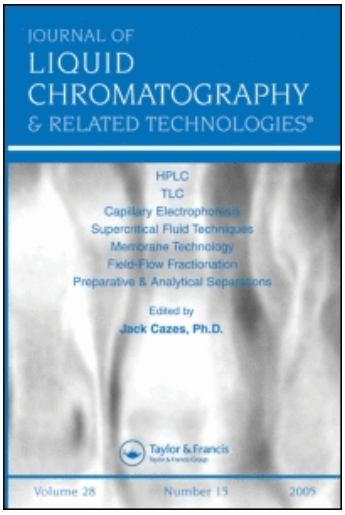

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Comparison of Different Analytical HPLC Columns for Determination of Furocoumarins in *Heracleum candicans* Fruits

Dhirendra P. Singh[a]; R. Govindarajan[a]; Ajay K. S. Rawat[a]
[a] Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute, Lucknow, India

To cite this Article Singh, Dhirendra P. , Govindarajan, R. and Rawat, Ajay K. S.(2008) 'Comparison of Different Analytical HPLC Columns for Determination of Furocoumarins in *Heracleum candicans* Fruits', Journal of Liquid Chromatography & Related Technologies, 31: 3, 421 — 427
To link to this Article: DOI: 10.1080/10826070701780821
URL: http://dx.doi.org/10.1080/10826070701780821

Journal of Liquid Chromatography & Related Technologies®, 31: 421–427, 2008

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701780821

Comparison of Different Analytical HPLC Columns for Determination of Furocoumarins in *Heracleum candicans* Fruits

Dhirendra P. Singh, R. Govindarajan, and Ajay K. S. Rawat

Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute, Lucknow, India

Abstract: Columns used in HPLC play a vital role, as the efficiency of separation and resolution determines the quantification of the markers. *Heracleum candicans* Wall. ex DC. (Apiaceae) is used extensively in the Indian system of medicines, being a rich source of furocoumarins, extensively used in the pharmaceutical industry for their photosensitizing property. Thus, keeping in view the importance of the plant, a comparison of the analytical HPLC columns has been carried out for the quality evaluation of *H. candicans* using furocoumarins viz. heraclenol and bergapten as markers. Four analytical columns (2 RP-8 and 2 RP-18) with different ligand chemistry were used for the analysis. The results indicate that the monolithic column, RP-18e has better efficiency in terms of time and cost, whereas the classical RP-18 column has better separation efficiency of the markers used, though there are some merged peaks. Also, by altering the flow rate and gradient separation, better resolution was achieved in monolithic columns. Thus, monolithic columns can be used for the quality control as it reduces the time and cost and are specific.

Keywords: RP-HPLC, Chromolith, *Heracleum candicans*, Monolithic, Furocoumarins

INTRODUCTION

Heracleum candicans Wall. ex DC. (syn. *H. lanatum* Michx, *H. nepalense* D. Don.) of the Apiaceae family is a large herb found in the temperate

Correspondence: Ajay K. S. Rawat, Scientist and Head, Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute, Lucknow 226001, India. E-mail: pharmacognosy1@rediffmail.com

forests of the Himalayan regions. In the Indian medicine system it is used as an aphrodisiac, nerve tonic, and also in the treatment of skin diseases.[1] The plant has been shown to possess a potent stimulatory effect on melanogenesis with significant enhancement of cell proliferation.[2] Dichloromethane extract of shoots of *H. candicans* has been reported for anti-inflammatory activity.[3]

Chemical investigation of the plant has revealed the presence of furocoumarins, viz., bergapten,[1] heraclenol, xanthotoxin, phellopterin, angelicin, imperatorin, xanthotoxol,[4] heraclenin,[5] candibirin,[6] and 8-gernoxypsoralen.[7] Activity guided isolation has also shown heraclinin to be the anti-inflammatory principle present in *H. candicans*.[3] Furocoumarins are polyphenolic compounds, synthesized from L-phenylalanine, which may occur in a linear form with the furan ring attached to the 6, 7 position of the benzo-2-pyrene nucleus. These are biologically active natural compounds found in many plants particularly in members of the Umbelliferae and Rutaceae family.[8,9] They are typical phototoxic compounds leading to photodermatitis upon exposure to UV light.[10] In humans and experimental animals, chronic furocoumarin treatment, in combination with UV light, is used in the therapy of psoriasis.[11] On the molecular level, furocoumarins bind to cellular constituents such as proteins, lipids, etc., and can damage lysosomes, leading to the formation of reactive oxygen species, and can contribute to the formation of novel antigens by covalent modification of proteins.[12] Furocoumarins of *H. candicans* are extensively used in the pharmaceutical industry for their photosensitizing activity on human skin.[13]

