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Analytica Chimica Acta 315 (1995) 145–151

ANALYTICA CHIMICA ACTA

Continuous kinetic method for the quantitative resolution of structural isomers of arginine and ornithine

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Received 24 April 1995; accepted 29 May 1995

Abstract

A flow injection method for the determination of L-arginine and L-ornithine based on the inhibition of L-histidine crystallization was developed. The open–closed system used permits turbidimetric multidetection of the signal in the crystallization of L-histidine in the presence of an organic solvent (2-propanol). The proposed method permits the selective determination of L-arginine (0.3–12 mg l^{-1}) and L-ornithine (0.5–20 mg l^{-1}) in the presence of their D-enantiomers and other L-amino acids without the need for a prior separation. The relative standard deviation was ca. 2.3% ($n = 11$). Both amino acids were determined satisfactorily in pharmaceutical preparations, and their recovery after addition to samples was $100 \pm 3\%$. The sampling frequency was 7 ± 1 h^{-1}.

Keywords: Turbidimetry; Flow system; L-Arginine; L-Ornithine; Pharmaceutical preparations

1. Introduction

About 150 amino acids are known to occur in free or bound form in various cell types and tissues, but never in proteins. Among them is ornithine, the precursor for arginine in many species that act as carriers for carbon and nitrogen in the urea cycle. On the other hand, arginine is an essential amino acid and an intermediate product in the urea synthesis [1].

In recent years, there have been impressive breakthroughs in analytical techniques for separation of enantiomers. Liquid chromatography (LC) is the separation technique most frequently used for their discrimination [2–5]. However, these techniques are not yet suitable for large-scale applications, so industrial resolution of racemic mixtures is still largely performed "Pasteur-like" through fraction crystallization of aggregated or diastereoisomers [6]. Various methods have been proposed for the kinetic resolution of amino acid enantiomers based on the interaction between a crystallizing substrate and a stereospecific "tailor-made" growth inhibitor [7–10]. Amino acids have also been determined by using flow injection (FI) methods coupled to LC with chemiluminescence [11,12] or electrochemical detection [13]. Several methods using FI systems have been reported for the turbidimetric determination of diphenhydramine [14] and sulphate [15]. A configuration for multidetection with a single detector where entrapment of the sample plug in a closed system

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SSDI 0003-2670(95)00301-0

allows the kinetic determination of partial reaction orders and rate constants has also been developed [16]. A continuous turbidimetric method for the discrimination of L- and D-lysine by spectrophotometric multidetection was recently reported [17].

The aim of this work was to develop a continuous-flow method for the discrimination of L- and D-enantiomers of arginine and ornithine based on the interaction between a crystallizing substrate and a stereospecific "tailor-made" growth inhibitor. The use of an open-closed system allows the derivation of kinetic parameters for the crystallization of L-histidine in the presence and absence of L-arginine and L-ornithine.

2. Experimental

2.1. Apparatus

A Unicam 8625 UV–visible spectrophotometer equipped with a Hellma 178.12 QS flow cell (10 mm light path, 1 mm i.d., 18 μl) and connected to a Radiometer REC-80 Servograph recorder was used. Turbidimetric measurements were made at 550 nm. Two Gilson Minipuls-2 peristaltic pumps fitted with poly(vinyl chloride) and Solvaflex pumping tubes for aqueous and organic solutions, respectively, two Rheodyne 5041 injection valves and PTFE tubing (0.5 mm i.d.) for coils were also used.

2.2. Reagents

L-Histidine, L-arginine, L-ornithine and the other amino acids used were supplied by Sigma (St. Louis, MO). The organic solvents (2-propanol, ethanol, methanol and acetonitrile), sodium hydroxide and hydrochloric acid were purchased from Merck (Darmstadt).

Solutions of L-histidine (10 g l^{-1}), L-arginine (1 g l^{-1}) and L-ornithine (1 g l^{-1}) were prepared in Milli-Q water (Millipore) and remained stable for at least a week.

