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Short communication

Simultaneous determination of adenine and guanine in ruminant bacterial pellets by ion-pair HPLC

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Abstract

An ion-pair reversed-phase high-performance liquid chromatography with gradient elution and UV detection was used to measure adenine (A) and guanine (G) in lyophilized bacterial pellets from ruminants using allopurinol as internal standard. The separation was performed on a Symmetry C18 column and the detection was monitored at 280 nm. Calibration curves were found to be linear in the concentration range from 5 to 50 mg/l with correlation coefficients (r^2) > 0.999. Mean recoveries of A and G standards added to bacterial samples were 102.2 and 98.2, respectively. The method proposed yielded sharp, well-resolved peaks within 25 min and was successfully applied for the determination of A and G in bacterial pellets.

Keywords: Purine bases: Adenine and Guanine; Ion-pair reversed-phase high-performance liquid chromatography

1. Introduction

Separation and determination of adenine (A) and guanine (G) is an interesting and challenging task, because of their involvement in a variety of biochemical processes. In the particular case of the ruminant nutrition field, the quantification of microbial protein synthesis and protein degradation in the rumen is critical in all the protein evaluation systems for these animals. Purine bases play a very important role in the study of the nutrition of the ruminants, because adenine and guanine were used, among others, like internal microbial markers to determine microbial protein synthesis [1].

The most widely used technique for the determination of purine bases in biological samples is liquid chromatography. The ion-exchange chromatography was used initially for the determination of purine nucleotides [2] and was also applied for the determination of nucleosides and bases [3]. However, its use presents several disadvantages such as large time analyses and elevated temperatures. The reversed phase is at present the most commonly used liquid chromatographic method for the determination of nucleic acid constituents using C8 or C18 columns [4–8].

The nucleotides, nucleosides and bases can be separated by reversed phase, but some of these could not be resolved simultaneously and required the ion-pairing mode [8–10]. The advantage of ion-pairing technique is the broad range of parameters, which may be conveniently adjusted to optimize the separation method, including the concentration of organic modifier in the mobile phase, the type and concentration of buffer in the mobile phase and the type and concentration of the counter-ion.

Within the last decade, capillary electroseparation methods, in particular capillary zone electrophoresis (CZE), have become versatile processes as well as quality control techniques and a viable alternative to commonly employed chromatographic methods [8,11,12].

UV detection has been usually employed, since it permits the measurement of the majority of nucleotides, nucleosides and purine bases with good sensitivity in most biological samples. However, both fluorescence and MS detection are increasingly popular [8].

The purpose of this work has been to apply a previous ion-pair RP-HPLC method [13] developed for the creatinine and purine derivatives which enables the simultaneous determination of adenine and guanine in ruminant bacterial pellets to contribute

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to the study of microbial protein synthesis and protein degradation in the rumen.

2. Experimental

2.1. Chemicals

All the chemicals were of analytical grade. Standards of adenine, guanine, allopurinol, decane-, octane- and hexanesulfonic acid (C10, C8, C6) sodium salts were supplied by Sigma (Madrid, Spain). Methanol HPLC-grade and the other chemicals were obtained by Merck (Barcelona, Spain). Buffers were prepared using ultrapure water (Milli-RO 15 reagent-grade water system, Millipore).

2.2. Standard solutions

Stock solutions of standards (1000 mg/l) were prepared by dissolving adenine and allopurinol (I.S.) in water and guanine in 1.2 M perchloric acid. These solutions were stable for at least 1 week at 4 °C. These stock solutions were diluted with deionized water to prepare working standard solutions of 25 mg/l for A, G and allopurinol. A 20 μl aliquot of these solutions was used daily as a control to check all conditions of the HPLC procedure.

Calibration graphs obtained with different concentrations ranging from 5 to 50 mg/l for A and G were constructed by plotting the peak areas of each compound against concentration. In all samples, the concentration of allopurinol was 25 mg/l. The calibration curves were linear over the concentration range studied. The peak area for every point was calculated as an average value of three injections, giving a relative standard deviation smaller than 3%.

2.3. Sample preparation

Before HPLC analysis, 15 mg of lyophilized bacterial pellet were hydrolized for 1 h at 100 °C with 2 ml 2 M-perchloric acid, adding 2 μmol of allopurinol and neutralized immediately with 4 M-KOH according to Martín-Orue et al. [14]. After cooling, the volume was made up to 10 ml with elution solvent A of the HPLC system. A portion (20 μl) was injected into the HPLC after centrifugation (3000 × *g*) and filtration (45 μm).

