Metabolic Fate of Siduron in the Animal

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Three metabolites of siduron [1-(2-methylcyclohexyl)-3-phenylurea] were found in the urine of a dog fed this herbicide at a dietary level of 2500 p.p.m. over a 14-month period. These metabolites were identified as the conjugates of 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea, 1-(4-hydroxy-2-methylcyclohexyl)-3-(*p*-hydroxyphenyl)urea, and 1-(4-hydroxy-2-methylcyclohexyl)-3-phenylurea. The free metabolites, after enzymatic hydrolysis and extraction, were isolated and characterized by thin-layer chromatographic, mass spectrometric, infrared spectrometric, and nuclear magnetic resonance techniques. A metabolic pathway for siduron is proposed.

Metabolism of selected, substituted phenylurea herbicides has been reported in the literature. The investigations of Ernst and Böhme (1965) and Böhme and Ernst (1965) on the biochemical transformation of 3-(*p*-chlorophenyl)-1,1-dimethylurea (monuron), 3-(*p*-chlorophenyl)-1-methoxy-1-methylurea (aresin), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron), and 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea (linuron) in the rat indicate that the majority of the excreted metabolites, resulting from the hydroxylation and dealkylation of the original compounds, still retained the intact urea grouping. Similarly, Hodge *et al.* (1967) reported on the excretion of 3,4-dichlorophenylurea, 3-(3,4-dichlorophenyl)-1-methylurea, 3,4-dichloroaniline, 3,4-dichlorophenol, and unchanged diuron in the urine and feces of rats and dogs fed diuron (25 to 2500 p.p.m.) over an extended period of time.

This study was aimed at determining the metabolic fate of another substituted phenylurea herbicide, 1-(2-methylcyclohexyl)-3-phenylurea (siduron) in a dog and comparing the metabolite content in the urine of this animal with that of rats fed this compound at the same dietary level of 2500 p.p.m. for 14 months.

MATERIALS AND METHODS

Analysis of Urine for Siduron and Its Metabolites Yielding Aniline on Caustic Hydrolysis. An established procedure (Bleidner *et. al.*, 1954; Bleidner, 1954; Dalton and Pease, 1962) was used for determining the urinary content of siduron and the siduron metabolite, 1-(4-hydroxy-2-methylcyclohexyl)-3-phenylurea. These two compounds were quantitatively hydrolyzed under reflux conditions in a strongly alkaline medium to aniline with simultaneous partitioning of this aromatic amine into an organic solvent. The aniline was then extracted into dilute acid and determined colorimetrically after diazotization and coupling reactions. The diazotization and coupling steps were carried out at 0° C. as a means of minimizing color interferences (Pease, 1964).

Analysis of Urine for Siduron Metabolites Yielding *p*-Aminophenol on Caustic Hydrolysis. This procedure was devised for determining the urinary content of siduron metabolites, 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea and 1-(4-hydroxy-2-methylcyclohexyl)-3-(*p*-hydroxyphenyl)urea.

A 25-gram urine sample, adjusted to pH 5.0, was incubated with 0.5 ml. of a β-glucuronidase preparation (Boehringer-Mannheim Corp., New York) at 35° C. for 24 hours and then extracted three times with 25-ml. portions of ethyl acetate. Centrifugation was necessary to facilitate phase separation. The ethyl acetate fractions were combined, reduced in volume to 2 to 5 ml., and refluxed with 20 ml. of phosphoric acid for 3 hours to effect hydrolysis of the metabolites to *p*-aminophenol. The pH of the hydrolysate was adjusted to 1.5, maintaining the solution temperature below 35° C. The volume was adjusted to 100 ml. with 1*M* H_3PO_4. An aliquot (up to 40 ml.) containing 2 to 100 μg. of *p*-aminophenol was transferred to a 50-ml. volumetric flask, to which was added 1 ml. of a solution containing 3% sodium nitrite and 5% sodium bromide. After 10 minutes, 1 ml. of 10% sulfamic acid was added, and the flasks were shaken to ensure destruction of all excess sodium nitrite. After an additional 10 minutes, 2 ml. of 2% *N*-(1-naphthyl)ethylenediamine dihydrochloride was added and the volume adjusted to 50 ml. with 1*M* H_3PO_4. The flasks were placed in a steam bath for 60 minutes for full color development. Color interferences were removed by passing an aliquot of the colored solution, when cool, through a cellulose powder column (Pease, 1962). The absorbance of the blue *p*-aminophenol azo dye solution, after elution from the column, was measured spectrophotometrically at a wave length of 570 mμ.

