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Simultaneous analysis of clivorine and its four microsomal metabolites by high-performance liquid chromatography

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Abstract

A specific high-performance liquid chromatographic assay was developed for a simultaneously qualitative and quantitative determination of clivorine, a hepatotoxic otonecine-type pyrrolizidine alkaloid, and its four putative hepatotoxicity-related metabolites, namely dehydroretronecine, 7-glutathionyldehydroretronecine, 7,9-diglutathionyldehydroretronecine, and clivoric acid, generated in rat microsomal incubation. This simultaneous determination was conducted by a direct analysis of aliquots of the supernatant of incubates using a specific two-column set-up. Impurities in the supernatant were firstly eluted out from the first PRP-1 guard column (50×4.1 mm) during an initial 5 min washing period with isocratic elution by mobile phase A (0.2% formic acid at pH 3.4 adjusted by ammonia). Subsequently, the guard column was then connected to the second PRP-1 analytical column (250×4.6 mm) and analytes were separated by a gradient elution with mobile phases A and B (acetonitrile). The assay provided good reproducibility and accuracy for all analytes tested with less than 12% of overall intra- and inter-day variations and higher than 87% of overall accuracy. This developed method was successfully applied to determine the intact clivorine and its four metabolites generated in rat microsomal incubation.

Keywords: Validation; Clivorine; Dehydroretronecines; Pyrrolizidine alkaloids; Alkaloids

1. Introduction

Clivorine (Fig. 1), an otonecine-type pyrrolizidine alkaloid found in various *Ligularia* species [1,2] including *L. hodgsonii* Hook, an antitussive traditional Chinese medicinal herb [3], is known to cause hepatic injury in rats [4–6]. Pyrrolizidine alkaloids (PAs) naturally occur in a wide variety of plant species worldwide and many of them are hepatotoxic to both livestock and human [7–9]. The toxic PAs are generally classified into two different types: retronecine-type and otonecine-type. The metabolism-induced hepatotoxicity by the former type PA has been extensively investigated. This type of PA is bioactivated by hepatic cytochrome P450 monooxygenases to the chemically reactive pyrrolic esters (the corresponding dehydro-PAs), which injure liver by a rapid covalent binding with hepatic cellular macromolecules [10–13]. However, the underlining mechanism of the latter type PA-induced hepato-

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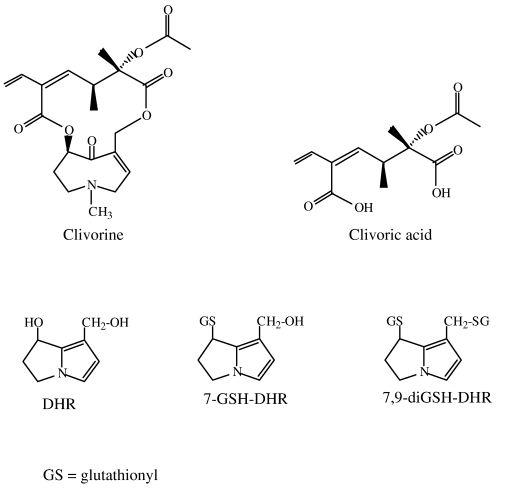

Fig. 1. Structures of clivorine, clivoric acid, dehydroretronecine, 7-glutathionyldehydroretronecine and 7,9-diglutathionyldehydroretronecine.

toxicity was not studied until a recent investigation of in vitro metabolites of clivorine was conducted by our research laboratory [14]. Like retronecine-type PA, a similar metabolic pathway of clivorine was observed in rat microsomal incubation, and four metabolites, namely dehydroretronecine (DHR), 7-glutathionyldehydroretronecine (7-GSH-DHR), 7,9-diglutathionyldehydroretronecine (7,9-diGSH-DHR), and clivoric acid, have been unequivocally identified in our recent study (Fig. 1). The former three are pyrrolic metabolites, and all these four metabolites are generated from further biotransformations of pyrrolic ester of clivorine and thus are putative hepatotoxicity-related metabolites [14]. Therefore, this result demonstrated that the reactive pyrrolic esters also play a key role in the hepatotoxicity induced by otonecine-type PA.

