Measurement of Diethylstilbestrol in Plasma from Patients with Cancer of the Prostate

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ABSTRACT

A specific radioimmunoassay has been developed for diethylstilbestrol (DES), using an antiserum raised against DES monocarboxymethyl ether and a tritium-labeled radioligand. Prior to radioimmunoassay, a fraction enriched in DES is obtained from a dichloroethane extract of plasma using Sephadex LH-20. The specificity of the assay is good, and the sensitivity (130 pg/ml) is adequate for accurate determination of DES in plasma from prostatic cancer patients treated with the drug. The precision is satisfactory, with an interassay coefficient of variation of approximately 10% at concentrations of approximately 1 ng/ml, and the blank values are negligible. Excellent agreement ($r = 0.96$) is observed between data obtained by radioimmunoassay and those obtained by a procedure using gas chromatography-high-resolution mass spectrometry. DES concentrations in the plasma of six treated (1 mg DES three times daily) patients were in the range 0.15 to 6.0 ng/ml. Increases in plasma concentration were observed within 2 hr of administration, with secondary rises occurring 5 to 6 hr later. Plasma testosterone concentrations were low in four of the patients; in a single subject, relatively high levels of testosterone were further elevated following administration of lutetizing hormone-releasing hormone.

INTRODUCTION

Although there is still controversy as to the most effective form of therapy for early prostatic cancer and on the relative merits of surgery and radiotherapy (16), it has long been accepted that endocrine therapy, generally in the form of DES (3), is the most effective for the management of the advanced progressive disease. There is still discussion concerning the precise mode of action of DES (10), though it is generally accepted that its principal effect is indirect, decreasing testicular synthesis and secretion of testosterone by inhibiting lutetizing hormone secretion from the pituitary. There is, however, evidence for a direct action of DES on the testis and on the prostate gland (6), and it is possibly this latter effect which prompted the use of large doses (>200 mg/day) of DES phosphate (Honvan) for the treatment of prostatic cancer. Endocrine studies of the type undertaken by this Institute (11), however, have clearly indicated that a dose of 1 mg DES tds effectively decreases plasma testosterone levels and does not result in concomitant increases in the concentrations of prolactin and growth hormone. Such observations, and the concern over side effects (3), have tended to direct clinicians towards treatment schedules using low-dosage DES.

Despite its extensive use, there is no information on the levels of DES in patients being successfully treated or in those who apparently fail to respond to such therapy. Initially, 70% of treated patients respond clinically to DES therapy, but the majority eventually relapse. It remains to be determined whether this is related to a change in the hormone sensitivity of the tumor resulting from dedifferentiation or to changes in the pharmacokinetics of DES in certain patients. RIAs with the potential to measure the plasma concentrations of DES have been described (2), but no data on the levels in patients have been reported. Here we report a sensitive RIA for DES and illustrate its application to the determination of concentrations in the plasma of patients receiving 1 mg tds.

MATERIALS AND METHODS

Materials. [monoethyl-$^3$H]DES (50 to 100 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, United Kingdom, and stored in either benzene or toluene solution (50 μCi/ml) at −20°. For use in the assay, an aliquot was taken, the solvent was evaporated under nitrogen, and the residue was dissolved in assay buffer (see below) containing antiserum. The working solution contained 35 to 40 pg/100 μl (about 0.125 μCi/ml). Nonradioactive DES, hexestrol, and various steroids were purchased from Sigma London Chemical Company, Poole, Dorset, United Kingdom; the sodium salt of DES diphosphate (Honvan) was obtained from Ward Blenkinsop & Co., Ltd., London, United Kingdom; ω-hydroxydiestrol, stored as the triacetate, and β-dienestrol were a gift from Dr. M. Metzler, Institut für Pharmakologie und Toxikologie der Universität Wurzburg, Wurzburg, West Germany; DES monoglucuronide and DES diglucuronide were kindly provided by Dr. K. Krohn, Institut für Organische Chemie und Biochemie, Hamburg, West Germany. Solutions of these compounds were stored in ethanol (1 mg/10 ml) at 4°. Dichloroethane, purchased from Koch-Light Laboratories, Ltd., Colnbrook, Buckinghamshire, United Kingdom, and dichloromethane from BDH Chemicals, Poole, Dorset, United Kingdom, were used without further purification. Ethanol and methanol were obtained from James Burroughs, Ltd., London. A solution of 0.01 M phosphate buffer (pH 7.4) containing 0.9% sodium chloride and 0.1% sodium azide was stored at 4°; the assay buffer additionally included gelatin (0.1%). Dextran-coated charcoal was prepared by dissolving dextran T-70 (0.06%), Pharmacia Ltd., Sweden, in ethanol (3 mg/10 ml). The suspension was stirred at 4° for 2 hr before use. Sephadex LH-20 from Pharmacia,
Lipidex 5000 (Packard, Downers Grove, Ill.) was converted to the triethylaminohydroxypropyl derivative by the procedure of Axelson and Sjövall (4). Lipid scintillant was prepared by dissolving PPO (5 g) in toluene (1 l) and Triton X-100 (500 ml). Other chemicals were Analar grade (BDH Chemicals). All glassware was washed overnight in detergent, rinsed, and soaked in sodium chromate-sulfuric acid before being rinsed in deionized water and dried.