Analysis of Urine for Aniline. A 25-gram sample of urine, adjusted to pH 8.5 to 9.0, was extracted 10 times with 40-ml. portions of HCl washed *N*-hexane. The combined hexane extracts were then washed twice with 50-ml. portions of 10% $NaHCO_3$ for 10 to 20 seconds. The aniline, if present, was extracted with six 15-ml. portions of 1*N* HCl, collecting the extracts in a 100-ml. volumetric flask. The volume was adjusted with 1*N* HCl. An aliquot up to 40 ml. containing 5 to 80 μg. of aniline was transferred to a 50-ml. volumetric flask and the aniline diazotized and coupled per colorimetric procedure for fenuron analyses (Dalton and Pease, 1962). This method is sensitive to 0.1 p.p.m. of aniline.

Extraction and Isolation of Siduron and Its Metabolites from Urine. A 25- to 100-ml. urine sample, adjusted to pH 5.0, was incubated with 0.5 to 2.0 ml. of the β-glucuronidase preparation at 35° C. for 24 hours. The sample was neutralized and extracted with a 1-to-1 mixture of diethyl ether and ethyl acetate for 24 hours in a continuous liquid-liquid extractor. The extract was streaked on TLC plates with 1000-micron Kieselgel coatings, and developed in a 9-to-1 chloroform/methanol mixture to the 15-cm. mark. The zones of Kieselgel, suspected of containing siduron and/or its metabolites, were removed from the plate and the adsorbed materials eluted with a 1-to-1 methanol and acetone mixture. The eluates were re-chromatographed, repeatedly if necessary, to effect the desired separation before characterization by mass spectrometry, infrared spectrophotometry, and nuclear magnetic resonance measurements.

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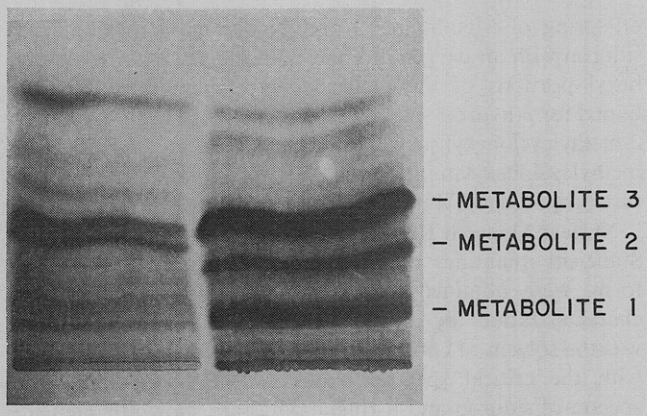

Figure 1. Thin layer chromatographic separation of siduron metabolites in a dog urine extract

Preparation of 1-(*p*-Hydroxyphenyl)-3-(2-methylcyclohexyl)urea. Ten grams of 2-methylcyclohexylisocyanate was added dropwise to a stirring mixture of 8.7 grams of *p*-aminophenol, a trace of "Dabco," dicyclopiperazine, and 3 drops of dibutyltin dilaurate in 75 ml. of tetrahydrofuran. The temperature rose spontaneously to 35° C. The mixture was refluxed for a few minutes and permitted to cool. When cool, the solids were filtered and washed with a small portion of ether. The solids (9.1 grams), were gray, m.p. 212° to 213° C. The material was recrystallized from a dioxane-isopropanol mixture to yield 2 grams of a white crystalline solid, m.p. 221° to 222° C. This material was again recrystallized from isopropanol to yield 1.3 grams of white crystalline solid, m.p. 223° to 224° C.

Anal. Calcd. for $C_{14}H_{20}N_2O_2$: C 67.8; H, 8.12; N, 11.3. Found: C, 67.5; H, 8.18; N, 11.1.

