3.8. HPAEC/PAD analysis of oligosaccharides from native and from neuraminidase treated rFVIIa

The HPAEC analysis of the oligosaccharides released from rFVIIa samples was performed at high pH using conditions similar to those originally described by Townsend and co-workers [7,34]. Using HPAEC it is possible to obtain a separation not only based on the content of Neu5Ac in the oligosaccharides but, in addition, a further separation based on the oxyanion formation of the neutral part of the oligosaccharides [5,7,8,34]. In HPAEC of the oligosaccharides from native rFVIIa and of the oligosaccharides from desialylated rFVIIa separation into 5–8 peaks is obtained for both samples (Fig. 6). The oligosaccharide peaks OS-A and OS-B (Fig. 6) were collected and subjected to neuraminidase treatment. While untreated OS-A and OS-B eluted as expected from the preparative run, the neuraminidase treated oligosaccharides for both oligosaccharides resulted in two peaks, one coeluting with Neu5Ac and one coeluting with a corefucosylated biantennary reference structure (not shown) (OS-C). Based on the relative amounts of Neu5Ac found after neuraminidase treatment, it is concluded that OS-A and OS-B are corefucosylated biantennary structures with two and one Neu5Ac residues, respectively. It is noteworthy that rFVIIa glycosylation shows considerably microheterogeneity also with regard to the desialylated structures (Fig. 6).

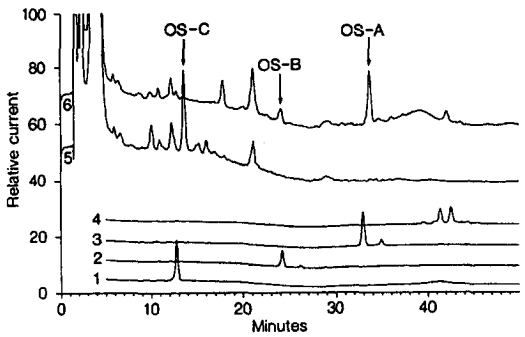

Fig. 6. High-pH anion-exchange HPLC separation of from top to bottom: (trace 6) oligosaccharides from native rFVIIa, (trace 5) oligosaccharides from neuraminidase treated rFVIIa, (trace 4) trisialylated triantennary oligosaccharide standard (C-335300), (trace 3) disialylated biantennary oligosaccharide standard (C-224300), (trace 2) monosialylated biantennary oligosaccharide standard (C-124300), (trace 1) Neu5Ac. Two oligosaccharides, labelled OS-A and OS-B, from native rFVIIa were purified and characterized as described in the text. The peak labelled OS-C corresponds to the desialylated forms of OS-A and OS-B. The oligosaccharide standards contain oligosaccharides with Neu5Ac linked either α2–3 or α2–6, and can therefore give more than one peak. Detection was pulsed amperometric. The separation conditions are described in Section 2.4.

4. Conclusion

Separation of the glycoforms of rFVIIa into six peaks or more has been obtained by CE, using phosphate as separation buffer with 1,4-diaminobutane as buffer additive. The separation has been shown to be related primarily to differences in the content of Neu5Ac of the rFVIIa glycoforms. Techniques for RP-HPLC separation of the rFVIIa tryptic glycopeptides containing the N-glycosylated sites and for HPAEC separation of the rFVIIa oligosaccharides released by hydrazinolysis have been established. The CE and the HPLC separations have been used for characterization of native and neuraminidase treated rFVIIa. Although the neuraminidase treatment, as expected, reduces the heterogeneity, significant peak separation is obtained by all three analyses for the neuraminidase treated sample. Characterization of this heterogeneity will be the subject of further studies using the potentials of CE and HPLC.

Acknowledgements

The authors wish to thank Anne-Lis Morsing Boeje and Mette Marie Thomsen for their excellent technical assistance.

