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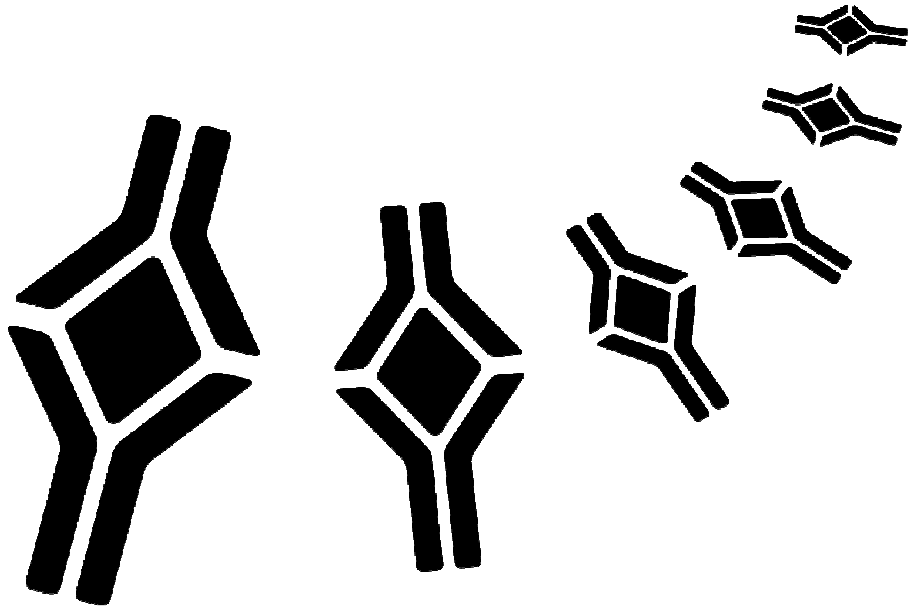


Human/Rat Angiotensin II ELISA

Product Data Sheet

Cat. No.: RA05880R

For Research Use Only

VERSION 51 190511 46

CONTENTS

» **This kit is manufactured by:**
BioVendor – Laboratorní medicína a.s.

» **Use only the current version of Product Data Sheet enclosed with the kit!**

1. INTENDED USE

Store the complete kit at -20°C. Under these conditions, the kit is stable until the expiration date (see label on the box).

2. REAGENT SUPPLIED

Kit Components	State	Quantity
Antibody Coated Microtiter Strips	ready to use	96 wells
Conjugate Solution	lyophilized	1 vial
Angiotensin II standard	lyophilized	2 vials
Quality Control sample	lyophilized	2 vials
Tween 20	liquid	1 vial
Dilution Buffer	lyophilized	1 vial
Wash Solution Concentrate	liquid	1 vial
Substrate Solution	lyophilized	2 vials
Glutaraldehyde	25% aqueous solution	1 vial
Borane Trimethylamine		2 vials

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 32 samples in duplicate.

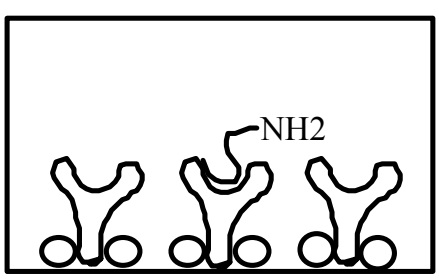
3. PRECAUTIONS FOR USE

Users are recommended to read all instructions for use before starting work.

Each time a new pipet tip is used, aspirate a sample or reagent and dispense it back into the same vessel. Repeat this operation two or three times before distribution.
For research laboratory use only.
Not for human diagnostic use.
Do not pipet liquids by mouth.
Do not use kit components beyond the expiration date.
Do not eat, drink or smoke in area in which kit reagents are handled.
Avoid splashing.
The total reagents contain less than 100 µg of sodium azide. Flush the drains thoroughly to prevent the production of explosive metal azides.
Borane-trimethylamine is highly toxic. Handle this reagent with care.

