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Short communication

Analysis of 12 different pentacyclic triterpenic acids from frankincense in human plasma by high-performance liquid chromatography and photodiode array detection

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Abstract

For the determination of pentacyclic triterpenes of the boswellic acid family in human plasma a novel sensitive method was developed combining serial extraction on diatomaceous earth and graphitized carbon black followed by reversed phase high-performance liquid chromatography (HPLC) and photodiode array detection. The overall average extraction yield of 12 different pentacyclic triterpenic acids was approximately 66%. The calibration graphs were linear with coefficients of correlation for all compounds greater than 0.999. The overall within-day and between-day coefficients of variation (CV) for the 12 pentacyclic triterpenic acids were 5.6 and 6.8%, respectively. This HPLC procedure delivers the analytical sensitivity, precision and accuracy required for clinical pharmacokinetic and therapeutic studies.

Keywords: Pentacyclic triterpenic acids

1. Introduction

Apart from the various constituents of frankincense, which are of commercial value for the cosmetic industry, the gum resins from various *Boswellia* species also contain pharmacologically active compounds. Different extracts from Indian frankincense (*Boswellia serrata*) marketed in Europe, e.g. as H15, are currently subject to clinical investigation [1–5]. Distinct boswellic acids have indeed been reported to possess both anti-inflammatory and anti-tumor activity. These effects might be due to inhibition of human leukocyte elastase [6] and/or 5-lipoxygenase inhibitory activity [7], as well as to topoisomerase inhibition [8] leading to apoptosis-related tumor cell death [9]. In order to evaluate the putative pharmacological and therapeutical potential of various boswellic acid derivatives from frankincense gum resins, we have initiated several lines of investigations. First we isolated and structurally characterized pure standard compounds [10] and measured their contents in phytopharmaceutical preparations [11]. Apart from pharmacological studies aiming at the molecular mechanisms [8,12], it is

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necessary to analyze the plasma levels of various boswellic acids in phases I and IIa clinical studies to gain insight into the pharmacokinetic parameters of these compounds. So far, this problem has only been partially addressed; thus, only methods for the determination of a single boswellic acid, i.e. 11-keto-β-boswellic acid, by solid phase or liquid–liquid extraction from plasma followed by high-performance liquid chromatography (HPLC)-UV or GC–MS detection have been reported [13,14]. Here we show that it is possible to analyze in a single HPLC run, up to 12 different pentacyclic triterpenic acids (Fig. 1) from *B. serrata* extracts in human plasma samples. The strength of our method is a highly efficient cleaning procedure combining a matrix-assisted 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. The carbon-based extraction is very efficient and provides extracts of sufficient purity for the detection of pentacyclic triterpenic acids in the UV short wave range.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent grade unless stated otherwise. Reverse osmosis type quality water, pureAqua (Schnaitsee, Germany), plus a Milli-Q station from Millipore (Eschborn, Germany) were used throughout. The standard compounds (Fig. 1, **1**–**12**) were obtained and characterized as described [10,11]. Methanol, ethyl acetate, tetrahydrofuran, cyclohexane, acetone, isopropanol, dimethylsulfoxide, Extrelut® NT and acetic acid 96% were purchased from Merck (Darmstadt, Germany). The test drug containing a defined extract from Indian frankincense (*B. serrata*) with a total pentacyclic triterpenic acid content (**1**–**12**) of 621.8 mg/g extract was provided by the central pharmacy of the University Hospital, Ulm; the detailed composition of the test drug is shown in Table 1. Carbograph solid phase extraction cartridges (150 mg, 4 ml) contained non-porous carbon with a total surface area of 100 m^2/g were purchased from Alltech (Unterhaching, Germany).

Table 1
Contents of pentacyclic triterpenic acids in the frankincense extract used

Compound	Content (mg/g extract)	Compound	Content (mg/g extract)
1	137.8	**7**	8.3
2	33.7	**8**	5.2
3	192.2	**9**	26.1
4	100.4	**10**	11.0
5	1.8	**11**	66.6
6	0.6	**12**	38.1

Total: 621.8 mg/g extract.

