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A microsphere-based duplex competitive immunoassay for the simultaneous measurements of aldosterone and testosterone in small sample volumes: Validation in human and mouse plasma

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ABSTRACT

Background: The small blood volumes available in rodent studies often limit adequate quantification of all hormones of interest. We report here the development of two new assays combining an extraction step with multiplex immunoassay (MIA) technology for the simultaneous determination of aldosterone and testosterone in 50 μl sample volume.
Methods: Following solvent extraction, aldosterone and testosterone competitive immunoassays are performed incorporating biotinylated tracers and antibody-coated beads each having a unique fluorescence. Quantification is via addition of streptavidin–R–phycoerythrin (SA–PE). The assays were validated and compared to established methods. Baseline hormone levels in mice from four different strains, and changes after ACTH and HCG stimulation in CD-1 mice are shown.
Results: The assays are sensitive (aldosterone 15 pg/ml, testosterone 12 pg/ml), reproducible (intra-/inter-assay imprecision aldosterone 5.1–15.6%/9.9–15.8% and testosterone 9.7–10.9%/7.7–11.4%) and correlate significantly to established assays ($r = 0.94–0.95$). Baseline aldosterone levels varied between strains, but not between the genders. Testosterone was significantly higher in male of all strains except in C57BL/6× NMRI mice. After ACTH injection, aldosterone (median, interquartile range) rose from 354 (261–396) pg/ml to 2008 (875–2467) in male and from 260 (210–576) to 1120 (734–1528) in female CD-1 mice. HCG injection in the same strain increased testosterone in male mice only (3.5 (0.4–8.3) ng/ml to 31.8 (30.4–33.9) ng/ml, $P < 0.01$).
Conclusions: We describe a MIA for the simultaneous measurement of aldosterone and testosterone in small volumes after extraction. In addition to presenting a new tool for steroid research in rodent models, our data show strain-dependent differences in steroid hormone metabolism in rodents.

1. Introduction

Accurate measurements of hormones are crucial for both the diagnosis of endocrine diseases and the monitoring of medications. Aldosterone, as one of the steroid hormones synthesized by the adrenal cortex, regulates ion and blood pressure homeostasis. In some of the hypertensive population, primary aldosteronism contributes up to 10% of the prevalence [1]. Besides this, further insight into the role of aldosterone in essential hypertension and metabolic syndrome has been provided by several recent studies [2,3]. Testosterone, another steroid hormone from the androgen group, is the principal male sex hormone and an anabolic steroid. The quantification of testosterone helps to determine the androgenic state [4,5]. Numerous immunoassays measuring aldosterone [6–10] and testosterone [4,5,11–14] in blood, saliva and urine samples have been reported, including radioimmunoassays (RIA), non-isotopic chemiluminescence immunoassays (CLIA), and more accurate liquid chromatography/tandem mass spectrometry (LC/MS) assays. RIAs provide rapid throughput, but require radioactivity and high sample volumes. LC/MS assays, though highly sensitive and considered the gold standard, are labor intensive and involve many manual steps. Considering the sample preparation, most of the assays available nowadays use direct measurement, with few assays involving an extraction step which helps to increase the accuracy and reproducibility. Taking all aspects into consideration, finding the optimal assay to use may be challenging, so

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assay development should be based on the "fit-for-purpose" principle.

Recent progress of the multiplex suspension array system has provided a novel platform for simultaneous measurement of several analytes in small volumes [15,16]. This benefits the research in which the specimen volume is limited and/or repeated analysis is necessary. Several MIAs have been described so far [17–21], however few utilize competitive immunoassays and most of the analytes being assessed are cytokines or immunoglobulins which are relatively large molecules with a high concentration in circulation. Both aldosterone and testosterone are comparatively small molecules and their concentrations are low in contrast to other steroid hormones such as cortisol. All these, together with some other interfering factors, make the measurements of both these hormones difficult and results from different assays vary significantly [8,14].

Here we describe two microsphere-based competitive immunoassays in the multiplex format for the simultaneous measurement of aldosterone and testosterone in small volumes of human or mice plasma after extraction. To further validate the assays, we investigated the levels of these two hormones in four different mouse strains and present means of baseline hormone concentrations of adult male and female mice from the different strains. In addition, the response of these two hormones to ACTH and HCG in adult CD-1 mice is also discussed.

2. Materials and methods

2.1. Reagents and materials

Carboxylated polystyrene microspheres (numbers 26 and 35) and Micro Bio-Spin™ 6 Tris chromatography columns were purchased from Bio-Rad (Munich, Germany). *N*-Hydroxysulfosuccinimide (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were by Pierce (Schwerte, Germany). SA–PE, steroids, organic and other materials were from Sigma–Aldrich (Taufkirchen, Germany). All purchased steroids were of analytical reagent grade or highest percent purity (>98%).

2.2. Antibodies

Antibodies specific for the analyte of interest were coupled to the beads after a primary coupling with immunoglobulins to correctly orientate the secondary, analyte-specific antibody. An affinity purified rabbit anti-mouse IgG was bought from DAKO (Hamburg, Germany), and a monoclonal mouse anti-rabbit IgG (1H7) was produced in our laboratory. The monoclonal antibody (mAb) against aldosterone was prepared as previously described [22], and cross-reactivities against other steroid hormones have been published elsewhere [22]. The antiserum raised against testosterone–CMO has also previously been presented by Tschop et al. [11]. Specificity of the antiserum was determined by measuring samples containing increasing concentrations of potentially cross-reacting substances. Cross-reactivity was defined at the point where the reduction in median fluorescent intensity (MFI) corresponds to 50% of the signal achieved for the blank (B/B_0 of 50%) and expressed as a percentage of the testosterone concentration giving the same reduction in MFI.

