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Title Page

Metabolism of boswellic acids *in vitro* and *in vivo*

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Running Title Page

Metabolism of boswellic acids

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Abbreviations: EMEA, European Medicines Agency; NFkB, nuclear factor-kappa B; BA, boswellic acid; KBA, 11-keto-β-boswellic acid; AKBA, 3-acetyl-11-keto-β-boswellic acid; αBA, α-boswellic acid; βBA, β-boswellic acid; AαBA, 3-acetyl-α-boswellic acid; AβBA, 3-acetyl-β-boswellic acid; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; UGT, uridine 5´-diphospho-glucuronosyl transferase; UDPGA, uridine 5´-diphospho-

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glucuronic acid; RLM, rat liver microsomes; HLM, human liver microsomes; HPLC-UV, high performance liquid chromatography-ultraviolet detection; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NL, neutral loss; GC-MS, gas chromatography-mass spectrometry; RP, reversed phase; APCI, atmospheric pressure chemical ionisation; MRM, multiple reaction monitoring; SIM, selected ion monitoring; IDA, information dependent aquisition; QqQLIT, hybrid triple quadrupole linear ion trap mass spectrometer; QqQ, triple quadrupole mass spectrometer.

Abstract

Boswellia serrata resin dry extract is among the few herbal remedies designated with an orphan drug status for the treatment of peritumoral brain edema. In addition, boswellic acids (BA), the main active ingredients of *Boswellia serrata* extracts, have potent anti-inflammatory properties, and may represent promising agents for the treatment of inflammatory diseases. Pharmacokinetic studies, however, revealed poor bioavailability, especially of 11-keto-β-boswellic acid (KBA) and 3-acetyl-11-keto-β-boswellic acid (AKBA), the most potent BAs. To address the question whether BAs are extensively metabolized, we determined the metabolic stability of KBA and AKBA *in vitro*, investigated the *in vitro* metabolism of BAs and compared the metabolic profiles of KBA and AKBA with those obtained in rats *in vivo*. In rat liver microsomes and hepatocytes as well as in human liver microsomes, we found that KBA but not AKBA undergoes extensive phase I metabolism. Oxidation to hydroxylated metabolites is the principal metabolic route. *In vitro*, KBA yielded similar metabolic profiles to those obtained *in vivo* in rat plasma and liver, whereas no metabolites of AKBA could be identified *in vivo*. Furthermore, AKBA is not deacetylated to KBA. This study indicates that the efficacy of *Boswellia serrata* extract may be enhanced by increasing the bioavailability of AKBA.

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Introduction

Pentacyclic triterpenes (PTs) have gained significant importance because their chemical structures resemble those of steroids. In particular, boswellic acids (BAs), the main active components of the gum resin extract of *Boswellia serrata* (Salai guggal), have received considerable attention. Based on positive data on the treatment of peritumoral brain edema accompanying gliomas (Winking *et al.* 2000), the European Medicines Agency (EMEA) designated an orphan drug status to *Boswellia serrata* dry resin extract in 2002, thus boosting the status of *Boswellia serrata* extract as an herbal remedy.

Traditionally, *Boswellia serrata* extract is used in Indian ayurvedic medicine for the treatment of inflammatory and arthritic diseases (Culioli *et al.*, 2003; Gupta *et al.*, 1998). BAs possess potent anti-inflammatory properties by inhibiting 5-lipoxygenase, human leukocyte elastase and the NFkB-pathway., without exerting the adverse effects known for steroids (Safayhi *et al.*, 1992, 1997; Gupta *et al.*, 1997; Poeckel *et al.*, 2006; Syrovets *et al*., 2005). Among the six most important derivatives of BAs (Figure 4), KBA and AKBA are the most potent inhibitors of 5-lipoxygenase with IC_{50} values of 2.8 and 1.5 µM, respectively (Sailer *et al.*, 1996; Safayhi *et al.*; 1992). In several clinical trials the efficacy of *Boswellia serrata* extract was comparable to that of sulfasalazine and mesalazine for the treatment of Crohn´s disease and ulcerative colitis, with a risk-benefit analysis in favour of BAs (Gupta *et al.*, 1997; Gerhardt *et al.*, 2001). Efficacy, however, could not be clearly demonstrated for other inflammatory diseases such as asthma and polyarthritis (Gupta *et al.*, 1998; Sander *et al.*, 1998). Moreover, *Boswellia serrata* extract also exhibited anti-proliferative and cytotoxic effects (Poeckel *et al.*, 2006).

Preliminary pharmacokinetic studies found only very low concentrations of KBA in human plasma after oral administration of *Boswellia serrata* extract, ranging from 0.17 µM following a single dose administration of 786 mg (Sterk *et al.*, 2004), to 1.6 µM after taking

1600 mg (Abdel Tawab *et al.*, 2001), to 2.7 µM subsequent to the intake of 333 mg (Sharma *et al.*, 2004). AKBA, the most potent BA, was determined at a concentration of 0.1 µM following single dose administration of 786 mg *Boswellia serrata* extract (Sterk *et al.*, 2004). In the pharmacokinetic study carried out by Sharma *et al.*, AKBA was not detected in plasma, possibly due to the deacetylation of AKBA to KBA *in vivo* (Sharma *et al.*, 2004). In rat dosed with 240 mg/kg *Boswellia serrata* extract, plasma levels of KBA and AKBA were determined at 0.4 µM and 0.2 µM, respectively, whereas they reached concentrations of 0.3 µM in the brain (corresponding to 99 and 95 ng KBA and AKBA per gram brain, respectively) (Reising *et al.*, 2005). The studies mentioned above clearly suggest a substantial potential of BAs for the treatment of inflammatory diseases and CNS malignancies, if sufficient systemic concentrations can be achieved.

