Application of Supercritical Fluid Chromatography to Characterize a Labile Digitalis-Like Factor

Steven W. Graves, Karen E. Markides, Norman K. Hollenberg

Abstract—A sodium pump inhibitor (digitalis-like factor), isolated from the peritoneal dialysate of volume-expanded, hypertensive patients with kidney failure who were treated with this dialysis modality, was further purified and characterized by means of supercritical fluid chromatography, a separation technique whose application to very-low-concentration biomolecules is new. Previous studies suggested that after high-performance liquid chromatography (HPLC) purification, this inhibitor was the only factor correlated with volume status and blood pressure in these patients. When this same HPLC fraction was furthered purified on 2-dimensional supercritical fluid chromatography, a single peak coeluted with [Na,K]ATPase inhibitory activity. When split specimens were used, there was a strict correlation between the peak area, measured by flame ionization detection, and activity (n=10, R=0.98, P=0.00001). Inhibitory activity after supercritical fluid chromatography was still correlated with the degree of volume expansion of donor patients (P=0.01). After HPLC purification, this volume-sensitive inhibitor was chemically labile. With further purification on supercritical fluid chromatography, the active peak was still labile with comparable half-life. Supercritical fluid chromatography coupled with flame ionization detection provided an estimate of the amount of the inhibitor present. Again using split specimens, we determined that the labile digitalis-like factor was 30-fold more effective than ouabain in inhibiting renal [Na,K]ATPase activity and 500 times more effective than ouabain in causing vascular smooth muscle contraction. The data suggest that we have purified to homogeneity a labile digitalis-like factor that is readily distinguished from ouabain or bufalin, based on chromatographic characteristics, chemical lability, and a much lower effective concentration for its biological activity. (Hypertension. 2000;36:1059-1064.)

Key Words: sodium pump ■ sodium ■ hypertension, sodium-dependent ■ ouabain

The recognition of the natural ligands of the opiate receptor1,2 raised the interesting possibility that other endogenous ligands exist, the action of which is mimicked by potent pharmacological agents. Among these, candidates have included the cardiac glycosides, which are known to interact with a highly conserved specific binding site on the active subunit of the sodium pump or [Na,K]ATPase. Inhibitors of the [Na,K]ATPase (termed digitalis-like factors, DLF) have not only been identified as present in plasma but have also been shown to increase after sodium loading.3–6 Elevations in plasma levels of this inhibitory activity have correlated well with the development of hypertension, both in experimental models and in patients.3–9

Over the years, a large number of candidates for the sodium pump inhibitor have been proposed.10 Recent attention has been focused primarily on an ouabain-like agent that was characterized from human plasma obtained from patients undergoing routine plasmapheresis.11 More recently, evidence for a bufogenin-like factor has also emerged.12 We have identified another candidate present in the peritoneal dialysate (PD) of volume-expanded patients with chronic kidney failure, which preliminary data suggest differs from ouabain, including the demonstration of its having chemical lability.13,14

To confirm the difference in the PD DLF and ouabain unambiguously and to evaluate the amount and representative activity of highly purified preparations of this additional candidate, we sought a separation method that provided robust separation capabilities, allowed for coupling to sensitive, quantitative detectors, and provided a less reactive environment during purification. The application of a novel biochemical separation technique, supercritical fluid chromatography (SFC, described in more detail in Methods below) has facilitated our characterization of this PD candidate in patients with chronic kidney failure and has allowed us to make assessments of the purity of this material, the amount present, and the concentrations at which this material is active in comparison to ouabain. This represents the central line of investigation in this report.

Methods

Clinical Protocol

Patients with kidney failure who are treated by chronic ambulatory peritoneal dialysis were recruited to a clinical protocol approved by

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the Committee on Human Research at the Brigham and Women’s Hospital. Each subject gave informed, signed consent before participation in the study. Patients were maintained on their current diuresis regimen; however, their sodium intake was increased and fluid intake was kept at 2.5 to 3.0 L per day as described in detail elsewhere. This brought about a gradual and sustained volume expansion, which has previously been shown to increase levels of a labile sodium pump inhibitor in their serum, and based on these previous studies, this same factor appeared in their PD.

