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Determination of ajulemic acid and its glucuronide in human plasma by gas chromatography–mass spectrometry

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Abstract

A method using gas chromatography–mass spectrometry (GC–MS) and solid-phase extraction (SPE) was developed for the determination of ajulemic acid (AJA), a non-psychoactive synthetic cannabinoid with interesting therapeutic potential, in human plasma. When using two calibration graphs, the assay linearity ranged from 10 to 750 ng/ml, and 750 to 3000 ng/ml AJA. The intra- and inter-day precision (R.S.D., %), assessed across the linear ranges of the assay, was between 1.5 and 7.0, and 3.6 and 7.9, respectively. The limit of quantitation (LOQ) was 10 ng/ml. The amount of AJA glucuronide was determined by calculating the difference in the AJA concentration before ("free AJA") and after enzymatic hydrolysis ("total AJA"). The present method was used within a clinical study on 21 patients suffering from neuropathic pain with hyperalgesia and allodynia. For example, plasma levels of 599.4 ± 37.2 ng/ml (mean ± R.S.D., $n = 9$) AJA were obtained for samples taken 2 h after the administration of an oral dose of 20 mg AJA. The mean AJA glucuronide concentration at 2 h was 63.8 ± 127.9 ng/ml.

Keywords: Ajulemic acid (AJA); CT-3; GC–MS; Human plasma levels

1. Introduction

Ajulemic acid (AJA), also known as CT-3 or IP-751, is a synthetic derivative of 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid (THC–COOH), the main metabolite of the Cannabis constituent delta-9-tetrahydrocannabinol (THC). Replacement of the *n*-pentyl side chain of THC–COOH by a dimethylheptyl group leads to the compound 1′,1′-dimethylheptyl-11-nor-delta-8-tetrahydrocannabinol-9-carboxylic acid (for structures see Fig. 1) [1,2]. In preclinical and clinical studies this novel cannabinoid exhibited analgesic, anti-allodynic and anti-inflammatory activity [2–6]. The absence of psychoactive properties was shown in 24 volunteers with doses of up to 10 mg of AJA [7]. In a mouse pain model, AJA was equipotent to morphine [8]. AJA also suppresses 5-lipoxygenase and cycloxygenase-2 activities, but unlike the non-steroidal anti-inflammatory drugs, it is not ulcerogenic [6]. AJA was also effective in experimental arthritis [9] and inhibited tumor growth in vitro and in vivo [10]. It is unlikely that these effects of AJA are mediated by CB-1 and CB-2 receptors, as it shows only low affinity for these cannabinoid receptors [11]. Recently it was reported that AJA binds to the peroxisome proliferator-activated receptor γ (PPARγ), which is involved in several important physiological processes including lipid metabolism and glucose homeostasis [12]. Based on these findings, AJA has been recently approved for a controlled trial on patients suffering from neuropathic pain [13]. In this randomized, double-blind study, AJA, given orally in two daily doses of 40 and 80 mg, was significantly more effective than placebo in reducing chronic

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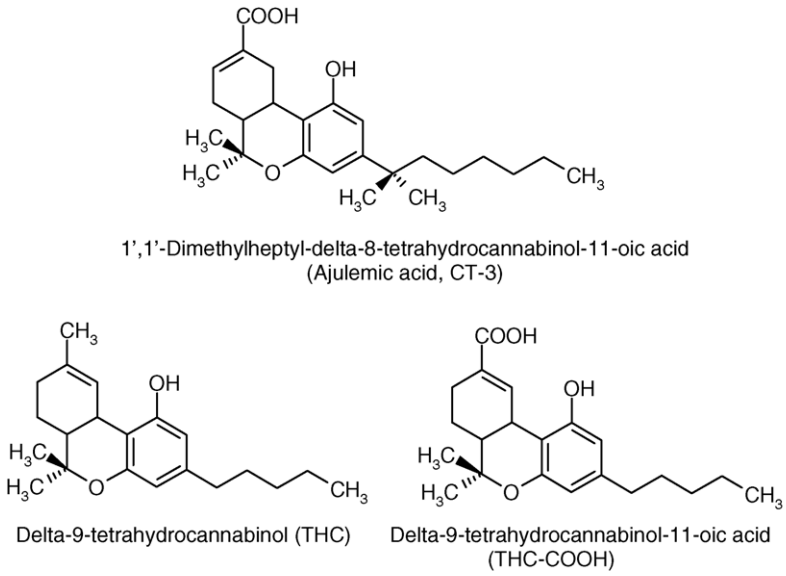

Fig. 1. Structures of ajulemic acid, 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid (THC─COOH, internal standard, I.S.), and delta-9-tetrahydrocannabinol (THC).

neuropathic pain measured by the visual analog scale (VAS).

