TITLE: Metabolite profiling and pharmacokinetic evaluation of hydrocortisone in a perfused 3D human liver bioreactor

Ujjal Sarkar, Dinelia Rivera-Burgos, Emma M. Large, David J. Hughes, Kodihalli C. Ravindra, Rachel L. Dyer, Mohammed Ebrahimkhani, John S. Wishnok, Linda G. Griffith, and Steven R. Tannenbaum.

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SUPPLEMENTAL METHOD

An RP-UHPLC-QTOF-MS/MS method was developed which enabled simultaneous determination of total and free hydrocortisone having a good separation and resolution of the chromatographic peaks. Reliability, extraction simplicity, good recovery of this RP-UHPLC-MS/MS method gives it an advantage over to the other reported methods for measuring hydrocortisone and its metabolites from hepatocytes culture media.

Method development and validation

The applicability and scope of the analytical method, e.g., the reagents, solvents, sample, standards, concentration range, equipment to be used, chromatographic conditions and calculations, were defined before validation. These included linearity, accuracy, precision, selectivity, limit of detection (LOD), limit of quantification (LOQ) and robustness.

Linearity: The linearity of the method was assessed by a series of three injections of six different concentrations of hydrocortisone (5, 10, 50, 75, 100 and 200 nM). Peak areas of the calibration LC-MS standards were plotted against their nominal concentrations (nM), yielding a linear relationship with a correlation coefficient (r^2) ≥ 0.999 (Supplemental Fig. 15).

Accuracy: The accuracy was evaluated by analyzing hydrocortisone at three different concentrations. Three replicates were made for each concentration (10, 75 and 100 nM). For this, a 50 μL aliquot of each LC-MS standard was spiked with 2 μL of the 1.25 μM d_4-HC internal standard solution and the peak areas corresponding to HC and d_4-HC

were obtained using Agilent QTOF 6530 mass spectrometer and Mass Hunter software. The percent of error obtained with this method was less than 5 % (Supplemental Table 4).

Precision: The intra-assay precision was measured by one operator using exclusively the Agilent QTOF over the course of one day. The experimental conditions were maintained as constant as possible and precision was expressed as the percent relative standard deviation of six replicates. The relative standard deviation for peak areas was 3.86, corresponding to less than 5 % variation (Supplemental Fig. 16). Also, the variation of retention times for overlapping chromatograms corresponding to these six samples was less than 2 %.

Selectivity: The selectivity was determined by comparing results from analyses of hydrocortisone samples containing impurities and degradation products with those obtained from analyses of a pure sample. A variation of less than 2 % was obtained after comparing peak heights and retention times for six replicates.

Limit of detection and limit of quantitation: The LOD of an analytical procedure is the lowest concentration of an analyte that can be detected but not necessarily quantitated; the LOQ is the lowest amount of the analyte that can be measured with suitable precision and accuracy {Armbruster, 2008 #57}. These values were determined by comparing measured signals from samples with known low concentrations of hydrocortisone with those of blank samples, and assessing the minimum concentration at which hydrocortisone could be reliably detected and measured. A S/N ratio of 3:1 was considered acceptable for estimating LOD; for LOQ a S/N ratio of 10:1 was considered appropriate. The LOD for HC was 1.4 nmol/L (1.4 femtomoles on-column for a 1 μL injection); the LOQ was 4.5 nmol/L (4.5 femtomoles on-column for a 1 μL injection).

Robustness: Robustness is the resistance of a method to modest changes in conditions {Garcia-Campana, 2000 #58}. For our assessment, we varied a number of chromatographic parameters including flow rate, column temperature, injection volume, and mobile phase. The overall influence of these variables on the peak areas and retention times was less than 10 %.

Also, as part of the validation process, the chromatographic reproducibility was assessed. Supplemental Fig. 17 shows that the method is highly reproducible based on retention time (min) and peak height.

Carryover is one of the biggest limitations of an analytical procedure and can have consequential effects in many areas where separation science is used. The issue is that unless the entire sample is removed from the analytical system the subsequent analysis will have residual compound from the previous injection, which could potentially lead to inaccurate data being produced. Supplemental Fig. 18 shows representatives chromatograms showing that the HC peak is absent in the blank runs, proving that there is no residual.

