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Direct quantitative digital autoradiography–thin-layer chromatography of 3α,3β- and 5α-reduced and 17β-dehydrogenated androgens derived from testosterone metabolism

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ABSTRACT

A digital autoradiographic–thin-layer chromatographic method involving simple steps is described for thorough separation of eight major androgens (testosterone, androstenedione, dihydrotestosterone, androsterone, epiandrosterone, androstanedione, 3α-androstanediol and 3β-androstanediol) derived from testosterone metabolism. Their direct quantification is performed by radioscanning, which avoids tedious plate-scraping and liquid scintillation counting. Satisfactory accuracy is obtained by both external standardization of plates by calibrated amounts of radiolabelled standard steroids and internal standardization of bioassays by radiolabelled squalane. Coefficients of variation are below 8.5% in the range 100–500 dpm. Some analytical criteria related to chromatographic conditions and quantification parameters depending on position-sensitive proportional counter are discussed.

INTRODUCTION

Evaluation of 5α-reductase, 3α,3β-hydroxysteroid dehydrogenase and 17β-dehydrogenase has always been under active investigation for various purposes including skin metabolic disorders such as acne and baldness, drug effects and other dermatological problems [1–6]. Indeed, evaluation of testosterone metabolism by sebaceous glands and hair follicles is an obvious way to a better understanding of these features [7–14]. However, the difficulty of sampling quantities of human material, in addition to the time-consuming methods such as scraping off thin-layer chromatographic (TLC) plates followed by liquid scintillation counting (LSC), or sophisticated techniques such as high-performance liquid chromatography (HPLC) with on-line radiodetection coupling [15,16] which are not always sensitive enough and not easily transposable in routine, led us to develop a TLC method [17–21] that permits a complete separation of the major testosterone metabolites and their direct quantification by digital autoradiography (DAR) combined with TLC. The various steps and the application of this procedure to sebaceous gland metabolism are developed, in order to obtain reproducible, sen-

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sitive and specific quantification by both external calibration of plates and internal standardization of samples. Moreover, certain technical features, such as the preparation of radiolabelled standard steroids, derived from the methods previously described [22–24], are also developed. Details of chromatographic conditions and analytical criteria are given.

EXPERIMENTAL

Preparation of standards

Radioinert steroids, such as testosterone (T, 4-androstene-17β-ol-3-one), dihydrostestosterone (DHT, 5α-androstane-17β-ol-3-one), androstenedione (Δ4, 4-androstene-3,17-dione), androstanedione (ANSTAN, 5α-androstane-3,17-dione), androsterone (ANDRO, 5α-androstane-3α-ol-17-one), epiandrosterone (EPIA, 5α-androstane-3β-ol-17-one), 3α-androstanediol (3α-DIOL, 5α-androstane-3α,17β-diol), and 3β-androstanediol (3β-DIOL, 5α-androstane-3β,17β-diol) were supplied from Sigma (St. Louis, MO, USA). Squalane (SQUA, 2,6,10,15,19,23-hexamethyltetracosane) was obtained from Fluka (Buchs, Switzerland).

Commercially available ^{14}C-radiolabelled standards, such as [4-^{14}C]testosterone (50–60 mCi/mmol), [4-^{14}C]dihydrotestosterone (50–60 mCi/mmol) and [4-^{14}C]androstenedione (50–60 mCi/mmol), were purchased from Amersham (Cardiff, UK) and [11,12,13,14-^{14}C]squalane (109 mCi/mmol) from C.E.A. (Gif-sur-Yvette, France). All the standards were purified by TLC prior to use. [4-^{14}C]Androstanedione was prepared from [4-^{14}C]dihydrotestosterone by chromic oxidation, and [4-^{14}C]-3β-androstanediol by $NABH_4$ reduction of [4-^{14}C]dihydrotestosterone in methanol. [4-^{14}C]Androsterone, [4-^{14}C]epiandrosterone and [4-^{14}C]-3α-androstanediol were obtained by enzymic transformation of [4-^{14}C]androstanedione or [4-^{14}C]dihydrotestosterone incubated in the presence of minced fragments of human skin as a source of 3α,3β-hydroxysteroid dehydrogenase and 17β-dehydrogenase. Separation, purification and recovery of biotransformed [4-^{14}C]steroids were performed as described below. The specificity of [4-^{14}C]standards was confirmed by a process of acetylation and oxidation [25]. The radiopurity of each compound was controlled by DAR before storage at -20°C in the form of $1.5 \cdot 10^3$ dpm per 10-ml solutions in methanol, chloroform or cyclohexane, according to the polarity.

