Journal of Chromatography, 426 (1988) 239–247
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4080

GAS CHROMATOGRAPHIC STUDIES OF THE CARBAMYLATION OF HAEMOGLOBIN BY METHYL ISOCYANATE IN RATS AND RABBITS

P.K. RAMACHANDRAN*, B.R. GANDHE, K.S. VENKATESWARAN, M.P. KAUSHIK, R. VIJAYARAGHAVAN, G.S. AGARWAL, N. GOPALAN, M.V.S. SURYANARAYANA and S.K. SHINDE

Defence Research and Development Establishment, Tansen Road, Gwalior 474 002 (India)

and

S. SRIRAMACHARI

Institute of Pathology, Safdarjung Hospital, New Delhi 110 029 (India)

(First received October 16th, 1987; revised manuscript received December 1st, 1987)

SUMMARY

Carbamylation of the N-terminal valine of haemoglobin with methyl isocyanate in rats and rabbits has been demonstrated in vitro and in vivo by gas chromatography. N-Methylcarbamylated haemoglobin, converted by cyclization into 3-methyl-5-isopropylhydantoin, has been quantified by gas chromatography. Standard hydantoin was synthesized, chemically characterized and used for calibration. The method is simple and reliable in the concentration range 0.06–2 nmol. Carbamylation of haemoglobin by methyl isocyanate in vivo in rats can be identified only above a dose of 1.05 mg/l in inhalation exposures. It is inferred that methyl isocyanate in the "active" form crosses the alveolar and erythrocyte membranes and carbamylates the haemoglobin.

INTRODUCTION

One of the hypotheses put forward for sudden deaths due to methyl isocyanate (MIC) exposure is tissue anoxia due to carbamylation of haemoglobin. Our attempts to substantiate this hypothesis by electrophoresis and isoelectric focusing of MIC-exposed haemoglobin did not give any positive conclusion and we therefore chose gas chromatography (GC) as a possible technique for elucidation of the hypothesis.

It has been shown by Manning et al. [1] that cyanates react with the N-terminal valine of haemoglobin, which can be converted into 5-isopropylhydantoin or valinehydantoin and determined by GC. The method was used for monitoring

0378-4347/88/$03.50

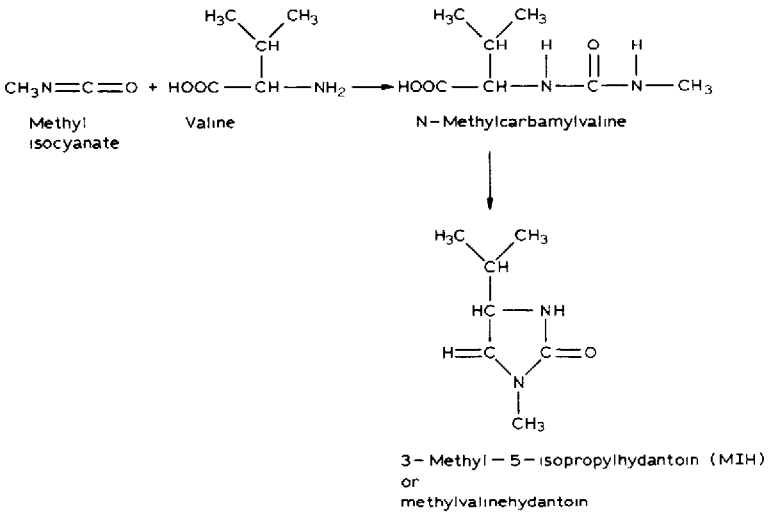

Fig 1. Reaction mechanism of haemoglobin carbamylation.

carbamylation levels in sickle cell anaemic patients subjected to cyanate therapy. Lee [2] extended the study to the anti-sickling properties of MIC, which he reported to be much faster. Lee clearly demonstrated that MIC reacts irreversibly with the α-amino group of the N-terminal valine in haemoglobin to the exclusion of sulphydryl and ϵ-amino groups. The above studies were carried out in vitro by the addition of cyanate or MIC to human blood. The in vivo carbamylation by cyanate has been reported by Crist et al. [3] and Papayannopoulou et al. [4], but no attempts at the in vivo determination of carbamylated valine with MIC have previously been reported.

