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# Metabolism of the Synthetic Prostaglandin Alfaprostol in the Cow

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The major metabolites formed from studies of alfaprostol in the cow and in vitro have been investigated. Incubation of 15-[$^{14}$C]alfaprostol (18,19,20-trinor-17-cyclohexyl-13,14-didehydro-PGF$_{2\alpha}$ methyl ester) with 9000*g* bovine liver supernatant yielded mainly one metabolite, alfaprostol acid (I), which is formed by hydrolysis of the methyl ester. This was also the main metabolite found in cow injection site muscle at 24 h after dosing (97–98%) and 5 days after dosing (66%). It was not detected in urine. The major pathway of alfaprostol metabolism in the cow is by way of $\beta$-oxidation following deesterification. The major metabolites identified in cow urine were dinor-5,6-dihydroalfaprostol acid (II) and tetranoralfaprostol acid (III). These two metabolites represented 87–88% of the urine metabolites (62–72% of the dose was excreted in urine by 48 h). Tetranoralfaprostol acid converts to the $\delta$-lactone (IV) under acidic conditions. The remaining excreted radioactivity consisted of several minor metabolites.

## INTRODUCTION

In 1972 the prostaglandin PGF $_{2\alpha}$ was reported to cause regression of the corpus luteum (luteolytic effect) in cattle (Rowson et al.). A number of compounds in this class have been used in recent years to induce estrus (heat), with subsequent ovulation, by shortening the life span of the corpus luteum. Controlling estrus with these compounds provided a highly effective means for timing insemination, and therefore, the use of artificial insemination has become a much more useful reproduction management aid.

Alfaprostol (18,19,20-trinor-17-cyclohexyl-13,14-didehydro-PGF$_{2\alpha}$ methyl ester) (Figure 1), a compound with greater stability and selectivity than PGF$_{2\alpha}$, has been shown to be a potent luteolytic agent for use in inducing estrus in cows (Maffeo et al., 1983) and mares (Howey et al., 1983). Intramuscular administration of alfaprostol in cows shortened the life span of the corpus luteum and induced estrus within 75–96 h.

The major metabolic pathways that have been identified for natural prostaglandins are (a) oxidation of the 15-hydroxy group, (b) reduction of the $\Delta^{13}$ double bond, (c) $\beta$-oxidation of the carboxylic acid side chain to yield dinor and tetranor derivatives, and (d) $\omega$-oxidation to produce hydroxy compounds and dicarboxylic acids (Bourne, 1979). Tetranor analogues have been found to be in a pH-dependent equilibrium with the corresponding $\delta$-lactone (Brash, 1980; Brash, 1982; Bourne et al., 1980).

This report describes studies conducted to isolate and identify the major radiolabeled metabolites formed from alfaprostol in the cow. The metabolites distributed in selected tissues and urine were identified. In addition, metabolism of alfaprostol by the 9000 *g* bovine liver supernatant fraction was investigated.

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## MATERIALS AND METHODS

**Chemicals and Tissues.** Unlabeled alfaprostol and unlabeled alfaprostol acid were obtained from Vetem SpA, Milano, Italy.

The compound 15-[$^{14}$C]alfaprostol was synthesized at Research Triangle Institute (RTI), Research Triangle Park, NC (Jeffcoat and Cook, 1979). The radiochemical purity was greater than 95%, and the specific activity was 132.0 $\mu$Ci/mg. Its identity was established by TLC, GC, HPLC, and MS and its radiochemical purity by TLC and HPLC.

The 15-[$^{14}$C]alfaprostol acid was prepared by saponification of 15-[$^{14}$C]alfaprostol in 0.1 N aqueous KOH at 90 °C for 30 min. After cooling, the solution was washed three times with 2 volumes of hexane. The pH was adjusted to 3.5 with HCl and the resultant mixture extracted three times with 2 volumes of ethyl ether. The ether solution was stored at 4 °C.

Injection site muscle and urine from young adult holstein cows (400–600 kg of body weight) dosed with approximately 1.5 mg/100 kg of 15-[$^{14}$C]alfaprostol were obtained from the gross distribution studies conducted at RTI (Jeffcoat and Cook, 1981). Urine collections were made between 0 and 120 h. The 16–24-h urine was used for metabolite isolation because it contained the highest concentration of radioactivity.

The 16–24-h urine containing unlabeled metabolites of alfaprostol was obtained from a separate experiment in which one holstein cow was dosed with unlabeled alfaprostol in the same manner as the radiolabeled study described above.

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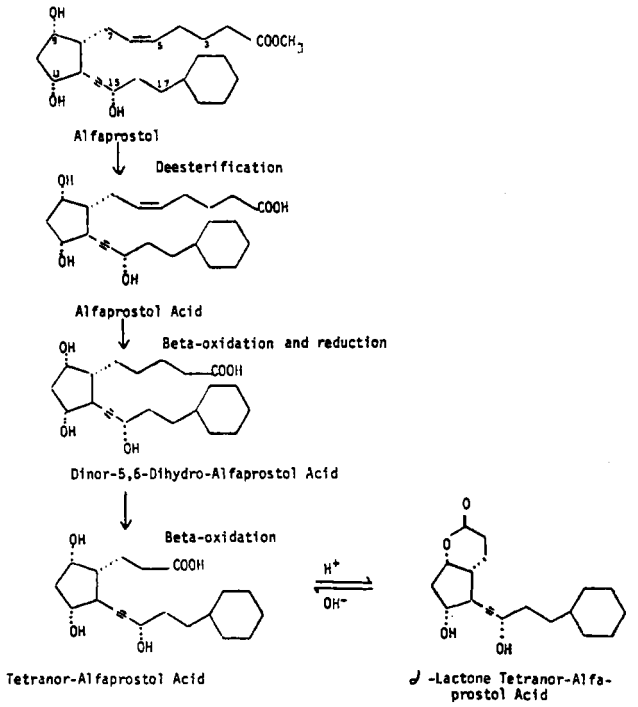