Incubation procedure

Microdissected human sebaceous glands (one to three), sheared from scalp skin biopsies, were incubated overnight in 100 μl of Dulbecco's buffer in the presence of $NADPH_2$ (2.4 m*M*) and 0.33 μCi [4-^{14}C]testosterone. Penicillin and streptomycin (0.1%) ensured sterile metabolism of the precursor. The same process was carried out for the preparation of standard [4-^{14}C]steroids, after adjustment of the incubation volume (5.0 ml) and the precursor amount (15.0

μCi) according to the amount of sliced or homogenized skin. For biological measurements, the reaction was stopped by introduction of 5 μg of each cold steroid. Concurrently, radiolabelled squalane was added as internal standard. Testosterone metabolites were extracted three times with 1.0 ml of diethyl ether, and organic phases were separated and then gently evaporated under a stream of nitrogen at 35°C. Previous controls showed that more than 95% of the radioactivity added was recovered whichever compound was extracted. Standard preparation incubation media were extracted twice with 20 ml of ethyl acetate–hexane–ethanol (7:2:1, v/v/v). After evaporation under vacuum, dried residues were submitted to preparative chromatography.

Chromatographic materials

Dichloromethane, diethyl ether, toluene and ethyl acetate were analytical grade (SBS, Peypin, France). Silica gel 60 F 254S plates (20 cm × 20 cm), with concentrating zone (4 cm × 20 cm) precoated for preparative TLC (thickness 2.0 mm), used for purification of standard [4-^{14}C]androstanedione and the first step in the preparation of enzymically prepared standard [4-^{14}C]steroids, were from Merck (Darmstadt, Germany). TLC plates (20 cm × 20 cm), coated with silica gel 60 F 254S, with concentrating zone (2.5 cm × 20 cm; layer thickness 0.25 mm) were used for the second step in the purification of [4-^{14}C]-3α,β-isomeric forms of 5α-reduced compounds and for any quantification purpose, related to the definition of analytical criteria and the evaluation of biological samples. Chromatographic separations were performed in 20 cm × 20 cm glass tanks with lateral channels (Desaga, Heidelberg, Germany).

Chromatographic procedures

Chemically prepared [4-^{14}C]androstanedione was chromatographed twice on preparative plates at room temperature in dichloromethane–diethyl ether (90:10, v/v) (system I). [4-^{14}C]-3β-Androstanediol was purified twice in system I, at 4°C, on a 0.25-mm TLC plate. [4-^{14}C]Androsterone and [4-^{14}C]epiandrosterone, which coeluted in system I on preparative plates, were extracted from silica gel, reconcentrated and chromatographed on a 0.25-mm TLC plate twice in the *y* and *x* directions in toluene–ethyl acetate (50:50, v/v) (system IIa). Analytical criteria and biological sample quantification were performed exclusively on 0.25 mm thickness plates by two methods: (1) double run, *y* direction, system I, 4°C, (up to ten spots per plate); (2) double run, *y* direction, system I, 4°C, then double run, *x* direction, system IIa, when single spot or system IIb (toluene–ethyl acetate, 70:30 v/v), 4°C, when two tracks were to be run.

Radiodetection equipment

The radio-TLC analyser employed in our studies was a Berthold LB 2840 linear analyser (Berthold, Wildbad, Germany) equipped with a high-resolution-head detector (LB 282/1) coupled with an HP Vectra ES12 computer allowing

detection, storage, reconstruction of bidimensional radiodistributions and their quantitative evaluation [26–30]. A Beckman LS1801 liquid scintillation counter was used for standardization of ^{14}C-radiolabelled steroid reference solutions.

RESULTS

Efficiency of separation of testosterone metabolites

System Ia does not achieve separation of [4-^{14}C]androsterone and [4-^{14}C]epiandrosterone (Fig. 1a). Complete separation of the eight [4-^{14}C]testosterone metabolites is easily obtained by a combination of system I and system IIa. After a double run in system I, [4-^{14}C]androstenedione and [4-^{14}C]testosterone are located under UV light and two x direction grooves are made in the silica gel, the first one as close as possible above the [4-^{14}C]testosterone spot, the second one as close as possible below the [4-^{14}C]androstenedione spot, in order to isolate [4-^{14}C]dihydrotestosterone and coeluted [4-^{14}C]androsterone and [4-^{14}C]epiandrosterone from other compounds. Silica gel is then removed from a 2.0-cm strip from outside the delimited central lane, allowing selective migration of [4-^{14}C]dihydrotestosterone, [4-^{14}C]androsterone and [4-^{14}C]epiandrosterone in system II. Fig. 1b shows the pattern of the metabolites separated. Under these conditions no overlapping is observed between [4-^{14}C]testosterone and [4-^{14}C]-3α,3β-androstanediols or between [4-^{14}C]androsterone and [4-^{14}C]epiandrosterone (Fig. 1c).

