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# A FLUORESCENCE DETECTOR FOR HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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## SUMMARY

We describe a fluorescence spectrophotometer adapted with a micro quartz flow cell to record the output of modern liquid chromatographs. The optical system is double beam in that the light source variations are cancelled out by a second photomultiplier, thus enhancing the sensitivity of the technique. The emission spectra may be scanned by stopping the flow in the chromatographic column and scanning the fluorescence detector. Many specific applications have been studied: polycyclic aromatic hydrocarbons, several vitamins, porphyrins, methyl anthranilate, etc. These are studied in natural samples and it is shown that the specificity of the fluorescence detector frequently obviates the need for sample preparation. The sensitivity available with the fluorescence detector for fluorescing compounds is often much greater than is available with variable-wavelength ultraviolet spectrophotometers. We report picogram-level detectability in real samples for many of the compounds that we have studied.

## INTRODUCTION

The development of sensitive and specific detectors has been an important factor in the growth of usefulness of high-pressure liquid chromatography (HPLC). Ultraviolet (UV) absorption has proven to be the most general detector in present use. Recent adaptation of variable-wavelength UV detectors has greatly increased both the sensitivity and the specificity, by permitting the choice of the specific wavelength that is optimal for a given compound or class of compounds. In addition, when the UV detector is used at very short wavelengths, near 200 nm, it becomes a general non-specific detector since almost all compounds exhibit very strong absorption in the far UV.

Spectroscopists have long recognized that fluorescence provides even greater

sensitivity than UV absorption for those compounds which fluoresce. Fluorescence enhances specificity because only those compounds will be observed which fluoresce. Thus fluorescence used as a liquid chromatographic (LC) detector provides additional flexibility to complement variable-wavelength UV. Generally, the UV and fluorescence detectors are used in series; the UV detector serving as a general detector and fluorescence to provide high sensitivity for quantitation of specific compounds.

There are numerous reports in the literature describing the adaptation of various fluorimeters for LC. Cassidy and Frei[1] adapted a commercial filter fluorimeter. They built a flow-cell of 7.5-$\mu$l capacity by drawing soft glass to a thin wall. A Lucite light pipe channelled the fluorescence emission to the detector. Their system was limited to the visible and near UV spectral regions by the materials that they used. They could detect 2 ppb quinine or about 15 pg.

A little later Pellizzari and Sparacino[2] adapted a commercial spectrophotofluorimeter to commercial HPLC equipment. The fluorimeter uses a xenon arc and a 50-$\mu$l quartz cell permitting measurements in the UV. These authors were particularly interested in achieving higher sensitivity for polycyclic aromatic hydrocarbons (PAH). They show emission and excitation spectra which permitted them to characterize compounds eluting from the column. Apparently they used relatively fast scanning techniques and obtained their spectra on the fly. We will show superior spectra obtained by stopping the chromatographic flow.

In 1972 Thacker[3], in Scott's lab at Oak Ridge, described a filter fluorimeter designed to be used as an LC detector. A mercury arc was used for excitation, and filters were used to select the appropriate mercury emission lines. Three-millimeter-I.D. quartz tubing was used for the flow cell. The fluorimeter was designed to be used as a detector for post-column reactions utilizing the reduction of Ce(IV) to Ce(III). The reduced Ce(III) is intensely fluorescent and acts as a quantitative indicator in the presence of reducible compounds. Katz *et al.*[4] used this equipment to study aromatic acids, principally in biological compounds. The post-column mixer used by Katz *et al.* has an 0.5-mm-I.D. glass tube fused concentrically within a 1-mm-I.D. tube. The column effluent passes through the smaller tubing and mixes with the Ce(IV) reagent. The reaction coil is made up of 8 m of 0.75-mm-I.D. PTFE tubing. These authors showed that the combination of UV and fluorescence detectors permitted a very large variety of compounds to be detected in biological samples. More recently Katz *et al.*[5] have used the Thacker fluorimeter and the Ce(IV) reagent to provide a very sensitive analysis for carbohydrates (sugars), particularly in biological samples.

