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Development of a high-performance liquid chromatographic method for the determination of 11-keto-β-boswellic acid in human plasma

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Abstract

A validated HPLC method for the determination of 11-keto-β-boswellic acid (KBA) in human plasma was developed. The method involves the solid-phase extraction of KBA from plasma followed by a separation with reversed-phase HPLC. Calibration was based on external standardisation and ranged between 0.1 and 2.0 μg KBA per ml plasma. Linearity was established over the entire calibration range and in each case the coefficient of correlation (r^2) was above 0.99. The recovery of KBA from plasma was 85.7%. It was further demonstrated that the method can be applied successfully to monitor the level of KBA in plasma. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Beside the corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs) including cyclooxygenase inhibitors (COX 1 and COX 2), leukotriene antagonists and lipoxygenase inhibitors are often used in the current treatment of inflammation. Since leukotrienes are important mediators in inflammatory, allergic and obstructive diseases, inhibitors of 5-lipoxygenase, the key enzyme of leukotriene biosynthesis, became the subject of intensive research [1]. Among the 5-lipoxygenase (5-LO) inhibitors of synthetic origin Zileuton (Zyflo™) was the first 5-LO inhibitor for the treatment of asthma. However it exerts undesirable side effects, because of its non selective mode of action [2]. For that reason, novel compounds selectively inhibiting the 5-LO pathway are gaining more and more importance.

The gum resin of *Boswellia serrata,* commonly known as Salai guggal, has been traditionally used in the Ayurvedic medicine of India for a variety of inflammatory diseases. In 1982 the crude extract of Salai guggal has been introduced in India under the trade name of Sallaki™, known in Europe as H15™ [3]. The main biologically active principles of *B. serrata* are boswellic acids [4]. 3-*O*-Acetyl-11-keto-β-boswellic acid (AKBA), with an IC_{50} of 1.5 μ*M*, and 11-keto-β-boswellic acid (KBA), with an IC_{50} of 2.8 μ*M*, proved to be the most potent 5-LO inhibitors [5]. Both, see Fig. 1 for structures, are found in *B. serrata* in nearly equal amounts (AKBA:KBA=1.3:1) [6]. They have been shown to

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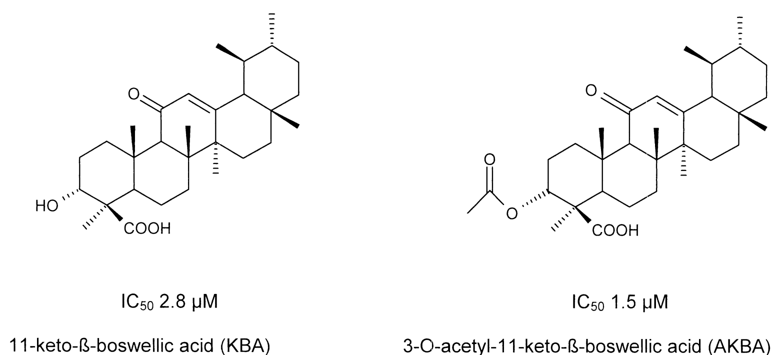

Fig. 1. Structures of KBA and AKBA.

be selective, non-competitive and non-redox inhibitors of the enzyme in in-vitro tests [4]. Therefore they possess little toxicity and limited side effects compared to other anti-inflammatory drugs [7]. Initial clinical studies with a limited number of patients have produced promising results, however further clinical studies involving a larger number of patients will have to be carried out [3,8].

Currently there is no validated analytical method for the analysis of boswellic acids in human plasma. Bearing in mind the possibility of deacetylation of AKBA upon oral administration, we concentrated our efforts on the development of a specific high-performance liquid chromatographic (HPLC) method for the determination of the metabolically more stable analyte KBA in human plasma.

2. Experimental

2.1. Chemicals and reagents

Pure KBA, used as the reference standard, and AKBA was generously donated by HWI Analytik (Dr. Wissel, Rheinzabern, Germany). Methanol and acetonitrile, both of HPLC grade, were obtained from Carl Roth (Karlsruhe, Germany). Orthophosphoric acid (85%) extra pure was purchased from Riedel-de Haen (Seelze, Germany). Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA) and used for all aqueous procedures. Human plasma used for method validation was donated from AAI Deutschland (Neu-Ulm, Germany).

