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QUANTIFICATION OF ARGININE IN DIETARY SUPPLEMENT TABLETS AND CAPSULES BY SILICA GEL HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY WITH VISIBLE MODE DENSITOMETRY

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SUMMARY

A quantitative method using silica gel HPTLC plates, automated band-wise sample application, detection with ninhydrin chromogenic reagent solution, and automated visible mode densitometry has been developed for determination of L-arginine hydrochloride in nutrition supplements containing a variety of other active and inactive ingredients. Accuracy was validated by analysis of spiked blank and standard addition samples and precision by performing replicate analyses on a single day and on different days. Recoveries from spiked blank and standard addition samples were 98.8% and 98.5%, respectively. Repeatability for two samples, each of which was analyzed six times on a single plate, was 1.92% and 0.674% relative standard deviation (*RSD*). The intermediate precision was 1.72% *RSD* for a sample analyzed in duplicate once per plate on four different days over a seven-day period. A survey was made of arginine content compared with label values for four commercial supplement products, using the new method. The method was shown to be suitable for routine analysis of nutritional supplements in a manufacturing quality-control or regulatory agency laboratory.

INTRODUCTION

The amino acid arginine is an important building block of proteins. Although it can be manufactured by the body with proper nutrition (i.e. it is a 'non-essential' amino acid), studies showing it to be involved in a number of important functions in the human body have led to the increased popularity of arginine nutritional supplements. These supplements are used

to treat high cholesterol, to improve immune responses to bacteria, viruses, and tumor cells, to promote healing and repair of soft tissue, to cause release of growth hormones, to facilitate optimum muscle growth and formation of collagen, and to build new bones and tendons. Arginine creates nitric oxide in the body and helps improve blood flow and maintain a proper nitrogen balance, all of which are important in male and female sexual function. A recent trend is, therefore, inclusion of arginine as an active ingredient in many new sexual-support products. Because of the proliferation of non-prescription dietary supplements containing arginine in the form of the free acid or the hydrochloride, both of which are being consumed in increasing amounts with and without the advice of a physician, rapid and simple qualitative and quantitative methods are required for their determination by manufacturers and government regulators.

The standard method for determination of arginine in dietary supplements involves use of an amino acid analyzer based on column ion-exchange chromatography or on precolumn derivatization then reversed-phase high-performance liquid chromatography (HPLC) [1,2]. In thin-layer chromatography (TLC) and high-performance TLC (HPTLC) studies in our laboratory, amino acid separations on normal-phase, reversed-phase, and ion-exchange layers were compared [3], and methods were developed for quantitative determination of amino acids in snails and snail parasites [4–9] and snail-conditioned water [10], and for assay of vitamins [11], creatine [12], and lutein [13] in nutritional supplements. A computer-assisted search of the literature through *Chemical Abstracts* and the *ISI Web of Science* found no papers on TLC or HPTLC analysis of arginine in amino acid dietary supplements. In this research, therefore, HPTLC methods described previously for biological samples were adapted for analysis of arginine supplements of varying complexity using a silica gel HPTLC layer, automated band-wise sample application, detection with ninhydrin spray reagent, and densitometric scanning. The new method was validated for sensitivity, linearity, accuracy, and precision and used in a survey of the content of four different supplement products.

EXPERIMENTAL

Preparation of Standard and Sample Solutions

A stock standard solution of L-arginine hydrochloride (#A-6969, Sigma, St Louis, MO, USA; CAS registry no. 1119-34-2; 99.4% purity) was

prepared at a concentration of 100 mg mL^{-1} in ethanol–deionized water (7:3). The TLC standard was prepared at 0.100 mg mL^{-1} in the same solvent mixture by direct weighing or by appropriate dilution of the stock solution.

