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Short communication

Analysis of barbiturates by micro-high-performance liquid chromatography with post-column photochemical derivatization

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Abstract

This study attempts to combine the advantages of microcolumn high-performance liquid chromatography (μ-HPLC) with those of post-column photochemical derivatization in barbiturate analysis. Some barbiturates are photochemically unstable, leading to photoproducts that show maximum absorption at 270 nm and not the typical one at ≈220 nm. For this purpose, a laboratory-built photoreactor has been developed to work with μ-HPLC instruments. Its performance is satisfactory in the forensic analysis of barbiturates. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Barbiturates are widely in use since the beginning of the century (barbital, 1903) especially as sedative hypnotics. With the advent of anxiolytic agents, the popularity of barbiturates has suffered although they are still less costly. However, those with specialized properties – such as the anticonvulsant phenobarbital – continue to be commonly used [1]. In addition, abuse of barbiturates is now widespread. Due to the international nature of the illegal drug market forensic laboratories encounter a vast range of such compounds. Complications arise from the fact that abused barbiturates often occur as complex mixtures and other drugs and/or excipients are also present [2]. This necessitates the continued development of methods for their efficient separation and precise identification. For the most part, detection by gas chromatography involves derivatization, whereas high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is relatively insensitive [3]. To overcome such problems, three different approaches have been employed. A marked spectral shift observed at extremely basic pH, allows UV detection at longer wavelengths. This approach however, requires a complex post-column reaction system [4]. Alternatively, improved electrochemical detection can be obtained after irradiation with UV light [3,5]. Finally, a shift of the UV maximum to 270 nm, after on-line post-column photochemical reaction, enhances both sensitivity and selectivity [6,7].

The photochemical instability of 5,5′-disubstituted barbiturates has been extensively studied [8,9]. It has been proposed that under UV-light barbiturates undergo a partial dealkylation at position 5. Consequently, the loss of an alkyl group leads to a bathochromic shift explained by a change in the equilibrium keto–enol. Wolf and Schmid [7] have postulated that ethyl-barbituric acid is a common

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photoproduct at least for pentobarbital and buthetal, justifying the post-column position of the photoreactor.

The present work is based on such an approach with the main modification involving the use of micro-HPLC (μ-HPLC), so as to take full advantage of well documented benefits of this method in comparison to the standard HPLC [10,11]. In the chromatographic analysis, there are two possibilities concerning the amount of sample available. For unlimited samples, the maximum sensitivity should be obtained for the maximum sample volume $(Vs)_M$, usually with a four-fold dilution with the mobile phase. For a sample with a limited volume, smaller than $(Vs)_M$, the dilution must be larger and results in a proportionally decreased sensitivity, a serious problem in trace analysis. A decrease in column diameter permits a considerable lowering of $(Vs)_M$, without changing instrument performance [10].

This is one of the most important advantages resulting from the use of microcolumns, especially benefiting clinical and forensic analysis where the analyst has to deal with small biological samples. Because of the lack of commercial photoreactors compatible with μ-HPLC systems, we have decided to design and to evaluate a new laboratory-made microphotoreactor. Its performance in the improved analysis of barbiturates is presented below.

2. Experimental

2.1. Apparatus and chromatographic conditions

The experimental set-up consisted of a conventional HPLC pump (PU 4100 Liquid Chromatograph, Philips, Cambridge, UK) modified with a fixed-ratio flow splitter Acurate IC-70 (LC-Packings, Zurich, Switzerland) which produced a constant mobile phase flow-rate of about 7 μl/min, at the 500 μl/min pump nominal flow. Samples were injected by means of a Valco (Vici Valco Europe, Schenkon, Switzerland) CI4W0 injector, with a 60-nl internal loop. Detection was carried out using a Waters (Waters, Milford, MA, USA) 484 UV programmable detector fitted with an UZ-View flow cell (LC-Packings). UV chromatograms were recorded at 220 nm (underivatized barbiturates) or at 270 nm (after photoderivatization). This system worked with a Maxima 820 Chromatographic Data Station (Waters).

Two different microcolumns were tested, in order to obtain the complete separation of the barbiturate mixture. These columns were: a Fusica II (LC-Packings) 15 cm×320 μm I.D., packed with Spherisorb ODS, 2.5 μm and a Fusica II 15 cm×320 μm I.D., packed with graphitized carbon Hypersil Hypercarb PH. All connections were made either with fused-silica capillaries (75 μm I.D.) (Composite Metal Services, Worcester, UK) and high-pressure fittings (LC-Packings) or using low-pressure PTFE unions (LC-Packings).

