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A sensitive LC–MS/MS method for the quantitative analysis of the *Echinacea purpurea* constituent undeca-2-ene-8,10-diynoic acid isobutylamide in human plasma

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ABSTRACT

Echinacea purpurea is one of the most popular herbal medicines and is known for its immunostimulatory effects. Alkylamides are the main lipophilic components of *E. purpurea* that contribute to its pharmacological actions. For quantification in human plasma of one of these alkylamides, undeca-2-ene-8,10-diynoic acid isobutylamide, a sensitive LC–MS/MS assay has been developed and validated. Plasma samples were pretreated using liquid–liquid extraction with a mixture of diethyl ether and n-hexane (50:50, v/v). Dried extracts were reconstituted in 50 μL of acetonitrile–water (50:50, v/v) after which 15 μL of sample was injected into the HPLC system. HPLC was performed using a Polaris 3 C18-A column (50 mm × 2 mm ID) and isocratic elution with acetonitrile–water (50:50, v/v) containing 0.1% formic acid at a flow rate of 0.3 mL/min. Subsequently, electrospray ionization in the positive ion mode followed by tandem mass spectrometry was performed for detection. The total run time was 3 min. The assay was validated over a concentration range from 0.05 to 50 ng/mL for undeca-2-ene-8,10-diynoic acid isobutylamide, with 0.05 ng/mL being the lower limit of quantification using 1.0 mL plasma samples. Inter-assay inaccuracy (±12.7%), within-day and between-day precisions (CV ≤ 8.23%) were acceptable. Further, undeca-2-ene-8,10-diynoic acid isobutylamide was found to be chemically stable under relevant conditions. Finally, the applicability of this assay has been successfully demonstrated in a pharmacokinetic experiment in which a human volunteer ingested a commercial extract of *E. purpurea*.

1. Introduction

Echinacea purpurea is one of the most popular herbal medicines, and is widely used because of its supposed beneficial effects on the immune system. Examples of reported indications are the common cold, flu and upper respiratory infections. The active ingredients considered responsible for the immunostimulatory and anti-inflammatory actions are caffeic acid derivatives, polysaccharides, glycoproteins and alkylamides [1]. Alkylamides are the main lipophilic constituents and so far 17 structures have been identified [2]. One of the alkylamides is undeca-2-ene-8,10-diynoic acid isobutylamide (UDAI, Fig. 1A), involved in the anti-inflammatory activity of *E. purpurea*. In combination with other *Echinacea* alkylamides, this compound was found to significantly inhibit TNF-α and nitric oxide production in mouse macrophage cells [3]. Further proof of the anti-inflammatory effects of UDAI was given by Hinz et al., who demonstrated that UDAI was able to inhibit COX-2-dependent prostaglandin E_2 formation [4]. Inhibition of COX-2 has been proven to effectively suppress pain and inflammation. In addition, UDAI was also reported to inhibit T-cell IL-2 inhibition [5]. In our clinic, the potential pharmacokinetic interaction between *E. purpurea* extract and an anticancer agent will be studied in patients. To check the compliance of *E. purpurea* intake, the *Echinacea* alkylamide UDAI was selected to be monitored in plasma collected from these patients. For this purpose, a bioanalytical assay has to be developed and validated.

Until now, few bioanalytical assays for the quantification of alkylamides in biological matrix (plasma, serum) have been developed. In one paper [6] a method was described for the quantification of eight alkylamides in human plasma using liquid chromatography coupled with mass spectrometry (LC–MS). Solid-phase extraction (SPE) was used for sample pretreatment. Drawbacks of this method

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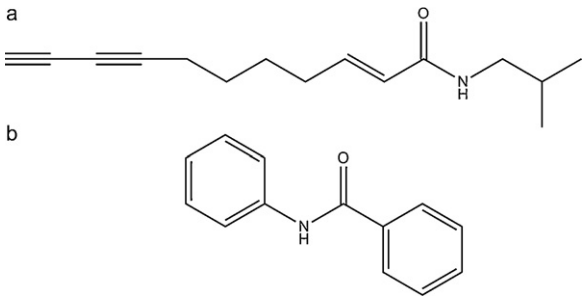

Fig. 1. Chemical structures of UDAI (A) and benzanilide (B).

were the large amount of solvents for SPE and a run time of 23 min. Furthermore, this method was executed in the single ion monitoring (SIM, LC–MS) mode, which is not as specific as *tandem* mass spectrometry (LC–MS/MS).

Another research group has also developed LC–MS/MS methods for the bioanalysis of alkylamides in human serum and plasma [7–9]. These methods were used for evaluation of the pharmacokinetics of alkylamides after ingestion of *Echinacea* preparations by human volunteers. All plasma and serum samples were pretreated using SPE before performing the LC–MS/MS analysis. Disadvantages of these LC–MS/MS methods were, however, a long run time of 20 min per sample [7–9] and the large solvent and sample volumes used (14 mL serum [8] and 16 mL plasma [9]).

