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PHOTOCHEMICAL DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO CANNABINOID ANALYSIS

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SUMMARY

A novel technique of on-line photochemical derivatization is described which can enhance considerably both the sensitivity and specificity of detection in high-performance liquid chromatography (HPLC). Material eluting from the column is irradiated with a high flux of UV light, which may induce a reaction to form fluorescent or highly UV-absorbing products. The irradiated eluent then passes into a suitable detector. The photochemical reactor has a negligible effect on resolution, and reaction is achieved in 1–5 sec.

An example of the use of this technique is in the detection of cannabinol (CBN), a component of cannabis, which is converted into a highly fluorescent compound on irradiation with UV light. Thus, if a sample containing CBN is chromatographed and the column eluent irradiated, CBN can be detected (as the fluorescent photoproduct) with a sensitivity of less than 1 ng. If the chromatogram is then repeated without UV irradiation, only naturally fluorescent products are detected. A comparison of the two chromatograms allows these to be eliminated and leads to a very high specificity for the method.

This approach is being developed as the basis of a rapid, sensitive and specific method for the detection of cannabinoids in body fluids. It is expected, however, that photochemical derivatization will extend the use of HPLC to many substances that cannot be satisfactorily detected at present.

INTRODUCTION

An important requirement in high-performance liquid chromatography (HPLC) is for a detector which is both sensitive and specific. Ultraviolet (UV) monitors and fluorescence detectors are commonly used, but it frequently happens that a substance of interest does not possess a suitable chromophore or fluorophore. This has led to the use of derivatization procedures which render the substance more readily detectable by chemical reaction either before (pre-column) or after chromato-

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TABLE I

DERIVATIZATION TECHNIQUES IN HPLC

Technique	*Advantages*	*Disadvantages*
Pre-column derivatization	Reaction conditions are not restricted. Eluent choice is unrestricted.	Each sample requires individual preparation. Reproducibility may be low. In quantitative work an internal standard is necessary for the derivatization.
Post-column derivatization	Sample preparation is minimal. Derivatization is automated and quantitative precision can be high.	Eluent must be a suitable solvent and reaction medium for the derivatization process. Reaction must be rapid. Resolution is usually adversely affected. Mixing and pumping pulses may affect sensitivity.

graphy (post-column). Conventional derivatization methods such as these suffer a number of disadvantages[1,2], which are summarized in Table I.

This paper describes a novel method of derivatization which combines many of the advantages of conventional methods, but with few of the disadvantages. The technique involves a photochemical reaction which takes place within a few seconds as the eluent leaves the column before passing into the detector.

This method of derivatization has been applied to the detection of cannabinol (CBN), a component of cannabis, with high sensitivity and specificity. Cannabis is a widely used illicit drug and its detection in body fluids is of considerable forensic importance. The complex metabolism of the drug[3] and the low levels found in body fluids have meant that no simple, rapid and specific method is yet available to the forensic toxicologist. Although a radioimmunoassay[4] has been developed for screening purposes and a technique based on gas chromatography–mass fragmentography[5] is available, the latter method requires extensive sample preparation and is not suitable for the analysis of large numbers of samples.

The application of photochemical detection to cannabinoid analysis is based on observations by Bowd *et al.*[6] that such substances, while having only a weak intrinsic fluorescence can be converted into highly fluorescent compounds on irradiation with UV light at 280 nm for up to 4½ h. For CBN (I), the photoproduct was postulated to be the substituted phenanthrene (II)[7].

I
CBN

II

The present study was intended as an investigation of the feasibility of on-line photochemical derivatization in HPLC, primarily for the detection of cannabinol in

body fluids. The use of the technique to enhance the specificity of detection of other substances is also briefly discussed.

EXPERIMENTAL

High-performance liquid chromatography

A Waters M-6000 pump (Waters Assoc., Northwich, Great Britain) was used to deliver eluent to a column of dimensions 10 cm × 4.6 mm I.D. slurry-packed with Spherisorb 5 ODS (Phase Separations, Queensferry, Great Britain), or 25 cm × 4.6 mm I.D. packed with Partisil 10 PAC (Whatman Ltd., Maidstone, Great Britain).

