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A rapid HPLC with evaporative light scattering method for quantification of forskolin in multi-herbal weight-loss solid oral dosage forms

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Received October 27, 2009, accepted November 17, 2009

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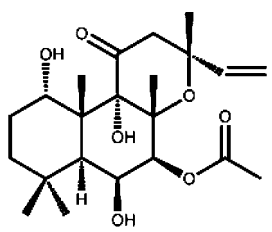
Pharmazie 65: 322–326 (2010) *doi: 10.1691/ph.2010.9346*

A rapid reverse-phase HPLC method with evaporative light scattering detection (ELSD) was developed for the determination of forskolin in weight loss multi-herbals products. The analysis was performed by water-acetonitrile gradient elution at a temperature of 40 °C and a flow rate of 1.0 mL/min. The evaporator tube temperature of ELSD was set at 35 °C, and with the nebulizing gas flow-rate (pressure) of 3.0 bar. The method was validated for linearity, accuracy, precision and limits of detection (LOD) and quantification (LOQ). Good linear relationships were obtained with correlation coefficients exceeding 0.9995. The average recovery of forskolin ranged from 99.4% to 100.4% with RSDs below 3%. The percent relative standard deviations (%RSD) of intra- and inter-day precision varied by less than 2.1%. LOD and LOQ were 0.95 μg/ml and 3.21 μg/ml, respectively. The validated ELSD method permits a shorter determination time without compromising accuracy and demonstrates that it can be used for quantification of forskolin incorporated in multi-herbal solid oral dosage forms.

1. Introduction

Forskolin is a diterpene compound isolated from the root of the perennial plant *Coleus forskohlii* Briq (Labiatae) native to India (Bhat et al. 1977). The root and its ethanolic extract of *C. forskohlii* have been used for centuries in Ayurvedic medicine to treat various diseases of the cardiovascular, respiratory, gastrointestinal, and central nervous systems (Ammon and Muller 1985). The labdane diterpenoids forskolin from this botanical have been extensively investigated and shown to have diverse therapeutic potential. The classic pharmacological response associated with forskolin is to increase cAMP and cAMP mediated functions, via activation of the enzyme adenylate cyclase (Metzger and Lindner 1981; Seamon et al. 1981). The right ring of the decalin portion of forskolin has a structure similar to alpha-D-galactose. As a result, forskolin binds to the glucose transporter to activate adenylate cyclase (Laurenza et al. 1989; Abbadi and Morin 1999). This characteristic of forskolin led to its extensive



Forskolin

use as a biochemical tool to increase intracellular cAMP concentration. Forskolin's ability to elevate cAMP has been shown to be of benefit as it stimulates lipolysis by promoting the breakdown of stored fats in animal (Okuda et al. 1992) and human fat cells (Litosch et al. 1982; Allen et al. 1986). Theoretically, levels of cAMP increased by forskolin will enhance lipolysis leading to elevation of fat degradation and fat usage in the body and therefore promote fat and weight loss. Godard et al. (2005) reported a significant decrease in both fat percentage and mass after oral ingestion of forskolin (250 mg of 10% forskolin extract twice a day) for a 12-week period in obese males. Recently multi-herbal formulas that contain *C. forskohli* extract are gaining popularity as they have been marketed as promoting a weight loss regimen due to their recognized fat-burning properties. In supplements available on the market a forskolin content of up to 20% from *C. forskohlii* extract have been claimed. In particular, its use in promoting lean body mass has drawn our attention towards this supplement. Treatment with *C. forskohlii* extract, although increasingly common, is contraindicated in combination with antihypertensives and anticoagulants due to the high potential for herb-drug interactions (Ding and Staudinger 2004). These interactions are likely to occur even before therapeutic levels of forskolin are achieved in patients using this powerful extract. It has therefore become an important issue to accurately determine the content of forskolin in weight loss promoting supplement products to ensure the safety of their use. Previous reported methods for the determination of forskolin include GLC (Inamdar et al. 1980), TLC, normal-phase HPLC (Inamdar et al. 1984) or reverse-phase LC with photodiode array detector (Schaneberg and Khan 2003). Quantification of forskolin from among six analytes in thermogenic weight loss herbals by a single method has also been demonstrated albeit with a running time of 45 min (Schaneberg and Khan 2004). Therefore, it would be desirable to develop a simple and rapid

