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Quantitation of insulin injection by high-performance liquid chromatography and high-performance capillary electrophoresis

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ABSTRACT

High-performance capillary electrophoresis (HPCE) was evaluated as a potential technique for the regulatory analysis of commercial dosage forms of insulin. A comparison was made to a liquid chromatographic analysis presently being proposed as an official monograph in the United States Pharmacopeia. The salient points of this comparison were accuracy, precision and ease of use. Both authentic (*i.e.* single blind, spiked) samples and commercial pharmaceutical formulations (injections) were examined.

Chromatographic analyses of both commercial formulations and authentic samples were characterized by good precision, with accuracy being supported by results from authentic (spiked) samples. Conventional HPCE (by which is meant a *non-micellar* electrolyte used with an *uncoated, unmodified* fused-silica capillary) achieved reasonable accuracy, but less than impressive precision, when applied to authentic samples. When used for commercial formulations, this type of HPCE did not produce a level of accuracy suitable for regulatory purposes, even with the use of an internal standard.

INTRODUCTION

High-performance chromatography (HPLC) is currently the most widely used technique for the quantitative analysis of pharmaceutical products in finished dosage form. Capillary electrophoresis, using a different separation mechanism, can also be applied to many pharmaceutical analyses now done by HPLC. With the advent of commercial high-performance capillary electrophoresis (HPCE) systems, a comparison of the two methods is appropriate.

There now exists a formidable body of literature devoted to HPCE. Since the publication of the seminal studies, first by Mikkers, *et al.* [1] and then by Jorgenson and Lukacs [2], well over 100 reports have appeared describing applications of capil-

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lary zone electrophoresis, capillary gel electrophoresis, and micellar electrokinetic capillary chromatography (MECC) [3].

However, the articles dealing specifically with quantitative analysis are limited. MECC has been used by Fujiwara and Honda to quantitate antipyretic/analgesic dosage forms [4]. In a previous paper these authors had employed free solution capillary electrophoresis (FSCE) to determine cinnamic acid analogues in canine plasma [5]. In that paper they mentioned the scarcity of reported applications of HPCE for quantitation and cited, as the only apparent example to that time, the measurement of nucleotide concentration in biological tissue by Tsuda *et al.* [6]. Recently Huang *et al.* [7] described the determination of low molecular weight carboxylic acids using FSCE.

All four of these reports relied upon the internal standard technique as a means of calibration and injection by siphoning for introduction of sample into the capillary. Except for Huang *et al.* [7], who employed conductivity detection, all the authors chose ultraviolet absorbance as a detection scheme.

Since insulin is a protein of great pharmaceutical and hence, regulatory significance, it was selected as an analyte upon which a comparison between HPLC and HPCE could be meaningfully based. HPCE separation conditions were established using buffer systems already described in the literature [8–11]. The HPLC procedure has been issued as an in-process revision in Pharmacopeial Forum and, according to the U.S. Pharmacopeial Convention (Rockville, MD, USA), has been collaboratively validated by insulin manufactures [12].

EXPERIMENTAL

Instrumentation

Capillary electrophoresis was performed using a Model M-1200 HPCE system (Microphoretic Systems, Sunnyvale, CA, USA) equipped with a polyimide-clad (exterior coating) fused-silica capillary approximaterly 65 cm in total length (60 cm effective length) with an internal diameter of 75 μm (Polymicro Technologies, Phoenix, AZ, USA). Schwartz *et al.* [13] have described this instrumentation and its performance characteristics in considerable detail. Electrokinetic injection was used exclusively, with typical parameters consisting of an application of 5 kV for 10 s. A running potential of 25 kV, generating roughly 70 μA of background current, was used for analytical determinations. Ultraviolet absorbance at 213 nm was employed for detection.

A modular HPLC system was assembled using, as components, a WISP 710B autosampler (Waters Chromatography Division/Millipore, Milford, MA, USA), a Waters/Millipore Model 6000A solvent delivery system (pump), a Model 7960 HPLC column heater (Jones Chromatography USA, Littleton, CO, USA), a Spectroflow 757 variable wavelength UV detector (Kratos Analytical Instruments, Ramsey, NJ, USA), and a Model SP4270 recording integrator (Spectra-Physics, San Jose, CA, USA). An Ultremex silica-based, 5-μm octadecylsilane column (Cat. No. 00G-00049-B0, Phenomenex, Rancho Palos Verdes, CA, USA) was used for HPLC separations.