Samples were introduced via a U6K valve injector (Waters Assoc.) and eluted components were detected by a variable-wavelength UV monitor (CE-212, Cecil Instruments, Cambridge, Great Britain) or, for CBN, a fluorimeter (MPF-2A, Perkin-Elmer, Beaconsfield, Great Britain) equipped with a home-made flow cell. The eluent for the reversed-phase (ODS) column was prepared from methanol and aqueous (0.025 *M*) disodium hydrogen orthophosphate and adjusted to pH 8 before use. For the normal-phase column (Partisil PAC), eluents consisting of iso-octane and dioxane were used.

Photochemical reactor

The photochemical reactor (PCR), which was designed and constructed at the Home Office Central Research Establishment, is illustrated in Fig. 1. A 100-W medium-pressure mercury arc (A) (Engelhard Hanovia Lamps, Slough, Great

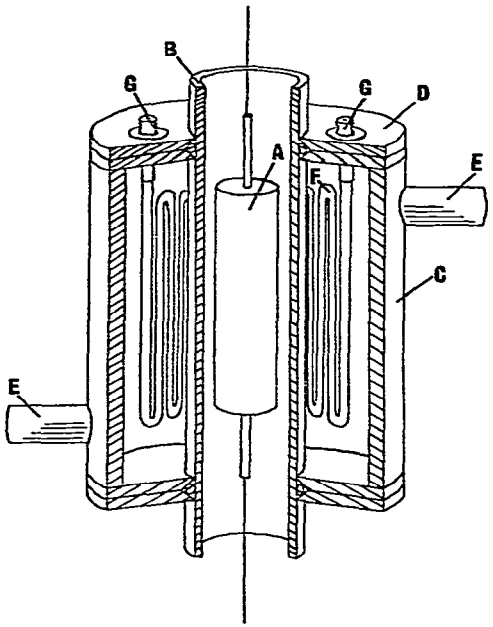

Fig. 1. Sectional drawing of the photochemical reactor for HPLC. A = lamp; B = fused silica tube; C = coolant jacket; D = end-plates; E = coolant connections; F = fused-silica irradiation coil; G = 1/16-in. Swagelok connectors.

Britain) was mounted co-axially within a fused-silica tube (B) (8.5 cm × 2.1 cm O.D., 1.8 cm I.D., Heraeus Quartz Fused Products Ltd., Byfleet, Great Britain). This tube was surrounded by an aluminium alloy cylindrical coolant jacket (C) (6.5 cm × 4.7 cm O.D.) fitted with alloy or stainless-steel end-plates (D), which sealed to tube (B) with O-ring seals. The inside surface of the coolant jacket was polished to form a reflective surface, and inlet and outlet connections for coolant were provided (E). Immersed in the annular coolant space was a convoluted irradiation tube of fused silica (F), O.D. 2 mm, I.D. 0.25 mm, length 70 cm (Heraeus Quartz Fused Products). The ends of the tube were attached by epoxy resin into drilled-out 1/16-in. Swagelok fittings (G) (Type 100-C, Phase Separations), which were brazed on to the end-plate (D).

The photochemical reactor was coupled between the HPLC column and detector (Fig. 2) using conventional 1/16-in. O.D. capillary-bore tubing attached to the connectors (G). Samples were chromatographed twice, both with and without UV irradiation. A single chromatogram would suffice if an additional detector were coupled between the column and the photochemical reactor and traces from the two detectors were compared.

The photochemical reactor is the subject of British Patent Applications[8,9].

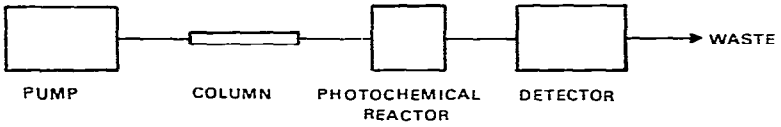

Fig. 2. HPLC with photochemical derivatization.

