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ON-LINE ELECTROCHEMICAL REAGENT PRODUCTION FOR FLUORESCENCE DETECTION OF PHENOTHIAZINES IN LIQUID CHROMATOGRAPHY

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SUMMARY

Thioridazine was detected by fluorimetry after separation by reversed-phase liquid chromatography, using a post-column derivatization system in which electrochemically generated bromine is applied as an oxidant. Analysis of plasma samples did not involve an extraction step. The detection limit was 0.5 ng or 5 ng/ml in plasma. The peak-area reproducibility was ±4.6% for plasma samples (n = 10). This inexpensive derivatization system compares well with alternative techniques. It can also be applied to the detection of other phenothiazines.

INTRODUCTION

As fluorescence measurement is generally the most sensitive and selective detection method for high-performance liquid chromatography (HPLC), much effort has been put into the development of derivatization techniques for compounds that lack a strong native fluorescence. Of these, pre-column methods have the advantage that the fluorescence labelling can be combined with an improvement in the separation properties of the compounds of interest and with sample clean-up. On the other hand, post-column techniques are also often applied as artefacts are less easily generated and automation is simpler. Several post-column derivatization techniques can be applied, such as the addition of reagent solutions, liquid–liquid phase reactions, solid phase reactions, including enzyme reactions, light irradiation and electrochemical conversion. Recently an extensive review on fluorescence derivatization methods for HPLC has been published[1].

In previous work[2,3] we have presented an electrochemical cell for the on-line production of reagents in the column effluent. It was used in conjunction with an amperometric detector, which monitored the excess of reagent. However, electrochemical reagent generation can be applied just as well with other detection princi-

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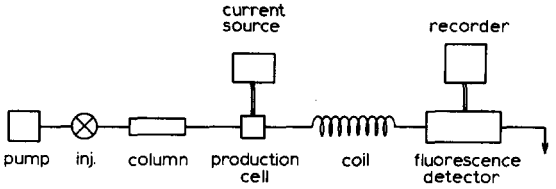
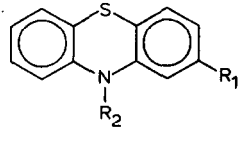


Fig. 1. Set-up for fluorescence detection in HPLC with on-line generated reagents.

ples, such as fluorimetry. Fig. 1 shows a scheme of this method. It has several advantages compared with other post-column techniques: (i) as opposed to the addition of reagent solutions, there is no extra peak dilution, no mixing problems, less pump noise and no need for an extra pump; (ii) in comparison with solid-phase reactors, the system is more versatile and the reactor does not wear out; (iii) with photochemical reactors the reaction time is usually long and the reaction often unspecific; as will be shown, this is not necessarily the case with electrochemically generated reagents; (iv) direct electrochemical conversion of a compound into a fluorescent product is also a promising technique, as has been shown[4] with vitamin K_1. When a coulometric cell is used, the compound of interest can be fully converted, and the same sensitivity may be expected as with the indirect method of electrochemical reagent generation, if the heterogeneous and the homogeneous reactions give the same products. An advantage of the indirect method presented in this paper is that it is less susceptible to electrode deactivation.

As a model compound we have chosen thioridazine (I), which is widely used as a psychotherapeutic drug. Its determination in plasma is difficult because of the low therapeutic concentrations (10–200 ng/ml). Normal- and reversed-phase HPLC separations of thioridazine and its metabolites have been used with UV[5,6], electrochemical[7,8] and fluorescence[9–11] detection. Thioridazine exhibits a weak native fluorescence, but it has been recognized in the past that oxidation of phenothiazines enhances the fluorescence intensity[12–14]. Determination by HPLC with fluorescence detection is therefore based on post-column oxidation. Muusze and Huber[9] oxidized thioridazine and its metabolites after normal-phase HPLC separation by addition of a permanganate solution and subsequent removal of the excess of permanganate with hydrogen peroxide. Wells *et al.*[10] modified this method. Scholten *et al.*[11] applied a post-column photochemically induced oxidation with fluorescence detection.

An attempt to effect the direct electrochemical conversion of thioridazine was not successful. Therefore, we studied a post-column system in which electrochemically generated bromine is the oxidant, and compared it with the above-mentioned methods for the determination of thioridazine in plasma. The applicability of the system to other phenothiazines has also been investigated.



