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Note

High-performance liquid chromatography of antifilarials

II. Determination of S-(2-carboxyethyl)-N-(2-*tert.*-butyl-5-methoxybenzthiazol-6-yl) dithiocarbamate and its active isothiocyanate metabolite in biological fluids*

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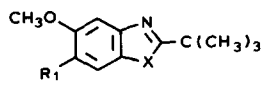
Filariasis is a major tropical infection for which the currently available drugs diethylcarbamazine and suramin have limited efficacy. S-(2-Carboxyethyl)-N-(2-*tert.*-butyl-5-methoxybenzthiazol-6-yl) dithiocarbamate (I, Fig. 1) is a new trial preparation, orally effective against micro- and macrofilariae and against schistosomal parasites in various experimental infections [1–3], which is now undergoing Phase I clinical trial [4]. The animal pharmacokinetic data of this compound have recently been presented [5]. At physiological pH, compound I



Compound	Mol. wt.	R_1	X
I (CGP 20 376)	384	$-NHCSS(CH_2)_2COOH$	S
II (CGP 20 308)	278	$-NCS$	S
III (CGP 24 914)	368	$-NHCSS(CH_2)_2COOH$	O
IV (CGP 24 915)	262	$-NCS$	O

Fig. 1. Structural formulae and relative molecular masses of compounds I and II and their internal standards.

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dissociates to its corresponding isothiocyanate metabolite (II, Fig. 1), which exhibits equipotent biological activity in the animal models [1].

This paper reports high-performance liquid chromatographic (HPLC) methods for the quantitation of compounds I and II in biological fluids by normal- and reversed-phase modes, respectively. The methods have been successfully used for animal studies [5] and are currently being applied to an investigation of human pharmacokinetics.

EXPERIMENTAL

Standards and solvents

Compounds I and II and their respective internal standards III and IV (Fig. 1) were synthesized in the Ciba-Geigy laboratories at Basle and Bombay. The purity of the synthesized compounds was ascertained by elemental and spectral analysis. Spectroscopic-grade methanol (Uvasol) and analytical-grade glacial acetic acid were obtained from E. Merck (Bombay, India) and used as such. Other reagent-grade solvents acetonitrile, cyclohexane, dichloromethane (E. Merck), chloroform (Glaxo and BDH, Bombay, India) and diethyl ether (Hyderabad Chemicals and Pharmaceuticals, Hyderabad, India) were distilled before use.

Liquid chromatography

For compound I, the equipment consisted of a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph equipped with two M-6000A pumps, a Model 680 solvent programmer, a Rheodyne valve injection system, an M-440 dual-channel UV–visible detector fitted with a 340-nm filter, and a Model C-R1B electronic data processor (Shimadzu, Tokyo, Japan). The separation was carried out on a LiChrosorb Si 60 (5 μm) normal-phase column (125×4 mm I.D.) using chloroform–methanol–acetic acid (99.5:0.5:0.01) as the mobile phase at a flow-rate of 1.5 ml/min.

For compound II, the equipment consisted of a Varian (Zug, Switzerland) Model 5000 ternary solvent-delivery system, a Rheodyne valve injection system, a UV–visible variable-wavelength detector (Spectromonitor D; LDC Milton Roy, Riviera Beach, FL, U.S.A.) set at 295 nm and an electronic data processor (Shimadzu, Model C-R3A). The reversed-phase column was a LiChrosorb RP-18 (5 μm; 150×4.6 mm I.D.) with acetonitrile–water (85:15) as the mobile phase at a flow-rate of 1.5 ml/min.

Standard solutions

Compound I and its internal standard III were dissolved and diluted in acetonitrile–water–glacial acetic acid (85:15:1) to obtain working standard solutions in the range 50–500 ng per 50-μl aliquot. Compound II and its internal standard IV were dissolved in chloroform to yield a final working standard concentration of 1 μg/ml.

Compound I: calibration and extraction procedure

A 50-μl aliquot of working standard solution corresponding to 50, 100, 200, 400 and 500 ng of I followed by another 50-μl aliquot containing 250 ng of internal standard III were carefully pipetted into 15-ml standard-joint glass tubes. Without evaporation of the solvent aliquots, 1 ml of drug-free acidified blood (5 μl of glacial acetic acid per ml of blood) was added, and the tubes were thoroughly vortex-mixed and allowed to stand for a few minutes for equilibration to be achieved. Then 1 ml of 0.17 *M* acetic acid was added, the tubes were vortex-mixed again, and a single extraction with 4 ml of diethyl ether–chloroform–cyclohexane (4:3:2) was carried out on a reciprocal shaker for 5 min. The tubes were centrifuged at 4000 *g* for 5 min and the organic phase was transferred to 10-ml conical glass tubes and evaporated to dryness under nitrogen at 37°C. The residual film was reconstituted in 0.1 ml of mobile phase and centrifuged at 4000 *g* for 2 min; 10–25 μl were injected into the chromatograph.

