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Development and validation of a sensitive assay for the quantification of arachidonoylcyclopropylamide (ACPA) in cell culture by LC–MS/MS

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Abstract

Synthetic and natural cannabinoid derivatives are highly investigated as drug candidates due to their antinociceptive, antiepileptic and anticancer potential. Arachidonoylcyclopropylamide (ACPA) is a synthetic cannabinoid with antiproliferative and apoptotic effects on non-small cell lung cancer and pancreatic and endometrial carcinoma. Thus, ACPA has a great potential for being used as an anticancer drug for epithelial cancers. Therefore, determining the levels of ACPA in biological fluids, cells, tissues and pharmaceutical dosage forms is crucial in monitoring the effects of various pharmacological, physiological and pathological stimuli on biological systems. However, the challenge in the quantification of ACPA is its short half-life and lack of UV signal. Therefore, we developed a liquid chromatography-tandem mass spectrometric (LC–MS/MS) method for sensitive and selective quantification of ACPA in cell culture medium and intracellular matrix. Multiple reaction monitoring in the positive ionization mode was used for detection with 344 → 203 m/z transitions. The separation of ACPA was performed on C18 column (50 × 3.0 mm, 2.1 µm) with the mobile phase run in the gradient mode with 0.1% formic acid (FA) in water and 0.1% FA in acetonitrile at a flow rate of 0.3 ml/min. The assay was linear in the concentration range of 1.8–1000 ng/mL ($r = 0.999$). The validation studies revealed that the method was linear, sensitive, accurate, precise, selective, repeatable, robust and rugged. Finally, the developed method was applied to quantify ACPA in cell culture medium and intracellular matrix.

Keywords ACPA, Cannabinoids, LC–MS/MS, Cell culture, Validation

Introduction

Cannabis sativa L. predominantly produces more than 100 psychoactive metabolites called cannabinoids (Chakravarti et al. 2014; Abrams and Guzman 2015) which are hydrophobic and terpenophenolic molecules (Chakravarti et al. 2014; Sledzinski et al. 2018; Klumpers and Thacker 2018). Major endocannabinoids which are known as anandamide and 2-arachidonoylglycerol exert physiological effects of cannabinoids (Velasco et al. 2016). Endocannabinoid system components are known to have wide expression patterns in human organ systems such as central nervous system (Pisanti et al. 2013; Howlett and Abood 2017), immune system (Jean-Gilles et al. 2015), genital system (Bilgic et al. 2017), digestive system

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(DiPatrizio 2021) and respiratory system (Boyacıoğlu et al. 2021; Ramer et al. 2014; Gkoumassi et al. 2007) by regulating mood, motor activity and appetite, immune modulation, fertility, food intake and airway functions, respectively. Recently, synthetic and natural cannabinoid derivatives are highly investigated as drug candidates due to their antinociceptive (Brunetti et al. 2020; Good et al. 2019; VanDolah et al. 2019), antiepileptic (Brunetti et al. 2020; VanDolah et al. 2019; Billakota et al. 2019) and anticancer potential (Boyacıoğlu et al. 2021; Milian et al. 2020; Preet et al. 2011; Donadelli et al. 2011; Dando et al. 2013; Brandi et al. 2013). Both endogenous and exogenous cannabinoids prevent proliferation and induce apoptosis in various types of epithelial cancers (Boyacıoğlu et al. 2021; Donadelli et al. 2011; Dando et al. 2013; Brandi et al. 2013; Roberto et al. 2019) that make them chemotherapeutic candidates for epithelial cancers such as lung cancer (Boyacıoğlu et al. 2021; Milian et al. 2020; Preet et al. 2011; Haustein et al. 2014; Ramer et al. 2012; Winkler et al. 2016). Both endogenous and exogenous cannabinoids induce their anticancer effects through G-protein-coupled CB receptors 1 (CB1) and 2 (CB2) (Chakravarti et al. 2014; Jaarsveld et al. 2016). Our group previously published the dose- and time-dependent antiproliferative and apoptotic effect of a synthetic specific CB1 receptor agonist ACPA (N-(Cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; $C_{23}H_{37}NO$; MW:343.555 g/mole) and ACPA-loaded polycaprolactone (PCL) on non-small cell lung cancer (NSCLC) cells with its downstream cascade (Patent pending for Turkish Patent and Trademark Office application no: TR2019/12451 and Patent Cooperation Treaty application no: PCT/TR2020/050618) (Boyacıoğlu et al. 2021). ACPA is also a drug candidate for other epithelial cancers including pancreatic (Donadelli et al. 2011; Dando et al. 2013; Brandi et al. 2013) and endometrial carcinoma (Bilgic et al. 2017). Therefore, determining the levels of synthetic cannabinoids such as ACPA in biological fluids, cells and tissues is crucial in monitoring the effects of various pharmacological, physiological and pathological stimuli on biological systems (Zou and Kumar 2018). However, it is difficult to quantify them accurately due to their short half-lives (Abrams and Guzman 2015).

