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Nanoelectrospray Mass Spectrometry and Precursor Ion Monitoring for Quantitative Steroid Analysis and Attomole Sensitivity

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Nanoelectrospray ionization (nanoESI) mass spectrometry was performed on naturally occurring steriod sulfates and unconjugated steroids derivatized to their sulfate esters using precursor ion monitoring. Initially, an extraction method was developed based on a combinatorial approach employed to obtain the most efficient liquid/liquid extraction protocol. The new method allowed unconjugated steroids and their sulfated analogues to be isolated separately in a two-step procedure using diethyl ether/hexane (90:10, v/v) in the first step to extract the unconjugated steroids and chloroform/2-butanol (50:50, v/v) in the second step to extract steroid sulfates. Precursor ion scanning performed with a triple-quadrupole mass spectrometer was used to examine quantitatively the extracted unconjugated and sulfated steroids, where the recovery efficiency averaged 70 and 87%, respectively. In addition, some steroids could be structurally elucidated by employing tandem mass spectrometry. The limit of detection for steroid sulfates from the biological matrix was 200 amol/μL (~80 fg/μL) with only 1 μL of sample being injected. Endogenous levels of the unconjugated and sulfated steroids were detected and quantified from physiological samples including urine and blood. Internal standards, pregnenolone-d_4 sulfate and dehydroepiandrosterone-d_2 (DHEA), were used for quantitation. Extraction and nanoESI analyses were also performed on cerebrospinal fluid where the neurosteroid DHEA sulfate was detected. The small amount of material consumed (typically less than 20% of the injection volume) suggests that nanoESI has even greater potential for high sensitivity when combined with nanoLC approaches, especially for monitoring reproductive and adrenal steroids, as well as for the analysis of the less abundant neurosteroids.

The analysis of steroids, especially those synthesized in the brain[1] which modulate neurotransmitter action[2], has provided new insights into animal behavior[3,4] and brain metabolism.[5] The detection and analysis of this class of molecules in tissues and biofluids such as cerebrospinal fluid, urine, blood, and feces have long posed a significant technical challenge due to their low concentrations, varied polarity, and presence in complex mixtures.[7] Normally, many biological samples must be pooled together to acquire enough material for the detection and study of steroids and their metabolites.[7] The analysis of steroids and their metabolites in biological fluids has traditionally been carried out by gas chromatography (GC), high-performance liquid chromatography (HPLC), gas chromatography/mass spectrometry (GC/MS), electrospray ionization (ESI) liquid chromatography/mass spectrometry (LC/MS), atmospheric pressure chemical ionization mass spectrometry (APCI-MS), and immunoassay.[8] While each of these techniques has its advantages, they all have their respective disadvantages. Steroid analysis by mass spectrometry has been complicated by the limited capability of mass spectrometers to ionize these compounds efficiently either by traditional electron ionization methods or with the newer approaches such as ESI or APCI. A standard mass spectrometric approach to analyzing steroids has been by negative ionization chemical ionization (NICI) GC/MS. NICI is performed in combination with derivatization of the molecule to enhance its ionization properties.[9] Although it is a very useful tool, the limitations to this approach include extensive chemical preparation time and relative instability

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(1) Robel, P.; Baulieu, E. E. *Crit. Dev. Neruosci.* **1995**, *9*, 383−394.
(2) Ma, Y. C.; Kim, H. Y. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 1010−1020.
(3) Robel, P.; Young, J.; Corpechot, C.; Mayo, W.; Perche, F.; Haug, M.; Simon, H.; Baulieu, E. E. *J. Steroid Biochem. Mol. Biol.* **1995**, *53*, 355−360.
(4) Bitran, D.; Purdy, R. H.; Kellogg, C. K. *Pharmacol. Biochem. Behav.* **1993**, *45*, 423−428.
(5) Uzunov, P.; Cooper, T. B.; Costa, E.; Guidotti, A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12599−12604.
(6) Masse, R.; Wright, L. A. *Clin. Biochem.* **1996**, *29*, 321−331.
(7) Watson, D.; Taylor, G. W.; Murray, S. *Biomed. Mass Spectrom.* **1985**, *12*, 610−615.
(8) Chard, T. *An Introduction to Radioimmunoassay and Related Techniques*, 5th ed., Elsevier: New York, 1995.
(9) Cheney, D. L.; Uzunov, D.; Costa, E.; Guidotti, A. *J. Neurosci.* **1995**, *15*, 4641−4650. Fitzgerald, R. L.; Herold, D. A. *Clin. Chem.* **1996**, *42*, 749−755.

