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Chromatographic methods used for characterization of boswellic acids

Abstract

Utilization of natural herbs and their drug products have been extensively increases worldwide because of lesser side effects of natural origin–based products in contrast with synthetic products. Gum resin of plant *Boswellia serrata* constitutes the major constituents Boswellic acids which are pentacyclic triterpenoids belonging to ursane group. Herbal medicines composed of various constituents and suffer from constraints of variability in their compositions. In order to make availability of herbal drugs, various chemical and instrumental methods have been used at regular intervals for the standardization and characterization of herbal drugs. Chromatography is very popular and mostly employed analytical technique for standardization and the separation of mixture of constituent's in herbal drug products. Current manuscript provides concise overview and trends of separation, identification and quantification of boswellic acids by modern chromatographic techniques including HPLC, HPTLC and LC/MS with significant research studies.

Keywords: boswellic acids, characterization, HPLC, HPTLC, LC/MS

Volume 2 Issue 4 - 2018

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Received: June 26, 2018 | **Published:** July 11, 2018

Introduction

Herbal medicines obtained from plants are an important source for discovery and development of new therapeutically active agents. Herbal products still remain as one of the best reservoirs of new drug molecules to make them available by using different approaches. The standardization is valuable tool for qualitative and quantitative estimation of plant extracts, which provides wide variety of prospective for novel drug molecules.[1] Despite the continuous advancements in area of medicinal field, public interest and acceptance towards herbal drug products in both developing and developed countries is increasing. World Health Organization (WHO) has made estimation that approximately 80% of the population globally still uses natural medicines and natural source based medicines for their primary health care. Herbal medicine and their preparations have been widely used for the thousands of years in developing and developed countries owing to its natural origin and lesser side effects or dissatisfaction with the results of synthetic drugs.[2–3] Herbal medicines continuously contributing an important role in production of new active agents as clinically effective therapeutic agents including starting materials to produce synthetic drugs.[4] Herbal products have reached widespread acceptability as therapeutic agents for various complex and life–threatening diseases.[5–6] *Boswellia serrata* plant belonging to the family *Burseracea* is one of the medicinal plants of the genus *Boswellia* and approximately about 20 species are known for their medicinal value.[7–8] An oleo–gum–resin obtained from *Boswellia serrata* BS (Salai guggal) also known as Frankincense (English) and Olibanum (Arabian). In India *B serrata* occurs in the dry hilly forests of Bihar, Madhya Pradesh, Gujarat, Rajasthan etc. Documented literature revealed the highly accepted medicinal applications for various diseases including cancer, inflammation, arthritis, asthma and hyperlipidemia. Among all species of genus *Boswellia*, *B serrata* is the most investigated in terms of phytochemical and bioactivity related studies.[9] Major phyto constituents of gum resin of *Boswellia* plant are Boswellic acids (BAs) with the molecular formulas of $C_{32}H_{52}O_4$ (pentacyclic triterpenoids) belongs to ursane group.[10] β–boswellic acid (BBA), acetyl–β–boswellic acid (ABBA), 11–keto–β–boswellic acid (KBA) and 3–O–acetyl–11–keto–β boswellic acid (AKBA) are well reported four main BAs, among them AKBA have good therapeutic value for anti–inflammatory diseases, anticancer, anti–arthritis, bronchial asthma, chronic colitis, ulcerative colitis.[11–14]

Need of phytochemical analysis

Assurance of quality, efficacy and safety of natural products has become crucial parameters for commercial acceptability of plant products in modern system of medicine. Genotypic, ecotypic drying and storage conditions variations of natural raw materials leads to variations in the constituents present in herbal drug product. Therefore, quantitative and qualitative characterization of herbal raw products, estimation of the biomarkers and/ or different chemical marker compounds and determination of fingerprinting profiles is necessary for adequate supply of phyto constituents. Both qualitative and quantitative analysis is done where an active constituent is not known, standard marker compound could be chosen for analysis use which should be specific for the particular active compound. In case, if therapeutically active constituent present in herbal raw extract is known then quantification of this compound is important characterization parameter from analytical point of view.[6,15] Selection of particularly effective standardization technique should take in to consideration which may affect assessment of the quality and quantity of constituents of the natural plant products. Today, modern analytical chemistry involves utilization of advanced analytical instruments for standardization of herbal drugs.[16,17] Drug discovery and development process includes modern scientific investigation at every step such as analysis of biological samples, bulk drug materials, intermediates of drug products, marketed products, formulations, impurities and degradation products.[15,18]

