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A Common Calibrator Does Not Secure Harmonisation of Commercial t-PA and PAI-1 Antigen Measurements

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within the frame work of the ISTH/SSC subcommittee on Fibrinolysis

Background. There is no common standardisation of different commercially available kits for both t-PA and PAI-1 antigen. Aim. The aim of this project was to study whether the exchange of the kit calibrator with the common calibration materials of the WHO would harmonise the results produced by five different commercially available t-PA and PAI-1 antigen kits when analysing the SSC secondary standard. Methods. WHO international standards were used as calibrator and the SSC secondary standard and a commercially available plasma standard were used as test plasma in 5 commercially available kits measuring total t-PA and PAI-1 antigen. For t-PA only, the SSC secondary standard was spiked with purified t-PA and recovery was studied. Results. There was a large variation in the concentrations of t-PA antigen (ranging from <0.5 to 6.6 ng/ml for the SSC secondary standard and from 3.3 to 10.9 ng/ml for the commercial plasma standard, respectively) produced by the different kits. Also, PAI-1 antigen results of the different kits showed a large variation (ranging from 20.3 to 51.2 ng/ml for the SSC secondary standard and from 41.8 to 89.7 ng/ml for the commercial plasma standard, respectively). Results of the two test samples and spiking with t-PA were not in agreement in all methods, indicating differences in specificity of tests. Data point to a specific effect of the matrix of standards. Conclusions. The use of a common calibration material does only marginally harmonise data for t-PA and PAI-1 antigen assays. There is a need for improvement of methods to cope with standards and standardisation.

INTRODUCTION

Increased protein concentrations of tissue-type plasminogen activator (t-PA) and increased activity and protein concentration of plasminogen activator inhibitor-1 (PAI-1) in plasma have been associated with the evolution of ischemic heart disease (IHD)(1-5). Therefore measurement of t-PA and PAI-1 antigen can be useful as a prognostic indicator of the risk of IHD.

Kits for total t-PA and PAI-1 antigen are commercially available, and we have previously demonstrated that the major problem with the measurement of e.g. PAI-1 antigen and activity is the large variation in the results obtained by the different commercially available kits (6,7). In these studies the calibrators provided with each kit were used, but even after harmonisation with the use of the NIBSC reference material a considerable variation in the results existed. Obviously such a situation is inconvenient when t-PA and PAI-1 are used in the clinical laboratory, because it makes it very difficult, if not impossible, to transfer data from scientific studies to daily clinical practice and also to compare results from one laboratory to another. In addition, the development of a secondary plasma standard by the SSC required decisions about the method to be used in conjunction with the WHO standards.

A logical step to try to reduce inter-assay variation and standardise measurement results would be to use the same calibrator in the different kits. Therefore, we studied within the framework of the Subcommittee of Fibrinolysis of the Scientific and Standardisation Committee (SSC) whether substitution of the kit calibrator by the NIBSC reference plasma would reduce or eliminate inter-kit variation.

We studied five different commercially available t-PA and PAI-1 antigen kits reported to measure total concentrations of each analyte, and in order to eliminate inter-laboratory variation we produced all the results at the same laboratory. The first batch of the SSC secondary standard and a commercial plasma standard were used as test plasmas.

MATERIALS AND METHODS

The NIBSC plasma t-PA (94/730), pure t-PA (86/670), and plasma PAI-1 (92/654)(Gaffney 1996) were used as calibrators (NIBSC,). The plasma t-PA standard (94/730) contains 25 ng t-PA per ampoule (by assignment). The purified second international standard for t-PA (86/670) is highly purified, 98% single chain t-PA from a Bowes melanoma cell line (8), and contains 2 µg/ml t-PA antigen (assigned by amino acid analysis). The PAI-1 plasma standard (92/654) is enriched with added reactivated recombinant PAI-1, lyophilised and assigned 185 ng/ml PAI-1 antigen (9). The calibration curves were constructed as dose-response curves by dilutions of the NIBSC plasma standards of t-PA and PAI-1, as described by the manufacturers; i.e. dilution buffer or plasma depleted of t-PA or PAI-1, respectively. The SSC secondary standard (Lot # 1)(Immuno, Vienna, Austria) and the Biopool reference plasma (Biopool, Umea,Sweden) were used as test materials.

Kits for t-PA antigen :

An EIA measuring total t-PA, i.e. free and complexed t-PA, single-chain and double-chain t-PA. Two monoclonal antibodies, directed against different epitopes of t-PA, are used for coating and a third monoclonal antibody for tagging. (Chromogenix, Molndal, Sweden) (10)

Two different monoclonal antibodies are used as capture and tagging antibody, directed against different epitopes of t-PA (Technoclone, Vienna, Austria)

A goat anti-human t-PA is used as coating antibody and anti-t-PA Fab fragments are used as tagging antibody measuring free and complex forms of t-PA (Tintelize t-PA, Biopool, Umea, Sweden)(11).

