A Simple High-Performance Liquid Chromatographic Method for the Estimation of Boswellic Acids from the Market Formulations Containing *Boswellia serrata* Extract

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Abstract

A simple, rapid, and reproducible reverse-phase high-performance liquid chromatographic method is developed for the estimation of boswellic acids, the active constituents in *Boswellia serrata* oleo-gum resin. The chromatographic separation is performed using a mobile phase consisting of acetonitrile–water (90:10, % v/v) adjusted to pH 4 with glacial acetic acid on a Kromasil 100 C18 analytical column with flow rate of 2.0 mL/min and detection at 260 nm. The elution times are 4.30 and 7.11 min for 11-keto β-boswellic acid (11-KBA) and 3-acetyl 11-keto β-boswellic acid (A-11-KBA), respectively. The calibration curve is linear in the 11.66–58.30 μg/mL and 6.50–32.50 μg/mL range for 11-KBA and A-11-KBA, respectively. The limits of detection are 2.33 μg/mL and 1.30 μg/mL for 11-KBA and A-11-KBA, respectively. The mean recoveries are 98.24% to 104.17% and 94.12% to 105.92% for 11-KBA and A-11-KBA, respectively. The inter- and intra-day variation coefficients are less than 5%. The present method is successfully applied for the estimation of boswellic acids from the market formulations containing *Boswellia serrata* extract.

Introduction

Sallaki is the oleo-gum resin obtained by incisions made on the trunk of the tree *Boswellia serrata* belonging to the family *Burseraceae* (1). It is popularly known as Indian olibanum, Frankincense, Salai gugal, Dhup, Loban, and Kundur. The oleo-gum resin of *Boswellia serrata* has been used in various Unani and Ayurvedic preparations as an anti-inflammatory agent (2–4). Sallaki is reported to contain monoterpenes, diterpenes, and triterpenes (2–4). The anti-inflammatory activity is attributed to the presence of four-pentacyclic triterpene acids [viz., β-boswellic acid (BA), 3-acetyl β-boswellic acid (ABA), 11-keto β-boswellic acid (11-KBA), and 3-acetyl 11-keto β-

boswellic acid (A-11-KBA)] (5) (Figure 1). 11-KBA and A-11-KBA have pronounced anti-inflammatory activity (5). These boswellic acid derivatives were reported to inhibit 5-lipoxygenase (5–7), human leukocyte elastase (HLE) (8), and human topoisomerases I and IIα (9) activities.

A survey of literature revealed that nonaqueous titration (10), reversed-phase high-performance liquid chromatographic (RP-HPLC) (11–12), high-performance thin-layer chromatographic (HPTLC) (13–16), and capillary electrochromatographic (17) methods are reported for the estimation of boswellic acids from Sallaki; whereas HPTLC (16), HPLC (18–20), gas chromatographic–mass spectrometric (21), and liquid chromatographic–mass spectrometric (22) methods are reported for their estimation in human plasma. The gradient HPLC (11) and HPTLC method (15,16) have so far been published for the estimation of 11-KBA and A-11-KBA from market formulation. The present paper aims at reporting a rapid
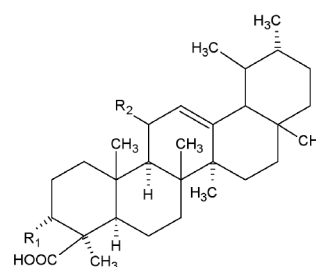
Chemical name	R₁	R₂
β-Boswellic acid (BA)	-OH	-H₂
3-Acetyl β-boswellic acid (ABA)	-OAc	-H₂
11-Keto β-boswellic acid (KBA)	-OH	=O
3-Acetyl 11-keto β-boswellic acid (AKBA)	-OAc	=O

Figure 1. Chemical structures of pentacyclic triterpenic acids.

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isocratic HPLC method for the estimation of boswellic acids from market formulation (tablets/capsules) containing *Boswellia serrata* extract (BSE).

