Liquid Chromatography Mass Spectrometric Analysis of Ouabainlike Factor in Biological Fluid

Yutaka KOMIYAMA, Noriko NISHIMURA, Xian Hui DONG, Shinji HIROSE*, Chiya KOSAKA, Hiroya MASAKI, Midori MASUDA, and Hakuo TAKAHASHI

Ouabainlike factor (OLF), assayed as ouabainlike immunoreactivity (OLI), is a probable endogenous digitalislike factor (EDLF). Liquid chromatography/mass spectrometry (LC/MS) is one of the most highly sensitive tools for obtaining structural information regarding low-molecular weight materials in a target compound, and to measure the concentrations of these materials. We have previously reported that OLI can be isolated from the culture supernatant of the rat pheochromocytoma cell line, PC12, by several reverse-phase chromatography and LC/MS techniques. The present study was performed to characterize OLF from biological fluids such as plasma and culture supernatant of PC12 cells by LC/MS. The previous applications of LC/MS to OLI in plasma have been limited to structural identification at the final stages of isolation, in which the starting volume of plasma has been over 10 l. In the present study, we tried to minimize the volume of plasma, and to develop a new preclearing step to gain adequate LC/MS characterization using MS/MS analysis. The plasma was acidified, and OLI was purified by ODS column chromatography. OLI in chromatographic fractions from plasma was assayed by a sensitive enzyme-linked immunosorbent assay for ouabain. After Sep-Pak treatment and two rounds of ODS column chromatography, OLI was identified from 80 ml of plasma. The structure of the purified OLI was identical to authentic ouabain and digoxin, as assessed by LC/MS. In conclusion, we identified the chemically or structurally clarified ouabain and digoxin as the circulating form in plasma by LC/MS. (Hypertens Res 2000; 23 Suppl: S21-S27)

Key Words: endogenous digitalis, ouabain, digoxin, plasma, liquid chromatography/mass spectrometry

Introduction

Recent progress in the development of techniques with liquid chromatography/mass spectrometry (LC/MS) enables us to obtain structural information for low-molecular weight compounds without any chemical modification, even with very small amounts of the target compound, on the order of µg to ng. In the MS/MS analysis, the comparison of parent to fragment ions clearly shows the structural information for the target compounds, including peptides, antibiotics, hormones, and other toxins (1).

Endogenous digitalislike factors (EDLFs) have been thought to be important modulators for salt and water metabolism (2). Extensive studies have indicated that the EDLF may be ouabain or its isomer (3, 4). Hamlyn et al. have extracted ouabain from a large volume of human plasma, and others have used the bovine hypothalamus as a source of OLF (3-5). Recently, Perrin and Schneider independently isolated ouabain from several kilograms of bovine adrenal glands, and identified it using LC/MS (6, 7). However, because exogenous ouabain can be ab-
sorbed in the adrenals and released into circulation in response to stimuli, it is possible that the purified OLF could be of plant origin from the contaminated diet of the cattle (8). Conditioned medium from cultured cells may be the simplest material to use for the purification of endogenous substances. However, because the amount of EDLF produced by these cultured cells is small, the chemical structure of EDLF from culture supernatant has not been determined. We recently confirmed that PC12 cells produce endogenous ouabain in the presence of progesterone (9).

Using an ELISA system for ouabain-like immunoreactivity (OLI), we have found that the hypothalamo-pituitary axis can be the source of circulating and excreted OLI in DOCA-salt hypertensive rats (10). Based on our findings with immunohistochemical staining, it appears that the adrenal medulla could also be the source of circulating OLI (11, 12). We recently described an increase in plasma OLI during the surgical removal of a pheochromocytoma, and determined that the elution volume for OLI in pheochromocytoma tissue is the same as that for authentic ouabain using high-performance liquid chromatography (HPLC) (13). Therefore, in the present study, structural characterization of OLF in human plasma was performed to prove the presence of a circulation form of OLF.

**Methods**

In an ELISA system for OLI, samples were precleared by Sep-Pak C18 cartridges (Waters, Milford, MA), reconstituted, and applied to a 96-well ELISA plate (MaxiSorp; Nalgen Nunc International, Tokyo, Japan) after 10-fold dilution with 0.1% gelatin containing phosphate-buffered saline, as described previously (10, 13). Characteristics of the anti-ouabain antibody have been described in our previous reports (10, 13).

In the preclear of plasma for LC/MS assay, we reconstructed a preclear system for OLI to obtain not only OLI but also other digitalislike factors, using a more hydrophobic extraction by organic sorbent. Eighty milliliters of ACD plasma from healthy volunteers, who had not been treated with digitalis glycosides, was used as the starting material. The plasma was acidified by trifluoroacetic acid (TFA) to pH 2.0 and stored overnight at 4°C. The supernatant was then applied onto a Sep-Pak C18 cartridge (2 ml/cartridge, with a total of 40 cartridges for 80 ml of plasma) and washed with 0.1% TFA. OLI fractions were eluted with 50% acetonitrile (3 ml/cartridge, with a total of 40 cartridges for 80 ml of plasma); the eluate was then concentrated and reconstituted with 1 ml of 0.1% TFA. After spin, an aliquot of the supernatant was applied onto a high-pressure ODS column (Shimpak ODS, 0.6×15 cm; Shimadzu Inc., Kyoto, Japan). The column was equilibrated with H₂O/0.1% TFA at a flow rate of 1.0 ml/min, and eluted with a 0 to 50% acetonitrile/0.1% TFA linear gradient over 60 min, monitored by the ultraviolet (UV) absorbance at 220 nm. ELISA detected OLI at the specific elution volume of ouabain. The ELISA-positive fractions were concentrated and reconstituted with 0.5 ml of 1% acetic acid.

