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On-line capillary isoelectric focusing–mass spectrometry for quantitative analysis of peptides and proteins

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Abstract

On-line capillary isoelectric focusing–mass spectrometry (cIEF–MS) was applied to determine concentrations of peptides and proteins using angiotensin II and human tetrasialo-transferrin as the model samples. The concentration of the carrier ampholyte was optimized for both resolution and ion intensity. cIEF–MS employing 1% Pharmalyte 3–10 and a sheath liquid containing water/methanol/acetic acid (50/49/1) resolved angiotensin I and II (5 μM each, $\Delta pI = 0.2$) at an Rs value of 2.29. The determined concentration of angiotensin II (0.1–5 μM) well correlated ($R = 0.999$) with that obtained by the conventional RP–HPLC method. The limit of detection was 0.22 μM, which was about 10 times lower than that by UV detection (2 μM). The repeatability and accuracy were <15 and <11%, respectively. cIEF–MS was also applied to determine human tetrasialo-transferrin concentration. The good linearity ($R^2 = 0.998$) was also observed between the transferrin concentration (0.5–1.2 g/L) and peak area ratio (IS; β-lactoglobulin B) with acceptable accuracy (<1.9%) and repeatability (∼10% at 1 g/L).

Keywords: Capillary isoelectric focusing; Mass spectrometry; Quantitative analysis; Angiotensin; Transferrin

1. Introduction

Capillary electrophoresis (CE) is one of the best methods for separation of ionic (and even non-ionic) compounds including proteins and peptides. Of several different CE modes, capillary isoelectric focusing (cIEF) is advantageous for the separation of zwitterionic compounds such as peptides and proteins. The separation is based on the focusing of analytes at locations of inherent isoelectric points within a pH gradient across the capillary. Therefore, the samples are highly concentrated into narrow zones [1], which is also advantageous for low amount samples such as biomolecules. To date, there have been several reports on the development and application of cIEF for bioanalysis of protein isoforms [2,3], quality control of monoclonal antibodies [4,5] and development of peptidic standards for accurate determination of isoelectric points [6]. Most cIEF applications do not aim to obtain quantitative information, even though the detection method employed is UV adsorption, which shows concentration-dependent responses. This was largely because the accurate quantification was disturbed by considerable noise due to co-existing carrier ampholytes. Shen et al. reported that 280 nm is the only detection-wavelength applicable for practical cIEF using Pharmalyte 3–10 [1], which is one of the carrier ampholytes most widely used. This suggests that accurate and sensitive quantification is not easy for analytes lacking a unique chromophore. Therefore, the application of cIEF with UV detection for quantitative

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analysis was limited to abundant and highly soluble proteins such as immunoglobulin [4,5]. A possible approach to overcome this problem is to use a mass spectrometric (MS) detector, because the migration pattern of the analyte can be selectively extracted from the total ion current containing the background carrier ampholyte. To date, cIEF–MS has been also applied to protein analyses by virtue of its high sensitivity and protein identification ability. For instance, the proteomic studies of cell lysates [7,8] and direct analysis of human cerebrospinal fluid [9] have been carried out. In addition, protein phosphorylation has also been studied [10]. However, to our knowledge, there are no reports dealing with the determination of protein concentrations by cIEF–MS with system validation parameters reliable enough for measurements such as linearity, range, precision and accuracy. In this study, to demonstrate the performance of cIEF–MS for quantitative analysis of peptides and proteins, model peptides (angiotensin I and II) and a protein (transferrin) were subjected to cIEF–MS, and resolution, LOD, linearity, accuracy and precision were evaluated. Also, compatibility with conventional HPLC quantification was demonstrated.

2. Experimental

2.1. Materials

Human angiotensin I (A9650), angiotensin II (A9525), [Lys^8]-vasopressin (V6879), β-lactoglobulin B (L8005, from bovine milk), carbonic anhydrase II (C2522, from bovine erythrocyte), human apo transferirn (T1147, Tf) and human myoglobin (M1882, from equine heart) were purchased from Sigma (St. Louis, MO). Their p*I* values, molecular masses and amino acid sequences [11], are listed in Table 1. Bovine serum albumin (01863, BSA) was obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Preparation of iron-saturated tetrasialo-transferrin

