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Characterization of immunoreactive acetyl–Ser–Asp–Lys–Pro in human plasma and urine by liquid chromatography–electrospray mass spectrometry

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Abstract

The tetrapeptide AcSDKP, a natural and specific substrate of angiotensin I-converting enzyme (ACE), is a negative regulator of hematopoiesis. AcSDKP has been measured in various biological media using an enzyme immunoassay (EIA), but its presence in human plasma and urine has not been formally established. By using immunoaffinity extraction and liquid chromatography–electrospray mass spectrometry, we demonstrate that AcSDKP-like immunoreactivity measured with EIA in plasma and urine samples from untreated, captopril- (an ACE inhibitor) and AcSDKP-treated subjects corresponds to AcSDKP. The present study confirms that AcSDKP is naturally present in human plasma and urine and that EIA is reliable for its measurement in such media.

Keywords: Acetyl–Ser–Asp–Lys–Pro; Angiotensin I-converting enzyme inhibitor

1. Introduction

The tetrapeptide acetyl–Ser–Asp–Lys–Pro (AcSDKP) is an endogenous negative regulator of hematopoiesis [1] that has been tested in humans as a bone-marrow protector against cytotoxic drugs during anticancer chemotherapy [2]. Angiotensin-converting enzyme (ACE), which plays a key role in cardiovascular homeostasis, has been shown recently to be involved in AcSDKP metabolism [3,4]. This may have two therapeutic consequences: (1) the administration of ACE inhibitors to cancer patients during antitumor chemotherapy in order to increase endogenous AcSDKP levels and sustain the bone-marrow protector effect of AcSDKP, which is otherwise limited by its short half-life [5,6] and (2) the use of plasma or urine AcSDKP levels as a reliable marker of ACE inhibition for evaluation of compliance in hypertensive therapy [7,8].

An enzyme immunoassay (EIA) with polyclonal antibodies has been developed for the measurement of AcSDKP in various biological media [9,10]. Because of their sensitivity, immunoassays represent a methodology of reference for the measurement of peptides, therapeutic proteins or biomarkers in biological media [11]. The major limitation of these methods is the potential lack of specificity, requiring an adequate validation study. This is usually

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achieved by cross-reactivity studies. As a complement, fractionation of the immunoreactivity by chromatography should indicate the presence of a single immunoreactive peak corresponding to the retention time of the analyte [12]. However, such a strategy failed to demonstrate the natural presence of AcSDKP in plasma, since immunoreactive material did not coelute with AcSDKP standards, suggesting that AcSDKP-like immunoreactivity might be attributed to a related peptide with other physico-chemical properties [9].

The aim of this study was to verify the specificity of the EIA for AcSDKP. A selective immunoaffinity extraction method [13,14] followed by liquid chromatography–electrospray mass spectrometry (LC–ESI–MS) was therefore developed for the formal identification of AcSDKP in human plasma and urine. Biological material was taken from untreated subjects and from subjects given an ACE inhibitor or synthetic AcSDKP. A correlation between AcSDKP levels measured by EIA and LC–ESI–MS in immunoextracted samples was then established in order to confirm the reliability of EIA for the measurement of AcSDKP in human plasma and urine.

2. Experimental

2.1. Reagents

Acetyl–Ser–Asp–Lys–Pro (AcSDKP) and acetyl–Thr–Asp–Lys–Pro (AcTDKP) were from Neosystem (Strasbourg, France). HPLC-grade acetonitrile, methanol and isopropanol were from Merck (Darmstadt, Germany). Captopril, acetic acid, propionic acid and trifluoroacetic acid (TFA) were from Sigma (St. Louis, MO, USA).

2.2. Biological samples

Plasma and urine pools were obtained from a clinical study in which healthy volunteers received no treatment, or captopril (50 mg twice a day), or an AcSDKP intravenous infusion (16.8 μg/kg in 15 min). The clinical study was conducted at the Broussais Hospital Clinical Investigation Center (Paris, France). Volunteers gave written consent to receive treatment and the protocol was approved by an Ethics Committee (Cochin Hospital, Paris, France). Blood samples were collected in the presence of $1 \cdot 10^{-6}$ *M* captopril, to prevent AcSDKP degradation by endogenous ACE and then were immediately centrifuged at 4500 rpm at 4°C. Plasma was stored at −20°C. Urine samples were centrifuged at $2000 \times g$ at 4°C and the supernatants were stored at −20°C until analysis. Human plasma (Etablissement de Transfusion Sanguine, Rungis, France), free of AcSDKP, was obtained after an overnight incubation at 37°C (optimal conditions for the degradation of AcSDKP by ACE).

