AMINO ACID DETERMINATION IN THE NANOMOLE RANGE BY tRNA CHARGING AND ISOTOPE DILUTION TECHNIQUE

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1. Introduction

The method developed almost 20 years ago by Moore and Stein [1] for determining amino acids with the aid of ion-exchange chromatography is excellently suited for the determination of all amino acids in one or in a few samples. However, in spite of extensive automation it is too complicated for measuring one or only a few amino acids in many samples. The enzymatic determination of individual amino acids is also usually complicated. With a few exceptions [2–5] the necessary enzymes are not easily accessible, in some cases poorly defined or lead to products, which are difficult to determine [6], as is true of the usually specific decarboxylases. Individual amino acids may be detected by microbiological methods [7]. However, this method is tedious and requires considerable expenditure and experience. For many amino acids, as for example Arg, His, Leu, Lys, Met, Phe, Thr, Trp and Val, a rapid and uniform determination method would be of advantage.

The method outlined in the following offers the possibility of determining all L-amino acids which take part in protein biosynthesis. The method utilizes the very specific charging reaction of transfer ribonucleic acids (tRNA) with amino acids by aminoacyl-tRNA synthetases according to the following reaction:

$$\text{Amino acid} + \text{tRNA} \xrightarrow[\text{ATP, } Mg^{2+}]{\text{Synthetases}} \text{Aminoacyl-tRNA}$$

combined with the isotope–dilution technique.

For this purpose an exactly known quantity of the amino acid to be determined is added in radioactive form to the amino acid mixture. The specific radioactivity of the labelled amino acid is reduced by the corresponding 'cold' amino acid of the sample, depending on its content and is transferred in this 'dilution' to the limiting tRNA. With an appropriate calibration curve the content of the amino acid desired is determined by comparing the test sample with a standard.

2. Material and methods

2.1. tRNA from *E. coli*, ATP, glutathione are commercial products of Boehringer Mannheim GmbH; [^{14}C]amino acids 'Stan STAR' of Schwarz/Mann.

2.2. Aminoacyl-tRNA synthetases (synthetases) (EC 6.1.1.–) may be obtained from *E. coli* according to [8] or by the following procedure: *E. coli* cells are disrupted and the extract centrifuged free from cell components, mixed with streptomycin sulfate (about 1.5 ml, 5% solution/10 ml extract). After centrifuging the supernatant is mixed with ammonium sulfate and the fractions between 30% and 50% saturation are

* After this paper was submitted for publication and was also discussed at the International Congress of Biochemistry, Stockholm (Abstracts, p. 124), we found, that already in 1970 J.B. Rubin and G. Goldstein had used the same principle for amino acid determination with similar results (Anal. Biochem. (1970) 33, 244).

We therefore considered withdrawing the paper, but then decided to resubmit it with this footnote, since we felt, that this very useful method had not become widely enough known.

centrifuged. The precipitate is dissolved in a small volume of buffer solution (1 M KCl; 0.01 M Tris–HCl; 0.01 M $MgCl_2$; 0.1 M dithiothreitol; 5% glycerol; pH = 7.9). The solution is transfered to a column of agarose A 1.5 M, equilibrated with the same buffer. Chromatography is performed with the same buffer. The ultraviolet extinction of the fractions is measured at 280 nm and 260 nm. The fractions of the second of the three main peaks are collected and made up to 80% saturation (about 3.2 M) with ammonium sulfate. In this form the mixture of the synthetases is stable for many weeks when stored at 0°C to +4°C.

2.3. The charging of the tRNA with amino adis was carried out according to known methods [8, 9]. The concentration range within which the individual amino acids can be measured is determined in a dilution experiment [10]. The I_0/I_x-values were determined according to [11], see also 2.4.

