Pine Needle Abortion in Cattle: Metabolism of Isocupressic Acid

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The rumen and hepatic metabolism of the cattle abortifacient compound isocupressic acid (ICA) was examined in vitro and in vivo. ICA was incubated for 56 h in bovine rumen inoculum and was found to be converted to three compounds identified as imbricatoloic acid, a structurally uncharacterized isomer of imbricatoloic acid, and dihydroagathic acid. In preparations of liver homogenates, ICA was found to be oxidized to agathic acid. No differences in ICA metabolites were detected in comparing the cow, sheep, pig, goat, guinea pig, and rat livers; however, guinea pig and rat liver homogenates were less efficient in converting ICA to agathic acid. ICA had been administered to cows orally and by intravenous infusion and induced abortions after either method of treatment. After intravenous infusion, agathic acid was identified as the major metabolite together with minor amounts of dihydroagathic acid. After oral administration, dihydroagathic acid was identified as the major metabolite with minor amounts of agathic acid, imbricatoloic acid, and a structurally uncharacterized metabolite tentatively identified as tetrahydroagathic acid.

Keywords: *Isocupressic acid; ponderosa pine; cattle abortions; metabolism*

INTRODUCTION

Isocupressic acid [15-hydroxylabda-8(17),13(*E*)-dien-19-oic acid] [**1** (ICA), Figure 1] is the principal abortifacient compound found in *Pinus ponderosa* (Laws) needles (Gardner et al., 1994). This compound has been demonstrated to induce abortions in late-term pregnant cattle after both oral and intravenous (iv) administration (Gardner et al., 1997). ICA was also shown to be the major diterpene acid in *Cupressus macrocarpa*, *Pinus contorta*, and *Juniperus communis*, all known, or demonstrated to be, abortifacient in cattle (Parton et al., 1996; Gardner et al., 1998). The biochemical mechanism of the abortions induced by ICA is still unknown. It is proposed that the primary physiological mechanism of pine needle-induced abortion is reduced uterine blood flow to the fetus from an increase in vascular tone (Ford et al., 1992, Christenson et al., 1993). However, ICA was not directly vasoactive when tested using an in vitro placentome assay procedure (Al-Mahmoud et al., 1995).

Two ICA derivatives, acetyl- and succinylisocupressic acid, are naturally present in ponderosa pine needles. Both compounds were abortifacient when given orally to cows but not when given by iv infusion (Gardner et al., 1996). Further in vitro research demonstrated that both compounds are rapidly hydrolyzed to ICA when incubated in the presence of cow rumen fluid (Gardner et al., 1996). Therefore, whereas acetyl- and succinyl-ICA contribute to the abortifacient activity of pine needles, they require rumen conversion to ICA to do so.

Analysis of serum samples from cattle treated by iv infusion of ICA provided preliminary evidence that this compound is rapidly metabolized after injection (Gardner et al., 1997). When serum was analyzed immediately after ICA infusion into a pregnant cow, several metabolites were observed and only a small amount of the infused ICA was detectable. One of these metabolites was identified as agathic acid (**2**). On the basis of the observed rapid metabolism of ICA we suggested that one of these metabolites is the chemical constituent producing the in vivo biochemical response that initiates abortions in cattle.

Recently, Lin et al. (1998) made a similar conclusion after demonstrating that ICA was metabolized to agathic acid (**2**) and dihydroagathic acid (**3**) when incubated in cow rumen fluid for 48 h. In addition, they reported that plasma from cows fed ponderosa pine needles contained a single metabolite identified as dihydroagathic acid (**3**). In contrast, we found no detectable degree of ICA metabolism during an initial 8 h in vitro incubation period in cow rumen fluid (Gardner et al., 1996) and subsequently hypothesized that ICA is probably absorbed before extensive metabolism in the rumen.

We have examined further the metabolism of ICA using the following experiments: (1) in vitro rumen incubation of ICA using extended incubation times and predetermined periodic sampling for metabolite analysis; (2) comparison of in vivo serum metabolites after oral dosage and intravenous infusion of ICA into cattle; (3) comparison of serum metabolites of ICA produced after oral administration of two plant materials containing ICA (*Pinus ponderosa* and *Juniperus communis*); and (4) further examination of hepatic metabolism of ICA by in vitro incubation in liver homogenates and liver homogenate supernatant fractions from cattle, sheep, pigs, goats, guinea pigs, and rats and in bovine liver microsome fraction.

MATERIALS AND METHODS

In Vitro Rumen Metabolism of ICA. Rumen fluid inoculum was collected from cattle (maintained on an alfalfa hay diet) through a rumen fistula, or by reverse pumping through a stomach tube, and filtered through cheesecloth. A 100 mL aliquot of rumen fluid was added to 400 mL of McDougal's

