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Improved electrochemical detection of diuretics in high-performance liquid chromatographic analysis by postcolumn on-line photolysis

M. Macher, R. Wintersteiger*

Institute of Pharmaceutical Chemistry, Karl-Franzens-University Graz, Schubertstrasse 1, A-8010 Graz, Austria

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Abstract

A sensitive, high-performance liquid chromatographic method including postcolumn on-line UV irradiation and electrochemical detection is described for the determination of pharmaceuticals with diuretic action. The investigations on this liquid chromatography–photolysis–coulometric detection approach indicate that, depending on the substituents of the compounds, oxidation of drugs containing an aromatic chloride and thiazide moiety occurs at potentials as low as +200 mV. Parameters influencing the electrochemical response are given to optimize the reaction conditions. The procedure presented allows the quantification of diuretics, and consequently of chemically related substances, with good reproducibility and high selectivity at the picogram level.

1. Introduction

UV–Vis detection is the technique most commonly used in determinations of pharmaceuticals. As its sensitivity and selectivity are very limited, better systems of detection are necessary in order to quantify low drug dosages. Fluorimetry, which meets these requirements, is usable only for a small group of drugs having native fluorescence. The same problem arises with electrochemical detection. To extend the advantages of this detection mode to a larger group of substances, electrochemical detection was combined with photolysis, because on-line photochemical derivatization or photochemical reaction detection has been found to be an extremely useful technique for converting electrochemically non-responding or poorly responding analytes into easily detectable derivatives [1–4]. In photochemical reaction detection, a photochemical reactor between the column and detector is included in the HPLC system, creating a series of differing derivatization processes [5–8].

Some studies [9–11] have shown that substances with an aromatic chloride are transformed by irradiation with UV light into electrochemically active derivatives. We investigated a group of drugs having this structure, hydrochlorothiazide, bendroflumethiazide, butizide, chlortalidone, furosemide and etacrynic acid. For a few of these diuretic substances, e.g. hydrochlorothiazide and furosemide, there are methods for quantitative analysis with UV [12–14] and electrochemical detection (ED) [15–17]. A disadvantage of these electrochemical methods is the high

* Corresponding author.

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working potential needed to produce electrochemical activity. The main thrust of our work was therefore to use photolysis to reduce the working potential and so to enhance selectivity and sensitivity. In addition, we also tested the influence of photolysis on UV absorption characteristics with a view towards possible improvement of detection limits.

2. Experimental

2.1. Drug standards and standard solutions

Hydrochlorothiazide and chlortalidone were obtained from Ciba-Geigy (Basle, Switzerland), bendroflumethiazide from Kwizda (Vienna, Austria), butizide from Boehringer (Mannheim, Germany), furosemide from Hoechst (Frankfurt, Germany) and etacrynic acid from Merck (Darmstadt, Germany). The substances were weighed with an analytical balance (Sartorius). A stock standard solution of each substance was prepared by dissolving the substance (hydrochlorothiazide 5.5 mg; bendroflumethiazide 5.5 mg; butizide 5.3 mg; chlortalidone 5.3 mg; furosemide 5.1 mg; etacrynic acid 5.1 mg) in doubly distilled methanol (Loba-Chemie, Linz, Austria) and diluted to 10 ml. A 1-ml volume of each solution was diluted to 50 ml with methanol–water (80:20). During our investigations on the influence of irradiation, the final concentration was about 60 ng in 6 μl. Only the working standard solutions of hydrochlorothiazide, bendroflumethiazide and butizide were further diluted 1:1 with methanol–water (80:20). Water for the mobile phase was purified with a Nanopur cartridge purification system (Barnstead, UK).

The HPLC mobile phase was prepared by mixing methanol and water (80:20) and adding 2 g/l of $LiClO_4$. The mobile phase was filtered through a 0.2-μm pore size membrane (Sartorius) and degassed with helium.