A one-step EIA measuring total, bound and free forms of molecules (12)(Organon Teknika, Turnhout, Belgium)

An EIA measuring total circulating t-PA, i.e. free and complexed t-PA, single-chain and double-chain t-PA. The EIA uses two different mouse monoclonal antibody against human t-PA for coating and tagging (Asserachrom ® t-PA, Stago, Asnieres-sur-Seine, France)

Kits for PAI-1 antigen:

An EIA with equal sensitivity for PAI-1/recombinant t-PA complex, PAI-1/melanoma t-PA complex, PAI-1/u-PA complex, inactive PAI-1 and active PAI-1 (Chromogenix, Molndal,Sweden) (13,14)

Two different monoclonal antibodies, directed against different epitopes of PAI-1, are used as capture and tagging antibody. The EIA measures free and complexed t-PA (Technoclone, Vienna, Austria)

An EIA using a combination of monoclonal and polyclonal antibodies that measures human PAI-1, endothelial type. It detects active and inactive (latent) forms of PAI-1, as well as that complexed as t-PA/PAI-1 and u-PA/PAI-1 (Tintelize PAI-1, Biopool, Umea, Sweden)(14,15).

A one-step EIA for PAI-1 measuring total, bound and free forms of molecules (16) (Organon Teknika, Turnhout, Belgium).

An EIA measuring total circulating PAI-1, i.e. free and complexed with t-PA, bound or unbound to vitronectin, active or inactive forms, and platelet PAI-1. The EIA uses two different mouse monoclonal antibody against human PAI-1 for coating and tagging (Asserachrom ® PAI-1, Stago, Asnieres-sur-Seine, France)

Experiments

In the first experiment (both t-PA and PAI-1) the calibration curve was constructed by means of the NIBSC plasma (matrix) standards, diluted according to the instructions given by the manufacturers (figure 1). The SSC secondary standard and the Biopool reference plasma were used as samples and the concentrations were determined using the NIBSC calibration curve. The experiment was performed eight times on two different days.

In the second experiment (for t-PA only) the SSC secondary standard was spiked in a dose-dependent manner with increasing amounts of NIBSC purified t-PA (figure 2). A dose-response curve was constructed and the absorbance at the cut-off point with the Y-axis was detected. The cut-off point represents the amount of t-PA in the SSC secondary standard and the absorbance was converted to concentration units with the use of the calibration curve, which goes through the point of origin and is parallel to the original dose-response curve. The experiment was performed six times on two different days.

RESULTS

t-PA antigen.

When the values for t-PA were determined in the SSC secondary standard, using the NIBSC t-PA reference plasma as a calibrator, the levels obtained with the different kits varied from <0.5 ng/ml to 5.2 ng/ml (Fig 3,4). Also in the second experiment, where the SSC secondary standard was spiked with NIBSC purified t-PA, the t-PA antigen levels measured for the SSC secondary standard varied widely from 1.6 ng/ml to 7.3 ng/ml. The tests that measured the highest and lowest levels in experiment 1 were similarly ranked in the second experiment (Fig 5).

When the values for t-PA were determined in the Biopool reference plasma, again using the NIBSC t-PA reference plasma as a calibrator, the levels obtained with the different kits also greatly varied, and the kits that measured relatively low levels in the SSC secondary standard were not the same as those that measured relatively low levels in the Biopool reference plasma (Fig 6).

There was no association between the variation in the levels measured in the SSC secondary standard with the different kits and the kind of reference material (buffer or plasma) originally provided in the kits. For all kits the slopes of the calibration curve in experiment 1 and the slope of the curve of the SSC secondary plasma standard spiked with purified t-PA in experiment 2 were different (results not shown).

PAI-1 antigen

When the values for PAI-1 were determined in the SSC secondary standard, using the NIBSC PAI-1 reference plasma as a calibrator, the concentrations obtained with the different kits varied from 22.0 ng/ml to 44.8 ng/ml (Fig 7). There was a weak relationship between concentrations measured in the SSC secondary plasma standard and the Biopool reference plasma (Fig 8).

There was no association between the variation in the levels measured in the SSC secondary plasma standard with the different kits and the kind of reference material originally provided with the kits.

DISCUSSION

We observed that the introduction of a common calibrator for determination of total t-PA and PAI-1 antigen concentrations did not harmonise results which was produced by the different available kits. To a great extent the problem is related to t-PA determination, while the variation of PAI-1 determination is fairly stable, at least when we include only kits 1, 2, 4 and 5.

