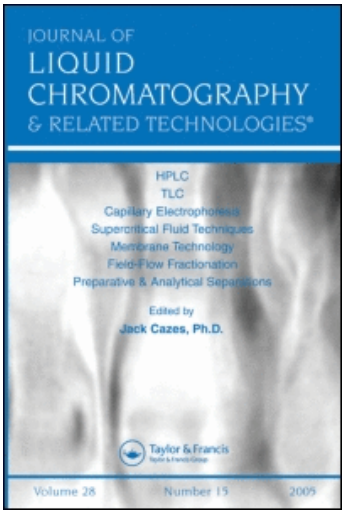

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Chemiluminescence High Performance Liquid Chromatography of Corticosteroids and *p*-Nitrophenacyl Esters Based on the Luminol Reaction

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CHEMILUMINESCENCE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF CORTICOSTEROIDS AND *p*-NITROPHENACYL ESTERS BASED ON THE LUMINOL REACTION

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ABSTRACT

Reducing agents, especially compounds having an α-hydroxycarbonyl group, give an intense chemiluminescence with luminol in the presence of catalyst in alkaline solution. Based on this reaction, the chemiluminescence detection of corticosteroids and *p*-nitrophenacyl esters of carboxylic acids has been developed. These compounds were separated by high performance liquid chromatography on a reversed phase column (Prodigy ODS) for corticosteroids with 30% acetonitrile and for *p*-nitrophenacyl esters with 50% acetonitrile as eluent, and detected by the chemiluminescence reaction with luminol-sodium hydroxide solution containing the catalyst as the post-column reagent. Hexacyanoferrate (III) and hexacyanoferrate (II) were used as catalyst of the reaction. The detection limits of cortisone and *p*-nitrophenacyl hippurate were 1.1 pmol and 0.8 pmol.

INTRODUCTION

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) has been a well-known organic chemiluminescence reagent, since it was first reported in 1928 by Albrecht.[1] The chemiluminescence has been mainly used to determine hydrogen peroxide, other oxidants, and metal ions. Recently, the chemiluminescence analytical methods with immobilized enzyme reactor, based on luminol reaction, have been reported and used to determine clinically important organic compounds such as glucose, ammonia, urea, uric acid, etc.[2-5] In all these methods, hydrogen peroxide produced by oxidizing enzymes has been detected.

We found that reducing agents, such as glucose, ascorbic acid, uric acid, corticosteroids, hydroxylamine, phenacyl alcohol etc., give a chemiluminescence with luminol in the presence of catalyst in an alkaline solution. Based on this finding, the chemiluminescence flow injection analysis (FIA) for the determination of reducing agents was developed. In these reducing agents, corticosteroids, and phenacyl alcohol produced intense light with luminol in alkaline solution.[6]

Carboxylic acids are widely distributed in nature and important as nutritional substrates and metabolites in living organisms. Therefore, there is a widespread interest in the isolation and quantitation of these compounds and a need for more convenient methods that are simple and reliable. Recently, several papers have been reported for the tagging of carboxylic acids with reagents that afford chromophores having UV and Vis absorbance.[7] Among those reagents, phenacyl bromide, and substituted phenacyl bromides[8-12] have been widely accepted for the determination of free fatty acids. We also found that these phenacyl bromides give the chemiluminescence.

In this paper, we describe the chemiluminescence detection system for corticosteroids and *p*-nitrophenacyl esters of carboxylic acids after separation by HPLC.

EXPERIMENTAL

Reagents

Luminol and all steroids were purchased from Tokyo Chemical Industry (Tokyo, Japan). Phenacyl bromide and its derivatives were also purchased from Tokyo Chemical Industry. All carboxylic acids and 18-Crown-6

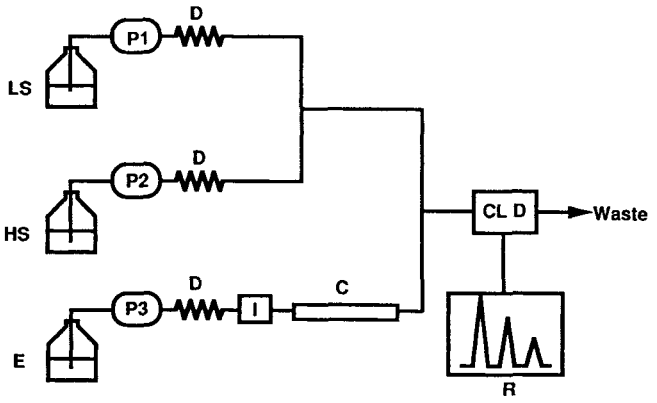


Figure 1. Flow diagram of chemiluminescence HPLC system. LS: luminol solution, HS: hexacyanoferrate (III) solution, E: eluent, P1, P2, P3 : pump, D: damper, I: injector, C: column, CLD: chemiluminescence detector, R : recorder.