4. PRINCIPLE OF THE ASSAY

The principle of this Enzyme ImmunoAssay (EIA) is summarised on the following page: a specific monoclonal anti-Angiotensin II is immobilised on a 96 well plate. After immunological reaction with Angiotensin II and washing, the trapped molecule is covalently linked to the plate by glutaraldehyde via amino groups. After washing and denaturing treatment, Angiotensin II can react again with the acetylcholinesterase-labelled mAb used as tracer.
The plate is then washed and Substrate Solution (enzymatic substrate for AChE and chromogen) is added to the wells.
The AChE tracer acts on the to form a yellow compound.
The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of tracer bound to the well and is proportional to the amount of Angiotensin II.

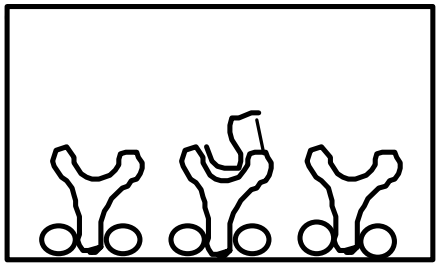


anti-Angiotensin II
monoclonal

BSA

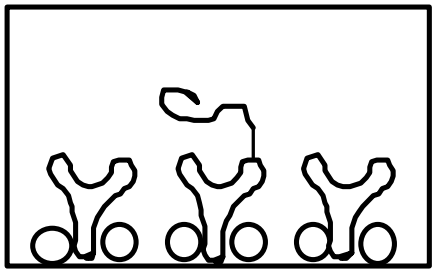
NH2 Angiotensin II

Step 1: immunological reaction



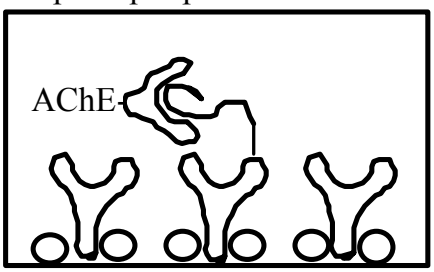
glutaraldehyde

Step 2: epitope immobilization



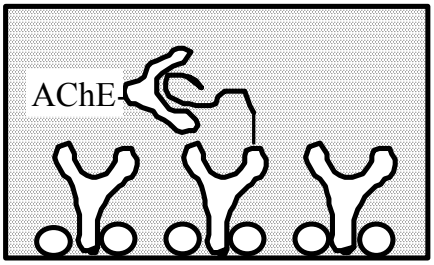
borane-trimethylamine in Hcl/MeoH

Step 3: epitope release



anti-Angiotensin II Fab'
monoclonal antibody
labelled

Step 4: enzymatic tracer



Step 5: enzymatic reaction & colorimetric assay

5. MATERIALS AND EQUIPMENT REQUIRED

In addition to standard laboratory equipment, the following material is required:

FOR SAMPLE PREPARATION

- Phenyl-cartridges
- Methanol
- HCl
- Distilled or deionized water

FOR THE ASSAY

- Precision micropipettes (20 to 1000 µL)
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or washbottles)
- Microplate shaker
- Distilled or deionized water
- Polypropylene tubes

6. SAMPLE COLLECTION & PREPARATION

CULTURE MEDIA SAMPLES

Tissue culture supernatants may be assayed directly. If the Angiotensin II concentration in the medium is high enough, the samples can be diluted with Dilution buffer. The assay can be performed without any modifications. When assaying less concentrated samples (where samples cannot be diluted with Dilution Buffer), dilute the standard curve in the same culture medium as that used in the experiment. This will ensure that the matrix for the standards is comparable to the samples. We recommend that a standard curve be run first to ensure that the assay will perform in the particular medium.

