

HPLC 法测定香菇中香菇嘌呤含量

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摘要: 建立 1 种运用高效液相色谱(HPLC)测定香菇(*Lentinula edodes*)中香菇嘌呤含量的方法。优化后的色谱条件为: Ultimate AQ-C₁₈柱(5 μm, 4.6 mm×250 mm), 流动相为甲醇-磷酸盐缓冲液(7:93, pH 4.67)等度洗脱, 流速 1 mL/min, 柱温 30 °C, 检测波长 259 nm, 进样量 10 μL。研究结果发现该方法准确、灵敏、重现性好, 适用于香菇嘌呤的定量分析。

关键词: 香菇; 香菇嘌呤; HPLC

香菇(*Lentinula edodes*)是世界第二大食用菌^[1], 具有独特香味, 也是我国传统食品之一。研究发现, 香菇中含有一种降血脂有效成分香菇嘌呤(ergitadenine), 其降血脂作用比常用降血脂药安妥明强 10 倍, 且口服有效^[2]。香菇嘌呤还具有降低血浆中高半胱氨酸浓度的作用, 从而可以防治高半胱氨酸浓度过高引发的血栓和血管疾病^[3,4], 同时香菇嘌呤还具有保肝^[5]作用。

目前, 有研究者用紫外分光光度法、气质联用法、纸电泳法等对香菇嘌呤进行定性、定量分析。其中紫外分光光度法前处理(供试品溶液制备)十分复杂, 且干扰成分不易去除^[6]; 气质联用法须对样品预先进行衍生化, 误差较大^[7]; 而纸电泳分析方法操作不方便、重现性较差^[8]。本文运用 HPLC 测定香菇中香菇嘌呤含量, 并进行方法学考察以寻找适合香菇中活性成分的定量分析方法。

1 材料与amp;方法

1.1 材料、试剂和仪器

1.1.1 供试样品

香菇(*L. edodes*)子实体(商品名金钱菇)购于上海百信食药菌科贸有限公司。

1.1.2 试剂

甲醇(色谱纯)为美国 Dikma 公司产品, 磷酸

二氢钾、磷酸、无水乙醇等均为国产分析纯。

1.1.3 主要仪器

Waters 600 系列高效液相色谱仪、Waters 717 自动进样器、Waters 2996 光敏二极管阵列检测器均为美国 Waters 公司产品, KQ-600B 型超声清洗器为昆山市超声仪器有限公司产品。

1.2 方法

1.2.1 香菇嘌呤标准品制备

参考 CHIBATA I 等方法^[9]运用阳离子和阴离子交换树脂的分离纯化方法得到香菇嘌呤粗品, 并通过反复重结晶得到无色针状结晶, 并进行核磁共振验证, 纯度大于 98% (另文报道)。

1.2.2 色谱条件优化

色谱柱: Ultimate AQ-C₁₈柱(5 μm, 4.6 mm×250 mm); 流动相: 甲醇-磷酸盐缓冲液(KH₂PO₄ 10 mmol/L, 85% H₃PO₄调节 pH); 检测器: UV 检测器检测; 检测波长: 259 nm; 流速: 1 mL/min; 柱温: 40 °C; 进样量: 10 μL。对甲醇-磷酸盐缓冲液的比例(甲醇所占比例分别为 3%、4%、5%、6%、7%、10%、15%和 20%)及 pH(4.45、4.67和 5.12)等条件进行优化, 确定色谱分析条件。

1.2.3 样品前处理方法优化

1.2.3.1 乙醇提取溶剂浓度筛选

称取 6 份香菇子实体粉 1 g, 分别加入 5%、10%、15%、20%、25%、30% 的乙醇溶液 100 mL, 超声提取 30 min, 放冷至室温, 用溶剂补足减失

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的重量, 过滤去除菌粉沉淀, 续滤液经 0.45 μm 膜过滤, 进行 HPLC 测定, 分析提取量。以加入蒸馏水 100 mL, 100 $^{\circ}\text{C}$ 热回流提取 1 h 为对照, 每个处理重复 3 次。