Different constraints affecting the quality of herbal drugs

a) Generally, mixture of different constituents is present within the raw plant extract.

b) Suitable analytical technique or standard marker compounds may not available commercially.

c) Mostly the active constituent is not known.

d) Chemical and natural variations affect the quality of herbal products.

e) Various factors such as harvesting method, drying, transportation, storage, and processing parameters including extraction technique and polarity of the solvent, extraction temperature etc. have great impact on quality and quantity of herbal drugs. Thus, for assurance of repeatability and reliability of phyto constituents of natural drug products and to identify their therapeutic activities and side effects it becomes necessary to develop suitable analytical approach for pharmacological and clinical research. Various chromatographic and spectrophotometric methods have been used for qualitative and quantitative analysis herbal medicines.[19]

Chromatographic analysis

Chromatography is mostly used suitable analytical technique to separate different compounds of a mixture on the basis of differences in their structural composition. Basic principle of this technique involves application of mixture of compounds to be separated over a stationary phase and different compounds have different interactions on the basis of their structural composition with the stationary support. Separating compounds which have greater affinity towards the stationary phase will move slowly while other having low affinity will move faster. Depending upon the affinity of test compounds towards stationary support a mixture of compounds can be separated from each other as they move over the stationary support.[19] Different stationary phases and mobile phases used in chromatography are represented in Table 1.

Thin layer chromatography

Thin Layer Chromatography (TLC) is one of the simple and most popularly employed chromatographic techniques for separation of a wide variety of organic and inorganic materials. TLC is effective fingerprint technique for both qualitative and semi quantitative in area of herbal drug technology. Generally, finely powdered alumina or silica particles adsorbent (polar absorbent), is coated onto a glass, plastic plate in the form of thin layer and mobile phase can be consists of a single solvent or combination of solvents.[20–23] Organic compounds can be separated by changing ratio of different solvents and a number of solvent mixtures can be utilized as the mobile phase for separation. Efficiency of chromatographic separation depends on various factors such as separating molecules selectivity towards adsorbent, which ultimately results in differences in the rate of elution of the substance from the mixture.

Advantages of thin layer chromatography

- Rapid analysis of herbal drug extracts with minimum sample clean–up requirement
- Effective approach for screening of unknown materials in bulk drugs
- TLC analysis gives qualitative and semi quantitative information
- Reaction progress can be monitored
- Determination of pharmaceuticals impurities
- A variety of solvents can be use
- Flexibility in sample distinction
- Effective technique to provide information in the early stage of drug development
- Low cost

Table 1 Commonly used stationary phase and mobile phase with increasing polarity

Stationary phase	Mobile phase
Polydimethylsiloxane	Helium
Methyl/Phenyl siloxane	Nitrogen
Cyanopropyl siloxane	Petroleum ether (pentanes)
Carbowaxes (Polyethylene glycol)	Hexanes
Hydrocarbon–coated silica e.g. C–18	Cyclohexane
Paper	Carbon tetrachloride
Cellulose	Toluene
Starch	Chloroform
Calcium sulfate	Dichloromethane (methylene chloride)
Silica (silica gel)	t–Butyl methyl ether
Florisil (magnesium silicate)	Diethyl ether
Magnesium oxide	Ethyl acetate
Alumina (aluminum oxide; acidic, basic or neutral)	Aniline
Activated carbon	Acetonitrile
C–18 (250cm × 4.6mm × 5µm)	0.1:99.9 v/v (Phosphoric acid:Water) and 0.1:99.9 v/v (Phosphoric acid:Acetonitrile
Kromasil 100 C18	Acetonitrile: water (90:10 v/v)
RP–18 column (2.1×100mm, 1.7µm)	Acetonitrile: water (0.1% Acetic acid)
RP–18, Merck column (4× 250mm, 5µm	Acetonitrile: 0.5% acetic acid in water (95:5)
RP C18 column (100×4mm; 3µm	Mobile phase A (methanol: water 90:10, 400 mg/L ammonium formate) and mobile phase B (methanol: water 80:20, 400 mg/L ammonium formate)
–	Ethanol
–	Methanol
–	Water
–	Acetic acid

