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Short communication

High-performance liquid chromatographic determination of acetyl-11-keto-α-boswellic acid, a novel pentacyclic triterpenoid, in plasma using a fluorinated stationary phase and photodiode array detection: Application in pharmacokinetic studies

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Abstract

A rapid, sensitive and selective HPLC separation with photodiode array detection was developed for the analysis of the novel pentacyclic triterpenoid acetyl-11-keto-α-boswellic acid. Complete baseline separation of acetyl-11-keto-α-boswellic acid from the corresponding isomer acetyl-11-keto-β-boswellic acid was achieved on a fluorinated stationary phase. The standard curve was linear from 0.98 nmol/l to 196 nmol/l acetyl-11-keto-α-boswellic acid. The compound was isolated from chick embryonic plasma using extraction on diatomaceous earth with an overall average extraction yield of 82%. This method was applied in a kinetic study on the chick chorioallantoic membrane model (CAM) and showed unequivocal separation between acetyl-11-keto-α-boswellic acid and acetyl-11-keto-β-boswellic acid unachievable so far.

Keywords: Pentacyclic triterpenoids; Acetyl-11-keto-α-boswellic acid; Chick chorioallantoic membrane; Pharmacokinetics; Pentafluorophenylpropyl-silica

1. Introduction

The gum resins from various *Boswellia* species contain boswellic acids, pharmacologically active compounds belonging to the family of pentacyclic triterpenoids. Distinct boswellic acids have been reported to possess both anti-inflammatory and anti-tumor activity. These effects might be due to pleiotropic effects including inhibition of human leukocyte elastase and/or of 5-lipoxygenase [1,2], as well as to topoisomerase inhibition [3] resulting in apoptosis-related tumor cell death [4]. Only recently, we were able to demonstrate that boswellic acids also inhibit the activity of the nuclear transcription factor, NF-κB, that is crucial for the expression of proinflammatory and other genes related to cancer cell survival and chemoresistance [5,6]. For the first time, these findings provided a molecular pharmacological basis for the dual efficacy of these triterpenoids in inflammatory and neoplastic disorders. To evaluate the putative pharmacological and therapeutic potential of various boswellic acid derivatives, we initiated several lines of investigations. Thus, we isolated and structurally characterized chemically pure standard compounds from frankincense gum resin [7], and analyzed their contents in phytopharmaceutical preparations [8]. Except for acetyl-11-keto-β-boswellic acid, most of the boswellic acid derivatives are known to exist either in an α- or a β-configuration. To clarify this exception, we synthesized and structurally elucidated acetyl-11-keto-α-boswellic acid from the corresponding acetyl-α-boswellic acid (submitted). Apart from pharmacological studies aiming at molecular mechanisms such as their antitumor efficacy [3,9], it is necessary to analyze the plasma levels of these compounds to gain insight into their pharmacokinetic parameters. So far, most studies dealing with pharmacokinetics used mammalian animal models or human clinical studies [10,11]. The analytical methods usually combine solid phase or liquid–liquid plasma extraction with HPLC-UV or GC–MS detection [12,13]. Here we show that a very efficient plasma extraction method on diatomaceous earth can be successfully combined with HPLC analysis on a fluorinated stationary phase that shows a high selectivity for acetyl-11-keto-α-boswellic; this

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method allowed to analyze the test compound in very small plasma samples collected from chick chorioallantoic membrane vessels. Specifically with regard to human cancer xenografts, this testing system offers several advantages such as: (i) reduction of mammalian animal experimentation due to pre-selection; (ii) consumption of only small amounts of limited and precious compounds; (iii) good reproducibility when used at a constant embryonic development [14]; and (iv) economic efficiency.

2. Experimental

2.1. Chemicals, animals

All chemicals were of analytical reagent grade unless stated otherwise. Reverse-osmosis type quality water, pureAqua (Schnaitsee, Germany) combined with a Milli-Q station from Millipore (Eschborn, Germany) was used throughout. The standard compounds acetyl-11-keto-α-boswellic acid and acetyl-11-keto-β-boswellic acid (Fig. 1) were obtained and characterized as described [7,8]. Methanol, ethyl acetate, tetrahydrofuran, isopropanol, dimethylsulfoxide, Extrelut® NT and acetic acid 96% were purchased from Merck (Darmstadt, Germany). The test substance acetyl-11-keto-α-boswellic was solubilized in physiological sodium chloride solution after complexation with γ-cyclodextrin from Wacker-Chemie (Burghausen, Germany). Fertilized chicken eggs from Hysex brown hens were obtained from a local supplier, Schuhmacher (Sinningen, Germany).

