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Note

High-performance liquid chromatography of antifilarials

I. Simultaneous determination of 1-4-(4-nitrophenylaminophenylthiocarbamido-4-methyl)piperazine and three of its metabolites in biological fluids*

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Filariasis is a collective name for several debilitating diseases that affect millions of people in the tropics. A major problem in its control has been the lack of safe and effective chemotherapeutic agents. 1-4-(4-Nitrophenylaminophenylthiocarbamido-4-methyl)piperazine (I, Fig. 1) is a new trial compound exhibiting potent activity against a variety of filarial and schistosomal parasites in several animal hosts [1–3] and is currently being evaluated in humans [4]. The pharmacokinetics and biotransformation of this compound in animals have recently been reported [5]. Two of the metabolites detected in rodent species, viz. the N-oxide (II) and desmethyl (III, Fig. 1) analogues of compound I, also possess intrinsic antifilarial activity [1,6].

In this paper we report a high-performance liquid chromatographic (HPLC) procedure for the simultaneous quantitation of compound I and three of its metabolites in biological fluids. The method has been applied to a pharmacokinetic investigation in animals, and would be also suitable for use in clinical pharmacokinetic studies.

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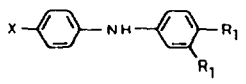
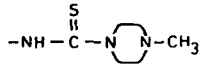
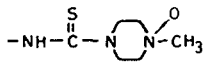
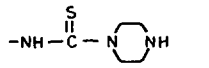
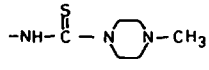


	Compound	M.W.	X	R_1	R_2
I	Parent compound	371	$-NO_2$		$-H$
II	N-oxide metabolite	387	$-NO_2$		$-H$
III	Desmethyl metabolite	357	$-NO_2$		$-H$
IV	Acetamido metabolite	383	$-NHCOCH_3$		$-H$
V	Internal standard	301	$-NO_2$	$-N=C=S$	$-OCH_3$

I : CGP 6140 1-4-(4-Nitrophenylamino-phenyl thio carbamido-4-methyl piperazine)
II : CGP 13231
III : CGI 18107
IV : BA 854
V : BA 130

Fig. 1. Structural formulae and molecular masses of 1-4-(4-nitrophenylaminophenylthiocarbamido-4-methyl)piperazine, its metabolites and the internal standard (V).

EXPERIMENTAL

Chemicals

Compound I and its metabolites II, III and IV and the internal standard (V) were synthesized in the Ciba-Geigy laboratories at Basle and Bombay [7,8]. The purity of the synthesized compounds was ascertained by elemental and spectral analysis. Synthesis-grade acetonitrile, spectroscopic-grade methanol and ammonia solution (25%, sp. gr. 0.91) were obtained from E. Merck (Bombay, India). Acetonitrile was distilled before use.

Liquid chromatography

The method was developed on a Waters Assoc. liquid chromatograph equipped with a Rheodyne valve injection system, two M-6000A pumps, a Model 680 solvent programmer, and an M-440 dual-channel fixed-wavelength UV–visible detector fitted with 313- and 405-nm filters. The separation was effected on a Partisil 10 ODS-3 (10 μm particle size) reversed-phase column (250 mm$\times$4.6 mm I.D.) obtained from Whatman (Clifton, NJ, U.S.A.). The mobile phase was acetonitrile–water–methanol–ammonia solution (64.5:28:7.5:0.001, v/v) pumped at a flow-rate of 1.5 ml/min. The chromatograms were recorded and integrated on an electronic data processor (Shimadzu, Model C-R1B) at an attenuation of 4 and a chart speed of 0.5 cm/min. The calibration and analysis were based on standard/internal standard height integration ratios.

Standards

The N-oxide metabolite (II) was dissolved in methanol, and all other compounds were dissolved in acetonitrile to yield stock solutions of 20 μg/ml. These were diluted five-fold to give working standards. Standard solutions of compound I and metabolite II were stored refrigerated in polypropylene bottles, and those of other compounds were stored in glass standard flasks.

Procedure

Aliquots of standard solutions in the range 100–500 ng each of compounds I, II, III and IV were evaporated to dryness in 15-ml standard-joint glass tubes. This was followed by the addition of 50 μl of internal standard solution in acetonitrile containing a fixed amount up to 400 ng. The tubes were briefly vortex-mixed, and drug-free samples of whole blood or urine in 0.5-ml aliquots were quickly added. The tubes were vortex-mixed thoroughly and 10 volumes (5 ml) of distilled acetonitrile were added. The tubes were vortex-mixed again, dipped in crushed ice for 5 min, and then centrifuged at 4000 *g* for 15 min. The aqueous acetonitrile supernatant was transferred to 10-ml conical glass tubes and evaporated to dryness at 40 °C under a stream of nitrogen. The residual film was reconstituted in 0.1 ml of mobile phase and centrifuged at 4000 *g* for 5 min. A 10–20 μl volume of the solution was injected into the liquid chromatograph.