Depending upon structural composition of compounds present within mixture some compounds strongly adsorbed on the adsorbent while others adsorbed poorly. An ideal solvent system used for chromatographic separation should travel all compounds of the mixture off the baseline, but does not put anything on the solvent front. Retention factor value of compounds should resolve between 0.15 and 0.85. Polar or non–polar nature of the mobile phase provides eluent

strength which affects the elution power of the mobile phase. Faster will be elution of the separating compound if compound have higher non polar nature because of poor affinity towards stationary phase or less time it will remain on the stationary phase.[24–27] have conducted pharmacognostical study of counting both macroscopic and powder microscopy of oleo gum resin of plant BS. TLC was carried out on different extractive samples after selection of appropriate solvent system for confirmation of presence of certain constituents in the extract. The spots obtained from both the extracts were observed under UV light of wavelength 254nm and 366nm. TLC confirmed the presence of different constituents in the test samples and is helpful for easy preliminary identification of phyto chemicals in the drug samples.[28]

High performance thin layer chromatography

Increasing advancements towards instrumental analysis high performance thin layer chromatography (HPTLC) arises as promising technique for rapid separation with flexibility for analysis a range of samples. Different samples can be analyzed simultaneously by using less amount of mobile phase as compared with High Performance Liquid Chromatography. HPTLC have number of advantages as it requires less time for analysis of crude or complex samples, easy to handle. Variety of analysis parameters can be evaluated by use of entire chromatogram without time limits. Simultaneously different samples and standards can be developed on each plate. This is effective technique to provide reliable results of test samples.[29,30] Chromatogram can be repeatedly scanning under same or different conditions by HPTLC. Multi compound formulation has been simultaneously assayed by this technique. HPTLC is advanced tool for authentication, consistency and stability analysis of plants along with their preparation from different manufacturers.[31,32]

Salient features of HPTLC as analytical technique

It shows better accuracy and precision for simultaneously processing of both sample and standard compounds.

a) Different samples analyzed at same time.

b) Economical process for analysis of different samples as low maintenance cost.

c) Pre–treatment like filtration and degassing is not required for solvents used and sample preparation is simple for different nature samples.

d) Low mobile phase consumption per sample.

e) There is no contamination occurs and free from interference of previous analysis.

f) HPTLC is an open system make availability of visual detection.

g) Compounds having absorption in UV resgion detected by post chromatographic derivatization.[33–36]

