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High-performance liquid chromatography using continuous on-line post-elution photoirradiation with subsequent diode-array UV or thermospray mass spectrometry detection

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ABSTRACT

The use of HPLC with continuous on-line post-elution photoirradiation followed by either diode-array UV or thermospray mass spectrometric detection is presented. These tandem techniques can greatly increase specificity of analysis, as demonstrated by select compounds whose UV or thermospray mass spectra are highly similar under lamp off conditions but significantly different after photoirradiation. The utility of the above approach for various compounds of forensic interest is presented.

INTRODUCTION

For HPLC analysis, retention data alone lack selectivity. As a result, additional on-line detectors are commonly used to increase specificity by providing complementary information [1,2]. Typical on-line detection modes include UV, MS, fluorescence and electrochemical. This approach is particularly useful in forensic drug analysis, where trace level analyses and establishment of peak identity and purity are very important [1,2].

Use of diode-array UV detectors can greatly increase selectivity by providing UV spectra; however, the spectra themselves are generally not unique. Similarly, thermospray (TSP) MS can also greatly increase selectivity by providing molecular weight information; however, usually little or no additional information beyond molecular weight is obtained. Furthermore, certain compounds do not give any detectable ion current at all using TSP-MS.

Continuous on-line post-elution photoirradiation has been previously employed as a means of converting an eluting compound into one or more photoproduct(s) prior to ether variable-wavelength UV [3,4], fluorescence [5–13] or electrochemical detection [14–23]. Photoirradiation of photosensitive solutes frequently results in formation of photoproducts with enhanced detectability. HPLC–photoirradiation followed by fluorescence or electrochemical detection has been previously reported for determination of drugs of forensic interest [5–8,14–17], herbicides [9,18], pesticides [13], sulfonamide

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diuretics [10], stilbene derivatives [11], vitamin K homologues [12], penicillins [19], explosives and related nitro compounds [20], foods [21], inorganic anions [22] and amino acids and proteins [23].

In this paper, the applicability of continuous on-line post-elution photoirradiation to increase the selectivity of diode-array UV and TSP-MS detection is described. The utility of this technique for the determination of various drugs of forensic interest is discussed.

EXPERIMENTAL

Equipment

Two HPLC systems were employed in this study. For diode-array UV detection, a Series 4 liquid chromatograph (Perkin-Elmer, Norwalk, CT, USA) fitted with an ISS autosampler (Perkin-Elmer), a Partisil-5 ODS-3 cartridge system (11.0 cm × 4.7 mm I.D., 5 μm, column) (Whatman, Clifton, NJ, USA) and a 1040M diode-array UV detection system (Hewlett-Packard, Waldbronn, Germany) were employed. For TSP-MS detection, a Series 4 liquid chromatograph fitted with a Model 7125 valve injector (Rheodyne, Cotati, CA, USA) and a Model 701C thermospray interface (Vestec, Houston, TX, USA) fitted to a 4530 quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA, USA) were employed. For both systems, a "Phred" photochemical reactor (Aura Industries, Staten Island, NY, USA) fitted with a KRC 10-50 knitted open tube (KOT) reactor coil (10 m × 500 μm I.D.) and a low-pressure 8-W mercury lamp with a strong 254-nm line (Aura Industries) were employed prior to the detection system.

Materials

Acetonitrile and ammonium acetate were HPLC grade. Water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). All other chemicals were reagent grade or better. Drug standards were obtained from the Reference Standards Collection of the Drug Enforcement Administration's Special Testing and Research Laboratory.

The HPLC mobile phase was internally mixed from two individual solvent reservoirs containing acetonitrile and 0.1 *M* ammonium acetate buffer, respectively.

Procedures

For flow injection analysis, 1 mg of standard was dissolved in 50.0 ml mobile phase prior to a 50- or 100-μl injection onto the liquid chromatograph. For the TSP-MS flow-rate optimization study, 1 mg of each standard was dissolved in 25.0 ml mobile phase prior to a 50-μl injection onto the liquid chromatograph. The mobile phase for both flow injection and HPLC with post-column analysis consisted of 0.1 *M* ammonium acetate–acetonitrile (60:40, v/v).

TSP conditions were optimized at a flow-rate of 1.0 ml/min (see below). The vaporizer had a tip orifice of 125 μm and was operated at 238–259°C with the source block temperature held at 266–282°C. The repeller was set at approximately 28 + V d.c. Data acquisition was from m/z 100 to 500 at 3 s/scan.

