CHROM. 6351

Determination of steroids by densitometry of derivatives

III. Micro-assay of estrogens as DANSYL derivatives*

The fluorodensitometry of the 1-dimethylaminonaphthyl-5-sulfonyl (DANSYL) derivatives of estrogens has been described in preceding communications[1,2]. By direct evaluation of thin-layer chromatograms, as little as 5 ng of estrogen could be determined with adequate accuracy and precision. In order to increase the sensitivity of this method, the separation of the DANSYL derivatives of estrogens by thin-layer chromatography (TLC) and the experimental conditions used for the ensuing fluorodensitometry were modified as described in this paper.

Methods

Preparation and thin-layer chromatography of the DANSYL derivatives of estrogens. DANSYL derivatives of estrone [3-hydroxy-1,3,5(10)-estratrien-17-one], estradiol [1,3,5(10)-estratriene-3,17β-diol] and estriol [1,3,5(10)-estratriene-3,16α,17β-triol] were prepared as described in earlier publications[1-3]. The separation of the free estrogens and DANSYL derivatives could be achieved by thin-layer chromatography on silica gel, using pre-coated plates of different brands and various solvent systems (Table I), as well as on polyamide in acetone–methanol–water (2:8:2). The following pre-coated plates were tested: silica gel (Woelm, Eschwege, G.F.R.), Silica Gel G (No. 1500; Schleicher & Schüll, Dassel, G.F.R.), Silica Gel F_{254} (Merck AG, Darmstadt, G.F.R.) silica gel alufoil (Woelm), polyamide (No. 1600 G; Schleicher & Schüll) and polyamide alufoil (Merck AG). Pre-treatment of the silica gel layers by ascending chromatography with chloroform–ethanol (3:1) or of polyamide layers with methylene chloride–carbon tetrachloride–methanol (1:1:1) and subsequent activation for 1 h at 110° were found to eliminate interfering substances in the sorbent material and to improve the reproducibility of the R_F values. After the application of samples on 1-cm wide lanes and development in the mobile phase, the chromatograms were dried in air, followed by drying for 24 h over blue gel, spraying with isopropanol–triethylamine (4:1)[4], drying in air and then for 24 h over blue gel.

Fluorodensitometry of DANSYL derivatives. When estrogen DANSYL derivatives on thin-layer chromatograms were submitted to fluorodensitometry in an SD 3000 spectrodensitometer (Schoeffel Instruments Corp., Westwood, N.J., U.S.A.), a wavelength of 362 nm was selected for excitation of fluorescence, which was then measured at 517 nm. The peaks recorded were evaluated by triangulation and determination of the peak areas.

When using the TLD 100 instrument (Vitatron, Dieren, The Netherlands) for fluorodensitometry, a primary filter with maximum transparency at 364 nm and a U-2 secondary filter were chosen, the peaks being evaluated by the above procedure or by digital intergration.

* Part of the Thesis of L. P. Penzes at the Mathematisch Naturwissenschaftliche Fakultät, Universität des Saarlandes, D-66 Saarbrücken, G.F.R.

TABLE I

R_F VALUES OF ESTROGENS AND DANSYL DERIVATIVES ON SILICA GEL LAYERS

Systems: (A) chloroform–benzene (8:2); (B) chloroform–dioxan (94:6); (C) chloroform–dioxan (8:2); (D) carbon tetrachloride–dioxan (9:1); (E) cyclohexane–ethyl acetate (3:1); (F) chloroform–benzene–ethanol (18:2:1); (G) chloroform–dioxan–carbon tetrachloride (3:2:5). All proportions are v/v.

