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Title: Mass Spectrometry Imaging for Dissecting Steroid Intracrinology within Target Tissues

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1. Supplementary Methods

***1.1 Screening of matrices*:** α-Cyano-4-hydroxycinnamic acid (CHCA): 4-chloro-α-cyanocinnamic acid (Cl-CCA; in-house synthesis [24], 2,5-dihydroxybenzoic acid (DHB), 2-(4'-cydroxybenzeneazo) benzoic acid (HABA): 3-indole acetic acid (IAA), ferulic acid and Buckminsterfullerene (C60) were screened as potential matrices for neutral corticosteroids. Matrix assessment was carried out by quantifying signal from spots, allowed to dry at room temperature (RT) (1µg; 1mg/ml in methanol: water (1:1)) of d_8CORT. (n=3 per matrix). Targeted matrices (1µl) were manually applied to spots and allowed to dry (RT). Samples were stored in a vacuum dessicator (RT) until MALDI analysis.

1.2 Optimising On-tissue Derivatization Conditions: Optimisation of reaction conditions was performed using a control tissue section with spots (5ng, 10µg/ml; methanol: water (1:1)) of d_8-corticosterone (d_8CORT) applied to the surface manually (n=3 per condition). CORT and 11-dehydrocorticosterone (11DHC) were also applied as spots to a tissue-free region as controls. Spots were allowed to dry, then Girard T (GirT) (1µl; 5mg/ml) in different solvent systems, (methanol and ethanol with trifluoroacetic acid (TFA) 0.2% v/v, formic acid (FA) 0.2%v/v, acetic acid (HA) 10%v/v (freshly prepared) was deposited on top. The MALDI glass slide was placed in a Petri dish containing a moist tissue and incubated (20, 60 or 120 min, 40°C), then allowed to cool and dry in a vacuum desiccator (RT, 20 min). Matrix (α-cyano-4-hydroxycinnamic acid (10mg/ml; (80%)) in acetonitrile + 0.2% v/v TFA) was applied by a pneumatic TLC sprayer (20ml per slide) and allowed to dry (RT) and stored in a dessicator until MALDI analysis.

1.3 Stability of Derivatized Steroid **:** The Girard T derivative of corticosterone (GirT-CORT) standard was prepared as reported previously [28] by reaction of GirT and the corresponding steroid in a 1:1 molar ratio, in a 10% v/v acetic acid solution in methanol. The reaction was incubated (3 h, room temperature (RT)). The derivative was extracted in toluene: dichloromethane (1:1) and the solvent evaporated to dryness. The residue was characterised using accurate mass measurement (460.31651 Da) and reconstituted in methanol and storage until MALDI analysis. On-tissue stability was performed using a control tissue section manually spotted with GirT-CORT standard solution (0.5 µl, 10µg/ml, 5ng) in of methanol: water (1:1). Spots (n=3, per time) were allowed to dry at RT. The matrix (α-cyano-4-hydroxycinnamic acid) was applied using a pneumatic TLC sprayer (25ml per slide) immediately after reaction (T=0, 10, 20 and 30 min, RT) to evaluate the stability of the corticosterone hydrazone on the tissue.

1.4 Saturation Studies: Adrenal gland sections were used as control tissue for saturation studies. The sections (n=2) were sprayed with 4, 6, 8 and 10 ml of GirT solution (5mg/ml) in methanol for post-coating experiments and with 0.15, 0.25, 0.35 and 0.5 mg/cm^2 for pre-coating slides. Both GirT steroids derivatives gradient distribution maps were acquired and evaluated. Results have shown that best sensitivity was achieved by using either 4ml of 5mg/ml of GirT solution (post-coating) or $0.15 mg/cm^2$ (pre-coating).

