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An Automated Fluorometric Method for the Measurement of Tryptophan in Plasma

The fluorometric analysis of tryptophan described by Hess and Udenfriend has been automated to facilitate the estimation of tryptophan in plasma. Details of manifold construction and reagent composition are presented along with data concerning the reliability of the method.

A variety of procedures (Hier and Bergeim, 1946; Spies and Chambers, 1948; Scott, 1961; Wapnir and Bessman, 1965) are available for the determination of tryptophan in biological materials, but the fluorometric method of Hess and Udenfriend (1959) has been found particularly suitable for blood plasma (Young et al., 1971; Tontisirin et al., 1973; Lewis and Speer, 1974). The method is based on the conversion of tryptophan to the fluorophore, norharman, in a two-step reaction: (a) the cyclization of tryptophan to a tetrahydronorharman derivative and (b) the subsequent oxidation to norharman. Norharman is then determined fluorometrically by activation at 365 nm and measurement at 440 nm. Modifications of the original method have been published by Denckla and Dewey (1967) and Peters et al. (1969). The various procedures are sensitive and specific, but are not convenient for the analysis of large numbers of samples. The purpose of this paper is to describe an automated procedure, based on the original method by Hess and Udenfriend (1959), capable of analyzing 30 samples per hour. In adapting the method for autoanalysis, only two basic changes were made from the original method: the concentration of formaldehyde was reduced from 18 to 15%, and the concentration of peroxide was increased from 5 to 30%.

MATERIALS AND METHODS

Reagents used included: hydrogen peroxide, 30% w/v; formaldehyde, 15% w/v; sulfuric acid, 0.2 N; wash solution, 0.5 ml of Brij-35 solution (30%) per liter of water containing 3.2% w/v trichloroacetic acid (Cl_3CCOOH). Standard solutions were prepared daily by combining 0.5, 1.0, 1.5, 2.0, and 2.5 ml of a stock solution of 2 mg/100 ml tryptophan with 4 ml of 8% w/v Cl_3CCOOH and diluting to 10 ml. When samples are prepared as described in the next section, the values of the standards correspond to 0.5, 1.0, 1.5, 2.0, and 2.5 mg of tryptophan per 100 ml of plasma. Cl_3CCOOH is added to the standards and the wash solution to provide a background similar to the samples.

Sample Preparation. Blood is withdrawn with heparinized syringes and centrifuged immediately and the plasma separated. Samples are prepared by combining 1 ml of plasma with 4 ml of 4% w/v Cl_3CCOOH and centrifuging at 19 000*g* for 15 min. The supernatant liquid is decanted and analyzed without further dilution. The deproteinizing agent, sulfosalicylic acid, produced a high fluorescence that interfered with the analysis.

Automated Assay. The flow diagram for the automated method is shown in Figure 1. The system was assembled with the following components: one Sampler II, one Proportioning Pump I, and one 95 °C Heating Bath (Technicon Instruments Corp., Tarrytown, N.Y.), one Model III Fluorometer (G. K. Turner Associates, Palo Alto, Calif.) fitted with a Corning No. 7-60 excitation filter and a Wratten No. 3 emission filter and with sensitivity set at 3×, and one vom 5 Recorder (Bausch and Lomb Inc., Rochester, N.Y.). Pump tubing and glass fittings are Technicon designations.

Deproteinized plasma solution was aspirated at the rate of 30 samples per hour using a wash to sample ratio of 2 to 1. A water wash cup was placed behind the highest standard and samples were then run consecutively without alternating wash cups. The heating bath was equipped with two 12.2-m coils. Each sample remained in the heating bath for approximately 7.5 min on the first pass and 7.0 min on the second pass. The tubing between the fluorometer debubbler and flowcell was kept short to minimize the possibility of bubble formation in this region.

RESULTS AND DISCUSSION

The effect of varying hydrogen peroxide and formaldehyde concentrations on fluorescence of tryptophan standards was investigated (Table I). Linear response to the higher concentrations of tryptophan was obtained only if 30% hydrogen peroxide was used. The highest con-

