Reaction of Acyl Glucuronides with Insulin *In Vitro:* Identification of an Imine Mechanism by Electrospray Ionization Mass Spectrometry

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Drugs having carboxylic acid groups are often excreted from the body following metabolism to acyl glucuronide conjugates. Previous studies have shown that these conjugates can either be hydrolyzed to the parent drug, rearranged into β-glucuronidase resistant forms (isomeric conjugates), or covalently bound to proteins (1,2). It has been postulated that proteins modified by addition of drug acyl glucuronide conjugates may cause immunological responses that could be associated with some of the observed toxicity of these drugs (3). Two mechanisms for the covalent binding reaction have been proposed. One is a nucleophilic displacement mechanism as proposed by van Breemen and Fenselau (4–6) and others (7) where the irreversible binding of carboxylic acids to protein via their glucuronides lead to an acylated protein and liberation of glucuronic acid. The other proposed mechanism involves glycation where irreversible protein binding of the isomeric conjugates of the acyl glucuronide occurs via imine formation between the free aldehyde of the acylic glucuronic acid and an amine (lysine or the N-terminus) in the protein molecule (8). Ding *et al.* (9,10) have more recently shown that the acyl glucuronide of tolmetin bonds covalently to human serum albumin in part via the imine mechanism. In the present experiments, insulin was chosen as a model protein to examine the mechanism for the formation of protein adducts by acyl glucuronide conjugates of five nonsteroidal anti-inflammatory drugs (NSAIDs). The selective reducing agent, $NaCNBH_3$, was also employed to trap putative unstable imine intermediates that may be formed by reactions of acyl glucuronides with insulin. The use of electrospray ionization mass spectrometry (ESI/MS) is demonstrated to be effective in the characterization of such adducts. This approach provides a rapid *in vitro* method to evaluate the reactivity of of acyl glucuronides which may be of value in drug development.

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MATERIALS AND METHODS

Bovine insulin (sodium salt) and recombinant human insulin (zinc salt) were purchased from Calbiochem (San Diego, CA). Acyl glucuronides of several NSAID's including suprofen, zomepirac, salicylic acid, etodolac and tolmetin were extracted from human or rabbit urine after oral dosing as described previously (11-14). All acyl glucuronide conjugates were purified by HPLC and characterized by FAB/MS. Based upon HPLC, the purity of the isolated glucuronic acid conjugates of suprofen, salicylic acid, zomepirac, etodolac and tometin were 98%, 100%, 100%, 98%, and 98%, respectively, with any remaining 0–2% present as polar impurities or the parent compound. These purified conjugates were estimated to be almost all present initially as the β-1 acyl glucuronide with 99%, 98%, 94%, 97% and 99% susceptible to hydrolysis with β-glucuronidase, respectively. Methanol and acetonitrile were HPLC grade from Fisher (Norcross, GA). Other chemicals used were reagent grade.

HPLC Chromatography

HPLC was performed with a Vydac protein and peptide C18 column (4.6 × 150 mm, Hesperia, CA) coupled with a Brownlee C18 guard column (OD-300, G18-GU, 4.6 × 30 mm). Two Hewlett Packard 3396A integrators were employed with two HP-1050 UV detectors in series to simultaneously observe and record the absorption of insulin and respective drugs at different wavelengths (Table I). Fractions were collected from HPLC with a Gilson FC 204 fraction collector. Aqueous 0.1% trifluoroacetic acid (TFA) (Solvent A) and acetonitrile-0.1% TFA (Solvent B) were employed as the mobile phase with gradient elution at a flow-rate of 1 ml/min. The gradient programs and UV detection wavelengths employed are listed in Table I.

