

采用高效液相色谱-高分辨质谱研究脂多糖诱导的兔炎症急性期模型代谢组学

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摘要: 本研究对新西兰白兔低剂量递增给予脂多糖(lipopolysaccharide, LPS), 诱导炎症急性反应期模型, 收集给予 LPS 前、给予 LPS 2 天和 7 天后的尿液样本, 采用超高效液相色谱-高分辨质谱(UPLC-HRMS)技术检测, 并进行非靶向代谢组学分析。利用主成分分析(PCA)和偏最小二乘法判别分析(PLS-DA)法筛选差异代谢物, 并通过 KEGG 数据库进行差异代谢通路分析。结果表明, 给予 LPS 2 天和 7 天后, 白兔尿液中的差异代谢物与给药前相比有显著差异, 分别鉴定出 41 和 161 个差异代谢物, 主要与类固醇激素生物合成通路显著相关($p < 0.01$)。炎症急性反应期发生了代谢紊乱, 类固醇激素生物合成通路上调, 这为炎症急性反应期有关靶标验证和疾病治疗提供了信息。

关键词: 代谢组学; 脂多糖(LPS); 差异代谢物; 类固醇激素; 高效液相色谱-高分辨质谱(UPLC-HRMS)

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Metabolomic Changes of Lipopolysaccharide-Induced Acute-Phase Response Rabbit Inflammation Model Using UPLC-HRMS

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Abstract: Inflammation can occur at any time in many diseases, and affect the activity of metabolism enzymes and transporters, so change the blood concentration and therapeutic effect of therapeutic drugs. Therefore, it is necessary to study the metabolic changes during the acute-phase response (APR) of inflammation. In this study, urine metabolomics was performed to investigate the possible differential metabolic pathways in New Zealand white rabbit APR inflammation model. An APR rabbit model with normal hepatic and renal function was induced by intravenous injection of lipopolysaccharide (LPS) in low-dose escalation, at 0.1, 0.2, 0.5, 1, 1.5, 2 and 2 $\mu\text{g/kg}$ from 1 to 7 days. The urine samples were collected at prior of dosing, 2-day and 7-day after the first dose of LPS, and detected using ultra-high liquid chromatography coupled with high resolution mass spectrometry

(UPLC-HRMS). The samples were separated by a Kinetex F5 column (150 mm×2.1 mm×2.6 μm) with the mobile phase of water containing 0.05% formic acid and acetonitrile containing 0.05% formic acid. ZenoTOF™ 7600 HRMS was operated under positive and negative ion modes to collect data, and the high-resolution mass spectral data was acquired by utilizing information dependent acquisition (IDA) and dynamic background subtraction (DBS). Non-targeted metabolomics analysis was completed by principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to search for differential metabolites. Then the differential metabolic pathway analysis was carried out using the metaboAnalyst 5.0 website and KEGG database. The analysis results indicated that the method has high stability and reproducibility with quality control samples clustered together in PCA. The quality control and APR model groups can be separated well by OPLS-DA, which showed that the metabolism in rabbits is significantly disturbed during APR period. Clustering heatmap analysis was performed on the screened differential metabolites which were judged by variable importance projection ($VIP>1$), significance ($p<0.05$), and folds of change ($FC>2$). The results showed differential metabolites in urine at 2-day and 7-day after LPS administration are significantly different from those before administration, 41 and 161 differential metabolites are identified in the two groups of samples, respectively. Furtherly, the differential metabolic pathway analysis revealed that the differential metabolites are significantly correlated with the steroid hormone biosynthesis pathway ($p<0.01$). Pregnenolone, 11-deoxycorticosterone, 17 α ,21-dihydroxypregnenolone, 11-deoxycortisol, dehydroepiandrosterone sulphate, 19-oxosteroids, cortisol, 16 α -hydroxyandrost-4-ene-3,17-dione, epinephrine, 19-oxoandrost-4-ene-3,17-dione, cortisone, 11 β -hydroxyprogesterone, 18-hydroxycorticosterone, 11-dehydrocorticosterone, estrone glucosinolate, and corticosterone are in an up-regulated trend on steroid hormone biosynthesis pathway. The study provides the important information for target verification and disease treatment during the APR of inflammation, and facilitates further understanding of inflammatory response mechanisms.