Chemicals, reagents and solvents

Reference standards of human, pork, and beef insulin were obtained from the United States Pharmacopeial Convention. Samples of insulin injection were collected

for investigational purposes only from normal channels of commercial distribution. Morpholine and tricine were purchased from Chemical Dynamics Corporation (South Plainfield, NJ, USA). Reagent grade potassium chloride and sodium sulfate were obtained from Fisher Scientific (Fairlawn, NJ, USA). Acetonitrile (HPLC grade) was supplied by J. T. Baker, (Phillipsburg, NJ, USA). Dansyl-l-phenylalanine and dansyl-l-glutamine (internal standards) were available from Sigma (St. Louis, MO, USA). Distilled water was produced in our laboratory using a Corning MP 6A distillation system (Corning, NY, USA).

Procedure for electrophoresis

Prior to its initial use, a fused-silica capillary was prepared by successive washings with 0.1 *M* sodium hydroxide, distilled water, and finally the running electrolyte. Once prepared, the capillary was flushed with running electrolyte between every injection. The M-1200 unit could be programmed to perform these functions automatically. The running electrolyte's composition was 10 m*M* tricine–5.8 m*M* morpholine–20 m*M* potassium chloride with an observed pH of between 8.0 and 8.1. The reference standard was dissolved in this buffer and samples of insulin injection were also diluted with it. The final concentration of both standard and sample preparations was approximately 0.15 mg/ml, corresponding to a 1:25 dilution for samples having a label declaration of 100 units (of biological activity) per ml. Lot F of USP Reference Standard Insulin (Pork), for example, had a declared activity of 26.2 units/mg. Thus, a sample of insulin injection, manufactured using bulk drug of this activity and formulated at a dosage level of 100 units/ml, would be equivalent to 3.817 mg of insulin per ml. Since there is no absolute guarantee that the insulin in a sample will possess the same biological activity (on a per-unit-of-weight basis) as a particular lot of reference standard, the actual and determined concentrations of insulin were expressed in terms of mg/ml. For those instances in which an internal standard was used, an appropriate aliquot (*e.g.* 3.0 ml of a 0.25 mg/ml solution of the internal standard) was combined with the sample aliquot prior to final dilution (to 25.0 ml) with buffer.

Procedure for HPLC

As previously mentioned, the method utilized in our studies has been published as an in-process revision [12]. The assay portion of this proposed monograph revision employs isocratic reversed-phase HPLC (25-cm, silica-based octadecylsilane column, maintained at 40°C.) with detection at 214 nm. The actual composition of the mobile phase is acetonitrile–0.2 *M* sodium sulfate (26:74), with the sodium sulfate component having been adjusted to a pH of 2.3 beforehand. Standard and sample preparation remained essentially the same as for HPCE, again with working concentrations of about 0.15 mg/ml, and either the tricine buffer or 0.01 *M* hydrochloric acid as a diluent.

RESULTS AND DISCUSSION

HPCE optimization

Nielsen *et al.* [9] have described an optimized buffer system for the characterization of human insulin, growth hormone, their derivatives, and related proteins.

Upon initial evaluation, this system proved to be suitable for our intended purposes (see Experimental Section). Hence no real optimization studies had to be conducted. We found that we were able to omit the morpholine component and obtain essentially