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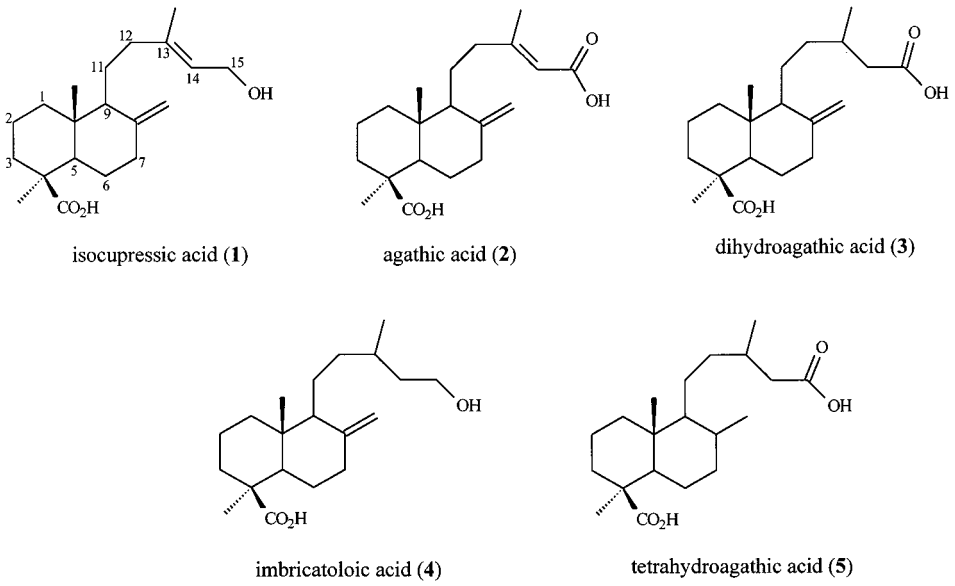

Figure 1. Chemical structures of ICA and related metabolic compounds identified in rumen and serum samples.

buffer at 39 °C maintained under anaerobic conditions by CO_2 suffusion. Alfalfa hay (0.5 g, dried and ground) was added, the headspace purged with CO_2, and the mixture allowed to incubate overnight. The next day, 0.5 g of an isocupressic acid/hay mixture (0.1 g of ICA/g of hay) was added to the flask and the preparation split into two 250 mL aliquots and incubated for 56 h. Duplicate samples (5 mL aliquots) were taken at 0, 4, 12, 24, 32, 48, and 56 h. Samples from the rumen preparations were acidified by the addition of 10 drops (Pasteur pipet) of 6 M HCl and frozen until analysis.

Each 5 mL rumen sample was extracted twice with $CHCl_3$ (4 mL, 2 mL), the extracts were combined and filtered through anhydrous Na_2SO_4, and the solvent was removed by evaporation under N_2 at 70 °C. The sample was derivatized by adding 1.0 mL of pyridine (with 200 μg of heptadecanoic acid as an external standard) and 100 μL of BSTFA (Pierce Chemical) silylation reagent and heating the sample for 30 min at 70 °C. A 200 μL aliquot was removed and diluted with 1 mL of $CHCl_3$, and 1.5 μL was injected for GC/MS analysis.

GC/MS was performed using a GCQ (Finnigan Corp.) equipped with a DB-5ms column (30 m × 0.25 mm i.d., J&W Scientific). Carrier gas was helium with a constant flow (40 cm/s). Samples (1.5 μL) were injected splitless (250 °C), and the column temperature was programmed as follows: 100 °C for 1.0 min, 100−200 °C at 40°/min; 200−300 °C at 5°/min; 300 °C for 5 min. The heated transfer line was at 275 °C, and the ion source temperature was 150 °C. Electron impact (70 eV) spectra were recorded for the mass range of *m*/*z* 50−650 at a scan rate of 0.5 s/scan.

Preparation of ICA Dose Materials. *Intravenous ICA Doses*. ICA was isolated from *P. ponderosa* bark using the methods previously described (Gardner et al., 1996). A known amount of ICA (30 mg/kg, ~13 g/dose) was dissolved in 250 mL of 0.75 M NaOH. Hydrochloric acid (6 M) was added slowly with mixing until the solution started to become turbid. The solution was filtered and diluted to 1.5 L with sterile phosphate-buffered saline solution (final pH 10). The ICA solution was administered by iv drip into the jugular vein at a rate of 75 mL/min (15−20 min/dose). Four cows with known breeding dates were treated on day 250 of gestation and dosed twice daily (morning and afternoon) until abortion or a maximum of 10 days. Two control cows received an iv dose of phosphate-buffered saline prepared as described above with no ICA added. For serum ICA and metabolite analysis, one cow received ICA (30 mg/kg/day) for three consecutive days by intravenous infusion. Serum samples were collected at scheduled times (0.3, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h).

Oral Dosage of ICA. Two cows received a single 30 g oral dose of purified ICA mixed with 1 kg of ground dry alfalfa via a stomach tube pump (Magrath cattle pump, Springer Magrath Co., McCook, NE). Blood samples were drawn via the jugular vein before treatment and every 4 h for 60 h after dosage. After clotting and centrifugation at 1150*g* for 20−30 min, the serum fraction was removed and stored at −4 °C until analysis.

