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Quantitation of insulin by capillary electrophoresis and high-performance liquid chromatography Method comparison and validation[1]

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Abstract

Two validated high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) assays for insulin are compared. Both methods are selective and robust, with best reproducibility in a 0.9% sodium chloride solution, pH 7–8, as the sample solvent. It is shown that, besides sample solvent and buffer pH, acetonitrile volatility is a crucial point for reproducibility. Its high importance for selectivity and ruggedness is also stressed. Trends have been extensively investigated and characterized. The separation efficiency is better for the CE method. Furthermore, the analysis time of the CE method is up to four times shorter than the respective parameter in HPLC and the acetonitrile consumption is more than 100-fold less. Earlier works stated that all relevant precision data, such as relative signal standard deviation (1.6% R.S.D. for peak areas, $n=20$), precision of the analytical result and the limit of quantitation, were about a factor of two worse than for corresponding HPLC data (0.8% R.S.D. for peak areas, $n=19$). This CE precision was further improved using relative instead of absolute peak areas, which compensate for the injection error (1.3% R.S.D. for relative peak areas, $n=20$). If acetonitrile evaporation is avoided, by covering the buffer with mineral oil, reproducibility is even better than with the HPLC assay (0.5% R.S.D. for relative peak areas, $n=60$). © 1997 Elsevier Science B.V.

Keywords: Validation; Pharmaceutical analysis; Insulin; Hormones; Peptides

1. Introduction

Capillary electrophoresis (CE) has been established as a technique that is suitable for a wide range of analytical problems. It offers a number of advantages, especially short analysis time, high separation efficiency and low sample and solvent consumption. Thus, performance of CE as a quantitative separation technique is of high relevance. However, in some cases there is concern about reproducibility.

For routine analyses in the pharmaceutical industry, where stable and reproducible methods are required, many still favour the use of LC. On the other hand, validated CE methods often perform the same as, or better than, the corresponding HPLC methods. Even if they are only second best, they can at least be used advantageously to cross-validate the LC assay.

A comparison of CE and LC on similar analytical problems has been done in several works, e.g. [1–4]. Very instructive inter-company CE cross-validation studies have also been described [5,6]. The analytical characterization of insulin is a task of major importance. Both HPLC and CE assays already exist [7–11].

In this work, optimized and validated assays of

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[1]Dedicated to Prof. Dr. H. Rüdiger on the occasion of his 60th birthday.

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both HPLC and CE have been developed and compared, focusing on reproducibility. Special emphasis is laid on the CE method, including the internal standard (I.S.). Influences of sample solvent and pH as well as of the organic modifier on selectivity and reproducibility are pointed out and discussed in detail.

Insulin is a peptide hormone that is involved in the regulation of the metabolism of carbohydrates, proteins and fatty acids. Human insulin (HI) consists of two peptide chains, A and B, which together contain 51 amino acids and which are connected by two disulphide bridges [12] (the A-chain contains 21 amino acids and the B-chain, 30 amino acids) (for a comparison, see [13]). Its relative molar mass is M_r=5808, the isoelectric point, pI=5.4–5.5 ([7] and literature cited therein). In order to quantify insulin, the degradation products and the commonly used preservative *m*-cresol have to be separated from the main compound. Two types of desamido-insulins, desamidated at different positions, are described as the main degradation products, being an acidic and a neutral desamido-insulin (desamidated at positions A-21 or A-21 and B-3, respectively) [7]. The relevant concentration range is between 0.038 mg/ml (≈1 I.U./ml) as the lowest usual concentration proposed for pharmaceutical preparations and 1.5 mg/ml (≈40 I.U./ml), which is the usually applied dose.

2. Experimental

2.1. Liquid chromatography

The apparatus consisted of a pump 2000 and an autosampler (Micromeritics 728) with an injection valve 7010 (Bischoff, Leonberg, Germany), equipped with a UV–Vis detector 73287 (Knaur, Böblingen, Germany). The column was a Nucleosil RP18, 5 μm, 250×4 mm I.D. (Macherey-Nagel, Düren, Germany). The injection volume was 20 μl, the detection wavelength was 214 nm, the flow-rate was 1 ml/min and the column temperature was 40°C. Data collection and analysis was performed using a laboratory-built interface and an HP217 computer (Hewlett-Packard, Böblingen, Germany), with evaluation software that has been described previously [14].