The UV spectra from barbiturates and their photoproducts were acquired with a conventional HPLC (Waters 600E)–diode array detection (DAD) (Hewlett-Packard 1100) system using a photoreactor described elsewhere [5].

2.2. Solvents and reagents

All standards used in this study (amobarbital, barbital, buthetal, pentobarbital and secobarbital) were obtained from Sigma–Aldrich (Madrid, Spain) and were of analytical purity. For all compounds stock solutions of 1000 μg/ml were prepared in methanol (HPLC grade, Merck, Darmstadt, Germany) and diluted to desired concentrations with methanol–water [purified with a Milli-Q system (Millipore, Milford, MA, USA)] (1:1, v/v). Before analysis all solutions were stored in the refrigerator.

The mobile phase represented mixtures of MeOH and a 10 m*M* phosphate buffer (pH 7) prepared combining KH_2PO_4 (99.5% purity, Merck) and $K_2HPO_4 \cdot 3H_2O$ (99% purity, Merck). For the flow-injection mode it was phosphate buffer–MeOH (50:50) at the pump flow of 300 μl/min corresponding to ca. 4.2 μl/min column flow-rate. For μ-HPLC analysis of barbiturate mixtures, several different multistep gradients were employed as specified, at the flow-rate of 500 μl/min corresponding to 7.0 μl/min column flow-rate.

2.3. Spiked serum sample

A 1-ml volume of human serum and 1 ml of an aqueous saturated solution of KH_2PO_4 were added to

a tube containing known aliquots of each barbiturate tested (stock solutions of 1000 μg/ml). After vortexing for 15 s, 5 ml of CH_2Cl_2 (analytical grade, Merck) was added. The serum was manually extracted for 3 min. The organic layer, containing the barbiturates, was transferred to a conical tube and carried to dryness at 45°C under a N_2 dry stream in a Turbovap system (Zymark) (pressure=1 bar). The residue was reconstituted in 1 ml of MeOH–Milli-Q water (1:1, v/v).

2.4. Photochemical reactor for μ-HPLC

Details about building material and light sources will be explain in the Results and Discussion section. This photoreactor worked with a ''supercool Peltier'' device (J.H. Roerden, AA 020-122-22, Spain), used for the efficient dissipation of the enormous heat generated by the lamp minimizing thermal decomposition that may accompany the photochemical reaction. All components were placed inside a stainless steel box (53 cm×28 cm×23 cm) whose front side had the main switch controlling both the lamp and the Peltier device, as well as the inlet and outlet connections to the chromatographic system.

All studies were carried out with a post-column derivatization by placing the microphotoreactor between the microcolumn and the UV detector.

3. Results and discussion

3.1. The photoreactor design

In designing a microphotoreactor, especially important is the selection of the material fulfilling important requirements such as transparency to UV light; small diameter enabling easy the connections to the chromatographic system, resistance to high pressures and temperatures, resistance to corrosive agents, flexibility, and finally an acceptable price. Good candidates were PTFE and fused-silica capillary. For the latter it is possible to make a transparent window by burning off the coating layer as commonly done in capillary electrophoresis. On the other hand, fused-silica capillaries are not sufficiently flexible thus limiting the useful length and the irradiation time. But fused-silica capillaries provide an important advantage as their small dimensions fit the μ-HPLC system and minimize dead volumes and leaks. On the other hand, PTFE is usually selected as a suitable material for conventional HPLC photoreactors [12]. Its typical O.D. is different from those commonly used in μ-HPLC complicating connection. As result of these considerations a fused-silica capillary of 75 μm I.D.×375 μm O.D. (Composite Metal Services) was selected for our reactor.