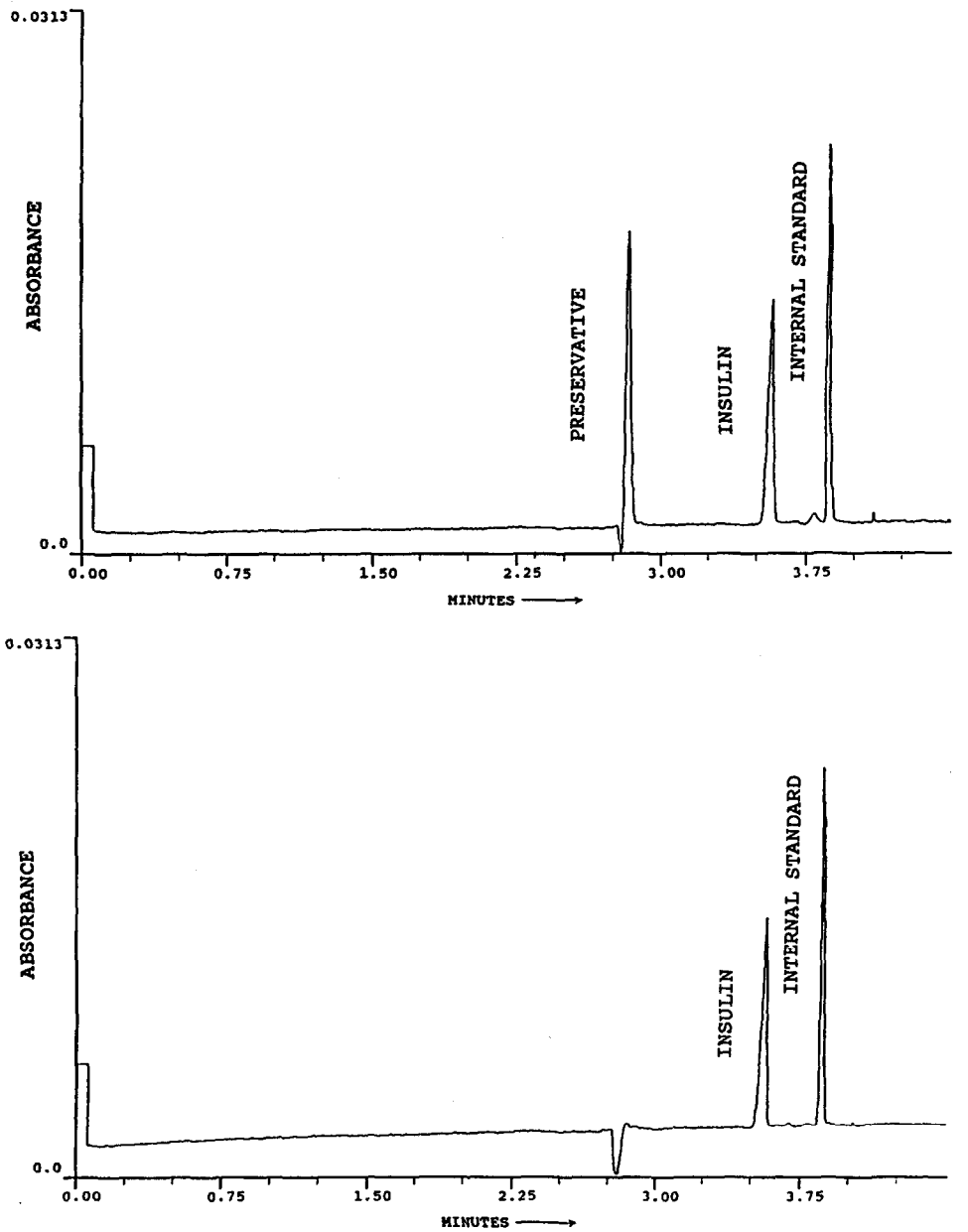

Fig. 1. Sample (upper) and standard (lower) electropherograms. Conditions are listed in Table I. Internal standard is dansyl-phenylalanine.

similar electropherograms of insulin. However, the addition of 0.1 *M* potassium hydroxide (to adjust the pH to 8.0–8.1) also produced elevated background current levels relative to the buffer containing morpholine. Little or no pH adjustment was necessary when the buffer was prepared using morpholine. Furthermore, since the lower background current required less heat to be dissipated from the capillary, we decided to include morpholine as a component. A typical set of electropherograms for a sample of insulin injection and the corresponding reference standard is presented in Fig. 1. In the case of the sample solution, the peak eluting at or near the electroosmotic flow front is attributable to phenol which is added to the formulation as a preservative.

Efficiency and replication of injection

For the purpose of our study, the choice between elecktrokinetic *vs.* vacuum injection centered on the two issues of efficiency and replication of injection. It simply proved to be the case that with our HPCE instrument superior peak shape and more reproducible peak response were observed for electrokinetic injection. As has been pointed out, analytes are introduced during electrokinetic injection with a bias based on differences in electrophoretic mobility [14]. Another point to keep in mind with this technique of sample introduction, is the fact that solutions being compared should be of similar composition and conductivity. Significant differences will result in different electric field strengths being generated by the sampling voltage, and hence different amounts of analyte being introduced into the capillary [15]. For this reason, samples were always diluted (a minimum of 25-fold) with running electrolyte prior to analysis. In Table I a representative series of electrokinetic injections for insulin standard and sample preparations are presented. It was our experience that, with a

TABLE I

REPRODUCIBILITY OF ELECTROKINETIC INJECTION FOR STANDARD AND SAMPLE PREPARATIONS OF INSULIN

65 cm × 75 μm I.D. fused-silica capillary, 5 kV/10 s injection, 25 kV running potential, 10 m*M* tricine–5.8 m*M* morpholine–20 m*M* potassium chloride buffer, 3 min purge with buffer between injections, detection at 213 nm, injection from 2 ml sample volume, *ca.* 0.15 mg/ml insulin.

