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Development of an updated assay for prekallikrein activator in albumin and immunoglobulin therapeutics

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Background Prekallikrein activator (PKA) is a contaminating enzyme found in therapeutic albumin and immunoglobulin products. The level is commonly measured using methods such as that defined by the European Pharmacopoeia (Ph Eur) with traceability to the WHO International Standard for PKA. This method generally works well, but problems are sometimes observed.

Materials and methods A simplified one-step method has been developed to replace the existing Ph Eur two-step method which consists of kallikrein generation followed by kallikrein measurement using a chromogenic substrate. Analysis of data from the one-stage method is simplified by the use of a dedicated online app.

Results The one-stage method was validated against the current Ph Eur method using batches of albumin and immunoglobulins. Problem batches of immunoglobulins were investigated using the one-stage method. Improved methodology using true initial rate determinations and use of acid-treated prekallikrein substrate (PKS) helped understand and reduce artefactual results.

Conclusions The one-stage method and associated app streamline real-time determination of PKA and promote good principles of enzyme assays to limit substrate depletion, while also conserving expensive PKS. Blanking steps and reproducibility are simplified.

Key words: prekallikrein activator, kallikrein, albumin, immunoglobulin, method development, online apps, reproducibility.

Introduction

Human albumin for therapeutic use is a long-established blood product with a good safety record [1], although there remain some questions around clinical benefit [2, 3]. Production and quality control includes determination of the level of contaminating prekallikrein activator (PKA), a fragment derived from Hageman factor, factor XII, responsible for the generation of kallikrein from prekallikrein [4]. Kallikrein catalyses the formation of the vasodilator bradykinin, leading to hypotensive reactions in animal models and patients [5, 6]. A widely used assay method for PKA is outlined in the European Pharmacopoeia (Ph Eur) [7]. The level of PKA must be below 35 IU/ml, traceable to the WHO International Standard (IS), which defines the international unit (IU) [8]. Similarly, immunoglobulins are tested for contaminating PKA, with activity traceable back to the same WHO IS. The WHO IS is a freeze-dried 20% albumin with a high level of PKA, and there is no specific IS for PKA in immunoglobulins. The Ph Eur test, (and other PKA pharmacopoeial methods, e.g. [9]), outline a relative potency determination against a standard in a two-step procedure: (i) an activation step where PK in prekallikrein substrate (PKS) made from plasma is activated for a fixed time to generate kallikrein; and ii) this solution or a subsample is mixed with chromogenic substrate to estimate kallikrein activity by measuring amidolytic activity. Variations

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within this basic method were investigated previously in the collaborative study that established the WHO 2nd International Standard (IS) for PKA in albumin [8]. This study identified some deviations from the Ph Eur monograph, for example in the ratio of PKS:sample volume, which is supposed to be 9:1 to avoid artefacts due to different salt concentrations between test samples or tests and standards. It was noted that this rule was not always adhered to, probably to conserve PKS, which is time-consuming to make in-house or costly to buy. The study [8] also showed variations in the way participants interpreted blanking in their protocols, which suggested that the general advice given in the Ph Eur method was not entirely clear.

Theoretically, it is possible to update the existing two-stage method as outlined in the Ph Eur [7] to a simplified one-stage method, as for instance used in the assay of plasminogen activators [10]. Recently, we have simplified this procedure by developing online apps that can be easily used in a computer browser with benefits for reproducibility and transparency [11, 12]. Such apps have been used successfully in a recent collaborative study to establish the WHO 4th IS for Streptokinase [13]. With this experience, we now propose a simplified one-stage method to determine PKA activity in albumin or immunoglobulin solutions which is facilitated by a dedicated online app to simplify rate calculations and associated blanking steps. The use of this new method also makes it easier to investigate a well-known problem in the measurement of PKA in some immunoglobulin solutions, where apparent negative values for PKA activity can be observed after blanking steps using the standard Ph Eur procedure [14]. Work is presented to compare the new one-stage method against the existing Ph Eur method, and an explanation and solution to problems associated with PKA measurements in immunoglobulins are offered.

