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Electrochemical and chromatographic properties of selected hydrazine and hydrazide derivatives of carbonyl compounds

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ABSTRACT

Electrochemical and chromatographic properties of selected hydrazine and hydrazide derivatives of carbonyl compounds were studied to find a new, highly sensitive and selective derivatization reagent for the determination of carbonyl compounds by high-performance liquid chromatography with electrochemical detection. Of the six hydrazines and five hydrazides investigated. 2,5-dihydroxybenzohydrazide was accepted as the most suitable reagent because (i) it and its hydrazones were stable and (ii) its hydrazones were sensitively detectable at a low oxidative potential. Seven ketosteroids derivatized with 2,5-dihydroxybenzohydrazide were satisfactorily separated on a reversed-phase column and detected at +0.20 V *vs.* Ag/AgCl. The detection limits were in the range of 60–500 fmol per injection.

INTRODUCTION

High-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been widely used to determine trace amounts of electroactive compounds because of its high sensitivity and selectivity. Also, chemical derivatization approaches in HPLC–ED have become commonplace [1].

Some reports have appeared on the HPLC–ED derivatization reagents for the determination of carbonyl compounds: 4-nitrophenylhydrazine (4-NPH) for 17-ketosteroids [2,3] and 2,4-dinitrophenylhydrazine (2,4-DNPH) for aldehydes [4,5]. Recently, Bond *et al.* [6] discussed the analytical and mechanistic aspects of the electrochemical oxidation of 3- and 17-ketosteroids derivatized with 4-NPH, 2,4-DNPH and phenylhydrazine (PH). However, carbonyl compounds derivatized with these reagents were detected at relatively high oxidative potentials ($\geqslant$ +0.80 V *vs.* Ag/AgCl) or in the reductive mode. In reductive-mode HPLC–ED, excluding oxygen from both the mobile phase and the sample is somewhat troublesome. On the other hand, use of a high oxidative potential causes drift of the baseline, high noise level and faster deterioration of the electrode surface, and also lowers selectivity, which is one of the most important advantages of HPLC–ED. To overcome these problems, the derivatives resulting from HPLC–ED derivatization reagents should be detectable at lower oxidative potentials.

In the present study, the electrochemical and chromatographic properties of carbonyl compounds derivatized with selected reagents containing a hydrazine group were examined to find a new, highly sensitive and selective derivatization reagent for the determination of carbonyl compounds by HPLC–ED. This is the first paper reporting systematic studies on the potency of hydrazine reagents for HPLC–ED. The chromatographic separation with sensitive ED of seven ketosteroids derivatized with the most suitable reagent, 2,5-dihydroxybenzohydrazide (2,5-DHBH), is also described.

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EXPERIMENTAL

Apparatus

The HPLC system consisted of a Model L-5000 solvent delivery pump (Yanagimoto, Kyoto, Japan), a Model 7125 syringe-loading sample injector with a 100-μl sample loop (Rheodyne, Berkeley, CA, USA) and a Model LC-4B amperometric electrochemical detector (Bioanalytical Sytems, West Lafayette, IN, USA).

The electrochemical detector consisted of a Model TL-5 thin-layer detector cell with a glassy carbon working electrode and an Ag/AgCl reference electrode. The surface of the working electrode was polished to a mirror finish with alumina powder (0.05 μm) on a glass plate each day before use.

Peak area and peak height were calculated using a model C-R2AX integrator (Shimadzu, Kyoto, Japan).

All melting points are uncorrected. Mass spectra were obtained on a Model M-68 mass spectrometer (Hitachi, Tokyo, Japan). IR spectra were measured on a Model A-702 spectrometer (Nihon Bunko, Tokyo, Japan), and absorption data are given in cm^{-1}. ^1H NMR spectra were recorded with a Model XL-200 spectrometer (200.06 MHz, Varian, Sunnyvale, CA, USA) with the samples in hexadeuterodimethyl sulfoxide (DMSO-d_6) with tetramethylsilane as an internal standard.

