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# Determination of 11-keto-boswellic acid in human plasma

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**Abstract**

A sensitive, specific, accurate, fast and reproducible GC/MS-method for the quantitative determination of 11-keto-boswellic acid in human plasma using 18α-glycyrrhetinic acid as the internal standard was developed and validated. 11-Keto-boswellic acid and the internal standard were separated from acidified plasma by liquid/liquid extraction. The extracted samples were methylated and analyzed by GC/MS in the negative ion chemical ionization mode (NICI) and selected ion monitoring (SIM). The total run time was 8 min between injections. The assay described in this paper demonstrates a validated lower limit of quantification of 0.0100 μg/ml using 1 ml of plasma. The calibration curves are linear in the measured range between 10.0 and 2000 ng/ml plasma. The overall precision (expressed as CV) and accuracy (expressed as bias) for all concentrations of quality controls and standards is better than 15%. No indications were found for possible instabilities of 11-keto-boswellic acid in plasma, in whole blood, in the extraction solvent or after repeated thawing/freezing cycles. The recovery of the extraction method is calculated as 84%. The assay was applied successfully to determine the plasma level of 11-keto-boswellic acid in a clinical pilot study. 

*Keywords:* 11-Keto boswellic acid; Human plasma; Quantitative determination; GC/MS

## 1. Introduction

At present, various preparations and extracts of Indian incense are gaining more and more scientific, therapeutic and, above all, public interest [1–5]. Since ancient times the gum resin from *Boswellia serrata* has been used in the Ayurvedic system of medicine for a variety of inflammatory diseases. In 1992 the active principle within the multi-component mixture of the resin could be identified, resulting in the recognition of boswellic acids [6] (Fig. 1).

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The most important are acetyl-11-keto-β-boswellic acid (AKBA), followed by 11-keto-β-boswellic acid (KBA). Both have been shown to be selective, non-competitive, non-redox and potent inhibitors of 5-lipoxygenase, which is the key enzyme of leukotriene biosynthesis from arachidonic acid [6,7]. Most inhibitors of this enzyme identified until now show a mechanism of redox based inhibition with the disadvantage of undesirable side effects because of its non-selectivity, whereas for boswellic acids a selective inhibition without contribution of redox processes was postulated. The high therapeutic importance of this drug is reflected in current efforts to characterize the biopharmaceutical parameters. Beside various experimental investigations, different clinical data from patients' treatment and several small studies have been published [8–16]. There is no discussion about the impact and therapeutic efficiency of *B. serrata*. Even more astonishing is that up till now no validated analytical method existed for the quantitative determination of boswellic acids in human matrices.

Regarding problems of possible deacetylation of AKBA upon oral application, a specific method for the determination of the metabolically more stable analyte, KBA, in human plasma was developed. The assay is suitable for the determination of KBA concentrations in samples generated from clinical studies evaluating the pharmacokinetic or bioavailability of the drug. The validation procedure was performed according to current guidelines for method validation [17,18].

## 2. Experimental

### 2.1. Chemicals and reagents

KBA (Lot No. 4801) was obtained by Fa. HWI (HWI Analytik GmbH, Rheinzabern, Germany), and the internal standard compound 18α-glycyrrhetinic acid (Lot No. 99H0742) was purchased from Sigma (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) (for comparison