(1,4,7,10,13,16-hexaoxacyclooctadecane) were purchased from Wako (Osaka, Japan). All other chemicals were of analytical-reagent grade. Standard solutions were prepared by dissolving in 30 % acetonitrile to give concentration of 200 μmole/L for corticosterone, 100 μmole/L for cortisone and phenacyl alcohol, and by dissolving in acetonitrile to give concentration of 50 μmole/L *p*-nitrophenacyl acetate and *p*-nitrophenacyl benzoate.

Preparation of Phenacyl Esters of Carboxylic Acids

Methanol solutions of the carboxylic acids were neutralized with a KOH-methanol solution. The solvent was removed under aspirator vacuum to give the potassium salts of the carboxylic acids. An excess solution of the phenacyl bromide / 18-Crown-6 (20 : 1 in acetonitrile) was then added and the mixture was refluxed for 30 min. The reaction mixture was evaporated under aspirator vacuum. The precipitated crude product was isolated by filtration and then recrystallized from ethanol. These esters were identified by NMR and MS spectra.

Apparatus

Figure 1 illustrates the flow diagram for a HPLC system. The pumps used were a Model 576 HPLC pump (GL Sciences, Tokyo, Japan) for the luminol

solution, a Hitachi Model L-6200 (Hitachi, Tokyo, Japan) for the hexacyanoferrate (III) solution, and a Model 501G HPLC pump (Nihon Waters Co., Tokyo, Japan) for the eluent. Each pump was attached to the damper. The injection device was U6K injector (Nihon Waters Co., Tokyo, Japan) and the column was a Prodigy 5μ ODS (3) (150 × 4.6 mm i.d., Phenomenex Co., California, USA).

The chemiluminescence detector equipped with a spiral type flow cell having a volume of 100 μL and with a photomultiplier tube biased at 750 V was a Soma S-3400 (Soma Optics LTD., Tokyo, Japan). The recorder was an Unicorder U-228 chart recorder (Nihon Denshi Kagaku Co., Kyoto, Japan).

Chromatographic Conditions

Eluents were 30 % acetonitrile and 50 % acetonitrile for the separation of corticosteroids and *p*-nitrophenacyl esters, respectively. Luminol solution was prepared in 0.4 M sodium hydroxide solution containing 1.0 mM luminol and 250 mM hexacyanoferrate (II). Hexacyanoferrate (III) solution was 0.2 mM hexacyanoferrate (III) aqueous solution. The flow rate of the eluent was 1.0 mL/min, and that of the luminol solution and the hexacyanoferrate (III) solution were 0.5 mL/min and 0.3 mL/min, respectively.

RESULTS

Conditions for Chemiluminescence in the FIA System

In order to determine the optimal conditions for the chemiluminescence detection system, concentrations of reagents and flow-rates were tested. The concentrations of reagents are shown in Figure 2, 3, 4, and 5. The optimal conditions of luminol, hexacyanoferrate (III), sodium hydroxide, and hexacyanoferrate (II) for all of the standard compounds were 1.0 mM, 0.2 mM, 0.4 M, and 250 mM, respectively.

Figures 6 and 7 illustrate the effect on varying the flow-rates of the luminol solution and hexacyanoferrate (III) solution with the eluent flow-rate constant at 1.0 mL/min. The maximum responses were obtained at 0.5 mL/min for the luminol solution and 0.3 mL/min for the hexacyanoferrate (III) solution.

