Development of Highly Sensitive Analytical Methods for Biologically Relevant Materials and Their Pharmaceutical Applications

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One important aspect of analytical chemistry research in the pharmaceutical sciences is the development of diagnostic and therapeutic analyses for disease, and the development of analytical methods for elucidating the causes of disease. I have been focusing on developing a highly sensitive method for measuring trace amounts of specific components in biological samples. This research can be roughly divided into three approaches: the use of immunoassays and DNA hybridization as methods utilizing specific affinities, the use of capillary electrophoresis as a highly sensitive and rapid separation method, and the use of chemiluminescence and bioluminescence reactions. The components being measured are compounds such as hormones, tumor markers, drugs, reactive oxygen species and genes in biological samples for the purpose of developing therapies for the prevention and treatment of diseases.

Key words chemiluminescence; bioluminescence; enzyme immunoassay; capillary electrophoresis; DNA probe; reactive oxygen

1. Introduction

I will describe the philosophy of analytical chemistry research and the flow of research in my laboratory. As shown in Fig. 1, an important goal of research in pharmaceutical analytical chemistry is “to develop new analytical methods for the medical diagnosis of diseases and the elucidation of biological functions.” Analytical chemical techniques require improved sensitivity, specificity, accuracy, precision, simplicity, and compact instrumentation. To address these issues, I have conducted the following research.

1. Analysis of biological components by chemiluminescence (CL) and bioluminescence (BL) detection.
2. Analysis of single nucleotide polymorphisms (SNPs) by capillary electrophoresis (CE) and microchip electrophoresis (ME).
3. Development of highly sensitive analytical methods for reactive oxygen.

At first glance, these studies appear to be independent of each other, but in reality, each analytical method is linked and flows between various aspects of related research. My research area was chosen in graduate school, and my doctoral dissertation in 1979 was entitled: “Enzyme immunoassay of cortisol using a CL method.” Enzyme immunoassay (EIA) and CL methods remain the basis of my research, and have led to the development of a series of novel analytical chemistry methods.

2. Analysis of Biological Components Using CL and BL Detection

This study focused on conducting immunoassays and DNA analyses.

Clinically applicable immunoassays require sensitivity, specificity, accuracy, and rapidity. In particular, since biological components are present in extremely small amounts, high sensitivity is essential; therefore, practical analysis requires increased sensitivity. We attempted to increase the sensitivity of immunoassays by developing methods for the highly sensitive detection of labeled compounds, and by adjusting the affinities between antibodies and their labeled antigens.

2.1. High Sensitivity Detection of a Labeled Enzyme in Immunoassays and DNA Probe Assays

Immunoassays originated from the radioimmunoassay (RIA) developed by Berson and Yalow in 1960 (1977 Nobel Prize). In an enzyme immunoassay (EIA), the radioisotope (RI) label is replaced with an enzyme to avoid the potential biohazards of RI. Therefore, in my research, the first study focused on how to approximate the detection sensitivity of the enzyme to RI. The first EIA was cortisol. In this method, peroxidase was chemically linked to cortisol, and the enzyme was detected by CL using luminol. The principle of CL is shown in Fig. 2.

In the presence of hydrogen peroxide, luminol reacts with peroxidase and emits light through an aminophthalic acid ion.
This luminescence method was first used in Bunseki Kagaku in 1977. Later, in 1979, the method was published as a Chemiluminescence EIA method for detecting cortisol. Since my report, this luminescence method has been widely used not only for EIA, but also for molecular biology research. The sensitivity of the CL reaction was improved by adding an enhancer to the luminescence method, and the resulting improved method is used globally. In 1991, we developed a highly sensitive assay for alkaline phosphatase (ALP) and β-D-galactosidase (β-gal) using indoxyl derivatives as substrates (Fig. 3). Furthermore, in 2002 we developed an ALP assay method based on lucigenin chemiluminescence using dihydroxyacetone phosphoric acid as a substrate (Fig. 4).

We obtained even higher sensitivity by subsequently developing a BL method using a luciferin luciferase reaction. The principle and the standard curve of luciferase are shown in Fig. 5. The CL and BL methods developed by us are presented in Table 1. Detection sensitivities of $1 \times 10^{-19}$ mol for CL and $1 \times 10^{-20}$ mol for BL have been obtained. These sensitivities are at least 100 times higher than that of RI, by which $3^\text{H}$ is $1 \times 10^{-15}$ and $13^1\text{I}$ is $1 \times 10^{-17}$ mol, respectively.