2.3. Samples

The contents of three oral ampoules (Dynamogén, Productos Farmacéuticos FAES; Sargenor, ASTA Médica) or injectable ampoules (Sedionbel, ALTER) were dissolved in 250 ml or 25 ml of Milli-Q water, respectively. Aliquots of 0.5–1.5 ml of these solutions were further diluted to 10 ml. For continuous flow-analyses, aliquots of 100–250 μl of these final solutions were placed in 10 ml calibrated flasks and diluted to the mark after the pH was adjusted to 7.0–8.2 with 0.01 M NaOH or HCl.

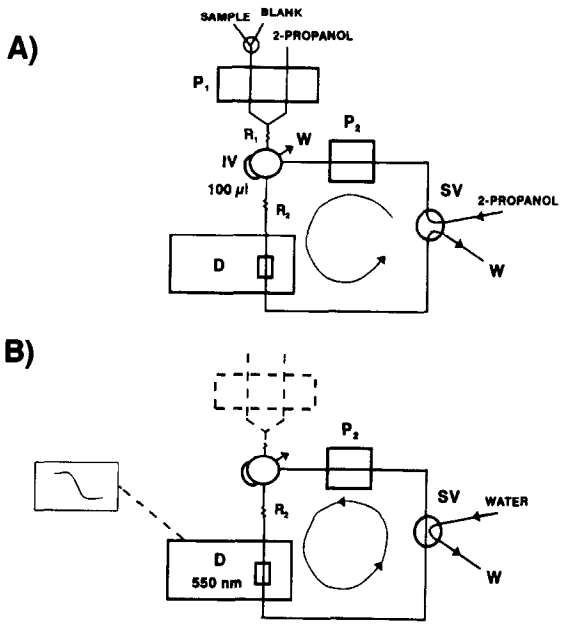

Fig. 1. Experimental set-up for the continuous determination of L-arginine and L-ornithine. (A) Introduction of the sample and 2-propanol streams into the system. (B) Closed system for signal multidetection. P, pump; R, reactor; IV, injection valve; W, waste; SV, switching valve; D, spectrophotometer.

2.4. Procedure

The continuous flow manifold used for the discrimination and determination of L-arginine and L-ornithine is depicted in Fig. 1. First (Fig. 1A), the pharmaceutical preparations or standard solutions containing between 0.3 and 12 mg l^{-1} L-arginine or 0.5–20 mg l^{-1} L-ornithine plus 1.8 g l^{-1} L-histidine (flow-rate 0.5 ml min^{-1}) at pH 7.0–8.2 were continuously merged with a stream of 2-propanol (flowing at 1.1 ml min^{-1}). Both solutions were mixed in coil R_1 (75 cm) and then passed through the injection valve (IV). Simultaneously, the second pump (P_2)

was used to propel a stream of 2-propanol (the carrier) at 1.0 ml min^{-1} through the open system. Second (Fig. 1B), the loop contents (100 μl) of IV were injected into the carrier and valve SV was switched in order to establish a closed circuit. At this stage, changes in crystal growth of L-histidine were recorded at 550 nm until physico-chemical equilibrium in the system was attained. A blank solution containing 1.8 g l^{-1} L-histidine was employed throughout. After each determination, SV was actuated to flush the open-closed system with Milli-Q water.

3. Results and discussion

L-Arginine and L-ornithine exhibit an inhibitory effect on the crystallization of L-arginine, which permits their indirect determination. Fig. 2 shows three typical signals obtained in the crystallization of L-histidine by using the manifold of Fig. 1 for a blank (Fig. 2A) and samples of L-arginine (Fig. 2B) or L-ornithine (Fig. 2C). The first part of the signal profile (t_1) in Fig. 2 corresponds to the homogenization of the closed loop and the signal variation is due to index refraction changes from pure 2-propanol to the 2-propanol–water (11:5, v/v) mixture. After t_2, L-histidine crystallization started; the induction period (t_3) was geometrically calculated as shown in Fig. 2A.

