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Review

Separation and detection of neuroactive steroids from biological matrices

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Abstract

This review is based on a selection of research papers published mainly in the last decade and it describes various analytical aspects of separation and detection of neuroactive steroids in biological matrices. © 2002 Published by Elsevier Science B.V.

Keywords: Reviews; Steroids; Steroid hormones; Lipids; Hormones

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1. General introduction

With the exception of retinoic acid, steroid hormones are biologically active low-molecular lipids naturally synthesized from cholesterol (3β-hydroxy-5-cholestene). These compounds, except the vitamin D metabolites, have the perhydrocyclopentenophenanthrene molecular backbone in common and atomic numbering system equal to cholesterol. The structures and the nomenclature of steroids have been well described by Kirk and Marples [1] and steroids with 21 carbon atoms are known as pregnanes, whereas those containing 18 and 19 carbon atoms are known as estranes and androstanes, respectively. Individual metabolites are characterized by the presence or absence of functional groups at certain positions in the molecular backbone, where hydroxy, keto, and aldehyde functionalities are by far the most common substituents, and are usually located at positions 3, 5, 11, 17, 18, 20, or 21. The functionalities can be oriented in either equatorial or axial position, yielding a vast number of possible stereoisomers. Apart from a few of the metabolites, these lipophilic molecules are essentially planar and exist in the boat, rather than in the chair form. Functional groups having β-configuration are located above the plane whereas those with α-configuration are situated below the plane of the molecular backbone. Metabolites containing a number of functional groups have rather complicated systematic names and often multiple trivial names, and to avoid confusion it is thus recommended that the IUPAC nomenclature [2] should be used to describe a steroid structure.

Despite their comparatively simple chemical structures, steroid hormones are potent and affect the endocrine, neuroendocrine, and behavioral functions via well-described intracellular receptors [3]. In 1929 Corner and Allen reported findings that indicated the presence of a fat-soluble hormone in extracts from swine corpora lutea [4], and the structure of this hormone was later elucidated and the compound was dubbed progesterone (pregn-4-ene-3,20-dione) [5,6]. Thereafter, in 1941 Selye reported that progesterone and deoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione) have sedative and anesthetic properties [7]. In the same study, the author also identified the ring A-reduced metabolites of progesterone and deoxycorticosterone as being highly potent compounds.

In 1981 Baulieu introduced the term *neurosteroid* to classify a steroid intermediate found in the brain, where its concentration is independent of the peripheral sources [8]. More recent studies have expanded this definition to cover all steroids that are de novo synthesized and stored in the brain [9–12]. However, the term *neuroactive steroid* refers to steroid hormones that are active on neuronal tissue and thus either synthesized endogenously in the brain or by peripheral endocrine organs, but with an action on neuronal tissue [13–16]. Consequently, a steroid hormone could be a *neuroactive neurosteroid* if it fulfils both these criteria. A common feature for the neuroactive steroids is that they rapidly alter the excitability of neurons by binding to membrane-bound receptors, such as those for inhibitory and/or excitatory neurotransmitters [16]. The time required to obtain physiological action is within seconds to

minutes [7,14,16,17], which is in contrast to classical steroid hormones such as the gonadal and the adrenal steroids where time lags of hours to days are more common in producing behavioral effects [3,18]. Because of these interesting properties, the amount of published research papers addressed to the use of neuroactive steroids has increased dramatically over the last decade. A literature search revealed that 558 unique papers were found containing both the words ''neuroactive'' and ''steroids'' [19]. If the term neurosteroid was added to the search, then the total number of references reached 1592. The first paper using the concept neuroactive steroid was published in 1980 and in total only 12 papers were published during the 1980s. A huge increase was seen during the 1990s going from six references in 1991 to 111 in 1999. Within this selection, only 18 steroid metabolites have been mentioned in more than 10 papers; see Table 1 and Fig. 1. The number of references obtained for each metabolite is obviously much larger if the search is based on the CAS number of each individual compound, and consequently numerous extensive reviews [12,14,15,20–23] and original papers [9,24–27] have been published elsewhere, dealing with various aspects of the *action* of the neuroactive steroids, which is thus not discussed herein.

1.1. Neuroactive steroids—chemistry and biosynthesis

Neuroactive steroids are endogenously synthesized metabolites, but synthetic analogues are available. The synthesis of new, artificial steroid hormones is of immense interest, and semi-synthetical pathways are often utilized starting from naturally occurring steroids such as cholesterol. Examples of artificial synthesis of steroids could be found elsewhere [28–30]. Known synthetic neuroactive steroids are D-norgestrel (13β-ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one) norethisterone (17β-hydroxy-17α-ethynyl-19-nor-Δ^4-androstan-3-one), mifepristone (RU-486), medroxyprogesterone (17α-hydroxy-6α-methyl-pregn-4-ene-3,20-dione) acetate, alfaxalone (3α-hydroxy-5α-pregnan-11,20-dione; compound **X**) [31], ganaxalone (3α-hydroxy-3β-methyl-5α-pregnan-20-one; compound **XIV**) [32], and various other A-ring reduced metabolites [33–35].

Nevertheless, the endogenously synthesized metabolites are the most commonly studied neuroactive steroids and Compagnone and Mellon have recently reviewed their function and biosynthesis [36]. The first step in the biosynthesis, which is also rate limiting, is for all C-18, C-19 and C-21 steroid hormones the conversion of cholesterol to preg-

Table 1
References to neuroactive steroids in chemical abstracts, or neuroactive steroids selected for discussion in this review

Compound	CAS number	CA index name	Selection[a]	Total
I	128-20-1	3α-Hydroxy-5β-pregnan-20-one	156	1062
II	57-83-0	Pregn-4-ene-3,20-dione	118	61,713
III	516-55-2	3β-Hydroxy-5α-pregnan-20-one	70	278
IV	145-13-1	3β-Hydroxypregn-5-en-20-one	65	5204
V	516-54-1	3α-Hydroxy-5α-pregnan-20-one	65	601
VI	567-03-3	3α,21-Dihydroxy-5β-pregnan-20-one	57	225
VII	53-43-0	3β-Hydroxyandrost-5-en-17-one	46	8852
VIII	64-85-7	21-Hydroxypregn-4-ene-3,20-dione	46	6097
IX	567-02-2	3α,21-Dihydroxy-5α-pregnan-20-one	46	141
X	23930-19-0	3α-Hydroxy-5α-pregnan-11,20-dione	43	456
XI	651-48-9	3β-Sulfooxyandrost-5-en-17-one	41	4044
XII	1247-64-9	3β-Sulfooxypregn-5-en-20-one	39	561
XIII	50-28-2	3,17β-Dihydroxyestra-1,3,5(10)-triene	32	74,881
XIV	38398-32-2	3α-Hydroxy-3β-methyl-5α-pregnan-20-one	26	37
XV	50-22-6	11β,21-Dihydroxypregn-4-ene-3,20-dione	25	23,535
XVI	566-65-4	5α-Pregnan-3,20-dione	16	759
XVII	50-23-7	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione	13	54,381
XVIII	58-22-0	17β-Hydroxyandrost-4-en-3-one	10	33,546

[a] Reference selection is the 558 references found containing both of the concepts ''neuroactive'' and ''steroids''.

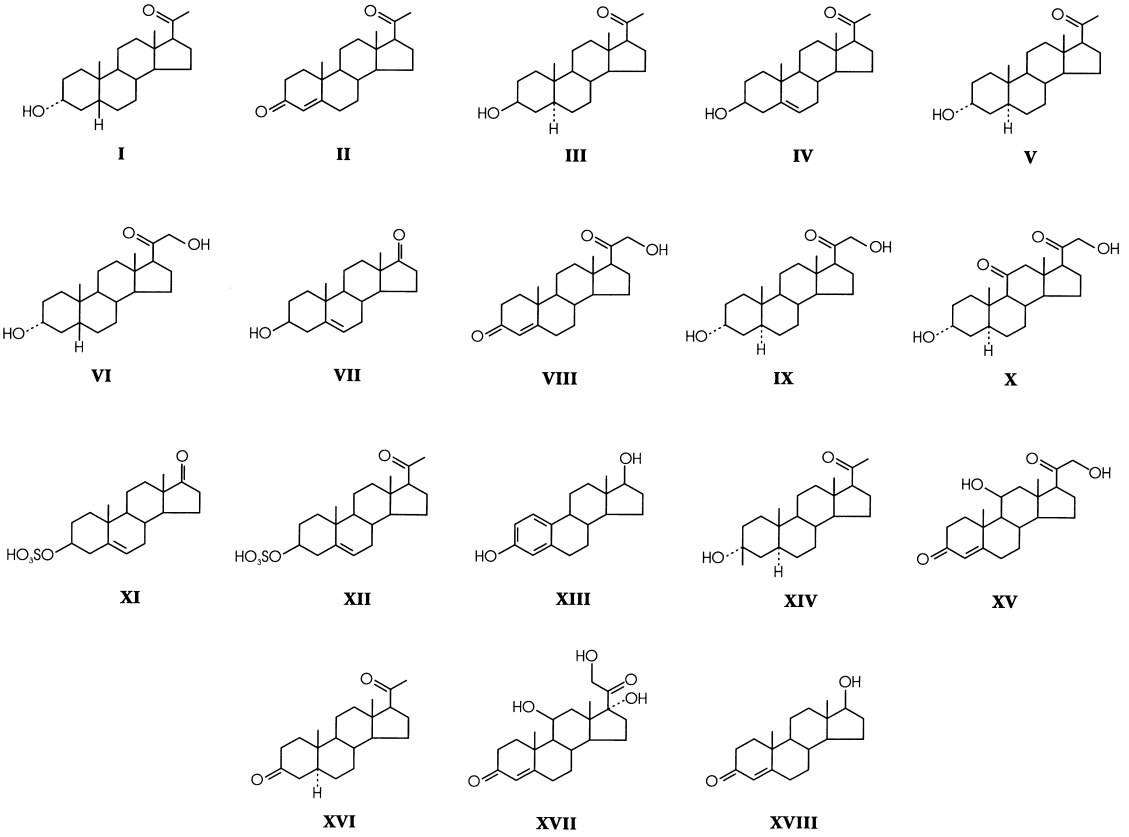

Fig. 1. Structures of some of the more central steroids dealt with in this review.

nenolone (3β-hydroxypregn-5-en-20-one; compound **IV**) through removal of a 6-carbon chain by the cytochrome P450 side chain cleavage enzyme (P450scc). Pregnenolone is thereafter converted to 17α-hydroxypregnenolone (3β,17α-dihydroxypregn-5-en-20-one), pregnenolone sulfate (3β-sulfooxy-pregn-5-en-20-one; compound **XII**), and progesterone (**II**). The main pregnenolone metabolite is progesterone, which can be converted to reduced progesterone metabolites but also to glucocorticoids, androgens, and estrogens according to Fig. 2.

It is generally assumed that these endogenously synthesized metabolites are immediately released into the blood circulation, and the concentration levels of steroid hormones are consequently directly correlated to the biosynthetic activity of the gland and to the blood flow-rate. However, some of the neuroactive steroids can be stored at the location of their synthesis and are thus available in various brain regions [21,37,38]. Moreover, due to their lipophilic nature, steroid metabolites are sparingly soluble in water and are consequently to a large extent found conjugated to ligands with hydrophilic functionalities (i.e. as sulfates or glucuronide derivatives), or bound to plasma proteins [39]. Steroids that are associated to plasma proteins commonly bind with low affinity through non-covalent bonds, where binding to albumin accounts for 20–50% of the bound fraction. Consequently, of the total steroid concentration in plasma, only 1–10% exists in the unconjugated and unbound form. This is however the most important fraction, since the free steroids are the species that are supposed to have activity on the neurons, and papers dealing with unconjugated and unbound steroids are thus the main focus of this review. There are also papers that specifically deal with steroid profiling, but this information should be sought elsewhere [40–45].

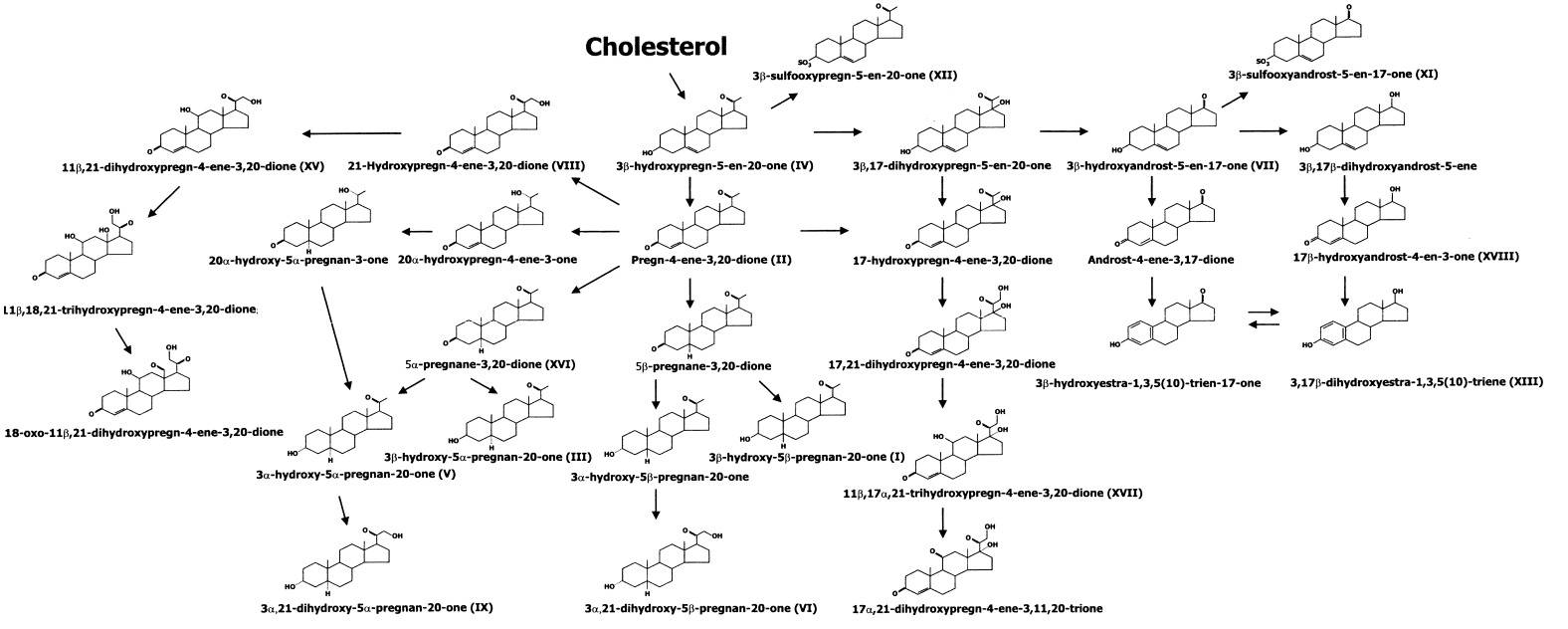

Fig. 2. Biological conversion pathways for some important neuroactive steroids, originating from cholesterol.

