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Determination of methyl isopropyl hydantoin from rat erythrocytes by gas-chromatography mass-spectrometry to determine methyl isocyanate dose following inhalation exposure

Brian A. Logue^{a,*}, Zhiling Zhang^a, Erica Manandhar^a, Adam L. Pay^a, Claire R. Croutch^b, Eric Peters^b, William Sosna^b, Jacqueline S. Rioux^c, Livia A. Veress^c, and Carl W. White^c

^aDepartment of Chemistry and Biochemistry, South Dakota State University, Avera Health and Science, Box 2202, Brookings, SD 57007

^bMRI-Global, 425 Volker Boulevard, Kansas City, MO 64110-2241

^cPediatrics-Pulmonary Medicine, University of Colorado-Denver, Denver, CO, 80045

Abstract

Methyl isocyanate (MIC) is an important precursor for industrial synthesis, but it is highly toxic. MIC causes irritation and damage to the eyes, respiratory tract, and skin. While current treatment is limited to supportive care and counteracting symptoms, promising countermeasures are being evaluated. Our work focuses on understanding the inhalation toxicity of MIC to develop effective therapeutic interventions. However, in-vivo inhalation exposure studies are limited by challenges in estimating the actual respiratory dose, due to animal-to-animal variability in breathing rate, depth, etc. Therefore, a method was developed to estimate the inhaled MIC dose based on analysis of an N-terminal valine hemoglobin adduct. The method features a simple sample preparation scheme, including rapid isolation of hemoglobin, hydrolysis of the hemoglobin adduct with immediate conversion to methyl isopropyl hydantoin (MIH), rapid liquid-liquid extraction, and gas-chromatography mass-spectrometry analysis. The method produced a limit of detection of 0.05 mg MIH/kg RBC precipitate with a dynamic range from 0.05–25 mg MIH/kg. The precision, as measured by percent relative standard deviation, was <8.5%, and the accuracy was within 8% of the nominal concentration. The method was used to evaluate a potential correlation between MIH and MIC internal dose and proved promising. If successful, this method may be used to quantify the true internal dose of MIC from inhalation studies to help determine the effectiveness of MIC therapeutics.

Keywords

methyl isocyanate; liquid-liquid extraction; gas-chromatography mass-spectrometry; inhalation exposure

*Corresponding Author. brian.logue@sdstate.edu, Phone: (605) 688-6698 Fax: (605) 688-6364.

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1. Introduction

Methyl isocyanate (MIC) is used for carbamylation of amines as an important precursor for the synthesis of carbamate pesticides and diisocyanates (i.e., intermediates in synthesis of plastics) (1–3). Although industrially important, MIC is also highly toxic, as tragically demonstrated by Bhopal disaster in 1984, where it is estimated that 8000 or more people died within minutes of exposure to MIC (4–6). Victims of MIC exposure suffer from severe health effects, both acutely and long-term, with MIC causing irritation and injury to the eyes, respiratory tract, and skin, with most damage occurring to the lungs and airways (7–9). MIC can enter the body via inhalation or skin contact with the toxicity of MIC stemming from its ability to readily react with electronegative groups in biological molecules (e.g., amino groups). Specifically, MIC mainly carbamoylates end-terminal amino acids of tissue proteins and side-chain amino groups of lysine (2, 10, 11), although about 20 other adducts have also been found (12). MIC-protein and DNA alkylation results in tissue hypoxia and cytotoxicity (10–15), targeting various organs, and leading to ophthalmic, respiratory, reproductive, neuromuscular, psychological, neural-behavioral, and other systematic problems (10, 16–24).

While current treatment is limited to supportive care and treatment of symptoms, promising drug treatments are being evaluated. New investigations are underway which focus on understanding the inhalation toxicity of MIC to develop effective therapeutic interventions. However, in-vivo inhalation exposure studies are limited by challenges in determining the actual respiratory dose, because the dose depends on many factors other than vapor concentration and exposure time, including respiratory rate, tidal volume, minute volume, and deposition rate within various regions of respiratory tract (25). These parameters are very difficult to estimate in animals, especially during active MIC exposure. Therefore, for inhalation exposures, it is very difficult to quantify how much MIC is internalized (i.e., the “internal dose”) from external parameters. To address this problem, quantification of a known MIC biomarker may allow better estimation of internal dose.

