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Determination of major boswellic acids in plasma by high-pressure liquid chromatography/mass spectrometry

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ABSTRACT

Until now, dexamethasone is the medication of choice to reduce peritumoral edema associated with primary and secondary brain tumors. Because of the severe side effects accompanying such a treatment the interest in alternative agents that may be co-administered with glucocorticoids and help to reduce the required dose is constantly increasing. *Boswellia serrata* gum resin extracts (*BSE*), which have been designated an orphan drug status by the European Medicines Agency (EMA) in 2002 for the treatment of peritumoral edema, may represent a promising supplemental herbal remedy. However, clinical studies on the effect of *BSE* on brain edema as well as analyzes of serum levels are very scarce. Based on that background a prospective, placebo controlled, and double blind clinical pilot trial was conducted on 14 patients applying for the first time a high dose of 4200 mg *BSE* per day and 13 patients receiving placebo. For monitoring the serum levels of all major boswellic acids (BAs) a highly sensitive HPLC–MS method has been developed that allows the determination of KBA and AKBA from 5.0 ng/ml to 3000 ng/ml and of αBA, βBA, AαBA and AβBA from 0.5 ng/ml to 12,000 ng/ml. It is the first validated method that covers such a wide concentration range, which makes it suitable to be used as standard method in clinical trials as it compensates for the great pharmacokinetic variability in the plasma levels of BAs observed in clinical practice. Average steady concentrations (ng/ml) in the range of 6.4–247.5 for KBA, 0–15.5 for AKBA, 36.7–4830.1 for αBA, 87.0–11948.5 for βBA, 73.4–2985.8 for AαBA and 131.4–6131.3 for AβBA were determined in the verum group. The here quantified steady state levels suggest βBA to be a possible candidate for the anti-inflammatory and anti-edemateous effects of *BSE*. In general, the serum level analysis underlines the promising clinical results of *BSE* on cerebral edema.

1. Introduction

Peritumoral edema not only contributes to symptoms associated with brain tumors but may also develop during radiotherapy, which plays a central role in the treatment of brain tumors [1,2]. Therefore reducing peritumoral edema constitutes a major issue in the treatment of primary brain tumors, which occur with an annual incidence of 7.3 per 100,000 habitants in the USA [3]. But also secondary tumors (brain metastases), making up 30% of all brain tumors, are associated with edema [4].

Until now, glucocorticoids, in particular dexamethasone, play a major role in the treatment of cerebral edema. Although glucocorticoids have facilitated the management of edema in patients with diagnosed brain tumors, these drugs are associated with a lot of side effects including immunosuppression, Cushing syndrome, and osteoporosis. Moreover glucocorticoids are supposed to interfere with the efficacy of chemotherapy by reducing tumor perfusion and inhibiting apoptosis [5,6]. As the observed side effects mainly depend on the dose and duration of administration, glucocorticoids should be generally used in a restrictive manner keeping the dose as low as possible. Therefore the interest for alternative agents that may be co-administered with glucocorticoids and help to reduce the required dose for the treatment of cerebral edema is constantly increasing.

Gum resin extracts of *Boswellia serrata* (Indian frankincense) have been found to represent a promising supplemental herbal remedy. For centuries *B. serrata* gum resin extracts (*BSE*) have been traditionally applied in folk medicine to treat various topical and

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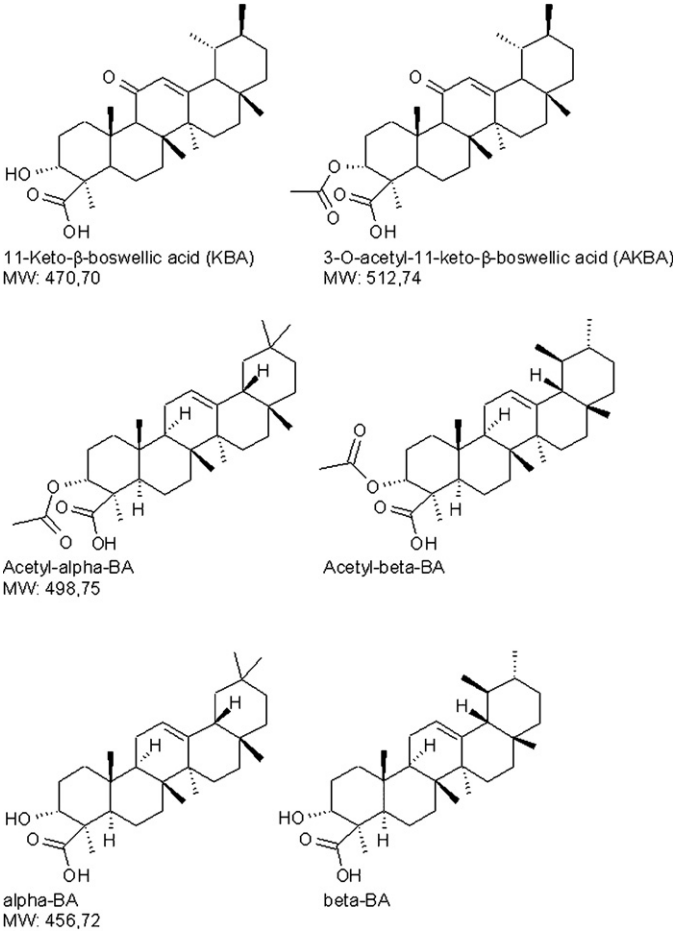

