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Subattomole Amino Acid Analysis by Capillary Zone Electrophoresis and Laser-Induced Fluorescence

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Subattomole analysis of fluorescein isothiocyanate (FITC) derivatives of amino acids is accomplished by combining capillary zone electrophoresis for high-efficiency separation with laser-induced fluorescence for high-sensitivity detection. Concentration detection limits range from 5×10^{-12} molar for alanine to 9×10^{-11} molar for lysine, injected in the column; 9×10^{-21} mole of alanine is contained within the ~1-nanoliter injection volume at the detection limit. The alanine detection limit corresponds to fewer than 6000 molecules injected onto the column and represents an improvement of four orders of magnitude in the state of the art for fluorescent detection of amino acids and an improvement of six orders of magnitude in the state of the art for the detection limit for isothiocyanate derivatives of amino acids.

ANALYSIS OF MINUTE QUANTITIES of amino acids is of broad interest. Because heroic efforts are often required to procure macroscopic quantities of biological samples, microanalysis schemes for amino acids are important. We report a relatively simple method for subattomole determination [1 attomole (amol) = 10^{-18} mol] of the FITC derivative of amino acids. Although routinely used as a fluorescent label for proteins, FITC is less commonly used as a derivatizing reagent for amino acids; however, the derivatives are relatively easy to form, have good electrophoretic properties, and generate strong fluorescence signals (*1–3*). Our amino acid analysis is based on the combination of capillary zone electrophoresis for the identification of amino acids with laser-induced fluorescence for detection of the amino acids. By this method, it is possible to determine subattomole quantities of 15 different FITC–amino acids in a 25-min separation period.

Capillary zone electrophoresis is an elegant technique for the separation of minute quantities of ionic species (*4*). In our instrument, a 99-cm length of fused silica capillary (inner diameter, 50 μm) is used for the separation. Before the separation, the capillary is filled with 5 m*M* aqueous carbonate buffer, *p*H 10. Sample is introduced by dipping the positive end of the capillary tube into a small vial containing the sample; the power supply is connected to the vial with a platinum electrode, and a 2-kV potential is applied for 10 s. Under these conditions, an injection precision of a few percent is possible; the precision is limited primarily by the reproducibility in the length of time for which the injection potential is applied. This injection technique introduces bias in the analysis; analytes with high electrophoretic mobility travel at a greater speed and thus are introduced to a greater extent than slower moving analytes. The results presented below have been corrected for this phenomenon (*5*). After injection, the sample vial is replaced by a vial containing the *p*H 10 buffer and high voltage is applied for separation of the amino acids. After each run, the high voltage is removed for a few minutes to allow the capillary to cool to ambient temperature. Although we have obtained separation efficiency of greater than 800,000 theoretical plates with a fresh capillary, the instrument more routinely produces 400,000 theoretical plates over a several month period.

Strong electroosmotic flow drives solvent from the positive to the negative end of the capillary; anions, cations, and neutral species may be detected in a single separation as they flow from the positive to the negative electrode. Detection occurs in a region after the capillary in a sheath flow cuvette (Fig. 1). This cuvette, commonly used in flow cytometry (*6*), provides good optical quality for high-sensitivity detection while virtually eliminating postcolumn band broadening that would degrade the separation performance; excellent laser-induced fluorescence detection limits have been achieved with this cuvette (*7*). In our system, the exit of the capillary tube is inserted into a quartz flow chamber 250 μm by 250 μm square. Sheath fluid, identical to the separation buffer, is introduced into the cuvette by a liquid chromatography pump at a flow rate of 9.3 μl/min. To complete the electrical circuit for electrophoresis, the stainless steel plumbing associated with the sheath stream is held at ground potential. Under the very low flow rate produced by electroosmosis, ~40 nl/min, the sample travels as a stream with a ~10-μm diameter through the center of the cuvette. Fluorescence is excited by a light-regulated, 1-W argon ion laser beam focused to a 10-μm spot about 0.2 mm downstream from the capillary exit. A laser wavelength of 488 nm both matches the absorbance spectrum of the FITC–amino acids (*2*) and provides a convenient spectral window wherein the fluorescence band falls between the excitation wavelength and the main water Raman band at 585 nm. Fluorescence is collected at right angles to both the sample stream and the laser beam with a 0.45 numerical aperture (NA), 18× microscope objective. A 495-nm, long-wavelength pass colored glass filter is used to block scattered laser light, whereas a 560-nm, short-wavelength pass interference filter is used to block the Raman band of water. A