The NIBSC matrix standards for t-PA and PAI-1 were used as calibrator materials for each of the kits, and all the measurements were performed in the same laboratory and by the same technician. In order to have optimal compliance to kit procedure

we used for each kit its own dilution reagent to dilute the NIBSC standard plasmas and the NIBSC purified t-PA. We used this experimental set-up in order to try to harmonise the calibration process of the different kits, but we cannot exclude that the matrix of the NIBSC plasma standards influenced the measurement results. It should be noted that some differences were observed for t-PA in the series of experiments in which the SSC secondary plasma standard was spiked with purified t-PA using a constant matrix back-ground. In figure 5, the kits 4 and 5 show a systematic deviation either due to differences in specificity for added t-PA and plasma t-PA or sensitivity for matrix effects.

Various monoclonal and polyclonal antibodies have been developed and used in the development of commercially available immunological enzyme-immuno-assays (EIA). Because the antibodies are directed against different epitopes and t-PA and PAI-1 can be found in various forms, the different EIA will have different specificities for the various forms. For each kit, it was stated by the companies that the total t-PA or PAI-1 antigen was measured. Data regarding sensitivity towards the different forms of t-PA and PAI-1 was only in the kits from Chromogenix, Biopool and Organon. Since no information was available for the other kits, it was not possible to evaluate whether the specificity of the antibodies explains the differences in values found with the different kits. It cannot be excluded that the additives of the calibrator and test plasmas or the lyophilisation method may affect the assays differently. We studied this by calculating for each kit the ratio between results of the SSC secondary standard and the Biopool reference plasma. This ratio would be expected to be equal for all kits, i.e. one kit would be expected to measure relatively high levels in both plasmas while another kit would be expected to measure relatively low levels in the two plasmas. However, we observed an inter-kit variation, in particular with respect to the t-PA measurements (Fig 6). These data indeed suggest that other factors (such as additives and lyophilisation) affect the measurement procedures - an observation which is also of importance for the preparation of future reference materials.

In our study the companies had the opportunity to comment on our results and conclusions and in general they were in agreement with our results. The companies also stressed that difference in the matrix of the calibration material, the lyophilisation and the specificity of the antibodies could be the factors causing the discrepancies. The only significant disagreement was that for kit number 3 the company reported that they had measured a higher t-PA concentration in the first experiment. This supports the assumption that matrix effects contribute to the observed variation in measured levels. For kit number 3 the company also reported interference in the assay by the buffer HEPES. Enquiry about the SSC secondary standard revealed that this standard contained HEPES (H. Lang, personal communication)

We have previously demonstrated a large inter-assay variation of PAI-1 measurements when the kit calibrators provided were used (6,7). The present study shows a similar result when a common reference material with an approved value is used and standard plasmas are measured Despite the fact that the information with all kits claimed to measure total protein concentration of t-PA and PAI-1, we observed a highly significant variance in measurement results of both t-PA and PAI-1 antigen in the standards in this study (Fig 3,7). We realise that we have increased the complexity by using lyophilised, stabilised standards, but only when kits also can cope with such standards, harmonisation becomes possible. It was observed that information on the additives to standards is meagre. It can be suggested that such information be improved to allow a better interplay in development between standards and methods.

We have now definitively demonstrated that the use of calibration materials with certified values is not sufficient to secure a low inter-kit variation of measurements. Standardisation efforts to improve the situation should, in addition to the improvement of reference materials, certainly focus on the evaluation of the specificity of kits. Work on the establishment of such criteria is ongoing within a working group of the SSC. Furthermore, we must focus on the development of reference methods. Such a strategy would be a first step in the development of a coherent measurement system (17) in which there is a close association between reference materials (primary, secondary etc.) and the analytical methods (definitive, reference and field methods). Such a procedure would also be of great help to manufacturers since so far there has been no general calibration system within fibrinolysis.

