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Development of Enzyme-Linked Immunosorbent Assays for Isocupressic Acid and Serum Metabolites of Isocupressic Acid

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The consumption of ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), common juniper (*Juniperus communis*), and Monterey cypress (*Cupressus macrocarpa*) causes abortions in pregnant cattle. Recent studies have identified isocupressic acid (**1**) as the primary abortificient compound in these plants. In vitro and in vivo studies using rumen and blood have shown isocupressic acid (**1**) is rapidly metabolized to agathic acid (**3**), dihydroagathic acid (**4**), and tetrahydroagathic acid (**5**). Rapid and sensitive diagnostic techniques are needed to identify poisoned animals, to study toxicokinetics, and to elucidate the mechanism of isocupressic acid-induced abortion in cattle. In this study, four competitive inhibition enzyme-linked immunosorbent assays for isocupressic acid and its sera metabolites were developed using polyclonal antibodies. One assay is specific to **1**, whereas the other three assays show cross-reactivity to **3**−**5** in addition to **1**. The assay specific to **1** had a limit of detection of 44.1 pg. The other assays which demonstrated cross-reactivity to the isocupressic acid blood metabolites also had comparably low limits of detection. One assay was used to follow the absorption and elimination profile of isocupressic acid metabolites in both cow serum and urine after oral dosage of a cow with common juniper.

KEYWORDS: Enzyme-linked immunosorbent assay; isocupressic acid; agathic acid; dihydroagathic acid; tetrahydroagathic acid; cattle abortions

INTRODUCTION

The consumption of ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), common juniper (*Juniperus communis*), and Monterey cypress (*Cupressus macrocarpa*) induces abortions in pregnant cattle that are in the second and third trimesters of gestation (*1*−*4*). Common poisoning scenarios occur in late winter and early spring when storms force cattle into pine tree stands for shelter and food. Under these conditions, pregnant cows often consume both pine needles and pine litter that can result in an increase in cattle abortions (*2*, *5*, *6*). Pine-needle-induced abortions can occur as early as 24 h or as late at 2 weeks after consumption. These abortions typically result in stillborn or small weak calves that generally die without extensive care. Cows that have aborted have retained placentas and develop endometritis and require expensive medical treatment for survival. The abortion and secondary infections affect subsequent reproduction as some cows become infertile and others require extended time periods to become pregnant again. The annual economic losses due to pine needle abortions have been estimated to be as high as $20 million (*7*, *8*).

Isocupressic acid (**1**) (**Figure 1**) has been identified as the primary abortificient compound in ponderosa pine, lodgepole pine, common juniper, and Monterey cypress (*3*, *4*, *9*, *10*). However, recent in vitro and in vivo studies have demonstrated the rapid metabolism of isocupressic acid (**1**) to several structurally related metabolites such as agathic acid (**3**), dihydroagathic acid (**4**), and tetrahydroagathic acid (**5**) (**Figure 1**) (*9*, *11*, *12*). These metabolites are present in the blood at parts per million levels. Currently, the biochemical role of isocupressic acid (**1**) and the more recently discovered metabolites in the abortion mechanism within the pregnant cow is unknown.

Because of the large economic losses due to pine-needle-induced abortions, the difficulty in obtaining a definitive diagnosis and predicting the outcome of poisoned cattle, and the ongoing research into the toxicokinetics and mechanism of abortion, it is important to develop rapid analytical and diagnostic methodologies capable of detecting and measuring isocupressic acid and its metabolites in animal tissues. Enzyme-linked immunosorbent assays (ELISAs) have been developed

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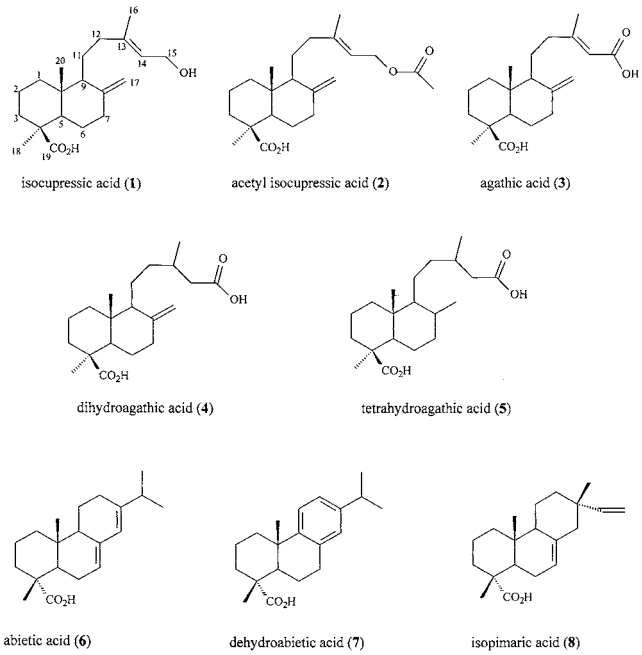

Figure 1. Chemical structures of isocupressic acid, serum metabolites of isocupressic acid, and abietane diterpene acids.

as alternatives to traditional analytical techniques for detection of natural products (*13*−*22*). ELISAs offer the advantages of minimal sample preparation and the ability to run many samples simultaneously.

