Journal of Chromatography, 306 (1984) 51—60
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1972

DETERMINATION OF THE STABLE ISOTOPE OF NITRITE FLUX IN THE BLOOD OF MICE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY WITH SELECTED ION MONITORING

AKIO TANAKA*, NORIHIDE NOSE, HIROYUKI MASAKI and HISAO IWASAKI

Saitama Institute of Public Health, 639-1, Kamiokubo-Higashi, Urawa, Saitama (Japan)

and

MISAO HARUTA

Department of Food Technology, College of Agriculture and Veterinary Medicine, Nihon University, 4-1, Shimouma 3-Chome, Setagaya-Ku, Tokyo (Japan)

(First received June 6th, 1983; revised manuscript received October 17th, 1983)

SUMMARY

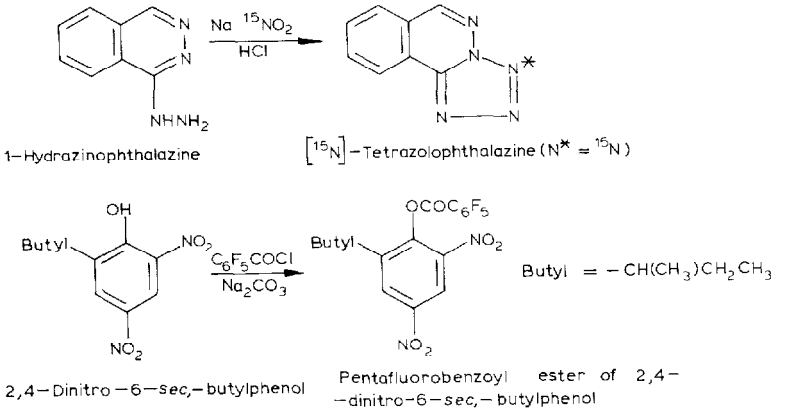
A simple and reliable method for the determination of the stable isotope of nitrite ($^{15}NO_2$) flux in the blood of mice is described. It is based on the reaction of $^{15}NO_2$ with 1-hydrazinophthalazine in acidic solution to form [^{15}N]tetrazolophthalazine, a stable compound which can be extracted with an organic solvent and then determined by gas chromatography—mass spectrometry with selected ion monitoring using the pentafluorobenzoyl ester of 2,4-dinitro-6-*sec.*-butylphenol as an internal standard. Amounts of 0.2—10 μg of $^{15}NO_2$ can be determined. The detection limit of $^{15}NO_2$ was 0.1 μg/ml. This is a specific method for $^{15}NO_2$. The procedure for determining $^{15}NO_2$ in the blood of mice involves extraction with solvent, followed by further clean-up by alumina column chromatography; the detection limit is 0.05 μg. With the new technique we were able to perform a metabolic fate study of nitrite in the blood of mice following a single oral dose of $^{15}NO_2$ or the stable isotope of nitrate ($^{15}NO_3$).

INTRODUCTION

The determination of nitrite in blood may need to be carried out in cases of intoxication by nitrite-containing drugs or ingestion of food containing nitrite or nitrate. Ingested nitrates may be reduced by intestinal flora to nitrite. Nitrite, when absorbed into the bloodstream, oxidizes haemoglobin to

0378-4347/84/$03.00

methaemoglobin, and if sufficient haemoglobin is converted the oxygen-carrying capacity of the blood may be markedly reduced. Therefore, the metabolic fate of nitrite in blood is of great interest. Nitrite in blood is usually determined by a colorimetric method based on the formation of an azo dye produced by diazotization of sulfanilic acid and subsequent coupling with 1-naphthylamine or N-(1-naphthyl)ethylenediamine [1—4]. However, these colorimetric methods are limited by the fact that occasionally turbid and slightly coloured sample extracts can affect the colour of the azo dye and, consequently, the accuracy of the nitrite determination. We found that tetrazolophthalazine [5] can be quantitatively prepared by reaction with nitrite and 1-hydrazinophthalazine in acidic solution and can be extracted with several organic solvents [6—9]. This is a specific reaction for nitrite (see Scheme 1, top).



Scheme 1.

