Journal of Chromatography, 252 (1982) 288–292
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,291

Note

Post-column air-segmentation reactor for the fluorometric detection of reserpine by liquid chromatography

J. R. LANG, I. L. HONIGBERG and J. T. STEWART*
Department of Medicinal Chemistry, School of Pharmacy, The University of Georgia, Athens, GA 30602 (U.S.A.)
(Received August 16th, 1982)

Reserpine is an important antihypertensive agent widely used in medical practice. Because of its low therapeutic dose, a sensitive analytical method to detect and quantify nanogram concentrations of the drug in biological samples is greatly needed. Presently available methods for reserpine include UV or VIS spectrophotometry[1], fluorescence[2], and high-performance liquid chromatography (HPLC)[3]. Even though the fluorometric methods are the most sensitive, none of the procedures were designed to assay reserpine in biological samples. Furthermore, many of the methods involved time-consuming solvent extraction and derivatization steps.

Reserpine has sufficient native fluorescence to allow direct analysis when present in milligram concentrations. However, in order to measure reserpine in the nanogram range, the drug must be converted to a more intense fluorophore via reaction of the drug with either light[4], acids[5], and/or oxidizing agents[6]. The fluorophore generated with any of these methods is thought to be 3,4-dehydroreserpine, although other reserpine derivatives can not be excluded since the reactions are complex.

In recent years, a new analytical technique based on the use of HPLC and post-column derivatization of the solute has been reported. The derivatization process usually involves forming the fluorophore in a post-column flow-through reactor by mixing appropriate chemicals with a drug in the mobile phase prior to detection.

The types of post-column reactors which have been utilized in conjunction with HPLC include tubular, bed, and air segmentation reactors. The development of post-column reactors during the past few years can be attributed to the lack of HPLC detectors with sufficient sensitivity and selectivity for certain analytical problems. The central problem with any post-column reactor is to avoid excessive dead volume which can lead to a loss of chromatographic resolution[7].

An important advantage of a post-column reactor is that the reaction does not have to go to completion or give well defined derivatives as long as the results are reproducible. The most serious disadvantage is the interdependence of the HPLC mobile phase and reaction medium.

In this paper, an air-segmentation post-column reactor has been designed which will allow the fluorometric measurement of reserpine in the 10–100 ng/ml range with good accuracy and precision. In the reactor, sulfuric acid and sodium nitrite are

0021-9673/82/0000–0000/$02.75 © 1982 Elsevier Scientific Publishing Company

mixed to yield nitrous acid which is used as the oxidizing agent for the conversion of reserpine to 3,4-dehydroreserpine in the mobile phase[5].

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model ALC-201 liquid chromatograph equipped with a Model U-6K injector, Model 6000A pump, a Schoeffel (Acton, MA, U.S.A.) Model FS-970 fluorometer set at an excitation wavelength of 395 nm and emission filter >470 nm, range, 0.5 μA, and sensitivity adjust, 5.4 units, and a post-column reactor (Fig. 1).

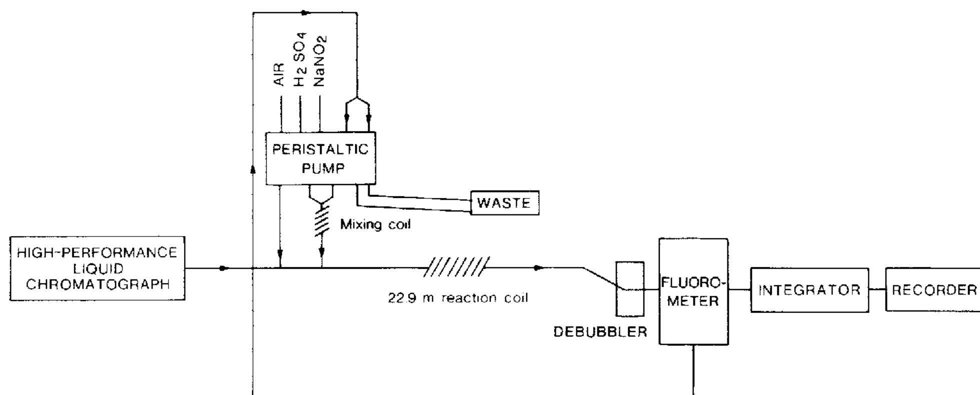


Fig. 1. Schematic of the HPLC post-column reactor. Flow-rates: mobile phase, 1 ml/min; air, 0.3 ml/min; sulfuric acid, 0.44 ml/min; sodium nitrite, 0.44 ml/min; waste, 1.67 ml/min; debubbler, 0.67 ml/min.