Chemicals

The following chemicals were purchased: phenylhydrazine, 4-nitrophenylhydrazine, 2,4-dinitrophenylhydrazine, 2-hydrazinobenzothiazole, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, isoamyl nitrite, hydrazine monohydrate, androsterone (Wako, Osaka, Japan); 4-hydroxybenzohydrazide, 4-aminophenol hydrochloride, 2,4-dihydroxybenzoic acid, acetophenone, sodium 1-pentanesulfonate, pregnenolone, dehydroisoandrosterone (Nacarai Tesque, Kyoto, Japan); 4-methoxyphenylhydrazine, 3,4-dihydroxyphenylacetic acid, testosterone, ethisterone, norethisterone (Tokyo Kasei Kogyo, Tokyo, Japan); corticosterone (Sigma, St. Louis, MO, USA).

Methanol for HPLC was of HPLC grade (Kanto, Tokyo, Japan). Water was deionized and distilled before use. All other chemicals and solvents were of analytical grade.

Chromatographic conditions

Chromatography was performed at room temperature. Each mobile phase was filtered with a Type FR-40 membrane filter (0.4 μm, Fuji Photo Film, Tokyo, Japan) and degassed under reduced pressure before use. In this study, five chromatographic conditions were used: (1) column, Nucleosil C_{18} (10 μm, 250 mm × 4.6 mm I.D., home-packing, Macherey-Nagel, Düren, Germany); mobile phase, methanol–0.5% ammonium dihydrogenphosphate (pH 4.5) (80:20, v/v); flow-rate, 0.8 ml/min; (2) column, same as (1); mobile phase, methanol–0.5% ammonium dihydrogenphosphate (pH 4.5) (90:10, v/v); flow-rate, 1.0 ml/min; (3) column, μBondapak NH_2 (300 mm × 3.9 mm I.D., Waters Assoc., Milford, MA, USA); mobile phase, 0.1 *M* phosphate buffer (pH 6.8)–methanol (90:10, v/v); flow-rate, 1.0 ml/min; (4) column, μBondapak C_{18} (300 mm × 3.9 mm I.D., Waters Assoc.); mobile phase, 0.1 *M* sodium dihydrogenphosphate containing 0.01 *M* sodium 1-pentanesulfonate (pH 4.4)–methanol (75:25, v/v); flow-rate, 1.0 ml/min; (5) column, Chemcosorb 5-ODS-UH (150 mm × 4.6 mm I.D., Chemco, Osaka, Japan); mobile phase, 0.05 *M* phosphate buffer (pH 7.0)–acetonitrile (64:36, v/v); flow-rate, 1.0 ml/min; applied potential, +0.20 V *vs.* Ag/AgCl.

Registry No.

2,4-DNPH, 119-26-6; 4-NPH, 100-16-3; PH, 100-63-0; 4-MPH, 3471-32-7; 4-HPH · HCl, 54049-23-9; HBT, 615-21-4; 4-HBH, 5351-23-5; 2,4-DHBH, 13221-86-8; 3,4-DHBH, 39635-11-5; 2,5-DHBH, 15791-90-9; 3,4-DHPAH, 1132-47-4; AP-2,4-DNPH, 1677-87-8; AP-4-NPH, 2675-22-1; AP-PH, 583-11-9; AP-4-MPH, 89671-57-8; AP-HBT, 59972-88-2; AP-4-HBH, 100969-27-5; AP-2,5-DHBH, 77163-30-5; testosterone, 58-22-0; ethisterone, 434-03-7; norethisterone, 68-22-4; androsterone, 53-41-8; dehydroisoandrosterone, 53-43-0; pregnenolone, 145-13-1; corticosterone, 50-22-6.

Synthesis

4-Hydroxyphenylhydrazine hydrochloride (4-HPH · HCl). 4-HPH was prepared in a similar manner to that described in the literature [7,8].