**Patients.** Blood was collected from 6 patients with histologically proven carcinoma of the prostate using an indwelling catheter over periods of approximately 8 hr (5 patients) or 16 hr (one patient). Five of the patients had received DES, 1 mg tds, for at least 3 months prior to the study. The sixth patient was investigated 6 days, 3 months, and 6 months after commencement of therapy. They were hospitalized for 48 hr and ate, slept, and drank as appropriate. DES was administered to 200 pg DES per 10 fil were prepared in ethanol. Triplicate aliquots and methanol (500 ,«l) was added.

**Chromatography** to correct for column blank effects. Aliquots (3 ml) were measured through a short column of Sephadex LH-20 (9).

**Extraction from Plasma.** Dichloroethane (3 ml) was added to plasma (0.5 ml). The tubes were shaken for 12 min and centrifuged (2.5 x 103 rpm) for 2 min, and 2 ml of the lower dichloroethane layer were removed to a clean tube. The solvent was evaporated under nitrogen.

**Septadex LH-20 Chromatography.** Each extract was dissolved in the chromatography solvent (dichloromethane:methanol, 94:6, v/v; 2 x 0.2 ml) and applied to a column (4 x 0.6 cm) of Sephadex LH-20, swollen in the same solvent. The first 3.0-ml eluate was discarded, and the DES fraction was collected in the next 3.0 ml. The fraction was dried and dissolved in methanol (500 µl). The reproducibility of the chromatographic separation was improved by the addition of a layer (1 to 2 mm) of sand to the top of each Sephadex LH-20 column. Replicate experiments using [3H]DES indicated excellent reproducibility: mean recovery was 96.3 ± 2.9% (n = 6). Eluate (30 ml) was collected from a large column (30 x 1.3 cm) containing swollen Sephadex LH-20. This eluate was used in the preparation of the standard curve to correct for column blank effects. Aliquots (3 ml) were measured into tubes, one for each point on the standard curve, and dried, and methanol (500 µl) was added.

**RIA.** Triplicate aliquots (100 µl) of the methanolic solution were dried under N2. Antiserum:radioiodide solution (200 µl) was added, and the mixture was incubated (37°C; 2 hr). For the standards, solutions of 20 to 200 pg DES per 10 µl were prepared in ethanol. Triplicate aliquots (10 µl) were mixed with aliquots (100 µl) of column solvent residue in methanol, dried, and treated as the samples. After equilibration, the free and bound fractions were separated by addition of a suspension of dextran-coated charcoal (1 ml) to all tubes except those used for determination of total counts, to which assay buffer (1 ml) only was added. The solutions were mixed, allowed to stand for 10 min, and centrifuged (12 min; 2.5 x 103 rpm). The supernatant was decanted into vials, scintillant (6.5 ml) was added, and the radioactivity was measured for 2 min; the tubes used for determination of total counts contained approximately 10,000 cpm. Plasma concentrations were calculated using a Hewlett-Packard 9810 A programmable desk-top calculator. The program used a rectangular hyperbola fit to the standard curve. Recoveries were monitored in preliminary assays by adding an internal standard of radioactive DES to the plasma aliquot before extraction. Recovery of DES (after allowance for the use of aliquots in the assay) was calculated to be 87.7% ± 6.8%; this was considered adequate, and addition of the internal standard was discontinued. Correction for recovery was not included in the calculation.

**Analyses by GC-MS.** A solution of DMS in drug-free plasma was prepared by addition of 50 µl of an ethanol solution (10 µg/ml) of DMS to 10 ml of plasma. After mixing and standing overnight, 50 µl were added as internal standard to each aliquot (0.25 to 1 ml) of plasma to be analyzed. The plasma was extracted with diethyl ether (8 ml), the aqueous layer was frozen, and the extract was removed and dried under a stream of nitrogen. The residue was dissolved in 3 x 100 µl methanol:water:chloroform (80:30:15, v/v; Solvent A) and applied to a column (2 x 0.6 cm) of triethylaminohydroxypropyl-Lipidex 5000 (4), swollen in Solvent A. Polar constituents were removed by elution with 2 ml of Solvent A. Nonpolar constituents were subsequently eluted with 2 ml of methanol:chloroform (80:15, v/v; Solvent B). Finally, DES and DMS, together with naturally occurring weakly acidic components, were recovered by elution with 2 ml of Solvent B, acidified by the addition of a small quantity of solid carbon dioxide. The final column eluate was dried under nitrogen, and the DES and DMS were converted to the TDBMS derivatives by reaction with tert-butylidimethylchlorosilane:imidazole:dimethylformamide (1:1:6, v/v; Applied Science Laboratories, State College, Pa.) Excess reagent was removed by filtration through a short column of Sephadex LH-20 (9).