Key words: metabolomics; lipopolysaccharide (LPS); differential metabolites; steroids; ultra-high liquid chromatography coupled with high resolution mass spectrometry (UPLC-HRMS)

炎症是对感染、创伤或组织损伤的急性反应,清除致病性物质从而保护机体,炎症可能出现在心脑血管、糖尿病和骨质疏松症等疾病的任意时期^[1]。在炎症过程中,许多关键的转录因子被激活,如NF- κ B信号通路进一步诱导IL-6、TNF- α 等炎症因子释放;转录因子对转运体和代谢酶的调节起着关键作用^[2-4],导致体内代谢紊乱,影响药物的血药浓度和治疗作用。因此,研究炎症状态下体内的代谢改变具有重要意义。

脂多糖(LPS)是革兰氏阴性菌外膜的组成部分,进入血液后会导致全身的炎症^[5],与临床炎症相似,是经典的炎症造模药物,常被用来建立动物的炎症模型或诱导细胞炎症^[6-7]。Puris

等^[8]研究了LPS诱导小鼠全身炎症对小鼠血浆、脑皮质和海马靶向代谢组、脂质组以及参与的脑皮质蛋白表达的短期影响,发现皮质和海马中的磷脂酰胆碱和磷脂酰乙醇胺水平显著增加,脑皮质中甜菜碱水平显著降低,小鼠大脑皮层GluN1受体蛋白表达显著增加。

新西兰白兔对LPS敏感,本研究拟利用实验室前期建立的兔7天炎症急性反应模型方法^[9],在造模的第2天及第7天收集兔尿液,采用超高效液相色谱-高分辨质谱(UPLC-HRMS)法进行炎症急性反应期代谢组学研究,分析差异代谢通路,以寻找急性炎症反应期的作用靶点,解释其对药代动力学的影响机制,希望为炎症期间疾病的治疗提供信息。

1 实验部分

1.1 主要仪器与装置

AB SCIEX ZenoTOF™ 7600 高分辨质谱仪: 美国 AB Sciex 公司产品; Acquity UPLC 液相色谱系统: 美国 Waters 公司产品; Centrifuge 5425 高速冷冻离心机: 德国 Eppendorf 公司产品。

1.2 主要试剂

乙腈(色谱纯): 德国 Merck 公司产品; 甲酸(色谱纯): 德国 Sigma 公司产品; 超纯水: 杭州娃哈哈集团有限公司产品; 脂多糖: 美国 Sigma 公司产品, 以生理盐水作为溶剂。

1.3 动物实验及样品收集

8 只新西兰兔: 购自绿叶制药有限公司实验动物中心, 体重 3~4 kg, 常规饲养于 SPF 级动物房, 自由进食进水。炎症急性反应期模型采用耳缘静脉注射递增剂量 LPS, 第 1~7 天给予 LPS 剂量分别为 0.1、0.2、0.5、1、1.5、2、2 $\mu\text{g/kg}$, 给药体积为 0.5 mL/kg。在给予 LPS 前、给予 LPS 2 天、给予 LPS 7 天进行 12 h 尿液收集(早晨 8:00~晚上 8:00), 分别作为空白组(B)、造模 2 天组(T)和造模 7 天组(S)。将尿液样本在 4 $^{\circ}\text{C}$ 以 3 000 r/min 离心 10 min, 取上清液于 Eppendorf (EP)管内, -80 $^{\circ}\text{C}$ 冰箱冻存, 待后续分析。检测前, 在 4 $^{\circ}\text{C}$ 冰箱解冻尿液样品, 于 4 $^{\circ}\text{C}$ 以 13 500 r/min 离心 10 min, 取上清液进行检测。所有样品取等体积上清液制成 6 个质控(QC)样品, 在相同条件下穿插在样品分析过程中检测。