Transferrin can bind two iron ions (Fe^{3+}), and the isoelectric point of transferrin decreases by ∼0.2 pH unit by the respective binding [12]. Therefore, in order to cancel the charge distribution caused by the iron load, saturation with ferric ion is necessary for cIEF analysis of transferrin. Ferric citrate (500 μM) was added to human apo transferrin (2 g/L) and left for 15 min to saturate its iron-binding site [13]. Then, the iron-saturated tetrasialo-transferrin was subjected to anion exchange chromatography according to a previous report using a Mono-Q HR 5/5 column (Amersham, Uppsala, Sweden) [14]. The fraction of tetrasialo-transfferin was collected and purified by dialysis and lyophilization. The iron saturation of the tetrasialo-transfferin was confirmed by the UV spectrometric method [15], revealing that the ferric ion shared ∼96% of the total capacity.

Table 1
Isoelectric points of model peptides and proteins

Peptide/protein	p*I*	Molecular mass	Sequence
Angiotensin I	6.9	1295.67[a]	DRVYIHPFHL
Angiotensin II	6.7	1045.53[a]	DRVYIHPF
Lysine-vasopressin	8.1	909.35[a]	CYQNCPKG[b]
Albumin (BSA)	4.9	66,500[c]	
Carbonic anhydrase	5.9	29,000[c]	
β-Lactoglobulin B	5.1	18,400[c]	
Myoglobin	7.2	16,900[c]	
Tetrasialo-transferrin	5.4	80,000[c]	

[a] Monoisotopic mass.
[b] With an intramolecular disulfide bond.
[c] Apparent mass.

2.3. cIEF–MS measurement

cIEF was carried out in the two-step mode using an EOF-suppressed fused silica capillary. A laboratory-built CE system was assembled with the capillary (length 50 cm, i.d. 75 μm) coated with linear polyacrylamide [16] and a high voltage power supply (HCE-30P, Matsusada Electronics, Kusatsu, Japan). The mass spectrometer employed was Mariner-E (Applied Biosystems, Foster City, CA). The outlet end of the capillary was put through a stainless steel tubing (22 gauge, 7 cm long), and was positioned ∼0.5 mm outside the outlet of the metal tubing. The coaxial sheath liquid was administered at a flow rate of 2 μL/min through the metal tubing without any auxiliary gas. The sample solution, containing sample peptides and the carrier ampholyte, was injected into the whole length of the capillary using a microsyringe. A focusing voltage (+20 kV) was then applied at the anolyte (1% acetic acid, pH 2.7), while the catholyte (0.28% ammonium hydroxide, pH 11.2) was continuously delivered at a flow rate of 2 μL/min through electrically grounded coaxial stainless steel tubing. Once the sample was focused (in 10 min), the catholyte was substituted by the sheath liquid (2 μL/min). The ESI voltage was between +3.3 and 3.5 kV while the voltage for chemical mobilization (+20 kV) was applied at the inlet of the capillary. At the electrospray interface, the flow rate of the curtain nitrogen gas to assist solvent vaporization was 0.6 L/min. The nozzle and quadrupole temperatures were fixed at 140 and 100 °C, respectively. cIEF analysis with UV detection was done by a CAPI-3000 CE system (Otsuka Electronics, Hirakata, Japan).

2.4. Determination of angiotensin II concentration by HPLC

To investigate the equivalence of the determined concentration between the cIEF–MS and conventional methods, the concentration of angiotensin II in the cIEF sample was also determined by a RP–HPLC method under the following conditions. Column; Inertsil-ODS-2 (15 cm × 4.6 mm, GL Sciences, Tokyo, Japan), pump; 880-PU (Jasco, Tokyo, Japan), UV detector; UV 1570 (Jasco), mobile phase; 10 mM sodium phosphate buffer (pH 7.5) containing 20% acetonitrile, flow

rate; 1 mL/min, column temperature; 40 °C, sample volume; 20 μL, detection wavelength; 200 nm.

3. Results and discussion

3.1. cIEF–MS for quantitative analysis of peptides

cIEF is known as a very sensitive technique, because the analytes initially dispersed in the capillary are focused into narrow zones. It was reported that a ~500 times concentration can be attained under typical conditions [1]. Therefore, cIEF seems suitable for analysis of amphoteric biomolecules present at low concentrations. Although an MS detector has excellent selectivity itself, determination of absolute concentration in the cIEF–MS format has not yet been reported, whereas determination of antibody concentration by cIEF–UV [4,5] and determination of expression ratio of isotopically depleted proteins using cIEF–MS [17] have been achieved. Improvements in cIEF in quantitative analysis, especially in determination of absolute concentration, will produce new demands for quality control of gene technology products and individual characterization of microheterogenetic proteins.