Abbreviations

rFVIIa	Activated recombinant human factor VII

RP-HPLC	Reversed-phase high-performance liquid chromatography
HPAEC	High-pH anion-exchange chromatography
PAD	Pulsed amperometric detection
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
EOF	Electroendosmotic flow
Neu5Ac	N-Acetyl-neuraminic acid
r-HuEPO	Recombinant human erythropoietin
rtPA	Recombinant tissue plasminogen activator

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JOURNAL OF CHROMATOGRAPHY A

Separation of tryptophan and related indoles by micellar electrokinetic chromatography with KrF laser-induced fluorescence detection

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Abstract

Micellar electrokinetic chromatography (MEKC) was applied for the separation of tryptophan and related indoles. Using a 5 m*M* sodium borate buffer (pH 9.2) containing 50 m*M* sodium dodecyl sulfate and 5% acetonitrile, eleven indoles were baseline separated in under 17 min. Most of the indoles were detected at the n*M* level by native fluorescence using KrF laser-induced fluorescence (LIF), which was approximately 100 times more sensitive than UV absorption detection at 200 nm. Preliminary results show that the MEKC–LIF with direct sample injection is a feasible method for assessing indole profiles in diluted urine and serum.

1. Introduction

In humans, tryptophan (TRP) is metabolized by two major pathways, either through kynurenine or a series of indoles [1]. Among the indoles (Fig. 1), 5-hydroxytryptamine (5-HT, serotonin) is pharmacologically the most active indole amine. These indoles are known to have important physiological and pathological properties. Abnormal metabolism of TRP has been observed in patients with bladder cancer [2], carcinoid syndrome [3], alcoholism [4], liver cirrhosis [5], psychiatric diseases [6], HIV infection [7], and acute rejection after renal transplant [8]. Therefore, measurement of TRP and/or its metabolites is essential in investigating and monitoring disease status.

High-performance liquid chromatography (HPLC) with various detection schemes has been widely used to determine TRP and related indoles in biological samples [9,10]. Using pre-column fluorescent derivatization with methoxyacetaldehydyde [11], phenylglyoxal [12,13], or 9-hydroxymethyl-β-carboline [14], TRP in serum, plasma, or brain tissue was selectively detected. Post-column fluorescent derivatization with benzylamine was also used for the selective detection of 5-hydroxyindoles in urine and serum [15,16]. However, these derivatization steps are not convenient and may not be quantitative. In general, TRP indoles have been detected mostly by electrochemistry [17–23] or native fluorescence [24,25]. Combinations of UV, electrochemical, or native fluorescence detection were

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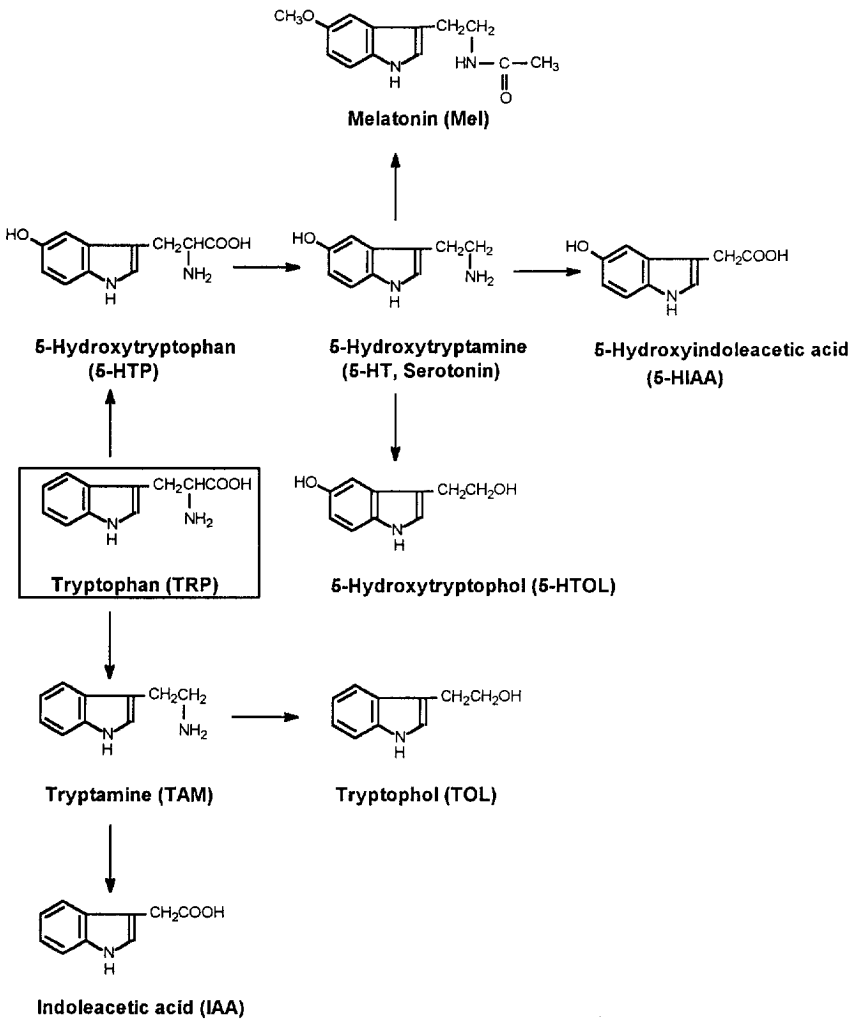