	Migration time, t_m (min)		Peak response (area)	
	Standard	Sample	Standard	Sample
	3.08	3.06	16754	16741
	3.07	3.07	16023	16139
	3.04	3.06	16380	16733
	3.07	3.05	16146	16381
	3.05	3.07	16104	16121
	3.04	3.07	16537	16433
	3.05	3.07	16947	16859
	3.03	3.09	16952	16460
	3.05	3.06	16489	16844
Average	3.05	3.07	16481	16523
R.S.D. (%)	0.54	0.36	2.41	1.72

TABLE II

ANALYSIS OF AUTHENTIC (SINGLE-BLIND, SPIKED) SAMPLES OF INSULIN (PORK) REFERENCE STANDARD [a] BY HPCE

Sample	Insulin content (mg/ml)	Average calculated value ($n=3$)
1	2.510	2.458 (97.9%, R.S.D. = 12.4%)[b]
2	2.692	2.612 (97.0%, R.S.D. = 13.1%)
3	2.823	2.801 (99.2%, R.S.D. = 0.44%)

[a] Instrumental conditions as in Table I, except for injection from 200 μl volume.
[b] Figures in parentheses represent % of actual amount (mg) in spiked sample, and relative standard deviation of replicate analyses. Single-level, external standard calibration.

well equilibrated system (the apparatus does not feature a thermostat), migration times exhibited a relative standard deviation (R.S.D.) on the order of 1% or less and peak responses were reproducible within roughly 2% R.S.D. These figures represent injections made from vials containing roughly 2 ml of solution. The apparatus also permits sampling from a 96-site microtiter tray. The sample wells in this tray can accommodate a maximum of about 200 μl of liquid. Replicate injections from this volume of solution resulted in reproducibility on the order of 5% R.S.D. for peak response and 2% R.S.D. for migration time. A typical efficiency (number of theoretical plates, $N = 5.54\, t_R^2 W_{1/2}^{-2}$, where t_R is the migration time for the peak of interest and $W_{1/2}$ is the peak width at half-height) for the insulin peak (65 cm × 75 μm I.D. capillary, 25 kV) was at least 40 000.

Quantitation

Our initial results at quantitation of commercial dosage forms of insulin (*i.e.* injection) were disappointing. A total of seven samples, six with a label declaration of 100 units (*ca.* 3.8 mg) per ml and one with a 500 unit/ml declaration, were examined. Overall results ranged from a low of 85.8% of declaration to a high of 108.1%. Compendial limits for this product are 95.0–105.0% of label claim. Even more discouraging was the poor repeatability of results. Triplicate analysis of one particular injection (discrete sample preparations) yielded results of 85.8%, 91.4% and 108.1%. Single level external standard calibration had been used for these determinations. A study of authentic (single blind, spiked) solutions of insulin reference standard produced a much more plausible, if not readily explicable outcome. The results of this study are summarized in Table II. Analytical accuracy was good in all three cases, but in only one instance was accuracy accompanied by an acceptable level of precision. Since accurate HPCE results were obtained only with single blind, spiked solutions (samples in which no matrix effects whatsoever existed) the poor accuracy encountered with commercial samples may be attributable to minor differences between standard and sample preparations.