1.2.3.2 料液比的筛选

称取 6 份香菇子实体粉 1 g, 分别加入筛选浓度乙醇溶液 50、100、150、200、250 和 300 mL, 超声提取 1 h, 后续操作同 1.2.3.1。

1.2.3.3 提取时间的优化

称取 6 份香菇子实体粉 1 g, 按照 1.2.3.2 和 1.2.3.1 试验筛选的料液比和浓度加入乙醇溶剂后, 超声提取 10、15、20、30、60、120 和 180 min, 后续操作同 1.2.3.1。

1.2.4 方法学考察

1.2.4.1 标准曲线的绘制

称取烘至恒重的香菇嘌呤标准品 10.0 mg, 置于 10 mL 容量瓶中, 加超纯水定容至刻度作储备液。将储备液稀释至不同浓度(10、15、20、25、30、35 和 40 $\mu\text{g}/\text{mL}$), 进样分析香菇嘌呤, 根据峰面积和香菇嘌呤含量绘制标准曲线。

1.2.4.2 精密度实验

吸取 20 $\mu\text{g}/\text{mL}$ 的标准品溶液 10 μL , 连续进样 5 次, 计算 RSD, 考察精密度。

1.2.4.3 重复性实验

取同一批样品, 按优化后的方法平行制备供试液 5 份, 分别进样, 测定峰面积。

1.2.4.4 稳定性实验

吸取 20 $\mu\text{g}/\text{mL}$ 的标准品溶液 10 μL , 分别于配制后的 0、5、10、15 和 20 h 时进样, 测定峰面积。

1.2.4.5 加样回收率实验

取香菇样品粉末 6 份, 每份 0.1 g, 按优化的样品前处理方法平行制备供试液, 分别加入 1 mg/mL 标准品溶液 0.2 mL, 并按优化色谱条件进行测定分析, 计算回收率。

1.2.5 香菇样品中香菇嘌呤含量的测定

取香菇样品粉末 5 份, 每份 0.1 g, 按优化的样品前处理方法平行制备供试液, 并按优化色谱条件进行测定分析。

2 结果与分析

2.1 色谱条件优化结果

利用 Ultimate AQ-C₁₈ 分析柱, 在柱温 30 $^{\circ}\text{C}$ 、流速为 1 mL/min、流动相甲醇-磷酸盐缓冲液条件下, 样品中香菇嘌呤的峰形及分离度较好, 随着甲醇比例的增加, 香菇嘌呤的保留时间提前, 当甲醇比例大于 10% 时, 香菇嘌呤的峰形和分离度不能达到分析的要求。综合考虑保留时间和分离效果, 选取甲醇-磷酸盐缓冲液 = 7:93 来进行等度洗脱。同时发现缓冲液 pH 对香菇嘌呤的峰形和保留时间也有较大影响, pH 4.67 时香菇嘌呤同其他成分可以达到最佳的基线分离且峰形好。

