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Rapid Separation and Identification of Urinary Metabolites of Zeranol by HPLC–UV Spectrophotometry

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Bovine urinary metabolites of zeranol (1), a growth stimulant, have been separated and identified by HPLC–UV spectrophotometry. Both free and conjugated zeranol (1) and taleranol (3), a C-7 epimer of zeranol, were readily separated and identified in the urine samples collected from a bull that received zeranol intramuscularly or intravenously. Only a trace amount of zearalanone (2), an oxidation product of zeranol (1) and reportedly a major metabolite in many animal species, has been found. On the other hand, none was found in the urine samples collected from a bull that received zeranol intraperitoneally. Ethyl acetate extracts of urine samples, following with or without β-glucuronidase treatment and silica gel column or TLC cleanup, were analyzed by GC–MS and HPLC. Zeranol (1) and taleranol (3) were indistinguishable by GC–MS when analyzed as trimethylsilylates. These two epimers were readily separated and identified by HPLC–UV spectrophotometry.

INTRODUCTION

Zeranol (1,7α-zearalanol or [35-(3*R**,7*S**)]-3,4,5,6,7,8,9,10,11,12-decahydro-7,14,16-trihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1-one) has been adopted as a growth stimulant and also employed to reduce stress in cattle (Bennet et al., 1974; Cole et al., 1984; Ingerowski et al., 1976). The radioactivity of subcutaneously implanted tritium-labeled zeranol pellets in cattle is excreted in feces (45%) and urine (10%) and remained at the site of implant (10%) (Sharp and Dyer, 1972). The metabolism of zeranol has been studied in vitro as well as in vivo, and zearalanone (2), an oxidation product, was reported to be the major metabolite. Ingerowski and Stan (1979) confirmed the presence of zearalanone (2) in the incubation medium by GC–MS when zeranol was incubated with microsomal preparations of bovine liver, uterus, and muscle. Migdalof et al. (1983) reported zearalanone in the urine and feces of the female rat, dog, rabbit, and Rhesus monkey by reverse isotope dilution analyses following oral administration of radioactive zeranol. The presence of taleranol (3), the C-7 epimer of zeranol, also has been reported in rabbit urine as a minor metabolite.

In order to investigate the biotransformation of zeranol (1), a rapid HPLC–UV spectrophotometric method was developed for the analysis and identification of urinary metabolites.

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MATERIALS AND METHODS

Zeranol (1), taleranol (3), and zearalanone (2) were gifts from the International Minerals and Chemical Corp., Terre Haute, IN. β-Glucuronidase (Type H-2) containing approximately 100 000 units/mL of β-glucuronidase and 1000–5000 units/mL of sulfatase activity and Amberlite XAD-4 were purchased from Sigma Chemical Co., St. Louis, MO, and Regisil, a trimethylsilylating reagent, was purchased from Regis Chemical Co., Morton Grove, IL.

Treatment of Animals. Zeranol (4.0 g) in dimethyl sulfoxide (Me_2SO), total volume 15.0 mL, was administered intraperitoneally to a bull (mixed breed) weighing 100 kg. The urine was collected via a urine bag for 48 h following treatment (urine sample 1).

Zeranol (4.0 g) in Me_2SO, total volume 19.0 mL, was administered intramuscularly (im, injected at four different sites) to a Holstein bull weighing 270 kg, and the urine was collected for 4 days following treatment. The bull was kept in a metabolic crate, and the urine samples collected were slightly contaminated with feces (urine sample 2).

The same Holstein bull was given zeranol (1.0 g) in propylene glycol, total volume 220 mL, intravenously (40 mL/h) 51 days after the previous im treatment. The urine was collected via a urine bag for 3 days following treatment (urine sample 3).

All urine samples were collected at room temperature and kept frozen in polyethylene bottles until further study.

Extraction of Urinary Metabolites. The first 24-h collection of urine samples 1 and 2 was filtered with the

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aid of Celite. The filtered urine (300 mL) was extracted with ethyl acetate (EtOAc), 2 × 300 mL, and the combined EtOAc extract was concentrated to a gummy residue in vacuo using a rotary evaporator (Brinkman) at or below 37 °C.

The remaining aqueous portion following EtOAc extraction was concentrated in vacuo to remove the residual EtOAc and then passed through a column (2.5 cm i.d., 3 mL/min) packed with Amberite XAD-4 (52 g) in water. The combined eluate was passed through the same column once again, and the eluate from the second elution was discarded. The adsorbed organic compounds including metabolites on the resin were desorbed with methanol until the eluate became colorless. The combined methanol washings (150 mL) were concentrated in vacuo, leaving a concentrated aqueous solution (24 mL).

