Urinary Metabolic Profiles for Choosing Test Animals for Chronic Toxicity Studies: Application to Naproxen

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Abstract □ Gradient elution ion-exchange chromatography was employed to determine urinary metabolic profiles of naproxen in test animals and humans. Retention molarities were found to be very reproducible, and the quantitation and column recovery were good. The profiles add another dimension to the basic information required to pick the most appropriate test animal for the chronic toxicological evaluation of a new drug. Based on the profiles, the mini pig and rat appeared to be the animals of choice for the chronic toxicological evaluation of naproxen.

Keyphrases □ Naproxen urinary metabolic profiles—used to determine appropriate test animal for toxicology evaluation, gradient elution ion-exchange chromatography □ Urinary metabolic profiles—determination of appropriate test animal for toxicology evaluation, naproxen □ Toxicology studies, naproxen—choice of appropriate test animal based on urinary metabolic profiles □ Ion-exchange chromatography, gradient elution—determination of naproxen urinary metabolic profiles, animals, humans

Naproxen [(+)-6-methoxy-α-methyl-2-naphthalene-acetic acid[1], I] is a new nonsteroidal anti-inflammatory agent as determined by the carrageenin-induced rat paw edema assay (1, 2). During the development of this new drug, it became important to generate information to determine which rodent and nonrodent test animals resemble the human most closely in the disposition of the drug. This information, which includes data on the absorption, excretion, distribution, rate of metabolism, and metabolic pattern of the drug, provides the rationale for the choice of the most appropriate test species in the chronic toxicological evaluation of a new drug (3, 4). A study of the absorption, distribution, and excretion of naproxen in various test animals and man was recently reported (5). The present report describes an approach taken for the determination of the urinary metabolic profiles of naproxen, based on gradient elution ion-exchange chromatography.

EXPERIMENTAL[2]

Species, Dosing, and Urine Collection—The test animals and humans employed in this investigation received intravenous injections of tritium-labeled naproxen. A description of the preparation of the dosing solutions, the method of dosing, and the method of urine collection was previously described (5). A description of the species and the dosing data employed in this investigation is summarized in Table I. The urine specimens were preserved with formaldehyde and stored in the frozen state.

Naproxen—The synthesis of naproxen[3] was previously reported (1). Tritiated naproxen[3] (6), labeled on the ring system, had a specific activity of 92 mc./mmole (5). Naproxen-3-^{14}C[3] had a specific activity of 25.6 mc./mmole.

2-(6'-Hydroxy-2'-naphthyl)propionic Acid[3] (II)—The structure of this compound was verified by its spectral properties and elemental analysis; IR (KBr): 3400, 1705, 1605, 1500, 1450, 1380, 1360, 1280, 1200, and 1145 cm.$^{-1}$; UV (CH$_3$OH): 225 (log ϵ 5.10), 256 (3.49), 265 (3.62), 274 (3.65), 322 (3.18), and 334 (3.24) nm.; NMR (d_6-acetone): δ 7.55–7.80 (m, 3), 7.36 (d of d, 1, J = 10 and 2.0 Hz.), 7.03–7.20 (m, 2), 3.83 (q, 1, J = 7 Hz.), and 1.49 (d, 3, J = 7 Hz.); mass spectrum (70 ev.) m/e: base peak 171, molecular ion 216.

Anal.—Calc. for C$_{13}$H$_{12}$O$_3$: C, 72.21; H, 5.59. Found: C, 72.26; H, 5.62.

Methyl 2-(6'-Methoxy-2'-naphthyl)propionate—To 4.08 g. (0.018 mole) of naproxen was slowly added 120 ml. (0.108 mole) of 14% (w/v) boron trifluoride-methanol[4]. The mixture was refluxed for 15 min., cooled to room temperature, and taken up in 1.2 l. of petroleum ether (30–60°). The solution was washed with water and dried over anhydrous sodium sulfate. The solution was filtered, and the petroleum ether was removed by rotary evaporation to give 3.97 g. (92%) of crystalline methyl 2-(6'-methoxy-2'-naphthyl)-propionate, m.p. 90–91°.