There are three ways to derive analytical information from the signals, namely: from the time required for crystallization to start (t_2), the induction period (t_3) or the crystallization rate (slope of the linear portion of the kinetic curve). The slopes were inadequately different at various concentrations analyte (L-arginine or L-ornithine) for implementing an analytical method. The induction period was thus selected as the analytical parameter because it resulted in better reproducibility in the measurements than the start of the crystallization time.

3.1. Optimization of chemical variables

An automated system similar to that depicted in Fig. 1 was used to optimize chemical variables. The solutions employed for this purpose were as follows: a blank containing 2.0 g l^{-1} L-histidine and samples containing 2.0 g l^{-1} L-histidine plus 5 or 10 mg l^{-1} L-arginine or L-ornithine, respectively.

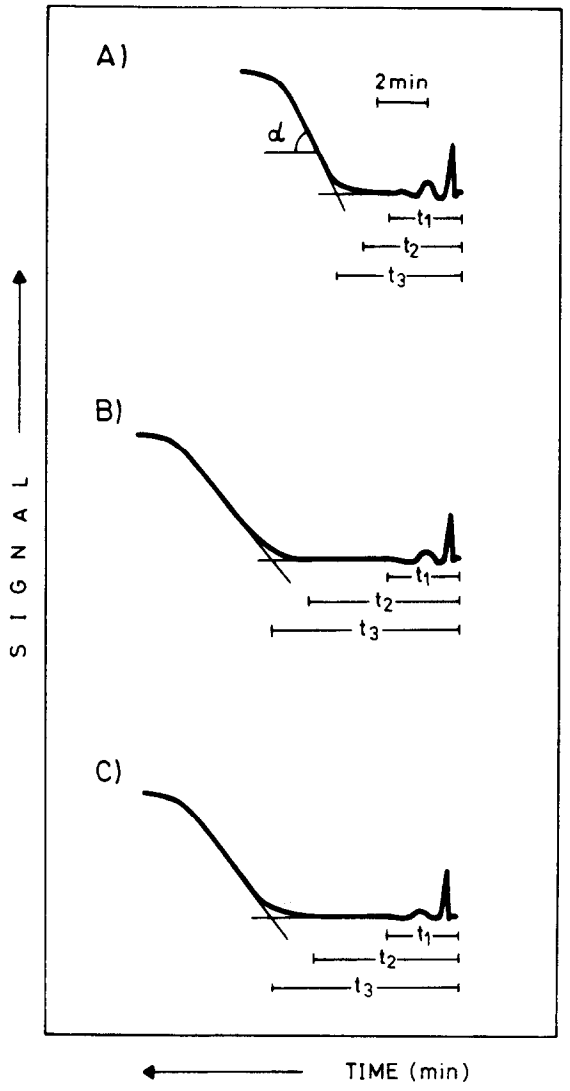

Fig. 2. Signal profile for the crystallization of 1.8 g l^{-1} L-histidine alone (A) and in the presence of 5 mg l^{-1} L-arginine (B) or 10 mg l^{-1} L-ornithine (C). t_1, homogenization time; t_2, start of crystallization time; t_3, induction period.

The effect of pH on L-histidine crystallization was studied between pH 6 and 10 (adjusted with 0.01 M HCl or 0.01 M NaOH). The optimal pH range was found to be from 7.0 to 8.2 (the isoelectric point for L-histidine was 7.5). Outside this range, the crystallization of L-histidine was significantly slower.

Therefore, a sample pH of 7.7, obtained by dilution of the blank and sample with water, was selected.