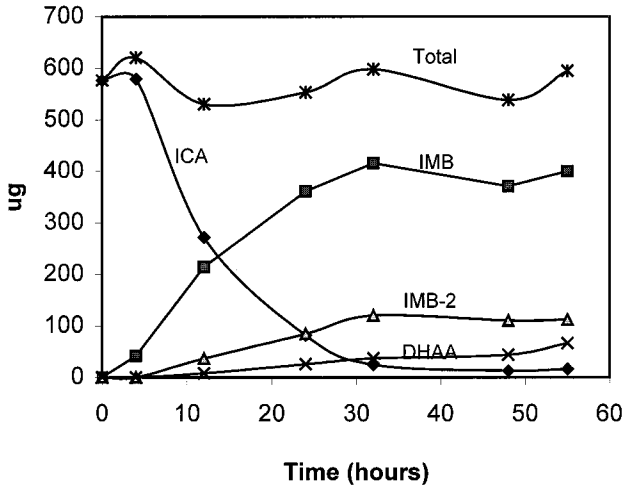

Figure 2. Levels of ICA and metabolites in 5 mL of in vitro rumen preparation for up to 56 h of incubation time: IMB (imbricatoloic acid); IMB-2 (possible isomer of imbricatoloic acid); DHAA (dihydroagathic acid); total (ICA + IMB + IMB-2 + DHAA).

Plant Material Doses Containing ICA. Plant materials (*P. ponderosa* and *J. communis*) were collected from the field, dried, and ground to pass a 2 mm screen (James et al., 1994; Gardner et al., 1998). A single dose of ground plant material (2.2 kg/animal) was given by oral gavage (via stomach tube) to two animals each. Blood samples were drawn before treatment, 1 h after treatment, and at 4 h intervals for 60 h postdosage. The serum fraction was recovered and stored at −4 °C for later analysis of ICA and metabolites.

Serum Analysis. A 5.0 mL aliquot of serum was taken and placed into a 15 mL screw-cap test tube. A saturated KH_2PO_4 solution (1 mL) was added, and the sample was extracted twice with $CHCl_3$ (4 mL, 2 mL) for 5 min each. The tubes were centrifuged to aid layer separation, and the bottom layer was removed and filtered through anhydrous Na_2SO_4. The solvent was removed by evaporation under N_2 at 70 °C. Samples were derivatized (silylated) and prepared for GC/MS analysis by adding 0.50 mL of pyridine containing 25 μg of heptadecanoic

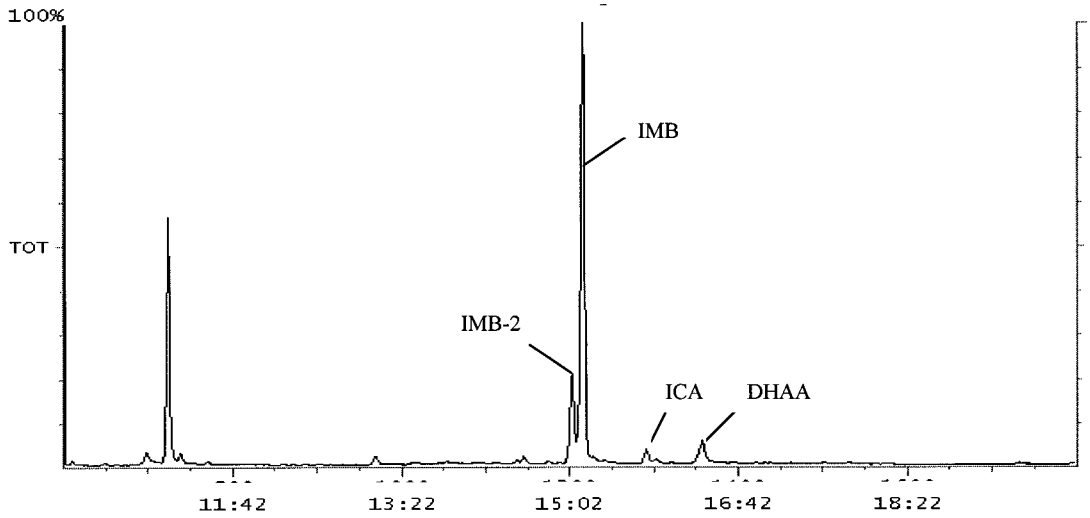

Figure 3. GC/MS chromatogram of ICA and metabolites after incubation in bovine ruminal fluid for 32 h: IMB (imbricatoloic acid); IMB-2 (possible isomer of imbricatoloic acid); ICA (isocupressic acid); DHAA (dihydroagathic acid).

Table 1. Results of Treating Pregnant Cows Starting on Gestation Day 250 with Intravenous Injections of ICA

cow	treatment	parturition	days
5745	15−30 mg/kg	abort	7
5737	30 mg/kg	abort	2
6034	30 mg/kg	term	35
5996	30 mg/kg	abort	7
5744	saline control	term	36
5999	saline control	term	27

acid (as an external standard) and 50 μL of BSTFA silylation reagent. Samples were heated at 70 °C for 30 min before injection of 1.5 μL of sample and analysis by GC/MS.

GC/MS Data for ICA Metabolites. *Agathic acid* (**2**): R_t = 17.6 min; ms data, *m/z* (relative abundance) 478 (8), 463 (18), 388 (120), 360 (32), 346 (100), 307 (20), 271 (50), 253 (85), 229 (60), 189 (82), 156 (55), 121 (50).

Dihydroagathic acid (**3**): R_t = 16.5 min; ms data, *m/z* (relative abundance) 480 (13), 465 (22), 390 (10), 362 (100), 347 (22), 293 (20), 272 (25), 255 (27), 239 (15), 161 (62), 121 (50).

Imbricatoloic acid (**4**): R_t = 15.17 min; ms data, *m/z* (relative abundance) 466 (15), 451 (8), 376 (15), 348 (50), 293 (20), 258 (38), 238 (35), 161 (48), 121 (98), 120 (100), 73 (85).

