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LASER FLUOROMETRIC DETECTOR FOR MICROCOLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

A fluorescence detector with a helium–cadmium CW laser as an excitation source has been constructed for high-performance liquid chromatography. The parameters of the detector were optimized with respect to the spectral characteristics of 5-dimethylaminonaphthalene-1-sulphonyl (Dns) amino acids. A method for the analysis of such compounds at the level of 10^{-16} mol has been developed based on microcolumn hydrophobic chromatography with the laser fluorometric detector. The separation of all components was achieved by use of an acetonitrile gradient. Ultra-high sensitivity of analysis is ensured by using capillary columns (0.5 mm I.D.) and a flow cell with detection volume variable between 0.1 and 0.4 μl and a post-column volume $\leqslant 0.05$ μl.

INTRODUCTION

From the methodological point of view, investigations of structure–function relationships of proteins and their fragments involve the isolation and identification of the component amino acids (in the form of chemically modified derivatives). One of the most promising methods for separating and subsequently analyzing micro quantities of amino acid derivatives is high-performance liquid chromatography (HPLC).

The main requirements of the chromatographic detectors may be formulated as follows. The maximum concentration sensitivity of detection, at the desired resolution, will be achieved when[1]

$$V = \leqslant 2\sigma_v = \frac{\pi d^2}{2} \cdot \frac{l}{N^{\frac{1}{2}}} \tag{1}$$

where V is the detection volume, σ_v is the standard chromatographic deviation of the peak in volume units, d is the inside diameter of the column, l is the working length and N is the number of theoretical plates involved in the chromatographic process.

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This suggests that for high efficiency and high chromatographic resolution with the minimum expenditure of the sample and the eluent, measuring cells with a small detection volume are required. In order to decrease extra-column band broadening the volume of the connection system between the column outlet and the detector cell should also be minimized.

The existing absorption detectors have a threshold sensitivity of no better than 10^{-9}–10^{-11} mol of the substance being analyzed[2,3]. Fluorometric detectors enable the detection threshold to be lowered to 10^{-13}–10^{-14} mol. For example, the BT 6630 series chromatograph of Biotronik (F.R.G.) has a fluorometric detector which enables measurement of some 5-dimethylaminonaphthalene-1-sulphonyl (Dns) amino acids at the 10^{-11} mol level and the FS-970 fluorometer of Kratos (U.S.A.) at the level of 10^{-12} mol. For the excitation source, gas-discharge lamps are used. However, their low spectral power density and omni-directional character of the emission do not fulfil the requirements placed upon detectors for HPLC.

The use of laser radiation for the fluorescence excitation offers new ways of improving the detection sensitivity and decreasing the size of the detection volume. Modern lasers can easily provide a radiation power of 10^{-2}–10 W in a narrow spectral region in the continuous wave (CW) mode, and hundreds of kilowatts in the pulse regime in a narrow collimated beam. This, in turn, offers the possibility of working with highly efficient columns of small diameter ($\leqslant 1$ mm).

It is interesting to estimate the detection limit of fluorescence analysis for the case where all the molecules are involved in the process of fluorescence. The fluorescence intensity is proportional to the number of photons emitted. The probability of photon emission is practically the same in all directions, because the fluorescence intensity is at least $4\pi/\omega$ times smaller than the brightness of the exciting source, where ω is the solid angle covered by the recording system. The minimum detectable number of photoelectrons, $Q = 10^2$–10^3, which related to the number of photons, N_p

$$Q = N_p \cdot \frac{\omega}{4\pi} \cdot \eta T \tag{2}$$

where η is the quantum yield of the detector photocathode and T is the transmission of the optical section of the recording channel.

Taking into account these factors, the efficiency of the recording channel is not greater than 1%[4]. Then the minimum detectable number of molecules will be 10^4–10^5, which corresponds to 10^{-20}–10^{-19} mol. This is the threshold sensitivity of laser fluorescence devices assuming that during recording each molecule will emit light only once.

DETECTOR OPTIMIZATION

Two versions of the laser detector can be used. In the first one, fluorescence is excited by CW laser radiation. The recording system measures the total light flux falling within its transmission band. This flux includes the fluorescence of the substance being analyzed, the fluorescence and luminescence of the eluent, upon laser irradiation, and of the material comprising the optical elements of the recording system.

