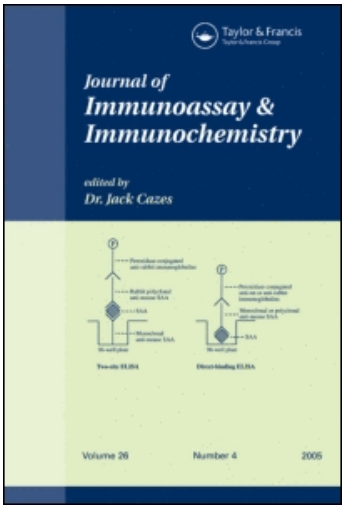

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ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR DETERMINATION OF PLASMA ALDOSTERONE USING HIGHLY SPECIFIC POLYCLONAL ANTIBODIES

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ABSTRACT

Two enzyme-linked immunosorbent assays were established and compared for the estimation of plasma aldosterone. In the first method immobilized aldosterone-protein complexes on the ELISA plates compete with aldosterone to be determined for the binding of certain amount of anti-aldosterone antibodies. The sensitivity of this method depends on the protein carrier used to conjugate with aldosterone. In the second method, anti-aldosterone antibodies adsorbed on ELISA plates compete for binding of known amount of the enzyme-labeled aldosterone and aldosterone to be determined. The highly specific rabbit anti-aldosterone antibodies were obtained by injection of aldosterone-oxime thyroglobulin. The detection limit of aldosterone in both methods ranged between 2-20 pg. The proposed assays are suitable for the determination of aldosterone in biological fluids compared with other reported ELISA assays, as well as with RIA.

KEY WORDS: Aldosterone, enzyme immunoassay

INTRODUCTION

Aldosterone, the most potent naturally occuring mineralocorticoid, is uniquely synthesized in the zona granulosa of the adrenal cortex. Its main physiological function is

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regulating the metabolism of sodium and potassium ions, mostly by stimulating sodium reabsorption in exchange for potassium and hydrogen ions in the distal tubule of the kidney. Determination of aldosterone in biological fluids is important for the diagnosis of a variety of diseases related to hypertension. The aldosterone concentration in human plasma and saliva are relatively low in comparison with other steroid hormones. The aldosterone level varies between 18 - 79 pg/ml, and after hormone stimulation between 38 - 140 pg/ml (1). Therefore, sensitive and specific assays are needed for its determination in biological fluids.

The earliest method for aldosterone determination involved the double isotope approach (2) which was followed by radioimmunoassay procedures (3 - 6). Due to the obvious inconveniences of the above methods an immunoenzymatic assay has been developed. This method was recently applied for the determination of several steroid hormones (7). Two articles have been published recently describing the establishment of an enzyme immunoassay for urinary and plasma aldosterone using monoclonal antibodies against aldosterone (8,9).

In the present study we developed and compared two different ELISA assays for plasma aldosterone using highly specific polyclonal anti-aldosterone antibodies. The sensitivity of the assays was evaluated according to the limit of detection of aldosterone, defined as the lowest concentration differing significantly from the zero standard. Concentration of aldosterone needed for 50% competitive inhibition of binding of a

known amount of antibodies by a certain amount of labeled aldosterone was used as another criterion for evaluation of sensitivity of the proposed assays. Both methods proposed are characterized by simplicity, reproducibility and high sensitivity required for their clinical applications.

MATERIALS AND METHODS

The following products were purchased from Sigma Chemical Co.: Bovine Thyroglobulin (TG) #T1001; Carboxypeptidase A (CPA) #C0386; Alkaline phosphatase (AP) #P5521; Alcohol dehydrogenase (ADH) #A3263 and Diaphorase (DI) #D2381. Horseradish peroxidase (HRP), and β-galactosidase labeled Goat anti-rabbit IgG, p-nitrophenyl phosphate (pNPP) and o-nitrophenyl β-D-galactopyranoside (oNPG) were purchased from Amersham.

Preparation of aldosterone-protein derivatives

Aldosterone-21-acetate and aldosterone 18,21-diacetate were prepared according to Bayard et al. (2).Aldosterone-3-carboxymethyloxime 18,21 diacetate was prepared as described by Janoski et. al. (10). Its purity was checked by TLC on silicagel using the following solvent system: acetone/methanol 1:8 and toluene/acetone/methanol 1:1:1.

