ELSEVIER

Journal of Chromatography B, 739 (2000) 301–312

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Validation of an analytical procedure to measure trace amounts of neurosteroids in brain tissue by gas chromatography–mass spectrometry

Philippe Liere[a,*], Yvette Akwa[a], Sébastien Weill-Engerer[a], Bernard Eychenne[a], Antoine Pianos[a], Paul Robel[a], Jan Sjövall[b], Michaël Schumacher[a], Etienne-Emile Baulieu[a]

[a]*INSERM U488 et Collège de France, 80 Rue du Général Leclerc, 94276 Le Kremlin-Bicêtre Cédex, France*
[b]*Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77, Stockholm, Sweden*

Received 16 September 1999; received in revised form 26 November 1999; accepted 29 November 1999

Abstract

A selective and extremely sensitive procedure has been developed and optimized, using high-performance liquid chromatography (HPLC), specific derivatization and gas chromatography–mass spectrometry (GC–MS), to simultaneously quantify very small amounts of different neurosteroids from rat brain. Unconjugated and sulfated steroids in brain extracts were separated by solid-phase extraction. The unconjugated fraction was further purified by HPLC, the steroids being collected in a single fraction, and the sulfated fraction was solvolyzed. All steroids were derivatized with heptafluorobutyric acid anhydride and analyzed by GC–MS (electron impact ionization) using selected-ion monitoring. High sensitivity and accuracy were obtained for all steroids. The detection limits were 1 pg for pregnenolone (PREG), dehydroepiandrosterone (DHEA) and their sulfate esters PREG-S and DHEA-S, 2 pg for progesterone (PROG) and 5 pg for 3α,5α-tetrahydroprogesterone (3α,5α-THP). In a pilot study on a rat brain, the concentrations of PREG-S and DHEA-S were 8.26±0.80 and 2.47±0.27 ng/g, respectively. Those of PREG, DHEA and PROG were 4.17±0.22, 0.45±0.02 and 1.95±0.10 ng/g, respectively. Good linearity and accuracy were observed for each steroid. The methodology validated here, allows femtomoles of neurosteroids, including the sulfates, found in small brain samples (at least equal to 10 mg) to be quantified simultaneously.

Keywords: Neurosteroids

1. Introduction

Steroids that are present in the central nervous system (CNS) can originate from steroidogenic endocrine glands (gonads and adrenals) directly or after the conversion of blood-borne precursors, or from local synthesis. The neurosteroids include those steroids whose accumulation in the CNS (and peripheral nervous system) occurs independently of peripheral steroidogenic sources and which can be synthesized by glial cells and neurons de novo from sterol precursors (for reviews see Refs. [1–3]). Using radioimmunoassays (RIA) with specific antibodies, the cerebral concentrations of pregnenolone (PREG), dehydroepiandrosterone (DHEA) and their sulfate esters PREG-S and DHEA-S have been found

*Corresponding author. Tel.: +33-1-4959-1894; fax: +33-1-4521-1940.
E-mail address: liere@kb.inserm.fr (P. Liere)

0378-4347/00/$ – see front matter
PII: S0378-4347(99)00563-0

to be larger in the rat brain than in plasma, and did not significantly change after removal of endocrine glands [4,5]. Progesterone (PROG) has also been assayed in the rat brain by RIA [6,7] and is now considered to be a neurosteroid. In the CNS, glial cells are responsible for most neurosteroid synthesis. PREG is obtained by the cleavage of cholesterol through the action of the cytochrome P-450 side chain cleavage enzyme [8], PROG is formed from PREG by the 3β-hydroxysteroid dehydrogenase-Δ_{5-4} isomerase and can further be reduced to 5α-dihydroprogesterone (5α-DHP) and 3α,5α-tetrahydroprogesterone (3α,5α-THP) [9–11].

Some neurosteroids influence CNS functions as paracrine/autocrine regulators (for reviews [12–14]). These neuroactive neurosteroids can alter neuronal excitability by modulating the activity of different neurotransmitter receptors, including $GABA_A$, NMDA and sigma receptors, and thus influence several types of behavior. Variations in the brain concentrations of neurosteroids can be induced under pharmacological conditions [15,16] and can be related to changes in behavior such as anxiety [17]. Such variations can also be observed in different physiological states, including stress [18,19] and premenstrual syndrome [20].

Although neurosteroids have originally been measured in brain by RIA, gas chromatography–mass spectrometry (GC–MS) is becoming increasingly used for identifying and measuring steroids in biological sources. Compared with RIA, GC–MS avoids some problems related to antibody specificity and lack of sensitivity. Quantification of neurosteroids in the rat brain using GC–MS with selected-ion detection has been described [21,22]. However in these reports, steroids were generally measured individually following high-performance liquid chromatography (HPLC) of relatively large brain samples (either the whole brain or pools of brain regions).

In the present work, we describe the validation of a methodology that allows rigorous identification and simultaneous quantification of several neurosteroids from small samples of rat brain (in the 10–40 mg range). In this procedure, steroid extraction and fractionation, as well as HPLC purification, were optimized and appropriate steroid derivatization and GC–MS analysis in the electron impact mode were used. Results indicated extremely high sensitivity (detection down to the low femtomole range), high reproducibility and accuracy for the detection of the main neuroactive neurosteroids. Thus, this methodology should be very suitable for determining, simultaneously, concentrations of different neurosteroids, including steroid sulfates, in very small regions of the CNS of single animals, and thus very useful for further studying physiopathological implications of neurosteroids in the modulation of CNS functions.

2. Experimental

2.1. Chemicals

The non-radioactive steroids PREG, DHEA, PROG, 3α,5α-THP, PREG-S (potassium salt) and DHEA-S (sodium salt) were kind gifts from Roussel-Uclaf (Romainville, France) and 3β,5β-tetrahydroandrostenedione (3β,5β-THA) was purchased from Sigma–Aldrich (Saint Louis, MO, USA). Tetradeuterated $^2H_4$-PREG-S ([17,21,21,21-^{2}H]-PREG-S, trimethyl ammonium salt) and $^2H_4$-PREG ([17,21,21,21-^{2}H]-PREG) were prepared by Dr. R.H. Purdy (La Jolla, CA, USA). The radioactive steroids ^{3}H-PREG ([7-^{3}H]-PREG, 25 Ci/mmol], ^{3}H-DHEA ([1,2,6,7-^{3}H]-DHEA, 92.5 Ci/mmol) and ^{3}H-DHEA-S ([1,2,6,7-^{3}H]-DHEA-S, 92.5 Ci/mmol) were provided by New England Nuclear (NEN, Boston, MA, USA), and ^{3}H-PROG ([1,2,6,7-^{3}H]-PROG, 92 Ci/mmol) by Amersham International (UK). ^{3}H-3α,5α-THP ([5,6-^{3}H]-3α,5α-THP, 52.2 Ci/mmol) was provided by Roussel-Uclaf (Romainville, France). ^{3}H-PREG-S ([7-^{3}H]-PREG-S, 25 Ci/mmol, potassium salt) was synthesized in the laboratory from ^{3}H-PREG using pyridine sulphur trioxide kindly given by Dr. R. Emiliozzi (Nice, France) [23]. The degree of purity of all steroids was at least 99%. All solvents were of analytical grade from Carlo Erba (Milan, Italy) and were used without further purification. The derivatization reagent heptafluorobutyric acid anhydride (HFBA) was purchased from Pierce (Rockford, IL, USA).