Tetrahydroagathic acid (**5**): R_t = 16.7 min; ms data, *m/z* (relative abundance) 482 (15), 467 (38), 392 (35), 364 (100), 349 (10), 302 (18), 275 (32), 257 (38), 163 (40), 122 (94).

Preparation of in Vitro Liver Metabolism of ICA. Fresh liver samples were immediately collected after slaughter at a local abattoir (cow and pig) or from euthanized animals (sheep, goat, guinea pig, and rat). The caudal lobes of the liver from the cow, pig, sheep, and goat and whole liver from guinea pig and rat were washed in cold physiologic saline solution and perfused with cold lactated Ringers solution (LRS) (Baxter Healthcare Corp., Deerfield, IL) through the portal vessels until the fluid was clear. Liver samples were stored (<4 h) in cold LRS until use.

Liver Homogenate. Ten grams of liver was washed with cold LRS and homogenized in 30 mL of LRS.

Liver Supernatant. The liver homogenate was centrifuged at 10000*g* for 30 min. The supernatant was drawn off and subsequently centrifuged at 110000*g* for 1 h. The second supernatant was removed to give the liver supernatant fraction used for ICA metabolism. The microsomal pellet was rinsed with LRS and saved.

Liver Microsomal Fraction. Microsomal fraction was prepared only from the cow liver. The remaining microsome fraction after the 110000*g* spin was rinsed and resuspended in 1 mL of LRS. A 0.100 mL aliquot of the suspended microsomes was added to a solution containing MgCl (0.15 mL, 0.4 M), KCl (0.15 mL, 1.65 M), glucose-6-phosphate (0.0375 mL, 1 M), NADP (0.30 mL, 0.1 M), and phosphate buffer solution (3.75 mL, 0.2 M, pH 7.4) similar to the Ames procedure (Ames et al., 1975). Oxygen was added by suffusion just prior to incubation.

Incubation of ICA with Liver Fractions. ICA was dissolved in buffered saline solution (pH 10, 1.3 mg/mL) and 0.100 mL added to each of the following: 7 mL of liver homogenate; 6 mL of liver supernatant; or liver microsomal fractions. Indi-

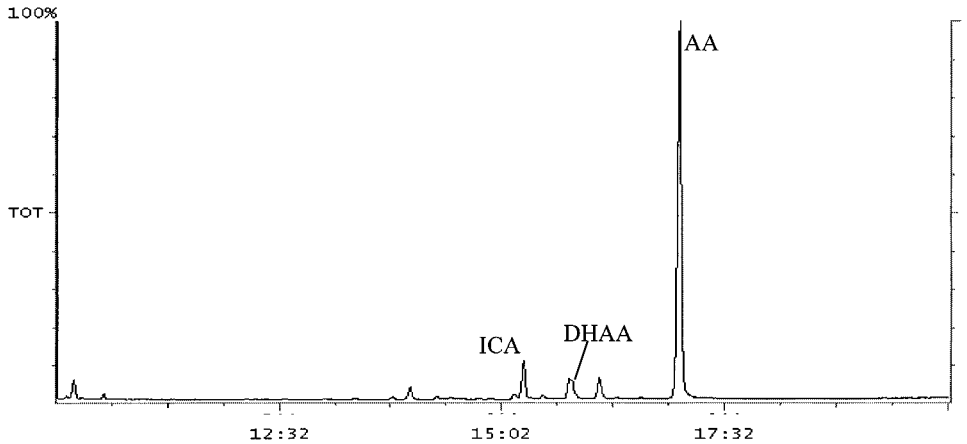

Figure 4. GC/MS chromatogram of metabolites in cow serum 30 min after iv dosage of ICA. Identified metabolites are agathic acid (AA) and dihydroagathic acid (DHAA).

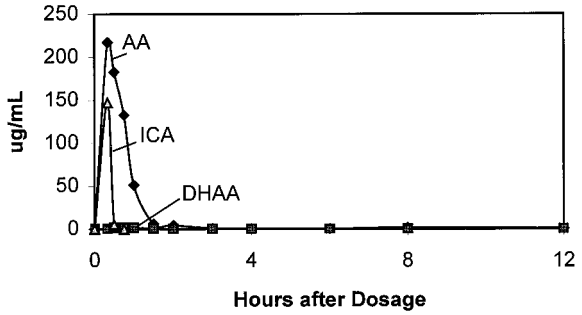

Figure 5. Concentration of ICA metabolites in serum after iv dosage of ICA: AA (agathic acid); DHAA (dihydroagathic acid).

vidual samples were incubated for either 0, 0.5, 2, or 18 h at 37 °C and then immediately frozen until chemical analysis.

To inhibit cytochrome P-450 activity, carbon monoxide was suffused through one set of liver homogenate and liver supernatant solutions and then 0.100 mL of the ICA solution added; the samples were allowed to incubate at the designated time periods.

Analysis of ICA/Liver Incubations. After thawing, the samples were centrifuged and the supernatant was removed; 1 mL of saturated KH_2PO_4 and 4 mL of $CHCl_3$ were added, and samples were extracted for 5 min. The $CHCl_3$ extract was removed and filtered through anhydrous Na_2SO_4 and evaporated to dryness under a stream of N_2 on the heating block (70 °C). Samples were analyzed by GC/MS after silylation using 0.5 mL of pyridine and 50 μL of BSTFA reagent (Pierce Chemical) and heating at 70 °C for 30 min.

