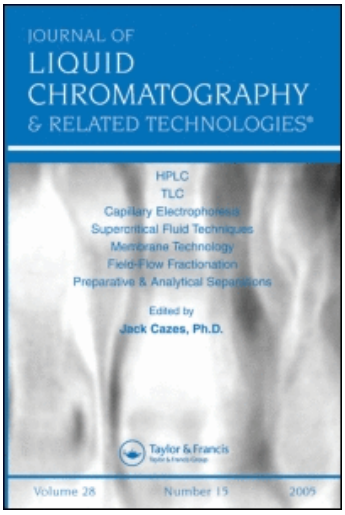

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ENHANCED UV-DETECTION OF BARBITURATES IN HPLC ANALYSIS BY ON-LINE PHOTOCHEMICAL REACTION

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ABSTRACT

After irradiation with UV-light a significant change in the UV-spectra of a number of barbiturates has been observed. The photochemical reaction results mainly in a bathochromic shift and appearance of a strong spectral band at 270nm, due to a dealkylation reaction in position 5 of the barbiturate molecule. By using an on-line photoreactor in HPLC analysis, barbiturates can be analyzed with much higher specificity in biological samples and, using absorbance ratios, identification of these drugs in unknown samples is facilitated.

INTRODUCTION

Low absorbance coefficients in the UV range above 230nm are characteristic for a number of barbiturates, making it necessary to choose shorter wavelengths to get sufficient sensitivity in HPLC-detection. A marked spectral shift can be observed under extremely basic pH conditions, but it requires a complex post-column reaction system for its use in HPLC detection (1). Krull et al. have demonstrated enhancement in electrochemical detection of barbiturates following irradiation with UV-light (2). Recently our group has reported (3,4), that certain barbiturates show a marked spectral shift after on-line photochemical reaction. In the present study this photochemical effects were studied further in detail and its

consequences for practical HPLC determination of this class of drugs in pharmaceutical and biomedical analysis were discussed.

METHODS AND MATERIALS

Chromatographic system

For chromatographic separations a HPLC system consisting of a Gilson 302 pump (GILSON, Villiers le Bel, France), a six-port injection valve (VALCO, Houston, TX, USA), a Shimadzu SPD-6A variable wavelength detector (SHIMADZU Europe, Duisburg, F.R.G) and a JASCO 820-FP fluorescence detector were used.

A photochemical reactor (Beam-Boost^{TM}, ICT, Frankfurt, F.R.G) was connected on-line in between the analytical column and the detector. Chromatograms were recorded using a LDC 10B integrator (Riviera Beach, FL, USA).

Chromatographic separations were generally done on a 11cm x 4.7mm Whatman cartridge packed with PartiSphere C-18, 5 micrometer silica material (WHATMAN, New Jersey, USA) at a flow of 0.8ml/min.

The mobile phase consisted of 30% acetonitrile and 70% 15mM phosphate buffer, pH 7.0 .

Description of the photochemical reactor

Up to 25m 1/16" heavy wall, narrow bore PTFE Teflon capillary tubing (1/16"O.D., 0.01" I.D.) in a cylindrically crocheted configuration is mounted around a tubular 8W low pressure mercury lamp. The light source emits the known mercury spectrum including the strong UV-line at 254nm. Because of the low heat production of the light source, no active cooling in the photoreactor is necessary.

Depending on the length of the crocheted reaction capillary used, irradiation times up to maximally 190 seconds (at nominal flow of 0.8ml/min) can be chosen using this photoreactor geometry.

For experiments, in which the spectral influence onto the photochemical reaction was studied, a 8W lamp with identical geometry but with emission maximum at approx. 366nm (SYLVANIA, F8-T5) was used.

In "off-line" photochemical experiments samples were irradiated by placing them in a quartz cuvette next to an identical tubular mercury lamp (SYLVANIA, G8-T5).