In this paper, we have monitored the in vivo disappearance of nitrite in the blood of mice by following a single oral dose of $^{15}NO_2$ or $^{15}NO_3$. In general, there are various methods for determining nitrogen-15, including mass spectrometry (MS) [10], chemical ionization MS [11] and emission spectrography [12]. However, these methods cannot distinguish whether $^{15}NO_2$ or $^{15}NO_3$ is being determined because they determine total nitrogen-15. Furthermore, the procedures are complex [13, 14]. We describe here an accurate, reliable, reproducible and selective determination of $^{15}NO_2$. In the present study deproteinized mice blood was analyzed by gas chromatography (GC)—MS with selected ion monitoring after reaction and clean-up by alumina column chromatography.

EXPERIMENTAL

Reagents and standards

Sodium nitrite (95 atom% ^{15}N) and potassium nitrate (99.5 atom% ^{15}N), obtained from Merck Sharp and Dohme (Montreal, Canada), were dried at 110°C for 1 h under vacuum immediately before use. A stock nitrite solution was prepared by dissolving 0.493 g of $Na^{15}NO_2$ in 100 ml of distilled water to give a concentration of 1 mg/ml of $^{15}NO_2$. A stock nitrate solution

was prepared by dissolving 0.718 g of $K^{15}NO_3$ in 100 ml of distilled water to give a concentration of 1 mg/ml $^{15}NO_3$. 1-Hydrazinophthalazine (Tokyo Kasei Kogyo, Tokyo, Japan) was of a special high grade and was used without further purification. A solution (1.0%, w/w) of it was prepared by dissolving 1.0 g in 100 ml of distilled water. The pentafluorobenzoyl (PFB) ester of 2,4-dinitro-6-*sec.*-butylphenol (2,4-dinitro-6SBP) as internal standard for GC—MS with selected ion monitoring was prepared as follows. A 0.1 g portion of 2,4-dinitro-6-*sec.*-butylphenol (ICN Pharmaceuticals, Plainview, NY, U.S.A.) was dissolved in 10 ml of 5% sodium carbonate solution; then 0.1 g of pentafluorobenzoyl chloride (Aldrich, Milwaukee, WI, U.S.A.) was added (see Scheme 1, bottom). After reaction at room temperature with shaking for 5 min, the reaction mixture was extracted with 50 ml of benzene and the benzene layer was separated and washed with 50 ml of distilled water. The benzene layer was dried with an adequate amount of anhydrous sodium sulphate and concentrated to 5 ml. The 5-ml solution was applied to the column (30 cm × 1.0 cm I.D., prepared with 10 g of activated alumina) and eluted with benzene. The 100 ml of effluent was collected, and then evaporated at 50°C. The substance obtained as mentioned above was prepared by dissolving 3 μg in 1 ml of ethyl acetate. Deproteinizing solution A was prepared by dissolving 90 g of ammonium thiocyanate and 80 g of mercuric chloride in 1000 ml of distilled water. Deproteinizing solution B was prepared by dissolving 125 g of zinc acetate in 500 ml of distilled water. All water used was triple distilled and deionized. All other reagents and solvents were of high purity and were obtained from Wako (Osaka, Japan).

Animals

Male ddY mice weighing 20—25 g were used for the experiments. They were housed in plastic cages on soft-wood bedding at room temperature (25°C) under a 12 h dark—light rhythm. The animals had free access to standard laboratory diet (Funabashi Farm, Chiba, Japan) and tap water.

Treatment of animals

A 100- or 500-μg amount of $^{15}NO_2$ was orally given to mice by gavage in 0.1 ml or 0.5 ml of the stock nitrite solution; also, $^{15}NO_3$ was given orally to mice in a similar manner. The mice were anesthetized with diethyl ether and killed by cervical dislocation 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after the dose had been given. Blood samples were collected in heparinized containers and were stored at 0—4°C for about 15 min.