The second version uses pulsed laser radiation where the duration of the pulse is less than that of the excited state of the substance being analyzed. In this case, the recording system measures the number of single-electron pulses only after the end of the pulse and the simultaneous count of the photons emitted[5]. In this manner it is possible partially to eliminate the scattered laser radiation, induced luminescence of the optical parts and fluorescence of the eluent. To achieve sufficiently rapid detection, the repetition frequency of the laser pulse must be not less than 10 kHz. At the present time, this requirement is satisfied only by metal vapour lasers operating in the visible spectral region, or the active mode-locked argon laser. These lasers can be used to excite the fluorescence of Dns derivatives of amino acids only if their output is converted into the ultraviolet radiation, which makes the excitation system much more complicated, enlarges its dimensions and decreases reliability.

In a liquid chromatograph, complex eluents are generally used consisting of several solvents differing in their chemical compositions and effects on the spectroscopic characteristics of Dns-amino acids. Since little information is available on this topic[6,7], the selection of appropriate spectral regions for fluorescence excitation and registration is difficult[8–11], and may lead to large errors in the quantitative analysis of Dns-amino acids. Therefore, we measured the absorption and fluorescence spectra and calculated the absolute quantum yields of 24 Dns-amino acids[12]. As solvents, doubly distilled water, methanol, acetonitrile and buffer solutions with different contents of acetonitrile were used.

The absorption spectra of Dns-amino acid solutions consist of three bands, while the fluorescence spectra comprise only one broad and structureless band. The ratio of the band intensities in the absorption spectrum and the positions of the absorption and fluorescence bands of Dns-amino acid solutions are practically independent of the nature of the amino acid (Fig. 1). However, the nature of the solvent has a significant effect on the positions of the absorption and fluorescence bands. On passing from acetonitrile to water, the fluorescence spectrum is shifted bathachromically on average by 50 nm with an hypsochromic shift of the long-wavelength absorption band by 10 nm. At the same time, the half-width of the fluorescence spectrum slightly increases (from 110 to 130 nm). Also of interest is the complete co-

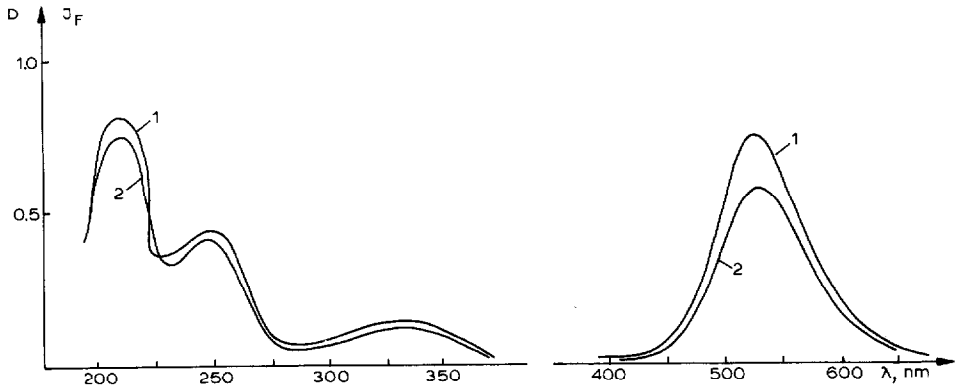

Fig. 1. Absorption and fluorescence spectra of Dns-Ala (1) and Dns-Gly (2) dissolved in acetonitrile.

incidence of the absorption and fluorescence spectra of Dns-amino acids in acetonitrile and methanol (Fig. 2). The luminescence efficiency of solutions of Dns-amino acids is practically independent of the nature of the amino acid, but is determined by the nature of the solvent. For example, the absolute quantum yields of Dns-amino acids in water were 5–8%, while in acetonitrile and methanol values up to 45% were found. The value of the absolute quantum fluorescence yield of a Dns-amino acid in a buffer solution is determined by the quantity of acetonitrile added.

The data obtained show that the optimum regions for excitation and recording of the fluorescence of Dns-amino acids are 325–335 and 540–570 nm, respectively.

