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Determination of medroxyprogesterone acetate in serum by HPLC with peroxyoxalate chemiluminescence detection using a fluorogenic reagent, 4-(*N*,*N*-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole*

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Abstract: The high-performance liquid chromatographic determination of medroxyprogesterone acetate (MPA) with peroxyoxalate chemiluminescence (PO-CL) detection is described. The spiked serum containing MPA was extracted on Bond-Elut C_{18} columns and derivatized with 4-(*N*,*N*-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H). The hydrazone of MPA with DBD-H was confirmed to be a mono-DBD-derivative. The reaction mixture was separated by direct injection onto a C_{18} analytical column, and quantified by PO-CL detection. The linear range of the standard curve, in serum, was 15.6–96.6 ng ml^{-1} with a detection limit of 9 ng ml^{-1} using only 100 μl of serum, while the detection limit of standard MPA derivatized with DBD-H was 8.7 fmol per injection. The relative standard deviation of the method was 7.4% at 19.3 ng and 1.7% at 77.3 ng ml^{-1}.

Keywords: *Medroxyprogesterone acetate; peroxyoxalate chemiluminescence detection; reversed-phase high-performance liquid chromatography; 4-(*N,N*-dimethylaminosulphonyl)-7-hydrazino-2; 1,3-benzoxadiazole; hydrazone; human serum.*

Introduction

Medroxyprogesterone acetate (MPA; 6α-methyl-17α-hydroxyprogesterone acetate) is a synthetic progestogen possessing high progestational activity. High dose MPA treatment is reported to be effective in advanced breast cancer, endometrial cancer, renal and prostatic carcinoma [1]. The effective blood concentration range for MPA in cancer therapy was found to be in the range 80–100 ng ml^{-1} [2] with wide inter-individual blood level variations [3]. Therefore, the measurement of MPA concentrations is essential for effective chemotherapy.

Concentrations of MPA in blood have been determined by radioimmunoassay (RIA) [4], gas–liquid chromatography (GLC) with electron capture detection [5], gas chromatography–mass spectrometry (GC–MS) [6] and high-performace liquid chromatography with UV detection (HPLC–UV) [7]. Among these methods, RIA is the most commonly used clinically because of its sensitivity, simplicity and commercial availability. However, the cross-reactivity of MPA metabolites limits the specificity of the RIA method and this has resulted in the overestimation of the MPA concentration with some antibodies [8]. GC–MS is generally regarded as the most specific method for measuring serum levels of hormones and drugs including MPA, but is not usually used for routine clinical application. GLC methods are employed in pharmacokinetic studies. However, they are less selective and sensitive than GC–MS. The low sensitivity of HPLC–UV methods still limits their use in clinical studies involving the quantitation of serum MPA.

Recently, 4-(*N*,*N*-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) has been developed as a fluorogenic labelling reagent for aldehydes and ketones [9]. The resultant hydrazones were separated on a reversed-phase column and detected at sub-pmol levels, fluorimetrically. Since the derivatives of amines with 4-(*N*,*N*-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole

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(DBD-F) were more sensitively detected by peroxyoxalate chemiluminescence (PO-CL) than by fluorescence [10], it appeared that hydrazones derived from DBD-H might also be detected more sensitively by PO-CL than by fluorescence. In the present study, derivatization of MPA, an oxosteroid, with DBD-H and HPLC–PO-CL detection were investigated for their suitability in providing a selective and sensitive method for the determination of serum MPA.