Considering the importance of furocoumarins and *H. candicans*, the same had been quantified using HPLC-PDA utilizing a simple RP-18 column.[14] The column used for the analysis plays a vital role in any HPLC analysis as the efficiency of separation and resolution determines the quantification of the markers. Different types of RP columns, based on the carbon chain length or the ligand chemistry of the columns, determines the efficiency and time of separation of the compounds through HPLC. There is a need to balance the efficiency of separation along with the cost and time involved while finalizing the protocol of the analysis. Thus, in the present paper, a comparison of 4 analytical columns (2 RP-8 and 2 RP-18) with different ligand chemistry has been carried out to find out which of these columns has better efficiency in separation, low cost, and has maximum precision.

EXPERIMENTAL

Chemicals

Bergapten was procured from Sigma Co. Ltd. Heraclenol was a generous gift from Dr. Khatewal of Kumaon University, Nainital. HPLC grade acetonitrile, water, and phosphoric acid were obtained from Merck (Darmstadt, Germany).

Plant Material and Extraction

The fruits of *H. candicans* were collected from Nainital, India during April–May, 2004, and the voucher specimen deposited in the herbarium of National Botanical Research Institute, Lucknow. The plant material was shade dried, powdered coarsely, and extracted at room temperature with methanol (100 mL × 3 times, 8 h each). The above extracts were pooled and concentrated at reduced temperature (45°C) on a rotary evaporator (Büchi, USA) and then freeze-dried (Freezone® 4.5, Labconco, USA under high vacuum (133 × 104 mBar) at a temperature of -40 ± 2°C to obtain 2.345% (DER). A simple mobile phase was used as the control to see the blank peaks.

HPLC-DAD System for Comparison of Columns

Analyses were performed in a liquid chromatograph with Waters (Milford, MA, USA) pumps (Waters 515) equipped with an online degaser, a Waters PCM, Rheodyne 7725 injection valve furnished with a 20 μL loop, a Waters 2996 photodiode array detector, and Waters Empower software. Separation was carried out using a Merck Purospher star® (250 × 4.6 mm, i.d., 5 μm pore size) RP-18 column, Sigma Supelco RP-8 column (250 × 4.6 mm, i.d., 5 μm pore size), Merck Chromolith RP-18e (100 × 4.6 mm), and Merck Chromolith RP-8e (100 × 4.6 mm) with guard columns of respective chemistries.

The HPLC finger print profile was established for furocoumarins. Elution was carried out at a flow rate of 0.5 mL/min with water: phosphoric acid (99.7:0.3 v/v) as solvent A and acetonitrile: water: phosphoric acid (79.7:20:0.3 v/v) as solvent B, using a gradient elution in 0–5 min with 30–20% A, 5–6 min with 20–19% of A, 6–7 min. with 19–17% of A, 7–10 min with 17–15% of A, 10–15 min with 15–12% A, 15–20 min with 12–0% A, and 20–25 min. with 0–70% A.

Calibration

The contents of the active furocoumarin compounds were determined using a calibration curve established with seven dilutions of each standard, at concentrations ranging from 2.5 to 200 μg/mL. Each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentration of the furocoumarins injected. Peak identification was achieved by comparison of both the retention time and UV absorption spectrum with those obtained for individual standards. The used reference substances were heraclenol and bergapten.

Linearity, Limits of Detection and Quantification

The linearity of the detector response for the prepared standards was assessed by means of linear regression regarding the amounts of each standard,

measured in μg, and the area of the corresponding peak on the chromatogram. Linearity was also confirmed for the extracts. After chromatographic separation, the peak areas obtained were plotted against the extract concentrations by linear regression. Limits of detection and quantification were determined by calculation of the signal-to-noise ratio. Signal-to-noise ratios of approximately 3:1 and 10:1 were used for estimating the detection limit and quantification limit, respectively, of the method.

Statistics

When applicable, one-way or two-way analyses of variance (SPSS11.0 for window) were used to assess the observed differences in the phenolic content. Differences were considered to be statistically significant when the P-value was <0.05.