Four different arginine dietary supplement products were purchased from local health and diet food stores. Products A and B were capsules containing only 500 mg L-arginine (free acid) as active ingredient; Product C was a supplement tablet containing 500 mg L-arginine hydrochloride plus vitamins A, C, E, B6, B12, thiamine, riboflavin, niacin, folic acid, biotin, pantothenic acid, zinc, selenium, ginseng, and ginko biloba; Product D was a tablet containing 453 mg L-arginine hydrochloride (equivalent to 375 mg arginine) plus glucosamine hydrochloride, vitamins C and B12, niacin, and boron. A sample stock solution of each was prepared by grinding one tablet or the contents of one emptied capsule to a fine powder by use of a mortar and pestle and transferring the powder through a funnel into a 100-mL volumetric flask by washing with ca. 70 mL ethanol–deionized water (7:3). The contents of the flask were stirred magnetically for 30 min and sonicated for 10 min to dissolve the arginine completely, after which the stirrer bar was removed by use of a magnetic rod. The solution was diluted to volume with ethanol–deionized water (7:3) and shaken. The flask was left to stand until any undissolved ingredients had settled to the bottom. Alternatively, undissolved ingredients were removed by filtering approx. 5 mL solution into a capped vial through a Pall Gelman (Ann Arbor, MI, USA) Acrodisc LC 13 mm syringe filter with 0.45 μm PVDF membrane. The theoretical arginine or arginine hydrochloride concentration of the solutions was 5.00 mg mL^{-1} except for Product D, which was 3.75 mg mL^{-1}, on the basis of the label declarations. TLC test solutions with theoretical concentrations of 0.0500 mg mL^{-1} and 0.0375 mg mL^{-1} (Product D) were prepared by pipetting 1.00 mL clear sample stock solution into a 100 mL volumetric flask and diluting to volume with ethanol–deionized water (7:3).

Thin Layer Chromatographic Analysis

Analyses were performed on 10 cm × 20 cm high-performance silica gel $60F_{254}$ GLP plates (no. 5613/6; EM Science, Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany). The plates were precleaned by development to the top with dichloromethane–methanol (1:1) and dried in a fume hood before use. Standard and sample solutions were applied to the plate by means of a Camag (Wilmington, NC, USA) Linomat IV automated spray-on band applicator equipped with a 100-μL syringe

and operated with band length 6 mm, application rate 10 s μL^{-1}, table speed 10 mm s^{-1}, distance between bands 4 mm, distance from the side edge 0.7 cm, and distance from the bottom 1.5 cm. The volumes applied for each analysis were 2.00 μL, duplicate 4.00 μL, and 8.00 μL of the arginine hydrochloride TLC standard (0.200–0.800 μg) and duplicate 8.00 μL of the sample solutions (0.400 μg theoretical content for all products except D, which was 0.375 μg).

Plates were developed to 6 cm beyond the origin with 1-butanol–glacial acetic acid–deionized water, 3:1:1, as mobile phase in a vapor-equilibrated Camag (Wilmington, NC, USA) twin-trough chamber containing a saturation pad (Analtech, Newark, DE, USA). The development time was approx. 1.5 h. After development the mobile phase was evaporated from the plate by drying in a fume hood for 10 min. The plate was then sprayed heavily and evenly with ninhydrin reagent (0.3 g ninhydrin dissolved in 100 mL 1-butanol plus 3 mL glacial acetic acid) and dried in a fume hood for approx. 10 min. Once dry, the plate was heated at 110°C for several min on a Camag plate heater to produce orange zones of arginine on a white layer background. Linear scanning at 495 nm using a Camag TLC Scanner II with tungsten source, slit length 4, slit width 4, and scanning rate 4.0 mm s^{-1} was used to measure the zones of standards and samples. The CATS-3 software controlling the densitometer produced a calibration curve by linear regression of the weights and areas of the standard zone scans and automatically interpolated the weights of the sample zones from their scan areas. Because an arginine hydrochloride standard was used, all results were obtained on the basis of this compound. Multiplication by the ratio 174.2/210.7 (molecular weight of arginine/molecular weight of arginine hydrochloride) converted the results to arginine for those products containing the hydrochloride of the amino acid. For each analysis, recovery (%) was calculated by dividing the average experimental weight of duplicate samples by the theoretical weight predicted by the label declaration and multiplying by 100.

Validation

The accuracy of the new method was validated by spiked blank and standard addition analyses. A 500 mg L-tyrosine capsule was chosen as the blank because it contained all of the inert ingredients (excipients) present in Products A and B (gelatin, povidone, silica, and vegetable magnesium stearate), and the tyrosine active ingredient separated from arginine on the layer. To prepare the blank solution, a tyrosine capsule test solution

was prepared as described above except that deionized water rather than ethanol–deionized water (7:3) was used as the solvent, and 5.00 mL of the arginine hydrochloride stock solution was added by pipet to the 100 mL volumetric flask before dilution to volume with water to simulate a 500 mg arginine hydrochloride capsule solution containing exactly the label amount. An unspiked solution of the blank capsule was prepared identically. The spiked and unspiked solutions were diluted 1:100 with ethanol–deionized water (7:3) and analyzed as described above. Recovery was calculated by comparing the mean analytical result for the spiked blank solution with the theoretical value based on the weight of arginine hydrochloride added.

