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AN UNUSUAL METABOLITE OF BENZYL *N*-BENZYL CARBETHOXYHYDROXAMATE (W-398)*

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Abstract—Increased urinary Porter–Silber values were found after administration of benzyl *N*-benzyl carbethoxyhydroxamate (W-398) to man. Investigation of this phenomenon showed that it was not due to steroidal compounds or to the known metabolites of W-398, hippuric and benzoic acids, but to an additional metabolite, α-(benzyloxyamino)benzyl glucuronide. The structure of this excretory product was established by the isolation and identification of its β-glucuronidase hydrolytic product, *O*-benzyl benzaldoxime. *O*-benzyl benzaldoxime accounted for about one-third of the excreted radioactivity in urine from patients receiving *N*-benzyl-α-^{14}C-labeled W-398.

BENZYL *N*-benzyl carbethoxyhydroxamate (W-398), an agent affecting lipid metabolism,[1–4] was shown to be metabolized by the rat to benzoic and hippuric acids exclusively.[5] In the rabbit the drug is converted to benzoic and hippuric acids and to several other metabolites.[5] During the clinical evaluation of W-398, it was found[6] that in the urine of patients receiving this compound there was a multifold increase in the values obtained by the Porter–Silber assay for 17-hydroxysteroids. A detailed analysis of corticosteroid metabolites in the urine of guinea pigs treated with W-398 indicated that the change in Porter–Silber values was not due to a greater excretion of corticosteroids.[7] Our investigations into the nature of this increase, which are described in this manuscript, have shown that this increase is due to a previously undescribed metabolite of W-398.

EXPERIMENTAL

Pooled human urine, obtained from subjects given W-398, 1 g *t.i.d.* orally, was brought to pH 6·4 with 85% phosphoric acid and extracted continuously with chloroform for 18 hr. The residual urine was either adjusted to pH 6·4 with phosphate buffer salts, treated with β-glucuronidase and incubated overnight at 37°, or brought to pH 1 with concentrated hydrochloric acid and allowed to remain overnight at room temperature. The urine was then continuously extracted with chloroform for 18 hr and the organic phase was washed once each with 1·0 M potassium hydroxide, 1·0 M hydrochloric acid, and water. The chloroform was evaporated under reduced pressure and the residue dissolved in benzene. The benzene solution was washed with alkali until the washings were colorless and then was washed once with acid and once with water. Removal of the benzene under reduced pressure left an oily residue which was dissolved in ethanol and chromatographed on a thick-layer glass plate coated with 2 mm

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silica gel G, with chloroform as the developing solvent. The major component, which had an R_f value of $\cong$ 0·8, was isolated by elution from the absorbent with water-saturated chloroform, and the solvent was removed under reduced pressure leaving a pale yellow oil.

Urine from one of the subjects receiving W-398 was extracted as described above. The ethanol-soluble material was treated with 2,4-dinitrophenylhydrazine in acid. The dinitrophenylhydrazone which formed was isolated and was found to have a melting point of 238–239°.

O-benzyl benzaldoxime was synthesized by refluxing 2·46 g (20 mM) benzyloxyamine and 2·12 g (20 mM) benzaldehyde in 50 ml toluene for 3 hr.[8] After removal of the toluene, the compound was obtained analytically pure by distillation at 153° (3 mm).

Anal. Calcd. for $C_{14}H_{13}NO$: C, 79·58; H, 6·20; N, 6·64. Found: C, 79·65; H, 6·03; N, 6·66.

Treatment of the O-benzyl benzaldoxime with 2,4-dinitrophenylhydrazine gave benzaldehyde dinitrophenylhydrazone (melting point 239–241°). Reactions of this type have been previously reported.[9]

The Porter–Silber assay[10] was performed on 1·0 ml urine adjusted to pH 6·4 with 0·1 N HCl, mixed with 4 vol. of pH 6·4 phosphate buffer containing 100 Sigma units of β-glucuronidase solution, and incubated for 18 hr at 37°. The mixture was shaken with 25 ml chloroform for 5 min and centrifuged; the chloroform extract was separated and washed with 2·0 ml of 0·1 N NaOH. To one 10-ml aliquot, 1·0 ml sulfuric acid phenylhydrazine reagent was added; another 10-ml aliquot received blank reagent, containing no phenylhydrazine. The samples were shaken vigorously and centrifuged. The chloroform layer was removed and the reagent layer heated at 60° for 30 min. Optical density of the resulting solution was measured at 410 mμ with the blank reagent utilized as the reference solution.