**Production of Purified Labile Sodium Pump Inhibitor**

The first 2 L of PD of the day were submitted to a series of rapid purification steps. Initially, 1 g of ascorbic acid was added to each liter of dialysate because of evidence of chemical lability. The PD was bubbled with argon while ultrafiltered through a 1000-D exclusion membrane with a tangential flow ultrafiltration device (Centrasert, Filtron) that filtered ~1 L/10 min of dialysate. The ultrafiltrate was submitted to solid-phase extraction by its being pumped through a preparative C18 reverse-phase guard column (45 mm × 11 cm, Dynamax, Rainin) at a rate of 20 mL/min. The active fraction was eluted with 25 mL of methanol, dried, and redissolved in a small volume of water (1.0 mL). This material was active fraction was eluted with 25 mL of methanol, dried, and redissolved in a small volume of water (1.0 mL). This material was then loaded onto a semipreparative C8 reverse phase column and eluted with an ethanol–water–0.1% TFA gradient as described elsewhere. This was followed by analytical high-performance liquid chromatography (HPLC) fractionation with a C8 reverse phase column eluted with a second ethanol–water–0.1% TFA gradient. The product of a single 2-L PD specimen was processed during the morning of the collection day and used for further experimentation within a few hours of its purification. The DLF used here was the only candidate that was undetectable in PD when patients were euvoletic and increased in proportion to the rise in body weight that followed the liberalization of salt and fluid intake. Blood pressure and serum activity of this factor rose in proportion to the rise in body weight and in proportion to DLF levels in the simultaneously acquired PD.

**Assessment of DLF Stability After HPLC Fractionation**

PD was submitted to HPLC fractionation and DLF was collected from the previously determined elution envelope and divided into 2 equal portions. Both halves were taken to complete dryness to remove the organic solvents present in the HPLC mobile phase (ethanol, 0.01% trifluoroacetic acid). Immediately thereafter, half was reconstituted in an aqueous assay buffer and was analyzed for its [Na,K]ATPase inhibitory activity. The other half was redissolved in distilled water and stored overnight frozen under argon at ~80°C. Storage was in silanized glass vials. The following day, fractions were thawed, dried quickly (rotary evaporator), and brought up in assay buffer, and the inhibitory activity was measured. Other less polar cardenolides (strophantidin or bufalin) were also processed identically to assess resolubilization in assay buffer and their stability.

**Two-Dimensional SFC**

SFC has features of both traditional gas chromatography (GC) and HPLC. The columns used can be either open tubular, similar to those used in HPLC. The mobile phase is very free of dissolved oxygen and is not particularly reactive and the mobile phase is easily and rapidly removed. SFC allows for the quantitative delivery of specimen to detectors such as flame ionization detection (FID), which can provide quantification of resolved materials with a sensitivity of ≤0.1 ng. The major disadvantages of SFC are its limited availability; highly polar molecules are not soluble in the mobile phase, and current commercial systems do not allow for the quantitative introduction of a specimen. Typically, commercial SFC moves a very small fraction of a much larger specimen from a precolumn onto the column. However, these limitations have been overcome through instrumental modifications that more appropriately address purifications of microscale and nanoscale quantities of physiological molecules. More sophisticated 2D systems (2D-SFC) allow for the interfacing of 2 SFC columns having different column coatings or packings and thus provide for orthogonal separation capabilities.

**Assay of Inhibition of [Na,K]ATPase Activity**

The assay monitored the influence of the labile sodium pump inhibitor on [Na,K]ATPase ATP hydrolysis. To determine the rate of hydrolysis, we used [γ32P]ATP (Amersham) and canine renal [Na,K]ATPase (Sigma Chemical Co) in a final volume of 130 μL of buffer (containing in mmol/L: Na 100, K 5, Mg 3, EGTA 1, Tris 80, pH 7.5, 37°C) in the absence or presence of labile DLF after correction for non-[Na,K]ATPase activity (residual activity in the presence of 10−6 ouabain). The reaction was started by adding 10 μL of 40 mmol/L [γ32P]ATP (Amersham) and run for 30 minutes. Ouabain in graded concentrations was used for comparison. DLF activity was expressed as the percent inhibition of [Na,K]ATPase hydrolysis.