1.5 Limits of Detection (LODs) of GirT derivatives of glucocorticoids applied to the surface of tissue: LODs (signal/noise≥3) were assessed using standards (CORT, 11DHC) applied to slides "off-tissue" and deuterated standard (d_8CORT) applied to the tissue surface as spots "on-tissue" using a dilution series of both steroids in methanol to achieve a range of 10µg to 100 pg/spot for neutral steroids and 1ng to

0.01 pg/spot for GirT-derivatives (n=3 spots per concentration). Matrix was applied and slides imaged by MALDI-FTICR-MS. Signal intensity response factors (CORT/11DHC) were estimated. Ions were detected using 250 laser shots with power optimized and fixed for the duration of the experiment

1.6 Extraction of steroids from whole brain: Analysis was adapted from Wang. [13] In brief**,** murine brain (~300mg) was homogenized in methanol–acetic acid (100:1 v/v, 10 mL) assisted by ultrasonication. The supernatant was retained and the pellet formed followed centrifugation (5000 × *g*, 10 min, 4 °C), further extracted with methanol–acetic acid (100:1 v/v, 10 mL) and the supernatants combined and stored at -80 °C. The extract was reduced to dryness and re-dissolved (3 mL, methanol/dichloromethane/water (7:2:1, v/v)) and enriched with internal standard (5 ng). The sample, prepared in methanol/dichloromethane/water (7:2:1, v/v), was passed through a diethylaminohydroxypropyl Sephadex LH-20 (GE Healthcare, Sweden) anion-exchange column in its acetate form (7 × 0.4 cm). The column was washed with 2 mL of the same solvent, followed by 1 mL of methanol/dichloromethane/water (2:2:1, v/v), and the “flow-through” and “wash” combined, dried and re-dissolved in methanol–acetic acid (2 mL, 100:1 v/v) with the final addition of water (2mL). The solution was passed through a Bond-elut (HLB, 2g, Waters, Manchester, UK) cartridge. After washing with water (2 mL), methanol–water (1:1, 3 ml) and hexane–ethyl acetate (5:1, 2 mL), the steroids were eluted with ethyl acetate (2 mL). The residue was evaporated, re-dissolved in 50:50 (methanol: H_2O with 0.1% of formic acid (FA)) and subjected to LC-MS/MS analysis.

1.6.1 *Quantitative LC-MS/MS analysis**:*** Solutions (0.1mg/mL in methanol) of CORT and 11DHC were serially diluted with water (as simulated media) to prepare calibration standards (0.1-50ng each steroid) with the addition of d_8CORT (5 ng),

and processed as tissue as above. The final extract was dissolved in (50:50) methanol: formic acid 0.1% v/v. Separation was achieved on an Inerstil ODS-3 100x 2.1mm, 3µm (GLSciences, Torrance, USA) column. Mobile phases, consisting of 0.1% v/v FA in H_2O (A) and 0.1% v/v formic acid in 90:10 (acetonitrile: methanol) (B), were used at a flow rate of 0.3 mL/min, with an initial hold of 3 min at 35% B followed by a linear gradient to 90% B in 8 min and re-equilibration at initial conditions (2 min). Column and autosampler temperatures were 45˚C and 10˚C respectively. Electrospray ionization was performed with an ion source voltage of 4500V, medium collision gas and source temperature 450°C). Data were acquired by multiple reaction monitoring (collision energy, declustering potential, cell exit potential): CORT *m/z* 347→121 (31,148,12V); 11DHC *m/z* 345→121 (30,155,15V); and d_8CORT *m/z* 355→125 (32, 119,12V). Peaks area ratios were calculated and compared with corresponding calibration samples.

1.7 Extraction of UE2316 from whole brain: Brain tissue (~150mg) were homogenized in 5ml of buffer (50:45:5 Hepes: Tris (0.1M): Sodium lauryl sulphate (SDS) (0.5%) pH 9.5 adjusted with NaOH containing 100µg/ml of Protein K enzyme. UE2346 was added (100ng) and homogenates were incubated at 37˚C in a rotating water batch for 60 minutes. Samples were cooled down to RT and centrifuged (3500g, 15minutes, 4˚C), supernatants were filtered through a 0.45µm nylon filters and extracted with 3 x 5ml of ethyl acetate. Organic layers were reduced to dryness under gentle stream of nitrogen at RT and residues were reconstituted in (acetonitrile: water 50:50 containing 0.1% formic acid) (100µl) and samples were then submitted to LC-MS/MS analysis.