Significant research work has been done in the arena of characterization of BAs. Here, we are describing some of the endeavor related to characterization of BAs by different chromatographic techniques. Conducted comparative analysis of HPTLC and HPLC for the estimation of KBA and AKBA in gum resin extract. Purity of KBA and AKBA was determined by UV detection assays by comparison of peaks obtained from test samples with standard marker compounds. Data obtained for the study revealed that there was no significant difference in results of both analytical methods between content of KBA and AKBA, and a slightly less quantity of KBA and was found by HPTLC method.[37] developed and characterize lipid based drug delivery system of *Boswellia serrata* extract for enhancement of solubility in turn to enhance the oral absorption. Suitable composition for lipidic formulation were screened via solubility and compatibility studies. Concentration of AKBA and release of AKBA from formulation was estimated by HPTLC method using chloroform and methanol as mobile phase. The concentration of AKBA in gum resin extract and formulation was found to be 3.94±0.22 and 0.95±0.17w/w respectively.[38] quantified boswellic acids content by HPTLC in the plant extract of BS by development of fingerprinting profiles of gum extract. Different solvents such as chloroform, methanol, and petroleum ether extracts were used for the fingerprint development. Linearity, correlation coefficient and least square regression equation was obtained by using peak area versus drug concentration from calibration curve. Different boswellic acids qualitatively and quantitatively in *Boswellia* extract were estimated by this analysis and this technique is able to separate the four boswellic acids in standard and samples.[39] Pawar and his coworkers developed a simple, rapid and selective HPTLC method for quantitative estimation of BBA in different samples of *Boswellia serrata* (BS). Standard and samples spots of BBA were scanned at 530nm wavelength. Recovery experiment was used for the establishment of the accuracy and reproducibility of the method. 100% mean recovery was obtained which indicates the accuracy of the method.[40] Identified pure AKBA in *Boswellia serrata* extract [BSE] by HPTLC technique using toluene–ethyl acetate 7:3 (*v/v*) as mobile phase and aluminium plates coated with silica gel as stationary phase. Presence of single sharp peak (Rf value 0.52) confirmed the purity of AKBA extracted from BSE.[41] Simple and rapid HPTLC method developed by for determination of BA, curcumin and piperine content in herbal formulation prepared for the treatment of arthritis. Sharp and intense peaks were obtained by densitometric analysis for BA, curcumin and piperine (Rf value of 0.61±0.03, 0.48±0.02 and 0.52±0.03 respectively). Developed HPTLC method was found to be accurate and reproducible and it would be an effective tool in the quality control method for poly herbal formulations.[42] separated and quantified BAs in BSE by HPTLC method. Separation of active BAs in the extract was done by TLC analysis by automated multiple development (AMD) using gradient method with the help of densitometric analysis.[43]

High performance liquid chromatography

In biological and chemical systems identification, estimation and separation of molecule and complexes of molecules is completed by use of advanced form of liquid chromatography recognized as High Performance Liquid Chromatography (HPLC). The technique, chromatography was originally developed by the Russian botanist M.S Tswett in 19031 but HPLC methods was appeared first time for the assay of bulk drug materials in 1980. Isolation, purification and standardization of herbal medicines in pharmaceutical industries are carried out by utilization of preparative and analytical HPLC methods. Basically low pressure HPLC especially under 5 bar and high pressure HPLC under pressure >20 bar have been employed for analysis of different components. Analytical and preparative HPLC is used for qualitative and quantitative estimation and isolation and purification of drug molecules respectively. Fast analysis, sensitivity and high resolution are the key parameters to be taken into consideration for effective qualitative and quantitative analysis by HPLC.[44]

Advantages of HPLC

- Sample resolution is high.
- Detection of very small sample quantity is possible by minimization of flow rates
- Small diameter (4.6mm), stainless steel, glass or titanium columns.
- HPLC can be used by normal and reverse phase methodology
- Column packing with very small (3, 5 and 10μm) particles.
- Well controlled flow rates and maintenance of relatively high inlet pressures provides efficient analysis of the samples
- Rapid qualitative and quantitative analysis of the samples.[45]

