Study for Catecholamine-2′-Deoxyguanosine Adduct Formation under Biomimetic Conditions Using Liquid Chromatography-Electrospray Ionization-Ion Trap Mass Spectrometry

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The adduct formation of 2′-deoxyguanosine (dG) with L-adrenaline under biomimetic conditions (pH 7.5, 37°C) with or without oxidant (MnO_2) was demonstrated in order to clarify the reaction mechanism and the structure. At least two adducts have been observed by liquid chromatography-electrospray ionization-ion trap mass spectrometry (LC-ESI-ion trap MS) and LC-photodiode array detection (LC-PAD) (compound **a**: more polar than dG, *m*/*z* 463 $(M+H)^+$, λmax: 230, 320 nm; compound **b**: less polar than dG, *m*/*z* 445 $(M+H)^+$, λmax: 220, 240, 320, 405 nm). Compound **a** appeared only in the early stage of the reaction prior to formation of compound **b**.

Key words —— DNA adduct, catecholamine, orthoquinone, adrenaline, 2′-deoxyguanosine, Michael addition

INTRODUCTION

Catecholamines are important biogenic amines that are well known to function as neurotransmitters,[1,2] and that have been suggested to have potential toxicities. In most cases, the toxicities result from the oxidative reaction of the catechol moiety, which generates semiquinones, quinones, oxygen radicals, and other reactive oxygen species.[3] In particular, dopamine-induced neurotoxicity has been well studied in terms of Parkinson's disease, with the result that covalent adducts between dopamine and cysteine or glutathione have been chemically identified.[4–6]

Generally, the orthoquinone form, which is the main oxidized form of catechol compounds, can act as an acceptor in Michael addition. In terms of breast cancer, it has recently been reported that DNA modification occurs in catechol estrogens through their quinone form.[7–12] Similarly, DNA modifications of 4-hydroxytamoxifen[13–15] through the quinone methide form and polycyclic aromatic hydrocarbons[16] through their orthoquinone forms are well studied. However, in the case of catecholamines, it is known that the intramolecular cyclization of the quinone form occurs dominantly and results in tetrahydro indoles, which eventually transform into melanin through adrenochrome under biological conditions. In the case of the above-mentioned catecholamine-cysteine adducts, the sulfhydril group, which is more nucleophilic than the amino group, might react ahead of the cyclization derived from the intermolecular reaction of the amino group on the side chain.[4–6] Recently, *in vitro* DNA modification with catecholamines has been also demonstrated.[17–19] These findings suggest that an intermolecular reaction, possibly a Michael addition, between a catecholamine quinone and DNA may occur fairly frequently, even *in vivo*. However, the reaction mechanism and the structure of the adduct remain unclear, because the adduct formation has been monitored only by radioactivity. Our interest has therefore been focused on the reaction mechanism, the structural elucidation, and ultimately the clinical utilization of these adducts as biomarkers.

The identification of DNA adducts requires so-

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phisticated analytical methodology, because such adducts are normally present in low concentrations as complicated structures in complicated matrices. Mass spectrometry (MS) coupled with high-performance liquid chromatography (HPLC) techniques would seem to be a preferred method for these purposes, because much structural information can be obtained after the chromatographic separation using only trace amounts of precious samples.[20]

In this paper, as our first model experiment, L-adrenaline, which is more widely distributed than dopamine, was reacted with 2′-deoxyguanosine (dG) with or without oxidant under biomimetic conditions (pH 7.5, 37°C). At least two DNA adducts were observed in the reaction mixture and were characterized using liquid chromatography-electrospray ionization-ion trap mass spectrometry (LC-ESI-ion trap MS) and photodiode array detection (PAD) as chemical evidences of the DNA modification by catecholamines.

MATERIALS AND METHODS

Reagents —— L-Adrenaline, D,L-adrenaline hydrochloride, adrenochrome, and dG were purchased from Tokyo Kasei Co. (Tokyo, Japan). MnO_2 was purchased from Wako Pure Chemical Industries (Osaka, Japan). All organic solvents were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan) as spectral or HPLC grade. Water used was purified by distillation and then deionized by ion exchange columns. Other general reagents were of analytical grade.