**Vascular Smooth Muscle Contraction**

The procedures used on animals complied with the guidelines of the Committee on Animals of the Harvard Medical School and have been described in detail previously. In brief, thoracic aorta were harvested freshly from 15 New Zealand White rabbits. Strips of tissue (~2 mm × 10 mm) were mounted on a pressure transducer in a 4.0-mL muscle bath containing modified Krebs-Henseleit solution (in mmol/L: NaCl 118.2, KCl 4.6, CaCl 2 1.25, KH2PO4 1.2, MgSO4 1.2, NaHCO3 24.8, and dextrose 10.0) and placed under 2.5 g tension at 37°C. The chamber and solution were aerated with 95% oxygen/5% carbon dioxide. Buffer solutions containing half of a specific preparation of the labile sodium pump inhibitor replaced the equilibration buffer, and changes in contractile force were recorded on a polygraph recorder (Grass Instruments). The other half of the labile DLF was used for quantitative assessment. Vascular smooth muscle cell (VSM) response to graded ouabain concentrations was also measured in duplicate.

**Relation Between Clinical Volume Status and Post-SFC DLF Activity**

Weight gain during the clinical protocol was taken as a measure of volume expansion. The increment in weight over baseline was...
determined at the time of PD collection and divided into 3 response ranges and compared with the inhibitory activity of labile PD DLF from the same dialysate after both HPLC and SFC. DLF activity was divided into 3 levels of inhibition.

Statistical Analysis

Data are expressed as mean±SEM. Comparisons of split DLF specimens measured immediately and after being maintained frozen for various periods >24 hours were made by paired t test. Correlation between two parameters was assessed by Pearson’s product moment correlation analysis. Comparison of weight gain to labile DLF activity used a 3×3 matrix evaluated by Spearman’s rank correlation test. Probability values <0.05 were considered statistically significant.

Results

Preparations of the labile DLF, representing the active HPLC fraction, were submitted to SFC. The only SFC peak that demonstrated consistent and significant inhibition occurred between 12.7 and 14.7 minutes of elution in the first dimension and at ~18.3 minutes in the second dimension for these experiments. A sample chromatogram is shown (Figure 1). Although this retention time was highly characteristic of this factor for columns and conditions used here, column or frit (needed for collection) replacement, even with comparable pieces, can result in small variations in elution time. Inclusion of internal control materials ensured that the same peak was identified and ation and at 

**Figure 1.** Representative 2D SFC chromatogram. SFC 1 represents FID output during linear gradient elution (from 70 to 180 atm) in first dimension. Two internal controls (IC 1, N-eicosane; IC 2, n-hexatriacontane) are identified. SFC 1 eluate between 12.7 and 14.7 minutes was moved to a second column representing second-dimension SFC 2. SFC 2 elution occurred over 40 minutes with linear pressure gradient from 70 to 340 atm. Eluate was again monitored by FID; starred peak at 18.3 minutes represents labile DLF.

and half assayed immediately after removal of HPLC solvents. Half was dried, dissolved in water, and stored frozen at −80°C under argon for 1 to 7 days and then assayed immediately after thawing. DLF activity was lost with a half-life of 25.3±9.1 hour (n=6). Strophanthidin and bufalin, both cardiac glycosides with comparable or reduced water solubility, showed no loss of activity when processed similarly (data not shown).

After the volume-sensitive, labile DLF had been isolated by HPLC and subsequently submitted to SFC FID, the single peak was still labile, showing a 58% reduction in amount (area under curve) when split specimens were analyzed, half on the first day of study and half 24 hours later (Figure 2, n=13; t=0: 408±80 U of area versus t=24 hours: 170±36 U of area, P=0.004) after being frozen (<−80°C) under argon in aqueous solution. The mean of the half-lives calculated for each pair was 34.7±13.0 hours, which was not different than the half-life of the HPLC-purified DLF (Figure 3, P=0.56).

The initial amount of inhibitor estimated by FID from individual purifications was variable but probably represented the degree of volume expansion. In other experiments, [Na,K]ATPase inhibitory activity purified from a single collection of dialysate was significantly related to the degree of volume expansion in the same patient at the time PD was obtained (Table, P<0.01).

The labile DLF, purified by SFC, produced a concentration-dependent inhibition of [Na,K]ATPase hydrolysis, decreased with time after partial purification on HPLC, as noted previously. In this study, those observations were confirmed with the use of additional specimens. The HPLC prepared labile DLF was divided in 2 equal portions

**Figure 2.** Chemical lability of labile DLF isolated from PD purified by HPLC and SFC. Region of HPLC eluate containing inhibitor of [Na,K]ATPase activity, correlated with volume status and blood pressure in patients on peritoneal dialysis, was divided, half applied to SFC immediately and half 24 hours later. Region of SFC eluate consistently demonstrating [Na,K]ATPase inhibition, represented by single peak, was monitored. This peak demonstrated significant reductions (n=13, P=0.01) in area under the curve (AUC) after 24-hour delay.

