Differential Spectrophotometric Determination of Tyrosine and Tryptophan in Pharmaceutical Amino Acid Solutions

J. DEMEESTER *x, **M. BRACKE** *, **R. VOCHTEN** ‡, and **A. LAUWERS** *

Received June 27, 1977, from the *Laboratory of General Biochemistry and of Physical Pharmacy, State University of Gent, Wolterslaan 12, B-9000 Gent, Belgium, and the ‡Laboratory of Industrial Technology, University of Antwerp, Middelheimlaan 1, B-2020 Antwerpen, Belgium. Accepted for publication August 30, 1977.

Abstract □ A method is proposed for the simultaneous determination of tyrosine and tryptophan in solutions by differential spectrophotometry. The concentrations are calculated from the measurement of the absorbance of the amino acid mixture in an alkaline medium and from the differential absorbance of the alkaline solution against the acid solution at 294.4 nm. A comparison with three other well-known methods is discussed.

Keyphrases □ Tyrosine—spectrophotometric analysis simultaneously with tryptophan in solutions □ Tryptophan—spectrophotometric analysis simultaneously with tyrosine in solutions □ Spectrophotometry, differential—simultaneous analyses of tyrosine and tryptophan in solutions □ Amino acids—tyrosine and tryptophan, simultaneous spectrophotometric analyses in solutions

For the determination of tyrosine and tryptophan in solutions, various widely used spectrophotometric methods are based on the fact that the absorption spectra of both amino acids differ in acid and in alkaline media. These methods have been used frequently for the analysis of proteins and peptides where wavelength shifts occur. The most important spectrophotometric routine methods that can be performed without computer-assisted least-squares or linear programming techniques (1–3) have been considered using known amino acid mixtures. When extreme tyrosine to tryptophan ratios are to be determined, large errors are observed. Therefore, a simple method without these inconveniences is desired.

The proposed method uses the same principles but takes advantage of differential absorbance measurements and uses only one wavelength to improve simplicity and accuracy. The objectives of this study were to develop a practical procedure for routine applications, to define general formulas for individual adaptations to special problems, and to evaluate the statistical errors. Furthermore, the proposed procedure was compared with the mentioned methods.

EXPERIMENTAL

General—The absorption spectra of vacuum-dried tyrosine[1] and tryptophan[2] were recorded in an alkaline medium (0.1 *M* NaOH), and an isosbestic point was found at 294.4 nm (ϵ^{OH^-}) corresponding to the literature value (1). The measurements were performed on a calibrated spectrophotometer[3] with a resolution of 0.1 nm. Molar absorptivities (ϵ) of vacuum-dried tyrosine and tryptophan at 294.4 nm were calculated by linear regression analysis from series of dilutions in 0.1 *M* HCl and 0.1 *M* NaOH (correlation coefficients were greater than 0.9998) and are expressed in M^{-1} cm^{-1} with their standard errors.

For tyrosine in an acid medium, the value $\epsilon_{tyr}^{H^+}$ was 25.07 ± 0.008; for tryptophan, $\epsilon_{trp}^{H^+}$ was 1330 ± 9. In an alkaline medium for both tyrosine and tryptophan, ϵ^{OH^-} was 2465 ± 6.

[1] Product 8371, Merck, Darmstadt, West Germany.
[2] Product 8404, Merck, Darmstadt, West Germany.
[3] Cary 14, Varian Instruments.

Table I—Absorbance Values, Calculated Tyrosine and Tryptophan Molar Concentrations (c_{tyr} and c_{trp}, Respectively), and Their Relative Errors in Percent [a]

Tyrosine to Tryptophan	A^{OH^-}	ΔA	$10^5\,c_{tyr}$	$10^5\,c_{trp}$	Percent Error Tyrosine	Percent Error Tryptophan
10:0	0.4134	0.4068	16.59	0.19	−0.5	—
9:1	0.4080	0.3809	14.79	1.76	−1.4	+5.6
8:2	0.4066	0.3515	12.66	3.75	−5.0	+12.6
7:3	0.4104	0.3377	11.40	5.25	−2.3	+5.0
6:4	0.4132	0.3274	10.51	6.25	+5.1	−6.2
5:5	0.4103	0.2985	8.40	8.25	+0.8	−1.0
4:6	0.4132	0.2784	6.76	10.00	+1.3	+0.1
3:7	0.4115	0.2547	5.00	11.70	0.0	+0.2
2:8	0.4103	0.2340	3.45	13.19	+3.6	−1.1
1:9	0.4150	0.2127	1.66	15.18	−0.6	+1.2
0:10	0.4104	0.1856	−0.26	16.91	–	+1.4

[a] The total amino acid concentration was 166 μM.

