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Improved gas chromatographic–mass spectrometric determination of the *N*-methylcarbamoyl adduct at the N-terminal valine of globin, a metabolic product of the solvent *N*,*N*-dimethylformamide

Jaroslav Mráz[a,*], Šárka Dušková[a], Eva Gálová[a], Hana Nohová[a], Pavla Krausová[a], Igor Linhart[b], Petr Šimek[c]

[a]*Centre of Industrial Hygiene and Occupational Diseases, National Institute of Public Health, Šrobárova 48, 100 42 Prague, Czech Republic*

[b]*Department of Organic Chemistry, Institute of Chemical Technology, Prague, Czech Republic*

[c]*Department of Analytical Biochemistry, Institute of Entomology, Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic*

Abstract

A sensitive method for determination of the *N*-methylcarbamoyl adduct at the N-terminal valine of globin, a new metabolic product of the industrial solvent *N*,*N*-dimethylformamide (DMF), has been developed and validated. The method includes conversion of the adduct by the Edman degradation to 3-methyl-5-isopropylhydantoin (MVH), which is followed by optimized gas chromatographic analysis with mass spectrometric detection at *m*/*z* 114. The recovery of MVH from terminal *N*-methylcarbamoylvaline was determined using a model dipeptide to be 90%. Calibration of the method is done with MVH, employing 3-methyl-5-isobutylhydantoin as the internal standard. The limit of detection is 0.2 nmol MVH/g globin when a 100-mg sample is used. Within- and between-day precision is 4–10%. The method has been used to determine the background levels of MVH in unexposed subjects. Further, toxicokinetic studies in volunteers laid the grounds for setting the reference value for biological monitoring of occupational exposure to DMF.

Keywords: Globin; *N*,*N*-Dimethylformamide; *N*-Methylcarbamoylvaline adduct

1. Introduction

N,*N*-Dimethylformamide (DMF, Fig. 1) is a widely used industrial solvent, known to cause a variety of occupational health problems. The primary metabolic reaction of DMF is oxidation to *N*-hydroxymethyl-*N*-methylformamide [1,2], which is mainly excreted in urine. A minor portion of *N*-hydroxymethyl-*N*-methylformamide and/or its decomposition product, *N*-methylformamide (MF), are further oxidized at the formyl group to a reactive intermediate, most probably methylisocyanate (MIC, Fig. 1) [3], which binds rapidly to endogenous nucleophilic sites. The conjugate formed by the reaction of MIC with the

*Corresponding author. Fax: +42-2-673-11236.
E-mail address: jmraz@szu.cz (J. Mráz).

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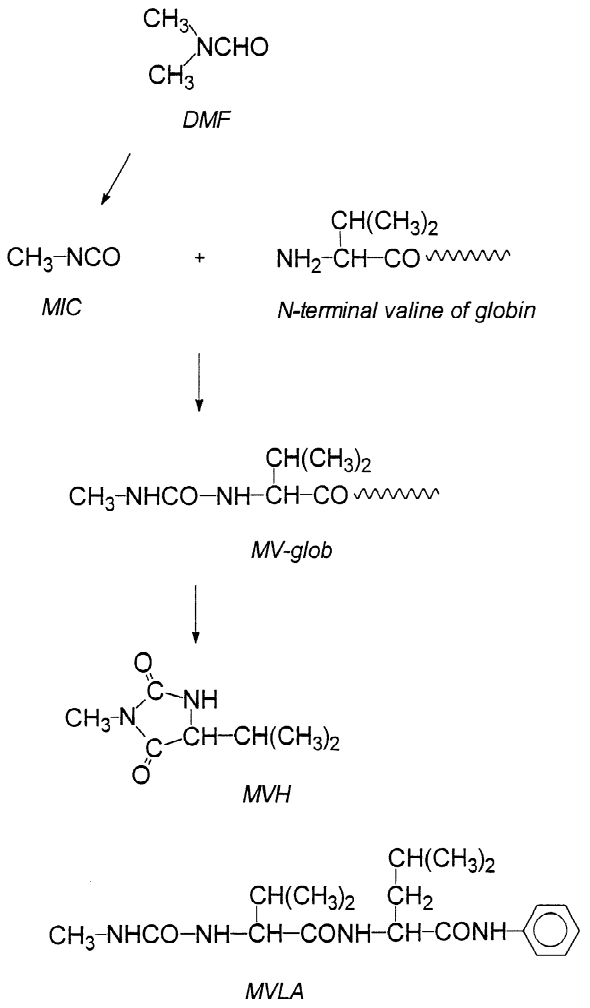

Fig. 1. Structure of compounds used in the study.

cysteinyl group of the tripeptide glutathione is decomposed in several steps to *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (AMCC) and excreted in urine [4,5].