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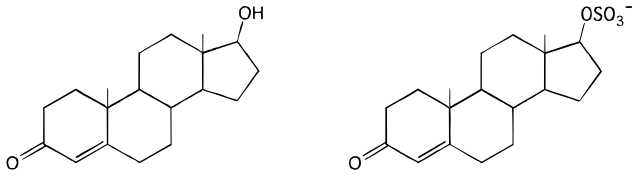
of the derivative. Immunoassays have been widely used for investigating steroids and their metabolites, and while much has been accomplished, the limitation of its sensitivity, selectivity, and accuracy are factors that bar more in-depth investigations.

Many steroids and their metabolites occur in nature in nanomolar and even picomolar concentrations.[10−11] An approach that allows for the detection and quantitation of steroids and their metabolites at biologically relevant concentrations will enhance the study of steroids and help us further understand the multitude of biological functions in which they are involved. The advent of nanoESI and its application to peptides[12] has clearly demonstrated outstanding sensitivity for charged molecules, as well as its quantitative capabilities with lipids.[13] The technique is accomplished by loading microliter volumes of sample solution into a glass capillary needle. Various types of capillaries are available, sealed or nonsealed, and in multiple sizes with different types of coatings for different types of analyses. In the instance of sealed capillaries, the needle is opened by gently touching the closed end against the orifice plate of the mass spectrometer to produce an opening with a diameter of ~10 μm. Once opened, the needle is positioned in front of the orifice at a distance of 1−2 mm. The sample is directed into the mass spectrometer by a gastight syringe that allows a static backing pressure to be applied. The resulting flow rate is approximately 20−30 nL/min,[12] allowing microliter sample volumes to be examined for extended periods of time with a minimal amount of sample consumed. One microliter of sample can last up to 30 min depending on the pressure applied to the syringe.

To adapt nanoESI for steroid analysis, we have employed precursor ion scanning[12] and sulfation[14,15] of steroids. We have also developed a novel method for extracting unconjugated steroids and steroid sulfates from biofluids based on a combinatorial approach previously developed for cyclosporin.[16] The utility of precursor ion scanning for the selective detection of small molecules is well recognized.[17] By employing precursor ion scanning, the interference from chemical noise is greatly reduced, thereby enhancing the signal-to-noise ratio. In negative nanoESI, the anionic species of steroid sulfates is observed and can be detected at concentrations lower than the protonated $[M + H]^+$ or $[M - H_2O + H]^+$ species of unconjugated steroids in positive mode. The formation of a sulfate functional group not only increases sensitivity but also makes precursor ion scanning possible because the sulfate (HSO_4^-) fragment ion is observed in collision-induced dissociation (CID) tandem mass spectrometry. With precursor ion scanning, the sulfate fragment m/z 97 is used to generate spectra of its precursors or "parent" ions. The combination of enhancements offered by precursor ion scanning, sulfation, and the novel extraction methods has allowed steroid sensitivity levels to approach those necessary for neurosteroid analysis.



Testosterone as an unconjugated steroid (left) and its sulfated conjugate (right)

RESULTS AND DISCUSSION

Extracting Steroids for NanoESI-MS. Prior to analysis, a combinatorial approach was used to establish the most efficient extraction solvent system[16] for steroids from urine, blood, and cerebrospinal fluid (CSF). Precursor ion scanning by nanoESI was then used to quantitate these steroids against an internal standard. Confirmation of the structures was accomplished by tandem mass spectrometry.

Extraction from biofluids is the first step in any naturally derived steroid analysis due to the crude biological medium and the intolerance of mass spectrometers to salts. Various extraction procedures have been developed over the years for the extraction of steroids from biological fluids, most of which are somewhat time-consuming. Steroids have commonly been removed from biofluids using a liquid/solid and/or liquid/liquid extraction[10−11,18−20] or by column extraction.[21,22] The experiments described here focused on finding a simple liquid/liquid extraction solvent system that would efficiently remove steroid sulfates from biofluids in one step. Further experiments were directed at discovering a separate solvent system that could effectively remove a high percentage of unconjugated steroids without concurrently removing the steroid sulfates present. These unconjugated steroids could then be derivatized by sulfation prior to analysis by nanoESI. The objective was to combine these two extraction procedures into an extraction technique that would increase efficiency for steroid analysis. To accomplish this, we employed a combinatorial approach to identify the most effective solvent systems using polarity as a basis for extraction. NanoESI was used to test the validity and efficiency of each solvent system.

