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Structural Determination of Glucuronide Conjugates and a Carbamoyl Glucuronide Conjugate of Carvedilol: Use of Acetylation Reactions as an Aid to Determine Positions of Glucuronidation†

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Carvedilol is metabolized via both oxidation and conjugation pathways in dog and rat to more than 12 different products. Several glucuronide conjugates of the parent drug were identified. The drug contains an aliphatic hydroxyl, an aliphatic amine and a carbazole amine, all of which are potential sites for conjugation with glucuronic acid. In order to determine the positions of glucuronidation, a strategy involving acetylation of the metabolites was devised. The metabolites were acetylated using acetic anhydride in either pyridine or aqueous solution, and the products were analyzed by fast atom bombardment mass spectrometry. Carvedilol was acetylated at both the hydroxyl and the aliphatic amine in pyridine and at only the aliphatic amine in aqueous solution. The carbazole nitrogen was unreactive under both conditions. Based on the acetylation patterns observed for the metabolites in pyridine or aqueous solution, the positions of conjugation were determined. Each of the five glucuronide metabolites of carvedilol formed in dog and rat was analyzed and the structures included two diastereomeric carbamoyl glucuronide metabolites formed from addition of CO_2 and glucuronic acid to carvedilol, a carbazole-*N*-linked glucuronide, and two diastereomeric *O*-linked glucuronides. This approach should be generally applicable in many cases to determine the structures of glucuronide conjugates for compounds which contain more than one potential glucuronidation site.

INTRODUCTION

Conjugation with glucuronic acid is a common route by which many drugs and xenobiotics are metabolized prior to excretion. Several different nucleophilic functional groups, including hydroxyls, phenols, thiols, amines and carboxylic acids, are susceptible to conjugation with glucuronic acid.[1,2] Determining the structures of glucuronide conjugates can be difficult for xenobiotics which contain more than one potential conjugation site.

Several approaches have been used previously to establish positions of glucuronidation. Determination of the stability of glucuronide conjugates under acidic conditions is sometimes used to distinguish between amine- and hydroxyl-linked glucuronides. Amine-linked glucuronides are labile under acidic conditions, but hydroxyl-linked glucuronides are generally stable.[1] Typically, the parent drug is measured following the reaction to determine the extent of hydrolysis, and thus, indirectly, the position of glucuronidation may be assigned. This approach requires the parent drug to be stable under the harsh reaction conditions used. In addition, the reactivity of glucuronides linked to less common functional groups is often unknown.

Spectroscopic techniques have also been used to characterize the structures of glucuronide conjugates. Mass spectrometric methods, including tandem mass spectrometric techniques, are frequently used to characterize glucuronide conjugates.[3,4] These approaches can provide useful molecular weight data, as well as important structural information. However, in our experience, the glucuronide linkage is typically the most labile bond in a metabolite and elimination of the glucuronyl moiety is the most prevalent fragmentation pathway. Charged fragments with the glucuronide still attached are often not observed and, thus, the position of attachment cannot be determined directly from the mass spectrum. Nuclear magnetic resonance (NMR) is also frequently useful to determine positions of glucuronidation;[5] however, sufficient amounts of purified metabolite and appropriate reference standards are necessary.

In this report, glucuronide conjugates of carvedilol were characterized using a novel selective acetylation approach to elucidate positions of glucuronidation. Carvedilol contains an aliphatic hydroxyl group, an aliphatic amine group, and a carbazole amine group, all of which are potential sites for glucuronidation. The approach was used to characterize several *O*- and *N*-

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linked glucuronide conjugates, including a carbamoyl glucuronide metabolite, which were identified in bile samples from Sprague–Dawley rats following oral gavage administration of (^{14}C)carvedilol.

Carvedilol, which possesses β-adrenoreceptor antagonism and non-specific vasodilation activities, is currently being developed for treatment of hypertension and angina. It is metabolized extensively in rat[6] and dog via both oxidation and conjugation pathways to a large number of products. Only selected glucuronide metabolites are discussed in this report. Although these glucuronide metabolites were relatively minor metabolites in the rat, they were major metabolites in other species (Schaefer, unpublished data). A more comprehensive description of carvedilol metabolism, including quantitative aspects and characterization of metabolites formed by oxidative pathways, will be presented in a separate report.

EXPERIMENTAL

Chemicals

Racemic (^{14}C)carvedilol (labeled at the chiral carbon, see structure in Table 1) was obtained from Boehringer-Mannheim GmbH and was purified prior to use by the Radiochemistry Department, SmithKline Beecham Pharmaceuticals (specific activity, 31.7 mCi mmol^{-1}; radiochemical purity, 98.2%; chemical purity, 97.1%). Unlabeled carvedilol used in this study was also a racemic preparation. All other chemicals and reagents used for these studies were of reagent grade or better. β-Glucuronidase from bovine liver was purchased from Sigma Chemical Company (St Louis, Missouri). High-performance liquid chromatography (HPLC)-grade solvents were obtained from J. T. Baker, Inc. (Phillipsburg, New Jersey). HPLC-grade water was obtained from a Milli-Q Water System (Millipore, Milford, Massachusetts).

Dosing solutions

A stock solution of (^{14}C)carvedilol (10 mg ml^{-1}, 78 μCi mg^{-1}) was prepared by dissolving (^{14}C)carvedilol in absolute ethanol. The dosing suspension used for oral administration was prepared by addition of 87.79 mg of non-radiolabeled carvedilol to 0.82 mol of the stock (^{14}C)carvedilol solution, followed by dilution of this mixture to 6.4 ml with 0.5% methyl cellulose.

Surgical preparation and dose administration

Male and female Sprague–Dawley ratios (Charles River, six rats per dose group, 300–400 g) were bile duct exteriorized prior to oral dosing with (^{14}C)carvedilol. In preparation for bile duct cannulation, the rats were anesthetized with Ketamine (60 mg kg^{-1})/Xylazine (8 mg kg^{-1}), i.p., and the surgery was performed under sterile conditions. The bile duct was exposed through a midline abdominal incision and a cannula of narrowed PE-50 tubing was installed into the duct. The peritoneum and skin were closed separately with 3-0 silk. The rats were given access to a post-operation drink solution containing 50 g of dextrose and 0.5 g KCl per liter of physiological saline. The animals were dosed the following day (17–22 h after surgery) by gavage with (^{14}C)carvedilol (30 mg kg^{-1}). The dextrose drink solution was replaced with physiological saline and rat chow approximately 1 h after dosing.

Sample collection

Bile was collected from rats for 1 h prior to dosing. Following administration of (^{14}C)carvedilol, bile was collected over the periods 0–2, 2–4, 4–6, 6–12, 12–24 and 24–48 h into tubes which were maintained on ice out of direct light. Urine and feces were also collected over 48 h. Each sample was frozen at −80 °C immediately after collection. At the completion of the experiment, the rats were euthanized with a methoxyflurane overdose.