The industrial disaster in Bhopal, India in 1984 prompted extensive toxicological investigations on MIC itself. In one such study, the interaction of MIC with blood, both in vitro and in vivo, was reported to produce the *N*-methylcarbamoyl adduct at the N-terminal valine of globin (MV–glob, Fig. 1) [6]. The adduct was detected by gas chromatography following the Edman degradation to 3-methyl-5-isopropylhydantoin (MVH[1], Fig. 1). Recently, the MV–glob has also been identified in blood of rats exposed to DMF or MF [7] and in volunteers and workers exposed to DMF [8,9]. This is an important finding, considering the attention given to blood protein adducts as a new type of biomarker of exposure. Analytical procedures for adducts of several other alkylating agents (e.g., ethylene oxide, acrylonitrile, dimethyl sulphate) have been currently standardized and recommended for biomonitoring in humans [10].

In previous studies, MVH formed from MV–glob was measured by the following detection methods: gas chromatography (GC) with a flame ionization detector (FID) [6], high-performance liquid chromatography (HPLC) with a diode-array detector [11] or GC–mass spectrometry (GC–MS) [9]. Only the last method was sufficiently sensitive to detect MVH in the general (unexposed) population. It was, nevertheless, still considered as provisional. In this paper, we described an improved method that includes the use of an internal standard and optimized operational parameters of GC analysis. We also evaluated the method's analytical reliability and crucial steps of its work-up. Further, the recovery of MVH from the terminal *N*-methylcarbamoylvaline was assessed.

2. Experimental

2.1. Chemicals and reagents

MIC was prepared in our laboratory from potassium cyanate and dimethyl sulphate [12]. MVH was prepared from MIC and valine [6]. Similarly, 3-methyl-5-isobutylhydantoin (MIH) was prepared from MIC and isoleucine. Valinyl–leucine hydrochloride and bovine albumin, fraction V, were bought from Sigma (St. Louis, MO, USA); *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydro-

[1]MVH has not always been used as the abbreviation for 3-methyl-5-isopropylhydantoin. In some earlier studies, MIH was employed [6,9,11]. Here we used MVH because it is consistent with the abbreviation system used in our laboratory for isocyanate derivatives: MVH = methylisocyanate–valine–hydantoin. The abbreviation MIH in this paper is for the internal standard 3-methyl-5-isobutylhydantoin (i.e., methylisocyanate–isoleucine–hydantoin).

chloride, di-*tert*-butyl dicarbonate and 1-hydroxybenzotriazole were bought from Fluka (Buchs, Switzerland). TLC analyses were carried out on plates with silica gel 60 F_{254} (0.2-mm layer) from Merck (Darmstadt, Germany).

2.2. Synthesis of N-methylcarbamoylvalinyl–leucine anilide

(a) Valinyl–leucine hydrochloride (500 mg, 1.88 mmol), sodium bicarbonate (160 mg, 1.90 mmol) and di-*tert*-butyl dicarbonate (700 mg, 3.21 mmol) were dissolved in tetrahydrofurane–water (2:1, v/v). The reaction mixture was stirred for 24 h at room temperature. TLC analysis showed a violet spot R_f = 0.94 (4:1:5, v/v/v 1-butanol–acetic acid–water) after spraying with ninhydrine reagent. The mixture was further reacted with 360 mg (1.88 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 50 mg (0.37 mmol) of 1-hydroxybenztriazole and 350 mg (3.76 mmol) of aniline by heating under reflux for 15 min. After cooling, the mixture was saturated with sodium chloride, the organic layer was separated and the aqueous phase was extracted with diethyl ether (20 ml). The combined organic phases were washed with 2 *M* hydrochloric acid (2×10 ml) and water, dried with magnesium sulphate, filtered and then evaporated in a vacuum to dryness. TLC analysis showed a product positive to ninhydrine, R_f = 0.85 (7:3 v/v chloroform–acetone). The product was dissolved in trifluoroacetic acid (3 ml). After the reaction ceased, toluene was added and the solvents were evaporated in a vacuum. The residue was dissolved in water and the pH was adjusted to 7 with diluted ammonia. The resulting solution was filtered, had diluted ammonia added until the solution was pH 9.7 and then allowed to crystallize. Crystals were dried over phosphorus pentoxide. The procedure yielded 112 mg (20%) of valinyl–leucine anilide, m.p. 154–156°C.