Several chromatographic methods are available for the analysis of endocannabinoids in a wide variety of biological matrices such as cell culture (Ottria et al. 2014; Gouveia-Figueira and Nording 2014; Ivanov et al. 2015; Bobrich et al. 2020), blood (Lin et al. 2012; Bilgin et al. 2015), plasma (Ottria et al. 2014; Gouveia-Figueira and Nording 2014, 2015; Zoerner et al. 2012; Balvers et al. 2013; Sergi et al. 2013; Thieme et al. 2014; Gachet et al. 2015; Marchioni et al. 2017; Ozdurak et al. 2010), serum (Lam et al. 2010; Kirkwood et al. 2016), urine (Ottria et al. 2014; Lam et al. 2010), milk (Gouveia-Figueira and Nording 2014, 2015; Lam et al. 2010), cerebrospinal fluid (Leweke et al. 2007; Kantae et al. 2017), tissue (Bobrich et al. 2020; Lin et al. 2012; Marczylo et al. 2010; Gong et al. 2015; Lehtonen et al. 2011; Muguruza et al. 2013; Han et al. 2013; Liput et al. 2014; Bystrowska et al. 2014; Qi et al. 2015) and hair (Mwanza et al. 2016) samples. For this purpose, liquid chromatography (LC) techniques combined with ultraviolet, fluorescence, mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are used. The MS/MS detection option has a great advantage in identifying endocannabinoids precisely (Zoerner et al. 2012; Keereetaweep and Chapman 2016). Gas chromatography-based methods were also used for endocannabinoids analysis after derivatization in order to improve analysis sensitivity and thermal stability (Zoerner et al. 2012; Qi et al. 2015; Keereetaweep and Chapman 2016). Recently, the LC–MS/MS method has become a reference technique in the quantitation of endocannabinoids as it allows analysis with high sensitivity and selectivity without the need for derivatization (Marchioni et al. 1044). However, there is no method reported for the quantification of ACPA.

In this study, we aimed to develop a rapid, selective, accurate, precise and robust LC–MS/MS method for the determination of ACPA in cell culture samples since ACPA quantitation in various matrices is important for NSCLC research. ACPA lacks a UV signal and a very low inhibitory concentration is used for cancer treatment, and thus, MS is required for its detection. The developed method was fully validated according to ICH bioanalytical method guideline (Bioanalytical Method Validation M10 2019). The applicability of the developed and validated LC–MS/MS method has been proven by determining ACPA in cell culture samples including medium and intracellular matrix.

Experimental

Chemicals and reagents

The analytical standard of ACPA (cat. no1318) and arachidonoyl ethanolamide (cat. no1339, internal standard, IS) was obtained from Tocris Bioscience (Bristol, UK). LC–MS grade acetonitrile, water and formic acid (FA) were purchased from Sigma-Aldrich (Darmstadt, Germany).

LC–MS/MS instrumentation and conditions

The LC–MS/MS system consisted of a LC system (Shimadzu LC-20AXR) coupled with a triple quadrupole tandem mass spectrometer (Shimadzu 8030, Japan). The chromatographic separations were carried out using a C18 column (GL Sciences, 50 × 3.0 mm, 2.1 µm) at 40 °C via gradient elution of 0.1% FA in water (phase A)

and 0.1% FA in acetonitrile (phase B) at a flow rate of 0.3 ml/min. The binary solvent gradient was as follows: 0.0–0.01 min at 40% B, 0.01–4.0 min from 40 to 80% B, 4.0–7.0 min 80% B, followed by 3 min of equilibration at initial conditions (total run time 10 min). The injection volume was set at 10 µl.