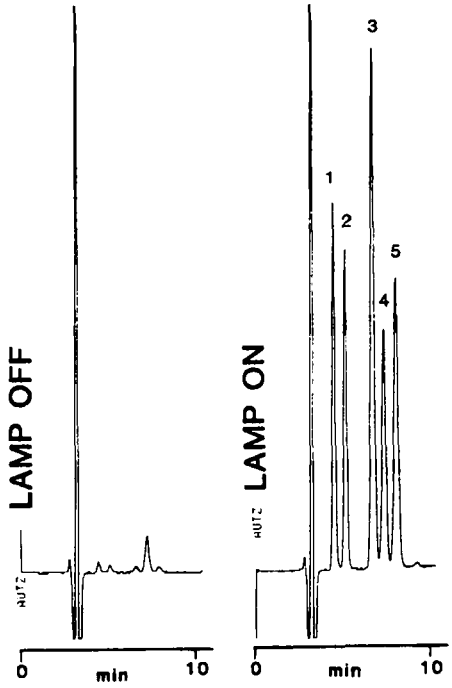

FIGURE 1 : Chromatogram of a standard sample of barbiturates detected at 270nm without (left) and with (right) on-line photochemical reaction.
1 Aprobarbital, 2 Butethal, 3 Pentobarbital,
4 Mephobarbital, 5 Secobarbital

Chemicals

Water and organic solvents used in HPLC were purchased in glass distilled quality and of HPLC grade (HiPerSolv, BDH, England), buffer salts were used in highest purity available (MERCK, Darmstadt, F.R.G).

All studied barbiturates were obtained as stock solutions dissolved in methanol (1mg/ml) from SUPELCO (Bad Homburg, F.R.G.) and diluted with mobile phase buffer to adequate concentrations.

Analyzing barbiturates in blood, a protein precipitation was performed by adding an equal volume of acetonitrile to the blood plasma. Precipitated proteins were centrifuged at approx. 10.000g in a Biofuge A (HARAEUS, F.R.G.) and after dilution of the supernatant with equal volumes of distilled water, 50 microliters were injected into the HPLC system.

