In Vitro Binding of Lorazepam and Lorazepam Glucuronide to Cholestyramine, Colestipol, and Activated Charcoal

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Received July 6, 1990; accepted November 8, 1990
KEY WORDS: lorazepam glucuronide; binding to cholestyramine, colestipol, and activated charcoal; enterohepatic recirculation.

INTRODUCTION

Lorazepam is a 3-hydroxy-1,4-benzodiazepine in wide clinical use as a sedative/hypnotic and antianxiety agent. In man, the drug is extensively conjugated by the liver to its 3-*O*-phenolic glucuronide and excreted in urine with only a small percentage recovered unchanged or as oxidized metabolites (1). However, glucuronides also appear in the bile and, as such, may participate in an enterohepatic circulation (2–4). Indeed, recent data from our laboratory suggest that chronic administration of cholestyramine along with neomycin increases the elimination of lorazepam in man by interruption of an enterocycling pathway (5). Cholestyramine, an anion-exchange resin, presumably binds the glucuronide in the gut, thereby facilitating its elimination, as has been shown for other cycling substrates (6–10). Neomycin is also highly cationic and may confer some binding, but more importantly it is thought to inhibit bacteria which produce the bulk of glucuronidases in the intestine (11). None of these mechanisms are proven, however. The present study was undertaken, therefore, to examine the binding of lorazepam and lorazepam glucuronide to cholestyramine to test the hypothesis that elimination is increased through clearance of a cycling glucuronide from the gut. Binding to related adsorbents such as colestipol and activated charcoal was also examined.

MATERIALS AND METHODS

Materials

All solvents were obtained from BDH Chemicals, Canada, Inc. Radiolabeled ^{14}C-lorazepam was synthesized by Amersham Ltd. (Arlington Heights, Il.), having a specific activity of 52 mCi/mmol and a purity of 98% by mass spectroscopy and thin-layer chromatography. Sodium phosphate buffer, 0.028 *M*, pH 7.4, was prepared in twice-distilled water and used to make cholestyramine (Questran, Bristol Laboratories of Canada, Belleville, Ont.) and colestipol (Colestid, Upjohn Company of Canada, Don Mills, Ont.) suspensions, each at a concentration of 6.67 mg/ml. Suspensions of activated charcoal (Laboratories Luvabec Inc., Montreal, Que.), 7 mg/ml, were also prepared using the same buffer.

Lorazepam glucuronide was isolated and purified from the urine of a human subject given 50 μCi of ^{14}C-lorazepam iv together with 4 mg of nonlabeled lorazepam by mouth. Urine was collected over 3 days, freeze-dried, and extracted with methanol. This was then applied to a 20 × 1 in silica gel column and eluted using differing mixtures of chloroform and ethanol at ratios, by volume, of 80:20 to 20:80. Fractions containing radioactivity were evaporated to dryness, reconstituted in methanol, and purified using semipreparative HPLC. Here, the solvent phase consisted of 40% acetonitrile in 0.1% sodium phosphate buffer, pH 3.0, and the column was a 25 × 0.10 cm C-18 μBondapak (Waters Associates, Milford, Mass.). Detection was by UV spectrophotometry at a wavelength of 230 nm, the absorption maximum for lorazepam. Effluent was collected from the column during elution of peaks, and those containing radioactivity were pooled and dried under nitrogen. The resulting precipitate was redissolved in distilled water and desalted using SEP-PAK C-18 cartridges (Waters Associates). A small volume was incubated with β-glucuronidase/sulfatase (from *Helix pomatia,* Sigma Chemical Co., St. Louis, Mo.), and the extract was subjected to HPLC analysis as described for the identification of lorazepam (5). This treatment resulted in a single peak corresponding to pure lorazepam standard, whereas a second sample not hydrolyzed with β-glucuronidase did not show a lorazepam peak, but retained all its radioactivity in the aqueous, nonextracted phase.

Methods

The binding of radiolabeled lorazepam and lorazepam glucuronide to the differing adsorbents was determined by equilibrium dialysis using Teflon microcells fitted with a semipermeable membrane; molecular weight cutoff, 12,000–14,000 daltons (Spectrum Medical Industries, Inc., Los Angeles, Calif.). One milliliter of cholestyramine, colestipol, or activated charcoal suspension was incubated together with varying concentrations of lorazepam (10–200 n*M*) or lorazepam glucuronide (1–10 μ*M*, lorazepam equivalents) against an equal volume of phosphate buffer, pH 7.4, for 4 hr at 37°C. Radioactivity was determined on 0.1 ml aliquots of buffer to which 10 ml of scintillation cocktail (Ready Micro, Beckman Instruments Inc., Mississauga, Ont.) had been added, and the resulting mixture counted using the external standardization procedure to correct for quenching. Extent of binding was calculated as

$$\% \text{ bound} = \frac{\text{total dpm added} - \text{free dpm recovered in the buffer}}{\text{total dpm added}} \times 100$$

