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Simultaneous determination of thiopental and its metabolite, pentobarbital, in blood by high-performance liquid chromatography and post-column photochemical reaction*

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Abstract: A novel approach for the simultaneous high-performance liquid chromatographic (HPLC) analysis of thiopental and its major metabolite, pentobarbital, in blood plasma is described. On-line irradiation of the column eluate with UV-light leads to a significant bathochromic shift in the absorbance spectrum of pentobarbital, allowing now sensitive UV-detection of both barbiturates at 270 nm. At this longer wavelength, plasma matrix constituents are less interfering in the analysis, thus less sample preparation is necessary and blood plasma can be directly injected into the HPLC system only after protein precipitation with acetonitrile. Because of the minimal sample handling, the described HPLC method has good accuracy and reproducibility and thiopental and pentobarbital can be determined in small plasma volumes down to 0.2 μg ml^{-1}.

Keywords: *HPLC analysis; thiopental; pentobarbital; photochemical reaction.*

Introduction

The barbiturate thiopental is frequently used clinically in the treatment of intensive care patients suffering from severe head injuries. Thiopental is metabolized in the body primarily by desulphuration to the analogue, pentobarbital [1] (Fig. 1). Because of their acute toxic side-effects, both drugs should be monitored in blood of patients chronically treated with thiopental to improve intensive care therapy.

For determination of thiopental and pentobarbital in blood, a number of high-performance liquid chromatography (HPLC) methods [2–6] have been described in the literature.

Although the determination of thiopental in clinical samples might not be considered to be a very complex task because of its relative high therapeutic concentration, when the metabolite pentobarbital is included into HPLC analysis, a number of problems have to be solved. For sensitive detection of both barbiturates in the same chromatographic run, detection at two wavelengths is necessary at 285 nm for thiopental and at <220 nm for

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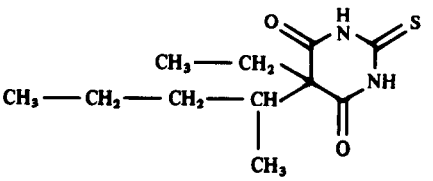
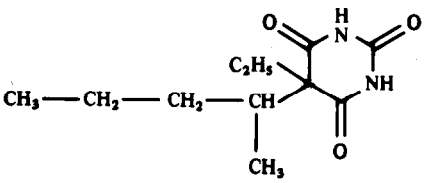


Thiopental

Figure 1



Pentobarbital

pentobarbital. But measurement in this low UV-region demands mobile phase systems of high purity and a good sample clean-up of the biological samples [2].

Recently, it has been shown by the authors that an on-line photochemical reaction in HPLC will cause a significant spectral shift in the UV-spectra of a number of barbiturates, including pentobarbital [7]. Based on this phenomenon, a new, fast and simplified HPLC method for the simultaneous determination of thiopental and its metabolite, pentobarbital, in blood plasma has been developed.

Experimental

On-line photochemical reaction

On-line *hv*-irradiation was performed with a photoreactor system built in the authors' laboratory [8]. In short, it is constructed of a tubular 8 W low pressure mercury lamp (Sylvania G8-T5, GTE, obtained from a local laboratory supplier) mounted in a small plastic housing. This light source emits the known mercury spectrum including the main line at 254 nm. Because of the low power rating of the mercury lamp active cooling is not necessary. As reaction capillary, 6 m of heavy wall, narrow-bore PTFE tubing (1/16″ o.d., 0.01″ i.d.; Pierce Europe, The Netherlands) has been crocheted, forming a rectangular pad of approx. 6 cm side length, which is mounted around the mercury lamp by means of a stainless steel mesh.

With this chosen reaction capillary geometry, irradiation time of the column eluate is approx. 25 s at a nominal mobile phase flow of 1.0 ml min^{-1}.

To evaluate the rate of reaction of the photochemical process, a 20 m reaction capillary in the same geometry was installed and partly covered with aluminium foil to give variable irradiation between 5–90 s approx.

