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Determination of arginine in dietary supplements

Michelle E Gange, Paul S Francis,* Jason W Costin, Neil W Barnett and Simon W Lewis

School of Biological and Chemical Sciences, Deakin University, Geelong, Victoria 3217, Australia

Abstract: We present a rapid and sensitive flow injection method for the determination of arginine in dietary supplements. Detection was based on the chemiluminescence reaction of arginine with alkaline hypobromite. The response is proportional to analyte concentration over the calibration range, from 2.5×10^{-6} to 1×10^{-4} M, and a relative standard deviation of 1% was calculated (1×10^{-5} M, $n = 12$). Samples required only aqueous dilution prior to analysis, and over 100 samples could be analysed per hour, which is superior to that achieved with conventional colorimetric and enzymatic procedures. The results obtained with the flow injection methodology were concordant with those achieved using high-performance liquid chromatography employing pre-column derivatisation and fluorescence detection.

Keywords: chemiluminescence; flow injection analysis; arginine; sports drinks; ergogenic aids

INTRODUCTION

L-Arginine is classified as a non-essential amino acid for humans, but it is considered essential for normal development of many other mammals until adulthood.[1] The biochemistry of this amino acid is complex and involves many key metabolic pathways.[1–3] L-Arginine is converted to L-ornithine and urea by arginase and is a precursor for creatine, which plays a crucial role in the energy metabolism of muscle, nerve and testis. It is also used in the synthesis of nitric oxide, which is involved in vascular relaxation. Arginine can act as a neurotransmitter and mediator of immune response and contributes to the cytotoxic activity of macrophages against tumour cells, bacteria and protozoa.[1–3]

As a pure powder or a component of free amino acid mixtures, L-arginine is marketed to athletes as a dietary supplement to improve cellular energy and muscle growth. For example, the Musashi™ range includes Arginine, a pure free-form amino acid, and mixed powders such as Chen, Energy Formula, Kuan and Muscle Gain Formula, which contain approximately 750, 350, 110 and 80 mg g^{-1} L-arginine respectively. The role of L-arginine in the production of nitric oxide and efficient blood flow has led to its incorporation in preparations that are designed to improve sexual performance.

Amino acids have been determined with a variety of techniques, including high-performance liquid chromatography (HPLC), ion exchange liquid chromatography, gas chromatography, thin layer chromatography, capillary electrophoresis, mass spectrometry and nuclear magnetic resonance spectroscopy.[4,5] In the case of liquid chromatography, which has been used extensively for this task, pre- or post-column derivatisation with ninhydrin, fluorescamine, *o*-phthaldialdehyde, dansyl chloride, dabsyl chloride, phenyl isothiocyanate or 9-fluorenylmethyl chloroformate is often employed to achieve the desired selectivity and sensitivity. Post-column or post-capillary chemiluminescence detection has been demonstrated with reagents such as luminol,[6–10] tris(2, 2′-bipyridine)ruthenium(III),[11] lucigenin[12] and peroxyoxalates.[13,14] In addition to the inherent high sensitivity of chemiluminescence detection, most of these approaches do not require pre-column derivatisation of the amino acids. Nevertheless, these detection systems have not been widely adopted, presumably owing to the plethora of readily available methods utilising UV-vis absorbance or fluorescence.

There are also numerous specific tests for individual amino acids. Most colorimetric approaches to the selective determination of arginine are based on the Sakaguchi reaction, involving hypohalites and 1-napthol or 8-hydroxyquinoline,[15–17] but other reagents have also been employed for the determination of free or bound arginine, including butane-2,3-dione monoxime,[18] 4-hydroxy-3-nitrophenylglyoxal,[19] *p*-nitrophenylglyoxal[20] and syringaldehyde.[21] Benzoin[22] and 9,10-phenanthrenequinone[23] have been used as fluoregenic reagents for

* Correspondence to: Paul S Francis, School of Biological and Chemical Sciences, Deakin University, Geelong, Victoria 3217, Australia
E-mail: psf@deakin.edu.au
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compounds with guanidine functionality, including arginine. Other approaches include second-derivative spectrophotometry[24] and selective enzymatic conversion of the analyte.[25,26]

Three related chemiluminescence reactions that exhibit some selectivity for arginine have been reported. In alkaline solution a weak chemiluminescence accompanies the reaction of *N*-bromosuccinimide with four amino acids (serine, threonine, glycine and arginine); this emission was sensitised with fluorescein.[27] Glycine and arginine have been determined in pharmaceutical formulations by oxidation with *N*-bromosuccinimide after reaction with the carbonyl functional groups of humic acid,[28] but this method is not sufficiently selective to determine arginine in the presence of other amino acids. We have recently shown that arginine is the only naturally occurring amino acid that produces an intense emission when oxidised with hypobromite.[29] The emission spectrum from this reaction is broadly distributed, with a maximum around 600–700 nm. In this paper we further explore this reaction and apply it to the determination of arginine in nutritional supplements that are marketed as ergogenic aids.

