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CHEMILUMINESCENCE WITH LUCIGENIN AS POST-COLUMN REAGENT IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CORTICOSTEROIDS AND *p*-NITROPHENACYL ESTERS

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SUMMARY

Compounds having an α-hydroxycarbonyl group give an intense chemiluminescence with lucigenin 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 (Zorbax ODS) with a methanol–water (7:3) mixture as eluent, and detected by the chemiluminescence reaction with lucigenin–potassium hydroxide solution as the post-column reagent. The detection limits of corticosteroids and *p*-nitrophenacyl esters were *ca.* 0.5 pmol per injection.

INTRODUCTION

In recent years, there has been an increasing interest in the development of chemiluminescence and bioluminescence methods for the analysis of biological substances because of their high sensitivities, selectivities and wide dynamic ranges[1]. In order to increase the sensitivity of high-performance liquid chromatography (HPLC), several investigators have reported chemiluminescence HPLC methods with the peroxyoxalate hydrogen peroxide system[2–7] and the lucigenin chemiluminescence reaction system[8,9]. In a previous paper[10], we developed a chemiluminescence HPLC analysis for carboxylic acids and amines with an isoluminol derivative as the pre-column reagent. Lucigenin (N,N′-dimethyl-9,9′-diacridinium nitrate) is another well-known organic chemiluminescent reagent. Chemiluminescence appears on oxidation by hydrogen peroxide in aqueous basic solution, and also on addition of various nucleophiles and reducing compounds[11]. Veazey and Nieman[12] reported the chemiluminescence analysis of clinically important organic reductants, such as glucose, ascorbic acid, uric acid, etc., and developed a chemiluminescence HPLC analysis for these compounds based on the lucigenin chemiluminescence reaction. We have also examined the chemiluminescence reaction of lucigenin with various biologically important substances and found that corticosteroids and phenacyl esters of carboxylic acids produced intense light with lucigenin in basic solution[13].

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

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EXPERIMENTAL

Reagents

Lucigenin was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All steroids were purchased from Sigma (St. Louis, MO, U.S.A.) and Steraloids (Wilton, NH, U.S.A.). Phenacyl bromide and its derivatives were purchased from Tokyo Chemical Industry (Tokyo, Japan) and all carboxylic acids also from Tokyo Chemical Industry and Wako (Osaka, Japan). All other chemicals were of reagent grade.

Solutions

Lucigenin (0.01%) and potassium hydroxide (1 mol/l) solutions were prepared by dissolving in redistilled water, and were kept in a refrigerator until use.

The luminogenic reagent solution was prepared by mixing 10 ml each of 0.01% lucigenin and 0.1 mol/l potassium hydroxide, followed by dilution with redistilled water to 100 ml just before use. This solution should be kept in a light-resistant bottle. The flow system of the HPLC should be washed with 0.1 mol/l potassium hydroxide and methanol after measurement.

All steroid stock solutions (1 mg/ml) were prepared by dissolving the steroids in methanol; they were stored at −20°C.

Preparation of phenacyl esters of carboxylic acids

Carboxylic acid (1 mmol) was dissolved in ethanol containing an equal amount of sodium ethoxide and 1.2 mmol of phenacyl bromide. After refluxing for 2 h, the reaction mixture was evaporated under reduced pressure. The precipitated crude product was isolated by filtration and then recrystallized from ethanol. These esters were identified by elemental analysis and IR spectra. The stock solutions of phenacyl esters (1 mg/ml) were prepared by dissolving them in methanol.

Apparatus

The flow diagram of the HPLC detection system, based on chemiluminescence, is shown in Fig. 1. The pumps used were a Shimadzu LC-5A (Shimadzu, Kyoto, Japan) for the eluent and a Hitachi Model 634 (Hitachi, Tokyo, Japan) for the luminogenic reagent. The injection valve was a Rheodyne 7125 and the column was a Zorbax ODS column (150 mm × 4.6 mm I.D.). A rotating-flow mixing device, reported by Kobayashi and Imai[14] (Kyowa Seimitsu, Tokyo, Japan), was used at the position where the two solutions were jointed with a mixing coil instead of a conventional joint. The chemiluminescence generated was monitored with a chemiluminescence detector, consisting of a photomultiplier tube (Hamamatsu TV, End-on

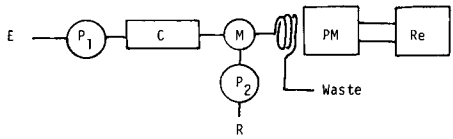


Fig. 1. Flow diagram of the HPLC detection system based on lucigenin chemiluminescence. E, eluent; P_1, Shimadzu LC-5A pump; C, column (Zorbax ODS); P_2, Hitachi 634 pump; R, luminogenic reagent; M, rotating-flow mixing device; PM, photomultiplier; Re, recorder.