The previously described LC–MS/MS assay for UDAI, has been used to study the pharmacokinetics of UDAI in volunteers [7]. In that study, 2.5 mL of an 60% ethanolic extract from the roots of *Echinacea angustifolia* was administered to 11 volunteers. The mean maximum plasma concentration of 1.87 ng/mL UDAI was found. The LLQ of this assay was 0.08 ng/mL. In our clinical study, 20 drops (approximately 1 mL) of an 65% ethanolic extract of *E. purpurea* will be administered. Compared to the pharmacokinetic study, a mean maximum plasma concentration lower than 1.87 ng/mL was expected due to a 2.5 times lower volume of the ingested dose. Furthermore, UDAI is more abundant in *E. angustifolia* than in *E. purpurea* [2]. Therefore, we hypothesized that a LLQ of 0.08 ng/mL may not be sufficient for our clinical study. Furthermore, for practical reasons a shorter run time and a smaller sample volume than the previously developed bioanalytical assays for UDAI [6–9] was desirable. Therefore, we have developed a fast and sensitive LC–MS/MS method for the quantification of UDAI in human plasma. In the present assay, liquid–liquid extraction (LLE) was used for sample preparation. As no isotopically labeled internal standard was commercially available, benzanilide was used as internal standard which was also used for this purpose in previous studies (Fig. 1B) [7–10]. Further, the presently described assay has been fully validated according to the FDA guidelines on Bioanalytical Method Validation [11].

2. Materials and methods

2.1. Reagents and chemicals

UDAI ($C_{15}H_{21}NO$) was purchased from ChromaDex, Inc (Irvine, CA, USA). Benzanilide ($C_{13}H_{11}NO$) originated from Acros Organics (Leicestershire, UK). LC–MS grade water, ethanol and methanol of HPLC quality and acetonitrile of HPLC-S gradient grade quality were obtained from Biosolve (Valkenswaard, The Netherlands). HPLC grade n-hexane and analytical grade diethyl ether stabilized with 2,6-di-tert-butyl-4-methylphenol (BHT) and formic acid were purchased from Merck (Darmstadt, Germany). Blank, drug-free human plasma, containing heparin (Li) as anti-coagulant, was obtained from Innovative Research Inc. (Novi, MI, USA).

2.2. Instrumentation

The LC–MS/MS equipment consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler, two LC10-ADvp-μ pumps (all from Shimadzu, Kyoto, Japan) and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) with electrospray ionization (ESI). For data acquisition and processing, Xcalibur software (version 1.4, Thermo Fisher Scientific) was used.

2.2.1. Chromatographic conditions

Sample injections (15 μL) were made on a Polaris 3 C18-A column (50 mm × 2 mm ID, particle size 3 μm, Varian, Middelburg, The Netherlands) with a Polaris 3 C18-A pre-column (10 mm × 2 mm ID, particle size 3 μm, Varian). The column temperature was maintained at 40 °C and the autosampler was set at 15 °C. The total run time was 3 min. The mobile phase consisted of acetonitrile–water (50:50, v/v) containing 0.1% formic acid, which was delivered at a flow rate of 0.3 mL/min.

2.2.2. Mass spectrometry

The mass spectrometer operated in the positive ion mode with both quadrupoles set at 0.7 full width at half height (FWHM, unit resolution) and with dwell times of 200 ms. For UDAI, the mass transitions from *m/z* 232 to 105 were optimized and for benzanilide, responses from *m/z* 198 to 105 were monitored. The optimized collision energies were −16 V for UDAI and −20 V for benzanilide. For both compounds, the tube lens voltage was set at 101 V. Furthermore, spray voltage was set at 4500 V with an ion tube temperature of 210 °C. Nitrogen sheath, ion sweep and auxiliary gasses were set at 49, 2.0 and 14 arbitrary units, respectively. Finally, the up-front collision-induced dissociation (CID) was set off and collision gas pressure (Ar) was set at 2.0 mTorr.

The robustness of the LC–MS/MS method was tested by monitoring the absolute area of UDAI and the ratio with the internal standard for two calibration samples at the concentration level of 5 ng/mL UDAI. One of these calibration samples was injected at the beginning of a run containing 55 plasma samples in total, whereas the other calibration sample was injected at the end of the same run. Relative standard deviations of the absolute signal of UDAI and the ratio were calculated.

2.3. Preparation of stock and working solutions

Two stock solutions of UDAI (0.1 mg/mL) from two independent weightings were prepared in methanol. One stock solution was used for the preparation of calibration standards, and the other solution was used to prepare quality control (QC) samples. For the calibration standards the stock solution was further diluted with control human heparinized plasma to obtain working solutions in a range from 2 to 10,000 ng/mL. The stock solution for QC samples was diluted with control human plasma to obtain a working solution of 10,000 ng/mL.