2.2. Steroid extraction and fractionation

The general analytical procedure is presented in Fig. 1. The entire brain (2 g) of a Sprague-Dawley adult male rat (9 months old, supplied by Janvier,

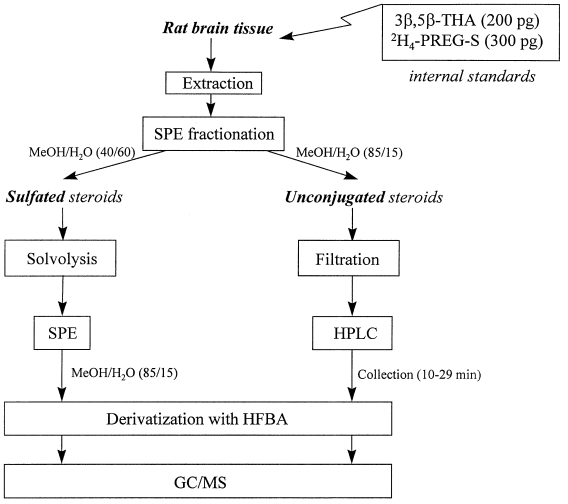

Fig. 1. Analytical procedure for the analysis of the unconjugated and sulfated neurosteroids from rat brain.

CERJ, Le Genest St-Isle, France) was collected in a polypropylene Falcon tube. A solution of methanol (MeOH) containing 1% acetic acid (CH_3CO_2H) (10 volumes, w/v) was added together with 200 pg of 3β,5β-THA and 300 pg of $^2H_4$-PREG-S. The former, a non-endogenous steroid, was systematically used as the internal standard for the four steroids PREG, DHEA, PROG and 3α,5α-THP, and the latter for PREG-S and DHEA-S. The sample was then sonicated in an ultrasonic bath for 5 min, left overnight at room temperature and centrifuged at 3000 *g* for 5 min. The organic phase was collected and the rest of the extract residue washed again with 20 ml of MeOH containing 1% CH_3CO_2H and centrifuged. The two organic phases were pooled and aliquots of 800 μl of the total extract (corresponding to 40 mg of brain tissue) were collected, evaporated to dryness under compressed air in a water bath at 60°C, taken up in 1 ml of MeOH–H_2O (40:60, v/v) and sonicated for 5 min.

An in vivo experiment was carried out to test the efficiency of steroid extraction. One hour following intraperitoneal injection of ^{3}H-PREG (33 μCi) into the rat, the brain was collected and divided into two hemispheres. One part, serving as a control, was homogenized in 5 ml of phosphate buffered saline and digested with 3 ml Soluene 100 (Packard Instruments, Warrenville, RI, USA) at 60°C for 20 min. The other part was extracted as described above. The radioactivity in each hemisphere was determined.

A clean-up step was performed by solid-phase extraction (SPE) on AMPREP silica minicolumns C_{18} (500 mg, Amersham International, UK). This C_{18} SPE method, previously described by Belanger et al. [24] and modified by De Wang et al. [25] allows sulfated and unconjugated steroids to be separated. Samples were deposited on columns which had been previously activated successively with 5 ml of MeOH, 5 ml of H_2O and 5 ml of MeOH–H_2O (40:60, v/v). The fraction of steroid sulfates was eluted with 5 ml of MeOH–H_2O (40:60, v/v) and taken to dryness. The unconjugated steroid fraction was eluted with 5 ml of MeOH–H_2O (85:15, v/v), filtered through a 0.45-μm Millipore PFTE membrane and dried.

2.3. Solvolysis and purification

The sulfated steroids fraction was subjected to solvolysis in 1 ml ethyl acetate containing 2 μl of 0.5 *M* H_2SO_4 [26] at room temperature for 1 h. The organic phase containing unconjugated steroids was washed with H_2O and evaporated. The residue was taken up in 1 ml of MeOH–H_2O (40:60, v/v) and purified by SPE as described above. The fraction eluted with 5 ml of MeOH–H_2O (85:15, v/v) was collected and dried.

2.4. High-performance liquid chromatography (HPLC)

The HPLC system from Thermoquest (San Jose, CA, USA) consisted of a P1000XR Thermo Separation Product (TSP) quaternary pump and an AS 100XR TSP autoinjector. A 202 model Gilson fraction collector was used. HPLC was achieved with a Lichrosorb Diol column (25 cm×4.6 mm, 5 μm) in a thermostated block at 30°C.

Only the unconjugated steroid fraction that had been purified by SPE and filtered, was submitted to HPLC. The analytical column was first equilibrated in a solvent system of hexane and mixture A (90:10, v/v), the latter being composed of hexane–isopropanol (85:15, v/v). One hundred and eighty μl of the steroid fraction dissolved in hexane–isopropanol (91:9, v/v) were then injected. The elution was performed at a flow-rate of 1 ml/min, first with

hexane and mixture A (90:10, v/v) for 15 min, then with a linear gradient to 30% mixture A in 2 min. This mobile phase was kept constant for 13 min, after which the proportion of mixture A was increased to 100% in 5 min and kept constant for 19 min. Finally, the column was washed with 100% MeOH for 5 min.

2.5. Derivatization reaction

The unconjugated steroids from the HPLC fraction and those obtained after solvolysis and purified by SPE were derivatized separately. They were transferred in 1 ml of anhydrous acetone to reaction vials under anhydrous conditions (the vial was previously heated and cooled down under nitrogen). After complete evaporation, 20 μl of HFBA and 200 μl of anhydrous acetone were added and the vial closed with a PFTE-coated cap. The reaction was then carried out at room temperature for 30 min. After evaporation, the residue was redissolved in 12 μl of hexane.

