AcSDKP

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For laboratory research use only. Not for human or veterinary diagnostic use.

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European patent # 89 139 552
U.S. patent # 50 47 330

AcSDKP
Enzyme Immunoassay kit
#A05881.96 wells

For research laboratory use only
Not for human diagnostic use

This assay has been developed & validated
by Bertin Pharma

Fabriqué en France
Made in France

A11881
Version: 0117

Table of contents

96 wells
Storage: -20°C
Expiry date: stated on the package

This kit contains:

Designation	Colour of cap	Item #	Quantity per kit	Form
Mouse anti-rabbit IgG precoated 96-well Strip Plate	Blister with zip	A08100.1ea	1	
AcSDKP Tracer	Green	A04881.100 dtn	1	Lyophilised
AcSDKP Antiserum	Red	A03881.100 dtn	1	
AcSDKP Standard	Blue with red septum	A06881.1 ea	2	Lyophilised
AcSDKP Quality Control	Green with red septum	A10881.1 ea	2	Lyophilised
EIA Buffer	Blue	A07000.1 ea	1	Lyophilised
Wash Buffer	Silver	A17000.1 ea	1	Liquid
Tween 20	Transparent	A12000.1 ea	1	Liquid
Quality Control stock solution	White whith red septum	D31881.1 ea	1	Liquid
Ellman's Reagent 50	Black with red septum	A09000_50.100 dtn	2	Lyophilised
Technical Booklet	-	A11881.1ea	1	-
Well cover Sheet	-	-	1	-

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 33 samples in duplicate.

If you want to use the kit in two times, we provide one additional vial of Standard, one of Quality Control and one of Ellman's Reagent.

Precaution for use

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

Each time a new pipette tip is used, aspirate a sample or reagent and expel it back into the same vessel. Repeat this operation two or three times before distribution in order to equilibrate the pipette tip.

- 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 amount of reagents contains less than 100 µg of sodium azide. Flush the drains thoroughly to prevent the production of explosive metal azides.

Wearing gloves, laboratory coat and glasses is recommended when assaying kit materials and samples.

Temperature

Unless otherwise specified, all the experiments are done at room temperature (RT), that is around +20°C. Working at +25°C or more affects the assay and decreases its efficiency.

Background

Acetylcholinesterase AChE® Technology

Acetylcholinesterase (AChE®), the enzymatic label for EIA, has the fastest turnover rate of any enzymatic label. This specific AChE is extracted from the electric organ of the electric eel, *Electrophorus electricus*, and is capable of massive catalytic turnover during the generation of the electrochemical discharges. The use of AChE as enzymatic label for EIA has been patented by the French academic research Institute CEA ***[1, 2, 3]***, and Bertin Pharma, formerly known as SPI-Bio, has expertise to develop and produce EIA kits using this technology.

AChE® assays are revealed with Ellman's Reagent, which contains acetylthiocholine as a substrate. The final product of the enzymatic reaction (5-thio-2-nitrobenzoic acid) is bright yellow and can be read at 405-414 nm. AChE® offers several advantages compared to enzymes conventionally used in EIAs:

- **Kinetic superiority and high sensitivity**: AChE® shows true first-order kinetics with a turnover of 64,000 sec^{-1}. That is nearly 3 times faster than Horseradish Peroxidase (HRP) or alkaline phosphatase. AChE® allows a greater sensitivity than other labeling enzymes.
- **Low background**: non-enzymatic hydrolysis of acetylthiocholine in buffer is essentially absent. So, AChE® allows a very low background and an increased signal/noise ratio compared to other substrate of enzymes which is inherently unstable.

- **Wide dynamic range**: AChE® is a stable enzyme and its activity remains constant for many hours as, unlike other enzymes, its substrate is not suicidal. This permits simultaneous assays of high diluted and very concentrated samples.
- **Versatility:** AChE® is a completely stable enzyme, unlike peroxidase which is suicidal. Thus, if a plate is accidentally dropped after dispatch of the AChE® substrate (Ellman's Reagent) or if it needs to be revealed again, one only needs to wash the plate, add fresh Ellman's Reagent and proceed with a new development. Otherwise, the plate can be stored at +4°C with Wash Buffer in wells while waiting for technical advice from the Bioreagent Department.