In this investigation four different labdane diterpene acid−protein conjugates were prepared and their polyclonal antibodies were generated. The antibodies were characterized by cross-reactivity studies. Four competitive inhibition enzyme-linked immunosorbent assays (CI-ELISA) were developed. One assay is specific to **1**, whereas the other three assays show cross-reactivity to **3**, **4**, and **5** in addition to **1**. The applicability of these assays for the detection of **3**−**5** in cow serum and urine is demonstrated.

MATERIALS AND METHODS

Chemicals. Fetuin from fetal calf serum, chicken egg albumin (OVA), succinic anhydride, 3,3′,5,5′-tetramethylbenzidine, thimerosal, polyoxyethylene sorbitan monolaurate (Tween 20), and phosphate buffered saline tablets were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Hydroxysuccinimide (NHS), 1,3-dicyclohexylcarbodiimide (DCC), and ethylene glycol dimethyl ether were purchased from Aldrich Chemical Co. (Milwaukee, WI). Pyridine and dimethyl sulfoxide were purchased from Mallinckrodt Specialty Chemical Co. (Paris, KY). Chloroform and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Bovine serum albumin (BSA) fraction V reagent grade was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). DEAE dextran was purchased from Pharmacia Biotech (Uppsala, Sweden). Quil A saponin was obtained from Superfos Biosector a/s (Frydenlundsvej, Denmark). Montanide 888 was purchased from Seppic (Paris, France). Carnation Nonfat Dry Milk was obtained from Nestle USA, Inc. (Solon, OH).

Isocupressic acid (**1**) was isolated from the bark of *Pinus ponderosa* using previously described methods (*9*). Acetyl isocupressic acid (**2**), agathic acid (**3**), dihydroagathic acid (**4**), and tetrahydroagathic acid (**5**) were synthesized from **1** using previously described methods (*12*, *23*). Abietic acid (**6**), dehydroabietic acid (**7**), and isopimaric acid (**8**) were purchased from Helix Biotech (New Westminster, BC).

Alkaloid−protein conjugates were filtered and concentrated using an ultrafiltration cell from Amicon, Inc. (Beverly, MA) and 30 000 MW cellulose ultrafiltration membranes purchased from Millipore Corp. (Bedford, MA). Enzyme-linked immunosorbent assays (ELISAs) were performed on 96-well NUNC F96 Maxisorp polystyrene microtiter plates purchased from VWR Scientific Products (Denver, CO). Microtiter plates were read at 450 nm with a model 3550-UV microplate reader (Bio Rad Laboratories, Hercules, CA). Atmospheric pressure chemical ionization (APCI) mass spectral data were acquired on an LCQ mass spectrometer (Finnigan Corporation, San Jose, CA). Samples were loop-injected into the APCI source in a methanol/ammonium acetate (20 mM) solution (1:1, v:v) at a flow rate of 0.5 mL/min. Nonlinear curve fitting was performed using SigmaPlot software purchased from Jandel Scientific (Sausalito, CA).

Isocupressic Acid Hapten. Isocupressic acid (**1**) (369.3 mg, 1.15 mmol) was dissolved in dry THF (10 mL), and NaH (106.9 mg of a 60% dispersion in oil) and methyl bromoacetate (0.230 mL, 2.45 mmol) were added to it in a 25-mL round-bottom flask, and the mixture was refluxed continuously with stirring (18 h). The THF was removed by rotary evaporation and the residue was taken up in ethyl acetate (20 mL). The organic layer was washed with saturated $NaHCO_3$ (15 mL) and distilled water (15 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated by rotary evaporation. The reaction product was isolated by column chromatography using silica as the stationary phase and a hexane/ethyl acetate/acetic acid (75:25:1, v:v:v) mixture as the mobile phase. The structure of the product (276.1 mg, 7.04×10^{-1} mmol, 61% yield) was confirmed by MS and ^{1}H NMR. 15-Methoxycarbonylmethyl-isocupressic acid (**9**): positive ion APCI-MS, $MH^+ = 392$; ^{1}H NMR (270 MHz, CD_3OD), 3.73 (s, 3H, H-23), 4.55 (d, 1H, H-21), 4.62 (d, 1H, H-21), other proton resonances were similar to those for isocupressic acid (*9*).

