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高效液相法同时测定大鼠脑组织中积雪草苷和羟基积雪草苷浓度

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摘要 目的：建立高效液相色谱（HPLC）同时测定脑组织中积雪草苷和羟基积雪草苷浓度的方法。**方法：**大鼠尾静脉注射积雪草苷、羟基积雪草苷和积雪草总苷，30 min 后用生理盐水进行心脏灌注，去除脑组织中残留血液，取脑组织，经甲醇沉淀蛋白后，利用 HPLC 测定脑组织中积雪草苷和羟基积雪草苷浓度。**结果：**积雪草苷和羟基积雪草苷在 0.5-12 μg/mL 线性关系良好，定量下限为 0.5 μg/mL，日内和日间精密度（RSD）均小于 15%，准确度（RE）在 -5.25%－9.16%，提取回收率在 70.54%－102.61%。**结论：**该方法符合生物样品测定要求，可以同时测定大鼠脑组织中积雪草苷和羟基积雪草苷的浓度。

关键词：积雪草苷；羟基积雪草苷；含量测定；高效液相；脑组织分布

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Simultaneous Determination of Asiaticoside and Madecassoside in Rat Brain by HPLC

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ABSTRACT Objective: To establish a determination method of asiaticoside and madecassoside concentration in rat brain by HPLC. **Methods:** After the rat caudal vein injection of total triterpene saponin of Centella asiatica, asiaticoside and madecassoside for 30 minutes, brain tissue was obtained by conducting the heart perfusion with physiological saline to remove residual blood in the brain. The brain tissue was extracted by methanol, then detected by HPLC for both asisticoside and madecassoside concentration. **Results:** The linear range was 0.5-12 μg/mL for asiaticoside and madecassoside. The lower limit of quantification was 0.5 μg/mL; The intra-day and inter-day variations of RSD were less than 15%, with relative error (RE) ranging from -5.25% to 9.16% and the extraction recovery ranging from 70.54% to 102.61%. **Conclusion:** This method can satisfy the requirement of biological determination of drugs and could be applied to concentration determination of asiaticoside and madecassoside.

Key words: Asiaticoside; Madecassoside; Content determination; HPLC; Brain drug distribution

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前言

现代研究表明积雪草(Centella Asiatica L.)在焦虑、抑郁、帕金森和阿兹海默症等中枢神经方面具有药理作用[1]，积雪草的乙醇 - 水（1:1）提取物可以降低阿茨海默症动物模型病变组织中的 β- 淀粉含量[2]，对神经退变性疾病如帕金森具有保护作用[3]，其甲醇、乙酸乙酯提取物则有抗焦虑作用[4]。积雪草苷(O-6-Deoxy-α-L-mannopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl (2α, 3β, 4α)-2, 3, 23-trihydroxyurs-12-en-28-oate, asiaticoside, AS, C48H78O19, 图 1.)、羟基积雪草苷 O-6-Deoxy-α-L-mannopyranosyl- (1→4)-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl (2α, 3β, 4α, 6β)-2, 3, 6, 23-tetrahydroxyurs-12-en-28-oate，madecassoside, MA, C48H78O20, 图 1)是从积雪草[5-7]全草提取得到的三萜皂苷类成分，为该植物的主要药效成分。动物实验表明，积雪草苷对脑缺血再灌注引起的炎症有保护作用[8-10]，对脊髓损伤[11]、阿兹海默症[12,13]、癌症[14]、皮肤烧伤[15]、认知障碍[16]，抑郁焦虑[17,18]和帕金森症[19]等均有效。羟基积雪草苷同样在阿兹海默症、认知障碍和帕金森症等方面具有确切药理活性[20-22]。为进一步研究积雪草中枢神经活性和脑组织分布关系，有必要研究积雪草苷、羟基积雪草苷吸收入脑情况。虽有文献报道脑组织中积雪草苷浓度的测定方法[23]，但是未见同时测定脑组织中积雪草苷和羟基积雪草苷浓度的方法。本文首次报告 HPLC 同时测定大鼠脑组织中积雪草苷和羟基积雪草苷浓度的方法。为同时研究二者在脑内药物动力学，探索药物脑组织分布与抗抑郁活性之间的关系奠定基础。