Chromatographic separation

The HPLC system consisted of Gilson Mod. 302 pump (Gilson, Villiers le Bel, France), a Valco six-port injection valve (Valco, Houston, TX, USA), a Shimadzu SPD-6A variable wavelength detector (Shimadzu Europe, Duisburg, FRG) and a LDC 10B integration system (LDC-Milton Roy, Riviera Beach, FL, USA).

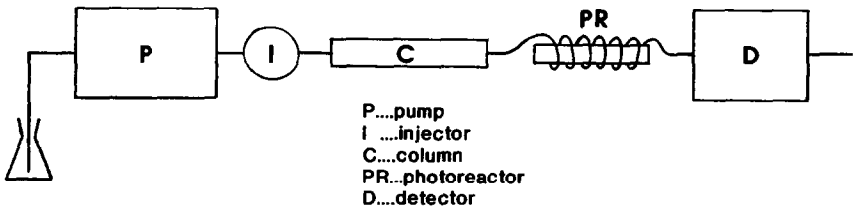


Figure 2
Scheme of the HPLC system with on-line photochemical reactor.

On-line spectra of the drugs were recorded with a Hewlett–Packard Mod. 1050 Multiple Wavelength detector (Waldbronn, FRG). For on-line post-column photochemical reaction the reactor was connected in the flow line of the HPLC system between the column outlet and the UV-detector (Fig. 2).

Separations were carried out on a Whatman PartiSphere C8 cartridge (Whatman, NJ, USA) with a mobile phase consisting of 50% acetonitrile in 120 mM phosphate buffer, adjusted to pH 6.2, and an eluent flow of 1 ml min^{-1}.

Chemicals

Water and acetonitrile used in HPLC and in sample preparation were obtained in glass distilled quality from BDH (Poole, England). Buffer salts were purchased in highest grade available from Merck (Darmstadt, FRG).

Thiopental and pentobarbital were obtained as stock solutions dissolved in methanol in concentrations of 1 mg ml^{-1}, from Sigma Chemicals (Deisenhofen, FRG).

Calibration curves in the range 0.2–50 μg ml^{-1} were prepared by adding appropriate amounts of the stock solutions to control blood plasma (obtained from the hospital's blood bank).

Sample preparation of plasma samples

Equal volumes of blood plasma and acetonitrile were mixed and vigorously shaken. Precipitated proteins were then centrifuged in a bench top centrifuge at approx. 10.000*g* for 3 min, and 20 μl of the supernatant was injected into the HPLC system.

Results

Photochemical reaction of pentobarbital

After on-line irradiation of the column eluate with UV-light, a significant shift in the absorbance spectrum of pentobarbital will be observed. While unirradiated pentobarbital shows no significant absorbance in the UV above 240 nm, after short irradiation a spectral band in the region at 270 nm appears (Fig. 3). This photochemical reaction may be observed after relatively short irradiation times of a few seconds. Analysing the rate of progress of this photochemical reaction revealed that the maximal absorbance increase for pentobarbital at 270 nm is seen after approx. 25–35 s under the chosen experimental conditions (Fig. 4), while longer irradiation will result in decreasing absorbance.

As reported in an earlier study [7], to observe this photochemical effect only a low power UV-light source is necessary, for example a mercury lamp.

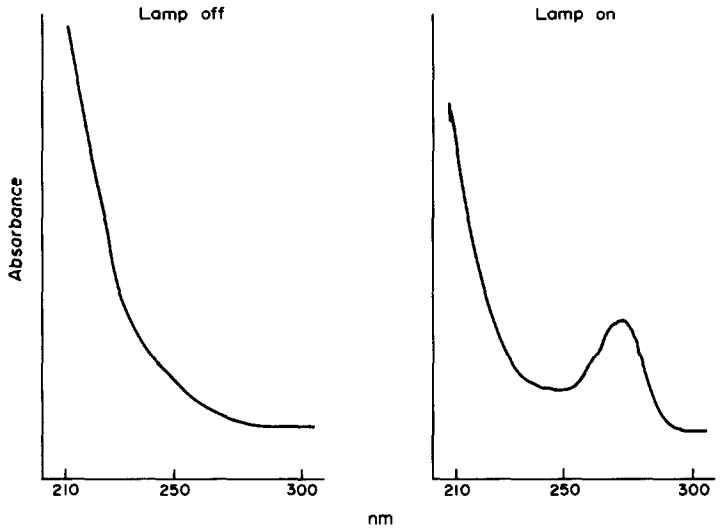


Figure 3
On-line spectrum of 10 μg pentobarbital. Left, without *hv*-irradiation; right, after 25 s on-line *hv*-irradiation.

