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Determination of corticosteroids in plasma by high-performance liquid chromatography after pre-column derivatization with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole

Masatoki Katayama, Yuichi Masuda and Hirokazu Taniguchi

Meiji College of Pharmacy, 1-35-23, Nozawa, Setagaya-ku, Tokyo 154 (Japan)

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ABSTRACT

2-(4-Carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) was used as a pre-column derivatization reagent for corticosteroids analysed by high-performance liquid chromatography with fluorimetric detection. Eight corticosteroids were derivatized with CDB to their esters in acetonitrile containing 4-piperidinopyridine and 1-isopropyl-3-(3-dimethylaminopropryl)carbodiimide perchlorate. The resulting CDB esters were extracted with a Sep-Pak C_{18} cartridge and the esters were separated on a reversed-phase column (Zorbax ODS) with water–methanol (25:75, v/v) containing 5 mmol/l tetramethylammonium hydrogensulphate as the mobile phase. The limits of detection for steroids were 0.06–0.3 pg per 100 μl of plasma (signal-to-noise = 3). The within-day relative standard deviations (n = 6) were 7.8–11.1%, and day-to-day relative standard deviations (n = 6) were 7.0–10.4%.

INTRODUCTION

Corticosteroids have been monitored widely in clinical analysis. For example, aldosterone, cortisone, cortisol, corticosterone, dexamethasone and triamcinolone have been determined by gas chromatography (GC) [1,2], gas chromatography–mass spectrometry (GC–MS) [3,4], radioimmunoassay (RIA) [5,6], enzyme immunoassay (EIA) [7] and high-performance liquid chromatography (HPLC) [8–16]. The last-mentioned technique, coupled with fluorimetric pre-column derivatization, has been developed for simultaneous assay of corticosteroids in biological samples [13–16]. These methods are specific and sensitive and do not require expensive reagents and apparatus. We have previously reported an HPLC method with fluorimetric pre-column derivatization for the determinations of alcohol using 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) [17]. Using this method, as little as 0.2–0.4 pg per 20 μl fatty alcohols and lipids can be determined. This method is more sensitive than other HPLC methods with photometric detection or derivatization [8–16]. CDB is stable and derivatization can be carried out under mild reaction conditions.

In this work, the determination of corticosteroids by HPLC after derivatization with CDB was therefore studied (Fig. 1).

EXPERIMENTAL

Sample and reagents

Corticosteroids (aldosterone, corticosterone,

Correspondence to: M. Katayama, Meji College of Pharmacy, 1-35-23. Nozawa, Setagaya-ku, Tokyo 154, Japan.

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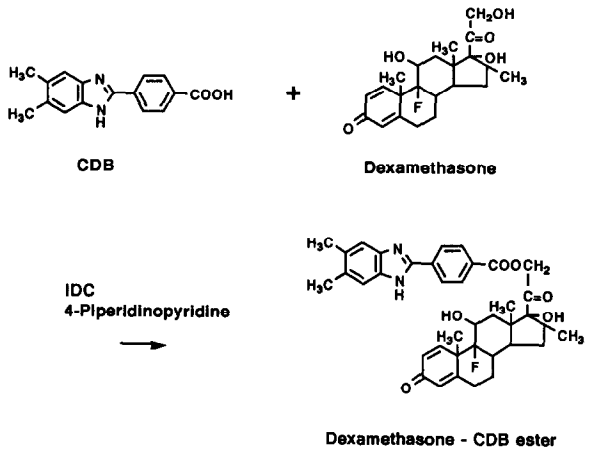

Fig. 1. Derivatization reaction of CDB with steroids.