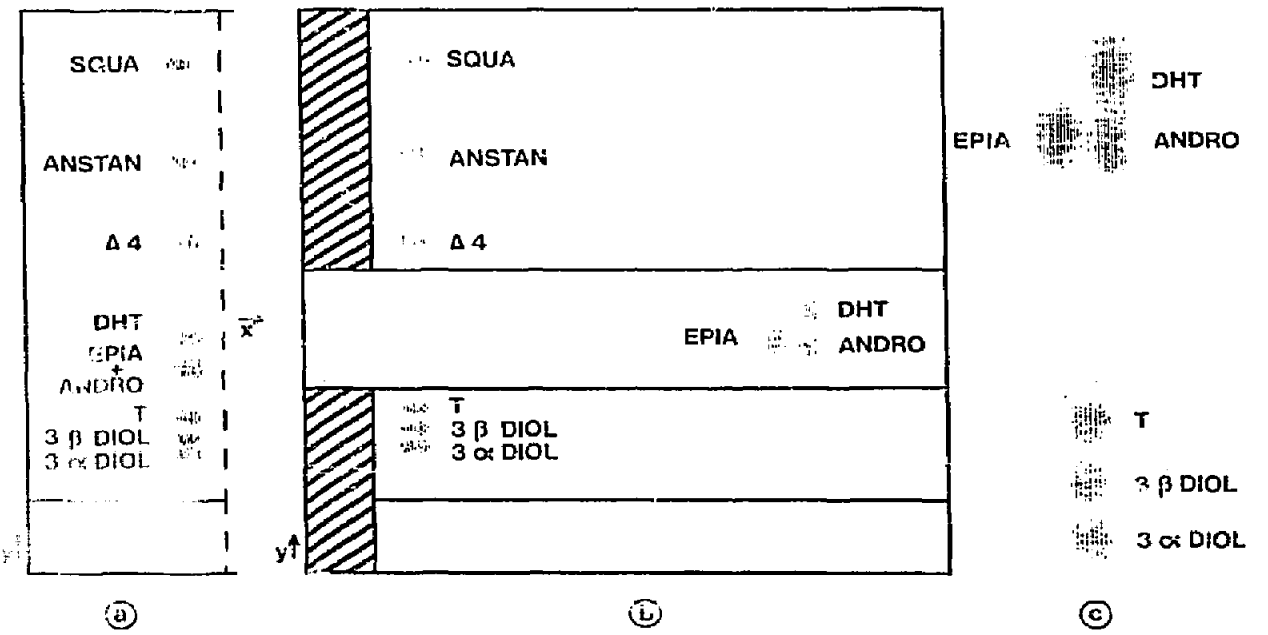

Fig. 1. Digital autoradiography (TLC) of standard 3α,3β- and 5α-reduced and 17β-dehydrogenated [4-^{14}C]steroids: (a) one-dimensional chromatography; (b) two-dimensional chromatography; (c) extension of 3α,3β-isomer migration areas. For abbreviations, chromatographic conditions and acquisition parameters, see text.

Analytical criteria

The reproducibility of direct radioscanning quantification was evaluated by intra-plate and inter-plate measurements of standard steroids. Acquisition was performed under the following conditions: gain, 2; step-width, 1.9 mm; slit-width, 2.0 mm; step number, 106; voltage, 1375 V; argon–methane (90:10) flow-rate, 600 cm^3/min; acquisition time, 6 h.

Intra-plate and inter-plate reproducibility. A mixture of standard [4-^{14}C]steroids and [11,12,13,14-^{14}C]squalane, $3 \cdot 10^3$ dpm each, was spotted six times on six plates, then chromatographed twice in system I at 4°C. [4-^{14}C]-3β-Androstanediol and [4-^{14}C]epiandrosterone were run independently. Quantitative measurements of each spot were performed after manual delimitation and automatic integration of radioactive areas. The method of calculation is given for one compound by the following equation:

$$RA = (RI_{cpmb} - BKG_1)/(IS_{cpmb} - BKG_2)$$

where RI is the region of interest, defined as the [4-^{14}C]steroid migration area, IS is region of interest, defined as the internal standard migration area, BKG is the background of the same surface as RI or IS and cpmb is counts per minute. RI, IS and BKG are from the same track.

Table I gives the coefficients of variation (C.V.) for each compound, calculated on six tracks from the same plate (Fig. 2). In no case does the C.V. exceed 6.9%. The average C.V., independent of compound specificity, is 6.03% (n = 6 plates, Table II). Table III shows that the C.V. calculated for the same compound from six different plates is much higher (16.7%). Consequently, inter-plate comparison is not valid if external calibration of each plate is not carried out beforehand.

TABLE I

INTRA-PLATE VARIABILITY OF RELATIVE DAR-TLC QUANTIFICATION OF STANDARD [4-^{14}C]STEROIDS CHROMATOGRAPHED IN SYSTEM I

IS = internal standard (SQUA); 3β-DIOL and EPIA were run independently. For abbreviations and chromatographic system composition, see text.

Compound	Mean ± S.D.	C.V. (%)
3α-DIOL/IS	1.194 ± 0.0589	4.93
3β-DIOL/IS	1.163 ± 0.0517	4.45
T/IS	1.365 ± 0.0577	4.23
ANDRO/IS	1.225 ± 0.0567	4.63
EPIA/IS	1.297 ± 0.0890	6.87
DHT/IS	1.299 ± 0.0458	3.53
Δ4/IS	1.379 ± 0.0833	6.04
ANSTAN/IS	1.600 ± 0.0757	4.73
Mean		4.93

