Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfda-online.com

Original Article

Quantitation of DNA reactive pyrrolic metabolites of senecionine – A carcinogenic pyrrolizidine alkaloid by LC/MS/MS analysis

Qingsu Xia [a], *Xiaobo He* [a], *Qiang Shi* [a], *Ge Lin* [b], *Peter P. Fu* [a,*]

[a] *National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, United States*
[b] *School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China*

ARTICLE INFO

Article history:
Available online 13 December 2019

Keywords:
Pyrrolizidine alkaloid
Senecionine
Pyrrolic metabolites
Carcinogenicity
LC/MS/MS analysis

ABSTRACT

Pyrrolizidine alkaloids (PAs) are carcinogenic phytochemicals, inducing liver tumors in experimental rodents. We previously determined that (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP), 7-glutathione-DHP, 7-cysteine-DHP, 7-*N*-acetylcysteine-DHP, and 1-CHO-DHP are DNA reactive pyrrolic metabolites potentially associated with PA-induced liver tumor initiation. In this study, we developed an LC/MS/MS multiple reaction monitoring (MRM) mode method to identify and quantify these metabolites formed from the metabolism of senecionine, a carcinogenic PA, by mouse, rat, and human liver microsomes, and primary rat hepatocytes. Together with the chemically prepared standards of these metabolites, this represents an accurate and convenient LC/MS/MS analytical method for quantifying these five reactive pyrrolic metabolites in biological systems.

1. Introduction

Pyrrolizidine alkaloids (PAs) are widespread and toxic phytochemicals [1,2]. There are more than 6000 PA-containing plants in the world, which are probably the most common poisonous plant species affecting livestock, wildlife, and humans [1–4]. PAs have been found to induce liver tumors in experimental animals [3,5–9]. Monocrotaline, riddelliine, and lasciocarpine are classified as possible human carcinogens by the International Agency for Research on Cancer (IARC) [7,8]. PAs require metabolic activation to exert toxicity, including carcinogenicity [1–4]. Metabolism of toxic PAs generates primary pyrrolic metabolites (dehydro-PAs), which are easily hydrolyzed to produce (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP [1–3]. Both dehydro-PAs and DHP can bind to cellular DNA to form exogenous DNA adducts leading to liver tumor initiation.

* *Corresponding author*. Fax: +1 870 543 7136.
E-mail address: peter.fu@fda.hhs.gov (P.P. Fu).
https://doi.org/10.1016/j.jfda.2019.12.001

Recently, we established a common genotoxic mechanism mediated by a set of DHP-DNA adducts leading to PA-induced liver tumor initiation (Fig. 1) [5,10–15]. This mechanism has been common, involved in the metabolism of a series of hepatocarcinogenic PAs and riddelliine *N*-oxide in rats *in vivo* [1,5,10,15], in the livers of male mice dosed retrorsine [16], in the livers of cows accidently fed with PA-contaminated hay [4], and in liver microsomal metabolism of PAs and pyrrolic metabolic metabolites *in vitro* [11,13,17]. Based on these findings, we proposed that these DHP-DNA adducts are potential biomarkers of PA exposure and liver tumor initiation [5,10,11,14,15].

We recently determined that besides DHP, four secondary pyrrolic metabolites, 7-glutathione-DHP (7-GS-DHP), 7-cysteine-DHP, 7-*N*-acetylcysteine-DHP (7-NAC-DHP), and 1-CHO-DHP are also DNA reactive metabolites. These results suggest that multiple activation pathways exist, leading to the formation of DHP-DNA adducts [5,10–12,15]. In the present study, with the use of the synthetically prepared standards of these five pyrrolic metabolites, we developed an LC/MS/MS selected reaction monitoring (SRM) mode method to accurately and conveniently quantify these metabolites formed *in vitro* and in rat primary hepatocytes. By this analytical method, the pyrrolic metabolites formed from the metabolism of senecionine, a carcinogenic PA, by rat, mouse, and human liver microsomes and by primary rat hepatocytes were identified and quantified.

2. Experimental procedures

2.1. Chemicals

Senecionine and senecionine *N*-oxide were purchased from Cerilliant Corporation, (Round Rock, TX). DHP, 7-glutathione-DHP (7-GS-DHP), 7-cysteine-DHP, 7-*N*-acetylcysteine-DHP (7-NAC-DHP), and 1-CHO-DHP were synthesized as previously described [11,12,18,19]. The structures of these pyrrolic compounds are shown in Fig. 2. Male Fischer 344 rat, male mouse, and male human liver microsomes were purchased from BioreclamationIVT (Baltimore, MD).

2.1.1. Animal care and isolation of rat hepatocytes

Male Fischer 344 rats at the age of 10–12 weeks were obtained from the U.S. FDA National Center for Toxicological Research (NCTR) breeding colonies. Animal care and experimental procedures were performed in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and was authorized by the NCTR Institutional Animal Care and Use Committee.