Fig. 1. Structures of major BA's.

systemic inflammatory diseases. Experimental data from animal and human studies confirmed the potential of *BSE* for the treatment of a variety of inflammatory disorders like inflammatory bowel disease (IBD), rheumatoid arthritis (RA), osteoarthritis (OA) and asthma [7]. Moreover *BSE* were found to significantly reduce peritumoral brain edema accompanying glioma [8]. In 2002, the European Medicines Agency (EMA) designated *BSE* an orphan drug status for the treatment of peritumoral brain edema. The pharmacological effects of *BSE* were attributed to the pentacyclic triterpenic boswellic acids, especially to 11-keto-β-boswellic acid (KBA) and 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA).

Although many patients treated for primary or secondary brain tumors are taking *BSE* in addition to glucocorticoids, only two studies have been published on the effect of *BSE* on brain edema. Streffer et al. investigated the effect of H15 (a commercial *BSE* preparation) on twelve patients with brain edema caused by tumor progression or radiotherapy-associated leukoencephalopathy. In this study eight of twelve patients showed a clinical or radiological response [9]. Similarly, Boker and Winking demonstrated significant dose-dependent reduction of peritumoral edema, associated with improvement of the clinical conditions of patients when administering *BSE* at the highest dose of 3× 1200 mg per day [8].

Based on that background a prospective randomized, placebo controlled, double blind pilot trial was conducted on 14 patients applying for the first time a high dose of 4200 mg *BSE* per day for the whole duration of radiotherapy and 13 patients receiving placebo. In this context it was interesting to monitor the serum levels of the major BA's, KBA, AKBA, βBA, acetyl-β-BA (AβBA), αBA and acetyl-α-BA (AαBA) (Fig. 1), in order to get an idea about the steady state concentrations achieved after the application of the hitherto highest dose of *BSE*. This is of special interest as previous pharmacokinetic studies revealed rather low plasma levels of KBA and AKBA which have long believed to represent the active BA's [10–16].

Only two analytical methods have been reported so far which determine the plasma levels of several BAs simultaneously [11,14]. Other methods concentrated on the determination of KBA and AKBA using LC/UV, GC/MS or LC/MS [10,17,18]. Only a RP-HPLC method with photodiode array detection was capable of determining all six major BAs, but this method was mainly limited by the comparatively high limit of quantification for KBA and AKBA not falling below 47 ng/ml [11]. Moreover, a recently reported LC/ESI-MS/MS determined four BAs, namely βBA in a range of 0.125–1250 ng/ml as well as acetyl-β-BA (AβBA), KBA and AKBA from 0.013 to 125 ng/ml in plasma [14]. However the calibration range of this LC/ESI-MS/MS is too narrow when higher doses of *BSE* are administered.

Therefore a new LC/MS method for the quantification of all six major BAs has been developed and validated which is capable of determining trace (down to 0.5 ng/ml) as well as high amounts (up to 12,000 ng/ml) of the individual BAs. In view of the generally higher doses of *BSE* that are expected to be administered to brain tumor patients this is the first analytical method that is capable of determining all major BAs over a wide concentration range covering thus the great pharmacokinetic variability observed in clinical practice. It is thus suitable to be used as standard analytical method for the analysis of BAs in clinical trials.

2. Experimental

2.1. Chemicals and reagents

KBA, AKBA, βBA, AβBA, αBA and AαBA were obtained from Phytoplan GmbH (Heidelberg, Germany, content >99.0%). The internal standard fluoxymesterone was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany, content >98.0%). Ammonium formate was obtained from VWR (Leuven, Belgium). All solvents used were of analytical grade or better quality. Methanol, tetrahydrofuran, ethyl acetate and n-hexane were purchased from Roth GmbH (Karlsruhe, Germany), 2-propanol, water and Extrelut® NT from Merck (Darmstadt, Germany).

2.2. Clinical study

The *B. serrata* product H15 (Hecht Pharma, Stinstedt, Germany) containing 350 mg *BSE* was chosen as study medication. The detailed BA composition of H15 was determined to be 35.3 mg KBA (10.6%), 6.7 mg AKBA (2%), 80.8 mg αBA (24.2%), 186.4 mg βBA (55.8%), 6.1 mg AαBA (10.6%) and 19.0 mg AβBA (5.7%). For blinding, the H15 capsules were sealed by another capsule (capsule in capsule method). The placebo capsules containing lactose were produced in the same way. Randomization was carried out in the pharmacy of the University Hospital Freiburg using a computer generated randomization schedule. Details regarding the conduction and outcome of the study are published elsewhere [19]. The study was done within all rules of the declaration of Helsinki and was submitted to the ethic committee of the Albert-Ludwigs University Freiburg giving a positive vote.