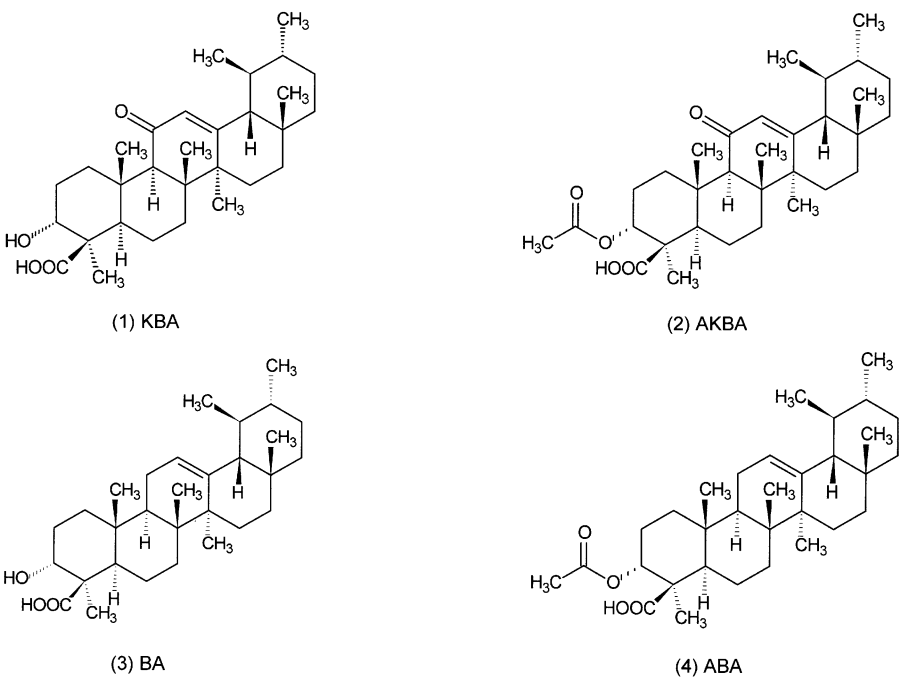
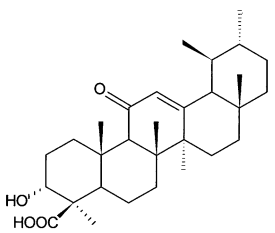



Fig. 1. Structure of β-boswellic acids. (1) KBA, (2) AKBA, (3) β-boswellic acid (BA) and (4) acetyl-β-boswellic acid (ABA).

Analyt (1)

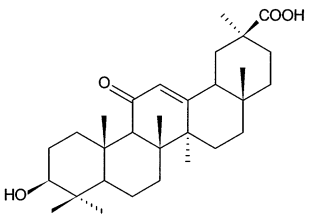



KBA

Molecular formula: $C_{30}H_{46}O_4$

Molecular weight: 470,7 g/mol

I.S. (2)




18α-Glycyrrhetinic acid

Molecular formula: $C_{30}H_{46}O_4$

Molecular weight: 470,7 g/mol

Fig. 2. Comparison of analyt (1) and internal standard (2).

of chemical structures see Fig. 2). All solvents used were of analytical grade or better quality. Diisopropylether, 0.1 N HCl and methanol were obtained from Merck (Merck Eurolab GmbH, Darmstadt, Germany), and toluene were purchased from J.T. Baker (J.T. Baker Chemikalien, Gross Gerau, Germany).

### 2.2. Stock solutions

A concentrated KBA stock solution was prepared by weighing 20.0 mg KBA and dissolving it in 20 ml methanol to give a 1.00 μg/ml stock solution.

For the internal standard stock solution, 10.0 mg of 18α-glycyrrhetinic acid were dissolved in 50 ml methanol to give a 200 ng/ml stock solution. A working solution with the concentration of 20.0 ng/μl was prepared by diluting the stock solution with methanol. Plasma samples were spiked with 25 μl from these working solutions. A concentration of 0.50 μg internal standard per ml plasma results. All solutions were stored in a refrigerator at $5 \pm 3$ °C.

### 2.3. Preparation of calibration standards and quality control samples

Calibration standards were prepared daily by spiking blank plasma with 25 μl appropriate KBA-working solutions. These working solutions were prepared by diluting the KBA stock solution with methanol to give the following concentrations: 2.00, 1.00, 0.500, 0.200, 0.100, 0.0500, 0.0200, and 0.0100 μg KBA per ml plasma.

All quality control samples were prepared at the beginning of the study. Three different pools of quality control samples were prepared by spiking 1 ml aliquots of blank plasma with 25 μl of spiking solutions freshly diluted from the stock solution. The QC samples were at different concentrations to the calibration standard samples. One set of quality control samples consists of three single samples with the following concentrations: 0.0300, 0.300 and 1.50 μg KBA per ml plasma. They were stored at $-20 \pm 5$ °C until the day of extraction.

### 2.4. Extraction procedure

One millilitre of plasma was aliquoted and spiked with 500 ng of the internal standard dissolved in 25 μl methanol. After addition of 1 ml 0.1 N HCl, the samples were extracted using 4 ml of a diisopropylether/toluene mixture. The organic phase was transferred into a glass tube and evaporated to dryness with a stream of nitrogen at 45 °C. The residue was methylated with 100 μl of an etheric diazomethane solution. The organic solvent was evaporated to dryness in a nitrogen stream at 45 °C. The residue was reconstituted in 30 μl toluene. The samples were analyzed by GC/MS using negative ion chemical ionization

(NICI) and selected ion monitoring (SIM). Fig. 3 shows a typical chromatogram obtained by the described extraction procedure.