15-Methoxycarbonylmethyl-isocupressic acid (**9**) (160.2 mg, 4.09×10^{-1} mmol) was dissolved in methanol (7.1 mL) containing sodium hydroxide (1.2 mL of a 12 N solution), and refluxed (2 h). The solution was acidified with 1 N HCl and extracted with ethyl acetate (3×30 mL). The organic layer was dried over magnesium sulfate, filtered, and concentrated by rotary evaporation. The isocupressic acid hapten was isolated by prep-scale reversed-phase HPLC. The structure of the product (60.0 mg, 1.60×10^{-1} mmol, 39% yield) was confirmed by HREI and ^{1}H NMR. 15-Carboxymethyl-isocupressic acid (**10**): HREI, 378.2399 (M^+), $C_{22}H_{34}O_5$ requires 378.2406; ^{1}H NMR (270 MHz, CD_3OD), 4.26 (d, 1H, H-21), 4.55 (d, 1H, H-21), other proton resonances were similar to those for isocupressic acid (*9*).

Hapten Activation and Protein Conjugation. 15-Carboxymethyl-isocupressic acid (**10**), agathic acid (**2**), dihydroagathic acid (**3**), and tetrahydroagathic acid (**4**) (referred to as haptens) were each conjugated to immunizing and coating proteins. The reaction scheme for the *N*-hydroxysuccinimide (NHS) activation, and subsequent protein conjugation of the haptens is shown in steps 3 and 4 of **Figure 2**. The hapten, *N*-hydroxysuccinimide, and dicyclohexylcarbodiimide were measured into equal molar ratios and added to anhydrous chloroform. The reaction was stirred (16 h, room temperature), the reaction flask was cooled on dry ice, and the solution was filtered through a Buchner funnel and transferred to a 20-mL round-bottom flask. The chloroform was removed (in vacuo) at ambient temperature. The transparent residue was stored in a desiccator until conjugation with the protein.

The NHS-activated haptens were dissolved in dimethyl sulfoxide. The appropriate amount of dimethyl sulfoxide solution was added slowly to a continuously stirred solution of OVA or fetuin dissolved in 0.1 M $NaHCO_3$. The remaining dimethyl sulfoxide solution was further diluted with dimethyl sulfoxide to obtain the desired concentration. An appropriate amount of the further-diluted NHS-activated hapten/dimethyl sulfoxide solution was added slowly to a continuously stirred solution of BSA dissolved in 0.1 M $NaHCO_3$. The targeted conjugation ratios of these reactions were ∼20:1 hapten to OVA or fetuin and ∼5:1 hapten to BSA. These reactions were stirred (16 h) and then filtered (0.45 μm syringe filter). The eluent was again filtered through a 30 000 MW cutoff filter (5×) with deionized distilled water (50 mL). The product was then diluted to a concentration of ∼1 mg/mL in deionized distilled water and stored (−120 °C) as 1-mL aliquots. The OVA and BSA conjugates of the 15-carboxymethyl-isocupressic acid hapten are designated as ICA−OVA and ICA−BSA, repectively. The fetuin and BSA conjugates of agathic acid are designated as AA−FET and AA−BSA, respectively. The fetuin and BSA conjugates of dihydroagathic acid are designated as DHAA−FET and DHAA−BSA,