cortisol, cortisone, dexamethasone, fluocinolone acetonide, triamcinolone and triamcinolone acetonide, Fig. 2) were purchased from Sigma (St. Louis, MO, USA). A stock solution of steroid (1000 μg/ml) was prepared by dissolving 10 mg of steroid in 1 ml of pyridine and subsequently diluting to 10 ml with acetonitrile. The CDB was synthesized according to a previous report [17]. CDB (0.3%, w/v) solution was prepared by dissolving 30 mg of the reagent in 3 ml of pyridine, adding 700 mg of 4-piperidinopyridine (Aldrich, Milwaukee, WI, USA) and subsequently diluting to 10 ml with acetonitrile. This solution was stable for three days in daylight at room temperature. The 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate (IDC) solution (7%, w/v) was prepared by dissolving 700 mg of the reagent (Kanto, Tokyo, Japan) in 10 ml of acetonitrile. This solution was stable for eight days at room temperature. Other reagents were of reagent grade.

Apparatus and HPLC conditions

The HPLC apparatus and conditions were as follows: pump, Shimadzu LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan); guard-column, Zorbax ODS (50 mm × 4.6 mm I.D., 7 μm, DuPont, Wilmington, DE, USA); analytical column, Zorbax ODS (250 mm × 4.6 mm I.D., 7 μm) sample solvent, 20 μl; column temperature, about 22°C; detector, Shimadzu RF-535 fluorescence spectromonitor (excitation 334 nm, emission 418 nm); mobile phase, water–methanol (25:75, v/v) containing 5 m*M* tetramethylammonium hydrogensulphate; flow-rate, 0.4 ml/min.

Pre-column derivatization of steroids

Aliquots of 10 μl of each of the CDB and IDC solutions were added to 100 μl of sample solution in a screw-capped vial. This mixture was heated at 70°C for 20 min then cooled to room temperature. Volumes of 100 μl of water and 200 μl of 50% (v/v) methanol were added to the reaction solution, which was then applied to the Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA). This vial was washed with 2.0 ml of 50% methanol and the washing applied to the cartridge. The col-

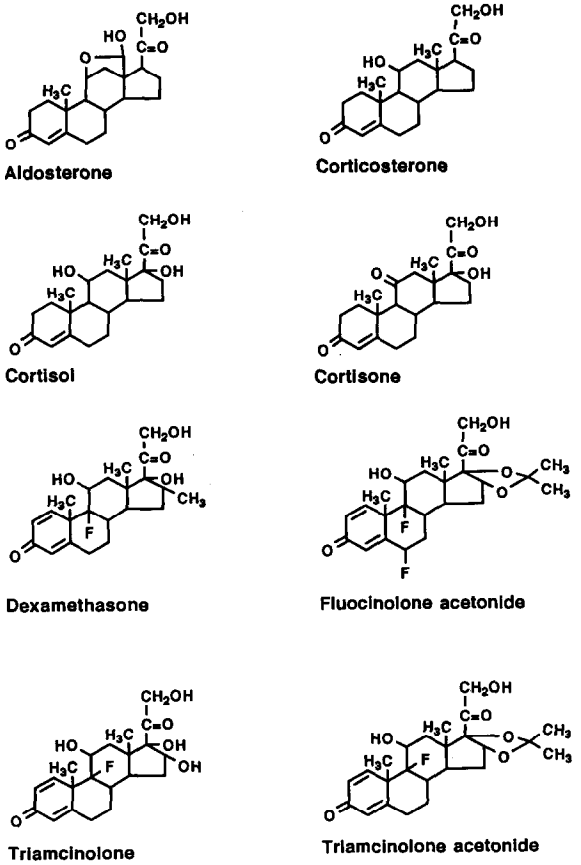

Fig. 2. Structures of corticosteroids.

umn was then washed with 40 ml of 50% methanol, and the resultant fluorescent derivatives were eluted with 5.0 ml of methanol. The eluate was concentrated to 500 μl by evaporation below 40°C under reduced pressure. A 20-μl aliquot of the eluate was injected into the HPLC system.

