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Research Article

Isolation of a Novel Morphinan 3-*O*-Diglucuronide Metabolite from Dog Urine

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Following oral administration of the narcotic antagonist nalmefene [17-(cyclopropylmethyl)-4,5α-epoxy-6-methylenemorphinan-3,14-diol] labeled with ^{14}C to the dog, approximately 50% of the dose was excreted in the urine as a highly polar water-soluble conjugate. Although this major metabolite could be hydrolyzed with β-glucuronidase to yield nalmefene, the intact conjugate was chromatographically more polar on reversed-phase high-performance liquid chromatography (HPLC) than authentic nalmefene 3-*O*-glucuronide. Milligram quantities of the metabolite were subsequently isolated and subjected to fast atom bombardment (FAB) mass spectral and nuclear magnetic resonance (NMR) analyses. The conjugate was identified as nalmefene 3-*O*-β-diglucuronide with a 1,2-β linkage between the two glucuronic acids. It is unlikely that this novel form of conjugate is unique to nalmefene and it is probably a metabolite of other morphinans and/or similar drugs in the dog. Nalmefene 3-*O*-diglucuronide is not a metabolite of nalmefene in man.

KEY WORDS: nalmefene; diglucuronide; nalmefene 3-*O*-diglucuronide; narcotic antagonist.

INTRODUCTION

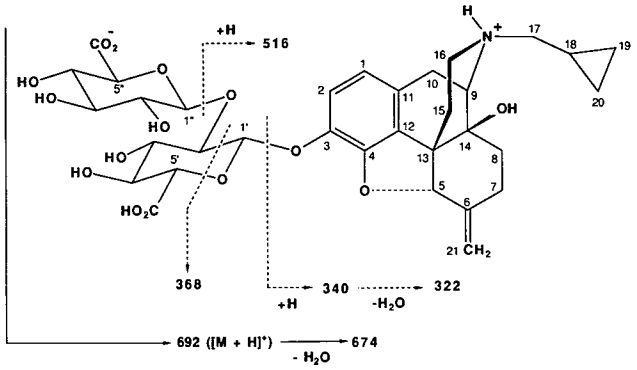
Nalmefene [17-(cyclopropylmethyl)-4,5α-epoxy-6-methylenemorphinan-3,14-diol] (Fig. 1) is a new opioid antagonist currently undergoing clinical evaluation following both parenteral and oral administration (1,2). During a routine investigation of the disposition of the drug in dogs using ^{14}C-nalmefene, a highly polar water-soluble metabolite was isolated from urine by adsorption onto Amberlite XAD-2 resin. The compound, which was the major metabolite of nalmefene excreted in the urine, could be hydrolyzed with β-glucuronidase enzyme to yield nalmefene and was initially presumed to be nalmefene 3-*O*-glucuronide. However, the fast atom bombardment (FAB) mass spectrum of the metabolite was inconsistent with the latter structure and indicated the presence of an additional glucuronic acid function in the molecule. Authentic nalmefene 3-*O*-glucuronide was subsequently synthesized for comparative purposes. On reverse-phase high-performance liquid chromatography (HPLC) the metabolite was considerably more polar than the authentic monoglucuronide. In an effort to identify this metabolite, milligram quantities were isolated for further spectral analysis.

The present report describes in detail the isolation and structural elucidation of a novel drug conjugate which has been identified as nalmefene 3-*O*-diglucuronide in which the two β-glucuronic acid functions are linked at the 1″ and 2′ positions (Fig. 1).

MATERIALS AND METHODS

[6-^{14}C-Methylene]Nalmefene HCl (sp act, 3.12 mCi/mmol). This was prepared by Midwest Research Institute, Kansas City, Mo. Radiochemical purity was estimated at 98% by thin-layer chromatography (TLC) on silica gel using two different solvent systems immediately prior to administration to the animals.

Radioactivity. Radioactivity was determined in a Beck-



Obsd.: m/e 692.2600 ([M + H]$^+$); calcd. for $C_{33}H_{42}NO_{15}$: m/e 692.2554

Fig. 1. Nalmefene 3-*O*-diglucuronide FAB mass spectra data.