Quantification was conducted with the multiple reaction monitoring (MRM) mode in positive ionization.

Optimum source-dependent parameters were 4.5 kV of interface voltage, -14 V of collision energy, 250 °C desolvation line temperature and 400 °C of heat block temperature. The gas flow rate of the nebulizer and drying was 3 l/min and 15 l/min, respectively. For MRM detection, precursor and the product ions, quadrupole voltages and collision energies were optimized for ACPA and IS in direct infusion mode. The transitions were 344 → 203 m/z for quantification of ACPA, 344 → 287 m/z for qualification of ACPA and 166 → 120 m/z for quantification of IS. The dwell time was 200 ms for both transitions. Data acquisition and processing were done with the LabSolutions software (version 5.72, Shimadzu).

Preparation of calibration standards and internal standard solutions

Stock solutions of ACPA and IS were prepared in acetonitrile at the concentration of 1000 ng/ml. Calibration solutions containing ACPA at different concentrations were prepared by serial dilution of stock standard solution with mobile phase A and B mixture (1:1, v/v). Calibration curve standards were daily prepared at ten different concentrations (1.8, 3.75, 7.50, 15, 31.25, 62.5, 125, 250, 500 and 1000 ng/ml) of ACPA, including 1000 ng/ml of IS by diluting of appropriate volume of stock solutions ACPA and IS.

Cell culture studies and ACPA analysis

L929 mouse fibroblast cell line (CCL-1™, ATCC) as a well-defined and suggested cell line for use per ISO Standard 10,993–5 was used for the current study (Wang et al. 2013; Jablonská et al. 2021). Cells were cultured in DMEM HG (Dulbecco's Modified Eagle Medium w/High Glucose) supplemented with 10% fetal bovine serum (Biological Industries) and 1% penicillin/streptomycin (Biological Industries) and cultured in 37 °C incubator with humidified atmosphere of 5% CO_2. Cells were tested in case of any mycoplasma contamination before the experiments. The fibroblasts were incubated with ACPA (0.1 mM) and normal medium for 24 h ($n=3$). Cell culture media were collected at the end of the incubation period and stored to determine ACPA amount in the media. Then, the cells were quickly washed twice with PBS and extracted using methanol/water mixture (9:1, v/v) to determine ACPA amount in the intracellular matrix. After precipitation of the proteins with centrifugation at 14,000 g for 10 min at 4 °C, amount of ACPA in supernatants of each sample was determined with the developed method.

Method validation

The LC–MS/MS method was validated for selectivity, linearity, sensitivity, matrix effect, carry over, precision, accuracy, robustness and ruggedness following ICH bioanalytical method guideline (Bioanalytical Method Validation M10. Committee for Medicinal Products for Human Use, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use 2019).

The selectivity of the LC–MS/MS method was investigated by comparing blank chromatograms obtained for cell culture medium, intracellular matrix and placebo with ACPA spiked chromatograms of them at limit of quantitation (LOQ) concentration (1.8 ng/ml).

The linearity studies were carried out by analyzing calibration solutions containing different concentrations of ACPA (1.8, 3.75, 7.50, 15, 31.25, 62.5, 125, 250, 500 and 1000 ng/ml) and constant concentration of IS (1000 ng/ml) using the proposed LC–MS/MS method. The calibration plots were created by plotting the peak area ratio of ACPA to IS against the concentrations with least-squares linear regression analysis.

The sensitivity of the developed method was evaluated with limit of detection (LOD) and LOQ values based on signal-to-noise ratio at 3:1 and 10:1, respectively.

The matrix effect on the ionization of analytes can be expressed as the ratio of peak area values obtained in the presence and absence of the sample matrix. ACPA (1.8–1000 ng/ml) and IS (1000 ng/ml) were spiked into both blank solution and water to evaluate the matrix effect. The samples were analyzed under the optimum analytical conditions. The matrix effect was determined by comparing the results between matrix spiked samples and water spiked standards.

The carryover was determined by injection of blank solutions immediately after the highest calibration standard (1000 ng/ml).

To evaluate dilution integrity, the stock solution of ACPA was prepared three times of the upper limit of quantification (3000 ng/ml) and then diluted 1:5 and 1:10 with the mobile phase. Each of the diluted samples ($n=6$) was analyzed by the LC–MS/MS method and compared to the nominal concentrations.