Calibration solutions had previously been derivatized with HFBA, under the same conditions as described above. A mixture containing PREG and DHEA (1, 2, 5, 10 and 20 pg/μl for each steroid), PROG (2, 5, 10, 20 and 40 pg/μl), 3α,5α-THP (5, 10, 20, 40 and 60 pg/μl) and the internal standard 3β,5β-THA (20 pg/μl) was used for unconjugated steroids, and a mixture containing PREG and DHEA (1, 2, 5, 10 and 20 pg/μl for each steroid) and the internal standard $^{2}H_{4}$-PREG (20 pg/μl) was used for steroids released from sulfate esters.

2.6. Gas chromatography–mass spectrometry (GC–MS)

Derivatized brain samples (2 μl) as well as derivatized calibration solutions (1 μl) were injected with an AS 800 autosampler (Carlo Erba, Milan, Italy) into the GC–MS system which consisted of a GC 8000 Top gas chromatograph (Carlo Erba) coupled with an Automass (model 150) mass spectrometer (Finnigan Automass, Argenteuil, France). The fused-silica capillary column (25 m×0.22 mm) was coated with 0.25-μm layer of cross-linked BPX5 [5% phenyl (equivalent), 95% dimethyl polysiloxane; SGE, Australia].

GC was performed in the splitless mode with a 1-min splitless-time. The temperature in the oven was initially 50°C for 1 min, further increased to 140°C at 30°C/min and then to 300°C at 10°C/min. The temperature in the injection chamber was 250°C and the flow-rate of helium (carrier gas) was maintained constant at 0.7 ml/min.

The mass spectrometer was operated in the electron impact (EI) mode with an emission current of 2000 μA and with an ionization energy of 70 eV. The temperatures in the ionization chamber and the interface were 170°C and 290°C, respectively. Identification was performed in the full scan mode in the m/z range 50–550. Quantification was done in the single-ion monitoring (SIM) mode.

2.7. Radioactivity analysis

Five ml of scintillation liquid (Picofluor 15) was added to counting vials containing dried radioactive samples, and the radioactivity was measured on a Tricarb 4660 liquid-scintillation spectrometer (Packard Instruments) equipped with quench correction.

2.8. Data analysis

Data were expressed as means±SEM (standard error of mean). Linear regression analysis using the least square method was calculated for the calibration curve of each neurosteroid.

3. Results and discussion

The aim of this work was to develop a general and very sensitive analytical procedure for the quantification of unconjugated and sulfated 3β-hydroxy-Δ_5-neurosteroids, using extraction, fractionation and purification steps sufficient to avoid sample overloading of the GC column, with acceptable recovery and reproducibility.

3.1. Extraction, fractionation and purification of neurosteroids

A pilot study was performed to optimize the extraction, SPE and HPLC methods and estimate the recoveries of all these steps. For this purpose, the

radioactive steroids ^{3}H-PREG, ^{3}H-DHEA, ^{3}H-PROG, ^{3}H-3α,5α-THP and ^{3}H-PREG-S (30 000 dpm of each steroid) were individually added to isolated brain samples.

Quantitative extraction of steroids from brain tissue is somewhat difficult to estimate [27]. Nevertheless, the same amounts of ^{3}H-PREG were found in both methanolic brain extract and digested brain (for details see the Experimental section), leading to the conclusion that the extraction recovery was 100%. This suggests that an isotopic equilibrium is reached within the brain following radioactive neurosteroid injection into the rat.

The fractionation method by SPE on C_{18} columns was chosen for its efficient and easy separation of unconjugated and sulfated steroids compared with other methods such as liquid–liquid extraction. Two fractions were obtained from brain extracts, the sulfated steroids being eluted with MeOH–H_2O (40:60, v/v) and the free steroids with MeOH–H_2O (85:15, v/v). The recoveries were 75±5% in both cases.

The general goal of the study was to be able to quantify steroids with closely related structures (3-hydroxy-, 3-oxo-Δ_4- and 3-oxo-steroids) and steroids of different classes (progestagens, estrogens, corticosteroids). Thus, unconjugated steroids need to be separated by HPLC prior to their chemical structure-specific derivatization.

In the present paper, the use of HPLC served to clean up a mixture of unconjugated steroids and to separate them into two fractions, the one of interest containing 3-hydroxy- and 3-oxo-Δ_4-steroids.

Unconjugated steroids were analyzed individually in the HPLC system consisting of a Diol column and eluted with a solvent sequence used for the separation of steroids with high resolution [21,22]. Fractions of 500 μl were collected and the radioactivity counted to determine the elution profile and retention times of steroids (Fig. 2). Under these conditions, the four radioactive steroids PROG, 3α,5α-THP, PREG and DHEA and the corresponding internal standard 3β,5β-THA (which is not an endogenous steroid) were eluted between 10 and 29 min (Fig. 2). The retention time of the derivatized internal standard 3β,5β-THA was determined by GC–MS because its radioactive form was not available commercially. The recovery after HPLC purification was 75±10% for all steroids. Such a simplification in the HPLC method avoids the use of an internal standard for each steroid, greatly shortens the time for sample preparation and also results in a gain of precision in quantification. Therefore, the global recovery was 54±10% for all steps of sample preparation (including extraction, SPE, and HPLC).

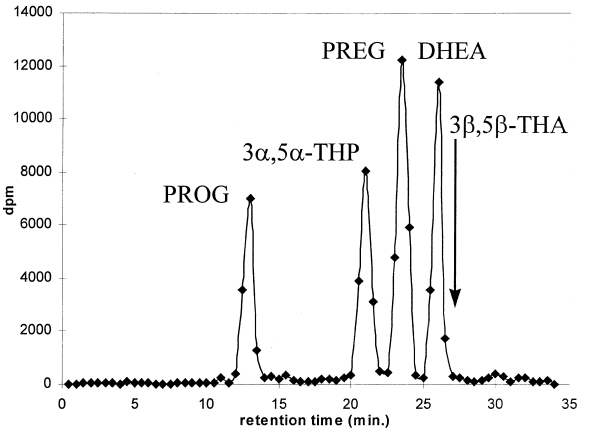

Fig. 2. HPLC elution profile of radioactive PROG, 3α,5α-THP, PREG and DHEA. The internal standard 3β,5β-THA was identified by GC–MS.

For the sulfated steroids PREG-S and DHEA-S, which were not purified by HPLC, the recovery of the entire analytical procedure using ^{3}H-PREG-S was 57±5%.

