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Note

Analysis of DL-*threo*-α-(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol phosphate (enpiroline), a new candidate antimalarial, in blood using high-performance liquid chromatography

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Enpiroline [DL-*threo*-α-(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol phosphate (Fig. 1)] belongs to one class of aminoalcohols under investigation by the U.S. Army Antimalarial Drug Development Program. This compound appears to be a promising schizonticidal agent against chloroquine- and other drug-resistant strains of *Plasmodium vivax* [1]. In order to optimize dosage regimen, pharmacokinetic studies were needed and these in turn necessitated reliable analytical methods. Two previously reported analytical methods [2,3] were not suitable for pharmacokinetic studies. The gas chromatographic method [2] required derivatization and 5 ml of blood per sample. In addition, insufficient information was provided for critical evaluation of the method. The high-performance liquid chromatographic (HPLC) method [3] was too complex and not readily suitable for analyzing biological extracts. Therefore, a relatively simple method was developed for the analysis of enpiroline in blood.

EXPERIMENTAL

Chemicals

Enpiroline phosphate (I, WR 180,409) and the internal standard [DL-2,8-bis(trifluoromethyl)-4-(1-hydroxy-3-(N-*tert.*-butylamino)propyl)quinoline

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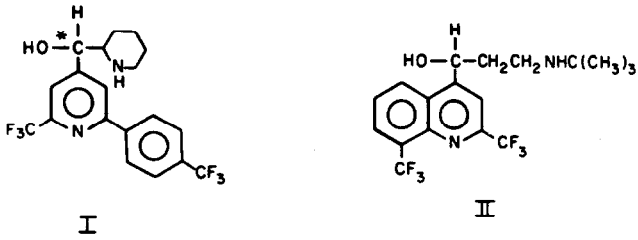

I II

Fig. 1. Chemical structures of enpiroline (I) and the internal standard (II). The asterisk denotes the position of the ^{14}C label in the radioactive compound.

phosphate, II, WR 184,806] were obtained from Walter Reed Stock. Their structures are depicted in Fig. 1. ^{14}C-Labeled enpiroline (39.1 μCi/mg; chemical and radiochemical purity > 98%; Fig. 1) was obtained from Research Triangle Institute (Research Triangle Park, NC, U.S.A.). Baker-analyzed reagents of greatest purity available were used (J.T. Baker, Phillipsburg, NJ, U.S.A.). Organic solvents were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Commercially available heptanesulfonate ion-pairing reagent, PIC B7, was purchased from Waters Assoc. (Milford, MA, U.S.A.).

Standard solutions

All concentrations refer to the free base form. Methanolic stock solutions of I and II were prepared to contain 1.0 μg/μl of the respective compounds. These stock solutions were then diluted in 0.05 *M* hydrochloric acid to yield 10.0, 2.0 and 0.50 ng/μl I and 10.0 and 2.0 ng/μl II.

Extraction procedure

To disposable glass centrifuge tubes (16 mm × 125 mm) with PTFE-lined screw caps were added 1 g of blood and 1 ml of deionized water. Contents were mixed by vortexing and appropriate amounts of I and II were added. Samples were mixed again by vortexing which was followed by the addition of 1 ml of 0.5 *M* dibasic sodium phosphate (pH 9.2) and vortex-mixing again to ensure homogeneity. All extractions were carried out by mixing for 15 min using Roto-Torque rotator (Cole Palmer, Chicago, IL, U.S.A.) and centrifugation for 10 min at 1400 *g* and 4 °C in a Sorvall Refrigerated RC-3B centrifuge (Dupont, Newton, CT, U.S.A.). Blood samples were extracted with 5 ml of ethyl acetate–hexane (3:2, v/v). The organic phase was extracted with 2 ml of methanol plus 3 ml of 0.5 *M* monobasic ammonium phosphate. To the methanolic aqueous phase were added 2 ml of 0.2 *M* perchloric acid and the resultant mixture was extracted with 6 ml of dichloromethane. The dichloromethane phase was transferred to clean tubes and evaporated to dryness under nitrogen at room temperature. The residue was redissolved in 100 μl of mobile phase just prior to analysis.

High-performance liquid chromatographic analysis

The chromatographic system consisted of a Model 6000A pump (Waters Assoc.), Model 440 absorbance detector (Waters Assoc.), Model BD-41 dual-

channel strip chart recorder (Kipp & Zonen, Delft, The Netherlands) and a 30 cm×3.9 mm I.D. 10-μm particle size μBondapak C_{18} column (Waters Assoc.). The column was at ambient temperature. Mobile phase consisted of 0.005 *M* heptanesulfonate PIC B7 ion-pairing reagent in methanol–water (70:30, v/v) and the flow-rate was 1.0 ml/min. Injections were made using 10–50 μl of the sample and the ultraviolet (UV) signal was monitored at 254 nm and 0.005 absorbance units full scale (a.u.f.s.).