Table 2. Summary of Detected Resin Acids or Metabolites of ICA in the Original Dose Material and in the Serum of Cattle after both Oral and Intravenous Administration[a]

dose material[b]	IMB	ICA	AA	DHAA	THAA
ICA	---[c]	++++	---	---	---
PN plant	++	++++	---	+	---
CJ plant	++	++++	---	---	---
IV dose					
ICA	---	+	++++	+	---
oral doses					
ICA	+	---	++	++++	+
PN	+	---	+	++++	+
CJ	+	---	+	++++	+

[a] Diterpene acids: IMB (imbricatoloic acid); AA (agathic acid); DHAA (dihydroagathic acid); THAA (tetrahydroagathic acid). [b] Dose materials: PN (*P. ponderosa*); CJ (*J. communis*). [c] (- - -) none detected; (+) trace; (++) minor; (++++) major.

RESULTS

In Vitro Metabolism of ICA by Cow Rumen Preparations. After incubation of ICA in cow rumen inoculum, no significant decrease in ICA was detected at 4 h but a decline of >50% of the original amount of ICA was observed by 12 h, 85% by 24 h, and >98% by 32 h (Figure 2). Analysis of rumen preparations by GC/

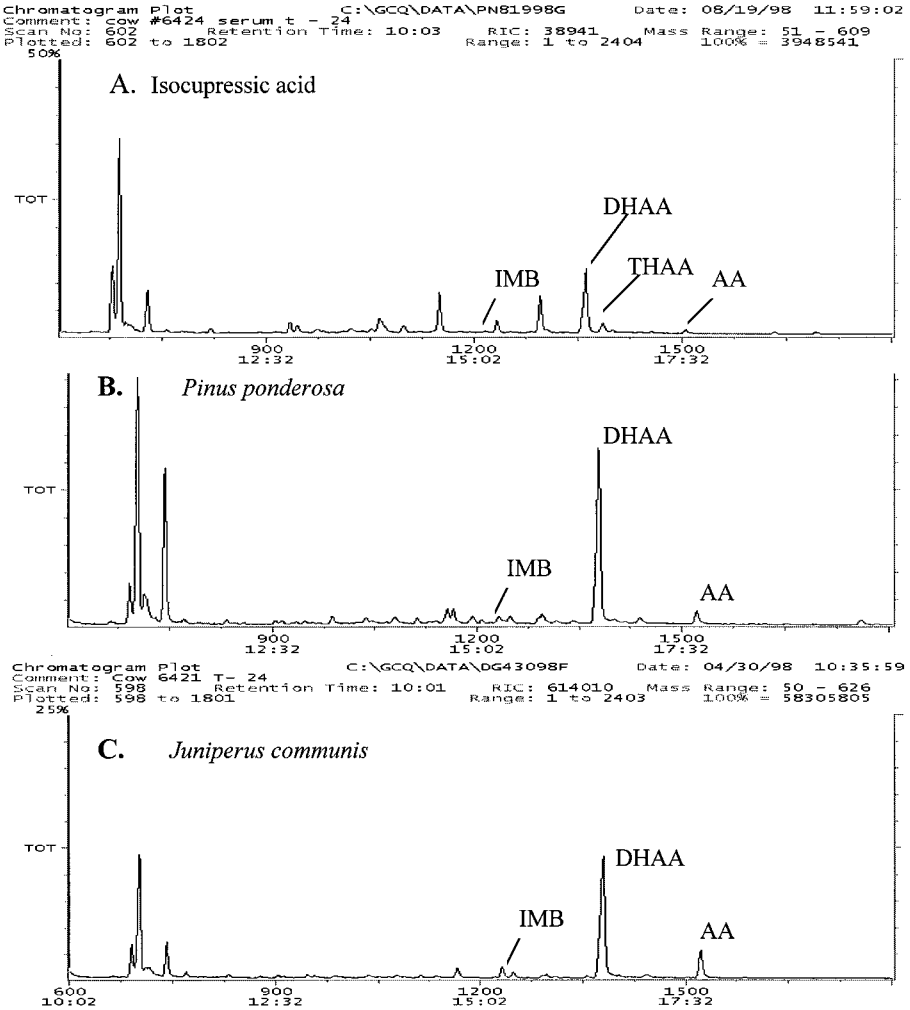

Figure 6. GC/MS chromatograms of ICA metabolites in serum from orally dosed cows 24 h after dosage: (A) purified ICA dose; (B) *P. ponderosa* dosage; (C) *J. communis* dosage; AA (agathic acid); DHAA (dihydroagathic acid); IMB (imbricatoloic acid).

MS identified three possible metabolites (Figure 3). The major metabolite identified was imbricatoloic acid (**4**) and accounted for 68% of the total extracted acids after 32 h of incubation. A second metabolite, which eluted just prior to imbricatoloic acid in the GC/MS chromatogram (Figure 3), is possibly an isomer of imbricatoloic acid resulting from isomerization of the C-13,14 double bond during reduction of ICA. The mass spectrum of this compound was identical to that of imbricatoloic acid and accounted for ~19% of the total extracted acids. Dihydroagathic acid (**3**) was the only other metabolite (11% of the total extracted acids) detected. No agathic acid (**2**) was detected.

