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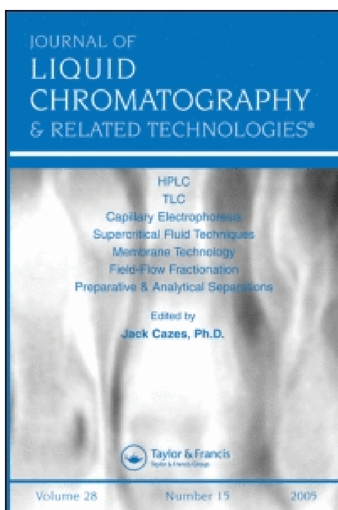

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RELIABLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION FOR MEMBRANE-BOUND ENZYMES; APPLICATIONS ONTO GUANYLATE CYCLASE AND 5'-NUCLEOTIDASE

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ABSTRACT

A convenient, simple and reliable HPLC assay method for membrane-bound enzymes, such as guanylate cyclase and 5'-nucleotidase, were developed. The membrane enzymes of guanylate cyclase had been assayed by determination of the product cyclic GMP by radioimmunoassay after column chromatographical separation of cyclic GMP, and 5'-nucleotidase had been assayed by colorimetrical phosphate assay method. The use of radioisotope is dangerous and requires a special laboratory, and photometrical assay method on membrane enzymes is difficult to be carried out reliably because membrane samples exhibit turbidity. RP-HPLC assay method for guanylate cyclase directly measures the product of cyclic GMP fluorimetrically, which afford a sensitive detection for cyclic GMP at pmol level. RP-HPLC assay method for 5'-nucleotidase also directly the product of adenosine by UV detection at 260 nm. Reliable stoichiometry was observed in both of enzyme assay, i.e., decrease in substrate of GTP and increase in product of cyclic GMP for guanylate cyclase, decrease in substrate of AMP and increase in product of adenine for 5'-nucleotidase. These assay method are successfully applied onto membrane samples and also onto serum samples.

INTRODUCTION

Membrane-bound enzymes were essential for energy transduction, such as electron transport systems (succinate oxidase system, NADH oxidase system, and photosynthetic photon-electron

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energy tranport system)(1), active transport systems (amino acid transport, sugar transport, and possibly vitamin and essential metal transport)(2), and receptor systems (insulin receptor, asialoglycoprotein receptor, hormone receptors)(3, 4) were present in the membrane structures.

Further, important membrane-bound and/or integral membrane enzymes were present; lipoamidase (5, 6), guanylate cyclase (7), acetylcholinesterase (4, 8), and 5'-nucleotidase (9) were present in the membranes and play the important roles in signal transduction or active transport.

These membrane enzymes were assayed by photometric and radioisotopic methods. However, membranes samples were not clear or transparent, and reliable photometric assays were usually difficult to perform. Radioisotopic method is sensitive, however the method is dangerous and special precaution is usually necessary. Radioisotopic method is often difficult to understand, because hydrophobic membranes sometimes enrols or entraps radioisotopic compounds nonspecifically to give a high level of background values.

Reversed-phase HPLC method is suitable for membrane samples because it separate various compounds essentially according to hydrophobicity of molecules (10). Reversed-phase HPLC separates substrates and products of enzyme reaction from membrane components, and free-from interference of turbidity or enrolment. We developed HPLC assay methods for two representative membrane enzymes by using reversed-phase octadecyl silane (ODS) column. Such method is also shown to be applicable to body fluid (composed of complex components) of human serum.

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MATERIALS AND METHODS

Chemicals and reagents: Adenosine 5'-monophosphate (AMP), adenosine, guanosine 5'-triphosphate (GTP), and cyclic guanosine 3',5'-monophosphate (cyclic GMP) were purchased from Boehringer-Mannheim Yamanouchi Co., Tokyo, Japan. 3-Isobuthyl-1-methyl-xanthine (IBMX) was from Sigma Chemical Co., St.Louis, MO, USA. Develosil ODS (5 micrometer particle diameter) was from Nomura Chemical Co., Seto, Aichi, Japan. Home-made Develosil ODS column (50 x 4.6 mm I.D.) was manually packed according to the procedure as previously described (11). Nucleosil 5C18 (250 x 4.6 mm I.D.) packed column, trifluoroacetic acid (TFA), phosphoric acid, and sodium dihydrogen phosphate were from Wako Pure Chemical Co., Osaka, Japan.

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Specimens: Membrane fractions were prepared from pig brain as described previously (6). Microsomal membranes (P3) were obtained after ultracentrifugation of the S2 fraction at 105,000 x g for 60 min in 0.32 M sucrose (specific gravity=1.05) containing 1 mM sodium phosphate buffer (pH 7.0). Human serum was obtained from National Children's Hospital, Setagaya-Ku, Tokyo, Japan. The specimens were stored at -20°C.