W-398, labeled in the alpha position of either the *O*-benzyl or the *N*-benzyl group with carbon-14, was synthesized as previously described.[5]

Paper chromatography was carried out with the descending technique, at room temperature, on Whatman No. 1 paper. The compositions of the solvents employed are given in Table 1. Radioactivity on the chromatograms was measured with a Vanguard model 880 Autoscanner equipped with an integrating recorder. *O*-benzyl benzaldoxime was visible as a quenched spot under u.v. illumination.

TABLE 1. R_f VALUES OF *O*-BENZYL BENZALDOXIME AND ISOLATE FROM ACID-TREATED URINE

Developing solvent	R_f value	
	O-Benzyl benzaldoxime	Isolate
n-Butanol–pyridine–sat. NaCl (1:1:2)	0·92	0·92
n-Butanol–1·5 M ammonia (1:1)	0·92	0·91
n-Propanol–ammonia–water (85:1:14)	0·85	0·85

^{14}C-labeled W-398, 150 mg/kg, in 1 ml propylene glycol was administered intraperitoneally to male guinea pigs, and their urine was collected under toluene for 24 hr. An aliquot of the urine was extracted by the corticosteroid assay technique described above, except that the phenylhydrazine was omitted and the various fractions were assayed for radioactivity with a liquid scintillation counter. Urine from patients receiving 50 μc of *O*-benzyl-α-^{14}C-labeled W-398 by capsule, collected over a period of 24 hr, was processed as described for the guinea pig. Pooled urine, 100 ml, from four patients given 1 g *O*-benzyl-α-^{14}C-labeled W-398, 50 μc, was adjusted to pH 1 with hydrochloric acid and extracted continuously with ether for 48 hr. The ether was removed by distillation and the residue was dissolved in 20 ml tetrahydrofuran. The hydrolyzed metabolites were quantitated by the paper chromatographic technique in the propanol–ammonia–water developing system.

RESULTS AND DISCUSSION

After oral administration of 3 g W-398 to man, there occurred an increase in the urinary Porter–Silber values customarily reported for 17-hydroxysteroids (Table 2). Initial investigations into this rise in clinical assay value indicated that the agent responsible was a glucuronide, since the O.D. of the solution containing the chromophore formed by reaction with phenylhydrazine increased from 0·020 to 0·260 after

TABLE 2. EFFECT OF ORAL ADMINISTRATION OF W-398 (1 G T.I.D.) ON URINARY PORTER–SILBER VALUES IN MAN*[6]

Patient No.	Porter–Silber values* (mg/24 hr)†	
	Predrug	Postdrug
1	4·8	30·9
2	9·1	46·1
3	7·8	45·5
4	6·8	30·2
5	6·8	61·2
6	2·3	40·2
Average	6·2	42·4

* Each value represents an average of 3 determinations at 2-day intervals for predrug values and 6 determinations at 2-day intervals for postdrug values.
† Calculated with hydrocortisone as reference chromophore.

β-glucuronidase treatment. Evidence was also obtained to indicate that the factor responsible for the increased values was not a corticosteroid, since the partition coefficient of the hydrolyzed urinary end-product in benzene–water (>10) was radically different from that of hydrocortisone (0·25). After splitting of the glucuronide moiety by either enzymatic or acid hydrolysis, the Porter–Silber reactive compound was isolated by solvent extraction and purified by thick-layer chromatography. It was identified as *O*-benzyl benzaldoxime.

Evidence for the identification of the isolated metabolite was obtained by comparing it to *O*-benzyl benzaldoxime as follows: 1) the urine isolate had the same infrared spectrum as *O*-benzyl benzaldoxime (Fig. 1); 2) both compounds gave benzalde-