1.7.1 *Quantitative LC-MS/MS analysis:* Stock solutions of 1 mg/ml of UE 2316 and UE 2346 (ISTD) were obtained by dissolving 1.0 mg of corresponding analytes in 1ml of methanol. Working standards of 5, 25, 100, 250, and 500ng were prepared by

serial dilution of the stock solution in methanol and were stored in vials at −20˚C protected from light. Standard curve was prepared using water as a simulated media and ion suppression was evaluated by standard addition experiments at 25 and 250ng respectively. The final extract was dissolved in mobile phase (50:50) acetonitrile: formic acid 0.1% v/v. Separation was achieved on a Kinetex PFP column 100x3mm, 2.6µm (Phenomenex, Macclesfield, UK,). Mobile phases, consisting of 0.1% v/v FA in H_2O (A) and 0.1% v/v formic acid in acetonitrile (B), were used at a flow rate of 0.35 mL/min, with an initial hold of 2 min at 20% B followed by a linear gradient to 100% B in 10 min and re-equilibration at initial conditions (2 min). Column and autosampler temperatures were 40˚C and 10˚C respectively. Electrospray ionization was performed with an ion source voltage of 5000V, medium collision gas and source temperature 450°C). Data were acquired by multiple reaction monitoring (collision energy, declustering potential, cell exit potential): UE2316 *m*/*z* 391→177 (31,148,12V) and UE 2346 *m*/*z* 482→194 (35, 115,10V). Peaks area ratios were calculated and compared with corresponding calibration samples.

Table S1

Screening of Derivatization reagents for MALDI-MSI.

Derivatization reagent	Intensity (cps*)	
	11-dehydrocorticosterone	Corticosterone
	Intensity of derivatives (cps)	
None (underivatized)	*0.9×10^4*	*1.3×10^4*
Aminoguanidine	ND	ND
Dansyl Chloride	ND	ND
Sulfonyl Chloride	1×10^4	2×10^4
Isonicotinoyl Chloride	5.4×10^5	3.1×10^5
Ferrocene Azide	ND	ND
Dansyl Hydrazine	6.7×10^6	8.1×10^6
Girard T	8.7×10^8	8.5×10^8
Girard P	1.7×10^7	2.1×10^7