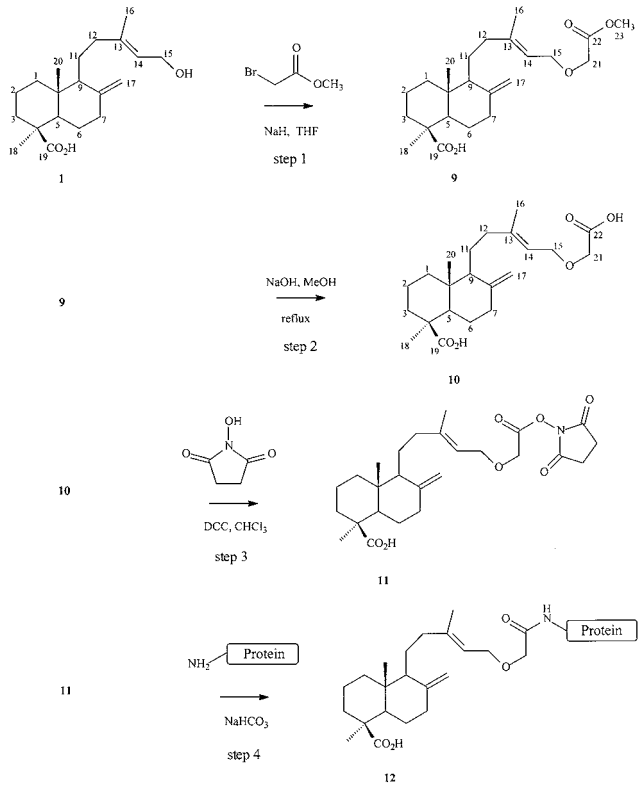

Figure 2. Synthesis of isocupressic acid protein conjugates.

respectively, while the fetuin and BSA conjugates of tetrahydroagathic acid are designated as THAA−FET and THAA−BSA, respectively.

Immunizations. The primary injection solution was prepared by adding a 1 mg/mL hapten−fetuin conjugate−distilled water solution (6 mL) to a 1.25% DEAE−dextran, 0.125% Quil A, distilled water solution (9.6 mL), and emulsified with Montanide 888 (14.4 mL) for a total volume of ∼30 mL. Two crossbred ewes were initially injected subcutaneously with primary injection solution (2 mL) or 0.4 mg of hapten−fetuin conjugate. Booster injections with one-half the concentration of hapten−fetuin conjugate (0.2 mg) in the above injection solution were given after three six-week intervals. Blood samples were drawn immediately before the initial injection and 14 days after the second booster injection. Sera were stored at −120 °C.

ELISA Procedure. Isocupressic acid (**1**)−, agathic acid (**3**)−, dihydroagathic acid (**4**)−, and tetrahydroagathic acid (**5**)−BSA conjugates were dissolved in carbonate buffer (0.05 M, pH 9.6), and 100 μL was added to each well of the microtiter plate. The microtiter plates were incubated for 2 h at ambient temperature and then 16 h at 4 °C. The plates were then inverted to remove excess coating solution. The plates were then covered with an adhesive plate sealer and stored in a plastic bag (−20 °C) for up to 6 months. The plates were washed (3×) with saline−Tween buffer (0.15 M NaCl, 0.5% Tween 20) and blotted dry. Next, 150 μL of assay buffer (0.1 M Tris, pH 7.5, 0.1% Tween 20, 5% Carnation Nonfat Dry Milk powder) was added and the were plates incubated (1 h). The assay buffer acted as a blocking buffer to reduce nonspecific binding of the antisera. The plates were washed (3×) and blotted dry. A 50-μL aliquot of samples or standards diluted in the assay buffer was added to each of the wells followed by 50 μL of antiserum diluted in the assay buffer. The plates were then incubated (2 h). After the-2 h incubation, the plates were washed (4×), and HRP-conjugated donkey anti-sheep IgG (100 μL), diluted 1/10 000 in the assay buffer, was added to all wells, and the plates were again incubated (1 h). The plates were then washed (4×), and 100 μL of tetramethylbenzidine/H_2O_2 substrate (pH 5.5, 30 °C) (*24*) was added to each well. After 10 min the reaction was stopped by the addition of 50 μL of 0.5 M H_2SO_4 to each well. The absorbances were measured at 450 nm (OD_{450}).

GC/MS Analysis of Cow Serum. Serum sample preparation procedures and subsequent GC/MS analysis have been described in detail previously (*10*). Briefly, 1 mL of saturated KH_2PO_4 was added to 5.0 mL of serum, and then extracted twice with 3 mL of $CHCl_3$ for 5 min. The combined $CHCl_3$ extracts were filtered through anhydrous sodium sulfate and the solvent was removed to dryness under a flow of N_2 in heat block (70 °C). Samples were derivatized for analysis by adding 0.50 mL of pyridine containing 210 μg/mL heptadecanoic acid and 50 μL of BSFTA silyation reagent and heated at 70 °C for 30 min. Samples were then analyzed by GC/MS using previous described conditions (*10*). The concentration of each detected metabolite was estimated based on peak area ratio with the heptadecanoic acid. Serum metabolite concentrations can be considered only an estimate as individual response factors were not determined.

Serum Preparation. Blood was collected from a cow by jugular venipuncture into sterile tubes. The blood was allowed to coagulate (45 min). The samples were then centrifuged (2100 rpm, 30 min), and the serum was removed with a pipet. Serum aliquots were spiked with **2**, **3**, or **4** at concentrations of 100, 500, 1000, and 6000 ppb (pg/μL), vortexed, and refrigerated for a minimum of 16 h. A 20-μL portion of the serum was diluted into 980 μL of assay buffer and 50 μL was applied to the microtiter plates.

