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Journal of Chromatography A, 890 (2000) 281–287

JOURNAL OF CHROMATOGRAPHY A

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Post-column oxidative derivatization for the liquid chromatographic determination of phenothiazines

Georg Diehl, Uwe Karst*

Westfälische Wilhelms-Universität Münster, Anorganisch-Chemisches Institut, Abteilung Analytische Chemie, Wilhelm-Klemm-Str. 8, 48149 Münster, Germany

Received 1 February 2000; received in revised form 9 May 2000; accepted 23 May 2000

Abstract

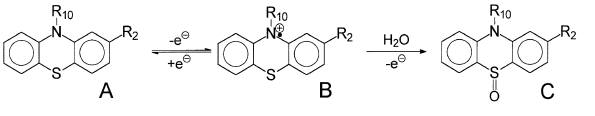
A first post-column chemical derivatization method for the liquid chromatographic determination of phenothiazines is presented. Peroxyacetic acid is introduced as a derivatizing agent for phenothiazines, yielding the colored radical cations or fluorescent sulfoxides, depending on reaction conditions. Both reaction products were successfully employed for the detection of the phenothiazines after their liquid chromatographic separation. The fluorescence spectroscopic detection of the sulfoxides proved to be the more robust and sensitive method. Limits of detection ranged from 4 n*M* for triflupromazine and trimeprazine to 300 n*M* for phenothiazine for the fluorescence spectroscopic detection of the sulfoxide and from 0.3 μ*M* for phenothiazine and triflupromazine to 2 μ*M* for trifluperazine for the UV–Vis spectroscopic detection of the radical cation. The calibration functions for the fluorimetric sulfoxide determination ranged from two to more than three decades, starting at the limit of quantification.

Keywords: Derivitization, LC; Phenothiazines; Peroxyacetic acid

1. Introduction

Phenothiazine and its derivatives possess a wide range of pharmaceutical properties that can be used for the treatment of patients with various diseases. Their main field of application are psychic diseases like schizophrenia [1]. Other useful properties like antihistaminic and antiemetic effects are utilized in different clinical fields [2]. The literature shows a wide variety of employed analytical techniques [3] for their determination. These include mass spectrometric [4], titrimetric [5], photometric [6], fluorimetric [7], electrochemical [8], flow injection [9] as well as gas [10,11] and liquid [12–15] chromatographic methods with different detectors. The official methods are mainly based on titrimetry and spectrophotometry [16].

Phenothiazine and its derivatives are characterized by low ionization potentials [17]. They are easily oxidized by different chemical, electrochemical, photochemical and enzymatic methods. The reaction scheme for the oxidation process [18] is the following:



*Corresponding author. Tel.: +49-251-8333-182; fax: +49-251-8333-169.

E-mail address: uwe.karst@uni-muenster.de (U. Karst).

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00607-5

The first step is a reversible electron abstraction from the phenothiazine (A) to the colored semiquinone radical cation (B) [19]. The radical is stable for a certain period of time depending on phenothiazine substitution, pH and buffer used [20]. In a second step, the corresponding phenothiazine sulfoxide (C) is formed [19,21]. The oxidation is interesting for the determination of the phenothiazines, because it improves the spectroscopic properties for UV–Vis and fluorescence detection. General approaches for the oxidation of the phenothiazines are based on either photochemical [22,23] or chemical oxidation [19,24–28]. The major disadvantage of the photometric or fluorimetric methods is their lack of selectivity: As soon as several phenothiazines are present in a sample, a sum parameter is obtained. Therefore, coupling the oxidative detection scheme to a chromatographic separation is an attractive approach to keep the sensitivity of the method and to gain additional selectivity. Only one method for HPLC analysis with post-column photochemical oxidative derivatization has been described yet [23].

Davidson [29] described the use of peroxyacetic acid (PAA), a strong oxidizer with frequent industrial use as disinfectant and bleaching agent, earlier for the oxidation of phenothiazines. Pinkernell et al. (microplate spectrophotometry) [30] and Effkemann et al. (HPLC with post-column derivatization) [31] on the other hand, used 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonate (ABTS) in the presence of iodide traces as a reagent for the determination of PAA and other peracids in industrial samples. ABTS is oxidized by PAA to a green colored radical cation. Therefore, the use of PAA with iodide traces appeared to be a promising approach for the determination of phenothiazines as well.