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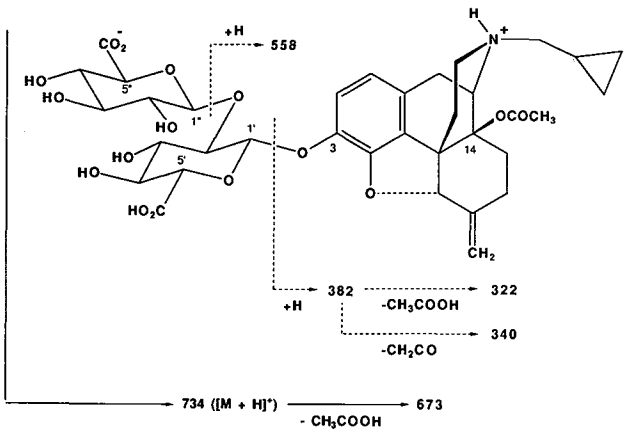

Fig. 2. Nalmefene 3-*O*-diglucuronide 14-acetate FAB mass spectral data.

man LS-1800 liquid scintillation counter using Aquasol (NEN Corp., Boston, Mass.) as the scintillation fluid.

Nalmefene 3-O-β-D-*Glucuronide.* This was synthesised from nalmefene HCl by the method of Berrang *et al.* (3) developed for the synthesis of morphine-3-*O*-β-D-glucuronide. Briefly, the lithium salt of nalmefene in ethanol solution was treated with methyl (tri-*O*-acetyl α-D-glucopyranosyl) bromide-uronate at room temperature, and after completion of the reaction, protective groups were removed by alkaline hydrolysis with a lithium hydroxide solution. Finally, the products were purified by ion-exchange chromatography on Dowex 50W-X8 (H form) with NH_4OH as the eluant and crystallized from water and an ethanol–water solution. The synthetic product exhibited the expected infrared (IR) and nuclear magnetic resonance (NMR) spectra; elemental analyses and physical properties were consistent with expected values.

High-Performance Liquid Chromatography (HPLC). This was performed using a selection of reverse-phase preparative, semipreparative, and analytical columns at various stages of the project. For reasons of clarity and space the actual column, solvent system, and chromatographic conditions employed are detailed in the text when necessary and relevant.

Fast Atom Bombardment (FAB). FAB mass spectra were obtained on a Finnigan MAT 312 double-focusing mass spectrometer operating at an accelerating voltage of 3 kV. Samples were dissolved in dimethylsulfoxide (~5 μg/ml) and deposited on a copper probe tip. A thin layer of glycerol/thioglycerol was applied to the probe tip containing the samples and mixed thoroughly with a Pasteur pipet before insertion into the source. The primary atom was comprised of xenon and was produced using a saddle-field ion source (Ion Tech, Ltd.) operating with a tube current of 2 mA at an energy of 6–8 keV. The temperature of the source was maintained at 25°C. High-resolution FAB mass spectra were carried out by peak matching at a resolution of 5000.