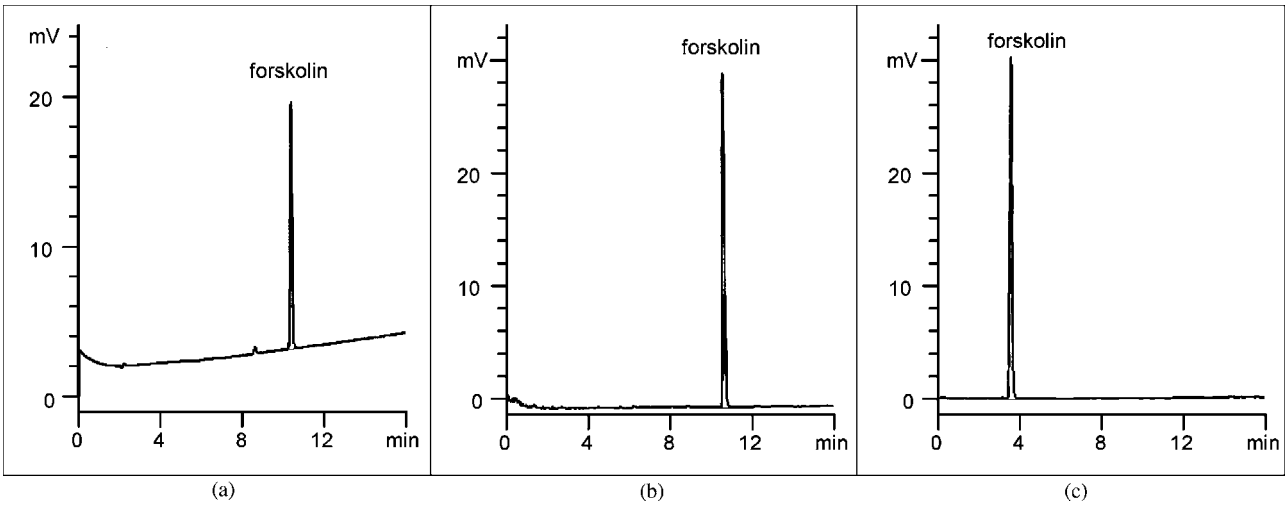


Fig. 1: Comparison of chromatograms of forskolin obtained from standard solution, (a) HPLC-UV (long column condition 1), (b) HPLC-ELSD (long column condition 1), (c) HPLC-ELSD (short column condition 2)

analytical method that could quantify forskolin content in products on the market. Recently detection of a forskolin-like molecule in cyst fluid using HPLC-MS or ELS detectors has been reported (Putnam et al. 2007). The ELSD system is an attractive alternative because of its versatility and good sensitivity, and can be connected in series with a UV detector. The aim of this investigation was to develop a rapid HPLC method that offers sufficient selectivity, accuracy and precision for the determination of forskolin in various weight loss formulations. In this study, mobile phase selection was developed using a slightly modified analytical condition based on the method established by Schaneberg and Khan (2003). UV and ELS detection were used to obtain two fingerprinting chromatograms. Comparison between the results obtained by the two different detectors showed that the HPLC-ELSD method was highly specific.

