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Capillary electrophoresis with laser-induced native fluorescence detection for profiling body fluids

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Abstract

Laser-induced native fluorescence detection with a KrF excimer laser (λ=248 nm) was used to investigate the capillary electrophoretic (CE) profiles of human urine, saliva and serum without the need for sample derivatization. All separations were carried out in sodium phosphate and/or sodium tetraborate buffers at alkaline pH in a 50-μm I.D. capillary. Sodium dodecyl sulfate was added to the buffer for micellar electrokinetic chromatography (MEKC) analysis of human urine. Although inherently a pulsed source, the KrF excimer laser was operated at a high pulse repetition rate of 553, 1001 or 2009 Hz to simulate a continuous wave excitation source. Detection limits were found to vary with pulse rate, as expected, in proportion to average excitation power. The following detection limits (3σ) were determined in free solution CE: tryptophan, 4 n*M*; conalbumin, 10 n*M*; α-lactalbumin, 30 n*M*. Detection limits for indole-based compounds and catecholamine urinary metabolites under MEKC separation conditions were in the range 7–170 n*M*.

Keywords: Tryptophan; Conalbumin; α-Lactalbumin; Indoles; Catecholamines

1. Introduction

Although capillary electrophoresis (CE) and its family of related separation techniques demonstrate remarkable resolution of analytes in a relatively short time, they continue to be plagued by poor detectability below micromolar concentrations. On-column UV absorbance is the simplest and least expensive detection scheme for CE since many detectors in use today are modified HPLC detectors. However, detection capability is directly proportional to the optical pathlength, which averages only 39 μm for a 50-μm I.D. capillary. This represents more than a 150-fold reduction in pathlength relative to a conventional HPLC flow cell. For biomolecules containing a UV absorbing residue, detection limits for CE separations are typically at the micromolar level (10^{-6} *M*) using on-column absorbance detection. These detection limits can be improved by 1 to, in some cases, 3 orders of magnitude (10^{-7}–10^{-9} *M*) using modified capillaries with extended pathlengths or by on-column stacking procedures [1]. Extreme care must be used in the latter methodology, however, to maintain precision of analysis. Furthermore, in the analysis of real samples in a complex matrix, extraction is usually necessary before stacking can be accomplished.

Fluorescence detection is an alternative method for improving detection limits. Unlike absorbance, pathlength and analyte concentration are not the only

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parameters that influence fluorescence signal intensity. Fluorescence is also proportional to the intensity of the light source incident on the sample volume viewed by the detector. Whereas fluorescence excited by an incoherent source (i.e., lamp) improves detection limits by a factor of about 10 compared to absorbance [2], the coherent nature of lasers allows them to be focused down to the internal diameter of the capillary permitting a higher photon flux for analyte excitation. Five orders of magnitude improvement in detection limit compared to absorbance can be achieved for highly fluorescing dyes that are well matched to the laser wavelength of excitation [3]. Further reduction in the limit of detection (10^{-21} mol or 10^{-12} *M*) has been demonstrated with the use of a sheath flow cuvette for amino acids labeled with tetramethylrhodamine isothiocyanate (TRITC) [4].

Unfortunately, these extraordinary detection limits are for amino acids diluted after derivatization with the amine reactive fluorescent probes. The chemistry of isothiocyanate derivatization to primary amines is slow and competing hydrolysis reactions limit the concentration of analyte that can be successfully labeled. If fact, amino acids are typically derivatized at relatively high concentration (10^{-5}–10^{-4} *M*) before dilution and analysis. Further complications arise when derivatizing proteins that contain multiple labeling sites from lysine residue ε-amino groups. Derivatization of every available primary amine is incomplete and heterogeneous within the analyte population being derivatized resulting in a range of protein products that differ in the number and distribution of attached fluorophores. CE can only partially separate these derivatives resulting in an analytically useless result [5].

An alternative to covalent derivatization is the use of native fluorescence, i.e., excitation of tryptophan (Trp) residues in proteins (λ_{em}~320–380 nm). Swaile and Sepaniak were the first to demonstrate the utility of laser-induced native fluorescence (LINF) for detection of proteins separated by CE [6]. They used an argon ion laser (514.5 nm, 7 W) that was frequency doubled to produce 257 nm radiation for excitation. Although this wavelength is not optimal for Trp excitation, detection of conalbumin was possible down to 10^{-8} *M* — an order of magnitude lower than pathlength-extended UV absorbance detection. Lee and Yeung improved upon this detection limit by using a water-cooled argon ion laser operating at 275.4 nm, which closely matches the wavelength of maximum excitation (280 nm) for Trp. With their fluorescence detector, a limit of detection (LOD) of 10^{-10} *M* conalbumin was realized [7]. Unfortunately, the water-cooled argon ion laser is expensive relative to lasers typically used with CE separations [3–5]. An economical alternative to this is a low power, high repetition rate KrF excimer laser (λ=248 nm), which represents a compromise between cost and nonideal wavelength of excitation. A detection limit (2σ) of 10^{-9} *M* for conalbumin has been demonstrated using such a KrF laser with LINF detection [8].

