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DETERMINATION OF ADRIAMYCIN AND DAUNORUBICIN IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH LASER FLUOROMETRIC DETECTION

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SUMMARY

A separation and detection scheme is presented for the determination of the antitumor drugs adriamycin and daunorubicin in human urine. Separation is accomplished by reversed-phase high-performance liquid chromatography and the drugs are detected down to the low picogram level by laser excited fluorescence using a unique fiber optic based flow-cell. Excellent detector selectivity and linearity are reported, and some of the factors influencing the performance of the detector are discussed. Possible extension of the procedure to other biologically important determinations are mentioned.

INTRODUCTION

Adriamycin (A_1) and daunorubicin (D_1) are antibiotic drugs shown to be effective in treating certain malignancies. Sensitive detection of the drugs and their metabolites in body fluids is essential in determining major metabolic pathways and safe clinical dosages[1,2]. Total fluorescence spectroscopy is incapable of resolving the drugs, which differ only slightly in structure (see Fig. 1). Current methods include high-performance liquid chromatography (HPLC) coupled with UV absorbance, visible absorbance, or radioimmunoassay (RIA) detection[2–4]. UV absorbance detection has not been selective enough for urine samples[3]. While leukemic plasma samples

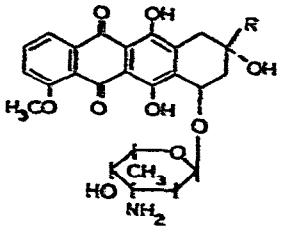

Fig. 1. Structural formula. R = –$COCH_2OH$, adriamycin; R = –$COCH_3$, daunorubicin.

were analyzed without interference at therapeutic levels (20–200 ng/ml plasma) using visible absorbance detection, the procedure includes an undesirable extraction step[4]. Furthermore, long-term studies may involve drug levels below the detection limits of that method. RIA is capable of quantitating the low drug levels present in urine without interference[3], but the high cost of counting equipment, difficulties associated with handling radioactive tags, and the need to collect and work up chromatographic fractions are all disadvantages of RIA, which should be avoided if an alternate method is available.

The limited number of organic compounds with appreciable fluorescence quantum efficiencies and the presence of both excitation and emission spectral differences to aid in resolving the compound of interest from its matrix, have resulted in many applications demonstrating the high selectivity possible with fluorometric HPLC detection[5–7]. The laser has certain properties, most notably its high intensity, which makes it an attractive light source for fluorescence detection[8,9]. Since fluorescence signals are proportional to the light intensity incident on the sample, excellent sensitivity is possible with laser excitation. Also, when coupled with a suitably designed detector, the spatial and temporal properties of the laser can result in reduced spectral interference and lower background levels.

We present here a procedure for the reversed-phase HPLC separation of A_1 and D_1 in human urine. Filtered samples are injected directly and low picogram quantities are detected fluorometrically, without interference, using an argon ion laser for excitation and fiber optics for fluorescence collection.

EXPERIMENTAL

Chromatography

Separations were performed on a 25 cm × 4.6 mm Alltech 10 μm C_{18} column. The drugs were supplied by Dr. Nicholas Bachur of the Baltimore Cancer Research Center. Samples were eluted with a 50% acetonitrile (Burdick & Jackson, Labs., Muskegon, Mich., U.S.A.; LC grade) in 0.01 *M* phosphoric acid solution. Injections were made with a Rheodyne fixed loop injector. A flow-rate of 1 ml/min was maintained with an LDC minipump.

Detection

Fluorometric detection of the drugs was accomplished with the apparatus shown in Fig. 2. Radiation from a Control Laser Model 553 argon ion laser was focused with a 100 mm focal length lens into a 1.05 mm I.D., 2.0 mm O.D. quartz capillary tube (Suprasil grade quartz from Amersil Company, Sayreville, N.J., U.S.A.). A laser power of 1.2 W was used for this work. The flow-cell was positioned with a Model FP-1 fiber optic positioner from Newport Research Corporation. The protective coating and optical cladding was stripped from the end of a 1.0 mm core diameter fused silica optical fiber (Model QSF-1000 from Math Assoc., Great Neck, N.Y., U.S.A.). The fiber was slipped into the capillary tube and held in position with a 1/16 in. stainless-steel Swagelok tee, leaving a clearance between the capillary tube and optical fiber of 60 μm. The volume of the flow-cell used in this work was approximately 20 μl, but this could be decreased to a few microliters by simply shortening the capillary tube. Narrower bore capillary tubing and smaller fiber-optic

diameters could produce submicroliter flow-cells, but a concomitant decrease in sensitivity may result. By placing the flow-cell in the vertical position shown in Fig. 3, bubbles resulting from eluent degassing are rapidly swept through the flow-cell.