High-performance liquid chromatography: (1) Apparatus for Guanylate cyclase (EC 4.6.1.2) assay; HPLC pump used was Waters model 600 (Waters Associates, Inc., Milford, MA, USA). Model U6K sample injection unit (Waters; 0.2 ml sample-loop) and Hitachi F-3000 Fluorometer (Hitachi Corp., Tokyo, Japan) were used with 50 mm long home-made ODS column. Solvent A was aqueous 0.1% TFA, and sovent B was methanol. A step gradient was used as shown in Table I. Column temperature was at ambient of 23°C. (2) Apparatus for 5'-nucleotidase (EC 3.1.3.31) assay; Combination of HPLC pump of LKB model 2150 (Pharmacia-LKB,

Table I. Typical elution programme routinely used in the guanylate cyclase assay.*

Time (min)	Flow-rate (ml/min)	Solvent A (%)	Solvent B (%)
Initial	1.00	100	0
7.00	1.00	100	0
7.01	2.00	0	100
10.00	2.00	0	100
10.01	2.00	100	0
13.00	2.00	100	0
13.01	1.00	100	0

* Guanylate cyclase assay per one cycle of determination required for 15 min.

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Uppsala, Sweden), an injector model SSC-EIE-005 (0.2 ml of loop for sample; Senshu Scientific Co., Tokyo Japan), a column oven model 655A-52 (Hitachi), and model 655A-21 variable wavelength UV monitor (Hitachi) was used. An isocratic elution by a solvent of 0.1 M sodium phosphate buffer (pH 2.0) was used, and a flowrate was 1.5 ml/min. Column temperature was 45°C.

Enzyme assay: (1) Guanylate cyclase; Guanylate cyclase activity was assayed in the reaction mixture essentially according to reference 7. The reaction mixture consisted of 20 mM Tris-hydrochloride (pH 8.0); 3 mM manganese chloride, 0.1 mM GTP, 0.2 mM IBMX, and enzyme 0.01 ml (membrane fraction, 10 mg of protein/ml; 10-fold diluted serum with distilled water, 6.5 mg

of protein/ml) in a total voume of 0.1 ml. As a control, boiled enzyme sample, which was heated at 100°C for 3 min, was also incubated. Reaction was started by incubating the reaction test tube (10 x 75 mm) at 37°C. After incubation, the reaction mixture was added 0.1 ml of 6 M hydrochloric acid to stop the reaction. The test tube was centrifuged at 1,500 x g for 15 min. A portion (0.01 ml) of the clear supernatant was injected into the HPLC-fluorimetric system. Produced cyclic GMP was measured by the intrinsic fluorescence (excitation wavelength at 290 nm and emission wavelength at 400 nm).

(2) 5'-Nucleotidase; The reaction mixture was essentially according to the reference 9. The rection mixture (total of 0.1 ml) was as follows; 20 mM Tris-hydrochloride (pH 8.0), 0.01 ml of membrane fraction (10 mg of protein/ml) or 10-fold diluted serum by water (6.5 mg of protein/ml), and 0.1 mM AMP. The reaction was started after addition of enzyme by incubating at 37°C. After the reaction, 0.1 ml of 6 M hydrochloric acid was added to stop the reaction. After the centrifugation at 1,500 x g for 15 min, 0.01 ml portion of the clear supernatant was injected into the HPLC system. The produced adenine was measured by UV adsoption at 260 nm. The amount of AMP and adenine injected were calculated by a data processor (Chromatopac model C-R1A, Shimadzu Co., Kyoto, Japan).

<u>Protein assay</u> Protein concentrations were determined by BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

(1) Guanylate cyclase assay: When a Develosil ODS column (50 x 4.6 mm I.D.) and the gradient programme shown in Table I

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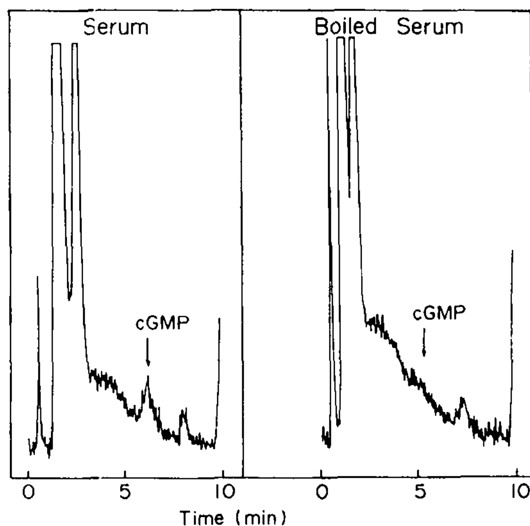

Figure 1. Typical chromatograms for the determination of guanylate cyclase activity in serum sample. Panel A; serum sample. Panel B; boiled serum control. The amount of cGMP in panel A was determined as 53 pmol by external calibration (peak height). Other conditions were as described in Materials and Methods.