### 2.5. Measurement

GC–separation were performed by using a fused silica capillary column of 15 m × 0.32 mm i.d., coated with a film of trifluoropropylmethyl polysiloxane. The MS was operated in the negative ion chemical ionization mode with ammonia as the reagent gas. Selected ion monitoring was performed for $m/z$ 483, which is the mass to charge ratio for derivatized KBA and for 18α-glycyrrhetinic acid (internal standard). Data acquisition and integration of the peak areas were achieved using the standard instruments selected ion recording software.

#### 2.5.1. MS conditions

| | | |
|---|---|---|
| Mass spectrometer | Fisons Trio 1000 | |
| Ion source temperature | 280 °C | |
| Transferline temperature | 320 °C | |
| Operation mode | NICI | |
| Reagent gas | Ammonia | |
| Quadrupole settings | KBA | $m/z$ 483.5 |
| | I.S. | $m/z$ 483.5 |

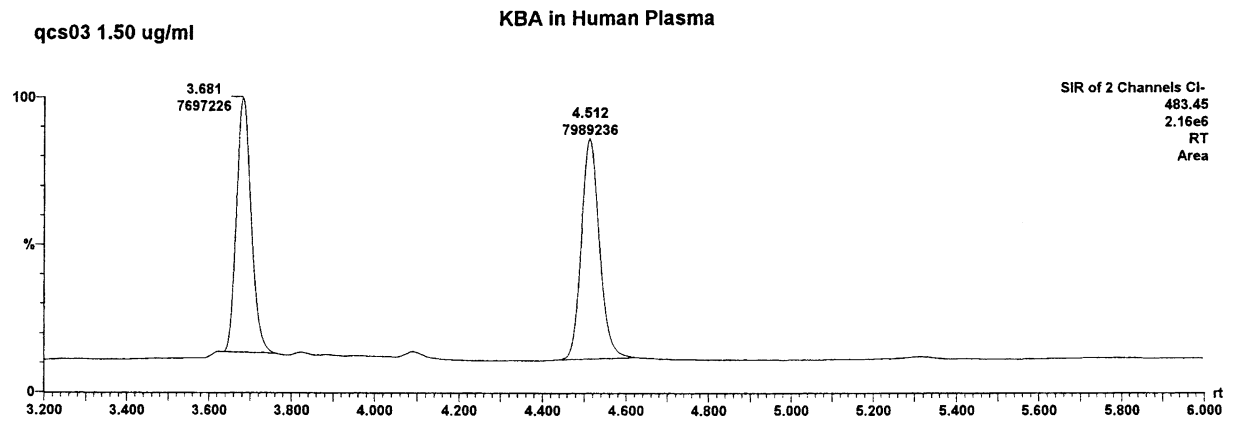



Fig. 3. SIM-chromatogram of KBA methyl ester (retention time 3.68 min) and 18α-glycyrrhetinic acid methyl ester (internal standard retention time 4.51 min) obtained by the described extraction procedure.