Rat hepatocytes were isolated by the *in situ* two-step perfusion method [20] with modifications [21]. After an intraperitoneal injection of pentobarbital, the liver was perfused with buffer containing 2 mM $CaCl_2$, 0.03% collagenase, and 0.01% soybean trypsin inhibitor. The digested liver

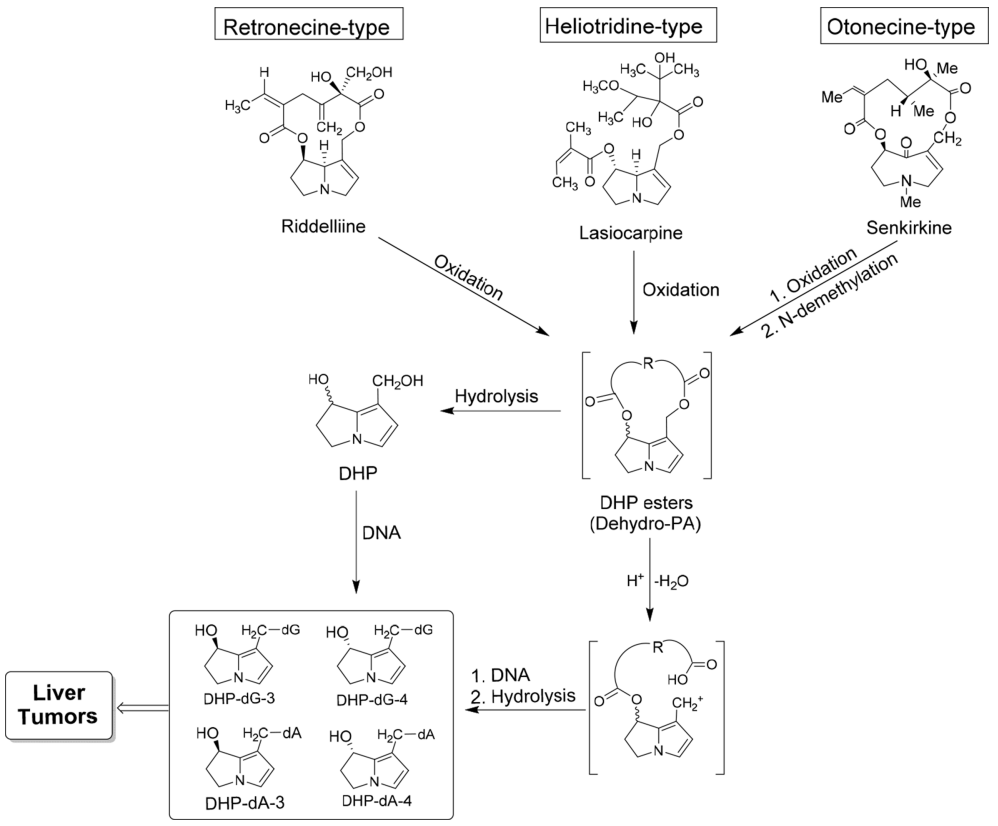

Fig. 1 – The proposed general metabolic activation pathways of carcinogenic PAs mediated by the formation of DHP-DNA adducts leading to PA-induced liver tumor initiation.

