Original Research Paper

Acta Chromatographica 29(2017)2, 235–252
DOI: 10.1556/1326.2017.29.2.7

Highly Sensitive UHPLC–MS/MS Method for Quantification of Ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl) Propanoic Acid Derivatives in Mouse Serum

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Summary. A series of new (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate esters has shown cytotoxic activity towards human leukemic cell lines. The aim of this study was to develop and validate a bioanalytical method for quantification of (*S*,*S*)-*O*,*O*-diethyl-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate dihydrochlorides (DE-EDCP) and its metabolite, substituted propanoic acid (EDCP), in mouse serum by ultra high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). Structural analog, derivative of 1,3-propanediamine, was used as an internal standard (IS). Sample preparation employed protein precipitation by acetonitrile and subsequent centrifugation. Optimal UHPLC separation conditions were set to achieve simultaneous determination of both compounds in a short run time of 6 min. Additionally, the selected reaction monitoring (SRM) mode developed in this method allowed a highly sensitive, accurate, and precise identification of compounds of interest. The lower limit of quantitation (LOQ) was 1.3 ng mL^{-1} for DE-EDCP and 0.3 μg mL^{-1} for EDCP. The calibration curves were linear over the concentration range of 1.3–26.7 ng mL^{-1} and 0.3–6.7 μg mL^{-1} for DE-EDCP and EDCP, respectively. Precision (%CV) and accuracy (%RE) for DE-EDCP and EDCP ranged from 3.5% to 16.0% and from 1.8% to 14.4%, respectively.

The validation process was performed in accordance with the regulatory guidance/guideline, and all of the obtained results met the established acceptance criteria. The newly developed and validated UHPLC–MS/MS method is rapid, sensitive, and selective, and it can be successfully applied to drug monitoring in nonclinical studies.

Key Words: UHPLC–MS/MS, protein precipitation sample preparation, (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl) propanoic acid esters, cytotoxic activity, metabolite

First published online: June 22, 2016 *ISSN 2083-5736 © The Author(s)*

Introduction

Novel ester derivatives of (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl) propanoic acid are toxic to leukemic cell lines [1]. It was demonstrated that methyl, ethyl, and *n*-propyl esters were toxic to HL-60, REH, MOLT-4, KG-1, JVM-2, and K-562 leucemic cell lines, while the nonesterified compound and *n*-butyl ester were devoid of cytotoxic action. The ethyl ester exhibited the highest cytotoxic activity (IC_{50} in the range of 11–45 μM) [1].

Platinum chemotherapeutic agents have been widely used in cancer treatment. Cisplatin was the first of the platinum-based chemotherapeutic agents and, therefore, has been extensively studied as an antitumor agent since the late 1960s [1]. It has revolutionized the treatment of various solid organ tumors [2], resulting in its widespread use among antineoplastic drugs [3].

It has been recently reported that octahedral platinum(IV) complexes with cyclohexyl-functionalized ethylenediamine-*N*,*N*′-diacetate-type ligands affected various cancer cell lines with higher efficiency than the prototypical platinum-based antineoplastic drug cisplatin [1, 4].

The high efficiency and unusual mechanism of antineoplastic action of Pt(IV) complexes could be at least partly ascribed to their organic ligands designed to incorporate the ethylenediamine group, which is known for its positive contribution to the cytotoxicity of various compounds [1, 4]. Indeed, the organic ligands alone exerted significant in vitro toxicity towards glioma, melanoma, and fibrosarcoma cell lines [1, 5].

Although no direct correlation was observed between the cytotoxic potency and the alkyl side-chain length of the compounds, the increase in alkyl side-chain length was apparently associated with the loss of activity of *n*-butyl ester [1].

(*S*,*S*)-*O*,*O*-diethyl-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate dihydrochlorides was chosen for further characterization since it exerted the strongest cytotoxic activity in HL-60 cell line.

The observed cytotoxic effect in HL-60 cells was associated with an increase in superoxide production and mitochondrial membrane depolarization, leading to apoptotic cell death characterized by phosphatidylserine externalization and deoxyribonucleic acid (DNA) fragmentation in the absence of autophagic response [1].

It has also been observed that compounds with ester structure are prone to degradation by esterases and nonspecific pseudoesterases; therefore, the possibility of hydrolytic biotransformation to the carboxylic acid metabolites was speculated. This has to be taken into an account during

method development and validation as well as interpretation of further preclinical pharmacokinetics studies.