Aliquots of each of 65 solvent systems, categorized into two groups, were added separately to measured volumes of artificial CSF with the addition of a steroid mixture consisting of the unconjugated steroids dehydroepiandrosterone-d_2 (DHEA) and pregnenolone and the sulfated conjugates of estradiol, DHEA, pregnenolone, and 3α-hydroxy-5α-pregnan-20-one (allopregnanolone). One set of solvent systems was aimed at only extracting the steroid sulfates. This set included hexane, dichloromethane, chloroform, ethyl acetate, and diethyl ether. These solvents were used singly or in varying ratios with either the organic base

(10) Corpechot, C.; Robel, P.; Axelson, M.; Sjovall, J.; Baulieu, E. E. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4704−4707.
(11) Guazzo, E. P.; Kirkpatrick, P. J.; Goodyear, I. M.; Shiers, H. M.; Herbert, J. *J. Clin. Endocrinol. Metab.* **1996**, *81*, 3951−3960.
(12) Wilm, M., Neubauer, G.; Mann, M. *Anal. Chem.* **1996**, *68*, 527−533.
(13) Brugger, B.; Erben, G.; Sandhoff, R.; Wieland, F. T.; Lehmann, W. D**.** *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2339−2344.
(14) Dusza, J.; Joseph, J.; Bernstein, S. *Steroids* **1968**, *12*, 49−61.
(15) Sanaullah; Bowers, L. D. *J. Steroid Biochem. Mol. Biol.* **1996**, *58*, 225−234.
(16) Wu, J.; Chatman, K.; Harris, K.; Siuzdak, G. *Anal. Chem.* **1997**, *69*, 3767−3771.
(17) Neubauer, G.; Mann, M. *J. Mass Spectrom.* **1997**, *32*, 94−98.
(18) Bean, K. A.; Henion, J. *J. Chromatogr. B* **1997**, *690*, 65−75.
(19) McGee, J. P.; Palin, K. J.; Shaw, P. N.; Potter, C. *J. Chromatogr.* **1991**, *567*, 282−287.
(20) Komatsu, F.; Morioka, M.; Fujita, Y.; Sugahara, K.; Kodama, H. *J. Mass. Spectrom.* **1995**, *30*, 698−702.
(21) Rizea Savu, S.; Silvestro, L.; Haag, A.; Sorgel, F. *J. Mass. Spectrom.* **1996**, *31*, 1351−1363.
(22) Lacroix, C.; Fiet, J.; Benais, J. P.; Gueux, B.; Bonete, R.; Villette, J. M.; Gourmel, B.; Dreux, C. *J. Steroid Biochem.* **1987**, *28*, 317−325.

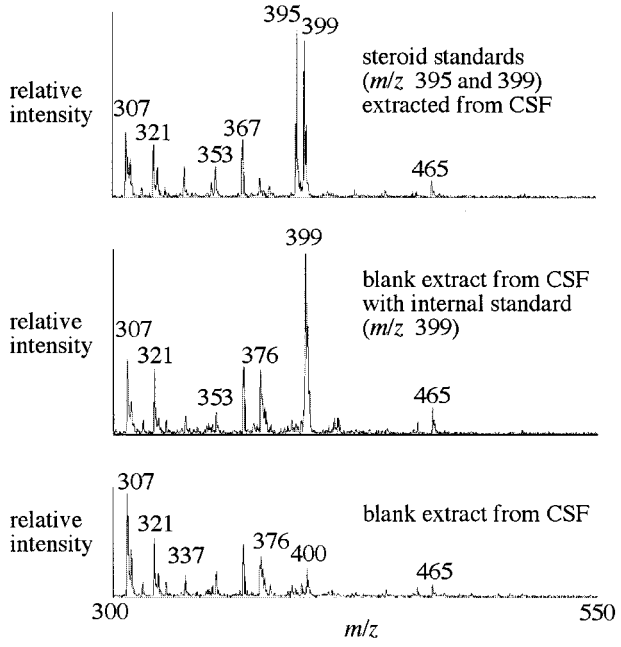


Figure 1. NanoESI mass spectra using precursor ion scanning of sulfate compounds recovered from human CSF by the two-step extraction method. (top) Extract from CSF blank spiked with pregnenolone sulfate (*m*/*z* 395) at 250 fmol/μL,and the internal standard pregnenolone-d_4 sulfate (*m*/*z* 399) at 250 fmol/μL. (middle) Extract from CSF blank with internal standard pregnenolone-d_4 sulfate (*m*/*z* 399) at 250 fmol/μL. (bottom) Extract from CSF blank.

trimethylamine or 2-butanol. Trimethylamine was added to artificial CSF in amounts of 0.5, 1, 3, 6, 10, and 30% to vary the pH from 8 to 13. The solvents were separately diluted with 2-butanol at ratios 90:10, 80:20, 70:30, 60:40, 50:50, and 40:60. Once the best system for sulfated steroid extraction was defined, focus was shifted to extracting the unconjugated steroids using the remaining set of solvent systems. This set consisted of the same solvents, but without the addition of trimethylamine or 2-butanol. In these experiments, 600 μL of each solvent system was added to 100-μL aliquots of artificial CSF with the addition of the steroid mixture. These solutions were then vortexed for 3 min and centrifuged for 1 min at 1000*g*. Finally, the organic layer was removed, evaporated to dryness, and resuspended in MeOH/H_2O (70:30, v/v) for analysis by nanoESI.