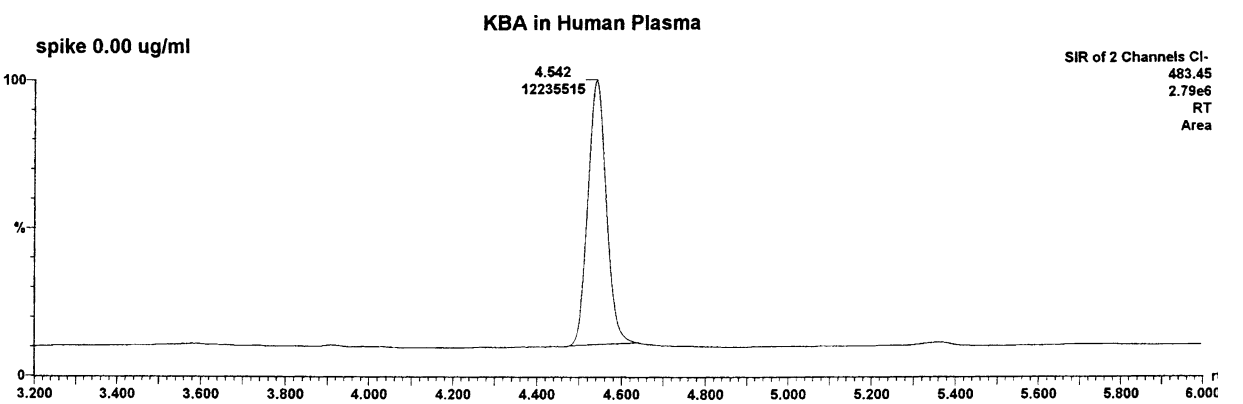



Fig. 4. Zero standard chromatogram (internal standard retention time 4.54 min).

*2.5.2. GC conditions*

| | |
|---|---|
| Column | Rtx 200 ($l = 15$ m, 0.32 mm i.d., 0.25 μm film thickness) Restek GmbH, Germany |
| Carrier gas | Helium |
| Injector temperature | 320 °C |
| Oven temperature program | 250–250 °C 1 min<br>250–320 °C 25 °C/min<br>320–320 °C 2.0 min |
| Injection mode | Splitless at 320 °C injector temperature, split closed for 30 s |
| Injection volume | 1 μl |

*2.6. Evaluation*

Analyte concentrations were evaluated using the internal standard method. The standard curves $y = a + bx$ ($a$ = intercept, $b$ = slope) were calculated from the peak area ratios of analyte/internal standard and the nominal analyte concentrations using linear regression with $1/x^2$ weighting.

The concentrations were reported in μg of KBA per ml of human plasma.

## 3. Validated parameters and performance data

*3.1. Selectivity*

*3.1.1. Blank matrix*

The selectivity is defined as the lack of interfering peaks at the retention times of the assayed drug and the internal standard in the chromatograms. This was confirmed by the injection of the extracts of blank plasma samples. Samples which neither contained KBA nor the internal standard generated from six different sources of plasma were analyzed. No significant interference peaks could be observed in the case of KBA as well for the internal standard. Additionally, six plasma samples from the same sources were spiked only with the internal standard. No significant interference peaks could be observed where KBA elutes (Fig. 4.)

*3.1.2. Blood withdrawal systems*

For possible interference peaks or adsorption phenomena, different blood withdrawal systems and two sorts of plasma storage tubes were tested. The tested systems were:

| | | |
|---|---|---|
| Glass tube | | |
| Polypropylene tube | | |
| Vacutainer: | Li-Heparin: | Becton-Dickinson |
| Vacutainer: | K-EDTA: | Becton-Dickinson |
| Vacutainer: | Na-Heparin: | Becton-Dickinson |
| Monovette: | K-EDTA: | Sarstedt |

One set of quality controls was pipetted into each of the six different tubes (double volume). The samples were shaken for 10 min and then 1 ml aliquots were pipetted into the glass-extraction tubes and treated as described in the extraction procedure. The samples were measured and calculated together with a standard curve.

No influence on chromatograms and on the results of the samples could be observed in all cases. Therefore, all these systems can be used for blood withdrawal and plasma storage.

*3.2. Inter-day precision and accuracy*

The inter-day precision and accuracy experiments were carried out by measuring different standard curves ($n = 8$) including blank and zero standard at different days. With each of the standard curves, two sets of quality control samples ($n = 6$) were analyzed. Summarized statistical data on the inter-day accuracy (expressed as bias: percent deviation of the back calculated versus the nominal values) and precision (coefficient of variation of the back calculated values) are shown in Tables 1 and 2.

The international acceptance criteria (CV better than 15% for precision and Bias better than 15%

Table 1
Inter-day precision and accuracy data: standards

| Number of measured standards | Nominal concentration (μg/ml) | Measured concentration mean (μg/ml) | Standard deviation | CV (%) | Bias (%) |
|---|---|---|---|---|---|
| 8 | 0.0100 | 0.0104 | 0.000296 | 2.9 | 3.7 |
| 8 | 0.0200 | 0.0192 | 0.00111 | 5.8 | −3.8 |
| 8 | 0.0500 | 0.0476 | 0.00486 | 10.2 | −4.8 |
| 8 | 0.100 | 0.0914 | 0.00614 | 6.7 | −8.6 |
| 7 | 0.200 | 0.193 | 0.0144 | 7.5 | −3.4 |
| 8 | 0.500 | 0.507 | 0.0305 | 6.0 | 1.3 |
| 7 | 1.00 | 1.10 | 0.0605 | 5.5 | 10.1 |
| 8 | 2.00 | 2.12 | 0.107 | 5.0 | 6.2 |