So far, the activity of the aforementioned compound has been investigated exclusively in vitro. Hence, the need for rapid, sensitive, and selective bioanalytical method has arisen in order to proceed to the nonclinical studies involving the selected animal model. The aim of this study was to develop and validate an ultra high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) bioanalytical method for determination of (*S*,*S*)-*O*,*O*-diethyl-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl) propanoate dihydrochlorides and its hydrolytic metabolite in mouse serum. The structural analog, derivative of 1,3-propanediamine, was used as an internal standard (IS). The developed method is supposed to be integrated in future nonclinical studies and drug monitoring.

Experimental

Solvents and Chemicals

(*S*,*S*)-*O*,*O*-diethyl-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl) propanoate dihydrochlorides (DE-EDCP × 2HCl, $C_{24}H_{46}O_4N_2Cl_2$; MW = 497.55 g mol^{-1}), (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid dihydrochlorides (EDCP × 2HCl, $C_{20}H_{38}O_4N_2Cl_2$; MW = 433.55 g mol^{-1}), and internal standard (*S*,*S*)-*O*,*O*-dibutyl-1,3-propanediamine-*N*,*N*′-di-2-(3-cyclohexyl) propanoate dihydrochlorides (DB-PDCP × 2HCl, $C_{29}H_{52}O_4N_2Cl_2$; MW = 567.67 g mol^{-1}) were provided by Faculty of Chemistry, University of Belgrade, Serbia (*S*,*S*-enantiomers of mentioned substances were synthesized from enantiopure (*S*)-2-amino-3-cyclohexylpropanoic acid as starting material) [5]. Acetonitrile, methanol, ethyl acetate, diethyl ether, triethanolamine, chloroform and trifluoroacetic acid (HPLC grade), ammonium acetate (CH_3COONH_4) and sodium fluoride (ACS grade) from Fluka (Sigma-Aldrich Co.), and deionized water (Gen Pure Ultrapure, Germany) were used. Mouse serum was purchased from Sigma-Aldrich (Saint Louis, USA).

Equipment

Method development and validation were carried out on Thermo ACCELA (Thermo Scientific, Waltham, Massachusetts, USA) UHPLC system coupled to a triple quad Mass Spectrometer Thermo TSQ Quantum Access Max

(Thermo Scientific, Waltham, Massachusetts, USA) with a heated electrospray ionization (HESI) interface. A reverse-phase Thermo Scientific Hypersil GOLD aQ column (100 × 2.1 mm, 1.9 μm, Thermo Scientific) and guard cartridge (Thermo Scientific Hypersil GOLD aQ, 10 mm L × 4 mm ID) were used. Solids were weighed using five-digit Mettler analytical balance (Mettler-Toledo International Inc., USA), and less sensitive weighting was performed on Adventurer Pro analytical balance (OHAUS, USA). Sample preparation was done using Eppendorf 5417R microcentrifuge (Eppendorf, Germany). Solid-phase extraction (SPE), as an alternative sample preparation method, was performed using Strata X-C (Phenomenex, USA) and OASIS HLB (Waters, USA) cartridges.

Software

LogD tool in MarvinSketch 4.1.13 (ChemAxon, Budapest, Hungary) was used to calculate $logD_{7.4}$ values of the investigated compounds.

Preparation of Standard Solutions

DE-EDCP, EDCP, and DB-PDCP (IS) were weighed from solid to an appropriate amount.

DE-EDCP stock solution was prepared by dissolving 5 mg of solid DE-EDCP with methanol in a 50-mL volumetric flask in order to obtain the concentration of 0.1 mg mL^{-1}.

IS stock solution was prepared by dissolving 5 mg of solid DE-PDCP with methanol in a 50-mL volumetric flask. The concentration of the aforementioned solution was 0.1 mg mL^{-1}.

Afterwards, the appropriate dilutions with methanol were made from IS stock solution to produce IS working solution (WS) of 10 ng mL^{-1}.

Another stock solution was made in methanol from 200 μL stock solution of DE-EDCP and appropriate amount of solid EDCP (5 mg) to obtain final concentrations of 0.2 μg mL^{-1} for DE-EDCP and 0.05 mg mL^{-1} for EDCP.

Standard Preparation

Stock solutions were diluted with methanol to obtain calibration solutions (CS) and four levels of quality control samples (QS). CS and QS were prepared in 2 mL microcentrifuge tubes by spiking 150 μL of mouse serum

with various concentrations of DE-EDCP ranging 2–40 ng mL^{-1} and of EDCP ranging 0.5–10 μg mL^{-1}. One hundred microliters of IS (10 ng mL^{-1} in methanol) and 750 mL acetonitrile (ACN) were added to each tested serum sample, and the microcentrifuge tubes were vortexed 1 min and then centrifuged (10 min, 25,000 rcf, 4 °C). Supernatants were transferred into glass vials and analyzed by UHPLC–MS/MS thereafter.