2,4-Dihydroxybenzohydrazide (2,4-DHBH), 3,4-dihydroxybenzohydrazide (3,4-DHBH), 2,5-dihydroxybenzohydrazide (2,5-DHBH), 3,4-dihydroxy-

phenylacetohydrazide (3,4-DHPAH). 2,4-DHBH, 3,4-DHBH, 2,5-DHBH and 3,4-DHPAH were prepared in a similar manner to that described for 2,5-DHBH in the literature [9,10].

Acetophenone hydrazone of 2,5-DHBH (AP-2,5-DHBH). To 2,5-DHBH (100 mg, 0.59 mmol) in a 30-ml flask, methanol (5 ml), acetic acid (2 ml) and acetophenone (0.2 ml, 1.72 mmol) were added. The mixture was stirred for 3 h at room temperature, and then water (30 ml) was added. A precipitate was collected, washed with water and dried. Recrystallization from ethanol–water gave AP-2,5-DHBH as white needles (65 mg, 40%), m.p. 265°C (decomposition, ethanol–water). Analysis: Calculated for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.56; H, 5.32; N, 10.29.

Acetophenone hydrazone of 4-HBH, 3,4-DHBH, 2,4-DHBH, 3,4-DHPAH, 2,4-DNPH, 4-NPH, PH, 4-MPH, 4-HPH, BTH (AP-4-HBH, AP-3,4-DHBH, AP-2,4-DHBH, AP-3,4-DHPAH, AP-2,4-DNPH, AP-4-NPH, AP-PH, AP-4-MPH, AP-4-HPH, AP-HBT). AP-4-HBH, AP-3,4-DHBH, AP-2,4-DHBH, AP-3,4-DHPAH, AP-2,4-DNPH, AP-4-NPH, AP-PH, AP-4-MPH, AP-4-HPH and AP-HBT were prepared in a similar manner to that described above for AP-2,5-DHBH. The m.p. and elemental analysis data for new compounds which could not be found in literature are described below.

AP-3,4-DHBH: 31% yield, m.p. 246–248°C. Analysis: Calculated for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.85; H, 5.36; N, 10.36.

AP-2,4-DHBH: 68% yield, m.p. 230–232°C. Analysis: Calculated for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.56; H, 5.20; N, 10.20.

AP-3,4-DHPAH: 64% yield, m.p. 185–187°C. Analysis: Calculated for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.37; H, 5.82; N, 9.82.

AP-4-HPH: 13% yield, m.p. 136–137°C. Analysis: Calculated for $C_{14}H_{14}N_2O$: C, 74.31; H, 6.24; N, 12.38. Found: C, 74.18; H, 6.18; N, 12.23.

Androsterone hydrazone of 2,5-DHBH (A-2,5-DHBH). To a mixture of androsterone (140 mg, 0.48 mmol) and 2,5-DHBH (80 mg, 0.48 mmol) in a 30-ml flask, methanol (5 ml) and acetic acid (2 ml) were added. The reaction mixture was stirred for 1 h at room temperature, and then water (30 ml) was added. A precipitate was collected, washed with water and dried. Recrystallization from ethanol–water gave A-2,5-DHBH as white plates (168 mg, 80%), m.p. 290–291°C. Analysis. Calculated for $C_{26}H_{36}N_2O_4$ (molecular weight, 440.56): C, 70.88; H, 8.24; N, 6.36. Found: C, 70.78; H, 8.23; N, 6.44. Mass spectrometry (MS) *m/e*: 440 (M^+). IR (KBr) ν_{max} (cm^{-1}): 1655, 1645. ^1H NMR (DMSO-d_6) δ: 0.77 (3H, s), 0.86 (3H, s), 6.80 (2H, m), 7.34 (1H, m).

Corticosterone, dehydroisoandrosterone, testosterone, norethisterone, ethisterone, pregnenolone hydrazone of 2,5-DHBH (C-2,5-DHBH, D-2,5-DHBH, T-2,5-DHBH, N-2,5-DHBH, E-2,5-DHBH, P-2,5-DHBH). C-2,5-DHBH, D-2,5-DHBH, T-2,5-DHBH, N-2,5-DHBH, E-2,5-DHBH and P-2,5-DHBH were prepared in a similar manner to that described for A-2,5-DHBH.

