Evidence for Glucuronide Conjugation of *p*-Nitrophenol in the Caco-2 Cell Model

Susan Bjorge,[1] Katherine L. Hamelehle,[1] Reynold Homan,[1] Steven E. Rose,[1,2] Daniel A. Turluck,[1] and D. Scott Wright[1]

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INTRODUCTION

Phase II conjugation accompanies the transit of many drugs across the intestinal epithelium. Phenolic drugs were shown to be conjugated by *in vitro* intestinal slices as early as 1952 (1). Several experimental models have been used to characterize intestinal conjugation of selected compounds. These include everted gut (2), perfused intestinal segments (2,3), and isolated intestinal epithelial cells (4). The usefulness of these experimental models is limited by poor viability or lack of direct information about epithelial transport processes (5).

Human adenocarcinoma of the colon (Caco-2) cells have proven to be useful as *in vitro* models of enterocyte function. Caco-2 cells, when grown to confluent, polar monolayers on semipermeable polycarbonate membranes, retain several properties of intact human enterocytes, including microvilli (6), tight junctions between cells (7), and epithelial-like electrical properties (8). Enterocytic biochemical markers (9) and transport carriers (10) are also present in Caco-2 cells. The use of Caco-2 cells in drug transport studies has recently been demonstrated (11,12), however, their usefulness as a model for gut wall drug metabolism has not been explored. Planar phenols are known to be susceptible to intestinal wall conjugation upon absorption. *p*-Nitrophenol, for example, has been shown to be conjugated in rat intestinal homogenates (13). Therefore, *p*-nitrophenol was selected to probe for the existence of UDPglucuronosyl transferase enzyme function in Caco-2 cells. This report describes the results obtained upon incubating *p*-nitrophenol with Caco-2 cells.

EXPERIMENTAL

Materials

Caco-2 cells, ATCC HTB 37, were grown from culture to a confluent monolayer on a microporous membrane which separates upper (apical) and lower (basolateral) chambers (Transwell, Costar Corp., Cambridge, MA). Nine wells containing Caco-2 cells (seeded) were used for the present study. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum at 5% CO_2 and 37°C and used on the eighteenth day after seeding. During studies, cells were kept in DMEM without bovine serum. [^{14}C]*p*-Nitrophenol (PNP), 11.7 mCi/mmol, was obtained from Sigma Chemical Company, St. Louis, MO. [^{14}C]Polyethyleneglycol (PEG) molecular weight 4000, 0.82 mCi/g, was purchased from E. I. du Pont de Nemours & Co., Inc., Wilmington, DE. β-Glucuronidase, sulfatase, and *p*-nitrophenyl β-D-glucuronide were obtained from Sigma. All solvents were HPLC grade. All other reagents were of analytical grade.

Methods

Integrity of Monolayer

Three Caco-2 seeded wells were randomly selected from the nine prepared for the current study to establish the integrity of the cell monolayer. A 50-μl aliquot of 10.7 μCi/ml [^{14}C]PEG aqueous solution was added to the apical chamber of each well. The same amount of [^{14}C]PEG solution was added to a control well containing no Caco-2 cells. Wells were agitated at 50 rpm on a rotating plate inclined at 5° and placed inside the incubator to maintain the conditions described above. Ten-microliter sample aliquots were taken from the apical and basolateral chambers at the time of, and at 30 and 240 min after, addition of [^{14}C]PEG. Samples were analyzed by liquid scintillation counting.

Metabolism of [^{14}C]PNP

Apical contents of the six remaining wells were replaced with 1.5 ml of a solution of 16 μCi/ml [^{14}C]PNP in DMEM. An identical volume of [^{14}C]PNP solution was added to a control well containing no cells. Wells were agitated as before. Ten-microliter sample aliquots were withdrawn from the basolateral chambers upon [^{14}C]PNP addition and 24 hr after [^{14}C]PNP addition. Samples were analyzed for ^{14}C components of [^{14}C]PNP using a radioactive flow detection (RFD) liquid chromatographic method. Samples were diluted with 100 μl of mobile phase (20% acetonitrile in 1% acetic acid). The entire diluted sample was injected onto a C-8 analytical column with an initial mobile-phase composition of 20:80 acetonitrile:acetic acid, followed by a linear gradient for 20 min to 80:20 acetonitrile:acetic acid. Mobile phase flow rate was maintained at 1 ml/min.

Two-hundred microliter samples of 24-hr basolateral chamber contents were incubated with either β-glucuronidase (18,000 units per 0.4 ml 0.1 *M* acetate buffer, pH 5.1) or sulfatase (2000 units per 0.4 ml 0.01 *M* Tris buffer, pH 7.5) at 37°C for 24 hr. In addition, 200-μl samples of the same basolateral contents were incubated with 0.4 ml of either acetate or TRIS buffer under identical conditions as controls. Aliquots of control and enzyme-treated samples were in-

[1] Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, Michigan 48105.
[2] To whom correspondence should be addressed.