Apparatuses —— Analytical HPLC-UV system consisted of a PU-1580 pump (Jasco, Tokyo, Japan), an LG-1580-2 low pressure gradient unit (Jasco, Tokyo, Japan), GT-103 degasser (Lab-Quatec Co., Ltd. Tokyo, Japan), and a UV detector (Hitachi, Tokyo, Japan). Samples were injected with a rotary injector (Rheodine, Coati, CA, U.S.A.) and 100 μl microsyringe (Hamilton, Reno, Nev, U.S.A.). The data were processed on a Chromatocorder21 recorder (TOSOH, Tokyo, Japan).

LC-ESI-ion trap MS was performed using an HPLC 1100 series (Hewlett-Packard, Wilmington, DE, U.S.A.) interfaced with an LCQ ion trap mass spectrometer (Finnigan Mat, San Jose, CA, U.S.A.) through an electrospray ion source (Finnigan Mat, San Jose, CA, U.S.A.). The HPLC system consisted of a degasser, a binary pump, a column oven, an auto injector, and a PAD. ESI-ion trap MS was performed in the positive mode using nitrogen as the sheath (80 psi) and auxiliary (40 psi) gas to assist with nebulization. A potential of 4 kV was applied to the ESI needle. The metal capillary was maintained at 270°C to provide desolvation. Collision-induced dissociation (CID) was performed using argon as the collision gas. PAD was used under the condition of 5 nm slit width.

LC separation for both analytical HPLC and LC-ESI-ion trap MS was performed using a Nucleosil 5C18 (Macherey-Nagel, Düen, Germany) or Inertsil ODS-2 (GL Sciences Inc., Tokyo, Japan) column (5 μ, 150 mm × 4.6 mm i.d.) maintained at 40°C at a flow-rate of 0.8 ml/min unless noted. Solvent A consisted of 5 mM ammonium acetate containing 0.1% (v/v) acetic acid in water, and solvent B consisted of 5 mM ammonium acetate containing 0.1% (v/v) acetic acid in methanol. Linear gradient elutions were performed as follows: gradient I, 1% B at 0 min, 1% B at 2 min, 4% B at 22 min, 95% B at 24 min, 95% B at 34 min, 1% B at 36 min, 1% B at 50 min; gradient II, 1% B at 0 min, 1% B at 2 min, 61% B at 10 min, 1% B at 12 min, 1% B at 30 min; gradient III, 1% B at 0 min, 16% B at 30 min, 95% B at 32 min, 95% B at 44 min, 1% B at 46 min, 1% B at 50 min.

General Reaction Procedure of Adrenaline Quinone and Deoxyguanosine Adduct —— L-Adrenaline (18.3 mg, 0.1 mmol) was dissolved in an equimolar amount of aqueous HCl (1 N, 100 μl) and diluted with 5 mM aqueous HCl (1.9 ml) in order to avoid the polymerization after the oxidation. The solution was oxidized with MnO_2 (ca. 1 mg) at room temperature for 5 min. The solution was centrifuged to remove MnO_2, and 100 μl of the purple supernatant (corresponding to 5 μmol of the quinone form) was added in a dropwise manner into the dG solution (5 mg, 18.7 μmol in 2 ml of 100 mM sodium phosphate buffer pH 7.5 unless noted). The resulting solution was incubated at 37°C for 24 hr unless noted. Two blanks without dG or adrenaline were performed under the same conditions.

RESULTS AND DISCUSSION

Characterization of the Adducts by LC-MS and LC-PAD

Typical chromatograms (254 nm) of the solution reacted at 37°C for 24 hr at pH 7.5 are shown in Fig. 1. At least two peaks appeared in the reaction mixture at t_R = 8.5 min (compound **a**) and 15 min

