Comparative analysis of the activity and content of different streptokinase preparations

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Aims The dosage of fibrinolytic agents such as streptokinase must be controlled carefully to maximize therapeutic activity while avoiding adverse effects. Therefore, the integrity and activity of streptokinase products is likely to be clinically relevant. This study was conducted to compare the in vitro characteristics of different streptokinase preparations.

Methods and results Sixteen streptokinase preparations (three of which were recombinant) were compared in a chromogenic substrate activity assay by native, and reducing, sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), and N-terminal sequencing. Deficiencies in streptokinase activity were observed in most of the products: only three fulfilled the minimum requirements of the European Pharmacopoeia. These were Icikinase™ (ICI Pharm Ltd, India, only one of two batches tested), Kabikinase™ (Pharmacia Upjohn, Sweden), and Streptase™ (Aventis Behring GmbH, Germany). The remaining products exhibited activities ranging from 20.8 to 86.6% of the label claim. Differences in composition and purity were demonstrated by both native and reducing SDS-PAGE. N-terminal sequencing of the recombinant preparations showed differences compared with the native protein — indeed, for one product, the 15 N-terminal amino acids bore no resemblance to streptokinase.

Conclusion There are wide variations in the activity, purity, and composition of the available streptokinase preparations.

KEYWORDS Infarction; Protein sequence; Streptokinase; Thrombolysis

Introduction

Streptokinase was the first thrombolytic drug to be described,1 and was introduced as a therapy for acute myocardial infarction more than 40 years ago.2,3 It is now the leading fibrinolytic agent in the treatment of thromboembolic conditions,4 and is included in the World Health Organization (WHO) Model List of Essential Medicines.5

Streptokinase is a 45–50 kilodalton glycoprotein that mediates the cleavage of a peptide bond in plasminogen, producing the active product plasmin, which cleaves fibrin polymers to promote thrombolysis. A large number of clinical trials and meta-analyses have shown that intravenous infusion of streptokinase is associated with reductions in both short-term and long-term fatality compared with placebo in patients with acute myocardial infarction.4,6–9 In addition to reducing mortality, treatment with streptokinase is cost-effective and associated with shorter hospital length of stay and decreased utilization of intensive care facilities compared with conventional therapy without thrombolysis.10,11

The dosage of fibrinolytic agents must be carefully controlled. Administration of too low a dose is associated with decreased rates of reperfusion of the infarct-related artery, and too high a dose with increased intracranial
haemorrhage. In the GUSTO-1 trial, the streptokinase dosing regimen deviated from the protocol, either by incorrect total dose or infusion duration, in 13.5% of participants. A 30-day mortality rate of 11.3% was observed in these patients, compared with only 6.4% in those with per-protocol dosing ($P$, 0.001). Therefore, administration of the incorrect dose increases the likelihood of serious adverse outcomes.

Current guidelines, based on several randomized, multi-centre clinical trials, recommend a 1 500 000 International Units (IU) intravenous infusion over 1 h as the appropriate streptokinase dosage for the treatment of acute myocardial infarction, with the European Pharmacopoeia demanding a fibrinolytic activity of 90–111% of the declared value. Given the narrow therapeutic window, it is likely that the integrity of streptokinase preparations will be of clinical relevance. However, although a large number of streptokinase products are available, the comparability of the different preparations in terms of activity, purity, and composition has not been assessed. Therefore, the

<table>
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Methods

Samples

Sixteen preparations of streptokinase, from 11 different manufacturers, were tested. In six cases, duplicate samples of the same product from different batches were included. Each preparation was available on the market in Brazil, India, Kingdom of Jordan, China, Pakistan, or Europe. The purchased (lyophilized) market products were kept at $+2$ to $+8$°C until use, ensuring that only freshly dissolved samples were used for activity determination, and enabling comparability of results. The dissolved samples were then kept at $-15$ to $-25$°C until their further analysis by native PAGE respectively SDS–PAGE, for which the samples have been diluted according to their actual activity determined in the chromogenic assay (with the exception of STPase, where native PAGE and SDS–PAGE were performed prior to activity determination, due to logistic reasons). For
native PAGE respectively SDS–PAGE, any freeze–thaw effects may be neglected (unpublished results).

An in-house streptokinase standard calibrated against the WHO international standard was used as a reference for activity determination. The purified drug concentrate of Streptase (SK-PDC), determined against the in-house standard, was used as a reference sample for native PAGE and SDS–PAGE.