Selective acetylation of the C14 tertiary alcohol function

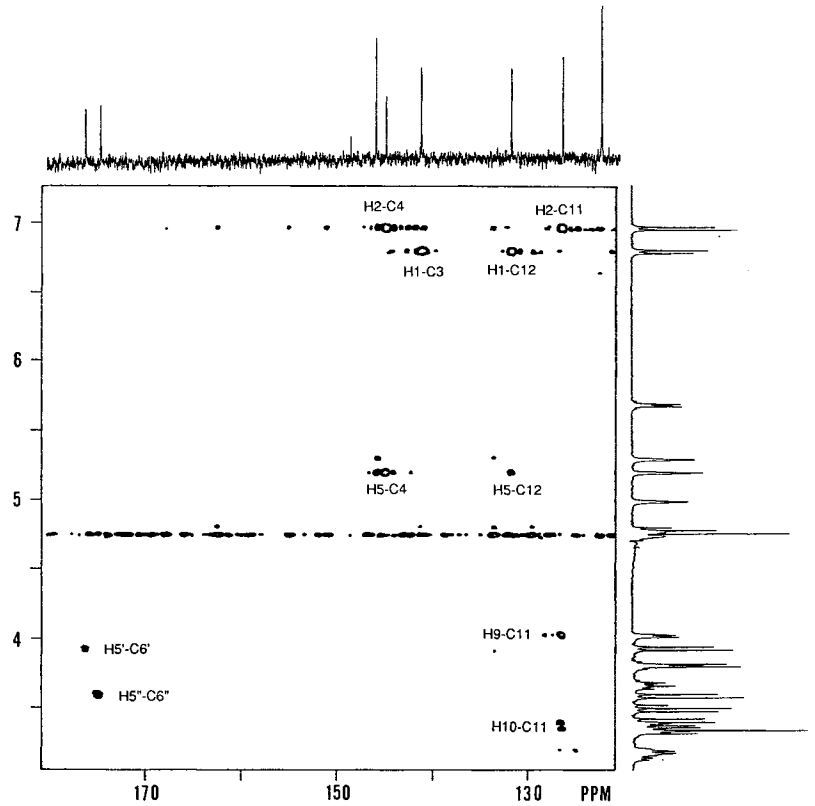

Fig. 3. HMBC spectrum of nalmefene 3-*O*-diglucuronide. On the top of the two-dimension spectrum is part of the carbon spectrum of nalmefene 3-*O*-diglucuronide (120 to 180 ppm). On the right side of the two-dimensional spectrum is part of the proton spectrum (3 to 7 ppm).

in the metabolite was obtained by dissolving 200 μg of the metabolite in 500 μl of acetone and adding 100 μl of acetic anhydride. This reaction mixture was mixed thoroughly and allowed to stand overnight. After evaporation to dryness under nitrogen, the sample was dissolved in the FAB matrix (above) and analyzed by linked-scan mass spectrometry (MS). In this technique, mass spectra are obtained by linking the scans of the magnetic and electric sector fields. By revealing which daughter ions are produced from which parent ions, linked-scan MS removes any ambiguities in the mass spectrum analysis which may be caused by the presence of extraneous peaks from other reaction products.

NMR Experiments. NMR experiments, except heteronuclear multiple-bond coherence (HMBC) spectra (4), were performed on a Varian XL-400 spectrometer. The HMBC spectrum, a two-dimensional $^{13}C-{}^{1}H$ correlation for long-range coupling, was obtained on a Bruker AM-500 spectrometer equipped with a probe that has a broad-band decoupling coil outside the ^{1}H observe coil. It was recorded with Δ_2 set to 60 msec. APT spectra (5) were used to establish the multiplicities of the ^{13}C resonances. $^{1}H-{}^{1}H$ correlated (COSY) spectra were recorded with the Jeener (6) pulse sequence. These spectra were used to establish J coupling between protons. In certain experiments, the cross peaks between protons with a small coupling were emphasized using additional delays of 50 msec before and after the second 90° pulse in the pulse sequence (7). Phase-sensitive COSY spectra were obtained in a pyridine-d_5/D_2O system, with standard Varian software (8,9). Two-dimensional $^{13}C-{}^{1}H$ correlation (HETCOR) spectra were obtained with the established sequence (10) modified for broad-band homonuclear decoupling (11,12). These HETCOR spectra were used to establish one bond correlation between the carbons and the protons. For long-range (two or three bonds) $^{13}C-{}^{1}H$ correlation, several one-dimensional, selective INEPT spectra were obtained (13). The selective 90° proton pulse used was 15 msec and the delays (Δ_1 and Δ_2) were both set to 40 msec.

Isolation of the Nalmefene Conjugate from Dog Urine. Portions of urine collections (0–48 hr) from two mongrel dogs (11 and 17 kg), each of which had received 10 mg of ^{14}C-nalmefene HCl orally, were combined with a 7-day pool of urine obtained from a third dog (15 kg) that had ingested 1 g of the drug daily for 5 days.

