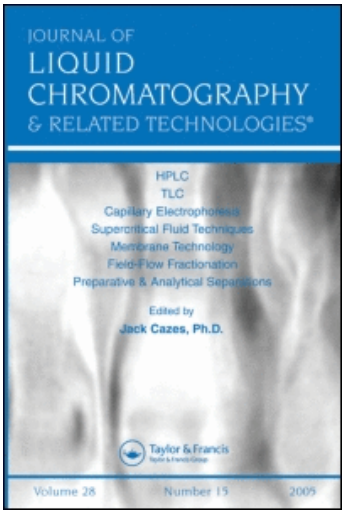

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ABSTRACT

Several steroidal hormones with an α,β-unsaturated carbonyl group sensitized the fluorescence of terbium(III) ions: medroxyprogesterone, medroxyprogesterone acetate, methyltestosterone, nandrolone, nandrolone decanoate, progesterone, testosterone, testosterone enanthate, and testosterone propionate, but other steroids which have this chromophore did not give any signal: clostebol acetate, dehydrotestosterone, dydrogesterone, methandienone, and methenolone enanthate. Sensitized fluorescence was used to detect the steroids when eluted with micellar mobile phases of sodium dodecyl sulphate (SDS)-acetonitrile and SDS-pentanol containing 0.01 M Tb(III), with 245 nm and 545 nm as excitation and emission wavelengths, respectively. The micelles favour the fluorescence process due to proximity of donor and acceptor, and shielding of the Tb(III) ions from the aqueous medium. Signal intensity increased linearly with the concentration of Tb(III), but decreased with acetonitrile and pentanol. The concentration of organic solvent in the mobile phase should, therefore, be limited. Mobile phases of 0.075 M SDS-12.4% acetonitrile and 0.13 M SDS-4.5% pentanol offered the highest sensitivities, but only pentanol permitted the elution of the most hydrophobic steroids at adequate retention times. With this mobile phase, the limits of detection were in the 0.07-0.2 μg/mL

range. The sensitivities did not change in the 25-60°C range, but the efficiencies of the chromatographic peaks were larger at higher temperature, and consequently, the resolution was improved.

INTRODUCTION

New applications are being reported in reversed-phase liquid chromatography (RPLC), which utilize novel mobile phases. In this field, during the last years, hybrid solutions containing micellized surfactant and several organic solvents have been examined.[1] One interesting feature of these media is that they permit the direct injection of physiological fluids, such as plasma,[2] serum,[3,4] and urine[5-8] samples, without obstruction or damage of the chromatographic system. Simplified procedures with reduced cost and analysis time are achieved.

In previous work, we showed the capability of mobile phases of sodium dodecyl sulphate (SDS) and acetonitrile or pentanol to resolve complex mixtures of steroids of diverse hydrophobicity, in a C_{18} column, with appropriate retention times.[9,10] Other alcohols usually employed as modifiers were too weak to elute the steroids. However, adequate detection systems are still needed to detect the steroids at the low concentrations found in physiological samples. Fluorimetric detection is a good option to enhance chromatographic sensitivity, but most steroids are non-fluorescent, and must be derivatized to be detected, making the procedures too laborious.[11,12] Also, the lack of selectivity and the formation of a large number of uncharacterized products limit the application of these methods in drug analysis.

Steroids with an α,β-unsaturated carbonyl group in the A-ring (Figure 1), such as cortisone, progesterone, and several hormones derived from testosterone, can however sensitize the fluorescence of lanthanide ions. Sensitized fluorescence is achieved when a molecule (the donor) transfers energy to the

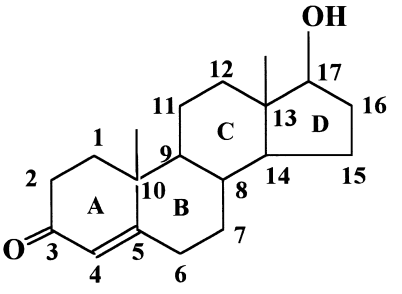

Figure 1. Structure of the steroids which sensitize the fluorescence of Tb(III).

low absorbing lanthanide ion (the acceptor), which enhances greatly its fluorescence. The efficiency of the energy-transfer depends on the proximity of the excited triplet level of the donor and the excited singlet level of the acceptor, which should be slightly lower. In these conditions, radiative deactivation competes favourably against other deactivation vias and the characteristic emission of the acceptor is produced. The first excited singlet level of steroids is at 41666 cm^{-1} (240.0 nm), but more important is the existence of a triplet level at 26041 cm^{-1} (384.0 nm), that also yields a phosphorescence signal at low temperature.[13] At room temperature, this triplet level excites efficiently the $^5d_3$ level of Tb(III) (26000 cm^{-1}, 384.6 nm). After relaxation to the $^5d_4$ level, a radiative transition to the ^7f ground level is produced, which results in the characteristic Tb(III) fluorescence. Fluorescence enhancements have also been reported for Eu(III), Sm(III), and Dy(III), but the energy of the triplet level of steroids matches better with Tb(III).