The intra-day and inter-day precision and accuracy of the method were estimated by analyzing three replicates containing ACPA (and IS) at four different concentration levels (1.8, 31.25, 250 and 500 ng/ml) on the same day and on three consecutive days, respectively. The coefficient of

variation (CV, %) for precision and bias for accuracy was calculated for each concentration level.

For the reinjection reproducibility study, QC samples at four different concentration levels (LOQ, low, medium and high OCs) were prepared and six replicate analyses for each level were performed while keeping the standards in the autosampler. The analysis was completed in 18 h. The accuracy and precision for each level were evaluated for reinjection reproducibility.

The robustness of analytical methods is checked by examining the effect of small deliberate changes in experimental conditions on the analysis results. An experimental design was applied with the simultaneous change of factors to determine the robustness of the LC–MS/MS method. Small changes were made in the percentage of FA in mobile phase A (0.09–0.11%), percentage of FA in mobile phase B (0.09–0.11%), acetonitrile percentage of the mobile phase (39–41%), column temperature (39–41 °C) and flow rate (0.295–0.305 ml/min). A thirteen-run fractional factor design with three experiments under optimized conditions was applied to determine whether a small deviation in experimental conditions is significantly affecting.

The ruggedness of the developed method was achieved by analyzing the ACPA standard solution (1000 ng/ml) by two different analysts (Analyst 1 and Analyst 2) under optimum conditions.

The stability of the developed method was investigated using samples (1000 ng/ml of ACPA) stored in refrigerator (5 ± 3 °C), freezer (-20 °C) and autosamplers. The stability was determined by comparing freshly prepared ACPA standard solutions.

Statistical analysis

The statistical calculations were carried out using Microsoft Excel software. *P* values of the regression coefficient and regression equation were calculated by ANOVA test. The robustness of analytical methods was evaluated by one-way analysis of variance (ANOVA) test. For ruggedness, Student's *t* test was used for the comparison of two different analysts.

Results and discussion

Method optimization

An IS usage in LC–MS/MS based bioanalytical methods is mandatory for repeatable results due to unexpected changes especially in ionization steps. The IS selected for analysis should have a similar chemical structure to the analyte to eliminate errors that may happen during sample preparation or analysis. Therefore, arachidonoyl ethanolamide, as an endocannabinoid, was selected as IS.

The optimization of the method was systematically studied for MS and LC conditions. Firstly, MS detection parameters were optimized by direct injection of ACPA and IS individually at a concentration of 1000 ng/ml to obtain the highest peak intensity. The MRM was operated in positive ionization mode, and the transitions of ACPA and IS were determined as m/z $344 \rightarrow 203$ and m/z $166 \rightarrow 120$, respectively (Fig. 1).

After the optimization of MS conditions, chromatographic conditions were optimized to achieve the best possible analyte separation within the shortest time. Analytical column, mobile phase composition and flow rate have been optimized to achieve precise and reproducible results. Different sizes of C18 columns and various gradients of mobile phase compositions were studied, and a C18 column (GL Sciences, 50×3.0 mm,