Streptokinase activity determination

Streptokinase samples were diluted to an activity of 25–500 IU/mL (based on the activity stated on the product label), in 1/15 M phosphate buffer pH 7.4, containing 3% (w/v) bovine serum albumin. Plasmin activity was suppressed by addition of 30 Kallikrein inactivator units/mL aprotinin (Aventis Behring GmbH, Germany). Cuvettes containing chromogenic substrate solution (980 µL; 0.57 mg/mL lys-p-nitroanilide, 0.05 M sodium phosphate buffer pH 7.0 ± 0.1), were carefully supplemented with diluted streptokinase samples (20 µL) and mixed with a stirring rod. The increase in absorbance at 405 nm was measured in a spectrophotometer with thermostable cuvette holder (U-2000; Hitachi, Japan) and thermostat (RM6; Lauda, Germany) over 3 min. A calibration curve was constructed from at least three measurements with the reference standard adjusted to 200 IU/mL (20 µL). The sample activities were determined from the calibration curve, using the value of the highest absorbance increase within 15 s. Incubations and measurements were carried out at 25 ± 0.5°C.

Sample purity and content determination

Native PAGE (8% pre-cast gels; Invitrogen GmbH, Germany) and reducing SDS–PAGE (4–20% pre-cast gels; Invitrogen GmbH, Germany) were carried out using a Mighty Small II electrophoresis chamber (SE 250; Amersham Biosciences Europe GmbH, Germany). The running buffers were 25 mM Tris–HCl pH 8.5, 192 mM glycine for native PAGE, and 25 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS for SDS–PAGE. Samples for SDS–PAGE were reduced in a buffer consisting of 63 mM Tris–HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 2.5 mg/mL dithiothreitol, and 0.0025% (w/v) bromophenol blue.

Samples were diluted with water to a concentration of 60,000 IU/mL (based on the activity determined in the chromogenic assay) and then mixed 1:1 (v/v) with sample buffer (resulting in 30,000 IU/mL), and boiled for 3 min. After 7 µL of sample were loaded per well, electrophoresis was carried out at 10°C. Pre-electrophoresis was performed for 30 min at 10 mA/gel, 3000 V, 150 W; main electrophoresis at 20 mA/gel, 3000 V, 150 W, until the bromophenol blue front reached the lower end of the gel.

The gel was placed in fixation solution [3% (v/v) acetic acid, 25% (v/v) ethanol] with gentle shaking at room temperature for 30 min, stained with Coomassie blue staining solution.
[0.01% (w/v) Serva Blue G, 7.5% (w/v) ammonium nitrate, 0.325% (v/v) nitric acid, and 0.2% (w/v) sodium acetate-3-hydrate] overnight at 40 °C, photographed, and scanned. Optical density patterns of scanned images were evaluated using 1-D-Elite Image Master 4.10 software (Amersham Biosciences Europe GmbH, Germany).

N-terminal sequencing

The composition of streptokinase products conspicuous in native PAGE was analysed by N-terminal sequencing. Samples to be analysed were subjected to native PAGE, followed by Western blotting onto polyvinylidine difluoride (PVDF) membranes. The regions of PVDF membrane corresponding to blotted streptokinase were excised and subjected to N-terminal sequencing via automated Edman degradation, using a Procise 492 sequencer from Applied Biosystems.

Results

Streptokinase activity determination

The results of the chromogenic assay are summarized in Table 1. The origin of the samples is shown in Table 2. The activity of the different products ranged from 20.8 to 107.4% of the activity claimed on the product label. For purified native streptokinase preparations, activities of 42.6–107.4% of the claimed activity were recorded. For the recombinant streptokinases, Heberkinase and STPase (two batches), the measured activity was only 37.2% (Heberkinase) respectively, 20.8 and 23.3% (STPase, two batches) of the label claim. The activity of the recombinant SK from China could not be determined as it produced abnormal reaction kinetics in the chromogenic assay. Only three streptokinases, i.e. Streptase®w, Kabikinase, and Icikinase (batch DK 1124), had activities within 90–111% of the declared value, while another batch of Icikinase (DK 0706) exhibited an activity of only 53.1% of the label claim.

Sample purity and content determination

In the native gel, SK-PDC produced the expected single band (Figure 1A, Figure 2A; see also Figure 3A and Figure 5A). The corresponding market product, Streptase® (Figure 1A, Figure 2A, see also Figures 3A, 4A and 5A) revealed an additional band in the lower region of...
the gel, attributable to the human albumin used as a stabilizer. In addition, a faint smear in the upper half of the lane was observed, attributable to the polygeline stabilizer. In the corresponding SDS–PAGE, SK-PDC (Figures 1B, 2B; see also Figures 3B and 5B) migrated as a single band, whereas Streptase® (Figures 1B, 2B; see also Figures 3B, 4B and 5B) exhibited two major bands, corresponding to albumin (higher molecular weight) and streptokinase (lower molecular weight), and some faint higher molecular weight bands.