1 材料与方法

1.1 仪器与试药

1.1.1 **实验仪器** Agilent 1200 型高效液相色谱仪系统：Agilent 1200 四元泵（G1311A），自动进样阀（G1329A），Chemstation6.01 色谱工作站，柱温箱（G1316A），检测器：Agilent 1200 二极管阵列检测器 DAD(G1315D)。超纯水仪(Millipore, Milford, MA, USA)。

1.1.2 **药品与试剂** 积雪草总苷（积雪草苷 15%、羟基积雪草

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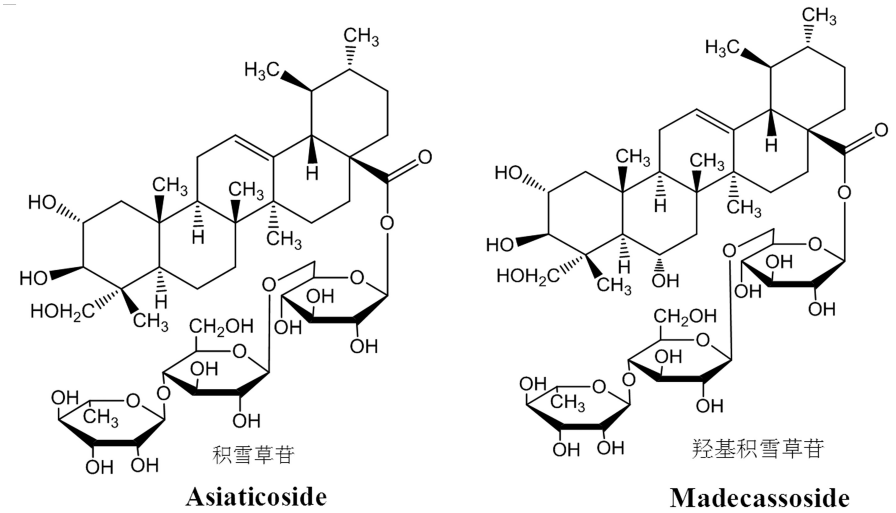

图 1 积雪草苷、羟基积雪草苷结构式

Fig.1 The chemical structure of asiaticoside and madecassoside

苷 32%)、积雪草苷(纯度为 90%,实验室自制)、羟基积雪草苷(纯度为 90%,实验室自制)、积雪草苷对照品(纯度大于 98%,购于阿拉丁试剂公司)、羟基积雪草苷对照品(纯度大于 98%,购于阿拉丁试剂公司)、酸枣仁皂苷 A (纯度大于 98%,购于阿拉丁试剂公司)、乙腈(色谱纯)、甲醇(色谱纯)、生理盐水,超纯水。

1.1.3 **实验动物** SD 大鼠 40 只(200 ± 10) g,上海斯莱克实验动物有限公司,清洁级饲养,恒温净化通风动物房,自由进食与饮水,(25 ± 2)℃,相对湿度 50%～70%。

1.2 **方法**

1.2.1 **色谱条件** Agilent TC-C18 (4.6 × 150 mm,5 μm);流动相:水(A)- 乙腈(B);梯度洗脱程序:0~15 min,20%~46% B;15~16 min,46%~100% B;16~21 min,100% B;21~22 min,100% ~20% B;22~27 min,20% B;柱温 25℃,检测波长 205 nm;流速 1.0 mL/min ;进样量 10 μL。

1.2.2 **对照品溶液配制** 取积雪草苷、羟基积雪草苷对照品各 5.0 mg 精密称定,置 25 mL 的容量瓶中,甲醇溶解定容,得 200 μg/mL 的对照品溶液;依次稀释配制成 120 μg/mL、80 μg/mL、36 μg/mL、24 μg/mL、15 μg/mL、9 μg/mL、3 μg/mL、1.5 μg/mL 对照品溶液,置 4℃冰箱保存。

