Some Aspects of Spectrophotometric Determination of Amino Acids Using o-phthalaldehyde as a Chromogenic Reagent

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Summary: A rapid, sensitive and convenient spectrophotometric method has been developed for the determination of Amino acids. At lower concentration (3×10^{-7} mol/ml) of amino acids the Beer-Lambert law is obeyed. The absorptivities of the *o*-phthalaldehyde (OPA)-adducts formed with different amino acids have been examined and calculated. The OPA-derivative is found to be composed of amino acid, OPA and a thiol in a molar ratio 1:2:1.

Introduction

Amino acids occurring in nature [1], amino acids in nutritional foods [2], amino acids in hydrolysates of purified protein [3], amino acids in tissue and tissue extracts, amino acids in physiological fluids [4] (e.g., blood and urine) and total amino acids in natural water [5] have been extensively studied and characterised during recent years [6,7]. Clinically important amino acids in blood serum or urine have been estimated using pre-column derivatisation followed by reversed-phase HPLC technique [8].

The usefulness of amino acids in physiologically important areas of biology and medicinal chemistry is so significant that it needs a routine method for the identification and quantification in a sample. Many procedures have been used for their determination using spectrofluorimetric methods [9-11] as well as spectrophotometric ones [12,13]. The procedure involves derivatisation of the amino acids with the fluorigenic reagents followed by measurement of emission or absorbance at the wavelength of excitation. The latter procedure is adopted when the laboratory can not afford to have the expensive fluorimeter.

Little has been reported, however, on the molar absorptivities for the amino group determined for a variety of amino acids and peptides. The use of standard methods such as chromatography is time consuming for detection purpose especially when amino acids are linked to the fluorigenic reagents.

In order to avoid expensive and time consuming techniques, we wish to report and describe detection and determination of amino acids using spectrophotometric method, involving *o*-phthalaldehyde (OPA) as the derivatising reagent. Following this procedure, it is desired to achieve these objectives., a) detection of amino acids via molar absorptivity b) quantitative estimation of amino acids through the measurement of absorbance of the chromophores at the wavelength of excitation (335 nm).

Results and Discussion

It was observed during a systematic investigation of a series of amino acids for their derivatisation reaction with *o*-phthalaldehyde (OPA) that

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the product (isoindole) [9] absorbed electromagnetic radiation at 335 nm. The reaction is stoichiometric i.e., the molar ratio for OPA to amino acid is 2:1. Further, the OPA-adducts were found to absorb maximally when the solution was made alkaline (pH 10). This result confirms the other reported work [14].

Since the reaction involved the use of thiol for derivatisation, it was deemed necessary to determine the molar ratio of amino acid to thiol. For this purpose, a series of solutions (buffer at pH 10) in which the molecular ratio of thiol to amino acid varied from 1.5:1 to 4:1 were prepared and their absorbancy (-log transmittancy) was measured at 335 nm. A plot of absorbance versus moles of thiol per mole of amino acid gave a curve (not shown) which had a sharp break at molar ratio of 1:1, showing that the absorbance of the derivative does not increase beyond the point. Thus the ratio of thiol to amino acid(s) in the OPA-adduct is 1:1. However, a molar ratio of 1:2 was found more suited for amino acid to OPA.

The studies also indicated that changes, in the absorbances observed with varying the type of thiols, were the results of the differences in the structure of thiols. The thiols e.g., 3-mercaptopropionic acid (3-MPOH) and 2-methyl-2-propanethiol (2-MPT) were found to give OPA-adducts which absorbed maximally in the desired region. It is believed that the intensity of the absorption is proportional to the number of insulated chromophores present in the molecule. Electronic interaction between chromophores can thus lead to π-π conjugation or n-π conjugation or both. The extended open chain conjugated system may have, in the present case, be responsible for increase in molar absorptivity. Further the abruptness with which the shift (bathochromic) in the position of maximum absorbancy occured indicating a definite change in the molecular structure of the OPA-derivatives.

The effect of temperature on the absorbance of OPA-adduct was also studied. It was observed that a temperature increase to 30°C or above resulted in a gradual decrease of intensities of absorption.

The OPA reagent specially reacts with primary amino groups, and the resulting absorbance represents the basis for amino acid assays. A number of amino acids were found to give absorbances proportional to concentrations, shown in Figs., 1 and 2 (Table-1). The Beer-Lambert law was obeyed at lower concentrations upto $3x10^{-7}$ moles/ml.

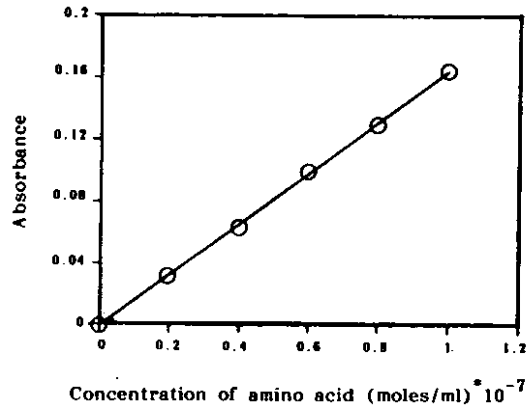

Fig.1: Relationship between the absorbance and concentration of glycine.