MATERIALS AND METHODS

A conventional flow injection analysis manifold was assembled (Fig 1). A peristaltic pump (Gilson Minipuls 3, John Morris Scientific, Balwyn, Victoria, Australia) with bridged PVC tubing (Protech Group, Coolum Beach, Queensland, Australia) was used to propel solutions through 0.8 mm id PTFE tubing (Protech). Solution lines were combined with a T-piece, samples were injected into the carrier stream with an automated six-port valve (Valco Instruments, Schenkon, Switzerland), and the light emitted from the reacting mixture was detected with a custom-built flow-through luminometer, which consisted of a coiled flow cell (PTFE tubing; Protech) mounted flush against the window of a photomultiplier tube (model 9828SB, Thorn-EMI, Ruislip, UK). The photomultiplier tube was operated at 900 V, which was provided by a stable power supply (model PM28BN, Thorn-EMI) via a voltage divider (C611, Thorn-EMI). The flow cell, photomultiplier tube and voltage divider were encased in a padded light-tight housing, and the output from the photomultiplier was documented with a chart recorder (YEW type 3066, Yokogawa Hokushin Electric, Tokyo, Japan). All experiments were carried out at room temperature.

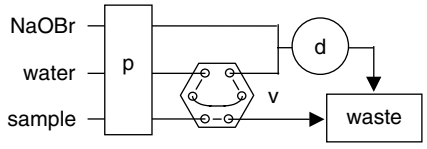

Figure 1. Flow injection manifold for determination of arginine, consisting of (p) peristaltic pump, (d) chemiluminescence detector and (v) six-port injection valve.

Table 1. Chromatographic conditions for FMOC amino acid analysis (eluent A, 6 mM sodium dihydrogen phosphate (pH 6.5); eluent B, pure acetonitrile)

Time (min)	Eluent A	Eluent B	Flow rate (ml min^{-1})
0	80	20	0.8
5	80	20	0.8
20	65	35	0.8
30	30	70	0.8
34.05	0	100	1
37	0	100	1
37.05	80	20	1
40	80	20	1

Amino acid determinations were performed with an HP 1100 series HPLC system (Agilent Technologies, Forest Hills, Victoria, Australia), which consisted of a solvent degasser system, a quaternary pump, an autosampler and a fluorescence detector operated at 270 nm (excitation) and 315 nm (emission). The system was controlled by a Hewlett-Packard Vectra X_m series 4 data analysis workstation. The column used was a C18 Lunar column (250 mm × 4.6 mm id, 5 μm; Phenomenex, Lane Cove, NSW, Australia). An injection volume of 10 μl was used for all chromatographic runs. Chromatographic separation was achieved using a binary solvent system according to Table 1.

The hypobromite reagent was prepared daily by disproportionation of 1 ml of bromine (Hopkin and Williams, Chadwell Heath, UK) in 250 ml of cold aqueous sodium hydroxide (0.4 M; APS, Auburn, NSW, Australia) and stored in Schott bottles covered with aluminium foil to prevent photodegradation. Solutions of L-arginine (APS) were prepared by dissolution of the solid in deionised water (Milli5Q Water System, Millipore, Bedford, MA, USA), followed by appropriate dilutions using an autodilutor. Stock solutions (0.87 g l^{-1}) of the samples—Arginine, Chen: The Arousal, Kuan: The Creative, Energy Formula and Muscle Gain Formula (Musashi, Notting Hill, Victoria, Australia)—were prepared by dissolving the powder in deionised water, and each stock was diluted 50-fold prior to analysis.