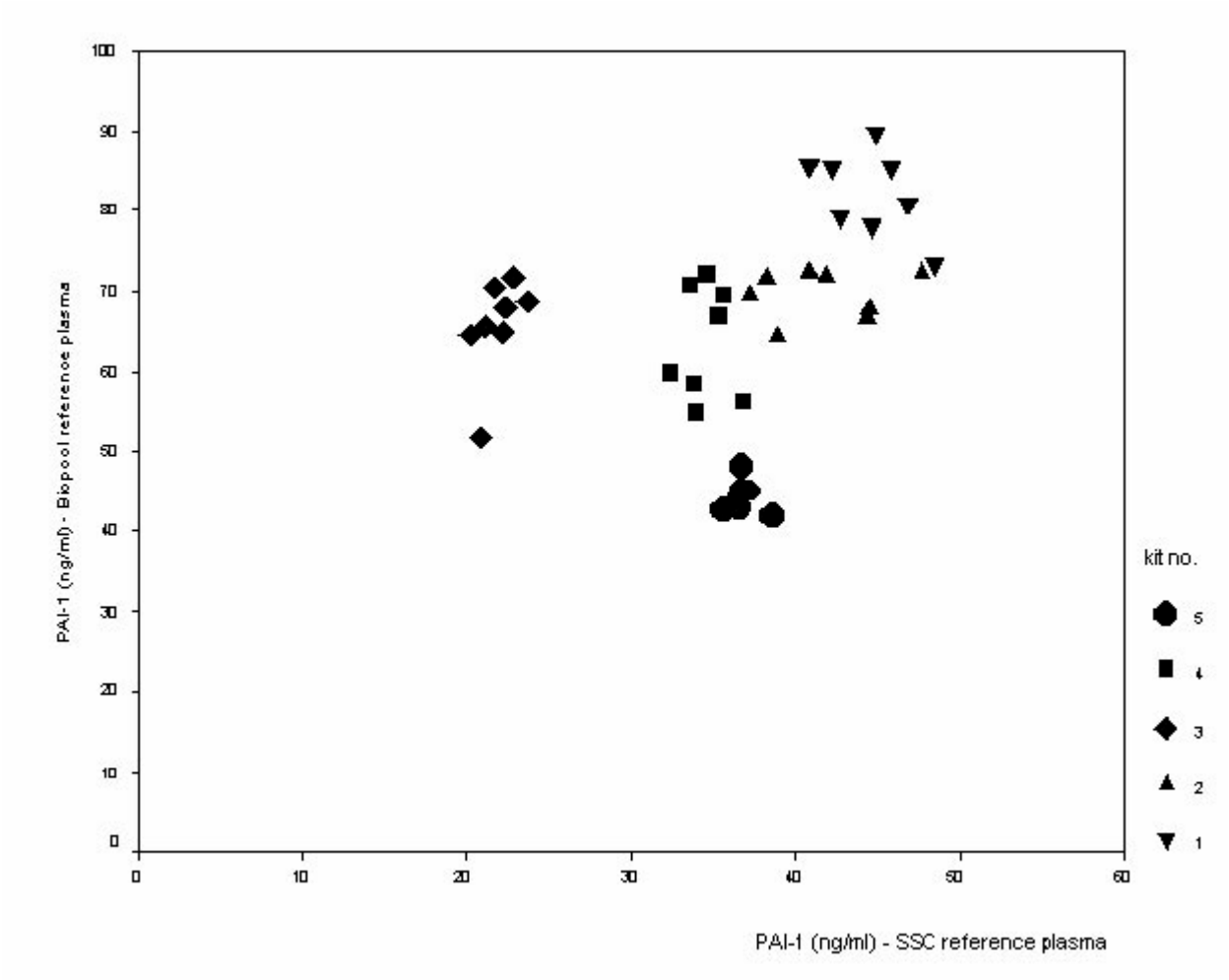

Figure 1: Design of the first experiment where a calibration curve was made using the NIBSC matrix standards. The SSC secondary standard or the Biopool reference plasma were then used as sample and the concentration was determined using the NIBSC calibration curve from the particular kit.

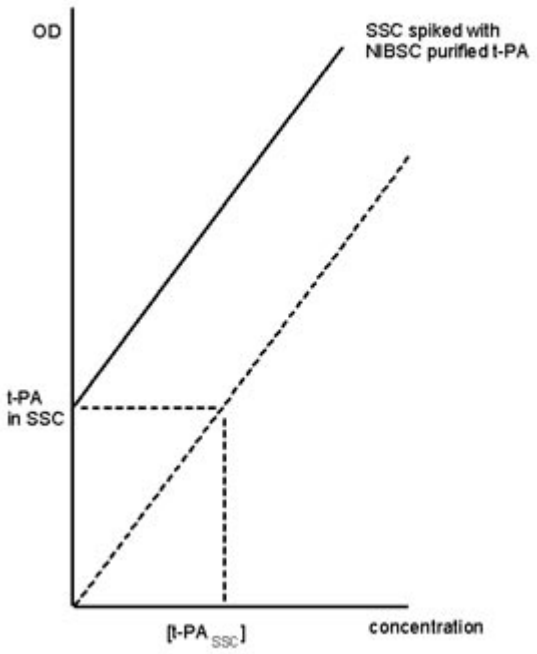

Figure 2: Design of the second experiment (for t-PA only) where the SSC secondary standard was spiked in a dose-dependent manner with increasing amounts of purified t-PA. A dose-response curve was then constructed and the absorbance at the cut-off point with the Y-axis was detected. The cut-off point represents the amount of t-PA in the SSC secondary standard.

