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Research Article

COMPARISON OF THE METABOLIC STABILITY OF SOLID LIPID BOSWELLIA SERRATA PARTICLES VERSUS PLAIN BOSWELLIA SERRATA EXTRACT IN HUMAN HEPATOCYTES (HHL-17)

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ABSTRACT

Boswellic acids (BAs) including 11-keto-β-boswellic acid (KBA) and 3-acetyl-11-keto-β-boswellic acid (AKBA) are the active principles of Boswelliaserrata extract (BSE) which is used in traditional Indian medicine for the treatment of inflammatory conditions. BAs are characterized by low oral bioavailability. In order to circumvent this problem, solid lipid boswelliaserrata particles (SLBSP) were developed which is essentially a complexation of BAs with phospholipids such as phosphatidyl choline. The objective of this study was to compare the metabolic stability of SLBSP versus plain BSE using HHL-17, a Human telomere inactivated hepatocyte cell line. The two formulations were incubated in HHL-17 for time points ranging from 30 minutes to 480 minutes. At the end of incubation period, cells were lysed and concentration of KBA and AKBA in the cell lysates was estimated using a validated LC-MS/MS technique. It was observed that KBA from plain BSE was cleared by the hepatocytes with a half-life of 5.8 hours, whereas, KBA from SLBSP exhibited lesser accumulation in hepatocytes and lowmetabolic clearance. No difference was observed in the rate of metabolism of AKBA from the two formulations. It can be concluded that phospholipid complexation confers metabolic stability to KBA by rendering it less permeable into human hepatocytes.

Keywords: Boswelliaserrata extract, metabolic stability, KBA, AKBA. SLBSP, LC-MS/MS

INTRODUCTION

Boswelliaserrata (Salai/Salaiguggul) is a moderate to large sized branching tree from the family Burseraceae and genus *Boswellia*. The tree grows in dry mountainous regions of India, Northern Africa and the Middle East.[1]*Boswelliaserrata*extract (BSE) is used in Indian ayurvedic medicine for the treatment of arthritis and other inflammatory conditions.[2-4] Recently, Gota*et al* reported the efficacy of solid lipid *Boswelliaserrata*particles (SLBSP) in Osteoarthritis of knee.[5]Boswellic acids (BAs) have been identified as the major active components of the gum resin extract of *Boswelliaserrata.* In recent years, these compounds have been the subject of various scientific investigations.[6,7] The pharmacological

effects of BSE have been mainly attributed to 11-keto-β-boswellic acid (KBA) and 3-acetyl-11-keto-β-boswellic acid (AKBA). Previous studies on KBA and AKBA have suggested low oral bioavailability. Sterk*et al.,* reported very low plasma concentration in human volunteers, while the study of Sharma *et al.* showed that AKBA was not detected in plasma.[8,9] In order to circumvent this problem, Solid lipid *boswelliaserrata*particle having phosphatidylcomplexation of BSE was developed for better bioavailability of BAs.

Metabolic stability studies evaluate the susceptibility of compounds to biotransformation or intrinsic clearance; it also helps in the prediction of pharmacokinetic parameters.[10] Information about the metabolic stability of a compound is important to develop formulations with enhanced bioavailability. *In vitro* methods for predicting metabolic information have over the years gained increasing attention and have also been applied in chemical risk assessment.[11] Human-liver derived *in vitro* systems have proven useful in early studies of metabolic stability, in identifying enzymes capable of metabolising, and interactions with drugs in use or under development. Here, we determined the metabolic stability of KBA and AKBA using the human hepatocytes HHL-17 cell line. The aim of the present study was to evaluate and compare the metabolic stability of KBA and AKBA in the Solid lipid *boswelliaserrata*particle (SLBSP) having phosphatidylcomplexation of BSE (WokVida™) and plain uncomplexed BSE (WokVel™) over different time points.

MATERIALS AND METHODS

Chemicals and cell line

SLBSP (WokVidaTM/WV) and plain BSE (WokVelTM/ WW) were procured from Pharmanza Herbal Pvt. Ltd. WW contained KBA and AKBA at 7% and 1% w/w respectively whereas WV contained KBA and AKBA at 2.3% and 0.3% w/w respectively. Human telomere inactivated hepatocytes HHL-17 was a kind gift of Prof. Arvind H Patel, MRC-University of Glasgow Centre for Virus Research, Scotland, UK.