2. Investigations, results and discussion

To obtain excellent chromatographic conditions, various proportions of water-acetonitrile, category of column (ODS 4.6 × 150 mm, 5 μm particle size; L column ODS 4.6 × 250 mm, 5 μm particle size), and flow rate of mobile phase (0.8, 1.0 or 1.2 mL/min) were all investigated and compared. ELSD connected in series with UV was optimized to obtain the best signals, and signal to noise (S/N) ratio. With the ELS detection, evaporating temperature and nebulizing gas flow-rate (pressure) two major instrumental adjustments are available for maximizing detector response efficiency. In this study, temperature and pressure of the gas for the detector was evaluated systematically from 25 to 45 °C, and from 2.0 to 4.5 bar, respectively. Since the sensitivity of the ELS detector is known to change in solvent gradients, mainly due to changes in droplet size, a UV detector is placed before the ELS detector for maximal sensitivity. The chromatographic conditions were optimized in order to obtain chromatograms with a good resolution of adjacent peaks within a short analysis time. Chromatographic separation was carried out by gradient elution with a mobile phase of water-acetonitrile (condition 1) at a flow-rate of 1.2 ml/min. The evaporating temperature of 35 °C and a pressure of 3.0 bar were selected. When these chromatographic conditions were used, forskolin was well separated within 10.4 min using the long column. The use of a short column at a flow-rate of 1.0 ml/min (condition 2) allows the ELSD system to increase the analytical speed without compromising peak resolution, and within 3.5 min forskolin eluted. In Fig. 1 representative HPLC-UV (Fig. 1a) and ELSD (Fig. 1b and c) chromatograms of a forskolin standard solution are presented. The linearity was determined by analysis of six appropriate concentrations of standard solutions (range 3.5–350 μg/ml) in triplicate. The HPLC-UV calibration curve constructed by plotting peak area against concentration was linear with a correlation coefficient (r^2) of 1.000. The regression equation was $y = 6.5748x - 0.075$, where y is the peak area and x is the concentration (μg/ml). The response for the ELS detector was known to be nonlinear. However, good linearity was achieved with calibration curves plotted logarithmically using peak area (y) versus concentration (x). The equation of the calibration curve was $Y = 1.5657X + 2.02$ (Y, X are the logarithmic values of area and concentration) and r^2 of the calibration curve was found to be 0.9995. The LOD and LOQ were experimentally verified by diluting known concentrations of forskolin with mobile phase until the average responses were approximately three or ten times, the standard deviation of the responses for three replicate determinations, respectively. The LOD and the LOQ of forskolin were 0.95 μg/ml and 3.21 μg/ml, respectively, in ELS detection. The precision of the developed method was determined for intra- and inter-day variations. The intra-day variation

Table 1: Accuracy of the HPLC-UV and -ELSD methods to determine forskolin in solid oral dosage forms

Detector	Added (μg)	Determined (μg ± S.D.)	Recovery (% ± S.D.)	% R.S.D.
UV	7.5	7.64 ± 0.14	101.39 ± 2.88	2.84
(condition 1)	22.5	22.18 ± 0.44	99.34 ± 2.08	2.10
	37.5	36.73 ± 0.65	98.29 ± 2.31	2.35
ELSD	7.5	7.45 ± 0.15	99.35 ± 2.01	2.02
(condition 2)	22.5	22.48 ± 0.52	99.90 ± 2.30	2.31
	37.5	37.64 ± 0.80	100.38 ± 2.13	2.14