For the internal standard, a stock solution of benzanilide in methanol (1 mg/mL) was further diluted with reconstitution solvent (acetonitrile–water (50:50, v/v)) to obtain working solutions of 200 and 10,000 ng/mL.

The stock solutions and the internal standard working solutions were stored at −30 °C until use, while the working solutions in plasma were used immediately after preparation.

2.4. Preparation of calibration standards and QC samples in plasma

Calibration standards were prepared freshly by diluting plasma working solutions with human plasma to obtain concentrations of

0.05, 0.1, 1, 5, 25 and 50 ng/mL UDAI. These standards were prepared and analyzed in duplicate.

QC samples were prepared in batches by diluting the QC working solution in volumetric flasks, obtaining QC samples at concentrations of 0.05 (LLQ), 0.15 (low), 5 (mid), 40 (high) and 50 (upper limit of quantification: ULQ) ng/mL UDAI. As no plasma concentrations of UDAI higher than 50 ng/mL were expected in patient samples, no dilution test sample above the ULQ (>ULQ sample) was prepared. QC samples were stored at −30 °C until analysis.

2.5. Sample preparation

To 1 mL plasma samples 50 μL of the internal standard benzanilide (200 ng/mL) was added in a conical glass tube. After vortex-mixing, LLE was performed with 8 mL diethyl ether/n-hexane (50:50, v/v). Subsequently, the samples were shaken with a rotary-mixer for 10 min at 50 rpm. After centrifugation for 10 min at 3200 × *g* (4 °C), the samples were stored at −30 °C for 60 min. Next, the organic layer was decanted into another conical glass tube and evaporized under a stream of nitrogen at 40 °C. The residue was reconstituted in 50 μL of acetonitrile–water (50:50, v/v) and vortex-mixed for approximately 15 s. After transferring the solution into 1.5 mL Eppendorf tubes the solution was centrifuged for 10 min at 13,500 rpm. Finally, the clear supernatant was transferred into a 250 μL glass insert placed in an autosampler vial.

3. Validation procedures

A full validation of the assay in human heparinized plasma was performed according to the current FDA guidelines on Bioanalytical Method Validation [11].

3.1. Linearity

Six non-zero calibration standards at concentrations of 0.05, 0.1, 1, 5, 25 and 50 ng/mL UDAI were prepared freshly in duplicate for each run and analyzed in three separate runs. Calibration curves (ratio of the areas of the analyte and internal standard peaks *versus* the nominal concentration) were fitted by least-squares linear regression and the reciprocal of the squared concentration ($1/x^2$) was used as a weighting factor.

Deviations from the nominal concentrations should be within ±20% for the LLQ and within ±15% for the other concentrations.

3.2. Accuracy and precision

Accuracy and precision of the assay were determined by analyzing five replicates of QC samples of UDAI at the LLQ, low, mid and high concentration levels together with duplicate calibration standards in three analytical runs. Inter-assay accuracy was calculated as the relative difference between the mean measured concentration after the three runs and the nominal concentration. The accuracy should be within 80–120% for the LLQ and 85–115% for the other concentrations. Intra- and inter-assay precisions were represented by the coefficient of variation (CV%), which should be less than 20% for the LLQ and less than 15% for the other concentrations.

3.3. Recovery and ion suppression

For the determination of ion suppression (matrix effect), 1 mL of blank human plasma was processed as a double blank plasma sample. After LLE and evaporation (as described in Section 2.5), the extracts were reconstituted with 50 μL of reconstitution solvent. Three different concentrations of reconstitution solvents (all prepared in acetonitrile–water (50:50, v/v)) were added in triplicate: solvent 1 (3 ng/mL UDAI + 200 ng/mL internal standard), solvent 2 (100 ng/mL UDAI + 200 ng/mL internal standard) and solvent 3 (800 ng/mL UDAI + 200 ng/mL internal standard).

For calculation of ion suppression, the analytical response of the reconstituted extracts was compared with the response of the directly injected reconstitution solvents (1, 2 and 3).

For the extraction recovery study, QC samples (0.15, 5 and 40 ng/mL UDAI) were prepared according to Section 2.5. The final concentration of UDAI in these QC samples was 3, 100 and 800 ng/mL (due to the 20 times concentration factor during sample preparation).

The analytical response of these QC samples was compared to the response of the blank plasma extracts reconstituted with the solvents 1, 2 and 3 mentioned above.

Determination of the overall recovery was performed by comparing the analytical response of the processed QC samples (0.15, 5 and 40 ng/mL) to the response of unprocessed reconstitution solvents 1, 2 and 3.