Experimental

Materials

4-(*N*,*N*-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole was purchased from Tokyo Chemical Industries (Tokyo, Japan). DBD-H was synthesized as described previously [9]. Medroxyprogesterone acetate (MPA), beclomethazone dipropionate, 16α-methylprogesterone, methyltestosterone and prednisolone were purchased from Sigma (St Louis, MO, USA). Zone-refined imidazole was donated by Tokyo Chemical Industries. Bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)-phenyl]oxalate (TDPO), nitric acid and hydrogen peroxide (30%) of analytical-reagent grade, distilled water, acetonitrile and ethyl acetate of HPLC-grade, Dotite Spectrozol tetrahydrofuran (THF) and trifluoroacetic acid (TFA) of amino acid sequence analysis-grade were obtained from Wako Pure Chemical Industries (Tokyo, Japan). The Bond-Elut C_{18} columns (1 ml capacity) were from Varian (Harbor City, CA, USA). Normal human serum, non-lipid stripped, was purchased from Funakoshi Chemicals (Tokyo, Japan). TLC plates, Kieselgel 60, were obtained from Merck (Darmstadt, Germany). Other chemicals were of analytical-reagent grade.

Apparatus

The HPLC system with UV and fluorescence detection consisted of an L-6200 pump (Hitachi, Tokyo, Japan), a sample injector with a 20 μl injection loop (Rheodyne, Cotati, CA, USA), an analytical column, TSK gel ODS $80T_M$ (5 μm, 150 or 250 × 4.6 mm i.d. for UV or fluorescence detection, respectively) which were maintained at room temperature, a L-4200 UV–vis detector or an F-1000 fluorescence spectrophotometer (Hitachi) and a C-R1A Chromatopac integrator (Shimadzu, Kyoto, Japan). In the HPLC system with PO-CL detection, a KHP-011 pump (Kyowa, Tokyo, Japan), equipped with a sample injector, Rheodyne 7125 with 100 μl injection loop (Rheodyne) was used. A guard column, TSKguardgel ODS-$80T_M$ (7 μm, 15 × 3.2 mm i.d.) (Tosoh) was inserted between the pump and the injector as a damper. The effluent from the analytical column, TSK gel ODS $80T_M$ (5 μm, 250 × 4.6 mm i.d.) (Tosoh) was mixed in a KZU-11 mixing device (Kyowa) with the post-column CL reagent solution delivered by a LC-6A pump (Shimadzu) through a TSK gel ODS $80T_M$ column (5 μm, 150 × 4.6 mm i.d.) as a damper. The generated CL was monitored by a S-3400 chemiluminescence detector (Soma, Tokyo, Japan), the signal from which was integrated by a C-R1A Chromatopac (Shimadzu). The analytical column and the mixing device were housed in a 655A-52 column oven (Hitachi) maintained at 30°C. Colour filter glasses, Y-47, Y-50, Y-52, and O-54 (Hoya Glass Works, Ltd, Tokyo, Japan) with sharp cut-off wavelengths at 470, 500, 520 and 540 nm, respectively, were placed just in front of the photomultiplier of the CL detector.

Liquid-chromatographic conditions

The mobile phases were acetonitrile–water (70:30, v/v) containing 0.05% TFA, THF–50 mM imidazole nitrate buffer (pH 6.0) (115:85, v/v) or THF–100 mM imidazole nitrate buffer (pH 6.0) (62:38, v/v) for UV, fluorescence or PO-CL detection, respectively [10]. The flow rates were 1.0, 0.8 or 0.5 ml min^{-1} for UV detection at 241 nm for MPA, fluorescence detection (excitation: 480 nm, emission: 570 nm) or PO-CL detection of DBH-H–MPA, respectively. The post-column PO-CL reagent solution comprised 0.5 mM TDPO and 25 mM hydrogen peroxide in acetonitrile and ethyl acetate (1:1, v/v). The flow rate was 1.0 ml min^{-1}.

Synthesis of the DBD-H hydrazone of MPA (DBD-H–MPA)

Medroxyprogesterone acetate (39 mg, 0.1 mmol) was dissolved in 10 ml of acetonitrile, mixed with DBD-H (26 mg, 0.1 mmol) dissolved in 7 ml of CH_3CN and a few drops of TFA. The mixture was stirred at room temperature in the dark for 5 h and then evaporated to dryness under reduced pressure. The residue was dissolved in 1 ml of chloroform and chromatographed on a Kiesdgel TLC plate

with the developing solvent, $CHCl_3$–CH_3CN (9:1, v/v). The band exhibiting orange fluorescence was scraped off and extracted twice with 10 ml of $CHCl_3$. After evaporation of the extract, the residue was purified again by TLC as described above to yield crystals.

