High-sensitivity HPLC Quantification of Nonfluorescent but Photolabile Analyte through Photoreversion in Fluorescence Detector

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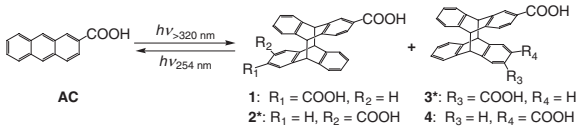
Ultrahigh sensitivity chiral HPLC analysis of nonfluorescent cyclodimers of 2-anthracenecarboxylic acid (AC) was achieved with much improved accuracy and reproducibility through a nonconventional on-detector photoreversion/re-excitation/detection mechanism. Excitation of cyclodimers at 254 nm in the detector cuvette caused photoreversion to AC, in situ excitation of which led to strong fluorescence at longer λ. The sensitivity was enhanced by a factor of ca. 2000 compared to usual UV detection.

High-performance liquid chromatography (HPLC) is one of the most prevalent instruments for analyzing a variety of inorganic, organic, and biological species. In particular, chiral HPLC has become more popular in recent years and a number of chiral stationary phases are now available for the separation of diverse chiral compounds.[1] Simultaneously, a range of optical detectors, sensing the change in refractive index, UV–vis absorption, circular dichroism, and fluorescence, have been developed for quantitative analysis with better sensitivity, accuracy, and reproducibility. Among them, fluorescence (FL) detection is practically the most sensitive and can analyze a trace amount (down to sub-pg) of sample with high signal-to-noise ratio, as long as the analyte fluoresces in reasonable quantum efficiency. However, if the analyte is nonfluorescent or poorly fluorescent, FL detection becomes useless unless the analyte is labeled with a fluorescent tag.[2]

In our study to reveal the detailed mechanism of supramolecular [4 + 4] photocyclodimerization of 2-anthracenecarboxylic acid (AC) with serum albumins,[3] we intended to precisely determine the relative yield and enantiomeric excess of photocyclodimers at very low conversions. For that purpose, we employed an FL detector in chiral HPLC analysis to find a remarkable enhancement of sensitivity, accuracy, and reproducibility, which is assignable not to the cyclodimer fluorescence but to the fluorescence of AC generated by photoreversion upon excitation of the cyclodimers in the detector cuvette. This new detection method, utilizing a series of photochemical and photophysical on-detector events, i.e. the photoreversion of cyclodimers to AC followed by the re-excitation and fluorescence of produced AC, provides us with a powerful and practical tool for significantly expanding the range of fluorescence detection without using tedious pretreatment or fluorophore-labeling techniques.

A THF solution of AC (7.5 mM) was irradiated for 12 h under Ar at >320 nm with a 300-W high-pressure Hg lamp fitted with a uranium filter to give a photolyzate (Scheme 1), which was subjected to chiral HPLC analysis on a JASCO LC-2000 Plus equipped with a tandem column of Cosmosil 5C18-AR-II (4.6 mm × 150 mm, Nacalai) and Chiralcel OJ-RH (4.6 × 150 mm, Daicel) at 35 °C. The column was eluted with a 64:36 (v/v) mixture of deionized water and acetonitrile containing 0.1% trifluoroacetic acid at a rate of 0.5 mL min^{-1}. The effluent from the column was analyzed by a triple detection system composed of the first UV–vis (UV1; JASCO UV-2075; detection at 254 nm), fluorescence (FL; JASCO FP-2025; excitation at 254 nm and emission at 420 nm), and the second UV–vis (UV2; JASCO UV-970; detection at 386 nm) detectors. The wavelengths were carefully chosen and set at 254 nm for detection of aromatics by the UV1 detector. The excitation for the FL detector was set at 254 nm to achieve the photoreversion of cyclodimers[4,5] and excitation of regenerated AC and at 420 nm for detection of regenerated AC. The UV2 detector was set at 386 nm for selective detection of regenerated AC. The photoreversion occurred not in UV1 but in the FL detector, because of the intense Xe lamp compared to the D_2 lamp used in the UV detector.



Scheme 1. Photocyclodimerization of 2-anthracenecarboxylic acid (AC) upon irradiation at >320 nm and photoreversion of cyclodimers upon irradiation at 254 nm.

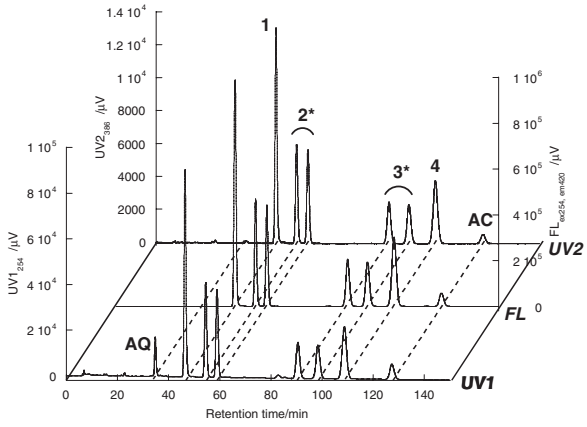


Figure 1. Typical chromatograms of a photolyzate, which contains AC, cyclodimers **1–4**, and by-product 2-carboxyanthraquinone (AQ), recorded by a series of UV1 (254 nm), FL (excitation at 254 nm, emission at 420 nm), and UV2 (386 nm) detectors (from bottom to top).