In an effort to improve precision, we next investigated the use of an internal standard. Our sample preparation did not require isolating insulin from a complex sample matrix, and merely involved diluting injections to a suitable level. For this reason we did not concern ourselves with identifying as an appropriate internal standard a compound with nearly identical physical properties (molecular weight, solu-

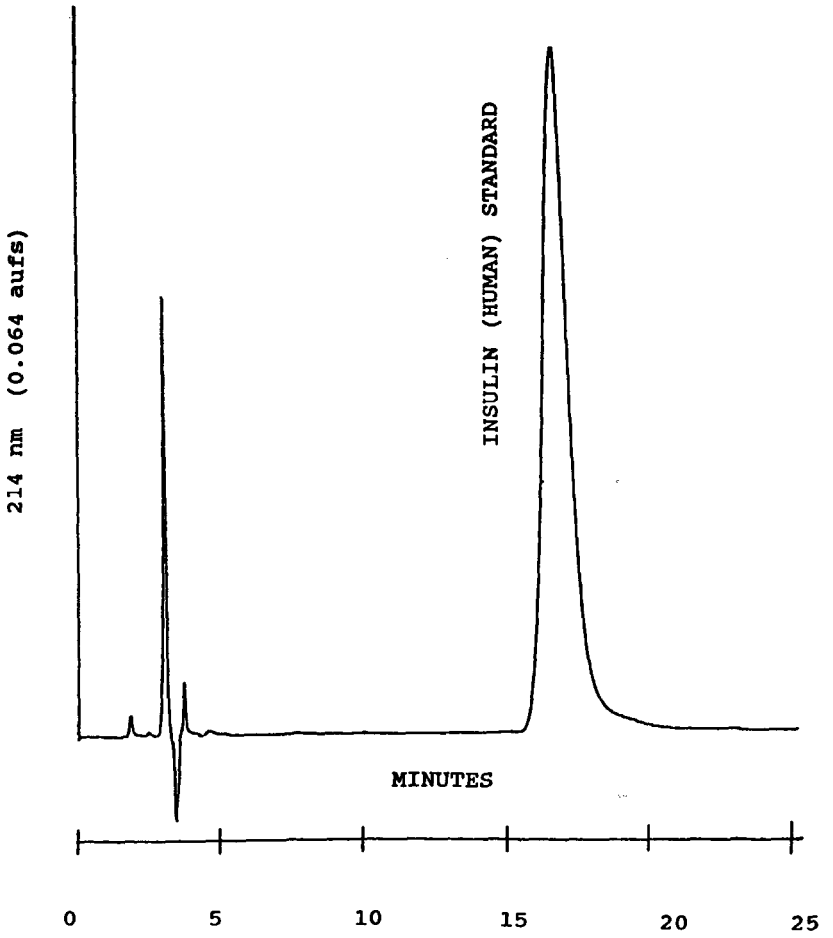

Fig. 2. Chromatogram of insulin (human) standard with UV detection (20 μl injection). Ultremex (Phenomenex) 5-μm C_{18} column, 25 cm × 0.46 cm I.D. Mobile phase: acetonitrile–0.2 *M* (pH 2.3) sodium sulfate (26:74). Flow-rate: 0.9 ml/min. Temperature: 40°C.

bility, isoelectric point) to those of insulin. We would be employing an internal standard for the express purpose of ascertaining whether its use resulted in improved analytical precision and accuracy. Two dansyl amino acids, dansyl-glutamine and dansyl-phenylalanine, were found to exhibit appropriate migration times relative to insulin and were commercially available in sufficiently pure form. Five of the pharmaceutical formulations were subjected to replicate analysis using the internal standard technique.

These samples were then reanalyzed by the previously described HPLC procedure in order to obtain comparative data. A typical chromatogram of insulin is presented in Fig. 2. Triplicate determinations were performed with single level external standard calibration being the only modification to the method. A statistical analysis of these results (omitting sample 399, the data for which were generated by

TABLE III

ANALYSIS OF INSULIN INJECTION BY HPCE VIA INTERNAL STANDARD TECHNIQUE

Sample	Species	Label claim (units/ml)	Amount found		R.S.D.[b] (%)
			mg/ml	units/ml[a]	
394	Human	100	3.359	89.01	2.51
395	Human	100	3.447	91.35	0.50
397	Pork	100	3.518	92.17	1.98
398	Pork	100	3.424	89.71	0.64
399	Human	100	3.678	97.47	1.33
399[c]	Human	100	3.572	94.66	0.37

[a] Based on assumed activities of 26.2 and 26.5 units per mg of pork and human insulin, respectively.
[b] Relative standard deviation, $n = 3$ (minimum).
[c] This series of analyses is based upon samplings from microtiter tray (200 μl volume). Otherwise, instrumental parameters as in Table I. Dansyl-*l*-phenylalanine internal standard.