The combined radioactive pool (13 liters) was adjusted to pH 9 with ammonium hydroxide, stirred, and allowed to stand overnight at room temperature. The urine was then decanted from the precipitated solids and percolated through a 2-kg column (10-cm i.d.) of Amberlite XAD-2 resin as described by Bradlow (14). The resin was washed with 5 liters of water to remove inorganic salts, and the adsorbed radioactivity eluted with 5 × 1-liter portions of methanol. The solvent was removed *in vacuo,* and the aqueous residue diluted to 200 ml with water and adjusted to pH 9 with ammonium hydroxide. The dark brown aqueous alkaline solution was extracted with 2 × 500 ml of methyl t-butyl ether to remove any unconjugated metabolites of nalmefene along with nonpolar impurities. The remaining aqueous phase was evaporated *in vacuo* to a brown oil by azeotropic distillation with *n*-butanol. The crude extract was dissolved in 150 ml of 1% acetic acid and pumped onto a Whatman Partisil M-20 10/50 ODS-2 column followed by a 200-ml wash of the same solution. The column was eluted in a stepwise gradient with 500-ml portions of 10, 20, and 25% methanol in 1% acetic acid at a flow rate of 9 ml/min. Fractions (50 ml) were collected and assayed for ^{14}C radioactivity. A major peak of radioactivity was eluted with 25% methanol, and the fractions were combined and evaporated to yield 600 mg of a brown amorphous powder. A portion of the crude material (300 mg) was dissolved in a small volume of aqueous methanol and precipitated by the careful addition of diethyl ether. The light brown precipitate was collected by centrifugation and subjected to three additional precipitations from methanol with ether. Following a final trituration with anhydrous ether, 40 mg of the nalmefene metabolite was obtained as an

Table I. ^{13}C and ^{1}H NMR Chemical Shifts for Nalmefene-3-*O*-Diglucuronide in D_2O

	D_2O	
C No.	^{13}C[a]	^{1}H[b]
1	122.38	6.77
2	120.34	6.94
3	141.19[c,d]	
4	144.82[c]	
5	90.27	5.20
6	145.89[e]	
7	28.15	2.23,2.48
8	32.97	1.40,1.73
9	63.87	4.01
10	24.99	3.18,3.36
11	126.46[c]	
12	131.77[c]	131.13
13	48.28	
14	73.37	
15	28.66	1.73,2.50
16	48.72	2.80,3.15
17	59.45	2.97,3.35
18	7.21	1.06
19, 20	4.05	0.44,0.80
	6.81	0.44,0.70
21	114.63	4.98,5.29
1′	100.47	5.68[e]
2′	83.64[f] (81.19)[g]	3.80 (4.41)[g]
3′	76.73 (77.89)[g]	3.80 (4.27)[g]
4′	73.00	3.66
5′	77.65	3.92
6′	176.00[c,h]	
1″	103.95	4.79[f]
2″	75.04	3.34
3″	76.92	3.50
4″	73.45	3.40
5″	76.84	3.58
6″	174.47[c]	

[a] All protonated carbons are assigned by HETCOR.
[b] All couplings between protons are confirmed by COSY.
[c] Assigned by HMBC spectrum.
[d] 1′→ *O*-C3 linkage determined by selective INEPT.
[e] Assigned by elimination, the other nonprotonated carbons being assigned by either HMBC or selective INEPT.
[f] 1″→ 2′ linkage determined by selective INEPT.
[g] D_2O/pyridine-d_5 solvent.
[h] Assigned by selective INEPT experiment.

amorphous white powder. Repeated attempts at crystallization were unsuccessful and the amorphous material was used for all subsequent analyses and structural elucidation.

RESULTS AND DISCUSSION

The 3-*O*-diglucuronide structure of the nalmefene metabolite shown in Fig. 1 was determined on the basis of its hydrolysis with β-glucuronidase, high-resolution FAB mass studies, the mass fragmentation obtained by FAB, ^{13}C and ^{1}H NMR chemical shift data, and selective INEPT NMR data. The ^{1}H and ^{13}C resonances were assigned by the use of an array of two-dimensional NMR experiments detailed below. The ^{13}C NMR data show the appropriate substitution shifts for the indicated structure. Additional proof of the structure was obtained through acetylation of the metabolite at the tertiary hydroxyl position.

Hydrolysis with β-Glucuronidase. Hydrolysis with bovine liver β-glucuronidase enzyme (Glucurase, Sigma Chemical Co., St. Louis, Mo.) provided the first evidence that the metabolite was a conjugate of nalmefene and β-D-glucuronic acid. Five milligrams of the pure conjugate was hydrolyzed at 37°C overnight in 10 ml of 0.2 *M* acetate buffer (pH 5) containing 1000 U/ml of β-glucuronidase. After adjustment to pH 9 and extraction with methyl t-butyl ether, unconjugated nalmefene was isolated by TLC and identified by IR (KBr) spectroscopy.