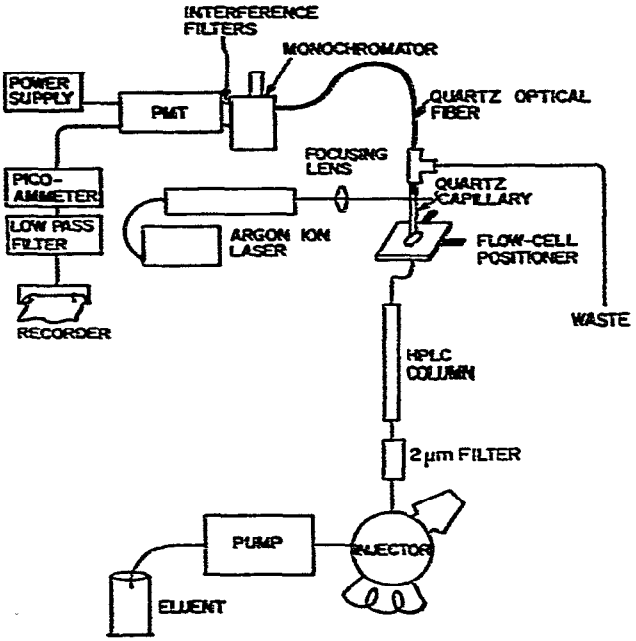

Fig. 2. Apparatus used for the laser fluorometric HPLC detection of adriamycin and daunorubicin.

The opposite end of the optical fiber was positioned at the entrance slit of a f/3.9 monochromator (Minichrom 1 from PTR Optics, Waltham, Mass., U.S.A.). The monochromator was fitted with 1 mm slits and had a bandpass of 6 nm. Further fluorescence isolation was provided by two Corion BB-6000 broadband interference filters. Signals were measured with an Amperex 56 DVP photomultiplier tube and photocurrents were fed to a Keithley Model 417 picoammeter. A simple low-pass filter, with a 2-sec time constant, was used to couple the picoammeter output to a chart recorder.

Detector optimization

The photocurrent was monitored while a dilute drug solution or pure solvent were alternately circulated through the flow-cell. Several parameters were then varied to optimize the ratio of fluorescence signal to background noise. The excitation and emission wavelengths were found to be optimum at 488 nm and 590 nm respectively. The discrete output of the laser limits the excitation tunability. While the 488 nm line of the laser is not at the excitation maximum of the drugs, the high intensity of the laser more than makes up for this shortcoming.

The path of a light ray emanating from near the flow-cell wall is traced in Fig. 3. The angle i_c must be greater than the critical angle for the light ray to be

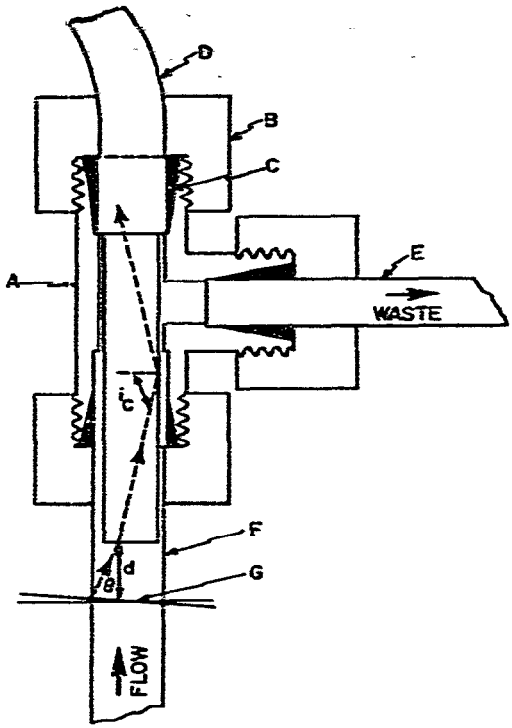

Fig. 3. Close-up of fiber-optic flow-cell. A = 1/16 in. stainless-steel tee; B = 1/16 in. stainless-steel nut; C = graphite ferrule, D = optical fiber (0.99 mm diameter core, 1.5 mm O.D.); E = 1/16 in. stainless-steel tubing; F = quartz capillary tube (1.05 mm I.D., 2 mm O.D.); G = focused laser beam.