Fig. 1. Metabolic pathways of tryptophan.

also reported [8,26]. Gas chromatography–mass spectrometry (GC–MS) was also used to determine 5-hydroxyindoleacetic acid (5-HIAA) and indoleacetic acid (IAA) in biological samples, but the sample clean-up procedures before derivatization for GC separation were tedious [27].

Recent advances in capillary electrophoresis (CE) have made it an attractive separation tool alternative to HPLC [28]. CE has the advantages of versatility, rapid analysis, low-mass detectability, high-efficiency separation, and ease of operation. Among various CE modes, capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are widely used to separate charged and neutral molecules, respectively. When CE is coupled to laser-induced fluorescence (LIF), detection of attomole amounts is achieved [29].

The aim of this study was to apply MEKC for the separation of TRP and its indolic metabolites. Since TRP-related indoles fluoresce when

excited by UV light (Fig. 2), the detection sensitivity of the indoles using LIF with a KrF laser (248 nm) as the excitation source was evaluated. Finally, the feasibility of using MEKC–LIF for achieving indole profiles in urine and serum was investigated.

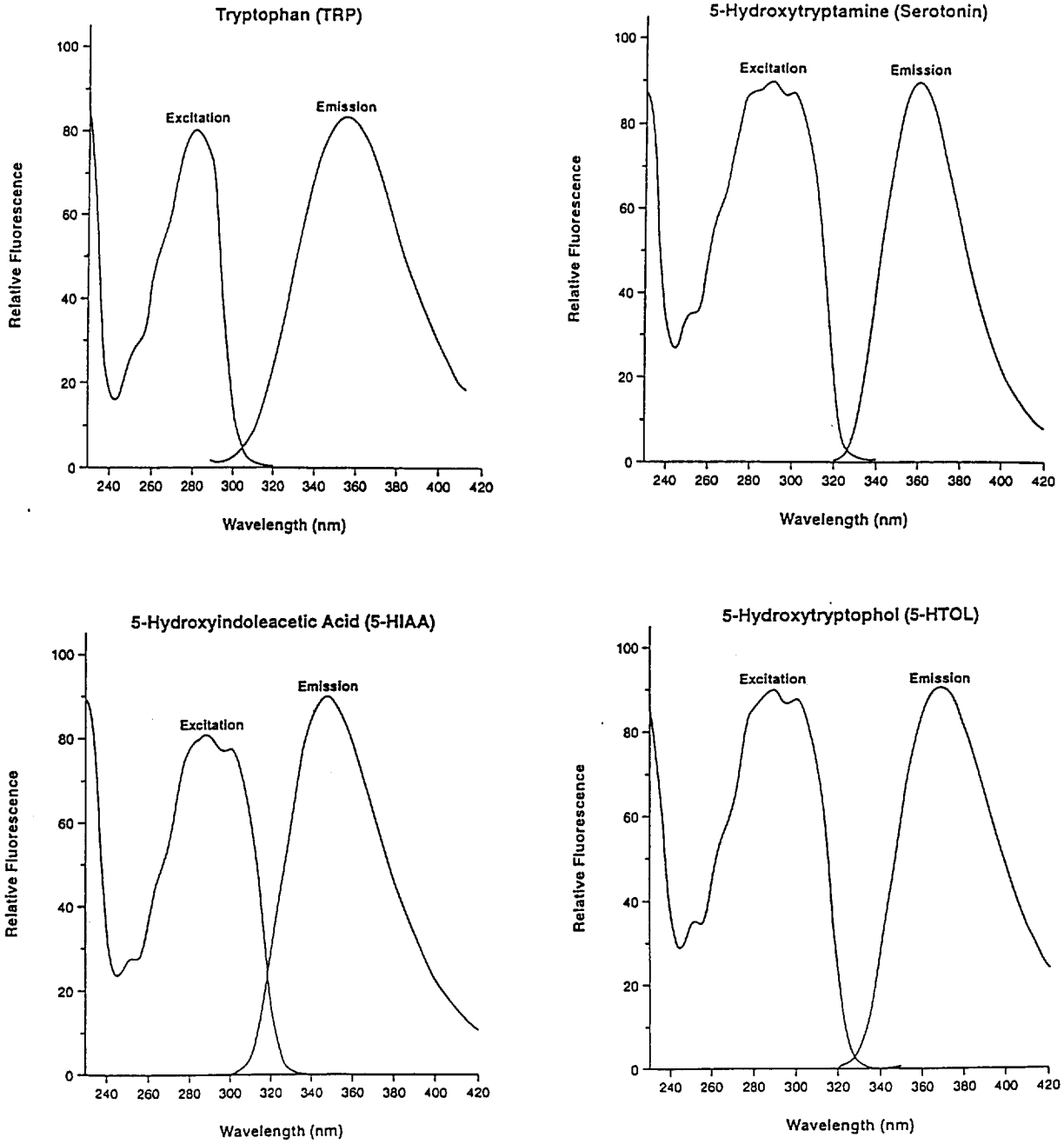

Fig. 2. Excitation and emission spectra of tryptophan and some related indoles.

2. Experimental

2.1. Apparatus

Experiments using UV detection were carried out with a Beckman P/ACE 2050 CE systems. Separations were performed at 25°C with a 67 cm × 50 μm I.D. (60 cm to detector) fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). Each new capillary was washed with 0.1 *M* NaOH and deionized water each for 15 min before use. Samples were injected into the capillary by applying pressure (0.5 p.s.i. = 3447.38 Pa) for 5 s. The capillary was then flushed with the running buffer for 1 min between runs.