Equally crucial was the selection of the UV lamp. It was reported before that the typical 254 nm light source can be substituted with a lamp with an emission maximum at 366 nm [7]. Under such conditions, however, the photochemical reaction of barbiturates does not take place, indicating that absorption occurs at lower wavelengths (i.e. 254 nm) promoting molecular transformation. The intensity of the light source was also considered. By increasing the photon flux, the irradiation time required for the increased sensitivity could be reduced. Consequently, in the first prototype, we tested a 50 W mercury lamp (Philips, HPL-N). This design consisted of a cylindrical lens concentrating the radiation in an horizontal line coinciding with the capillary, fitted just under the lens [13]. A lens equipped with the three-dimensional movement permitted focusing, enabled, in turn, by a visible halogen lamp (to avoid UV damage). In spite of the satisfactory result obtained in a preliminary phase, focusing turned out to be unstable affecting reproducibility. This led to elimination of the lens and the lamp power was increased enough to ensure the efficient photoreaction. It was obtained with a 250 W mercury lamp (Philips HPL-N) after the removal of the external glass bulb.

3.2. Initial experiments

First, the photochemical behavior of selected barbiturates was investigated in a flow-injection mode experiment. Thus, six barbiturates including amobarbital, pentobarbital, buthetal, barbital, secobarbital and phenobarbital in concentrations of 50 μg/ml (methanol–water, 1:1) were injected in triplicate through the 60-nl fixed-loop injection valve, with no column in the system, and with the photoreactor switched off and on respectively, under conditions described in Experimental. A previous irradiation time optimization study had shown a

plateau in the UV response at a pump flow-rate range from 300 to 600 μl/min before a splitter. At lower flow-rates the band dispersion minimized efficiency whereas at higher rates, the irradiation time was affected. Detection was carried out at 270 nm. The peak height relationship obtained with the photoreactor OFF/ON for each particular barbiturate was, respectively: barbital (≈11), buthetal (≈7), phenobarbital (≈4), pentobarbital (≈6), amobarbital (≈11) and secobarbital (≈8). Phenobarbital was the least affected compound with an increment of about fourfold in its response and both barbital and amobarbital the most affected, with an 11-fold improvement.

The UV spectral analysis confirmed the increase in absorbance at longer wavelengths, especially in the region of 265–270 nm. Although detectability increase is not apparent in Fig. 1 (in comparison to measurements below 220 nm without irradiation), it must be noted that spectral analysis was carried out using a conventional photoreactor with a low-power lamp and DAD. The use of a 250 W lamp allows increased detectability and, in any case detection at longer wavelengths simplified the HPLC analysis of these compounds: by reducing the sample clean-up and by giving a nearly interference-free chromatogram after injection of routine plasma samples [6].

3.3. Microcolumn experiments

With the improved detection by the photoreaction, the next step involved separation of model mixtures. This was done because several barbiturates can appear together in the real sample [2], and their efficient separation would enable the effective forensic analysis under standard and well optimized conditions.

Analyses were carried out using reversed-phase microcolumn chromatography, already proven for the separation of members of homologous barbiturates (e.g., 5,5′-disubstituted barbiturates) [14]. First attempts were done employing a C_{18} silica-based bonded phase (ODS). With the isocratic elution, there were no mobile phase combinations enabling resolution of the mixture (data not shown). With gradient elution the separation was better but isomeric amobarbital and pentobarbital overlapped (Fig. 2).

Next, we tried porous graphitized carbon (PGC). Encouraged by the previous reports of the isomers resolution with this stationary phase [15] we attempted the resolution of barbiturates. However, the best isocratic elutions were worse than those provided by the ODS column, and phenobarbital (the barbiturate most affected for changes in retention) also coeluted with amobarbital and pentobarbital. Under the optimized gradient elution phenobarbital was clearly separated. Peak width and shapes for separated compounds appeared of better quality than those given by ODS column while the run time for the mixture was the same. In this case, however amobarbital and pentobarbital continued to fully

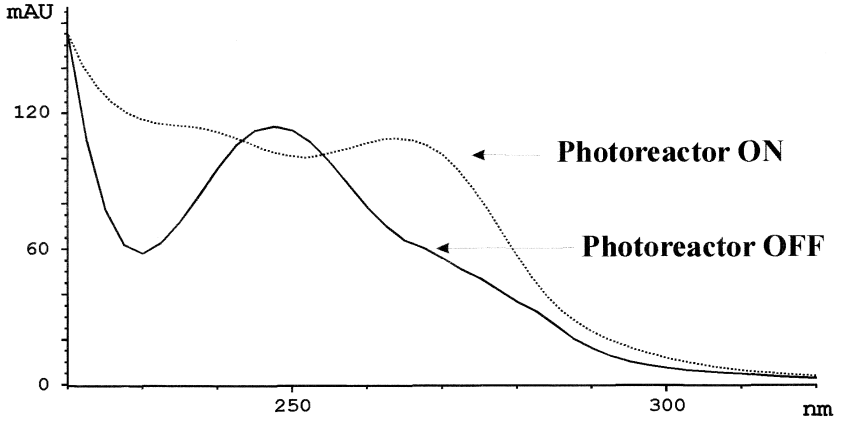

Fig. 1. Overlaid spectra for phenobarbital and its photoproduct; similar behavior was observed for the rest of the studied compounds.

