Supplementary information

Mass spectrometry imaging (MSI) demonstrates the regional brain distribution patterns of three first-line antiretroviral drugs.

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LC-MS/MS

Figure S1. MS spectra of all the analytes.
Figure S2A. The proposed MS fragmentation pathways for EFV and CBB (IS).
Figure S2B. The proposed MS fragmentation pathways for TFV and ADV (IS).
Figure S2C. The proposed MS fragmentation pathways for FTC and ZDV (IS).
Sample extraction method development and optimization

SPE is used as a clean-up to remove endogenous substances from biological matrices, which may affect the analysis. Recoveries were determined by using different cartridges and solvents (MeOH or ACN), as protein precipitants. The best recoveries were achieved using the Discovery® DSC-PS/DVB SPE C18 (100 mg, 1 mL) and MeOH for plasma and Supel™ - Select HLB (30 mg, 1 mL) and ACN for brain homogenate.

| Table S1. Extraction recoveries of EFV, TFV and FTC for different SPE cartridges (n = 6). |
|---------------------------------------------------------------|
| SPE cartridge | EFV | TFV | FTC |
|                | Plasma | Brain | Plasma | Brain | Plasma | Brain |
| HLB SPE (30 mg, 1 mL) | 73 ± 2 | 79 ± 3 | 85 ± 1 | 87 ± 2 | 80 ± 1 | 79 ± 2 |
| HybridSPE-Phospholipid (30 mg, 1 mL) | 61 ± 1 | 58 ± 3 | 76 ± 1 | 81 ± 3 | 78 ± 2 | 73 ± 2 |
| C18 (50 mg, 1 mL) | 75 ± 2 | 57 ± 2 | 81 ± 1 | 78 ± 3 | 72 ± 1 | 65 ± 2 |
| C18 (100 mg, 1 mL) | 69 ± 3 | 55 ± 3 | 75 ± 3 | 80 ± 2 | 74 ± 1 | 68 ± 1 |

Linearity

Three calibration curves were constructed from each biological matrix (plasma and brain) and repeated on three consecutive days. The regression equations were:

- For EFV: $y = 0.4246x + 0.0017$ ($r^2 = 0.9992$)
- For TFV: $y = 0.2441x + 0.0217$ ($r^2 = 0.9983$)
- For FTC: $y = 0.3864x - 0.0045$ ($r^2 = 0.9996$)

All the $r^2$ values were above 0.99, indicating good linearity.
Matrix effect and extraction recovery

Matrix effect was tested for six different lots of drug-free rat plasma and brain homogenates. The matrix effect for each lot was evaluated at LQC, MQC, and HQC (n = 6). According to EMA guidelines, the variability of matrix factor should be within ± 15 %. The extraction recovery of each drug was determined by comparing the peak area from the extracted samples with the peak area from un-extracted samples.

| Drug | Sample | QC level | Mean recovery (%) | % RSD | Matrix effect (%) | % RSD |
|------|--------|----------|-------------------|-------|-------------------|-------|
| EFV  | Plasma | Low      | 76                | 3     | -2                | 4     |
|      |        | Mid      | 73                | 1     | -2                | 2     |
|      |        | High     | 77                | 1     | -4                | 4     |
|      | Brain  | Low      | 79                | 5     | -4                | 3     |
|      |        | Mid      | 76                | 3     | -3                | 2     |
|      |        | High     | 80                | 4     | -2                | 4     |
| TFV  | Plasma | Low      | 87                | 2     | -4                | 1     |
|      |        | Mid      | 84                | 1     | -4                | 1     |
|      |        | High     | 89                | 2     | -3                | 1     |
|      | Brain  | Low      | 81                | 4     | -5                | 2     |
|      |        | Mid      | 79                | 7     | -2                | 4     |
|      |        | High     | 83                | 2     | -2                | 2     |
| FTC  | Plasma | Low      | 86                | 3     | -5                | 3     |
|      |        | Mid      | 88                | 3     | -4                | 3     |
|      |        | High     | 85                | 1     | -2                | 4     |
|      | Brain  | Low      | 88                | 1     | -2                | 6     |
|      |        | Mid      | 91                | 4     | -1                | 2     |
|      |        | High     | 90                | 1     | -3                | 2     |
Accuracy and precision

