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Short communication

Assay of soluble guanylate cyclase activity by isocratic high-performance liquid chromatography

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Abstract

A HPLC method alternative to labelled or unlabelled procedures was developed for the assay of guanylate cyclase (GC) activity. The substrate (GTP) and the product (cGMP) of the enzymatic reaction were separated in the isocratic mode on a μBondapak C_{18} column. The activity of GC was linearly dependent on the amount of cGMP produced in the presence of sodium nitroprusside. This approach was applied to follow the purification of GC from bovine lung and to evaluate its stability in different storage conditions.

Keywords: Guanylate cyclase; Enzymes

1. Introduction

Guanylate cyclase (EC 4.6.1.2) catalyzes the formation of 3′,5′-cyclic GMP (cGMP) from 5′-GTP in the presence of a divalent cation [1]. cGMP is considered a mediator in vascular smooth muscle relaxation, platelet anti-aggregation, intestinal secretion and retinal phototransduction [2,3]. For these reasons, the study of the guanylate cyclase system is very important in therapeutic approaches to hypertension, enterotoxigenic diarrhoea and vasospasm. Guanylate cyclase exists in two main isoenzyme forms: soluble or cytosolic and particulate or membrane-bound; the relative amounts of each form differ with type of tissue and physiological state [2]. The soluble enzyme is a heme protein and consists of two subunits with molecular mass of about 70 000 and 75 000 [4]; this form is activated by nitric oxide and/or NO-containing compounds, as sodium nitroprusside [5].

Guanylate cyclase can be assayed by radioimmunoassay [6]; however, these methods require caution, because of the use of radioactivity and are quite laborious. In recent years, high-performance liquid chromatography (HPLC) has been reported as a valuable alternative for the assay of enzyme activity [7]. This approach is based on the assessment of a chromatographic separation of the substrate and products of the enzymatic reaction, which has to be performed under time and reaction stopping conditions compatible with HPLC runs. This method has been used for measuring the activity of different classes of enzymes [8,9], including also retinal guanylate cyclase [10]. The described procedure is

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adapted from a previous method [11] for adenylate cyclase and involves a gradient elution. This approach requires column washing–reequilibration and cannot be considered the best for routine analysis. Moreover, only a chromatographic separation of standard nucleotide mixture is reported in the case of adenylate cyclase, and no chromatograms are given concerning the guanylate cyclase assay.

To overcome these limits, we developed a simple isocratic HPLC method, which allows the quantitation of cGMP produced enzymatically from GTP without any interference from, i.e. GMP and GDP. This procedure has been used to follow the purification of guanylate cyclase from lung tissues and to control its stability in different storage conditions.

2. Experimental

2.1. Materials

DEAE 52 cellulose was obtained from Whatman (Maidstone, UK). All chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Partial purification of soluble guanylate cyclase

Bovine lung (0.5 kg), previously trimmed of larger blood vessels and airways, was thawed and homogenized with two volumes of cold 25 m*M* triethanolamine hydrochloride (TEA·HCl), 25 m*M* 2-mercaptoethanol (buffer) containing 0.5 m*M* phenylmethylsulfonylfluoride with the aid of a Waring Blendor. All subsequent procedures were conducted at 4°C.

2.2.1. Batch purification

The homogenate was centrifuged at 14 000 *g* for 20 min, the supernatant was filtered through glass wool, and added to 400 g of DEAE 52 cellulose pre-equilibrated with buffer. After 45 min stirring, the resin was filtered and washed 4 times with 3 volumes of extract buffer and 3 times with 3 volumes of 100 m*M* NaCl in extract buffer. Each wash was performed by slowly stirring the resin in buffer for 15 min. Guanylate cyclase activity was eluted with one volume of 250 m*M* NaCl in extract buffer. Samples of this eluate were used immediately to detect the activity. The bulk solution (550 ml) was concentrated (50 ml) with an Amicon ultrafiltration apparatus (PM-30 membrane) and stored in 50% (v/v) glycerol at −80°C.