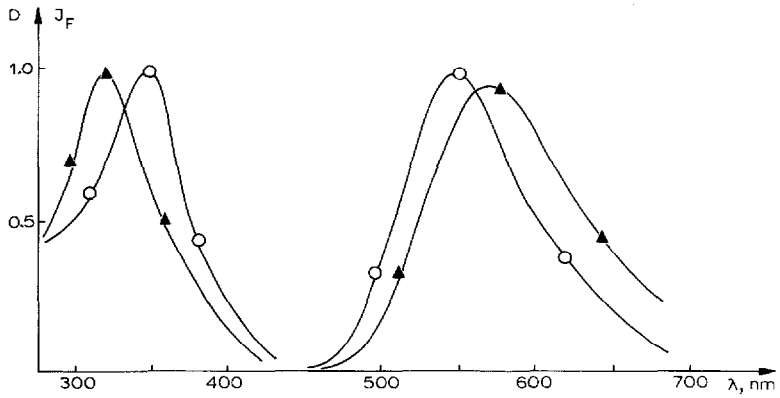

Fig. 2. Absorption and fluorescence specta of Dns-L-Pro in methanol or acetonitrile (○), and in water (▲).

It should be noted that in previous work the fluorescence of Dns-amino acids was recorded mainly at wavelengths shorter than 500 nm[8–11] where its intensity is sometimes one order of magnitude weaker than at the band maximum.

Most suitable for the fluorescence excitation of Dns-amino acids in organic eluents was the helium–cadmium laser generating radiation at 325 nm. This is because its radiation corresponds to the maximum of the long-wave absorption band, and excites the fluorescence of the solvent to a smaller extent than does excitation at longer wavelengths. These industrially produced lasers provide radiation power in the UV spectral region of 7–30 mW as a narrow collimated beam.

EQUIPMENT

We chose a helium–cadmium LGN-504 laser as the excitation source. A block diagram of the detector is given in Fig. 3. The laser provides a rated power of up to 10 mW and a beam divergence of $3 \cdot 10^{-3}$ rad. The laser beam passes through the lens and forms a 0.5 mm × 0.5 mm spot which illuminates the centre of a rectangularly shaped jet of the effluent under investigation.

The fluorescence radiation was collected by two recording channels with spectral transmission maxima of 550 nm for Dns derivatives of amino acids and 440 nm for the eluent, respectively. The photodetectors of both channels (PMT-79) were

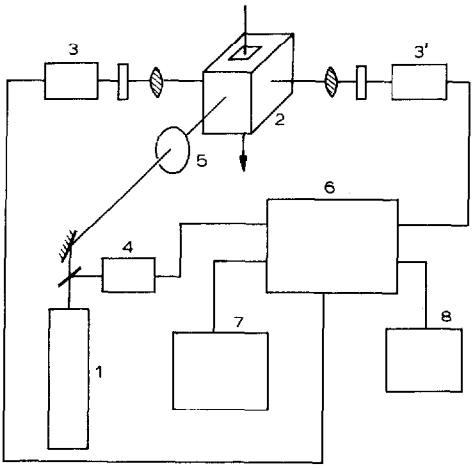

Fig. 3. Optical layout of the laser fluorometric detector. 1 = helium-cadmium CW laser; 2 = quartz flow-cell; 3,3' = operating channel of PMT; 4 = reference channel of PMT; 5 = laser beam focusing optics; 6 = preliminary data processing; 7 = computer; 8 = recorder.

operated in the single-electron pulse mode, which enables an high sensitivity of recording, low intrinsic noise and reduces the power requirement (the voltage stability). To take into account the instabilities in the laser power output, the detector was provided with a reference channel. The time required for single-pulse accumulation by the recording system was 0.1–1 s, the threshold sensitivity of the photodetectors being 10^2 pulses/s. The recording system was equipped with a recorder and a computer interface.

The detector has the following characteristics: threshold sensitivity in a 100-nl measuring cell, 10^{-17} mol for fluorescein and $2 \cdot 10^{-16}$ mol for Dns-alanine; variable detection volume from 0.1 to 0.4 μl; post-column volume $\leqslant 0.05$ μl; zero signal (baseline) drift after the detector has been set into operation, $\leqslant 4\%$; standard deviation of the baseline from the limiting value of the recorder scale in the range of minimum sensitivity, $\leqslant 3\%$; fluctuation of the zero signal from the limiting value of the recorder scale in the minimum sensitivity range $\leqslant 1\%$; linearity coefficient, 1 ± 0.03; measurement accuracy, 3%.

EXPERIMENTAL

Dns-amino acids were separated on fluoroplastic microcolumns (30 cm × 0.5 mm I.D., 2.5 mm O.D.) packed with 7-μm Silasorb SPH-C_{18} silica gel (Lachema, Brno, Czechoslovakia). The columns were packed with a 10% (v/v) sorbent suspension in pure tetrachloromethane at a pressure of 10–11 MPa, and subsequently washed with methanol–water (1:1). The efficiency of these columns was 14000–16000 plates at a flow-rate of 5 μl/min in acetonitrile–water (8:5:1.5), *i.e.*, HETP = 20 μm $\approx 3\ d_p$.

