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ENHANCED ULTRAVIOLET DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DRUGS BY "ON-LINE" PHOTOCHEMICAL REACTION

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SUMMARY

The effect of on-line photochemical reaction on UV detection in high-performance liquid chromatography (HPLC) has been studied. For on-line irradiation a photoreactor utilizing a low pressure mercury light source and a crocheted reaction capillary has been used by which the chromatographic performance of the HPLC separation is only slightly influenced. On-line irradiation of the drugs methadone, methoin, nirvanol and several barbiturate analogues results in a significant increase in their UV absorptivity, either due to the appearance of new spectral bands or due to absorption enhancement at longer UV wavelengths. By these photochemical effects, the detectability of drugs at longer UV wavelengths will be increased, in some cases more than 80-fold. Thus routine analysis of these drugs in biological samples may be simplified considerably, allowing more specific detection and less complex sample preparation.

INTRODUCTION

Pre- or postcolumn chemical modification of an analyte is a common approach to increase the sensitivity and specificity in high-performance liquid chromatographic (HPLC) trace analysis of pharmacological substances in biological fluids. At the same time there are a number of drawbacks. While in precolumn derivatizations increased variability occurs due to variable reaction times and rates, in postcolumn derivatization, for on-line addition of reagent(s), an additional pumping system is required introducing pulsation, dilution and peak broadening into the chromatographic system.

Recently, photochemical reaction has been demonstrated to be an efficient postcolumn reaction system[1]. Because light is the only "reagent" which has to be "added" in on-line photochemical reactions, many properties of an ideal on-line reaction system are exhibited.

Although a wide range of different chemical reactions have been described in photochemistry[2], on-line photochemical reactions in HPLC have been described only

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for a small number of substances, nearly all of them leading to an fluorescent or electrochemically active product after irradiation[3]. Extending this principle of postcolumn photochemical reaction to other HPLC detection systems, we have investigated whether by on-line irradiation drugs of various molecular structures are modified in their spectral properties, in the search for a more specific or sensitive HPLC determination of these drugs in biological samples.

EXPERIMENTAL

The photochemical reactor

For on-line, postcolumn irradiation experiments, a photochemical reactor was used which was constructed in the laboratory and has been described in detail before[3]. Up to 40 m of 1/16-in. heavy wall, narrow bore PTFE tubing (1/16 in. O.D., 0.01 in. I.D.; Pierce Europe, Oud-Beijerland, The Netherlands) was crocheted into a rectangular pad of side approximately 6 cm. It was fixed on a stainless-steel mesh and mounted around a tubular 8-W low pressure mercury lamp (GTE, Sylvania G8-T5). This light source emits the known mercury spectrum including the strong line in the UV at 254 nm.

To increase the light yield and the efficiency of the photochemical reaction, the inner side of the reactor housing may be covered by reflecting aluminium foil. Because of the low power rating of the light source, no active cooling in the photoreactor is necessary.

Depending on the length of the crocheted reaction capillary, irradiation times of up to 240 s (at a nominal flow-rate of 0.8 ml/min) are possible in this photoreactor geometry. For certain experiments in which the influence of the irradiation spectrum on the photochemical reaction was studied, the mercury light source was exchanged for an identical lamp with a spectral cut-off at *ca.* 366 nm (Sylvania, F8-T5).

In "off-line" irradiation experiments, samples were irradiated in a quartz cuvette using a low pressure mercury pen-ray lamp (Analamp, BHK, Monrovia, CA, U.S.A.).

Chromatographic system

The HPLC system used consisted of a 302 pump (Gilson, Villiers le Bel, France), a six-port injection valve (Valco, Houston, TX, U.S.A.) and a SPD-6A variable wavelengh detector (Shimadzu Europe, Duisburg, F.R.G.). The photochemical reactor was connected between the analytical column and the UV detector by means of zero dead volume unions (Valco).