AcSDKP

AcSDKP is a new reliability marker of chronic ACE inhibition ***[4, 5]***. The tetrapeptide N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is an endogenous regulatory factor of hematopoiesis which reverses stem cells and normal early progenitors into S-phase ***[6, 7]***.

Angiotensin I-Converting Enzyme (ACE) has two homologous active NH2- and COOH-terminal domains and displays activity toward a broad range of substrates. The AcSDKP has been shown to be hydrolyzed by ACE and to be a preferential substrate for its NH2-terminal active site ***[8]***.

In healthy subjects, the acute administration of the ACE inhibitor captopril increases the AcSDKP plasma levels ***[9]***.

Several studies aimed to measure plasma or urine AcSDKP levels during treatment with various ACE inhibitors and to confirm its relevance as a marker of ACE inhibition ***[10]***.

Principle of the assay

This Enzyme Immunometric Assay (EIA) is based on the competition between unlabelled (free) AcSDKP (standards / QC / samples) and acetylcholinesterase (AChE) linked to AcSDKP (Tracer) for limited specific rabbit anti-AcSDKP antiserum sites.

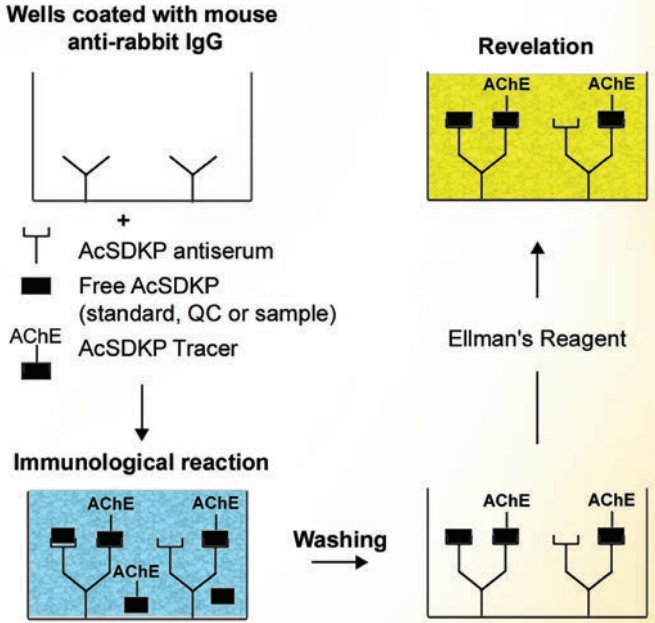
The complex rabbit antiserum-AcSDKP (free AcSDKP or Tracer) binds to the mouse anti-rabbit antibody coating the well.

The plate is then washed to remove any unbound reagent and Ellman's Reagent (enzymatic substrate for AChE and chromogen) is added to the wells.

AChE acts on the Ellman's Reagent to form a yellow compound that strongly absorbs at 414 nm.

The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of Tracer bound to the well and is inversely proportional to the amount of free AcSDKP present in the well during the immunological incubation.

The principle of the assay is summarised below:



Materials and equipment required

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

For sample preparation:

- Methanol
- Heparine tube and Captopril (for blood samples only)

For the assay:

- Precision micropipettes (20 to 1000 µL)
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or washbottles)
- Orbital microplate shaker
- Multichannel pipette and disposable tips 30-300µL
- UltraPure water #A07001.1L
- Polypropylene tubes

Water used to prepare all EIA reagents and buffers must be UltraPure (deionized & free from organic contaminant traces).
Otherwise, organic contamination can significantly affect the enzymatic activity of the tracer Acetylcholinesterase (AChE).
Do not use distilled water, HPLC-grade water or sterile water.
UltraPure water may be purchased from Bertin Pharma: item #A07001.1L.

Sample collection and preparation

This assay may be used to measure AcSDKP in plasma, serum, urine, tissue or cell samples.

General precautions

- All samples must be free from organic solvents prior to assay.
- Samples should be assayed immediately after collection or should be stored at -20°C.

Urine samples

Dilute urine sample more than five fold in EIA buffer (no extraction needed).

Blood samples

Plasma or serum samples should be measured after extraction.