The concentrated aqueous solution (24 mL) containing metabolites was diluted with 2 M NaOAc (2.4 mL), adjusted to pH 5.0 with HOAc, and incubated with β-glucuronidase (0.6 mL) for 16 h at 37 °C. The cooled, hydrolyzed solution was extracted with EtOAc, 3 × 200 mL, and the combined extracts were washed with saturated brine and concentrated in vacuo, leaving a reddish brown residue with a strong odor of HOAc. The residual HOAc was removed as a benzene–HOAc azeotrope; benzene was repeatedly added to the residue and evaporated in vacuo until no odor of HOAc could be detected.

Urine sample 3 (100 mL of the first 24-h collection) was diluted with 2 M NaOAc (10 mL), adjusted to pH 5.0 with HOAc, and incubated with β-glucuronidase (0.5 mL) for 19 h at 37 °C. The hydrolyzed urine was cooled to room temperature and extracted with EtOAc (3 × 80 mL), and the combined EtOAc extract was washed with saturated brine and then concentrated in vacuo. Another aliquot of the same urine (sample 3, 100 mL) was extracted with EtOAc, 2 × 100 mL, and the combined extracts were concentrated in vacuo.

Separation of Urinary Metabolites. All the EtOAc extracts (with or without β-glucuronidase hydrolysis) of the three urine samples collected were separated by either preparative layer (0.5-mm-thick) silica gel plates (20 × 20 cm glass plates coated with silica gel 60 HF254, E. Merck) or silica gel column chromatography. A glass column equipped with a Teflon stopcock and a fritted disk was packed with silica gel 60 (70–230 mesh, E. Merck) in benzene (2.5 × 15 cm). The extracts to be analyzed (EtOAc extracts of urine sample 1 or 2) in a minimum volume (less than 5 mL) of EtOAc were charged to the column and eluted (2 mL/min) with benzene followed by benzene–EtOAc (1:1, v/v), EtOAc, and a methanol wash (approximately 150 mL of each). The elution of metabolites was monitored with analytical thin-layer (0.2-mm-thick) chromatography (TLC) utilizing plastic plates coated with silica gel 60 (E. Merck), 2.5 × 7 cm, by developing with benzene–acetone (9:1, v/v), and visualizing under short-wave UV light. One or more reference standards (zeranol, taleranol, zearalanone) were spotted on the same plate, and the R_f values were compared with that of the samples. Fractions containing a fluorescent spot corresponding to zeranol or taleranol on TLC (eluates of benzene–EtOAc and EtOAc) were combined, and other polar and nonpolar fractions were grouped according to their TLC behavior for further analysis by GC–MS and/or HPLC.

The EtOAc extracts of urine sample 3, both enzyme hydrolyzed and nonhydrolyzed, were separated by preparative layer silica gel plates developed once with benzene–acetone (10:1) and repeated once again with benzene–acetone (4:1). A band corresponding to taleranol centered at R_f 0.32 (blue fluorescent under short-wave UV light) was scraped off, eluted with methanol, concentrated, and then analyzed by HPLC and GC–MS (following Me_3Si derivatization). All other zonal scrapings were also eluted and analyzed by GC–MS following derivatization with Regisil and by HPLC (bands located below the one centered at R_f 0.32).

Direct Analysis of Urine. To three centrifuge tubes with glass stoppers was placed 10-mL each of filtered urine sample 3. Samples in tubes 1 and 2 were diluted with 2 M NaOAc (1.0 mL) and adjusted to pH 5.0 with HOAc. β-Glucuronidase (0.1 mL) and water (0.1 mL) were added to tubes 1 and 2, respectively, the urine in tube 3 was diluted with water (1.1 mL), and all three samples were incubated for 16 h at 37 °C. The incubated samples were allowed to cool to room temperature, and a small portion of clear supernatant (0.5 mL) of each sample was diluted with an equal volume of methanol and analyzed by HPLC. The remaining portion of each sample was extracted with EtOAc (3 × 20 mL) for further study.

Preparation of Trimethylsilyl (Me_3Si) Derivatives. A large excess of Regisil (0.1 mL) was added to each analytical standard (zeranol, taleranol, zearalanone) or each fractioin (about 0.5 mg each in acetonitrile, 0.3 mL) obtained from various urinary extracts by either preparative layer chromatography or silica gel column chromatography and then allowed to react overnight at room temperature before GC–MS analysis.