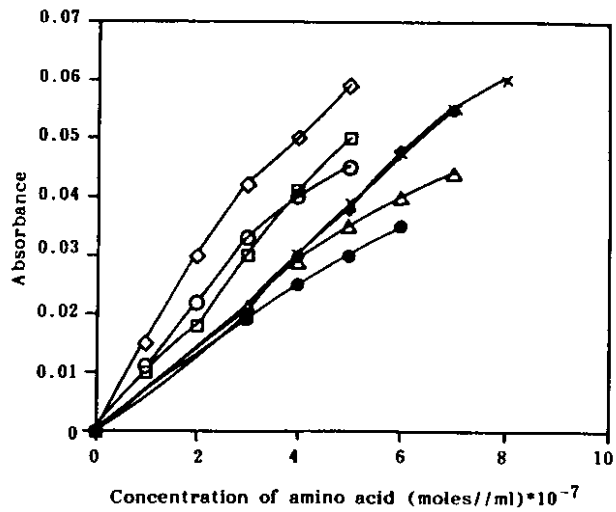

Fig.2: Relationship between the absorbance and concentration of amino acids in the presence of different thiols.

Although the reaction does not discriminate between one amino acid and the other having the primary amino group, yet there are the molar absorptivities which are different for OPA-adducts formed with different amino acids. Therefore, the absorptivity for this adduct was also examined. The resulting calculated molar absorptivities and E (1%

Table-1: Effect of concentration on the absorbance in presence of Thiols

Amino acid/Thiol(s)	Concentration (moles/ml)x10^{-7}	Abosrbance
Gly/3-MPOH+2-MPT	1.0	0.010
	2.0	0.018
	3.0	0.030
	4.0	0.041
	5.0	0.050
Gly/Ethanethiol	0.2	0.032
	0.4	0.064
	0.6	0.100
	0.8	0.130
	1.0	0.165
Val/2-MPT	3.0	0.019
	4.0	0.025
	5.0	0.030
	6.0	0.035
Tryp/3-MPOH	1.0	0.011
	2.0	0.022
	3.0	0.033
	4.0	0.040
	5.0	0.045
Glu/3-MPOH	3.0	0.020
	4.0	0.030
	5.0	0.038
	6.0	0.048
	7.0	0.055
Met/3-MPOH+2-MPT	4.0	0.030
	5.0	0.039
	6.0	0.047
	7.0	0.055
	8.0	0.060
Tryo/2-MPT	1.0	0.015
	2.0	0.030
	3.0	0.042
	4.0	0.050
	5.0	0.059
Arg/3-MPOH+2-MPT	3.0	0.021
	4.0	0.029
	5.0	0.035
	6.0	0.040
	7.0	0.044

Table-2: Molar absorptivities of *o*-phthalaldehyde-adducts of various amino acids measured at 335 nm.

Amino acid/Thiol(s)	E(1 %,/1 cm)	$E_{335}(M^{-1}Cm^{-1})$
Gly/Ethanethiol	225	1783
Gly/3-MPOH	66	508
Gly/2-MPT	66	508
Gly/2-MPT+3-MPOH	66	508
Arg/2-MPT+3-MPOH	19	331
Met/2-MPT+3-MPOH	262	5600
Tryp/3-MPOH	24	490
Tryp/2-MPT	33	674
Glu/3-MPOH	26	382
Lys/3-MPOH	-	-
Lys/2-MPT	76	1111
Val/3-MPOH	-	-
Val/2-MPT	25	293

1cm) i.e., the extinction of 1% solution, are presented in Table 2. The changes of absorptivities for OPA-amino acid adducts demonstrate the convenience of OPA spectrophotometric assay for monitoring the amino acids and the hydrolysis of milk proteins [15].

Experimental

Instrumentation

A Hitachi Model U-2000 double beam Spectrophotometer was employed for measuring the absorbance. A Spectronic-20 of Bausch and Lamb was also used to obtain the absorbances of *o*-phthalal derivatives. A Genway Model-3020 pH meter was used for pH adjustment.

Reagents and solutions

Borate buffer (pH-10)

Borax (4.8 g) and sodium hydroxide (0.8 g) were used to prepare 1L solution in water. Final pH adjustment before diluting to 1L was made using sodium-hydroxide (0.5 N) or hydrochloric acid (2N).

Amino acid (10^{-3}M) and o-phthalaldehyde (10^{-3}M) solution

Amino acid solution was obtained by dissolving the requisite amount of amino acid in ethanol (5 ml) and made upto the desired volume with distilled water.

o-Pthalaldehyde (OPA) solution was made similarly in distilled water.

Thiol solution (0.1% v/v)

Ethanethiol, 2-methyl-2-propanethiol or 3-mercapto-priopionic acid was dissolved in distilled water to obtain the solution.

Procedure

The reaction was carried out as described as Roth [9].

An aliquote of 0.5-10 ml solution of amino acids (10^{-3}M) was taken into a 50 ml measuring flask. For each ml of sample solution there were added buffer solution (3 ml), thiol solution (0.5 ml) and OPA solution (2 ml) respectively. The volume was made upto the mark and the absorbance of the product (1-alkylthio-2-alkyl-substituted isoindole) [10] was noted at .335 nm (the wavelength of excitation).

Conclusion

The reaction is applicable to all the amines and amino acids which have the primary amino (-NH_2) group(s). Only those amino acids which have been studied are reported. The beauty of this OPA-based method is that the reaction is specific, rapid, reproducible and quantitative.

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