2. Experimental

2.1. Chemicals

All phenothiazine salts, ammonium formate and peroxyacetic acid (32% solution in dilute acetic acid; **caution:** strong oxidizer, dilute before mixing with organic substances) were purchased from Sigma (Deisenhofen, Germany) or from Aldrich Chemie (Steinheim, Germany) in the highest quality available. Potassium iodide and formic acid were obtained from Fluka (Buchs, Switzerland). Solvent for liquid chromatography was acetonitrile LiChroSolv gradient grade from Merck (Darmstadt, Germany).

2.2. UV–Vis spectroscopy

A HP 8453 diode array spectrophotometer (Hewlett-Packard, Waldbronn, Germany) with software HP Chem Station 845x-biochemical UV–Vis system was used. The spectrophotometric measurements were carried out as follows:

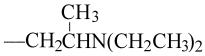
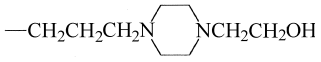
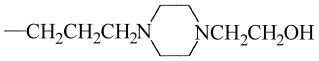
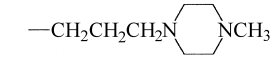
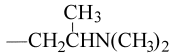
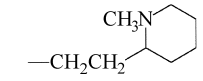
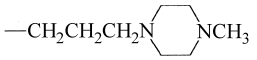
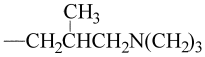
A 1 m*M* stock solution of the phenothiazine in a mixture of 50% (v/v) each of eluent A (see HPLC conditions) and acetonitrile was prepared. (a) Phenothiazines: Two ml of the stock solution were diluted with 1.2 ml of the mixture of 50% (v/v) each of eluent A and acetonitrile. The UV–Vis spectrum was recorded in the range from 200 nm to 600 nm. (b) Radical cations: 1.2 ml of a 50 m*M* solution of PAA in water and 20 μl of a 200 mg/l solution of KI in water were added to two ml of the stock solution. The stationary and time-resolved UV–Vis spectra in the range from 200 nm to 600 nm were recorded immediately after mixing the reactants. (c) Sulfoxides: The mixture obtained for the radical cations was left to react for 30 min. Afterwards, the spectrum was recorded as described for (a).

2.3. Fluorescence spectroscopy

Fluorescence spectra were recorded using a Shimadzu (Duisburg, Germany) RF-5301PC spectrofluorophotometer with RF-5301PC software (version 1.10). The fluorescence spectroscopic measurements were carried out as follows:

A 20 μ*M* stock solution of the phenothiazines in 1 *M* acetic acid was prepared. (a) Phenothiazines: 2.8 ml of the stock solution were diluted with 0.7 ml of 1 *M* acetic acid. Fluorescence spectra were recorded according to the properties of the individual phenothiazines which are displayed in Table 1. (b) Radical cations: 200 μl of 200 mg/l potassium iodide solution and 500 μl of 50 m*M* PAA solution were added to 2.8 ml of the stock solution. The fluorescence spectra were recorded immediately after mixing the solution. (c) Sulfoxides: The mixture obtained for the radical cation was left to react for 10 min.

Table 1
Structure and spectroscopic data of the relevant phenothiazines and their derivatives

Substance	Substitution		λ_{max}(UV) sulfide (nm)	λ_{max}(UV) radical (nm)	λ_{max}(UV) sulfoxide (nm)	Fluorescence (sulfoxide)	
	R_{10}	R_2				λ_{ex} (nm)	λ_{em} (nm)
Acetopromazine	$-CH_2CH_2CH_2N(CH_3)_2$	$-COCH_3$	277	515	373	378	502
Chlorpromazine	$-CH_2CH_2CH_2N(CH_3)_2$	$-Cl$	256	529	340	345	393
Ethopropazine		$-H$	248	520	333	338	374
Fluphenazine		$-CF_3$	258	500	347	353	401
Perphenazine		$-Cl$	256	525	340	343	381
Phenothiazine	$-H$	$-H$	252	517	335	344	389
Prochlorperazine		$-Cl$	256	525	340	344	389
Promazine	$-CH_2CH_2CH_2N(CH_3)_2$	$-H$	252	515	340	345	380
Promethazine		$-H$	250	520	333	339	376
Thioridazine		$-SCH_3$	262	638	343	351	436
Trifluperazine		$-CF_3$	258	500	347	352	401
Triflupromazine	$-CH_2CH_2CH_2N(CH_3)_2$	$-CF_3$	258	500	347	352	400
Trimeprazine		$-H$	252	515	340	344	379

Afterwards, the fluorescence spectrum was recorded as described for (a).

2.4. HPLC and post-column instrumentation

The HPLC system from Shimadzu consisted of the following components: CBM-10 A controller unit, GT-154 degasser, two LC-10 AS pumps, SIL-10 A autosampler, CTO-10 AC *VP* column oven, SPD-M10 A *VP* UV–Vis diode array detector, RF-10 A XL fluorescence detector, and Class LC 10 software.