To the best of our knowledge, no metabolism studies and methods for AJA plasma analysis have yet been published. It was therefore the aim of this study to establish the bioanalytical methodology enabling the determination of AJA and AJA glucuronide in clinical samples.

2. Experimental

2.1. Chemicals

AJA was produced by Creapharm (Le Haillan, France) with a purity of 98.1% (determined by high performance liquid chromatography, HPLC). Identity and purity of AJA were checked in our lab by HPLC and gas chromatography–mass spectrometry (GC–MS). β-Glucuronidase, type IX-A from *Escherichia coli* was supplied by Sigma (Buchs, Switzerland), Bakerbond octadecyl solid-phase extraction (SPE) columns (3 ml, 500 mg) by Stehelin (Basel, Switzerland), and 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid (THC–COOH, internal standard, I.S.) by LCG Promochem (Molsheim, France). *N*,*O*-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Fluka (Buchs, Switzerland). All other solvents and chemicals were of analytical quality obtained from Merck (Basel, Switzerland) or Fluka (Buchs, Switzerland).

2.2. Calibrators and controls

For the calibrator samples, nine working solutions were prepared in ethanol at the following concentrations: 0.12, 0.3, 1.2, 3.0, 6.0, 9.0, 12.0, 24.0 and 36.0 ng AJA/μl and 3.0 ng I.S./μl. For the control samples, six working solutions were prepared in ethanol at concentrations of 0.6, 3.6, 7.2, 10.2, 18.0 and 30.0 ng AJA/μl and 3.0 ng I.S./μl. The working solutions were prepared from two different ethanolic AJA stock solutions and were stored at −20 °C when not in use. Calibration samples were prepared by the addition of 50 μl of working solution to 0.6 ml of blank plasma, giving final concentrations of 10, 25, 100, 250, 500, 750, 1000, 2000 and 3000 ng/ml for AJA and 250 ng/ml for I.S. Low, medium and high control samples were prepared in the same way, giving final concentrations of 50, 300, 600, 850, 1500 and 2500 ng/ml for AJA and 250 ng/ml for I.S.

2.3. Sample preparation and derivatization

Frozen plasma samples (stored at −20 °C) were thawed and centrifuged at 2000 rpm before further processing. For the determination of free AJA, 0.6-ml aliquots were pipetted into a 10-ml tube, spiked with 50 μl of an ethanolic solution containing 3 ng/μl I.S. and followed by addition of 2620 μl of a 0.1 M potassium phosphate buffer (pH 6.8). The samples were then extracted on Bakerbond octadecyl SPE columns by using a robotic sample preparation system (Aspec XL; Gilson, Villiers Le Bel, France). The SPE procedure was as follows: after conditioning with 6 ml of methanol and 3 ml of bidistilled water, an aliquot of 2725 μl of the mixture (corresponding to 0.5 ml of plasma) was applied on the SPE column. Then the column was washed with 3 ml of bidistilled water, 3 ml of 0.25 M acetic acid, 3 ml of bidistilled water and 75 μl of acetone. Elution was performed by three portions of 0.5 ml of acetone. The eluate was evaporated to dryness at 50 °C under nitrogen. The residue was then derivatized by adding 60 μl of BSTFA + 1% TMCS, vortexing, and heating

at 70 °C for 30 min. A 2 μl-aliquot was injected splitless into the GC–MS.

For the determination of "total" AJA, 50 μl of an ethanolic solution containing 3 ng/μl I.S., 2420 μl of a 0.1 M potassium phosphate buffer (pH 6.8) and 200 μl of a 25,000 units/ml solution of β-glucuronidase in 0.1 M potassium phosphate buffer, corresponding to a total of 5000 units, were added to 0.6-ml plasma aliquots. The tubes were capped, vortexed and incubated at 37 °C for 16 h. The hydrolysis conditions were the same as already used and optimized earlier in our lab and by other groups for the determination of 11-nor-9-carboxy-delta-9-tetrahydrocannabinol glucuronide, the main THC metabolite, which is structurally related to AJA glucuronide [14–17]. After cooling to room temperature, extraction and derivatization were performed as for the free AJA. A 2 μl-aliquot was injected splitless into the GC–MS.

