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Note

Raman spectroscopic detection of haemoproteins in the eluate from high-performance liquid chromatography

KEIJI IRIYAMA* and YUKIHIRO OZAKI
Division of Biochemistry, Central Research Laboratory, The Jikei University School of Medicine, 3-25-8 Nishi-Shinbashi, Minato-ku, Tokyo 105 (Japan)
and
KIYOKATSU HIBI and TERUKI IKEDA
Japan Spectroscopic Co. Ltd., Ishikawa-cho, Hachioji-shi, Tokyo 192 (Japan)
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The development of new liquid chromatographic detectors (*e.g.*, Fourier transform infrared, nuclear magnetic resonance and Raman spectrometers) for practical use has been required for several years and has been studied by several workers, as reviewed elsewhere[1]. These detectors are believed to be promising for molecular structure determinations.

Recently, the application of resonance Raman spectrometry to effluent analysis in high-performance liquid chromatography (HPLC) has been studied by Saito *et al.*[2]. Substituted aminoazobenzenes in a methanol eluent were monitored at 1406 cm^{-1} and it was possible to obtain characteristic resonance Raman spectra of these aminoazobenzene derivatives by using a stop flow method[2]. However, there has been no report on the analysis of biomacromolecules by HPLC with Raman spectrometric detection.

In this work, we have extended an HPLC–Raman method to the detection of haemoproteins in an artifical fluid, not in a physiological fluid.

EXPERIMENTAL

Horse heart cytochrome *c* (cyt c) and equine skeletal muscle myoglobin (Mb) were purchased from Sigma (St. Louis, MO, U.S.A.) and were used as test materials without further purification. These haemoproteins were dissolved into a buffer solution (0.005 *M* potassium dihydrogen phosphate–disodium hydrogen phosphate, pH 6.8) containing 0.2 *M* sodium sulphate. A 20-μl volume of each haemoprotein solution as a test solution was usually injected for HPLC examination using Raman spectrometry. The buffer solution was used as the eluting solution.

Fig. 1 illustrates the system employed. A Twincle constant-flow pump (JASCO, Tokyo, Japan) was used to pump the eluting solution through a column (JASCO, Finepak SIL AF-102). The pump was operated at a pressure of 40 kg/cm^2 and delivered at a flow-rate of 1.0 ml/min. Test solutions were injected into the column with a microsyringe. The effluent from the column entered a Raman cuvette

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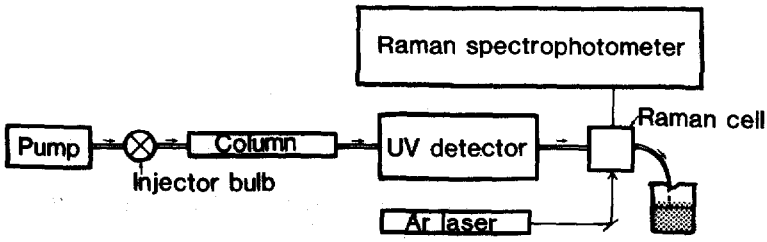

Fig. 1. Schematic diagram of the combined HPLC–Raman system.

cell via a UV detector (UVIDEC-100-III, JASCO). As a multi-channel detector system for Raman measurements was not available, a scanning-type laser Raman spectrophotometer (JASCO, Model R-800) equipped with a photomultiplier tube and photon-counting detection was used. The excitation wavelength used was the 480.0 nm line from an argon laser. The laser power at the Raman cell was about 400 mW. Raman peak frequencies are believed to be accurate to ± 1 cm^{-1} for well resolved bands under the present experimental conditions. The haemoprotein solutions injected were not photodecomposed by the laser power of 400 mW and could be recovered as purified haemoproteins.

RESULTS AND DISCUSSION

Fig. 2a and b show the high-performance liquid chromatograms and resonance Raman spectra of cyt c and Mb, respectively. The chromatograms were recorded at a

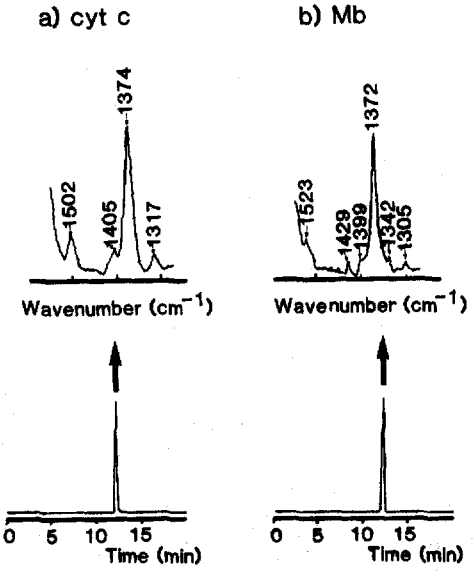

Fig. 2. Chromatograms and corresponding resonance Raman spectra of (a) cytochrome *c* and (b) myoglobin HPLC: wavelength of UV detector, 280 nm; flow-rate, 1.0 ml/min; sample concentration, 1.6 m*M*; sample injection volume, 20 μl. Resonance Raman spectra: excitation wavelength, 488.0 nm; laser power, 400 m*M*; time constant, 0.5 sec; scan speed, 60 cm^{-1}/min; spectral slit width, 8 cm^{-1}.