**Figure 1.** Cow metabolites of alfaprostol.

All solvents used were glass distilled (Burdick & Jackson). Water was deionized–distilled (Hydro-Service). Other materials: Amberlite XAD-2 (Rohm and Haas); cofactors for the in vitro metabolism studies (Sigma); pentafluorobenzyl bromide (Pierce); Rigisil (Regis); HEPES (Sigma); 18-crown-6-ether (Regis); Sep-Pak $C_{18}$ cartridges (Waters); phosphomolybdic acid (EM Laboratories); buffer, pH 8.0 (Fisher); diazomethane (prepared from diazoid, Aldrich); polytron homogenizer (Brinkmann Instruments); 2V filter paper (Whatman); syringe microfilters (Rainin).

**Analysis.** Radioactive measurements were made in 10 mL of BBOT (Packard) scintillation cocktail with either a Model 460C or Model 4640 (Packard) liquid scintillation counter (LSC). Quench correction was automatically performed by the Spectral Index of the external standard method.

A Packard 7500 radioactive monitor (RAM) equipped with a flow-through cell containing glass-encased solid scintillant was used for detection of radioactivity in HPLC effluents. A Packard Model 7201 radiochromatogram scanner was used to detect radioactive spots on 5 × 20 cm TLC plates. A Birchover radiochromatogram spark chamber, Model 986-010, was used to detect radioactive zones on 20 × 20 cm TLC plates.

Proton NMR spectra were recorded in $CDCl_3$, at 90 MHz, on Varian XL-200 and XL-400 NMR spectrometers using tetramethylsilane as internal reference.

IR spectra were obtained on KBr pellets or $CHCl_3$ solutions with a Digilab FTS-15E spectrophotometer.

The field-desorption mass spectrum (FD MS) was run at the Midwest Analytical Consultants Laboratory, Champaign, IL.

Negative chemical ionization mass spectra (NCI) were obtained on a Finnegan Model 1015 mass spectrometer coupled to a Finnegan GC, Model 9500.

The gas chromatographic system consisted of a 30-m J & W DB-1 fused-silica capillary column, hydrogen carrier gas pressure regulated at 25 PSI, and a dropping-needle solid injector. The interface between the chromatograph and the mass spectrometer was a capillary glass transfer line with methane makeup gas added coaxially at the GC column end. The initial GC column temperature was 200 °C. The column temperature was manually changed during the run to 250 °C at 4 min and 300 °C at 8 min after injection.

The Finnegan 6000 data system was used to collect full scan data ($m/z$ 400–700, 3-s scans) under negative chemical ionization conditions. The tuning and calibration compound was perfluorotributylamine (PFTBA). The resolution of the mass spectrometer was adjusted to maintain unit resolution over the mass range of interest. This was done to ensure that the expected doublets separated by 2 amu would be clearly resolved.

For alfaprostol–pentafluorobenzyl ester–trimethylsilyl ether (Tri-$Me_3Si$–alfaprostol-COO–PFBE), the major ion between $m/z$ 200–700 in the NCI mass spectrum is at $m/z$ 607, corresponding to the loss of the pentafluorobenzyl radical upon electron capture. Since one of the possible degradation and/or metabolic pathways for alfaprostol would be oxidation, the free acid of 15-oxoalfaprostol was used as a test compound. Under the NCI GC/MS conditions, Di-$Me_3Si$–15-oxoalfaprostol-COO–PFBE forms an abundant fragment ion corresponding to the loss of the pentafluorobenzyl radical at $m/z$ 533. The results with this ketone-containing compound demonstrated that it is possible to chromatograph metabolites that contain a carbonyl group without further derivatization. The metabolite samples were expected to contain approximately equal amounts of $^{12}C$ and $^{14}C$ from the 15-position of alfaprostol. a set of possible metabolite $[M–PFB]^-$ ions was calculated from the structures of possible metabolites based on previous data on the in vivo metabolism of $PGF_{2\alpha}$ (Brash, 1980).

HPLC was performed on analytical (4.6 mm i.d. × 25 cm) and semiprep (9.4 mm i.d. × 25 cm) Zorbax ODS columns (Du Pont). A Waters Intelink system was used consisting of a Model 720 System Controller, a 730 Data Module, and a WISP 710B Auto Injector.

The following HPLC solvent systems were used: (1) A, acetonitrile–water (10:90); B, acetonitrile; A:B (60:40). (2) A, water–acetonitrile–tetrahydrofuran (585:268:147); B, water–acetonitrile–tetrahydrofuran (360:496:144); A (30 min) followed by B (30 min). (3) acetonitrile–water (70:30). (4) A, water–acetonitrile–tetrahydrofuran (900:50:50); B, water–acetonitrile–tetrahydrofuran (585:268:147); A (15 min) followed by B (45 min).

