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Note

Determination of diethylcarbamazine in blood using gas chromatography with alkali flame ionization detection*

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Diethylcarbamazine, 1-diethylcarbamyl-4-methylpiperazine, (DEC), is an antifilarial drug showing good microfilaricidal activity. Pharmacokinetic studies on this drug also are few probably due to lack of sufficient sensitivity of the methods reported in the literature for the determination of DEC concentration in biological fluids, viz. spectrophotometric methods [1—3] and the more recent improved gas chromatographic (GC) methods [4, 5].

The present report describes a GC procedure using an alkali flame ionization detector, for the quantitative determination of DEC in blood samples. The method can be used for measuring 50 ng of DEC (free base) and above in blood with a good degree of precision and accuracy. 1-Diethylcarbamyl-4-ethylpiperazine was used as the internal standard in the analytical procedure.

EXPERIMENTAL

Solvents, standards and reagents

Reagent grade toluene, methylene chloride, and methyl ethyl ketone were obtained from E. Merck (Bombay, India); hexane and ethyl acetate were from Sarabhai M. Chemicals (Baroda, India). Hexane and toluene were purified as described earlier [6].

All other chemicals used were of analytical reagent grade. Diethylcarbamyl chloride, N-methylpiperazine and ethyl *p*-toluene sulphonate were purchased from Fluka (Buchs, Switzerland).

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Apparatus

The gas chromatograph was a Varian Model 2700 equipped with an alkali flame ionization detector. Nitrogen was used as the carrier gas at a flow-rate of 40 ml/min. Hydrogen and air flow-rates were 40 and 268 ml/min, respectively. The range and attenuator settings corresponded to $4 \cdot 10^{-12}$ A for full scale deflection. The column was operated at 180° C, the injector at 200° C, and the detector at 270° C. Peak height measurements were used to quantitate the chromatograms. The samples (3 μl) were injected on-column using a 10-μl syringe with a long needle.

The glass column (2.7 m × 4 mm I.D.) was packed with 5% SP-2401 DB (base deactivated) on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The packed column was conditioned, silylated using Silyl 8 (Pierce, Rockford, IL, U.S.A.) and primed using authentic standards.

Standards

DEC (Fig. 1, IIIa) and the internal standard (Fig. 1, IIIb) were conveniently prepared by the general carbamylating procedures [7] using diethylcarbamyl chloride and N-monoalkyl piperazines (Fig. 1, IIa and IIb). IIb was synthesized by a procedure different from that reported [8]. The free bases (Fig. 1, IIIa and IIIb) were converted to the corresponding crystalline citrate salts by reacting with equimolar quantities of citric acid in acetone solution.

The general synthetic procedures are outlined in Fig. 1.

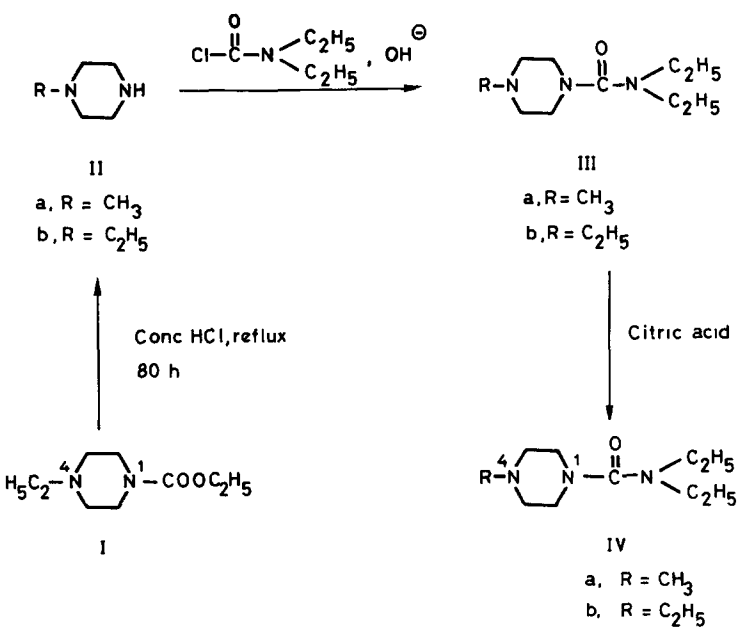

Fig. 1. Summary of reactions used in the synthesis of DEC and internal standard.