Insulin-Drug Adduct Preparation

Solutions of bovine insulin or recombinant human insulin, 2.0 mg in 0.2 ml 150 mM NaH_2PO_4 buffer, pH 7.4, were incubated with 0.15 mg/ml of the respective drug acyl glucuronide (expressed in equivalents of the aglycone) and 20 mM $NaCNBH_3$ at 37°C for 24 hours. This provided about a three fold molar excess of insulin relative to acyl glucuronide. The concentrations employed allowed for ease of measurement of adduct formed in a 24 hr period and the ratio used reduced the likelihood of multiple additions of acyl glucuronide to each insulin molecule. Insulin was also incubated with each respective parent drug and reducing agent, $NaCNBH_3$, to serve as controls. After incubation the pH of each solution was adjusted to 5.3 to precipitate insulin. After centrifugation the supernatant was discarded. To resuspend the insulin pellet for further washing, 0.5 ml of 150 mM NaH_2PO_4 buffer (pH 5.3) was added. The steps of centrifugation, decanting of supernatant and resuspension were repeated three times to remove unbound acyl glucuronide and parent drug. Finally, the suspension of insulin was adjusted with 150 mM H_3PO_4 to pH 3–4 to dissolve the protein material. The solubilized protein was injected on HPLC; those fractions having absorbance response at the λ_{max} of the drug chromophore (wavelength 2) were collected, lyophilized and

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Table I. HPLC Systems for the Separation of Glucuronide-insulin Adducts and Results of Electrospray Mass Spectrometry of the Isolated Adducts

Drug acyl glucuronide	Wavelength (nm) 1[a]	2[b]	Gradient program[c] (% Solvent B)	MW of drug acyl glucuronide	Calculated MW of insulin adduct[d]	Observed MW of insulin adduct
Suprofen	214	295	20%——20%——40% 10 min. 30 min.	436.5	6228.1	6227.8
Salicylic acid	214	313	20%——20%——40% 10 min. 30 min.	314.1	6105.7	6105.4
Zomepirac	214	313	10%————70% 40 min.	467.9	6259.5	6259.5
Etodolac	214	280	30%——30%——70% 10 min. 35 min.	463.5	6255.1	6254.8
Tolmetin	214	315	10%————70% 35 min	432.4	6224.0	6225.1

[a] Wavelength for the detection of insulin peptide.
[b] Wavelength for the detection of acidic drug.
[c] Solvent A and B as described in the text.
[d] Human insulin has a molecular weight of 5807.6 daltons.

stored at –80°C for later ESI/MS studies. A representative chromatogram of the separation obtained with insulin adducted with suprofen glucuronide is shown in Figure 1. Adduct formed is expressed on the basis of the estimated percentage of total insulin based upon HPLC peak area at 214 nm.

To determine the effect of reducing agent on the covalent binding, solutions of bovine insulin, 2.0 mg in 0.2 ml 150 mM NaH_2PO_4 buffer, pH 7.4, were incubated with 0.15 mg/ml respective drug acyl glucuronide for 24 hours without NaCN BH_3. After incubation, $NaCNBH_3$ was added to give a final concentration of 20 mM followed by an additional 30 minutes of incubation. The samples were then prepared for HPLC using the same procedures described above. Similar experiments were also conducted with all five acyl glucuronides and human insulin without the addition of reducing agent.

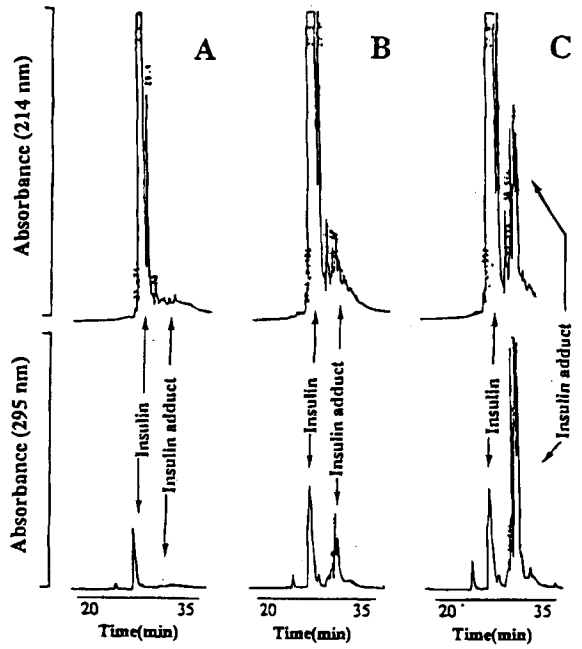

Fig. 1. HPLC chromatogram of insulin and suprofen glucuronide-insulin adduct, A: insulin incubated with suprofen glucuronide at 37°C for 24 hours without reducing agent $NaCNBH_3$; B: addition of 20 mM $NaCNBH_3$ for 30 min after 24 hr incubation without reducing agent. C: insulin incubated with suprofen glucuronide and 20 mM $NaCNBH_3$ at 37°C for 24 hours.