I will next describe EIA using a BL reaction, which is the most sensitive assay in Table 1. The first report of a method for measuring biotin labeled with luciferase was published by us in 1992. This method was the first report using luciferase as a labeling enzyme, but the luciferase was chemically unstable. As a result, luciferase was not used as a label enzyme of EIA for some years. However, biotinylation by microorganisms can be used to label biotin. The discovery of inosine triphosphate (ITP) and pyrophosphate as enhancers of luciferase, and the study of pH conditions during luminescence, revived...
the viability of using luciferase in EIA (2009). Figure 5 shows the results of applying this method to the EIA of hepatitis B virus.

In the previously mentioned report, we compared the sensitivity of EIA using different detection methods: BL, and ALP CL using 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy) phenyl-1,2-dioxetane (AMMPD) as a substrate. The same antibody as used for the EIA of hepatitis B virus was used. We found that bioluminescent EIA (BLEIA) was about 50 times more sensitive than chemiluminescence EIA (CLEIA) using ALP as a label enzyme as shown in Fig. 6. This increased sensitivity is important clinically because treatment can be started earlier, as compared with the conventional CL detection method, since infectious viruses can be detected in about 11 d.

2.2. High Sensitivity of EIA Achieved by Adjusting the Affinity between the Antibody and the Labeled Antigen

For the experiments shown in Fig. 7, bovine serum albumin (BSA) was used as a carrier protein and was linked to the 3rd, 6th or 21st positions of cortisol. Three antibodies were obtained following the immunization of rabbits using these immunogens. At the same time, the enzyme horseradish peroxidase (HRP) was labeled at these three cortisol positions to obtain three labeled antigens. To select the most sensitive
immunoassay, nine immunoassays combining these antibodies and labeled antigens were examined. We selected a labeled antigen having an appropriate affinity for the produced antibody. The combination of the antibody to the immunogen linked at position 6 with the antigen labeled with HRP at position 21 showed the highest sensitivity (100 pg) at the 50% replacement value (midpoint) on the standard curve. The standard curve in Fig. 7 schematically shows improved sensitivity by this combination.

This method was applied to the EIA of thyroxine. As shown in Fig. 8, thyroxine has an amino group and a carboxyl group, and thus four antibodies and four labeled antigens were prepared using these derivatives, resulting in 16 types of EIA from each combination. This approach established a high sensitivity EIA. The results are shown in Table 2. The midpoint value for each combination of immunoassays is considered the sensitivity of the assay. We found that the midpoint value of a combination with an antigen in which glucose oxidase (GOD) was labeled with glutaraldehyde, and the antibody obtained from BSA linked with a glutaric derivative at the position of the amino group of thyroxine, showed a sensitivity of 14 pg, and the detection sensitivity was 2.5 pg.

Table 3 shows the results of steroid and thyroxine EIAs developed by us in the 1980s and 1990s. 17-α-Hydroxyprogesterone (17-α-OHP) showed a detection sensitivity of 1 pg, and thyroxine a detection sensitivity of 2.5 pg. These analytical methods have been used for neonatal mass screening by the Japan Ministry of Health, Labor and Welfare since the 1980s. 17-α-OHP has been used to diagnose congenital adrenocortical hyperplasia, and thyroid-stimulating hormone (TSH) and thyroxine (T₄) have been used to treat hypothyroidism. In this method, four drops of blood from the heel of a neonate are placed on filter paper (Guthrie cards), dried, then a 3 mm diameter disk is punched from the dried blood on the filter paper using a punching machine; hormone contained in one disk is analyzed. The volume of whole blood contained in one punched disk is 3.6 µL, corresponding to 1.6 µL serum. Our ultra-sensitive method is now used to ex-
amine the blood of all neonates in Japan.