2.4. Gas chromatography–mass spectrometry

The GC–MS system consisted of a Hewlett Packard (HP) GC 5890A series II gas chromatograph with electronic pressure control, a HP 7673 autosampler, a HP 5972 mass selective detector, and a HP 5895A ChemStation software. For the separation of the derivatized extracts, a DB-1 MS column was used (30 m × 0.25 mm i.d., 0.25 μm film; J&W Scientific, supplied by MSP Kofel, Bern, Switzerland) with helium as the carrier gas at a constant flow rate of 1.2 ml/min. The injector and transfer line temperatures were 250 and 280 °C, respectively. The oven temperature was programmed from 200 °C (0.5 min) to 250 °C at 10.0 °C/min, from 250 to 280 °C at 3 °C/min and held at 280 °C for 5 min. The MS system was operated in selected ion monitoring mode (SIM). The qualifier ions *m*/*z* 222 and 544 were used for identifying, and *m*/*z* 460 for quantifying AJA. In the case of I.S., *m*/*z* 473 and 488 were used as qualifying ions, and *m*/*z* 371 as the quantifying ion. Peak assignment was achieved by retention times, the characteristic ions and the ion ratios of the analytes compared to those of standards (control samples; 50-, 300-, 1500- and 2500-ng/ml level; mean). The accepted tolerances were ±0.1 min and ±15%.

2.5. Calibration and quantitation

Calibration of AJA was performed by using the internal standard method and linear regression analysis over the concentration ranges of 10–750 ng/ml and 750–3000 ng/ml. For the low concentration range (calibration graph 1), pooled blank plasma from a blood bank was spiked with 250 ng/ml of I.S., and 10, 25, 100, 250, 500 and 750 ng/ml of AJA. In the high concentration range (calibration graph 2), the concentrations were 250 ng/ml of I.S., and 750, 1000, 2000 and 3000 ng/ml of AJA (calibration graph 2). Triplicates of each concentration level were extracted and analyzed by GC–MS as described above. Quantitation of AJA was based on the peak area of *m*/*z* 460 versus the peak area of the I.S. ion *m*/*z* 371. The amount of AJA glucuronide was calculated by the difference of the AJA concentration before (free AJA) and after enzymatic hydrolysis (total AJA).

2.6. Validation

2.6.1. Selectivity

To evaluate the method's selectivity, i.e. to check for matrix peaks interfering with AJA and I.S., pooled blank plasma as well as baseline plasma specimens from each patient were analyzed as described before but without adding the analyte and I.S.

2.6.2. Limits of quantitation and detection

Validation samples ($n = 3$) were used to determine the limit of detection (LOD) and quantitation (LOQ) of AJA. The LOD was the lowest concentration at which the ion signal-to-noise ratio (peak height) was ≥3:1 with acceptable peak shape and resolution. The LOQ was the lowest concentration fulfilling LOD criteria and reaching the AJA target value within ±20%.

2.6.3. Accuracy and precision

Intra- and inter-day accuracy and precision for the AJA compound were determined at six concentration levels over the linear ranges (low, medium and high). For each concentration level, blank plasma samples were spiked with a known amount of AJA and analyzed as described before. Intra-day accuracy and precision ($n = 5$) were established within the same day whereas inter-day values ($n = 8$) were based on measurements done over a period of 1 month. Data were also evaluated using one-way analysis of variance (ANOVA).

2.6.4. Recovery

The absolute overall recoveries of AJA and I.S. were determined at the low (50 ng/ml) and high (1500 ng/ml) concentration levels ($n = 5$). A first set of non-hydrolyzed samples was analyzed as described before. A second set was analyzed the same way but without extraction, using working solutions of the same concentration levels. The absolute extraction efficiency was determined by comparing the analyte and I.S. peak areas of the first and second set of samples.

2.6.5. Stability

The AJA stability in the matrix and in plasma extracts was checked at low, medium and high concentration levels. For the determination of AJA stability in the matrix, a set of unextracted spiked samples was stored at −20 °C for 30 days prior to extraction and analysis. AJA concentrations in this set were calculated and compared with freshly prepared control samples. The AJA stability in plasma extracts was assessed from two other sets of samples; one set of derivatized extracts which was analyzed after being stored for 30 days at −20 °C and the other set which was analyzed after the extracts were left at room temperature for 36 h. Concentrations of AJA in stored samples were compared to the ones of freshly prepared control samples.