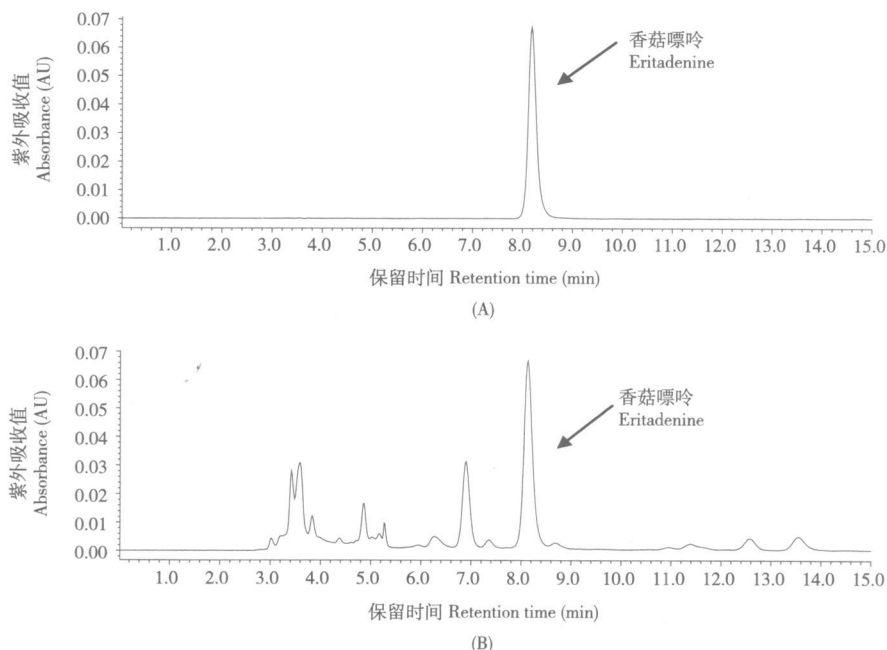


图 1 标准品(A)及样品(B)的 HPLC 图谱

Fig. 1 RP-HPLC chromatograms of eritadenine standard (A) and test samples (B)

2.2 样品前处理优化方法

由于乙醇具有较好的穿透细胞壁作用, 同时香菇嘌呤具有水溶性的特点, 考察不同浓度乙醇超声提取和蒸馏水加热回流的提取效率, 结果发现 5% ~ 20% 乙醇超声提取的效率优于蒸馏水加热回流, 由于超声提取方式简便易操作, 且使用 5% 乙醇提取时提取液所含杂质最少, 所以确定最佳提取方式为 5% 乙醇超声提取。

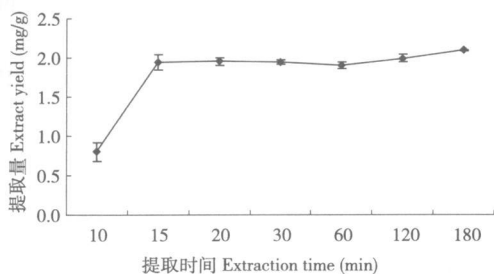


图 2 不同提取时间的香菇嘌呤提取量

Fig. 2 Effect of extraction time on eritadenine yields

2.3 方法学考察

2.3.1 标准曲线

以标准品的进样浓度($\mu\text{g}/\text{mL}$)为横坐标, 色谱峰的面积纵坐标, 得回归方程: $y = 40200x - 15600$, $r^2 = 0.99987$ 。线性范围为 5~40 $\mu\text{g}/\text{mL}$ 。

2.3.2 精密性、重复性和稳定性实验

精密性、重复性和稳定性实验中香菇嘌呤含量的 RSD 分别为 0.40%、0.08% 和 0.46%, 说明运用建立的 HPLC 分析方法测定香菇嘌呤含量, 精密性、重复性和稳定性均良好。

2.3.3 加样回收率

加样回收率实验结果表明, 加样回收率在 95.31% ~ 97.85% 之间, 平均回收率为 96.42%, RSD = 0.86%。

2.4 样品中香菇嘌呤含量

采用优化的前处理方法及色谱条件平行测定 5 份香菇样品, 其香菇嘌呤的平均含量为 1.9936 mg/g, RSD = 0.08%。

3 讨论

卫生部发布的《2002 年中国居民营养与健康现状调查报告》中就宣布: 全国血脂异常人数估计达 1.6 亿, 其中约有 9000 万人患有高血脂症(俗称高血脂)。目前高血脂症是导致心脑血管疾病的元凶, 发病率高, 已成为威胁中老年人生

命的主要祸首。早在 60 年代, 日本就发现香菇具有降血脂作用, 并得到降血脂活性成分香菇嘌呤。近年来, 对其降血脂的作用机制也进行了大量深入研究, 发现香菇嘌呤通过调控脂肪酸代谢^[10, 11]及改变磷脂酰胆碱分子谱图的组成^[10, 12-14]来降低血液胆固醇的含量。香菇是日常食品之一, 毒副作用小, 香菇嘌呤含量较高, 因此开发降血脂的香菇嘌呤保健品或药品具有很好的应用前景。

