CHARACTERIZATION OF RAT LIVER MICROSMAL METABOLITES OF CLIVORINE, AN HEPATOTOXIC OTONECINE-TYPE PYRROLIZIDINE ALKALOID

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ABSTRACT:
The metabolism of the hepatotoxic otonecine-type pyrrolizidine alkaloid (PA), clivorine, was investigated using rat liver microsomes. The metabolites dehydroretronecine (DHR), 7-glutathionyl-dehydroretronecine (7-GSH-DHR), 7,9-diglutathionyldehydroretronecine (7,9-diGSH-DHR), and clivoric acid were identified using chromatographic and mass spectrometric analyses. NMR characterizations were also performed on the isolated clivoric acid and the synthetic 7-GSH-DHR and 7,9-diGSH-DHR. The results indicated that the two glutathione (GSH) conjugates were formed by reaction of the unstable toxic pyrrolic ester with GSH added in the microsomal incubation system, whereas DHR was generated from hydrolysis of the unstable pyrrolic ester, and that clivoric acid was produced from all these further conversions of the unstable pyrrolic ester. Furthermore, tissue-bound pyrroles were also determined to be present after microsomal incubation of clivorine. Clivoric acid has not been previously identified, and DHR and 7,9-diGSH-DHR were found, for the first time, as metabolites of an otonecine-type PA, while 7-GSH-DHR was previously reported by us to be a microsomal metabolite of clivorine. In vitro metabolic pathway of clivorine was delineated to be the initial formation of the unstable pyrrolic ester, which then may undergo hydrolysis, GSH conjugations, or covalent binding with hepatic tissues that may lead to hepatotoxicity. The present definitive identification of four pyrrolic ester-related metabolites of clivorine and indirect determination of bound pyrroles provide the strongest evidence to date to support the hypothesis that the formation of an unstable pyrrolic ester plays a key role in otonecine-type PA-induced hepatotoxicity.

Clivorine (Fig. 1), an hepatotoxic otonecine-type pyrrolizidine alkaloid (PA1), clivorine, is present in various Ligularia species (Klásek et al., 1967; Birnbaum et al., 1971; Kuhara et al., 1980; Mori et al., 1985), for example, the antitussive traditional Chinese medicinal herb L. hodgsonii Hook (Lin et al., 1998b, 2000). It is well documented that human and farm animal poisonings through consumption of PA-containing plants, herbal teas, and herbal medicines (Mattocks, 1968; McLean, 1970; White et al., 1973; Huxtable, 1980; Mattocks, 1986; Huxtable, 1989). These toxic PAs are generally classified structurally into two types, namely, retronecine-type [or heliotridine-type, a 7(S)-isomer of retronecine] and otonecine-type (Mattocks, 1986), in which characteristically the 8-membered heterocyclic necine base is bicyclic and monocyclic, respectively (Fig. 1).

The mechanism of hepatotoxicity induced by retronecine-type PAs has been extensively investigated. It is established that the oxidative biotransformation of this type of PA to the corresponding dehydro-PAs, the chemically reactive pyrrolic esters, plays a key role in causing hepatotoxicity, since the generated unstable pyrrolic esters rapidly form covalent adducts with cellular macromolecules in the liver (Mattocks, 1968, 1986; Mattocks and White, 1971; White and Mattocks, 1971; Huxtable, 1989; Hinson et al., 1994; Castagnoli et al., 1997). Mattocks and White (1971) also proposed a similar mechanism for otonecine-type PAs. This mechanism has gained support from our recent discovery of 7-glutathionyldehydroretronecine (7-GSH-DHR) as a metabolite of clivorine (Lin et al., 1998a). This finding has prompted more thorough investigation into the mechanism of otonecine-type PA-induced hepatotoxicity, using clivorine as model substrate. The aim of the present study was to investigate the in vitro metabolic profile of clivorine in male rat liver microsomes with emphasis on the formation of the toxic pyrrolic ester and its related metabolites.

