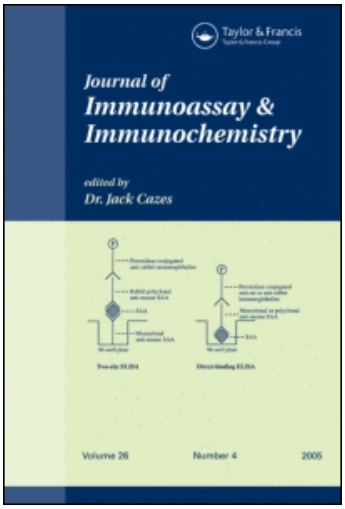

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Development and Validation of an ELISA Method for Detection of Growth Arrest Specific 6 (GAS6) Protein in Human Plasma

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Abstract: Gas6 protein is possibly involved in human diseases, but a validated plasma assay is lacking. So, we developed a sandwich enzyme-linked immunosorbent assay (ELISA) method using commercially available reagents. An appropriate plasma-based matrix was prepared to optimize the assay. The ELISA method showed inter- and intra-assay coefficients of variation lower than 15%. Recoveries all fell within 15% of expected values. Plasma Gas6 concentration in 61 healthy donors was 20.3 $\pm$ 3.8 ng/mL. Our assay meets FDA requirements for precision and accuracy for the validation of bioanalytical methods and it is suitable for research or diagnostic purposes.

Keywords: Gas6, ELISA, Development, Assay, Normal concentration, Validation

INTRODUCTION

The Growth Arrest Specific gene 6 (Gas6), described to be up-regulated during the G_0-G_1 phase of the cell cycle,[1] codify for a 70 KDa protein of 678 amino acids. The Gas6 protein contains three domains: an N-terminal (Gla) domain of γ-carboxyglutammic acid residues, four epidermal growth factor(EGF)-like repeats, and a C-terminal steroid hormone-binding globulin(SHBG)-like site with two globular regions involved in receptor binding. Gas6 protein displays a 46% structure homology with protein S, a negative

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regulator of the coagulation cascade; the homology being higher for the EGF-like sequences.[2] Gas6 gene maps on chromosome 13q34, close to other genes involved in the coagulation cascade.[3] However, it seems not to participate directly in the coagulation process, since it lacks the thrombin-sensitive cleavage site which is, instead, present in protein S.[4]

A number of in-vitro studies evidenced that Gas6 protein is expressed in several human cells and tissues, such as endothelial (EC) and vascular smooth muscle cells (VSMCs),[5,6] normal and malignant haemopoietic cells,[7] brain tissue,[8] and Sertoli cells.[9] It has been demonstrated that, as secreted protein,[2] Gas6 displays mitogenic/survival activity on several mesenchimal and epithelial cell lines, mostly exerted by an autocrine pathway;[10] moreover, the same activity has been shown on different human tumour cell lines.[11–13] Gas6 is the only known ligand for Axl, Sky, and Mer, all members belonging to the same receptor tyrosine kinase family. Vitamin K dependent γ-carboxylation of the Gla residues is crucial for Gas6 activity and receptor binding.[14,15]

In the past few years, reports concerning Gas6 or Axl/Sky/Mer recombinant mice raised attention towards Gas6 protein as an important factor in some complex pathological processes. Gas6 knock out (-/-) mice resulted to be protected from venous thrombosis when challenged with prothrombotic substances; these data were confirmed on wild type mice treated with anti Gas6 antibodies.[16,17] On the other hand, Gas6 is a chemotactic factor for VSMCs[18] and, recently, Fisher et al. found its up-regulation in VSMCs isolated from atherosclerotic plaques.[15] All these data suggest a relevant potential role of Gas6 in vascular pathology.

