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Note

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

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An air-segmentation post-column reactor compatible with a high-performance liquid chromatographic (HPLC) system and useful for the fluorometric detection of reserpine, an important antihypertensive agent, was recently developed[1]. The reactor employed a 6-min nitrous acid (produced *in situ* by mixing sodium nitrite and sulfuric acid) derivatization reaction which oxidized reserpine to the proposed 3,4-dehydroreserpine fluorophore in the mobile phase. Sensitivity of the method for reserpine was 200 pg (signal-to-noise ratio, S/N = 2).

The adaptation of the UV photochemical process to drug analysis in dynamic flow systems such as HPLC has been reported by several investigators[2–7]. Since it has been reported that UV irradiation *versus* native fluorescence of reserpine results in a 20-fold increase in spectrophotofluorometric sensitivity[7–9], it was decided to investigate a post-column photochemical reactor for the fluorometric detection of reserpine by liquid chromatography. The advantages of the photochemical reactor might include a faster fluorophore development time than our 6-min reactor. This should result in decreased band broadening with a concurrent increase in sensitivity due to minimal residence time of the fluorophore in the post-column reactor.

In this paper, an HPLC post-column air-segmentation photochemical reactor for the fluorometric analysis of reserpine is reported. The reactor utilizes a 1-min mixing and reaction time of reserpine and nitrous acid prior to a 1-min UV irradiation time. It is an improvement over our previous non-photochemical reactor design in that it provides a 3-fold faster fluorophore development time along with a 2–3-fold concurrent decrease in band broadening of the reserpine peak. This improves the sensitivity of the fluorometric method to 80 pg (S/N = 2) compared to the previously reported 200 pg.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model ALC-201 liquid chromatograph equipped with a Model U6K 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 cutoff filter >470 nm, range 0.5 μA, and

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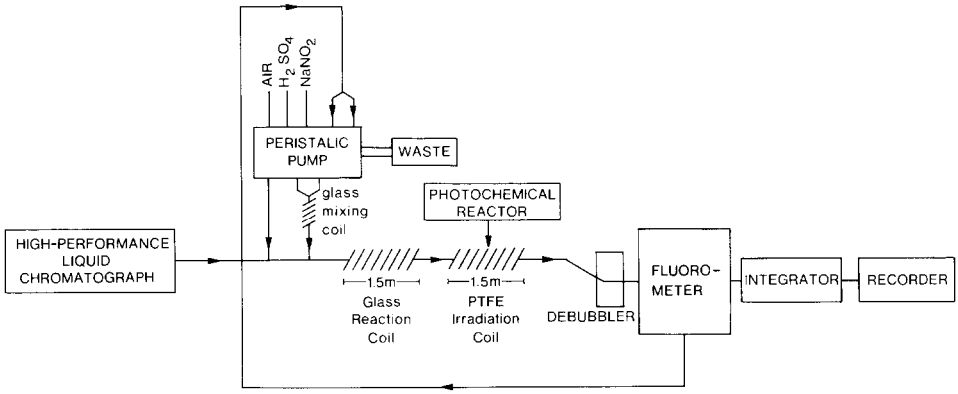

Fig. 1. Schematic of the HPLC post-column air-segmentation photochemical reactor. Flow-rates: mobile phase, 1 ml/min; air, 0.3 ml/min; sulfuric acid, 0.44 ml/min; sodium nitrite, 0.44 ml/min; waste, 1.67 ml/min; debubbler, 0.67 ml/min.

sensitivity adjust, 5.4 units, and a post-column reactor (Fig. 1).

The chromatographic 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.

A Perkin-Elmer (Norwalk, CT, U.S.A.) Model MPF-4 spectrophotofluorometer equipped with a corrected spectra accessory was also utilized in certain preliminary fluorescence experiments.