Materials and methods

The Ph Eur method

The method used follows Ph Eur guidelines [7]. PKA potencies of test and internal control (IC) samples were determined relative to the WHO 2nd IS for PKA (02/168, NIBSC, UK), a freeze-dried 20% albumin preparation with an assigned potency of 29 IU/ml. The IC sample (NIBSC code 00/488) was a freeze-dried 5% albumin batch with a potency of 10 IU/ml. Test albumin batches were from routine testing and coded AL1-AL4. For the first stage of PKA activation, 10 µl of test solution was incubated with 90 µl of PKS (prepared in-house, see below) at 37°C for 10 min. The second step of kallikrein measurement was started with the addition of 100 µl of 0·4 mM S2302 chromogenic substrate (Pro-Phe-Arg-pNA, Chromogenix, Milan, Italy) to the reaction well. All reactions were carried out in a microtitre plate, and each reaction had a corresponding blank for subtraction on the plate consisting of 10 µl of test solution plus 90 µl of buffer to replace the PKS in the first step. Dose–response curves for standards and tests were performed in duplicate, and results analysed by parallel line bioassay methods as outlined in the Ph Eur and previously [7, 8].

PKS preparation

Preparation of PKS followed Ph Eur guidelines [7]. The dialysis and column buffer used was 0·05 M Tris–HCl, pH 8·0, containing 0·02 M NaCl and 50 µg/ml of polybrene (hexadimethrine bromide). Briefly, blood (approximately 45 ml) was collected fresh into 3·8 % w/v sodium citrate containing 1 mg/ml polybrene and PKS isolated using a DEAE-Sephacel column. The PKS fraction does not bind to the column. This fraction was pooled, divided into aliquots and flash-frozen.

One-stage method

The reaction mixture consisted of 10 µl of sample, 50 µl of 1 mM S2302 and 40 µl of PKS, all warmed to 37°C and added to the wells of a microtitre plate in that order, using multichannel pipettes. The plate was divided into + and – PKS sections as for the Ph Eur method. Absorbance at 405 nm was read as soon as possible for at least 60 min. Exported results as time versus absorbance were read into a dedicated app [15], which subtracts background absorbance changes from pre-existing kallikrein activity (–PKS wells) from rates of kallikrein generation (+PKS wells) and calculates PKA activity from slopes of absorbance due to pNA generation versus time squared. Rates were calculated over a change in absorbance value of 0·1 or a maximum of 100 points, for a read interval of 30 s. Detailed instructions and a link to the app are available online [16]. Relative potencies of PKA were calculated from rates of absorbance versus time squared using the same parallel line bioassay methods as for the Ph Eur method, above. Immunoglobulins coded IG1-IG4 were used to investigate PKA measurement in immunoglobulins and acid-treated PKS.

PKS acid treatment

Acid treatment of the PKS substrate described above was as outlined in the US Pharmacopoeia [9]. Briefly, 2 ml of PKS at 37 °C was added to 1 ml of 0·33 M HCl and the

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resulting solution was incubated at room temperature for 15 min, before addition of 1 ml of 0·33 M NaOH and 100 μl of 1M Tris, buffer pH 8·0. Any precipitate was removed by centrifugation at 3000 × *g* for 3 min.

Software and statistical analysis

Parallel line analysis to calculate relative potencies of PKA against a standard were performed using Combistats v 5 [17]. Software designed to automate the calculation of rates of absorbance vs. time squared and deal with blanking steps in PKA assays was developed using the programming language R [18]. A browser-based user interface (the app) was written in R using the Shiny package [19]. Statistical tests were performed using R or Minitab v 18 (Minitab LLC, PA, USA).

Results

One-stage PKA assay using a dedicated app

The starting point for method development was the current Ph Eur method for the determination of PKA in albumin and immunoglobulin solutions [7]. The main change in the new method is to use a one-stage process to measure the generation of kallikrein. Thus, test or standard is mixed with PKS and chromogenic substrate and the reaction is followed immediately, avoiding the two-step process. In line with other similar reactions [10, 13, 20] this means that the activity of PKA, the desired measurand, can be calculated from plots of absorbance versus time squared. To simplify this process, software has been developed using the programming language R [18] and data may be analysed using a browser by means of an app, developed using the Shiny package [19]. The app also handles blank subtraction of pre-existing kallikrein enzyme activity in samples from test wells. In contrast to the current Ph Eur method, subtraction of background kallikrein activity is performed in real time, point by point, rather than at the end as final calculated slopes. A working version of the app may be explored [15], and additional detailed help notes are available online [16].

The one-stage method was investigated over a number of assays that included a common sample, internal control code 00/488 and several other albumin products, coded AL1 to AL4. Results are shown in Fig. 1 as a box plot with pairs of results for each test sample, using the one-stage method and the current Ph Eur method.

The results shown in Fig. 1 suggest that the one-stage method gives equivalent results to the current Ph Eur method as performed at NIBSC.

