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LIQUID CHROMATOGRAPHY OF DEMOXEPAM AND PHENOTHIAZINES USING A POST-COLUMN PHOTOCHEMICAL REACTOR AND FLUORESCENCE DETECTION

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SUMMARY

Upon irradiation with short-wavelength UV light the tranquillizer demoxepam is converted into a highly fluorescent product. This reaction serves as a sensitive and selective means of detection of low levels of demoxepam in, *e.g.*, serum after separation by means of reversed-phase high-performance liquid chromatography. The effect of mobile phase composition and time of irradiation on the intensity of the fluorescence signal has been studied. A residence time of about 2 min in the post-column photochemical reactor is optimal, band broadening being efficiently suppressed (σ_t *ca.* 1 sec) by means of air segmentation. Linear calibration graphs are obtained over a three-orders of magnitude concentration range; the detection limit for demoxepam is about 100 pg.

Further work has demonstrated that detection limits of between 40 and 100 pg can also be obtained for the photoproducts of the phenothiazines fenergan, largactil, levopromazine and nedaltran. The calibration graphs show good linearity and the analysis of spiked serum samples was successful.

INTRODUCTION

In 1947, Brodie *et al.*[1] developed a photochemical procedure for the conversion of the naturally fluorescent chloroquine into another fluorescent product because the excitation wavelength of chloroquine itself did not correspond with a strong emission line of mercury. More recently, it has been shown[2] that this procedure does not bring about an increase in sensitivity. In the 1960s, Goodyear and Jenkinson[3] reported a highly specific and sensitive method for the determination of diethylstilbestrol following UV irradiation with a high-pressure arc lamp. About 10 years later, Wright and Tang[4] elucidated the structure of the final photoproduct of reserpine, the reaction itself having been described[5] as early as 1958. In our laboratory, batch experiments have shown that, under proper conditions, UV irradiation of reserpine causes a 20-fold increase in sensitivity. Increased sensitivity and selectivity were reported by Hadjú and co-workers in the analysis of pharmaceuticals such as clobazam[6] and

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fendosal[7] with short-wavelength UV light. The high potential of combined photochemical–fluorimetric methods has been further demonstrated for the determination of five phenothiazines[8], tamoxifen and two of its metabolites (in human plasma)[9,10] and vitamin K_1[11].

All of the above procedures were batch techniques, in which a fluorescent product is formed upon irradiation. The principle of adapting the photochemical process to detection in dynamic flow systems, and particularly to on-line connection with high-performance liquid chromatography (HPLC), was first applied by Iwaoka and Tannenbaum[12]. They used a post-column reactor system in which N-nitroso compounds were hydrolysed by irradiation with long-wavelength UV light, the nitrite formed being detected via a Griess reaction. The time of irradiation was long and the sensitivity not very high. More promising results were obtained by Twitchett *et al.*[13] who determined cannabinol in body fluids without any previous clean-up with a detection limit of about 0.5 ng. Their experimental set-up was relatively simple and contrasted favourably with, for example, the complicated system described for the analysis of volatile nitrosamines[14]. Lastly, we mention that in some studies chromatographic techniques were used for, *e.g.*, monitoring the photochemical reaction[7] or examining the kinetics of the photochemical conversion[9].

Recent work from our group[15,16] has dealt with aspects such as suitable mobile phase composition and time of irradiation on the intensity of the fluorescence signal, and application of the principle to the determination of clobazam, desmethylclobazam and the phenothiazines thio-, meso- and sulphoridazine in serum and urine. Optimal residence times in the photochemical reactor were found to be around 20–30 sec in methanol–water mixtures, with a sudden breakdown of signal intensity for longer irradiation times. The intensity of the fluorescence signal was more or less independent of the carrier-stream composition, but a severe decrease in signal intensity occurred upon replacing the aqueous–organic solvent mixtures with pure water. Detection limits of the phenothiazines were about 0.5 ng and those of clobazam and desmethylclobazam were below 0.05 ng. As an important conclusion we note that PTFE coils were found to be highly suitable for use in photochemical reactors. The transparency of PTFE towards UV light, which is probably based on a diffuse radiation transfer, was excellent even in the 200–300-nm region. Compared with the much more expensive and less flexible quartz capillaries, similar or even higher signal intensities were observed with the PTFE coils, and the peaks showed better symmetry and less tailing.