Method

Step 1. DEC citrate (1 mg) and 1 mg of internal standard citrate were dissolved separately in 50 ml of methanol; 1 ml of this stock solution was

diluted with methanol to 10 ml. The stock solutions were kept refrigerated and dilutions from stock solutions were made fresh.

Step 2. A 100-μl volume of the diluted internal standard corresponding to 200 ng of internal standard citrate and 50, 100, 150 and 200 μl of the diluted DEC solution corresponding to 100, 200, 300 and 400 ng of DEC citrate were pipetted into a set of silanized glass-stoppered tubes. Then 1 ml of blood was added and the tubes were swirled by hand. This was followed by the addition of 3 ml of 0.1 *M* sodium hydroxide and after gentle mixing the contents of the tube were allowed to equilibrate for 5 min at room temperature. Then 3 ml of an ethyl acetate—methyl ethyl ketone (4:1, v/v) mixture was added. The stopper was sealed with a drop of water and the tube was mounted horizontally on a reciprocal shaker and shaken at full speed for 5 min.

Step 3. The tube was removed from the shaker and centrifuged at 4000 *g* for 10 min. The organic layer was transferred to a 10 ml glass-stoppered tube and the extraction was repeated as described above.

Step 4. The free bases in the pooled organic layer were back-extracted into citric acid by the addition of 1 ml of 0.1 *M* citric acid using a 10 min shaking period on a reciprocal shaker. The tube was centrifuged and the organic layer was aspirated and discarded.

Step 5. The aqueous layer was washed first with 2 ml of ethyl acetate, followed by 2 × 3 ml of hexane, and the organic phases were discarded.

Step 6. The aqueous layer was basified by the addition of 1 ml of 2 *M* sodium hydroxide and extracted three times using a mixture of toluene—methylene chloride (5:2, v/v). The pooled organic layer was evaporated to dryness under a gentle stream of nitrogen and resuspended in 100 μl of acetone. A 2—4 μl aliquot was injected on-column in duplicate. Peak height ratios were calculated by dividing the peak height of DEC by the height of the internal standard peak. Calibration curves were constructed by plotting the peak height ratio as a function of DEC concentration. Ten to twelve samples were analysed for each calibration point. This calibration curve was used subsequently to calculate unknown concentrations of DEC in blood.

RESULTS AND DISCUSSION

Use of 1-diethylcarbamyl-4-ethylpiperazine as the internal standard in the quantitation of DEC was largely responsible for the success of the analytical method. The internal standard was ideal in its extraction and GC resolution behaviour when compared to DEC. The availability of pure internal standard and DEC paved the way for optimizing the response of the alkali flame ionization detector.

Among the solvents and solvent mixtures tried, the solvent pair ethyl acetate—methyl ethyl ketone (4:1, v/v) was found to be superior in extracting DEC from blood. This extract was unsuitable for direct GC and therefore several clean-up procedures were tried. Back-extraction of the bases into citric acid and successive washings with ethyl acetate and hexane followed by re-extraction using the solvent mixture toluene—methylene chloride emerged as the ideal purification step. The overall recovery of DEC and the internal standard added to blood ranged from 45% to 50%.