C-2,5-DHBH: 70% yield, m.p. 181–183°C (methanol–water). Analysis. Calculated for $C_{28}H_{36}N_2O_6$ (molecular weight, 496.60): C, 67.72; H, 7.31; N, 5.64. Found: C, 67.61; H, 7.55; N, 5.31. MS *m/e*: 496 (M^+). IR (KBr) ν_{max} (cm^{-1}): 1705, 1645, 1615. ^1H NMR (DMSO-d_6) δ: 0.77 (3H, s), 1.24 (3H, s), 5.81 and 6.06 (1H, s and s), 6.81 (2H, m), 7.35 (1H, m).

D-2,5-DHBH: 64% yield, m.p. 295–297°C (methanol). Analysis. Calculated for $C_{26}H_{34}N_2O_4$ (molecular weight, 438.57): C, 71.21; H, 7.81; N, 6.39. Found: C, 71.23; H, 8.03; N, 6.62. MS *m/e*: 438 (M^+). IR (KBr) ν_{max} (cm^{-1}): 1635, 1615. ^1H NMR (DMSO-d_6) δ: 0.89 (3H, s), 0.99 (3H, s), 5.28 (1H, s), 6.80 (2H, m), 7.35 (1H, m).

T-2,5-DHBH: 29% yield, m.p. 294–296°C (acetone). Analysis. Calculated for $C_{26}H_{34}N_2O_4$ (molecular weight, 438.57): C, 71.21; H, 7.81; N, 6.39. Found: C, 71.03; H, 8.04; N, 6.52. MS *m/e*: 438 (M^+). IR (KBr) ν_{max} (cm^{-1}): 1620. ^1H NMR (DMSO-d_6) δ: 0.68 (3H, s), 1.06 (3H, s), 5.89 and 6.14 (1H, s and s), 6.80 (2H, m), 7.36 (1H, m).

N-2,5-DHBH: 45% yield, m.p. 196–198°C (acetone–hexane). Analysis. Calculated for $C_{27}H_{32}N_2O_4$ (molecular weight, 448.56): C, 72.30; H, 7.19; N, 6.25. Found: C, 72.10; H, 7.33; N, 6.36. MS *m/e*: 448 (M^+). IR (KBr) ν_{max} (cm^{-1}): 1635. ^1H NMR (DMSO-d_6) δ: 0.79 (3H, s), 5.98 and 6.24 (1H, s and s), 6.81 (2H, m), 7.36 (1H, m).

E-2,5-DHBH: 63% yield, m.p. 290–293°C (decomposition, ethanol–water). Analysis. Calculated for $C_{28}H_{34}N_2O_4$ (molecular weight, 462.59): C, 72.70; H, 7.41; N, 6.06. Found: C, 72.34; H, 7.55; N,

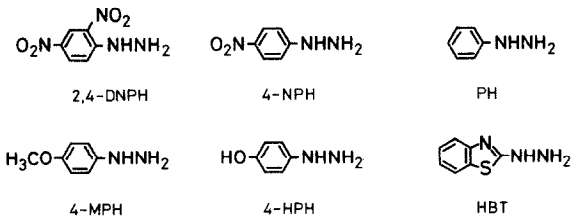

Fig. 1. Chemical structures of hydrazine reagents. 2,4-DNPH, 2,4-dinitrophenylhydrazine; 4-NPH, 4-nitrophenylhydrazine; PH, phenylhydrazine; 4-MPH, 4-methoxyphenylhydrazine; 4-HPH, 4-hydroxyphenylhydrazine; HBT, 2-hydrazinobenzothiazole.

5.91. MS *m/e*: 462 (M^+). IR (KBr) ν_{max} (cm^{-1}): 1645, 1620. ^1H NMR (DMSO-d_6) δ: 0.77 (3H, s), 1.06 (3H, s), 5.89 and 6.15 (1H, s and s), 6.80 (2H, m), 7.35 (1H, m).