2.3. Immunoaffinity extraction procedure

Rabbit antiserum containing polyclonal antibodies directed against AcSDKP [9] was immunopurified by passage through a column containing immobilized AcSDKP on Sepharose 4B (Pharmacia Biotech, Orsay, France). An affinity gel was prepared by coupling the immunopurified antiserum with CNBr-activated Sepharose 4B (Pharmacia Biotech). The couplings were performed according to the manufacturer's instructions. Individual cartridges containing 200 μl of affinity gel were prepared and stored at 4°C in 0.1 *M* phosphate buffer, pH 7.4, containing 0.1% bovine serum albumin, 0.001% sodium azide, 2.3% sodium chloride and 0.2% EDTA.

Immunoaffinity extraction and concentration of biological samples were performed as follows. Pooled plasma samples (6 ml) were mixed with methanol, centrifuged and the supernatants were evaporated to dryness. The dried residues were reconstituted in 2 ml of 0.1 *M* phosphate buffer, pH 7.4. Reconstituted plasma samples (2 ml) or pooled urine samples (2 ml) were applied to each affinity gel cartridge and mixed end-over-end for 1 h at room temperature. The unbound material was successively washed away with 9 ml of 0.1 *M* phosphate buffer, pH 7.4, 3 ml of 0.2 *M* acetate buffer, pH 4.0, and 3 ml of 0.2 *M* formate buffer, pH 3.0. The immunoreactive material was eluted with 6 ml of methanol, which was evaporated to dryness. The dried residues were dissolved in 0.2 ml of 0.1% trifluoroacetic acid (TFA) in water containing 30 ng/ml of the internal standard AcTDKP.

2.4. LC–ESI–MS

2.4.1. Liquid chromatography

HPLC was performed using an HP 1100 system (Hewlett-Packard, Les Ulis, France) with a 0.2-μm on-line filter, a 10×4.0 mm modulo-cart pre-column (Interchim, Asnières, France) and a Kromasil C_{18}, 5 μm, 100 Å (150×2.1 mm) column (Touzart et Matignon, Courtaboeuf, France). The mobile-phase consisted of acetonitrile–water (30:70, v/v) containing 0.1% TFA and was delivered at a flow-rate of 0.1 ml/min.

2.4.2. Post-column derivation

Propionic acid–2-isopropanol (75:25, v/v) was added post-column at a flow-rate of 50 μl/min, in order to limit the signal suppression effects induced by TFA in electrospray ionization [15].

2.4.3. Mass spectrometry (MS)

The mass spectrometer was a Nermag R1010C (Quad-Services, Poissy, France) with an Analytica electrospray interface (Brandford, CT, USA). Both HPLC and MS were controlled by the HP Chemstation software (Hewlett-Packard), allowing simultaneous instrument control, data acquisition and data analysis. The drying gas heater was fixed at 350°C. The capillary, end plate and cylinder voltages were fixed at −4500, −3700 and −2500 V, respectively. An electron multiplier voltage of 750 V was used. The dwell time was 100 ms. The instrument was operated in the positive-ion mode. The $[M+H]^+$ ion at m/z 488 for AcSDKP and 502 for AcTDKP (internal standard) were selected by the quadrupole. A standard calibration curve was prepared in the mobile phase for the quantification of AcSDKP in the extracts. Linear regression was performed between the ratio of the peak areas of AcSDKP to that of the internal standard and AcSDKP concentrations.

2.5. Enzyme immunoassay of AcSDKP

AcSDKP was quantified in immunoextracted plasma and urine by means of a competitive enzyme immunoassay with a detection limit of 0.1 ng/ml [9].

2.6. Statistical analysis

Calculations were done using Sigmastat statistical software (Jandel Corporation, San Raphael, CA, USA). After logarithmic transformation, data obtained with the LC–ESI–MS system were compared to the EIA measurements by linear regression analysis. Student's *t*-test was used to assess any statistically significant differences between the slope and one, and between the intercept of the generated straight line and zero.