DBD-H–MPA: orange crystals; m.p. 170–180°C (decomp.); yield, 16%; ^1H NMR ($CDCl_3$) δ: 2.05 (s, 3H, 17-CH_3), 2.10 (s, 3H, 21-CH_3), 0.69 (s, 3H, 18-CH_3), 1.13 (s, 3H, 19-CH_3), 1.22 (d, 3H, J = 6.16 Hz, 6-CH_3), 6.07 (d, 1H, $J < 1.5$ Hz, 4-H), 7.06 (d, 1H, H^a, J = 7.91 Hz), 7.96 (d, 1H, H^b, J = 7.91 Hz), 2.88 (s, 6H, H^c), 8.51 (s, 1H, H^d); IR (KBr), 1730 cm^{-1} (C=O); UV λ_{max} (in ethanol), 228.6, 304, 466.6 nm; EI–MS, *m/z* 625 (M^+); calculated for $C_{32}H_{43}O_6N_5S \cdot 1/2\ CHCl_3$, calc.: C, 56.94; H, 6.40; N, 10.22; S, 4.68; Cl, 7.76, found: C, 57.24; H, 6.46; N, 9.62; S, 4.45; Cl, 7.92.

Derivatization of MPA with DBD-H for HPLC analysis

A 20 μl volume of 1 mM DBD-H in CH_3CN, 20 μl of 1% TFA in CH_3CN and 40 μl of MPA solution ranging from 20 nM to 50 μM in CH_3CN were mixed and left at approx. 4°C in a refrigerator for 5 h or overnight.

Solid-phase extraction and derivatization procedure

A 100 μl volume of serum and 5 μl of MPA in methanol were mixed in an Eppendorf tube and to this, 200 μl of CH_3CN was added. After vortex-mixing for 30 s and sonication for 1 min, the mixture was centrifuged at 10 000*g* for 1 min. The supernatant solution was mixed with 300 μl of H_2O and applied to a Bond-Elut C_{18} column which was prewashed with CH_3CN and conditioned with 40% CH_3CN (v/v) before use. A 50 μl of 40% CH_3CN was added to the Eppendorf tube to dissolve the supernatant solution remaining and then applied to the Bond-Elut column. The column was washed with 500 μl of 40% CH_3CN and 500 μl of 45% CH_3CN and elution was performed with 1 ml of 70% CH_3CN. The extract was evaporated under reduced pressure at 35°C. The residue obtained was dissolved in 40 μl of a solution of 0.25 mM DBD-H and 0.25% TFA in CH_3CN, and left at approx. 4°C for 5 h or overnight. A 1.5 μl aliquot of the reaction mixture was subjected to HPLC analysis. The recoveries of the derivative extracted from the Bond-Elut column were obtained by comparing the peak heights of extracted MPA with those of the corresponding amount of standard MPA by HPLC with UV detection. The recoveries through the whole procedure were determined in the same manner, except with PO-CL detection after derivatization with DBD-H.