2.7. Subjects and clinical study

Twenty-one outpatients (8 females, 13 males), aged 29–65 years (mean 51 years), who suffered from neuropathic pain with hyperalgesia and allodynia, participated in a randomized, placebo-controlled, double-blind, crossover trial conducted at the Hannover Medical School from May to September 2002. Written informed consent was obtained from all patients. Present or past recreational and/or therapeutic Cannabis use was excluded by performing baseline urine tests. This study was approved by the Hannover Medical School institutional review board, Hannover, Germany, and the German Federal Institute for Drugs and Medical Devices. After an initial 7-day baseline period (baseline week 1), two daily doses of 20 mg AJA (patients A–I) or placebo (patients J–R) were administered orally as capsules (packed by Creapharm, Le Haillan, France) at 08:00 and 20:00 h from day 8 to 11, followed by two daily doses of 40 mg AJA (patients A–I) or placebo (patients J–R) from day 12 to 14 (treatment week 1). After a washout (washout week 1) and baseline phase (baseline week 2) of 7 days each, patients crossed over to the second 7-day treatment period (treatment week 2) from day 29 to 32 and 33 to 35. For this period the same dose regimen was used as for treatment week 1. The study ended with a 7-day washout phase (washout week 2). Blood samples (10 ml) were collected at 10:00 h, i.e. 2 h after the first daily dose, on day 1, 8, 12, 14, 22, 29, 33, 35 and 42. The heparinized blood specimens were immediately centrifuged at 3000 rpm for 10 min at 4 °C, and the plasma samples stored at −20 °C until required for analysis. Further details of the study are described elsewhere [13].

3. Results and discussion

3.1. Method

Two calibration graphs were established to cover the broad range of AJA concentrations. The correlation coefficients r of 0.9915 (calibration graph 1, low range) and 0.9988 (calibration graph 2, high range) indicate good linearity from 10 to 750 ng/ml and 750 to 3000 ng/ml AJA, respectively (see Table 1). The SPE procedure resulted in overall AJA recoveries from plasma of 52.0 ± 10.9 and 51.4 ± 3.6% (mean ± R.S.D., $n = 5$) determined at the 50- and 1500-ng/ml concentration levels, respectively. The I.S. recoveries were 51.4 ± 9.6 and 48.1 ± 5.7%. The most suited I.S. would be deuterated AJA. However, this standard is (not yet) commercially available. In cases, where Cannabis use by patients cannot be excluded, deuterated THC–COOH should be the preferred I.S. However, within the present clinical study setting, including basal urine tests for cannabinoids, Cannabis consumption could be excluded. Recovery and selectivity experiments with d3-THC–COOH instead of THC–COOH have shown that the proposed method can without major modifications be used with this alternate deuterated standard. The limit of quantitation was 10 ng/ml corresponding to the lowest calibrator. The lowest concentration of AJA still detectable (limit of detection, LOD) was about 1 ng/ml. With a loss of between 0 and 9%, AJA proved to be stable in the matrix after 30 days of storage at −20 °C. When stored as a derivatized extract, the AJA content varied from 0 to 2% and 3 to 8% after 36 h at room temperature and 1 month at −20 °C, respectively. The validation data are summarized in Table 2.

Table 1
Linearity data for the determination of AJA in plasma obtained by measuring triplicates of each of the nine calibrator concentrations (10, 25, 100, 250, 500, 750, 1000, 2000 and 3000 ng/ml)

Concentration range (ng/ml)	Linearity data ($n = 3$)		
	Slope (mean ± S.D.)	Intercept (mean ± S.D.)	r (mean ± S.D.)
10–750	0.3315 ± 0.0190	−0.0107 ± 0.0177	0.9915 ± 0.0056
750–3000	0.3825 ± 0.0207	−0.1654 ± 0.0916	0.9988 ± 0.0009

Table 2
Recovery, intra- and inter-day precision and accuracy (±% of concentration added), and limit of quantitation of AJA in plasma determined at the 50, 300, 600, 850, 1500 and 2500 ng/ml-AJA concentration levels

AJA (ng/ml)	Recovery ($n = 5$) (mean ± R.S.D.[a])	Intra-day ($n = 5$)		Inter-day ($n = 8$)		Limit of quantitation (ng/ml; S:N = 10:1)
		Precision (R.S.D.)	Accuracy[b] (%)	Precision (R.S.D.)	Accuracy (%)	
50	52.0 ± 10.9	2.1	−9.1	5.3	−7.8	
300		7.0	3.9	6.3	2.6	
600		4.9	1.0	7.8	−2.9	
850		1.8	−0.2	5.0	0	
1500	51.4 ± 3.6	4.3	12.2	8.3	10.0	
2500		1.5	−2.7	5.8	−1.2	10

[a] Percent relative standard deviation.
[b] Percent difference between mean and target concentrations.