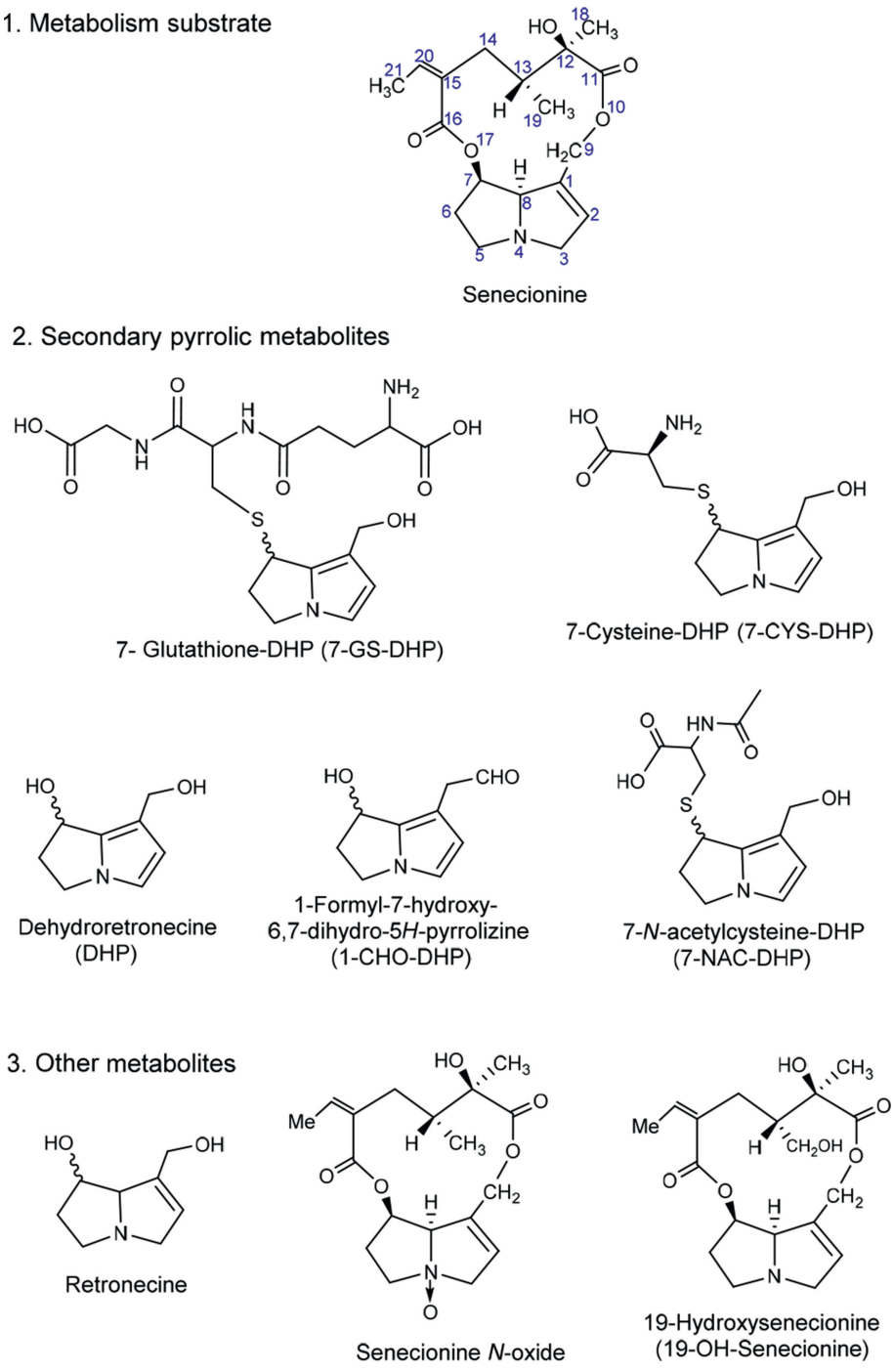

Fig. 2 – Structures of senecionine, its five secondary pyrrolic metabolites, and three other identified metabolites.

was collected and the resulting suspension was centrifuged to collect the hepatocytes, which were further purified using a 90% Percoll solution. Cell viability was routinely >90% as measured by trypan blue exclusion, and the average cell yield was usually 2–3 × 10^8 cells per rat.

2.1.2. Metabolism of senecionine by male rat, mouse, and human liver microsomes

The metabolism of senecionine by male rat, mouse, and human (50-donor pooled) liver microsomes was conducted in a 1 mL incubation volume containing 100 mM sodium phosphate buffer (pH 7.4), 5 mM magnesium chloride, 1 mM NADPH, 500 μM senecionine, and 1 mg of human, mouse or rat liver microsomes at 37 °C for 30 min. After incubation the mixture was centrifuged at 105,000 *g* for 30 min at 4 °C to remove microsomal proteins. The supernatant was collected and analyzed via HPLC or LC/MS/MS.

2.1.3. Metabolism of senecionine by male primary rat hepatocytes

The isolated hepatocytes were cultured in William's E medium supplemented with 10 mM HEPES, 1% linoleic acid-albumin, 5 μg/mL insulin, 5 μg/mL holo-transferrin, 25 nM dexamethasone, 5 ng/mL sodium selenous acid, and 5% FBS. The cells were

Table 1 – Regression equation, correlation coefficients, linearity range and limit of detection (LOD) for the five PA metabolites.

Compounds	Regression equation	Linear range (nM)	R^2	LOD (nM)
DHP	Y = −55575.2 + 19962.5X	6.1–3125	0.9941	3.3
7-GS-DHP	Y = −205265 + 48877.3X	12.2–6250	0.9975	1.5
7-cysteine-DHP	Y = 2365.2 + 1932.4X	48.8–100000	0.9964	22.7
7-NAC-DHP	Y = −90630.4 + 109046X	3.1–6250	0.9963	0.4
1-CHO-DHP	Y = −380014 + 256738X	12.2–3125	0.9968	3.1

seeded at a density of 4×10^5 cell/mL into 150 cm^2 culture dishes, which were coated with 1 mg/ml PureCol (bovine collagen I) 24 h prior to use. The cells were allowed to attach for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2, then the cells were exposed to vehicle control (0.1% DMSO in DMEM) or test chemicals at 50 μM for 24 h. After exposure the cells were washed quickly with 10 mL PBS three times, then were scraped into 6 mL H_2O and disrupted using a freeze-thaw lysis method (cells were frozen in liquid nitrogen and subsequently thawed cyclically three times). After removing protein by adding two volumes of acetonitrile, the resulting suspension was centrifuged at 8000 rpm and the supernatant was collected and concentrated for LC/MS/MS SRM analysis.

