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Development and Validation of Improved HPLC Method for the Quantitative Determination of Curcuminoids in Herbal Medicament

Richa Malasoni[a], Akansha Srivastava[a], Rishi Ranjan Pandey[a], Pradeep Kumar Srivastava[b] and Anil Kumar Dwivedi[a*]

[a]Pharmaceutics Division, [b]Medicinal and Process Chemistry Division, Central Drug Research Institute, (CSIR) Lucknow 226001, India

Correspondence-*Dr. A. K. Dwivedi, Pharmaceutics Division, CSIR-Central Drug Research Institute, Post Box No.173, MG Road, Lucknow – 226001, India. *Email- anilcdri@gmail.com.* ***Fax- +91-522-****91-522-2623938, 91-522-2623405*

Abstract

A simple and precise high performance liquid chromatography (HPLC) method was developed and validated for simultaneous trace analysis of the content of pharmacologically important active curcuminoids: curcumin (C), demethoxycurcumin (DMC) and bisdemethoxy curcumin (BDMC) in a novel standardized herbal preparation derived from the hexane soluble extract of *Curcuma longa,* tentatively assigned the generic nomenclature "herbal medicament" (HM). HPLC separation of active constituents was achieved on a Chromolith (100 x 4.6 mm, 2 µm, Merck) column using water-acetonitrile-acetic acid (60:40:1, v/v/v) as mobile phase, with UV detection at 425 nm. The method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), robustness, recovery, precision and accuracy. An empirical equation enabling calculation of the percentage content of DMC and BDMC from a calibration curve of C, without relying on reference standards of DMC and BDMC, is proposed on the basis of available evidence, suggesting that pharmacologically important curcuminoids may be accurately reported as proportions of a single constituent assayed quantitatively. Hence, quantitative estimation of trace constituents on the basis of empirical evidence for proportionate composition is suggested as a means of quantifying the key constituents in herbal preparation intended for pharmacological intervention in patients requiring treatment with HM.

Keywords: Chromolith column, Curcuminoids, Curcumin, Herbal Medicament, HPLC

Introduction

Curcuma longa (Zingiberaceae) rhizomes, commonly called Haldi, are used frequently in Ayurvedic and Unani system of medicine. Turmeric powder has been used for coloring, flavoring, cosmetics, digestive and for the treatment of various diseases and disorders particularly for urticaria, skin allergy, viral hepatitis and inflammatory condition for joints, sore throat and wounds. Recent studies have found several new and useful properties such as anticancer and antiviral. The bile output is increased by using turmeric, and it scavenges free radicals[1-5].

The essential oils of turmeric had shown to possess antimicrobial[6], antifungal[7,8], antiviral[9] and anti-inflammatory activity[10,11]. The curcumin have potential in the prevention of cancer and in the treatment of infection with human immunodeficiency virus (HIV) is the subject of intensive laboratory and clinical research[12-15]. The effect of curcuminoids on

the proliferation of MCF-7 human breast tumor cells was also examined and it was found that demethoxycurcumin was the best inhibition of MCF cells.

Our institute has developed a new standardized herbal preparation, Herbal Medicament, (HM) from the hexane soluble extract of *Curcuma longa*[16]. HM contains about 25 highly lipophilic compounds, in which, these three curcuminoids are present in trace amount.

Numbers of methods for the quantification of the curcuminoids in the alcoholic and other extracts have been reported in the literature. Some of these are spectrophotometric method, expressing the total color content of sample. HPLC[17], HPTLC[18-20] and UPLC[21] methods have been reported for the quantitative estimation of these curcuminoids in *Curcuma longa*. No method was reported in the literature for estimation of these curcuminoids in the hexane soluble portion of *Curcuma longa.* Therefore, the present method was developed by using a Chromolith column (MERCK), in which all the three curcuminoids shown base line separation without any interference from other components of HM. In this paper we had also derived equations capable of providing an alternative method of estimation of other two curcuminoid even without having their own reference standards (RS).

Materials and methods

Samples of HM from different batches were provided by the Medicinal and Process Chemistry Division of CSIR-CDRI, Lucknow, India. Acetonitrile, methanol and acetic acid were purchased from Merck (Mumbai, India). All other chemicals used were of analytical/HPLC grade. The working standard of C, DMC and BDMC were synthesized and purified by standard methods (*see below*).