RESULTS AND DISCUSSION

Determination of the optimum photochemical reaction time (i.e., flow-rate)

It was of interest to determine whether the optimum flow-rate for TSP-MS analysis (*i.e.*, approximately 1.0 ml/min) also represented the optimum exposure time for photochemical reaction. Six representative compounds of forensic interest (acetaminophen, heroin, noscapine, pentobarbital, methapyrilene and diphenhydramine) were subjected to photoirradiation at several different flow-rates (effectively giving different photochemical reaction times). Following diode-array UV detection, peak heights were measured at wavelengths where the photoproduct(s) have significantly higher absorbances than the parent compounds. Acetaminophen was measured at 380 nm, heroin and noscapine at 340 nm and pentobarbital, methapyrilene and diphenhydramine at 270 nm. As shown in Fig. 1, comparison of peak height response *vs.* flow-rate indicates unchanged or only slightly reduced sensitivity levels (compared with the maximum response) when the optimum TSP-MS flow-rate of 1.0 ml/min is used for acetaminophen, heroin, pentobarbital and methapyrilene, with greater reductions for noscapine and diphenhydramine. However, there are still significant and sufficient photoinduced reactions occurring at 1.0 ml/min for each of the photosensitive substrates examined, and this flow-rate was therefore utilized for all studies.

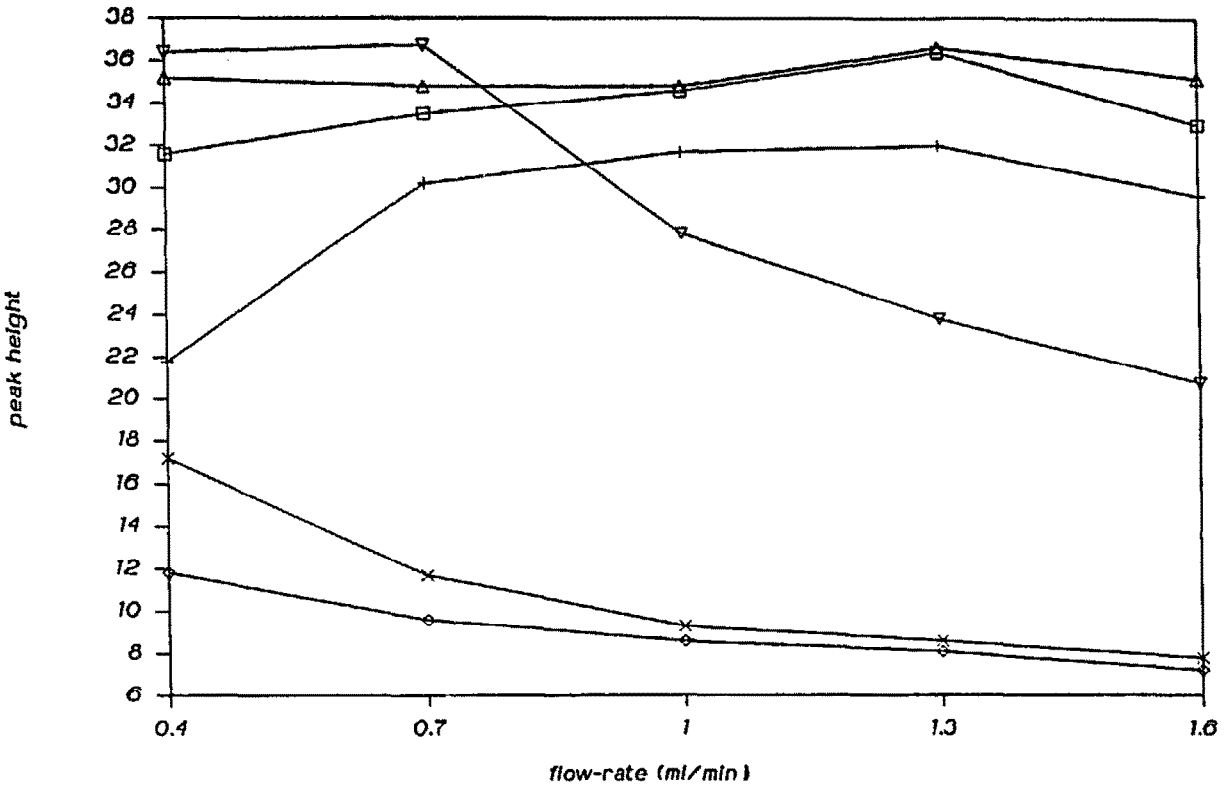


Fig. 1. Peak height response *versus* flow-rate for pentobarbital (+), heroin (◇), methapyrilene (△), diphenhydramine (×), acetaminophen (□) and noscapine (▽) after post-column photochemical reaction and diode-array detection.