Urine Preparation. For spike and recovery experiments, aliquots of urine were spiked with **2**, **3**, or **4** at concentrations of 100, 500, 1000, and 6000 ppb (pg/μL), vortexed, and refrigerated for a minimum of 16 h. A 20-μL aliquot of the urine was diluted into 980 μL of assay buffer and 50 μL was applied to the microtiter plates.

Plant Material Doses. *Juniperus communis* was collected from Gibson Basin, 20 miles east of Logan, Utah, in the Cache National Forest. The pine needles were dried, ground to pass a 2-mm screen, analyzed by GC/MS, and found to contain 2.5% w/w isocupressic acid (*25*). A single dose of ground plant material (1.8 kg) was given by oral gavage (via stomach tube) to one cow. Blood and urine samples were collected prior to treatment, and at 4-h intervals for 40 h postdosage. From 40 to 80 h postdosage, blood and urine samples were collected at 8-h intervals. The serum fraction was recovered and serum and urine samples were stored at −20 °C until ELISA and GC/MS analysis.

RESULTS AND DISCUSSION

Hapten Synthesis and Conjugation. Isocupressic acid (**1**), agathic acid (**3**), dihydroagathic acid (**4**), and tetrahydroagathic acid (**5**) are not large enough compounds to be immunogenic. These compounds were therefore conjugated to a high-molecular-weight protein to elicit an immune response. Isocupressic acid (**1**) was conjugated to the carrier and coating proteins through an α alkoxyl acetamide linker. The α alkoxyl acetamide linker was intoduced by reacting methyl bromoacetate with **1** to form 15-methoxycarbonylmethyl-isocupressic acid (**9**) which was then demethylated, resulting in 15-carboxymethyl-isocupressic acid (**10**). 15-Carboxymethyl-isocupressic acid (**10**) has carboxylic acid groups at C-22 and C-19. The carboxylic acid at C-22 is α to a primary carbon and is much more reactive than the carboxylic acid at C-19 which is α to a quaternary carbon and is sterically hindered. When 15-carboxymethyl-isocupressic acid is activated with NHS in a 1:1 molar ratio, the NHS ester preferentially forms at C-22 resulting in **11**. Compound **11** was then reacted with the protein to form a 2-atom-length α alkoxyl acetamide linker **12** (**Figure 2**). Agathic acid (**3**), dihydroagathic acid (**4**), and tetrahydroagathic acid (**5**) contain two carboxylic acid groups. Because these compounds each possessed carboxylic acid groups α to a primary carbon, no spacer groups were introduced, and these compounds were directly conjugated to carrier and coating proteins by activation of the carboxylic acid groups α to the primary carbon and subsequent reaction with the proteins to form 0-atom-length linkers.

Antisera. Antibody titers were determined by titration of serial dilutions (1/500−1/256 000) of sheep antisera raised

Table 1. Analytical Figures of Merit for Assays 1, 2, 3, and 4

	I_{50} (pg)	slope	LOD (I_{80}) (pg)	I_{50} (pg)	slope	LOD (I_{80}) (pg)
		Assay 1			Assay 2	
isocupressic acid (**1**)	794	2.85	44.1	391	5.22	76.4
acetyl isocupressic acid (**2**)	4.87×10^4	5.55	4.13×10^3	NP[a]	NP	NP
agathic acid (**3**)	1.23×10^5	3.96	2.64×10^3	989	6.16	191
dihydroagathic acid (**4**)	6.42×10^4	5.42	1.19×10^3	333	3.60	40.8
tetrahydroagathic acid (**5**)	4.23×10^5	2.96	2.96×10^3	893	3.90	104
		Assay 3			Assay 4	
isocupressic acid (**1**)	692	5.19	132	443	4.24	63.3
acetyl isocupressic acid (**2**)	NP	NP	NP	NP	NP	NP
agathic acid (**3**)	738	4.38	106	736	5.59	181
dihydroagathic acid (**4**)	276	4.02	53.0	312	4.37	62.0
tetrahydroagathic acid (**5**)	626	4.26	94.0	264	4.85	68.1

[a] NP, Competitive inhibition was not performed on Assays 2, 3, and 4 with acetyl isocupressic acid.

against all four injection conjugates with 250 ng/well of corresponding BSA conjugates used as well coatings. All sheep injected with conjugates produced antibodies. The sera were compared from the two sheep that were injected with the same immuno-conjugate. The sera from the sheep that resulted in the highest titers was selected for further ELISA development.