Dibella et al.[14] first suggested the use of lanthanide ions in the post-column detection of compounds that enhance or inhibit their fluorescence, yielding positive or negative peaks, respectively. More recently, sensitized terbium fluorescence was proposed in RPLC for the detection of mixtures of testosterone, methyltestosterone, bolasterone, progesterone, and testosterone acetate, eluted with micellar solutions of SDS-acetonitrile,[15] and in micellar electrokinetic chromatography for mixtures of testosterone and progesterone, eluted with SDS-methanol.[16] This type of detection has also been applied to the chromatographic analysis of theophylline, eluted with a normal phase of reversed micelles of sodium bis(2-ethylhexyl)sulphosuccinate (AOT) in ethyl acetate-hexane.[17]

The fluorescence yield of Tb(III) depends on the spatial proximity to the donor. Micellar media can favour this proximity since both donor and acceptor can be accommodated simultaneously in the micelles, and therefore, be found in a very small volume. Neutral Tb(III) dodecyl sulphate ($Tb(DS)_3$) is solubilized in the micelle, likely in its palisade layer, whereas the steroids are included in the micelle core. The exclusion of water from the micelle and shielding of Tb(III) ions from the aqueous medium is another factor for the enhanced fluorescence. Coupling of sensitized terbium fluorescence with micellar media in RPLC seems to be a good alternative for steroid analysis. The performance of this technique in the detection of several steroidal hormones, eluted with micellar mobile phases of SDS-acetonitrile or SDS-pentanol is examined in this work.

EXPERIMENTAL

Reagents and Columns

The following steroids were studied (Figure 2): clostebol acetate, dehydrotestosterone, mesterolone, methandienone, methenolone enanthate, methyl-

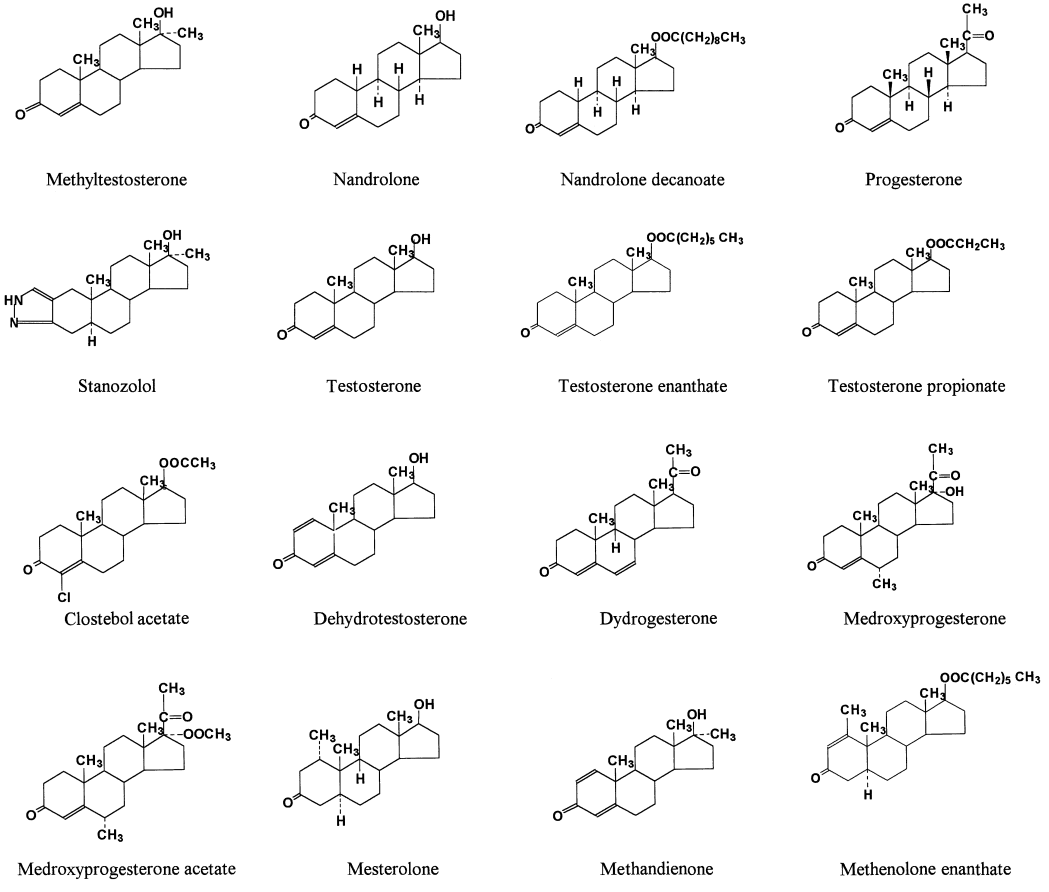

Figure 2. Studied steroids.