3.4. Carry-over

Carry-over was assessed by injecting two processed blank plasma samples immediately after injecting a sample at the ULQ (50 ng/mL). The response of the first blank sample at the retention time of UDAI should be less than 20% of the response of a processed LLQ sample.

3.5. Specificity and selectivity

Potential interference between analyte and endogenous matrix components was investigated by analyzing six batches blank human plasma from different origin. From each batch, a double blank and a LLQ sample were prepared, processed and analyzed according to the described procedures. To assess potential interference between internal standard and analyte, blank samples spiked separately with analyte (at the ULQ) and internal standard were processed and analyzed.

Peak areas of compounds co-eluting with UDAI should be less than 20% of the peak area of the LLQ sample. Areas of peaks that co-elute with the internal standard, should not exceed 5% of the mean internal standard peak area.

The calculated concentrations of the LLQ samples should not deviate more than 20% from the nominal concentrations.

3.6. Stability

Stability of stock solutions of UDAI and internal standard was determined by analyzing aliquots of stock solutions in triplicate which were kept at room temperature for 24 h. Peak areas of these samples were compared with those of stock solutions that were stored at −30 °C and represented freshly prepared stock solutions. Furthermore, stability of UDAI stock solution was assessed after storage for 2 months at −30 °C.

To determine short-term temperature stability, QC samples kept at room temperature for 6 h were analyzed in triplicate and compared to the initial concentrations.

Long-term stability of UDAI was determined in triplicate by analyzing QC samples after storage at −30 °C.

Freeze (−30 °C)/thaw stability of UDAI in human heparin plasma was assessed by comparing QC samples after three freeze/thaw cycles with the concentrations determined at time zero.

Stability of reconstituted extracts was assessed in quadruplicate after storage for 48 h at 2–8 °C and compared with the initial concentrations.

QC samples analyzed in the above mentioned stability experiments were prepared at two concentration levels: 0.15 and 40 ng/mL UDAI. Concentrations of UDAI in the biological matrix