Photoirridiation with subsequent diode-array UV detection

The utility of photoirradiation diode-array UV detection is demonstrated by select compounds whose UV spectra are highly similar under lamp off conditions but which exhibit quite different spectra after photoirradiation. In this vein, it is particularly illustrative to compare structurally closely related compounds, *e.g.*, butalbital *vs.* talbutal, O6-monoacetylmorphine (O6-MAM) *vs.* morphine and 3,4-methylenedioxyamphetamine (MDA) *vs.* 3,4-methylenedioxymethamphetamine (MDMA). As shown in Figs. 2–4, these pairs display similar spectra under lamp off conditions but significantly different spectra following photoirradiation. Butalbital and talbutal display no discernible UV maxima under lamp off conditions; following photoirradiation, however, both compounds displayed distinct UV maxima at 265 nm, with talbutal undergoing the significantly more pronounced hyperchromic effect (Fig. 2). Similarly, both O6-MAM and morphine gave slight blue shifts (286 → 284 nm) following photoirradiation, with morphine undergoing the greater hyperchromic effect (Fig. 3). Finally, both MDA and MDMA developed a shoulder at 318 nm following photoirradiation, with MDMA undergoing the more pronounced hyperchromic effect at 318 nm and more pronounced hypochromic effect at 286 nm.

Molecular consequences of photoirradiation on UV spectra

UV absorption and photochemical reaction both involve excitation of photosensitive substrates via photoirradiation. However, the former process involves only a fully reversible absorption of energy raising a specific chromophore from its ground level to a low lying excited electronic energy level. The resulting excited state quickly returns to the ground state via various intra- and intermolecular vibra-

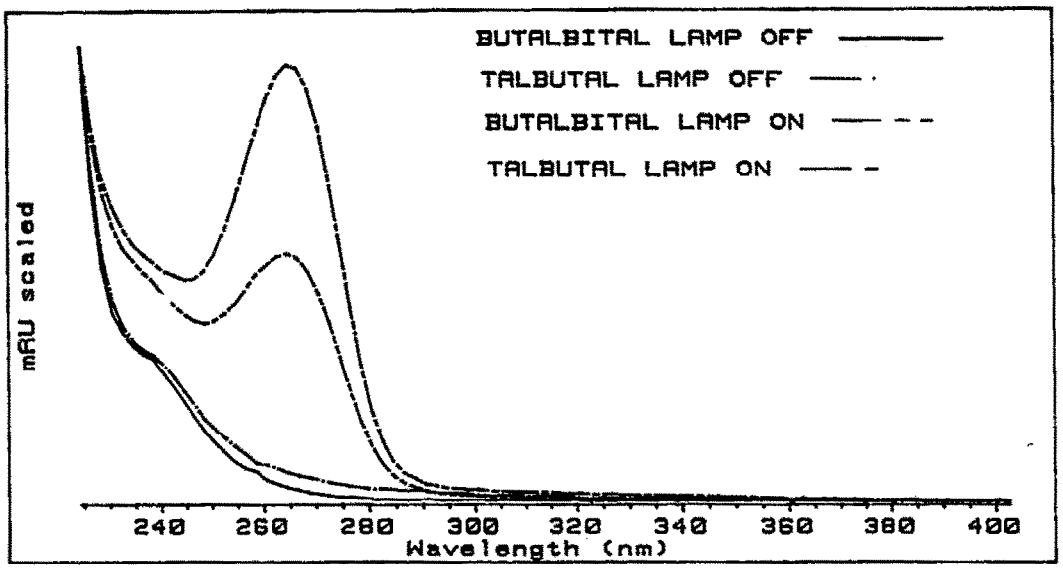


Fig. 2. UV spectra of butalbital and talbutal before and after photoirradiation.

tional and rotational interactions (thereby end-resulting only in simple heating of the solution). In contrast, the latter process involves absorption of very high energy photons raising a specific chromophore from its ground level to an upper excited electronic energy level. As with standard UV absorption, the resulting photoexcited molecule *may* return to the ground state via vibrational rotational interactions, but can alternately act as a high energy transition state to a new molecule (or molecules) via (usually) intra- or (rarely) intermolecular reactions. The resulting photoproducts, whose formation and structure(s) can be highly dependent on the presence of nearby functional groups and/or stereochemistry, can have different chromophores (and therefore dramatically different UV spectra)