3.2. Identification of neurosteroids

The choice of the derivatization reaction was based on the presence of a hydroxyl group in the steroid structure. Trifluoroacetyl, pentafluoropropionyl and heptafluorobutyryl (HFB) anhydrides are mostly used to derivatize alcohol functions. The formation of such steroid derivatives results in a decrease of polarity and an increase of volatility and thermal stability, which are crucial advantages for GC. Furthermore, such derivatization reactions give improved detection properties in the electron-capture negative ionization mode (ECNI) [22,28,29]. A highly sensitive analysis can then be obtained because of the high response of halogenated derivatives and the selectivity of ionization. However, the ECNI detection mode lacks robustness, so that EI is more often used for quantification of perfluoroacylated

derivatives of steroids in biological fluids or tissue samples. Indeed, with this type of derivatives, EI mass spectra are generally characterized by abundant ionic species of high mass. Thus, the perfluoroacylation, and particularly the formation of heptafluorobutyrate derivatives, is considered as a method of choice for steroid analysis by GC–MS [30,31].

PROG with its 3-oxo-Δ_4 group reacts similarly to 3-hydroxy steroids in the derivatization reaction with HFBA. For this reason, all of the unconjugated steroids can be purified and collected together by HPLC. The derivatization reaction of PROG with HFBA was optimized using specific conditions (Fig. 3). In these conditions, two dienol 3-HFB isomers (2,4- and 3,5-dienols) were formed with the major isomer (95%) being the 3,5 dienol 3-HFB resulting in an enhancement of sensitivity. Any changes in reaction time or reagent quantity can strongly affect the ratio between the two isomers [31]. Such a derivatization method has previously been reported for testosterone-17β-acetate, which was found to be mainly converted to 3,5-dienol-3-HFB when it occurred in acetone containing 10% of HFBA for 30 min [31].

In the present experiments, all steroids were completely converted into derivatives by reaction with HFBA (100% recovery). The 3-hydroxy groups formed 3-HFB esters as it was found by others for steroids hydroxylated at the C_3 and C_{17} positions [21,22,32,33]. The 3-oxo-group of PROG gave 3-HFB enol esters as described by Cheney et al. [21].

The reconstructed ion chromatograms of the unconjugated fraction of the reference compounds, i.e. PREG-HFB, DHEA-HFB, PROG-HFB, 3α,5α-THP-HFB and the internal standard 3β,5β-THA-HFB are shown in Fig. 4A. The chromatograms of the fraction

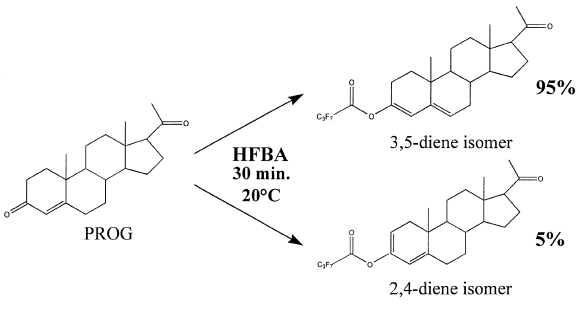

Fig. 3. Derivatization reaction pathways of PROG with HFBA.

of the reference sulfated steroids after solvolysis i.e. PREG-HFB, DHEA-HFB and $^2H_4$-PREG-HFB are shown in Fig. 4B. The EI mass spectra of reference internal standards are represented in Fig. 5 and those of reference neurosteroids are shown in Fig. 6. The GC conditions gave identical retention times for PROG-HFB and PREG-HFB. However, since ions of different mass were monitored, i.e. m/z 298 for PREG-HFB and m/z 510 for PROG-HFB, no interference could occur in the quantification of these steroids. Furthermore, the retention time of PREG-HFB was 3 s longer than that of the internal standard $^2H_4$-PREG-HFB and this facilitated identification and integration calculations. The other steroids were clearly separated by the BPX5 column.

The fragmentation pathways of the HFB-steroids were generally favourable. For example, the molecular ion of the internal standard 3β,5β-THA ($M^{+\cdot}$ 486) was relatively abundant. The derivatives of the 3β-hydroxy-Δ_5-steroids underwent similar fragmentations. The molecular ions of PREG-HFB and DHEA-HFB were absent in their EI mass spectra and the major ions came from the loss of heptafluorobutyric acid (214) from the molecular ion (Fig. 6). The diagnostic ion m/z 302 is the base peak in the EI mass spectrum of $^2H_4$-PREG-HFB (Fig. 5) indicating that there was no or very little H/^{2}H exchange during the derivatization reaction with HFBA under our conditions. The extent of fragmentation of 3α,5α-THP-HFB was higher than those of other steroids, the abundance of the ionic species of high m/z being low. The EI mass spectrum of the acylated PROG was the most favourable case, the molecular ion ($M^{+\cdot}$ 510) being the base peak.

3.3. Quantification of neurosteroids

For the quantification of neurosteroids in brain tissue, the high sensitivity obtained with our analytical procedure is a great advantage, in particular when only a small amount of material (10 mg) is available.

3.3.1. Sensitivity

It is well known that the sensitivity can be increased by selected monitoring of relevant ions. Ionic species of high intensity and high m/z ratio are generally chosen to increase the selectivity of the