System	*R_F value*					
	Estrone		*Estradiol*		*Estriol*	
	Free	*DANSYL*	*Free*	*DANSYL*	*Free*	*DANSYL*
A	0.15		0.07		0.00	
B	0.55	0.70	0.29	0.48	0.02	0.04
C	0.60	0.73	0.48	0.58	0.16	0.14
D	0.14		0.07		0.00	
E	0.44	0.16	0.17	0.076	0.02	0.00
F	0.48	0.65	0.34	0.50	0.05	0.11
G	0.55		0.46		0.13	

TABLE II

FLUORODENSITOMETRY OF THE DANSYL DERIVATIVE OF ESTRIOL ON SILICA GEL IN THE SD 3000 INSTRUMENT

Amount of estriol (ng)	*Peak area (cm^2)*			
	A[a]	*B*[b]	*C*[c]	*D*[d]
Silica gel (Woelm)				
1.0				
2.5				0.45
5.0	0.62	0.50	0.95	1.10
7.5	1.34	1.00	2.10	2.52
10.0	1.60	1.25	2.90	3.10
25.0	3.78	2.72	7.75	7.85
Silica Gel G (No. 1500; Schleicher & Schüll)				
1.0				
2.5				0.72
5.0		0.27	0.85	1.26
7.5	0.60	0.47	1.44	2.15
10.0	0.72	0.69	1.98	2.52
25.0	1.92	1.95	5.95	6.55
Silica Gel F_{254} (Merck)				
1.0				
2.5	Not detectable			Not measured
5.0				
7.5			1.15	
10.0			1.40	
25.0			4.45	
Silica gel, alufoil (Woelm)				
1.0				
2.5	Not detectable			Not measured
5.0			1.10	
7.5			1.44	
10.0			1.89	
25.0	0.66	0.64	4.90	

[a] A = after drying the chromatogram in air.
[b] B = after drying for 24 h over blue gel.
[c] C = after spraying with isopropanol–triethylamine (4:1) and drying in air.
[d] D = after drying treated chromatograms for 24 h over blue gel.