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 340S (Dionex, Idstein, Germany) connected to a personal computer equipped with Chromeleon Software version 6.11 (Dionex, Idstein, Germany). Statistical calculations were carried out with the software package Valoo (Applica, Bremen, Germany). The separation was performed on a ReproSil-Pur 120 ODS-3 column (250 mm × 3.0 mm i.d., particle size 5 μm; Dr. Maisch, Ammerbuch, Germany). Solid phase extraction was performed with a Lichrolut extraction manifold (VWR International, Darmstadt, Germany).

2.3. Standards and stock solutions

Standard stock solutions were prepared from each compound (**1**–**12**) by dissolving 1 mg substance in 0.25 ml dimethylsulfoxide. For further preparation of standard solutions, the stock solutions were diluted with dimethylsulfoxide yielding concentrations from 0.03 to 2 μg substance/80 μl depending on the corresponding UV absorption characteristics. Standard solutions of the test drug in human control plasma were prepared in the same range.

2.4. Sample preparation

Blood samples anticoagulated with EDTA 5 mM (final concentration) were collected from the antecubital

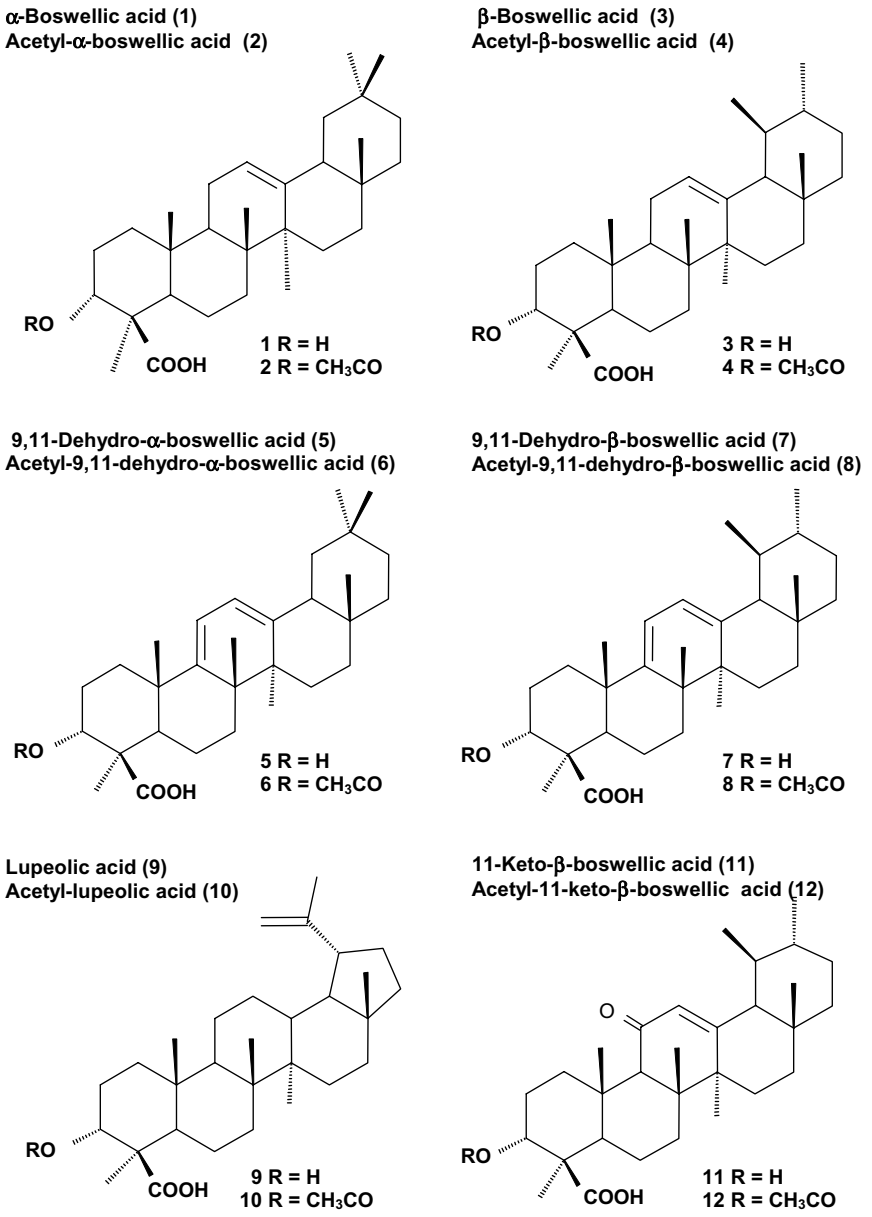

Fig. 1. Structures of the pentacyclic triterpenic acids analyzed.