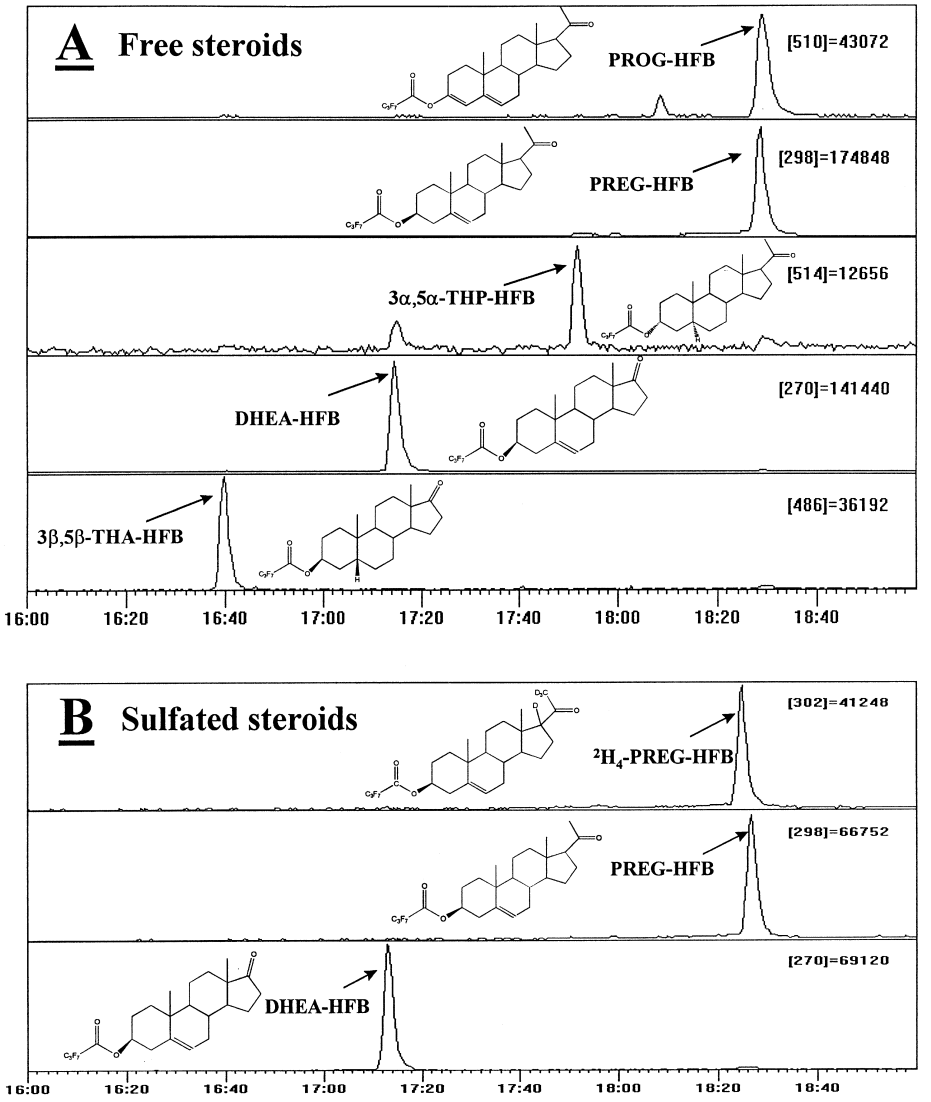

Fig. 4. Reconstructed ion chromatograms of reference compounds (1 ng) in full scan mode: (A) the unconjugated steroid fraction containing 3β,5β-THA-HFB (*m*/*z* 486), DHEA-HFB (*m*/*z* 270), 3α,5α-THP-HFB (*m*/*z* 514), PREG-HFB (*m*/*z* 298) and PROG-HFB (*m*/*z* 510), and (B) the sulfated steroid fraction after solvolysis containing $^2H_4$-PREG-HFB (*m*/*z* 302), PREG-HFB (*m*/*z* 298) and DHEA-HFB (*m*/*z* 270).

detection relative to the electronic and chemical noise. The diagnostic ions for each steroid analyzed by GC–MS are given in Table 1.

The ions selected for DHEA-HFB and PREG-HFB were *m*/*z* 270 and *m*/*z* 298, respectively. These ions were the base peaks of the respective mass spectra (Fig. 6) and originated from an analogous fragmentation pathway as discussed below. Identification was further ascertained by analyzing the *m*/*z* 255 and *m*/*z* 283 ions of DHEA-HFB and PREG-HFB, respectively. These ionic species were formed by the loss of a methyl radical from *m*/*z* 270 and *m*/*z* 298, respectively. The detection limits were 1 pg for PREG and DHEA (3.2 and 3.5 fmol, respectively), and also 1 pg for PREG-S and DHEA-S (2.3 and 2.5 fmol, respectively).

The respective detection limits for PROG-HFB and 3α,5α-THP-HFB were 2 pg (6.4 fmol) and 5 pg

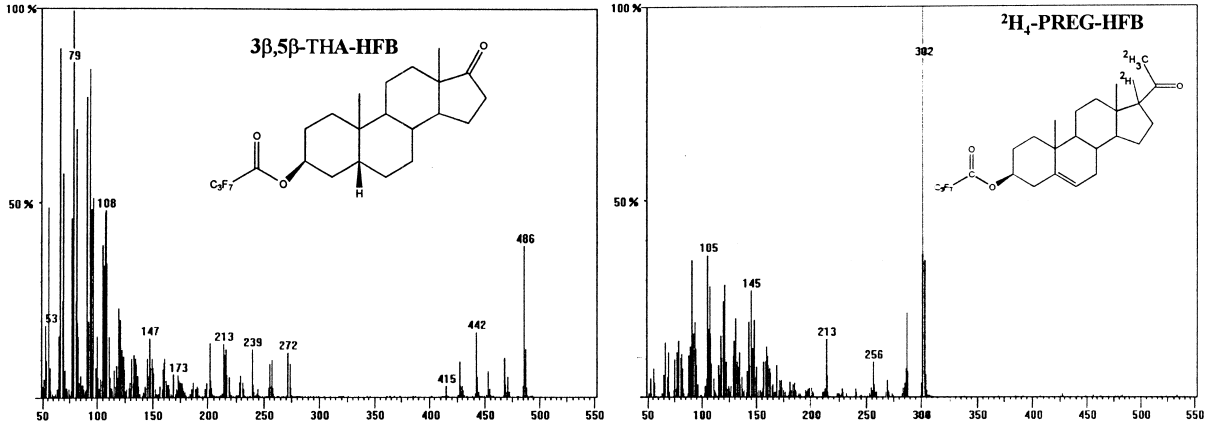

Fig. 5. EI mass spectra of the reference internal standards 3β,5β-THA-HFB and $^2H_4$-PREG-HFB from the unconjugated and sulfated fractions, respectively.