Aldosterone-3-carboxymethoxime 18,21-diacetate was conjugated using N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (11,12) to the following proteins: thyroglobulin, carboxypeptidase A and alkaline phosphatase. The conjugates were denoted as Aldo-TG, Aldo-CPA and Aldo-AP. Determination of the number of steroid

residues per one protein molecule was carried out by spectrometric analysis at 240 nm of the steroid-protein conjugates in PBS, compensated by the subtraction of the absorption due to the amount of the protein present in the complex (13).

The aldosterone-alkaline phosphatase complex, in addition as a coating antigen, was used as enzymatic marker for determination of unknown amounts of plasma aldosterone.

The enzyme activity of conjugated alkaline phosphatase was measured by both, the conventional assay using pNPP as substrate and the amplified method (14). This was done by adding 10 µl samples containing a range of concentrations of the conjugate to microtitre plate wells and assaying them as follows:

Conventional assay: 80 µl of 5 mmol/l pNPP in 0.9 mol/l diethanolamine buffer, pH 9.8, containing 0.5 mmol/l $MgSO_4$ was added to each well and incubated for 30 nmin at 25°C. The enzyme reaction was then stopped by addition of 270 µl of 2 mol/l NaOH and the increase in absorbance at 405 nm over a reagent blank recorded.

Enzyme amplified assay: 80 µl of substrate consisting of 0.2 mmol/l NADP in 50 mmol/l diethanolamine buffer, pH 9.5, containing 1.0 mmol/l $MgCl_2$ was added to each sample to be assayed by the amplified method and incubated for 15 min at 25°C. Further phosphatase activity was then inhibited and cycling commenced by the addition of 220 µl of a solution consisting of: 0.4 mg/ml ADH, 0.4 mg/ml diaphorase and 0.55 mmol/l p-iodonitrotetrazolium violet (INT) in 25 mmol/l sodium phosphate buffer, pH 7.2, containing 4% (v/v) ethanol. The colour development was stopped

after a further 15 min incubation by addition of 50 µl 0.4 mol/l HCl and the increase in absorbance at 492 nm over a reagent blank recorded using a Titertek Multiskan MC microtitre plate reader.

Preparation of TG-aldosterone antiserum

Three rabbits were immunized with the Aldo-TG conjugate according to Vaitukaitis et. al. (15). Each rabbit was intramuscularly injected with a total of 2.5 mg of the conjugate emulsified in Freund's complete adjuvant. After two and three months booster injections of 1.5 mg of conjugate each in Freund's incomplete adjuvant were given. The antiserum was partially purified by successive precipitation with saturated ammonium sulfate, dialysed and the amount of protein determined by spectrophotometric measurement at 280 nm. The apparent binding constant of these antibodies was calculated from the free concentration of the antibodies which bind 50% of total amount of coated antigen (16). The antibody titers of the antiserum were measured by an ELISA method using Aldo-CPA, Aldo- AP, CPA and TG as coating antigens. The amount of bound antibodies was monitored using a second anti-rabbit immunoglobulin labeled with β-galactosidase. Enzyme activity of β-galactosidase was determined according to manufacturer. The specificity of the antiserum for aldosterone was determined by measuring the cross-reactivity with various steroids by radioimmunoassays (RIA).

Preparation of aldosterone sample from human plasma

Frozen male plasma was allowed to thaw and a 0.5 ml sample was shaken with 12.5 ml of dichloromethane. The extract was evaporated with nitrogen and the residue was dissolved in PBS

containing 0.1% of gelatin. The extraction was repeated twice and amount of extracted aldosterone was determined by measuring the optical density at 240 nm. The E_{240} -extinction coefficient of pure aldosterone is 17000. Recovery experiments were performed as follows. Aldosterone (5-10 pg/ml) was added to human plasma and extracted in a similar way. The detection of added aldosterone, as well as the aldosterone from human plasma, were measured and compared. The calibration curve with native aldosterone and steroid-free human plasma were performed under similar experimental conditions.