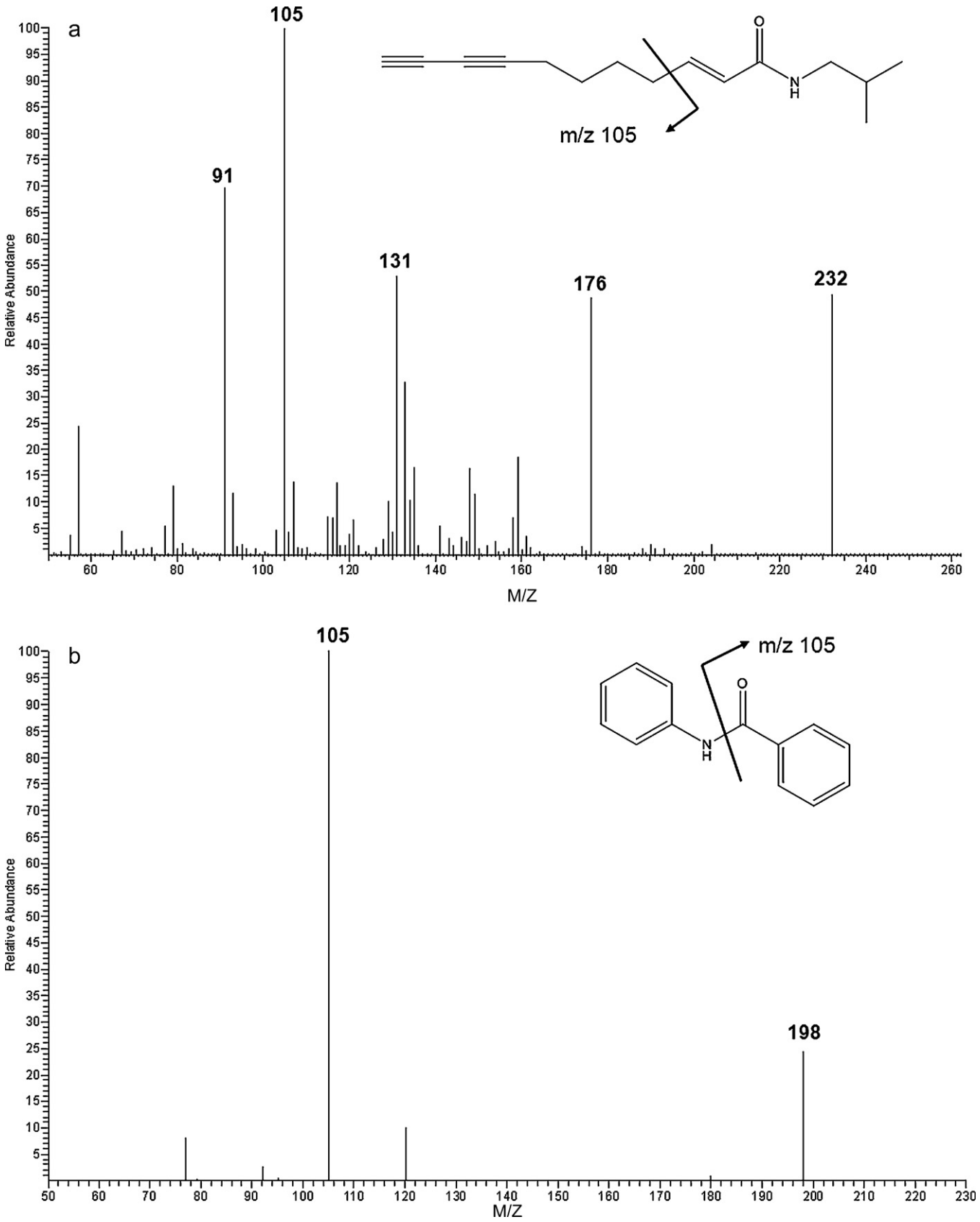

Fig. 2. (A) MS/MS product ion scan of UDAI (precursor ion *m*/*z* 232). (B) MS/MS product ion scan of benzanilide (precursor ion *m*/*z* 198).

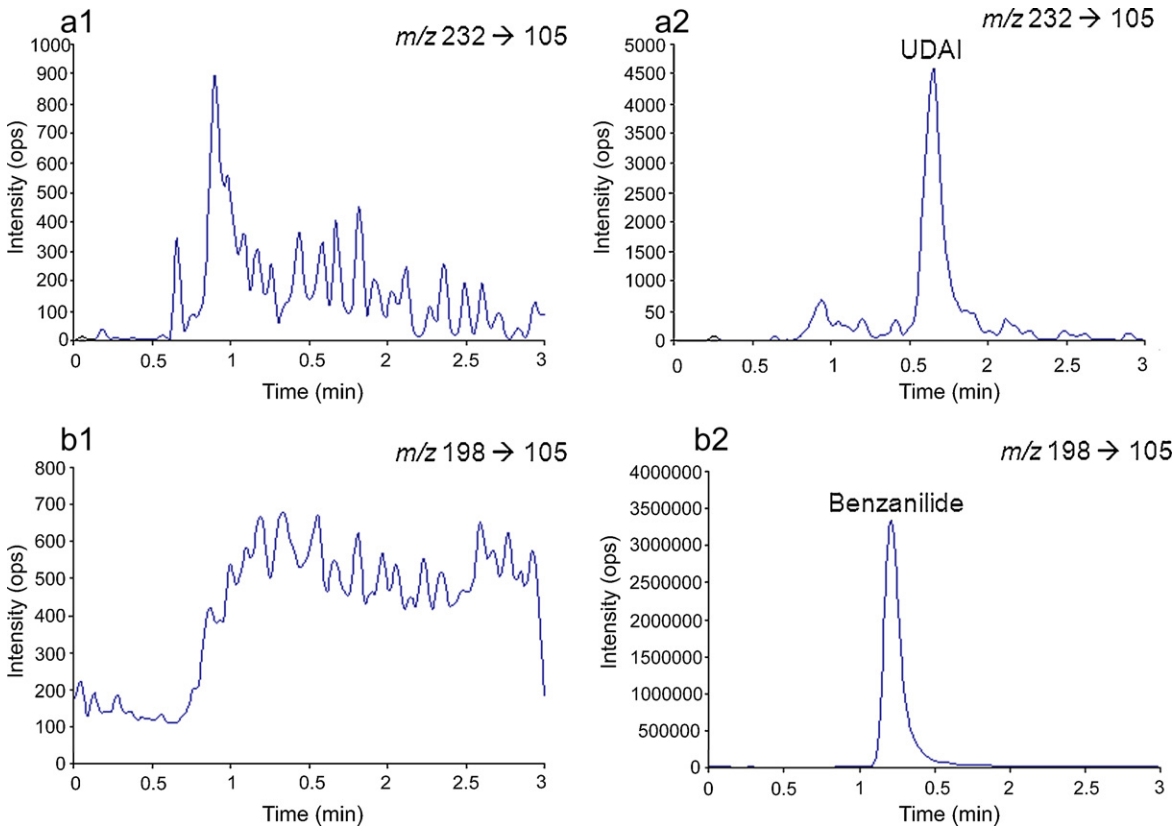

Fig. 3. Representative LC–MS/MS chromatograms of a blank human heparinized plasma sample (A1, B1) and of a spiked human heparinized plasma sample at the LLQ level of 0.05 ng/mL (A2, UDAI, t_r = 1.6 min; B2, internal standard benzanilide, t_r = 1.2 min).

samples should not deviate more than 15% from the initial concentrations. UDAI and internal standard were considered stable in stock solution if 95–105% and 80–120% of the initial peak area is recovered for UDAI and benzanilide, respectively.

