Fluorescence Stability of Human Albumin Solutions

D. L. PARSONS

Received June 24, 1981, from the *Department of Pharmaceutical Sciences, College of Pharmacy, University of Arizona, Tucson, AZ 85721.* Accepted for publication July 23, 1981. Present address: School of Pharmacy, Auburn University, Auburn, AL 36849.

Abstract □ The fluorescence stability on aging of three types of human albumin solutions was investigated. For the first 8 hr after preparation, the magnitude of the fluorescence of the albumin solutions examined did not undergo the large fluctuations with time that had been previously observed for bovine albumin solutions. The fluorescence intensity of each of the human albumin solutions examined appeared to undergo a small but consistent decrease with time for the length of each study. Based on these results, the practice of preparing human albumin solutions several hours prior to use simply to achieve fluorescence stability is unnecessary.

Keyphrases □ Fluorescence, stability—human albumin solutions □ Human albumin—evaluation of fluorescence stability □ Stability, fluorescence—human albumin solutions

The quenching of the intrinsic fluorescence of serum albumin and other proteins upon the binding of a ligand has been used for many years to study ligand–macromolecule interactions (1). In such studies it is important to correct the collected data for variations in fluorescence intensity that are due to factors other than the ligand–macromolecule interaction, such as the inner filter effect. Another factor that may affect the magnitude of the intrinsic fluorescence of a protein molecule is the age of the protein solution. The magnitude of the fluorescence of freshly prepared solutions of crystallized bovine serum albumin at pH 7.45 varies regularly as a function of time until the solutions are ~5-hr old (2). Fluorescence values for one bovine serum albumin solution varied from an initial value of ~72–65 after 2 hr, and to 85 after 3 hr before stabilizing at a value of 78 after 5 hr. To avoid these time-dependent fluctuations in fluorescence intensity, solutions of bovine serum albumin were not used for ligand binding studies until they were 5–6-hr old. It was suggested that these fluctuations in fluorescence intensity may be the result of a slow obtainment of equilibrium among various conformational species of this protein or to hydration of the bovine serum albumin molecules followed by a rearrangement to native structures. Similar events, which were termed conformational oscillations, were observed for other protein macromolecules (3).

BACKGROUND

In most ligand binding studies utilizing quenching of intrinsic fluorescence of human serum albumin, the possible effect of time was not discussed. However, the results of the study of the fluorescence stability of bovine serum albumin (2) have been applied to studies utilizing human serum albumin. In one binding study, solutions of both bovine and human serum albumin were not used for fluorescence quenching studies until the solutions were at least 6-hr old (4). In another study, measurements of the fluorescence of human serum albumin solutions were not taken until the solutions were 2–3-hr old (5), even though this is the time period when bovine serum albumin appears to undergo the greatest fluctuations in fluorescence (2). A different approach to the problem of fluorescence stability was used in another binding study (6). A reference solution of human albumin was used to correct the fluorometer for drifts in instrument response and for variations in intrinsic protein fluorescence during the titration of a second cell of human albumin with ligand. The magnitude of any variation observed in the fluorescence of these albumin solutions with time was not given. Of course, any variation observed in the magnitude of fluorescence in this study could be the result of instrument drift and/or photodecomposition rather than, or in addition to, the variation in intrinsic albumin fluorescence. In addition, if serum albumin undergoes large fluctuations in fluorescence with time as a result of a time-dependent obtainment of an equilibrium conformation, the binding of ligand could also be time dependent.

Since most ligand binding studies are currently conducted utilizing human, rather than bovine, serum albumin, the stability of the magnitude of fluorescence of human albumin solutions was investigated. The fluorescence stability of three types of human albumin was investigated, since any observed fluctuations in fluorescence could be the result of a time-dependent obtainment of an equilibrium conformation. The time required to obtain this equilibrium conformation could possibly depend on the method used to purify the protein.