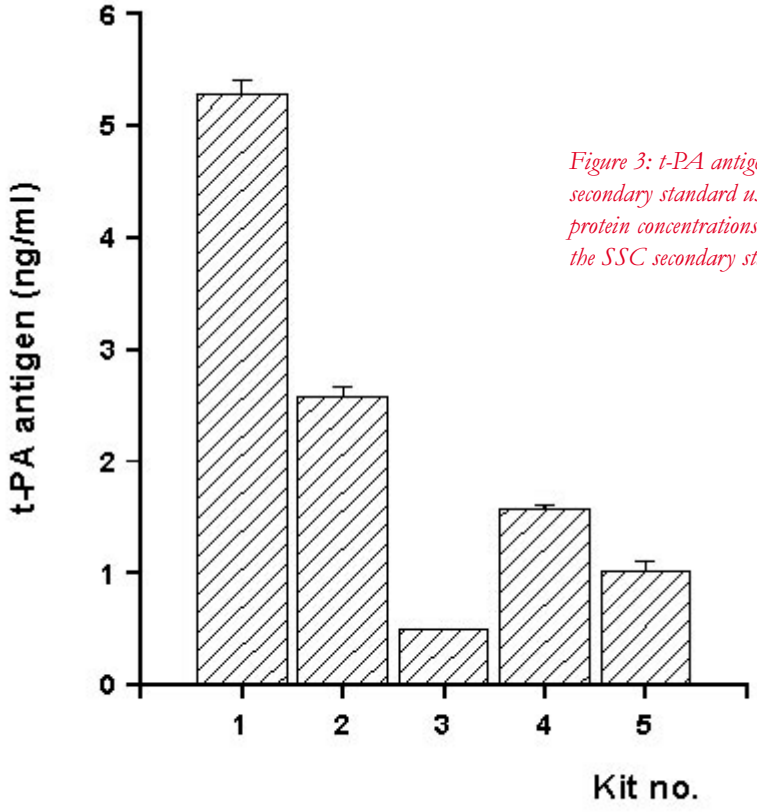

Figure 3: t-PA antigen concentrations measured in the SSC secondary standard using five different kits that measure total t-PA protein concentrations (first experiment with direct measurement of the SSC secondary standard)(mean and 1 SEM).