2-(6'-Methoxy-2-naphthyl)propionuric Acid (III)—The synthesis of this compound was based on Ford's (7) procedure for the synthesis of phenaceturic acid. A solution of sodium methoxide in methanol was prepared by the reaction of 0.51 g. (0.022 g.-atom) of sodium metal in 20 ml. of anhydrous methanol. A 1.31-g. (0.018 mole) portion of glycine was added to the alkoxide solution, and the solution was refluxed for 20 min. to dissolve the glycine. A 3.87-g. (0.016-mole) portion of methyl 2-(6'-methoxy-2'-naphthyl)propionate was then added to the glycine solution, and the mixture was refluxed (91°) for 5 days. The methyl alcohol was removed by distillation through a short Vigreux column, and the residue was taken up in 200 ml. of 5% sodium bicarbonate and washed with two 50-ml. portions of ether. The aqueous solution was adjusted to pH 2 with hydrochloric acid, precipitating 3.41 g. of crystalline material. TLC on silica gel GF, using benzene-methanol-acetic acid (90:16:8), showed this material to be approximately a 50:50 mixture of naproxen and the desired product. Preparative TLC on 0.51 g. of the crude material, using toluene-acetic acid-water (50:50:5) followed by elution with methanol, recrystallization from methanol-water (50:50), and drying over phosphorus pentoxide (0.01 mm.), gave 0.24 g. of the desired product, m.p. 156–157°; IR (KBr): 3350, 1750, and 1625 cm.$^{-1}$; UV (CH$_3$OH): 232 (log ϵ 4.84), 253 (3.58), 262 (3.67), 272 (3.67), 324 (3.09), 303 (2.88), 316 (3.14), and 331 (3.24) nm.; NMR (d_6-dimethyl sulfoxide): δ 8.30 (broad t, 1), 7.68–7.86 (m, 3), 7.46 (d of d, 1, J = 8.5 and 1.7 Hz.), 7.28 (d, 1, J = 2.0 Hz.), 7.15 (d of d, 1, J = 9.0 and 2.5 Hz.), 3.86 (s, 3), 3.76 (m, 3), and 1.41
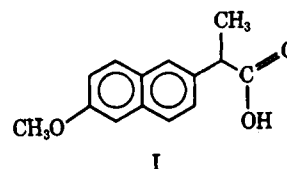
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Table I—Species and Dosing Data

Species	Number, Sex	Weight, kg.	Urinary Half-Life, hr.[a]	Urine Specimen Used, hr.	Dose, Cold, mg./kg.	Dose, Hot, μc./kg.
Human	2M 1F	49–86	13	1–3	93[b]	40[c]
Mini pig	2F	63–70	5	0–24	2.5	11.6
Rhesus monkey	2F	5–6	2	0–0.5	10.0	200
Guinea pig	1M	0.82–1.1	10	0–24	5.0	49.0
Rat (Sprague–Dawley)	3M	0.45–0.50	8	0–24	2.88	28.8

[a] Obtained from *Reference 5*. [b] The total cold human dose was 93 mg. [c] The total hot human dose was 40 μc.

Table II—Retention Data for the Biotransformed Products of Naproxen in Test Animals and Man

Compound Number	Rhesus Monkey	Guinea Pig	Rat	Mini Pig	Human Male	Human Female	Authentic III	Authentic II
Retention Molarity								
1	0	0	0	0	0	0	—	—
2	0.01	0.01	0.01	—	—	—	—	—
3	—	0.09	—	0.09	—	—	—	—
4	0.14	0.16	0.16	0.14	—	—	—	—
5 (Naproxen, I)	0.20	0.20	0.20	0.20	0.19	0.20	0.20	0.20
6	0.22	—	—	0.22	0.23	0.25	—	—
7 (III)	0.25	0.27	0.27	0.27	0.26	—	0.26	—
8 (II)	—	—	—	—	—	0.29	—	0.29
9	0.31	0.31	0.31	0.30	0.30	0.31	—	—
10	—	—	0.36	0.35	0.35	0.37	—	—
11	—	—	0.40	0.40	0.40	0.41	—	—
12	0.46	0.45	0.45	0.45	—	—	—	—
13	—	—	0.49	0.48	0.48	0.50	—	—
14	—	0.57	—	0.58	—	—	—	—
15	0.81	—	0.80	—	—	—	—	—
16	0.85	—	0.84	—	—	—	—	—
Retention pH								
17	—	—	3.85	—	—	—	—	—
18	4.45	—	4.45	4.45	—	—	—	—
19	—	4.65	4.65	—	—	4.65	—	—
20	—	—	—	—	6.50	—	—	—

(d, 3, J = 7.0 Hz.); mass spectrum (70 ev.) m/e: base peak 185, molecular ion 287.