Preparation and analysis of mice blood extracts

A 1-ml blood sample was placed in a 10-ml test-tube (11.5 cm × 1.5 cm I.D.). A 1-ml volume of each deproteinizing solution A and B was added and then the mixture was shaken for 1 min followed by centrifugation at 1400 *g* for 5 min. A 2-ml aliquot of the supernatant was placed in a 10-ml test-tube and 1 ml of 1-hydrazinophthalazine solution and 2 ml of 2 *M* hydrochloric acid were added. The mixture was heated at 70°C in a water-bath with occasional shaking for 20 min. After cooling to room temperature, the reaction mixture was extracted with 5 ml of toluene with shaking for 3—5 min. The toluene

extract was pipetted into a 10-ml test-tube and then the procedure was repeated. After drying with an adequate amount of anhydrous sodium sulphate, the combined extracts were subjected to alumina column chromatography as described previously [6] to remove interfering substances from the blood. The effluent was then evaporated in a stream of nitrogen gas at 40°C. The residue was dissolved in an adequate volume of internal standard solution. A 5-μl volume of the final solution was analyzed by GC—MS with selected ion monitoring.

Gas chromatography—mass spectrometry

GC—MS was carried out using a Shimadzu LKB-9000 combined gas chromatograph—mass spectrometer equipped with a multiple ion detector-peak matcher. The column was a glass tube (0.5 m × 3 mm I.D.) packed with 3% OV-225 on Chromosorb W HP (80—100 mesh) and was conditioned at 230°C; injector temperature was 180°C. The flow-rate of helium carrier gas was 30 ml/min. MS conditions were as follows: separator temperature 235°C; ion source temperature 290°C; trap current 60 μA; electron energy 70 eV; accelerating potential 3.5 keV. For selected ion monitoring, the following ions were used: *m/e* 171 for tetrazolophthalazine, *m/e* 172 for [^{15}N]tetrazolophthalazine, and *m/e* 167 for the PFB ester of 2,4-dinitro-6SBP as internal standard.

Preparation of calibration curve

A series of working standard $^{15}NO_2$ solutions was prepared by diluting the stock solution with distilled water. Aliquots were placed in 10-ml test-tubes. After the addition of 1-hydrazinophthalazine and hydrochloric acid and subsequent reaction, the reaction mixtures were extracted with toluene and the solvent removed by evaporation according to the procedure described above. The residues were dissolved in 1 ml of internal standard solution and 5-μl aliquots of the resulting solutions were injected for GC—MS with selected ion monitoring. The concentration range of the $^{15}NO_2$ standard was 0.2—10.0 μg/ml. As shown in Fig. 1, the retention time of [^{15}N]tetrazolophthalazine

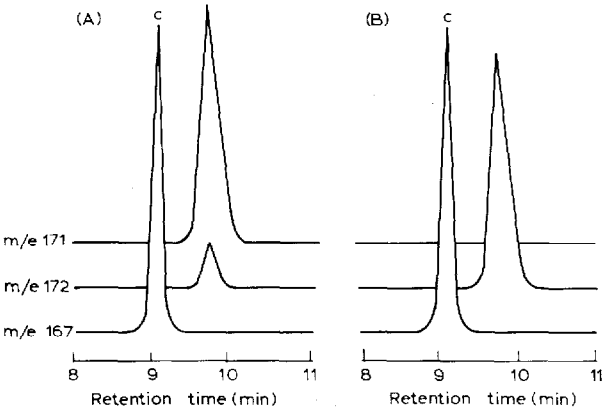


Fig. 1. Selected ion chromatograms of tetrazolophthalazine (A) and [^{15}N]tetrazolophthalazine (B) with the pentafluorobenzoyl ester of 2,4-dinitro-6-*sec.*-butylphenol as internal standard (C). Aliquots of 5 μl of a mixture of the internal standard solution and tetrazolophthalazine or [^{15}N]tetrazolophthalazine were directly injected for GC—MS with selected ion monitoring. For conditions, see text.

relative to that of internal standard was 1.07. The peak height ratios of [^{15}N]-tetrazolophthalazine to internal standard were plotted against the amount of $^{15}NO_2$ analyzed. The ratio (R) of the peak height of [^{15}N] tetrazolophthalazine to internal standard was calculated as follows:

$$R = (a - 0.219b)/c \quad (1)$$

where a and b are the peak heights, of [^{15}N] tetrazolophthalazine at m/e 172 derived from $^{15}NO_2$ and that of tetrazolophthalazine at m/e 171 derived from $^{14}NO_2$, and c is the peak height of internal standard at m/e 167. The typical standard curve was linear.

