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**Nano-Electrospray Mass Spectrometry for the Analysis of Neurosteroids and  
Related Molecules**

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**To my family**

## ABSTRACT

Neurosteroids are steroids synthesised in the central and peripheral nervous systems. Known neurosteroids include pregnenolone, dehydroepiandrosterone (DHEA), progesterone and its reduced metabolites. It has been demonstrated that neurosteroids modulate neurotransmission by binding to neurotransmitter receptors, and exert physiological functions that are clearly different from those of endocrine steroids. The effects of neurosteroids on improving the memory of cognitively impaired aged rats, on the inhibition of aggressiveness in castrated male mice, and trophic effects on neuronal regeneration and remyelination have been documented. The local synthesis, selective interaction with neurotransmitter receptors and behavioural effects of neurosteroids strongly suggests that they may have important physiological or pathophysiological roles. There is an increasing need to develop methods to analyse these hormones with high sensitivity and high specificity. In this thesis I focused on the development of methods combining nano-electrospray (ES) mass spectrometry with capillary column liquid chromatography (CLC) for the analysis of profiles of neurosteroids in rat brain. It was also an aim to make the methods applicable to a broad range of lipophilic biomolecules.

Initially, synthetic steroid sulphates and unconjugated oxosteroids (ketosteroids) were studied by nano-ES and tandem mass spectrometry. Steroid sulphates could be detected as deprotonated molecules in full range scanned spectra at a level of 1 pg/ $\mu$ L. Information about steroid structure was obtained from collision-induced dissociation (CID) spectra of 1 ng of steroid sulphate, while characterisation of the sulphate ester group required only 3 pg of material. Unconjugated oxosteroids were converted into their oximes which were detected as protonated molecules with 20 times higher sensitivity than the underivatized steroids. The detection limits for the oximes of 3-oxo- $\Delta^4$ , 20-oxo and 17-oxo steroids were 2.5, 5, and 25 pg/ $\mu$ L, respectively in full range scans. CID spectra of the protonated oximes provided valuable information regarding the position of oxo and hydroxyl group(s). These studies established a basis for determination and structure characterisation of neurosteroids from brain samples

A procedure for CLC-ES mass spectrometry was then developed. A double splitter method was introduced which made it possible to use a pre-column for analyte focusing from large sample volumes. It also made it possible to operate the solvent pumps at flow rates compatible with gradient elution while the flow rates through the analytical column were compatible with micro-electrospray. The method was designed to be generally applicable to the analysis of biomolecules and its utilities were demonstrated by the analysis of steroid sulphates in human plasma.

In the course of these studies, certain CLC-ES conditions were found to cause on-column chemical transformations of 3 $\beta$ -hydroxy- $\Delta^5$  steroid sulphates. Radical species generated from electrolysis of water and methanol in the solvent are proposed to be responsible for the formation of oxidised and methoxylated products of these steroids. Other analytes with double bonds were also transformed under these conditions. Thus, on-column electrochemistry can be an important source of artefacts in analyses by CLC-ES mass spectrometry. The reactions could be prevented by appropriate grounding.

The analysis of neurosteroids in rat brain required the development of an extraction, purification and subfractionation procedure. Brain steroids were extracted, and unconjugated neutral steroids and sulphated steroids were separated. The steroid sulphate fraction was then analysed by CLC-ES mass spectrometry. Endogenous sulphates of pregnenolone and DHEA were not detected at levels above the detection limit, 0.3 ng/g wet brain, while pregnenolone sulphate, added to brain extract at a level of 6.6 ng/g, was easily detected. The unconjugated oxosteroids were converted to their oximes, selectively isolated on a cation

exchanger, and analysed by CLC-ES tandem mass spectrometry. The chromatograms showed the presence of progesterone, pregnenolone, pregnanolone isomers, DHEA and testosterone in rat brain. These steroids were characterised by tandem mass spectrometry. Based on the results of CLC-ES tandem mass spectrometry, the levels of C<sub>21</sub> and C<sub>19</sub> steroids were estimated in the range of 0.04 – 20 ng/g wet brain. The levels of progesterone and testosterone showed a sex difference.

During the development of the above analytical methods, nano-ES mass spectrometry was applied to the characterisation of a lipophilic modulatory factor isolated from mouse brain. The factor, which activated the retinoid X receptor (RXR), was extracted from mouse brain incubates, purified by HPLC and analysed by nano-ES and tandem mass spectrometry. Accurate mass measurement and CID spectra of the purified active compound revealed that it was cis-4,7,10,13,16,19-docosahexaenoic acid.

In conclusion, the methods developed and described in this thesis are suitable for the analysis of sulphated steroids and oxosteroids, as well as other related compounds. With their high sensitivity the methods enable highly specific analysis of these important compounds from small amounts of sample.

## LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. William J. Griffiths, **Suya Liu**, Yang Yang, Robert Purdy and Jan Sjövall. Nano-Electrospray Tandem Mass Spectrometry for the Analysis of Neurosteroid Sulphates. *Rapid Commun. Mass Spectrom.* **13**, 1595-1610 (1999).
- II. **Suya Liu**, Jan Sjövall and William J. Griffiths. Analysis of Oxosteroids by Nano-Electrospray Mass Spectrometry of Their Oximes. *Rapid Commun. Mass Spectrom.* **14**, 390-400 (2000).
- III. **Suya Liu**, William J. Griffiths and Jan Sjövall. Capillary Liquid Chromatography/Electrospray Mass Spectrometry for the Analysis of Steroid Sulphates in Biological Samples. *Anal. Chem.* **75**, 791-797, (2003).
- IV. **Suya Liu**, William J. Griffiths and Jan Sjövall. On-Column Electrochemical Reactions Can Accompany the Electrospray Process. *Anal. Chem.* **75**, 1022-1030, (2003).
- V. **Suya Liu**, Jan Sjövall and William J. Griffiths. Neurosteroids in Rat Brain: Extraction, Isolation, and Analysis by Capillary Liquid Chromatography-Electrospray Mass Spectrometry. Manuscript.
- VI. Alexander Mata de Urquiza, **Suya Liu**, Maria Sjöberg, Rolf H. Zetterström, William Griffiths, Jan Sjövall, and Thomas Perlmann. Docosahexaenoic Acid, a Ligand for the Retinoid X Receptor in Mouse Brain. *Science*, **290**, 2140-2144 (2000).

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## ABBREVIATIONS

APCI	atmospheric pressure chemical ionisation
CID	collision-induced dissociation
CLC	capillary column liquid chromatography
CNS	central nervous system
DHEA	dehydroepiandrosterone
ES	electrospray
GABA	gamma-aminobutyric acid
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
LC	liquid chromatography
MRM	multiple reaction monitoring
NMDA	N-methyl-D-aspartate
RIA	radioimmunoassay
RIC	reconstructed ion chromatogram
SPE	solid phase extraction
Th	Thomson ( $m/z$ )
TIC	total ion chromatogram

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## ACKNOWLEDGMENTS

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## INTRODUCTION

### Steroids and neurosteroids

Steroid hormones are a class of compounds with structures based on the cyclopentanoperhydrophenanthrene nucleus with or without a side chain and with hydroxyl or oxo (ketone) groups attached. They are synthesised from cholesterol in different cells via the intermediate, pregnenolone (3 $\beta$ -hydroxypregn-5-en-20-one) (Fig. 1). They exist in the free form, as fatty acid esters, as conjugates with glucuronic or sulphuric acid, and also in other forms. According to their physiological functions steroid hormones are classically divided into adrenal hormones, including glucocorticoids and mineralocorticoids, and sex hormones, including androgens, estrogens and progestins.

Some steroids are also synthesised in the central and peripheral nervous system, the so-called neurosteroids (Baulieu, 1997). The term neurosteroid does not signify a particular class of steroids but only refers to those steroids that are synthesised in the nervous system rather than in adrenal glands or gonads. Some steroids, like estrogens, are not considered as neurosteroids even though they are neuroactive, because they are synthesised from blood-borne precursors and disappear from the CNS after the removal of steroidogenic glands (Robel et al. 1999). So far, dehydroepiandrosterone (DHEA, 3 $\beta$ -hydroxyandrost-5-en-17-one) and pregnenolone, in free or sulphated forms, progesterone (pregn-4-ene-3, 20-dione) and its reduced metabolites, e.g. 5 $\alpha$ -pregnane-3,20-dione and 3 $\alpha$ /3 $\beta$ -hydroxy-5 $\alpha$ /5 $\beta$ -pregnan-20-one, have been considered as neurosteroids (Fig.1). The distribution of neurosteroids in brain is heterogeneous. In rat brain the levels range from 0.24 to 15 ng/g and vary in different parts of the brain (Baulieu, 1997).

### *Biosynthesis*

Steroid hormones are synthesised in the adrenal cortex, ovaries, testes, and during pregnancy in the placenta. Cholesterol first undergoes side chain cleavage to form pregnenolone, a step that is mandatory in the synthesis of all steroid hormones (Fig. 1). Pregnenolone can be converted directly to progesterone (progestin), which requires the cytoplasmic enzyme, 3 $\beta$ -hydroxy- $\Delta^5$ -steroid dehydrogenase/4, 5-isomerase. Progesterone can then be converted to cortisol (glucocorticoids), aldosterone (mineralocorticoids) and testosterone (androgen). Pregnenolone is also converted into DHEA, which can then be converted to testosterone and further to estradiol (estrogen). The pathways for the conversion of cholesterol to adrenal cortical steroids and sex hormones are shown in Fig.

1. The rate-limiting step in steroid hormone biosynthesis is the transfer of cholesterol into the mitochondria and subsequent side chain cleavage accomplished by enzymes collectively known as the cytochrome P450 side chain cleavage enzyme complex (P450<sub>scc</sub>).