Blood samples were collected at different time points and immediately centrifuged. The serum was frozen at −80 °C until the time of analysis. Samples were obtained from the University Hospital Freiburg, Dept. of Radiation Oncology and included 27 samples of 14 patients of the verum and 24 samples of 13 patients of the placebo group. In addition the serum levels of all BAs were monitored over

Table 1
Correlation coefficients for the calibration curves generated during validation.

Day of validation	KBA	AKBA	αBA	βBA	AαBA	AβBA
1	0.9977	0.9992	0.9934	0.9949	0.9957	0.9964
2	0.9978	0.9968	0.9972	0.9954	0.9935	0.9942
3	0.9992	0.9992	0.9944	0.9960	0.9963	0.9953

a period of 6 h in a volunteer person taking a single dose of 1400 mg of H15.

2.3. Determination of BAs in plasma

2.3.1. Sample preparation

Concentrated stock solutions of all used boswellic acids (AKBA, KBA, αBA, βBA, Aα- and AβBA) for standards and quality controls as well as the internal standard fluoxymesterone were prepared at a concentration of 1 mg/ml diluted in methanol. Different working solutions containing all BA's were prepared as well as an internal standard solution at 4 μg/ml by diluting the stock solutions with methanol. Calibration standards were prepared daily by spiking 1 ml of blank plasma with 25 μl of the internal standard solution and 40 μl of the corresponding working solution resulting in concentrations of 0.5, 1.0, 5.0, 10.0, 50.0, 100, 500, 1000, 1500, 3000, 6000 and 12,000 ng/ml plasma for βBA, AβBA, αBA and AαBA and 5.0, 10.0, 50.0, 100, 500, 1000, 1500, 3000 ng/ml plasma for KBA and AKBA. QC pools at different concentration levels (15.0, 800 and 2500 ng/ml plasma for AKBA and KBA and 1.5, 15.0, 800, 2500 and 10,000 ng/ml plasma for βBA, AβBA, αBA and AαBA) were prepared by spiking blank plasma with the corresponding spike solution. Afterwards the QC samples were aliquoted and stored at −20 °C until analysis. Based on the method of Buechele and Simmet [11], the patient samples and QC's were carefully thawed and 1 ml of the plasma homogenate was spiked with 40 μl pure methanol corresponding to the volume of the spike solution for calibration samples and 25 μl internal standard solution containing 1 μg fluoxymesterone in methanol. All samples were mixed briefly using a vortex while 0.8 g of Extrelut® NT was filled into an 8 ml glass column for each sample. The plasma homogenates were transferred onto the columns for a matrix-based liquid–liquid-extraction. After 15 min the boswellic acids were eluted with 8 ml of an elution mixture consisting of tetrahydrofuran–n-hexane–ethyl acetate–2-propanol (160:160:160:15, v/v/v/v) into clean centrifuge tubes. After that the solvent was evaporated to dryness using a nitrogen stream at 40 °C. The residue was reconstituted in 100 μl mobile phase A and 50 μl was injected into the HPLC/MS-system.

2.3.2. Determination of BAs in H15

Concentrated stock solutions of BA's were prepared as described above. Calibration solutions were prepared by diluting the stock solutions in methanol with mobile phase A to obtain concentrations of 10, 50, 100, 500, 1000, 2500, 10,000 and 15,000 ng/ml. Of these calibration solutions 150 μl was mixed with 150 μl of an internal standard solution containing 3000 ng of the internal standard fluoxymesterone.

After weighting the capsule content of ten capsules of the H15 preparation the average mass was determined. After that the capsule content was mixed and the average weigh of one capsule was diluted in 20 ml methanol (ultrasonic bath for 30 min). Then an aliquot of 1.0 ml was centrifuged for few minutes and 100 μl of the supernatant were filled up to 100 ml with mobile phase A to obtain the sample solution. 150 μl of the sample solution was mixed with 150 μl of an internal standard solution containing 3000 ng of the internal standard fluoxymesterone.

2.3.3. Instrumental and chromatographic conditions for the determination of BAs

Liquid chromatography was performed on an Agilent instrument 1200 series (Agilent Technologies Waldbronn, Germany). A Hypersil BDS RP C18 column (100 mm × 4 mm, 3 μm) (Thermo Sci-