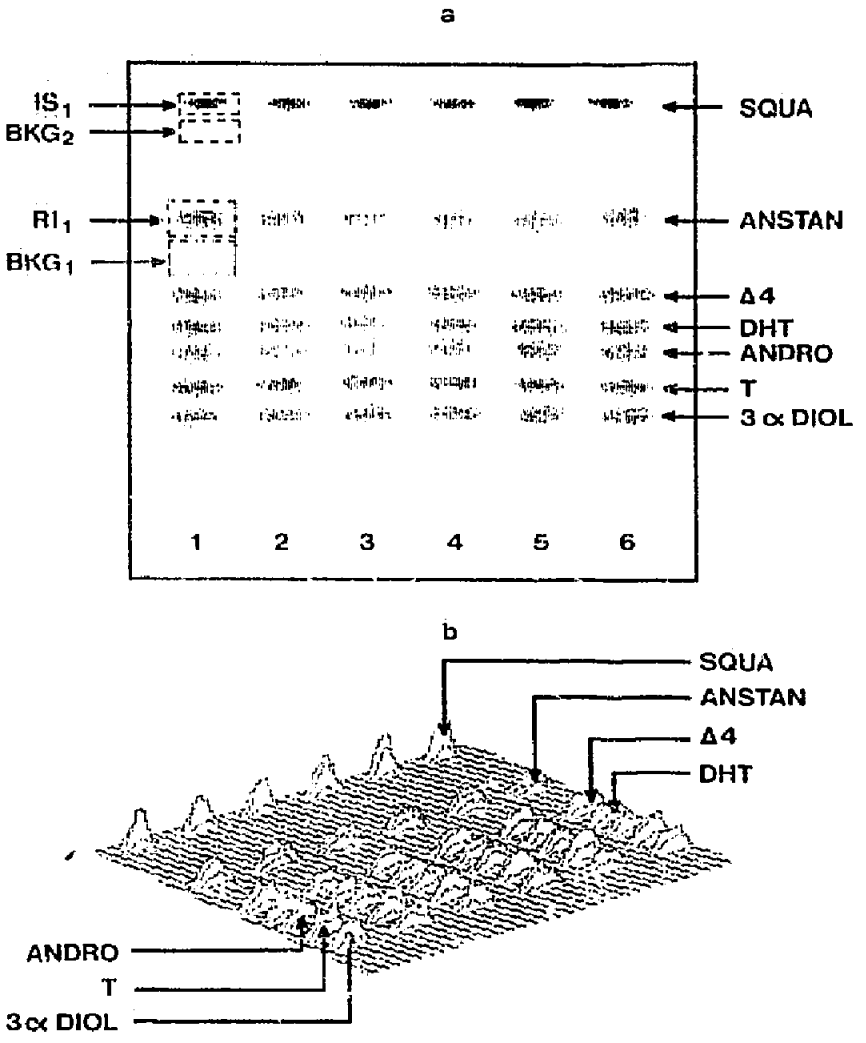

Fig. 2. Intra-plate reproducibility: digital autoradiography of six tracks of standard 3α,3β- and 5α-reduced and 17β-dehydrogenated [4-^{14}C]steroids (a) and its three-dimensional view (b). For abbreviations, chromatographic conditions and acquisition parameters, see text. IS_1 = internal standard ($SQUA_1$) region of interest; RI_1 = $ANSTAN_1$ region of interest; BKG_1 = background 1 substracted from RI_1; BKG_2 = background 2 substracted from IS_1.

Calibration curves. Solution of standard [^{14}C]steroids were adjusted to 10^{5}, 200, 300, 400 and 500 dpm in order to obtain the recovery between theoretical curve (*t*) and liquid scintillation measurements (m). Simultaneous determination of the ratios of standard [4-^{14}C]steroids to the internal standard (250 dpm) by the two techniques was performed for the same range value (Fig. 3a), and curves were plotted (LSC, DAR–TLC) for each compound. Fig. 3b shows the calibration curve for [4-^{14}C]testosterone. Fig. 3c shows the correlation between LSC and DAR–TLC for the whole range of [4-^{14}C]steroids under investigation.

Reproducibility of two-dimensional DAR–TLC. Fig. 4 shows double-track migration of standard [4-^{14}C]steroids in the two-dimensional system. The C.V.,

TABLE II

AVERAGE COEFFICIENT OF VARIATION OF RELATIVE DAR-TLC QUANTIFICATION OF STANDARD [4-^{14}C]STEROIDS CHROMATOGRAPHED IN SYSTEM I (INTRA-PLATE VARIABILITY, SIX PLATES)

Plate	C.V. (%)
1	4.93
2	4.84
3	7.18
4	5.15
5	8.78
6	5.30
Mean	6.03

calculated by Snedecor's method (S.D. = $[\Sigma(x_i - x_j)^2/2n]^{1/2}$) and recorded in Table IV, do not differ from those calculated from one-dimensional chromatography.

Accuracy of biological sample quantification. After incubation of human sebaceous glands as described in Experimental and addition of the internal standard, the organic phase extract was divided into four aliquots and the dried residues were chromatographed independently on four plates. Fig. 5a and b shows double-track chromatography of one aliquot and is three-dimensional view. The [4-^{14}C]testosterone spot of the bioassay track was removed before radioscanning

TABLE III

INTER-PLATE VARIABILITY OF RELATIVE DAR-TLC QUANTIFICATION OF STANDARD [4-^{14}C]STEROIDS CHROMATOGRAPHED IN SYSTEM I (SIX PLATES)

IS = internal standard (SQUA); 3β-DIOL and EPIA were run independently. For abbreviations and chromatographic system composition, see text.