Materials and Methods

Chemicals. The reduced form of glutathione (GSH), monocrotaline, retorsine, and all other chemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, MO). Clivorine was isolated from Ligularia hodgsonii Hook by a standard alkaloid extraction procedure for PAs (Lin et al., 2000). The identity of isolated clivorine was confirmed by UV, infrared, NMR,
and MS analyses, and its purity was determined to be higher than 99% by thin-layer chromatography, HPLC, and NMR spectroscopy.

**Apparatus and Conditions.** The on-line HPLC/MS analyses for the identification of the GSH conjugates were performed on a Hewlett Packard 1100 liquid chromatograph connected to a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer coupled with an electrospray ionization (ESI) interface. The analytes were eluted on a Hamilton PRP-1 reversed phase HPLC column (5 μ, 150 × 4.0-mm i.d.) (Hamilton Co., Reno, NV), with the mobile phase consisting of 1% acetic acid (A) and acetonitrile (B), using the following operating conditions: initial 0 to 5 min, 100% A; 5 to 35 min, linear change to 75% A, and maintained for 5 min. The flow rate was kept constant at 0.8 ml/min.

ESI-MS was performed under the operating conditions of 5 kV of spray voltage, sheath gas setting at 50 psi, 15 units of auxiliary gas, and a heated capillary temperature at 250°C. Full scan mass spectra were obtained over the mass range of m/z 150 to 850 in the negative ion mode.

Both positive and negative ion ESI-MS analyses with direct loop injection were also carried out in the Finnigan TSQ 7000 mass spectrometer under conditions similar to HPLC/MS, but with the solvent system consisting of MeOH and H2O (1:1 containing 1% acetic acid at a flow rate of 0.8 ml/min. In addition, negative ion source collision induced dissociation (CID) ESI-MS with a potential of 50 eV was also measured for the structural elucidation of clivorine. The Hewlett Packard 1100 chromatographic system coupled with a photodiode array (PDA) multiple wavelength UV detector set at 230 nm was utilized for all HPLC quantitation studies, while UV spectra for the initial 0 to 5 min, with the guard column directly connected to a waste bottle, isocratic elution was performed with mobile phase A (0.2% formic acid and H2O) was recorded by the PDA detector for the identification of dehydroretronecine (DHR) and intact clivorine. 1H and 13C NMR spectra in D2O were recorded on a Bruker ARX-500 spectrometer using the peak of TMS as reference.

**Preparation of Authentic Samples.** DHR was synthesized from monocrotaline by adoption of a reported method (Mattocks, 1982). 7-GSH-DHR was obtained using a previously described method that involved o-chloranil oxidation of monocrotaline to dehydromonocrotaline followed by reaction of dehydromonocrotaline with a limited amount of GSH in phosphate buffer (pH 7.4) (Mattocks et al., 1989, 1991). 7,9-Diglutathionyldehydroretronecine (7,9-diGSH-DHR) was prepared similarly from monocrotaline, but an excess of Na2S2O4 was added. The resultant precipitate was collected and dissolved in 20% aqueous acetonitrile as the mobile phase to remove ammonium formate. Subsequently, the collected fraction was evaporated under reduced pressure to remove acetonitrile, and the aqueous fraction was lyophilized to give pure clivorine as a white powder.

**Identification of Metabolites.** The supernatants of incubates were directly subjected to HPLC/MS analysis. The identities of 7-GSH-DHR, 7,9-diGSH-DHR, and clivorine generated in microsomal incubation were confirmed by a direct comparison of the retention time and mass spectrometric data with that for authentic standards. The isolated clivorine acid was also characterized by NMR and direct loop injection MS analyses. Although DHR and the unchanged clivorine present in the incubated mixture characteristically could not be detected by the negative ion mode HPLC/MS analysis, their identities were verified by comparing the HPLC retention time and UV spectrum with those obtained for the corresponding authentic standards.

**Preparation of Rat Liver Microsomes.** Adult male Sprague-Dawley rats (body weight, 200–250 g) supplied by the Laboratory Animal Services Center at the Chinese University of Hong Kong were used. Microsomes were prepared by a standard procedure (Williams et al., 1989), and protein content was determined using a modified procedure of the method of Lowry et al. (1951).