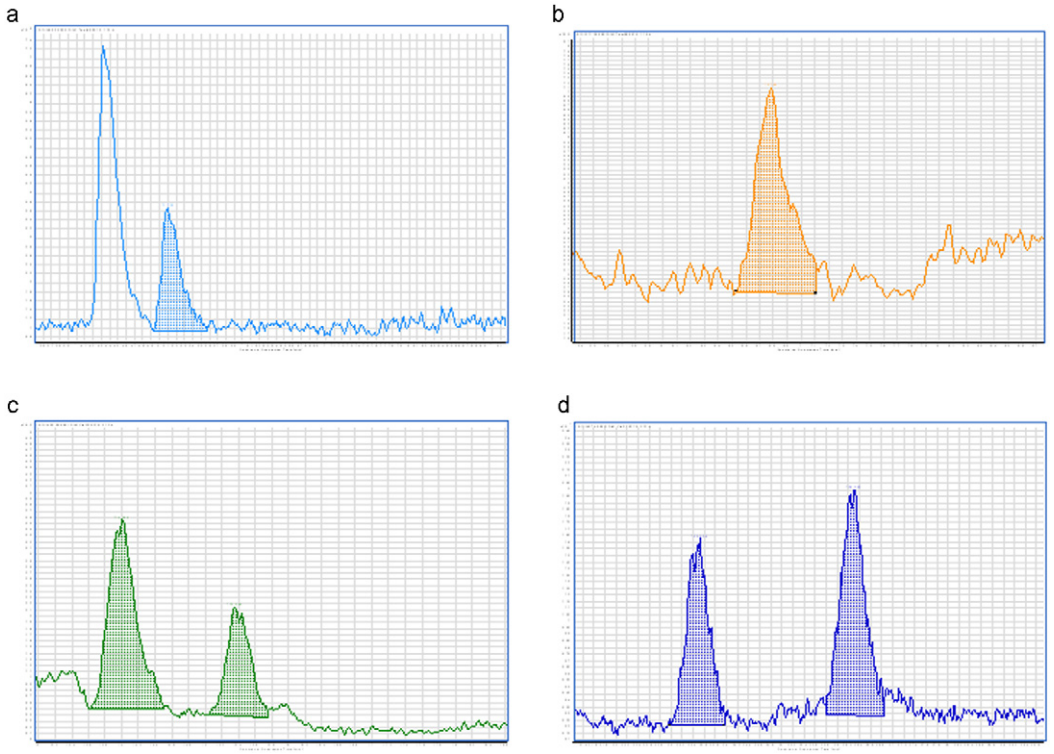

Fig. 2. HPLC-SIM-chromatogram of spiked serum (a) with 5.0 ng/ml KBA, (b) with 5.0 ng/ml AKBA, (c) with 0.5 ng/ml αBA and βBA and (d) with 0.5 ng/ml AαBA and AβBA.

Table 2a
Results of precision and accuracy for the determination of AKBA and KBA in human serum (data represent the mean of six replicates on the same day and on 3 days, respectively).

Nom. conc. in ng/ml	Calc. conc. in ng/ml		Intra-day RSD (%)		Inter-day RSD (%)		Intra-day accuracy (%)		Inter-day accuracy (%)	
	AKBA	KBA	AKBA	KBA	AKBA	KBA	AKBA	KBA	AKBA	KBA
15	15.6	16.1	3.7	6.3			4.2	7.6		
	14.5	14.4	6.3	3.1	5.4	7.5	−3.7	−4.3	−0.6	−0.3
	14.7	14.3	2.3	3.3			−2.2	−4.8		
800	741.8	790.0	6.4	10.7			−7.3	−1.3		
	777.7	805.8	5.9	1.7	6.4	8.3	−2.8	0.7	−6.5	−3.9
	725.2	711.7	5.5	3.3			−9.4	−11.0		
2500	2178.7	2376.1	1.7	7.8			−12.9	−5.0		
	2287.0	2471.0	3.8	0.5	4.5	5.5	−8.5	−1.2	−8.8	−4.5
	2371.7	2313.5	2.9	4.1			−5.1	−7.5		

Table 2b
Results of precision and accuracy for the determination of α- and β-BA in human serum (data represent the mean of six replicates on the same day and on 3 days, respectively).

Nom. conc. in ng/ml	Calc. conc. in ng/ml		Intra-day RSD (%)		Inter-day RSD (%)		Intra-day accuracy (%)		Inter-day accuracy (%)	
	α-BA	β-BA	α-BA	β-BA	α-BA	β-BA	α-BA	β-BA	α-BA	β-BA
1.5	1.6	1.5	5.9	9.0			8.5	1.2		
	1.6	1.6	3.2	5.7	4.6	8.4	9.3	7.1	7.6	0.4
	1.6	1.4	4.1	4.9			5.1	−6.0		
800.0	814.4	841.5	10.6	9.6			1.8	5.2		
	761.3	704.4	8.4	1.4	9.5	11.5	−4.8	−11.9	−1.3	−1.7
	793.2	809.6	9.7	11.7			−0.9	1.2		
10000.0	9853.0	9522.0	5.7	9.8			−1.5	−4.8		
	9250.3	9270.9	5.7	6.0	6.6	8.8	−7.5	−7.3	−6.5	−2.1
	8954.5	10535.0	4.9	5.1			−10.5	5.4		

Table 2c
Results of precision and accuracy for the determination of AαBA and AβBA in human serum (data represent the mean of six replicates on the same day and on 3 days, respectively).

