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Chem. Res. Toxicol., **Just Accepted Manuscript** • DOI: 10.1021/acs.chemrestox.6b00120 • Publication Date (Web): 07 Jul 2016

ACS Publications

Chemical Research in Toxicology is published by the American Chemical Society.
1155 Sixteenth Street N.W., Washington, DC 20036

Pyrrolizidine Alkaloid-Protein Adducts - Potential Non-Invasive Biomarkers of Pyrrolizidine Alkaloid-Induced Liver Toxicity and Exposure

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Running Title: Blood Protein Adducts from Pyrrolizidine Alkaloids as a Biomarker

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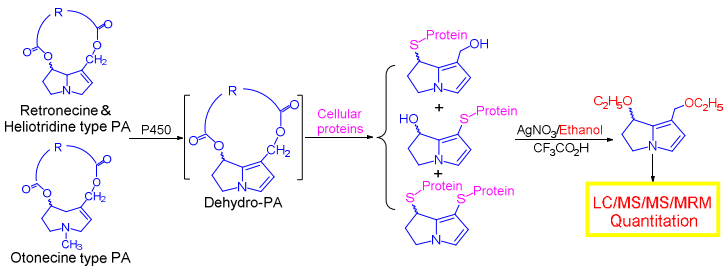

ABSTRACT:

Pyrrolizidine alkaloids (PAs) are phytochemicals present in hundreds of plant species from different families widely distributed in many geographical regions around the world. PA-containing plants are probably the most common type of poisonous plants affecting livestock, wildlife, and humans. There have been many large-scale human poisonings caused by the consumption of food contaminated with toxic PAs. PAs require metabolic activation to generate pyrrolic metabolites to exert their toxicity. In this study, we developed a novel method to quantify pyrrole-protein adducts present in the blood. This method involves the use of $AgNO_3$ in acidic ethanol to cleave the thiol linkage of pyrrole-protein (DHP-protein) adducts, and the resulting 7,9-di-C_2H_5O-DHP is quantified by HPLC-ES-MS/MS multiple reaction monitoring analysis in the presence of a known quantity of isotopically-labeled 7,9-di-C_2D_5O-DHP internal standard. Using this method, we determined that diester-type PAs administered to rats produced higher levels of DHP-protein adducts than other types of PAs. The results suggest that DHP-protein adducts can potentially serve as a minimally invasive biomarker of PA exposure.

Keywords: Pyrrolizidine alkaloid; DHP; DHP-protein adducts; HPLC-ES-MS/MS.

■ INTRODUCTION

Pyrrolizidine alkaloids (PAs) are common secondary phytochemical metabolites of hundreds of plant species from different families that are widely distributed in many geographical regions around the world.[1-11] To date, more than 660 structurally different PAs and PA *N*-oxides have been identified in over 6,000 plants worldwide and about half of them are hepatotoxic.[2, 8-10] About 3 - 5% of the flowering plants in the world contain toxic PAs.[11,12] PA-containing plants are the most common type of poisonous plants affecting livestock, wildlife, and humans.[2, 5, 8, 13-16] Humans are exposed to toxic PAs through the consumption of contaminated food staples, herbal medicines, herbal dietary supplements, teas, herbal teas, honey, and milk.[1, 6, 8, 18-21] There have been many large scale human poisonings in many countries caused by the intake of food contaminated with toxic PAs.[7, 8, 17, 20,22]

PAs and PA *N*-oxides that contain a double bond at the C1 and C2 positions of the necine base (Figure 1) are toxic. These PAs undergo metabolic activation to generate

dehydropyrrolizidine alkaloids (dehydro-PAs) that react with cellular proteins and DNA, leading to hepatotoxicity, genotoxicity, and tumorigenicity.[5, 7, 8, 15, 21, 23-29] Acute poisoning causes massive hepatotoxicity, including haemorrhagic necrosis, hepatomegaly, ascites, and endothelial proliferation.[7, 8, 19] Further liver damage can lead to occlusion of hepatic veins, resulting in veno-occlusion disease, also called hepatic sinusoidal obstruction syndrome, which is a characteristic histological sign of PA-induced poisoning.[1, 7, 8, 19] PA-induced DNA damage includes DNA strand breakage, unscheduled DNA synthesis, DNA-DNA cross-linking, DNA-protein cross-linking, and DNA adduct formation.[2, 5, 7, 8, 15, 24-26, 28, 30-42]

We recently reported that riddelliine, a representative tumorigenic PA, induces liver tumors through a genotoxic mechanism mediated by covalent binding of the pyrrolic metabolite, dehydroriddelliine, to cellular DNA to form a set of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP)-derived DNA adducts.[2, 15, 16] The DHP-DNA adduct levels correlated closely with the riddelliine-induced tumorigenic potencies in the rats fed with riddelliine; also, the metabolic pattern and DNA adduct profiles are highly relevant to humans.[2, 5, 39] Our further mechanistic studies demonstrated that different types of hepatotumorigenic PAs generated the same set of DHP-derived DNA adducts (designated as DHP-dG-3, DHP-dG-4, DHP-dA-3, and DHP-dA-4), but these DNA adducts were not formed from non-hepatotumorigenic PAs (Figure 1).[15] These results indicate that this set of DNA adducts can serve as a common biological biomarker of PA tumorigenicity and exposure.

Pyrrole-protein (DHP-protein) adducts have long been considered to be responsible for PA-induced hepatotoxicity, and can be used as biomarkers of toxic PAs exposure.[1, 8] Thus, an important goal has been to develop accurate and sensitive analytical methods to detect and quantify DHP-protein adducts *in vivo*. In the early 1990's, Mattocks and co-workers developed an analytical method to detect DHP-protein adducts in the blood and liver of rodents, as well as in livestock.[43-46] Briefly, blood or liver samples were reacted with silver nitrate in acidic ethanol to cleave the thiol linkage of DHP-protein adducts at the C7 and C9 positions, leading to the protein moiety being replaced by ethoxyl groups. The resulting 7,9-di-C_2H_5O-DHP was then identified by GC/MS measurements or by a colorimetric test by using Ehrlich's reagent, 4-dimethylaminobenzaldehyde (DABA), to form a 7,9-di-C_2H_5O-DHP-DABA product that has an intense yellow color.[47] In 2011, Lin and co-workers used a similar approach, coupled with

LC/MS/MS analysis, to detect DHP-protein adducts in patients who suffered from hepatic sinusoidal obstruction syndrome due to the consumption of the PA-containing herbal plant *Gynura segetum*.[48] This represented the first time that DHP-protein adducts had been detected in human blood samples. This method was further modified, by using 7,9-diglutathionyl-DHP (7,9-di-GS-DHP) to construct a calibration curve to quantify the 7,9-di-C_2H_5O-DHP-DABA derived from DHP-protein adducts in the blood of the 15 liver-injured patients who ingested *Gynura segetum.*[49]

In the present study, we report the development of a method to quantify DHP-protein adducts formed *in vivo*. This method involves: (i) following the experimental procedure of Mattocks and Jukes,[45] treatment of blood samples with silver nitrate in acidic ethanol to convert DHP-protein adducts into 7,9-di-C_2H_5O-DHP; and (ii) utilizing a synthetically prepared isotope-labeled 7,9-di-C_2D_5O-DHP as an internal standard, with quantification being conducted by LC/MS/MS multiple reaction monitoring (MRM) analysis. The method was then applied to quantify the DHP-protein adducts formed *in vivo* in two separate animal experiments: (i) female rats orally gavaged with different concentrations of riddelliine for 5 or 30 consecutive days; and (ii) a series of PAs (Figure 1)[15] orally gavaged to female rats for 3 consecutive days. This quantitation study indicates that diester-type PAs (Figure 1) produced higher levels of DHP-protein adducts than other types of PAs. These results indicate that the developed HPLC-ES-MS/MS method is accurate and precise for the detection and quantitation of DHP-protein adducts in the blood and suggest that DHP-protein adducts can potentially serve as minimally invasive biomarkers of PA exposure.

■ EXPERIMENTAL PROCEDURES

Caution: *Riddelliine, retrorsine, monocrotaline, lycopsamine, retronecine, lasiocarpine,* clivorine, *senkirkine, heliotrine, and riddelliine N-oxide are carcinogenic in laboratory animals. They should be handled with extreme care, using proper personal protective equipment and a well-ventilated hood.*

Chemicals. Monocrotaline, silver nitrate, trifluoroacetic acid, formic acid, sodium bicarbonate, diethyl ether, heparin, deuterated ethanol (99.5%), Protein Quantification Kit, and

Hemoglobin Assay Kit were purchased from the Sigma-Aldrich (St. Louis, MO). Absolute ethanol (200 proof) was purchased from AAPER Alcohol and Chemical Co. (Shelbyville, KY). Riddelliine was obtained from Dr. Po-Chan, National Toxicology Program, National Institute of Environmental Health Sciences (Research Triangle Park, NC). Dehydromonocrotaline was synthesized from the reduction of monocrotaline by *o*-chloroanil in chloroform as described previously.[50] All chemicals used for the animal studies were analyzed by HPLC and found to be >97% pure. All solvents used were HPLC grade.