The serum concentrations of CS were 1.3, 3.3, 6.7, 10.0, 13.3, 20.0, and 26.7 for DE-EDCP and 0.3, 0.8, 1.7, 2.5, 2.3, 5.0, and 6.7 for EDCP.

The serum concentrations of QC samples were 1.3, 3.3, 10.0, and 20.0 ng mL^{-1} and 0.3, 0.8, 2.5, and 5.0 μg mL^{-1} for DE-EDCP and EDCP, respectively.

All working and stock solutions were stored at 5 °C, in darkness.

CS and QC were freshly prepared for each batch.

Sample Preparation

Fresh and thawed frozen sera containing enzyme-inhibitor sodium fluoride (40 mM) were used.

Solid-Phase Extraction

Initially, we tested the following SPE procedure: SPE column was activated with 1 mL of 5% triethanolamine–methanol (TEA–MeOH), followed by 1 mL of 5% TEA–water; afterwards, SPE column was loaded with 50 μL of serum, 50 μL of methanol, 50 μL of IS, and 500 μL of 5% TEA–water; the elution step was performed with 500 μL of 5% TEA–MeOH, and the eluate was collected and analyzed by UHPLC–MS/MS. Afterwards, the same SPE procedure with 1% trifluoracetic acid–water instead of 5% TEA–water was tested. We have also tried out SPE procedure with serum being pretreated in the following manner: 50 μL of serum, 250 μL water, 250 μL 0.1 M $ZnSO_4$, 50 μL IS, and 450 μL of methanol were mixed together, vortexed for 30 s, and centrifuged for 5 min at 16,000 rcf. The supernatant was then used in the aforementioned SPE procedure with 5% TEA–water. All of the procedures were tested on both Strata X-C (Phenomenex, USA) and OASIS HLB (Waters, USA) SPE cartridges.

However, in all conducted procedures, the obtained recovery was up to 50%.

Liquid-Liquid Extraction

The following liquid–liquid extraction (LLE) procedure was also tested: 150 μL of sample, 250 μL of water, 100 μL of methanol, 100 μL IS, 100 μL methanol–water mixure (60:40, *v*/*v*), and 3 mL of ethyl acetate were mixed, briefly vortexed, and centrifugated for 15 min at 2500 rcf. Afterwards, the upper layer was transferred into the clean glass tube and evaporated under nitrogen at 40 °C. Dried debris was redissolved in 500 μL of methanol and vortexed prior to UHPLC–MS/MS analysis. Ethyl acetate, diethyl ether, chloroform, and ACN were also tested as extraction solvents.

Protein Precipitation

The same preparation procedure was applied for all samples, CS, and QS. One hundred and fifty microliters of mouse serum were transferred into clean 2 mL microcentrifuge tube. One hundred microliters of IS (10 ng mL^{-1} in methanol), 100 μL of methanol, and 750 mL of ACN were added, and the microcentrifuge tubes were vortexed for 1 min. Samples were centrifuged (10 min, 25,000 rcf, 4 °C), and supernatants (300 μL) were transferred to clean glass autosampler vials for UHPLC–MS/MS analysis.

Chromatographic and Mass Spectrometric Conditions

DE-EDCP, EDCP, and IS were eluted using a mobile phase composed of ammonium acetate (5 mM)–trifluoroacetic acid (99.9:0.1, *v*/*v*) and methanol–trifluoroacetic acid (99.9:0.1, *v*/*v*) according to the following gradient program: 50% buffer A and 50% buffer B were held from 0 to 1 min, and then, the buffer A was linearly decreased to 10% over 1.5 min and remained constant for 3.5 min when these analytes were eluted, followed by reequilibration to initial condition via a step gradient from 5 to 6 min. The flow rate was 0.3 mL min^{-1}.

Quantitation was achieved by MS–MS detection in positive ionization mode for DE-EDCP, EDCP, and IS. The MS operating conditions were optimized as follows: the spray voltage was 4500 V with a tube lens offset of 107 V and skimmer offset of 0 V. The capillary temperature was set to 300 °C. Nitrogen was used as the sheath gas (50 units) and auxiliary gas (10 units). Detection of the ions was performed in the selected reaction monitoring (SRM) mode using the following transitions of *m*/*z*

425.220 → 197.800 and 226.070 for DE-EDCP, m/z 369.256 → 152.130 and 198.100 for EDCP, and m/z 495.339 → 166.034 and 268.062 for DB-PDCP (IS), respectively, with a scan time of 0.1 s per transition. TSQ Tune Software (Thermo Electron Corporation, Hemel Hepstead, UK) was used for the automatic optimization of tuning parameters. Data acquisition was performed using Xcalibur 1.3 software (Thermo Electron Corporation, Hemel Hepstead, UK). Peak integration and calibrations were performed using LC Quan™ software (Version 2.5.6, Thermo Electron Corporation, Hemel Hempstead, UK).