In most of the studies on on-line systems mentioned above, the irradiation times were relatively short and band broadening in the post-column reactor consequently was not particularly large. The major exception is the study by Iwaoka and Tannenbaum[12], where the residence time was of the order of several minutes and band broadening was rather severe. In the present study we selected a test compound, demoxepam, with which an irradiation time of a few minutes is required, and studied the possibility of using segmented flow to suppress band broadening. Also, work on an earlier class of model compounds, the phenothiazines, has been continued.

EXPERIMENTAL

Apparatus

The photochemical reactor equipped with a mercury–xenon lamp and air cooling has been described earlier[16]. The reaction spiral was a piece of PTFE capillary of appropriate geometry.

A schematic diagram of the instrumental arrangement of a segmented-flow system has been given elsewhere[17]. Air bubbles were introduced via a Technicon (Tarrytown, NY, U.S.A.) A-10 glass tee-piece by using a Technicon AutoAnalyzer pump. Debubbling occurred in a modified[17] tee-piece of 1/16-in. I.D. to allow insertion of the PTFE capillaries of 1/16-in. O.D. The flow through the debubbler to waste and to the detector cell of the fluorospectrometer was regulated with an Ismatec (Zürich, Switzerland) peristaltic pump (although this can also be done with the Technicon pump).

The chromatographic system consisted of an Altex (Berkeley, CA, U.S.A.) Series 100 pump and a six-port Valco (Houston, TX, U.S.A.) injection valve. Perkin-Elmer (Norwalk, CT, U.S.A.) Model 204A or 3000 (for the low excitation wavelengths) fluorospectrometers were used for detection and the signals were recorded on a Moseley (Pasadena, CA, U.S.A.) Model 2DR-2AM recorder.

Chemicals

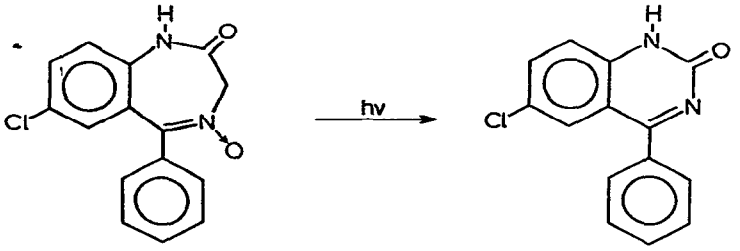
Gifts of demoxepam were obtained from Hoffmann-La Roche (Mijdrecht, The Netherlands) and from R. Jochemsen (State University of Leiden, The Netherlands). The phenothiazines were gifts from the Academic Hospital of the Free University (Amsterdam, The Netherlands). All other chemicals were of normal analytical-grade quality. The solvents used in HPLC were degassed before use.

RESULTS AND DISCUSSION

Demoxepam

Demoxepam (7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one-4-oxide) is known as a metabolite of the anxiolytic chlordiazepoxide[18] and is currently of interest as a tranquillizer in its own right. The best method of analysis developed to date is based on a photochemical reaction. Upon irradiation of the drug (I), which does not display native fluorescence, with UV light[18] a highly fluorescent product is formed which, according to Strojny and De Silva[19], is a quinazolinone (II) (see Fig. 1). The excitation and emission maxima of the quinazolinone in 0.1 *M* sodium hydroxide solution are 380 and 460 nm, respectively. In the laser-induced fluorescence assay, detection limits of 10–20 ng/ml were obtained with good linearity over about two orders of magnitude. We should add that preliminary results from gas chromatographic–mass spectrometric measurements show the chlorine atom to be absent from the main photoproduct formed in our studies, and suggest the presence of a hydroxy group in the 2-position. In other words, the product is not identical with the quinazolinone (II) and, indeed, has different excitation and emission maxima (see below).