P-2,5-DHBH: 5% yield, m.p. 294–296°C (ethanol). Analysis. Calculated for $C_{28}H_{38}N_2O_4$ (molecular weight, 466.62): C, 72.07; H, 8.21; N, 6.00. Found: C, 71.89; H, 8.25; N, 5.84. MS *m/e*: 466 (M^+). IR (KBr) ν_{max} (cm^{-1}): 1635, 1620. ^1H NMR (DMSO-d_6) δ: 0.56 (3H, s), 0.95 (3H, s), 1.90 (3H, s), 5.28 (1H, s), 6.80 (2H, m), 7.35 (1H, m).

RESULTS AND DISCUSSION

Figs. 1 and 2 show the chemical structures of the six hydrazine and five hydrazide reagents, respectively, used for this study.

Hydrazine reagents

First, six acetophenone hydrazones of the hydrazine reagents, AP-2,4-DNPH, AP-4-NPH, AP-PH, AP-4-MPH, AP-4-HPH and AP-HBT, were prepared to find the one most suitable for HPLC–ED

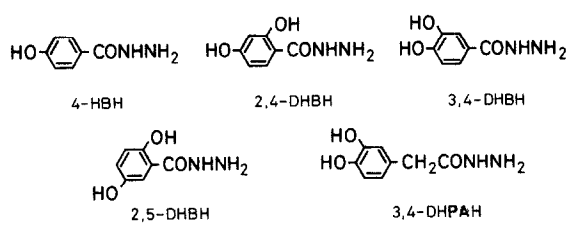

Fig. 2. Chemical structures of hydrazide reagents. 4-HBH, 4-hydroxybenzohydrazide; 2,4-DHBH, 2,4-dihydroxybenzohydrazide; 3,4-DHBH, 3,4-dihydroxybenzohydrazide; 2,5-DHBH, 2,5-dihydroxybenzohydrazide; 3,4-DHPAH, 3,4-dihydroxyphenylacetohydrazide.

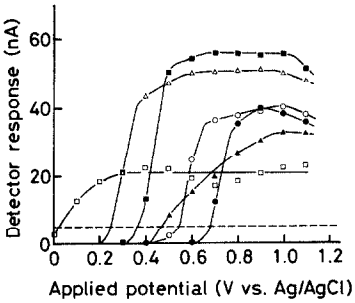

Fig. 3. Relationship between detector response and applied potential for 200 pmol of hydrazones. Symbols: ● = AP-2,4-DNPH; ○ = AP-4-NPH; ■ = AP-PH; △ = AP-4-MPH; □ = AP-4-HPH; ▲ = AP-HBT. HPLC conditions: column, Nucleosil C_{18} (10 μm, 250 mm × 4.6 mm I.D.); mobile phase and flow-rate, methanol–0.5% ammonium dihydrogenphosphate (pH 4.5) (80:20, v/v), 0.8 ml/min for AP-4-HPH; methanol–0.5% ammonium dihydrogenphosphate (pH 4.5) (90:10, v/v), 1.0 ml/min for the others.

derivatization. Electrochemical properties of the hydrazones were examined with their hydrodynamic voltammograms (Fig. 3). Fig. 3 indicates that the order of the potential at which each hydrazone shows a response of 5 nA per 200 pmol (dashed line) is AP-4-HPH < AP-4-MPH < AP-PH < AP-HBT < AP-4-NPH < AP-2,4-DNPH. AP-4-HPH responds even at about 0 V *vs.* Ag/AgCl. This order of the ease of electrochemical oxidation of those hydrazones agrees with the electron-donating ability of their substituent(s) (4-hydroxy > 4-methoxy > 4-hydrogen > 4-nitro > 2,4-dinitro), except for AP-HBT, which is not a phenylhydrazone derivative.

TABLE I

DETECTION LIMITS OF ACETOPHENONE HYDRAZONES OF AROMATIC HYDRAZINE DERIVATIVES

Signal-to-noise ratio = 2. HPLC conditions were the same as in Fig. 3.