Extraction of steroids from plasma

Plasma (100 μl, containing 0.1–1 ng of dexamethasone, triamcinolone and triamcinolone acetonide) was mixed with 10 μl of internal standard (fluocinolone acetonide in water) and transferred to a screw-capped vial. This mixture was then extracted twice by shaking for 1 min with 1.2 ml of diochloromethane. A 2.0-ml volume of the dichloromethane layer was collected, the dichloromethane layer was evaporated at a temperature below 40°C under reduced pressure, and the residue was dissolved in 100 μl of acetonitrile. This reaction solution was then used as a sample solution and derivatized with CDB.

RESULTS AND DISCUSSION

Pre-column derivatization

Dexamethasone has been widely used as an anti-inflammatory agent, and many pharmacokinetic studies have been reported. However, sensitive HPLC methods with pre-column fluorimetric derivatization for dexamethasone have not been reported [13–16]. We therefore used dexamethasone as a model corticosteroid compound for the development of a sensitive HPLC method with pre-column fluorimetric derivatization.

Derivatization solvent. Acetone, acetonitrile, benzene, chloroform, dichloromethane, dioxane, N,N-dimethylformamide, dimethyl sulphoxide and pyridine were tested as reaction solvents for the pre-column derivatization of steroids (Table I). The highest detector response was obtained with acetonitrile, which was therefore chosen as the reaction solvent.

Effect of CDB concentration. The highest detector response was obtained in the range 0.2–1.0% (w/v) CDB. Therefore, 0.3% CDB was used.

Effect of catalyst. It has been reported that esterification of alcohol progresses favourably in the presence of 4-dimethylaminopyridine as a catalyst and dicyclohexylcarbodiimide as an esterification reagent [18]. The effect of catalyst was tested by using 4-dimethylaminopyridine, 4-piperidinopyridine, 4-pyrrolidinopyridine, pyridine, triethylamine, tributylamine and quinuclidine (Table II). The highest detector response was ob-

TABLE I

EFFECT OF REACTION SOLVENT ON THE DERIVATIZATION REACTION OF CDB AND STEROID

Concentration of dexamethasone: 100 ng/ml. The derivatization reaction conditions were as follows: CDB 0.3% (w/v), 4-piperidinopyridine 7% (w/v), IDC 7% (w/v); reaction time, 20 min; temperature, 70°C. Average values were obtained from six runs. The detector response of the CDB derivative in acetonitrile was taken as 100.

Solvent	Detector response
Acetone	10
Acetonitrile	100
Benzene	–
Chloroform	21
Dichloromethane	23
Dioxane	95
N,N-Dimethylformamide	7
Dimethyl sulphoxide	3
Pyridine	3

TABLE II

EFFECT OF CATALYST ON THE DERIVATIZATION REACTION OF CDB AND STEROID

Concentration of catalyst: 0.5 *M*; dexamethazone, 100 ng/ml. Average values were obtained from six runs. The detector response of the CDB derivative using 0.5 *M* (*ca.* 8%, w/v) 4-piperidinopyridine was taken as 100. Other derivatization conditions as in Table I.

Catalyst	Detector response
4-Dimethylaminopyridine	66
4-Piperidinopyridine	100
4-Pyrrolidinopyridine	47
Pyridine	–
Triethylamine	1
Tributylamine	–
Quinuclidine	–

tained with 4-piperidinopyridine. The highest peak height was obtained when 4-piperidinopyridine was used in the concentration range 5–20% (w/v); the concentration of 7% (w/v) 4-piperidinopyridine was therefore chosen.

Effect of dicyclohexylcarbodiimide. For the derivatization of steroids with CDB, the following compounds were tested as esterification reagents: dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride, 1-cyclohexyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate, IDC, 1-hydroxybenzotriazole, N-ethyl-5-*m*-sulphophenylisoxazolium hydrochloride and 2-bromo-1-ethylpyridinium tetrafluoroborate (Table III). The highest detector response was obtained with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride perchlorate and IDC. In view of their solubilities in acetonitrile, IDC was selected. The highest detector response was obtained in the range of 5–10% (w/v) IDC solution; the concentration of 7% (w/v) IDC was therefore chosen in the procedure.