Apparatus. Instruments used in the characterization of the siduron metabolites were the Bendix Time-of-Flight Model 12-107 mass spectrometer, the Beckman Infra-Red Spectrophotometer IR-12, the Varian, Model A-60 nuclear magnetic resonance spectrometer, and the F & M Carbon-Hydrogen-Nitrogen Analyzer, Model 185.

RESULTS AND DISCUSSION

Based on thin-layer chromatographic separations and spectrophotometric analysis of Kieselgel eluates, no siduron was detected in the dichloromethane extracts of urine from a dog fed siduron at a level of 2500 p.p.m. in the diet. Similarly, the analysis of urine for aniline, using a procedure sensitive to 0.1 p.p.m. as outlined in Materials and Methods section of this paper, also failed to show detectable amounts of aniline.

Initial attempts to extract siduron metabolites from urine with the usual organic solvents revealed that metabolites for the most part remained in the aqueous phase. Qualitative tests for glucuronides were strongly positive. After treatment of urine with β-glucuronidase, the siduron metabolites were extractable in 1-to-1 mixture of ethyl acetate and diethyl ether.

A typical thin-layer chromatographic separation of the crude extracts of urine of the control animal and siduron-fed animal, photographed under ultraviolet light, is shown in Figure 1. In the chromatogram of urine extract of the treated animal, there were three distinct bands of strong ultraviolet absorption, which were not present in the control chromatogram. The zones of Kieselgel with these strongly ultraviolet absorbing materials were removed from the plate. The metabolites were eluted with a methanol and acetone mixture and rechromatographed separately as a means of minimizing the presence of interfering materials.

For discussion purposes, the siduron metabolites isolated from each of the bands of strong ultraviolet absorption will be referred to in the text that follows as Metabolites 1, 2, and 3, Metabolite 1 being closest to, and Metabolite 3 being furthest from the origin.

Mass Spectrometric Analysis. Mass spectra were obtained on the three metabolites and compared with several known compounds suspected of being metabolites. The spectra