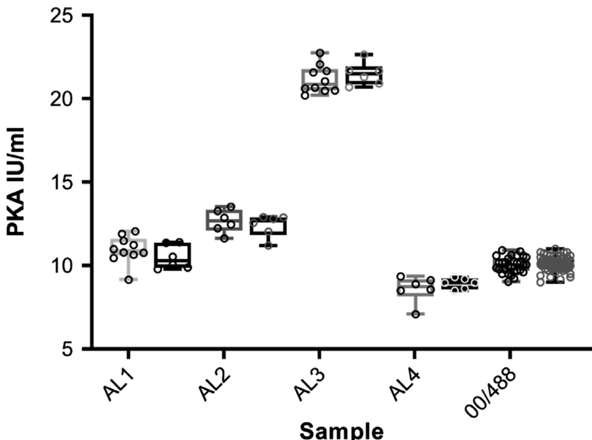

Fig. 1 Comparison of one-stage method and Ph Eur method for PKA determination in albumin samples. Each pair of boxes shows results from assays using the one-stage method (grey box/ black circles) or Ph Eur method (black box/grey circles). There was no significant difference in mean values between Ph Eur and one-stage methods using these samples (Student's *t*-test results were $P = 0{\cdot}28$, 0·52, 0·41, 0·38, 0·4, for AL1-AL4 and 00/488, respectively).

Acid-treated PK substrate

Some methods for PKA determination recommend an acid treatment step for the PKS [9], and acid treatment of the PKS was explored first of all with batches of albumin. In fact, the results shown in Fig. 1 include one-stage assay results using both normal PKS and acid-treated PKS. The results shown in Fig. 1 for 00/488 are elaborated in Fig. 2 to show more clearly the overlap between methods and differentiate normal and acid-treated PKS results. Statistical analysis applying a Shapiro–Wilk normality test to these results showed there was no significant deviation from a normal distribution for 00/488, for the two methods, Ph Eur and one-stage. A full breakdown of results by method and substrate is shown in Table 1 for the total of 168 determinations shown in Figs.1 and 2.

Acid treatment of PKS did not appear to affect assay results but has the disadvantage of adding time and extra manipulations to substrate preparation. Furthermore, there were problems caused by continued precipitation of proteins in the reaction wells after the acid treatment protocol, adding noise to the time courses of kallikrein generation. This developing precipitate may make the acid-treated PKS less suitable for the Ph Eur method which incorporates more PKS in the reaction mixes, especially the first activation step. Therefore, it is not recommended for routine PKA assays for albumin batches.

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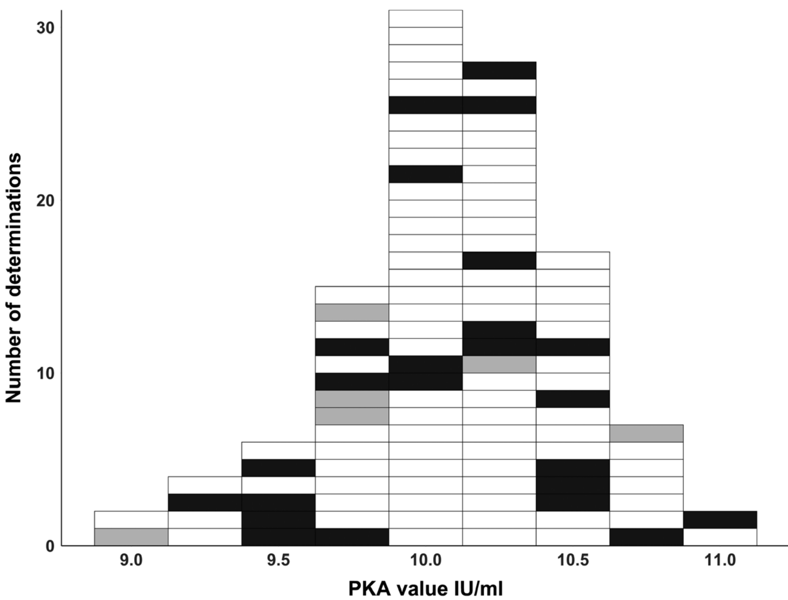

Fig. 2 Summary of PKA determinations in sample 00/488 in different assay formats. Each box in the stacked histogram represents the result from an individual determination of PKA value for the same sample, 00/488, using the Ph Eur method (open boxes), or the one-stage method (shaded boxes), where normal PK substrate (dark shading) or acid-treated PK substrate (light shading) was used. The vertical shading shows the distribution of results within each PKA value bin.