It has been also described that mice lacking the Gas6 receptor Sky displayed the interesting tendency to develop polyclonal lymphocyte proliferation and diffuse autoimmune aggression to tissues[19] and that the vascular endothelial growth factor (VEGF) induced angiogenesis, both in vivo and in vitro, is heavily inhibited by Gas6.[20]

All these evidences of Gas6 involvement in in-vitro and in-vivo animal models need to be confirmed in humans.

In the present study we report the development and the validation procedure of an Enzyme-linked Immunosorbent Assay (ELISA) method for the quantitative detection of Gas6 in human plasma. We also report the measurement of this analyte in plasma obtained from healthy donors as reference values for normality in Italy.

EXPERIMENTAL

Reagents

— Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 in distilled water. Working solution, pH 7.3;

— Tris-buffered saline (TBS): 20 mM Trizma base, 150 mM NaCl. Working solution, pH 7.3;
— Tween 20 (EuroClone, Trieste, Italy);
— Washing Buffer: Tween 20, 0.05% in PBS;
— Blocking/Incubation Buffer: Albumin from bovine serum, further purified fraction V, $\geq$98% (agarose gel electrophoresis), lyophilized powder (BSA) (Sigma, St. Louis, MO, USA). Working solution: BSA 2.5% in Washing Buffer;
— Recombinant human (rh) Gas6 (R&D Systems, Minneapolis, USA): Stock solution: 2.5 mg/mL 1% BSA in PBS;
— Anti-human Gas6 Antibodies: A) capture antibody (CA): goat polyclonal affinity purified IgG (R&D Systems, Minneapolis, USA): produced in animals immunized with rhGas6 and further purified by affinity chromatography. Stock solution: 0.1 mg/ml in PBS. Working solution: 0.35 μg/mL in PBS. (Other antibodies tested as capturers in preliminary experiments: mouse monoclonal anti Gas6 IgG antibody (R&D Systems, Minneapolis, USA) and mouse monoclonal anti Gas6 IgM antibody purified by affinity chromatography from hybridoma Ag8.28 cell line[5]). B) Biotinylated Antibody (R&D Systems, Minneapolis, USA) produced in goats immunized with rhGas6 and purified by affinity chromatography. Stock solution: 50 μg/mL, 0.1% BSA in TBS. Working solution: 15 ng/mL in incubation buffer;
— Streptavidin peroxidase conjugate (Sigma, St. Louis, MO, USA): stock peroxidase conjugated streptavidin solution 1 mg/mL in PBS. Working solution: 400 ng/mL in incubation buffer;
— 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma, St. Louis, MO, USA): liquid substrate system, ready to use solution for ELISA;
— Sulphuric Acid (H_2SO_4) (Sigma, St. Louis, MO, USA): 1.8 M in distilled water;
— Samples: the blood obtained from healthy donors was collected in 3% sodium citrate, centrifuged at 2,000$\times$g for 10 minutes and 100 μL aliquots were stored at -80°C until ELISA was performed. Every sample was defrosted once only. For each assay, samples were diluted 1:50 v/v in incubation buffer and then tested. Since samples collected in sodium citrate gave the most uniform results in preliminary tests, this reagent was employed as standard anticoagulant.
— Axl-IgG complex (kindly donated by Brian Varnum). Stock solution: 4 mg/mL in 1% BSA in PBS. Working solution 10 μg/mL in 1% BSA in PBS . Final concentration in samples, 100 ng/mL;
— Calcium chloride ($CaCl_2 \cdot 2H_2O$) (Sigma, St. Louis, MO, USA). stock solution: 0.2 M in distilled water. Final concentration in samples 2 mM.

Special Equipment

— ELISA plates: NUNC ImmunoPlates MaxiSorp F96 (NUNC, Hereford, UK);

— ELISA reader: Spectra Count, BS1000 (Packard Instrument, Meriden, CT, USA).