2.2. Instrumentation

The HPLC–photolysis–electrochemical system includes a Perkin-Elmer Model 250 binary LC pump and a Rheodyne Model 7125 loop injector with a 6-μl loop. The precolumn (5 × 4 mm I.D.) was packed with LiChrosorb RP-8 (Merck), 10 μm particles. The analytical column was a Brownlee Lab RP-18 (Bartelt, Graz, Austria), Spheri 5 μm, 100 × 4.6 mm I.D. The photochemical reactor was the Beam Roost Reaction Unit (BB) (ICT, Vienna, Austria), which employs a Sylvania GTE 8 W low-pressure UV lamp, emission maximum 254 nm. The irradiation coil was constructed from a PTFE tubing. We used both commercial PTFE coils from ICT, with I.D. 0.3 mm and O.D. 1.3 mm and lengths of 10 and 20 m, and a laboratory-knitted PTFE coil, I.D. 0.33 mm, and O.D. 0.77 mm, 20 m long. Injections were made via a 100-μl syringe (Hamilton, Bonaduz, Switzerland).

Electrochemical detection was effected with a Model 5100A Coulochem electrochemical detector (ESA, Bedford, MS, USA), a model 5020 guard cell and a Model 5010 analytical cell. The working potential of the guard cell was +950 mV vs. palladium as reference electrode. The operating potential of the main cell was between +200 and +500 mV for injections under irradiation and was extended to +900 mV for injections without irradiation. The range was 50 × 10, the response time 0.4 s and the full output scale 10 mV.

The UV detector was a Perkin-Elmer LC 235 diode-array detector with a fixed wavelength of 240 nm, bandwidth 15 and sensitivity 0.05. This detector was connected on-line to the BB and the drug to be tested flowed directly from the BB to the detector. The connections between the column and irradiation coil were Upchurch fingertight fittings.

The integration of the peak areas was carried out with the Omega 235 software package and the results were plotted on an Epson LQ 850.

2.3. Optimization of photochemical reaction conditions

The irradiation time was varied either by mounting two fixed-length PTFE irradiation coils (10 and 20 m) to the photoreactor, or by varying the flow from the normally used 0.5 ml/min to

0.25 ml/min. The influence of the thickness of the coil on the effect of irradiation was tested with the original coil from ICT and with the laboratory-knitted thinner spaghetti coil. The working standard solution of each tested drug was injected into the HPLC–photolysis–electrochemical or HPLC–photolysis–UV detection system and the responses were recorded under the different conditions with and without irradiation. To shorten the equilibration time of the analytical cell, investigations were started at the lowest potential of +200 mV and then raised in steps of +100 mV.

3. Results and discussion

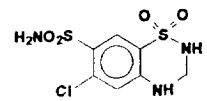
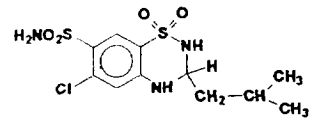
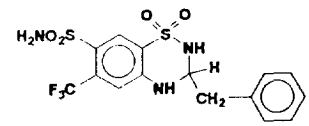
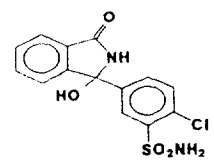
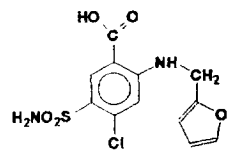
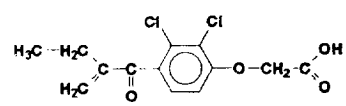
3.1. Electrochemical detection

Influence of chemical structure

The structures of the compounds investigated are given in Table 1. It is known that the chloride substituent in hydrochlorothiazide is replaced by H, OH or OCH_3 in aqueous or methanolic solutions on photolysis [9,10]. Additionally, hydrolysis of the thiazide ring occurs before and after photosubstitution reactions. To investigate the influence of different substituents on the electrochemical response, we chose substances with similar structures, such as bendroflumethizide and butizide.

As the substances flow through a 20-m coil of I.D. 0.3 mm and O.D. 1.3 mm, they are irradiated at 254 nm and then detected electrochemically. The three benzothiazides show an electrochemical response at a working potential of +200 mV vs. palladium. At this potential, these drugs are not electrochemically active without irradiation. Comparing the intensities of the signals for hydrochlorothiazide, bendroflumethiazide and butizide, it could be observed that the signal for bendroflumethiazide is about 20% smaller and that for butizide is about 50% smaller than the signal for hydrochlorothiazide. If these three substances are detected electrochemically at a higher working potential of +500 mV with irradiation, the intensity of the peaks is reversed. This means that the peak for butizide is higher than that for bendroflumethiazide; however, neither reaches the intensity of hydrochlorothiazide. Fig. 1 shows the assumed reactions of butizide after UV irradiation.