LEGENDS FOR FIGURES

Supplemental Figure 1: Quality control. A) CYP3A, B) total protein levels, C) albumin and D) urea were measured at day 7. n=3 replicates, single donor. Results are reported as mean ± SD.

Supplemental Figure 2: Anti-inflammatory effects of hydrocortisone on 10:1 cocultures in LiverChip. Cocultures were carried out in the presence of increasing concentrations of hydrocortisone for up to 8 days in culture. TNFα and IL6 were measured in coculture after 24 h stimulation with 1 µg/mL LPS. n=3 replicates, single donor. Results are reported as mean ± SD.

Supplemental Figure 3: Anti-inflammatory effects of glucocorticoids with varying potency on 10:1 cocultures in LiverChip. Cocultures were carried out in the presence of no glucocorticoid (no GC), 100 nM hydrocortisone (HC) and 100 nM dexamethasone (Dex) for up to 8 days in culture. TNFα and IL6 were measured in coculture after 24 h stimulation with 1 µg/ml LPS. n=3 replicates, single donor. Results are reported as mean ± SD.

Supplemental Figure 4: Representative chromatogram of the aqueous layer after liquid extraction. Hydrocortisone was not found which confirms that there is no loss of the analyte during the extraction process.

Supplemental Figure 5: Experimental workflow used for the analysis of glucuronides. The method consists of spiking with internal standard, solid phase extraction (SPE) and mass spectrometry analysis in negative ion mode.

Supplemental Figure 6: Sample Preparation. For technical variation assessment, each well was extracted two times and each extraction was run twice consecutively in positive ion mode in RP-UHPLC-QTOF-MS.

Supplemental Figure 7: Non-specific binding assessment. The cell-free bioreactor was exposed to 100 nM hydrocortisone for 4 hours. There was no detectable adsorption of HC to the materials of the bioreactor.

Supplemental Figure 8: Kinetics of hydrocortisone release. $ln\frac{HC}{HC_0}$ vs time post-dosing. HC clearance follows first order kinetics.

Supplemental Figure 9: Clearance rate of three donors. Donor-to-donor variability was assessed and the %RSD was ≤ 15%.

Supplemental Figure 10: Effect of LPS induced inflammation in HC clearance over time. Different concentrations of LPS were used to simulate inflammation in hepatocytes.

Supplemental Figure 11: Representative MS/MS spectra using 20 eV as the collision induced dissociation energy. A) hydrocortisone and B) cortisone**.**

Supplemental Figure 12: Characterization of metabolites using human urine as a model. Chromatogram comparison of tetrahydrocortisone found in LiverChip and in human urine. Comparison based on retention time.

Supplemental Figure 13: Characterization of metabolites using human urine as a model. Spectra comparison of tetrahydrocortisone found in bioreactor medium and in human urine. Comparison based on MS/MS pattern.

Supplemental Figure 14: Potential interconversion of tri- and tetra-deuterated hydrocortisone via labeled cortisone by 11β-HSD pathways.

Supplemental Figure 15: Regression plot. The linearity of the method was assessed by a series of three injections of six different concentration of hydrocortisone (5, 10, 75, 100 and 200 nM). n=3 replicates**.** Results are reported as mean ± SD.

Supplemental Figure 16: Precision of the HPLC-MS method used in the quantification of hydrocortisone. The intra-assay precision was measured by one operator using exclusively the Agilent QTOF over the course of one day. The precision was expressed as the percent relative standard deviation of six replicates.

Supplemental Figure 17: Chromatographic reproducibility of the LC/MS system (Agilent 1200 Infinity LC interfaced with a 6530 Quadrupole-Time-of-Flight (QTOF) mass spectrometer. Ten overlayed extracted ion chromatograms of hydrocortisone.

Supplemental Figure 18: Chromatograms of blank runs extracted for hydrocortisone. A) Blank 1, B) Blank 2 and C) Blank 3. There is no residual hydrocortisone being carrying from run to run.

TABLES

Supplemental Table 1: Number of seeded hepatocytes and Kupffer cells in cocultures in LiverChip.