Several analytical methods including UV photometric assay [15] and HPLC analyses [16–18] have been previously reported for the determination of metabolites of retronecine-type PAs including the intact PA, its corresponding N-oxides, and DHR in both biological samples and in vitro incubated mixtures. A HPLC assay for the analysis of 7-GSH-DHR generated in microsomal incubation with senecionine was also documented [19]. However, all these published HPLC methods are not able to simultaneously quantify the parent PA and its various metabolites. Furthermore, there are no reports on the simultaneous determination of otonecine-type PA and its metabolites. Therefore the present study, for the first time, reports the development of a specific HPLC method for a simultaneous qualitative and quantitative analysis of clivorine and its four putative hepatotoxicity-related metabolites, namely DHR, 7-GSH-DHR, 7,9-diGSH-DHR and clivoric acid. In addition, a successful application of the developed HPLC method to analyze these four metabolites of clivorine generated in rat liver microsomal incubation is described.

2. Experimental

2.1. Chemicals and materials

Monocrotaline, retrorsine, glutathione (GSH), and all other chemicals were purchased from Sigma (St. Louis, MO, USA). HPLC-grade solvents were obtained from BDH (Poole, UK). Clivorine was isolated from *Ligularia hodgsonii* Hook by a standard procedure for alkaloid extraction [4]. DHR was synthesized from monocrotaline by adoption of a reported method [20]. 7-GSH-DHR and 7,9-diGSH-DHR were obtained by *o*-chloranil oxidation of monocrotaline to dehydromonocrotaline [21] followed by reaction of dehydromonocrotaline with limited amount or excess of GSH, respectively. While clivoric acid was isolated from scale-up rat microsomal incubation of clivorine and purified by preparative HPLC [4]. The detailed synthetic and metabolic approaches for the preparation of four putative metabolites will be reported separately. The purity and identity of both synthesized and isolated putative metabolites were determined by MS, IR and NMR spectroscopic analyses [4].

2.2. Apparatus and chromatographic condition

A Hewlett-Packard (HP) 1100 system with a photodiode-array multiple-wavelength UV detection (DAD) system and two six-port valve Rheodyne injectors was utilized in the present study. The column configuration consisted of a Hamilton PRP-1 guard column (50×4.1 mm I.D., 5 μm) and a

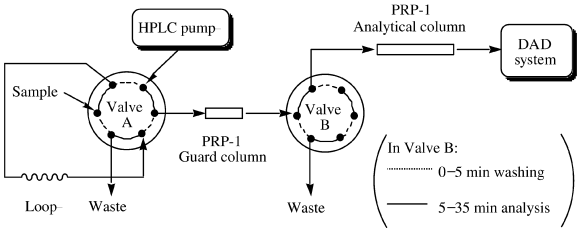

Fig. 2. Schematic set-up of the HPLC system.

Hamilton PRP-1 analytical column (250×4.6 mm I.D., 5 μm) at controlled operating temperature of 30°C.

A schematic set-up of the HPLC system with two PRP-1 columns is shown in Fig. 2. The first injector (valve A) was equipped with a 200-μl loop and connected to the guard column. The second injector (valve B) was connected between guard column and analytical column or waste bottle as a switching valve. At the initial 0–5 min, valve B was set at the position of a direct connection to waste bottle, and isocratic elution with mobile phase A (0.2% formic acid at pH 3.4 adjusted by ammonia) was performed to wash out the aqueous soluble impurities. At 5 min valve B was switched to the position connecting to the analytical column, and analytes were then separated by a gradient elution with mobile phase A and B (acetonitrile) as follows: at 5–35 min, linear change from 100% A to 75% A; at 35–40 min, linear change from 75% A to 70%. The flow-rate was kept constant at 1.0 ml/min for a complete analysis. Peak responses were measured at 230 nm by DAD for the routine analysis or with a full spectrum at 200–400 nm for the identification of metabolites.