Competitive assay with immobilized antigen

Aldo-AP or Aldo-CPA or Aldo-TG (0.1 - 7.3) µg/ml (100 µl) were added to each well of an ELISA plate (Nunc) and incubated overnight at 4°C. After washing the plate with PBS containing 0.05% Tween 20 non-specific sites were blocked with 0.1% of gelatin. The aldosterone antiserum (2µg/ml) was added at a dilution corresponding to the inflection point of the titration curve, together with known amounts of aldosterone, 1-1000 pg/well, or with the sample to be determined in a total volume of 100 µl. The reaction mixtures were incubated for 1 hour at 37°C. The amount of bound antibody was measured by the addition of either β-GAL or HRP conjugated anti-rabbit IgG. The enzymic activity of β-GAL or HRP were determined according to the manufacturer's instructions. The standard calibration curve was obtained with known amounts of aldosterone.

Competitive assay with immobilized antibody

Aldosterone-TG antibodies (1 μg/ml, 100 μl in PBS) were added to the wells of ELISA plates and incubated at 4°C. After washing and blocking, as described above, a known amount of aldosterone treated similar to the sample was incubated with a constant amount of Aldo-AP conjugate in PBS containing 0.1% gelatin. After incubation for 1 hour in 37°C the wells were washed and the amount of AP corresponding to the amount of bound aldosterone was measured either by a standard method employing p-nitrophenyl phosphate as substrate, or by the amplified method of Johannsson et al (14) using iodonitrotetrazolium violet as previously described.

RESULTS

Aldosterone derivatives were conjugated with various protein carriers, such as CPA, AP and TG, in order to elicit the production of highly specific antibodies, for the labeling of aldosterone, as well as for adsorption of aldosterone on the ELISA plates.

Conjugation with TG yielded a product containing an average of 44 aldosterone (Table 1) residues per protein molecule. This conjugate was injected into rabbits in order to elicit production of antibodies. The titer of antibodies was determined by the ELISA assay using Aldo-CPA and/or Aldo-AP as shown in Fig. 1. As can be deduced from the titration curve, 50% binding occurs at a dilution of 1:9000 for Aldo-AP and 1:20,000 for Aldo-CPA (Fig. 1).

TABLE 1

Comparison of the sensitivity of the different enzyme immunoassays for determination of plasma aldosterone.

A.

Aldosterone conjugate	Molecular weight of the carrier (kD)	Moles of Aldosterone per mol of protein	Transformation number(+)	Detection* limit pg	Concentration* of Aldosterone which inhibits 50% of binding pg/well
Aldo-CPA	35	22	0.63	500 +/- 50	5000 +/- 50
Aldo-TG	660	44	0.067	20 +/- 2	70 +/- 7
Aldo-AP	116	7	0.060	2 +/ -0.2	20 +/- 2

B.

Anti-aldosterone				5 +/- 0.5	50 +/- 5

* Each figure in the table represents the mean data obtained from five different experiments with a standard deviation varying between 4-10%.

(+) moles of aldosterone per kilodalton of protein

The calculated apparent binding constant of the polyclonal antibodies to aldosterone was found to be $3 \times 10^{9-1} M$ (see Fig. 1 insert). The antibody titer obtained from these titration curves was relatively high as compared to data reported in literature, probably due to the high content of aldosterone molecules bound to