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 (1.5 m × 1.0 mm, Universal Scientific, Atlanta, GA, U.S.A., No. 25-GCB-UM) served as the reaction coil for the mobile phase, drug, and chemical reagents. The irradiation coil was PTFE tubing (1.5 m × 1.0 mm I.D., 18 lightweight, Penntube Plastics, Clifton Heights, PA, U.S.A.) wrapped around the quartz well of UV light source assembly (Model 679A36, Ace Glass, Vincland, NJ, U.S.A.) which was maintained at 25°C with circulating tapwater (see Fig. 2). The air-segmented post-column mobile phase was debubbled with a Technicon debubbler-tee (Part No. 195-G172-02). The output from the fluorometer cell to waste was further regulated by the peristaltic pump. The inlet and outlet tubing to the Schoeffel fluorometric cell was increased to 0.02 in. I.D. to decrease back pressure. The peristaltic pump tubing was either Technicon acid or organic resistant depending upon the chemical reagent. Tygon tubing was used for transporting waste solvents and for connecting the glass mixing coil, glass capillary reaction coil, and PTFE coils in the reactor.

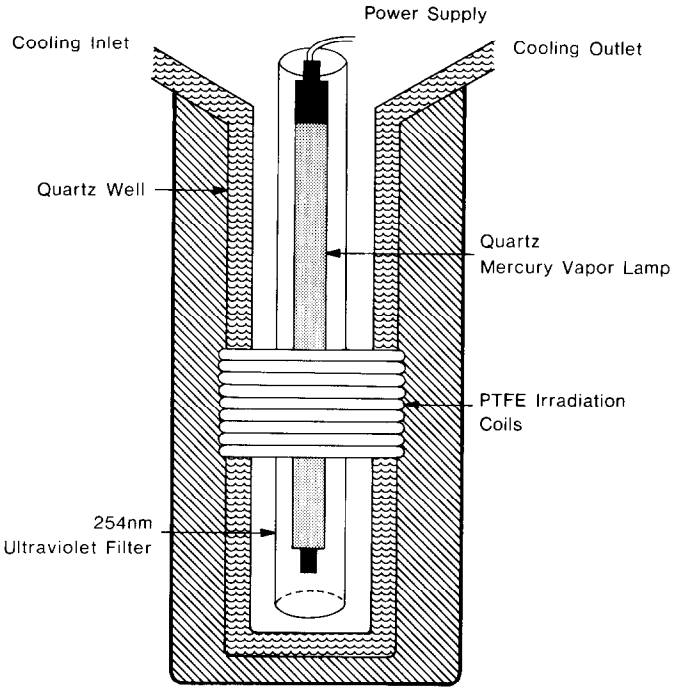

Fig. 2. Schematic of photochemical irradiation coil apparatus.

Chemicals and reagents

Reserpine powder was 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 aid in decreasing back pressure in the reactor mixing coils.

RESULTS AND DISCUSSION

The aim of this study was to design and evaluate a post-column photochemical reactor for the analysis of trace levels of reserpine. In a previous report from this laboratory[1], an air segmentation reactor was developed that utilized a 6-min post-column reaction time of nitrous acid and reserpine to create a highly intensely fluorescent reserpine fluorophore. While the reactor allowed the determination of reserpine in the low ng/ml range with good accuracy and precision, UV irradiation of

the reaction mixture in the mobile phase might be expected to further increase reserpine sensitivity by decreasing band broadening due to a faster fluorophore development time.

The chromatographic parameters utilized for reserpine in our initial report were also employed in this study[1]. The mobile phase was acetonitrile–0.05 *M* sodium dihydrogen phosphate buffer (adjusted to pH 6 with sodium hydroxide) (70:30). At a flow-rate of 1.0 ml/min, reserpine and methyl reserpate, its major metabolite, can be separated on a bonded phase phenyl column with capacity factors (k') of 3.3 and 1.7, respectively.

In order to design a suitable photochemical reactor, the effect of UV irradiation on the fluorescence intensity of the reserpine fluorophore as a function of time was studied using a spectrophotofluorometer. The fluorophore was formed in a quartz cuvette by mixing the appropriate quantities of sodium nitrite and sulfuric acid solutions with 10–100 ng/ml levels of reserpine for 30 sec and irradiating the solution in the cuvette for 1–5 min with a mercury vapor lamp adapted with a 254-nm cutoff filter (see Fig. 2). Fig. 3 shows the effect on reserpine fluorescence as irradiation time increased. Maximum fluorescence intensity occurred when irradiation time was restricted to 1 min. The decreased fluorescence readings obtained beyond the 1-min irradiation time is probably due to decomposition or rearrangement of the reserpine fluorophore by the UV light.