Because of its reactivity, MIC is quickly eliminated from biological systems, mainly via its reaction with protein-based amino groups. Therefore, biomarkers used to verify exposure to MIC are typically based on protein adducts, with the most common based on the reaction of MIC with the N-terminal valine in hemoglobin (Hb) (2, 15, 26–29). In fact, the MIC-N-terminal valine adduct has been used to verify exposure of individuals during the Bhopal disaster (15). Detection and quantification of the MIC-N-terminal valine Hb adduct is accomplished by the Scheme shown in Figure 1, featuring acid hydrolysis, cyclization of the resulting N-(methylcarbamoyl)valine, and subsequent extraction of the methyl isopropyl hydantoin (MIH) using liquid-liquid extraction. This process is performed in each of the methods available for the analysis of the MIC-N-terminal hemoglobin adduct listed in Table 1. Although each of these methods are effective, most of the methods are arduous, lengthy, complex, generate a relatively large amount of organic waste, and consume considerable amounts of energy (Table 1). Mráz et al. (28) simplified the typical sample preparation as compared to the other methods in Table 1. Although the method was simplified to 20 steps and approximately 9 hr, it still was lengthy, consumed considerable amounts of energy, and generated a large amount of organic waste. Wang et al. (29) also simplified the sample

preparation of MIH from MIC-adducted hemoglobin by using a 96-well protein precipitation and phospholipid removal plate. This method featured similar hydrolysis and MIH formation steps as the other methods in Table 1, but instead of liquid-liquid extraction (LLE), a sorbent plate was used to isolate the MIH for UHPLC-MS-MS analysis. While this method was rapid, it still necessitated many steps (i.e., 18) and specialized equipment for the sample preparation.

The objective of the current study was to develop a more rapid, simpler, and greener method than currently available for evaluation of MIC exposure via analysis of MIH following hydrolysis of carbamylated N-terminal valine of hemoglobin. A secondary objective was to evaluate the potential of the method to estimate the internalized dose of MIC from inhalation exposures.

2. Experimental

2.1. Reagents and standards

All reagents were at least HPLC grade unless otherwise noted. Methanol, hydrochloric acid (certified ACS), acetic acid (certified ACS), acetone, ethyl acetate, sodium hydroxide (certified ACS) and sodium bicarbonate (certified ACS) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Reverse-osmosis water was purified to 18.2 MΩ-cm using a Lab Pro polishing from Labconco (Kansas City, KS, USA). Methyl isocyanate (MIC) was synthesized for the analytical work in our lab by the method of Kaushik et al. (32) based on the Curtius Rearrangement method, which involved refluxing acetyl chloride with sodium azide in toluene until all starting materials were consumed. The MIC was collected via distillation. Purity was confirmed using ^1H -NMR and at 95%. MIC for the animal exposures was prepared at MRIGlobal (Kansas City, MO) by the same general procedure. The MIC prepared at MRIGlobal was 99.2% pure, as verified by gas chromatography-flame ionization detection. Methyl isopropyl hydantoin (MIH) was synthesized in our lab according to Ramachandran et al. (15) by the reaction of MIC with L-valine in acidic solution. MIH purity was verified by ^1H -NMR and GC-MS analysis and was 97% pure. The internal standard, MIH- $^{13}\text{C}_5$ ^{15}N , was synthesized by the same procedure using valine- $^{13}\text{C}_5$ ^{15}N , purchased from Sigma Aldrich (St. Louis, MO). MIH- $^{13}\text{C}_5$ ^{15}N purity was verified by ^1H -NMR and GC-MS as 99.8% pure. MIH and internal standard stock solutions (1 g/mL) were prepared and stored in a -30 °C freezer. For each experiment, they were brought to room temperature by leaving them on bench top and were diluted to the desired concentration.

2.2. Biological fluids

For method development and validation, Sprague-Dawley rat red blood cells (RBCs) with K2EDTA as anticoagulant, were purchased from Bioreclamation IVT (Westbury, NY, USA) and immediately stored at -80 °C until used.

