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Identification of the mononitro derivative of dapsone as a product from an oxidation *in vitro*

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ALTHOUGH the metabolism of the antileprotic drug dapsone, 4,4′-bis (aminophenyl)sulfone, has been studied extensively *in vivo*,[1-3] there appear to have been relatively few studies of its metabolism *in vitro*. However, the oxidation *in vitro* of one of the amino nitrogens of dapsone to form mono-*N*-hydroxydapsone has been reported recently.[4,5] We shall present evidence in this paper to show that dapsone is oxidized to its mononitro derivative in the presence of rat liver microsomal enzyme preparations.

The method of microsome preparation was essentially that of Fouts.[6] Liver microsomes from rats were used unless otherwise indicated. All operations in preparation of microsomes were carried out at 0°. Dapsone, uniformly labeled with ^{14}C, was incubated at a concentration of 5×10^{-4} M with 30 ml of 0·05 M phosphate buffer (pH 7·4) and 10 ml of the microsomal preparation (equivalent to 8 g of the original liver) in a total volume of 50 ml. Mg^{2+} was present at a final concentration of $1{\cdot}8 \times 10^{-4}$ M, nicotinamide at $1{\cdot}2 \times 10^{-4}$ M, NADP at 5×10^{-4} M and glucose 6-phosphate at 5×10^{-4} M. The NADPH generating system was completed with 36 i.u. of glucose 6-phosphate dehydrogenase. Incubations were carried out in 125 ml Erlenmeyer flasks within 6 hr after sacrifice. The flasks were held at 37° for 60 min and swirled at a rate of 100 rev/min.

After incubation, proteins were precipitated with 5 ml of 10% $ZnSO_4$. The pH was adjusted to 7·25 and the mixture centrifuged at 25,000 *g* for 10 min. The aqueous supernatant was extracted with ethyl acetate (3×20 ml). The organic phase contained about 65 per cent of the total radioactivity and was evaporated to dryness at room temperature in a stream of N_2. The residue was dissolved in 0·1 ml methanol and then taken to a final volume of 8 ml with 0·05 N HCl. This solution was extracted with methylene chloride (3×2 ml). Most of the parent compound remained in the aqueous phase, while 35 per cent of the radioactivity went into the organic phase. Thin-layer chromatography of an aliquot of this extract on Silica gel (benzene–ethanol 80:20), revealed two zones of radioactivity, one

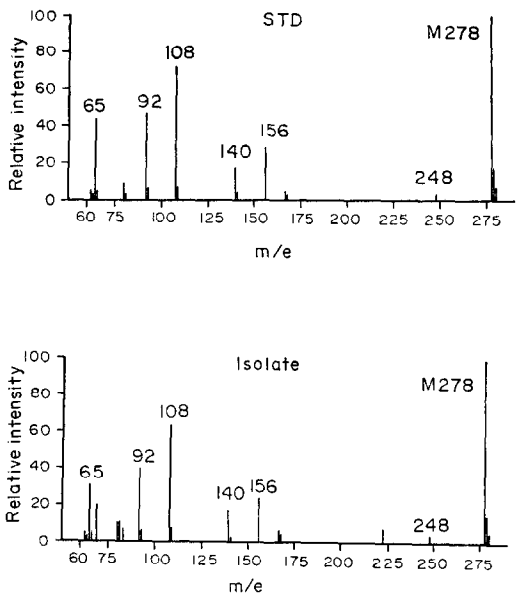

FIG. 1. Mass spectra of 4-amino-4′-nitrodiphenylsulfone (STD) and an isolate from the incubation of dapsone with rat liver microsomes. Instrument conditions: source temperature, 270°; electron energy, 70 eV; accelerating potential, 3·5 kV; trap current, 60 μA.

with an R_f of 0·40, identical to that of dapsone, and the other with an R_f of 0·55. The latter zone contained about 12 per cent of the starting activity. This material was completely separated from dapsone by column chromatography on Silica gel (isooctane–isopropanol, 75:25). Further purification was achieved using high pressure chromatography on Silica gel (isooctane–isopropanol, 90:10). Three peaks were observed by ultraviolet monitoring at 254 nm. The radioactivity was associated with the largest peak, which was recovered and analyzed by mass, ultraviolet and infrared spectrometry.