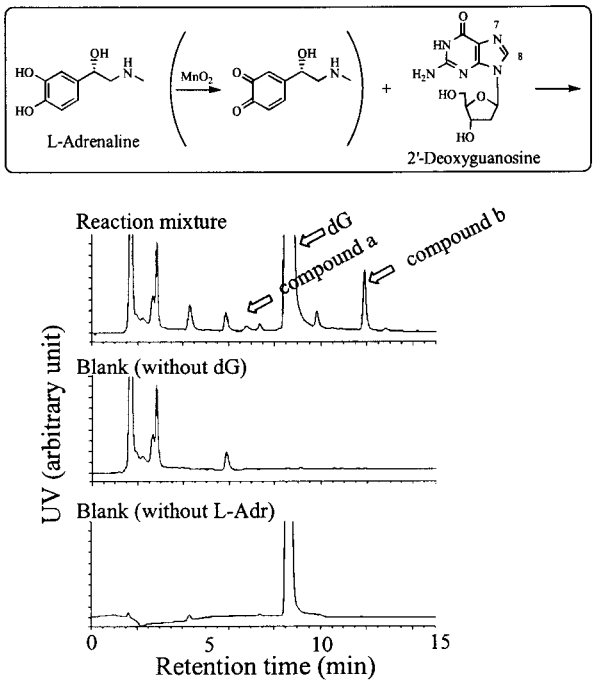

Fig. 1. Typical Chromatograms of the L-Adrenaline-2′-Deoxyguanosine Reaction Mixture

LC conditions: Column, Nucleosil 5C18; gradient, #I; detection UV 254 nm (other information is in the experimental section). Reaction condition: L-Adrenaline, 5 μmol; 2′-deoxyguanosine, 18.7 μmol. The reaction was carried out in 2 ml of phosphate buffer (pH 7.5) at 37°C for 24 hr. Compounds **a** and **b** are indicated by arrows.

(compound **b**) using gradient I. Each peak was analyzed by ESI-ion trap MS. The mass spectra obtained with MS^1 and the interpretations of the multiple stage MS-MS (MS^n) analyses are shown in Figs. 2 and 3, respectively. In the case of compound **a**, several intense ions, *m/z* 463, 347, 329, and 210, were observed even without CID; these ions were thought to be due to source dissociation. In the case of compound **b**, two intense ions, *m/z* 445 and 329, were observed without CID. By the subsequent MS^n analysis of compound **a**, fragmentations of *m/z* 463 $(M+H)^+$ on $M^1 \rightarrow$ 329 $(M+H\text{-}dR\text{-}H_2O)^+$ on $M^2 \rightarrow$ 286 $(M+H\text{-}dR\text{-}H_2O\text{-}CH_2NHCH_3)^+$ on M^3 were observed. In the case of compound **b**, the sequential fragmentations of *m/z* 445 $(M+H)^+$ on $M^1 \rightarrow$ 329 $(M+H\text{-}dR)^+$ on $M^2 \rightarrow$ 311 $(M+H\text{-}dR\text{-}H_2O)^+$, 286 $(M+H\text{-}dR\text{-}CH_2NHCH_3)^+$, 283, 270 or 256 on M^3, were observed. Compound **b** seemed to be a dehydrated compound deriving from compound **a**. According to the loss of deoxyribose moiety (*m/z* 116) from both compounds, neither of the two adducts was modified on the sugar moiety. Both adducts were designated as 1 : 1 adducts, because the nitrogen rule explains that compounds containing an odd number of nitrogens show an odd-numbered molecular weight (dG contains 5 nitrogens and adrenaline con-