RBCs from MIC-exposed male Sprague-Dawley rats were received from MRIGlobal (Kansas City, MO) with all procedures performed according to approved Institutional Animal Care and Use Committee protocols there. Male Sprague-Dawley rats (250–300 g) were obtained from Charles River (Kingston, NY). Animals were acclimated to laboratory

conditions for 5–7 days prior to study, including acclimation to nose-only exposure chambers at variable time intervals for 72 hours before study. Food and water were provided ad libitum. A nose-only (CH Technologies; Westwood, NJ) inhalation system was used for the exposure of rats with the entire inhalation system placed in a fume hood. A proprietary custom vapor diffusion system (MRIGlobal) was used to generate MIC vapor. Rats were acclimated to the exposure system for three sequential days prior to MIC exposure. The vapor was then delivered to the plenum of the exposure system in a mixture of dry nitrogen (50 mL/min ultra-high purity nitrogen) that was then blended with high efficiency particulate air (HEPA)-carbon-filtered dry air (10–15 L/min, depending on the desired concentration). Downstream from the site of mixing and prior to the plenum, gas constituents were monitored by Fourier transform infrared spectroscopy by accessing the system via a 3-way valve. Exhaust from the system was scrubbed in 10% NaOH and delivered to the fume hood. Conscious rats were exposed for 30 min, followed by in-cage recovery for 1, 2, 4, or 8 hr. Blood samples were collected just prior to euthanasia (under ketamine/xylazine anesthesia), by placement of a butterfly catheter in the descending abdominal aorta of each rat. Blood (5 mL) was collected into a pre-citrated 5-mL syringe (0.5 mL of 3.2% sodium citrate) and transferred to a centrifuge tube. Citrated blood was centrifuged at 750 rpm for 15 minutes at 2–8 °C. The blood was again centrifuged at 3600 rpm for an additional 10 minutes at 2–8 °C. Plasma was removed, and the remaining red blood cells were frozen and stored at –80 °C until shipment. The RBCs were shipped on dry ice to South Dakota State University where they were stored at –80 °C until ready for analysis.

2.3. Sample preparation

RBCs (3 mL) were added to a 15-mL centrifuge tube. (Note: Although 3 mL of RBC lysate was used to produce enough protein for triplicate analysis, only 700–800 µL of RBC lysate was typically necessary to provide 200 mg of protein.) Acetone with 1% HCl (v/v), was added to the RBCs with a 3:1 volume ratio of acetone to RBC. The mixture was vortexed, and then centrifuged at 800 x g for 10 mins at 20 °C. The supernatant was discarded. The precipitate was washed once by adding 6 mL of acetone and breaking the pellet with a clean spatula. The mixture was again centrifuged, as above, and the supernatant was discarded. The precipitate was dried in a centrifugal evaporator (Labconco, Kansas City, USA) equipped with a rotary vacuum pump (Edwards, Glenwillow, USA) at 30 °C until dry. If not analyzed immediately, the precipitate was stored at –80 °C until ready for use. If the precipitate was stored at –80 °C, it was thawed, unassisted, at room temperature prior to continuing sample preparation.

A portion of the precipitate (200 mg) was added to a clean 15 mL centrifuge tube. A mixture of 1:1 (v/v) hydrochloric acid and acetic acid (1.5 mL total volume) was added to the precipitate to produce MIH from MIC-adducted protein. The centrifuge tube was capped and heated at 110 °C for 1.3 hours. The sample was then removed from heat and cooled to room temperature. Aqueous NaOH (1.4 mL of 10 M) was added to neutralize the acids. Prior to finalizing the method, multiple combinations of NaOH concentration and volume were tested for their ability to adjust the pH of the solution to 3–5 and 1.4 mL of 10 M NaOH was found to consistently produce the desired pH. After pH adjustment, ethyl acetate (1.5 mL)

was added to the vial to extract the MIH. The sample was capped and shaken vigorously for 1.5 minutes, then left on the bench top for 15 mins. The sample was centrifuged at 2000 x g for 5 mins and the upper organic layer was transferred to a new 15-mL centrifuge tube. The ethyl acetate was washed by adding 2.5 mL of 1 M aqueous NaHCO₃ to neutralize residual base in the ethyl acetate. Following addition of NaHCO₃, the vial was shaken for 1.5 mins, stored at room temperature for 15 mins, and centrifuged at 2000 x g for 5 mins. An aliquot of the organic layer (approximately 1 mL) was then transferred to a 2 mL GC vial and the vial was capped with a Teflon-coated septa screw-cap prior to GCMS analysis.