Type Model HT 614) and spiral coil flow-cell of silica (120 mm × 4 mm I.D.). The photomultiplier tube was biased at 400–500 V by a Jasco Model JA-2001A Regulated DC Supply and the output was amplified and quantized by a home-made electronic amplifier. A Hitachi Model 056 recorder (Hitachi) was used for recording the chromatograms.

The HPLC system without the column was used for the measurement of relative chemiluminescence intensities of various compounds.

Chromatographic conditions

The mobile phase was methanol–water (7:3) and the flow-rates were 0.5 ml/min and 0.8 ml/min for the separation of corticosteroids and *p*-nitrophenacyl esters, respectively. The luminogenic reagent solution (0.001% lucigenin in 0.1 mol/l potassium hydroxide solution) was pumped at a flow-rate of 0.7 ml/min.

RESULTS AND DISCUSSION

Nieman and co-workers[8,9,12] reported the chemiluminescence reaction of lucigenin with organic reducing compounds (reducing sugars, ascorbic acid, uric acid, etc.) found at significant levels in blood and urine, and applied it to an HPLC detection system. We have also investigated the chemiluminescence reaction of lucigenin with various biological compounds. Table I summarizes the data obtained by the chemiluminescence reaction with lucigenin from our experiments[13]. Reducing sugars produced light, while glycosides gave no chemiluminescence because they have no reducing group. Glyceraldehyde, glycolaldehyde and dihydroxyacetone also gave an intense chemiluminescence. On the other hand, formaldehyde, acetaldehyde and acetone produced no light. Cortisol and related steroids having 17,21-dihydroxy-20-one side-chains gave an intense chemilumescence. Other α-hydroxycarbonyl compounds (2-hydroxyacetophenone, benzoin, phenacyl alcohol and phenacyl esters of various carboxylic acid) also emitted light with lucigenin. From these results, we

TABLE I

RELATIVE CHEMILUMINESCENCE INTENSITIES OF VARIOUS COMPOUNDS

Compound	*Relative chemiluminescense intensity*	*Compound*	*Relative chemiluminescense intensity*
Glucose	1.0	Formaldehyde	0.0
Galactose	1.0	Acetaldehyde	0.0
Mannose	1.0	Cortisol	41.0
2-Deoxyglucose	0.0	Tetrahydrocortisol	47.0
2-Deoxygalactose	0.0	Cortisone	42.0
Glucosamine	1.9	17α-Hydroxyprogresterone	0.0
N-Acetylglucosamine	0.0	Progesterone	0.0
Methyl α-D-glucoside	0.0	Dihydroxyacetone	212.0
Methyl β-D-galactoside	0.0	Acetone	0.0
Glyceraldehyde	138.0	2-Hydroxyacetophenone	267.0
Glycolaldehyde	181.0	Benzoin	173.0

concluded that an α-hydroxycarbonyl group is essential for the chemiluminescence of lucigenin.

In the chemiluminescence reaction of lucigenin, many side-reactions occur but the main chemiluminescence reaction pathway is the formation of the dioxetane intermediate, which decomposes to produce N-methyl acridone in the excited state[15]. Recently, Veazey *et al.*[16] suggested that the chemiluminescence reaction of lucigenin with reducing sugars is based on the formation of the 1,2-enediol tautomer. This intermediate enediol is oxidized by lucigenin in subsequent reaction steps. The various α-hydroxycarbonyl compounds tested here also convert easily to the 1,2-enediol type structures, which are strong reductants for reacting with lucigenin. Although uric acid and creatinine have no α-hydroxycarbonyl moiety, they are strong reductants like the 1,2-enediols, and react with lucigenin to yield intense light. The detailed mechanisms are not well understood at present.

Conditions for chemiluminescence in the flow detection system

In order to determine the optimal conditions for the chemiluminescence detection system, some parameters (concentrations of regents and flow-rate) were tested. The dependence of peak height on the concentrations of potassium hydroxide and lucigenin are shown in Fig. 2A and 2B, respectively. Constant chemiluminescence was observed at potassium hydroxide concentrations >0.1 mol/l, and the most effective concentration was 0.001% for lucigenin (equivalent to 0.02 mmol/l). Fig. 2C illustrates the effect on the peak height of varying the luminogenic regent flow-rate with the HPLC flow-rate constant at 0.7 ml/min. Optimization involved adjusting the luminogenic reagent flow-rates and the delay time between mixing of the reagent and observation of the light emitted from the reaction. As shown in Fig. 2C, the maximum response was obtained at *ca.* 0.7 ml/min.