Table 1 Summary of PKA assay results for albumin samples using Ph Eur method or one-stage method with normal PK substrate or acid-treated PK substrate.

Sample	Method	Substrate	Mean PKA IU/ml	SD	N
AL1	Ph Eur	Normal	10·51	0·73	6
	One-stage	Normal	10·81	1·22	4
	One-stage	Acid-treated	11·05	0·55	6
AL2	Ph Eur	Normal	12·41	0·67	6
	One-stage	Normal	12·05	0·59	2
	One-stage	Acid-treated	12·98	0·56	4
AL3	Ph Eur	Normal	21·50	0·69	6
	One-stage	Normal	21·11	0·53	5
	One-stage	Acid-treated	21·20	1·11	5
AL4	Ph Eur	Normal	8·93	0·31	6
	One-stage	Normal	7·80	1·08	2
	One-stage	Acid-treated	8·99	0·33	4
00/488	Ph Eur	Normal	10·12	0·37	82
	One-stage	Normal	10·08	0·46	24
	One-stage	Acid-treated	9·85	0·55	6

PKA assays in immunoglobulins

While PKA assays are generally reliable and reproducible with good dose–responses with albumin products, this is not always the case with immunoglobulins. Complications are reported, for example [14], when using the Ph Eur assay where subtraction of the no-PKS blank wells can result in apparent negative values for PKA in some immunoglobulin batches. The reasons for this artefact are not fully understood but may be explained by observations laid out below. First of all, the problem is illustrated in Fig. 3a where several different preparations of therapeutic immunoglobulins were assayed alongside the IS for PKA (02/168) and the internal control sample (00/488), using the one-stage assay. Similar problems were seen using the Ph Eur assay. The standard and control albumin samples behaved as expected, but the immunoglobulin samples were characterized by apparent negative PKA values, lack of dose–response and variable results. Figure 3b shows clearly that the negative results were associated with high background in immunoglobulin samples, which may be termed 'kallikrein-like' activity as there may be other proteases present that cleave the chromogenic substrate S2302. It is likely that subtracting very high background rates, seen in Fig. 3b in the presence of low levels of PKA would contribute to errors and variability shown in Fig. 3a.

A theoretical contributing factor to apparent negative calculated values for PKA is the presence of an inhibitor of kallikrein-like activity in PKS, and this was explored using acid-treated PKS. Figure 4 shows a summary of results using one of the immunoglobulin samples also used in Fig. 3, IG4 (dark grey bars). Figure 4 presents results from the one-stage method over the 3-point

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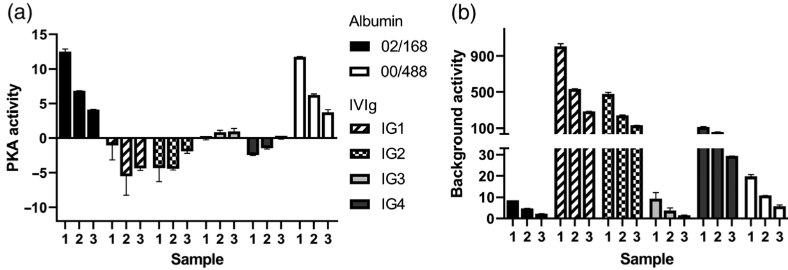

Fig. 3 Attempted determination of PKA in batches of immunoglobulin. Panel a shows results from a one-stage PKA assay using normal PKS, including results from the WHO 2nd IS (02/168, black bars) and internal control (00/488, white bars), where 3 doubling dilutions of samples are numbered 1–3. PKA activity is presented as rate of chromogenic substrate cleavage as absorbance change per $s^2 \times 10^9$, after subtraction of absorbance from no-PKS wells according to the normal protocol. The hatched and shaded bars are results from 4 different immunoglobulin batches, IG1 to IG4. Panel b shows the results for the same samples from no-PKS wells run in parallel for background kallikrein-like activity against chromogenic substrate as absorbance change per s $\times 10^6$. All results are means with SD error bars, $n = 2$.