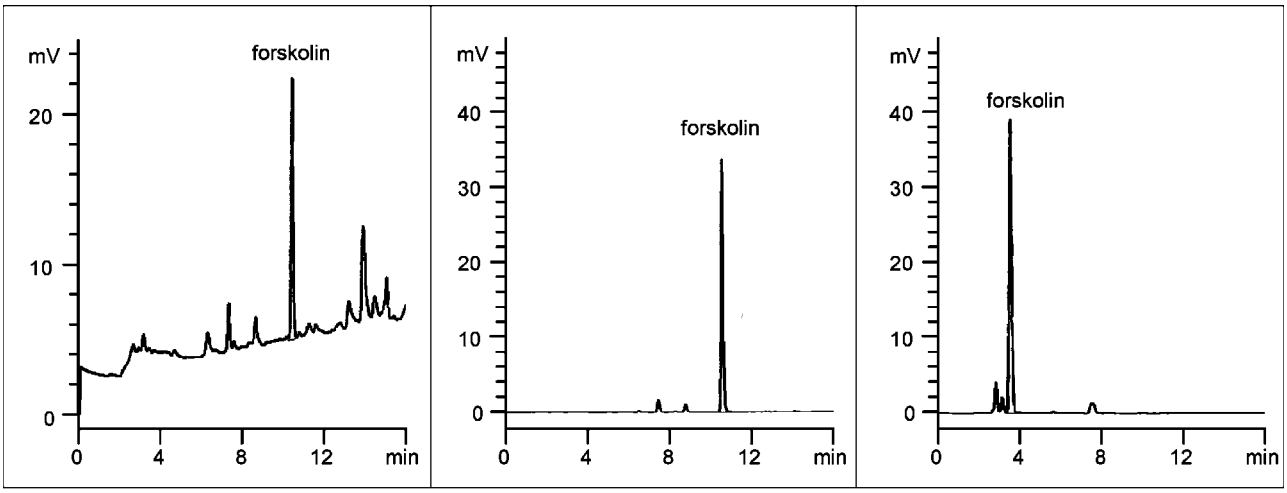


Fig. 2: Comparison of chromatograms of forskolin obtained from *C. forskohlii* root extract, (a) HPLC-UV (long column condition 1), (b) HPLC-ELSD (long column condition 1), (c) HPLC-ELSD (short column condition 2)

was determined by analyzing the same mixed standard solution three times within one day. While for the inter-day variability test, the solution was examined in triplicate for 3 consecutive days. The RSD of the peak area was taken as the measure of precision. The RSD values of intra- and inter-day precisions were 0.98% and 1.35% for UV detector, respectively, and 1.52% and 2.04% for the ELSD detector, respectively. The low values of intra- and inter-day %RSD indicate good instrumental precision. In general, the RSD was smaller for UV areas than for ELSD areas as other studies have also indicated. Recovery test was used to evaluate the accuracy of this method. The recoveries were performed by adding suitable amounts of the forskolin standard to sample #1 before extraction. The mixture was then prepared in accordance with the sample preparation procedure and the mean recovery was calculated for three assays of each preparation. Recoveries of forskolin by both detectors were over 98% with low RSD (Table 1). The UV detector ranged from 98.3% to 101.4% with an RSD less than 3%. Similarly high results were obtained from the ELSD method which ranged between 99.4–100.4% with an RSD also less than 3%. To confirm the repeatability, six different working solutions prepared (in parallel with recovery test) from sample #1 were analyzed. The RSD was taken as the measure of repeatability. RSDs of both detectors for the six measurements were within 2.0%. Therefore, the chromatographic system utilizing ELSD for quantitative determination of forskolin was appropriate and that the method had a high level of accuracy and repeatability. A typical chromatogram of *C. forskohlii* root extract (Fig. 2) shows that forskolin was detected and there were no major interfering absorbances from other compounds from the *C. forskohlii* matrix. The chromatographic peaks were identified by comparing their retention time with that of standard. In addition, spiking samples with the reference standard showed no additional peaks, which further confirmed the identities as that of forskolin.

The established HPLC-ELSD method was used to analyse forskolin content in various oral solid dosage form weight loss products. Eight formulations marketed for weight loss were quantified for their forskolin contents. In addition to *C. forskohlii* root extract the products comprised medicinal materials and various extracts from *Garcinia cambogia*, *Camellia sinensis*, *Gymnema sylvestre*, *Zingiber officinale*, *Citrus aurantium*, *Morinda citrifolia*, *Rhodiola rosea* and *Ginkgo biloba* (Table 2). The forskolin was successfully separated on top of the complex sample matrix with excellent peak shape and achieved in short time. A typical product chromatogram (Fig. 3) shows that other constituents used in the products did not interfere with the analysis. With the ELS detection, the method provided clean chromatograms with a strong response that shows the suitability of ELSD for the analysis of complex matrices. It can be seen that the ELSD profile always reveals fewer peaks than the UV profile. Extract components might evaporate and will not be seen in the ELSD signal. However, the ELSD signal has the advantage of having a flatter baseline, while in the UV chromatogram baseline drift can occur due to mobile phase UV absorption. Contents of forskolin in the analyzed weight loss samples are given in Table 2. The low standard deviation values indicate that the developed method was successfully applied for assessing forskolin in various oral weight loss dosage forms. In recent years, the technique of ELSD has also shown good compatibility to different sample matrices encountered in such diverse products as pharmaceutical dosage forms, beverages, oil seed, and dietary supplement products (Ganzera et al. 2001, 2004). The present results show that the content of forskolin in the market products meets the requirements (91–111% of the label claim, mean value 98%).