***cps**: count per second

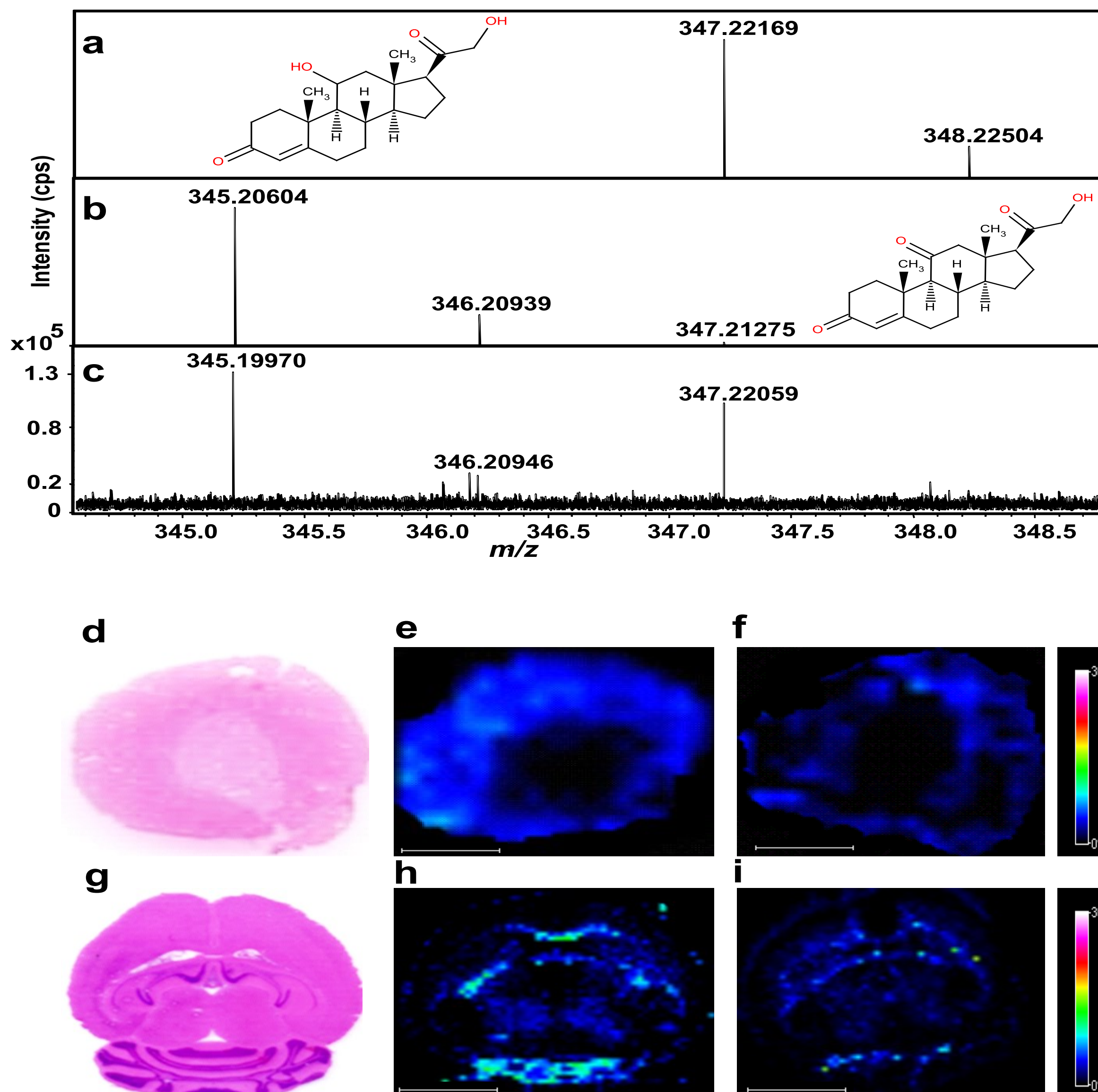

Figure S1: MALDI-FTICR-MS imaging (MSI) of neutral steroids in rat adrenal gland sections. MSI of neutral glucocorticoids using CHCA as matrix showed very low ionization yields in both adrenal and brain tissue sections. Simulated theoretical isotopic distribution pattern of: (**a**) corticosterone; and (**b**) 11-dehydrocorticosterone. (**c**): MALDI-FTICR-MS spectra of adrenal gland of neutral corticosteroids of corticosterone at *m/z* 347.22059Da and 11-dehydrocorticosterone at *m/z* 345.19970Da. (**d**) Histological image of adrenal gland stained with haematoxylin and eosin. MSI heat map distribution of: (**e**) corticosterone at *m/z* 347.22169±0.025Da; and (**f**) 11-dehydrocorticosterone at *m/z* 345.20604Da in rat adrenal gland. **(g)** Histological image of murine brain stained with haematoxylin and eosin. MSI heat map distribution of: (**h**) corticosterone at 347.22169±0.025Da; and (**i**) 11-dehydrocorticosterone at *m/z* 345.20604Da in murine brain. Signal intensity is depicted by colour as per the scale shown. **cps**: count per second. Scale bar (2mm) for adrenal section and (5mm) for brain section.