Metabolite isolation

Bile samples containing ^{14}C-labeled carvedilol metabolites were subjected to solid-phase extraction using a 12 ml Analytichem C_{18} Meg-elute column. The column was preconditioned with 40 ml of acetonitrile–water (80:20), followed by 40 ml of water. Bile (1 ml) was diluted with 1 ml of water and applied to the column. The column was washed successively with 10 ml of water, 10 ml of acetonitrile–water (80:20), and 10 ml of acetonitrile–water–trifluoroacetic acid (80:20:1). Typically, <1% of the radioactivity from bile eluted with the water wash, 96% eluted during the acetonitrile–water (80:20) wash, and 1–3% eluted during the acetonitrile–water–trifluoroacetic acid (80:20:1) wash.

Metabolites isolated by solid-phase extraction were separated further by HPLC using a Brownlee RP-300 column (C8; 7 × 250 mm) and the following linear gradient conditions: solvent A, 0.1 M ammonium acetate pH 5.0; solvent B, acetonitrile–water (80:20); 0 min, 10% B; 70 min, 45% B; 75 min, 100% B; 80 min, 100% B; flow, 2.0 ml min^{-1}; ultraviolet (UV) detection, 285 nm. Fractions (0.5 min) were collected and a radiochromatogram was generated by liquid scintillation counting. Metabolites were numbered in the order that they eluted from the HPLC column and only glucuronide metabolites M2, M9, M10 and M11 are discussed in this report. These chromatographic conditions resolved carvedilol glucuronide diastereomers M9 and M10, but did not separate the diastereomers of the carbamoyl glucuronide metabolite, M11.[7] In order to completely resolve the diastereomers, M11a and M11b, an isocratic HPLC method was developed with the following conditions: Beckman ODS column (10 × 250 mm), solvents A and B (as above) 65:35 (v/v); flow, 3.0 ml min^{-1}; detection, 285 nm. The stereochemistry of M2 was not determined.

Metabolites purified by HPLC were desalted prior to structural characterization using a 4.6 × 30 mm Brownlee RP-300 column and the following linear gradient conditions: solvent A, water; solvent B, acetonitrile–

water (80:20); 0 min, 0% B; 5 min, 0% B; 10 min, 100% B; 20 min, 100% B; flow, 1.0 ml min^{-1}; UV detection, 285 nm. At 15 min, 250 µl of acetonitrile–water–trifluoroacetic acid (80:20:0.2) was injected in order to elute any highly retained products.

Synthesis of carvedilol ethylcarbamate

Carvedilol (200 mg, 0.492 mmol) was dissolved in 3 ml of pyridine and cooled to 0–4 °C in an ice bath. Ethyl chloroformate (56.07 mg, 0.517 mmol) was added dropwise with stirring over a period of 1h. Stirring was continued for 3h, and the solvent was evaporated under reduced pressure. The residue was dissolved in 6 ml of water (the resulting aqueous solution had a pH ~2), and the product was extracted twice with 10 ml of chloroform. The combined chloroform extracts were treated with charcoal, filtered, and concentrated. Recrystallization of the residue from ethanol yielded 175 mg of carvedilol ethylcarbamate. Analyses using proton NMR (COSY spectrum obtained in d_6-dimethylsulfoxide (d_6-DMSO), Table 1) and desorption chemical ionization (DCI) mass spectrometry (discussed below) showed that the carbamate was on the aliphatic nitrogen. The carbazole NH was observed in the NMR spectra at 11.25 ppm. The chemical shift (4.25 ppm) of the proton (H-b) on the chiral carbon of the carbmate product was very similar to that of carvedilol (4.17 ppm), indicating that the ethyl chloroformate had not reacted at the hydroxyl of carvedilol.

Ethanolysis of metabolite M11a

The ethanolysis reaction was conducted essentially as described by Straub *et al.*[8] Briefly, approximately 100 mg of sodium metal was dissolved in 10 ml of anhydrous ethanol to give a 0.4 M solution of sodium ethoxide. A 100 µl aliquot of this was added to an ethanol solution of metabolite M11a (about 100 µg in 0.5 ml), and the mixture was allowed to stand at room temperature for 2.5 h; 1 ml of water was added and the pH was adjusted to 7. The product was extracted twice with 500 µl of ethyl acetate. The combined ethyl acetate extracts were concentrated and the solvent was removed under a stream of nitrogen. The synthetic carvedilol ethylcarbamate was also subjected to ethanolysis under the same conditions. The formation of the cyclic carbamate (described below) from both M11a and carvedilol ethylcarbamate was confirmed by mass spectrometry.

Acetylation of metabolites

Metabolites were acetylated in pyridine solution by dissolving approximately 10 µg of metabolite in 50 µl of pyridine–acetic anhydride (1:1) and reacting 15 min. Acetylation in aqueous solution was accomplished by dissolving approximately 10 µg of metabolite in 50 µl H_2O–ethanol–diisopropylethylamine (50:50:1), adding 1 µl of acetic anhydride–acetonitrile (1:9), and reacting for 15 min. The products were analyzed directly by fast atom bombardment (FAB) mass spectrometry.

Mass spectrometry

FAB mass spectra were obtained using a VG-7070E-HF mass spectrometer (6 kV accelerating potential) equipped with a saddle-field fast atom gun (operated at 8 kV, 1 mA). Spectra were obtained by automatically switching between positive and negative ion modes. Samples were ionized from a matrix of either gycerol or a mixture of dithiothreitol and dithioerythritol (3:1). Some samples were analyzed using a linked *B/E* scan technique for analysis of daughter ions produced from metastable parent ions.

Bile was analyzed directly by liquid chromatography/mass spectrometry (LC/MS) using thermospray ionization on a Finnigan TSQ-70 mass spectrometer operated in alternating positive and negative ion modes. Bile samples were diluted with water, injected directly onto the HPLC instrument, and separated using the linear gradient described above except using a 250 × 4.6 mm Brownlee RP-300 column and a flow rate of 1.5 ml min^{-1}. Ion source conditions were: block temperature 220 °C, and vaporizer temperature 120 °C. Ion current profiles were reconstructed for the major ions which were observed in each spectrum to help ensure that these ions corresponded to a single compound.

DCI experiments were conducted on a Finnigan 3625 mass spectrometer using several reagent gases, including methane, ammonia, d_3-ammonia and NF_3.

NMR spectroscopy

Proton NMR spectra were obtained using a Bruker AM-400 NMR spectrometer. Purified, desalted carvedilol metabolites were dried over P_2O_5 *in vacuo*, handled under dry argon, and dissolved in d_6-DMSO for analysis. Protons were assigned based on decoupling and COSY experiments, as appropriate, as well as by comparing their chemical shifts to those of authentic carvedilol.