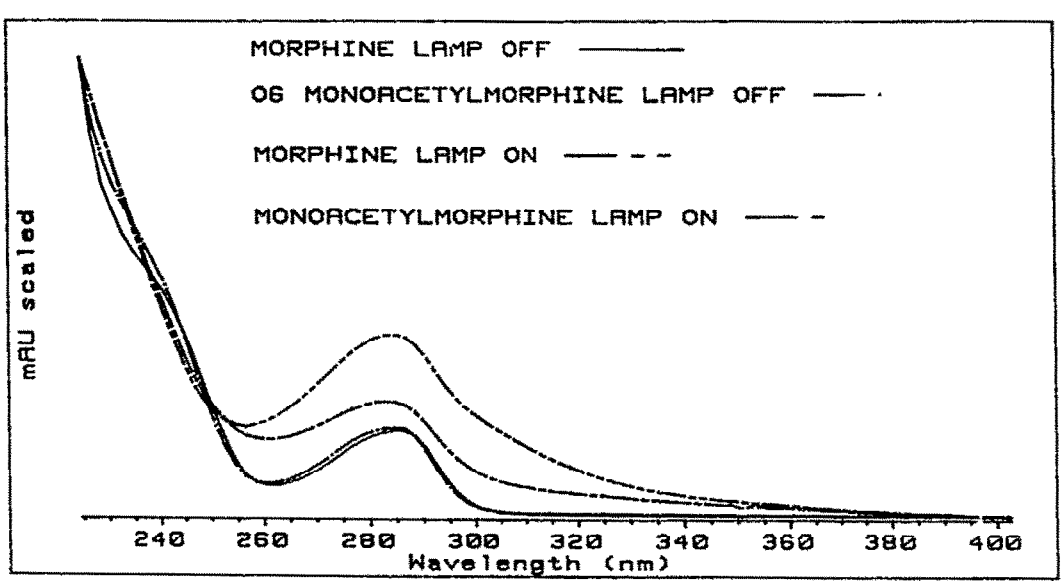


Fig. 3. UV spectra of morphine and O6-monoacetylmorphine before and after photoirradiation.

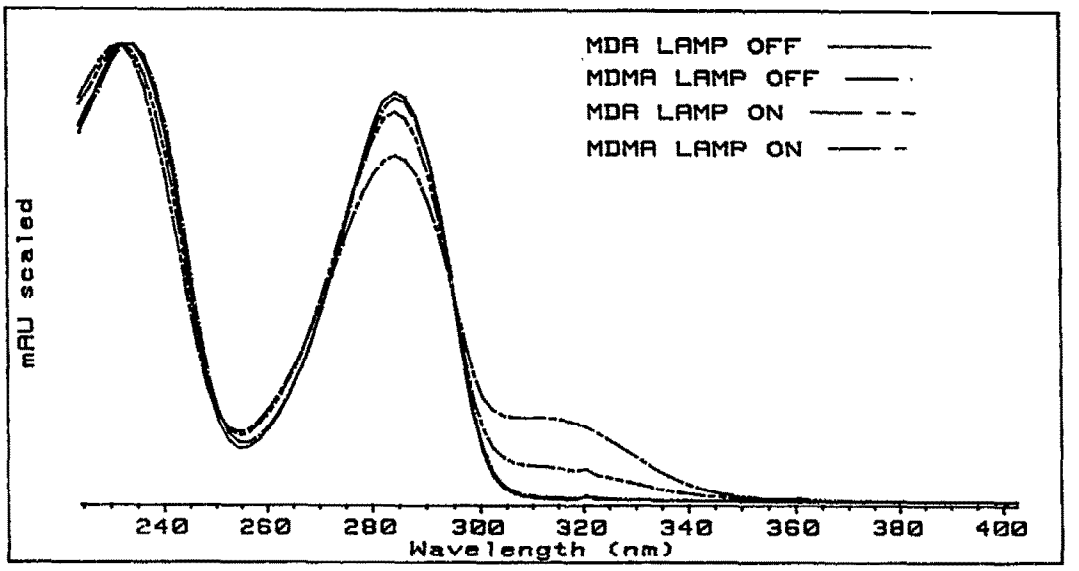


Fig. 4. UV spectra of MDA and MDMA before and after photoirradiation.

vs. the original substrate. As the examples detailed above illustrate, even minor structural and/or stereochemical differences can lead to significant changes in UV spectra.