Compound	Mean ± S.D.	C.V. (%)
3α-DIOL/IS	1.433 ± 0.1853	12.93
3β-DIOL/IS	1.464 ± 0.2599	17.75
T/IS	1.708 ± 0.2734	16.01
ANDRO/IS	1.523 ± 0.2364	15.52
EPIA/IS	1.607 ± 0.3111	19.36
DHT/IS	1.596 ± 0.2759	17.29
Δ4/IS	1.656 ± 0.2870	17.33
ANSTAN/IS	1.753 ± 0.3028	17.27
Mean		16.69

The amount of each [4-^{14}C]steroid derived from [4-^{14}C]testosterone metabolism is given in dpm in Table V. Variability of quantification calculated by the crossed Snedecor's method (comparison of plates 1–2, 1–3, 1–4, 2–3, 2–4 and 3–4) gives an average C.V. of 8.72%. Minor or structurally undefined metabolites, such as 2,6-ζ-hydroxylated androstanediols, appearing as the most polar materials were not investigated.

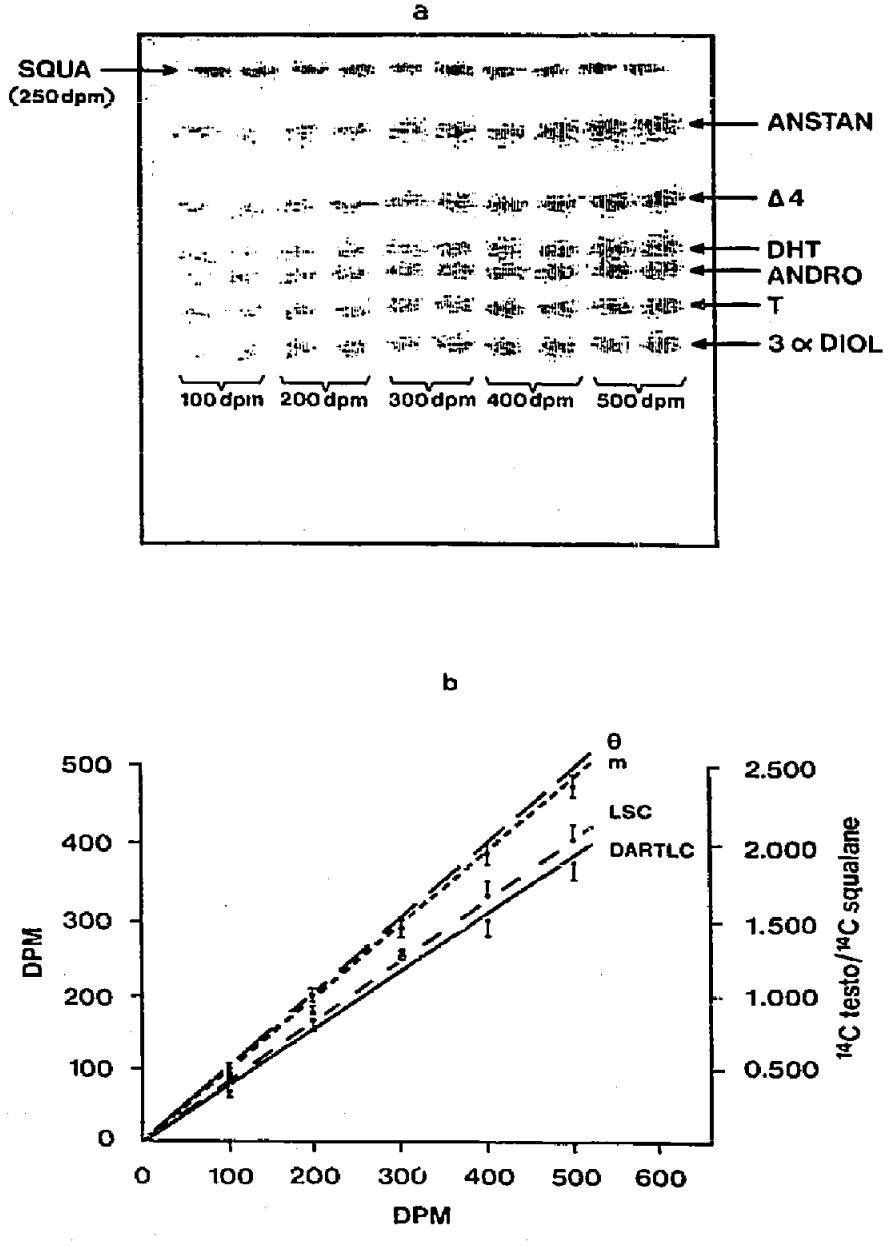

Fig. 3.