1.4 实验条件

1.4.1 色谱条件 Kinetex F5 色谱柱(150 mm $\times$ 2.1 mm $\times$ 2.6 μm); 流动相: A 为 0.05%甲酸-水溶液, B 为 0.05%甲酸-乙腈溶液; 梯度洗脱程序: 0~1.5 min(0%~2%B), 1.5~3 min(2%~25%B), 3~13 min(25%~60%B), 13~15 min(60%~95%B), 15~17 min(95%B), 17~17.1 min(95%~2%B); 流速 0.3 mL/min, 柱温 35 $^{\circ}\text{C}$, 进样体积 1 μL 。

1.4.2 质谱条件 采用正、负离子扫描模式, 雾化气压强 379 kPa, 辅助气压强 379 kPa, 气帘气压强 206 kPa, 离子源温度 550 $^{\circ}\text{C}$, 喷雾电压 5 000 V(正离子模式)/-4 500 V(负离子模式), 去簇电压 60 V, 碰撞能量(35 $\pm$ 15) eV, 质量扫描范

围 m/z 60~1 000。通过信息依赖采集(IDA)和动态背景扣除(DBS)功能获得高分辨质谱数据。

1.5 数据处理

利用 R 语言软件包对原始谱图进行峰提取, SCIEX OS 软件的本地数据库和 MetDNA 网络数据库对二级质谱数据进行匹配。使用 MarkerView™软件进行主成分分析(PCA)和偏最小二乘法判别分析(PLS-DA), 得到变量重要性投影(VIP)值、显著性(p)和差异倍数(FC)值。利用 metaboAnalyst 5.0(<https://www.metaboanalyst.ca/>)进行代谢通路分析, 使用 KEGG 数据库筛选出具有显著差异的代谢通路。

2 结果与讨论

2.1 数据质量评价

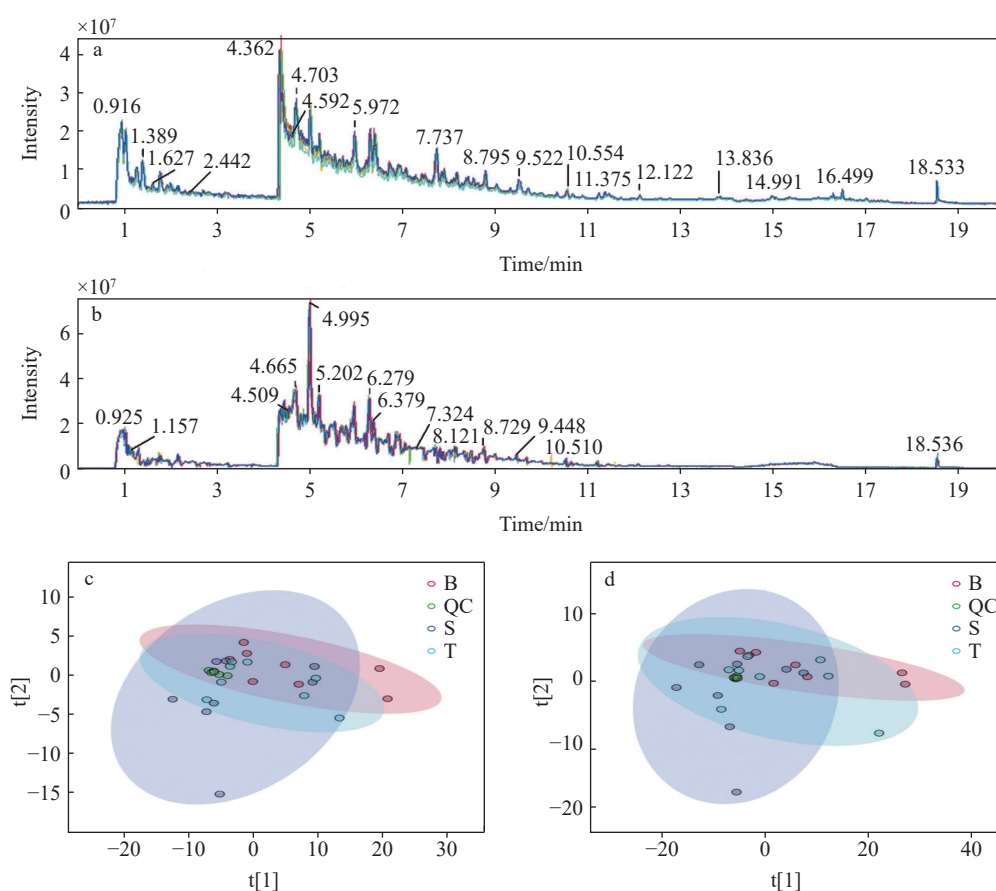
在分析批数据采集过程中穿插 QC 样品测定, 正、负离子模式下, QC 样品的保留时间和信号强度的重现性良好, 示于图 1a、1b。对待测样本及 QC 样品进行 PCA 分析, 结果示于图 1c、1d, 可以看出, QC 均聚集在一起, 表明数据稳定性和重现性较好。