3. Results and discussion

3.1. LC–ESI–MS

The LC–ESI–MS system was operated in the selected ion monitoring mode. The analog AcTDKP was chosen as the internal standard because of its structural analogy with AcSDKP and its AcSDKP-like behavior during the analytical process. The internal standard could not be added before the immunoaffinity extraction, since it was not recognized by the antibodies. Nevertheless, it has been used in order to correct variability of the electrospray ionization process. The retention times of AcSDKP and AcTDKP were 4.8 min. The standard calibration curve was linear for concentrations of AcSDKP ranging from 5 to 100 ng/ml. The slope was 0.0318±0.0005 (mean±SE), the intercept was −0.0322±0.0240 (mean±SE), and the correlation coefficient (r) was 0.9995 (n=7). This method has not been fully validated as recommended by Shah et al. [12] since it was not designed for pharmacokinetic or bioequivalence studies. However, the coefficients of variation (C.V.) obtained from the repeated analysis of immunoextracted standards and samples were always within 5 and 10% for the intra-day (n=3) and the inter-day (n=3) experiments, respectively. The limit of quantification (L.O.Q.) was set at the lowest standard value (5 ng/ml, S/N=8). The C.V. of the back-calculated concentrations at the L.O.Q. was 15% and the accuracy was within 20%.

3.2. Immunoaffinity extraction

Since the immunoreactivity related to AcSDKP was less than 0.5 ng/ml in the least concentrated biological sample to be analyzed (plasma basal level), and since the lowest standard value was 5

ng/ml, sample concentration by at least a factor of ten was required. Procedures involving liquid–liquid extraction with organic solvents such as methanol, or solid-phase extraction on various C_{18} sorbents, were tested, but resulted in insufficient sample clean-up. An affinity gel was therefore tested and evaluated by extracting plasma and urine samples that were previously spiked with various amounts of AcSDKP.

