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Short Communication

Micellar electrokinetic chromatography of hydroxyproline and other secondary amino acids in biological samples with laser-induced fluorescence detection ☆

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ABSTRACT

Micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence (LIF) was used for the rapid and sensitive detection of hydroxyproline in serum and hydrolyzed urine that were pre-column derivatized with 9-fluorenylmethyl chloroformate (FMOC). The application of the combined *o*-phthalaldehyde (OPA)/FMOC derivatization in MEKC for the selective detection of secondary amino acids in biological samples is investigated.

INTRODUCTION

Hydroxyproline (Hyp) is a secondary amino acid that reflects the metabolism of collagen [1]. The determination of Hyp is important because it has been suggested as a marker for certain cancers [2–4] and bone diseases [5,6]. The detection of Hyp in biological samples is difficult due to its relatively low concentration compared to other amino acids and its lack of any strong spectroscopic property. Sensitive detection is possible by modifying the molecule using chemical derivatization. Employing pre-column derivatization, Hyp labeled with phenylisothiocyanate, dabsyl chloride, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride, or radioisotopes has been detected with absorption [7–9], fluorescence [10–13], electrochemistry [14], or radiometry [15] in HPLC separation. Gas chromatography–mass spectrometry also has been used to detect Hyp after trifluoroacetylation and methanol esterification [16,17]. All these methods provide adequate results; however, the derivatizations are time-consuming or the experimental set-ups were complicated.

Recently Guzman *et al.* [18] employed capillary zone electrophoresis to separate fluorescamine-labeled Hyp and proline (Pro). They used on-column absorption detection in their

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study because fluorescamine-derivatized secondary amines do not fluoresce. However, the usefulness of Guzman's method has not yet been demonstrated with biological samples. In this study, our major goal is to apply micellar electrokinetic chromatography (MEKC) [19] and laser-induced fluorescence (LIF) for the rapid and sensitive detection of Hyp in biological samples after pre-column FMOC derivatization. In addition, the application of the combined OPA/FMOC derivatization [20] in MEKC for the selective detection of secondary amino acids will be discussed.

EXPERIMENTAL

Apparatus

The home-built capillary electrophoresis and LIF detection system used in this study has been described in detail previously [21,22]. Briefly, separations were performed in a 60 cm × 50 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with a +30 kV high-voltage supply (Glassman, Whitehouse Station, NJ, USA). Gravity injection was carried out by raising the high-voltage end to 10 cm for 15 s. Fluorescence excitation was provided by a compact KrF excimer laser (248 nm) (Model GX-500, Potomac Photonics, Lanham, MD, USA). Emission light was collected by a glass or a UV-graded microscope objective (Model Fluar, Carl Zeiss, Thornwood, NY, USA) and detected by a photomultiplier tube. A 310-nm bandpass and UV cut-off filters were used to reduce background light.

Chemicals

Acidic neutral, and basic physiological amino acid standards, 4-hydroxyproline, proline, OPA reagent solution, FMOC, and other analytical grade chemicals were obtained from Sigma (St. Louis, MO, USA). FMOC reagent solution was prepared daily as a 5 m*M* solution in acetonitrile. Sodium dodecyl sulfate (SDS) was obtained from Kodak (Rochester, NY, USA) and was washed with diethyl ether before use to remove the fluorescing organic impurities [22].

Sample preparation

Serum was deproteinized by mixing 100 μl of serum and 100 μl of acetonitrile. After centrifugation at 1000 *g* for 15 min, the supernatant was diluted fivefold with water before derivatization. Urine samples were hydrolyzed by mixing 100 μl of urine and 100 μl of 6 *M* hydrochloric acid in a screw-capped test tube, followed by heating at 110°C for 20 h in an aluminum heating block. The reaction mixture was then neutralized by adding 4 ml of 400 m*M* sodium borate buffer (pH 9.5). This diluted urine solution was then derivatized.

Diluted amino acid standard mixtures, urine or serum were derivatized by mixing 20 μl of sample, 40 μl of 200 m*M* borate buffer (pH 9.5), and 40 μl of FMOC reagent in a 1.5-ml conical tube for 1 min. Excess FMOC reagent was extracted twice with 1 ml of pentane. The aqueous portion was then diluted with water before injection. For the combined OPA/FMOC derivatization, 20 μl of sample, 20 μl of borate buffer, and 10 μl of OPA reagent solution were mixed for 2 min, then 10 μl of iodoacetamide (70 mg/ml) was added. After 2 min, 40 μl of FMOC solution was added. This final mixture was allowed to react for 2 min and subsequently extracted twice with 1 ml diethyl ether. The aqueous portion was then diluted with water before injection. Peak identification of the secondary amino acids was performed by spiking a sample with the standard component of interest.