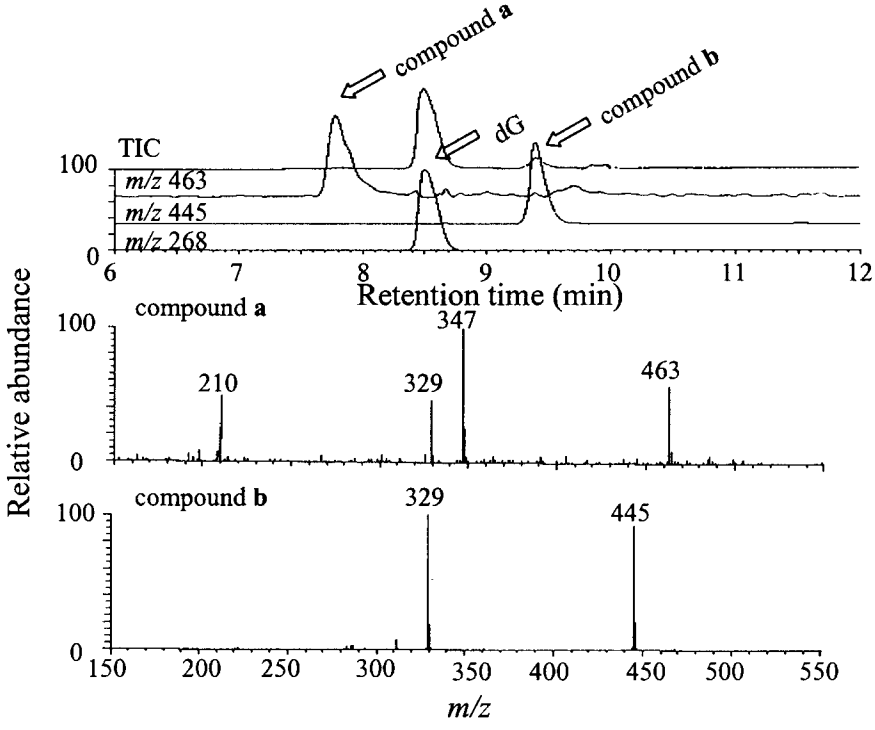

Fig. 2. LC-ESI-MS Chromatogram and ESI-MS Spectra (without CID) of the L-Adrenaline-2′-Deoxyguanosine Reaction Mixture

LC ESI-MS conditions: Column, Nucleosil 5C18; gradient, #II (other information is in the experimental section). Reaction conditions: L-Adrenaline, 5 μmol; 2′-deoxyguanosine, 18.7 μmol. The reaction was carried out in 2 ml of phosphate buffer (pH 7.5) at 37°C for 24 hr.

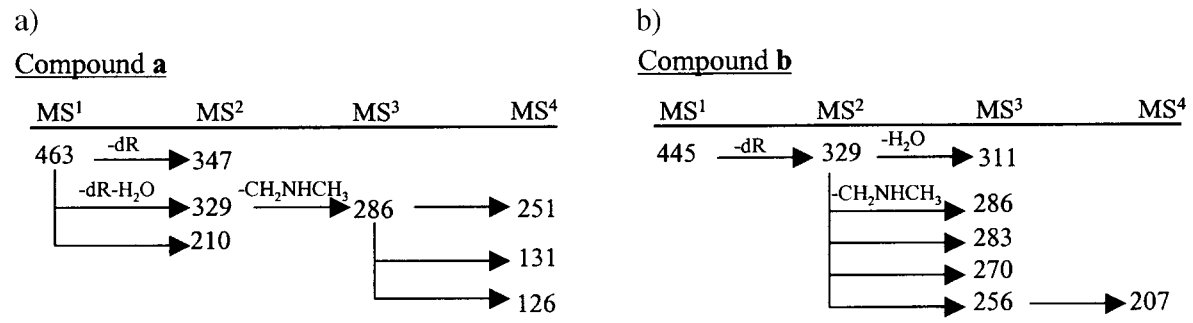

Fig. 3. ESI-MS^n Spectral Data of Compounds **a** and **b**
a) compound **a**; b) compound **b**.