2.2 代谢组学多元统计分析

排除 QC 并对 B、S、T 组样本的数据进行 PCA 分析, 结果表明, 在正、负离子模式下, 各组间分类趋势不明显, 示于图 2a、2b, 其原因是生物样本组内、组间存在变异, 仅通过 PCA 难以得到显著的组间分类效果。为了使组间差异更显著, 使用 PLS-DA 进行分析, 结果示于图 2c、2d。可见, 造模 2 天组和造模 7 天组与空白组对比均分类明显。PLS-DA 对造模组和空白组的区分能力优于 PCA, 有助于发现 LPS 诱导兔炎症急性模型体内的代谢特性变化。

2.3 差异代谢物的筛选和鉴定

对空白组和造模组进行差异代谢物分析, 差异代谢物筛选标准为同时满足 $VIP > 1$ 、 $p < 0.05$ 和 $FC > 2$ 。与空白组相比, 造模 2 天组尿液中共发现 41 个差异代谢物, 其中正、负离子模式分别为 22、19 个; 造模 7 天组共发现 161 个差异代谢物, 其中正、负离子模式分别为 53、108 个。通过聚类热图分析差异代谢物, 结果示于图 3, 发现造模 2 天组和造模 7 天组与空白组之间存在聚类相关性, 且代谢物在造模组中高



注: a.c. 正离子模式; b.d. 负离子模式

图1 QC样本的总离子流图(a, b)和QC、空白组、造模2天组和造模7天组PCA图(c, d)
Fig. 1 Total ion chromatograms of quality control (QC) sample (a, b), PCA plots of QC, blank, 2-day and 7-day modeling groups (c, d)

表达。

2.4 代谢通路分析

将筛选得到的差异代谢物进行代谢通路分析,具有显著性差异($p < 0.01$)的代谢通路主要为类固醇激素生物合成(steroid hormone biosynthesis)通路,在造模2天组和7天组, p 值均小于0.01;而其他代谢通路差异并不明显,示于图4。与空白组相比,在类固醇激素生物合成通路上,造模2天组和7天组中分别有15、14个代谢物处于上调趋势,结果列于表1。