Table 2
Inter-day precision and accuracy data: quality control samples

| Number of measured standards | Nominal concentration (μg/ml) | Measured concentration mean (μg/ml) | Standard deviation | CV (%) | Bias (%) |
|---|---|---|---|---|---|
| 16 | 0.0300 | 0.0289 | 0.00300 | 10.4 | −3.7 |
| 16 | 0.300 | 0.301 | 0.0289 | 9.6 | 0.3 |
| 16 | 1.50 | 1.62 | 0.0838 | 5.2 | 8.1 |

Table 3
Intra-day precision and accuracy data: quality control samples

| Number of measured standards | Nominal concentration (μg/ml) | Measured concentration mean (μg/ml) | Standard deviation | CV (%) | Bias (%) |
|---|---|---|---|---|---|
| 4 | 0.0300 | 0.0265 | 0.00161 | 6.1 | −11.8 |
| 5 | 0.300 | 0.294 | 0.0147 | 5.0 | −2.0 |
| 5 | 1.50 | 1.66 | 0.125 | 7.5 | 10.9 |

Table 4
Overall precision and accuracy data: standards

| Number of measured standards | Nominal concentration (μg/ml) | Measured concentration mean (μg/ml) | Standard deviation | CV (%) | Bias (%) |
|---|---|---|---|---|---|
| 11 | 0.0100 | 0.0104 | 0.00261 | 2.5 | 3.6 |
| 11 | 0.0200 | 0.0192 | 0.00106 | 5.5 | −4.0 |
| 10 | 0.0500 | 0.0474 | 0.00431 | 9.1 | −5.2 |
| 11 | 0.100 | 0.0939 | 0.00745 | 7.9 | −6.1 |
| 10 | 0.200 | 0.191 | 0.0161 | 8.4 | −4.3 |
| 11 | 0.500 | 0.512 | 0.0289 | 5.6 | 2.4 |
| 10 | 1.00 | 1.08 | 0.0681 | 6.3 | 7.5 |
| 11 | 2.00 | 2.12 | 0.102 | 4.8 | 5.9 |

Table 5
Overall precision and accuracy data: quality control samples

| Number of measured standards | Nominal concentration (μg/ml) | Measured concentration mean (μg/ml) | Standard deviation | CV (%) | Bias (%) |
|---|---|---|---|---|---|
| 22 | 0.0300 | 0.0288 | 0.00283 | 9.8 | −4.2 |
| 22 | 0.300 | 0.294 | 0.0296 | 10.1 | −2.1 |
| 22 | 1.50 | 1.62 | 0.102 | 6.3 | 8.0 |

deviation from the nominal value for accuracy) are clearly met.

### 3.3. Intra-day precision and accuracy

The intra-day data (repeatability) express precision and accuracy of the method under the same conditions within 1 day. For repeatability, five different quality control sample sets were analyzed and calculated with a standard curve (including two sets of quality control samples). The statistical evaluation for the resulting concentrations is shown in Table 3.

The statistical data fulfil international acceptance criteria (CV better than 15% for precision and Bias better than 15% for accuracy) for intra-day experiments.

### 3.4. Linearity—overall precision and accuracy

The standard curves $y = a + bx$ were calculated from the peak area ratios of analyte/internal standard with weighted linear regression. To select the suitable weighting model, the standard curves were calculated without, with $1/x$ and $1/x^2$ weighting. The chosen $1/x^2$ weighting model gives slightly better results for accuracy and precision in the lower concentration range than $1/x$ weighting. Calculation without weighting could not be used due to an unacceptable relative deviation near the LOQ. With the recommended $1/x^2$ model the values for $r^2$ indicate linearity over the whole calibration range (see Table 6). During this validation procedure, a total of 11 standard curves and 22 sets of quality control standards were analyzed. Tables 4 and 5 show the statistical data for all these standards and quality controls.

As can be seen from Table 6, the values for $r^2$ (overall mean > 0.99) indicate linearity over the whole calibration range.