A limited number of analytical methods are available for the analysis of boswellic acids. The HPLC (11) and HPTLC (15,16) methods estimate boswellic acids in the marketed dosage form.

Indian Pharmacopoeial (12) and Ganzera et al. (11) reported an HPLC method for the estimation of boswellic acids in the crude gum resin of *boswellia serrata*. The Pharmacopoeial method (12) utilized an isocratic mobile phase at acidic pH of 2.8. The acidity of the mobile phase reduces the life-span of analytical columns. Another method (11) utilizes a gradient elution technique at an elevated temperature of 60ºC for better separation, and PDA as the detector, though the retention time obtained for 11-KBA and A-11-KBA was very high compared to proposed method.

Apart from that, the marketed dosage forms of BA do not use crude gum resin for their preparation, but gum resin extract utilized for the purpose. Therefore, the applicability of the previously mentioned method for the estimation of 11-KBA and A-11-KBA in marketed formulations is in question.

An RP-HPLC method (18–20,22) was reported for the estimation of only 11-KBA in plasma. They (19–20) required solid-phase extraction and other techniques for the isolation of 11-KBA from plasma, followed by gradient elution and a PDA detector with a higher retention time. Sharma et al. (18) utilized an isocratic technique for estimation in plasma and did not publish details of the method. Reising et al. (22) estimated BA in brain and plasma using a matrix-assisted liquid–liquid extraction technique on Extrelut NT, followed by isocratic RP-HPLC and tandem mass spectrometry. The elution time for boswellic acids is double than that obtained with the proposed method.

Experimental

Instrumentation

The HPLC system (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) consisting of a Rheodyne Syringe loading sample injector (Cotati, CA), an LC-10AS solvent delivery module, a UV-visible spectrophotometric detector SPD-10A/10Ai, and a Class-CR10 Version 1.2 workstation and Hamilton Microliter Syringe (25 μL capacity; Reno, NV) was used for chromatographic studies.

The chromatographic separation was accomplished on a Kromasil 100 C_{18} column (250 mm × 4.6 mm i.d.; particle size 5 μm); Flexit Jour Laboratories Pvt. Ltd., Pune, India) protected by a guard column of the same phase.

Chemicals

A working standard of BSE (Composition: 18.74% β-boswellic acid, 12.59% acetyl-β-boswellic acid, 5.83% 11-KBA, 3.25% A-11-KBA) was procured from M.P. Shah Medical College (Jamnagar, India). Methanol for HPLC, acetonitrile for HPLC, glacial acetic acid, chloroform AR, and anhydrous sodium sulphate were purchased from S.D. Fine-Chem Ltd.

(Mumbai, India). Triple distilled water was prepared at L.M. College of Pharmacy.

Chromatographic conditions

The mobile phase consisted of acetonitrile–water (90:10, % v/v) adjusted to pH 4 with glacial acetic acid. Samples were analyzed using the following parameters: flow rate, 2.0 mL/min; injection volume, 20 μL; run time 18 min; temperature, 27 ± 2ºC; detection wavelength, 260 nm.

Preparation of stock solution of standard BSE

Accurately weighed 50 mg standard BSE powder were dissolved in 25 mL of methanol to get a BSE stock solution (2 mg/mL; corresponding to 116.6 μg/mL of KBA and 65 μg/mL of A-11-KBA).

Preparation of sample solution for analysis of tablet/capsule formulation

Ten tablets/capsules were sampled. Tablets were weighed accurately and finely powdered. Capsules were emptied. Tablet/capsule powder equivalent to 100 mg of BSE was accurately weighed and transferred to a 50-mL volumetric flask, and 20 mL of methanol were added. The mixture was sonicated for 30 min, diluted to the mark with methanol, and filtered through Whatman filter paper (No. 41, 0.45 μm).