Four independent competitive inhibition ELISA assays were developed with the antisera raised against ICA−OVA (assay 1), AA−FET (assay 2), DHAA−FET (assay 3), and THAA−FET (assay 4) with their corresponding BSA conjugates used as coating conjugates. The optimum dilutions for both the coating conjugate and antiserum were determined by checkerboard titrations. The concentration of the coating conjugate and antiserum were selected based on the combination at which the greatest difference in optical density were observed at 450 nm (OD_{450}s) between the wells of no free isocupressic acid metabolite and the presence of isocupressic acid metabolite at levels of 0.1 to 10 ng/well, and yet still giving an optical density between 0.9 and 1.3 in the absence of the isocupressic acid metabolite.

Cross-Reactivity. To evaluate the cross-reactivity of typical diterpene resin acids found in ponderosa and lodgepole pine, five labdane and three abeitane diterpene acids were selected for cross-reactivity studies: isocupressic acid (**1**), acetyl isocupressic acid (**2**), agathic acid (**3**), dihydroagathic acid (**4**), tetrahydroagathic acid (**5**), abietic acid (**6**), dehydroabietic acid (**7**), and isopimaric acid (**8**) (**Figure 1**). The compounds were tested for cross reactivity over the range of 2.0 pg/well to 1.0×10^6 pg/well. The data for each compound were fit to the four-parameter equation

$$y = (a - d)/[1 + (x/c)^b] + d \qquad (1)$$

to yield inhibition curves. In this equation a and d are the upper and lower asymptotes, b is the slope of the linear portion of the curve, and c is the midpoint of the linear portion of the curve. **Table 1** reports the I_{50} values, the limit of detection (LOD) (I_{80}), and the slope (b) of the curves for each compound. The I_{50} values were calculated from the equation of the curve. The limit of detection is conservatively defined in this study as the I_{80} values, as calculated from the equation of the curve.

Assay 1. Checkerboard assays using the antiserum raised against the ICA−OVA immunogen with ICA−BSA coating conjugate resulted in an optimum dilution of antisera of 1/8000 with the coating conjugate at 200 ng/well for a competitive inhibition ELISA assay. The I_{50} values reported in **Table 1** show compounds **2**−**5** are recognized and cross-react with isocupressic acid (**1**) only at masses 2 orders of magnitude greater than **1**. The LOD for isocupressic acid (**1**) in this assay is 44.1 pg which is an order of magnitude lower than the limit of detection of 360 pg which we measured by GC/MS (*26*).

Assay 2. The optimum dilution of AA−FET antisera and AA−BSA coating conjugate was determined to be 1/3000 and 100 ng/well for this assay. The I_{50} values reported in **Table 1** show compounds **1**, **3**, **4**, and **5** cross react. Abietic acid (**6**), dehydroabietic acid (**7**), and isopimaric acid (**8**) were tested, but showed no cross-reactivity to the antibodies over the concentration range studies in this assay. The LODs for **1**, **3**, **4**, and **5** in this assay are 76.4, 191, 40.8, and 104 pg, respectively.

Assay 3. The optimum dilution of DHAA−FET antisera and DHAA−BSA coating conjugate was determined to be 1/10 000 and 250 ng/well for this assay. The I_{50} values reported in **Table 1** show compounds **1**, **3**, **4**, and **5** cross react. Compounds **6**−**8** showed no cross-reactivity to the antibodies over the concentration range studies in this assay. The LODs for **1**, **3**, **4**, and **5** in this assay are 132, 106, 53.0, and 94.0 pg, respectively.

Assay 4. The optimum dilution of THAA−FET antisera and THAA−BSA coating conjugate was determined to be 1/128 000 and 50 ng/well for this assay. The I_{50} values reported in **Table 1** show compounds **1**, **3**, **4**, and **5** cross react. Compounds **6**−**8** were tested, but showed no cross-reactivity with the antibodies over the concentration range studies in this assay. The LODs for **1**, **3**, **4**, and **5** in this assay are 63.3, 181, 62.0, and 68.1 pg, respectively.