Chromatographic separations were generally done on a 11 cm × 4.7 mm Whatman cartridge packed with PartiSphere C_{18}, 5-μm silica material (Whatman, Clifton, NJ, U.S.A.). Mobile phase compositions (methanol or acetonitrile–phosphate buffer mixtures) were chosen depending on the compounds to be separated.

For off-line spectral analysis a Perkin-Elmer Lambda 7 spectral photometer (Bodenseewerk, F.R.G.) was used.

Chemicals

Water and organic solvents used for HPLC were distilled from glass apparatus and of HPLC grade (HiPerSolv; BDH, Poole, U.K.); buffer salts were of the highest purity available (Merck, Darmstadt, F.R.G.). Methadone hydrochloride was ob-

tained from Heilmittelwerke (Linz, Austria). Mephenytoin and nirvanol were gifts from Gerot Pharmaceuticals (Vienna, Austria). Barbiturates were obtained from Merck.

Analysis of blood plasma sample

To 100 μl blood plasma an equal volume of acetonitrile was added. Precipitated proteins were centrifuged at *ca.* 10000 *g* in a Biofuge A (Haraeus, F.R.G.). After dilution of the supernatant in an equal volume of distilled water, 50 μl were injected into the HPLC system.

RESULTS

Influence of the photochemical reactor on chromatographic performance

One basic question, when on-line postcolumn reactions are used in HPLC, is to what extent the postcolumn reactor influences the chromatographic performance of the separation system. When connected to the HPLC system in front of the UV detector, the photochemical reactor described results in only a minor deterioration of the chromatographic resolution. Because of the crocheted geometry of the reaction capillary, by which the turbulent flow within the capillary is induced[4,5], the contribution to extracolumn peak broadening by the photochemical reactor is very low (<1%/m), even when longer capillaries (irradiation times >90 s) were chosen. This was tested by injection of C_6-alkylphenone as a test substance. The apparent plate counts dropped by only 14% when the photoreactor with a 15-m crocheted reaction capillary was installed ($N=6135$ without, 5279 with the photoreactor). In contrast, when using a different (non-crocheted) reaction capillary design, the efficiency of the chromatographic system drops dramatically by more than 60% ($N=2385$, using a loosely coiled reaction capillary).

Influence of irradiation on UV absorbance of drugs

Methadone. This drug (Fig. 1), of great importance in clinical opiate substitution programmes, is generally analyzed in blood by HPLC, with UV detection below 235 nm. At lower wavelengths the purity of the mobile phase systems becomes an important factor, excluding certain buffer systems, for example nitrate buffers (as needed in highly selective separations on unmodified silica[6].

Photochemical irradiation of methadone has a pronounced effect onto its absorbance spectrum (Fig. 2), which results in the appearance of a spectral peak in the region of 250 nm. When using on-line photochemical reaction in HPLC separation of

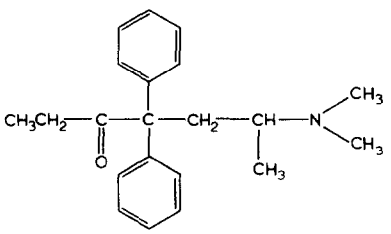

Fig. 1. Chemical structure of methadone.

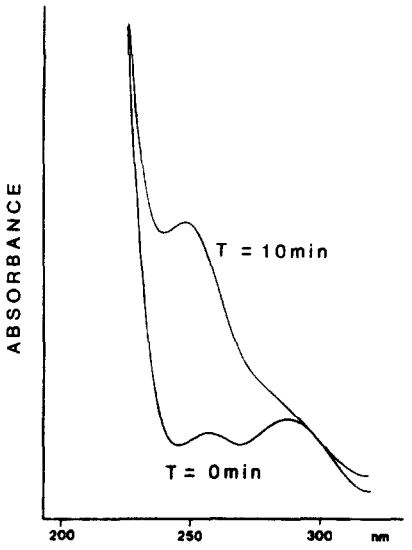

Fig. 2. UV spectrum of methadone before and after off-line irradiation with a low pressure mercury light source.