RESULTS AND DISCUSSION

In order to investigate an obvious difference between tetrazolophthalazine derived from $^{14}NO_2$ and [^{15}N] tetrazolophthalazine derived from $^{15}NO_2$, each product was analyzed by GC—MS. As shown in Fig. 2, the parent peak (m/e 171) for tetrazolophthalazine and that of m/e 172 for [^{15}N] tetrazolophthalazine correspond to the molecular weight of each compound. However, the shift of the peaks from m/e 115 to 76 for [^{15}N] tetrazolophthalazine was in agreement with that of tetrazolophthalazine (Fig. 2C). Therefore, for GC—MS with selected ion monitoring, we adopted m/e 171 for tetrazolophthalazine and m/e 172 for [^{15}N] tetrazolophthalazine.

Columns containing DC-200 (5%, w/w), SE-30 (3%, w/w), OV-1 (5%, w/w), OV-101 (2%, w/w) and OV-225 (3% w/w) on Chromosorb W HP were tested. Except for OV-225, all the columns caused peak tailing of [^{15}N] tetrazolophthalazine. Good peak characteristics and sensitivity were achieved with OV-225 under the conditions described above. A high temperature and a short column were preferable for the GC—MS with selected ion monitoring of [^{15}N] tetrazolophthalazine.

At 235°C a 0.5-m column containing OV-225 on Chromosorb W HP gave a good ion chromatogram (see Fig. 1). On the other hand, the PFB ester of 2,4-dinitro-6SBP was chosen as internal standard since it had favourable properties for the OV-225 column. Furthermore, as blood extract contents interfered with the base peak (m/e 195), we adopted m/e 167 as the monitoring ion for the internal standard. The retention time of [^{15}N] tetrazolophthalazine relative to that of internal standard was 1.07 (Fig. 1).

$^{14}NO_2$ is widespread in nature. Therefore, to investigate its influence on the determination of $^{15}NO_2$ 1.0—10.0 μg of $^{15}NO_2$ were added to 1.0—10.0 μg of $^{14}NO_2$ as shown in Table I, and each mixture was analyzed by GC—MS with selected ion monitoring after reaction and extraction according to the described procedure. The peak height ratio of [^{15}N] tetrazolophthalazine to internal standard increased with an increase in the amount of tetrazolophthalazine added, and, at the same time, the accuracy decreased (Table I). On the other hand, tetrazolophthalazine derived from $^{14}NO_2$ gave a peak at m/e 172 beside the parent peak at m/e 171 (Fig. 1). Therefore, by using the ratio (k) of the peak height at m/e 172 to that at m/e 171 of tetrazolophthalazine, the determinable peak height of [^{15}N] tetrazolophthalazine (x) in the blood extract of mice can be calculated by the equation $x = a - kb$, where a and b are the peak heights at m/e 172 and m/e 171, respectively, in the analysis

