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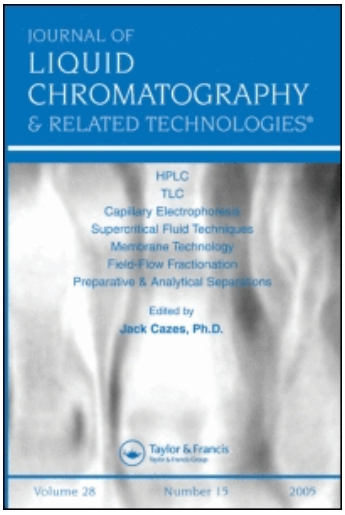

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Simultaneous Determination of Purine and Pyrimidine Bases Using High-Performance Liquid Chromatography with Electrochemical Detection: Application to DNA Assay

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SIMULTANEOUS DETERMINATION OF PURINE AND PYRIMIDINE BASES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION: APPLICATION TO DNA ASSAY

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ABSTRACT

A sensitive method for simultaneous determination of purine and pyrimidine bases was developed using high performance liquid chromatography with electrochemical detection (ECD). Pyrimidines as well as purines were detectable by ECD under alkaline conditions, and the order of susceptibility to electrochemical oxidation was guanine > adenine > thymine > cytosine. These bases were separated on a vinyl alcohol copolymer gel column and determined by ECD. The detection limit for each base was picomole level. This method was applied to the quantitation of bases in the acid hydrolysates of DNA and oligodeoxynucleotides.

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INTRODUCTION

High-performance liquid chromatography has been widely used in the determination of nucleic acid constituents (1). While the most frequently used detection mode for HPLC is ultraviolet absorption, several methods using HPLC coupled with electrochemical detection (ECD) have been developed for the determination of nucleotides, nucleosides and nucleobases. Thus, the HPLC-ECD method was applied to quantitate guanine nucleotides (2,3), guanosine (3), guanine (3-5), adenine (4), xanthine (3), and hypoxanthine (3). The ECD of nucleic acid constituents and related derivertives is highly sensitive and selective; however, its availability is limited to the easily oxidizable compounds such as purine nucleotides, nucleosides and bases. We have, therefore, extended the applicability of ECD to pyrimidine bases and have applied the HPLC-ECD method to the simultaneous determination of purine and pyrimidine bases in the acid hydrolysates of DNA.

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MATERIALS AND METHODS

Materials

Guanine hydrochloride, adenine hydrochloride, cytosine, thymine, and disodium hydrogen phosphate were purchased from Katayama Chemical Industry (Osaka, Japan). Salmon testes DNA (Type III) and calf thymus DNA (Type I) were obtained from Sigma Chemical (St.Luis, MO, U.S.A.). Oligodeoxynucleotide 1 (5'-AGCCGGCCCTTCAATGGGTCA-3') and 2 (5'-GATCTGGAGAGGCA-GAACTGG-3') were synthesized by the phosphite triester method and purified by electrophoresis on a preparative 20% polyacrylamide gel after the separation of failed sequences by Sep-Pak C18 cartridges (Waters Assoc., Milford, MA, U.S.A.) (6).

Water throughout the experiments was distilled twice over permanganate.

Apparatus

The HPLC system consisted of a Tosoh CCPM pump (Tokyo, Japan), a Rheodyne 7125 injector with a 20 µl sample loop (Berkeley, CA, U.S.A.), a 25 cm x 7.6 mm ID. Asahipak GS-320H analytical column (Asahi Chemical Industries, Tokyo, Japan), packed with vinyl alcohol copolymer gel, having a mean particle diameter of 9.0 µm and an exclusion limit of 40,000 Da., and a LC-4B Amperometric Detector (Bioanalytical Systems Inc., West Lafayette, IN, U.S.A.) equipped with a glassy carbon electrode. The peaks were monitored with an Chromatocorder 12 computing integrator (System Instrument, Tokyo, Japan).

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HPLC-ECD conditions

The mobile phase was 0.1 M disodium hydrogen phosphate (pH 9.2) and the flow rate was 2.0 ml/min. The electrode potential was set at + 1.1 V relative to a Ag/AgCl reference electrode. The analytical column and ECD system were maintained at 25°C.