Adsorption isotherms for the interaction of lorazepam and lorazepam glucuronide with the various binding agents were examined using the Langmuir equation:

$$\frac{c}{x/m} = \frac{1}{a\,b} + \frac{c}{b}$$

where c represents the free equilibrium concentration of

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0724-8741/91/0400-0538$06.50/0

lorazepam glucuronide (or lorazepam); x, the mass of glucuronide per gram of adsorbent, m; b, the adsorption capacity of the binder; and a, its adsorption coefficient. Thus, a plot of $c/x/m$ versus c should be linear, with the adsorptive capacity of the solid, b, equal to the reciprocal of the slope and the adsorption coefficient, a, equal to the slope divided by the intercept.

RESULTS AND DISCUSSION

Time Course for Equilibrium of Lorazepam and Lorazepam Glucuronide Binding to Cholestyramine, Colestipol, and Activated Charcoal

The equilibrium profiles of lorazepam glucuronide binding to cholestyramine and colestipol are shown in Fig. 1. The amount bound by the two resins reached a maximum plateau level within 3 hr. Equilibrium for lorazepam binding to cholestyramine and colestipol was also reached in 2 to 3 hr. Binding by activated charcoal was extremely rapid, with no measurable free drug in the incubate at 10 min.

Extent of Binding of Lorazepam and Lorazepam Glucuronide to Cholestyramine, Colestipol, and Activated Charcoal

The extent of lorazepam and lorazepam glucuronide binding to the various adsorbent is shown in Table I. The percentage of lorazepam bound approached 24 for cholestyramine, 11 for colestipol, and 100 for activated charcoal. Similarly, lorazepam glucuronide binding was 74% to cholestyramine, 21% to colestipol, and 100% to activated charcoal. These values were independent of substrate concentrations, and buffers of pH 5 and 6 had no significant effect. While it is possible that the binding characteristics differed outside the ranges examined, the study was designed to reflect physiological conditions expected in the body on standard dosing. The rather low ionic strength of the buffer (0.15 M approximates the lower limit of tonicity in the gut) was used because of the inherent difficulties in dialyzing concentrated suspensions. Low ionic strength might lead to an overestimation of drug binding *in vivo*.

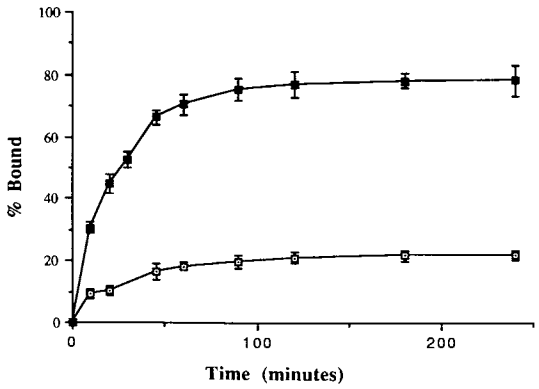

Fig. 1. Time course for the attainment of equilibrium for binding of lorazepam glucuronide to cholestyramine and colestipol. Binding to cholestyramine is represented by the filled squares, and colestipol by the open symbols. The concentration of lorazepam glucuronide used in these experiments was 1.06 μM.

Table I. Percentage Binding of Lorazepam and Lorazepam Glucuronide to Various Binding Agents at Equilibration[a]

	Lorazepam	Lorazepam glucuronide
Cholestyramine	23.67 ± 3.01	74.30 ± 1.21
Colestipol	11.33 ± 1.51	20.83 ± 3.87
Activated charcoal	100[b]	100[b]

[a] Concentration of lorazepam in the incubates was 196.56 nM, and that of lorazepam glucuronide 1.06 μM.
[b] No measurable radioactivity was present in the buffer.

Lorazepam Glucuronide Binding to Cholestyramine and Colestipol as a Function of Lorazepam Glucuronide Concentration

Figure 2 is an adsorption isotherm for the binding of lorazepam glucuronide to cholestyramine and colestipol. Amount bound versus amount free in each case showed a straight-line relation with no signs of a plateau, indicating nonsaturable binding for the two adsorbents. A strong linear relation was also observed using the Langmuir transformation (Fig. 3), with an approximate adsorption capacity for cholestyramine of 1.82 μg/mg adsorbent and an adsorption coefficient of 0.13 μM. Values for colestipol were 1.23 μg/mg and 0.28 μM, respectively, whereas binding to activated charcoal was too great to permit accurate measurements of percentage bound. These values are in the range of previously published adsorption parameters for these resins (8,12).