Among many factors affecting bioavailability, poor absorption and/or extensive metabolism may play a crucial role in limiting the systemic availability of BAs. The present study will focus on the contribution of hepatic metabolism to the low bioavailability observed with KBA and AKBA. To date, no data describing the metabolism of PTs, including BAs, is available. To our knowledge, only the metabolism of oleanolic acid, leading to the formation of hydroxylated metabolites upon incubation with rat liver microsomes, has been described (Jeong *et al.*, 2007). Furthermore, the fate of the most potent BA, AKBA, is not known. It is unclear whether AKBA is predominantly deacetylated to the pharmacologically active KBA or whether it is metabolized via other pathways. Based on the particular therapeutic importance of *Boswellia serrata* extracts, the present study has three objectives: First, to investigate the metabolic stability of KBA and AKBA in rat liver microsomes (RLM), rat hepatocytes and human liver microsomes (HLM). Second, to determine whether AKBA is deacetylated to KBA *in vivo*; and finally, to identify the metabolites of BAs *in vitro* and to compare the *in vitro* metabolic profiles of the most potent BAs, KBA and AKBA, with those in rat plasma, liver and brain after oral administration.

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Methods and Materials

Chemicals and reagents. Boswellic acids [α-boswellic acid (αBA), (3α)-3-Hydroxy-olean-12-en-23-oic acid; β-boswellic acid (βBA), (3α,4β)-3-Hydroxyurs-12-en-23-oic acid; 3-acetyl-α-boswellic acid (AαBA), (3α)-3-Acetoxy-olean-12-en-23-oic acid; 3-acetyl-β-boswellic acid (AβBA), (3α)-3-Acetoxy-urs-12-en-23-oic acid; 3-acetyl-11-keto-β-boswellic acid (AKBA), (3α)-3-Acetoxy-urs-12-en-11-keto-23-oic acid; 11-keto-β-boswellic acid (KBA), (3α)-3-Hydroxy-urs-12-en-11-keto-23-oic acid] (purity > 99 %) were purchased from Phytoplan (Heidelberg, Germany). All solvents were of analytical grade. Methanol, acetonitrile, tetrahydrofurane, *n*-hexane, 2-propanol and ethyl acetate were from Caledon (Georgetown, ON, Canada). Water was collected from a Milli-Q organic free water system (Millipore, Bedford, MA, USA). Extrelut NT was obtained from VWR (Darmstadt, Germany). Solid phase extraction cartridges were obtained from Waters (Milford, MA, USA). Tris buffer was acquired from Applichem (Darmstadt, Germany). Pooled human liver microsomes (HLM, >25 donor livers), NADPH regenerating solutions A and B, as well as UGT Reaction Mix solutions A and B were purchased from BD Biosciences (San Jose, CA, USA). Potassium phosphate monobasic, Krebs-Henseleit-Buffer, 7-ethoxycoumarin, 7-hydroxycoumarin, 7-hydroxycoumarinsulfate, asiatic acid and pooled male rat liver microsomes (RLM) were from Sigma (St. Louis, MO, USA).

Microsomal incubation and sample preparation. Stock solutions of each BA were prepared at concentrations of 500 µM in methanol:water (50:50, v/v). The NADPH regenerating system (NRS) was used in this study because it provides constant NADPH levels over the entire incubation period. The final incubation solutions consisted of 50 mM potassium phosphate buffer containing 1 mg/mL microsomal protein, 10 µM BA, 1.3 µM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$ and 0.4 [U]/mL glucose-6-phosphate dehydrogenase. The total volume was 250 µL. Controls were incubated without NRS. The solutions were incubated at 37 °C in a water bath. After 60 min, the metabolic reaction was stopped by adding 250 µL ice-cold acetonitrile. The solutions were vortex-mixed, put on ice for 15 min and centrifuged for 10 min at 8000 g at 4 °C. Aliquots of the supernatants were diluted to 500 µL with water. The sample solutions containing 20 % or less acetonitrile were transferred to a solid phase extraction cartridge. After washing with 1 mL water, the metabolites were eluted from the cartridges with 1 mL methanol. After solvent evaporation, the samples were redissolved in 200 µL methanol:water (50:50, v/v) and used for LC-MS/MS analysis.

Metabolic stability. The incubation conditions for the phase I reactions were the same as described above but the incubation time was extended to two hours, in order to allow sufficient time for the reactions. For the glucuronidation reactions, solutions containing 1 mg/mL microsomal protein, 10 µM KBA or AKBA, 2 mM uridine 5´-diphospho-glucuronic acid (UDPGA) cofactor, 50 mM Tris-HCl, 8 mM $MgCl_2$ and 25 µg/mL alamethicin in deionized water were incubated, as previously described. The total volume was 250 µL. Aliquots (50 µL) of the reaction mixtures were drawn at 15, 30, 60 and 120 minutes and the reaction was stopped by adding equal amounts of ice-cold acetonitrile containing asiatic acid (10 µM) as internal standard, followed by centrifugation for 10 min at 8000 g at 4 °C. The supernatants were analyzed directly by LC-MS/MS in the MRM mode. Controls were incubated without the cofactors.