2.3. Aldosterone and testosterone tracers

Biotinylated aldosterone and testosterone were used as tracers in the immunoassays for aldosterone and testosterone, respectively. The tracers were produced and purified as previously described [6,11]. For use in the assay, biotinylated aldosterone and testosterone were diluted in assay buffer (PBS, 1% BSA, 0.05% NaN_3, pH 7.4).

2.4. Bead coupling

2.4.1. Antibody preparation

Removal of azide, TRIS, glycine, or other nitrogen-containing compounds from the antibody-containing solution by Micro Bio-Spin 6 Tris chromatography was followed by a buffer exchange to PBS (pH 7.4), concentrations of both the rabbit anti-mouse IgGs and the mouse anti-rabbit IgGs were determined by spectrophotometer.

2.4.2. Covalent coupling of the first antibodies to fluorescent microspheres

Covalent coupling was performed following the protocol recommended by Bio-Rad with some modifications. Briefly, after vortexing and sonicating, 100 μl (1.25×10^6) spectrally differentiable carboxylated microspheres were transferred into a 1.5 ml tube and centrifuged at 14,000 g for 4 min. The pellet was washed with 100 μl activation buffer (0.1 mol/L NaH_2PO_4, pH 6.2) and resuspended by gentle vortexing and sonication in 80 μl activation buffer. 10 μl of freshly prepared EDC and sulfo-NHS solutions (each at 50 mg/ml in activation buffer) were sequentially added to activate the microspheres. Suspensions were incubated for 20 min at room temperature in the dark with continuous shaking. After 2-fold washing with PBS, 12 μg of the respective first antibodies in 500 μl PBS was added to the beads, i.e. rabbit anti-mouse IgG to beads 26 (aldosterone assay), and 1H7 to beads 35 (testosterone assay). After incubation for 120 min in the dark at room temperature with continuous shaking, the reaction was terminated by adding 250 μl blocking buffer (PBS–TBN: PBS containing 0.1% BSA, 0.02% Tween 20, and 0.05% NaN_3, pH 7.4).

2.4.3. Coupling of the specific anti-aldosterone and anti-testosterone antibodies

After washing with assay buffer, microspheres were incubated overnight with 500 μl of the respective specific antibodies (4 °C, continuous shaking in the dark). Titration experiments revealed that a 1:100 dilution of the anti-aldosterone mAb and a 1:250 dilution of the testosterone antiserum in PBS were best suited for the duplex assay. The following day, beads were washed twice with blocking buffer to remove any unbound antibody and then counted on the hemacytometer. After adjusting concentrations to 5.5×10^3/ml with blocking buffer, beads were stored at 4 °C until use.

2.5. Final assay procedure

2.5.1. Sample extraction

Organic solvent extraction (dichloromethane (DCM)/polyethylene glycol (PEG) 10,000 (100 mg/L)) of samples (50 μl) was carried out as previously described [6]. After phase separation, the organic phase was transferred to glass tubes and evaporated to dryness under a nitrogen stream. Samples were reconstituted in a salt matrix buffer (4.2 g/l $NaHCO_3$, 0.5 g/l NaCl, 0.2 g/l K_2CO_3) which is also used for preparation of calibrators. Concentrations obtained upon measurements were multiplied by four to compensate for the dilution factor.

2.5.2. Assay procedure

All calibrators and samples were assayed in duplicate. Calibrators were prepared from an ethanolic stock of aldosterone or testosterone by serial dilution in the buffer described above. 5000 beads from each with coupled antibodies were added into each

well of a 96-well multi-screen BV filter plate (Millipore, Schwalbach/Ts, Germany). After 2-fold washing (PBS, 0.05% Tween 20), 50 μl of calibrators or reconstituted samples was added into the corresponding wells. Subsequently, 100 μl tracer was added and the plate was sealed with aluminum foil before an overnight incubation (4 °C, continuous shaking). The incubation was terminated on the following day by three times washing. 50 μl SA–PE (2 μg/ml) was added and incubated for 30 min on a shaker at room temperature. Finally, after 3-fold washing, beads were resuspended in 125 μl assay buffer and the whole plate was read on a Bio-Plex 200 (Bio-Rad, Munich, Germany). The specific MFI of the beads corresponds with the amount of biotinylated aldosterone or testosterone bound to the respective beads. For standard curve fitting and subsequent calculation of the analyte concentrations, data were transferred to the Wiacalc software (Perkin-Elmer LAS, Jügesheim, Germany). For assay development, both assays were first established as individual aldosterone or testosterone assays, and only the respective beads, calibrators and tracer were present in the incubation mixture. In the final duplex format of the assays, both sets of beads, calibrators and tracers were added in parallel.

2.6. Assay validation and comparison

Validation and method comparison experiments were completed using human samples to avoid the need to obtain large volumes of mouse serum or plasma.