**Derivatizations.** Pentafluorobenzyl ester (PFBE) derivatives were prepared by reacting 1 μg of the sample with 100–150 μL of a solution consisting of 6.5 mg of 18-crown-6 ether, 21 μL of pentafluorobenzyl bromide, and 6 mL of acetonitrile at 45 °C for 15 min or 1 h at room temperature. potassium acetate (four to five crystals) was added at the beginning of the reaction.

Trimethylsilyl ethers ($Me_3Si$) of the PFBE derivatives were formed by dissolving the dried residues in 100 μL of Regisil (BSTFA + 1% TMCS) and heating at 60 °C for 30 min. Samples were evaporated to dryness with a stream of nitrogen and redissolved in an appropriate amount of heptane.

**In Vitro Metabolism Studies.** The methods described by Schwartz and Postma (1966) were adhered to except that pH 7.4 HEPES buffer was substituted for the phosphate buffer in the incubation medium because of its superiority in this system (Good et al., 1966). The 9000 $g$ bovine liver supernatant was prepared from liver removed from the animal at the slaughterhouse and immediately frozen. The following day, a 50-g portion of the liver was thawed, minced, and placed in a Petri dish over ice.

Ten-gram portions of the minced tissue were homogenized with 20 mL of ice-cold 1.15% KCl in a Tembrock glass homogenizing apparatus dipped in crushed ice. The homogenates were centrifuged at 9000 *g* for 20 min at 2 °C. Fifteen-milliliter portions of the supernatant were stored in 60-mL polyethylene bottles at −70 °C.

Alfaprostol was incubated aerobically at 37 °C in a shaking water bath (Model RW-650, New Brunswick Scientific) in the presence of the 9000 *g* bovine liver supernatant and the cofactors. Several incubations were performed, with incubation times from 0 to 24 h. After cooling to room temperature, the incubation medium was transferred into 250-mL centrifuge bottles and the pH adjusted to 3.5 with 1 N HCl. It was extracted twice with 2 volumes of ethyl ether, followed by two extractions with 2 volumes of ethyl acetate. The extracts and the aqueous solution were assayed for [$^{14}$C] activity by LSC.

The ether and ethyl acetate solutions were concentrated under $N_2$ to 1.0 and 0.5 mL, respectively, spotted on silica gel 60F TLC plates (Merck, 0.25 mm), and developed in benzene–dioxane–acetic acid (20:20:1). Alfaprostol and [$^{14}$C]alfaprostol free acid were spotted alongside for reference. After development, the plates were scanned on a radiochromatogram scanner. They were then sprayed with phosphomolybdic acid solution (3.5%) and heated on a hot plate for a few minutes. The phosphomolybdic acid revealed the prostaglandins as gray spots on a green background.

The major alfaprostol metabolite found in the ether extract was separated on a preparatory silica gel G TLC plate (Analtech, 2 mm), using the same solvent system mentioned above. Detection of the radioactive zones on a 20 × 20 cm plate was accomplished by photographing the plate in a Birchover spark chamber. The radioactive zone was scraped, eluted with methanol, and concentrated for HPLC analysis.

The major metabolite eluted from TLC was further purified by HPLC on a semiprep Zorbax ODS column using HPLC solvent system 1 at a flow rate of 2 mL/min.

**Muscle.** Well-ground and well-mixed samples of injection site muscle from cows killed at 0.5 and 24 h and 5 days after dosing were extracted for HPLC analysis. Initially, four 25-g aliquots from different locations of each frozen tissue sample were each homogenized in 100 mL of pH 7.4 phosphate buffer and extracted twice with 200 mL of petroleum ether. The buffer solution was then adjusted to pH 4.5 with 3 N HCl and extracted twice with 200 mL of ethyl acetate. It has been reported that pH 4.5 yields the most effective recovery of prostaglandins from the tissues examined and the least extraction of pigment (Goswami et al., 1981). The ethyl acetate extracts of the 0.5-h and 5-day cow tissue were chromatographed on a Zorbax ODS column.

The tissues were also assayed for carbon-14 by direct combustion. Triplicate aliquots of 200 mg each from four different locations of the frozen tissue were assayed after allowing the aliquots to dry at room temperature for 3 days.

The 0.5-h muscle sample was extracted with four 100-mL volumes of methanol, each time homogenizing with a Polytron homogenizer for 1 min at high speed, shaking on a reciprocal shaker for 15 min, and centrifuging at 2000 rpm for 20 min. Because of the low [$^{14}$C] activity present in the 24-h and 5-day tissue, two 25-g aliquots of each tissue were extracted and the methanol extracts combined.

Also, because of the polar nature of the metabolites in the 5-day tissue, additional extractions were performed with three 100-mL volumes of 0.1 N methanolic hydrochloric acid. The extracts were rotoevaporated at 25 °C to remove the methanol. The residue was transferred to a 50-mL graduated centrifuge tube with two 15-mL portions of water and two 10-mL portions of ethyl acetate. The ethyl acetate was evaporated under nitrogen at 25 °C in a water bath. The aqueous concentrate was adjusted to pH 2.0 with 2 N HCl except for the already acidic 5-day tissue extract that required adjustment to pH 2.0 with 8 N NaOH.