1.2. Definition of biological matrices

The liquid part of blood, devoid of cells and platelets, is termed either plasma or serum depending on how the sample has been prepared. Since some confusion seems to exist in the literature, a definition could be appropriate. Blood *plasma* is the liquid portion remaining after the cellular components have been removed from the blood by centrifugation. An anticoagulant must be added to prevent the blood from clotting before the separation takes place, and the kind of anticoagulant used may be important to know for the analyst. Anticoagulants usually interfere with the clotting process by binding calcium ions, and examples of anticoagulants are sodium citrate, EDTA, potassium oxalate, and heparin. If no anticoagulant is added, clotting will start within minutes and the fibrin clot formed will contain within it the cellular components of the blood. The liquid remaining when the clot is removed is blood *serum*, and is equivalent to blood plasma, except that it lacks the plasma components that have taken part in the clotting process, mainly the protein fibrinogen. *Tissue* is defined as a group or layer of similar cells united to perform specific functions. Since the neuroactive steroids currently discussed are associated with nervous substance per definition, the availability of tissue samples from healthy humans is quite limited for obvious reasons. *Urine* is the excrementitious fluid secreted from the blood by the kidneys, passed through the ureters, stored in the bladder, and discharged through the urethra, in which a wide variety of metabolic products (androgens, estrogens, corticoids, pregnanes, etc.) in both conjugated and unconjugated form are present.

1.2.1. Sample storage

Plasma, serum and tissue samples are comparatively stable, therefore no special precautions must be taken, and samples are normally stored in plastic containers at temperatures down to −20 °C. Urine is perhaps the most complicated biological matrix available and will normally need the addition of preservatives to prevent bacterial degradation of the steroids and/or hydrolysis of steroid conjugates.

1.3. Concentrations of neuroactive steroids in various matrices

Reported concentration levels for neuroactive steroids are varying in the literature, and the variations seem to depend on the sample matrices and applied analytical techniques. It is consequently strongly recommended that relevant certified reference ma-

Table 2
Concentration levels in nanomolar (nmol/l) of neuroactive steroids in humans

CA index name	Plasma (n*M*)	Serum (n*M*)	Brain (nmol/kg)
3α-Hydroxy-5β-pregnan-20-one	0.2–104 [61,79,180,181]	1.2–236 [156]	n.f.[a]
Pregn-4-ene-3,20-dione	0.1–835 [93,180–182]	0.45–663 [156,183–186]	1.1–146 [184,187–189]
3β-Hydroxy-5α-pregnan-20-one	8–60 [79]	0.05–7.6 [93]	n.f.
3β-Hydroxypregn-5-en-20-one	0.8–70 [93,190]	1.6–10.5 [186,191]	3.2–549 [187,189]
3α-Hydroxy-5α-pregnan-20-one	0.1–312 [61,79,93,128,180–182]	0.1–182 [185,191]	47–66 [184]
3α,21-Dihydroxy-5β-pregnan-20-one	n.f.	n.f.	n.f.
3β-Hydroxyandrost-5-en-17-one	0.3–161 [134,192]	9.4–29 [186]	8–207 [187,189]
21-Hydroxypregn-4-ene-3,20-dione	0.08–3.3 [182,192]	0.1–0.2 [186]	n.f
3α,21-Dihydroxy-5α-pregnan-20-one	0.2–1.6 [182,193]	n.f.	n.f.
3α-Hydroxy-5α-pregnan-11,20-dione	Synthetic hormone		
3β-Sulfooxyandrost-5-en-17-one	2000–10,000 [192]	1800–6200 [191]	1.6–201 [187]
3β-Sulfooxypregn-5-en-20-one	11.2–15.2 [190]	97–529 [191]	8–259 [187]
3,17β-Dihydroxyestra-1,3,5(10)-triene	0.2–0.3 [180,190]	0.06–0.8 [156,183,186]	0.3–7.3 [187,194]
3α-Hydroxy-3β-methylpregnan-20-one	Synthetic hormone		
11β,21-Dihydroxypregn-4-ene-3,20-dione	2.3–23 [192]	14–30 [195,196]	n.f.
5α-Pregnan-3,20-dione	0.03–180 [93,181,182]	4.8–17.5 [184]	35–105 [184]
11β,17α,21-Trihydroxypregn-4-ene-3,20-dione	174–550 [182,192]	50–1500 [84,186,197]	n.f.
17β-Hydroxyandrost-4-en-3-one	0.5–10.9 [61,134]	0.6–25 [186]	0.9–14 [187,188]

[a] n.f., not found.

terials (CRMs) are analyzed alongside the analytical samples whenever possible to reduce systematic errors in the quantitation process (both intra-laboratory and between laboratories). However, as a guideline for selecting appropriate analytical techniques a few examples of reported concentrations are summarized in Table 2. To summarize, it is worth mentioning that apart from the obvious biological interest in neuroactive steroids, this class of substances is interesting from a purely analytical standpoint as model compounds, considering their limited solubility in most solvents, their interesting pharmacodynamic profiles, and the low levels by which they occur in complicated matrices.

2. Steroid analysis

The establishment of new analytical methods involves choosing, optimizing, and validating all the techniques used to perform the analytical unit operations. This includes sampling, preliminary operations (i.e. sample pretreatment), measurement (which in this context means separation and detection), and data handling. To obtain accurate quantitative measurements, a suitable sampling technique must first be chosen to ascertain that representative samples are gathered and pretreated into a format that allows analysis with a reliable and rugged analytical technique. The data thus acquired are then evaluated and presented according to predetermined procedures. All these steps are interconnected and thus if non-selective sampling and extraction techniques are chosen, higher demands are imposed on the analytical separation and quantitation procedures, and vice versa. Consequently the most fruitful way is to utilize selective and efficient techniques for sampling and sample pretreatment to yield enriched samples that can be determined using analyte-specific detection techniques. Although several reviews have been written with relevance to this topic [46–48], only a single book has been found. This monography is titled *Steroid analysis*, and was edited by Makin et al. in 1995 [49].

2.1. Sample pretreatment

A number of different approaches are available for carrying out sample pretreatment and purification of neuroactive steroids, and some are reviewed here. A common feature for all these techniques is that loss of analyte during the work-up procedure is inevitable, and the actual analyte recoveries must thus be determined while developing a new method, or otherwise the data produced by the method will be questionable. The most common practice for determining the analyte recovery is to utilize internal standards (I.S.), which should be added to the matrix at the start of the analysis. When working with biological matrices, one important criterion for the suitability of an I.S., is that binding strength of the analytes and the I.S. to proteins and other components of the matrix should be similar. The I.S. should moreover have physiochemical properties that are comparable to the analyte and thus behave similar to the analyte during sample pretreatment (i.e. extraction and purification), yet the properties must be sufficiently unique to allow the I.S. to be clearly discerned from the analyte in the quantitation step. As highly sensitive mass spectrometers have become readily available, isotope dilution has emerged as the preferred route for determining analyte recoveries. Deuterated analytes are the ideal internal standards, whose identical chemical properties make them behave exactly as the analytes during the preliminary operations, but the mass difference makes them easily identifiable in the quantitation procedure. A problem is the limited availability of suitable standards at affordable prices.

2.1.1. Liquid–liquid extraction

During the early ages of steroid analysis, liquid–liquid partitioning was the predominant technique for extraction of steroids from biological matrices. The technique is still widely used but the popularity has declined as other, more efficient, techniques have emerged. If liquid–liquid extraction (LLE) is contemplated as a sample pretreatment alternative, the polarity of the steroids and their ability to bind to proteins are important aspects when selecting solvents for the extraction procedure. Thus, to quantitatively extract the analyte(s), the chosen solvent should not only dissolve the compounds of interest completely, but also be capable of breaking associations to proteins. These requirements are seldom satisfactorily met in practice and low extraction

efficiencies are therefore often seen. Another obstacle is that LLE is an inherently non-selective procedure whereby other non-steroidal lipids are likely to be co-extracted. These compounds may well cause interferences in the ensuing operations. Co-extracted lipids can be removed to some extent, but when LLE is used, the user should expect a significant presence of non-steroidal lipids, even in a purified sample. The limited compatibility of solvents with plastic vessels is another problem, which makes glass one of the few extraction reservoir materials possible. But since several steroids tend to bind to glass surfaces, it is recommended that all glassware should be silanized prior to use. Another problem often associated with LLE is the necessity of using large amounts of toxic and/or flammable solvents, leading to complications with handling and waste disposal. Despite all drawbacks, numerous papers have been and will probably continue to be published where LLE is the technique chosen for sample purification of neuroactive steroids.

2.1.1.1. Plasma, serum, tissue and urine samples

LLE of pregnanes in plasma or serum is often carried out using a two-phase system comprising diethyl ether and water, in which the steroids are extracted to the ether phase and then decanted and evaporated to dryness, after which the residue can be resolved in various matrices for different operations. Yamada et al. [50] have investigated diethyl ether, tetrahydrofuran (THF), *n*-decane, *n*-hexane, cyclohexane, and ethyl acetate for extraction of 17β-estradiol (3,17β-dihydroxyestra-1,3,5(10)-triene; compound **XIII**) from plasma whereby ethyl acetate was found to be best suited. Visser et al. [51] have quantified alphaxalone, pregnenolone, and 3α-hydroxy-5β-pregnan-20-one (**I**) in plasma using a precipitation technique, whereby acetonitrile was added to the plasma sample and the proteins precipitated and removed after centrifugation. However, sample work-up of steroids from tissue normally requires a mixture of solvents (methanol, ethanol, isopropanol, acetone, hexane, or chloroform) to satisfactorily purify the analyte(s) of interest. A number of pregnanes have successfully been extracted from brain tissue merely by allowing the samples to stand in 95% aqueous ethanol for 7 days at +4 °C [52–54]. In spite of all the drawbacks, LLE can be successfully used as a pre-purification technique for steroids in complex matrices. Marchand et al. [55] employed LLE to separate phenolic steroids from androgens and progestagens prior to solid-phase extraction (SPE) on silica columns. In that study it was shown that non-phenolic steroids could be readily separated from phenolic steroids using extraction with non-polar solvents such as hexane and diethyl ether, provided sodium hydroxide was used to convert the phenolic steroids into the phenolate forms. Moreover, residues of 17β-estradiol have been extracted and quantified in kidney fat [56]. After a de-lipidation step, the authors used dichloromethane to extract the relatively polar steroid metabolites including 17β-estradiol. Despite the successful use of halogenated solvents, such as dichloromethane and chloroform in several studies, such solvents should be avoided when new methods are developed, since these solvents are banned from use in a number of countries because of their negative effects on the environment.

2.1.2. Solid-phase extraction

Over the last two decades, the use of SPE has gained in popularity and is today considered as being the technique of choice for sample work-up. SPE offers several benefits over LLE, such as improved recoveries, less solvent consumption, smaller sample volumes, and increased sample throughput. SPE is relatively easy to use and the technique is often incorporated in dedicated fully automized sample preparation systems of various formats, a subject recently reviewed by Rossi and Zhang [57]. Another important advantage with SPE is the wide variety of sorbents commercially available with a variety of functionalities that allow users to choose from different modes of interaction when performing sample purification. Despite the wide variety of sorbents available, the SPE material most commonly used for clean-up of neuroactive steroid samples is bonded silica with either C_8 or C_{18} functionality, where the first application related to the extraction of steroids was published as early as 1980 [58]. The development of new sorbents of various formats (i.e. discs, syringes, membranes, etc.) is still of interest as the number of analyses demanding highly specific and efficient sample work-up increases. When imple-

menting SPE into an analytical method some general guidelines should be considered.

Sample characteristics: analyte pK_a, molecular mass, polarity, matrix, interferents, etc.

Sorbent selection: reversed phase, normal-phase, ion-exchange, adsorption, molecularly imprinted networks, etc.

Solvent selection: prepare elution profiles for different solvents.

Method development can thereafter be carried out according to these four steps.

(i) Conditioning of the solid-phase bed.
(ii) Application of the sample.
(iii) Pretreatment of the sample (multiple washing steps) to remove loosely bound matrix constituents.
(iv) Elution of the analyte, preferentially with a minimal amount of matrix compounds co-eluting.

When a suitable procedure has been established for the compounds of interest, the breakthrough volume of the sorbent should be examined. Knowing the maximum extractable sample amount, both volumetrically and gravimetrically, linearity, repeatability, reproducibility, and recoveries may be determined in order to validate the work-up procedure. An excellent source of detailed information about SPE method development can be found elsewhere [59].