The results showed chloroform/2-butanol (50:50, v/v) to be the best solvent system for extracting steroid sulfates from artificial CSF. The chloroform/2-butanol (50:50, v/v) extraction was also tested on human CSF samples (Figure 1). Analysis of the unconjugated steroid extractions revealed diethyl ether to be the most effective solvent tested for removing unconjugated steroids, although it also removed a substantial amount of the sulfated steroid conjugates. Hexane proved to be the most efficient for removing the unconjugated steroids without removing the steroid sulfates. This inspired further testing with combinations of diethyl ether and hexane at various ratios in an attempt to improve the extraction recoveries. These tests found diethyl ether/hexane (90:10, v/v) to be the best binary solvent system for removing unconjugated steroids, leaving behind the sulfated steroid conjugates (the unconjugated steroid extraction must be performed prior to the sulfated steroid extraction in order to achieve maximum recoveries from both phases).

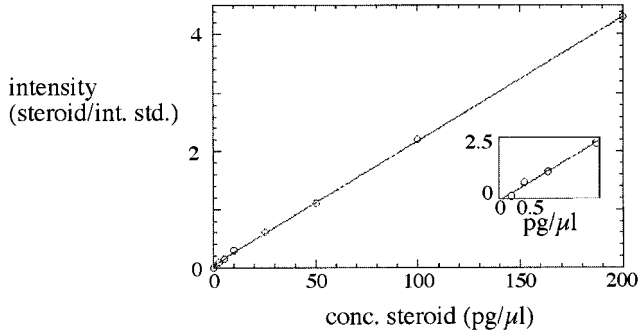


Figure 2. Standard curve of pregnenolone sulfate with internal standard, pregnenolone-d_4 sulfate, generated using precursor scanning with negative ion nanoESI. Quantitative data obtained in separate calibration experiments ranging from 100 amol/μL to 500 fmol/μL (40 fg/μL−200 pg/μL). The limit of quantiation was 250 amol/μL where the accuracy was found to be within ±20%.

The complete extraction procedure was then tested on 100-μL aliquots of artificial CSF spiked with the steroid mixture. This was accomplished by adding 600 μL of diethyl ether/hexane (90:10, v/v) to each sample. These were vortexed for 3 min and centrifuged for 1 min, after which the organic phase was collected and evaporated to dryness. The aqueous phase was recovered and again extracted by adding 600 μL of chloroform/2-butanol (50:50, v/v), vortexing, centrifuging, collecting the organic phase, and evaporating. The residue was then resuspended in MeOH/H_2O (70:30, v/v) for analysis by nanoESI. The time required for the entire extraction procedure was ~15 min. The same experiments were also performed from the three different biological matrixes where the limit of detection was found to be 200 amol and the limit of quantitation was 250 amol.

Sulfation of Unconjugated Steroids. The procedure for sulfating free steroids was based on known procedures;[14,15] the reason for preparing sulfates (instead of using other modifications) is that they are well-characterized natural products which are readily synthesized in pure form to serve as standards. This technique was tested on free steroid standards of pregnenolone and DHEA which were sulfated separately and as a mixture. Free steroid fractions in ethyl acetate were spiked with a recovery standard (tetradeuterated pregnenolone sulfate) and evaporated to dryness under N_2. The reagent (sulfur trioxide *N*,*N*-dimethylformamide complex in dimethylformamide/pyridine) was added and heated for 1 h at 40 °C. The samples were then evaporated under nitrogen at 40 °C. Next, 500 μL of H_2O was added and the steroid sulfates were extracted with 600 μL of chloroform/2-butanol (1:1), following the extraction procedure described above. Recoveries averaged 70%.

Quantitative Steroid Analysis with NanoESI-MS. An internal standard, pregnenolone-d_4 sulfate at 250 fmol/μL, was added after extraction but before evaporation to dryness. Then the dried extracts, including the internal standard, were redissolved in MeOH/H_2O (70:30, v/v), loaded into glass capillary needles, and analyzed by negative ion nanoESI. At the lower concentrations of sulfated steroid (<25 fmol/μL), it was necessary to use a smaller amount (1.0 fmol/μL) of internal standard to obtain quantitative data. A calibration curve was obtained by precursor ion scanning using the plot of the ratio of steroid/internal standard versus the concentration of steroid present. The data showed a linear