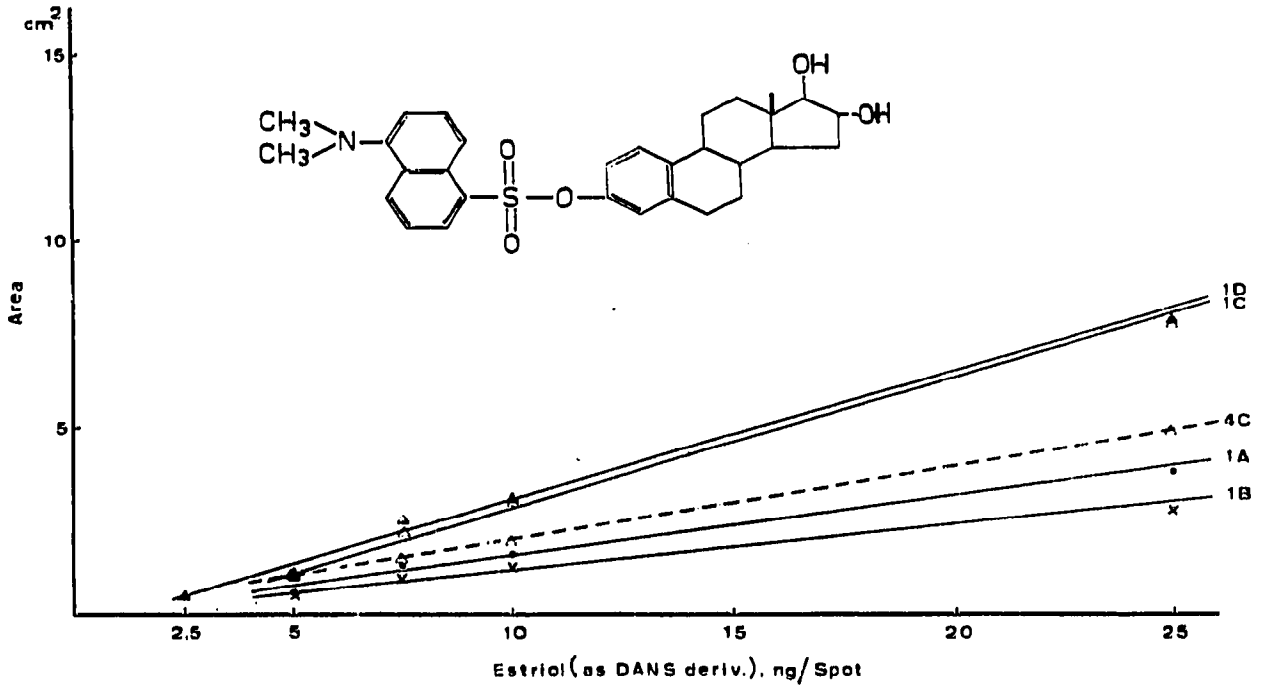

Fig. 1. Calibration curves obtained by fluorodensitometry in the SD 3000 instrument of DANSYL derivatives on silica gel (Woelm) before and after treatment with isopropanol–triethylamine (see Table II). 1A = after drying the chromatogram in air; 1B = after drying for 24 h over blue gel; 1C = after spraying with isopropanol–triethylamine and drying in air; 1D = after drying treated chromatogram for 24 h over blue gel; 4C = as 1C.

TABLE III

FLUORODENSITOMETRY OF THE DANSYL DERIVATIVE OF ESTRIOL ON POLYAMIDE IN THE SD 3000 INSTRUMENT

Amount of Estriol (ng)	*Peak area (cm²)*			
	A[a]	*B*[b]	*C*[c]	*D*[d]
Polyamide No. 1600 G (Schleicher & Schüll)				
1.0	0.60			
2.5	1.62	1.52	1.98	
5.0	3.90	3.12	4.18	
7.5	5.50	5.05	5.82	
10.0	8.30	6.40	8.40	
25.0	12.40	16.00	15.10	7.50
Polyamide alufoil (Merck)				
1.0	0.52	0.49	0.58	0.49
2.5	1.95	1.82	1.89	1.60
5.0	4.10	3.40	3.80	2.96
7.5	5.80	5.10	5.92	4.80
10.0	7.55	6.80	7.80	5.25
25.0	16.80	12.70	16.10	13.70

[a] A = after drying the chromatogram in air.
[b] B = after drying for 24 h over blue gel.
[c] C = after drying for 72 h in air.
[d] D = after spraying with isopropanol–triethylamine (4:1, v/v) and drying in air.