Supersaturated solutions of L-histidine were prepared by altering the solvent composition. Thus, addition of an organic solvent to an aqueous solutions of this amino acid provided a supersaturated solution. Three organic solvents (methanol, ethanol and 2-propanol) were mixed with the aqueous sample to induce the crystalline growth of L-histidine. The start of crystallization time for L-histidine in methanol and ethanol was longer (ca. 30 and 15 min, respectively) than in 2-propanol (ca. 4 min). Therefore, 2-propanol was selected as the organic solvent and also as the carrier for the open-closed system. Once the organic solvent was selected, several calibration graphs for each amino acid (L-arginine and L-ornithine) were constructed in order to choose the best L-histidine concentration. Fig. 3A and 3B show the calibration graphs run for L-arginine and L-ornithine, respectively, at three different concentrations of L-histidine (1.6, 1.8 and 2.0 g l^{-1}). As can be seen, the induction period for L-histidine increased with decreasing L-histidine concentration. Crystallization of L-histidine was more favoured by the presence of L-ornithine than by that of L-arginine; therefore, the slope of the calibration graph was steeper and the sensitivity greater in the determination of L-arginine. A concentration of 1.8 g l^{-1} was selected as a compromise between adequate sensitivity and sampling frequency.

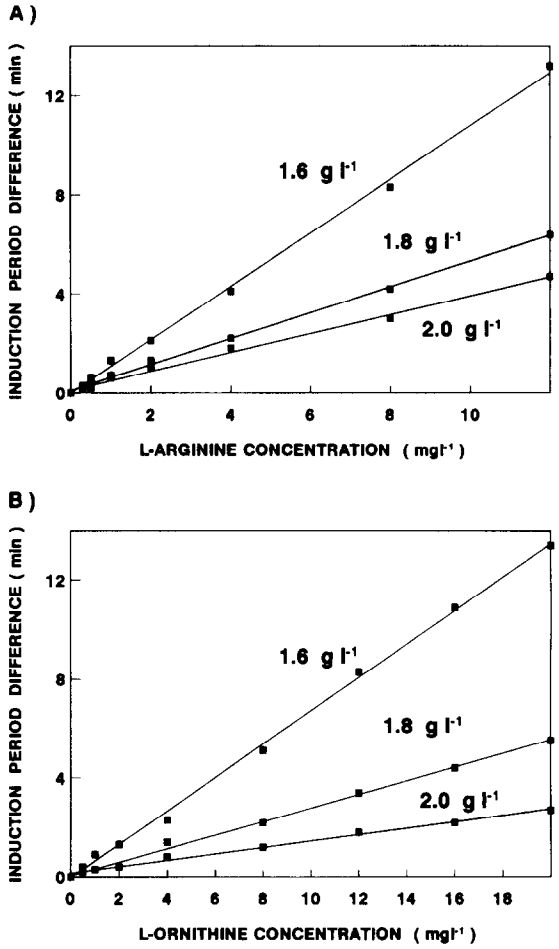

Fig. 3. Calibration graphs for L-arginine (A) and L-ornithine (B) at different concentrations of L-histidine.

3.2. Optimization of flow-injection variables

The effect of flow variables related to the L-histidine crystallization, viz. flow-rates, coil lengths and injected volume, was investigated. For this purpose, a blank containing 1.8 g l^{-1} L-histidine at pH 7.7 or a sample containing 1.8 g l^{-1} L-histidine plus 5 or 10 mg l^{-1} of L-arginine or L-ornithine, respectively, at pH 7.7 was merged with the 2-propanol stream.

The influence of the flow-rates of the aqueous sample and 2-propanol streams was studied by changing the 2-propanol flow-rate while keeping that of the sample constant at 0.5 ml min^{-1}. The effect of increasing the organic phase flow-rate was similar to that of increasing the organic solvent volume in the final mixture and also of sample dilution. Fig. 4 shows the variation of the induction period as a function of the flow-rate ratio of sample to 2-propanol. As expected, the length of the induction period decreased with increasing flow-rate ratio for the blank and samples. However, the reproducibility of the measurements decreased with decreasing 2-propanol flow-rate. A sample flow-rate of 0.5 ml min^{-1} and an organic flow-rate of 1.1 ml min^{-1} were chosen (i.e., a flow-rate ratio of 0.45) taking into account the mutual influence of reproducibility and sampling frequency. The effect of flow-rate of carrier (2-propanol) in the closed system was studied over the range 0.6–1.4 ml min^{-1}. Decreasing flow-rates slightly decreased the induction period for L-histidine crystallization through increased dispersion of the sample in the closed loop. The analytical