testosterone, stanozolol (Sigma, Madrid, Spain), dydrogesterone (Kalifarma, Barcelona, Spain), medroxyprogesterone (Frumtos-Zyma, Barcelona), medroxyprogesterone acetate (Cusí, Barcelona), nandrolone (Fher, Barcelona), nandrolone decanoate (Organon, Barcelona), progesterone (Seid, Barcelona), testosterone, testosterone enanthate, and testosterone propionate (Schering, Madrid). The steroids, except those of Sigma, were kindly donated by the pharmaceutical laboratories. Stock solutions of 100 μg/mL were prepared for all the steroids. The compounds were dissolved with the aid of an ultrasonic bath (Selecta, Model 617, Barcelona), in a small amount of methanol and diluted with an aqueous solution of sodium dodecyl sulphate (99% purity, Merck, Darmstadt, Germany). No decomposition of the steroids solutions was observed during at least one month. Cellulose acetate membranes (Micron, Westboro, MA) of 0.45 μm were used to filter the solutions before their injection into the chromatograph.

A Spherisorb ODS-2 column (Scharlau, Barcelona, 5 μm, 120 mm x 4.6 mm i.d.) was used to analyze the steroids. A similar shorter column (Scharlau, 35 mm x 4.6 mm i.d.), which acted as guard pre-column to saturate the mobile phase with silica, was placed before the injector. The mobile phases consisted of solutions of SDS, acetonitrile (HPLC grade, Scharlau) or 1-pentanol (analytical grade, Merck), and terbium(III) nitrate pentahydrate (99% purity, Aldrich, Milwaukee, WI). Methanol and chloroform (HPLC grade, Scharlau) were used to wash the column and eliminate strongly retained compounds. Nanopure water (Barnstead, Sybron, Taunton, MA) was used throughout to prepare steroids solutions and mobile phases. When the surfactant was added to a terbium nitrate solution, precipitation of $Tb(DS)_3$ was produced initially, which was redissolved with further addition of SDS.

Apparatus

Absorption spectra were obtained with a Hewlett-Packard diode-array spectrophotometer (Model 8452A, Palo Alto, CA). The fluorescence studies in static were performed with a Hitachi fluorimeter (Model F-4500, Tokyo, Japan), equipped with a Xenon lamp and a 1-cm pathlength cell. The excitation and emission slits were both set at 10 nm.

The liquid chromatograph from Hewlett-Packard (Model HP 1050, Palo Alto, CA) was equipped with an isocratic pump, an autosampler (Model HP 1100), and a fluorescence detector (Model HP 1046A). The delay and gate times were 0.03 ms and 0.8 ms, respectively, and the response time, 6 ms. The excitation and emission wavelengths were 245 nm and 545 nm, respectively. The injection volume was 20 μL and the flow-rate, 1 mL/min. A thermostatic bath (Barrington, Cambridge, United Kingdom) was used to control the temperature of the chromatographic column. The chromatographic signal was

acquired through an integrator (Model HP 3396A) connected to a computer, using the Peak-96 software of Hewlett-Packard (Avondale, PA, USA).

RESULTS AND DISCUSSION

Absorbance Spectra of the Steroids

It is known that micellar media can modify the spectra of solutes. For this reason, the absorption spectra of the steroids were obtained in 0.10 M SDS solution. The compounds showed a single band with maximum absorption at 245 nm, except methenolone enanthate (248 nm), mesterolone (192 nm), stanozolol (228 nm), clostebol acetate 260 nm), and dydrogesterone (292 nm). The maximum wavelength at 245 nm should be assigned to the α,β-unsaturated carbonyl group (Figure 2). The longer wavelengths of clostebol acetate and dydrogesterone are due to the chloro substituent in position α, and the conjugated double bonds, respectively. In contrast, the double bonds of dehydrotestosterone and methandienone are not conjugated, and the absorption at 245 nm is not affected. Otherwise, mesterolone lacks the double bond and stanozolol possess a different chromophor. Table 1 shows the molar absorptivities of the steroids (except for mesterolone and stanozolol). The molar absorptivity of Tb(III) at 245 nm, in 0.10 M SDS, was only 2 mol^{-1} 1 cm^{-1}. The

Table 1

Molar Absorptivities of the Steroids at Their Maxima

Compound	Molar Absorptivity (Mol^{-1} 1 cm^{-1})
Clostebol acetate	7650 ± 160
Dehydrotestosterone	14350 ± 80
Dehydrogesterone	24900 ± 500
Medroxyprogesterone	14790 ± 30
Medroxyprogesterone acetate	15520 ± 20
Methandienone	14640 ± 70
Methenolone enanthate	12860 ± 12
Methyltestosterone	14640 ± 120
Nandrolone	10730 ± 110
Nandrolone decanoate	15840 ± 110
Progesterone	15740 ± 50
Testosterone	16360 ± 110
Testosterone enanthate	12860 ±12
Testosterone propionate	15090 ± 60

absorbance for most steroids was, therefore, approximately 6000-8000 fold that of Tb(III).