	Cell Type Ratio	Hepatocytes (x 10^6)	Kupffers (x 10^6)
Co-	2.5:1	0.6	0.24
Co-	10:1	0.6	0.06
Co-	15:1	0.6	0.04

Supplemental Table 2: Hepatocytes and Kupffer cells from three different donor pairs were plated as cocultures under non-inflammed conditions and treated with 100 nM hydrocortisone.

Pair 1	**Hepatocyte**	**Kupffer**
Vendor	Life Technologies	Life Technologies
Lot Number	Hu8150	HK8160
Age (Year)	21	69
Gender	Female	Male
BMI	25	23
Pair 2	**Hepatocyte**	**Kupffer**
Vendor	Life Technologies	Life Technologies
Lot Number	Hu1583	HK8180
58	58	62
Gender	Female	Female
BMI	22	20
Pair 3	**Hepatocyte**	**Kupffer**
Vendor	Life Technologies	Life Technologies
Lot Number	Hu8179	HK8202
58	55	58
Gender	Female	Female
BMI	24	31

Supplemental Table 3: Scaling parameters used to calculate the *in vivo* intrinsic clearance ($CL_{int\text{-}in\ vivo}$)

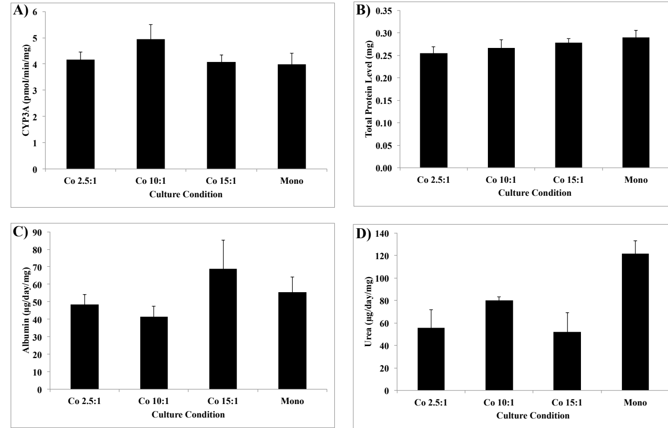
Physiological Parameter	Value	Unit
Hepatocytes per well (LiverChip)	600000	cells
Incubation volume	2.2	mL
Number of *in vivo* hepatocytes/gram	120×10^6	cells/g
Healthy human liver weight	1.8×10^3	g
Human body weight	70	kg
Human hepatic blood flow	20	mL/min/kg
Unbound fraction in blood	0.1	--

Supplemental Table 4: Accuracy assessment of the method for hydrocortisone quantification.

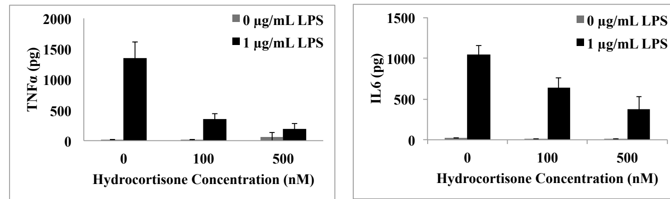
Experimental Concentration (nM)	Calculated Concentration (nM)	Percentage Error (%)
10	9.51	4.9
75	75.20	0.27
100	101.14	1.14

FIGURES

Supplemental Figure 1



Supplemental Figure 2



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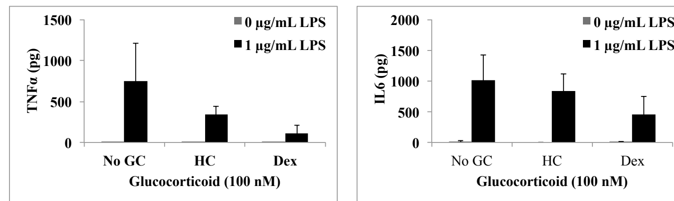

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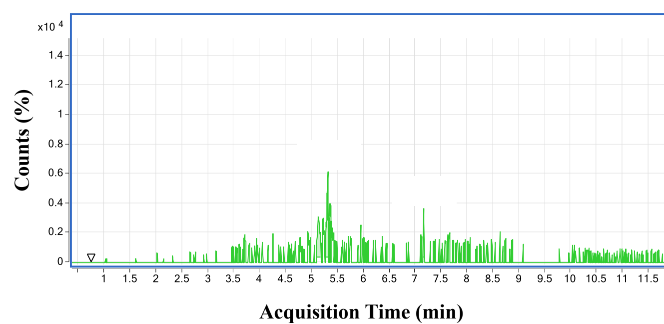