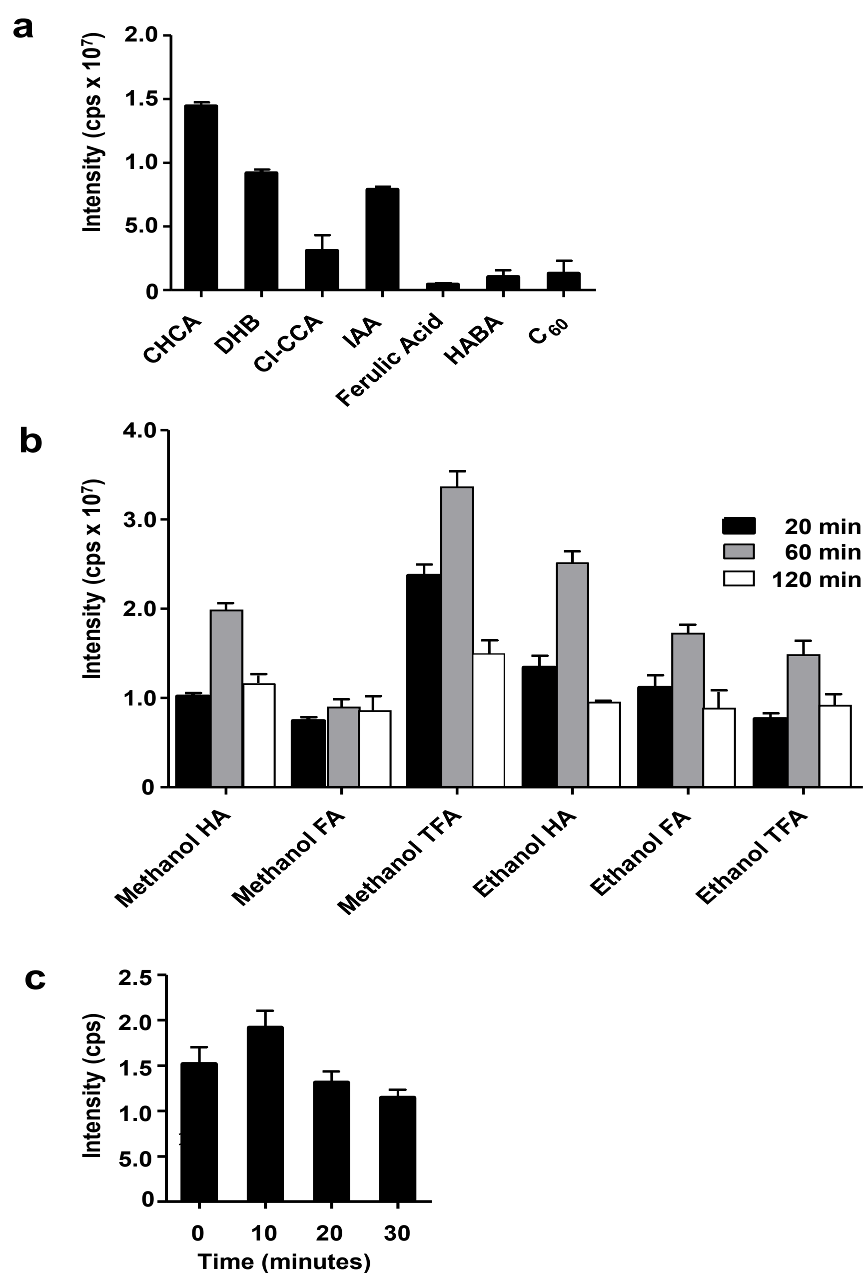

Figure S2: **Sample preparation and chemical derivatisation reaction optimisation**. (**a**) Matrix screening for detection of neutral corticosteroids**.** The best yield of ions was achieved using CHCA as a matrix. **CHCA** = α-Cyano-4-hydroxycinnamic acid, **DHB** = 2, 5-dihydroxybenzoic acid, **Cl-CHCA** = 4-Chloro-α cyano cinnamic acid, **IAA** = 3-indole acetic acid**, HABA** = 2-(4'-Hydroxybenzeneazo) benzoic acid, $\mathbf{C_{60}}$ = Buckminsterfullerene. (**b**) Optimisation of on-tissue reaction conditions for derivatisation. The highest yield was achieved at 60 minutes using methanol/TFA as solvent/catalyst system. (**c**) Stability of Girard T-steroid derivative. The Girard T-hydrazone was stable for 30 minutes at RT. **HA** = Acetic acid, **FA** = Formic acid, **TFA** = Trifluoroacetic acid. **cps** = Counts per second.