Sensitization of Terbium(III) Fluorescence

Amin et al.[15] reported enhancements in lanthanide fluorescence in SDS micellar solutions of several steroids. This seemed to indicate that the neutral terbium dodecyl sulphate complex was solubilized inside the micelle, probably in its palisade. RMN and fluorescence studies have however suggested that the energy transfer from steroids to lanthanides in micellar solutions does not arise from the formation of a complex of both species which would guarantee the proximity of donor and acceptor.[15]

We found that nine of the sixteen steroids included in this work sensitized the fluorescence of Tb(III): medroxyprogesterone, methyltestosterone, medroxyprogesterone acetate, nandrolone, nandrolone decanoate, progesterone, testosterone, testosterone enanthate, and testosterone propionate. Mesterolone and stanozolol which lack an α,β-unsaturated carbonyl group, and five steroids (clostebol acetate, dehydrotestosterone, dydrogesterone, methandienone, and methenolone enanthate), which have this chromophor did not give any signal. Possible reasons for the absence of fluorescence are the chloro substituent in position 4 of clostebol acetate, the additional double bond in positions 1-2 in the A-ring of dehydrotestosterone and methandienone, and the double bond in positions 6-7 in the B-ring of dydrogesterone (Figure 2). Methenolone enanthate has an α,β-unsaturated carbonyl group in the A-ring, but in positions 1-2.

The excitation spectrum of Tb(III) showed maximum wavelength at 255 nm, whereas two maxima were observed in its emission spectrum at 332 nm and 545 nm, the latter more intense. Figure 3 depicts the emission spectra obtained for a micellar solution of 0.05 M Tb(III) in the presence of methyltestosterone, using as excitation wavelength the maximum of Tb(III) at 255 nm (Figure 3a), or the maximum of the steroid at 245 nm (Figure 3b). The fluorescence intensity was higher by exciting at 255 nm, but the emission band overlapped the wide band of the armonic at 510 nm. Excitation at 245 nm allowed, therefore, lower limits of detection for the steroid.

Effect of Several Chemical Variables on Sensitized Fluorescence

A study was first performed in static to investigate the effect of several reagents (Tb(III), SDS, acetonitrile and pentanol), on the fluorescence intensity. A linear increase was observed in the signal at increasing concentrations of Tb(III) in the 0.001-0.01 M range, for several solutions containing 0.10 M SDS and 20% acetonitrile, or 7% pentanol (maximal concentrations of the modifiers

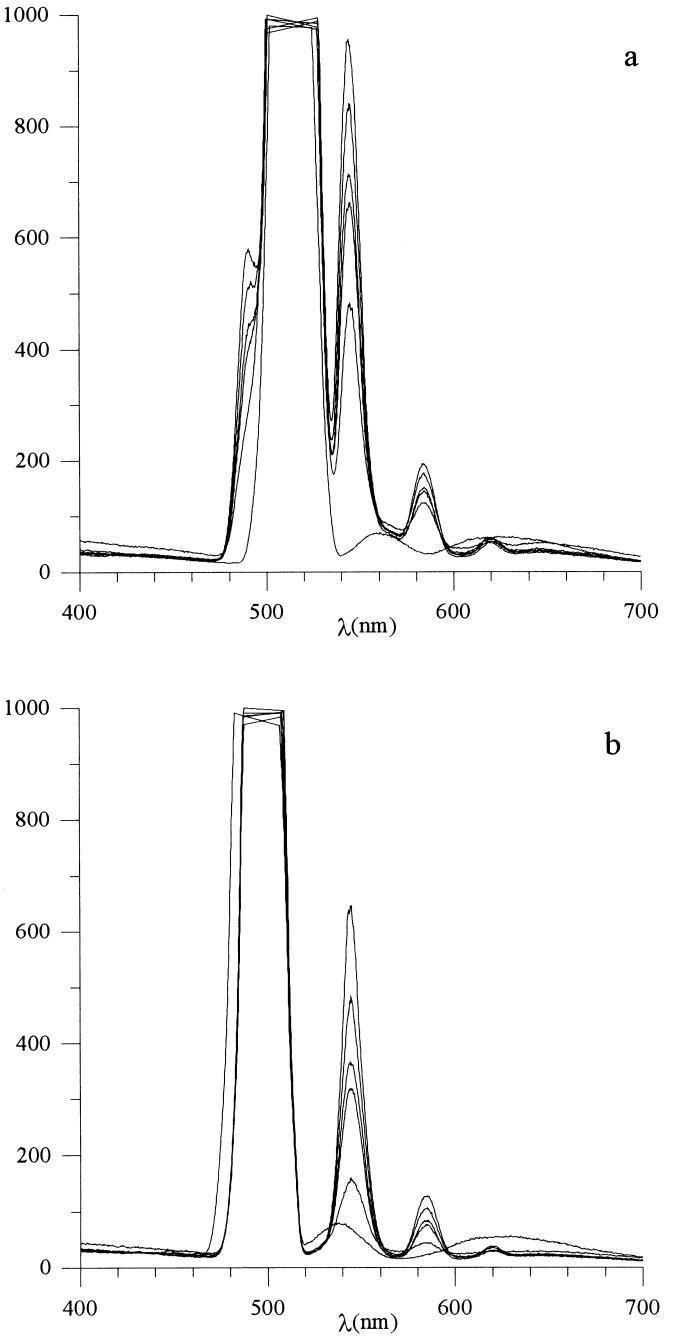

Figure 3. Emission spectra of 0.01 M Tb(III) solutions in micellar media of 0.10 M SDS, in the absence and presence of increasing concentrations of methyltestosterone in the 1-8 μg/mL range. Excitation wavelength at: a) 255 nm, and b) 245 nm.

that guaranteed the formation of micelles), and a fixed concentration of methyltestosterone (1 μg/mL).