RESULTS AND DISCUSSION

General strategy

Glucuronide conjugates of carvedilol were initially characterized by thermospray LC/MS and FAB mass spectrometry. The spectra showed a characteristic fragment ion which was 176 u less than the pseudomolecular ion, confirming the presence of a glucuronyl moiety on each of the metabolites, but did not reveal the position of glucuronidation on carvedilol. Thus, a series of acetylation experiments with subsequent FAB mass spectral analyses was devised to aid in determining the positions of glucuronidation. The approach relies on the selective acetylation of hydroxyl and amine groups under different conditions. Nucleophilic groups, such as amines and hydroxyls, are readily acetylated in non-aqueous solution by acetic anhydride in the presence of a base, such as pyridine. In aqueous solution, the more nucleophilic amine groups are rapidly acetylated by acetic anhydride, but water (which is present in

great molar excess) competitively prevents acetylation of metabolite hydroxyl groups. Amine and hydroxyl moieties of metabolites which are conjugated to glucuronic acid are blocked and, thus, will not be acetylated. After completion of the acetylation reaction, the reaction mixture is analyzed directly by FAB mass spectrometry. Based on the number of acetyl groups added in each reaction, the position of glucuronidation can be established.

First, carvedilol was reacted with acetic anhydride in pyridine and aqueous solutions in order to determine the reactivities of the various functional groups of the compound. The results were used as a reference for interpreting the results obtained for isolated carvedilol glucuronide metabolites. Following reaction with pyridine–acetic anhydride (1:1), an intense $[M + H]^+$ ion was observed at *m/z* 491, which was 84 u higher than that for carvedilol (*m/z* 407), indicating that two acetyl groups were added to carvedilol. In a *B/E* linked scan of the ion at *m/z* 491, shown in Fig. 1(a), daughter ions observed at *m/z* 449, 431, 367 and 308 corresponded to loss of CH_2CO, acetic acid, methylcatechol and hydroxycarbazole, respectively. Thus, these data indicated that both the hydroxyl and aliphatic secondary amine moieties were acetylated. It is important to note that the carbazole nitrogen was not acetylated under these conditions. The carbazole nitrogen was expected to be a relatively poor nucleophile due to delocalization of its electrons into the aromatic rings. The reaction of carvedilol with acetic anhydride in water–ethanol–diisopropylethylamine (50:50:1) added only one acetyl group to the molecule as indicated by the $[M + H]^+$ ion observed at *m/z* 449. The *B/E* linked scan of *m/z* 449 (Fig. 1(b)) showed a daughter ion at *m/z* 407, corresponding to loss of CH_2CO, but lacked an ion resulting from loss of acetic acid. The presence of an ion corresponding to loss of H_2O (*m/z* 431) and the absence of a fragment ion in the mass spectrum resulting from the loss of acetic acid were consistent with the acetylation of the amine. NMR data for these products, summarized in Table 1, confirmed the structures. The diacetylated product formed in pyridine showed proton Hb to be shifted downfield due to acetylation of the hydroxyl. The singlets expected for both *N*-and *O*-linked acetyl groups each appeared as two discrete resonances, presumably due to slow rotation of the *N*-acetyl group. Several aliphatic protons appeared as complex multiplets due to non-equivalence and/or slow rotation of the acetyl group.

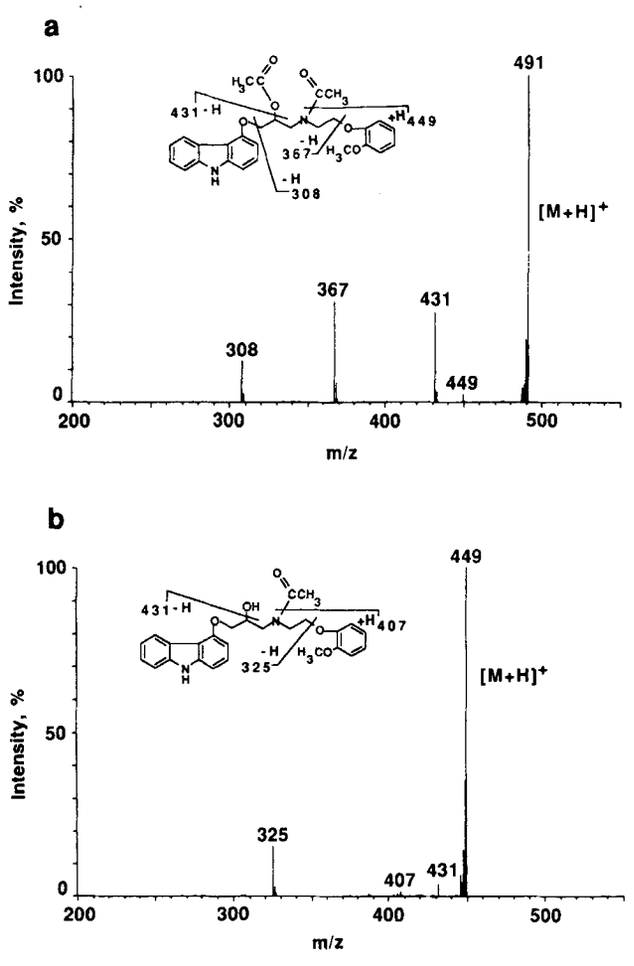


Figure 1. (a) Positive ion linked-scan (constant *B/E*) FAB mass spectrum of carvedilol following acetylation in pyridine and acetic anhydride. Daughter ions produced from the $[M + H]^+$ ion at *m/z* 491 are shown. (b) Positive ion linked-scan (constant *B/E*) FAB mass spectrum of carvedilol following acetylation with acetic anhydride in aqueous solution. Daughter ions produced from the $[M + H]^+$ ion at *m/z* 449 are shown.

As mentioned above, carvedilol has three sites which could, theoretically, be conjugated with glucuronic acid. Structures for the possible *O*- and *N*-glucuronides of carvedilol are shown in Table 2. Based on the acetylation pattern observed for carvedilol, the results expected from acetylation of each of these structures with acetic anhydride in pyridine or aqueous solution are also shown. As is evident in Table 2, each of these structures will yield a unique pattern of acetylation which can be used to differentiate between the possible structures.

Metabolites 9 and 10

Metabolites 9 and 10 (M9 and M10) from rat bile were both shown to be carvedilol glucuronides by mass spectral analysis. The mass spectrum for M10 in Fig. 2(a) obtained using thermospray ionization showed intense signals at *m/z* 583 and 407 which were consistent with the $[M + H]^+$ ion and aglycone, respectively. The negative ion spectrum in Fig. 2(b) displayed the corresponding ions at *m/z* 581 and 405. Similar results were obtained using FAB ionization. Larger quantities of these products were prepared and isolated from incubations with dog liver microsomes fortified with uridine 5′-diphosphoglucuronic acid (UDPGA).[7] The microsomal products were used to optimize the acetylation reactions, since only limited amounts of the metabolites were isolated from *in vivo* samples. The results from characterization of the microsomal products using the acetylation approach were in complete agreement with the acetylation results obtained for M9 and M10 isolated from bile from animals. The COSY NMR spectra for M9 and M10 formed using dog liver microsomes are summarized in Table 1. Because these data did not indicate, unequivocally, the position of glucuronidation, M9 and M10 were acetylated as described above.