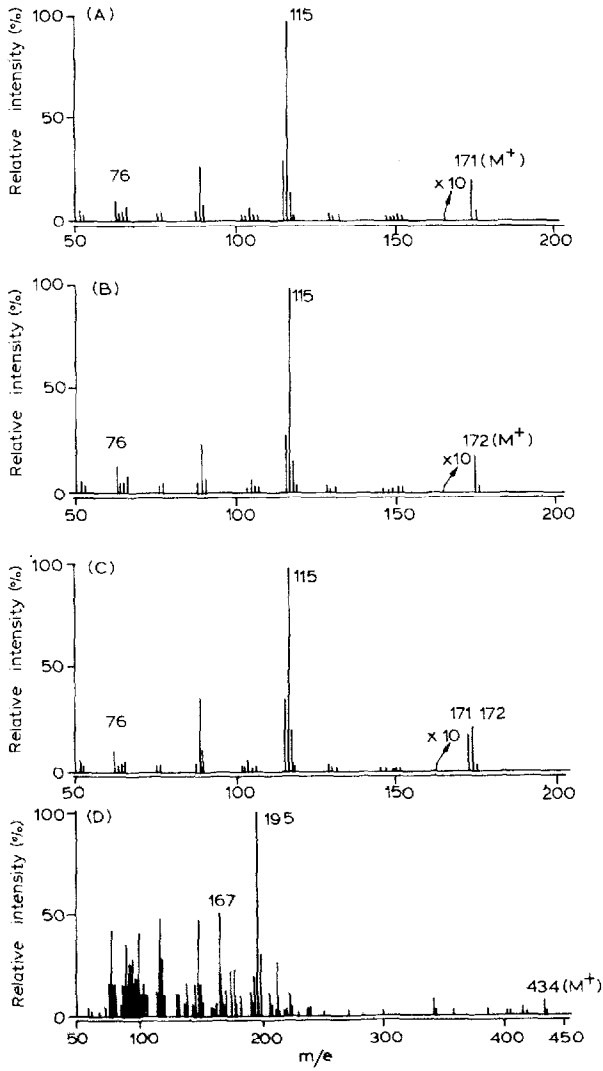


Fig. 2. Mass spectra of tetrazolophthalazine (A), [^{15}N]tetrazolophthalazine (B), a mixture of tetrazolophthalazine and [^{15}N]tetrazolophthalazine (C), and the pentafluorobenzoyl ester of 2,4-dinitro-6-*sec.*-butylphenol (D) as internal standard.

of the sample digest by GC—MS with selected ion monitoring. Therefore, in order to measure the k value, aliquots were placed into test-tubes to give amounts of 0.5, 1, 3, 5, 7 and 10 μg of $^{14}NO_2$. After reaction and extraction according to the described procedure, each extract was evaporated in a stream of nitrogen gas at 40°C. The residues were dissolved in 1 ml of ethyl acetate and dried with a small amount of anhydrous sodium sulphate, then analyzed by GC—MS with selected ion monitoring. As shown in Table II, the k value for each amount of $^{14}NO_2$ ranged from 0.201 to 0.221 with an average of 0.219. Thus, in practice 0.219 was adopted. Since the presence of $^{14}NO_2$ did affect the determination of $^{15}NO_2$, as shown in Table II, the results were re-calculated using eqn. 1. These results are shown in Table I with values in parentheses, and are in fair agreement with the additional amounts of $^{15}NO_2$.

TABLE I

INFLUENCE OF $^{14}NO_2$ ON THE DETERMINATION OF $^{15}NO_2$

For each amount of $^{15}NO_2$ were added various amounts of $^{14}NO_2$ and 1% 1-hydrazinophthalazine. The reaction procedure and GC—MS with selected ion monitoring conditions are as described in the text. The amounts of $^{15}NO_2$ were determined by comparing with the calibration curve obtained from eqn. 1 with $b = 0$; the amounts of $^{15}NO_2$ in parentheses were determined in the same way, after recalculation using eqn. 1 as in the text.

$^{15}NO_2$ added (μg)	$^{14}NO_2$ added (μg)				
	1.0	3.0	5.0	7.0	10.0
1.0	1.21 (0.98)	1.68 (1.01)	2.13 (1.03)	2.46 (0.93)	3.11 (0.92)
3.0	3.19 (0.98)	3.68 (3.01)	4.14 (3.04)	4.50 (2.97)	5.19 (3.00)
5.0	5.16 (4.94)	5.64 (4.98)	6.15 (5.03)	6.54 (5.01)	7.15 (4.95)
7.0	7.26 (7.04)	7.63 (6.96)	8.10 (7.00)	8.49 (6.96)	9.20 (7.00)
10.0	10.15 (9.93)	10.64 (9.98)	10.95 (9.85)	11.45 (9.92)	12.25 (10.04)

TABLE II

STUDY OF SET OF k VALUES

Each amount of $^{14}NO_2$ was added to 1.0 ml of 1% 1-hydrazinophthalazine. The reaction procedure and analysis by GC—MS with selected ion monitoring conditions are as described in the text. Each value is the mean ± standard deviation of five replicate determinations.

$^{14}NO_2$ added (μg)	k (peak height at m/e 172 / peak height at m/e 171)
0.5	0.225 ± 0.011
1.0	0.219 ± 0.018
3.0	0.212 ± 0.019
5.0	0.220 ± 0.010
7.0	0.215 ± 0.014
10.0	0.221 ± 0.013

On the other hand, analysis of the blood digest by the proposed method after the formation of [^{15}N]tetrazolophthalazine is shown in Fig. 3. Besides the peak of tetrazolophthalazine at m/e 171, there was another peak, the retention time of which relative to that of internal standard was 0.98. The intensity of the peak was sufficient to interfere in the determination of tetrazolophthalazine. Therefore, a clean-up stage was needed. However, use of the alumina column as described previously [6] was effective in removing this interfering substance (Fig. 3).