2.2. Instrumentation and software

The HPLC system consisted of a low pressure gradient LC-9A Shimadzu pump (Kyoto, Japan), an automatic sample injector Aspec XL (Abimed, Langenfeld, Germany), a column oven IWN CH100 (Junedis, Gröbenzell, Germany) and a photodiode array detector UVD 340U (Dionex, Idstein, Germany) connected to a personal computer equipped with Chromeleon Software version 6.6 (Dionex, Idstein, Germany). Statistical calculations were carried out with the software package Valoo (Applica, Bremen, Germany). The separation was performed on a Discovery HS F5 column (150 mm × 4.0 mm I.D., particle size 5 μm; Sigma–Aldrich, Taufkirchen, Germany). Solid phase extraction was performed with a Lichrolut extraction manifold (VWR International, Darmstadt, Germany).

2.3. Standards and sample solutions

Standard stock solutions were prepared by dissolving 1 mg substance in 1 ml dimethylsulfoxide. For further preparation of standard solutions, the stock solutions were diluted with a mixture of methanol–water (80:20, v/v) yielding concentrations from 9.8 nmol/l to 1.96 μmol/l. Standard solutions of acetyl-11-keto-α-boswellic acid for the validation of the method in chick embryonic plasma were prepared in the same range. For the pharmacokinetic study, acetyl-11-keto-α-boswellic acid was first complexed with γ-cyclodextrin according to standard procedures [6], which allowed it to be dissolved in appropriate concentrations in 0.9% sodium chloride solution.

2.4. Sample preparation

The test compounds were applied to the CAM as described previously [6,14]. Blood samples anticoagulated with EDTA 5 mmol/l (final concentration) were collected on day 10 after fertilization from the embryonic vessels using 0.3 mm × 13 mm

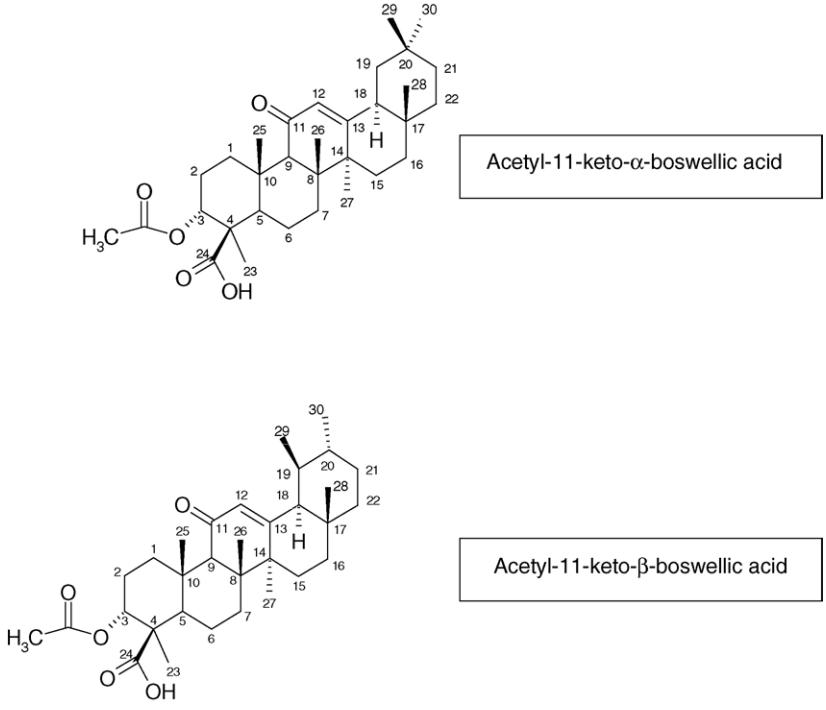

Fig. 1. Structures of the acetyl-11-keto-boswellic acids analyzed.

needles. All CAM tests were performed in full compliance with the German animal protection act. The blood samples were centrifuged immediately at 2340 × *g* at 4 °C for 10 min. The plasma samples were analyzed immediately. For matrix-assisted liquid–liquid extraction, 1.0 g Extrelut® NT was filled into an 8 ml glass column fitted with PTFE® frits. The plasma samples were diluted with water to 1 ml and transferred onto an Extrelut® NT column. After adsorption for 15 min, the columns were rinsed with 9 ml of a solvent mixture consisting of tetrahydrofuran–hexane–ethyl acetate–isopropanol (32:32:32:3, v/v/v/v). The solvent was evaporated under nitrogen protection in a water bath at 60 °C. For reconstitution, 100 μl solvent consisting of methanol–water–dimethylsulfoxide (64:16:20, v/v/v) was added and 80 μl were injected for analysis.

