.0231X - 0.0115$, $r^2 = 0.9995$, respectively.

The samples were free of interfering peaks as seen in the blank sample (Fig. 2A). Baseline separation is achieved between compound I and the internal standard and the analysis time is about 11 min (Fig. 2B). Following a single 60-mg oral dose to human volunteers, compound I was detected in human plasma 24 h after administration (Fig. 2C). Analysis of plasma from volunteers receiving a placebo revealed no interfering peak in the region of interest (not shown). Furthermore, Fig. 3 demonstrates that the presence of the N-deethylated metabolite (III) of compound I in plasma would not interfere with the quantitation of compound I. This metabolite has similar chromatographic mobility as that of compound I and is found in in vitro rodent hepatic microsomal metabolism experiments [8]. Therefore, the formation of this metabolite in vivo is likely.

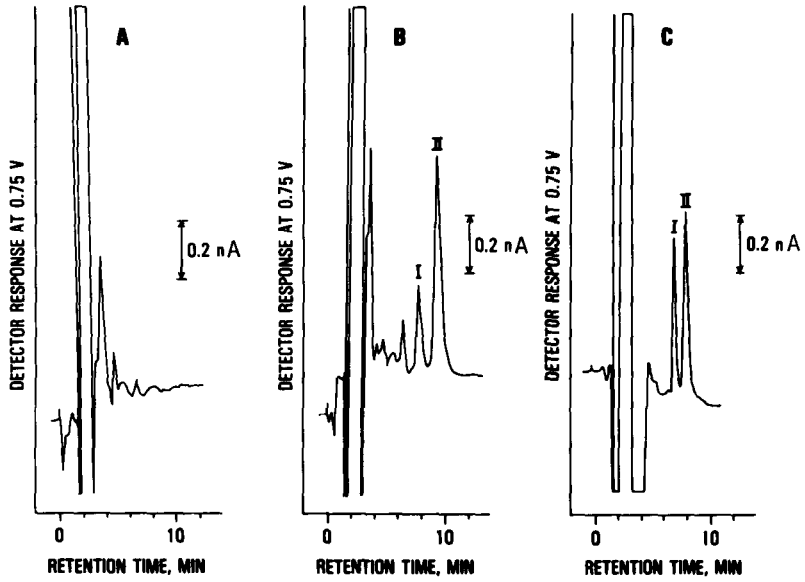

Fig. 2. HPLC chromatograms of extracts from: (A) blank canine plasma; (B) canine plasma (1.0 ml) spiked with 10 ng of compound I (I) and 50 ng of the internal standard (II); (C) patient sample drawn 24 h after oral administration of compound I, spiked with 50 ng of the internal standard (II). A 38-ng amount of compound I (I) per ml of human plasma was detected. In both instances 50 μl of a total 250 μl were injected. For additional information, see Experimental.

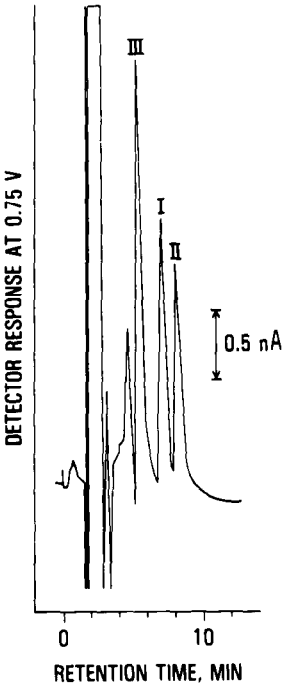

Fig. 3. HPLC chromatogram of extract from canine plasma (1.0 ml) spiked with 500 ng of compound I (I), internal standard (II), and the N-deethylated metabolite of compound I (III). In this instance, 25 μl of a total 250 μl was injected. For additional information, see Experimental.

DISCUSSION

Quantitating this antileishmanial drug by this method has two unique features. First, the liquid—solid extraction of compound I from plasma using disposable prepackaged extraction columns, silica gel Bond-Elut columns, simplifies the extraction by allowing the binding of the drug and internal standard to a solid matrix. This allows the bound sample to be selectively washed to remove unwanted contaminates, and then eluted off the column with the use of aqueous salt and organic mixture. This is less cumbersome, time-consuming and uses less glassware than classical liquid—liquid extraction methods.

The second feature is the use of oxidative electrochemical detection (ED) rather than ultraviolet (UV) detection which provides 50% greater sensitivity and greater selectivity. This observation was determined by the analysis of compound I from plasma and detected simultaneously by both ED and UV at their maximum useable sensitivities (2 and 0.001 nA full scale, respectively) in our laboratory. Oxidative HPLC—ED is an amperometric determination involving the heterogenous electron transfer from the electrode surface to the solute as it passes through the low volume thin layer cell of the electrochemical detector. Therefore the ability to detect a compound by oxidative ED is dependent on its strength as an oxidizing agent which is a more specific process than detection by UV absorption at 254 nm. This selectivity became useful due to the fact that interfering compounds which were detected occasionally by UV were not detected by ED at all, thereby eliminating a major obstacle in the development of this assay.

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