A calibration curve for $^{15}NO_2$ was prepared by adding to mice plasma (2 ml) known amounts of $^{15}NO_2$ (0.2—10 μg) followed by GC—MS analysis with selected ion monitoring, and plotting the ratio of the peak height of [^{15}N]-tetrazolophthalazine to that of internal standard against concentration. The calibration curve was linear with a linear regression equation $y = 0.932x - 0.281$ (correlation coefficient of 0.9962). The average relative standard devia-

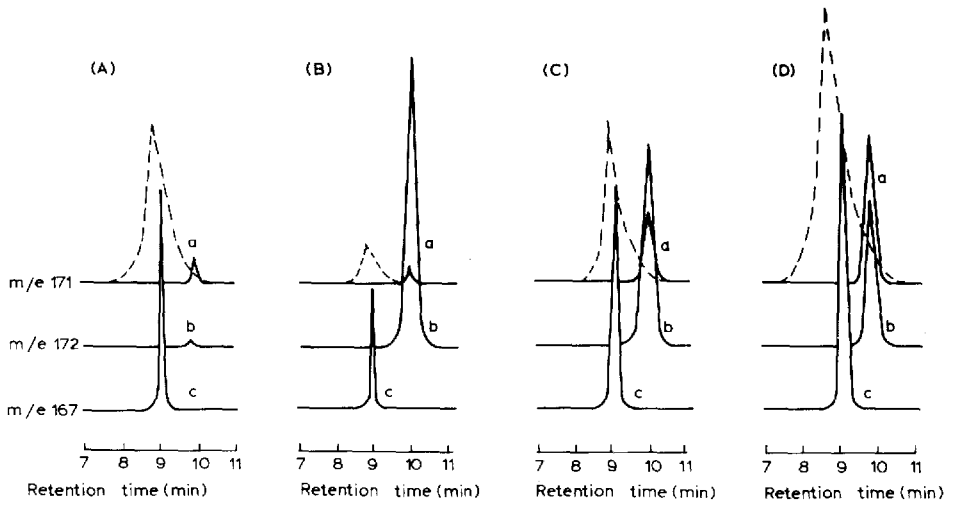


Fig. 3. Selected ion chromatograms of ethyl acetate extracts of mice blood. The sample size was 5 μl. Chromatogram A is control; B and C are obtained with a single oral dose of 500 and 100 μg of $^{15}NO_2$, respectively; D is obtained with a single oral dose of 500 μg of $^{15}NO_3$. Peaks: a = tetrazolophthalazine; b = [^{15}N]tetrazolophthalazine; c = pentafluorobenzoyl ester of 2,4-dinitro-6-*sec.*-butylphenol. The dotted lines show the shape of chromatograms obtained for blood before clean-up by alumina chromatography. For GC—MS with selected ion monitoring conditions, see text.

TABLE III

RECOVERY OF NITRITE FROM MOUSE BLOOD PLASMA

Results represent the mean of five determinations at each level

$^{15}NO_2$ added (μg)	Mean $^{15}NO_2$ found (μg)	C.V. (%)	Mean recovery (%)	Recovery range (%)
0.5	0.46	5.3	92.8	87.6—94.3
1.0	0.96	4.1	95.7	91.7—96.1
3.0	2.92	1.7	97.2	96.3—98.0
5.0	4.88	2.0	97.6	95.8—98.1
7.0	6.78	1.3	96.8	96.0—97.3
10.0	9.70	1.5	97.0	96.4—98.0

tion of five determinations was 2.1% for 0.5 μg or 1.0 μg and 3.7% for 5.0 μg or 10 μg of $^{15}NO_2$ and the reproducibility was considered satisfactory.

Recovery data from whole blood were not evaluated because haemoglobin reacts immediately with nitrite. However, the residual nitrite in blood is also present in plasma as reported by Shechter et al. [4]. In order to check the validity of the proposed procedure for the determination of $^{15}NO_2$ in mice plasma, recoveries of $^{15}NO_2$ added to plasma (2 ml) were measured at each concentration; the results are presented in Table III. The mean recovery varied between 92.8% and 97.6% (the coefficient of variation, C.V., varied between 1.3 and 5.3%). The detection limit was 0.05 μg of $^{15}NO_2$.