Studied comparative TLC and HPLC analysis on the composition of different dry and aqueous extracts of BS gum resin as a tool for the evaluation of the quality of the extracts. At 260nm, the majority of extracts presented two major peaks: the first one with retention time of 13.2min, and the second one, identified as AKBA by the use of an analytical standard, with retention time of 26min. Combination of TLC and HPLC analyses can be considered as a multidimensional analytical approach combining fast qualitative screening with an accurate and precise quantification of specific compounds.[46] Sharma and his co–workers developed HPLC method for quantitative estimation of main BAs in BS gum resin extract. Various extraction processes were used by researchers for separation of BAs from gum resin. Calibration curves were plotted and showed good linear regression with R^2 value of about 0.997 within the limits with good precision and overall recoveries. The developed HPLC method provides quantitative estimation of BAs in appreciable amounts in the marketed formulations and extracts.[47] In another study prepared AKBA nano particles for the management of cerebral ischemia–reperfusion injury. HPLC method was used for the calculation of the encapsulation efficiency and loading capacity of prepared nano particles. Non–compartment model was used for the evaluation of pharmacokinetic parameters. Trapezoidal rule was used for the estimation of the area under plasma concentration time.[48] further studied anti–inflammatory activity in the form of herbal gel for management of gout. The physicochemical properties of drug were evaluated on the basis of solubility, UV, FTIR, HPLC and DSC. Analysis of drug sample was done by HPLC technique with an attached UV detector, on a reverse phase column with Mobile phase in 90:10 ratio (Acetonitrile: water). The drug interaction FT–IR studies and DSC indicated that there was no chemical interaction between the drugs and the additives used in gel formulations.[49] developed HPLC method for simultaneous estimation of BA and myristicin in herbal formulation used for the management of rheumatoid arthritis. Developed method was validated in terms of its accuracy, precision, selectivity, repeatability and recovery. Good linear correlation coefficients values for calibration curves were obtained (r^2>0.995). Estimation of two marker compounds in market formulation was done with respect to boswellic acid and myristicin. The developed method leads to simultaneous determination of two markers in prepared formulation and marketed formulation.[50] Moreover estimated concentration of different BAs extracted from callus cultures treated with different biotic and abiotic elicitors by HPLC method. HPLC chromatogram of standards with test samples were compared for retention time and peak area.[51] A rapid and sensitive HPLC method was developed and validated for the quantitative analysis of BAs in formulation containing BSE by. The limit of detection and limit of quantification were found to be (0.01740, 0.05273, 0.01739 and 0.05270 mg^3/mL) for α and β boswellic acid, respectively. The developed HPLC method was successfully utilized for the assay of marketed preparations having BSE.[52] Shah and his co–workers developed reverse phase HPLC method for the quantification of BAs present in extract of gum resin. Calibration curves were constructed for the quantification of components and retention time for KBA and AKBA was 4.3 and 7.11min respectively. Developed method was highly useful for estimation of BAs and total run time was reduced by this method and makes it economically effective.[53] explored pharmacokinetic parameters by using (Wok Vel.) BSE capsules. Twelve healthy adult men volunteers were selected for the study and KBA concentration was quantified in plasma by HPLC method. Different kinetic parameters were then calculated from the plasma concentrations of volunteers.[54] HPLC method was employed for separation of BAs, from BSE as active constituents by Ganzera and Khan. Six BAs were accurately determined in the BSE and in multi constituents' formulation. Analysis of marketed products revealed considerable difference in the content of these pharmacologically active compounds in commercial samples.[55–60] Various patents on HPLC technique are represented in Table 2.

Liquid chromatography–mass spectroscopy

LC–MS stands for Liquid chromatography–mass spectrometry is combination of two techniques and involves the principle of liquid chromatography or HPLC (phenomena of physical separation) along with mass spectroscopy (mass analysis of separating compounds). This technique possesses significant applications in the field of drug chemical analysis and powerful technique in terms of selectivity and sensitivity and frequently employed for pharmacokinetic evaluation of drug molecules in the field of bio analysis.[61–64] Various merits of LCMS/MS are:

Significant combined effect of both liquid chromatography and mass spectroscopy for physical separation and identification of pharmaceuticals

Detection limits are low

Capability of generation of structural information

Having wide ability to analyze different polar and non polar analytes

Minimum quantity is required for sample analysis

Highly useful analytical technique for bio analysis.[65,66]