2.2. *Quantitation of pyrrolic metabolites by LC-ES-MS/MS analysis*

LC-ES-MS/MS analysis was conducted as previously described [12].

2.3. *Standard characterization and calibration curves*

For quantitation of each pyrrolic metabolites, the standard curves (8 points) of the metabolites were generated by plotting the amounts of synthetic standard compounds against the peak-area ratios at the maximum absorption wavelengths (Table 1). Injection volume was 10 μL for each sample (Table 1).

3. Results

3.1. *Metabolism of senecionine by male rat, mouse, and human liver microsomes and primary rat hepatocytes*

The metabolism of senecionine by male mouse liver microsomes was conducted and the metabolites were separated by reversed-phase HPLC (Fig. 3). The chromatographic peak eluting at 38.7 min in Fig. 3 was the recovered senecionine substrate. The material in the chromatographic peak eluting at 34.1 min had the HPLC retention time, UV–visible-absorption spectrum, mass full scan profile, and mass product ion scan profile identical to those of the synthetic standard senecionine *N*-oxide. By comparison to the mass and spectral data previously reported by Eastman and Segall (1982) [22], the material eluted at 34.7 min was identified as 19-hydroxysenecionine (19-OH-senecionine). Similarly, based on the comparison of their HPLC-UV retention times, UV–visible spectra, and mass spectrometric data, the metabolites eluted at 21.8, 22.5, 24.4, 24.8, and 26.4 min were identified as 7-CYS-DHP, 7-GS-DHP, DHP, 7-NAC-DHP, and 1-CHO-DHP, respectively.

The identification of all the above-described metabolites from the metabolism of senecionine by rat and human liver microsomes, as well as from primary rat hepatocytes, was similarly conducted (data not shown).

3.2. *Quantitation of pyrrolic metabolites formed from the metabolism of senecionine by LC/MS/MS SRM mode analysis*

The pyrrolic metabolites obtained from the metabolism of senecionine by male rat, mouse, and human liver microsomes was identified by comparison of their LC/MS/MS SRM mode profiles with those of the synthetic standards (Fig. 4). LC/MS SRM chromatograms of the synthetic standards 7-cysteine-DHP, DHP, 1-CHO-DHP, 7-GS-DHP, and 7-NAC-DHP are shown in Fig. 4A. Fig. 4B shows the four of five pyrrolic metabolites formed from the metabolism of senecionine by rat liver microsomes. The metabolite of 7-NAC-DHP was not formed. Similar results were generated from the metabolism by mouse (Fig. 4C) and human liver microsomes (Fig. 4D). Differently,

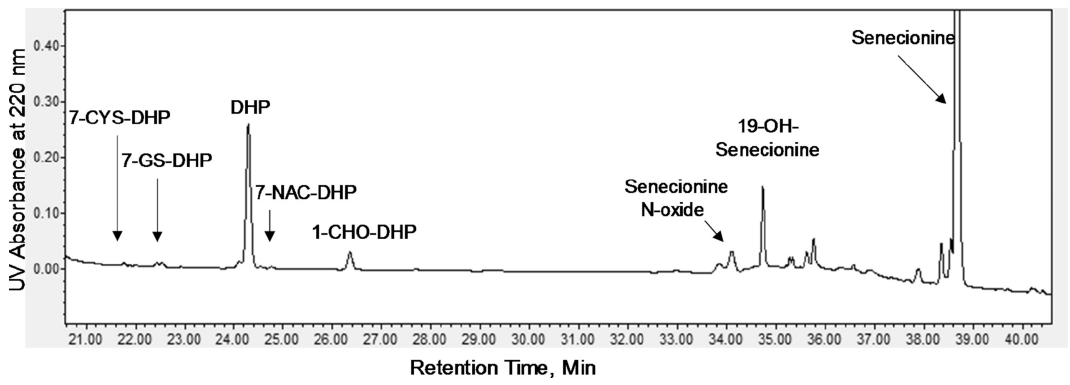

Fig. 3 – HPLC-UV profile of identified metabolites produced from the mouse liver microsomal metabolism of senecionine measured at 220 nm.

incubation of senecionine with primary rat hepatocytes generated all five pyrrolic metabolites (data not shown).

For quantitation, the LC/MS SRM chromatograms of each pyrrolic metabolite were compared to the corresponding standard characterization and calibration curves. The summarized data are listed in Table 2. The levels of secondary pyrrolic metabolites formed from the metabolism of senecionine with rat, mouse, and human liver microsomes are in the order: mouse > human > rat.