2.5. Optimal HPLC conditions

Due to the optimized extraction procedure allowing the efficient elution of matrix compounds by the gradient program as well as to the high stability of the Discovery HS F5 column filled with pentafluorophenylpropyl-silica (150 mm × 4.0 mm I.D., particle size 5 μm), guard columns were not required. For the elution of the compounds, the following mobile phase and gradient program was used. Mobile phase A: methanol–water–acetic acid (80:20:0.2, v/v/v); mobile phase B: methanol–acetic acid (100:0.2, v/v). Initial conditions were 100% A at a flow rate of 1 ml/min. Isocratic elution at 100% A over 10 min, then linear gradient to 5% A until 12 min, 5% A until 17 min, 100% A until 19 min. At the end of this program, all remaining matrix compounds were eluted from the column. In order to stabilize the chromatographic system, the column was maintained at 28 °C. A new sample injection was feasible after 24 min.

2.6. Identification and quantification

The eluent was continuously monitored at 250 nm and the scanned three-dimensional data fields were recorded. In the plasma samples, acetyl-11-keto-α-boswellic acid was positively identified by three different parameters: (i) the retention time of the peak was required to be in the range of ±0.1 min of the corresponding standard; (ii) the resulting photodiode array spectrum of the sample was online compared with a stored reference spectrum of the corresponding standard at a concentration of 1.22 μmol/l; and (iii) the peak area of the tested peak had to be enlarged at the same retention time after adding the corresponding pure standard substance to the real sample. Calibration curves of the standards ranging from 0.5 ng/injection up to 100 ng/injection revealed linearity with R values exceeding 0.9999 (peak area versus concentration). Generally, quantification was performed on the basis of external standards with several concentrations that were analyzed before and after six samples.

2.7. Validation of the method

The validation was performed as previously described [8]. The regression and limit of detection were calculated with Valoo software (Applica, Bremen, Germany) based on standardization criteria DIN 32645 as defined by the German standardization committee [8]; this procedure warrants that the limit of detection is with 99% probability different from the background.

Table 1
Pharmacokinetic parameters after topical application of 20 μl of a 100 μM acetyl-11-keto-α-boswellic acid γ-cyclodextrin complex solution in 0.9% sodium chloride

Pharmacokinetic parameters	Mean	S.E.M.
C_{max} (μmol/l)	0.466	0.098
T_{max} (min)	46.7	6.7
K_{el} (per h)	0.135	0.005
$T_{1/2}$ (h)	5.1	0.25
$AUC_{(0–8)}$ (μmol/l × h)	1.0158	0.046

2.8. Pharmacokinetics protocol and analysis

To evaluate the pharmacokinetic profile, 20 μl of a 100 μmol/l acetyl-11-keto-α-boswellic acid γ-cyclodextrin complex solution was topically applied. The plasma levels were determined at 20 min, 40 min, 60 min, 1.5 h, 2 h, 3 h, 4 h 6 h, 8 h after application in triplicate. The concentrations of acetyl-11-keto-α-boswellic acid in chick embryonic plasma were calculated from the corresponding calibration curve. Pharmacokinetic calculations were processed by the non-compartmental method [16]. The highest plasma concentration (C_{max}) and the time of the maximum plasma concentration (T_{max}) were obtained directly from the original data. The area under the concentration–time curve (AUC) was calculated using the linear trapezoidal rule. The terminal elimination rate constant (K_{el}) was calculated as the negative slope of the non-weighted least squares curve fit to logarithmically transformed concentration versus time. The elimination half-life was determined by the equation: $T_{1/2} = \ln 2/K_{el}$. Various software packages were used and data were calculated as previously described [10]. All data are represented as means ± standard error of the mean (S.E.M.) (Table 1).