EXPERIMENTAL

Materials—Fraction V[1], crystalline[2], and fatty acid-free fraction V[3] human albumin, and propranolol hydrochloride[4] were used as received. Human albumin solutions were prepared using 0.1 *M* phosphate buffer (pH 7.4). The buffer was prepared from analytical grade dibasic sodium phosphate[5], monobasic potassium phosphate[5], and water purified by reverse osmosis followed by distillation.

Methods—Measurements of the magnitude of fluorescence of 2×10^{-6} *M* solutions of human albumin were made at various time intervals after preparation on a spectrofluorometer[6] at excitation and emission wavelengths of 283 and 351 nm, respectively. Excitation and emission slit widths of 2 and 10 nm, respectively, were used for all fluorescence measurements. The fluorescence sample cells had a pathlength of 1 cm and were oriented in the same direction in the cell holder for each fluorescence measurement during an experiment. The zero time point was set as the time at which the human albumin was first brought into contact with the buffer solution. No fluorescence measurements were made for the following 30 min to ensure complete dissolution of the protein.

Fluorescence measurements were made at ambient temperature (23.4 ± 0.6°). However, the temperature of the cell compartment of the spectrofluorometer was found to increase steadily for the first 2 hr after the instrument was turned on. Therefore, an instrument warm-up time of at least 2 hr was used before any fluorescent measurements were taken. After temperature equilibration, the cell compartment temperature was ~4° higher than room temperature. A time of 5 min was found to be sufficient for the temperature equilibration of samples placed inside the cell compartment. The effect of fluctuations in ambient temperature was apparently less than random error, since changes in the magnitude of fluorescence did not follow the variations in temperature observed during these studies.

A solution of propranolol hydrochloride (1.3 μg/ml) in 0.01 *N* HCl was used as a reference solution for each fluorescence measurement to correct for drifts in spectrofluorometer response and to correct for variations in

[1] Lot 903653, Calbiochem-Behring Corp.
[2] Lot 001395, Calbiochem-Behring Corp.
[3] Lot 15, Miles Laboratories, Inc.
[4] Ayerst Laboratories, Inc.
[5] Fisher Scientific Co.
[6] Model 650-10M, Perkin-Elmer Corp.

0022-3549/82/0300-0349$01.00/0

Table I—Results of Linear Regression Analysis of the Fluorescence Stability Data

Human Albumin	r^2	y-Intercept	Slope	95% Confidence Interval of the Slope
Fraction V	0.179	64.3	-2.14×10^{-3}	-7.79×10^{-4} to -3.49×10^{-3}
Crystalline	0.466	51.9	-3.76×10^{-3}	-2.88×10^{-3} to -4.63×10^{-3}
Fatty acid-free fraction V	0.202	42.3	-2.45×10^{-3}	-1.48×10^{-3} to -3.43×10^{-3}
Fraction V[a]	0.464	63.7	-3.18×10^{-3}	-1.67×10^{-3} to -4.69×10^{-3}

[a] Based on the data of Fig. 2.

the fluorescence sample cells used. No detectable change in the magnitude of the fluorescence of the reference solution occurred after 3 hr of continuous irradiation at the wavelength and slit width of excitation used in this study. The total irradiation time of the reference solution was not more than 15 min in subsequent experiments.

To prevent photodecomposition of the protein solutions, aliquots of the same solution of human albumin were irradiated in the spectrofluorometer only once for the few seconds required for a fluorescence measurement. Except as otherwise noted, fluorescence sample cells were rinsed six times with water followed by two rinses in spectrophotometric grade acetone between each use.

RESULTS AND DISCUSSION

The fluorescence intensities of human albumin solutions were determined at various times for 480 min after preparation. The results obtained for fraction V, crystalline, and fatty acid-free fraction V human albumin are presented in Fig. 1. The three data points obtained at each time point were obtained for three different protein solutions. To allow sufficient drying time after rinsing, two different fluorescence sample cells had to be used for each albumin solution when fluorescence measurements were made every 15 min for crystalline and fatty acid-free fraction V human albumin. In an attempt to decrease data variability, fluorescence measurements were only made every 30 min using the same fluorescence sample cell for each solution of fraction V human albumin.