Nom. conc. in ng/ml	Calc. conc. in ng/ml		Intra-day RSD (%)		Inter-day RSD (%)		Intra-day accuracy (%)		Inter-day accuracy (%)	
	AαBA	AβBA	AαBA	AβBA	AαBA	AβBA	AαBA	AβBA	AαBA	AβBA
1.5	1.6	1.5	4.8	6.4			3.6	−3.6		
	1.4	1.4	8.0	7.7	7.6	6.3	−5.8	−4.8	−3.0	−4.6
	1.4	1.4	5.5	5.7			−6.6	−5.6		
800.0	748.3	772.8	7.9	9.5			−6.5	−3.4		
	895.1	832.7	1.2	6.9	9.8	7.9	11.9	4.1	1.4	0.2
	780.5	794.1	7.6	7.3			−2.4	−0.7		
10000.0	10251.4	10177.8	6.4	2.9			2.5	1.8		
	9400.6	10323.4	4.2	3.0	6.5	4.7	−6.0	3.2	0.2	5.3
	10410.0	11098.0	3.9	2.3			4.1	11.0		

entific) with a Gemini C18 security guard cartridge (Phenomenex, Germany) 4 mm × 3 mm was used for chromatography. Separation was achieved using a gradient program starting with 90% mobile phase A (methanol:water 90:10, 400 mg/l ammonium formate) and 10% mobile phase B (methanol:water 80:20, 400 mg/l ammonium formate) raising to 100% mobile phase A within 20 min. This was kept constant for 14 min before returning to the initial conditions during 1 min. The total run time was 35 min at a flow rate of 0.4 ml/min. The column oven was set to 35 °C and the autosampler was kept at room temperature.

MS analysis was performed in the negative single ion mode (SIM) on an Agilent Triple Quadrupole LC/MS 6410 series (Agilent Technologies, Waldbronn, Germany) equipped with an electro spray ionization source (ESI). Dwell time was chosen to be 200 ms.

The detected ions in single ion mode were *m/z* 511.5 for AKBA at a retention time (RT) of 6.7 min, *m/z* 469.3 for KBA (RT 5.2 min), *m/z* 497.4 for AαBA and AβBA (RT 23.3 min and 26.0 min, respectively), *m/z* 455.5 for αBA and βBA (RT 13.8 and 15.2 min, respectively) and *m/z* 381.2 for the internal standard fluoxymesterone (RT 2.7 min). Data acquisition and integration of the peak areas were achieved using the standard instruments selected ion recording software (Masshunter).

Analyte concentration of the BA's was evaluated using the internal standard method. The standard curves were calculated from the peak area ratios of the analyte/internal standard and the nominal analyte concentration using second order regression with $1/x^2$ weighting.

Table 3
Results of relative recovery.

Nom. conc. in ng/ml	Rel. recovery (%)		
	15 ng/ml	800 ng/ml	3000 ng/ml
KBA	73.8	84.0	78.1
AKBA	72.7	93.7	93.7
αBA	75.7	84.5	80.2
βBA	79.6	81.0	100.6
AαBA	76.1	72.9	70.7
AβBA	62.0	73.3	64.2
I.S.	80.2	84.6	97.6

Table 4a
Results of stability (freeze–thaw cycles) for the determination of AKBA and KBA in human serum (data represent the mean of three replicates on the same day).

Nom. conc. in ng/ml	Cycle	Calc. conc. in ng/ml		Intra-day RSD (%)		Bias (%)	
		AKBA	KBA	AKBA	KBA	AKBA	KBA
15	1	14.6	14.3	8.8	6.6	−3.0	−4.9
	2	14.7	14.0	4.5	6.7	−2.3	−6.9
	3	14.6	14.7	4.5	6.2	−3.0	−1.9
800.0	1	787.5	770.3	8.0	6.5	−1.6	−3.7
	2	729.8	688.0	6.0	1.1	−8.8	−14.0
	3	724.2	732.3	8.2	5.5	−9.5	−8.5
2500	1	2199.8	2255.7	1.5	5.1	−12.0	−9.8
	2	2174.5	2155.5	0.3	0.9	−13.0	−13.8
	3	2341.7	2419.1	6.4	3.1	−6.3	−3.2

Table 4b
Results of stability (freeze–thaw cycles) for the determination of αBA and βBA in human serum (data represent the mean of three replicates on the same day).

Nom. conc. in ng/ml	Cycle	Calc. conc. in ng/ml		Intra-day RSD (%)		Bias (%)	
		αBA	βBA	αBA	βBA	αBA	βBA
15	1	13.7	14.5	11.4	8.4	−8.8	−3.6
	2	13.0	13.8	0.1	1.6	−13.6	−7.8
	3	13.6	15.4	7.0	12.3	−9.0	3.0
800.0	1	851.8	794.5	5.3	12.3	6.5	−0.7
	2	724.7	900.9	3.3	1.0	−9.4	12.6
	3	728.2	829.1	3.2	11.0	−9.0	3.6
2500	1	2769.3	2519.7	3.1	9.3	10.8	0.8
	2	2286.5	2847.8	2.6	0.4	−8.5	13.9
	3	2770.8	2844.2	2.3	0.9	10.8	13.8