With the above method, the determination of demoxepam in biological samples demands elaborate extraction procedures in order to eliminate interferences. We



I II

Fig. 1. Photoconversion of demoxepam according to Strojny and De Silva[19].

have therefore attempted an on-line combination of reversed-phase HPLC with photochemical–fluorimetric detection. As an irradiation time of 30 min[18,19] might cause too much band broadening even in a segmented-flow system, special attention was devoted to the selection of optimal irradiation conditions. These studies were performed under stop-flow and dynamic conditions, sample plugs being introduced into a 0.3-mm I.D. PTFE coil mounted in the photochemical reactor. The advantage of a stop-flow technique at this stage is its convenience, especially as regards varying the residence time in the reactor while using the same conditions as in dynamic operation.

Reaction conditions. The influence of pH on fluorescence yield was studied in methanol–water (1:1), which approximates actual mobile phase conditions for reversed-phase HPLC; 0.05 *M* acetate buffers were used for the pH range 3–5 and 0.05 *M* phosphate buffers for the pH range 6–10.

The relative fluorescence signals obtained after a 1-min irradiation time for various pH values are shown in Fig. 2. A pH 8 buffer gave the best results. Under

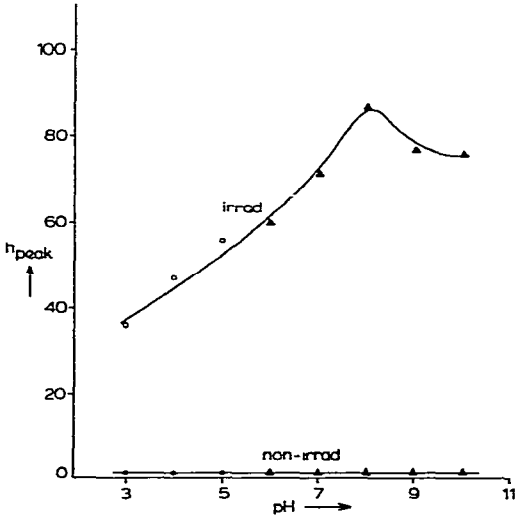


Fig. 2. Dependence of the relative fluorescence intensity of irradiated demoxepam on the pH of the carrier stream. ▲, Methanol–phosphate buffer (1:1); ○, methanol–acetate buffer (1:1).

these conditions, the excitation and emission maxima for uncorrected spectra were 340 and 410 nm, respectively. Two further buffers were tested at pH 8, *viz.*, a Tris and a borate buffer. Their use resulted in a high background fluorescence and consequently they were rejected.

Next, the stop-flow technique was used to determine the optimal time of irradiation under appropriate conditions [pH 8; methanol–water (1:1); reactor temperature 50–60°C]. An irradiation time of 100–110 sec was found to be optimal, with about a 2-fold enhancement of fluorescence intensity due to the presence of the phosphate buffer. Fig. 3 shows the relative fluorescence intensities obtained with an identical experimental set-up but using dynamic conditions. Loop injections of 20 μl were made into the non-segmented carrier stream, with flow-rates varying from 0.1 to 1.0 ml/min, which corresponds to residence times from 220 to 22 sec. An optimal residence time of about 110 sec was again observed. Obviously, results obtained with the convenient stop-flow technique can reliably be transferred to dynamic conditions.