*Anal.*—Calc. for $C_{16}H_{17}NO_4$: C, 66.89; H, 5.96. Found: C, 66.72; H, 6.00.

Ion-Exchange Resin—All ion-exchange separations described here were carried out with portions of the same batch of diethylaminoethyl Sephadex in the formate form prepared as follows. A 200-g. portion of diethylaminoethyl Sephadex (chloride) A-25[6] in a 12 × 60-cm. chromatography column was treated successively with 1 M NaOH until chloride was not detectable in the eluate, with distilled water until the eluate was neutral, with 0.5 M formic acid until the pH of the eluate was equivalent to that of 0.5 M formic acid, and finally with distilled water until formate ion was not detectable in the eluate. Formate ion was monitored by the method of Feigl (8).

Ion-Exchange Chromatography—The procedure employed was similar to that described by Lethco and Brouwer (9). Composite urine specimens were prepared by combining the specimens (same percent by volume of each) of all animals of the same species. Aliquots (5–40 ml.) of the composite specimens were taken for chromatography so as to contain about 3 × 10^6 d.p.m. of tritium for the animal specimens and about 7.5 × 10^5 d.p.m. of tritium for the human specimens. An internal standard of about 3.5 × 10^5 d.p.m. of ^{14}C-naproxen was introduced into each specimen used for chromatography by mixing the specimens in a centrifuge tube in which a solution of ^{14}C-naproxen had been evaporated. Male and female human urine specimens were chromatographed separately; however, the two female specimens were combined.

Urine composite aliquots were pipeted onto a 35 × 1.8-cm. i.d. column charged with 35 meq. of ion-exchange resin[6]. The biotransformed components related to naproxen were separated by successive elution with distilled water (200 ml.), pH 3.5 ammonium

formate buffer (4000 ml.) over a linear concentration gradient from 0 to 1.0 M, 1.0 M ammonium formate buffer (2000 ml.) over a pH gradient from 3.5 to 5.5, and 1 M ammonium formate buffer (1000 ml.) at pH 6.5. Final washing of the resin in a conical flask with methanol gave less than 0.5% of the initially present tritium radioactivity in each case.

The eluate was collected in 10-ml. fractions, and 5-ml. aliquots of every other fraction were evaporated to dryness, diluted with scintillation fluid, and subjected to double isotope counting using an external standard for quench correction. Plots of tritium radioactivity *versus* fraction number gave the unperturbed metabolic profiles shown in Fig. 1. Retention molarities and retention pH's (Table II) were determined from fraction number *versus* formate molarity and fraction number *versus* pH calibration curves, respectively. Every fraction of each biotransformed product (as defined by the profile)

II

III
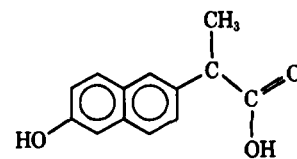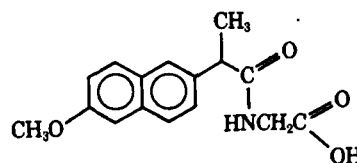
Table III—Quantitative Distribution of the Biotransformed Products of Naproxen in Test Animals and Man[a]

Compound Number	Rhesus Monkey	Guinea Pig	Rat	Mini Pig	Human Male	Human Female
1	12.1	0.3	0.2	19.7	0.3	0.2
2	0.6	0.8	0.7	—	—	—
3	—	0.3	—	0.5	—	—
4	0.6	0.5	0.2	0.9	—	—
5 (Naproxen, I)	6.7	39.8	2.8	3.6	11.5	7.3
6	6.4	—	—	1.3		
7 (III)	31.9	4.5	8.2	16.9		
8 (II)	—	—	—	—	28.0[b]	22.7[b]
9	19.9	8.2	1.0	7.7		
10	—	—	0.5	2.3	38.2	40.1
11	—	—	1.1	16.1	7.9	6.6
12	2.2	2.6	2.1	1.2	—	—
13	—	—	0.5	3.0	10.3	13.9
14	—	1.8	—	8.2	—	—
15	0.9	—	7.4	—	—	—
16	1.2	—	4.1	—	—	—
17	—	—	2.2	—	—	—
18	2.9	—	24.4	1.7	—	—
19	—	15.1	32.8	—	—	2.5
20	—	—	—	—	5.6	—