4. Results and discussion

4.1. Mass spectrometry

The protonated molecule of UDAI at *m*/*z* 232 was used to generate a product ion spectrum. For selected reaction monitoring (SRM), the most abundant fragment ion (*m*/*z* 105) was selected and the fragmentation conditions were optimized. The conditions of *m*/*z* 198–105 were optimized for monitoring the internal standard benzanilide. MS/MS product scans and the proposed fragmentation pathways of UDAI and benzanilide are depicted in Fig. 2.

4.2. Chromatography

Woelkart et al. developed a method using a reversed phase column and the analytes were eluted from the column with a gradient consisting of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) [7–9]. A flow rate of 0.25–0.3 mL/min was used [7–10]. We have tested isocratic elution (A:B (50:50, v/v)) at a flow rate of 0.3 mL/min to shorten the total run time. These conditions resulted in symmetric peaks and a total run time of only 3 min. Representative chromatograms of a blank sample and a human plasma sample spiked at the LLQ are given in Fig. 3. In the robustness test, the relative standard deviation of the absolute area of UDAI was less than 2% and less than 5% for the ratio with the internal standard, demonstrating the robustness of the method.

4.3. Sample pre-treatment

After administration of a single oral dose of an ethanolic extract of *E. angustifolia* to 11 healthy volunteers, a C_{max} of 1.87 ng/mL in plasma was found for UDAI [7]. This finding indicated the need for a sensitive bioanalytical assay. Clean sample extracts were required to achieve a LLQ of 0.05 ng/mL. LLE was tested in the present study. Several ratios of diethyl ether/n-hexane were investigated: the highest overall recovery was found for diethyl ether/n-hexane (50:50, v/v).

Subsequently, the optimal volume of diethyl ether/n-hexane (50:50, v/v) was assessed by LLE with organic phase volumes rang-

Table 1
Assay performance data for UDAI (n = 15).

Nominal concentration (ng/mL)	Mean measured concentration (ng/mL, ±SD)	Inter-assay accuracy (%)	Within-day precision (% CV)	Between-day precision (% CV)
0.0500	0.0533 (±0.00454)	107	7.99	8.23
0.150	0.163 (±0.00686)	108	3.71	4.08
5.00	5.17 (±0.155)	103	2.56	2.90
40.0	34.9 (±1.27)	87.3	2.89	3.51