Cell Culture

i) Reagents

Dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), gentamycin sulphate, L-glutamine penicillin streptomycin solution (GPSS), Dulbecco Modified Eagle Medium (DMEM) trypan blue and trypsin-EDTA were obtained from Invitrogen INDIA. All chemicals/reagents were of analytical grades.

ii) Maintenance of Cell Lines

The adherent human telomere inactivated hepatocytes (HHL-17) cell line was maintained according to the protocols as described by Celis (1998) and Freshney (2010).[12,13]

iii) Thawing of Cell lines

The cryovials containing the cell lines were placed in water bath at 37 ^{o}C for 1-2mins and contents were immediately transferred into centrifuge tubes (15mL) containing pre-warmed media. After gentle mixing, the tubes were balanced and centrifuged for 10 mins at 1000 rpm. The supernatant was discarded and the cell pellet was dislodged by gentle tapping and re-suspended in media for counting and determination of viability. The cell line was cultured according to standard cell culture procedure.

iv) Trypsinization of Monolayer

The culture medium was discarded and 1x PBS (phosphate buffer saline) was added to remove serum and dead cells followed by addition of trypsin-EDTA (0.05 %, 2 mL for 25 cm2 flask) and kept at 370C for 2-3 minutes in the CO2 incubator. The trypsin was inactivated by the addition of media (5 - 8mL) for 25 cm2 flask. The cell suspension was centrifuged at 1000 rpm for 10 mins at room temperature and the cell pellet was resuspended in media.

Treatments of Cell line

HHL-17 cells were grown in DMEM supplemented with 10 % (v/v) fetal bovine serum (FBS), 1 % (v/v) L-glutamine, 100 U penicillin and 100 μg/mL streptomycin at 37 ˚C in a humidified atmosphere with 5 % CO2. The cells were passaged twice weekly. WW and WV were initially dissolved in culture medium at a final concentration of 50ug/ml. The mixture was freshly prepared before adding to cell cultures. WW and WV were added to cells in complete medium after 24 hrs of growth. The experiments were done in triplicates. The normal incubation conditions for the hepatocytes were maintained after the addition of drugs. The incubation time points were 30 minutes, 1hour, 2hours, 4hours, 6hours and 8hours.

Cell lysate preparation for LCMS/MS analysis

The experiment was terminated by aspirating the media from the culture plate and the plate was rinsed twice with ice cold PBS, followed by detachment of cells with trypsin-EDTA into 1ML PBS and subsequent transfer of cell suspension into 1.5ml tube. Cells pellet was obtained by centrifuging at 3000 rpm for 5minutes, the

supernatant was removed and the pellet suspended in 500 microliter PBS and vortex for 10-15 minutes. The cell suspension was transferred to the cryovial, and then to the liquid nitrogen for 1 minute, this was immediately transfered to 370C water bath for 1 minute. The last two steps were repeated three times for proper cell lysing. This is referred to as the freeze thawing method of cell lysing. The cell lysates obtained were kept in -200C freezer and finally to -800C until samples were required for analysis.

Analysis of the samples by liquid chromatography–tandem mass spectrometry (LCMS/MS)

In order to determine the concentration of BAs in cell lysates, isocratic separation was performed on a PerkinElmer series 200 high-performance liquid chromatography system with a reversed-phase 18 column (Hypersil BDS, 150 4 mm, 5 m 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). Mass spectrometric analysis was performed on a triple quadrupole mass spectrometer (API 300; ABSciex, 4500Q-Trap Concord, Singapore. Equipped with an atmospheric pressure chemical ionization interface operating in positive ionization mode at 425°C. Multiple reaction monitoring was used to quantify KBA and AKBA in hepatocytes.

Solutions containing 50 ug/ml KBA or AKBA were prepared with the media out of which 250 ul was added to each plate. The metabolic reaction was stopped by aspirating the media at the aforementioned time points and subsequent lysing of the cells according to the protocol earlier described. The cell lysates were transferred to autosampler vials and analyzed directly by LC-MS/MS. The metabolic stability of KBA and AKBA was monitored by determining the ability of hepatocytes to biotransform these compounds. The metabolic stability of KBA and AKBA was calculated on the basis of three determinations, at each time point respectively.

Statistical Analysis

The results were expressed as mean ± Standard deviation. Differences between the groups were analyzed by two-way analysis of variance (ANOVA) with Sidak's*post hoc* test using Prism version 6 (GraphPad Software Inc.) USA. P-values less than 0.05 were considered statistically significant for differences in mean.