As the mobile phase, a mixture of 240 g of acetonitrile (LiChrosolv HPLC-grade) and 760 g of phosphate buffer was used. The latter was prepared by dissolving 9800 mg of H_3PO_4 (85%, w/w, analytical-reagent grade) and 28.4 g of Na_2SO_4 (analytical-reagent grade) in 1000.0 ml of HPLC-grade water (Millipore, Eschborn, Germany); prior to use, the buffer was degassed with helium for 5 min. Acetonitrile, H_3PO_4 and Na_2SO_4 were purchased from Merck (Darmstadt, Germany).

Each standard was injected six times.

2.2. Capillary electrophoresis

CE experiments were carried out using three different instruments. A detailed overview of the different instrumental features is given in some reviews [15,16].

CE validation experiments were performed on P/ACE 2050 and 2100 systems (Beckman Instruments, Palo Alto, CA, USA). The capillaries were from fused-silica (Polymicro Technologies, Phoenix, AZ, USA) with a standard length of 30 cm (inlet to detector) and with a 50 μm I.D. (375 μm O.D.). The detection wavelength was 214 nm; injection was by pressure for 4 s with 0.5 p.s.i (=34.5 mbar). The separation voltage was 20 kV, with the cathode at the buffer outlet side (resulting current, 53 μA). The thermostat was set to 30°C. Prior to their first use, the capillaries were conditioned with 0.1 *M* NaOH for 30 min at 50°C followed by equilibration with running buffer for 40 min, applying the above-mentioned separation conditions. Before each run, the capillary was rinsed with buffer for 2 min.

CE experiments with internal standard were performed (A) on a SpectraPhoresis 1000 system (Thermo Separation Products, San Jose, CA, USA) and (B) on a Crystal 310 system (ATI Unicam, Cambridge, UK). In the case of (A), the fused-silica capillaries had a length of 35 cm (inlet to detector, corresponding to a total length L=43 cm), hydrodynamic injection was done by vacuum (0.75 p.s.i= 51.115 mbar) and the resulting current was 32 μA (E=233 V/cm). In the case of (B), the capillary length was 45 cm (inlet to detector, corresponding to a total length L=60 cm), hydrodynamic injection

was done by pressure (50 mbar) and the separation voltage was 30 kV (resulting current, 20 μA; E=333 V/cm). The system was run at ambient temperature. Apart from the procedure given above, capillary equilibration with both systems (A) and (B) lasted 120 min, instead of 40 min.

All other parameters are the same as mentioned under CE validation.

A buffer system developed by Mandrup [7] [50 mmol/l acetate, 850 mmol/l 2-(N-cyclohexylamino)ethanesulfonic acid (CHES), 10% acetonitrile, virtual pH=7.8] was used. The buffer is prepared by dissolving 820 mg of sodium acetate (analytical-reagent grade) in 200.0 ml of HPLC-grade water. A 17.62-g amount of CHES (analytical-reagent grade) is dissolved in 100.0 ml of sodium acetate solution and 10.000 g of acetonitrile are added; the virtual pH, measured using a freshly calibrated pH electrode, is adjusted to 7.8 by adding triethylamine. In the series *insulin4*, the buffer in the vial was covered by adding two drops of mineral oil, which gave a film of approximately 1 mm.

Sodium acetate, CHES and mineral oil (German pharmacopoeia quality) were obtained from Merck.

Each standard was injected ten times.

2.3. Samples

The samples for HPLC and CE validation were prepared by dissolving the appropriate amount of insulin (Boehringer Mannheim, Mannheim, Germany, pharmacopoeia quality) in 0.9% sodium chloride solution. Concentrations below 0.24 mg/ml were prepared from a stock solution, diluting it ten-fold with the same solvent. A clearly detectable amount of degradation products was found in insulin solutions that had been stored for two weeks at 8°C; these solutions were used to validate the separations. The standard solutions were injected in a randomized order. The W_sA statistics program (see [17]) was used to evaluate the data presented in Table 1.

Sample solutions for I.S. investigations were prepared by dissolving appropriate amounts of insulin (porcine insulin CRS, European Pharmacopoeia quality, 89% insulin peptide, batch No. 1; Merck, or bovine insulin, Fluka, assay ≥98% HPLC; Fluka, Neu-Ulm, Germany) and neostigmine bromide (German pharmacopoeia quality), as I.S., in a 0.9% (w/v) sodium chloride solution to give final concentrations of about 0.6 mg/ml (≈16 I.U./ml) for insulin and of about 1 mg/ml for the I.S., respectively. To improve the solubility of insulin, a few drops of 0.1 *M* NaOH or 0.1 *M* HCl were added. Sample solutions as well as buffer solutions were filtered through 0.22 μm membrane filters (Optex by Wepa, Höhr-Grenzhausen, Germany).