wavelength of 280 nm by the UV detector. The resonance Raman spectra were recorded by stopping the flow of the HPLC system at the retention times of cyt c and Mb, and measuring the characteristic Raman bands. It took 4.5 min to measure the resonance Raman spectra of a haemoprotein in the region of 1275–1550 cm^{-1}. The resonance Raman spectra of cyt c and Mb were identical with those previously reported[3,4]. The medium band is seen at 1317 cm^{-1} in the resonance Raman spectrum of cyt c, whereas the weak band is observed at 1305 cm^{-1} in the spectrum of Mb. This band, which is mainly due to the in-plane CH deformation mode at the methine bridges, can be used as a practical indicator for distinguishing B- and C-type haemoproteins[3]. The intense Raman bands observed at 1373 cm^{-1} in the spectra in Fig. 2a and b are the so-called oxidation-state marker of haeme iron[3]. Usually this marker band is located between 1355 and 1365 cm^{-1} for iron(II) haemoproteins and between 1370 and 1375 cm^{-1} for iron(III) haemoproteins. Thus, it can be concluded that the haemoproteins injected into HPLC system are in an oxidized form. The spin-state marker of haeme iron is located in the region of 1470–1505 cm^{-1}. For low-spin haemoproteins this marker is observed near 1500 cm^{-1} (Fig. 2a), whereas the corresponding band is seen weakly near 1480 cm^{-1} for high-spin haemoproteins (Fig. 2b).

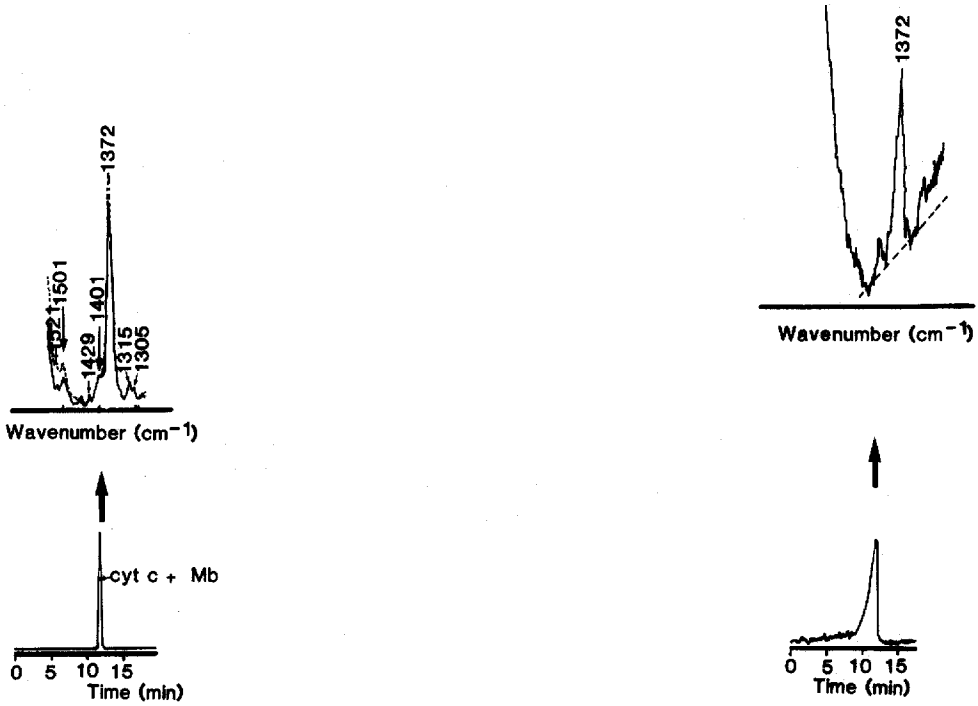

Fig. 3. Chromatogram and corresponding resonance Raman spectrum of a mixture of cytochrome *c* and myoglobin; 40 μl of a sample solution containing 0.8 m*M* cytochrome *c* and myoglobin was injected. Other conditions as in Fig. 2.

Fig. 4. Chromatogram and corresponding resonance Raman spectrum of the peak component (cytochrome *c*) in the chromatogram; 20 μl of a sample solution containing 0.4 m*M* cytochrome *c* were injected and the flow-rate was 1.0 ml/min. The chromatogram was monitored at a wavenumber of 1372 cm^{-1} with a Raman detector.

In this way, Raman detection can provide molecular structural information about the haeme group.

Fig. 3 shows a chromatogram obtained when 20 μl of a test solution containing 1.6 m*M* cyt c and 1.6 m*M* Mb was injected. The chromatogram shows a single peak, probably containing cyt c and Mb. The resonance Raman spectrum of the peak component(s) was recorded and is also presented in Fig. 3. The spectrum can be thus predicted to be the sum of the spectra of cyt c and Mb. The Raman spectrum synthesized by adding Fig. 2b to Fig. 2a using a computer is shown as a broken line in Fig. 3. The synthesized Raman spectrum is very close to the spectrum of the peak component(s) illustrated in the same figure as a solid line.

As described above, it may be possible to predict the type of haemoproteins, the oxidation and spin states and the presence of contaminants in a peak in a chromatogram.

In addition, the chromatogram was also monitored by the Raman detector at a wavenumber of 1372 cm^{-1} and is shown in Fig. 4 for the injection of 20 μl of a test solution containing 0.4 m*M* cyt c. The resonance Raman spectrum of the peak component (cyt c) is also shown in Fig. 4. In order to determine the detection limit of the Raman detector employed the chromatograms and the corresponding resonance Raman spectra were obtained as a function of concentrations of cyt c in test solutions. The band at 1372 cm^{-1} could be detected up to 20 μg of cyt c.

We chose a combined HPLC–Raman system for the following reasons: (i) the Raman technique is free from water interference; (ii) the technique is non-destructive; (iii) the laser beam, the spectroscopic sensor, does not require a large amount of sample to be injected; and (iv) a commercial HPLC instrument can be connected with a Raman cell without any interface. These merits in monitoring high-performance liquid chromatograms with a Raman detector have been demonstrated in this study. Probably this combined HPLC–Raman system can be applied to various kinds of biological materials.

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