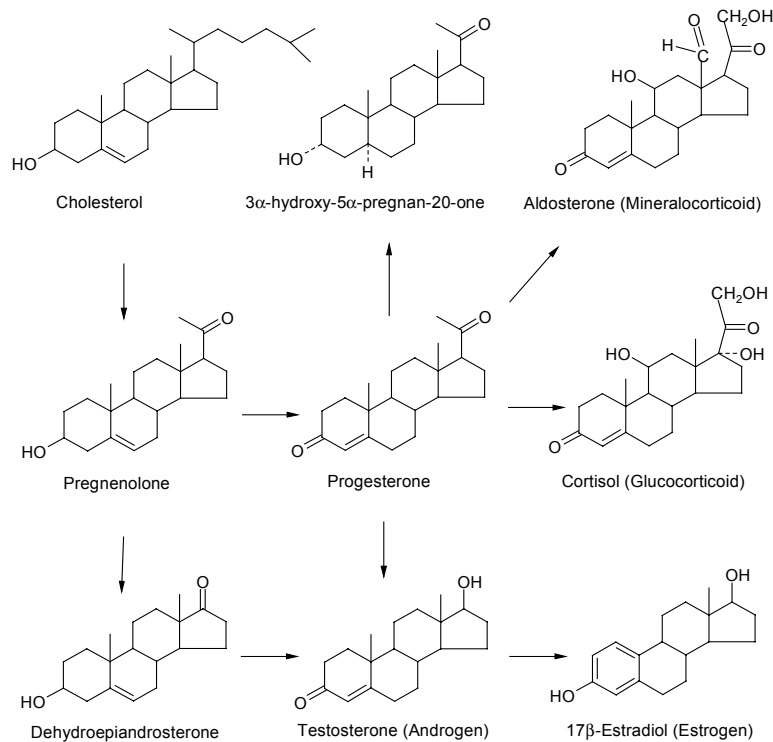


Fig. 1. Synthetic pathways of steroid hormones.

Some steroids are synthesized within the central and peripheral nervous system. DHEA sulphate (Corpechot et al. 1981) and pregnenolone sulphate (Corpechot et al. 1983) were reported to be present in rat brain at much higher levels than in blood. This finding could not be explained by the cerebral retention of the circulating hormone, as pregnenolone sulphate and DHEA sulphate were maintained at high levels in the brain for weeks after castration and adrenalectomy, given that the cerebral clearance of the circulating hormone was very rapid (Corpechot et al. 1983). It was then shown by immunocytochemistry that the cytochrome P450<sub>scc</sub> that converts cholesterol into pregnenolone is expressed in the white matter throughout the brain (Le Goascogne et al. 1987). The biosynthesis of pregnenolone was demonstrated by incubating glial cells from newborn rats in the presence of [<sup>3</sup>H]-mevalonolactone, a precursor of cholesterol, which easily enters cells and mitochondria (Jung-Testas et al. 1989). Also, the P450<sub>scc</sub> mRNA has been detected by reverse transcription polymerase chain reaction both in rat brain and

cultured glial cells (Mellon and Deschepper, 1993, Strömstedt and Waterman, 1995, Sanne and Krueger, 1995).

Progesterone was also detected in male rat brain and mouse sciatic nerves at a level of about 2 and 10 ng/g wet tissue weight, respectively (Baulieu, 1997, Koenig et al. 1995). The levels remained high after adrenalectomy and gonadectomy, while the levels of progesterone in plasma fell below detection limits after adrenalectomy (Koenig et al. 1995, Corpechot et al. 1993). The formation of progesterone from pregnenolone catalysed by  $3\beta$ -hydroxy- $\Delta^5$ -steroid dehydrogenase/4,5-isomerase in myelinating glial cells is well established (Jung-Testas et al. 1989). Progesterone can be further converted to  $5\alpha$ -dihydroprogesterone ( $5\alpha$ -pregnane-3,20-dione) catalysed by a  $\Delta^4$ -3-oxosteroid  $5\alpha$ -reductase and to allopregnanolone ( $3\alpha$ -hydroxy- $5\alpha$ -pregnan-20-one) by a  $3\alpha$ -hydroxysteroid dehydrogenase. The above enzymatic reactions occur in cultures of oligodendrocytes and astrocytes (Jung-Testas et al. 1989, Kabbadj et al. 1993)

To date, pregnenolone, progesterone and their reduced metabolites are the only steroids that have been shown to be formed *de novo* from cholesterol within the brain. Although DHEA was the first to be called a neurosteroid, its pathway of synthesis is not clear since the  $17\alpha$ -hydroxylase, which is the first enzyme in the conversion of pregnenolone to DHEA, has not been detected in the nervous system. However, an unconventional pathway may exist (Prasad et al. 1994, Cascio et al. 1998)

### *Physiological functions*

It is well understood that endogenous steroid hormones exert their functions by binding to specific intracellular receptors and regulate target gene transcription. In this way, steroid hormones, which themselves are regulated by other hormones and/or signal molecules, regulate the synthesis of metabolic enzymes, receptors and other proteins, thus affect metabolism, reproduction and development. For example, cortisol, an adrenal steroid hormone, generally stimulates the degradation of proteins to amino acids in skeletal muscle and the promotion of gluconeogenesis as a response to stress. The effects of steroid hormones usually require hours or days to become evident. It should be noted that classical steroid hormones also have intracellular receptors in the nervous system. These receptors, e.g. those of glucocorticoids and estrogens are localised to specific areas of the brain (Fuxe and Gustafsson 1981).

In contrast to traditional steroid hormones, neurosteroids exert their functions by binding to neurotransmitter receptors. They can either stimulate or inhibit neurotransmission rapidly, in seconds to minutes. Neurosteroids like allopregnanolone

selectively enhance the interaction of GABA with the GABA<sub>A</sub> receptor by binding to the GABA<sub>A</sub> receptor (Lambert et al. 1995). They are active at the nM level (Woodward et al. 1992). Their effects are to prolong the open time of the GABA<sub>A</sub> receptor ion channel and to increase the frequency of ion channel opening. Pregnenolone sulphate is a weaker enhancer of GABA-evoked currents in the nM range, but it is an inhibitor in  $\mu$ M range (Majewska et al. 1988). Besides interaction with the GABA<sub>A</sub> receptor, neurosteroids or their synthetic analogues interact with other neurotransmitter receptors, i.e. NMDA receptor, glycine receptor, ionotropic glutamate receptor, nicotinic receptor, 5-HT<sub>3</sub> receptor, and Sigma receptor (Baulieu, 1997).

Pregnenolone has recently been reported to bind to microtubule-associated protein 2 and to stimulate microtubule assembly (Murakami et al. 2000), showing a possible way by which neurosteroids can affect the development of the nervous system. So far, nuclear receptors for DHEA, pregnenolone or allopregnanolone have not been demonstrated.

Although the physiological functions of neurosteroids are not well understood, effects of neurosteroids on behaviour have been demonstrated. DHEA and its synthetic analogues have been found to inhibit the aggressiveness of castrated male mice (Schlegel et al. 1985). This effect was not mimicked either by DHEA sulphate or by its estrogenic metabolite androst-5-ene-3 $\beta$ ,17 $\beta$ -diol. The inhibitory effect on aggressiveness induced by DHEA is related to a significant decrease of pregnenolone sulphate in the brain of DHEA-treated castrated mice (Young et al. 1991). Interestingly, a linkage of pregnenolone sulphate levels in the hippocampus of rats and memory performance in the water maze has been observed and it suggested that pregnenolone sulphate in the hippocampus plays a physiological role in memory (Vallee et al. 1997). This was further supported by the correction of the memory deficit of cognitively impaired aged rats after injection of pregnenolone sulphate. Neurosteroids also have some trophic effects on neurons and glial cells. When DHEA and DHEA sulphate were added to culture medium, they were found to enhance the survival and differentiation of neurons prepared from embryonic mouse brain (Bologa et al. 1987). Pregnenolone and progesterone have been found to help regeneration of injured spinal cord and the survival of motor neurons, respectively (Guth et al. 1994, Yu, 1989). Progesterone has been shown to promote peripheral myelination which may also occur in the CNS (Koenig et al. 1995).

It should be noted that in the studies referred to above all of sulphated steroids were not directly characterised. Instead, they were estimated by RIA, or the free steroids released by solvolysis analysed by GS-MS.

## **Analytical methods**

Although the analysis of steroids in biological samples has a long history, and there are many methods currently used, the analysis of neurosteroids in brain remains a challenge to analytical chemists. First, because of the low level of steroids in brain, their measurement requires analytical methods with high sensitivity. In addition, because of the local synthesis of neurosteroids and probable local function (paracrine or autocrine), a crucial requirement for an analytical strategy is its ability to analyse the steroids in small amounts of brain tissue from specific areas, such as from hippocampus, amygdala and olfactory bulb. Second, many steroid isomers may be present, both structural and geometric isomers, and a differentiation of these isomers is required. Third, neurosteroids exist in free and conjugated forms, so it is desirable that they are analysed in their intact forms. Fourth, because of their lipophilic nature, a severe contamination problem from the lipid constituents of brain tissue is expected, so that high specificity of the analytical method is needed. The ability to perform multicomponent analysis is also important, particularly for studies of the biochemistry of neurosteroids. A method for the comprehensive analysis of steroid profiles could serve as a basis to increase our understanding of the nature and functions of these steroids in brain. The following sections briefly review possible analytical steps and sample preparation procedures for analysis of steroids in brain.

### *Extraction, isolation and purification of steroids from brain*

Extraction of steroids from tissues like brain often starts with homogenisation of the brain tissue, which can be performed in water or organic solvents. If homogenised in water or saline, the steroids in the brain homogenate must be extracted with organic solvents like ethyl acetate (Corpechot et al. 1983, Cheney et al. 1995, Uzunov et al. 1996) or chloroform-methanol (2:1, v/v) (Shimada and Mukai 1998) since most of the steroids are poorly soluble in water. In many studies, brain tissue has been homogenised in organic solvent, and steroids extracted into the organic solvent. Alcohols were used either alone or in combination with water or with other less polar solvents like acetone or chloroform. (Shimada and Yago 2000, Corpechot et al. 1981, Liere et al. 2000, Vallée et al 2000). Conjugated steroids may demand an ion-pairing reagent to aid such extraction (Sjövall and Axelson, 1982). A general procedure using hexane-isopropanol for homogenisation has been developed by Andersson and Sjövall (1985) for characterisation and quantitation of unconjugated steroids in testis using GC-MS. Although selectivity can be achieved to

some extent (Corpechot et al. 1983), liquid-liquid extraction methods are considered as non-selective methods and further purification steps are required.