Blood collections

To do so, collect blood samples in tubes containing Heparin (do not use EDTA) and Captopril to 10^{-5} M final concentration (e.g.: 10 µl of 10^{-3} M Captopril solution for 1 ml of blood). Centrifuge the samples at 1 600 g for 20 minutes.

Collect and keep plasma at -20°C until assay.

> Extraction

Thaw the plasma/serum on ice water bath and keep it on ice. Centrifuge 1 mL of plasma during 15 minutes at 4,500 rpm at 4°C.
Collect 0.15 mL of supernatant and add 1 mL of methanol. Vortex the solution twice during 10 seconds and centrifuge it during 15 minutes at 4,500 rpm at 4°C.Decant the supernatant into a clean test tube and dry it by vacuum centrifugation. Reconstitute the sample with 0.45 mL of EIA buffer (dilution of initial plasma/serum sample = three fold).

> Recovery and calculation

To determine the recovery factor after extraction, use the QC Stock solution #D31881 (500 nM) provided in this kit.

- > Thaw 1 mL of plasma/serum.
- > Dilute 0.1 mL of QC Stock Solution (500 nM) with 0.9 mL of plasma/serum to prepare a QC Working Solution at the concentration of 50 nM.
- > Collect 0.15 mL of the QC Working Solution (50 nM) and add 1 mL of methanol. Vortex the solution twice during 10 seconds and centrifuge it during 15 minutes at 4,500 rpm at 4°C.
- > Decant the supernatant into a clean test tube and dry it by vacuum centrifugation. Reconstitute the sample with 3 mL of EIA buffer (dilution of initial QC Working Solution = 1:20 = dilution factor of the extraction).
- > Determine by EIA the concentration of the extracted QC Working Solution.

The recovery is determined by comparing the concentration of the extracted QC Working Solution determined by EIA multiplied by 20 (the dilution factor of the extraction) to the concentration of the initial QC Working Solution (50 nM).

▷ Tissue or Cell samples

For heterogeneous samples, like cell pellets, cell lysis supernatant or tissue homogenate supernatant, we advise to extract samples before asssay.

Pease refer to literature for additional information ***[11, 12, 13]***.

Reagent preparation

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 33 samples in duplicate.

If you want to use the kit in two times, we provide one additional vial of Standard, one of Quality Control and one of Ellman's Reagent.

All reagents need to be brought to room temperature, around +20°C, prior to the assay.

EIA Buffer

Reconstitute the vial #A07000 with 50 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at 4°C: 1 month

AcSDKP Standard

Short immunological reaction for urine samples only

Reconstitute one AcSDKP Standard vial #A06881 with 1 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

The concentration of the first standard (S1) is 25 nM.

Prepare seven polypropylene tubes (for the seven other standards S2 to S8) and add 500 µL of EIA buffer into each tube. Then

prepare the standards by serial dilutions as follow:

Standard	Volume of Standard	Volume of Assay Buffer	Standard concentration
S1	-	-	25 nM
S2	500 µL of S1	500 µL	12.5 nN
S3	500 µL of S2	500 µL	6.25 nN
S4	500 µL of S3	500 µL	3.12 nM
S5	500 µL of S4	500 µL	1.56 nM
S6	500 µL of S5	500 µL	0.78 nM
S7	500 µL of S6	500 µL	0.39 nM
S8	500 µL of S7	500 µL	0.19 nM

Stability at 4°C: 24 hours.

Long immunological reaction for any type of sample

Reconstitute one AcSDKP Standard vial #A06881 with 2 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

The concentration of the first standard (S1) is 12.5 nM.

Prepare seven polypropylene tubes (for the seven other standards S2 to S8) and add 500 µL of EIA buffer into each tube. Then prepare the standards by serial dilutions as follow:

Standard	Volume of Standard	Volume of Assay Buffer	Standard concentration
S1	-	-	12.5 nM
S2	500 µL of S1	500 µL	6.25 nM
S3	500 µL of S2	500 µL	3.12 nM
S4	500 µL of S3	500 µL	1.56 nM
S5	500 µL of S4	500 µL	0.78 nM
S6	500 µL of S5	500 µL	0.39 nM
S7	500 µL of S6	500 µL	0.19 nM
S8	500 µL of S7	500 µL	0.09 nM

Stability at 4°C: 24 hours.

