

A SOLID PHASE RADIOIMMUNO ASSAY FOR URINE ALDOSTERONE USING ANTIBODIES LINKED TO NYLON NETS

A. SCHÄFER and H. FAULSTICH

Max Planck Institut für Medizinische Forschung, Abteilung Chemie, D69 Heidelberg, Jahnstr. 29, W-Germany

and

P. VECSEI and H. HOBLER

Pharmakologisches Institut der Universität, D69 Heidelberg, W-Germany

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1. Introduction

Since the pioneering work of Erlanger et al. [1] and Mayes et al. [2], a large number of radioimmunoassays for aldosterone in urine or plasma have been described [3–17]. Most of these assays depend on chromatographic procedures to separate aldosterone from other steroids. Only in the last year have sera against aldosterone been produced that are specific enough to allow aldosterone assays omitting chromatographic purification steps [14,18]. Recently, an aldosterone assay on cellulose support has been described [16].

In this study we conjugated sheep serum proteins against 21-aldosterone-hemisuccinate to surfaces of nylon nets. The nylon surfaces were activated as *N*-hydroxysuccinimide esters, a procedure, which guarantees a minimum damage of the biospecific properties of the proteins and also permits the recovery of non reacted protein material [19]. Small pieces of 0.65 m² of nylon coated with 0.08 µl of serum proved most sensitive for the valuation of aldosterone in a solid phase radioimmunoassay (type A). Nylon nets reacted with a ten-fold volume of serum (type E) showed maximum binding capacity for aldosterone and were used to extract the steroid specifically from aqueous solutions. This extraction was suited to substitute chromatographic procedures. A combination of the two methods may one day allow the automatization of aldosterone radioimmunoassays.

2. Material and methods

Serum proteins against aldosterone-21 hemisuccinate were prepared in sheep according to Vecsei [17] and used without further purification. Hydrolysis of nylon nets, succinylation, and activation as *N*-hydroxysuccinimide esters have been described by us [19]. In the case of assay nets type A, 5 µl of antiserum of sheep 3 [17] in 20 ml of 0.154 M NaCl were coupled to 40 cm² of activated nylon surface under magnetic stirring for 18 hr at room temperature. After washing twice in 0.154 M NaCl the nets were stored in the same medium at 4°C. For preparation of extraction nets type E 50 µl serum proteins were reacted analogously.

Radioimmunoassay for aldosterone in 0.05 M borate buffer solutions of pH 8 was performed by adding 73–84 pg of [1,2-³H]aldosterone (specific activity 53 Ci/mmol, Amersham), which corresponded to 4350–5000 cpm, to 1 ml of the solution of aldosterone to be assayed in an Eppendorf vessel. The mixture was incubated with 0.65 cm² of the nylon assay net A for 1 hr at room temperature under shaking in an Eppendorf rotation mixer. After incubation the nets were taken out, rinsed twice with water, put into 15 ml of dioxane scintillation liquid and counted. Cross reaction with other steroids was determined according to Abraham et al. [20].

In urine samples the 18-*O*-glucuronide of aldosterone was estimated. The free steroids were removed from

urine by extraction with dichloromethane. Aldosterone was released from its glucuronide by acidic hydrolysis at pH 1 for 24 hr and extracted with dichloromethane, followed by partitioning between benzene and water. From the aqueous phase the aldosterone was extracted by nylon nets type E and measured after elution with dichloromethane following the conventional procedure of radioimmunoassay [5].

The thermostability of the antibodies attached to nylon was checked either by the release of bound $[1,2-^3\text{H}]$ aldosterone from nylon nets, or by determination of the $[1,2-^3\text{H}]$ aldosterone binding capacity of nylon nets type E after 1 hr exposure in 0.154 M NaCl to temperatures between 20 and 100°C. Stability in organic solvents was measured by determination of the $[1,2-^3\text{H}]$ aldosterone binding capacity after exposing the nylon nets type E under shaking to different organic solvents for 5 min at room temperature.