An acetonitrile gradient in 0.01 *M* sodium formate at pH 3.5 was used for the separation of Dns-amino acids. The shape of the gradient is shown in Fig. 4. Elution was carried out by pumps and the gradient device of a Kh Zh-1311 microcolumn

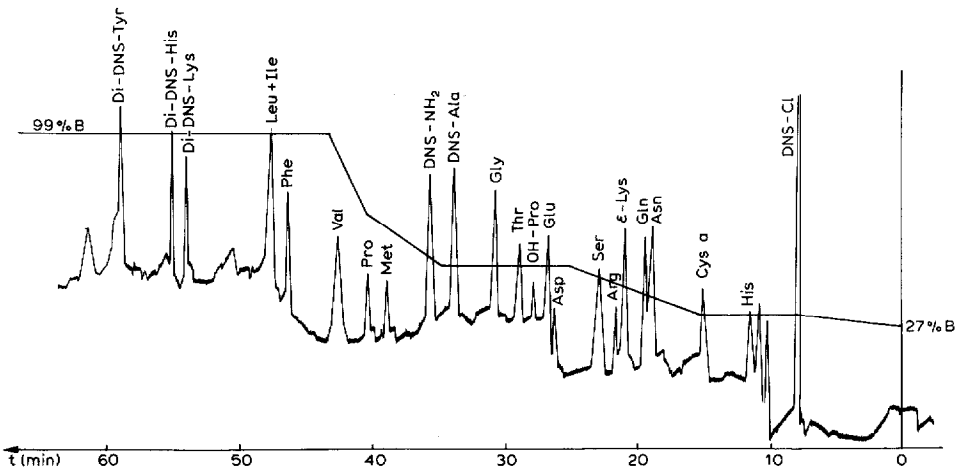

Fig. 4. Ultrasensitive analysis of Dns-amino acids by mean of microcolumn HPLC with laser-induced fluorescence detection. Sample mass: $5 \cdot 10^{-16}$ mol of each Dns-amino acid. Sample volume: 0.17 μl. Flow-rate: 5 μl/min. Gradient: from 27 to 99% acetonitrile in 0.01 *M* sodium formate (pH 3.5).

chromatograph (Special Design Bureau of Analytical Instruments, Academy of Sciences of the USSR, Leningrad).

Since the analysis of Dns-amino acids is very sensitive, high purity eluents should be used because the rise of the baseline in gradient elution prevented the identification of Dns-amino acids eluted at the end of the chromatogram. Initially, a double gradient of pH and acetonitrile content[13] was used for the separation of Dns-amino acids. The buffer solutions required for gradient elution were 0.01 *M* sodium formate at pH 3.5 and 0.01 *M* phosphate buffer at pH 7.0. These solutions as well as 0.1 *M* hydrochloric acid necessary for the preparation of Dns-amino acid samples were purified by passage through a 250 mm × 4.6 mm column packed with LiChrosorb RP-18, 10 μm. After the purification of each 0.5 l of buffer, the column was regenerated successively with 100 ml of doubly distilled water, 100 ml of methanol (Merck), 10 ml of dichloromethane (Merck), 100 ml of methanol and 100 ml of doubly distilled water. However, even with this method of eluent purification, it was not possible to attain a sensitivity higher than $1 \cdot 10^{-14}$ mol of Dns-amino acid because after the phosphate buffer is mixed with acetonitrile it develops an intense fluorescence in the blue spectral range.

Hence, a pH gradient could not be used, and an acetonitrile gradient in 0.01 *M* sodium formate at pH 3.5 was used. Prior to use, the eluents were deaerated under reduced pressure.

A 0.17-μl sample of Dns-amino acid was injected with a Rheodyne 7410 injector from a 10% solution of acetonitrile in 0.1 *M* hydrochloric acid in order to ensure a narrow initial zone. The injection was carried out by turning the valve to the "inject" position for 2 s at a flow-rate of 5 μl/min. The amount of each Dns-amino acid introduced was $5 \cdot 10^{-16}$ mol. The analysis time was 1 h.

Fig. 4 shows the results of analysis of a mixture of Dns-amino acids carried out under the above conditions.

When the method of quantitative high-speed dansylation of free amino acids[14]

is used, which permits the preparation of Dns derivatives in 2 min, this method of analysis may also be applied to the analysis of amino acids with pre-column derivatization.

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