Synthesis of 7,9-Di-C_2H_5O-DHP. 7,9-Di-C_2H_5O-DHP was synthesized following the method of Mattocks and Jukes[43] with modification. Briefly, absolute C_2H_5OH (20 mL) was added to dehydromonocrotaline (200 mg, 0.62 mmol), and the reaction mixture was stirred for 24 hr at ambient temperature. To the reaction mixture was added 10 mL 8% sodium bicarbonate solution, the ethanol was removed under reduced pressure, and the remaining material was extracted with diethyl ether (20 mL) three times. The ether extracts were combined and the solvent was removed under reduced pressure, resulting in practically pure 7,9-di-C_2H_5O-DHP (85 mg; 65.7% yield).

The 7,9-di-C_2H_5O-DHP was further purified by reversed phase HPLC (a Waters HPLC system consisting of a Model 600 controller, a Model 996 photodiode array detector, and a 600 pump) (Milford, MA), using a Phenomenex Luna C18 (2) column (250 x 4.6 mm) (Phenomenex, Inc. CA) by eluting with acetonitrile at 800 µL/min and monitoring at 220 nm (Figure S1 in the Supporting Information). The product, which eluted at 4.3 min, was collected and identified as 7,9-di-C_2H_5O-DHP by MS and ^{1}H NMR spectral analyses.

The ^{1}H NMR spectral assignments of 7,9-di-C_2H_5O-DHP in acetone-d_6 are: δ 1.15 (3H, t, CH_3, $J_{CH2\text{-}CH3}$ = 7 Hz), 1.16 (3H, t, CH_3, $J_{CH2\text{-}CH3}$ = 7 Hz), 2.46 (1H, m, H6b), 2.71 (1H, m, H6a), 3.45 (4H, m, CH_2), 3.62 (4H, m, CH_2), 3.93 (1H, m, H5b), 4.07 (1H, m, H5a), 4.35 (1H, d, H9b, $J_{9a,9b}$ = 11.4 Hz), 4.41 (1H, d, H9a, $J_{9a,9b}$ = 11.4 Hz), 4.83 (1H, dd, H7, $J_{6,7}$ = 6.1 and $J_{5,7}$ = 1.6 Hz), 6.12 (H, d, H2, $J_{2,3}$ = 2.7 Hz), and 6.63 (1H, d, H3, $J_{2,3}$ = 2.7 Hz).

The 7,9-di-C_2H_5O-DHP was stable in petroleum ether, diethyl ether, acetone, acetonitrile, and ethanol, but unstable in dichloromethane and chloroform.

Synthesis of Isotopically Labeled 7,9-Di-C_2D_5O-DHP Adduct. Isotopically labeled 7,9-di-C_2D_5O-DHP was similarly synthesized by the reaction of dehydromonocrotaline (20 mg) with 2 mL of C_2D_5OH. After the reaction, the7,9-di-C_2D_5O-DHP was purified by reversed phase HPLC using the conditions described above. Its structure was determined based on analysis of its HPLC retention time, UV-visible spectrum, mass, and ^{1}H NMR spectra. Its ^{1}H NMR spectrum is shown in Figure S2 in the Supporting Information.

The ^{1}H NMR spectral assignments of 7,9-di-C_2D_5O-DHP in acetone-d_6 are: δ 2.46 (1H, m, H6b), 2.71 (1H, m, H6a), 3.93 (1H, m, H5b), 4.07 (1H, m, H5a), 4.35 (1H, d, H9b, $J_{9a,9b}$ = 11.4 Hz), 4.41 (1H, d, H9a, $J_{9a,9b}$ = 11.4 Hz), 4.83 (1H, dd, H7, $J_{6,7}$ = 6.1 and $J_{5,7}$ = 1.6 Hz), 6.12 (H, d, H2, $J_{2,3}$ = 2.7 Hz), and 6.63 (1H, d, H3, $J_{2,3}$ = 2.7 Hz).

Treatment of Rats with Pyrrolizidine Alkaloids. Procedures involving care and handling of rats were reviewed and approved by the National Center for Toxicological Research (NCTR) Laboratory Animal Care and Use Committee. Female F344 rats (8-10 weeks of age) were obtained from the NCTR breeding colony and maintained on a 12-hr light-dark cycle. There were two separate sets of animal studies.

1. Treatment of rats with different doses of riddelliine - Female rats (4 per group) were orally gavaged for 5 or 30 consecutive days at daily doses of 0, 0.1, 1, 2, or 5 mg riddelliine/kg body weight (bw) in 2.5 mL of 0.1 M sodium phosphate (pH 7.4). Twenty-four hr after the last dose, the rats were sacrificed by exposure to CO_2, followed by exsanguination. The livers were removed, rinsed with cold saline, and stored at -70 ºC before isolation of DNA for DNA adducts analyses. Blood samples were collected by cardiac puncture into heparin treated tubes. After centrifugation, each sample was separated into two fractions, the supernatant (plasma fraction) and precipitated blood cells, the latter of which contained mainly erythrocytes and was designated the hemoglobin fraction. All blood samples were stored at -70 ºC before protein adducts analyses.
2. Treatment of rats with eleven individual pyrrolizidine alkaloids and derivatives – As reported previously,[15] riddelliine, retrorsine, monocrotaline, lycopsamine, retrornecine, riddelliine *N*-oxide, lasiocarpine, heliotrine, senkirkine, clivorine, and platyphylliine (a mixture of 70% platyphylliine and 30% neoplatphylliine) were orally gavaged to female rats

(4 per group) for 3 consecutive days, at daily doses of 24 (high dose) and 4.5 (low dose) μmol/kg bw in 500 μL of 10% DMSO in water. Rats for vehicle control group were treated with 500 μL of 10% DMSO in water. Twenty-four hr after the last dose, the rats were sacrificed by exposure to CO_2 followed by exsanguination.[15] Liver and blood samples were collected and stored as described above. The DNA adducts in the liver of these rats were identified and quantified by LC/MS/MS analysis; these results were published previously.[15]

Reaction of DHP-Protein Adducts in Blood of Rats with Silver Nitrate in Alcohol. The reaction of silver nitrate in alcohol with DHP-protein adducts contained in the blood samples was assessed by using many different experimental conditions, including different pHs, solvents (ethanol and methanol), with or without an acid (trifluoroacetic acid, trichloroacetic acid, formic acid), reaction time, and isolation methods. Plasma and hemoglobin fractions of blood samples were tested and the results were compared. The optimal solvent system for the reaction was determined to be ethanol in the presence of trifluoroacetic acid. The experimental procedure that provided the optimal reaction yield is described below.

Each blood sample (100 μL of plasma or hemoglobin) was mixed with 500 μL acetone, vortexed, and centrifuged at 1000 g for 5 min. The supernatant was removed and the residue was washed with 500 μL of absolute ethanol and then 100 μL of a freshly prepared 2% silver nitrate/ethanol solution containing 5% of trifluoroacetic acid was added. After the mixture was shaken for 1 hr at room temperature, the mixture was adjusted to ~pH 7 with 60 μL of 25% K_2CO_3, and then 100 μL of the supernatant was transferred for LC-MS/MS analysis. To each 100 μL sample was added 10 μL internal quantitation standard (200 ng/mL 7,9-di-C_2D_5O-DHP).

For comparison, the yields of 7,9-di-C_2H_5O-DHP produced from several other experimental conditions are shown below:

(i) When methanol was used in place of absolute ethanol, the yield of 7,9-di-CH_3O-DHP was about 90% of 7,9-di-C_2H_5O-DHP obtained with absolute ethanol (both reactions were conducted in the presence of trifluoroacetic acid).

(ii) When 5% trifluoroacetic acid was replaced by formic acid or trichloroacetic acid, the yields of 7,9-di-C_2H_5O-DHP were about 95 and 80%, respectively, of that obtained with trifluoroacetic acid.

(iii) When an acid was not added to the silver nitrate/ethanol solution, the yield of 7,9-di-C_2H_5O-DHP was about 10% of that obtained in the presence of acid, accompanied by the production of 7-C_2H_5O-DHP as the major product. These results are consistent with the findings previously described by Mattocks et al.[51] and by Lame et al.[52]

Detection and Quantitation of 7,9-Di-C_2H_5O-DHP Adducts in Rat Blood Samples by LC-MS/MS. Bioanalytical methods were developed to determine the concentration of 7,9-di-C_2H_5O-DHP in the blood samples using atmospheric pressure ionization LC-MS/MS in positive electrospray ionization mode. The liquid chromatography system consisted of a Shimadzu Prominence HPLC system, including a CBM-20A controller, two LC-20AD pumps, a SIL-20AC HT autosampler, a SPD-20A UV/VIS detector (Shimadzu Scientific Instrument, Columbia, MD), and an automated switching valve (TPMV, Rheodyne, Cotati, CA). The switching valve was used to divert the column effluent to either a waste collector or to the MS instrument. A Shimadzu Prominence HPLC system was used for sample injection and separation. Each sample (10 μL) was loaded onto a reverse phase column (ACE AQ 5 μm, C18, 50 x 2.0 mm, MAC-MOD Analytical, Chadds Ford, PA) with acidic aqueous buffer/acetonitrile gradient at 500 μL/min, and the sample components were eluted into the mass spectrometer. The column chamber temperature was set to 40 °C and the autosampler temperature was set at 5 °C. The mobile phases were 2 mM ammonium acetate in water containing 0.1% formic acid and acetonitrile. The initial gradient consisted of 15% acetonitrile for 0.3 min followed by a linear gradient up to 95% acetonitrile over 2 min. After holding 95% acetonitrile for 1 min, the instrument was reset to the initial conditions in 0.1 min. The analytical column was equilibrated with the mobile phase for 1.2 min. The total run time for a sample analysis was 4.6 min.