The combination of HPLC with ELSD looks promising, easy to implement and complementary to UV detection. Furthermore, analysis times were on average decreased by two thirds for ELSD compared to the conventional UV method. The reduction in retention time was made possible by performing the separation using a shorter analytical column. With the shorter analysis time, lower solvent consumption with a concomitant reduction in operating costs and satisfactory resolution, the ELSD method is effective for routine analysis of a large number of samples.

3. Experimental

3.1. Chemicals and materials

Forskolin was purchased from Biomol (USA), HPLC grade acetonitrile was from Kanto Chemical Co. Ltd. (Tokyo, Japan). Purified deionised water for HPLC analysis was prepared using a Milli-Q water purification system (Millipore, MA, USA). Sample of dried roots extract of *C. forskohlii* were kindly provided by Tokiwa Phytochemical Co. Ltd. (Chiba, Japan). Weight-loss products claiming to contain forskolin, *C. forskohlii,* were purchased from local drug stores.

3.2. Sample preparation

The weight-loss products analyzed in this work were in tablet and capsule dosage forms. For tablets, a minimum of 20 tablets were weighed and then pulverized with a mortar and pestle. For capsules (hard gelatin), ten samples were weighed, opened and the contents were emptied and weighed

Table 2: Forskolin content in weight-loss solid oral dosage form

Sample no.	Claimed active ingredients	Type	Label claim (%)	Detected (%)
1	*Coleus forskohlii* extract, *Camellia sinensis*, extract	Hard capsule	3.39	3.16 ± 0.07
2	ForsLean®, *Gymnema sylvestre* extract	Hard capsule	2.60	2.64 ± 0.07
3	ForsLean®, alpha lipoic acid, citric acid, and various amino acids	Hard capsule	1.86	1.83 ± 0.05
4	*Coleus forskohlii* extract, *Garcinia cambogia* extract, and various vitamins and minerals	Hard capsule	1.75	1.68 ± 0.04
5	*Coleus forskohlii* extract, *Morinda citrifolia* extract	Hard capsule	0.50	0.46 ± 0.01
6	*Coleus forskohlii* extract, and various vitamins and minerals	Tablet	6.20	5.66 ± 0.15
7	ForsLean®, *Coleus forskohlii*, *Citrus aurantium*, *Ginkgo biloba*, vitamin mix	Tablet	1.92	1.93 ± 0.05
8	ForsLean®, *Camellia sinensis* extract, *Rhodiola rosea* extract, *Zingiber officinale* extract	Tablet	1.21	1.34 ± 0.04

The results are expressed as the mean of triplicate determinations, SD: standard deviation of the mean, ForsLean® is registered trade mark of Sabinsa Corporation and is a standardized *Coleus forskohlii* root extract with minimum 10% forskolin