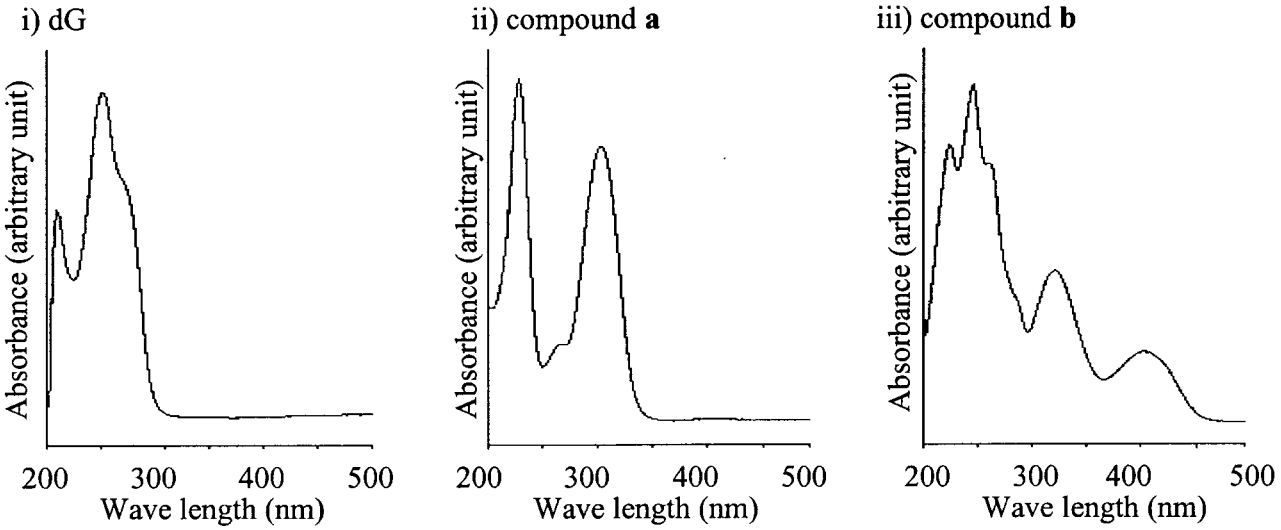

Fig. 4. UV/Visible Spectra of Compounds **a** and **b** Monitored by LC-PAD
i) dG; ii) compound **a**; iii) compound **b**.

tains one nitrogen). Also, because a part of the adrenaline moiety [CH_3-NH-CH_2-(*m*/*z* 43)] could be observed in the fragmentation, intermolecular cyclization did not seem to occur through the adduct formation. Because no fragment ions derived from guanine moiety (*m*/*z* 152) could be observed even under condition of 95% CID, another stable ring system might have been produced through the cyclization following another reaction, as is often seen between the exocyclic amine and the adjacent amine on guanine moiety.[21–23] No spontaneous depurinated adduct, which would appear in the case of N7 adducts resulted from localization of the purine moiety,[24] was observed in the reaction mixture.

UV/Visible spectra by LC-PAD are also shown in Fig. 4. In both cases, absorptions having a longer λmax than that of dG were observed (λmax: compound **a**: 230, 320 nm; compound **b**: 220, 240, 320, 405 nm) due to lengthening of the conjugated system. In particular, the spectrum of compound **b** resembled a benzenoid band, possibly due to the aromatization.

Reaction with Adrenochrome or Racemic Adrenaline

In order to determine whether this reaction was due to Michael addition, the reaction was repeated under the same conditions but using adrenochrome derived from the intramolecular cyclization of adrenaline quinone. As shown in Fig. 5, neither of the previously observed peaks appeared. It suggested that the adduct formation did not occurred after intramolecular Michael addition, but followed intermolecular Michael addition toward adrenaline-quinone.

A similar reaction was demonstrated using D,L-

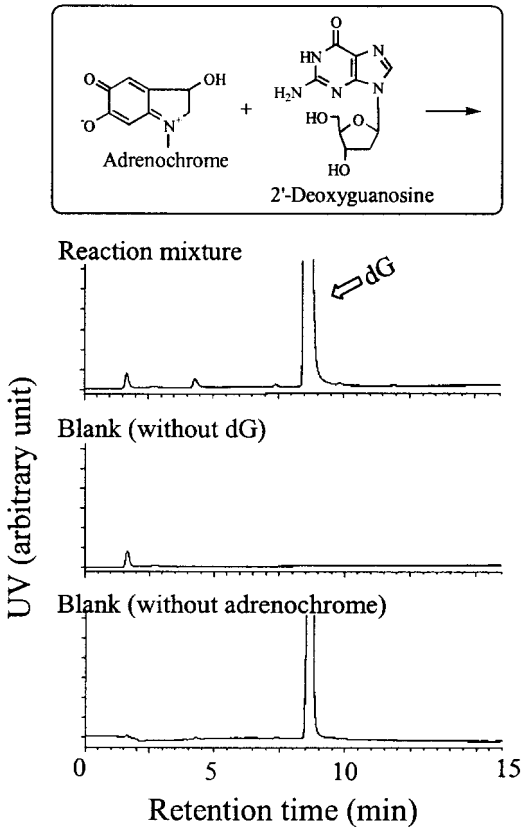

Fig. 5. Chromatograms of the Adrenochrome-2′-Deoxyguanosine Reaction Mixture

LC conditions: Column, Nucleosil 5C18; gradient, #I; detection UV 254 nm (other information is in the experimental section). Reaction conditions: Adrenochrome, 5 μmol; 2′-deoxyguanosine, 18.7 μmol. The reaction was carried out in 2 ml of phosphate buffer (pH 7.5) at 37°C for 24 hr.