Table 1
Structures of the compounds

Substance	Formula
Hydrochlorothiazide	
Butizide	
Bendroflumethiazide	
Chlortalidone	
Furosemide	
Etacrynic acid	

The different electrochemical signal intensities of bendroflumethiazide and butizide under UV irradiation conditions can be attributed solely to the structures of these drugs. Because there is a similar phenomenon for two neuroleptics, i.e., fluphenazine, which is substituted with CF_3 and not with a chlorine, as in perphenazine, the CF_3 group could be responsible for the reduced signal [18]. The decrease in intensity occurs at higher operating potentials and it appears that chloride

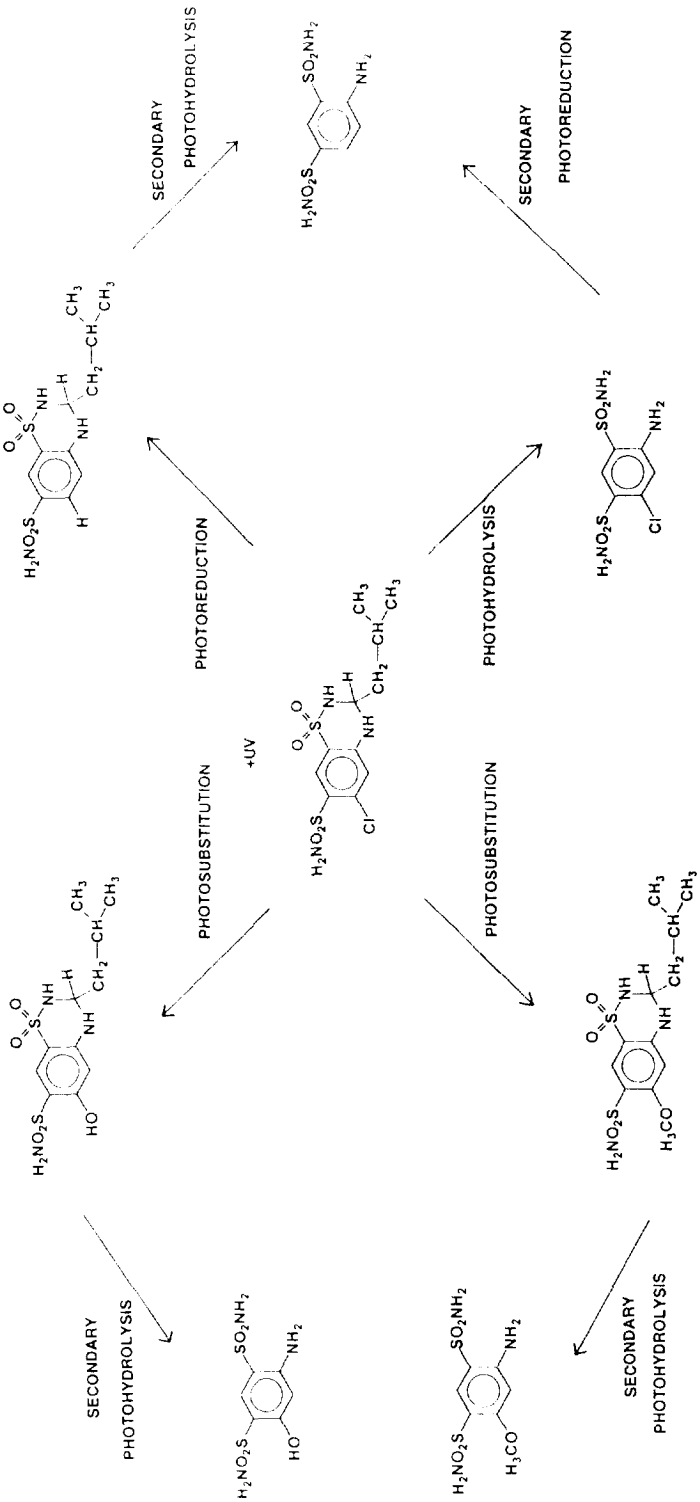


Fig. 1. Proposed butizide reactions induced by photolysis.

could be more easily replaced than CF_3. The substituent at position 3 of the thiazide ring, which is common to both drugs, is a steric hindrance for opening the thiazide ring, thus reducing the signals compared with hydrochlorothiazide.