2.1.2.1. Plasma, serum, tissue, and urine samples

In general, to prevent clogging of the sorbent, such liquid biological samples should be pretreated to disrupt the steroid–protein bindings prior to addition to an SPE device. This may be done by adding an equal amount of acetonitrile or methanol to the sample, after which the mixture is centrifuged. This is normally sufficient to remove the protein fraction of the sample. Another procedure is to simply dilute the samples in a large quantity of buffer prior to sample application. Draisci et al. [60] have analyzed progesterone and testosterone (17β-hydroxyandrost-4-en-3-one; compound **XVIII**) along with a few metabolites, in urine and bovine serum using C_{18} SPE columns for sample work-up. The same type of extraction device has furthermore been used for purification of 3α-hydroxy-5α-pregnan-20-one (**V**) and related metabolites from both human cerebrospinal fluid (CSF) and plasma [61]. SPE columns with C_{18}-functionality have also been used by Vallée et al. [62] to extract progesterone, pregnenolone, 3α-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one (**III**) and dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one or DHEA; compound **VII**) from both plasma and brain tissue. Que et al. [63] have extracted a number of steroids into different classes from urine, using a thorough extraction procedure, encompassing both C_{18} cartridges and ion-exchange columns. However, a drawback often associated with SPE as sample preparation devices is their rather high dead volume, which can cause loss of sample and leads to unwanted dilution of applied samples.

An alternative, that might be equally suited for clean up and pre-concentration, is particle-loaded membranes. Lensmeyer et al. [64] have, e.g., shown that cortisol (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione; compound **XVII**), cortisone (17α,21-dihydroxypregn-4-ene-3,11,20-trione), corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione; compound **XV**), prednisone (17α,21-dihydroxypregna-1,4-diene-3,11,20-trione), and prednisolone (11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione) can be extracted from serum using a syringe reservoir packed with a PTFE membrane loaded with octyl (C_8)-bonded silica. Even more beneficial is to utilize a sample preparation device based on a planar sorbent format. Thus, using a disc rather than a column, packed with either a sorbent or a particle loaded membrane the user can retain the sample loading capacity but with a decreased dead volume as the net result. Accordingly, Van Vyncht et al. [65] have used Empore brand C_{18} discs to simultaneously extract 17β-estradiol and a number of anabolic steroids from hydrolyzed urine samples. The 96-well format is another technology that has been implemented in conjunction with SPE, and Rule and Henion [66] have shown that it is possible to extract and quantify progesterone and equilenin (3-hydroxy-1,3,5(10),6,8-estrapentaen-17-one), from 384 human urine samples in only 24 h by using this concept.

2.1.3. Molecular imprinted polymers

Another technique with a potential of becoming part of an efficient sample preparation scheme is the use of molecular imprinted polymers (MIPs), see Fig. 3 [67–69]. MIPs packed in discs or syringes can

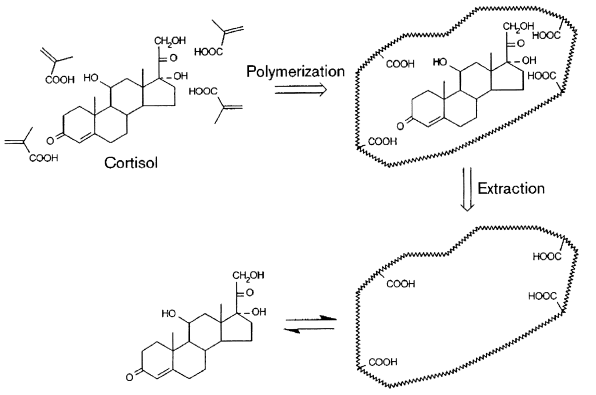

Fig. 3. Schematic representation of the ''molded'' molecular cavity of a molecularly imprinted polymer, binding to the template molecule. From Ref. [68] with permission.

be utilized as SPE devices, but these materials can also be packed in ordinary chromatography columns and thus used for direct injection of biological samples. The number of works dealing with steroid applications is unfortunately limited and a Chemical Abstract search revealed only 23 references (including patents) where MIPs have been used with steroids. In relation to sample preparation of neuroactive steroids, Haginaka and Sanbe [70] have developed a uniformly sized MIP material selective for 17β-estradiol, using 4-vinylpyridine and ethylene dimethacrylate as monomers. The material exhibited molecular recognition properties when evaluated as a chromatographic stationary phase and allowed efficient discrimination among estrogen metabolites and distinctively separated estrogens from other steroids such as testosterone and corticosterone. Rachov et al. [71] have also synthesized a material with recognition properties for 17β-estradiol, and in another paper the same group has shown the synthesis of molecular imprinted polymers selective to six different steroids (testosterone, androst-4-ene-3,17-dione, androst-1,4-diene-3,17-dione, 17β-estradiol, progesterone, and testosterone) [72]. In that study, the authors concluded that the most efficient templates for methacrylic acid-containing imprinted polymers are steroids with a relatively rigid structure and the OH group located at the C-17 position.

2.1.4. Restricted access materials

The use of restricted access materials (RAMs) is perhaps the most interesting alternative for sample clean-up and pre-concentration of neuroactive steroids, since RAMs allow direct injection of biological fluids onto a column. In practice this means that no pretreatment of the sample is necessary, and columns packed with restricted access materials can be used either as SPE columns or more beneficially as pre-columns in coupled column chromatographic systems and thus enable fully automated solid-phase extraction of drugs and metabolites from untreated biological fluids via common valve-switching technologies. Restricted access materials are characterized by different surface chemistries on the particle perimeter and in the internal pore system. This facilitates fast and efficient clean-up of complex biological samples such as whole blood, serum, or urine by excluding matrix components of macromolecular nature, while low molecular mass compounds of interest are extracted. In a recent review, Boos and Grimm [73] have described the utility of high-performance liquid chromatography integrated solid-phase extraction in bioanalysis using restricted access pre-column packings. However, this technique is still rather immature and no references were found, where RAM had been applied for extraction of neuroactive steroids in biological matrices.

2.2. Separation techniques

Based on molecular mass alone, steroids are well within the molecular size range that is amenable to gas chromatographic separation, and separations by GC are—as a general rule—both faster and more efficient than separations by liquid chromatography. However, the presence of polar functional groups in steroids calls for derivatization, an operation that sometimes leads to artifact formation, as discussed below. Liquid chromatography is therefore a valuable alternative for many steroid separations.

2.2.1. Gas chromatography

Due to its high resolving power, excellent performance, and its relative ease of use, gas chromatography is the most commonly used separation technique for neuroactive steroids. In the early ages of GC, packed columns with an internal diameter up to 4 mm were used, and the stationary phase was coated onto a porous solid support such as, e.g., acid

washed Celite. These columns typically suffered from stationary phase bleeding and unwanted adsorption of analytes due to difficulties of completely deactivating the support material. The peak shapes of eluting analytes were therefore often poor and loss of analyte was frequently seen. The introduction of capillary wall-coated open tubular columns with chemically bonded stationary phases dramatically improved the chromatographic resolution and reduced the effect of adsorption to the support material. These columns can be used at temperatures well in excess of 300 °C for sustained periods, although some column bleeding should still be expected, especially when working in the upper extreme region of the temperature range. The improved bleeding has had a strong impact on the use of GC in steroid analysis, as compounds belonging to this class have rather low volatility and thus elute at high temperatures. Due to this improvement, certain metabolites among the androgens and the estrogens became susceptible to analysis without being modified. However, it is still far more common that steroids require some form of derivatization to be amenable to GC separation, and the most common labeling techniques are summarized below.

2.2.1.1. Derivatization for gas chromatography

A number of reviews [74–76] and books [77,78] dealing with the labeling of steroids prior to GC separation have been published, and thus only a selection of the most commonly used reactions and reagents will be discussed here.

Steroid metabolites having one or multiple hydroxyl groups in the molecular backbone are labeled to increase the volatility by reducing the polar interactions of the parent compounds. Numerous reagents are commercially available that facilitate modification of compounds bearing hydroxyl functionality, but the main reagents are based on the formation of trimethylsilyl (TMS) derivatives, using trimethylhalosilanes, TMS-amines, TMS-amides, or TMS-esters. The reaction scheme is convenient and the formed derivatives exhibit excellent chromatographic properties. Commonly used reagents are *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), see Fig. 4, due to their high volatility and strong silylating power. Trimethyliodosilane (TMIS), trimethylsilylimidazole (TMSIM), and trimethylchlorosilane (TMCS) have also been used as reagents, but they are more commonly utilized as catalysts in conjunction with the aforementioned reagents, in order to increase the silylating power, enable labeling of sterically hindered functionalities, or merely to enhance the reaction kinetics. Specific reagent mixtures (with universal silylating power) are furthermore commercially available, and an example thereof is TMSI-S, which is composed of BSTFA, TMCS, and TMIS. An important objective to bear in mind when silylation reactions are to be performed, is that strictly anhydrous experimental conditions are required since both the reagents and the formed derivatives are sensitive towards moisture and water.

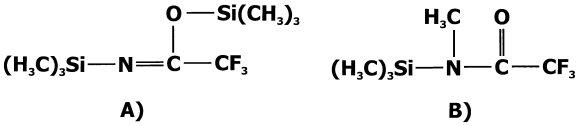

Fig. 4. Some reagents used for the formation of TMS-derivatives of compounds bearing hydroxy functionality; (A) *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) and (B) *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA).

Insertion of a *tert*.-butylsilyl chain onto the molecular backbone is another important labeling scheme for compounds bearing hydroxyl functionalities, and it is well suited for applications where mass spectrometric detection is carried out, see Fig. 5. The formed *tert*.-butylsilyl derivatives enhance the hydrolytic stability of the steroids and provide specific fragmentation patterns since an addition of a *tert*.-butylsilyl group increases the molecular mass of the parent compound with 114 amu. Moreover, fragment losses of 57 amu are seen, and examples of suitable reagents are *tert*.-butyldimethylsilylchlorosilane (TBDMCS), *tert*.-butyldimethylsilylimidazole (TBDMSIM), and *N*-*tert*.-butyldimethylsilyl-*N*-trifluoroacetamide (MTBSTFA).

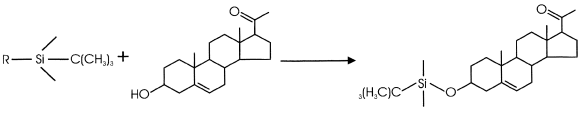

Fig. 5. *tert*.-Butylsilylation for formation of derivatives with characteristic mass spectroscopic fragmentation patterns.

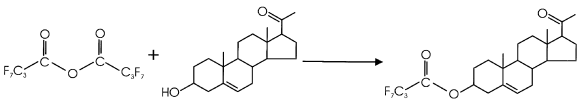

Fig. 6. Perfluoroacylation reactions leading to stable derivatives with good stable derivatives with suitable properties for chromatography and detection.

Conversion of parent compounds into perfluoroacyl derivatives such as trifluoroacetyl, pentafluoropropyl, and heptafluorobutyryl (HFB) derivatives is another labeling procedure; Fig. 6. Numerous reagents are commercially available, but steroids are preferably converted into the HFB-derivatives, using heptafluorobutyric anhydride (HFBA). This reagent is rather mild, in comparison to other acylation reagents, and produces stable derivatives with good chromatographic and detection properties, that are especially useful for isotope dilution mass spectrometric detection.

2.2.1.1.1. *N,O-Bis(trimethylsilyl) trifluoroacetamide:* Steroids having unhindered 3β-hydroxyl groups are readily derivatized with BSTFA (mixed with 1% TMCS), with or without the addition of pyridine as base catalyst. Hill et al. [79] have shown an example of this procedure, where a commercial reagent containing 99% BSTFA and 1% TMCS was used to label 3α-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5β-pregnan-20-one, and 3β-hydroxy-5β-pregnan-20-one in the presence of pyridine.

2.2.1.1.2. *N - Methyl - N - trimethylsilyltrifluoracetamide:* Bowers and Borts [80] have labeled a wide range of anabolic steroids including testosterone by adding MSTFA–1-propanethiol–ammonium iodide (1000:2:3, v/v/w) to dried steroid extracts and allowing the reaction to proceed at 60 °C for 15 min. Hartmann and Steinhart [81] have used MSTFA–TMIS–1,4-dithioerythritol (1000:2:2, v/v/w) for several androgens, estrogens, progestagens, and corticoids. In that study, the derivatization mixture was added to dried steroid extracts and the reaction was performed at 60 °C for 15 min. However, the authors claimed that the reaction should proceed overnight at 4 °C to obtain complete derivatization for steroids bearing a reactive group at position 17. Moreover, Marchand et al. [55] have used a similar derivatization mixture, MSTFA–TMIS–dithiothreitol (DTT) (1000:5:5, v/v/w), for labeling of androgens, estrogens, and progestagens. However, for labeling of the estrogenic metabolites, 17α-estradiol and 17β-estradiol, MSTFA was used alone. Van Vyncht et al. [65] have also used MSTFA and TMIS mixed with DTT for labeling of testosterone and estradiol, among other steroids, but with a slightly different composition (100:10:5).

2.2.1.1.3. *Trimethylsilylimidazole:* Trimethylsilylimidazole is considered as the strongest silylation reagent available for labeling of hydroxyl groups, and Kwan et al. [82] have formed TMS derivatives from a number of androgens, including testosterone, merely by adding TMSIM to a dry residue and allowing the reaction to proceed at 50 °C for 1 h.

2.2.1.1.4. *Heptafluorobutyric anhydride:* Liere et al. [83] have used HFBA to label a number of unconjugated and sulfated steroids, including pregnenolone, DHEA, progesterone, 3α-hydroxy-5α-pregnan-20-one, and pregnenolone sulfate. The reaction was allowed to proceed for 30 min at room temperature, using anhydrous acetone as solvent. Thienpoint et al. [84] have converted 24 steroids into corresponding heptafluorobutyryl derivatives and quantified the formed derivatives with isotope dilution mass spectrometry.