Briefly, liver samples were homogenized in 3 volumes of ice-cold 0.25 M sucrose solution buffered with 0.1 M Tris buffer (pH 7.4). Microsomes and cytosol were isolated after centrifugation of the whole liver homogenate at 10,000g for 30 min. The resuspended microsomes were reincubated at 105,000g for 60 min. The microsomal pellets were suspended in 0.25 M sucrose solution (1:1, volume/liver weight) and stored at −80°C until use.

**Microsomal Incubation and Treatment of Incubated Samples.** A typical microsomal incubation mixture (10 ml) in potassium phosphate buffer (100 mM, pH 7.4) contained liver microsomes (2 mg of protein/ml), 0.25 mM clivorine, 1 mM NADH, 4 mM MgCl2, 1 mM NADP, 10 mM glucose-6-phosphate, 1.0 unit/ml glucose-6-phosphate dehydrogenase, and 2.0 mM GSH. Incubations were initiated by the addition of glucose-6-phosphate dehydrogenase, performed at 37°C for 60 min, and terminated by chilling in an ice bath. A similar incubation system without the addition of GSH was also performed in the same manner. Furthermore, incubation in the absence of the NADPH-generating system was performed for the confirmation of cytochrome P450-dependent enzymatic biotransformation. Incubations with various concentrations of GSH and incubation times and the presence or absence of NADH (1 mM) were conducted for the assessment of the optimal incubation condition. In addition, various controls, including the use of denatured microsomes and incubation in the absence of substrate, were also conducted.

The resultant incubates were centrifuged at 105,000g at 2°C for 20 min. Aliquots (200 μl) of the supernatant were directly subjected to HPLC/MS for qualitative studies and coupled-column HPLC for quantitative analysis. In addition, the resultant pellets for all different incubations were used for the determination of hepatic tissue-bound pyrroles. Incubations for each individual condition were conducted in triplicate.

For the preparation of a large quantity of clivoric acid, a scaled-up incubation system (200 ml) without addition of GSH was performed similarly. The resultant supernatant was lyophilized, and the residues were extracted by ethyl acetate (2 × 200 ml). The organic layers were filtered, combined, and concentrated under reduced pressure. The residues were reconstituted into 50% aqueous acetonitrile and subjected to preparative HPLC with a C18 column (5 μ, 250 × 10-mm i.d.) and the mobile phase of acetonitrile:0.2% ammonium formate (2:8) at a flow rate of 2.0 ml/min. Fractions containing clivoric acid were collected, concentrated, and subjected to further HPLC purification with 20% aqueous acetonitrile as the mobile phase to remove ammonium formate. Subsequently, the collected fraction was evaporated under reduced pressure to remove acetonitrile, and the aqueous fraction was lyophilized to give pure clivoric acid as a white powder.

**Quantification of Metabolites.** The HPLC assay recently developed in our research laboratory (Cui and Lin, 2000) using two Hamilton PRP-1 columns (guard column: 50 × 4.1 mm, 5 μ; analytical column: 250 × 4.6 mm, 5 μ) was adopted for the quantitative studies of each individual incubation in triplicate. For the initial 0 to 5 min, with the guard column directly connected to a waste bottle, isocratic elution was performed with mobile phase A (0.2% formic acid adjusted to pH 3.4 by ammonia) to wash out the aqueous soluble impurities. At 5 min, using a six-port valve, the guard column was switched to connect the analytical column. Subsequently, analytes were separated by gradient elution with mobile phases A and B (acetonitrile) as follows: at 5 to 35 min, linear change from 100% A to 75% A; and at 35 to 40 min, linear change from 75% A to 70% A. The flow rate was kept constant at 1.0 ml/min for the complete analysis. Peak responses were measured at 230 nm by a PDA detector. The data for the calibration curves of clivoric acid and each of its four metabolites, i.e., DHR, 7-GSH-DHR, 7,9-diGSH-DHR, and clivorine acid, were obtained using an internal standard method with retorosine, a retronecine-type PA, as the internal standard. The quantity of each analyte present in different incubates was determined from the corresponding calibration curve.