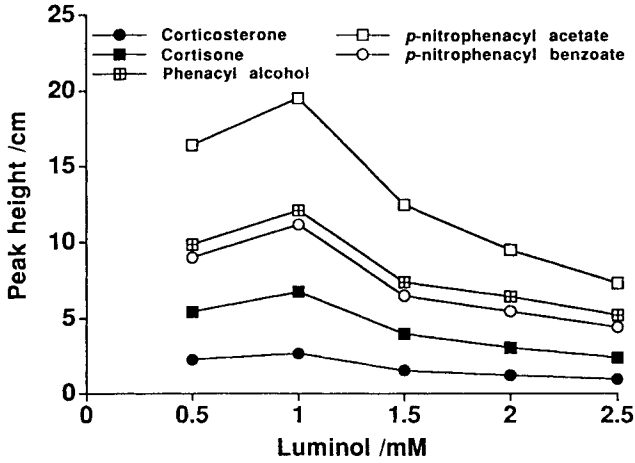


Figure 2. Effect of luminol solution. Conditions: luminol solution containing 0.4 M NaOH and 250 mM $K_4Fe(CN)_6$, hexacyanoferrate (III) solution containing 0.2 mM $K_3Fe(CN)_6$.

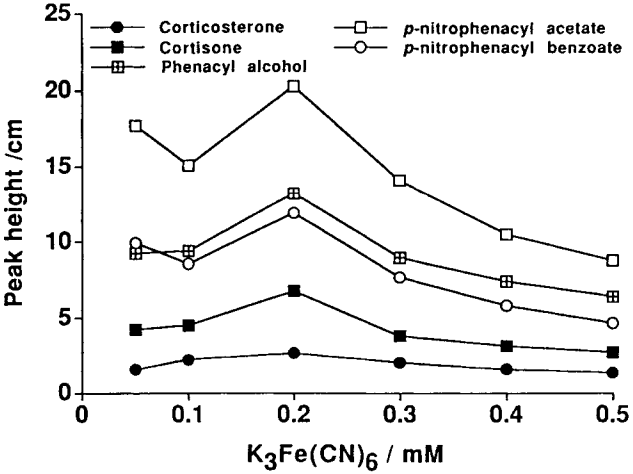


Figure 3. Effect of hexacyanoferrate (III) concentration. Conditions: luminol solution containing 1.0 mM luminol, 0.4 M NaOH and 250 mM $K_4Fe(CN)_6$.

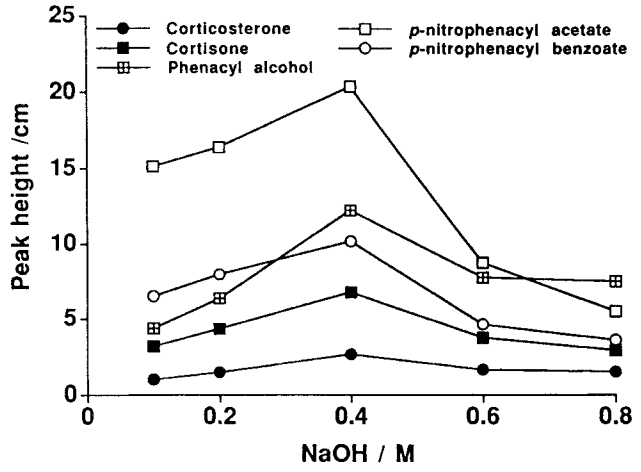


Figure 4. Effect of sodium hydroxide concentration. Conditions: luminol solution containing 1.0 mM luminol and 250 mM K4Fe(CN)6, hexacyanoferrate (III) solution containing 0.2 mM $K_3Fe(CN)_6$.

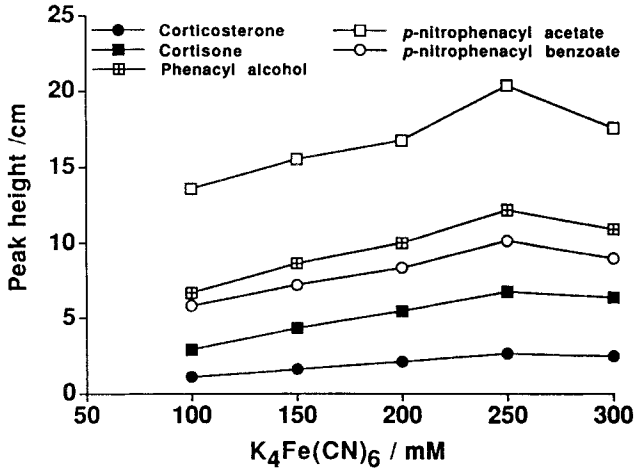


Figure 5. Effect of hexacyanoferrate (II) concentration. Conditions: luminol solution containing 1.0 mM luminol and 0.4 M NaOH, hexacyanoferrate (III) solution containing 0.2 mM $K_3Fe(CN)_6$.