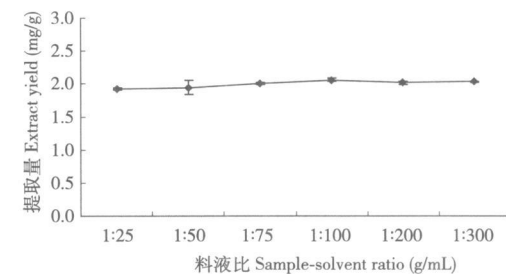


图 3 不同料液比的香菇嘌呤提取量

Fig. 3 Effect of sample-solvent ratio on eritadenine yields

命的主要祸首。早在 60 年代, 日本就发现香菇具有降血脂作用, 并得到降血脂活性成分香菇嘌呤。近年来, 对其降血脂的作用机制也进行了大量深入研究, 发现香菇嘌呤通过调控脂肪酸代谢^[10, 11]及改变磷脂酰胆碱分子谱图的组成^[10, 12-14]来降低血液胆固醇的含量。香菇是日常食品之一, 毒副作用小, 香菇嘌呤含量较高, 因此开发降血脂的香菇嘌呤保健品或药品具有很好的应用前景。

香菇嘌呤是由腺嘌呤上接一个丁酸组成的, 因此极性较大, 运用传统的核苷分析条件即水与甲醇梯度洗脱^[15], 分离度和峰形都不好。笔者采用单核苷酸的色谱条件, 使香菇嘌呤与其它的核苷类成分得到了很好的分离。本文所建立的运用 HPLC 测定香菇嘌呤的分析方法, 操作简单、重复性好、准确度高, 应用此方法对样品进行测定的结果显示数据稳定, 误差小, 适用于香菇嘌呤的定量分析。

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[本文编辑] 于荣利

Determination of Eritadenine in *Lentinula edodes* Fruit bodies Using HPLC

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Abstract: A simple and sensitive HPLC-based method for the accurate determination of eritadenine in *Lentinula edodes* fruit bodies is described. Optimization of sample work-up procedures showed that the efficiency of eritadenine extraction was highest when powdered fruit bodies were suspended in 5% (w/v) ethanol and subjected to ultrasound for 30 min using a 1: 100 sample-solvent ratio. Optimal separation of eritadenine in the extracts was achieved by loading 10 μ L samples on to an Ultimate AQ-C₁₈ column (5 μ m, 4.6 mm \times 250 mm) and eluting with methanol-phosphate buffer (7: 93, pH 4.67) at 30 $^{\circ}$ C using a flow rate of 1.0 mL/min. Eritadenine in eluted fractions was monitored at 259 nm.

Key words: *Lentinula edodes*; eritadenine; HPLC

Lentinula edodes ranks among the top three mushroom species available on the international market^[1], mushroom fruit bodies contain eritadenine, the cholesterol-lowering effects of which are reported to be ten times stronger than the widely used lipid-lowering drug, clofibrate^[2]. Eritadenine also reduces homocysteine concentrations in plasma, thereby preventing high-level homocysteine-induced thrombosis and vascular disease^[3,4], and exhibits hepatoprotective activity^[5].

Currently, UV spectrophotometry, GC-MS or paper electrophoresis are used for the qualitative and quantitative determination of eritadenine, although each of these methods has inherent deficiencies. Preparation of the test samples for UV spectrophotometry is complex and it is often difficult to remove interfering components^[6], the required pre-derivatization of samples for GC-MS causes large errors^[7], and poor reproducibility is a regular problem with the paper electrophoresis method^[8]. In this paper, we have used HPLC

to determine eritadenine levels in *L. edodes* fruit bodies, and have examined various features of the methodology (i. e. accuracy, reproducibility, eritadenine stability and recovery rate) to establish if the method is suitable for the routine quantitative analysis of this bioactive component.

1 Materials and methods

1.1 Materials

L. edodes fruit bodies was purchased from Shanghai Baixin Bio-Tech Co., Ltd.

1.2 Reagents

Methanol (HPLC grade) was from Dikma Technologies Inc (CA, USA), while other reagents were from Chinese sources and of analytical grade.