2.6.1. Cross-reactivity

Specificity of the monoclonal antibody against aldosterone has been demonstrated before [22]. For the testosterone assay, cross-reactivity was tested for a wide spectrum of endogenous and synthetic steroids (at *x*-fold the endogenously found or administered concentrations).

2.6.2. Analytical sensitivity and linear range

Analytical sensitivity or lower limit of detection (LLOD) of the assays was determined by 20-fold measurements of the respective zero calibrators. The concentration corresponding to the mean − 2SD of the respective MFI was calculated from its intercept with the displacement curve.

2.6.3. Precision

The intra-assay coefficients of variation were determined by 20-fold measurements of three plasma pools with low, medial and high levels of aldosterone and testosterone on one plate. Another three pools (also with low, medial and high aldosterone and testosterone concentrations) were determined in 10 consecutive assays and the mean values of duplicate wells in each run were then calculated for the inter-assay CV.

2.6.4. Recovery and linearity

Recovery, expressed as a percentage of the expected values, was determined in two plasma samples within the normal range (aldosterone: 93 pg/ml and 277 pg/ml; testosterone 636 pg/ml and 831 pg/ml). Linearity was determined in two spiked plasma samples (aldosterone: 704 pg/ml and 838 pg/ml; testosterone: 1602 pg/ml and 2142 pg/ml) which were serially diluted in standard matrix.

2.7. Comparison to established assays

An in-house time-resolved fluorescence immunoassay (TR-FIA) for aldosterone with a same extraction step [6] was used for comparison of the aldosterone measurements. Testosterone assay results were compared to those obtained by a commercial testosterone enzyme immunoassay (EIA) kit (IBL International, Hamburg, Germany). This is a direct assay without extraction and uses a testosterone horseradish peroxidase conjugate as tracer. The analytical sensitivity of this assay is 83 pg/ml.

2.8. Animal husbandry

Mice were maintained on a 12 h light/dark cycle at 22.3 °C (95% interval: 22.1–22.5 °C) and at a humidity of 68.7% (95% interval: 65.8–71.6%). All mice had free access to a standard rodent pellet diet (V1534; Ssniff, Soest, Germany) and tap water ad libitum. The diet used contained 0.59% sodium chloride and 0.97% potassium, which is within the range of standard rodent pellet diets. Baseline aldosterone and testosterone levels were measured in 12-week-old male and female mice of different strains. The strains used include CD-1 mice ($n = 12$); C57BL/6× NMRI hybrid mice ($n = 11$), i.e. offspring of a crossing performed with C57BL/6 and NMRI mice; C3H mice ($n = 11$, Harlan, Borchen, Germany), and DUC mice ($n = 10$) from a long term selection mouse line, which had been generated by phenotypic selection for high reproductive performance over 144 generations [23]. For the stimulation experiments, 12-week-old male ($n = 12$) and female ($n = 12$) CD-1 mice were used.

Mice were anesthetized individually in a glass jar containing saturated isoflurane vapour, and then decapitated. Blood was collected within 2 min into 1.5 ml tubes containing 10 μl EDTA (0.5 mol/L). For the stimulation experiments, CD-1 mice were injected with ACTH (Novartis, Nürnberg, Germany, 1 μg/g body weight) or 10 IU/mouse HCG (Calbiochem, Darmstadt, Germany) intraperitoneally without anesthesia. An hour later, blood samples were collected as described above. To investigate the physiological stimulation of the RAAS, 12-week-old female C3HeB/FeJ mice (Jackson Laboratories, Maine) received drinking water ad libitum supplemented with 2% KCl. After 1 and 7 days, respectively, 5 mice per time point were euthanized for collection of trunk blood. For baseline controls, another group of mice receiving normal water was euthanized. To avoid stress induced changes in aldosterone concentrations, the blood sampling procedure was performed in less than 60 s. All blood samples were centrifuged immediately after collection and the supernatant was transferred into new tubes and stored at −20 °C until use. All procedures were approved by the Upper Bavarian Government's ethical committee for animal experiments.

Table 1
Cross-reactivities of anti-testosterone antiserum (%).

Steroid	Anti-testosterone antiserum
Testosterone	100
Aldosterone	<0.00003
Cortisol	<0.00003
DOC	<0.00003
18-OH-corticosterone	<0.00003
Corticosterone	<0.00003
Progesterone	<0.00003
17α-Hydroxyprogesterone	<0.00003
17α-Hydroxypregnenolone	<0.00003
Estradiol	0.00249
Estrone	<0.00003
Androstenedione	<0.00003
Epitestosterone	<0.00003
Dehydroisoandrosterone	<0.00003
Cortisone	<0.00003
Danazol	<0.00003
17α-Methyltestosterone	0.03
19-Nortestosterone	7.33
Dexamethasone	<0.00003
17α-Ethynylestradiol	<0.00003
D-Norgestrel	<0.00003

Table 2
Intra-assay and inter-assay imprecision of duplex assays.

	Intra-assay		Inter-assay	
	Concentrations (mean ± SD; pg/ml)	CV (%)	Concentrations (mean ± SD; pg/ml)	CV (%)
Duplex aldosterone assay	35.3 ± 5.5	15.6	24.1 ± 3.8	15.8
	95.9 ± 4.9	5.1	59.6 ± 7.0	11.7
	197.1 ± 14.3	7.2	135.9 ± 13.5	9.9
Duplex testosterone assay	177.6 ± 19.4	10.9	185.4 ± 21.1	11.4
	512.1 ± 57.4	11.2	494.6 ± 46.7	9.4
	1357.9 ± 131.2	9.7	1338.0 ± 103.0	7.7

Table 3
Recovery of two plasma samples spiked with calibrators.