Compound II: calibration and extraction procedure

For the calibration curve of compound II, concentrations of the working standard solution corresponding to 25, 50, 100, 200 and 300 ng and 200 ng of the internal standard (IV) were placed in 15-ml standard-joint glass tubes. The solvent layer was evaporated and 1 ml of drug-free acidified blood (5 μl of glacial acetic acid per ml of blood) was added. The tubes were vortex-mixed and allowed to stand for a few minutes before extraction. Extraction was carried out only once with 4 ml of cyclohexane–dichloromethane (2:1) on a reciprocal shaker for 5 min. The tubes were centrifuged at 4000 *g* for 5 min. The upper organic phase was transferred to 10-ml conical glass tubes and evaporated to dryness at 37°C under nitrogen. The residual film was reconstituted in 0.1 ml of acetonitrile–water–glacial acetic acid (85:15:1), and 20–30 μl were injected into the chromatograph.

Stability of compound I in acidified blood

The stability of compound I was checked in blood samples spiked with compound I, and also in rat blood samples obtained after the oral administration of 25 mg/kg. Samples of rat blood were acidified with acetic acid and frozen immediately, whereas spiked blood samples were prepared by adding a known amount of I to acidified blood, which was vortex-mixed, frozen and stored at −20°C.

RESULTS

Chromatography

Under the chromatographic conditions described for a normal-phase column, compound I and its internal standard III eluted at 2.7 and 3.2 min, respectively. Compound II and its internal standard IV were also well resolved on a reversed-phase column, eluting at 4.4 and 3.4 min, respectively. Compounds I and II elute with the solvent front on a reversed-phase and normal-phase column, respectively, and hence neither interferes with the chromatography of the other. As shown in Fig. 2, when standards were spiked to drug-free blood, there was no interference from endogenous components in the blank profile, and the spiked

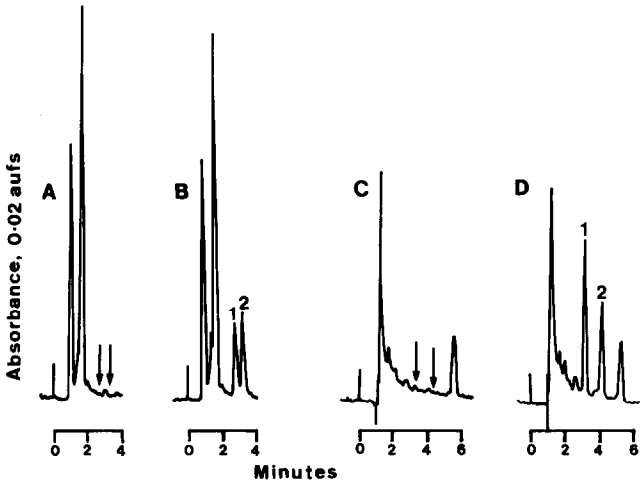

Fig. 2. Chromatograms of human blood extracts for compound I assay: (A) blank; (B) spiked with 200 ng/ml of compound I (1) and 250 ng/ml of internal standard III (2); and for compound II assay: (C) blank; (D) spiked with 200 ng/ml of internal standard IV (1) and 100 ng/ml of compound II (2).

TABLE I

REGRESSION PARAMETERS FOR HUMAN BLOOD CALIBRATION CURVES

Mean values from four calibration sets.

Compound	Concentration range (ng/ml)	Intercept	Slope	Correlation coefficient (r)
I	50–500	−0.0003	0.0044	0.9997
II	25–300	−0.0026	0.0068	0.9997

samples showed the peaks of compounds I and II to be well resolved from the respective internal standards.

Recovery and precision

Six replicate injections of human blood samples spiked with 100 ng/ml of standards gave mean percentage recovery values ($\pm$coefficient of variation, C.V.) of 83 ± 3.4 and 84 ± 1.1 for compound I and its internal standard III, and 54 ± 4.5 and 78 ± 4.3 for compound II and its internal standard IV, respectively. The C.V. values of the inter- and intra-assay for compound I were 2.9 and 3.0%, and that for compound II 5.5 and 0.9%, respectively, at a spiked blood concentration of 100 ng/ml.

Linearity

The linearity of the method was established with human blood calibration curves in the concentration range 50–500 ng/ml for compound I and 25–300 ng/ml for compound II. Table I lists the regression parameters obtained with four replicate

analyses of each of the five concentrations. The correlation coefficients of 0.9997 indicate good linearity.

Accuracy and limit of detection

The methods were applied to an assay of human blood samples spiked with compound I in the concentration range 25–150 ng/ml and with compound II at 10–150 ng/ml. Results recorded in Table II indicate that concentrations in these ranges can be estimated with good precision, with a C.V. within 7% of the spiked concentrations. Based on a signal-to-noise ratio of more than 3, the limit of detection of compound I is ca. 15 ng/ml and that of compound II is ca. 5 ng/ml.

The blood-to-plasma ratio was determined by administering 25 mg/kg of compound I orally to six rats. A 0.3-ml volume of blood was collected retroorbitally from each rat over 6 h post administration. The ratio of plasma to blood ranged between 0.55 and 0.74, with a mean ratio of 0.64.