However, since the molecular backbone of most neuroactive steroids comprises both hydroxyl groups and keto-functionalities, special precautions must be taken prior to labeling to avoid the formation of artifacts during the silylation process. A common problem for steroids with unprotected keto functionalities is the formation of enol-ethers via enolisation of the carbonyl group. Thus, if the keto groups are unblocked when adding the TMS-labeling reagent, formation of unstable enol TMS ethers (derivatives that are difficult to prepare quantitatively) are obtained. Nevertheless, a feasible approach to exclude this risk is to mix reagents in order to simultaneously form derivatives at both functional groups; Fig. 7. By

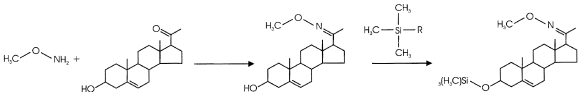

Fig. 7. Simultaneous derivatization of the keto and hydroxy functions in a ketosteroid, in order to avoid the formation of an unstable enol-ether.

converting the keto groups into oxime or alkyloxime derivatives (most commonly through methoximation; MO), prior to the TMS labeling, stable derivatives may be obtained. Unfortunately, formation of multiple derivatives due to *syn* and *anti* addition during the oxime or alkyloxime labeling hampers this approach, but despite this drawback the technique has proven to be a reliable tool for analysis of hydroxysteroids.

2.2.1.1.5. *Methoxytrimethylsilyl derivatives:* Ichimura et al. [85] have converted 14 steroids (including DHEA, pregnenolone, 17β-estradiol, testosterone, progesterone, corticosterone, and progesterone) into TMS or methoxytrimethylsilyl (MO-TMS) derivatives using methyloxime and trimethylsilyl derivatization. Hämäläinen et al. [86] have determined several neutral steroids, including DHEA, in urine by initial conversion of parent compounds into methoxime derivatives using 4% methoxyamine hydrochloride in pyridine and thereafter applied TMS-silylation using TMSIM. In another study, Thienpoint et al. [87] analyzed progesterone and cortisol as their corresponding HFB and MO-TMS derivatives, respectively.

2.2.1.1.6. *Flophemesyl chloride:* Choi and coworkers [88–90] have presented a procedure where both MSTFA and pentafluorophenyldimethylchlorosilane (flophemesyl chloride) are used for labeling of steroids such as testosterone, epitestosterone (17β-hydroxy-4-androsten-3-one), dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), androstenedione (4-androstene-3,17-dione), eticholanolone (3α-hydroxy-5β-androstan-17-one), DHEA, progesterone, pregnenolone, and androsterone (3α-hydroxy-5α-androstan-17-one), to produce trimethylsilyl derivatives. Using this procedure it is possible to form mixed derivatives, i.e. 3-trimethylsilyl-17-pentafluorophenyldimethylsilylethers, see Fig. 8, that

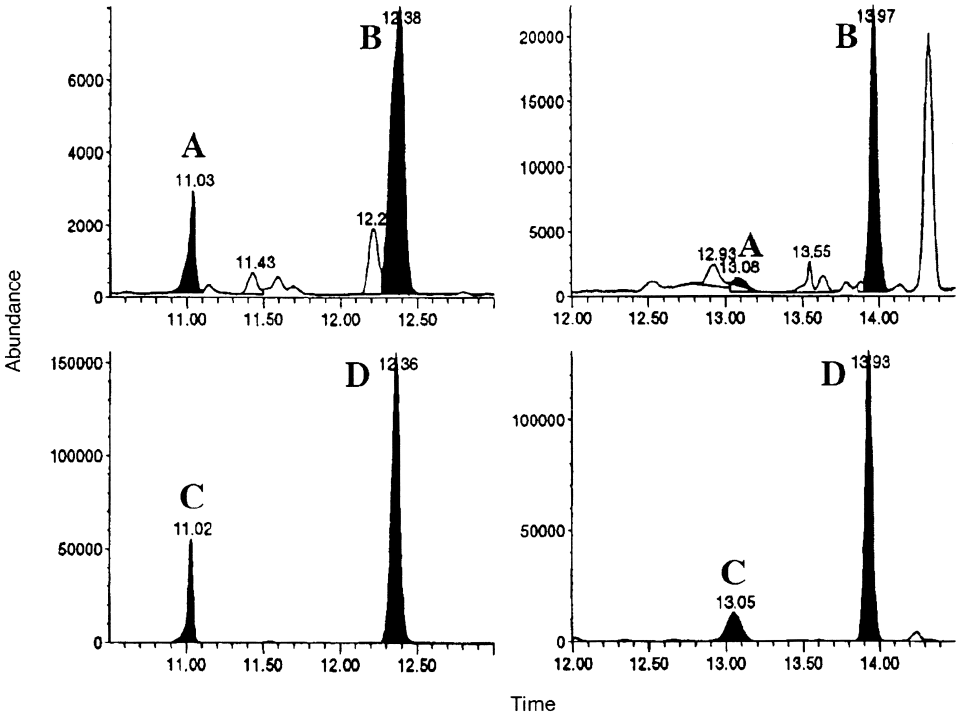

Fig. 8. Formation of mixed derivatives from steroids with multiple functions through the use of both MSTFA and pentafluorophenyldimethylchlorosilane as derivatization reagents. The SIM chromatograms show the separation of epitestosterone (A), testosterone (B), [$^2H_4$]epitestosterone (C) and [$^2H_4$]testosterone (D) after mixed TMS-flophemesyl (left) and trimethylsilyl (right) derivatization schemes. From Ref. [89] with permission.

allow determination of steroids in matrices such as urine [89], human hair [88], and nails [90].

2.2.2. High-performance liquid chromatography

HPLC is probably the most versatile separation technique for neuroactive steroids and, as it is a non-destructive technique, it can be used for preparative, qualitative, and quantitative purposes. In 1981 Purdy et al. [91] reviewed the analysis of progesterone metabolites, and in 1990 Robards and Towers [92] summarized the analysis of steroids. Thus, early developments in HPLC are not included in this overview. Today the common practice is to use short and narrow columns packed with 5 or 3 μm particles that offer high chromatographic resolution and a decreased overall solvent consumption. Although a wide variety of stationary phases (packed in various column formats) are commercially available, RP-HPLC using octadecylsilica (ODS or C_{18}) columns is the dominating technique for small non-volatile organic compounds. This technique offers a high chromatographic resolution and columns with up to 200,000 theoretical plates/m are common. RP-HPLC is therefore considered as the primary choice when developing new methods today, independent of compound class. However, normal-phase chromatography is still used, as it is easy to directly transfer existing off-line sample preparation methods to a HPLC system. In particular, this is common for those who on a routine basis have used Celite chromatography in combination with LLE as sample work-up technique. In a recent paper, Pearson Murphy and Allison [93] have shown a separation profile for progesterone, pregnenolone, and five ring A-reduced metabolites including 5α-pregnan-3,20-dione (**XVI**), 5β-pregnan-3,20-dione, 3β-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5α-pregnan-20-one, and 3β-hydroxy-5β-pregnan-20-one, using a silica column and an eluent consisting of 0.18% ethanol in dichloromethane, see Fig. 9. Complete baseline resolution of five of the model compounds was achieved, although the chromatographic efficiency was rather limited. In another study, Liere et al. [83] have shown the possibility of using gradient elution on a diol column for separation of several neuroactive steroids.

Although there is a heritage of normal-phase applications, RP-HPLC with C_8 or C_{18} columns is

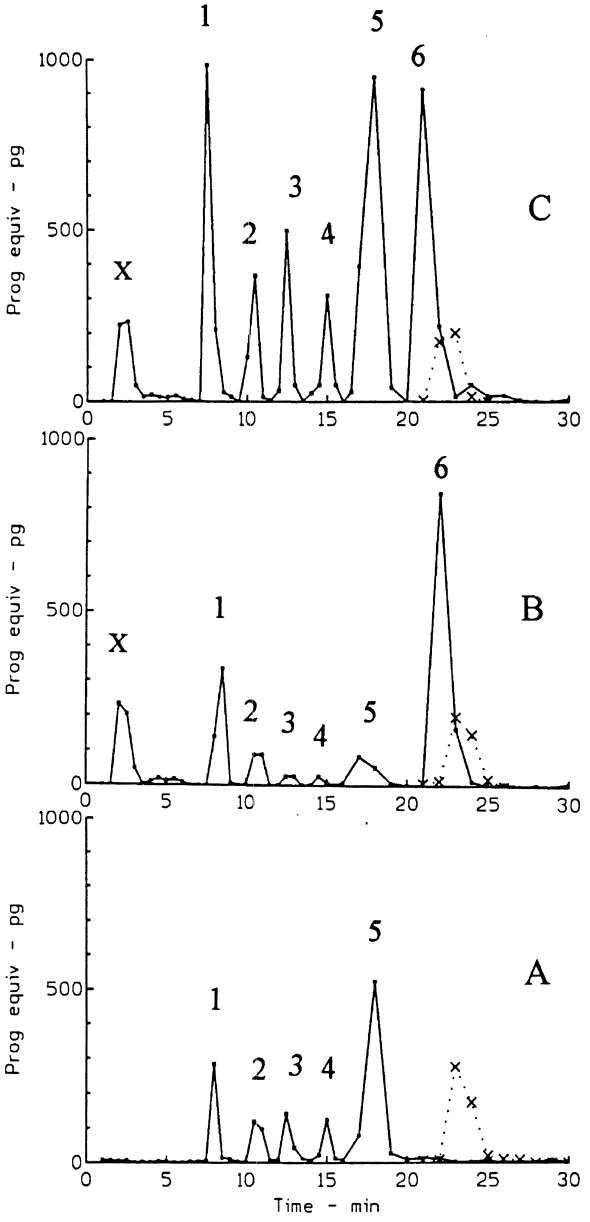

Fig. 9. Raw data patterns of steroids determined by RIA after normal-phase HPLC separation. (A) Pure steroid standards (1 ng of each metabolite). (B) Serum pattern of a patient in early pregnancy. (C) Serum sample spiked with 1 ng of each steroid metabolite. The retention order was: 5α-pregnan-3,20-dione (1), 5β-pregnan-3,20-dione (2), 3β-hydroxy-5β-pregnan-20-one (3), 3α-hydroxy-5α-pregnan-20-one (4), 3β-hydroxy-5α-pregnan-20-one and pregnenolone (5), and progesterone (6) using an eluent consisting of 0.18% ethanol in dichloromethane and a 5×100 mm Nova-Pak silica column. X denotes unidentified material. From Ref. [93] with permission.

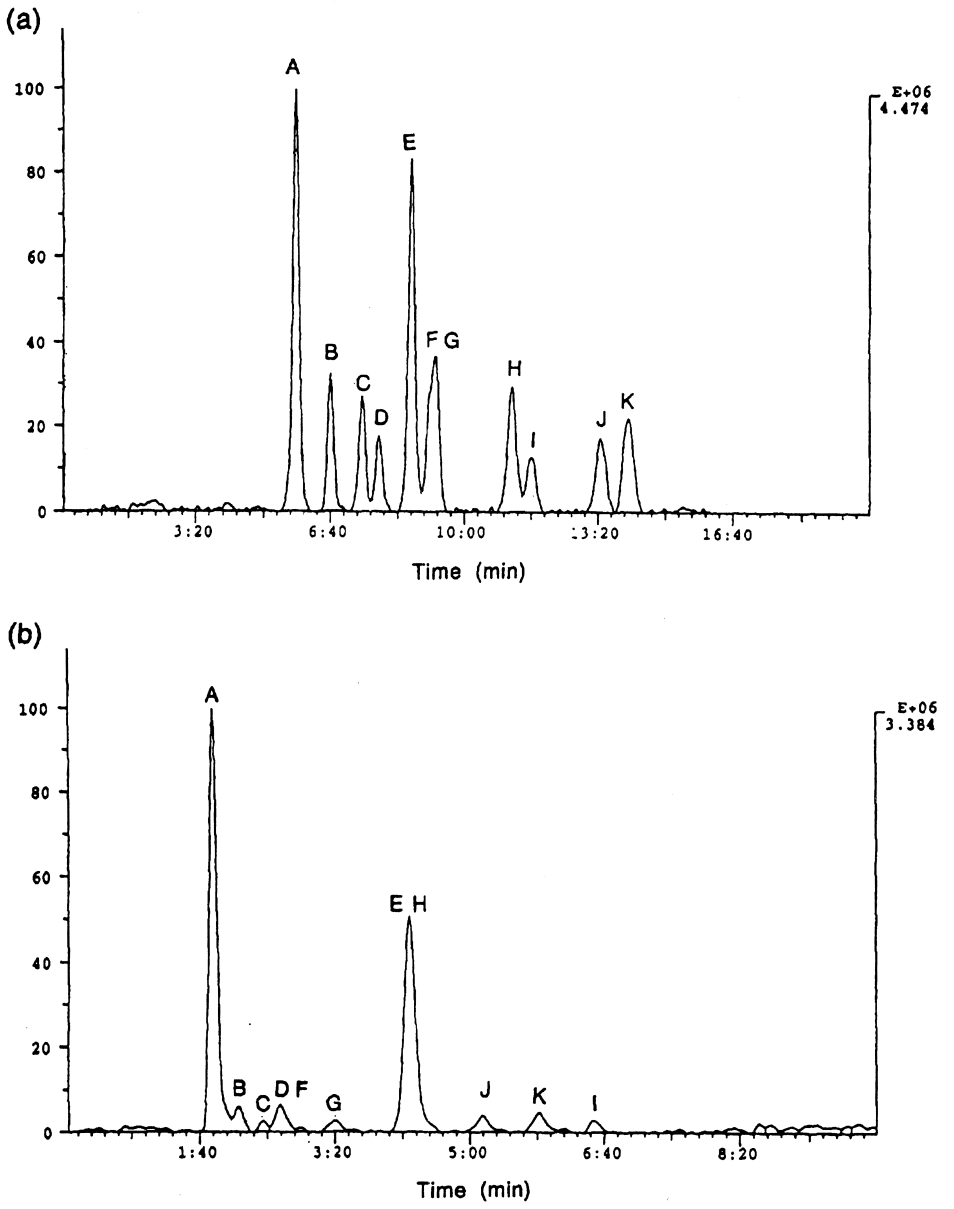

Fig. 10. Total ion current profiles from gradient LC–MS (APCI mode) analysis of a steroid mixture containing: testosterone (A), dehydroepiandrosterone (B), epiandrosterone (C), 20α-hydroxy-5α-pregnan-3-one (D), progesterone (E), 3α,21-dihydroxy-5α-pregnan-20-one (F), androsterone (G), pregnenolone (H), 5α-pregnan-3,20-dione (I), 3α-hydroxy-5β-pregnan-20-one (J), 3α-hydroxy-5α-pregnan-20-one (K). The LC gradients constituted of mixtures of 1% acetic acid in water (Y) and in (a) methanol and in (b) acetonitrile as organic modifier (Z). In both cases a linear gradient of Z/Y changing from 60:40 to 73:27 in 4 min was employed. After holding at 73:27 for 8 min, the gradient was changed linearly to 100:2 over the next 2 min. The total eluent flow-rate was 400 μl/min through a 2.1×150 mm C_{18} reversed-phase column with 5 μm particles. From Ref. [94] with permission.