I $R_1 = -S-CH_3$
II $R_1 = -SO-CH_3$
III $R_1 = -SO_2-CH_3$
$R_2 = -CH_2CH_2-$ (N-CH_3)

EXPERIMENTAL

Apparatus

Excitation and emission spectra were registered with a Perkin-Elmer 3000 scanning spectrofluorimeter with 1 × 1 cm quartz cuvettes. The chromatographic equipment consisted of a Perkin-Elmer 601 pump, a Rheodyne injection valve with 20- and 200-μl loops, a 250 × 4.6 mm I.D. column packed with LiChrosorb RP-8 (5 μm), a Kontron SFM 23 spectrofluorimeter, a laboratory-built integrator and a Kipp BD-41 recorder. In optimization studies a Metrohm 1096/2 amperometric detector was used with a 3-mm glassy carbon working electrode and a silver–silver chloride–1 *M* lithium chloride reference electrode. For on-line electrochemical reagent production a KOBRA-cell, manufactured in the Free University Chemistry Department workshop, and described in the literature[2], was used with a home-made constant-current source. Electrochemical batch experiments were performed with a rotating platinum electrode, silver–silver chloride–1 *M* lithium chloride reference electrode and a platinum auxiliary electrode in a three compartment vessel.

Reagents and solutions

Thioridazine(I), mesoridazine besylate(II) and sulforidazine(III) were obtained from Sandoz. Other phenothiazines were obtained from various sources. The other chemicals used were of analytical-reagent grade. Phenothiazine stock solutions were prepared in methanol and stored at 4°C in the dark. The mobile phase was 70% (v/v) methanol–water containing 0.1 *M* lithium nitrate, 0.05 *M* tetraethylammonium perchlorate, 1 m*M* potassium bromide and 0.1 m*M* EDTA. The EDTA was added shortly before use.

Plasma sample preparation

Human pooled plasma was stored at −18°C. After thawing it was spiked with the desired amount of thioridazine and incubated for 30 min. For deproteination 1 ml of methanol containing 0.1 *M* sulphuric acid was added to 1 ml of plasma. The mixture was shaken vigorously and centrifuged for 10 min in a table-top centrifuge. Of the supernatant 200 μl were injected.

RESULTS AND DISCUSSION

Electrochemical and chemical oxidation

In first instance we investigated the direct electrochemical oxidation of thioridazine. With a rotating platinum electrode in a 0.1 *M* acetic acid buffer a well defined, diffusion-limited wave was obtained with a half-wave potential of +0.53 V *vs.* silver–silver chloride. Under the assumption that the electrode reaction is reversible, from the slope of the wave a value of $n = 1$ for the number of electrons transferred was calculated. On prolonged electrolysis at +0.8 V, a compound was produced with a dark blue colour. This compound was unstable, especially when exposed to light. It is likely that under the conditions employed direct electrochemical oxidation yields initially the radical cation of thioridazine[15]. Probe measurements showed that the 320/465 nm fluorescence of the unoxidized thioridazine decreased during electrolysis, and a new, stable fluorescent compound with maxima at 320/390 nm was found.

However, the intensity was not higher than that of the original thioridazine. Fluorescence signal enhancement was thus not possible by direct electrochemical conversion, and we directed our study to the on-line generation of bromine as homogeneous oxidant.

We studied the bromine oxidation of thioridazine and two of its metabolites, mesoridazine(II) and sulforidazine(III). When an excess of bromine was added to 10^{-4} *M* solutions of these compounds in an acetic acid buffer, the fluorescence intensity increased drastically. As can be seen in Fig. 2, the excitation spectra of the oxidized compounds show the typical crescendo-like wave paterns with three maxima such as have been reported for oxidation with potassium permanganate[13]. The spectra of the oxidation products of thioridazine and mesoridazine are identical and that of sulforidazine deviates slightly. Maximum fluorescence intensity with sulforidazine was obtained with a stoichiometric amount of bromine and for the other compounds an excess of bromine had to be added. Although the absolute fluorescence intensity for thioridazine is lower than that of the other compounds, the relative gain was largest for thioridazine, as the fluorescence of the unoxidized form is weak.