RESULTS AND DISCUSSION

The concentration of heraclenol and bergapten was found to be 0.059 and 0.281%, respectively. The results indicate that there was a reduction in the RT of heraclenol from 5.4 min in a classical RP-18 column to 4.6 in the monolithic RP-18 column, similarly there was a reduction of the RT from 5.1 to

Table 1. Regression curves, linearity, limit of quantification (LOQ), limit of detection (LOD) and recovery of heraclenol

Column	*t*R (min)	R^2	LOD[a]	LOQ[a]	Recovery (%)
RP-18	5.491 ± 0.12	0.986	1.68	3.8	98.56 ± 1.45
RP-8	5.132 ± 0.25	0.975	1.54	3.9	97.45 ± 1.23
Chromolith RP-18e	4.645 ± 0.19	0.983	1.36	3.9	98.21 ± 1.51
Chromolith RP-8e	3.505 ± 0.16	0.977	1.21	4.1	98.67 ± 1.96

The retention times (*t*R) are the mean of 10 replicates ± S.D.
[a]Values expressed in μg/mL.

Table 2. Regression curves, linearity, limit of quantification (LOQ), limit of detection (LOD) and recovery of bergapten

Column	*t*R (min)	R^2	LOD[a]	LOQ[a]	Recovery (%)
RP-18	9.543 ± 0.06	0.998	2.92	4.9	95.79 ± 2.48
RP-8	9.108 ± 0.12	0.987	2.82	5.1	96.76 ± 1.98
Chromolith RP-18e	7.689 ± 0.23	0.975	2.56	5.3	98.24 ± 1.23
Chromolith RP-8e	4.719 ± 0.19	0.989	2.47	5.3	98.54 ± 0.87

The retention times (*t*R) are the mean of 10 replicates ± S.D.
[a]Values expressed in μg/mL.

3.50 min from classical RP-8 to monolithic RP-8 column. Also, there was a consistency in the R^2 and also in the recovery (Table 1). Similarly, there was a change in the RT of bergapten from RT 9.543 to 7.689 min in the case of RP-18 and from 9.108 to 4.719 in the case of RP-8, with no change

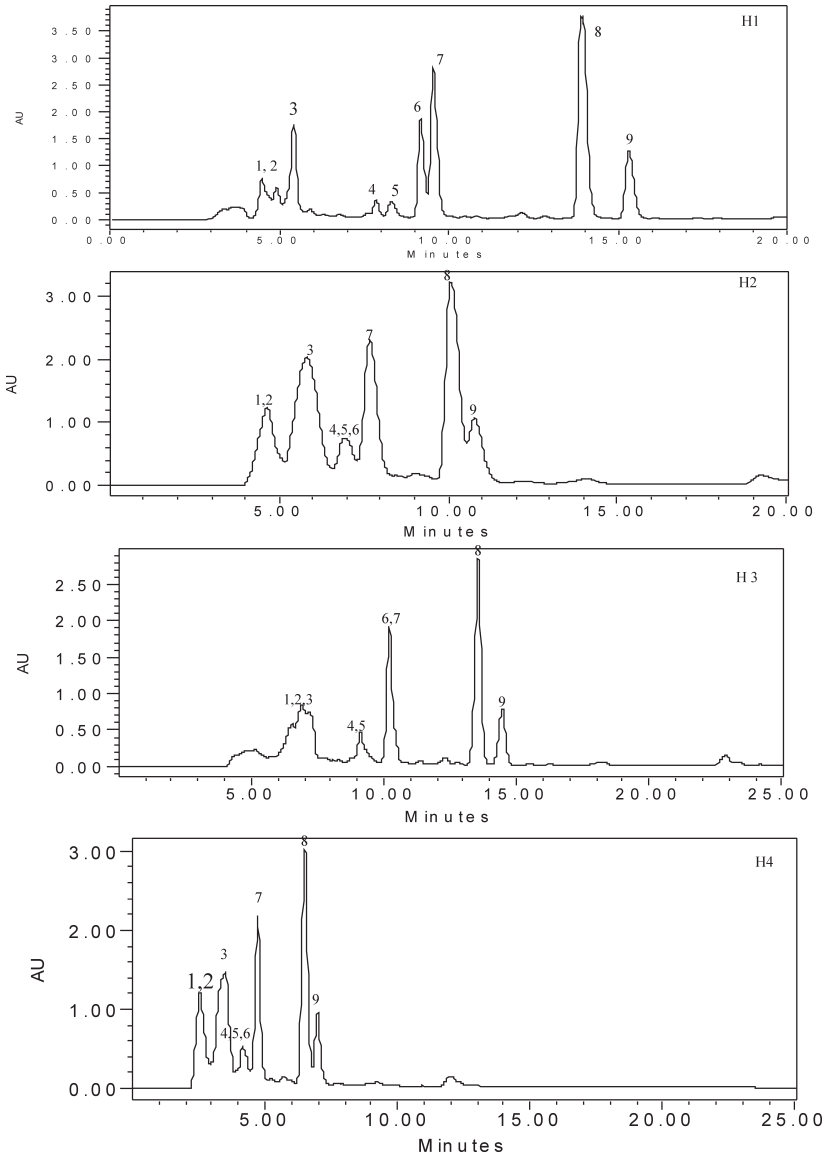

Figure 1. Chromatograms of the methanolic extract of *H. candicans* in various analytical columns. H1: RP-18, H2: Chromolith RP-18e; H3: RP-8; H4: Chromolith RP-8e; 3: Heraclenol; 7: Bergapten.