Neostigmine bromide, sodium chloride (analytical-reagent grade), sodium hydroxide pellets (analytical-reagent grade) and 0.1 *M* hydrochloric acid (for volumetric analysis) were purchased from Merck.

3. Results and discussion

3.1. Reproduction and confirmation of preceding results

As a first step, the HPLC and the CE method were tested for robustness. The HPLC assay is based on the German pharmacopoeia monography [8]. Different parameters were varied, such as the percentage of acetonitrile, the buffer pH and molarity, temperature, flow-rate and sample solvent. Selectivity depends strongly on the percentage of acetonitrile and on the column temperature, while other experimental parameters have a minor influence on it. These results confirmed and essentially completed the findings of Salem et al. [18] and led to the standard method described above. A typical chromatogram is depicted in Fig. 1. The separation is accomplished within 13 min. Insulin and its degradation product as well as the preservative *m*-cresol are well separated.

In CE, different buffer systems have been described to separate insulin from side components up to now [7,10,11]. The work of Mandrup [7] was most promising because good reproducibility was reported. As insulin is a large peptide, it tends to stick to the capillary wall. From a number of possible solutions, one is to add a zwitterion that is effective in reducing peptide–wall interactions. This zwitterion provides several benefits: It has no net charge, thus, it does not contribute to the buffer's conductivity. It not only associates with the negatively charged surface of the capillary, but also with the charged sites of the peptide. Hence, it allows for a moderate buffer pH that is near the physiological

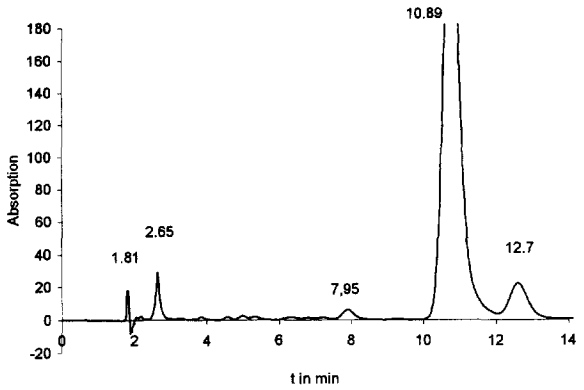

Fig. 1. Chromatogram of a human insulin solution (c=0.6 mg/ml), which was allowed to stand at room temperature for two weeks in order to form hydrolysis products. The main compound (t_R = 10.89 min) was separated from the major degradation product, A-21-desamido-insulin (t_R = 12.7 min), by the HPLC standard method. The peaks at 1.81 and 2.65 min are system peaks, the peak at t_R = 7.95 min corresponds to the preservative *m*-cresol. Column, Nucleosil RP18, 5 μm, 250×4 mm I.D.; mobile phase, 240 g of acetonitrile + 760 g of phosphate buffer (9800 mg of 85% H_3PO_4 + 28.4 g of Na_2SO_4 in 1000.0 ml of water); λ = 214 nm; column temperature, 40°C; flow-rate, 1 ml/min. For further details, see Section 2.

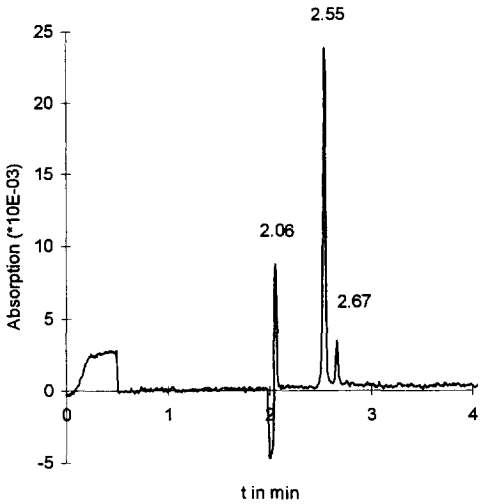

Fig. 2. Electropherogram of a human insulin solution (c=0.6 mg/ml), which was allowed to stand at room temperature for two weeks in order to form hydrolysis products. The main compound (t_M = 2.55 min) was separated from the major degradation product, A-21-desamido-insulin (t_M = 2.67 min), by the CE standard method. The preservative *m*-cresol, being a neutal species, migrates with the EOF (t_M = 2.06 min). Capillary, fused-silica, l = 30 cm (inlet to detector), 50 μm I.D.; 20 kV (≈53 μA), normal polarity (detector at cathode side); hydrodynamic injection, 0.5 p.s.i. (=34.5 hPa) for 4 s; λ = 214 nm; capillary temperature, 30°C; buffer (as described in [7]): 50 m*M* acetate, 850 m*M* CHES, 10% (w/w) acetonitrile, virtual pH = 7.8. For further details, see Section 2.2.