The peak area for reserpine was integrated with a Columbia Scientific Industries (Austin, TX, U.S.A.) digital integrator, Model CRS-204. A Fisher (Pittsburgh, PA, U.S.A.) Series 5000 recorder was used for tracing the detectable peaks from the post-column detector system.

Post-column reaction system

A Brinkmann (Westbury, NY, U.S.A.) peristaltic pump, Model 131900, was used to deliver chemical reagents and air to the mobile phase at the flow-rates shown in Fig. 1. The sulfuric acid and sodium nitrite reagents were mixed in a 14-turn 4-in. Technicon (Tarrytown, NY, U.S.A.) mixing coil (Part. No. 116-0127-02) prior to addition into the mobile phase via a Technicon fitting (Part No. 195-G183-01). Coiled borosilicate glass capillary tubing (25 m $\times$ 1.0 mm I.D., Universal Scientific, Atlanta, GA, U.S.A., No. 25-GCB-UM) served as the reaction coil for the mobile phase, drug, and chemical reagents. Debubbling of the mobile phase was achieved using a Technicon debubbler-tee (Part No. 195-G172-02). The flow from the fluorometer cell to waste was further regulated by the peristaltic pump. The fluorometric cell was modified by Schoeffel with 0.020 in. I.D. inlet and outlet tubing to aid in decreasing backpressure associated with the increased flow-volume through the reactor system. The peristaltic pump tubing was either Technicon acid-flex or SMA depending upon the

chemical reagent. Tygon tubing was used for carrying waste solvents and for connecting mixing coils and capillary reaction coils in the reactor.

Chemicals and reagents

Reserpine and methyl reserpate powders were obtained from Ciba-Geigy Pharmaceuticals (Summit, NJ, U.S.A.). All other chemicals were of analytical-grade quality. The solvents used in the preparation of the mobile phase were HPLC grade. The mobile phase was degassed immediately before use.

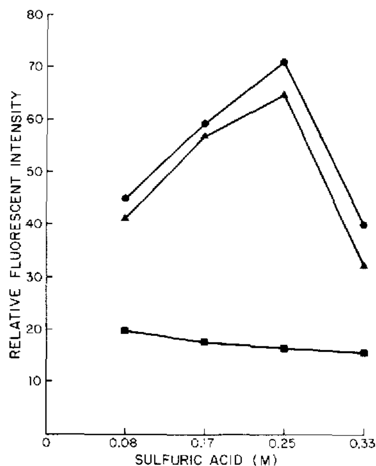
A reserpine stock solution (1 mg/10 ml) was prepared by dissolving a weighed quantity of the powder in absolute methanol. Aliquots of this solution were then used to prepare reserpine solutions in the 10–100 ng/ml range. Injections of these solutions into the liquid chromatograph were performed with the aid of a 25-μl syringe (Hamilton, Reno, NV, U.S.A.). Aqueous stock solutions of sulfuric acid (0.25 M) and sodium nitrite ($2.42 \cdot 10^{-5}$ M) were also prepared for use in the post-column reactor. One drop of Triton X-100 detergent was added to the sodium nitrite solution to decrease back-pressure in the reactor mixing coils.