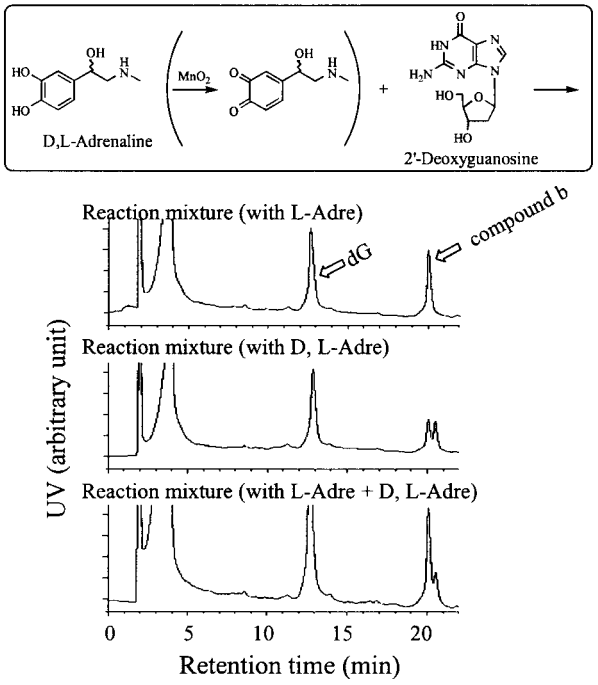

Fig. 6. Chromatograms of the D,L-Adrenaline-2′-Deoxyguanosine Reaction Mixture

LC conditions: Column, Inertsil ODS-2; gradient, #III; detection, UV 320 nm (other information is in the experimental section). Reaction conditions: D,L-adrenaline, 5 μmol; 2′-deoxyguanosine, 18.7 μmol. The reaction was carried out in 2 ml of phosphate buffer (pH 7.5) at 37°C for 24 hr.

adrenaline. In this case, a diastereomeric pair, which gave the same mass spectrum and UV spectrum as compound **b**, appeared at around 20 min, as shown in Fig. 6. The retention time of the former peak corresponded to that of compound **b**. No racemization resulting from the 1,4-quinone methide therefore occurred on the benzyl hydroxyl position of adrenaline through the adduct formation.

Time Course for the Adduct Formation

We examined the time course of the reaction under condition of 37°C and pH 7.5 in the presence or absence of MnO_2 (Fig. 7). Ten microliters of the reaction mixture was injected into the HPLC system at the appropriate interval. Generation of compound **a** was first observed in the early stage of the reaction, followed by generation of compound **b**, which increased in concentration along with the decrease in compound **a**. Compound **a** therefore seemed to be a precursor of compound **b**. The adduct formation without MnO_2 proceeded slowly but still gave the same pattern after spontaneous oxidation of adrenaline. The oxidation with MnO_2 therefore seemed to give the same oxidative product from adrenaline as one after spontaneous oxidation. The reaction yield was estimated as less than 5% according to the calculation of remaining dG.

The pH Effect of the Adduct Formation

The pH effect was demonstrated in 100 mM sodium phosphate buffer between pH 6 and 9 with or without MnO_2 (Fig. 8). The adduct formation was found over pH 6.5. The generation of compound **b** was increased with the pH value, but pH value did not have as pronounced an effect on the generation of compound **a** through pH 6.5 to 9.

^{1}H-NMR for Compound b

Compound **a** was too unstable to isolate: the collecting peak by HPLC was easy to reform to compound **b**. Compound **b** was stable enough to isolate at least under weak acidic conditions. However, our preliminary ^{1}H-NMR (500 MHz, in CD_3OD) analysis did not give enough information to identify the