Identification of boswellic acids

Standard BSE powder (100 mg) was hydrolyzed with aqueous sodium hydroxide solution (20 % w/v, 25 mL) by heating on a steam bath for 2 h. The content was cooled to room temperature, neutralized with hydrochloric acid, and extracted with chloroform (10 mL × 3). The chloroform extract was dried with anhydrous sodium sulphate and evaporated to dryness. The obtained solid (5 mg) was dissolved in methanol (100 mL) and analyzed by the proposed method.

Calibration curve of standard 11-KBA and A-11-KBA

Aliquots (1, 2, 3, 4, and 5 mL) of the standard solution of BSE were diluted up to 10 mL with methanol (corresponding to 11.66, 23.32, 34.98, 46.64, and 58.30 μg/mL of KBA and 6.50, 13.00, 19.50, 26.00, and 32.50 μg/mL of A-11-KBA). Twenty microliters of solution from each flask were injected manually into the chromatographic system. The calibration curve was prepared by plotting peak areas of 11-KBA and A-11-KBA against respective concentrations.

Validation of the proposed method

Validation of the proposed method was carried out in terms of linearity, accuracy, precision, limit of detection, and limit of quantitation as per ICH guidelines.

The linear responses for 11-KBA and A-11-KBA in the range 11.66–58.30 μg/mL of 11-KBA and 6.50–32.50 μg/mL of A-11-KBA were assessed in terms of slope, intercept, and correlation coefficient values.

The accuracy was determined by standard addition method. To a fixed amount of pre-analyzed sample of BSE, increasing amount of standard BSE solution was added in all the levels of calibration curve. The recovery of 11-KBA and A-11-KBA was

calculated at each level (n = 3). The inter-day precision (RSD) was determined by analyzing a standard solution of BSE over the entire calibration range for five days. The intra-day precision (RSD) was determined by analyzing a standard solution of BSE over the entire calibration range for five times on the same day.

For determinations of limit of detection, the concentrations of standard lower than that of the lowest point of calibration curve were injected to the HPLC and responses were measured.

Analysis of market formulations

The sample solution of tablets or capsules (20 µL) was injected into the chromatographic system three times and mean peak area of 11-KBA and A-11-KBA were noted. The quantity of 11-KBA and A-11-KBA in the market formulation was estimated using respective calibration curve.

Results and Discussion

The goal of this study was to develop a rapid and specific isocratic HPLC method for the estimation of boswellic acids (11-KBA and A-11-KBA) from their market formulations. This HPLC method is not intended to be used to determine BA and ABA because of the high UV background of the mobile phase due to the acetic acid.

The proposed method utilizes an isocratic technique at room temperature without tedious sample preparation procedure and PDA detection. It estimates both 11-KBA and A-11-KBA, the active acids among the boswellic acids. The mobile phase with pH 4.0 gives greater stability to the analytical column. The real advantage of the method is its low retention time: 4.30 and 7.11 min for 11-KBA and A-11-KBA, respectively. It reduces total run time for HPLC, leads to low solvent consumption, and makes the method more economical.

BSE has good solubility in methanol; therefore, methanol was selected for extraction from dosage form (tablet/capsule). The formulation powder was sonicated with methanol for 30 min to ensure complete dissolution of boswellic acids. The chromatographic system was pre-washed with a methanol and methanol–water (50:50, % v/v) mixture to remove any retained impurities in the column. The wavelength maxima of 11-KBA and A-11-KBA were found to be 260 nm; hence it was used for the detection of 11-KBA and A-11-KBA.

The chromatogram of the standard

BSE solution shows two major peaks at 4.30 min and 7.11 min retention times. To confirm the peak of A-11-KBA, the standard BSE was hydrolyzed with sodium hydroxide and analyzed. The hydrolyzed BSE solution showed only one peak at 4.30 min, while the peak at 7.11 min was abolished, confirming that the peak at 7.11 min corresponds to A-11-KBA, which hydrolyzed on treatment with sodium hydroxide to 11-KBA. Thus, it was confirmed that the peaks at 4.30 min and 7.11 min were attributed to 11-KBA and A-11-KBA, respectively (Figure 2).