pH to maintain optimum stability of the peptide. Best results were reported with CHES: Other ampholytes, such as AMPSO [3-(1,1-dimethyl-2-hydroxyethyl-amino)-2-hydroxypropanesulfonic acid], CAPSO [3-(N-cyclohexylamino)-2-hydroxy-propanesulfonic acid] or betaine, were not satisfactory. The buffer pH and the percentage of acetonitrile are the dominating parameters and slight variations severely deteriorate the separation. The buffer composition could be used without changes, but the preparation procedure was improved with respect to the critical parameters mentioned above; the resulting method is referred to as the CE standard method in the following. A typical electropherogram is depicted in Fig. 2. Insulin and its degradation product are well separated in less than 3 min.

3.2. Comparison of validated capillary electrophoresis and liquid chromatography methods

The CE data that are compared here were recorded on the Beckman instrument; no internal standard was used.

The selectivity of both methods is sufficient because they manage to separate the major degradation product, A-21-desamido-insulin, from the main compound. The CE method is even more accurate because it separates B-3-desamido-insulin as well, which runs with the main peak in HPLC. However, this difference is of minor importance since significant amounts of B-3-desamido-insulin were barely detectable in any of the samples. Both assays are robust, with one limitation due to the use of acetonitrile: due to its volatility, the buffer has to be changed after each calibration series. However, the consumption of organic solvent is enormously different, that is, more than 100-fold less for CE. The separation efficiency is higher with the CE method, which leads to shorter analysis times.

Table 1 summarizes precision and validation data of insulin obtained with the CE and HPLC calibrations. The HPLC results are more reproducible. In the table, long-term reproducibilities are given, cor-

Table 1
Summary of precision and validation data for insulin, obtained with CE and HPLC calibrations

	CE	HPLC
Concentration range x (mg/ml)	0.024–0.240	0.0172–0.270
Signal range (arbitrary units)	0.13–2.60	72–2479
SD(y) Eq. (1)	0.116	120
Slope a_1 (ml/mg)	10.1	19 400
SD(x) (mg/ml); Eq. (2)	0.0115	0.0062
LOQ (mg/ml); Eq. (3)	0.115	0.062
R.S.D.(x)% x=0.038 mg/ml	30.2	16.3
n (calibration data)	50	30
LOD (mg/ml) [19]	0.02	0.0015

CE data obtained with Beckman instrument, see Section 2.

responding to 19/20 data measurements in a time period of 15/14 h for HPLC/CE, respectively. In the case of HPLC, the percentage relative standard deviation [R.S.D. (%)] of retention times was 0.3 and the R.S.D. of peak areas was 0.8%. The corresponding data for CE were 1.5% for migration times, which is worse than reported in other cases [7,10,11]. The reproducibility of corrected peak areas was R.S.D. (%)=1.6, better than described in [10,11] and similar to that described in [7]; better values are likely to be reached with higher UV-absorbing compounds rather than insulin and, in fact, have been obtained, see e.g. [19,20].

On recording data calibration sets for either technique, other interesting quantitative parameters have been investigated (see Table 1). In order to avoid severe systematic errors caused by heteroscedasticity, the concentration ranges have been restricted. The standard deviations did not increase by more than a factor of two within the working ranges. This increase is still statistically significant, but the systematic error using ordinary least squares regressions is small compared to the overall error and can be tolerated [21]. The linearity was excellent in both cases and the residues were distributed normally [17].

In an analogy to the standard deviation of repeated measurements, the standard deviation of a calibration line SD(y) is a measure for deviations from the estimated regression line. It is the square root of the variance, var(y), given by Eq. (1).

$$\mathrm{var}(y) = \frac{1}{n-2} \cdot \sum_{i=1}^{n} (y_i - \hat{y}(x_i))^2 \quad (1)$$

Here, the mean is substituted by the expected value $\hat{y}(x_i)$ obtained by the calibration function at position x_i. However, it is difficult to compare calibration sets by using SD(y), because the signal range can be very different. Thus, it is better to compare SD(x) (Eq. (2)), a measure for the error made when the concentration is estimated by signals using the calibration function [22,23].

$$\mathrm{SD}(x) = \frac{\mathrm{SD}(y)}{a_1} \quad (2)$$

The value SD(x) is always given in units of concentration and can easily be compared. The precision-dependent limit of quantitation (LOQ) is readily obtained from SD(x) (Eq. (3)).

$$\mathrm{LOQ} = \frac{\mathrm{SD}(x)}{\mathrm{R.S.D.}_{\max}\%} \cdot 100\% \quad (3)$$

The parameter $\mathrm{R.S.D.}_{\max}$ (%) is the maximal tolerated percentage standard deviation; this precision is strongly dependent on the particular analytical problem and was set to 10% in the present case. So far, for all quantitative parameters, HPLC was about a factor of two better than CE. The advantage of the latter is the short analysis time, which allows two analyses to be run for every one HPLC analysis.