In the LC/MS analysis, the ODS fraction described above was the material for the assay. The HPLC conditions were as follows: system, LC-10 series (Shimadzu Inc., Kyoto); column, Shimpak ODS, 0.6×15 cm (Shimadzu Inc., Kyoto); flow rate, 0.5 ml/min in the main pump; and gradient, 0 to 50% acetonitrile/1% acetic acid over 30 min and to 100% in the next 10 min. For a highly sensitive detection of ouabain in MS/MS analysis, the post-column HPLC pump was connected between the HPLC column and the MS detector to mix the chloroform (0.1 ml/min). The MS conditions were as follows: system, LC/MS ESI system (ion trap LC/MS system LCQ; Thermo Quest Co. Ltd., Tokyo, Japan); probe, ESI negative; and collision-induced dissociation energy, 30%.

**Results**

To study the circulating form of EDLF in plasma by the LC/MS method, we analyzed both authentic ouabain and digoxin, used as a standard, using ODS columns, acetonitrile gradient elution, post-column chloroform mixing, and negative mode scan. In the total ion scan mode, 125 pg of ouabain was found at 20.1 min, but peaks representing ions other than ouabain were detected, as shown in Fig. 1. In the selected ion scan mode (SIM) of LC/MS analysis, ouabain, a hydrophilic steroid, was recovered at 20.1 min (below 20% acetonitrile) as an M-H ion (m/z 583.0) in the first MS analysis. In the next MS/MS analysis of the M-H ion, specific fragment ions such as m/z 437.2, 419.1, 371.3, and 353.1 were detected, as shown in Fig. 2. Digoxin, a relatively hydrophobic steroid, was eluted at 32.4 min as an M-H ion (m/z 779.2) in the first MS analysis. In total ion scan mode, a high background was detected, as shown in Fig. 3, but this background was reduced, and the m/z 779.2 peak became clear in the SIM analysis, as shown in Fig. 3. Therefore, we selected this as the parent ion. In the MS/MS analysis, the parent ion (m/z 779.2) and its specific fragment ions were identified as m/z 605.1 and 475.2, as shown in Fig. 4. In the MS/MS analysis, the collision energy and isolation width were tuned and selected according to the instruction manual. Isolation width 2 gave the most specific MS pattern. In measuring the collision energy, the highest fragment ion energy was detected in 30%.

Plasma contains large amounts of proteins and low-molecular weight substances that interfere with LC/MS analysis. In the preclear step using Sep-Pak, most proteins were removed from the plasma samples, and the ex-
Fig. 1. Total ion chromatogram of authentic ouabain. Authentic ouabain (500 pg) was applied onto an LC/MS system eluted by an acetonitrile gradient (0-50% in 30 min, and 50-100% in the next 10 min) from an ODS column, and directly analyzed by mass spectrometry. The lower panel indicates the MS/MS chromatogram of m/z 583.

Fig. 2. ESI(−)MS/MS analysis of authentic ouabain. Experimental conditions were the same as those described for Fig. 1. Left panels indicate MS (the top panel) and MS/MS chromatogram of m/z 583.1, 437.2, 419.1, and 371.3, as indicated in the panels. The right panel shows the daughter ion spectrum of the 20.1-min peak.
**Fig. 3.** Total ion chromatogram of authentic digoxin. Authentic digoxin (2 ng) was analyzed according to the method described in Fig. 1. The lower panel indicates the MS/MS chromatogram of m/z 279.2.

**Fig. 4.** ESI(−)MS/MS analysis of authentic digoxin. Experimental conditions were the same as those described for Fig. 1. Left panels indicate the MS (the top panel) and MS/MS chromatogram of m/z 779.2, 605.2, and 475.2, as indicated in the panels. The right panel shows the daughter ion spectrum of the 32.4-min peak.
tract was evaporated at neutral pH. The residue was reconstituted and applied onto the first ODS HPLC column. The eluate was collected in glass tubes (1 ml/tube) using a fraction collector, and the elution volume of OLI was detected by ELISA. A large OLI-positive fraction was eluted at 20 min, which gave the same elution volume as that of the authentic ouabain. This peak was eluted just after a UV peak. Another large OLI-positive fraction was eluted at 29 min, which gave the same elution volume as that of authentic digoxin. After this OLI peak, a large UV peak was observed. This first ODS step was repeated eight times. Finally, two ELISA-positive peaks were collected and evaporated at neutral pH.