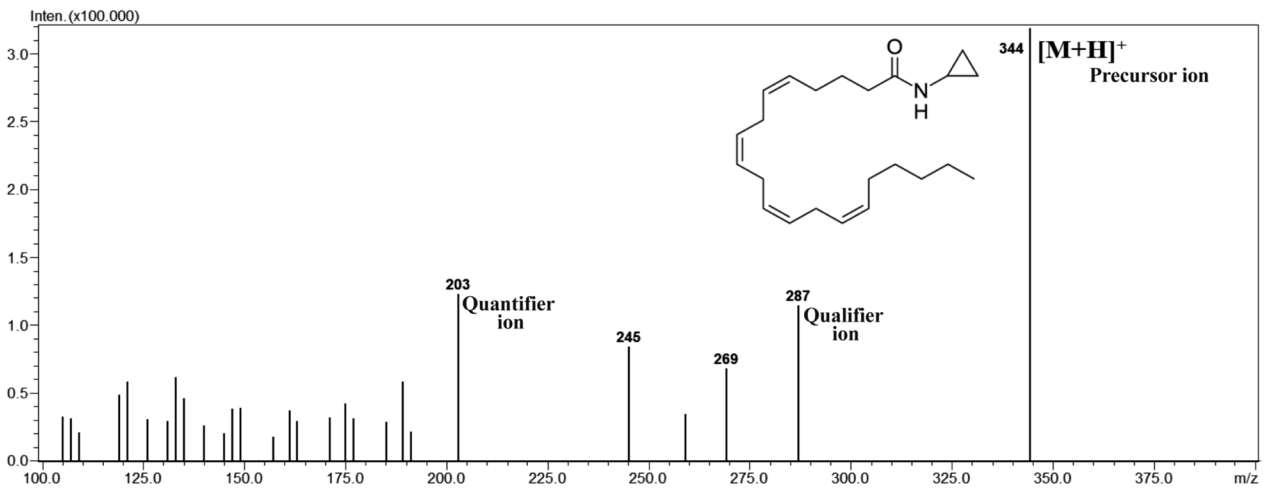

Fig. 1 MS/MS fragmentation pattern of ACPA (Quantifier ion: 203 m/z, qualifier ion: 287 m/z, CE: − 14 eV)

2.1 µm) was chosen as stationary phase because of symmetric peaks and short retention time. The best ionization was achieved when 0.1% FA in water and 0.1% FA in acetonitrile were used as the mobile phase. The gradient elution was applied to the mobile phase as follows: 0.0–0.01 min at 40% B, 0.01–4.0 min from 40 to 80% B, 4.0–7.0 min 80% B, followed by 3 min of equilibration at initial conditions.

The total run time was 10 min, and the retention time of ACPA and IS was 6.4 min and 5.1 min, respectively (Fig. 2).

The suitability of the LC–MS/MS method under optimum analysis conditions was evaluated in terms of asymmetry (10%), column efficiency (theoretical plate number, N), capacity factor (k') and tailing factor parameters. The values obtained (CV = 0.20%, N = 319,230, k' = 6.77 and tailing factor = 1.25) are within the specified limits (Bioanalytical Method Validation M10 2019), and it has been determined that the system is suitable for the analysis of ACPA.

Method validation

The LC–MS/MS method was validated for selectivity, linearity, sensitivity, matrix effect, carry over, precision, accuracy, robustness and ruggedness following ICH bioanalytical method guideline (Bioanalytical Method Validation M10 2019).

The selectivity of the LC–MS/MS method was investigated by comparing blank chromatograms of the matrix and ACPA spiked chromatograms (Fig. 3). There is no significant interference higher than 20% of the peak area of ACPA at LOQ level and 5% of the peak area of IS. This indicates that the developed method is selective for the analysis of ACPA from cell culture and placebo samples.

The linearity of the calibration curves was determined over the concentration range of 1.8–1000 ng/ml with a correlation coefficient value which is 0.999 ± 0.0002. The values (mean ± SE; n = 6) of the slope and intercept were 0.0021 ± 0.0001 and 0.0102 ± 0.0029, respectively. The LC–MS/MS method showed an acceptable linearity range from 1.8 to 1000 ng/ml for ACPA. The LOD was 0.6 ng/ml, and the LOQ calculated in this work was 1.8 ng/ml with acceptable accuracy and precision (Table 1). The developed method was highly sensitive for estimating ACPA in the cell culture.

The matrix effect was evaluated based on ACPA analysis in the presence and absence of the sample matrix. The samples were prepared triplicate and analyzed under the optimum analytical condition. The matrix effect was calculated as 98.60% ± 1.67 for ACPA and 100.49% ± 1.60 for IS. These results showed that the developed method was not affected by the sample matrix. The carryover was not observed in any of the blank matrix samples (≤ 20% of the analyte response at the LOQ and ≤ 5% of the mean IS response of the accepted calibration standards) after the highest calibration standard injection (1000 ng/ml).