The set-up for the HPLC system with post-column oxidative derivatization module is the same as in earlier publications [31]. The post-column instrumentation included a non-commercial Siemens (Karlsruhe, Germany) HPLC pump as reagent pump, an Upchurch (Oak Harbor, WA, USA) mixing tee and an 8 m knitted Teflon capillary [32] with an inner diameter of 0.3 mm.

The reagent solution (900 μl PAA (32%) in 100 ml 0.01 N sulfuric acid, prepared shortly before the measurements) was delivered by an HPLC pump at a flow-rate of 300 μl/min for the formation of the sulfoxides and 25 μl/min for the radicals and was

mixed with the HPLC effluent using a low dead volume mixing tee. For detection of the sulfoxides, an 8 m knitted Teflon capillary with an inner diameter of 0.3 mm (internal volume of 0.57 ml) was integrated in an oven (30°C) and used as a manifold for the post-column derivatization. Under these conditions, 26 s are available for the reaction to the sulfoxides.

2.5. HPLC conditions

All separations were performed using a Discovery C_{18} column (Supelco, Deisenhofen, Germany) equipped with a guard column of the same material with the following dimensions: particle size, 5 μm; pore size, 100 Å; inner diameter, 4.6 mm; length, 20 mm (guard column) and 150 mm (analytical column). Eluent A of the mobile phase was a solution of 575 mg ammonium formate and 1.3 ml formic acid in 500 ml deionized water (pH≈3); Eluent B was acetonitrile. A binary gradient at a flow-rate of 1.0 ml/min with the following profile was used:

t (min)	0.01	3	11	12	13	14	15
$c(CH_3CN)$ [%]	40	40	80	100	40	40	stop

The injection volume was 20 μl and the oven temperature 25°C.

For time-programmed fluorescence detection, the following program was used:

t (min)	0.01	4.75	8.35	10
λ (excitation) (nm)	378	344	351	344
λ (emission) (nm)	502	380	436	389

3. Results and discussion

As a first step to optimize the detection system, the UV–Vis and fluorescence spectra of the phenothiazines were recorded. Similar spectroscopic properties were observed for all analytes, with the exception of acetopromazine and thioridazine. The respective data are presented in Table 1. The phenothiazines and their corresponding sulfoxides are characterized by similar UV–Vis spectroscopic properties, with a slightly red-shifted absorption maximum for the sulfoxides e.g., at 340 nm for perphenazine. However, the properties are too similar to be used for post-column derivatization. On the other hand, the radical cation exhibits an additional absorption maximum at 525 nm with small molar absorptivity, which should be suitable for photometric detection. The fluorescence properties of the sulfoxides also allow post-column derivatization. Although there is a slight fluorescence of the native phenothiazines, the oxidation to the sulfoxides results in strong fluorophores, which can be used for post-column derivatization.

When mixing the oxidant and the phenothiazines in solution, the formation of a red color is observed, which is associated with the additional absorption band as described above. This color quickly fades away again. To quantify this effect, with the goal of using it for post-column derivatization, the time-dependency of the reaction of perphenazine was recorded with a spectrophotometer. While the absorption at 340 nm is constantly high after a few seconds, the band at 525 nm reaches its maximum after a reaction time of 38 s. However, high absorption is observed already very early. After 3 s, 80% of the maximum absorbance is recorded. As mixing the solutions rapidly is a crucial factor in spectrophotometry, but not in a HPLC post-column reaction system with a low dead volume mixing tee, the use of an additional mixing coil appears not to be necessary in the post-column system.

In Fig. 1, dependence of the peak areas for the post-column derivatization of thioridazine with PAA on the length of the reaction coil is observed. To confirm the assumption that mixing is better in the post-column system compared to cuvette spectrophotometry for another phenothiazine, it is observed that the highest peak area of the radical cation is observed without inserted reaction loop. Therefore, the use of a reaction coil for all measurements of the radical cations was avoided. On the other hand, the strongest fluorescence of the sulfoxide is observed with the longest reaction loop. For this reason, an 8 m reaction capillary heated to 30°C was used for all measurements of the sulfoxide.