RESULTS AND DISCUSSION

From a metabolic standpoint, a good drug or drug candidate should be relatively stable, maintaining an effective concentration in the blood for a reasonable period of time. Under the provided experimental condition, metabolic stability studies with human hepatocytes HHL-17 show that the hepatocytes are more permeable to KBA in WokVel (WW) as compared to WokVida (WV). The highest concentration of KBA was approximately four fold higher in cell lysates following incubation with WW as compared to WV. This suggests that higher proportion of drug is exposed to first pass effect in case of uncomplexed BAs as compared to those complexed with phospholipids. Peak concentration of KBA in the hepatocyte lysate was observed at 120 minutes of incubation with WW followed by a steady decline (Figure 1). The half-life of elimination of KBA from the lysate was found to be 5.8 hours. On the other hand, the concentration of KBA following incubation with WV did not change appreciably during 8 hours of incubation suggesting that it did not undergo significant metabolism. Thus KBA complexed with phospholipids exhibited greater metabolic stability as compared to uncomplexed KBA. This is in line with the earlier observation of Sharma *et al.* who reported low bioavailability of KBA in the plasma of human volunteers (Sharma *et al.,* 2004) following administration of uncomplexed BSE. Therefore it could be expected that the bioavailability of KBA in WV formulation would be hugely enhanced.

Interestingly, the concentrations of AKBA in the hepatocyte culture were comparable between WW and WV at all time points (Figure 2). Peak concentration in the hepatocyte lysate was observed at 60 minites and 120 minutes for WV and WW respectively, and the concentration did not change appreciably even after 480 minutes of incubation. This suggests that both the complexed and uncomplexed AKBA are able to permeate into the hepatocytes in equal measure and a steady state exists between the rate of entry and the rate of elimination through metabolism. The reason for differential metabolic stability of AKBA and KBA following phospholipid complexation is not very clear. The implications of this finding is that phospholipid complexation is unlikely to enhance the bioavailability of AKBA, unless it is enhancing the intestinal permeability of the compound.

BAs, particularly KBA, are primarily metabolised by oxidation to hydroxylated metabolites**.** Krüger P et al have shown in liver microsomes and rat hepatocyte systems that KBA is metabolically unstable. On the other hand, it was

also shown that AKBA has an extremely low hepatic clearance.[7]Both KBA and AKBA are poorly absorbed from the gastrointestinal tract. In our experiment, the metabolic profile of KBA from BSE is very similar to that reported by Krüger P et al. However, the phospholipid complexed KBA in SLBSP had reduced accumulation in the hepatocytes as well as low clearance. Thus, SLBSP may be expected to have higher oral bioavailability of KBA compared to plain BSE. Gota P et al have demonstrated the efficacy of WV in patients with osteoarthritis of the knee (OA knee).[5] Treatment with WV resulted in improvement in OA symptomatology as determined by VAS and WOMAC scores. In addition, a comparative study of WW versus WV in the same patient population showed that patients on WV had fewer painful episodes and required lesser rescue analgesics as compared to WW (unpublished data). It is plausible that patients on WV achieved higher plasma concentration of KBA which could have lead to this effect. In a separate study, Gota V et al demonstrated good oral bioavailability of curcumin administered as solid lipicccucumin particles (SLCP) in a phase I clinical trial, which once again underscores the benefit of lipid complexation.[14] On the other hand, AKBA was metabolically stable in both formulations suggesting that future studies should evaluate the intestinal permeability of AKBA in SLBSP using Caco-2 cells.

The exact measure of metabolic stability is possible only if the rate of formation of metabolites is estimated. A limitation of this study is that we did not quantify the rate of formation of metabolites of KBA and AKBA. Rather, we relied entirely on the clearance of the parent compounds from hepatocyte lysates. While this allows us to quantify the net difference in the rates of cellular uptake of the drug and its elimination, the individual rates cannot be measured separately.

CONCLUSION

KBA from the SLBSP formulation of WV was found to concentrate less in the hepatocytes thereby avoiding hepatic clearance whereas AKBA intrinsically has low hepatic clearance. Since absorption across the gastrointestinal tract is a major factor limiting the bioavailability of AKBA, studies using Caco-2 cells are likely to yield useful information about the bioavailability of AKBA from SLBSP formulation.