3. Results and discussion

In plasma samples Martin and Nugent [21] and Gomez-Sanchez et al. [13] separated aldosterone from other steroids by binding it to antibodies and by isolating the aldosterone antibody complex. In urinary extracts prepared for the estimation of acid-labile aldosterone 18-glucuronide, these free steroids are absent, because they were extracted with dichloromethane prior to the acidic hydrolysis. Since the other substances in urinic extracts, which interfere with the aldosterone radioimmunoassay, are not yet identified, we compared directly the purification of aldosterone achieved by nylon net extraction with those achieved either by a paperchromatographic procedure or by simple partition between benzene and water. In fig. 1 the values of aldosterone obtained by net extraction (black points) and those obtained by partition (white

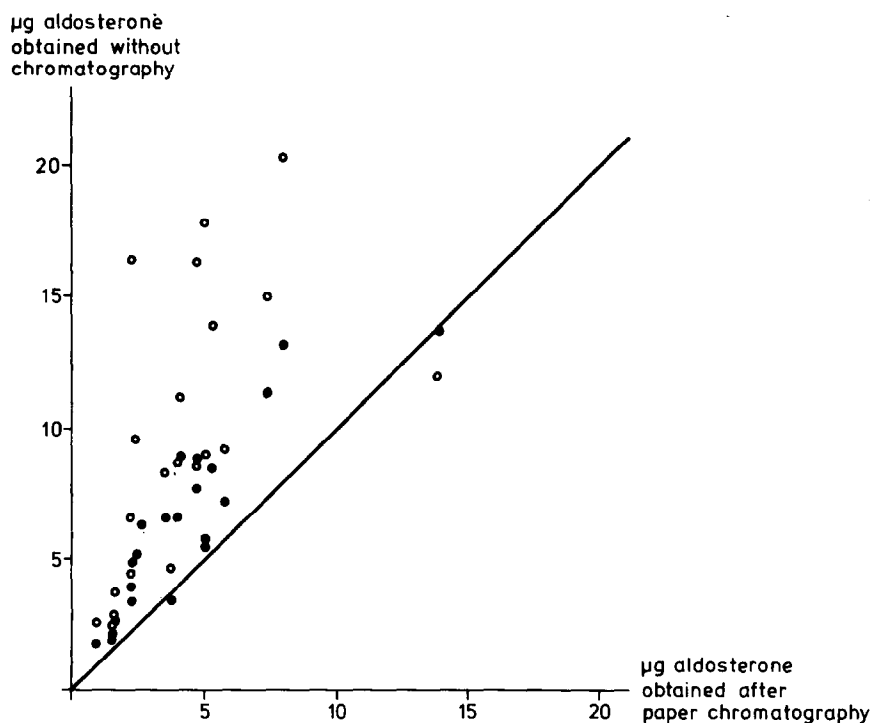


Fig. 1. Values of aldosterone obtained after extraction with nylon attached antibodies (●) and values of aldosterone obtained after partition in benzene/water (○) plotted against values of aldosterone obtained after paperchromatography (in µg aldosterone/12 or 24 hr).