Compound	Detection limit (fmol per injection)		
	+0.9 V	+0.5 V	+0.3 V
AP-2,4-DNPH	100	520 000	–
AP-4-NPH	50	500	–
AP-PH	40	30	4800
AP-4-MPH	60	50	60
AP-4-HPH	100	40	20
AP-HBT	100	240	45 000

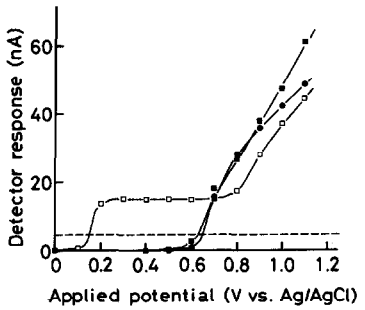

Fig. 4. Relationship between detector response and applied potential for 200 pmol of hydrazones. Symbols: ● = AP-4-HBH; ■ = AP-2,4-DHBH; □ = AP-2,5-DHBH. HPLC conditions: column, μBondapak NH_2 (300 mm × 3.9 mm I.D.); mobile phase, 0.1 *M* phosphate buffer (pH 6.8)–methanol (90:10, v/v); flow-rate, 1.0 ml/min.

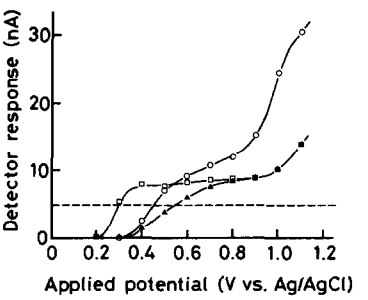

Fig. 5. Relationship between detector response and applied potential for 200 pmol of hydrazones. Symbols: ○ = AP-3,4-DHBH; □ = AP-2,5-DHBH; ▲ = AP-3,4-DHPAH. HPLC conditions: column, μBondapak C_{18} (300 mm × 3.9 mm I.D.); mobile phase, 0.1 *M* sodium dihydrogenphosphate containing 0.01 *M* sodium 1-pentanesulfonate (pH 4.4)–methanol (75:25, v/v); flow-rate, 1.0 ml/min.

Table I shows the detection limits of the hydrazones measured at the applied potentials of +0.9, +0.5 and +0.3 V *vs.* Ag/AgCl by HPLC–ED. Two hydrazones, AP-4-HPH and AP-4-MPH, have low detection limits even at +0.3 V *vs.* Ag/AgCl, but these two and also 4-HPH · HCl are unstable in air at room temperature.

Hydrazide reagents

Next, five hydrazide reagents were prepared. Each reagent was designed to have one or two carbon unit(s) between a hydrazine group as the chemical reaction part with carbonyl compounds, and a hydroxyphenyl group as the electrochemical reaction part for electrochemical detection in order to improve stability. All of these hydrazide reagents were stable in air at room temperature for six months. Five acetophenone hydrazones were synthesized with the hydrazide reagents (AP-4-HBH, AP-2,4-DHBH, AP-3,4-DHBH, AP-2,5-DHBH, AP-3,4-DHPAH), and all of these hydrazones were also stable in air at room temperature for six months.

As shown in Figs. 4 and 5, hydrodynamic voltammograms of the hydrazones were examined under the two HPLC conditions necessary to obtain appropriate retention and good peak shapes for all of them. For comparison of the data in Figs. 4 and 5, the hydrodynamic voltammograms of AP-2,5-DHBH were obtained under both conditions. The results indicate that the order of the potential at which each hydrazone shows a response of 5 nA per 200 pmol (dashed line) is AP-2,5-DHBH < AP-3,4-DHBH < AP-3,4-DHPAH < AP-2,4-DHBH ⩽ AP-4-HBH. This sequence is the same as that of the half-oxidative potentials for some of the hydroxy-substituted benzenes in acetonitrile containing 0.1 *M* lithium perchlorate with reference to a saturated calomel electrode [1,4-dihydroxybenzene (+0.75 V) < 1,2-dihydroxybenzene (+0.93 V) < 1,3-dihydroxybenzene (+1.13 V) < hydroxybenzene (+1.31 V)] [11,12].