4. Discussion

The primary pyrrolic metabolites, dehydro-PAs, are highly unstable and cannot be isolated from the metabolism or any other biological systems. In the past, DHP was the only reactive pyrrolic metabolite that could be isolated from the metabolism. Recently, we demonstrated that five DNA reactive pyrrolic metabolites (DHP, 7-GS-DHP, 7-CYS-DHP, 1-CHO-

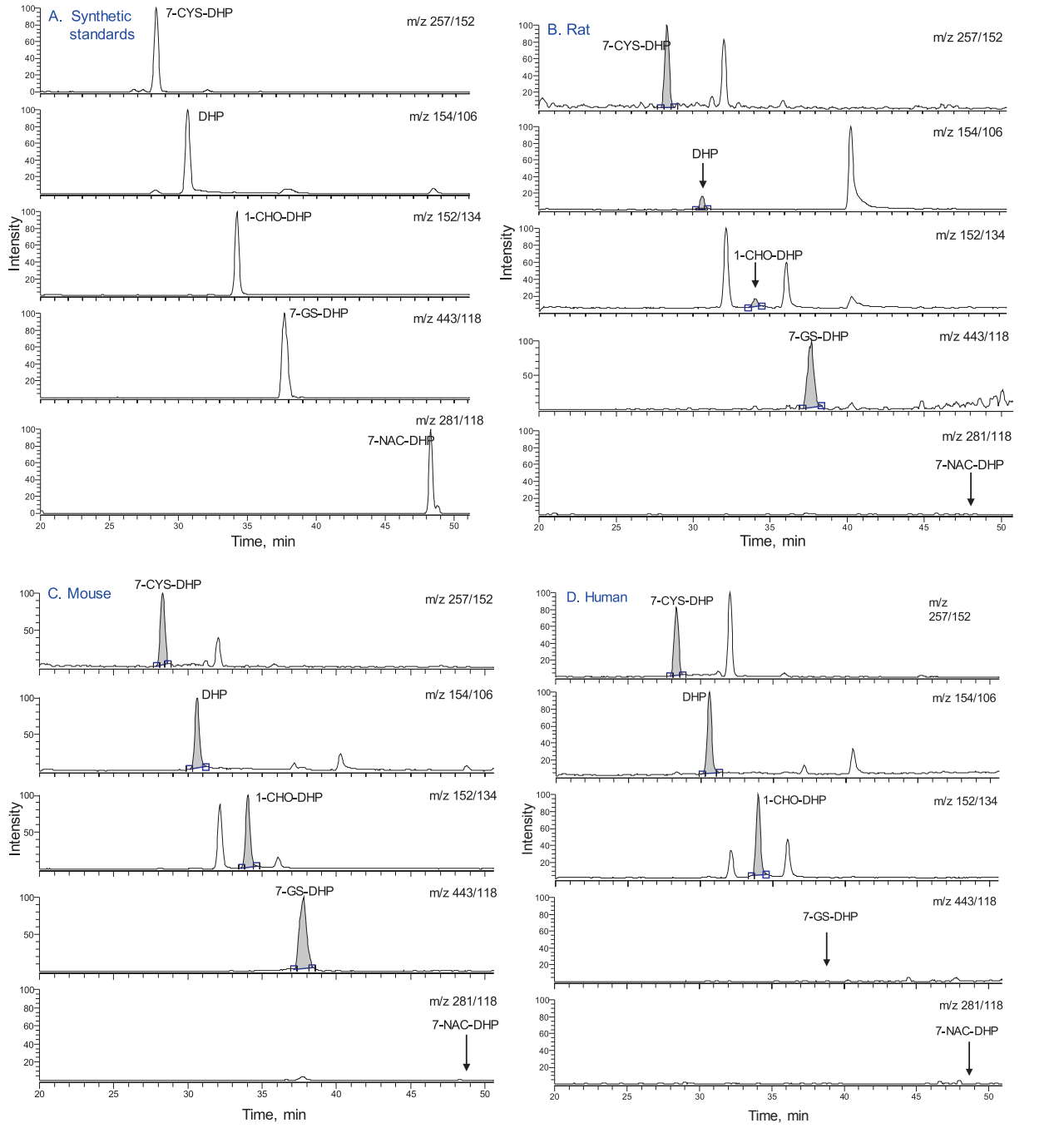

Fig. 4 – LC/MS SRM chromatograms of 7-CYS-DHP, DHP (DHR), 1-CHO-DHP, 7-GS-DHP, and 7-NAC-DHP from (a) synthetically prepared, and formed from the male (b) rat, (c) mouse, and (d) human liver microsomal metabolism of senecionine. The SRM scheme was employed involving transitions of the $[M + H]^+$ precursor ions to selected product ions with the values: *m/z* 257 → 152 for 7-CYS-DHP; 154 → 106 for DHP; *m/z* 152 → 134 for 1-CHO-DHP; *m/z* 443 → 118 for 7-GS-DHP; and *m/z* 281 → 118 for 7-NAC-DHP.