2.3. Validation of the developed assay

Buffered solution containing all five analytes and the internal standard was prepared by mixing appropriate amounts of each compound obtained from the corresponding stock solutions in 0.01 *M* phosphate buffer (pH 7.4) to give the concentration of each analyte near the highest level formed in the metabolic study (50 μg/ml for DHR, 7-GSH-DHR and 7,9-diGSH-DHR, 100 μg/ml for clivorine and clivoric acid). The same volume of the prepared sample was analyzed by HPLC with or without initial 5 min washing, respectively. The resultant peak areas of each analyte were compared to evaluate the loss of each analyte during the initial 5-min washing period.

2.4. Calibration curves

Stock solutions of 7-GSH-DHR (0.50 mg/ml), 7,9-diGSH-DHR (0.50 mg/ml), clivoric acid (1.0 mg/ml) and clivorine (1.0 mg/ml) were prepared by directly dissolving in 0.1 *M* phosphate buffer (pH 7.4). Retrorsine, an internal standard (0.50 mg/ml), and DHR (0.50 mg/ml) were firstly dissolved in a small volume of acetonitrile followed by appropriate dilution with phosphate buffer. All stock solutions were stored at −20°C until use.

Aliquots of the stock solutions containing five analytes were spiked into the typical microsomal incubation system to give a final volume of 1.0 ml and appropriate ranges of concentrations for the calibrations. Microsomes were denatured by preheating at 80°C for 5 min. Each calibration curve was constructed with five different concentrations (Table 1) in triplicate. Concentration of the internal

Table 1
Calibration curves for clivorine, clivoric acid, DHR, 7-GSH-DHR and 7,9-diGSH-DHR

Analyte	Retention time (min)	Standard curve[a]	R^2	Test range (μg/ml)
DHR	16.7	$y = 0.0369x - 0.0168$	0.9937	5.0–40.0
7-GSH-DHR	18.9	$y = 0.0361x + 0.0066$	0.9962	5.0–70.0
7,9-diGSH-DHR	20.3	$y = 0.0297x - 0.0238$	0.9823	5.0–40.0
Clivoric acid	29.8	$y = 0.1114x - 0.1380$	0.9987	10.0–120.0
Clivorine	35.5	$y = 0.1147x - 0.1743$	0.9970	10.0–100.0

[a] y, peak area ratio (analyte/internal standard); x, concentration of analyte (μg/ml).

standard, retrorsine, was 25.0 μg/ml for all analyses. The resultant samples were mixed thoroughly and centrifuged at 105 000 *g* at 4°C for 30 min. Aliquots of the supernatant (200 μl) were directly subjected to HPLC analysis. Calibration curves were constructed by plotting concentration of each analyte as a function of peak area ratio (analyte/internal standard).

2.5. Accuracy and precision

The measurements of intra- and inter-day variability were utilized to determine the accuracy and precision of the developed assay. Three concentrations of each analyte which were at low, medium, and high levels of the corresponding calibration curves were chosen to test both intra-day and inter-day variations. Known quantities of five analytes and internal standard (25 μg/ml) were spiked into the pre-heated microsomal incubation system. The resultant samples were centrifuged and analyzed by coupled-column HPLC method as described in Section 2.4. Peak area ratio for each analyte was measured, and quantity of each analyte was subsequently determined from the corresponding calibration curve. Relative standard deviation (RSD) was taken as a measure of precision and percentage difference between amounts determined and spiked was considered as a measure of accuracy. Samples at each given concentration were analyzed in triplicate for intra-day variation. While, the inter-day reproducibility was examined on 5 separate days. Furthermore, three samples spiked with all five analytes with the concentrations unknown to analyzer were also tested for the assessment of quality control (QC) of the developed assay.

2.6. Limits of detection

Aliquots of analytes were spiked into the pre-heated microsomal system to provide concentrations with a range of 0.2–10 μg/ml. The samples were treated, and analyzed similarly as described in Section 2.4. Limit of detection for each analyte was determined when the ratio of the testing peak signal-to-noise ratio was greater than 5.