Results and Discussion

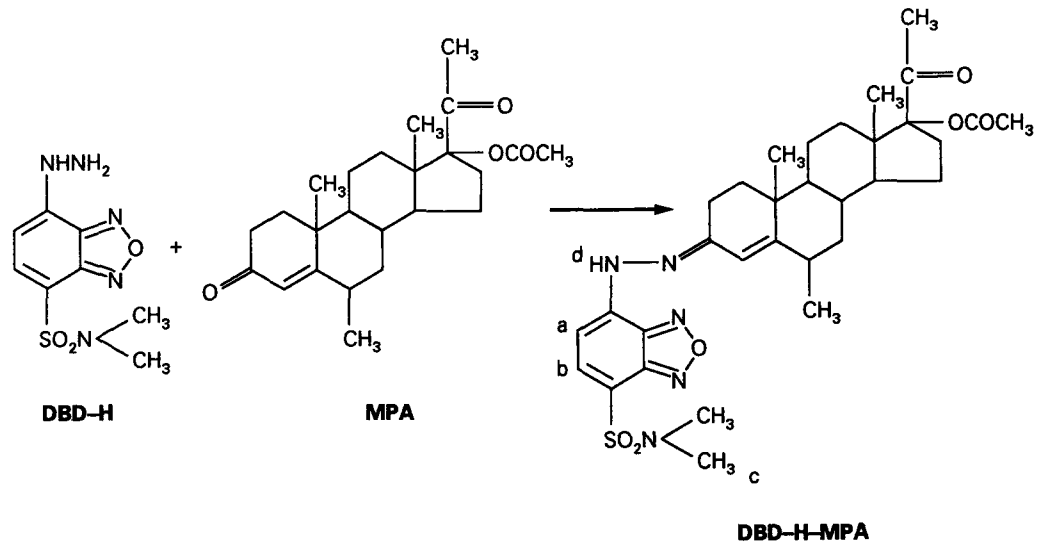
Medroxyprogesterone acetate (MPA) has no native fluorescence and thus has to be converted to a fluorescent derivative. It has two carbonyl groups at the 3- and 20-positions which may possibly react with DBD-H. Instrumental analysis by UV, IR, NMR spectrometry, MS and elemental analysis of synthetic DBD-H–MPA indicates that only the unsaturated carbonyl group at the 3-position reacted with the hydrazino group of DBH-H to yield the monohydrazino derivative (Scheme 1).

The derivatization of MPA for HPLC analysis was carried out by a previously published method [9]. Since the reaction proceeds even at approx. 4°C with the formation of fewer by-products than at room temperature, the reaction was performed in a refrigerator for 5 h. It was confirmed that the retention time of DBD-H–MPA obtained by this method was the same as that of authentic DBD-H–MPA. The resulting hydrazone was stable for 1 day in the reaction medium containing 0.25 mM DBD-H and 0.25% TFA in CH_3CN at 4°C, but thereafter gradually decomposed.

In the derivatization of DBD-H with other labelling reagents for carbonyl groups, such as dansyl hydrazine (DNS-H), the anti- and syn-isomers of the hydrazone of oxo-steroids are formed and are separated on a reversed-phase column when methanol or acetonitrile is used as the organic modifier in the mobile phase. Only THF allows the elution of the isomers as a single peak [11].

The reaction mixture (4 μl) was directly chromatographed by HPLC with fluorescence detection. The calibration curve was linear between 0.15 and 5 μM corresponding to 0.6 and 20 pmol per injection and the correlation coefficient (r) was 0.9999. The detection limit was 0.38 pmol per injection (signal to noise ratio = 3).

With CL detection, the chromatographic separation of DBD-H–MPA from the by-product derived from DBD-H and impurities



Scheme 1
The derivatization reaction of medroxyprogesterone acetate with DBD-H.