The HPLC flow-rate also affects the peak height of analyte. The peak height increased on increasing the flow-rate of the HPLC mobile phase to the constant flow-rate of luminogenic reagent. This result indictes that there is definitely a dilution factor of major significance influencing the chemiluminescence intensity of analyte. The rate of analyte entering the mixing device linearly increases with the HPLC flow-rate, resulting in a corresponding decrease in the dilution of analyte in the mixing device. Therefore, a linear increase in analyte concentration would be expected to yield a linear increase in the chemiluminescence intensity of analyte. However, the flow-rates of the mobile phase in HPLC for good separation of steroids and *p*-nitrophenacyl esters of fatty acids were 0.5 ml/min and $\leqslant 0.8$ ml/min, respectively. There is thus a compromise between being able to obtain a good separation and obtaining the maximum sensitivity of detection.

Chemiluminescence HPLC for corticosteroids

Table II lists the chemiluminescence intensities obtained by injecting standard steroid solutions into the HPLC system without the separation column. The intensities are normalized so that the signal of cortisol has a value of 1.00. Cortisol, cortisone, 11-deoxycortisol and their tetrahydro derivatives all show a value of *ca.* 1.00. These steroids have the 17,21-diol-20-one side-chain, which forms the tautomeric 1,2-enediol in alkaline medium. The chemiluminescence intensities of corticosterone and deoxycorticosterone (having no hydroxy group at C-17) were only one-

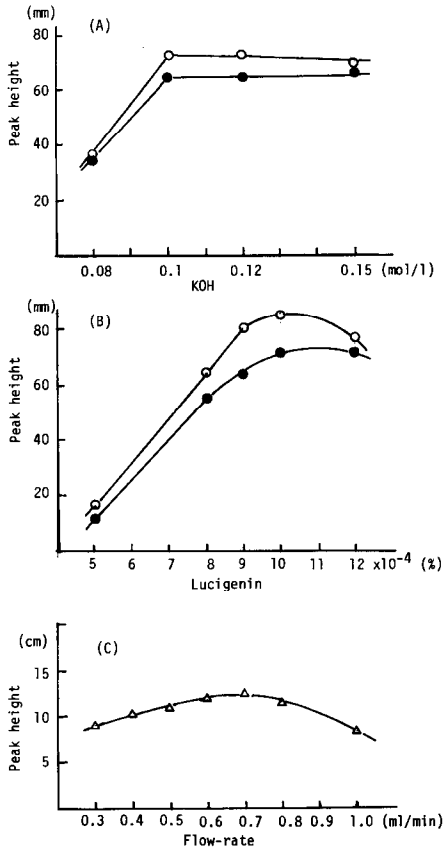


Fig. 2. Effects of concentration of potassium hydroxyde and lucigenin, and flow-rate of luminogenic reagent. (A) Potassium hydroxide, (B) lucigenin, (C) flow-rate of luminogenic regent. (●): *p*-Nitrophenacyl benzoate, 50 ng/ml; (○): *p*-nitrophenacyl acetate, 10 ng/ml; (△): *p*-nitrophenacyl acetate, 17 ng/ml.

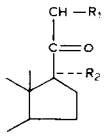
third or one-quarter that of cortisol. 17α-Hydroxyprogesterone and 21-deoxycortisol, having a hydroxy group at C-17, gave no chemiluminescence with lucigenin. Although both steroids have an α-hydroxycarbonyl group in the side-chain, the hydroxy group at C-17 is tertiary and therefore cannot be converted to a 1,2-enediol structure. Progesterone, having no hydroxy group at the α-position of the carbonyl group, gave no lucigenin chemiluminescence reaction.

Six corticosteroids were separated on a reversed-phase column within 25 min and detected by the proposed detection system with lucigenin, as shown in Fig. 3. Retention times are listed in Table II. Linear calibration curves were obtained in the range 2–14 pmol per injection. The detection limit was 2 pmol per injection (S/N = 5), which was comparable to that of fluorescence HPLC with dansyl hydrazine as a pre-column reagent[17,18].