Prior to cIEF–MS analysis, optimization of the carrier ampholyte concentration was carried out with respect to ion intensity and separation efficiency using the infusion–MS method. In cIEF–MS, the use of cellulose derivatives (e.g. hydroxypropyl methylcellulose and methyl cellulose) is disadvantageous because of ionization disturbance and contamination in the mass analyzer. Cellulose derivatives of high viscosity, which are used as dynamic coating agent for cIEF, are known to improve resolution by decreasing sample diffusion [18]. Therefore, the omission of the derivatives for MS detection can increase the optimum concentration of the carrier ampholyte. On the other hand, a high concentration of the carrier ampholyte (containing various zwitterionic compounds) can suppress sample ionization leading to an increase in the LOD. Thus, the optimization of carrier ampholyte concentration is essential for cIEF–MS. A sample solution containing angiotensin I, II, [Lys^8]-vasopressin (30 μM each) dissolved in 0.5–4% Pharmalyte 3–10 solution (200× to 25× dilution of the commercial product) was delivered into the mass spectrometer at 0.5 μL/min, and the mass spectra were integrated for 120 s. As shown in Fig. 1A, the intensity of the trivalent angiotensin I signal was strongly decreased at Pharmalyte concentrations higher than 1%. Angiotensin I and II (100 μM each) were then subjected to cIEF UV analysis in order to study how separation efficiency depends on Pharmalyte concentration. In the electropherograms detected by UV absorption at 280 nm (Fig. 1B), the resolution was calculated as follows:

$$\mathrm{Rs} = 1.18(T_2 - T_1)/(w_{\mathrm{h}1} + w_{\mathrm{h}2})$$

where T_1, T_2, $w_{\mathrm{h}1}$ and $w_{\mathrm{h}2}$ represent the migration times and the peak widths at the half height for angiotensin I and II, respectively. The resolution increased with Pharmalyte concentration as shown in Fig. 1A. Pharmalyte solution at high concentrations gave good resolution, but caused low ionization efficiency. In the following study, the Pharmalyte concentration was fixed at 1 or 2.5% compromising between signal intensity and separation.

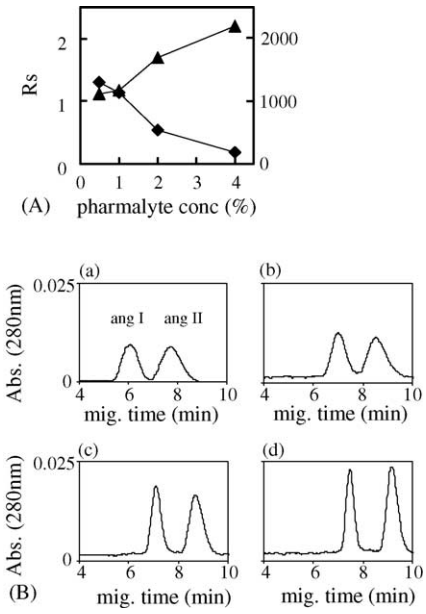

Fig. 1. Optimization of the carrier ampholyte concentration. (A) Effect of concentration of the carrier ampholyte on ionization efficiency (♦) and cIEF resolution (Rs, ▲). Ionization efficiency is indicated as ion intensity of angiotensin I (+3). The Rs value was determined using peaks corresponding to angiotensin I and II in UV electropherograms. (B) cIEF electropherograms of angiotensin I and II. Pharmalyte concentration was 0.5% (a), 1% (b), 2% (c) and 4% (d). The concentration of each peptide was 30 μM. capillary; total length 50 cm, separation length 40 cm, i.d. 75 μm, temperature 25 °C.

Angiotensin I, II and [Lys^8]-asopressin were then subjected to cIEF–MS analysis. Fig. 2 shows the reconstructed ion electropherograms (RIEs) corresponding to the respective peptides. [Lys^8]-vasopressin migrated first, followed by angiotensin I and II, which was in good accordance with the order of their pI values. These peptides were completely separated from each other, and the resolution (Rs) between angiotensin I and II was estimated to be 2.29, which was significantly better than that observed in cIEF with UV detection (Fig. 1B). This was probably due to the smaller sample amount than that in the cIEF UV analysis. At this resolution, almost baseline separation was obtained for peptides with ΔpI = 0.1, even if the m/z values were identical. In this study, no cellulose derivatives were used to prevent contamination in the mass analyzer. Nevertheless, peptides with a small pI difference (0.2) were well separated under these conditions. The relationship between migration time and pI value was also studied using myoglobin and bovine serum albumin in addition to the three peptides. A good correlation ($R = -0.996$) was obtained between pH 8.1 and 4.9, as shown in Fig. 3.