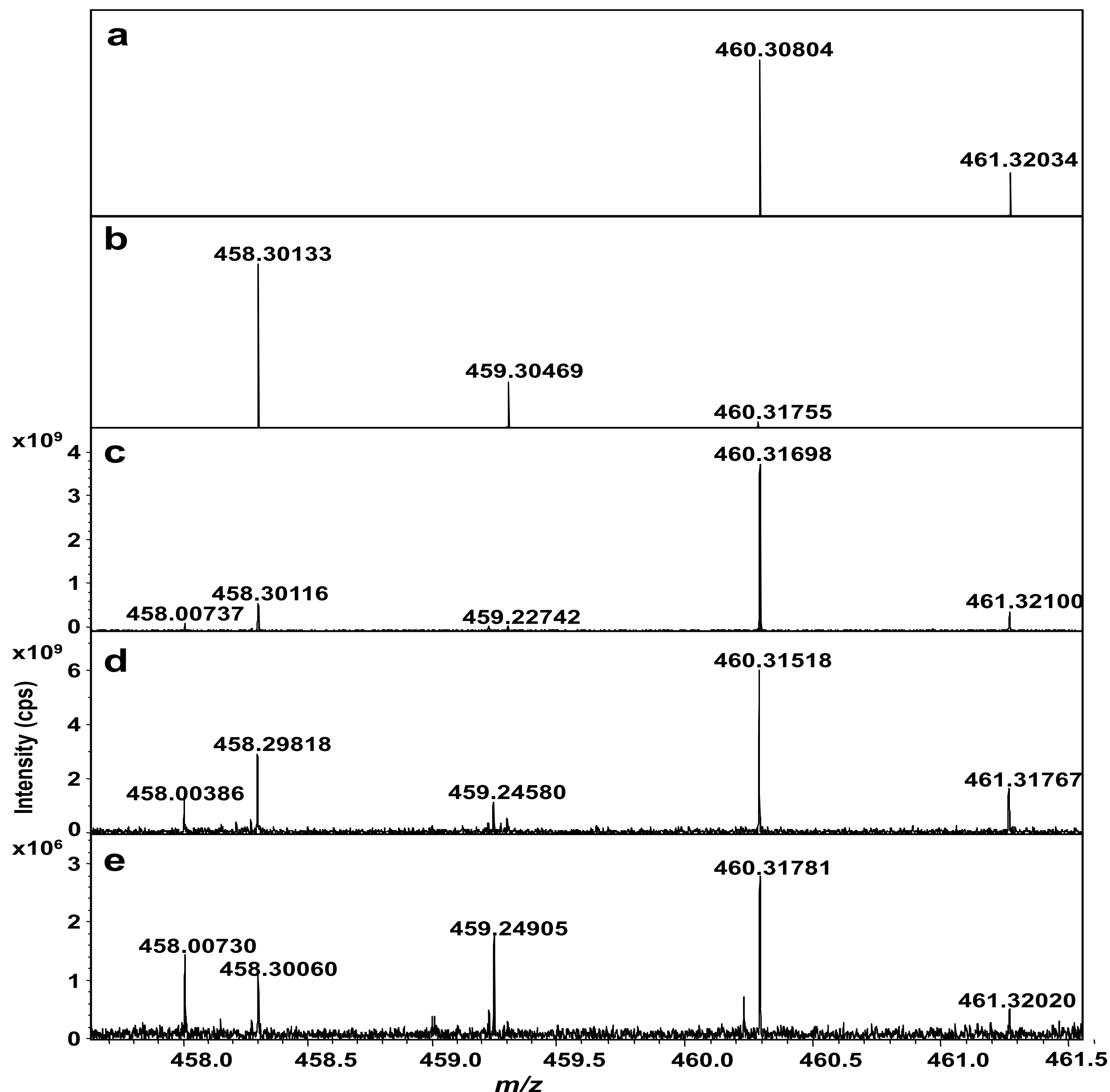

Figure S3: Simulated and observed MALDI-FTICR-MS spectra of corticosteroids derivatives. Simulated theoretical isotopic distribution patterns of (**a**) Girard T (GirT)-corticosterone. (**b**) GirT-11-dehydrocorticosterone. Representative MALDI-FTICR-MS spectra of GirT hydrazone derivatives of corticosteroids in tissue; acceptable mass accuracy was achieved with measured *m*/*z* differing by <5 ppm from theoretical monoisotopic masses in (**c**) rat adrenal gland, (**d**) murine brain (wild-type mouse) and (**e**) brain (mouse deficient in 11β-HSD1). **cps**: counts per second.