**Determination of Bound Pyrroles.** The total tissue-bound pyrroles formed at the Chinese University of Hong Kong were used. Microsomes were prepared by a standard procedure (Williams et al., 1989), and protein content was determined using a modified procedure of the method of Lowry et al. (1951).
after incubation was estimated by modification of a reported method (Yan and Huxtable, 1995). Briefly, the pellets obtained after centrifugation of incubates were homogenized in 6.0 ml of ethanol and centrifuged at 900g for 5 min. The residue obtained was washed with ethanol (3.0 ml) and reconstituted into ethanolic silver nitrate (1.0 ml). The resultant mixture was shaken for 30 min and then centrifuged at 900g for 5 min. The residue obtained was extracted with ethanol (2 × 1.0 ml). Ehrlich reagent (0.5 ml) was added to the combined ethanol extract (1.5 ml), and the resultant mixture was heated at 55°C for 10 min. Absorbance of the sample was measured at both 562 and 625 nm, respectively. Adjusted absorbance (A) was determined from $A = 1.1(A_{441} - A_{GSH})$ Bound pyrrolic metabolite levels were calculated using a molar absorbivity of 60,000 (Yan and Huxtable, 1995).

### Results

**Characterization of the Synthetic Putative Metabolites.** Structures of the synthetic 7-GSH-DHR and 7,9-diGSH-DHR were elucidated by negative ion ESI-MS with direct loop injection and 1H NMR spectroscopic analyses. The MS spectrum of 7-GSH-DHR showed the pseudomolecular ion ([M – 1]) at m/z 441 as the base peak. Similarly, the MS spectrum of 7,9-diGSH-DHR exhibited the highest mass unit ion at m/z 730 with 100% intensity, which corresponds to the pseudomolecular ion ([M – 1]) of dehydroretronecine conjugated with two glutathione molecules. 1H NMR results also confirmed their structural identities. The NMR data assignments for these two compounds were compatible with the published 1H NMR data for several dehydropyrroloidizine alkaloids (Logie et al., 1994), including DHR (Robertson, 1982), as well as GSH obtained in the present study (Table 1). Compared with DHR, a significant 0.6-ppm upfield shift of the H-7 proton (δ 4.33–4.46) and the absence of a marked shift of the H-9 signals for 7-GSH-DHR clearly indicated that GSH conjugation occurred at the 7 position. For the NMR spectrum of 7,9-diGSH-DHR, compared with 7-GSH-DHR, the signal for H-7 was comparable, whereas the H-9 signals (δ 3.56–3.65) significantly shifted upfield 0.8 ppm due to a shielding effect of glutathionyl substitution, indicating that a second GSH moiety was present at the 9 position. Furthermore, the signals for the other hydrogen atoms of 7-GSH-DHR and 7,9-diGSH-DHR were consistent with those obtained for both DHR and GSH. Therefore, the structures of both synthetic pyrrolic and 7,9-diGSH-DHR were consistent with those obtained for both DHR and GSH incubations, the negative ion ESI mass spectra of the components corresponding with the first two chromatographic peaks showed the base peaks at the highest mass unit ion at m/z 441 and 730 that corresponded to the quasi-molecular ions ([M – H]–) of 7-GSH-DHR and 7,9-diGSH-DHR, respectively. For direct comparison, the denatured micromsomal incubate spiked with the synthetic 7-GSH-DHR and 7,9-diGSH-DHR was also analyzed by HPLC/MS under the same conditions to provide standard chromatographic patterns and mass spectra. As shown in Table 2, the chromatographic behaviors and MS data for the first two eluted metabolites were identical with those for the two synthetic GSH conjugates. Therefore, these two metabolites were unequivocally identified as the pyrrolic alcohol glutathione conjugates 7-GSH-DHR and 7,9-diGSH-DHR, respectively.

In the case of HPLC/MS analysis of the third peak found in the incubates with addition of GSH and the only metabolite generated in the absence of GSH, the ESI-MS spectra showed an indicative quasi-molecular ion ([M – 1]) at m/z 255. In the absence of a synthetic standard, further confirmation of its identity was conducted by negative ion ESI-MS with direct loop injection and NMR analyses of the sample isolated from micromsomal incubates. The negative ion in-source CID MS spectrum of the isolated compound (Fig. 2) exhibited the base peak at m/z 255, corresponding to the pseudomolecular ion ([M – H]–) of the assigned structure, and other fragmentation ions at m/z 195, 151, 123, and 107. Both 1H and 13C NMR data assignments (Table 1) are in good agreement with those obtained for the acidic moiety of clorivone (Lin et al., 2000). Therefore, this unknown metabolite was definitively characterized as 2-acetoxy-5-carboxy-2,3-dimethyl-4,6-heptadienoic acid, which corresponds to the necic acid moiety of clorivone. This novel metabolite has not been reported previously and thus was named clivoric acid (Fig. 3).