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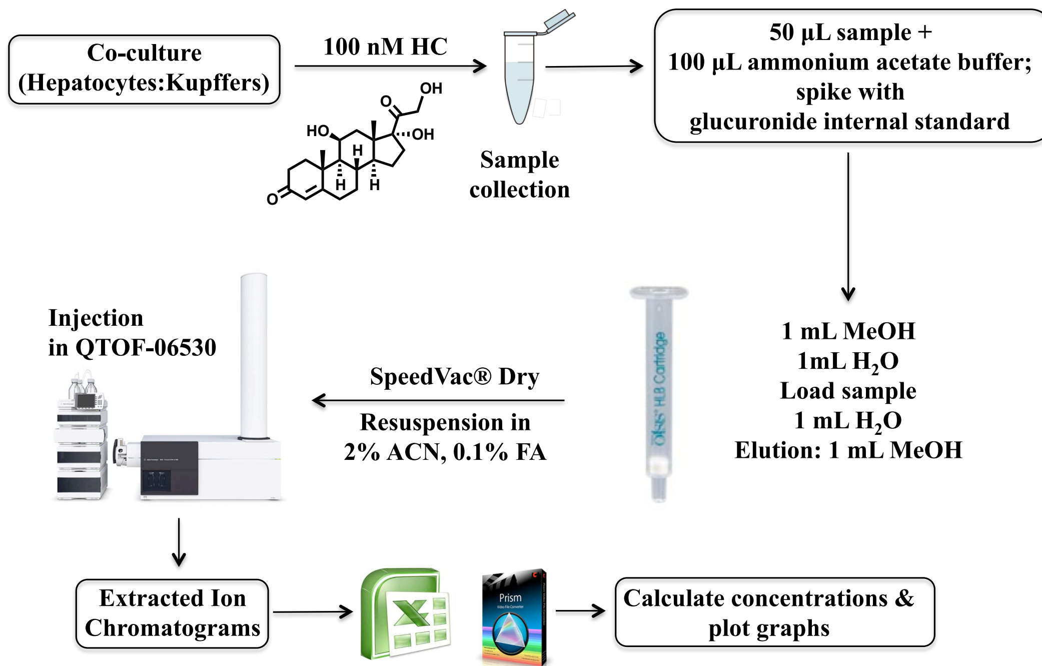

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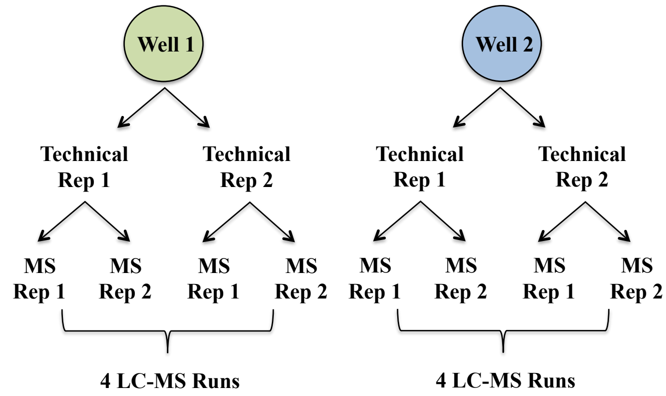

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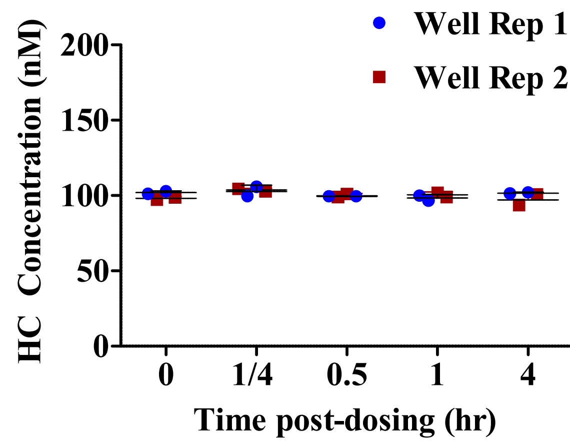