Mass Spectrum Studies. The FAB mass spectral fragmentation scheme for the metabolite is illustrated in Fig. 1. The molecular formula, derived from the high-resolution mass spectrum, was consistent with a diglucuronic acid conjugate structure. A weak fragment ion at $M/Z = 516$ corresponds to the loss of one glucuronyl unit, with retention of the anomeric oxygen. This fragmentation pathway is consistent with a secondary, rather than a tertiary, carbinol as the second site of glycosidation. The major fragment at $M/Z = 340$ corresponds to loss of both glucuronic acid units, yielding the unconjugated nalmefene ion. Linked-scan metastable MS data were obtained on the acetylated metabolite to establish the parent ion and fragmentation pathway (Fig. 2) and provide a chemical proof of structure.

Assignment of NMR Spectra. The proton resonances of nalmefene diglucuronic acid are assigned from the COSY spectrum. The resonances of all the protonated carbons are assigned by the HETCOR spectrum. The resonances of the nonprotonated carbons (established by the APT and HETCOR spectra), C14 and C13, are assigned by their chemical shifts. The other nonprotonated carbons at C3, C4, C11, C12, C6′, and C6″ are assigned by the HMBC spectrum, part of which is shown in Fig. 3. The pertinent cross peaks are identified. The majority of these cross peaks arises from three-bond proton–carbon coupling (H10 – C11, H5′ – C6′, and H5″ – C6″). The HMBC spectrum does not show cross peaks for all multiple-bond proton–carbon couplings. The low-intensity peaks are due to T_1 noise. The chemical shift of the proton and carbon resonances of nalmefene diglucuronide in D_2O are summarized in Table I.

The carbon resonance at 83.6 ppm (C2′) is about 6 ppm downfield from the corresponding resonance in nalmefene monoglucuronic acid and phenolphthalein monoglucuronic acid (Chan, unpublished results). This downfield shift is consistent with a β substituent at this carbon. The peak at 73.4 ppm is assigned to C14 of the nalmefene moiety. The chemical shift for this carbon is 72.9 ppm for nalmefene and 73.3 ppm for synthetic nalmefene 3-*O*-glucuronic acid. A glucuronic acid linked to the C14 position would shift the carbon resonance about 3 ppm downfield (15). These results are consistent with the second glucuronic acid being linked to the first, which, in turn, is linked to the phenolic hydroxyl group of the nalmefene moiety. The second glucuronic acid is not linked to C14 of nalmefene.

Proton spectra were obtained in pyridine-d_5/D_2O, to eliminate the degeneracy of the H2′ and H3′ resonances. COSY and HETCOR experiments in this mixed solvent system yielded assignments for H2′, H3′, and C2′. The downfield shift of C2′, 81.2 ppm, indicated glycosidation at this site.

Selective INEPT experiments were used to determine

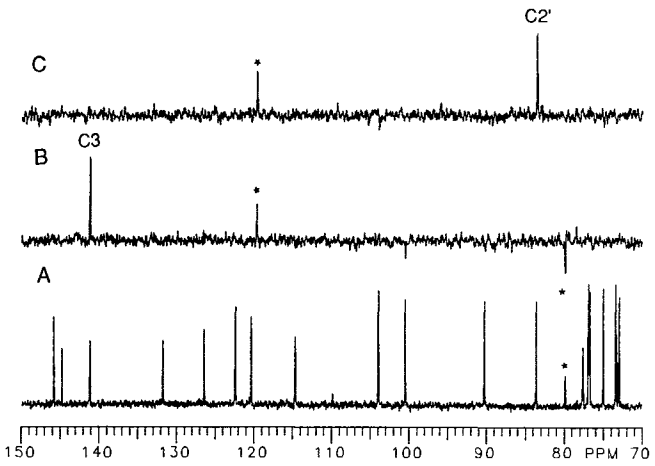

Fig. 4. Selective INEPT spectra of nalmefene 3-*O*-diglucuronide. (A) Part of the carbon spectrum of nalmefene 3-*O*-diglucuronide (70 to 150 ppm). (B, C) The same region of two selective INEPT spectra. Peaks marked with an asterisk are spikes in the spectra.