From this point on, each tissue required a different treatment to minimize background interference for HPLC and maximize [$^{14}$C] recovery. Four-milliliter aliquots of the 0.5- and 24-h acidic concentrates were passed through three Sep-Pak $C_{18}$ cartridges in series. The cartridges were washed with 4 mL of water passed through in series, and then each cartridge was eluted with 4 mL of methanol.

The methanol eluates from each cartridge were combined and evaporated to dryness under nitrogen at 25 °C. The residue from the 0.5-h tissue was dissolved in 200 μL of acetonitrile, 200 μL of tetrahydrofuran, and 1.6 mL of water and the resultant mixture analyzed by HPLC. The residue from the 24-h tissue was partitioned between water and ethyl acetate (1:5). The ethyl acetate was evaporated to dryness, and the residue was dissolved in the same manner as the 0.5-h sample for HPLC.

The 5-day tissue aqueous concentrate contained very heavy background material that made it difficult to obtain a sample clean enough for HPLC using only Sep-Pak cartridges. To be able to look at both the polar and the medium-polarity metabolites in this sample, the aqueous concentrate was extracted two different ways: (1) A 10-mL portion of the aqueous concentrate was extracted with two 10-mL portions of ethyl acetate (93% of carbon-14 recovered). The ethyl acetate was evaporated to dryness at 25 °C under nitrogen and the residue dissolved in 2 mL of acetonitrile. The acetonitrile solution was washed with 2 mL of hexane and concentrated to 1 mL for HPLC analysis. (2) A 15-mL portion of the aqueous concentrate was extracted with three 15-mL portions of 2-propanol–hexane (5:95) mixture (94% of carbon-14 recovered). The extracts were combined and evaporated to dryness under nitrogen at 25 °C. A dark, oily residue remained that was extracted with three 3-mL portions of acetonitrile and evaporated to 1 mL for HPLC analysis.

HPLC solvent system 4 at a flow rate of 1 mL/min was employed for separation of all muscle extracts.

**Urine.** The HPLC profiles of urine collections from 0–48 h at pH 2.0 were similar. Collections up to 48 h accounted for 95–99% of the radioactivity excreted in urine. Metabolites were isolated from the 16–24-h urine collection after dosing, since it contained the highest level of radioactivity. Urine from two separate cows, one treated with [$^{12}$C] alfaprostol and the other with [$^{14}$C]alfaprostol, was mixed in a 1:1 ratio. The total volume was 2 L. It was assumed that these cows would metabolize the compounds in similar fashion, since they were of the same breed and on the same diet. This technique was previously employed (Bourne et al., 1980) for identification of cloprostenol metabolites. The fragments detected in the mass spectrum contained characteristic [$^{12}$C] to [$^{14}$C] isotope doublets, distinguishing them from endogenous material.

Initially, it was noticed there were three dominant peaks and two of these interconverted during purification. This behavior led us to suspect that we were dealing with tetranoralfaprostol acid that was in a pH-dependent equilibrium with its lactone form. Therefore, an extraction scheme was used to isolate the lactone and confirm its presence by conversion back to its acid form for deriva-

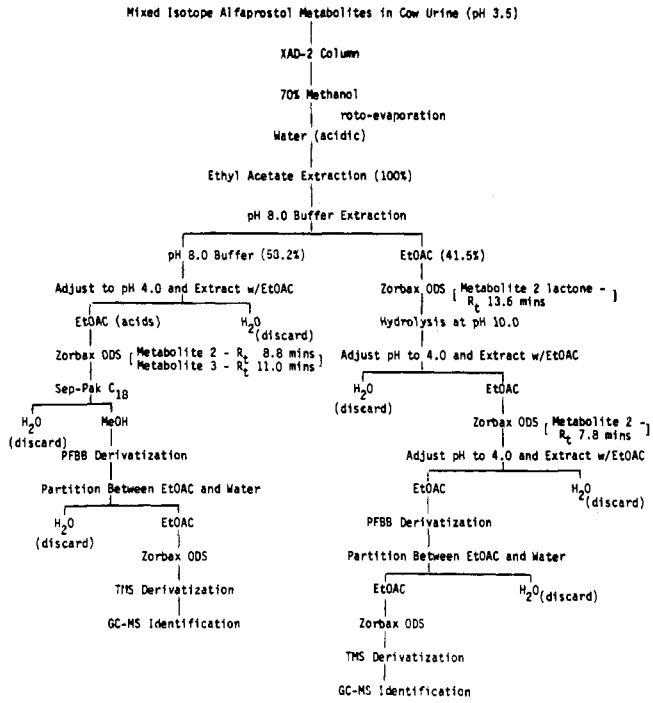


**Figure 2.** Isolation of mixed-isotope alfaprostol metabolites.

tization and GC–MS analysis.

The metabolites were isolated as shown in Figure 2. The mixed-isotope cow urine (pH 9.1) was acidified to pH 3.5 with HCl, filtered through 2V filter paper, and chromatographed on a large column (53 × 750 mm) of Amberlite XAD-2 resin (resin volume 530 $cm^3$).

The column was washed with 3 L of water, followed by 2 L of petroleum ether, and then eluted with 600 mL of 70% methanol. The 70% methanol eluate was rotoevaporated at 25 °C to remove all the methanol. The acidic aqueous solution remaining was extracted twice with 200 mL of ethyl acetate and twice with 100 mL of ethyl acetate. An aliquot (100 mL) of the combined ethyl acetate extracts was extracted three times with 100 mL of pH 8.0 buffer to remove acidic metabolites, and the combined solution was back-washed with 50 mL of ethyl acetate. The combined ethyl acetate solutions were immediately washed with 50 mL of 0.1 N HCl to prevent hydrolysis of lactones during concentration.