RESULTS AND DISCUSSION

The objective of this research was to develop a sensitive HPLC post-column fluorescence derivatization reactor that would be useful in the detection and quantitation of ng/ml levels of reserpine. Fluorescence derivatization procedures previously reported in the literature for reserpine used such reagents as *p*-toluenesulfonic acid[2], vanadium pentoxide[6], and nitrous acid[5] to induce fluorophore formation. Both toluenesulfonic acid and vanadium pentoxide procedures were reported to give good accuracy, precision, and sensitivity data. However, they involved reaction of the respective reagent with reserpine in a concentrated acid medium. A pre-column derivatization approach using vanadium pentoxide has been applied to the HPLC analysis of reserpine, but it was not shown if the method would distinguish between reserpine and its major metabolite, methyl reserpate[8]. A more suitable analytical approach would involve a separation step followed by post-column derivatization. Thus, the nitrous acid method was selected as the derivatization reaction for this work since it would not only provide ng/ml sensitivity, but it would utilize reagents that were compatible with aqueous–organic mobile phases and the reaction would be essentially complete within 30 min at ambient temperature.

The chromatographic parameters necessary for the HPLC separation of reserpine and methyl reserpate were investigated so that a suitable mobile phase could be utilized in the further development of the post-column reactor. It was found that a mixture of acetonitrile–0.005 M sodium dihydrogen phosphate buffer (70:30) adjusted to pH 6 with sodium hydroxide, would separate reserpine and methyl reserpate on a bonded phase phenyl column at a flow-rate of 1.0 ml/min. The capacity factors (k') for reserpine and methyl reserpate was 3.3 and 1.7, respectively.

A study of the fluorescence intensity of the reserpine reaction mixture as a function of reagent (sulfuric acid and sodium nitrite) concentration was performed in a spectrophotofluorometer to determine the respective concentration of each reagent necessary to produce maximum fluorescence of the reserpine fluorophore. It was found that the nitrous acid produced *in situ* from a mixture of 0.25 M sulfuric acid



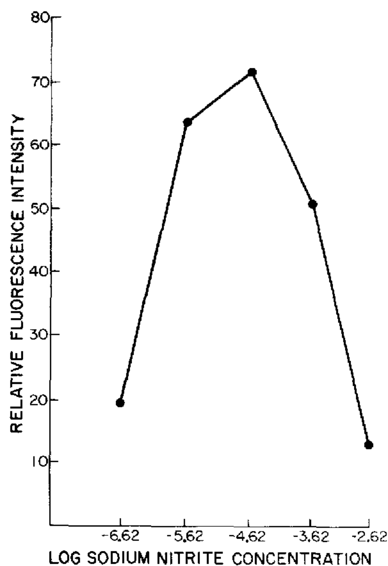


Fig. 2. Fluorescence intensity of the reserpine fluorophore as a function of sulfuric acid concentration. Reserpine concentration was $2.74 \cdot 10^{-8}$ *M*. Legend: ●, $2.42 \cdot 10^{-5}$ *M* sodium nitrite; ▲, $2.42 \cdot 10^{-4}$ *M* sodium nitrite; ■, $2.42 \cdot 10^{-3}$ *M* sodium nitrite.

Fig. 3. Fluorescence intensity of the reserpine fluorophore as a function of sodium nitrite concentration. Reserpine and sulfuric acid concentrations were $2.74 \cdot 10^{-8}$ and 0.25 *M*, respectively.

and $2.42 \cdot 10^{-5}$ *M* sodium nitrite would react with reserpine to give optimum fluorescence within 30 min (Figs. 2 and 3). As initially reported by Haycock *et al.*[5], it was also observed that increases in sodium nitrite concentration resulted in quenching of fluorescence of the fluorophore. The excitation and emission wavelengths for the fluorophore in the nitrous acid solution were found to be 395 and 505 nm, respectively. These wavelengths did not change appreciably when aliquots of the acidic fluorophore solution were added to the HPLC mobile phase.

Based on a predicted reaction time of greater than 5 min, a post-column reactor system was designed based on the air-segmentation principle[7]. As shown in Fig. 1, a peristaltic pump was employed to deliver fixed flow-rates of air and nitrous acid-producing reagents into the mobile phase. Initially, it was necessary to determine the optimum reaction time required for maximum fluorescence intensity using the reactor. This was studied by using different lengths of glass capillary coils (1.0 mm I.D.) up to a maximum coil length of 39.6 m or a 15 min residence time. It was found that maximum fluorescence was achieved using a coil length of approximately 22.9 m or a residence time of approximately 6 min (Fig. 4). Further increases in capillary coil length did not cause a significant increase in fluorescence intensity. It was also apparent from examination of the fluorophore peaks that band broadening also reached a plateau around 6 min and was not affected by further increases in coil length. A preliminary investigation using a non-segmented version of the above described reactor system showed that there was a diminution of fluorophore peak height and increased band broadening compared to the air-segmentation reactor.