Table 1. Summary of NMR data for carvedilol glucuronide metabolites and reference compounds (in DMSO-d_6)

	Chemical shift, ppm																			
Metabolite	NH	H8	H7	H6	H5	H3	H2	H1	CAT[a]	Ha	Hb	Hc	Hd	He	Me	H1′	H2′	H3′	H4′	H5′
Carvedilol	11.23	7.44	7.32	7.12	8.21	6.68	7.28	7.07	6.81–6.97	4.11, 4.15	4.17	2.82, 2.93	2.93	4.01	3.72	—	—	—	—	—
2	—	7.66	7.37	7.65	8.27	6.77	7.33	7.29	6.85–7.01	[b]	[b]	[b]	3.15	4.12	3.73	5.79	[b]	[b]	[b]	[b]
9	11.30	7.43	7.32	7.08	8.15	6.66	7.28	7.08	6.84–7.03	4.37, 4.40	4.57	3.51, 3.55	3.48	4.23	3.70	4.67	3.18	3.21	3.34	3.71
10	11.30	7.44	7.32	7.09	8.18	6.70	7.30	7.10	6.84–7.03	4.37	4.59	3.48, 3.58	3.48	4.28	3.68	4.69	3.17	3.19	3.32	3.69
11a	11.22, 11:23	7.41	7.28	7.07	8.25, 8.30	6.66, 6.69	7.25	7.05	6.78–6.99	[b]	[b]	[b]	[b]	[b]	3.66	[b]	[b]	[b]	[b]	[b]
11b	11.23	7.42	7.30	7.07, 7.16	8.25, 8.29	6.66, 6.70	7.28	7.06	6.79–6.98	[b]	[b]	[b]	[b]	[b]	3.67, 3.68	[b]	[b]	[b]	[b]	[b]
Carvedilol ethyl carbamate	11.25	7.43	7.30	7.08, 7.09	8.24, 8.29	6.65, 6.66	7.28	7.05	6.79–6.97	3.97–4.07	4.25	3.63, 3.88	3.63, 3.88	3.97–4.07	3.67	4.01,[c] 4.04	1.08,[d] 1.14			
N-Acetyl carvedilol	11.24, 11.26	7.44	7.30	7.09	8.24, 8.30	6.65, 6.70	7.30	7.09	6.81–6.99	4.10–4.18	4.30	3.42–3.46	3.83	3.63, 4.10	3.67, 3.71	2.11,[e] 2.19				
N,O-Diacetyl carvedilol	11.28, 11.30	7.44, 7.45	7.33	7.10	8.13, 8.15	6.65, 6.70	7.28	7.09	6.81–6.98	4.23–4.44	5.65	3.72–3.92	3.54–3.72	4.09	3.68, 3.70	2.13,[e] 2.17	2.03,[f] 2.07			

[a] CAT = protons on the methyl catechol ring.
[b] Signal not assigned due to limited amounts of sample and complexity of the spectrum from multiplicity caused by non-equivalence and / or slow rotation.
[c] Corresponds to $-CH_2CH_3$ of the ethyl carbamate moiety.
[d] Corresponds to $-CH_2CH_3$ of the ethyl carbamate moiety.
[e] Corresponds to $-CH_3$ of the *N*-acetyl moiety.
[f] Corresponds to $-CH_3$ of the *O*-acetyl moiety.

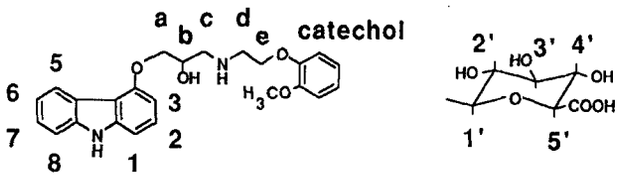
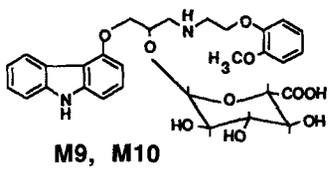
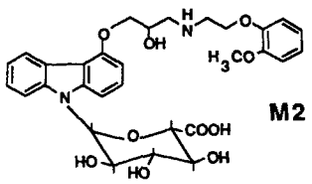
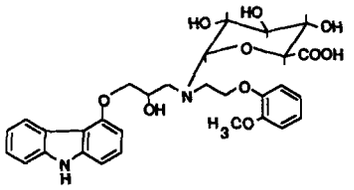


Table 2. Structures of the possible carvedilol *O*- and *N*-glucuronides and the unique patterns of acetylation expected for each

Structure	Number of acetyl groups added	
	Pyridine	Aqueous
 M9, M10	4	1
 M2	5	1
	4	0

Following reaction with acetic anhydride in pyridine, the FAB mass spectrum of M10 (Fig. 3(a)) showed an $[M + H]^+$ ion at *m/z* 751, 168 u higher than that of the unreacted metabolite. The corresponding $[M - H]^-$ ion at *m/z* 749 was observed in the negative ion spectrum (data not shown). These data were consistent with addition of four acetyl groups to the metabolite. The fragment ion observed at *m/z* 449 corresponded to monoacetylated carvedilol and resulted from elimination of the triacetylated glucuronide moiety. This result indicated that the glucuronide moiety was linked to either the hydroxyl group or the aliphatic amine group of carvedilol. When M10 was reacted with acetic anhydride in an aqueous solution, the FAB mass spectrum for the product (Fig. 3(b)) displayed an $[M - H]^-$ at *m/z* 623, 42 u higher than that of the unreacted metabolite, indicating the addition of one acetyl group. Therefore, the aliphatic amine of M10 was not conjugated. Thus, the glucuronide of M10 was conjugated to the hydroxyl group of carvedilol. Further support for this assigned was evident in the NMR spectrum of M10 (Table 2). Indeed, the chemical shift of H1′ of the sugar moiety at 4.69 ppm in the NMR spectrum for M10 was also consistent with glucuronide linkage to an aliphatic hydroxyl group.