In the native PAGE, Akinase (Figure 1A), Durakinase (Figure 1A), and Icikinase (Figures 2A and 4A) produced very similar migration patterns, which was confirmed via SDS–PAGE (see corresponding lanes, Figures 1B, 2B and 4B). When compared by optical density pattern evaluation, the traces in native PAGE produced by these three samples were indistinguishable (Figure 6A). Unitinase (Figure 1A) and Kabikinase (Figure 1A) also produced similar native migration patterns, although at an altered SK/albumin ratio, which was confirmed in the corresponding SDS–PAGE (Figure 1B).

Similarly, Streptokinase TTK, Eskinase, and Thrombosolv (Figure 2A) from two German manufacturers (Table 2) were comparable by native PAGE, which was confirmed by SDS–PAGE (see Figure 2B). These three products contained less albumin than the Streptase® control sample (native PAGE, Figure 2A; SDS–PAGE, Figure 2B) or than Akinase, Durakinase, and Icikinase (Figures 1 and 2).

The similarity of Streptokinase TTK, Eskinase, and Thrombosolv (Figure 2A) was confirmed by optical density pattern evaluation (Figure 6B). Notably, two samples of Thrombosolv, both purchased in India, but manufactured at two separate sites (Germany and Korea), produced different migration patterns in both native PAGE and SDS–PAGE (Figures 2 and 4).

Unitinase and Strek (Figure 3A), both purchased in Brazil, were indistinguishable by visual inspection, which was confirmed by optical density pattern evaluation (Figure 7A). In native PAGE, a double band was observed in the streptokinase region, of which only the upper band co-migrated with streptokinase, which was likewise seen in SDS–PAGE (Figure 3B).

In Thrombosolv, manufactured in Korea (Figure 4A), the region corresponding to streptokinase also contained two bands of which only the upper co-migrated with streptokinase. This migration pattern was similar to that of Unitinase (Figure 3A), which was manufactured by the same Korean company, and Strek (Figure 3A), the manufacturer of which was not indicated on the label.

Two batches each of Solustrep and Streptonase, each purchased in Brazil, produced similar migration patterns in both native PAGE (Figure 3A) and SDS–PAGE (Figure 3B), despite these products being manufactured by two different Korean companies. The similarity of these samples was again confirmed by optical density pattern evaluation (Figure 7B).

The three recombinant streptokinase samples, recombinant Streptokinase from China (Figure 3A), Heberkinease (Figure 4A), and STPase (two batches; Figure 5A), produced two respectively three (STPase) major bands in native PAGE, where the lowest band presumably corresponded to albumin. The recombinant Streptokinase from China revealed an upper band that migrated more slowly...
than streptokinase (which points to a biochemical difference), whereas the corresponding SDS–PAGE pattern was comparable to Streptase® (Figure 3B). The presumed active ingredient of Heberkinase (Figure 4A) migrated slightly more rapidly than streptokinase in native PAGE, but co-migrated with streptokinase in SDS–PAGE, suggesting that it may carry an additional negative charge or lack a positive charge compared with streptokinase. The presumed active ingredient of STPase (Figure 5A; two batches) produced two major bands in native PAGE, of which the lower band co-migrated with streptokinase, while further (more slowly migrating) bands of unknown origin could be identified. The intensity of the bands corresponding to streptokinase was much lower than in the other samples. This is due to the fact that (unlike the other samples) the two STPase samples were diluted following the concentration stated on the label claim (rather than awaiting the results of the chromatographic assay that indeed confirmed an activity of only 20% of the label claim, using a second i.e. fresh vial of the same batch).

N-terminal sequencing

The composition of samples abnormal in native PAGE (Figures 3A–5A), i.e. recombinant SK from China (Figure 3A), recombinant Heberkinase (Figure 4A) and recombinant STPase (Figure 5A) as well as Unitinase, and Strek (Figure 3A) and Thrombosolv manufactured in Korea (Figure 4A), was further analysed by N-terminal sequencing of bands excised from PVDF membranes following Western blotting of native PAGE gels. N-terminal sequencing of Thrombosolv revealed two sequences, corresponding respectively to the 15 N-terminal residues, and amino acids 85 onwards, of streptokinase (data not shown). These two sequences were both obtained from the same band, which migrated more rapidly than the SK in Streptase® (Figure 4A). This suggests that the band consisted of a mixture of streptokinase altered at an unknown location and a degradation product, which had a relative frequency of 30%.

The first 15 amino acid residues of the lower bands of Unitinase and Strek (Figure 3A) corresponded to those of streptokinase (data not shown). However, the faster migration of these proteins in native PAGE suggests that they may carry a higher negative charge, and may therefore contain differences elsewhere in the sequence.

N-terminal sequencing of the first 15 amino acid residues revealed significant differences for all recombinant products compared with the sequence of Streptococcus pyogenes streptokinase (Table 3). The N-terminus of the recombinant SK from China (Figure 3A) was elongated by six amino acid residues (VKPVQA), identical to the last six amino acids of the signal sequence of streptokinase A of S. pyogenes.18 Furthermore, a substitution of proline to serine corresponding to position 4 of S. pyogenes streptokinase was present in this sample. The additional positive charge due to the lysine residue in the signal sequence may contribute to the migration pattern of this protein in native PAGE.