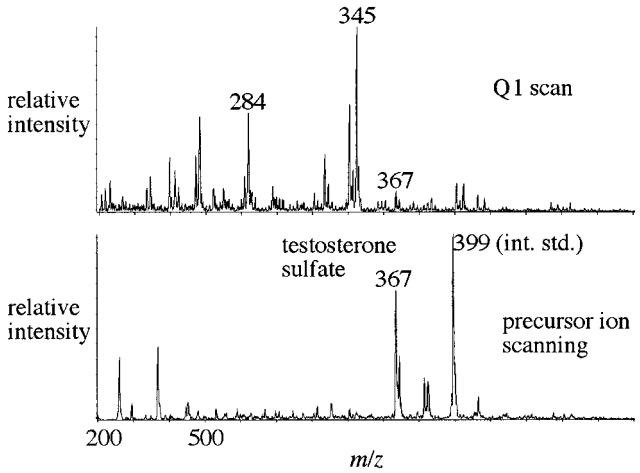


Figure 3. Mass spectra of male bonobo urine extract, obtained by negative ion nanoESI. Q1 scan (top) and precursor ion scan (bottom) was used for steroid sulfates and the internal standard (*m*/*z* 399) at 12.5 pmol/μL. Testosterone sulfate anion detected at $[M - H]^- = m/z$ 367 and estimated at 9.5 pmol/μL.

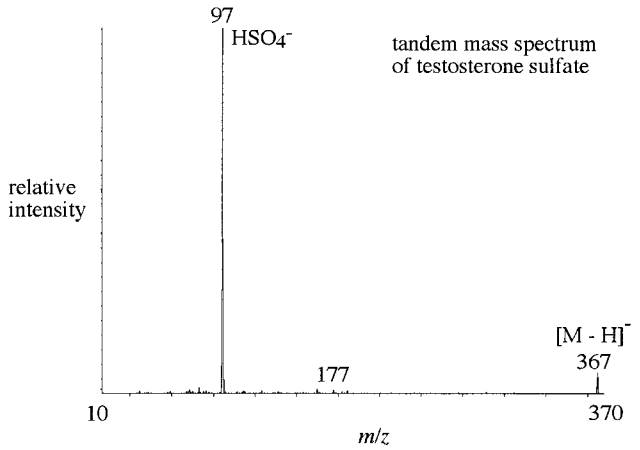


Figure 4. Negative ion nanoESI MS/MS spectrum of testosterone sulfate (*m*/*z* 367) from extract of male bonobo urine. Base peak (*m*/*z* 97) is the sulfate anion [HSO_4^-].

relationship in the range of 25−500 (10−200 pg/μL) and 0.10−1.0 fmol/μL (40−400 fg/μL) (Figure 2). The equations of the first and second line had correlation coefficients of $R = 0.998$ and 0.990, respectively, and the standard deviations of the slopes was 4 and 7%, respectively. Quantitation of steroids in blood, urine, and CSF was accomplished by comparing the intensities of the precursor ions of *m*/*z* 97, specific to each sulfated steroid present in the steroid mixture, with the internal standard.

NanoESI of Steroid Sulfates from Urine and Plasma. Urine and plasma samples from various male and female primates, including human, chimpanzee, bonobo, lowland gorilla, and mountain gorilla, were extracted and analyzed by nanoESI according to the described techniques. The purpose of these experiments was simply to test the validity of the techniques employed and not specifically to identify and quantitate steroids found in primate biofluids. While these primates were found to have similar steroidal makeup, differences were observed for the levels of individual steroids.

The results from the extraction technique were positive, showing the recovery of a variety of compounds. The new extraction technique proved to be simple and fastidious, allowing the removal of steroid sulfates from biofluids and analysis by nanoESI in ~15 min with this simple methodology. These techniques are consistent, reliable, and far less time-consuming than techniques described in the literature.[10,11,18−22] The Q1 scan of the male bonobo urine (Figure 3) displayed a crude mixture of all compounds extracted, some sulfated and some nonsulfated. In Figure 3, testosterone sulfate is less distinguishable in the Q1 scan than in the precursor ion scan. The Q1 scan provides a complex spectrum, making it difficult to ascertain which, if any, peaks represent steroid sulfates. By employing the precursor ion scan for "parents" of *m*/*z* 97, the sulfated compounds are easily observable and steroid sulfates can be quickly determined by tandem mass spectrometry. By scanning for parent ions of the sulfated compounds only, the undesired compounds are eliminated and the spectra are less complex. Not all steroid sulfates are detected by searching for parents of the sulfate group *m*/*z* 97. For instance, phenolic steroids such as natural estrogens are more stable, and these natural estrogen 3-sulfates lose their sulfate group as [M − 80] instead of [M − 97]. In either case, the utility of the precursor ion scan for steroid sulfates shows great potential for analyzing a wide variety of compounds out of crude mixtures, on the basis of their specific fragmentation patterns.