Representative calibration curves of 11-KBA and A-11-KBA were obtained by plotting the mean peak area of 11-KBA and A-11-KBA against concentration over the range of 11.66–58.30 µg/mL and 6.50–32.50 µg/mL, respectively. The linear regression equations were $y = 66552x - 13407$ and $y = 66997x +$

Table I. Content of 11-KBA and A-11-KBA in Market Formulations (n = 3)

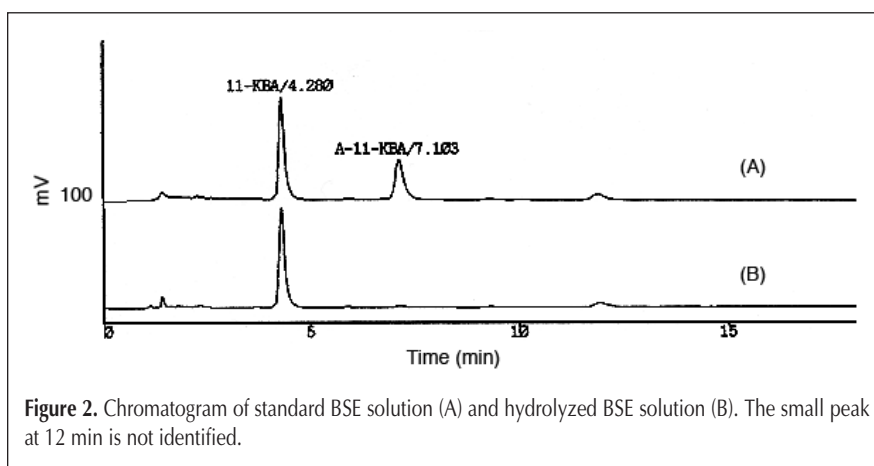| Formulation	% of 11-KBA Mean ± S.D.	% of A-11-KBA Mean ± S.D.
Tablet 1	3.14 ± 0.176	2.55 ± 0.215
Tablet 2	3.02 ± 0.151	1.52 ± 0.045
Capsule 1	4.20 ± 0.018	1.03 ± 0.036

Figure 2. Chromatogram of standard BSE solution (A) and hydrolyzed BSE solution (B). The small peak at 12 min is not identified.
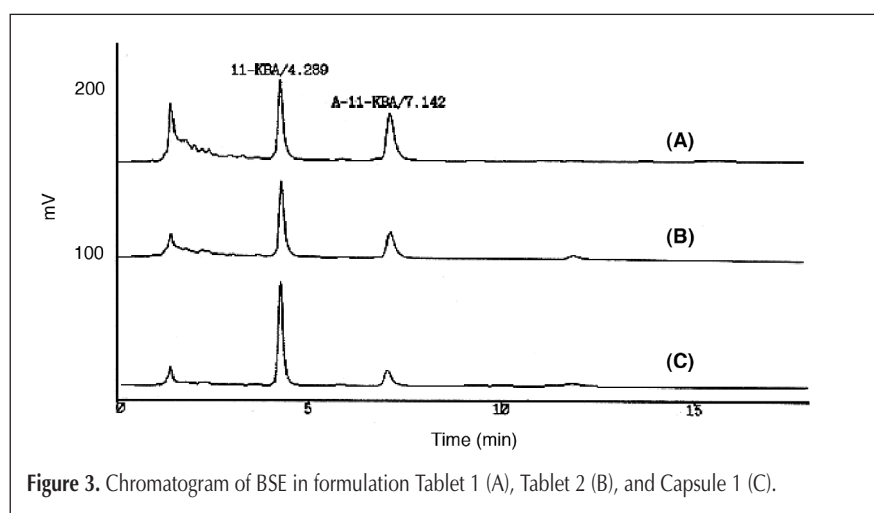

Figure 3. Chromatogram of BSE in formulation Tablet 1 (A), Tablet 2 (B), and Capsule 1 (C).