the principal separation mode of small non-volatile compounds such as the neuroactive steroids. Using these stationary phases, enhanced resolution is obtained and with the more stable and efficient packing materials available, the column formats have decreased. For instance, Rule and Henion [66] have separated progesterone and equilenin in 2 min on a 2×20 mm guard column packed with 5 μm Betasil C_8 particles, using isocratic elution at a flow-rate of 400 μl/min. Ma and Kim [94] have used linear gradients to separate 11 neuroactive steroids within 20 min on a 2.1×150 mm Prodigy C_{18} column packed with 5 μm particles, see Fig. 10. Kuronen et al. [95] have developed a screening method for multisteroid monitoring using gradient elution, and retention indices for 24 steroids were presented along with UV absorbance spectra recorded online with a diode-array detector. Generally, when optimizing a separation process on a C_{18} column, either methanol or acetonitrile mixed with buffer or water in suitable proportions is the initial choice for the mobile phase, but if several metabolites have to be completely baseline resolved, a more complex eluent based on multiple solvents might be needed. Wei et al. [96] have presented an optimization procedure for RP-HPLC separation of steroids using factorial design and computer simulation.

A prevailing problem with neuroactive steroid separations by liquid chromatography is the lack of simple and generally applicable detection techniques with detection limits sufficiently low to permit direct determination at physiological concentrations. The perhydrocyclopentenophenanthrene molecular backbone is typically saturated or contains a solitary unconjugated unsaturation, which provides no useful absorption in the ultraviolet spectral range. Nor are such properties introduced by the functional groups commonly attached to the molecular backbone of neuroactive steroids, i.e. hydroxy, keto, and aldehyde groups. Most neuroactive steroids therefore have low absorptivity in the UV range, and few alternative detection techniques are available. The multitude of structural isomers of the metabolites of interest also decreases the applicability of RP-HPLC, since the chromatographic profiles become very complex with overlapping peaks. Thus, in most studies where direct detection is utilized the compounds of interest have to be derivatized prior to separation.

2.2.2.1. Derivatization for high-performance liquid chromatography

Even though a number of reagents are commercially available that may facilitate modification of compounds bearing hydroxyl functionalities in the molecular backbone, rather few applications are seen where steroids with one or multiple hydroxyl groups are labeled prior to HPLC separations. The most rational explanation is that these compounds are preferably analyzed with GC. Nonetheless, Nakajima et al. [97] have synthesized 2-[2-(isocyanato)ethyl]-3-methyl-1,4-naphthoquinone to facilitate sensitive detection of hydroxysteroids as corresponding carbamic acid esters. Using cholesterol as model compound, the reaction conditions were optimized and it was found that the reagent was suitable for labeling of relatively unhindered hydroxyl groups in the molecular backbone. Shibata et al. [98] have developed a method for quantitation of certain glucocorticoids, including cortisol and cortisone, in plasma or urine using 9-anthroyl nitrile in the presence of triethylamine and quinuclidine (1-azabicyclo[2.2.2]octane) to form fluorescent esters at the 21-hydroxyl group, see Fig. 11a and b. Nozaki et al. [99] have used dansyl chloride to label 17α-estradiol (3,17α-dihydroxyestra-1,3,5(10)-triene) from serum samples, see Fig. 12, and Kurosawa et al. [100] have derivatized 18-hydroxycortisol (11β,17α,18,21 - tetrahydroxypregn - 4 - ene - 3,20-dione), 18-hydroxycortisone (17α,18,21-trihydroxypregn - 4 - ene - 3, 11, 20 - trione), and 18-oxocortisol (11β, 17, 21-trihydroxypregn-4-ene-3, 18, 20-trione) with 1-anthroyl nitrile in the presence of quinuclidine, Fig. 13a and b.

Derivatization of the ketone functionality in the molecular backbone of neuroactive steroids is more common and preferentially carried out by hydrazone formation, using a hydrazine label under acidic conditions, see Fig. 14 [77]. A reagent frequently used for this purpose is 5-(dimethylamino)-naphthalenesulfonic hydrazide (dansylhydrazine or DNSH). This reagent has a high quantum yield and it forms fluorescent derivatives. Visser et al. [51] have used DNSH to develop a method for quantitation of alphaxalone and 3β-hydroxy-5α-pregnan-20-one in rat plasma, and Higashidate et al. [102] have shown the possibility of converting 3α- or 3β-hydroxysteroids into 3-ketosteroids using a column packed

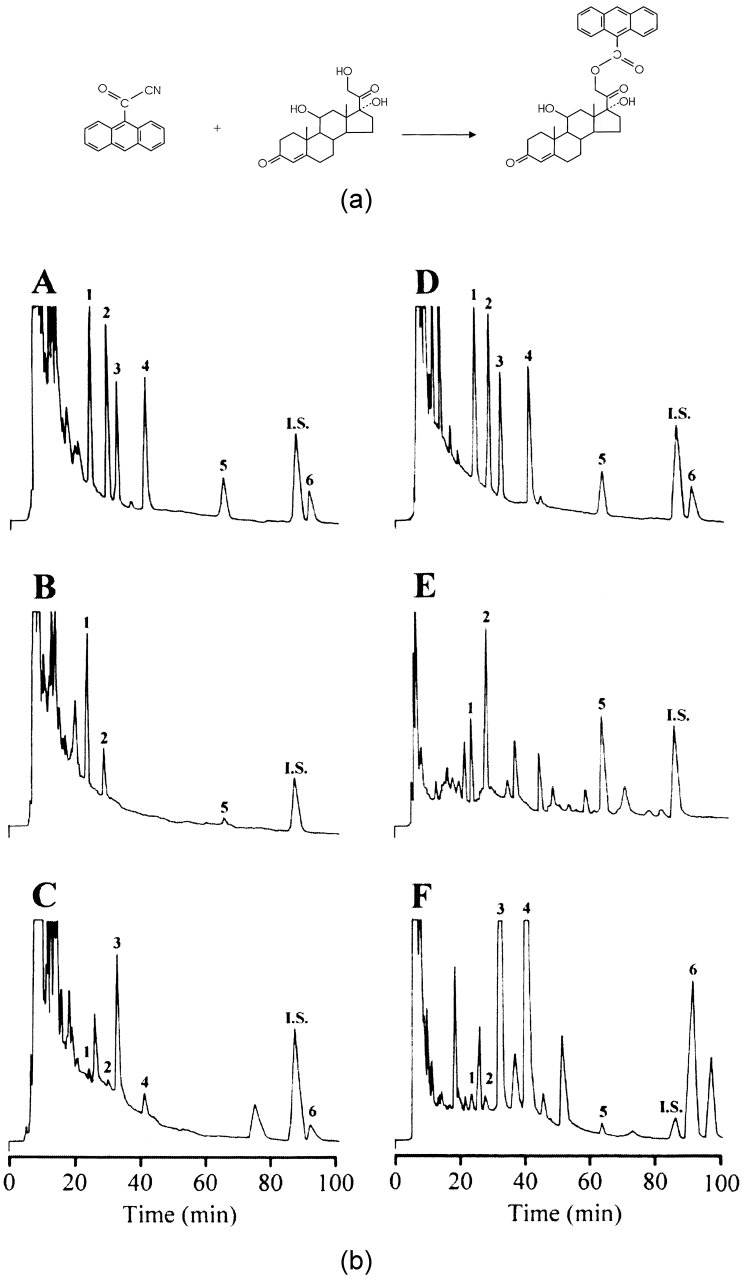

Fig. 11. (a) Formation of fluorescent esters at the 21-hydroxy group of glucocorticoids using 9-anthroyl nitrile. (b) Chromatograms obtained with (A) standard plasma and (D) standard urine, (B) plasma from a healthy volunteer, (C) plasma from a renal transplant patient, (E) urine from a healthy volunteer and (F) urine from a renal transplant patient. Peaks: 1, cortisol; 2, cortisone; 3, prednisolone; 4, prednisone; 5, 6β-hydroxycortisol; 6, 6β-hydroxyprednisolone. From Ref. [98] with permission.

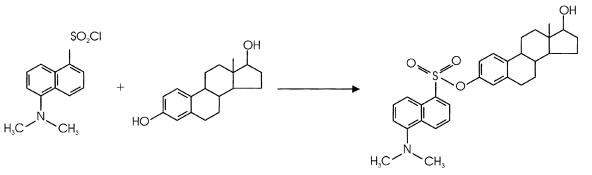

Fig. 12. Formation of the fluorescent dansyl derivative of 17β-estradiol.

with immobilized hydroxysteroid dehydrogenase and thereafter labeled the oxidized products with DNSH to form the corresponding dansylhydrazones. However, it has been found that different catalysts influence both the rate and yield of this hydrazone formation, and thus several acids such as hydrochloric acid [103], acetic acid [104], trichloroacetic acid [105], and trifluoroacetic acid [106] have been studied as catalysts. In addition to enhancing the kinetics of the reaction, the catalyst should also be selective in the reaction it promotes. Several of the neuroactive steroids have multiple carbonyl groups and usually also have multiple stereo centers in the molecular backbone. It is therefore important to ascertain that the reaction is group-specific. Recent studies have shown that almost all of these prerequisites can be fulfilled by using trifluoromethanesulfonic acid (TFMSA) as catalyst, an acid that yields much faster reaction kinetics compared to previous catalysts [107]. Selective labeling of the steroids can moreover be achieved merely by using optimized experimental conditions for TFMSA catalysis and quantitation of a number of neuroactive steroids is feasible in both plasma [108] and serum [109]. Unfortunately, TFMSA spontaneously forms highly toxic and carcinogenic methyl triflate when diluted in anhydrous methanol [110], a required component of the derivatization reagent. Catalysis by TFMSA is therefore not suitable for routine analysis. A way of circumventing this problem is to use Nafion, a perfluorinated polymer that can be considered as a solid-phase analogue of TFMSA, as catalyst [111]. By using this polymeric acid, the formation of triflic esters will occur on the polymeric solid sorbent, and thus the exposure to hazardous chemicals is minimized compared to solution-based TFMSA catalysis. Appelblad et al. [112] have recently presented a new derivatization procedure where these features are utilized to facilitate the formation of hydrazones in pre-column labeling of keto-containing compounds. In that study, a novel material for solid-phase enrichment and enhanced pre-column dansylation (SPEED) was prepared by modifying a porous polyethylene sorbent with Nafion and DNSH to act as catalyst and reagent, respectively. Using the SPEED device it was possible to label

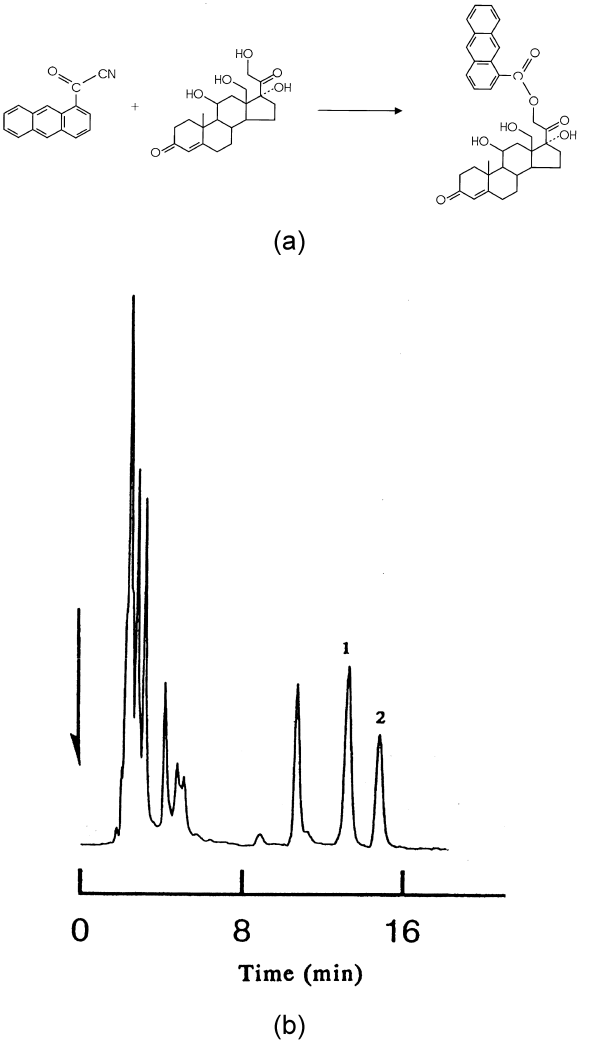

Fig. 13. (a) Formation of fluorescent derivatives of hydroxysteroids through the reaction with 1-anthroyl nitrile. (b) Chromatogram showing separation of fluorescent 1-anthroyl nitrile derivatives of hydroxysteroids (1 indicates pregnenolone and 2 the internal standard, 3β-hydroxy-16-methyl-pregna-5,16-dien-20-one). The separation was carried out on a 4.6×150 mm C_{18} reversed-phase column with 4 μm particles using an eluent consisting of acetonitrile–water (15:2) at a flow-rate of 1 ml/min. From Ref. [101] with permission.