## THE FLUORESCENCE DETECTOR

A fluorimeter (Perkin-Elmer Model 1000) has been adapted as an LC detector[6,7]. A schematic of the optical system is shown in Fig. 1. A xenon lamp is imaged onto the flow cell. Filters, usually interference filters, control the excitation wavelength. Fluorescence of the material in the flow cell is observed at 90° to the excited beam. The flow cell is imaged by a lens onto a photomultiplier. The emission wavelength is varied by scanning a continuously graded interference filter past a vertical slit.

The continuously graded interference filter, driven by a scan motor, provides

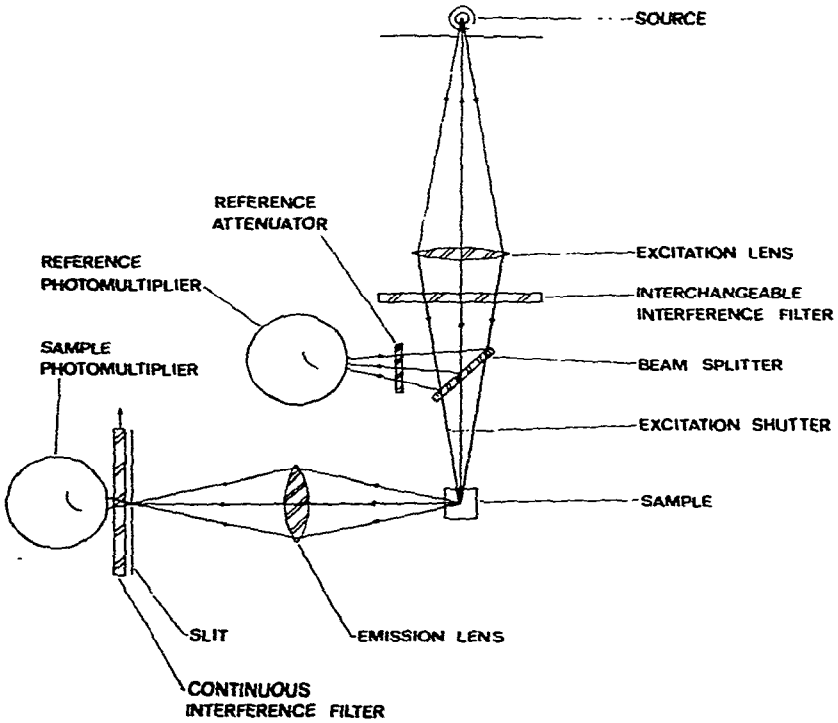


Fig. 1. Optical arrangement of the fluorescence spectrometer.

a convenient, simple monochromator. The filter is made very much like an ordinary interference filter, with a transparent dielectric material between two partially transparent metal films. To make the wavelength continuously variable, the dielectric layer is deposited as a wedge of increasing thickness. The wavelength range of this monochromator is from 390 to 750 nm. With the narrowest available slit, the spectral bandpass is about 14 nm.

The excitation interference filter is easily exchanged to be optimum for the particular compound being studied. By rotating the interference filter through a small angle the excitation wavelength can be tuned over a range of about 10 nm.

A xenon capillary flash lamp is used as the light source. The lamp is very similar to those used for high-speed flash photography. Since the flash occurs within the capillary, the geometry is particularly suitable for imaging upon a narrow flow cell. A portion of each flash is reflected by a quartz plate to a reference photomultiplier. After several flashes have occurred, the integrated digital signals from the two photomultipliers are compared in a ratio circuit to correct the fluorescence signal for any variations of the separate flashes of the xenon lamp. Xenon is traditionally preferred as a light source for fluorescence spectroscopy, since it provides a continuum spectrum of remarkably high intensity from 260 nm to 650 nm.

It is very useful to provide variable emission wavelengths because compounds have characteristic emission spectra. Failure to provide continuous adjustment of the emission wavelength, as in filter instruments, is a significant handicap to the versatility of fluorescence as an LC detector.