Among the sample work-up techniques, solid phase extraction (SPE) has become the most popular method for isolation and purification of lipids. It can be applied directly to urine and blood samples to extract and purify steroids of interest, provided that conditions are chosen to minimise protein binding (Sjövall and Axelson, 1982). For the analysis of steroids in tissues SPE is very often used as an isolation and purification step. Reversed-phase SPE has been widely used in analyses of steroids in brain. (Wang et al. 1997, Liere et al. 2000, Nakajima et al. 1998, Mitamura et al. 1999, Shimada and Yago 2000). It is possible to separate steroid sulphates from unconjugated steroids by applying different washing and eluting solvents. However, the high lipid content of the brain must be considered. In a highly aqueous solution, which is often used for application of a sample solution to a reversed-phase SPE, formation of micelles or lipid aggregates is very likely to occur when large amounts of phospholipids are also present. This may result in loss of steroids in the effluent and wash fractions. One should always keep in mind that sorption of analytes can occur only when the analytes are soluble in the solvent applied. When the sample contains compounds of widely different polarity and solubility a recycling SPE method can be used (Axelson and Sjövall 1985). Alternatively, normal phase SPE may have some advantages in this respect, as the sample can be applied to and eluted with organic solvents. Sjövall and Vihko (1966) have separated steroid sulphates from unconjugated steroids and phospholipids on a Sephadex LH-20 column. Silicic acid chromatography is a classical method for purification of neutral steroids and silica gel columns were recently used to purify DHEA and pregnenolone and its 3-stearate from brain (Shimada and Yago 2000, Shimada and Mukai 1998).

Ion-exchange chromatography has been extensively used for the group isolation and purification of steroids, bile acids and other metabolites from biological samples (Sjövall and Axelson 1979, 1982, Fotsis, et al. 1981, Meng and Sjövall, 1997, Yang et al. 1997). It has also been used in the analysis of steroid sulphates in brain (Mitamura et al. 1999). Compared to other chromatographic techniques like partition chromatography, ion-exchange chromatography is more suitable for the subsequent analysis of steroids by mass spectrometry as it separates steroids into groups based on their charge state. The charge state influences the choice of ionisation mode in mass spectrometry.

Because of the complexity of biological samples and the variety of steroids of interest, usually a combination of two or more chromatographic techniques is required to fulfil a satisfactory purification. In several studies, preparative HPLC has been used to

selectively purify the steroids of interest (Andersson and Sjövall 1985, Cheney et al. 1995, Liere et al. 2000).

### *Radioimmunoassay*

RIA has been widely used in the analysis of steroids, especially in clinical chemistry, largely because of its high sensitivity and simplicity of use. RIA is commonly regarded as a specific method because it is based on the specific interaction between a molecule and its antibody. However, in the case of steroid analysis, specificity is questionable as cross-reactions may occur. HPLC separation is usually required prior to RIA. In addition, non-specific interactions become serious when the level of steroid to be analysed is low. Although neurosteroids in brain were first analysed by RIA (Corpechot et al. 1981, 1983, 1993), RIA is not a method of choice for profile analysis, especially for a complex sample like brain.

### *Mass spectrometry*

Recent developments in mass spectrometry have made it the method of choice for the analysis of a wide range of chemical and biological compounds. The features that make it stand out from other techniques, are the high specificity, high sensitivity, and capability to characterise unknown compounds, as well as the capability for multicomponents analysis in a complex sample matrix. Mass spectrometry has for many years been used in the analysis of steroids in biological samples.

For a sample to be analysed by mass spectrometry, it must be transferred into the gas phase and ionised. The ions are then directed to a mass-to-charge ratio ( $m/z$ ) analyser and the  $m/z$  of the ions determined. Many ionisation methods have been developed over the years and it is the recent developments in ionisation methods that have brought mass spectrometry to the focal point of biological research. Electron impact (EI) ionisation is a classical method and suitable for the ionisation of small, volatile, and thermostable molecules, including most unconjugated steroids after derivatisation. Upon EI ionisation, steroids usually give molecular ions and fragment ions, enabling both quantitative and qualitative analysis to be made. Chemical ionisation (CI), an alternative to EI, results in a spectrum with fewer fragment ions, which can make it more sensitive for quantitative applications. These two ionisation methods are often used in combination with GC since both require the analytes to be vaporised prior to ionisation.

The development of fast atom bombardment (FAB) ionisation (Barber et al. 1981) provided a means to analyse steroid conjugates directly and was extensively used to study

steroid metabolites in biological fluids and to determine their structures (Griffiths et al. 1996, Griffiths et al. 1993, Shackleton and Straub 1982, Shackleton, 1983, Tomer and Gross, 1988).

Electrospray (ES) was first coupled with mass spectrometry in the mid-1980s (Yamashita and Fenn 1984a, 1984b). By applying a high potential to a small capillary containing the sample solution, a very fine spray of droplets of the sample solution is generated, which contains an excess of ions of one polarity. As they follow a potential and pressure gradient, these droplets will decrease in size, as solvent evaporates, and cleave into smaller droplets, which will eventually contain only one ion, or an ion may be desorbed from the small droplets. With the improvements in ES interface design it has become a common ionisation mode and has been used in a diverse array of applications. In ES mass spectra the dominant ions are protonated or de-protonated molecules, depending on the ionisation mode. In general, ES is a preferable method for the analysis of pre-charged compounds like steroid sulphates. Atmospheric pressure chemical ionisation method (APCI) is more favoured by some workers for the analysis of neutral steroids. The utility of ES and APCI mass spectrometry for the analysis of steroids and steroid conjugates has been demonstrated by several groups (Bean and Henion 1997, Zhang and Henion 1999, Chatman et al. 1999, Yang et al. 1997, Griffiths et al. 1999, Schackleton et al. 1997, Ma and Kim 1997). A most valuable feature of ES and APCI is their compatibility with liquid separation techniques like HPLC, and electrophoresis.

Although mass spectrometry is a very powerful analytical tool, it does not alone allow the full characterisation of neurosteroids in brain. Different geometric isomers of pregnanolone,  $3\alpha$ -hydroxy- $5\alpha$ -pregnan-20-one and  $3\beta$ -hydroxy- $5\alpha$ -pregnan-20-one, have different effects on GABA<sub>A</sub> receptor modulation and have to be analysed individually. Although tandem mass spectrometry is able to distinguish between structural isomers of steroids, it is less well equipped to differentiate geometric isomers of steroids. A combination of mass spectrometry with separation techniques will solve this problem to a large extent.

#### *Gas chromatography-mass spectrometry*

The coupling of gas chromatography with mass spectrometry has been a perfect combination, combining the advantages of both techniques, e.g. high separation efficiency of GC, and high sensitivity and specificity of mass spectrometry. GC-MS with EI or CI has been used for the characterisation and quantitative or determination of neurosteroids in brain (Corpechot et al. 1981, 1983, 1993, Cheney et al. 1995, Uzunov et al. 1996, Liere et



al. 2000, Vallee et al. 2000, Shimada and Yago 2000, Kim et al. 2000). Wolthers and Kraan (1999) have recently reviewed the clinical applications of GC-MS in steroid analysis.

One limitation of GC-MS in steroid analysis is the demand for derivatisation to increase volatility and thermal stability. Conjugated steroids need to be solvolysed/hydrolysed before analysis. This precludes the direct analysis of neurosteroid sulphates, so their analysis must rely on a selective isolation of steroid sulphates in the sample preparation procedure.

#### *Liquid chromatography-mass spectrometry*

Both ES mass spectrometry and APCI mass spectrometry, coupled with LC, have been used for the analysis of steroids and steroid conjugates (Bean and Henion 1997, Zhang and Henion 1999, Ma and Kim, 1997, Ghulam et al. 1999). These combinations improve the sensitivity and specificity of analysis. LC-MS has also been applied to the analysis of steroids in biological samples (Bean and Henion, 1997, Mikšík et al. 1999, Yang et al. 1997), including neurosteroids in brain (Shimada and Nakagi 1996, Shimada and Mukai 1998, Shimada et al. 1998, Nakajima et al. 1998). For the analysis of neutral steroids, derivatisation was used to increase sensitivity (Shackleton et al. 1997, Shimada et al. 1998).

#### *Capillary column liquid chromatography-electrospray mass spectrometry*

The fact that ES is a concentration dependent process at low flow rate indicates that a combination of low flow rate CLC with ES should give higher sensitivity than conventional LC with ES. The CLC-ES combination provides higher sensitivity compared to conventional LC-ES mass spectrometry, because of the higher analyte concentration in eluting peaks when using a CLC column and the inherent gain in ionisation efficiency when using low-flow rate ES (Griffiths 2000). Numerous studies of the CLC-ES coupling have been published (Hyllbrant et al. 1999, Vanhoutte et al. 1997, Oosterkamp et al. 1998, Alexander, IV et al. 1999, Licklider et al. 2002).

## **AIMS OF THE PRESENT STUDY**

- 1) To investigate the use of nano-ES mass spectrometry in the analysis of steroid sulphates.
- 2) To study oxime derivatives of oxosteroids for the analysis of neurosteroids by nano-ES mass spectrometry.
- 3) To design a capillary column liquid chromatography-micro-ES mass spectrometry system for the analysis of neurosteroid sulphates and neutral neurosteroids in brain tissue.
- 4) To develop a sample preparation procedure for the isolation and purification of neurosteroids from brain tissue.
- 5) To apply the developed methods to brain samples for detection and characterisation of neurosteroid sulphates and neutral oxosteroids, as well as other lipophilic compounds.