For determining the metabolic stability in rat hepatocytes, 100 µM KBA or 100 µM AKBA were added to freshly isolated hepatocyte suspensions, which were previously incubated for 15 min at 37 °C in an atmosphere of 5 % CO_2 and 95 % O_2. The incubation mixture was then kept for 2 h under the same incubation conditions. The metabolic capacity was monitored by determining the ability of the hepatocytes to metabolize 7-ethoxycoumarin. CYP, UGT and sulfotransferases were shown to be active over the entire incubation period.

Incubations were stopped at 0, 15, 30 60 and 120 min by adding 200 µL cold methanol. Afterwards, the terminated incubation samples were vortex-mixed, put on ice for 15 min and centrifuged for 10 min at 8000 g at 4 °C. The supernatants were stored at -20 °C until they were analyzed by LC-MS/MS. The metabolic stability of KBA and AKBA, was calculated on the basis of six determinations, at each time point respectively.

Isolation of rat hepatocytes. A male Wistar rat was anesthetized with pentobarbital prior to abdominal midline incision and liver cannulation. Hepatocytes were then isolated by collagenase perfusion (Berry *et al.*, 1969). Isolated hepatocytes were suspended in Krebs-Henseleit-Buffer (KHB) supplemented with 10 mM fructose and 3 mM glycine (pH 7.4), at a cell density of 1 x 10^6 cells/mL. The viability of hepatocytes, based on trypan blue exclusion, was > 85 %.

Animal study. Female albino Wistar rats ranging from 101 to 125 g in body weight were supplied by Charles River Laboratories (Wilmington, UK). The animals were housed under standard conditions, with standard chow diet and water freely available. Fifteen mg of KBA and AKBA were suspended in 3 g of aqueous agarose gel 0.2 % (w/v). In order to enhance bioavailability, 30 µL of neutral oil was added as a lipophilic component (Sterk *et al.*, 2004). The control group was given only agarose gel. The second group was given KBA suspension at a dose of 12.5 mg/kg (corresponding to the dose of 240 mg *Boswellia serrata* extract/kg applied in previous studies, Reising *et al.*, 2005, Winking *et al.* 2000) and the third group received AKBA suspension at the same dose. Each group consisted of four rats. The treatment was administered once by oral gavage via a pharyngeal tube with a maximal application volume of 0.43 mL. Oral application was chosen since it is the most common application route of frankincense. All experiments were carried out by appropriately trained persons according to the guidelines of the German Protection of Animals Act (Deutsches Tierschutzgesetz, BGBI 1998, Part I, No. 30, S. 1105ff.), in accordance to the Declaration of

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Helsinki. Two hours after the oral administration, the rats were decapitated and brain and liver were isolated. The brain samples (weight range = 1.18 to 1.35 g) and 1.2 g of the livers were rinsed and homogenized in 5 mM Tris-HCl buffer (pH 7.4), yielding suspensions containing 200 mg brain and 200 mg liver per mL buffer, respectively. The homogenates were stored at -20 °C prior to sample preparation. The blood samples were collected from the trunk immediately after decapitation and transferred to tubes containing 0.03 mL heparin to avoid coagulation. They were centrifuged at 2000 g for 10 min to obtain the plasma fractions, which were then stored at -20 °C until analysis.

Preparation of *in vivo* samples. Based on the method described by Büchele and Simmet (Büchele *et al.*, 2003), 0.8 g Extrelut NT per mL plasma was used for sorbent assisted liquid-liquid extraction in Extrelut glass columns. For the extraction, 6 mL brain or liver homogenate were passed through the Extrelut NT3 glass columns containing 5 g Extrelut. After 15 min, KBA and AKBA were eluted with a solvent mixture consisting of tetrahydrofurane: n-hexane: ethyl acetate: 2-propanol (160:160:160:15, v/v/v/v). The plasma samples were eluted with 6 mL solvent, whereas 30 mL were used for the elution of brain and liver samples. The eluates were dried under a stream of nitrogen at 40 °C and the residues were reconstituted in 150 µL of methanol and cooled on ice for 15 min prior to centrifugation at 2000 g (4 °C) for 10 min. The clear supernatants were used for LC-MS/MS analysis.

LC-MS/MS. For the experiments on the metabolic stability of KBA and AKBA, isocratic separation was performed on a Perkin-Elmer Series 200 HPLC system with a RP 18 column (Hypersil BDS, 150×4mm, 5 µm particles; MZ-Analysentechnik, Mainz, Germany) at 40 °C and a flow rate of 1 mL/min. The mobile phase consisted of methanol: water: glacial acetic acid (8:1:0.4 v/v/v). MS analysis was performed on a triple quadrupole mass spectrometer (API 300, MDS Sciex, Concord, ON, Canada) equipped with an APCI interface operating in positive ionization mode at 425 °C. Multiple reaction monitoring (MRM) was used to quantify KBA and AKBA in RLM, HLM and rat hepatocytes, using asiatic acid as the internal standard. The details of the method have been published elsewhere (Reising *et al.*, 2005). Screening for glucuronides was performed in the full scan mode followed by product ion scanning. Additionally, neutral loss scans (for glucuronides, sulfates, and amino acid conjugates via neutral losses of 176, 80, 129, 75, 57 u) and precursor ion scans (based on the KBA fragment ions at *m/z* 391, 407, 423, 421 and the AKBA fragment ion at *m/z* 59) were performed in the negative ionization mode. Data acquisition and processing was conducted using the Analyst 1.4.1 software.