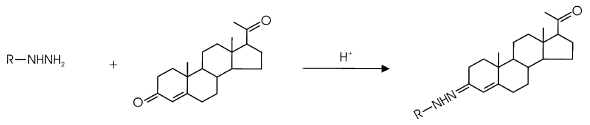

Fig. 14. Formation of hydrazone derivatives of ketosteroids, using a hydrazine label under acidic conditions.

picomole amounts of ketosteroids within 10 min at room temperature. Another improvement compared to classical solution phase hydrazone labeling procedures, is that the SPEED device is remarkably insensitive to water in the reaction mixture, and thus it can be concluded that heterogeneous catalysis is a preferred route in performing these types of derivatization reactions. Nonetheless, there are other reagents available for labeling the carbonyl functionality, and Katayama et al. [113] have used 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionohydrazide (BODIPY FL hydrazide) to derivatize six steroids having a keto-functionality at the 3-position, including progesterone, androstenedione, DHEA, 17-hydroxyprogesterone (17-hydroxypregn-4-ene-3,20-dione), 17-methyltestosterone (17β-hydroxy-17α-methyl-androst-4-en-3-one), and testosterone; see Fig. 15a and b. Furthermore, Shimada et al. [114] have studied the labeling of 17-oxosteroids, epiandrosterone (3β-hydroxy-5α-androstan-17-one), DHEA, androsterone, 5β-androsterone (3α-hydroxy-5β-androstan-17-one), and 3β-hydroxy-5β-androstan-17-one using DNSH, 4-(*N*,*N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD), or *p*-nitrophenylhydrazine, and studied the retention behavior of the formed derivatives. DBD has also been used for labeling of several neuroactive steroids, including pregnenolone, 3β-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5β-pregnan-20-one, 3α-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5β-pregnan-20-one, and corresponding sulfates, through labeling at the 20-position in the molecular backbone, see Fig. 16 [115]. Shimada et al. [116] have also used *O*-methyl hydroxylamine to form oximes from pregnenolone, its 3-sulfate, and DHEA in rat brain samples.

2.2.3. Capillary electrophoresis

Since Jorgenson and Lukacs [117] showed the advantages of performing electrophoretic separations

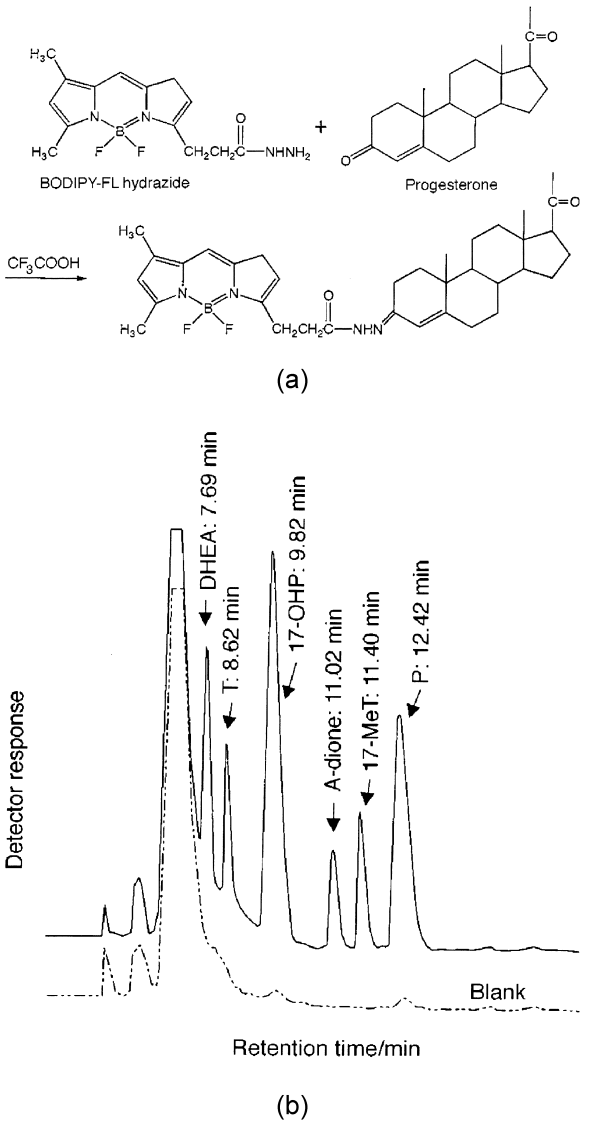

Fig. 15. (a) Formation of fluorescent derivative of the steroid carbonyl functionalities using 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-*s*-indacene-3-propionohydrazide (BODIPY FL hydrazide). (b) Chromatogram of steroids derivatized with BODIPY FL hydrazide. Each steroid was added to serum from a non-pregnant woman (100 ng/ml). The eluting peaks correspond to dehydroepiandrosterone (DHEA), testosterone (T), 17-hydroxyprogesterone (17-OHP), androstenedione (A-dione), 17α-methyltestosterone (17-MeT), and progesterone (P). (a) and (b) from Ref. [113] with permission.

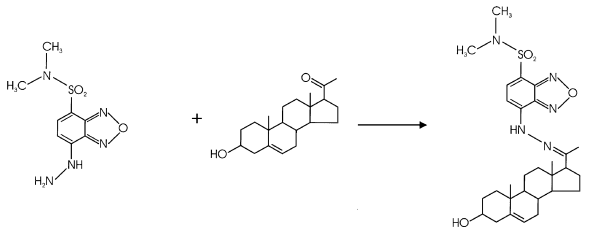

Fig. 16. Formation of fluorescent derivatives of steroids with 20-keto functionality through the reaction with 4-(*N*,*N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD).

in fused-silica capillaries in the early 1980s, various sub-techniques have emerged and today a wide range of separation techniques are available that involve the application of high voltages across buffer-filled capillaries to achieve separations of charged and uncharged species. In relation to steroid analysis, two major electrophoretic separation modes have proven particularly efficient as tools, namely micellar electrokinetic capillary chromatography (MECC or MEKC) and capillary electrochromatography (CEC).

2.2.3.1. Micellar electrokinetic chromatography

MEKC can be regarded as a hybrid between capillary electrophoresis and chromatography, based on partitioning of analyte species between the aqueous electrophoretic buffer and micelles formed from an ionic surfactant, added to the buffer at a concentration above the critical micelle concentration. The micelles are charged and thus mobile, and their internal form a hydrophobic entity that moves relative to the electroosmotically driven bulk buffer flow at a rate mainly determined by the properties of the detergent. Partitioning into the hydrophobic micelle interior permits separation of electrically neutral compounds otherwise not amenable to electrically driven separation in free solution, but also adds an additional separation dimension for ionic species that are undergoing dissociation or protonation. In 1996 Nishi and Terabe [118] reviewed micellar electrokinetic chromatography for drug applications. The review includes a number of applications and the possibilities of using MEKC for separation of steroid hormones and corticosteroids are discussed in one section. Salo et al. [119] published the same year an article that dealt with structure–retention relationships of steroid hormones in RP-HPLC and MEKC. Furthermore, Quirino and Terabe [120] have shown that it is possible to perform online concentration of neutral analytes using micellar electrokinetic chromatography. A model of the sample stacking was described and the technique was applied to the analysis of progesterone and testosterone from urine. Abubaker et al. [121] have used MEKC to separate clinically relevant steroids from serum. In their study, examples were given showing the separation of seven steroids and it was demonstrated that baseline resolution can be obtained for all model compounds within 6 min. There are also some drawbacks associated with MEKC, such as incompatibility with certain detection systems, a limited choice of surfactant systems available for formation of the pseudo-stationary phase, and limited separation efficiency for neutral analytes.

2.2.3.2. Capillary electrochromatographic separation

CEC utilizes applied voltages across capillaries that are filled with silica gel stationary phases that comprise both a hydrophobic group interaction and a low charge density that enables an electroosmotically driven mobile phase flow to be established through the stationary phase. The separation is thus a combination of both electrophoretic and chromatographic processes. CEC is still in development, but it has a promising potential for separation problems that require very high resolution and high selectivity, and is consequently a good candidate for the separation of neuroactive steroids in complex matrices. In 1999, Dermaux and Sandra [122] reviewed various applications with CEC, including packed column CEC, open tubular CEC, and pressure-assisted CEC. Que et al. [63] have demonstrated the potential of separating various groups of steroids, both in free form and as conjugates, using a hydrophobic CEC column. Using gradient elution, five derivatized steroid conjugates were baseline resolved within 14 min and the separation of seven dansylated steroids was accomplished in 16 min, see Fig. 17. They also made an attempt at coupling the CEC separation to a mass spectrometer for the analysis of unlabeled steroids. Huber et al. [123] have shown that it is possible to separate corticosterone, testosterone, androst-4-ene-3,17-dione, androstan-3,17-dione, and progesterone

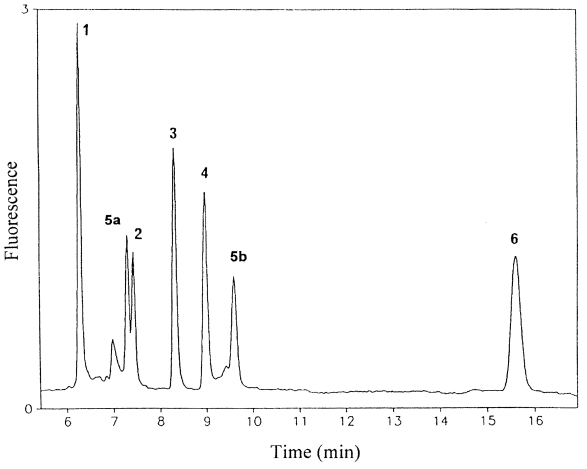

Fig. 17. Separation of derivatized neutral steroids using capillary electrochromatography in a 35 cm×100 μm (I.D.) with 360 μm O.D. capillary having an effective column length of 25 cm. The mobile phase consisted of acetonitrile–water–240 m*M* ammonium formate buffer (pH 3) (55:40:5, v/v/v) and separation was achieved by applying a field strength of 600 V/cm and an 11 μA current. The samples were injected using 100 V/cm for 10 s. The eluting peaks corresponds to: excess derivatization reagent (1), 11β-hydroxyandrosterone (2), dehydroisoandrosterone and equiline (3), androsterone (4), 19-hydroxy-4-androstene-3,17-dione (5) and 5α-androstan-17-one. From Ref. [63] with permission.

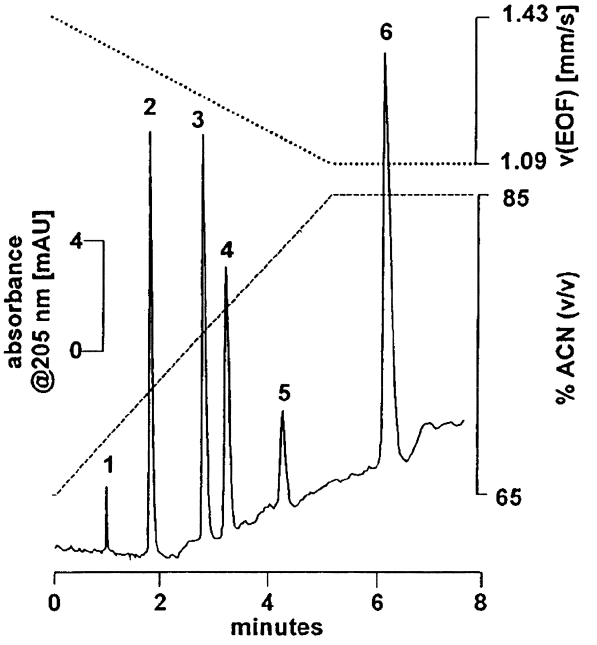

Fig. 18. Separation of steroids using capillary electrochromatography with gradient elution. The column (50 μm×17.6 cm (effective length 9.6 cm)) was packed with 6 μm Zorbax ODS particles having a mean average pore size of 80 Å. Starting eluent (A), 10 m*M* borate, pH 8.0, 65% acetonitrile; gradient former (B), 10 m*M* borate, pH 8.0, 85% acetonitrile; flow-rate of the mobile phase through the mobile phase reservoir, 0.1 ml/min; gradient 0–100% B in 5 min, and then 100% B for 3 min; voltage 14 kV; current 1 μA; electroosmotic flow velocity 1.43–1.09 mm/s; temperature 25 °C, electrokinetic injection 0.5 s, 1 kV, using a UV detector at 205 nm. The eluting peaks correspond to formamide (1), corticosterone (2), testosterone (3), androstene-3,17-dione (4), androstan-3,17-dione (5), and pregnan-3,20-dione (6). From Ref. [123] with permission.

by CEC within 7 min, using gradient elution, see Fig. 18. In another study, Stead et al. [124] have shown that it is possible to transfer an existing RP-HPLC method for the analysis of progesterone and its major metabolites in plasma to a CEC system. A comparison between the two methods revealed that the superior separation obtained with CEC is maintained even with a complex biological sample matrix. It was also shown that CEC enhanced the efficiency and substantially reduced the overall time of analysis.