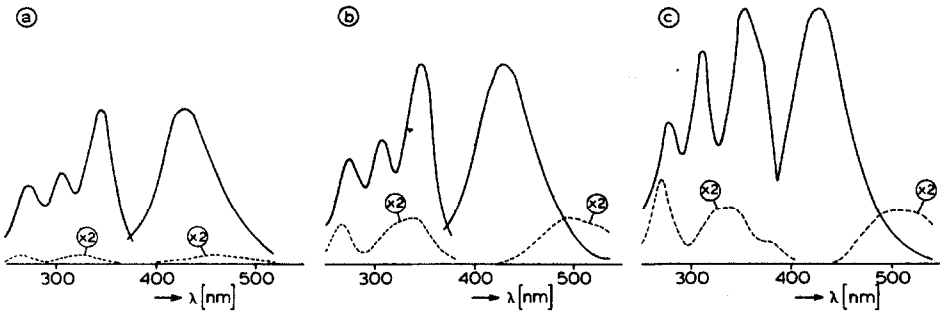


Fig. 2. Fluorescence excitation and emission spectra of thioridazines, before (--------) and after (———) oxidation with bromine. (a) Thioridazine; (b) mesoridazine; (c) sulforidazine. For the underivatized thioridazines the signal is amplified twice.

Optimization of the post-column system

The optimization of the post-column system was carried out with flow-injection experiments. In the early stages of our experimental work we used 0.1 *M* potassium bromide in methanol–water mixtures with acetate or formate buffers as the solvent in which bromine was generated electrochemically. In contrast to the batch experiments, the gain in fluorescence intensity was disappointingly low. High generating currents (1 mA, corresponding to a bromine concentration of 0.31 m*M* at a flow-rate of 1 ml/min) and long reaction capillaries (1 min) were needed to oxidize thioridazine to a sufficient extent. Only under these conditions was the fluorescence enhanced. By thermostating the reaction capillary at 60°C the reaction was accelerated, and the capillary could be shortened to reduce band broadening. A considerably faster reaction was observed with the unbuffered methanol–water mixtures containing only 1 m*M* KBr, which we used in subsequent studies. The decrease in bromide concentration leads to less formation of tribromide ion, which is less reactive than

bromine. Apart from this, the buffers appeared to have an inhibitory effect on the oxidation reaction.

The relationship between bromine consumption and fluorescence intensity for the three thioridazine derivatives was investigated by varying the reaction time and generating current. The bromine consumption was calculated from peak areas obtained with an amperometric detector instead of the spectrofluorimeter. This amperometric detector monitored the excess of bromine after the reaction coil. The reaction time multiplied by the concentration of bromine generated was taken as an indication of the "oxidation strength" of the derivatization system. As can be seen in Fig. 3, mesoridazine and sulforidazine react with at most one stoichiometric amount of bromine, mesoridazine with the higher reaction rate. The fluorescence intensity, measured at 345/425 nm, is proportional to the progress of the reaction. Thioridazine reacts with more than one equimolar amount of bromine at higher oxidation strengths. The largest gain in fluorescence intensity is in the first stage of the reaction.

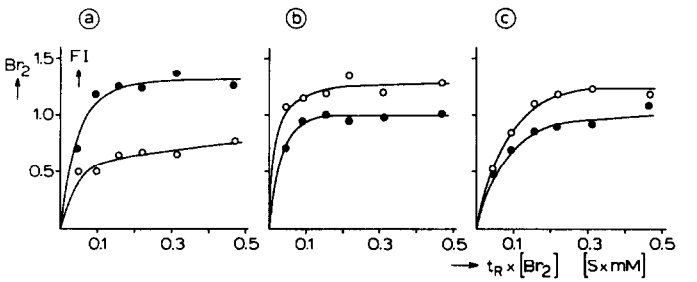


Fig. 3. Dependence of the amount of bromine reacted (●) and fluorescence intensity of thioridazines (○) on the reaction time and generating current. (a) Thioridazine; (b) mesoridazine; (c) sulforidazine. The bromine uptake is in moles of Br_2 per mole injected. For details see text.

Although there is still some ambiguity between the results of the batch and flow-injection experiments, some general conclusions on the mechanisms involved may be drawn. (i) Oxidation of the ring sulphur to a sulphoxide, the first step in the reaction of the thioridazines, is essential to obtain a high fluorescence. (ii) Oxidation of the side-chain sulphur enhances the fluorescence further. (iii) With oxidized side-chain sulphurs, as in sulforidazine, the rate of oxidation of the ring sulphur is decreased.