AcSDKP Quality Control

Reconstitute one vial #A10881 with 1 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at +4°C: 24 hours.

AcSDKP Tracer

Reconstitute the vial #A04881 with 5 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at +4°C: 1 month.

AcSDK Antiserum

Reconstitute the vial #A03881 with 5 mL of UltraPure water for **short immunological reaction** or 10 mL of UltrPure water for **long immunological reaction**. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at +4°C: 1 month.

Quality Control stock solution

The QC stock solution contained in the vial #D31881 is ready to use.

Wash Buffer

Dilute 2 mL of concentrated Wash Buffer #A17000 with 800 mL of UltraPure water. Add 400 µL of Tween 20 #A12000. Use a magnetic stirring bar to mix the content.
Stability at +4°C: 1 month.

Ellman's Reagent

5 minutes before use (development of the plate), reconstitute one vial of Ellman's Reagent #A09000_50 with 50 mL of UltraPure water. The tube content should be thoroughly mixed.
Stability at +4°C and in the dark: 24 hours.

Assay procedure

It is recommended to perform the assays in duplicate and to following 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.
Stability at +4°C: 1 month.

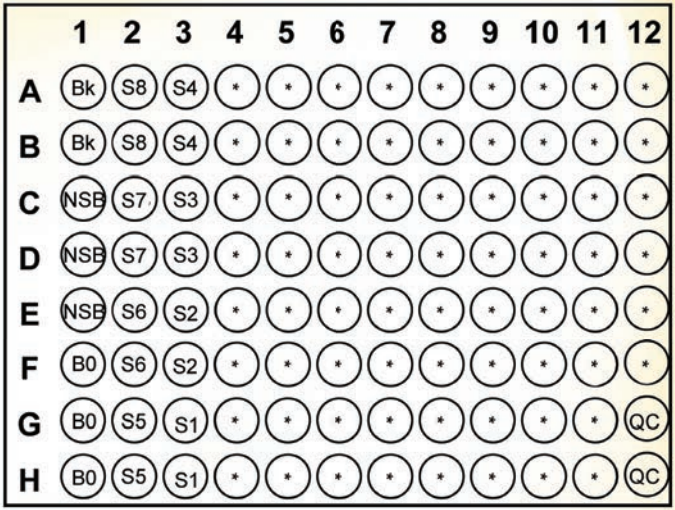
Rinse each well 5 times with Wash Buffer (300 µL/well).

Just before distributing the reagents and samples, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel.

Plate set-up

A plate set-up is suggested hereafter.

The contents of each well may be recorded on the template sheet provided at the end of this technical booklet.



Bk : Blank

NSB : Non Specific Binding

*/QC : Samples or Quality Controls

B0: Maximum Binding

S1-S8 : Standards 1-8

▷ Pipetting the reagents

All samples and reagents must reach room temperature prior to performing the assay. Use different tips to pipet the buffers, standards, samples, Tracer, antiserum and other reagents.

Before pipetting, equilibrate the pipette tips in each reagent.

Do not touch the liquid already in the well when expelling with the pipette tip.

> **EIA Buffer**
Dispense 100 µL to Non Specific Binding (NSB) wells and 50 µL to Maximum Binding (B0) wells.

> **AcSDKP Standard**
Dispense 50 µL of each of the eight standards (S8 to S1) in duplicate to appropriate wells.
Start with the lowest concentration standard (S8) and equilibrate the tip in the next higher standard before pipetting.

> **AcSDKP Quality Control and Samples**
Dispense 50 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in EIA Buffer.

> **AcSDKP Tracer**
Dispense 50 µL to each well, **except** Blank (Bk) wells.

> **AcSDKP Antiserum**
Dispense 50 µL to each well, **except** Blank (Bk) wells and Non Specific Binding (NSB) wells.

▷ Incubating the plate

Cover the plate with the cover sheet and incubate for:

- 3 hours (short immunological reaction) at room temperature (22°C), for urine samples only.

or

- 18 hours (long immunological reaction) at +4°C for any type of sample.

Developing and reading the plate

- Reconstitute Ellman's Reagent as mentioned in the Reagent preparation section.