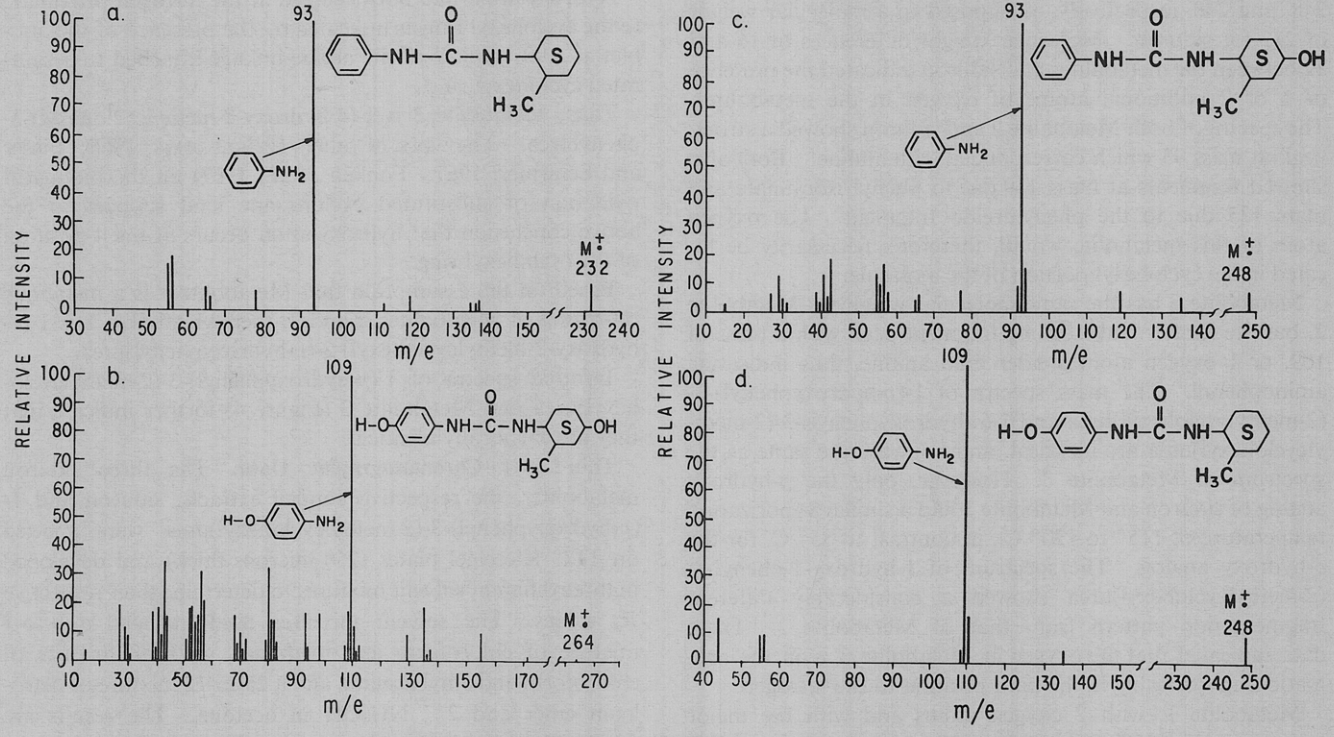

Figure 2. Mass spectra of (*a*) siduron, (*b*) metabolite 1, (*c*) metabolite 2, and (*d*) metabolite 3

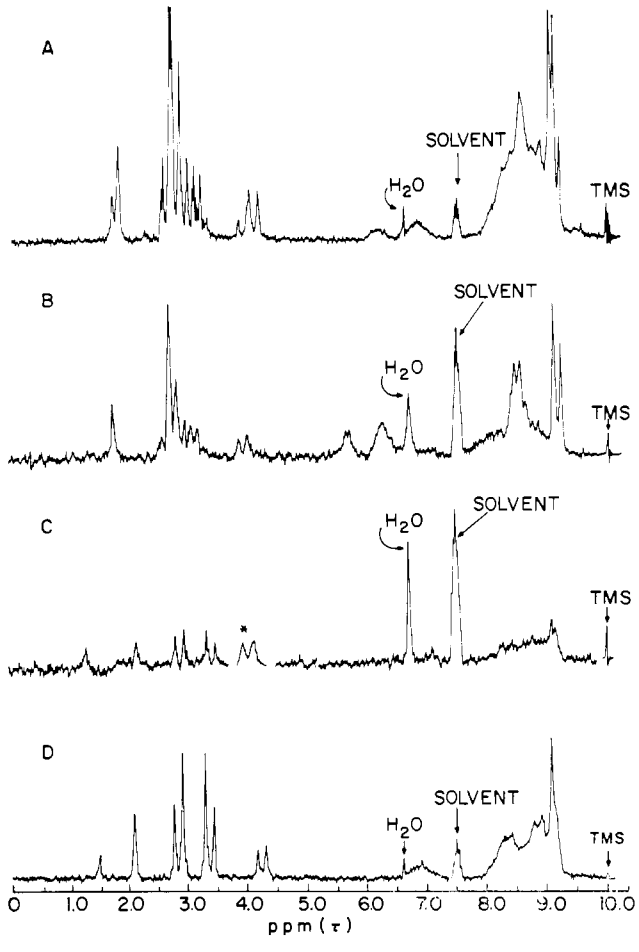

Figure 3. NMR spectra of (*A*) siduron, (*B*) metabolite 2, (*C*) metabolite 3, and (*D*) 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea

* Doublet amplified by 75 passes with a C-1024 time averaging computer

of siduron and the three metabolites are shown in Figure 2. The molecular weights of Metabolites 1, 2, and 3 were 264, 248, and 248, respectively, as opposed to a molecular weight of 232 for siduron. Molecular weight differences of 16 and 32 between the metabolites and siduron indicated the presence of 1 or 2 additional atoms of oxygen in the metabolites. The spectra of both Metabolite 2 and siduron showed a strong peak at mass 93 which corresponded with aniline. Both also showed fragments at mass 119 due to phenyl isocyanate and mass 135 due to the phenylureido fragment. The oxygen atom of this metabolite would, therefore, necessarily be located in the cyclohexyl portion of the molecule.