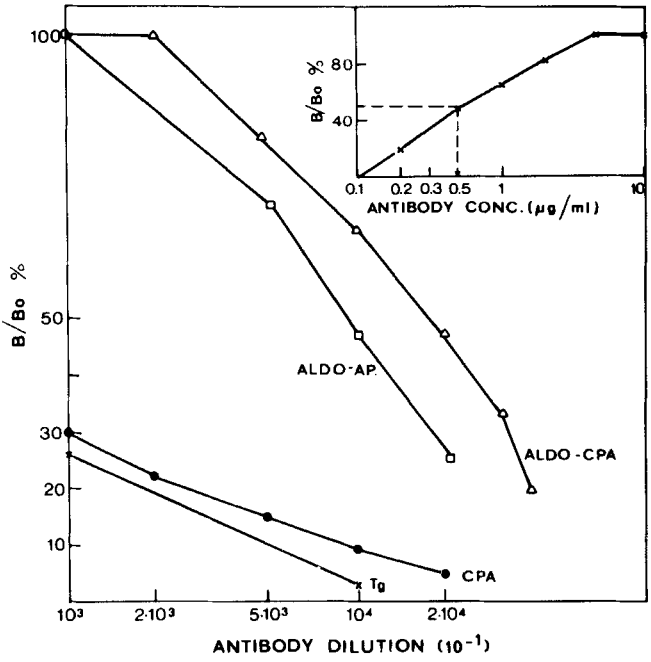

FIGURE 1

Antibody dilution curves obtained with different aldosterone-protein conjugates coated on the ELISA plates. The curves show the percentage of binding of decreasing concentrations of anti-aldosterone antibodies to the complexed aldosterone (B), relative to the saturation value (Bo) AP (not shown), TG and CPA were used for comparison as coating antigens. The amount of bound antibody was followed by determination of enzymic activity of B-galactosidase labeled anti-rabbit immunoglobulin.

In the insert of the Figure, the binding constant of anti-aldosterone to Aldo-CPA is measured, based on the concentration of free antibody which binds 50% of the total antigen.

TABLE 2

Cross-reactivity of anti-aldosterone antibodies with various steroids measured by radioimmunoassy. These data were obtained from Professor Vecsei, Department of Pharmacology, University of Heidelberg, FRG.

Steroids	% Cross-reactivities with anti-aldosterone
Aldosterone	100
5a-Dihydroaldosterone	64.4
Tetrahydroaldosterone	4.6
Corticosterone	0.4
18-Hydroxycorticosterone	0
Cortisol	0
Progesterone	0

the TG. CPA, AP and TG were used for coating as negative control. Evaluation of the enzyme-immuno assays proposed in this paper for the determination of plasma aldosterone and aldosterone itself were characterized regarding specificity, sensitivity, precision and accuracy. (17). Specificity of the antiserum for aldosterone was determined by measuring the cross-reactivity with various steroids by RIA assay and results are presented in Table 2. The cross-reaction with corticosterone, cortisol and progesterone were below the detection limit of the assay, suggesting the high specificity of those antibodies to aldosterone, confirmed also by the high binding constants of the immuno complex ($\sim 10^{+9} M^{-1}$) of

aldosterone and its antibodies. Sensitivity of these immunoassays are evaluated according to curve sensitivity which is defined by detection limit for identification of pure analyte in buffered solutions, whereas the assay sensitivity is defined by a detection limit for the analyte in the presence of a variety of possible interferring components, both steroidals and non-steroidals. In extraction immunoassays, solvent extraction may sometimes minimize this interference and provide a system in which overall sensitivity and curve sensitivity do not differ significantly. In all the experiments performed free aldosterone was treated in the same way as aldosterone of human plasma to avoid this inconvenience. Another parameter used in this work to characterize the sensitivity of the assay was the concentration of aldosterone that induced 50% competitive inhibition of binding a fixed amount of antibodies by a known amount of labeled aldosterone.

Determination of plasma aldosterone, as well as the calibration curves of aldosterone itself, in buffer and/or extracted in a similar way to plasma aldosterone, was performed in two different ways. In the first method the calibration curves of aldosterone, as well as the plasma aldosterone to be determined, were carried out by competitive assay with immobilized antigens. Aldo-CPA (10 μg/ml), containing 22 moles aldosterone per mole of protein, adsorbed on the ELISA plates was reacted with a mixture of unknown amounts of free plasma aldosterone, preincubated with anti-aldosterone antibodies and quantitated by