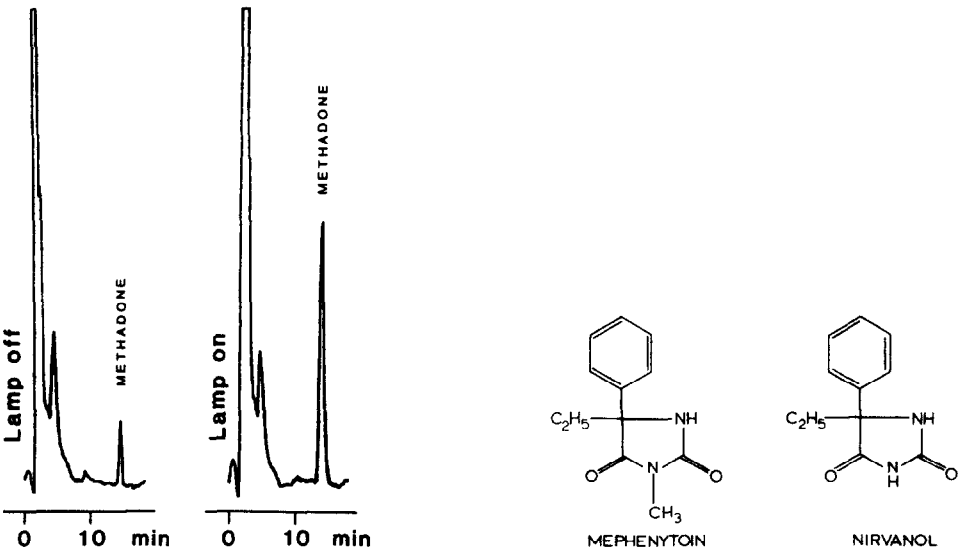

Fig. 3. Chromatogram of a blood plasma sample containing 75 ng/ml methadone. Column: Whatman PartiSphere Si, 110 mm × 4.7 mm. Mobile phase: acetonitrile–methanol–ammonium nitrate buffer, pH 9.5 (55:35:10). Flow-rate: 1.4 ml/min. Detection: 254 nm. Plasma sample extracted by a two-step solid phase extraction. Left chromatogram: without photochemical reaction. Right chromatogram: with photochemical reaction, reaction capillary 15 m (irradiation time 65 s).

Fig. 4. Chemical structures of methoin (mephenytoin) and nirvanol.

methadone a four-fold enhancement in UV detection at 254 nm was observed after only 65 s of irradiation (Fig. 3).

By chosing longer wavelengths for detection in comparison to 235 nm without on-line irradiation, a much higher specificity and less interference from the sample matrix were seen when analyzing biological samples.

Methoin and nirvanol. The hydantoin methoin is clinically used as an anti-convulsant drug and, because of strong side-effects, methoin and its pharmacological active metabolite, 5-ethyl-5-phenylhydantoin (nirvanol) (Fig. 4), should be routinely determined in blood of patients treated with this drug. In drug monitoring with HPLC, methoin is generally detected[7] in the UV at 211 nm.

Although structurally totally different from methadone, after on-line photochemical reaction similar effects on their absorbances are observed for methoin and nirvanol: irradiation of methoin and nirvanol leads to a strong increase in the absorption in the region of 254 nm, which is the result of a bathochromic shift, as revealed by spectral analysis (Fig. 5). In on-line irradiation in HPLC, a net gain of more than 20-fold in the detection of mephenytoin and nirvanol at 254 nm was observed. Because of the increased sensitivity and specificity, when measuring at longer wavelengths, the sample preparation of blood samples can be simplified: in combination with photochemical reaction, only deproteinization is necessary in routine HPLC analysis of plasma samples, allowing the two hydantoins to be determined from less than 50 μl of blood sample by a much more rapid and simplified HPLC procedure (Fig. 6).