1:1 Dilution with calibrators (pg/ml)		Recovery (%)		
		Sample A	Sample B	Mean
Aldosterone	0	106.6	98.9	102.8
	10	116.6	114.7	115.7
	20	110.5	111.1	110.8
	50	117.6	106.8	112.2
	100	112.0	106.3	109.2
	200	113.8	107.1	110.5
	500	103.7	111.5	112.6
	1000	107.5	104.0	105.8
Mean recovery (%)		111.1	107.6	109.3
Testosterone	0	97.5	92.6	95.1
	25	102.0	111.4	106.7
	50	113.8	112.2	113.0
	125	92.5	105.9	99.2
	250	108.6	99.3	104.0
	500	132.0	113.2	122.6
	1250	114.0	111.9	113.0
	2500	90.2	99.1	94.7
Mean recovery (%)		106.3	105.7	106.0

Table 4
Linearity of two plasma samples serially diluted in DIN.

Analyte	Dilution factor	Measured/expected (%)		
		Sample A	Sample B	Mean
Aldosterone	1:2	97.0	107.4	102.2
	1:4	90.0	115.6	102.8
	1:8	98.9	105.1	102.0
	1:16	105.8	98.3	102.1
	1:32	106.6	110.9	108.8
Mean recovery (%)		99.7	107.5	103.6
Testosterone	1:2	99.6	100.5	100.1
	1:4	109.4	101.9	105.7
	1:8	108.1	104.5	106.3
	1:16	91.2	94.7	93.0
	1:32	96.3	88.3	92.3
Mean recovery (%)		100.9	98.0	99.5

2.9. Statistical analysis

All results are expressed as median with interquartile range. As the data were not normally distributed, groups were compared using non-parametric Mann–Whitney test and a $P < 0.05$ was considered significant. The relationship between the results obtained from two different assays was analyzed using Spearman correlation coefficient (two-tailed hypothesis).

3. Results

3.1. Assay validation

Most of the endogenous and synthetic steroids except estradiol, 17α-methyltestosterone and 19-nortestosterone did not cross-react with anti-testosterone antiserum. The results of cross-reactivity are summarized in Table 1. In the duplex assay, the analytical sensitivity of the aldosterone was 15 pg/ml and that of testosterone was 12 pg/ml. The upper limit of the linear range of the assay was 1000 pg/ml for aldosterone and 2500 pg/ml for testosterone. The intra-assay and inter-assay coefficients of variation of the two assays for plasma pools with low, medial and high levels of aldosterone and testosterone were from 5.1% to 15.8% (Table 2). Recovery determined in two plasma samples was found to be between 94.7% and 122.6% (Table 3). Linearity was between 92.3% and 108.8% (Table 4).

3.2. Comparison to established assays

Human plasma samples measured by the new MIA for aldosterone in the duplex format including testosterone correlated well with the aldosterone results obtained by the TR-FIA (Fig. 1A, $Y = 1.0451X - 19.396$, $R = 0.9532$, $P < 0.0001$, $n = 70$). Bland–Altman plot analysis (Fig. 1B) showed that results were scattered symmetrically around the zero line, indicating random deviations with no systematic difference. We also correlated aldosterone results obtained by both assay methods in mouse plasma samples, revealing a very similar correlation (Fig. 1C, $Y = 1{,}1463X + 1{,}0731$, $R = 0{,}9634$, $P < 0.0001$, $n = 20$). Results of the MIA testosterone assay in the duplex format including aldosterone also showed

Table 5
Aldosterone concentrations in adult male and female mice from three different strains (pg/ml). Statistical significance: $^{*}P < 0.001$ vs. CD-1 mice with same gender.

	CD-1	C57BL/6× NMRI	C3H	DUC
Male	354 (261–396) ($n = 6$)	136 (89–185)* ($n = 7$)	199 (146–288) ($n = 6$)	70 (57–191)* ($n = 5$)
Female	260 (210–576) ($n = 6$)	N/A	203 (78–454) ($n = 5$)	311 (105–461) ($n = 5$)

Table 6
Testosterone concentrations in adult male and female mice from three different strains (pg/ml). Statistical significance: $^{*}P < 0.05$, $^{\#}P < 0.01$ vs. female mice from same strain. $^{\ddagger}P < 0.01$ vs. mice from other strains with same gender.

