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The in vitro metabolism of thalicarpine, an aporphine–benzyltetrahydroisoquinoline alkaloid, in the rat API-MS/MS identification of thalicarpine and metabolites

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Abstract

The in vitro metabolism of an antitumor, hypotensive, and antimicrobial aporphine–benzyltetrahydroisoquinoline alkaloid, thalicarpine was studied after incubation with rat hepatic S9 fraction in the presence of an NADPH-generating system. Unchanged thalicarpine (46% of the sample) plus eight metabolites were profiled, quantified, and tentatively identified on the basis of API (ionspray)-MS/MS/MS data. The proposed metabolic pathways for thalicarpine are proposed, and the three metabolic pathways are: (1) *N*-demethylation; (2) aporphine ring oxidation; and (3) benzylic oxidation/reduction. Pathway 1 formed *N*-desmethyl thalicarpine (M1, 6%). Pathway 2 produced three minor keto/hydroxy metabolites (M2–M4, each 2–7%). Pathway 3 formed a major (M6, 28%) and three minor (M5, M7 and M8, each 2–3%) benzylic-cleavage metabolites. Thalicarpine is substantially metabolized by this rat hepatic system.

Keywords: Thalicarpine; Aporphine–benzyltetrahydroisoquinoline alkaloid; Rat hepatic S9; In vitro metabolism; API-MS/MS/MS

1. Introduction

Natural products are an important source of novel therapeutic agents in a drug discovery program. Isoquinoline alkaloids exibit antitumor, antimicrobial, hypotensive, analgesic, muscle relaxant, antifibrillatory, and antiprotozoal activities [1–6]. Thalicarpine, (Fig. 1), is an antitumor, hypotensive, and antimicrobial aporphine–benzyltetrahydroisoquinoline alkaloid, first isolated and synthesized by Kupchan and Tomita [5–10]. Microbial transformation of thalicarpine was investigated by Rosazza [12,13]. The objectives of the current study were to investigate the in vitro metabolism of thalicarpine in rat hepatic S9 fraction using API-MS/MS/MS techniques. This resulted in the profiling characterization, and identification of unchanged thalicarpine and eight metabolites. Preliminary results of this study have been reported previously [14].

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2. Experimental

2.1. Materials

Thalicarpine was previously isolated from *Thalitrum sp.* (Ranunculaceae) with a purity >97% (API-MS/MS/MS, HPLC) [8]. HPLC-grade solvents were obtained from the Fisher Scientific Co. (Fairlawn, NJ, USA) and glass-distilled solvents were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI, USA). The incubation components for hepatic S9, Tris, potassium chloride, magnesium chloride, $NADP^+$ and glucose-6-phosphate, were purchased from Sigma (St. Louis, MO, USA).

2.2. Hepatic S9 fraction

The rat hepatic S9 fraction was generated from a male, Crl:CD®(SD)IGSBR VAF/Plus®, rat at Johnson & Johnson Pharmaceutical Research and Development (Spring House, PA, USA).

2.3. Rat hepatic S9 incubation

Chilled, freshly-made components were added to each flask (on ice) in the following order: 1.15%