2222 for 11-KBA and A-11-KBA, respectively. Correlation coefficients were found to be 0.9974 and 0.9988 for 11-KBA and A-11-KBA, respectively. The percentage coefficient of variation (C.V.) calculated for slope and intercept was 4.94 and 5.52 for A-11-KBA and 3.85 and 5.83 for 11-KBA, respectively. The precision of the method in terms of % C.V. values were ranging from 1.34–4.51% and 1.97–5.78% for 11-KBA and A-11-KBA respectively. The accuracy of the method was evaluated by recoveries of the added sample. They were obtained in range of 98.24% to 104.17% and 94.12% to 105.92% for 11-KBA and A-11-KBA, respectively. Interday precision (% C.V.) was found to be 2.67–4.60% and 3.63–4.61% for 11-KBA and A-11-KBA, respectively. Intraday precision (% C.V.) was found to be 1.11–3.80% and 1.68–3.48% for 11-KBA and A-11-KBA, respectively. The limit of detection for 11-KBA and A-11-KBA found to be 2.33 µg/mL and 1.30 µg/mL, respectively.

The developed HPLC method was used to estimate 11-KBA and A-11-KBA from its marketed formulations (Tablet 1, Tablet 2, and Capsule 1; Table I). No interfering peaks were found in the chromatogram, indicating that formulation excipients did not interfere in the estimation of boswellic acids by the proposed HPLC method (Figure 3).

Conclusion

The method is simple, rapid, precise, and accurate for the analysis of boswellic acids from its marketed formulations. It can be used for the routine quality control of the formulation in pharmaceutical industry.

The proposed method utilizes isocratic technique at room temperature without a tedious sample preparation procedure or a PDA detector. It estimates both 11-KBA and A-11-KBA, the active acids among the boswellic acids. The mobile phase with pH 4.0 gives greater stability to the analytical column. The real advantage of the method is its low retention times, 4.30 and 7.11 min for 11-KBA and A-11-KBA, respectively. It reduces the total run time for HPLC, leads to low solvent consumption, and makes the method more economical.