The flow cell is quartz tubing of square cross section, 1 mm on each edge, inside. This square cross-section is carefully merged to a circular cross-section at each end. Plastic tubing connects this quartz tube to the narrow-bore steel tubing used to transport the eluent from the column to the fluorescence detector. The illuminated portion of the tubing provides a flow cell of smaller than 20-$\mu$l capacity.

Previous fluorescence detectors have used circular cross-section material. Our experience indicates that the rectangular geometry produces much less stray light. Since the sensitivity of the fluorescence detector is generally limited by stray light, we believe that this design has considerable merit. The skillful merging of the circular to square cross-section with no portions of the system having gaps or corners, preserves peak sharpness and shape. The eluent from the column enters the cell from the bottom and flows upward.

When the Perkin-Elmer Model LC-55 variable-wavelength UV detector is used in series with the fluorescence detector, the fluorescence detector is last, so that the outlet of the detector is at atmospheric pressure.

## TEST OF THE SYSTEM

The fluorescence instrument is described in some detail by its inventor, West[6] of the Royal Institution in England. His paper[7] contains test information and also provides the design characteristics. As a fluorescence spectrophotometer the Model 1000 can detect less than 0.5 ng/ml of quinine sulfate in dilute sulfuric acid[7], thus less than 10 pg of the compound in the 20-$\mu$l cell.

We have tested the system as an LC detector. Linearity, repeatability and specificity are reported in the specific application papers which are discussed below.

### *Detector sensitivity*

To test the improvement that can be expected with different photomultipliers, chromatograms of a mixture of three porphyrins, 2 ng each, are shown in Fig. 2 for three Hamamatsu photomultipliers, R777, R446 and R105. For the R777 a second chromatogram was prepared at reduced sensitivity to bring the porphyrin peak on scale. The R105 is less sensitive than the R446 by a factor of about 0.75 and its noise level is at least twice as great. The R777 has much greater sensitivity than the R446, but unfortunately the noise is also greater. The R105 displays about one fifth of the signal-to-noise of the R777 and about one third of that of the R446. Obviously, there are significant differences from unit-to-unit for each of these detectors.

### *Instrument response*

The Model 1000 has two response times. The fast response provides half-scale signals in about 0.4 sec and the slower response in about 1 sec. The effect on the records is shown in Fig. 3, yielding a signal-to-noise ratio of about 2.5. The important factor to note is that the slow response does not distort the peak height of the chromatogram, while reducing the noise, thus providing better analytical sensitivity.

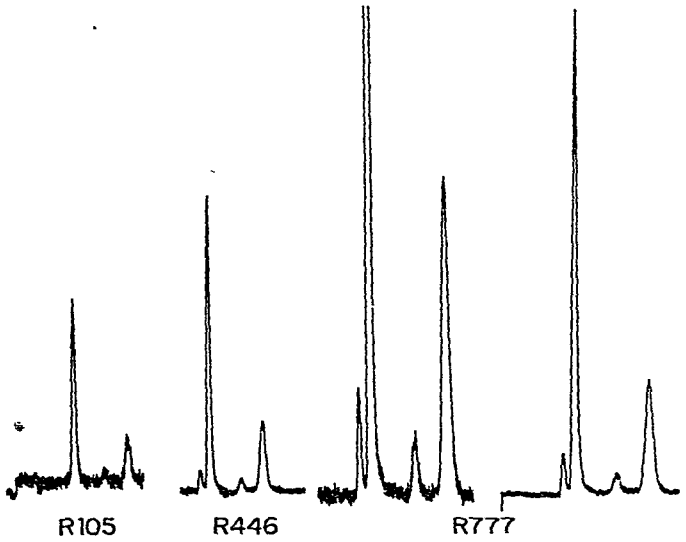


Fig. 2. Test of different photomultipliers for porphyrin analysis. Excitation wavelength is at 403 nm and emission is at 627 nm. The right-hand panel represents a scale compression on the R777 detector. The chromatographic conditions were also changed slightly, but this did not affect relative sensitivity to the porphyrin compounds.

