Journal of Chromatography, 262 (1983) 367–372
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,804

Note

Determination of 4,4′-diaminodiphenylmethane in blood by gas–liquid chromatography with electron-capture detection

M. TORTORETO, P. CATALANI, M. BIANCHI, C. BLONDA and C. PANTAROTTO*
Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)
and
S. PAGLIALUNGA
Division of Occupational Health, Montedison S.p.A., Via Appiani 12, 20121 Milan (Italy)
(Received February 24th, 1983)

4,4′-Diaminodiphenylmethane (DDM) is a compound of considerable commercial importance as it is widely used in the manufacture of plastic, fibres and elastomers. The toxicity of this compound has been the object of different studies. DDM was found to be mutagenic *in vitro* towards *Salmonella* TA 98 and TA 100 strains[1,2]. It has also been reported that DDM is hepatotoxic in rats, dogs and man[3,4]. The data on its carcinogenicity are controversial and, at present, long-term cancerogenesis studies in mice and rats are in progress[5,6].

Interest in the mechanisms of toxicity of different compounds used in the plastic and rubber industry prompted us to work out a sensitive and specific procedure for the determination of DDM in biological specimens to enable us to make kinetic and distribution studies in laboratory animals.

EXPERIMENTAL

Chemicals

4,4′-Diaminodiphenylmethane (DDM) was supplied by Montedison (Milan, Italy) and 4,4′-diamino-1,2-diphenylethane (DDE), used as internal standard for quantitation, by Aldrich Europe (Beerse, Belgium). Trifluoroacetic anhydride was a product of Fluka (Buchs, Switzerland). All other reagents and solvents were of the purest grade commercially available.

Animals

Male CD_2F_1 mice (body weight, 20–22 g) were obtained from Charles River Italy (Calco, Como, Italy). Animals were injected intraperitoneally with a single dose of DDM (200 mg/kg dissolved in corn oil). Groups of five animals were killed by decapitation 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240, 300, 360, 480, 720 and 1440 min after treatment and blood samples were collected in heparinized tubes and stored at −20°C until required for analysis.

Extraction

To 1 ml of mouse blood, 1 ml of 0.05 *M* sodium hydroxide and 10 μl of an

0021-9673/83/$03.00

acetone solution of DDE (1 mg/ml or 10 ng/ml, depending on the expected DDM concentration range and the type of detector used) as internal standard were added. The appropriate amount of DDE to be added to the biological sample was determined from a preliminary experiment as that which gave a suitable ratio between the peak areas. The samples were extracted twice with 5 ml of *n*-heptane–isoamyl alcohol (99:1). After shaking for 30 min and centrifugation at 2000 *g* for 10 min, the organic phase was transferred to a second test-tube containing 1 ml of 0.1 *M* hydrochloric acid. The tubes were shaken, centrifuged and the organic phase was discarded. The aqueous layer was then extracted twice with 5 ml of diethyl ether. The aqueous phase was then brought to an alkaline pH by addition of 200 μl of 1 *M* sodium hydroxide and extracted twice with 5 ml of the *n*-heptane–isoamyl alcohol mixture. The samples were shaken, centrifuged and the organic phase was transferred to conical glass tubes and evaporated to dryness under a gentle stream of nitrogen. The extraction recovery of DDM from blood was 72 ± 3%.

Trifluoroacetylation

The dry residue was redissolved in 100 μl trifluoroacetic anhydride–ethyl acetate (1:2) and the stoppered tubes were heated at 80°C in a sand-bath for 30 min. The samples were then dried under nitrogen, 100 μl of ethyl acetate were added and 1–3 μl were injected into the gas chromatographic column.

Gas–liquid chromatography (GLC)

GLC was carried out on a Carlo Erba Fractovap Model G1 gas chromatograph, equipped with a flame ionization detector (FID) or a ^{63}Ni electron-capture detector (ECD). In both cases the column was a glass tube (1 m × 4 mm I.D.) packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh (Supelco, Bellefonte, PA, U.S.A.). Nitrogen was used as carrier gas at a flow-rate of 30 ml/min. The air and hydrogen flow-rates were adjusted to give maximum response when operating with the FID. The column oven was maintained at 190°C, the injection port heater at 230°C and the ECD at 250°C. The ECD was used with a pulse current: excitation voltage, 10 V; pulse width, 3 μsec; period, 30 μsec; scavenger gas (nitrogen) flow-rate, 30 ml/min.