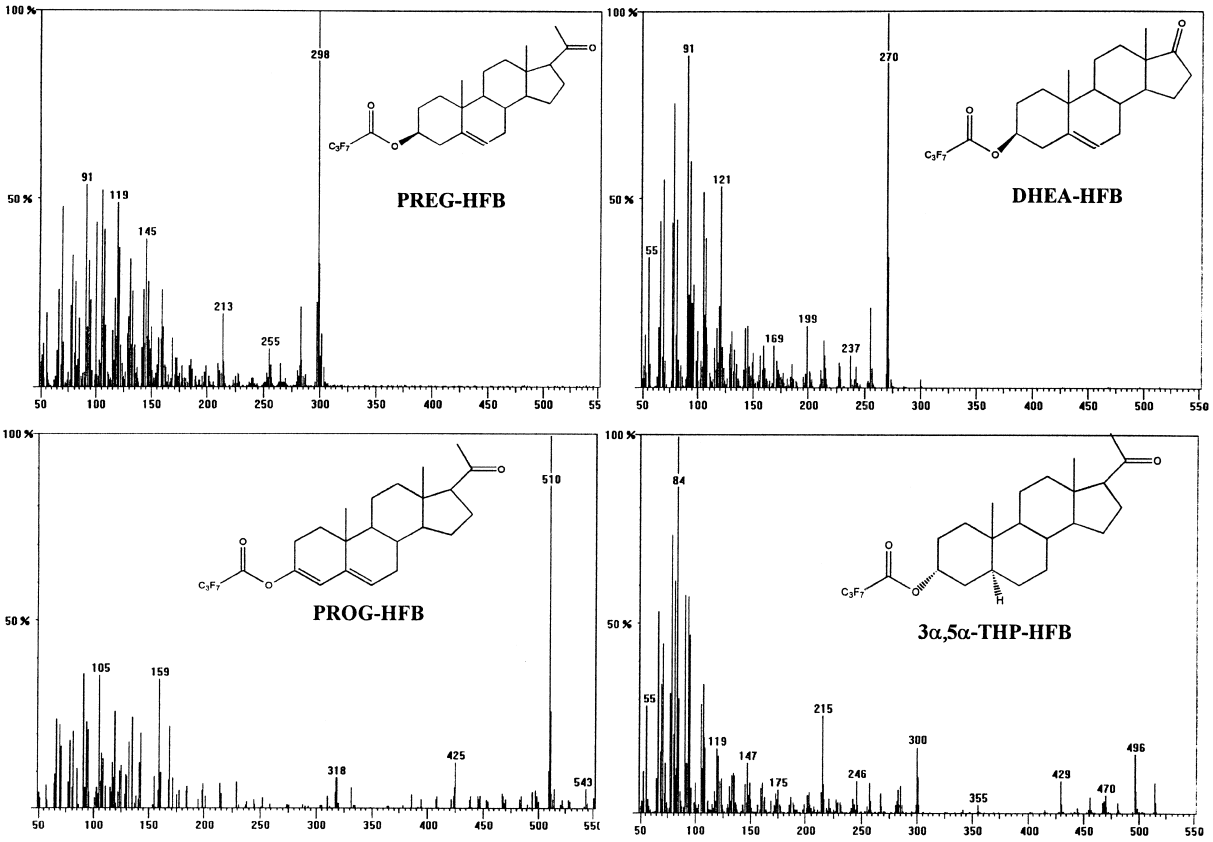

Fig. 6. EI mass spectra of the reference neurosteroids PREG-HFB, DHEA-HFB, PROG-HFB and 3α,5α-THP-HFB.

Table 1
Molecular mass of steroids and derivatized steroids with values of the respective diagnostic ions analyzed by GC–MS

Steroids	Molecular mass (Da)	Derivatized steroids	Molecular mass of derivatized steroids (Da)	Diagnostic ions m/z
PREG	316	PREG-HFB	512	298[a]
DHEA	288	DHEA-HFB	484	270[a]
PROG	314	PROG-HFB	510	510
3α,5α-THP	318	3α,5α-THP-HFB	514	514
3β,5β-THA	290	3β,5β-THA-HFB	486	486
$^2H_4$-PREG	320	$^2H_4$-PREG-HFB	516	302[a]

[a] Formed by the loss of heptafluorobutyric acid (214 Da) from the molecular ion. Note: PREG-S and DHEA-S were analyzed as PREG and DHEA, respectively, after solvolysis of the sulfated fraction.

(15.7 fmol), respectively, based on the relative intensity of the respective target ions m/z 510 and m/z 514 in the corresponding mass spectra (Fig. 6). The m/z 495 ($M^{+\cdot}$-$CH_3^{\cdot}$) and m/z 496 ($M^{+\cdot}$-H_2O), respectively, were also monitored to definitely identify the PROG-HFB and 3α,5α-THP-HFB signals.

The calibration curves for the unconjugated neurosteroids (towards the internal standard 3β,5β-THA-HFB) and for the sulfated neurosteroids (towards the internal standard ^{2}H-PREG-HFB) are suitable, according to good linearity and correlation coefficients for PREG ($y=0.8787x-0.2844$, $r=0.9998$), DHEA ($y=0.6037x-0.2766$, $r=0.9986$), PROG ($y=0.0724x+0.2361$, $r=0.9944$), 3α,5α-THP ($y=0.0096x-0.008$, $r=0.9958$), PREG-S ($y=0.0372x+0.0021$, $r=0.9927$) and DHEA-S ($y=0.0512x-0.0557$, $r=0.9979$). PREG, DHEA, PREG-S and DHEA-S would be detected at levels higher than 0.3 ng/g when starting with 40 mg brain tissue, based on their detection limits. On the other hand, PROG and 3α,5α-THP would be detected at concentrations ≥0.6 ng/g and 1.5 ng/g, respectively.

Preliminary experiments on another neurosteroid namely 5α-dihydroprogesterone (5α-DHP, a metabolite of PROG) were performed. 5α-DHP was analyzed under the same HPLC conditions as described here, and was eluted separately in a less polar fraction (3–10 min) (data not shown). As this steroid is in the 3-oxo-5α configuration, it does not react with HFBA and a specific derivatization reaction with methoxyamine reagent was used. Indeed, methyloxime formation is the most popular derivatization reaction for ketosteroids [34,35]. 5α-DHP forms a dimethyloxime derivative by reaction at C_3 and C_{20}. Two isomers (*syn* and *anti*) are formed at C_3 in the same proportions [21,36], thus decreasing sensitivity two fold. In our study, a suitable fragmentation pathway was obtained in EI with these oximes. Indeed, in spite of the low abundance of the molecular ion ($M^{+\cdot}$ 374), high amounts of the ionic species m/z 343 were formed by EI. This ion originates from the loss of a methoxy radical (−31) from the molecular ion. Unfortunately, the detection limit of 5α-DHP was as high as 50 pg (158 fmol). Taking into account the detection limit, the dilution factor and the recovery, it appears that 5α-DHP cannot be detected at levels lower than 15 ng/g in 40 mg tissue. The low sensitivity is probably due to the formation of two isomers and the relatively high chemical background with the methoxyamine reagent.