Extraction of urine samples

As no samples were available from subjects who had smoked or ingested cannabis or CBN, urines from non-drug users were collected and spiked by addition of 20 ng·ml^{-1} of CBN. It was expected that CBN would be present in the urine of cannabis users as a glucuronide or sulphate conjugate, and urines (10 ml) were therefore hydrolysed by treatment with an equal volume of cold methanolic sodium hydroxide (1.0 *M*) for 5 min. The urine was then diluted with water (10 ml) and extracted into *n*-hexane (10 ml; BDH, Poole, Great Britain, spectroscopic grade). Aliquots of the extract were injected directly on to the Partisil PAC column.

RESULTS AND DISCUSSION

Detection of cannabinol by photochemical derivatization

The photochemical detection of cannabinol is shown in Fig. 3, which illustrates the specificity of the method: CBN was detected only after irradiation of the eluent. A high sensitivity was achieved and the technique has been found to be suitable for the detection and quantitation of CBN in the range 0.5–500 ng injected on-column.

The value of this specificity for the analysis of biological samples is shown in the chromatogram of urine extracts (Fig. 4). Although several endogenous urinary components are naturally fluorescent, only CBN is photolabile and forms a fluorescent

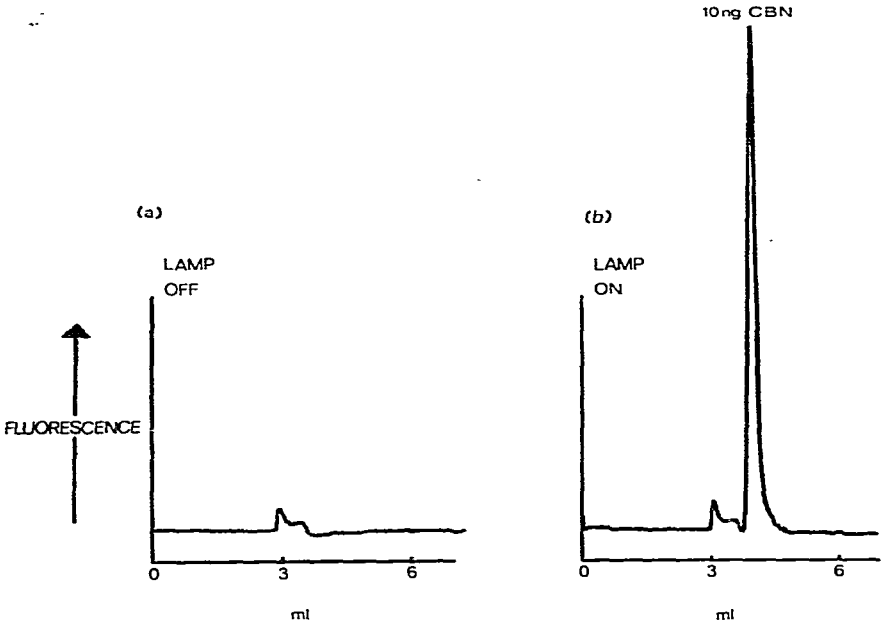

Fig. 3. Photochemical detection of 10 ng of CBN: (a) without UV irradiation; (b) with UV irradiation. Column, Partisil PAC; eluent, isooctane–dioxane (3:2); flow-rate, 1 ml/min^{-1}; detector, MPF-2 fluorimeter, λ_{ex} = 258 nm, λ_{em} = 362 nm, slits 12 nm, sensitivity ×4.