Spike and Recovery of Isocupressic Acid Metabolites in Cow Serum and Urine. We evaluated the applicability of assay 4 by a series of spike and recovery experiments for known serum metabolites of isocupressic acid (**3**−**5**) in cow serum and urine. Assay 4 was chosen because the average I_{50} value for the isocupressic acid metabolites (**3**−**5**) was lower in this assay than those in assays 1, 2 and 3. In addition, assay 4 had the lowest limit of detection for tetrahydroagathic acid (**5**) which is observed for longer periods of time in the serum than the other metabolites (*10*, *12*) making it the most useful metabolite to use as a diagnostic marker for pine-needle-induced abortion. Samples spiked with **3**−**5** were quantified against 10-point standard curves prepared the day of analysis in cow serum or urine consisting of agathic acid (**3**), dihydroagathic acid (**4**), and tetrahydroagathic acid (**5**), respectively, over the range of 15.6 to 8000 pg. The standard curves were determined by fitting the standards to eq 1. The r^2 values for the standard curves were >0.9953 in all cases. **Figure 3A** shows the spike and recovery results for four different levels of agathic acid (**3**) in serum and urine. Average recoveries for 100, 500, 1000, and

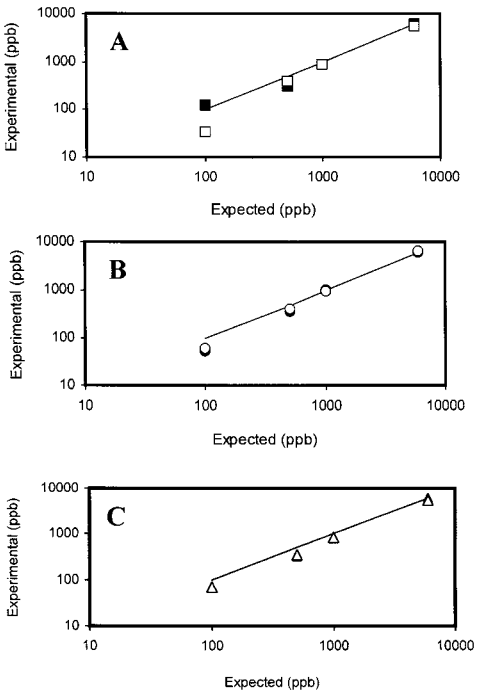

Figure 3. Correlation between spiked levels of (A) agathic acid and the level determined by assay 4 in cow serum (■) and urine (□); (B) dihydroagathic acid and the level determined by assay 4 in cow serum (●) and urine (○); and (C) tetrahydroagathic acid and the level determined by assay 4 in cow serum (▲) and urine (Δ). The line in each graph represents the theoretical correlation of agathic acid, dihydroagathic acid, and tetrahydroagathic acid levels in cow serum and urine. All points are the average of 3 replicates.

6000 ppb of agathic acid (**3**) in serum and urine ranged from 62 to 127% and 33 to 93%, respectively, while relative standard deviations ranged from 6.7 to 37% and 6.4 to 12%, respectively. **Figure 3B** shows the spike and recovery results for the same four concentrations of dihydroagathic acid (**4**) in blood and urine. Average recoveries for dihydroagathic acid (**4**) in blood and urine ranged from 54 to 104% and 65 to 105%, respectively, while relative standard deviations ranged from 6.5 to 21% and 1.6 to 16%, respectively. **Figure 3C** shows the spike and recovery results for the same four concentrations of tetrahydroagathic acid (**5**) in blood and urine. Average recoveries for tetrahydroagathic acid (**5**) in blood and urine ranged from 64 to 103% and 69 to 90%, respectively, and relative standard deviations ranged from 5.9 to 22% and 2.9 to 11%, respectively.

Estimation of Isocupressic Acid Metabolites in Cow Serum and Urine after Oral Dosage of Common Juniper. Assay 4 was also used to follow the absorption and elimination profile of the total isocupressic acid metabolites such as agathic acid (**3**), dihydroagathic acid (**4**), and tetrahydroagathic acid (**5**) in cow serum and urine. Serum samples were quantified against a 10-point agathic acid (**3**) standard curve prepared the day of analysis in cow serum over the range of 0−13 650 pg. Urine samples were quantified against a 10-point agathic acid (**3**) standard curve prepared the day of analysis in cow urine over the range of 0−20 480 pg. The standard curves were determined by fitting the standards to eq 1. The r^2 values for the standard curves were >0.9939 in both cases. **Figure 4** shows the results for the estimation of the total isocupressic acid metabolites in cow blood and urine by assay 4 after oral dosage of common juniper. **Figure 4** also shows the absorption and elimination profile of the total isocupressic acid metabolites in cow serum by GC/MS. With both the GC/MS and ELISA measurements,