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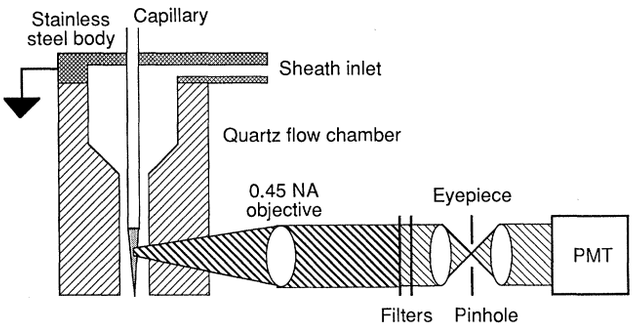

Fig. 1. Laser-induced fluorescence detector. The fused silica capillary (inner diameter, 50 μm) used for electrophoresis is placed about 1 cm into the flow chamber of the sheath flow cuvette. The sheath stream surrounds the sample as it exits from the capillary, forming a thin stream in the center of the flow chamber. A focused laser beam, not shown, excites fluorescence. The fluorescence is collected at right angles with a 0.45 NA microscope objective, spectrally filtered to reduce Raman and Raleigh scatter, and passed through an eyepiece fitted with a pinhole 200 μm in radius. The pinhole restricts the field of view of the PMT to the illuminated sample stream. The stainless steel body of the cuvette and the associated plumbing are held at ground potential.

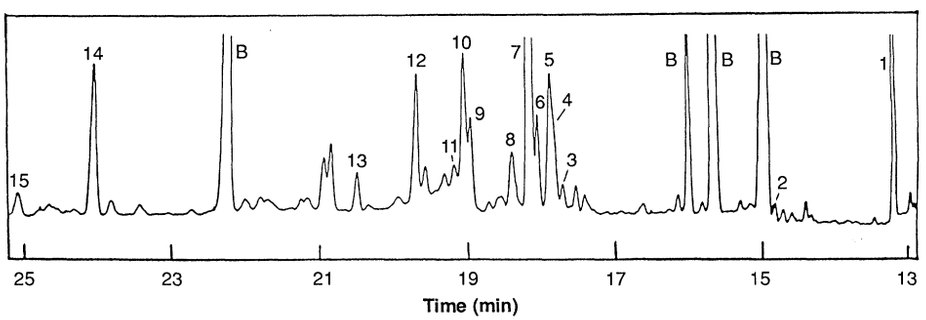

Fig. 2. Separation of between 2 and 7 amol of 18 amino acids. The separation is driven by a 25-kV potential, and a *p*H 10 buffer is used for both the separation and the sheath stream. Injection was for 10 s at 2 kV. Amino acids identified: peak 1, Arg; 2, Lys; 3, Leu; 4, Ile; 5, Trp; 6, Met; 7, Phe, Val, His, and Pro; 8, Thr; 9, Ser; 10, Cys; 11, Ala; 12, Gly; 13, Tyr; 14, Glu; and 15, Asp; peaks marked B are associated with the reagent blank.

Table 1. Detection limits for FITC–amino acid analysis (*10*). Detection limits are defined as the amount of analyte injected onto the capillary column that produces a signal three times as large as the estimated standard deviation of the blank signal, evaluated over a time period given by the peak width. All units refer to samples injected onto the column.