2.4. GC–MS analysis of MIH

The extracted MIH was analyzed using an Agilent GC–MS system equipped with a 6890N gas chromatograph and a 5975B inert XL electron ionization (EI)/chemical ionization (CI) mass selective detector. The sample (1 µL) was injected into a heated injection port at 250 °C in splitless mode, and two minutes after injection, the split vent was opened at a flow rate of 50 mL/min. The compound was ionized using EI at 70 eV. A DB-5MS bonded phase column (30 m x 0.25 mm I.D., 0.25 m film thickness; J & W Scientific, Santa Clara, CA) was used. Hydrogen, produced by a hydrogen generator (Parker Manufacturing LTD; Gateshead, United Kingdom), was used as the carrier gas at a flow rate of 1.5 mL/min. The initial oven temperature was set at 70 °C for 1 min, and then increased at 50 °C/min to 150 °C and held for 1 minute. The temperature was then increased to 180 °C at 20 °C/min, and finally increased to 250 °C at 100 °C/min and held for 3 mins. The temperatures of the MS transfer line, the source, and the quadrupole were 250, 230, and 150 °C, respectively. Selected ion mode (SIM) was used to quantify MIH for the fragments shown in Figure 1 using 114 m/z ([C₄H₆N₂O₂]⁺, quantification) and 156 m/z (C₇H₁₂N₂O₂]⁺, identification). The ions monitored for the internal standard were m/z ([¹²C₂¹³C₂H₆¹⁴N¹⁵NO₂]⁺, quantification) and 162 m/z ([¹³C₅¹²C₂H₁₂¹⁴N¹⁵NO₂]⁺, identification). Each ion was monitored with a dwell time of 100 ms and the solvent delay was 2.85 minutes.

2.5. Calibration, quantification, and limit of detection

Validation of the method was performed by generally following Food and Drug Administration guidelines (33–35). To determine the limit of detection (LOD), MIH stock solution (1 g/L) was diluted in water to create standards of 0.02, 0.05, 0.1, 0.2, 0.5, 1, 10, and 100 mg/L. A standard solution was spiked (50 µL) into a vial with 200 mg RBC precipitate prepared from rats which were not exposed to MIC. The spiked rat RBC precipitate was then prepared as described above via acid hydrolysis and extraction with ethyl acetate. The extracted MIH was then analyzed via GCMS. The lowest MIH concentration that reproducibly produced a signal-to-noise ratio of 3 was defined as the LOD for MIH. Noise was measured by averaging the peak-to-peak noise in prepared, unspiked RBC samples over the retention time of the analyte.

After the LOD was determined, the calibration curve dynamic range was established. For the calibrators, rat RBC precipitate (200 mg) was spiked with 100 µL of a mixture of MIH internal standard (50 µL, 10 mg/L) and MIH (50 µL) at concentrations ranging from the 0.05 to 25 mg/kg RBC precipitate. Each sample was prepared in triplicate. The average peak area ratios of MIH to IS were plotted as a function of MIH concentration to determine the

linearity, accuracy, and precision of the calibration. Peak areas were calculated by manual integration from baseline to baseline. The accuracy was quantified as the ratio of the back-calculated concentration to the nominal concentration for all calibration standards. The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were selected as the lowest and highest concentrations with less than 15% relative standard deviation (as a measure of precision) and with $100 \pm 15\%$ accuracy.

After the linear dynamic range was established, the accuracy and precision of the method was evaluated over three days (within 10 calendar days). Calibration standards within the linear dynamic range were created in triplicate and QC standards of 0.188 mg/kg (low QC), 1.88 mg/kg (medium QC), and 8.75 mg/kg (high QC) were prepared in quintuplicate. Calibration curves were created each day and used to calculate the concentration of each QC standard. QCs were not used as part of the calibration curve. The intraassay (within same day) and interassay (over three separate days) accuracy and precision for the developed method were determined from the QCs.

2.6. Selectivity and stability

The ability to differentiate and quantify MIH in the presence of other RBC protein components following sample preparation (assay selectivity) was determined by comparing unspiked and MIH-spiked blood RBC proteins. The absence of signals above the baseline of the blank over the elution time of MIH was indicative of high selectivity. Resolution of the MIH peak was also calculated as a measure of selectivity by standard methods (36).

To evaluate the stability of MIH or hemoglobin-MIC adducts (Hb-MIC) over the short and long-term, the following experiments were performed: auto-sampler stability, bench top stability, and long-term stability at -80°C . Auto-sampler stability was carried out by spiking RBC precipitate with MIH and preparing for analysis. The samples were placed on the auto-sampler (at ambient temperature) and analyzed at approximately 0, 1.3, 2.6, 5, 15, and 24 h following preparation. Internal standard was not used for the auto-sampler stability experiment, as it would correct for the loss of MIH during the storage.