transmitted and this will be true if $\sin\theta \leqslant (n_f^2 - n_c^2)^{1/2}/n_e$, where n_f, n_c, and n_e are the indices of refraction of the fiber, fiber cladding, and eluent. θ is generally smaller for light rays emanating from the center of the flow-cell. Hence, the fiber tends to select fluorescence over specular scattering at the flow-cell walls. It was found that the distance between the focused laser beam and the fiber (d) could be varied from less than 1 mm to greater than 3 mm with almost no change in the fluorescence signal. The detector was also found to be insensitive to small changes in the horizontal position of the laser beam.

Procedure

Standard solutions in the range of 5.2 ng/ml to 50 μg/ml were prepared by successive dilutions of a stock 50 μg/ml A_1 and D_1 in methanol (Burdick & Jackson, LC grade) solution. A series of spiked samples were prepared in the range of 12.5 ng/ml to 0.20 μg/ml by adding 2 ml of various standard solutions to 8 ml of filtered urine. The prepared solutions were then injected directly using the chromatographic and detection conditions previously mentioned.

RESULTS AND DISCUSSION

Detection limits

The detection limits of A_1 and D_1, based on a signal to noise ratio of 3, are 10 pg and 15 pg respectively. The observed baseline noise is a function of the background level when eluent is flowing. Improvement in detectability can result from reducing this functionality, which in the best case should approach the shot-noise limit. Pumping and other flow irregularities common to HPLC greatly increase the functionality. A reciprocating pump was used for this work, without pulse dampening, and improvements could be expected with a more sophisticated pumping system.

An alternate approach to decreasing the baseline noise is to minimize the background light level. Common sources of background in fluorometric HPLC detection are Rayleigh scattering, specular scattering off flow-cell walls, eluent Raman scattering, and eluent impurity fluorscence. During the optimization process described in the experimental section the various contributions to the background were investigated. It was found that the flow-cell design and the good stray light rejection of the monochromator combine to make the background contributions of Rayleigh scattering and specular scattering negligible. The contribution of eluent Raman scattering is minor in this case mainly because the largest Raman shift for the eluent is still considerably smaller than the 3540 cm^{-1} shift between the excitation and emission wavelengths used in this work. Since Raman linewidths are at least as great as the excitation bandwidth, the monochromatic output of the laser will generally result in better Raman rejection than conventional light sources[10]. Additional Raman rejection is accomplished by locating the flow-cell such that fluorescence is collected perpendicular to the laser polarization, thereby diminishing background resulting from polarized Raman transitions. The majority of the observed $6 \cdot 10^{-9}$ A photocurrent background resulted from eluent impurity fluorescence. LC-grade eluents were used in order to minimize this contribution to the background, but further purification is recommended for extremely low level work.

Linearity

Calibration plots drawn for the A_1 and D_1 standards exhibited linearity up to the 50 μg/ml stock solution. Linear regression constants of 1.000 were obtained for both drugs indicating that the inner filter effect, which often produces non-linear calibration plots in fluorometric analysis[11], is not a significant problem. This is not surprising considering the design of the detector and its insensitivity to small changes in the relative positions of the flow-cell and the focused laser beam. Calibration plots were also drawn for the urine solutions yielding linear regression constants of 0.999 and 0.997 for A_1 and D_1 respectively.

Selectivity

The excellent selectivity of this procedure is demonstrated in Fig. 4 where the chromatogram of a spiked urine sample reveals almost no extraneous peaks when injected directly.