The evaluation of intra-day and inter-day precision and accuracy involved analysis of four QC samples in six replicates. Accuracy and precision were expressed in terms of percentage of concentration found to the nominal concentration and percentage relative standard deviation (% RSD), respectively. According to EMA guidelines, the mean concentration should be within ± 15% of the nominal concentration value for the QC sample, except for the LLOQ which should be within ± 20% of the nominal concentration value.

Intra-day and inter-day accuracy and precision in both plasma and brain was within the limits set by EMA. See Tables S3A-C below.

| Sample | Parameter | LLOQ | LQC | MQC | HQC |
|--------|-----------|------|-----|-----|-----|
| Plasma | Nominal concentration (ng/mL) | 15 | 60 | 750 | 1800 |
| Intra-day (n = 6) | Average concentration found | 14.1 | 60.8 | 730.2 | 1760.1 |
| | Accuracy (%) | 94.0 | 101.3 | 97.4 | 97.8 |
| | % RSD | 1.6 | 1.2 | 1.8 | 2.2 |
| Inter-day (n = 6) | Average concentration found | 14.5 | 59.9 | 730.9 | 1764.2 |
| | Accuracy (%) | 96.7 | 99.8 | 97.5 | 97.8 |
| | % RSD | 2.5 | 3.8 | 2.9 | 2.5 |
| Brain | Nominal concentration (ng/g) | 5 | 20 | 600 | 1500 |
| Intra-day (n = 6) | Average concentration found | 4.8 | 19.4 | 587.2 | 1482.1 |
| | Accuracy (%) | 96.0 | 97.0 | 97.9 | 98.8 |
| | % RSD | 2.8 | 1.3 | 2.5 | 1.1 |
| Inter-day (n = 6) | Average concentration found | 4.9 | 18.9 | 566.2 | 1489.5 |
| | Accuracy (%) | 98.0 | 94.5 | 94.4 | 99.3 |
| | % RSD | 3.2 | 4.7 | 3.1 | 1.8 |
**Table S3B.** Accuracy and precision for TFV in plasma and brain homogenates samples.

| Sample  | Parameter                      | LLOQ | LQC | MQC  | HQC  |
|---------|--------------------------------|------|-----|------|------|
| Plasma  | Nominal concentration (ng/mL)  | 20   | 100 | 750  | 1400 |
|         | Intra-day (n = 6)              |      |     |      |      |
|         | Average concentration found    | 20.2 | 90.5| 745.1| 1368.5|
|         | Accuracy (%)                   | 101.0| 90.5| 99.3 | 97.8 |
|         | % RSD                          | 3.1  | 1.1 | 1.8  | 4.2  |
|         | Inter-day (n = 6)              |      |     |      |      |
|         | Average concentration found    | 18.2 | 95.5| 743.1| 1387.5|
|         | Accuracy (%)                   | 91.0 | 95.5| 99.1 | 99.1 |
|         | % RSD                          | 1.5  | 2.1 | 1.6  | 2.4  |
| Brain   | Nominal concentration (ng/g)   | 2.5  | 20  | 750  | 1400 |
|         | Intra-day (n = 6)              |      |     |      |      |
|         | Average concentration found    | 2.3  | 18.4| 738.9| 1379.2|
|         | Accuracy (%)                   | 92.0 | 92.0| 98.5 | 98.5 |
|         | % RSD                          | 2.5  | 3.6 | 2.8  | 1.7  |
|         | Inter-day (n = 6)              |      |     |      |      |
|         | Average concentration found    | 2.4  | 19.3| 610.5| 1381.1|
|         | Accuracy (%)                   | 96.0 | 96.5| 99.0 | 98.7 |
|         | % RSD                          | 3.5  | 1.5 | 2.6  | 2.6  |
### Table S3C: Accuracy and precision for FTC in plasma and brain homogenates samples.