M9 showed a retention time on HPLC which was very similar to that of M10 and the COSY NMR spectra for these metabolites were essentially identical. The results of the acetylation reactions for M9 were also

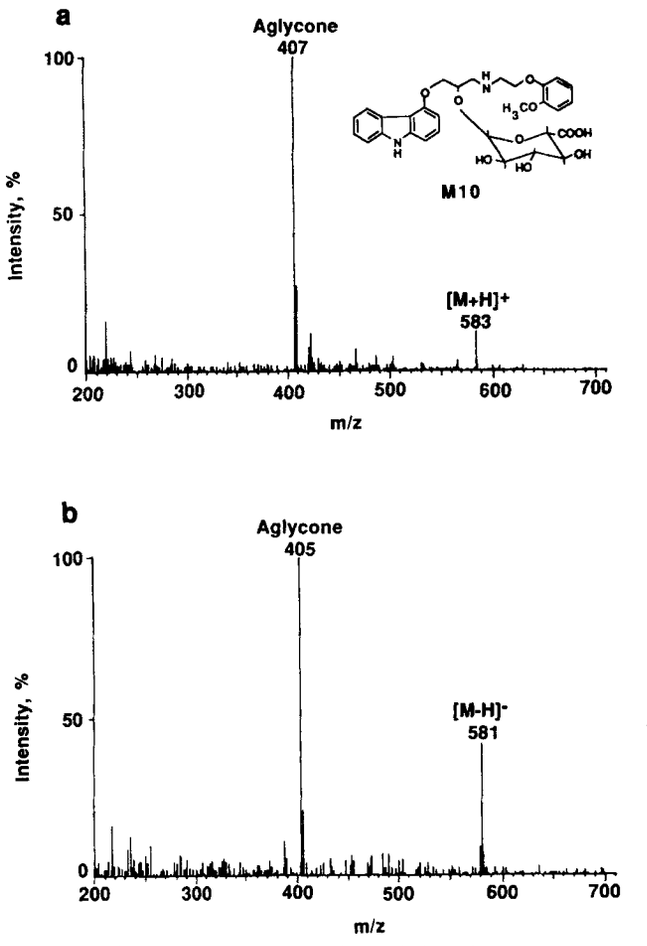


Figure 2. Positive (a) and negative (b) ion thermospray mass spectra for M10.

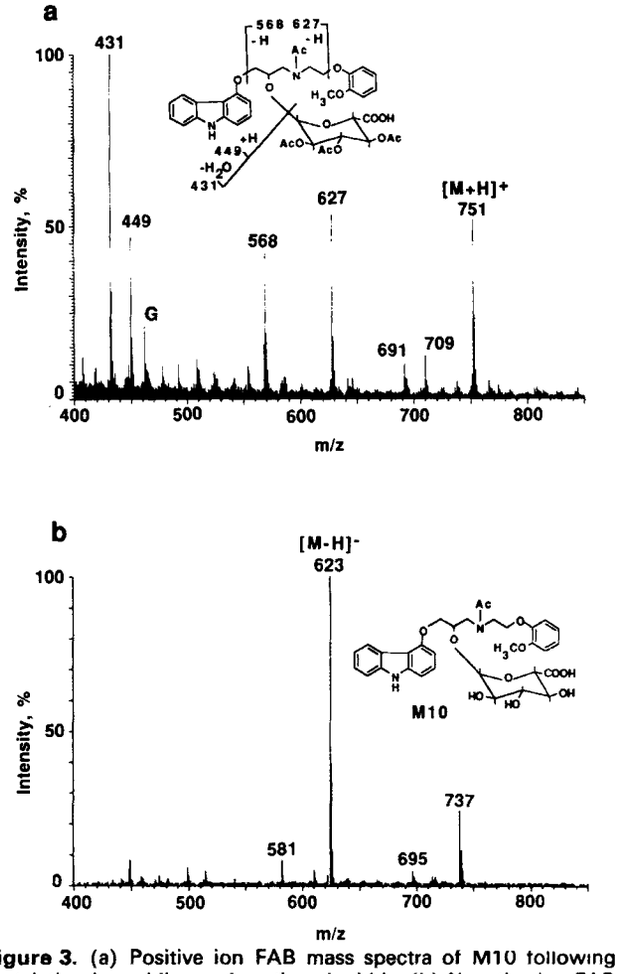


Figure 3. (a) Positive ion FAB mass spectra of M10 following acetylation in pyridine and acetic anhydride. (b) Negative ion FAB mass spectrum of M10 following acetylation with acetic anhydride in aqueous solution. 'G' denotes glycerol matrix ions.

identical to those obtained for M10. Based on these results, M9 and M10 were identified as diastereomers which resulted from *O*-glucuronidation of racemic carvedilol. Incubation of the individual *R*(+) and *S*(−) enantiomers of carvedilol with dog liver microsomes fortified with UDPGA indicated that M9 was *R*-carvedilol glucuronide and M10 was *S*-carvedilol glucuronide.[7]

Metabolite 2

Metabolite 2 (M2) was identified as a relatively minor metabolite in rate bile. It had a considerably shorter retention time (24 min) than M9, M10 or M11 (46, 49 and 53 min, respectively, suggesting a more polar structure. Mass spectra for M2 obtained using FAB and thermospray ionization (data not shown) were essentially identical to those observed for M9 and M10, consistent with a glucuronide conjugate of carvedilol. However, these spectra did not indicate the position of glucuronidation.

Consequently, M2 was subjected to further characterization using the acetylation strategy described above. Following acetylation with acetic anhydride in pyridine solution, the positive ion FAB mass spectrum showed an $[M + H]^+$ ion at *m/z* 793 and $[M + Na]^+$ ion at *m/z* 815 (Fig. 4(a)). The corresponding ions $[M - H]^-$ at *m/z* 791 and $[M - 2H + Na]^-$ at *m/z* 813, were observed in the negative ion spectrum data (not shown). Since more extensive fragmention was observed in the positive ion spectrum, the data were useful for structural assignment. The $[M + H]^+$ ion for the acetylated metabolite (*m/z* 793) was 210 u higher than that for the unreacted metabolite. This shift in mass was consistent with the addition of five acetyl groups to the metabolite. The fragment ion observed at *m/z* 491, which corresponded to diacetylated carvedilol, was formed from elimination of the triacetylated glucuronyl moiety. The presence of two acetyl groups on carvedilol, in addition to the three acetyl groups incorporated in the glucuronyl moiety, indicated that neither the aliphatic amine nor the hydroxyl group was the site of glucuronide attachment in M2. Thus, the carbazole nitrogen was the only other possible glucuronidation site. A fragment ion corresponding to loss of hydroxycarbazole (183 u) was not observed for M2, although this fragment ion was intense for acetylated carvedilol, M9 and M10. However, an intense fragment ion was observed at *m/z* 308, which corresponded to loss of both hydroxycarbazole and triacetylated glucuronic acid. Fragment ions at *m/z* 431 and 367 corresponded to loss of acetic