2.4. Establishment of calibration lines and carrying out the measurements.

The following quantities of the amino acid to be determined are employed for a measurement in 50 µl: 5, 10 and 20 nmoles [^{12}C]L-amino acid of a standard solution and per calibration sample 2 nmoles [^{14}C]L-amino acid (50 µCi/µmole); 0.1 ml ATP solution (10 mM) and 0.2 ml buffer solution (0.1 M Tris–HCl pH = 7.4; 10 mM $MgCl_2$: 10 mM KCl; 2 mM glutathione); 50 µl tRNA solution (4 mg tRNA/ml). The measurement is started in each case with 50 µl synthetases solution (3 mg protein/ml); total volume = 0.5 ml. After 10 min incubation at 37°C the reaction is stopped by adding 3 ml 5% trichloroacetic acid and cooled in the ice-bath. Thereafter, the mixture is filtered through glass filters (Whatman GF/C), washed, dried and the radioactivity measured on the filter in a liquid scintillator. The quotient of I_0 (= cpm of the standard) and I_x (= cpm of the sample) is plotted against the predetermined amount of amino acid. The amino acid content of the test sample is determined by using 50 µl of the solution to be investigated, in which volume 2 and 25 nmoles of the amino acid should be contained. It is of course in principle possible to carry out the measurements in substantially smaller volumes (for example 0.1 ml instead of 0.5 ml total volume).

2.5. Serum samples are deproteinized prior to the measurement by addition of 5% trichloroacetic acid, centrifuging the precipitate, extracting the supernatant with ether after addition of HCl (0.1 M), drying of the test sample and dissolving the residue in the test buffer.

3. Results

Optimization of the analysis conditions for individual amino acids are regards the buffer type and concentration, salt content, tRNA charge and synthetases activities or pH is not at all a prerequisite of a generally applicable method for determining many different amino acids. The sole requirement was that of obtaining a linear calibration line of adequate slope. This slope may even vary from test series to test series without influencing the accuracy of the determination.

Some of these calibration curves are shown in fig. 1. When using 50 µl of the sample they permit determination of the amino acid investigated in the range of 2–25 nmoles/measurement (0.4–0.5 µmoles/ml).

To test the method under the minimum requirements accepted as regards the test optimization, five amino acids not readily accessible to enzymatic measurement were first determined: Phe, Lys, Val. Met and Ser. The results of two determinations in each case are shown in table 1.

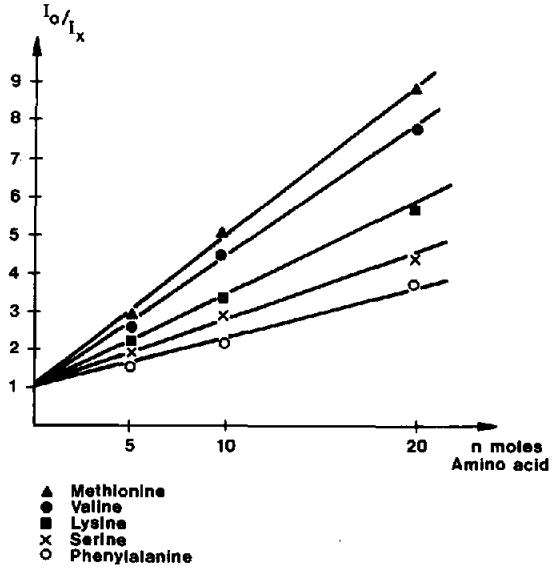

Fig. 1.

Table 1
Determination of five amino acids from standard solutions.

Amino acid/50 μl	Phe		Lys		Val		Met		Ser	
nmoles given	5.0	10.0	5.0	10.0	5.0	10.0	5.0	10.0	5.0	10.0
nmoles found	5.4	10.4	5.3	9.9	5.2	9.7	5.0	9.8	4.7	9.3

By the same method various amino acids were determined in artificial mixtures, a pharmaceutical preparation, an insulinhydrolysate and in serum samples. The results of these measurements are shown in table 2.

4. Discussion

After the initial tests the first determination of interest was that of the so-called essential amino acids because they are of special significance in nutrition. Only secondary investigations were then made of other amino acids. The results are shown in table 2.

According to the present results it appears possible to determine those amino acids participating in protein biosynthesis, with this method rapidly and with an accuracy of at least ±15% in the nmole range. No mutual interference was observed.