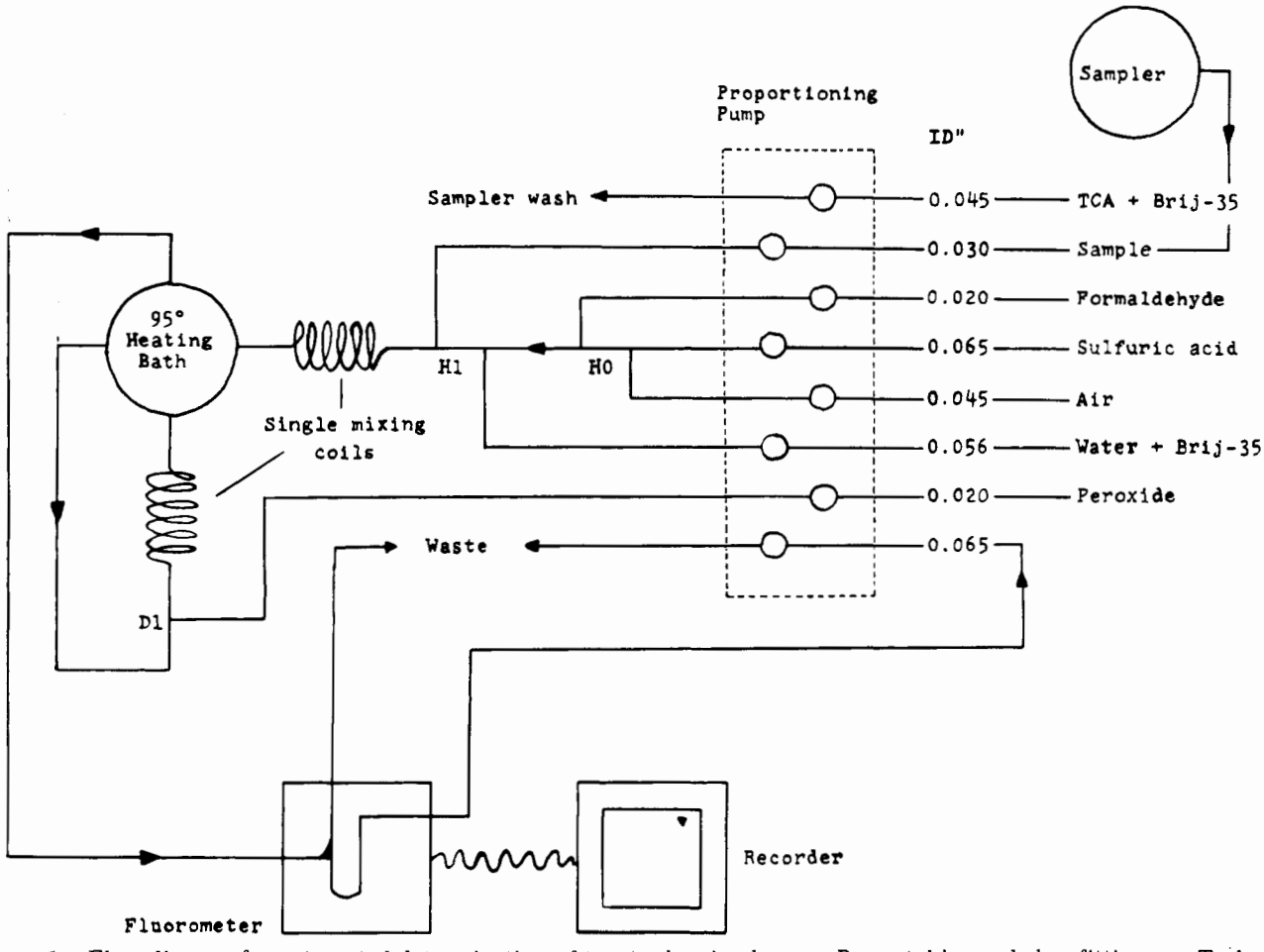

Figure 1. Flow diagram for automated determination of tryptophan in plasma. Pump tubing and glass fittings are Technicon designations.

