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メタデータ	言語: eng 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	http://hdl.handle.net/2297/7072

J.Pharmacobio-Dyn., **8**, 64–68 (1985)

A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ASSAY OF Na^+, K^+-ADENOSINE TRIPHOSPHATASE INHIBITION[1]

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(Received July 13, 1984)

A new method for the assay of Na^+, K^+-adenosine triphosphatase (Na^+, K^+-ATPase) inhibition has been devised involving the determination of enzymatically produced adenosine diphosphate (ADP) and unchanged adenosine triphosphate (ATP) by high-performance liquid chromatography (HPLC). The substrate, ATP, was incubated with the enzyme preparation in the presence of an inhibitor. The incubation mixture was filtered through a membrane filter, and ADP and ATP in the filtrate were separated by (HPLC). The inhibitory effect of a cardiac steroid on the enzymic reaction was estimated by measuring the peak area ratio of ADP to ADP plus ATP on the chromatogram. The proposed assay method has proved to be satisfactory with respects to simplicity, sensitivity, and reproducibility.

Keywords — Na^+, K^+-ATPase; ADP; cardiac steroid; HPLC; paired-ion chromatography; ion-exchange chromatography

INTRODUCTION

It is well known that Na^+, K^+-adenosine triphosphatase (Na^+, K^+-ATPase, EC 3.6.1.3) is the enzyme responsible for the active transport of Na^+ and K^+ across the cell membrane. Cardiac steroids inhibit both the sodium pump and Na^+, K^+-ATPase in most of the tissues. Estimation of the inhibitory effect on the enzymic reaction is required for the evaluation of cardiotonic activity.[2,3] The enzymic activity is usually assayed by the radiochemical,[4] coupled optical,[5] or Fiske-Subbarow method.[6] The first method has high sensitivity and selectivity but necessitates the use of a radioactive ligand. The second one involves continuously recording nicotinamide adenine dinucleotide (NADH) oxidation in the presence of lactate dehydrogenase, pyruvate kinase, and phosphoenolpyruvate. Altough this procedure is capable of measuring the activity continuously, it lacks reliability of the result and also needs several expensive reagents.[4,5] The last one consists of quantification of molybdenum blue which is reductively formed by the reaction of molybdate with orthophosphate produced from adenosine triphosphate (ATP). The method does not reveal the high affinity process; as mentioned by De Pover *et al.*,[7] measurement of the cumulative amount of inorganic phosphate released during the non-steady state period leads to underestimate of cardiac glycoside potency, especially for the low concentrations. In addition, this procedure is interfered by protein and other compounds. Recently, Tashima reported that protein interference with this method can be overcome by addition of sodium dodecyl sulfate,[8] but the method has still disadvantages *i.e.*, unstability of the coloring matter, low sensitivity, and lack of reliability.[4]

This paper describes a simple and sensitive method for the assay of Na^+, K^+-ATPase inhibition by determining adenosine diphosphate (ADP) and ATP by paired-ion or ion-exchange high-performance liquid chromatography (HPLC).

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MATERIALS AND METHODS

Materials — All nucleotides, digoxin, and ouabain were purchased from Sigma Chemical Co. (St. Louis, MO). Digoxigenin was prepared from digoxin in these laboratories. All the chemicals used were of analytical reagent grade. Solvents were purified by distillation prior to use.

High-Performance Liquid Chromatography — The apparatus used was a Toyo Soda 803A high-performance liquid chromatograph (Toyo Soda. Co., Tokyo) equipped with a SF-770 ultraviolet (UV) detector (260 nm) (Toyo Soda Co.). A stainless steel column (25 cm ×0.4 cm i.d.) was packed with TSKgel ODS-120T (Toyo Soda Co.) or PARTISIL-10 SAX (Whatman Co., Cliffon, NJ).

Na^+, K^+-ATPase Preparation — Hearts of male guinea pigs weighing 600–800 g were used. The enzyme preparation (10 μmol Pi/mg protein h) was obtained according to the procedure of Nakao *et al.*[9] Protein was measured by the method of Lowry *et al.*[10] using bovine serum albumin as a reference.