Table 2 – Quantitation of metabolites from metabolism of senecionine with liver microsomes by LC-ESIMS/MS.[a]

Metabolite	Quantitation of Metabolite (nM)			
	Rat	Mouse	Human	Primary rat hepatocytes
DHP	81 ± 11	392 ± 53	257 ± 39	154 ± 13
7-CYS-DHP	10 ± 1.5	17 ± 2.1	162 ± 27	994 ± 25
7-GS-DHP	6.4 ± 0.7	58 ± 10	ND[b]	628 ± 44
9-CHO-DHP	3 ± 0.5	387 ± 41	119 ± 10	109 ± 10
7-NAC-DHP[c]	ND	ND	ND	1138 ± 63

[a] The data are represented as the mean ± SD, n = 3.
[b] Not detected.
[c] 7–NAC–DHP detected when NAC was added in the reaction system.

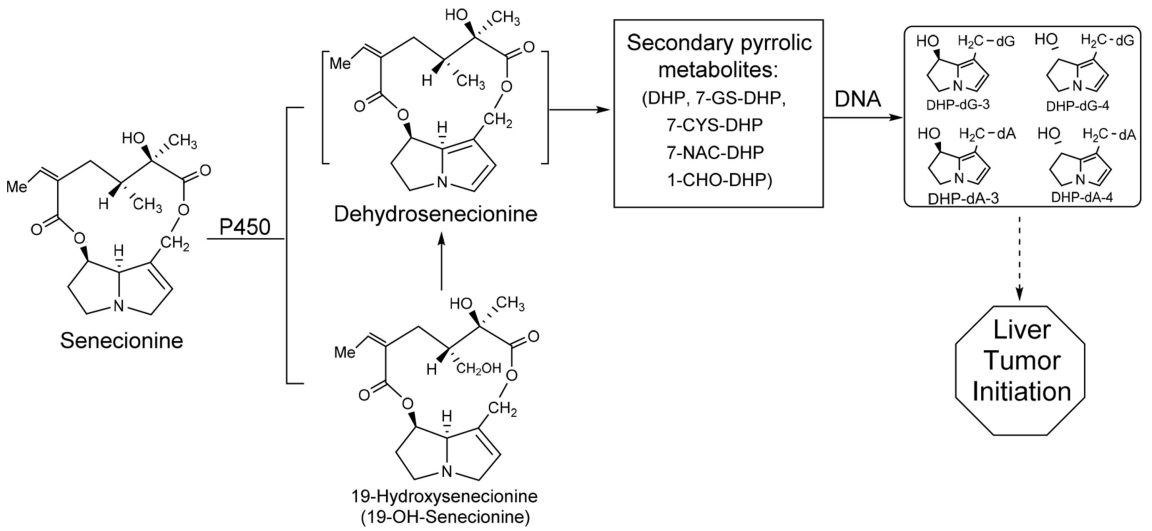

Fig. 5 – The proposed metabolic activation of senecionine by male rat, mouse, and human liver microsomal metabolism leading to the formation of five DNA reactive secondary pyrrolic metabolites resulting in the PA-induced liver tumor initiation.

DHP, and 7-NAC-DHP) are commonly generated from the metabolism of toxic PAs [10–13,15,17]. In this study, we developed a convenient LC/MS/MS SRM mode method for the quantitation of these DNA reactive pyrrolic metabolites produced from the metabolism of senecionine *in vitro*, by rat, mouse, and human liver microsomes; and from the incubation of senecionine in primary rat hepatocytes. This method requires the use of synthetically-prepared pyrrolic standards to construct calibration curves so that the pyrrolic metabolites can be chemically characterized and accurately quantified. Besides the metabolism of senecionine, this method can be conveniently utilized to quantify these five DNA reactive pyrrolic metabolites produced from the metabolism of other toxic PAs as well.

To date, the reports on the quantitative metabolism of PAs are few. The most popular quantitation method so far reported is to use the carbon-14 labelled parent PAs, specifically [^{14}C] monocrotaline [23–26]. The preparation of carbon-14 labelled PAs requires PA-containing plants grown with $^{14}CO_2$ in a closed system. In addition, the radioactive materials in both plant growth and biological studies require careful handling. Thus, our LC/MS/MS SRM method is more convenient and much safer to use.