The three types of human albumin were quite different with respect to the magnitude of their fluorescence. The magnitude of the fluorescence of each type of albumin was not corrected for differences in moisture content and purity. The greater fluorescence of fraction V as compared to the crystalline form of human albumin was observed previously for bovine serum albumin (4). The fatty acid-free fraction V human albumin exhibited the smallest fluorescence intensity of the human albumins examined. This decrease in fluorescence intensity may not be related to the decrease in the fatty acid content of the preparation. The fluorescence intensities of two crystalline human albumin preparations were found to differ, but the preparation containing the lowest content of fatty acid was more fluorescent (7). The difference in the emission spectra for three different crystalline human albumin samples which had equal absorbance values at the wavelength of excitation was apparently due to impurities other than fatty acids (8).

The magnitude of the fluorescence of the three types of human albumin examined in this study did not undergo the large fluctuations with time that had been previously observed for a bovine albumin solution (2). Therefore, it is not necessary to prepare these albumin solutions several hours prior to fluorescence measurements to allow the intrinsic protein fluorescence to stabilize. Of course, the proteins examined could have undergone conformational changes during this time period which were not reflected by changes in fluorescence intensity.

While large fluctuations in fluorescence intensity with time were not observed with the human albumin solutions used in this study, the magnitude of the fluorescence of the solutions appeared to undergo a small but continuous decrease with time for the length of each study. Much of the variability in the data for each type of protein was due to differences between the individual protein solutions used. For each type of human albumin at each time point, one albumin solution consistently had the highest fluorescence intensity while another albumin solution consistently had the lowest fluorescence. The apparent decrease in the magnitude of the fluorescence of each human albumin solution observed 480 min after preparation is not much greater than the experimental

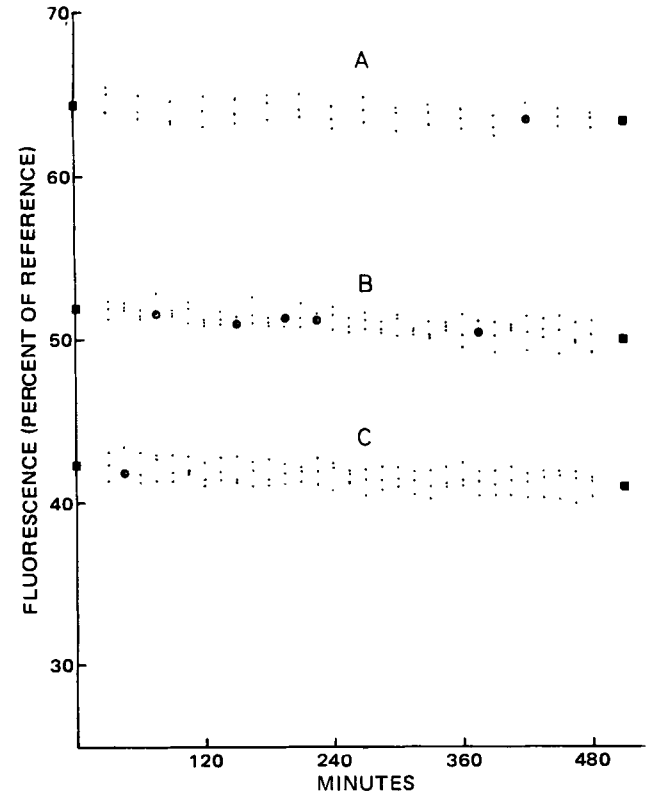

Figure 1—*The magnitude of fluorescence of human albumin solutions upon aging, where ● represents two identical experimental points and ■ represents the linear regression line of the data. Key: A, fraction V; B, crystalline; and C, fatty acid-free fraction V.*