Assays for DNA and oligodeoxynucleotides

The solutions (10 µl) of DNA (0.5 - 1 mg/ml in sterile water) and oligonucleotides (0.2 - 0.3 mg/ml in 10 mM Tris-HCl buffer containing 1 mM EDTA, pH 7.5) were hydrolyzed by addition of 100 µl of formic acid (98 %). The hydrolysis was performed in a sealed glass tube at 175°C for 2 hours. After the hydrolysates were lyophilized, the lyophilized residues were dissolved in 0.2 - 1.0 ml of the mobile phase, and aliquots of 20 µl were injected for the HPLC-ECD analysis. The concentrations of DNA and

oligonucleotides were determined by UV absorbance (1.0 A_{260} ; 50 μg/ml, average mass per base; 330 g/mole).

RESULTS AND DISCUSSION

Simultaneous determination of purine and pyrimidine bases

Since pyrimidine bases are not susceptible to the electrochemical oxidation at low pH (5), we tried to raise pH of the mobile phase and applied potentials. Figure 1 shows a chromatogram of a standard mixture of adenine, guanine, cytosine and thymine obtained using the mobile phase of 0.1 M disodium hydrogen phosphate (pH 9.2) and the applied potential of + 1.1 V. The pyrimidine bases were detectable under these conditions, although the ECD response of cytosine was smaller than those of other bases.

The hydrodynamic voltammograms for these bases are shown in Figure 2. Purine bases were easily oxidized, and the half-maximal potentials were + 0.55 V for guanine and + 0.84 V for adenine. The oxidation of pyrimidine bases required much higher potentials, and thymine was more efficiently oxidized than cytosine. The oxidation current for pyrimidines did not reach the limiting current plateau up to the maximal potentials examined. These results are supported by the order of the electrondonor capacity for bases, guanine > adenine > thymine > cytosine, which is expected from the energy of the highest filled molecular orbital (7).

To optimize the chromatographic separation and ECD response, the hydrodynamic voltammograms for the bases were measured at the pH range of 8 - 10 (TABLE 1). The pH dependence of the half-maximal potentials indicated that all bases were more efficiently oxidized at higher pH. However, the higher pH increased the background current. In the pH range examined, the four bases were successfully resolved, and the separation could be

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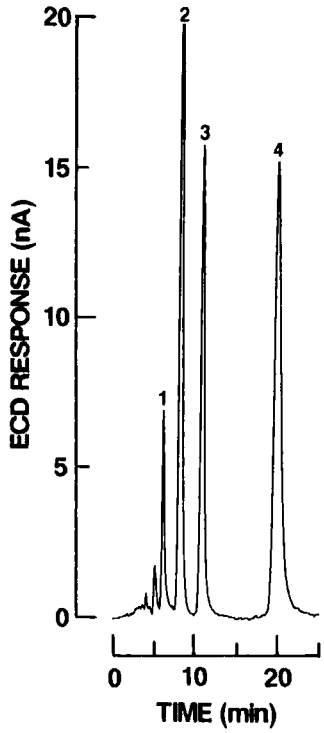

FIGURE 1. Chromatograms of nucleobases. An aliquot (20 µl) of the standard mixture containing 4.8 µM cytosine (1), 5.5 µM thymine (2), 3.5 µM guanine (3) and 3.7 µM adenine (4) was injected into HPLC-ECD. Chromatographic conditions are described in Materials and Methods.

TABLE 1
Effects of pH on retention time and half-maximal potential of nucleobases

	Cytosine		Thymine		Guanine		Adenine	
pH	RT [a]	$E_{1/2}$ [b]	RT	$E_{1/2}$	RT	$E_{1/2}$	RT	$E_{1/2}$
8.0	6.48	1.13	9.20	1.09	13.29	0.60	21.71	0.88
9.0	6.41	1.09	8.82	1.04	12.20	0.58	20.88	0.85
9.5	6.37	1.04	8.13	1.01	10.51	0.55	19.42	0.78
10.0	6.07	1.04	6.85	1.00	8.41	0.54	16.54	0.77

[a]RT; retention time (min)
[b]$E_{1/2}$; half-maximal potential (volt)