Abortifacient Activity of ICA after Dosage by Intravenous Infusion. Four cows were treated with ICA by iv infusion to determine if rumen metabolism is required for ICA to be abortifacient in cattle. After iv infusion (twice daily) into four pregnant cows starting on day 250 of gestation, three of the four cows aborted between 2 and 7 days and retained their placentas (Table 1). Two additional cows received a phosphate-buffered saline solution (pH 10) dose as control and calved at the expected normal stage of gestation and did not retain their placentas. The resulting abortions from iv infusion of ICA demonstrate that ruminal metabolism of ICA is not required for abortions to occur in cattle.

Analysis of Serum Metabolites after Intravenous Infusion of ICA. Intravenous infusion of ICA was repeated and the blood serum sampled over time (up to 24 h). Metabolism of ICA was rapid, and metabolites were detected (Figure 4) in the first sample (20 min) after infusion. Only a residual amount of ICA was detected in the serum after treatment. Agathic acid (**2**) was identified as the major serum metabolite along with trace amounts of dihydroagathic acid (**3**). Initially, ICA and the metabolites were quickly removed from the serum (Figure 5); however, trace amounts of **2** and **3** were detected in the serum for up to 12 h after the infusion.

Analysis of Serum Metabolites after Oral Dosage of ICA. Serum was collected from cows orally dosed with ICA by the following three treatments and subsequently analyzed for ICA metabolites: (1) with purified ICA mixed with alfalfa hay; (2) with *J. communis* plant material containing ICA as the major diterpene resin acid; and (3) with *P. ponderosa* needles that contain ICA, acetylisocupressic acid, and succinylisocupressic acid (see dose materials, Table 2).

ICA was not detected in any serum samples taken from orally dosed cows. Agathic acid (**2**) and dihydroagathic acid (**3**) were readily detected (Figure 6), and two other possible metabolites were detected at very low levels. The two minor components were identified as imbricatoloic acid (**4**) and a structurally uncharacterized compound tentatively identified as tetrahydroagathic acid (**5**) (see mass spectral data under Materials and Methods). Imbricatoloic acid (**4**) was present as a minor component in both the *P. ponderosa* and *J. communis* plant materials but not in the purified ICA. In the serum, the trace amounts of imbricatoloic acid (**4**) could originate from two possible sources: direct absorption from plant material and from the rumen after enzymatic reduction of ICA. Tetrahydroagathic acid (**5**) was not present in any of the original dosage materials and was not detected in serum samples until after ~30 h postdosage.

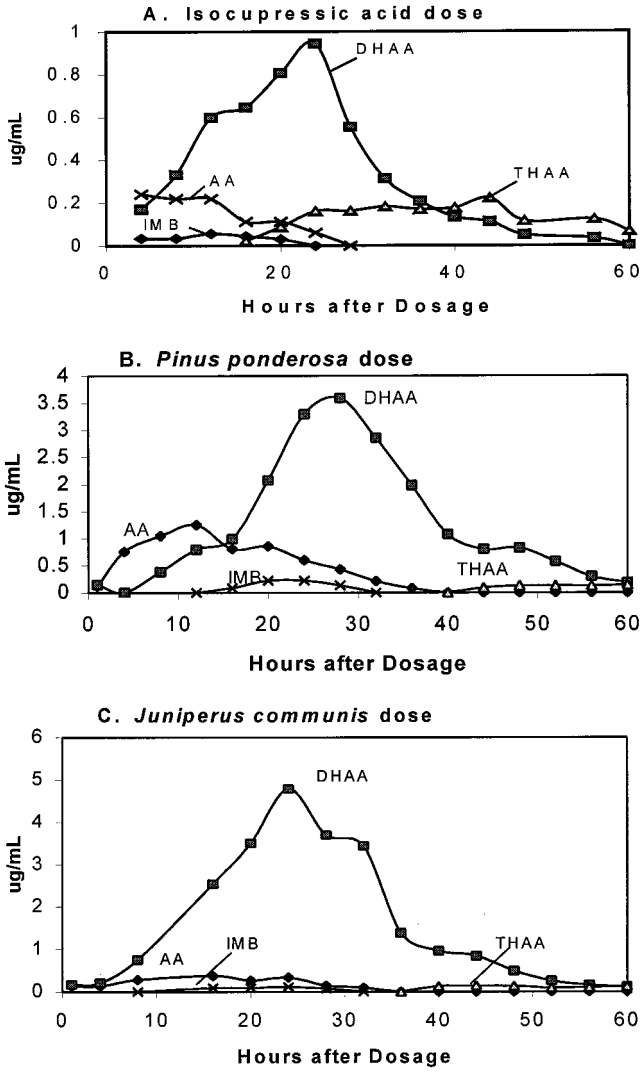

Figure 7. Concentration of ICA metabolites in serum of cows treated by oral dosage of (A) ICA, (B) *P. ponderosa*, and (C) *J. communis*.