HPLC Analyses. Each analytical standard (zeranol, taleranol, zearalanone) or each fractionated urinary extract in methanol was analyzed isocratically. One of the HPLC instruments consisted of a Waters Model 6000 pump, a U6K injector, a UV detector (254 nm), and a reversed-phase column (C_{18}, 5-μm, Regis, 4.6 mm × 25 cm). The mobile phase composition was methanol–water (60:40). HPLC–UV spectrophotometry was carried out with a Hewlett-Packard Model 1090 with a diode array detector, a C_{18} column (Supelco, 5-μm, 4.6 mm × 25 cm), and the same solvent system. The UV spectrum of each component was recorded as it eluted and compared with that of the reference standards as they eluted. Samples were analyzed at room temperature with the former instrument, at a flow rate of 2 mL/min, and the column temperature was 40 °C with the latter instrument at 1.5 mL/min.

Gas Chromatography–Mass Spectrometry (GC–MS). The GC–MS was performed with a Hewlett-Packard Model 5992 B instrument with data system using a 180 cm × 2 mm glass column packed with 3% OV-1 on gas chrome Q (100–120 mesh). The injector temperature was 250 °C; the column temperature initially was maintained at 150 °C for 1 min and then was increased at 12°/min to 270 °C. The flow rate of carrier gas (helium) was 20 mL/min, and the ion beam was monitored at m/e 538, for taleranol–Me_3Si or zeranol–Me_3Si and at m/e 464 for zearalanone–Me_3Si.

RESULTS AND DISCUSSION

The presence of zeranol (1) and taleranol (3) was readily confirmed in the urine samples (urine samples 2 and 3) collected from the zeranol-treated bull, by both intramuscular and intravenous administration. However, neither of these were found in the urine sample (urine sample 1) collected following intraperitoneal (ip) administration of zeranol probably because of poor solubility of this compound in water, which likely resulted in precipitating it in the ip cavity. Two metabolites, zeranol (1) and taleranol (3), were found in the EtOAc extracts of enzyme-hydrolyzed as well as nonhydrolyzed urine (samples 2 and 3), to some extent due to the presence of bacterial

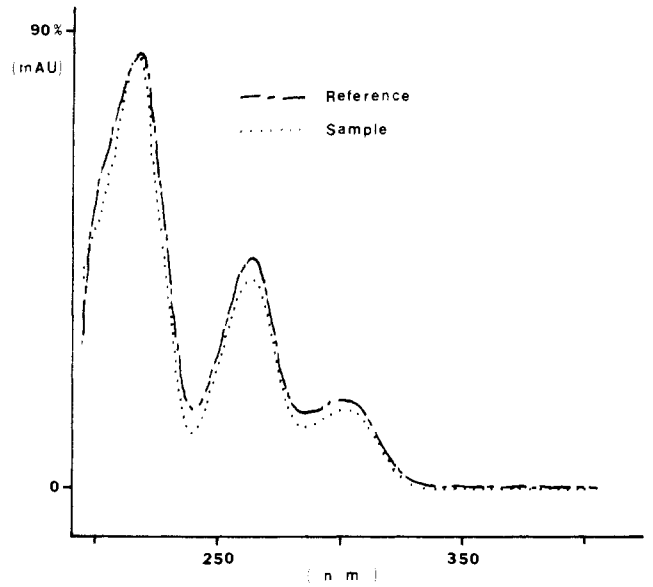

Figure 1. UV spectra of zeranol (1).

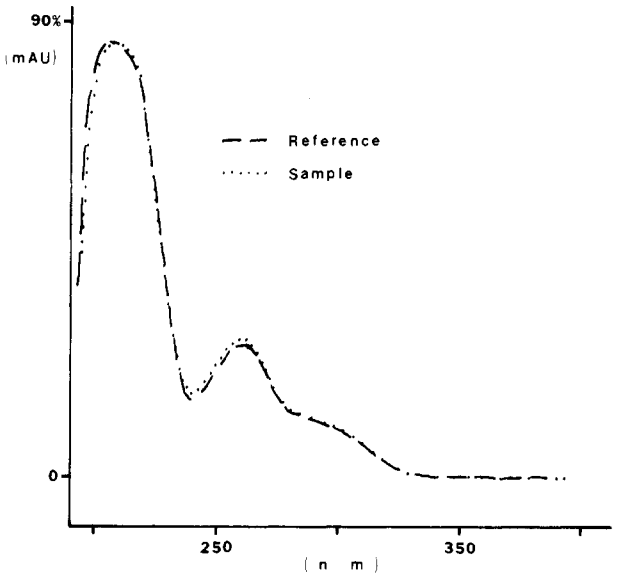

Figure 2. UV spectra of taleranol (3).