BLOOD COLLECTION

Blood samples are collected in tubes kept on ice at 4°C and usually containing EDTA. We suggest using a inhibitor cocktail to prevent generation and/or degradation of Angiotensin II. It should contain o-phenanthroline 0.44 mM, EDTA 25 mM, p-hydroxy-mercuribenzoic acid 1mM and pepstatin A 0.12 mM (aforementioned concentrations are in the final sample volume). The samples are centrifuged at 3000 g for 20 minutes at 4°C. Samples should be immediately extracted or stored at -20°C until extraction. Avoid thawing samples more than one time.

EXTRACTION PROTOCOL

- Pre-wash phenyl cartridges with 1 mL of methanol, followed by 1 mL of water.
- Pass 2 ml of plasma through the cartridge and then wash it with 1 mL of water.
- Elute absorbed Angiotensin peptides with 0.5 mL of methanol.
- Evaporate the methanol to dryness either by vacuum centrifugation or by evaporation under a stream of dry nitrogen.
- Add 0.5 mL of Dilution Buffer, vortex and centrifuge at 3000 g for 10 minutes at 4°C.

RECOVERY AND CALCULATION

To determine the recovery, the sample may be split into two equal aliquots and one spiked with a known amount of Angiotensin II (approximately equal to the expected amount in the sample).
The recovery will be determined after purification by comparing the concentration of the spiked and unspiked samples.
Either the original concentration of the sample or the recovery factor can be determined by solving the following equations simultaneously:

z = recovery factor
X/a = original concentration of the unspiked sample in a volume known (a)
(X+Y)/b = concentration of spiked sample (pg/ml) after adding a known amount (Y) in a final volume (b)
The concentration of the unspiked and spiked samples determined by the ELISA are respectively equal to (X/a)z & [(X+Y)/b]z.

EXAMPLE

Volume of the unspiked sample: a = 1 mL
Final volume of the spiked sample: b = 2 mL
Concentration determined by ELISA for the unspiked sample: (X/a)z = 8 pg/mL
Concentration determined by ELISA for the spiked sample: [(X+Y)/b]z = 16 pg/mL
Quantity of spike: Y = 30 pg in 1 mL

$Xz = 8 \Leftrightarrow\Leftrightarrow z=8/X$
$[(X+30)/2]z = 16 \Leftrightarrow\Leftrightarrow [(X+30)]z = 32$
thus,
$[(X+30)]8/X = 32 \Leftrightarrow\Leftrightarrow X+30 = 4X \Leftrightarrow\Leftrightarrow 3X = 30 \Leftrightarrow\Leftrightarrow X = 10$
And
$Xz = 8 \Leftrightarrow\Leftrightarrow z = 0.8$

NOTE

To minimise calculations, the standard should be concentrated enough so that the addition of the standard does not alter the volume of the sample (a = b) to any great degree (i.e., the assumption is made that the volume is not changed by the addition of the standard).

7. REAGENT PREPARATION

The coated microtiter plates and reagents are provided ready to use.

- Dilution Buffer

Reconstitute one vial with 50 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 month.

- Angiotensin II standard (calibrated with the standard WHO 86/538)

Reconstitute the vial with 1 mL of distilled or deionized water in a polypropylene tube. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the first standard is 125 pg/mL. Prepare seven propylene tubes (for the seven other standards) and add 500 µL of Dilution Buffer into each tube. Add 500 µL of the first tube (containing the first standard) to the second tube. Continue this procedure for the other tubes. Thus, standard concentrations are: 125 (S1), 62.5 (S2), 31.25 (S3), 15.63 (S4), 7.81 (S5), 3.91 (S6), 1.95 (S7) and 0.98 pg/mL (S8), respectively.

- Quality Control

Reconstitute one vial with 1 mL of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

- Conjugate Solution

Reconstitute one vial with 10 mL of Dilution Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 1 month.

- Glutaraldehyde

Prepare 5 ml of diluted buffer by adding 0.125 mL of the Wash Solution Concentrate in distilled or deionized water. Just before use add 100 µL of glutaraldehyde then mix thoroughly by gentle inversion. Stability at 4°C: 24 hours.