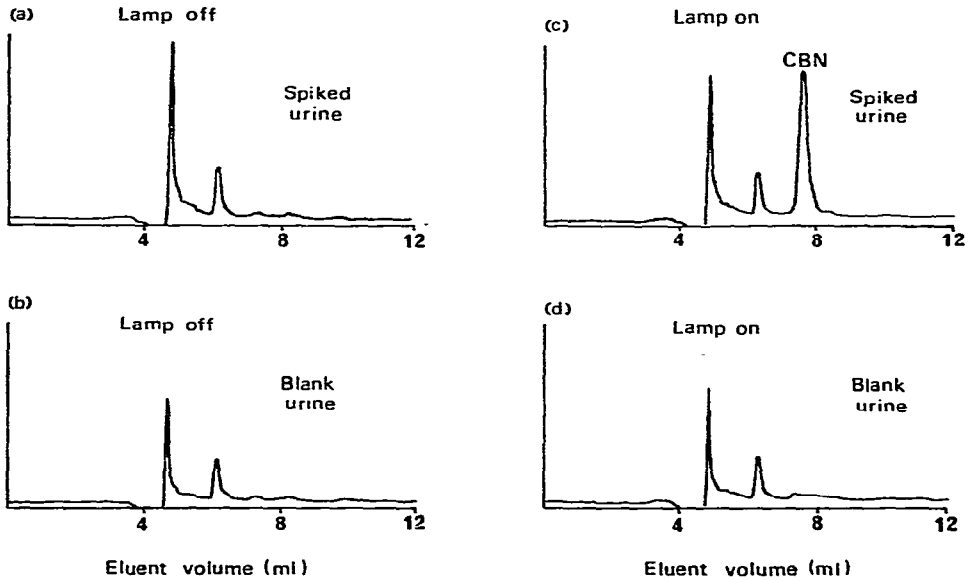

Fig. 4. Photochemical detection of CBN in urine extracts. (a) Extract (500 μl) of urine containing 20 ng·ml^{-1} of CBN; (b) extract of blank urine; (c) and (d), as (a) and (b), respectively, but chromatographed with UV irradiation. Column, Partisil PAC; eluent, isooctane–dioxane (82.5:17.5); flow-rate, 1 ml·min^{-1}; detector, MPF-2A fluorimeter, λ_{ex} = 258 nm, λ_{em} = 362 nm, slits 12 and 15 nm, sensitivity ×5.

product, and it can be readily detected by comparison of the chromatograms with and without UV irradiation. In contrast, the chromatograms of blank urine extracts are identical, with and without irradiation. Hence, by disconnecting or by-passing the photochemical reactor, each sample can be made to serve as its own blank.

Effect of the photochemical reactor on resolution

The efficiency of the chromatographic system (with the Partisil PAC column) was determined by measurement of the number of theoretical plates using the CBN photoproduct (II) as a convenient solute. Samples were chromatographed using the eluent and conditions given in Fig. 3 with the PCR connected but with the lamp switched off. The measurements were repeated with the column eluent flowing directly into the detector in the conventional fashion. The conventional chromatographic system gave an average of 4710 theoretical plates, which was reduced to 4650 plates when the PCR was incorporated into the system. Thus, for all practical purposes, the effect of the PCR on column resolution is negligible. This is thought to be due to the narrow bore of the irradiation coil (0.25 mm) and the absence of unswept dead volumes which would cause eluent mixing. In contrast, most conventional methods of post-column derivatization involve coils of wide (2 mm) bore tubing for mixing and reaction to take place before detection.

Effect of chromatographic parameters on the derivatization process

Eluent flow-rate. The eluent flow-rate has a fundamental effect on the yield of photoproduct (II), as the flow-rate regulates the dwell time of a chromatographic peak in the photochemical reactor. Using an irradiation coil of length 70 cm, irradiation times of 0.5–4.1 seconds were achieved by variation of the flow-rate from 4 to 0.5 $ml \cdot min^{-1}$. The optimal sensitivity of detection for CBN was achieved at flow-rates between 0.5 and 1.0 $ml \cdot min^{-1}$. Presumably, at higher flow-rates, the sample receives insufficient irradiation, while at flow-rates below 0.5 $ml \cdot min^{-1}$, the photoproduct itself may undergo photodecomposition to a non-fluorescent product. Thus although the PCR can be used at flow-rates above 1 $ml \cdot min^{-1}$, the sensitivity of detection for CBN is reduced unless an irradiation coil longer than 70 cm is fitted. At a flow-rate of 2 $ml \cdot min^{-1}$, for example, the sensitivity for CBN was 60% of that found at 1 $ml \cdot min^{-1}$ when a 70-cm irradiation coil was used.