Interference of compound I in the analysis of II and vice versa

There was no interference observed in the estimation of compound I in the presence of compound II. However, compound I can readily dissociate to compound II at mildly basic pH and thereby vitiate the analysis of the metabolite. Therefore, the extent of the dissociation, under the experimental conditions described for the determination of compound II, was studied by spiking different concentrations of compound I to blood and analysing the amount converted into compound II. As indicated in Table III, there was no dissociation of compound I at spiked concentrations up to 200 ng/ml. At 300 ng/ml and above, the conversion was in the range 1.9–3.4% (mean 2.6%). In the presence of a fixed concentration of compound I (500 ng/ml), compound II was spiked to blood in the concentration range 20–300 ng/ml. On analysis of compound II, higher recoveries were obtained owing to dissociation of compound I. The mean conversion value of 2.6% of the spiked compound I was subtracted as an equimolar amount of compound II from the total analysed concentration. The corrected concentrations of compound II showed a C.V. within 6% of the spiked concentrations.

Blood samples acidified with acetic acid did not degrade, which was confirmed

TABLE II

ACCURACY AND PRECISION OF THE PROCEDURES FOR THE DETERMINATION OF COMPOUND I AND ITS METABOLITE FROM SPIKED HUMAN BLOOD

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

Compound I (ng/ml)		Compound II (ng/ml)*	
Spiked	Determined	Spiked	Determined
25	26.0 ± 6.3	10	9.6 ± 6.3
75	76.8 ± 3.4	20	20.9 ± 2.9
150	146.5 ± 2.9	75	78.6 ± 2.3
		150	153.2 ± 3.7

*In the absence of compound I.

TABLE III

ESTIMATION OF COMPOUND II IN THE PRESENCE OF COMPOUND I IN BLOOD

Each value represents the mean of four determinations.

Compound I concentration		Compound II concentration		
Spiked (ng/ml)	Converted into compound II (%)	Spiked in presence of compound I (ng/ml)*	Total analysed (ng/ml)	Corrected ±C.V. (%) (ng/ml)**
100	0	20	30.1	20.7±3.7
200	0	75	86.3	76.9±5.3
300	3.4	300	312.9	303.5±0.9
400	1.9			
500	2.5			
Mean (300-500)	2.6			

*At a fixed compound I concentration of 500 ng/ml.

**Mean conversion value (2.6%) of spiked compound I (mol.wt. 384) converted into compound II (mol. wt. 278) is subtracted from the total analysed concentration of compound II (2.6% of 500 ng = 13 ng of I = 9.4 ng of compound II).

by repeated freezing and thawing of the spiked blood samples. Spiked blood samples were frozen at −20°C, and on analysis after 1 week, 2 weeks and 4 weeks had concentrations within 5% of the original, indicating that compound I is stable on freezing and thawing provided that blood samples are acidified immediately on collection. These results were observed with spiked as well as with rat blood samples obtained after treatment with a 25 mg/kg dose of compound I, suggesting that spiked samples are representative of biological samples since the concentrations observed were reproducible over 1 month.

DISCUSSION

The parent compound I is acidic and polar in nature while the isothiocyanate metabolite II is neutral and lipophilic. These characteristics necessitated the development of separate HPLC methods for their determination.

Compound I was found to degrade to compound II in solutions prepared in chloroform, methanol, acetonitrile and acetonitrile–water. However, a standard solution prepared in acetonitrile–water–acetic acid (85:15:1) remained stable for 2 weeks on refrigeration. In order to minimize the degradation of compound I during extraction procedures, the glassware was rinsed with this acidified solvent mixture. Blood was also acidified with 5 μl/ml acetic acid before spiking the standards.

Compound I was found to be stable on both normal-phase and reversed-phase columns under the chromatographic conditions described for the estimation of both the compounds. However, in the analysis of compound II in the presence of compound I, the dry extracts of blood required reconstitution in acidified mobile phase (acetonitrile–water–acetic acid, 85:15:1) before injection. Despite the use of glacial acetic acid as a stabilizer at various stages during the analysis of com-

pound II, the parent drug I, when present in concentrations above 200 ng/ml, dissociated to the extent of 2.6% during sample work-up. A correction factor was therefore applied. It is recommended that all biological fluid samples are preserved by quickly adding 5 μl/ml acetic acid, freezing immediately over dry ice and storing at $-20°$C.

The analysis of both compounds should preferably be carried out on the same day in order to avoid repeated thawing and freezing of samples. Both methods for the estimation of compound I and its metabolite II are rapid, sensitive and specific. The procedure for the estimation of compound II is applicable to plasma and urine without modification. For compound I, plasma is extracted with cyclohexane–dichloromethane (2:1) in the presence of 0.5 ml of 1.7 *M* acetic acid, and urine is acidified with 1 ml of 0.17 *M* acetic acid and extracted with the solvent mixture used for plasma.

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