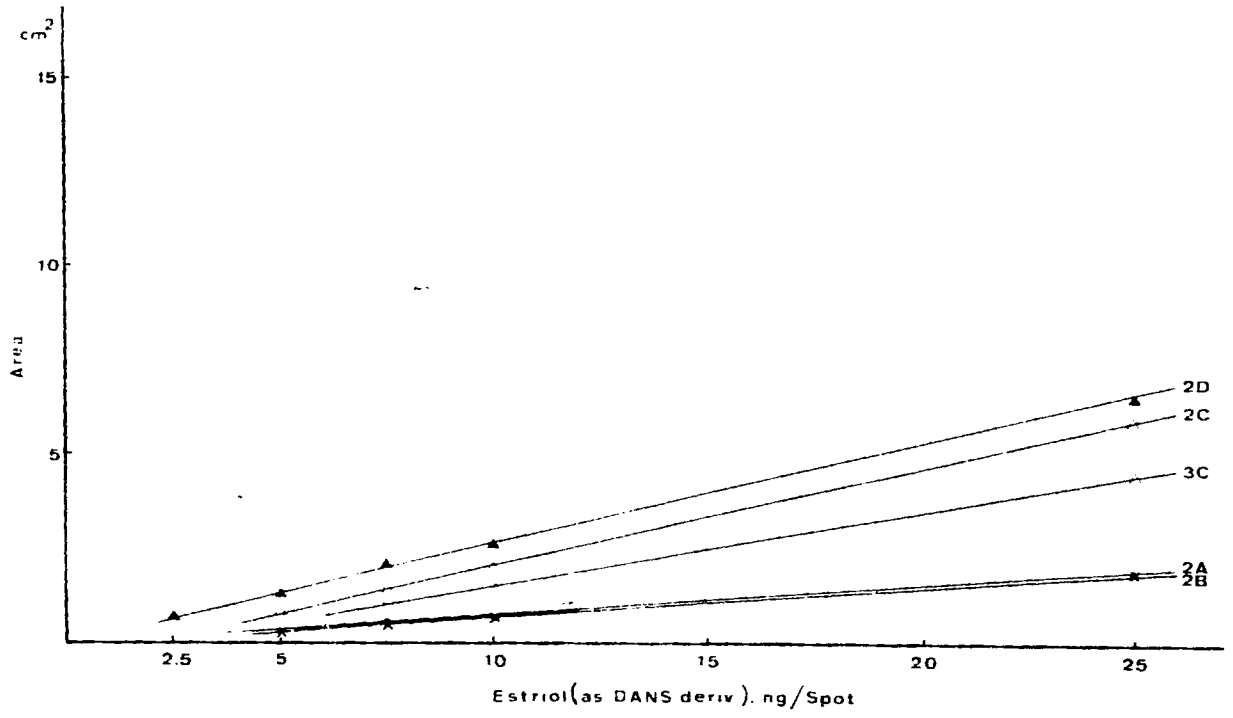

Fig. 2. Calibration curves obtained by fluorodensitometry in the SD 3000 instrument of DANSYL derivatives on Silica Gel G No. 1500 (Schleicher & Schüll) (see Table II). 2A = after drying the chromatogram in air; 2B = after drying for 24 h over blue gel; 2C = after spraying with isopropanol-triethylamine and drying in air; 2D = after drying treated chromatogram for 24 h over blue gel; 3C = as 1C or 2C.