Eluent composition. The eluent composition had only a small effect on the efficiency of the photochemical derivatization of CBN, and both normal- and reversed-phase eluents have been used successfully. Some variation in the sensitivity of detection is to be expected, however, as factors such as the eluent polarity, pH, hydrogen bonding and proton-donor ability may influence the rate and course of the photochemical reaction and the fluorescence quantum efficiency of the photoproduct. Eluents with a UV absorbance above 250 nm also act as an "inner filter" in both the derivatization and detection stages of the process.

Other applications of the photochemical reactor

The PCR can be used to enhance the specificity of detection other than by the formation of fluorescent derivatives. A shift in the wavelength of UV absorbance can be advantageous, as interferences due to extraneous UV-absorbing substances are generally minimized at longer wavelengths of detection. An example of this is the use

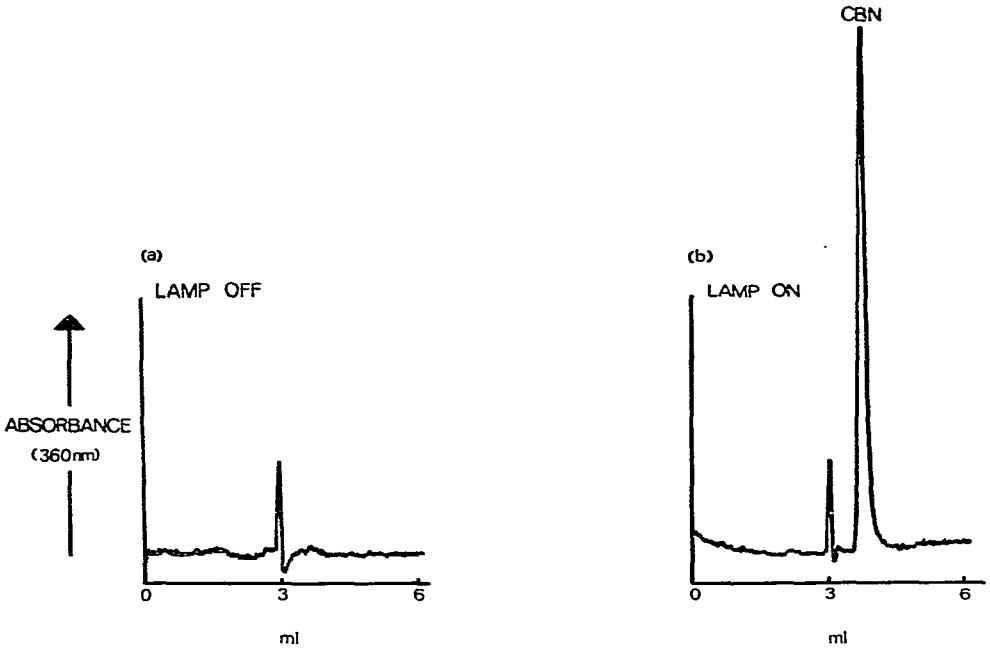

Fig. 5. Photochemical detection of CBN (180 ng) by UV absorbance at 360 nm. Conditions as in Fig. 3 except: flow-rate, 0.5 ml·min^{-1}; detector, Cecil CE-212, 360 nm, 0.02 a.u.f.s.

of a UV monitor in place of a fluorimeter for the detection of CBN, when the drug can be detected by its UV absorbance at 360 nm (Fig. 5).

The specificity of detection of the hallucinogen LSD may also be enhanced. Upon irradiation at 320 nm, the naturally fluorescent LSD is converted into the non-fluorescent *lumi* derivative[10,11] (Fig. 6), and HPLC with UV irradiation may be valuable in distinguishing the drug from other fluorescing material with similar chromatographic properties.