The HPLC workstation was equipped with LC-10AT VP twin pumps, SPD-10A VP UV-VIS detector with SCL-10AVP system controller (Shimadzu, Japan) and a Rheodyne injector with a 20µl injection loop. Separation was achieved on a Chromolith column (100 x 4.6mm, 2µm, Merck, Germany).

Synthesis and purification of working standards of curcuminoids

Curcumin (C) and bisdemethoxycurcumin (BDMC)

Curcumin (1,7-*bis*-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione) and BDMC (1,7-*bis*-(4-hydroxyphenyl)-hepta-1,6-diene-3,5-dione) were synthesized by condensation of vanillin or 4-hydroxybenzaldehyde (2mM) and acetyl acetone (pentane-2,4-dione) (1mM) in presence of boric anhydride/tributyl borate (2mM) and butyl amine (1.5mM). The crude products so obtained were purified by column chromatography using silica as stationary phase and finally recrystallized using a mixture of methanol and chloroform to give yellow needle-like crystals, which were filtered and dried in a desiccator.

Demethoxy curcumin (DMC)

DMC (1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-hepta-1,6-diene-3,5-dione) was synthesized by condensation of vanillin (1mM) and acetyl acetone (1mM) as above, followed by treatment of the purified product with an equimolar proportion of 4-hydroxy benzaldehyde in the presence of boric anhydride/tributyl borate (1mM) and butyl amine. The mixture so obtained was purified by column chromatography followed by successive

recrystallizations using ascending proportions of methanol and chloroform and yielded an amorphous, dark orange solid.

Characterization and Purity

The purified compounds were characterized by IR, Mass and ^{1}H and ^{13}C NMR spectrometry. These compounds were subjected to TLC on precoated plate of silica gel (10x20 cm, Merck, Germany) with chloroform-methanol (99:1, v/v) as the developing solvent, visualized under UV or by exposure to iodine vapour. Their purity was also established by HPLC.

Standard stock solutions and sample preparation

About 2 mg RS of each C, DMC and BDMC were individually dissolved in 10 ml methanol. Working standards of different concentrations were prepared by serial dilution to obtain solutions in the range of 0.5-100 µg/ml. HM (~20 mg) was accurately weighed and dissolved in 2 ml methanol. Volume was made up to 10 ml with methanol in a volumetric flask. Blank samples were spiked with known concentrations of HM.

Validation

The accuracy of the method was calculated from the difference in the mean calculated and added concentrations and the precision by calculating the inter-day relative standard deviations (RSD). The absolute recovery was calculated as the percentage of the response observed in case of a processed spiked sample *versus* pure RS. Recovery was calculated from the peak areas of processed samples containing expected concentrations of C, DMC and BDMC.

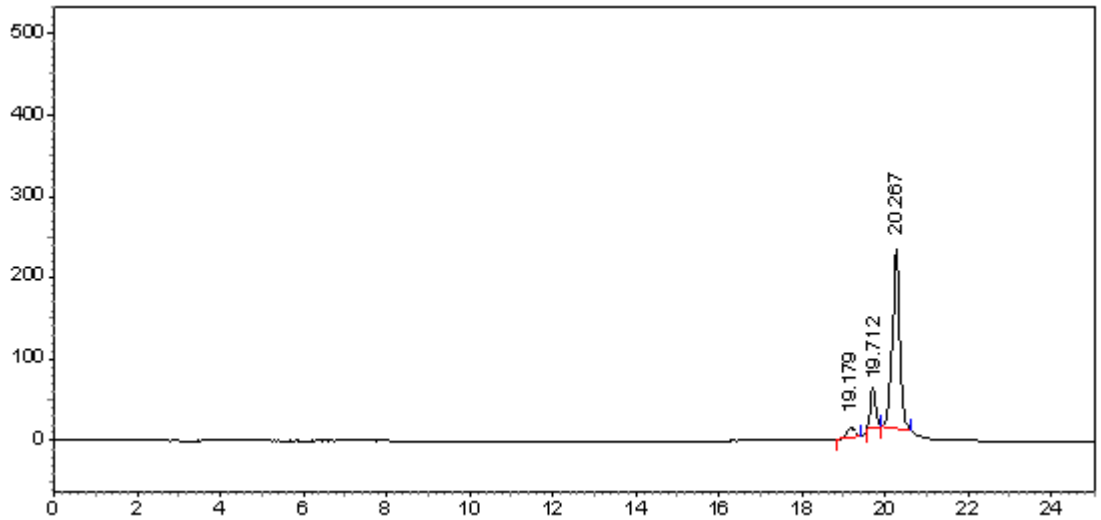

Fig. 1- Separation of Bisdemethoxycurcumin (BDMC), Demethoxycurcumin (DMC) and Curcumin (C) in standard curcuminoids at 425nm.