Next, we will describe the ongoing development of an EIA for oxytocin. Oxytocin is a female hormone with a molecular weight of 1007. It is synthesized in the hypothalamus and secreted from the posterior pituitary gland. An RIA method was developed in 1960. This hormone is present in men, but details of its action has long remained unknown. Recently, it was reported that this hormone is related to social activity reliability, affection, etc., and it has attracted attention as a marker and treatment for conditions such as autism. Oxytocin levels increase during pregnancy, but otherwise oxytocin levels are typically low and it was difficult to measure by RIA. The small mass of oxytocin and its identical sequence in all animals makes it difficult to produce antibodies with high titer and to then develop a highly sensitive EIA. 17)

Three antibodies have been raised in which a carrier protein is bound to oxytocin as an immunogen. The antigen and carrier are linked to either 0, 2, or 4 lysine residues between the antigen and carrier protein. Three antibodies were obtained following the immunization of rabbits. Seven biotin-labeled antigens linked via 0, 1, 2, 3, 4, 5, or 6 lysines were prepared.

### Table 3. Steroids and Thyroxine EIA's Developed by Us

| Hormone                        | Detection method | Sensitivity | Application                        | Reference |
|--------------------------------|------------------|-------------|------------------------------------|-----------|
| Cortisol                       | Chemiluminescence| 10 pg       | Cushing's syndrome, Stress marker  | 3)        |
| 21-Deoxycortisol               | Fluorescence     | 0.5 pg      | Congenital adrenal hyperplasia     | 13)       |
| Dehydroepiandrosterone (DHEA)  | Chemiluminescence| 25 pg       | Adrenal function                   | 14)       |
| 17-α-Hydroxyprogesterone (17-OHP)| Fluorescence    | 1 pg        | Congenital adrenal hyperplasia     | 15, 16)   |
| Thyroxine (T₄)                 | Chemiluminescence| 2.5 pg      | Congenital hypothyroidism          | 11)       |
| Free thyroxine (F-T₄)          | Fluorescence     | 1 fg (calculated value) | Congenital hypothyroidism | 12)       |

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[Fig. 9. Standard Curves for Oxytocin and Assay Conditions](#)

[Fig. 10. Principle of Pyrophosphate Assay and Its Standard Curve](#)
between the antigen and biotin. Twenty-one immunoassays for oxytocin were constructed from combinations of antibody (three kinds) and labeled antigen (seven kinds), resulting in a highly sensitive EIA with a detection sensitivity of 2.3 pg. This competitive EIA method, a standard curve, and analytical validation are shown in Fig. 9.

Clinical analysis using this method provided the following new results. Oxytocin in the blood was analyzed by this EIA following gel filtration. We found that a high molecular weight polymeric state of oxytocin is present in the blood, in addition to a low molecular weight peptide. This polymeric form does not exhibit dilution linearity in this proposed EIA, making accurate clinical application difficult. We are therefore investigating pretreatments, such as enzymatic digestion, to develop an analytical method for the polymeric form. We are also studying the clinical significance of the polymeric form.

3. Highly Sensitive Analysis of DNA and RNA by Bioluminescence

This study is based on the highly sensitive analysis of pyrophosphate by a bioluminescence (BL) reaction. In 1988, Hyman reported a method which is the basis of the pyrophosphate method, in which pyrophosphoric acid produced by DNA synthetase is converted to ATP by ATP sulfurylase and is detected by a bioluminescence reaction with luciferin luciferase. Although this method was applied to DNA anal-
sis, the stability and sensitivity of the assay were inadequate due to the use of ATP sulfurylase.\(^9\) We therefore developed a new pyrophosphate measurement method using pyruvate phosphate dikinase (PPDK) to replace ATP sulfurylase.\(^{20}\) The principle behind this assay and the calibration curve obtained are shown in Fig. 10.

Our methodology made it possible to detect as little as 1 fmole of pyrophosphate, and this method was applied to the analysis of SNPs.\(^{21}\) The principle is shown in Fig. 11. As an example, when adenine (A) in Fig. 11 is detected, a specific primer within each aliquot possessing a short 3’-end of the base of interest is hybridized to the single-stranded DNA template (the base (A) of the SNP), then four bases (2’-deoxyadenosine 5’-triphosphate (dATP), 2’-deoxythymidine-5’-triphosphate (dTTP), 2’-deoxyguanosine-5’-triphosphate (dGTP), or 2’-deoxycytidine-5’-triphosphate (dCTP)) are sequentially added in the presence of (exo-) Klenow DNA polymerase. When thymine (T), which is complementary to (A), is reacted, the sequence is extended by a single base, and pyrophosphate is generated. The pyrophosphate emits light via the PPDK-luciferin luciferase reaction, and signals that a single base elongation has occurred. Since luminescence does not occur in the presence of dATP, dCTP, or dGTP, it can be determined that the SNP is an adenine.