- Borane trimethylamine

Just before use, reconstitute it with 5 ml of 2N HCl/Methanol (50/50, v/v). The tube contents should be vortexed until complete dissolution. At this step, bubble formation could be observed. **Warning**: once reconstituted, Borane trimethylamine should be used immediately as mentioned above.

- Substrate Solution

Five minutes before use, reconstitute it with 1 mL of Wash Solution Concentrate and 49 mL of distilled or deionized water. The tube contents should be thoroughly mixed. Stability at 4°C and in the dark: 4 days.

- Wash buffer

Dilute 2 mL of to 0.8 L with distilled or deionized water. Add 400 µL of tween 20 (Use a magnetic stirring bar to mix the contents). Stability at 4°C: 1 week.

8. ASSAY PROCEDURE

It is recommended to perform the assays in duplicate and to follow the instructions hereafter.

PLATE PREPARATION

Prepare the wash buffer as indicated in the reagent preparation section. Open the plate packet and select the sufficient strips for your assay and place the unused strips back in the packet (stored at 4°C). Rinse each well five times with the wash buffer (300 µL/well).
Just before distributing reagents and samples, remove the buffer from the wells by inverting the plate and shaking out the last drops.

DISTRIBUTION OF REAGENTS AND SAMPLES

A plate set-up is suggested on the following page. The contents of each well may be recorded on the sheet provided with the kit.

PIPETTING THE REAGENTS

Note that the first column should be left empty for blanking Substrate Solution. All samples and reagents must reach room temperature prior to performing the assay. Use different tips to pipet the buffer, standard, sample, conjugate solution and other reagents.

	A	B	C	D	E	F	G	H
12	*	*	*	*	*	*	*	*
11	*	*	*	*	*	*	*	*
10	*	*	*	*	*	*	*	*
9	*	*	*	*	*	*	*	*
8	*	*	*	*	*	*	*	*
7	*	*	*	*	*	*	*	*
6	*	*	*	*	*	*	*	*
5	*	*	*	*	*	*	*	*
4	S5	S5	S6	S6	S7	S7	S8	S8
3	S1	S1	S2	S2	S3	S3	S4	S4
2	NSB	NSB	NSB	NSB	NSB	NSB	NSB	NSB
1	B	B	B	B	B	B	B	B

B : Blank
NSB : Non Specific Binding
S1-S8 : Standards 1-8
* : Samples or Quality Controls

- Dilution Buffer: Dispense 100 µL to Non Specific Binding (NSB) wells.
- Angiotensin II standard: Dispense 100 µL of each of the eight standards (S1 to S8) in duplicate to appropriate wells. Start with the lowest concentration standard (S8) and equilibrate the tip in the next higher standard before pipetting.
- Samples: Dispense 100 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in Dilution Buffer.

INCUBATING AND WASHING THE PLATE

- Incubate for 1 h at room temperature with gentle agitation.
- Dispense 50 µL of glutaraldehyde to each well (except Blank (B) wells) and incubate for 5 minutes at room temperature with gentle agitation.
- Dispense 50 µL of borane-trimethylamine to each well (except Blank (B) wells) and incubate for 5 minutes at room temperature with gentle agitation.
- After washing each well five times with the wash buffer (300 µL/well), remove the liquid from the wells by inverting the plate and shaking vigorously. Dispense 100 µL of conjugate solution to each well (except Blank (B) wells).
- Cover the plate with a plastic film and incubate overnight at +4°C.
- After washing each well five times with the wash buffer (300 µL/well), remove the liquid from the wells by inverting the plate and shaking vigorously. Add 300 µL of wash buffer.
- Incubate for 10 minutes at room temperature with gentle agitation.

DEVELOPING AND READING THE PLATE

- Reconstitute Substrate Solution as indicated in reagent preparation section. After washing each well five times with the wash buffer (300 µL/well), empty the plate by turning over and shaking, dispense 200 µL of Substrate Solution to the 96 wells. Incubate in the dark (plate covered with an aluminium sheet) for 30 min to 1 hour at room temperature. Optimal development is obtained using an orbital shaker. The plate should be read between 405 and 414 nm (yellow colour).