For the isolation of the lactone metabolite, the ethyl acetate was evaporated to dryness under nitrogen at room temperature and dissolved in HPLC solvent mixture 2A. This solution was filtered through a disposable syringe microfilter (0.45 μm, nylon) and chromatographed on a Zorbax ODS semiprep column using HPLC solvent system 2 at 4 mL/min.

A major peak eluted (>90%) that had a radioactive monitor retention time of 13.6 min. This peak was concentrated under nitrogen at room temperature until only water remained.

A 20-mL aliquot of 0.1 M borate buffer (pH 10.0) solution was added and allowed to sit overnight at room temperature for selective hydrolysis of the lactone to its corresponding acid. The solution was adjusted to pH 4.0 with 0.1 N HCl and extracted with ethyl acetate to isolate the acid. The ethyl acetate solution was back-washed with water to prevent relactonization upon concentration. The ethyl acetate was evaporated under nitrogen, and the residue was dissolved in HPLC mobile phase and rechromatographed on Zorbax ODS using HPLC solvent system 3 at a flow rate of 0.5 mL/min. The major peak eluted ($R_t$ 7.8 min) was concentrated and derivatized with pentafluorobenzyl bromide (PFBB).

**Table I. Relative Radioactivity in Extracts of Incubation Medium at Various Time Intervals**

| | rel % | | |
|---|---|---|---|
| incubn time, h | ether | EtOAc | water |
| 0 | 95.0 | 2.2 | 2.8 |
| 1 | 93.2 | 2.4 | 4.4 |
| 2 | 90.6 | 3.2 | 6.1 |
| 5 | 80.3 | 9.2 | 10.5 |
| 24 | 75.1 | 11.6 | 13.3 |

The pentafluorobenzyl ester (PFBE) derivative was partitioned between ethyl acetate and water, and the ethyl acetate solution was evaporated to dryness. The PFBE derivative was dissolved in acetonitrile and chromatographed on Zorbax ODS column (4.6 mm × 25 cm) using HPLC solvent system 3 at a flow rate of 0.5 mL/min. The PFBE derivative ($R_t$ 18.8 min) was evaporated to dryness under nitrogen and converted to its trimethylsilyl ether ($Me_3Si$) for analysis by GC–MS.

For the isolation of the acidic metabolites, the pH 8.0 buffer solution (refer to Figure 2) was adjusted to pH 4.0 with 0.1 N HCl and extracted with ethyl acetate. The ethyl acetate was removed in vacuo, and the residue was chromatographed on Zorbax ODS column using the same system used to separate the lactone. The separated acids ($R_t$ 8.8 and 11.0 min) were further purified by evaporating the organic solvents under nitrogen, passing each aqueous solution through a Sep-Pak $C_{18}$ cartridge, and eluting with 5 mL of methanol (Pollante et al., 1982).

Each of the eluted compounds from the Sep-Paks was derivatized with PFBB and partitioned between ethyl acetate and water. The ethyl acetate was evaporated, and the PFBE derivatives were chromatographed on a Zorbax ODS column using HPLC solvent system 3 at 0.5 mL/min. Each of the PFBE derivates eluted ($R_t$ 18.8 and 24.6 min) was converted into trimethylsilyl ethers ($Me_3Si$) for analysis by GC–MS.

## RESULTS

**In Vitro Metabolism.** Alfaprostol was rapidly (at most a few minutes were needed) and almost completely (>95%) metabolized in the presence of the 9000*g* bovine liver supernatant supplemented with an NADPH-generating system. The majority of the products formed at 0–24-h incubation time partitioned into ether (Table I). An apparent increase in the more polar products was observed as incubation time increased.

The 5-h-incubated extracts by TLC and radiochromatogram scanning revealed that the major product in the ether extract was also the major product in the ethyl acetate and the water extracts, indicating the other metabolites were minor in comparison.

The main radiolabeled component in the ether extract was alfaprostol acid (I) which is formed by hydrolysis of the methyl ester in alfaprostol (Figure 1). Its mobility on HPLC (24.75 min) and TLC, field-desorption mass spectra, and infrared and nuclear magnetic spectra data are compatible with alfaprostol acid. The FD spectra show $m/z$ 392 $[M]^+$ and 415 $[M + Na]^+$. The $[M + Na]^+$ ion is often observed in FD spectra.

**Muscle Metabolites.** The total radioactivity as percent of dose and the distribution in each solvent extract are listed in Table II. HPLC analysis of the ethyl acetate fractions showed that alfaprostol acid (I) accounted for 96.1% and 12.3% of the 0.5-h and 5-day muscle radioactivity, respectively.