2.2.2. Column purification

The resin was poured into a column (80×2.5 cm I.D.) and packing was achieved by washing the column with buffer at a constant rate of 200 ml/h. The homogenate, after centrifugation and filtration, was loaded on the column. After continuing the wash for 90 min (150 ml/h), 1.3 l of a linear NaCl gradient (0–0.4 *M*) in buffer was passed through the column (100 ml/h). Fractions with peak activity (130 ml, 0.23–0.27 *M* NaCl) were pooled, concentrated and stored as described in Section 2.2.1.

2.3. Guanylate cyclase activity.

Enzymatic reactions were initiated by addition of 10–50 μl of enzyme to the reaction mixture (final volume 100 μl) containing 50 m*M* TEA·HCl pH 7.6, 3 m*M* dithiothreitol, 1 m*M* 1-methyl-3-isobutylxanthine, 1 m*M* GTP, 4 m*M* $MnCl_2$ and a GTP-regenerating system containing 15 m*M* creatine phosphate and 0.1 mg/ml creatine kinase (800 units/mg), in the presence or absence of 0.1 m*M* sodium nitroprusside, which had been brought to 37°C for 2 min prior to the addition of the enzyme. Sodium nitroprusside was dissolved immediately before use and kept in the dark. Assay incubations were routinely conducted for 10 min at 37°C, terminated by addition of 0.2 ml sodium acetate 0.25 *M* pH 4.0 and centrifuged at 12 000 *g* for 3 min. Protein concentrations were determined by the Coomassie Brilliant Blue G-250 method as described by BioRad Laboratories (Hercules, CA, USA) using bovine serum albumin as a standard.

2.4. HPLC conditions

Chromatographic runs were performed using a M590 pump and a U6K Universal injector connected to a 10 μm Bondapak C_{18} (300×3.9 mm I.D.) with a precolumn Bondapak C_{18} Corasil (20×3.9 mm I.D.) (all from Waters, Milford, MA, USA). The eluent

was 100 m*M* phosphate buffer pH 4.3 (by triethylamine)–methanol (90:10), flow-rate was 1.5 ml/min. Peaks were monitored with a 1040 diode array detector (Hewlett–Packard, Waldbronn, Germany).

3. Results and discussion

Nucleotides are normally separated by a gradient mode [12], but this approach is unsuitable for routine assays since each analysis requires re-equilibration and it is quite long. On the contrary, it has been possible to develop an isocratic method which allows the separation of the substrate (GTP) and the product (cGMP) of the guanylate cyclase reaction on a C_{18} column in less than 15 min. A typical chromatogram of the reaction mixture is shown in Fig. 1A, where peaks A and B are due to buffer and sodium nitroprusside, respectively. Peak identity was assessed both by co-chromatography, with reference standards, and by "on-line" UV detection (Fig. 1B). Under these conditions, GMP and GTP elute at 3.1 and 3.4 min, respectively. Moreover, GTP and cGMP were stable in each assay step.

The amount of cGMP produced during the enzymatic reaction was determined by external standardization, being the linearity between peak areas and masses of cGMP injected in the range 0.025–4 nmol (r=0.998). The limit of detection was 10 pmol, which is higher than that achievable by labelled methods [6].

As shown in Fig. 2, the amount of cGMP produced was linearly dependent on the guanylate cyclase activity up to 15 min. Therefore, the assay was routinely performed by one-point determinations, stopping the reaction after 5 or 10 min. It is important to note that at t=0 no cGMP was detected in the assay mixture, consequently all the measured cGMP arises from the enzymatic reaction. Moreover, our results are in agreement (89%) with the scintillation proximity assay kit (Amersham, Milan, Italy) [13].

The potential of this analytical approach was assessed in the course of the purification of guanylate cyclase from bovine lung. The enzymatic activity was checked first in the crude extract to ensure tissue homogeneity. Then, the extract was purified using