The effects of pH and flow-rate of the PAA solution are presented in Fig. 2. The graph shows that using a reagent solution of pH 2 results in the largest peak area for both UV and fluorescence detection. The best flow-rate of the reagent solution

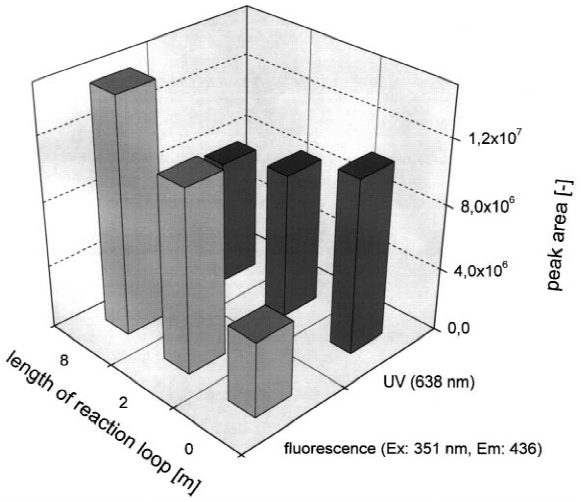


Fig. 1. Dependance of UV and fluorescence peak areas on the length of the reaction loop.

is different for the two detection modes. A higher flow-rate correlates to a larger concentration of PAA, but also to a reduced reaction time. As a short reaction time is sufficient for the formation of the desired products (see above), the increase in the PAA concentration has a greater influence on the signal intensity. Therefore, more sulfoxide is produced with increased PAA flow-rate, thus resulting in a high fluorescence signal. The radical cation, however, can be best detected UV–Vis spectroscopically at low flow-rates with corresponding low PAA concentrations. The flow-rate of 25 μl/min could not be further reduced and was therefore selected for all following measurements of the radical cation. For the analysis of the phenothiazine sulfoxides with fluorescence detection, the flow-rate of the oxidant was raised to 300 μl/min to guarantee complete oxidation.

The HPLC separation of a series of phenothiazines, with subsequent UV–Vis (340 nm) and time-programmed fluorescence detection after oxidation to the sulfoxides, is presented in Fig. 3. It should be noted that the time delay of a few seconds between the two traces in the chromatogram is caused by connecting the detectors in series.

The respective chromatogram with detection of the corresponding radical cations at 515 nm after post-column derivatization is characterized by small peak areas due to the low molar absorption of the radical cations and their low stability.

A comparison of the analytical parameters of the developed methods yields the following results:

The calibration functions are linear for three

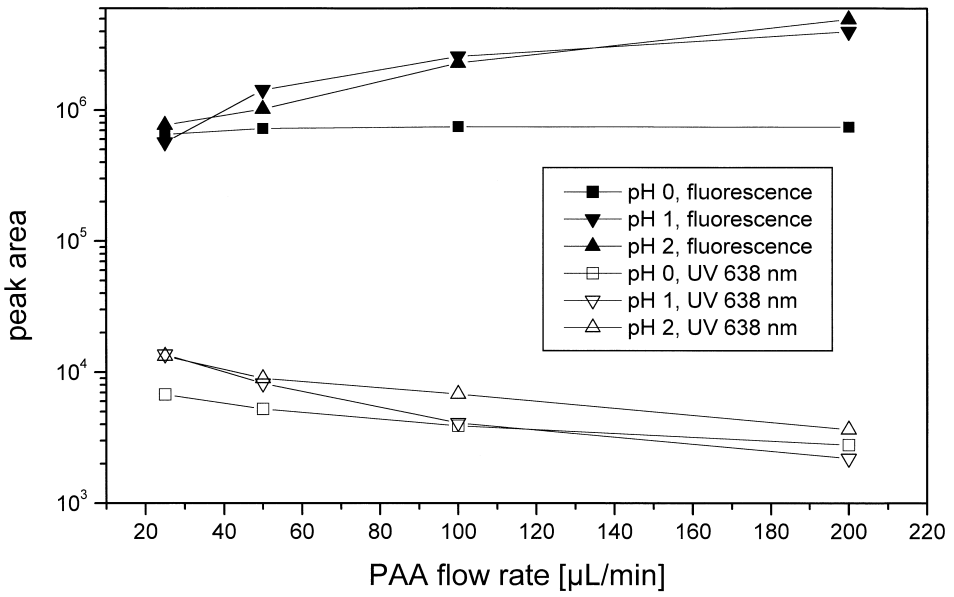


Fig. 2. Influence of pH and flow of the reagent solution on the signal intensity.