Table II shows the detection limits of the hydrazones measured at the applied potentials of +0.9, +0.5 and +0.3 V *vs.* Ag/AgCl by HPLC–ED. AP-2,5-DHBH is the only hydrazone which has a low detection limit even at +0.3 V *vs.* Ag/AgCl.

From the above results, 2,5-DHBH is the most

TABLE II

DETECTION LIMITS OF ACETOPHENONE HYDRAZONES OF HYDRAZIDE DERIVATIVES

Signal-to-noise ratio = 2. HPLC conditions were the same as in Fig. 4 (AP-4-HBH, AP-2,4-DHBH and AP-2,5-DHBH) and Fig. 5 (AP-3,4-DHBH and AP-3,4-DHPAH).

Compound	Detection limit (fmol per injection)		
	+0.9 V	+0.5 V	+0.3 V
AP-4-HBH	100	2100	–
AP-2,4-DHBH	30	21 000	–
AP-3,4-DHBH	20	90	82 000
AP-2,5-DHBH	60	40	40
AP-3,4-DHPAH	60	400	5600

suitable derivatizing reagent of the hydrazines and the hydrazides investigated because (i) it and its hydrazone are stable in air at room temperature and they are easy to operate and (ii) its hydrazone is detectable at a lower oxidative potential which causes higher selectivity and a lower detection limit.

Properties of 2,5-DHBH derivatives of ketosteroids

The electrochemical and chromatographic properties of ketosteroids derivatized with 2,5-DHBH were also studied to prove its suitability. Seven ketosteroids were used: three 3-ketosteroids, testosterone (T), ethisterone (E), norethisterone (N); two 17-ketosteroids, androsterone (A), dehydroisoandrosterone (D); one 20-ketosteroid, pregnenolone (P); and one 3,20-diketosteroid, corticosterone (C). All ketosteroids were derivatized to hydrazones with 2,5-DHBH (T-2,5-DHBH, E-2,5-DHBH, N-2,5-DHBH, A-2,5-DHBH, D-2,5-DHBH, P-2,5-DHBH, C-2,5-DHBH). All hydrazone structures were verified by MS, IR, ^{1}H NMR and elemental analysis as described in the Experimental section: each carbonyl group of the monoketosteroids (T, E, N, A, D and P) was derivatized with 2,5-DHBH; only one carbonyl group was derivatized with 2,5-DHBH at the 3-position of the diketosteroid (C). The position presented for the derivatization of C seems to be reasonable because the 3-position of the steroid keto group has a higher reactivity than the 20-positon [13,14]. Several HPLC conditions were investigated and then 2,5-DHBH and all the hydrazones of ketosteroids were clearly separated on a reversed-phase column (Fig. 6). The hydrodynamic voltammograms of the hydrazones of ketosteroids were obtained under the conditions described in Fig. 6 (Fig. 7). Fig. 7 shows that: (i) all voltammograms have very similar shapes though they have slightly different responses caused by diverse retention times; (ii) all the hydrazones show responses even at about +0.1 V *vs.* Ag/AgCl; (iii) the responses show a plateau in the potential range from +0.2 to +0.7 V *vs.* Ag/AgCl; (iv) the responses increase with increasing potential in the range from +0.7 to +1.1 V *vs.* Ag/AgCl.

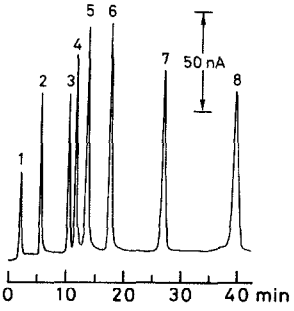

Fig. 6. Chromatogram of a synthetic mixture of 2,5-DHBH and its hydrazones of ketosteroids by HPLC–ED. Peaks (concentration injected): 1 = 2,5-DHBH (260 pmol); 2 = C-2,5-DHBH (650 pmol); 3 = D-2,5-DHBH (570 pmol); 4 = T-2,5-DHBH (830 pmol); 5 = N-2,5-DHBH (1330 pmol); 6 = E-2,5-DHBH (1280 pmol); 7 = A-2,5-DHBH (1260 pmol); 8 = P-2,5-DHBH (1710 pmol). HPLC conditions: column, Chemcosorb 5-ODS-UH (150 mm × 4.6 mm I.D.); mobile phase: 0.05 *M* phosphate buffer (pH 7.0)–acetonitrile (64:36, v/v), flow-rate: 1.0 ml/min, applied potential: +0.20 V *vs.* Ag/AgCl.