1.2.3 **内标溶液配制** 取酸枣仁苷 A 2.0 mg 精密称定,置 10 mL 容量瓶中,甲醇溶解定容。得 200 μg/mL 的内标溶液,依次稀释配制成 15 μg/mL、3 μg/mL,置 4℃冰箱保存。

1.2.4 **供试品溶液配制** 取积雪草苷、羟基积雪草苷各 10 mg ,精密称定,置 10 mL 量瓶中,37 ℃恒温水浴中超声 15 min,注射用生理盐水定容,分别配成 1.0 mg /mL 的溶液。供大鼠尾静脉注射使用。同法配制积雪草总苷溶液,浓度为 10 mg/mL。现配现用。

1.2.5 **脑组织获取和处理** (1) 空白脑匀浆液制备:取大鼠 31 只,适应一周。实验前禁食 24 h,经麻醉,打开胸腔,生理盐水心脏灌注后取脑组织(图 2)。锡箔纸包裹,放入液氮中冷冻 5 min 后,称重后放入玻璃研磨器中加入 2 倍量的生理盐水研磨。(2) 脑匀浆液处理:取脑匀浆液 0.3 mL,加 0.6 mL 甲醇,涡旋 2.0 min,离心(14000 r/min) 25 min。取上清液置 1.5 mL EP 管中,于室温轻柔氮气吹干,残渣用 100 μL 甲醇复溶,涡旋 2.0 min,离心(14000 r/min)25 min ,取上清液 10 μL 进样。记录积雪草苷、羟基积雪草苷和内标的峰面积,采用内标标准曲线法以峰面积比值定量。

2 结果

2.1 **方法学考察**

2.1.1 **专属性考察** 在本试验条件下,积雪草苷和羟基积雪草

图 2 脑组织未灌注(左)、灌注后的脑组织(右)

Fig.2 Brain tissues without perfusion (left), and with perfusion (right)

苷有较强的色谱峰和较好的分离度，脑组织中杂质峰和代谢物峰不干扰样品的测定，积雪草苷、羟基积雪草苷和内标酸枣仁苷 A 的保留时间分别为 10.2、8.7 和 14.2 min (图 3)，本方法具有较高的专属性，可以作为积雪草苷和羟基积雪草苷在脑组织中的测定方法。

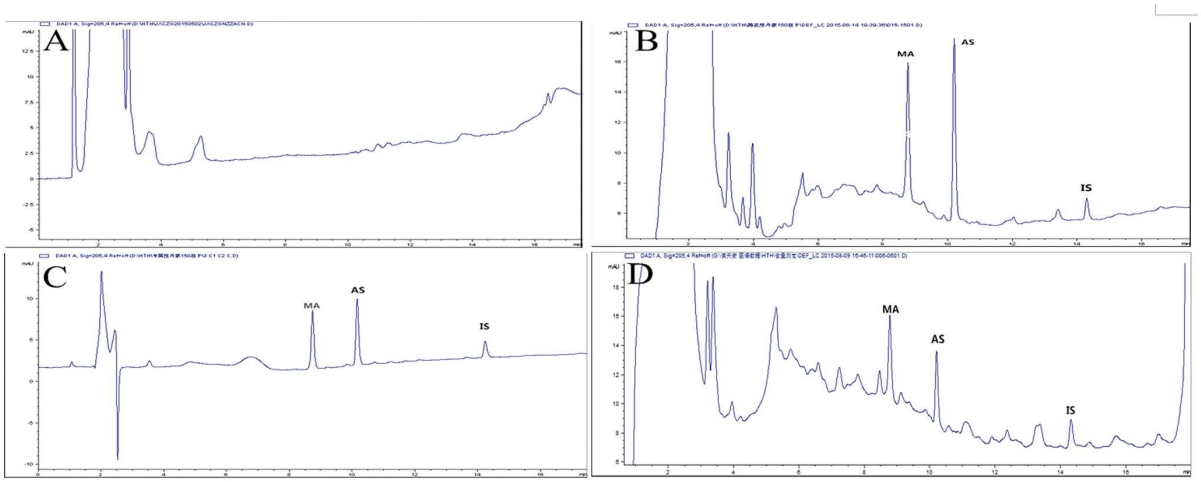

图 3 空白脑匀浆液(A)、空白脑匀浆液 + 对照品 + 内标(B)、对照品 + 内标(C)、含药脑匀浆液(D)HPLC 色谱图

Fig.3 HPLC chromatogram of rats blank brain tissue (A), reference standard in the blank brain tissue (B). reference standard(C) and administration (D)