| Sample | Parameter                     | LLOQ | LQC  | MQC  | HQC  |
|--------|-------------------------------|------|------|------|------|
| Plasma | Nominal concentration (ng/mL) | 10   | 20   | 750  | 1800 |
|        | Intra-day (n = 6)             |      |      |      |      |
|        | Average concentration found   | 10.2 | 18.8 | 730.7| 1705.1|
|        | Accuracy (%)                  | 102.0| 94.0 | 97.4 | 94.7 |
|        | % RSD                         | 2.3  | 2.9  | 3.6  | 1.4  |
|        | Inter-day (n = 6)             |      |      |      |      |
|        | Average concentration found   | 9.4  | 18.5 | 738.3| 1755.4|
|        | Accuracy (%)                  | 94.0 | 92.5 | 98.4 | 97.5 |
|        | % RSD                         | 3.2  | 2.2  | 1.5  | 0.9  |
| Brain  | Nominal concentration (ng/g)  | 10   | 20   | 750  | 1800 |
|        | Intra-day (n = 6)             |      |      |      |      |
|        | Average concentration found   | 9.2  | 20.4 | 725.9| 1774.1|
|        | Accuracy (%)                  | 92.0 | 102.0| 96.8 | 98.6 |
|        | % RSD                         | 5.1  | 2.8  | 3.2  | 5.4  |
|        | Inter-day (n = 6)             |      |      |      |      |
|        | Average concentration found   | 9.8  | 20.6 | 730.3| 1801.3|
|        | Accuracy (%)                  | 98.0 | 103.0| 97.4 | 100.1|
|        | % RSD                         | 2.5  | 5.4  | 3.2  | 2.7  |
Pharmacokinetics and statistical calculations were computed using Stata 13 (StataCorp, College Station, TX). Table S4 and Table S5 show the calculated pharmacokinetic parameters in plasma and brain, respectively.

### Table S4. Plasma PK data (n = 3).

| Time (h) | EFV Conc (ng/mL) | SEM | TFV Conc (ng/mL) | SEM | FTC Conc (ng/mL) | SEM |
|----------|------------------|-----|------------------|-----|------------------|-----|
| 0        | 0                | 0   | 0                | 0   | 0                | 0   |
| 0.25     | 3246.075         | 480.5378 | 5651.718         | 672.8655 | 6470.335         | 500.5745 |
| 0.5      | 1786.029         | 170.6117 | 4042.005         | 228.6711 | 5201.166         | 818.5461 |
| 1        | 1214.336         | 245.5418 | 2535.999         | 263.1711 | 3183.055         | 413.3275 |
| 2        | 874.0536         | 190.3906 | 846.7313         | 143.3949 | 1557.083         | 238.4424 |
| 4        | 163.736          | 29.92175 | 205.7547         | 112.2346 | 445.3789         | 149.2692 |
| 6        | 148.162          | 25.68639 | 142.8576         | 24.39166 | 284.9283         | 129.9462 |
| 8        | 28.47397         | 2.057882 | 119.1999         | 25.86489 | 177.9213         | 83.53159 |
| 24       | .                | .     | .                | .     | .                | .     |