2.3. Detection techniques

As is evident from Table 2, naturally occurring neuroactive steroids are commonly present at very low (nanomolar to picomolar) levels in complex matrices, and sensitive and robust detection techniques are therefore needed to correctly quantitate these compounds. In the early age of steroid analysis this was not straightforward, as the available techniques were lacking in sensitivity. Large sample amounts were therefore needed. Fortunately, a number of new techniques with excellent sensitivity have been developed over the last few decades, and the most common techniques are summarized below.

2.3.1. Radioimmunoassay

The use of immunoassays was introduced in the 1970s as a new analytical tool for the determination of steroids, and because of its high sensitivity it rapidly became regarded as the technique of choice for these purposes. The technique is still one of the most commonly used for quantitation of neuroactive steroids (at least in clinical chemistry) and papers are

regularly published where radioimmunoassay (RIA) is used. Barnard et al. [125] have presented a detailed description and a summary of applications using immunobased detection systems for steroid quantitation prior to 1995. Unfortunately, the specificity of RIA is limited due to antibody cross-reactions. The RIA technique is furthermore time-consuming and labor-intensive, and therefore not suitable for high-throughput analysis. Due to its lack of specificity, thorough and efficient sample preparation techniques are required prior to the detection step. Among recent work using RIA is a study by Leb et al. [126] on the metabolic conversion of progesterone by human lymphocytes by using a method based on progesterone-3-(carboxymethyl)-oxime bovine serum albumin (CMO-BSA). Pearson Murphy and Allison [93] have improved this procedure and shown that by using the same antiserum it is possible to quantitate five ring A-reduced metabolites of progesterone, namely 5α-pregnan-3,20-dione, 5β-pregnan-3,20-dione, 3α-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, and 3β-hydroxy-5β-pregnan-20-one. Although the analyte recoveries were rather low, ranging between 22 and 51% with a mean overall recovery of only 35±6%, the authors could prove that picogram to nanogram/ml amounts of these five metabolites are present in human sera. For 3β-hydroxy-5α-pregnan-20-one, higher recoveries can be attainable merely by using pregnenolone-3-CMO-BSA instead [127]. Purdy et al. [128] have raised polyclonal antibodies in rabbits against 3α-hydroxy-5α-pregnan-20-one-11α-yl-CMO-BSA for quantitation of 3α-hydroxy-5α-pregnan-20-one in rat and human plasma. In another paper by Purdy et al. [129], specific RIAs have been used for the quantitation of 3α-hydroxy-5α-pregnan-20-one and 3α,21-dihydroxy-5α-pregnan-20-one (**IX**) in the cerebral cortex and hypothalamus of the brain, and in rat plasma. Corpechot et al. [130] have developed an RIA procedure for quantitation of progesterone, 5α-pregnan-3,20-dione, and 3α-hydroxy-5α-pregnan-20-one in the brain, plasma, and steroidogenic glands of male and female rats.

As mentioned, cross-reactivity towards the antibody is a frequent problem, therefore synthesis of more specific antibodies is of central interest and the antibody used should ideally only react with the analyte of interest. A way of decreasing the cross-reactivity is to utilize a saturation procedure where excess of cross-reacting species are added directly to the analytical system. The antibodies recognize the analyte as well as the cross-reactant and thus become saturated, whereby the contribution of the cross-reacting species in the sample is decreased. Hill et al. [131] have raised polyclonal antiserum against 3β,17α-dihydroxypregn-5-en-20-one-19-O-CMO-BSA in rabbits and it showed 107% cross-reaction with 17α-hydroxyprogesterone. This unspecificity was nearly eliminated by adding excess of the cross-reactant. In their study, the RIA results were compared with data obtained by using the unspecific antiserum alone, having removed the cross-reacting species with HPLC. Samples of human plasma (125 in all) were run in parallel and no significant difference was seen in the results between the two methods. Moreover, Dibbelt et al. have developed sensitive and highly specific RIA methods for the determination of 3α-hydroxy-5α-pregnan-20-one [132] and estrone (estra-1,3,5(10)-trien-17-one) [133] in human serum. A coordinated set of RIAs for 11-deoxycortisol (17,21-dihydroxy-pregn-4-ene-3,20-dione), 21-deoxycortisol (11β,17α-dihydroxy-pregn-4-ene-3,20-dione), testosterone, 11β-hydroxy-androstenedione (11β-hydroxyandrost-4-en-3,17-dione),17α-hydroxypregnenolone androstenedione, DHEA, and 17α-hydroxyprogesterone have been developed and validated by Fiet et al. [134] as a tool for diagnosis of hirsutism and (or) acne. Carpene et al. [135] have shown that it is possible to simultaneously measure corticosterone, deoxycorticosterone (**VIII**), 18-hydroxydeoxycorticosterone (18,21-dihydroxypregn-4-ene-3,20-dione), 18-hydroxycorticosterone (11β,18,21-trihydroxypregn-4-ene-3,20-dione), 11-deoxycortisol, and cortisol in human plasma merely by using high-performance liquid chromatography and RIA. Belanger et al. [136] have used RIA to measure C-19 steroids in rat and guinea pig adrenals, and have furthermore quantified a series of C-21 and C-19 steroids including pregnenolone, 17-hydroxypregnenolone, androstenedione, progesterone, 17-hydroxyprogesterone, DHEA, 11β-hydroxyandrostenedione and testosterone, in both adrenals and plasma [137].

2.3.2. Ultraviolet light absorption

A majority of the neuroactive steroids comprise

functionalities with UV light absorbtion properties, cf. Fig. 1. However, these functionalities are usually only weak absorbing, such as a hydroxyl group, or medium absorbing, such as the ketone or aldehyde groups. Some of the compounds are bearing α,β-unsaturated keto functionalities in the molecular backbone. This conjugation makes π–π* transitions within reach and useful absorption is therefore seen in the wavelength range between 220 and 350 nm. Theoretical approaches exist in calculating the absorption maximum for steroidal compounds [138], but it is more convenient to use a scanning UV detector to determine the observation wavelength for a compound of interest dissolved in an appropriate solvent mixture. Reduced progesterone metabolites absorb light below 220 nm and certain precautions must be taken if these wavelengths are to be used. Dissolved oxygen is one problem, but more generally the high UV-light cut-off of most solvents hinders sensitive detection for these compounds. Only a very limited number of solvents have low UV cut-off in combination with good steroid solvability. Water has a cut-off below 210 nm, but does not dissolve the neuroactive steroids. An alternative may be diethyl ether or acetonitrile, as they easily dissolve a number of polar steroids and also have UV cut-offs around or below 210 nm. Hexane and cyclohexane are even better with UV transparency down towards 200 nm. Unfortunately, these solvents are only suitable for normal-phase chromatography. Consequently, the most commonly used solvents are lower unbranched aliphatic alcohols such as ethanol and methanol, as they readily dissolve most steroids and offer UV cut-offs around 205 nm. Huber et al. [123] have monitored a capillary electrochromatographic separation of a standard solution containing corticosterone, testosterone, androst-4-ene-3,17-dione, androstan-3,17-dione, and progesterone at 205 nm. Ketosteroids with α,β-unsaturation such as progesterone are commonly monitored in the wavelength range 240–245 nm [139] whereas estrogenic compounds are generally detected at 280 nm. RP-HPLC separations coupled with UV detection at 254 nm have been used to monitor clinically relevant levels of 11-deoxycortisol, cortisol, cortisone, and 17-hydroxyprogesterone in serum and urine [140–142]. Although the low sensitivity only allows unsaturated steroids to be quantitated at physiological levels, UV spectroscopy is well suited for screening purposes, for stability studies of drug formulations, and for method development using relatively high concentration standards.

2.3.3. Electrochemical detection

Relatively few functional groups have useful electrochemical properties, although high specificity in the detection could be expected if such groups are present in a molecule. Electrochemical detectors are furthermore associated with low dead volumes, fast and linear response, and low cost. Consequently HPLC separation coupled with electrochemical detection has been investigated as a possible analytical tool for sensitive quantitation of neuroactive steroids, but electrochemical detection is not widely used in steroid analysis today despite the envisioned advantages. Nonetheless, Wang [143] has edited a book where various electroanalytical techniques are described in relation to clinical chemistry, and a review has been compiled by Nagatsu and Kojima [144] summarizing applications of electrochemical detection coupled to HPLC in order to assay biologically active compounds. Electrochemical detection has also been used in the quantification of 17β-estradiol and estrone in serum [139]. The electrochemical and chromatographic properties of seven derivatized ketosteroids, including pregnenolone, androsterone, testosterone, and corticosterone have been investigated by Ueno and Umeda [145]. Detectable electrochemical properties were introduced into the steroids by reaction with several different hydrazines and hydrazides, of which 2,5-dihydroxybenzohydrazide was found to be superior as it formed stable hydrazones that satisfactorily separated on a reversed-phase column. Sensitive detection was obtained at a low oxidative potential, with detection limits ranging between 60 and 500 fmol. Draisci et al. [146] have developed an electrochemical enzyme immunoassay (ELISA) for the quantitation of 17β-estradiol. Using anti-17β-estradiol monoclonal or polyclonal antibodies, two assay schemes were optimized and the authors found that a more sensitive assay was obtained using the polyclonal antibody, resulting in a detection limit of 20 pg/ml.

2.3.4. Fluorescence detection

An alternative detection mode for quantitation of neuroactive steroids is fluorescence spectroscopy, where absorbed light leads to light emitted at a

longer wavelength, detected at an angle perpendicular to the excitation source. Since the emitted light is a direct function of the analyte concentration and the incident light intensity, the background noise can be considerably reduced compared to absorption measurements. For analytes that are good fluorophores, the detection limits are normally one to two orders of magnitude better than UV detection, and quantitation of nanogram to picogram amounts is feasible, even in complex matrices. In general, molecules with rigid and highly conjugated structures are favored in fluorescence detection, but although many steroids have conjugations in the molecular backbone leading to absorption in the mid UV range, almost none have native fluorescent properties. A derivatization reaction is therefore necessary prior to detection to introduce fluorescent properties into the molecule. Fluorescence can be utilized for direct determination, but more commonly for flow-through detection, and steroids with carbonyl moieties in the molecular backbone have readily been monitored in various matrices. Katayama et al. [113] have quantified 17-hydroxyprogesterone, progesterone, androstenedione, testosterone, 17-methyltestosterone, and DHEA in serum as corresponding BODIPY-FL hydrazide derivatives, and Visser et al. [51] have shown that dansylated alphaxalone and 3α-hydroxy-5β-pregnan-20-one could be quantified in rat plasma. Laser-induced fluorescence (LIF) detection provides for even more sensitive detection, and Que et al. [63] have shown that attomole levels of dansylated ketosteroids can be determined. Fluorescence detection is, as previously mentioned, a rather uncommon alternative for quantitation of hydroxysteroids. Nonetheless, DeSilva et al. [147] have used conventional and LIF to monitor pyrene sulfonyl chloride labeled estrogens in human serum, and the detection limit for 17β-estradiol was found to be 4 p*M*. The compounds 18-hydroxycortisol, 18-hydroxycortisone and 18-oxocortisol have been quantified in urine as corresponding to their 1-anthroylnitrile derivatives by Kurosawa et al. [100], and detection limits near 0.1 pmol were obtained. Fluorescence can indeed provide sensitive detection limits, yet there are certain drawbacks with the technique and mechanisms that inhibit fluorescence are known as quenching phenomena. Internal and external conversion, whereby the excited electrons return to ground state without emission of light, is a competitive route and explains why most compounds show no fluorescence. Heavy atoms such as Cl, Br, I or paramagnetic species such as O_2 favor inter-system crossing, with decreased fluorescence quantum yield as a net result. Temperature, pH, solvent viscosity, and polarity of the used solvents are other physical properties with effect on the fluorescent response, and since the selection of chemicals also affect the noise in the analytical system, only chemicals with highest purity should be used.

2.3.5. Chemiluminescence

An even more interesting alternative for steroid quantitation is chemiluminescence detection (CL), as it yields sensitivities comparable to mass spectrometry along with very low noise and background levels, high selectivities, and a wide linear range of detector response. The technique is rather inexpensive and can be utilized for both stand-alone detectors and combined with various flow systems such as HPLC, CEC, and flow injection analysis (FIA). Dodeigne et al. [148] have extensively reviewed the principles of CL and the possibilities of using the technique as a diagnostic tool, and Campaña and Baeyens [149] have edited a book on chemiluminescence in analytical chemistry where current theories, mechanisms and technologies are discussed along with future trends. For quantitation of neuroactive steroids, few variants of CL are applicable and for flow systems, only the peroxyoxalate (PO) and the 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol)-mediated reaction schemes have been used. Detailed descriptions of these reaction schemes can be found elsewhere [148,149]. The PO-CL-mediated reaction scheme is used for quantitation of derivatized steroids whereas luminol and its derivatives can be used for unlabeled steroids. The latter technique has been used for quantitation of several corticosteroids, including cortisone and corticosterone in bovine liver, and sample extracts separated with HPLC yielded detection limits ranging between 0.13 and 8.40 ppb [150]. Toriba and Kubo [151] have used the luminol-mediated reaction detection scheme in conjunction with RP-HPLC separation to quantify cortisone, but also with FIA for cortisone and corticosterone quantitation [152]. In the latter study the corresponding detection limits were found to be 4 and 16

pmol, respectively. The peroxyoxalate-mediated reaction is the more commonly used CL detection scheme for steroids, and has been the subject of reviews by De Jong and Kwakman [153] and by Orosz [154]. With the PO-CL reaction, Koziol et al. [106] have quantified traces of fluocortin butyl, a 3α-ketocorticosteroid, in plasma using pre-column labeling with DNSH and post-column PO-CL detection with bis-(2,4,6-trichlorophenyl) oxalate (TCPO) and hydrogen peroxide. The detection limit was found to be 100 pg/ml or 7.5 pg injected. Nozaki et al. [99] have shown that PO-CL allows quantitation of 17α-estradiol in serum as corresponding dansyl derivatives and a detection limit of 50 pg could be obtained. Yamada et al. [50] have developed a similar method for quantitation of 17β-estradiol in plasma but by carrying out the separation in RP-HPLC mode. Four oxalates, namely TCPO, bis(2,4-dinitrophenyl) oxalate (DNPO), bis(pentafluorophenyl) oxalate (PFPO), and bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)-phenyl] oxalate (TDPO), were evaluated in terms of sensitivity and it was found that DNPO yielded the highest sensitivity with a detection limit for dansylated estradiol of 44 fmol in rat plasma. Higashidate et al. [102] have used TDPO and H_2O_2 to enable PO-CL detection of dansylated 3α- or 3β-hydroxysteroids that initially had been converted into 3-ketosteroids through oxidation by immobilized hydroxysteroid dehydrogenase, followed by pre-column labeling with DNSH. The authors showed that detection limits around 15 fmol could be obtained. The same group has also quantified ketosteroids and bile acids in urine as the corresponding dansyl derivatives with similar detection limits [155]. The reagent 1,1′-oxalyldiimidazole (ODI) has been used instead of TCPO by Appelblad et al. [109] for the quantitation of 3α-hydroxy-5β-pregnan-20-one in serum, using a coupled column chromatographic system, see Figs. 19 and 20. The detection limit was found to be 1.6 pmol for 3α-hydroxy-5β-pregnan-20-one, and the results from the analysis of 45 serum samples corresponded well with the results from a comparable RIA method [109,156].