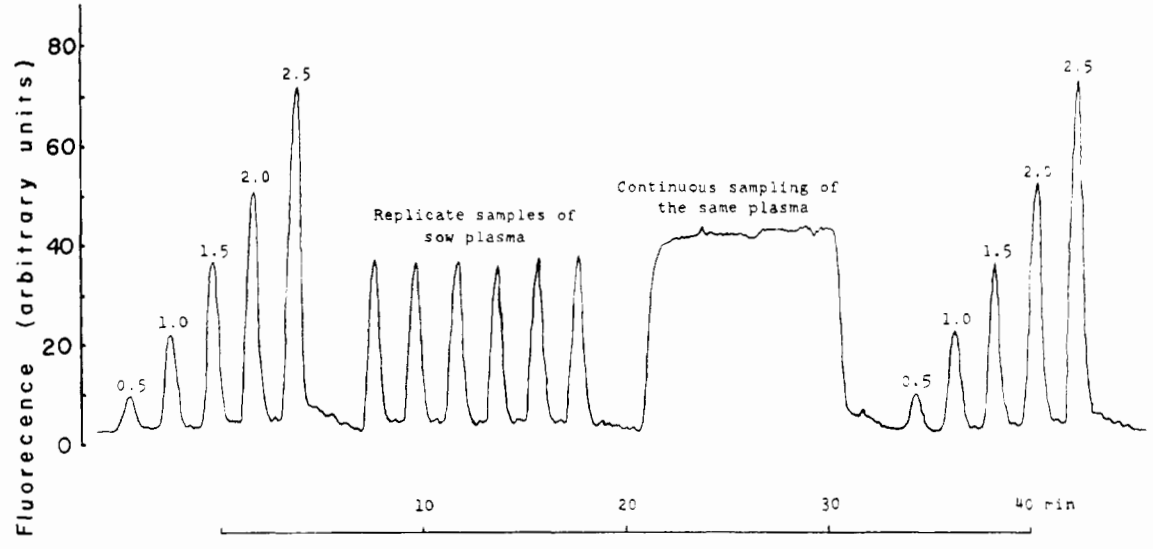

Figure 2. Recording of the analysis of tryptophan using the manifold of Figure 1. Trace shows standard solutions with concentrations equivalent to 0.5, 1.0, 1.5, 2.0, and 2.5 mg of tryptophan per 100 ml of plasma, and an example of sow plasma. The recording illustrates the reproducibility of sampling and the constancy of fluorescence with continuous sampling.

centration of formaldehyde (37%) inhibited fluorescence. This effect was particularly evident at a tryptophan concentration of 5 mg per 100 ml. A concentration of 15% formaldehyde was adequate to elicit a maximum fluorescence response.

A recording of the analysis of tryptophan standards and a sample of sow plasma are illustrated in Figure 2. This figure shows the characteristics of the peaks and demonstrates that over 90% of steady state was obtained. It is evident from Figure 2 that fluorescence is essentially linear over the range of normal plasma tryptophan concentrations (0.5 to 2.0 mg/100 ml).

The recovery of various amounts of tryptophan added to swine plasma was examined in experiments with two sets of plasma (Figure 3). The slope of the regression line fitted to these data by the method of least squares was

Table I. Effect of Concentrations of Hydrogen Peroxide and Formaldehyde Solutions on Fluorescence

Tryptophan, mg/100 ml	Fluorescence, arbitrary units						
	10%[a]		20%[a]		30%[a]		
	25%[b]	37%[b]	15%[b]	25%[b]	15%[b]	25%[b]	37%[b]
0.5	4.9	4.4	7.0	6.3	7.6	7.0	6.8
1.0	11.5	9.4	12.9	11.9	14.4	12.8	13.3
2.0	22.4	17.2	26.9	21.3	28.7	26.9	26.4
3.0	30.3	24.2	36.9	35.3	41.5	41.7	39.4
4.0	38.0	29.2	39.5	39.5	52.7	49.8	50.1
5.0	45.1	34.6	43.7	48.4	66.6	65.2	58.2

[a] Hydrogen peroxide (w/v). [b] Formaldehyde (w/v).

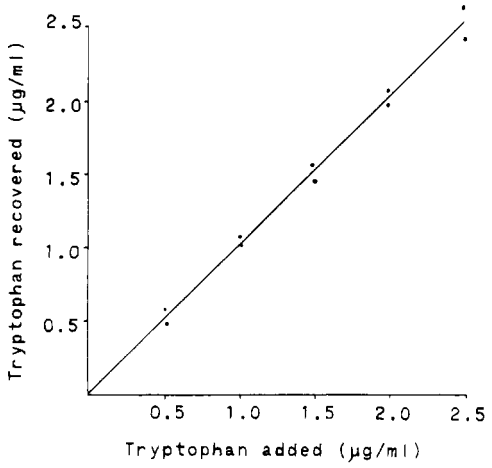

Figure 3. Recovery of tryptophan added to plasma.

0.97, indicating that on the average 97% of the added tryptophan was recovered. Therefore, there was essentially no quenching of fluorescence by sow plasma. However, Peters et al. (1969) observed a quenching of 20% with human plasma. The correlation between added and recovered tryptophan was 0.999.

To compare the automated method with the original manual procedure, 21 plasma samples obtained from sows receiving normal diets were analyzed by the automated method described herein and by the manual method of Hess and Udenfriend (1959). The mean tryptophan concentration found by the automated method was 1.05 mg/100 ml (range 0.68–1.49 mg/100 ml) and, by the manual method, the mean was 1.04 mg/100 ml (range 0.67–1.34 mg/100 ml). The correlation coefficient between methods was 0.89. This range of values for swine is similar to those reported by various workers (Denckla and Dewey, 1967; Peters et al., 1969; Young et al., 1971; Tontisirin et al., 1973) for humans and rats.

In the automated procedure described here, the total tryptophan in plasma was determined. However, the method could probably be adapted to differentiate bound and unbound tryptophan in plasma.

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