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Note

High-performance liquid chromatography of antifilarials

III. Determination of 2-*tert*.-butyl-6-isothiocyanato-5-(piperidin-1-yl)-benzthiazole in biological fluids*

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The search for new chemotherapeutic agents to combat filariasis has, in recent years, led to ivermectin [1,2], 1-4-(4-nitrophenylaminophenyl thiocarbamido-4-methyl)piperazine (CGP 6140) and the 2-*tert*.-butyl benzthiazole class of synthetic compounds, viz. S-2-carboxyethyl-N-(2-*tert*.-butyl-5-methoxybenzthiazol-6-yl) dithiocarbamate (CGP 20376) and its isothiocyanate metabolite (CGP 20308) [3–7] showing potent antifilarial activity. 2-*tert*.-Butyl-6-isothiocyanato-5-(piperidin-1-yl)benzthiazole (I, Fig. 1) is a new preparation orally active against both microfilaria and adult parasites in several experimental infections, and also against infective larvae in vitro [7]. The compound is in Phase I clinical trial in India [8].

Preliminary animal pharmacokinetic data on compound I have been reported recently [9]. In this paper we present a reversed-phase high-performance liquid chromatographic (HPLC) method for the quantitation of unchanged drug in

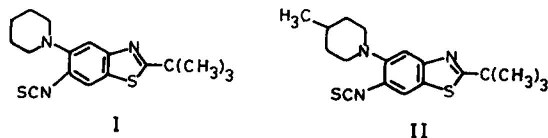

Fig. 1. Structural formulae of compound I (CGI 16343, mol.wt. 331) and its internal standard compound II (CGI 17658, mol.wt. 345).

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blood. The method is currently being applied for monitoring clinical pharmacokinetics.

EXPERIMENTAL

Standards and solvents

Compound I and the internal standard (II, Fig. 1) were synthesized at Hindustan Ciba-Geigy Research Centre, Bombay, India. The purity of the synthesized compounds was ascertained by elemental and spectral analysis. Reagent-grade acetonitrile and hexane were the products of E. Merck (Bombay, India) and were distilled before use.

Liquid chromatography

The equipment consisted of a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph equipped with a Rheodyne valve injection system, two M-6000 A pumps, a Model 680 solvent programmer and an M-440 dual-channel fixed-wavelength UV–visible detector set at 254 nm. The method was developed on a Nucleosil C_{18} (5 μm) reversed-phase column (125$\times$4.6 mm I.D.) packed at Ciba-Geigy (Basle, Switzerland). The mobile phase was acetonitrile, pumped at a flow-rate of 1.5 ml/min. The detector signal was recorded and integrated on an electronic data processor (Shimadzu, Model C-R1B) at an attenuation of 4 and a chart speed of 5 mm/min. Calibration and analysis were based on standard/internal standard height integration ratios.

Standard solutions

Compound I and the internal standard (1 mg each) were dissolved separately in 50 ml of acetonitrile to yield stock solutions of 20 μg/ml strength. These were diluted 2–20 fold with acetonitrile to provide working standards in the range 50–500 ng per 50 μl for compound I and 250 ng per 50 μl for the internal standard.

Calibration and extraction procedure

For the calibration curve, 50-μl aliquots of working standard solutions, corresponding to 50, 100, 200, 400 and 500 ng of compound I and 250 ng of internal standard, were carefully pipetted into 15-ml standard joint glass extraction tubes. Without evaporation of the solvent aliquots, 1 ml of drug-free blood was added, and the tubes were vortex-mixed and allowed to equilibrate. Extraction was carried out twice with 3 ml of hexane, for 5 min each time on a reciprocal shaker. The tubes were centrifuged at 4000 *g* for 5 min, and the combined hexane layers were transferred to 10-ml conical glass tubes and evaporated to near dryness at 37°C under nitrogen. The residual film was reconstituted in 0.1 ml of the mobile phase and, after a brief centrifugation, 10–20 μl were injected into the liquid chromatograph.

Animal experiment

Six male Charles Foster strain albino rats weighing 180–200 g were administered orally a suspension of compound I in 0.2% carboxymethyl cellulose at a

dose of 15 mg/kg. The rats had free access to food and water. About 0.3 ml of blood was collected from the retroorbital sinus of each rat at 0.5, 1, 2, 4, 6, 8, 12 and 24 h post medication into heparinized vials. Equal aliquots from all six rats were pooled in chilled glass tubes, and suitable aliquots were processed as described. The amount of unchanged drug in plasma and blood was also determined.

RESULTS AND DISCUSSION

Compound I eluted on a reversed-phase column only when 100% acetonitrile was used as the mobile phase. Addition of water to the acetonitrile led to protracted retention of the compound on the column. Methanol cannot be substituted for acetonitrile since compound I is unstable in alcohol: the free isothiocyanate group in the molecule results in the formation of a urethane.

As shown in Fig. 2, under the chromatographic conditions described, compound I elutes at 2.8 min followed by the internal standard II at 3.1 min. The two components exhibited sharp and symmetrical peaks that were well resolved from the early endogenous peaks. The total run time was only 4.5 min.