## APPLICATIONS

We have looked at several applications where the special qualities of fluorescence ought to improve LC separations. To illustrate the power of the combination of fluorescence for LC, we will discuss the application to PAH, important in environmental studies; to the analysis of a variety of materials for vitamins, particularly riboflavin; and to the determination of porphyrins in clinical samples. We have also developed a method for methyl anthranilate, the flavorant of grape juice, which does not require any sample preparation and achieves a detection limit better than 0.05 $\mu$g/ml (ref. 8).

### *Polycyclic aromatic hydrocarbons*

PAH are very important air pollutants and must be detected at very low concentrations. The aromatic rings provide very strong fluorescence emission and these compounds have been determined frequently by fluorescence. Using reversed-phase ODS columns, we were easily able to record strong signals for anthracene and pyrene[9]. In Fig. 4, only a few compounds are barely visible in the UV chromatogram, while the fluorescence runs give easily quantitated peaks for each compound. We were able to detect close to 40 pg of benzo[*a*]pyrene.

By changing the fluorescence conditions, selectivity can be found between the various compounds as shown in Fig. 5 where the emission and excitation wavelengths have been changed to produce very different chromatograms with the same analytical system.

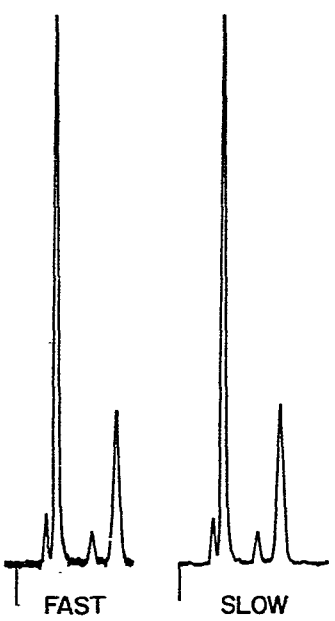


Fig. 3. Comparison of the two response times available. The porphyrin analysis is used here as the example; conditions are as in Fig. 2.

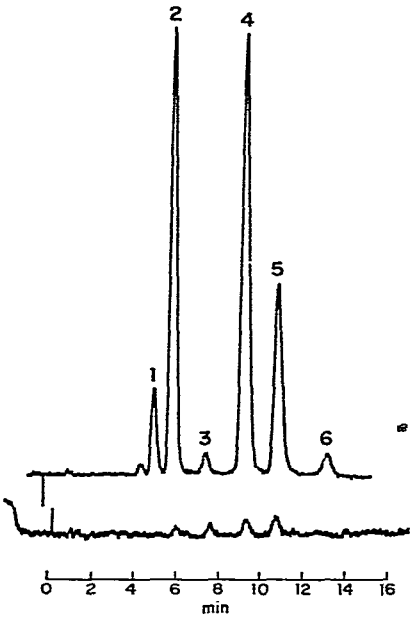


Fig. 4. Analysis of a PAH mixture. Standards for several environmentally important hydrocarbons were mixed approximately in proportion to their expected occurrence as urban pollutants; the amounts present range from less than 5 ng for pyrene to about 0.7 ng for coronene. Conditions: column, ODS SIL-X-I, 0.26 × 25 cm; eluent, linear gradient, for 100 min from acetonitrile–water (45:55) to 100% water; flow-rate, 1 ml/min; detector, fluorescence at an excitation of 338 nm and emission at 385 nm —UV at 280 nm, 0.02 AUFS. Peaks: 1 = anthracene, 2 = pyrene, 3 = chrysene, 4 = benzo[*a*]pyrene, 5 = benzoperylene, 6 = coronene.

The qualitative capability of the fluorescence detector is illustrated in Fig. 6, the emission spectrum of perylene. By stopping the chromatographic flow as each peak reaches the cell, the emission spectra were obtained. The figure illustrates the chromatography of 140 pg of perylene.