Table 1. Responses of serial UV1, FL, and UV2 detectors upon chiral HPLC analysis of stereoisomeric AC photocyclodimers **1–4**

Detector[a]	Injected volume[b] /μL	Peak area[c]/V s (Product distribution/%)					
		1	**2+**[d]	**2−**[d]	**3+**[d]	**3−**[d]	**4**
$UV1_{254}$	5	0.526 (31.1)	0.262 (15.6)	0.258 (15.3)	0.170 (10.0)	0.169 (10.0)	0.303 (18.0)
	45	4.78 (31.1)	2.39 (15.5)	2.35 (15.3)	1.55 (10.0)	1.56 (10.1)	2.78 (18.0)
$FL_{ex254,em420}$	5	7.19 (31.4)	3.37 (14.7)	3.34 (14.5)	2.35 (10.2)	2.36 (10.3)	4.35 (18.9)
	45	56.8 (29.5)	28.4 (14.8)	28.3 (14.7)	20.7 (10.8)	20.8 (10.8)	37.4 (19.4)
$UV2_{386}$	5	0.086 (31.2)	0.041 (14.7)	0.040 (14.6)	0.028 (10.2)	0.028 (10.0)	0.054 (19.3)
	45	0.736 (29.8)	0.365 (14.9)	0.362 (14.7)	0.257 (10.4)	0.263 (10.7)	0.475 (19.5)

[a]UV1, FL, and UV2 detectors were installed in series; UV1 operated at 254 nm, FL excitation set at 254 nm and emission at 420 nm, and UV2 at 386 nm. [b]The sample solution that contained **1–4** in ca. 0.05 mM total concentration (in addition to by-product AQ and remaining AC) was prepared by dissolving the evaporated photolyzate in the eluent solution. [c]Integrated recorder response (in μV) for the duration (in s) of each peak. [d]The positive/negative signs indicate the first/second-eluted enantiomers of chiral products **2** and **3**.

Figure 1 illustrates typical responses of UV1, FL, and UV2 detectors upon chiral HPLC analysis of the photolyzed sample. $UV1_{254}$ (bottom) indicates the presence of cyclodimers **1–4** and by-product 2-carboxyanthraquinone (AQ) as well as the remaining AC, while $FL_{ex254,em420}$ (middle) shows only the cyclodimer and AC peaks (due to the nonfluorescent nature of AQ) and $UV2_{386}$ (top) displays a tiny additional peak of AQ. It is to note that the origin of the intense peaks appearing in the FL trace is not the direct fluorescence of cyclodimers (which are practically nonfluorescent) but the fluorescence of AC regenerated by the photoreversion of cyclodimers upon excitation at 254 nm in the FL detector. This is supported by the fact that, in the analysis of isolated **1** under the same conditions, no corresponding peak was detected by the UV2 detector when the FL detector was turned off (see Figure S1).[5]

For the practical application of this method where the fluorescent photoproduct is generated in the detection cuvette from a nonfluorescent analyte, it is essential to guarantee the qualitative accuracy and reproducibility of the detector responses before and after the in situ photoreaction. In the present case, the agreement of the product distribution of cyclodimers **1–4** determined by conventional UV–vis detection with that by in situ photodecomposition-fluorescence detection is an absolute requirement. In Table 1, the peak areas of cyclodimers **1–4** determined by UV1, FL and UV2 detectors are listed. The product distributions, shown in parentheses, are fully consistent with each other within the experimental error (±1%), irrespective of the detector used or the injected sample volume. This indicates that the molar extinction coefficients and the photoreversion efficiency are comparable for cyclodimers **1–4** (including enantiomeric **2** and **3**), and hence validates the use of the post-column/on-detector photolysis-fluorometry method for analyzing the photoreversible product mixture. Thus, combining the inherently high sensitivity and low baseline drift of the FL detector with the photoreversion of analytes to highly emissive AC, the detection limit was expanded down to a one pg level, which is more than 2000 times lower than that of conventional UV detection (see Figure S6).[5]

We have demonstrated in the present study that nonfluorescent but photolabile AC cyclodimers can be quantified with much enhanced sensitivity, accuracy, and reproducibility by simply using a FL detector attached to a conventional HPLC apparatus. This method is not restricted to the anthracene photodimers but is also applicable in principle to any photolabile and photoreversible analytes, as long as the photoproduct generated in situ in the FL detector is more emissive than the original analyte. For example, this method could be used with dearomatized compounds derived from fused aromatic compounds such as naphthalene, anthracene, coumarin, and acenaphthylene.

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5 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

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