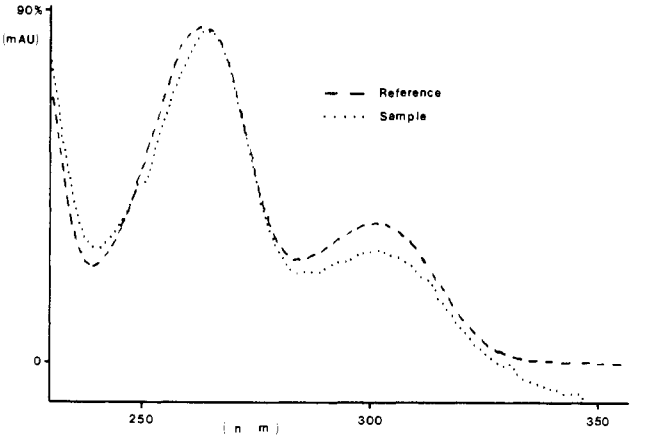

Figure 3. UV spectra of zearalanone (2).

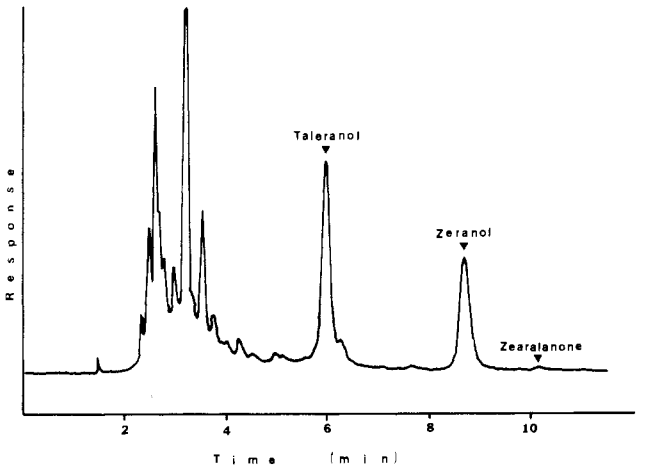

Figure 4. Typical HPLC chromatogram of a urinary extract.

β-glucuronidase introduced through feces contamination. The isolation of free metabolites was readily achievable without enzyme hydrolysis of urine samples 2 or 3; however, the extracts from the nonhydrolyzed urine samples contained more impurities and smaller amounts of metabolites than hydrolyzed samples as determined by HPLC and GC, indicating that glucuronides and/or sulfates do excrete. This was further supported by the direct analysis of urine by HPLC: taleranol and zeranol were readily detected from the enzyme-hydrolyzed urine of urine sample 3 (as little as 0.5 mL was sufficient) whereas the other urine samples incubated without enzyme contained a much lower concentration of free metabolites.

The HPLC retention time of reference compounds ranged from 4.4 min for taleranol to 7.8 min for zearalanone when analyzed by the HPLC system of Waters Associates. The HPLC chromatograms of four urinary extracts showed the two peaks corresponding to taleranol and zeranol. A small peak with the same retention time as zearalanone also was present.

Further confirmation of the presence of the two urinary metabolites, zeranol and/or taleranol, was carried out by gas chromatography–mass spectrometry (GS–MS), and the EtOAc extracts of urine (sample 2 and 3) showed the presence of zeranol–Me_3Si and/or taleranol–Me_3Si (molecular ion at m/e 538). However, it is practically impossible to distinguish between these two derivatives since their GC retention times are very close (17.18 and 17.56 min, respectively), and the mass spectra of the two Me_3Si derivatives are essentially identical.

Zeranol and taleranol were readily distinguished, however, by the comparison of their HPLC retention times and UV spectra using the HP 1090 LC system. UV spectra of zeranol, taleranol, and zearalanone obtained from the urinary extracts with the diode array detector were identical with those of reference standards recorded under the identical chromatographic conditions (Figures 1–3). The retention times of taleranol, zeranol, and zearalanone were 6.0, 8.9, and 10.6 min, respectively, with the HP 1090 LC System (Figure 4).