1.3 Methods

1.3.1 Eritadenine standard

A sample of eritadenine, shown by nuclear magnetic resonance imaging to be 98% pure, was further purified by chromatography using cation and anion exchange resins^[10] followed by

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repeated recrystallization to give colorless needle-like crystals (details to be reported elsewhere).

1.3.2 HPLC

HPLC was carried out using a Waters 600 Series high-performance liquid chromatograph, attached to a Waters 717 autosampler and a Waters 2996 photodiode array detector. The chromatograph was fitted with an Ultimate AQ-C18 column (5 μm , 4.6 mm \times 250 mm), and the mobile phase was methanol-phosphate buffer (10 mmol/L KH_2PO_4 adjusted with 85% H_3PO_4 to give different pH values). Operating conditions were as follows: column temperature, 40 $^\circ\text{C}$; injection volume, 10 μL ; flow rate, 1 mL/min; detection wavelength, 259 nm. The shape of the eritadenine peak and the degree of separation varied according to the methanol-phosphate buffer ratio. In order to optimize the HPLC conditions, the effects of using different proportions (3%, 4%, 5%, 6%, 7%, 10%, 15% and 20%) of methanol in the mobile phase, and different pH values (4.45, 4.67 and 5.12), were evaluated.

1.3.3 Optimization of sample pre-treatment

1.3.3.1 Concentration of extraction solvent

L. edodes fruit body powder (1 g) was suspended in 100 mL aqueous ethanol (5%, 10%, 15%, 20%, 25% or 30%) and subjected to ultrasound (KQ-600B ultrasonic cleaner, Kunshan Ultrasonic Instrument Co., Ltd, Kushan, China) for 30 min. After cooling to room temperature and addition of solvent to compensate for losses during extraction, suspensions were coarse-filtered to remove residual solids. Filtrates were then membrane filtered (0.45 μm) and the extraction yields determined using HPLC. Controls were prepared by suspending 1 g fruit body powder in 100 mL distilled water and extracting under reflux at 100 $^\circ\text{C}$ for 1 h. Each treatment was carried out in triplicate.

1.3.3.2 Solid-liquid ratio

L. edodes fruit body powder (1 g) was

suspended in different volumes (50, 100, 150, 200, 250 or 300 mL) of 5% aqueous ethanol and extracted using ultrasound as above. Subsequent work-up procedures were the same as described in Section 1.3.3.1.

1.3.3.3 Extraction time

L. edodes fruit body powder (1 g) was suspended in 100 mL of 5% aqueous ethanol and extracted using ultrasound for 10, 15, 20, 30, 60, 120 or 180 min. Subsequent work-up procedures were the same as described in Section 1.3.3.1.

1.3.4 Analysis of methodology

1.3.4.1 Standard Curve

A standard curve relating peak area with eritadenine concentration was prepared using different concentrations of eritadenine (10, 15, 20, 25, 30, 35 and 40 $\mu\text{g}/\text{mL}$) dissolved in ultra-pure water.

1.3.4.2 Precision test

The precision level of the methodology was assessed from the RSD value calculated after five replicate determinations of the peak area obtained following HPLC of a 10 μL sample of a standard (20 $\mu\text{g}/\text{mL}$) eritadenine solution.

1.3.4.3 Reproducibility

Reproducibility was assessed from the RSD value calculated from the peak areas obtained following HPLC of five test solutions prepared from the same batch of fruit bodies using the optimized pre-treatment and HPLC conditions.

1.3.4.4 Stability test

Eritadenine stability was assessed from the RSD value calculated from peak areas obtained following HPLC of five replicate 10 μL samples of a standard (20 $\mu\text{g}/\text{mL}$) eritadenine solution assayed after 0, 10, 15 and 20 h.

1.3.4.5 Recovery rate

Eritadenine recovery rates were determined by adding 0.2 mL aliquots of a standard eritadenine solution (1 mg/mL) to six powdered fruit body samples prior to extraction and analysis adopting the optimized pre-treatment and HPLC procedures.