Since the main issue of this study is the quantitation of insulin, the limit of detection (LOD) was estimated with just one instrument (Beckman; see Section 2 and Table 1). The LOD was defined as the sample concentration that allowed for an unambiguous detection of the corresponding peak signal in seven consecutive experiments [24]. In the light of new instrumental developments, this LOD of 20 mg/l should be considered as an upper limit.

3.3. CE with internal standard

The first CE experiments were carried out before 1995. Since then, instrumentation has been further improved, the detection system in particular. Peak interpretation as a major error source has been reduced using a laboratory-built CE integration program (K.I.S.S., [25]). Therefore, injection became the main error source. Thus, the use of an internal standard, which compensates for this error, should further improve precision.

Generally, an I.S. needs to be applied at a concentration (signal-to-noise ratio ≥ 30) that is sufficient to minimize integration errors [26] and it should have a similar chemical structure to that of the analyte. Due to difficulties in finding an appropriate peptide for this purpose, neostigmine bromide was established as a suitable I.S. Fig. 3 shows a typical electropherogram. As a quaternary ammonium salt, the I.S. migrates in front of the electroosmotic flow (EOF).

Table 2 depicts a comparison of precision and the limit of detection (LOD) for CE and HPLC for different analytical methods, including the present one. Special emphasis is laid on the comparison of precision in CE with and without the use of an I.S. For CE without an I.S., a value of 1.6% R.S.D. ($n=20$) is obtained for peak areas; this corresponds well to the findings of Mandrup [7], who reported a peak area reproducibility of 1.75% R.S.D. ($n=18$). Due to the fact that these results date from 1995 and earlier (Ref. [7] was published in 1992), it was an encouraging task to further improve this precision. Thus, a value of the order of 1% R.S.D. or better was desired, such as that found for other CE methods [15,16]. In fact, using an I.S., the reproducibility just increased to 1.3% R.S.D. (see Table 3). Hence, other factors that might also influence reproducibility have to be considered.

3.4. Influences on selectivity and reproducibility

In former investigations, it was found that the sample solvent strongly influences reproducibility. A physiological sodium chloride solution (0.9% w/v), which was used in the present study, proved to be

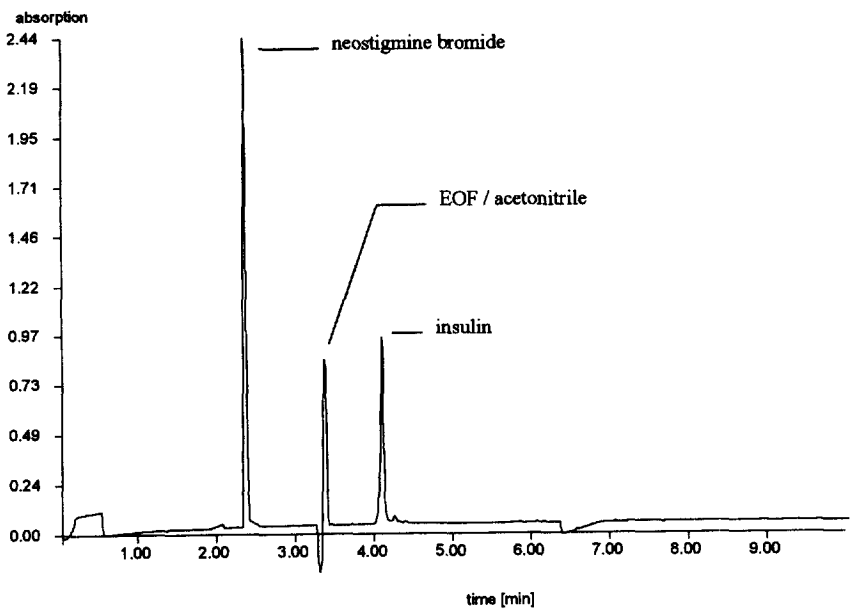

Fig. 3. Electropherogram of a porcine insulin solution ($c=0.724$ mg/ml). Capillary, fused-silica, $l=35$ cm (inlet to detector), 50 μm I.D.; 20 kV (≈32 μA), normal polarity (detector at cathode side); hydrodynamic injection, 0.75 p.s.i. (=51.115 kPa) for 4 s; $\lambda=214$ nm; capillary temperature, 30°C; buffer (as described by Mandrup [7]): 50 m*M* acetate, 850 m*M* CHES, 10% (w/w) acetonitrile, virtual pH=7.8. The amount of A-21 desamido-insulin is not as high as in the examples given above, because the sample was only stored for one day. For further details, see Section 3.3.