The same selectivity can be expected for other biologically important determinations in urine. The determination of porphyrins in urine is important in the diagnosis of liver diseases[7]. Porphyrins are highly fluorescent and can be excited with

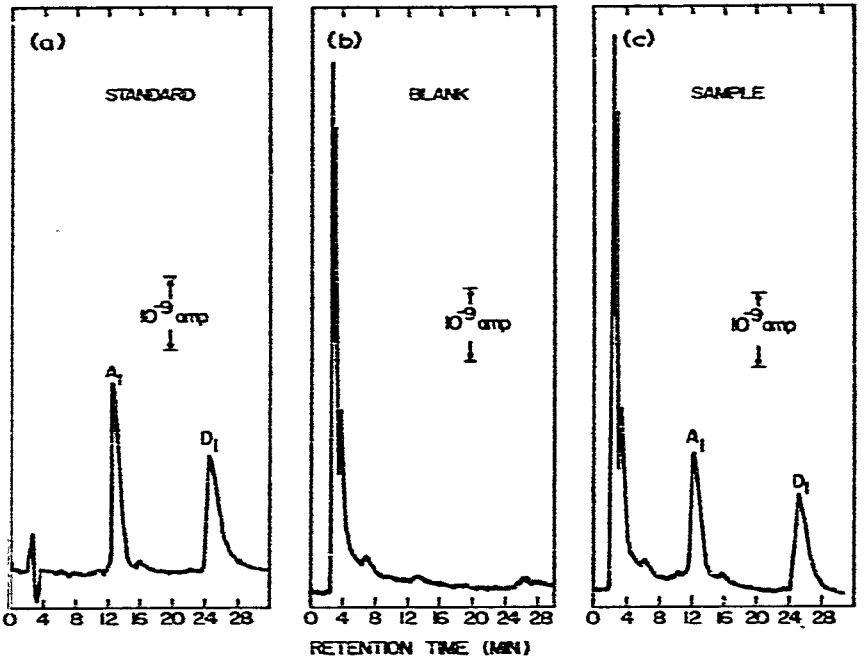

Fig. 4. Typical chromatograms: (a) A_1 and D_1 standards in methanol (94 pg of each injected), (b) urine blank, and (c) spiked urine sample (63 pg of A_1 and D_1 injected). Chromatographic and detection conditions are listed in the text.

the visible output lines of the argon ion laser. Likewise, the determination of riboflavin in urine, important in nutritional studies, can be performed with the detector used in this work. Hulhoven and Desager[12] have determined D_1 in plasma using an extraction procedure and HPLC with visible absorbance detection. A lower detectable concentration of 10 ng/ml plasma was obtained. Although we have not checked our procedure for interferences, the direct injection of 1 μl of plasma can be tolerated chromatographically. At 10 ng/ml this corresponds to 10 pg of D_1, which is measurable by the detector used in this work. Such a procedure will vastly reduce the volume of plasma needed for analysis. Moderately intense UV lines at 350 and 360 nm make this detector also applicable to the determination of Dns-amino acid derivatives in body fluids.

Laser fluorometric HPLC detection possesses some general advantages over conventional fluorometric HPLC detection, and this particular detector possesses some advantages over previously reported laser fluorometric HPLC detectors[13–15]. The selectivity observed in the determination of A_1 and D_1 in urine is mainly due to the uniqueness of visible fluorescence. When UV exictation is used, biological samples can be expected to show some interferences. When this happens, selectivity can be improved by narrowing the slits and adjusting excitation and emission wavelengths to minimize interferences. The monochromatic and intense output of the laser is more amenable to these measures than conventional fluorometric light sources, which require larger slit widths to compensate for their lower intensity. Bandpasses of about

10 nm are typical for conventional fluorometric HPLC light sources compared to subnanometers for lasers.

An earlier report of a laser fluorometric HPLC detector, using a flowing droplet as a windowless flow-cell, has shown low detection limits in the determination of aflatoxins[14]. Unfortunately the laminar-flow condition, required for stable operation, can be disturbed by trapped gas bubbles. In addition, the fluorescence signal observed there is affected by the shape of the droplet, which depends on the physical properties of the eluent. This can cause problems when gradient elution is employed. The detector used in this work does not exhibit these problems and has the additional advantage of requiring extremely simple alignment. The path of the excitation source through other types of flow-cells is usually magnified and imaged onto the entrance slit of the emission monochromator. Small deviations in the position of the focused beam resulting from refractive index gradients (pressure changes in the pumping cycle and thermal lens effects) are also magnified, and can cause the image to at least partially miss the entrance slit. The detector used here is insensitive to small changes in the position of the focused laser beam and the collected light is always aligned with the entrance slit of the monochromator.

The argon ion laser has become a routine tool for the spectroscopist and can be found in most Raman spectrometers. It is reliable, moderately priced (about the same as a good HPLC gradient system), and easy to operate. For these reasons the detector described in this paper can be considered a reasonable addition to an analytical laboratory.

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