Measured BAs concentration in the blood and synovium of mice after treatment with topical or oral BAs by liquid chromatography/mass spectrometry (LC/MS). Mouse model of osteoarthritis was used to find out whether topical or oral administration of BAs is useful for treatment of joint damage. Two to six fold higher synovial BAs concentrations was found of than that plasma of plasma concentration.[67] compared bioavailability of BSE standardized soy lecithin formulation (Casperome™) with its corresponding non formulated extract. In the present study previously developed LC–MS/MS method was used for the quantification of BAs. Enhanced BAs distribution in tissues with increased systemic availability increases possibilities of Casperome™ for further clinical development and make potential candidate for therapeutic use.[68] To investigate the details whether BAs have high metabolism rate in the body, explored

the pharmacokinetic evaluation of BAs *in vitro* in comparison with *in vivo* metabolic profiles of KBA and AKBA obtained after studies on animals (Rats). A rapid and sensitive LC–MS/MS method was developed resulted in effective better screening of metabolites. Human liver microsomes, rat liver microsomes and hepatocytes were used in the study and that KBA showed higher phase I metabolism rate than AKBA (means AKBA more stable toward phase I enzymes in comparison with KBA).[69] Frank and Unger explored inhibiting property of cytochrome P450 (CYP) enzymes of different *Boswellia* species. Different *Boswellia* species (*Boswellia serrata, Boswellia carteri*, *Boswellia frereana*, and *Boswellia sacra*) are equally potent, non–selective inhibitors of the CYP enzymes. BAs extracted from commercially obtained frankincense samples were standardized by Electro Spray Ionization and tandem Mass Spectrometry (LC/LC/ESI–MS) fingerprint analyses. Data obtained from study revealed that BAs could act as moderate to potent inhibitors of the applied CYP enzymes.[70] used HPLC–DAD coupled to ESI–MS qualitative and quantitative analysis of BAs extracted from the gum resins of different species of *Boswellia* (*B sacra* and *B serrata*.) Coupling of HPLC with mass spectrometry allow the accurate identification and precise quantification of BAs in *Boswellia* extracts.[71]

Table 2 Patents on hplc used for boswellic acids characterization

Patent no	Filling date	Publication date	Column	Patent title
EP1173162 A1	28 Apr, 2000	23 Jan, 2002	(C18 250 x 4.6mm)	Compositions of boswellic acids derived from Boswellia serrata gum resin, for treating lymphoproliferative and autoimmune conditions 56
US 20040073060A1	5 Mar, 2002	#########	C18 column (Phenomenex, Luna, 250mmx21.2mm,	Process for producing a fraction enriched up to 100% of 3–0–Acetyl–11–keto–beta boswellic acid from an extract containing a mixture of boswellic acids 57
Indian patent 205269	16 Jan, 2004	29 Jun, 2007	CI 8 column (Phenomenex, Luna, 250mmx21.2mm	A process for producing a fraction enriched up to 100% of 3–o– acetyl–11–keto–beta–boswellic acid from an extract containing a mixture of boswellic acids 58
WO2011080579 A2	30 Dec, 2010	7–Jul–11	CI8 (250X4.6mm, 5m) Phenomenex column	A herbal composition for inflammatory disorders 59
EP2536288 A1	12, Apr 2010	26 Dec, 2012	CI8 silica	A novel Boswellia low polar gum resin extract and its synergistic compositions 60
WO2015166462 A1	30 Apr,2015	5 Nov, 2015	–	Method of purifying 3–o–acetyl–11–keto–beta–boswellic acid (AKBA) 61
US9101599 B2	14 Sep, 2009	11 Aug, 2015	Phenomenex Luna Phenyl–Hexyl analytical column (4.6×250mm, 5µ)	Synergistic anti–inflammatory compositions comprising Boswellia serrata extracts 62
US9795646 B2	5 Aug, 2014	24–Oct–17	Reversed phase C18 silica column	Boswellia oil, its fractions and compositions for enhancing brain function 63

Conclusion

Utilization of herbal drug products industry is growing in a tremendous rate. Qualitative and quantitative analysis of natural drug products is fundamental step for their utilization in the field of medicine. Standardization and characterization ultimately affects the safe and effective use of herbal medicines. BAs obtained from herbal sources used have various therapeutic purposes including cancer. HPLC, HPTLC and LC/MS have been frequently used analytical techniques for identification and quantification of BAs.

Acknowledgments

None.

Conflict of interest

Authors declare that there is no conflict of interest.

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