Focusing Review

Development of Precise Chromatographic Separation, Preparation and Detection Tools for Biomedical Use

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Received for review February 20, 2008. Accepted February 27, 2008

Abstract

This review describes the author's various developed analytical tools (reagents and methods) for the precise chromatographic separation, preparation and determination of trace amounts of biologically important compounds in mammals. Especially, the development of tools combining highly sensitive fluorescence/chemiluminescence detection and highly selective HPLC are my central objective as in the following research studies: (1) fluorescent azole formation for the determination of aromatic aldehydes and catecholamines, (2) new hetero-bifunctional reagents for protein-protein conjugation, (3) fluorescent aminopyrazine reagent for the determination of saccharides (4) non-radioisotopic assay of insulin using insulin labeled at a selective position, (5) new concept of detachable amino-labeling reagents, which contain an acid anhydride moiety (6) highly sensitive HPLC for the determination of melatonin, and (7) highly sensitive HPLC of D-amino acids.

Keywords: HPLC, fluorescence, azole, hetero-bifunctional reagent, aminopyrazine, melatonin, D-amino acid

1. Introduction

For the analysis of small amounts of a component in biological samples, we need to measure only the target molecules in a complicated mixture of various components. To obtain this objective, we developed analytical methods and reagents which have both a highly sensitive fluorescence/chemiluminescence detection and highly selective separation methods such as HPLC. This strategy includes (1) the development of a fluorescence reagent, (2) utilization of a highly selective enzymatic reaction and product-accumulating function of the enzyme, (3) highly selective and sensitive immunoreaction, and (4) development of HPLC systems with fluorescence/chemiluminescnce detection. In this review, various methods and reagents, developed in our laboratory, related to HPLC used in the biomedical or biochemical field are described.

2. Reagents (1,2-DNS and DTAN) for fluorescence detection of aromatic aldehydes and chemiluminescence reagent (*m*-CED) for catechol amines

1,2-Diaminonaphthalene monosulfate (1,2-DNS), an aromatic 1,2-diamino compounds, was first found to react with aromatic aldehydes in dilute sulfuric acid to form 2-substituted naphtho [1,2-*d*] imidazoles [1, 2] (Figure 1). The fluorescence produced by the aromatic aldehydes in this method is generally higher than those of the fluorescence from aliphatic aldehydes. Since then, various 1,2-diamino benzene derivatives have been developed as fluorogenic reagents for aromatic aldehydes. 2,2 ' -Dithiobis (1-aminonaphthalene) (DTAN) reacts with aromatic aldehydes in an acidic medium in the presence of tributylphosphine, which serves to reduce DTAN to 1-amino-2-thionaphthol, to produce the 2-substituted naphtho [1,2-*d*] thiazoles [3, 4] (Figure 1).

DTAN has been applied to the sensitive HPLC assay of catechol-*O* -methyl transferase in rat liver by determination of the 3 -and 4-*O* -methylated products (vanillin and isovanillin, respec-

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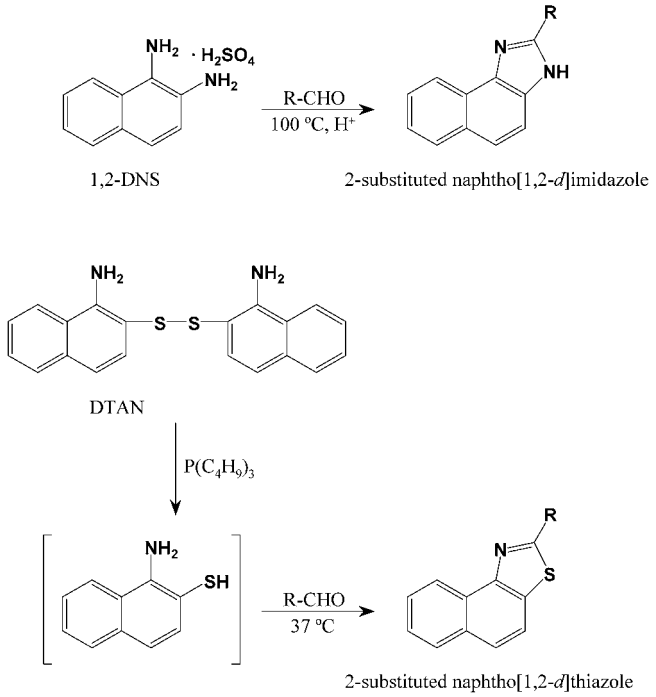