- Empty the plate by turning it over. Rinse each well 5 times with 300 µL of Wash Buffer. At the end of the last washing step, empty the plate and blot the plate on a paper towel to discard any trace of liquid.

- Add 200µL of Ellman's Reagent to each well. Cover the plate with an aluminium sheet and incubate in the dark at room temperature. Optimal development is obtained using an orbital shaker.

- Wipe the bottom of the plate with a paper towel, and make sure that no liquid has splashed outside the wells.

- Read the plate at a wavelength between 405 and 414nm (yellow colour).
  After addition of Ellman's Reagent, the absorbance has to be checked periodically (every 30 minutes) until the maximum absorbance (B0 wells) has reached 0.2-0.8 unit (blank subtracted).

Enzyme Immunoassay Protocol (volumes are in µL)					
Volume / Wells	Blank	NSB	B0	Standard	Sample or QC
EIA Buffer	-	100	50	-	
Standard	-			50	-
Sample or QC					50
Tracer	-	50	50	50	50
Antiserum	-		50	50	50
Cover plate, incubate 3 hours at room temperature (for urine sample only), or 18 hours at +4°C (for any type of sample)					
Wash strips 5 times & discard liquid from the wells					
Ellman's Reagent	200				
Incubate with an orbital shaker in the dark at RT					
Read the plate between 405 and 414 nm					

Data analysis

Make sure that your plate reader has subtracted the absorbance readings of the blank wells (absorbance of Ellman's Reagent alone) from the absorbance readings of the rest of the plate. If it is not the case, please do it.

- Subtract the average absorbance of NSB for each B0, standards, quality control and samples.
- Calculate the average absorbance for each B0, standard, QC and sample.
- Calculate the B/B0 (%) for each standard, QC and sample (average absorbance of standard, QC or sample divided by average of B0) & multiplied by 100
- For each standard, using a semi-log graph paper, plot the B/B0 (%) on y axis versus the concentration on x axis. Draw a best-fit line through the points.
- To determine the concentration of your samples, the corresponding B/B0 (%) value has to fall within the linear range of the standard curve (usual range of 20%-80%). Find the B/B0 (%) value of each sample on the y axis.
- Read the corresponding value on the x axis which is the concentration of your unknown sample.
- Most plate readers are supplied with a curve-fitting software capable of graphing these data (4-parameter logistic fit 4PL). If you have this type of software, we recommend using it. Refer to it for further information.

Two vials of Quality Control are provided with this kit.

Your standard curve is validated only if the calculated concentration of the Quality Control obtained with the assay is +/- 25% of the expected concentration (see the label of QC vial).

Acceptable range

- B0 absorbance >0.200 A.U. blank subtracted in the conditions indicated above.
- NSB absorbance <0.035 A.U.
- IC50:
  - 2.0 nM, after short immonological reaction
  - 0.5 nM, after long immonological reaction
- QC sample: ± 25% of the expected concentration (see the label of QC vial)

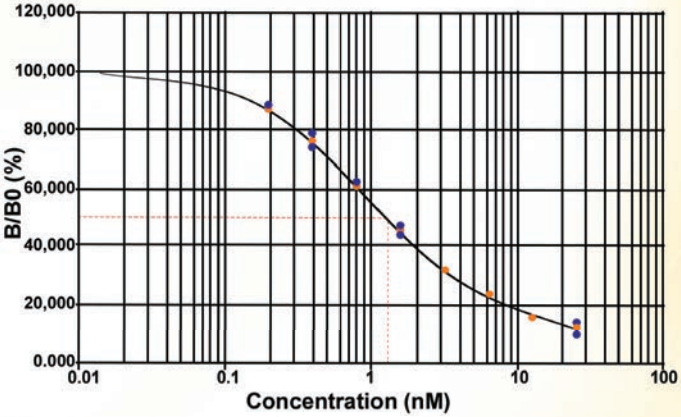
Typical results

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: 90 minutes developing at +20°C, reading at 414 nm. A 4-parameter curve fitting was used to determine the concentrations.