Amino acid	Concentration ($\times 10^{-11}M$)	Moles ($\times 10^{-20}$)	Molecules
Ala	3.4	4.6	27,000
Arg*	<0.5	<0.9	<5,700
Asp	6.6	6.8	41,000
Cys	2.4	3.3	20,000
Glu	2.6	2.8	17,000
Gly	2.8	3.7	22,000
Ile	1.7	2.5	15,000
Leu	7.0	10	61,000
Lys	8.6	15	90,000
Met	1.6	2.3	14,000
Ser	1.6	2.3	13,000
Thr	2.6	3.7	22,000
Trp	1.1	1.7	9,900
Tyr	1.1	1.4	8,400

*The values represent upper bounds because the Arg peak is off-scale in Fig. 2.

pinhole 200 μm in radius is located in the reticle position of a 20× microscope objective to restrict the field of view of the photodetector to the illuminated sample stream. Fluorescence is detected with a 1P28 photomultiplier tube (PMT) that was wired for fast response (*8*). The output of the PMT is directed across a 2.9-kilohm resistor in parallel with a 220-μF capacitor and then to a strip-chart recorder. The sheath flow cuvette interacts with the electrophoresis, producing back-pressure and slowing the separation. It is important not to perform injection while the sheath stream is pressurized; the pressure provided by the pump will drive the sample from the capillary tube. The interaction between capillary zone electrophoresis and the sheath flow cuvette is complicated and will be described in detail elsewhere (*9*).

The FITC–amino acids were prepared as follows (*3*). A $5.5 \times 10^{-4}M$ solution of FITC–isomer 1 was prepared in acetone containing a trace of pyridine, and a $10^{-4}M$ solution was prepared for each amino acid in 0.2*M* aqueous carbonate buffer, *p*H 9.0. Each amino acid solution (2 to 5 ml) was allowed to react with 20 μl of the FITC solution for 2 to 4 hours in the dark. We prepared the mixture of 18 amino acids by mixing small amounts of the individual FITC–amino acid solutions and then diluting them with the 5 m*M*, *p*H 10, carbonate buffer to the desired concentration. Samples were analyzed within 24 hours of preparation. We calculated the amount of analyte formed in the reaction with FITC as the limiting reagent, assuming complete reaction and pure reagents. Peaks associated with the underivatized reagent attest to both incomplete reaction and reagent impurities; the amounts of analyte reported below are conservative upper bounds on the actual amounts used.

The results of the analysis of 18 FITC–amino acids, ranging from 2 to 7 amol injected, are shown in Fig. 2. Of the 18 amino acids analyzed, 14 were separated. Phenylalanine, histidine, valine, and proline coeluted. It is anticipated that these four amino acids may be separated by manipulation of the composition of the separation buffer. Separation efficiencies, measured from the full width at half height, were greater than 400,000 theoretical plates for this mixture. We investigated the reproducibility with respect to peak height and retention time by use of multiple injections. A 10% variation in peak height was typical and appears to be a result of variation in the manual injection time. An automated injection timer might improve peak height reproducibility. The elution time for peaks shifted slightly during the day; this shift appears to be associated with variations in room temperature. The analyte's velocity in the capillary is inversely related to the viscosity of the separation buffer, which, in turn, varies by ~1% per degree Celsius temperature change. A constant-temperature oven or water bath would prove useful in the regulation of the capillary temperature.

Detection limits for the FITC–amino acid derivatives were calculated by the method of Knoll and are summarized in Table 1 (*10*). The mass detection limits correspond to less than 9×10^{-21} mol of arginine, the best case, and to 1.5×10^{-19} mol of lysine, the worst case, injected onto the column. The concentration detection limit corresponds to less than $5 \times 10^{-12}M$ arginine and to $9 \times 10^{-11}M$ lysine injected onto the column. Differences in the detection limits probably reflect differences in both the extent of reaction and the quantum yield of fluorescence for the derivatives. Because arginine produces a peak that is off-scale in this separation, the reported value represents an upper bound on the detection limit. Note that 9×10^{-21} mol corresponds to fewer than 6000 analyte molecules injected onto the column.

A detection limit of 9×10^{-21} mol represents an improvement of four orders of

magnitude over the previous state of the art for amino acid analysis; the best previous amino acid detection limits cluster around 10^{-16} mol for both fluorescence (*11*) and thermo-optical absorbance (*12*) detection. The present detection limits were obtained with a derivatizing reagent that may be used in a modified Edman degradation scheme (*1*). State-of-the-art detection limits with the conventional Edman degradation reagent, phenylisothiocyanate, fall near 10^{-12} mol (*13, 14*), and earlier work with FITC–amino acids has produced detection limits near 10^{-14} mol (*14*).

