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Short communication

The bioanalysis of the major *Echinacea purpurea* constituents dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides in human plasma using LC–MS/MS

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ABSTRACT

Alkylamides are a group of active components of the widely used herb *Echinacea purpurea* (*E. purpurea*), which have immunostimulatory and anti-inflammatory effects. For the most abundant alkylamides, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (DTAI), an LC–MS/MS assay has been developed and validated for quantification in human plasma. This assay will be used to support a clinical interaction study with *E. purpurea*. A 300 μL plasma aliquot underwent liquid–liquid extraction with diethylether-n-hexane (50:50, v/v). After evaporation and reconstitution in 100 μL of acetonitrile–water (50:50, v/v) 20 μL of sample were injected into the HPLC system. Chromatographic separation was achieved with a Polaris 3 C18-A column (50 mm × 2 mm ID, particle size 3 μm), a flow rate of 0.3 mL/min and isocratic elution with acetonitrile–water (50:50, v/v) containing 0.1% formic acid during the first 5 min. Hereafter, gradient elution was applied for 0.5 min, followed by restoration of the initial isocratic conditions. The total run time was 7.5 min. The assay was validated over a concentration range from 0.01 to 50 ng/mL for DTAI, with a lower limit of quantification of 0.01 ng/mL. Validation results show that DTAI can be accurately and precisely quantified in human plasma. DTAI also demonstrated to be chemically stable under relevant conditions. Finally, the applicability of this assay has been successfully demonstrated by measuring the plasma concentration of DTAI in patients after ingestion of a commercial extract of *E. purpurea*.

1. Introduction

Nowadays, *Echinacea purpurea* (*E. purpurea*) is one of the most often used herbal medicines. Alkylamides, caffeic acid derivatives, polysaccharides and glycoproteins are considered to be the components responsible for *E. purpurea*'s immunostimulatory and anti-inflammatory effects [1]. For alkylamides, the main lipophilic constituents, seventeen compounds have been identified in *E. purpurea* [2]. The main alkylamides are the isomeric dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides (DTAI) [3], which are likely to cross the intestinal barrier [4]. Oral bioavailability of DTAI has been demonstrated in rats [5] and humans [6–9]. Pharmacologically, DTAI have immunomodulatory and anti-inflammatory actions [3,10,11], that are presumably mediated by binding to cannabinoid receptors [12].

This article focuses on the quantitative analysis of DTAI in human plasma using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). The presented assay will be used to support a clinical study in which the potential pharmacokinetic interaction between *E. purpurea* extract and the anticancer drug docetaxel will be studied. In this clinical study plasma levels will be monitored to assess the adherence of *E. purpurea* intake by the patients and the pharmacokinetics of DTAI.

Previously, few bioanalytical assays for DTAI in human plasma or serum have been published [6–9]. Matthias et al. developed an assay for the quantification of eight alkylamides, including DTAI, in human plasma [6]. Drawbacks of this assay, however, are the large amount of solvents used during solid-phase extraction (SPE) and a run time of 23 min. Also, the single ion monitoring (SIM,

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LC–MS) mode used in this assay is not as specific as *tandem* mass spectrometry (LC–MS/MS).

Sensitive quantitative assays for DTAI in human plasma or serum with a lower limit of quantification (LLQ) down to 0.008 ng/mL have been developed by Woelkart et al. [7–9]. However, large sample volumes of up to 16 mL plasma [9] and long run times of 20 min were required for these assays [7–9]. Regarding practical issues such as patient convenience and time efficiency, a faster assay requiring less sample volume was desirable for application in our clinical study. Therefore, our objectives were to develop a sensitive validated assay according to the FDA guidelines on Bioanalytical Method Validation [13], which would require less sample volume and a shorter run time.