Figure 1. Reactions of aldehydes with 1,2-DNS and DTAN

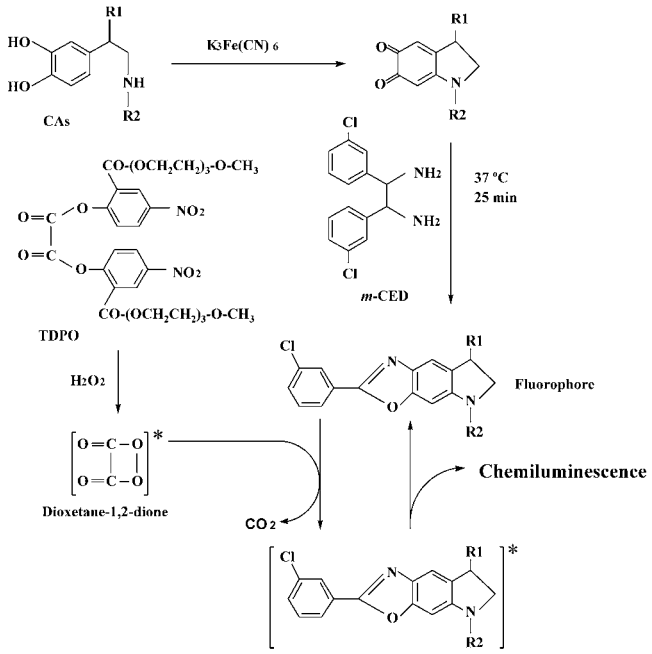


Figure 2. Possible reaction of CAs with *m*-CED and proposed mechanism of chemiluminescence development

tively) of the 3,4-dihydroxybenzaldehyde substrate [5, 6]. The products are converted into the fluorescent 2-aryl naphtho[1,2-*d*] thiazole. The compounds, after extraction with *n*-hexane-chloroform, are separated by normal-phase HPLC onto the in-house Lichrosorb Si 100 column [5].

Monoamine oxidases (MAOs) A and B in rat brain mitochondria can be assyed by measuring the benzaldehyde enzymatically produced from benzylamine (a substrate for MAO A) and 4-sulfamoylbenzaldehyde enzymatically formed from 4-sulfamoylbenzylamine (a substrate for MAO B), which are simultaneously derivatized into fluorescent 2-substituted naphthothiazoles with DTAN and separated by reversed-phase HPLC onto a μ Bondapak CN column [7].

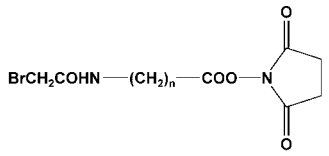
DTAN has also been applied to the HPLC assay of dopamine β-hydroxylase in human serum, rat serum and adrenal medulla [8, 9]. Octopamine, enzymatically formed from the substrate tyramine, is separated by a Dowex 50 W-X 4 open column and oxidized with periodate to 4-hydroxybenzaldehyde, which is converted to a fluorescent 4-hydroxyphenylnaphtho[1,2-*d*]thiazole. The derivative, after extraction with *n*-hexane-chloroform, is separated by normal-phase HPLC on an in-house Alox T aluminum column. This method requires as little as 5 μL of rat serum and 2 μL of human serum [9].

A highly sensitive chemiluminescence method for the determination of the human plasma catecholamines (CAs: norepinephrine, epinephrine and dopamine) was developed [10]. The amines, derivatized with 1,2-bis(3-chlorophenyl)ethylene diamine (*m*-CED), were separated on a reversed-phase TSKgel ODS-120 T column (250 mm x 4.6 mm I.D.; Tosoh Co.) with isocratic elution using a mixture of imidazole buffer, MeOH and MeCN reaction system using bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate (TDPO) and hydrogen peroxide (Figure 2). The detection limits (S/N=3) for each catecholamine are 40-120 amol in a 100 μL injection volume. This method allowed the determination of CAs in 20 μL of human blood plasma [11].