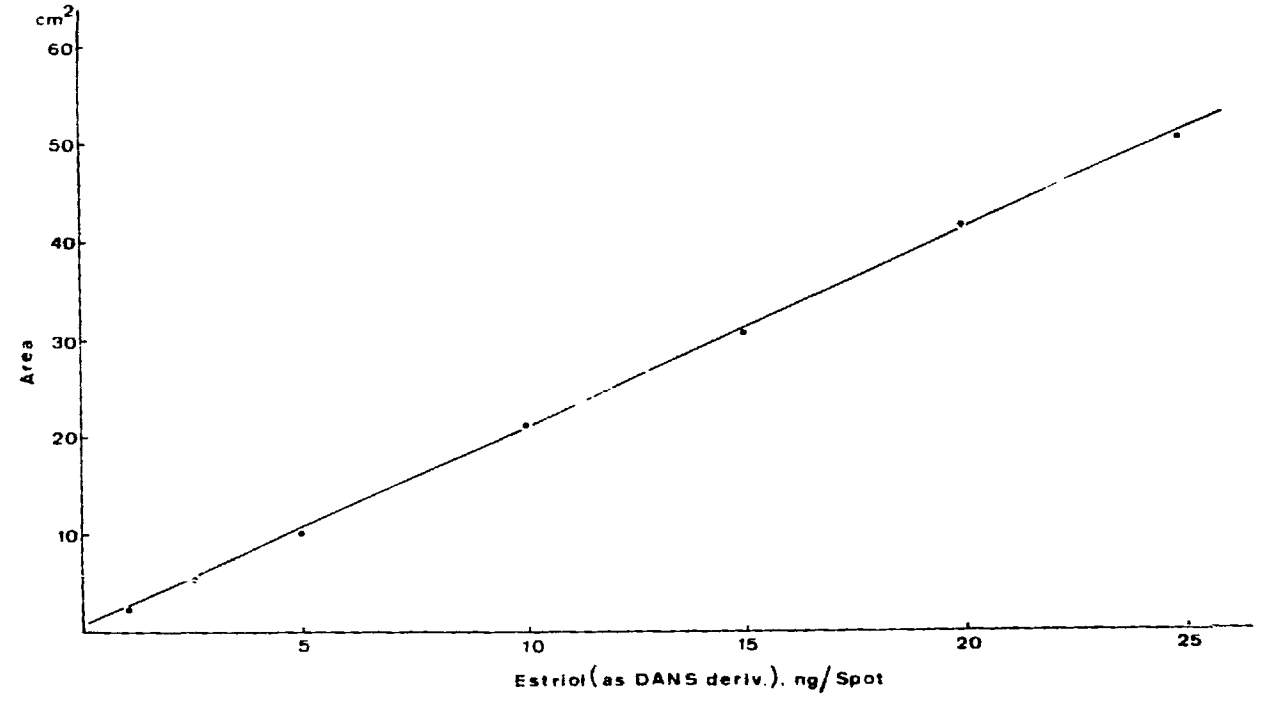

Fig. 3. Calibration curve for the micro-assay of the DANSYL derivatives of estrogens by fluorodensitometry on polyamide in the SD 3000 instrument (see Table III).

TABLE IV

MICRO-ASSAY OF THE DANSYL DERIVATIVE OF ESTRIOL BY FLUORODENSITOMETRY ON POLYAMIDE IN THE SD 3000 INSTRUMENT

Sensitivity setting of the instrument = 0.1.

Amount of estriol (ng per spot)	*Peak area (cm^2) and standard deviation from the mean*	
	n[a]	*Mean ± standard deviation (%)*
1.0	6	2.4 ± 5.0
2.5	6	5.4 ± 5.2
5.0	5	10.2 ± 4.7
10.0	6	21.0 ± 3.9
15.0	6	31.2 ± 3.5
20.0	6	40.8 ± 3.4
25.0	6	50.1 ± 3.1

[a] n = number of determinations.

Results and discussion

It can be seen from Table I that satisfactory resolution of the DANSYL derivatives of estrogens can be achieved by thin-layer chromatography on silica gel. When 1–25 ng of the DANSYL derivative of estriol were chromatographed on different pre-coated plates in chloroform–benzene–ethanol (18:2:1), direct measurement of fluorescence in the SD 3000 instrument yielded the results presented in Table II. The corresponding calibration curves, shown in Figs. 1 and 2, indicate a linear relationship between the recorded fluorescence and the concentration of the DANSYL derivative of estriol used. Whereas prolonged drying of the chromatograms over blue

TABLE V

DETERMINATION OF THE DANSYL DERIVATIVES OF ESTRIOL AND ESTRONE ON SILICA GEL BY USE OF THE TLD 100 INSTRUMENT

Amount (ng)	*Digital integral units*		*Peak area (cm^2)*	
	n[a]	*Mean ± standard deviation (%)*	n[a]	*Mean ± standard deviation (%)*
Estriol				
0.005			3	0.21
0.010			3	1.36
0.025			3	3.66
0.050			3	7.75
0.100	6	22 ± 2.7	6	0.52 ± 3.5
0.250	6	63 ± 2.7	6	1.24 ± 3.4
0.500	6	128 ± 2.4	6	2.47 ± 2.9
0.750	5	194 ± 2.4	6	3.70 ± 2.6
1.000	6	259 ± 2.3	6	4.86 ± 2.6
Estrone				
0.10	6	87 ± 3.2	6	1.30 ± 3.4
0.25	6	309 ± 3.0	6	4.96 ± 2.9
0.50	6	667 ± 2.9	6	11.40 ± 2.7
0.75	5	1032 ± 2.9	5	18.04 ± 2.4
1.00	6	1386 ± 2.7	6	24.42 ± 2.2

[a] n = number of determinations.

gel caused a noticeable decrease in fluorescence, treatment of chromatograms with isopropanol–triethylamine and drying over blue gel more than doubled the emitted fluorescence, thus increasing the sensitivity of the assay to approximately 1 ng.