We report here both in vivo and in vitro studies of haemoglobin carbamylation with MIC in rats and rabbits. The reaction sequences considered in this study are depicted in Fig. 1.

EXPERIMENTAL

Materials

All the reagents used were of analytical-reagent grade and L-valine was obtained from Sigma (St. Louis, MO, U.S.A.).

Synthesis of MIC

Acetyl chloride and sodium azide were reacted in benzene in the presence of a phase-transfer catalyst as described previously [5]. The purity of the product (b.p. 38.0°C) as checked by GC was 99.5%.

Preparation of standard 3-methyl-5-isopropylhydantoin (MIH)

L-Valine (468 mg; 4 mmol) was dissolved in 20 ml of distilled water and the pH adjusted to 8.0 with 0.1 *M* sodium peroxide. Chilled MIC (228 mg; 4 mmol) was added and allowed to react for 2 h with stirring at room temperature. Glacial

acetic acid (7 ml) and concentrated hydrochloric acid (13 ml) were then added and the solution was heated to 100°c for 1 h. The solvent was removed under reduced pressure and the residue dried overnight over sodium hydroxide in a vacuum desiccator. The melting point of the product was 210°C. The identity of MIH was established by mass spectrometry (MS) [$M^+ = m/z$ 156, $(M-42)^+ = m/z$ 114]. The purity of the compound as checked by GC was 99.5%.

MIH was also prepared by methylation of 5-isopropylhydantoin by the method of Carington and Waring [6]. 5-Isopropylhydantoin for this purpose was prepared as reported [1] by carbamylation of L-valine with potassium cyanate.

Exposure chambers

An all-glass static exposure chamber of volume 21.5 l was specially designed and has been described in detail elsewhere [7]. Required concentrations of MIC were obtained by introducing known amounts of MIC into the chamber through a detachable side-arm. A circulating pump enabled a uniform concentration to be attained. In a separate experiment, the concentration of MIC was monitored by GC and found to be constant over a period of 30 min. The exposed doses were calculated in terms of LC_{50} for 30 min for Wistar rats of weight 200 ± 20 g. Rabbits were exposed in an all-stainless-steel static exposure chamber of volume 35 l. The exposure dose was 3 mg/l for 30 min. New Zealand–Dutch cross rabbits of weight 1000 ± 50 g were exposed one at a time.

Blood was collected from rats and rabits 1 h prior to and 30 min after the exposure in heparinized centrifuge tubes. The haemolysate was prepared as described [8]. The haemoglobin concentration was measured spectrophotometrically [9].

Processing of haemolysate from in vivo experiments

To 1.0 ml of haemolysate prepared from exposed and control animals were added 5 ml of 2% hydrochloric acid in acetone at 4°C and mixture was vortexed thoroughly. The tubes were centrifuged at 2000 *g* for 3 min and the supernatant liquid was discarded. The whole globin pellet was resuspended, washed four times with cold acetone as above and finally with 5 ml of cold diethyl ether, then centrifuged again. The precipitate was dried by placing the tubes in a beaker of warm water at 45°C.

The carbamylated product was dissolved in a mixture of 0.5 ml of 50% glacial acetic acid and 0.5 ml of concentrated hydrochloric acid and kept at 100°C for 1 h, cooled immediately, neutralised by adding 0.8 ml of 10 *M* sodium hydroxide solution and 0.5 ml saturated sodium chloride solution and the contents were mixed. The pH of the solution was maintained between 3 and 5. Then 5 ml of ethyl acetate were added to the hydrolysate and MIH was extracted by mixing the contents of the tube for 1 min on a vortex mixer. After separation of the phases by centrifugation, 4 ml of the ethyl acetate phase were placed in a heavy-walled Pyrex tube. Ethyl acetate was removed by evaporation to dryness and the tube was placed overnight in an evacuated desiccator over sodium hydroxide for removal of traces of water. The dried residue was dissolved in a known volume of

ethyl acetate and 1 μl of the solution was injected on to the GC column. A similar procedure was followed for all other samples.

Processing of haemolysate from in vitro experiments

The haemoglobin concentration was adjusted to 160 mg/ml of normal rabbit haemolysate. In every instance 2.5 μmol of haemoglobin were taken and various amounts of MIC, as given in Table II, were added. The reaction was allowed to proceed for 2 h at room temperature with constant stirring. The haemolysates were then processed for extraction of the carbamylated product and cyclization to hydantoin as described above. Similarly, in vitro MIC-treated samples of rat haemolysates were processed to study the carbamylation. In vitro studies with excess of MIC (two-fold) were also carried out.