This method was applied to SNPs of the k-ras gene, the detection of a cariogenic bacteria (the mutans streptococcus gene),\(^{20}\) and the measurement of telomerase activity as a tumor marker.\(^{22}\) The apparatus for this method is compact, as shown in Fig. 12 (Hitachi, Central Research Laboratory), because no excitation light for the luminescence reaction is necessary.
4. DNA Analysis by Capillary Electrophoresis (CE) and Microchip Electrophoresis (ME)

CE is an analytical method for performing electrophoresis in fused-silica capillary tubing (50 cm × 50–100 µm inner diameter), and its resolution is higher than that of high performance LC as theoretical plates. It is therefore possible to separate macromolecules such as DNA and protein, but because the tube is thin, sensitivity is low, making its clinical application difficult.

We examined DNA analysis methods utilizing CE and ME, and compensated for the low sensitivity by combining amplification reactions such as PCR. The DNA analysis method was used primarily to examine the base length analysis of double stranded DNA, and analysis of single stranded conformational polymorphism (SSCP). A separating agent capable of directly analyzing the PCR product without pretreatment was developed for the base length analysis of double stranded DNA. We found that when dextran sulfate was added to carboxymethyl cellulose (CMC) exhibiting a molecular sieving effect, the separation of double stranded DNA was remarkably improved, and the four deoxynucleotides and synthesized double stranded DNA fragments (>60 bp DNA fragment) contained in the PCR product could be completely separated.

Allele-specific PCR using the specificity of PCR was then developed to analyze SNPs in human genes. The principle of allele specific PCR is that oligonucleotides (primer) with a mismatched 3′-residue do not function. This method was applied to the diagnosis of phenylketonuria (PKU) using the phenylalanine hydroxylase gene, and medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency using the MCAD gene. In the case of MCAD analysis, the first set consisted of sense primer M31 and antisense primer M34. It is capable of amplifying only the normal sequence, producing a 202 base pairs (bp) fragment, because the 3′-end of primer M31 is complementary to the normal MCAD sequence at nucleotide 985. The second set of primers consisted of M32 and M19. This second set is capable of amplifying only the mutant sequence, generating a 175-bp fragment, because the 3′-end of primer M32 is complementary to the mutant sequence at nucleotide 985. The PCR product obtained was analyzed by capillary gel electrophoresis at 11.5 min (175 bp) and 12 min (202 bp) of migration time, respectively. The principle of the allele specific PCR of the MCAD gene is illustrated in Fig. 13. Next, for the analysis of SSCP, double stranded DNA fragments amplified by PCR were dissociated to single strands by heating, and the secondary structures resulting from self-annaling were separated using the high analytical capability of CE. The SSCP principle is shown in Fig. 14. In this method, a mutation point (indicated by ·) different from VT1 (verotoxin 1) is present in the VT2 gene (verotoxin 2), so the single strand conformational polymorphism differs by base composition and can be separated. The analytical method developed based on these methods is shown in Table 4. The phenylalanine hydroxylase gene can now be used to diagnosis phenylketonuria (requiring 10.8 µL whole blood) using three discs (3 mm diameter) of dry blood on filter paper. This method is now used for neonatal mass screening. SSCP has made it possible to analyze SNPs implicated in k-ras genes. The schema of the k-ras gene containing a point mutation at

\[
\text{Polyphenol-} (\text{OH})_n + \text{OH} \rightarrow \text{Polyphenol-} (\text{OH})_n + 2e^- + 2H^+ \\
2e^- + O_2 + 2H^+ \rightarrow H_2O_2
\]
Table 5. The Antibacterial Action of Catechin

| Organism (1×10^6/mL) | Bactericidal action |
|-----------------------|---------------------|
| E. coli (ATCC25922)   | 100% 95%             |
| MRSA (F-51)           | 100% 100%            |
| B. bronchiseptica (IID929) | 100% 99%       |
| S. aureus (ATCC25923) | 100% 98%            |
| S. marcescens (IID5518) | 99% 98%          |
| K. pneumoniae (IID5207) | 94% 0% 100%   |
| B. subtilis (ATCC6623) | 100% 99%            |
| S. enteritidis (87–350) | 99% 99%            |
| Ps. aeruginosa (42)   | 57% 0% 70% 100%     |
| P. mirabilis          | 17% 0% 0% 100%      |

