Transfer of Orally Administered [^{3}H]Seneciphylline into Cow's Milk

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The presence of pyrrolizidine alkaloids in dairy products could pose a hazard to human health. To investigate the transfer into cow's milk, a single dose of 1 mg of [^{3}H]seneciphylline/kg of body weight was given orally to a dairy cow. The appearance of radioactivity derived from this compound was monitored in the blood and milk. Calculated as seneciphylline, over 100 ng/mL was found in the blood during the first 18 h. After 54 h, 11 ng/mL was still present. Alkaloid levels were similar in milk. After 64 h, the concentration was still at 5 ng/mL. In total, 0.16% of the dose was excreted in the milk. In the liver, 40 ng/g (0.06% of the dose) was found 3 weeks after treatment. In addition to unchanged seneciphylline and retronecine, *N*-oxides were detected in the milk as metabolites (11.2% at 27 h). The results indicate that the highest possible concentration of seneciphylline in the milk of unaffected cows can hardly exceed 10–20 μg/L.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) can be found in a wide range of plant species from many botanical families. They are known hepatotoxins with mutagenic and carcinogenic properties (Mattocks, 1986a,b). Poisoning of domestic animals by PAs has been reported from various parts of the world. The plant species involved in these incidents were from the three genera, *Senecio*, *Crotalaria*, and *Heliotropium* (Lüthy et al., 1981; Mattocks, 1986c; Molyneux et al., 1988). PA intake by livestock is not only due to grazing. Experiments with *Senecio alpinus*, a widespread plant in alpine meadows, show that the PA concentration in hay is stable for several months but slowly decreases in silage (Candrian et al., 1984a).

Contamination of cow's milk with PAs is a potential source of human exposure. In 1959 Schoental reported the transfer of lasiocarpine and retrorsine or their toxic metabolites into the milk of lactating rats. The alkaloids were detected by the appearance of liver lesions in the sucklings. These observations were confirmed by Bhattacharyya (1965) and extended to the PAs heliotrine and monocrotaline. These results raise the question of a possible contamination of dairy products by PAs and the subsequent exposure of humans. Experiments with radioactively labeled senecionine and seneciphylline in mice (Eastman et al., 1982) and rats (Lüthy et al., 1983) showed that only a small percentage of the radioactivity applied can be found in the milk. Up to 3.8 μg/mL of PAs has been detected in the milk of goats fed large amounts of *Senecio jacobaea* (Dickinson and King, 1978; Deinzer et al., 1982; Goeger et al., 1982; White et al., 1984). In these studies, the PA dosage was only approximately known. When cows were fed chronic lethal doses of *S. jacobaea* and their milk was subsequently given to calves and rats, no histopathologic changes of the liver were detected (Johnson, 1976). Cows fed highly toxic amounts of PAs from *S. jacobaea* gave milk in which relatively low concentrations of PAs, between 94 and 167 ng/mL, were found (Dickinson et al., 1976). In our study, the transfer of a single dose of [^{3}H]seneciphylline into cow's milk was investigated under well-defined experimental conditions. Seneciphylline is one of the major PAs of *S. jacobaea* (Dimenna et al., 1980; Ramsdell and Buhler, 1981) and the principal alkaloid of *S. alpinus* (Lüthy et al., 1981). *S. alpinus* was discovered as the cause of pyrrolizidine alkaloidosis in three herds of dairy cattle in Switzerland (Pohlenz et al., 1980). *S. jacobaea* has been responsible for loss by death of cattle and horses in the western United States (Dickinson and King, 1978).

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EXPERIMENTAL PROCEDURES

Animal. The dairy cow (Swiss brown, 9820-14) used for this study was 2 years and 9 month old, weighed 549 kg, and was in its first lactation period. The animal was scheduled to be slaughtered because of low milk production (<10 L/day). It was killed 21 days after the peroral single dose of [^{3}H]seneciphylline was administered. The liver was collected and kept at 4 °C for a few hours until frozen at −20 °C.

[^{3}H]Seneciphylline (See Figure 1). The biosynthesis, isolation, and characterization of [^{3}H]seneciphylline have been described (Candrian et al., 1985). Its analytical data were as follows: chemical composition, 96.8% seneciphylline and 3.2% integerrimine; radiochemical purity, >97%; radioactivity in the retronecine moiety, >99%; specific radioactivity, 22 mCi/mmol, 66 μCi/mg.