Fig. 2 shows a comparison of the peak intensities of the three substances at different working potentials. Chlortalidone has the same substituted benzene ring, which a chlorine atom, as butizide and hydrochlorthiazide and it is assumed that photosubstitution occurs here. The acid amide is hydrolysed [19] and a benzophenone and carboxylic group result. Fig. 3 shows the product formed. Without irradiation, chlortalidone has no electrochemical activity at any potential. With photolysis it becomes electrochemically active and is detectable at a working potential of +200 mV. The signal intensity in this case is also only 50% of the intensity of hydrochlorothiazide, which can be explained by the absence of the benzothiazide structure. At higher potential this percentage decreases without reaching the electrochemical response of hydrochlorothiazide.

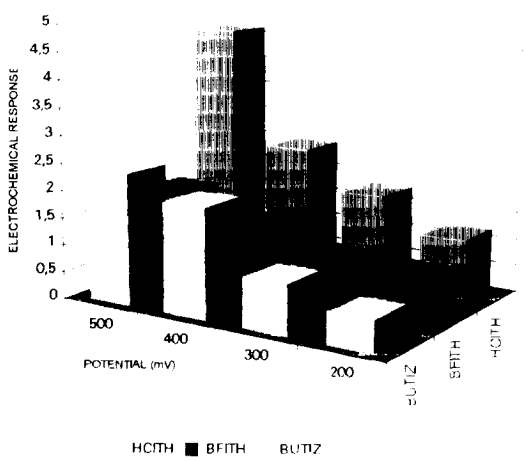


Fig. 2. Comparison of peak intensities of hydrochlorothiazide, bendroflumethiazide and butizide at different working potentials. Hydrochlorothiazide (HCITH) (concentration 30 ng per 6 μl), bendroflumethiazide (BFITH) (30 ng per 6 μl) and butizide (BUTIZ (30 ng per 6 μl). Column, Brownlee RP-18, Spheri 5 μm, 100 × 4.6 mm I.D.; mobile phase, MeOH–H_2O (80:20) + 2 g/l of $LiClO_4$; flow-rate, 0.5 ml/min; coil, 20 m × 1.3 mm O.D.

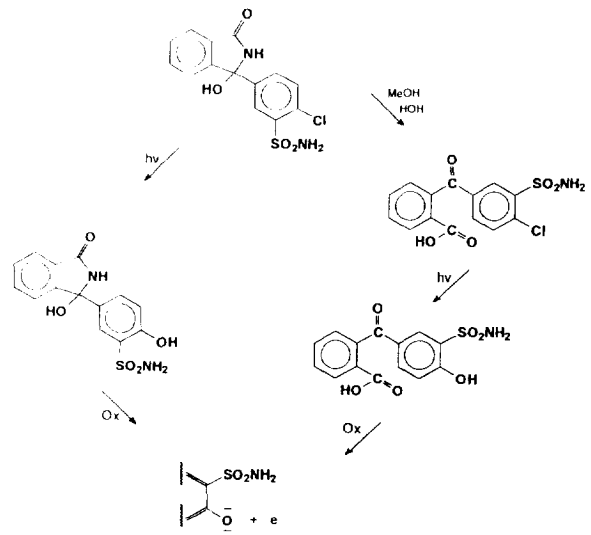


Fig. 3. Proposed formation of chlortalidone derivative by photolysis–ED.

Furosemide was recognized as a photosensitive drug which is photolysed at room temperature under daylight conditions [20]. The chlorine atom is also substituted in this molecule by OH. This characteristic can be utilized for sensitive and selective determination. Furosemide was transformed by photolysis from an electrochemically inactive substance into an electrochemically active derivative at a working potential as low as +200 mV. Etacrynic acid has similar feature. This drug is not electrochemically active without irradiation, but may be detected with irradiation at the same potential of +200 mV.

Stability of different PTFE tubing

The coils for the on-line postcolumn photolyic derivatization are made of PTFE tubing. They display good transparency to UV irradiation in the 230–400 nm range commonly used in organic photochemistry, are resistant to chemical solutions and have oxygen permeability. The reactor coils are made in a specific way by hand [21] or by machine. This, however, produces deformed tubes; this deformation is necessary to reduce the development of parabolic flow profiles and

subsequent peak broadening in the tubing. The commercially available coil is tested for a maximum pressure of 350 p.s.i. The coulometric analytical cell of the detector used produces a back-pressure depending on age and duration of utilization and if this back-pressure is too high, the coil may burst. The ICT coil provides higher stability against fluctuations in pressure because of the greater thickness. The thinner spaghetti coil reacts more quickly to fluctuations in pressure, leading to leakage. Hence the thicker tubing is to be preferred.