1) ANS solution: 8-anilinonaphthalene sulfonate ammonium salt (ANS) 20 mg, BSA 0.1g in 0.2 mol/L barbital buffer (pH 9.0) 100 mL
2) TCPO: bis(2,4,6-trichlorophenyl) oxalate (TCPO) in ethyl acetate (5 mmol/L)

Fig. 19. Peroxalate Chemiluminescence Assay for Hydrogen Peroxide

Fig. 20. Hydrogen Peroxide Concentration in Green Tea

Fig. 21. Relative Chemiluminescent Intensity (H₂O₂) of Natural Medicine Extracts
Table 6. The Concentration of Hydrogen Peroxide Generated from Natural Medicines and Its Main Physiological Action

| Natural medicines (1 mg/mL) | \( \text{H}_2\text{O}_2 \) (mol/L) | Major effect on stomach and intestines |
|---------------------------|-----------------|-------------------------------------|
| *Gallae chinensis*        | \(2.4 \times 10^{-4}\) | Anti-diarrhoic                       |
| *Gambir*                  | \(1.4 \times 10^{-4}\) | Anti-diarrhoic, intestinal regulator |
| *Myricae cortex*          | \(7.3 \times 10^{-5}\) | Anti-diarrhoic, intestinal regulator |
| *Gerani herba*            | \(3.4 \times 10^{-5}\) | Anti-diarrhoic                       |
| *Malloti cortex*          | \(2.2 \times 10^{-5}\) | Intestinal regulator                 |
| *Cinnamomi cortex* (Indonesia, Vietnam, Konan, Toko) | \(2.3 \times 10^{-5} - 1.6 \times 10^{-5}\) | Stomach medicine                     |

Table 7. Profile of Bacterial Strains

| Bacterial strain | Characteristic | Gram |
|------------------|----------------|------|
| *Escherichia coli* | Diarrhea (pathogenic *E. coli*) | (−)  |
| *Staphylococcus aureus* | Food poisoning, skin infection | (+)  |
| *Campylobacter sputorum biovar sputorum* | Food poisoning, diarrhea | (−)  |
| *Bacteroides thetaiotaomicron* | Opportunistic infection | (−)  |
| *Streptococcus salivarius ssp. thermophilus* | Maintain homeostasis, yogurt | (+)  |
| *Bifidobacterium longum ssp. infantis* | Maintain homeostasis, intestinal remedy | (+)  |
| *Lactobacillus casei* | Maintain homeostasis, intestinal remedy | (+)  |
| *Enterococcus faecalis* | Maintain homeostasis, intestinal remedy | (+)  |

Table 8. The Difference in Susceptibility to Hydrogen Peroxide

| \( \text{H}_2\text{O}_2 \) (mol/L) | *E. coli* | *S. aureus* | *C. sputorum* | *B. thetaiotaomicron* | *S. thermophilus* | *B. longum* | *L. casei* | *E. faecalis* |
|-----------------|---------|------------|-------------|---------------------|-----------------|-------------|-----------|-------------|
| 1\( \times 10^{-5}\) | +++     | +++        | +++         | +++                 | +++             | +++         | +++       | +++         |
| 5\( \times 10^{-5}\) | +++     | +++        | +++         | +++                 | +++             | +++         | +++       | +++         |
| 1\( \times 10^{-4}\) | +++     | +++        | ++          | +++                 | +++             | +++         | +++       | +++         |
| 2\( \times 10^{-4}\) | +++     | −          | −           | −                   | +               | +++         | +++       | +++         |
| 3\( \times 10^{-4}\) | +++     | −          | −           | −                   | +               | +++         | +++       | +++         |
| 4\( \times 10^{-4}\) | +++     | −          | −           | −                   | +               | +++         | +++       | +++         |
| 5\( \times 10^{-4}\) | +       | −          | −           | −                   | +               | +++         | +++       | +++         |
| 6\( \times 10^{-4}\) | −       | −          | −           | −                   | +               | +++         | +++       | +++         |
| 1\( \times 10^{-3}\) | −       | −          | −           | −                   | +               | +++         | +++       | +++         |
| 5\( \times 10^{-3}\) | −       | −          | −           | −                   | +               | +++         | +++       | +++         |
| 1\( \times 10^{-2}\) | −       | −          | −           | −                   | +               | +++         | +++       | +++         |
| Control         | +++     | +++        | +++         | +++                 | +++             | +++         | +++       | +++         |

+++ , Dense growth; ++ , Density reduced compared to control, but too numerous to count; + , Number of colonies can be counted; − , No colonies.