For metabolite identification, a sensitive LC-MS/MS method was established, providing a rapid and comprehensive screening for metabolites with simultaneous data-dependent acquisition of MS/MS data for potential candidates. Liquid chromatography was carried out on an Agilent 1100 (Agilent Technologies, Palo Alto, CA, USA) HPLC system using a RP 18 column (Ultracarb ODS (30), 30×3.2 mm, 5 µm; Phenomenex, Torrance, CA, USA) for the separation of BAs and their metabolites at a flow rate of 0.6 mL/min. The injection volume was 10 µL. The mobile phase consisted of water (A) and methanol (B). Three gradient programs were used to achieve adequate retention behaviour for the different BAs. The gradient programs started with 50 % B and changed to 100 % B within 12 min for KBA and AKBA, and within 10 min for αBA, βBA, AαBA and AβBA, respectively. This gradient was then held for 4 min for KBA and AKBA and 6 min for the remaining four BAs. Finally it was changed to 50 % B within 1 min followed by 5 min of equilibration at 50 % B prior to the next injection. The MS experiments were conducted on a hybrid triple quadrupole linear ion trap (QqQLIT) mass spectrometer (QTRAP 4000, MDS Sciex, Concord, ON, Canada) equipped with a Turbo V atmospheric pressure chemical ionization (APCI) interface operating in negative ionization mode with the following source parameters: needle current (NC), –3 µA; curtain gas (CUR), 20 psi; temperature, 450 °C; gas 1 (GS1), 30 psi. Data acquisition and processing was conducted using the Analyst 1.4.1 software. The mass spectrometric conditions were optimized by infusing standard solutions of the respective BA using a syringe pump (Harvard Apparatus, South Natick, MA, USA). The following parameters were obtained for the declustering potential (DP, volts) and the collision energy (CE, volts): KBA, -120, -55; AKBA, -85, -20; αBA, -170, -50; βBA, -170, -50; AαBA, -65, -20; AβBA, -65, -20. Screening for metabolites was conducted in the linear ion trap mode, with information dependent acquisition (IDA) in the enhanced product ion mode (EPI). In the EPI mode, the precursor ion was selected in Q1 and the collision-induced dissociation (CID) was conducted in the collision cell q2. The product ions were detected in the linear ion trap, providing enhanced sensitivity over traditional triple quadrupole experiments. Some metabolites were not seen in the IDA experiments because of matrix interferences. These metabolites could be observed, however, by narrowing the Q1 isolation window to increase selectivity.

Identification of metabolites was based on comparing the MS/MS spectra with those of BAs. Only if the same fragmentation pattern was obtained, the metabolites were assigned to the corresponding BA. Furthermore the peaks of the metabolites should not be detectable in the respective control group.

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Results

Metabolic stability of KBA and AKBA. After 15 minutes of incubation with RLM, more than 80 % of the initial KBA concentration was metabolized and less than 1 % of the starting concentration remained after 120 minutes. An extensive phase I metabolism was also observed in HLM. After 15 minutes, more than 60 % of the initial KBA concentration was metabolized and less than 10 % of the starting concentration remained after 120 minutes (Figure 1). On the other hand, AKBA was less susceptible to phase I metabolism than KBA, with about 80 % of the starting concentration still remaining 120 minutes after incubation with RLM and HLM, respectively (Figure 1).

The direct glucuronidation experiments carried out in RLM and HLM in parallel revealed no decrease in the initial concentrations of KBA and AKBA. Furthermore, KBA and AKBA glucuronides could not be detected upon screening with LC-MS/MS (data not shown).This clearly indicates that KBA and AKBA are mainly subjected to phase I metabolism. Moreover, no substantial differences between RLM and HLM were observed in their metabolic turnover rates.

Upon incubation of KBA and AKBA with rat hepatocytes, similar metabolic turnover rates were obtained as described above for RLM and HLM (Figure 1). Also, for rat hepatocytes more than 80 % of the initial KBA was metabolized after 30 min, whereas approximately 80 % of the starting AKBA concentration still remained after 120 minutes.

Identification of the KBA metabolites *in vitro*. The initial screening of the *in vitro* RLM reaction medium revealed the formation of metabolites with mass shifts of +16, +32 and +14 u in comparison to KBA. Upon screening for *m/z* 485.4 (corresponding to mass shifts of +16 u), three major metabolites eluting earlier than KBA (Figure 2A) were detected (Figure 2B), indicating the presence of derivatives with more hydrophilic properties than the parent compound. Since these peaks were absent in the control incubations, they can be clearly assigned to metabolic conversions of KBA.

This assumption is further supported by comparing the MS/MS spectra of KBA and its metabolites, providing consistent structure-specific fragmentation patterns. Whereas the MS/MS of KBA produced fragments at *m/z* 376.4, 391.3, 407.3 and 451.3 (Figure 2E), the three metabolites yielded the same fragment species with a characteristic mass shift of 16 u at *m/z* 392.3, 407.2, 423.2, 467.3 (Figure 2F). The peaks at 10.9 and 11.1 min could not be related to KBA, as they were also present in the control group and did not show the characteristic fragmentation pattern of KBA.