(b) Valinyl–leucine anilide (61 mg, 0.2 mmol) was dissolved in acetonitrile (2 ml), a 10% solution of MIC in acetonitrile (150 μl, 0.25 mmol) was added and the mixture was left overnight. The precipitated solid was filtered, washed with acetonitrile and recrystallized from water–methanol (1:1, v/v). The resulting fine white needles were filtered, washed with water and dried in a dessiccator. The procedure yielded 38 mg (52%) of *N*-methylcarbamoylvalinyl–leucine anilide (MVLA). The purity of the MVLA standard, as measured by HPLC–MS (*m*/*z* 100–500) and HPLC with a UV detector (250 nm), was estimated to be 97%.

Positive electrospray MS: *m*/*z* 363.1 $[M+H]^+$; CID-MS^2 of 363.2: *m*/*z* 270.1 $[M-C_6H_5NH]^+$; CID-MS^3 of 363.2→270.1: *m*/*z* 157.0 $[CH_3NHCO-NHCH(C_3H_7)CO]^+$; CID-$MS^4$ of 363.2→270.1 →157.0: 128.9 $[CH_3NHCONHCH(C_3H_7)]^+$.

Negative electrospray MS: *m*/*z* 361.2 $[M-H]^-$; CID-MS^2 of 361.2: *m*/*z* 205.2 $[NHCH(C_4H_9)CONH(C_6H_5)]^+$.

^{1}H-NMR (δ in ppm, DMSO-d_6): CH_3: 0.92 (m, 12H); $C\underline{H}_2$–$C\underline{H}$: 1.59 (m, 3H); val–$CH_3C\underline{H}$: 1.93 (m, 1H); $C\underline{H}_3NHCO$: 2.57 (d, ^3J = 4.4 Hz, 3H); leu–$C\underline{H}NH$: 4.0 (t, ^3J = 6.6 Hz, 1H); val–$C\underline{H}NH$: 4.42 (m, 1H); aromatic ring CH: 7.03 (t, ^3J = 7.1 Hz, 1H), 7.28 (t, ^3J = 8.0 Hz, 2H) and 7.62 (d, ^3J = 7.7 Hz, 2H); NH: 6.0, 6.04, 8.07 and 9.82 (exchangeable with D_2O).

^{13}C-NMR (δ in ppm, DMSO-d_6): CH_3: 17.4, 18.9, 21.1 and 22.7; $\underline{C}HCH_3$: 24.0 and 25.9; CH_3N: 30.4; CH_2: 40.1; CHN: 51.5 and 58.0; aromatic ring CH: 119.0, 123.0 and 128.3; aromatic ring C: 138.6; NHCONH: 158.5; CO: 170.7 and 171.9.

2.3. Exposure

Male Wistar rats were injected, i.p., with DMF or MF in saline (dose = 1000 mg/kg, injected volume = 8 ml/kg). The animals were killed 3 days later and blood (5–10 ml) was collected over a heparin solution (500 μl). Human volunteers were exposed by immersion of their left hands in pure DMF for 15 min. Blood samples (5 ml) were taken from the vena cubitalis and mixed with heparin (100 μl). One volunteer provided 300 ml of blood for the method reliability testing.

2.4. Isolation of the globin

Blood (5 ml) was centrifuged at 1000 *g* for 15 min. The plasma was discarded, the erythrocytes were washed twice with saline (25 ml) and hemolyzed with distilled water (1 ml) using sonica-

tion (30 s). Ice-cold acetone, containing 2% of concentrated HCl, (15 ml) was added slowly. The precipitated globin was spun down at 200 *g* for 5 min, the supernatant was discarded, the globin was washed three times with ice-cold acetone (15 ml) and once with diethyl ether (15 ml) and dried in a vacuum concentrator (Speedvac) at room temperature for 30 min and then at 60°C for 60 min.