2.7. Microsomal incubation and quantification of metabolites

Male Sprague–Dawley rats (body mass 200–220 g) supplied by Laboratory Animal Services Centre at the Chinese University of Hong Kong were pre-treated with phenobarbitone (80 mg/kg/day, i.p.) for 3 consecutive days. Microsomes were prepared by a standard two-step centrifugation procedure and finally resuspended in 0.25 *M* sucrose solution [14]. Liver microsomes were stored at −70°C until use. Protein contents were determined using a modified Lowry method [22]. A typical microsomal incubation mixture (10 ml) in potassium phosphate buffer (100 m*M*, pH 7.4) contained liver microsomes (2 mg protein/ml), 0.25 m*M* clivorine, 1 m*M* $NADP^+$, 10 m*M* glucose-6-phosphate, and 10 units glucose-6-phosphate dehydrogenase in the presence or absence of GSH, respectively. Incubation was performed at 37°C for 60 min. The resultant mixtures were centrifuged at 105 000 *g* for 30 min at 4°C. Aliquots of the supernatant were directly analyzed by HPLC as described in Section 2.4. Quantity of each metabolite was determined from the corresponding calibration curve.

2.8. Stability test

The stability of each metabolite of clivorine generated from rat microsomal incubation was determined. After centrifugation of the incubated samples at 105 000 *g* for 30 min at 4°C, the supernatants were divided into two portions. One portion was kept at ambient temperature, while the other was stored at 4°C till analysis. Each sample in both portions was directly analyzed by the coupled-column HPLC at 6, 12 and 24 h after termination of the incubation in triplicate.

3. Results and discussion

The representative HPLC chromatograms of the spiked standards and incubated sample are shown in Fig. 3. PRP-1 column packed with styrene-divinylbenzene resin is compatible with a relatively wide range of pH 2–13, and generally exhibits a good separation for the basic compounds. PRP-1

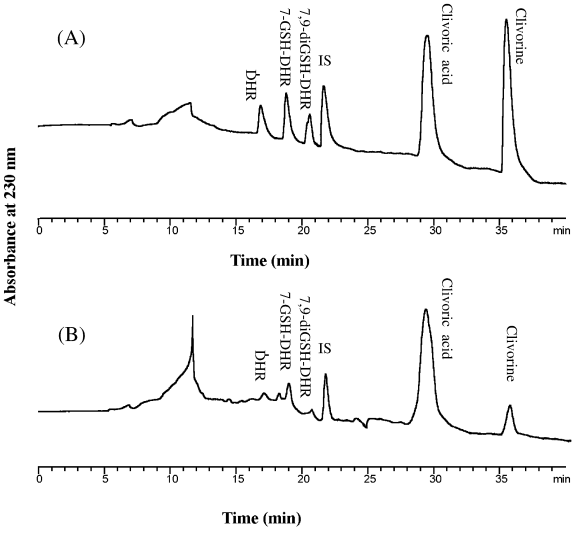

Fig. 3. Representative HPLC chromatograms of the supernatants of the incubated mixture (A) spiked with authentic samples and (B) incubated with clivorine. DHR, dehydroretronecine; 7-GSH-DHR, 7-glutathionyldehydroretronecine; 7,9-diGSH-DHR, 7,9-diglutathionyldehydroretronecine; I.S., internal standard.

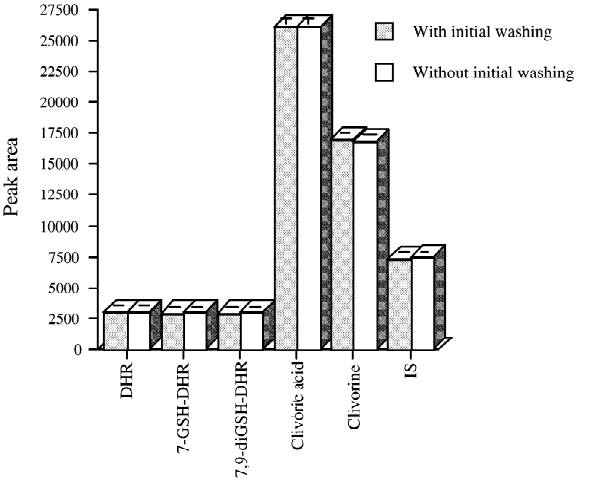

Fig. 4. Comparison of peak areas of each analyte obtained by HPLC analyses with and without initial 5 min washing ($n=6$). DHR, dehydroretronecine; 7-GSH-DHR, 7-glutathionyldehydroretronecine; 7,9-diGSH-DHR, 7,9-diglutathionyldehydroretronecine; I.S., internal standard.

column has been reported as a suitable column for the analysis of several retronecine-type PAs although the peak shapes are usually broader than those resolved in conventional reversed-phase analytical columns [16,23]. In the present study, using two coupled RPR-1 columns under the developed optimal condition, clivorine, its four metabolites, and the internal standard were resolved well with baseline separation (Fig. 3).