2.1.2 **标准曲线及线性范围** 取 30 个 1.5 mL 的 EP 管，按 "1.2.5" 项下操作。在脑匀浆液处理之前加入积雪草苷对照品、羟基积草苷对照品和内标，使积雪草苷、羟基积雪草苷的终浓度均为：0.5、1.0、3.0、5.0、8.0、12.0 μg/mL，进样分析。以积雪草苷、羟基积雪草苷脑组织浓度为横坐标，积雪草苷、羟基积雪草苷峰面积与内标峰面积比值为纵坐标，得积雪草苷、羟基积雪草苷的线性回归直线方程分别为：Y=0.4363X+0.1131 (R^2 = 0.9992)、Y=0.3940X+0.0341 (R^2 = 0.9984)（图 4）。结果表明积雪草苷、羟基积雪草苷在 0.5~12 μg/mL 线性良好，定量下限(LLOQ)均为 0.5 μg/mL。

2.1.3 **准确度和精密度** 按 2.1.2 项下操作，制备积雪草苷、羟基积雪草苷浓度分别为 1.0 μg/mL、8.0 μg/mL、12.0 μg/mL 的质控样品(QC)，用当日的随行标准曲线测定 QC 样品浓度，每个浓度 5 个样本，连续测定 3 天，计算日内和日间精密度(RSD)和准确度(RE)。结果见表 1。

2.1.4 **提取回收率** 按 2.1.2 项下操作，制备积雪草苷、羟基积雪草苷终浓度分别为 1.0 μg/mL、8.0 μg/mL、12.0 μg/mL 的质控样品(QC)，记录积雪草苷、羟基积雪草苷的峰面积和内标酸枣仁苷 A 的峰面积，计算样品峰面积与内标峰面积比值 X1，每个浓度 5 个样本；同法取空白脑组织匀浆 0.3 mL，按照按 "(2)"

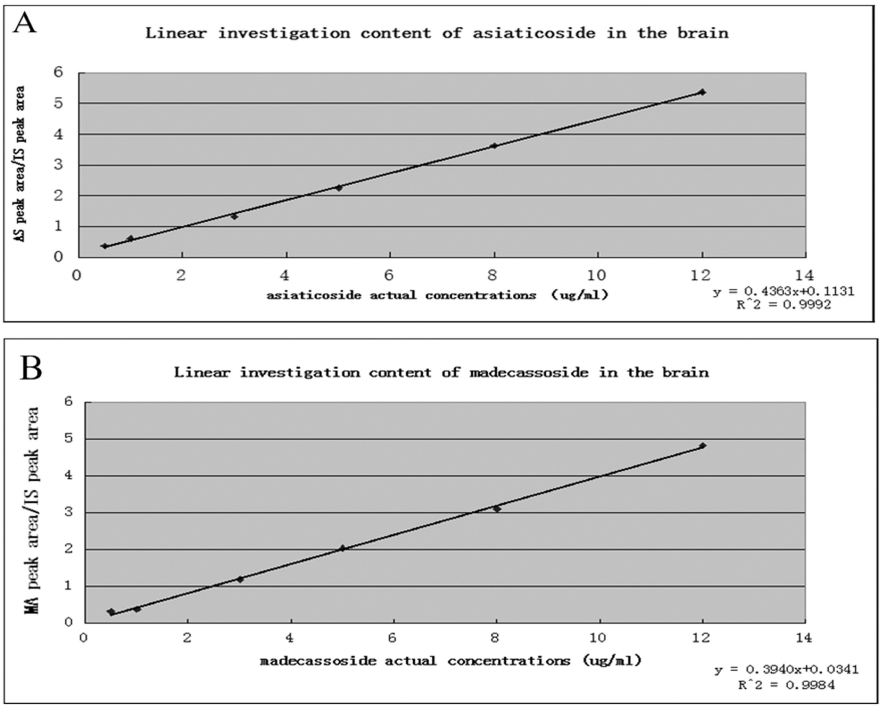

图 4 积雪草苷(A)、羟基积雪草苷(B)线性回归

Fig.4 The line regression of AS(A)and MA(B)