Dilution integrity was tested for fivefold and tenfold dilution, and the low bias (≤ 1.70%) and CV (≤ 1.40%)

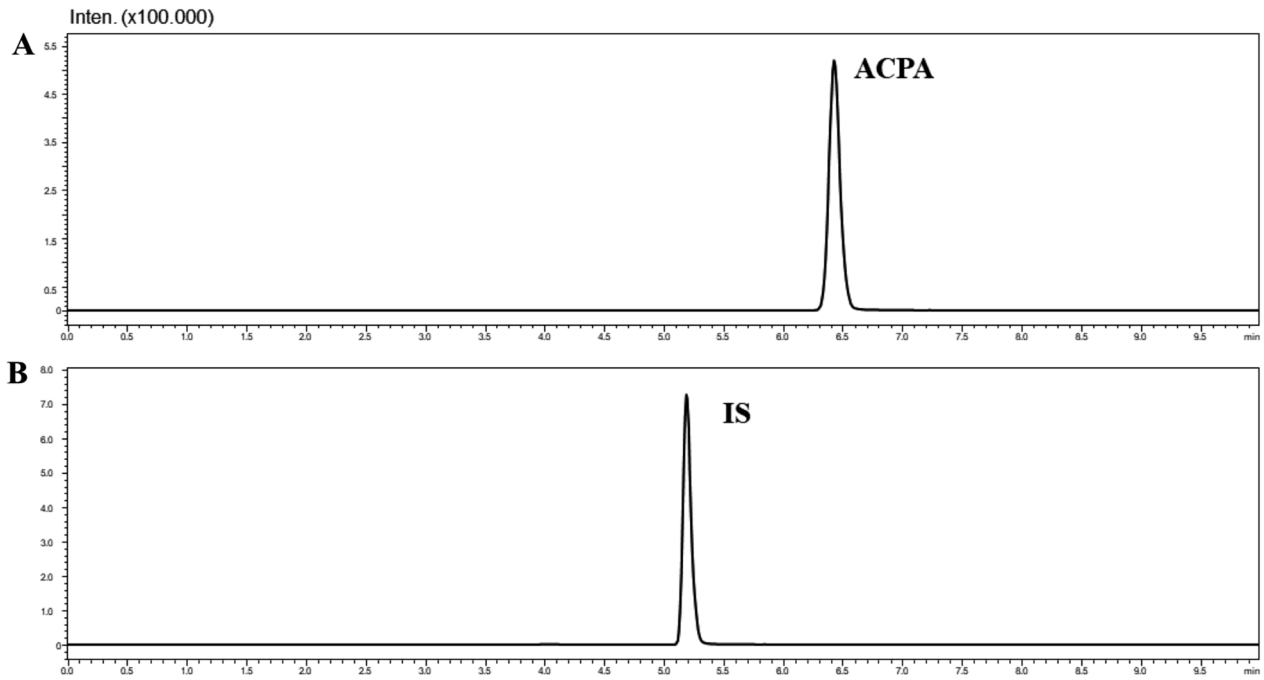

Fig. 2 Representative chromatograms of **A** ACPA (1000 ng/mL) and **B** IS (1000 ng/mL) at optimum chromatographic conditions