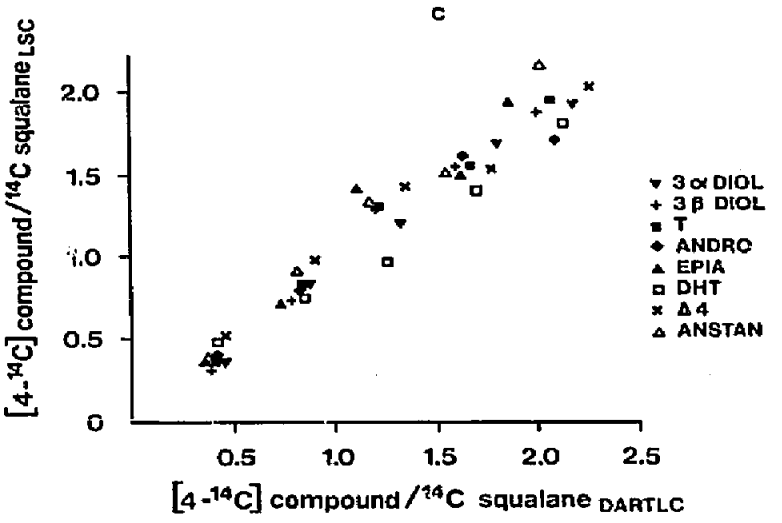

Fig. 3. (a) Digital autoradiography (log mode) of calibrated amounts of standard 3α,3β- and 5α-reduced and 17β-dehydrogenated [4-^{14}C]steroids. For abbreviations and chromatographic conditions, see text. Acquisition parameters are as described in text, except for the acquisition time (10 h). (b) [4-^{14}C]Testosterone calibration curve: (0) theoretical curve; (m) liquid scintillation measurements; (LSC) [^{14}C]testosterone/[^{14}C]squalane (liquid scintillation counting); (DARTLC) [^{14}C]testosterone/[^{14}C]squalane (digital autoradiography). (c) Correlation between LSC and DAR-TLC quantification of standard 3α,3β- and 5α-reduced and 17β-dehydrogenated [4-^{14}C]steroids.

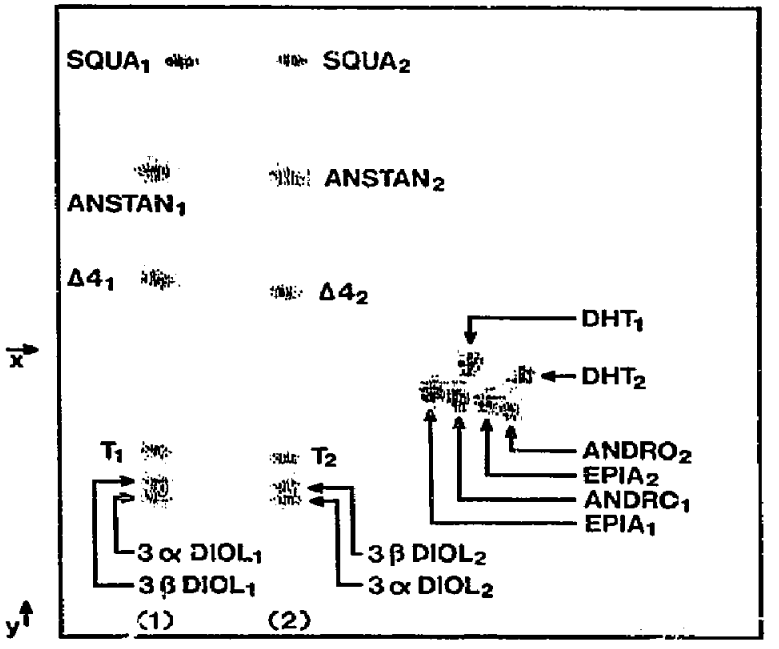

Fig. 4. Digital autoradiography of double track migration of standard 3α,3β- and 5α-reduced and 17β-dehydrogenated [4-^{14}C]steroids. For abbreviations, chromatographic conditions and acquisition parameters, see text.

TABLE IV

VARIABILITY OF MEASUREMENTS BY DAR–TLC OF DOUBLE-TRACK TWO-DIMENSIONAL CHROMATOGRAPHY OF STANDARD [4-^{14}C]STEROIDS CALCULATED BY SNEDECOR'S METHOD

For abbreviations and statistical methods, see text.

Plate	Mean ± S.D.	C.V. (%)
1	1.022 ± 0.06852	6.70
2	1.078 ± 0.05744	5.33
3	1.097 ± 0.08060	7.35
4	0.9759 ± 0.11437	11.72
Mean		7.78

DISCUSSION

Amongst the methods developed for quantifying the patterns of radiolabelled testosterone metabolites after chromatographic separation, HPLC coupled with on-line radiodetection seems to be the most suitable in terms of resolution and praticability, since neither derivatization nor sample collection is required, as in