GC analysis

A Perkin-Elmer Model 3920-B instrument was used with flame-ionization detection (FID). A stainless-steel column (180 cm × 2 mm I.D.) packed with 15% OV-225 (Analabs, North Haven, CT, U.S.A.) coated on 100–120 mesh Chromosorb W HP was employed. The injection port and detector block were maintained at 230 and 225 °C, respectively, and the column oven temperature was 220 °C. Nitrogen was used as the carrier gas (at a flow-rate of 30 ml/min). Air for FID was supplied at 300 ml/min and hydrogen at 30 ml/min. In all analyses, 1-μl samples were injected and peaks recorded on Shimadzu Chromatopac C-R3A data processor. The FID amplifier attenuation was 8 × 10 and the C-R3A × 1 or × 2.

Calibration graph for standard MIH

A stock solution of standard MIC was prepared at a concentration of 1 mg/ml. Solutions of various concentrations in the range of 0.06–2.0 nmol/μl were prepared by taking known volumes of stock solution and diluting it with freshly dried ethyl acetate. A 1-μl volume of each was then injected on to the GC column and the MIH peak height was recorded. Average peak heights from replicates were plotted against concentration of MIH.

Calculations

In every instance the concentration of MIH in μmol/ml of blood/haemolysate (C) was calculated using the observed peak height, the corresponding MIH concentration and the dilution factor. The degree of carbamylation was expressed in terms of the percentage of total haemoglobin utilized for carbamylation using the equation:

$$\text{percentage carbamylation} = \frac{C\ (\mu\text{mol})/4}{\mu\text{mol haemoglobin/ml blood}} \cdot 100$$

RESULTS AND DISCUSSION

The chromatogram of standard MIH is shown in Fig. 2. The retention time for MIH was 2.65 ± 0.15 min. The peak was narrow and symmetrical. The FID response was linear in the range of concentrations used for constructing the calibration graph. The peak-height reproducibility was within ± 2 mm. Hence the determinations were considered to be satisfactory. The recovery of MIH in the ethyl acetate layer was found to be better than 92%. The losses in the extraction procedure were not taken into account in the calculations. The detection limit of MIH was 5 ng (0.03 nmol) at a signal-to-noise ratio of 8.

Typical chromatograms obtained for blood samples processed from control and exposed rats are shown in Fig. 3a and b and for rabbits in Fig. 4a and b. Figs. 3 and 4 clearly indicate that no other product interferes with the MIH peak. Data from in vivo experiments given in Table I for the exposed animals indicate that all the exposed animals showed a positive test for carbamylation, as confirmed by the presence of MIH. The detection limit of MIH was 1 LC_{50} exposure dose for rats. For lower than 1 LC_{50} doses, no carbamylation was detectable. As MIC is a sensory irritant, the rate of breathing of the animal would be reduced by reflux inhibition. Further, as MIC is a highly reactive moiety, interacting with all nucleophiles, the amount of MIC reacting with haemoglobin would be only a small fraction of the total MIC entering the biological system. The data in Table I for percentage carbamylation show less than 2% in all doses studied from 1 to 3 LC_{50}.

Fig. 2. Chromatograms of (a) solvent (ethyl acetate) and (b) 2 nmol of standard MIH. Retention time, 2.65 min; column, stainless-steel tubing (180 cm × 2 mm I.D.) packed with 15% OV-225 coated on 100–120 mesh Chromosorb W HP; temperature, 220 °C. Other conditions as described in the text.

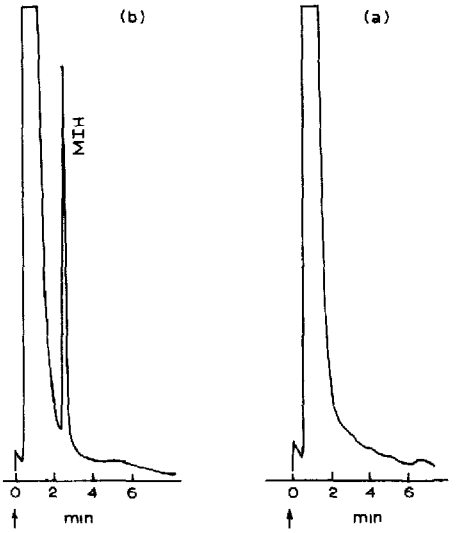

Fig. 3. Chromatograms of processed rat blood, (a) collected before exposing the rat to MIC and (b) collected after exposing the rat to a 3 LC_{50} dose of MIC for 30 min. In both instances 1 ml of blood was taken for processing and the final residue was diluted to 100 μl with ethyl acetate. Experimental conditions as in Fig. 2.