Table 3. Metabolism of ICA to Agathic Acid in Liver Homogenate Samples [Relative Percent (Agathic Acid/ICA) after Incubation in Liver Homogenate Preparations]

reaction time (h)	cow	sheep	pig	goat	guinea pig	rat
0.5	92	94	100	93	17	31
2	100	100	100	100	30	50
18	100	100	100	100	80	100

Serum metabolite concentrations after the three oral treatments are displayed in Figure 7. Peak metabolite concentrations for dihydroagathic acid occur near 24 h postdosage, whereas agathic acid concentrations peaked at ~10 h. Tetrahydroagathic acid concentrations appear to peak at ~40 h postdosage. The identified metabolites and approximate relative amounts found in the serum after both iv and oral dosages are summarized in Table 2.

In Vitro Liver Metabolism of ICA. ICA was incubated in liver homogenate and liver supernatant fractions from cow, sheep, pig, goat, guinea pig, and rat livers and the microsomal fraction of the cow liver. The results were similar for all six animal species, although the guinea pig and rat liver preparations were less efficient in metabolizing ICA (Table 3). The liver homogenate and liver supernatant fraction metabolized

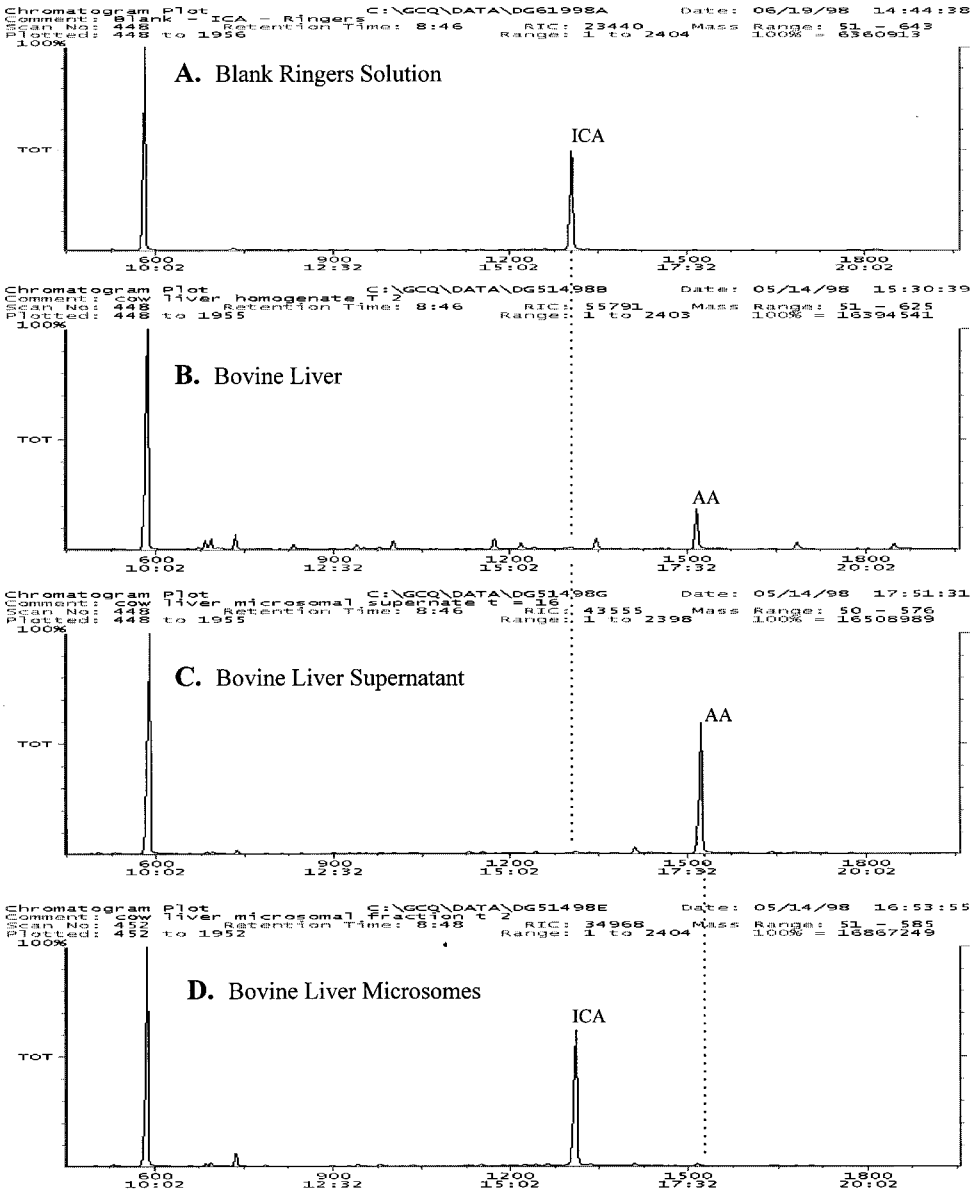

Figure 8. GC/MS chromatograms of ICA and metabolites from in vitro incubation of ICA with (A) lactated Ringers solution, (B) bovine liver homogenate, (C) bovine liver supernatant, and (D) bovine liver microsomal fraction: AA (agathic acid); ES (external standard).

ICA to agathic acid (Figure 8), and conversion was typically complete after a 30 min incubation period in the cow, sheep, pig, and goat liver preparations. The liver microsomal fraction from the cow showed no metabolic activity; essentially 100% of the ICA was recovered even after an 18 h incubation time. Subsequently, the addition of a cytochrome P-450 inhibitor (carbon monoxide) to a cow liver supernatant fraction also did not inhibit metabolism of ICA to agathic acid.