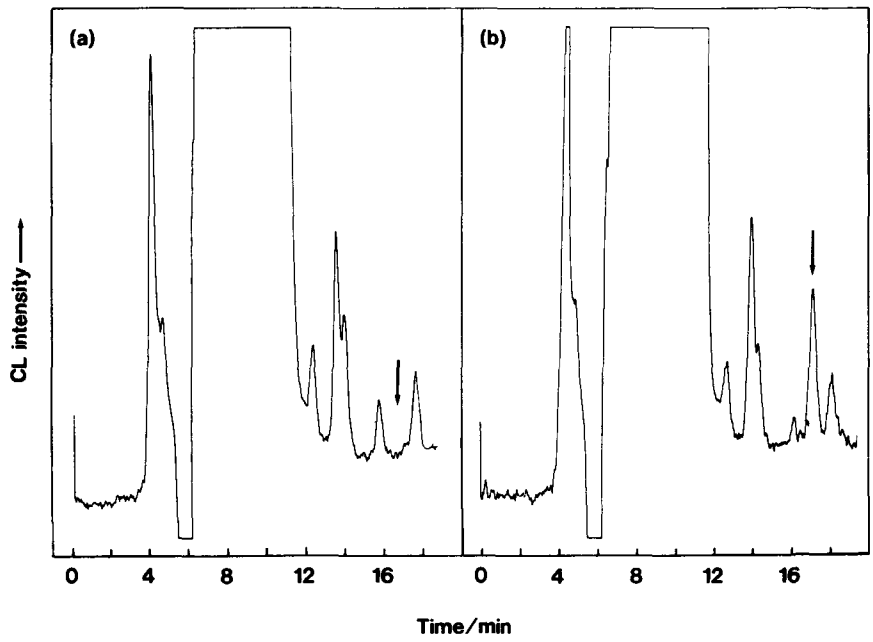


Figure 1
Chromatograms of medroxyprogesterone acetate derivatized with DBD-H with PO-CL detection. (a) Reaction mixture without medroxyprogesterone acetate. (b) Reaction mixture of medroxyprogesterone acetate (250 fmol per injection) with DBD-H. The arrows indicate hydrazone of medroxyprogesterone acetate with DBD-H.

in the reaction medium were difficult under the separation condition adopted for fluorescence detection because of the high sensitivity of the detector. Therefore, the mobile phase composition which had to be changed for CL detection as described in the Experimental section gave sufficient separation of DBD-H–MPA from the large interfering peaks eluting with short retention times and from other peaks eluting close to DBD-H–MPA (Fig. 1).

The CL post-column reaction conditions were studied. The CL reagent solution composed of 0.5 mM TDPO and 25 mM H_2O_2 gave the highest signal to noise ratio (S/N). To reduce the noise level, a colour filter glass was placed in front of the photomultiplier. Of the

four sharp cut-off filters tested, a Y-52 filter was the most efficient in reducing the noise level without affecting the signal intensity. This may be because the wavelength of maximum emission of DBD-H–MPA occurs at 570 nm (fluorescence range: 500–650 nm) while that of imidazole, as the catalyst for the PO-CL reaction, included in the mobile phase occurs at 330 nm. The fluorescence of imidazole may be one of the factors that increases the background CL. By using the filter, a concentration of up to 100 mM imidazole buffer could be used to give the highest *S/N* although generally 50 mM was used. The CL reaction time was regulated by changing the length of the reaction coil placed between the mixing device and the CL detector. The highest *S/N* was achieved at the reaction time of 3.6 s by using a 40 cm reaction coil (0.25 mm i.d.). Under the conditions obtained above, the calibration curve of standard MPA was linear between 10 nM and 0.5 μM with a correlation coefficient (r) or 0.9998. The detection limit of 8.7 fmol per injection of 2 μl reaction mixture was 40 times lower than that given by fluorescence detection.

Since the derivatives of amines with DBD-F were more sensitively detected (10–100 times) by CL than by fluorescence [10], it is suggested that the DBD-moiety might be commonly adopted for CL detection. Also, carboxylic acid derivatives such as prostaglandins could be sensitively detected by derivatization with DBD-piperazine and DBD-cadaverine with PO-CL detection [12].

In the case of DNS-H, purification with gel permeation chromatography after derivatization was necessary for application of the HPLC PO-CL detection procedure [13]. However, in the case of DBD-H, direct injection was possible for the determination of DBD-H–MPA without any chromatographic interference (Fig. 1).