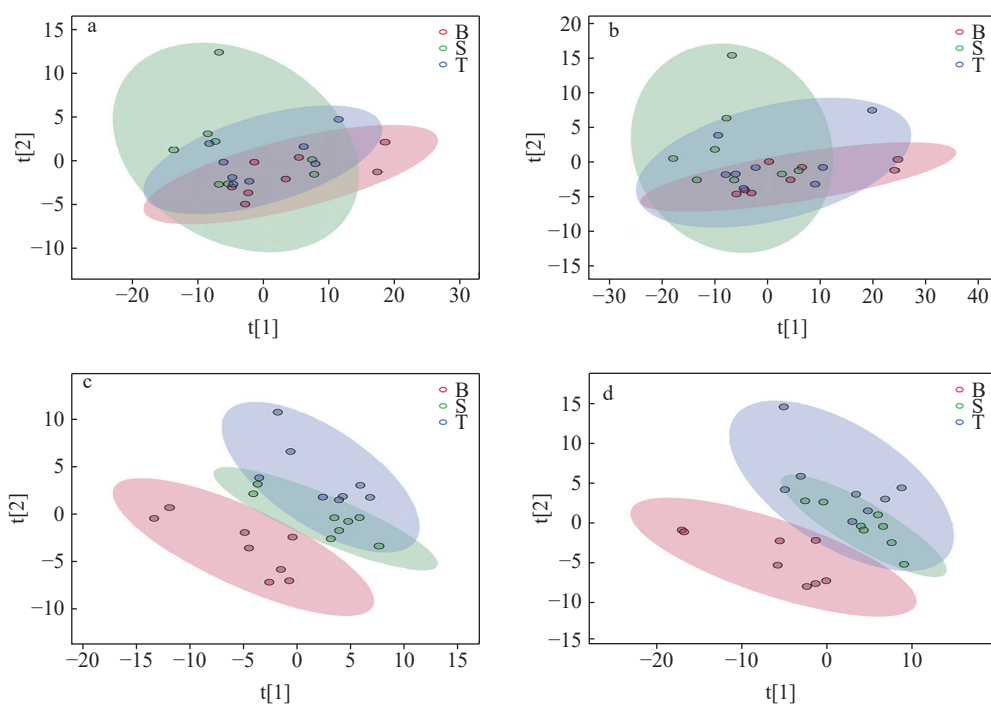
2.5 给予LPS后兔体内的作用机制分析

采用低剂量递增注射LPS方法建立新西兰白兔炎症急性反应期模型,在此期间,新西兰白兔的肝肾功能正常^[9],能很好地模拟临床炎症急性反应期状态。通过以上研究发现,类固醇激素生物合成为兔炎症期间的主要差异代谢通路。

类固醇激素通过肾上腺皮质、性腺和胎盘

生成^[10-11]。胆固醇可以生成孕烯醇酮,是类固醇激素生物合成的前体^[12]。孕烯醇酮通过抑制破骨细胞的形成来保护小鼠免受LPS诱导的体内炎症性骨破坏^[13]。硫酸脱氢表雄酮和孕烯醇酮可以抑制LPS诱导的肿瘤坏死因子(TNF)的产生^[14]。皮质醇具有抗炎作用^[15]。实验表明,给药LPS后,尿液中硫酸脱氢表雄酮、孕烯醇酮和皮质醇均出现显著升高,可能是机体自发性调节LPS给药后带来的炎症反应。

类固醇激素代谢主要通过细胞色素P450(CYP450)酶和羟基类固醇脱氢(HSD)酶完成^[16-17]。当机体产生炎症时,CYP450和HSD会参与合成类固醇激素以达到抗炎目的,同时,CYP450酶的活性会受到炎症因子的调控^[18]。该研究提供了急性炎症期有关靶标的验证,同时在候选药物的测试期间为其提供使用急性炎症模型的重要信息。



注: a.c. 正离子模式; b.d. 负离子模式

图2 空白组、造模2天组和造模7天组在正、负离子模式下的PCA图(a, b)和PLS-DA图(c, d)

Fig. 2 PCA (a, b) and PLS-DA (c, d) plots of blank, 2-day and 7-day modeling groups under positive and negative ion modes