Decomposition study

When studying the decomposition of antimalarials during the extraction, an HPLC radioactivity detector (Radiomatic Instruments, Tampa, FL, U.S.A.) was connected in series with the HPLC–UV detector. [^{14}C]Enpiroline was used in the decomposition studies. A decrease in the radioactivity and UV signal responses concommitant with an appearance of UV-absorbing radioactive peaks was indicative of a decomposition of the parent compound. The decomposition was especially dramatic when the evaporation was carried out in ether or acetate solvents; in some cases the decomposition was virtually complete.

Quantitation

For each analysis, a standard curve was generated by adding known, varying amounts of I to outdated human blood (Walter Reed Army Medical Center Blood Bank, Washington, DC, U.S.A.) prior to extraction. Known and constant amounts (250 or 600 ng) of II were also added to all samples prior to extraction. Standards (10–150 ng or 25–1500 ng) were selected to bracket the anticipated range of experimental values. Spiked samples were treated as unknowns to evaluate the accuracy and precision of the method. Quantitation was achieved by using peak-height ratios of I to II.

Determination of extraction efficiency

Four 1-g blood samples were spiked with 220 ng of [^{14}C]enpiroline and the extraction efficiency was determined by liquid scintillation counting (Model Mark II, Nuclear Chicago, Des Plaines, IL, U.S.A.) of the evaporate of the final extracts.

Stability of I in blood on storage

Aliquots of blood (75 ml) were spiked with I to give final concentrations of 50 and 150 ng/ml, stirred for 1 h at room temperature, divided into 1-ml portions and frozen at −20°C and −80°C. A freshly spiked standard curve was used for each week's analysis.

RESULTS AND DISCUSSION

A relatively simple method was developed for quantitation of I in blood requiring only 1 g of sample. The extraction procedure was essentially an adaptation of that developed for mefloquine, a quinoline-methanol antimalarial [4]. Both of these compounds are weak bases, as are many other antimalarials, and a three-step extraction procedure was developed to take advantage of this physicochem-

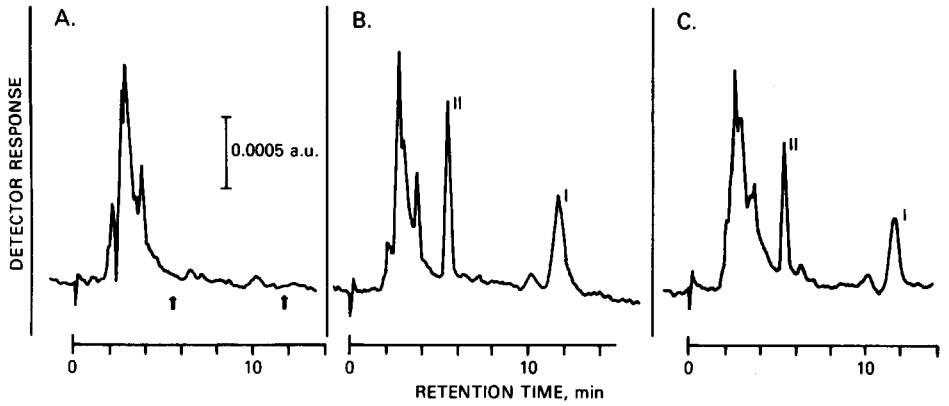

Fig. 2. Typical chromatograms of extracts from (A) blank blood, (B) blood of a patient containing 115 ng/g I and (C) blank blood spiked 125 ng/ml I. The arrows in chromatogram A denote the retention times for I and II.

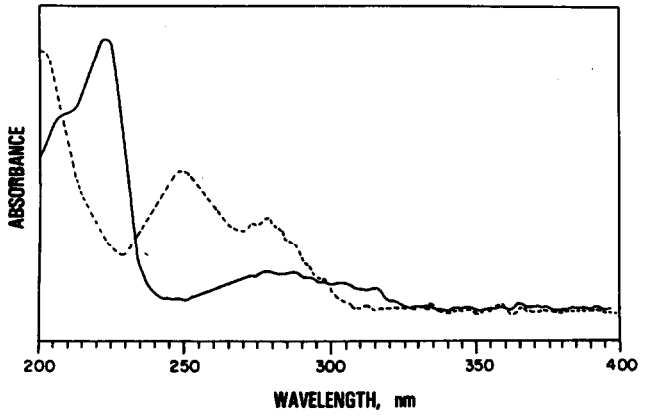

Fig. 3. Ultraviolet absorption spectra of I (- - -) and II (———). The spectra were obtained during an HPLC run using an HPLC spectrophotometric detector (Model 1040A, Hewlett-Packard, Avondale, PA, U.S.A.).

ical characteristic. After the initial lysis of red blood cells with water, the samples were made alkaline and the weakly basic antimalarials were extracted into a mixture of ethyl acetate–hexane. Next, the compounds were extracted from the organic phase into methanolic acidic buffer. In the last step, perchloric acid was added to methanolic buffer extract in order to facilitate ion-pair extraction into dichloromethane. The overall extraction efficiency for I from blood was 72%.