2.2. Instrumentation and chromatographic conditions

Liquid chromatography was performed with a Varian ProStar 210 pump, a Varian ProStar 410 autosampler, a Varian ProStar photo diode array detector Model 330 and a Degasys DG-1210 degasser. The system operation as well as data acquisition and integration were controlled with a Varian Star Chromatography Workstation.

A LiChrospher 100, RP-18 Merck column, 5 μm (125×4 mm) was used throughout the experiments. The corresponding guard columns LiChrospher 100, RP-18, 5 μm (4×4 mm) manufactured by Merck were replaced after 50–60 injections.

Different mobile phases were tested in order to find the best conditions for separating KBA from other extract constituents and endogenous plasma contaminants. The optimal composition of the mobile phase was determined to be: mobile phase A: water–methanol–orthophosphoric acid (85%) (90:9.5:0.5, v/v); mobile phase B: methanol–acetonitrile–water–orthophosphoric acid (85%) (55:40:4.5:0.5, v/v).

For the first 11 min the composition of the mobile phase was kept constant at 90% B, than changed to 100% B in 4 min. The column was reequilibrated for 10 min until the next sample was injected. The flow-rate was set to 1.0 ml/min, resulting in a back pressure of 50 bar and UV detection was carried out at 250 nm, the absorption maximum of KBA. The detection limit (LOD) of the method for KBA was approximately 0.040 μg/ml at a signal-to-noise ratio of 3.

2.3. Sample preparation

2.3.1. Stock solutions

KBA stock solutions were prepared in methanol at 1.00 μg/μl (SS1) and stored in a refrigerator at 5±3°C. Different working solutions were prepared by diluting the stock solution with methanol, resulting in KBA concentrations of: 0.8, 0.4, 0.2, 0.08, 0.04, 0.02, 0.008 and 0.004 μg/μl. Defined volumes, not exceeding 25 μl, of these working solutions were added to 1 ml plasma samples before the extraction procedure to prepare the calibration standards with the following concentrations: 0.1, 0.4, 0.8, 1.2 and 2.0 μg/ml.

2.3.2. Quality control samples

Three different pools of quality control (QC) samples were prepared by spiking 1 ml aliquots of blank plasma with KBA solutions freshly diluted from the stock solution (resulting concentrations: 0.2, 1.0 and 1.8 μg KBA per ml plasma). These solutions were stored at −20±5°C until the day of extraction.

In each sequence, QC samples of the above mentioned concentrations were analyzed in duplicate. The results of these QC samples provided the basis for accepting or rejecting the individual run. At least four of the six QCs had to be within ±15% (QCs close to the limit of quantification within ±20%) of their respective nominal value. Up to two of the QCs could fail this criteria, as long as they were not prepared at the same concentration. Analytical runs not meeting this criteria were repeated.

2.4. Extraction of KBA from plasma

Blank human plasma samples were thawed, thoroughly vortex-mixed for 15 s, and aliquots of 1 ml plasma were spiked with KBA at different concentrations.

For solid-phase extraction (SPE), several cartridges from Varian including Bond Elut C_{18} (100 and 200 mg), C_8 200 mg, C_2 200 mg and Abselut Nexus SPE columns were tested in order to extract KBA from plasma. The most satisfactory results with regard to separation performance and recovery were obtained by using Bond Elut C_8 200 mg cartridges (Varian).

Volumes of 1-ml of plasma spiked with KBA were centrifuged for 3 min at 1100 *g* in a Varifuge 3.0 R (Heraeus Sepatech) to discard any protein particles which may clog the cartridge. After equilibrating Bond-Elut C_8 200 mg cartridges with 3 ml methanol and 3 ml water, 1 ml of the spiked plasma was pipetted onto the column. Under gentle vacuum the liquid was passed through the cartridge, which was then washed five times with water and vacuumed to dryness. Finally, the column was eluted twice with 500 μl of methanol. The methanolic extract was directly collected in autosampler vials and a volume of 50 μl was injected into the HPLC apparatus.

2.5. Quantification

Calibration was based on external standardisation, with the peak area as assay parameter. Standard calibration curves were constructed by plotting the corresponding peak area against five standard concentrations of KBA, measured in triplicate. The quality of fit was evaluated by comparing the calculated concentrations with the nominal values.

All statistical data were calculated using Microsoft Excel 97 software.