3. Results and discussion

In our hands, attempts to separate acetyl-11-keto-α-boswellic acid from the corresponding isomer acetyl-11-keto-β-boswellic acid with a regular C18-column and various mobile phases were not successful. By contrast, the relatively new Discovery HS F5 column with a pentafluorophenylpropyl-moiety yielded sufficient resolution between the two isomers (Fig. 1). A typical separation of the standard compounds under these optimized conditions is shown in (Fig. 2). Whereas AKαBA was clearly detected in plasma samples after application of the compound, no peak was found in the blank plasma samples (Fig. 2). To demonstrate the significantly different selectivity of the new fluorinated HS F5 column (Fig. 3a) in comparison with the commonly used C18 columns (Fig. 3b), specifically with respect to the separation of acetyl-11-keto-α-boswellic acid and acetyl-11-keto-β-boswellic acid, we also used our previously published

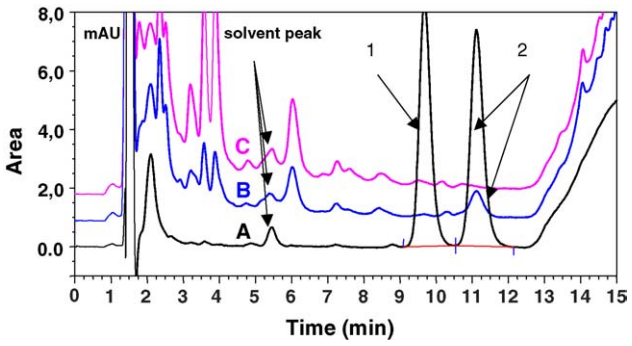

Fig. 2. Separation of a plasma sample. (1) acetyl-11-keto-β-boswellic acid (AKβBA) and (2) acetyl-11-keto-α-boswellic acid (AKαBA). (A) Standard compounds, (B) plasma sample at 90 min after application, (C) blank plasma sample. Discovery HS F5 column (150 mm × 4.0 mm I.D., particle size 5 μm), flow rate 1.0 ml/min, column temperature 28 °C, mobile phase gradient methanol–water–acetic acid, detection at 250 nm.

method [15]; it became obvious that only the fluorinated HDS F5 column warrants the desired separation.

To test the linearity of the compound, we used standard solutions in the range from 0.5 ng per injection to 100 ng per injection. Each sample was measured in triplicate (Table 2). The precision and accuracy of the method was tested with plasma pools containing acetyl-11-keto-α-boswellic acid in a lower and a higher concentration range of 19.6–196 nmol/l, respectively. Each series consisted of a set of six samples with repeated measurements on two additional different days. The mean coefficients of variation for the within-day variability was 2.8% and 23% at 196 nmol/l and 19.6 nmol/l, respectively, and 4.2% and 27% at 196 nmol/l and 19.6 nmol/l for the between-day variability. The within-day precision was 2.8% and 23% at 196 nmol/l and 19.6 nmol/l, respectively. The mean extraction efficiency (19.6–196 nmol/l) under these conditions, calculated by comparison of the peak area of the extracted samples with those of standards with the same concentrations, was 82%. The data obtained on different days were invariably found to be in good agreement (Table 3). The analysis of the stability of acetyl-11-keto-α-boswellic acid in plasma samples over a time period of 10 h did not reveal a significant loss of substance. The lower limit of detection for acetyl-11-keto-α-boswellic acid in plasma defined as a signal-to-noise ratio of three was 9.8 nmol/l plasma.