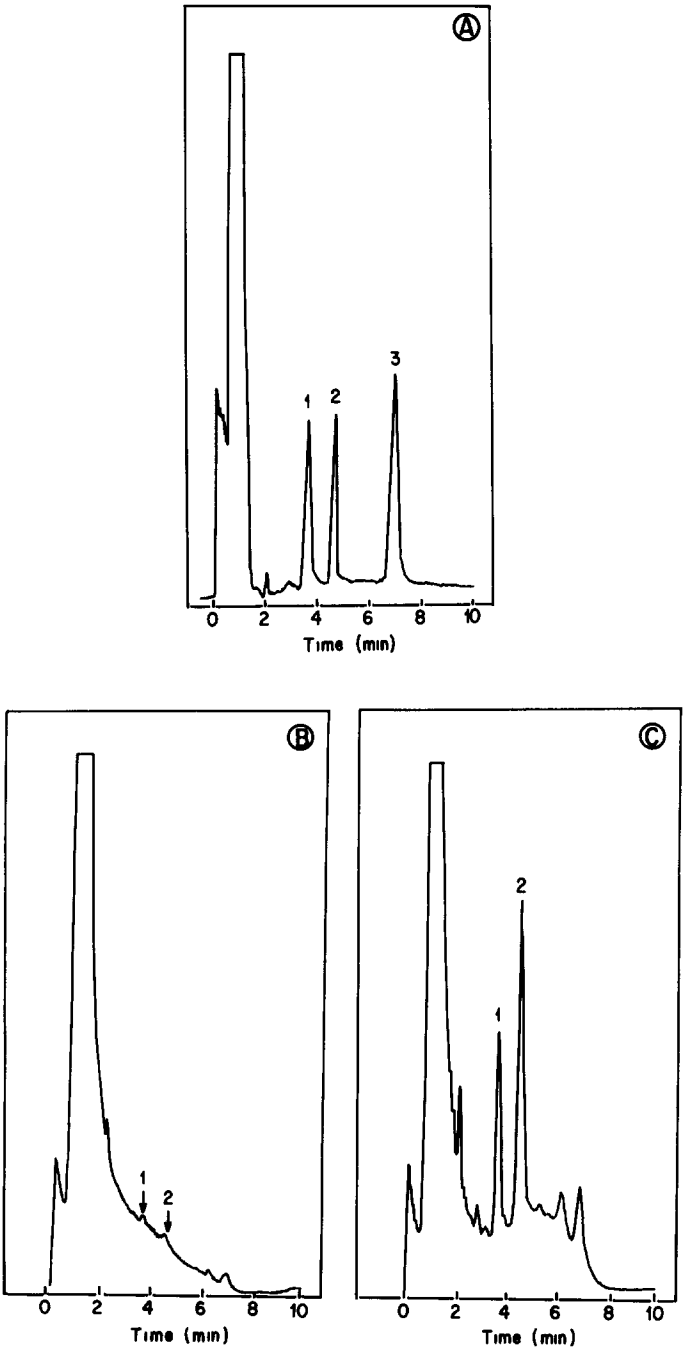

Fig. 2. (A) Separation of DEC, internal standard and desethyl metabolites of DEC. (B) Chromatogram of human blood blank. (C) Human blood sample spiked with 100 ng of DEC free base. Peaks: 1 = DEC; 2 = internal standard; 3 = desethyl DEC.

TABLE I

PRECISION OF THE METHOD APPLIED TO SPIKED HUMAN BLOOD SAMPLES

Amount of DEC (free base) added (ng/ml)	Amount found (ng/ml) (mean ± S.D.; n = 4)	Precision/reproducibility (C.V., %)
58.2	60.75 ± 3.7	6.09
77 6	79.75 ± 4.0	5.02
126.1	127.5 ± 6.5	5.1
174.6	171.5 ± 6.6	3.85

Fig. 2 illustrates the separation of DEC and some of its major metabolites [9] along with the internal standard. The mono- and di-desethyl metabolites of DEC overlap and therefore cannot be separated under the conditions used in the present study, but they are completely separable from both DEC and the internal standard.

Typical gas chromatograms obtained with the method using blood samples are also shown in Fig. 2. The calibration curve constructed by adding known amounts of DEC and a constant amount of internal standard to blood was