**Table 1**

<table>
<thead>
<tr>
<th>Position</th>
<th>7-GSH-DHR δH (D2O)</th>
<th>7,9-diGSH-DHR δH (D2O)</th>
<th>GSH δH (D2O)</th>
<th>DHR δH (CDCl3)</th>
<th>Clivoric Acid</th>
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</thead>
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<tr>
<td>2</td>
<td>6.10 (1H, d)</td>
<td>6.09 (1H, d)</td>
<td>5.95 (1H, d)</td>
<td>6.47 (1H, d)</td>
<td>177.26 (0)</td>
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<td>3</td>
<td>6.55 (1H, d)</td>
<td>6.58 (1H, d)</td>
<td>7.36 (1H, m)</td>
<td>7.36 (1H, m)</td>
<td>85.23 (0)</td>
</tr>
<tr>
<td>5</td>
<td>3.84 (1H, m), 3.94 (1H, m)</td>
<td>3.82 (1H, m), 3.92 (1H, m)</td>
<td>3.95 (1H, m)</td>
<td>3.95 (1H, m)</td>
<td>43.20 (3)</td>
</tr>
<tr>
<td>6</td>
<td>2.70 (0.5H, m), 2.82 (0.5H, m)</td>
<td>2.72 (0.5H, m), 2.82 (0.5H, m)</td>
<td>2.19 (1H, m)</td>
<td>4.56 (1H, d)</td>
<td>132.81 (1)</td>
</tr>
<tr>
<td>7</td>
<td>4.33–4.46 (1H, m)</td>
<td>4.26 (0.5H, dd), 4.48 (0.5H, dd)</td>
<td>4.95 (1H, m)</td>
<td>5.63 (1H, d)</td>
<td>140.05 (0)</td>
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<tr>
<td>9</td>
<td>4.33–4.46 (2H, m)</td>
<td>3.56–3.65 (2H, m)</td>
<td>4.29 (2H, m)</td>
<td>4.03 (1H, d)</td>
<td>135.88 (1)</td>
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<tr>
<td>2*</td>
<td>2.35 (1H, m), 2.82 (1H, m)</td>
<td>2.35 (1H, m), 2.72 (1H, m)</td>
<td>2.94 (2H, t)</td>
<td>7.51 (1H, d)</td>
<td>116.55 (2)</td>
</tr>
<tr>
<td>3*</td>
<td>4.33–4.46 (1H, m)</td>
<td>4.31–4.36 (2H, m)</td>
<td>4.65 (1H, t)</td>
<td>5.16 (1H, d)</td>
<td>20.32 (3)</td>
</tr>
<tr>
<td>6*</td>
<td>2.35 (2H, m)</td>
<td>2.35 (4H, m)</td>
<td>2.55 (2H, t)</td>
<td>1.08 (3H, d)</td>
<td>15.44 (3)</td>
</tr>
<tr>
<td>7*</td>
<td>1.95 (2H, m)</td>
<td>2.00 (4H, m)</td>
<td>2.17 (2H, q)</td>
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<td>17.45 (0)</td>
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<tr>
<td>8*</td>
<td>3.52 (1H, t)</td>
<td>3.56–3.65 (2H, m)</td>
<td>3.83 (1H, t)</td>
<td>1.08 (3H, d)</td>
<td>21.87 (3)</td>
</tr>
<tr>
<td>12*</td>
<td>3.62 (2H, s)</td>
<td>3.56–3.65 (4H, m)</td>
<td>3.98 (2H, s)</td>
<td>1.08 (3H, d)</td>
<td>21.87 (3)</td>
</tr>
</tbody>
</table>