The HPLC eluate was coupled to an AB Sciex 4000 QTrap LC/MS/MS system (AB Sciex, Foster City, CA), equipped with a Turbo V™ ion source and a desolvation temperature of 450 °C. Nitrogen was used as curtain gas, nebulizer gas, heater gas, and collision gas. The samples were acquired in positive electrospray ionization mode using multiple reaction monitoring methods (MRM). The MRM method for detection and quantitation of 7,9-di-C_2H_5O-DHP adducts in the samples used a combination of two ion transitions: (i) *m/z* 227 → *m/z* 164,

$[M+NH_4]^+ \rightarrow [M+H\text{-}NH_3\text{-}C_2H_5OH]^+$; and (ii) *m/z* 210 → *m/z* 164, $[M+H]^+ \rightarrow [M+H\text{-}C_2H_5OH]^+$, at collision energies of 27 and 15 eV, respectively (Figure 2). The internal isotope-labeled standard was 7,9-di-C_2D_5O-DHP, the ion transition of the MRM method was *m/z* 237 → *m/z* 169 ($[M+NH_4]^+ \rightarrow [M+H\text{-}NH_3\text{-}C_2D_5OH]^+$), and the collision energy was 12 eV. The declustering potential was 25 V.

7,9-Di-C_2H_5O-DHP and the internal standard, 7,9-di-C_2D_5O-DHP, were quantified by using a reference standard calibration curve. One set of standard curve and quality control samples was run before the blood and plasma sample analysis; quality control samples were run each day again at the end of sample analysis period. The lower limit of quantification (LLOQ) was 1 ng/mL for 7,9-di-C_2H_5O-DHP in the plasma and hemoglobin fractions. A linear calibration curve ranging 1-2000 ng/mL for 7,9-di-C_2H_5O-DHP was fitted to 1/x weighted linear regression model (Figure S3 in the Supporting Information). Accuracy and precision of the standard curve and quality control samples were all in the acceptable range for bioanalytical analysis. The coefficient (r^2) of determination for the standard curve of 7,9-di-C_2D_5O-DHP was > 0.99.

Validation of the LC-MS/MS Quantitation Method. To ensure the reliability of the LC-MS/MS method, the LC-MS/MS method for quantification of 7,9-di-C_2H_5O-DHP was partially validated. The selectivity of the method, calibration standard curve (including inter-day calibration curve) linearity, concentration dynamic range, accuracy, and precision were evaluated. No matrix interference of the analyte peak was observed at LLOQ. The LLOQ was 1 ng/mL. A linear calibration curve ranging from 1 ng/mL to 2000 ng/mL was fitted to 1/x weighted linear regression model. Inter-day standard calibration curves of 7,9-di-C_2H_5O-DHP are presented in Figure S4. The accuracy, precision, and the standard calibration curve parameters of the LC-MS/MS methods for 7,9-di-C_2H_5O-DHP are shown in Table S1. Accuracy and precision of the standards used for standard curve generation are summarized in Table S2. The coefficient (r^2) of determination for all standard curves was ≥ 0.995 (Table S1). The results of quality control samples of 7,9-di-C_2H_5O-DHP for the standard calibration curves are shown in Table S3. Quality control (QC) samples (low, mid, and high concentrations at 2.5, 50, and 1600 ng/mL)

and the accuracy of the individual QC samples ranged from 85-115% of the nominal concentration. The precisions were within a CV% value of ≤8.1%.

Incurred rat blood samples were also analyzed during the LC-MS/MS method validation. Four aliquots of the pooled rat blood after reaction with silver nitrate in alcohol were quantified for 7,9-di-C_2H_5O-DHP with each standard calibration curve. The summary result of the 7,9-di-C_2H_5O-DHP concentrations in the pooled blood samples is showed in Table S4, with the relative standard derivation (RSD) of 6.89, 7.59, and 14.2%, respectively, indicating the method with good reproducibility.

Statistical Analyses. Statistical analyses were performed by analysis of variance, with comparisons between groups being conducted by the Holm-Sidak method. When necessary, the data were log transformed to maintain an equal variance or normal data distribution. Dose-related trends were assessed linear regression analysis. Correlations between DHP-protein levels and DHP-DNA adduct levels were assessed by Pearson product moment analysis.

RESULTS

Synthesis of 7,9-Di-C_2H_5O-DHP and Isotopically Labeled 7,9-Di-C_2D_5O-DHP.

In our developed quantitation method, the reaction of silver nitrate in acidic ethanol with DHP-protein adducts in the plasma and hemoglobin fractions of blood from rats produced 7,9-di-C_2H_5O-DHP, which was identified and quantified by LC/MS/MS MRM analysis (Figure 3). For this purpose, 7,9-di-C_2H_5O-DHP was synthesized for structural identification of the product and to construct a calibration curve. Isotopically labeled 7,9-di-C_2D_5O-DHP was synthesized for use as a quantitation standard in the LC/MS/MS MRM analysis.

7,9-Di-C_2H_5O-DHP was previously synthesized by Mattocks and Jukes,[43] but the reaction yield and NMR spectral data were not provided. In our study, 7,9-di-C_2H_5O-DHP was synthesized in a 65.7% yield and its structure was fully characterized by mass and ^{1}H NMR spectral analysis. Isotopically labeled 7,9-di-C_2D_5O-DHP was similarly synthesized by reaction of dehydromonocrotaline with deuterated ethanol. As described in the Experimental Procedures section, its structure was determined by mass spectral data and ^{1}H NMR spectroscopic analysis.

LC-ES-MS/MS Analysis of DHP-Protein Adducts Formed in the Blood of Rats Dosed Riddelliine and Other Pyrrolizidine Alkaloids.

Blood Samples of Rats Dosed Riddelliine. Female rats (4 per group) were dosed by oral gavage at 0, 0.1, 1.0, 2.0, or 5.0 mg riddelliine/kg bw for 5 or 30 days. Blood samples were collected and separated into plasma and hemoglobin fractions. The levels of DHP-protein adducts, identified as 7,9-di-C_2H_5O-DHP in the plasma and hemoglobin fractions, were quantified by LC-ES-MS/MS MRM analysis.

LC/MS/MS MRM quantitation of 7,9-di-C_2H_5O-DHP formed from DHP-protein adducts contained in the hemoglobin fraction of rats dosed with 5 mg riddelline/kg bw for 30 consecutive days is shown in Figure 4. Panel A is MRM total ions of the 7,9-di-C_2H_5O-DHP. Panels B and C are the MRM ion transitions for *m/z* 210 → 164 and *m/z* 227 → 164, respectively. Panel D is the MRM ion transition for *m/z* 237 → 169 of the isotopically-labeled standard 7,9-di-C_2D_5O-DHP.

LC/MS/MS MRM chromatograms of 7,9-di-C_2H_5O-DHP formed from DHP-protein adducts contained in the hemoglobin fractions of rats dosed with: (A) 0 mg; (B) 0.1 mg; (C) 1.0 mg; (D) 2.0 mg; and (E) 5 mg riddelliine/kg bw for 30 consecutive days are shown in Figure 5. The left panels represent the level of DHP-protein adducts (measured as 7,9-di-C_2H_5O-DHP) from control rats or riddelliine dosed rats. The right panels are the signals for 50 fmol 7,9-di-C_2D_5O-DHP internal standard added to each sample. In the MRM method for quantitation of 7,9-di-C_2H_5O-DHP in the samples, the two ion transitions were monitored: (i) *m/z* 227 → 164 ($[M+H]^+$ → $[M+H-C_2H_5OH]^+$) and (ii) *m/z* 210 → 164 ($[M+NH_4]^+$ → $[M+H-NH_3-C_2H_5OH]^+$) at collision energy 27 and 15 eV, respectively. The combined peak area of the selected ion current peaks of these two ion transitions was used in the quantitation.

There was a significant ($P < 0.05$) dose- and time-dependent increase in the levels of DHP-protein adducts with both plasma and hemoglobin, with the extent of protein binding being significant at all doses of riddelliine (Figure 6 and Table S5 in the Supporting Information). A comparison of the plasma and hemoglobin binding indicated a more dynamic response with

hemoglobin due, in part, to the lower background levels obtained with the control hemoglobin samples. Based upon the more dynamic response observed with hemoglobin, subsequent work with a series of PAs focused on DHP-hemoglobin adducts.

