Mass Spectral Characterization of an Endogenous Digitalislike Factor From Human Plasma

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A sodium pump inhibitor has been isolated from human plasma and extensively purified. This material, endogenous digitalislike factor, was examined by a variety of mass spectrometric techniques. A low-resolution fast atom bombardment mass spectrometric analysis of a sample of purified endogenous digitalislike factor revealed a single unique molecular ion in the mass range 100-2,500. The accurate mass was determined to be 585.295 Da in a second high-resolution fast atom bombardment mass spectrometric experiment. Based on this accurate mass, the elemental composition of endogenous digitalislike factor was determined and found to be identical to the elemental composition of the known cardenolide ouabain. Direct comparison of ouabain and endogenous digitalislike factor by linked scan tandem mass spectrometry, derivatization with acetic anhydride coupled with fast atom bombardment mass spectrometry, and analytical high-performance liquid chromatography failed to reveal any differences. We conclude that the endogenous digitalislike factor isolated from human plasma is ouabain or a closely related isomer. (Hypertension 1991;17:930-935)
FIGURE 1. Low-resolution fast atom bombardment mass spectra of endogenous digitalislike factor. Spectra were acquired over a mass range of 100-2,500, only the mass range 200-800 is shown. A single unique protonated molecular ion is seen in the active fraction (panel B) at m/z 585 (*) and not in either of the adjacent fractions (panels A and C). Cluster ions from the glycerol matrix are indicated (G).
FIGURE 2. Magnetic field/electric field (B/E) linked scan tandem mass spectra of the m/z 535 parent ions of ouabain (panel A) and endogenous digitalislike factor (panel B). Inset: Structure of ouabain. A, portion of molecule responsible for ions observed at m/z 403 and m/z 439.

solved in glycerol for FABMS. A second set of samples, approximately 1 µg each of EDLF and ouabain, were acetylated with 50 µl of a 1:1 mixture of acetic anhydride and DMAP (15 mg/ml in pyridine) for 2 hours at room temperature. After excess reagent was removed under vacuum, the samples were dissolved in glycerol/thioglycerol for FABMS.

High-Performance Liquid Chromatography

HPLC was carried out on a Series 4 system (The Perkin-Elmer Corp., Norwalk, Conn.) equipped with a Model 783A UV detector (Applied Biosystems, Foster City, Calif.). Samples were chromatographed using a 4.6×250 mm Bakerbond octadecyl column (J.T. Baker Inc., Phillipsburg, N.J.) eluted isocratically with water/acetonitrile/TFA (90:10:0.1) at a flow rate of 1 ml/min and a 4.6×250 mm ASTEC Cyclobond II (gamma) column (Advanced Separation Technologies, Inc., Whippany, N.J.) eluted with water/methanol/t-butanol (94:5:1) at a flow rate of 1 ml/min.

Results

EDLF was purified from human plasma as described. Material from the final purification step, reversed-phase HPLC, was used for mass spectral characterization. The active fraction and the two adjacent fractions were concentrated, dissolved in glycerol matrix, and analyzed by FABMS. FAB mass spectra were acquired over a mass range of m/z 100–2,500. This mass range bracketed the molecular weight of EDLF, 300–900 Da, that had been estimated based on gel filtration chromatography. The FAB mass spectra of the three fractions from m/z 200 to 800 are shown in Figure 1. A single unique protonated molecular ion at m/z 585.2 was seen in the active fraction (Figure 1B) and not in the adjacent fractions (Figures 1A and 1C). A small ion at m/z 607.2 corresponding to the sodium adduct ion also was seen in the active fraction. No other unique ions were seen in the mass range 100–2,500.

A second sample of purified EDLF was used to obtain elemental composition information. The accu-
rate mass of EDLF was determined to be 585.295 Da by matching the peak at m/z 585 to glycerol cluster ion peaks at m/z 553.292 and 645.339 at a resolution of 4,000. The elemental composition, C_{29}H_{48}O_{12}, of the protonated form of EDLF was determined based on the accurate mass. The elemental composition suggested a cardiac glycoside-type structure, and inspection of the cardenolide literature revealed that the experimentally determined molecular weight and elemental composition of EDLF matched those of the cardenolide ouabain. The theoretical accurate mass of ouabain is 585.291 Da (protonated form). Subsequent experiments were designed to compare EDLF and ouabain.

To obtain additional structural information, linked scan tandem mass spectrometry was carried out on the protonated molecular ions of EDLF and ouabain. Linked scan tandem mass spectrometry is a technique that can be used to obtain fragments (or daughter ions) from molecular ions (or parent ions) of compounds ionized by FABMS and other soft ionization methods in which fragmentation usually is not observed. As shown in Figure 2, the daughter ion spectra of EDLF and ouabain are identical. Both exhibit a major fragment ion at m/z 439.2, corresponding to the aglycone portion of the molecule with addition of 2H⁺. Ions corresponding to successive losses of H₂O from the intact species (m/z 567, 549, 531, 513, 495) and the aglycone peak (m/z 421, 403, 385) also are seen. The aglycone peak results from cleavage of the glycoside bond and loss of the sugar moiety and indicates that sugar in EDLF, like ouabain, is a deoxyhexose. These daughter ion spectra are very similar to the FAB spectra of cardiac glycosides reported by Isobe et al.

