Ultraviolet-Excitation Photothermal Heterodyne Interferometer as a Micro-HPLC Detector

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The first demonstration of a photothermal heterodyne interferometer (PHI) combined with micro-HPLC (high-performance liquid chromatography) is reported. A semiconductor laser (375 nm) was used for excitation, and the temperature change caused by heat released from photoexcited species was detected with a He-Ne laser (632.8 nm). The temperature-dependent refractive index change of the solvent modified the optical path of the probe beam. The phase difference between two arms of the interferometer, one passing through the heated sample and another as a reference, was sensitively detected with the PHI. The nitro-polycyclic aromatic hydrocarbon and vitamin mixture separated via micro-HPLC was successfully detected with the PHI as well as a UV detector. The detection limit of the PHI for riboflavin in the absorbance units was 77 times better than that of the commercial UV detector. The detection limit of the PHI with a small flow cell (6 nL) was the same as that with a large flow cell (18 nL) for 1-nitropyrene.

Keywords Photothermal heterodyne interferometer, micro-HPLC, 375 nm excitation, nitro-polycyclic aromatic hydrocarbons, vitamins

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Introduction

High-performance liquid chromatography (HPLC) is a powerful analytical technique for separating compounds in a solution. Micro-HPLC equipped with a smaller diameter column, smaller diameter tubing, and smaller flow cell than conventional HPLC can improve separation performance by reducing on-column sample dilution and the amount of solvent waste. Ultraviolet-visible (UV-VIS) absorption detection is most widely applicable because all compounds of practical importance have optical absorption in the UV and some in the VIS regions. However, its sensitivity is sometimes insufficient. Furthermore, the short optical path length in a small flow cell results in poor sensitivity, since the absorbance is proportional to the optical path length. Highly sensitive fluorescence detection can be applied even at the single molecule detection level but is not applicable to all molecules of practical importance. Thus, when less fluorescent or nonfluorescent molecules, such as a large fraction of biologically and environmentally related molecules, are targets, fluorescence detection requires pre- and post-column derivatization or an indirect detection scheme with less sensitivity. The derivation method is sometimes troublesome and time consuming. A highly sensitive method without derivation is ideal for HPLC analysis.

Photothermal spectroscopy, including photothermal lensing, photothermal deflection spectroscopy, and photoacoustic spectroscopy, is one of the most sensitive optical methods for observing nonfluorescent molecules. The photothermal method is based on the detection of physical property changes by heat generation via optical absorption and subsequent nonradiative relaxation. This photothermal heating can be observed by probing the changes in the refractive index of a solvent. Tokeshi et al. reported a detection limit of 0.4 molecules of Pb (2) octaethylporphyrin in a 7-fL detection volume using a thermal lens technique. Photothermal spectroscopy has been combined with separation techniques such as HPLC,2–4 capillary electrophoresis,5,6 and microdevices.7,8 Zare et al. reported that a thermal lens is 140 times more sensitive than a commercial UV-HPLC detector for anthraquinone dyes.3

McLean combined a photothermal method with a homodyne interferometer, which is one of the most sensitive optical techniques for measuring optical path lengths. Many applications and theories of this technique have been discussed.10,11 Instead of the divergence change or deflection of the probe beam, the photothermal interferometric method uses a phase shift between two beams of the interferometer. One arm of the interferometer passes through the laser-heated portion of the sample solution where the optical path length changes with the refraction index changes of the solvent. The phase change relative to the beam passing through another arm of interferometer is monitored. Seidel et al. applied a photothermal homodyne interferometer to an HPLC detection device.12

The heterodyne interferometer (two different frequencies) is a more sensitive detection method than the homodyne interferometer (single frequency). Takahashi et al. has developed heterodyne interferometric photothermal equipment.13,14 The heterodyne interferometer uses two beams, each having slightly different frequencies, to produce a beat signal after their combination. The advantage of a heterodyne interferometer is to realize sensitive detection of the phase shift of the beat signal

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even under beam intensity fluctuation. This technique might have great potential as an HPLC detector utilizing photothermal phenomena.