References

1. K.R. Kirtikar and B.D. Basu. *Indian Medicinal Plants,* 2nd ed. M/s Periodical Experts, Delhi, India, Vol. I, 1935, p. 521.
2. R.S. Pardhy and S.C. Bhattacharya. Structure of serratol, a new diterpene cembranoid alcohol from *Boswellia serrata* Roxb. *Ind. J. Chem.* **16B:** 171–173 (1978).
3. R.S. Pardhy and S.C. Bhattacharya. Tetracyclic triterpene acid from the resin of *Boswellia serrata* Roxb. *Ind. J. Chem.* **16B:** 174–175 (1978).
4. R.S. Pardhy and S.C. Bhattacharya. β-Boswellic acid, acetyl β-boswellic acid, 11-keto β-boswellic acid, acetyl 11-keto-β-boswellic acid, four pentacyclic triterpene acids from the resin of *Boswellia serrata* Roxb. *Ind. J. Chem.* **16B:** 176–178 (1978).
5. H. Safayhi, T. Mack, J. Sabieraj, M.I. Anazodo, L.R. Subramanian, and H.P.T. Ammon. Boswellic acids: Novel, specific, non-redox inhibitors of 5-lipoxygenase. *J. Pharmcol. Exp. Biol.* **261:** 1143–1146 (1992).
6. H. Safayhi, E.R. Sailer, and H.P.T. Ammon. Mechanism of 5-lipoxygenase inhibition by acetyl-11-keto-beta-boswellic acid. *Mol. Pharmacol.* **47:** 1212–1216 (1995).
7. E.R. Sailer, L.R. Subramanian, B. Rall, R.F. Hoernlein, H.P.T. Ammon, and H. Safayhi. Acetyl-11-keto-β-boswellic acid (AKBA): structure requirements for binding and 5-lipoxygenase inhibitory activity. *Br. J. Pharmacol.* **117:** 615–618 (1996a).
8. H. Safayhi, B. Rall, E.R. Sailer, and H.P.T. Ammon. Inhibition by boswellic acids of human leukocyte elastase. *J. Pharmacol. Exp. Ther.* **281:** 460–463 (1997).
9. T. Syrovets, B. Buchele, E. Gedig, J. R. Slupsky, and T. Simmet. Acetyl-boswellic acids are novel catalytic inhibitors of human topoisomerases I and IIα. *Mol. Pharmcol.* **58:** 71–81 (2000).
10. R.K. Gupta, V.N. Gupta, V.K. Gupta, and C.K. Atal. Non-aqueous method for estimation of total triterpene acids in Salai guggal and its different forms. *Indian Drugs* **21:** 523–25 (1984).
11. M. Ganzera and I.A. Khan. A reversed-phase high-performance liquid chromatography method for the analysis of boswellic acids in *Boswellia serrata. Planta Med.* **67:** 778–780 (2001).
12. *Indian Pharmacopoeia 1996*, addendum. The Indian Pharmacopoeia Commission, Ghaziabad, India, 2005, p. 986.
13. K. Krohn, M.S. Rao, N.V. Raman, and M. Khalilullah. HPTLC analysis of anti-inflammatory triterpenoids from *Boswellia serrata* Roxb. *Phytochem. Anal.* **12:** 374–376 (2001).
14. R.T. Sane, M.M. Phadke, P.S. Hijli, M.C. Shah, and P.H. Patel. A high-performance thin-layer chromatography method for the simultaneous quantitative analysis of Gugul (*Boswellia serrata*) & Turmeric (*Curcuma longa*) from an Ayurvedic preparation. *Indian Drugs* **35:** 286 (1998).
15. O.N. Pozharitskaya, S.A. Ivanova, A.N. Shikov, and V.G. Makarov. Separation and quantification of terpenoids of *Boswellia serrata* Roxb. extract by planar chromatography techniques (TLC and AMD). *J. Separation Sci.* **29:** 2245–2250 (2006).
16. S.A. Shah, I.S. Rathod, B.N. Suhagia, D.A. Patel, V.K. Parmar, B.K. Shah, and V.M. Vaishnavi. Estimation of boswellic acids from market formulations of *Boswellia serrata* extract and 11-keto β-boswellic acid in human plasma by high-performance thin-layer chromatography. *J. Chromatogr. B.* **848:** 232–238 (2007).
17. M. Ganzera, W.M. Stöggl, G. K. Bonn, I.A. Khan, and H. Stuppner. Capillary electro-chromatography of boswellic acids in *Boswellia serrata* Roxb. *J. Separation Sci.* **26:** 1383-1388 (1993)
18. S. Sharma, V. Thawani, L. Hingorani, M. Shrivastava, V.R. Bhate, and S.R. Khiyane. Pharmacokinetic study of 11-Keto beta-boswellic acid. *Phytomedicine* **11:** 255–260 (2004).
19. M.A Tawab, A. Kaunzinger, U. Bahr, M. Karas, M. Wurglics, and M. Schubert-Zsilavecz. Development of a high-performance liquid chromatographic method for the determination of 11-keto-beta-boswellic acid in human plasma. *J. Chromatogr. B Biomed Sci Appl.* **761:** 221 (2001).
20. B. Büchele and T. Simmet. Analysis of 12 different pentacyclic triterpenic acids from frankincense in human plasma by high-performance liquid chromatography and photodiode array detection. *J. Chromatogr. B* **795:** 355–362 (2003).
21. A. Kaunzinger, A. Baumeister, K. Cuda, N. Haring, and B. Schug. Determination of 11-keto-boswellic acid in human plasma. *J. Pharm. Biomed. Anal.* **28:** 729–739 (2002).
22. K. Reising, J. Meins, B. Bastian, G. Eckert, W.E. Mueller, M. Schubert-Zsilavecz, and M.A. Tawab. Determination of boswellic acids in brain and plasma by high-performance liquid chromatography–tandem mass spectrometry. *Anal. Chem.* **77:** 6640–6645 (2005).