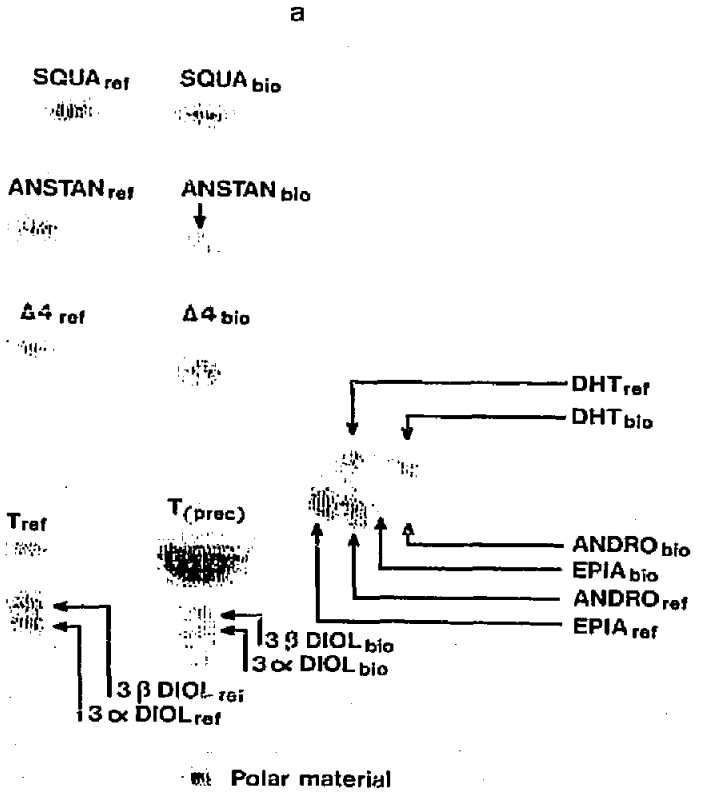

Fig. 5.

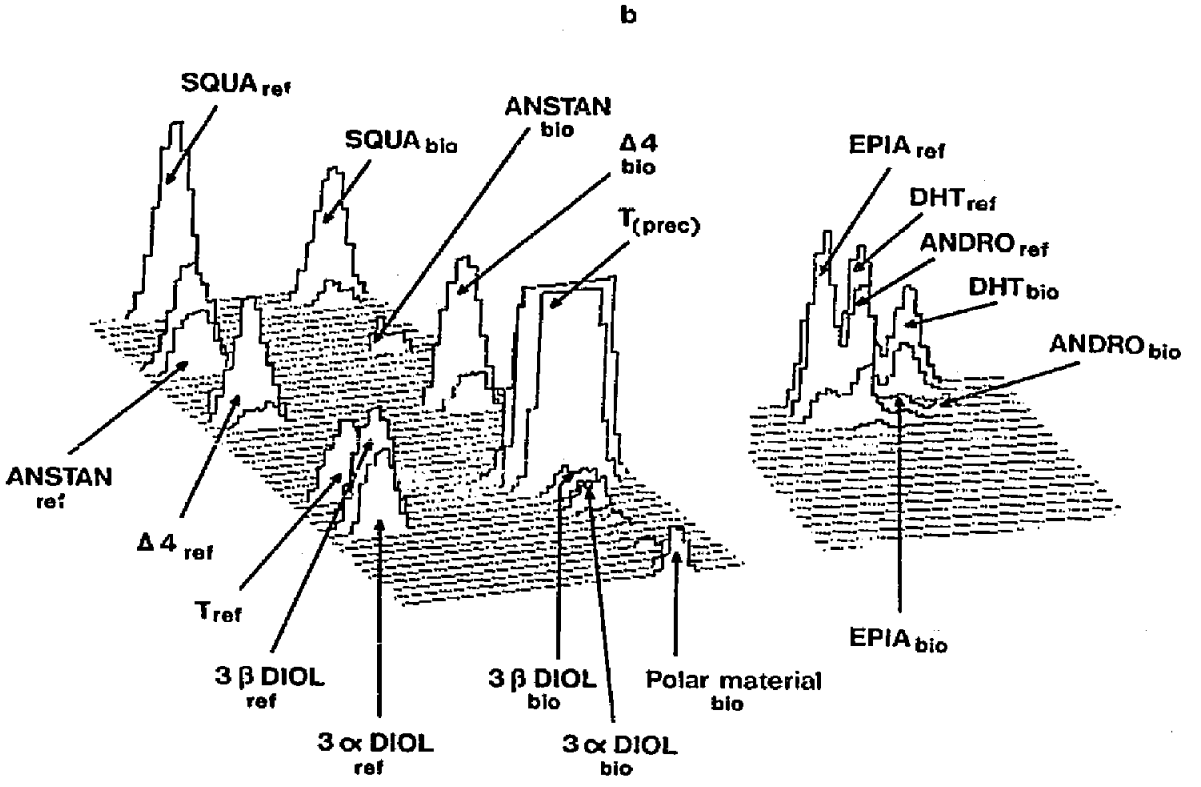

Fig. 5. (a) Digital autoradiography of 3α,3β- and 5α-reduced and 17β-dehydrogenated [4-^{14}C]androgens derived from *in vitro* [4-^{14}C]testosterone metabolism, incubated in the presence of human sebaceous glands; (b) three-dimensional view. For abbreviations and chromatographic conditions, see text; ref = calibration track; bio = bioassay track. Acquisition parameters were as described in text except for the acquisition time (10 h).