In this study, we investigate the performance of the KrF excimer laser CE–LINF detection system operated in a quasi-CW (continuous wave) mode, rather than with gated integration as used by Chan et al. [8–11], for profiling body fluids. Detection limits (3σ) were evaluated for several biofluid analytes, under free solution CE conditions and surfactant-modified CE (micellar electrokinetic chromatography, MEKC) conditions, which were used for urine analysis.

2. Experimental

2.1. Apparatus

All experiments were performed on a CE–LINF system built in-house. CE separations were carried out at ambient temperature in a 50 cm (44 cm effective length)×50 μm I.D., 185 μm O.D. untreated fused-silica capillary, unless otherwise stated. High voltage (model CZE1000R, Spellman, Plainview, NY, USA) to drive the separation was applied at the anode (inlet) from within a Plexiglas safety box (fabricated in-house). Samples were introduced into the capillary electrokinetically at the anodic end by applying 1 kV for 5 s, timed with a stopwatch.

The LINF system (Fig. 1) was constructed on an optical breadboard (Melles Griot, Nepean, ON, Canada) to facilitate alignment of the optical components and to dampen mechanical vibrations. A model SGX-500 KrF excimer laser (Potomac Photonics, Lanham, MD, USA) was used for excitation. The 248-nm output beam was reflected from an

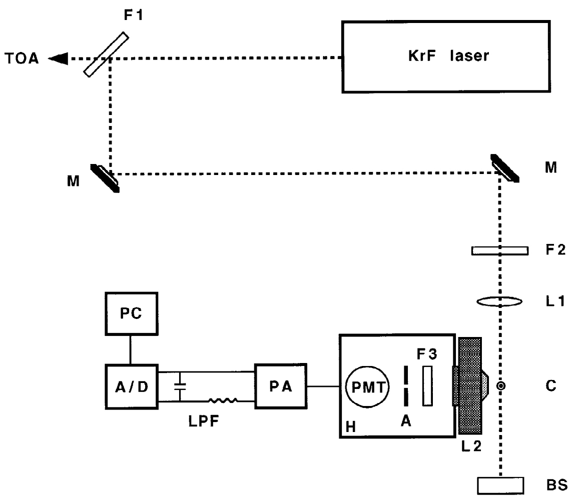

Fig. 1. Schematic of the LINF detection system with components labeled as follows: F1, F2=1.0 and 0.5 absorbance filters, respectively; M=mirror; L1=plano-convex fused silica lens; C=capillary cross-section; BS=beam stop; L2=reflective microscope objective; F3=UG1 glass filter; A=1-mm aperture; PMT=photomultiplier tube; H=PMT housing; PA=picoammeter; LPF=low pass filter; A/D=data acquisition card; PC=Pentium computer; TOA=beam path to thermo-optical absorbance detector.

A=1.0 neutral density filter (Melles Griot) and two aluminum-coated mirrors, then passed through an A=0.5 filter to reduce the average laser power to <2 mW. The attenuated laser beam was focused onto the capillary using a UV-grade synthetic fused-silica plano-convex lens (f=10.0 mm, ø=5.0 mm, Melles Griot). The average laser power, which increases with pulse repetition rate, was measured at various points along the optical train and the overall transmission efficiency was found to be 5% (i.e., a 95% attenuation of power) from the laser output to the separation capillary 125 cm away. Therefore, the average excitation power reaching the capillary was ~0.6 mW when the laser was operated at 553 Hz, 0.9 mW at 1001 Hz and 1.7 mW at 2009 Hz.

Fluorescence was collected normal to the excitation beam using a 15× Reflachromat reflective microscope objective (N.A.=0.58, f=11.5 mm, Spectra-Tech, Stamford, CT, USA). Spectral filtering of Raman and incident laser scatter was achieved using a UG1 filter (60% T at λ=350 nm, FWHM=80 nm) from Melles Griot. Rayleigh scatter and silica fluorescence were spatially filtered by a 1-mm pinhole aperture located in the focal plane of the reflective objective. After optical filtering, the emitted fluorescence signal was detected by a model 1P28 photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA) and amplified with a picoammeter (model 414 Microammeter, Keithly Instruments, Cleveland, OH, USA). The PMT was housed in a side-on, light-tight box (5×8×13 cm) and socket from Products For Research (Danvers, MA, USA). For the majority of data presented here, the picoammeter voltage output was further conditioned with a passive low pass filter (RC time constant=0.3 s) and collected at 10 Hz on a Pentium computer via an A/D interface (National Instruments, Austin, TX, USA) with the aid of LABVIEW acquisition software (National Instruments). The other data, human serum electropherogram and protein LODs, were obtained by strip chart recorder (model 056-1001, Hitachi, Tokyo, Japan) connected directly to the picoammeter voltage output.