ELISA Methodology

If not otherwise specified, all the steps were performed in static incubation. In preliminary experiments, we optimized the reagent concentrations and the incubation times. The definitive procedures to measure Gas6 concentration are the following: a 96-well ELISA plate was coated with 50 μL CA (0.35 μg/mL) overnight (O/N) at room temperature (RT). To prevent evaporation, the plate was covered with laboratory film (Parafilm, American National Can Group Inc., Chicago, USA); this procedure was performed for all the following incubations. The day after, the plate was washed with washing buffer and vacuum dried; free binding sites were then blocked with the addition, to each well, of 50 μL of blocking buffer for 2 h at RT. After three passages with washing buffer, the plates were gently vacuum dried and 50 μL of standard solutions or of samples were added to the wells and shaken at 300 rpm, on a horizontal plate shaker, for 2 h at RT. The plate was then washed five times with washing buffer and gently vacuum dried. Furthermore, 50 μL of biotinylated anti-human Gas6 antibody (15 ng/mL in incubation buffer) was added to each well. After 90 min of RT incubation, the plate was newly washed five times with washing buffer and gently vacuum dried. 50 μL of streptavidin peroxidase conjugate (400 ng/mL in incubation buffer) were added to each well and incubated for 15 min at RT; the plate was then washed with washing buffer and gently vacuum dried and, finally, 50 μL of TMB was dispensed into each well. After 30 min, the reaction was stopped by adding 50 μL of 1.8 M H_2SO_4 and, immediately, the plates were subjected to absorbance measurement at 450 nm (reference wavelength set at 570 nm) in a plate reader (Spectra Count, BS1000, Packard Instrument, Meriden, CT, USA).

Healthy Subjects

We recruited 61 volunteers who gave informed consent, all of them being medical students, medical trainees, hospital staff employees, or hospital assistance volunteers. To explore age influence, we divided donors according to the following age ranges: young subjects from 20 to 35 years old and older subjects from 54 to 79. Subjects who declared to be affected by any disease were excluded with the exception of arterial hypertension for subjects older than 54 years old. All subjects recruited did not consume any medication in the previous week before, with the exception of pressure lowering drugs.

Preparation of Gas6 Depleted Plasma Matrix

Three different plasma pools were prepared, mixing plasma obtained from three sets of ten donors. Thereafter, a 96-well plate was coated with CA (1 μg/mL) O/N at RT. The next day, the plate was washed three times with washing buffer and then gently vacuum dried. Aliquots of 50 μL of diluted plasma (1:50 v/v in incubation buffer) were distributed into wells. The plate was then incubated at RT for 2 h and shaken at 300 rpm on a horizontal plate shaker. Once reaction was completed, all samples were harvested and pooled, aliquoted and stored at −80°C for no more than 6 months.

Standards

A standard curve was prepared by serial dilution of rhGas6 in Gas6 depleted plasma matrix. Standard concentrations were 0, 0.26, 0.40, 0.60, 0.88, 1.32, and 2.00 ng/mL.

Assay Validation

We assessed the following ELISA validation requirements:[21]

— Intra-assay precision and accuracy: determined using 8 replicates of four validation samples. Coefficient of variation (%CV, defined as the ratio between standard deviation and mean value) should be lower than 20% and accuracy within 20% of nominal values. Validation samples were prepared by diluting rhGas6 in the assay matrix to a concentration of 0.26, 0.45, 0.90, and 1.80 ng/mL.
— Inter-assay precision and accuracy: determined with 4 independent assay runs of each validation sample (see above).
— Lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were established by the evaluation of %CV of measured standard values. %CV was considered reliable if lower than 20.
— Recovery: we performed recovery analysis adding two different known concentration of rhGas6 (10 and 20 ng/mL) to 10 plasma samples derived from 5 males and 5 females. Percent differences of measured from expected values should be within a 20% range of variability.
— Freeze-thaw stability: up to 3 freeze and thaw cycles were performed on 5 different samples before rhGas6 concentration was determined.