Preparation of [^{3}H]Seneciphylline for Treatment. Inactive PAs (605 mg) extracted from *Adenostyles alliariae* (89% seneciphylline, 7% senecionine, and 4% of an unidentified PA as determined by high-performance liquid chromatography; data not shown) were added to a solution of 117 μCi (2.6 mg) of [^{3}H]-seneciphylline in 630 μL of 0.1 M potassium phosphate buffer, pH 5.5. After 3 mL of distilled water and 1 mL of concentrated ammonium hydroxide were added, the solution was extracted five times with 3 mL of chloroform. The chloroform extracts were pooled, dried over magnesium sulfate, and evaporated to dryness. The radioactivity of this PA preparation was 4.71×10^5 dpm or 0.21 μCi/mg; 471 dpm corresponded to 1 μg of PAs, mainly seneciphylline. The radiochemical purity was greater than 95% as determined by thin-layer chromatography on precoated silica gel plates 60 F254 (Merck, Darmstadt, FRG) with chloroform/methanol/ammonia (84:15:1).

Treatment and Collection of Blood and Milk Samples. [^{3}H]Seneciphylline (547 mg; 2.57×10^8 dpm, 117 μCi) was filled into a gelatin capsule for a single peroral administration. The dose the cow received was approximately 1 mg of PAs (89% seneciphylline)/kg of body weight. Blood samples were drawn 1, 3, 18, 30, and 54 h later. The cow was milked 2, 16, 27, 40, 51, and 64 h after the seneciphylline was administered.

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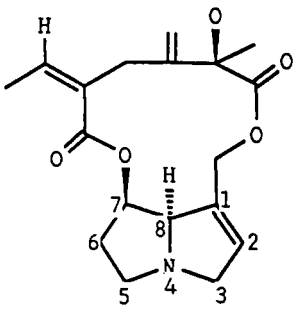

Figure 1. Chemical structure of seneciphylline. The compound used in this study was labeled with tritium at positions 2, 6, and 7 of the retronecine moiety.

Measurement of Radioactivity. All ^3H radioactivities were measured in triplicate in 10 mL of Insta-Gel with a Packard Tricarb 460 CD liquid scintillation counter (Packard Instruments, Downers Grove, IL). Prior to counting, milk samples (10–22 mL) were defatted with ether (no radioactivity detectable in ether phase, detection limit 0.5 ng of PAs), lyophilized, and burned on a Packard 306 oxidizer (Packard Instruments). Blood and liver samples (2–5 mL and 10–11 g, respectively) were lyophilized and then burned. In addition, the radioactivity of the water from each lyophilization was determined.

Extraction of Milk Samples. Free PAs were extracted as follows: Two hundred milliliters of absolute ethanol and 100 mL of 0.5 M sulfuric acid were added to 500 mL of milk. The mixture was heated until it began to boil and kept at this temperature for 20 min. Subsequently, proteins were allowed to precipitate at room temperature for 24 h. After the precipitate was filtered off with Celite 545 (Merck), the water phase was extracted once with 400 mL of petroleum ether (boiling range 60–80 °C) and twice with 400 mL of dichlormethane. The organic phases were discarded. The water phase was basified with concentrated ammonia to pH 10 and extracted twice with 250 mL of dichlormethane and twice with 250 mL of acetic ester. The organic phases were pooled, dried over sodium sulfate, and evaporated to dryness. Residues were dissolved in 200 μL of chloroform/ethanol (1:1). To isolate the *N*-oxides of PAs, the water phase was acidified with concentrated sulfuric acid to pH 1. After zinc powder was added, the mixture was stirred at room temperature for 3 h. Subsequently, concentrated ammonia was added to pH 10, and the reduced PA *N*-oxides were extracted and treated as the originally free alkaloids. The extracts were separated by thin-layer chromatography on precoated silica gel plates 60 (Merck) with chloroform/methanol/ammonia (84:15:1) as mobile phase. PAs were detected by the colorimetric method developed by Mattocks (1967). In addition, radioactivity of PA-containing zones was determined.

Extraction of the Liver. One hundred grams of liver tissue was homogenized in a blender (Waring Blendor, Dynamics Corp. of America, New Hartford, CT) with 200 mL of ethanol and 200 mL of 1 M sulfuric acid. The homogenate was heated until it began to boil and kept boiling for 20 min. After cooling to room temperature for 4 h, the mixture was filtered through Celite 545 (Merck). Subsequently, 200 mL of distilled water was added, and the clear solution was extracted twice with 250 mL of dichlormethane. The organic phases were discarded. The water phase was basified with concentrated ammonia to pH 10 and extracted three times with 200 mL of dichlormethane. The organic phases were pooled, dried over sodium sulfate, and evaporated to dryness. The residue was dissolved in 10 mL of Insta-Gel. The radioactivity was determined as described above.