Figure 2 shows the chromatograms of serum extracts. The extraction of MPA from serum was performed with Bond-Elut C_{18} columns since a cleaner extract was obtained for CL detection after derivatization than with Bond-Elut C_8 and C_1 columns. A preliminary study involving a relatively high concentration of MPA showed that the recovery from the column of MPA in a 100-μl injection of 10 μM standard MPA solution was quantitative. The recovery through the whole procedure, including extraction and derivatization, was 82.2% at 40 nM MPA in serum. The calibration curve obtained with MPA concentrations from 40 to 250 nM (15.6 to 96.6 ng ml^{-1}) in serum was

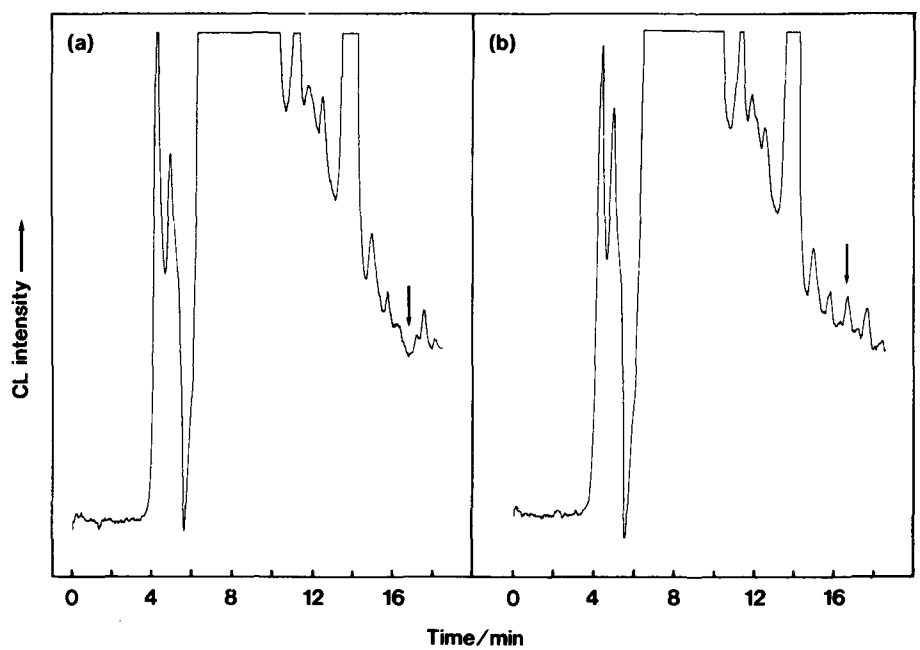


Figure 2
Chromatograms of spiked medroxyprogesterone acetate in serum. Medroxyprogesterone acetate was extracted from 100 μl of serum, derivatized with DBD-H and determined with PO-CL detection. (a) Serum, (b) 19.3 ng ml^{-1} medroxyprogesterone acetate spiked in serum. The arrows indicate hydrazone of medroxyprogesterone acetate with DBD-H.

linear, with a correlation coefficient of 0.998. The detection limit was 9 ng ml^{-1} using 100 μl of serum and injecting 1.5 μl of the reaction mixture. The eight identical aliquots of serum (50 and 200 nM; 19.3 and 77.3 ng ml^{-1}) was measured and gave relative standard deviations of 7.4 and 1.7%, respectively, representing the intra-assay reproducibility.

Several oxo-steroids (beclomethazone dipropionate, 16α-methylprogesterone, methyltestosterone and prednisolone) were examined for their potential as an internal standard. However, they were eluted at retention times similar to those of the by-products or impurities derived from the reagent and the reaction solvent. Thus, in the present method, the determination of MPA was performed without an internal standard. The use of an appropriate internal standard may improve the accuracy of the procedure.

The most serious problem encountered in the present analytical method for MPA was the large peak derived from serum components that eluted at earlier retention times and interfered in the determination of trace amounts of DBD-H–MPA. Column-switching was found to be very efficient in removing such interfering peaks. Thus an immunosuppressive agent, FK 506 in serum was determined by PO-CL derivatized with DNS-H with a detection limit in the fmol range [14]. It is expected that further studies on the extraction from serum and on-line clean-up procedures after derivatization with DBD-H will lead to the sensitive analysis of oxo-steroids with detection limits of sub ng ml^{-1} of serum using 100 μl serum.

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