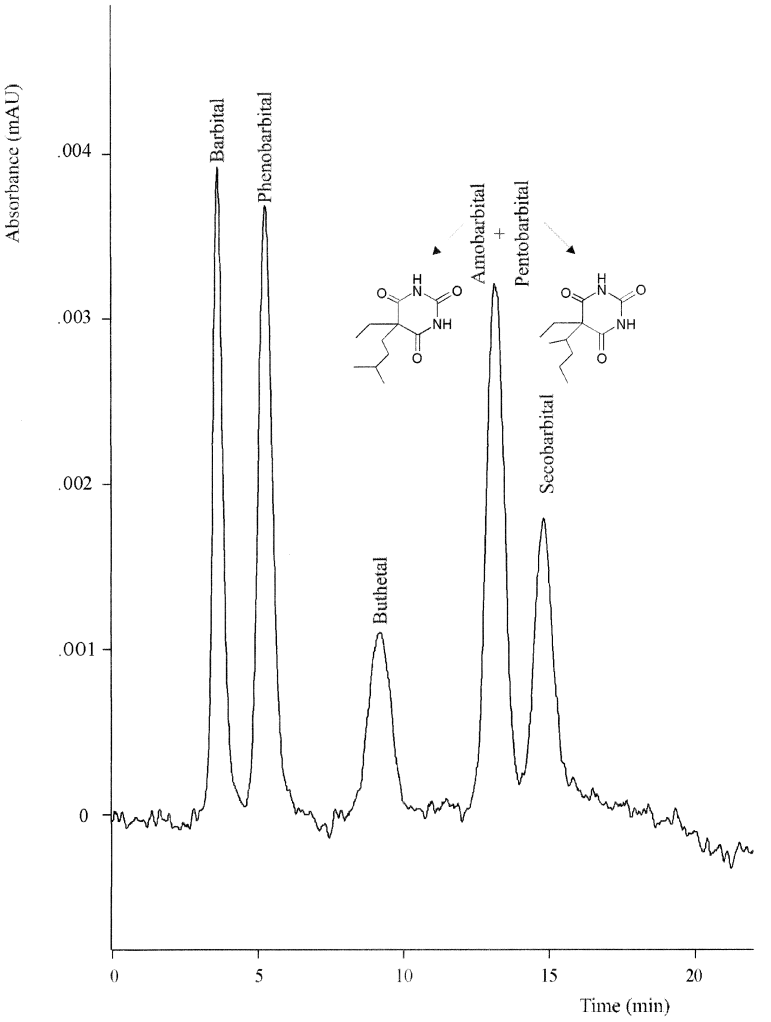

Fig. 2. Chromatogram obtained with ODS microcolumn and the stepwise gradient: 0–5 min hold at MeOH–buffer (25:75); 5–8 min from initial conditions to MeOH–buffer (60:40); 8–16 min hold at MeOH–buffer (60:40); pump flow-rate: 500 μl/min; λ=220 nm; 50 μg/ml.

overlap. In view of these facts we decided to use the PGC column to analyze spiked serum samples.

Fig. 3 shows a chromatogram from a spiked serum using the graphitized carbon microcolumn in gradient elution mode. Spiking level in this case was 50 μg/ml for each barbiturate. Although a noisy baseline is evident, all added barbiturates can be easily integrated. Linear dynamic range extent for all studied barbiturates up to 500 μg/ml and repeatability of injections was 5%. For the proposed method to be useful in forensic analysis of real samples from poisoned patients, the detection limits (LODs) for each particular barbiturate should be lower than the blood level reached at the moment of regained consciousness and small enough that the gradual recovery of patients could be controlled. Table 1 summarizes LODs obtained with the method described above (based on data of spiked samples), calculated at the S/N of 6 in comparison to barbiturate levels that are relevant in clinical conditions.

