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LASER FLUORIMETRY FOR CAPILLARY COLUMN LIQUID CHROMATOGRAPHY: HIGH-SENSITIVITY DETECTION OF DERIVATIZED BIOLOGICAL COMPOUNDS

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SUMMARY

A high-sensitivity, low-dead-volume laser-induced fluorescence detector has been designed for use in capillary column liquid chromatography. This detector exhibits femtogram detection limits and a linear dynamic range spanning five orders of magnitude. Derivatization schemes were utilized to add a fluorescent moiety to biologically important compounds such that their excitation maxima corresponded well with the output wavelength of the laser. Sample chromatograms are presented for picogram amounts of standard bile acid and steroid hormone derivatives. In addition, a high-efficiency separation of picogram amounts of the solvolyzable steroid hormones from human serum is illustrated.

INTRODUCTION

As the advantages of capillary column liquid chromatography (LC) become increasingly evident in the analysis of highly complex mixtures[1–5], the need for a variety of suitable detection techniques is gradually being recognized. Interest is growing in the use of miniaturized LC systems incorporating packed capillary or open tubular columns, since the the successful separation of complex samples, such as those of biological or fossil fuel origin, requires very high chromatographic efficiencies[6–8]. Indeed, theoretical plate numbers in excess of 250,000 have recently been obtained in reasonable analysis times[5,9] with slurry-packed capillary columns[10–13], which, unlike conventional-scale columns, can be effectively packed with microparticulates in lengths of up to 3 m. As these packed capillary columns are reduced in size to have inner diameters of 250 μm or less, and as interest grows in open tubular columns whose optimal inner diameters are at most 10 μm (refs. 14 and 15), detection volumes must be similarly reduced to be at or below the nanoliter range if the full separation power of the columns is to be maintained[16].

However, in conjunction with these necessarily small volumes, high detection sensitivities and/or selectivities are often desirable. Several novel detection techniques, such as direct liquid introduction into the mass spectrometer and flame-based detection techniques, have operational parameters which are such that they clearly benefit from the reduced flow-rates of miniaturized LC[17–20]. However, there is also

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a group of concentration-sensitive detectors which exhibit an enhanced mass sensitivity at very low flow-rates[21,22]. Effective detection volumes on the order of picoliters have been suggested in recent work with electrochemical[23,24] and laser-based[25–39] detectors, a development which is also significant in the general handling of very small biological specimens[40].

Fluorescence is one of the most sensitive detection techniques available to liquid chromatography. A relatively recent development in the area of fluorimetric detection is the use of lasers as excitation sources[25–39]. The intensity of the fluorescence signal produced is directly proportional to the intense laser light illuminating the cell, and the highly collimated nature of the beam allows it to be focused onto a very small area. Its monochromaticity also allows stray light arising from reflections or from Rayleigh or Raman scattering to be readily suppressed.

Many flow-cell configurations have been utilized in conjunction with laser fluorescence detectors for conventional and microbore LC. Zare and co-workers[25–29] and Voigtman *et al.*[30] employed windowless flow cells, based on the formation of a flowing droplet bridge suspended between a stainless-steel capillary tube and a solid rod beneath. These flow cells facilitated the rejection of scattered light when the laser beam was focused on a small spot inside the liquid column and eliminated contamination and memory effects from sample adsorbed on the cell walls. Their volume was, however, on the order of 4 μl. Sepaniak and Yeung[32,33] utilized a fiber-optic inserted into the end of a quartz capillary tube just above the focal point of the laser beam. The column effluent moved upwards in the capillary, passing through the illuminated region and around the fiber. Emission from the sample was, thus, collected with minimal interference due to scattered or fluorescent light from the capillary tube walls. Folestad *et al.*[31] developed both a flow cell for conventional LC, based on a freely falling effluent stream with a configuration similar to that described by Zare and co-workers[25–29], and also a microbore cell, constructed from a small segment of fused-silica capillary from which the polyimide had been removed, as is frequently done in optical detectors for microcolumn LC[11]. To eliminate scattering influences from the cell walls, the authors placed the emission optics at an angle of 30° relative to the horizontal plane of the laser beam.