2. Materials and methods

2.1. Reagents and chemicals

DTAI ($C_{16}H_{25}NO$, mixture of 2*E*,4*E*,8*Z*,10*Z* and 2*E*,4*E*,8*Z*,10*E* isomers) was purchased from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany). The internal standard benzanilide ($C_{13}H_{11}NO$) originated from Acros Organics (Leicestershire, UK). Docetaxel ($C_{43}H_{53}NO_{14}$) and dexamethasone ($C_{22}H_{29}FO_5$) were purchased from Sigma–Aldrich (St. Louis, MO, USA). LC–MS grade water, methanol and n-hexane of HPLC quality, acetonitrile of HPLC-S gradient grade quality and analytical grade diethyl ether stabilized with 2,6-di-tert-butyl-4-methylphenol (BHT) were obtained from Biosolve (Valkenswaard, The Netherlands). Formic acid was purchased from Merck (Darmstadt, Germany). Blank, drug-free human plasma, containing lithium-heparin as anticoagulant, was obtained from Sera Laboratories International Ltd. (Haywards Heath, UK). *E. purpurea* drops originated from A. Vogel (Echinaforce®, batch 08K0302, Biohorma BV, Elburg, The Netherlands) and were labeled to contain 95% aerial parts and 5% roots of *E. purpurea* (contents of DTAI and other alkylamides not specified).

2.2. Liquid chromatography

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). Sample injections (20 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. Mobile phase A consisted of water, containing 0.2% formic acid, and mobile phase B consisted of acetonitrile. The flow rate was 0.3 mL/min and the total run time was 7.5 min. During the first 5 min of the run, isocratic elution was applied with 50% B. From 5.01 until 5.5 min, the eluent composition was changed to 90% B. Hereafter, the isocratic system of 50% B was restored until the end of the run at 7.5 min.

2.3. Mass spectrometry

The Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) with electrospray ionization (ESI) operated in the positive ion mode with both quadrupoles set at 0.7 full width at half maximum (FWHM, unit resolution) and with dwell times of 200 ms. For DTAI, the mass transitions from *m*/*z* 248 to 167 were optimized and for benzanilide, responses from *m*/*z* 198 to 105 were monitored. The optimized collision energies were −17 V for DTAI and −20 V for benzanilide. Tube lens voltages were 124 V for DTAI and 101 V for benzanilide. Further, 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 argon collision gas pressure was set at 1.8 mTorr. For data acquisition and processing, Xcalibur software (version 1.4, Thermo Fisher Scientific) was used.

2.4. Preparation of stock and working solutions

Two stock solutions of DTAI (1.0 mg/mL) from two independent weightings were prepared in methanol. One stock solution was used for the preparation of calibration standards (CS), and the other solution was used to prepare quality control (QC) samples. For preparation of CS and QC working solutions, stock solutions were diluted with acetonitrile–water (50:50, v/v). For the internal standard, a stock solution of benzanilide in methanol (1 mg/mL) was diluted with acetonitrile–water (50:50, v/v) to obtain a final working solution of 100 ng/mL benzanilide.

All stock solutions were stored at −80 °C until use, while the working solutions were used immediately after preparation.

2.5. Preparation of CS and QC samples

CS samples were prepared freshly by spiking CS working solutions to human plasma. Two calibration curves were validated: one curve for low DTAI concentrations (0.01–0.05 ng/mL) and another curve for higher DTAI levels (0.05–50 ng/mL). The 'low DTAI' curve consisted of CS with concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 ng/mL, while the 'high DTAI' curve included concentrations of 0.05, 0.1, 1, 5, 25 and 50 ng/mL DTAI. For each validation run, these standards were prepared and analyzed in duplicate.

QC samples for the 'low DTAI' curve had concentrations of 0.02 (low), 0.03 (mid) and 0.04 (high) ng/mL DTAI. For the 'high DTAI' curve QC samples were prepared in concentrations of 0.05 (LLQ), 0.15 (low), 5 (mid), 40 (high) and 250 (>upper limit of quantification) ng/mL DTAI. The QC sample with 250 ng/mL DTAI was used for a dilution test above the upper limit of quantification (ULQ). QC samples, prepared in batches, were stored at −30 °C until analysis.

2.6. Sample preparation

To 300 μL plasma samples, 50 μL of the internal standard benzanilide (100 ng/mL) was added in a 2.0 mL polypropylene reaction tube. After vortex-mixing, LLE was performed with 1.6 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 13,500 rpm (4 °C), the samples were stored at −30 °C for 60 min. Next, the organic layer was decanted into another 2.0 mL polypropylene reaction tube and evaporated under a stream of nitrogen at 40 °C. The dry extract was reconstituted in 100 μL of acetonitrile–water (50:50, v/v). After vortex-mixing, the solution was centrifuged for 10 min at 13,500 rpm (4 °C). Finally, the clear supernatant was transferred into a 250 μL glass insert placed in an autosampler vial.