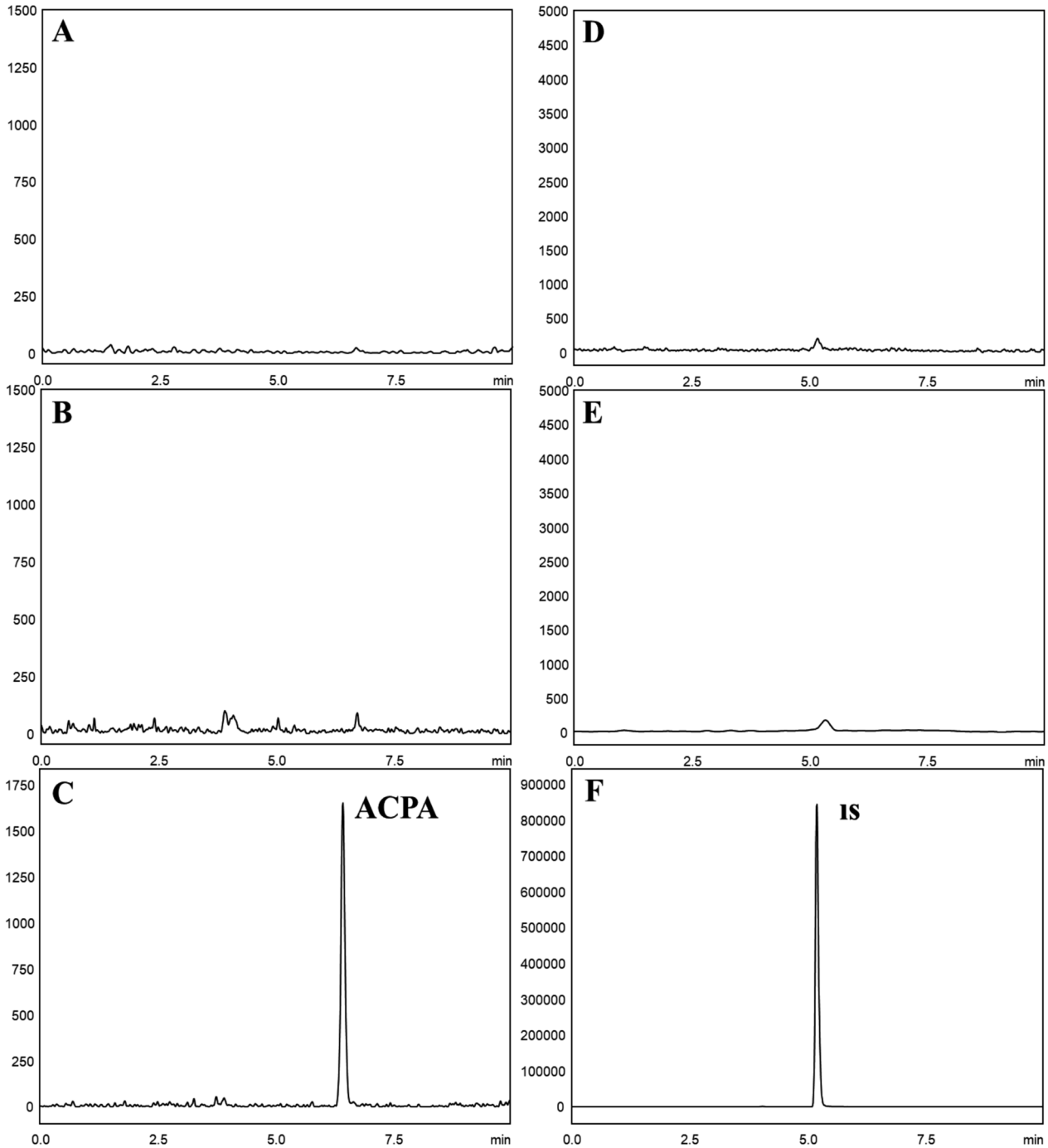

Fig. 3 Chromatograms obtained under optimum chromatographic conditions: **A** blank for ACPA (in cell mixture), **B** blank for ACPA (in cell supernatant mixture), **C** ACPA spiked matrix at LOQ concentration, **D** blank for IS (in cell mixture), **E** blank for IS (in cell supernatant mixture), **F** IS spiked matrix (1000 ng/mL)

values were that the method has the ability to accurately quantify samples containing high concentrations of analytes diluted 1:10 within acceptance criteria ($\leq$ 15.0%) (Table 1).

Intra-day and inter-day precision and accuracy and reinjection reproducibility were estimated by analyzing three or six replicates at four different concentration levels (Table 1). The low CV and bias values

Table 1 Intra- and inter-day accuracy, precision and reinjection reproducibility of the method

Concentration (ng/mL)	Intra-day ($n=3$)		Inter-day ($n=3$)		Reinjection reproducibility ($n=6$)	
	Accuracy (Bias, %)	Precision (CV[a], %)	Accuracy (Bias, %)	Precision (CV[a], %)	Accuracy (Bias, %)	Precision (CV[a], %)
1.80	1.48	1.07	−1.25	3.12	1.73	0.92
31.25	−0.23	1.97	−0.17	1.07	1.67	1.79
250.00	1.49	1.73	−0.37	0.09	1.66	1.37
500.00	−0.23	0.77	0.42	0.79	3.36	0.14

[a] *CV* Coefficient of variation

Table 2 The experimental design and results for robustness study

Exp. No	FA % in MP(A)	FA % in MP(B)	Organic phase %	Temp. (°C)	Flow rate (ml/min)	Peak ratio ($n=3$)
1	−1	1	1	−1	1	2.15 ± 0.01
2	−1	−1	1	1	1	1.92 ± 0.03
3	1	−1	1	1	−1	2.39 ± 0.01
4	1	1	−1	1	−1	2.15 ± 0.01
5	1	−1	−1	−1	1	2.51 ± 0.01
6	−1	1	1	1	−1	2.14 ± 0.01
7	−1	−1	−1	−1	−1	2.14 ± 0.02
8	1	1	−1	1	1	2.29 ± 0.01
9	−1	1	−1	−1	−1	2.13 ± 0.01
10	1	−1	−1	1	1	2.53 ± 0.01
11	1	−1	1	−1	−1	2.48 ± 0.01
12	1	1	1	−1	1	2.37 ± 0.02
13	0	0	0	0	0	1.84 ± 0.01
p values	0.344	0.538	0.985	0.346	0.958	