### 3.5. Sensitivity/lower limit of quantitation

For validation of the LOQ, six different lots of plasma were spiked with the analyte at the lowest level of the standard curve (0.0100 μg/ml) and recalculated with a normal standard curve. Table 7 shows the results obtained for this experiment.

The data listed in Table 7 show that the values for accuracy (bias = −0.7%) and precision (CV = 5.5%) of the method at LOQ are acceptable and fulfil the requirements of bioanalytical studies.

Table 6
Overall precision and accuracy data: regression parameters

| Standard curve | Intercept | Slope | $r^2$ |
|---|---|---|---|
| 1 | −0.00253 | 0.66400 | 0.99130 |
| 2 | −0.00029 | 0.65878 | 0.99011 |
| 3 | 0.00021 | 0.67459 | 0.99039 |
| 4 | 0.00115 | 0.75448 | 0.99098 |
| 5 | 0.00189 | 0.79972 | 0.98408 |
| 6 | 0.00303 | 0.74998 | 0.99145 |
| 7 | −0.00117 | 0.60751 | 0.98568 |
| 8 | −0.00134 | 0.59415 | 0.99141 |
| 9 | −0.00144 | 0.59127 | 0.99424 |
| 10 | −0.00088 | 0.56191 | 0.99193 |
| 11 | −0.00270 | 0.66680 | 0.99420 |
| Mean | | 0.67574 | 0.99052 |
| S.D. | | 0.07609 | |
| CV (%) | | 11.4 | |

Table 7
Sensitivity

| Nominal concentration (μg/ml) | Measured concentration (μg/ml) |
|---|---|
| 0.0100 | 0.01070 |
| 0.0100 | 0.00948 |
| 0.0100 | 0.00991 |
| 0.0100 | 0.00993 |
| 0.0100 | 0.01040 |
| 0.0100 | 0.00919 |
| Mean | 0.00993 |
| S.D. | 0.000546 |
| CV (%) | 5.5 |
| Bias (%) | −0.7 |

### 3.6. Stability tests

All stability tests were examined by extracting and measuring four sets of quality control samples. After the test period, the samples were proceeded and analyzed with a freshly prepared standard curve and compared with their nominal values.

#### 3.6.1. Stability in plasma at room temperature

The stability of KBA in plasma at room temperature was determined by extracting and measuring four sets of quality controls after incubation at room temperature for 24 h.

#### 3.6.2. Long-term stability in plasma at $-20 \pm 5$ °C

The long-term stability of KBA in plasma at $-20 \pm 5$ °C was examined by extracting and measuring four sets of quality controls after storage at $-20 \pm 5$ °C for 28 days.

#### 3.6.3. Stability after repeated thawing/freezing cycles

Three pools of control samples with concentrations of 0.0300, 0.300 and 1.50 μg/ml for KBA were subjected to repeated freezing and thawing cycles. Just before freezing the pools, each of the four 1 ml-aliquots were pipetted into a glass tube (thaw/freeze cycles $n = 0$). Then the pools were frozen a first time and after the following thawing four new 1 ml-aliquots of each pool were pipetted (thaw/freeze cycles $n = 1$). Then the pools were subjected twice more to this cycle. After the third cycle, four additional sets of 1 ml-aliquots of these control samples were taken (thaw/freeze cycles $n = 3$).

#### 3.6.4. Stability in the extraction solvent at room temperature

In order to check the stability of KBA in the extraction solvent at room temperature, a total of four sets of control samples were extracted and stored at room temperature for 48 h.

#### 3.6.5. Autosampler stability of the processed samples

In order to check the stability of KBA in the extracted samples under autosampler conditions, a total of four sets of control samples were extracted and prepared for the measurement. Then they remained in an autosampler vial for 48 h in the autosampler rack.

#### 3.6.6. Stability in the extraction solvent at $5 \pm 3$ °C (refrigerator storage)

In order to check the stability of KBA in the extraction solvent, a total of four sets of control samples were extracted and stored in a refrigerator for 8 days.

#### 3.6.7. Stability during sample collection

To test the stability of KBA during sample collection and processing, whole blood was spiked with the analyte concentrations 0.0300, 0.300 and 1.50 μg KBA/ml whole blood. The whole blood was aliquoted and stored at room temperature for 0, 30 and 60 min. Then aliquots were processed into plasma by centrifugation. 300 μl portions of that plasma were added to 700 μl blank plasma, extracted and measured.