The benchtop stability of the MIC-protein adduct was evaluated by initially spiking MIC in RBCs at levels which produced MIH signals generally equivalent to the low and high QCs: $1.61\text{ }\mu\text{mol/g}$ and $80.9\text{ }\mu\text{mol/g}$, respectively. The MIC-spiked rat RBC samples were vortexed for 1 min and shaken overnight in a Benchmark INCU-SHAKER™ 10-L Shaking Incubator at 290 rpm and room temperature. The samples were then stored on the bench top for 0, 2, 5, 12, and 24 hours. They were then precipitated, dried, and stored in -80°C freezer until all the protein samples were ready for analysis. Prior to analysis, the samples were removed from the freezer and allowed to reach room temperature. The remaining sample preparation and analysis steps described above were then completed. The experiment for the long-term stability of the MIC-protein adduct was similar to the bench-top stability experiment, except MIC-spiked RBCs were stored at -80°C and 4°C for 0, 1, 2, 5, 15, and 30 days before they were prepared using the developed method.

2.7. Recovery and matrix effect

To determine the recovery of MIH, a set of samples were prepared in ethyl acetate which contained the amount of MIH representative of 100% recovery. Specifically, triplicates of low, medium, and high QC concentrations in MeOH (50 μ L) were dried in air, then 1.5 mL ethyl acetate was added and vortexed to dissolve the MIH prior to GCMS analysis. These samples were analyzed alongside a set of low, medium, and high MIH QCs prepared in rat RBC precipitate prepared by the current method. Following GCMS analysis, the recovery (i.e., signal recovery) for each QC was quantified as the average percentage of the peak areas of the MIH-spiked RBC samples to that of the corresponding MIH QCs in ethyl acetate. It is important to note that the recovered signal obtained in this manner is influenced by both matrix effects and recovery.

To evaluate the matrix effect of the RBC precipitate on the analysis of MIH, calibration curves of MIH in both aqueous solution and RBC-precipitate were established. The samples were prepared using the identical procedures as described above, except the MIH and MIH internal standard were added directly to the acetic acid:HCl mixture with no RBC precipitate for the non-matrix calibration standards. The ratio of the slopes of the calibration curves in RBC-precipitate compared to the slope obtained for aqueous calibration standards was used to quantify the matrix effect.

3. Results and discussion

3.1. GC–MS analysis of MIH

The method presented here shortens the time needed for analyzing MIC exposed RBCs from 7 hours and over 18 steps for the methods listed in Table 1 to 4–5 hours and 14 steps. The steps associated with the Kafferlein et al. (27) method are shown in Figure 2 for comparison with the method presented here (Figure 3). Both the Kafferlein et al. (27) method and the method presented here were conducted for the same samples in our lab and the analytical parameters achieved for the current method were comparable, but our method was much more rapid, simpler, and greener.

The main highlights of the current method and most methods listed in Table 1 (15, 26–28) are the same. RBCs are precipitated and hydrolyzed via treatment with strong acid to produce MIH from MIC adducted N-terminal valine. LLE with ethyl acetate is then used to extract the MIH from the aqueous solution. The extract is then analyzed by GC-FID (15), GC-NPD (31), GC-MS (26–28), HPLC-Diode Array Detector (DAD) (30), or UHPLC-MS-MS (29). Although the major features are the same, there are many differences in the current method that simplify the analysis of MIH. First, during precipitation, both protein and cell debris are simultaneously precipitated, whereas in most other methods, cell debris is precipitated first, then proteins in the supernatant are precipitated. The simplification of this process did not affect the precision and accuracy of the current method, but greatly reduced the preparation time and effort. After the RBC precipitate is dried and weighed, it is hydrolyzed by a mixture of HCl and acetic acid. This resulting solution is highly acidic. Some methods prepare this solution via several lengthy steps, including addition of NaOH to adjust the pH to 7, addition of saturated NaCl, and adjustment of the pH to 3–5 using HCl.

In the simplified method, the pH of the solution was adjusted to 3 to 5 directly by adding a constant volume of 10 M aqueous NaOH to each sample. In the Kafferlein et al. (27) method, the solution was extracted twice using ethyl acetate (4 mL each), with the solvent subsequently evaporated to approximately 3 mL. In the simplified method, the neutralization and extraction of the hydrolyzed solution were accomplished in one step by adding NaOH and ethyl acetate to the vial at the same time, with no subsequent drying step. For the previous and the current method, the organic phase was further neutralized with sodium bicarbonate before GCMS analysis. Most previous methods further centrifuged the resulting solution, transferred the supernatant to a clean vial, and evaporated it to dryness using nitrogen gas. The residue was then dissolved in ethyl acetate (1 mL) for GCMS analysis.