in the elution order of the components, although there is merging of some of the peaks (Table 2).

The use of HPLC columns containing the classic 3 or 5 μm small silica particles often results in high back pressure, which causes damage both to the column and the HPLC system, therefore, the classical columns have a limited length and limited number of theoretical plates. Particularly in the industry, workers are interested in finding ways to balance the need to analyze more samples with the limited financial and human resources available. Many of today's scientists wish to accelerate the entire separation process reducing the time, while at the same time getting the compound of interest separated.

The current analysis shows that reduction in the carbon chain length (from RP-18 to RP-8) causes a decrease in total analysis time from RT 15.309 to 14.460 min (compound 9) though there is a merger in some of the peaks, although the standard peaks are well resolved. Change in the ligand chemistry from the classical column to the monolithic columns caused a reduction in total analysis time from 15.309 min in the classical RP-18 to 6.988 min in the monolithic RP-8 column (compound 9) with no change in the elution order. The same is the case in the change in the carbon chain length within the monolithic columns (RT 10.776 to 6.998 min). Chromatograms in different columns showing the analysis time is depicted in Figure 1.

Thus, the present analysis clearly shows the importance of choosing the correct column and correct flow rate for achieving the desired efficiency in separation of the compounds, at the same time increasing the cost effectiveness and reducing the time of analysis.

ACKNOWLEDGMENTS

The authors are thankful to Dr. Khatewal of Kumaon University, Nainital for providing the standard heraclenol. DPS is thankful to CSIR, New Delhi for providing Senior Research Fellowship.

REFERENCES

1. Nath, Y.; Nazir, B.N.; Handa, K.L. Indian J. Pharm. **1961**, *23*, 303–304.
2. Matsuda, H.; Hirata, N.; Kawaguchi, Y.; Yamazaki, M.; Naruto, S.; Shibano, M.; Taniguchi, M.; Baba, K.; Kubo, M. Biol. Pharm. Bull. **2005**, *28*, 1229–1233.
3. Tembe, B.S.; Joshi, D.D.; Lakdawala, A.D. Indian J. Chem. B **1996**, *35*, 518–519.
4. Bandopadhyay, M.; Malik, S.B.; Seshadri, T.R. Indian J. Chem. **1973**, *11*, 410–412.
5. Sharma, Y.N.; Zaman, A.; Kidwai, A.R. Tetrahedron **1964**, *20*, 87–90.
6. Doi, M.; Nakamori, T.; Shibano, M.; Taniguchi, N.H.; Wang, K.; Baba, A.; Candibirin, A. Acta Crystallogr. **2004**, *60*, 833–835.

7. Sharma, Y.N.; Zaman, A.; Kidwai, A.R.; Bates, R.B.; Thalacker, V.P. Tetrahedron **1966**, *22*, 3221–3225.
8. Wagstaff, D.J. Reg. Toxicol. Pharmacol. **1991**, *14*, 261–272.
9. Guo, L.Q.; Yamazoe, Y. Acta Pharmacol. Sin. **2004**, *25*, 129–136.
10. Smith, A.M.J.; Poulton, G.A.; Barker, M.; Mildenberger, M. Nature **1980**, *285*, 407–409.
11. Baumgart, A.; Schmidt, M.; Schmitz, H.J.; Schrenk, D. Biochem. Pharmacol. **2005**, *69*, 657–667.
12. Schmitt, I.M.; Chimenti, S.; Gasparro, F.P. J. Photochem. Photobiol. **1995**, *B27*, 101–107.
13. Devagiri, G.M.; Chand, R.; Singh, J.M.; Pujar, G.S. Indian J. Chem. **1996**, *B35*, 878–879.
14. Govindarajan, R.; Singh, D.P.; Singh, A.P.; Pandey, M.M.; Rawat, A.K.S. Chromatographia **2007**, *43*, 401–405.

Received August 20, 2007
Accepted September 6, 2007
Manuscript 6192