For the HPLC procedure, the amino acids were derivatised with 9-fluorenylmethyl chloroformate (FMOC; Sigma-Aldrich, Castle Hill, NSW, Australia) according to the method described by Bauza *et al*[30] with slight modifications. The standard or sample (100 μl) was mixed with borate buffer (100 μl of 200 mM boric acid, pH 8.5). The derivatisation reagent (1.6 mg of FMOC in 200 μl of tetrahydrofuran) was then added and allowed to react for 3 min. This was followed by addition of cleavage solution (100 μl of 0.5 M NH_3), which was left to stand for a further 3 min. The reaction was terminated by addition of quenching solution (480 μl of tetrahydrofuran, 120 μl of acetic acid).

RESULTS AND DISCUSSION

The arginine contents in four nutritional supplement powders were determined using flow injection analysis with chemiluminescence detection and by HPLC with fluorescence detection. Prior to chromatographic separation the amino acids in the sample were derivatised with FMOC. A time of 50 min was required to complete each separation. The relationship between fluorescence peak area and arginine concentration was linear ($R^2 = 0.9945$) over the calibration range (from 2.5×10^{-6} to 1×10^{-4} M). The overall precision of this method was examined using 10 individual arginine solutions (1×10^{-5} M), which produced a relative standard deviation of 4%. The relatively poor precision of HPLC was most probably a consequence of the small volumes used during the derivatisation step. The sample powders each contained a variety of amino acids and other additives, such as glucosamine, inosine, adenosine triphosphate and flavouring, and therefore numerous peaks were observed on each chromatogram (Fig 2). In three of the powders, arginine was the main L-amino acid; however, in Muscle Gain Formula, others such as lysine, leucine, glutamine, histidine and taurine were found in similar or higher quantities.

The same samples and standard solutions were used for the flow injection procedure, but, unlike with HPLC, amino acid derivatisation was not required. A linear relationship ($R^2 = 0.9998$) between chemiluminescence intensity (peak height) and arginine concentration was observed over the calibration range (from 2.5×10^{-6} to 1×10^{-4} M). The precision of this technique was examined using 12 replicate injections of an arginine standard (1×10^{-5} M), which were found to have a relative standard deviation of 1%. Over 100 samples could be analysed per hour. The limit of detection was determined to be 1×10^{-7} M when using a 300 µl sample injection loop.

The results from the analysis of the four dietary supplements using both methodologies are summarised in Fig 3; these values were in good agreement with each other and with those described as a 'typical profile'

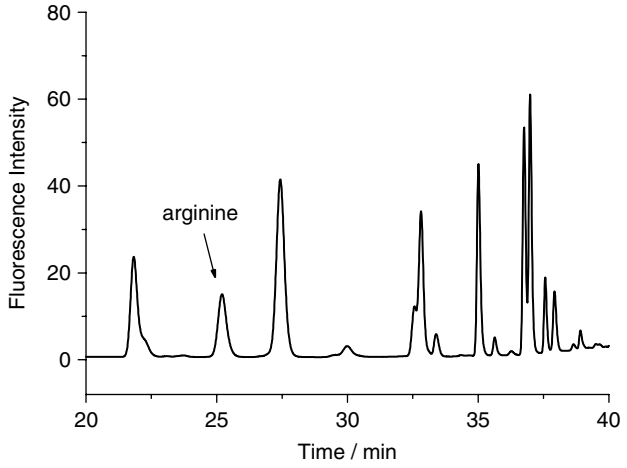

Figure 2. Chromatogram of Muscle Gain Formula sample after derivatisation with FMOC.

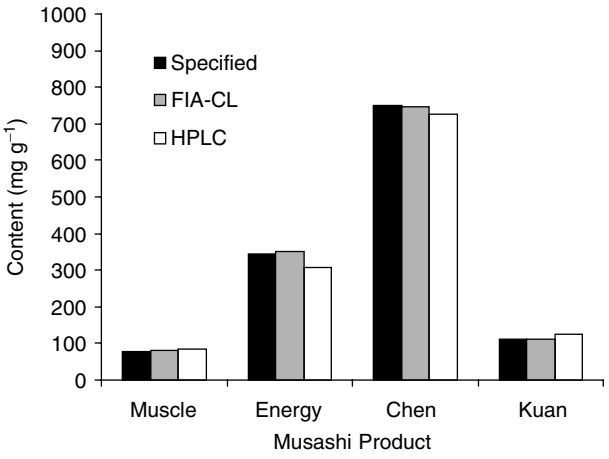

Figure 3. Arginine contents in nutritional supplement powders.