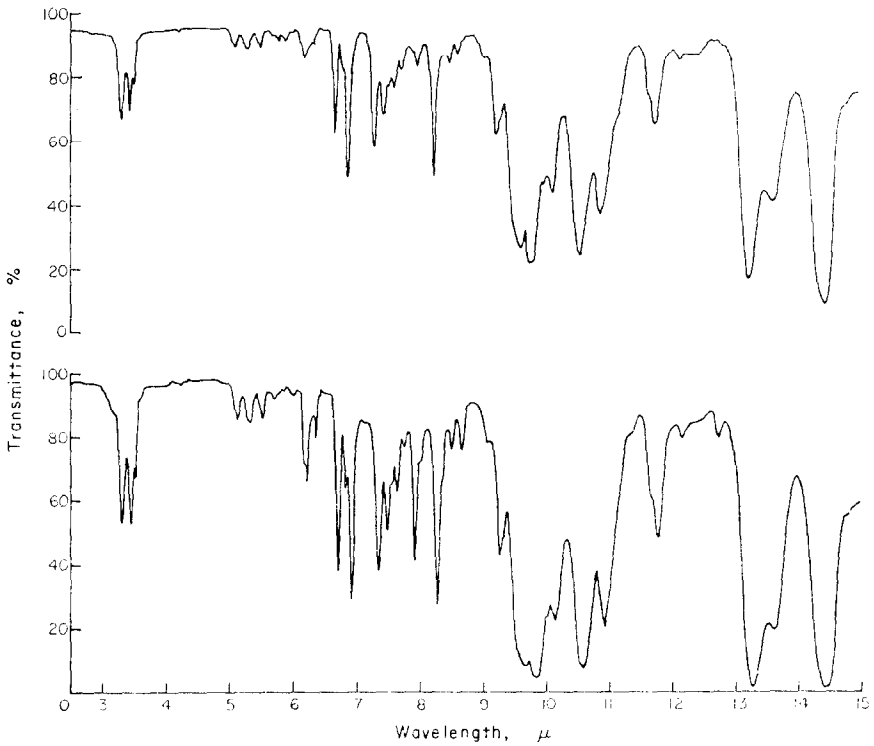

FIG. 1. Infrared absorption spectra of *O*-benzyl benzaldoxime (upper) and urine isolate (lower).

hyde 2,4-dinitrophenylhydrazone on the basis of their identical melting points and infrared spectra (Fig. 2); 3) the authentic synthetic compound and the urine isolate gave identical R_f values when subjected to paper chromatographic analysis in three different solvent systems (Table 1). In addition, *O*-benzyl benzaldoxime reacted readily in the 17-hydroxysteroid assay, yielding a product with a strong absorption at the characteristic Porter–Silber wavelength of 410 mμ (Fig. 3).

The formation of *O*-benzyl benzaldoxime on removal of the glucuronide moiety can arise from a labile hydroxyl group which spontaneously dehydrates to form a double bond (—CHOH—NH— → —CH=N— + H_2O). The compound must be stable toward dehydration prior to glucuronide conjugation however, or substantial amounts of *O*-benzyl benzaldoxime would be excreted in the urine. This stabilization may be provided by the *N*-carbethoxy moiety of W-398. We propose that the urinary excretion product which gives the typical Porter–Silber color response is α-(benzyloxyamino)benzyl glucuronide. Its formation from W-398 and subsequent conversion to *O*-benzyl benzaldoxime and benzaldehyde phenylhydrazone are represented in Fig. 4.

The sequence of reactions presented in Fig. 4 indicates that, of the two benzyl groups in W-398, only the *N*-benzyl (depicted by the asterisk) leads to a phenylhydrazone in the Porter–Silber reaction. This view is supported by two findings. None of the radioactivity from the urine of patients receiving *O*-benzyl-α-^{14}C-labeled W-398 is extracted into the Porter–Silber color reagent (Table 3) after hydrolysis, although there