Reaction time and temperature. The reaction time was varied from 0 to 60 min and the temperature from 30 to 90°C (Fig. 3). The highest detector response was obtained by heating for 15 min at 70°C. Heating for 20 min was therefore chosen as optimal.

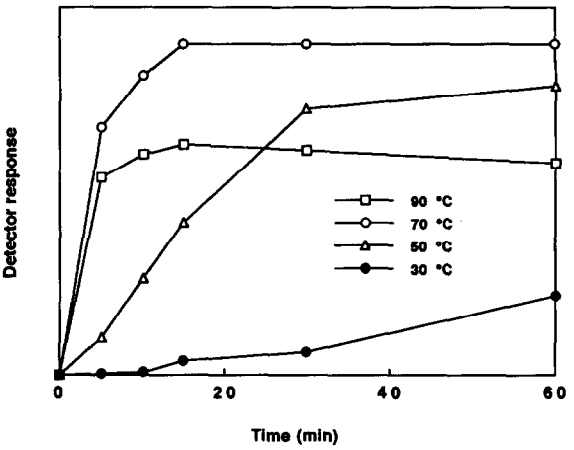

Fig. 3. Effect of reaction time and temperature on the derivatization reaction. Concentration of dexamethasone used: 100 ng/ml.

Extraction of CDB derivatives

To avoid interferences, CDB derivatives were extracted from the reaction solution by a short column. Silica gel, florisil, alumina, octyl (C_8) and octadecyl (C_{18}) cartridges were tested with mixtures of organic solvents. The interferences could be effectively removed by a Sep-Pak C_{18} cartridge with 50% (v/v) methanol as eluent, and this solvent was used for further work.

Separation of CDB derivatives

The separation of mixtures of eight corticoste-

TABLE III

EFFECT OF DCC DERIVATIVE ON THE DERIVATIZATION REACTION OF CDB AND STEROID

Concentration of DCC derivative: 0.1 *M*; dexamethasone, 100 ng/ml. Average values were obtained from six runs. The detector response of the CDB derivative by 0.1 *M* (*ca.* 2.7%, w/v) IDC was taken as 100. The other derivatization conditions were as in Table I.

Condensing agent	Detector response
Dicyclohexylcarbodiimide (DCC)	1
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride	100
1-Cyclohexyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate	88
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate	100
1-Isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate (IDC)	100
1-Hydroxybenzotriazole	–
N-Ethyl-5-*m*-sulphophenylisoxazolium hydrochloride	–
2-Bromo-1-ethylpyridinium tetrafluoroborate	–

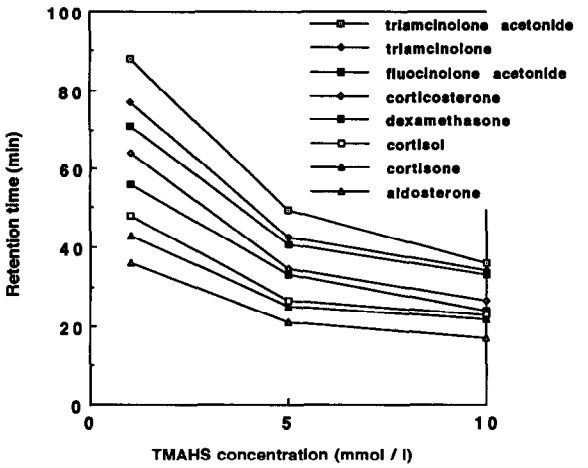

Fig. 4. Effect of TMAHS concentration. Concentration of steroid used: 100 ng/ml.

roids, aldosterone, corticosterone, cortisone, cortisol, dexamethasone, fluocinolone acetonide, triamcinolone and triamcinolone acetonide, was studied. Amino, trimethyl (TMS), octyl (C_8), octadecyl (C_{18}) silica gel and styrene polymer columns were tested. Acetonitrile, tetrahydrofuran, methanol and 2-propanol were tested as mobile phases. However, these conditions did not allow the separation of the eight corticosteroids. Thus, the addition of other organic solvents and surface-active agents was further studied. It was found that the Zorbax ODS column with water–methanol containing tetramethylammonium hydrogensulphate (TMAHS) was the most suitable for the separation of corticosteroids. TMAHS concentration was varied in the range 1–10 m*M*; the concentration of 5 m*M* was selected as optimal (Fig. 4).