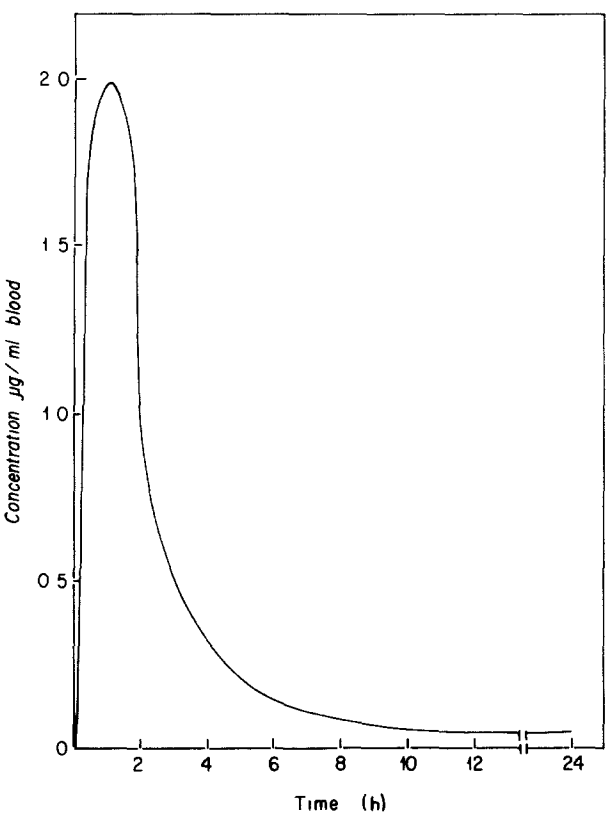

Fig. 3. Concentration of DEC in dog blood after the administration of an oral dose of 200 mg DEC (banocide).

linear in the range 50—200 ng DEC (free base). The equation for the calibration curve from blood was calculated and found to be $Y = 0.0061X + 0.025$ with an excellent correlation ($r = 0.999$). Table I shows the results obtained on spiked blood samples. The results demonstrate good reproducibility of the method.

The specificity and sensitivity of the method for pharmacokinetic studies was checked by an in vivo experiment in a 12-kg mongrel dog following oral administration of 200 mg of DEC citrate (Banocide®). The results of this study are shown in Fig. 3. A C_{max} of 2.03 μg/ml was reached in 1 h followed by a very rapid decline to 0.094 μg/ml at 8 h. The levels beyond this time were around 50 ng/ml, the minimum detection limit of the present method. The

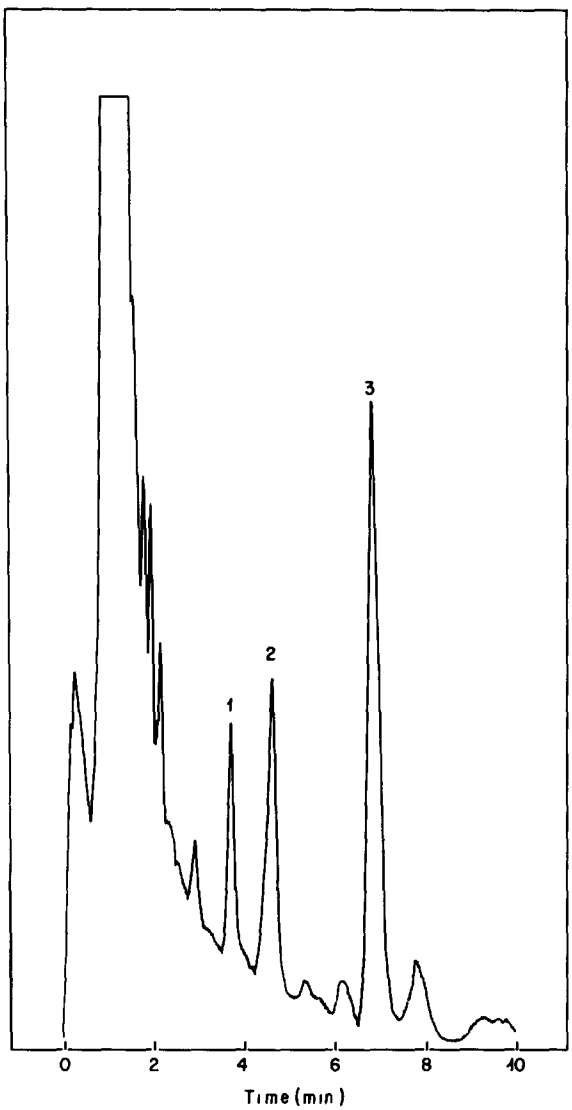

Fig. 4. Chromatogram of a steady-state human blood sample. 1 = DEC; 2 = internal standard; 3 = desethyl metabolites of DEC.

suitability of the method for measuring steady-state levels of DEC in man was ascertained by determining DEC levels in one subject after seven days on a 100-mg t.d.s. dosage. A level of 0.605 μg/ml was found on the eighth day. Studies are in progress to establish potential interference if any from other concomitantly prescribed drugs.

Fig. 4. presents the gas chromatogram of the steady-state blood sample. Besides DEC considerable amounts of the desethyl metabolites are present in the blood and their non-interference with the reported method of assay of DEC in blood is also quite apparent.

Experiments on the application of this analytical method to study single- and multiple-dose pharmacokinetics of this antifilarial drug are in progress.

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