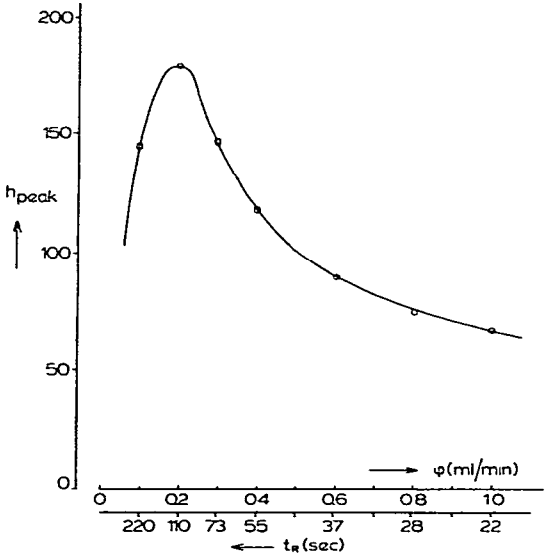


Fig. 3. Dependence of the relative fluorescence intensity of irradiated demoxepam on the residence time in the photochemical reactor. Methanol–pH 8 buffer (1:1).

Here, one should realise[16] that an optimal reaction time is dependent on the design of the photochemical reactor and the geometry of the reaction capillary as well as on lamp characteristics and age. Consequently, with each type of reactor optimal conditions have to be evaluated and periodical tests are recommended for checking lamp performance.

Dynamic reaction system. From the above, it is clear that good fluorescence yields can be obtained for demoxepam with irradiation times considerably shorter than those reported in the literature. One reason may be the use of a high-power lamp source in our studies. Another factor may be differences in reaction medium and reaction conditions plus the use of PTFE instead of Pyrex glass. Unfortunately, even a residence time of about 2 min (instead of 30 min) in the post-column reactor will

cause prohibitively large band broadening in a non-segmented system. In recent papers[17,20] we have shown that for capillaries of, *e.g.*, 0.25 and 0.8 mm I.D. the use of segmented flow systems is to be preferred to non-segmented flow for residence times longer than about 8 and 1 sec, respectively. In other words, segmented flow is mandatory in the present situation. In principle, both solvent and air segmentation are applicable in the photochemical reactor. Unfortunately, at the high temperatures prevalent in the system, segmentation with *n*-hexane caused the formation of tiny droplets which floated around in the carrier stream and caused an inadmissibly high noise level. With air segmentation no such problems were encountered: baseline stability was excellent and phase separation was very efficient, with about 90% of the carrier stream passing through the detector cell of the fluorospectrometer.

For the sake of convenience, a relatively short (3.8 m) and, therefore, wide-bore (1.1 mm I.D.) PTFE capillary, with a coil diameter of 6 cm, served as the reactor spiral. Such a capillary gives a low back-pressure and can easily be accommodated around the lamp source. It yielded the optimal residence time at a carrier-stream flow-rate of 1 ml/min; the air stream was maintained at 0.7 ml/min. [For completeness, it was experimentally verified that the optimal residence time in the segmented system was equal to that in the (stop-flow) non-segmented system.] As for band broadening in the post-column reactor system, previous studies[17] have demonstrated unequivocally that the major contribution is attributable to the phase separator and tee-piece, the contribution of the capillary itself being negligibly small. In the present work, the variance contribution, σ_t^2, of the miniature phase separator (plus tee-piece) was found to be 210 $\pm$ 20 μl^2 in both air and solvent segmentation, which corresponds to σ_t = 1 sec at the prevalent flow-rates. These values agree very satisfactorily with our earlier results[17] (150 μl^2).

Application. HPLC of demoxepam was carried out on a 15 cm × 4.6 mm I.D. stainless-steel 5-μm LC-18 Supelcosil (Supelco, Bellefonte, PA, U.S.A.) reversed-phase column, with methanol–0.1 *M* phosphate buffer (pH 8) (3:2) as the mobile phase. Using the photochemical reactor under the conditions stated above, the detection limit for demoxepam, at a signal-to-noise ratio of 2:1, was 100 pg, which is about 10-fold better than with UV detection (1 ng at 237 nm). Linearity was good (r = 0.999) over a concentration range of three orders of magnitude (10 μg/ml–10 ng/ml). The repeatibility of the method in serum was $\pm$ 2.3% (n = 7).