TABLE IV

COMPARISON OF DETERMINATIONS OF INSULIN INJECTION (100 UNITS/ml) BY HPCE AND HPLC

Sample	Species	Amount found		mg/ml and [units/ml][a]			
		HPLC			HPCE		
394	Human	3.574	(0.91)	[94.71]	3.359	(2.51)	[89.01]
395	Human	3.719	(0.24)	[98.55]	3.447	(0.50)	[91.35]
397	Pork	3.871	(0.72)	[101.4]	3.518	(1.98)	[92.17]
398	Pork	3.662	(0.73)	[95.94]	3.424	(0.64)	[89.71]
399	Human	3.560	(2.99)	[94.34]	3.678	(1.33)	[97.47]
399	Human[b]				3.572	(0.37)	[94.66]

[a] Single level external standard calibration for HPLC data. Figures in parentheses represent relative standard deviation ($n = 3$). Based on assumed activities of 26.2 and 26.5 units per mg of pork and human insulin, respectively.
[b] This series of analyses is based upon samplings from microtiter tray (200 μl nominal volume). Otherwise, instrumental parameters are as in Table I. Dansyl-l-phenyl-alanine used as internal standard for HPCE.

TABLE V

COMPARATIVE ANALYSES OF AUTHENTIC, SINGLE-BLIND SPIKED SAMPLES[a]

Sample	Insulin content	Amount found, mg/ml, (R.S.D.), [% of actual content]					
		HPLC			HPCE		
1	2.07	2.077	(0.71)	[100.3]	2.019	(1.94)	[97.5]
2	1.19	1.172	(0.53)	[98.5]	1.219	(5.13)	[102.4]
3	4.25	4.309	(0.53)	[101.4]	4.160	(3.10)	[97.9]

[a] Single-level external standard calibrations for HPLC and HPCE. Relative standard deviation values based on 4 determinations per sample. HPCE conditions as in Table I. Refer to text for details of HPLC procedure.

sampling from two different size reservoirs) was carried out [16]. Critical values for t (Student's t-test) were exceeded (with at least a 95% confidence level) in each instance, indicating a statistically significant difference in results obtained by the two approaches, HPLC and HPCE. Tables III and IV summarize these findings.

Our final study involved a repetition of the comparison between HPLC and HPCE, but this time in a single blind format using authentic samples. The samples were prepared independently, using USP reference standard insulin (human) as the analyte and tricine buffer as the diluent. In generating the HPCE data we chose not to repeat the use of an internal standard, as it had been of no obvious advantage in improving accuracy. As Table V indicates, we were able to achieve reasonable accuracy using HPCE, although HPLC exhibited a clear advantage in terms of both accuracy and precision. Statistical evaluation revealed however that critical t-values for the two sets of data were not exceeded (95% confidence level) for any of the three samples. From a statistical standpoint then, the data are equivalent. It is worthy of note however, that relative standard deviation and variance are directly related. Since variance figures prominently in the calculation of t-values, it is in fact the relative imprecision of the data obtained by HPCE that results in a finding of statistical equivalence between the two sets of analyses.

CONCLUSIONS

Our experience —based upon the results in Tables II and V— with the analysis of single-blind spiked samples has shown that with identical standard and sample matrices, single level external standard calibration can provide reasonable accuracy (97.0% to 102.4%) at a sacrifice in precision (R.S.D. as high as 13.1%). The addition of an internal standard —refer to Tables III and IV— does impart added precision (R.S.D. of 2.51% or less) to HPCE with electrokinetic injection. At least in the case of actual sample determinations however, where even 25-fold dilution with running electrolyte leaves some disparity between standard and sample matrices, the internal standard technique did not achieve high accuracy.

It is our intention to pursue and more fully understand the issues of accuracy and precision in the FSCE of proteins. Modifications of the capillary surface (to overcome or minimize the problem of adsorption–desorption) are one possible approach to this problem. Such modifications could be either dynamic (achieved with additives, *e.g.* surfactants, to the electrolyte) or permanent (involving actual chemical transformations in order to produce a deactivated surface). It may prove necessary to use MECC rather than FSCE to resolve insulin types of different mammalian origin. In this report however, we have chosen not to focus on these matters. If indeed these issues are ever fully resolved, they clearly would merit publication in their own right. Our purpose in this study was first to examine the question of quantitation of proteins using FSCE in its simplest form.

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This is contribution N. 477 from the Barnett Institute at Northeastern University.

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