Results and Discussion

Characterization and purity of the curcuminoids

The melting points of C, DMC and BDMC were 182–184 °C, 171-174 °C and 222-225 °C respectively; in close correspondence to reported[22]. λ_{max} was 425 nm for all three. These prepared standards curcuminoids were also characterized by IR, Mass, ^{1}H and ^{13}C

NMR spectral analysis and the values were identical with reported compounds. The purity of these curcuminoids was established by TLC and HPLC. TLC and quantitative assay of the curcuminoids by HPLC showed a single peak for each standard. The purity of the three curcuminoids was more than 99%.

Validation of HPLC Method

HM contains more than 25 highly lipophilic compounds. Many of them have lipophilicity very close to curcuminoids and hence separation of these curcuminoids in HM by reported methods was not achieved. We tried Lichrospher RP-18 (250mm, 4mm, 5µM, Merck) column, which was not optimal for proper separation of curcuminoids from the other peaks. Broad tailing of peaks along with elevation of baseline was observed. Satisfactory separation was achieved with the Chromolith column (Merck). To optimize the HPLC parameters, different mobile phase compositions were evaluated. Finally, the optimized mobile phase was comprised of a mixture of water-acetonitrile-glacial acetic acid (60: 40: 1, v/v/v). The flow rate was 1ml/min and the eluents were monitored at 425 nm. The retention times of BDMC, DMC and C were 19.3, 19.8, 20.5 minutes respectively. Sharp peaks with a baseline separation were obtained as shown in Fig. 1. The peak symmetry was 1.2, 1.10 and 1.3 respectively. External standardization method was used for quantitation. The LOD and LOQ of analyte curcuminoids were calculated by diluting known concentrations until the signal to noise ratio was three and ten times respectively. The LOD and LOQ were 50 ng/ml and 100 ng/ml respectively for all three curcuminoids. Precision and accuracy, determined between concentration ranges of 0.5-10 µg/ml, were within the acceptable limits at all calibration points[23-25]. The calibration curves were linear within the concentration ranges of 0.5-10 µg/ml (r^2 = 0.9998, 0.9992, 0.9996 respectively). Recovery was within 98-103 %.

Extrapolation of DMC and BDMC from direct determination of C

The ratio of the areas of DMC or BDMC peaks versus the area of C at identical concentration levels was calculated. Within the concentration range of 2-10 µg/ml, mean values of 0.90 and 0.93 were obtained in the case of DMC and BDMC respectively. Therefore the amount of the other two curcuminoid can be calculated from the peak area of the parent curcuminoid, C, using the following equations:

Amount of DMC = amount of DMC calculated from calibration curve of C/0.90

Amount of BDMC = amount of BDMC calculated from calibration curve of C/0.93

These equations are valid for DMC and BDMC at concentrations > 4 µg/ml.

Several samples of HM bulk preparation were analyzed using the reported method. Therefore, this method can be applied to calculate the amount of DMC and BDMC in HM samples using a calibration curve of curcumin alone if reference standards of DMC and BDMC are difficult to procure and store.

Conclusion

A **s**ensitive and selective isocratic HPLC method was developed and validated for simultaneous determination of curcuminoids based on the calibration curve of curcumin

in the samples of HM. This method obviates the need for reference standards of DMC and BDMC.

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References

1 Tonnesen H H, Studies on curcumin and curcuminoids. XIII. Catalytic effect of curcumin on the peroxidation of linoleic acid by 15-lipoxygenase, *Int J Pharmacol*, **50** (1989) 67-69.

2 Kunchandy E & Rao M N A, Oxygen radical scavenging activity of curcumin, *Inter J Pharmacol*, **58** (1990) 237.

3 Sreejayan & Rao M N A, Nitric oxide scavenging by curcuminoids, *J Pharm Pharmacol*, **49** (1997) 105-107.

4 Chiranjib D B, Kumar K P S, Chandira M & Jayakar B, Turmeric: a herbal and traditional medicine, *Arch Appl Sci Res*, **1** (2009) 86-108.