Drug disposition study

In order to test the ability of the method to measure I and its metabolites II, III and IV in the course of pharmacokinetic studies, compound I was administered orally to ten male albino Charles Foster strain rats at a dose of 25 mg/kg. About 0.3 ml of blood was collected from each rat through the retroorbital sinus into heparinized vials from 0.5 to 24 h, pooled for analysis and preserved at −20 °C. In another set of four animals receiving a similar dose, 0–24 h urine samples were collected in chilled flasks and frozen. Aliquots (0.05–0.5 ml) of blood and urine were processed as described.

RESULTS

Chromatography

The acetamido metabolite (compound IV), being devoid of the nitro group, shows maximum absorption at ca. 313 nm and hence was monitored at this wavelength. All the other components were simultaneously detected at 405 nm in the dual-channel detector. As shown in Figs. 2 and 3, under the chromatographic conditions employed, drug-free blood and urine blanks exhibit neat profiles at both wavelengths. Spiked samples show well-resolved peaks of the five components, including internal standard. The acetamido metabolite (IV) eluted at 2.7 min (Figs. 2B and 3B) followed by compounds I, II, V and III at 4.1, 4.9, 5.8 and 7.5 min, respectively.

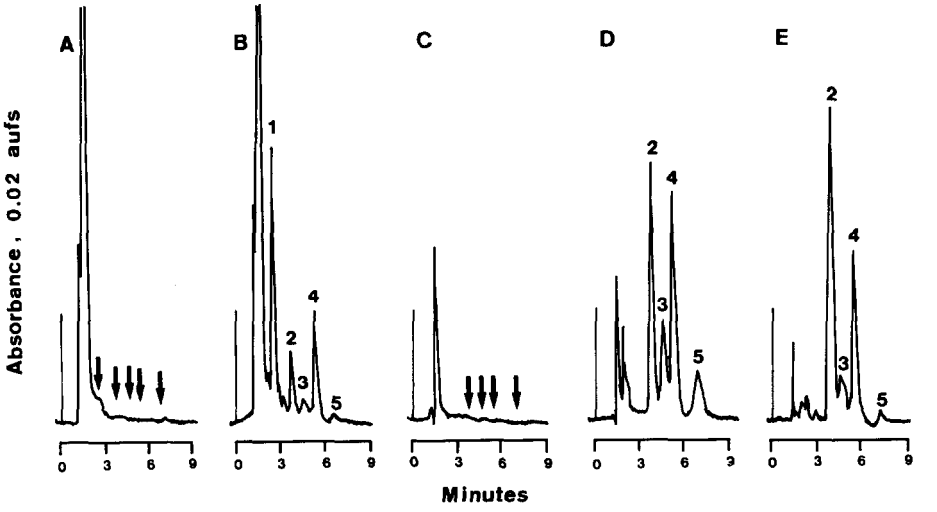


Fig. 2. Representative chromatograms of: (A) rat blood blank monitored at 313 nm; (B) blood spiked with 500 ng of acetamido metabolite IV (1), 500 ng of I (2), 500 ng of N-oxide metabolite II (3), 400 ng of internal standard V (4) and 500 ng of desmethyl metabolite III (5) monitored at 313 nm; (C) as for (A), but monitored at 405 nm; (D) as for (B), but monitored at 405 nm; (E) 3-h pooled blood sample from rats receiving 25 mg/kg p.o. of I; unchanged drug, 8.54 μg/ml (2), N-oxide metabolite, 2.95 μg/ml (3), internal standard, 400 ng (4) and desmethyl metabolite III, 800 ng/ml (5) were monitored at 405 nm.