RESULTS

One dairy cow was treated with seneciphylline labeled in the retronecine moiety with tritium (Figure 1). The single, orally applied dose was 547 mg of [^3H]seneciphylline (117 μCi) which corresponded to 1 mg of PA/kg of body weight. Radioactivity in the blood and the milk, which were lyophilized for measurements (no radioactivity was detectable in the water removed, detection limit expressed as PAs = 0.5 ng/mL), was monitored during the following 64 h. As shown in Table I and Figure 2, blood radioactivity levels expressed as nanograms of seneciphylline per milliliter reached values around 100 ng/mL within 1 h after treatment, remained in this range for about 20 h, and then decreased rapidly. After 54 h, 11 ng/mL were still present in the blood. Milk levels were 11 ng/mL 2 h after treatment and reached a maximum of 102 ng/mL 14 h later. Sixty-four hours after the oral administration, radioactivity corresponding to 5 ng/mL seneciphylline was detectable in milk. During the observation period, 22.9 L of milk were collected. The total amount of radioactivity found in the milk expressed as seneciphylline was 900 μg or 0.16% of the dose given. Sixty-five percent of this amount was found in 5.8 L of milk collected 16 h after treatment.

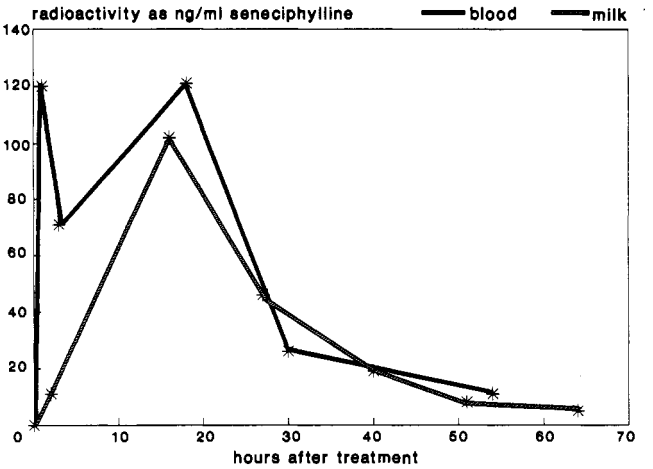

Figure 2. Blood and milk levels of radioactivity calculated as seneciphylline. At hour 0, the cow received orally 1 mg of [^3H]-seneciphylline/kg of body weight (547 mg, 117 μCi).

Table I. ^3H Radioactivity in Cow's Blood and Milk following a Single Oral Dose of [^3H]Seneciphylline (1 mg/kg of Body Weight)

time after treatment, h	blood levels: dpm[a]/mL	blood levels: calcd as seneciphylline, ng/mL	milk levels: dpm[a]/mL	milk levels: calcd as seneciphylline, ng/mL	cum ^3H act. in milk as % of dose
1	56	120			
2			5	11	0.005
3	33	71			
16			48	102	0.112
18	57	121			
27			22	46	0.143
30	12	26			
40			9	19	0.155
51			4	8	0.160
54	5	11			
64			2	5	0.164

[a] Decays per minute.

The cow was slaughtered 21 days after the seneciphylline was given. At this time, the liver was collected. The total weight was 8400 g. The radioactivity measured in the lyophilized liver corresponded to a seneciphylline concentration of 40 ng/g in the fresh liver. This was 0.06% of the seneciphylline dose or 340 μg of PA. No radioactivity was detectable in the water removed (detection limit expressed as PAs = 0.5 ng/mL).

The milk samples collected at 16 and 27 h after treatment had the highest radioactivity levels. These samples were extracted with organic solvents and analyzed as described under Experimental Procedures. At 16 h, 11.3% of the radioactivity was extractable as free alkaloids and 2.9%

as *N*-oxides. At 27 h, these values were 15.1% and 11.2%, respectively. Analysis of the extracts by thin-layer chromatography indicated the presence of unchanged seneciphylline and retronecine. Seneciphylline was detected by the colorimetric reaction according to the procedure of Mattocks (1967), and radioactivity was measured in the zone of the chromatogram that corresponds to seneciphylline. Detection of retronecine was by radioactivity measurement only. From the liver, 13.0% of the radioactivity present could be extracted with organic solvents. There was not enough activity present to determine the portion of *N*-oxides and to analyze the extract by thin-layer chromatography.