Standard mixtures of DES and DMS for the preparation of the standard curve were obtained in 2 ways. First, aliquots of stock solutions of DES and DMS in ethanol were mixed in appropriate proportions. Second, solutions of DES in plasma were prepared, and appropriate aliquots were diluted to 1 ml with drug-free plasma to give total amounts of DES equal to those in standard mixtures prepared from stock ethanolic solutions. The plasma samples were then treated as for samples of unknown DES concentration.

GC-MS was performed using a Varian MAT 731 double-focusing instrument. Separations were achieved on a glass column (length 3 m, inside diameter 3.5 mm) packed with 1% OV-1 on Gas-Chrom Q (100 to 120 mesh) at 280°C. The mass spectrometric resolution was 8000. The ion source temperature was 200°C, and the electron energy was 70 eV. Analyses were carried out in the single-ion monitoring mode with sequential detection of ions of m/z 468.2880 for cis- and trans-DMS bis-TDBMS ether and m/z 496.3193 for cis- and trans-DES bis-TDBMS ether. (Each mass:charge ratio corresponded to the respective

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>% of cross-reactiona</th>
</tr>
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<tbody>
<tr>
<td>DES</td>
<td>100</td>
</tr>
<tr>
<td>β-Diestanol</td>
<td>0.25</td>
</tr>
<tr>
<td>ω-Hydroxydienestrol</td>
<td>0.10</td>
</tr>
<tr>
<td>meso-Dihydroxybutylstilbestrol</td>
<td>0.06</td>
</tr>
<tr>
<td>Hexestrol</td>
<td>10.9</td>
</tr>
<tr>
<td>DES monoglucuronide</td>
<td>67.4</td>
</tr>
<tr>
<td>DES diglucuronide</td>
<td>7.8</td>
</tr>
<tr>
<td>DES diphosphate (Horvan)</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.44</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;0.04</td>
</tr>
</tbody>
</table>

* Determined according to the criteria of Abraham (1).
molecular ion.) The ratio (R) of peak areas corresponding to the DES and DMS derivatives were determined using the expression $R = (HT_{CDES} + HT_{DDES})/(HT_{CDMS} + HT_{TDMS})$, where $HT_{CDES}$ is the product of the height of the peak attributed to cis-DES TBDMS and the gas chromatographic retention time (which is proportional to peak width) of that component. Values for peak area ratios were converted to DES concentrations by reference to the standard curves derived as described. Linear regression of ratio values derived from analyses of "spiked" plasma samples (y) on values derived from analyses of standard mixtures (x) gave the equation $y = 1.067x - 0.038$ ng/ml (correlation coefficient, $r = 0.997$), indicating excellent agreement between the 2 methods and confirming the ability of the assay procedure to determine accurately the concentrations of DES in plasma supplemented with the drug. The standard curve derived from mixtures of aliquots of stock ethanolic solutions of DES and DMS was used for the determination of unknown concentrations.

RESULTS

Characteristics of the RIA. The cross-reactions of the anti-serum (used at a final dilution of 1:8000) are listed in Table 1. There is a negligible degree of cross-reaction with the naturally occurring steroids and only a small degree of cross-reaction with the major metabolites (13) β-dienestrol and ω-hydroxydienestrol, indicating acceptable specificity for the hex-3-ene substructure of DES. The degree of cross-reaction with the glucuronides of DES is high, but, since an extraction step is included in the analytical procedure, no glucuronides are expected in the RIA. The cross-reaction with hexestrol (11%), although relatively high, is clinically irrelevant.

A typical standard curve for the DES assay, over the range of 0 to 200 pg, with a corresponding precision profile, is shown in Chart 1. Addition to the standards of column solvent eluate, in methanol, decreases the binding by approximately 8% and is a necessary part of the procedure. The sensitivity of the assay, calculated using the expression $ts/>/N$ (5), gave a curve value of 8.8 pg corresponding to 0.132 ng/ml. The accuracy of the method, determined by repeatedly measuring 3 pools of plasma, spiked with known amounts of DES, was similar for high, medium, and low values (Table 2). Assay precision, determined by calculating within-assay and between-assay coefficients of variation for 3 quality control plasma pools, is shown in Table 3.