From several blood samples, the globin was also isolated by the alternative method of Bader et al. [13], slightly modified as described by Deutsche Forschungsgemeinschaft [10]. The modified procedure included removal of cell debris by centrifugation of the hemolyzate at 30 000 *g*, addition of 50 m*M* hydrochloric acid solution in 2-propanol, precipitation of the globin by ethyl acetate and, finally, washing of the globin with ethyl acetate and hexane and drying in a Speedvac.

2.5. Analytical procedure

Samples of globin (100 mg) were placed in 10-ml ampoules, and then concentrated HCl–acetic acid (2:1, v/v, 5 ml) and an aqueous solution of the internal standard MIH (20 μ*M*, 100 μl) were added. The unsealed ampoules were heated in a heating block at 100°C for 1 h (the elongated top of the ampoule served as a vertical condenser). Care was taken at the beginning of the heating, since the samples may develop foam until the globin is dissolved. After cooling, the contents of the ampoules were poured into 25-ml centrifugation tubes and ca. 3 g of $(NH_4)_2SO_4$ and one drop of phenol red were added. The mixture was then neutralized by the stepwise addition of 10 *M* NaOH to pH 7.0–7.5 (indicated by the change of the phenol red colour from pink to yellow and by an indicator paper strip), and shaken with ethyl acetate (8 ml) on a vortex mixer for 2 min (the yield of MVH extraction = 91%). After centrifugation, the resulting clear organic phase was transferred and evaporated in a Speedvac at 60°C to dryness. The residue was dissolved in ethyl acetate (50 μl) and an aliquot (0.6 μl) was analyzed by GC–MS.

2.6. Calibration of MVH determination

Stock solutions of MVH and MIH (10 m*M*) were prepared by dissolving MVH (15.6 mg) or MIH (17.0 mg) in water–MeOH (1:1, v/v, 10 ml). Further dilution to working solutions of MVH (0.1, 0.2, 0.4, 0.6, 1, 2, 4, 6, 10, 20, 40, 60 and 100 μ*M*) and to a working solution of MIH (20 μ*M*) was done with distilled water. The working solutions of MVH (100 μl) or water (to prepare a blank sample; 100 μl) and the working solution of MIH (100 μl) were pipetted to 10-ml ampoules containing 5 ml of concentrated HCl–acetic acid (2:1, v/v) with dissolved bovine serum albumin (20 mg/ml). The ampoules were placed in a heating block (100°C) for 1 h and the procedure continued as described in Section 2.5.

Following the GC–MS analysis, the ratios of the peak areas for MVH and MIH were plotted against the MVH amounts added to the sample. The calibration line was constructed separately in three concentration ranges, namely, 0–1, 1–10 and 10–100 nmol MVH per sample. In all three ranges, the relationship between the amount of MVH and the ratio of the peak areas for MVH and MIH (R) was found to be linear, and was described as: MVH (nmol/sample) = $a + b \cdot R$. For the MIH amount of 2 nmol (the amount added to the samples), the value of b was typically about 1.60. The coefficient a was usually not significantly different from 0.

The amounts of MVH in the unknown samples were determined from these calibration lines and finally expressed in nmol MVH/g globin: MVH (nmol/g globin) = 10 × MVH (nmol/sample).

2.6.1. Interfering peak from ethyl acetate

Since MVH and MIH are poorly soluble in distilled water, dissolution in methanol or ethyl acetate to prepare the stock and working solutions may appear to be suitable. However, presence of these solvents during the Edman degradation, i.e., heating of globin or other proteins with concentrated acid, was found to generate high levels of phenylalanine methyl- and ethyl ester, respectively. Whereas the former eluted between MVH and MIH, the latter coeluted with MIH and interfered with its determination. Thus, the use of ethyl acetate for dissolving the hydantoin standards should be avoided.