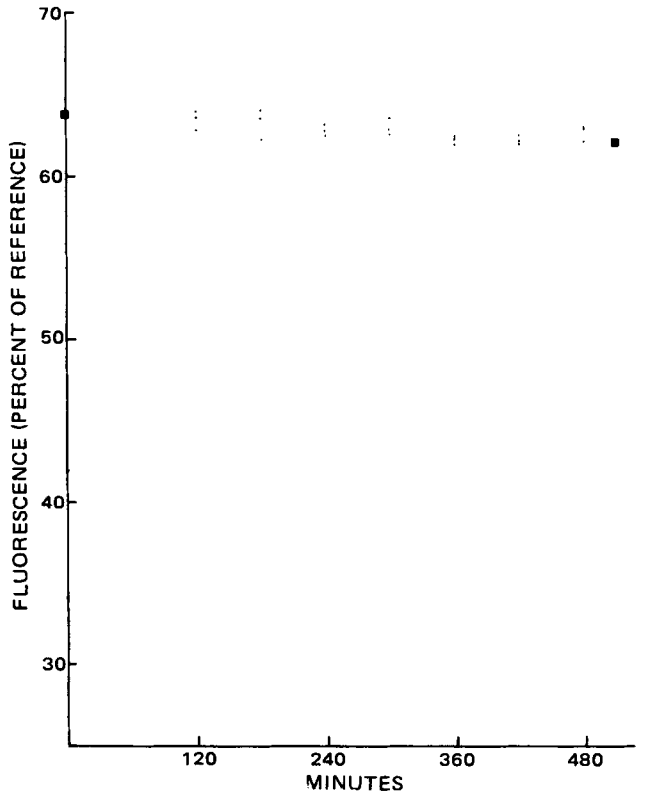

Figure 2—*The magnitude of fluorescence of fraction V human albumin solutions upon aging when fluorescence sample cells are cleaned with sulfuric acid–dichromate solution between measurements. Key: ■, the linear regression line of the data.*

variability observed in the fluorescence intensity of the different albumin solutions at each time point.

The results of linear regression analysis of the data for each type of human albumin are presented in Table I. While the r^2 value for each set of data is very low, the slope of each set of data does have a small negative value and zero is not included in the 95% confidence interval of the slope. The points at $t = 0$ and $t = 510$ min in Fig. 1 represent the linear least-squares fit of the experimental data. The actual lines were not drawn so that the experimental data could be examined without obstruction.

To determine if the small decrease in fluorescence intensity with time was due to inadequate washing of fluorescence sample cells between sample measurements, the experiment with fraction V human albumin was repeated. In this second study, fluorescence measurements were taken every hour to decrease the use of each sample cell. After each fluorescence measurement the cells were rinsed once with water, filled with sulfuric acid–dichromate cleaning solution for 15 min and rinsed using the procedure used in the previous experiments. The results are presented in Fig. 2. The fluorescence intensities of the solutions again appeared to undergo a small decrease with time. The results of linear regression analysis of the data are presented in Table I. The slope has a small negative value which is within the 95% confidence interval of the slope previously obtained for fraction V human albumin. As observed previously, zero is not within the 95% confidence interval of the slope. Thus, this different method of sample cell treatment between fluorescence measurements had no significant effect on the apparent decrease in fluorescence intensity of these protein solutions with time.

In conclusion, the intrinsic fluorescence intensity of each of the solutions of human albumin examined in this study was very stable for the first 8 hr after preparation. Based on these results, the practice of preparing solutions of human albumin several hours prior to use simply to achieve fluorescence stability is unnecessary. Since the fluorescence intensity of each of the albumin solutions examined underwent a small, consistent decrease with time, it may be advisable to use a reference solution of human albumin, as described previously (6), to correct binding data obtained from the quenching of intrinsic human albumin fluorescence by ligand.

REFERENCES

(1) C. F. Chignell, *Methods Pharmacol.*, **2**, 33 (1972).