2.4. Method validation

The analytical method was developed and validated using human plasma as matrix. The specificity of the method was verified by comparing the chromatograms of six different batches of blank plasma samples with the corresponding plasma samples spiked with 1000 ng/ml fluoxymesterone (I.S.) and 0.5 ng/ml βBA, AβBA, αBA and AαBA, respectively, and 5.0 ng/ml for AKBA and KBA, respectively. No peaks from blank plasma should coelute with the BA's or the internal standard. Linearity was assessed by weighted second order regression of standard curves prepared in triplicate on three separate days based on the analyte/I.S. peak area ratios. The correlation coefficient of each curve should be greater than 0.98. Precision [expressed as relative standard deviation (R.S.D.)] and accuracy [expressed as relative error (R.E.)] were determined by analysis of six plasma samples spiked with 1.5 ng/ml (QC-T), 800 ng/ml (QC-M) and 10,000 ng/ml (QC-H) for βBA, AβBA, αBA and AαBA as well as 15.0 ng/ml (QC-T), 800 ng/ml (QC-M) and 2500 ng/ml (QC-H) for KBA and AKBA on the same day (intra-day) and additionally on two other days (inter-day). The R.S.D. and R.E. should not exceed 15% at each concentration level, except for the lowest concentration level, a deviation of 20% is allowed. Moreover precision and accuracy at the lower limit of quantification (LLOQ) was determined in six plasma samples. R.S.D. and R.E should be less than or equal to 20%. Furthermore the signal at this concentration level should be at least 5 times higher than the blank response. The relative recovery of fluoxymesterone was evaluated by comparing the peak areas in post-extraction blank plasma with the peak areas in processed samples. Similarly the relative recovery of the I.S. fluoxymesterone was determined by comparing the peak areas of post-extraction blank plasma spiked with fluoxymesterone with the respective areas of the I.S. in processed samples. The stability of the BA's was assessed using QC samples at the concentration levels 1.5 ng/ml, 800 ng/ml and 10,000 ng/ml for βBA, AβBA, αBA and AαBA and 15.0 ng/ml, 800 ng/ml and 2500 ng/ml for KBA and AKBA subjected to the following conditions: (I) storage at room temperature for 24 h and (II) three freeze/thaw cycles. The samples were considered stable if R.S.D and R.E. at each concentration level did not exceed 15%, except for the lowest concentration level where a deviation of 20% is allowed. For testing the robustness, the BA's were determined in plasma samples processed three-fold at the concentration level of 800.0 ng/ml using two different column

Table 4c
Results of stability (freeze–thaw cycles) for the determination of AαBA and AβBA in human serum (data represent the mean of three replicates on the same day).

Nom. conc. in ng/ml	Cycle	Calc. conc. in ng/ml		Intra-day RSD (%)		Bias (%)	
		AαBA	AβBA	AαBA	AβBA	AαBA	AβBA
15	1	15.1	15.4	4.4	3.8	0.3	2.4
	2	13.7	13.6	4.1	7.3	−8.8	−9.4
	3	14.1	14.4	9.1	14.1	−6.0	−4.2
800.0	1	772.7	802.9	6.2	4.5	−3.4	0.4
	2	690.2	691.4	1.1	1.6	−13.7	−13.6
	3	719.5	702.7	5.6	3.3	−10.1	−12.2
2500	1	2276.5	2436.4	3.9	6.0	−8.9	−2.5
	2	2193.8	2139.1	2.7	0.5	−12.2	−14.4
	3	2327.9	2458.8	6.6	9.7	−6.9	−1.6

temperatures that are 30 °C and 40 °C. The method was considered robust, if R.S.D. and R.E. did not exceed 15% (Tables 2a-2c).

3. Results and discussion

3.1. Development and validation of the LC/MS method

Despite their structural similarities BAs differ a lot in their solubility, lipophilicity and adsorptive behavior to surfaces as well as in their chromatographic properties and fragmentation. Thus the more lipophilic non-ketylated BAs show lower solubility in aqueous buffer solutions and greater adsorption to plastic surfaces compared to the ketylated BAs KBA and AKBA, which may affect sensitivity of analysis. Moreover they do not fragment easily like KBA and AKBA. The here described LC/MS method was adopted to take into account the different properties of the BAs allowing the simultaneous monitoring of all six BAs with high sensitivity over a wide concentration range. The present method is based on a previously published method which combines matrix-based liquid–liquid extraction on diatomaceous earth with solid phase extraction on a graphitized carbon black surface followed by reversed phase gradient separation and photodiode array detection [11]. The method we developed now was adapted to clinical practice with regard to the calibration range and easier sample preparation using MS instead of UV detection. Thus purification of the biological sample was limited to liquid–liquid extraction with Extrelut® NT in a single step. Additional purification steps were not necessary resulting in simple and proper performance as well as saving of time, material and solvent. Also higher recoveries could be achieved compared to the HPLC–UV method including several purification steps (Table 3).

Representative chromatograms of blank plasma spiked with 0.5/5.0 ng/ml of the respective BA's (LLOQ) are presented in Fig. 2. The retention times were 2.7 min for the I.S., 5.2 min for KBA, 6.7 min for AKBA, 13.8 min for αBA, 15.2 min for βBA, 23.3 min for AαBA and 26.0 min for AβBA, respectively. No interfering peaks were detected at those retention times neither in samples spiked with BA's nor in samples spiked only with I.S. The standard curve using twelve concentration levels, a double blank and blank with I.S. was linear in the range of 0.5–12,000 ng/ml plasma for βBA, AβBA, αBA and AαBA and 5.0–3000 ng/ml plasma for AKBA and KBA. The coefficients of correlation (r) were always above 0.98, resulting in values of $r = 0.9934–0.9992$ (Table 1). As can be deduced from Tables 2a–2c all values for intra- and interday accuracy and precision met the requirements.