In keeping with the desire for a laser fluorescence flow cell that is compatible with miniaturized LC columns, several researchers have utilized the sheath-flow detection principle[34–38] commonly used in flow cytometry[41]. The quest for picoliter detection volumes in which to conduct biochemical analyses on many cellular and sub-cellular organelles by microfluorescence techniques[42] led to the use of hydrodynamic focusing techniques to produce what is essentially an enclosed free-falling effluent stream of micron dimensions. By maintaining laminar flow conditions, the sample stream can be ensheathed within a larger solvent stream of similar refractive index so that a well-focused laser beam illuminates the sample in a very small detection volume, which is far removed from the cuvette walls and, thus, virtually devoid of interferences from scattering or sample adsorption. Probed volumes as low as 11 pl have been reported[38], yielding a detection limit of 22,000 molecules for Rhodamine 6G at an excitation power of 500 mW (ref. 38). While no detection volumes below 4 nl have been reported for LC applications[37], the sheath-flow cell has yet to be tested in conjunction with capillary column chromatography, for which it appears ideally suited. Indeed, only recently have Guthrie *et al.*[39] published work utilizing

laser-fluorimetric detection for capillary-sized columns.

The limited wavelength range available for sample excitation is, unfortunately, one of the disadvantages inherent in the use of a laser as an excitation source. This restriction frequently makes it necessary to alter the chemical structure of the compounds of interest so that their excitation cross-section at the wavelength of the laser is increased. Adding an appropriate fluorescent moiety to the molecule through chemical derivatization is one solution[2,6,7,43]. A judicious choice of derivatization reagent can also add further selectivity to the analysis, since many compounds in the sample matrix may be excluded from the chemical reaction. Because volumetric considerations are significant, precolumn derivatization methods appear most appropriate for capillary column LC[2].

The object of the present study was to combine many of the appropriate components from existing systems into a simple and relatively inexpensive laser-based fluorescence detector compatible with capillary LC columns. Both fiber-optic and sheath-flow detection cells were modified to have minimal volumes for use with the microcolumns, and the system was designed to have sufficient flexibility so that detection directly on the column packing[44,45] could also be accomplished. While both the sheath-flow and the fiber-optic cells were constructed to minimize the amount of scattered or reflected light reaching the emission optics, the former, an extensively altered version of the cell from a Model 50 flow cytometer (Ortho Diagnostic Systems, Westwood, MA, U.S.A.), provided a virtually dead-volume-free detection cell capable of achieving picoliter detection volumes, while the latter exhibited slightly greater detectabilities at some cost to detection volume. In addition, precolumn derivatization was employed to add a fluorescent moiety to organic molecules containing either a hydroxy[7] or a carboxylic acid[2] functionality such that their excitation maxima closely corresponded to the output of the helium–cadmium laser. High-efficiency separations were, thus, achieved for biological compounds which typically occur at trace levels in body fluids.

EXPERIMENTAL

Chromatographic system

Separations were performed on 250 μm I.D. slurry-packed capillary columns, prepared as described previously[13].

A high-pressure syringe pump (Model 314, ISCO, Lincoln, NE, U.S.A.) was modified to operate in the pressure-controlled mode, providing a constant, pulsation-free solvent flow at operating pressures below 2300 p.s.i. At higher pressures, a Shimadzu (Kyoto, Japan) Model LC-5A pump was used in the constant-pressure mode.

A direct sampling method[46] was utilized in the determination of the sensitivity and linearity of the detector in order to minimize sample carry-over between injections. Chromatograms were obtained by using a low-volume Valco injection valve with a 0.2-μl internal sample loop (Model CI4W.2, Valco Instruments, Houston, TX, U.S.A.). A miniaturized continuous-gradient device[47] was employed for the separations requiring gradient elution.