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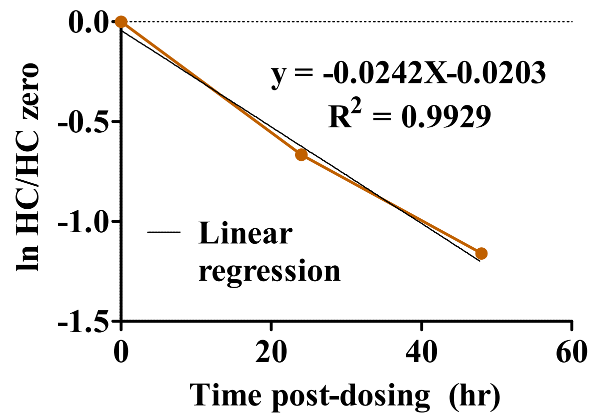

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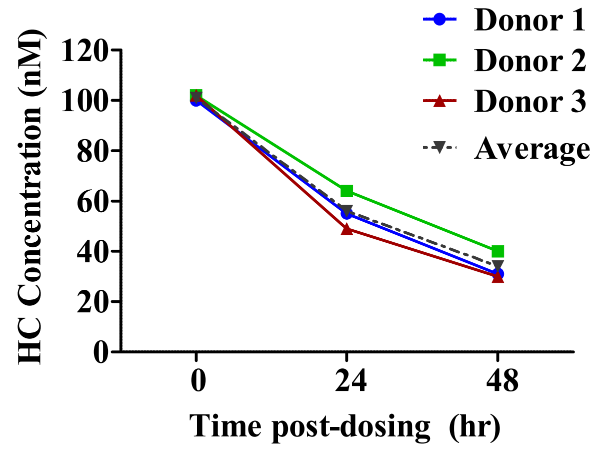

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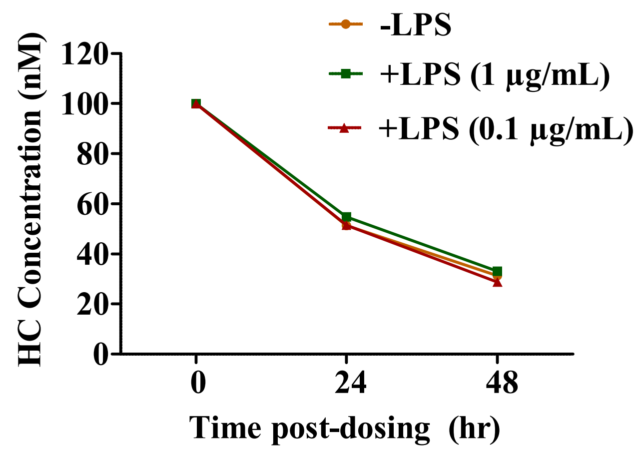

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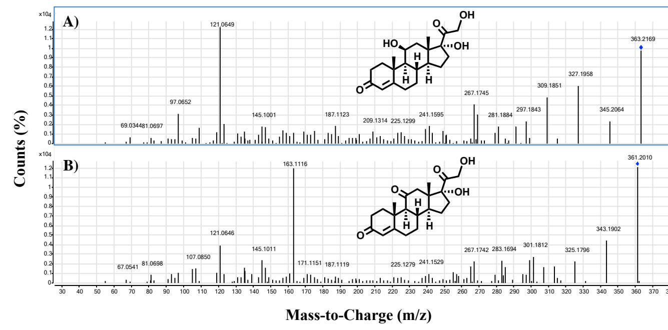

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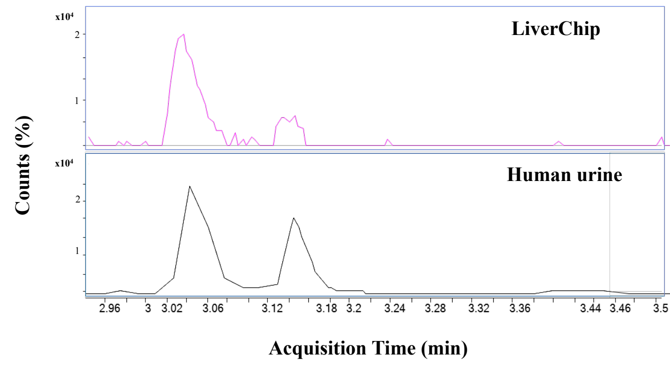