Photoirradiation with subsequent TSP-MS detection

As is the case for diode-array detection, photoirradiation frequently increases specificity of pure TSP-MS analyses. For compounds having the same molecular mass, pure TSP mass spectra are frequently the same, however, as shown in Table I. For example, O3- and O6-MAM, both of which give only *m/z* 328 ions (MH^+) under lamp-off conditions, give significantly different spectra containing multiple ions under lamp-on conditions which formally can be interpreted. O3-MAM has an intense *m/z* 345 ion (probably $[M + NH_3]H^+$) and a weak *m/z* 314 and *m/z* 304 (probably $[M - CH_2CO + H_2O]H^+$) ion; in contrast, O6-MAM has strong *m/z* 346 (probably $[M + H_2O]H^+$), *m/z* 302 and *m/z* 300, 284, 270, 268, 257 and 256 ions. Both compounds retain very intense *m/z* 328 ions (which almost certainly represent the unreacted parent compound MH^+'s) and also display common *m/z* 286 (most probably $[M - CH_2CO]H^+$) ions.

In another pertinent example, cannabidiol and Δ^9-tetrahydrocannabinol (Δ^9-THC) also give identical TSP mass spectra under lamp off conditions but different spectra after photoirradiation. Under lamp-off conditions, both compounds exhibit *m/z* 315 ions (MH^+). After photoirradiation, cannabidiol gives only the *m/z* 315 ion (Note, however, that the absolute intensity of the UV spectrum was significantly higher, suggesting that at least some photoproducts were produced); in contrast, Δ9-THC gives three ions [an intense *m/z* 315 (= MH^+) and smaller *m/z* 373 and 333 (probably $[M + H_2O]H^+$) ions].

TABLE I

MASS SPECTRA (THERMOSPRAY IONIZATION) OF COMPOUNDS OF FORENSIC INTEREST BEFORE AND AFTER PHOTOCHEMICAL REACTION

Compound	*m/z* (relative abundance)	
	Lamp off	Lamp on
O3-Monoacetylmorphine	328(100)	345(34), 328(100), 314 (3), 304(3), 286(51)
O6-Monoacetylmorphine	328(100)	346(36), 328(70), 302 (10), 300(8), 286(40), 284(40), 270(18), 268(12), 257(100), 256(12)
Cannabidiol	315(100)	315(100)
Δ^9-THC	315(100)	373(6), 333(12), 315 (100)

TABLE II

MASS SPECTRA (THERMOSPRAY IONIZATION) OF BARBITURATES BEFORE AND AFTER PHOTOCHEMICAL REACTION

Compound	m/z (relative abundance)	
	Lamp off	Lamp on[a]
Pentobarbital	–	261(12)^{B2b}, 244(100)^{B2a,B1b}, 227(4)^{B1a} 218(4)^{Cb}, 201(25)^{Ca}, 157(14)^{AaR1}
Butalbital	–	242(49)^{B2a,B1b}, 225(6)^{B1a}, 207(100)^{Da} 199(15)^{Ca}
Barbital	–	219(24)^{B2b}, 202(100)^{B2a,B1b}, 185(4)^{B1a} 159(8)^{Ca}
Talbutal	–	259(8)^{B2b}, 242(37)^{B2a,B1b}, 207(100)^{Da} 199(20)^{Ca}, 155(12), 143(10)
Allobarbital	–	243(6)^{B2b}, 232(5), 226(16)^{B2a,B1b} 209(4)^{B1a}, 201(4), 191(100)^{Da}
Amobarbital	–	261(15)^{B2b}, 244(100)^{B2a,B1b}, 227(8)^{B1a} 218(4)^{Cb}, 201(20)^{Ca}
Secobarbital	–	256(68)^{B2a,B1b}, 239(8)^{B1a}, 221(100)^{Da} 213(45)^{Ca}, 169(19)^{AaR1}
Phenobarbital	–	250(96)^{B2a,B1b}, 233(100)^{B1a}, 207(56)^{Ca} 204(8), 164(4)

[a] A, B1, B2 and C: see Fig. 5 and refs. 25–27 for postulated structures; D: see Fig. 6 for postulated mechanism and structure; lower case a and b denotes H^+ and NH_4^+ ion adducts respectively; Rx references the C-5 substituent lost to form ion A.