The pH of the mobile phase was varied in the range 2.2–7.5. However, the pH of the mobile phase had little effect on the separation of each steroid, and peak widths were broadened when a buffer solution was used. Therefore, water–methanol was used. Finally, water–methanol (25:75, v/v) containing 5 m*M* TMAHS was selected as the best mobile phase.

Excitation and emission spectra of 100 ng/ml dexamethasone are shown in Fig. 5. The excitation maxima of the fluorescent products of steroids were at around 334 nm, and the emission maxima were at around 418 nm. Thus, each steroid was detected at 334 nm excitation and 418 nm emission wavelength.

Determination of corticosteroids

A chromatogram of eight CDB derivatives of corticosteroids (100 ng/ml) is shown in Fig. 6. Retention times and detection limits are shown in Table IV. Detection limits of each steroid were 0.2–10.0 pg per 20 μl of acetonitrile (signal-to-

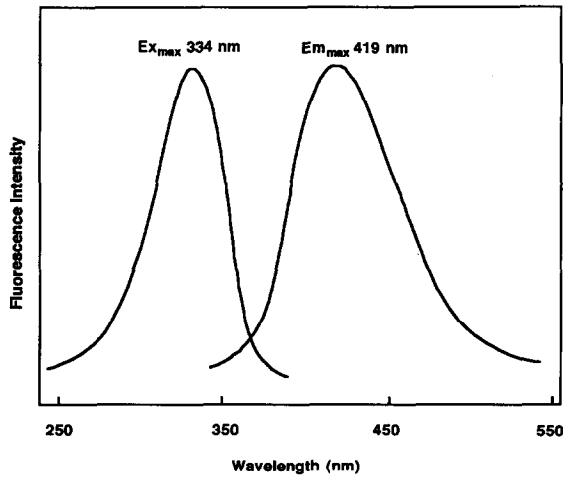

Fig. 5. Excitation and emission spectra of the CDB derivative of dexamethasone in HPLC eluent. Concentration of dexamethasone used: 100 ng/ml.

TABLE IV

RETENTION TIME AND LIMIT OF DETECTION OF STEROID

Average values obtained from six runs.

Steroid	Retention time (min)	Limit of detection (pg per 20 μl)
Aldosterone	21.1	5
Corticosterone	34.7	1
Cortisol	26.5	10
Cortisone	25.2	5
Dexamethasone	33.1	0.2
Fluocinolone acetonide	40.7	0.2
Triamcinolone	43.7	0.2
Triamcinolone acetonide	49.4	1