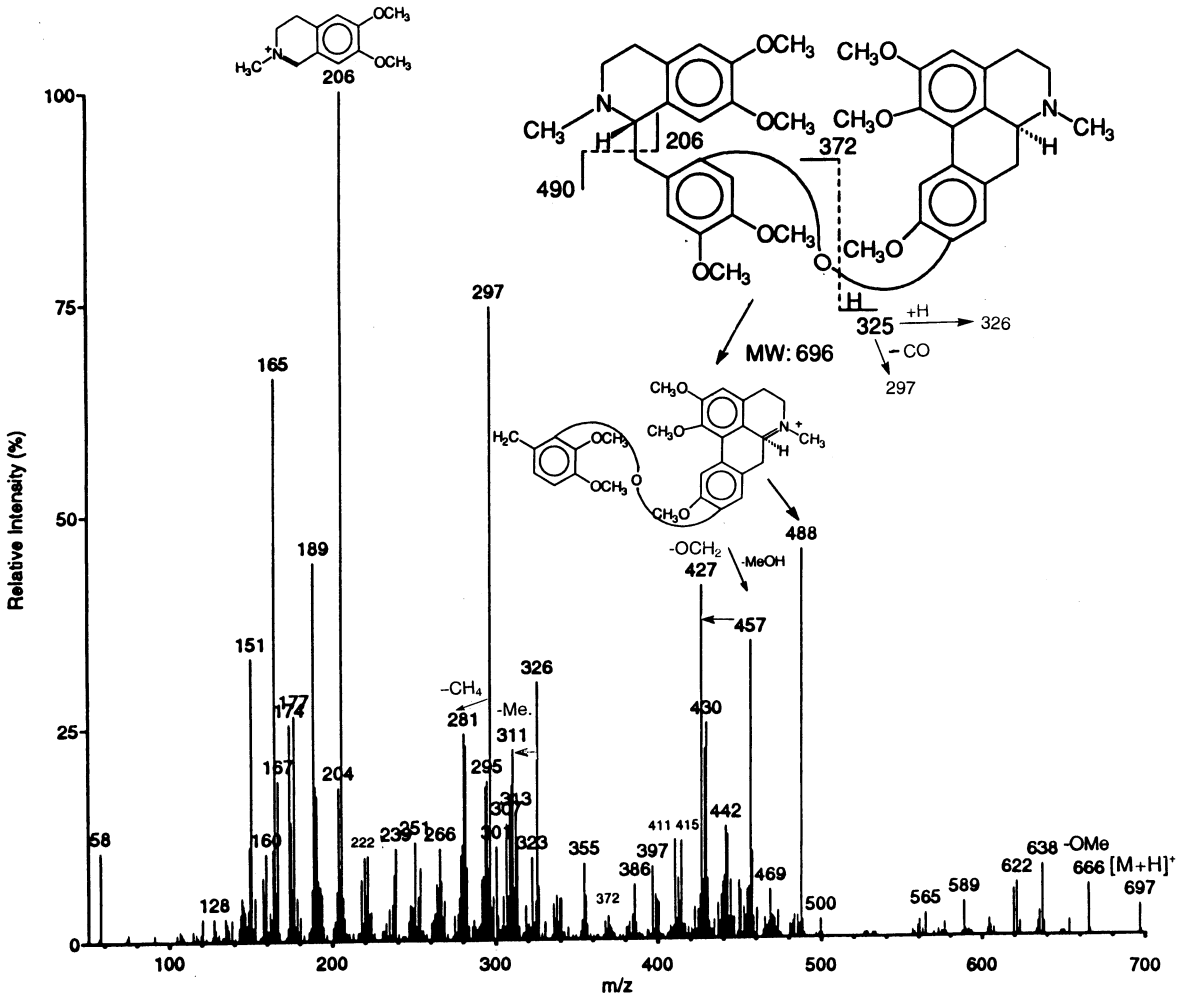

Fig. 1. Structure and MS/MS spectrum of thalicarpine.

Table 1
Hepatic metabolism of thalicarpine in the rat

Percent of rat hepatic S9 sample			
Analyte	0 (min)	30 (min)	60 (min)
Thalicarpine	100	60	46
M1 (*N*-desmethyl thalicarpine)	–	4	6
M2 (OH–thalicarpine)	–	5	7
M3 (Oxo-thalicarpine)	–	2	3
M4 (Oxo-OH–thalicarpine)	–	<2	2
M5 (hernandaline)	–	2	3
M6 (hernandalinol)	–	22	28
M7 (OH–hernandalinol)	–	<2	3
M8 (Oxo–hernandalinol)	–	<1	<2

Data are derived from the integrated ion chromatograms via Q1 scan MS determinations.

KCl in 0.05 M Tris buffer (pH 7.4), 5 mM $MgCl_2$, glucose-6-phosphate, 0.5 mM $NADP^+$, test substrate (hepatic S9), and a thalicarpine spike, to obtain a final volume of 5 ml and a thalicarpine concentration of 100 μg/ml. After the addition of the last component, each flask was incubated at 37 °C in a Dubnoff Metabolic Shaker Incubator (Precision Scientific, Chicago, IL, USA). Samples were removed at 0, 30, and 60 min. Control flasks were incubated without rat subcellular fraction, to determine drug stability under incubation conditions.

2.4. Sample storage

Immediately following removal from the incubator, aliquots were transferred to prelabelled storage vials, deactivated by the addition of ethyl acetate, and placed in a dry-ice/acetone bath to terminate and freeze the reaction. Samples were stored at approximately −20 °C.

2.5. Metabolite profiling, quantifying, and identification

Following ethyl acetate (3 ml) extraction of each ammonium hydroxide-basified (pH ~ 9) incubate (1 ml), the extract residue was reconstituted in buffer (0.5 ml of acetonitrile–water (50/50, v/v) with 2 mM ammonium acetate buffer, pH 4.0) and then analyzed via 20 μl flow-injection using the PE Sciex API III-Plus MS (Perkin–Elmer Sciex Instuments, Thornhill, Ontario, Canada), a triple quadrupole mass spectrometer, interfaced to a Hitachi HPLC solvent delivery system (L-6200 A Intelligent pump) via an ion-sprayer using nitrogen as a curtain and nebulizing gas and argon as a collision gas for MS/MS analysis. The mobile phase for this system was the same buffer as described for the residue reconstitution, at a flow rate of 0.1 ml/min. For each sample, the relative percentage of unchanged thalicarpine and its metabolites were estimated using the integrated chromatograms generated by the Sciex API-III Q1 scan MS (TIC total ion chromatogram). Unchanged thalicarpine, its metabolites, and reference standards (thalicarpine, *N*-desmethyl thalicarpine (northalicarpine)) [9,11] were elucidated on the basis of MS and MS/MS data.

2.6. Methyl derivatization

Each extract residue was dissolved in 1 ml of methanol, an excess amount of ethereal diazomethane was added, allowed to react at room temperature overnight, and subsequently evaporated to yield a residue. Each residue was analyzed for further confirmation of metabolites using the same method as described above.

3. Results and discussion

The in vitro metabolism of thalicarpine was conducted in rat hepatic S9 fractions. Unchanged thalicarpine (60 min incubate; 46% of the sample) and a total of eight metabolites (M1–M8), were profiled, quantified, characterized, and tentatively identified in the 60 min incubate based on API

ionspray-MS and MS/MS data. The structures of thalicarpine (Fig. 1), its metabolites, reference standards, and their MS data are presented, and the percent of unchanged thalicarpine and each metabolite are listed in Table 1. Control incubates revealed unchanged thalicarpine only.