Enzyme Immunometric Assay Protocol			
Steps	Blank	NSB	Standard and Samples
Wash the plate 5 times			
Step 1: Incubation (1 h. RT)	—	100 µL of Dilution Buffer	100 µL of standard or sample
Step 2: Epitope immobilization (5 min. RT)	—	50 µL of glutaraldehyde in phosphate buffer	
Step 3: Glutaraldehyde Neutralization (5 min. RT)	—	50 µL of borane-trimethylamine in HCl 2N/Methanol (50/50, v/v)	
Wash the plate 5 times			
Step 4: Incubation (overnight 4°C)	—	100 µL of mAb tracer	
Wash the plate 5 times			
Step 5: Washing (10 min. RT)	300 µL of Wash Solution		
Wash the plate 5 times			
Step 6: Enzymatic reaction	200 µL of Substrate Solution		
Incubate with an orbital shaker in the dark at room temperature			
Read the plate between 405 and 414 nm			

DATA ANALYSIS

Make sure that your plate reader has subtracted the absorbance readings of the blank well (absorbance of Substrate Solution) from the absorbance readings of the rest of the plate. If not, do it now.

- Calculate the average absorbance for each NSB, standards and samples.
- Using linear graph paper, plot the absorbance of each standard point (y axis) versus the concentration of that point (x axis).
- Results are calculated by interpolation from the standard curve. The standard curve may be used for the determination of the unknown concentrations in samples assayed at the same time as the standards.
- Most plate readers are supplied with curve-fitting software capable of graphing this data (linear curve fit). If you have this type of software, we recommend using it. Refer to it for further information.

9. TYPICAL DATA

EXAMPLE DATA

The following data are for demonstration purpose only. Your data may be different and still correct. These data were obtained using all reagents as supplied in this kit under the following conditions: 1 hour developing at 20°C, reading at 414 nm. A linear curve fitting was used to determine the concentrations.

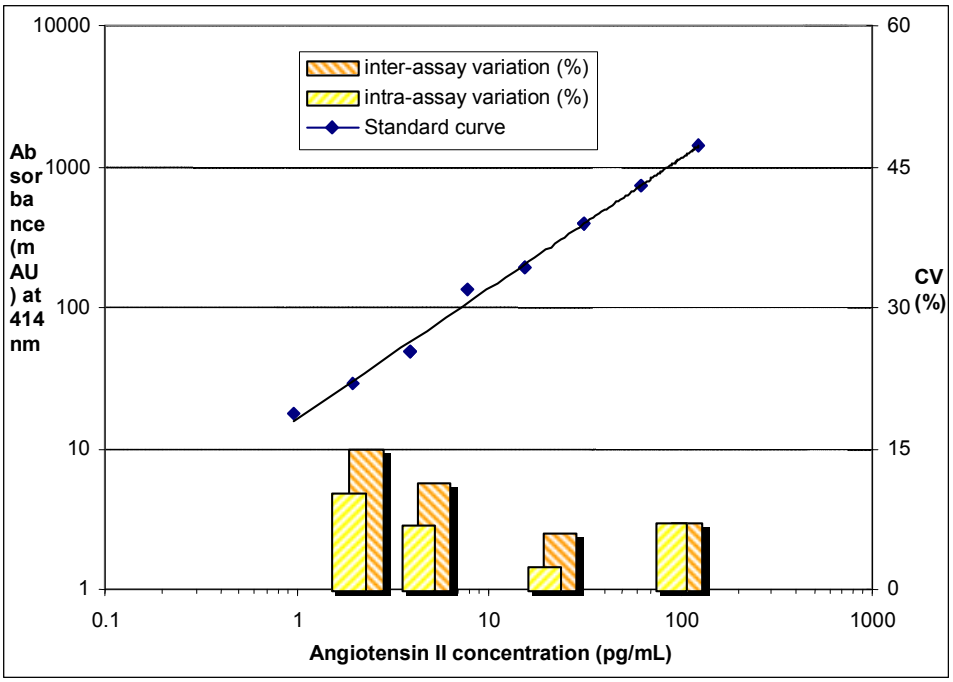
Angiotensin II	Absorbances (mAU)
NSB	104
Standard 125 pg/mL	1396
Standard 62.5 pg/mL	743
Standard 31.25 pg/mL	397
Standard 15.63 pg/mL	193
Standard 7.81 pg/mL	136
Standard 3.9 pg/mL	48.5
Standard 1.95 pg/mL	29.0
Standard 0.98 pg/mL	18.0