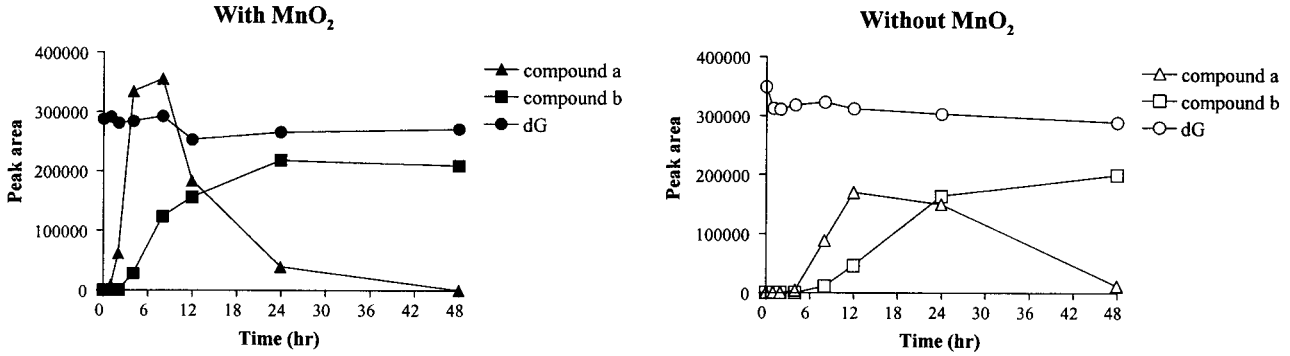

Fig. 7. Time Courses for L-Adrenaline-2′-Deoxyguanosine Adduct Formation

Reaction conditions: L-Adrenaline, 5 μmol; 2′-deoxyguanosine, 18.7 μmol. The reaction was carried out in 2 ml of phosphate buffer (pH 7.5) at 37°C.

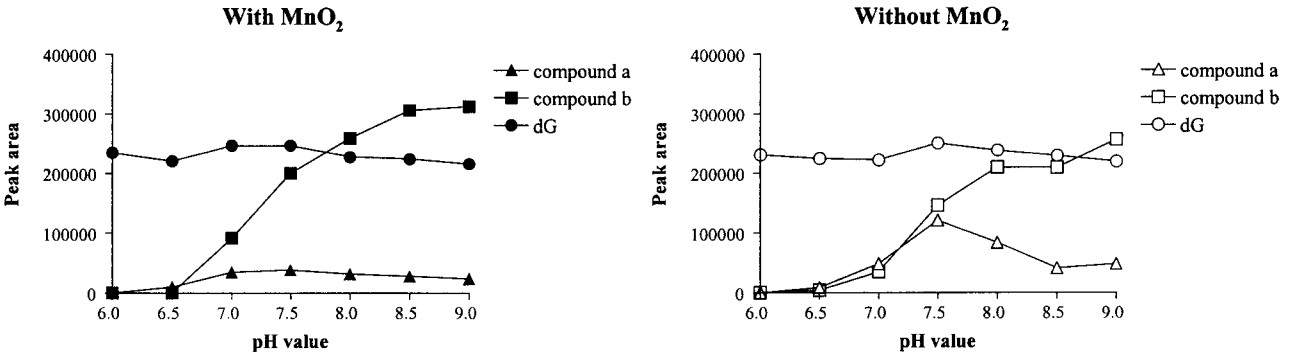

Fig. 8. Effect of pH on L-Adrenaline-2′-Deoxyguanosine Adduct Formation

Reaction conditions: L-Adrenaline, 5 μmol; 2′-deoxyguanosine, 18.7 μmol. The reaction was carried out in 2 ml of phosphate buffer at 37°C for 24 hr.

whole structure. In fact, ^{1}H-NMR revealed only that the characteristic 8-H on the guanine moiety was still observed (1H, s, 8.15 Hz). Attempts to make a final identification of these compounds using ^{13}C-NMR and advanced techniques like HMQC are currently underway in our laboratories.

In conclusion, the adduct formation of dG by adrenaline quinone under biomimetic conditions (pH 7.5, 37°C) was demonstrated in this study, and at least two adducts were characterized by LC-ESI-ion trap MS and LC-PAD. The mechanistic and structural information obtained was as follows: i) both compound **a** and compound **b** were 1 : 1 adducts; ii) no cyclization of the side chain occurred; iii) both compounds had a stable ring system; iv) neither compound was an N7 adduct; v) both showed a longer conjugated system than dG; vi) no racemization on the benzyl moiety of adrenaline occurred; vii) compound **a** was a precursor, which was hydrated somewhere on compound **b**; and viii) C8 was not the binding position.

To our knowledge, that is the first chemical information on the adduct formation between DNA and catecholamines, with the exception of data derived by radioisotope.

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