### Table S5. Brain distribution data (n = 3).

| Time (h) | EFV Conc (ng/g) | SEM | TFV Conc (ng/g) | SEM | FTC Conc (ng/g) | SEM |
|----------|-----------------|-----|-----------------|-----|-----------------|-----|
| 0        | 0               | 0   | 0               | 0   | 0               | 0   |
| 0.25     | 385.9712        | 29.34078 | 51.0665         | 29.22901 | 591.5768        | 46.28393 |
| 0.5      | 428.543         | 33.33848 | 48.3366         | 23.45138 | 342.466         | 37.6826  |
| 1        | 363.5287        | 31.04823 | 30.7281         | 16.23854 | 201.5936        | 53.42299 |
| 2        | 258.5461        | 29.34518 | 28.49667        | 19.05553 | 71.50143        | 7.45932  |
| 4        | 140.0169        | 29.40835 | 10.8512         | 2.607479 | 56.00918        | 9.056186 |
| 6        | 94.68181        | 26.3346 | .               | .     | 23.26041        | 3.289221 |
| 8        | 57.67899        | 26.76648 | .               | .     | .               | .     |
| 24       | .               | .     | .               | .     | .               | .     |
Mass spectrometry imaging (MSI)

The MALDI-MS method was optimized for the detection of the three compounds by spotting freshly prepared standards on a ground steel MALDI target plate and then on an untreated brain tissue section. Figure S4 shows the imaged standard spots of each drug from the untreated brain tissues.

**Figure S3.** MALDI-MS spectrum for EFV ($m/z$ 316.673) in brain tissue.

The MALDI-MS spectra for EFV ($m/z$ 316.673), TFV ($m/z$ 288.121) and FTC ($m/z$ 248.463) in brain tissues are shown in Figure S4, S5, and S6 respectively.
Figure S4. MALDI-MS spectrum for EFV (m/z 316.673) in brain tissue.

Figure S5. MALDI-MS spectrum for TFV (m/z 288.121) in brain tissue.
Figure S6. MALDI-MS spectrum for FTC (m/z 248.463) in brain tissue.

**LIFT MS/MS confirmation**

The precursor ions monitored for MS imaging were confirmed by LIFT MS/MS fragmentation and the resultant product ions are shown in Table S6 below.

| Analyte | Precursor ion [M+H]$^+$ | Product ions                                      |
|---------|-------------------------|---------------------------------------------------|
| EFV     | m/z 316.673             | m/z 298.640 and m/z 244.037                      |
| TFV     | m/z 288.121             | m/z 270.058, m/z 206.104 and m/z 176.253         |
| FTC     | m/z 248.463             | m/z 130.280                                       |
Table S7. Drug intensities (SUM [a.u.]) in different brain regions

| Brain region | EFV SUM [a.u.] ± SD | TFV SUM [a.u.] ± SD | FTC SUM [a.u.] ± SD |
|--------------|---------------------|---------------------|---------------------|
| CC           | 545.37 ± 61.98      | 31.15 ± 3.25        | 456.87 ±20.65       |
| HPF          | 218.10 ± 30.55      | 35.70 ± 4.67        | 97.21 ± 9.01        |
| CTX          | 1081.69 ± 185.20    | 428.81 ± 32.25      | 78.67 ± 30.67       |
| cst          | 266.53 ± 10.26      | 11.43 ± 6.08        | 175.26 ± 16.20      |
| CP           | 285.28 ± 6.00       | 167.20 ± 6.02       | 34.88 ± 12.92       |
| TH           | 578.68 ± 36.70      | 44.69 ± 10.96       | 1077.92 ± 73.34     |
| GP           | 123.82 ± 14.42      | 12.59 ± 3.60        | 28.54 ± 6.61        |
| HY           | 242.21 ± 7.77       | 10.92 ± 9.80        | 578.64 ± 49.14      |
| BFB          | 338.01 ± 24.17      | 63.17 ± 10.81       | 37.21 ± 4.12        |
| Whole brain  | 3679.69 ± 129.88    | 805.68 ± 34.50      | 2565.19 ± 91.37     |
| Whole brain (control) | 1.04 ± 0.24 | 0.34 ± 0.63 | 0.95 ± 0.14 |

Figure S7. A labeled H&E stained brain tissue section and MALDI-MS brain images (100 µm spatial resolution) of the three drugs showing their spatial distribution in different brain regions.