Barbiturates. For sensitive HPLC determination of barbiturates (Fig. 7) either

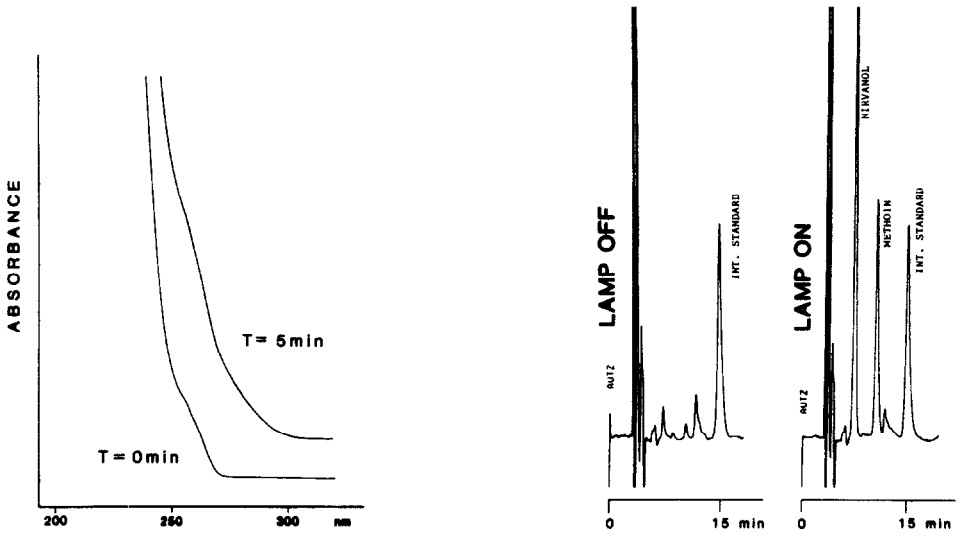

Fig. 5. UV spectrum of methoin before and after off-line irradiation.

Fig. 6. Chromatogram of a plasma sample containing methoin (9 μg/ml), nirvanol (14 μg/ml) and internal standard (mephobarbital, 30 μg/ml). Column: Whatman PartiSphere C_{18}, 110 mm × 4.7 mm. Mobile phase: acetonitrile–20 m*M* phosphate buffer, pH 7 (30:70). Flow-rate: 0.8 ml/min. Detection: 254 nm. Injection of 50 μl of deproteinized and diluted plasma sample. Left chromatogram: without photochemical reaction. Right chromatogram: with photochemical reaction, reaction capillary 10 m (irradiation time 75 s).

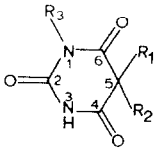

Fig. 7. Structures of barbiturates.

	R_1	R_2	R_3
Aprobarbital	Allyl	Isopropyl	H
Butethal	Ethyl	Butyl	H
Pentobarbital	Ethyl	1-Methylbutyl	H
Secobarbital	Allyl	1-Methylbutyl	H

UV detection at very low wavelengths(< 220 nm)[8] or complex postcolumn procedures have to be used[9].

When considering the structural similarities to the hydantoins, a similar positive spectral effect is expected for barbiturates. Photochemical reaction has been performed with several barbiturates: on-line UV irradiation with the photoreactor described showed a pronounced effect on the UV absorbance. However, except for the hydantoins, a characteristic increase in absorbance at longer wavelengths, in the region of 270 nm was seen. The resulting gain in absorbance at 270 nm is only two-fold for hexobarbital, but in the case of the barbiturate pentobarbital this increase may be as large as 80-fold after only 45 s of on-line UV irradiation (Table I). For different barbiturates the extent of observed spectral changes is different. Their reactivity upon irradiation cannot directly be associated with their molecular structures, preventing prediction as to the photochemical effects to be expected for an individual barbiturate.