Mass spectrometry

An LKB 2091 mass spectrometer equipped with a computer system Model 2130 for data acquisition and calculation was used. The GLC conditions were as described before except that helium was used as carrier gas. Spectra were recorded at 70 eV (energy of the ionization beam): ion source temperature, 290°C; accelerating voltage, 3.5 kV; trap current, 60 μA.

RESULTS AND DISCUSSION

Typical gas chromatograms obtained by flame ionization detection and referring to the analysis of DDM in the blood of untreated (A) and DDM treated (B) mice are shown in Fig. 1. DDE was chosen as internal standard for quantitative purposes because of its similarity to DDM as regards both structure and physicochemical properties. The chemical identity of peaks a and b was checked by combined gas chromatography–mass spectrometry and the resulting mass spectra are reported

in Fig. 2. The mass spectrum of DDM shows an intense molecular ion at *m/e* 390, indicating that two trifluoroacetyl groups entered the molecule. A similar derivatization reaction occurs for DDE and, in its spectrum, a molecular ion was observed at *m/e* 404. The other fragments present in both the DDM and DDE spectra arise as schematized in the figure. When required, an increase in sensitivity could be achieved by using the ECD, and gas chromatograms of mouse blood extracts obtained with this type of detector are shown in Fig. 3. Interference from endogenous substrates was never observed and good linearity in both FID and ECD response was found over a range of DDM concentrations from 10 ng/ml of blood to 100 μg/ml.

The precision of the procedure was calculated from the percentage coefficients of variation (% C.V.) as standard deviation/mean × 100. Assays were made in quintuplicate at six different DDM concentrations, thus covering the range expected *in vivo*. The precision of the method is illustrated by the low % CV values reported in Table I.

The validity of this procedure for the determination of DDM in biological specimens was checked by studying the distribution of the compound in mouse blood. Fig. 4 gives a semilogarithmic plot of the time course of mouse blood DDM concentrations. DDM is rapidly absorbed, reaching a peak of 78.4 ± 8.8 μg/ml at 10 min, after which it disappears following, for the times considered, a monoexponential pattern of decay with a calculated half-life of 3.2 h. The parameters describing

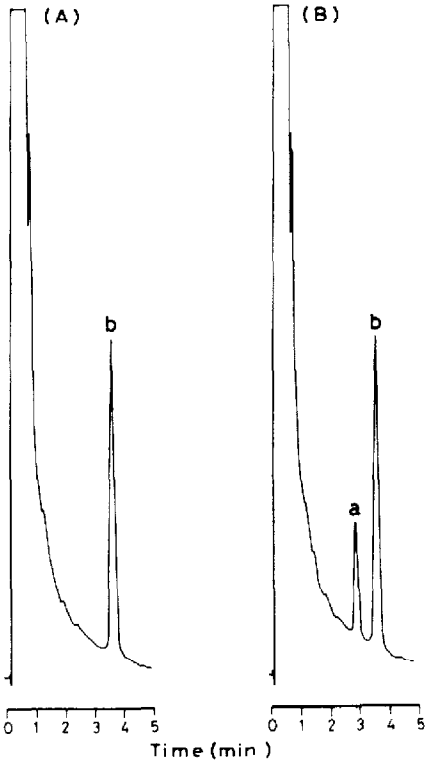

Fig. 1. Gas chromatographic analysis with flame ionization detection of the blood of an untreated mouse (A) and of the blood of a DDM treated mouse (B). Peaks: a = DDM; b = DDE.