The results of the mean relative recoveries of BA's and I.S. are summarized in Table 3. The results of the stability studies are summarized in Tables 4a–4c. Under all tested conditions no decrease greater than 15% in the analyte concentration was observed, indicating stability of all BA's.

Moreover the analytical method was found to be robust with regard to changes in column temperature, as the difference in the calculated analyte concentration did not exceed 1.5%.

3.2. Serum level analysis

The pharmacokinetic profiles for the individual BAs measured in one volunteer following single oral administration of 1400 mg *BSE* are shown in Fig. 3. The highest serum levels were determined for βBA. Also the steady state concentrations were highest for βBA. The higher serum level of βBA may be either attributed to the higher content in the capsules or to better permeability properties. Studies on the permeability behavior of βBA are going on. In fact, this is the first study that provides data on the steady state concentrations of all major six BA's on a big scale. The determined concentrations are listed in Table 5.

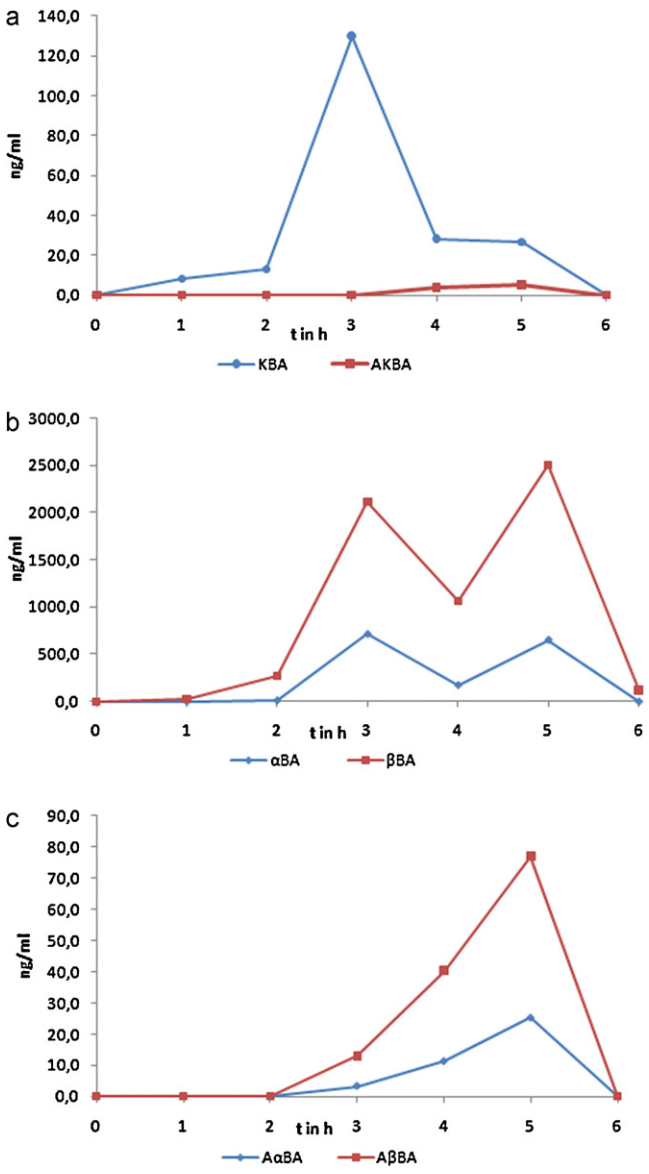

Fig. 3. Serum profile after administration of H15 capsules (Hecht) (a) KBA and AKBA, (b) αBA and βBA and (c) AαBA and AβBA.

Despite the extremely high administered *BSE* dose AKBA could not be detected at all except in one verum subject out of 14 at a concentration level of 15.5 ng/ml (0.03 μM). A previous study of Sterk et al. demonstrated that the average maximum plasma levels increase when taking the extract with a standardized high fat meal (28.8 ng/ml) compared to fasted condition (6 ng/ml) [12]. The here observed AKBA steady state level determined in one subject is lower than the maximum plasma levels of Sterk et al. in the fed state but corresponds to the steady state concentrations determined by Tausch et al. in 3 subjects after 4 weeks of daily intake of 2400 mg *BSE* during or immediately after a meal. In the present study, the subjects were given the medication at home and were asked to take three times four capsules à 400 mg *BSE* extract (corresponding to 4200 mg) daily. No instructions were given regarding intake of medication or standardization of food. Moreover blood samples were taken during weekly routine medical check-ups irrespective of the time of the medication intake. Therefore it is not known whether the absence of AKBA in all but one subject taking the verum

Table 5
Results of the clinical study (average steady state concentration in ng/ml).