In contrast, the signal decreased with the concentrations of surfactant and organic solvents (acetonitrile and pentanol). It was found that terbium sensitized fluorescence, measured in micellar solutions of 0.10 M SDS-0.01 M Tb(III) at varying concentrations of acetonitrile in the 0-20% range, decreased linearly with increasing acetonitrile. It has been reported that the number of micelles is critical for the observation of fluorescence.[15] At concentrations below the critical micellar concentration (cmc), no micelles are present and quenching of Tb(III) fluorescence by water leads to a weak emission. Approaching the cmc, pre-micellar aggregation gives an increasing protection against quenching, leading to an increase in emission intensity which is maximal close to the cmc. Above the cmc, further quenching of the fluorescence is produced due to the lower probability of finding the donor and acceptor in the same micelle, as the concentration and size of micelles increase.

It should be taken into account that the cmc of SDS solutions increases with the concentration of acetonitrile (cmc is 0.008, 0.010, and 0.030 M for 0, 10 and 20% acetonitrile, respectively.[18] The smaller number of micelles with increasing acetonitrile can be the reason for the decreased fluorescence. However, for pentanol, another reason should exist since the cmc of SDS decreases with increasing pentanol (cmc is 2.5×10^{-3} M and 2.0×10^{-3} M for 4.5% and 7% pentanol, respectively.[18] The nature of the aqueous-organic solvent can be important, but it should be also noted that pentanol is inserted in the micellar assembly producing swollen micelles. This alcohol is first accommodated in the intermonomer spaces of the micelle structure, the polar hydroxyl group orientated towards the Stern layer and the alkyl chain located in the non-polar micelle core.[19] Above 1.5-2% pentanol, the extra pentanol molecules added to the micellar solution seem to be solubilized in the micelle core.[18] Sensitized fluorescence is clearly weaker in the modified micelles.

The linearity of the response with respect to the concentration of the steroids was also checked. As an example, the calibration straight-line for methyltestosterone in a micellar medium of 0.10 M SDS-20% acetonitrile-0.01 M Tb(III), in the 0.5-3 μg/mL concentration range was:

$$y = -40\ (\pm 30) + 181\ (\pm 13)\ C\ (\mu g/mL)$$

with a regression coefficient, r = 0.990.

Fluorimetric Detection of Steroids in Micellar Liquid Chromatography

The chromatographic peaks of the steroids were measured using 245 nm and 545 nm as excitation and emission wavelengths, respectively. In the litera-

ture, Tb(III) has been added as post-column reagent in micellar medium or to the micellar mobile phase to sensitize the fluorescence of the eluted steroids.[15] We preferred the second mode due to its instrumental simplicity and the elimination of the background noise due to post-column mixing of the reagents. The minimal concentration of surfactant in the mobile phase was kept above 0.05 M to well exceed the cmc of SDS in the presence of acetonitrile. On the other hand, at lower concentrations of SDS, the retention times of the steroids were too high and the C_{18} column was not sufficiently protected against the adsorption of proteins when urine samples were injected.[4]

The fluorescence intensity of lanthanides increases with temperature.[20,21] The energy transfer process implies the collision of the organic molecules and lanthanide ions, which is favoured at higher temperature. However, the temperature increase can also originate non-radiative deactivation of Tb(III), and consequently, quenching of the fluorescence. Matovich and Suzuki[21] investigated the fluorescence of Eu(III) in acetophenone and observed sensitivity enhancements up to 90°C. These authors reported that, at high temperature, the energy transfer between certain organic molecules and lanthanide ions is greater than the fluorescence diminution produced by non-radiative deactivation.

In liquid chromatography, the working temperature depends on the volatility and thermal stability of organic solvent and analytes, respectively, in the mobile phase. The temperature range of the column packing should also be considered. A study of the effect of temperature on the chromatographic peaks detected using sensitized fluorescence was thus performed. The chromatographic column was thermostatized at different temperatures in the 25 - 60°C range, and a mixture of medroxyprogesterone, testosterone, methyltestosterone, medroxyprogesterone acetate, progesterone, testosterone propionate, and testosterone enanthate, was eluted with a mobile phase of 0.13 M SDS-7% pentanol-0.01 M Tb(III). As observed in Figure 4, the efficiencies of the peaks increased with temperature, but their areas scarcely changed. This enhanced the resolution of the chromatograms. As an example, the efficiencies of the peaks were 925, 1415, and 1945 for medroxyprogesterone, 1920, 2120, and 2950 for progesterone, and 1715, 2050, and 3870 for testosterone enanthate, at 25, 40, and 60°C, respectively. The areas of the peaks at the same temperatures were 1.80, 1.95, and 2.08 for medroxyprogesterone, 1.93, 1.95, and 2.30 for progesterone, and 1.26, 1.29, and 1.40 for testosterone enanthate.