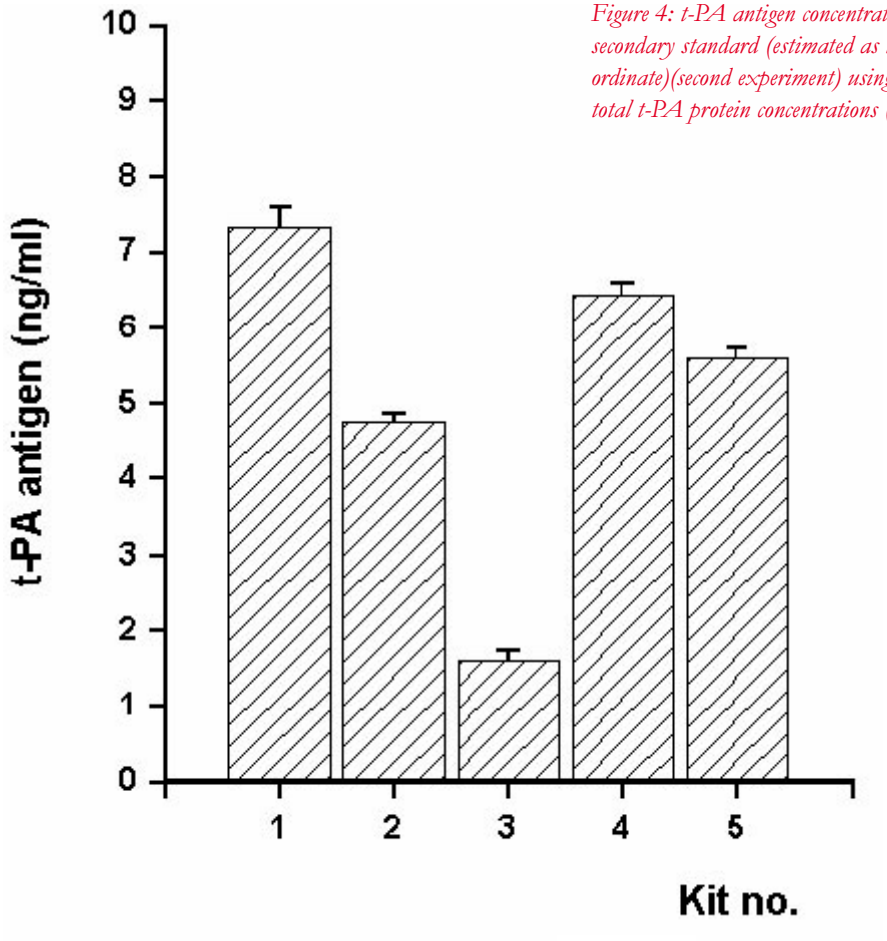

Figure 4: t-PA antigen concentrations measured in the SSC secondary standard (estimated as the cut-off point on the ordinate)(second experiment) using five different kits that measure total t-PA protein concentrations (mean and 1 SEM)

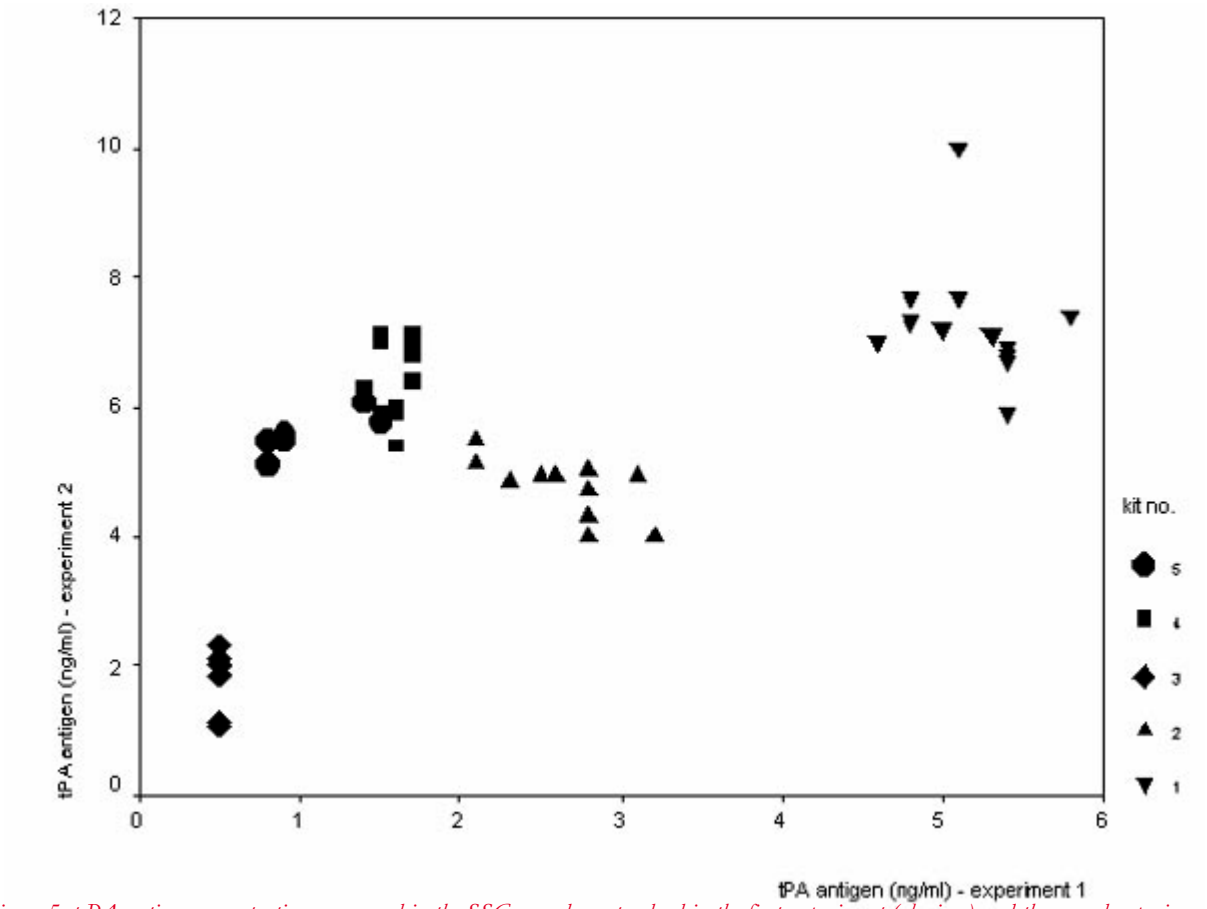

Figure 5: t-PA antigen concentrations measured in the SSC secondary standard in the first experiment (abscissa) and the second experiment (ordinate) using five different kits that measure total t-PA protein concentrations.