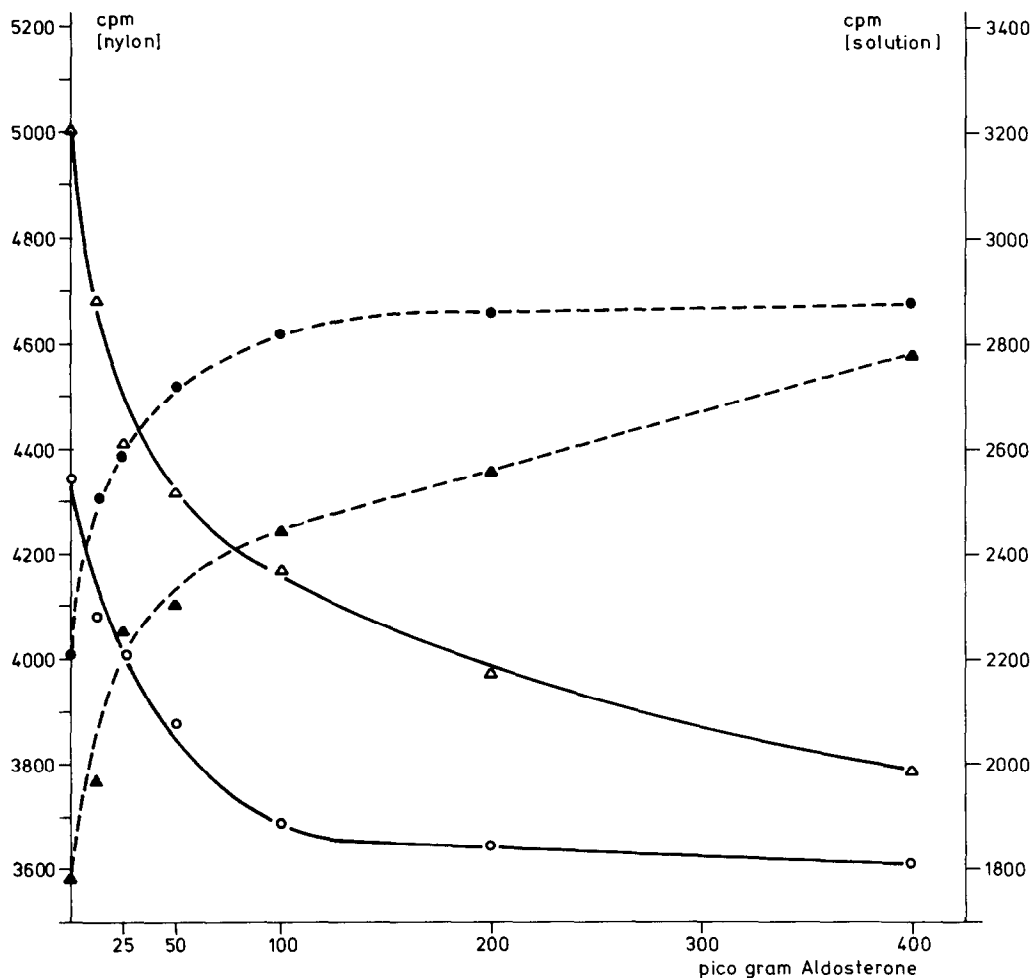


Fig. 2. Typical calibration curves of radioimmunoassays of aldosterone with antibodies attached to nylon. Radioactivities extracted with nylon bound antibodies (full lines), rest radioactivities in the incubation buffer (dotted lines).

points) are plotted against the values obtained by chromatography. It is evident that the values obtained by nylon extraction are a little higher than those obtained by chromatography, but they are nearer to the ideal (45°) line than those without the biospecific extraction.

Determination of aldosterone with nets of type A by substitution of labelled aldosterone from the nylon bound antibodies proved to be an insensitive and slow procedure. Even at elevated temperature, e.g. 40°C , the exchange rate was low: 50 pg aldosterone substituted only 5%, 100 pg aldosterone 15%, and 10 ng aldosterone 55% of the labelled aldosterone. There-

fore the aldosterone was diluted with labelled aldosterone and hereafter incubated with the nylon attached antibodies. The conditions for the incubation were not critical with respect to medium, temperature or time. Using water instead of 0.05 M borate buffer of pH 8 the value of bound aldosterone was only 8% lower. Lowering the temperature of incubation from 25° to 4°C gave exactly the same values of bound aldosterone. Extension of the incubation time from 1 to 20 hr raised the yield of bound aldosterone by only 16%.

Fig. 2 gives two typical calibration curves, obtained by counting the radioactivity of the 0.65 cm^2

nylon net in dioxan scintillation fluid directly. The mirror-image curves were obtained when non-bound radioactivity in the incubation fluid was measured for control. We preferred the nylon elution curve, because it was obtained more easily and the values were less scattered. Aldosterone could be determined down to 10 pg. The limit of sensitivity for the same antibodies in solution was 5 pg aldosterone.

Specificity of the nylon attached antibodies against aldosterone was investigated in competition with other steroids. The values of the cross reactions, according to Abraham [20], were 5% for corticosterone and 0.25% for cortisol. The corresponding values of cross reaction in solution were 0.3% and < 0.1%, respectively. It is evident that by coupling to nylon surfaces the specificity of the antibodies is affected. The simple separation, however, of bound and of free aldosterone qualifies this solid phase method especially for screening assays. The application of the solid phase method in biological samples is in progress.