Table 1
Detection limits (LODs) of the proposed method and the relationship of average blood concentrations (μg/ml) of barbiturate to clinical condition (from Ref. [16])

Barbiturate	LOD (μg/ml)	Awake, mild sedation	Sedated, reflexive	Comatose, reflexive	Comatose, (circulatory and respiratory difficulties)
Barbital	26	80			
Buthetal	40	30			
Phenobarbital	20	35	44	65–100	
Pentobarbital/	}12	10	20		
amobarbital		15	25	62	86
Secobarbital	21	8	15	25	

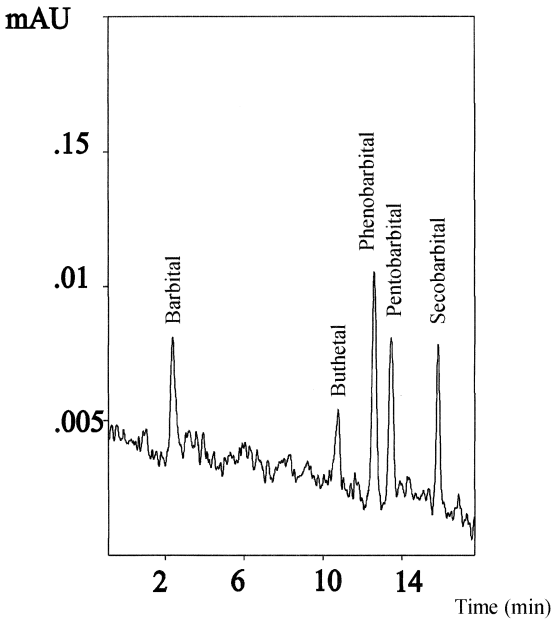

Fig. 3. Chromatogram of a spiked serum sample obtained with graphitized carbon microcolumn and the stepwise gradient: 0–20 min from MeOH–buffer (40:60) to 100% MeOH; pump flow-rate: 500 μl/min; photoreactor ON; λ=270 nm.

This comparison shows suitability of μ-HPLC combined with the post-column photoreactor to the requirements stated above.

References

[1] A. Gringauz, in: Introduction to Medicinal Chemistry, Wiley–VCH, 1997, pp. 568–569.
[2] R. Gill, A.H. Stead, A.C. Moffat, J. Chromatogr. A 204 (1981) 275.
[3] C.M. Selavka, I.S. Krull, I.S. Lurie, J. Chromatogr. Sci. 23 (1985) 499.
[4] C.R. Clark, J.L. Chan, Anal. Chem. 50 (1978) 635.
[5] M. Lores, D. Bonfiglio, R. Cela, Lab. Rob. Autom. 9 (1997) 185.
[6] R.W. Schmid, Ch. Wolf, J. Chromatogr. A 478 (1989) 369.
[7] Ch. Wolf, R.W. Schmid, J. Liq. Chromatogr. 13 (1990) 2207.
[8] H. Barton, J. Bojarski, Pharmazie 38 (1983) 38.
[9] H. Barton, J. Bojarski, J. Mokrosz, Tetrahedron Lett. 23 (1982) 2133.
[10] P. Kucera (Ed.), Microcolumn High-Performance Liquid Chromatography, Elsevier, Amsterdam, 1985.
[11] M. Novotny, D. Ishii (Eds.), Microcolumn Separations – Columns, Instrumentation and Ancillary Techniques, Elsevier, Amsterdam, 1985, pp. 57–104.
[12] A.H.M.T. Scholten, P.L. Welling, U.A.Th. Brinkman, R.W. Frei, J. Chromatogr. A 199 (1980) 239.
[13] O. Cabaleiro, M. Lores, R. Cela, presented at the 4th International Symposium on Hyphenated Techniques (HTC4), Bruges, 1996, poster.
[14] U.D. Neue, in: HPLC Columns – Theory, Technology and Practice, Wiley–VCH, 1997, Chapter 10.
[15] J. Schlatter, A. Astier, Biomed. Chromatogr. 9 (1995) 302.
[16] H.E. Spiegel, Barbiturates, ultraviolet spectrophotometric method, in: W.R. Faulkner, S. Meites (Eds.), Selected Methods of Clinical Chemistry, Vol. 9, AACC Inc., USA, 1982, p. 110.