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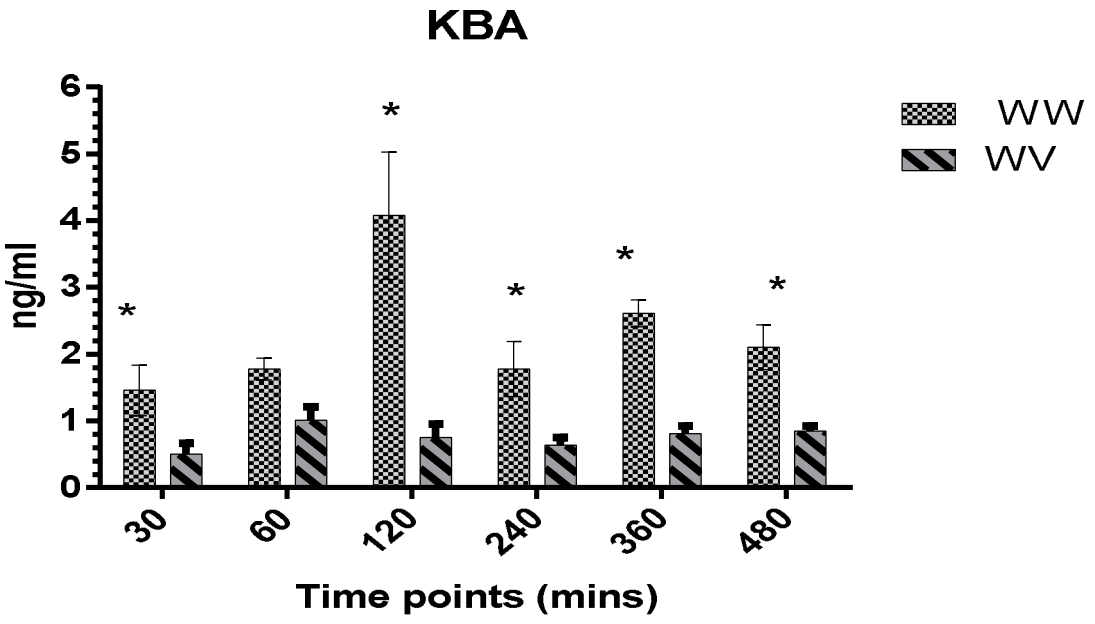

Fig. 1: Concentration-time profile of KBA in the hepatocyte lysate following incubation with WW and WV at different time points. Data shown as mean +SD for each time point (N=3). * Mean is significant when KBA WW is compared with KBA in WV

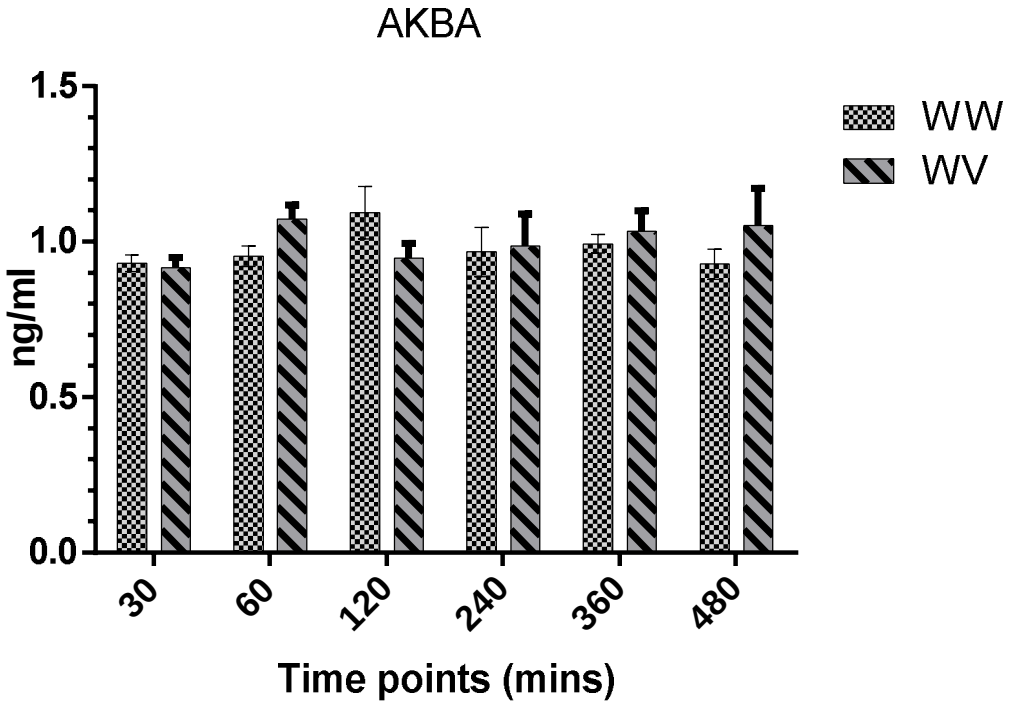

Fig. 2: Concentration-time profile of AKBA in the hepatocyte lysate following incubation with WW and WV at different time points.
Data shown as mean +SD for each time point (N=3)

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