Metabolite 3 has the same molecular weight as Metabolite 2, but the former had a strong fragment peak with a mass of 109, or 1 oxygen atom greater than aniline, thus indicating aminophenol. The mass spectra of 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea and 1-(*o*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea are identical, and both are the same as the spectrum of Metabolite 3. However, only the *p*-hydroxy analog of siduron and Metabolite 3 had a similar vaporization temperature of 115° to 120° C., in contrast to 85° C. for the *o*-hydroxy analog. The spectrum of 1-hydroxy-1-phenyl-3-(2-methylcyclohexyl)urea showed a considerably different fragmentation pattern from that of Metabolite 3. These data indicated that the oxygen in Metabolite 3 is in the aromatic ring, probably in the para-position to the nitrogen.

Metabolite 1, with 2 oxygen atoms and with the major fragment peak of 109 (aminophenol), appears to be a common off-spring of Metabolites 2 and 3; that is, a metabolite of siduron with an oxygen atom in both the aromatic and cyclohexyl portions of the molecule. Fragment ions were obtained for *p*-hydroxy phenylisocyanate (mass 135), 4-hydroxy-2-methylcyclohexylisocyanate (mass 155), and 4-hydroxy-2-methylcyclohexylamine (mass 129), which support the structure proposed for Metabolite 1.

Nuclear Magnetic Resonance (NMR) Spectrometric Analysis. Sufficient quantities of Metabolites 2 and 3, in crystalline form, were obtained from the dog urine to permit their characterization by NMR. Deuterated dimethylsulfoxide was the solvent of choice, since it did not interfere in any way with the critical spectral bands of the metabolites. The spectra of siduron and its metabolites are shown in Figure 3. The spectrum of siduron depicts the characteristic hydrogen peaks of an aromatic ring, of the amide-linkage proximate to the aromatic ring, of the cyclohexyl structure, and of the methyl substitution on the cyclohexyl ring. The similarity of spectra of 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)-urea and Metabolite 3 is evidence that these two materials are chemically identical. The spectrum of Metabolite 2 showed the following:

The methyl group attached to the cyclohexyl ring was intact, as indicated by the position and multiplicity of the absorption at about 9.15τ. This also shows that the C_2 position still bears a hydrogen.

Hydroxylation had definitely not occurred at the C_1 position of the cyclohexyl ring because of the presence of the doublet at 5.65τ.

Hydroxylation had probably not occurred at either the C_3 or C_6 position, since the methyl hydrogen absorption did not shift relative to that of siduron. The general shift of the absorption of the cyclohexyl methylene groups in the spectrum of Metabolite 2 is viewed as good evidence for the positioning of the hydroxyl group at either the C_4 or C_5 position of the cyclohexyl ring. In view of this shift and its extent, together with the lack of changes in the rest of the spectrum, relative to that of siduron, the hydroxyl group is assigned to the 4-position of the cyclohexyl ring.

Hydroxylation had not occurred at the nitrogen proximate to the cyclohexyl ring as indicated by the presence of the doublet at 3.90τ typical of the amide-linkage attached to a saturated cyclohexyl ring.

Thus, Metabolite 2 is 1-(4-hydroxy-2-methylcyclohexyl)-3-phenylurea. The work of others (Elliott *et al.*, 1965; Fieser and Schirmer, 1967; Fonken *et al.*, 1967) on the biological oxidation of substituted cyclohexane lend support to the above conclusion that hydroxylation occurs at the 4-position of the cyclohexyl ring.

Based on the assumption that Metabolite 1 is a metabolic off-spring of Metabolites 2 and 3, then Metabolite 1 is 1-(4-hydroxy-2-methylcyclohexyl)-3-(*p*-hydroxyphenyl)urea.

Infrared spectra of 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea and Metabolite 3 (Figure 4) further indicate that they are chemically identical.

Thin-Layer Chromatographic Data. The three siduron metabolites, the respective control extracts, siduron and 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea were spotted on TLC Kieselgel plates (250 microns thick) and developed in three different solvent mixtures to determine their respective R_f values. The solvent mixtures used included a 9-to-1 mixture of chloroform and methanol, a 7-to-8 mixture of chloroform and ethyl acetate, and a 25-to-12 mixture of petroleum ether and 2% NH_4OH in acetone. The results are tabulated in Table I. Here again, the R_f values obtained