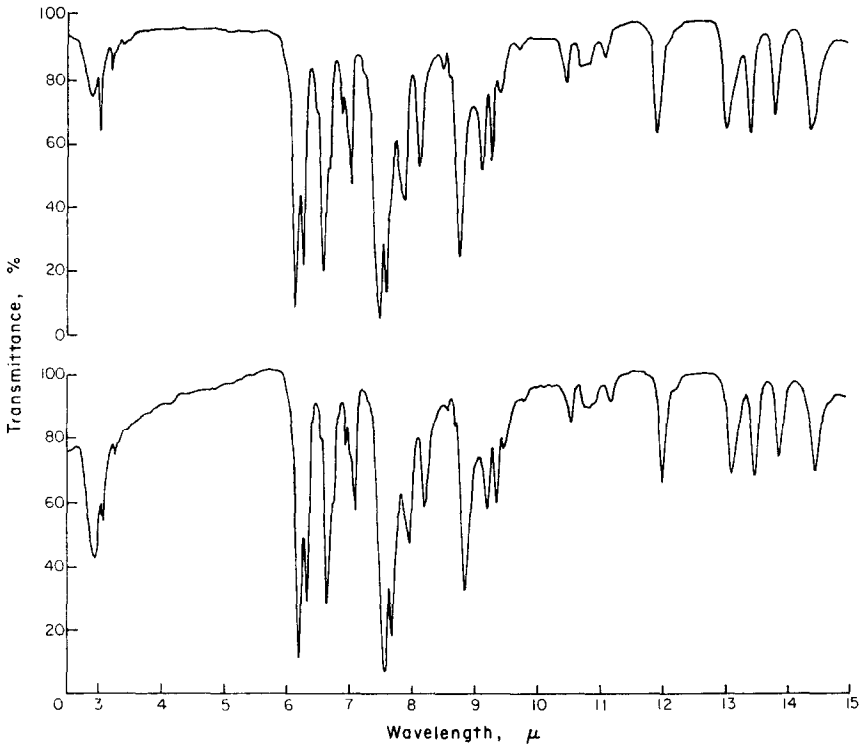

FIG. 2. Infrared absorption spectra of the 2,4-dinitro-phenylhydrazones from reaction with *O*-benzyl benzaldoxime (upper) and urine isolate (lower).

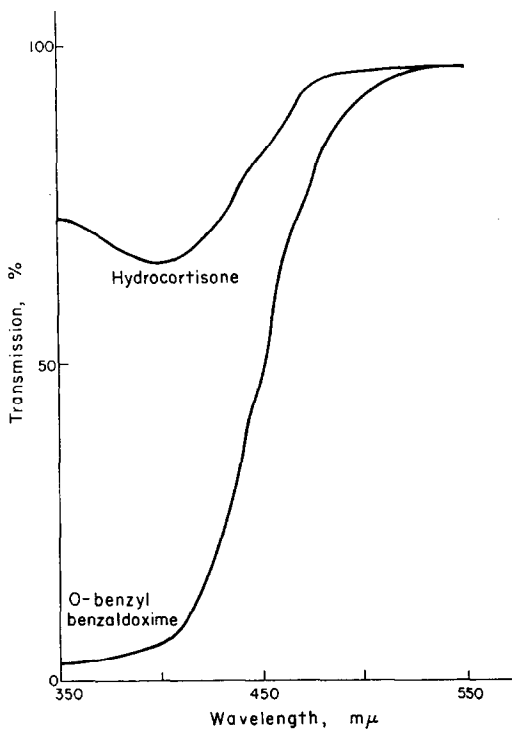

FIG. 3. Spectrum of the product formed when *O*-benzyl benzaldoxime or hydrocortisone is subjected to the Porter–Silber assay procedure.