## METHODOLOGY

### *Extraction, isolation and purification of neurosteroids from brain tissues*

The sample preparation procedure for the analysis of neutral oxosteroids and sulphated steroids from rat brain is outlined in Fig. 2. Brain samples ranging from 50 mg to 300 mg wet weight was used (paper V). Either the entire brain or isolated amygdala or hippocampus regions was homogenised and aliquots analysed. Homogenisation was performed in ethanol with a glass homogeniser and followed by ultrasonication for 10 min. Then water was added to dilute the ethanol solvent to 70% and the sample was ultrasonicated for a further 5 min. The mixture was then centrifuged and the residue was extracted again with 1 mL of 70 % ethanol. This second extract was also centrifuged and the supernatant combined with the first. The combined supernatants were applied to a Bondesil C18 bed (100 mg) packed in a Pasteur pipette followed by a lipophilic cation exchanger column (SP-LH-20, 5 cm × 0.4 cm, in H<sup>+</sup> form) packed in a glass column (Axelson and Sjövall, 1979). The effluent from this sequence of column beds was combined with a 2 mL 70 % methanol wash, and applied to a 4 cm × 0.4 cm column of the lipophilic anion exchanger Lipidex-DEAP in the acetate form (Packard instruments Co, Downers Grove, IL USA). The effluent and a wash with 3 mL of 70 % methanol constituted the neutral steroid fraction. The column was further washed with 2 mL of 0.25 M formic acid in 70 % methanol, and steroid sulphates were then eluted in 4 mL of 0.3 M ammonium acetate buffer, pH 6.5, in 70 % methanol (Meng and Sjövall 1997).

The neutral steroid fraction was reacted with 100 mg of hydroxyammonium chloride at 70 °C for 3 h (paper V). The reaction solution was evaporated to almost dryness under a stream of nitrogen and redissolved in 2 mL of 20% methanol. The resulting solution was applied to a 30 mg bed of Bondesil C18. After a wash with 2 mL of 20% methanol, the Bondesil C18 bed was superficially dried by a stream of nitrogen. Steroid oximes were then eluted with 1 mL of methanol. This eluate was applied to an 8 cm × 0.4 cm column of SP-LH-20 in the H<sup>+</sup>-form (paper II). Following a wash with 5 mL of methanol to remove unretarded compounds, for example neutral non-oxosteroids, steroid oximes were eluted with 4 mL 0.3 M ammonium hydroxide in 70 % methanol. This solution was evaporated to dryness and reconstituted in 100 µL of 20% methanol, ready for injection into the CLC-ES mass spectrometer system.

The steroid sulphate fraction was evaporated to almost dryness under a stream of nitrogen, dissolved in 20% methanol and applied to a bed of Bondesil C18, 10 mg, packed

in a Pasteur pipette. Following a wash with 1 mL of water, steroid sulphates were eluted with 100  $\mu$ L of methanol. This solution was evaporated to dryness under a nitrogen stream and dissolved in 100  $\mu$ L of 10 % methanol, prior to injection into the CLC-ES mass spectrometry system.

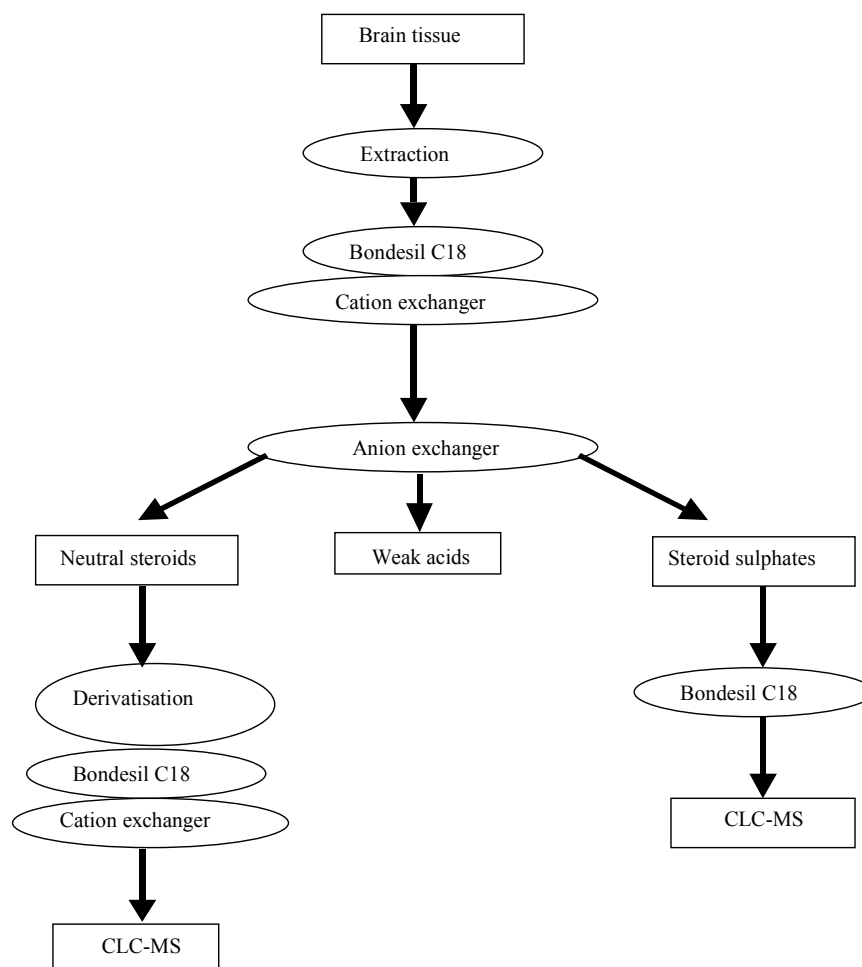


Fig. 2. Scheme of extraction and isolation procedure.

### *Nano-electrospray mass spectrometry and tandem mass spectrometry*

ES mass and tandem mass (MS/MS) spectra were recorded on an AutoSpec-OATOFFPD hybrid double focusing magnetic sector orthogonal acceleration time-of-flight instrument (Micromass, Manchester, England), a Quattro Ultima triple quadrupole instrument (Micromass), and a Quattro Micro triple quadrupole instrument (Micromass).

In nano-ES mass spectrometry experiments (papers I and II), gold-coated capillaries (Protana AS, Odense, Denmark) were used as electrospray emitters. The sample (2-5  $\mu$ L, 1 ng–1 pg steroid/ $\mu$ L) in methanol was loaded into a gold-coated capillary whose tip was cracked against a metal stopper on the stage of a light microscope to give a

spraying orifice of about 5  $\mu\text{m}$ . The capillary was then installed in the nano-ES interface. Using the AutoSpec instrument in the negative-ion mode, the voltages on the capillary and cone were approximately -5.3 kV and -4.3 kV, respectively, and in the positive-ion mode, approximately 7.0 and 4.3 kV, respectively. The accelerating potential was either -4 kV or +4 kV. The resolution was set at about 3000 (10 % valley definition). Mass spectra were recorded at a scan rate of 10 s/decade. CID spectra were recorded with the OATOF mass analyser. The monoisotopic  $[\text{M}-\text{H}]^-$  ions of the steroid sulphates or the monoisotopic  $[\text{M}+\text{H}]^+$  ions of the steroid oximes were selected by the double focusing sectors of the instrument, decelerated to 400 eV and focused into the 4<sup>th</sup> field-free region collision cell. Xenon or methane was used as collision gas in CID experiments of steroid sulphates and steroid oximes (papers I, II and III). The pressure of the collision gas was sufficient to give about 75% attenuation of the selected ion beam. The resulting fragment ions and undissociated precursor ions were  $m/z$  analysed by the TOF analyser.

Accurate mass measurements were performed on the AutoSpec instrument. The resolution was tuned to 8000 (10% valley definition). A voltage scan over a range of 80 Thomson (Th,  $m/z$ ) was used to record the spectra of the samples. Mass calibration was with internal standards.

For the triple quadrupole instruments (papers III, IV and V), typical capillary and cone voltages were about -1.2 kV and -90 V, respectively, in the negative-ion mode, and 1.1 kV and 40 V, respectively, in the positive-ion mode. A cone gas flow of 30 L/h was used. In CID experiments the collision energy was varied between 20 and 40 eV and argon was used as the collision gas at a pressure reading of  $3 \times 10^{-3}$  mbar on the gas cell gauge. Multiple reaction monitoring (MRM) experiments were carried out with a dwell time of 0.5 s and an interscan delay of 0.05 s.

In the CLC-ES mass spectrometry experiments (papers III IV and V), gold-coated fused silica capillaries (PicoTip, 15  $\mu\text{m}$ , New Objective Inc., Cambridge, MA, USA) were used as electrospray emitters. On the AutoSpec instrument, the typical voltage on the capillary was -5.5 kV in the negative-ion mode. On the Quattro Ultima triple quadrupole instrument the typical voltage on the capillary was -2.0 kV in the negative-ion mode and 1.8 kV in the positive-ion mode.

#### *Capillary column liquid chromatograph-electrospray mass spectrometry*

Capillary columns were packed using a packing procedure similar to that described by Alborn and Stenhagen (1985). A small amount of coarse packing material (Bondesil C18) was transferred to a fused silica capillary tubing (100  $\mu\text{m}$  i.d., 375  $\mu\text{m}$  o.d.) the end of

which had been shrunk to 10-20  $\mu\text{m}$  i.d. using a torch. This packing formed a 3-5 mm support on which the column was packed using a slurry (10 mg/mL) of Genesis C18, particle size 3  $\mu\text{m}$ , in chloroform/methanol, 80:20 (v/v). Methanol was used as the pumping medium and the pressure was increased to 400 bar in one minute with a pneumatic pump (Maximator, Schmidt, Krantz & Co, Zorge, Germany). Upon completion of the packing, the methanol was replaced by water, which was pumped through the column overnight to compress the packing. The column was finally inspected under a microscope to check the homogeneity of the packing.