The main band excised from the native PAGE of recombinant Heberkinase (Figure 4A) revealed two sequences, present in approximately equal amounts, one of which appeared to be identical to the first 15 N-terminal residues of streptokinase (Table 3), while the other had an additional N-terminal methionine. These two sequence variants were indistinguishable from each other in native and SDS–PAGE (Figure 4). However, the observation that they migrated more rapidly than the regular streptokinase in native PAGE strongly suggests that this recombinant protein may differ elsewhere from native streptokinase (e.g. sequence errors).

STPase (Figure 5A) also yielded two distinct N-terminal sequences, with relative frequencies of 60 and 40%. Neither of these sequences resembled streptokinase in the first 15 N-terminal residues. One possible explanation of this finding is that STPase could be a different product.

Discussion

The most striking finding of this study was the significant discrepancy between claimed and measured streptokinase activity of the majority of the tested products. The European Pharmacopoeia demands that streptokinase preparations contain a fibrinolytic activity of 90–111% of the declared value. Most of the products failed to fulfil these requirements by a substantial margin, exhibiting activities in the range of only 20.8 to 86.6% of the label claim. Only Streptase® (Aventis Behring GmbH, Germany), Kabikinase® (Pharmacia Upjohn, Sweden), and a distinct batch of Icikinase® (ICI Pharm Ltd, India), were in line with these requirements, while a second batch of Icikinase® (ICI Pharm Ltd, India) exhibited an activity of only 53.1% of the label claim.

However, the history of the samples prior to purchase, which may be relevant to activity determination, is unknown, and any previous improper handling or storage of the products cannot be excluded.
Furthermore, it is unknown which activity assay has been used by the respective manufacturer and whether these assays would meet pharmacopoeial standards. As we have only analysed one sample of most batches, the results cannot be generalized, but they indicate that careful product selection should be made for clinical use.

Accurate reporting of the characteristics of streptokinase preparations is essential for safe and effective fibrinolytic therapy. Our study suggests quality deficiencies in several streptokinase products. Some preparations showed additional bands by PAGE or SDS–PAGE, indicating greater heterogeneity (i.e. lower purity) of the samples. In some products, the drug substance appeared to migrate as a double band, suggesting that the streptokinase protein may have been subject to biochemical alterations or degradation. Moreover, the streptokinase/albumin ratio of the products varied considerably.

The amino acid sequences of the three recombinant products tested (recombinant SK from China, Heberkinase, and STPase) all deviated from the published S. pyogenes streptokinase sequence. Furthermore, the behaviour of the recombinant streptokinase proteins in PAGE differed considerably from that of the purified native protein. Two of the recombinant products [i.e. Heberkinase and STPase (two batches)] exhibited very low biological activity [37.2% (Heberkinase) respectively 20.8% and 23.3% (STPase, two batches) the label claim], and the activity of the third recombinant product (recombinant SK from China) resisted determination. Therefore, the observed differences in recombinant
protein sequence and behaviour in PAGE are correlated with alterations in the activity of the drug. It must also be considered that the differences noted may have further effects not detectable by an in vitro activity assay. While the bacterial origin of the different purified streptokinase products is not known, one possible explanation for the differences in migration, sequence, and activity of the different streptokinase preparations is that they may have been cloned or purified from different *Streptococcus* strains.

There are potentially serious clinical implications of the low activity with respect to the label claim of most of the streptokinase preparations examined in this study. The dose of streptokinase must be carefully controlled to provide adequate thrombolysis without increasing the risk of intracranial haemorrhage. If the activity of the drug is significantly lower than the label claim, there is a risk that an appropriate dose will not be administered and reperfusion will not be achieved. Therefore, the discrepancy between claimed and actual activity of many streptokinase preparations may cause life-threatening situations in thrombolytic therapy of severely ill patients.

It is clear from this study that there are wide variations in the available streptokinase preparations in terms of activity, purity, and composition.

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ment of Acute Myocardial Infarction of the European Society of Car-

### Table 3 N-terminal sequencing of recombinant streptokinase samples

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| ~40    |                | XKKXKEVAVFKFKDLX   |<|>

For sample designation see Table 1.

SK, sequence of N-terminus of streptokinase from *S. pyogenes*. 19

X, No amino acid detectable.

X, No amino acid detectable (traces of W were detected; however, W is known to be highly unstable as it is easily oxidized).

( ), These amino acid residues were detected in approximately equal amounts and could therefore not be assigned unequivocally.

( ), These amino acid residues are the most likely candidates but have not been assigned unequivocally.