Table 2
Comparison of precision and limit of detection (LOD) for CE and HPLC using different analytical methods

Analyte	CE/ HPLC[a]	R.S.D. (%)[b]	λ (nm)	c (mg/l)	n	LOD (mg/ml)	λ (nm)	Reference
Carbohydrates		2.2/0.8	[c]	400/100	7/10	45/0.13	[2]	[1]
Felbamate[d]		3.5/3.2	214/205	50/55	20/10	4/3	214/205	[2]
Quats[e]		≈5/≈3	205/257[f]	0.08	8	≈0.004	205/257[5]	[3]
Anthraquinone-1-sulphonate		2[g]/0.5	254	100	33/15	0.7/0.006	254	[4]
Insulin	Area	1.6[h]/0.8	214	700/190	20/19[i]	20/1.5	214	This study
	Rel. area (CE with I.S.)	1.3	214	704	20			
	ditto[j]	0.5	214	750	60			

[a]First value refers to CE data, second value to HPLC data, respectively.
[b]Intra-day; sometimes mean of different values.
[c]Indirect UV detection in CE was compared to amperometric detection in HPLC.
[d]Anticonvulsant drug, measured in serum.
[e]Paraquat, diquat, difenzoquat, used as pesticides.
[f]310 nm in one case.
[g]CE: 0.5% using an internal standard.
[h]Corrected peak areas.
[i]Data correspond to long-term reproducibility: HPLC, $n=19$, $c_{Insulin}=0.19$ mg/ml, time period=15 h; CE, $n=20$, $c_{Insulin}=0.7$ mg/ml, time period=14 h.
[j]Additionally, buffer vials were covered with a layer of mineral oil.

Table 3
Analysis of insulin with CE using an internal standard

Name of series (instrument)	Number of runs (n) R.S.D. (%)	Remarks
(A) CE (I.S.) — insulin in basic sodium chloride solution (pH 8)		
Insulin1 (TSP)	$n=20$	
t_M	3.80%	
Relative area	1.26%	
Insulin2 (TSP)	$n=51$	Compare also Fig. 4A–C
t_M	0.85%	
Relative area	1.64%	
Insulin3 (TSP)	$n=10$	
t_M	0.20%	80 runs of the insulin standard method
Relative area	1.00%	had already been carried out on this capillary
Insulin4 (TSP)	$n=60$	
t_M	1.64%	Buffer vials were covered with mineral oil
Relative area	0.53%	to avoid evaporation of acetonitrile
(B) CE (I.S.) — insulin in acidic sodium chloride solution (pH 4)		
Insulin5 (ATI Unicam)	$n=16$	
t_M	2.48%	
Relative area	1.54%	
Insulin6 (TSP)	$n=10$	
t_M	2.32%	Last 10 runs of a series of 42
Relative area	2.14%	

much better than 0.01 m*M* hydrochloric acid [8] or a 150 mg/l EDTA solution [27]. Due to solubility problems of the pure substance at pH 4.5 to 7 [28], it is necessary to either increase or decrease the pH. In a part of the series, a few drops of a 0.1 *M* sodium hydroxide solution were added to the solvent (Table 3A, *insulin1* and *insulin2*). For the latter, an inter-day series with 51 runs, a R.S.D. of 1.6% was obtained for relative peak areas. Since insulin is reported to be more stable in acidic than in basic solution [28], solubilization was also done in the presence of a few drops of 0.1 *M* hydrochloric acid. Insulin preparations usually contain HCl (e.g. Insulin Hoechst), resulting in a pH of about 3 to 4. However, reproducibility worsened rather than improved; this was checked with two CE instruments (Table 3B). Good reproducibilities are obtained: 0.2% R.S.D. for migration times and 1.0% for relative peak areas ($n=10$) (see Table 3A, *insulin3*, recorded after 80 runs of insulin with the standard method).

The crucial parameters for robustness in CE are the buffer pH and the concentration of the additive, acetonitrile. Very small changes in the pH can influence migration behavior in CE; however, it is easy to prepare buffers with constant pH by using non-volatile reagents and buffer recipes [29].