In Fig. 1, the arrangement of optical components for fluorescence collection is not to scale. In fact, the reflective objective was threaded directly into the PMT housing and the UG1 filter and 1-mm pinhole, both 2.54-cm diameter components, fit snugly between the objective and PMT in a self-aligned fashion. As a result, no additional light-tight box was needed and the CE–LINF system was always operated with the room lights on. Care was taken, however, to block incident sunlight because the UG1 filter transmits at >700 nm. The PMT housing and capillary were mounted on xyz translation stages (Newport, Mississauga, Canada). Rapid alignment of the CE–LINF system was achieved by first centering the capillary in the excitation beam via visual inspection of the far field profile on the beam stop. Secondly, the microscope objective/PMT housing was translated across the capillary until a signal maximum was obtained for 3 μM tryptophan in buffer flowing through the capillary at 300 V/cm.

2.2. Materials

Uric acid (UA), tryptophan (Trp), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5HIAA), 3-indoxyl sulfate (3IXS), vanillylmandelic acid (VMA), α-amylase, conalbumin, α-lactalbumin and human serum as well as ACS grades sodium tetraborate, sodium phosphate (tribasic) and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). ACS grade hydrochloric acid and sodium hydroxide were from BDH (Toronto, ON, Canada). In-house distilled water was purified with a multi-cartridge Millipore water filtration/deionization system before use. Fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA). Platinum wire for electrodes and microcentrifuge tubes (1.5 ml and 600 μl) for buffers were obtained from Fisher Scientific (Montréal, QC, Canada). Nylon membrane syringe filters, 0.22-μm pore size, were purchased from Chromatographic Specialties (Brockville, ON, Canada).

2.3. Buffer and sample preparation

Buffers were prepared using the Millipore-purified water (18 MΩ) and pH was adjusted with the appropriate volume of hydrochloric acid or sodium hydroxide solution. SDS was added to buffers before pH adjustment for separations performed in the MEKC mode. All buffers were filtered through 0.22-μm membrane filters before installation at the capillary inlet and outlet. Where indicated, MEKC buffers were irradiated for 120 min with 254 nm light in a UV Cross-linker instrument (UVC-515 UV Multilinker, Ultra-Lum, Carson, CA, USA) to photobleach fluorescent impurities. The capillary was rinsed first with 0.1 *M* NaOH then with running buffer (5 column volumes for 1 min for each solution) before each injection of body fluid or standard. Fresh urine and saliva samples collected from volunteers and human serum from Sigma were filtered through 0.22-μm nylon filters before being diluted with running buffer. Standards were prepared in the same buffer used for the body fluid analysis.

Isolation of saliva proteins from a twofold diluted saliva sample was performed using a Microcon-10 microconcentrator (Amicon, Beverly, MA, USA) and repetitive washing of the retenate with running buffer after each centrifugation period (3×10 min at 5585 *g*). The washed retenate was reconstituted in running buffer to the initial sample volume (~600 μl) before analysis.

3. Results and discussion

The advantage of LINF detection for CE is apparent: no sample derivatization is required. The LINF detection of endogenous compounds in body fluids requires that they have an appreciable molar absorptivity at the excitation wavelength and an appreciable fluorescence quantum yield in the appropriate separation buffer. For detection of proteins in body fluids by LINF, the requirement is that they contain aromatic residues, preferably Trp. The molar absorptivity is an order of magnitude higher for Trp than for either tyrosine (Tyr) or phenylalanine (Phe) at an excitation wavelength of 248 nm [12]. In addition, the intensity of Trp fluorescence is more than 100 times that of Tyr or Phe at 248-nm excitation [8]. Phe has a low fluorescence quantum yield, even when excited at its wavelength of maximum absorbance, and Tyr is easily quenched or can readily undergo energy transfer [12]. Tryptophan fluorescence also depends on the surrounding environment in that quenching can lead to a decrease in intensity, but not to the same extent as Tyr.

Sensitive detection of Trp is important in diagnostic applications because it is an intrinsic fluorophore in proteins and is excreted in urine as the free amino acid and as various metabolized forms indicative of disease [13]. Free Trp fluorescence, when excited at 280 nm, is highly pH dependent, in that a twofold increase in fluorescence intensity is seen when increasing pH from 8 to 10.2 [14]. Therefore, a working range of pH 9–10 was used in all our work to take advantage of this pH dependence of free Trp fluorescence and also to minimize protein adsorption to the capillary wall.

3.1. LINF detection

Nanomolar detection limits for tryptophan-containing polypeptides [8] and other fluorescing analytes [9,11,15] have been demonstrated for LINF using the same pulsed KrF laser used in this work along with a gated integration (boxcar) detection scheme. Gated integration is a phase sensitive detection technique for low duty-cycle modulated signals such as those obtained with pulsed laser excitation (high duty-cycle signals are best handled by phase-locked amplifiers). Phase sensitive detection is typically used to enhance the S/N ratio of modulated signals, provided the dominant noise is additive. Gated integration provides an additional advantage in that it permits temporal discrimination of the laser scatter from the fluorescence, as long as the temporal profiles of the two differ to some extent. Most pulsed lasers, including the KrF laser used in this work, suffer from relatively large pulse-to-pulse fluctuations that contribute in a multiplicative fashion to the overall noise in fluorescence intensity. As well, in the absence of analyte, the measured intensity for on-column laser fluorescence is generally dominated by laser scatter and/or background fluorescence. Even under ideal conditions, the fundamental (shot) noise would still be multiplicative rather than additive. Consequently, the S/N ratio improvements expected for phase sensitive detection would be limited.