	Short immunological reaction		Long immunological reaction	
Standard	**mAU**	**B/B0 (%)**	**mAU**	**B/B0 (%)**
NSB	0	0	2	0
B0	292	100.0	347	100.0
25.0 nM	28	9.6		
12.5 nM	45	15.4	23	6.6
6.25 nM	76	25.9	36	10.4
3.12 nM	119	40.7	66	19.0
1.56 nM	163	55.9	97	28.0
0.78 nM	197	67.6	154	44.4
0.39 nM	232	79.8	223	64.3
0.19 nM	258	88.5	283	81.6
0.09 nM			296	85.3
QC	140	48	93	26.8

Typical AcSDKP standard curve



Assay validation and characteristics

For additional information regarding the validation of immunoassay for protein biomarkers in biological samples, please refer to bibliography ***[14, 15]***.

> Cross-reactivity [5]

Number	Peptide sequence	Abbreviated	CR (%)
1	Acetyl-Ser-Asp-Lys-Pro	AcSDKP	100
2	Acetyl-Ser-Asp-Orn-Pro	AcSDOP	500
3	Acetyl-Ser-Asp-Arg-Pro	AcSDRP	100
4	Acetyl-Ala-Asp-Lys-Pro	AcADKP	6
5	Acetyl-Ser-Asp-Lys	AcSDK	0.03
6	Ser-Asp-Lys-Pro	SDKP	0.5
7	Acetyl-Ser-Asp-Lys-Pro-Asp-Cys	AcSDKPDC	<0.01
8	Acetyl-Ser-Asp-Lys-Pro-Tyr	AcSDKPY	<0.01
9	Thymosin B4	TB4	<0.25
10	rTumor Necrosis Factor	rTNFa	<0.01

Cross-reactivity was determined by comparing the molar concentration at B/B0 50% of each peptide to that of AcSDKP and expressing it as a percentage of AcSDKP immunoreactivity.

Immunochromatograms (plasma sample)

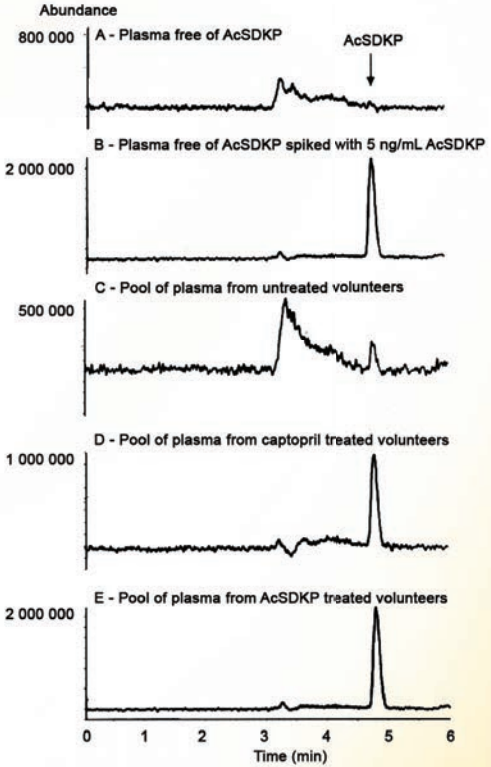


Fig. 1: chromatograms obtained from plasma samples with selected ion monitoring. Plasma free from AcSDKP (A), plasma free from AcSDKP but spiked with 5 ng/mL AcSDKP (B), pooled samples from untreated volunteers (C), pooled plasma from captopril-treated volunteers (D) and pooled plasma from AcSDKP-treated volunteers (E)
The internal standard peak is not shown.

Immunochromatograms (urine sample)