1.3.5 Determination of eritadenine in *L. edodes* fruit bodies

Eritadenine levels were determined in five powdered *L. edodes* fruit body samples (0.1 g) adopting the optimized pre-treatment and HPLC procedures.

2 Results and Analysis

2.1 Optimized HPLC conditions

An Ultimate AQ-C18 analytical column operated at 30 °C, and using a methanol-phosphate buffer mobile phase and a flow rate of 1 mL/min, gave a well-defined eritadenine peak and a good degree of separation. Eritadenine retention times were shorter with increasing concentrations of methanol in the mobile phase but peak characteristics and the degree of separation made analysis more difficult, especially when the proportion of methanol in the mobile phase > 10%. A methanol: phosphate buffer ratio of 7: 93 was selected as optimal based on retention time and degree of separation. Peak features and retention times were also greatly affected by the pH of the buffer, with pH 4.67 providing the best baseline separation and a well-defined eritadenine peak (see Fig. 1 in the Chinese version).

2.2 Optimized sample pre-treatment conditions

In view of the higher cell wall penetrating properties of ethanol and the water-soluble nature of eritadenine, two extraction methods were compared in terms of eritadenine extraction efficiency: (1) extraction with ultrasound using different concentrations of ethanol as the solvent, and (2) refluxing at 100 °C using distilled water. Extraction with ultrasound using 5%–20% ethanol was more efficient than refluxing with boiling water and 5% ethanol was selected on the basis of easy operation and lowest impurity levels. Maximum eritadenine yields were achieved after 15 min ultrasound treatment (see Fig. 2 in the Chinese version) but, since the extraction efficiency at

30 min was more reproducible, the longer extraction time was adopted. The ratio of fruit body sample to extraction solvent had no significant effect on eritadenine yields over the range tested (Fig. 3 in Chinese version) and the solid-liquid ratio of 1: 100 was selected as standard.

2.3 Methodology data

2.3.1 Standard curve

The following regression equation was derived from the experimental data: $y = 40\,200x - 15\,600$, $r^2 = 0.99987$, linear range 5–40 µg/mL, x: standard sample concentration (µg/mL), y: peak area.

2.3.2 Precision, reproducibility and stability

RSD values for eritadenine levels determined in precision, reproducibility and stability tests were 0.40%, 0.08% and 0.46%, respectively, indicating that the HPLC method gave accurate, reproducible and stable results.

2.3.3 Recovery rate

The rates of recovery ranged between 95.31% and 97.85%, with an average value of 96.42% (RSD = 0.86%).

2.4 Content of eritadenine in *L. edodes* fruit bodies

The average eritadenine content of *L. edodes* fruit bodies was 1.993 6 mg/g (RSD = 0.08%).

3 Discussion

According to the “2002 China National Nutrition and Health Survey Report” issued by the Ministry of Health, there are approximately 160 million dyslipidemic people living in China, of which about 90 million are suffering from hyperlipidemia. Hyperlipidemia is a leading cause of cardiovascular disease and a major life-threatening factor affecting the elderly. The lipid-lowering effect of *L. edodes* fruit bodies was first recorded in the 1960s and the active lipid-lowering component was subsequently identified as eritadenine. More recent studies on the mechanism underlying the

lipid-lowering effects have shown that eritadenine reduces blood cholesterol by regulating fatty acid metabolism^[10,11] and changing the molecular composition of phosphatidylcholine^[10, 12-14]. Eritadenine has wide application and exploitation potential for use in cholesterol-lowering nutraceutical and pharmaceutical products.

Conventional methods for nucleotide analysis based on gradient elution using water and methanol^[15] are not particularly suited for separating eritadenine due to the highly polar nature of a molecular structure consisting of adenine and butyrate. We have now demonstrated that eritadenine can be readily separated from other purine nucleoside components adopting chromatographic conditions suitable for separating single nucleotides. Our HPLC-based determination method is straightforward, highly accurate and reproducible, and is therefore suitable for the quantitative determination of this bioactive component.

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