Sample	Calc. conc. in ng/ml						
	Verum/placebo	KBA	AKBA	αBA	βBA	AαBA	AβBA
1	v	6.4	n.d.	322.4	623.4	187.0	343.0
2	p	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	p	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4	v	23.9[a]	n.d.	429.7[a]	989.1[a]	148.8[a]	288.1[a]
5	v	64.2	n.d.	1299.2	4513.5	651.7	1923.7
6	p	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	v	247.5[a]	n.d.	4791.9[a]	11948.5[a]	846.5[a]	1479.7[a]
8	p	n.d.	n.d.	n.d.	3.63	n.d.	n.d.
9	v	56.0	n.d.	4830.1	10548.7	2985.8	6131.3
10	p	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	p	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	p	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	v	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14	p	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	p	n.d.	n.d.	0.74	2.04	3.99	2.76
16	v	n.d.	n.d.	1562.7	4225.4	204.8	316.0
17	v	57.2	n.d.	1713.9	4579.6	73.4	199.6
18	p	n.d.	n.d.	n.d.	0.85	n.d.	n.d.
19	v	29.6	n.d.	1380.6	4004.3	112.7	183.4
20	v	47.6	n.d.	1494.9	4554.8	324.5	590.9
21	p	n.d.	n.d.	0.68	0.81	n.d.	n.d.
22	v	n.d.	n.d.	1328.2	1664.4	118.9	131.4
23	p	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24	v	14.3	n.d.	3899.0	10294.5	314.8	648.6
25	p	n.d.	n.d.	n.d.	0.81	n.d.	n.d.
26	v	107.8[a]	n.d.	1291.7[a]	3378.8[a]	90.7[a]	160.0[a]
27	v	n.d.	15.5	36.7	87.0	177.1	144.5

n.d.: not quantifiable, <LOQ; p: placebo group; v: verum group.
[a] The patient exhibited strong variation of the individual steady state values that may be caused by difficulties with the medication compliance during the clinical study [19].

may be attributed to the intake of *BSE* extract in the fasting state or is due to the bad absorption of AKBA. In general, the present first big scale study confirmed previous results suggesting lower plasma levels for AKBA compared to KBA. In fact, KBA steady state levels ranged between 6.4 and 247 ng/ml (0.01–0.5 μM), indicating high pharmacokinetic variability depending on the individual and the conditions of intake. Similar observations were made in an *in vitro* study on Caco-2 cells that revealed higher permeability for KBA compared to AKBA [15]. With regard to the other BA's βBA and AβBA, also revealed comparable average steady state serum levels to those reported by Tausch et al. [14]. Nevertheless individual patients showed up to five times higher serum levels for βBA. In the light of these high plasma levels the role of βBA as another potential active ingredient of *BSE* extract should be taken into account in further pharmacological and clinical studies. These insights are of special interest, as it could be shown recently, that also βBA is able to pass the blood brain barrier achieving higher concentrations than AKBA and KBA (results are published elsewhere).

Also the steady state concentrations of αBA reaching up to 14 μM were also found to be remarkably high. Maybe synergistic effects of βBA and αBA may not be excluded and should be investigated in further studies.

In general a significant reduction in cerebral edema was observed in the *BSE* group compared to placebo. Thus at the end of radiotherapy 60% of the patients who received *BSE* reached a decrease in edema to less than 25% of the baseline values or showed no edema at all. In the placebo group only 26% of the patients reached this optimal outcome. These results underline the therapeutic efficacy of *BSE* in the reduction of peritumoral edema. It should be pointed out, that the observed edema reduction may not be attributed to the intake of dexamethasone during radiotherapy, because the placebo and *BSE* group needed on average the same amount of dexamethasone (24.6 mg for placebo and 18.6 mg for *BSE* group). In the follow-up i.e. 4 weeks after the end of therapy and after stopping *BSE* or placebo, the edema volumes in both groups converged again. This may be explained by the missing effect of *BSE* on edema [19].

In summary, this clinical study strongly suggests that *BSE* could be given beside glucocorticoids for anti-edematous treatment. The prospective, randomized, placebo controlled, strictly double blind, and additionally rater blinded design of this study makes the results highly reliable.

4. Conclusion

For the first time a highly sensitive HPLC–MS-method was developed that allows the simultaneous quantitative monitoring of all major BA's over a wide concentration range from 5.0 ng/ml to 3000 ng/ml for KBA and AKBA and from 0.5 ng/ml to 12,000 ng/ml for αBA, βBA, AαBA and AβBA. Precision and accuracy as major control parameters met the international criteria for bioanalytical method validation. This method covers the great pharmacokinetic variability observed in clinical practice and is thus suitable to be used as standard analytical method for the analysis of BAs in clinical trials. It was successfully applied in a clinical study monitoring the steady state concentrations of all major BA's. The here quantified steady state serum levels for βBA encourage further studies on the role of βBA as further potential active ingredient of *BSE*. In general, the insight gained in this study underlines *BSE* as promising anti-edematous agent for the treatment of cerebral peritumoral edema in addition to glucocorticoids and provides interesting hints with regard to the therapeutic active BA and the underlying mechanism of action.

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