It was then necessary to determine if additional mixing of reserpine and nitrous acid prior to the 1 min irradiation step would result in a further increase in reserpine fluorescence. Drug samples mixed in a cuvette with sodium nitrite and sulfuric acid solutions for 0.5-, 1-, 2-, and 5-min intervals were irradiated for 1 min with the UV light source. A 2-min total reaction and irradiation time was selected as the best combination for maximum fluorophore formation, since only slight increases in fluorescence intensity were obtained beyond that point (see Fig. 4).

The post-column photochemical reactor system based on a 2-min reaction–

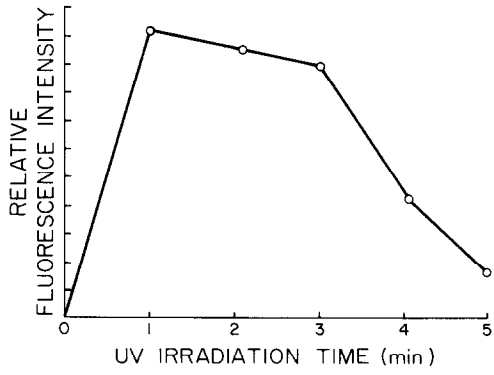

Fig. 3. Fluorescence intensity of the reserpine fluorophore as a function of UV irradiation time. Reserpine concentration was $1.6 \cdot 10^{-6}$ *M*.

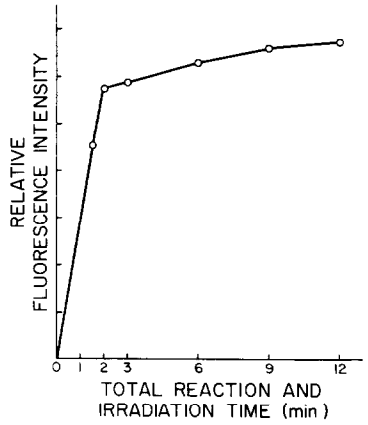

Fig. 4. Fluorescence intensity of the reserpine fluorophore as a function of reaction time including 1 min of UV irradiation. Reserpine concentration was $1.6 \cdot 10^{-6}$ *M*.

irradiation time and utilizing the principle of air-segmentation was then constructed. 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 containing reserpine. The drug and nitrous acid were mixed in a 1.5 m length of glass capillary coils (1.0 mm I.D.) followed by UV irradiation in a 1.5 m length of PTFE tubing (1.0 mm I.D.) coiled around the UV light assembly (see Fig. 2). A 254-nm cutoff filter was employed on the light source so that only wavelengths of mercury >254 nm were used to irradiate the reserpine fluorophore. The mobile phase containing the fluorophore was then passed through a fluorometric cell where the fluorescence was measured using an excitation wavelength of 395 nm and emission >470 nm. It was apparent from examination of the chromatographic peaks that band broadening was minimized in this photochemical reactor (5–10 mm) compared to our previous chemical derivatization reactor (20–25 mm)[1].

Linearity of drug concentration with fluorescent intensity was then evaluated. 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 and 100 ng/ml reserpine solutions for preparation of a standard curve, the slope, intercept, and correlation coefficient (r) were calculated to be 706.44, -165.1, and 0.9995 ($n = 9$), respectively. Aqueous samples containing 20 and 80 ng/ml of reserpine were then analyzed using the photochemical reactor and the constants (slope and intercept) from the standard curve were used to solve for drug concentration. It was calculated that the 20 and 80 ng/ml samples contained 19.34 $\pm$ 1.55 ($n = 3$) and 82.03 $\pm$ 3.12 ($n = 3$) ng/ml, respectively. Percent error and precision were found to be 3.3 and 8.0%, respectively, for the 20 ng/ml samples and 2.5 and 3.8%, respectively, for the 80 ng/ml samples. Sensitivity of the post-column photochemical reactor system for reserpine was found to be 80 pg (S/N = 2).

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