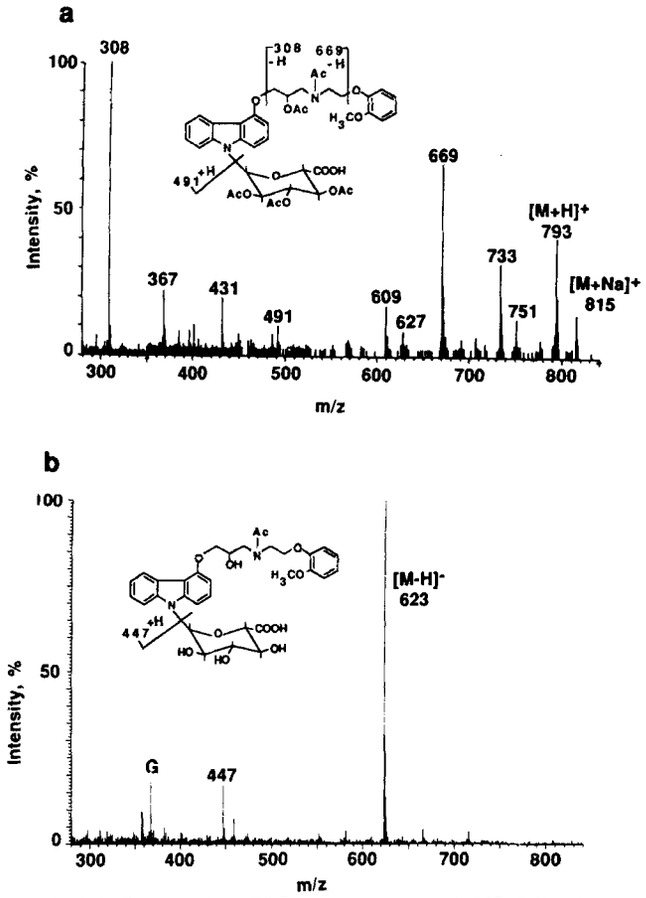


Figure 4. (a) Positive ion FAB mass spectra of M2 following acetylation in pyridine and acetic anhydride. (b) Negative ion FAB mass spectrum of M2 following acetylation with acetic anhydride in aqueous solution. 'G' denotes glycerol matrix ions.

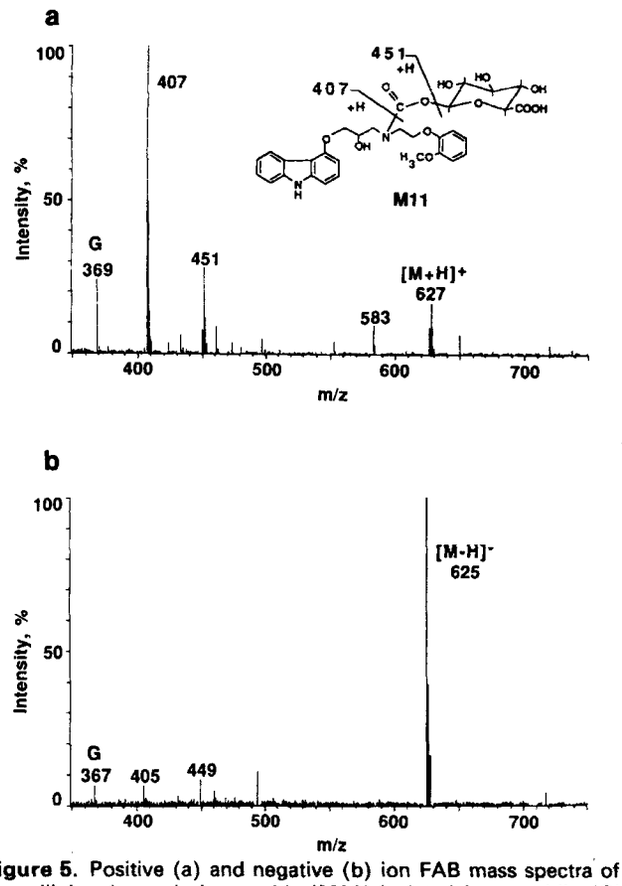


Figure 5. Positive (a) and negative (b) ion FAB mass spectra of carvedilol carbamoyl glucuronide (M11) isolated from rat bile. 'G' denotes glycerol matrix ions.

acid and methylcatechol, respectively, from the *m/z* 491 ion. Acetylation of M2 in aqueous solution resulted in the addition of only one acetyl group, which confirmed that the glucuronide was not linked to the aliphatic amine group. The negative ion FAB mass spectrum of the reaction product (Fig. 4(b)) showed an $[M - H]^-$ ion at *m/z* 623 and a fragment ion at *m/z* 447, which corresponded to monoacetyl carvedilol following elimination of the glucuronyl moiety. Further evidence for a carbazole *N*-linked glucuronide was provided by the proton NMR spectrum of M2 (Table 1). A signal for the carbazole NH (at ~11.2 ppm) was not observed in this spectrum. In addition, the anomeric proton (H1') of the glucuronic acid moiety was shifted downfield (5.79 ppm), consistent with linkage at an aromatic ring system.

Metabolite 11

Metabolite 11 (M11), a significant metabolite excreted in rat bile, was assigned as a carbamoyl glucuronide conjugate of carvedilol (see below). Figure 5 shows the mass spectra for M11 obtained using FAB ionization. The ion at *m/z* 627 corresponded to the $[M + H]^+$ ion and the fragment ions at *m/z* 451 and 407 represented protonated carvedilol carbamic acid and protonated carvedilol, respectively. In negative ion mode, an $[M - H]^-$ at *m/z* 625 was observed with little fragmentation. A *B/E* linked scan of daughter ions of *m/z* 627 (data not shown) displayed ions which corresponded to loss of the glucuronide (*m/z* 451) and loss of both CO_2 and the glucuronide (*m/z* 407). Interestingly, an ion was also observed which corresponded to loss of CO_2 alone (*m/z* 583). Pearson *et al.*[9] have also reported the loss of CO_2 from benzyl and *t*-butyl carbamate ester derivatives of glutathione conjugates during FAB mass spectrometric analyses, suggesting the CO_2 was eliminated from the carbamates via an intramolecular rearrangement. The thermospray ionization mass spectra for M11 were quite different from the FAB mass spectra. The positive ion spectrum in Fig. 6(a) showed a very weak ion at *m/z* 627. More intense fragment ions at *m/z* 407 and 433 were assigned to protonated carvedilol and a cyclic carbamate product formed from elimination of the glucuronide moiety. This cyclic product was most likely formed as a thermal degradation product in the thermospray interface and is discussed in more detail below. The corresponding ions at *m/z* 625, 431 and 405 were observed in the negative ion spectrum (Fig. 6(b)). Improved HPLC separation (using isocratic conditions, see Methods) resolved two diastereomers (see below and ref. 7). M11a and M11b were readily hydrolyzed by β-glucuronidase from bovine liver to yield carvedilol. Previous studies done *in vitro* using liver microsomes indicated that M11b was produced from *S*(−)-carvedilol and M11a was produced from *R*(+)-carvedilol.[7]