Linearity of drug concentration with fluorescence intensity was then investi-

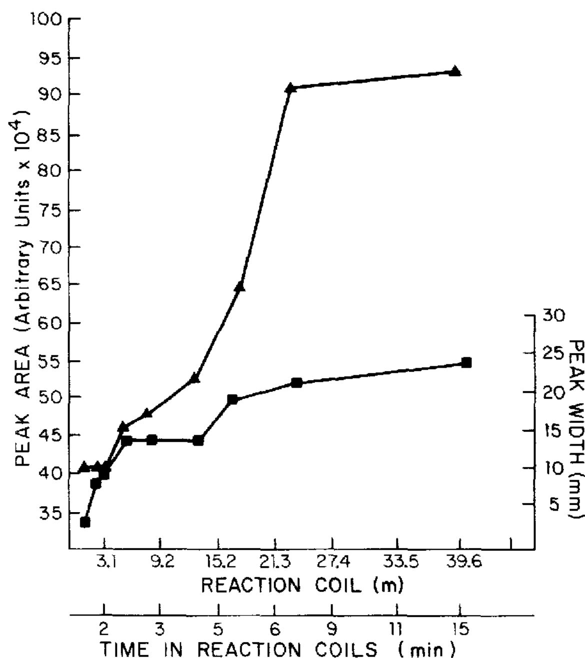


Fig. 4. Effect of reaction coil length on fluorescent intensity (▲) and peak width (■) of reserpine fluorophore.

gated. Linear regression analysis of drug concentration *versus* mean peak area data showed that the best fit was obtained in the 10–100 ng/ml range for reserpine. Using 20-μl injections of 10, 40, 60, and 100 ng/ml reserpine solutions for preparation of a standard curve, the slope, intercept and correlation coefficient (r) were calculated to be 767.5, −816.1, and 0.9993 ($n = 12$), respectively. Spiked aqueous samples containing 20 and 80 ng/ml of reserpine were then analyzed using the postcolumn reactor. The constants (slope and intercept) from the standard curve were used to solve for drug concentration in the spiked samples. It was calculated that the 20 and 80 ng/ml samples contained 20.85 ± 1.34 ($n = 3$) and 78.73 ± 4.57 ($n = 3$) ng/ml, respectively. Accuracy and precision were found to be 4.25 and 6.44%, respectively, for the 20 ng/ml samples and 1.59 and 5.80%, respectively, for the 80 ng/ml sample. Sensitivity of the post-column reaction system for reserpine was found to be 200 pg (signal-to-noise ratio = 2).

REFERENCES

1 B. C. Bose and R. Vliayvargiya, *J. Pharm. Pharmacol.*, 11 (1959) 456.
2 I. M. Jakovijecic, J. M. Fose and N. R. Kunzel, *Anal. Chem.*, 31 (1962) 410.
3 I. L. Honigberg, J. T. Stewart, A. D. Smith, R. D. Plunkett and D. W. Hester, *J. Pharm. Sci.*, 63 (1974) 1962.
4 G. E. Wright and T. Y. Tany, *J. Pharm. Sci.*, 61 (1972) 299.
5 R. P. Haycock, P. B. Sheth, T. Higuchi, W. J. Mader and G. J. Papariello, *J. Pharm. Sci.*, 55 (1966) 826.
6 T. Urbanyi and H. Stober, *J. Pharm. Sci.*, 59 (1970) 1824.
7 R. W. Frei, *Proc. Anal. Div. Chem. Soc.*, 16 (1979) 289.
8 R. Sams, *Anal. Lett.*, B11(9) (1978) 697.