#### 3.6.8. Stability test results

As seen in Tables 8 and 9, no indications were found for possible instabilities of KBA in plasma, whole blood, in the extraction solvent or after repeated thawing/freezing cycles.

For this experiment the whole blood samples (1 ml) were processed into plasma by centrifugation. 300 μl portions of these samples were added to

700 μl blank plasma, extracted and measured. The measured peak area ratios of samples from whole blood without storage time were set to 100% and the stored samples were regarded in relation to this 100% value. No indications were found for possible instabilities of KBA in plasma, whole blood, in the extraction solvent or after repeated thawing/freezing cycles.

Table 8
Summarized statistical data-stability test results

| Stability test | Nominal concentration (μg/ml) | Measured concentration mean ($n = 4$) (μg/ml) | CV (%) | Bias (%) |
|---|---|---|---|---|
| Plasma at r.t. 24 h | 0.0300 | 0.0261 | 4.9 | −13.1 |
| | 0.300 | 0.264 | 1.3 | −12.0 |
| | 1.50 | 1.53 | 5.2 | 2.0 |
| Plasma at −20 ± 5 °C 28 d | 0.0300 | 0.0281 | 4.8 | −6.4 |
| | 0.300 | 0.282 | 4.9 | −5.8 |
| | 1.50 | 1.56 | 5.8 | 4.3 |
| Thawing/freezing Cycle 0 | 0.0300 | 0.0283 | 2.70 | −5.70 |
| | 0.300 | 0.277 | 7.70 | −7.70 |
| | 1.50 | 1.52 | 5.60 | 1.10 |
| Thawing/freezing Cycle 1 | 0.0300 | 0.0257 | 3.30 | −14.4 |
| | 0.300 | 0.263 | 3.80 | −12.4 |
| | 1.50 | 1.47 | 3.00 | −2.20 |
| Thawing/freezing Cycle 3 | 0.0300 | 0.0258 | 10.1 | −14.0 |
| | 0.300 | 0.270 | 7.70 | −10.0 |
| | 1.50 | 1.47 | 3.00 | −2.20 |
| Extraction solvent at r.t. 48 h | 0.0300 | 0.0312 | 3.9 | 4.1 |
| | 0.300 | 0.264 | 5.1 | −12.1 |
| | 1.50 | 1.56 | 0.9 | 3.8 |
| Autosampler stability 48 h | 0.0300 | 0.0281 | 5.8 | −6.4 |
| | 0.300 | 0.292 | 1.3 | −2.5 |
| | 1.50 | 1.75 | 1.9 | 16.6 |
| Extraction solvent at 5 ± 3 °C 8 d | 0.0300 | 0.0281 | 8.8 | −6.2 |
| | 0.300 | 0.289 | 3.2 | −3.7 |
| | 1.50 | 1.72 | 5.1 | 14.4 |

Table 9
Statistical data stability during sample collection

| Stability test | Nominal concentration (μg/ml) | Measured concentration mean (μg/ml) | CV (%) | Rel. (%) |
|---|---|---|---|---|
| Whole blood | 0.0300 | 0.0183 | 10.4 | 100 |
| $t = 0$ min | 0.300 | 0.2295 | 6.2 | 100 |
| | 1.50 | 1.4521 | 5.2 | 100 |
| Whole blood | 0.0300 | 0.183 | 8.0 | 100.1 |
| $t = 30$ min | 0.300 | 0.2022 | 3.1 | 88.1 |
| | 1.50 | 1.4221 | 12.4 | 97.9 |
| Whole blood | 0.0300 | 0.0204 | 8.3 | 111.5 |
| $t = 60$ min | 0.300 | 0.2481 | 7.8 | 108.1 |
| | 1.50 | 1.6296 | 6.7 | 112.2 |

Table 10
Results, out of range QCs, diluted by factor 2 and 4

| Dilution-factor | Original concentration (μg/ml) | Back calculated concentration (mean) (μg/ml) | Standard deviation | CV (%) | Bias (%) |
|---|---|---|---|---|---|
| 2 | 3.00 | 2.95 | 0.0359 | 1.2 | −1.5 |
| 4 | 6.00 | 5.75 | 0.328 | 5.7 | −4.2 |