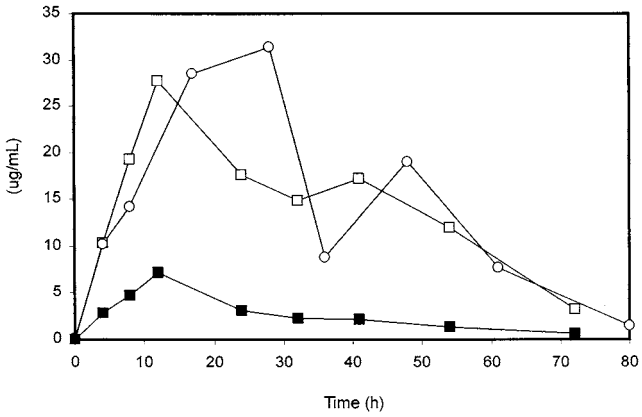

Figure 4. Concentration of total isocupressic acid metabolites in cow serum after oral dosage of common juniper as determined by assay 4 (□) and GC/MS (■); and concentration of total isocupressic acid metabolites in cow urine after oral dosage of common juniper as determined by assay 4 (○). All points are the average of 3 replicates.

the concentration of isocupressic acid metabolites in serum increases from the time of dosage and reaches a maximum at 12 h, after which the isocupressic acid metabolite concentration in the blood decreases over the next 58 h (12−70 h). In **Figure 4** the estimation of isocupressic acid metabolites in cow blood by the ELISA appears 5−6 times higher than the measurement of the known isocupressic acid metabolites by GC/MS. The elevated estimation of metabolites by assay 4 as compared to that by the GC/MS measurements may be attributed to several factors. One reason the ELISA estimation is higher is because the individual metabolites are all quantified against the agathic acid (**3**) standard curve. In assay 4 the agathic acid (**3**) standard curve has a higher limit of detection and a higher I_{50} value than the limits of detection and I_{50} values for dihydroagathic acid (**4**) and tetrahydroagathic acid (**5**) (**Table 1**). This results in mass values that may be inflated 2−3 times for the absorbances contributed by dihydroagathic (**4**) and tetrahydroagathic acid (**5**). Another reason that the estimation of metabolites by ELISA may be higher than that of the GC/MS measurement is that, because of its class specificity, the ELISA may be recognizing and measuring metabolites present in the serum that have not yet been identified by the GC/MS method. The GC/MS method relies on the relative quantitation of metabolite peak areas to an internal standard (heptadecanoic acid) peak area. While the GC/MS method is consistent within itself, recovery information and standard curves for each of the metabolites were not performed. A direct quantitative comparison between the two methods is impossible because of the inability of the ELISA method to measure the metabolites individually.

The concentration of total isocupressic acid metabolites in cow urine increases and peaks at 28 h after an oral dosage of common juniper, then the concentration decreases over the next 52 h (28−80 h). The ability to detect and estimate relative concentrations of isocupressic acid metabolites in cow urine is significant because the sample preparation and measurement of isocupressic acid metabolites in urine by GC/MS has proven to be more difficult than those in sera and have not yet been accomplished.

The simple ELISA methods described in this study demonstrate the potential of using these techniques for the rapid screening of biological samples for the presence and levels of isocupressic acid (**1**) and its blood and urine metabolites, and would be beneficial in the diagnosis of animal poisonings and pharmacological studies.

ABBREVIATIONS USED

BSA, bovine albumin; OVA, chicken egg albumin; CI−ELISA, competitive inhibition−enzyme linked immunosorbent assay; APCIMS, atmospheric pressure chemical ionization mass spectrometry; GC/MS, gas chromatography−mass spectrometry; HREI, high-resolution electron impact mass spectrometry; HPLC, high-performance liquid chromatography; LOD, limit of detection; NHS, *N*-hydroxysuccinimide; I_{50}, the mass of compound at which the absorbance at 450 nm is 50% of the maximum absorbance; I_{80}, the mass of compound at which the absorbance at 450 nm is 80% of the maximum absorbance; NMR, nuclear magnetic resonance spectrometry; OD_{450}, optical density at 450 nm; PBS, phosphate buffered saline solution (pH 7.4); ICA−OVA, isocupressic acid chicken egg albumin conjugate; ICA−BSA, isocupressic acid bovine albumin conjugate; AA−FET, agathic acid fetuin conjugate; AA−BSA, agathic acid bovine albumin conjugate; DHAA−FET, dihydroagathic acid fetuin conjugate; DHAA−BSA, dihydroagathic acid bovine albumin conjugate; THAA−FET, tetrahydroagathic acid fetuin conjugate; THAA−BSA, tetrahydroagathic acid bovine albumin conjugate; Tween 20, polyoxyethylenesorbitan monolaurate.

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