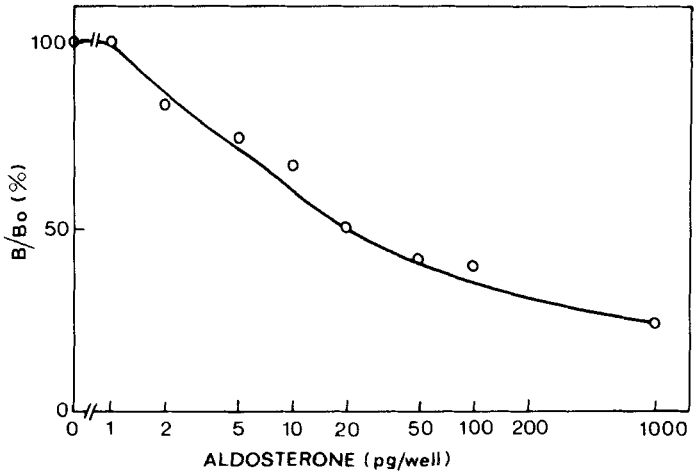

FIGURE 2

A typical standard curve for the displacement of aldosterone antibodies from immobilized aldosterone-protein conjugates by different amounts of aldosterone in solution. Plasma aldosterone dilutions, as well as free aldosterone extracted in a similar way as plasma samples, are in good correlation. The coefficient of variation for the important points of the curve (detection limit and concentration of aldosterone at 50% inhibition) are given in Table 3. The wells coated with the Aldo-AP (200 ng/ml) (100 ul) and the aldosterone antibodies dilution used was 1:9000.

addition of enzyme labeled anti-rabbit IgG. Fifty percent inhibition occurs at relatively high concentrations of aldosterone. The detection limit of aldosterone observed in this system was 500 pg (see Table 1). The measurements of aldosterone concentration with another immobilized antigen, Aldo-TG (44 moles aldosterone per mol protein) (10 µg/ml), by using anti-rabbit Ig antibodies conjugates with HRP, improved the sensitivity of the assay. Fifty percent inhibition occured at 70 pg/well of

aldosterone. The sensitivity obtained by this assay is sufficient for the measurement of aldosterone in body fluid. The best results were obtained by coating with Aldo-AP (0.2 µg/ml) containing 7 mole of aldosterone/mol protein (Table 1a, Fig 2), followed by sequential addition of free aldosterone and/or plasma dilutions previously incubated with polyclonal anti-aldosterone. The residual amount of antibody which remains after binding of aldosterone to be determined was measured with commercial second antibody labeled with HRP . This assay has the higher sensitivity, a detection limit of 2 pg - and 50% competition between the free and bound aldosterone on the plate occured at 20 pg/well. The results are shown in Fig. 2 and summarized in Table 1a.

In another type of assay the aldosterone concentration was measured by coating with anti-aldosterone antibodies, followed by competition between aldosterone to be measured and Aldo-AP conjugates (Table 1b). The enzymic activity of alkaline-phosphatase complex was found to be 80% as compared with the activity of the amount of native enzyme measured under same experimental conditions. By this method 50% inhibition was obtained at 50 pg/well and the detection limit of aldosterone was 5 pg.

In a variant of this method, the activity of the AP was detected not only through the use of the standard substrate but by the use of an amplifying chain of enzymes, as was previously described by Johannsson et. al. (14) (Fig. 3). The use of this

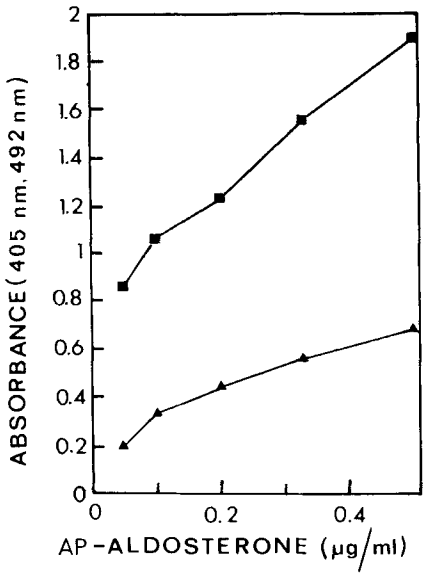

FIGURE 3

Determination of enzymatic activity of Aldo-AP complex bound to anti-aldosterone antibodies measured by standard methods (Δ Δ) and by amplified enzymatic method ( ). Enzyme activity of alkaline phosphatase, determined by standard method, was followed by increase in optical density at 405nm and the amplified enzymic method by increase in optical density at 462nm (for details see experiment).

modification resulted in overall higher OD values, increasing the sensitivity of the assay for low concentrations of aldosterone.

Precision - The precision of the standard curve of human plasma aldosterone and aldosterone itself are appreciated by intra-assay and interassay coefficients of variation and results summarized in Table 3. The standard deviation of the reading of the mid-point of the standard curve was +- 10%. Both developed assays are

TABLE 3

The reproducibility of the enzyme immunoassay for determination of aldosterone.