Blood Samples of Rats Dosed Different PAs. Female rats (4 per group) were dosed by oral gavage daily with riddelliine, retrorsine, monocrotaline, lycopsamine, retrornecine, riddelliine *N*-oxide, lasiocarpine, heliotrine, senkirkine, clivorine, or platyphylliine (a mixture of 70% platyphylliine and 30% neoplatphylliine) at 4.5 or 24 μmol/kg bw in 0.5 mL of 10% DMSO in water or vehicle for three consecutive days. One day after the last treatment, blood samples were collected and separated into plasma and hemoglobin fractions. Using the analytical method described previously, each hemoglobin sample was converted into 7,9-di-C_2H_5O-DHP adducts that were identified and quantified by LC-MS/MS MRM analysis.

The results of the 7,9-di-C_2H_5O-DHP adducts formed from the hemoglobin fractions, expressed as ng 7,9-di-C_2H_5O-DHP/g hemoglobin/mg PA/kg bw, are summarized in Figure 7 and Table S6 in the Supporting Information. The lowest level of binding was detected platyphylliine, a non-tumorigenic PA that lacks a double bond in the nicine ring. This was followed by retronecine, heliotrine, and lycopsamine, PAs that contain a hydroxyl substituent at C7. The next highest hemoglobin binding was observed with clivorine and senkirkine, PAs that contain an N-methyl substituted nicine ring. The greatest extent of DHP hemoglobin adduct formation was obtained with riddelliine *N*-oxide, retrorsine, lasiocarpine, riddelliine, and monocrotaline, PAs that are cyclic diesters.

Pearson Product Moment Correction Coefficients and P Values. With both the PA DHP-DNA adducts reported previously[15] and the PA DHP-hemoglobin adducts available, we determined the correlation between (i) the PA DHP-DNA adducts and (ii) the PA DHP-DNA adducts and PA DHP-hemoglobin adducts. As the summarized in Table 1, there is an excellent correlation between any two of the PA DHP-DNA adducts (*i.e.*, among the DHP-dG-3, DHP-dG-4, DHP-dA-3, and DHP-dA-4 adduct), with correlation coefficients of 0.951 - 0.998 and P values of 1×10^{-5} - 9×10^{-12} (Table 1). A representative correlation plot is shown in Figure 8. A more modest correlation was observed between PA DHP-hemoglobin adducts and PA DHP-

DNA adducts, with correlation coefficients of 0.573 - 0.702 and P values of 0.07 - 0.02 (Table 1 and Figure 8).

DISCUSSON

Mattocks and co-workers previously developed a method to detect pyrrole-protein (DHP-protein) adducts present in the blood and liver of rodents administered toxic PAs.[44, 45] This method specifically detects pyrrole-protein adducts that are formed through binding of the proteins' thiol group at the C7 and/or C9-position of the necine base by using the $AgNO_3$ in acidic ethanol to cleave the thioether group (at the C7 and/or C9-position). The resulting 7,9-di-C_2H_5O-DHP product was then separated by TLC and identified by GC/MS; however, the 7,9-di-C_2H_5O-DHP product was not quantified. In our study, based on this method, we developed a novel approach to quantify the DHP-protein adducts formed in the blood of rats administered PAs. In the developed method, blood samples were treated with $AgNO_3$ in acidic ethanol, and the resulting 7,9-di-C_2H_5O-DHP was identified and quantified by HPLC-ES-MS/MS MRM analysis in the presence of a known quantity of an isotopically-labeled 7,9-di-C_2D_5O-DHP internal standard. This HPLC-ES-MS/MS method was accurate and precise, with the lower limit of quantification (LLOQ) at 1 ng/mL for 7,9-di-C_2H_5O-DHP. The coefficient (r^2) of determination for the standard curve of 7,9-di-C_2H_5O-DHP ranging 1 - 2000 ng/mL was over 0.99.

We determined that when the reaction of DHP-protein adducts in blood of rats with silver nitrate in alcohol was conducted without the addition of an acid, the yield of 7,9-di-C_2H_5O-DHP was about 10% of that obtained in the presence of acid and this was accompanied by the production of 7-C_2H_5O-DHP as the major product. This result indicates that under experimental conditions, the DHP-protein adducts isolated as 7-C_2H_5O-DHP are the predominant DHP-protein adducts, while DHP-di-protein adducts (protein-protein cross-link) are formed no more than 10%. PA-induced protein-protein cross-linking and DNA-protein cross-linking have been suggested to cause animal toxicity and may lead to tumor formation.[25,26,31,32] Further investigations are clearly necessary to determine the role of protein-protein cross links, DNA-protein cross links, and DHP-protein adducts in PA-induced toxicity and tumorigenicity.

With this developed method, we were able to detect and quantify pyrrole-protein (DHP-protein) adducts formed in the blood of rats dosed by gavage with riddelliine of different concentrations for 5 and 30 consecutive days (Figure 6 and Table S1 in the Supporting Information) and with a series of different PAs for 3 consecutive days (Figure 7 and Table S2 in the Supporting Information). The results indicate that the levels of DHP-protein adducts formed in the hemoglobin and plasma fractions are time dependent (Figure 6). Their formation was also dose dependent; with the DHP-protein adducts formation in the hemoglobin fraction having a more dynamic dose response than that in the plasma fraction. This suggests that for the quantitation of the DHP-protein adduct formation, the use of DHP-hemoglobin fraction is a better choice.

Drug-induced liver injury (DILI) is an important disease in humans. In the United States, it is one of the leading causes of acute liver failure.[53] PA-induced liver injury has been recognized for a long time and it may be one of the most common causes for drug-induced liver injury.[48, 54, 55] In a clinical study, Ruan et al.[56] tested 31 DILI patients with unknown etiology and found DHP-protein adducts in 12 (39%) of the patients. It is noteworthy that the LC/MS/MS method reported in our study can potentially be utilized as a mechanism-based clinical bioassay to quantify DHP-protein adducts present in human blood samples, and consequently DHP-protein adducts can potentially serve as minimally invasive biomarkers of PA exposure in humans.

In the present study we determined that: (i) the most extensive DHP-hemoglobin adduct formation occurred with cyclic diester PAs, including retrorsine, lasiocarpine, riddelliine, monocrotaline, and riddelliine *N*-oxide; (ii) the next most extensive DHP-hemoglobin adduct formation occurred with clivorine and senkirkine, otonecine-type PAs containing an *N*-methyl substituted nicine ring; (iii) this was followed by retronecine, heliotrine, and lycopsamine, PAs that contain a hydroxyl group at C7 of the necine base; and (iv) the lowest extent of binding was with platyphylliine, a non-tumorigenic PA that lacks a double bond in the nicine ring (Figure 7). We have previously used the livers of these rats to quantify the DHP-DNA adduct formation and found that the levels of DHP-DNA adducts (DHP-dG-3, DHP-dG-4, DHP-dA-3, and DHP-dA-4) are in the order: retrorsine > lasiocarpine > riddelliine ~ monocrotaline > riddelliine *N*-oxide > senkirkine > heliotrine ≥ clivorine >>> lycopsamine ~ retronecine ~ platyphylliin ~ vehicle control.[15] Although the Pearson product moment correction coefficients between DHP-protein adducts and DHP-DNA adducts were moderate (Table 1 and Figure 8), when the comparison is

based on the structural features of the parent PAs, the same general relationship holds for both DHP-DNA adducts in the liver and DHP-hemoglobin adducts in the blood with cyclic diester PAs > otonicine-type PAs > monoester PAs (and retronecine) > PAs lacking a double bond in the nicine ring.

The Edman rearrangement reaction is a commonly used method for quantifying metabolite-bound protein adducts *in vivo*.[57, 58] This method uses the phenyl isothiocyanate (PITC) or its derivatives to bind to the adducted terminal valine group of hemoglobin (in our case, e.g. DHP-hemoglobin adducts), resulting in the replacement of the hemoglobin moiety by the phenylthiohydantoin group followed by structural identification and quantitation. We have attempted to use this method to quantify DHP-protein adducts, by measuring the resulting phenylthiohydantoin-DHP adducts by LC/MS/MS.[29] We found that reaction of PITC with DHP-hemoglobin adducts that were prepared from the reaction of rat hemoglobin with dehydromonocrotaline generated four isomeric phenylthiohydantoin (DHP-valine-PITC) products.[29] Quantitation of four products by LC/MS/MS would be less favorable (and probably less accurate) than the quantitation of a single product (7,9-di-C_2H_5O-DHP). In addition, we were unable to find a practical method to prepare synthetically the four deuterium-labeled DHP-valine-PITC adducts for use as quantitation standards for LC/MS/MS MRM analysis. Mattocks and Jukes[44, 45] have reported that reactive pyrrolic metabolites, dehydro-PAs, preferentially bind to proteins at thiols rather than nitrogens, which suggests that DHP-protein adducts results from reactions with thiols should be formed in a higher yield than those from reactions with nitrogens. Consequently, we conclude that PA-protein binding measurements based upon the detection of 7,9-di-C_2H_5O-DHP have distinct advantages over the detection of DHP-valine adducts.