3. Cross-linking reagents containing ω-amino acid for protein-protein conjugation

Bifunctional cross-linking reagents are essential for the preparation of protein-protein conjugate, protein-low molecular compound conjugates, ligand-linked solid matrices and immobilized enzymes, which have been used in various bio-analytical research studies, such as enzyme immunoassays. New heterobifunctional reagents, *N*-(β-bromoacetamido-*n*-alkanoyloxy)succinimides having a glycine or ω-amino acid (β-alanine, γ-aminobutyric acid, δ-aminovaleric acid or ε-aminocaproic acid) residue were synthesized (Figure 3). The bromoacetamido-*n*-alkanoyl group could be introduced into horseradish peroxydase (HRP) and hen egg-white lysozyme. The number of bromoacetamido-*n*-alkanoyl groups introduced into the proteins could be successfully estimated from the amount of glycine or ω-amino acid produced from the introduced group by acid hydrolysis and subsequent amino acid analysis [12].

Because of our interest in the use of enzyme labeled insulin for enzyme immunoassays, we prepared the HRP-insulin conjugate



n = 1	*N*-(bromoacetamidoacetoxy)succinimide	(B A A S)
n = 2	*N*-(β-bromoacetamido-*n*-propionoyloxy)succinimide	(B A P S)
n = 3	*N*-(γ-bromoacetamido-*n*-butyryloxy)succinimide	(B A B S)
n = 4	*N*-(δ-bromoacetamido-*n*-pentanoyloxy)succinimide	(B A P E S)
n = 5	*N*-(ε-bromoacetamido-*n*-caproyloxy)succinimide	(B A C S)

Figure 3. *N*-(ω-bromoacetamido-n-alkanoyloxy) succinimides

using *N*-(β-bromoacetamido-*n*-propionoyloxy)succinimide. The HRP was selectively linked to the ε-amino group of the Lys(B 29) residue of the insulin. In the first step, a single β-bromoacetamido-*n*-propionoyloxy (BAP) group was introduced into the Gly(A 1), Phe(B 1)-dicitraconyl insulin [13] to obtain Gly(A 1), Phe(B 1)-dicitraconyl-BAP-insulin. This conjugate was separated from various citraconylated insulins using anion exchange HPLC (TSKgel DEAE-2 SW column) (Figure 4). The citraconyl groups of Gly(A 1), Phe(B 1)-dicitraconyl-BAP-insulin were then eliminated with 1 M acetic acid to obtain the BAP-insulin. The reaction of HRP-SH with the BAP- insulin produced the HRP-insulin (1:1) conjugate. The principle of this method should be applicable for estimating the molar ratio of the protein to protein conjugate. In this study, these protein-protein conjugates release ω-amino acids which can be separately measured from proteinic amino acid using an amino acid analyzer in which the step-wise elution of the amino acids and post column derivatization with ninhydrin were carried out.

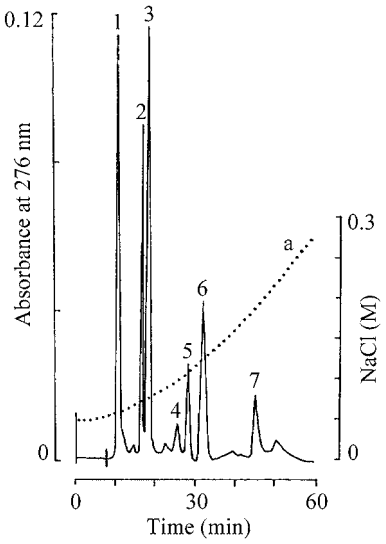


Figure 4. Preparative separation of citraconyl insulins and intact insulin

Curve a: the concentration of NaCl in the eluent during the gradient elution.

Peaks: 1, intact insulin; 2, Phe(B 1)-citraconylinsulin;
3, Lys(B 29)-citraconylinsulin;
4, Gly(A 1) Phe(B 1)-dicitraconylinsulin;
5, Phe(B 1) Lys(B 29)-dicitraconylinsulin;
6, Gly(A 1) Lys(B 29)-dicitraconylinsulin;
7, Gly(A 1) Phe(B 1) Lys(B 29)-tricitraconylinsulin.