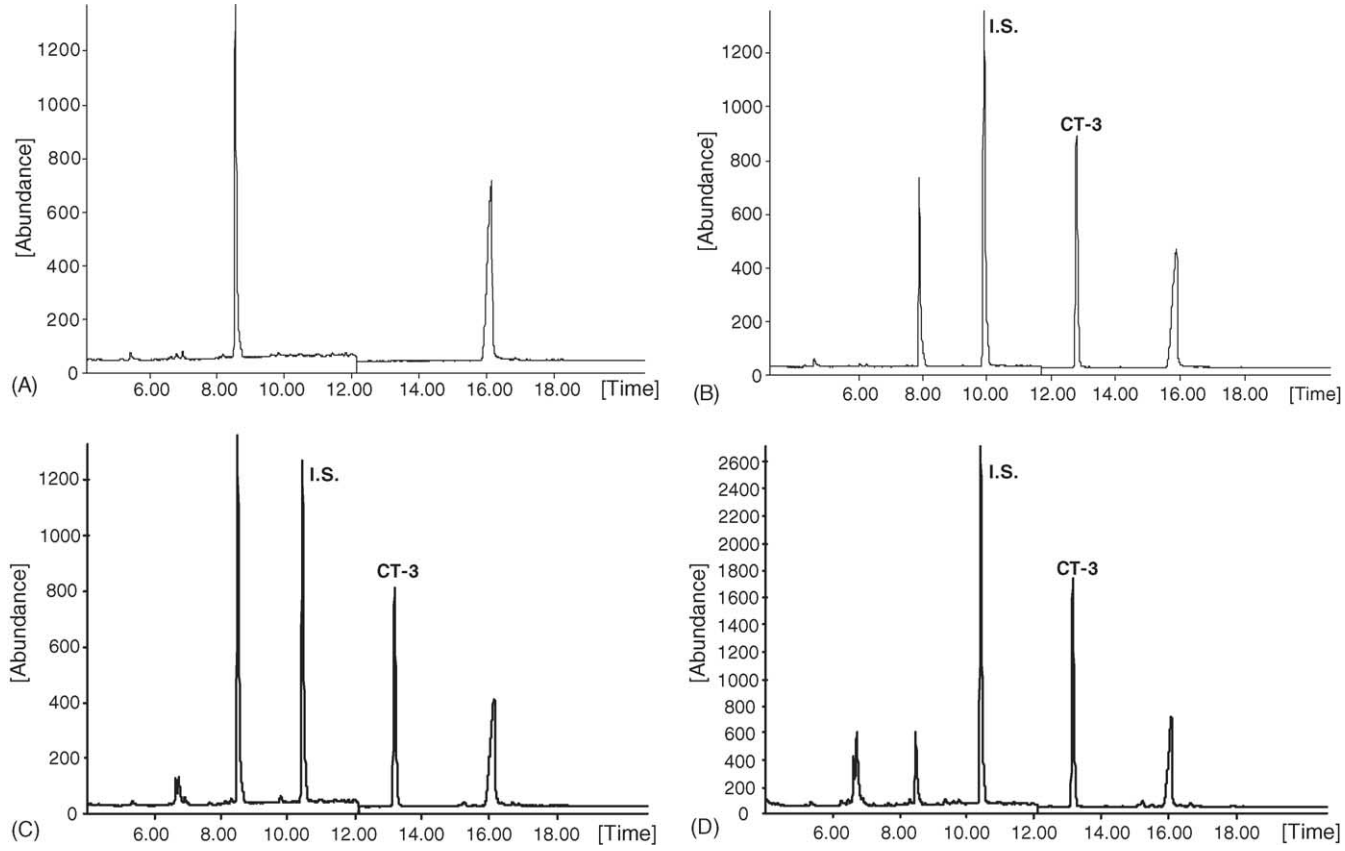

Fig. 2. GC–MS–SIM profiles of plasma extracts; the ions recorded were *m/z* 460 for AJA, and *m/z* 371 for I.S. (A) blank plasma; (B) blank plasma spiked with 500 ng/ml AJA and 250 ng/ml I.S.; (C) non-hydrolyzed plasma specimen of patient 7 collected on day 29, containing 519.7 ng/ml free AJA; (D) hydrolyzed plasma specimen of patient 7 collected on day 29, containing 547.4 ng/ml total AJA (free + glucuronidated AJA).