The HPLC system set-up with two columns (Fig. 2) allowed an initial 5-min washing out of the aqueous soluble impurities present in the incubates, such as soluble proteins, coenzymes and compounds used for NADPH generating system, directly form the first guard column, while clivorine and its metabolites remained on the guard column. After 5 min, with valve B switching the guard column was connected to the second analytical column, and the purified mixture of analytes was then loaded on and separated by the analytical column. Peak areas of all analytes obtained by either initial 5 min washing or directly passing through two columns without initial 5 min washing were determined and compared. The results demonstrated that no significant reduction of the peak area responses was observed for all analytes examined with initial 5 min washing (Fig. 4). Therefore, this specific two-column set-up was utilized with at least three advantages in the present study. Firstly, the incubated mixture was directly subjected to HPLC analysis without time-consuming extraction. Secondly, the direct analysis was able to determine thermal and pH sensitive pyrrolic metabolites, which maybe degrade during solvent extraction with alteration of pH and/or temperature. Finally, majorities of impurities present in the mixture were removed from the guard column before reaching the analytical column, thus separation of the analytes by the analytical column was more easily achieved and the life of the expensive analytical column was markedly prolonged. Furthermore, the results showed that the initial 5 min washing was adequate to routinely remove the contaminating materials in the centrifuged incubates from the HPLC system, which allowed, under the current condition, about 230–250 and 950–1000 injections on the guard and analytical column, respectively.

All five calibrations for the parent clivorine and its four metabolites exhibited good linear regressions. Their regression equations, correlation coefficients, and the concentration ranges tested are summarized in Table 1. The developed HPLC method has been

fully validated. The results indicated a good reproducibility with overall intra- and inter-day variations of less than 12%, and acceptable precision with overall accuracy higher than 92% for DHR, 87% for 7-GSH-DHR, 90% for 7,9-diGSH-DHR, 96% for both clivoric acid and clivorine, respectively. Furthermore, accuracy for all five analytes measured for QC samples was between 85 and 97% (Table 2). The limits of detection for DHR, 7-GSH-DHR, 7,9-diGSH-DHR, clivoric acid and clivorine were determined to be 2.5 μg/ml, 1.5 μg/ml, 4.0 μg/ml, 0.4 μg/ml and 0.5 μg/ml, respectively, which gave adequate sensitivities for their determination in the incubated microsomal system.

Previous investigation has reported that at physiological or higher pH, the formation of pyrrolic GSH conjugates occurred primarily via reacting GSH with dehydro-PA, the corresponding pyrrolic ester, generated in microsomal incubation, but not with the stable pyrrolic alcohol, DHR [23]. Thus after termination of metabolic incubation the contents of DHR and its two GSH conjugates present in the supernatant of the incubated system at pH 7.4 should remain unchanged. The stability of all four metabolites tested was examined for 24 h at ambient temperature and −4°C, respectively. The results demonstrated that in the presence of phosphate buffer at pH 7.4, DHR, 7-GSH-DHR, and 7,9-diGSH-DHR were stable for longer than 12 h at −4°C and for at least 6 h at room temperature, while clivoric acid was stable for longer than 24 h at room temperature. Furthermore, all analytes were stable for more than 24 h when stored at −20°C. Therefore, in the present study all samples including the incubated mixtures and the stock solutions were kept in phosphate buffer (pH 7.4) at −4°C for the same day analysis. Whereas samples were stored at −20°C when analysis was conducted next day.