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jected as above onto the RFD/UV system for analysis. Samples were also examined using a liquid chromatographic/photodiode array/mass spectroscopic (LC/PDA/MS) method. Chromatographic conditions for mass spectral analysis were modified as follows: 0.1 *M* ammonium acetate (pH 4.0) was substituted for 1% acetic acid and the mobile phase was held at 20:80 acetonitrile:buffer for 5 min, followed by a linear gradient from 5 to 25 min to 80:20 acetonitrile:buffer. All other conditions were as detailed above.

RESULTS AND DISCUSSION

Radioactive counts attributable to [^{14}C]PEG were evenly distributed between apical and basolateral sides in the control well, demonstrating unrestricted and bidirectional penetration of [^{14}C]PEG through the polycarbonate membrane. In contrast, radioactivity was restricted to the apical side in all Caco-2 seeded wells tested, indicating the impermeability of Caco-2 cells to the macromolecule and the integrity of the monolayers under investigation.

RFD analysis established the retention time of [^{14}C]PNP at approximately 12 min (Fig. 1). No additional radioactive peaks were observed in control samples in which [^{14}C]PNP was added to wells containing no Caco-2 cells. RFD analysis of basolateral samples from Caco-2 seeded wells revealed a radioactive component eluting at approximately 6 min (Fig. 1). This component disappeared upon incubation with β-glucuronidase but was unchanged after treatment with sulfatase. Furthermore, no reduction in peak height of this component occurred in control incubates containing no β-glucuronidase or sulfatase.

Under the conditions used in the LC/PDA/MS analysis, the presumed metabolite and unchanged PNP eluted at approximately 4 and 19 min, respectively. A hypsochromic shift from 320 to 295 nm in the LC/PDA metabolite spectrum was observed, indicating a metabolism-induced perturbation of the UV chromaphore. The thermospray negative ion mass spectrum of the presumed metabolite is given in Fig. 2. Observed in the spectrum is a molecular ion at *m/z* 314 and a molecular ion minus glucuronide (M-176) at *m/z* 138. These observations matched those obtained using authentic PNP-β-glucuronide. Under identical conditions, authentic compound eluted at approximately 4 min. No differences in photo diode array and thermospray spectra between authentic standard and sample were evident.

These results provide compelling evidence that the observed putative metabolite is a β-glucuronide conjugate of *p*-nitrophenol. Previous work by Borchardt (unpublished data) demonstrated phenol sulfotransferase activity in Caco-2 cells. Confirmation of UDPglucuronosyl transferase (EC 2.4.1.17) activity in the current qualitative study underscores the potential utility of Caco-2 monolayers in drug gut wall metabolism studies.

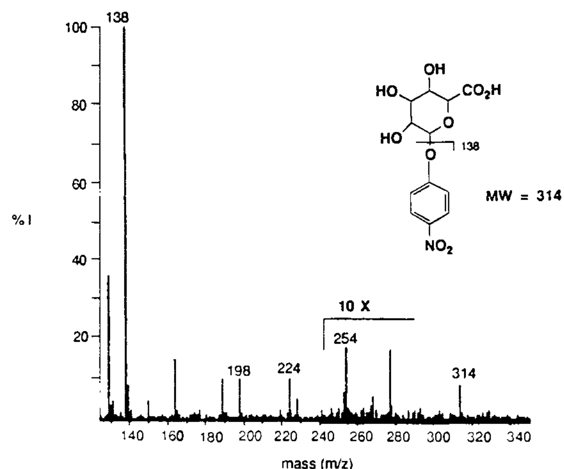

Fig. 2. Thermospray negative ion mass spectrum of PNP-glucuronide.

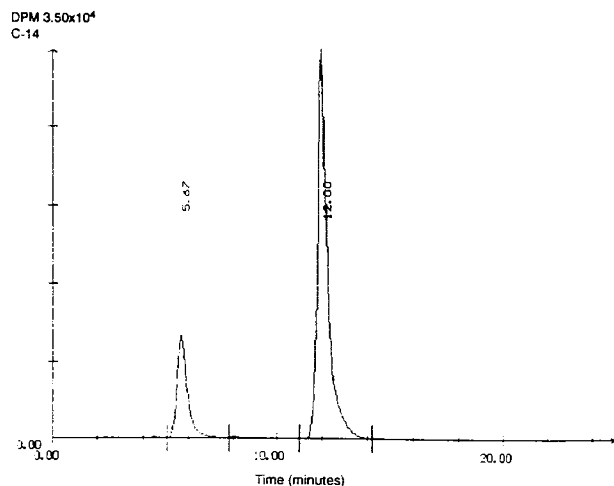

Fig. 1. RFD chromatogram of basolateral sample from a well seeded with Caco-2 cells 24 hr after replacement of apical medium with 1.5 ml of 16 μCi/ml [^{14}C]PNP in DMEM.

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