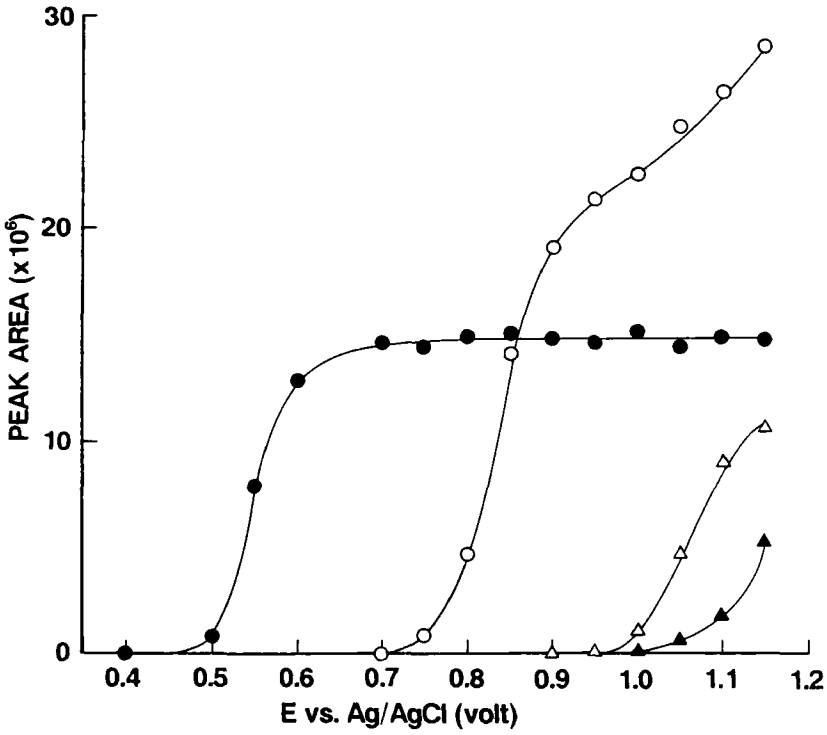

FIGURE 2. Hydrodynamic voltammograms of nucleobases. Twenty μl of the standard mixture containing 5 μM adenine (○), guanine (●), thymine (△) and cytosine (▲) were injected into HPLC-ECD, and the ECD response for each base was determined as function of applied potentials. Chromatographic conditions are described in Materials and Methods.

achieved less than 25 min (TABLE 1). Both purine and pyrimidine bases exhibited decreases in the retention time with increases in the pH of the mobile phase, and the separation of cytosine and thymine were not enough at pH 10 when large amounts of the samples were applied.

Silica-based column cannot be used because of the high pH of the mobile phase necessary to effectively oxidize the pyrimidine bases. Therefore, we employed the Asahipak GS-320H column packed with vinyl alcohol copolymer gel for the analysis of purine

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and pyrimidine bases. This column is stable in the pH range of 2 - 12 and suitable for the separation of nucleic acid components at low pH. Noguchi et al. reported that nucleotides, nucleosides and bases were consistently separated on the GS-320H column by a hydrophobic adsorption mechanism, permitting the simple, efficient analysis of these mixtures by isocratic elution (8). The vinyl alcohol copolymer gel column was also available for the analysis of purine and pyrimidine bases under alkaline conditions.

From the pH dependence of retention time and ECD response for the nucleobases, we decided to use 0.1 M disodium hydrogen phosphate (pH 9.2) as the mobile phase. Furthermore, an applied potential of + 1.1 V relative to a Ag/AgCl reference electrode was chosen for lowering the background current.

The ECD response was proportional to the amount of bases (Figure 3), and the minimum detection limits for the pyrimidines and purines were picomole levels. The sensitivity for purine bases was lower than that of the previous results obtained by the ECD at low applied potential and low pH (3-5), since the higher potential and pH resulted in the increase of baseline noise. However, the sensitivity of the HPLC-ECD method for nucleobases was higher than that of spectrophotometric method (1).

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Assays for DNA and oligodeoxynucleotides

The acid hydrolysates of DNA and oligodeoxynucleotides were analyzed under the standard condition (Figure 4). The acid treatment of DNA produced four major peaks emerging with the same retention times as authentic cytosine, thymine, guanine and adenine. Interfering substance was not observed in the hydrolysates of salmon testes and calf thymus DNA. Similarly, these four bases could be detected in the hydrolysates of oligonucleotides, but the elution pattern of cytosine was slightly modified by the major interfering peak. This interfering substance, which was identified as EDTA from the stock solution of oligonucleotides, did not seriously influence the quantitation of cytosine.