The representative metabolic profile using Q1 scan MS (TIC) for the 60 min incubate of rat S9 is presented in Fig. 2. Representative MS/MS spectra for unchanged thalicarpine, metabolites 1, 2 and 6 are presented in Figs. 1, 3–5. The isolation, profiling, quantification, characterization, and identification of unchanged thalicarpine and each metabolite are discussed below. Unchanged thalicarpine was isolated, and identified from all incubates (0, 30 and 60 min) by solvent extraction and MS and MS/MS techniques, and by comparison to a reference standard (Figs. 1 and 2). Mass spectral analysis of thalicarpine revealed an intense protonated molecular ion at m/z 697 ($[M + H]^+$) (Figs. 1 and 2). MS/MS analysis of m/z 697 exhibited prominent product ions at m/z 666 ($[M + H]^+ - OMe$, 6%), 488 (45%), 457 (35%), 427 (41%), 326 (30%), 297 (74%), 206 (100%), 189 (45%),165 (66%), and 151 (33%) (Fig. 1). Unchanged thalicarpine was present in major quantities (46% of the sample) in the 60 min S9 incubate (Table 1). Metabolite 1 was present as a minor metabolite (6% of the sample) (Table 1). The MS and MS/MS spectral data showed an intense protonated molecular ion at m/z 683 (14 amu less than thalicarpine), and intense as well as diagnos-

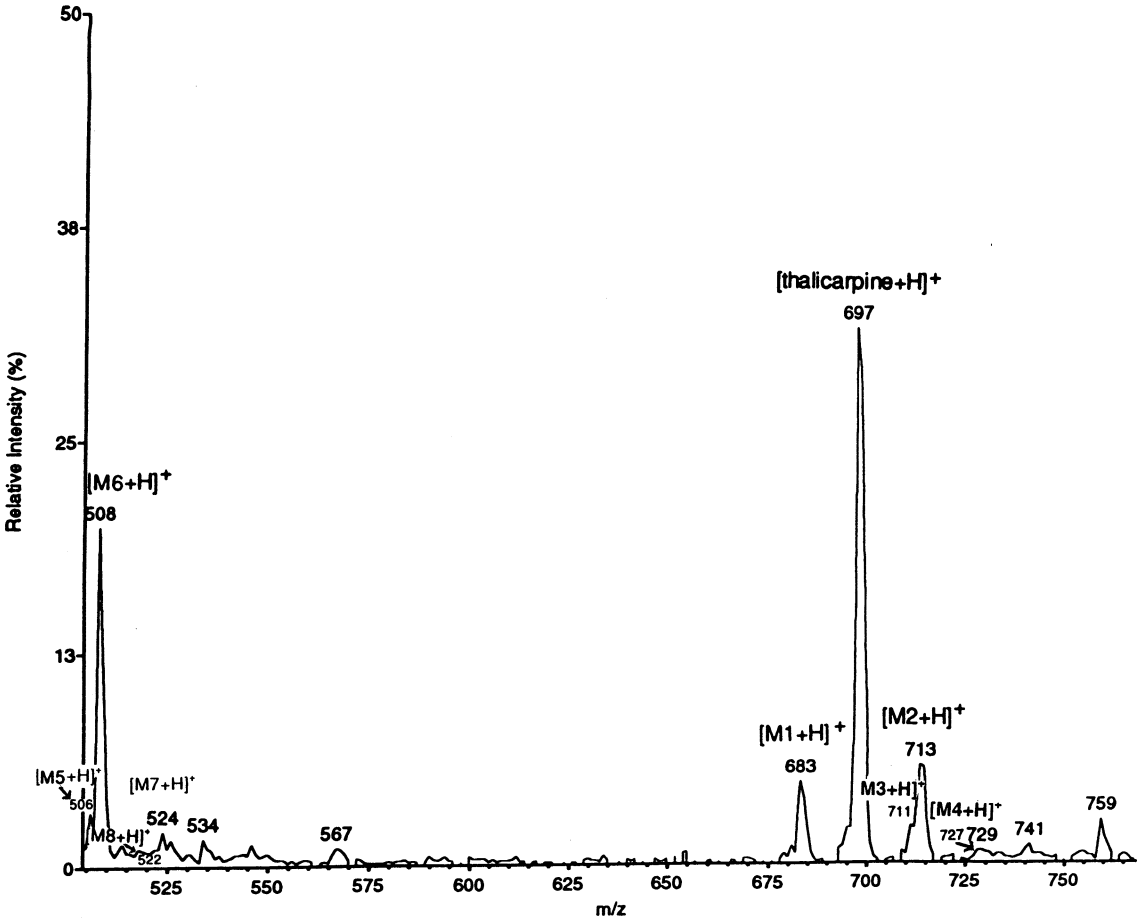

Fig. 2. Q1 scan MS (TIC) of unchanged thalicarpine and its metabolite.