**Time Interval between Measurements**
magnitude) to those of ouabain but at concentrations 30 times lower. Fifty percent inhibition occurred at a concentration estimated as $1.7 \times 10^{-28}$ mol/L for the labile inhibitor compared with $5.4 \times 10^{-27}$ mol/L for ouabain. There was a strict relation between the amount of material after 2D SFC estimated by FID of half the specimen and the inhibition produced by the other half ($n=10$, $R=0.98$, $P=0.00001$, Figure 4).

Other specimens of the labile DLF were split, half assayed against VSM and half assessed by SFC FID for quantity. Graded concentrations of ouabain were applied to other strips of aorta for comparison. The labile DLF and ouabain produced concentration-dependent increases in contraction of rabbit aortic rings (Figure 5). The response threshold for the labile DLF was $1.5 \times 10^{-28}$ mol/L as compared with $1 \times 10^{-27}$ mol/L for ouabain. One gram of contraction was achieved at a concentration of $1 \times 10^{-28}$ mol/L for the labile DLF and $5 \times 10^{-27}$ mol/L for ouabain.

### Discussion

Supercritical fluid chromatography provides a powerful purification technique that has greater separation efficiency than liquid chromatography and uses a mobile phase that is readily removed by decompression of the liquefied gas, allowing it to be coupled easily to sensitive and quantitative ionization techniques such as FID or mass spectrometry. The mobile phase, because it is a gas, is very

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**Comparison of Weight Gain of Subject at Time of PD Collection and DLF Activity Recovered From Dialysate After SFC Purification**

<table>
<thead>
<tr>
<th>[Na,K]ATPase Inhibition</th>
<th>$\Delta$Wgt≤1.0 kg</th>
<th>$\Delta$Wgt 1.1–2.0 kg</th>
<th>$\Delta$Wgt&gt;2.0 kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤15%</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>15%–25%</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>&gt;25%</td>
<td>0</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

Numbers in the matrix represent the number of independent collections of PD and are classified according to the subject’s weight gain during protocol vs the amount of DLF activity, measured as inhibition of [Na,K]ATPase activity, present in the SFC DLF fraction purified from a collection.

$\Delta$Wgt indicates increment in weight over baseline (as a measure of volume expansion) of PD patients during the clinical protocol. $P<0.01$ Spearman’s rank correlation test.
clean, which is highly desirable when working with trace quantities of an unknown. In this particular application, HPLC-purified specimens of the labile DLF were applied to SFC, and, as described in previous work, gave rise to a single region of DLF activity, which coincided with one peak completely resolved from other peaks present in the SFC eluate. When we divided HPLC-purified DLF specimens in half, the area of the peak obtained from half the specimen, measured by its ionization response, was strictly correlated with the inhibitory activity of the other half. The close relation between a single region of activity and the composition of the two tissues and to an apparent preferential effect of the labile DLF for rabbit $\alpha_1$ isoform compared with its effects on canine $\alpha_1$. In addition, the labile DLF demonstrated enhanced inhibition when compared with comparable concentrations of ouabain for both tissues, especially so against rabbit aorta. This probably is caused by the labile DLF having somewhat greater affinity for the $\alpha_1$ (dog kidney) but a markedly greater affinity for the $\alpha_2$ isoform (abundant in rabbit vascular smooth muscle) compared with ouabain.

Both ouabain and bufalin were applied to SFC. Ouabain was insufficiently soluble in the supercritical CO$_2$ to allow detection, consistent with its being more polar than the labile DLF. Bufalin was readily soluble and provided a consistent and stable FID peak, the location of which was several minutes later in the first SFC chromatogram.

In summary SFC provided a useful tool in the purification of the labile DLF from residual impurities present even after multiple HPLC purifications. It also easily distinguished the labile DLF from other cardenolides or bufadienolides, specifically ouabain and bufalin. The ability to couple SFC with FID allowed us to characterize the labile DLF concentration-activity profile and demonstrate that this factor has a profound contractile effect on rabbit VSM, with detectable changes achieved at subnanomolar concentrations, a concentration-response remarkably different than that of ouabain. One final but future consideration in the use of SFC is that it can be coupled to a mass spectrometer and allow for important further characterization of this inhibitor of [Na,K]ATPase despite its inherent instability.

Acknowledgments

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References


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