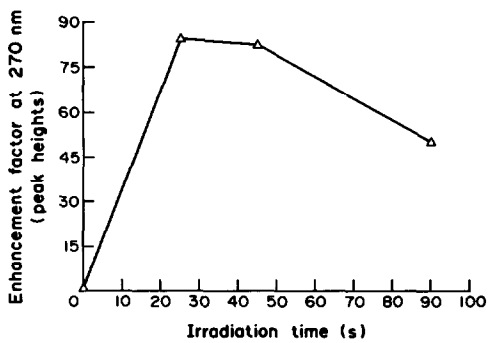


Figure 4
Time course of absorption enhancement for pentobarbital at 270 nm with increasing on-line irradiation time.

Thiopental already shows a native spectral band in the region of 285 nm, and no significant change in its UV-spectrum will result following on-line irradiation.

Utilizing this photo-effect for UV-detection has significant positive consequences for HPLC analysis: while otherwise only pentobarbital can be analysed with sufficient sensitivity at wavelengths below 220 nm, the short on-line photochemical reaction allows the detection at 270 nm with a nearly 100-fold increase in sensitivity (Fig. 4; middle and right). Although this wavelength is not optimal for the detection of thiopental, it still offers more than sufficient sensitivity when both barbiturates are analysed in clinical blood samples.

Chromatographic separation of thiopental and pentobarbital

A fast HPLC separation for thiopental and pentobarbital was established on a short 110 × 4.6 mm Whatman PartiSphere C8 cartridge with 50% acetonitrile in 120 mM phosphate buffer (pH 6.2) as the mobile phase. Under these chromatographic conditions

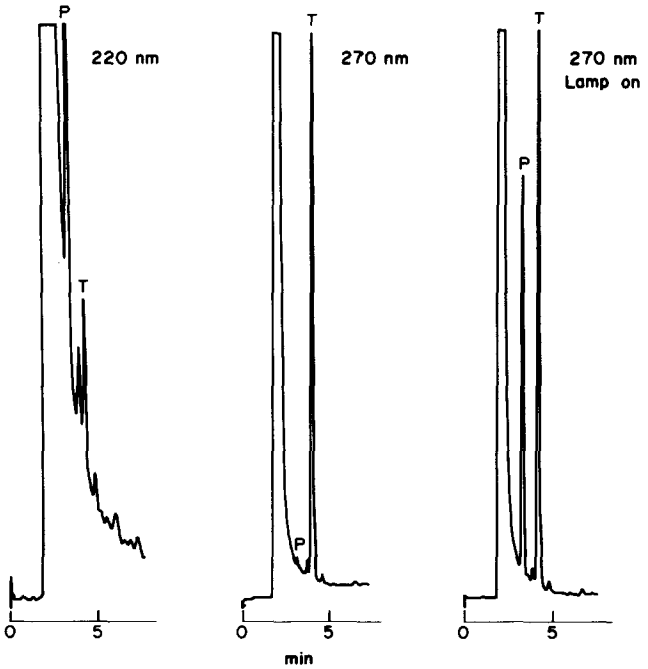


Figure 5
HPLC chromatogram of a patient plasma sample containing 4.3 μg thiopental (T) and 8.7 μg pentobarbital (P). Left, detection at 220 nm without photochemical reaction; middle, detection at 270 nm, photoreactor off; right, detection at 270 nm, photoreactor on, 25 s irradiation. Chromatographic conditions as in Experimental section.

pentobarbital elutes with a retention time of 3.28 min, well separated from thiopental (retention time of 4.25 min) as can be seen from the chromatogram of a patient's sample (Fig. 5, right).