The following simple equations can be set up when differential measurements of absorbance, ΔA, are made of the alkaline mixture against the acid mixture with molar concentrations of tyrosine and tryptophan, c_{tyr} and c_{trp}, respectively:

$$\Delta A = \Delta A_{tyr} + \Delta A_{trp} = c_{tyr}(\epsilon^{OH^-} - \epsilon_{tyr}^{H^+})l + c_{trp}(\epsilon^{OH^-} - \epsilon_{trp}^{H^+})l \quad \text{(Eq. 1)}$$

$$A^{OH^-} = (c_{tyr} + c_{trp})\epsilon^{OH^-}l \quad \text{(Eq. 2)}$$

From these equations follows:

$$c_{tyr} = \frac{\Delta A - A^{OH^-}\dfrac{\epsilon^{OH^-} - \epsilon_{trp}^{H^+}}{\epsilon^{OH^-}}}{l(\epsilon_{trp}^{H^+} - \epsilon_{tyr}^{H^+})} \quad \text{(Eq. 3)}$$

By substituting the calculated ϵ values into Eqs. 2 and 3, the formulas to calculate the molar concentrations of both amino acids are obtained:

$$c_{tyr} = \frac{\Delta A - 0.4605A^{OH^-}}{1305} \quad \text{(Eq. 4)}$$

$$c_{trp} = \frac{A^{OH^-}}{2465} - c_{tyr} \quad \text{(Eq. 5)}$$

The most probable errors on the molar concentrations were calculated using the classical partial differential formulas and yielded, for tyrosine:

$$s_{tyr}^2 = \frac{1.212s^2 + (0.00782)^2A^2}{(1305)^2} + \frac{(15)^2}{(1305)^4}(\Delta A - 0.460A^{OH^-})^2 \quad \text{(Eq. 6)}$$

and for tryptophan:

$$s_{trp}^2 = \frac{1.980s^2 + (0.00628)^2A^2}{(1305)^2} + \frac{(15)^2}{(1305)^4}(\Delta A - 0.990A^{OH^-})^2 \quad \text{(Eq. 7)}$$

where s is the standard error on the absorbance measurements.

Procedure—Dilute the amino acid mixture until a total concentration of tyrosine and tryptophan of about 200–800 μM is obtained; this is Solution U.

Measure (in triplicate) in 1-cm UV cells in a wavelength-calibrated dual-beam spectrophotometer at 294.4 nm and in absorbance scale:

Table II—Relative Errors in Percent on the Concentrations of Tyrosine and Tryptophan Determined with the Four Spectrophotometric Methods[a]

Tyrosine to Tryptophan Ratio	Reference 1		Reference 2		Reference 3		Present Method	
	Tyrosine	Tryptophan	Tyrosine	Tryptophan	Tyrosine	Tryptophan	Tyrosine	Tryptophan
1:9	+51	+0.7			+400	−39	+6.0	−0.7
2:8	−20	+11			+120	−33	+3.0	−1.5
3:7	−3	−2.2			+83	−36	−3.0	+0.4
4:6	+0.8	−1.5	+4.1	−1.3	+50	−34	+1.6	−1.0
5:5	−4.8	−1.2	+6.4	−7.1	+29	−35	+1.2	−1.8
6:4	0	−1.5	−13	−1.4	+23	−36	+0	−1.5
7:3	+3.9	−6.1			+15	−32	−5.6	+3.0
8:2	+1.5	−9.1			+6	−27	−1.5	+3.0
9:1	−0.7	−18			+1.3	−36	−1.3	+12.0

[a] The total amino acid concentration was 330 μM.

1. A mixture of 10 ml of Solution U with 10 ml of 0.2 M NaOH (sample beam) against a mixture of 10 ml of 0.2 M NaOH with 10 ml of clean water (reference beam); this gives the absorbance value A^{OH^-}.