As an application, human serum was spiked with 8 μg/ml of demoxepam. After the addition of 1 ml of methanol and shaking, the mixture was centrifuged for about 7 min at 7000 *g*, and 20-μl aliquots were injected on to the analytical column. The chromatogram obtained is shown in Fig. 4; the recovery of demoxepam was 95%. With a blank serum sample no peak was observed in the t_R = 8–10 min region.

Phenothiazines

The separation of thio-, meso- and sulphoridazine on an apolar chemically bonded stationary phase with mixtures of methanol and aqueous acetate solutions containing a small amount of peroxodisulphate as eluent has been reported[16]. In the present work five further phenothiazines were tested (*cf.*, Table I), all of which display weak native fluorescence with excitation maxima at around 260 and 305 nm, and an emission maximum at about 450 nm. With the exception of thiodiphenylamine, these compounds are converted into highly fluorescent photoproducts upon irradia-

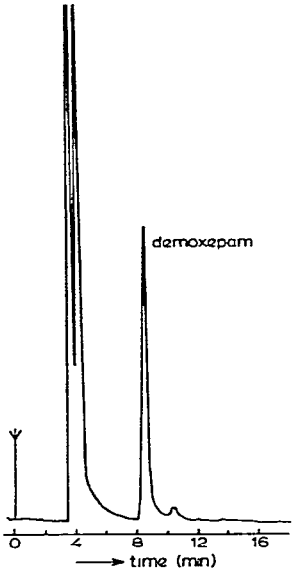
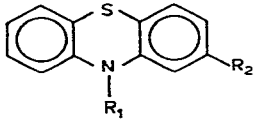


Fig. 4. HPLC trace of human serum spiked with 8 ppm of demoxepam. Detection conditions: gain, 3; sensitivity range, 1; 2 mV full scale.

TABLE I

STRUCTURES AND DETECTION LIMITS OF THE PHENOTHIAZINES TESTED



Compound	R_1	R_2	*Detection limit (ng)**
Fenergan	$-CH_2-CH(CH_3)-N(CH_3)_2$	$-H$	100
Largactil	$-CH_2-CH_2-CH_2-N(CH_3)_2$	$-Cl$	100
Levopromazine	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	$-OCH_3$	40
Nedaltran	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	$-H$	50
Thiodiphenylamine	$-H$	$-H$	—

* For experimental conditions, see text.

tion with short-wavelength UV light. Irradiation was carried out under dynamic conditions in mixtures of methanol and aqueous sodium acetate; the irradiation times were 35, 55, 70 and 110 sec.

The photoproducts of fenergan, largactil, levopromazine and nedaltran were found to exhibit two main excitation maxima at around 235 and 290 nm. In both instances, the emission maximum was in the 360–380 nm region. Excitation of the photoproduct with 235-nm light led, however, to fluorescence signals which generally were about 5-fold higher than those obtained upon excitation with 290-nm light. Plots of fluorescence intensity *versus* irradiation time in the photochemical reactor displayed fairly flat maxima for fenergan and nedaltran. For all four compounds, the signal intensity remained essentially the same with irradiation times between 35 and 70 sec. Under optimal conditions, *i.e.*, with $\lambda_{ex} = 235$ nm, results obtained in the absence and presence of peroxodisulphate showed a less than 2-fold difference in fluorescence intensity. The sensitivity increased in the order largactil < fenergan < nedaltran < levopromazine, with detection limits recorded for a 35-sec residence time in the reactor and in the absence of peroxodisulphate being in the 40–100-pg range (see Table I).