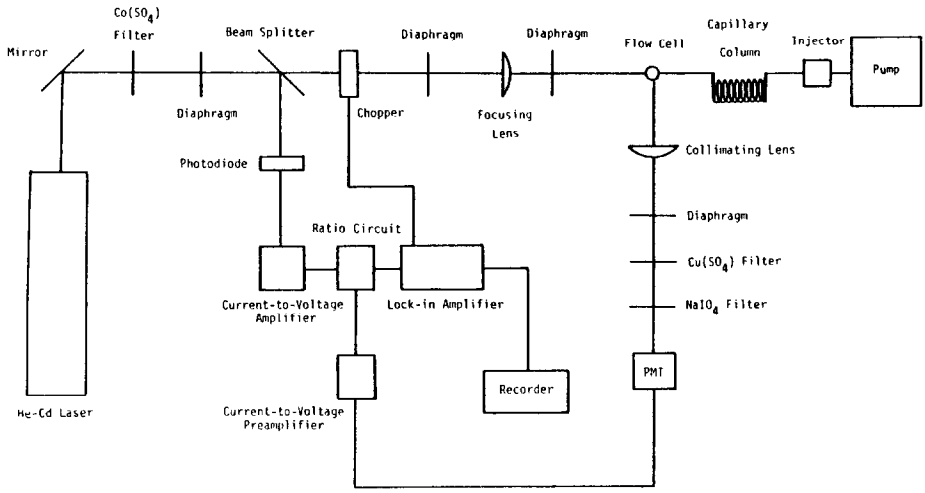

Fig. 1. Schematic diagram of the chromatographic system, laser-induced fluorescence detector, and ancillary electronic equipment. PMT = Photomultiplier tube.

Laser fluorescence detector

A schematic diagram of the analytical system appears in Fig. 1. The 325-nm output of a helium–cadmium laser (Model 4240 BUV, Liconix, Sunnyvale, CA, U.S.A.) passed through a $CoSO_4$ solution filter (296 g/l, 1.0 cm path length), an iris diaphragm, and a beam splitter, before being chopped at 800 Hz (Rofin Optics and Electronics, Marlboro, MA, U.S.A.). The beam then passed through a second diaphragm, a focusing lens (f/2, Melles Griot, Irvine, CA, U.S.A.), and a third diaphragm, before entering the flow cell. The filter and initial diaphragm served to eliminate background radiation from the dominant, non-lasing 442-nm line of the helium–cadmium discharge. The beam was chopped so that lock-in amplification (Model 128, Princeton Applied Research, Princeton, NJ, U.S.A.) could be used to eliminate background due to stray light. The two final diaphragms removed back reflections from the focusing lens and the flow cell, respectively. Fluorescence from the sample was collected by an optical train, consisting of an f/1 spherical collimating lens (Melles Griot), an iris diaphragm, a $CuSO_4$ filter (saturated solution, 1.0 cm path length), and an $NaIO_4$ filter (11.7 g/l, 1.0 cm path length). The two emission filters were designed to eliminate trace contaminant fluorescence above 530 nm, while also removing any scattered laser light.

The output from the photomultiplier tube (RCA Model 1P28) passed through a current-to-voltage preamplifier (Model 181, Princeton Applied Research) and was ratioed with the amplified output of a photodiode (Type 5D/PSB, United Detector Technology, Culver City, CA, U.S.A.), situated to monitor the signal split from the laser beam. Fluctuations in the output power of the laser were, thus, compensated before the fluorescence signal entered the lock-in amplifier and appeared on a strip-chart recorder (Series 5000 Recordall, Fisher Scientific, Cincinnati, OH, U.S.A.). A 0.3-sec time constant was used for all measurements.

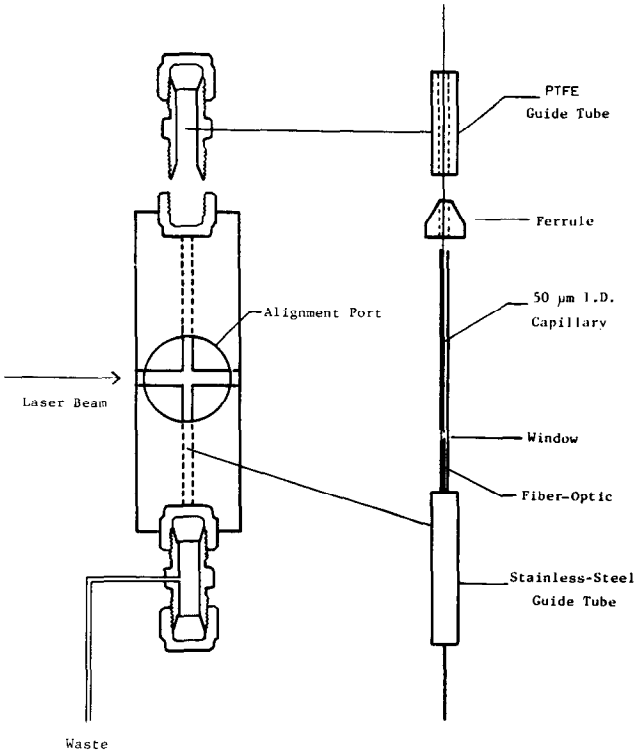

Fig. 2. Cross-sectional view of flow cell body, showing expanded detail of cell interior.