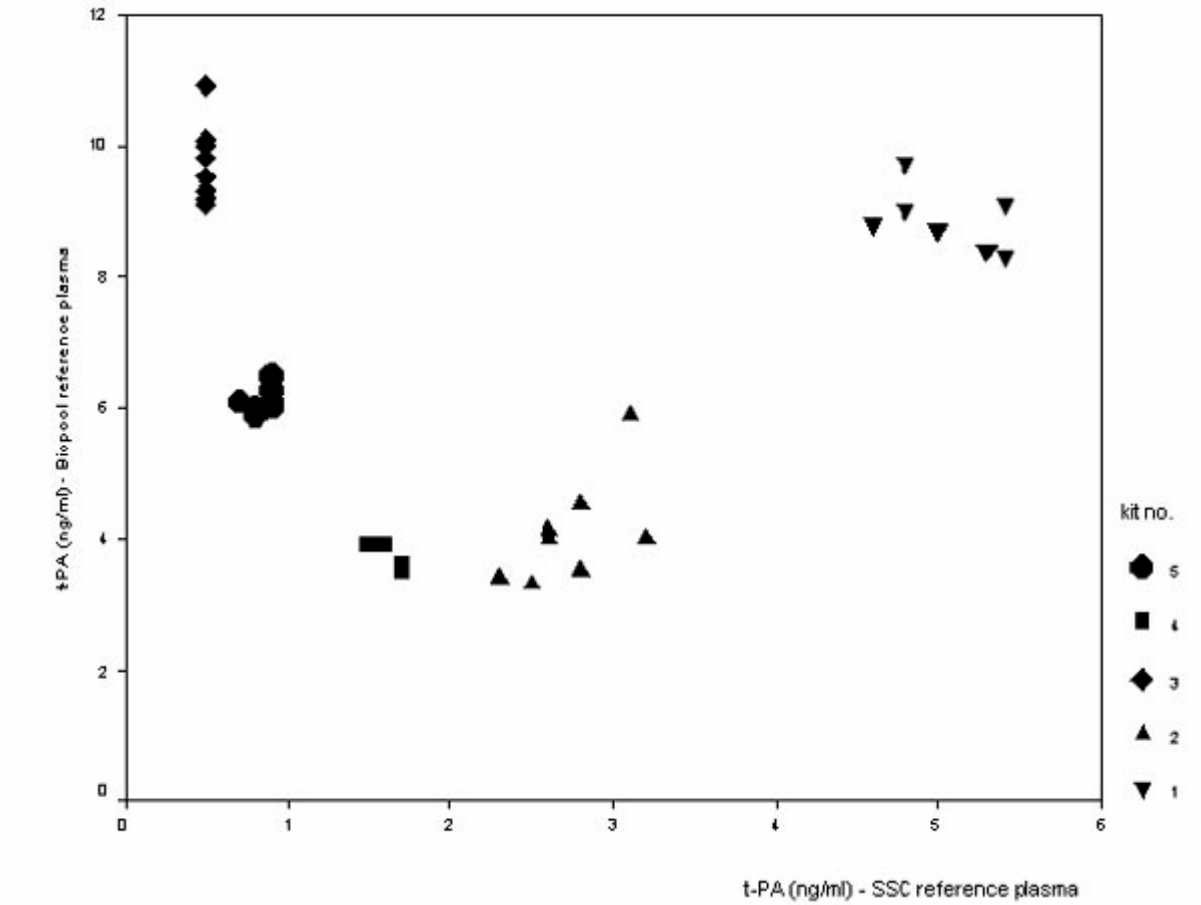

Figure 6: t-PA antigen concentrations in SSC secondary standard and Biopool reference plasma measured by the use of the different kits and the NIBSC t-PA plasma standard as calibrator (first experiment).