2. A mixture of 10 ml of Solution U with 10 ml of 0.2 M NaOH (sample beam) against a mixture of 10 ml of Solution U with 10 ml of 0.2 M HCl (reference beam); this gives the absorbance value ΔA.

From Eqs. 4 and 5, the molar concentrations of tyrosine and tryptophan after dilution are obtained so that it is a simple procedure to obtain the original concentration by multiplying by 2 and by the dilution factor used. Other experimental procedures (*e.g.*, other cells) can be considered when using Eqs. 2 and 3.

RESULTS

The method was controlled experimentally using known amino acid mixtures with different concentration ratios. Table I gives the experimental values, and Fig. 1 shows the deviation of the experimentally found concentrations from the real concentrations. Figure 1 also shows the computed plots of the theoretical 2s most probable error limits, calculated from Eqs. 6 and 7; the value of s was taken as 0.003, corresponding to the spectrophotometer specifications.

All experimental values were means of three absorbance measurements.

This method was compared with the most important spectrophotometric methods previously described (1–3). Table II gives the relative errors in percent of the concentrations in the four methods.

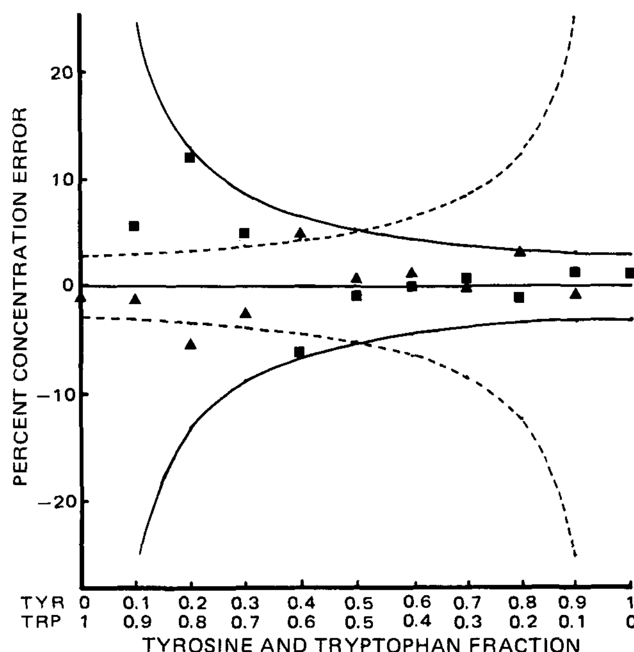

Figure 1—*Plot of the ratios of tyrosine to tryptophan (the total concentration equals 166* μM) versus *the deviations in percent of the concentrations of tyrosine* (▲) *and tryptophan* (■). *The 2s (95%) most probable error limits for tyrosine (- - -) and tryptophan (—) were calculated from Eqs. 6 and 7.*

DISCUSSION

Good results were found with the method of Goodwin and Morton (1) except at extreme ratios (Table II). Two wavelength settings are needed. The method of Bencze and Schmid (2) gives good results only with approximately equimolar solutions. With more extreme ratios, many difficulties are encountered. The method of Brown *et al.* (3) is less accurate in the whole range.

The proposed method, in general, gives less error than the other methods, especially at higher amino acid ratios. Moreover, the found errors are in good agreement with the expected values of the general error theory, so that when a determination is performed, the expected error in concentration can be calculated easily from Eqs. 6 and 7. Only one wavelength is needed for the assay, so no supplementary scatter in the results occurs from bad wavelength settings. The wavelength accuracy eventually can be checked with known amino acid solutions.

This proposed method is less accurate than some other methods when the tyrosine and tryptophan content of unhydrolyzed protein solutions is measured because of the wavelength shifts of the absorbance spectra of both amino acids in proteins. The only interfering amino acids are thyroxine and the thyronines, which do not occur in normal protein hydrolysates or amino acid mixtures. The present method can be very useful when it is important to control the amino acid composition of intravenous and other pharmaceutical preparations, because no interference exists from other amino acids at this high wavelength.

REFERENCES

(1) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40,** 628 (1946).
(2) W. L. Bencze and K. Schmid, *Anal. Chem.*, **29,** 1193 (1957).
(3) J. R. Brown, N. Greenshields, M. Yamasaki, and H. Neurath, *Biochemistry*, **2,** 867 (1963).