Validation of UHPLC–MS/MS Method

The developed quantitative UHPLC–MS/MS method was validated by taking into account selectivity, linearity, accuracy, precision, limit of quantitation (LOQ), recovery, matrix effects, and stability in accordance with the regulatory guidance/guideline [6, 7]. Thereafter, a validation was carried out for DE-EDCP and EDCP analysis in mouse serum samples.

Selectivity

The selectivity of the method was evaluated by analyzing six independent drug-free mouse serum samples with the corresponding spiked plasma at LOQ.

Coeluting components should not be present in the amount greater than 20% of the analyte and 5% of the corresponding IS as seen by comparing relevant peak areas.

Linearity of calibration dependencies

Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of DE-EDCP and EDCP, respectively, in the standard samples. Fresh calibration solutions (CS) were extracted and assayed as described above on three different days and in duplicate. Calibration curves for DE-EDCP were represented by the plots of peak area ratio (DE-EDCP/DB-PDCP) versus the nominal concentration of the DE-EDCP in CS. Calibration curves for EDCP were represented by the plots of the peak area ratio (EDCP/DB-PDCP) versus the nominal concentration of the EDCP in CS. The coefficient of the correlation should exceed 0.99.

Accuracy and precision

The accuracy and precision were evaluated using the spiked samples at four concentration levels of 1.3 ng mL^{-1} and 0.3 μg mL^{-1} (LOQ), 3.3 ng mL^{-1} and 0.8 μg mL^{-1} (low), 10 ng mL^{-1} and 2.5 μg mL^{-1} (medium), and 20 ng mL^{-1} and 5 μg mL^{-1} (high) for DE-EDCP and EDCP, respectively. The spiked samples were analyzed in six replicates on three different days. Assay accuracy was calculated as the relative error to the nominal concentration (%RE). Assay precision was calculated as the coefficient of variation (%CV). The acceptance criteria were set at within ±15% of the %RE except for the LOQ where it should be within ±20% and at <15% of the %CV except for the LOQ where it should be <20%.

Limit of quantitation

The lower limit of quantitation (LOQ), defined as the lowest concentration on the calibration curve, was evaluated by analyzing the samples prepared in six replicates on three consecutive days.

The signal-to-noise ratio should be larger than 5.

Recovery (%)

The recovery of analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in solvent. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of internal standard should be consistent, precise, and reproducible [6].

Recovery experiments were performed by comparing the analytical results for extracted samples and four concentrations (LOQ, low, medium, and high) with unextracted standards that represent 100% recovery.

The recovery was evaluated for DE-EDCP and EDCP at four concentration levels of 1.3 ng mL^{-1} and 0.3 μg mL^{-1} (LOQ), 3.3 ng mL^{-1} and 0.8 μg mL^{-1} (low), 10 ng mL^{-1} and 2.5 μg mL^{-1} (medium), and 20 ng mL^{-1} and 5 μg mL^{-1} (high) for DE-EDCP and EDCP, respectively. The peak area ratios obtained from extracted samples at the above mentioned concentrations were compared with those of the corresponding unextracted standard solutions (LOQ, low, medium, and high).

Matrix effects

Blank mouse serum was extracted and then spiked with analyte and IS to assess the matrix effect. The corresponding peak area ratios of the analyte to IS in spiked serum postextraction (A) at low and high QC levels were then compared with those of the corresponding standard samples (B) at equivalent concentrations. The ratio $A/B \times 100\%$ is defined as the matrix effect.

Stability

It was tested in vitro stability of stock and standard solutions.

Stock solution stability

The stability of DE-EDCP, EDCP, and IS in methanol was evaluated at room temperature, 5 °C, and −20 °C. Stock solutions with concentrations of 100.0 μg mL^{-1} for IS and DE-EDCP and concentration of 50 μg mL^{-1} for EDCP were prepared in methanol. Three aliquots of each of the stock solutions were kept at room temperature, 5 °C, and −20 °C, respectively, for 7 days. After diluting the stored stock solutions in injection solvent to 100.0 ng mL^{-1}, the stability of DE-EDCP, EDCP, and IS was assessed by comparing the peak areas obtained from the stored stock solutions with peak areas of the freshly prepared stock solutions. For stock solution results to be acceptable, the percentage reference value should not exceed 15%.