A standard addition validation analysis was performed for Product C. A solution of the tablet was prepared in a 100-mL volumetric flask, as described above, and 1.00 mL was placed in a second 100-mL volumetric flask and mixed with arginine hydrochloride stock solution (100 mg mL^{-1}, 50.0 μL), measured with a 100-μL Drummond (Broomall, PA, USA) digital dispenser. After 1:100 dilutions to prepare the test solutions, they were analyzed on the same plate by applying the usual standard volumes, duplicate 8.00 μL volumes of the unspiked solution, and duplicate 4.00 μL volumes of the spiked solution. The mean result from the spiked sample was compared with the theoretical result (the mean result from the unspiked sample plus the added weight) to calculate the recovery.

The precision (repeatability) of the method was evaluated by analyzing two Product B test solutions six times on one plate and calculating the relative standard deviation (*RSD*) of the recovery. Intermediate precision was determined by analyzing a single sample on different plates on four days over a seven-day period.

RESULTS AND DISCUSSION

Ethanol–deionized water (7:3) was an excellent solvent for arginine standard compound and was found to dissolve arginine from crushed nutritional supplements completely after 30 min magnetic stirring and 10 min ultrasonic mixing. Longer mixing or sonication times did not lead to greater recovery. After initial dissolution of the products in a 100-mL volumetric flask and removal of any undissolved excipients by settling or filtration, 1:100 dilution was required to bring the sample zone concentrations within the calibration range of the standards. This dilution was also performed with ethanol–deionized water (7:3), which is a suitable solvent for rapid application of compact initial zones using the Linomat. The tyro-

sine capsule used as a blank had to be dissolved in water, because of the poor solubility of this amino acid in ethanol.

Arginine formed a narrow, flat, band-shaped zone with an R_F of 0.18 on the silica gel HPTLC layer developed with 1-butanol–acetic acid–water, 3:1:1, as mobile phase (Fig. 1). Detection and quantification limits were approximately 150 ng per zone. The scanning wavelength of 495 nm was the absorption maximum of the in-situ spectrum of an arginine standard zone measured by use of the spectral mode of the Camag scanner. No other zones were detected in sample chromatograms except for that of tyrosine (R_F 0.58), present in the blank used in the validation study, and glucosamine (R_F 0.50), in Product D. Preparation of calibration curves by use of linear regression led to superior analytical results than use of polynomial regression. The value of r (correlation coefficient) for linear regression of the calibration plots (of scan area against weight for the four standards) was typically 0.997.

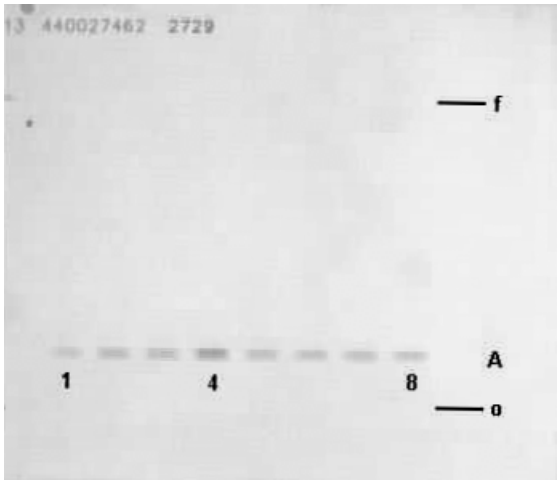

Fig. 1

Chromatograms obtained from 500-mg arginine tablets 1 and 2 (Product C) analyzed on an EM Science high-performance silica gel $60F_{254}$ GLP plate developed by the method described. The plate was photographed under white light using a Camag VideoStore image-documentation system. f, mobile phase front; o, origin; lanes 1–4, arginine (A) standards; lanes 5–8, duplicate (n = 2) samples of the two tablets

Table I shows results from analyses of the four arginine supplements by use of the new HPTLC method. The values for all the products

were within 90 to 110% of the label value, which is the range usually specified for pharmaceutical dosage forms in the USP [14] and found in most of our earlier surveys of commercial pharmaceutical products, e.g. Ref. [15]. Published ranges of legally acceptable ingredient content comparable with those in the USP are not available for amino acid or other nutritional supplements.