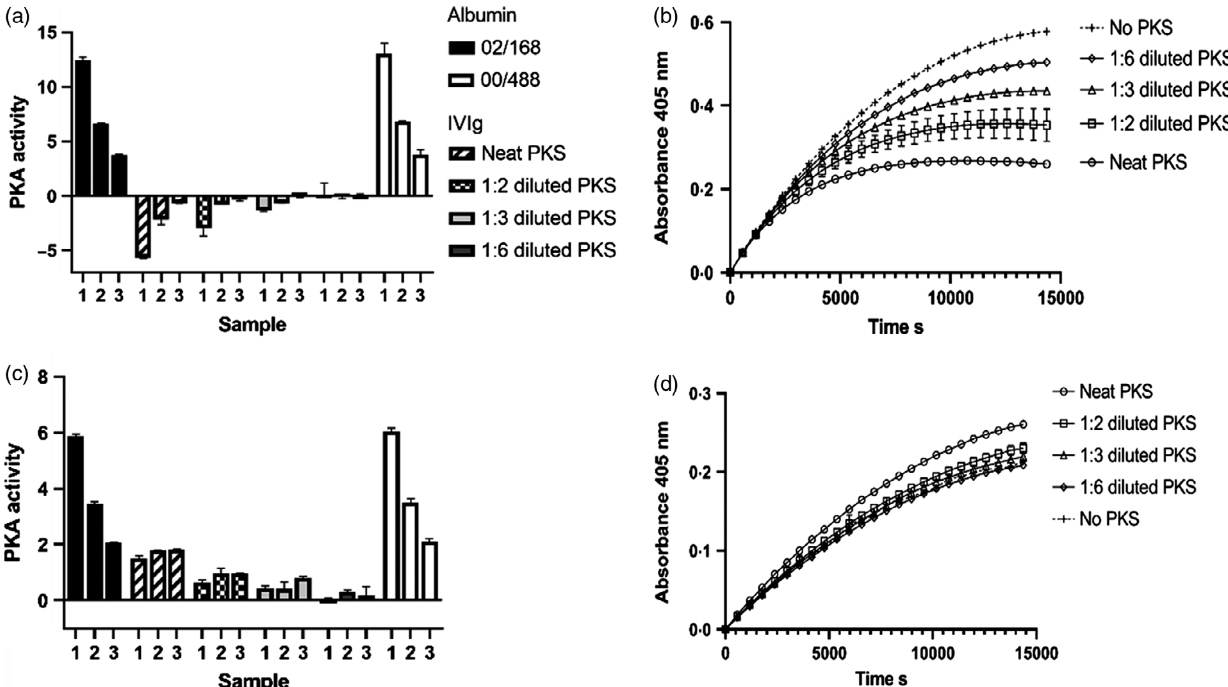

Fig. 4 Measurement of PKA in immunoglobulin and the effect of acid-treated PKS. Panel a shows results from a one-stage PKA assay using normal PKS, including results from the WHO 2nd IS (02/168, black bars) and internal control (00/488, white bars) alongside immunoglobulin sample IG4 using PKS neat (hatched bars), 2x dilution (checks), 3× dilution (light grey) and 6× dilution (dark grey). Each bar 1–3 is a 2-fold dilution of the albumin or immunoglobulin sample. PKA activity is presented as rate of chromogenic substrate cleavage in absorbance change per $s^2 \times 10^9$. Panel b shows the kinetics of kallikrein generation in a one-stage assay as absorbance at 405 nm versus time in s. A single concentration of IG4 is shown (5× dilution of stock 10% solution), over a range of PKS: neat, 2×, 3×, 6× dilutions and no PKS, as circles, squares, triangles diamonds and crosses, respectively. Panels c and d are identical to a and b but using acid-treated PKS. The bars and kinetic traces results are means with SD error bars, $n = 2$.

sample dilutions as shown in Fig. 3, but now also including a range of PKS concentrations so the influence of an enzyme inhibitor might be seen. Figure 4 shows both a summary of apparent final calculated values, after subtraction of background (panels a and c) and the raw kinetic data of all kallikrein-like activity before any subtraction of background rates (panels b and d) from a one-stage assay. Panels a and b are with normal PKS; c and d are with acid-treated PKS. Panel 4a again illustrates the issue of apparent negative values after background subtraction, which is dependent on the sample and PKS concentrations. Panel b explains the phenomenon. Without PKS (crosses), there is a high rate of kallikrein-like activity that rapidly hydrolyses