[a] The percentage values are based on the total tritium radioactivity applied to the column. [b] These values represent the total concentration of products eluted after Compound 5 and before Compound 10.

was combined, and an aliquot was counted to determine the quantitative distribution of each product (Table III).

The elution of authentic II and III was followed by monitoring their UV absorption at 225 and 232 nm., respectively.

RESULTS

The data in Table IV demonstrate the good total recovery using the specified column. The mean recovery based on seven separations was 93.8%.

The urinary metabolic profiles presented in Fig. 1 show that at least 20 biotransformed products of naproxen were detected. For purposes of comparison, each biotransformed product, defined by a retention molarity or pH, was given a compound number. These data are presented in Table II.

Compounds 10 and 13 were identified[•] as the glucuronides of naproxen and II, respectively, by incubation with β-ketodase. Naproxen, II, and III (Compounds 5, 8, and 7, respectively) were identified in the various urine specimens by comparison of their retention molarities with those of authentic compounds.

The reproducibility of the retention molarities from run to run is demonstrated by the data in Table II for naproxen (Compound 5), in which identification is unambiguous because of the presence of the naproxen-^{14}C internal standard. For naproxen, a retention molarity of 0.20 was obtained in each of the eight runs (Table II), except for the human male for which the value was 0.19. Additionally, the data in Table V show that except for Compound 9 (retention molarity 0.30 and 0.29) the retention molarities are identical for duplicate runs on the human male specimen.

The retention molarity range for each compound in Table II is 0.02, except for Compound 6 for which the retention molarity varied from 0.22 in the mini pig to 0.25 in the human female (range 0.03).

The assignment of compound numbers for most of the biotransformed products based on retention molarities was straightforward. Some very minor components, however, were not given compound numbers, such as the shoulder on the descending side of Compound 6 and on the ascending side of Compound 12 in the rhesus monkey.

The assignment of compound numbers for the biotransformed products in the human specimens between the retention molarities of 0.22 and 0.31 was difficult due to the poor resolution of the products in this region. However, an attempt was made to assign some compound numbers to the components in this region, but these assignments should be considered with reservation. This is the region where II and III elute.

Although reference compounds were not employed in the retention pH region, the data in Table II indicate that the retention pH's are reproducible. The only exception to this is the retention pH

values of 6.5 and 5.1 (Table V) obtained from duplicate runs on the human male urine specimens. Since in this specimen only one component eluted in the retention pH range and since the remainders of both profiles were virtually identical, it has been assumed for convenience that the two retention pH's represent the same component. Following a similar line of reasoning, Compound 20 found in the human male may be equivalent to Compound 19 found in the human female, rat, and guinea pig.

Since the column may not be selective when distilled water is used as the eluting solvent, the radioactive naproxen-related materials eluted in the first 200 ml. in all of the urine specimens may or may not represent the same substance. For convenience, however, the material eluted in this region was assigned as Compound 1.

Inspection of the percent distribution data in Table V shows that the quantitative capabilities of the method are sufficiently reproducible to make quantitative comparisons of the naproxen-related products produced by the different species. The percent distribution data in Table III show that less than 1% of the biotransformed products of naproxen was eluted with water in the human. However, the material eluted in this region represents about 12 and 20% of the naproxen products in the rhesus monkey and mini pig, respectively. Compounds 2, 3, and 4 were not found in the human samples and represent less than 1% of the products found in the test animals. Naproxen (Compound 5) was found in every specimen. It represents about 7–12% of the products in the human specimen and was the major product (about 40%) in the guinea pig. Compounds 6, 7, 8, and 9, which were poorly resolved in the human runs, represent a total of about 23–28% of the products. The glycine conjugate of naproxen (Compound 7) was a major product in both the rhesus monkey (about 32%) and the mini pig (about 17%). Compound 9, unknown, represents about 20% of the products in the rhesus monkey.