2.7. Validation procedures

A full validation of the assay in human heparinized plasma was performed according to the current FDA guidelines on Bioanalytical Method Validation [13]. Parameters that were validated were linearity, accuracy, precision, recovery, matrix effect, specificity, selectivity, and stability.

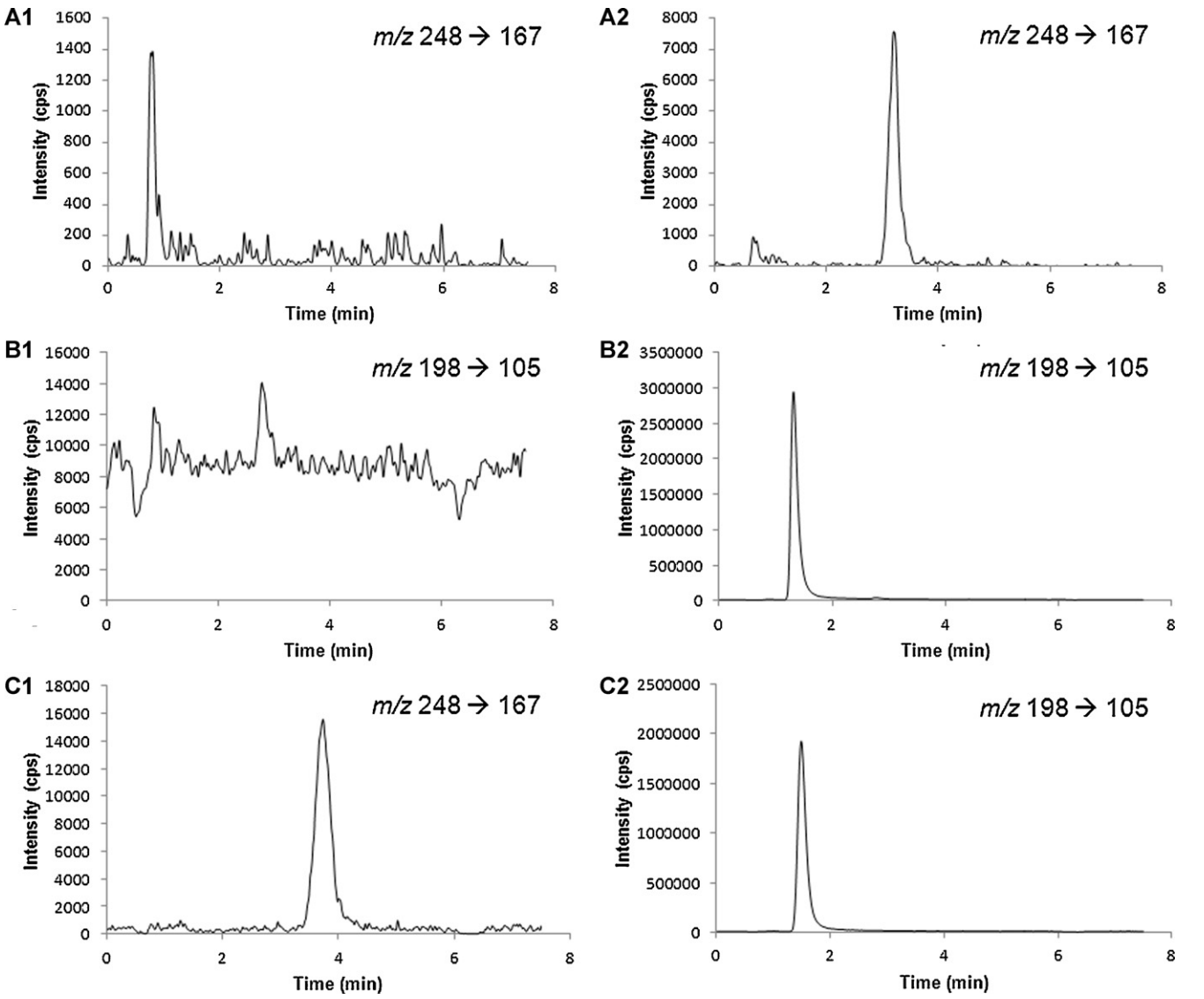

Fig. 1. Representative chromatograms of a blank human plasma sample (A1 and B1) and of a human plasma sample spiked at the level of 0.05 ng/mL DTAI (A2, DTAI, t_r = 3.2 min; B2, internal standard benzanilide, t_r = 1.3 min). Representative chromatograms of DTAI (C1) and the internal standard benzanilide (C2) in a patient sample obtained 30 min after intake of 20 oral drops of Echinaforce® extract.