Aldosterone	Coefficient of variation % Intra-assay (n = 5)	Inter-assay (n = 5)
20 pg/well	0.32	0.04
2 pg/well	6.32	1.28

sensitive enough for the assay of plasma aldosterone level and the reproducibilities adequate for clinical assays.

Accuracy of the assay was checked by the linearity of data obtained by assaying several dilutions of sample containing known concentrations of steroid, or monitoring the recovery of known amounts of steroid added to biological samples. Five-ten pg aldosterone added to 100 ul of plasma or buffer increased the measured aldosterone content by 5-10 pg +/- (0.5-1). Recovery of aldosterone is quantative, indicating that in normal conditions no interference of other components with anti-aldosterone occured.

DISCUSSION

Many investigators have been turning to enzyme immunoassay to replace radioimmunoassay since it frees them from the problems

and hazards of handling radioisotopes and the necessity for expensive counting instruments. Several such assays have been developed for various steroids and recently for aldosterone (7-9). An evaluation of sensitivity of various ELISA determinations on plasma aldosterone, with regard to detection limit and its concentration in biological fluids that induce 50% competitive binding inhibition of a fixed amount of antibodies by a known amount of labeled aldosterone, as well as specificity, precision and accuracy of the assays, are discussed. Comparison of the records obtained in each ELISA using immobilized aldosterone complexed with various proteins (CPA, AP, TG) indicated that the transformation number (the number of aldosterone residues per kilodalton of protein) seems to determine the sensitivity of the assay. Apparently, low density of aldosterone on the protein carrier yields a more sensitive assay. Presumably this conclusion can be argued as follows: Each antibody molecule has two binding sites for its antigen. When exposed to a protein aldosterone conjugate containing many aldosterone residues (high transformation number) the anti-aldosterone antibody would bind to the conjugate via both its binding sites and therefore with a higher apparent binding affinity to the conjugate than to the free diluted aldosteroned solutions. This would result in lower sensitivity measurements of free aldosterone. When exposed to a conjugate with low transformation number, (ideally one aldosterone molecule per protein) only one of the binding sites of the antibody would bind to the conjugate and under such conditions the

affinity of the antibody for the conjugate should be similar to the affinity of the antibody for the free aldosterone, resulting in higher detection sensitivity of free aldosterone.

The production of anti-aldosterone antibodies using Aldo-TG complex containing 44 moles of aldosterone per mole of protein as on immunogen was a good choice with respect to the high specificity and affinity of the antibodies. However, for coating the ELISA plate, Aldo-AP complex containing only 7 moles aldosterone per mole of protein increased at least ten times the sensitivity of the assay as compared with data obtained with Aldo-TG or Aldo-CPA.

The established assays for plasma aldosterone, using immobilized antigen (Aldo-protein complexes) or immobilized anti-aldosterone antibodies, possess high sensitivity, as mentioned for RIA (3-6), and possess all the advantages of ELISA assays, such as simple non-hazardous materials and reproducibility. The normal range of plasma aldosterone, which varied between 18-79 pg/ml (1), as well as the elevated values observed after hormone stimulation associated with pathological states, can be measured at high accuracy by both methods.

A comparison between the two assays proposed in this paper showed that they differ primarily in the nature of the reagents used, which make the first type of assay more convenient. In this assay (with immobilized antigen) only one type of aldosterone protein conjugate needs to be prepared. In principle, the same conjugate can be used for both immunization of the rabbits and for

running the assay. The biological activity of the conjugated protein is of no importance and conjugates with high and low transformation number may be easily prepared, and the rabbit antiserum used as a second antibody is a commercial preparation. Moreover the nature of the second antibody may be easily changed and different methods for signal amplification compared e.g. the use of the avidin biotin system. The second type of assay (with immobilized antibody) is more difficult to set up. Two conjugates are needed, one with high transformation number for immunization, and another with low transformation number for testing. The protein used for testing has to be an enzyme and its enzymatic activity has to be retained after conjugation. The antibody used for the coating should be at least partially purified. Altogether, this comparison indicates that the first type of assay is far easier to set up and is also easier to manipulate and improve.

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