3.3.2. Reproducibility

The inter-assay variations for neurosteroid quantification were investigated on ten fractions of the same rat brain extract, each corresponding to 40 mg tissue. The SEM and coefficients of variation (C.V.) values for all steroids indicate a satisfactory reproducibility. As shown in Table 2, sample A, PREG

Table 2
Reproducibility of quantification of the unconjugated (A) and sulfated (B) neurosteroids in rat brain by GC–MS

	A ($n=10$)			B ($n=5$)	
	PREG	DHEA	PROG	PREG-S	DHEA-S
Mean (ng/g)	4.17	0.45	1.95	8.26	2.47
SEM	0.22	0.02	0.10	0.80	0.27
C.V. (%)	17	13	17	21	23

was the neurosteroid characterized by the highest concentration in rat brain (4.17±0.22 ng/g). PROG and DHEA were also measured, albeit at lower levels (1.95±0.10 and 0.45±0.02 ng/g, respectively), but 3α,5α-THP was not detected in this brain sample probably because of its higher detection limit compared with that of the other steroids. Our data show that the levels of PROG and DHEA found in rat brain (Table 2) are in good agreement with the results of others obtained with large amounts of tissue using either RIA [4,5] or GC–MS [21]. PREG-S and DHEA-S levels in rat brain were 8.26±0.80 ng/g and 2.47±0.27 ng/g, respectively with C.V.s slightly larger than those for the unconjugated steroids (Table 2, sample B). Previous studies have shown that PREG-S is present in adult male rats, at 15.8±3 ng/g and 5.7±2.1 ng/g in anterior and posterior brain, respectively [5]. In addition, the brain concentrations of PREG, PREG-S and DHEA-S were similar to those reported by De Wang et al. [25] and Corpéchot et al. [4].

3.3.3. Linearity and accuracy

Linearity and accuracy are other criteria to validate an analytical method. For the assessment of linearity, increasing amounts of rat brain fractions (10, 20, 40, 70 and 100 mg) were assayed in triplicate. The linearity was satisfactory for all steroids studied since the measured amounts of neurosteroids increased as expected with increasing amounts of brain tissue (Fig. 7). Remarkably, PREG, PROG and PREG-S were measured accurately in brain fractions weighing as little as 10 mg. However, due to low concentrations of DHEA and 3α,5α-THP in rat brain and the relatively high detection limit for 3α,5α-THP, these two neurosteroids were measurable only from samples weighing at least 40 mg.

The criterion of accuracy is the relationship between the amounts of added steroid and the amounts detected by the GC–MS assay. In the present work, 100 pg of unconjugated steroids were added to an aliquot of brain extract corresponding to 40 mg, whereas 200 pg of sulfated steroids were added to 50 mg of tissue. The accuracy values were expressed as percentage of assayed concentration relative to calculated concentration. They were 108±9%, 105±1%, 113±8%, 112±5%, 98.3±5% and 96.2±5% for PREG, DHEA, PROG, 3α,5α-THP, PREG-S and DHEA-S, respectively.

4. Conclusion

We have developed a methodology for neurosteroid quantification in very small samples of brain tissue. The optimization of extraction, fractionation by SPE, purification by HPLC of unconjugated neurosteroids and solvolysis of sulfated neurosteroids, together with the combination of the derivatization with HFBA and GC–MS analysis by SIM provides a highly selective and sensitive analytical procedure. This should contribute to improving the quality of trace analysis of neurosteroids from complex tissue such as brain. The results of the reproducibility, linearity and accuracy experiments indicate that the procedure is reliable for quantification of neurosteroids at low levels using a wide range of brain sample sizes. Although PREG-S and DHEA-S were previously analyzed by LC–MS [26,37–40], this is, to our knowledge, the first report showing a methodology for the determination of the levels of both unconjugated and sulfated forms of PREG and DHEA in the same biological sample by GC–MS.

Thus, the highly reliable procedure that is validated here, is potentially of extensive use to perform serial assays of neurosteroids in small brain structures of individual animals, under different pathophysiological conditions.

Acknowledgements

We thank Dr. Robert H. Purdy (Scripps Research Institute, La Jolla, CA, USA) for the synthesis of deuterated PREG and PREG-S, and the Del Duca Foundation which contributed to his financial support. This research was supported by the European Community BioMed Program (contract BMH4-CT-97-2349), and the Fondations Gould (New York) and Leila Y. Mathers (New York). P.L. was also supported by the BioMed Program. Y.A. was the recipient of a fellowship from the Foundation Prince Louis de Polignac.

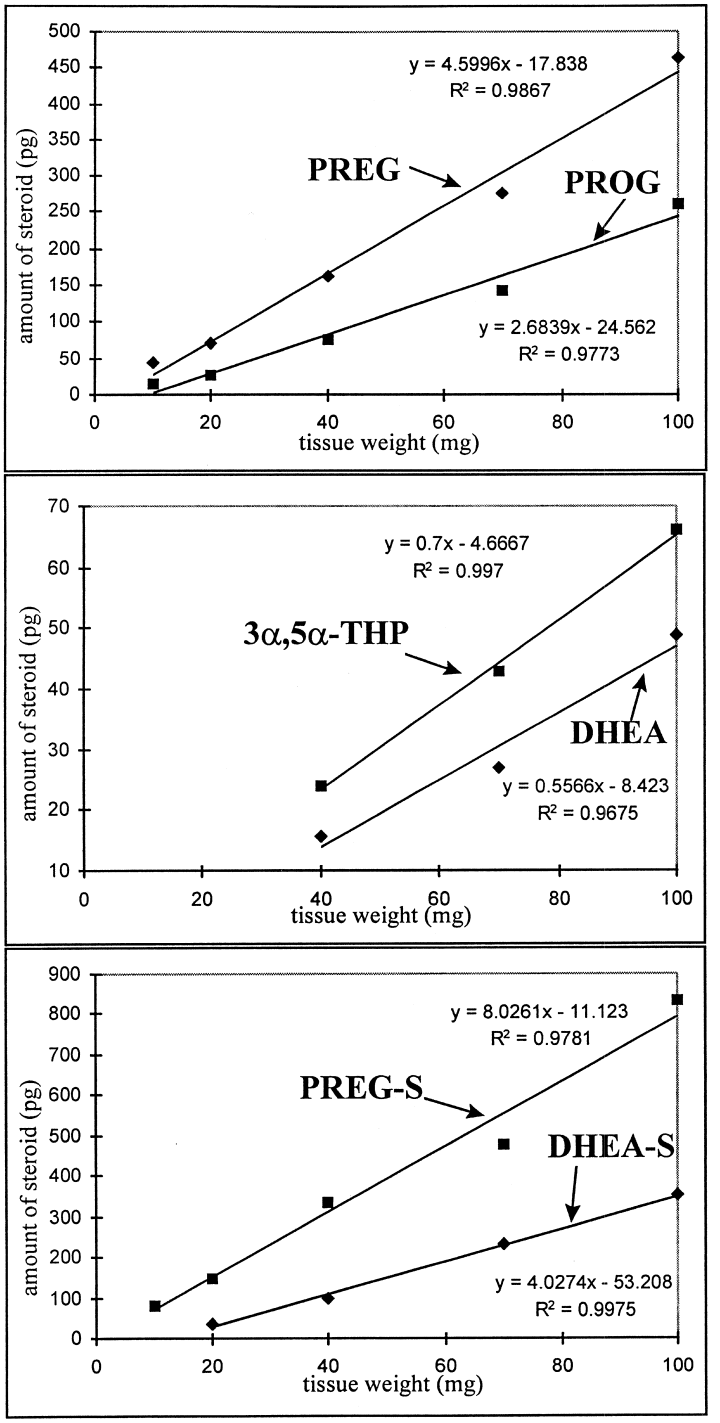

Fig. 7. Linearity studies of neurosteroid quantification in rat brain. The amounts of rat brain fractions were assayed in triplicate.