*Porphyrins*

We have completed preliminary experiments[10] intended to lead to the development of a clinical method for the determination of porphyrins in urine. With this method, using the fluorescence detector, we are able to detect as little as 30 pg of porphyrins in urine, and we could do that with very little sample preparation. In Fig. 7 is shown the run of standards containing two isomers of uroporphyrin, two of coproporphyrin, protoporphyrin and deuteroporphyrin. The chromatographic conditions had to be adjusted carefully to resolve the isomers, but fluorescence provided a technique which permitted analysis to be independent of the other compounds present in the urine.

The system was tested for linearity, for repeatability and for specificity. An example of the clinical results is shown in Fig. 8, two different patients with erythro-

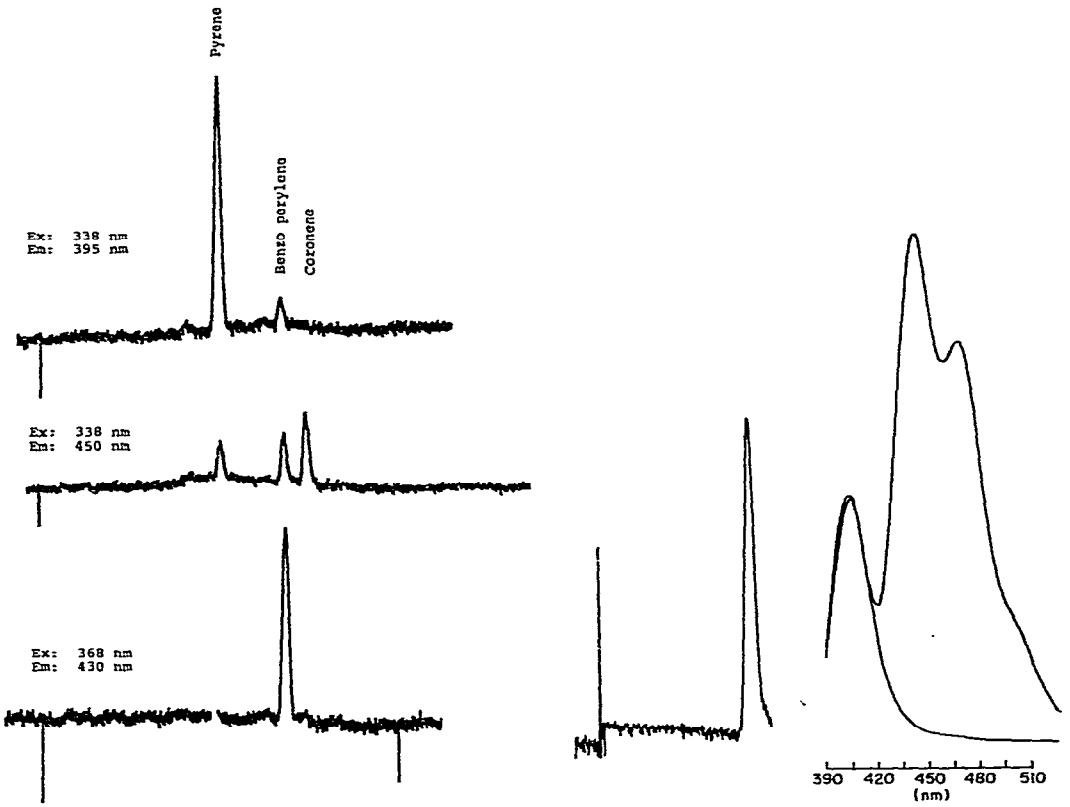


Fig. 5. Specificity for PAH. By changing the fluorescence conditions different compounds in a mixture could be enhanced and others suppressed. Amounts present: perylene, 464 pg; pyrene, 876 pg; and coronene, 528 pg. Conditions as in Fig. 4.

Fig. 6. Qualitative scan of perylene in the chromatograph cell. 140 pg of perylene were chromatographed and the flow stopped when the perylene peak appeared in the fluorescence detector cell. Conditions as in Fig. 4, except that the fluorescence excitation wavelength was 403 nm and the emission was at 450 nm.

poietic urines. The specificity and simplicity of the LC–fluorescence technique should help these clinical analyses greatly.