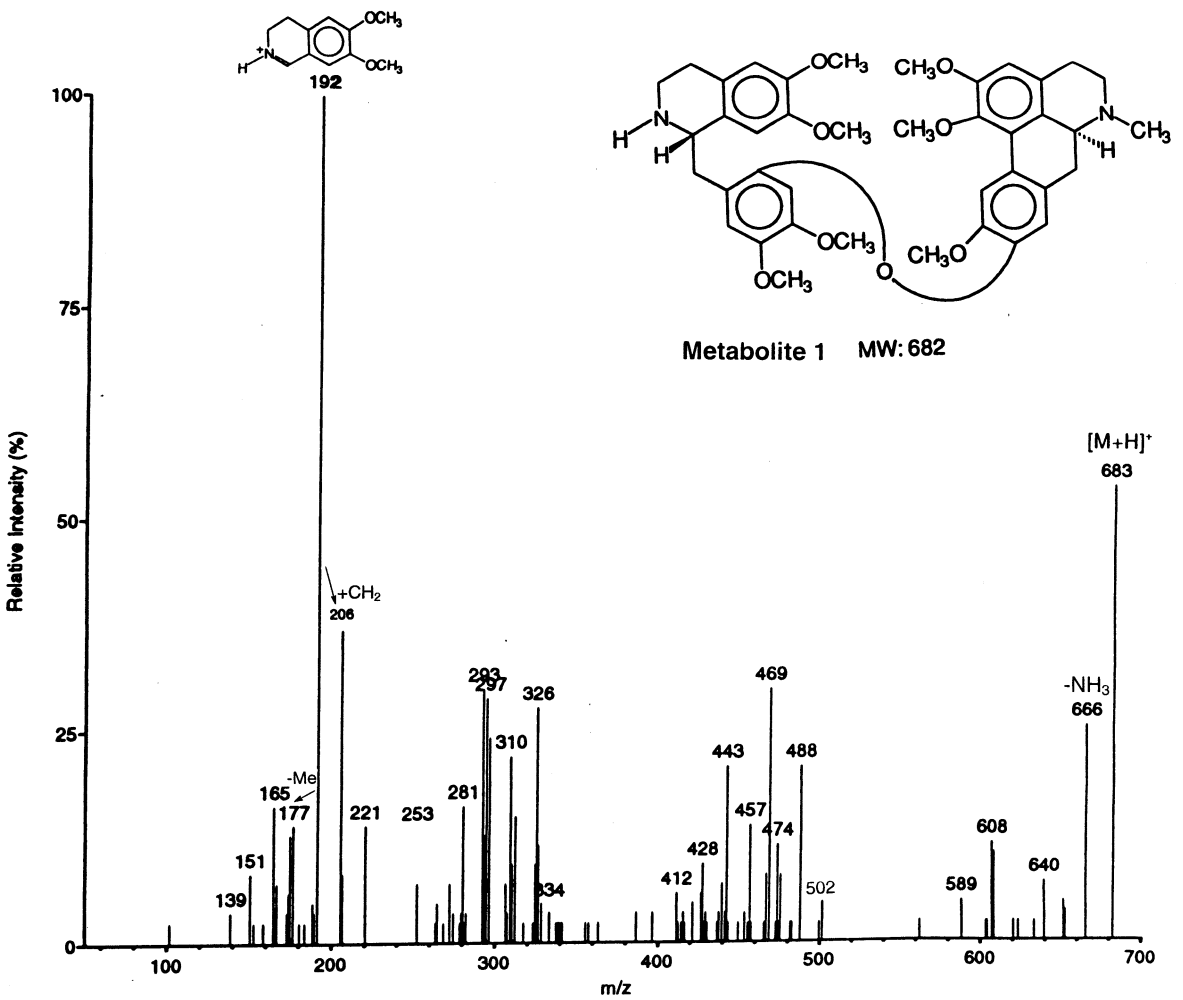

Fig. 3. MS/MS spectrum of metabolite 1 and northalicarpine.

tic product ions at m/z 666 ($[M + H]^+ - NH_3$, 25%), 488 (20%), 469 (29%), 457 (13%), 443 (21%), 326 (27%), 297 (29%), 293 (30%), 281 (17%), 192 (100%), 177 (14%), and 165 (16%) (Figs. 2 and 3). The MS data clearly assigned M1 as *N*-desmethyl thalicarpine as well as by comparison to northalicarpine isolated previously from *Thalictrum sp*. [11]. It remained unchanged by a reaction with diazomethane.

Metabolite 2 was present in small amounts in the 60 min incubates (7%) (Table 1). The structure of M2 was tentatively identified on the basis of MS and MS/MS data. The MS data displayed an apparent protonated molecular ion at m/z 713 (16 amu more than thalicarpine) (Figs. 2 and 4). MS/MS analysis of the protonated molecular ion revealed important product ions at m/z 695 ($[M + H]^+ - H_2O$, 51%), 504 (3%), 486 (8%), 428 (15%), 415 (22%), 370 (3%), 340 (6%), 297 (9%), 206 (66%), 204 (45%), and 175 (6%) (Figs. 2 and 4) together with an intense protonated molecular ion (100%). M2 did not react with diazomethane, and was identified as an alcoholic (aporphine ring) metabolite.