2.4. Equipment

Waters Model (Milford, MA, USA) 600-E instrument equipped with a Waters Model 717 plus injector and a 484 UV detector was used. The detection wavelength was set at 280 nm. The separation was carried out with a Symmetry C18 column (250 mm × 4.6 mm I.D., 5 μm) (Waters, Milford, MA, USA). Quantification was based on integration of peak areas using Borwin 1.5 software (JMBS Development).

2.5. Chromatographic conditions

The mobile phase composition was phosphate buffer 10 mM with 3 mM 1-octanesulfonic acid, sodium salt, pH 4, mixed with methanol: eluent A (5%) and eluent B (20%). Before use, the mobile phase was always filtered through an HA 0.45 μm pore size filter (Millipore, Bedford, MA, USA) and degassed by ultrasonication.

The gradient program was: 0–12 min, 100% A, flow-rate of 0.5 ml/min; 12–13 min, 0–100% B, flow-rate of 0.5–1.5 ml/min; 13–25 min, 100% B, flow-rate of 1.5 ml/min. The gradient was then immediately returned to 100% A and the initial conditions were restored in 10 min. The purity of every compound was tested by comparison of the peak areas obtained at wavelengths 254 and 280 nm. The injection volume was 20 μl. The column temperature was set at 30 °C.

3. Results and discussion

3.1. Optimization of chromatographic conditions

In a previous paper [13], we found the optimum chromatographic conditions for the separation of creatinine and purine derivatives (PD): allantoin, uric acid, hypoxantine using allopurinol as internal standard. In this analysis, we tested different variables to optimize the simultaneous determination of creatinine and PD: alkyl chain length of the pairing-ion agent (C6, C8, C10), buffer concentration, pH and percentage of methanol of the mobile phase and column temperature. In this work, we tested these conditions for the simultaneous determination of adenine and guanine. The retention time of both compounds was not significantly influenced by the pH of the mobile phase over the range 4–6 [13,15]. The influence of the concentration of methanol on the retention was used to establish the gradient elution system and the absorbance detector was set at 280 nm. According to these studies, we decided to adopt the chromatographic conditions specified under Section 2, which represented a good compromise between a good separation and a reasonable analysis time.

The chromatograms resulting from the injection of pure standards and pellet samples under the chromatographic conditions finally adopted are presented in Fig. 1. The retention times for allopurinol (I.S.) guanine and adenine were 12.5, 15.1 and 23.9 min, respectively. A high reproducibility in the retention time was obtained with relative standard deviations (R.S.D.s) less than 5% in all cases studied.

3.2. Analytical variables

Linearity of the standards was checked by measuring various concentrations in the ranges 5–50 mg/l for both analytes, adenine and guanine. Linear relationships between the peak areas and the concentrations tested were found. The equations calculated were: $y = (0.173 \pm 0.002)x + (0.083 \pm 0.061)$; S.D. = 0.101 for adenine and $y = (0.292 \pm 0.002)x + (0.078 \pm 0.069)$; S.D. = 0.114 for guanine. In all instances, the correlation coefficients were greater than 0.999. The standard addition method (standard plus hydrolized bacterial pellet samples) was used in determining chemical interferences of different analytes. The equations calculated were: $y = (0.180 \pm 0.003)x + (5.413 \pm 0.133)$; S.D. = 0.227 for adenine

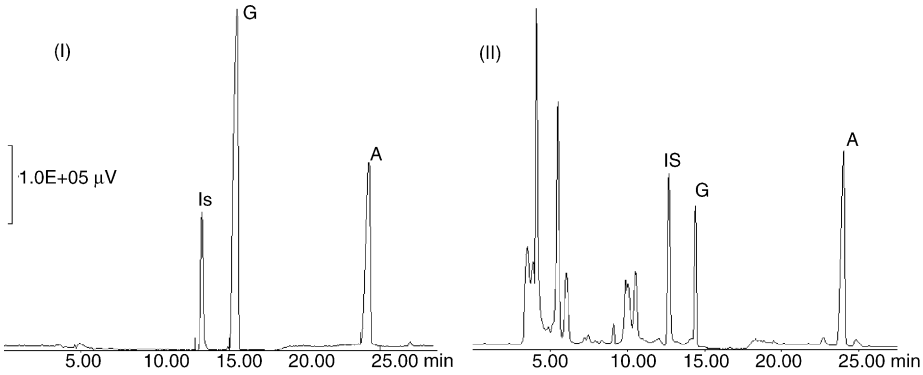

Fig. 1. Chromatograms of (I) standards and (II) bacterial pellet sample. Conditions: column: Symmetry C18 (250 mm × 4.6 mm I.D., 5 μm); mobile phase: phosphate buffer 10 mM with 3 mM 1-octanesulfonic acid, sodium salt, pH 4, mixed with methanol: eluent A (5%) and eluent B (20%); detection: λ = 280 nm. Peaks: A, adenine; G, guanine and I.S., allopurinol.