Further comparison of EDLF and ouabain is shown in Figure 3. Ouabain and EDLF were acetylated with acetic anhydride/pyridine, and the products were analyzed by FABMS. After acetylation, EDLF and ouabain gave nearly identical spectra. In each case, ions corresponding to partial acetylation products, fragments due to loss of H₂O, and adduct ions were observed. The ions were assigned as follows (where M is the parent molecule, Ac is the acetyl group, and R is a cyclohexylamine contaminant): m/z 669 [M(584) +2Ac+H]⁺, m/z 693 [M+3Ac−H₂O+H]⁺, m/z 711 [M+3Ac+H]⁺, m/z 735 [M+4Ac−H₂O+H]⁺, m/z 753

Figure 3. Mass spectra of ouabain (panel A) and endogenous digitalislike factor (panel B) after acetylation with acetic anhydride/pyridine. Ions are labeled as described in text. Ac, acetyl group; R, cyclohexylamine contaminant, introduced by the glassware used for the acetylation reaction.
FIGURE 4. Comparison of endogenous digitalislike factor (EDLF) and ouabain by analytical high-performance liquid chromatography using a reversed-phase column (panels A–C) and a Cyclobond column (panels D–F). A, absorbance.

[M+4Ac+H]+, m/z 777 [M+5Ac−H2O+H]+, m/z 795 [M+5Ac+H]+, m/z 810 [M+3Ac+R+H]+, m/z 852 [M+4Ac+R+H]+, and m/z 894 [M+5Ac+R+H]+. Sodium adduct ions at m/z 733 [M+3Ac+Na]+ and m/z 775 [M+4Ac+Na]+ were observed in the EDLF sample but not the ouabain sample, consistent with the presence of sodium in the EDLF sample (see Figure 1). Because these acetylation conditions were not sufficient to drive the reaction to completion, a second set of samples was acetylated using more rigorous reaction conditions. Acetylation of EDLF and ouabain with acetic anhydride/pyridine/DMAP resulted in identical spectra, with major ions corresponding to the addition of six acetyl groups and minor ions corresponding to the addition of seven and eight acetyl groups, respectively (data not shown). The acetylation results are consistent with the structure of ouabain, which has six reactive primary or secondary OH groups and two hindered tertiary OH groups.

As a final comparison, the chromatographic behavior of EDLF and ouabain was investigated. Two different supports were selected as being likely to be able to resolve ouabain isomers. A C18 reversed-phase column was used, because Cassels reported that ouabain and acolongifloroside K, isomers that differ only in the stereochemistry of a single sugar OH, could be resolved on this support. Cyclobond columns have unique cyclo-dextrin solid supports that have been reported to be very useful for resolving a wide variety of geometric, stereo, and structural isomers. EDLF and ouabain were analyzed on a Bakerbond C18 reversed-phase column (Figures 4A–C) and a Cyclobond II gamma column (Figures 4D–F). In each case, a single peak was obtained when the samples were coinjected.

Discussion

EDLF purified from plasma from volume-expanded human patients has been extensively characterized. After initial mass spectrometric data had indicated that EDLF shared the same elemental composition and accurate mass as the known cardiac glycoside ouabain, we compared the two compounds to see if we could discover any differences. We were not able to detect any physicochemical differences between EDLF and
ouabain. In addition to the low-resolution and high-resolution mass spectral data, the linked scan tandem mass spectra were identical. Acetylation with acetic anhydride/pyridine or acetic anhydride/pyridine/DMAP resulted in identical products as determined by FABMS. Finally, EDLF and ouabain cochromatographed on two different analytical HPLC columns. Together, these results strongly suggest that EDLF either is identical to ouabain or is a closely related isomer of ouabain.

Although EDLF clearly is very similar to ouabain, the present data do not rule out the possibility that EDLF may be a closely related isomer. However, several observations argue against this possibility. First, the acetylation experiments indicate that both compounds have OH groups of similar reactivity, because both gave the same distribution of partial acetylation products. Acetylation using the DMAP catalyst indicates that both have two hindered OH groups and six more reactive OH groups. Second, EDLF and ouabain cochromatograph on two different HPLC columns. Third, no significant biochemical differences between EDLF and ouabain have been detected (unpublished observations). Finally, a specific polyclonal antibody to ouabain cross-reacts strongly with EDLF.

Until now we have referred to the inhibitor of the sodium pump we isolated from human plasma as EDLF. We used this general nomenclature because we found our inhibitor to possess many of the properties of the cardiac glycosides. Our current findings that the biochemical, physicochemical, and immunological properties of this substance are identical to those of ouabain suggest that more specific nomenclature such as ouabainlike compound (OLC) be used.

The identification of OLC as a known cardenolide raises the question of the origin of this compound. Ouabain is found in Strophanthus gratus, Acocanthera ouabaio, and possibly other plants. Ouabain is very polar and is not orally active. Therefore, it is not likely that diet is a source of OLC. It also is not likely that ouabain was introduced as a contaminant during the sample purification. Ouabain has been used clinically as an intravenous solution but is no longer in common usage. None of the patients from whom volume-expanded plasma was obtained for this study was taking ouabain or other cardiac glycosides. Furthermore, using a specific antibody to ouabain, we have shown that plasma OLC levels in the rat decrease after adrenalectomy and increase with deoxyoxygen corticosterone acetate salt treatment (unpublished observations), results that argue for an endogenous source of OLC.

The steroid nature of OLC suggests that the adrenal may be a likely site of synthesis or secretion. Several groups have shown that an EDLF with chromatographic properties similar to the one identified here is derived from the adrenal gland. Preliminary data based on the immunological studies indicate that the adrenal is a rich source of immunoreactivity and that adrenalectomy reduces plasma OLC levels (unpublished observations). Proof of the endogenous nature of OLC will require the demonstration that humans have the biosynthetic capability to produce the material.

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