Comparison of Analytical Data from RIA and GC-MS. Twelve plasma samples from patients treated with DES, representing a spread of concentrations across the range observed in this study, were analyzed by both RIA and GC-MS procedures (Chart 2). Linear regression of RIA values (y) on GC-MS results (x) gave the equation $y = 0.878x + 0.051$ (correlation coefficient, $r = 0.96$; if a single outlier is omitted). Agreement between the methods was thus excellent, particularly since no allowance for recovery was made in the RIA. The single discrepancy when the DES concentration was high may suggest incomplete extraction in the RIA in this example. With

Table 2

<table>
<thead>
<tr>
<th>Added (ng/ml)</th>
<th>Measured (ng/ml)</th>
<th>n</th>
<th>% of recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00</td>
<td>1.69</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>1.17</td>
<td>0.97</td>
<td>11</td>
<td>83</td>
</tr>
<tr>
<td>0.60</td>
<td>0.53</td>
<td>16</td>
<td>88</td>
</tr>
</tbody>
</table>

* Number of replicate analyses.

Table 3

<table>
<thead>
<tr>
<th>Plasma pool</th>
<th>Intraassay CV (%)</th>
<th>Interassay CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>10.3</td>
<td>11.7</td>
</tr>
<tr>
<td>Medium</td>
<td>11.4</td>
<td>10.0</td>
</tr>
<tr>
<td>Low</td>
<td>19.3</td>
<td>19.3</td>
</tr>
</tbody>
</table>

* Number of replicate analyses.

a CV, coefficient of variation.

b Mean ± S.D.
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Few samples of relatively high concentration, it has not, however, been possible to evaluate this further.

**DES Levels in Treated Patients with Prostatic Cancer.** Plasma samples from 6 patients were assayed for levels of DES and testosterone. Patient F. C. showed an expected marked rise in the concentration of DES approximately 1.5 to 2.0 hr after DES administration (1-mg tablet; 6:30 a.m.) and a less significant rise 6 hr later (Chart 3). The first peak was also observed on Day 2. Similar patterns of DES concentration were observed with Patient J. W. (Chart 4) and 3 others not illustrated.

Plasma testosterone concentrations in Patient F. C. (Chart 3) were higher than expected after DES therapy for several months. The circadian rhythm (18) suggests a substantial adrenal contribution to the plasma androgen level, although in this patient an LH-RH stimulation test also resulted in a significant elevation in the concentration of plasma testosterone (Chart 3), indicating a responsive pituitary-testicular axis. Low levels of testosterone were found in Patient J. W. (Chart 4) and in the 3 other patients studied; there was no response in any of these to the administration of LH-RH.

Plasma DES concentrations in a single patient (A. F.) throughout the night, 6 days, 3 months, and 6 months after commencing therapy are shown in Chart 5. The rise in DES levels after the 6:30 a.m. DES tablet was the same at each period. Rises in DES concentration were in each case observed 4 to 6 hr after administration of the 5 p.m. tablet, presumably corresponding to the secondary rises noted above.

**DISCUSSION**

The development of this routine RIA for DES will permit studies of the relationships between DES concentration in plasma, hormone secretion, and clinical response of treated prostatic cancer patients. The assay described has satisfied accepted performance criteria and has been validated by reference to a highly specific GC-MS procedure. The antiserum displayed high specificity, although inclusion of a Sephadex LH-20 chromatography step in the procedure was essential to remove interfering plasma constituents.

The range of DES concentrations during the day in the 6 patients studied was 0.15 to 5.9 ng/ml. The observed second peak of DES concentration in plasma after the p.o. dose could arise when DES glucuronides are hydrolyzed in, or reabsorbed from, the large intestine approximately 5 hr after the tablet has been taken (14). Administration p.o. of [14C]DES glucuronide results in a rise in plasma radioactivity 3 to 6 hr later (8).

The data from Patient F. C. clearly indicate that, although treatment at the level of 1 mg DES tds is sufficient to suppress testicular activity in most patients (11), higher doses may be required for others. A responsive pituitary-testicular axis was demonstrated in Patient F. C., and furthermore, rhythmic se-
creatin of testosterone was observed. This testosterone profile may be related to the secretion of adrenal androstenedione, the precursor of plasma testosterone. These studies again illustrate the difficulties of analyzing single daily samples for the assessment of the endocrine status of patients with prostatic cancer (19). The development of a specific RIA procedure for the measurement of DES allows the investigation of plasma levels of the drug in relation to changes in the rhythmic secretion of hormones during treatment and will facilitate a study of patient variability in relation to response.

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REFERENCES


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