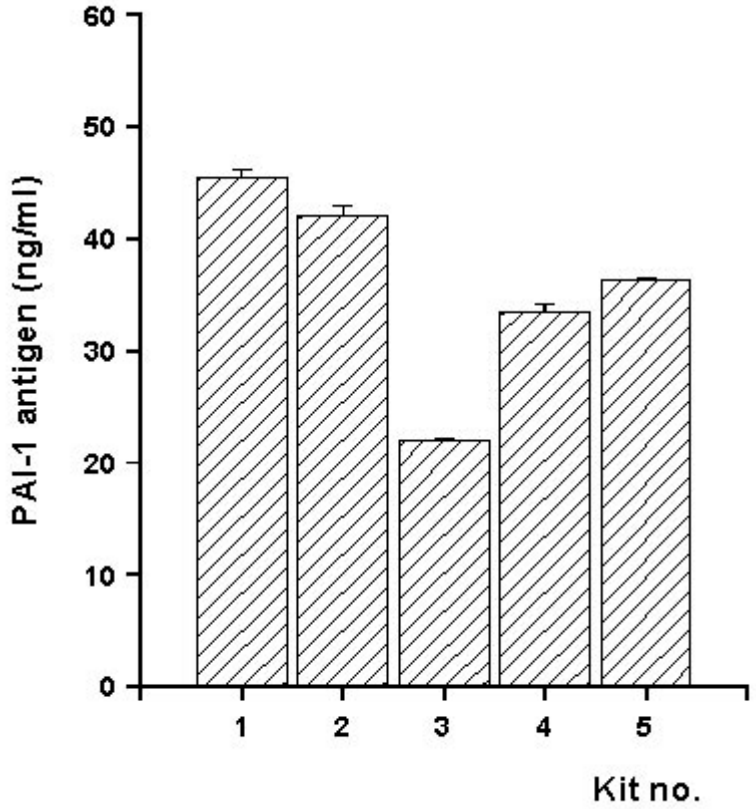

Figure 7: PAI-1 antigen concentrations measured in the SSC secondary plasma standard (first experiment) using five different kits that measure total PAI-1 protein concentrations(mean and 1 SEM)

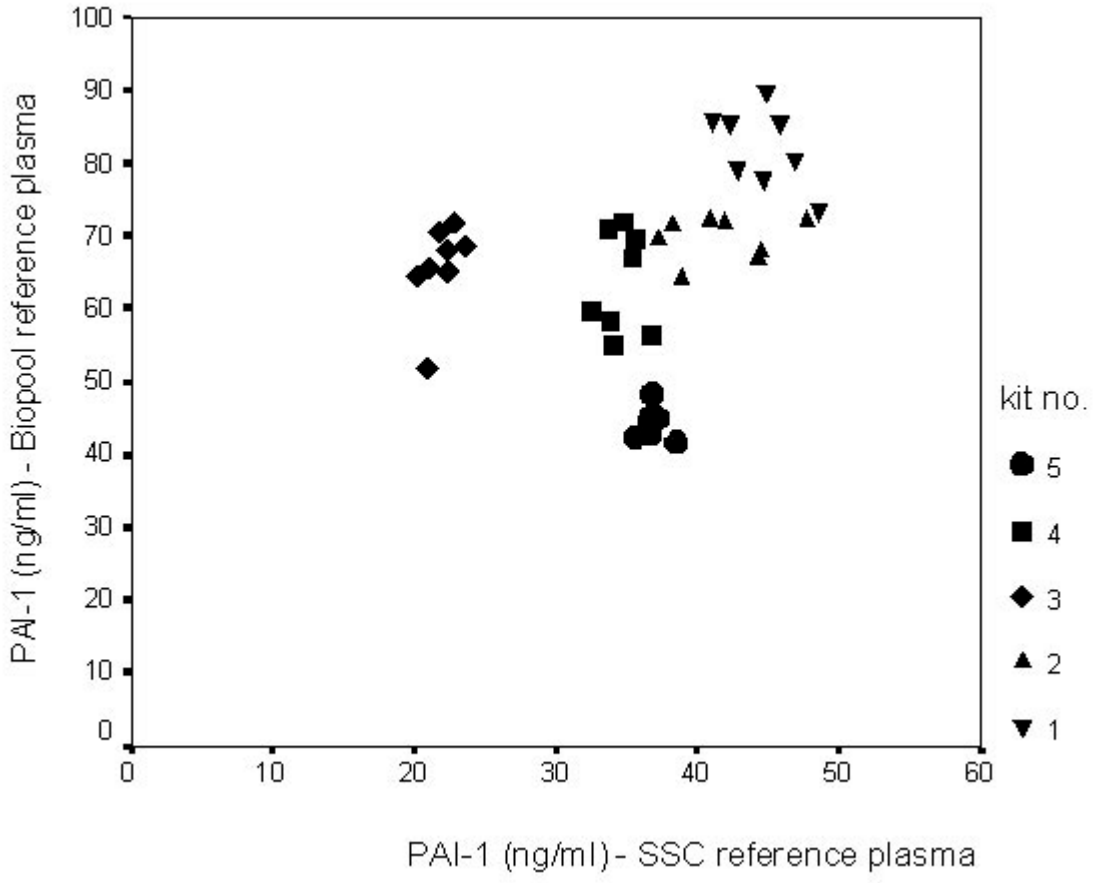

Figure 8: PAI-1 antigen concentrations measured using the different kits in SSC secondary plasma standard and Biopool reference plasma and using the NIBSC PAI-1 plasma standard as calibrator.

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