For nedaltran and largactil, calibration graphs were constructed under dynamic conditions, using 50-μl injections. Good linearity was observed ($r = 0.9986$ and 0.9974, respectively) for concentrations of between 2 and 100 ng/ml. These two phenothiazines were also used to spike serum. After deproteination with methanol, HPLC was carried out on a 10 cm × 4.6 mm I.D. Polygosil 60-10 CN (Macherey, Nagel & Co, Düren, G.F.R.) column with methanol–aqueous sodium acetate (4:1) as the mobile phase. With 50-μl injections, a detection limit of about 10 ng/ml of phenothiazine in serum could easily be obtained.

CONCLUSION

Combining the results presented above with those reported in our previous papers[15,16], one can draw the following conclusions.

The use of a post-column photochemical reactor opens the route to the rapid, sensitive and selective determination of an ever increasing number of pharmaceuticals. The detection limits of the fluorescent photoproducts of demoxepam, clobazam, desmethylclobazam and seven phenothiazines typically are between 0.05 and 0.5 ng. Current research indicates that photoconversion of reserpine and vitamins K_1 and K_3 probably will also lead to sensitive methods of analysis.

Optimal irradiation times, in methanol–water mixtures, are generally between 20 and 35 sec. If a longer residence time in the photochemical reactor is required (demoxepam; about 2 min), air segmentation is a feasible approach for the efficient suppression of band broadening. For most of the compounds tested, the potential of the combined photochemical–fluorimetric method has been demonstrated by the analysis of spiked serum and urine samples with a minimum of sample handling.

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REFERENCES

1 B. B. Brodie, S. Udenfriend, W. Dill and T. Cheukin, *J. Biol. Chem.*, 168 (1947) 319.
2 R. J. Lukasiewicz and J. M. Fitzgerald, *Appl. Spectrosc.*, 28 (1974) 151.
3 J. M. Goodyear and H. R. Jenkinson, *Anal. Chem.*, 32 (1960) 1203 and 33 (1961) 853.
4 G. E. Wright and T. Y. Tang, *J. Pharm. Sci.*, 61 (1972) 299.
5 J. Bayer, *Pharmazie*, 13 (1958) 468.
6 P. Hadjú and D. Damm, *Arzneim.-Forsch.*, 26 (1976) 2141.
7 H. M. Hill, J. Chamberlain, P. Hadjú and D. Damm, *Biophys. Drug Dispos.*, 1 (1979) 1.
8 V. R. White, C. S. Frings, J. E. Villafranca and J. M. Fitzgerald, *Anal. Chem.*, 48 (1976) 1314.
9 D. W. Mendenhall, H. Kobayashi, F. M. L. Shih, L. A. Sternson, T. Higuchi and C. Fabian, *Clin. Chem.*, 24 (1978) 1518.
10 Y. Golander and L. A. Sternson, *J. Chromatogr.*, 181 (1980) 41.
11 J. J. Aaron, *Methods Enzymol.*, 67 (1980) 140.
12 W. Iwaoka and S. R. Tannenbaum, *I.A.R.C. Sci. Publ.*, 14 (1976) 51.
13 P. J. Twitchett, P. L. Williams and A. C. Moffat, *J. Chromatogr.*, 149 (1978) 683.
14 B. G. Snider and D. C. Johnson, *Anal. Chim. Acta*, 106 (1979) 1.
15 A. H. M. T. Scholten, U. A. Th. Brinkman and R. W. Frei, *Anal. Chim. Acta*, 114 (1980) 137.
16 A. H. M. T. Scholten, P. L. M. Welling, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.*, 199 (1980) 239.
17 A. H. M. T. Scholten, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.*, 205 (1981) 229.
18 M. A. Schwartz and E. J. Postma, *J. Pharm. Sci.*, 55 (1966) 1358.
19 N. Strojny and J. A. F. de Silva, *Anal. Chem.*, 52 (1980) 1554.
20 A. H. M. T. Scholten, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, submitted for publication.