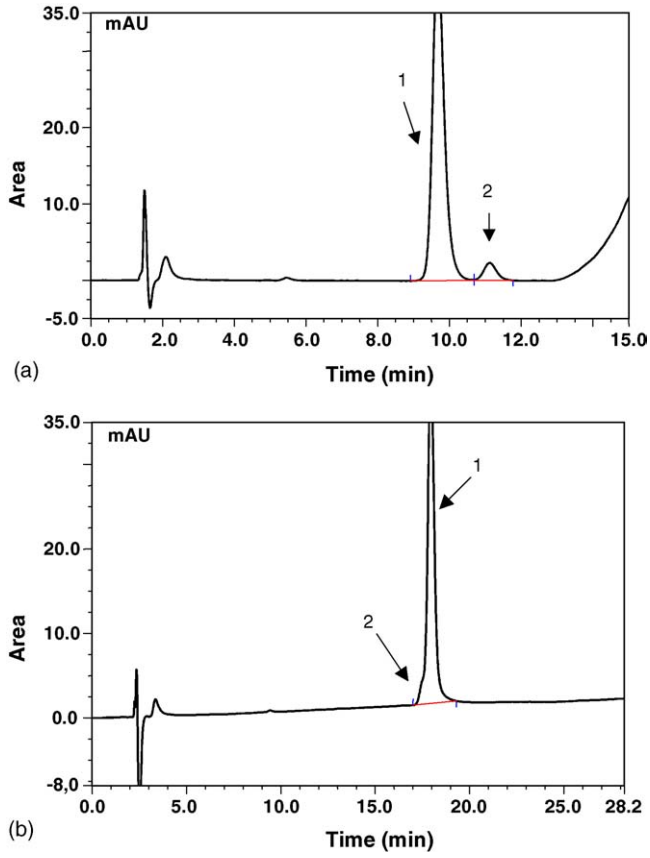

Fig. 3. Separation of the same solution of AKβBA (1) with a 5% (w/w) impurity of AKαBA (2) on different stationary phases: (a) separation on HS F5 (150 mm × 4.0 mm I.D., particle size 5 μm), flow rate 1.0 ml/min, mobile phase gradient methanol–water–acetic acid, detection at 250 nm; (b) separation on Reprosil PUR ODS-3 (250 mm × 3 mm I.D., particle size 5 μm), flow rate 0.56 ml/min, mobile phase gradient methanol–water–acetic acid, detection at 250 nm (according to reference [15]).

The molecular separation mechanism on this new stationary phase is not fully elucidated yet [17]. There is evidence that the strong electronegativity of the fluorine atom contribute specifically to the retention by a polar attraction mechanism.

Table 2
Calibration data for acetyl-11-keto-α-boswellic acid (AKαBA)

Compound	Slope (±S.E.M.)	Intercept (±S.E.M.)	Correlation coefficient	LOD (ng/injection) (±S.E.M.)
AKαBA	0.016 (0.0001)	−0.007 (0.002)	0.99999	1.7 (0.7)

Table 3
Within-day and between-day variations for acetyl-11-keto-α-boswellic acid (AKαBA) determinations

Compound	Within-day				Between-day			
	Low		High		Low		High	
	Recovery (%)	C.V.	Recovery (%)	C.V.	Recovery (%)	C.V.	Recovery (%)	C.V.
AKαBA	71.7	23	93.4	2.8	73.2	27	93	4.2

Reported values for within-day are the means of six repeated analyses measured at the same day; between-day values are the means of each six repeated analyses measured at two different days. Test range low was 19.6 nmol/l plasma, high was 196 nmol/l plasma. C.V.: coefficient of variation.

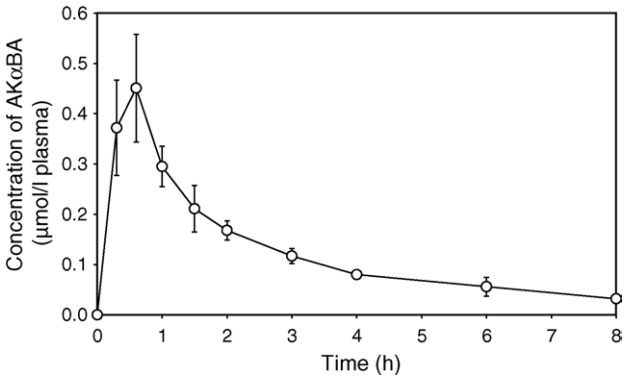

Fig. 4. Time-course of plasma concentrations of acetyl-11-keto-α-boswellic acid in the embryonic circulation after a single topical application of 20 μl of a 100 μM acetyl-11-keto-α-boswellic acid γ-cyclodextrin solution on the chick chorioallantoic membrane. Data shown are mean ± S.E.M.