Typical standard curves are shown on the following picture.

ACCEPTABLE RANGE

- Limit of detection: 0.5-1.5 pg/mL.
- Quality Control sample: see the label on the vial.

ANGIOTENSIN II STANDARD CURVE



10. ASSAY VALIDATION AND CHARACTERISTICS

The Enzyme Immunoassay of Angiotensin II has been fully validated for its use in plasma after extraction.

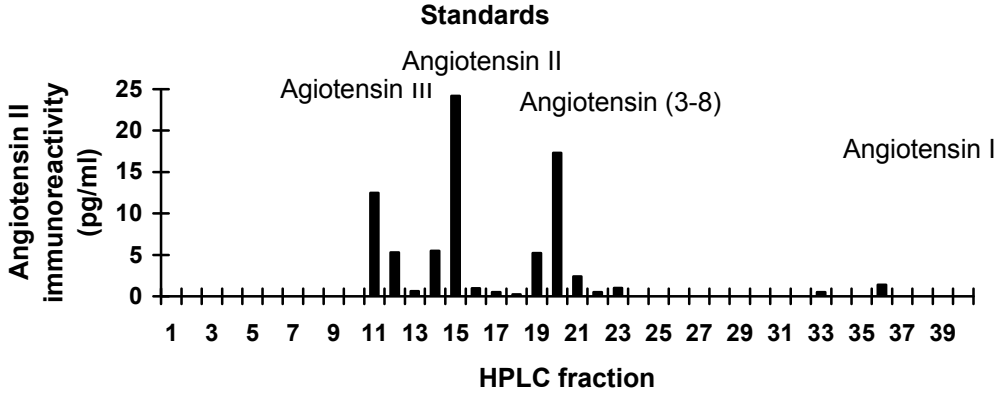
- The Minimum Detectable Concentration (MDC) of Angiotensin II corresponding to the NSB average plus three standard deviations (n = 8) is: 1 pg/mL.
- Quality Ccontrol (QC) samples intra & inter-assay variation: established by measuring each QC five times per assay and in six different assays (i.e. 30 assays per QC):

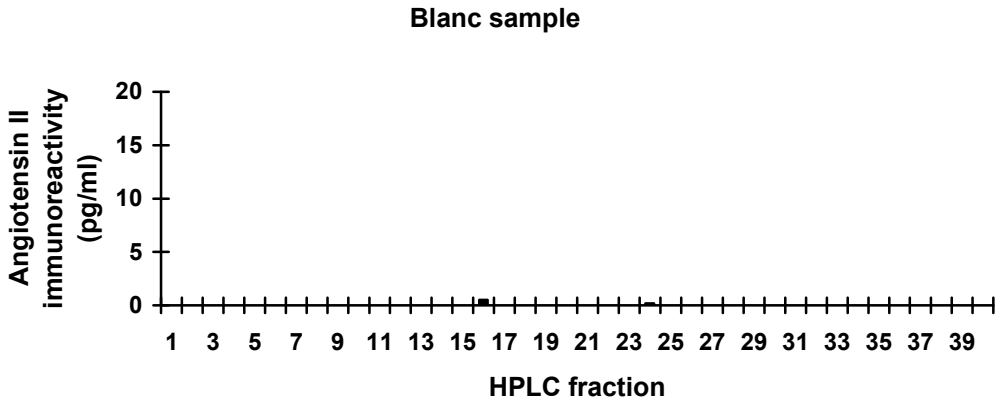
pg/mL	Intra-assay coefficient of variation	Inter-assay coefficient of variation
100	7 %	7 %
20	2 %	5 %
5	6 %	10 %
2	10 %	15 %