The utility of the proposed method was demonstrated by using it in a metabolic fate study of nitrite in mice blood. Mice were injected with a single

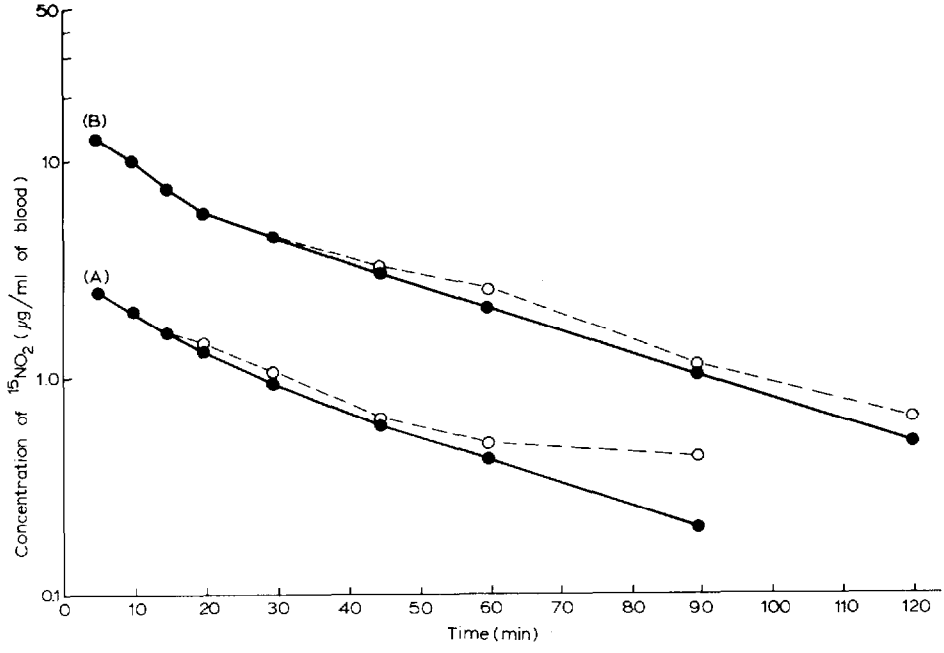


Fig. 4. Concentration of $^{15}NO_2$ in blood of mice vs. time after a single oral dose of 100 μg (A) or 500 μg (B) of $^{15}NO_2$. Each point represents the mean value for five mice. The dotted lines show data obtained using the GC method [6].

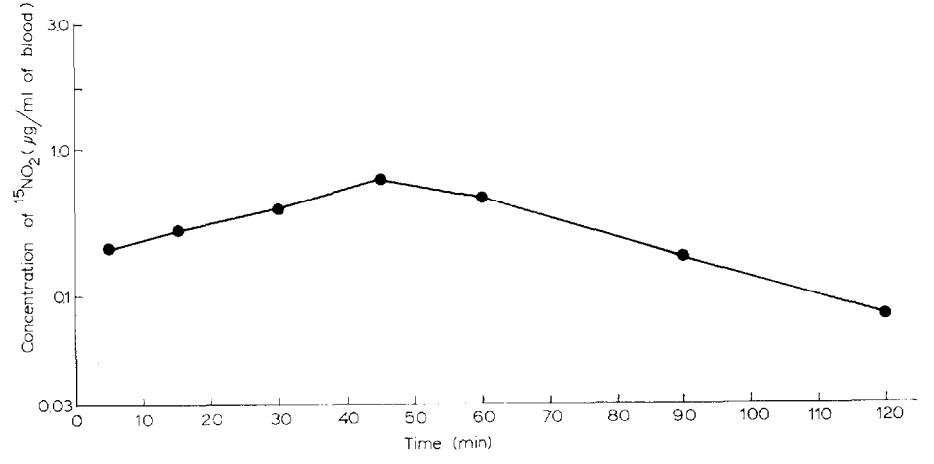


Fig. 5. Concentration of $^{15}NO_2$ in blood of mice vs. time after a single oral dose of 500 μg of $^{15}NO_3$. Each point represents the mean for five mice.