Upon screening for *m/z* 501.4 (corresponding to a +32 u mass shift), various signals were observed between retention times t = 2.4 and 7.8 min (Figure 2C), exhibiting characteristic fragment ion shifts of +32 u, in comparison to those of KBA at *m/z* 408.2, 423.1, 439.3 (Figure 2G). Furthermore, the two peaks at t = 5.4 and 8.0 min (Figure 2D; $[M-H]^-$ at *m/z* 483.4) were not present in the control, suggesting metabolites corresponding to a mass shift of +14 u. As expected, the fragmentation pattern of these products (fragment ions at *m/z* 390.2, 405.2 and 421.3) again resembled that of KBA, indicating a link to KBA. The peak at 12.7 min could not be related to KBA.

Incubation of KBA with hepatocytes yielded only phase I metabolites, represented by KBA derivatives with mass shifts of +16 u (t = 5.6, 7.7, 7.9 min) and +32 u (t = 2.5, 4.4, 7.8 min) as well as +48 u (t = 2.0 min). No phase II metabolites could be identified, neither by screening for glucuronides, sulfates and amino acid conjugates nor by precursor ion scanning.

Metabolites of KBA *in vivo* in rat plasma, liver and brain in comparison to its metabolites *in vitro*. Following the identification of the KBA metabolites *in vitro*, the metabolites of KBA were further explored *in vivo* in the course of a feeding study. In order to ensure sufficient metabolic conversion of KBA, screening for the *in vivo* metabolites in rats was carried out two hours after oral administration of KBA suspension. This time interval was chosen on the basis of previous pharmacokinetic studies (Reising *et al.*, 2005) and the rapid metabolism of KBA observed *in vitro* upon incubation with RLM.

In rat plasma, two derivatives with a mass shift of +16 u (at t = 5.7 and 8.1 min) and one derivative with a mass shift of +32 u (t = 7.8) were detected, in addition to the parent compound KBA. All MS/MS spectra of these metabolites correlated well with the corresponding spectra of KBA and its metabolites obtained *in vitro* after microsomal incubation, indicating that these metabolites were identical.

The analysis of the rat liver revealed the formation of various metabolites showing the same fragmentation pattern as KBA and its metabolites *in vitro*. In addition to KBA, three metabolites with a mass shift of +16 u (t = 5.7, 7.7 and 8.0 min) and four KBA derivatives exhibiting a mass shift of +32 u (t = 2.8, 3.4, 4.4 and 7.8 min) were detected as well as one derivative corresponding to KBA +48 u (t = 1.8 min). These results suggest that the liver plays an important role in the metabolism of KBA. The analysis of the rat brain revealed the presence of KBA, but no metabolites could be detected.

Moreover, the rat plasma, liver and brain were screened for the occurrence of phase II metabolism of KBA and /or KBA metabolites but no conjugates were found.

In the control group consisting of rats fed with agarose suspension only, neither KBA nor any metabolite were identified in rat plasma, liver or brain.

Table 1 illustrates an overview of all metabolites of KBA detected *in vivo* in comparison to those identified *in vitro*.

Identification of AKBA metabolites *in vitro*. The experiments on the metabolic stability of AKBA in the liver microsomes described in the previous section showed that the transformation rate of AKBA is much lower than that of KBA. Hence, the initial screening of the RLM *in vitro* reaction medium revealed the formation of derivatives with mass shifts of only +16 u. Upon screening for *m/z* 527.3, corresponding to a +16 u mass shift, three different metabolites with retention times of t = 7.7, 8.2 and 9.4 min were detected, which were absent in the control incubations. As the MS/MS spectra of AKBA as well as those of the metabolites observed showed a characteristic fragment at *m/z* 59 (acetic acid anion), these metabolites can be assigned to AKBA. In hepatocyes, AKBA yielded only one AKBA derivative with a mass shift of +16 u (t = 8.1). Again, no phase II metabolites could be detected.

Metabolites of AKBA *in vivo* in rat plasma, liver and brain in comparison to its metabolites *in vitro*. Only AKBA but no metabolites were detected in rat plasma, liver and brain two hours after oral administration of AKBA suspension. A representative selected ion monitoring (SIM) chromatogram and product ion spectrum of AKBA (t = 12.3) in the rat liver is shown in Figures 3A and 3B. The peaks at t = 12.8, 10.6 and 9.5 are not derived from AKBA, since they were also present in the control animals and did not show the characteristic fragmentation pattern of AKBA. Screening the plasma, liver and brain samples for the occurrence of phase II metabolism of AKBA and AKBA metabolites, no evidence for phase II metabolism could be found, as in the case of KBA.

Checking for deacetylation as an alternative metabolic pathway for AKBA. Since AKBA represents an acetyl derivative of KBA, it could be metabolized to KBA via deacetylation. AKBA incubated with rat hepatocytes, however, yielded only very small amounts of KBA, not exceeding 2 % of the initial AKBA concentration. Also, the plasma, liver and brain of the rats fed with AKBA were screened for the presence of KBA. In the plasma, KBA was almost undetectable. As shown in the SIM chromatogram of KBA in Figure 3C, KBA (t = 11.2) was detected only in negligible amounts in the liver, not exceeding those determined in hepatocytes, indicating that deacetylation of AKBA to KBA takes place in the liver to a minor extent only. The peaks at t = 9.7 and 9.9 were also present in the control group and did not show the characteristic fragmentation pattern of KBA represented in Figure 3D. Therefore they cannot be related to KBA. Furthermore, no KBA was detected in the brain samples of the rats fed with AKBA.

Identification of metabolites of αBA, βBA and their acetylated derivatives *in vitro*. The incubation of αBA and βBA with rat liver microsomal preparations and its subsequent analysis by LC-MS/MS revealed the formation of various metabolites. In addition to metabolites with +16 u and +32 u mass shifts seen for both BA derivatives, metabolites with a mass shift of +48 u were observed for βBA. No metabolites were detected upon incubation of AαBA and AβBA with microsomal enzymes.