Increasing concentrations of angiotensin II were applied to the system to investigate the effect on the performance

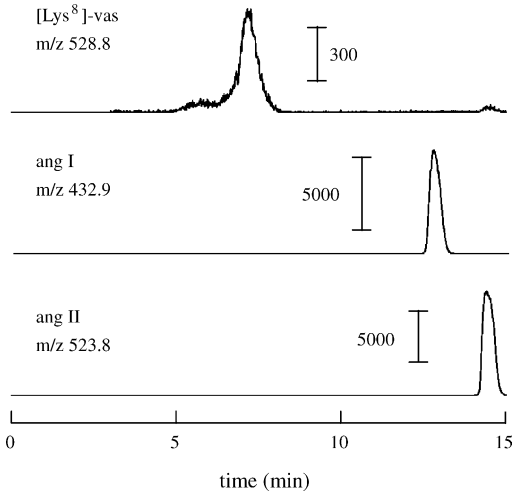

Fig. 2. Reconstructed ion electropherograms of [Lys^8]-vasopressin (30 μM, *m*/*z* 528.8), angiotensin I and II (5 μM each, *m*/*z* 432.9 and 523.8, respectively) mixed solution obtained by cIEF–MS analysis. Linear polyacrylamide coated capillary (50 cm), Pharmalyte 3–10 (1%), sheath liquid; 0.1 M acetic acid containing 3% methanol (2 μL/min), focusing; +20 kV (15 min), mobilization; +20 kV, ESI voltage; +3.5 kV.

for quantitative analysis. The anolyte reservoir was raised up 4 cm higher than the orifice of the mass spectrometer during mobilization to generate a gravity driven flow for ESI stabilization [19]. The resolution was not deteriorated (Rs ∼2) by the laminar flow because of Pharmalyte addition (2.5%, 40× dilution). Table 2 lists the calibration data of angiotensin II concentration and peak area ratio (IS, angiotensin I). Good linearity (R^2 = 0.999) was obtained within 0.1–5 μM. Other parameters of the system validation were as follows: LOD (intercept + 3$S_{y/x}$), 0.22 μM (∼4.9 pmol); LOQ (intercept + 10$S_{y/x}$), 0.75 μM; precision (CV%), <15%; accuracy (relative error) at 0.5 and 3 μM, +11 and +0.9%, respectively. For comparison, LOD (S/N = 3) obtained in UV detection was roughly estimated as 2 μM. The correlation between concentrations determined by cIEF–MS and RP–HPLC was also studied (Fig. 4). Not only a good correlation (R = 0.999), but also the regression line passing though the origin at 95% confidence was observed. These results suggest that cIEF–MS is compatible with the HPLC method, and is applicable to quantitative analysis of peptides at lower concentration ranges than those previously studied by cIEF with UV detection.

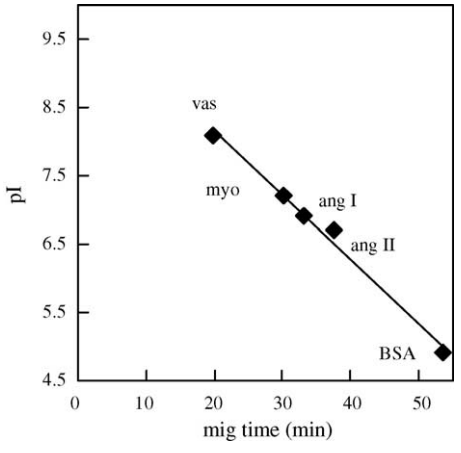

Fig. 3. Correlation between the migration times of the samples and their isoelectric points. [Lys^8]-vasopressin (vas, p*I* = 8.1), myoglobin (myo, 7.2), angiotensin I and II (ang, 6.9 and 6.7, respectively) and bovine serum albumin (BSA, 4.9). Slope, −0.0984; intercept, 10.1; R^2, −0.996.