2.6. Validation

2.6.1. Specificity

The specificity of the assay was verified by comparing the chromatograms of six blank plasma samples of different origin processed by the analytical procedure described above before and after spiking with KBA. Further, the peak purity of KBA was checked by a photo diode array detector in the range of 220–300 nm.

2.6.2. Linearity of calibration curves and method sensitivity

Linearity was checked using eight calibration curves (each with five concentration levels from 0.1 to 2.0 μg/ml) prepared and assayed in triplicate. The resulting regression curve, the corresponding correlation coefficient and the relative deviation of the backcalculated standard concentrations from their nominal values were used to evaluate the linearity of the detector response. The slope of the standard curve was used to evaluate sensitivity.

2.6.3. Lower limit of quantification (LLQ)

The LLQ of the method is defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy and precision. The precision is expressed in terms of the relative standard deviation (RSD) and the accuracy is given by the relative deviation (bias). Both should be in the range ±20% [9].

2.6.4. Intra- and inter-day precision and accuracy

The intra-day precision and accuracy of this method was obtained by measuring five replicates of each QC sample (in the lower, in the middle and in the upper range) on the same day.

The inter-day precision and accuracy were evaluated by constructing a calibration curve together with QC samples on 8 different days. From the resulting concentrations the mean, the standard deviation, the RSD and bias were determined. RSD and bias values for the intra- and inter-day precision and accuracy should be better than 15%, whereas in the lower range of quantification 20% is acceptable [10].

2.6.5. Stability

The stability of KBA in human plasma was assessed with four sets of QC samples (each set consisting of three concentration levels: low, medium and high) after storage at room temperature for 48 h, after three freeze and thaw cycles and after storage below −20°C for 14 days. Further, the stability of KBA in processed samples was determined after storage at room temperature for 48 h, after storage in the refrigerator for 8 days and after storage in the autosampler at room temperature for 72 h. Evaluation of the stability of QC samples was based on the comparison of the calculated mean of the respective sample with its nominal value.

2.6.6. Recovery

The absolute recovery of KBA from human plasma was tested at three concentration levels ($n=4$) by comparing QC samples prepared in plasma with unprocessed QC samples prepared in methanol. The absolute recovery was then determined as the amount of KBA in spiked plasma compared to the amount of KBA in pure methanol (expressed in percent) [11].

2.7. Results

2.7.1. Specificity

The HPLC chromatogram of a *Boswellia* extract in Fig. 2a indicates the complete separation of KBA from AKBA, the only boswellic acid which also has an absorption maximum at 250 nm. The retention time of KBA was about 5.8 min. Fig. 2b shows a typical chromatogram obtained after spiking plasma with 1.0 μg/ml KBA. The absence of interfering signals at the retention time of KBA in blank plasma is illustrated in Fig. 2c. There was no interference detected when comparing the chromatograms of blank and spiked plasma samples indicating the specificity of the method.

Identical spectra obtained with the photo diode array detector at the maximum, ascending and descending part of the peak eluting at 5.8 min were a further indication of the peak purity of KBA.

2.7.2. Linearity and sensitivity

A total of eight calibration curves were assayed and calculated. Linearity of the assay was given over the whole calibration range of 0.1–2.0 μg/ml. The coefficient of correlation (r^2), being above 0.992 in each case, yielded a mean value of 0.9978. The intercept ranged between −0.06698 and 0.01963. The average value of the individual slopes, which was calculated as 2.66 with an RSD of 8.1%, indicates a high sensitivity of the method. The deviation of standards, other than the LLQ, from their nominal values did not exceed 8%. Furthermore the deviation of LLQ from its nominal concentration never exceeded 18%, therefore fulfilling the requirements of bioanalytical method validation suggested in the literature [9].

2.7.3. Lower limit of quantification

The LLQ was 0.1 μg/ml. A chromatogram at this level is shown in Fig. 2d. This was validated by analyzing five 1-ml aliquots of plasma samples spiked with 0.1 μg KBA. The calculated mean value was 0.102 μg/ml±5.8% and the bias was 2%. The values for accuracy and precision thus clearly met the acceptance criteria.