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Discussion

To date only one study exists on the microsomal incubation of *Boswellia* species, which focused on investigating the inhibitory effect of *Boswellia* extract on cytochrome P450 enzymes (Frank *et al.* 2006). Our study represents the first investigation of BA metabolism. An overview of all phase I metabolites obtained *in vitro* from different BAs and their respective retention times and mass shifts is given in Figure 4.

In general, the incubation of BAs with phase I enzymes *in vitro* revealed that oxidation, yielding derivatives with mass shifts of +16 u, +32 u and in some cases of + 48 u, corresponding to mono-, di- and trihydroxylated metabolites, respectively, is the principal metabolic route of KBA, AKBA, αBA and βBA. The phase I metabolites of BAs identified in this study are in agreement with the phase I metabolites described for oleanolic acid, which have been characterized as hydroxyl- and di-hydroxyloleanolic acid (Jeong *et al.*, 2007). Together, this previous investigation and the present study represent the first detailed insight into the metabolism of pentacyclic triterpenes.

The incubation of KBA with RLM yielded three monohydroxylated and six di-hydroxylated metabolites as well as two additional metabolites, exhibiting a +14 u mass shift. Taking the chromatographic elution behaviour of these metabolites into consideration, indicating more hydrophilic properties in comparison to KBA, the mass shift of +14 u may be attributed to ketone formation or dehydrogenation and hydroxylation of KBA. The incubation of KBA with hepatocytes yielded similar metabolites represented by three mono- and three di-hydroxylated KBA derivatives as well as one tri-hydroxylated metabolite.

In vivo hydroxylated derivatives of KBA were detected only in rat plasma and liver. The observed absence of KBA metabolites in rat brain may be attributed to the increased hydrophilicity of the hydroxylated derivatives, not allowing them to pass the blood brain barrier. Both the *in vitro* experiments in RLM and hepatocytes as well as the *in vivo* feeding study revealed the formation of the same metabolic profiles suggesting that no phase II metabolism is taking place.

In case of AKBA, only derivatives with a mass shift of +16 u, corresponding to mono-hydroxylated metabolites were detected *in vitro* and no metabolites were identified *in vivo*. The limited metabolism of AKBA by phase I enzymes coincides with observations made for other acetylated BAs (AαBA and AβBA), where no phase I metabolites were identified at all *in vitro*. Obviously, the acetyl group at position 3 is responsible for the impeded phase I metabolism. Nevertheless, some hydroxylated metabolites were identified for AKBA *in vitro* in contrast to AαBA and AβBA. This may be attributed to the presence of a keto group at position 11.

In general, microsomes and hepatocytes are not only used for the elucidation of drug metabolism, but are also typically applied during the drug screening process for determining metabolic stability (Shearer *et al.*, 2005). Since the pharmacological effects of *Boswellia serrata* are mainly attributed to KBA and AKBA, this study focused on the metabolic stability of these two BAs in rat liver microsomes and hepatocytes. In addition the metabolic stability was also determined in HLM, in order to get a preliminary idea about the hepatic transformation rate of KBA and AKBA in humans.

The present study revealed that KBA undergoes extensive hepatic phase I metabolism. This might represent one of the major reasons for the low systemic KBA bioavailability observed in rats and humans after the oral administration of very high doses of *Boswellia* extract. In contrast, the pharmacokinetic behaviour of AKBA raises several questions. Although the concentration of AKBA is equivalent or even exceeds that of KBA in *Boswellia* extracts, AKBA was detected in rat and human plasma at even lower concentration levels than KBA (Büchele *et al.*, 2003; Reising *et al.*, 2005). This is surprising, since AKBA is more lipophilic than KBA and should therefore be easier absorbed. A possible explanation could be an extensive first pass metabolism. However, the present study revealed that although AKBA differs from KBA only by acetylation of the hydroxyl-group at position 3, its metabolic behaviour is completely different. AKBA was more stable towards phase I enzymes than KBA. Therefore, extensive hepatic metabolism could not explain the low systemic availability of AKBA.

Furthermore, the present study revealed that deacetylation of AKBA to KBA takes place only to a minor extent and thus cannot be responsible for the low systemic availability of AKBA as previously assumed (Abdel Tawab et al., 2001; Sharma et al., 2004).

A further reason for the lower plasma levels of AKBA compared to KBA might be the greater volume of distribution of AKBA associated with its greater lipophilicity. Indeed, the brain-to-plasma ratio determined for AKBA (0.8) in a previous study (Reising *et al.*, 2005) was higher than that of KBA (0.5), although only half the plasma levels of KBA were determined for AKBA. These are very promising results, as they suggest a higher brain penetration of AKBA compared to KBA. Based on these findings, the metabolic stability determined for AKBA is of particular importance. At last, the influence of intestinal metabolism and poor absorption on limiting the systemic availability of BAs should not be underestimated. Initial results of subsequent *in vitro* permeability studies using Caco-2 cells, which are still in progress, suggest poor absorption of KBA and AKBA from the gastrointestinal tract. It seems that the poor systemic availability of AKBA may be mainly attributed to its poor absorption and the low bioavailability of KBA results from its metabolic instability and poor absorption (Krüger *et al*, work still in progress). In a previous study *Boswellia serrata* extract as well as KBA and AKBA, were identified as potent inhibitors of P-glycoprotein (Pgp) in porcine brain capillary endothelial cells (PBCEC) and human lymphocytic leukemia parenteral cell lines (VLB) (Weber *et al.*, 2006). AKBA produced a significant inhibition of Pgp at a concentration of 3 µM and 10 µM in PBCEC and

VLB cells, respectively, whereas KBA exerted significant Pgp inhibition at a concentration of 10 µM in PBCEC but not in VLB cells compared to untreated cells. As this study could not differentiate whether substrate inhibitor or allosteric effects were responsible for the observed Pgp inhibition, the relevance of P-gp efflux, as a possible factor affecting the bioavailability of KBA and AKBA, cannot be assessed at present.