Assay of Na^+, K^+-ATPase Inhibition — The assay medium (0.2 ml; 100 mM NaCl, 5mM KCl, 3mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethylether)-*N*, *N'*-tetraacetic acid and 50 mM Tris·HCl buffer (pH 7.4)) was preincubated with the enzyme preparation (0.05 ml; 10–30 μg protein) and various concentrations of a cardiac steroid in 0.05 ml H_2O at 37 °C for 5 min. After addition of ATP (0.05 ml; 6 mM), the mixture was incubated at 37 °C for 1 h. The incubation mixture was ice-cooled, filtered through a membrane filter (Column Guard®, 0.45 μm) (Nihon Milipore Ltd., Tokyo), and an aliquot of the filtrate was applied to HPLC. The inhibitory activity (I) of a cardiac steroid on the enzymic activity was expressed as follows:

$$I = \frac{X_0 - X_i}{X_0 - X_{100}} \times 100\ (\%)$$

X : ADP/(ADP+ATP) (peak area ratio)

X_0 : without addition of an inhibitor

X_{100} : in the presence of 1 mM ouabain

X_i : in the presence of an inhibitor

Recovery Test for ATP, ADP, and AMP — For the determination of ATP, ADP, and adenosine monophosphate (AMP), uridine triphosphate was chosen as an internal standard. The internal standard appeared between peak 1 and 2 on the chromatogram in Fig. 1a. The calibration graph was constructed by plotting the ratio of the peak height of ATP, ADP, or AMP to that of the internal standard against the amount of ATP, ADP, or AMP. The linear response to each nucleotide was observed in the range of 1.5–20 μg/0.1 ml incubation medium. The standard incubation mixture without the substrate was denatured with heat, fortified with known amounts of ATP, ADP, and AMP and filtered through Column Guard® in the manner described above. After addition of the internal standard to the filtrate, an aliquot of the solution was applied to HPLC. The recovery rate was determined according to the calibration graph.

RESULTS AND DISCUSSION

An initial effort was directed to the separation of ATP, ADP, and AMP by HPLC. Several attempts have previously been made on the separation of these nucleotides by paired-ion HPLC[11] and ion-exchange HPLC.[12] No satisfactory resolution in a short retention time could be attained when ion-exchange HPLC was carried out under isocratic conditions.[12] Accordingly, paired-ion HPLC was examined on a TSKgel ODS-120T column employing methanol-0.125 M KH_2PO_4 (1:5, v/v) containing 4 mM tetra-*n*-butylammonium hydroxide as the mobile phase. The three nucleotides were distinctly resolved within 13 min as illustrated in Fig. 1a. These results prompted us to employ paired-ion HPLC for the assay.

The deproteinized incubation mixture exhibited no interfering peaks due to endogeneous

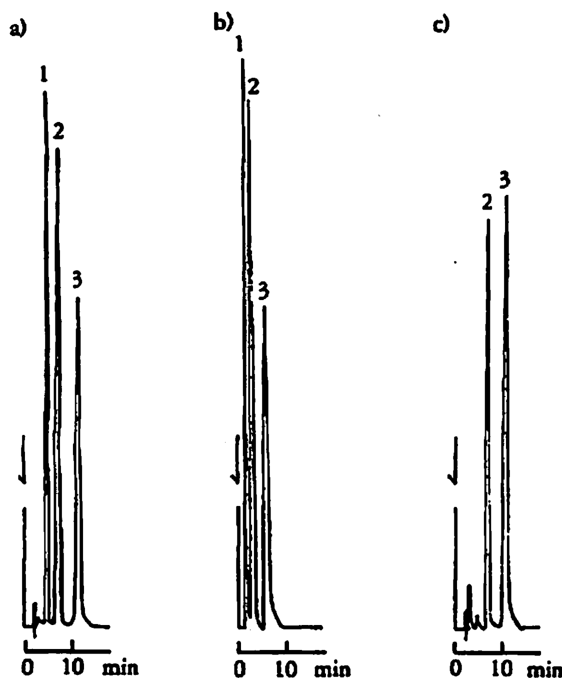

FIG. 1. *Separation of Nucleotides by HPLC*
a) TSKgel ODS-120T, 1 ml/min; 1. AMP, 2. ADP, 3. ATP.
b) PARTISIL-10 SAX, 0.25 M KH_2PO_4 (pH 6.5; adjusted with NaOH), 2 ml/min; 1. solvent front (AMP), 2. ADP, 3. ATP.
c) Nucleotides from incubation mixture. Conditions were the same as in a).