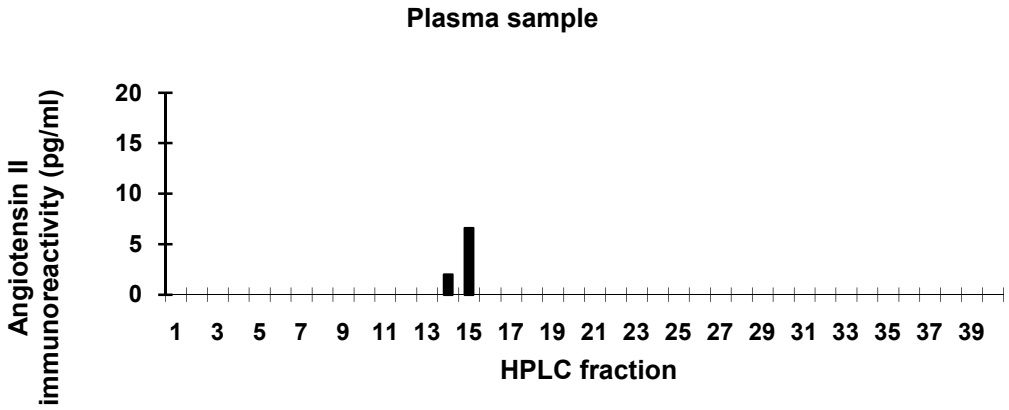
- Limit of quantification: 2 pg/mL
- Cross-reactivity:

Angiotensin II: 100 %
Angiotensin I: 4 %
Angiotensin III: 36 %
Angiotensin 3-8: 33 %
Angiotensin 1-7: <0.01 %

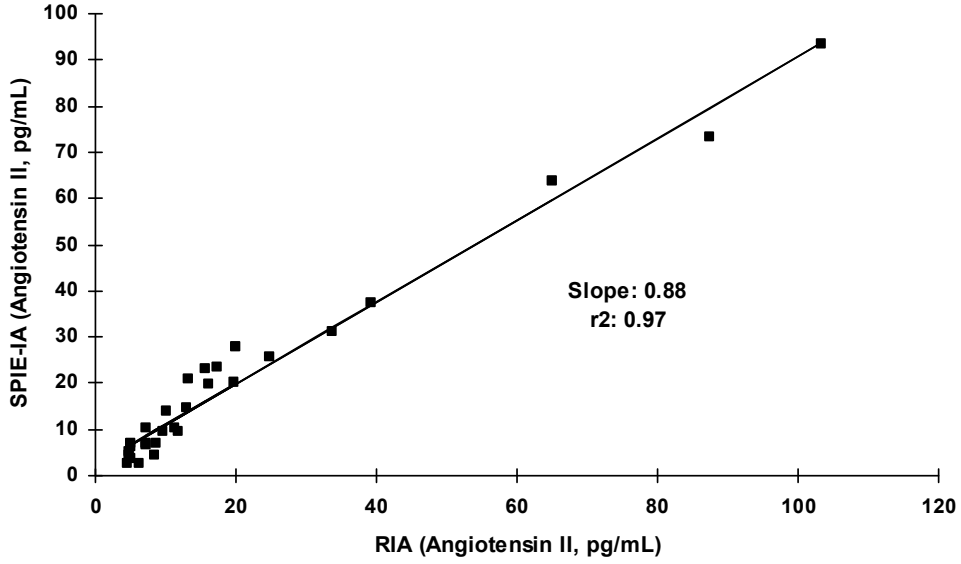
- Specificity: comparison of HPLC profiles of Angiotensin standards, a blank sample and a plasma sample.