Finally, in contrast to the findings of Heeremans *et al.* [24] for heptabarbital, most barbiturates give *no* detectable TSP mass spectra at all under lamp off conditions, but (as detailed for eight typical barbiturates in Table II) numerous ions after photoirradiation. These results were expected in light of previously reported photoproducts for secobarbital, pentobarbital and barbital [25–27]. All eight barbiturates displayed an intense $[M + NH_3]H^+$ ion (consistent with the previously postulated protonated photoproduct B2 shown in Fig. 5). In addition, a previously unreported photoproduct (designated photoproduct D) was observed at M − 17 for all barbiturates having a 5-allyl subsituent; D appears to be the end result of a combined photo-Fries rearrangement–dehydration reaction mechanism (Fig. 6) and represents a highly specific and convenient marker for identification of 5-allyl barbituric acids [28].

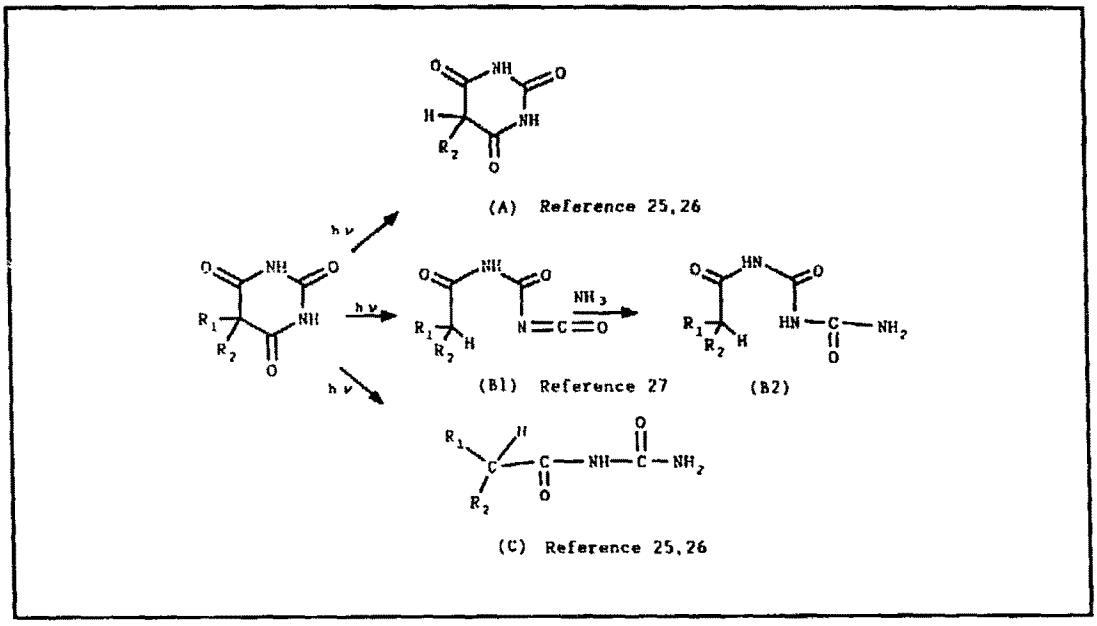
Molecular consequences of photoirradiation on TSP mass spectra

Under pure TSP-MS conditions, the ions which are typically observed represent combinations of proton, ammonium and solvent adducts; the formation of these adducts essentially depends on the ability of a compound to act as a Bronsted base. As shown earlier, any compounds with the same molecular mass and closely similar structures will typically have similar Bronsted base properties and therefore essentially identical TSP mass spectra.

As previously discussed, photoirradiation can result in the formation of new photoproducts, which will result in clear differences in TSP mass spectra *if* those new compounds have different molecular masses and/or altered Bronsted base properties. In addition, dramatically increased fragmentation may be observed, even with photoproducts of identical molecular mass and Bronsted base properties, if those compounds are (as expected) thermodynamically less stable than the original substrate (thermodynamically unstable photoproducts are more susceptible to fragmentation under standard TSP-MS conditions). As the examples detailed above again illustrate, even minor structural and/or stereochemical differences can result in significant changes in TSP mass spectra.