Statistical Analysis

Optical density was fitted versus nominal concentration applying a four-parameter logistic regression to the calibration curve. Average, standard

deviations, %CV, and differences from theoretical values were calculated for all standards and samples. All statistical analysis was performed using an appropriate software.

RESULTS

Linearity and Calibration Standards: Evaluation of Matrix Effects

We performed several preliminary experiments to establish the adequate assay conditions for antibodies and streptavidin peroxidase concentrations, standards concentration, time-course, and temperature of antigen-antibody incubation. As CA, we tested two monoclonal and one polyclonal antibodies; the affinity purified goat polyclonal anti Gas6 antibody was chosen for its elevated sensitivity and lower background. With the aim to obtain the highest assay sensitivity with the lowest background, we tested several CA and biotin-conjugated antibody concentrations, beginning with 10 μg/mL and 2 μg/mL, respectively, tapering till 0.35 μg/mL and 15 ng/mL. The latter were chosen as the CA and biotin conjugated antibody working concentrations. Since the calculations of analyte concentrations gave superimposable results, and were reproducible, both applying 50 and 100 μL reactions volume conditions, the 50 μL volume was chosen for all antigen-antibodies incubations in 96-well plates (data not shown).

During these preliminary experiments, we also evidenced that known amounts of rhGas6, added to different plasma samples, resulted in being underestimated, in all experiments performed, when plotted to standards prepared in incubation buffer. Thus, we hypothesized that these differences could be determined by a matrix effect.[21] To overcome this problem, a plasma pool depleted of Gas6 was prepared (see methods); then, the standard concentrations chosen were diluted in incubation buffer and compared to those prepared in Gas6 depleted plasma. In five different assays, we evidenced that, as long as standard concentrations increased, the absorbance values rose quite differently, depending upon the matrix: a steeper curve was obtained if samples were diluted in incubation buffer. Two representative curves are shown in Figure 1. Since assay accuracy resulted matrix dependence, all the validation procedures were then performed with samples diluted in Gas6 depleted human plasma. We compared Gas6 depleted human plasmas belonging to three sets of multiple donors (10 for each plasma pool). We did not find any relevant differences in background signals and in standard curve performance between all of them; moreover, effective Gas6 depletion from plasma was verified with an ELISA assay with standards diluted in incubation buffer: all Gas6 depleted plasma pools had a residual Gas6 concentration that was lower than 0.26 ng/mL (data not shown). We also verified that Axl-IgG 100 ng/mL and calcium chloride 2 mmol/L addition to Gas6

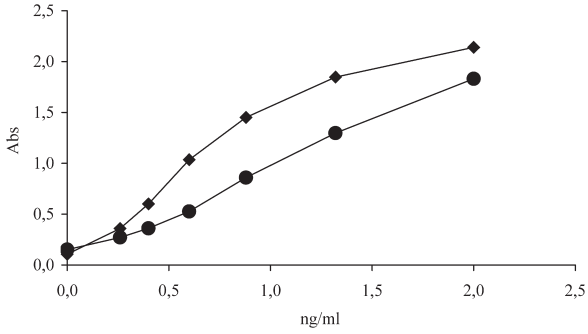

Figure 1. Comparison between the standard curves performed in incubation buffer (square points) and in Gas6 depleted plasma matrix (assay matrix) (circle points). The concentration values are reported in the abscissa and the absorbance values in the ordinate axis. Results of one representative experiment out of five is shown.

depleted plasma did not influence standard curve performance (data not shown).

The standard curve calibrators (in number of 6, run in duplicate) ranged from 0.26 to 2.00 ng/mL. The linearity of the method was evaluated using a four-parameter logistic regression algorithm to fit the optical density versus Gas6 concentration. The average correlation coefficient (goodness of fit) was 0.9978. A typical standard curve is shown in Figure 2. Accuracy (deviation of the mean of measured values from nominal concentration) was within 10%. The mean % difference from nominal concentration ranged from −6.00 to 4.00%, and %CV, as an estimate for precision, ranged from 2.83 to 8.63 (data not shown).