Senecionine is one of the most studied hepatotoxic and carcinogenic Pas [1–3,10,22,27]. In the present study, up to five DNA reactive pyrrolic metabolites were formed from the metabolism of senecionine. Since these five secondary pyrrolic metabolites can bind to DNA to form the same set of DHP-dG-3/4 and DHP-dA-3/4 adducts [11,13,18,28], these results further support our previous findings that there exist multiple activation pathways leading to the formation of DHP-DNA adducts *in vitro* and in cells [10,28]. Based on this study, as shown in Fig. 5, we propose the metabolic activation of senecionine leading to the formation of DNA reactive secondary pyrrolic metabolites and potentially resulting in PA-induced liver tumor initiation.

Metabolism of senecionine produced 19-OH-senecionine (Fig. 3) formed from mouse hepatic microsomes *in vitro* [22]. It is apparent that further metabolism of 19-OH-senecionine can also produce dehydrosenecionine, which, in turn, can be metabolized into DHP, 7-GS-DHP, 7-CYS-DHP, 7-NAC-DHP, and 1-CHO-DHP (Fig. 4). Thus, the metabolic formation of these pyrrolic metabolites should be contributed by the metabolism of both senecionine and 19-OH-senecionine.

The P450-catalyzed oxidative metabolism at the necine base to form pyrrolic metabolites is considered the

predominant activation pathway; whereas the others, such as hydrolysis of the ester linkage to produce DHP and N-oxidation of the necine base to produce the corresponding PA N-oxides, are detoxification pathways [1–3,10,29]. It has been established that PA-induced cytotoxicity is highly species specific in livestock and laboratory animals [1,2]. In general, rats, mice, and humans are highly susceptible to PA intoxication. Thus, species difference in susceptibility to the PA toxicity is that the susceptible species show high formation rates for producing pyrrolic metabolites [30,31].

As the results summarized in Table 2 indicate, the levels of secondary pyrrolic metabolites formed from the metabolism of senecionine with rat, mouse, and human liver microsomes are in the order: mouse > human > rat. This study represents the first to report the species specificity in liver microsomal metabolism of the carcinogenic PA, senecionine, leading to the formation of five different DNA reactive secondary pyrrolic metabolites.

Acknowledgments

This research was supported in part by an appointment (X.H.) 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 U.S. Food and Drug Administration (FDA). This article is not an official FDA guidance or policy statement. No official support or endorsement by the FDA is intended or should be inferred.

The authors declare no competing financial interest.