From Table III it is evident that in the thin-layer chromatography of the same amounts (1–25 ng) of the DANSYL derivative of estriol on different pre-coated polyamide plates and the fluorometric determination of the derivative in the SD 3000 instrument, as little as 0.5 ng of steroid was equivalent to *ca.* 0.5 cm^2 of peak area. In contrast to fluorodensitometry on silica gel, the treatment of polyamide layers with isopropanol–triethylamine after chromatography of the derivatives led to a significant decrease in the fluorescence. A linear dependence of fluorescence on steroid concentration was found for the range 1–25 ng (Fig. 3). The precision of such assays is shown by the results of multiple determinations (Table IV). At a concentration of 1 ng of the DANSYL derivative of estriol per spot, the standard deviation from the mean was only ± 5.6%. Hence, the fluorodensitometry of the DANSYL derivatives of estrogens on polyamide appears to be well suited to the determination of such steroids in small volumes of non-pregnancy urine or even in peripheral plasma[5].

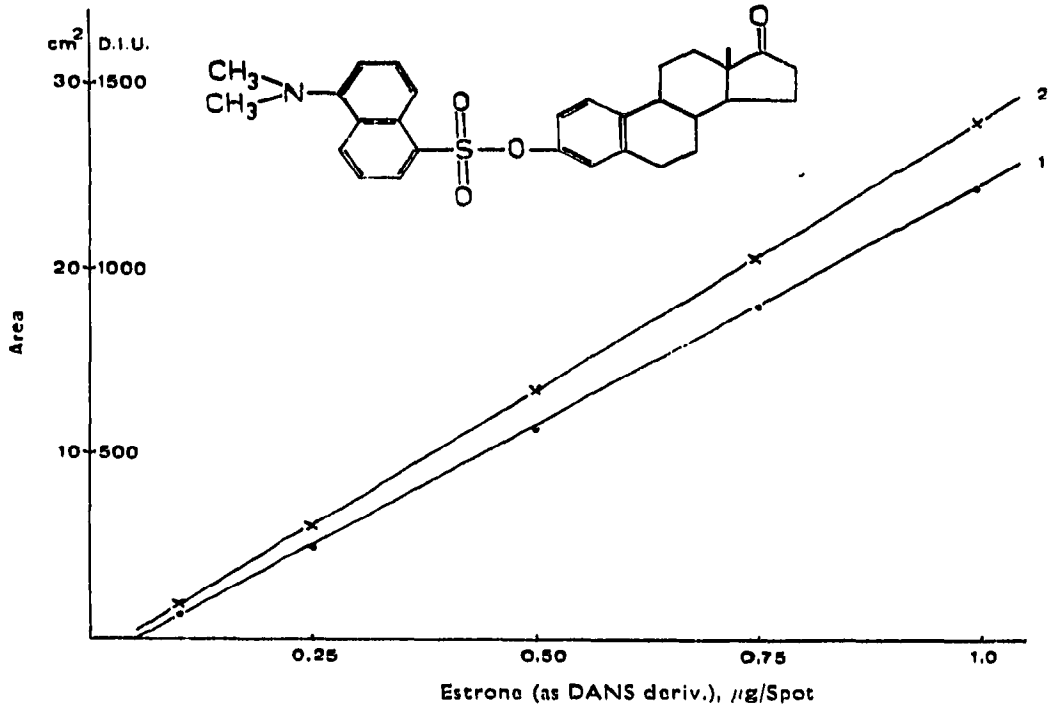

Fig. 4. Calibration curves for the determination of the DANSYL derivative of estrone by fluorodensitometry on Silica Gel G No. 1500 (Schleicher & Schüll) in the TLD 100 instrument (see Table V). 1 = Evaluation by triangulation ($H \times B(1/2\ H)$, where H = extended peak height and B $(1/2\ H)$ = peak-width at $1/2\ H$); 2 = evaluation by digital integration of curves.

Similar results were obtained for the fluorodensitometry of 5–1000 ng of the DANSYL derivative of estriol and 100–1000 ng of the derivative of estrone on precoated Silica Gel G (No. 1500) plates following ascending chromatography in chloroform–benzene–ethanol (18:2:1) and measurement in the TLD 100 instrument (Table V). Again, a linear dependence of the recorded fluorescence on the concentra-

tion of the DANSYL derivative was observed, whether the evaluation was carried out by triangulation of the peaks or by direct integration in the instrument (Fig. 4).

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