2.6.2. Protein matrix in calibration samples

In the first step of preparation of the calibration

samples, MVH and MIH solutions were added to the mixture of acids containing dissolved albumin. If the albumin was not present, the ratio of peak areas of MVH and MIH was less reproducible and usually lower than if protein was present. This effect was probably due to MVH losses during evaporation of the ethyl acetate extract. The extracts of the protein-containing samples afford a solid residue in which the analyzed compounds are absorbed, thus, prevented from losses. (Prolonged drying of the residue must still be avoided.) Serum albumin was employed as a matrix protein instead of globin because of a measurable physiological level of MV–glob in the latter.

2.7. Determination of recovery of MVH from MVLA

The MVH and MVLA standards (20 μ*M* aqueous solutions, 100 μl each) were placed separately in 10-ml ampoules and an aqueous solution of the internal standard MIH (20 μ*M*, 100 μl) and concentrated HCl–acetic acid (2:1, v/v, 5 ml) were added. The samples were then carried through the analytical procedure as described in Section 2.5 and recoveries of MVH were compared. Assuming that the recovery (of MVH) from MVH was 100.0% [±6.2% (*N*= 20)], that from MVLA was found to be 90.3±7.6% (*N*=20).

2.8. GC–MS

Instrument: GC–MS ion trap mass spectrometer GCQ (Thermoquest, San José, CA, USA); column: DB-5 ms, 30 m×0.25 mm, 0.25 μm film thickness (J&W; Folsom, CA, USA); carrier gas: helium: 4.6, linear velocity: 30 cm/s (0.88 ml/min); temperatures: injector: 225°C, column: 60°C held for 1 min, increase of 20°C/min to 250°C, then isothermal 5 min, transfer line: 250°C, ion source: 180°C; injection splitless, splitter closed for 1 min; ionization mode: electron impact (EI); electron energy: 70 eV; detection mode: SI*M*, *m*/*z* 114; speed: 2 scans/s.

2.9. Mass spectrometry

Instrument: ion trap mass spectrometer LCQ (Thermoquest, San Jose, CA, USA) equipped with an electrospray ion source operated at 4.5 kV and a capillary tube kept at 200°C. Collisionally induced dissociation (CID) mass spectra were obtained at an appropriate collision energy within the mass spectrometer, using a standard software procedure. Samples were introduced into the ESI probe with a stream of acetonitrile −0.4% formic acid (40:60) mobile phase at 50 μl/min. A 5-μl sample loop was used for the sample injection. The mass range of 50–500 was scanned every second.

2.10. NMR

NMR spectra (300 MHz for ^{1}H) were measured on a Gemini 2000 spectrometer (Varian, Palo Alto, CA, USA).

3. Results and discussion

3.1. Identification of the N-methylcarbamoylvaline adduct

GC–MS analysis of globin from rats exposed to DMF or MF and from human volunteers exposed to DMF revealed a notable peak with the retention time and EI mass spectrum (base peak at *m*/*z* 114) identical with those of the MVH standard. Further, collision-induced dissociation of the fragment ion *m*/*z* 114 from both the MVH standard and the globin-derived peak afforded a dominant daughter ion *m*/*z* 57, corresponding to $[CH_3NCO]^+$. This is a convincing evidence of MVH in the biological samples. Somewhat surprisingly, a small peak of MVH was also observed in most globin samples from persons with no known exposures to DMF or MF (Fig. 2).

The identification of MVH released from globin heated with strong acid is an evidence of the presence of *N*-methylcarbamoyl adduct at the N-terminal valine of globin.

3.2. Optimization of the procedure

3.2.1. Isolation of globin

Two different procedures for the isolation of globin from the hemolyzed erythrocytes have been reported. It was either precipitated with acetone