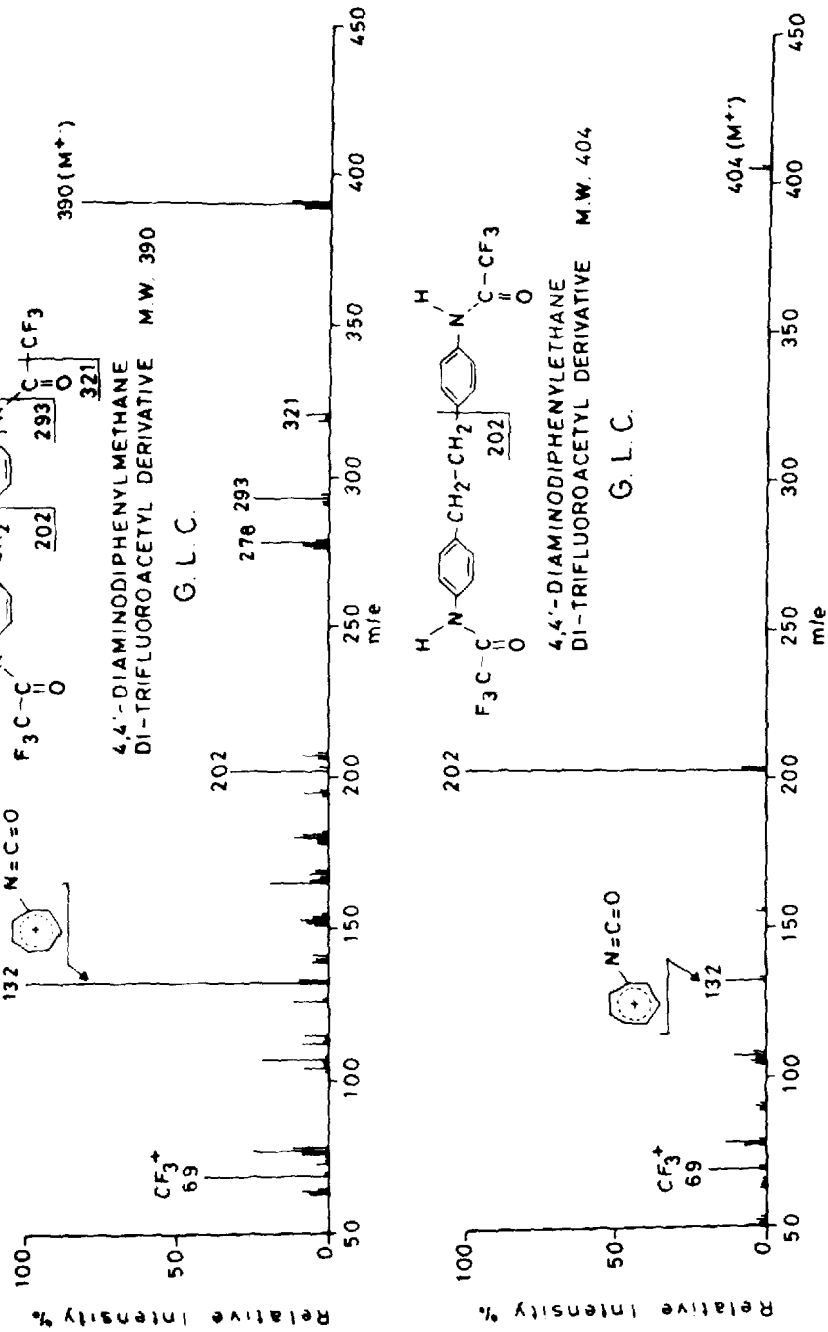

Fig. 2. Mass spectra of *DDM* and *DDE* as their di-trifluoroacetyl derivatives at 70 eV after GLC analysis.