Given the high repetition rate of the KrF laser (up to 2009 Hz), and in view of the above considerations, we have investigated LINF detection performed in a quasi-CW mode rather than with gated integration of the fluorescence pulses. This approach was recently used to monitor the refolding pathway of a large protein [16], although the authors did not provide the necessary data to compare the performance of gated to nongated integration detection schemes. Fig. 2 shows the relationship between the excitation pulse rate and fluorescence emission before (Fig. 2A–C) and after (Fig. 2D–F) conditioning with a low pass filter (RC=0.3 s) for a continuous flow of 30 n*M* Trp in buffer (500 V/cm, 6 μA). Fig. 2A–C clearly show the pulse-to-pulse variations in emitted light. These large fluctuations mimic the incident laser profile and appear in the scattered light, background fluorescence and analyte fluorescence. Such variations are not discriminated against by phase sensitive detection, as mentioned above. The emitted pulses in Fig. 2A–C are broad (~0.8 ms at half height) compared to the excitation pulses (~60 ns), due to the response of the picoammeter. The traces in Fig. 2D–F show the effect of low-pass filtering of the fluorescence pulses, using a 0.3-s time constant. Data were collected at 10 Hz and plotted on the same scale for each pulse repetition rate. While the average fluorescence intensity increased as a function of the pulse repetition rate (i.e., the average excitation power), the noise on the Trp signal stayed essentially constant. The same trend was seen for the background signal from tetraborate buffer in the absence of Trp (data not shown) with the net result of a higher S/N ratio for higher pulse repetition rates.

Fig. 3 shows the electropherogram of a 2-nl (apparent volume [17]) injection of 30 n*M* Trp. The Trp LOD (3σ) for our CE–LINF detection system (KrF laser operated at 1001 Hz) was determined to be 4 n*M*. This compares favorably with Chan's LOD (2σ) of 3 n*M* Trp for a 15-nl injection and KrF excimer LINF detection with gated integration in a 75-μm I.D. capillary [8], and the LOD of Lee and Yeung, also at 2σ, of 2 n*M* Trp obtained by argon-ion LINF at 275.4 nm without gated integration [7]. We found that our LOD improved to 1 n*M* when the KrF laser was operated at 2009 Hz, taking advantage of the higher incident power versus noise characteristics described above. However, LOD measurements at 2009 Hz were obtained using a shorter capillary (33 cm total length, 758 V/cm, 10 μA) to ensure that Trp eluted (data not shown) before the capillary cracked under the stress of the high incident laser power and separation voltage. In our laboratory, the KrF laser is used concurrently for the LINF work presented here and for thermo-optical absorbance (TOA) detection of peptides [18]. Because it is a shared laser (Fig. 1), excitation was performed at a pulse rate of 553 Hz (the optimum for TOA detection) instead of 1001 or 2009 Hz for profiling body fluids.

Detection limits for proteins depend on the number of Trp residues present and, to some extent, the local environment of those residues. For example, the LODs (3σ) of egg white conalbumin and bovine α-lactalbumin were determined to be 10 n*M* and 30 n*M*, respectively, for our CE–LINF system. Fan et al. reported a similar LOD for P22 tailspike endorhamnosidase protein detected with their KrF excimer LINF system [16]. Conalbulmin has ten Trp residues whereas α-lactalbumin has four (obtained from NCBI Entrez protein query database). Therefore, we were surprised to find that both of our protein detection limits were worse than that of Trp. It can be postulated that the proteins interacted with