Photochemical derivatization may be applicable to the HPLC analysis of other drugs which are at present difficult to detect with the required sensitivity or specificity. An example is dextropropoxyphene, a widely prescribed analgesic which has been implicated in many drug overdose fatalities[12]. The drug has only a weak UV absorbance, and detection by UV spectroscopy or HPLC is difficult. Irradiation of the drug with UV light leads to the formation of a photoproduct with an increased UV absorbance[13], and preliminary results indicate that the use of the PCR described here leads to a 10-fold increase in the sensitivity of detection by HPLC using a UV monitor. Further work on this aspect is in progress.

It is envisaged that the application of the technique can be further extended by the use of derivatization reagents which photoreact with the substance of interest. Such reagents can conveniently be added to the eluent prior to chromatography.

CONCLUSION

HPLC with photochemical derivatization and fluorimetric detection has been shown to provide a highly specific means of detection of nanogram amounts of

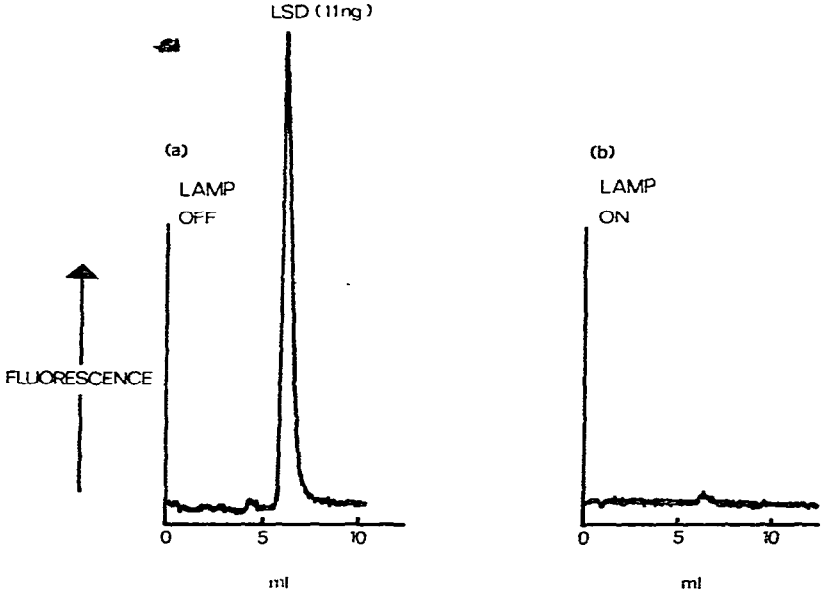

Fig. 6. Specific detection of LSD (11 ng) using photochemical derivatization (a) without and (b) with UV irradiation. Column, Spherisorb ODS; eluent, methanol–aq. (0.025 *M*) Na_2HPO_4 (65:35) adjusted to pH 8; flow-rate, 0.5 ml·min^{-1}; detector, MPF-2A fluorimeter, λ_{ex} = 320 nm, λ_{em} = 400 nm, slits 10 nm, sensitivity ×5.

cannabinol. A general method based on this principle for the detection of cannabinoids in body fluids is under development. The photochemical technique is also applicable to the specific detection of several other drug substances, such as dextropropoxyphene, for which conventional HPLC with UV detection gives poor sensitivity.

Photochemical derivatization offers several advantages over other pre- and post-column derivatization techniques. Time-consuming sample preparation procedures associated with pre-column methods are eliminated, and the retention volume measured is that of the parent substance rather than that of a derivative. In contrast with conventional post-column derivatization methods, photochemical derivatization has a negligible effect on the resolution of the chromatographic system and imposes few practical restrictions on the eluent composition. As no reagents need to be added to the eluent stream, problems of mixing and pulsating flow do not arise. The principal advantage of photochemical derivatization is, however, the specificity of detection that it provides, and the simple means by which the substance of interest can be differentiated from non-photolabile interfering compounds. It is expected that photochemical derivatization will form a valuable addition to available derivatization methods owing to its simplicity, speed of operation and very high specificity.

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