The extraction scheme produced HPLC profiles that showed the dominant metabolites in the 0.5- and 24-h

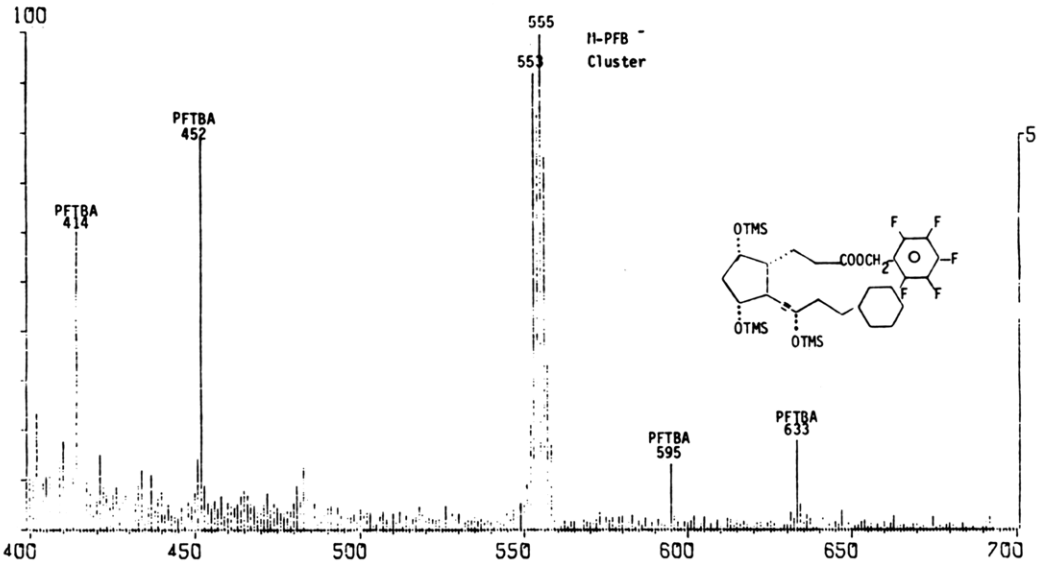


**Figure 3.** Methane NCI mass spectrum of $Me_3Si$–PFBE derivative of tetranoralfaprostol acid.

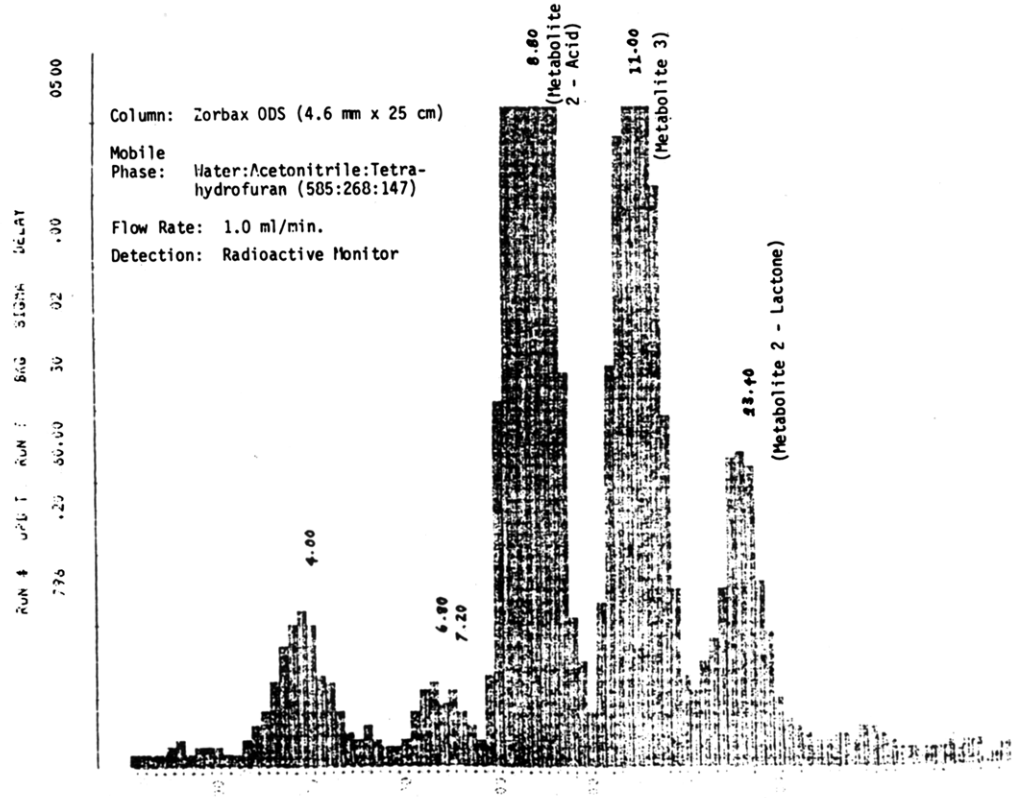


**Figure 4.** HPLC of cow urinary metabolites extracted in pH 8.0 buffer.

tissues were alfaprostol acid (97–98%) and a slightly more polar metabolite (2–3%). The same percentage of this minor compound was detected by HPLC when alfaprostol was either saponified or hydrolyzed by porcine liver esterase. Methyl ester formation of this compound by reaction with diazomethane gave a product that cochromatographed with alfaprostol by HPLC.

After purification by HPLC, the compound was identified as the 5,6 trans isomer of alfaprostol acid by comparison of their NMR spectra. Since the compound showed a singlet for the olefin protons, a trans coupling constant for the double bond was obtained by observing the $^{13}C$ satellites of the olefin protons. The observed 15-Hz coupling indicated a trans double bond. The IR spectra showed little distinction between the two compounds. Thus, this evidence strongly suggests that the trans isomer of alfaprostol acid was derived from the trans isomer of alfaprostol that is originally present in the dosage material.