Table 2
Angiotensin II concentrations and peak area ratios determined in cIEF–MS analysis

Angiotensin II concentration (μM)	Peak area ratio[a]	
	Mean	CV (%)
0.1	0.547	11.2
0.5	1.26	12.9
1.0	2.45	13.4
3.0	5.96	4.69
5.0	9.50	8.06

[a] n = 4, angiotensin I (0.5 μM) as internal standard (slope, 1.82 ± 0.033; intercept, 0.444 ± 0.136; R^2, 0.998).

3.2. Application of cIEF–MS to quantitative analysis of protein

Finally, the cIEF–MS system was applied to protein quantification. Human transferrin was used as a model protein. The protein shows microheterogeneity in the degree of sialylation ranging from 0 to 8 sialyl residues [20]. Transferrin possessing two or fewer sialyl residues is referred to as carbohydrate deficient transferrin (CDT). The plasma level of CDT increases under pathological states such as chronic alcohol abuse or carbohydrate-deficient syndrome. Therefore, CDT is used for detection and follow-up of chronic alcohol abuse [21]. The quantification of CDT is clinically carried out using ion-exchange chromatography on a small disposable microcolumn followed by an immunological assay. Although several kinds of diagnostic kits for this two-step immunoassay are commercially available, the

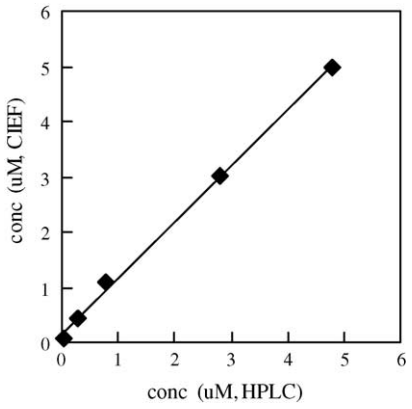

Fig. 4. Correlation between concentrations determined by cIEF–MS and RP–HPLC. Slope, 1.01 ± 0.044; intercept, 0.166 ± 0.247; R^2, 0.999.

selectivity of CDT differs between kits, and the reference value has not been standardized [22]. A possible approach to overcome this problem is to use a separation system of high resolution enough to distinguish CDT from non-CDT. The IEF in gel format is widely used for laboratory analysis [23] due to its reliable resolution. The isoelectric point of transferrin decreases by ∼0.1 pH unit in the presence of a single sialic acid residue [12]. Although gel-IEF can resolve these Tf isoforms, reliable quantitative measurement is not easy by gel visualization. The capillary-based separation methods including CZE [13], cIEF [24] and cIEF–MS (for bovine transferrin analysis) [25] have also been demonstrated successfully, although there are few CE applications. In this study, human transferrin was applied to cIEF–MS for absolute quantification. To reduce complexity in the electropherogram, the most abundant sialoform, tetrasialo-transferrin, was isolated by anion-exchange chromatography, and subjected to cIEF measurement. The composition of the sheath liquid was optimized, and the best separation and strongest ion intensity were obtained at a composition of water/acetonitrile/acetic acid = 49/49/2 (data not shown). Fig. 5 shows the electropherograms of tetrasialo-transferrin and p*I* marker proteins (carbonic anhydrase II (p*I* 5.9) and β-lactoglobulin B (p*I* 5.1)) in addition to a typical mass spectrum of tetrasialo-transferrin. In the RIE of Tf, a small peak was also detected at 9 min, which would correspond to a Tf isoform of high p*I* value, while its molecular mass was similar to that of tetrasialo-Tf. There are three possible reasons for the high p*I* value: desialylation, iron loss and lowly sialylated Tf. However, there is no report suggesting desialylation of Tf during isoelectric focusing. Indeed, slight release of ferric ion with a long analysis time in gel-IEF has been reported previously [23], but this is not likely with a very short analysis time in cIEF analysis as compared to gel-IEF. Taking this into account, the small peak at 9 min would be lowly sialylated Tf, which was co-eluted with tetrasialo-Tf from the anion-exchange column due to its low resolution. In Fig. 5, the migration order of the three proteins (carbonic anhydrase, tetrasialo-Tf, β-lactoglobulin B) was in good accordance with the p*I* values; the estimated p*I* value of tetrasialo-Tf was 5.6 (±1%, $n = 6$). The charge number ranged from +26 to +41, while the most abundant was +33. However, the charge distribution envelope varied slightly between runs; for instance, +33 and +32 sometimes indicated almost equivalent intensities. To improve precision in quantitative analysis the areas of RIEs of nine major charge states were combined [26]. A calibration line between transferrin concentration and peak area ratio (IS, β-lactoglobulin B, summed area of six major charge states) showed good linearity ($R^2 = 0.998$) in a range of 0.5–1.2 g/L, which roughly corresponds to plasma CDT levels under pathological conditions [27,28]. The LOD (intercept + $3S_{y/x}$) and LOQ (intercept + $10S_{y/x}$) were evaluated as 0.05 and 0.17 g/L, respectively. Precision and accuracy as well as determined concentrations are listed in Table 3. The precision was almost equivalent to that of the IEF and immunoaffinity methods [28,29] in the high concentration range (10–20%; 1–1.2 g/L, $n = 6$), though it was large within the low concentration range (∼30%; 0.5–0.8 g/L). The accuracy (relative error to the calibration line) was less than 2%. Although a further improvement in repeatability is desired for the wide spread application of this system, the present results suggest that cIEF–MS can be also applied to quantitative analysis of proteins at physiological concentrations.