In addition to using fluorescence for its very high sensitivity and its discrimination against the non-fluorescent concomitants in the sample, fluorescence can, as we have already shown, identify peaks in the chromatogram. In the fluorescence emission spectra in Fig. 9, the three principal urine porphyrins are shown by scanning each peak as it reached the fluorescence detector. The three porphyrins show similar spectra, but there are enough individual differences to provide a distinction between them.

*Riboflavin*

We have established the chromatographic and fluorescence conditions for the determination of riboflavin (vitamin $B_2$) by LC and fluorescence. This material has

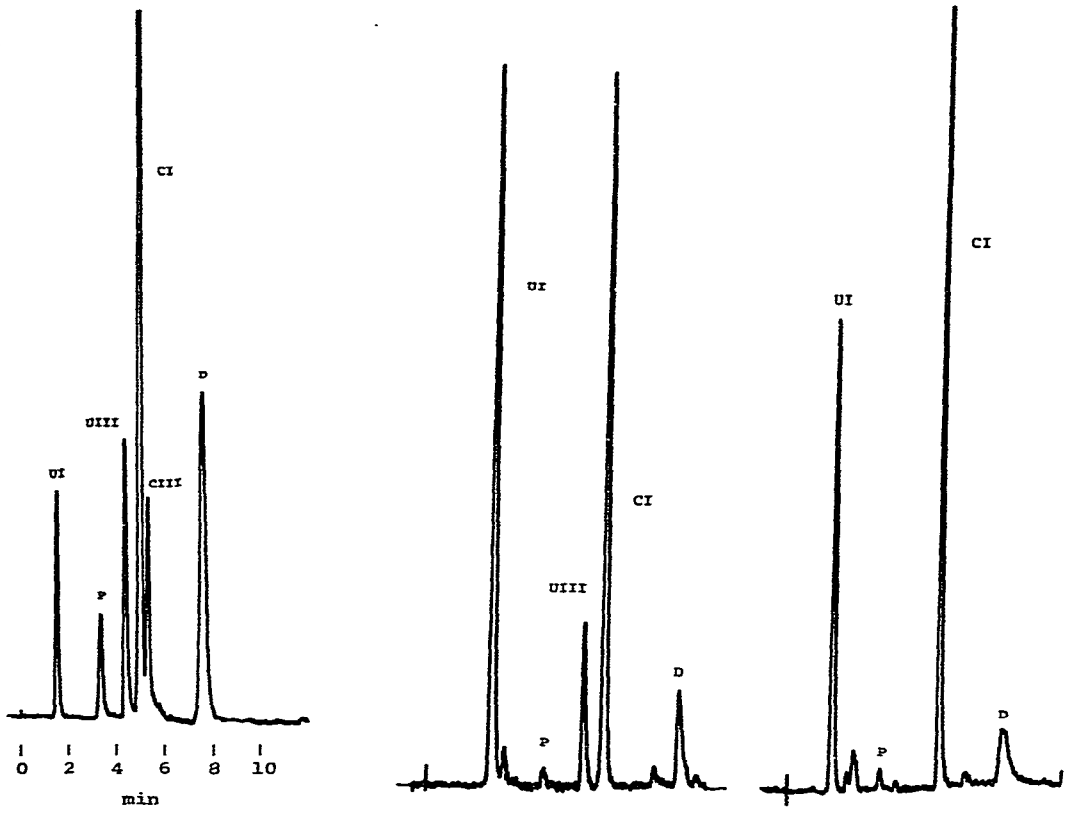


Fig. 7. Analysis of a mixture of porphyrin standards. Conditions: column, 13-$\mu$m silica A, 0.46 × 25 cm; eluent, 0.3% water in acetone, pH 7.60 using tributylamine, isocratic; flow-rate, 2 ml/min; temperature, 40°; detector, LC Model 1000, excitation at 403 nm (8 nm bandpass), emission at 627 nm. Peaks: UI and UIII = uroporphyrin isomers, P = protoporphyrin, CI and CIII = coproporphyrin isomers, D = deuteroporphyrin.