**Table II. Total Radioactivity in Injection Site Bovine Muscle and Distribution in Solvent Extracts**

| | | | [$^{14}C$] distribn,[a] % | | |
|---|---|---|---|---|---|
| cow | h postdose | % [$^{14}C$] of dose | EtOAc | pet. ether | spent aq homog |
| 2 | 0.5 | 99.1 | 89.6 | 1.7 | 8.7 |
| 3 | 24.0 | 3.2 | 70.4 | 4.4 | 25.2 |
| 4 | 120.0 | 0.52 | 30.2 | 5.0 | 64.8 |

[a] Percent of direct-combustion $^{14}C$ radioactivity.

The highly polar peak ($R_t$ 3.8 min) revealed in the 5-day injection site muscle chromatogram was collected, hydrolyzed with glusulase, and characterized by HPLC. This

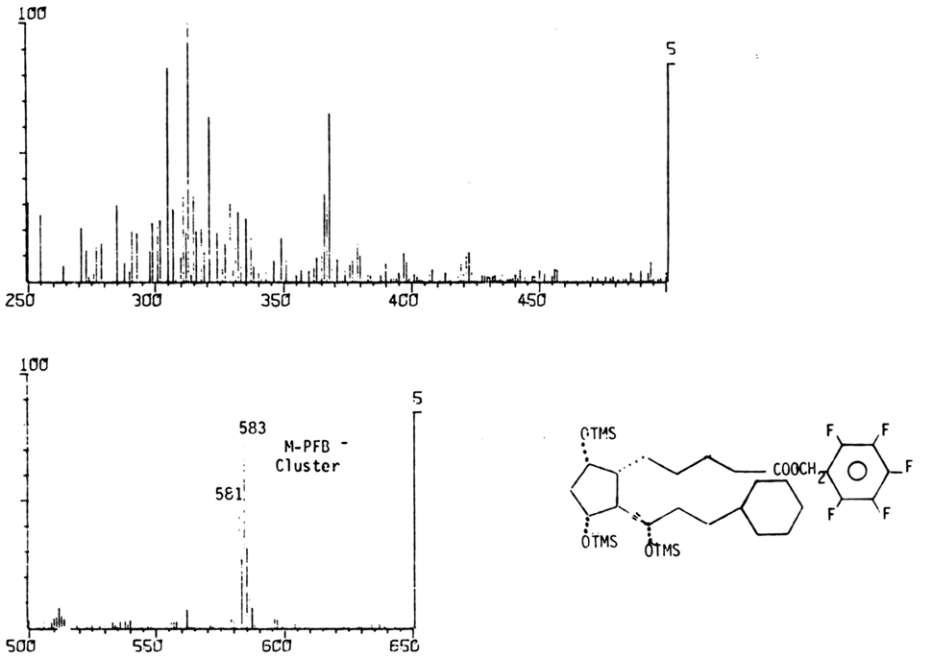


**Figure 5.** Methane NCI mass spectrum of $Me_3Si$–PFBE derivative of dinor-5,6-dihydroalfaprostol acid.

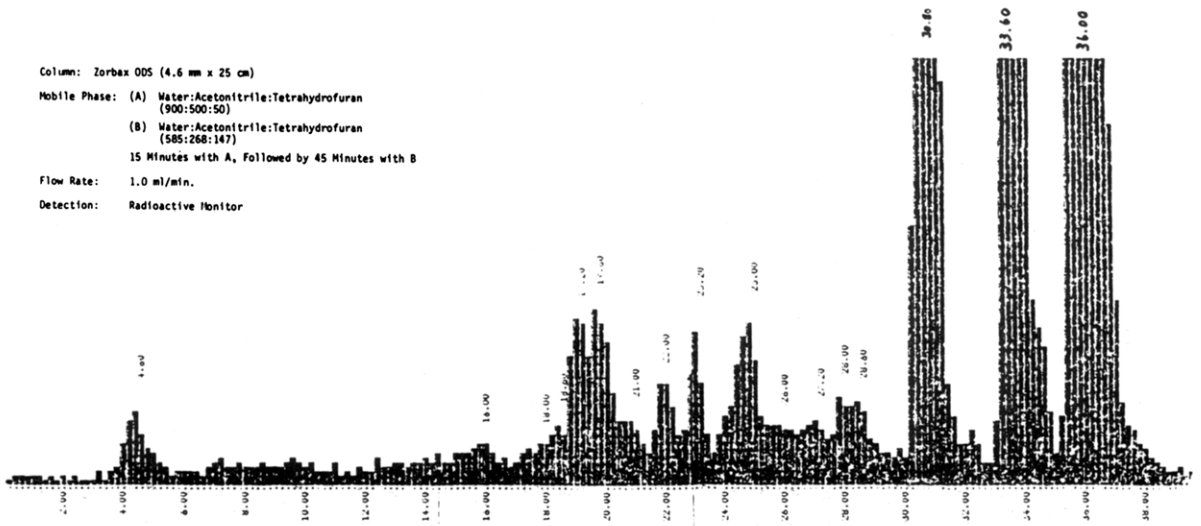


**Figure 6.** HPLC of 16–24-h urine from cow dosed with alfaprostol.

chromatogram revealed the presence of three metabolites coeluting in their conjugated form. This group represented 7% of the total injection site muscle radioactivity 5 days after dosing (0.04% of the dose) and consisted of dinor-5,6-dihydroalfaprostol acid (II), tetranoralfaprostol acid (III), and alfaprostol acid (I). The other peaks in the 5-day chromatogram were alfaprostol acid (I), its trans isomer, and a minor unknown. The unidentified metabolite only represents 0.1% of the carbon-14 dose. It was not found in the urine even after glusulase treatment.