* Data cited from Robertson (1982).
** Number of hydrogen attached is indicated in parentheses.
* Overlapping signals.
olites were mainly catalyzed by NADPH-dependent cytochrome P450somal metabolism of clivorine and the formation of pyrrolic metab-
of NADH (data not shown). These results indicated that the micro-
the addition of NADH, the amount of clivorine metabolized and
generation of the glutathione metabolites were investigated. Regard-
Lin, 2000). The optimal microsomal incubation conditions in the
were determined by our previously developed HPLC assay (Cui and
metabolites formed in various incubations in the presence of GSH
incubation without the addition of GSH. Furthermore, the bound
addition to the metabolites found by HPLC/MS analyses, the further
metabolite in the incubations was identified as DHR by comparing the
addition, the pyrrolic metabolites, solely as DHR and the tissue-bound
amount of GSH conjugates formed did not significantly increase
trapped by GSH, and thus less bound pyrroles and DHR were formed.
DHR and bound pyrroles significantly decreased. This result sug-
noteworthy, with an increase in GSH concentration the formations of
addition of 0.5 M GSH resulted in enhanced metabolism of clivorine
the concentration of GSH in the incubation system
significantly affected the metabolic profile of clivorine (Fig. 5B). The
the addition of 0.5 M GSH resulted in enhanced metabolism of clivorine
with increased levels of clivoric acid, but no further marked changes
occurred with these two analytes at higher GSH concentration. Most
worthy, with an increase in GSH concentration the formations of
the two pyrrolic GSH conjugates increased, whereas the formations of
DHR and bound pyrroles significantly decreased. This result sug-
gested that in the presence of GSH, the reactive pyrrolic ester was
trapped by GSH, and thus less bound pyrroles and DHR were formed.
The decrease in bound pyrolole level was maximal at 2 mM GSH, and
the amount of GSH conjugates formed did not significantly increase
when the concentration of GSH was higher than 1 mM. Consequently,
the addition of NADH (1 mM) and GSH (2 mM) at 37°C for 1 h
was chosen as an appropriate incubation condition to further study the in
vitro metabolism of clivorine, including formation of glutathione
metabolites.

The amount of clivorine remaining and metabolites formed after
incubation of substrate in the presence and absence of added GSH for
1 h is summarized in Table 3. In the absence of added GSH, 25%
clivorine remained, and the predominant metabolite was identified as
clivoric acid, which accounted for 59% of the incubated clivorine. In
addition, the pyrrolic metabolites, solely as DHR and the tissue-bound

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<td></td>
<td>HPLC Rt min m/z</td>
<td>HPLC Rt min m/z</td>
</tr>
<tr>
<td>Clivorine</td>
<td>35.2 N.D.</td>
<td>35.2 N.D.</td>
</tr>
<tr>
<td>DHR</td>
<td>16.2 N.D.</td>
<td>16.7 N.D.</td>
</tr>
<tr>
<td>7-GSH-DHR</td>
<td>19.2 441 (100%)</td>
<td>19.2 441 (100%)</td>
</tr>
<tr>
<td>7,9-DiGSH-DHR</td>
<td>21.6 730 (100%)</td>
<td>21.6 730 (100%)</td>
</tr>
<tr>
<td>Clivoric acid</td>
<td>34.0 255 (100%)</td>
<td>34.0 255 (100%)</td>
</tr>
</tbody>
</table>

N.D., not detected by HPLC/MS with negative ion electrospray ionization; Rt, retention time.
*a R was obtained by HPLC/UV analysis.
*b Reference sample was isolated from scaled-up microsomal incubation.