Electrospray Ionization Mass Spectrometry

Identification of insulin and acyl glucuronide adducts was accomplished by ESI/MS. Samples (ca. 5 to 10 pmol/μl) were dissolved in methanol containing 0.5% acetic acid and introduced into the ESI/MS at a rate of 1 μl/min. Mass spectra were acquired on a Finnigan-MAT model SSQ700 using an electrospray energy of –3.5 kV. The ESI/MS spectra were obtained after signal averaging for approximately 1 min.

RESULTS AND DISCUSSION

Preliminary studies had shown that suprofen glucuronide became covalently bound equally well *in vitro* to bovine insulin as it did with human serum albumin *in vitro* which was studied earlier (11). The gradient system with dual UV detection was able to separate and distinguish suprofen, insulin and suprofen acyl glucuronide-insulin adducts. Analysis by HPLC as shown in Figure 1 revealed a cluster of three major peaks corresponding to the adducts which had higher UV absorption at 295 nm than did insulin (Figure 1C). The adduct peaks generated when suprofen glucuronide or either of the four other acyl glucuonides was incubated with insulin were enhanced by the presence of the imine trapping reagent, $NaCNBH_3$. These adduct peaks and the insulin peak were collected from HPLC for later ESI/MS analysis. No attempt was made to separate these adduct peaks that eluted as a cluster of peaks on HPLC. These studies were done off-line due to the limited availability for direct access to ESI/MS, however, such experiments should be possible using on-line methods if a desalting step or other adjustments of the incubation or HPLC are optimized. Identification of the reduced adducts was accomplished by ESI/MS which showed that the molecular weight

(MW) was equal to bovine or human insulin, respectively, plus suprofen acyl glucuronide conjugate minus 16 ($-H_2O+2H$). The MW of insulin which was isolated from the same incubation matrix by HPLC was unaltered. Figure 1 shows sections of a typical HPLC chromatogram of insulin, suprofen acyl glucuronide-insulin adduct and its controls. Figure 2 shows a representative deconvoluted ESI mass spectrum. The peaks at m/z of 5807.2 and 6227.8 correspond to molecular weights for human insulin and its reduced adduct formed with the putative isomeric conjugates of suprofen glucuronide. The deconvulated spectrum had multiply charged insulin or abducts with charges corresponding to +4 to +6. Similar results were obtained using acyl glucuronides of suprofen and four other NSAIDs incubated with recombinant human insulin. The ESI/MS results of individual drug acyl glucuronide-insulin adducts are given in Table I.

The reducing agent, $NaCNBH_3$, can dramatically enhance the formation of adducts by reducing the labile imine to a stable amine to permit workup and subsequent ESI/MS. Being labile, it was not possible to isolate imine intermediates or to identify such intermediates by ESI/MS, though with an on-line method such identification may be possible. Although it was not possible to accurately quantify the degree of adduct formation when no reducing agent was employed due to the small peaks present on HPLC at 214 nm (Figure 1A), comparisons with HPLC chromatograms from control incubations (insulin and suprofen) indicated that adducts were present when acyl glucuronides were incubated without reducing agent in solution with insulin. Based upon HPLC peak area ratios at 214 nm, the percentage of suprofen glucuronide-adduct increased from less than 1.0% of total insulin when no $NaCNBH_3$ was added, to 2.8% when $NaCNBH_3$ was added after 24 hrs of incubation, and finally to 24% when $NaCNBH_3$ was added at the beginning of 24 hrs of incubation. For etodolac the percentage glucuronide-adduct increased from less than 1.0% of total insulin when no $NaCNBH_3$ was added to 3.6% when $NaCNBH_3$ was added after 24 hrs of incubation and to 8.5% when $NaCNBH_3$ was added at the beginning of 24 hrs of incubation. The other three acyl glucuronides studied behaved similarly with results within the range obtained with the acyl glucuronides of suprofen and etodolac. The acyl glucuronides studied vary in their stability in aqueous solution at physiological pH with half-lives of the respective β-1 conjugates ranging from 0.4 hr for zomepirac (3) and tolmetin (12), 1.3 hr and 1.4 hr for salicylate (13) and suprofen (11), and up to 21 hr for etodolac (14). As reported previously for numerous acyl glucuronides, though initial loss of the β-1 conjugate may be rapid under physiological conditions, the primary degradation products are the isomeric conjugates that have much longer half-lives and hydrolyze slowly to the parent compound (2,3,12–14). The lower reactivity of etodolac acyl glucuronide with insulin *in vitro* appeared to correlate here with its higher stability (14) in aqueous solutions relative to the other acyl glucuronides studied.