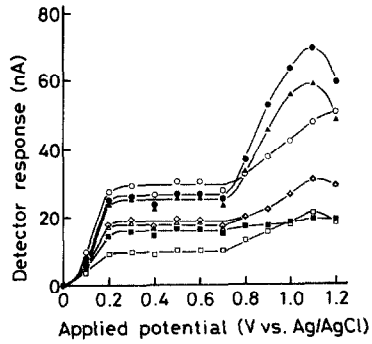

Fig. 7. Relationship between detector response and applied potential for 200 pmol of hydrazones. Symbols: ● = C-2,5-DHBH; ○ = D-2,5-DHBH; ▲ = T-2,5-DHBH; △ = N-2,5-DHBH; ◇ = E-2,5-DHBH; ■ = A-2,5-DHBH; □ = P-2,5-DHBH. HPLC conditions were the same as in Fig. 6.

TABLE III

DETECTION LIMITS OF 2,5-DHBH HYDRAZONES OF KETOSTEROIDS

Signal-to-noise ratio = 2. HPLC conditions were the same as in Fig. 6.

Compound	Detection limit (fmol per injection)				
	+0.8	+0.6	+0.5	+0.3	+0.2
C-2,5-DHBH	60	150	150	150	150
D-2,5-DHBH	100	200	150	150	150
T-2,5-DHBH	80	300	150	200	150
N-2,5-DHBH	200	400	250	300	300
E-2,5-DHBH	200	400	250	300	300
A-2,5-DHBH	200	400	400	300	500
P-2,5-DHBH	300	500	500	400	500

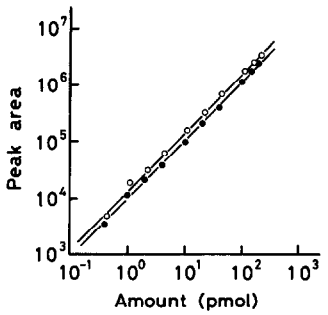

Fig. 8. Linearity of C-2,5-DHBH (●) and D-2,5-DHBH (○). Applied potential, +0.30 V *vs.* Ag/AgCl; other conditions were the same as in Fig. 6. C-2,5-DHBH: $b = 1.0138$, $t_b = 1.125 < t$ (9, 0.05) = 2.262, $r = 0.999$; D-2,5-DHBH: $b = 1.0148$, $t_b = 0.875 < t$ (9, 0.05) = 2.262, $r = 0.999$.

Table III shows detection limits of the ketosteroid hydrazones measured at the applied potentials of +0.8, +0.6, +0.5, +0.3 and +0.2 V *vs.* Ag/AgCl by HPLC–ED using authentic samples. The results shown in Tabnle III are as follows: (i) the detection limits are in the range of 60–500 fmol per injection; (ii) the detection limits of each hydrazone are not significantly different when the applied potentials are changed from +0.2 to +0.6 V *vs.* Ag/AgCl, as expected from the results of hydrodynamic voltammograms described above. As shown in Fig. 8, a good linear relationship was observed between the peak area and the concentration in the range of 0.4–200 pmol of C-2,5-DHBH and D-2,5-DHBH.

In conclusion, 2,5-DHBH is a suitable derivatization reagent for sensitive and selective HPLC–ED determination of carbonyl compounds because of its electrochemical properties, detection ability and stability. In order to demonstrate the usefulness of this reagent, further study to determine carbonyl compounds in biological fluids is in progress and will be reported elsewhere in the near future.

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