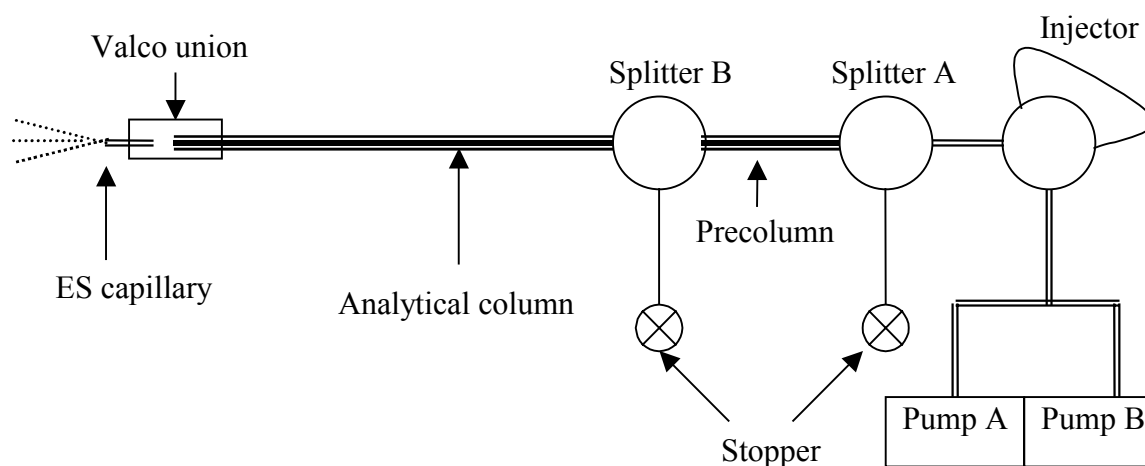


Fig. 3. Schematic drawing of the capillary liquid chromatography-micro-electrospray system. (Reproduced with permission from Analytical Chemistry, 2003. Copyright (2003) American Chemical Society).

Fig. 3 shows a schematic drawing of the chromatography system. It consists of two syringe pumps (ISCO Model 100 DM, ISCO, Inc. Lincoln, NE), a Valco C6 injector with an external 20  $\mu\text{L}$  loop (Valco, Houston, TX, USA), a Valco T (ZT1C, Splitter A), a pre-column, a second Valco T (ZT1C, Splitter B), the analytical column mounted in the ES probe of an AutoSpec mass spectrometer, and a Valco zero dead volume union (ZU1XC) coupling the analytical column to the ES emitter. The column, injector and splitters were mounted on an adjustable table, which could be readily and precisely positioned for insertion of the ES emitter into the ES interface on the AutoSpec instrument. When the Quattra Ultima (or Quattro Micro) instrument was used, a transfer capillary (30 cm, 25  $\mu\text{m}$  i.d.) was used to connect the column end to the ES emitter.

Splitter A had a 5 m fused silica capillary (50  $\mu\text{m}$  i.d., 375  $\mu\text{m}$  o.d.) connected to its third outlet to provide an approximately 1:100 split against the pre- and analytical columns. This capillary ended with a Valco ZU1XC union that could be stoppered with a steel plug.

Splitter A was stoppered during sample injection and was open during sample elution. Splitter B also had a 5 m fused silica capillary (50  $\mu\text{m}$  i.d., 375  $\mu\text{m}$  o.d.) connected to its third outlet to provide an approximately 1:110 split against the analytical column. It was opened during sample injection and stoppered during sample elution. As an alternative to the unions and stoppers, the two fused silica capillaries were connected to a 6-port valve (Valco) in such a way that one capillary was closed when the other was opened and *vice versa*. Flow rates were measured at the exit of the column and the splitter capillaries using an empty 10  $\mu\text{L}$  Hamilton syringe (Hamilton Co, Reno, NV, USA).

The pumps were operated as follows. Before sample injection, pump A was run in the constant pressure mode to deliver solvent A through the entire column system for at least half an hour with both splitters stoppered. This resulted in a flow rate of about 0.2  $\mu\text{L}/\text{min}$  through the pre- and analytical columns. Then splitter B was opened giving a flow through the pre-column of about 2  $\mu\text{L}/\text{min}$ , and 20  $\mu\text{L}$  (or less) of sample was injected. Pump A continued to pump solvent A through the pre-column for 20 min to allow transfer, sorption and desalting of the sample. At the end of this time splitter B was stoppered and splitter A opened. Then pump A was stopped and elution was initiated by starting either pump B alone or a gradient program. In the latter case the ISCO pumps were run at a total flow rate of 20-30  $\mu\text{L}/\text{min}$ , the flow rate through the columns being 0.2-0.3  $\mu\text{L}/\text{min}$ .

Mixtures of reference compounds were used to establish the optimal conditions for separation, and to determine the retention times of different steroid sulphates and steroid oximes.

To test recoveries, 100  $\mu\text{L}$  of [ $^3\text{H}_4$ ]-DHEA sulphate (550 pg, about 100000 cpm) was added to 100  $\mu\text{L}$  of the test mixture above. Twenty  $\mu\text{L}$  of the resultant solution was injected, and the effluents from the columns and the splitters were collected. The radioactivity was determined and the recovery calculated.

#### *HPLC isolation of a lipophilic modulatory factor from mouse brain*

Mouse brain tissue was incubated with cell culture medium overnight. The resultant conditioned medium was extracted with hexane in the presence of 0.1 M HCl. The hexane extract was taken to dryness and the residue was redissolved in 200  $\mu\text{L}$  hexane. After centrifugation an aliquot of 150  $\mu\text{L}$  was injected onto a normal-phase HPLC column. Elution was performed by a linear gradient from hexane to hexane/ dichloromethane/isopropanol (85:10:5, by volume), both containing 1% acetic acid, in 30 min at a flow rate of 0.5 ml/min. Fractions of 0.25 mL were collected and aliquots were taken for activity assay. The active

fractions were pooled and taken to dryness. The residue was redissolved in 50  $\mu$ l of 80% methanol and 30  $\mu$ l were injected onto a reversed phase HPLC column. The separation was made by isocratic elution with methanol/isopropanol/water (80:10:10, by volume) containing 1% acetic acid at a flow rate of 0.3 ml/min. Fractions were collected and aliquots were taken for activity assay. The active fractions and preceding and following fractions were analysed by ES mass spectrometry.



## RESULTS AND DISCUSSION

### *Analysis of steroid sulphates by nano-electrospray mass spectrometry*

In paper I we have evaluated the potential of nano-ES tandem mass spectrometry for structural analysis and detection of steroid sulphates, with particular focus on compounds potentially present in brain. Twenty-four steroid sulphates with different structures were studied. The intensity of the  $[M-H]^-$  ion signal was approximately linearly proportional to analyte concentration over the range of 1 ng/ $\mu$ L to 1 pg/ $\mu$ L. The limit of detection (signal/noise 3:1) was 1 pg/ $\mu$ L. From CID spectra, structural information could be obtained. Typical charge-remote fragment (CRF) ions (Tomer and Gross 1988) were observed in the CID spectra as well as  $[M-H-SO_3]^-$ ,  $[SO_3]^-$ ,  $[SO_4]^-$  and  $[HSO_4]^-$  ions. The peaks corresponding to  $SO_3^-$  and  $HSO_4^-$  ions were about 10 times more intense than other ion peaks and could be very useful in a precursor ion scan. Substituents on the steroid skeleton changed the  $m/z$  of the CRF fragmentation ions making it possible to determine the location of substituents. Detailed structural information about the steroid skeleton could be obtained from 1 ng (3 pmol) of steroid sulphate, while fragment ions characteristic of the sulphate ester group could be obtained from only 3 pg (10 fmol) of sample.

### *Analysis of oxosteroids as their oximes by nano-electrospray mass spectrometry*

A method for the analysis of neutral oxosteroids by nano-ES mass spectrometry is described in paper II. Conversion of the oxosteroids into their oximes was chosen as a method to increase the proton affinity of the steroids. In addition, oximes can be isolated from nonaqueous biological extracts by sorption on a lipophilic cation exchanger, thus permitting selective isolation from a biological matrix (Axelson and Sjövall, 1979). A previous method (Thenot and Horning, 1972) for the preparation of methyloximes for GC-MS analysis was modified to suit our procedure for the isolation of neurosteroids from brain, which utilized aqueous ethanol/methanol as the solvent. Thus, the oxosteroids were converted into their oximes by treatment with hydroxylamine hydrochloride in aqueous methanol. Most of the known neutral oxosteroids can be quantitatively converted into oximes using this method. Oxo groups not reacting under these conditions include hindered sites i.e. at C-11, and at C-20 in steroids substituted both at C-17 and C-21.

Derivatisation of oxo groups into oximes improves the sensitivity of analysis of oxosteroids by ES mass spectrometry in the positive ion mode. Unlike underivatized steroids, which are detected as protonated and sodiated molecules (Ma and Kim, 1997), steroid oximes are predominantly found as protonated molecules in nano-ES mass

spectra. In mass scans over the range of 200-1000  $m/z$ , the detection limits for the oximes of progesterone, pregnenolone and DHEA were 2.5, 5, and 25  $\text{pg}/\mu\text{L}$ , respectively, approximately 20 times lower than for the underivatized steroids.  $[\text{M}+\text{H}]^+$  ion intensities were found to be proportional to the concentration of steroids in the range of 500 to 2.5  $\text{pg}/\mu\text{L}$ . The detection limits, 2.5-25  $\text{pg}/\mu\text{L}$ , should be sufficient for the analysis of oxosteroids in 100 mg of brain at levels of 0.25-2.5  $\text{ng}/\text{g}$  provided that the oximes can be isolated in a sufficiently pure form and be concentrated into a small volume (10  $\mu\text{L}$ ).