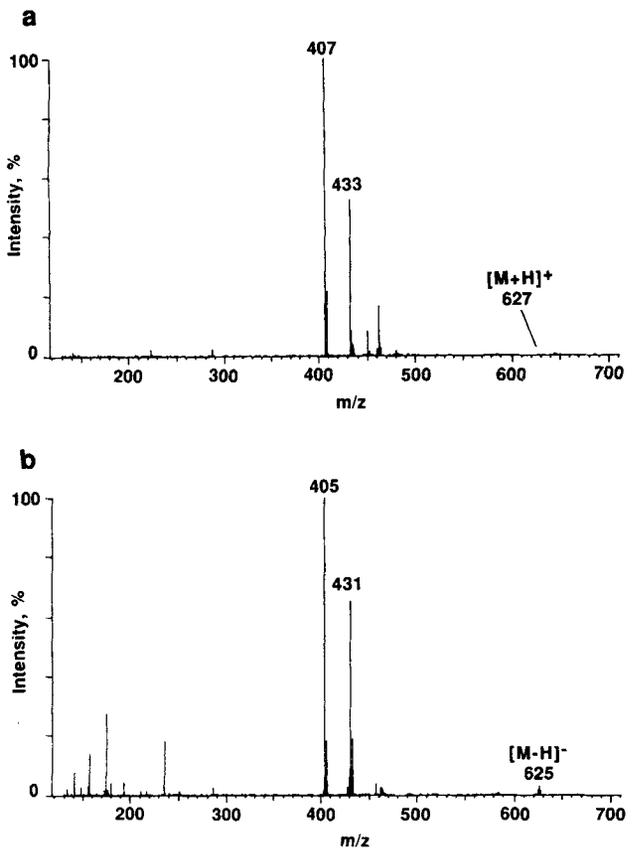


Figure 6. Positive (a) and negative (b) ion thermospray mass spectra for M11.

Proton NMR analyses of M11a and M11b, summarized in Table 1, yielded very complicated spectra. The aromatic region of the spectra for M11a and M11b showed the carbazole NH at 11.23 ppm, indicating that metabolism had not occurred at the carbazole nitrogen. However, many of the other aromatic and aliphatic proton signals appeared as two discrete resonances or appeared as complex multiplets. A similar pattern of complex aromatic signals was observed in the proton NMR spectrum for carvedilol ethylcarbamate, which has only one chiral center. This phenomenon was attributed to slow rotation about the carbamate C—N bond. Consequently, the region of the NMR spectra which described the aliphatic protons for M11a and M11b was too complex to allow interpretation on such a limited amount of material. Thus, the position of metabolism could not be determined from the NMR spectra.

M11a was characterized further using the ethanolysis reaction described by Straub and co-workers for characterization of a benzazepine carbamoyl glucuronide.[8] The metabolite was reacted with sodium ethoxide and was expected to yield carvedilol ethylcarbamate through displacement of the glucuronic acid. Authentic carvedilol ethylcarbamate was prepared for use as a reference. HPLC analysis of the ethanolysis reaction showed that a single product was formed, but this product had a slightly different retention time from that of the authentic carvedilol ethylcarbamate. Analysis of these compounds by DCI mass spectrometry confirmed that the ethanolysis product was not carvedilol ethylcarbamate. As expected, authentic carvedilol ethylcarbamate showed an $[M + H]^+$ ion at m/z 479 (Fig. 7(a)). The fragment ion at m/z 461, corresponding to elimination of H_2O, and the absence of a fragment ion resulting from elimination of ethylcarbonic acid (as well as other NMR data, Table 1), confirmed that the ethoxycarbonyl group was linked to the aliphatic secondary amine of carvediolol. However, the ethanolysis product of M11a showed an $[M + H]^+$ at m/z 433 (Fig. 7(b)) using methane as a reagent gas and an $[M - H]^-$ ion at m/z 431 using NF_3 (data not shown). Thus, the ethanolysis product weight 26 u more than carvedilol. The DCI mass spectrum in Fig. 7(c), obtained using deuterated ammonia as the reagent gas, indicated that the product had only one exchangeable proton which was on the carbazole nitrogen. The cyclic carbamate structure in Fig. 7(b) is in agreement with these data. These data also confirmed that metabolism had occurred on the aliphatic chain, but did not indicate the position unequivocally. Further investigation demonstrated that authentic carvedilol ethylcarbamate also yielded the same cyclic product (as determined from the

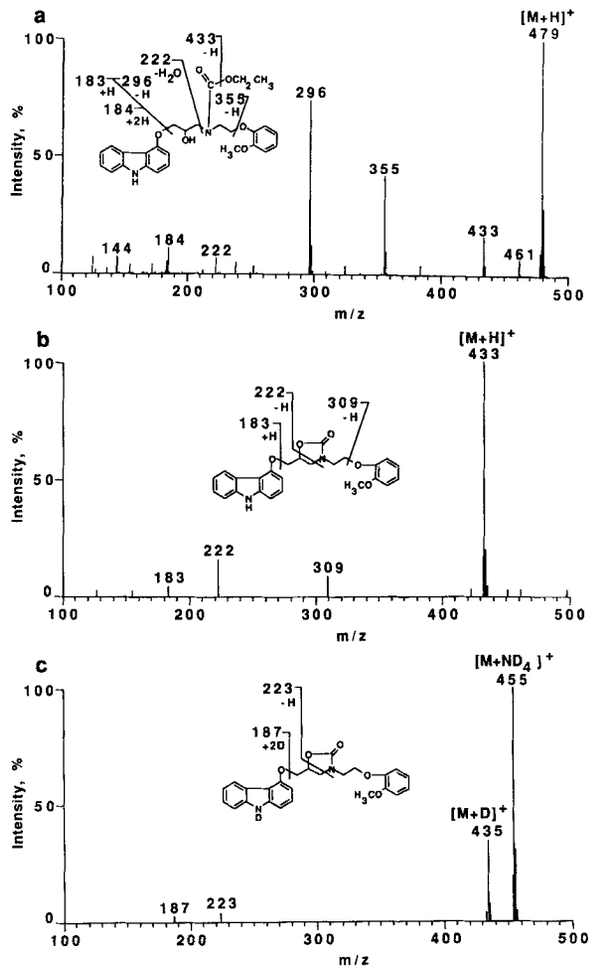


Figure 7. (a) Positive ion DCI mass spectrum of authentic carvedilol ethyl carbamate obtained using methane as a reagent gas. (b) Positive ion DCI mass spectrum of the ethanolysis product of M11 obtained using methane as a reagent gas. (c) Positive ion DCI mass spectrum of the ethanolysis product of M11 obtained using d_3-ammonia as a reagent gas.