vein of patients; all subjects had given informed written consent and the study reviewed by the Ethics Committee of the University was performed in agreement with the declaration of Helsinki and its subsequent modifications. The blood samples were centrifuged immediately at 562 × *g* at 4 °C for 20 min. The plasma was removed and stored frozen at −20 °C until analyzed (not longer than 3 months). For matrix-assisted liquid–liquid extraction 1.5 g Extrelut® NT was filled into a 8 ml glass column fitted with PTFE® frits. The plasma samples were thawed, thoroughly mixed and 1 ml of undiluted

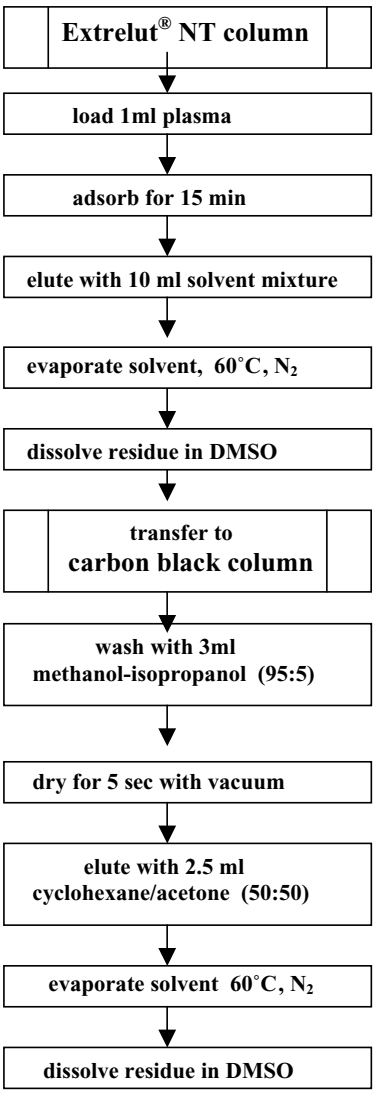

Fig. 2. Flow chart of plasma sample processing for pentacyclic triterpenic acid determination.

plasma was transferred onto each Extrelut® NT column (Fig. 2). After adsorption for 15 min the columns were rinsed with 10 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, 80 μl dimethylsulfoxide was added and the solution was transferred to a carbograph column preconditioned with 6 ml methanol. For complete sample transfer, the vial was rinsed twice with 1 ml methanol–isopropanol (95:5, v/v). The carbograph column was washed with 3 ml methanol–isopropanol (95:5, v/v). To dry the cartridge, a vacuum of 50 kPa was applied to the manifold for 5 s. After this, the pentacyclic triterpenic acids were eluted from the carbograph column with 2.5 ml cyclohexane–acetone (50:50, v/v); the samples were eluted by gravity only. The solvent was evaporated under nitrogen protection in a water bath at 60 °C. For reconstitution of the residue, 80 μl dimethylsulfoxide was added (Fig. 2).

