BIOSYNTHETIC PREPARATION OF DIETHYLSTILBESTROL MONOGLUCURONIDE - (GLUCURONYL-U- ^{14}C)

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SUMMARY

The production of diethylstilbestrol monoglucuronide - (glucuronyl-U-^{14}C) by incubation of uridine diphospho-D- glucuronic acid-U-^{14}C with diethylstilbestrol in the presence of guinea pig liver microsomes is described. The specific activity of the product was 9.1 mCi/mmole and the overall radiochemical yield was 13%, based on UDPGA - (glucuronyl-U-^{14}C).

Key Words: Diethylstilbestrol monoglucuronide - (glucuronyl-U-^{14}C), Biosynthesis, Metabolite, Microsomes.

INTRODUCTION

The synthetic estrogen diethylstilbestrol (DES) is metabolized in mammals to form the monoglucuronide as the primary product [1]. In order to conduct certain studies on the metabolic disposition of DES, it became necessary to prepare the monoglucuronide of DES with a carbon-14 label in the sugar moiety.

DES monoglucuronide has been prepared by isolation from the urine of rabbits treated with DES [2]. Although this *in vivo* method is the one of choice for the preparation of unlabeled material, or the monoglucuronide labeled in the aglycone portion, it is not applicable for placing a label

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in the sugar group. However, the in vitro incubation of DES with uridine diphospho-D-glucuronic acid-U-^{14}C in the presence of microsomes appeared to offer a tenable means to accomplish the desired synthesis since glucuronosyltransferase [E.C.2.4.1.17] activity present in microsome fractions had been previously employed in the preparation of monoglucuronides [3, 4]. Further, a recent modification describes the use of UDP glucuronosyltransferase immobilized on agarose gel for the preparation of glucuronides [5].

Initial studies were patterned after the method described by Mehendale and Dorough for the preparation of the glucuronide of 1-naphthol in 90% yield on a μg scale [4]. Thus, in preliminary runs utilizing rat liver microsomes, yields of 10-20% of DES monoglucuronide were realized as determined by TLC analyses. Variation of the pH or the addition of activators such as digitonin [6] and Triton X-100 [7] failed to increase the yield. However, the substitution of guinea pig liver microsomes for those from rat liver resulted in a 50% conversion to the monoglucuronide (based on UDPGA).

Subsequently, ten simultaneous incubations were carried out, using guinea pig liver microsomes, each containing 0.25mCi of UDPGA (glucuronyl-U-^{14}C). The combined average incorporation of carbon-14 into DES monoglucuronide was found to be 33% as determined by reverse isotope dilution [8]. The isolation of the radiolabeled product proved to be very tedious with only 39% of the product produced being isolated with a radiochemical purity of $\geq$99%. The overall radiochemical yield was 13%.

EXPERIMENTAL

Uridinediphospho- D-(U^{14}-C) glucuronic acid ammonium salt was obtained from Amersham/Searle Corporation, Arlington Heights, Illinois. Diethylstilbestrol was obtained from Sigma Chemical Company, St. Louis, Missouri. IR spectra were determined with a Beckman Acculab I, using KBr. UV spectra were recorded with a Cary 118 spectrophotometer. Radioactivity was determined on a Packard Model 3003 liquid scintillation counter using Hydromix (Yorktown Research) as the counting medium. Radiochemical purity was determined using a Packard Model 7201 radiochromatogram scanner.

Unlabeled Diethylstilbesteol Monoglucuronide

The unlabeled DES-monoglucuronide required for dilution purposes was prepared, employing the general procedure described by Dodgson et al. [2] Thus, by processing the urine collected for four days following the intubation of 1.0g of DES into a single 10 lb giant chinchilla rabbit, approximately 200 mg of the sodium salt of DES monoglucuronide was obtained. Final crystallization was from water yielding a white crystalline solid, TLC, R_f 0.20, silica gel/chloroform: methanol: acetic acid (8:2:1). The UV spectra was consistent with literature values [2], UV max (95% EtOH) 239 nm ($\log \varepsilon$ 4.19), inflection 275 to 280 nm ($\log \varepsilon$ 3.67).