We proceeded with a reaction coil with a 12 s hold-up time and a generating current of 100 μA, corresponding to a bromine concentration of 3.1×10^{-5} *M* at a flow-rate of 1 ml/min. Under these conditions the fluorescence intensities of the three compounds virtually reach a plateau value.

Determination of thioridazine in plasma

Separation of thioridazine from interfering compounds was achieved with an RP-8 column and an aqueous mobile phase containing 70% (v/v) methanol, 0.1 *M* lithium nitrate, 0.05 *M* tetrathylammonium perchlorate, 1 m*M* potassium bromide and 0.1 m*M* EDTA. The addition of the quaternary ammonium salt to the mobile phase was necessary to reduce peak tailing. A guard column in front of the injection

valve improved the stability of the analytical column, probably by preventing the accumulation of metal salts from the pump on the top of the analytical column. Sulforidazine, mesoridazine and thioridazine eluted with k' values of 0.35, 0.6 and 2.3, respectively. The signal obtained with standard thioridazine injections was linear with the amount injected up to 40 ng. Above this amount signals were lower than expected. This may be caused by an internal fluorescence quenching effect, or by a depletion of the available reagent. The improvement of the sensitivity by the post-column system was determined by comparing signal-to-noise ratios obtained with underivatized and oxidized thioridazine at the appropriate wavelengths. As can be seen from Table I, the bromine generation system improves the detection limit by a factor of 16. A signal-to-noise ratio of 2 will be obtained with 0.5 ng injected.

TABLE I

SENSITIVITY OF FLUORESCENCE DETECTION OF THIORIDAZINE WITH AND WITHOUT POST-COLUMN OXIDATION WITH BROMINE

i_G (μA)	Detection wavelengths		Signal*,**	Noise*	Signal-to-noise ratio
	λ_{exc} (nm)	λ_{em} (nm)			
0	325	465	3.5	0.8	4.4
0	345	425	<2	1.1	<2
100	325	465	19	0.8	24
100	345	425	76	1.1	69

* Fluorescence intensity in arbitrary units.
** 0.1 nmole of thioridazine injected.

The clean-up procedure for plasma samples was a simple deproteination technique. An equal volume of acidified methanol was added to the plasma and the mixture was centrifuged. Of the supernatant 200 μl were injected. Fig. 4 shows the chromatograms of a standard solution, a blank plasma and a plasma sample spiked with 20 ng/ml of thioridazine.

The recovery of thioridazine from spiked plasma samples was determined by comparing peak areas with those of standard solutions (Table II). The recoveries are close to 100%. No interfering compounds were found in plasma samples, and the detection limit is the same as with standard solutions, *viz.*, 0.5 ng, corresponding to a plasma concentration of 5 ng/ml. The peak-area variance was $\pm 3.8\%$ ($n = 4$) for a standard solution and $\pm 4.6\%$ ($n = 10$) for a plasma supernatant, both containing 100 ng/ml of thioridazine.

The above investigations were performed with a pulseless pump and a custom-made current source. We also tested a reciprocating pump and replaced the constant-current source by a simple 12 V d.c. supply, with a 100 kΩ resistance in series with the cell. As the voltage drop over the production cell was approximately 2 V, the current was close to 100 μA. The signal-to-noise ratios were unchanged. Therefore, apart from the reagent production cell, the extra investment for the post-column derivatization system can be minimal.

TABLE II

RECOVERY OF THIORIDAZINE FROM PLASMA AFTER DEPROTEINATION WITH AN EQUAL VOLUME OF ACIDIFIED METHANOL

Thioridazine concentration added to plasma (ng/ml)	*Recovery (%) (mean ± S.D., n = 3)*
200	95 ± 2
100	101 ± 6
20	93 ± 9

Other phenothiazines

Preliminary experiments were performed to establish the applicability of the bromine derivatization system to the determination of other phenothiazines. Excitation and emission spectra of these compounds were measured before and after addition of an excess of bromine. Chromatograms were recorded with the fluorescence detector at the optimal wavelength for the underivatized and oxidized compounds, with the generating current on and off, respectively. No effects were made to optimize the separation or the derivatization; the same conditions as for thioridazine were applied. Table III summarizes the results. Changes in the noise level of the detector are not accounted for in this table. Although the increase in the fluorescence intensity is not as large as with thioridazine, the sensitivity for the phenothiazines can be improved 2–14 times with the post-column system. Further improvements may be possible by optimization of the reaction time or generating current for the individual compounds.