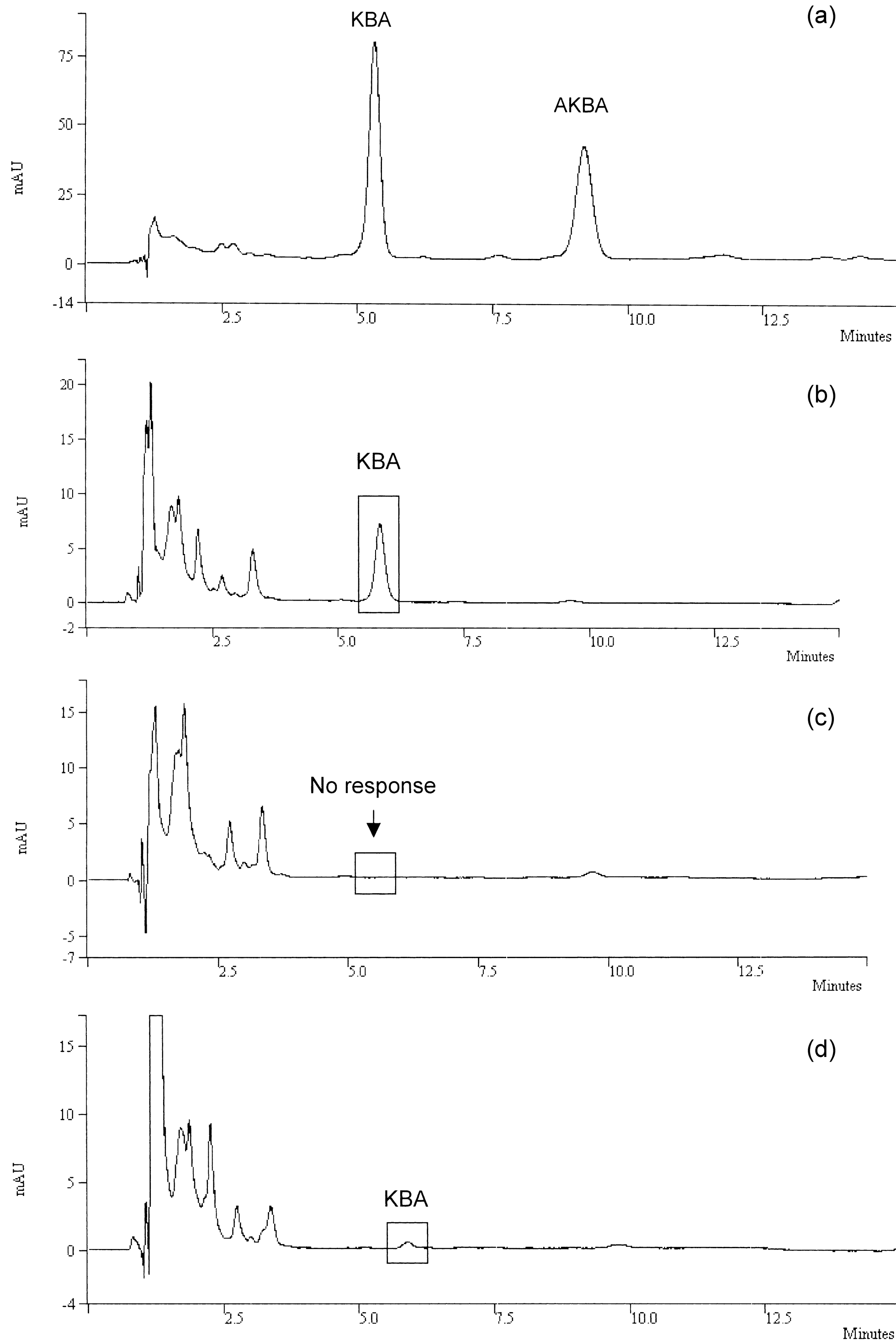

Fig. 2. (a) HPLC chromatogram of *Boswellia* extract (250 nm). (b) HPLC chromatogram of plasma spiked with KBA (250 nm). (c) HPLC chromatogram of blank plasma (250 nm). (d) HPLC chromatogram of plasma spiked with KBA at the lower limit of quantification (250 nm).

Table 1
Data for intra- and inter-day precision and accuracy in human plasma

Nominal concentration (μg/ml)	Intra-day (n=5)		Inter-day (n=16)	
	RSD (%)	Bias (%)	RSD (%)	Bias (%)
0.200	5.8	9.8	4.3	−5.7
1.000	5.5	5.9	3.4	−4.6
1.800	5.5	3.7	3.3	−4.4

2.7.4. Precision and accuracy

The values for the inter-day precision of the method given by RSD lay between 3.3 and 4.3%. The inter-day accuracy expressed as bias lay between −4.4 and −5.7%. The results for intra- and inter-day precision and accuracy are summarised in Table 1. All values were within the limits recommended for bioanalytical method validation.