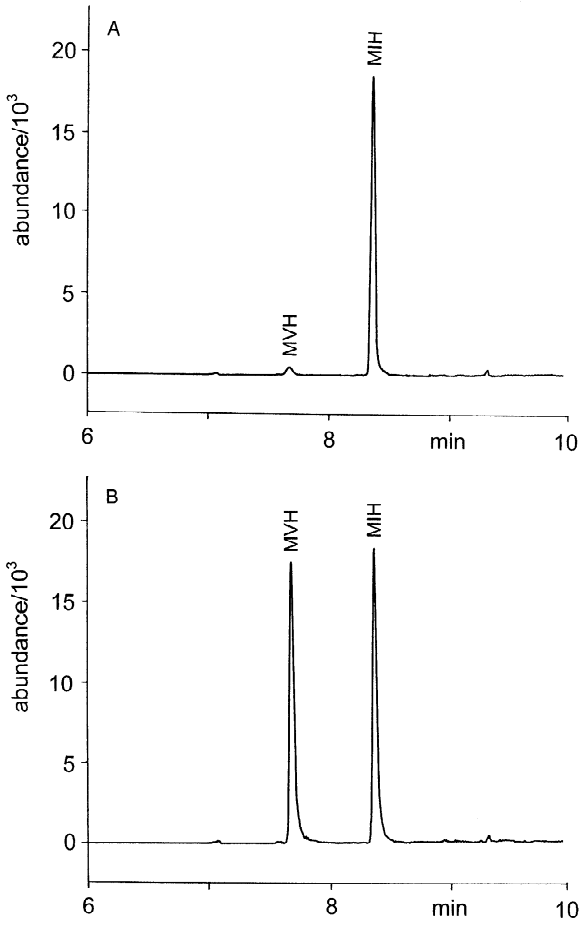

Fig. 2. GC–MS chromatograms (m/z 114) of the extracts of processed globin from a person without previous exposure to DMF (A) and from the same person collected 3 days after immersion of hand to pure DMF (B). MVH, $t_R = 7.7$ min; MIH (internal standard), $t_R = 8.3$ min. The MVH levels in (A) and (B) were 0.5 and 15.0 nmol/g globin, respectively. Experimental conditions are given in the text.

containing 2% of concentrated HCl [6, this study], or the hemolysate was mixed with a 50 m*M* solution of hydrochloric acid in 2-propanol and the globin was precipitated with ethyl acetate [9,13]. In this study, blood from a DMF-exposed volunteer was used to isolate globin by both procedures, and both globin batches were then analyzed as described in Section 2.5. The MVH levels were 14.4±0.4 nmol/g protein ($N = 6$) using acetone and 15.6±0.8 nmol/g protein ($N = 6$) using ethyl acetate. Thus, the (less laborious) procedure with acetone, which is taken here as a reference one, affords a product containing slightly lower level of MV–glob (probably because of the lower purity of the isolated globin). This data allows for evaluation of differences in the MVH levels obtained by exploiting either of the above isolation procedures.

3.2.2. The effect of hemolysis

The standard procedure of the isolation of globin includes washing of the erythrocytes in order to remove plasmatic proteins before hemolysis. In one clinical study in volunteers who received DMF (not published), the erythrocytes were frozen by mistake after the separation of the plasma, washing being omitted. Since the defrosted erythrocytes were hemolyzed and could not be washed, the level of MVH in the subsequently precipitated globin was diminished due to the presence of the contaminating plasmatic proteins. To evaluate the extent of this contamination, globin from unwashed erythrocytes of the DMF-exposed volunteer was precipitated (using acetone–concentrated HCl, 98:2, v/v) and analyzed. The MVH level found was 13.1±0.4 nmol/g globin ($N = 6$), i.e., 91% compared to the standard isolation procedure.

3.2.3. Duration of hydrolysis

The effect of the duration of the hydrolysis of MV–glob on the recovery of MVH was tested with rat globin (150 nmol MVH/g). The hydrolysis for 20, 40 or 60 min provided virtually the same values, whereas its duration for 4 or 8 h resulted, in some instances, in 10–25% losses of MVH. The period of 60 min was employed in the standard procedure.

3.2.4. Composition of the acid

The Edman degradation of MV–glob in previous investigations was carried out with various acid compositions such as concentrated HCl–acetic acid: 2:1 [6] or 1:1 [9] (v/v) and or 6 *M* HCl [14]. In this study, the variation in the recovery of MVH from rat globin (150 nmol MVH/g) was tested with concentrated HCl–acetic acid (2:1, v/v), concentrated HCl and 6 *M* HCl. The differences in the recovery of MVH were below 5%. The concentrated HCl–acetic acid solution was used in further studies.

3.2.5. Operational parameters for GC

The parameters of the GC analysis described above (2.7) permitted fast and reliable MVH determination. In comparison with the previous method [9], the use of less polar columns and faster temperature programming reduced the MVH retention from 28 to 8 min, while the use of the splitless injection mode lowered the detection limit by one order of magnitude.