Preparation of Microsomes

The livers of six freshly decapitated English Shorthair guinea pigs were processed in pairs as follows at temperatures < 8°C. Two livers were combined (combined weights were 27,27 and 29g for the three pairs) and blended with a fourfold volume of pH 7.0, (0.05M) tris buffer for 1 min. then homogenized in portions with a Potter Elvejehm tube. The

combined homogenates were centrifuged at 9,800g for 30 min. and the supernatent filtered through cheesecloth to remove fat particles. Subsequent centrifugation at 92,700g for 90 min. gave the microsomal pellet which was frozen overnight under a layer of tris buffer. Immediately prior to use in the incubations, the pellets were thawed, suspended with a twofold (V/W) amount of pH 7.0 (0.05M) tris buffer, then mixed to give a uniform representation of microsomal preparation from the six animal livers. A total of 166 ml of a microsomal suspension corresponding to 500 mg tissue equivalents per ml was obtained.

Microsomal Incubation Procedure

The aqueous solution (12.5 ml) of 5.1 mg of uridine diphospho-D-(U-^{14}C) glucuronic acid ammonium salt, specific activity 318 mCi/mmole, 2.5 mCi total activity, was combined with 55 mg of unlabeled uridine diphospho-D-glucuronic acid sodium salt and 2.0g of magnesium chloride, then diluted to 250 ml with pH 7.4 (0.05M) tris buffer. Ten 125 ml flasks were each charged with 15.0 ml of the microsomal suspension and 25.0 ml of the uridine diphospho-D-(U-^{14}C) glucuronic acid solution (0.25 mCi). Diethylstilbestrol, 2.7 mg in 0.10 ml of 95% ethanol, was then added to each flask and the resulting solutions incubated with shaking at 37°C for two hours.

Isolation Procedure for Diethylstilbestrol Monoglucuronide-(glucuronyl-U-^{14}C)

The ten incubations were grouped into two equal batches for initial extraction. The pH of each solution was adjusted to 3-4 with 5% hydrochloric acid, followed by the addition of granular sodium chloride until saturation was achieved. The aqueous mixtures were then extracted with 2 x 200 ml of ether. To each aqueous solution was added 11.1 mg of "cold" DES-monoglucuronide and they were again extracted with ether,

3 x 200 ml. The ethereal extracts were then combined, 200 ml of benzene was added, and the resulting solution dried (Na_2SO_4) and evaporated in vacuo yielding a dark oil which was partitioned between 30 ml of ether and 10 ml of saturated sodium bicarbonate solution. The resulting ether layer was washed with an additional 10 ml of water, and the aqueous layers combined, leaving the ethereal extract for additional processing. The aqueous layer was then acidified with 5% hydrochloric acid and subsequently extracted with 3 x 50 ml of ether. These ethereal extracts were combined, evaporated in vacuo and the residue triturated with 3 ml of saturated sodium bicarbonate and 5 ml of ether. The layers were separated and the precipitate present in the aqueous layer (which increased upon cooling at 6°C) was collected by centrifugation. The ether extract was combined with the similarly obtained ethereal layer described above and recycled through the partition sequence. In all, three precipitates were obtained which were combined and subsequently recrystallized from water to yield 15.1 mg (13% based on UDPGA) of diethylstilbestrol monoglucuronide (glucuronyl-U-^{14}C) sodium salt as a white crystalline solid; TLC, Rf 0.20, silica gel/chloroform: methanol: acetic acid (8:2:1). The specific activity was 9.1 mCi/mmole and the radiochemical purity was $\geq$ 99% as indicated by radiochromatography. The UV, IR spectra and TLC characteristics were identical to the unlabeled DES monoglucuronide isolated from rabbit urine.

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8. Such a result suggests a radiation induced partial inactivation of the glucuronosyltransferase present in the microsome preparation. Yields on "cold" runs ran consistently at about 50% incorporation under identical conditions.

9. Immediately before the incubations utilizing labeled UDPGA were initiated, a single "cold" run was made to verify enzyme activity. Estimation by TLC analysis indicated the expected degree of glucuron-osyltransferance activity, i.e. an approximately 50% production of the monoglucuronide.