Quantitation of the sulfated steroid conjugates is simplified by combining the precursor ion scan with deuterated analogues. Here, pregnenolone-d_4 sulfate was added as an internal standard (12.5 pmol/μL) to determine the concentration of testosterone in male bonobo's urine. Using the ratio of peak intensities in the precursor ion spectrum, we determined the level of testosterone was 9.5 pmol/μL for the male bonobo, while the male chimpanzee had 2.0 pmol/μL of testosterone.

The usefulness of tandem mass spectrometry for the structural confirmation of known steroids as well as for identifying new steroids is favorable, but has limitations. In some situations, where multiple isomers of the same mass are present, MS/MS spectra can be unclear due to identical fragmentation patterns. In these cases, the application of microLC/MS may eliminate ambiguities by separating the isomers and allowing MS/MS of each individual isomer. The presence of testosterone sulfate in bonobo urine was identified from other isoforms such as DHEA sulfate because of the characteristic *m*/*z* 177 fragment that was present in the MS/MS spectrum (Figure 4).

The extraction technique described above was also applied to human plasma to explore the efficacy of extracting steroids from blood using these methods. Steroid sulfates were isolated and observed by precursor ion scanning (Figure 5). These findings suggest that the new extraction protocol is not limited to urine but is applicable to other biofluids.

Sensitivity of NanoESI and Precursor Ion Scanning. To further demonstrate the sensitivity afforded sulfated steroid analysis by nanoESI and precursor ion scanning, we established a limit of detection in the attomole range using a mixture of three sulfated steroid standards. A mixture of pregnenolone sulfate, allopregnanalone sulfate, and pregnenolone-d_4 sulfate was prepared with a concentration of 2.5 μmol/μL (1.0 mg/mL) for each steroid. This solution was diluted by serial dilution to concentrations of 1.0 fmol/μL, 500 amol/μL, 100 amol/μL, and 50 amol/μL. Single microliters of each solution were loaded into glass

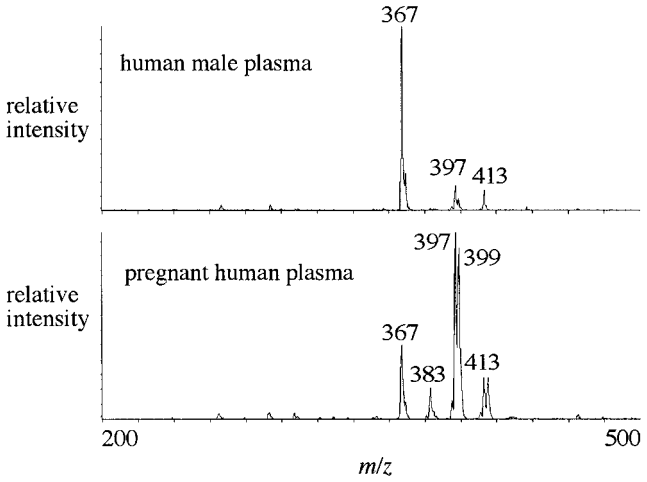


Figure 5. Negative ion nanoESI mass spectra (precursor ion scanning) of human male plasma (top) and female pregnancy plasma (bottom) generated by precursor ion scanning for parents of *m*/*z* 97 fragment. Steroid sulfates are DHEA sulfate, testosterone sulfate (*m*/*z* 367), and allopregnanalone sulfate (*m*/*z* 397).

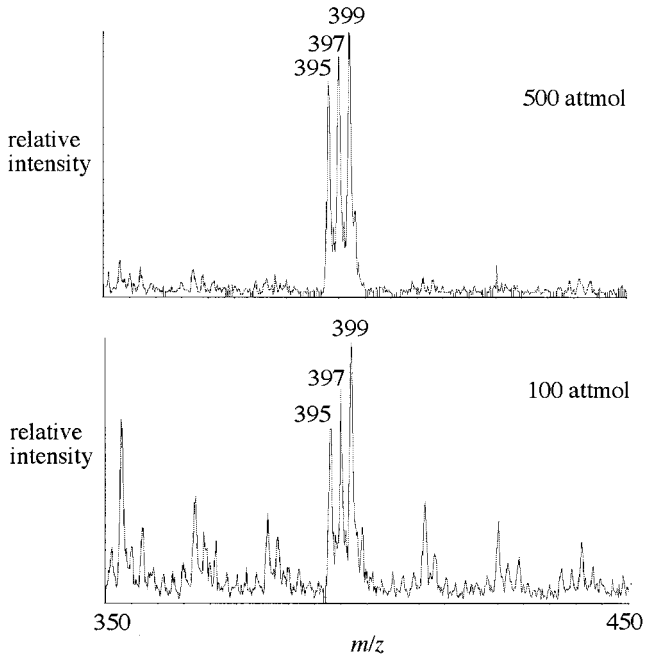


Figure 6. (top) Negative ion nanoESI mass spectra (precursor ion scanning) of a mixture of pregnenolone sulfate (*m*/*z* 395), allopregnanalone sulfate (*m*/*z* 397), and pregnenolone-d_4 sulfate (*m*/*z* 399) at 500 amol/μL. (bottom) The same analysis performed at a concentration of 100 amol/μL. Both spectra demonstrate the sensitivity of nanoESI using precursor ion scan negative ionization. The limit of detection from CSF and artificial CSF was found to be 200 amol/μL.