**Urine Metabolites.** Recovery of radioactivity at all stages of the isolation process shown in Figure 2 was essentially quantitative. The ethyl acetate extracted 41.5% of the radioactivity from pH 8.0 solution. This radioactivity consisted mainly of the least polar metabolite ($R_t$ 13.6 min), since the scheme was tailored to extract lactone(s). This substance was collected (metabolite 2—lactone) from the column. Specific hydrolysis of the lactone moiety at pH 10.0 allowed the corresponding acid that was formed (metabolite 2) to be extracted from acidic solution (pH 4.0) with ethyl acetate. The ethyl acetate solution was concentrated and chromatographed on Zorbax ODS using HPLC solvent system 3.

The eluate containing the corresponding acid was collected, concentrated, acidified, and extracted with ethyl acetate for derivatization with PFBB. The electron-capture detection properties of the pentafluorobenzyl ester of $PGF_{2\alpha}$ have been demonstrated (Wickramasinghe et al., 1973). The optimum capillary column gas chromatographic conditions for separation and detection of pentafluorobenzyl derivatives of prostaglandins occurring in biological tissues and confirming their identity by gas chromatography–mass spectrometry have been established (Mai et al., 1982). Derivatization of urinary metabolites of alfaprostol with pentafluorobenzyl bromide was carried out not only to enhance the selectivity of separation but more importantly to increase the specificity and sensitivity of detection by negative chemical ionization mass spectrometry (Min et al., 1980).

The PFBE of metabolite 2 acid ($R_t$ 18.8 min) was purified by HPLC and silylated before NCI GC–MS. The NCI spectrum of this compound had a doublet at $m/z$ 553, 555 (Figure 3). This spectrum is not background subtracted and contains peaks at $m/z$ 414, 452, 595, and 633

that originate from the NCI tuning compound PFTBA. These calibration peaks show that the cluster at $m/z$ 553 is significantly wider than would be expected for a single mass ion under these conditions. The molecular weight of the unlabeled metabolite is thus 553, consistent with the tetranoralfaprostol acid (III).

The pH 8.0 buffer solution, containing the remaining 58.2% of the radioactivity (Figure 2), was acidified to pH 4.0 and extracted with ethyl acetate. The two major peaks separated on Zorbax ODS, $R_t$ 8.8 min (metabolite 2, acid) and 11.0 min (metabolite 3) (Figure 4), were collected separately. They were further purified on Sep-Pak $C_{18}$ cartridges before derivatization with PFBB. The 8.8-min HPLC peak corresponds to the retention time of the hydrolyzed lactone product. Their PFBE derivatives also had identical retentions (18.8 min).

The 11.0-min peak, after PFBB reaction, chromatographed at 24.6 min with solvent system 3. This peak was collected and silylated for NCI GC–MS identification. The NCI mass spectrum of this compound showed a cluster of peaks at $m/z$ 581 and 583 (Figure 5). These ion $m/z$ values are those expected for the tri-$Me_3Si$–dinor-5,6-dihydroalfaprostol-COO–PFBE (II) derivative under these NCI conditions. There were also smaller clusters of peaks in this spectrum at $m/z$ 509, 511 and $m/z$ 365, 367 that may be due to fragment ions.

The three minor polar peaks shown in Figure 4 ($R_t$ 4.0, 6.8, 7.2 min) were resolved by employing the more polar HPLC solvent system 4 at 1 mL/min. A 16–24-h cow urine sample was injected directly onto the HPLC column using this same solvent system after pH adjustment to 2.0 and centrifugation. This chromatogram (Figure 6) revealed that the three polar peaks were eight or nine peaks, totaling 12–13% of the $^{14}C$-labeled metabolites. No single peak represented more than 2% of the $^{14}C$-labeled metabolites.

## DISCUSSION

The major pathway of alfaprostol metabolism is by way of $\beta$-oxidation following deesterification (Figure 1). The major product of alfaprostol in vitro with 9000$g$ bovine liver supernatant was alfaprostol acid (I). It was also the major product found in cow injection site muscle at 0.5 and 24 h after dosing (97-98%), indicating that this is the first, rapid step in the metabolism.

Two major metabolites, dinor-5,6-dihydroalfaprostol acid (II) and tetranoralfaprostol acid (III), were eliminated in urine. At acidic pH, tetranoralfaprostol acid establishes equilibrium with its corresponding $\delta$-lactone (IV). These amounted to 87–88% of the urine metabolites (62–72% of the dose excreted in urine by 45 h). Alfaprostol acid (I) and its isomers were not detected in urine in either the free or conjugated form.

No change in the chromatographic profile of the minor polar metabolites was detected when the composite urine sample (0–24-h collections) was incubated with $\beta$-glucuronidase and sulfatase (glusulase).

No metabolites were found to indicate a metabolic change at the 13,14 triple bond and the C-15 position, or products of $\omega$-oxidation. This suggests that introduction of a cyclohexyl group at C-17 protects the alfaprostol molecule from these metabolic pathways just as the oxyaryl function does for cloprostenol at C-16 (Bourne et al., 1980).

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