Development and Preliminary Application of a New Assay for Aldosterone Stimulating Factor

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SUMMARY A new assay method has been developed to measure aldosterone stimulating factor (ASF) quantitatively. This method utilizes a combination of affinity chromatography and high pressure liquid chromatography (HPLC). Antibodies raised against ASF, coupled to Affi-Gel 10, were used as the affinity column, and adsorbed ASF was eluted with 4 M urea. Quantitation was based on the external standard method of analysis using the area of the standards as controls, which were previously tested by HPLC for purity. The amount of ASF in the unknown samples was calculated on the basis of the area of the peak emerging at the retention time of ASF. A parallel bioassay using adrenal glomerulosa cells was also done to assess the biological activity. In eight normal volunteers, the urinary ASF was 146 ± 4.6 ng/24 hr urine, whereas in plasma it was 70.5 ± 6.5 ng/100 ml. The method is reproducible, specific for ASF, and showed less than 3% inter- or intraassay variability. In a preliminary study, when ASF was quantified from the 24-hour urine of two patients with adrenal hyperplasia, a significantly higher level of ASF was found (750 and 1020 ng/24 hr urine). These data suggest that ASF may be of pathological significance in certain hypertensive patients, especially in the hypertension associated with hyperaldosteronism. (Hypertension 5 (supp I): 1-27—1-31, 1983)

KEY WORDS • aldosterone stimulating factor • quantitation • bioassay • high pressure liquid chromatography • adrenal hyperplasia • affinity chromatography

At least four humoral factors have been shown to play important roles in the regulation of aldosterone production, namely, angiotensin II, ACTH, sodium, and potassium.1 2 In addition, experimental evidence has suggested that other as yet unidentified factor(s) may also play a role in controlling aldosterone production. Mulrow et al.3 isolated a substance from human urine that stimulated aldosterone production from rat adrenal glomerulosa tissue. However, they did not pursue this study because that substance failed to produce a dose-related steroidogenic response. Other investigators4-6 also reported evidence implicating the central nervous system as a possible source of an aldosterone stimulator. Recently Sen et al.7 reported the isolation of a protein fraction from normal human urine which stimulated aldosterone production and, upon chronic administration, produced hypertension in rats. The hypertension was associated with hypervolemia and hyperaldosteronism, and appeared dependent on the presence of adrenal glands, since it failed to produce hypertension when administered to adrenalectomized animals on maintenance doses of corticosterone. Subsequently, we reported8-10 the complete purification of the protein fraction and partially defined its mechanism of action. The compound has been identified as a glycoprotein with a molecular weight of 26,000 daltons. The compound has been named aldosterone-stimulating factor (ASF).

ASF stimulated aldosterone production in a dose-related manner both in vivo and in vitro8 and has been localized in the anterior lobe of the pituitary gland by immunohistochemistry.10 The experimental evidence obtained to date suggests that ASF is defined as a hormone. To evaluate its physiological significance, it is important to develop an assay system to quantitate ASF in plasma, urine, and tissue.

This paper describes the development of an assay system using affinity chromatography in combination with high pressure liquid chromatography (HPLC) to quantitatively measure ASF. This method was validated by a parallel bioassay using adrenal glomerulosa cells to determine the steroidogenic effect of ASF by measuring aldosterone production in vitro.
Preparation and Purification of Antibodies

Antibody against ASF was prepared in a goat by intradermal injection of 150 μg of ASF in combination with complete Freund's adjuvant. The goat was immunized at 3-week intervals; the first three intervals with complete Freund's adjuvant and from then on with incomplete Freund's adjuvant. Blood samples were taken prior to each immunization, and antibody titer was determined before each immunization. After 16 weeks of immunization, the plasma obtained from the goat was found to contain antibody against ASF, which was confirmed by Ouchterlony's technique. The antibody was purified and concentrated in the following manner.

The plasma was diluted 1:1 with water and the protein precipitated by ammonium sulfate (40% saturation) and kept overnight at 4°C. It was then centrifuged and the precipitate washed three times with saturated ammonium sulfate solution. At the end of this procedure, the precipitate was dissolved in one fourth the original volume of 0.05 M sodium acetate, pH 5.0. This was then dialyzed against the same buffer for 24 hours at 4°C and then centrifuged. The supernate containing the antibodies was stored at −40°C for future use. By Ouchterlony's technique, this antibody has been found to form a single precipitin line and does not cross-react with ACTH, angiotensin II, or β-lipotropin, as has been reported in earlier publications.