Table 3
Mean plasma levels of AJA and AJA glucuronide (expressed as the difference between the AJA concentration before and after enzymatic hydrolysis) after the oral administration of placebo, two daily doses of 20 mg AJA, or two daily doses of 40 mg AJA; each treatment group consisted of nine patients

Day	Patient group	Treatment (mg AJA or placebo)	AJA (ng/ml, mean ± R.S.D., $n = 9$[a])	AJA glucuronide (ng/ml, mean ± R.S.D., $n = 9$[a])
1	A–R	No (baseline week 1)	n.d.	n.d.
8	A–I	2 × 20	599.4 ± 37.2	63.8 ± 127.9
	J–R	Placebo	0	0
12	A–I	2 × 40	541.3 ± 71.7	54.7 ± 177.8
	J–R	Placebo	0	0
14	A–I	2 × 40	1271.2 ± 54.4	<LOQ
	J–R	Placebo	0	0
22	A–R	No (baseline week 2)	0	0
29	A–I	Placebo	0	0
	J–R	2 × 20	479.7 ± 65.6	37.8 ± 99.6
33	A–I	Placebo	0	0
	J–R	2 × 40	708.6 ± 64.0	30.7 ± 133.1
35	A–I	Placebo	0	0
	J–R	2 × 40	792.6 ± 63.9	38.9 ± 145.4
42	A–I	No (washout week 2)	0	0
	J–R	No (washout week 2)	<LOQ	<LOQ

n.d.: not determined.

[a] Number of patients per patient group; two drop outs due to side effects on second day of treatment week 1; samples of one patient not analyzed because of rejection of informed consent.

These data show that the method is reproducible, accurate and sensitive. One-way ANOVA analysis indicated no statistically significant difference ($p \geq 0.12$) between intra- and inter-assay data sets, respectively. The good selectivity of the method can be seen in Fig. 2 depicting the GC–MS–SIM profile of a blank patient plasma (A), a plasma spiked with 250 ng/ml I.S. and 500 ng/ml AJA (B), and a patient sample before and after enzymatic hydrolysis (C and D). Only two prominent endogenous compounds were detected and these did not interfere with the analytes.

3.2. Plasma levels

An example of a clinical plasma sample, collected from patient 7 on day 29 (2×20 mg AJA) is shown in Fig. 2C and D. It contained 519.7 ng/ml free, unconjugated AJA and 27.9 ng/ml AJA in conjugated, glucuronidated form, corresponding to 5.1% of total AJA (free + conjugated AJA). The concentrations of free and glucuronidated AJA in plasma samples collected under different treatment conditions (baseline, placebo, AJA, washout) are summarized in Table 3. Samples of day 1 (baseline week 1) were not analyzed. At day 8 (treatment week 1, AJA, patients A–I) and day 29 (treatment week 2, AJA, patients J–R), the levels of free AJA were 599.4 ± 37.2 and 479.7 ± 65.6 ng/ml (mean ± R.S.D., $n = 9$), respectively. 63.8 ± 127.9 and 37.8 ± 99.6 ng/ml, respectively, were present as AJA glucuronide. It has to be considered, that on these days AJA was given for the first time and blood sampling was at 10:00 h, i.e. 2 h after the administration of the first of the two daily doses of 20 mg AJA. With 1271.2 ± 54.4 ng/ml the levels of free AJA of patient group A–I were increased by a factor of 2 on day 14 (high dose regimen) compared to day 8 (low dose regimen). A similar difference was observed for patient group J–R (day 35 versus day 29). No AJA could be detected in the baseline two samples (day 22), following the 7 days washout phase, but surprisingly, 7 days after the last administration of AJA to patient group J–R (day 42), two patient samples still contained significant amounts of free AJA (15.8 and 12.3 ng/ml). An extended pharmacokinetic study should show if this phenomenon is due to distribution and accumulation of AJA in fat tissue. Overall, the percentage of AJA glucuronide related to the total amount of AJA (free + conjugated AJA) varied enormously from 0 to 100% ($7.6 \pm 203.9\%$).

In conclusion, the present method using SPE and GC–MS-SIM is suited to quantify AJA and AJA glucuronide in clinical plasma samples.

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