Determination of psilocybin in hallucinogenic mushrooms

The use of diode-array UV and TSP-MS detection under lamp-off/lamp-on conditions provides for both the identification of psilocybin and its differentiation from psilocin. The UV spectra of psilocybin and psilocin are quite similar under lamp off conditions, with UV maxima at 265 nm (Fig. 7). After photoirradiation, both compounds exhibit pronounced hyperchromic effects throughout the entire UV spectra 220–400 nm; however, psilocybin displays no discernible UV maxima, while psilocin displays a new maxima at 233 nm. The TSP mass spectra of psilocybin displays only an abundant *m/z* 285 MH^+ ion and a more intense *m/z* 205 ion (the latter almost certainly representing the MH^+ ion for psilocin) under lamp off conditions (Table III).



pentobarbital	R_1– $CH_3CH_2CH_2CH(CH_3)$	R_2– C_2H_5
butalbital	R_1– $(CH_3)_2CHCH_2$	R_2– $CH_2{=}CHCH_2$
barbital	R_1– C_2H_5	R_2– C_2H_5
talbutal	R_1– $CH_3CH_2CH(CH_3)$	R_2– $CH_2{=}CHCH_2$
allobarbital	R_1– $CH_2{=}CHCH_2$	R_2– $CH_2{=}CHCH_2$
amobarbital	R_1– $(CH_3)_2CHCH_2CH_2$	R_2– C_2H_5
secobarbital	R_1– $CH_3CH_2CH_2CH(CH_3)$	R_2– $CH_2{=}CHCH_2$
phenobarbital	R_1– C_6H_5	R_2– C_2H_5

Fig. 5. Correlation of 5,5-disubstituted barbituric acid photoproducts [25–27].

After photoirradiation, however, a highly complex TSP mass spectra is obtained containing 27 ions; the spectrum is vastly different than that obtained for the structurally similar psilocin under identical conditions. The new base peak for psilocybin is *m/z* 221, while for psilocin is *m/z* 219. In all, there are 22 unique ions in the photoirradiated TSP mass spectra of psilocybin (*vs.* psilocin), including intense ions at *m/z* 164, 162, 128 and 118. The intense *m/z* 205 ion present in both spectra is consistent with the MH^+ ion for psilocin. Finally, since the ion of *m/z* = 285 (representing the MH^+ for psilocybin) is *not* present after photoirradiation, the aquisition of preliminary lamp off TSP mass spectra further increases the specificity of analysis.

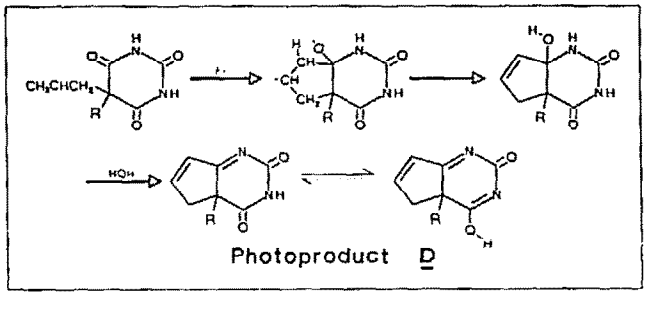


Fig. 6. Postulated mechanism for photoproduct D.

For actual sample analysis the chromatographic system reported by Borner and Brenneisen [29] appears to be viable. The reversed-phase gradient system which was used (containing ammonium acetate buffer and methanol) would be expected to be compatible with photoirradiation and diode-array UV and TSP-MS detection. Borner and Brenneisen [29] showed the homogeneity of the psilocybin peak from a mushroom sample by overlaying upslope.