Cholestyramine is a strongly basic cation in which quaternary ammonium functional groups are attached to a styrene divinyl–benzene copolymer (12). A series of drugs that exist as anions at physiologic pH are known to interact with the resin, although the amount adsorbed and stability of the complex do not seem to be related to the acidity of the solute (13). Glucuronides are weak acids with pK_a's ranging between 3.0 and 4.0. At the pH of the intestines (fasting—2.5–7.5 for the duodenum, 6.5–7.5 for the jejunum, and 3.5–8.0 for the ileum; fed—7.0–8.0 throughout) (14,15), these would be expected to be almost entirely ionized. Lorazepam glucuronide, therefore, when present together with cholestyramine in the intestine may act as a counterion to the quarternary ammonium groups of the resin. Indeed, this

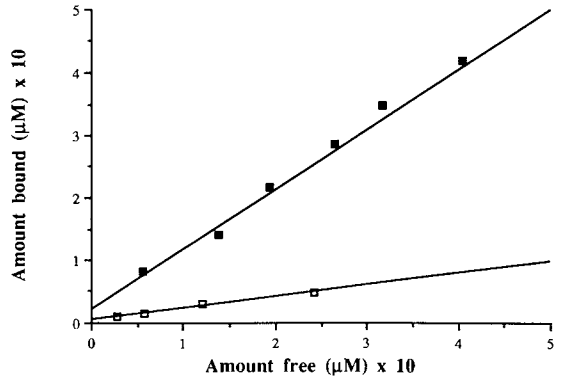

Fig. 2. Binding of lorazepam glucuronide to cholestyramine and colestipol as a function of the amount of free lorazepam glucuronide. Binding to cholestyramine is represented by the filled squares, and colestipol by the open symbols.

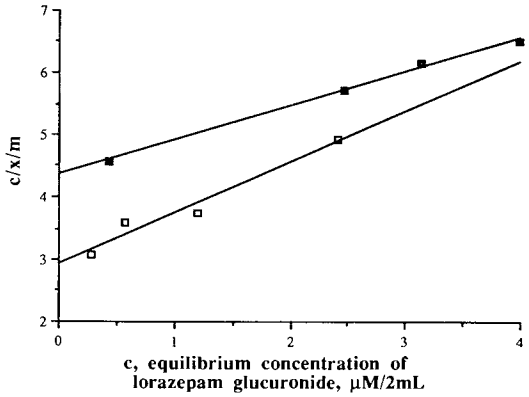

Fig. 3. Binding of lorazepam glucuronide to cholestyramine and colestipol as described by the Langmuir equation. Binding to cholestyramine is represented by the filled squares, and colestipol by the open symbols.

study indicates that cholestyramine has a relatively high binding capacity for lorazepam glucuronide. Since cholestyramine is not absorbed into the systemic circulation and since, under similar conditions of resin concentration, temperature, and pH, it does not interact appreciably with unchanged lorazepam, the 30–100% increases in lorazepam clearance observed in man on administration of cholestyramine and neomycin (5) are likely due to binding of the glucuronide by cholestyramine in the gut. This implies interruption of an enterocycling loop. Activated charcoal, on the other hand, binds strongly to both unchanged as well as glucuronidated drug, so that interruption of enterohepatic recirculation could not be discriminated from enteric dialysis of free lorazepam using this sequestrant (16,17). Its application, therefore, as an *in vivo* interruptor of enterohepatic recirculation would be inappropriate. Colestipol also may not be particularly useful since it binds both the glucuronide and the unchanged drug, and neither to a very great extent. A significant binding interaction between neomycin and lorazepam or lorazepam glucuronide is not expected, and hence, the neomycin effects are probably due to inhibition of glucuronidase containing intestinal microflora as proposed by other investigators. The reason that both cholestyramine and neomycin are required to interrupt effectively lorazepam enterocycling may be that binding to the adsorbent does not prevent subsequent hydrolysis and release of lorazepam back into the circulation and that the resin facilitates removal of the glucuronide before it can be acted on by other, presumably nonbacterial, glucuronidases.

This study supports our initial clinical observations that combined neomycin and cholestyramine produce a significant increase in free clearance of lorazepam because of interruption of an enterohepatic circulation. Although the data are indirect, anion-exchange resins are known to block the reabsorption of other cycling substrates (6,9,18), and the nature of the interaction is identical to that seen with lorazepam. Also, this investigation validates our choice of cholestyramine over activated charcoal and colestipol as the enterically acting agent.

ACKNOWLEDGMENTS

This work was supported by The Canadian Diabetic Association. The authors wish to thank Dr. J. W. Hubbard, College of Pharmacy, for lending the Spectrum Equilibrium Dialyzer for an extended period of time.

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