Table 1
Inter-day precision and accuracy

Compound	Concentration added (mg/l)	Concentration found (mg/l) mean ± S.D. ($n = 5$)	R.S.D. (%)	Relative error (%)
Adenine	5	5.24 ± 0.11	2.10	4.2
	15	15.64 ± 0.32	2.04	4.3
	25	26.62 ± 0.24	0.90	6.5
Guanine	5	4.76 ± 0.21	4.40	4.8
	15	14.84 ± 0.32	2.16	0.3
	25	24.54 ± 0.24	0.98	1.8

Table 2
Determination of adenine and guanine in bacterial pellets

Compound	n	Concentration range (mg/l)	Mean ± S.D.
Adenine	27	9.79–29.74	5.62 ± 4.14
Guanine	27	11.50–50.81	18.68 ± 9.64

and $y = (0.313 \pm 0.005)x + (7.666 \pm 0.211)$; S.D. = 0.358 for guanine. The slopes for the calibration and standard addition graphs were similar for both compounds. The detection limits were 1.75 mg/l for adenine and 1.17 mg/l for guanine with a 20 μl injection. The detection limit was determined from the calibration curves according to the method described by Miller and Miller [16].

The analytical recovery was determined by triplicate analyses of bacterial pellet samples spiked with standards of the analytes at concentrations ranging from 5 to 50 mg/l for both compounds. The mean recovery was 102.16 ± 1.83 (R.S.D. = 1.80%) for adenine and 98.22 ± 2.22 (R.S.D. = 2.26%) for guanine. For each analyte, the inter-day precision and accuracy were assessed by analysing five times per day for 1 week bacterial pellets samples spiked at three concentrations. The results are given in Table 1.

The quantitative data for adenine and guanine obtained with this method are given in Table 2. The range of the values obtained is due to the variability between animals.

4. Conclusions

We have applied a sensitive ion-pair RP-HPLC method for the simultaneous determination of adenine and guanine using allopurinol as internal standard in ruminant bacterial pellets, in which a simple mobile phase is used and a good reproducibility is achieved. The final chromatographic conditions adopted were a compromise between analysis time, peak shapes and symmetry and the resolution of these compounds from interfering substances. This method should be applicable to studies designed to reveal a better understanding of the quantification of microbial protein synthesis and protein degradation in the rumen.

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References

[1] M.D. Carro, E.L. Miller, Anim. Sci. 75 (2002) 214.
[2] W.E. Cohn, Science 109 (1949) 377.
[3] P.R. Brown, S. Bobick, F.F. Hanley, J. Chromatogr. 99 (1974) 587.
[4] J. Balcells, J.A. Guada, J.M. Peiró, J. Chromatogr. 575 (1992) 153.
[5] M. Piñeiro-Sotelo, A. Rodríguez-Bernaldo de Quirós, J. López-Hernández, J. Simal-Lozano, Food Chem. 79 (2002) 113.
[6] M. Katayama, Y. Matsuda, K. Shimokawa, S. Tanabe, S. Kaneko, I. Hara, H. Sato, J. Chromatogr. B 760 (2001) 159.
[7] A. Rodríguez-Núñez, E. Cid, J. Rodríguez-García, F. Camiña, S. Rodríguez-Segade, M. Castro-Gago, Brain Dev. 25 (2003) 102.
[8] P.R. Brown, C.S. Robb, S.E. Geldart, J. Chromatogr. A 965 (2002) 163.
[9] V. Stocchi, I. Cucchiarini, F. Canestrari, M.P. Piacentini, G. Fornani, Anal. Biochem. 167 (1987) 181.
[10] S.L. Blanco, J. Moal, F. San Juan, J. Chromatogr. A 891 (2000) 99.

[11] C.W. Klampfl, M. Himmelsbach, W. Buchberger, H. Klein, Anal. Chim. Acta 454 (2002) 185.
[12] G. Chen, Q. Chu, I. Zhang, J. Ye, Anal. Chim. Acta 457 (2002) 225.
[13] P. García del Moral, M.T. Díez, J.A. Resines, I.G. Bravo, M.J. Arín, J. Liq. Chrom. Rel. Technol. 26 (17) (2003) 2961.
[14] S.M. Martín-Orue, J. Balcells, J.A. Guada, C. Castrillo, Br. J. Nutr. 73 (1995) 375.
[15] R. Boulieu, C. Bory, J. Chromatogr. 339 (1985) 380.
[16] J.N. Miller, J.C. Miller, in: I. Capella (Ed.), Estadística y Quimiometría para Química Analítica, Pearson Education, S.A. Madrid, 2002, p. 11.