2.8. Pharmacokinetic application

The applicability of the present assay was assessed in three cancer patients of the clinical interaction study with *E. purpurea* and docetaxel. The clinical study had been approved by the Medical Ethics Committee of the Antoni van Leeuwenhoek Hospital – The Netherlands Cancer Institute and all patients gave written informed consent. The cancer patients ingested 20 drops of a commercial extract of *E. purpurea* (A. Vogel Echinaforce®, batch 08K0302, Biohorma BV, Elburg, The Netherlands) three times daily for fourteen days. After the last ingestion in the morning of day 15, blood samples for pharmacokinetics of DTAI were drawn at t = 0, 30, 60 and 120 min.

3. Results and discussion

3.1. Mass spectrometry

The protonated molecule of DTAI at m/z 248 was used to generate a product ion spectrum (Supplemental Fig. 1A). For selected reaction monitoring (SRM), the most abundant product ion (m/z 167) was selected and the collision conditions were optimized. The conditions of m/z 198–105 were optimized for monitoring the internal standard benzanilide (Supplemental Fig. 1B). MS/MS product ion spectra and the proposed fragmentation patterns of DTAI and benzanilide are depicted in Supplemental Fig. 1A and B.

3.2. Chromatography

Chromatographic conditions were adopted from our previously validated LC–MS/MS assay for undeca-2-ene-8,10-diynoic acid isobutylamide (UDAI) [14]. At first, an isocratic elution (A:B (50:50, v/v)) at a flow rate of 0.3 mL/min was applied for 5 min. After observing an increasing noise in the MS/MS signal after multiple injections, a gradient was introduced after each injection by rapidly increasing % B from 50 to 90% within 30 s followed by stabilization at 50% B for 2 min. Consequently, the noise was significantly reduced.

In accordance with other bioanalytical assays for DTAI [7–9], the 2*E*,4*E*,8*Z*,10*Z* and 2*E*,4*E*,8*Z*,10*E* isomers were not separated in the present assay. Thus, peak areas of DTAI represent the sum of both isomers.

Representative chromatograms of a double blank sample, a human plasma sample spiked at 0.05 ng/mL DTAI and a patient plasma sample obtained 30 min after ingestion of Echinaforce® extract are given in Fig. 1.

3.3. Sample pre-treatment

LLE was performed with 1.6 mL diethyl ether/n-hexane (50:50, v/v). The composition of the organic phase was optimized in our previous UDAI assay [14], in which the highest overall recovery was found for diethyl ether/n-hexane (50:50, v/v). With a small plasma volume of 300 μL, high and reproducible overall recoveries (87.7%, range 80.4–95.5%) were found for DTAI after LLE with 1.6 mL of organic phase and reconstitution in 100 μL acetonitrile–water (50:50, v/v).

3.4. Validation

3.4.1. Linearity

For DTAI concentrations ranging from 0.01 to 0.05 ng/mL and from 0.05 to 50 ng/mL, linearity was assessed by preparation and analysis of duplicate CS samples in three separate runs. The assay was linear over both concentration ranges for DTAI in human plasma. Using least-squares linear regression (area ratio versus the concentration $1/X^2$), the lowest total bias and the most constant bias across the range were obtained. The average regression parameters of the linear regression functions ($n = 3$) for the low and high calibration range were $y = -0.0000905(\pm 0.0000198) + 0.0171(\pm 0.0040) \times x$ ($r^2 = 0.9781(\pm 0.007937)$) and $y = 0.000375(\pm 0.000469) + 0.0497(\pm 0.0174) \times x$ ($r^2 = 0.9887(\pm 0.0071784)$), respectively.

At all concentration levels, deviations of measured from nominal concentrations were between −14.9% and 14.7%. Thus, FDA acceptance criteria (±20% deviation from nominal concentration for LLQ and ±15% for other concentrations) were met.

3.4.2. Accuracy and precision

Accuracy and precision of the lower level calibration curve (0.01–0.05 ng/mL) were validated by analysis of QC samples with DTAI concentrations of 0.02, 0.03 and 0.04 ng/mL (five replicates per concentration level in three analytical runs).