As outlined above, the current method does not include drying, which greatly reduces the analysis time, energy use, and waste since ethyl acetate has a relatively high boiling point. The omission of the drying step did not significantly affect the precision, accuracy, or sensitivity (LOD) of the current method. In addition to reducing the time and complexity of the analysis, the current method produces less chemical waste than most other methods. For example, the chemicals needed for the Kafferlein et al. (27) method were 2-propanol, ethanol, ethyl acetate, acetic acid, n-hexane, sodium chloride, sodium hydroxide, sodium bicarbonate, and HCl (10 total). The simplified method requires six (acetone, ethyl acetate, acetic acid, sodium hydroxide, sodium bicarbonate, and HCl) and eliminates the most toxic organic chemical used, n-hexane. Furthermore, the amount of chemical waste is drastically reduced by both eliminating the requirement for solvent drying and reducing the extraction and washing steps necessary.

For the current method, the isolated MIH was analyzed via GCMS to produce the chromatograms represented in Figure 4, with MIH eluting at approximately 4.18 min. The method produced adequate peak shape, although the MIH peak tailed slightly (i.e., the peak asymmetry factor, A_s , was 2.18). The selectivity was good, although there was a small peak at approximately 4.2 min in the non-MIC-exposed rat RBCs, likely due to endogenous N-methylcarbamoylated valine (37–39). The closest consistent peak eluted at about 4.4 min, producing a resolution (R_s) of 3.56.

3.2 Endogenous MIH

Endogenous levels of N-methyl carbamoylated N-terminal valine residues in Hb are known to be present (37–39). Therefore, in order to assess potential interference from MIH not associated with MIC exposure, RBCs from different animals (rabbit, swine, and rat) were evaluated for MIH produced during sample preparation. The MIH produced in the animals evaluated followed the trend: rabbit RBCs >> swine RBCs > rat RBCs. Although the MIH produced from swine RBCs was much less than that from rabbit RBCs, it was still significant enough to affect determination of low concentrations of MIH. Therefore, rat RBC was chosen as the matrix to validate the developed method.

3.3. LOD, dynamic range, and sensitivity

Following method optimization, the LOD of MIH was evaluated at a S/N of 3. The LOD was 0.05 mg/kg RBC precipitate), which compared favorably to the other methods listed in

Table 1, only being higher than the Mráz et al. (28) method. There was a small rise in baseline around the retention time of MIH for the naïve samples, which was likely due to endogenous carbamylation of the N-terminal valine, but even considering this as part of the noise, the LOD was still excellent. Following the determination of the LOD, the calibration curve of MIH was established between 0.05 and 25 mg/kg RBC precipitate. The calibration curve was determined using a $1/x^2$ weighted fit of the data (the ratio of the peak area of MIH to MIH IS versus the calibration standard concentration). The R^2 was 0.993, and residual analysis indicated that the linearity was acceptable with no obvious concentration bias. The true ULOQ was not determined since the calibration curve was linear up to the highest concentration tested. This method produced a large dynamic range of almost 3 orders of magnitude for measuring MIH, which is typically considered excellent for analysis of biological samples (40–42).

The sensitivity and calibration repeatability were evaluated over three separate days. The results are shown in Table 2. Each of the three calibration curves produced an $R^2 > 0.999$ and residual analysis indicated no consistent bias in the calibration curves for the 3 days. Although the linearity was consistently excellent, the slopes for each day were not consistent. Therefore, calibration curves should be created for each day of analysis. Evaluation of the peak shape and sensitivity of the method over several months for MIH showed no deleterious effects of multiple injections of the final sample solution.

3.3. Accuracy and precision

The accuracy and precision of the method were estimated by analysis of quintuplicate low, medium and high QCs on three different days (within a 10-day period) and are summarized in Table 3. The intraassay accuracy and precision were $100 \pm 8.0\%$ and $<8.1\%$ RSD, respectively. The interassay accuracy and precision were $100 \pm 5.7\%$ and $<4.5\%$ RSD, respectively. Evaluation of the aggregate data according to Krouwer and Rabinowitz (43) indicates that the within run precision is 6.4, 4.6, and 3.4% RSD for the low, medium and high QC standards, respectively, with total %RSDs, for the method, of 6.8, 5.4, and 3.7% RSD for the low medium and high QC standards, respectively. These values indicate the precision and accuracy of the method are excellent and are well within the FDA method validation guidelines (35). Although most methods from Table 1 do not report accuracy and precision, Mráz et al. (28) reported a precision between 4.1–7.3% for intraassay precision and approximately 10% for interassay precision. Mráz et al. (28) did not report accuracy since they did not utilize spiked MIH to simulate the “true” concentration as we did here. Käßerlein et al. (27) reported slightly worse precision than the current method: intraassay precision of 4.4–14.2% and interassay precision of 4.9–8.9%. They also reported an accuracy of $100 \pm 7\%$, which is very similar to the current method.