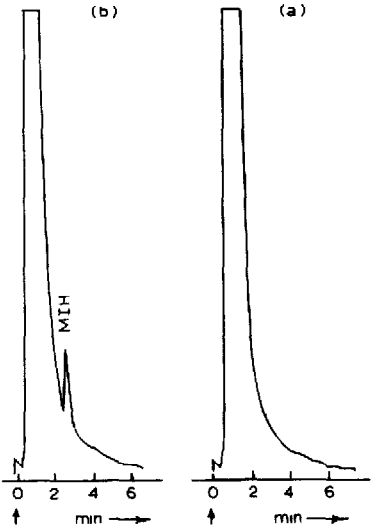

Fig. 4. Chromatograms of processed rabbit blood, (a) collected before exposing the rabbit to MIC and (b) after exposing it to MIC at a 3 mg/l inhalation dose. In both instances 1 ml of blood was taken for processing and the final residue was diluted with 300 μl ethyl of acetate. Experimental conditions as in Fig. 2.

For rabbits, the degree of carbamylation after exposure to an arbitrary dose of 3 mg/l is only 1%, confirming the above facts.

In vitro experiments were also carried out to study the dose dependence of the degree of carbamylation using rabbit haemolysates. The data given in Table II on the degree of carbamylation clearly indicate that the extent of carbamylation is high in vitro as compared to in vivo exposures.

The degree of carbamylation seems to be dose-dependent from the data given in Tables I and II. However, in the in vivo experiments it is not exactly proportional to the exposure dose. This is as expected, because MIC would enter the bloodstream from the lungs after reacting with the lung proteins, which reaction will be dependent on the residence time of MIC in the lungs; in the bloodstream also it is difficult to predict the amount reacting with haemoglobin and other proteins.

Work in this laboratory [10] with ^{14}C-labelled MIC administered by inhalation to rats showed that radioactivity was widely distributed in the liver, kidney and brain. This was also identified as the protein-bound radioactivity, thereby establishing the passage across the blood–tissue interface by MIC in the "active" form. A better correlation and enhanced carbamylation observed in the in vitro experiments further confirm the above.

The electrophoretic techniques carried out in our laboratory [11] and reported by Troup et al. [12] failed to help in identifying the carbamylation of red blood cells with MIC; the GC technique reported here could effectively establish carbamylatin in vivo. The sensitivity of the technique can be improved further by using a capillary column coupled with a nitrogen-selective detector. The method can then be successfully used for monitoring the lower degrees of carbamylation that may occur as a result of lower exposures in a chemical plant.

TABLE I

IN VIVO STUDIES ON CARBAMYLATION OF HAEMOGLOBIN IN RATS AND RABBITS AT VARIOUS EXPOSURE DOSES OF MIC

Exposed species	MIC exposure dose	Number of animals exposed	Number of animals showing carbamylation (detected by GC)	Average MIH formation in 1 ml of blood (determined by GC) (mean ± S.D.*) (nmol)	Degree of carbamylation (% of haemoglobin carbamylated)
Wistar rats	1 LC_{50}**	4	4	20.44 ± 1.96	0.2
	2 LC_{50}	5	5	80.09 ± 2.94	0.8
	3 LC_{50}	4	4	151.59 ± 3.40	1.5
Rabbits (New Zealand-Dutch cross)	3 mg/l	2	2	102.50 ± 2.24	1.0

*Losses in MIH extraction were < 8% and neglected in the calculations. Average values for each animal for every exposure were obtained from replicate analyses and were used to find average MIH formation for a given dose for a given animal, as shown in this column.