Fragmentation by CID of  $[\text{M}+\text{H}]^+$  ions at 400 eV was studied using oximes of 28 model steroids. Fragment ions were observed which yielded useful structural information. Upon CID, protonated oximes of 3-oxo- $\Delta^4$ -steroids produced abundant ions by cleavage through the B-ring ( $m/z$  112, 124, and 138) and by loss of the side chain. For  $[\text{M}+\text{H}]^+$  ions of oximes of 20-oxosteroids, fragmentation through the D-ring ( $m/z$  86) was predominant. Protonated oximes of steroids containing only a 17-oxo group gave ions representing the ABC and ABCD rings after loss of the 3-hydroxyl group and the oxime group ( $m/z$  213 and  $m/z$  253, respectively). The intensities of these two ions were similar to the intensities of many other ions which appeared as clusters. The protonated molecule, the fragment ions at  $m/z$  112 and 124 formed from oximes of 3-oxo- $\Delta^4$ -steroids and at  $m/z$  86 formed from oximes of 20-oxosteroids were used for detection of oxosteroids in brain using MRM.

#### *Capillary column liquid chromatography-electrospray mass spectrometry*

Paper III describes a new procedure for CLC-ES mass spectrometry. As discussed in the introduction, a combination of low flow rate CLC and ES mass spectrometry is needed for the analysis of neurosteroids in brain, because of the low levels of these steroids and the probable existence of isomers. For our purpose, we needed a simple and versatile CLC-ES system that could be used for the injection of 10-20  $\mu\text{L}$  of sample, could be operated with gradient elution, and could be run at a flow rate of  $\sim 0.2$   $\mu\text{L}/\text{min}$ .

In the system we developed, two splitters are used (Fig. 3). Splitter A is placed between the injector and the pre-column and is closed during sample injection, while splitter B is positioned between the pre-column and the analytical column and is opened during sample injection. During this operation pump A is run under pressure control giving a flow rate of 2-4  $\mu\text{L}/\text{min}$  through the pre-column. After analytes have been sorbed onto the pre-column, but before starting the gradient program, splitter A is opened and splitter B closed. Then gradient elution is carried out at typical total flow rates of 20-30  $\mu\text{L}/\text{min}$  from the pumps, while the flow rates through the columns are about 0.2-0.3  $\mu\text{L}/\text{min}$ . In this way

the reproducibility of the gradient elution is improved when the total flow rate is above 20  $\mu\text{L}/\text{min}$ . The variation in retention time of reference steroids was less than 3.5 % (RSD,  $n=5$ ).

Recovery experiments showed that 85 % of injected  $[7\text{-}^3\text{H}]\text{-DHEA}$  sulphate reached the end of the analytical column. The loss of sample through splitters A and B during the sample injection was less than 5%. It is probable that the other losses occurred in the injector and connectors.

The chromatographic efficiency is somewhat reduced by the introduction of the two splitters and the pre-column, but this is acceptable in view of the gains in time and practicability in analysis of biological samples.

Washing the precolumn and the analytical column with a strong solvent after each injection of biological sample is important to maximize the lifetime of the column. It also helps to reduce the variation of retention times between different injections by removing phospholipids and other nonpolar compounds. This wash step was carried out easily by an injection of 20  $\mu\text{L}$  (9 times the column volume) of a mixture of methanol and isopropanol (1:1) with the two splitters closed. In this way the need for a change of solvent in the syringe pumps was avoided.

ES performance was tested with different kinds of ES emitters. Among the tested ES emitters of different design, metal coated tapered fused silica capillaries (New Objective PicoTip, 8  $\mu\text{m}$  and 15  $\mu\text{m}$  orifice) were found to produce a stable spray over a wide range of solvent composition, i.e. above 20% methanol. No sheath gas was required. When the orifice of the PicoTips was 8  $\mu\text{m}$  a more intense signal was generated than with the 15  $\mu\text{m}$  emitters. However, the 8  $\mu\text{m}$  tips were more prone to clogging. The 15  $\mu\text{m}$  tips were thus chosen for the analysis.

Using the AutoSpec instrument a detection limit (signal/noise ratio 10) of 3 pg (7.5 fmol) injected on column was achieved for pregnanolone sulphate isomers when scanning the mass range of 416-360 Th. The peak area response was linear from 2 pg to 1 ng injected on the column.

Using the Quattro Ultima instrument, a detection limit of 0.2 pg (500 amol) of  $[^2\text{H}_3]\text{-allopregnanolone}$  sulphate injected on the column was obtained using single ion monitoring. The detection limit was 0.1 pg (250 amol) when single reaction monitoring (monitored transition  $m/z$  400 $\rightarrow$ 97) was used.

The potential of the CLC-ES system in metabolome analysis, where numerous isomeric compounds will require identification, is illustrated by the application of the system to the analysis of steroid sulphates in plasma as shown in Fig. 4.

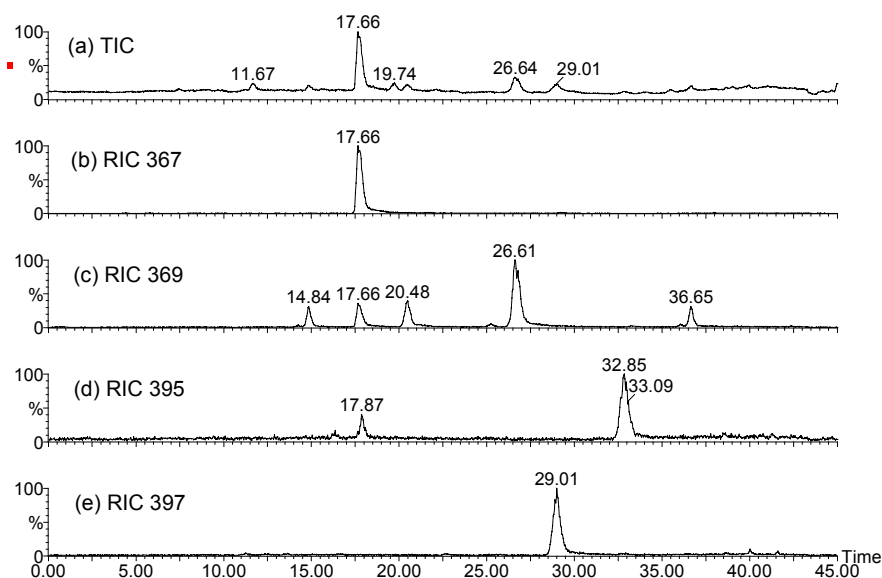


Fig. 4. TIC and RICs obtained from an analysis of a plasma sample. (a) TIC, (b) RIC of  $m/z$  367, (c) RIC of  $m/z$  369, (d) RIC of  $m/z$  395, (e) RIC of  $m/z$  397. Twenty  $\mu\text{L}$  of sample solution corresponding to 1  $\mu\text{L}$  of plasma was injected onto the pre-column. (Reproduced with permission from Analytical Chemistry, 2003. Copyright (2003) American Chemical Society).

The CLC-ES system was also applied to the analysis of steroid oximes (paper V). Separation of the relevant steroid oximes was achieved except between pregnenolone oxime and progesterone bisoxime. Using mass scans over the range of 280-380 Th on the triple quadrupole instruments, a detection limit of 1 pg injected was obtained for  $[\text{M}+\text{H}]^+$  ions of  $[\text{}^2\text{H}_3]$ -testosterone oxime ( $\text{S}/\text{N} = 10$ ), 1.5 pg for DHEA oxime, pregnenolone oxime and allopregnanolone oxime, and 3 pg for progesterone bisoxime.

Sensitivity was improved when using multiple reaction monitoring on the triple quadrupole instruments. A detection limit of 0.1 pg injected was obtained for  $[\text{}^{19,19,19}\text{-}^2\text{H}_3]$ -testosterone oxime when using the transition  $m/z$  307  $\rightarrow$  115; 0.3 pg for pregnenolone oxime using  $m/z$  332  $\rightarrow$  86; 0.5 pg for DHEA oxime using  $m/z$  304  $\rightarrow$  213, pregnanolone oxime using  $m/z$  334  $\rightarrow$  86 and progesterone using  $m/z$  345  $\rightarrow$  124.

#### *On-column electrochemical reactions in capillary column liquid chromatography-electrospray mass spectrometry*

In paper IV, we show that electrochemical processes can occur on a CLC column coupled to an ES mass spectrometer. This is important because it constitutes a potential source of error in analysis performed by CLC-ES mass spectrometry.

The CLC-ES system described in paper III had been used in the analysis of steroid sulphate standards for a period of one year. Then, after it had been used in a study of plasma steroids, a chemical conversion of some injected steroid sulphate standards was observed. It seemed likely that some material in the injected plasma extracts had activated the pre-column. The mass spectra of the reaction products revealed a series of oxidised compounds. On-column oxidation of steroid sulphates was found to require a reactive double bond in the steroid structure. On-column oxidation reactions of peptides possessing a site of unsaturation were also shown. Experimental results suggested that the site of oxidation of the steroid sulphates injected was the precolumn. The potential difference and the current across the precolumn apparently resulted in electrolysis of the solvent to generate free radicals, which subsequently initiated analyte oxidation. These reactions could be prevented by grounding the precolumn.

Structures of the oxidation products were determined by means of accurate mass measurement and CID. B-ring oxygenated and methoxylated steroid sulphates were found to be the major products.

#### *Analysis of steroid sulphates and oxosteroids in brain tissue*

Paper V describes studies of a sample preparation method for the analysis of steroids in brain by the CLC-ES mass spectrometry. The main feature of the method is to separate the steroids into two groups, steroid sulphates and neutral steroids, and then analyse the groups separately by CLC-ES mass spectrometry. The reason for doing so is that the neutral unconjugated steroids and the steroid sulphates have very different physical and chemical properties, and they cannot be analysed with sufficient sensitivity in a single mass spectrometric method. In this study, the lipophilic anion exchanger Lipidex-DEAP was used to separate the neutral unconjugated steroids and steroid sulphates.

For the sulphate fraction, after a micro SPE step to remove the salts and to concentrate the sample to a small volume, the steroid sulphates were analysed by CLC-ES mass spectrometry. Recovery of steroid sulphates through the whole procedure was studied with  $^3\text{H}$ -labelled DHEA sulphate added to tissue extracts and was found to be 80%.

The neutral steroid fraction was derivatised with hydroxylamine hydrochloride and the resulting oximes were isolated by cation exchange chromatography. Recoveries of  $^3\text{H}$ -labelled progesterone, DHEA and pregnenolone added to the neutral steroid fraction were above 90 % through the derivatisation and isolation procedure. The recovery of  $^3\text{H}$ -labelled DHEA was 73% (n=4) through the whole procedure, starting with the extraction.