Compound 10, the glucuronide conjugate of naproxen, was the major biotransformed product of naproxen in the human (about

Table IV—Column[a] Recovery for the Biotransformed Products of Naproxen in Test Animals and Humans

Species	Recovery, %[b]
Rhesus monkey	92.5
Mini pig	89.9
Rat	91.3
Guinea pig	86.1
Human male	102.8, 99.9
Human female	94.2
Mean	93.8

[a] Diethylaminoethyl Sephadex (formate). [b] Based on tritium radioactivity.
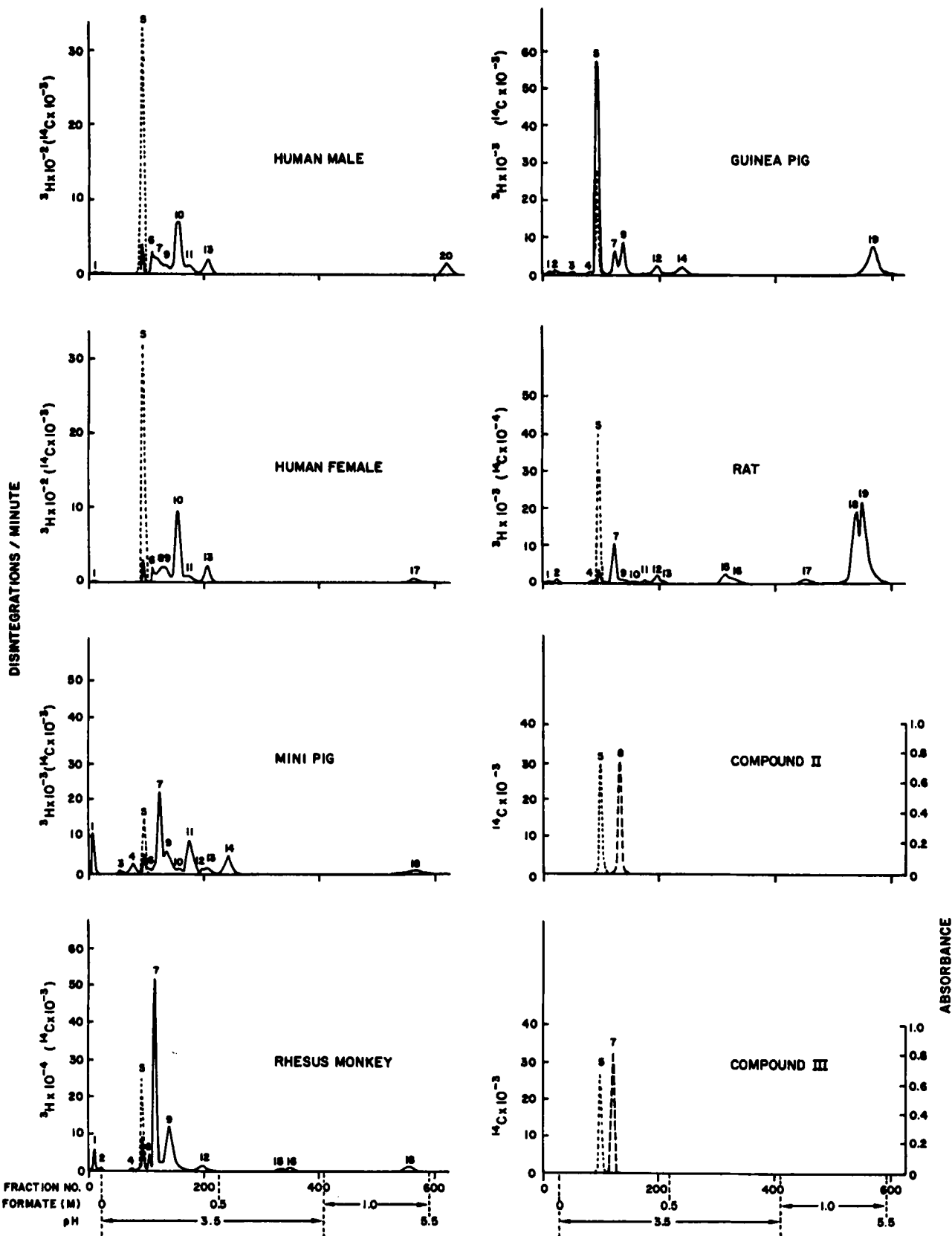
[•] To be published.