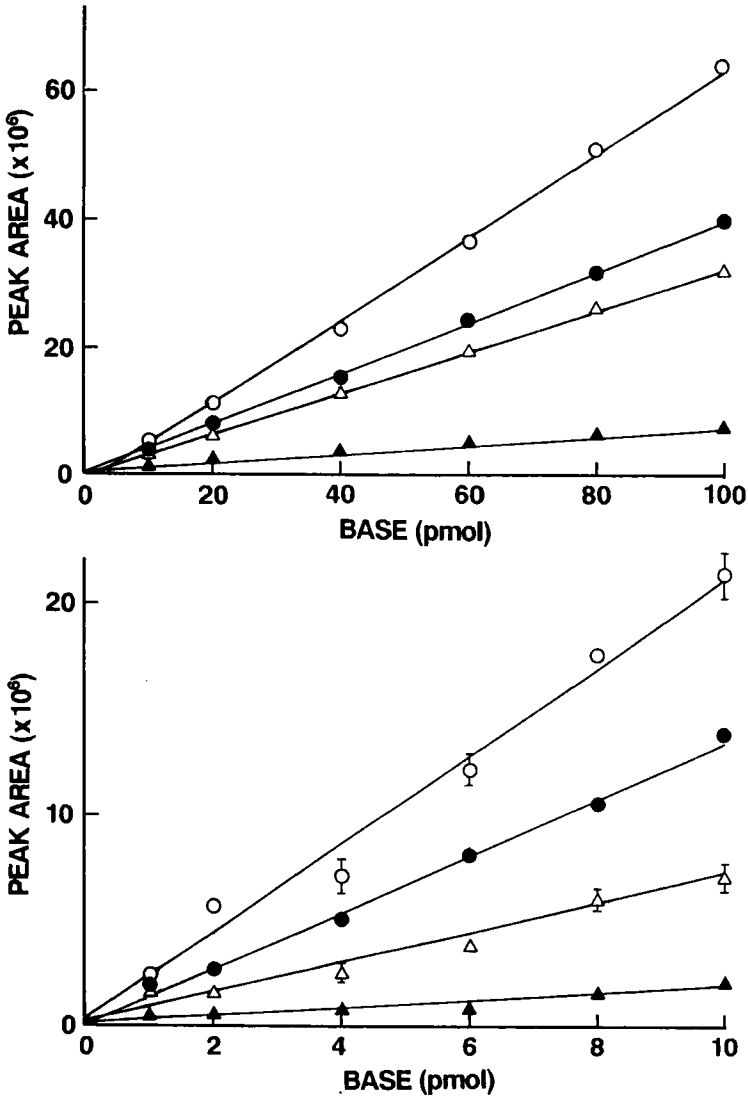

FIGURE 3. Linearity of ECD response for nucleobases. Twenty μl of the standard mixture containing 0.5 - 5 μM (upper) or 0.05 - 0.5 μM (lower) adenine (○), guanine (●), thymine (△) and cytosine (▲) were injected into HPLC-ECD. Each point represents mean ± S.E.M. of triplicated assays.