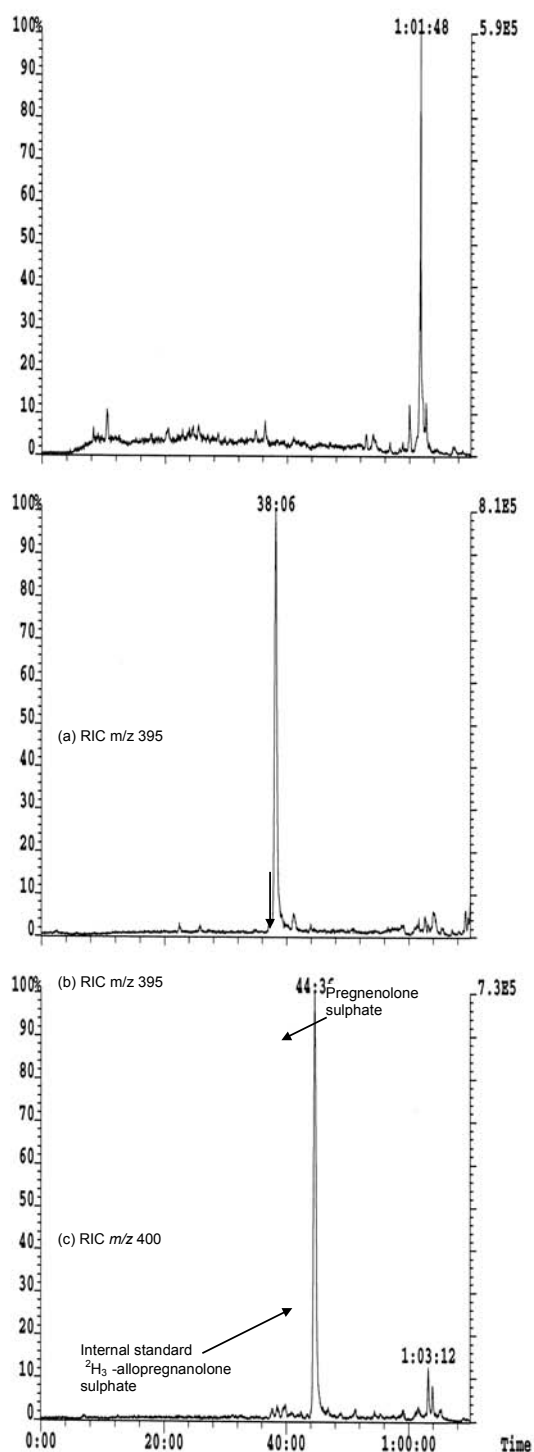


Fig. 5. (a) RIC for pregnenolone sulphate ( $m/z$  395) from a brain sample. The arrow indicates where pregnenolone sulphate is expected to elute. (b) RIC for pregnenolone sulphate ( $m/z$  395) from a spiked brain sample to which 2 ng of the steroid had been added to 300 mg brain. (c) RIC for [ $^2\text{H}_3$ ]-allopregnanolone sulphate ( $m/z$  400) added as internal standard to a brain sample (1.74 ng to 300 mg brain). Spectra were recorded on an AutoSpec instrument. Steroid sulphates were extracted and purified from 300 mg of brain. Twenty  $\mu\text{L}$  of sample solution (corresponding to 60 mg brain) were injected onto the capillary column.

As pregnenolone sulphate and DHEA sulphate have previously been analysed by indirect methods (GC-MS or RIA) and apparently been found to be present in rat brain (Corpechot et al. 1981, 1983, Baulieu 1997), their detection in the intact form was our initial aim. Given the detection limit of our method (~ 0.3 ng/g wet brain), both pregnenolone sulphate and DHEA sulphate should be detected. Surprisingly, neither of them was detected, either in whole brain or in isolated areas of brain (amygdala or hippocampus), when brain samples from 50-300 mg were extracted. Internal standards, including pregnenolone sulphate, added to the sample were recovered, whereas endogenous pregnenolone sulphate and DHEA sulphate were not detected. Fig. 5 shows the results from analyses of a brain sample and of a spiked brain sample. Cholesterol sulphate was detected at a level of 1.2 µg/g. When human plasma samples were analysed using the same sample preparation method two major steroid sulphates, pregnenolone sulphate and DHEA sulphate, as well as other sulphated steroids such as sulphated androstanolones, androstenediols and pregnenediol were also detected. Our result about the levels of pregnenolone sulphate and DHEA sulphate in brain (< 0.3 ng/g wet brain) is consistent with the result recently published by Shimada et al. (Higashi et al. 2001, 2003) in which they showed the levels of pregnenolone sulphate and DHEA sulphate to be below 0.4 ng/g. The reasons for the discrepancy between the results obtained with our direct method and indirect methods are probably methodological.

The neutral unconjugated steroid fraction was derivatised with hydroxylamine hydrochloride to convert oxosteroids into their oximes. The oximes were isolated using cation exchange chromatography and were analysed by CLC-ES tandem mass spectrometry. These analyses confirmed the presence in rat brain of pregnenolone, pregnanolone isomers, progesterone, testosterone and DHEA, which were characterised by their retention times, masses of the protonated molecules, and characteristic fragment ions in MS/MS spectra/chromatograms. The approximate levels of the steroid oximes from rat brain samples were estimated using [<sup>13</sup>C<sub>2</sub>]-progesterone as an internal standard. Table 1 summarises the results of these quantitations. Progesterone levels were lower in the samples from male rats than in the samples from female rats, while testosterone was found at a higher level in male rat samples than in female rat samples. Since these are sex hormones, this difference is not surprising. However, there was also a difference in the presence of pregnanolone isomers between the male and the female brain samples. In the male rat brain samples, three pregnanolone isomers were observed, and they were assigned as epiallopregnanolone/epipregnanolone, pregnanolone, and allopregnanolone. No peak corresponding to pregnanolone was seen in any of the female rat brain samples.

Confirmation of these differences between male and female rat brain samples will require analysis of a larger number of rat brains.

Table 1 Approximate levels of some oxosteroids in whole rat brain (ng/g wet brain).

		progesterone	pregnenolone	epipregnanolone or epiallopregnanolone	pregnanolone	allopregnanolone	DHEA	testosterone
Rat 1 Male	Sample 1 <sup>a</sup>	1.2	1.2	0.14	0.15	0.55	0.07	0.4
	Sample 2 <sup>a</sup>	1.9	0.63	0.11	0.14	0.51	0.07	0.5
Rat 2 Male	Sample 1 <sup>a</sup>	1.0	0.60	0.06	0.12	0.42	0.05	0.48
	Sample 2 <sup>a</sup>	3.4	0.73	0.05	0.16	0.57	0.11	0.37
Rat 3 female	Sample 1 <sup>a</sup>	5.1	2.7	1.1	Not detected	2.1	0.04	0.04
	Sample 2 <sup>a</sup>	4.4	4.3	1.3	Not detected	2.3	0.07	0.06
Rat 4 female	Sample 1 <sup>a</sup>	21	3.9	2.5	Not detected	11	0.08	0.11
	Sample 2 <sup>a,b</sup>	20	12	11	Not detected	38	---	0.32

<sup>a</sup> Samples 1 and 2 were taken from the same brain homogenate.

<sup>b</sup> This sample was stored in the oxime form for 6 weeks before analysis and had precipitate.

The design of our method of sample preparation and CLC-ES mass spectrometry should be applicable for the characterisation of sulphated steroids and free oxosteroids in brain. For accurate quantitation it will be necessary to add appropriate steroids labelled with stable isotopes. The limited availability of isotope-labelled steroids might be a problem if a profile analysis is desired. To circumvent this problem in the analysis of steroid oximes, a mixture of reference steroids can be derivatized with <sup>15</sup>N-labelled hydroxylamine hydrochloride and used as internal standards. This mixture can be then added to the brain samples after the derivatisation of the neutral fraction with non-labelled hydroxylamine hydrochloride. This method will correct for the variations in response factors for different steroid oximes caused by variation of flow rate and ES performance during the CLC-ES. However, the possibility of oxime exchange reaction should be considered and studied.

#### *Characterisation of docosahexaenoic acid in mouse brain as a ligand for the retinoid X receptor*

The hexane extract of the cell culture medium (MEM) conditioned with adult mouse brain was fractionated using normal-phase HPLC and reversed-phase HPLC, and aliquots



were taken from each fraction for activity assay (Fig. 6). Negative-ion nano-ES mass spectra were recorded of the active fraction as well as pre- and post-active fractions. The

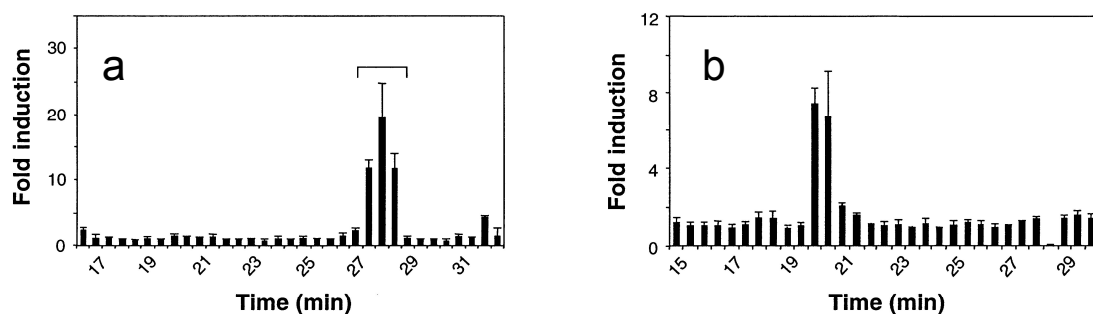


Fig. 6. Chromatograms for (a) normal-phase HPLC and (b) reversed-phase HPLC. The Y axis represents the fold induction of activity.