DISCUSSION

The determination of the transfer of PAs into the milk of dairy cows should allow the estimation of the possible human PA exposure through dairy products, which are part of the daily diet in many countries. In milk from goats fed *S. jacobaea* up to 3.8 μg/mL PAs was found (Dickinson and King, 1978). Because of the carcinogenicity of PAs, such amounts pose a danger to humans drinking regularly contaminated goat's milk. In contrast, PA levels in cow's milk are lower. This is probably caused by the higher sensitivity of cattle to acute toxic effects of PAs which does not allow ingestion of large amounts of these alkaloids. PA concentrations ranging from 94 to 167 ng/mL were found in the milk of cows fed dried *S. jacobaea* at a highly toxic dose of 16 mg of PAs/kg of body weight per day for 20 days (Dickinson et al., 1976). In our study, radiolabeled seneciphylline was given as a single dose of 1 mg/kg of body weight to a dairy cow. The maximum alkaloid concentration found was 102 ng/mL in the milk collected 14 h after treatment. Fifty hours later, radioactivity corresponding to 5 ng/mL seneciphylline was still detectable. These data indicate that the biological half-life of seneciphylline is in the range of a few hours. Repeated ingestion of PAs at 1 mg/kg per day, which is tolerated by cattle for weeks, might therefore cause a slight accumulation (less than a factor of 2) of alkaloids in the milk.

There is limited information available that concerns the presence of possible PA metabolites in milk. It is likely that the majority of PAs in milk are metabolized to highly polar compounds and/or are covalently bound to macromolecules and therefore present in biologically inactive form since less than 20% of the total radioactivity was extractable with organic solvents in this study. However, the determination of the mutagenicity in *Drosophila* of milk from rats given seneciphylline gave a mutagenicity pattern consistent with that of unchanged seneciphylline (Candrian et al., 1984b). In another study with lactating rats, it could be shown that radioactivity applied as a mixture of labeled seneciphylline and senecionine was excreted in the milk mainly as unidentified water-soluble retronecine-derived metabolites and only about one-fourth as unchanged PAs (Lüthy et al., 1983). In our experiment we found that PA *N*-oxides are an important fraction of metabolites in cow's milk. In addition to unchanged seneciphylline and retronecine, 2.9% *N*-oxides were detected 16 h after the application of seneciphylline. This value increased to 11.2% at 27 h. There was not enough material, in respect to total radioactivity as well as chemical amount, for further determination of metabolites. The percentage of radioactivity extractable with organic solvents from the liver (13.0%) was similar to the percentage found with milk.

The minimum dose level for macrocyclic unsaturated PAs causing liver damage and even death in cattle is between 1 and 2 mg of PAs/kg of body weight per day (Johnson, 1976). The analysis of greenfodder, hay, and silage and the occurrence of livestock losses due to pyrrolizidine alkaloidosis have shown that intake of PAs in this dose range can occur naturally (Pohlenz et al., 1980; Candrian et al., 1984a). Considering our data and the experiments of Dickinson (1980), the expected possible concentration of PAs (like seneciphylline) in the milk of not obviously intoxicated cows can hardly exceed 10–20 μg of biologically active PAs/L. The analytical detection limit of PAs with presently available methods is in about the same range. An earlier performed risk assessment for humans for these carcinogens recommends that the daily intake be less than 1 μg (Lüthy, 1987). Therefore, milk containing up to 20 μg of PAs/L cannot be considered as safe for regular consumption.

An issue not raised so far is the possible presence of unchanged PAs or toxic pyrrolic PA metabolites in the meat of cattle. Radioactivity corresponding to 40 ng of seneciphylline/g of liver tissue was still detectable 3 weeks after the application of 1 mg/kg of body weight of radiolabeled seneciphylline. As only a small fraction of this radioactivity was extractable with organic solvents, unchanged seneciphylline contributes only to a minor extent to the total radioactivity found in the liver. It is generally accepted that the electrophilic pyrrole metabolites of PAs are capable of alkylating nucleophiles and may become covalently linked to tissue constituents (Mattocks, 1986d). However, a water-soluble pyrrolic glutathione conjugate metabolite of the PA monocrotaline was recently detected in the bile of rats (Lamé et al., 1990). At this time, it cannot be excluded that electrophilic compounds can be generated in vivo from these pyrrole conjugates (Lamé et al., 1990). It has been demonstrated that reactive pyrrolic metabolites from PAs can alkylate soluble and tissue-bound thiol groups in rats (Mattocks and Jukes, 1990). Persistent thiol-trapped pyrrolic metabolites were detected by these authors in liver, kidney, and lung tissues and in the urine and blood of rats treated with the PAs retrorsine or monocrotaline. Preliminary experiments with glutathionyldehydroretronecine (a glutathione–pyrrole conjugate) showed that this compound is capable of mimicking some of the cardiopulmonary actions of the PA monocrotaline (Mattocks and Jukes, 1990). The toxicological significance of the high liver ^3H activity derived from [^3H]-seneciphylline that we found in our experiment remains therefore to be determined.

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