4. Reversed-phase HPLC of sacccharides using aminopyrazine as fluorescence derivatizing reagent

Various precolumn fluorescence derivatizing reagents, such as 2-aminopyridine (origin: [14]) have been used for the assay of saccharides. These methods have some disadvantages with respect to the analytical time, production of by-products and separation of the saccharide derivatives. A more sensitive and convenient precolumn fluorogenic reagent of saccharides was needed. Thus a sort of 2-aminopyridine analog, aminopyrazine, was evaluated as a reagent [15, 16]. The separation and determination of monosaccharides were carried out by anion-exchange chromatography using a borate buffer as the eluant. The eight tested monosaccharides were completely separated with a high sensitivity. The detection limits of the eight monosaccharides under optimized conditions were less than 1 pmol/injection, even for Fuc and Gal, which were insensitive in the system. On the other hand, the detection limits for GalNAc and GlcNac were very sensitive and 10 fmol on the column [16]. In this study, the nine tested monosaccharides were separated within 45 min. The Glc oligomers and saccharides having GlcNAc at the reducing end were also separated. The reducing ends of the sugar chains of the glycoprotein are GlcNac or GalNAc in many cases. Thus, this method should be valuable for the carbohydrate analysis of glycoproteins. However, the method requires a very expensive anion-exchange HPLC column. This led us to develop a reversed-phase HPLC method of monosaccharides in glucoproteins. The results obtained for Gal, Man, GlcNAc and GalNAc by this method were consistent with that obtained by this anion-exchange HPLC [17]. Furthermore, 2-amino-3-phenylpyrazine (3 APP) was selected as a more efficient reagent [18]. This pyrazine derivative gave a significantly strong fluorescence and its intensity did not significantly change under the various pH conditions. In addition, the fluorescence excitation maximum wavelength of 3 APP was 331 nm, and therefore, this reagent is expected to be suitable for use with the He-Cd laser beam (325 nm) as an excitation light source. Actually, the 3 APP reagent was successfully applied for the capillary zone electrophoresis (CZE) with the laser-induced fluorescence detection (He-Cd laser). The electropherogram showed the separation of the derivatives for 15 amol of GalNAc and Gal (Figure 5). The sensitivities are about 100 times higher than that obtained by a conventional fluorescence detector.

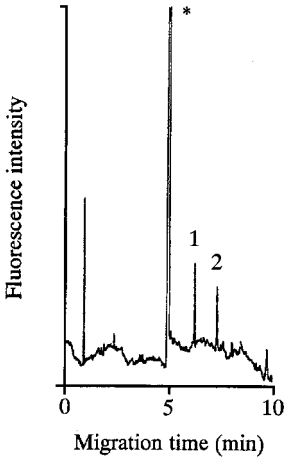


Figure 5. Electropherogram of Gal and GalNAc derivatized with 2 -amino-3-phenylpyrazine obtained by laser-induced fluorescence detection

The amounts of the monosaccharides used are 15 amol for both Gal and GalNAc.

Peaks: 1, GalNAc derivative; 2, Gal derivative. The reagent peaks are denoted by the asterisks.

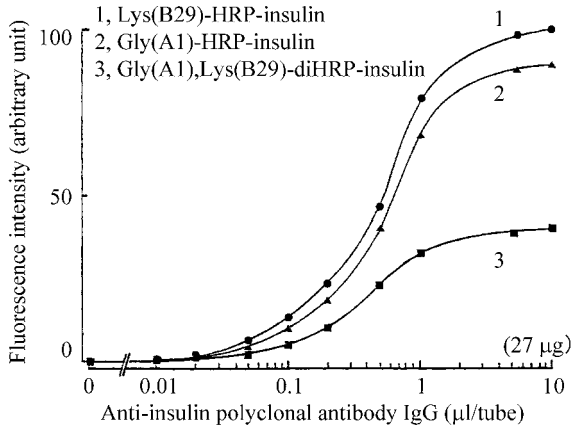


Figure 6. Standard curves for anti-human insulin polyclonal antibody IgG with HRP-insulins

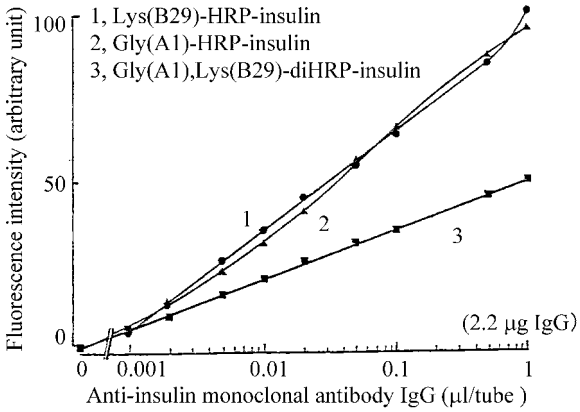


Figure 7. Standard curves for anti-human insulin monoclonal antibody IgG with HRP-insulins

5. Methods for the controled modification of amino group (s) of insulin

Insulin labeled with a reactive amino group (s) at definite site (s) on the molecule should be useful for the preparation of site-specific insulin conjugates of enzyme and fluorescent substances, which may serve to improve the sensitivity for the enzyme- and fluoro-immunoassays of insulin and insulin antibodies. This was first verified by the following study using various insulin conjugates prepared by HPLC.