The LLOQ, as the lowest analyte concentration value that can be quantitatively determined with suitable precision and accuracy, was evaluated with the preparation of two samples at the final concentrations of 0.26 and 0.16 ng/mL, respectively, in Gas6-depleted plasma matrix. The minimal concentration

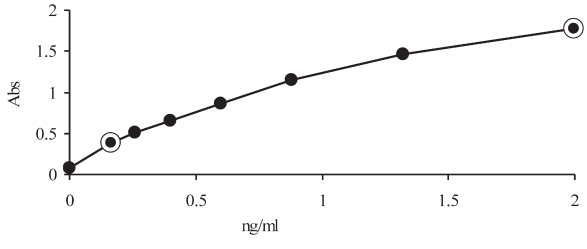

Figure 2. Typical standard curve. The concentration values are reported in the abscissa and the absorbance values in the ordinate axis. LLOQ and ULOQ values are indicated by white rings on the left and on the right, respectively.

which fulfilled the criteria for being considered LLOQ, was 0.26 ng/mL. LLOQ absorbance was 5 times or more higher than that of blank in all experiments performed (data not shown).

Accuracy (Recovery)

The accuracy was determined by spiking 10 different plasma samples with known amounts of rhGas6. Every sample was evaluated at least four times. Recovery analysis was performed to evaluate a nominal increase of 10 and 20 ng/mL in Gas6 concentration. The mean recoveries were 94 ± 5% and 96 ± 5% of the theoretical Gas6 values, respectively. The complete recovery analysis is shown in Figure 3. All the recoveries were very close to 100%, and all were well within the recommended range of 20%.

Precision (Intra-Assay Variability and Inter-Assay Variability)

Intra-assay variability was determined by evaluating eight replicates of four standard samples: 0.26–0.44–0.94–1.88 ng/mL. The mean %CVs were 6.06–4.52–10.59–10.27, respectively.

Inter-assay mean % CV, determined with the same standard samples reported above, was repeated in five different plates and gave the values of 10.44 ± 2.83 (Table 1).

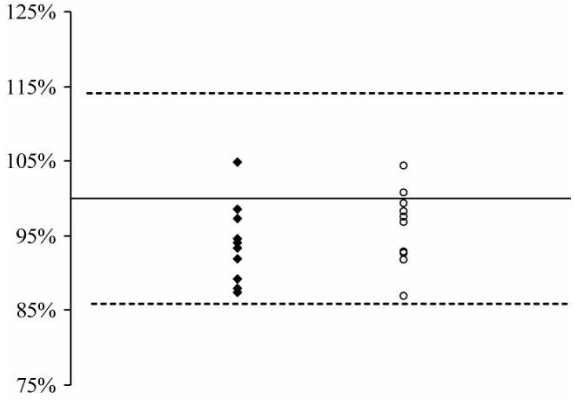

Figure 3. Results of the recovery analysis; the values are expressed as percentage of the expected concentration values. The recovery values obtained when 10 ng/mL and 20 ng/mL of Gas6 were added are indicated by the black rhombus and the white circles, respectively. The dashed lines set the range of ± 15% accepted for the method validation.

Table 1. Inter-assay variability

Theoretical concentration ng/mL	Calculated concentration ng/mL	Standard deviation	Coefficient of variation (%)	Theoretical value (%)
0.234	0.235	0.028	11.91	100.43
0.469	0.464	0.044	9.48	98.93
0.938	0.963	0.067	6.96	102.67
1.876	1.859	0.249	13.39	99.09

Freeze-Thaw and Room Temperature Stability

Three cycles of freezing and thawing were performed with four standards spiked in Gas6 depleted plasma matrix. We observed a 10% average reduction in measured Gas6 concentration. Even though this value is included within precision and accuracy ranges, we discourage defrosting samples more than once. We suggest to store small frozen aliquots of samples in order to repeat measures on the same sample.