3.4. Matrix effects and recovery

To evaluate the effect of the RBC matrix on the analysis of MIH, calibration curves of MIH in both aqueous solution and RBC matrix were compared. The slope of the calibration curve for the samples in RBC matrix was higher than that for the aqueous samples (15800 kg/mg versus 10700 kg/mg, respectively). Because this behavior is unusual for biological matrices, the experiment was repeated, and the results were confirmed. Moreover, the experiment was

repeated by spiking extracted RBC matrix with MIH and measuring the slope of the calibration curve. The calibration curve slope in RBC matrix was (20600 kg/mg). The enhancement due to the matrix may be due to a “salting out” effect when the RBC protein precipitate is hydrolyzed, creating a large amount of charged amino acids and peptides (44, 45). Since the method presented does not include the addition of NaCl, as with the Kafferlein et al. (27) method, the hydrolyzed RBC proteins may increase the extraction efficiency of the ethyl acetate for MIH as compared to the aqueous solution by salting out the MIH. It is not clear as to why spiking the MIH prior to sample preparation and following sample preparation produces a difference in the slope, but it is clear that the matrix increases the signal of MIH for this method.

The recoveries for low, medium and high QCs were 88%, 96%, and 98%, respectively. The recovery was excellent for the mid and high QCs, and relatively high for the low QC, although each was below 100%. The loss of MIH during the sample preparation steps is likely due to incomplete extraction of MIH from the blood matrix into the ethyl acetate. This is corroborated, at least partially, by the increase in slope for the RBC matrix during the matrix effect experiment. Comparing to other methods reporting recovery, Ramachandran et al. (15) and Kafferlein et al. (27) reported a recovery of 91% and >93%, respectively. Therefore, the recovery of the current method is comparable to previous methods.

3.5. MIH and MIC protein adduct storage stability.

To determine the stability of the prepared samples on an auto-sampler, LQC and HQC standards were prepared and spiked into rat RBC precipitate (50 μ L to 200 mg) along with 50 μ L of 10 mg/L MIH IS solution. After the samples went through the sample preparation procedure, they were placed on the auto-sampler and were analyzed at 0, 1.3, 2.6, 5, 15, and 24 hours. The MIH signals were stable for the tested period of 24 hours. The MIH internal standard also corrected for raw signal instability.

We also tested the stability of the N-methyl carbamoylated hemoglobin by spiking MIC directly onto isolated hemoglobin, incubating, washing, and drying the protein. Analysis of MIH following storage on the bench top for different time periods up to 30 days showed that the adduct was stable for all time periods tested. Mraz et al. (28) also tested the stability of the adduct in blood and after isolation of the globin and found similar results, in that the adduct was stable for all conditions tested (i.e., up to 21 days in blood, and up to 2 years as an Hb adduct when stored at 4 $^{\circ}$ C).

3.6. Evaluation of MIH correlation with MIC exposure

Correlation of MIH to MIC exposure was first evaluated by spiking multiple concentrations of MIC onto hemoglobin and incubating for 24 hours. The resulting hemoglobin was analyzed for MIH. The MIH signal correlated linearly with MIC exposure over the concentration range of 0.08–4.1 mmol (Figure 5) but was generally constant over the concentration range of 1.6–82 μ mol. This is likely due to the use of rabbit RBCs producing a large and consistent endogenous MIH concentration.

The MIH was then analyzed for multiple exposure concentrations of MIC (125, 250, and 500 ppm) for rats (N = 3 at each concentration). Although the actual dose of MIC cannot be

definitively measured, the relationship shown in Figure 6 is very promising for the potential to use MIH to document exposure and to estimate MIC internal dose. More animals and MIC concentrations must be evaluated to gain a true understanding of the correlation between MIH produced and MIC exposure, but the correlations derived from Figures 5 and 6 show the potential of this analytical technique to determine the inhalation exposure of individual animals to MIC.