3.3. Reliability of the method

3.3.1. Precision

Human globins with MVH levels of 5 and 18 nmol/g were repetitively ($N=10$) processed and analyzed. Within-day precision, expressed by RSD, was 7.3 and 4.1%, respectively. Between-day precision, determined after processing and analyzing the above two globin batches for 5 consecutive days (one analysis per day), was 9.9 and 9.5%, respectively.

Within-day precision of analysis was also calculated in Section 3.3.4.1. The mean value of five determinations of the RSD (each obtained by repeated analysis of six globin samples within 1 day) was 6.1%.

3.3.2. Accuracy

Rigorously, the accuracy of the method, defined by means of the deviation of the analytical results from the true value, cannot be determined, since the true value of the MV–glob level is not accessible. This is because neither globin with the defined level of MV–glob, nor the pure MV–glob standard, nor a definitive or alternative method of MV–glob determination are at present available.

3.3.3. Detection limit

The detection limit of MVH (three times the signal-to-noise ratio) for a 100-mg globin sample was 0.2 nmol/g globin.

3.3.4. Stability of the adduct

3.3.4.1. Stability in native blood. The blood of a volunteer exposed to DMF was collected over citrate and divided into 30 portions of 5 ml each, six portions being worked up to isolate the globin on the day of blood collection (day 0) and 24 portions being stored at 4°C with the globin isolated on days 3, 7, 14 and 21 (six portions per day). All globin samples were analyzed after day 21 (those isolated on the same day were always analyzed within 1 day). The following results were obtained (in nmol MVH/g globin): 14.4 ± 0.4 (day 0), 14.8 ± 1.2 (day 3), 14.9 ± 1.1 (day 7), 14.3 ± 0.8 (day 14) and 14.9 ± 1.0 (day 21).

3.3.4.2. Stability in isolated globin. Human globin samples, 5–12 nmol MVH/g ($N=12$), were analyzed as follows: within a few days after isolation — after storage for 1 year at 4°C and after another year of storage at 4°C. The matching values obtained after 1 and 2 years were 94.9 ± 6.9 and 104.5 ± 6.3%, respectively, compared to reference values.

The above results demonstrate a high stability of MV–glob: no significant changes in MVH levels were observed after 3 week's storage of full blood or after 2 year's storage of dried globin.

3.4. Discussion of the calibration method

The Edman degradation (the conversion of carbamoyl adducts at protein N-termini to the corresponding hydantoins) has two major applications in protein chemistry: the identification of N-terminal amino acids in proteins and the determination of the extent of carbamoylation thereof. Here it was used to determine the *N*-methylcarbamoyl adduct at the N-terminal valine of globin. Since protein standards with known levels of the adducts are not available, the essential methodological problem of quantitation of any protein adduct is calibration. This problem has been encountered especially in the ''modified'' Edman degradation in which *N*-alkyl valines at the N-terminus of globin (produced on the exposure to alkylating xenobiotics) are reacted with an isothiocyanate agent to produce 1-alkylthiohydantoins [15]. The recoveries of the latter do not exceed 40–60% and are sensitive to various factors [10]. The use of 1-alkylthiohydantoin standards for calibration allows the liberated compounds to be determined. However, because their liberation is not included in the calibration procedure and their recoveries from the adducts are generally not known, the actual level of the adduct cannot be determined. Neither the free

N-alkylvalines proved to be suitable calibration standards for the modified Edman degradation since they afforded thiohydantoins at recoveries different from the *N*-alkylvaline adducts at globin [10]. The problem of the calibration standards was successfully solved only by using peptides simulating the N-terminal sequence of the modified globin, *N*-alkylvalinyl–leucine–anilides [10]. With regard to the currently used, i.e., the ''original'' Edman degradation, however, no data on the hydantoin recovery has been to our knowledge available.

To evaluate the recovery of MVH from MV–glob in the presented method, the peptide MVLA simulating the N-terminus of the MV–glob was prepared and the degree of its conversion to MVH during the standard work-up was evaluated. (In both MVLA and MV–glob, *N*-methylcarbamoylvaline is attached to leucine). The value obtained, 90%, was then assumed to represent the recovery of MVH from the MV–glob.