	CD-1	C57BL/6× NMRI	C3H	DUC
Male	5494 (430–8309)* ($n = 6$)	181 (68–257)‡ ($n = 7$)	5328 (1710–11230)$^{\#}$ ($n = 6$)	3509 (471–10894)$^{\#}$ ($n = 5$)
Female	56 (46–274) ($n = 6$)	N/A	81 (73–250) ($n = 5$)	41 (22–106) ($n = 5$)

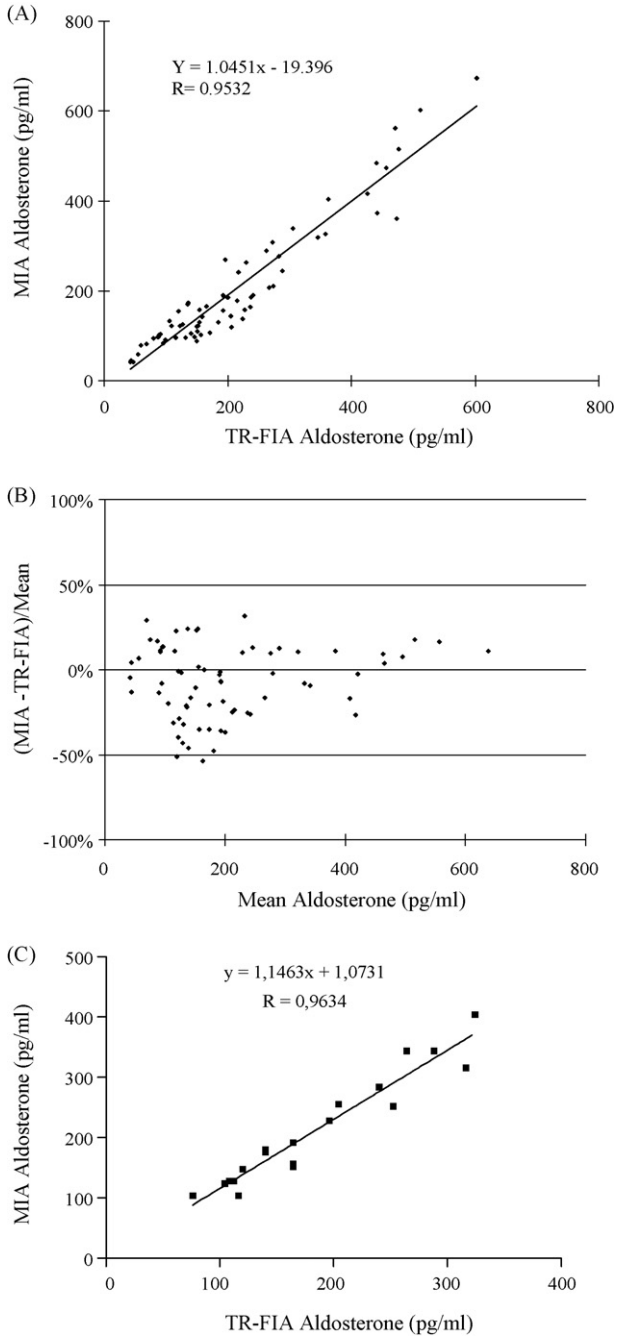

Fig. 1. (A) Comparison of aldosterone levels in 70 extracted human plasma samples measured by the duplex assay and the in-house TR-FIA. (B) Bias plot of the same data. (C) Comparison of aldosterone levels in 20 extracted mouse plasma samples measured by the duplex assay and the in-house TR-FIA.

an overall acceptable correlation to the results from the commercial EIA kit (Fig. 2A: $Y = 0.8314X + 373.18$, $R = 0.9385$, $P < 0.001$, $n = 82$). However, Bland–Altman plots revealed a systematic discrepancy at lower concentrations (Fig. 2B). Results for samples below 1000 pg/ml (Fig. 2C), give a much wider scatter with concentrations from the extraction MIA being on average approximately 50% higher than those from the direct EIA. Results above 1000 pg/ml (Fig. 2D), however, show a symmetrical distribution around zero.

3.3. Measurements in rodents

3.3.1. Baseline aldosterone concentrations: Impact of gender and strain

Aldosterone and testosterone levels for four mouse strains are listed in Tables 5 and 6. For aldosterone, no gender difference was observed in CD-1, C3H or DUC mice. Comparison between strains revealed that aldosterone levels in male C57BL/6× NMRI and male DUC mice were lower than the levels in male CD-1 mice ($P < 0.01$).

3.3.2. Baseline testosterone concentrations: Impact of gender and strain

Male mice had a significantly higher level of testosterone than female mice from the same strains ($P < 0.05$ in CD-1 mice, $P < 0.01$ in C3H and DUC mice). Male CD-1, C3H, and DUC mice had levels of testosterone within the physiological range reported for men [24]. In contrast, male C57BL/6× NMRI mice had much lower plasma testosterone concentrations than all other three strains ($P < 0.01$).

3.3.3. Short term stimulation of aldosterone in adult CD-1 mice

No gender difference was found in aldosterone levels of CD-1 mice either at baseline or after ACTH and HCG treatment. Aldosterone values in both genders increased significantly one hour after intraperitoneal ACTH injection (male: 354 (261–396) pg/ml vs. 2008 (875–2467) pg/ml, $P < 0.01$; female: 260 (210–576) vs.1120 (734–1528) $P < 0.01$), whereas HCG had no effect on aldosterone levels (Fig. 3).

3.3.4. Short term stimulation of testosterone in adult CD-1 mice

No significant effect of ACTH was found on testosterone levels of either male or female CD-1 mice. HCG did not increase the levels of testosterone in female CD-1 mice 1 h after i.p. injection, but raised those in male mice nearly 9-fold (Fig. 4, 3.5 (0.4–8.3) ng/ml vs. 31.8 (30.4–33.9) ng/ml, $P < 0.01$).