2.3.6. Mass spectrometry

Mass spectrometry is the only quantitative detection technique that allows positive identification of unknown compounds, and for many years it has been the state-of-the-art technique for many classes of compounds. Today, a variety of mass spectrometers are available, from low-end quadrupole benchtop models to advanced instruments having both time-of-flight and multiple fragmentation features. Recent instrumental development has improved the general applicability of this technique, allowing connection to a multitude of separation processes, such as GC, LC, CE and CEC and three recent review articles describe the use of mass spectrometry in clinical laboratories [157], and separations combined with MS [158,159].

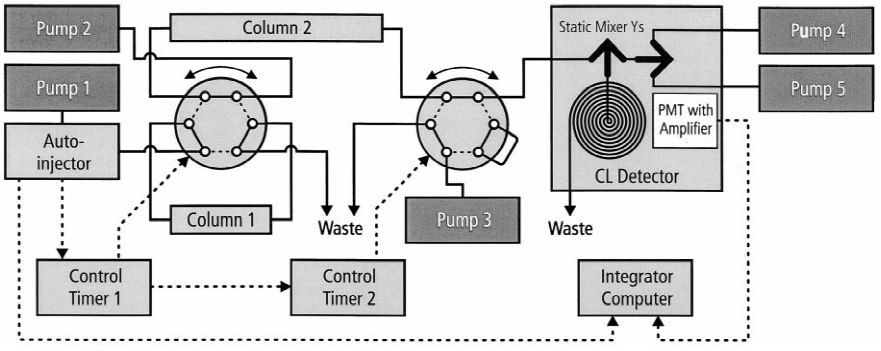

Fig. 19. Schematic drawing showing the coupled column chromatography system used in this study. The valves are shown in their unactivated position (timers off). Dotted lines indicate electrical control and signal pathways. See experimental section for details. From Ref. [109] with permission.

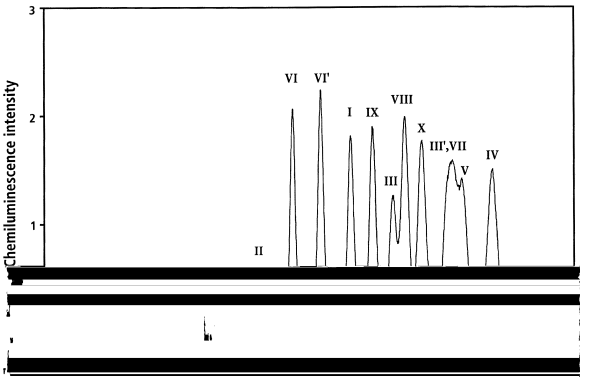

Fig. 20. Chromatogram showing a derivatized mixture containing 1 μ*M* of each steroid (**I**–**X**); 3β-hydroxy-5-pregnen-20-one (**I**), 3α,21-dihydroxy-5β-pregnan-20-one (**II**), 4-pregnene-3,20-dione (**III**), 5α-pregnan-3,20-dione (**IV**), 5β-pregnan-3,20-dione (**V**), 20α-hydroxy-4-pregnen-3-one (**VI**), 3α-hydroxy-5α-pregnan-20-one (**VII**), 3β-hydroxy-5α-pregnan-20-one (**VIII**), 3α-hydroxy-5β-pregnan-20-one (**IX**), 3β-hydroxy-5β-pregnan-20-one (**X**). The double peaks for the steroids **III** and **VI** are due to *syn* and *anti* bonding of the fluorescent label. The peak appearing at 24.7 min was found to be an impurity from the standard solution of **III**. Time window **A** indicates the loading and injection for derivatized solutions from the enrichment block into the separation block. Window **B** illustrates the duration that the separation column is connected to waste. From Ref. [109] with permission.

2.3.6.1. Gas chromatography with mass spectrometric detection

The combination of mass selective detection with gas chromatography is a commonly used analytical set-up for the quantitation of neuroactive steroids. The GC–MS technique was introduced as a way of analyzing steroids in the mid-1960s, and over the last four decades instruments have been immensely improved and the procedures refined. GC–MS is thus a mature technique with numerous quantitative and qualitative methods available [75], capable of serving as rugged and reliable tools for the validation of other techniques that lack the option of positive identification [130,160–162]. Examples showing the utility of GC–MS as a tool for trace analysis have been shown by a number of researchers and, for instance, Liere et al. [83] have developed and optimized a procedure for quantitation of pregnenolone, pregnenolone sulfate, progesterone, 3α-hydroxy-5α-pregnan-20-one, DHEA, and DHEA sulfate (3β-sulfooxyandrost-5-en-17-one; compound **XI**) in rat brain samples. Following initial treatment by SPE and preparative NP-HPLC, the steroids were converted into their corresponding HFB derivatives and quantified using electron impact ionization (EI) and selected-ion monitoring (SIM). Cheney et al. [163] have also studied some of the model steroids just mentioned. Both Marchand et al. [55], and Hartmann and Steinhart [81], have presented quantitative methods for steroids including androgens, estrogens, and progestagens, in meat, see Fig. 21. Kim et al. [61] have used GC combined with electron capture negative chemical ionization mass spectrometry (GC–ECNCI–MS) to quantitate androsterone, testosterone, 3α-hydroxy-5α-pregnan-20-one, and pregnenolone in CSF and plasma. The model steroids were converted to carboxymethoxime, pentafluorobenzyl and trimethylsilyl derivatives and thereafter quantified as $(M-181)^-$ fragments with detection limits in the low picogram range, see Fig. 22. Matsumoto et al. [164] have used GC–NCI–MS for quantitation of 3α-hydroxy-5α-pregnan-20-one. Ueki and Okano [165] combined GC with combustion-isotope ratio mass spectrometry to monitor excretion of DHEA in urine, and Shackleton et al. [166] have monitored the intake of testosterone, epitestosterone, dihydrotestosterone, and DHEA with the same technique. Choi and Chung [88,89] have developed a method for quantitation of androstenedione, dihydrotestosterone, testosterone, androsterone, DHEA, etiocholanolone, progesterone, and pregnenolone as corresponding pentafluoro-

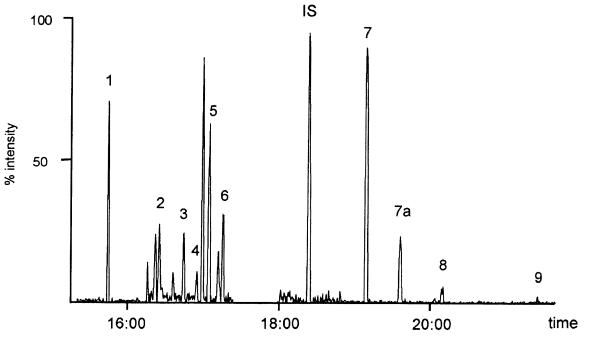

Fig. 21. The total GC–MS ion chromatogram showing a separation of neutral steroids in meat. The eluting peaks correspond to α-androsterone (1), dehydroepiandrosterone (2), epitestosterone (3), dihydrotestosterone (4), androstenedione (5), testosterone (6), methyl testosterone (I.S.), pregnenolone (7, 7a), progesterone (8), hydroxyprogesterone (9). From Ref. [81] with permission.

functionalities (III). Using APCI mode, best sensitivity and lowest detection limit for all three groups were obtained with an eluent based on methanol and 1–2% acetic acid in water. Using electrospray ionization, the best sensitivity and lowest detection limit for steroids of groups I and II were obtained with an eluent based on methanol and water only, and detection limits in the 5–15 pg range were obtained, see Fig. 10. In addition, it was also found that an addition of ammonium acetate and/or acetonitrile to the eluent decreased the sensitivity. Since the mass spectrometers are rapidly becoming more advanced and less expensive, reports have started to appear where HPLC is utilized in combination with tandem mass spectrometry for steroid analysis. Lagana et al. [174] have monitored common biologically active natural and synthetic steroids using RP-HPLC separation in combination with tandem mass spectrometry with atmospheric pressure chemical ionization (LC–APCI–MS–MS). Estriol (3,16α,17β-trihydroxy-estra-1,3,5(10)-triene), 17β-estradiol, 17α-ethynylestradiol (3,17β-dihydroxy-17α-ethynyl-estra-1,3,5(10)-triene), estrone, progesterone, trenbolone (17β-hydroxyestra-4,9,11-trien-3-one), boldenone (17β-hydroxyandrosta-1,4-dien-3-one), nandrolone (17β-hydroxyestra-4-en-3-one), testosterone, 17α-methyltestosterone (17β-hydroxy-17α-methyl-androst-4-en-3-one), and stanozolol (17β-hydroxy-17-methyl-5α-androstano[3,2-c]-pyrazole) were studied using positive ionization mode, and the protonated ions and dehydrated ions were used as precursor ions for CID. The LC–APCI–MS–MS combination has also been used for the quantitation of cortisol [175], 17β-estradiol [176], progesterone [66,177], and 17-hydroxyprogesterone [178]. In the latter study the authors compared data acquired from patient samples with those obtained using a corresponding GC–MS–MS method, and they found no statistically significant difference between the results produced by the two methods. However, although LC–MS has proven to be a promising analytical technique, the detection limits attainable at present are insufficient for certain interesting neuroactive steroids, and derivatization is therefore still required to enable quantitation in biological samples. Mitamura and Shimada [179] reviewed suitable labeling reaction schemes.

2.3.6.3. Capillary electrochromatography with mass spectrometric detection

Electroosmotically driven chromatographic separation in the capillary format are conceptually ideal for coupling to MS detection, and although this is a rather immature technique, it has the advantage of having very high separation efficiency and high sensitivity with small samples. Que et al. [63] have used hydrophobic macroporous monolithic columns for separation of both neutral and conjugated steroids and determined the steroid profiles with electrospray-ion-trap mass spectrometric detection. Good column efficiency along with femtomole detection limits were attained. However, the technique can only tolerate small sample volumes and efficient sample pre-concentration steps are therefore necessary.

3. Conclusions

Having summarized a large portion of the research on neuroactive steroid analysis published during the last decade, our opinion is that, although many systematic investigations have been carried out to establish new analytical methods for these compounds in complex biological matrices, there are still problems in analyzing at the trace levels. Clinically relevant concentrations of neuroactive steroids vary between high p*M* and low to medium n*M* concentrations, as shown in Table 2, and the small sample volumes (ml or mg) typically available call for sensitive and selective validated analytical methods for reliable quantitation. Numerous papers claim that ‘‘methods’’ have been developed, but when the reports are critically reviewed it is apparent that validation data for one or more unit operations are commonly missing. It is therefore our recommendation that the research community should be more restricted in using the word *method*, so that it is not used to cover technical improvements or merely potential analytical applications. Another recommendation is that *real samples* should always be analyzed for the compounds of interest if detection limits should be presented, since determination of analytes in standards or other artificial solutions seldom reveal more than instrumentation variability. In the important area of sample pretreatment, surpris-

ingly little improvement has been made during the last decades, but recent developments such as molecularly imprinted polymers and restricted access materials are likely to become important tools to improve the selectivity in the determination of trace amounts of steroids in complex biological samples. Historically, only the GC technique could produce a sufficient resolution for these compounds, but today LC is considered as an equally suitable separation technique. There are detection techniques available that offer high sensitivities without proper selectivity (i.e. RIA and fluorescence), but only schemes that comprise efficient sample pretreatment and separation steps in combination with positive identification can be regarded as state-of-the art. Based on the above prerequisites, only GC–MS and LC–MS qualify as general platforms on which rugged methods can be developed and used without questioning the retrieved analytical data. These techniques do, furthermore, allow the use of isotope labeled standards that increase the method reliability and enable very low detection limits. Nonetheless, RP-HPLC coupled with either fluorescent (in particular laser excited) or chemiluminescent detection can qualify as suitable analytical techniques, provided they can be validated against other methods, preferentially involving mass spectroscopy for positive identification of analytes. For screening purposes, in stability studies, and for initial method development, and other applications where the limited sensitivity is sufficient, UV detection should be considered because of its ruggedness, ease of use and cost-efficiency.

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