Metabolite 3 was detected in minor amounts (3%) (Table 1). The MS data for this metabolite

gave an apparent protonated molecular ion at m/z 711 (Fig. 2), and the MS/MS analysis of the protonated molecular ion exhibited prominent as well as informative product ions at m/z 693 ($[M+H]^+ - H_2O$, 64%), 666 ($693 - CO + H$, 41%), 636 ($666 - CH_2O$, 54%), 502 ($[M+H]^+ -$ isoquinoline ion, 18%), 488 ($502 - CH_2$, 53%), 457 ($488 - OMe$, 67%), 338 (oxo-aporphine ion, 9%), 310 ($338 - CO$, 28%), 308 ($338 - CH_2O$, 28%), 293 (29%), 220 (45%), 206 (isoquinoline ion, 50%), 204 ($206 - 2H$, 61%), 192 ($206 - CH_2$,76%) and 58 [$CH_2N(CH_3)_2^+$, 45%], together with a protonated molecular ion (100%). MS data of M3 characterized the metabolite as oxo-thalicarpine. Methylation of M3 remained unchanged.

Metabolite 4 was found as a minor metabolite in the 60 min incubate (2%) (Table 1). The ion-spray-MS and MS/MS analysis of the incubate extract contained a protonated molecular ion at m/z 727, and important product ions at m/z 695 ($[M+H]^+ - MeOH$, 7%), 682 ($[M+H]^+ - NH_3 - CO$, 68%), 484 (15%), 473 (15%), 428 (17%), 417 (21%), 297 (24%), 218 (48%), 206 (isoquinoline ion, 100%), 192 ($206 - CH_2$, 7%),

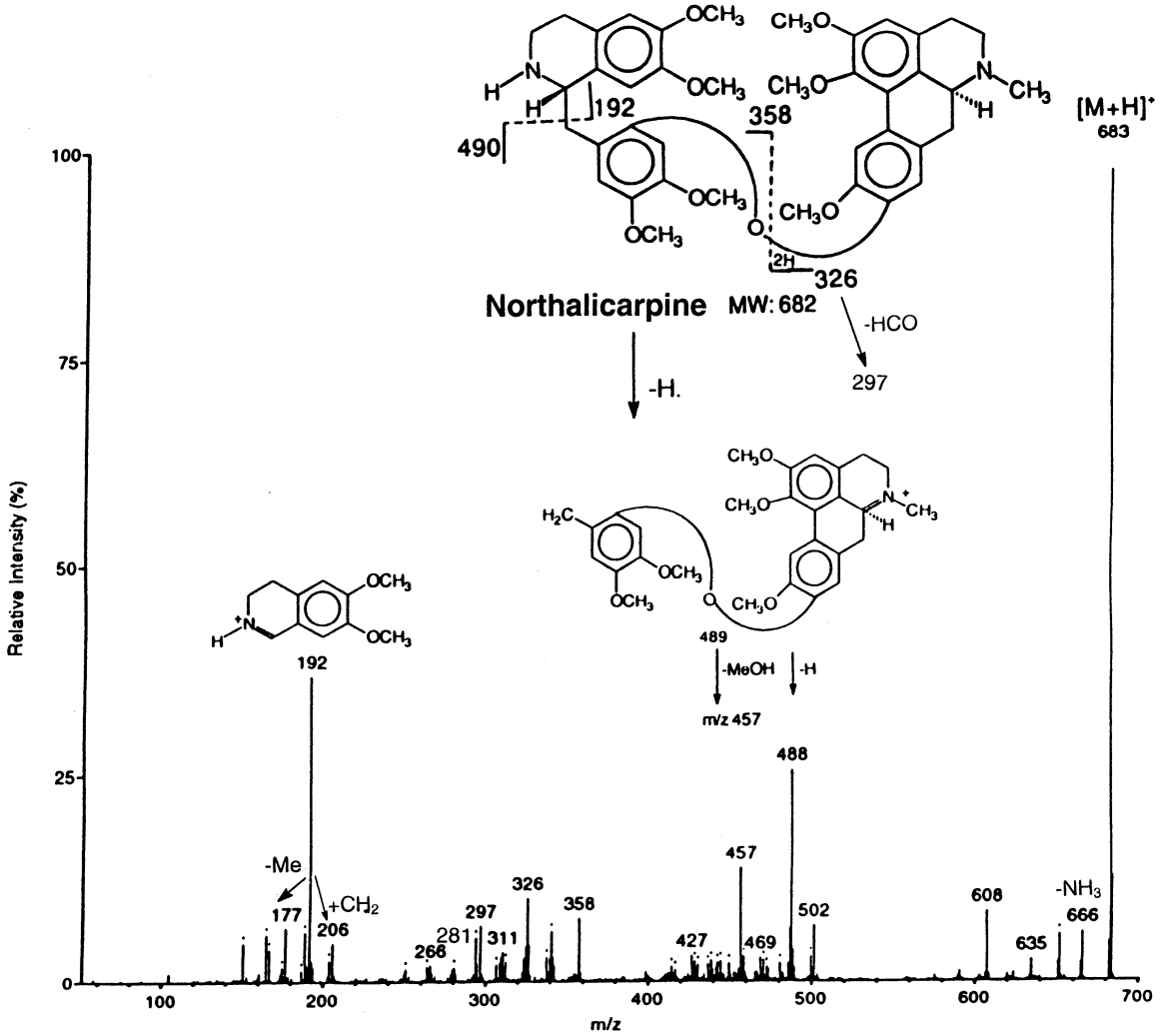

Fig. 3. (*Continued*)