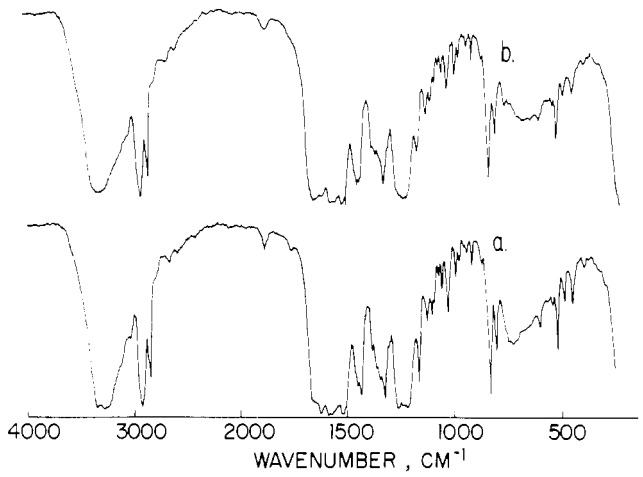

Figure 4. IR spectra of (*a*) 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea and (*b*) metabolite 3

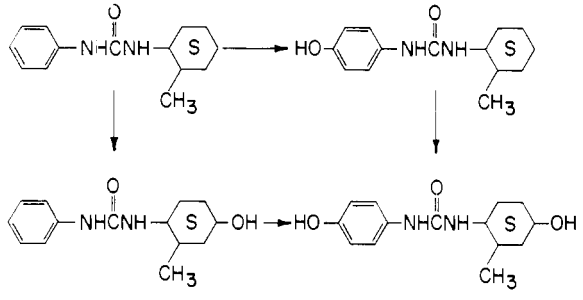

Figure 5. A proposed metabolic pathway for siduron

were further proof that Metabolite 3 and 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea are identical.

Microelemental Analysis. The results of microelemental analysis showed excellent agreement between the calculated and analytical values for both Metabolites 2 and 3.

Anal. Calcd for $C_{14}H_{20}N_2O_2$: C, 67.8; H, 8.1; N, 11.3. Found for Metabolite 2: C, 66.9; H, 8.0; N, 11.1. Found for Metabolite 3: C, 67.5, H, 8.0, N, 11.3.

The crystal structures of Metabolite 3 and 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea are identical. The melting point of Metabolite 3 is 221° C., as opposed to 223° to 224° C. for the synthetic compound.

The foregoing results point to a possible metabolic pathway of siduron in animals. It appears that siduron is hydroxylated to form either 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea or 1-(4-hydroxy-2-methylcyclohexyl)-3-phenylurea. Both these metabolites are further hydroxylated to form 1-(4-hydroxy-2-methylcyclohexyl)-3-(*p*-hydroxyphenyl)urea (Figure 5). All three metabolites apparently exist in the urine as highly water-soluble conjugates which can be eliminated at high concentrations.

Analysis for Aniline- and *p*-Aminophenol-Yielding Compounds. The urine from rats and dogs, both control and those on a diet with 2500 p.p.m. siduron, were analyzed for their total content of siduron metabolites. The results of these analyses (Table II) showed that urine of the dog fed siduron contained more of the aniline-yielding metabolite than the rat's urine (305 *vs.* 239 p.p.m. siduron equivalents), but considerably less of the *p*-aminophenol-yielding residues (467 *vs.* 757 p.p.m. siduron equivalents). The total metabolite level in the urine from both species on a diet with 2500 p.p.m. siduron approximated 800–1000 p.p.m. siduron equivalents.

Table I. R_f Values (×100) of Siduron and Its Metabolites on Kieselgel D-O

Developing Solvent Mixture	Ratio	Siduron	Metabolites 1	2	3	1-(*p*-Hydroxyphenyl)-3-(2-methylcyclohexyl)-urea
Chloroform/methanol	9/1	63	13	27	38	38
Chloroform/ethyl acetate	7/8	59	3	6	28	28
Pet. ether/2% NH_4OH in acetone	25/12	59	0	10	18	18

Table II. Analysis of Urine from Animals Fed Siduron[a]

Urine from	Siduron Equivalents of *p*-Aminophenol-yielding metabolites, p.p.m.	Aniline-yielding metabolites, p.p.m.
Control dog	<0.4	<0.1
Siduron-fed dog	467	305
Control rats	<0.4	<0.1
Siduron-fed rats	757	239

[a] 2500 p.p.m. in diet for 14 months.

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