Detection cells

The fiber-optic cell utilized in the present study was similar in design to that described by Sepaniak and Yeung[32] for conventional-scale LC and by Fjeldsted and Lee[48] for supercritical-fluid chromatography. A short segment (approx. 5–10 cm) of 50 μm I.D. (210 μm O.D.) fused-silica tubing was cemented to the end of the capillary column just past the PTFE end-frit used to retain the column packing[49]. This tubing was then inserted into a 5-cm piece of 250 μm I.D. fused-silica capillary from which 6 mm of the polyimide coating had been removed, as shown in Fig. 2. The column effluent, thus, entered the flow cell 1 mm above the 5-μm laser spot. A 10-cm segment of mid-UV single optical fiber (P/N WF00200, Maxlight Fiber Optic Division, Raychem Corp. of Arizona, Phoenix, AZ, U.S.A.) was inserted through the bottom of the flow cell to a distance 1 mm below the laser beam so that fluorescence from the column effluent was collected with a minimal amount of interference from scattered or reflected light. The probed volume was, thus, only 3 pl, as defined by the size of the illuminated area. The actual cell volume, however, was 98 nl from the entrance point of the column effluent to the tip of the fiber-optic, a value which was found to contribute very little to the dispersion of a typical chromatographic peak.

Reagents

The linearity and sensitivity of the detector were determined with pyrene

(99.9% pure, Aldrich, Milwaukee, WI, U.S.A.) in a 90% aqueous acetonitrile mobile phase and coumarin (reagent grade, Sigma, St. Louis, MO, U.S.A.) in a 100% acetonitrile mobile phase, respectively.

Bile acid standards were purchased from Sigma and were used without further purification. Chenodeoxycholic, cholic, deoxycholic, glycochenodeoxycholic, glycocholic, glycodeoxycholic, glycolithocholic, lithocholic and urodeoxycholic acids were employed in this investigation. The bile acids were derivatized with bromomethylcoumarin (Sigma), according to the procedure of Okuyama *et al.*[50].

Steroid standards were obtained from various commercial and private sources and were used without further purification. Their hydroxy functionality was derivatized with 7-(chlorocarbonylmethoxy)-4-methylcoumarin as previously reported[7]. 5α-Androstan-3α-ol-17-one, 5β-androstan-3α,17β-diol, 5β-pregnane-3α,11β,17α,20α-21-pentol, 5β-pregnane-3α,11β,17α-20β,21-pentol, 5β-androstan-3α,11β-diol-17-one, and 5α-pregnane-3β,20β-diol were the standards utilized in this study.

A small aliquot of pooled human plasma was analyzed according to the procedure of Axelson and Sahlberg[51].

All solvents used were HPLC grade (Fisher Scientific), and all water was passed through a six-cartridge cation, anion, and activated charcoal purification system (Continental Water Systems, El Paso, TX, U.S.A.) to give a minimum resistivity of 17 MΩ.

RESULTS AND DISCUSSION

The ability to achieve detection sensitivities at or below the picogram level without significantly contributing to the dispersion of a chromatographic peak is extremely important for the analysis of trace components in complex biological samples, which are frequently only available in nanoliter or smaller volumes[40]. Capillary LC columns coupled with a laser fluorescence detector provided the requisite high detection sensitivities in very small detection volumes, especially when precolumn derivatization techniques were utilized to add appropriate fluorescent moieties to naturally occurring biological molecules. This combination of factors, therefore, allowed the efficient separation and high-sensitivity analysis of naturally occurring hydroxy- and carboxylic acid-containing steroid hormones and bile acids.

Linearity

The linear dynamic range of the laser-induced fluorescence detector was determined by using pyrene in an acetonitrile–water (90:10) mobile phase as a model solute (capacity factor, $k' = 2.7$). Based on duplicate injections of increasingly concentrated standard solutions prepared by serial dilution, the signal was found to be linear over 5 orders of magnitude, as shown in Fig. 3. The laser power was monitored immediately prior to and following the chromatographic analyses, and peak height measurements were corrected for minor variations in output power by normalizing them to correspond to an average excitation power of 6.45 mW.