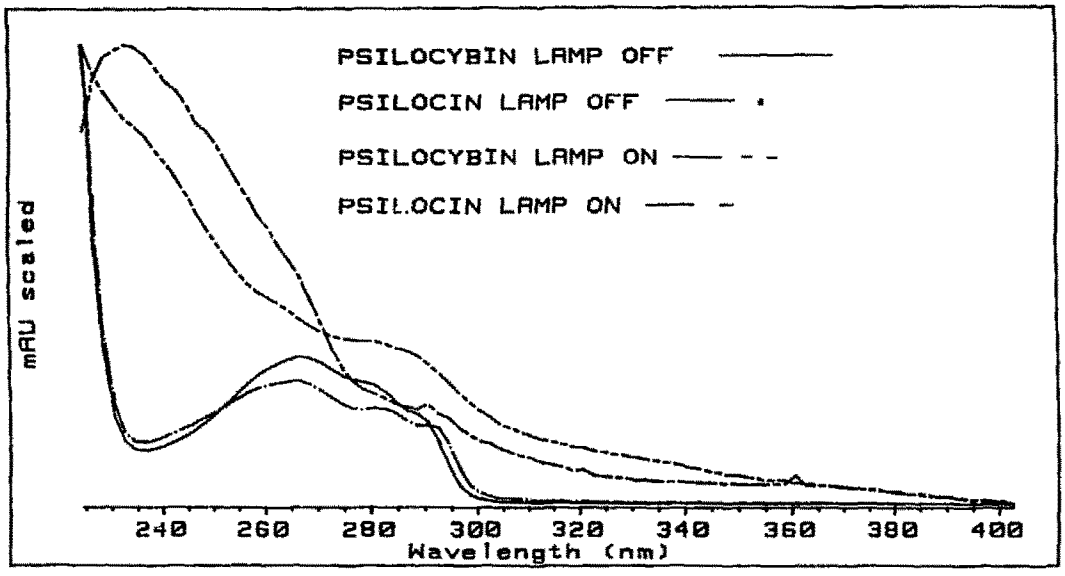


Fig. 7. UV spectra of psilocybin and psilocin before and after photoirradiation.

apex and downslope spectra obtained using a diode array UV detector.

Limitations of photoirradiation

The two reactor coils used in this study lasted for 150 and 80 injections, respectively, before developing leaks (approximately equal injections for each detector). It has been previously reported that fluoride liberated from PTFE (polytetrafluoroethylene) tubing during UV irradiation causes the tubing to turn brittle and eventually rupture [30,31]. This problem might be alleviated if ice-bath cooling is provided for the lamp and the KOT assembly [31]. Another possible factor contributing to coil rupture may be the back pressure generated by the TSP apparatus (4.4–6.1 MPa), since the PTFE tubing used in this study is only rated for 3.4 MPa. Working at lower flow-rates (e.g., 0.7 ml/min) would significantly lower back pressure —although at the expense of TSP detector sensitivity (approximately 2× loss). However (as shown in Fig. 1), there would still be sufficient generation of photoproducts for routine analytical analyses even at this lower flow-rate. Alternately, use of different lengths and/or internal diameters of PTFE tubing for the KOT assembly could maintain or increase sensitivity even at lower flow-rates.

TABLE III

MASS SPECTRA (THERMOSPRAY IONIZATION) OF PSILOCYBIN AND PSILOCIN BEFORE AND AFTER PHOTOCHEMICAL REACTION

Compound	*m/z* (relative abundance)	
	Lamp off	Lamp on
Psilocybin	205(100) 285(18)	391(4), 348(3), 301(3) 300(3), 283(4), 240(4) 237(3), 221(100), 219(17) 217(4), 212(11), 205(30) 203(39), 192(5), 191(9) 190(3), 178(5), 177(5) 167(5), 164(14), 162(26) 128(36), 126(6), 125(3) 118(12), 116(4), 114(8)
Psilocin	205(100)	266(13), 247(8), 235(5) 221(31), 219(100), 217(5) 212(3), 205(39)

CONCLUSIONS

Continuous on-line post-elution photoirradiation followed by diode-array UV or TSP-MS detection is a readily performed technique which results in significantly increased specificity of analysis. Although flow injection analysis was primarily used to acquire data for this study, the approach presented is applicable to HPLC with post-column analysis.

The UV and TSP-MS data obtained are highly complementary. In addition, the possibility exists for the direct coupling of the diode-array UV and/or TSP-MS detectors with a high-pressure-rated UV flow cell.

Additional drugs of forensic interest which undergo changes in either their TSP-mass spectra and/or UV after photoirradiation include morphine, codeine, acetylcodeine, heroin, lysergic acid diethylamide, phencyclidine, N-hydroxy-MDA, fentanyl, noscapine, quinine, diphenhydramine, phenacetin, acetaminophen, antipyrene, methapyrilene and nicotinamide. Further applications can be reasonably expected for analysis of, *e.g.*, penicillin, sulfonamide diuretics, herbicides, stilbene derivatives, vitamin K homologues, explosives, foods, inorganic anions, amino acids and proteins; in all of the latter examples, photoproducts have been previously reported using either fluorescence or electrochemical detectors (see above). Ballard and Grinberg [32] are presently investigating the use of flow injection–photoirradiation–TSP-MS for analyses of benzophenone, heptylphysotigmine (used for the treatment of Alzheimer's disease) and the immuno-suppressant drug FK-520. Finally, diode-array UV and/or TSP-MS detection may be used for continuous on-line monitoring of photochemical reactions.

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