The developed HPLC assay was applied to a simultaneous determination of clivorine and its four metabolites generated in rat microsomal incubation. A representative chromatogram for the incubated

Table 2
Intra- and inter-day variation for the assay of clivorine and its four metabolites

Analyte spiked (μg/ml)	Intra-day variation			Inter-day variation		
	Detected ($n=3$)	RSD (%)[a]	Accuracy (%)[b]	Detected ($n=5$)	RSD (%)[a]	Accuracy (%)[b]
DHR						
4.0	3.82±0.44	11.6	95.5	3.68±0.39	10.7	92.0
16.0	16.29±0.22	1.4	98.2	15.96±0.49	3.1	99.7
32.0	32.35±1.08	3.3	98.9	31.25±1.36	4.3	98.5
7-GSH-DHR						
5.0	4.61±0.51	11.0	92.2	4.36±0.49	11.3	87.2
20.0	19.45±0.54	2.8	97.3	19.95±1.14	5.7	99.7
70.0	69.31±2.01	2.9	99.0	70.02±2.60	3.7	99.97
7,9-diGSH-DHR						
5.0	4.68±0.28	5.9	95.6	4.49±0.33	7.4	89.8
30.0	30.45±1.80	5.9	98.5	30.48±2.12	6.9	98.4
40.0	40.15±1.94	4.8	99.6	39.05±2.03	5.2	97.6
Clivoric acid						
10.0	10.33±0.07	0.7	96.7	10.17±0.28	2.7	98.3
40.0	40.54±1.60	4.0	98.6	40.38±1.17	2.9	99.0
120.0	119.61±3.10	2.6	99.7	120.22±2.42	2.0	99.8
Clivorine						
10.0	10.40±0.18	1.8	96.0	10.33±0.23	2.2	96.7
40.0	41.56±1.48	3.6	96.1	40.78±1.55	3.8	98.1
100.0	101.06±3.17	3.1	98.9	100.58±2.92	2.9	99.4

[a] RSD (%) (relative standard deviation) = (SD/mean) × 100.
[b] Accuracy (%) = [1 − (mean concentration measured − concentration spiked)/concentration spiked] × 100.

sample is also shown in Fig. 3B. Identification of metabolites was confirmed by a direct comparison of HPLC retention time and UV spectrum of each analyte with those of authentic sample. The results demonstrated a successful application of the developed HPLC method for a simultaneous identification and quantification of clivorine and its four metabolites in the incubated mixture. Although the chemically reactive pyrrolic ester could not be directly determined, two metabolites formed from further biotransformation of this pyrrolic ester [4,14], namely DHR (77.82±5.00 nmol/ml) and clivoric acid (188.00±15.63 nmol/ml), were found in the microsomal incubation in the absence of GSH. In the presence of GSH in addition to DHR (29.74±0.61 nmol/ml) and clivoric acid (289.50±8.40 nmol/ml), two more pyrrolic alcohol GSH conjugates: 7-GSH-DHR (31.79±0.92 nmol/ml) and 7,9-diGSH-DHR (7.04±0.18 nmol/ml), were determined. The results suggested that the unstable pyrrolic ester formed in microsomal incubation rapidly reacted with GSH to form GSH conjugates. Furthermore, the in vitro biotransformation of clivorine significantly increased in the presence of GSH. The intact clivorine found after incubation in the presence or absence of GSH was 9.24±0.55 and 63.87±5.07 nmol/ml, respectively, which was probably due to the protective effect of GSH against inactivation of hepatic metabolizing enzymes by the toxic pyrrolic ester [23]. Investigations of metabolic pathways of clivorine and a correlation between the formation of pyrrolic metabolites and clivorine-induced hepatotoxicity are currently under progress in our laboratories and the results will be reported separately.

4. Conclusions

A specific HPLC method has been developed and fully validated for a simultaneous determination of clivorine and its four putative hepatotoxicity-related metabolites, namely DHR, 7-GSH-DHR, 7,9-diGSH-DHR, and clivoric acid. The assay is simple, selective and accurate, and is the first reporting method for a simultaneous analysis of the parent PA and its four hepatic metabolites for any type PA. An application for the analysis of in vitro metabolites of clivorine in rat live microsomal studies was successfully demonstrated. Thus the developed HPLC method can be either readily adopted or utilized with a minor modification to investigate the metabolic profiles and thus delineate the mechanism of metabolism-induced hepatotoxicity for both types of hepatotoxic PAs.

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