Here we report a newly developed photothermal heterodyne interferometer (PHI) combined with micro-HPLC and demonstrate, for the first time, separation and sensitive detection with nonderivatized chemical species having environmental and biological importance. Nitro-polycyclic aromatic hydrocarbons (NPAHs) as environmental, mutagenic, and carcinogenic chemicals and vitamins as model metabolites were selected as target species. Incomplete combustion processes of fossil fuels release NPAHs into the living environment, and NPAHs are present in low concentrations in the environment as compared with polycyclic aromatic hydrocarbons (PAHs). Since NPAHs exhibit only weak fluorescence, the reduction of NPAHs to their corresponding amino-PAHs or the reaction with chemiluminescence reagents after their reduction is usually performed for high-sensitivity analysis of NPAH separation and detection with HPLC/fluorescence or chemiluminescence, respectively. The analysis of multiple metabolites in biofluids is essential for biomedical diagnoses.

Experimental

Reagents and chemicals

Nitro-polycyclic aromatic hydrocarbons and vitamins were used as received without further purification. 1-Nitronaphthalene, riboflavin (vitamin B2), folic acid (vitamin B9), and cyanocobalamin (vitamin B12) were purchased from Wako Pure Chemical. 1,6-Dinitropyrene and 6-nitrochrysene in toluene (100 μg/mL) were obtained from AccuStandard Inc. 1-Nitropyrene was obtained from Acros Organics. Water was deionized and purified with a Millipore Milli-Q system. Acetonitrile of HPLC grade was purchased from Sigma-Aldrich. All of the solvents were filtered through membrane filters of 0.20 μm pore size supplied by Advantec Co., Ltd.

Apparatus

A schematic illustration of the experimental setup and the combination of PHI and micro-HPLC is shown in Fig. 1 (the principle and advantages of PHI are described in Supporting Information). The excitation beam was emitted from a GaN diode laser (wavelength, 375 nm; maximum power, 70 mW; TC20-03750070-45, Neoark Co.). The beam was intensity modulated at 150 Hz with a function generator (FG-273, Kenwood). A frequency-stabilized He-Ne laser (633 nm, 1.5 mW, Research Electro-Optics, Inc.), whose frequency corresponds to 474 THz, was used as the light source of the interferometer and split into two beams (sample beam and reference beam) of equal intensity using a nonpolarizing cube half mirror (NPCH-10-6328, Sigma Koki Co., Ltd.). The sample and reference beams were frequency shifted by two acousto-optic modulators (AOMs) operating at 110 and 80 MHz, respectively. The excitation beam and the sample beam were coaxially combined by a dichroic mirror and allowed to irradiate a U-shaped capillary flow cell connected to a micro-HPLC. Two types of commercially available capillary flow cells for an HPLC/UV detector were used for PHI detection: one has a cell volume of 18 nL (Cat. No. 6001-70167, GL Sciences, Inc.), and the other has a cell volume of 6 nL (Cat. No. 6001-70170, GL Sciences, Inc.). They have optical pass lengths of 4 mm and volumes that are well matched to a micro-HPLC.

The sample beam passing through the flow cell was mixed with the reference beam by a nonpolarizing cube half mirror (NPCH-10-6328, Sigma Koki Co., Ltd.). When two beams interfered, a beat signal at 30 MHz was detected with an avalanche photodiode (3-dB bandwidth; 25 kHz - 125 MHz; New Focus 1611-AS-FS, Newport, Ltd.). The 30 MHz beat signal was demodulated to generate optical phase-dependent voltage, with the electrical circuits designed by Kobe Steel, Ltd. The output was connected to the lock-in amplifier (5610B, NF Corporation) to measure the signal amplitude synchronized with the intensity modulation of the excitation beam at 150 Hz. The time constant was set to 300 ms. This optical PHI system was set on a non-air desktop active vibration

Fig. 1 Experimental apparatus of a photothermal heterodyne interferometer combined with a micro-HPLC.
isolation table (TS-140, Herz Co., Ltd.) in an acoustic enclosure (AEK-3000, Herz Co., Ltd.) to remove vibrational and acoustic noises from the outside.