A total of 135 μg (determined by radioactivity) of the compound was isolated from the microsomal incubation, which amounted to a final yield of 3·3 per cent. Direct probe mass spectrometry (LKB model 9000) was carried out on 2 μg of the sample. The spectrum (Fig. 1, lower panel) was characterized by an intense molecular ion with an m/e of 278, which was 30 units greater than the parent compound. Trimethylsilylation of the product resulted in a shift in molecular ion from m/e 278 to 350, a change of 72 mass units, indicating the presence of one derivatizable functional group. The spectrum of an authentic sample of 4-amino-4′-nitrodiphenylsulfone was virtually identical (Fig. 1, upper panel) to that of the isolated substance.

The product had an ultraviolet spectrum considerably altered from that of the dapsone, characterized by a shoulder at 330 nm and a maximum at 266 nm (dapsone with maxima at 297 and 261 nm). The ultraviolet spectra of the isolate and an authentic sample of 4-amino-4′-nitrodiphenylsulfone were the same, with the exception of a slight difference in the $A^{1\%}_{1cm}$, which was probably the result of small quantities of impurities in the isolate. The infrared spectra of the reaction product and the mononitro derivatives of dapsone were nearly identical. Thin-layer chromatography using neutral, acidic and basic systems gave indistinguishable R_f values for the isolate and the model compound (Table 1).

Although identification of the 4-amino-4′-nitrodiphenylsulfone isolated from microsomal incubations of dapsone was unequivocal, the possibility existed that an impurity present in the starting material had been isolated. The latter was thus carefully examined by mass spectrometry and thin-layer chromatography. A maximum of 0·5 per cent of the mononitro compound could have been present in the dapsone, as calculated from radioactivity measurements on thin-layer chromatograms. There was no evidence of any nitro contaminant in the mass spectrum of the starting material. Studies utilizing thin-layer chromatographic analysis show that heating the microsomal preparation at 80° for 5 min resulted in an 85 per cent reduction in the formation of product. Furthermore, formation of the product was time dependent, offering additional evidence that this compound results from an enzymatic degradation of dapsone.

TABLE 1. THIN-LAYER CHROMATOGRAPHY OF 4-AMINO-4′-NITRODIPHENYL SULFONE AND AN ISOLATE FROM THE INCUBATION OF DAPSONE WITH RAT LIVER MICROSOMES*

System	4-Amino-4′-nitrodiphenyl sulfone (R_f)	Isolate (R_f)
Benzene–ethanol (80:20)	0·54	0·56
Ethyl acetate–acetic acid (95:5)	0·72	0·72
Benzene–dioxane–NH_4OH (10:80:10)	0·67	0·67

* Silica gel G plates (Analtech).

We have also obtained evidence by reverse isotope dilution analysis for the formation of the nitro product by microsomal preparation from chicken liver. This mode of oxidation is not confined to dapsone. Upon incubation with microsomes from either rat or chicken liver, a dapsone analog, 4-amino-4′-ureidodiphenyl sulfone (amidapsone), has also yielded the analogous product, 4-nitro-4′-ureidodiphenyl sulfone. Further, there is evidence that both amino groups of dapsone may be oxidized in these systems. The base peak in the mass spectrum of 4,4′-bis(nitrophenyl)sulfone is found at m/e 170. A crude isolate from an incubation of radiolabeled dapsone with rat liver microsomal enzymes and separated by thin-layer chromatography produced a mass spectrum which, although clearly resulting from a mixture of compounds, did show the m/e values expected for the authentic dinitro compound, including the characteristic base peak at m/e 170. Evidence from thin-layer chromatography coupled with radioactivity measurements also suggested the formation of the dinitro product.

The oxidation of dapsone[4,5] and other aromatic amines[7] to their hydroxylamines has previously been reported. It is possible that the conversion of dapsone to the nitro compound is the result of a nonenzymatic oxidation of its enzymatically formed hydroxylamine. Indeed, we have observed that the hydroxylamine degrades to several products, including the nitro compound (thin-layer chromatography, ultraviolet and mass spectral data) when incubated with or without microsomal protein. Jackson[8] has found that mono-*N*-hydroxydapsone is oxidized in aqueous organic solvents by atmospheric oxygen to yield 4,4′-bis(*p*-aminobenzenesulfonyl)azobenzene. Feller *et al.*,[9] have observed the auto-oxidation of an *N*-hydroxylamine to the corresponding nitro compound. Kuwai *et al.*[10] have reported the oxidation of several aromatic amines to their corresponding nitro compounds by *Streptomyces thioluteus*. The biological significance of this conversion remains to be demonstrated.

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