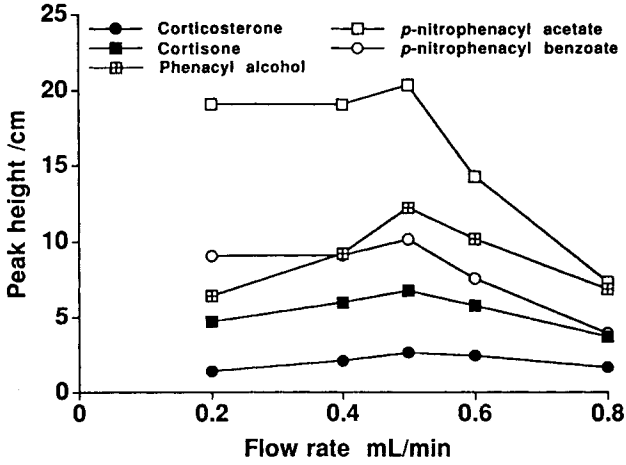


Figure 6. Effect of flow-rate of luminol solution. Conditions: luminol solution containing 1.0 mM luminol, 0.4 M NaOH and 250 mM $K_4Fe(CN)_6$, hexacyanoferrate (III) solution containing 0.2 mM $K_3Fe(CN)_6$.

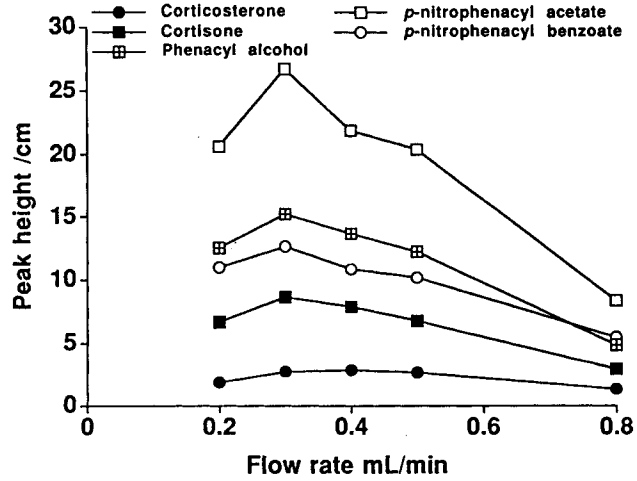
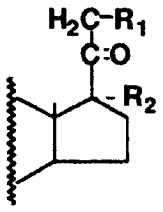


Figure 7. Effect of flow-rate of hexacyanoferrate (III) solution. Conditions: luminol solution containing 1.0 mM luminol, 0.4 M NaOH and 250 mM $K_4Fe(CN)_6$, hexacyanoferrate (III) solution containing 0.2 mM $K_3Fe(CN)_6$.

Table 1

Chemiluminescence Intensities of Steroids



Steroids	Relative Intensity	R_1	R_2
Cortisone	2.62	OH	OH
Methylprednisolone	1.45	OH	OH
Dexamethasone	1.38	OH	OH
Hydrocortisone	1.00	OH	OH
Tetrahydrocortisol	0.99	OH	OH
Corticosterone	0.65	OH	H
Deoxycorticosterone	0.20	OH	H
Betamethasone	0.16	OH	OH
Progesterone	0.00	H	H
11α-Hydroxyprogesterone	0.00	H	H
17α-Hydroxypregnenolone	0.00	H	OH

Chemiluminescence HPLC for Corticosteroids

Table 1 lists the chemiluminescence intensities obtained by injecting standard steroids solutions into the FIA. The intensities are normalized so that the signal of hydrocortisone has a value of 1.00.

Figure 8 shows a chromatogram from a standard mixture of corticosteroids. The separation of the compounds was obtained within 20 min and detected by the proposed detection system.