For the higher level calibration curve (0.05–50 ng/mL), accuracy and precision of the assay were determined by analyzing five replicates of QC samples of DTAI spiked at 0.05, 0.15, 5 and 40 ng/mL DTAI in three analytical runs.

Assay performance data for DTAI in human plasma are summarized in Table 1. With a within-day inaccuracy ≤ ±14.0% and a between-day inaccuracy within ±8.40% for all concentrations, the required inaccuracy criterion of ±15% (±20% for LLQ) was met [13]. Within-day and between-day precisions, represented by the coefficient of variation (CV%), did not exceed the required criterion of ±15% (±20% for LLQ) [13].

Further, a QC sample >ULQ (250 ng/mL) can be diluted 10 and 100 times in human plasma with acceptable inaccuracy (≤15%) and precision (≤15%).

3.4.3. Recovery and matrix effect

Based on the mean peak areas of DTAI and benzanilide, the mean matrix effect was 101% (range 96.8–106.9%) for DTAI and 92.7% (range 85.5–99.2%) for benzanilide. Mean LLE recovery was 86.7% (range 83.1–89.3%) for DTAI and 84.0% (range 81.0–88.5%) for benzanilide, and mean total recovery was 87.7% (range 80.4–95.5%) and 77.7% (range 75.7–81.8%) for DTAI and benzanilide, respectively. These results were found to be reproducible and acceptable.

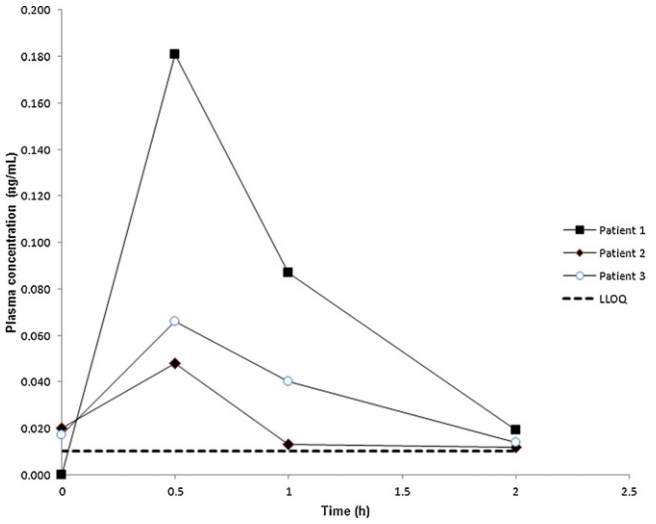

Fig. 2. Plasma concentration–time curves of DTAI in three patients after oral administration of 20 drops of Echinaforce® extract. The dotted line indicates the LLQ of 0.01 ng/mL.

3.4.4. Specificity and selectivity

Potential interference between the analyte and endogenous matrix components was investigated by analyzing a double blank and a sample with 0.05 ng/mL DTAI for six individual batches blank human plasma. The six double blank batches did not contain co-eluting peaks >20% of the analyte peak area at 0.05 ng/mL DTAI, or co-eluting peaks >5% of the area of the internal standard. In the six batches spiked at 0.05 ng/mL DTAI, deviations from the nominal concentrations were between −12.5 and −2.36% for DTAI and were approved.

To assess potential interference between the internal standard and analyte, blank samples spiked separately with analyte (at the ULQ) and internal standard were processed and analyzed. Eventually, no cross-analyte/internal standard interference was observed, as no co-eluting peaks of DTAI and benzanilide were detected.

Assessment of potential interference of co-medication (docetaxel and dexamethasone) revealed no co-eluting peaks >20% of the DTAI peak area at 0.05 ng/mL or >5% of the benzanilide peak area in double-blank plasma samples containing only docetaxel (5000 ng/mL) or dexamethasone (200 ng/mL). Furthermore, the accuracy of plasma samples containing 0.05 ng/mL DTAI were also within accepted ranges in the presence of docetaxel or dexamethasone. Thus, no interference of co-medication was observed.

3.4.5. Stability

The stability data for DTAI are summarized in Table 2. Stability data regarding the internal standard benzanilide have been described previously by our group [14].

Table 2 shows that a stock solution of DTAI was stable for at least 18 h at ambient temperatures and after storage for 3 months at −80 °C.