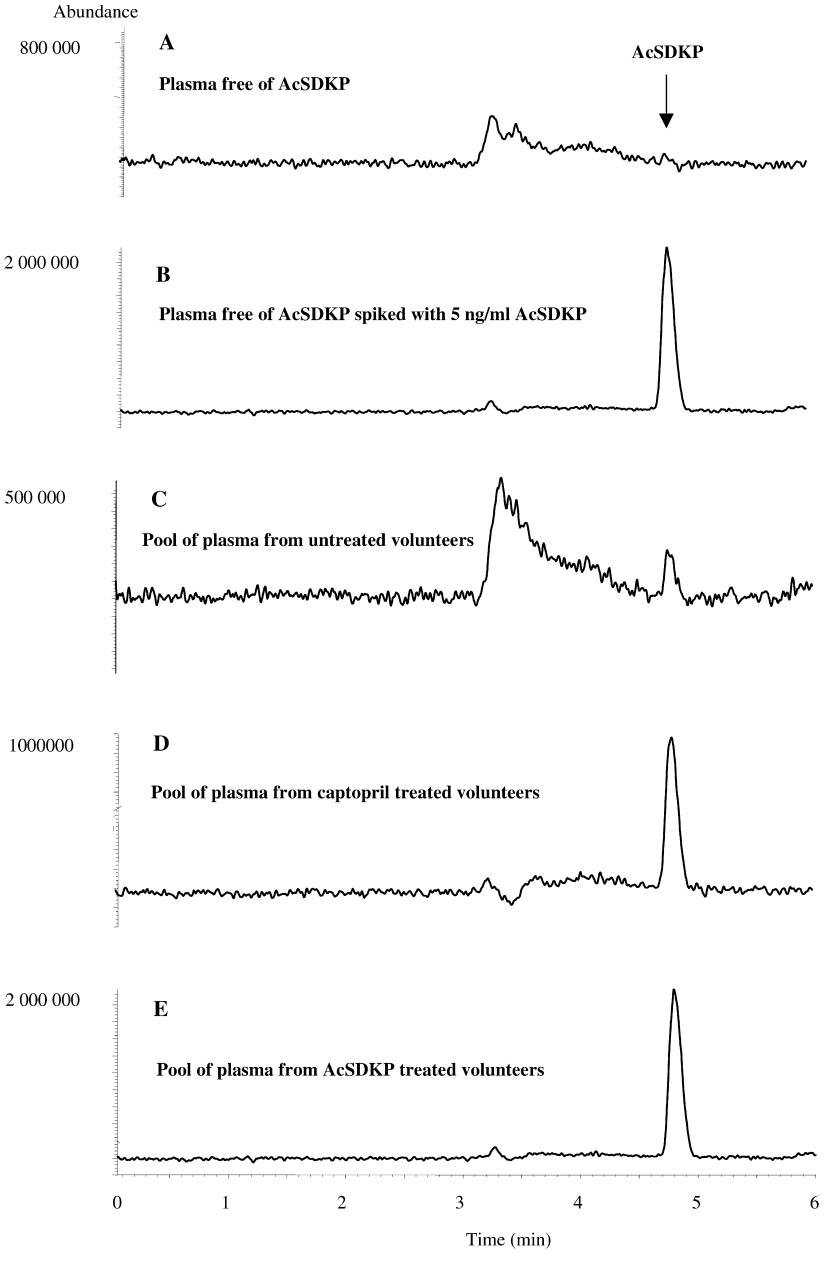

Fig. 1. Chromatograms obtained from plasma samples with selected ion monitoring. Plasma free of AcSDKP (A), plasma free of AcSDKP but spiked with 5 ng/ml AcSDKP (B), pooled plasma samples from untreated volunteers (C), pooled plasma from captopril-treated volunteers (D) and pooled plasma from AcSDKP-treated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

The mean recovery in plasma was 64% for concentrations ranging from 2.5 to 25 ng/ml AcSDKP. In urine, the mean recovery was 88% for the same range of AcSDKP concentrations, but fell to 67 and 15% for 250 and 1000 ng/ml of spiked AcSDKP, respectively, indicating saturation of the affinity gel by an excess of AcSDKP. Immunoextraction recoveries were found to be lower in plasma than in urine because plasma samples were previously extracted using methanol, with a recovery that ranged from 70 to 80%.

Using the immunoextraction procedure, we demonstrated that LC–ESI–MS analysis of a plasma sample free of AcSDKP (plasma incubated overnight at 37°C in the absence of ACE inhibitor) did not show any signal at m/z 488 at a retention time of 4.8 min (Fig. 1A). The occurrence of a specific signal was demonstrated when the same plasma was spiked with AcSDKP (Fig. 1B).

3.3. Identification of AcSDKP in biological samples

The immunoaffinity extraction procedure was then applied to eight pools of human biological samples from various sources: plasma and urine from untreated volunteers (one and two samples, respectively), plasma and urine from captopril-treated vol-