Keeping samples at RT up to 24 hours determines a reduction of Gas6 measured concentration that averages around 17%. On the other hand, samples permanence, on ice up to 6 hours, did not modify Gas6 concentration (data not shown).

Gas6 Concentration in Human Plasma

We measured Gas6 concentration in plasma samples obtained from healthy donors. Out of the 61 subjects recruited, 20 male and 41 female, Gas6 mean plasma concentration was: 20.3 ± 3.8. Considering the 31 young healthy donors, 15 males and 16 females, with a mean age of 27.2 ± 3.1 years, the mean concentration measured was 20.4 ± 4.2 ng/mL. A higher, though not significantly different, concentration was found in females (21.4 ± 3.8) respect to males (19.4 ± 4.5). Focusing on the group of the 30 donors, 5 males and 25 females, with a mean age of 64.4 ± 7.2, the mean concentration of Gas6 was 20.2 ± 3.4 ng/mL. Also, in this case, the Gas6 concentration in females' plasma (20.3 ± 3.4) is not significantly different with respect to males (19.4 ± 3.5).

DISCUSSION

There is an increasing research interest in the Gas6 protein activity, since recent in vitro and in vivo studies pointed out several Gas6 physiological

functions and potential implications in several pathologies. On the basis of data obtained through in vitro and in vivo studies with recombinant mice, it appears that Gas6 protein could be implicated in vascular pathology.[17] Curiously its effect could be directed both to atherosclerotic plaque formation and on thrombus formation in the venous system. Gas6 protein involvement has been also described in other important pathological events like neoangiogenesis blockage,[20] cancer cell proliferation/survival,[13] and inhibition of tissue inflammation.[5] Since all these experimental evidence derives from in vitro or in vivo animal studies, investigations with human subjects are necessary to confirm these hypotheses. Our study was designed with the aim to develop and validate an ELISA method for the detection of Gas6 protein in human plasma. This method would be useful for the exploration of Gas6 involvement in human pathology.

The measurement of Gas6 protein in human plasma with a sandwich immunoassay has been previously described by Balogh et al.,[22] however, in our opinion, this method shows some limitations. The procedure described is not fully reproducible, since both the standards and the secondary antibody used are not commercially available. Moreover, the procedure was not discussed in full detail and recovery experiments were executed only on two plasma samples while a matrix influence in the assay performance has not been ruled out. Moreover, freezing-thawing stability and the temperature of the experiments were not described. Because of the lack of these elements, the method described by Balogh et al. cannot satisfy criteria for a full validation.

In our experimental procedures, we used only commercially available materials. We tested several anti-human Gas6 antibodies; the monoclonal antibodies assayed as CA had an inadequate sensitivity to be used in this immunoassay, so an affinity purified polyclonal antibody was chosen as capturer. The only biotin-conjugated antibody commercially available was used as a secondary antibody, since all unconjugated ones displayed unacceptable sensitivity and too high background.

During the assay development, we evidenced several factors that could influence a correct Gas6 quantification. The principal interference we encountered was a very high matrix effect, since the absorbance grew steeply when standards were spiked in BSA solution with respect to plasma. Thus, we concluded that assays based on a BSA matrix may underestimate Gas6 concentration in human plasma and should not be used. We overcame this problem with the preparation of a human-based plasma matrix, derived from pooled Gas6 depleted plasma from healthy donors. The preparation of a standard curve in this matrix allowed us to obtain results similar to those obtained by spiking Gas6 in untreated plasma. Since different plasma pools, obtained from multiple donors (10 donors for each plasma pool), showed a superimposable matrix effect, with analogous standard curve performance, we consider adequate accuracy for the preparation of the assay matrix if plasma is obtained by pooling 10 healthy donors. If healthy donors were not

available as matrix sources, commercially available lyophilized healthy donor plasma may be, alternatively, used; in that case, however, the accuracy of the method should be re-evaluated.