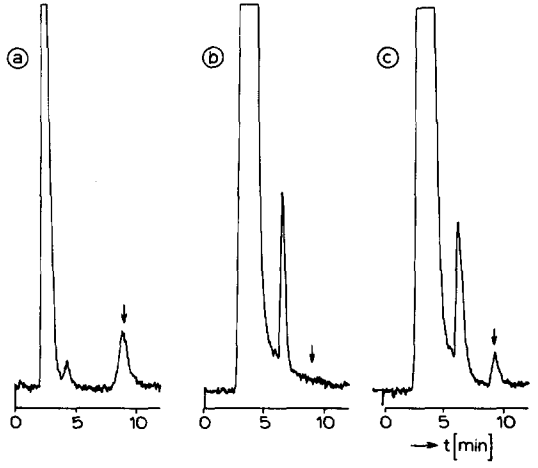
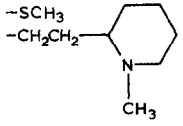
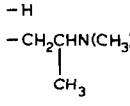
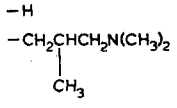
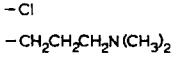
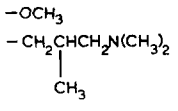
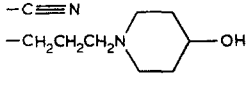


Fig. 4. Chromatograms of (a) standard solution of thioridazine (20 ng/ml); (b) blank plasma, deproteinated; (c) plasma, spiked with 209 ng/ml thioridazine and deproteinated with an equal volume of acidified methanol. For details see text.

TABLE III

FLUORESCENCE INTENSITY OF PHENOTHIAZINES WITH AND WITHOUT POST-COLUMN OXIDATION WITH BROMINE

Phenothiazine	R_1, R_2	*Unoxidized*			*Oxidized*			*Gain*
		λ_{ex}	λ_{em}	*F.I.**	λ_{ex}	λ_{em}	*F.I.**	
Thioridazine		325	465	1.00	345	425	19	19
Promethazine		315	440	0.8	335	375	3.2	4
Alimemazine		315	445	1.4	340	375	16	12
Chlorpromazine		315	440	0.45	340	375	6.2	14
Levomepromazine		315	445	1.8	340	375	3.5	1.9
Propericiazine		340	510	8.6	375	435	16	1.8

* F.I. = fluorescence intensity relative to unoxidized thioridazine.

TABLE IV

COMPARISON OF HPLC DETECTION METHODS FOR THIORIDAZINE

*Separation**	*Detector*	*Post-column oxidation*	*LOD*** (ng)*	*Plasma extraction****	*Lowest plasma concn. (ng/ml)*	*Ref.*
NP	UV, 254 nm	–	2	5	10	5
RP	UV, 254 nm	–	1	2	1	6
RP	LC–ED, +0.9 V	–	1	3	–	7
RP	LC–ED, +0.9 V	–	0.1	4	1	8
NP	Fluorescence, filters	Permanganate/H_2O_2	0.8	2	2	10
RP	Fluorescence, 340/378 nm	Photochemical	0.5	–	50	11
RP	Fluorescence, 345/425 nm	Bromine	0.5	–	5	This work

* NP = normal phase; RP = reversed phase.
** LOD = limit of detection, signal-to-noise ratio = 2.
*** Number of extraction steps in clean-up procedure.

CONCLUSION

In Table IV our detection system is compared with other techniques reported in the literature. Fluorescence detection after post-column oxidation is not superior to other detection modes in respect of absolute detection limits, but the selectivity is such that elaborate sample extraction procedures are not necessary. Other workers determined lower thioridazine concentrations in plasma than we can by concentrating the sample as part of the clean-up procedure. Although it was not attempted, the results indicate that our system is also compatible with sample concentration methods, *e.g.*, pre-column techniques, as the instrumental noise overrules any chemical noise from trace sample compounds. In this way lower plasma concentrations can be determined if necessary. Table IV shows that our inexpensive derivatization method compares well with alternative techniques. Studies on the electrochemical generation of other reagents for optical signal enhancement are under way.

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