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chromogenic substrate (the decreasing rate over time is most likely due to chromogenic substrate depletion). However, as PKS is added in increasing concentrations, the rate of chromogenic substrate hydrolysis is depressed. The profiles seen in Fig. 4b are entirely consistent with a slow-binding inhibitor (e.g. a serpin) which gradually knocks out the activity of background amidolytic activity (e.g. see [21]). After acid treatment of the PKS, negative values of PKA are not calculated (Fig. 4c), but there is still no good dose–response for PKA determination with increasing immunoglobulin concentration, in each set sample dilutions 1–3, as there is for the albumin samples. The kinetics using the acid-treated PKS (Fig. 4d) do show a different pattern of amidolytic activity (the order of the curves is reversed in Fig. 4b and d), suggesting that the inhibitor proposed in 4b is much reduced after acid treatment of PKS. Increasing the concentration of acid-treated PKS does lead to a small increase PKA activity, as expected. It is apparent from Fig. 4b and d that under the conditions used in this assay, there would be minimal interference of the PKS inhibitor(s) for time-points below 45 min. Importantly, because more PKS is used in the first step in the Ph Eur assay, and this step has no competing chromogenic substrate present, there is more scope for inhibitor binding to existing or generated kallikrein, which could lead to an underestimate of PKA.

Discussion

The results presented above suggest that the newly developed one-stage assay for PKA works as well as the existing two-step assay to give the same results, but has a number of practical, economic and theoretical advantages, summarized below.

- Fewer manipulations and time critical steps
- Measures initial rates before significant substrate depletion (both PKS and S2302)
- Lower use of PKS: 40 µl per reaction in one-stage method, 90 µl in Ph Eur
- True rate of kallikrein generation is estimated using time squared transformation
- Reactions monitored throughout the measurement procedure (no blind first step)
- Large dilution of sample in well, by PKS and S2302 so efficient use of PKS
- True initial rates measured so less time for slow-binding inhibitors in the PKS
- Reaction wells and background wells can be observed in real time side by side
- The app performs time squared transformation and all rate calculations
- Plate layout can be varied to avoid plate edge or heating artefacts
- Blanking is clear and easy to understand and can be easily recorded
- The app performs a check on a set of data before each run
- Settings are recorded by the app and can be stored with data to aid reproducibility.

Testing, data analysis and results interpretation are usually straightforward for albumin, using existing methods or the one-stage variation, but problems have been noted when PKA is measured in some immunoglobulins [14]. Part of the problem may be due to a matrix effect because of the lack of a standard for PKA in immunoglobulins and the use of the albumin standard. However, the current work suggests that apparent negative results for PKA values after performing the expected blanking steps, are caused by high background levels of protease (‘kallikrein-like’ activity) and the presence of a corresponding protease inhibitor in the PKS. Albumin products rarely have high background rates, and the problem of apparent negative PKA activities, as seen in Figs 3 and 4 with immunoglobulins, is not observed. The subject of contaminating proteases in immunoglobulin preparations was a topic of major interest a few years ago when an increased rate of thromboembolic events (including strokes and venous or arterial thrombosis) was found to be associated with some batches of therapeutic immunoglobulins from some manufacturers. Subsequent research and collaborative efforts by manufacturers, regulators and researchers established that the procoagulant agent in the high-risk batches was activated factor eleven (FXIa) [14, 22, 23]. These studies also showed that the background ‘kallikrein-like’ activity routinely seen in PKA assays with albumin or immunoglobulins is dominated by kallikrein, but this is unlikely to contribute to prothrombotic activity. As none of the immunoglobulin samples we explored in the current work were implicated in thromboembolic events, it is likely that the ‘kallikrein-like’ activity we observed was in fact kallikrein. Nevertheless, it has been noted that high levels of contaminating kallikrein may be a cause for some concern and deserves more attention [13].

As mentioned above in Results, the suppression of amidolytic activity in Fig. 4 in the presence of PKS could be explained by the presence of a slow-binding inhibitor in the PKS, which can be largely eliminated by acid treatment. Currently, the identity of this inhibitor is not known. One of the advantages of the one-stage method is that the whole reaction showing the development of kallikrein and cleavage of chromogenic substrate is monitored, so problems can be observed. Furthermore, early

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initial rates are used, thus reducing the effect of remaining slow-binding inhibitors in the PKS. The traditional Ph Eur method includes the first, blind step of PK activation in the presence of a high concentration of PKS. Issues such as precipitate formation or interference by inhibitors in the PKS can occur during this step.

Overall, the updated one-stage method and associated app provides several theoretical, economical and practical advantages over the existing widely used two-stage Ph Eur method and should provide more reliable results when measuring PKA in immunoglobulin samples. If this approach is adopted more widely, we anticipate that variability of results obtained by different laboratories should be reduced.

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