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Der Pharmacia Lettre, 2010, 2(6): 316-320
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

A validated HPTLC method for estimation of Eucalyptol in polyherbal formulations

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ABSTRACT

'Amukkara choornam' is a popular polyherbal Siddha formulation used for gastritis, spleen enlargement, leucorrhoea, hiccup, anemia, tuberculosis and kappa diseases. Eucalyptol in one of the prevalent phytoconstituent present in the ingredients of this formulation. A sensitive, selective and precise thin-layer chromatographic method has been developed and validated for the analysis of eucalyptol in Amukkara choornam. Separation and quantification was achieved by TLC using mobile phase of ethanol-ethyl acetatate (9:2, v/v), (R_F 0.38) on precoated silica gel 60F_{254} aluminium plates and densitometric determination was carried out in reflection/absorption mode at 343 nm. The calibration curve was linear in the concentration range of 4.612ng-55.344ng spot $^{-1}$. The method was validated for precision, repeatability and accuracy. The proposed method was found to be simple, precise, specific, sensitive and accurate for the quantification of eucalyptol.

Key words: Eucalyptol; *Amukkara choornam;* Siddha formulation; HPTLC.

INTRODUCTION

Standardization of herbal formulations is essential in order to assess the quality of drugs, based on the concentration of their active principles. The increasing demand of the population and the chronic shortage of authentic raw material have made it incumbent. Therefore, the uniformity in the manufacture of herbal medicines is prerequisite [1]. *Amukkara choornam* is a popular marketed siddha formulation used for gastritis, spleen enlargement, leucorrhoea, hiccup, anemia, tuberculosis and kappa disease, with different ingredients like *Syzygium aromaticum* Linn. (Myrtaceae), *Cinnamomum wightii* Blume. (Lauraceae), *Elettaria cardamomum*(Linn.) Maton (Zingiberaceae), *Piper nigrum* Linn. (Piperaceae), *Piper longum* Linn. (Piperaceae), *Zingiber*

officinale Rosc. (Zingiberaceae), *Withania somnifera* (Linn.) Dun. (Solanaceae) and Cane sugar in geometric proportion [2].

Preliminary phytochemical screening, TLC fingerprinting and co-TLC studies (with eucalyptol) of *Amukkara choornam* revealed the presence of a fairly high content of volatile constituents and an identical spot as that of standard eucalyptol was observed. Further it was confirmed by R_F comparison, multi wavelength scanning and spectral overlay. Eucalyptol (1, 3, 3-trimethyl- 2-oxabicyclo [2, 2, 2] octane), is one of the most prevalent volatile constituent, which is found in numerous plants. Eucalyptol has an amazing array of scientifically acknowledged benefits for key areas of health in the treatment of respiratory ailments, mucus hypersecretion, asthma via anti-inflammatory cytokine inhibition [3,4], nonpurulent rhinosinusitis [5] and reduces inflammation and pain when applied topically [6]. Further due to its pleasant spicy aroma taste, eucalyptol is used in flavorings, fragrances, and cosmetics. Further more eucalyptol is used as insecticide, insect repellent and toxic if ingested at higher than normal doses [7, 8]. So quantification of this active principle is essential in food products, crude drugs and in polyherbal formulations. It has been investigated that there is only GC and HPLC method is available for determination of eucalyptol [9, 10]. In this context we have developed a validated HPTLC method for the analysis of eucalyptol. Once the method is developed, extraction parameters optimized to obtain a fast and complete extraction of the eucalyptol in the compound herbal formulation, method validated on the basis of its linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy according to ICH requirements, it can successfully be applied for the standardization of '*Amukkara choornam*' for the content of eucalyptol.

MATERIALS AND METHODS

Collection of Samples

'*Amukkara choornam*' samples were collected from different manufacturers of India viz., Aravindh Herbal laboratories (P) Ltd. (sample-1); Rajapalayam, south India (sample-2); SKM Siddha and Ayurvedic Medicines India, Pvt. Ltd. (sample-3); In House preparation (sample-4). For in house preparation the ingredients were collected from the local raw traders and tribal belt of Ranchi, Jharkhand and authenticated by Dr. S. Jha, Department of Pharmaceutical sciences, BIT, Mesra, Ranchi.