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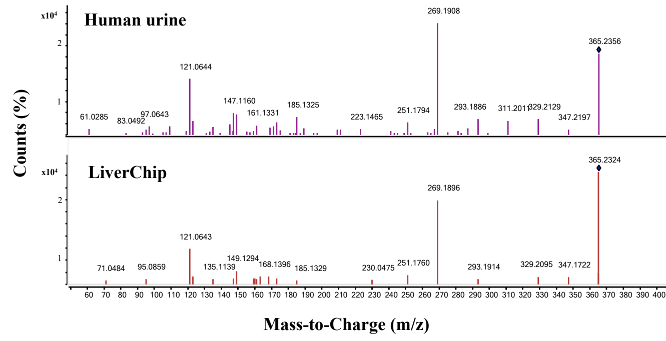

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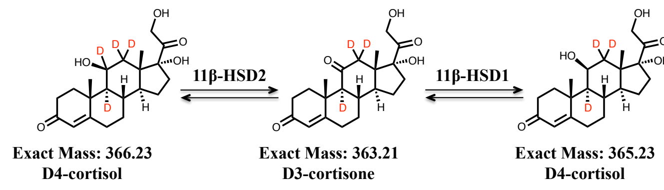

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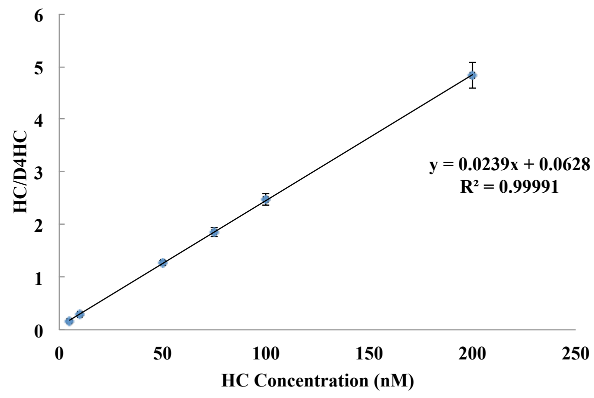

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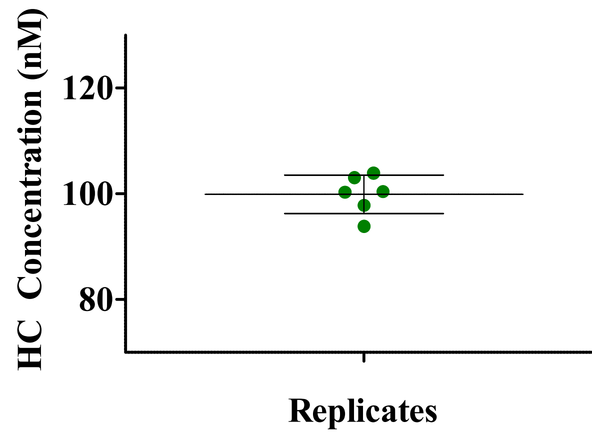

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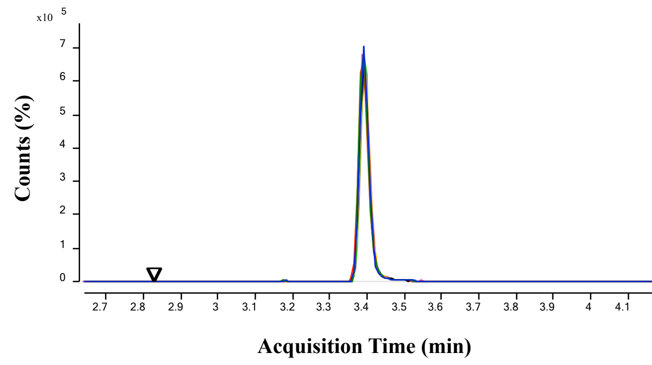

Supplemental Fig. 18