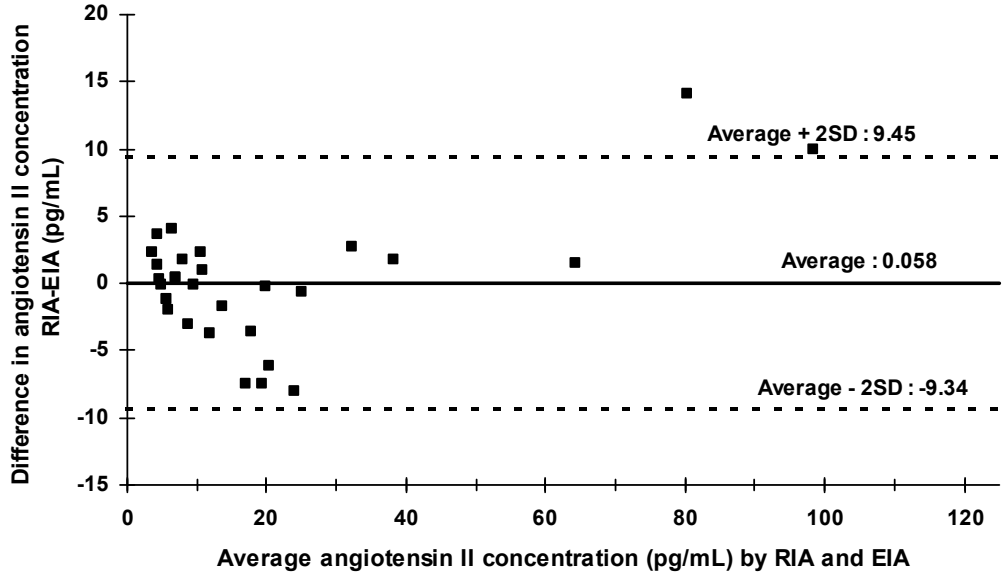






- Comparison with RIA on 29 samples:





11. ASSAY TROUBLE SHOOTING

- Absorbance values too low: incubation in wrong conditions (time or temperature) or reading time too short or mAb-AChE tracer, glutaraldehyde, wash buffer or borane trimethylamine have not been dispensed.
- NSB value too high: contamination of NSB wells with Angiotensin II standard, inefficient washing or borane trimethylamine has been dispensed not on time or not at all.
- High dispersion of duplicates: poor pipetting technique or irregular plate washing.

12. REFERENCES

- **Volland H., Pradelles Ph., Ronco P., Azizi M., Simon D., Créminon C. & Grassi J.**
  A solid-phase immobilized epitope immunoassay (SPIE-IA) permitting very sensitive and specific measurment of angiotensine II in plasma.
  Jounal of Immunological Methods, 228, 37-47, 1999
- **Grassi J. & Pradelles Ph.**
  Compounds labelled by the acetylcholinesterase of *Electrophorus Electricus*. Its preparation process and its use as a tracer or marquer in enzymo-immunological determinations. *United states patent, N°1,047,330. September 10; 1991*
- **Pradelles Ph.**
  Immunometric determination of an antigen or hapten.*United states patent, N°5,476,770. December 19; 1995*

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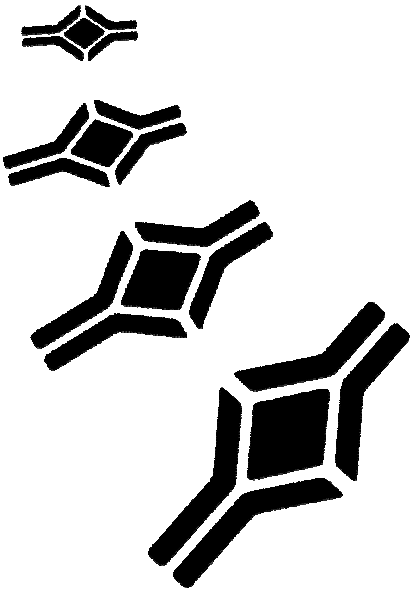

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