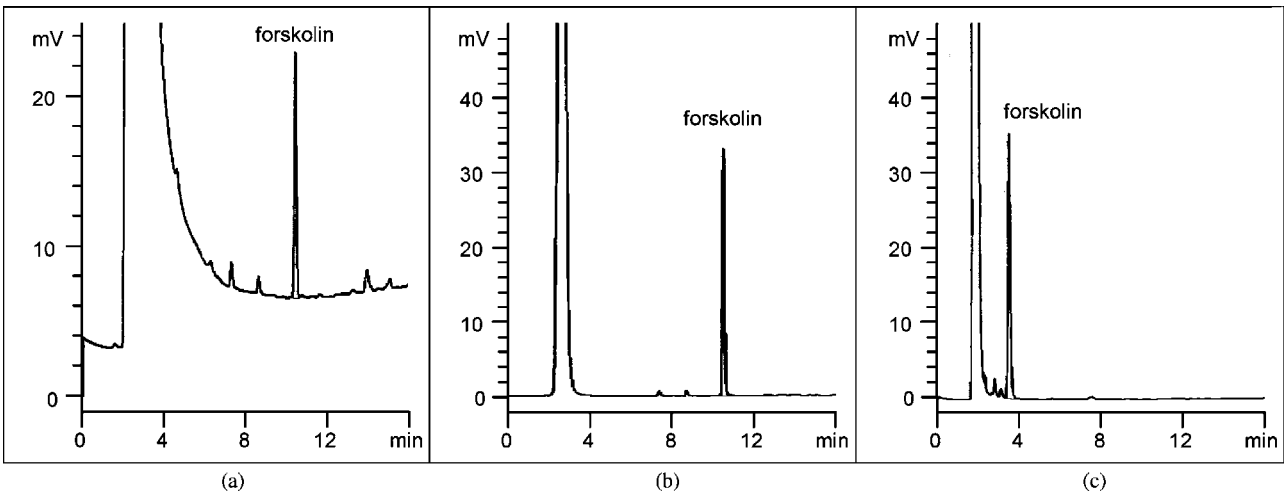


Fig. 3: Comparison of chromatograms of forskolin obtained from real product sample, (a) HPLC-UV (long column condition 1), (b) HPLC-ELSD (long column condition 1), (c) HPLC-ELSD (short column condition 2)

accurately, then mixed and triturated in a mortar and pestle. A dried powdered root extract of *C. forskohlii* (0.300 g) or ground materials from each weight loss product (range 0.500–1.000 g) were accurately weighed into the centrifuge tube, and sonicated in 3 ml acetronitrile for 15 min. After centrifugation the supernatant was then transferred to a 25 ml volumetric flask. The procedure was repeated two more times and the respective supernatants were combined. It was concluded that the method described by Schaneberg and Khan (2003) was the most suitable and efficient extraction method since it extracted the largest amounts of forskolin. After being repeated a fourth time, forskolin was undetectable. The final volume was adjusted with acetonitrile to 25 ml. Prior to injection all sample solutions were filtered through a 0.45 μm Millipore filter (Advantec). Each sample solution was assayed in triplicate with an injection volume of 10 μl.

3.3. Standard preparation

Standard stock solution of pure forskolin was prepared by dissolving in acetronitrile to obtain a concentration of 350 μg/ml. Five addition calibration levels (350–3.5 μg/ml) were prepared by diluting this solution with acetronitrile. All the solutions were kept at 4 °C before analysis.

3.4. Apparatus and chromatographic conditions

The Shimadzu HPLC-VP system (Shimadzu Corporation, Kyoto, Japan) coupled with double detectors of UV (SPD-10A) and ELSD (ELSD-LT). The HPLC system was equipped with system controller (SCL-10AVP), pump (LC-10AT VP), a autosampler (SIL-10A), and a column oven (CTO-10ACVP) set at 40 °C. The sample injection volume was 10 μl. The detectors monitored the eluent at 210 nm. Two optimized HPLC conditions were selected for the separation of forskolin. Under condition 1, the sample was applied to L column ODS 4.6 × 250 mm, 5 μm particle size (Chemical Inspection & Testing Institute, Japan) and eluted with a linear gradient of water (A) and acetonitrile (B). The gradient protocol was 0–12.0 min, 50–80% B; 12.1–16.0 min, 100% B; 16.1—20.0 min, 50% B at a flow rate of 1.2 ml /min. Under condition 2, the sample was applied to a short L column ODS 4.6 × 150 mm, 5 μm particle size with a linear gradient elution system of water (A) and acetonitrile (B). The gradient protocol was 0–4.5 min, 70% B; 4.51–10.0 min, 100% B; 10.1–14.0 min, 70% B at a flow rate of 1.0 ml /min. The optimized evaporator temperature for the ELSD that connected to LC system was set at 35 °C with the nebulizing gas flow-rate of 3.0 bar. Data acquisition was performed using a data processor (chromatopac CR7A plus, Shimadzu, Japan).

Acknowledgement: This research was conducted under the project entitled "*Project for Collaborative Research on the Safety and Efficacy of Herbal Medicines*" was financially supported in part by a research grant from the Ministry of Health, Labour, and Welfare in Japan.

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