Scott[52] has defined a response function, Y, for varying concentrations, C, as

$$Y = AC^r \tag{1}$$

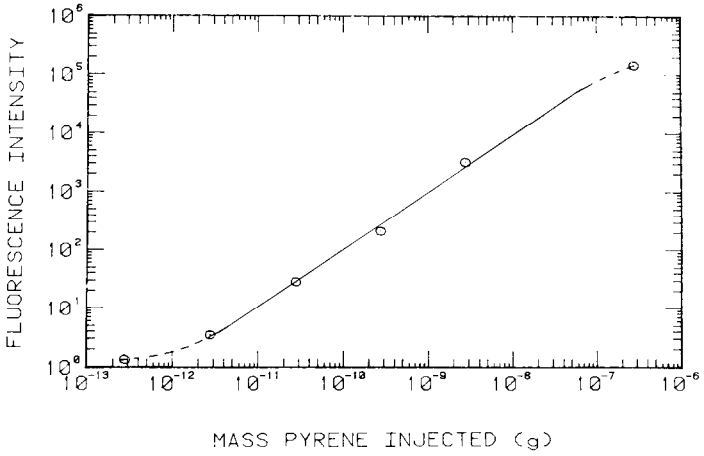

Fig. 3. Linearity of the fluorescence response for 0.2-μl injections of different concentration solutions of pyrene (k' = 2.7) in acetonitrile–water (90:10).

where both A and r are constants. Converting to logarithms yields the equation of a straight line

$$\log Y = \log A + r \log C \tag{2}$$

with slope r over the linear portion of the response curve. Deviations in r from 0.98 to 1.02 were arbitrarily set by Scott as the acceptable limits for a linear response. A linear least-squares regression of the pyrene data yielded an r value of 0.99 for the curve from an injected amount of 2.76 pg to approximately 100 ng of pyrene, at which point the signal began to level off.

Detection limits

The minimum detectable quantity was determined by using Student's t-test and the procedure described by Hieftje[53] to be 24 fg of pyrene, injected in an acetonitrile–water (90:10) mobile phase and 8.4 fg of coumarin in both 100% acetonitrile and acetonitrile–water (75:25) at an excitation power of 6.45 mW. These detection limits, at signal-to-noise ratios of 3 (99% confidence level), corresponded to a peak maximum of only 67 attograms (10^{-18} g) of pyrene and 24 attograms of coumarin in the probed volume during the 0.3-sec time constant of the detector.

Applications

Most biologically or medically important molecules appear in complex matrices which require highly efficient analytical separation techniques. While gas chromatography has been highly successful in the analysis of many such samples[54–56], biologically important molecules are frequently thermally labile or too large or polar to be effectively analyzed by gas chromatography. Although derivatization methodology has been extensively developed to increase sample volatility[54,57], there are many instances where LC techniques appear more appropriate. Capillary column LC provides a natural solution to this problem. However, suitable detection techniques,

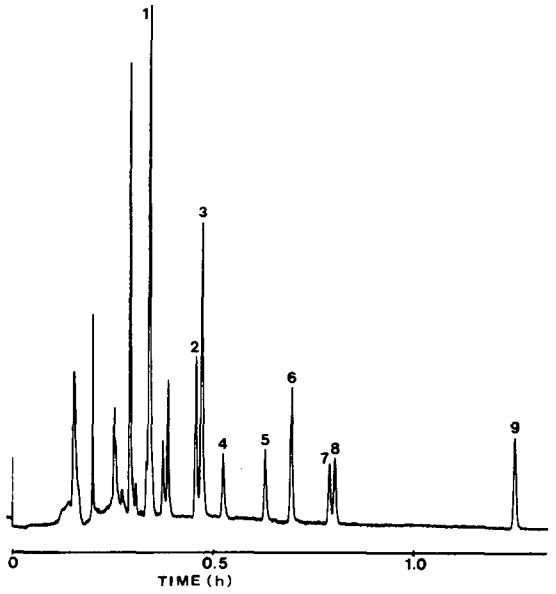

Fig. 4. Chromatogram of standard bile acids, derivatized with bromomethylcoumarin: column, 0.66 m × 250 μm I.D. packed with 5-μm Spherisorb ODS; mobile phase: continuous gradient 75–100% aqueous acetonitrile (1.5 μl/min); injection, approximately 50 pg of each acid in 0.2 μl. Solutes: 1 = glycocholic acid; 2 = glycochenodeoxycholic acid; 3 = glycodeoxycholic acid; 4 = cholic acid; 5 = urodeoxycholic acid; 6 = glycolithocholic acid; 7 = deoxycholic acid; 8 = chenodeoxycholic acid; 9 = lithocholic acid.