It is generally accepted that the stability of proteins, especially that of enzymes, is increased by attachment to solid supports. In the case of antibodies high stability under ethanol treatment has recently been communicated by Updike et al. [22] for gel-trapped antibodies to proteohormones. When antibodies against aldosterone were bound to nylon surfaces, full binding capacity was retained after 5 min treatment with methanol (table 1). Under these conditions the complex of aldosterone with its antibodies was fully dissociated. Ethanol or acetonitril did also not affect the binding capacity, but released only 90% of the bound aldosterone. Dichloromethane, which is commonly used for extraction of aldosterone from aqueous solutions, released only 40% of the aldosterone and we suggest that organic solvents, which are not miscible with water, do not penetrate into the water-surrounded hydrophobic regions of the protein, which are suggested to be involved in the binding of the steroid. 1,4-Dioxane dissociated the aldosterone-antibody complex only to 75%. Nevertheless occurred the liquid scintillation counting of [^3H]aldosterone in homogenous solution, because the scintillation liquid contains naphthalene and fluorescent substances besides dioxane and thus affords full dissociation of the antibody-aldosterone complex. A severe damage of the nylon attached antibodies was found after treatment in dimethylformamide (table 1).

Table 1
Effects of 5 min treatment with organic solvents on antibodies attached to nylon

Solvent	A	B	B-(100-A)
0.05 M Borate buffer, pH 8	0.0	100.0	0
Acetonitril	87.4	99.6	87.0
Methanol	99.2	96.7	95.9
Ethanol	89.9	97.9	87.8
Dichloromethane	39.5	89.1	28.6
1,4-Dioxane	75.0	88.3	63.3
Dichloromethane/ methanol/water (30/60/10)	95.5	74.0	73.5
Dimethylformamide	66.4	62.0	28.4
1,4-Dioxane scintillation liquid	99.4	64.0	63.4

A: % of [^3H]aldosterone released from its nylon attached antibodies by organic solvents.

B: resting binding capacity of nylon attached antibodies after treatment with organic solvents (in %).

B-(100-A): free binding capacity of nylon attached antibodies after treatment with organic solvents (in %).

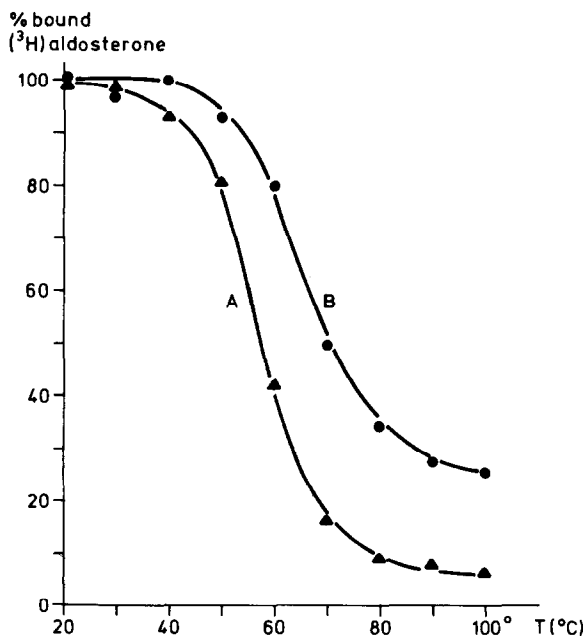


Fig. 3. Thermostability of antibodies attached to nylon. Release of bound [^3H]aldosterone after 30 min at different temperatures (A). Binding capacity of [^3H]aldosterone at 25°C after 30 min treatment at different temperatures (B).

Fig. 3 shows that also thermostability is increased by immobilisation of the antibodies on nylon surfaces. After 30 min treatment at 60°C the binding capacity decreased by only 20%. Even after 30 min treatment at 100°C, 25% of the binding capacity remained, which, however, could be of an unspecific nature.

A relatively high rate of denaturation was found after thorough desiccation of the nylon attached antibodies in vacuo over phosphorous pentoxide. The decrease in binding capacity was 50%, also when the nylon nets were washed free from buffer with ethanol before desiccation.

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