showed that the method was precise and accurate. In addition, the accuracy of the developed method was also examined with recovery studies from placebo, cell culture medium and intracellular matrix. To evaluate the recovery, cell culture matrixes were spiked with ACPA at different concentrations and compared with the same amount of spiked water samples. The recoveries of ACPA were found as between 95.70 and 97.25%. The result indicates the method has high accuracy and recovery.

The robustness of analytical methods was evaluated with a thirteen-run fractional factor design with three experiments under optimized conditions. The results of the analysis were statistically compared with ANOVA test, and p values of the regression coefficient were calculated (Table 2). The p values of each variable were higher than 0.05 indicating that small changes do not have a statistically significant effect on the peak area ratio and robustness of the method.

The ruggedness of the method was evaluated by comparing two different analyst's results. It was found that there was no statistically significant difference ($p>0.05$) between them (Table 3). Thus, the method was found rugged.

Table 3 Ruggedness data of the developed method

	Retention time*	Found*
Analyst 1	6.413 ± 0.01	0.99 ± 1.49
Analyst 2	6.420 ± 0.01	0.91 ± 0.95

*Mean ± standard error ($n=3$), *P* value: 0.41 > 0.05

The stability of the developed method was found at least 3 days in the refrigerator, two months in the freezer and 8 h in the autosampler.

Cell culture studies

L929 mouse fibroblast cells (CCL-1™, ATCC) as a well-defined and suggested cell line for use per ISO Standard 10,993–5 were spiked with 0.1 mM ACPA solution. The intracellular matrix and cell culture medium were

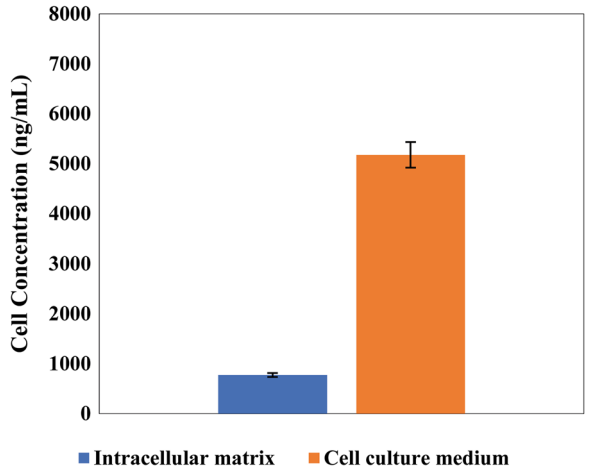

Fig. 4 The ACPA concentrations in cell culture medium and intracellular matrix of L929 mouse fibroblast cells line samples ($n = 3$)

analyzed with the developed method, and ACPA amounts in the intracellular matrix and cell culture medium were found 773 ± 46.17 and 5175 ± 906.85 ng/ml, respectively (Fig. 4). The amount of ACPA in the intracellular matrix was found 15% of cell culture medium after 24 h incubation.

Conclusion

The LC–MS/MS method developed is simple, fast, sensitive and reliable for the quantification of ACPA. The method was validated according to the ICH bioanalytical method guideline and was found selective, linear, sensitive, precise, accurate, robust and rugged. The developed and validated method was successfully applied for the quantification of ACPA in the cell culture medium and intracellular matrix. As a result, it can be recommended to use the LC–MS/MS method for monitoring of ACPA in drug development studies and pharmacokinetic studies to be performed in different biological matrices.

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Author contributions
ÖB contributed to investigation, methodology, formal analysis and writing—original draft. TR was involved in methodology, formal analysis and writing—original draft. SK contributed to investigation, conceptualization, methodology and writing—review and editing. PK was involved in investigation, conceptualization, methodology, writing—review and editing, and supervision. EN contributed to investigation, conceptualization, methodology, writing—review and editing, and supervision. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this article.

Declarations

Competing interests
The authors state no conflict of interest.

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