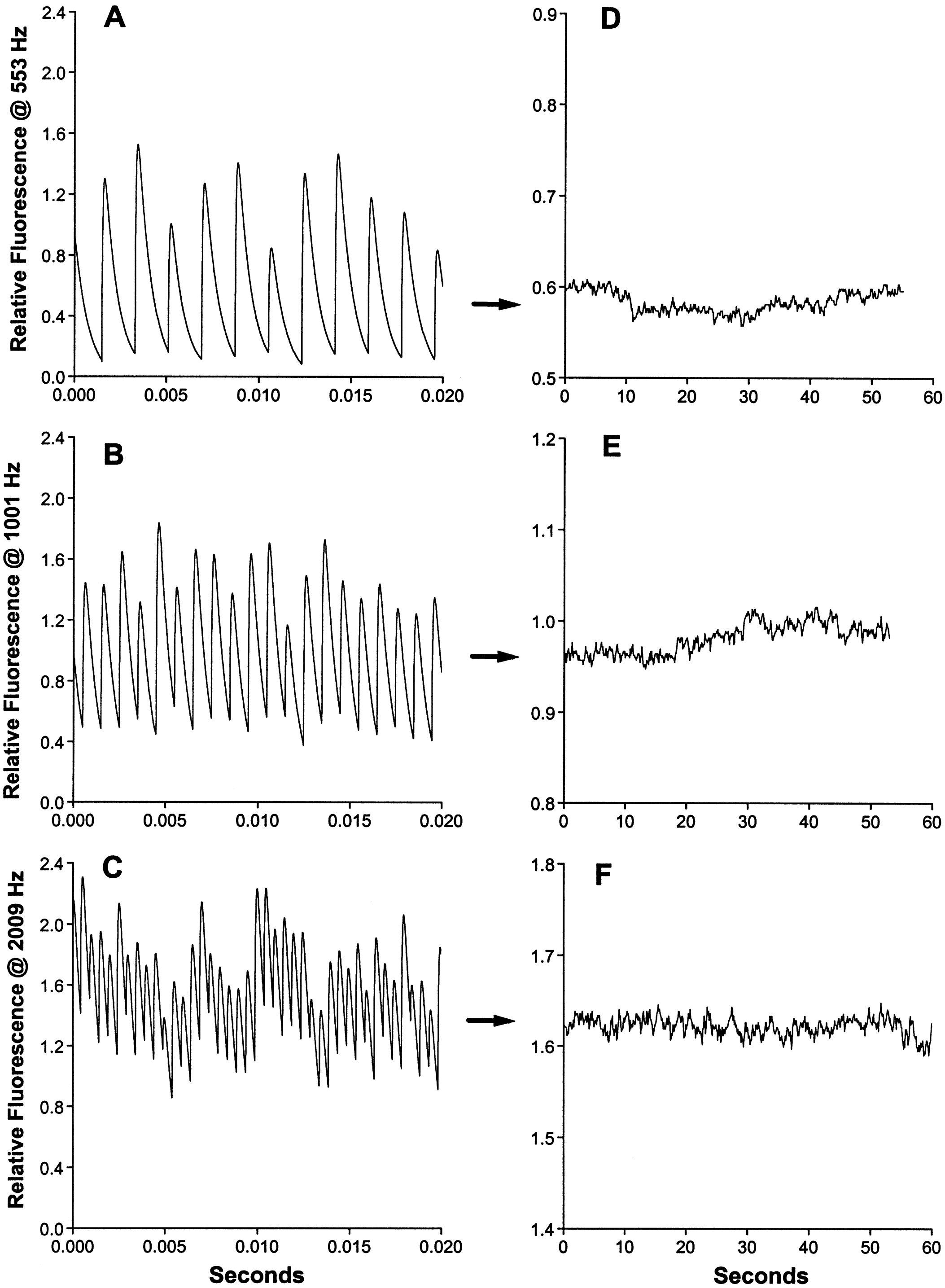

Fig. 2. Representative traces of the picoammeter signal output (A–C) and low-pass filtered output (D–F) from the CE–LINF detector operated at 553 Hz (A and D), 1001 Hz (B and E), 2009 Hz (C and F) for 30 n*M* Trp in 5 m*M* sodium tetraborate buffer (pH 10.0), 500 V/cm, 6 μA.

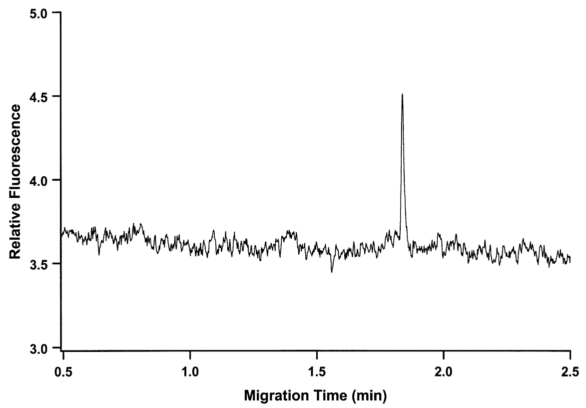

Fig. 3. Capillary electropherogram of 30 n*M* Trp at 500 V/cm in a 5 m*M* sodium tetraborate (pH 10.0) buffer. Laser excitation was at a pulse rate of 1001 Hz.

the capillary wall and that adsorptive broadening of peaks increased the LOD, which we had calculated based on peak height. Also, Trp residues would be in a different microenvironment than free Trp amino acid, thus the fluorescence characteristics would vary.

We observed blue and white luminescence of the capillary at all pulse repetition rates, which was also reported by Lee and Yeung for excitation at 275.4 nm [7], and nonspecific fluorescence from the borate buffer. Both sources of background signal were not completely eliminated by the UG1 filter after being efficiently transmitted by the Reflachromat reflective objective, which is a mirror-based microscope objective. After prolonged exposure of the capillary to 248-nm excitation, we could distinctly see red fluorescence, which may be due to damage of the fused-silica [19]. This red luminescence was not caused by heating of the capillary.