DISCUSSION

ICA induces abortions in late-term pregnant cattle when given either orally or by iv infusion (Gardner et al., 1994, 1996, 1997). Induced abortions after iv infusion of ICA indicate that abortifacient activity is not dependent on ruminal metabolism of ICA and yet does not preclude the importance of the rumen in metabolism of acetyl- and succinyl-ICA (Gardner et al., 1996). We previously suggested that ICA was not significantly metabolized in the rumen (Gardner et al., 1996); however, additional experiments by Lin et al. (1998), and those here, have shown ICA to be metabolized in vitro. It has been further demonstrated in the current work that ICA is rapidly metabolized in the liver to agathic acid most likely via soluble enzymes (alcohol and aldehyde dehydrogenase) and that liver metabolism of ICA is not specific to cattle.

Our results on the incubation of ICA in cow rumen preparations for time periods >8 h are slightly different from those reported by Lin et al. (1998). We observed enzymatic reduction of ICA to imbricatoloic acid as the major rumen metabolite and detected very little oxidation to dihydroagathic acid or the direct oxidation of ICA to agathic acid. It is not evident what factors account for the difference between these two experiments. However, one possible explanation would be diet and differences in the source rumen microflora. Animals used in this study were maintained on an alfalfa hay diet. Animals maintained on a higher percentage of

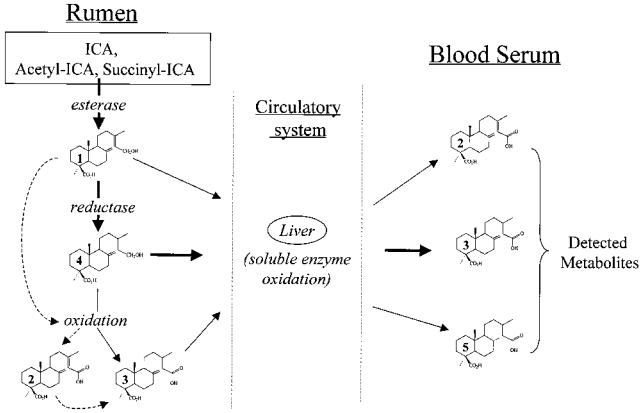

Figure 9. Summary of proposed metabolism of ICA after ingestion by cattle: (**1**) ICA; (**2**) agathic acid; (**3**) dihydroagathic acid; (**4**) imbricatoloic acid; (**5**) tetrahydroagathic acid. Bold arrows indicate the major pathway found under the reported experimental conditions. Dashed arrows indicate the pathway as proposed by Lin et al. (1998) with ICA oxidized to **2** and then reduced to **3**.

grass hay or grain diets would differ in rumen microflora, which might favor an oxidative endpoint in the rumen metabolism of ICA. Lin et al. (1998) did not report the diet of the source animals used in that study but did mention that grass hay was added to the in vitro rumen preparations as opposed to alfalfa hay reported here.

Whatever preferred metabolic transformation occurs in the rumen, it is likely that the resulting serum metabolites will eventually be the same. For example, Lin et al. (1998) found dihydroagathic acid to be the sole metabolite in plasma of cows fed ponderosa pine needles, and in our current experiments dihydroagathic acid was again the major metabolite detected in serum samples from cows receiving oral treatments of purified ICA or whole plant material. The additional metabolites of ICA (**2**, **4**, and **5**) identified in our serum samples and not reported by Lin et al. (1998) might be accounted for with differences in sample incubation time, rumen microflora, or analytical method sensitivity between the two investigations. More importantly, the major metabolite formed in both experiments was dihydroagathic acid.

We have attempted to summarize what is now known on the metabolism of ICA in cattle schematically in Figure 9. We hypothesize that some portion of ICA is absorbed directly from the rumen and then metabolized to agathic acid (**2**) by the liver. This would account for the initial levels of agathic acid in the serum. A second portion of ICA is metabolized in the rumen to dihydroagathic acid (**3**) [as proposed by Lin et al. (1998)] and then absorbed with no further metabolism. In addition, ICA in the rumen could be metabolized to imbricatoloic acid (**4**), absorbed, and then oxidized to dihydroagathic acid (**3**) via the liver, thus providing a second pathway for conversion of ICA to dihydroagathic acid. The transformation of ICA to tetrahydroagathic acid (**5**) requires an additional reduction of the exomethylene group, and it is not clear if this occurs in the rumen or postabsorption.

It is evident that ICA is rapidly metabolized by the liver but can also be metabolized by ruminal microorganisms given long residence times in the rumen. After iv administration of ICA, the major metabolite formed is agathic acid, whereas after oral dosage of ICA, the major metabolite formed is dihydroagathic acid. After either method of treatment (oral or iv) with ICA, a cow may abort her calf. It is possible that both agathic and dihydroagathic acid are abortifacient in the cow as their structures differ only in the C-13,14 bond. It could also be asked what additional metabolites of ICA might be present but not detected using the current methods. In the in vitro rumen experiments essentially all of the ICA added was accounted for by the sum of unmetabolized ICA and the detected metabolites. However, such a mass balance experiment has not been completed in vivo. In addition, terminal metabolites and the mechanism of elimination are not known.

ACKNOWLEDGMENT

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