RESULTS AND DISCUSSION

FMOC reacts with primary and secondary amino acids to form highly stable and fluorescing derivatives [23]. The advantages of FMOC derivatization include short reaction time, high yield, simplicity, and ease of automation. Identification of peaks in separation methods is usually performed by matching the elution or migration times with those of standards and requires an efficient separation of the peaks of interest from other matrix components; this can be difficult to achieve when complex samples are analyzed. To demonstrate the unique applicability of MEKC for the determination of Hyp, FMOC-derivatized acidic–neutral and basic physiological amino

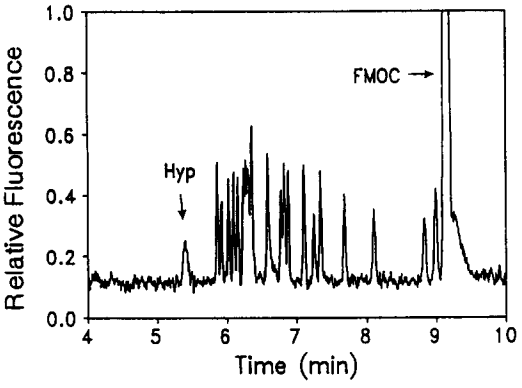

Fig. 1. Electropherogram of a FMOC – derivatized acidic/neutral amino acid standard mixture ($\sim 5 \cdot 10^{-8}$ *M*). Capillary: 60 cm × 50 μm I.D. Buffer: 20 m*M* sodium borate (pH 9.2) containing 25 m*M* SDS. Applied voltage: 22 kV. Injection: 15 s, gravity.

acid standard mixtures were subjected to MEKC separations using a borate buffer containing SDS micelles, as shown in Figs. 1 and 2, respectively. In Fig. 2, no Hyp peak was detected because the basic standard mixture does not contain Hyp. Using the indicated separation conditions, complete separation of the acidic, neutral, and basic FMOC derivatives (>40) in a single run is not possible or necessary because FMOC-Hyp migrates ahead of all other tested FMOC-amino acids. The identity of the first peak (*i.e.* Hyp) was also confirmed by the selective detection of secondary amino acids present in the acidic/neutral standard mixture using the OPA/FMOC derivatization (see below).

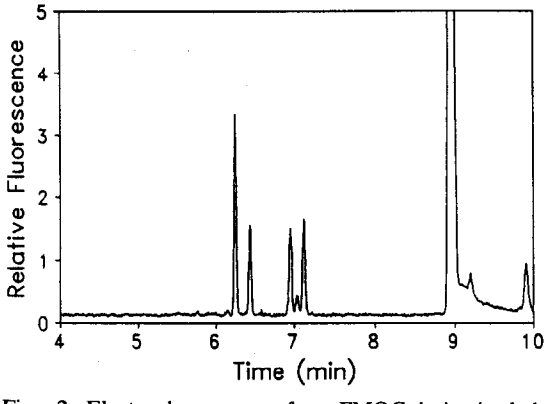

Fig. 2. Electropherogram of a FMOC-derivatized basic amino acid standard mixture ($\sim 1 \cdot 10^{-7}$ *M*). Running conditions were the same as in Fig. 1.

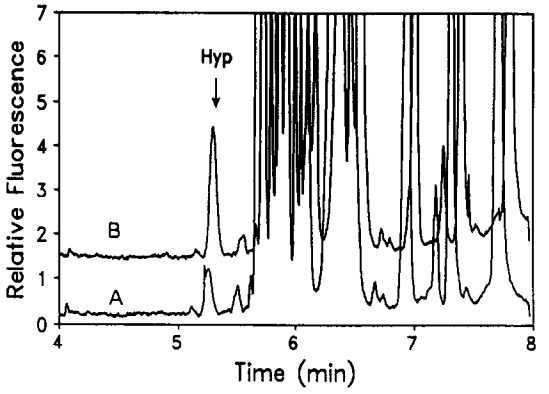

Fig. 3. Electropherograms of a FMOC-derivatized serum (A), and a Hyp-spiked ($\sim 6 \cdot 10^{-8}$ *M*) serum sample (B). The serum was diluted *ca.* 500-fold. Running conditions were the same as in Fig. 1.