For our test compounds acetyl-11-keto-α-boswellic acid and acetyl-11-keto-β-boswellic acid, the forces of interaction were so strong that a transition from reversed phase behaviour to normal phase behaviour occurred, whereas there was still water present in the mobile phase. A typical sign for this behaviour is first, the polar solvent dimethylsulfoxide used in our method had a very strong elution power, exact opposite to a reversed phase mode, and secondly the elution order of the two isomers is completely reversed in comparison to reversed phase mode. For this reason, the dimethylsulfoxid content of the standard and sample solution may not exceed 10–20%. With this novel method, we tested our acetyl-11-keto-β-boswellic acid standards as well as native frankincense samples for acetyl-11-keto-α-boswellic acid contents. All of our tested acetyl-11-keto-β-boswellic acid standards showed a contamination of about 0.5–1.0% acetyl-11-keto-α-boswellic acid, which remained undetected upon separation on C18 columns. Therefore, commercially available batches of acetyl-11-keto-β-boswellic acid require purity testing, specifically with respect to acetyl-11-keto-α-boswellic acid contaminations. The native frankincense samples, on the other hand, displayed contents of approximately 4% acetyl-11-keto-α-boswellic acid after sample preparation as previously described [8] (data not shown). To test the pharmacokinetic profile of the novel compound acetyl-11-keto-α-boswellic acid (Fig. 4) in the chick chorioallantoic membrane model (CAM), we used this novel stationary phase. In earlier studies, we tested the optimal conditions for drug application with respect to embryonic and chorioallantoic membrane development [18]. If the embryonic development is taken into account, the model is very reproducible. The strengths of this model for testing new pharmacologically active substances are several-fold. First, it saves mice, further it is quick to perform, economic and it requires only very small quantities of precious novel compounds. Upon successful pretesting in this model, additional experiments are required to confirm the pharmacotherapeutic correlation between the xenotransplantation models in mice and fertilized chicken eggs.

In conclusion, the method described in this paper allows separation and quantitative detection of a previously unknown boswellic acid isomer, namely acetyl-11-keto-α-boswellic acid, in standards and frankincense gum resins. Importantly, the procedure can also be easily applied to pharmacokinetic studies as exemplified with the chick chorioallantoic model that offers distinct advantages in cancer xenotransplantation studies.

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References

[1] H. Safayhi, B. Rall, E.R. Sailer, H.P.T. Ammon, J. Pharmacol. Exp. Ther. 281 (1997) 460.
[2] H. Safayhi, T. Mack, J. Sabieraj, M.I. Anazodo, L.R. Subramanian, H.P.T. Ammon, J. Pharmacol. Exp. Ther. 261 (1992) 1143.
[3] T. Syrovets, B. Büchele, E. Gedig, J.R. Slupsky, Th. Simmet, Mol. Pharmacol. 58 (2000) 71.
[4] R.F. Hoernlein, Th. Orlikowski, C. Zehrer, D. Niethammer, E.R. Sailer, Th. Simmet, G.E. Dannecker, H.P.T. Ammon, J. Pharmacol. Exp. Ther. 288 (1998) 613.
[5] T. Syrovets, B. Büchele, C. Krauss, Y. Laumonnier, Th. Simmet, J. Immunol. 174 (2005) 498.
[6] T. Syrovets, J.E. Gschwend, B. Büchele, Y. Laumonnier, W. Zugmaier, F. Genze, Th. Simmet, J. Biol. Chem. 280 (2005) 6170.
[7] K. Belsner, B. Büchele, U. Werz, T. Syrovets, Th. Simmet, Magn. Res. Chem. 41 (2003) 115.
[8] B. Büchele, W. Zugmaier, Th. Simmet, J. Chromatogr. B. Anal. Technol. Biomed. Life Sci. 791 (2003) 21.
[9] T. Syrovets, B. Büchele, A. Schüle, J. Marinacci, Th. Simmet, Naunyn-Schmiedeberg's Arch. Pharmacol. 367 (Suppl. 1) (2003) 302.
[10] V. Sterk, B. Büchele, T. Simmet, Planta Med. 70 (2004) 1155.
[11] K. Vipul, M. Nitin, R.C. Gupta, J. Chromatogr. B. Anal Technol. Biomed. Life Sci. 820 (2005) 221.
[12] M.A. Tawab, A. Kaunzinger, U. Bahr, M. Karas, M. Wurglics, M. Schubert-Zsilavecz, J. Chromatogr. B 761 (2001) 221.
[13] A. Kaunzinger, A. Baumeister, K. Cuda, N. Häring, B. Schug, H.H. Blume, K. Raddatz, G. Fischer, M. Schubert-Zsilavecz, J. Pharm. Biomed. Anal. 28 (2002) 729.
[14] K. Kunzi-Rapp, F. Genze, R. Küfer, E. Reich, R.E. Hautmann, J.E. Gschwend, J. Urol. 166 (2001) 1502.
[15] B. Büchele, Th. Simmet, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 795 (2003) 355.
[16] M. Gibaldi, M. Perrier, Pharmacokinetics, second ed., Marcel Dekker, New York, 1982.
[17] D.S. Bell, A.D. Jones, J. Chromatogr. A 1073 (2005) 99.
[18] B. Büchele, W. Zugmaier, F. Genze, K. Kunzi-Rapp, Th. Simmet, ALTEX 20 (2003) 163.