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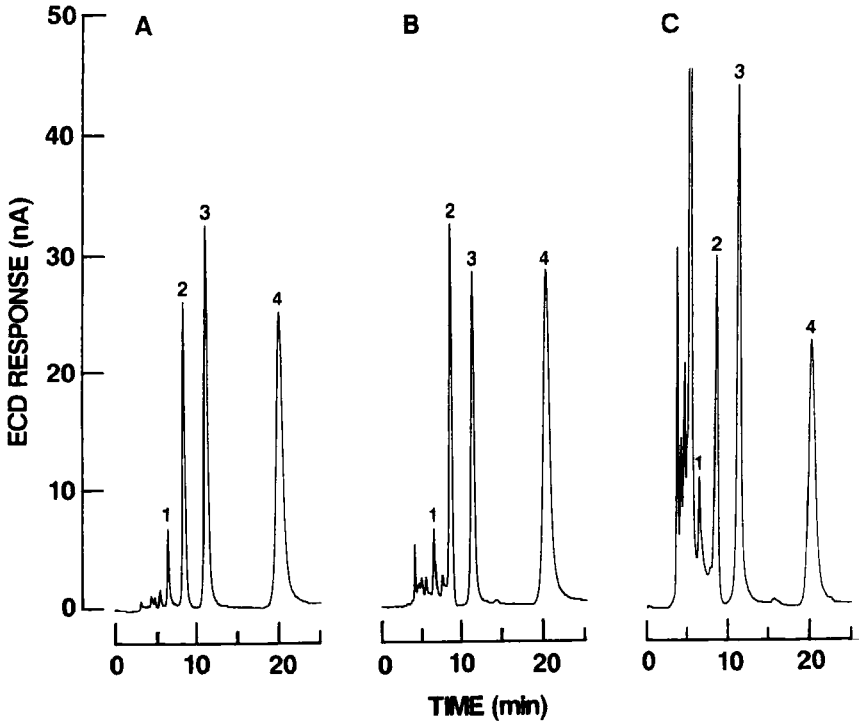

FIGURE 4. Chromatograms of standard mixture of bases (A) and acid hydrolysates of salmon testes DNA (B) and oligodeoxynucleotide 1(C). Twenty μl of the standard mixture containing 5 μM of each bases were injected into HPLC-ECD. Salmon testes DNA (7.3 μg) and oligodeoxynucleotide 1 (2.9 μg) were hydrolyzed with formic acid at 175°C for 2 hours, and their lyophilized residues were dissolved in 1.0 ml (DNA) and 0.2 ml (oligodeoxynucleotide) of the mobile phase, Aliquots (20 μl) of these solutions were injected into HPLC-ECD. Peaks: 1; cytosine, 2; thymine, 3; guanine, 4; adenine.

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TABLE 2
Analysis of nucleobases in acid hydrolysates of DNA and oligodeoxynucleotides

	Amount added (pmol)	Amount found (pmol)	Base composition (mole %) found				Base composition (mole %) reported or calculated			
			A	G	C	T	A	G	C	T
DNA salmon testes	442	408 ±4	28.9 ±0.2	21.2 ±0.4	21.1 ±0.3	28.7 ±0.4	28.7	20.4	19.9	29.3
DNA calf thymus	318	292 ±1	28.3 ±0.8	23.0 ±0.5	19.2 ±0.3	29.6 ±0.2	27.3	21.2	20.9	29.0
Oligodeoxy-nucleotide 1	878	531 ±21	16.9 ±0.2	28.5 ±0.2	32.6 ±0.4	21.9 ±0.3	19.0	28.6	33.3	19.0
Oligodeoxy-nucleotide 2	383	293 ±16	28.7 ±0.5	43.0 ±1.1	13.5 ±2.9	14.8 ±1.5	28.6	42.9	14.2	14.2

Each value is mean ± S.E.M. of three different samples.
A; adenine, G; guanine, C; cytosine, T; thymine.

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The determinations of bases in the hydrolysates of DNA and oligonucleotides are summarized in TABLE 2. The base compositions of salmon testes and calf thymus DNA determined by HPLC-ECD were almost identical with other results (9,10). The total amounts of bases were 92% of the amounts of bases estimated by the UV absorption of DNA. The base compositions of synthesized oligonucleotides were also consistent with those calculated from their sequences, whereas the total amounts of bases were 23 - 40% less than those determined by UV absorption. The difference in the total amounts of bases between HPLC-ECD and UV spectroscopy might be due to the UV absorbing impurity (4).

The absolute amount of DNA analyzed by the HPLC-ECD method (0.10 μg of calf thymus DNA and 0.15 μg of salmon testes DNA) was less than that required for the spectrophotometric method (1 - 100 μg)(1).

Previously, we determined DNA and RNA in various tissues by the ECD response of guanine liberated by acid hydrolysis (5,11).

These results were expressed as the relative values obtained with calf thymus DNA or calf liver RNA as a standard. The present method has an advantage that the major nucleobases of DNA can be simultaneously determined by HPLC-ECD. This procedure is suitable for the estimation of base compositions of small amount of DNA and DNA fragments or applicable to the samples with compounds which interfere with spectrophotometric assay.

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