As indicated above, the sensitized fluorescence signal is affected by the presence of organic solvent in the micellar solutions. For this reason, its concentration should be controlled. Figure 5 illustrates the sensitized fluorescence signal achieved in four solutions of varying composition, used as mobile phases. The larger intensity was achieved for 0.075 M SDS-12% acetonitrile. This mobile phase showed good resolution for mixtures of medroxyprogesterone,

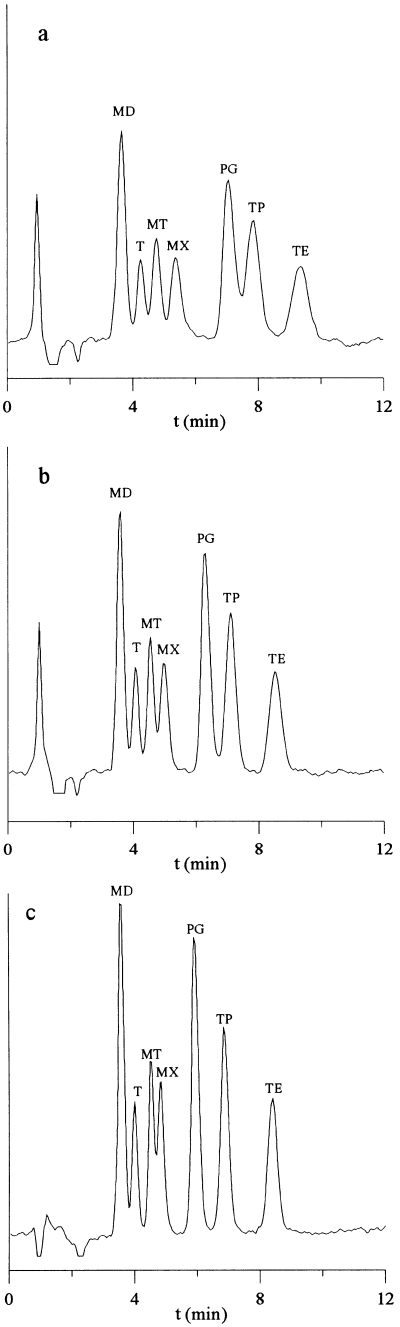

Figure 4. Chromatograms of a mixture of medroxyprogesterone (MD), testosterone (T), methyltestosterone (MT), medroxyprogesterone acetate (MX), progesterone (PG), testosterone propionate (TP), and testosterone enanthate (TE), eluted with 0.13 M SDS-7% pentanol at: a) 25°C, b) 40°C, and c) 60°C. Sensitized fluorescence was detected at λ_{ex} = 245 nm and λ_{em} = 545 nm. The ordinates scale is the same for the three chromatograms.