Fig. 3 shows the electropherogram for a diluted serum sample which was derivatized with FMOC (A). The sample spiked with FMOC-Hyp is also shown in Fig. 3(B). A small variation in the migration times between these two runs was observed which was probably caused by a change in the electroosmotic flow during the experiment. Nevertheless, the Hyp in the serum sample was uniquely identified. More than 90% of Hyp in urine is peptide-bound; thus, acid hydrolysis of urine is needed for the determination of total Hyp [24]. Fig. 4 shows the electropherogram for a diluted urine sample which was derivatized with FMOC (A), and the FMOC-Hyp spiked sample. Determination of Hyp can

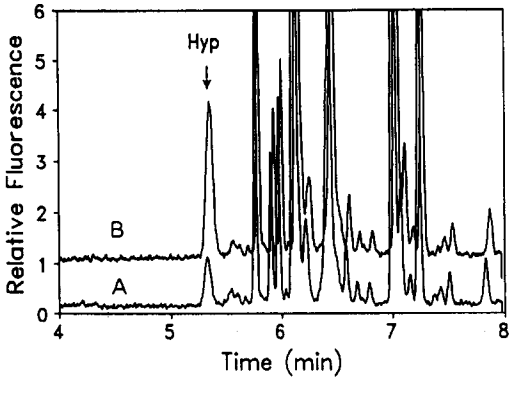

Fig. 4. Electropherograms of a FMOC-derivatized urine (A), and a Hyp-spiked ($\sim 6 \cdot 10^{-8}$ *M*) urine sample (B). The urine was diluted *ca.* 8000-fold. Running conditions were the same as in Fig. 1.

be facilitated by applying a high separation voltage, using a short capillary, and flushing the capillary with running buffer as soon as the FMOC-Hyp has passed the detector.

OPA reacts with primary amines while FMOC reacts with both primary and secondary amines. These two types of derivatives fluoresce at different wavelengths that can be spectroscopically isolated by using the appropriate emission filters. The combined OPA/FMOC derivatization has been used in HPLC for the selective detection of secondary amino acids in biological samples, fibroblast cultures and protein hydrolyzates [20,25–28]. In this procedure, primary amino acids are first removed by the OPA reaction, and the secondary amino acids are then selectively reacted with FMOC. Using the combined derivatization with MEKC, Pro in serum was also selectively detected (Fig. 5); this procedure is simpler than a recent published procedure for the determination of Pro by HPLC using the chloramine-T, sodium borohydride, and OPA reaction [29]. In addition, Hyp, Pro, and another secondary amino acid sarcosine (Sar) were simultaneous detected in hydrolyzed urine (Fig. 6). The stability of FMOC-amino acids in the presence of OPA adducts has been questioned [14] and this item has not been investigated further [20,23]. Our results indicate that the FMOC-derivatized Hyp, Pro, and Sar are stable for at least 24 h at room temperature in the presence of OPA adducts. In this study, excess FMOC reagent was removed by extraction according to the published procedure for HPLC analysis [23]. However, this may not be necessary in this study because the FMOC peak does not interfere with the electropherogram used for the determination of the secondary amino acids of interest.

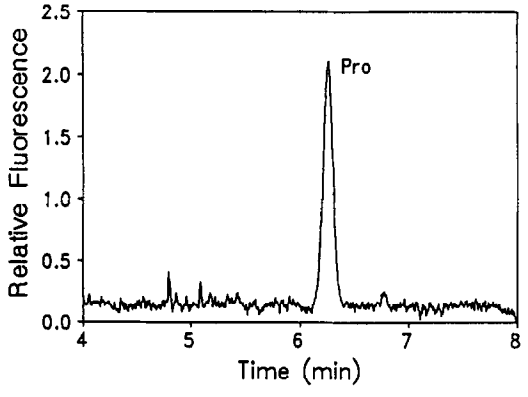

Fig. 5. Electropherogram of a OPA/FMOC-derivatized serum. The serum was diluted *ca.* 5000-fold. Running conditions were the same as in Fig. 1.

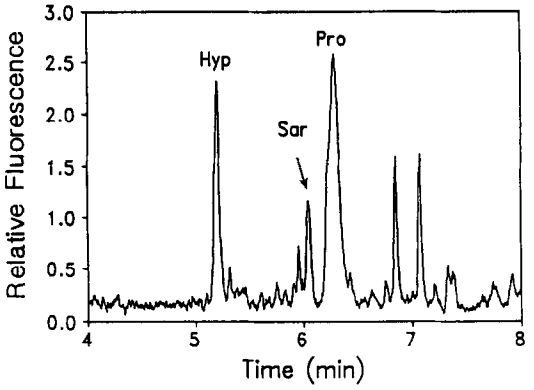

Fig. 6. Electropherogram of a OPA/FMOC-derivatized urine. The urine sample was diluted *ca.* 5000-fold. Running conditions were the same as in Fig. 1.

CONCLUSION

The described MEKC-LIF method allows rapid and sensitive detection of Hyp in serum and hydrolyzed urine that were derivatized with FMOC. The combined OPA/FMOC derivatization simplified the method for the selective detection of Pro in serum and the simultaneous detection of Hyp, Pro, and Sar in hydrolyzed urine. Quantitation, reproducibility, and accuracy of the described method will be studied in this laboratory.

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