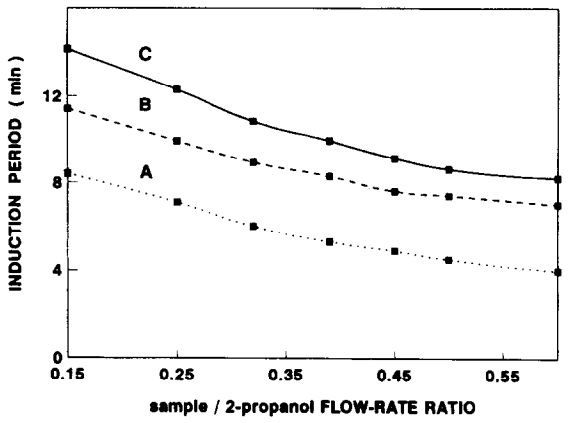

Fig. 4. Influence of the flow-rate ratio of sample to 2-propanol on the crystallization of a blank containing 1.8 g l^{-1} L-histidine alone (A), and 1.8 g l^{-1} L-histidine plus 10 mg l^{-1} L-ornithine (B) or 1.8 g l^{-1} L-histidine plus 8 mg l^{-1} L-arginine (C). Sample flow-rate, 0.5 ml min^{-1}.

signal for the sample and blank remained constant above a 2-propanol flow-rate of 0.8 ml min^{-1} because the sample was rapidly dispersed. A 2-propanol flow-rate of 1 ml min^{-1} was thus chosen to introduce the sample loop contents into the closed system.

The injection valve (IV in Fig. 1) could theoretically be placed in various locations in the flow system; however, the best results were achieved when it was placed between pump 2 and the detector. The sample volume injected into the 2-propanol line had a significant effect on the induction period for L-histidine crystallization. The induction period decreased with increasing injected volume (between 50 and 180 μl), however, the reproducibility decreased as a result of increasing homogenization time of the sample plug. An injected volume of 100 μl was chosen as compromise for all subsequent experiments. The tubing length between the mixing point for the sample and 2-propanol stream (R_1 in Fig. 1) and the injection valve did not affect the signal above 50 cm at an inner diameter of 0.5 mm. A homogenization coil of 75 cm was thus selected. The influence of the residence time before detection was studied by changing the length of coil R_2 between 25 and 200 cm. The induction period increased with increasing length of R_2 because the residence time in the closed system also increased (the sample and blank were more diluted because the overall volume of the closed system increased with coil length), which delayed L-histidine crystallization. A coil length of 50 cm was chosen for further experiments. Under the above optimal conditions, L-histidine crystal formation was clearly inhibited by the presence of L-arginine and L-ornithine.

Temperature can affect crystal growth. We therefore studied its effect on L-histidine crystallization in the absence (blank) and presence of other amino acids (sample) by immersing the reaction coil (R_2) in a thermostated bath that was held at different temperatures over the range 10–50°C. The temperature had no effect on the induction period for L-histidine crystallization (neither in the blank nor in the sample) above 15°C. Therefore, it is quite acceptable to work at room temperature.

3.3. Analytical figures of merit

The figures of merit of the calibration graphs (induction period versus concentration) obtained for each amino acid assayed with the proposed continuous method were calculated from results obtained by introducing a sample containing 1.8 g l^{-1} L-histidine plus different amounts of L-arginine (0.3–12 mg l^{-1}) or L-ornithine (0.5–20 mg l^{-1}). The equations for the graphs, obtained from 7 points in each instance, were as follows:

$$T = 0.09 + 0.52[\text{L-Arginine}] \quad (r = 0.997)$$

$$T = 0.04 + 0.27[\text{L-ornithine}] \quad (r = 0.998)$$

where T is the difference between the induction period of the sample and the blank, in min, and concentrations are given in mg l^{-1}.