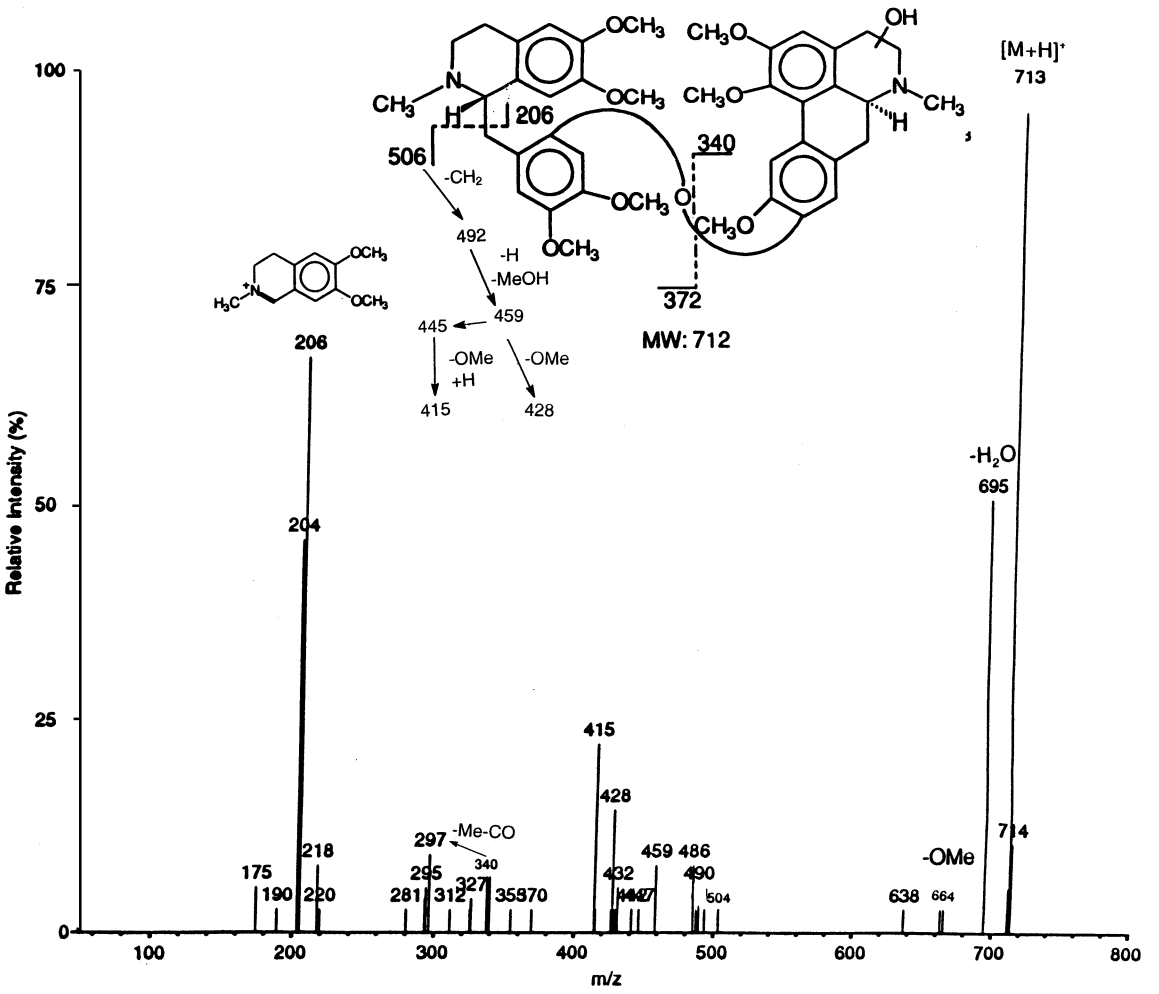

Fig. 4. MS/MS spectrum of metabolite 2.

172 (10%), and 58 ($CH_2N(CH_3)_2^+$, 27%), along with an intense protonated molecular ion at m/z 727 (100%) in the MS/MS data (Fig. 2). On the basis of the MS data, metabolite 4 was tentatively proposed to be oxo-OH–thalicarpine.

Metabolite 5 was present in trace amounts (3%) (Table 1). This metabolite showed a protonated molecular at m/z 506 in ionspray-MS (Fig. 2), and significant product ions at m/z 491 ($[M + H]^+ -$ Me, 5%), 475 ($[M + H]^+ -$ OMe, 34%), 457 (475 $-$ H_2O, 85%), 357 (25%), 349 (57%), 337 (74%), 294 (21%), 183 (oxybenzaldehye ion, 41%) and 144 (23%) together with an intense protonated molecular ion at m/z 506 (100%). The structure of metabolite 5 was tentatively proposed to be a benzylaldehyde metabolite via benzylic-cleavage of parent compound. M5 was previously reported as a hernandaline metabolite in a study on the microbial transformation of thalicarpine [12,13].

Metabolite 6 was detected in major amounts in the 60 min incubate (28%). An apparent protonated molecular ion at m/z 508 exhibited in the MS spectrum indicated a molecular weight of 2 amu higher than metabolite 5. The addition of 2 amu could be explained by the reduction of the formyl group of M5 after benzylic cleavage of thalicarpine. MS/MS analysis of the protonated molecular ion, m/z 508, provided prominent as well as informative

product ions at m/z 477 ($[M+H]^+ - OMe$, 26%), 462 ($477 - Me$, 41%), 444 ($462 - H_2O$, 57%), 428 ($444 - CH_4$, 100%), 413 ($428 - Me$, 95%), 397 ($413 - CH_4$, 46%), 385 ($413 - CO$, 28%), 369 ($385 - CH_4$, 36%), 293 (55%), 278 (10%), 167 (benzyl alcohol ion, 71%) and 139 (17%) along with an apparent protonated molecular ion (Figs. 2 and 5). The structure of M6 was tentatively assigned on the basis of MS/MS data. M6 remained unchanged by reaction with diazomethane. M6 was previously identified as a major microbial transformation metabolite of thalicarpine, hernandalinol [12,13].