Sample preparation of blood plasma samples

The possibility to choose a longer wavelength in HPLC detection gives the possibility to reduce sample preparation in analysis of plasma samples drastically: extensive extraction of the drugs from the blood plasma matrix may be avoided and sample preparation is limited to precipitation of plasma proteins with acetonitrile. Because only few sample matrix constituents will be interfering when detecting at 270 nm, even without extensive sample clean-up a relative "clean" chromatogram will be obtained (Fig. 5, middle). In comparison, measuring the same sample at 220 nm, which would be necessary to analyse pentobarbital without a photochemical reaction, this simplified sample clean-up step is not sufficient for a reliable analysis (Fig. 5, left).

Linear standard curves for thiopental and pentobarbital have been routinely obtained with spiked blood plasma in the range 0.2–50 μg ml^{-1} with very good correlation. Typical regression calculations for areas vs concentrations gave for pentobarbital a slope of 1.572 and intercept of −0.02 μg ml^{-1}, and for thiopental a slope of 4.439 and intercept of 0.01 μg ml^{-1}.

Because preparation of blood samples is kept minimal, the overall variability of the method is excellent with a within-day variation coefficient of <3.7% ($n = 8$ at concentrations of 20 μg ml^{-1}). Maximal day-to-day variability is similarily low with a relative standard deviation of 4.4% ($n = 8$ at concentrations of 20 μg l^{-1}).

Many other drugs, commonly co-medicated during thiopental therapy and encountered in a similar concentration range as thiopental and pentobarbital in blood, either do not show significant UV-absorbance at the longer wavelength setting of 270 nm, or elute at a different retention window under the chosen chromatographic conditions. Having analysed approx. 200 clinical samples, no substance has yet been found to interfere in routine thiopental analysis.

Using the described sample preparation, sensitivity of analysis of thiopental and pentobarbital in blood plasma can be as low as approx. 0.2 μg ml^{-1}, which is more than adequate for therapeutic drug monitoring.

Discussion

Although therapeutic levels of thiopental and pentobarbital in clinical samples are relatively high, the low absorbance coefficient of pentobarbital above 220 nm, requires HPLC detection at low wavelengths, with its negative consequences for sample extraction and for the purity of the mobile phase system [2]. Also, due to its spectral characteristics at shorter wavelengths, thiopental cannot be detected with sufficient sensitivity.

Our observations [7], that by using an on-line, post-column photochemical reaction, the absorbance coefficient is significantly enhanced for pentobarbital at longer wavelengths gives the basis for a simplified routine HPLC assay of thiopental, allowing both barbiturates to be detected at the same wavelength of 270 nm. What kind of a photoproduct is formed in HPLC during on-line irradiation of pentobarbital has not yet been identified. But it can be assumed, that the bathochromic shift might be due to a photolytic dealkylation of pentobarbital, allowing keto–enol tautomerization in the barbituric acid molecule [9].

Although detection enhancement at 240 nm has been shown for certain barbiturates through a pH-shift by post-column addition of a basic buffer [10], an on-line photochemical reaction does not require any complex instrumentation for the post-column addition of reagent, and it only minimally influences the performance of the chromatographic separation. Installing the described photoreactor with a 6-m reaction capillary into the HPLC system will decrease column efficiencies by <6% [7].

The possibility of measuring both barbiturates at the longer wavelength of 270 nm not only simplifies detection for routine HPLC analysis, it additionally has the consequence that complex sample clean-up procedures for biological samples may be avoided. Minimal sample handling means not only greater speed of analysis and higher sample throughput, it also increases the accuracy and reproducibility of the analysis, even when no internal standardization is used.

Because sample preparation is limited to protein precipitation, the described HPLC method is especially suited to handle small blood samples as in pediatric laboratory medicine.

By including an on-line photochemical reaction into HPLC, a fast and reliable routine procedure has become available, allowing the sensitive determination of thiopental and pentobarbital in blood down to 0.2 μg ml^{-1}.

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