A linear regression analysis of the working curves which were obtained in the range 0.1 ~ 3 nmol per injection yielded the equation Y = 17.376X - 0.086 (r = 0.997) for predonisolone, Y = 15.664X - 0.100 (r = 0.996) for hydrocortisone, Y = 47.017X - 0.340 (r = 0.996) for cortisone, Y = 11.458X - 0.097 (r = 0.996) for methylpredonisolone, Y = 1.349X - 0.008 (r = 0.997) for betamethasone, Y = 8.643X - 0.044 (r = 0.999) for dexamethasone and Y =

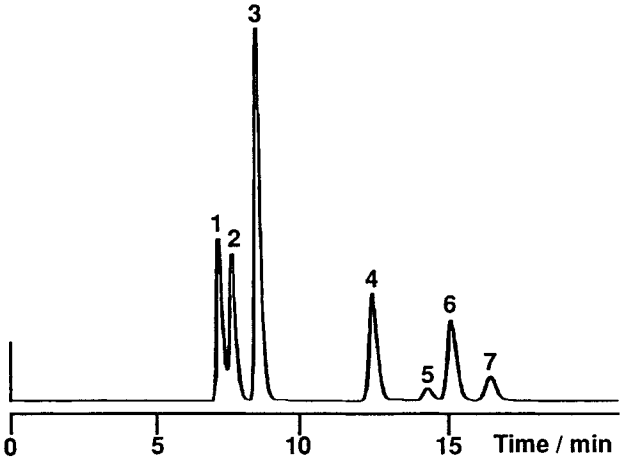


Figure 8. Chromatogram obtained with a mixture of corticosteroids. Peaks: 1 = prednisolone, 2 = hydrocortisone, 3 = cortisone, 4 = methylprednisolone, 5 = betamethasone, 6 = dexamethasone and 7 = corticosterone, 1 mg each.

2.566X - 0.024 (r = 0.994) for corticosterone. The chemiluminescence, peak height (Y), was related to the moles (X) with high linearity. The relative standard deviation was 1.0 ~ 2.5 % for the injection analysis of standard solution repeated 10 times. The limits of detection (S/N = 3) for predonisolone, hydrocortisone, cortisone, methylpredonisolone, betamethasone, dexamethasone, and corticosterone were 2.9 pmole, 3.2 pmole, 1.1 pmole, 4.3 pmole, 34.9 pmole, 5.5 pmole, and 20.5 pmole, respectively.

Chemiluminescence HPLC for Carboxylic Acids

The chemiluminescence of four phenacyl esters with luminol was examined by the FIA system. The results are shown in Table 2. *p*-Nitrophenacyl esters gave a higher chemiluminescence than other phenacyl esters. Therefore, *p*-nitrophenacyl bromide was selected as the pre-column reagent for chemiluminescence HPLC of carboxylic acids.

Figure 9 shows a chromatogram obtained from a standard mixture of synthesized *p*-nitrophenacyl esters of various carboxylic acids. The separation of these esters was achieved within 30 min.

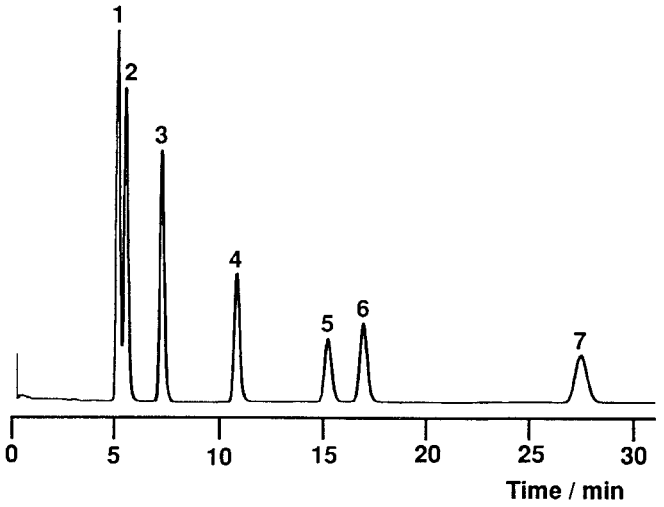
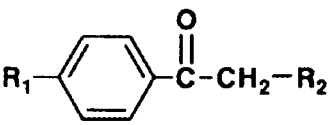


Figure 9. Chromatogram obtained with a mixture of p-nitrophenacyl esters of carboxylic acids. Peaks: 1 = acetic acid, 2 = hippuric acid, 3 = propionic acid, 4 = butyric acid, 5 = benzoic acid, 6 = valeric acid and 7 = caproic acid, 100 ng each.