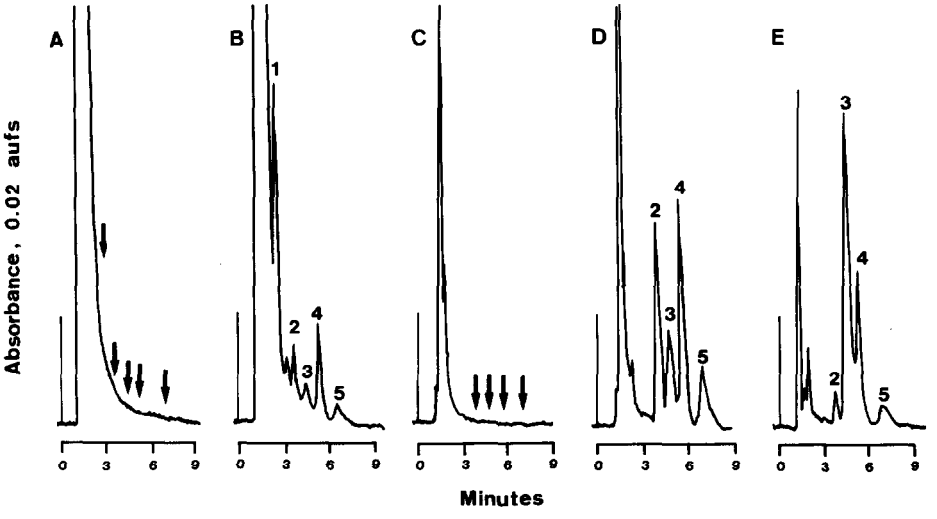


Fig. 3. Representative chromatograms of: (A) rat urine blank at 313 nm; (B) urine spiked with 500 ng of acetamido metabolite IV (1), 500 ng of I (2), 500 ng of N-oxide metabolite II (3), 400 ng of internal standard (4) and 500 ng of desmethyl metabolite III (5) monitored at 313 nm; (C) as for (A), but monitored at 405 nm; (D) as for (B), but monitored at 405 nm; (E) 24-h urine sample of a rat receiving 25 mg/kg p.o. of compound I; unchanged drug, 3.28 μg/ml (2), N-oxide metabolite II, 56.0 μg/ml (3), internal standard, 200 ng (4) and desmethyl metabolite III, 2.56 μg/ml (5) were monitored at 405 nm.

Sample processing

Although the parent compound I, its metabolites III and IV, and the internal standard could be readily extracted by an organic solvent in a biphasic system, metabolite II was not amenable owing to its high polarity. Several solvent systems yielded very poor recoveries. Eventually, a single step deproteinization–extraction with acetonitrile resulted in a very simple sample preparation procedure that permitted the simultaneous determination of the parent compound I and its metabolites. While large fluid volumes can be processed, smaller (0.05–0.5 ml) aliquots were preferred to reduce the evaporation time of the aqueous acetonitrile.

Recoveries and precision

Six replicate injections of human blood samples spiked with 500 ng/ml of each of the components gave recovery values of 65, 55, 77, 60 and 77% for compounds I, II, III, IV and V, respectively. These values were constant over a wide range of concentrations. The coefficient of variation (C.V.) was less than 10% for both inter- and intra-assay for all the components at a spiked blood concentration of 500 ng/ml.

Linearity

The linearity of the method was established with human blood calibration curves in the concentration range 200–1000 ng/ml, based on 0.5-ml sample volume. Least-squares regression analysis ($y=a+bx$) showed correlation coefficients in the range of 0.9949–0.9988, indicating good linearity for all the components analysed (Table I).

Stability of compounds I, II, III and IV

Samples of normal blood spiked with compounds I, II, III and IV, and also the blood samples collected from rats treated with compound I, were stored at −20°C. These samples were thawed and reanalysed after 1 week and after 1 month. The concentrations after 1 month deviated by less than 5% from those in the fresh samples. This indicates that the compounds are stable on storage, freezing and thawing, and that spiked samples are true representatives of biological samples.

TABLE I

REGRESSION PARAMETERS FOR HUMAN BLOOD CALIBRATION CURVES

Mean values from six calibration sets with five concentrations in the range 200–1000 ng/ml for each component.

Component	Intercept	Slope	Correlation coefficient (r)
I	−0.024	0.0018	0.9986
II (N-oxide metabolite)	−0.011	0.0007	0.9988
III (desmethyl metabolite)	−0.007	0.0004	0.9949
IV (acetamido metabolite)	−0.130	0.0090	0.9978

Accuracy and limit of detection

Human blood samples spiked with different amounts (200–1000 ng/ml) of compound I and metabolites II, III and IV were submitted to the assay procedure. Results presented in Table II demonstrate that concentrations in this range can be estimated with good accuracy and precision. Based on a signal-to-noise ratio of more than 3, the limit of detection is ca. 25 ng/ml for compound I and metabolite IV, 50 ng/ml for the N-oxide (II), and 100 ng/ml for the desmethyl metabolite (III).