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 stability of the ReproSil-PUR ODS-3 column, 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 62% phase A and 38% phase B at a flow rate of 0.56 ml/min. Linear gradient to 51% phase A over 20 min, then 39% phase A until 35 min, 32% phase A until 40 min, 31% phase A until 45 min, and 100% phase B until 50 min. At the end of this program all remaining matrix compounds were eluted from the column with 100% phase B at a flow rate of 0.9 ml/min. At 60 min, the flow rate was reduced to 0.56 ml/min with 62% phase A. In order to stabilize the chromatographic system, the column was maintained at 28 °C. A new sample injection was feasible after 66 min.

2.6. Identification and quantification

The eluent was continuously scanned at 210, 250, and 280 nm and the three-dimensional data fields were recorded. In the plasma samples, the pentacyclic triterpenic acids were 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 compared online with a stored reference spectrum of the corresponding standard at a concentration of 0.05–3 μg/80 μl, and (iii) the 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 per injection to 6 μg per injection revealed linearity with *R* values exceeding 0.999 (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 described earlier [11]. To test the linearity of the compounds, we used standard solutions in the range from 0.1 ng per injection to 6 μg per injection. Each sample was measured in duplicate. 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 [11]; this procedure warrants that the limit of detection is with 99% probability different from the background. Precision and accuracy of the method were determined by preparing pools of plasma containing the pentacyclic triterpenic acids in a lower and a higher concentration range 0.02–1.1 and 0.16–9 μg/ml; the individual concentration range for each compound is shown in Table 2.

Table 2
Concentration range of individual pentacyclic triterpenic acids used for the validation of the plasma method

Compound	Concentration range	
	Low	High
1	1125	9000
2	1125	9000
3	1125	9000
4	1125	9000
5	19	160
6	19	160
7	19	160
8	19	160
9	1125	9000
10	1125	9000
11	47	376
12	47	376

Values correspond to triterpenic acids contents in ng/ml plasma.

Each series consisted of six sample values. The mean coefficients of variation for 12 pentacyclic triterpenic acids for the within-day and between-day variability, respectively, are shown in Table 3. The mean extraction efficiency over all 12 compounds and different conditions, calculated by comparison of the peak area of the extracted samples with those of dimethylsulfoxide standards of same concentrations,

Table 3
Within-day and between-day variations for pentacyclic triterpenic acid determinations

Compound	Within-day				Between-day			
	Low		High		Low		High	
	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV
1	61.5	8.5	62.1	5.2	64.1	11.9	67.3	2.1
2	53.7	4.7	48.9	7.3	59.3	12.6	65.5	6.6
3	66.0	1.6	62.9	5.4	67.6	4.5	69.5	3.1
4	66.8	6.9	56.3	5.9	67.8	9.4	65.5	6.6
5	60.3	3.6	68.7	5.8	65.7	7.9	68.7	4.1
6	57.1	6.8	56.6	16.4	59.2	9.6	60.2	12.8
7	59.0	1.5	65.6	5.7	63.8	9.9	68.8	3
8	63.9	5.5	62.1	5.4	62.7	2.8	65.7	3.9
9	61.2	2.4	67.5	2.2	63.7	11.6	59.9	3.7
10	60.0	7.1	93.7	6.9	65.4	13.3	61.5	6.9
11	75.1	2.1	78.4	5.9	79.2	2.2	77.9	2.5
12	89.7	8.6	73.8	4.7	82.7	8.1	78.2	4.6
Mean	64.5	4.9	66.4	6.4	66.8	8.7	67.4	5.0

Reported values for within-day are the mean of six repeated analyses measured at the same day; between-day values are the mean of each six repeated analyses measured at two different days and two different graphitized carbon batches. Test range: low was 0.02–1.1 μg/ml plasma, high was 0.16–9 μg/ml plasma. CV: coefficient of variation.