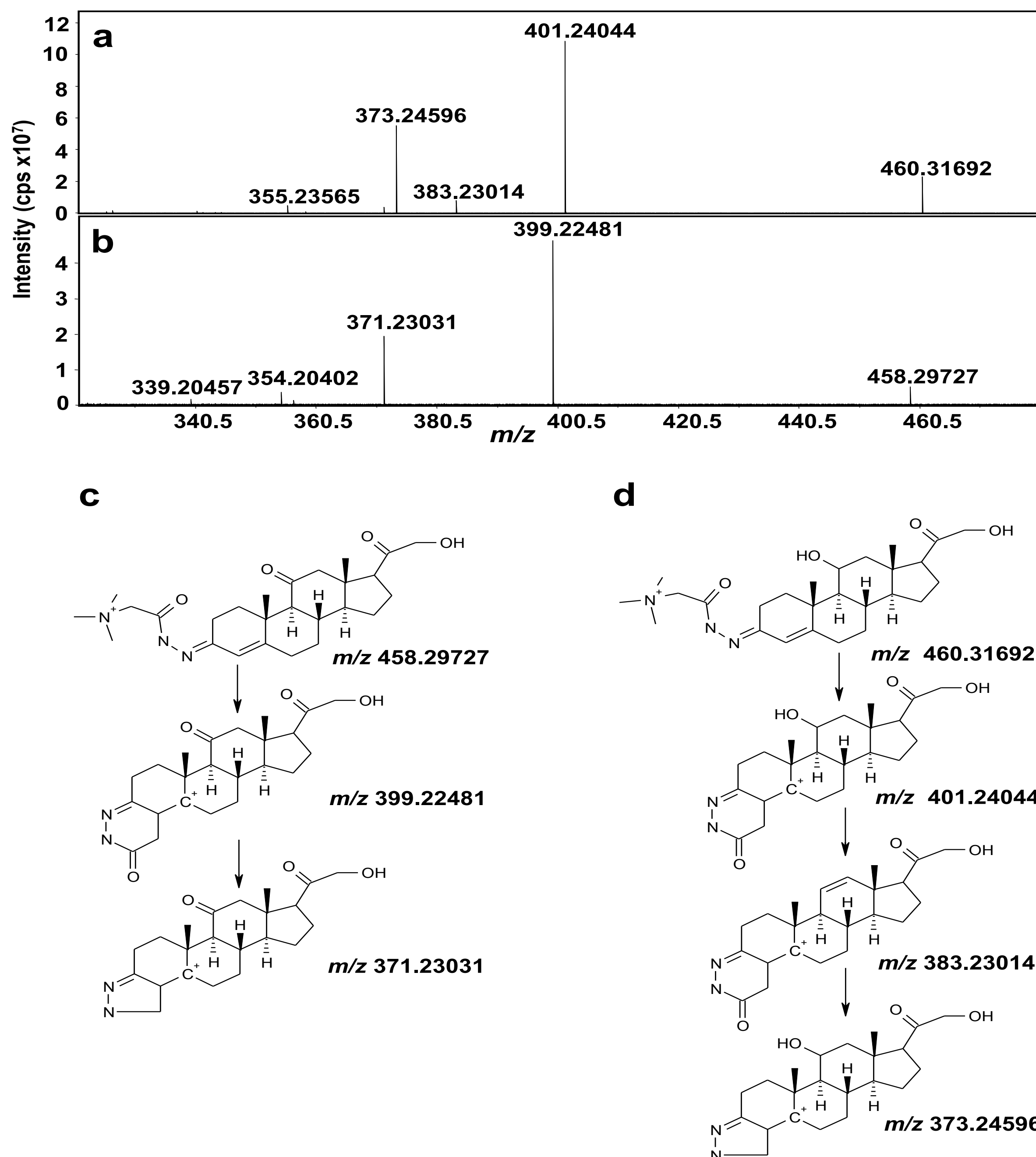

Figure S4: Liquid extraction surface analysis (LESA) with nanoESI-FTICR collision induced dissociation mass spectra of Girard T derivatives of corticosteroids. Collision induced dissociation mass spectra and proposed fragmentation patterns of Girard T (GirT) derivatives were in agreement with the structures of steroid hydrazones, extrapolating from Girard P (GirP) analogues[13]. Precursors (**a**) at m/z 460.31692Da (GirT-corticosterone) and (**b**) at m/z 458.29727Da (GirT-11-dehydrocorticosterone) (**d**) Proposed