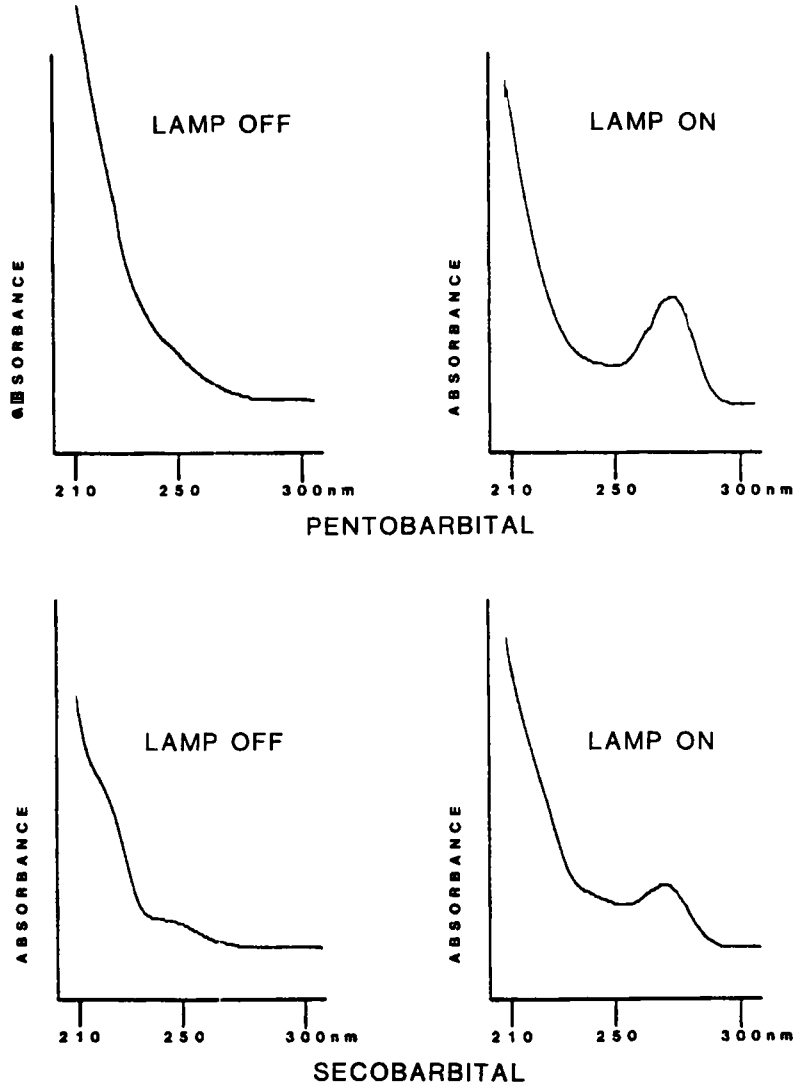

FIGURE 2 : On-line spectra of pentobarbital and secobarbital before and after on-line photochemical reaction.

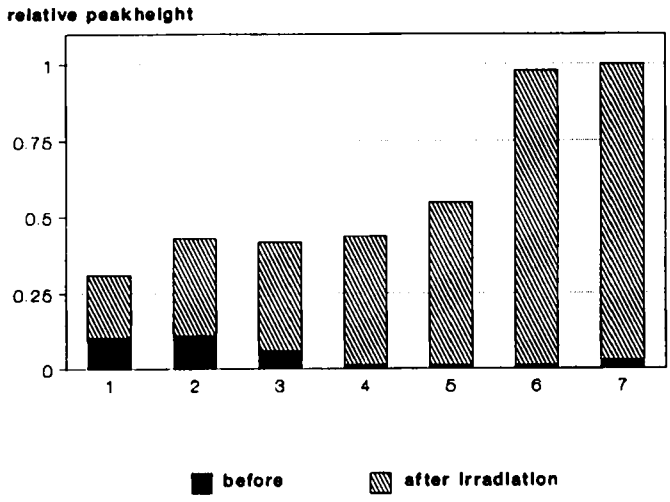

FIGURE 3 : Relative absorbance at 270nm of barbiturates with and without on-line photochemical reaction.
1 Cyclobarbital, 2 Hexobarbital, 3 Mephobarbital, 4 Butethal,
5 Secobarbital, 6 Pentobarbital, 7 Aprobarbital

RESULTS AND DISCUSSION

As most barbiturate derivatives show no significant absorbance coefficients in the UV above 230nm, analysis wavelengths in the range between 200-220nm must be chosen to achieve sufficient sensitivity.

As preliminary reported by the authors (3), irradiation of the column eluate with UV-light leads to a significant increase in UV absorbance at longer wavelengths for a number of barbiturates (Fig.1).

Spectral analysis of this effect shows that this photochemical reaction leads to a significant change in their absorbance spectra: Fig.2 shows that after short irradiation with UV light in spectra of secobarbital and pentobarbital a new spectral band with $lambda_{max}$ at 270nm appears, whereas no or less change in absorbance at shorter wavelengths is seen. Further investigations showed, that this amplification effect at 270nm could also be observed for other members of this drug class.