The main reason for the above-mentioned restrictions in reproducibility (see Section 3.3) is obviously the use of acetonitrile. This organic modifier strongly influences the selectivity of insulin and related compounds and is essential for a good separation. However, acetonitrile is highly volatile. Thus, trends occur with both HPLC and CE and, for the latter, they also occur with acidic as well as with basic sample solvent (see Fig. 4). These trends are characterized by decreases in retention/migration times and peak areas; they are observed for the I.S., neostigmine bromide, as well. Diminishing migration times can be explained by a change in selectivity; an additional factor might be an increasing EOF. The trends in peak areas are probably caused by an increase in the viscosity of the buffer, due to the decreasing acetonitrile concentration. By applying the same injection pressure at each injection, a smaller sample volume is going to be injected into the capillary during a sample series, and all peak areas decrease. Since these trends occur both in acidic and in basic solution, possible degradation of insulin can be excluded as a reason. The evaporation effect can additionally be verified by a slightly increasing current during a series of runs (approx. 1.2–1.5 μA for a series where $n=50$).

Thus, evaporation of acetonitrile should be avoided. There are different means of so doing, depending on the respective instrumental features ([15,16], see also Section 2.2). Vial caps are often suggested for this purpose. However, depending on the instrument that was used in this study, the vial caps had either cross-shaped slits or were of a solid synthetic material. In the second case, a little hole had to be pierced into the cover, because reproducibility was found to be significantly worse when using new, non-pierced vial caps. This is most likely due to uncontrollable processes that occur when the capillary pierces the material.

Buffer cooling should also help to reduce evaporation. One of the instruments (SpectraPhoresis 1000, Thermo Separation Products) offers a passively cooled sample and buffer tray. However, the trends still remained, because the cooling capacity was found to be insufficient.

In principal, solvents other than acetonitrile could be used. These could be either isosteric compounds (like CH_3CF_3; [31]) or compounds with similar polarity and hydrogen bond donor–acceptor properties (e.g. aliphatic ketones and esters, dioxane, other nitriles or aniline; [32]). These compounds are only of theoretical interest, as most of them are also highly volatile. It was reported that acetonitrile improves efficiency and resolution very well, while methanol shows only minor effects [7]. This agrees with our own results.

Instead of using vial caps, evaporation could also be avoided by finding liquids that are lighter than the buffer and do not dissolve in any of the buffer constituents (as demonstrated in [30]). Mineral oil proves to be a suitable substance for this purpose. On using mineral oil, the trends that occur due to evaporation are no longer observed and the method is very stable. A reproducibility of 0.53% R.S.D. was obtained for relative peak areas ($n=60$).

By reducing the capillary length, the analysis times of about 15–16 min, as reported by Mandrup [7], were improved to about 3–4 min in the present study. Thus, analysis times are about a factor of four faster. This is an important argument for precision.