Although the absolute detectability for barbiturates at 270 nm will not be increased by on-line photochemical reaction, in comparison to measurements below 220 nm without irradiation, again, detection at longer wavelengths has the effect of simplifying the HPLC analysis of barbiturates: on reducing the sample clean-up to protein precipitation only, in contrast to measuring at 220 nm, a nearly interference-free chromatogram was obtained, after injection of a blood plasma sample, by on-line photochemical reaction and detection at 270 nm (Fig. 8).

TABLE I

GAIN IN UV DETECTION BY POSTCOLUMN PHOTOCHEMICAL REACTION

Substance	*Enhancement factor*	*Wavelength (nm)*	*Irradiation time (s)*
Aprobarbital	35	270	45
Buthetal	44	270	45
Methadone	4	254	50
Methoin	29	254	240
Nirvanol	33	254	240
Pentobarbital	85	270	45
Secobarbial	40	270	45

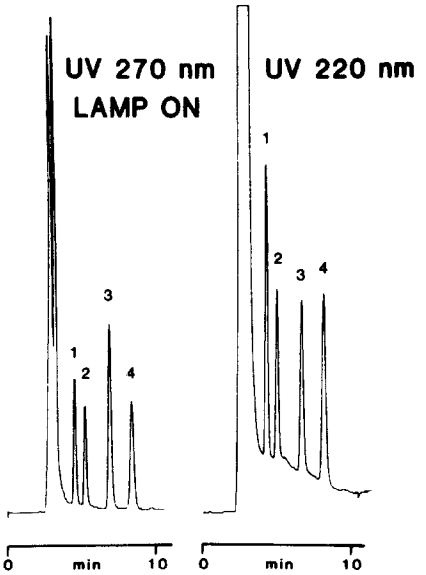

Fig. 8. Chromatogram of a spiked plasma sample containing aprobarbital (1), butethal (2), pentobarbital (3) and secobarbital (4), each 10 μg/ml. Column: Whatman PartiSphere C_{18}, 110 mm × 4.7 mm. Mobile phase: acetonitrile–20 m*M* phosphate buffer, pH 7 (30:70). Flow-rate: 0.8 ml/min. Injection of 50 μl of deproteinized and diluted plasma sample. Right chromatogram: detection at 220 nm without photochemical reaction. Left chromatogram: detection at 270 nm with photochemical reaction, reaction capillary 7.5 m (irradiation time 45 s).

Influence of the reaction time in postcolumn irradiation

Although the spectral changes described are generally seen after very short irradiation times (few tens of seconds), the rate change of absorbance as a function of the irradiation time has been studied in detail for the two hydantoins methoin and nirvanol. Variable irradiation periods were obtained with the standard photoreactor system by installing the longest available reaction capillary (32 m, representing an irradiation time of 240 s at a nominal flow-rate of 0.8 ml/min) and by protecting fractions of the reaction capillary from light, resulting in irradiation of the column effluent between 20 and 240 s (at constant flow-rates). For methoin and nirvanol a steady increase in absorbance at 254 nm was found when the irradiation time was increased. Under our experimental conditions, no maximum in the formation of the photoproduct was reached, even after irradiation for as long as 4 min (Fig. 9).

Photochemical reaction will be observed for methoin and nirvanol only if light of a certain minimum energy level is utilized for irradiation: exchanging the light source in the photoreactor for a mercury lamp of identical geometry but with an UV-emission cut-off at 366 nm, no absorbance increase at 254 nm was seen for the two hydantoins, indicating that mainly the major mercury UV line at 254 nm is responsible for the observed photochemical effect. For the other drugs studied no detailed time dependance has been established, but individual irradiation times were optimized for the maximum absorbance increase at a certain wavelength.