For numerous drugs, the duration and intensity of action is determined by their metabolic rate. Characterizing the metabolic behaviour is therefore an important issue to assess the therapeutic effect of drugs. Considering the results of this study, it can be concluded that the observed extensive hepatic metabolism of KBA strongly contributes to its low bioavailability. However, the low bioavailability of the more lipophilic AKBA, which may not be attributed to an extensive hepatic metabolism, will surely represent a pivotal question in further studies addressing the permeability of boswellic acids.

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Legend for Figures

Figure 1. Metabolic stability of KBA (■) and AKBA (▲) in rat liver microsomes (RLM), human liver microsomes (HLM) and rat hepatocytes (RH). The error bars represent the standard deviations of three individual experiments for RLM and HLM and six individual experiments for RH. The metabolic stability curves in RLM and HLM result from phase I metabolism of KBA and AKBA, whereas the metabolic stability curve in RH is based on phase I and II metabolism.

Figure 2. SIM chromatograms and product ion spectra of KBA (A, E) and the corresponding metabolites for mass shifts of +16 u (B, F), +32 u (C, G) and +14 u (D, H) *in vitro*.

Figure 3. SIM chromatograms and product ion spectra of AKBA (A, B) (t = 12.3) and KBA (C, D) (t = 11.2) in the rat liver samples from the group given AKBA, showing that only negligible amounts of KBA were produced from AKBA by deacetylation.

Figure 4. Chemical structures of the six most important derivatives of BAs and their corresponding phase I metabolites, KBA, AKBA, αBA, βBA, AαBA and AβBA, *in vitro*.

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Legend for Tables

Table 1. Overview of all metabolites of KBA and AKBA detected *in vivo* in comparison to those identified *in vitro*.

Table 1

Boswellic acid	***In vitro***		***In vivo***		
	RLM	**Hepatocytes**	**Plasma**	**Liver**	**Brain**
KBA	+	+	+	+	+
Mono-hydroxylated KBA derivatives	+	+	+	+	-
Di-hydroxylated KBA derivatives	+	+	+	+	-
Tri-hydroxylated KBA derivatives	-	+	-	+	-
Hydroxylated and Dehydrogenated KBA derivatives	+	-	-	-	-
AKBA	+	+	+	+	+
Mono-hydroxylated AKBA derivatives	+	+	-	-	-

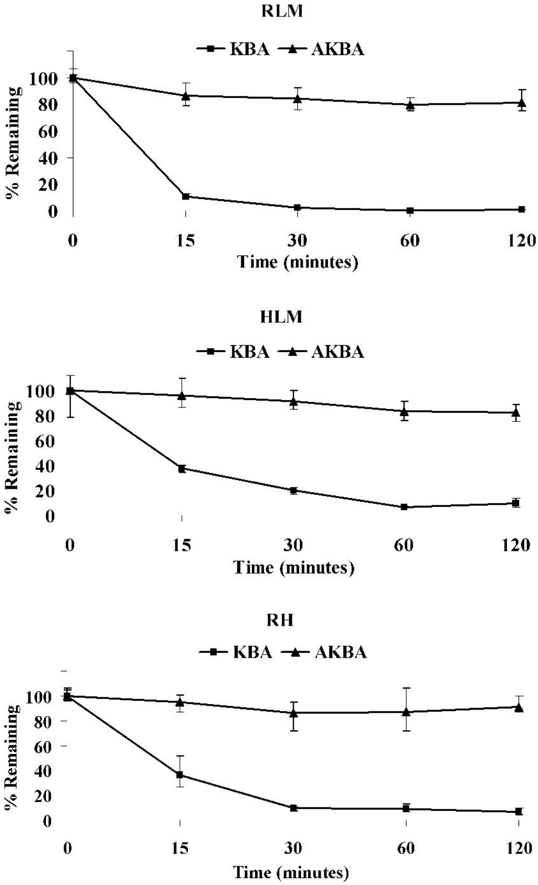

RLM
KBA
AKBA
% Remaining
100
80
60
40
20
0
0
15
30
60
120
Time (minutes)
HLM
KBA
AKBA
% Remaining
100
80
60
40
20
0
0
15
30
60
120
Time (minutes)
RH
KBA
AKBA
% Remaining
100
80
60
40
20
0
0
15
30
60
120
Time (minutes)