In the LIF experiments, high voltage and pressure for CE operations were supplied by a Crystal 310 CE module obtained from ATI/Unicam (Boston, MA, USA). Separations were performed at room temperature with a 67 cm × 50 μm I.D. (60 cm to detector) fused-silica capillary. Samples were injected into the capillary by applying pressure (30 mbar) for 6 s. The home-built LIF detection system was described previously [30]. Fluorescence excitation was provided by a compact, pulsed laser operating at 248 nm (Model GX-500, Potomac, Lanham, MD, USA). The laser beam was first passed through an interference filter centered at 248 nm and then focused onto a capillary with a 50-mm focal-length bi-convex lens. The emission was collected at a 90° angle to the incident laser beam with a 10×, N.A. = 0.5, microscope objective (Carl Zeiss, Thornwood, NY, USA). After passing through an UG-1 bandpass filter (Melles Griot, Irvine, CA, USA), the collected emission was detected by a photomultiplier tube (PMT, Model 70680, Oriel, Stratford, CT, USA). The current output of the PMT was fed into a boxcar averager (Model 4100, EG&G, Princeton, NJ, USA) and its voltage output was displayed on a PC computer via an A/D interfacing module (Model 406, Beckman Instruments, Fullerton, CA, USA). In a typical experiment, a laser pulse rate of 1 kHz with an averaged excitation power of 0.5 mW was used. Excitation and emission spectra of the indoles (in water) were obtained with a Perkin-Elmer LS3 spectrofluorometer.