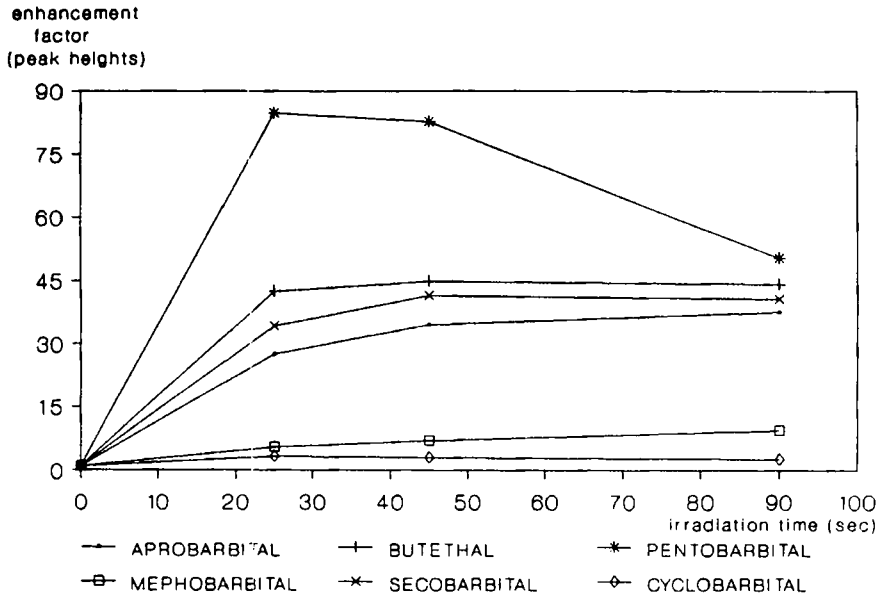

FIGURE 4 : Dependance of absorbance enhancement on irradiation time.

Spectral effects after on-line UV-irradiation were seen for the barbiturates pentobarbital, secobarbital, aprobarbital, butethal, mephobarbital and cyclobarbital, but with varying intensity: Comparing peak heights of these seven barbiturates in equimolar concentrations detected at 270nm, pentobarbital and secobarbital showed the largest amplification effect following photochemical reaction for 45sec, hexobarbital and cyclobarbital the least. Furthermore this absorbance enhancement seems to be inversely related to their "native" absorbance at 270nm (Fig.3). In case of cyclobarbital, for which only a moderate photoeffect was found, still short on-line irradiation may result in an approx. three to four-fold amplification of the detector signal, in case of pentobarbital this can be as much as an approximately 90-fold increase.

If the light source is exchanged by a lamp with an emission maximum at 366nm, the described photochemical reaction for barbiturates will not take place, indicating that the 254nm line of the mercury lamp seems to be mainly responsible for this photochemical effect.

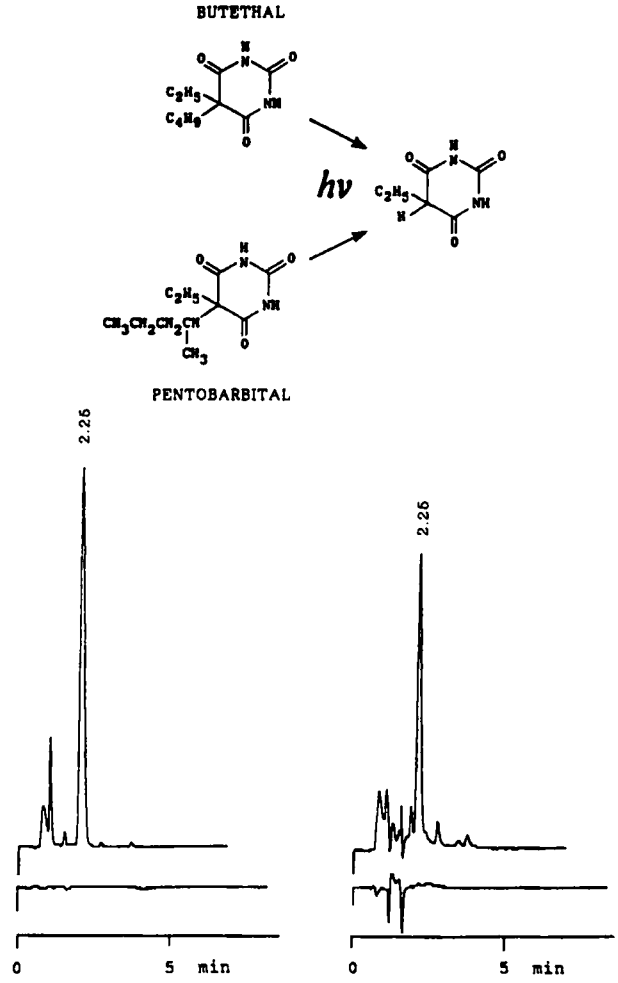

FIGURE 5 : Chromatograms of (off-line) UV-irradiated solutions of pentobarbital (left) and butethal (right) and reaction scheme of the proposed photolytic reaction.