oral dose of 100 μg or 500 μg of $^{15}NO_2$. The mice were killed as described above and blood samples analyzed by the described procedure. The data were used to construct semilogarithmic plots of concentration vs. time as shown in Fig. 4. The in vivo disappearance of nitrite in mice blood was rapid, the essential reaction being completed within 30—45 min and showed an almost linear regression. Although in the case of the 100-μg dose the residual $^{15}NO_2$ disappeared at 90 min, with the 500-μg dose it was still detected in blood at 120 min

after injection. On the other hand, both the proposed method and the GC method [6] were approximately in agreement with the data for 15—20 min after injection. However, the data obtained by the GC method were higher than those obtained with the proposed method at 20—120 min after injection (Fig. 4). It is assumed that the GC method determines both $^{14}NO_2$ and $^{15}NO_2$. Therefore, the proposed method is specific for $^{15}NO_2$. On the other hand, nitrate is hardly absorbed in the stomach compared with nitrite [15]. However, the formation of nitrite may occur if nitrite-reducing bacteria are present in the stomach; also, it has been reported that nitrate is converted to nitrite by nitrate-reducing bacteria in saliva [16]. Therefore, to investigate the presence of $^{15}NO_2$ in mice blood in cases of $^{15}NO_3$ intake, mice were injected with a single oral dose of 100 μg or 500 μg of $^{15}NO_3$ and the subsequent experiments were carried out as for $^{15}NO_2$. The results obtained are shown in Fig. 5. The detectable amount of $^{15}NO_2$ derived from $^{15}NO_3$ was very low. The formation of $^{15}NO_2$ reached a maximum 45 min after $^{15}NO_3$ injection (Fig. 5) and then gradually decreased. From these results the in vivo formation of nitrite from ingested nitrate is clearly demonstrated in mice. For the 100-μg dose, however, it was impossible to determine $^{15}NO_2$ because of the lower limit of detection.

CONCLUSION

The determination of trace levels of nitrite in biological samples such as blood has posed many difficulties. In this study, $^{15}NO_2$ was determined as [^{15}N]tetrazolophthalazine by GC—MS with selected inon monitoring after sample clean-up with alumina column chromatography. The method is simple, and has been successfully applied to a metabolic fate study of nitrite in mice blood using $^{15}NO_2$ or $^{15}NO_3$.

REFERENCES

1 E.J. Stieglitz and A.E. Palmer, J. Pharmacol. Exp. Ther., 11 (1934) 368.
2 R.H. Diven, W.J. Pistor, R.E. Reed, R.J. Trautman and R.E. Watts, Amer. J. Vet. Res., 23 (1962) 94.
3 M.H. Lichfield, Analyst (London), 92 (1967) 132.
4 H. Shechter, N. Gruener and H.I. Shuval, Anal. Chim. Acta, 60 (1972) 93.
5 J. Darey and B.H. Ringier, Helv. Chim. Acta, 34 (1951) 195.
6 A. Tanaka, N. Nose, F. Yamada, S. Saito and A. Watanabe, J. Chromatogr., 206 (1981) 531.
7 A. Tanaka, N. Nose and A. Watanabe, Shokuhin Eiseigaku Zasshi, 22 (1981) 14.
8 A. Tanaka, N. Nose, H. Masaki, S. Saito and A. Watanabe, Bunseki Kagaku (Jap. Anal.), 30 (1981) 269.
9 A. Tanaka, N. Nose, H. Masaki, Y. Kikuchi and H. Iwasaki, J. Ass. Offic. Anal. Chem., 66 (1983) 260.
10 H. Sakai, O. Mastubaya and Y. Nakajima, Shisturyo Bunseki, 18 (1970) 1195.
11 C.V. Ludeen, A.S. Viscomi and F.H. Field, Anal. Chem., 47 (1973) 1288.
12 C. Veillon and M. Margoshes, Spectrochim. Acta, 23B (1968) 503.
13 C.F. Larson, V.A. Fassel, R.H. Scott and R.N. Kniseley, Anal. Chem., 47 (1975) 235.
14 K. Kumazawa, Radioisotopes, 21 (1972) 37.
15 J.P. Witter, S.J. Gratley and E. Balish, Science, 204 (1979) 411.
16 S.R. Tannenbaum, A.J. Sinskey, M. Weisman and W. Bishop, J. Nat. Cancer Inst., 53 (1974) 79.