Preparation of Affinity Column

The antibody was coupled to Affi-Gel 10, N-Hydroxysuccinimide ester agarose, by the following method: Affi-Gel 10, obtained from Bio-Rad Laboratories, was washed with reagent grade isopropanol (three bed volumes) followed by three bed volumes of deionized water at 4°C. The gel cake was suspended in Hepes buffer (0.1 M, pH 7.5) at 1 g/ml, transferred to a flask, and cold antibody ligand added. The ratio of ligand to gel was 0.5 ml of ligand, prepared as described above, per ml gel suspended in Hepes buffer, and it was agitated sufficiently to make a uniform suspension. The mixture was then agitated on a rocker for 4 hours at 4°C. The unreacted sites on the agarose were blocked by the addition of 0.1 ml of 1 M ethanolamine HCl, pH 8; 1 hour was allowed for completion of the blocking reaction. The gel was then transferred to a column and washed with 0.1 M Hepes buffer, pH 7.5, until the gel was free of reactants which were detected by monitoring the absorption at 280 nm. The gel column was then washed with 0.1 M ammonium formate buffer at pH 7.0. At this stage the gel was ready for absorption.

Either filtered urine (150 ml) or plasma (20 ml) was passed through this column, and the column washed with three bed volumes of 0.1 M ammonium formate buffer, pH 7.0. The ASF was eluted from the column with 4 M urea. A known aliquot of the eluate (100 to 250 μl) was applied to the HPLC column for quantitation. When the affinity column was not in use, it was stored at 4°C after washing thoroughly with 0.1 M ammonium formate buffer containing 0.02% sodium azide. Prior to using the column again, the column was thoroughly washed with the ammonium formate buffer before application of the next sample.

Quantitation by High Pressure Liquid Chromatography

The quantitative measurement of ASF by HPLC was conducted using four prepacked columns in a series I-60, I-125, I-125, and I-250, which resulted in the best chromatographic resolution for ASF. The mobile phase was freshly prepared 0.1 M ammonium formate buffer, pH 5.5. Peaks eluting from the column system, were detected by monitoring absorbance at 280 mM with a model 440 UV detector (Waters Associates), and the peak area electronically integrated using a Data Module (Waters Associates). Quantitation was based on the external standard method of analysis using standards which had been previously tested by HPLC for purity and stability. The amounts of ASF in the unknown samples were calculated on the basis of the area of the peak emerging at the retention time of ASF in comparison with the peak area of the ASF standards.

Methods

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Isolation and Purification of Aldosterone-Stimulating Factor

Pure ASF has been used as the external standard for quantitation. The method for isolation and purification of ASF has been described previously. It involved precipitation of ASF from 24-hour human urine collections which have been kept refrigerated during collection and frozen at −5°C while awaiting processing. ASF was adsorbed by benzoic acid, and then the benzoic acid was removed by dissolving it in Tris-ethanol buffer at −10°C. ASF was purified by conventional biochemical techniques including ion exchange chromatography, gel filtration, affinity chromatography, and HPLC. After this purification, ASF has been demonstrated to be homogenous by several criteria.

Bioassay of Aldosterone-Stimulating Factor

The detailed method for dispersion and preparation of adrenal glomerulosa cells for bioassay has been described previously. About 150,000 cells per tube were used for assay, and each assay was done at least in duplicate. The cells were incubated for 2 hours at 37°C under 95% O₂ and 5% CO₂ in the presence of different concentrations of ASF. The aldosterone produced at the end of the 2 hours of incubation was measured by radioimmunoassay.

Extraction of Aldosterone-Stimulating Factor

ASF was extracted from pituitary and other tissues by homogenization first in 2 ml of 1:1 ethanol and saline per gram of tissue and then with 2 ml of saline per gram of tissue. After each extraction, the mixture was centrifuged at 10,000 rpm for 20 minutes. The supernatants were combined, lyophilized, and then ASF extracted and measured by affinity chromatography and HPLC, with the result expressed as ng ASF per mg organ protein as measured by the Lowery method.
MEASUREMENT OF ASF/Sen el al.

Results

Quantitation of Aldosterone-Stimulating Factor by High Pressure Liquid Chromatography

When pure ASF was applied to the HPLC column at different concentrations, a linear dose-related curve was obtained, as shown in figure 1. The curve is linear from 1 to 50 ng, and when 5 to 50 ng of ASF were applied to the HPLC column good recovery was obtained. This method of quantitation has been found to be extremely reproducible. The percent recovery has been found to average 85%, as summarized in table 1. ASF, after its passage through the HPLC column, retains full steroidogenic activity, suggesting that quantitation of ASF in this manner does not destroy its biological activity. The reproducibility of the retention time was found to be excellent (less than 1% difference in retention time upon multiple injections in a calibrated system).

Isolation of Aldosterone-Stimulating Factor from Urine and Plasma

The basis of this assay method for quantitation of ASF in biological samples is affinity chromatography using antibodies raised against ASF. Therefore, the specificity of the antibody is extremely important. The antibodies raised against ASF in goat have been checked for cross-reactivity for all other known aldosterone secretagogues. Antibodies raised against human ASF fully cross-react with ASF isolated and purified from dog and rat urine. However, they do not cross-react with ACTH (both 1-24 and 1-39), β-lipotropin, angiotensin II, or renin, showing that this antibody is specific for ASF. The efficiency and specificity of the affinity column is demonstrated in figure 2. When crude ASF was applied to HPLC, it separated into at least 6 peaks of UV absorbing material. However, when crude ASF preparation was initially purified...
with the affinity column, only one peak emerged at the specified retention time of ASF (18.0 minutes). When this peak was collected from the HPLC column, it was found to be fully active.