3.3.5. Physiological stimulation of the RAAS in adult C3HeB/FeJ mice

Plasma aldosterone measured in the duplex format of MIA after the first day of exposure to potassium supplemented water was significantly higher than in the untreated control group (day 1: 300 ± 18 pg/ml, vs. untreated: 137 ± 12 pg/ml, $P < 0.01$). Aldosterone values on day 7 remained also significantly higher than under baseline conditions (day 7: 222 ± 24 pg/ml, $P < 0.05$).

4. Discussion

We present a new method to measure aldosterone and testosterone simultaneously in only 50 μl of plasma or serum. To achieve this, conventional DCM extraction of samples was combined with the multiplex immunoassay technology. The multiplex suspension array system allows the application of immunoassays on a spectrum of microspheres distinguishable by labeling with internal fluorescence dyes. The ratio of the intensity of the two dyes allows the identification of each distinct set of beads. Each set of beads is coupled with antibodies specific for a particular analyte, so that using different sets of beads in a sample allows the simultaneous measurement of various analytes at the same time.

Several bead-based sandwich type immunoassays have been developed, mainly for cytokines and adipokines. Recently, a set for the simultaneous quantification of six pituitary hormones has been described [15]. However, very few such assays have been developed for the measurement of steroid hormones, which in contrast to the larger peptide hormones require a competitive assay format involving labeled hormone as a tracer. In addition, according to the principle of competitive immunoassays, the amount of the

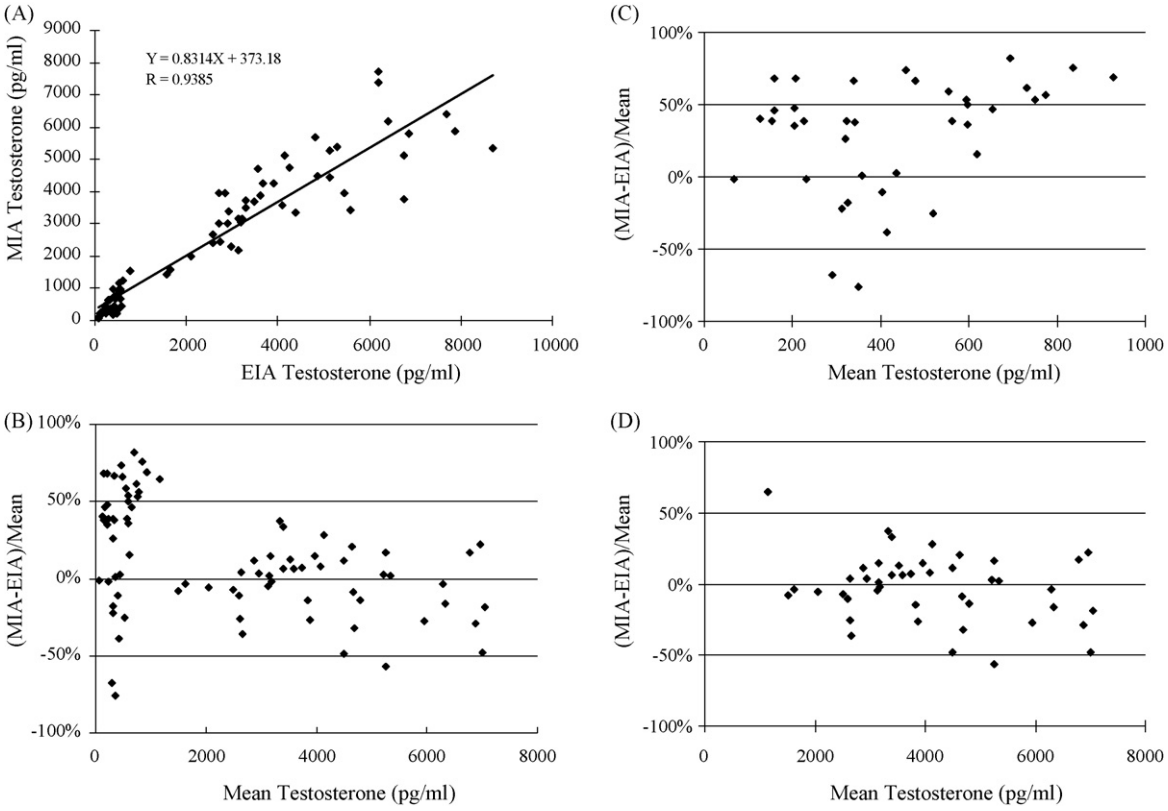

Fig. 2. (A) Comparison of testosterone levels in 82 extracted plasma samples measured by the duplex assay and the commercial EIA. (B) Bias plot of the same data. (C) Bias plot of 38 samples with testosterone concentrations less than 1000 pg/ml. (D) Bias plot of 44 samples with testosterone concentrations higher than 1000 pg/ml.

specific antibody available for binding is limited. Both aspects provide problems for signal generation in the multiplex assay format: small hormone molecules have to be labeled for use as a tracer, but the labeling must not change their structure significantly. This inherently limits the amount of fluorescence which can be attached to each molecule, even when the streptavidin–biotin system is used to enhance the signal. Furthermore, the need to restrict the amount of specific antibodies in competitive assays further limits the total signal which can be generated through binding of the tracer. Accordingly, the only steroid included in some commercial multiplex panels is corticosterone, which circulates at relatively high concentrations. In contrast, aldosterone and testosterone both circulate at very low concentrations. It is well established that accurate quantification of such steroids is better achieved after extraction, a procedure not yet applied to bead-based assays. To our knowledge, the assays we describe here are the first ones to combine an extraction procedure with the simultaneous measurement of two low abundance steroid hormones.