At present, there is much interest in the sequence determination of minute quantities of proteins (*14*). Because FITC has been used as a modified Edman degradation reagent, it is, in principle, possible to use this reagent to determine the amino acid sequence of low and subattomole quantities of proteins. However, extrapolation of current technology by six orders of magnitude is fraught with difficulty. Before attomole quantities of proteins can be sequenced, at least two significant issues must be addressed. First, the sample produced by the sequenator must be transferred to the electrophoresis column. Conventional sequenators produced sample volumes of a few microliters, whereas it is necessary to use samples of nanoliter or smaller volume in capillary zone electrophoresis. Second, reagent purity must be drastically improved to minimize the reagent blank. The first problem might be avoided by concentrating the amino acid before it is introduced into the separation capillary. Unfortunately, any technique that concentrates the sample also concentrates impurities. Instead, it may be profitable to redesign the solid-phase sequenator to match the volume requirements of the electrophoresis separation. Miniaturization of the sequenator offers several advantages. For example, preliminary concentration of the sample is not necessary, because the sequenator would produce samples matched in size to the requirements of the electrophoresis. Also, miniaturization of the sequenator would result in a proportional decrease in the volume of reagent required for the Edman degradation steps; because only a few nanoliters of reagent might be required for the degradation, costly and very high purity reagents can be used. The development of this miniaturized sequenator will require significant attention to detail to avoid sample loss and contamination. However, techniques developed for the manipulation of minute samples in capillary chromatography, electrophoresis, and flow injection analysis should provide a useful guide in the development of this attomole sequenator.

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Xeroderma Pigmentosum Group E Cells Lack a Nuclear Factor That Binds to Damaged DNA

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The disease xeroderma pigmentosum is characterized by deficient repair of damaged DNA. Fusions of cells from different patients have defined nine genetic complementation groups (A through I), implying that DNA repair in humans involves multiple gene products. In this report, an extension of the gel electrophoresis binding assay was used to identify at least one nuclear factor that (i) bound to DNA damaged by ultraviolet radiation or the antitumor drug cisplatin, but (ii) was notably absent in cells from complementation group E. Therefore, the factor appears to participate in a versatile DNA repair pathway at the stage of binding and recognition.

INDIVIDUALS HOMOZYGOUS FOR XERODERMA pigmentosum (XP) are hypersensitive to ultraviolet (UV) radiation and have a high incidence of skin cancers (*1*). XP cells are defective in the repair of damaged DNA containing UV-induced pyrimidine dimers. So far, the molecular basis for the XP defect has remained undefined. By contrast, the uvrABC endonuclease system in *Escherichia coli*, which catalyzes the excision repair of pyrimidine dimers, is well characterized. UvrA protein binds to UV-irradiated DNA by itself (*2*), but uvrB protein does not (*3*). However, both proteins together form a stable complex of uvrA, uvrB, and DNA. UvrC protein then catalyzes the enzymatic nicking of the damaged DNA. Motivated by the possibility that proteins analogous to uvrA and uvrB might be found in human cell extracts, we extended the gel electrophoresis binding assay (*4*) to the identification of proteins that bind to damaged DNA rather than to specific DNA sequences.

XP cells are defective in the repair of many forms of DNA damage in addition to UV damage. In particular, they are defective in the repair of transfected plasmid DNA that has been cross-linked in vitro by the antitumor drug cisplatin, cis-$Pt(NH_3)_2Cl_2$ (*5*). To find proteins that participate in the DNA repair system defined by the XP mutations, we searched for factors that would bind specifically to DNA damaged by either UV radiation or cisplatin.

DNA probes f65 and f103 were constructed as substrates for UV- and cisplatin-induced damage, respectively (Fig. 1). Fragment f65 contained a string of eight consecutive thymine residues that was a target for UV-induced thymine dimers. Fragment f103 contained a string of 14 consecutive guanine residues as a target for cisplatin, which shows a preference for forming intrastrand cross-links at the N7 position of adjacent guanine residues (*6*).

Nuclear extracts were prepared from HeLa cells by methods previously used to

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