Table 2. Influence of halides in 1×10^{-4} M arginine standard

Conc (M)	Relative intensity		
	Chloride	Bromide	Iodide
0.001	1.01	0.97	1.04
0.01	0.97	0.98	0.82
0.1	0.98	0.96	0.17

on the product packaging. Interferences observed with this chemiluminescent reagent have been investigated with respect to the determination of urea,[31,32] but it was also suspected that halides might interfere with the reaction. This was examined by adding 0.001, 0.01 or 0.1 M NaCl, NaBr or KI to an arginine standard (1×10^{-4} M). Only iodide produced a significant change in emission intensity (Table 2). Unlike chloride and bromide, iodide reacts with hypobromite to form hypoiodite, which rapidly disproportionates in alkaline solution to iodide and iodate ions[33] and, as a consequence, consumes the reagent.

The flow injection/chemiluminescence method described herein was compared with other means of determining arginine, which have been summarised in Table 3. Although the colorimetric and fluorimetric techniques are well established and compatible with common laboratory instrumentation, they suffer from relatively long reaction times and often require elevated temperatures. The enzymatic techniques provide superior selectivity but are dependent on strict control of conditions such as pH and the degradation of the enzymes over time. The earlier chemiluminescence approaches exhibit the best analytical throughput and are well suited to automation; however, these assays do not possess sufficient selectivity for the determination of arginine in many matrices without physical separation. The proposed methodology overcomes this deficiency and has a limit of detection that is comparable to that of previous studies.

CONCLUSION

The proposed flow injection/chemiluminescence methodology is inherently simple and affords a rapid

Table 3. Comparison of some selective reactions for determination of arginine

Method	Detection limit or range	Samples	Comments	Ref
Absorbance				
Hypobromite + thymol	1–13 μg ml^{-1} (6–75 μM)	Protein hydrolysates	Manual procedure with reaction time of 1 min	17
Hypobromite + 1-naphthol	Not stated	Raw and roasted peanuts	Automated procedure (segmented flow) with complicated manifold. Fifty samples analysed per hour	16
p-Nitrophenylglyoxal	0.03–0.33 mM	Arginine residues in proteins	Incubation at 30 °C for 30 min. Interference (~24%) by free histidine	20
Fluorescence				
Benzoin + 2-mercaptoethanol	5–15 pmol	Arginine-containing peptides separated by HPLC	Post-column derivatisation in 15 m reaction coil at 76 °C. Reaction time of 40 s	34
9,10-Phenanthrenequinone	Not stated	Arginine content of intact proteins	Reaction time of minutes for free arginine and hours for proteins. Excess reagent produces blank signal	23
2,3-Naphthalenedicarbaldehyde	1–100 μM	Blood serum after deproteinisation	Reaction time of 25 min at 20 °C. Thiol compounds such as glutathione and cysteine interfere unless blocked	35
Enzymatic				
Octopine dehydrogenase	Not stated	Protein hydrolysates	Photometric detection of NADH consumption. Reaction time of 10 min	25
Arginase + urease	0.03–3 mM	Human blood serum and bovine insulin hydrolysates	Ammonia electrode with gas-permeable membrane. Response time of 5 min	36
Arginase + urease	2 μM	Pharmaceutical preparation	Surface acoustic wave conductance sensor. Analysis time of ~15 min	37
Arginine kinase + pyruvate kinase + lactate dehydrogenase	Up to 3 mM	Human blood serum	Photometric measurement of NADH consumption at 60 and 180 s. Fifteen analyses achieved per hour	38
Arginine decarboxylase	0.1–10 mM	Suggested for biological samples	Manual and segmented flow methods with incubation at 37 °C for 25 min and an ion-selective membrane electrode	39
Chemiluminescence				
N-Bromosuccinimide + fluorescein	0.03 μM	Synthetic mixtures of arginine and serine	FIA. Similar response from serine, threonine, glycine and arginine	27
Humic acid + *N*-bromosuccinimide	0.25 mg l^{-1} (1 μM)	Pharmaceutical formulations	FIA. Mixed with humic acids prior to injection. Greater response from glycine and threonine	28
Hypobromite	0.1 μM	Nutritional supplements	FIA. High sample throughput. Interference from urea, guanidino compounds and iodide	

and selective means of determining arginine in dietary supplements containing other amino acids.

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