Our study focused on characterizing DHP-hemoglobin adducts in the blood of rats treated with PAs. Rat hemoglobin has ten free thiol groups (from the cysteinyl residues), six from the two α-chains and four from the two β-chains.[59] In contrast, human hemoglobin has six free thiol groups, one from each of the two α-chains and two from each of the two β-chains.[59] This suggests that at equal levels of exposure, more DHP-hemoglobin adducts may be detected from rats as compared to humans. Furthermore, the binding capability and the stereochemical activity of the thiol groups may differ between human and rat hemoglobin,[59-62] which could also affect the extent of adduct formation from PAs between rats and humans. Additional experiments are clearly necessary to demonstrate the utility of our LC/MS/MS with human hemoglobin samples;

nonetheless, as noted above, DHP-protein adducts have been detected in the blood of patients consuming the herbal plant *Gynura segetum*.[48]

SUPPORTING INFORMATION

Tables S1 and S2. LC-MS/MS analysis calibration curve parameters, curve linearity, accuracy, and precision data of 7, 9 di-C_2H_5O-DHP. Table S3. LC-MS/MS analysis quality control results for 7, 9 di-C_2H_5O-DHP standard calibration curve from inter-day. Table S4. Summary of 7, 9 di-C_2H_5O-DHP concentration in pooled blood sample containing pyrrolizidine alkaloids. Tables S5 and S6. The levels of DHP-protein adducts (ng/g protein) identified as 7,9-di-C_2H_5O-DHP in hemoglobin and plasma fractions of female rats. Figure S1. HPLC profile of 7,9-di-C_2H_5O-DHP prepared from reaction of dehydromonocrotaline with ethanol. Figure S2. ^{1}H NMR spectra of 7,9-di-C_2H_5O-DHP and 7,9-di-C_2D_5O-DHP. Figures S3 and S4. Standard calibration curve of 7,9-di-C_2H_5O-DHP. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This research was supported, in part, by the appointment (Y.Z.) to the Postgraduate Research Program at the NCTR administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the FDA.

Notes

This article is not an official U.S. Food and Drug Administration (FDA) guidance or policy statement. No official support or endorsement by the U.S. FDA is intended or should be inferred. The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Ms. Linda S. Von Tungeln, Ms. Michelle Vanlandingham, and Mr. Gene White for assistance with the animals, Dr. Po-Chuen Chan of NTP for supplying riddelliine, Dr. John A. Edgar, CSIRO Livestock Industries, Australia for supplying lasiocarpine, and Dr. H.S. Chen, the Secondary Military Medical University, Shanghai, China for supplying platyphylliine.

ABBREVIATIONS

PAs, pyrrolizidine alkaloids; dehydro-PAs, dehydropyrrolizidine alkaloids; DHR, dehydroretronecine or (−)-R-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine); DHP, (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine; HPLC-ES-MS/MS, high-performance liquid chromatography-electrospray ionization tandem mass spectrometry; NCTR, National Center for Toxicological Research; MRM, multiple reaction monitoring; LOD, limit of detection

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Table 1. Pearson Product Moment Correction Coefficients and P Values between (i) PA DHP Hemoglobin Adducts and PA DHP-DNA Adducts and (ii) PA DHP-DNA Adducts.

PA hemoglobin or DNA adduct	ng DHP/g hemoglobin/mg PA/kg BW	DHP-dG-3/mg PA/kg BW	DHP-dG-4/mg PA/kg BW	DHP-dA-3/mg PA/kg BW	DHP-dA-4/mg PA/kg BW
ng DHP/g hemoglobin/mg PA/kg BW					
DHP-dG-3/mg PA/kg BW	0.631[a] 0.04[b]				
DHP-dG-4/mg PA/kg BW	0.702 0.02	0.947 1×10^{-5}			
DHP-dA-3/mg PA/kg BW	0.573 0.07	0.956 4×10^{-6}	0.951 7×10^{-6}		
DHP dA-4/mg PA/kg BW	0.633 0.04	0.955 5×10^{-6}	0.951 7×10^{-6}	0.998 9×10^{-12}	

[a]Correlation coefficient
[b]P-value

Figure legends

Figure 1. Structures of pyrrolizidine alkaloids and derivatives used in this study.

Figure 2. The ion transitions of the MRM method for specific detection of 7,9-diethoxyl-DHP adducts in the samples were obtained through a combination of two routes: (i) $[M+H]^+$ *m/z* 227→ $[M+H-C_2H_5OH]^+$ *m/z* 164 and (ii) $[M+NH_4]^+$ *m/z* 210 → *m/z* 164, and collision energy 27 and 15 eV, respectively.

Figure 3. The quantitation of protein-DHP adducts in blood samples by converting the DHP-protein adducts into 7,9-di-C_2H_5O-DHP adduct by reaction of the protein adducts with silver nitrate in acidic ethanol.

Figure 4. LC/MS/MS MRM chromatograms of 7,9-di-C_2H_5O-DHP formed from the DHP-protein adducts contained in the hemoglobin fraction of rats dosed with 5 mg/kg body weight of riddelline for 30 consecutive days: (A) MRM total ions; (B) the MRM *m/z* 210 – 164; (C) the MRM *m/z* 227 – 164; and (D) the MRM *m/z* 237 - 169.

Figure 5. LC/MS/MS MRM chromatograms of 7,9-di-C_2H_5O-DHP formed from the DHP-protein adducts contained in the hemoglobin fraction of rats dosed with: (A) 0 mg; (B) 0.1 mg; (C) 1.0 mg; (D) 2.0 mg; and (E) 5 mg/kg body weight of riddelline for 30 consecutive days.

Figure 6. The levels of DHP-protein adducts (ng/g protein) identified as 7,9-di-C_2H_5O-DHP in hemoglobin and plasma fractions of female rats dosed with 0, 0.1, 1.0, 2.0, or 5.0 mg riddelliine/kg bw for 5 (circle) or 30 (triangle) days (4 rats per group).

Figure 7. The mean levels of DHP-protein adducts identified as 7,9-di-C_2H_5O-DHP in the hemoglobin fraction of female rats dosed with PAs at 4.5 and 24 μmol/kg bw/day for three consecutive days. The data are normalized to ng 7,9-di-C_2H_5O-DHP/g hemoblobin/mg PA/kg bw.

Figure 8. Pearson Product Moment Correction coefficient and P value; (A) between DHP-hemoglobin adducts and DHP-dA-4; and (B) between DHP-dA-3 and DHP-dA-4 of female rats dosed with PAs at 4.5 or 24 μmol/kg bw/day for three consecutive days.

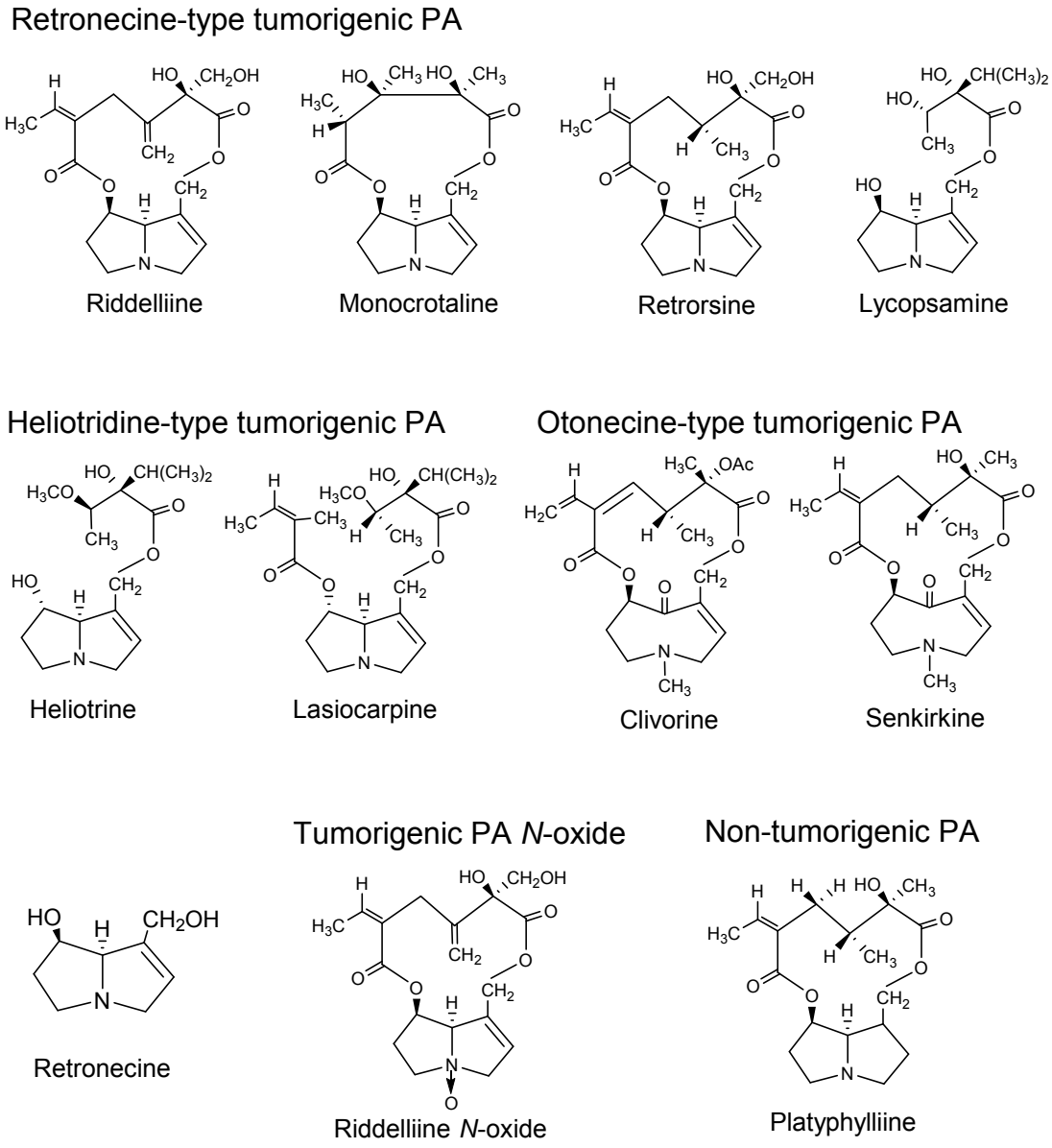
Retronecine-type tumorigenic PA
Riddelliine
Monocrotaline
Retrorsine
Lycopsamine
Heliotridine-type tumorigenic PA
Heliotrine
Lasiocarpine
Otonecine-type tumorigenic PA
Clivorine
Senkirkine
Retronecine
Tumorigenic PA N-oxide
Riddelliine N-oxide
Non-tumorigenic PA
Platyphylliine