Standard solutions stability

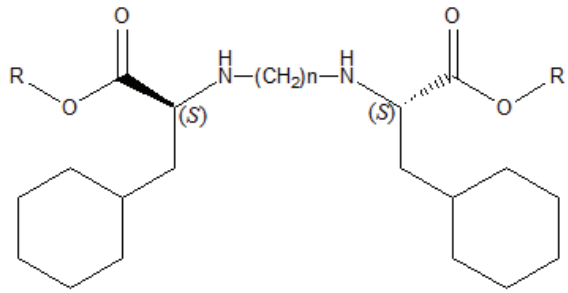
The stability of DE-EDCP, EDCP, and IS in methanol was evaluated at room temperature, 5 °C, and −20 °C. Standard solutions with concentrations of 200 ng mL^{-1} for DE-EDCP, 50 μg mL^{-1} for EDCP, and 10 ng mL^{-1} for IS were prepared in methanol. Three aliquots of each of the standard solutions were kept at room temperature, 5 °C, and −20 °C, respectively, for 3 days. The stability of DE-EDCP, EDCP, and IS was assessed by comparing the peak areas obtained from the stored stock solutions with peak areas of the freshly prepared standard solutions. For stock solution results to be acceptable, the percentage reference value should not exceed 15%.

Autosampler stability of DE-EDCP, EDCP, and IS was determined at four low, medium, and high QC concentrations. Autosampler stability of extracted samples was determined by comparing DE-EDCP, EDCP, and DB-PDCP concentration in freshly prepared samples and samples kept in autosampler at 4 °C for 24 h.

Results and Discussion

Method Development

The developed and validated bioanalytical method that has been presented in this article is a new ultra high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (UHPLC–ESI–MS/MS) method for the quantification of recently synthesized substance DE-EDCP and its metabolite EDCP in biological material. The structures of DE-EDCP, EDCP, and DB-PDCP are given in *Fig. 1*. Since substances do not possess maximum absorption in the UV part of the spectrum, we have opted for the mass detector. Maximum absorption with molar absorptivity of DE-EDCP and DB-PDCP is λ = 209 nm (a = 0.127 dm^3 cm^{-1} mol^{-1}) and λ = 215 nm (a = 0.085 dm^3 cm^{-1} mol^{-1}), respectively. As regards initial physicochemical characterization of DE-EDCP and EDCP, we found that the two compounds



Substance	n	R
DE-EDCP	2	-C_2H_5
EDCP	2	-H
DB-PDCP	3	-C_4H_9

Fig. 1. Chemical structures of DE-EDCP, EDCP, and DB-PDCP

differ in polarity; logarithm values of the distribution coefficient (D) in octanol–water at pH 7.4 (log $D_{7.4}$) were 4.04 and −1.8 for DE-EDCP and EDCP, respectively, and isoelectric point value for EDCP was 5.49 (by MarvinSketch 4.1.13).

Using sodium-fluoride-coated blood collection tubes in preanalytical phase proved successful in achieving esterase inhibition [8].

Internal standard DB-PDCP was selected according to structural similarity (structural analog). A small number of drugs used in medicine have ethylenediamine structure. The compounds previously analyzed by a group of researchers led by T. Sabo were actually ligands of Pt-complexated ethylenediamine and 1,3-propanediamine [9–12].

Extraction method from biological material was based on protein precipitation by addition of five volumes of ACN [13] and centrifugation at low temperature. Our preliminary exploration has shown that acetonitrile-induced precipitation has provided higher extraction recovery and cleaner samples than methanol. Protein-binding property of DE-EDCP was overcome by optimizing vortexing time to exactly 1 min since longer vortexing resulted in loss of linearity.

While developing the extraction procedure, SPE was considered for extracting DE-EDCP from mouse serum. Although both basic and acidic washing and elution reagents as well as two different SPE columns were tested, each time, unsatisfactory extraction yields for both compounds (recovery was less than 50%) were obtained. We have also included protein precipitation procedure with 0.1 M $ZnSO_4$ solution preceding the SPE extraction, in order to improve the extraction yield. Nevertheless, it proved unsuccessful.

We also tried to carry out the LLE using diethyl ether, chloroform, and ACN, but none of those solvents managed to achieve satisfying extraction yield of both DE-EDCP and EDCP in the same liquid phase because of the difference in polarity of the observed substances. In addition, the LLE method proved to be quite time-consuming.