The background noise encountered during the assay development was resolved by applying an adequate dilution of the samples (1:50, final dilution), performing ELISA plate blockade with blocking buffer and using a biotin conjugated antibody as revelatory. Sample pre-treatment, e.g., addition of goat immunoglobulin and/or polyethylene glycol (PEG)[23,24] did not modify the matrix absorbance or the recovery performance in our assay. Since Gas6 protein can bind calcium,[2] we verified that calcium chloride at physiological concentrations did not influence the assay performance. Moreover, due to the recent description that a soluble form of Axl receptor can associate with Gas6 in mouse serum,[25] we also excluded that Axl-IgG complex addition to samples could influence analyte determination in our assay. At the end of the assay development, we obtained a low matrix absorbance (<0.09 Units of absorbance), and LLOQ absorbance 5-fold higher than that observed with the matrix alone, as required by the validation procedural guidelines.[21,26]

Precision and accuracy criteria dictated by FDA were satisfied. Our method displays an adequate accuracy, since all samples spiked with known amounts of Gas6 evidenced values between plus or minus 10% range of the nominal value; that is within the requested range for validation ($\pm 20\%$ of the nominal value).[21] These data assure specificity to the method, rendering very improbable the presence of significant interferences from other substances in the assay. Moreover, the intra-assay and inter-assay %CVs all resulted within the requested value of 15% for all standards tested, granting a suitable precision. Since Gas6 protein was evidenced to be unstable if stored one day at room temperature and frozen and thawed more than once, we suggest that all plasma samples to be assayed should be frozen in small aliquots and, once thawed, kept in ice during assay preparation.

Applying our method to healthy human donors, we evidenced a Gas6 concentration in human plasma ranging from 14.3 to 26.5 ng/mL without any significant difference between males and females. Moreover, we could not find any variation of this protein according to age. So, Gas6 seems to be a stable circulating protein in healthy subjects, independent of age; this value may be considered a reference for future analysis concerning its pathological modification. These results confirm those obtained by Balogh and colleagues, but are very different from those reported by Borgel and co workers. These authors, in fact, have recently described one other ELISA method to measure Gas6 in human plasma; they found an average Gas6 plasma concentration of 54 ng/mL in healthy subjects, slightly different from those we obtained. The method described by Borgel et al.[27] does not fulfill validation requests, since only inter- and intra-assay CV's are presented, while matrix effect evaluation, recovery analysis, standard calibration, freeze-thaw analysis are not described. Moreover, some details of the procedure are

lacking, such as peroxidase conjugate incubation conditions. In addition, in preliminary experiments, while testing the same capture and biotin conjugated antibodies, at the conditions presented by Borgel et al., we obtained Gas6 protein concentrations similar to that which they found (around 50 ng/mL). However, in these preliminary conditions, recovery analysis gave inadequate results and the background was unacceptable (data not shown). We conclude that the method presented by Borgel et al., despite the fact that it would probably be adequate to point out Gas6 *concentration differences* among subjects affected by different diseases, does not reach the accuracy for absolute measurement of Gas6 protein.

CONCLUSIONS

In summary, we have developed and validated a new ELISA method for the quantitative determination of Gas6 protein concentration in human plasma. Based on a validation study, this method is suitable for Gas6 plasma determination in human subjects, both for research and diagnostic purposes.

ABBREVIATIONS

BSA	Bovine serum albumin
CVC	oefficient of variation
CAC	apture antibody
EC	Endothelial cell
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and drug administration
Gas6	Growth arrest specific 6
LLOQ	Lower limit of quantification
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
SHBG	Steroid hormone-binding globulin
TBS	Tris-buffered saline
TMB	Tetramethylbenzidine
ULOQ	Upper limit of quantification
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

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