substances and decomposed products from ATP on the chromatogram (Fig. 1c). Known amounts of ATP, ADP, and AMP at three levels were added to the incubation medium, and their recovery rates were estimated using uridine triphosphate as an internal standard. It is evident from the data in Table I that each nucleotide spiked was recovered at a satisfactory rate with good reproducibility. The nucleotides in the deproteinized sample were stable and no significant change was observed in the chromatographic pattern at least 3 h after deproteinization. The detection limits (signal to noise=5 at 0.01 AUFS) of AMP, ADP, and ATP for the injected amounts were 5, 10, and 14 ng, respectively. The sensitivity of the present method is at least 100−200 times higher than that of the Fiske–Subbarow method where the detection limit of Pi is 2 μg.[13)]

When the peak area ratio of ADP to ATP plus ADP formed by Na^+, K^+-ATPase was plotted against the concentration of digoxin, a linear relationship was observed between these two in the range $10^{-7}-3\times10^{-6}$ M (Fig. 2). In this procedure, the addition of an internal standard to the incubation medium was not required. The enzyme inhibition of digoxin increased with the incubation time and reached a plateau in 45 min. Therefore, the suitable incubation time was set at 60 min.

TABLE I. *Recovery of Nucleotides Added to Incubation Medium*

Added	Found (μg/0.1 ml)[a)]		
	1.5 μg	8 μg	20 μg
AMP	1.44±0.03 (95.8±2.0)[b)]	7.38±0.19 (92.3±2.4)	–
ADP	1.31±0.05 (87.5±3.5)	7.50±0.16 (93.8±2.0)	–
ATP	–[c)]	–	18.76±0.64 (93.8±3.2)

a) Mean ±SD (n=10).
b) Figures in parentheses represent the recovery rate (%).
c) Not carried out.

The paired-ion HPLC on a reversed phase column provided satisfactory results as described above. However, it is pointed out that the use of a paired-ion system may shorten the life time of a reversed phase column. The present result revealed that ATP underwent no nonenzymic decomposition under the assay condition where only two peaks corresponding to ATP and ADP appeared on the chromatogram. Therefore, ion-exchange chromatography on a PARTISIL-10 SAX column was also employed for this assay. The two nucleotides could be resolved within 10 min (Fig. 1b).

The inhibitory activity of digoxin on Na^+, K^+-ATPase was determined by means of ion-exchange chromatography. The inhibition curve thus constructed was superimposable with that obtained by paired-ion chromatography illustrated in Fig. 2. The dose-response curves were also obtained with ouabain and digoxigenin (Fig. 2). These results are compatible with the findings previously reported.[7] Thus, both paired-ion and ion-exchange chromatographic methods have proved to be applicable to the assay.

The present method for the assay of Na^+, K^+-ATPase by means of HPLC with UV detection is simple in the procedure and requires only a small amount of precious cardiac steroids. The similar principle may also be applicable to the assay of ATP pyrophosphatase (EC 3.6.1.8) by determining simultaneously AMP formed and the unchanged substrate by paired-ion HPLC. The proposed method is more favorable than the known methods with respects to simplicity, sensitivity, and reproducibility. The application of this method to the assay of inhibitory activities of cardiac steroids from toad venom[14] on Na^+, K^+-ATPase is being conducted in these laboratories, and the details will be reported elsewhere.