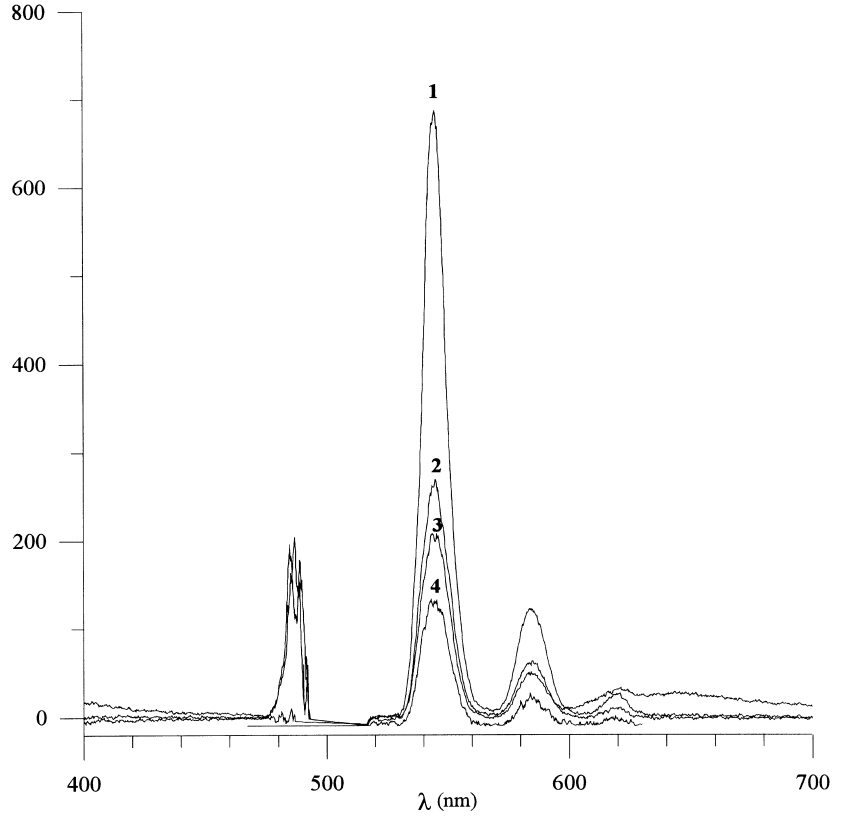

Figure 5. Sensitized Tb(III) fluorescence signal in the presence of 1 μg/mL of methyltestosterone and 0.01 M Tb(III) for diverse media: 1) 0.075 M SDS-12% acetonitrile, 2) 0.10 M SDS-20% acetonitrile, 3) 0.17 M SDS-15% acetonitrile, and 4) 0.10 M SDS-7% pentanol. The excitation wavelength was 245 nm and the signal in the absence of methyltestosterone was subtracted.

methyltestosterone, medroxyprogesterone acetate, and testosterone, but the retention time of the last eluted steroid was 40 min. Also, the retention times of nandrolone, nandrolone decanoate, progesterone, testosterone enanthate, and testosterone propionate were > 60 min. For these compounds, a mobile phase of larger elution strength was required, such as solutions with a larger concentration of acetonitrile or with pentanol.

Two series of calibration curves were obtained for the steroids in the 0.05-3 μg/mL range, using two mobile phases of 0.13 M SDS-0.01 M Tb(III) at two different pentanol concentrations: 4.5% and 7%. In both cases the plots were linear. As expected, the sensitivity was greater at the lower concentration of modifier.

Analytical Figures of Merit

Calibration curves were obtained in the 0.05-8 μg/mL range for the nine steroids that sensitized the fluorescence of Tb(III). Tables 2 and 3 show the parameters of the equations of the fitted straight-lines, and the regression coefficients, for mobile phases of SDS-acetonitrile and SDS-pentanol, respectively. As expected, the sensitivities (slopes of the calibration straight-lines) were greater (approximately three-fold) for the mobile phase containing the lower SDS and acetonitrile contents (0.075 M SDS-12.4% acetonitrile), in comparison with the mobile phase of 0.15 M SDS-15% acetonitrile. The sensitivities ranged between 1 and 2.8 (arbitrary units) for the latter mobile phase, and between 1.6 and 3.2 for 0.13 M SDS-4.5% pentanol. The mobile phase of pentanol, with a larger elution strength, allowed the determination of the most hydrophobic steroids.

Table 2

Calibration Parameters, Repeatabilities, and Limits of Detection for the Steroids Eluted with SDS-Acetonitrile Mobile Phases*

Compound	$b \pm s_b$	$a \pm s_a$	r	CV (%)	LOD (μg/mL)
Medoxyprogesterone	2.76 ± 0.09^a	-0.8 ± 0.4^a	0.998^a	8.4^a	0.13^a
Mexroxyprogesterone	1.008 ± 0.014^a	-0.50 ± 0.07^a	0.9997^a	8.4^a	0.87^a
Acetate	2.84 ± 0.10^b	-0.3 ± 0.4^b	0.997^b	4.7^b	0.38^b
Methyltestosterone	1.294 ± 0.024^a	0.14 ± 0.10^a	0.9990^a	6.9^a	0.41^a
Progesterone	2.12 ± 0.05^a	-0.2 ± 0.2^a	0.9990^a	8.3^a	0.55^a
Testosterone	1.78 ± 0.08^a	-0.03 ± 0.3^a	0.993^a	5.9^a	0.19^a
	5.54 ± 0.17^b	-2.1 ± 0.6^b	0.997^b	4.1^b	0.17^b
Testosterone Propionate	1.80 ± 0.08^a	1.3 ± 0.4^a	0.990^a	4.1^a	0.55^a

* Calibration parameters: $F = a + bC$. [a] Mobile phase: 0.15 M SDS-15% acetonitrile-0.01 M Tb(III). [b] Mobile phase: 0.075 M SDS-12.4% acetonitrile-0.01 M Tb(III).

Table 3

Calibration Parameters, Repeatabilities, and Limits of Detection for the Steroids Eluted with SDS-Pentanol Mobile Phases*

Compound	$b \pm s_b$	$a \pm s_a$	r	CV (%)	LOD (μg/mL)
Medroxyprogesterone	3.01 ± 0.03	0.25 ± 0.09	0.9997	5.7	0.074
Medroxyprogesterone Acetate	2.64 ± 0.04	0.04 ± 0.13	0.9992	5.8	0.20
Methyltestosterone	1.286 ± 0.018	0.38 ± 0.06	0.9994	6.2	0.19
Nandrolone	2.82 ± 0.05	0.08 ± 0.12	0.9990	5.8	0.14
Nandrolone Decanoate	1.72 ± 0.08	0.2 ± 0.2	0.994	9.2	0.17
Progesterone	2.32 ± 0.04	0.12 ± 0.13	0.9992	10.6	0.19
Testosterone	1.639 ± 0.017	0.15 ± 0.05	0.9996	12.8	0.22
Testosterone Enanthate	3.23 ± 0.04	0.32 ± 0.15	0.9995	6.3	0.19
Testosterone Propionate	2.28 ± 0.04	0.37 ± 0.12	0.9991	8.1	0.11

* Calibration parameters: F = a + bC. Mobile phase: 0.12 M SDS-4.5% pentanol-0.01 M Tb(III).