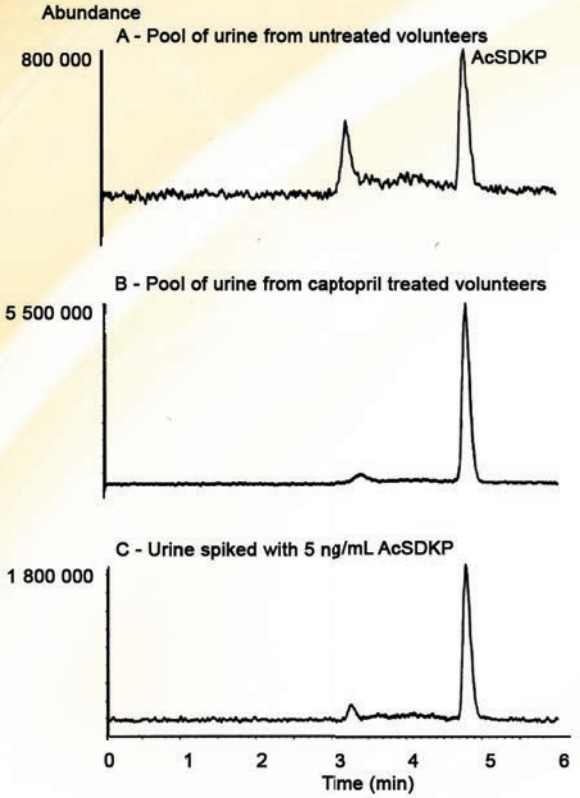


Fig. 2: chromatograms obtained from urine samples with selected ion monitoring. Pooled urine samples from untreated volunteers (A), pooled urine from captopril-treated volunteers (B), and urine from untreated volunteers but spiked with 5 ng/mL AcSDKP (C).
The internal standard peak is not shown.

Correlation

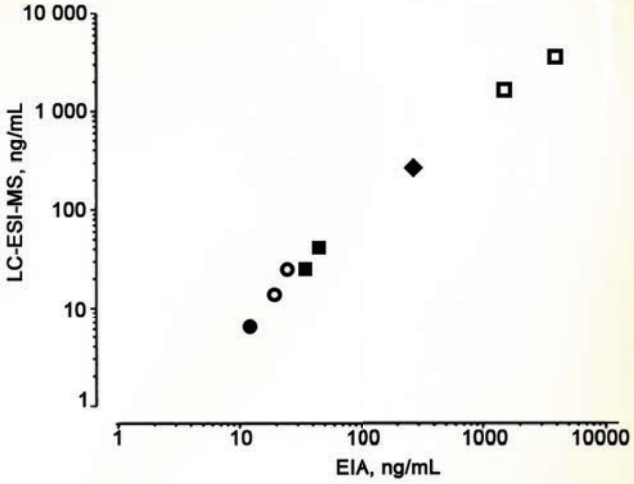


Fig. 3: correlation between EIA and LC-ESI-MS for AcSDKP measurements in immunoextracted samples. Pooled plasma from untreated volunteers (1 sample) (●), pooled plasma free from captopril-treated volunteers (2 samples) (■), pooled plasma from AcSDKP-treated volunteers (1 sample) (◆), pooled urine from untreated volunteers (2 samples) (○), and pooled urine from captopril-treated volunteers (2 samples) (□).

Troubleshooting

- **Absorbance values are too low**:
  - organic contamination of water,
  - one reagent has not been dispensed,
  - incorrect preparation / dilution,
  - assay performed before reagents reached room temperature,
  - reading time not long enough.

- **High signal and background in all wells**:
  - inefficient washing,
  - overdeveloping (incubation time should be reduced),
  - high ambient temperature.

- **IC50 or QC concentrations not within expected range**: wrong preparation of standards.

- **High dispersion of duplicates**:
  - poor pipetting technique,
  - irregular plate washing.

- **Analyses of two dilutions of a biological sample do not agree**: Interfering substances are present. Sample must be purified prior to EIA analysis (excepting plasma samples).

- **If a plate is accidentally dropped after dispatch of the AChE® substrate (Ellman's Reagent) or if it needs to be revealed again:**
  - one only needs to wash the plate, add fresh Ellman's Reagent and proceed with a new development.

- otherwise, the plate can be stored at +4°C with Wash Buffer in wells while waiting for technical advice from the Bioreagent Department.

These are a few examples of troubleshooting that may occur. If you need further explanation, Bertin Pharma will be happy to assist you. Feel free to contact our technical support staff by phone (+33 (0)139 306 036), fax (+33 (0)139 306 299) or E-mail (bioreagent@bertinpharma.com), and be sure to indicate the batch number of the kit (see outside the box).

Bertin Pharma proposes EIA Training kit #B05005 and EIA workshop upon request. For further information, please contact our Marketing Department by phone (+33 (0)139 306 260) or E-mail (marketing@bertinpharma.com).

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