3.2. Body fluid profiles

Reports on the use of CE with UV absorbance detection for body fluid analysis are numerous [20,21]. For some applications, the analyte of interest is at sufficient concentration that direct injection and quantitation by CE–UV is reliable. However, sensitivity issues arise in the determination of less abundant proteins and protein precursors, which may be indicators of a diseased state [22]. Accurate quantitation by CE–UV becomes problematic at the micromolar level. Therefore, 100-fold better detectability achieved with CE–LINF is advantageous and increases the working range for analyte determination. Of equal importance is the selectivity that LINF detection affords. Caslavska et al. [23] demonstrated the utility of fluorescence detection in CE as a means of simplifying urine profiles compared to CE–UV, where electropherograms become crowded by the vast number of endogenous urinary compounds.

3.2.1. Human serum

The detection selectivity offered by CE–LINF with the KrF excimer laser was investigated using various body fluids obtained commercially or from volunteers working in our laboratory. Fig. 4 shows the CE–LINF profile at pH 10.0 of a 60-fold dilution of standard human serum. Such profiles obtained using UV absorbance detection, and their use for identifying gammopathies and other protein disorders, have been reported numerous times [24–27]. To the best of our knowledge, this is the only example of a serum profile by CE–LINF. Although the use of LINF detection for profiling serum may not provide many distinct advantages over UV absorption, its use in quantifying prealbumin is one

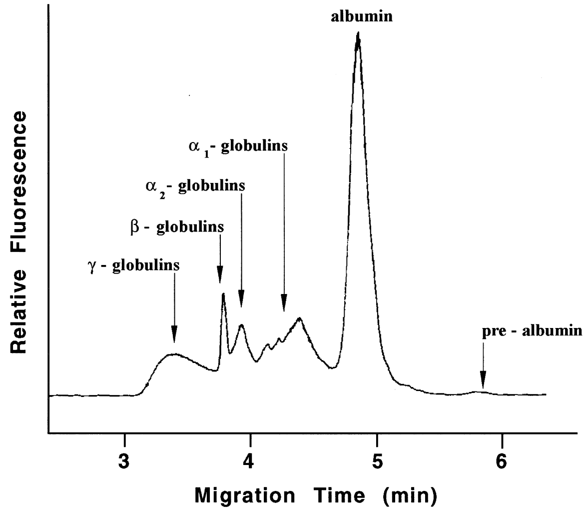

Fig. 4. Capillary electropherogram of human serum, filtered (0.22 μm) then diluted 60-fold in buffer before injection. Separation was carried out at 300 V/cm in a buffer consisting of 5 m*M* sodium tetraborate (pH 10.0).

possible application. Prealbumin, which can be an important indicator of nutritional status, inflammation, malignancy, cirrhosis of the liver and Hodgkin's disease [25,28], is generally found in serum at micromolar concentrations, i.e., close to the LOD for CE with UV absorbance detection [25,29]. Because prealbumin is rich in Trp, facile analysis by CE–LINF with the KrF excimer laser is expected. We are investigating this application further.

3.2.2. Human saliva

There are few reports on the CE analysis of endogenous components in saliva [30]. Fig. 5 shows the CE–LINF profile of human saliva before (Fig. 5A) and after (Fig. 5B) filtration to remove compounds of nominal M_r<10 000. One of the main components secreted in saliva is α-amylase, the enzyme responsible for hydrolysis of amylose and amylopectin [31]. The saliva sample was spiked with an α-amylase standard to confirm its migration time at 2.35 min. Trace B in Fig. 5, which is the retenate from filtration and should contain only high-M_r compounds, showed that smaller species, perhaps Trp-containing peptides, coeluted with α-amylase and may contribute in part to the shoulders seen on the main peak in Fig. 5A. The source of some of these peptides may be proteolytic degradation of saliva proteins because no protease inhibitory pretreatment was implemented, such as addition of PMSF (phenylmethylsulphonyl fluoride) [32]. Identification of other salivary components such as lysozyme, kallikrein [31] and trace endogenous species by CE–LINF may be undertaken in the future.