As for the hydantoins, for methadone, saturation of the photochemical effect was not seen even after on-line irradiation for 4 min. A further absorbance increase

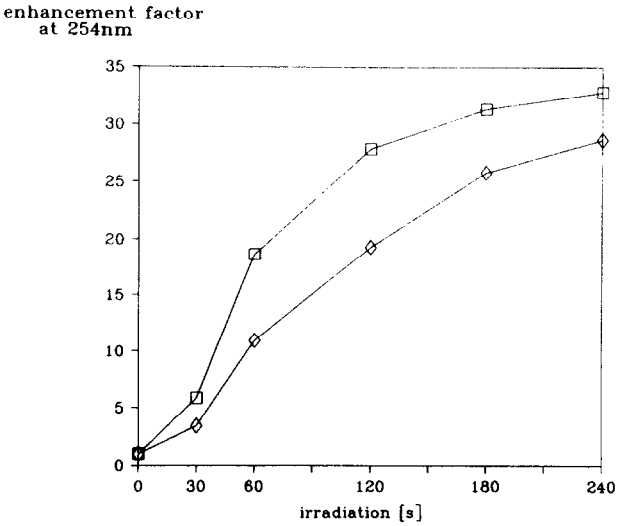

Fig 9. Time dependence of the UV absorbances of (◇) methoin and (□) nirvanol with increasing on-line irradiation of the column eluate.

might be obtained with longer irradiation, but under such conditions the adverse influence of the photoreactor on the chromatographic separation would become dominant.

In the case of the barbiturates, a maximum "photoproduct" formation is reached after much shorter irradiation periods: optimum irradiation times were found after *ca.* 25–45 s, varying for the different members of this drug class.

DISCUSSION

In Table I the drugs studied, the experimental conditions of on-line post-column photochemical reaction and its effect on the absorbance spectra are summarized. It is seen that a significant absorbance increase is observed generally after a relatively short period of irradiation, a precondition to integrate photochemical reactions into HPLC in the on-line mode. On-line irradiation of the column eluate leads in the cases of the drugs studied either to a bathochromic shift or to the appearance of an additional spectral band in the longer wavelength UV region. While all the drugs exhibit a hyperchromic shift, the type of photochemical reaction which takes place or the kind of photochemical product(s) formed during on-line irradiation has still to be clarified. However, from the structural differences between the drugs studied it must be assumed that more than one type of photochemical reaction is responsible for the absorbance changes. In the case of barbiturates and possibly also of methoin, it might be based on a photochemical modification of the molecule by which one tautomeric form of the barbiturate structure is more favoured, as can be observed[10] after a basic shift of the pH.

Nevertheless the generally observed absorbance increase in the longer wavelength range has a significant practical consequence for HPLC analysis of drugs: the use of longer wavelengths for UV detection after photochemical reaction generally

allows more specific but also more sensitive detection of the drugs studied with the positive consequence that lower sample volumes or less complex sample preparation procedures are required in routine HPLC analysis of biological samples.

Although only a small number of drugs have been described in this study, similar photochemical effects are expected for a much larger number of substances. Because of the simplicity in the use of on-line irradiation and because of the fact that it places nearly no constraints on the chromatographic performance a much wider application of post-column photochemical reaction in HPLC is indicated.

REFERENCES

1 I. S. Krull and W. R. LaCourse, in I. S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986, Ch. 7, p. 303.
2 A. Zweig, *Pure Appl. Chem.*, 33 (1973) 389.
3 Ch. Kikuta and R. Schmid, *Pharm. Biomed. Anal.*, 7 (1989) 329.
4 B. Lillig and H. Engelhardt, in I. S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986, Ch. 1, p. 1.
5 K. Hofmann and I. Halász, *J. Chromatogr.*, 173 (1979) 211.
6 R. Schmid and Ch. Wolf, *Chromatographia*, 24 (1987) 713.
7 A. Küpfer, R. James, K. Carr and R. Branch, *J. Chromatogr.*, 232 (1982) 93.
8 P. M. Kabra, H. Y. Koo and L. J. Martin, *Clin. Chem.*, 24 (1978) 657.
9 C. R. Clark and J. L. Chan, *Anal. Chem.*, 50 (1978) 635.
10 L. A. Gifford, W. P. Hayes, L. A. King, J. N. Miller, D. Thorburn Burns and J. W. Bridges, *Anal. Chem.*, 46 (1974) 94.