Chemicals

All the chemicals, including solvents, were of analytical grade from Sigma Aldrich, India. Eucalyptol was obtained from Shree Bankey Behari Lal Board Mills, Ghaziabad, Uttar Pradesh, India. The HPTLC plate silica gel $60F_{254}$ (20cm×20cm) was purchased from E.Merck, Darstadt, Germany, supplied by anchrom technologies, Mumbai.

Preparation of extract for analysis of eucalyptol in Samples

To determine the content of eucalyptol in the formulation 2g powder was extracted with ethanol. To ensure complete extraction of the drug, it was sonicated for 30 min and volume was made up to 30 ml. The resulting solution was centrifuged at 3000 rpm for 5 min and supernatant was analyzed for drug content. The filtered solution was applied on TLC plate followed by development and scanning. Analysis was repeated in triplicate [11].

Preparation of standard solution

A stock solution of 0.05 ml (0.046125mg)/10ml of eucalyptol was prepared in toluene, and different amounts 1,5,10 and 12 µl were spotted in triplicate on TLC plate, using Camag Linomat IV sample applicator (Camag, Muttenz, Switzerland) and a 100µl Hamilton syringe for preparing five point calibration curve.

HPTLC instrumentation

The HPTLC system (Camag, Muttanz, Switzerland) consisted of (i) TLC scanner connected to a PC running WinCATS software under MS DOS; (ii) Linomat IV sample applicator using 100µl syringes and connected to a nitrogen tank. Each plate accommodated 8 tracks of samples and standards, applied according to following settings: band width 5 mm; distance between bands 10 mm; application volume 1-12 µl; gas flow 150 nl/s. The plates were developed to 7 cm in a twin trough glass chamber presaturated with the upper layer of mixture ethanol-ethyl acetate (9:2, v/v). Antimony (III) chloride was sprayed on the developed plate, for full colour development the plate was kept at 100^0c for 10 mins in oven. The scanner was set for maximum light optimization and with the following settings: slit dimension, 5.00 mm × 0.45 mm, micro; scanning speed, 20 mm/s; data resolution, 100µm/step; scanning wave length, 343 nm in absorbance reflectance mode. All remaining measurement parameters were left at default settings. Regression analyses and statistical data were generated by the WinCATS Planar chromatography version 1.1.4.0 software.

Development of the optimum mobile phase

The composition of the mobile phase for development of chromatographic method was optimized by testing different solvent mixtures of varying polarity.

Calibration plot of eucalyptol

A stock solution of 0.05 ml (0.046125mg)/10ml of eucalyptol was prepared in toluene. Different volumes of stock solution, 1,5,10 and 12 µl were spotted in triplicate on TLC plate to obtain concentrations of 4.612, 23.060, 46.125 and 55.344ng $spot^{-1}$ of eucalyptol, respectively. The data of peak area versus drug concentration were treated by linear least-square regression.

Purity of Spot in chromatogram

The spot obtained on the chromatogram were analysed at wavelength 343 nm at three points in the standard as well as in sample i.e. in the point start to middle, middle and finally in the middle to end.

Validation of the method

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 ng, and the area of the corresponding peak on the chromatogram (n=4). Linearity was also confirmed for '*Amukkara choornam*' extract. 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.

Accuracy

Recovery studies were carried out to check accuracy of the method. Recovery experiments were performed by adding three different amounts of eucalyptol i.e., 25, 50 and 75% of the amount of eucalyptol analysed from different formulations and result was analysed (n=6).

Precision

The intra-day precision was evaluated by analysing eucalyptol repeatedly at concentration range of 5-50 ng/spot (n=5). The inter-day precision was evaluated by analysing eucalyptol at concentration range of 5-50 ng/spot over a period of 10 days (n=5).

RESULTS AND DISCUSSION

The mobile phase ethanol: ethyl acetate (9:1, v/v) provided good resolution with R_F value 0.17 for eucalyptol but typical peak nature was missing. Finally, the mobile phase consisting of ethanol-ethyl acetate (9:2, v/v) provided a sharp and well defined peak at R_F value 0.38 **(Figure 1A, 1C).** The well defined spot was obtained when the chamber was saturated with the mobile phase for 15 min at room temperature.