By performing two assays concurrently in a single sample, as in the multiplex assay system, antibody specificity gains even greater importance compared to conventional immunoassay setups. Cross-reaction in our setting had to be excluded at two levels: To achieve a reproducible coating of the beads with a defined and limited amount of specific antibody, we used the indirect procedure through anti-mouse and anti-rabbit IgGs for the mouse anti-aldosterone antibody and the rabbit anti-testosterone antiserum, respectively. These IgGs themselves were from mice and rabbits, respectively. However, although theoretically possible, we did not observe significant aggregation of the two sets of beads after coating with the specific antibodies. More importantly for assay specificity, we carefully characterized the antibodies used against aldosterone and testosterone with respect to their cross-reaction with a large panel of endogenous and synthetic steroids. The anti-aldosterone mAb has been proven to be very specific, with no detectable cross-reaction to cortisol, corticosterone, testosterone or any other steroid tested. For the testosterone assay we used a polyclonal antiserum which also showed no cross-reaction with most of the steroids tested. The highest cross-reaction from an endogenous steroid was found for estradiol (0.00249%). Given the expected physiological concentrations of estradiol even in samples collected from female mice around the time of ovulation, testosterone results would not be affected significantly. Overall, the highest cross-reactivity (7.33%) was observed for 19-nortestosterone, a synthetic steroid which only needs to be taken into account in case it is used in an experimental context.

A major advantage of the multiplex technology is the possibility to simultaneously measure several analytes in an individual sample. In this respect, the introduction of an extraction step obviously provides a limitation – although the simultaneous measurement of steroids is possible, many other analytes including peptide hormones are removed through this procedure. However, it is our opinion that measurement of steroid hormones, especially those at very low concentrations, is more reliable after extraction. This is in agreement with findings from many previous studies, demonstrating the poor performance of direct steroid assays compared to reference methods [14], and also to assays involving extraction [8]. We compared the values obtained in the aldosterone/testosterone MIA to those obtained by other established assays. For aldosterone, a recently published, in-house immunofluorometric assay served as the reference method [6], whereas for testosterone we used a commercially available direct enzyme immunoassay. As expected, the assays showed overall significant correlation (Figs. 1A and 2A, $r = 0.94–0.95$; $P < 0.0001$). However, the agreement was much closer between the aldosterone assays (both involving extraction and the same monoclonal antibody) than between the testosterone assays. To examine this further we carried out Bland–Altman plot analysis.

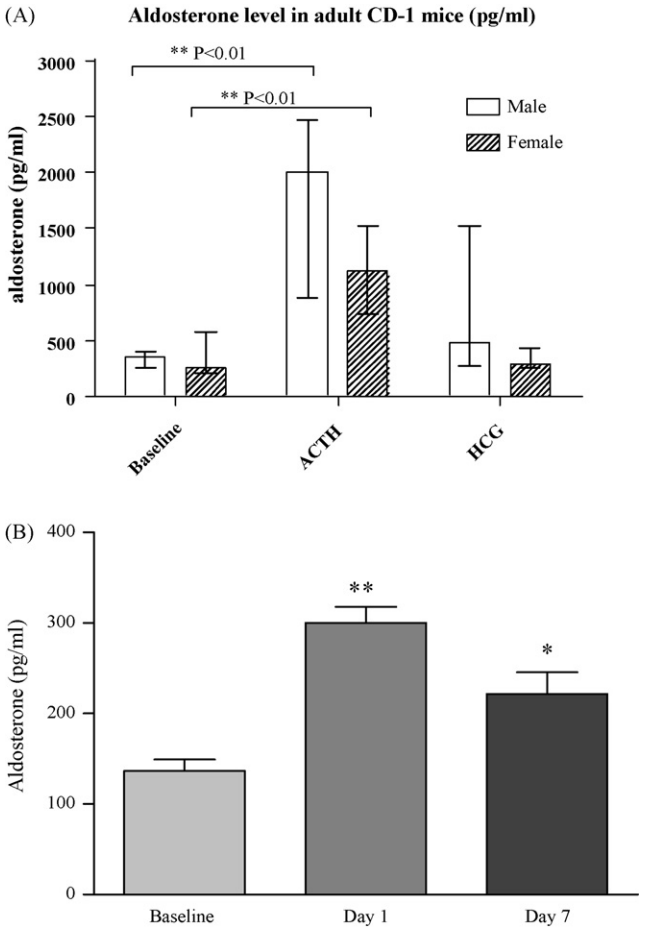

Fig. 3. (A) Aldosterone levels in grouped adult male and female CD-1 mice treated with ACTH or HCG. ACTH injection increases aldosterone concentration significantly ($P < 0.001$) an hour after injection, but no change after HCG injection. (B) Plasma aldosterone levels in age-matched adult C3HeB/FeJ female wild type mice after 1 and 7 days of potassium supplementation.