TABLE II

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

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

Component	Concentration spiked (ng/ml)			
	300	400	500	600
I	318 ± 4.3	408 ± 3.9	502 ± 4.1	616 ± 1.7
II	303 ± 4.3	400 ± 4.8	506 ± 5.1	613 ± 3.3
III	306 ± 3.8	388 ± 3.1	498 ± 2.9	601 ± 4.7
IV	291 ± 5.8	–	–	598 ± 8.2

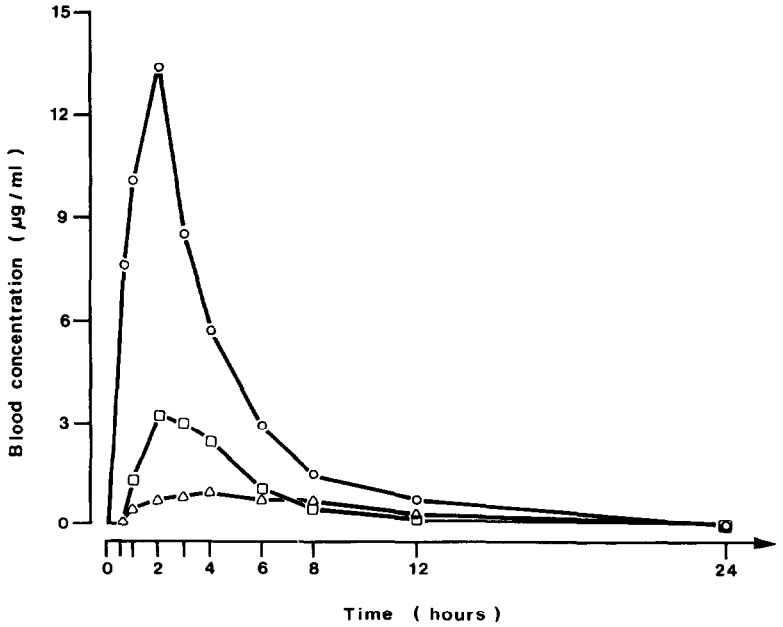


Fig. 4. Blood concentration profiles of compound I (○), N-oxide metabolite (□) and desmethyl metabolite (△) after a single oral dose of 25 mg/kg to rats. Blood was collected retroorbitally from ten rats and pooled for analysis.

Application

This assay was applied to a pharmacokinetic study of compound I and its metabolites in rats after a single oral dose of 25 mg/kg. The representative chromatograms of extracts of blood and urine samples are presented in Figs. 2E and 3E, respectively. The blood concentration–time profiles (Fig. 4) indicate that compound I was rapidly absorbed, reached a peak of 13.44 μg/ml at 2 h, and was eliminated rapidly with a half-life of ca. 3 h. While the areas under the blood concentration–time curve of the N-oxide (II) and desmethyl (III) metabolites were 27 and 16%, respectively, of that of the parent drug I, blood levels of the acetamido (IV) metabolite were below the limit of detection. The plasma-to-blood ratios per millilitre in the treated samples collected up to 6 h after administration were in the range 1.24–1.49 (mean 1.39). The mean renal excretions (0–24 h) of compounds I, II and III were 0.21, 19.98 and 0.63% of the dose, respectively. The quantitation of the acetamido metabolite in rat urine was hindered by the presence of another metabolite peak, which coeluted at 2.8 min at 313 nm.

DISCUSSION

Compound I and the N-oxide metabolite (II) readily adsorb on glass and therefore standard solutions need to be stored in polypropylene bottles. Analytical recoveries of these components can be improved if samples are processed in polypropylene or silanized tubes. Furthermore, N-oxides of tertiary amines are known to be unstable. They can be easily reduced to the parent compound under certain conditions [7,8]. Urine samples, in particular, were found to be more suscept to the reduction of the N-oxide metabolite to compound I, and hence needed to be collected in ice-chilled containers over the 24-h period. Once quickly frozen, preserved blood and urine samples gave reproducible analysis over several weeks.

CONCLUSION

The HPLC procedure described for compound I, a new filaricidal trial drug, is rapid and sensitive, requires few and inexpensive solvents and materials, and has an extremely simple work-up procedure. The method can be easily applied to 0.05–0.5 ml specimens of blood to monitor the pharmacokinetics of the unchanged drug and three of its metabolites simultaneously. In rat urine, the acetamido metabolite cannot be determined owing to interference from another coeluting metabolite.

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