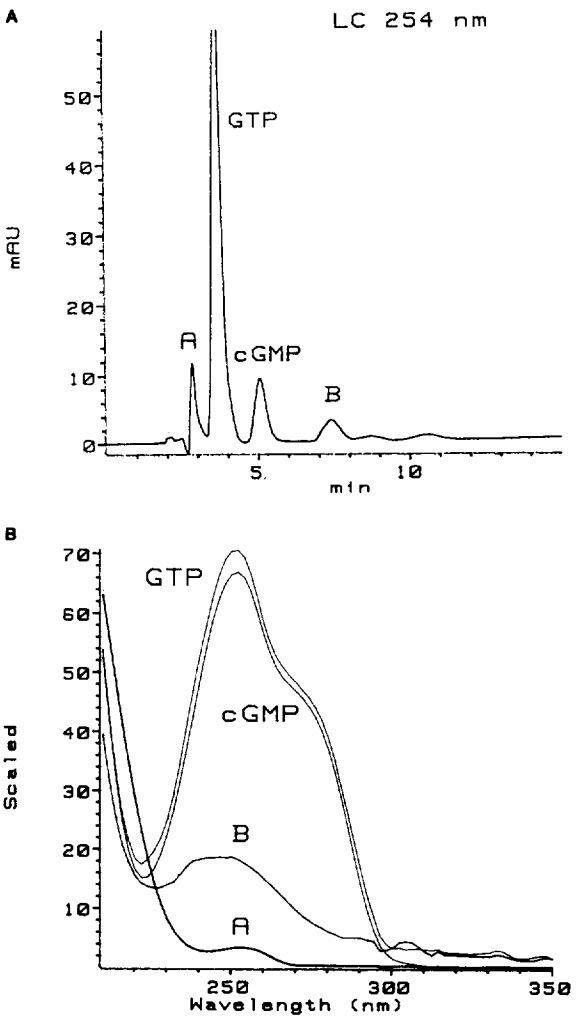

Fig. 1. (A) Typical HPLC chromatogram of a reaction mixture of guanylate cyclase and (B) related on-line UV spectra. Peak A is due to the buffer; peak B is from sodium nitroprusside. Column: μBondapak C_{18}; eluent: 100 m*M* phosphate (pH 4.3)–methanol (90:10); detection: 254 nm; flow-rate: 1.5 ml/min.

DEAE-cellulose, both in batch or in a column. The first approach was preferred since it is more rapid and yields a higher specific activity, even if in greater volumes (Table 1). Furthermore, the stability of guanylate cyclase in different storage conditions was investigated by determining the activity at different intervals. As shown in Table 2, the best storage conditions are 30% glycerol at −20°C.

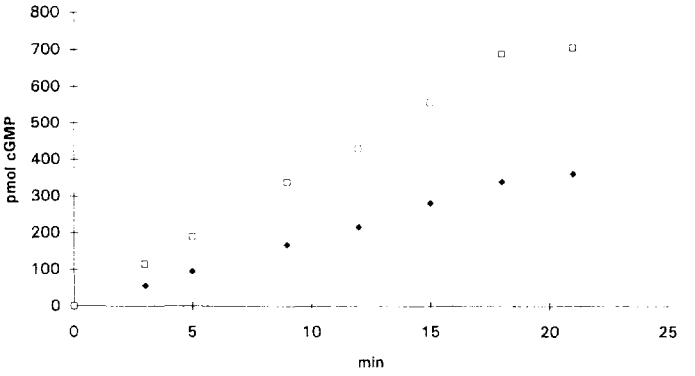

Fig. 2. Amount of cGMP produced from GTP by guanylate cyclase purified by batch procedure as a function of time. (□) 20 μl of enzyme solution; (♦) 10 μl of enzyme solution.

Table 1
DEAE purification: comparison between column and batch procedures

	Column	Batch
Eluate (ml)	130	550
Protein content (mg/ml)	12.6	2.85
Reaction volume for the enzymatic assay (μl)	49	22349
Activity (pmol cGMP/min mg)	286.6	328.3

However, the activity is reduced to one-third after seven days.

From these results, it may be concluded that the described HPLC method is a valid alternative to radiolabelling or radioimmunoassay for routine assay of guanylate cyclase activity. It allows a rapid and direct quantitation of cGMP, and represents a safe analytical support to follow guanylate cyclase purification and stability.

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Table 2
Guanylate cyclase stability under different storage conditions

Day	Activity (nmol cGMP/min)		
	0.25 *M* NaCl (4°C)	Glycerol 30% (−20°C)	0.25 *M* NaCl (−20°C)
0	1024	1024.4	1024
1	662	945	723
2	76	481	204
4	70	472	94
7	45	342	67

See Section 2.3 for experimental conditions.

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