As another parameter of the photochemical reaction the influence of the irradiation time was studied. The enhancement of the detection signal at 270nm reaches a maximum followed by an attenuation, indicating further reaction of the formed photoproducts at longer exposure times (Fig.4).

Based on these results we tried to find out the mechanism of this photochemical reaction. In their pharmaceutical synthesis work Barton et al. (5) have studied the photolysis of a number

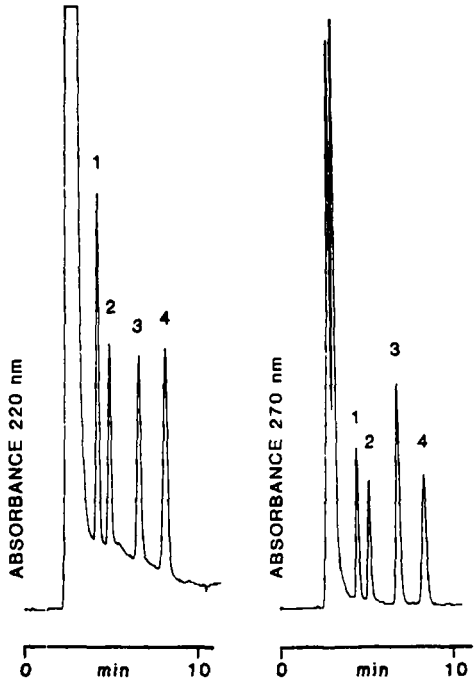

FIGURE 6 : Chromatogram of a spiked plasma sample after protein precipitation at 220nm (left) and at 270nm with on-line photochemical reaction (right).
1 Aprobarbital, 2 Butethal, 3 Pentobarbital, 4 Secobarbital.

of barbiturates and proposed a possible reaction scheme: Under UV-light barbiturates undergo dealkylation of one substituent at the position 5. To prove this reaction scheme two barbiturates with one identical side chain at position 5, pentobarbital (ethyl-methylbutyl-barbituric acid) and butethal (ethyl-butyl-barbituric acid), were irradiated off-line in mobile phase buffer and analyzed by HPLC with detection at 270nm without further on-line irradiation.

From both off-line irradiated solutions only one major peak with identical retention time appears in the chromatogram with a lower k'-value than the two barbiturates themselves. These are strong indications, that by UV-irradiation from both barbiturates the identical dealkylation product, ethyl-barbituric acid, is preferentially formed (Fig.5).

These results are in accordance with the work of Barton et al. and the above spectral data. The loss of an alkyl group in the barbiturate molecule leads to a bathochromic shift, which may be explained by a change in equilibrium of the keto-enol tautomerisation.

These studied photochemical reactions for the barbiturates have significant implications for practical HPLC work. The photochemically induced absorbance-enhancement at 270nm gives the possibility for a sensitive and significantly more specific detection at this wavelength. In comparison to detection at 220nm or lower, less interferences from the biological matrix are detected in the chromatogram, an advantage in HPLC-analysis of barbiturates in biological samples, especially when no or little sample preparation is performed (Fig.6).

The described photochemical effects also offer another aspect in the HPLC analysis of barbiturates: Because the spectral changes after UV-irradition are characteristic for different barbiturates, the photochemical enhancement by on-line photo-chemical reaction can be utilized for identification of these drugs in unknown samples. Comparison of spectra or the absorbance ratio of two selected wavelengths (for example 220nm and 270nm), with and without on-line photochemical reaction, together with the k'-value are very strong confirmations for the presence or absence of a certain barbiturate in a sample, a fact which is especially valuable in toxicological analysis.

CONCLUSIONS

Photochemical reaction of barbiturates has been demonstrated to be a simple post column reaction procedure to enhance UV-absorbance at longer wavelengths. A photoreactor is easily integrated into a HPLC system with nearly no consequence on the chromatographic performance. Photoreaction not only allows a simplified and faster routine analysis, additionally gives the analyst a valuable tool for identification of these drugs in unknown samples.

With the knowledge about the photochemical mechanism of barbiturates a prediction of similar reactions for other pharmaceutical substances becomes possible and in a number of cases detection will be improved.

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