Metabolite 7 was detected in minor amounts (3%) in the 60 min incubate, and showed a protonated molecular ion at m/z 524 (Fig. 2), which exhibited important product ions at m/z 506 ($[M+H]^+ - H_2O$, 22%), 475 ($[M+H]^+ - H_2O - OMe$, 29%), 358 (oxyaporphine ion, 3%), 338 (5%), 334 (6%), 184 (oxybenzyl alcohol ion, 100%), and 104 (51%) in MS/MS data. The MS data tentatively assigned M7 as OH-M6. Metabolite 8 was profiled as a trace metabolite ($< 2\%$) in the 60 min incubate. The Q1 scan MS (Fig. 2) and MS/MS showed a protonated molecular ion at m/z 522 and product ions at m/z 504 ($[M+H]^+ - H_2O$, 7%), 491 ($[M+H]^+ - OMe$, 75%), 473 ($[M+H]^+ - OMe - H_2O$, 29%), 444 ($473 - CHO$, 50%), 350 (18%), 184 (oxybenzyl alcohol ion, 64%) and 104 (24%). The structure of M8 was tentatively assigned as oxo-M6 by means of MS/MS data.

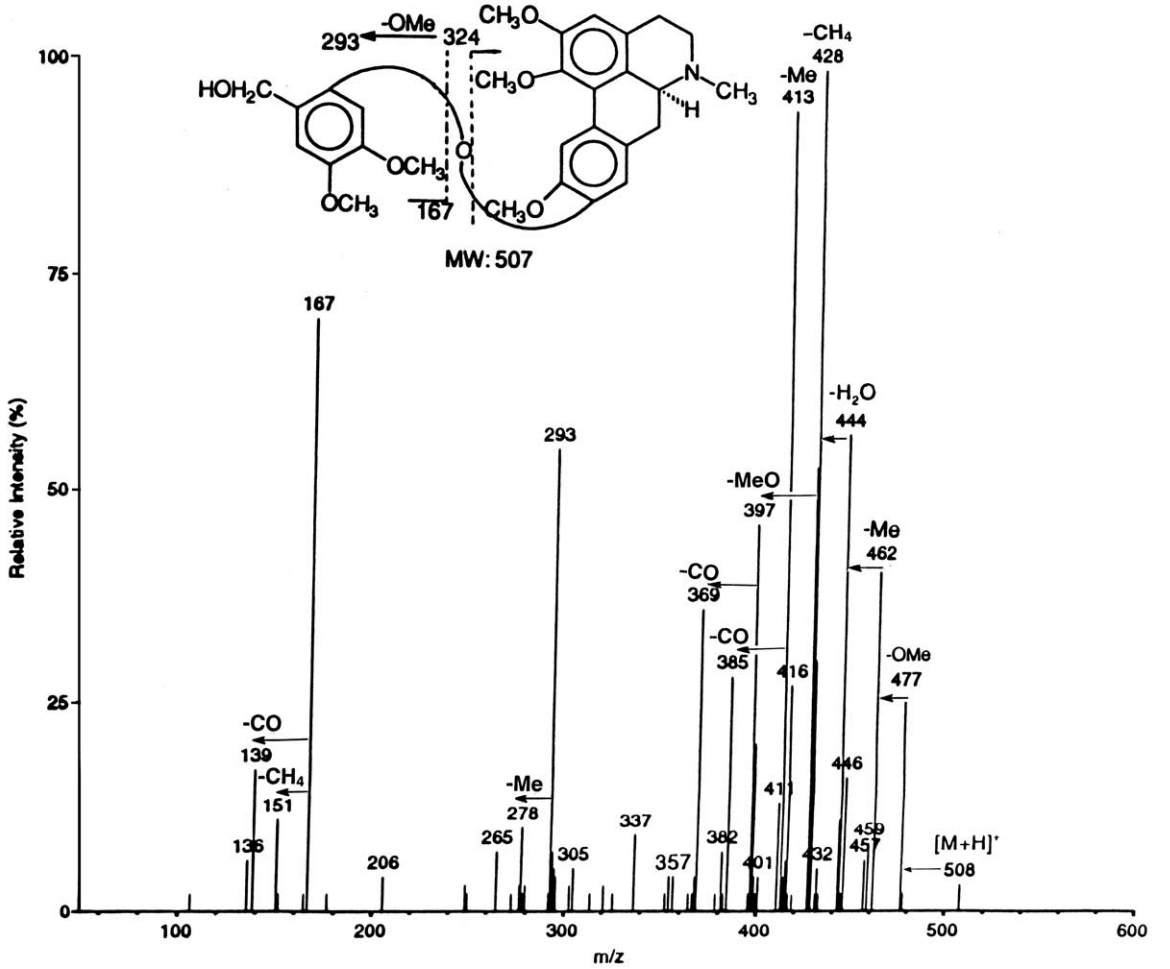

Fig. 5. MS/MS spectrum of metabolite 6.