So far, Ala, Asp, Asn, Glu, Gln have not been determined because they can be measured with simpler enzymatic analyses [2–5]. Cysteine has also not been measured because it is present in the mixture partly or completely in oxidized form as cystine or cysteate and consequently cannot be directly measured with the method described.

The measured values obtained so far for Gly and Trp are subject to pronounced fluctuation. This is possibly due to instability of the synthetase preparation used or too low content of the specific tRNA. In this case, in order to obtain a quantitative analysis the test conditions must be further improved. The results recently reported by Bonnet and Ebel should be taken into account here [13].

Since amino acid values, for example in serum, are subject to pronounced variations – the values given in the literature differ by a factor of 2–3 [13, 14] – the error tolerance of the present method is sufficiently small for a routine determination. The accuracy of the tRNA charging reaction, on which the determination is based, is itself ±7% [9]. Consequently, repeated measurement of a sample can hardly be expected to result in any appreciable reduction actively of the error tolerances.

It is very important to use the tRNA in limiting ammount. The specific tRNA must be present in the test only in such a small quantity that it is completely charged, even by the undiluted radioactively-labelled amino acid alone. If this is not the case the calibration line does not intersect the ordinate at the point 1 or is not straight. This can result in considerable errors in the measurements.

The advantage of the method is to be seen in particular in the fact that all amino acids mentioned can be determined by the same procedure. The method is particularly suitable for determining one or only a few amino acids in several samples because for a measuring series per amino acid only one calibration line has to be plotted. Another advantage is the small amount of sample which required. Thus, the amino acids specified in table 2 can readily be determined in only 0.5 ml serum (amino acid content of the serum: 0.1–0.3 μmoles/amino acid/ml serum) [13, 14].

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Table 2

Amino acid	Artificial mixture (1)		Synthetic mixture (2)		Amino acid infusion solution (3)			Serum		Insulin hydrolysate (4)		
	nmole Given	nmole Found	μmole Acc. to Moore and Stein	μmole Found	Manufacturer's figures g/ℓ	nmole/50 μl	Found nmole/50 μl	μmole Acc. to Moore and Stein/ 0.8 ml serum	μmole Found	nmole by weighting	nmole found by hydrolysis (a)	(b)
Arg	8.0	8.5	0.143	0.160	6.0	(a) 8.65	9.1	0.184	0.168	10	8	9.8
His					1.5	(b) 4.83	5.7	0.103	0.124			
Ile	15.0	15.1	0.228	0.256						20	21	24.7
Leu	15.0	15.1	0.231	0.236	3.7	(a) 7.05	7.2			60	52.8	62.1
Lys	8.0	8.0	0.165	0.176	3.3	(a) 5.6	5.6	0.196	0.192	10	9.1	10.7
Met	15.0	14.3	(0.203)		2.15	(a) 3.6	3.9	0.022	0.023	0	0	0
			0.102	0.112								
Phe	8.0	7.5			2.55	(a) 3.4	3.8	0.101	0.112	30	25.6	–
Ser	8.0	7.7	(0.286)					0.330[5]		30	29.2	34.3
			0.143	0.132				(0.274)	0.270			
Thr	8.0	7.0	(0.264)		2.2	(a) 4.6						
Tyr	15.0	12.9	0.167	0.160				0.093	0.088	40	–	44.0
Val	8.0	8.5	(0.259)		3.1	(a) 6.1	5.0	0.356	0.379	40	31.2	36.7
			0.130	0.140								

(1) In the artificial mixture all twenty natural amino acids were used to test for any mutual interference. Only the amino acids indicated were measured.
(2) Met, Ser, Thr and Val were present as D,L-amino acids (values in brackets); only the L-amino acids can be determined.
(3) The manufacturer's figures (g/ℓ) were converted to nmoles/50 μl.
(4) Crystallized insulin of the company Hoechst AG was used.
(5) This value is too high because it includes asparagin; corrected value according to[1] below in brackets.
(a) Starting solution diluted 1:200.
(b) Starting solution diluted 1:100.

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