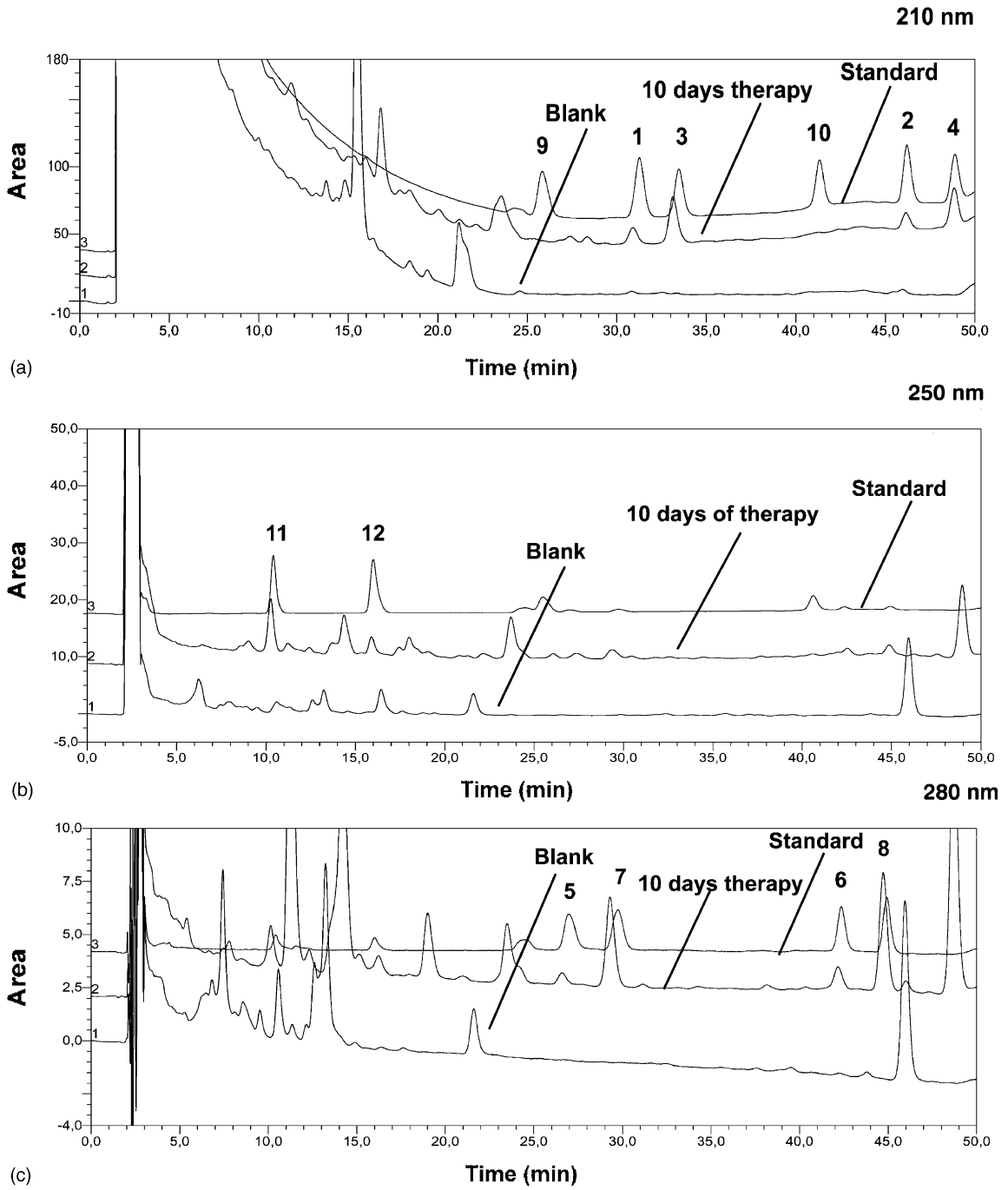

Fig. 3. Profiles of pentacyclic triterpenic acids in plasma from an untreated brain tumor patient (glioblastoma multiforme, WHO grade IV) (blank) and after 10 days of therapy with an extract from Indian frankincense (4 × 786 mg extracts per day). ReproSil-PUR ODS-3 column (250 mm × 3 mm i.d.); mobile phase gradient methanol–water; detection at (a) 210 nm, (b) 250 nm, and (c) 280 nm.