Optimization of the Microsomal Incubation System and Quan-
tification of Metabolites. The amount of intact clivorine and the four
metabolites formed in various incubations in the presence of GSH
were determined by our previously developed HPLC assay (Cui and
Lin, 2000). The optimal microsomal incubation conditions in the
generation of the glutathione metabolites were investigated. Regard-
ing the addition of NADH, the amount of clivorine metabolized and
clavoric acid formed markedly increased, whereas the generation of
the three pyrrolic metabolites was similar in the presence or absence
of NADH (data not shown). These results indicated that the micros-
metabolism of clivorine and the formation of pyrrolic metabol-
olites were mainly catalyzed by NADPH-dependent cytochrome P450
monooxygenase. NADH may also be a cofactor in the catalytic cycle
of cytochrome P450 and/or flavin adenine dinucleotide-containing
monooxygenases mediating the metabolism of clivorine; however,
such NADH-dependent enzymes likely only play a minor role in the
catalysis of the observed metabolism of clivorine. In the case of
incubation time (Fig. 5A), the metabolism of clivorine and the for-
mations of DHR, 7-GSH-DHR, and clavoric acid were formed in the
first 15 and 30 min, respectively. Therefore, to obtain relatively high
amounts of all the metabolites generated for both qualitative and
quantitative analyses, the incubation time was chosen to be 1 h for the
present study. The concentration of GSH in the incubation system
significantly affected the metabolic profile of clivorine (Fig. 5B). The
addition of 0.5 M GSH resulted in enhanced metabolism of clivorine
with increased levels of clivoric acid, but no further marked changes
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The amount of clivorine remaining and metabolites formed after
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1 h is summarized in Table 3. In the absence of added GSH, 25%
clivorine remained, and the predominant metabolite was identified as
clavoric acid, which accounted for 59% of the incubated clivorine. In
addition, the pyrrolic metabolites, solely as DHR and the tissue-bound
pyrroles, were determined to be 31 and 0.16% of the incubated clivorine, respectively. With the addition of GSH, less clivorine remained (4%), and the amount of clivoric acid generated markedly increased to 90% of the incubated clivorine. Regarding pyrrolic metabolites, less DHR (12%) and bound pyrroles (0.09%) were generated, whereas 7-GSH-DHR and 7,9-diGSH-DHR were found to be about 13 and 3% of the incubated clivorine, respectively.

**Discussion**

Previous investigations of the metabolism of retronecine-type PAs have clearly indicated that the pyrrolic esters, which play the key role in PA toxicity, are unstable and cannot be directly isolated. Once formed, they either rapidly react with water and other soluble nucleophiles, such as GSH, to form DHR and metabolites, such as glutathione conjugates, or covalently bind nucleophilic macromolecules to generate tissue-bound pyrroles (Hinson et al., 1994; Castagnoli et al., 1997). The tissue-bound pyrroles, especially those having a thioether bond between the pyrrolic nucleus and tissue constituents, can be determined by established methods (Mattocks and Jukes, 1990; Yan and Huxtable, 1995). In the present study the total amount of bound pyrroles formed after incubation of clivorine was determined by adoption of a published method (Yan and Huxtable, 1995). Furthermore, three pyrrolic metabolites, i.e., DHR, 7-GSH-DHR, and 7,9-diGSH-DHR, which are formed from further biotransformations of dehydroclivorine, the corresponding pyrrolic ester, were also identified. Therefore, firm evidence was obtained for the formation of the pyrrolic ester in the metabolism of otonecine-type PAs.

The two pyrrolic alcohol GSH conjugates, 7-GSH-DHR and 7,9-diGSH-DHR, were only detected in the microsomal incubation after the addition of GSH. Furthermore, this addition of GSH resulted in decreased amounts of both DHR and tissue-bound pyrroles (Table 3). These results indicated that a significant proportion of the reactive pyrrolic ester formed was rapidly trapped by GSH to form the corresponding conjugates. Thus, employment of a GSH-containing microsomal incubation system is a useful in vitro method to demonstrate the existence of a reactive pyrrolic ester. DHR and 7-GSH-DHR have been previously reported to be both in vivo (Buhler et al., 1990; Lamé et al., 1990) and in vitro (Mattocks et al., 1991; Reed et al., 1992; Lin et al., 1998a) metabolites of retronecine-type PAs. Moreover, DHR has been suggested to be a rat liver microsomal metabolite of an otonecine-type PA, specifically otosenine, based only on the chromatography and color reactions of this metabolite (Culvenor et al., 1971).
Fig. 4. The representative chromatograms of HPLC/UV analysis of control microsomal incubate (A), control microsomal incubate spiked with authentic samples (B), microsomal incubate in the presence of GSH (C), and microsomal incubate in the absence of GSH (D).
In the present study, both 7,9-diGSH-DHR and the previously proven 7-GSH-DHR (Lin et al., 1998a) were identified as in vitro microsomal metabolites of clivorine. Previous reports on the identification of 7-GSH-DHR were based primarily on mass spectrometric analysis (Buhler et al., 1990; Lame´ et al., 1990; Mattocks et al., 1991; Reed et al., 1992), and in fact, the 1H NMR data for only the two pyrrolic protons (nonspecifically assigned as doublets at δ 6.15 and 6.60) have been reported previously (Robertson et al., 1977). 7-GSH-DHR is an unstable compound with both pH- and temperature-dependent sensitivity and hence difficult to isolate when formed either synthetically or metabolically.