5.1. Preparation of the conjugates of insulin for enzyme-immunoassay

Five [3-(2-pyridyldithio)propionyl] insulins (PDP-insulins) were initially separated from the reaction mixture of porcine insulin with *N*-succinimidyl-3-(2-pyridyldithio)propionate by anion-exchange HPLC on a TSK gel DEAE-SW column. From the mixture of the random reaction, the HRP-insulin conjugates (Gly(A 1)-HRP-insulin, Lys(B 29)-HRP-insulin, Gly(A 1)Lys(B 29)-diHRP-insulin) were prepared by the reaction of the thiolated HRP and the corresponding PDP-insulins, and purified by gel-permeation HPLC on a TSK gel G 3000 SW column [19]. The three HRP-labelled porcine insulins, which have definite labeling site (s), were compared in the first time regarding the sensitivity in a solid-phase EIA of human insulin [20] and anti-insulin-antibodies [21]. The standard curves obtained with the mono-HRP-insulins were steeper than that with the diHRP-insulin for both the polyclonal and monoclonal antibodies (Figures 6 and 7). The importance of the HRP-labeling site in insulin and the number of labeled HRPs was first demonstrated using HRP-labeled insulin having definite labeling site (s). In this study, HPLC plays an essential role in preparing the pure HRP-labeled insulins. This kind of experiment can be done with only the pure labeled conjugates prepared by the preparative HPLC.

5.2. Preparation of low molecular fluorophore labeled-insulin

Verious insulin derivatives, which have protected sulfhydryl group (s) at definite site (s) on the insulin molecule, were prepared. Porcine insulin reacts with *N*-succinimidyl-*S*-acetylthioacetate (or *S*-acetylmercaptosuccinic anhydride) to afford four species of insulin derivatives that have *S*-acetylthioglycoloyl (ATG) (or acetylmercapto-3-carboxypropanoyl (AMCP)) group (s) at the i) Gly(A 1), ii) Gly(A 1) Phe(B 1), iii) Gly(A 1) and Lys(B 29), and iv) Gly(A 1) Phe(B 1) and Lys(B 29) positions [22, 23]. Separation of these derivatives by anion-exchange HPLC was successfully completed. The four derivatives are all readily deacetylated to give the corresponding sulfhydryl insulins. Theses sulfhydryl insulins

should react with enzyme (s) and fluorophore (s) substituted with a halogenated moiety or maleimide moiety to yield the insulin conjugate. For example, 7-chloro-4-sulfobenzoxadiazole, a fluorogenic reagent, afforded the Gly(A 1)- and Lys(B 29)-[*S*-(4-sulfobenzoxadiazole-7-yl)-thioglycoloyl]-insulins [24]. The fluorescence labeled insulins were compared to the immunoreactivity. Gly(A 1)-labeled insulin provides a 1.6 times greater immunoreactivity and canafford a higher sensitivity in the assay. This part of the study first con firmed that a labeled position [25] and spacer groups [26] on the fluorescent moiety in the insulin molecule affect the immunoreactivity of the labeled insulin.

6. New detachable amino-protecting and amino-labeling reagents

A few reagents can be used for the labeling of functional groups with a detachable reagent at definite site (s) on a protein molecule in a moderate reaction medium. Deprotection of such a detachable reagent will be a key for expanding the precise labeling on the insulin molecule to much larger protein molecules. As the-first step for the expansion, various dicarboxylic acid anhydrides such as 3,4,5,6-tetrahydrophthalic anhydride, were tested to find the best reagent for practical use in the reversible amino-protection of insulin in terms of the rapidity of acid-deprotection. Twelve Gly (A 1), Phe(B 1), Lys(B 29)-triacyl-insulins were prepared by the reaction of porcine insulin with the dicarboxylic acid anhydrides, and the time courses for the deprotection of the acylated insulins with dilute acetic acid were investigated using CZE, and the tetrahydrophthalyl (THP)-insulin was the most rapidly deacylated [27]. The preparative and analytical separations by anion-exchange HPLC and CZE of the product from the reaction of insulin with *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride were also presented [28]. Based on these technical backgrounds, heme-undecapeptides (HUP) labeled on Gly(A 1), Phe(B 1) or Lys(B 29) of insulin were successfully prepared [29] (Figure 8). The catalytic activity was demonstrated using Phe(B 1)-HUP-insulin for the chemiluminescence detection during the immunoassay of insulin [29]. Furthermore, detachable fluorescence amino-labeling and chiral amino-labeling reagents were developed. A new derivatizing reagent, dansylaminomethyl maleic acid (DAM), was synthesized and utilized for the detachable fluorescence labeling of amino groups. The reagent DAM was dehydrated and the resulting anhydride reacted with amino compounds. With this reagent, both benzylamine and insulin were derivatized and successfully regenerated under mild acidic conditions at pH 5.0 [30, 31].