Figure 1.

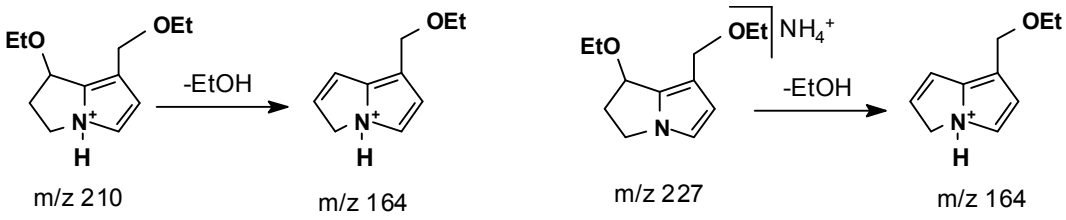
EtO
OEt
-EtOH
OEt
N+
H
m/z 210
m/z 164
OEt
NH4+
EtO
N
-EtOH
OEt
N+
H
m/z 227
m/z 164

Figure 2.

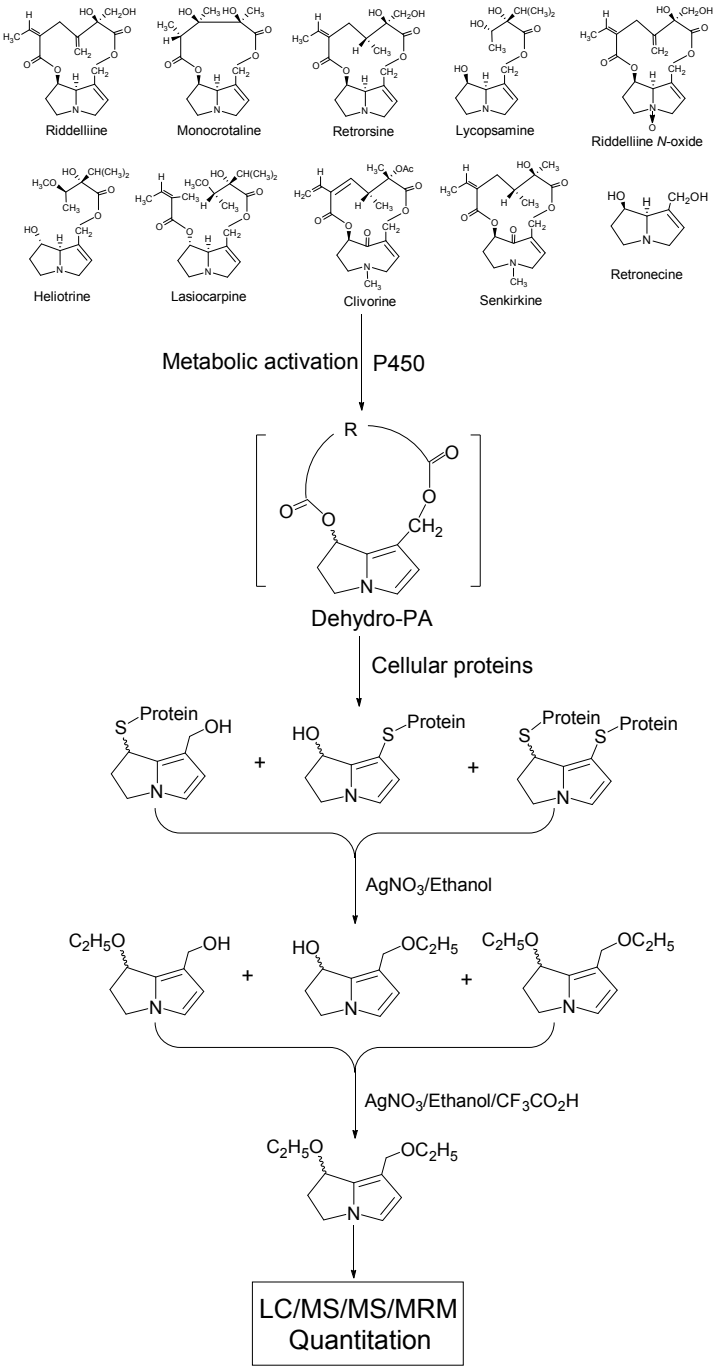
Riddelliine
Monocrotaline
Retrorsine
Lycopsamine
Riddelliine N-oxide
Heliotrine
Lasiocarpine
Clivorine
Senkirkine
Retronecine
Metabolic activation
P450
Dehydro-PA
Cellular proteins
Protein
AgNO3/Ethanol
AgNO3/Ethanol/CF3CO2H
LC/MS/MS/MRM
Quantitation

Figure 3.

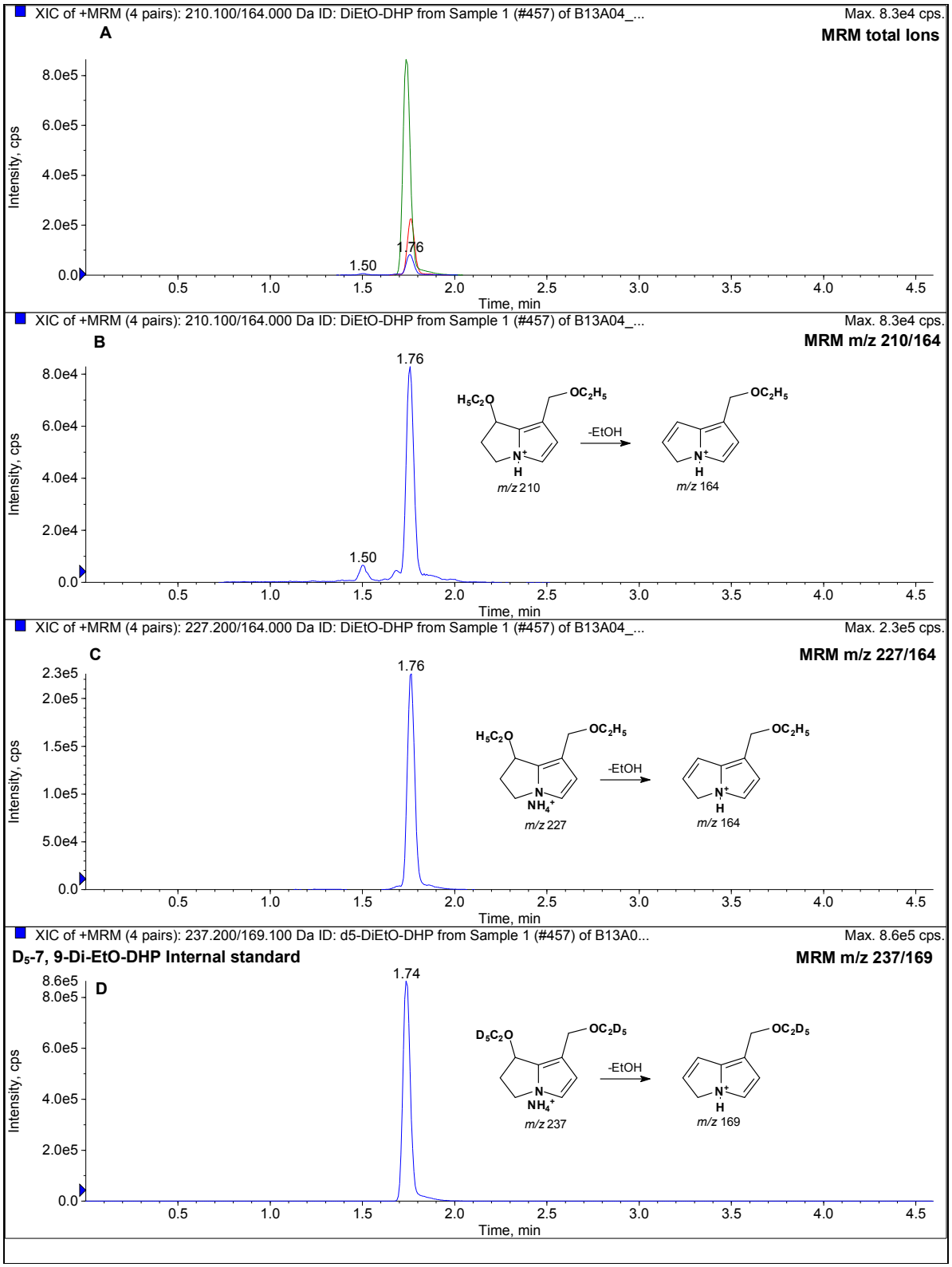
XIC of +MRM (4 pairs): 210.100/164.000 Da ID: DiEtO-DHP from Sample 1 (#457) of B13A04_...
Max. 8.3e4 cps.
A
MRM total Ions
Intensity, cps
Time, min
1.50
1.76
B
MRM m/z 210/164
m/z 210
m/z 164
-EtOH
XIC of +MRM (4 pairs): 227.200/164.000 Da ID: DiEtO-DHP from Sample 1 (#457) of B13A04_...
Max. 2.3e5 cps.
C
MRM m/z 227/164
m/z 227
XIC of +MRM (4 pairs): 237.200/169.100 Da ID: d5-DiEtO-DHP from Sample 1 (#457) of B13A0...
Max. 8.6e5 cps.
D5-7, 9-Di-EtO-DHP Internal standard
MRM m/z 237/169
1.74
m/z 237
m/z 169