2.2. Chemicals

TRP, 5-hydroxytryptophan (5-HTP), 5-HIAA, 5-hydroxytryptophol (5-HTOL), 5-HT, IAA, 5-methoxytrytophan (5-MTP), tryptophol (TOL), melatonine (MEL), tryptamine (TAM), and N-acetyl-tryptophan (NAT) were obtained from Sigma (St. Louis, MO, USA). A stock solution of individual indole was prepared in methanol (2 mg/ml) and stored at −20°C when not in use. The injected standard mixture was prepared by mixing the individual stock, followed by dilution with water. Sodium tetraborate and sodium dodecyl sulfate (SDS) was obtained from Fluka (Ronkonkoma, NY, USA). Urine and serum samples were diluted 200-fold with water before injections.

3. Results and discussion

The tryptophan metabolites contain either acidic, basic, neutral, or zwitterionic groups. Partial separations of some of indoles were achieved by CZE using a 5 m*M* sodium borate buffer (pH 9.2), as shown in Fig. 3A. The basic indoles (TAM and 5-HT) migrated first, while the acidic indoles (5-HIAA and IAA) migrated last. TRP, 5-HTP, and 5-MTP were not separated due to their similar electrophoretic mobilities. Also, the neutral indoles (5-HTOL, MEL, TOL) all migrated as one peak because uncharged molecules are not separated by CZE. MEKC was developed initially for the resolution of neutral molecules [31]. The method is based on the differential affinities of analytes for the hydrophobic core of micelles which serves as a pseudo-stationary phase for separation. Because the TRP-related indoles have different natures, they were effectively separated by MEKC. Using a borate buffer containing 50 m*M* SDS, ten of the eleven indoles were separated (Fig. 3B). In comparison to CZE, 5-HT and TAM migrated last in MEKC, probably due to the strong electrostatic interaction between the negatively charged SDS micelles and the positively charged 5-HT and TAM.

The addition of an organic solvent is commonly used to enhance resolution in MEKC [32–34].