REFERENCES

[1] Fu PP, Xia Q, Lin G, Chou MW. Pyrrolizidine alkaloids–genotoxicity, metabolism enzymes, metabolic activation, and mechanisms. Drug Metab Rev 2004;36:1–55.
[2] Mattocks AR. Chemistry and toxicology of pyrrolizidine alkaloids. London, NY: Academic Press; 1986.
[3] Edgar JA, Molyneux RJ, Colegate SM. Pyrrolizidine alkaloids: potential role in the etiology of cancers, pulmonary hypertension, congenital anomalies, and liver disease. Chem Res Toxicol 2015;28:4–20.
[4] Fu PP, Xia Q, He X, Barel S, Edery N, Beland FA, et al. Detection of pyrrolizidine alkaloid DNA adducts in livers of cattle poisoned with heliotropium europaeum. Chem Res Toxicol 2017;30:851–8.
[5] Fu PP, Chou MW, Churchwell M, Wang Y, Zhao Y, Xia Q, et al. High-performance liquid chromatography electrospray ionization tandem mass spectrometry for the detection and quantitation of pyrrolizidine alkaloid-derived DNA adducts *in* vitro and in vivo. Chem Res Toxicol 2010;23:637–52.
[6] Fu PP, Xia Q, Chou MW, Lin G. Detection, hepatotoxicity, and tumorigenicity of pyrrolizidine alkaloids in Chinese herbal plants and herbal dietary supplements. J Food Drug Anal 2007;15:400–15.
[7] IARC. Pyrrolizidine alkaloids. IARC Monograph on the evaluation of carcinogenic risk of chemicals to man - some naturally occurring substance. Lyon, France: International Agency for Research in Cancer; 1976.
[8] IARC. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC monographs on the evaluation of carcinogenic risks of chemicals to humans. Lyon, France: International Agency for Research in Cancer; 2002. p. 153–68.
[9] Pyrrolizidine IPCS. Alkaloids Health and safety guide. Health and safety criteria guide 26. International programme on chemical safety. Geneva, Switzerland: WHO; 1989. p. 19.
[10] Fu PP. Pyrrolizidine Alkaloids: metabolic activation pathways leading to liver tumor initiation. Chem Res Toxicol 2017;30:81–93.
[11] He X, Xia Q, Fu PP. 7-Glutathione-pyrrole and 7-cysteine-pyrrole are potential carcinogenic metabolites of pyrrolizidine alkaloids. J Environ Sci Health, Part C 2017;35:69–83.
[12] He X, Xia Q, Gamboa da Costa G, Lin G, Fu PP. 1-Formyl-7-hydroxy-6,7-dihydro-5 H-pyrrolizine (1-CHO-DHP): a potential proximate carcinogenic metabolite of pyrrolizidine alkaloids. Chem Res Toxicol 2019;32:1193–203.
[13] He X, Xia Q, Ma L, Fu PP. 7-Cysteinyl-pyrrole conjugate-a new potential DNA reactive metabolite of pyrrolizidine alkaloids. J Environ Sci Health Part C 2016;34:57–76.
[14] Xia Q, Zhao Y, Lin G, Beland FA, Cai L, Fu PP. Pyrrolizidine alkaloid-protein adducts-potential non-invasive biomarkers of pyrrolizidine alkaloid-induced liver toxicity and exposure. Chem Res Toxicol 2016;29:1282–92.
[15] Xia Q, Zhao Y, Von Tungeln LS, Doerge DR, Lin G, Cai L, et al. Pyrrolizidine alkaloid-derived DNA adducts as a common biological biomarker of pyrrolizidine alkaloid-induced tumorigenicity. Chem Res Toxicol 2013;26:1384–96.
[16] Zhu L, Xue J, Xia Q, Fu PP, Lin G. The long persistence of pyrrolizidine alkaloid-derived DNA adducts in vivo: kinetic study following single and multiple exposures in male ICR mice. Arch Toxicol 2017;91:949–65.
[17] He X, Ma L, Xia Q, Fu PP. 7-N-Acetylcysteine-pyrrole conjugate—a potent DNA reactive metabolite of pyrrolizidine alkaloids. J Food Drug Anal 2016;24:682–94.
[18] Xia Q, Ma L, He X, Cai L, Fu PP. 7-Glutathione pyrrole adduct: a potential DNA reactive metabolite of pyrrolizidine alkaloids. Chem Res Toxicol 2015;28:615–20.
[19] Yang Y-C, Yan J, Doerge DR, Chan P-C, Fu PP, Chou MW. Metabolic activation of the tumorigenic pyrrolizidine alkaloid, riddelliine, leading to DNA adduct formation in vivo. Chem Res Toxicol 2001;14:101–9.
[20] Seglen PO. Preparation of rat liver cells. I. Effect of Ca 2+ on enzymatic dispersion of isolated, perfused liver. Exp Cell Res 1972;74:450–4.
[21] Shi Q, Yang X, Greenhaw J, Salminen WF. Hepatic cytochrome P450s attenuate the cytotoxicity induced by leflunomide and its active metabolite A77 1726 in primary cultured rat hepatocytes. Toxicol Sci 2011;122:579–86.
[22] Eastman DF, Segall HJ. A new pyrrolizidine alkaloid metabolite, 19-hydroxysenecionine isolated from mouse hepatic microsomes in vitro. Drug Metab Dispos 1982;10:696–9.
[23] Estep JE, Lame MW, Morin D, Jones AD, Wilson DW, Segall HJ. [14C]monocrotaline kinetics and metabolism in the rat. Drug Metab Dispos 1991;19:135–9.
[24] Lame MW, Jones AD, Morin D, Segall HJ. Metabolism of [14C] monocrotaline by isolated perfused rat liver. Drug Metab Dispos 1991;19:516–24.
[25] Lame MW, Jones AD, Morin D, Segall HJ, Wilson DW. Biliary excretion of pyrrolic metabolites of [14C]monocrotaline in the rat. Drug Metab Dispos 1995;23:422–9.
[26] Reid MJ, Lame MW, Morin D, Wilson DW, Segall HJ. Involvement of cytochrome P450 3A in the metabolism and covalent binding of 14C-monocrotaline in rat liver microsomes. J Biochem Mol Toxicol 1998;12:157–66.

[27] Huan JY, Miranda CL, Buhler DR, Cheeke PR. Species differences in the hepatic microsomal enzyme metabolism of the pyrrolizidine alkaloids. Toxicol Lett 1998;99:127–37.
[28] Xia Q, He X, Ma L, Chen S, Fu PP. Pyrrolizidine alkaloid secondary pyrrolic metabolites construct multiple activation pathways leading to DNA adduct formation and potential liver tumor initiation. Chem Res Toxicol 2018;31:619–28.
[29] Ruan J, Yang M, Fu PP, Lin G. Metabolic activation of pyrrolizidine alkaloids: insights into the structural and enzymatic basis. Chem Res Toxicol 2014;27:1030–9.
[30] Cheeke PR. Toxicity and metabolism of pyrrolizidine alkaloids. J Anim Sci 1988;66:2343–50.
[31] Cheeke PR. A review of the functional and evolutionary roles of the liver in the detoxification of poisonous plants, with special reference to pyrrolizidine alkaloids. Vet Hum Toxicol 1994;36:240–7.