The presence of zearalanone (2) has been confirmed by HPLC–UV analysis but failed by GC–MS, probably because of very low concentration of this metabolite in urine samples collected. It is interesting that the concentration of zearalanone in the urinary extracts found in this study is much lower than the other two metabolites: the ratio of zeranol (1), zearalanone (2), and taleranol (3) is approximately 54:1:82 estimated from the area percentage of the HPLC chromatogram (Figure 4). The area percentage ratio exhibited by a mixture consisting of equivalent amounts (w/w) of the three compounds is approximately 1.1:1.6:1 under the HPLC conditions employed. It has been reported (Migdalof et al., 1983) that urinary zearalanone comprised 13.0, 4.0, 21.4, and 4.2% of total doses administered in man, monkey, rabbit, and rat, re-

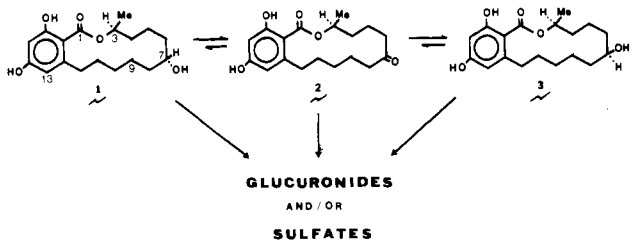

Figure 5. Structures and a possible biotransformation of zeranol (1).

spectively. The ratio of zeranol (1), zearalanone (2), and taleranol (3) has been reported (Migdalof et al., 1983) to be 30.1, 26.8, and 14.8% of the total urinary radioactivity following oral administration of [^{3}H]zeranol to a rabbit. It is conceivable that zeranol (1) is oxidized to zearalanone (2), in vivo, and then reduced to both zeranol (1) and taleranol (3) as proposed previously (Figure 5).

The analysis of urinary extracts by HPLC or TLC alone could be misleading unless the presence of the assumed metabolites is confirmed by spectral methods. A peak corresponding to taleranol in a fraction separated from urine sample 1 (which was collected from a bull treated with zeranol intraperitoneally) was detected by HPLC, but the identity could not be established by GC–MS. In addition, a fluorescent fraction separated from another extract by repeated preparative TLC did not contain either zeranol or taleranol by GC–MS.

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Identification of Arsenobetaine and Arsenocholine in Canadian Fish and Shellfish by High-Performance Liquid Chromatography with Atomic Absorption Detection and Confirmation by Fast Atom Bombardment Mass Spectrometry

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The major organic forms of arsenic in fresh marine fish such as haddock, halibut, cod, herring, mackeral, sole, lobster, scallops, and shrimp obtained throughout Canada were identified as arsenobetaine and, in shrimp only, arsenocholine. Freshwater fish including pike, bass, carp, pickeral, whitefish, yellow perch, and striped perch contained no arsenobetaine or arsenocholine but did contain methanol-extractable arsenic, which has not yet been identified. Salmon obtained from British Columbia contained arsenobetaine and an unknown arsenic compound that eluted later from the reversed-phase HPLC system employed. The arsenobetaine levels for marine fish ranged from 0.15 to 15.8 μg/g of fresh weight. The sample extraction included freeze drying the tissue and then Soxhlet extracting with chloroform (which was discarded) and then with methanol. The methanolic extract containing the organoarsenic compounds was purified by alumina and ion-exchange chromatography then subjected to reversed-phase high-performance liquid chromatography with off-line graphite furnace atomic absorption detection. The compounds were confirmed by mass spectrometry using fast atom bombardment. Arsenobetaine was also confirmed by derivatization to the ethyl ester with further characterization by HPLC and mass spectrometry.

INTRODUCTION

Arsenic is of concern as an environmental pollutant because it is known to give rise to adverse health effects involving respiratory, gastrointestinal, cardiovascular, and nervous systems. Effects of arsenic exposure range from reversible to cancer and acute death (Weinstein, 1978).

The World Health Organization has recently lowered the maximum tolerable daily intake of arsenic from 50 μg/kg of body weight to 2 μg/kg of body weight with the recommendation that more work be done to elucidate the nature of arsenical compounds that occur in food and in particular in seafood, where arsenic levels are generally high (Food Chemical News, 1984). In Canadian fish, levels of total arsenic have been observed in the range of 0.1–90 mg/kg and vary with species and location (Kennedy, 1976; Zook et al., 1976).

The concentrations of arsenic found in fish and shellfish are higher than in the surrounding water. Unpolluted fresh water generally contains up to 1 μg/kg of arsenic, while

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