5 Anand P, Thomas S G, Kunnumakkara A K, Sundaram C, Harikumar K B, Sung B, Tharakan S T, Misra K, Priyadarsini I K, Rajasekharan K N & Aggarwal B B, Biological activities of curcumin and its analogues (congeners) made by man and mother nature, *Biochem pharmacol*, **76** (2008) 1590-1611.

6 Patil S D & Kamble V A, Antibacterial activity of some essential oil against food borne pathogen and food spoilage bacteria, *IJPBS*, **2** (2011) 143-150.

7 Pundir R K & Jain P, Comparative studies on the antimicrobial activity of the black pepper (*Piper nigrum*) and turmeric (*Curcuma longa*) extracts, *IJABPT*, **1** (2010) 491-501.

8 Schraufstatter E & Bernt H, Antibacterial action of curcumin and related compounds, *Nature*, **164** (1949) 456-457.

9 Kim H J, Yoo H S, Kim J C, Park C S, Choi M S, Kim M, Choi H, Min J S, Kim Y S, Yoon S W & Ahn J K, Antiviral effect of *Curcuma longa* Linn Extract Against Hepatitis B Virus Replication, *J Ethnopharmacol***, 124** (2009) 189-196.

10 Shrimal R C & Dhawan B N, Pharmacology of diferuloyl methane (curcumin), a non-steroidal anti-inflammatory agent, *J Pharm Pharmacol*, **25** (1973) 447-452.

11 Ammon H P T & Wahl M A, Pharmacology of *Curcuma longa*, *Planta Med*, **57** (1991) 1-7.

12 Suz L, Craik C S, Oritz T & Montanello P R, *Proc 205th ACS National Meeting* (Denver Colo) 28 March-2 April 1993.

13 Soudamini K K & Kuttan R, Inhibition of chemical carcinogenesis by curcumin, *J Ethnopharmacol*, **27** (1989) 227.

14 Take Y, Inoyya H, Nakamura S, Alauddin H S Kuba A, Comparative studies of the inhibitory properties of antibiotics on human immunodeficiency virus and avian myeloblastosis virus reverse transcriptases and cellular DNA polymerases, *J Antibiot*, **42** (1989) 107-118.

15 Desai K, Curcumin cyclodextrin combination for preventing or treating various diseases, *US Patent 20100179103,* 15 July 2010.

16 Ray M, Pal R, Singh S & Khanna N M, *US Patent US 6,991,814 B2,* 31 January (2006).
17 Jayaprakasha G K, Rao L J M & Sakariah K K, An improved HPLC method for the determination of curcumin, demethoxycurcumin and bisdemethoxycurcumin, *J Agric Food Chem*, **50** (2002) 3668-3672.
18 Paramasivam M, Akhtar M D W, Poi R, Banerjee H & Bandyopadhyay A, Occurrence of curcuminoids in *Curcuma longa*: A quality standardization by HPTLC, *Bangladesh J Pharmacol*, **3** (2008) 55-58.
19 Gantait A, Barman T & Mukherjee P K, Validated method for estimation of curcumin in turmeric powder, *IJTK*, **10** (2011) 247-250.
20 Gupta A P, Gupta M M & Kumar S, Simultaneous determination of curcuminoids in *Curcuma* samples using high performance thin layer chromatography, *J Liq Chrom & Rel Technol*, **22** (1999) 1561-1569.
21 Cheng J, Weijun K, Yun L, Jiabo W, Haitao W, Qingmiao L & Xiaohe X, Development and validation of UPLC method for quality control of *Curcuma longa* linn, fast simultaneous quantitation of three curcuminoids, *J pharmaceut biomed anal*, **53** (2010) 43-49.
22 Karnes H T & March C, Precision, accuracy and data acceptance criteria in biopharamceutical analysis, *Pharm Res*, **10** (1993) 1420-1426.
23 Shah V P, Midha K K, Findlay J W A, Hill H M, Hulse J D, Mcgilveray I J, Mckay G, Miller K J, Patnaik R N, Powell M L, Tonelli A, Viswanthan C T &Yacobi A, Bioanalytical method validation-a revisit with a decade of progress, *Pharm Res*, **17** (2000) 1551-1557.
24 ICH guidelines on validation of analytical procedures: methodology, *Federal Register*, **60** (1995) 11200-11262.
25 ICH guidelines on validation of analytical procedures: definition and terminology, *Federal Register,* **62** (1997) 27464-2746.