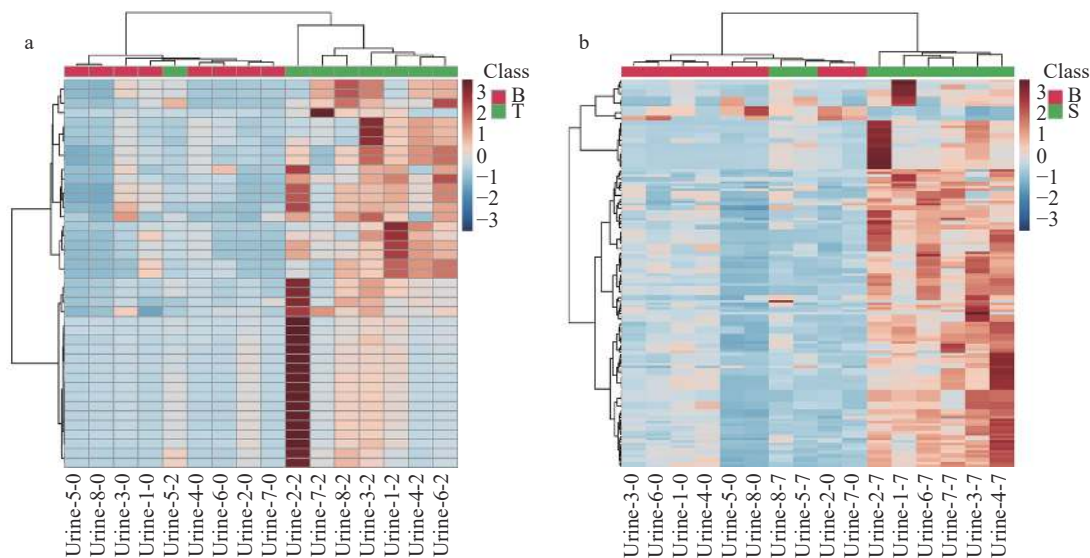


图3 造模2天组(a)和造模7天组(b)差异代谢物的聚类分析热图

Fig. 3 Clustering heat maps of differential metabolites in 2-day (a) and 7-day (b) modeling groups

3 结论

本研究采用UPLC-HRMS法对LPS诱导的兔炎症急性反应期模型进行尿液非靶向代谢组学研究,在造模2天组和造模7天组分别发现41和161个差异代谢物,主要为类固醇激素生物

合成通路。孕烯醇酮等14个代谢物在类固醇激素生物合成通路处于上调趋势,这为炎症急性反应期有关靶标验证和疾病治疗提供了重要信息,有利于进一步理解炎症反应机制及候选药物评价。

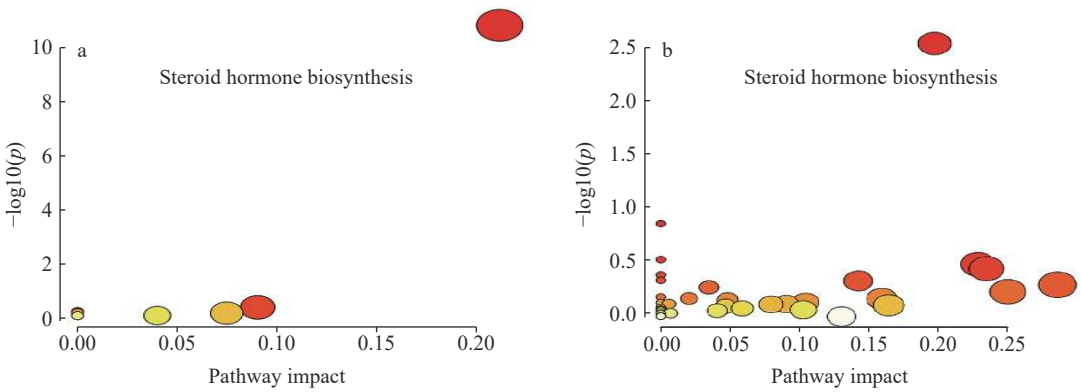


图 4 造模 2 天组(a)和造模 7 天组(b)的代谢通路分析
Fig. 4 Metabolic pathway analysis of 2-day (a) and 7-day (b) modeling groups