three-bond carbon–proton connectivity. A selective INEPT spectrum contains only the carbon resonances which are coupled to the pulsed proton with a coupling constant of about 4 Hz (13). Two selective INEPT spectra are shown in Fig. 4. When the H1′ resonance at 5.68 ppm was pulsed, only the C3 carbon resonance at 141.2 ppm appeared in the spectrum (Fig. 4B). This result established the anomeric proton resonance at 5.68 ppm as belonging to H1′ and the site of conjugation to nalmefene as C3. When the other anomeric proton, H1″, at 4.79 ppm, is pulsed, only the C2′ carbon at 83.6 ppm appears in the selective INEPT spectrum (Fig. 4C). Thus the second site of glycosidation is established as C2′, rather than C14 or the amine function.

In conclusion, the novel structure of nalmefene 3-*O*-diglucuronide was established by NMR and MS analyses. All carbon-13 and proton NMR assignments were determined by two-dimensional homonuclear and heteronuclear NMR methodologies. The 1′ → 3-*O* and 1″ → 2′-*O* glycosyl linkages were established unequivocably by selective INEPT NMR studies.

The high-resolution and FAB mass spectral data are consistent with the molecular formula and expected fragmentation for this structure. Chemical proof of the sites of glycosidation was provided by selective *in situ* acetylation of the C14 hydroxyl group. The assignments of the molecular ion and major fragments of the acetylated metabolite in the mass spectrum were established by linked-scan MS studies.

At the present time, the metabolic pathway from nalmefene leading to the formation of this novel metabolite has not been established and it is highly improbable that this type of conjugate is unique to nalmefene. Recent studies by Jacqz *et al.* (16) have indicated that morphine undergoes extensive intra- and extrahepatic glucuronidation in the dog but the exact nature of the conjugate(s) was not established. As is so often done in studies of the latter type, the plasma concentrations of "morphine glucuronide" were estimated by subtracting the unconjugated morphine plasma concentration prior to Glusulase (β-glucuronidase/sulfatase enzyme obtained from *Helix pomatia*) hydrolysis from the total morphine concentration obtained following the enzymic hydrolysis. In this way the nature of the morphine conjugate(s) would be unknown.

Our present results with nalmefene point to the importance of establishing the true chemical identity of drug conjugates prior to assumptive conjecture as to their physiologic relevance and origin.

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REFERENCES

1. R. Dixon, J. Howes, J. Gentile, H. Hsu, J. Hsiao, D. Garg, D. Weidler, M. Meyer, and R. Tuttle. *Clin. Pharmacol. Ther.* 39:49–53 (1986).
2. R. Dixon, J. Gentile, H. Hsu, J. Hsiao, J. Howes, D. Garg, and D. Weidler. *J. Clin. Pharmacol.* 27:233–239 (1987).
3. B. Berrang, C. Twine, G. Hennessee, and Fl. Carroll. *Synth. Comm.* 5:231–236 (1975).
4. A. Bax and M. F. Summers. *J. Am. Chem. Soc.* 2093–2094 (1986).
5. S. L. Patt and J. N. Shoolery. *J. Magn. Res.* 46:535–539 (1982).
6. J. Jeener. Ampere International Summer School, Bosko Polje, Yugoslavia (1971).
7. A. Bax and R. Freeman. *J. Magn. Res.* 42:164–168 (1981).
8. L. Mueller and R. R. Ernst. *Mol. Phys.* 38:963–992 (1979).
9. D. J. States, R. A. Harberkorn, and D. J. Reubin. *J. Magn. Res.* 48:286–292 (1982).
10. A. Bax and G. A. Morris. *J. Magn. Res.* 42:501–505 (1981).
11. A. Bax. *J. Magn. Res.* 53:517–520 (1983).
12. J. A. Wilde and P. H. Bolton. *J. Magn. Res.* 59:343–346 (1984).
13. A. Bax. *J. Magn. Res.* 57:314–318 (1984).
14. H. L. Bradlow. *Steroids* 11:265–269 (1968).
15. S. Morimoto, Y. Takakashi, Y. Watanabe, and S. Omura. *J. Antibiot.* 37:187–189 (1984).
16. E. Jacqz, S. Ward, R. Johnson, S. Schenker, J. Gerkens, and R. A. Branch. *Drug Metab. Disp.* 14:627–630 (1986).