Figure 4.

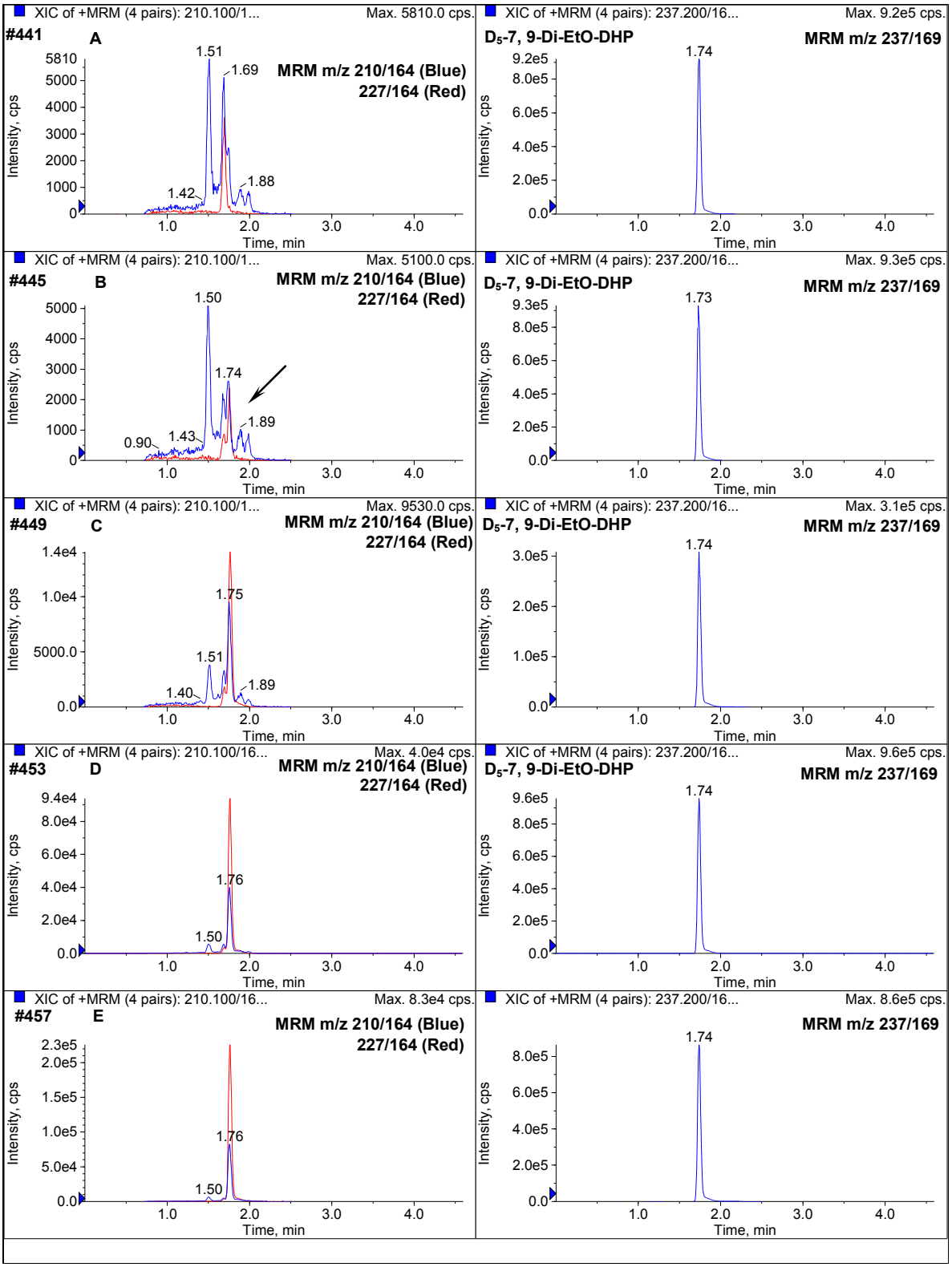
XIC of +MRM (4 pairs): 210.100/1... Max. 5810.0 cps.
#441 A
MRM m/z 210/164 (Blue)
227/164 (Red)
1.51
1.69
1.42
1.88
XIC of +MRM (4 pairs): 237.200/16... Max. 9.2e5 cps.
D5-7, 9-Di-EtO-DHP
MRM m/z 237/169
1.74
XIC of +MRM (4 pairs): 210.100/1... Max. 5100.0 cps.
#445 B
MRM m/z 210/164 (Blue)
227/164 (Red)
1.50
1.74
1.89
0.90
1.43
XIC of +MRM (4 pairs): 237.200/16... Max. 9.3e5 cps.
D5-7, 9-Di-EtO-DHP
MRM m/z 237/169
1.73
XIC of +MRM (4 pairs): 210.100/1... Max. 9530.0 cps.
#449 C
MRM m/z 210/164 (Blue)
227/164 (Red)
1.75
1.51
1.40
1.89
XIC of +MRM (4 pairs): 237.200/16... Max. 3.1e5 cps.
D5-7, 9-Di-EtO-DHP
MRM m/z 237/169
1.74
XIC of +MRM (4 pairs): 210.100/16... Max. 4.0e4 cps.
#453 D
MRM m/z 210/164 (Blue)
227/164 (Red)
1.76
1.50
XIC of +MRM (4 pairs): 237.200/16... Max. 9.6e5 cps.
D5-7, 9-Di-EtO-DHP
MRM m/z 237/169
1.74
XIC of +MRM (4 pairs): 210.100/16... Max. 8.3e4 cps.
#457 E
MRM m/z 210/164 (Blue)
227/164 (Red)
1.76
1.50
XIC of +MRM (4 pairs): 237.200/16... Max. 8.6e5 cps.
MRM m/z 237/169
1.74
Intensity, cps
Time, min

Figure 5.

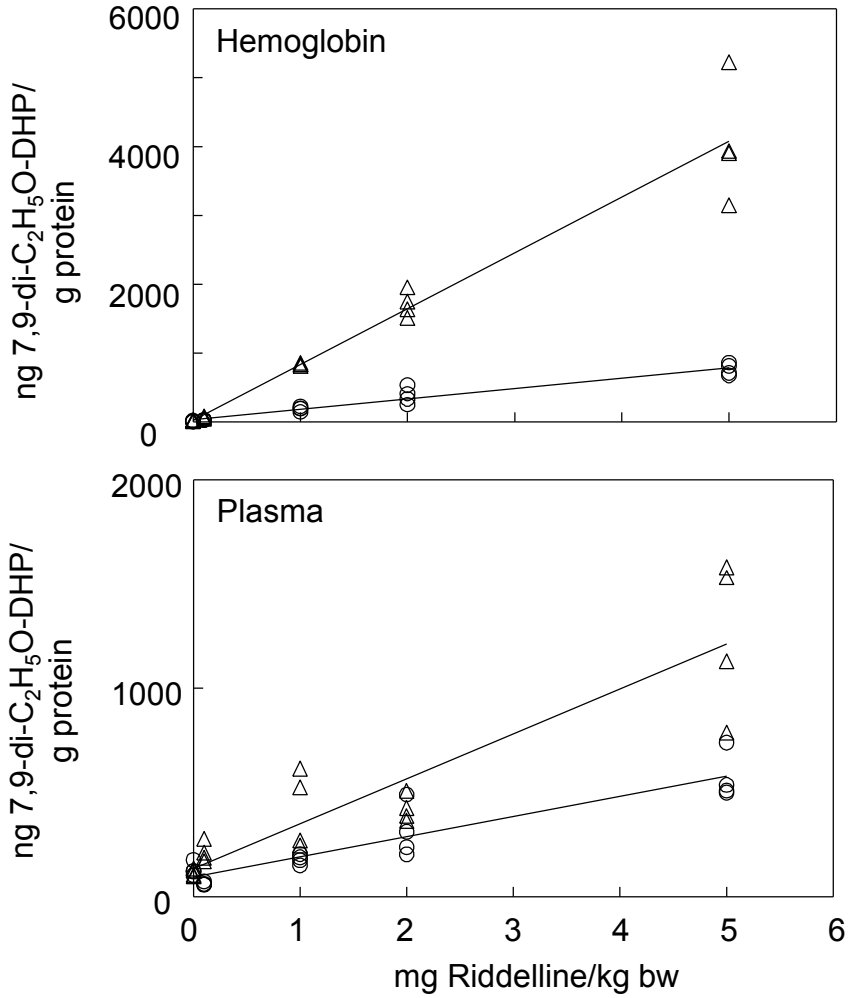
Hemoglobin
Plasma
ng 7,9-di-C2H5O-DHP/ g protein
mg Riddelline/kg bw
0
2000
4000
6000
1000
1
2
3
4
5
6