Optimization of photolysis conditions

The irradiation intensity influences the production of electrochemically detectable compounds and depends on four parameters: the energy of the lamp used, the distance from the reaction coil to the light source, the thickness of the coil and the duration of irradiation. The energy of the lamp is constant; an 8 W low-pressure lamp was always used. The knitted reaction coil was slipped over the light source to minimize the distance to the coil. Therefore, only the thickness of the coil and the duration of irradiation were considered as parameters that could be varied to optimize the photochemical conditions.

Two coils with different thicknesses were available. They had the same inner diameter to guarantee identical conditions for peak form and flow characteristics. The examinations with all the diuretics resulted in an increased signal intensity when the thicker reaction coil was used for irradiation. These findings are opposite to those produced in additional investigations with benzaldehyde, which produces significantly higher signals when thinner tubing is used. The most advantageous thickness of the coil depends on the structure of the substance. If the highest sensitivity is essential, each compound to be analysed should be tested for optimum thickness of the coil.

The duration of irradiation was tested first by varying the length of the reaction coil; we alternated fixed lengths of 10 and 20 m with an O.D. 1.3 mm, and second by changing the flow to extend or shorten the irradiation time. As photochemical derivatization is an excellent method for improving selectivity in the determination of pharmaceuticals and this advantage is obtained with electrochemical detection at lower potentials, the drugs to be determined were tested for the effect of duration of irradiation at +200, +300 and +400 mV (Table 2).

By comparing the peak areas obtained with the 10- and 20-m coils, the factor increasing signal intensity (F_1) can be calculated. F_1 is the ratio of the value for the 20-m coil to that for the 10-m coil. With the exception of butizide at a

Table 2
Influence of the duration of irradiation

Potential (mV)	Coil length (m)	Hydrochlorothiazide	Bendroflumethiazide	Butizide	Chlortalidone	Furosemide	Etacrynic acid
200	10	1.9	1.2	1.8	0.8	0.4	0.06
200	20	2.3	2.0	1.1	1.1	0.9	0.03
F_1		1.2	1.7	0.6	1.4	2.3	0.5
300	10	1.9	0.9	1.8	0.7	0.5	0.6
300	20	3.2	2.2	2.3	2.0	1.6	0.3
F_1		1.7	2.4	1.3	2.9	3.2	0.5
400	10	1.9	1.3	1.9	1.0	0.5	0.8
400	20	4.6	3.0	5.1	4.2	1.4	0.8
F_1		2.4	2.3	2.7	4.2	2.8	1.0

Column, Brownlee RP-18, Spheri 5 μm, 100 × 4.6; I.D. mobile phase, MeOH–H_2O (80:20) + 2g/l of $LiClO_4$; flow-rate, 0.5 ml/min; coil, 20 m × 1.3 mm O.D

potential of +200 mV and etacrynic acid at +200 and +300 mV, an increase in the detector response was recognized using the longer reaction coil. It appeared that a longer coil corresponding to a longer irradiation time generally produces a higher detector response, but the F_I is individual and depends on the substance. Fig. 4 shows the different signals of bendroflumethiazide.

The effect of varying the flow-rate was tested with chlortalidone. The irradiation time was doubled by reducing the flow-rate from 0.5 to 0.25 ml/min. Under these conditions the peak area obtained with longer irradiation is only 23% larger than the peak response obtained with a flow-rate of 0.5 ml/min. The resulting peak broadening is not significant but, together with the longer retention, the sensitivity and selectivity decrease. Therefore, the first method is to be preferred, i.e., influencing the irradiation time by varying the length of the reaction coil.