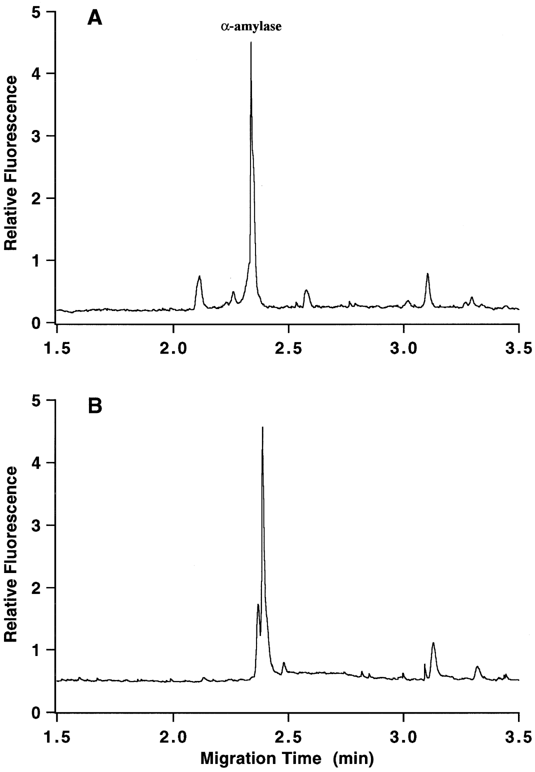

Fig. 5. Capillary electropherograms of human saliva. Separation was carried out at 400 V/cm in a buffer consisting of 5 m*M* sodium phosphate (pH 10.2). (A) Saliva sample was filtered (0.22 μm) then diluted tenfold in buffer before injection. (B) Saliva sample was filtered (0.22 μm), diluted twofold, then passed through a 10 000 M_r cut-off filter and the retenate was reconstituted in buffer before injection.

3.2.3. Human urine

Urine is a complex biological matrix. Rapid screening to give a chromatographic fingerprint of urine or any other mammalian biological fluid can provide useful qualitative and semiquantitative information, but usually only for one or a few classes of compounds at a time. GC and HPLC are perhaps the most widespread analytical techniques for screening urine [33–35]. MEKC–LINF represents a complimentary technique for the selective detection of endogenous and exogenous compounds in urine. MEKC can potentially provide better separation selectivity than free solution CE because many urinary components, such as Trp and 5-hydroxytryptophan, are not well resolved by the latter technique (data not shown).

The application of CE and MEKC to monitoring underivatized fluorescent compounds, either endogenous or exogenous, in body fluids has been reported by several research groups [10,11,15,23,36,37]. Fig. 6 shows the MEKC–LINF electropherogram of diluted urine. Native fluorescence detection simplifies the urine profile, compared to multi-wavelength absorbance detection [38], because only that class of analytes that fluoresce are specifically detected. The peaks for six endogenous species known to be in urine were identified by spiking urine samples with the appropriate standards and monitoring their peak comigration. This method is not necessarily conclusive, nor does it exclude the

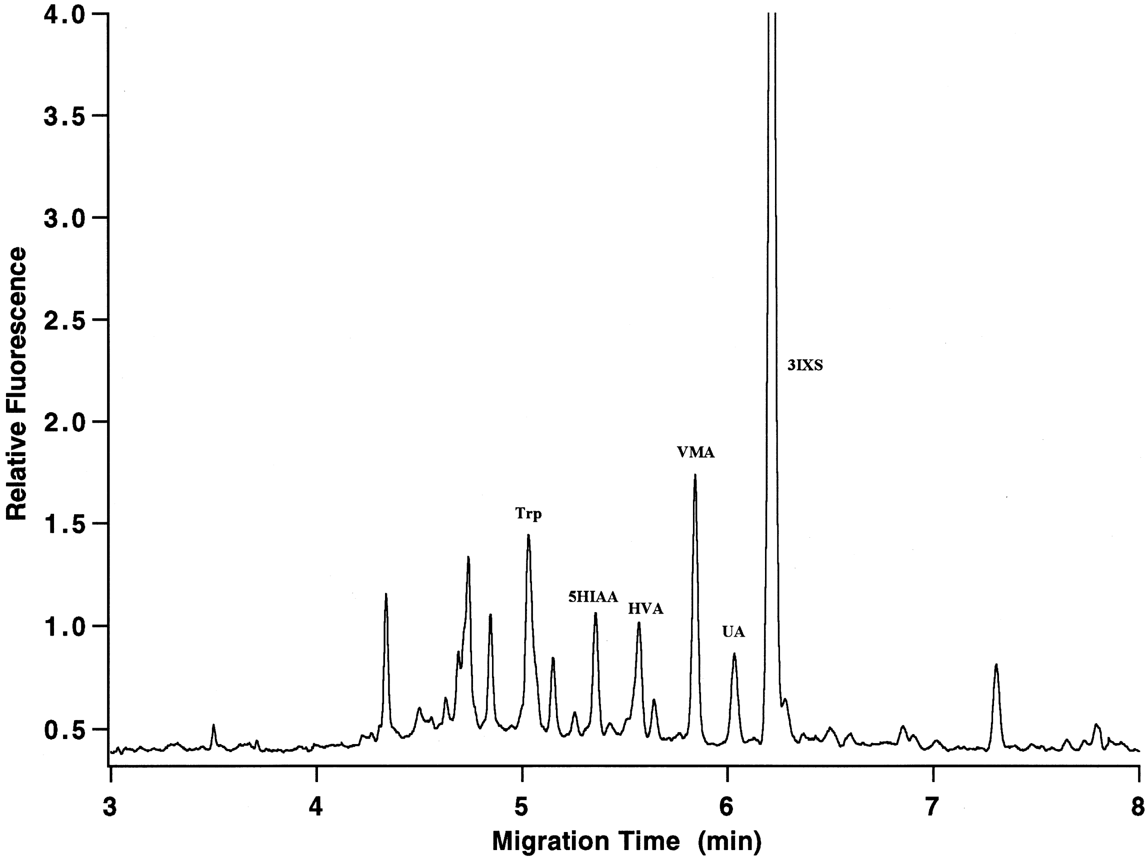

Fig. 6. MEKC electropherogram of human urine. Separation was carried out at 300 V/cm in a buffer consisting of 6 m*M* sodium tetraborate, 10 m*M* sodium phosphate, 75 m*M* SDS (pH 9.3). The following endogenous compounds were identified by spiking with standards: UA, uric acid; Trp, tryptophan; 5HIAA, 5-hydroxyindole-3-acetic acid; 3IXS, 3-indoxyl sulfate; HVA, homovanillic acid; VMA, vanillylmandelic acid.