The detection limits (0.2 and 0.3 mg l^{-1} for L-arginine and L-ornithine, respectively) were calculated as three times the standard deviations of the induction periods for 11 injections of blank (1.8 g l^{-1} of L-histidine). The relative standard deviation, obtained by measuring 11 samples of L-arginine (5 mg l^{-1}) or L-ornithine (10 mg l^{-1}) was 2.3 or 2.6%, respectively. The sampling frequency was 7 ± 1 h^{-1}.

3.4. Selectivity

The influence of a number of common amino acids accompanying L-arginine and L-ornithine in

pharmaceutical preparations was studied in order to detect potential interferences. For this purpose, variable volumes of stock solutions of the different potential interferents were added to the samples (containing 1.8 g l^{-1} L-histidine plus 5 or 10 mg l^{-1} L-arginine or L-ornithine, respectively) in order to determine tolerated levels. As can be seen from Table 1, most of the species tested are tolerated at high concentrations by the proposed flow method. The D-enantiomers of L-arginine and L-ornithine did not interfere in the determination of the analytes at the concentrations assayed. Only L-glutamic acid posed a serious interference. Because the proposed method is more sensitive to L-arginine, L-ornithine is tolerated at similar concentration levels.

3.5. Application to pharmaceutical preparations

The proposed method cannot be applied to L-ornithine in pharmaceutical products also containing L-arginine at a similar concentration. The above procedure described for determining L-arginine and L-ornithine was applied to several commercially available preparations. Samples were dissolved as described under Experimental. The results obtained in five determinations of L-arginine or L-ornithine and their standard deviations are listed in Table 2. All of them were consistent with the nominal contents, which confirms the high accuracy and precision of the proposed method.

Finally, in order to test the reliability of the proposed method for the determination of both amino acids, their recovery was determined in three additions of standards to each pharmaceutical preparation prior to dissolution in water (the final concentration of L-arginine or L-ornithine in the diluted samples were 2, 4 and 6 mg l^{-1}). The recoveries obtained in the individual additions of equal amounts of L-arginine or L-ornithine were close to 100% (97.3–102.9%) in all instances.

Table 1
Maximum tolerated weight ratios for amino acids in the determination of L-arginine (5 mg l^{-1}) and L-ornithine (10 mg l^{-1})

Amino acid	L-Arginine	L-Ornithine
D-Ornithine	–	> 60
D-Arginine	> 120	–
L-Leucine, L-methionine, L-isoleucine, L-phenylalanine, L-alanine, L-tyrosine	> 20	> 10
L-Valine, L-serine, L-asparagine	8	3
L-Glutamine, L-aspartic acid, L-threonine, L-cysteine	3	2
L-Lysine	2	1.5
L-Glutamic acid	0.6	0.5
L-Ornithine	1	–
L-Arginine	–	0.2

Table 2
Determination of L-arginine and L-ornithine in pharmaceutical preparations

Trade name	L-Arginine		L-Ornithine	
	Nominal content (g l^{-1})	Found [b] (g l^{-1})	Nominal content (g l^{-1})	Found [b] (g l^{-1})
Dynamogén [a]	56.7	58.6 ± 2.0	–	–
Sargenor [a]	113.4	109.7 ± 3.6	–	–
Sedionbel	–	–	7.1	6.8 ± 0.2

[a] L-Arginine is present as aspartate in the original sample.
[b] Mean ± standard deviation ($n = 5$).

4. Conclusions

Amino acids can be determined by turbidimetric methods based on adsorption processes during crystalline growth of other amino acids [7–10]. The proposed method has the same foundation as these manual methods, but is implemented in a continuous configuration, which facilitates automation and handling, thereby reducing sample and reagent consumption and manipulation, and increasing the precision. The high tolerance to D-enantiomers in the determination of L-arginine and L-ornithine by the proposed method can be used to detect potential adulteration of samples with such enantiomers.

Acknowledgements

This work was supported by Grant PB94-0450 from the Spanish CICyT.

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