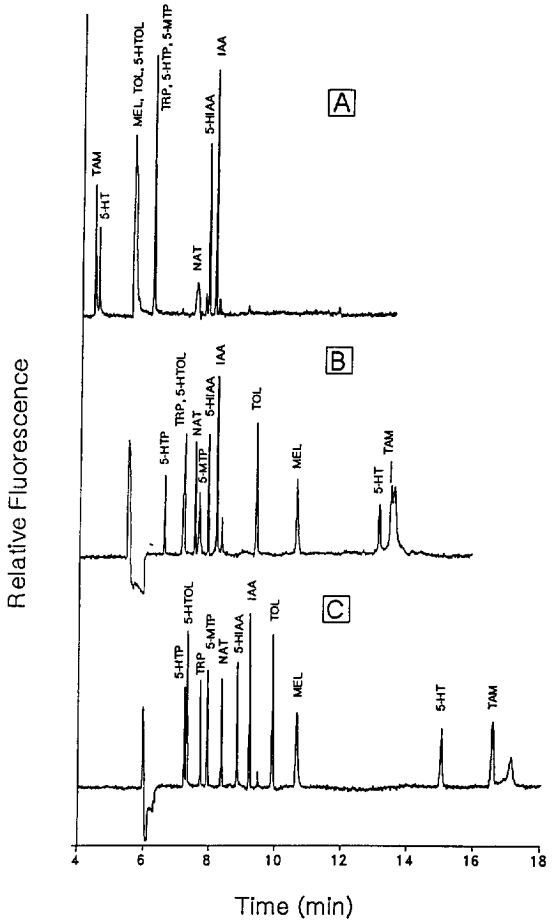

Fig. 3. Electropherograms for the separation of eleven tryptophan-related indoles using different buffers. Buffers: (A) 5 m*M* sodium borate (pH 9.2); (B) 5 m*M* sodium borate (pH 9.2) and 50 m*M* SDS; and (C) 5 m*M* sodium borate (pH 9.2), 50 m*M* SDS, and 5% acetonitrile. Capillary: 67 cm × 50 μm. Voltage: 20 kV.

Organic solvent reduces electroosmotic flow by increasing the viscosity of the running buffer, consequently widening the migration window. In this study, addition of 5% acetonitrile to the micellar buffer allowed the separation of TRP from 5-HTOL, and improved the resolution of most indoles (Fig. 3C). The distributions of NAT and 5-MTP into the SDS micelles were also affected by acetonitrile, resulting in their migration order reversal. In addition, the 5-MTP peak was sharpened, which was probably due to its higher solubility in organic solvent. All of the eleven indoles were baseline separated in about 17 min. Although these indoles also were separated by HPLC [3], the analysis time was rather long (ca. 50 min).

When excited by UV light, TRP and its related indoles fluoresce with excitation and emission maximums at ca. 288 nm and 350–370 nm, respectively (Fig. 2). Since the KrF laser operates at 248 nm, it is not an ideal excitation source for the indoles. Nevertheless, TRP was detected at the n*M* level by KrF-LIF in our previous CZE study using a 75 μm I.D. capillary [30]. In this MEKC study with a 50-μm capillary, high sensitivity detection for the indoles was also obtained. Fig. 4A shows the KrF-LIF detection of 90 ng/ml of each indole, which is about 100-fold more sensitive than the absorption detection at 200 nm (Fig. 4B). In Fig. 4A, the signal-to-noise ratio (*S*/*N*) ranged from 62 to 190, which

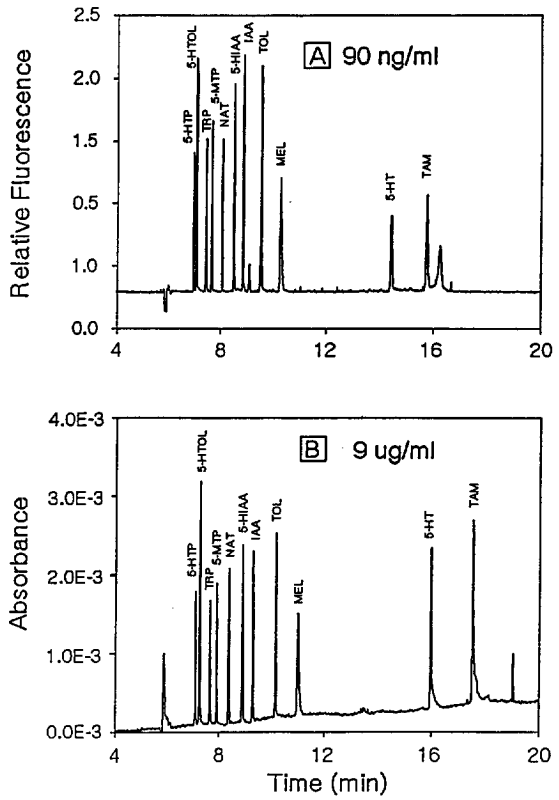

Fig. 4. Detections of tryptophan and related indoles. (A) KrF-LIF detection, 90 ng/ml of each indole; and (B) UV detection (200 nm), 9 μg/ml of each indole. Buffer: 5 m*M* sodium borate (pH 9.2), 50 m*M* SDS, and 5% acetonitrile. Capillary: 67 cm × 50 μm. Voltage: 20 kV.