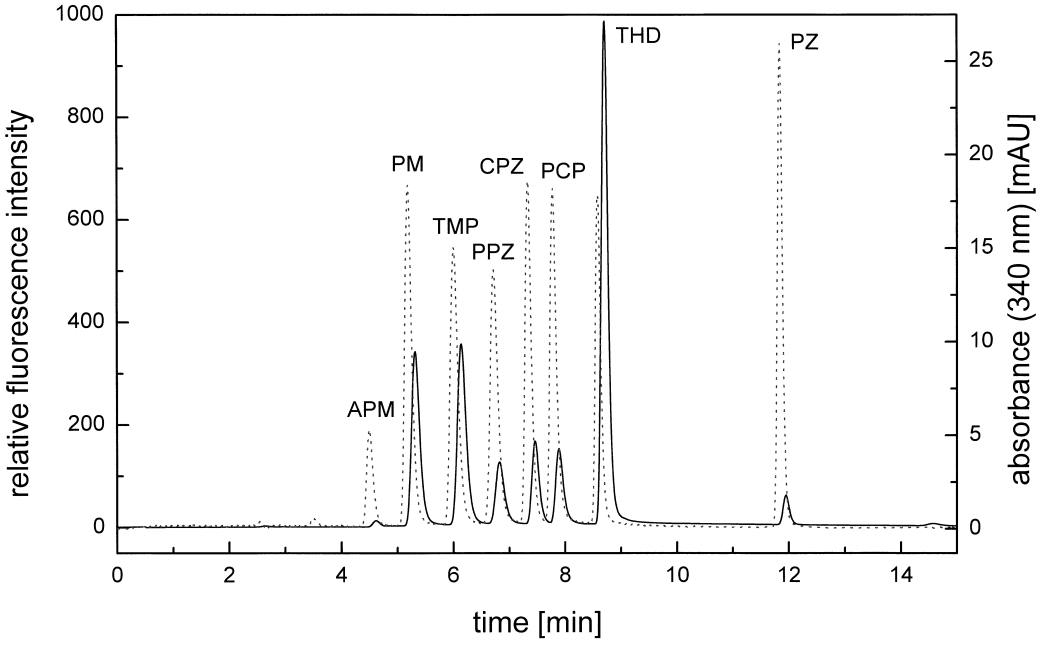


Fig. 3. Chromatogram of seven phenothiazine derivatives (APM: acetopromazine, PM: promazine, TMP: trimeprazine, PPZ: perphenazine, CPZ: chlorpromazine, PCP: prochlorperazine, THD: thioridazine) and phenothiazine (PZ); time-programmed fluorescence (———) and UV detection at 340 nm (· · · · · ·) of the corresponding sulfoxides after post-column derivatization.

decades in the case of UV detection of the native phenothiazines and fluorescence detection of the sulfoxides, approximately two decades for the UV detection of the sulfoxides and 1.5 decades for the detection of the radical cation. The coefficients of linear regression are above 0.994 in all cases except one. Table 2 shows the limits of detection of the different determination methods for thirteen phenothiazines. It is obvious that the limits of detection are typically best for fluorescence detection of the sulfoxides or for direct UV–Vis spectroscopic detection of the phenothiazines. However, selectivity can be claimed to be superior for the post-column detection scheme compared to direct UV–Vis detection at short wavelengths. The UV–Vis detection of the sulfoxides is not attractive, as the respective limits of detection are inferior to the direct UV–Vis detection of the phenothiazines. The UV–Vis spectroscopic detection of the radical cation provides for excellent selectivity due to strongly red-shifted absorption wavelengths, but the limits of detection are high due to the low molar absorptivity of the radical cations.

It is therefore concluded that the chemical post-column oxidative derivatization offers a wide variety of useful detection schemes for phenothiazines after their liquid chromatographic separation. As expected,

Table 2
Comparison of limits of detection for phenothiazine sulfides (SU, UV 254 nm), radical cations (R, UV 525 nm) and sulfoxides (F, time-programmed fluorescence; SO, UV 340 nm)

Substance	Limit of detection (n*M*)			
	SU	R	F	SO
Acetopromazine	20	500	10	300
Chlorpromazine	20	600	20	300
Ethopropazine	30	800	100	800
Fluphenazine	30	800	8	100
Perphenazine	20	800	6	300
Phenothiazine	70	300	300	600
Prochlorperazine	80	800	20	300
Promazine	20	900	20	300
Promethazine	40	800	40	300
Thioridazine	60	500	20	90
Trifluperazine	40	2000	8	100
Triflupromazine	40	300	4	100
Trimeprazine	80	900	4	400

the instability of the phenothiazine radicals makes the determination based on the sulfoxide fluorescence the most reliable and sensitive method.

Acknowledgements

Financial support of parts of this work by the Fonds der Chemischen Industrie and the European Commission (EUROPEROX project, SMT4-CT97-2153) is gratefully acknowledged.

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