Chromatographic separation was achieved by reversed-phase HPLC utilizing heptanesulfonate ion-pairing reagent. Representative chromatograms of extracts from blank blood, patient blood and spiked blood are depicted in Fig. 2. The absorbance spectra for I and II are depicted in Fig. 3. Based on this, a detection wavelength of 254 nm was selected for monitoring, since it is near a maximum for I and is a commonly used wavelength. Poor absorbance of the internal standard at this wavelength necessitated using a relatively large amount of II.

In order to cover a large concentration range (10–1500 ng I per g blood) without

TABLE I

PRECISION AND ACCURACY DATA FOR ANALYSIS OF LOWER CONCENTRATIONS OF I IN BLOOD

Data represent a compilation of four separate experiments. Spiked unknowns were bracketed by a standard curve ranging from 10 to 150 ng of I.

Actual (ng)	Experimental (mean ± S.D.) (ng)	Coefficient of variation (%)	n
15.0	17.9 ± 1.10	6.1	16
30.0	30.7 ± 1.75	5.7	16
50.0	50.0 ± 2.55	5.1	16
90.0	88.2 ± 6.93	7.8	11

sacrificing accuracy or precision, it was advisable to use two standard curves. The low standard curve was designed for 10–150 ng/g and the high curve for 25–1500 ng/g. Each standard curve consisted of at least eight points bracketing the anticipated experimental concentration range. Excellent linearity and a negligible y-intercept were found routinely. Using a least-squares linear regression analysis, representative equations of the line and the regression coefficient (r^2) for the low and high standard curves were $y=0.0128x+0.0055$ ($r^2=0.995$) and $y=0.00487x+0.0265$ ($r^2=0.998$), respectively.

Method was validated by using spiked blood. The results showed good accuracy and precision in the 30–1400 ng/g concentration range (Tables I and II). Other antimalarials (chloroquine, primaquine, quinine, pyrimethamine, sulfadoxine and mefloquine), as well as several candidate antimalarials, were shown not to interfere with the analysis. I was found stable in blood on storage at either −20°C or −80°C for at least four weeks (Table III).

The method avoided evaporation of extracts in diethyl ether, ethyl acetate or isopropyl acetate since it has been our experience that the evaporation in these solvents resulted in a decomposition of various aminoalcohol candidate antimalarials including I [4]. There was no detectable decomposition of I during the analysis described here as examined using the radioactive compound.

In subsequent studies, this analytical method was shown to provide the nec-

TABLE II

PRECISION AND ACCURACY DATA FOR ANALYSIS OF HIGHER CONCENTRATIONS OF I IN BLOOD

Data represent a compilation of four separate experiments. Spiked unknowns were bracketed by a standard curve ranging from 25 to 1500 ng of I.

Actual (ng)	Experimental (mean ± S.D.) (ng)	Coefficient of variation (%)	n
50.0	51.2 ± 6.87	13.4	16
90.0	93.1 ± 6.38	6.9	12
300.0	304.7 ± 20.9	6.9	15
1400.0	1461.2 ± 72.3	4.9	12

TABLE III

STABILITY OF I IN BLOOD ON STORAGE

Each data point represents analysis of three replicate samples.

Week	Measured (mean ± S.D.) (ng)			
	−20°C		−80°C	
	50.0 ng	150.0 ng	50.0 ng	150.0 ng
0	52.9 ± 4.86	161 ± 10.7	56.1 ± 6.81	160 ± 7.00
1	58.5 ± 6.97	168 ± 7.02	62.9 ± 0.66	159 ± 4.93
2	62.7 ± 1.81	162 ± 10.5	54.6*	158 ± 13.2
4	53.6 ± 1.75	154 ± 6.03	58.7 ± 5.72	155 ± 5.00

*Duplicate samples.

essary sensitivity and was found suitable for clinical pharmacokinetic studies. As suggested earlier [4] and confirmed here, the method could be easily modified for analysis of other aminoalcohol candidate antimalarials.

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