(2) N. A. Attallah and G. L. Lata, *Biochim. Biophys. Acta*, **168**, 321 (1968).

(3) S. E. Shnoll and E. P. Chetverikova, *ibid.*, **403**, 89 (1975).

(4) D. V. Parke and W. E. Lindup, *Ann. N.Y. Acad. Sci.*, **226**, 200 (1973).

(5) H. Uchida and M. Hanano, *Chem. Pharm. Bull.*, **22**, 1571 (1974).

(6) R. L. Levine, *Clin. Chem.*, **23**, 2292 (1977).

(7) D. S. Lukas and A. G. DeMartino, *J. Clin. Invest.*, **48**, 1041 (1969).

(8) R. F. Chen, *J. Biol. Chem.*, **242**, 173 (1967).

ACKNOWLEDGMENTS

The author thanks Ms. M. DiMatteo for technical assistance.

Separation of Penicillin and Its Major Degradation Products by Ion-Pair Reversed-Phase High-Pressure Liquid Chromatography

ISAAC GHEBRE-SELLASSIE *, STANLEY L. HEM ‡, and ADELBERT M. KNEVEL *x

Received March 12, 1981, from the **Department of Medicinal Chemistry and Pharmacognosy and the ‡Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907.* Accepted for publication June 11, 1981.

Abstract □ An ion-pair reversed-phase high-pressure liquid chromatographic technique capable of separating penicillin and its major degradation products within 8 min was developed. The influence of pH, counterion concentration, buffer concentration, and organic modifier content was studied and the observed behavior of the compounds during the chromatographic process was discussed.

Keyphrases □ Penicillin—major degradation products, separation by ion-pair reversed-phase high-pressure liquid chromatography □ High-pressure liquid chromatography—ion-pair reversed-phase, separation of penicillin and its major degradation products □ Degradation products—penicillin, separation by ion-pair reversed-phase high-pressure liquid chromatography

Various analytical methods for the detection, separation, and/or quantification of penicillin in the presence of its degradation products have been reported (1–7). The most recent papers deal with anion-exchange chromatography (5), reversed-phase chromatography (6), and NMR spectroscopy (7). NMR generally lacks sensitivity while the chromatographic approaches result in relatively long analysis times. However, the availability of small-particle size packings for reversed-phase chromatography suggests that very high chromatographic efficiencies and shorter analysis times can be expected with this procedure (8, 9). The present paper describes a high-pressure liquid chromatographic (HPLC) method that utilizes an ion-pair reversed-phase technique to separate penicillin and its three major degradation products: penillic acid, penicilloic acid, and penilloic acid in <8 min.

EXPERIMENTAL

Chemicals and Reagents—Penicillin G potassium[1] and tetrabutylammonium chloride[2] were obtained commercially and used without further treatment. Penillic acid, penicilloic acid, and penilloic acid were synthesized by standard methods (10). Acetonitrile was HPLC grade[3] while all other chemicals were either USP or reagent grade. Double-distilled water was used to prepare buffer solutions.

Apparatus—A liquid chromatograph[4] equipped with a fixed-wavelength UV absorbance detector set at 254 nm was used. A commercial stainless steel column[5] (150-mm × 4.6-mm i.d.) prepacked with 5-μm particles, with the silanol groups chemically bonded to a monomolecular layer of octadecylsilane, was used. A strip-chart recorder[6] recorded the detector output.

Chromatographic Conditions—The mobile phase was composed of acetonitrile, phosphate buffer, and a counterion. The ion-pair reagent, tetrabutylammonium chloride, was dissolved in the phosphate buffer

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Waters Associates, Milford, Mass.
[4] Model ALC 202, Waters Associates, Framingham, Mass.
[5] Ultrasphere-ODS, Beckman Instruments, Irvine, Calif.
[6] Omniscribe recorder, Houston Instruments, Houston, Tex.

0022-3549/82/0300-0351$01.00/0