Table 2

Relative Chemiluminescence Intensities of Phenacyl Esters



R_1	R_2	Relative Intensity
H	OH	1.00
H	$OCOCH_3$	1.09
Br	$OCOCH_3$	2.07
NO_2	$OCOCH_3$	3.61
C_6H_5	$OCOCH_3$	1.11

A linear regression analysis of the working curves which were obtained in the range 10 ~ 350 pmol per injection yielded the equation Y = 153.091X + 0.329 (r = 0.999) for acetic derivative, Y = 130.364X + 0.266 (r = 0.999) for hippuric derivative, Y = 104.427X + 0.214 (r = 0.999) for propionic derivative, Y = 50.969X + 0.110 (r = 0.999) for buthyric derivative, Y = 25.748X + 0.051 (r = 1.000) for benzoic derivative, Y = 31.298X + 0.048 (r = 1.000) for valeric derivative, and Y = 19.551X - 0.024 (r = 1.000) for caproic derivative. The chemiluminescence, peak height (Y), was related to the moles (X) with high linearity. The relative standard deviation was 1.0 ~ 2.5 % for the injection analysis of standard solution repeated 10 times. The limits of detection (S/N = 3) for acetic derivative, hippuric derivative, propionic derivative, butyric derivative, benzoic derivative, valeric derivative, and caproic derivative were 1.0 pmole, 0.8 pmole, 1.5 pmole, 2.9 pmole, 5.0 pmole, 4.5 pmole, and 6.8 pmole, respectively.

DISCUSSION

In the proposed system, hexacyanoferrate (III) as cooxidant was used as the catalyst for the chemiluminescence reaction. The chemiluminescence intensity is very high, but the background intensity is large in the presence of hexacyanoferrate (III). Shevlin and Neufeld[13] have reported that the addition of hexacyanoferrate (II) onto the luminol reaction in the presence of hexacyanoferrate (III) decreases the chemiluminescence intensity. Therefore, the background intensity could be controlled by the addition of hexacyanoferrate (II) to the luminol solution.

Among the previously observed reducing agents for the chemiluminescence intensity with luminol, there was a different relative response for each compound. There was no obvious correlation potential for each compounds. In previous papers,[14-18] the chemiluminescence reaction of lucigenin with reducing agents was reported. Maeda et.al.[19,29] described that α-hydroxycarbonyl group is essential for the chemiluminescence and α-hydroxycarbonyl compounds convert easily to the 1, 2-enediol tautomer, which are strong reductants for reacting with lucigenin. Our results of the chemiluminescence of luminol with reducing agents were similar to their results. The agreement between the chemiluminescence spectra produced with the reducing agents and the fluorescence spectrum of an aminophthalic acid indicated that aminophthalic acid is chemiluminescent species.

As shown in Table 1, there is a different relative response for each corticosteroids. Cortisone, methylprednisolone, dexamethasone and hydrocortisone, having 17, 21-diol-20- one side-chain, gave an intense

chemiluminescence. 17 α-Hydroxypregnenolone, having a hydroxy group at C-17 and progesterone and 11 α-hydroxyprogesterone, having no hydroxy group at the α- position of the carbonyl group, gave no chemiluminescence with luminol. Although 17 α-hydroxypregnenolone has an α-hydroxycarbonyl group in the side - chain, the hydroxy group at C-17 is tertiary and therefore cannot be converted to a 1, 2- enediol structure.

As mentioned above, the phenacyl esters of carboxylic acids gave an intense chemiluminescence with luminol. Phenacyl alcohol is obtained by the hydrolysis of the phenacyl esters in the alkaline medium and the phenacyl alcohol gives a chemiluminescence.

The proposed chemiluminescence detection system for HPLC with luminol in the presence of catalyst in alkaline solution proved to be applicable to the determination of corticosteroids without derivatization, and of carboxylic acids following pre-column derivatization with *p*-nitrophenacyl bromide.

The detailed mechanisms are not yet elucidated at present about the luminol reaction by the reducing agents. Further studies on the application of this system to the assay of other reducing agents are in progress in our laboratory.

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