Conclusion

A rapid, greener, and simpler GC-MS method for analysis of MIH isolated from N-methyl carbamoylated hemoglobin was developed and validated. This method is promising for quantifying the internal dose of MIC from inhalation studies. The availability of this method may allow better estimation of the relative exposure level and/or MIC internal dose than external parameters, such as tidal volume, minute volume, and concentration of MIC in the exposure system.

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Figure 1.

Reaction of methyl isocyanate with the N-terminal valine of hemoglobin to produce a carbamoylated hemoglobin. The carbamoylated hemoglobin is hydrolyzed by Edman degradation to form N-(methylcarbamoyl)valine which cyclizes under acidic conditions to form MIH for subsequent analysis. Structures from electron ionization fragmentation and the resulting mass spectrum are also shown. Asterisks denote the locations of the stable isotopes in the internal standard for each of the fragments.

Figure 2.

Schematic representation of the sample preparation procedure of the Kafferlein et al. (27) method. This method takes at least two days and over 37 steps to prepare blood for analysis of MIH from MIC-adducted hemoglobin. EA – Ethyl Acetate.

Figure 3.

Schematic representation of the sample preparation procedure used for the current study.

This method takes at about 4–5 hours and 14 steps to prepare blood for analysis of MIH from MIC-adducted hemoglobin.

Figure 4. SIM mode GCMS chromatograms with selected m/z of 114 of MIH produced via Edman degradation from MIC spiked rat RBCs, RBCs from rats exposed to MIC, MIH spiked rat RBCs, and the non-MIC exposed rat RBCs.

Figure 5.

MIH signal as a function of spiked MIC concentration in rabbit RBCs. The error bars represent standard deviation. Note that a higher concentration of internal standard was used with this experiment than used for the final method.

Figure 6.

Correlation of MIH concentration with MIC concentration during an inhalation exposure in ppm. The error bars represent standard error of the mean for the analysis of MIH isolated from rat hemoglobin for each exposure group (N = 3 for each dose).

Table 1.

Comparison of methods of analysis for methyl isopropyl hydantoin produced from Edman degradation of carbamylation of the N-terminal valine of hemoglobin.

Reference	Year	Analytical Method	LOD	Approximate Estimated Time (hr)	Steps
Ramachandran et al. (15)	1988	EI-GC-FID	0.0094 mg/L lysate ^b	20 ^c	22
Venkateswaran et al. (30) ^a	1992	HPLC-DAD (200 nm)	0.016 mg/L lysate ^b	20 ^c	22
Angerer et al. (26) ^a	1998	EI-GC-MS	0.30 mg/kg	20 ^c	22
Mráz et al. (28)	2002	EI-GC-MS	0.031 mg/kg	9	20
Mráz et al. (31) ^e	2006	EI-GC-NPD	0.16 mg/kg	9	20
Käfferlein et al. (27)	2009	EI-GC-MS	0.078 mg/kg	60 ^d	>37
Wang et al. (29)	2014	UHPLC-MS-MS	1.6 mg/kg	7,19 ^c	18

^aSame sample preparation as Ramachandran et al. (15).

^bDid not report the amount of red blood cell precipitate mass per mL of lysate.

^cIncludes an overnight drying step.

^dIncludes two overnight steps.

^eSame sample preparation as Mráz et al. (28).

Table 2.

Calibration equations and coefficients of determination (R^2) for calibration curves created over 3 days.

Day	Equation	R^2
1	$y = 0.09679x + 0.006831$	1
2	$y = 0.08767x + 0.01078$	0.9996
3	$y = 0.08252x + 0.008578$	0.9999

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Table 3.

Intra and interassay accuracy and precision of MIH produced by acid hydrolysis of an MIC-protein adduct.

Concentration (mg/kg)	Intraassay (within run)						Interassay (between run)	
	Accuracy (%) ^a			Precision (%RSD) ^a			Accuracy (%) ^b	Precision (%RSD) ^c
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3		
0.188	100±1.8	100±3.9	100±2.1	3.6	6.3	8.1	100±2.6	2.3
1.88	100±7.1	100±1.4	100±0.9	5.3	4.9	3.1	100±3.1	2.9
8.75	100±8.0	100±4.1	100±5.1	3.7	2.3	3.9	100±5.7	1.4

^aQC method validation (N = 5).

^bMean of three different days of QC method validation (N = 15).

^cBetween run precision was calculated based on Krouwer and Rabinowitz (43) (N = 15).