References

[1] P. Robel, E.-E. Baulieu, Trends Endocrinol. Metab. 5 (1994) 1.
[2] E.-E. Baulieu, Recent Prog. Horm. Res. 52 (1997) 1.
[3] P. Robel, M. Schumacher, E.-E. Baulieu, in: E.-E. Baulieu, P. Robel, M. Schumacher (Eds.), Contemporary Endocrinology: Neurosteroids: A New Regulatory Function in the Nervous System, Humana Press Inc, Totowa, NJ, 1999, p. 1.
[4] C. Corpéchot, P. Robel, M. Axelson, J. Sjövall, E.-E. Baulieu, Proc. Natl. Acad. Sci. USA 78 (1981) 4704.
[5] C. Corpéchot, M. Synguélakis, S. Talha, M. Axelson, J. Sjövall, R. Vihko et al., Brain Res. 270 (1983) 119.
[6] M. Bixo, T. Bäckström, S. Cajander, B. Winblad, Acta Pathol. Microbiol. Immunol. Scand [A] 94 (1986) 297.
[7] C. Corpéchot, J. Young, M. Calvel, C. Wehrey, J.N. Veltz, G. Touyer et al., Endocrinology 133 (1993) 1003.
[8] Z.-Y. Hu, E. Bourreau, I. Jung-Testas, P. Robel, E.-E. Baulieu, Proc. Natl. Acad. Sci. USA 84 (1987) 8215.
[9] I. Jung-Testas, Z.-Y. Hu, E.-E. Baulieu, P. Robel, Endocrinology 125 (1989) 2083.
[10] K. Kabbadj, M. El-Etr, E.-E. Baulieu, P. Robel, Glia 7 (1993) 170.
[11] Y. Akwa, N. Sananès, M. Gouézou, P. Robel, E.-E. Baulieu, C. Le Goascogne, J. Cell Biol. 121 (1993) 135.
[12] E.-E. Baulieu, in: K. Fuxe, J.A. Gustafsson, L. Wetterberg (Eds.), Steroid Hormone Regulation in the Brain, Pergamon Press, Oxford, 1981, p. 3.
[13] S.M. Paul, R.H. Purdy, FASEB J. 6 (1992) 2311.
[14] E.-E. Baulieu, P. Robel, J. Endocrinol. 150 (Suppl.) (1996) S221.
[15] J. Young, C. Corpéchot, F. Perché, B. Eychenne, M. Haug, E.-E. Baulieu, Steroids 61 (1996) 144.
[16] V. Uzunova, Y. Sheline, J.M. Davis, A. Rasmusson, D. Uzunov, P.E. Costa et al., Proc. Natl. Acad. Sci. USA 95 (1998) 3239.
[17] D. Bitran, M. Shiekh, M. McLeod, Neuroendocrinology 7 (1995) 171.
[18] R.H. Purdy, A.L. Morrow, P.H.J. Moore, S.M. Paul, Proc. Natl. Acad. Sci. USA 88 (1991) 4553.
[19] M.L. Barbaccia, G. Roscetti, M. Trabucchi, R.H. Purdy, M.C. Mostallino, A. Concas et al., Br. J. Clin. Endocrinol. Metab. 81 (1996) 1076.
[20] M. Wang, L. Seippel, R.H. Purdy, T. Bäckström, J. Clin. Endocrinol. Metab. 81 (1996) 1076.
[21] D.L. Cheney, D. Uzunov, E. Costa, A. Guidotti, J. Neurosci. 15 (1995) 4641.
[22] D.P. Uzunov, T.B. Cooper, E. Costa, A. Guidotti, Proc. Natl. Acad. Sci. USA 93 (1996) 12599.
[23] E.E. Baulieu, R. Emilliozzi, Bull. Soc. Chim. Biol. 44 (1962) 823.
[24] A. Belanger, J. Couture, S. Caron, R. Roy, Ann. NY Acad. Sci. 595 (1990) 251.
[25] M.D. De Wang, G. Wahlström, T. Bäckström, J. Steroid Biochem. Mol. Biol. 62 (1997) 299.
[26] K. Shimada, Y. Kukai, K. Yago, J. Liq. Chrom. Rel. Technol. 21 (1998) 765.
[27] J. Sjövall, M. Axelson, Vitam. Horm. 39 (1982) 31.
[28] M.H. Choi, B.C. Chung, M. Kim, J. Choi, Y. Kim, Rapid Commun. Mass Spectrom. 12 (1998) 1749.
[29] M.H. Choi, B.C. Chung, W. Lee, U.C. Lee, Y. Kim, Rapid Commun. Mass Spectrom. 13 (1999) 376.
[30] S.J. Gaskell, B.G. Brownsey, G.V. Groom, Clin. Chem. 30 (1984) 1696.
[31] L. Dehennin, R. Scholler, Tetrahedron 29 (1973) 1591.
[32] L. Dehennin, A. Reiffsteck, R. Scholler, J. Chromatogr. Sci. 10 (1972) 224.
[33] L. Siekmann, J. Steroid Biochem. 11 (1979) 117.
[34] J.P. Thenot, E.C. Horning, Anal. Lett. 5 (1972) 801.
[35] K. Blau, J. Halket (Eds.), Handbook of Derivatives for Chromatography, John Wiley and Sons Ltd, Chichester, 1993, p. 40.
[36] K.T. Koshy, D.G. Kaiser, A.L. VanDerSlik, J. Chromatogr. Sci. 13 (1975) 97.
[37] S.J. Gaskell, Steroids 55 (1990) 458.
[38] C.H. Shackleton, C. Kletke, S. Wudy, J.H. Pratt, Steroids 55 (1990) 472.
[39] M. Nakajima, S. Yamato, K. Shimada, Biomed. Chromatogr. 12 (1998) 211.
[40] W.J. Griffiths, S. Liu, Y. Yang, R.H. Purdy, J. Sjövall, Rapid Commun. Mass Spectrom. 13 (1999) 1595.