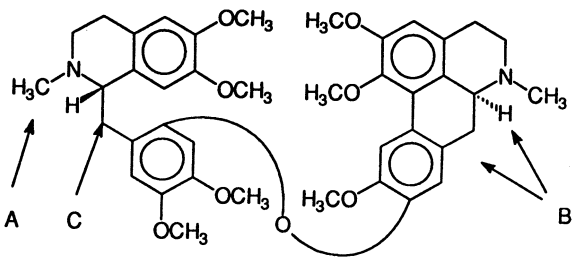

Fig. 6. Sites of metabolic reactions for thalicarpine.

4. Conclusion

The in vitro metabolism of thalicarpine was conducted in the rat hepatic S9 fraction. Unchanged thalicarpine plus eight metabolites were profiled, quantified, characterized, and tentatively identified by means of MS data. API ionspray-MS and MS/MS exhibited apparent protonated molecular ions, and prominent, as well as important, fragment product ions for the structural elucidation of thalicarpine, its metabolites and reference standards. Formation of these metabolites in the rat hepatic S9 can be explained by three pathways: (1) *N*-demethylation; (2) aporphine ring oxidation; (3) benzylic oxidation/reduction (Fig. 6). Pathways 1 and 2 appeared to be minor steps producing four minor *N*-desmethyl (M1) and oxygenated (M2–4) metabolites. Pathway 3 appeared to be a most important pathway, forming a major benzylic-cleavage metabolite, M6

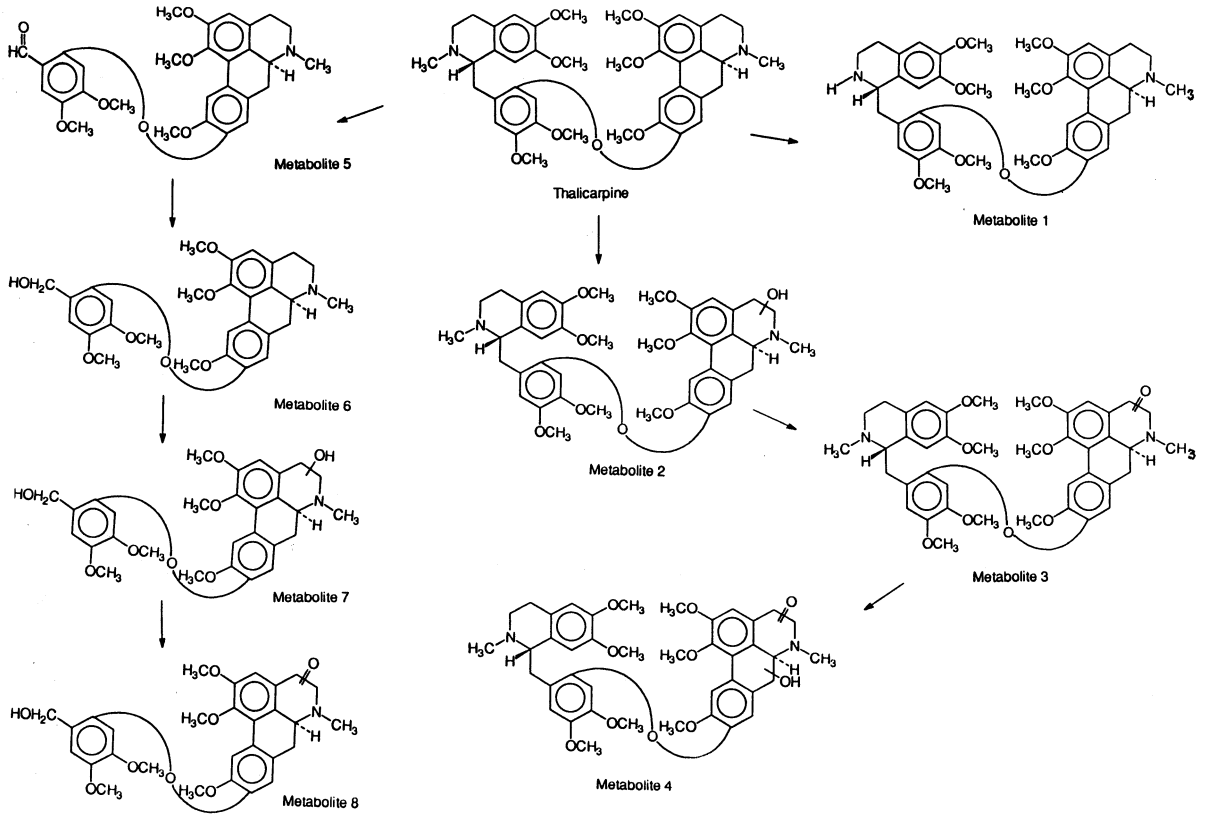

Fig. 7. Proposed in vitro metabolic pathways for thalicarpine in the rat.

(28%), and three minor cleavage metabolites (M5, M7, M8) in conjunction with pathway 2. The proposed in vitro metabolic pathways for thalicarpine in rat hepatic S9 fraction are depicted in Fig. 7. In conclusion, thalicarpine is substantially metabolized in rat hepatic S9 fraction.

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