Table I

Recoveries (%) of arginine in products analyzed relative to the label values

Product	Sample	Recovery (%)	*n*	*RSD* (%)
A	Capsule 1	90.0	2	
	Capsule 2	103	2	
	Capsule 3	105	2	
B	Capsule 1	99.1	2	
	Capsule 2	106	6	1.92
	Capsule 3[a]	101	6	0.674
C	Tablet 1	94.1	2	
	Tablet 2	91.0	2	
	Tablet 3	94.2	2	
D	Tablet 1	109	2	
	Tablet 2	100	2	
	Tablet 3	90.9	2	

[a]Trial 3 of the intermediate precision study
n is the number of sample zones applied to the layer
RSD is the relative standard deviation

Preparing and analyzing a spiked blank sample as described above validated the accuracy of the method. Recovery of added arginine from the spiked tyrosine capsule solution was 98.8%, an error of 1.2%. Analysis of the unspiked blank solution showed that no interference occurred at the R_F of arginine, and therefore, no correction of the scan areas of the spiked blank solution was required.

Accuracy was also validated by standard addition analysis of a fourth tablet of Product C for which no suitable blank was available because of the complexity of its active and inactive ingredients. Spiked and unspiked solutions of this sample were analyzed on the same plate as described above. The tablet solution initially assayed at 98.5% recovery compared with the label declaration, and recovery of arginine spike from this solution was within 101.5% of the amount added (1.5% error).

Repeatability was determined by spotting six 8.00 μL aliquots ($n = 6$) of Product B sample solution instead of the usual duplicate sample aliquots ($n = 2$). As shown in Table I, the *RSD* was 1.92%. As a second measure of precision, percentage differences between experimental weights of duplicate samples and the peak areas of duplicate standards spotted in each analysis averaged 1.47%, with a range of 0.303–3.64%. Intermediate precision was evaluated by performing four replicate analyses of a third capsule of Product B on different plates, on the day of sample preparation, on the next two days, and on the seventh day after preparation. The average recovery of this sample was 101% relative to the label value, and the *RSD* of the analytical results was 1.72%. Analysis on day three was performed with six spotted aliquots to obtain a second value for repeatability; the *RSD* of this analysis ($n = 6$) was 0.674%. All these precision values are excellent considering that application of detection reagent by spraying was a step in the method.

It is apparent from the results given above that validation data for this new quantitative HPTLC method for analysis of arginine meet the acceptance criteria for accuracy, precision, linearity, and detection and quantification limits set by the International Conference on Harmonization (ICH) for assay of pharmaceutical products [16]. Further, the validation data are at least as good as values reported regularly in the literature for HPTLC and HPLC analysis of pharmaceutical products [17–20] and nutritional supplements [21]. For example, in the last reference repeatability was reported as 1.86% and intermediate precision 5.2% for HPTLC densitometric quantitative analysis of hyperforin in herbal drug products.

The new method will be valuable for use by manufacturing companies in qualitative or quantitative quality assurance analysis and by government laboratories for survey and regulation of a variety of commercial formulations and brands of arginine supplements. It can also be adapted for analysis of supplements containing mixtures of two or more amino acids if they can be separated adequately by HPTLC. R_F data we have published for numerous amino acids on silica gel, cellulose, reversed-phase C_{18}, and ion-exchange resin layers [3–6,10] will aid analysts in choosing appropriate systems to test for analysis of these mixed formulations.

The selectivity of the ninhydrin reagent for compounds containing an NH_2 group enables determination of arginine without interference in complex formulations, as demonstrated with Product C, which contained 17 additional active ingredients and eight excipients that did not give chromatographic zones. The method is easier to perform than column ion-

exchange chromatography or HPLC, and up to 16 individual samples, or eight samples spotted in duplicate, can be analyzed on a single plate along with the minimum of three standards needed to produce a calibration curve. This capability leads to increased accuracy, speed, and sample throughput and lower cost of solvent use and disposal per sample.

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