capillaries and analyzed by nanoESI using precursor ion scanning, with 50−200 nL consumed per analysis. The limit of detection was found to be 100 amol/μL with less than 20 amol consumed (Figure 6). Steroids were noticeable at the 50 amol/μL concentration, but interference from background noise made the spectrum less distinct than that of the 100 amol/μL solution. A second study was performed which analyzed extracts of the same steroid mixture from artificial CSF and urine using the aforementioned extraction procedure. The result of these experiments was a detection limit of 200 amol/μL, with less than 40 amol consumed. Interestingly, the fact that only 20% of the sample was actually

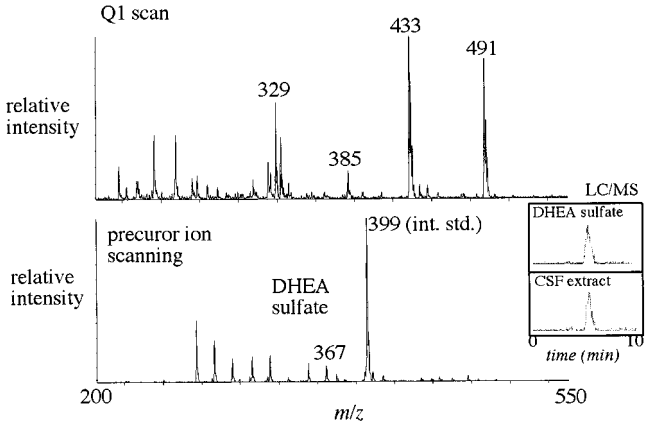


Figure 7. Negative ion nanoESI Q1 scan (top) and precursor ion scan (bottom) mass spectral data of steroid sulfates extracted from CSF (pregnant human) containing an internal standard (*m*/*z* 399) at 1.2 pmol/μL. The DHEA sulfate anion was detected at $[M - H]^- = m/z$ 367 and estimated at 100 fmol/μL. The selected ion chromatogram of DHEA sulfate standard (inset, top) and a sample extracted from an actual CSF sample (inset, bottom) are shown.

consumed during these experiments offers opportunities employing nanoLC/MS for more profound studies involving steroids and their metabolic pathways, including the investigation of neurosteroids.

Analysis of Steroids Extracted from CSF. Neurosteroids were extracted from human CSF by the technique described and analyzed by nanoESI using precursor ion scanning. The neurosteroid DHEA sulfate was observed in the CSF extract and separately confirmed by LC/MS (Figure 7). We also observed parents of *m*/*z* 97 at 397 and 411 which we could not confirm by LC/MS because of the low levels of material present, yet the observation of these ions indicates the greater sensitivity of nanoESI.

The detection of neurosteroids in CSF by mass spectrometry suggests that these techniques are very promising for future investigations. Typically, the analyses of neurosteroids have been challenging due to their low-nanomolar concentrations in vivo, and therefore, neurosteroids have been primarily analyzed using radioimmunoassay. With the methods described here for extraction and analysis, it is possible to detect steroids at endogenous levels using nanoESI without having to concentrate or pool samples.

CONCLUSION

NanoESI mass spectrometry is an accurate and sensitive analytical technique for the detection, quantitation, and structural confirmation of steroid sulfates found in biofluids. Combined with precursor ion scanning, nanoESI provides for low femtomole to attomole detection limits for charged compounds. By comparison, APCI and ESI are far less sensitive (high picomoles), consume more material (30−80 μL), and have the possibility of carryover from previous samples which can interfere with detection and quantitation. While MALDI is relatively sensitive (low picomoles), matrix interference in the mass ranges below 500 Da make it an undesirable approach. The combinatorial approach to determining optimal extraction conditions was successful in reducing the process to a two-phase, one-step extraction procedure and improving overall efficiency. Using this approach, we determined that

diethyl ether/hexane (90:10, v/v) is an effective solvent system for extracting unconjugated steroids from artificial CSF. In addition, we conclude that (50:50, v/v) chloroform/2-butanol effectively extracts steroid sulfates from artificial CSF. The new extraction procedure also proved to be an excellent tool for extracting steroids from blood and urine. The small amount of sample actually consumed in these studies suggests that nanoLC/MS, in concert with nanoESI, can further increase sensitivity (excellent sensitivity has recently been achieved using LC/MS on a related compound[23]) and eliminate potential biological matrix effects. These applications toward the study of neurosteroids are very promising and warrant continued investigation.