Fig. 22. Major Components of Natural Medicines
codon 12 and in the PCR region are illustrated in Fig. 15. Figure 16 shows an electropherogram for SSCP analysis of seven kinds of k-ras (normal(Gly) and mutated (Ala, Arg, Cys, Val, Asp, Ser). All seven SSCP variations could be separated within 31 min. The principles underlying microchip electrophoresis and capillary electrophoresis are similar, the separation mechanisms are almost the same, and the separation time using microchip electrophoresis is several minutes. Microchip electrophoresis was applied to the analysis of allele-specific PCR to identify verotoxin \cite{50,51} and aptamers. \cite{33,34}

5. Development of Highly Sensitive Analytical Methods for Reactive Oxygen

The principle of chemiluminescence (CL) analysis is based on the reaction of reactive oxygen and free radicals, and thus these reactions have been applied to the analysis of bio-related substances. Research has been conducted on the antibacterial action of catechin, crude intestinal drugs and reactive oxygen, and on the production of nitric oxide (NO) from an antiangiinal drug.

5.1. Antibacterial Action of Catechin

We initially developed a CL analysis method for catechin based on the generation of reactive oxygen. We subsequently expected that hydrogen peroxide is generated during the reaction process, \cite{35} and that this hydrogen peroxide is related to the observed antibacterial action. \cite{36} There are many chemical structures of catechins, as shown in Fig. 17. Epigallocatechin gallate (EGCg) has a pyrogallol group and is the main component of tea.

In our study using CL, we focused on this pyrogallol group and developed a method to generate hydrogen peroxide in basic solution, then detect it using peroxyoxalate chemiluminescence \cite{2000}. Subsequently, we found that EGCg reacts with dissolved oxygen under neutral conditions to produce hydrogen peroxide. This led us to speculate that hydrogen peroxide might be related to the antibacterial action of catechin. The hydrogen peroxide production reaction is shown in Fig. 18, and the principle behind hydrogen peroxide measurement is shown in Fig. 19.

Using our methodology, the hydrogen peroxide level in green tea was found to be $2 \times 10^{-4}$ for dilute green tea and $1 \times 10^{-3}$ mol/L for strong green tea, as shown in Fig. 20.

Next, the antibacterial action of catechin was investigated on 10 bacterial species and the results are shown in Table 5. The catechin contained in green tea showed antibacterial activity towards many bacteria, but had little effect towards *Pseudomonas aeruginosa* and *Proteus mirabilis*. The antibacterial action of hydrogen peroxide solution similarly showed low antimicrobial activity towards *Pseudomonas aeruginosa* and *Proteus mirabilis*; however, when the amount of hydrogen peroxide was increased, its effect increased, and it showed almost 100% antibacterial action. These results revealed that the antimicrobial action of catechin is due to hydrogen peroxide, and the strength of the antimicrobial action of catechin against each bacteria may be due to differences in susceptibility of these bacteria to hydrogen peroxide. This study was used to analyze reactive oxygen generated from herbal medicines and to clarify the bactericidal action of hydrogen peroxide.

5.2. Natural Medicine (Intestinal Medicine) and Reactive Oxygen \cite{37} The purpose of this study was to understand the efficacy of natural medicines that act on the gastrointestinal tract as intestinal regulators, with stomachic properties and anti-diarrheic actions, but in many cases the mechanism of action of a natural medicine is unknown. Relative chemiluminescence (CL) intensity (corresponding to the hydrogen peroxide concentration generated) from various natural medicines was examined, and the results are shown in Fig. 21. We then compared the concentration of hydrogen peroxide generated from these herbal medicines and the main physiological action of the herbal medicine. As shown in Table 6, *Cinnamomum cortex* and *Malloti cortex* show a low concentration of hydrogen peroxide, and are used as intestinal regulators and stomach medicines. *Gallae chinensis*, *gambir*, and *Myricae cortex* show a higher concentration of hydrogen peroxide and are used as...
anti-diarrheics and intestinal regulators. From these findings we inferred that hydrogen peroxide is involved in the action of these natural medicines.