The mobile phase, composed of 5 mM CH_3COONH_4 with 0.1% trifluoroacetic acid and MeOH with 0.1% trifluoroacetic acid, was chosen since the investigated compounds and ethambutol both share the diethylamine-based structure [14]. Excellent chromatographic separation in six-minute run period was obtained by applying a suitable gradient. The appearance of the chromatogram is shown in *Fig. 2*.

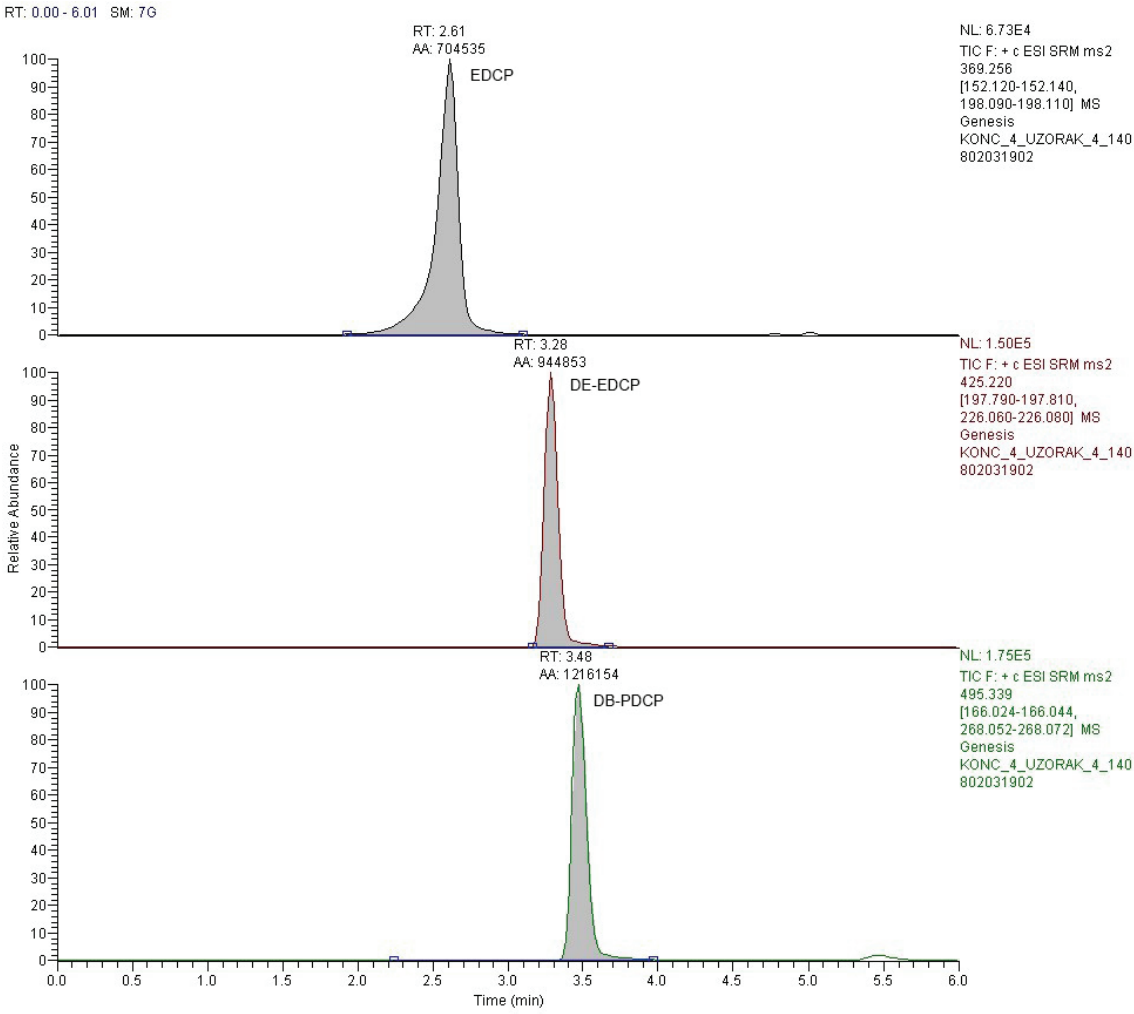


Fig. 2. Appearance of chromatogram (chromatographic separation of DE-EDCP, EDCP, and DB-PDCP)

Both compounds were quantified following the appropriate mass transitions for DE-EDCP (m/z 425.220 → 197.800, 226.070), EDCP (m/z 369.256 → 152.130, 198.100), and DB-PDCP (m/z 495.339 → 166.034, 268.062). Proposed SRM fragmentation patterns and structures are given in *Figs. 3–5*.