表 1 类固醇激素生物合成通路的差异代谢物

Table 1 Differential metabolites of steroid hormone biosynthesis pathway

分组 Group	代谢物 Metabolite	分子式 Molecular formula	准分子离子 Quasi-molecular ion	质荷比 Mass charge ratio (<i>m/z</i>)	特征碎片离子 Characteristic fragment ion (<i>m/z</i>)	差异 倍数 <i>FC</i>	显著性 <i>p</i>
造模 2天	孕烯醇酮	C ₂₁ H ₃₂ O ₂	[M+H] ⁺	317.25	281.22, 159.10	3.63	0.003
	11-脱氧皮质酮	C ₂₁ H ₃₀ O ₃	[M+H] ⁺	331.23	109.06, 97.06	3.60	0.024
	17 α , 21-二羟基孕烯醇酮	C ₁₁ H ₃₂ O ₄	[M+H] ⁺	349.24	281.22	3.14	0.047
	11-脱氧皮质醇	C ₂₁ H ₃₀ O ₄	[M-H] ⁻	345.21	327.21, 121.06	5.09	0.015
	硫酸脱氢表雄酮	C ₁₉ H ₂₈ O ₅ S	[M+CH ₃ COOH-H] ⁻	427.18	97.30	18.6	<0.001
	19-氧代甾酮	C ₁₉ H ₂₆ O ₃	[M+CH ₃ COOH-H] ⁻	361.20	117.46	8.26	0.003
	皮质醇	C ₂₁ H ₃₀ O ₅	[M+H] ⁺	363.22	327.20, 121.06	6.38	<0.001
	16 α -羟基雄甾-4-烯-3,17-二酮	C ₁₉ H ₂₆ O ₃	[M+CH ₃ COOH-H] ⁻	361.20	173.99	8.36	0.003
	肾上腺素	C ₁₉ H ₂₄ O ₃	[M+CH ₃ COOH-H] ⁻	359.19	121.20	8.15	0.003
	19-氧代雄甾-4-烯-3,17-二酮	C ₁₉ H ₂₄ O ₃	[M+CH ₃ COOH-H] ⁻	359.19	169.60	8.15	0.003
	可的松	C ₂₁ H ₂₈ O ₅	[M+H] ⁺	361.20	163.11, 121.06	8.38	0.002
	11 β -羟孕酮	C ₂₁ H ₃₀ O ₃	[M+H] ⁺	331.23	313.22, 121.06	3.60	0.024
	18-羟基皮质酮	C ₂₁ H ₃₀ O ₅	[M+H] ⁺	363.22	191.01	6.38	<0.001
	11-脱氢皮质酮	C ₂₁ H ₂₈ O ₄	[M+H] ⁺	345.21	327.14	7.14	<0.001
	雌酮葡萄糖苷酸	C ₂₄ H ₃₀ O ₈	[M-H ₂ O+H] ⁺	427.18	269.13	19.1	<0.001
造模 7天	孕烯醇酮	C ₂₁ H ₃₂ O ₂	[M+H] ⁺	317.25	281.22, 159.10	3.31	0.028
	17 α , 21-二羟基孕烯醇酮	C ₁₁ H ₃₂ O ₄	[M+H] ⁺	349.24	281.22	2.44	0.049
	11-脱氧皮质醇	C ₂₁ H ₃₀ O ₄	[M+H] ⁺	347.22	329.21, 119.06	2.90	0.021
	硫酸脱氢表雄酮	C ₁₉ H ₂₈ O ₅ S	[M+CH ₃ COOH-H] ⁻	427.18	97.30	35.3	<0.001
	19-氧代甾酮	C ₁₉ H ₂₆ O ₃	[M+CH ₃ COOH-H] ⁻	361.20	117.46	18.7	<0.001
	皮质醇	C ₂₁ H ₃₀ O ₅	[M+H] ⁺	363.22	327.20, 121.06	15.3	<0.001
	16 α -羟基雄甾-4-烯-3,17-二酮	C ₁₉ H ₂₆ O ₃	[M+CH ₃ COOH-H] ⁻	361.20	173.99	18.7	<0.001
	肾上腺素	C ₁₉ H ₂₄ O ₃	[M+CH ₃ COOH-H] ⁻	359.19	121.20	14.4	<0.001
	19-氧代雄甾-4-烯-3,17-二酮	C ₁₉ H ₂₄ O ₃	[M+CH ₃ COOH-H] ⁻	359.19	169.60	14.4	<0.001
	可的松	C ₂₁ H ₂₈ O ₅	[M+H] ⁺	361.20	163.11, 121.06	14.5	<0.001
	皮质酮	C ₂₁ H ₃₀ O ₄	[M+H] ⁺	347.22	109.06, 97.06	2.90	0.021
	18-羟基皮质酮	C ₂₁ H ₃₀ O ₅	[M+H] ⁺	363.22	191.01	15.3	<0.001
	11-脱氢皮质酮	C ₂₁ H ₂₈ O ₄	[M+H] ⁺	345.21	327.14	14.4	<0.001
	雌酮葡萄糖苷酸	C ₂₄ H ₃₀ O ₈	[M-H ₂ O+H] ⁺	427.18	269.13	36.4	<0.001

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