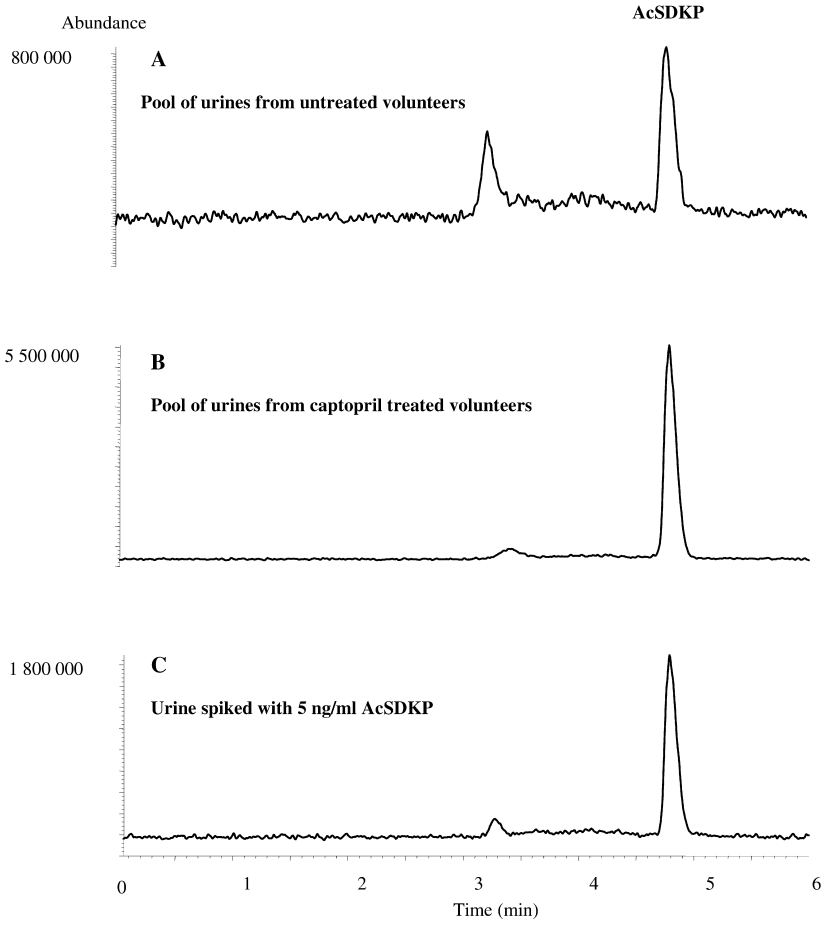

Fig. 2. Chromatograms obtained from urine samples with selected ion monitoring. Pooled urine from untreated volunteers (A), pooled urine from captopril-treated volunteers (B), and urine from untreated volunteers that was spiked with 5 ng/ml AcSDKP (C). The internal standard peak is not shown.

unteers (two samples for each), and plasma from AcSDKP-treated volunteers (one sample).

Selected chromatograms obtained from plasma and urine samples are presented in Figs. 1 and 2, respectively. Urine samples from captopril-treated volunteers were diluted before analysis. Chromatograms corresponding to endogenous immunoreactivity (untreated and captopril-treated subjects, Figs. 1C, D and 2A, B, for plasma and urine samples, respectively) showed a single peak at a retention time of 4.8 min, indicating the natural presence of AcSDKP. The signal was stronger in the sample from subjects treated with captopril.

AcSDKP was also identified from a pooled plasma sample from subjects who received AcSDKP intravenously (Fig. 1E), confirming previous results obtained after chromatographic separation and immunoassay detection [5].

3.4. LC–ESI–MS and EIA measurements of AcSDKP in immunoextracted biological samples

AcSDKP levels in immunoextracted samples were quantified by EIA and the corresponding results were then compared to LC–ESI–MS values by means of linear regression analysis (Fig. 3). The linear regression analysis equation was Log (LC–ESI–MS)= 1.077 Log (EIA)−0.555, with a correlation coefficient (r) of 0.998. The slope and the intercept did not significantly differ from the values of one and zero, respectively, indicating that the two methods gave similar results. Due to the small quantity of plasma available from the clinical study, we analyzed a small number of samples, but the correlation that has been established between EIA and LC–ESI–MS covered a large range of concentrations.

The aim of this work was not to develop a new analytical method for the determination of AcSDKP, but rather to report the natural presence of this peptide in human plasma and urine, where its endogenous presence has never been formally assessed. Due to the formal identification of AcSDKP by the use of immunoaffinity extraction followed by LC–ESI–MS analysis, and the strong correlation between the concentrations measured by EIA and those obtained by LC–ESI–MS in immunoextracted samples, our study demonstrates that AcSDKP is naturally present in human plasma and urine and can be reliably quantified by EIA. Finally, this study

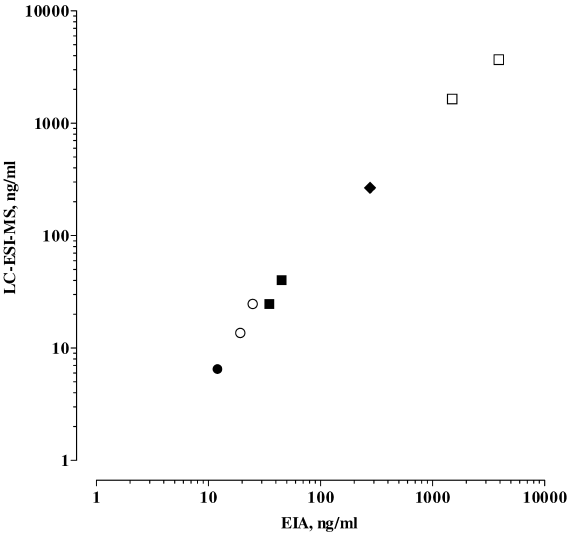

Fig. 3. Correlation between EIA and LC–ESI–MS for AcSDKP measurements in immunoextracted samples. Pooled plasma from untreated volunteers (one sample) (●), pooled plasma from captopril-treated volunteers (two samples) (■), pooled plasma from AcSDKP-treated volunteers (one sample) (♦), pooled urine from untreated volunteers (two samples), (O) and pooled urine from captopril-treated volunteers (two samples) (□).

underlines the use of LC–ESI–MS in immunoanalysis, in order to confirm the specificity of the antibodies.

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