EXPERIMENTAL SECTION

NanoESI-MS. The steroid analyses were performed on a PE Sciex API III (Alberta, Canada) modified with a nanoESI source from Protana A/S. Orifice was set at −115 V, and ESI voltage was set at −650 V. A curtain gas of ultrapure nitrogen was pumped into the interface at a rate of 0.6 L/min to aid evaporation of solvent droplets and prevent particulate matter from entering the analyzer region. Desolvated ions entered the analyzer via the vacuum interface and were guided by entrance optics. Normal-sized palladium-coated, borosilicate glass capillaries from Protana A/S were used for sample delivery. The resolution was typically maintained at 280 or higher. The CID experiments were performed with ultrapure argon as a collision gas. The precursor ion spectra were acquired by scanning the first quadrupole, while collisions with argon (target thickness of 30×10^{14} atom/cm^2) in the second quadrupole produced ion dissociation. The third quadrupole was used to mass select the fragment ion (m/z 97). Spectra were the result of averaging from 50 to 200 scans depending on the number of scans necessary to obtain a signal-to-noise ratio greater than 50. "Cross-talk"[23] between MS/MS channels used for detecting the internal standard and other steroids was not observed.

APCI-MS. APCI-MS analyses were performed on a Finnigan LCQ ion trap mass spectrometer. Samples were delivered by LC flow injections at 200 μL/min flow rate using MeOH/H_2O (50:50, v/v). Vaporizer temperature was 350 °C, capillary temperature was 150 °C, and the discharge current was 5.00 μA. Steroid standards, including unconjugated steroids, pregnenolone, testosterone, allopregnanolone, and DHEA and their sulfated conjugates were analyzed by APCI-MS to investigate the utility of this technique for the study of steroids at biologically relevant concentrations. Analyses of the unconjugated steroids were performed in the positive ionization mode, observing the $[M + H]^+$ and $[M - H_2O + H]^+$ cations, while analyses of the steroid sulfates were performed in negative ionization mode, observing $[M - H]^-$ anions. While the signal for low-nanomole quantities of material was good, the signal-to-noise ratio was poor for low-picomole quantities of the steroids. Therefore, we opted to continue our focus on nanoESI.

CSF. The human CSF samples were obtained for research purposes from the UCSD Medical School with the consent of the donors. Steroids were extracted by adding 600 μL of diethyl ether/hexane (90:10, v/v). The extract was vortexed, centrifuged, collected, evaporated, and prepared for nanoESI analysis following the protocol described above. After recovery of the biological fraction, the steroids were again extracted with 600 μL of chloroform/2-butanol (50:50, v/v) by the procedure described above for the second-phase extraction and were prepared for analysis by nanoESI.

HPLC/MS. LC/MS analysis of human CSF was performed using a Hewlett-Packard 1090 liquid chromatograph coupled with a Finnigan LCQ ion trap mass spectrometer. Negative-mode ESI was used with a capillary temperature of 220 °C, capillary voltage −15 V, and spray voltage 4.2 kV. Nitrogen was used as the nebulizing gas. The acetonitrile/water (30:70, v/v) mobile phase was maintained at a flow rate of 200 μL/min through a Zorbax C_{18} (300 SB 2.1 mm × 15 cm) column into the mass spectrometer. Injections (10 μL) of sulfated DHEA and sulfated testosterone standards (50 pmol) were injected in sequence with the extracted human CSF.

Primate Biofluids Experiments. Urine and plasma samples were collected from male and female primates. These specimens were extracted in 100-μL aliquots following the described techniques and analyzed by nanoESI. Biological samples were obtained from primates housed at the San Diego Zoo using an approved protocol and were provided by the Center for the Reproduction of Endangered Species (CRES) laboratory of the San Diego Zoo.

Artificial CSF Experiments. Artificial CSF was provided by Scripps neuropharmacology composed of 145 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 5.4 mM glucose-*d*. Aliquots of 100 μL of CSF were spiked with steroids and extracted according to the techniques described.

Stock Solutions. Stock solutions of steroids synthesized in our laboratories were prepared at 5ug/μL and stored at −20 °C. Steroid sulfates were dissolved in ethanol, and unconjugated steroids were dissolved in ethyl acetate. Desired dilutions were prepared with MeOH/H_2O (70:30, v/v) for working solutions and stored at −20 °C.

Chemicals and Solvents. Methanol, dichloromethane, chloroform, ethyl acetate, diethyl ether, hexane, trimethylamine, 2-butanol, dry pyridine, acetic acid, and triethylamine sulfur trioxide complex were purchased from Sigma Chemical Co. (St. Louis, MO). Ethyl alcohol was purchased from Quantum Chemical Co. (Tuscola, IL).

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