As shown in Table I, a dual extraction with hexane at physiological pH resulted in a simple extraction procedure that gave optimal and reproducible recoveries for both compound I (76%) and the internal standard (67%). The intra- and

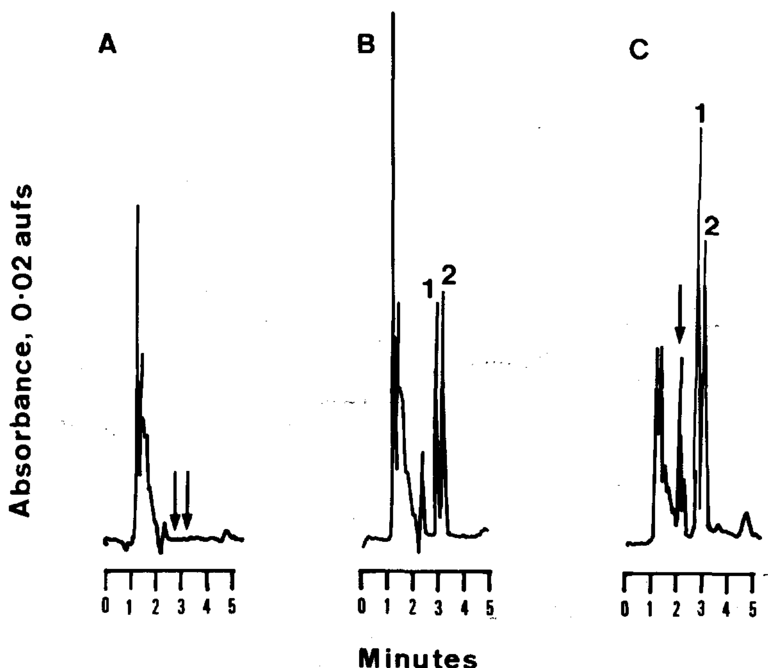

Fig. 2. Representative chromatograms of: (A) 1 ml of rat blood (blank); (B) rat blood spiked with 100 ng/ml compound I and 250 ng/ml internal standard; and (C) 0.5 ml of a 2-h post-treatment blood sample from rat receiving 15 mg/kg compound I orally (unchanged drug 436 ng/ml). Peaks: 1 = compound I; 2 = internal standard.

TABLE I

RECOVERY AND WITHIN-DAY PRECISION OF THE METHOD FOR THE DETERMINATION OF COMPOUND I FROM SPIKED HUMAN BLOOD

Each value represents the mean of six determinations.

Compound	Concentration (ng/ml)	Recovery (mean ±C.V.) (%)	Precision (C.V., %)	
			Intra-assay	Inter-assay
I	100	76±1.2	0.51	0.53
	500	76±2.2		
II	250	67±2.2		

TABLE II

ACCURACY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF COMPOUND I FROM SPIKED HUMAN BLOOD

Each value represents the mean ± C.V. (%) of four determinations per concentration.

Concentration of I (ng/ml)	
Spiked	Determined
25	25±2.0
75	72±3.1
150	148±1.4
400	382±0.7
800	797±1.0

inter-assay coefficients of variation (C.V.) of blood samples spiked with 100 ng/ml I were 0.51% and 0.53% ($n=6$), respectively.

Calibration curves generated in the concentration range 50–500 ng/ml, with 250 ng of internal standard, showed good linearity (intercept = −0.0058; slope = 0.0051; $r=0.9999$). The minimum detectable concentration of compound I, which gave a peak three times the baseline noise, corresponded to 5 ng/ml.

The assay was applied to human blood samples spiked with compound I in the range 25–800 ng/ml. Results presented in Table II indicate that levels in this range can be estimated with good accuracy and precision with a C.V. of less than 4%.

The procedure was also applied to study the blood concentration profile of compound I in rats following a 15 mg/kg oral dose (Fig. 3). A maximum concentration of 452 ng/ml, recorded at 2 h, declined rapidly to 43 ng/ml at 12 h. The ratio of unchanged drug in rat blood to plasma collected 2–8 h post-administration was found to be 0.61 (range 0.58–0.63). An extra but clearly resolved peak eluting at 2 min (Fig. 2C), not seen in the chromatogram of blank blood (Fig. 2A), could be ascribed to an unknown metabolite that is more polar than the parent compound. The method developed for blood is simple, rapid and inexpensive and has been applied to plasma and urine without modification.

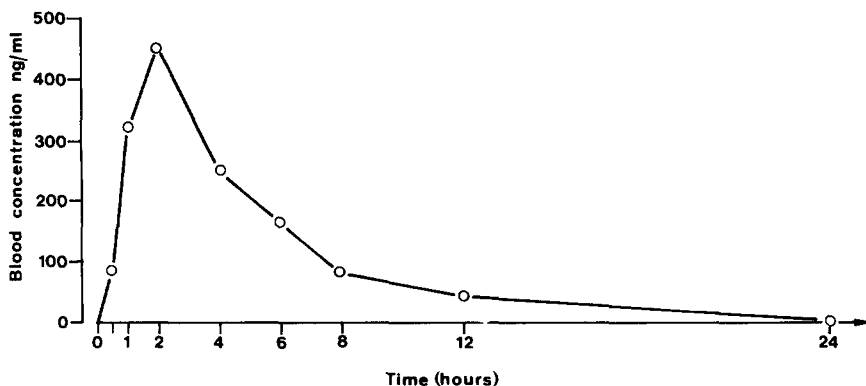

Fig. 3. Blood concentration profile of I after a single oral dose of 15 mg/kg to rats. Blood was collected retroorbitally from six rats and pooled for analysis.

Compound I has a reactive thiocyanate group in its molecule, which can covalently bind with amino groups of free amino acids, peptides and proteins in biological fluids. To minimize this reaction, it is imperative that all biological fluid samples, once collected, be immediately frozen over dry ice and preserved at −20°C till analysed. To check the stability of the compound on storage, rat blood samples were analysed immediately on collection and six weeks after freezing at −20°C. Results indicated no change in concentrations of compound I. However, blood samples frozen and thawed three times showed loss of ca. 27% (range 19–38%) over three weeks. Hence it is suggested that frequent thawing and freezing should be avoided as it would lead to erroneous results.

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