This assumption raised the possibility that “crude drugs that generate high concentrations of hydrogen peroxide kill pathogenic bacteria, but at the same time kill bacteria acting on the intestines.” Therefore, we studied the difference in susceptibility to hydrogen peroxide of the following pathogenic bacteria and intestinal bacteria in Table 7, and the results are shown in Table 8.

We found that $1 \times 10^{-2}$ mol/L of hydrogen peroxide showed antibacterial action against all the bacteria listed in the Table 8, whereas concentrations from $5 \times 10^{-3}$ to $5 \times 10^{-4}$ mol/L showed antibacterial action against pathogenic bacteria but not against bacteria involved in intestinal regulation. This result indicates that natural (herbal) medicines with antidiuretic action show antibacterial action against pathogenic bacteria, but not antibacterial activity towards bacteria involved in intestinal regulation. This finding suggested that natural medicines acting on the gastrointestinal tract are effective due to the difference in susceptibility of bacteria to hydrogen peroxide.

The chemical structure of the main component in each natural medicine studied is shown in Fig. 22. We presume that each principal component is a polyphenol and produces hydrogen peroxide such as catechin.

![Fig. 25. A Schema of the Generation of NO from Antianginal Drugs](image)

![Fig. 26. The Fluorescence Intensity Obtained from Nicorandil and Isosorbide Dinitrate](image)

![Fig. 27. The Measurement Principle of Bioluminescence Method for NO](image)

![Fig. 28. Measurement of NO Release from Nitrate Medicine on This Bioluminescence Assay](image)
5.3. Production of NO from Antianginal Drugs

NO relaxes smooth muscle and is thus considered to be the mechanism of action of antianginal drugs. However, the direct production of NO from antianginal drugs has not been reported to date, and it is generally thought that NO is released only by living cells. Neither electron spin resonance nor fluorescence methods using diaminofluorescein (DAF) have detected NO from aqueous solutions of antianginal drugs. We therefore developed two NO measurement methods based on new principles, and studied NO production from drugs. The two methods are a fluorometric assay using sesamol (38,39) a sesame seed component, and a BL method using soluble guanylate cyclase.40 The measurement principle behind sesamol is shown in Fig. 23, and the method and standard curve are shown in Fig. 24.

When sesamol reacts with NO, sesamol dimerizes, resulting in a fluorescent compound with an excitation wavelength of 365 nm and a fluorescence wavelength of 447 nm. Using this method, it is possible to detect 4×10^{-13} mol of NOC 7 (a NO releasing agent). This method was used to analyze the generation of NO from antianginal drugs, as shown in Fig. 25.

This result shows that NO is generated directly from these drugs because the fluorescence intensity increases as the concentrations of nicorandil and isosorbide dinitrate increase, as shown in Fig. 26.

We simultaneously developed a different approach for NO analysis—a measurement method using BL which allowed us to confirm NO production from these antianginal drugs. The measurement principle is shown in Fig. 27. In this method, NO reacts with guanylate cyclase, the enzyme activity increases, and guanosine 5′-triphosphate (GTP) is converted to cGMP. Since pyrophosphoric acid is generated, NO was measured using the BL assay for pyrophosphoric acid developed for gene analysis, as described above. When this method was applied to nicorandil, the emission intensity increased as the concentration of nicorandil increased, as shown in Fig. 28. This revealed that NO was produced directly from nicorandil. We believe that these NO analysis methods are useful for drug discovery research into antianginal drugs.

6. Summary

We have developed immunoassays based on CL/BL methods, and by adjusting the affinity between antibody and antigen. Both approaches provided highly sensitive EIAs. DNA analysis for SNPs was performed by BL DNA analysis and capillary electrophoresis. We developed a specific method based on hydrogen peroxide and NO for active oxygen/free radical analysis. This specific analysis method makes it possible to elucidate the mechanism of the antibacterial action of catechin and of the production of NO from antiangial drugs.

I have introduced my research in this paper. As I mentioned at the beginning, I believe that analytical chemistry applied to pharmaceutical science will help establish more innovative analytical methods for elucidating pathological conditions and their diagnoses. The disease state becomes more complicated as medical science progresses and new diseases are identified. The discovery of new causative compounds and the development of accurate measurement methods are required for the advancement of analytical chemistry. Analytical chemistry can contribute to medical care by providing more sensitive, more specific, and more accurate techniques by combining and integrating previously known proven methods and by developing novel methods as well.

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Conflict of Interest

The author declares no conflict of interest.

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