Eucalyptol showed a good linear relationship over the concentration range 4.612ng-55.344ng per spot with respect to peak area (n=3). The linearity was observed with the regression coefficient being 0.9996 with Standard error of the mean (SEM) of 0.0034. No significant differences were observed in the slopes of standard curve. Purity of each spot which is scanned at wave length 343nm with value of r (S, M) within the range, 0.998-0.999 and r (M, E) within the range, 0.997-0.998**(Figure 1B).**

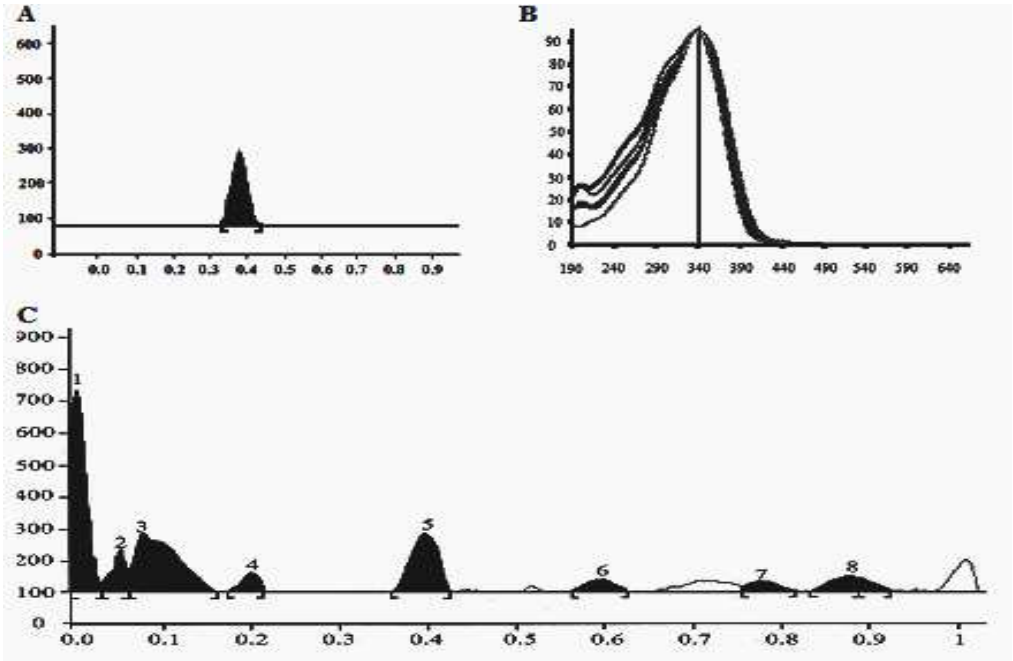

Figure 1A. HPTLC chromatogram of eucalyptol at 343 nm (R_F = 0.38) B. Absorption spectra of eucalyptol (λmax= 343nm. C. Chromatogram for '*Amukkara choornam* ethanolic extract' at 200-400 nm, showing eucalyptol (R_F = 0.38)

LOD, LOQ, accuracy and precision were evaluated for quantitative purposes. LOD (0.101ng) and LOQ (0.639 ng) suggests high efficiency of the process. Accuracy in terms of % recovery (97.19±1.204) which shows high extraction efficiency of eucalyptol from formulation components. The % coefficient of variance for intra-day and inter-day precision was found to be 4.2 and 5.2 respectively which is comparable and within the limits. Hence, the proposed method can be used for estimation of eucalyptol in polyherbal formulations. The eucalyptol content in different formulations was found to be 3.37±0.046 (sample-1), 3.78±0.052 (sample-2), 4.13 ±0.063 (sample-3) and 3.66±0.075 (sample-4) in µg /g sample. Which clearly indicates that there is no uniformity in preparation of formulations, it may be due to varied geographical locations where these plants grow, coupled with the problem of different vernacular names these plants are known by, a great deal of adulteration or substitution is encountered in commercial market [12].

The proposed HPTLC technique is found to be precise and accurate. Further, the method is sensitive for the analysis of eucalyptol in pharmaceutical formulations. Since some of plants used in the *Amukkara choornam* also contain eucalyptol for example *Elettaria cardamomum* Maton var, which can also be standardized by using this method for the content of eucalyptol. With the growing demand of herbal drugs in the herbal drug market and with the increased belief in the usage of green medicine (herbal drugs), this standardization tool will help in maintaining the quality and batch to batch consistency of this important siddha preparation.

CONCLUSION

The HPTLC method developed here for the quantification of eucalyptol in *Amukkara choornam* is simple, rapid, cost-effective and easily adaptable for screening and quantitative determination than any other analytical technique.

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