Figure 6.

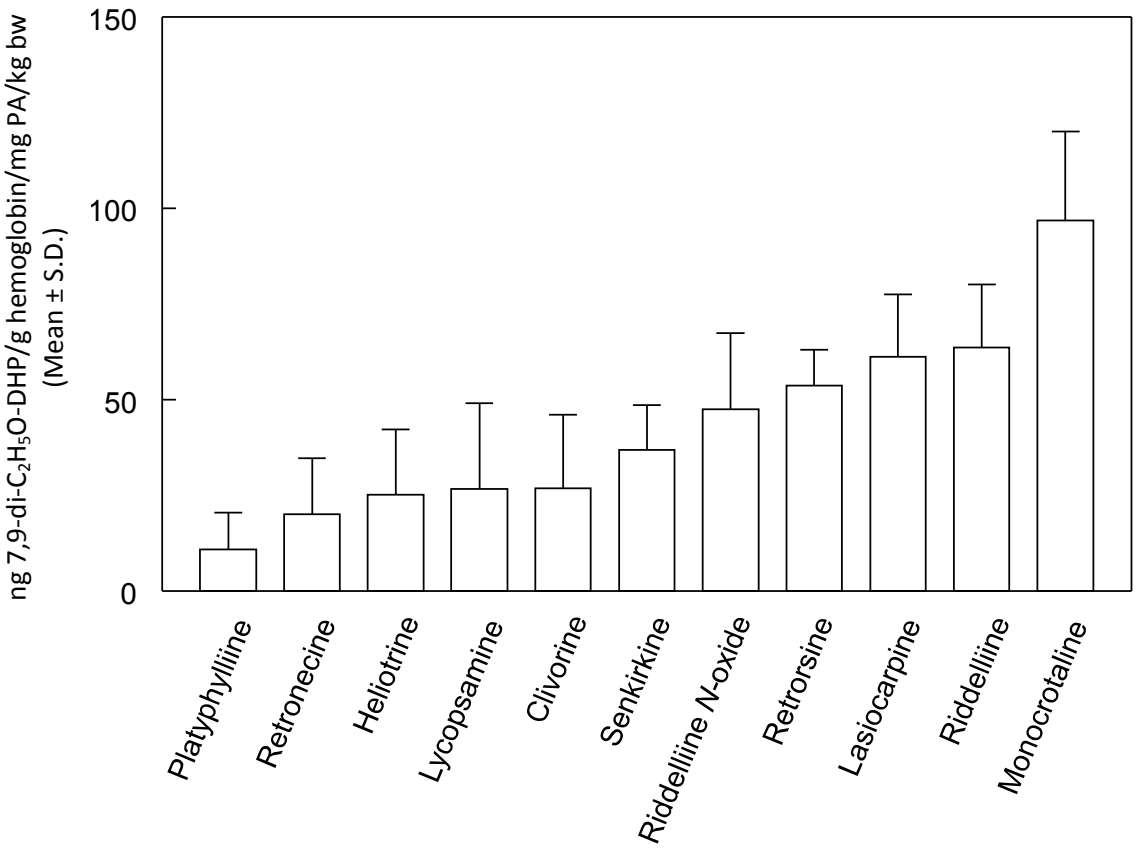
ng 7,9-di-C2H5O-DHP/g hemoglobin/mg PA/kg bw
(Mean ± S.D.)
150
100
50
0
Platyphylliine
Retronecine
Heliotrine
Lycopsamine
Clivorine
Senkirkine
Riddelliine N-oxide
Retrorsine
Lasiocarpine
Riddelliine
Monocrotaline

Figure 7.

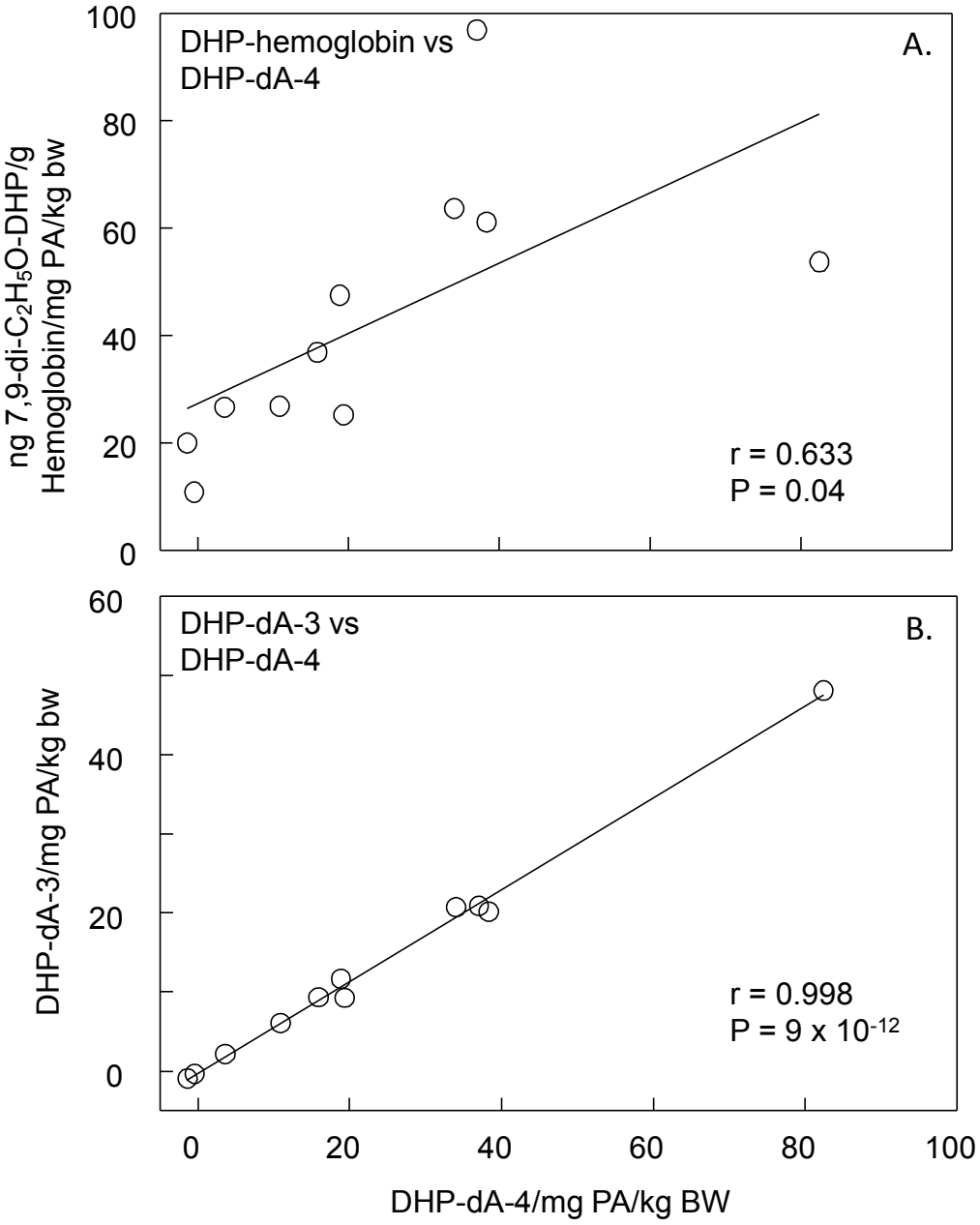
DHP-hemoglobin vs
DHP-dA-4
A.
ng 7,9-di-C2H5O-DHP/g
Hemoglobin/mg PA/kg bw
r = 0.633
P = 0.04
DHP-dA-3 vs
DHP-dA-4
B.
DHP-dA-3/mg PA/kg bw
r = 0.998
P = 9 x 10-12
DHP-dA-4/mg PA/kg BW

Figure 8.