In the present study, purification of the two synthetic GSH conjugates by preparative HPLC immediately after their formation enabled samples to be obtained for 1H NMR analysis (Table 1). Four signals, each having a peak integration equivalent to half a proton, were observed for the two H-6 atoms in both spectra. Furthermore, the H-7 proton in 7,9-diGSH-DHR also exhibited two signals with a 0.5:0.5 proton integration ratio, although the same signal pattern in the spectrum of 7-GSH-DHR overlapped with other protons and could not be definitively assigned. These data indicate that a racemic mixture of 7(S)- and 7(R)-GSH-DHR enantiomers was formed from reaction of dehydro-PA with GSH that might initially involve S₉₁ reaction at the 7 position of dehydro-PA, such as ester hydrolysis, followed by GSH substitution. 7,9-DiGSH-DHR may form either by further glutathionyl substitution at the 9 position of 7-GSH-DHR or by two simultaneous GSH substitutions at both the 7 and 9 positions of the corresponding dehydro-PA (Fig. 3). In a previous study, evidence was obtained for the in vitro formation of 7(S)- and 7(R)-DHR-deoxyguanosine enantiomers as the DNA adducts of the reactive pyrrolic ester (Robertson, 1982). Furthermore, it has been demonstrated that the extent of formation of 7-GSH-DHR via reaction of GSH directly with DHR is very low at physiological pH, while 7,9-diGSH-DHR is not obtained after direct reaction of DHR with GSH at physiological pH. Consequently, pyrrolic alcohol GSH conjugates are formed primarily by reaction of GSH with unstable dehydro-PAs, the corresponding reactive pyrrolic esters, generated enzymatically (Buhler et al., 1990; Reed et al., 1992). Therefore, a similar S₉₁ reaction is suggested to occur in the present metabolic formations of the two GSH conjugates to give the corresponding racemic mixtures of 7-enantiomers. However, confirmation of this suggestion is awaited, since large enough samples of enzymatically produced GSH conjugates could not be obtained to enable 1H NMR analysis in the present study.

Clivorine acid, the corresponding necic acid metabolite of clivorine, has not been previously identified. Although appropriate configurational spectroscopic analyses were not conducted in the present study, the two chiral centers are most likely s configurations (Fig. 3), since the configuration of the corresponding acidic moiety in clivorine would unlikely change during the hydrolysis process. The formation of clivorine acid may arise via two alternate pathways, i.e., either direct hydrolysis of clivorine or further hydrolysis of the pyrrolic ester. However, the former was ruled out because clivorine acid could not be detected when clivorine was incubated in the microsomal system without the added NADPH-generating system. Furthermore, clivorine acid was not found when clivorine was incubated in rat liver cytosolic fractions and rat liver microsomes in the presence of cytochrome P450 inhibitors (unpublished work performed in this laboratory). Therefore, as shown in Fig. 3, the necic acid arises primarily from further metabolism of the pyrrolic ester, such as in concurrent formation of DHR, glutathione conjugates, and bound pyroles.

It is interesting to note that the rate of metabolism of clivorine signif-

![Fig. 5. Effects of incubation time (A) and concentration of GSH (B) on the rat liver microsomal metabolic profile of clivorine.](image-url)
tissue-bound pyrroles were quantified. These results provide the strongest evidence to date in support of the hypothesis that, like retronecine-type PA, otonecine-type PA intoxication may also involve the formation of chemically reactive pyrrolic ester(s) and covalent binding of such ester(s) to cellular macromolecules.

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References