Figure 1—*Metabolic profiles for naproxen in humans and test animals. Key:* ——, ^3H *measurements;* - - -, ^{14}C *measurements; and* — — *absorbance measurements.*

40%). It was present in very small quantities in the mini pig and rat but not present in the guinea pig or rhesus monkey. Unidentified Compound 11, which represents about 7% of the products in the human was a major product (about 16%) in the mini pig. Unknown

Compound 12 was not found in the human and represents less than 3% of the products in each of the test animals. Compound 13, a glucuronide of II, was a major product in the human (10–14%) and was also found in the mini pig (3%) and rat (0.5%). Unknown Com-

Table V—Comparison of Retention and Percent Distribution Data for Duplicate Separations (Human Male) of the Same Specimen[a]

Compound Number	Retention Molarity Run 1	Run 2	Percent Distribution[b] Run 1	Run 2
1	0	0	0.3	0.3
2	—	—	—	—
3	—	—	—	—
4	—	—	—	—
5	0.19	0.19	11.5	11.5
6	0.23	0.23		
7	0.26	0.26		
8	—	—	28.0[c]	27.5[c]
9	0.30	0.29		
10	0.35	0.35	38.2	32.7
11	0.40	0.40	7.9	8.0
12	—	—	—	—
13	0.48	0.48	10.3	10.4
14	—	—	—	—
15	—	—	—	—
16	—	—	—	—
	Retention pH Run 1	Run 2		
17	—	—	—	—
18	—	—	—	—
19	—	—	—	—
20	6.50	5.70	5.6	9.3

[a] Run 1, column eluted with an ammonium formate buffer; run 2, column eluted with a sodium formate buffer. [b] The percentage values are based on the total tritium radioactivity applied to the column. [c] These values represent the total concentration of products eluted after Compound 5 and before Compound 10.

pounds 14, 15, 16, 17, and 18 were not found in the human. Compound 18 was a major product in the rat. Unknown Compound 19 was found to the extent of 2.5% in the human female and was the major product in the rat. About 5% of Compound 20, which may or may not be equivalent to Compound 19, was found in the human male.

DISCUSSION

When employing metabolic profiles as the only criterion, the ideal test animal for chronic toxicity studies is that test animal whose metabolic profile is identical with that of the human. Since this is a very unlikely or even impossible goal for most drugs, the next best criterion is to pick that animal whose profile is most similar to that of the human. When using the metabolic profiles as the only criterion, the mini pig appears to be the best nonrodent test animal (of those studied) for the chronic toxicological evaluation of naproxen. This choice is based on the presence in the mini pig of Compounds 10, 11, and 13. These are the three major biotransformed products of naproxen in the human and are not found in the rhesus monkey. Again, based only on the profiles, the rat appears to be the choice as a rodent test species. This choice is also based on the presence in the rat (albeit smaller quantities) of Compounds 10, 11, and 13 which are not present in the guinea pig. Additionally, the guinea pig contained about 40% free naproxen whereas the rat contained only about 3%, a value more comparable to that found in the human specimens.

Since the biotransformed products of most drug substances are amenable to ion-exchange chromatography, the method appears to

have general applicability. The good column recovery allows the development of a complete unperturbed picture of the number and quantity of biotransformed products produced. This method appears to have advantages over other methods that include extraction and derivatization procedures which may perturb the urinary products. The generally good reproducibility of the retention data demonstrates the utility of the profiles for both comparison purposes and for the tentative identification of the drug-related components when authentic compounds are available.

In addition to supplying part of the information for choosing the best test animals, metabolic profiles form a good basis for the planning and development of experiments to give additional metabolic information. The method provides separated samples of the individual biotransformed products which can be subjected to other analytical techniques.

The major limitation of the method is that it is fairly time consuming. It was primarily for this reason that the urine specimens from the same test animals were pooled. Although it can be argued that this results in an average picture for a particular test animal, it does not give a picture of animal-to-animal variation. It appears at this point that one method of circumventing the time requirement would be the use of linear gradient high pressure chromatography monitored by a flowthrough scintillation (and UV) detector such as was described by Hunt (10).

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