Table 1
The limits of detection of tryptophan and related indoles using KrF-LIF detection

	Analyte	LOD (n*M*)
1	5-HTP	7.4
2	5-HTOL	5.5
3	TRP	7.3
4	5-MTP	5.6
5	NAT	6.1
6	5-HIAA	5.8
7	IAA	5.5
8	TOL	6.3
9	MEL	8.7
10	5-HT	13.7
11	TAM	11.9

Buffer: 5 m*M* sodium borate (pH 9.2), 50 m*M* SDS, and 5% acetonitrile. Capillary: 67 cm × 50 μm. Voltage: 20 kV. Signal-to-noise ratio = 2.

correspond to the limits of detection (LOD) ranging from 5.5 to 13.7 n*M* for the indoles ($S/N = 2$, Table 1). The detection sensitivity is expected to improve with an UV laser operating at 284 nm [35], which is currently not available in this laboratory.

Kema et al. [3] have recently used HPLC to assess the indoles profiles in urine, platelet-rich plasma, and gut-tissue, and they found that the amounts of TRP, 5-HIAA, and 5-HT in carcinoid patients were significantly different from that of normal patients. Alternatively, it is also feasible to use MEKC to obtain the indole profiles in biological samples. Fig. 5A shows the MEKC electropherogram of a diluted normal urine sample with direct sample injection. A few major peaks were observed. The TRP and 5-HIAA peaks in the urine were tentatively identified by matching the migration times of standards and by spiking the sample with standards (Fig. 5B). Other indoles in the diluted normal sample were not detected because of their low concentrations. Fig. 6A shows the MEKC electropherogram of a diluted normal serum with direct sample injection. The broad peak at 11 min was serum protein. As with the urine sample, TRP in the serum sample was tentatively identified by matching the migration times of standards and by spiking (Fig. 6B). The fluores-

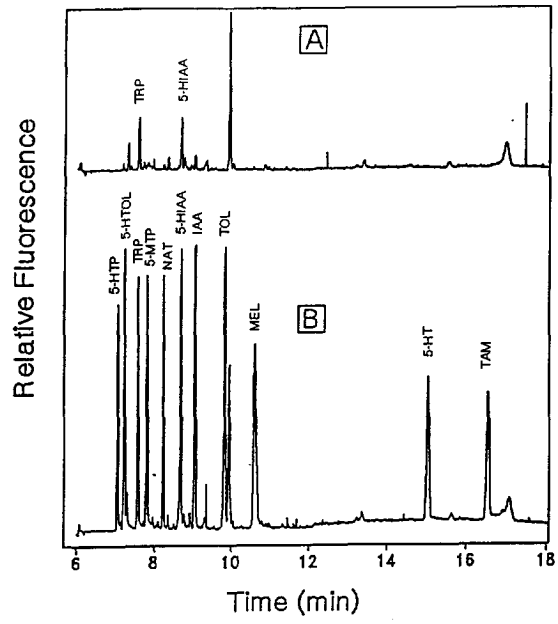

Fig. 5. Electropherograms for the MEKC of (A) urine, and (B) urine fortified with 90 ng/ml of each indole standard. The urine was diluted 200-fold with water before injection. Running conditions were the same as in Fig. 4A.

cence intensity of an emitting molecule is easily affected by the matrix, which may be significant for the direct injection of biological samples. Since diluted samples (200-fold) were used in this study, the above-mentioned effect was minimal, as judged by the similar fluorescence intensity of the indoles in the standard and spiked samples. Also, serum protein did not interfere with the detection of the indoles because most of them were separated from the protein.

4. Conclusion

MEKC using SDS micelles is a suitable method for the separation of TRP and related indoles. Most of the indoles were detected by native fluorescence at the n*M* level using KrF-LIF, which eliminates the need for chemical derivatization to obtain sensitive detection. Preliminary results show that MEKC–LIF is a feasible method for assessing the indole profiles in biological samples such as urine and serum.