The presented results indicate that the recovery of MVH from MV–glob is almost complete and reasonably stable under variable experimental conditions. Therefore, the determined levels of MVH and the actual levels of MV–glob are assumed to parallel closely. This has practical consequences. Whereas the determination of *N*-alkyl globines by the modified Edman degradation requires the calibration to be always carried out using compounds simulating the particular globin N-termini, this appears unnecessary in the case of MV–globin. Instead, we recommend that the calibration be based on the MVH standard itself and the adduct levels be expressed by means of an index nmol MVH/g globin. This will make the method more available and convenient for the occupational biomonitoring for which it is intended.

Unlike the previous studies on MVH [6,9,11], the presented analytical procedure included the use of an internal standard. MIH is structurally very close to MVH; both compounds are together carried through the analytical procedure, have close retention times and undergo analogous fragmentation in EI ionisation, affording the diagnostic fragment ion at m/z 114.

3.5. Applications

The method has already been employed in several investigations. MVH levels were determined in the unexposed subjects [0.7 ± 0.5 nmol/g globin ($N = 20$)] [8] as well as in human volunteers following immersion of one hand to DMF for 15 min [14.4 ± 4.1 nmol/g globin ($N = 8$)] [8]. Further, the toxicokinetics of MV–glob in rats and humans were described in detail [7,8]. The data obtained is the basis for setting the reference value for biological monitoring of occupational exposure to DMF.

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References

[1] C. Brindley, A. Gescher, D. Ross, Chem. Biol. Interact. 45 (1983) 387.
[2] J. Mráz, H. Nohová, Int. Arch. Occup. Environ. Health 64 (1992) 85.
[3] M.D. Threadgill, D.B. Axworthy, T.A. Baillie, P.B. Farmer, K.C. Farrow, A. Gescher, P. Kestell, P.G. Pearson, A.J. Shaw, J. Pharmacol. Exp. Ther. 242 (1987) 312.
[4] J. Mráz, F. Turecek, J. Chromatogr. 414 (1987) 399.
[5] P. Kestell, A.P. Gledhill, M.D. Threadgill, A. Gescher, Biochem. Pharmacol. 35 (1986) 2283.
[6] P.K. Ramachandran, B.R. Gandhe, K.S. Venkateswaran, M.P. Kaushik, R. Vijayaraghavan, G.S. Agarwal, N. Gopalan, M.V.S. Suryanarayana, S.K. Shinde, J. Chromatogr. 426 (1988) 239.
[7] J. Mráz, E. Gálová, H. Nohová, Š. Boušková, M. Hornychová, in: Abstracts of the Fourth International Symposium on Biological Monitoring in Occupational and Environmental Health, Seoul, September 1998, p. 125.
[8] J. Mráz, Š. Dušková, E. Gálová, H. Nohová, M. Brabec, in preparation.
[9] J. Angerer, T. Göen, A. Krämer, H.U. Käfferlein, Arch. Toxicol. 72 (1998) 309.
[10] J. Angerer, K.H. Schaller (Eds.), Analyses of Hazardous Substances in Biological Materials, Deutsche Forschungsgemeinschaft (DFG), Vol. 5, VCH, Weinheim, 1997, p. 181.
[11] K.S. Venkateswaran, C.D. Raghuveeran, N. Gopalan, G.S. Agarwal, M.P. Kaushik, R. Vijayaraghavan, Biochem. Int. 28 (1992) 745.

[12] K.H. Slotta, L. Lorenz, Chem. Ber. 58 (1925) 1320.
[13] M. Bader, J. Lewalter, J. Angerer, Int. Arch. Occup. Environ. Health 67 (1995) 237.
[14] C.K. Lee, J. Biol. Chem. 251 (1976) 6226.
[15] M. Törnqvist, J. Mowrer, S. Jensen, L. Ehrenberg, Anal. Biochem. 154 (1986) 255.

Jaroslav Mráz was born in 1960 in Kralovice, Czech Republic. He studied analytical chemistry at the Faculty of Science, Charles University, Prague. After graduation in 1983 he joined a research group of toxicological analysis at the Centre of Industrial Hygiene and Occupational Diseases, National Institute of Public Health, Prague. Here he is now in the position of a senior scientist. In the meantime he had