fragmentation patterns for GirT-corticosterone (**c**) and GirT-11-dehydrocorticosterone. **cps** = counts per second. Cell isolation was 20sec and collision energy was set to 28eV.

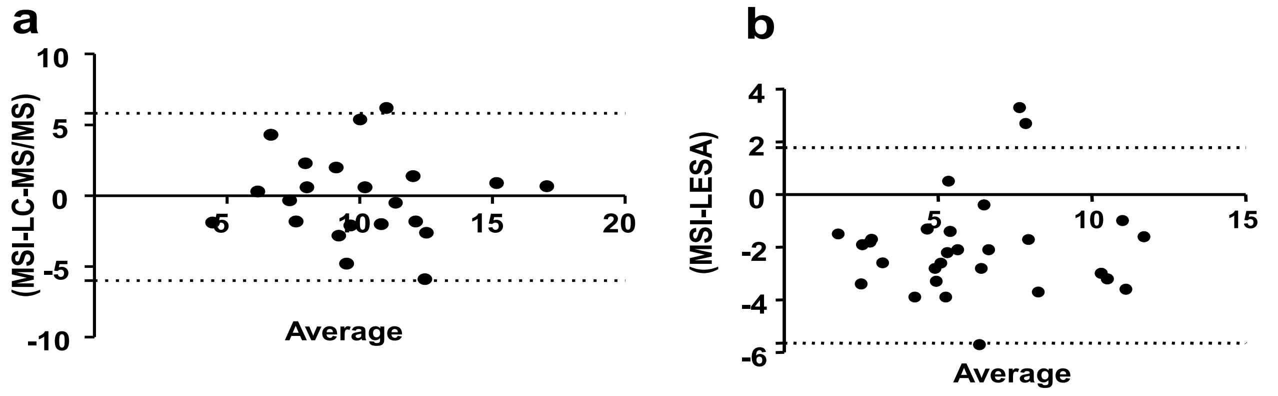

Figure S5: Agreement between MSI and alternative methods for measuring corticosteroids. Bland-Altman plots with 95% confidence intervals for agreement for ratios of corticosterone to 11-dehydrocorticosterone (CORT/11DHC) measured in wild type mouse brain by: (**a**) MSI and LC-MS/MS; (**b**) MSI and LESA.