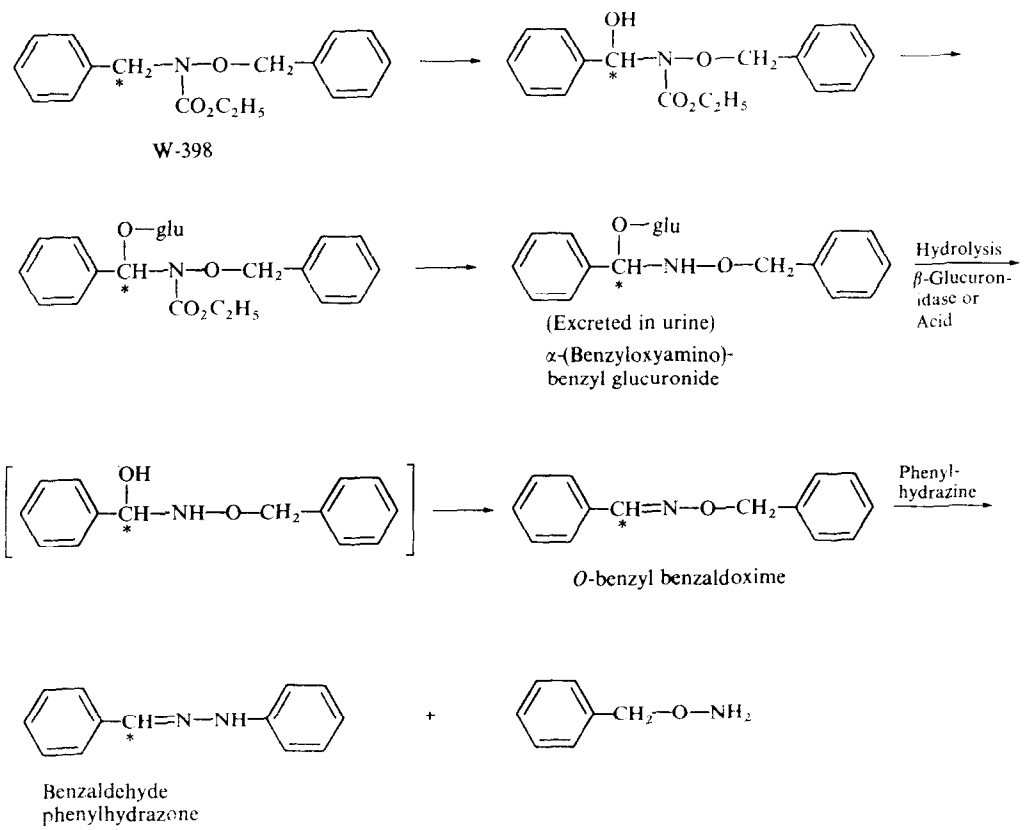

FIG. 4. Proposed metabolic pathway of W-398 and reaction of the urinary end-product in the Porter–Silber assay procedure; glu is glucuronic acid.

TABLE 3. DISTRIBUTION OF RADIOACTIVITY IN CORTICOSTEROID ASSAY FRACTIONS AFTER TREATMENT OF URINE FROM PATIENTS RECEIVING O-BENZYL-α-^{14}C-LABELED W-398, ORALLY

Fraction	dpm Recovered/ml urine	
	Sample 1	Sample 2
Extracted urine	17,100	15,750
Sodium hydroxide extract	3060	330
Residual chloroform	515	1800
Color reagent	0	0

is a considerable quantity of *O*-benzyl benzaldoxime generated. Furthermore, when *O*-benzyl- or *N*-benzyl-α-^{14}C-labeled W-398 is administered to guinea pigs and the urinary radioactivity in the various fractions of the corticosteroid assay is measured, only the *N*-benzyl-labeled moiety is converted to a labeled phenylhydrazone (Table 4).

TABLE 4. DISTRIBUTION OF RADIOACTIVITY IN CORTICOSTEROID ASSAY FRACTIONS AFTER TREATMENT OF URINE FROM GUINEA PIGS RECEIVING ^{14}C-LABELED W-398 INTRAPERITONEALLY

Fraction	dpm Recovered/ml urine	
	O-benzyl-α-^{14}C-labeled W-398	*N*-benzyl-α-^{14}C-labeled W-398
Extracted urine	30,250	13,600
Residual chloroform	4820	11,000
Sodium hydroxide extract	4900	2100
Color reagent	<10	1600

Hippuric and benzoic acids, the known metabolites, are not extracted into the Porter–Silber solvent.

The quantitation of urinary metabolites in man receiving *O*-benzyl-α-^{14}C-labeled W-398 was studied by using paper chromatographic techniques. After acid hydrolysis, it was found that approximately two-thirds of the urinary radioactivity was derived from benzoic acid, one-third from *O*-benzyl benzaldoxime, and a small amount from hippuric acid.

Other animal species were investigated to ascertain their ability to metabolize W-398 to *O*-benzyl benzaldoxime. Of the six species studied—man, rat, monkey, cat, guinea pig, and rabbit—only the rat was unable to metabolize W-398 to produce a substantial increase in urinary Porter–Silber values.

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REFERENCES

1. F. M. BERGER, J. F. DOUGLAS, B. J. LUDWIG and S. MARGOLIN, *Proc. Soc. exp. Biol. Med.* **114** 337 (1963).
2. J. F. DOUGLAS, *Proc. Soc. exp. Biol. Med.* **117**, 190 (1964).
3. J. F. DOUGLAS, B. J. LUDWIG, S. MARGOLIN and F. M. BERGER, *J. Atheroscler. Res.* **6**, 90 (1966).
4. J. F. DOUGLAS, B. J. LUDWIG, S. MARGOLIN and F. M. BERGER, *Prog. Biochem. Pharmac.* **2**, 422 (1967).
5. J. F. DOUGLAS, *J. Pharmac. exp. Ther.* **150**, 105 (1965).
6. C. MOSES, personal communication.
7. S. BURSTEIN, personal communication.
8. O. EXNER, *Chem. Listy* **48**, 1543 (1954), in *Chem. Abstr.* **49**, 11603 (1955).
9. G. MINUNNI and G. CORSELLI, *Gazz. chim. ital.* **22** (2), 149 (1892).
10. R. H. SILBER and C. C. PORTER, *J. biol. Chem.* **210**, 923 (1954).