2.7.5. Stability

From the results shown in Table 2 it can be concluded that KBA is stable in human plasma and in processed samples under all described conditions.

2.7.6. Recovery

The mean recovery of the method was calculated as 85.7%. The precision of the recovery at each

Table 2
Stability data of KBA (n=4 per test and concentration)

	Nominal concentration (μg/ml)	RSD (%)	Bias (%)
Stability of KBA in human plasma			
Short-term stability at room temperature (48 h)	0.200	14.5	−17.0
	1.000	4.2	−5.6
	1.800	1.9	−2.6
Long-term stability at −20±5°C (14 days)	0.200	1.5	2.0
	1.000	4.0	6.0
	1.800	6.4	9.9
Freeze–thaw stability, 0 cycle	0.200	10.8	−13.5
	1.000	1.4	−2.0
	1.800	1.8	2.7
Freeze–thaw stability, 1 cycle	0.200	1.7	2.5
	1.000	6.4	10.0
	1.800	7.3	11.4
Freeze–thaw stability, 3 cycles	0.200	1.4	−2.0
	1.000	0.3	0.4
	1.800	0.1	−0.1
Stability of KBA in the extraction solvent			
At room temperature (72 h)	0.200	5.3	−7.5
	1.000	0.8	−1.2
	1.800	3.5	−4.9
At 5±3°C (8 days)	0.200	5.7	−7.5
	1.000	0.8	−1.2
	1.800	3.7	−4.9
In the autosampler at room temperature (72 h)	0.200	12.3	−15.0
	1.000	7.4	−9.5
	1.800	4.0	−5.3

Table 3
Recovery of KBA (n=4 at each concentration level)

Original concentration (μg/ml)	Recovery (%)
0.200	85.2
1.000	88.1
1.800	83.8
Mean	85.7
SD	2.16
RSD (%)	2.5

concentration level was 2.5%. Due to the good recovery it was not necessary to improve the extraction procedure described above. The results are listed in Table 3.

2.7.7. Clinical suitability of the assay

After completing validation, the assay was tested for its clinical suitability by monitoring the level of KBA in plasma of a volunteer (one of the authors) who took a single dose of 1600 mg of a commercial *Boswellia* extract formulated in our laboratories as hard gelatine capsules. A standard curve was generated for this run in order to determine the concentrations of KBA in the plasma. The coefficient of correlation (r^2) was 0.997. The plasma concentration versus time is presented in Fig. 3. KBA plasma levels could be determined up to 8 h after medication, with the C_{max} occurring after 1 h. The peak purity of KBA was verified with the photo diode array detector in the manner described before in order to exclude any interference caused by metabolites eluting at the same time as KBA. AKBA was not detectable in plasma with this method.

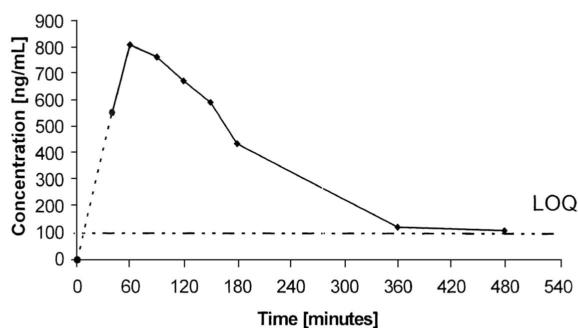

Fig. 3. Plot of KBA concentrations in human plasma after administration of 1600 mg of commercial *Boswellia* extract.

2.8. Conclusion

In most herbal plants, with the exception of a few such as *Silybum marianum* and *Piper methysticum,* the biologically active constituents are not yet known. In *B. serrata* the active principles have been identified as the boswellic acids. To facilitate clinical studies we developed and validated a specific HPLC method for the determination of KBA, one of the major boswellic acids found in *B. serrata*, in human plasma. We were able to extract KBA from a complex biological matrix by introducing a single SPE step prior to HPLC analysis, thus obviating the need for time-consuming derivatisation procedures. Using this assay a linear quantification range from 0.100 to 2.000 μg/ml was established. Precision and accuracy determined during the validation procedure were within acceptable limits, and the method was shown to be suitable for monitoring the level of KBA in humans.

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