was approximately 66%. Data obtained on different days were invariably found to be in good agreement (Table 3). To test the stability of the plasma samples, data obtained with fresh plasma were compared with those from samples stored frozen at −20 °C for a period of 3 months; the results showed no differences. The lower limit of detection of the individual pentacyclic triterpenic acids in plasma defined as a signal-to-noise ratio of the three varied from 3 to 80 ng/ml plasma depending on their respective UV absorption.

3. Results and discussion

Attempts to extract boswellic acids and related pentacyclic triterpenes from human plasma with a C18 solid phase extraction method were unsuccessful in our hands, primarily because the elimination of interfering matrix material required for undisturbed UV detection between 210 and 280 nm was insufficient. We therefore introduced the step of a matrix-assisted liquid–liquid extraction with Extrelut® NT, which indeed allowed detection of boswellic acids at 250 and 280 nm. However, this additional purification step still did not permit undisturbed recordings at the wavelength of 210 nm urging us to search for additional purification measures. We developed a new procedure for the extraction of pentacyclic triterpenic acids based on solid phase extraction with graphitized carbon black. The use of this special material as a sorbent for solid phase extraction began already in the 1980s. Graphitized carbon black allows multiple interactions for retention of analytes, by hydrophobic, electronic and ion-exchange forces [15]. In our experiments, we observed very strong retention of all analyzed pentacyclic triterpenic acids as the prerequisite for the separation of plasma compounds from our analytes. By contrast, in comparable C18 solid phase extraction experiments, where hydrophobic forces are mostly responsible for retardation, we never observed those characteristics. The reason for these remarkable properties may be due to functional groups at the surface, like hydroquinone, quinone, chromene and benzpyrilium salts. These groups are able to interact very strongly with acidic compounds making it hard for conventional solvent systems to desorb them [16,17]. Typical UV tracings of HPLC eluents from standards, from a drug-free control plasma, and from a plasma sample of a patient suffering from a highly malignant brain tumor (glioblastoma multiforme, WHO IV) who had been orally treated with an extract from Indian frankincense gum resin 4 × 786 mg per day for 10 days is shown in Fig. 3; the absolute contents of pentacyclic triterpenic acids in plasma of this patient are shown in Table 4. Using blank plasma, no endogenous compounds or metabolites eluted near the retention times corresponding to boswellic acid or related pentacyclic triterpenic acid standards. In plasma samples from patients undergoing long-term therapy one can observe typical variations in the composition of the early and late eluting compounds, which are not detectable in patients obtaining placebo or volunteers after a single oral dosing of the frankincense extract (data not shown); these compounds might possibly reflect metabolites of the pentacyclic triterpenic acids and are currently subject to further investigation.

Table 4
Contents of pentacyclic triterpenic acids in plasma from a brain tumor patient (glioblastoma multiforme) treated with the frankincense extract for 10 days

Compound	Content (μmol/l plasma)	Compound	Content (μmol/l plasma)
1	3.5	**7**	0.47
2	4.0	**8**	0.29
3	10.1	**9**	n.d.
4	2.4	**10**	n.d.
5	0.06	**11**	0.34
6	0.1	**12**	0.1

n.d.: not detectable.

In conclusion, the method described in this paper introduces a highly selective procedure of solid phase extraction with graphitized carbon black for the analysis of plasma samples in pharmacokinetic and therapeutic studies with pentacyclic triterpenic acids.

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