Table 11
Statistical data: extraction recovery

| KBA | | Internal standard | |
|---|---|---|---|
| Nominal concentration (μg/ml) | Recovery (mean) (%) | Nominal concentration (μg/ml) | Recovery (mean) (%) |
| 0.0300 | 85.7 | 0.0300 | 91.3 |
| 0.300 | 82.2 | 0.300 | 95.9 |
| 1.50 | 84.2 | 1.50 | 91.1 |
| Mean | 84.2 | | 92.8 |
| S.D. | 1.4 | | 2.7 |
| CV (%) | 1.7 | | 2.9 |

### 3.7. Dilution of out-of-range specimen

An out-of-range QC pool with a concentration of 3.00 μg KBA/ml plasma was prepared. Four samples out of this pool were diluted with blank plasma by a factor of two (DF = 2) to result in a sample concentration of 1.50 μg KBA/ml.

Furthermore, an out-of-range QC pool with a concentration of 6.00 μg KBA/ml plasma was prepared. Four samples out of this pool were diluted with blank plasma by a factor of four (DF = 4) to result in a sample concentration of also 1.50 μg KBA/ml.

All these samples were analyzed and calculated with an undiluted (“normal”) standard curve. The recalculated concentrations of these diluted samples agreed with their original nominal concentrations, which proves the ability to dilute specimen by a factor of both two and four in a linear fashion (Table 10).

### 3.8. Recovery

The recovery of the extraction for KBA and the internal standard was examined by a comparison of three sets of spiked plasma samples:

(a) Each of the four samples spiked with 0.0300, 0.300 and 1.50 μg/ml plasma extracted and treated as described in Section 2.

(b) Each of the four samples spiked with internal standard before extraction. After extraction a 4 ml aliquot of the sample extracts were spiked with the working solutions for the 0.0300, 0.300 and 1.50 μg/ml concentrations (KBA recovery).

(c) Each of the four samples spiked with the 0.0300, 0.300 and 1.50 μg/ml plasma before extraction. After extraction a 4 ml aliquot of the sample extracts were spiked with the internal standard working solution (18α-glycyrrhetinic acid recovery).

The measured peak area ratios of these samples from (b), respectively (c) were set to 100% recovery.

The recovery of the extracted samples was calculated with the following equation:

$$\text{recovery } (\%) = \frac{\bar{x}_{\text{extr.}}}{\bar{x}_{\text{ref.}}\, f} 100 \qquad (1)$$

with: $f = 1$ (fraction of total volume).

Table 11 shows the extraction recovery data for KBA and the internal standard.

Due to this good recovery, the sensitivity and the reproducibility of the assay is acceptable. The

Table 12
Validation results

| Validated parameters | Results |
|---|---|
| Calibration range | 0.0100–2.00 μg/ml |
| LOQ | 0.0100 μg/ml |
| $r^2$ (overall mean) | 0.99052 |
| Inter-day precision* | CV = 10.4% |
| Inter-day accuracy* | Bias = −3.7% |
| Intra-day precision* | CV = 6.1% |
| Intra-day accuracy* | Bias = −11.8% |
| Selectivity | No problems |
| Stability during sample collection | No problems |
| Stability of plasma samples after three thawing/freezing cycles | No problems |
| Stability in plasma at room temperature | At least 24 h |
| Stability in plasma at $-20 \pm 5$ °C (freezer) | At least 4 weeks |
| Stability of extracted samples at $5 \pm 3$ °C (refrigerator) | At least 8 days |
| Stability of extracted samples at r.t. | At least 48 hours |
| Stability of processed samples in autosampler vials | At least 48 hours |
| Dilution procedures | No problems |
| Recovery | Mean = 84.2% |

* At the lowest QC level.

precision of the recovery at each level is $\leq \pm 15$ ($\leq \pm 20\%$ at the low levels).

## 4. Assay validation results

A summary of the validation results is given in Table 12.

## 5. Conclusion

A GC/MS-method for the quantitative determination of KBA in human plasma was developed and validated. The method has been shown to provide good sensitivity, accuracy and precision. The validation procedure of the assay was performed according to current guidelines for method validation [17,18]. The results obtained during the validation fulfill all requirements and recommendations generally accepted for bioanalytical studies [17,18]. The analytical method has been used successfully for the analysis of clinical samples.

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