Chromatographic separation was performed on a micro-HPLC system (Micro21 series, Jasco Co., Ltd.) equipped with a binary pump and an autosampler. A hydrophilic interaction chromatography (HILIC) column (SeQuant ZIC HILIC, 5 μm particle size, 1 mm inner diameter, 150 mm long, Merck Millipore Ltd.) was selected for vitamins, and a reversed-phase microcolumn (Inertsil ODS-P, guard column; 5 μm particle size, 0.3 mm inner diameter, 50 mm long, analytical column; 5 μm particle size, 0.3 mm inner diameter, 150 mm long, GL Sciences, Inc.) was selected for NPAHs. The mobile phase of both vitamins and NPAHs was 80% (v/v) acetonitrile–water. The injection volume was 1.0 μL in all of the experiments. The mobile phase was filtered through a membrane with 0.2 μm pores. Isocratic elution was performed at a flow rate of 20 μL/min for vitamins and 10 μL/min for NPAHs and a column temperature of 30°C for vitamins and 40°C for NPAHs. To compare the sensitivity, a commercial UV detector (Micro21UV-01, Jasco Co., Ltd.; cell volume, 16 nL) was used. The absorbance was monitored at 270 nm for vitamins and at 375 nm for NPAHs. Eluent from the separation column was connected directly to the capillary flow cell for PHI measurements or commercial UV detection by using the fused silica capillary (50 μm inner diameter, 375 μm outer diameter). The union joint (P-779, swept volume 8 nL, IDEX Health & Science, LLC) was used for minimized dead volumes to connect the separation column and the PHI flow cell. This joint prevents peak dispersion. The UV detector values and the amount of output voltage of the lock-in amplifier in the PHI equipment were sent to a computer using the Jasco LC-NET/ADC. The chromatograms were recorded at a data-sampling rate of 1.7 Hz and analyzed with JASCO-BORWIN software. All measurements were taken under steady-state conditions at room temperature (25 ± 2°C).

Results and Discussion

Figure 2 shows an HILIC phase chromatogram of a mixture of three vitamins using a PHI. All of the peaks have been separated successfully. Hydrophilic interaction chromatography is designed for the separation of polar and hydrophilic compounds and can use high concentrations of organic solvents in the mobile phase. The magnitude of the photothermal signal is dependent on the physical properties of the mobile phase, such as thermal conductivity and the temperature derivatives of the refractive index. Most organic solvents produce sensitivity tens of times higher than that of water. Separation using a HILIC column improves the sensitivity of PHI detection.

Figure 3 shows a reversed-phase chromatogram of a mixture of 4 NPAHs using a PHI and a UV detector. The Inertsil ODS-P microcolumn used was selected to increase retention for compounds with a planar structure because of densely covered octadecyl groups as compared to a common reversed-phase column. Four kinds of NPAHs have been separated successfully and analyzed with a PHI and a commercial UV detector. The wavelength 375 nm of PHI excitation and UV detection was chosen because the sum of the absorbance of four NPAHs is maximal at 375 nm. The chromatographic results are summarized in Table 1, together with the octanol/water partition coefficient \( K_{ow} \), defined as the equilibrium ratio of a chemical’s concentrations in a two-phase system of n-octanol and water. The log \( K_{ow} \) determination from chromatographic retention times has been explored extensively in the reversed-phase C18 column.17,18 As expected, Table 1 shows that NPAHs with low log \( K_{ow} \) values may be eluted earlier than those with high log \( K_{ow} \) values. All of the peaks were closely eluted but remained separate with a resolution >1.5. Note that resolution

### Table 1 Chromatographic parameters of the peaks of NPAHs for PHI detection

| Analyte         | log \( K_{ow} \) | Retention time/min (UV) | Retention time/min (PHI) |
|-----------------|------------------|-------------------------|--------------------------|
| 1-Nitronaphthalene | 3.19 ± 0.22     | 2.53                    | 2.68                     |
| 1,6-Dinitropyrene  | 4.44 ± 0.21     | 3.73                    | 3.85                     |
| 1-Nitropyrene      | 5.29 ± 0.21     | 4.94                    | 5.05                     |
| 6-Nitrochrysene    | 5.41 ± 0.21     | 5.91                    | 6.02                     |
shows the ability to separate two peaks and that two peaks with a resolution value greater than 1.5 are generally considered to be well separated.