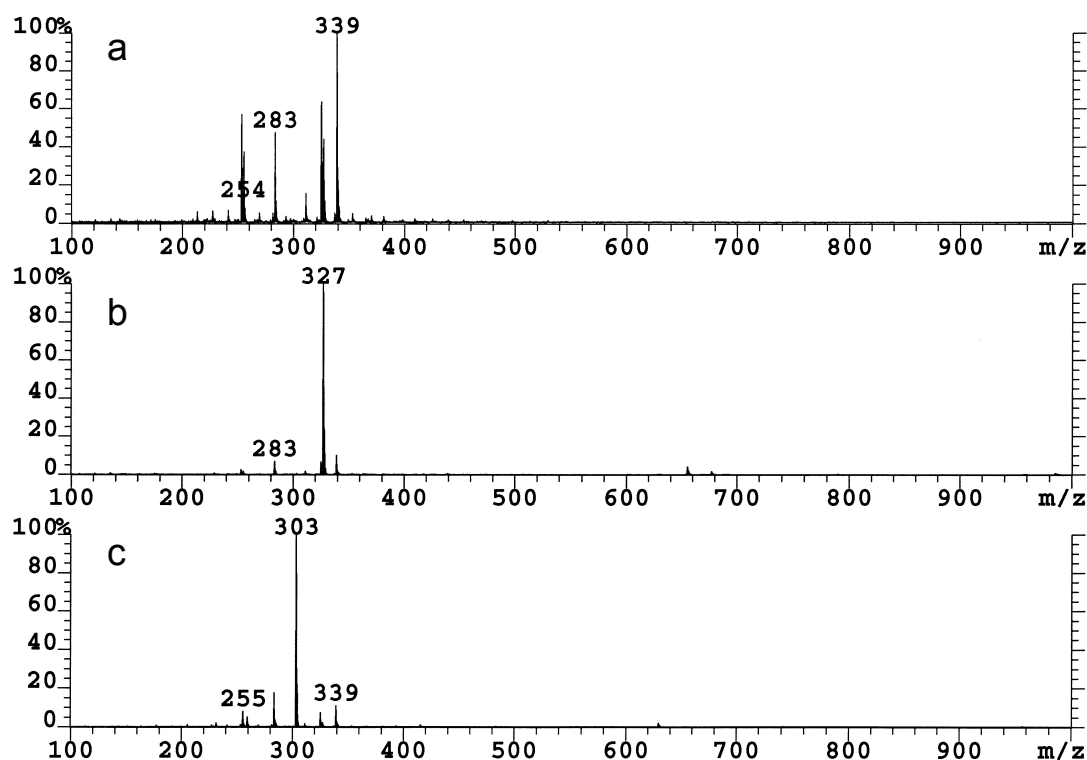


Fig. 7. Negative-ion ES mass spectra of (a) pre-active, (b) active and (c) post-active fraction.

ES mass spectrum of the active fraction was dominated by a very intense ion at  $m/z$  327.2, with minor peaks being also observed at  $m/z$  283.2, 339.2 and 655.5 (Fig. 7). The pre-active fraction gave abundant ions at  $m/z$  339.2, and the post-active fraction abundant ions at  $m/z$  303.2. As expected low amounts of the ion at  $m/z$  327.2 were also observed in the pre- and post- fractions closest to the active fraction. Thus the compound giving ions at  $m/z$  327.2 was thought to be responsible for the activity. The accurate mass was

determined to be 327.2316 Th corresponding to a molecular formula of  $C_{22}H_{31}O_2$ , the anion of docosahexaenoic acid. The CID spectra of the ion at  $m/z$  of 327.2 from the active fraction and the  $[M-H]^-$  ion of cis-4,7,10,13,16,19-docosahexaenoic acid were very similar as shown in Fig.8. It was concluded that the active compound is cis-4,7,10,13,16,19-docosahexaenoic acid.

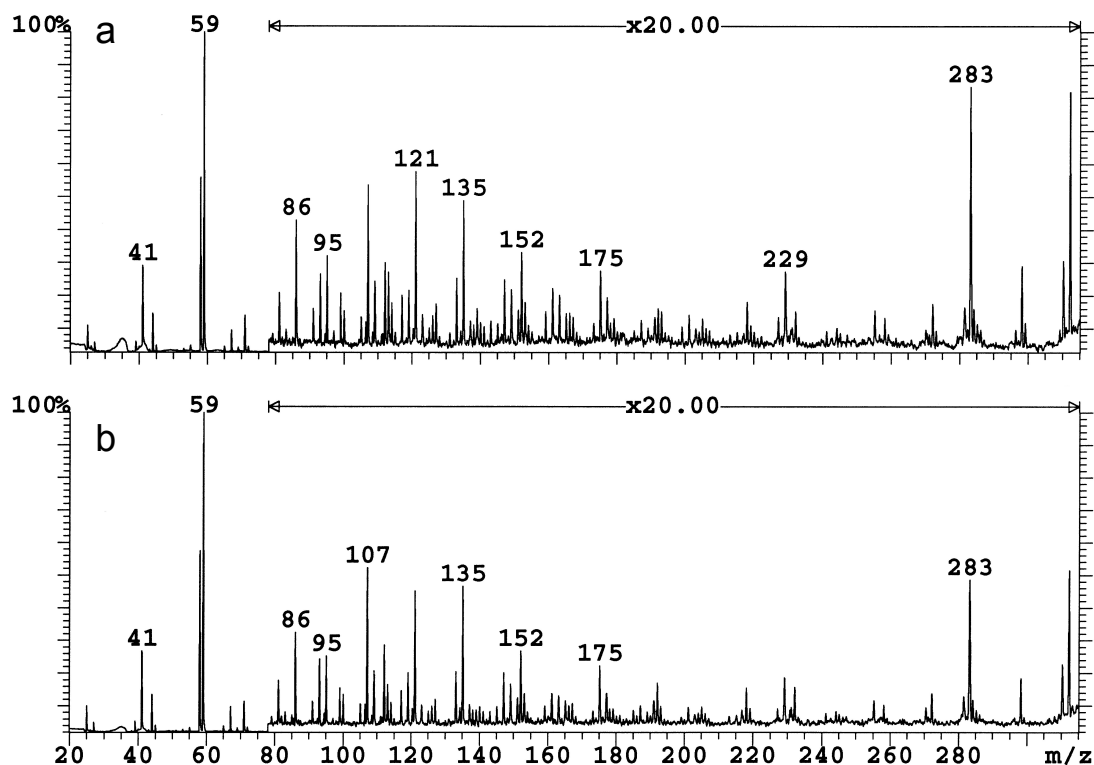


Fig. 8. CID spectra of  $[M-H]^-$  ion at  $m/z$  327.2 from (a) active fraction and (b) from the reference fatty acid cis-4,7,10,13,16,19-docosahexaenoic acid.

Cis-4,7,10,13,16,19-docosahexaenoic acid and other polyunsaturated fatty acids were tested for their activities on RXR using a cell-based assay. Cis-4,7,10,13,16,19-docosahexaenoic acid was found to bind and activate the RXR. Thus cis-4,7,10,13,16,19-docosahexaenoic acid is an endogenous ligand.

## CONCLUDING REMARKS

The aim of this study was to develop a method for the analysis of neurosteroid profiles in rat brain. The goal was achieved by combining a CLC-ES mass spectrometric method with a selective sample preparation method.

The main reasons for choosing CLC-ES mass spectrometry were: 1) it permits a direct analysis of steroid sulphates; 2) ES ionisation gives a high yield of protonated or deprotonated molecules which can be fragmented by CID to give structural information from the MS/MS spectra. The fragments can also be used in precursor or product ion scanning procedures that increase the specificity and sensitivity of the analyses; 3) the sample capacity of a capillary LC column system is higher than that of a capillary GC column so that the sample preparation procedure can be simplified and partly carried out on-line with a precolumn; 4) the choice of a CLC-ES mass spectrometric method is in line with the increasing availability in many laboratories of LC-ES instruments for quantitative and qualitative analysis of non-volatile biomolecules. It is convenient to cover several analytical needs using the same instrument. Our system for CLC-ES mass spectrometry is simple and efficient. The sensitivity in analyses of steroid sulphates and oximes of oxosteroids is sufficiently high and is comparable to that obtained by GC-MS, although the separation efficiency in CLC is less than that in capillary GC. Our method should increase the possibilities to detect and identify known and unknown neurosteroids in complex biological samples and should also be applicable to the analysis of related metabolites.

The sample preparation method was aimed at the isolation of neurosteroids according to polarity, acidity and the nature of functional groups. The first step after extraction is the removal of nonpolar lipids in a reversed-phase SPE. The choice of solvent in this step determines the least polar steroid included in the analysis. Steroid sulphates are separated from less acidic compounds and neutral compounds on an anion exchanger and are then directly analysed by CLC-ES mass spectrometry. Compared to the analysis of steroid sulphates by GC-MS after solvolysis and derivatisation, this method avoids the possible problems inherent from the solvolysis method, e.g. release of steroid from other conjugated forms or chemical transformations of the steroid nucleus. A discrepancy was observed between the levels of steroid sulphates determined by our method and the indirect GC-MS method. This discrepancy shows the importance of analysing the neurosteroids in brain in their intact form. Oxosteroids in the neutral fraction were analysed by CLC-ES mass spectrometry after their derivatisation into oximes. The results confirmed the presence of neutral C<sub>21</sub> and C<sub>19</sub> steroids in rat brain at levels comparable to those

previously reported using GC-MS methods. Differences in the steroid profile pattern between individual rats were observed, demonstrating the capability of the method to distinguish the steroid profile patterns of the rats under different physiological conditions. Application of the method may lead to an increased understanding of the functions of neurosteroids.

With some modifications the method will also permit analysis of non-oxosteroids in the neutral fraction. For example, conversion of  $3\beta$ -hydroxysteroids into 3-oxo- $\Delta^4$  steroids catalysed by cholesterol oxidase will permit these compounds to be analysed by ES mass spectrometry using the above derivatisation into oximes or Girard hydrazones.

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