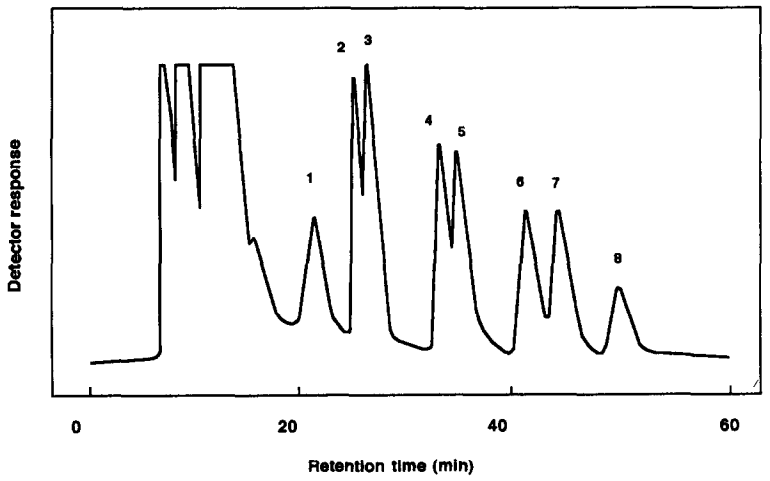

Fig. 6. Chromatogram of CDB derivatives. Concentration of steroid used: 100 ng/ml. Peaks: 1 = aldosterone; 2 = cortisone; 3 = cortisol; 4 = dexamethasone; 5 = corticosterone; 6 = fluocinolone acetonide; 7 = triamcinolone; 8 = triamcinolone acetonide.

noise ratio of 3). Calibration graphs were linear up to 5000–10000 ng/ml. The relative standard deviations (n = 6) were about 6.0% at 10 ng/ml of each eight steroids. The proposed HPLC method is 5–5000 times more sensitive than other HPLC methods using photometric detection [8–12] and HPLC methods using pre-column fluorimetric derivatization [13–16]. This is especially important for dexamethasone, triamcinolone and triamcinolone acetonide, which are not detectable by other HPLC methods with pre-column fluorimetric derivatization [13–16]. The efficiency of the derivatization was studied with dexamethasone by comparing the detector response given by the isolated reaction product. The conversion was 46%.

Determination of dexamethasone, triamcinolone and triamcinolone acetonide in plasma

Dexamethasone, triamcinolone and triamcinolone acetonide are synthetic corticosteroids that are used as anti-inflammatory drugs [3–6, 8–10].

TABLE V

RECOVERY TEST ON THE APPLICATION TO THE DETERMINATION OF STEROID

Plasma (100 μl) was spiked at each indicated concentration. Average values were obtained from six runs.

Steroid	Expected concentration (ng/ml)	Found concentration (mean ± S.D.) (ng/ml)	n	Relative standard deviation (%)
Dexamethasone	1.0	0.8 ± 0.1	6	13.2
	10.0	9.5 ± 1.1	6	11.1
	100.0	94.8 ± 5.0	6	5.1
Triamcinolone	1.0	0.8 ± 0.2	6	14.9
	10.0	9.6 ± 0.8	6	7.8
	100.0	91.3 ± 4.5	6	4.8
Triamcinolone acetonide	1.0	0.9 ± 0.1	6	13.6
	10.0	9.0 ± 0.9	6	8.7
	100.0	94.8 ± 6.6	6	6.6

A sensitive and selective method is required for monitoring the concentration of dexamethasone, triamcinolone and triamcinolone acetonide in plasma and tissue samples. Several GC–MS [3,4], RIA [5,6], and HPLC with photometric detection [8–10] techniques have been employed for monitoring drug level of dexamethasone, triamcinolone and triamcinolone acetonide. However, the HPLC methods are not sufficiently sensitive for the determination of low concentrations of these compounds in plasma or tissue extracts. The proposed HPLC method with CDB was therefore applied to the determination of dexamethasone, triamcinolone, triamcinolone acetonide in plasma.

Recovery test. The recoveries of 0.1–10 ng (1.0–100 ng/ml) of three steroids added to 100 μl of plasma are shown in Table V. The average recovery (n = 6) was 90.2%. The within-day relative standard deviations (n = 6) were 7.8–11.1%, and day-to-day relative standard deviations (n = 6) were 7.0–10.4% at 10 ng/ml of three steroids. The detection limits of each steroid were 0.06–0.3 pg per 100 μl of plasma (signal-to-noise ratio of 3).

The proposed CDB method was found to be useful for the separation and determination of eight corticosteroids at 0.06–0.3 pg per 100 μl of plasma. Further application of this method to monitoring drug levels in plasma and tissue samples from dexamethasone-dosed patients is in progress.

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