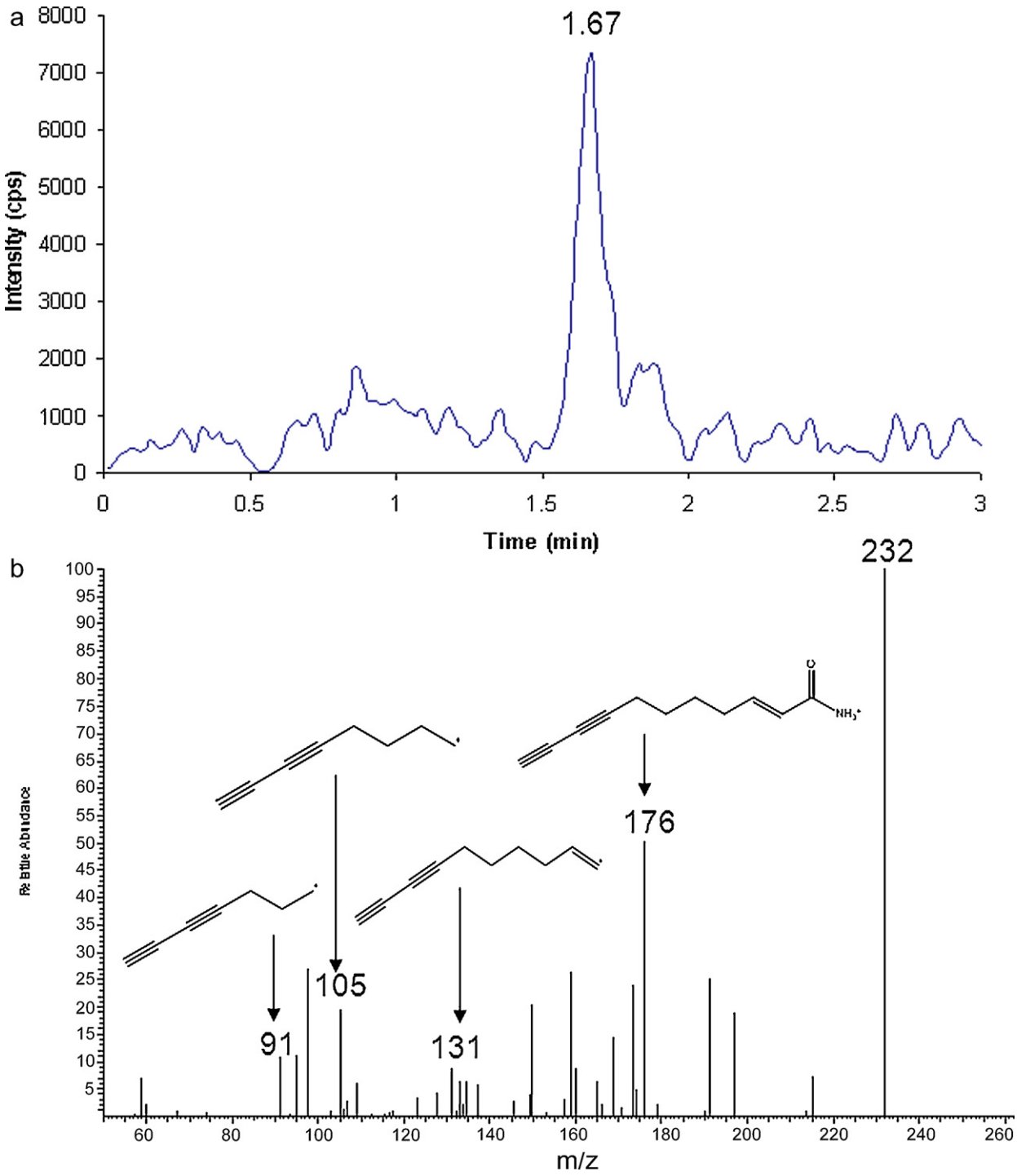

Fig. 4. (A) LC–MS/MS chromatogram of a plasma sample taken from a human volunteer 60 min after ingestion of 20 drops of A. Vogel Echinaforce® (t = 60 min). (B) MS/MS product ion scan of UDAI (t_r = 1.67 min) of the human plasma sample at t = 60 min. Proposed chemical structures are depicted for product ions m/z 91, 105, 131 and 176.

ing from 2 to 9 mL and selecting the volume with the highest absolute MS/MS response of analyte and internal standard. Eight milliliters of diethyl ether/n-hexane (50:50, v/v) was found to give the best results and was therefore selected.

4.4. Validation

4.4.1. Linearity

The assay was linear over a concentration range of 0.05–50 ng/mL for UDAI in human plasma.

Using least-squares linear regression (area ratio *versus* the concentration) with a weighting factor of $1/x^2$, the lowest total bias and the most constant bias across the range were obtained. The average regression parameters of the linear regression functions (n = 3) were $y = 7.97 \times 10^{-5}(\pm 6.30 \times 10^{-5}) + 0.0292(\pm 0.0176)x$ with an average correlation coefficient of 0.994(±0.00395).

At all concentration levels deviations of measured from nominal concentrations were between −17.9 and 8.15% for LLQ and between −12.2 and 12.1% at the other concentration levels. CV values varied between 3.46 and 18.1% at LLQ and between 0.148 and 12.4% at the remaining concentration levels.

Table 2
Stability data for UDAI and the internal standard benzanilide.

Conditions	Matrix	Initial concentration (ng/mL)	Found concentration (ng/mL)	Dev (%)	CV (%)	No. of replicates
UDAI						
Ambient, 24 h	Methanol	1.10×10^5	1.14×10^5	4.12	17.4	3
−30 °C, 2 months	Methanol	1.01×10^5	1.07×10^5	5.52	3.04	3
Ambient, 6 h	Plasma	0.154	0.163	5.58	6.97	3
		41.2	44.5	8.01	3.03	3
−30 °C, 1 month	Plasma	0.176	0.188	7.28	13.7	3
		40.4	44.1	8.93	6.25	3
3 freeze (−30 °C)/thaw cycles	Plasma	0.154	0.159	3.31	2.53	3
		41.2	40.6	−1.33	4.91	3
2–8 °C, 48 h	Final extract	0.154	0.145	−6.09	5.20	4
		5.15	4.87	−5.40	1.87	4
		41.2	37.5	−9.07	5.66	4
Benzanilide						
Ambient, 24 h	Methanol	9.70×10^5	9.04×10^5	−6.84	12.1	3
−30 °C, 4 months	Methanol	1.07×10^6	1.16×10^6	8.49	3.25	3

Dev: deviation from the initial concentration and CV: coefficient of variation.

4.4.2. Accuracy and precision

Assay performance data for UDAI in human plasma are summarized in Table 1. The inter-assay inaccuracy was within ±12.7% for all concentrations, thus meeting the required ±15% (±20% for LLQ) [11]. The within-day and between-day precisions did not exceed 8.23% for all concentrations and therefore fulfilled the required criterion of ±15% (±20% for LLQ) [11].