Weinberger *et al.*[7] have reported higher detectability in the peroxyoxalate chemiluminescence reaction than in the fluorescence method could be obtained, without increasing the background signal, by raising a photomultiplier tube voltage. When we raised the photomultiplier tube voltage from 400 to 500 V in our system,

TABLE II

RELATIVE CHEMILUMINESCENCE INTENSITIES AND RETENTION TIMES OF STEROIDS



Steroid	R_1	R_2	*Relative chemiluminescence intensity*	*Retention time (min)*
Cortisol	OH	OH	1.00	4.0
11-Deoxycortisol	OH	OH	0.94	8.0
Cortisone	OH	OH	1.10	5.2
Tetrahydrocortisol	OH	OH	0.94	8.8
Tetrahydro-11-deoxycortisol	OH	OH	0.80	20.0
Tetrahydrocortisone	OH	OH	1.10	8.4
Corticosterone	OH	H	0.30	8.0
Deoxycorticosterone	OH	H	0.25	14.0
17α-Hydroxyprogesterone	H	OH	0.00	*
21-Deoxycortisol	H	OH	0.00	*
Progesterone	H	H	0.00	*

* No peak by chemiluminescence detection.

the detection limit fell to 500 fmol per injection and a linear calibration curve was obtained between 500 fmol and 5 pmol per injection. Corticosteroids exhibiting weak UV absorption or no native fluorescence could be detected at pmol or fmol levels without pre-column derivatization by the proposed detection system.

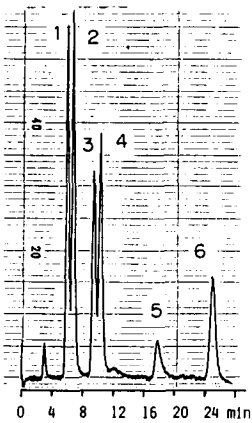
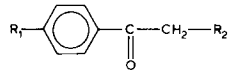


Fig. 3. Chemiluminescence HPLC profile of corticosteroids. Peaks: 1 = cortisol, 2 = cortisone, 3 = tetrahydro-11-deoxycortisol, 4 = tetrahydrocortisone, 5 = deoxycorticosterone, 6 = tetrahydro-11-deoxycortisol. Conditions: Zorbax ODS column (150 × 4.6 mm I.D.); mobile phase = methanol–water (7:3); flow-rate = 0.5 ml/min.

TABLE III

RELATIVE CHEMILUMINESCENCE INTENSITIES OF PHENACYL ESTERS



R_1	R_2	*Relative chemiluminescence intensity*	R_1	R_2	*Relative chemiluminescence intensity*
H	OH	1.00	NO_2	CH_3COO	1.51
H	CH_3COO	0.83	NO_2	C_6H_5COO	1.25
H	C_6H_5OO	0.65	C_6H_5	CH_3COO	0.39
Br	CH_3COO	1.54	C_6H_5	C_6H_5COO	0.39
Br	C_6H_5COO	0.13			

Chemiluminescence HPLC for carboxylic acids

As mentioned above, phenacyl esters of carboxylic acids gave an intense chemiluminescence with alkaline lucigenin. The chemiluminescence of four phenacyl bromides with lucigenin was examined by the flow injection system. The results are shown in Table III. *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 using lucigenin. A typical chromatogram of a standard mixture of synthesized *p*-nitrophenacyl esters of various carboxylic acids is shown in Fig. 4. These esters could be separated on a reversed-phase column within 30 min. Calibration curves were linear between 2 and 20 pmol per injection. The detection limit (S/N = 5) was *ca.* 500 fmol per injection. This value is lower than the UV detection for various phenacyl esters of carboxylic acids[19,20] and comparable to that obtained by fluorescence detection of bile acids with 1-bromoacetyl pyrene as a pre-column reagent[21].

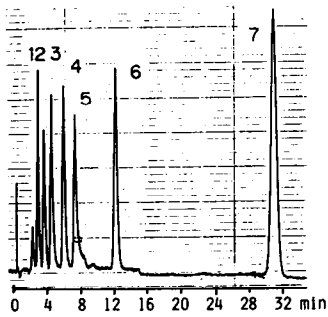


Fig. 4. Chemiluminescence HPLC profile for *p*-nitrophenacyl esters of carboxylic acids. Peaks: 1 = hippuric acid, 2 = acetic acid, 3 = propionic acid, 4 = butyric acid, 5 = benzoic acid, 6 = caproic acid, 7 = caprylic acid. Peaks 1–5, 20 pmol; peak 6, 30 pmol; peak 7, 40 pmol. Conditions: same as in Fig. 3 except flow-rate = 0.8 ml/min.

Conclusion

The proposed chemiluminescence detection system for HPLC with lucigenin in alkaline medium proved to be applicable to the determination of steroids, without pre-column derivatization, and of carboxylic acids following pre-column derivatization with *p*-nitrophenacyl bromide. The sensitivity of the method described in this system was superior to that of other HPLC methods using a UV detector and comparable to those of fluorescence HPLC methods. Further studies on the application of this system to the assay of biologically important substances are in progress in our laboratory.

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