The difference in retention times for each sample between the PHI and UV detector was 0.1 min. This time delay corresponds to the difference in the distance of the PHI or UV detector from the separation column. The chromatogram of the UV detector showed a large, fluctuating baseline noise, while that of the PHI provided a stable baseline. The PHI peaks were slightly sharper than those detected by UV. The smaller flow-cell volumes (6 nL) of the PHI will result in less chromatographic broadening.

The PHI system shows greater sensitivity than commercial UV detection. The HPLC calibration curves for the peak height of the chromatogram of riboflavin according to the PHI and UV detector are shown in Fig. S1 (Supporting Information). The concentration ranges examined were 0.8 - 60 μM with the UV detector and 0.04 - 1 μM with the PHI. Both plots show good linearity with correlation coefficients $R^2$ of 0.9958 using the UV detector and 0.9338 using the PHI. The PHI calibration curves of 1-nitropyrene are shown in Fig. S2 (Supporting Information). The signal intensity was linear with the concentration over four orders of magnitude and correlation coefficients of 0.9904.

The UV and PHI detection limits are examined as the values of three times the standard deviation (3σ) of the blank. The blank signal is analogous to the chromatographic baseline. The standard deviation (σ) of the blank signal was estimated from half of the short-term peak-to-peak noise in 2 min on a chromatogram. The detection limits (3σ) determined for riboflavin and 1-nitropyrene are summarized in Table 2, together with the molar absorptivity, excitation wavelength, optical length, and cell volume. For the UV detection, 270 nm of the absorption maximum of riboflavin was chosen. Detection at 270 nm is better for riboflavin than at 375 nm when the detection limits in concentration are considered, but for detection limits in absorbance, no large merit is expected among different wavelengths because the difference in molar absorptivity has no direct relation. The concentration detection limits of riboflavin with the PHI are 17 times better than those with the conventional UV detector. On the other hand, excluding the factors of molar absorptivity and optical length, and comparing these results with those for a similar cell volume, the detection limit in the absorbance unit was 77 times better with the PHI than with a UV detector for riboflavin. The detection limit of 1-nitropyrene measured with a 6-nL cell is similar to that of riboflavin with an 18-nL cell.

The gradient separation mode in HPLC, in which the composition of mobile phase as well as its refractive index is changing in time, is powerful for highly efficient separation. However, photothermal techniques are ill suited for the gradient separation mode: the detection sensitivity under gradient separation is generally lower than that under isocratic separation. This is mainly because locally incomplete mixing of different solvents results in turbulence in the thermal conductivity and temperature coefficient of the refractive index, causing noise in the photothermal signals. Molecular diffusion-dominated uniform mixture formation is quadratically faster in a smaller space. Thus, for photothermal detection, a micro-HPLC system with a smaller inner-diameter flow channel better suits the gradient separation mode than does a normal HPLC system. The technique using a micro-HPLC combined with a PHI provided high sensitivity, is widely applicable for the detection of various compounds, and has enormous potential for the analysis of small-volume samples, such as detection in microdevices.

### Conclusions

An ultraviolet-excitation photothermal heterodyne interferometer (PHI) combined with a micro-HPLC has been developed. The separation and detection of unlabeled species are for the first time, demonstrated. Mixtures of nitro-polycyclic aromatic hydrocarbons or vitamins were successfully separated and detected. The chromatogram of the PHI detector showed a stable baseline as compared with that of the commercial UV detector. The sensitivity of the PHI was nearly 77 times better than that of the commercial UV detector. There was no prominent difference in the sensitivity at different sample volumes of the flow cell, 6 and 18 nL in the PHI, indicating excellent PHI capabilities for analyzing small-volume samples. Although the 375 nm ultraviolet laser was operationally convenient for excitation, excitation at a short wavelength near 220 nm will greatly enhance versatility. The system of ultraviolet-excitation HPLC/PHI is expected to become a powerful tool for the direct analysis of environmental and biorelated substances.

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### Supporting Information

The principle of the photothermal heterodyne interferometer, mentioned in the Experimental section, and Figs. S1 and S2, mentioned in the Results and Discussion section, are available free of charge on the Web at http://www.jsac.or.jp/analsci/. Some descriptions of the principle and advantages of the PHI are given. The way to access Ref. 16 is noted as well.
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