4.4.3. Recovery and ion suppression

Ion suppression has been demonstrated for both UDAI and benzanilide. The mean ion suppression for UDAI was −15.0% (range −22.0 to −8.60%), and for benzanilide a mean ion suppression of −14.1% (range −19.8 to −11.2%) was observed.

After comparing the analytical response of processed QC samples to that of blank processed plasma samples reconstituted with solutions containing analyte and internal standard, mean LLE recoveries of 53.5 and 62.8% were found for UDAI and benzanilide, respectively.

The mean total recovery was 45.5% for UDAI and 53.9% for benzanilide and were found to be reproducible.

4.4.4. Carry-over

In the first processed blank, the carry-over was 6.84 and 0.687% of the areas of the compounds in a processed LLQ sample for UDAI and benzanilide, respectively. These percentages were far below the required 20% and were thus found to be acceptable.

4.4.5. Specificity and selectivity

SRM chromatograms of six batches of control drug-free heparinized plasma contained no co-eluting peaks >20% of the analyte peak area at the LLQ level, and no co-eluting peaks >5% of the area of the internal standard. The six batches showed deviations from the nominal concentrations at the LLQ level between −12.9 and 19.6% for UDAI and were approved.

Further, no cross-analyte/internal standard interference was observed, as no peaks were detected at the retention time of UDAI when analyzing a sample containing only internal standard. Additionally, no peaks were detected at the retention time of benzanilide when a sample containing only UDAI was processed.

4.4.6. Stability

The stability data for UDAI and the internal standard benzanilide are summarized in Table 2. These data show that stock solutions of UDAI and benzanilide were stable for at least 24 h at ambient temperatures. The stock solution of benzanilide was stable for at least 4 months at −30 °C. After storage for 2 months at −30 °C, the stock solution of UDAI was just outside the range of 95–105% recovery of the initial measured concentration, indicating that UDAI in stock solution is stable for up to 2 months under the tested conditions.

Short-term stability experiments demonstrated stability of UDAI in human heparinized plasma at ambient temperatures for at least 6 h.

Regarding long-term stability, UDAI was stable in plasma for up to 1 month at −30 °C.

Freeze/thaw stability experiments showed that UDAI was stable in human heparinized plasma for at least three freeze (−30 °C)/thaw cycles.

Finally, in the final extract stability of UDAI was demonstrated for at least 48 h at 2–8 °C.

5. Application of the method

To demonstrate the applicability of the present assay, a single pharmacokinetic experiment was conducted in which a human volunteer ingested a single low dose of 20 drops of a commercial extract of *E. purpurea* (Echinaforce®, A. Vogel). The presence of UDAI was detected in the blood sample taken at 60 min (Fig. 4A). The identity of UDAI was confirmed by the retention time of the peak at 1.6 min, absent in the blank sample, and which correlates with that of the reference standard of UDAI. Additionally, in the product ion spectrum of *m/z 232* at 16.0 V the major product ions *m/z* 91, 105, 131 and 176 were observed (Fig. 4B) which are identified as those in the reference compound of UDAI (Fig. 2A). The plasma concentration of UDAI at $t = 60$ min was quantified as 0.060 ng/mL. The chromatogram and product ion scan of UDAI in the human sample at $t = 60$ min are depicted in Fig. 4.

6. Conclusions

This paper describes the development, validation and application of a LC–MS/MS assay for the quantitative analysis of UDAI in human plasma. This is the first quantitative assay for an alkylamide which has been fully validated according to the FDA guidelines

on Bioanalytical Method Validation [11]. To extract UDAI from human plasma, a LLE method was performed. Validation results show that this assay is accurate, precise, selective and reproducible. Using 1 mL of plasma aliquots, a concentration range from 0.05 to 50 ng/mL UDAI could be quantified. This sample volume is less than used in previously published LC–MS/MS assays for alkylamides of *E. purpurea* in biological matrices. Furthermore, with a LLQ of 0.05 ng/mL this assay is more sensitive than the previously described bioanalytical assay for UDAI in human plasma [7]. At last, in the pharmacokinetic experiment a peak plasma concentration of 0.06 ng/mL UDAI was measured 60 min after ingestion of 20 drops of a commercial extract of *E. purpurea*. This low concentration of UDAI demonstrated the need for the present, sensitive, assay for UDAI to be used in the clinical study. In our clinical trial, however, we expect to detect higher levels of UDAI in patients, as the patients will use the extract more frequently than our volunteer: 3 times daily 20 drops during a period of 14 days. Thus, this pharmacokinetic experiment showed that the LLQ was sufficient for our purpose to monitor the adherence of *E. purpurea* in a clinical setting.

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