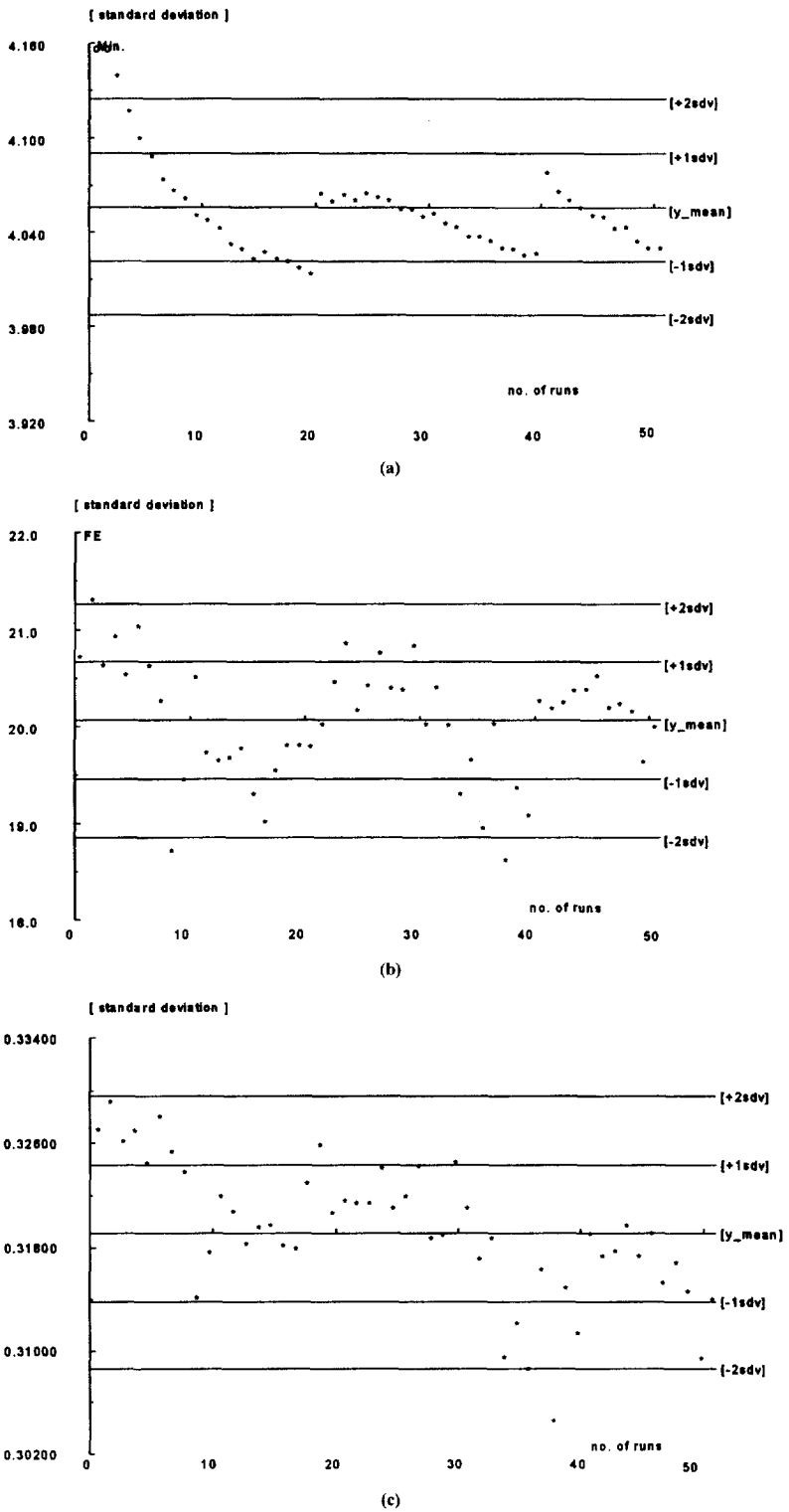

Fig. 4. Trends seen for insulin in basic solution (pH 8). [With an acidic sample solution (pH 4) the same trend phenomena are observed (see Section 3.4)]. (a) *Insulin2*, migration times vs. number of runs. Interruptions after the 20th and the 40th run are caused by changing the buffer vial. (b) *Insulin2*, absolute areas vs. number of runs. (c) *Insulin2*, relative areas vs. number of runs.

Meanwhile, about four analyses can be done in the same time as it took for one run in the work reported in [7]. The mean of these CE results can be treated as one measured value. The precision of a mean value is given as Eq. (4), where n stands for the number of repeated measurements.

$$SD(\bar{x}) = \frac{SD(x)}{\sqrt{n}} \quad (4)$$

This advantage of high speed is an additional parameter that influences reproducibility and can be utilized to obtain better precision (compare with Ref. [30]). Thus, all values, R.S.D.(y)%, SD(x), LOQ and R.S.D.(x)%, can be divided by a factor of $\sqrt{4}=2$. Considering this, HPLC reproducibility should be compared with the new value of 0.27% (with an I.S. and with the buffer covered with mineral oil).

4. Conclusions

A validated CE method for the quantitation of insulin has been presented. From the starting-point of 1.75% R.S.D. ($n=18$) for peak areas in CE, as reported by Mandrup [7], a reproducibility of 1.3% ($n=20$) was obtained using an internal standard. Considering the evaporation effects of acetonitrile, standards and samples should be compared in a limited time window; this leads to values of 0.2% R.S.D. for migration times and 1.0% for relative peak areas ($n=10$).

Precision can be improved radically by covering the buffer with a layer of mineral oil. The above-mentioned restrictions no longer remain and the system can be run for unlimited time periods, leading to a reproducibility of 0.53% R.S.D. for relative peak areas ($n=60$).

Due to the four-fold shorter migration times compared to those presented in Ref. [7], reproducibility can be calculated from the means, which leads to a further improvement by a factor of two, i.e. 0.27%. Thus, short analysis times can consequently be sacrificed to obtain better reproducibility. Extremely short migration times can be reached using special instrumentation [33,34], which was not commercially available until now. Reproducibility of peak areas in HPLC is 0.8% R.S.D. ($n=19$), with CE now being significantly better. The consumption of organic solvent in CE is about 100-fold less than in HPLC.

In summary, the precision of the CE assay is equivalent to, or even better than, that of the HPLC method. Since there is a HPLC pharmacopoeia method [8] similar to the method described here, HPLC will still be applied often for routine analysis, even if the CE method offers a higher sample throughput with the same precision. Both methods are complementary because of their different selectivities and, thus, are very suitable for cross-validation studies.

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