**1 LC_{50} = 1.05 mg/l.

TABLE II

IN VITRO STUDIES ON CARBAMYLATION OF HAEMOGLOBIN IN RABBIT HAEMOLYSATE SHOWING THE DEPENDENCE OF THE DEGREE OF CARBAMYLATION ON THE AMOUNT OF MIC ADDED TO BLOOD LYSATE

In all instances 1 ml of haemolysate was processed.

Haemoglobin concentration in haemolysate taken for carbamylation* (10^3 nmol/ml)	Concentration of MIC added to lysate (10^3 nmol/ml)	Concentration of MIH in injected solution** (mean ± S.D.) (nmol/μl)	MIH determined by GC per ml of haemolysate*** (10^3 nmol)	Degree of carbamylation (% of haemoglobin carbamylated)
2.5	11.8	3.48 ± 0.15	6.9	69.0
2.5	5.8	1.54 ± 0.32	3.1	31.6
2.5	3.9	1.09 ± 0.20	2.2	22.5

*1 mol of haemoglobin = 4 mol of valine.
**The final residue was diluted with 2 ml of ethyl acetate and 1 μl of the solution was injected on to the GC column.
***Losses in MIH extraction were < 8% and neglected in the calculations.

CONCLUSIONS

Different workers had expressed and reiterated their belief that MIC would be completely hydrolysed to dimethylurea by the lung fluids and hardly any "active" MIC can enter the bloodstream [13,14]. Studies with radiolabelled MIC in this laboratory [10], and those of Hill et al. [15] using ^{14}C-labelled toluene diisocyanate, have confirmed the interaction of alkyl and aryl isocyanates with blood proteins. Our work clearly shows that MIC in the "active" form crosses the lung–blood barrier and reacts with haemoglobin in the circulating blood by carbamylating it.The degree of carbamylation by MIC is less than 2% in rats and rabbits by the inhalation route. Hence, at least one of the toxic symptoms after exposure to MIC, namely tissue anoxia, because of increased oxygen affinity of the carbamylated haemoglobin, is a possibility. The sensitivity of the technique can be improved by using a capillary column coupled with a nitrogen-selective detector.

ACKNOWLEDGEMENTS

The keen interest and encouragement of Brig. K.M. Rao, Additional Director, Defence R&D Establishment, Gwalior, India, during this study are gratefully acknowledged. Acknowledgements are due to Mr. R. Raghunandanan and Mr. A. Daniel for typing the manuscript.

REFERENCES

1 J.M. Manning, C.K. Lee, A. Cerami and P.N. Gillette, J. Lab. Clin. Med., 81 (1973) 941.
2 C.K Lee, J. Biol. Chem., 251 (1976) 6226.

3 R. Crist, S. Grisolia, C. Beths and A. Diederich, Fed. Proc. Fed. Am. Soc. Exp. Biol., 3 (1972) Abs. 231.
4 T. Papayannopoulou, G. Stamatoyannopoulous, E.G. Giblett and J. Anderson, Life Sci., 12 (1973) 127.
5 M.P Kaushik, A.K. Sikder and D.K. Jaiswal, Current Sci., 56 (1987) 1008.
6 H.C. Carington and W.S. Waring, J. Chem. Soc., (1950) 350.
7 R. Vijayaraghavan and M.P.Kaushik, Indian J. Exp. Biol., 25 (1987) 531.
8 A. Cerami and J.M. Manning, Proc. Natl. Acad. Sci. U.S.A., 68 (1971) 1180.
9 H. Varley, A.H. Gowenlock and M. Bell, in H. Varley (Editor), Practical Clinical Biochemistry, Heinemann Medical Books, New Delhi, 1976, p. 585.
10 B.K. Bhattacharya, S.K. Sharma and D.K. Jaiswal, Biochem. Pharmacol., in press.
11 K.S. Venkateswaran and K. Zachariah, unpublished data.
12 C.M. Troup, D.E. Dodd, E.H. Fowler and F.R. Frank, Environ. Health Perspect., 72 (1987) 21.
13 D.R. Varma, in J. Saxena (Editor), Anatomy of Methyl Isocyanate Leak in Bhopal, Hazard Assessment of Chemicals, Vol. 5, Hemisphere, Wshington, DC, 1987, p. 255.
14 R. Dagami, Chem. Eng. News, 63 (1985) 37.
15 B.L. Hill, M.H. Karol and W.E. Brown, Toxicologist, 6 (1986) 15.