TABLE V

VARIABILITY OF MEASUREMENTS OF [4-^{14}C]TESTOSTERONE BIOTRANSFORMED INTO ITS METABOLITES AFTER DOUBLE-TRACK TWO-DIMENSIONAL CHROMATOGRAPHY AND DAR–TLC QUANTIFICATION

For abbreviations, incubation conditions and statistical methods, see text.

Compound	Amount (dpm)				
	Standard	Bioassay$_1$	Bioassay$_2$	Bioassay$_3$	Bioassay$_4$
3α-DIOL	3375	886	1197	920	1168
3β-DIOL	3115	338	561	519	597
ANDRO	3030	587	557	924	593
EPIA	3180	218	180	160	128
DHT	3065	3868	4820	4074	4782
Δ4	3270	6194	5623	6253	5408
ANSTAN	3175	1323	1195	1297	1064

Mean 1979.78 dpm; S.D. 172.55 dpm; C.V. 8.72%

gas chromatography and LSC. However, on-line radiodetection does not reach the sensitivity needed in our experiments, in which microamounts of biological tissue are not able to metabolize more than 0.02% of 0.33 μCi of precursor added to specific classes of radiometabolites (3α,3β-androstanediols, androsterone or epiandrosterone). Indeed, the conception of an on-line radiodetection cell does not permit cumulative measurements like a position-sensitive proportional counter, which reduces the limit of detection to 10 dpm ^{14}C within 60 h. In addition, if the radioprecursor is not removed before introduction of the sample into the radiodetection cell, an excess of β-emission could mask the weak radiosignal emitted by minor compounds of similar retention time. In the case of DAR–TLC this problem is avoided by removing the main part of the radioprecursor spot, visualized under UV light after the first runs in system I. The manual scraping-off procedure followed by LSC is time-consuming for series of plates on which more than 70 spots per plate are to be recovered, with the risk of losses and inadequate delimitation of areas of interest. Moreover, in DAR–TLC the background count-rate is low (1.3 cpm/cm^2) and can be thoroughly estimated by the integration of any area selected on the plate. It allows both increased precision and acceptable acquisition time for low activities: a 100-dpm signal is collected in 11.85 min. Radiorecording of one track takes 35 min for spots of 40 mm^2 (10 mm × 4 mm), with a signal-to-background ratio of 3.5 and precision of 2.8%.

Finally, TLC samples can be kept and eventually recovered after measurements for other analytical purposes and, except for the calibration of standard [4-^{14}C]steroid solutions, the use of scintillation fluid is not necessary. Radioscanning has previously been described for the quantification of testosterone metabolites, but no analytical parameters were given [31,32]. Apparent difficulties encountered during this kind of measurement, especially in terms of reproducibility, can be solved since this parameter depends more on chromatographic constraints than on ones of detection. An important difficulty is linked to the differential gradient pressure on lateral tracks, leading to a distorted separation pattern (Fig. 5a, ref. track). Linear radiochromatograms reconstructed after one-step acquisition are uncertain, insofar as radioemitting spots may be positioned partially outside the window of the detector. Consequently, multi-step acquisition followed by two-dimensional reconstruction is the only way to ensure complete collection of β-emission.

Slight discrepancies observed between LSC slope values and DAR–TLC ones (Table VI) can be interpreted as a silica gel quenching effect inherent to plate quality and relative depth diffusion of each steroid within it, which could also explain the important C.V. observed in inter-plate comparison (Table III). Indeed, since linearity and low intra-plate C.V. are observed, this quenching factor appears as an internal plate constant. DAR–TLC values can therefore be expressed directly in dpm, provided that one standard track per plate is chromatographed.

In conclusion, when inter-plate and multi-sample analyses are to be made, the

TABLE VI

SLOPE COMPARISON BETWEEN LSC AND DAR-TLC MEASUREMENTS OF STANDARD [4-^{14}C]STEROIDS

For abbreviations, see text.

Compound	LSC			DAR-TLC		
	a	*b*	r^2	*a*	*b*	r^2
3α-DIOL	1.08	+0.003	1.00	1.00	+0.002	0.99
3β-DIOL	1.00	−0.002	1.00	0.99	−0.004	0.98
T	1.03	+0.100	0.99	0.98	+0.030	0.99
EPIA	1.03	−0.003	1.00	0.86	+0.140	0.96
ANDRO	0.97	−0.020	1.00	0.99	0.000	0.96
DHT	1.05	+0.004	1.00	0.83	+0.090	0.98
Δ4	1.11	+0.020	1.00	0.90	+0.220	0.97
ANSTAN	1.00	−0.010	1.00	1.05	+0.020	0.97

main problems of quantification are easily worked out by an adequate combination of both internal and external standardization. In this way, the advantages of radioscanning can be optimized in terms of sensitivity, specificity and practicability, which are not always achieved simultaneously with other techniques. In the case of 3α,3β- and 5α-reduced and 17β-dehydrogenated testosterone radiometabolites, which are completely separated on the same TLC silica gel plate following the method described above, quantitative DAR appears to be a suitable tool for research and routine analyses. The procedure described has been successfully employed in our laboratory for investigations involving studies from subfractions of sebaceous glands and hair follicles, in which the formation and metabolization of steroids is a field of considerable interest [33].

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