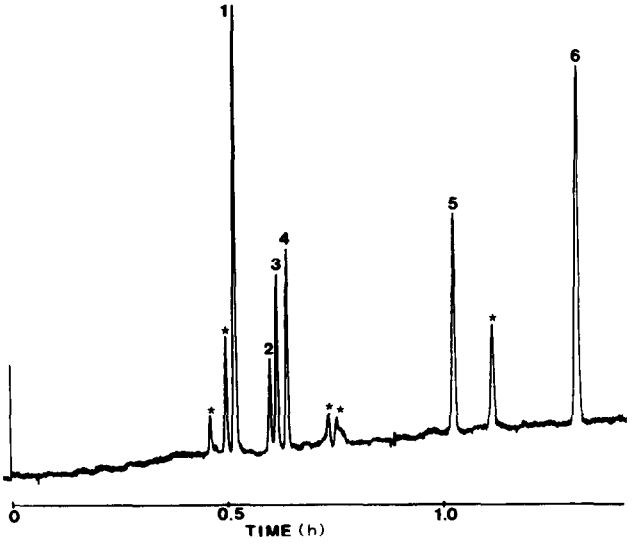

Fig. 5. Chromatogram of standard steroid hormones derivatized with CMMC. Chromatographic conditions as in Fig. 4; injection, approximately 50 pg of each steroid in 0.2 μl. Solutes: 1 = 5β-androstan-3α,11β-diol-17-one; 2 = 5β-pregnane-3α,11β,17α-20β,21-pentol; 3 = 5β-pregnane-3α,11β,17α,20α,21-pentol; 4 = 5α-androstan-3α-ol-17-one; 5 = 5β-androstan-3α,17β-diol; 6 = 5α-pregnane-3β,20β-diol; ★ = sample impurity.

which offer both sensitivity and selectivity, are frequently lacking in condensed-phase separations. It was for this reason that the authors chose to develop a laser fluorescence detection system for the microcolumn LC analysis of such samples.

Many biochemically important compounds, such as the prostaglandins, steroid hormones, and bile acids, contain hydroxy and/or carboxylic acid functions. Two generally applicable derivatization reagents exist for the addition of a coumarin moiety, which has an excitation maximum at 317 nm, to these molecules. Bromomethylcoumarin has frequently been utilized in the derivatization of compounds containing a carboxylic acid group[50], and a novel reagent, 7-(chlorocarbonylmethoxy)-4-methylcoumarin (CMMC), has recently been developed in this laboratory[7] for the deri-