In the LC/MS analysis, the detection was divided into three parts. During the first 10 min the eluate from the HPLC column was discarded. Between 10 and 25 min, ouabain was found in MS and MS/MS mode, because SIM using the molecular ion of ouabain (m/z 583) in a pg range had a relatively low signal/noise ratio, as shown in Fig. 5 (right panel). Specific fragment ions (m/z 437.2, 419.1, 371.3, and 353.1) from a ouabain-negative ion (m/z 583.1) were detected at a collision energy of 20% and isolation width 2 through the use of the ODS precleared plasma-derived fraction (19-min fraction), as shown in Fig. 5 (left panel), and these data corresponded with the results shown in Fig. 2. At between 26 and 35 min, digoxin was found in the MS and MS/MS mode, from the ODS precleared plasma-derived fraction (35-min fraction). The molecular ion of digoxin (m/z 779.2) and its specific fragment ions were identified as m/z 605.2 and 475.2 in the SIM detection system, as shown in Fig. 6. The collision-induced dissociation patterns of ouabain and digoxin from the plasma-derived fractions were the same as those of their authentic substances.

Discussion

The structural analysis of small amounts of a low-molecular weight compound is carried out through the use of various methods such as nuclear magnetic resonance (NMR); gas-liquid chromatography/mass spectrometry (GC/MS); LC/MS; ultraviolet spectrometry; and CD spectrometry. Although GC/MS in EI mode is a sensitive and informative method, the sample should be volatile or be changed to volatile derivatives. Recent LC/MS can analyze a target compound, even if it is not volatile, in one run using fragmentation (MS/MS analysis), similar to GC/MS analysis on a pg to ng scale. Therefore, this is one of the most sensitive and informative methods available. We analyzed plasma OLI on a pg to ng scale using LC/MS, and thus ouabain and digoxin could be identified in very small amounts.

In our previous study of PC12-derived OLI, we used the positive ion mode to characterize the structure in the purified form (9). Generally the positive ion mode provides high energy in ESI MS detection; this high energy
means, however, that all of the substances in the chromatography fraction are ionized and detected in the ESI MS detector, and give a high background in a highly sensitive detection. Therefore, in the present LC/MS study, in which MS detection in a mixture form such as the plasma-derived fraction was performed, we used the negative mode in the MS detection to minimize the noise of other contaminants from the plasma. We changed the acid in the chromatographic separation from TFA to acetic acid to increase the sensitivity. Moreover, we added chloroform between the chromatographic separation and the MS analyzer to increase the sensitivity of the MS detector. Then, in the analysis of the plasma-derived fraction, we identified the signal of both the molecular ion peak (m/z 583.1 for ouabain in the left panel of Fig. 5, and m/z 779.2 for digoxin in the left panel of Fig. 6) and their specific daughter ion spectrum (m/z 437.2, 419.1, 371.3, and 353.1 for ouabain in the right panel of Fig. 5, and m/z 779.2, 605.2, and 475.2 for digoxin in the right panel of Fig. 6). The negative ion of m/z 779.2 in the daughter ion spectrum was not the fragment ion but the parent ion of digoxin. The collision energy of 30% was the most useful for dissociating digoxin because the signal strength of the daughter ions was reduced when more energy was applied.

Structural information in the MS study revealed the following points. In the previous study, MS/MS analysis of PC12-derived OLI gave the same daughter ion spectrum for authentic ouabain (9). In particular, the m/z 439.0 fragment was the same molecular size as ouabagenin, a positive ion of aglycone of ouabain. In the present study, we detected the same aglycone-derived negative ion ouabagenin-, m/z 437.2 fragment. These data indicate that the OLI molecule in the circulating form consists of a glycosylated steroid. Furthermore, the molecular size of the sugar was the same as that for rhamnose. In the case of digoxin, the daughter ion spectrum showed fragment ion similar to digoxigenin and mono- and di-carbohydrate-moiey-deleted molecules in the circulating form. Tracqui et al. have used a similar elution pattern for LC/MS analysis of cardiotonic glycosides, although they did not assay endogenous substances (14). Collectively, from these findings as described above, EDLF, of the circulating form of EDLF and the secreted form from PC12 cells, was identified to be ouabain and digoxin not only immunochemically but also structurally. Both ouabain and digoxin were confirmed to consist of a steroid moiety such as aglycone and carbohydrate moieties based on the daughter ion spectrum from the MS/MS analysis.

Various investigators, including us, have studied the pathophysiological significance of EDLF/OLF. In most studies, EDLF/OLF immunoassay has been used, and the circulating form of EDLF/OLF has until now been chemically ambiguous. This study is the first in which the circu-
lating form of EDLF/OLF has been chemically or structurally clarified by an LC/MS technique.

In the present study, LC/MS proved to be a useful tool for the assay of OLF in various biological fluids. OLF seems to be an essential component of EDLF that regulates electrolyte and water metabolism as well as blood pressure. The development of sensitive assay methods for OLF in a chemically proven form would promote research in this field. LC/MS is a definite candidate method to this end, and can be a useful tool in elucidating the clinical significance and synthetic pathway for OLF, which will lead to a further understanding of the pathophysiology of hypertension.

References