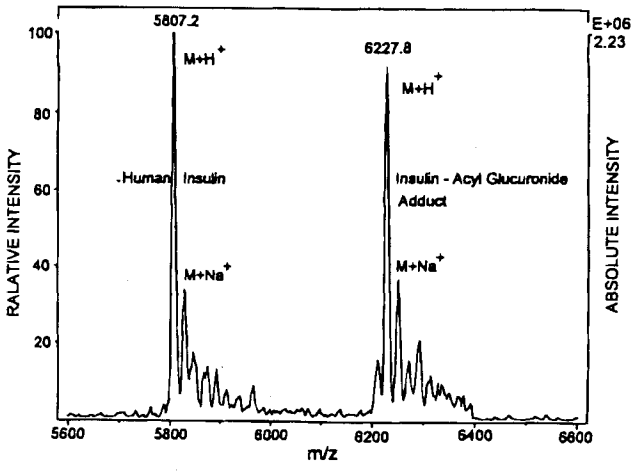

Fig. 2. Deconvoluted electrospray mass spectrum of reduced adducts formed by the reaction of suprofen glucuronide and human insulin (infusion of approx. 7–8 pmol/μl into the ESI source).

Insulin was chosen as a model protein for these studies because it is well characterized, homogeneous, has a lysine residue as well as two free amino termini, is inexpensive and of moderate size, allowing easy measurement by ESI/MS. Moreover, it was expected that the molecular weight of insulin and its physical properties would be significantly altered by covalent modification by a reactive acyl glucuronide. This change in physical properties of insulin permitted the easy separation of the adducted insulin from native insulin by HPLC. The adducts exhibited three peaks on HPLC as shown for the adduct cluster in Figure 1. The results of ESI/MS analysis of this group of peaks indicated that they contained the same molecular weight components which suggests that the ε-amino group of lysine or the N-terminus of the A and B chains of insulin may be separately reacting with the acyl glucuronide in a nonspecific manner. This behavior is similar to that observed when glucose reacts and glycates multiple sites on albumin (15) and multiple reactions sites were also identified for the reaction of tolmetin glucuronide with albumin (9,10). If the multiple peaks on HPLC were due to attachment of acyl glucuronide to different sites on insulin, ESI/MS would not be expected to discern such isomeric adducts formed via the imine mechanism since all would have the same molecular weight. However, the use of MS/MS may permit specific identification of amino acid sites (9,10), if such instrumentation is available as it is at most large pharmaceutical companies.

The formation of covalent adducts was enhanced by the addition of reducing agent which supports the hypothesis that an imine is involved in the formation of adduct. Only one species was apparent in the ESI/MS and this corresponded to the addition of acyl glucuronide to insulin with the loss of 16 daltons due to elimination of water and reduction of a putative imine intermediate (Table 1). The spectra exhibited no evidence of adducts that might be formed by the addition of only acidic drug via addition to insulin by a nucleophilic displacement mechanism where glucuronic acid is the leaving group (9) though human insulin does contain four tyrosine residues as well as several threonine and serine amino acids. This does not rule out the existence of nucleophilic displacement for the reactions of acyl glucuronides with proteins since reactions with larger proteins may behave differently and the reducing agent employed here selectively enhances one mechanism to levels that facilitated identification by ESI/MS. These results clearly show that numerous acyl glucuronides of NSAIDs react similarly with a model protein, insulin, and that the imine mechanism is common to a wide range of acyl glucuronides. All evidence from these studies indicate that the adduct is produced through formation of an imine between an amine of insulin and

the free aldehyde of glucuronic acid that is exposed following acyl migration of the glucuronide to yield isomeric conjugates. These results suggest that insulin, available at low cost may be a good model protein for *in vitro* evaluation of the reactivity of acyl glucuronides in drug development.

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