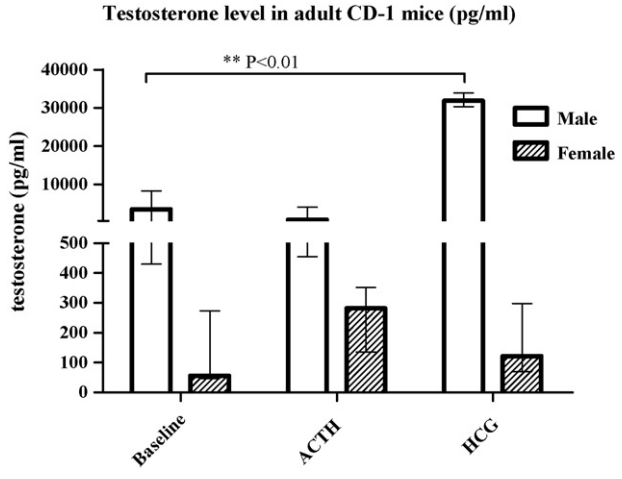

Fig. 4. Testosterone concentrations in grouped adult male and female CD-1 mice after ACTH or HCG stimulation. An hour after injection, HCG increases testosterone level significantly only in male mice ($P < 0.01$). ACTH has no effect on the testosterone levels in both genders.

For aldosterone, the values for the differences between the methods for each sample are scattered almost symmetrically around the *x*-axis (Fig. 1B), indicating random deviations and confirming the overall good agreement throughout the whole concentration range. In contrast, the plots for the testosterone assay show a biased distribution above zero at low concentrations (Fig. 2B). This becomes more obvious if the data are plotted separately for low (<1000 pg/ml) and high concentrations. At high concentrations, the scatter is wider than for aldosterone assays, but still symmetrically distributed around the *x*-axis (Fig. 2D). Analyzing only testosterone values from females and patients with hypogonadism (low range, Fig. 2C) reveals a much wider distribution. Most of the samples in this range are measured approximately 50% higher by the MIA compared to the direct EIA, but for a few samples the EIA gives higher readings. Such obvious discrepancies at very low testosterone concentrations are not unexpected, and have been reported by several studies [14,25,26]. The exact reason is not clear, but interference from matrix effects has been discussed [27]. In this respect, one might discuss if the extraction procedure involved in our MIA allows detection of testosterone at low concentrations, where matrix components impair the measurements with the direct EIA. Furthermore, differences in the sensitivities of the assays and in the precision profile at low concentration might contribute to the discrepancies, e.g., the sensitivity claimed by the manufacturer of the direct EIA is approximately 100 pg/ml, but 10 pg/ml for the testosterone MIA described here. Final proof of the accuracy of the respective methods could only come from comparison with LC/MS data, which unfortunately were not available for our samples.

One important application for methods capable of measuring hormones in small volumes is research in rodent models. In order to demonstrate the applicability of our method in this context, we measured samples from four different mouse strains currently used in our laboratory. Plasma aldosterone and testosterone concentrations in these mice were well within the dynamic range of the duplex assay. Although the presentation of reference ranges for male and female mice from different strains was not the main purpose of our study, our preliminary data on baseline aldosterone and testosterone levels in the four strains, including two strains very commonly used in biomedical research (CD-1 and C3H), confirm the need for careful interpretation of hormone data in animal studies: there were no gender dependent differences in aldosterone concentrations in any of the strains we examined. Though we could not include any female mice of the C57BL/6× NMRI in this instance, we have previously shown that concentrations do not vary between genders in this strain of mouse [6,28]. In addition, we found the levels were significantly higher in male CD-1 mice compared to male mice from C57BL/6× NMRI and DUC strains. Differences between common mouse strains have only been reported for corticosterone [29,30] and insulin levels [31]. In contrast, so far there have been no in-depth studies describing strain related differences in aldosterone plasma levels. Differences in modifying genes, e.g., at the level of feedback mechanisms to the renin–angiotensin–aldosterone system or the hypothalamus–pituitary axis regulating the aldosterone secretion [29] could explain these findings.

As expected, testosterone concentrations were significantly higher in male than in female CD-1, C3H, and DUC mice. In contrast, male C57BL/6× NMRI mice exhibited very low testosterone levels, which were not different from those in the females from the same strain. This is in agreement with a recent publication linking chronic androgen deficiency in male C57BL/6 mice to a shortened ventricular repolarization [32].

We also used the new MIA to investigate short time dynamic changes of aldosterone and testosterone after stimulation in CD-1 mice. ACTH and HCG are known stimulators of aldosterone and male testosterone secretion, respectively [1]. As expected, in our

study aldosterone levels in male and female mice increased significantly after ACTH injection (Fig. 3a) and after exposure to high potassium in drinking water (Fig. 3b), whereas HCG injection led to an increase in testosterone in male mice only (Fig. 4). In this setting, we were able to demonstrate that the duplex MIA is also an appropriate tool for use in pharmacological intervention studies and in studies using physiological stimulation of the RAAS.

In summary, we have established and validated a new multiplex suspension array based assay for the simultaneous measurement of aldosterone and testosterone after extraction. We have demonstrated that competitive immunoassays can be performed on this platform in combination with extraction. The assay was applied to samples from different mouse strains, where significant differences in the hormone levels between genders and strains were found. In addition to presenting a promising new tool for steroid hormone related research in rodent models, our data highlight the need for gender and strain specific normative data for hormones in rodents.

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