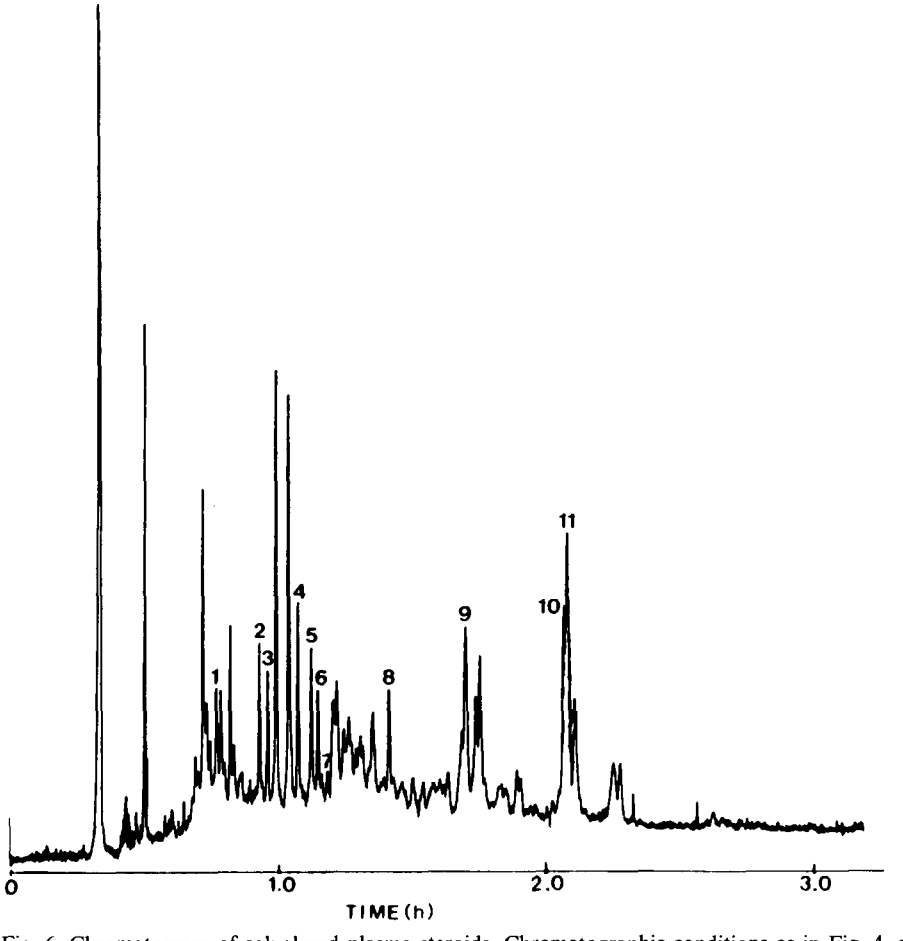

Fig. 6. Chromatogram of solvolyzed plasma steroids. Chromatographic conditions as in Fig. 4, except column 2.25 m × 220 μm I.D. Approximately 50 pg of each steroid was injected. Tentatively identified components: 1 = 5α-androstan-3α,11β-diol-17-one; 2 = 5β-androstan-3α,11β-diol-17-one; 3 = 5β-pregnane-3α,11β,17α,21-tetrol-20-one; 4 = 5β-pregnane-3α,17α,20β,21-tetrol-11-one; 5 = 5β-pregnane-3α,-11β,17α-20β,21-pentol; 6 = 5β-pregnane-3α,17α,20α,21-tetrol-11-one; 7 = 5β-pregnane-3α,11β,17α,20α,-21-pentol; 8 = 5α-androstan-3α-ol-17-one; 9 = 5-androstene-3β-ol-17-one; 10 = 5β-pregnane-3α,20α,21-triol; 11 = 5β-androstan-3α,17β-diol.

vatization of hydroxy compounds. A chromatogram of nine standard bile acids, which were derivatized with bromomethylcoumarin and then analyzed at the picogram level, appears in Fig. 4. Fig. 5 illustrates a similar separation of six steroid hormone standards following derivatization with CMMC.

In addition, samples of naturally occurring body fluids, such as blood plasma, may be purified and quantitatively analyzed for trace amounts of many important biological compounds. Such an analysis appears in the chromatogram of Fig. 6, which illustrates a separation of the solvolyzable steroid hormones from human serum after derivatization with CMMC. The steroid peaks correspond to approximately 50 pg (approximately $6–10 \cdot 10^{-14}$ moles or $3–5 \cdot 10^{-7}$ M solution) of injected compound.

The laser-induced fluorescence detector is, therefore, an integral component in the trace analysis of compounds occurring in complex sample matrices. Although biologically relevant applications are presented here, the detector is equally well suited to the analysis of any fluorescent molecule which can be excited at the 325 nm wavelength of the laser, such as several of the polyaromatic hydrocarbons. Indeed, the monochromatic nature of the excitation beam can be a ready source of desirable detection selectivity.

The small focal spot of the laser beam has allowed relatively large excitation powers to be focused within the small detection volume required for capillary column LC, and, indeed, is compatible with several flow-cell designs, such as the sheath-flow cell for extremely small volumes, or a cell for direct detection on the column packing, which can yield increases in efficiency, as well as sensitivity.

Thus, in conclusion, both the separations and the detection sensitivities obtained in this study serve to illustrate how well the appropriate combination of chemical and instrumental technologies can work to enhance the power of either methodology alone in the high-sensitivity analysis of complex samples.

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