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were used, the two standards (GTP and cyclic GMP) were eluted at 1.2 min (injection point) and 6.4 min, respectively. The relationship between the amount of GTP and cyclic GMP injected and the peak height was linear in the concentration range 5-5000 pmol. The coefficient of variation (C.V.) for determination of GTP and cyclic GMP were less than 1%.

We applied this method to membrane fraction (P3) and diluted serum samples. A typical example (serum sample) of chromatographic separation is shown in Figure 1. GTP was usually co-eluted other interfering compoiunds in serum, however cyclic GMP was clearly separated in this HPLC system. In

membrane samples there are less interfering compounds than serum, and similar stoichiometrical correlation between the decrease in the amount of substrate (GTP) and the increase in the product (cyclic GMP) was also obtained similarly as the previous reference 12. As shown in Figure 1 (panel B), boiled serum did not produce the product of cyclic GMP at all. The production of cyclic GMP was linear between 0 to 15 h. The specific activities of guanylate cyclase activity found in pig brain microsomal membranes (P3) and human serum were 167 ± 6.2 (n=3) and 6.4 ± 1.2 (n=3) pmol/min per mg of protein, respectively. This method was shown to be applicable to samples of the membrane and also human serum.

(2) 5'-Nucleotidase assay: When a Nucleosil 5C18 column (250 x 4.6 mm I.D.) and an isocratic elution by acidic phosphate buffer (0.1 M sodium phosphate buffer, pH 2.0) were used, the two standards (AMP and adenosine) were eluted at 4.0 and 8.7 min, respectively. A typical example for serum sample was shown in Figure 2, where simultaneous determinations for the substrate (AMP) and the product (adenosine) by a data processor were also indicated. As shown in Figure 2 (panel B), boiled serum also showed no production of the product of adenosine. The coefficient of variation (C.V.) was also within 1%. The relationship between the amounts of AMP and adenosine injected and the peak heights was linear in the concentration range 1-100 nmol. In the case of 10-fold diluted serum and membrane samples in 5'-nucleotidase assay, there is a clear stoichiometric decrease in substrate of AMP and increase in product of adenosine. Adenosine peak was rather asymmetric as shown in Fig. 2, although the column used was a newly obtained column. This reason is not yet clear at this time, however deletion of

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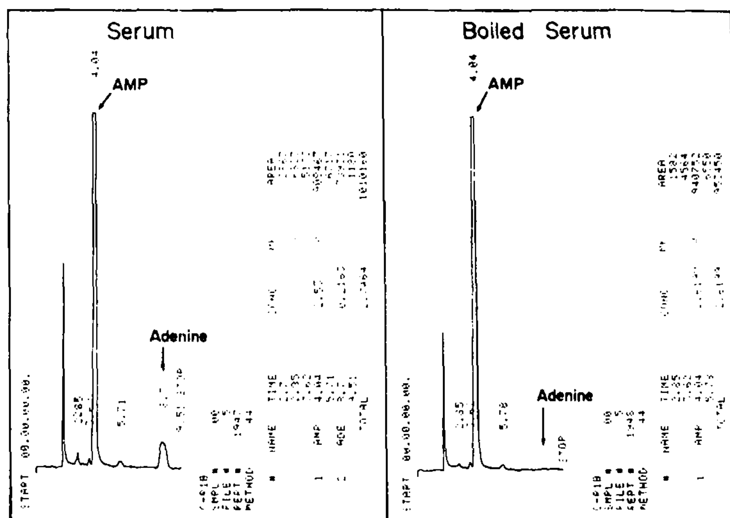

Figure 2. Typical chromatograms for the determination of 5'-nucleotidase activity in serum sample. Panel A; serum sample. Panel B; boiled serum control. CONC in panel A and B represents the amount of AMP and adenine injected at nmol. Other conditions were as described in Materials and Methods.

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phosphate from AMP induces adenosine molecule more hydrophobic having complex partition nature; i.e. such as glychyrhitin (a glycosilated molecule) exhibit such a complex solvophobic phenomenon. However, the peak obtained was satisfactory in the peak area measurment by a data processor.

5'-Nucleotidase activities in pig brain microsomal membranes (P3) and human serum were 275 ± 12.3 (n=3) and 46.8 ± 7.8 pmol/min per mg of protein, respectively. The method was also shown to be applicable to membrane samples and human serum.

5'-Nuclease is located on the outer side (exterior side) of plasma membranes, on the other hand guanyl cyclase is located in

the inner side (cytoplasmic side) of the plasma membrane. The membrane preparations of microsomes exhibited both of 5'-nucleotidase and guanyl cyclase activities. This indicates that both directions of inverted and normal vesicles were present in microsomes. The precise determination method by HPLC is expected to be applicable on the determination of vesicle direction.

5'-Nucleotidase activity has been known to be present in serum as alkaline phosphatase does. However, the presence of guanylate cyclase in serum is the first to demonstrate. There remained to be elucidated the reason why such enzymes as lactate dehydrogenase, which reside in the internal space of the cells, and guanylate cyclase are present in serum.

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