The repeatability of the chromatographic method was obtained from triplicate injections of five solutions contaning 1.5 μg/mL of the steroids. The limits of detection (LODs) were evaluated by measuring the area of chromatographic peaks achieved with a solution at a low concentration of the steroid (close to the LOD), and using the 3s criterion (n = 10). The results are also given in Tables 2 and 3 for mobile phases of acetonitrile and pentanol. The LODs were restricted by the background noise of the mobile phase containing Tb(III), which was weakly fluorescent.

The results reported in this work show that the mobile phase of 0.13 M SDS-4.5% pentanol succeeded in eluting steroids of diverse hydrophobicity (octanol-water coefficients, log P_{ow} in the 3.4-8.1 range),[22] with good resolution and low analysis times. It was also an appropriate medium to allow the sensitization of Tb(III) fluorescence, which is very selective for the detection of the steroids in the presence of other drugs. However, when a urine sample spiked with a mixture of steroids was injected directly into the chromatograph, without previous treatment, the peaks of the less retained steroids, medroxyprogesterone, testosterone, and methyltestosterone, were overlapped by the wide band of proteins, which appears at the head of the chromatograms. Previous extraction of these steroids is, thus, convenient. The extraction also will reduce the

LODs needed to detect the steroids at very low concentration, in the physiological samples. Further work is being done in our laboratory to optimize the extraction procedure.

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REFERENCES

1. S. López-Grío, J. J. Baeza-Baeza, M. C. García-Alvarez-Coque, Anal. Chim. Acta, **381**, 275-285 (1998).

2. B. Ameer, M. B. Burlingame, Anal. Lett., **21**, 1589-1601 (1988).

3. R. A. Grohs, F. V. Warren, Jr., B. A. Bidlingmeyer, Anal. Chem., **63**, 384-390 (1991).

4. K. B. Sentell, J. F. Clos, J. G. Dorsey, BioChromatography, **4**, 35-40 (1989).

5. L. J. Cline-Love, J. J. Fett, J. Pharm. Biomed. Anal., **9**, 323-333 (1991).

6. E. Bonet-Domingo, M. J. Medina-Hernández, M. C. García-Alvarez-Coque, Quim. Anal., **12**, 167-172 (1993).

7. S. Carda-Broch, J. S. Esteve-Romero, M. C. García-Alvarez-Coque, Anal. Chim. Acta, **375**, 143-154 (1998).

8. I. Rapado-Martínez, R. M. Villanueva-Camañas, M. C. García-Alvarez-Coque, Anal. Chem., **71**, 319-326 (1999).

9. S. Torres-Cartas, R.M. Villanueva-Camañas, M. C. García-Alvarez-Coque, Anal. Chim. Acta, **302**, 163-172 (1995).

10. S. Torres-Cartas, R. M. Villanueva-Camañas, M. C. García-Alvarez-Coque, Anal. Chim. Acta, **333**, 31-40 (1996).

11. F. M. Schnepel, in **Fluorometric Analysis in Biomedical Chemistry,** J. D. Winefordner, ed., Wiley, New York, 1985, p. 105.

12. T. Kawasaki, T. Maedo, A. Tsuji, J. Chromatogr., **233**, 61-68 (1982).

13. G. Marsh, D. R. Kearns, K. Schaffner, J. Am. Chem. Soc., **93**, 3129 (1971).

14. E. E. Dibella, J. B. Weissman, M. J. Joseph, J. R. Schultz, T. J. Wenzel, J. Chromatogr., **328**, 101-109 (1985)

15. M. Amin, K. Harrington, R. von Wandruszka, Anal. Chem., **65**, 2346-2351 (1993).

16. R. E. Milofsky, M. G. Malberg, J. M. Smith, J. High Resolut. Chromatogr., **17**, 731-732 (1994).

17. A. G. Mwalupindi, I. M. Warner, Anal. Chim. Acta, **306**, 49-56 (1995).

18. S. López-Grío, J. J. Baeza-Baeza, M. C. García-Alvarez-Coque, Chromatographia, **48**, 655-663 (1998).

19. **Micellization, Solubilization and Microemulsions**,**Vol. 1**, K. L. Mittal, ed., Plenum Press, New York, 1979.

20. W. J. McCarthey, J. D. Winefordner, Anal. Chem, **38**, 848 (1966).

21. E. Matovich, C. K. Suzuki, J. Chem. Phys., **39**, 1442 (1963).

22. ACD software. Advance Chemistry Development, Toronto, Canada, 1996.

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