7. A highly sensitive method for the determination of melatonin in biological samples

Although few fmol of melatonin could be determined by various methods, a more sensitive method is needed to determine the low levels of melatonin in the central nervous system and periphery. Therefore, we tried to establish a highly sensitive method for melatonin in biological samples. Melatonin was derivatized under alkaline conditions in the presence of hydrogen peroxide (Figure 9). The resultant fluorophore, *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]acetamide (6-MOQMA), was excited at 247 nm and the emission wavelength was 384 nm. The melatonin derivative was separated by reversed-phase HPLC. The detection limit was 500 amol (S/N=5). This method was successfully used for the determination of melatonin in the rat pineal gland [32]. This method was used for the measurement of the circadian rhythm in rats and mice [33, 34]. The calibration curve of melatonin was linear over the range of 1-500 fmol (injection amounts/20 μL). The detection limit of added melatonin was 1 fmol (S/N=5). Using this method, circadian changes of the melatonin content in the rat (Wistar) and mouse (C 3 H) pineal glands were determined, because the pineal melatonin of many inbred mice strains, such as the ddY

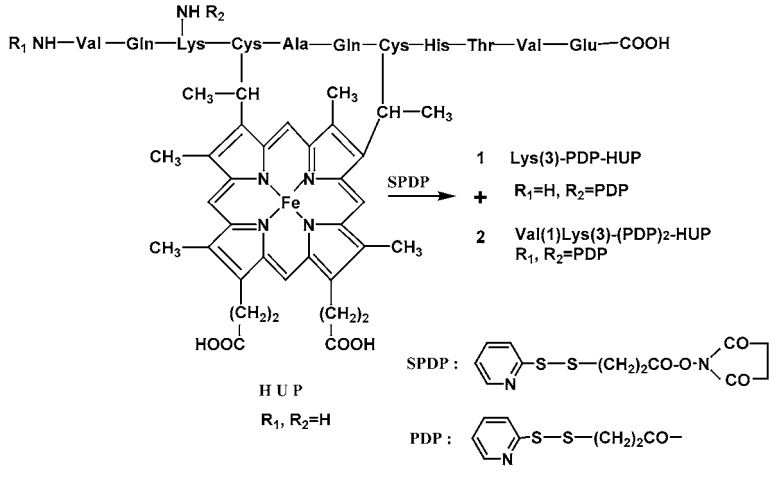


Figure 8. Synthesis of Lys (3)-3-(2-pyridyldithio) propionoyl-HUP

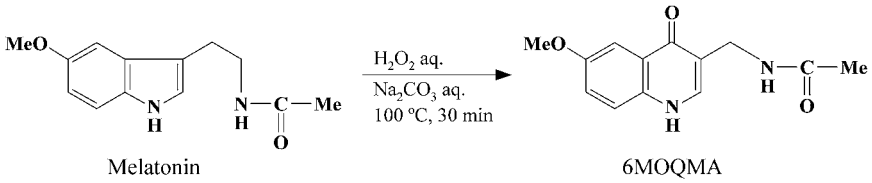
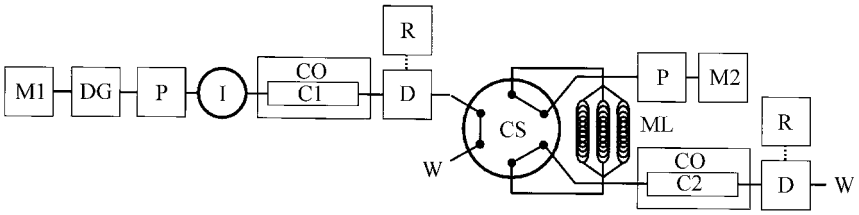