表 1 大鼠脑匀浆液积雪草苷、羟基积雪草苷浓度日、日间精密度和准确度(n = 6)

Table 1 The intra- and inter-day precisions and accuracies of AS, MA and IS in rat brain (n = 6)

Compound		Spliked μg/mL	Measured (mean ± SD) μg/mL	(RE%)	(RSD%)
AS	Intra-day	1	0.96 ± 0.11	-4.00	11.46
		8	7.58 ± 0.31	-5.25	4.09
		12	12.32 ± 0.73	2.67	5.93
	Intra-day	1	1.01 ± 0.11	1.00	10.89
		8	7.89 ± 0.43	-1.38	5.45
		12	12.35 ± 0.47	3.25	3.80
MA	Intra-day	1	1.08 ± 0.06	8.00	5.56
		8	7.78 ± 0.42	-2.75	5.80
		12	12.17 ± 0.45	1.42	3.70
	Inter-day	1	1.09 ± 0.08	9.16	7.68
		8	8.43 ± 0.78	5.37	9.25
		12	12.04 ± 0.87	8.67	8.49

项下操作,向获得的残渣中加入 100 μL 甲醇(内含积雪草苷、羟基积雪草苷、内标)复溶,使其终浓度与 QC 相同。记录积雪草苷、羟基积雪草苷的峰面积和内标酸枣仁苷 A 的峰面积,计算样品峰面积与内标峰面积比值为 X2,每个浓度 5 个样本。以 X1/X2 的比值作为积雪草苷、羟基积雪草苷的提取回收率。结果见表 2。

表 2 积雪草苷、羟基积雪草苷、内标在脑组织中提取回收率(n = 6)

Table 2 The recoveries of AS, MA and IS from rat brain (n = 6)

Compound		Recoveries (%)		
		1 μg/mL	8 μg/mL	12 μg/mL
AS	Measured (mean ± SD)	102.61 ± 13.51	88.01 ± 9.02	93.29 ± 2.31
	RSD%	13.16	10.25	2.48
MA	Measured (mean ± SD)	70.54 ± 3.07	92.07 ± 9.68	95.83 ± 8.43
	RSD%	4.35	10.52	8.79
IS	Measured (mean ± SD)	72.65 ± 9.11	91.27 ± 8.81	83.69 ± 7.83
	RSD%	12.53	9.65	9.36

2.1.5 **稳定性考察** 质控样品(QC),每个浓度 5 个样品,-80℃冰箱中保存 30 天,室温解冻后测定样品中积雪草苷和羟基积雪草苷浓度,积雪草苷、羟基积雪草苷(1.0 μg/mL、8.0 μg/mL、12.0 μg/mL)解冻后测定结果分别为:(0.994 ± 0.006)、(8.422 ± 0.099)和(12.673 ± 0.441)μg/mL;(0.969 ± 0.029)、(8.267 ± 0.315)和(11.758 ± 0.304)μg/mL,结果表明积雪草苷和羟基积雪草苷冻融稳定。

2.2 脑组织中积雪草苷和羟基积雪草苷含量测定

取 9 只大鼠随机分 3 组,每组 3 只,积雪草总苷低剂量组(100 mg/kg)、积雪草总苷高剂量组(200 mg/kg)、积雪草苷和羟基积雪草苷组联合给药组(各 10 mg/kg),适应一周。实验前禁食 24 h,以积雪草总苷浓度为 100、200 mg/kg 尾静脉给药,积雪草苷 10 mg/kg 和羟基积雪草苷 10 mg/kg 同时尾静脉给药。30 min 后麻醉[11],生理盐水心脏灌注后取脑组织。按 2.1.2 方法处理,进样分析。结果见表 3。