Fig. 8. Analysis of two erythropoietic urine samples. Specimens from two different erythropoietic patients, 10 $\mu$l urine in each case, were analysed. Conditions and peak identification as in Fig. 7.

been published elsewhere[11]. We then studied the determination of riboflavin in several practical samples. In Fig. 10 the UV detector is compared with the fluorescence detector for the determination of riboflavin in milk. Note that in the milk sample (prepared simply by deproteinization) riboflavin is just barely detectable, even when using an optimal UV wavelength, 266 nm. The fluorescence detector shows a high sensitivity to riboflavin. The large variety of other compounds recorded by the UV detector coming off early in the run masks a riboflavin phosphate ester peak. Fluorescence permitted this compound to be measured quantitatively and reliably by discriminating against the non-fluorescent compounds with similar retention times. This is another illustration of the advantage of fluorescence with respect to selectivity.

We have measured riboflavin clearance in urine, important in nutritional studies. Figs. 11 and 12 compare the chromatographic detection by UV and fluo-

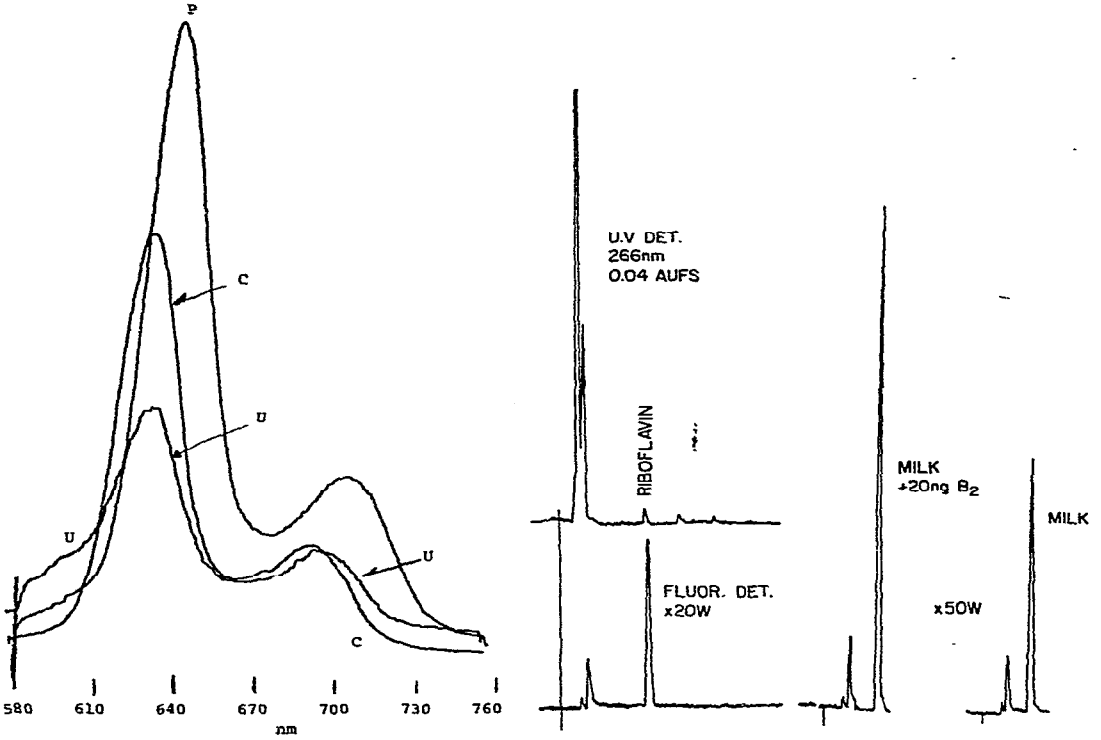


Fig. 9. Emission spectra for several porphyrins. The sample consisted of 40 ng of uroporphyrin (U), protoporphyrin (P) and coproporphyrin (C). Conditions as in Fig. 7.