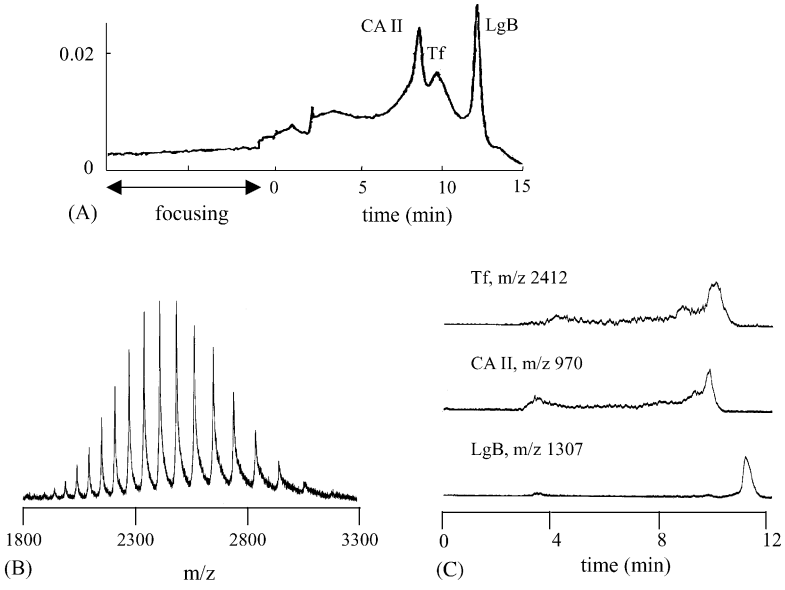

Fig. 5. Electropherograms and mass spectrum of human tetrasialo-transferrin. (A) UV electropherogram (280 nm) obtained by a stand-alone CE system without gravity flow. Tetrasialo-transferrin (Tf, 0.2 g/L), carbonic anhydrase (CA II, 0.1 g/L) and β-lactoglobulin B (LgB, 0.2 g/L) were injected together with an equivalent mixture of Pharmalyte 5–6 and 5–8 (1%). $L = 50$ cm, $l = 40$ cm, 25 °C. The catholyte was provided in a reservoir. (B) Mass spectrum of tetrasialo-transferrin. (C) Reconstructed ion electropherograms of tetrasialo-transferrin, carbonic anhydrase II and β-lactoglobulin B obtained by cIEF–MS.

Table 3
Tetrasialo-transferrin concentrations and accuracies determined in cIEF–MS analysis

Theoretical concentration (g/L)	Determined concentration[a] (g/L, ±CV (%))	Accuracy (%)[b]
0.5	0.496 (±33.7)	−0.8
0.8	0.798 (±35.4)	−0.3
1.0	1.02 (±12.8)	+1.9
1.2	1.19 (±22.6)	−1.1

[a] Determined by the regression line (slope, 0.177 ± 0.006; intercept, -0.013 ± 0.005; R^2, 0.998).
[b] Relative error to the regression line.

4. Conclusion

Applicability of on-line cIEF–MS for absolute quantification of angiotensin II and human tetrasialo-transferrin was demonstrated. In angiotensin II analysis, the LOD was 0.22 μM, which was about 10 times lower than conventional UV detection. The determined concentrations correlated well with those measured by conventional HPLC. Human transferrin (at physiological concentrations) could be quantified by cIEF–MS with good accuracy and acceptable repeatability.

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