Stability of DTAI in human plasma has been demonstrated at ambient temperatures for at least 18 h, at −30 °C for up to 3 months and for at least three freeze (−30 °C)/thaw cycles.

Further, stability of DTAI in the final extract and reinjection reproducibility was confirmed after storage for 24 h at 15 °C.

3.5. Application of the method

The plasma concentration–time curves of three patients are shown in Fig. 2. DTAI could be quantified in all plasma samples and therefore, it is expected that the described assay can be successfully applied in support of the intended clinical interaction

Table 1
Assay performance data for DTAI ($n = 15$ per concentration level).

Run	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Inaccuracy (%)	Precision (%)	No. of replicates
1	0.0200	0.0197	−1.66	15.0	5
2	0.0200	0.0180	−10.1	14.6	5
3	0.0200	0.0199	−0.407	13.6	5
Between-day	0.0200	0.0192	−4.05	13.6	15
1	0.0300	0.0301	0.237	9.95	5
2	0.0300	0.0323	7.64	14.0	5
3	0.0300	0.0289	−3.69	8.59	5
Between-day	0.0300	0.0304	1.40	11.1	15
1	0.0400	0.0453	13.4	6.41	5
2	0.0400	0.0410	2.50	7.33	5
3	0.0400	0.0411	2.67	12.1	5
Between-day	0.0400	0.0425	6.17	9.23	15
1	0.0500	0.0570	14.0	3.28	5
2	0.0500	0.0566	13.2	6.20	5
3	0.0500	0.0490	−2.00	5.20	5
Between-day	0.0500	0.0542	8.40	8.15	15
1	0.150	0.150	0.133	9.30	5
2	0.150	0.141	−5.87	1.69	5
3	0.150	0.143	−4.93	3.89	5
Between-day	0.150	0.145	−3.56	6.08	15
1	5.00	4.48	−10.4	6.66	5
2	5.00	5.30	5.92	4.51	5
3	5.00	5.00	−0.116	2.09	5
Between-day	5.00	4.92	−1.55	8.02	15
1	40.0	35.7	−10.7	6.61	5
2	40.0	40.6	1.54	11.7	5
3	40.0	37.5	−6.34	2.80	5
Between-day	40.0	37.9	−5.18	9.09	15

Table 2
Stability data for DTAI.

Conditions	Matrix	Initial conc. (ng/mL)	Mean measured conc. (ng/mL)	Dev (%)	CV (%)
Ambient, 18 h	Methanol	1.00×10^6	9.66×10^5	−3.38	3.69
−80 °C, 3 months	Methanol	1.00×10^6	1.02×10^6	2.02	9.29
Ambient, 18 h	Plasma	0.150	0.150	−0.113	0.408
		40.0	38.4	−4.08	1.97
−30 °C, 3 months	Plasma	0.150	0.158	5.41	2.80
		40.0	39.3	−1.64	3.41
3 freeze (−30 °C)/thaw cycles	Plasma	0.150	0.153	1.89	8.40
		40.0	41.4	3.40	4.25
Processed sample stability (15 °C, 24 h)	Final extract	0.150	0.142	−5.52	6.99
		40.0	38.4	−3.93	3.17
Reinjection reproducibility (15 °C, 24 h)	Final extract	0.150	0.149	−0.959	6.61
		5.00	4.95	−1.11	1.61
		40.0	38.1	−4.65	3.17

study. For all three patients the plasma concentration–time curves showed a similar time course with a maximum plasma concentration of DTAI achieved at 1 h after ingestion. The measured plasma concentrations were all in the calibration range (0.01–50 ng/mL), with 0.181 ng/mL as the maximum and 0.012 ng/mL as the lowest obtained DTAI plasma concentration.

4. Conclusions

In this paper, the development, validation and application of a LC–MS/MS assay for the quantification of DTAI in human plasma have been described. Compared to previously published bioanalytical assays for DTAI in human matrix [6–9], this assay requires the lowest sample volume (300 μL plasma) and has the shortest run time (7.5 min). Furthermore, with an LLQ of 0.01 ng/mL this is the most sensitive assay for DTAI that has been validated according to the FDA guidelines on Bioanalytical Method Validation [13]. Finally, the applicability of the present assay has been demonstrated in three patients for the intended clinical study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.06.022.

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