Fig. 10. Analysis of riboflavin in milk. Riboflavin is just barely visible in the UV detector record while very strong signals are available from the fluorescence detector. The amount of riboflavin was quantitated by the method of additions, adding 20 ng to an aliquot of the milk sample. Conditions: column, ODS SIL-X-I, 0.26 × 25 cm; temperature, 55°; detector, LC Model 1000, excitation at 453 nm, emission at 520 nm.

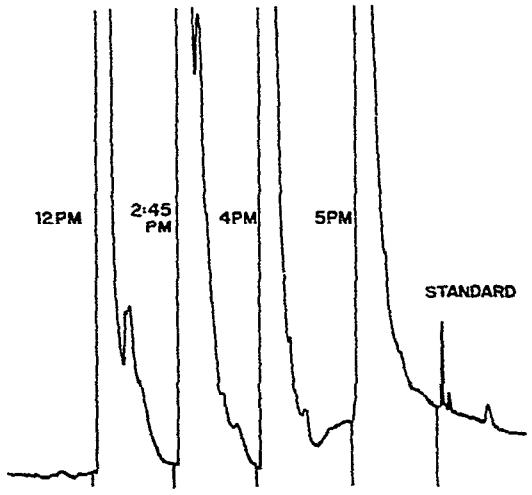


Fig. 11. Riboflavin clearance from urine, I. UV detector. Note that data cannot be extracted from the massive urine background. Conditions as in Fig. 10; 10-$\mu$l urine sample was injected directly on to the column.

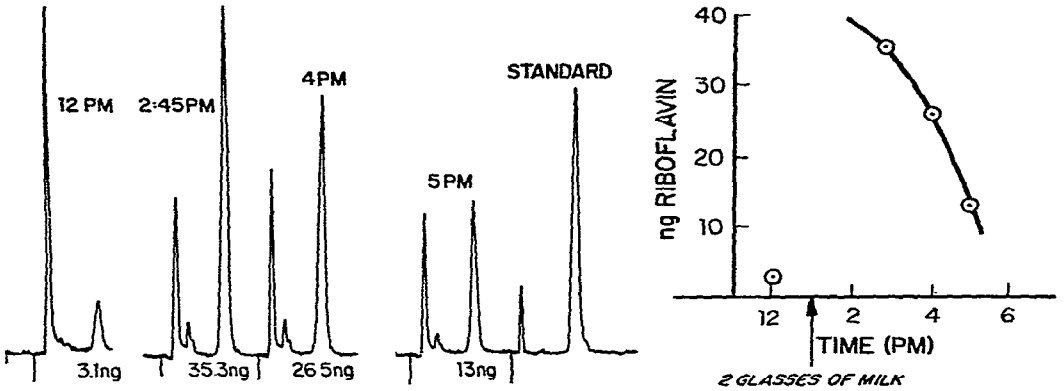


Fig. 12. Riboflavin clearance from urine, II. Fluorescence detector. Conditions as in Fig. 10; 10-$\mu$l urine sample was injected directly on to the column.

Fig. 13. Riboflavin clearance from urine, III. The plot of riboflavin clearance from urine, using the data calculated from Fig. 12.

rescence for these samples. In the UV run (Fig. 11) the very large number of compounds eluting approximately at the same time provides an unseparated scramble that cannot be used for analytical purposes. When the same urine is detected by fluorescence (Fig. 12), the riboflavin peak stands out sharply and clearly and is very easily quantitated. Two glasses of milk containing riboflavin were consumed by the subject at about 1 p.m. The urine samples were taken at noon, just prior to ingestion of milk and at several times in the afternoon after ingestion. Ten-microlitres aliquots of the urine were injected at the head of the column to produce the runs shown in Figs. 11 and 12. It is clear that owing to the massive amount of many different materials found in urine, it is impossible to measure riboflavin by UV detection. The values obtained when using fluorescence detection were calculated against the standard run and plotted *vs.* time. This curve is given in Fig. 13. It is clear that the specificity of the fluorescence detection has made the solution of this analytical problem very fast and simple.

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