3 讨论

现代药理研究表明,积雪草苷在炎症、脊髓损伤和糖尿病等方面具有确切药理作用,近年中枢神经系统药效尤其受到关注,如改善记忆,抗抑郁、阿兹海默症[12,13]等。作为皮肤方面的药物,积雪草总苷已经在市场上销售,其主要成分为积雪草苷和羟基积雪草苷。但在中枢疾病方面,相关积雪草苷的研究已经非常多,但一直未成药,主要原因就是积雪草苷的理化性质不良。积雪草中的五环三萜皂苷类成分分子量大,不易透过生物膜,普遍吸收较差,入血困难,更难以到达脑组织,不能达到有效的治疗浓度。有关积雪草苷、羟基积雪草苷的血液药动学、代谢等已有研究,但有关积雪草苷、羟基积雪草苷脑组织内分布、代谢情况研究极少。

为了研究二者吸收入脑情况,进一步探究其量效关系,采用尾静脉给药,建立脑组织药物浓度的测定方法。文献报道[23]

表 3 大鼠尾静脉给药后 30 分钟脑内积雪草苷、羟基积雪草苷浓度测定(n=3)

Table 3 The concentration of AS, MA and IS in rat brain tissue of 30 minutes after the rat caudal vein injection (n=3)

Groups		Dose	Content%	Measured μg/mL
Total triterpene	AS	200 mg/kg	15%	1.6837
	MA		32%	6.7491
Total triterpene	AS	100 mg/kg	15%	5.4965
	MA		32%	11.5536
	AS	10 mg/kg	90%	1.7384
	MA	10 mg/kg	90%	1.7167

的测定的方法未进行心脏灌注无法去除脑组织内血管里的血液。脑组织内部血管丰富,残留血液相对较多,无法排除给药后血液中积雪草苷和羟基积雪草苷的干扰。积雪草苷和羟基积雪草苷透过血脑屏障进入脑组织量很少,测量结果不能反映药物进入脑组织的真实水平,并且仅测定积雪草苷一种成分。现有报道大多数前处理方法是使用固相萃取小柱, 相对成本较高;乙腈沉淀蛋白效果好于甲醇,但积雪草苷和羟基积雪草苷在甲醇的溶解度要好于乙腈,本研究先用甲醇沉淀蛋白,再离心,成本低,且快速方便易操作。本研究建立了同时测定大鼠脑组织积雪草苷和羟基积雪草苷浓度的方法。方法简单,专属性、稳定性、提取回收率和重现性好。为研究积雪草苷和羟基积雪草苷脑组织分布、代谢提供了参考依据。

本实验发现积雪草总苷低剂量组静脉给药时,大鼠脑组织积雪草苷、羟基积雪草苷测定浓度高于积雪草总苷高剂量组,提示可能存在脑组织吸收阈值。积雪草总苷以 100 mg/kg 浓度给药时,测得的脑组织中积雪草苷和羟基积雪草苷浓度比同浓度下积雪草苷、羟基积雪草苷联合给药时测得的浓度高,表明积雪草总苷中可能有其他成分辅助了两者的脑内吸收。积雪草总苷以 100 mg/kg 浓度给药时,羟基积雪草苷和积雪草苷在大鼠脑组织中测定浓度基本一致。积雪草总苷以 200 mg/kg 浓度给药时,羟基积雪草苷的给药量是积雪草苷的 2.2 倍,但羟基积雪草苷在大鼠脑组织中的测定浓度是积雪草苷测定浓度的 4 倍,表明羟基积雪草苷的透过血脑屏障进入脑组织的量要大于积雪草苷,而羟基积雪草苷的极性大于积雪草苷,这和分子极性越大越难透过血脑屏障入脑似乎矛盾,更进一步说明可能有其他因素辅助了羟基积雪草苷进入脑组织,对于两者的转运情况有待进一步研究。

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