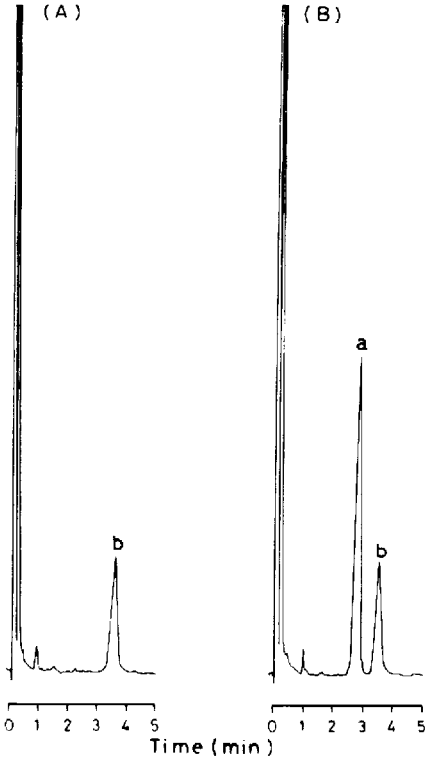

Fig. 3. Gas chromatographic analysis with electron capture detection of the blood of an untreated mouse (A) and of the blood of a DDM treated mouse (B). Peaks: a = DDM; b = DDE.

the kinetics of DDM in mouse blood are given in the legend to Fig. 4 and were calculated by the method of residuals[7].

In conclusion, a simple and rapid method for DDM determination in biological specimens has been developed. In view of its high specificity and sensitivity, we believe this procedure can easily be applied in experimental toxicological studies and might even prove useful for monitoring human exposure.

TABLE I

PRECISION OF DDM ASSAY METHOD IN MOUSE BLOOD

Parameter	*Concentration (μg/ml)*					
	ECD			*FID*		
	0.01	*0.1*	*1*	*2.5*	*25*	*100*
$\bar{X}$	0.011	0.097	1.015	2.38	24.63	98.74
S.D.	0.001	0.004	0.039	0.15	1.33	2.57
% C.V.	9.1	4.1	3.8	6.3	5.4	2.6
n	5	5	5	5	5	5

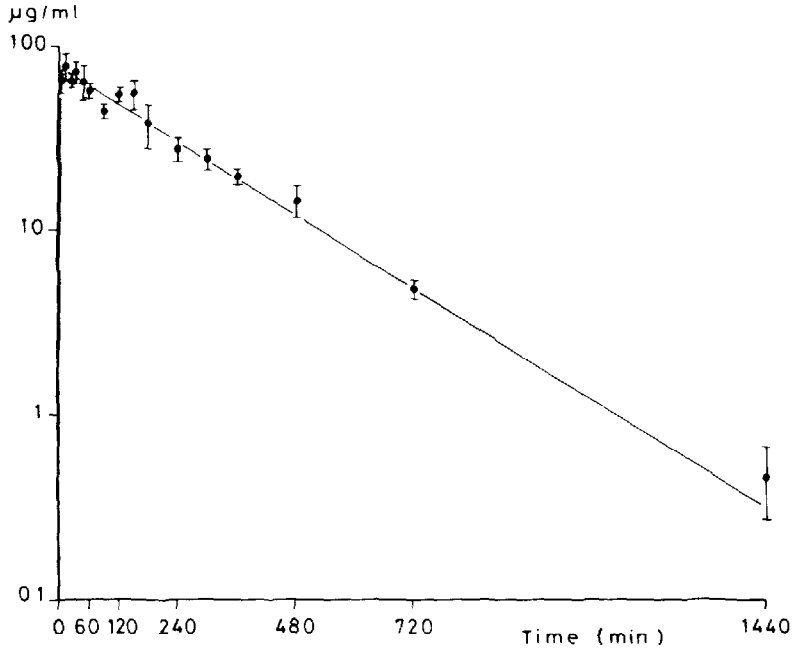

Fig. 4. Blood concentrations of DDM at different times after intraperitoneal injection of 200 mg/kg. Each point is the average of five determinations. Kinetic parameters: k_a (rate constant of absorption) = 1.96 h^{-1}; k_{el} (rate constant of elimination) = 0.22 h^{-1}; $t_{\frac{1}{2}}$ (half-life) = 3.2 h; c_0 (concentration extrapolated to time 0) = 76.7 µg/ml; V_d (apparent volume of distribution) = 2.60 l/kg; AUC (area under the curve) = 352.2 µg/ml · h.

ACKNOWLEDGEMENT

This study was supported by Montedison S.p.A., Milan, Italy.

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