Figure 9. Oxidation of melatonin and the fluorescent product, 6-MOQMA



M1: THF / TFA / H_2O
=25 : 0.05 : 75 (v / v)
flow rate, 75 μL / min
C1: Capcell pak C18 MG II
(150 mm x 1.0 mm I.D., 40 °C)

M2: MeCN / MeOH = 50 / 50 (v / v)
containing 10 mM citric acid
flow rate, 1.5 mL / min
C2: Chiralpak QN-AX
(150 mm x 4.0 mm I.D., 40 °C)

Figure 10. The multi-loop HPLC system for on-line 2 D-determination of four D-amino acids

CS, column selection unit; ML, multi-loop unit; PG, degasser; P, pump; I, injector; CO, column oven; D, detector; R, integrator W, waste.

mouse has been reported to be lower than the detection limit [33]. To improve the sensitivity of this method, a reversed-phase micro-HPLC (Shiseido Nanospace SI-2, 1.0 mm i.d. column) system was constructed. 5-Methoxyindole-3-acetic acid was used as the internal standard for the precise determination. The highest value of 170 S/N for 1 fmol melatonin was attained [34], and the lowest quantification limit of melatonin was 200 amol [35]. The fluorescence product was identified as 6-MOQMA. The fluorescence properties of the formed fluorophore, the 4-quinolone derivative, were also studied using computational chemistry, and developed as an anion sensor [36] and labeling reagent [37]. For the accurate and reliable determination, several melatonin analogs were designed and utilized as the internal standard for the HPLC [38]. A precise and reliable determination of melatonin in human blood samples are now under investigation.

8. A highly sensitive method for the determination of D-amino acids in biological samples

The determination of small amounts of D-amino acids in mammalian tissues is a challenging issue in the separation sciences and bio-medical sciences. As described in a previous paper [39], though some D-amino acids has been noted as the candidates of physiologically active substances, the determination of most D-amino acids is difficult because their amounts in mammalian tissues are extremely low and various interfering substances are present in the tissues. Therefore, for the accurate and sensitive fluorescence determination of minute amounts of D-amino acid, a column-switching system using a micro-ODS column and an enantioselective column was adopted. After pre-column derivatization of D- and L-Leu [40] with the known reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [41], the derivatives of the enantiomers were fractionated on a micro-ODS column as a D+L-amino acids mixture. The eluted D+L-mixture was then introduced into the enantioselective column, and each enantiomer was determined. This procedure has also been conducted to determine other D-amino acids (D-Ala [42], D-Pro [43, 44], D-Thr and D-*allo*-Thr [45]) in rats and mice. Furthermore, a new automated two-dimensional column-switching HPLC system for the analysis of small amounts of branched aliphatic D-amino acids in mammalian tissues and physiological fluid has been developed. A narrow bore reversed-phase column and an enantioselective column were connected with an integrated multi-loop peak fraction storage device (Figure 10) [46]. On the other hand, mouse monoclonal anti-D-Ala antibodies have been raised and very interesting results were obtained. Immunohistochemical localization of D-Ala in the rat pancreas was visualized and specific staining for D-Ala has been observed only in the Langerhans islets. The co-localization of D-Ala immunoreactive cells with insulin producing β-cells is also shown by the double staining technique [47]. The relation between D-Ala and the blood glucose level is under investigation.

9. Conclusion

The author describes highly sensitive and selective chromatographic methods for the determination of biologically important materials and also for the preparation of various conjugates used in several assay systems, such as enzyme-immunoassays. In the near future, the two-dimensional column switching HPLC systems for the analysis of small amounts of melatonin and D-amino acids will direct us to some new bio-medical fields.

Acknowledgements

I would like to express my gratitude to Emeritus Professor Yosuke Ohkura for his kind supervision. I also gratefully acknowledge all my collaborators, coworkers, and colleagues for their enthusiastic research work.

Financial supports from the Ministry of Education, Culture, Sports, Science and Technology, the Japan Society for the Promotion of Science (JSPS), the Japan Science and Technology Agency (JST), New Energy and Industrial Technology Development Organization (NEDO) of Japan, Kyushu University and also from private companies are gratefully acknowledged.

The author is grateful to the Society for Chromatographic Sciences for presenting him with the 2007 Award of the Society for Chromatographic Sciences.

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