To assess the reliability and reproducibility of the assay method, ASF was measured from six aliquots of pooled normal human urine samples, and less than 2% intraassay variability was observed. To check the day-to-day assay variability, ASF was measured from the same pool on 3 successive days; again, less than 3% variability was found.

The above data suggest that the assay method developed is reproducible, reliable, and has exceedingly good recovery. The lowest reliable range for assay for this HPLC method is 2.5 ng of ASF.

Quantitation of Aldosterone-Stimulating Factor from Normal Human Urine

When 24-hour urine samples were collected from eight normal volunteers and ASF measured, the average amount of ASF present was found to be 146.8 ± 4.7 ng/24 hr. The individual data from the eight volunteers are summarized in table 2. Each sample was measured in duplicate and the mean of each duplicate is presented. The lowest value of ASF in this particular group was found to be 120 and the highest was 162 ng/24 hr urine.

When 20 ml of human plasma was processed in the same fashion to measure circulating ASF, an average of 70.5 ± 6.5 ng/100 ml plasma was obtained from five normal individuals, with a range of 45–95 ng/100 ml of plasma.

Distribution of Aldosterone-Stimulating Factor in Other Organs

ASF was extracted from pituitary glands from dogs and human beings and found in the pituitaries in a concentration of 465 ng/ml dog pituitary protein and 385 ng/mg human pituitary protein (table 3). However, when other organs, namely, human posterior pituitary, heart, muscle, and adrenals were extracted in a similar fashion and measured by the HPLC and affinity chromatography method, no detectable ASF was found.

Bioassay of Aldosterone-Stimulating Factor

When ASF isolated from urine was measured by bioassay, the amount found was similar to the HPLC results. The data are summarized in table 2. A correlation coefficient of \( r = 0.789, \ p < 0.01 \), indicates a good correlation between bioassay and direct affinity-HPLC assay.

Quantitation of Aldosterone-Stimulating Factor from 24-Hour Urine of Patients with Adrenal Hyperplasia

When two patients with known adrenal hyperplasia (a detailed description of the patients is given elsewhere in the journal) were tested for ASF, the amount of ASF in the 24-hour urine was found to be significantly higher than in normal volunteers. The data are summarized in table 4. As shown, the 24-hour urine of patient G.H. contained 750 ng/24 hr ASF vs 146 ng/24 hr found in normal volunteers. In patient G.F. (with bilateral adreneneral hyperplasia), the ASF was 1020 ng/24 hr urine. The ASF was undetectable in urine from a hypophysectomized patient.

Discussion

These studies demonstrate the development of a new assay system to measure ASF from urine, plasma, and other tissues using a combination of affinity chromatography and HPLC. The method appears to be specific for ASF, reproducible, and reasonably sensitive. The recovery of ASF has been found to be excellent (85%) with little intra- and interassay variability.
Using this assay system we have demonstrated that ASF is present in the anterior lobe of the pituitary gland (387 ng/mg protein), that it circulates in the blood of normal human volunteers (70 ng ± 6.5/100 ml plasma), and that it is excreted in the urine (146 ± 4.6 ng/24 hr urine). This suggests that ASF could possibly be a new hormone that may modulate aldosterone production.

In our previous publications, we have shown that ASF is readily distinguishable from other known aldosterone secretagogues ACTH, angiotensin II and β-lipotropin. We have demonstrated that ASF is a potent stimulus for aldosterone biosynthesis in isolated rabbit, rat, cat and human (unpublished observations) adrenal capsules. We have also shown that ASF shares some similar requirements for its steroidogenic activity as angiotensin II and ACTH. However, the finding that specific competitive antagonists of angiotensin II and ACTH do not inhibit its activity suggests that ASF is different from any aldosterone stimulating factor described so far. Brown et al. and Kern et al. also reported the isolation of an aldosterone stimulating factor from human urine. Whether it is similar to ASF has not yet been determined.

In a preliminary study, when ASF was measured in the urine from two patients with aldosterone disorders (adrenal hyperplasia), a fivefold increase in urinary ASF (146 ± 4.6 vs 885 ± ng/24 hr) demonstrated a possible pathological significance of ASF. The present study provides evidence that ASF could possibly be a new circulating hormone and may play an important role in the regulation of aldosterone production. However, its role in different forms of hypertension, especially those associated with aberrations of aldosterone production has not yet been determined. With the development of this assay system, it is now possible to define the regulatory mechanism of ASF production in humans as well as in experimental hypertension.

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