Acknowledgement The authors are indebted to Professor Y. Tashima, School of Medicine, Akita University, and Professor Y. Suzuki of this Institute for their helpful discussions. This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

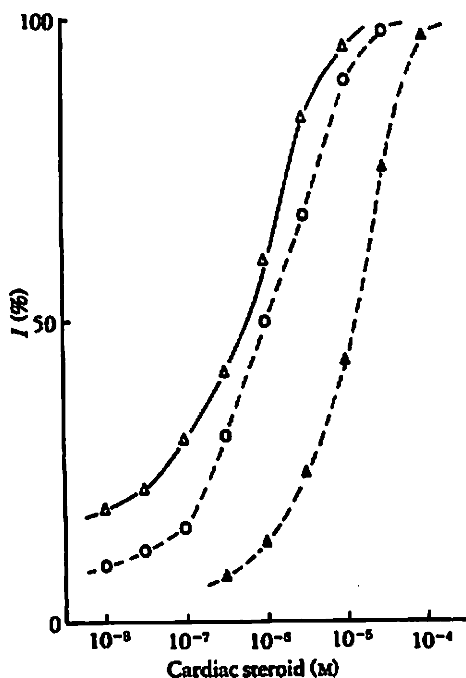

FIG. 2. *Inhibition of* Na^+, K^+*-ATPase from Guinea Pig Heart by Digoxin (△), Ouabain (○), and Digoxigenin (▲)*
—: *by paired-ion and ion-exchange chromatography,*
- - -: *by ion-exchange chromatography.*
Each point represents a mean value of duplicate determinations.

REFERENCES

1) Part CCV of "Studies on Steroids" by T. Nambara; Part CCIV: K. Shimada, K. Ohishi and T. Nambara, *Chem. Pharm. Bull.*, 32, 4396–4401 (1984).
2) R. L. Post, C. R. Merritt, C. R. Kinsolving and C. D. Albright: Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte, *J. Biol. Chem.*, 235, 1796–1802 (1960).
3) J. C. Skou: Further investigations on a Mg^{2+}+Na^+-activated adenosintriphosphatase, possibly related to the active, linked transport of Na^+ and K^+ across the nerve membrane, *Biochim. Biophys. Acta*, 42, 6–23 (1960).

4) F. Noel and T. Godfraind: Heterogeneity of oubain specific binding sites and ($Na^+ + K^+$)-ATPase inhibition in microsomes from rat heart, *Biochem. Pharmacol.*, 33, 47−53 (1984).
5) T. Godfraind and T. Lutete: Inhibition by digoxin and SC4453 of ($Na^+ + K^+$)-ATPase prepared from human heart, guinea-pig heart and guinea-pig brain, *Eur. J. Pharmacol.*, 60, 329−336 (1979).
6) C. H. Fiske and Y. Subbarow: The colorimetric determination of phosphorus, *J. Biol. Chem.*, 66, 375−400 (1925).
7) A. De Pover and T. Godfraind: Influence of 16β formylation on Na, K-ATPase inhibition by cardiac glycosides, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 321, 135−139 (1982).
8) Y. Tashima: Removal of protein interference in the Fiske-Subbarow method by sodium dodecyl sulfate, *Anal. Biochem.*, 69, 410−414 (1975).
9) T. Nakao, Y. Tashima, K. Nagano and M. Nakao: Highly specific sodium-potassium-activated adenosine triphosphatase from various tissues of rabbit, *Biochem Biophys. Res. Commun.*, 19, 755−758 (1965).
10) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J Randall: Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 193, 265−275 (1951).
11) E. J. M. Pennings and G. M. J. van Kempen: Analysis of 3′-phosphoadenylylsulphate and related compounds by paired-ion high-performance liquid chromatography, *J. Chromatogr.*, 176, 478−479 (1979).
12) J. T. Axelson, J. W. Bodley and T. F. Walseth: A volatile liquid chromatography system for nucleotide *Anal. Biochem.*, 116, 357−360 (1981).
13) H. Hettler: Paper chromatography of phosphorus compounds. I. Inorganic phosphorus compounds, *J. Chromatogr.*, 1, 389−410 (1958).
14) K. Shimada, Y. Fujii, E. Yamashita, Y. Niizaki, Y. Sato and T. Nambara: Studies on cardiotonic steroids from the skin of Japanese toad, *Chem. Pharm. Bull.*, 25, 714−730 (1977).