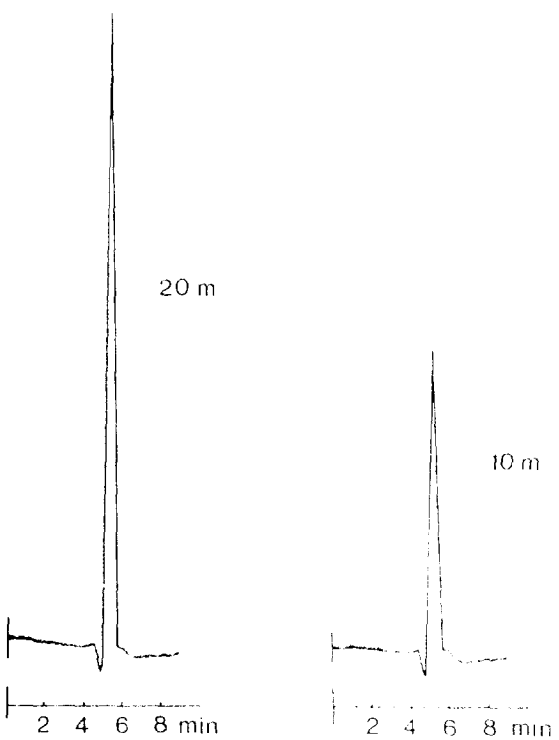


Fig. 4. Influence of coil length on detector response. Bendroflumethiazide concentration, 65 ng per 6 μl; column, Brownlee RP-18, Spheri 5 μm, 100 × 4.6 mm I.D.; mobile phase, MeOH–H_2O (80:20) + 2 g/l of $LiClO_4$; flow-rate, 0.5 ml/min; working potential, +400 mV; with irradiation, coils, 10 and 20 m × 1.3 mm O.D.

Reproducibility and limit of detection (LOD)

The reproducibility of the method under irradiation conditions was determined using 60 ng per 6μl of chlortalidone ($n = 8$). The relative standard deviation was 2.9%. The LOD was measured at a signal-to-noise ratio of 3. The LODs at a potential of +400 mV with UV irradiation were the following: hydrochlorothiazide 0.8, butizide 1, bendroflumethiazide 1.6, chlortalidone 1.6 and etacrynic acid 8 ng per 6 μl. The LOD of hydrochlorothiazide at a potential of +900 mV without UV irradiation is 1 ng per 6 μl and for the other analytes about 2 ng per 6 μl. For etacrynic acid a comparison of the LOD with and without irradiation is impossible because of its lack of electrochemical response without irradiation.

For all substances, a linear response is obtained over the range 2 ng–2 μg, with the exception of etacrynic acid (10 ng–10 μg). The correlation coefficient for the function $y = ax + b$ was between 0.997 and 0.999.

3.2. Influence of irradiation on UV detection

Photolysis may improve the detectability of substances not only by enhancing the electrochemical activity but also by influencing the UV absorption behaviour. Although electrochemical detection is more selective than UV detection, a comparison with respect to sensitivity by irradiation was of interest.

The drugs were detected by UV spectrophotometry at 240 nm without and with irradiation using a 20-cm ICT coil and the peak areas were compared. Only the detector response for bendroflumethiazide increased by about 30% with irradiation. The signals for the other diuretics generally decreased with irradiation. The decrease of the signal of butizide by 40% was the highest and for etacrynic acid by 4% was the lowest.

Comparing the sensitivities of these two detection methods, UV and ED with irradiation, it was found that ED is more sensitive, because the

LOD of hydrochlorothiazide with UV detection was 4 ng per 6 μl. We found the same result for the other substances. This LOD was lower than the LOD for ED with irradiation only for etacrynic acid. Therefore, photolysis in combination with UV detection has no advantages over electrochemical detection and is not recommended for these substances.

4. Conclusion

HPLC–photolysis–ED does not require expensive reagents and is a precise, convenient method for determining diuretics which can be transformed by on-line post-column photolysis into electrochemically active derivatives. The diuretics become electrochemically active by UV irradiation at very low working potentials. Normally they become active at potentials of +800 and +900 mV. This increases the selectivity. HPLC–photolysis–UV detection usually leads to a lower sensitivity.

The expectation that the chloride atom on the benzene ring is replaced and that this substitution is responsible for electrochemical detectability by photolysis could be verified. However, comparison of the signals of hydrochlorothiazide, bendroflumethiazide and butizide indicated that hydrolysis of the benzothiazide ring also has an influence on the transformation into an electrochemically active substance. The effect of the UV irradiation depends on the preparation, thickness and length of the coils and the flow-rate. The method presented will provide the basis for pharmacokinetic studies on real samples and will be published in a subsequent paper.

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