possibility that another unidentified fluorescent component of urine may also coelute with the spiked standard. However, conditions for the MEKC separation were chosen based on the work from Thormann's group [23] who confirmed the assignment of these peaks by matching the multi-wavelength absorption spectra collected during electrophoresis. We did not attempt further optimization of this separation.

Detection limits (3σ) were initially determined for 5HIAA, HVA and VMA in the same MEKC separation buffer as that used in Fig. 6. Only a tenfold improvement in detectability was seen for all three species with respect to non-laser-induced methods of fluorescence detection (λ_{ex}=220 nm) [23]. In fact, our detection limits were on the same order of magnitude (~1 μM) as those reported by Simon and Nicot [39] for phenylglyoxylic and mandelic acids, analytes similar to HVA and VMA, determined by CE with UV absorbance detection (λ=255 and 210 nm). Impurities in SDS have been shown to contribute to background fluorescence [9] and the presence of micelles has been observed to increase the LOD by a factor of two [8,9,11]. Indeed, we found a sixfold improvement in the LODs of 5HIAA, HVA and VMA when the MEKC buffer was irradiated with UV light for 120 min to photobleach fluorescent impurites. Further improvements in LOD were seen when the concentration of SDS was lowered from 75 to 50 m*M* and when sodium phosphate was elimi-

Table 1
Normal level, medical importance and buffer-dependent LOD of three fluorescent components found in urine

Compound	Normal level[a]	Relevance	LOD at 3σ (n*M*)		
			Buffer A[e]	Buffer B[f]	Buffer C[g]
5HIAA (5-hydroxyindolacetic acid)	2 mg/day (<50 μ*M*)[b]	Carcinoid tumor screening[c]	33	15	7
HVA (homovanillic acid)	6 mg/day (<40 μ*M*)	Neuroblastoma and hepatic encephalopathy screening[d]	800[h]	–	170
VMA (vanillylmandelic acid)	3.5 mg/day (<45 μ*M*)	Neuroblastoma and hepatic[d] encephalopathy screening[d]	500[h]	–	150

[a] [40].
[b] Based on 1 l/day typical volume of urine excreted [40].
[c] [23,41].
[d] [33,34,42,43].
[e] Buffer A: 6 m*M* borate, 10 m*M* phosphate, 75 m*M* SDS (pH 9.1), UV irradiated for 120 min.
[f] Buffer B: 6 m*M* borate, 10 m*M* phosphate, 50 m*M* SDS (pH 9.1), UV irradiated for 120 min.
[g] Buffer C: 5 m*M* borate, 50 m*M* SDS (pH 9.1), UV irradiated for 120 min.
[h] Extrapolated from background S/N obtained for 5HIAA measurement.

nated. These results are summarized in Table 1 along with the normally excreted amounts of these compounds. Our LOD of 7 n*M* for 5HIAA (Table 1) compares well with that obtained by Chan et al. [11] (5.8 n*M* at $S/N=2$) for the same buffer, excluding 5% acetonitrile. The improvements in LOD for 5HIAA in buffers B and C (Table 1) were observed to be inversely proportional to the current generated in the capillary (data not shown). Apparently, Joule heating in the capillary affects the analyte emitted fluorescence. We are investigating this relationship further. Although the three metabolites in Table 1 have high normal excretion levels that could be quantitated by CE–UV, the inaccuracy associated with closely eluting peaks from other UV-absorbing components makes fluorescence detection, a selective technique, an attractive alternative for urine analysis.

4. Conclusions

Native fluorescence is an attractive approach for detecting biological compounds because the need for derivatization chemistry associated with visible–LIF detection is eliminated. LINF detection with a KrF excimer laser, operated at high repetition rates without gated integration, was used. Applications of this detection system were demonstrated by obtaining free solution CE profiles of human saliva and serum and an MEKC profile of human urine. It was shown that the selective detection offered by this technique could provide qualitative and, in future, quantitative information on various endogenous species in these body fluids.

Although comparatively low detection limits for Trp and urinary compounds were obtained, we are continuing to investigate further improvements in LOD. For example, ratioing the KrF excitation pulses to the fluorescence emission pulses will eliminate pulse-to-pulse fluctuations and should substantially improve S/N. Secondly, better spatial discrimination of silica fluorescence from analyte fluorescence might be achieved with a smaller pinhole aperture (ideally 0.75 mm) and/or the use of sheath flow detection techniques.

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