Figure 1

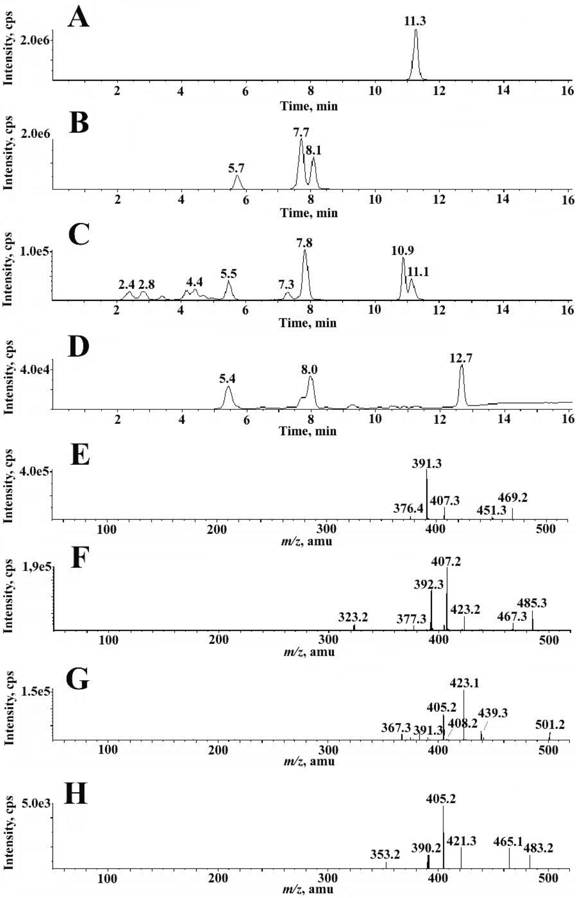

A
Intensity, cps
2.0e6
11.3
2
4
6
8
10
12
14
16
Time, min
B
Intensity, cps
2.0e6
5.7
7.7
8.1
Time, min
C
Intensity, cps
1.0e5
2.4
2.8
4.4
5.5
7.3
7.8
10.9
11.1
Time, min
D
Intensity, cps
4.0e4
5.4
8.0
12.7
Time, min
E
Intensity, cps
4.0e5
391.3
376.4
407.3
451.3
469.2
100
200
300
400
500
m/z, amu
F
Intensity, cps
1,9e5
407.2
392.3
323.2
377.3
423.2
467.3
485.3
m/z, amu
G
Intensity, cps
1.5e5
423.1
405.2
367.3
391.3
408.2
439.3
501.2
m/z, amu
H
Intensity, cps
5.0e3
405.2
353.2
390.2
421.3
465.1
483.2
m/z, amu

Figure 2

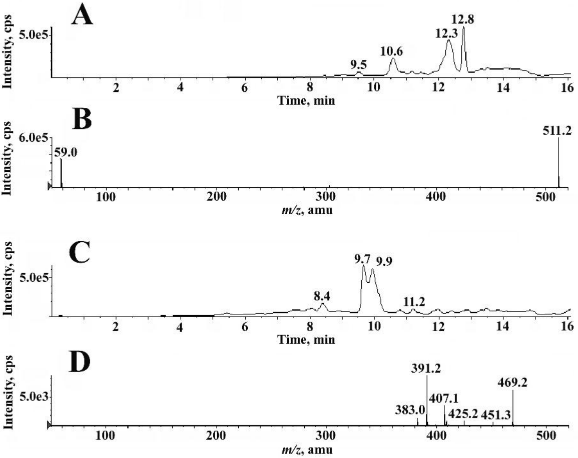
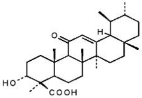

A
Intensity, cps
5.0e5
9.5
10.6
12.3
12.8
2
4
6
8
10
12
14
16
Time, min
B
Intensity, cps
6.0e5
59.0
511.2
100
200
300
400
500
m/z, amu
C
Intensity, cps
5.0e5
8.4
9.7
9.9
11.2
2
4
6
8
10
12
14
16
Time, min
D
Intensity, cps
5.0e3
383.0
391.2
407.1
425.2
451.3
469.2
100
200
300
400
500
m/z, amu

Figure 3

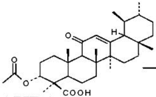


KBA
m/z 469.4 $[M-H]^-$ t=11.3min

Three monohydroxylated metabolites
***m/z* +16; t=5.7, 7.7, 8.1**

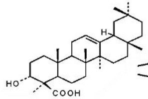
Six dihydroxylated metabolites
***m/z* +32; t=2.4, 2.8, 4.4, 5.5, 7.3, 7.8**

Two monohydroxylated+dehydrogenated metabolites *m/z* +14; t=5.4, 8.0



AKBA
m/z 511.4 $[M-H]^-$ t=12.2min

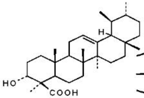
Three monohydroxylated metabolites
***m/z* +16; t=7.7, 8.2, 9.4**



αBA
m/z 455.3 $[M-H]^-$ t=11.6min

Three monohydroxylated metabolites
***m/z* +16; t=8.4, 9.2, 9.6**

Three dihydroxylated metabolites
***m/z* +32; t=5.3, 6.6, 7.3**

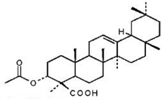


βBA
m/z 455.3 $[M-H]^-$ t=11.8min

Three monohydroxylated metabolites
***m/z* +16; t=8.5, 8.9, 9.3**

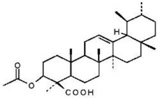
Three dihydroxylated metabolites
***m/z* +32; t=5.0, 6.5, 7.0**

Three trihydroxylated metabolites
***m/z* +48; t=4.5, 5.6**



AαBA
m/z 497.3 $[M-H]^-$ t=12.5min

No metabolites



AβBA
m/z 497.3 $[M-H]^-$ t=12.7min

No metabolites

Figure 4