FAB mass spectrum, data not shown) when subjected to the ethanolysis reaction. Carvedilol ethylcarbamate was, however, stable under acidic conditions. This base-catalyzed intramolecular reaction was facilitated by the ability of the aliphatic hydroxyl group of carvedilol to form a five-membered cyclic carbamate (see below). Similar base-catalyzed cyclization with elimination of glucuronic acid was reported for tocainide carbamoyl glucuronide.[13]

To locate unequivocally the position on carvedilol where metabolism had occurred, both M11a and M11b were subjected to the acetylation methods described above. Although M11 was formed by addition of CO_2 and glucuronic acid to carvedilol, the CO_2 and glucuronic acid could, hypothetically, be linked to either the aliphatic hydroxyl or amine group of carvedilol. As described for the glucuronide metabolites above, determination of the numbers of acetyl groups added to M11 could distinguish between these possible structures. Figure 8(a) shows the negative ion FAB mass spectrum for M11b following acetylation in pyridine. The $[M - H]^-$ ion at m/z 793, a shift of 168 u, indicated the addition of four acetyl groups to M11b. The fragment ion at m/z 491 in the negative ion FAB mass spectrum was consistent with monoacetylated carvedilol carbamic acid, which resulted from elimination of the tri-acetylated glucuronyl moiety. In comparison, M11b was found to be unreactive with acetic anhydride in aqueous solution as evidenced by the $[M - H]^-$ ion at m/z 625 (Fig. 8(b)). This spectrum was identical to that observed for the untreated metabolite. The results for M11b indicated that the aliphatic amine group of carvedilol was blocked from acetylation and the hydroxyl group of carvedilol had reacted in the pyridine reaction. Thus, both the CO_2 and the glucuronide were attached to the amine of carvedilol to form a carbamoyl glucuronide structure. Identical results were obtained for the diastereomer M11a. Although carvedilol carbamoyl glucuronide was unstable under the basic conditions used for ethanolysis, it was stable under the weakly basic conditions used for acetylation in pyridine or aqueous solution.

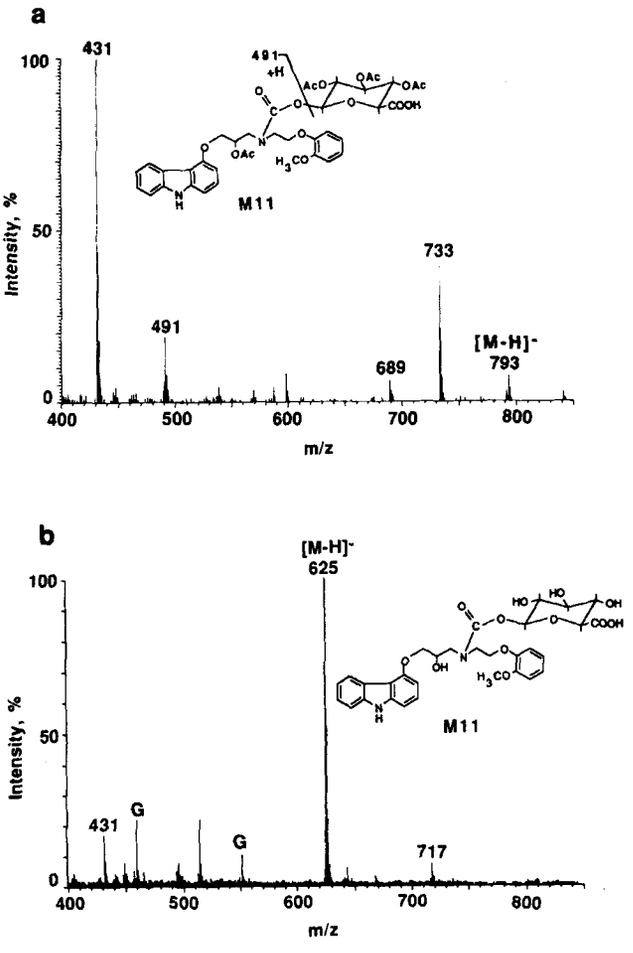


Figure 8. (a) Negative ion FAB mass spectrum of M11 following acetylation in pyridine and acetic anhydride. (b) Negative ion FAB mass spectrum of M11 following acetylation with acetic anhydride in aqueous solution. 'G' denotes glycerol matrix ions.

Several other carbamoyl glucuronides have recently been identified as metabolites of primary and secondary amine-containing compounds. These include tocainide,[13,14] the benzazepine SK&F 86466,[8] sertraline[15] and rimantadine.[16] Carbamoyl glucuronide metabolites have been hypothesized to be formed by reaction of CO_2 with a primary or secondary amine to form a carbamic acid, and subsequent glucuronidation of this carbamic acid.[8,13] Results from experiments done *in vitro* support this mechanism.[7] In the past, polar conjugated metabolites were routinely hydrolyzed prior to mass spectral analysis. Upon enzymatic hydrolysis with β-glucuronidase, carbamoyl glucuronide metabolites generate parent drug due to the instability of free carbamic acids, and could be erroneously identified as direct glucuronide conjugates. Because spectroscopic methods are currently available which are better suited for characterization of polar metabolites, many more carbamoyl glucuronide metabolites of amines are likely to be identified in the future.

CONCLUSIONS

An approach employing selective acetylation of amine and hydroxyl groups of glucuronide conjugates with acetic anhydride in conjunction with FAB mass spectrometry was used to determine positions of glucuronidation of carvedilol metabolites. Acetylation with acetic anhydride in pyridine is a common reaction for derivatization of nucleophilic amine and hydroxyl groups. Based on mass spectral analysis, the reaction of carvedilol glucuronides with acetic anhydride in pyridine reached completion in minutes. Each acetylation was readily identified in the mass spectrum of the reaction product by the addition of 42 u to the molecular ion. Additions other then 42 u (or multiples of 42 u) would indicate that additional chemical reactions or degradations had occurred. Selective acetylation of aliphatic amine groups was accomplished using acetic anhydride in aqueous solution in the presence of base. This reaction has been utilized in classical synthetic chemistry,[10] as well as for acetylation of N-termini of peptides which may contain serine and/or threonine residues.[11,12] The reaction conditions described in this report were optimized to give complete reaction on a small scale.

Without base (diisopropylethylamine), the reaction only reached approximately 60–77% completion (as determined by HPLC). In this case, the reaction was likely halted as the hydrolyzed acetic anhydride rendered the reaction mixture acidic. If too much acetic anhydride (>0.5% final concentration, v/v) was used, excessive acetylation of hydroxyl groups resulted.

Phenolic hydroxyl groups are also sites of glucuronidation and are frequently introduced via phase I metabolism. In order to determine the reactivity of phenols under the acetylation conditions described above, the acetylation of labetalol was examined as a model phenol-containing compound (data not shown). The results indicated that the phenol was acetylated in both pyridine and aqueous solutions. Thus, acetylation in aqueous solution may be used to distinguish between aliphatic hydroxyl- and phenol-linked glucuronides, but not between amine- and phenol-linked glucuronides. In cases such as this where simply determining the number of acetyl groups added yields ambiguous results, the use of tandem mass spectrometric techniques could provide additional structural information. Thus, acetylation would serve to modify or label positions which were not conjugated.

The acetylation strategy described above was a valuable aid for determining positions of glucuronidation. Although this approach was designed primarily for carvedilol glucuronides, it may be generally applicable to other glucuronide conjugates.

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