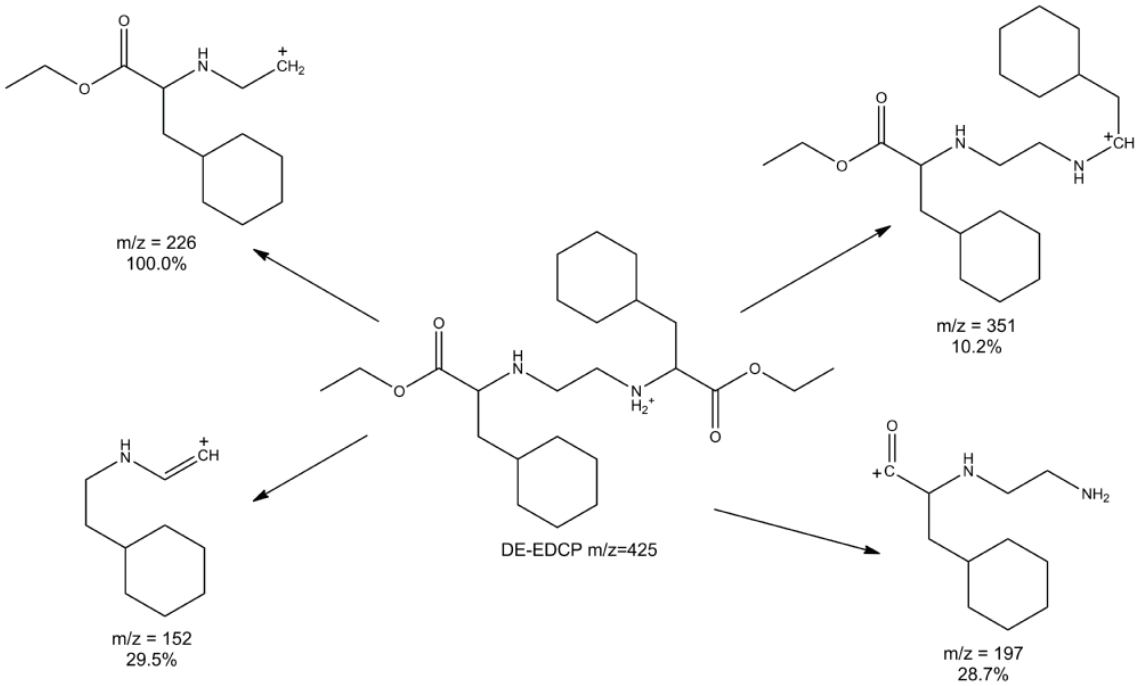


Fig. 3. The proposed SRM fragmentation patterns of DE-EDCP

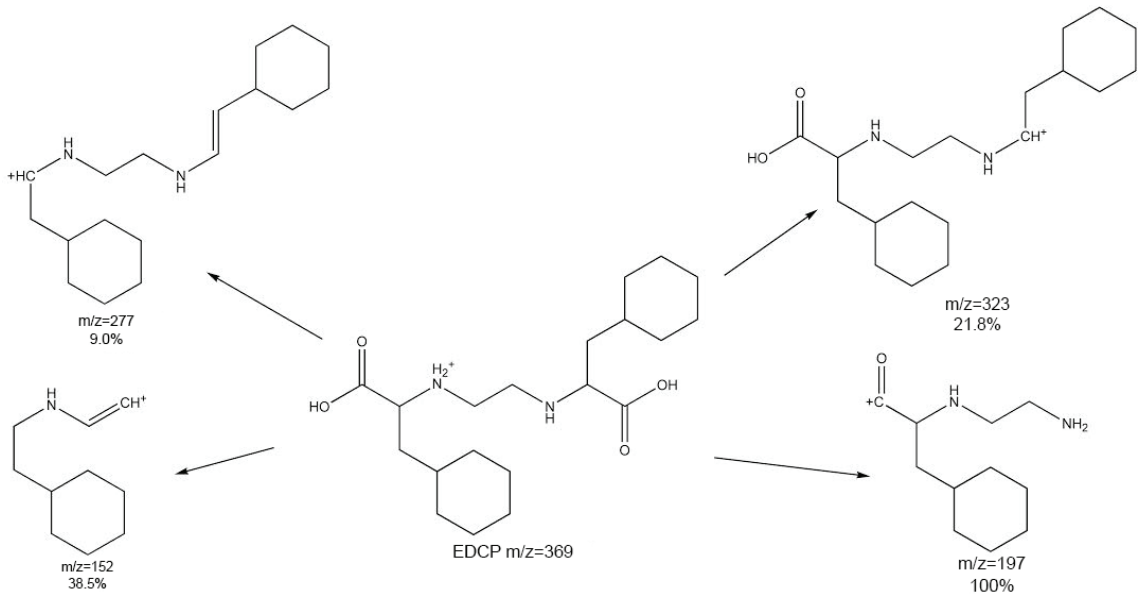


Fig. 4. The proposed SRM fragmentation patterns of EDCP

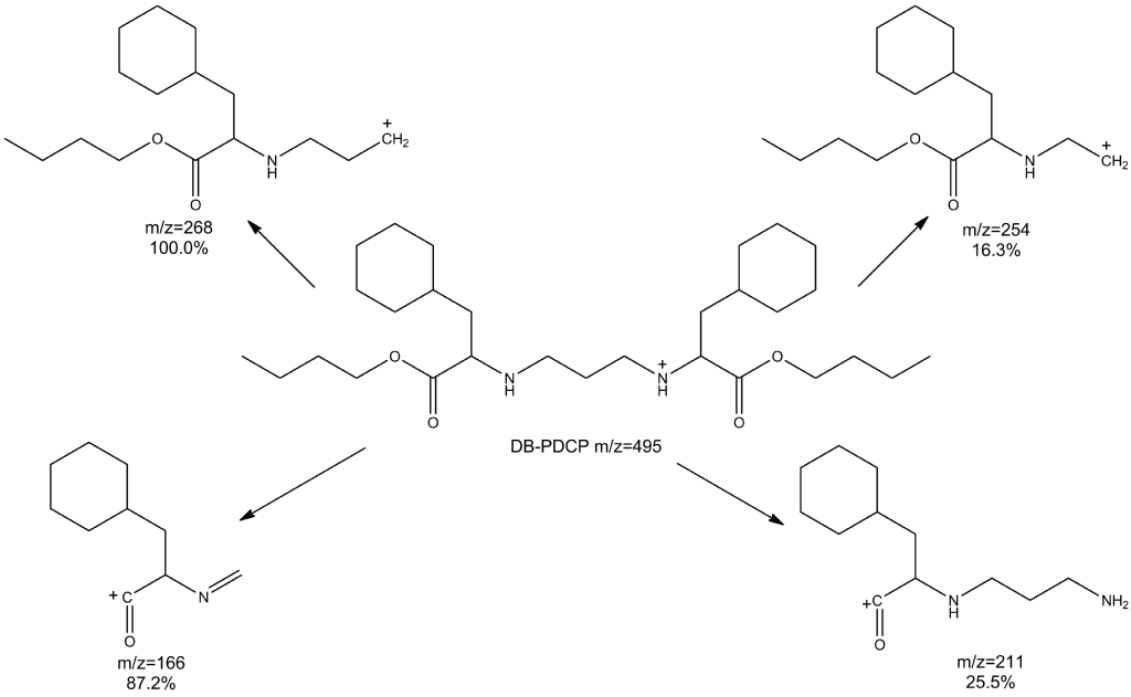


Fig. 5. The proposed SRM fragmentation patterns of DB-PDCP

In the MS–MS spectra, the most abundant daughter ions (m/z = 226 for DE-EDCP and m/z = 268 for DB-PDCP) resulted from loss of corresponding ester of 2-amino-3-cyclohexylpropanoic acid from only one side of molecules *(Figs. 3* and *5)*. The loss of corresponding alkoxy group from one side of both molecules and the loss of corresponding ester of 3-cyclohexylpropanoic acid from other side of both molecules at the same time produced daughter ions $m/z = 197$ and $m/z = 211$ for DE-EDCP and DB-PDCP, respectively. The breaking of C–C bond inside of 1,3-propanediamine chain in parent ion can be seen as specific fragmentation pattern for DB-PDCP *(Fig. 5)* to form two ions (m/z = 254 and m/z = 168). The similar fragmentation pattern occurs both in DE-EDCP and EDCP but with additional cleavage of ethoxycarbonyl or carboxylic groups to form daughter ion $m/z = 152$ *(Figs. 3* and *4)*. The daughter ions $m/z = 351$ and m/z = 323 are formed by only from the one side cleavage of ethoxycarbonyl and carboxylic groups in DE-EDCP and EDCP, respectively.

The presented method was applied in real sample, in mice, and we found that EDCP exists as one of metabolites.

The presented method of extraction and UHPLC–MS/MS analysis may be successfully applied for analyzing serum, plasma, liver, and brain homogenate for determining the preliminary pharmacokinetic profile of DE-EDCP and EDCP in mice.

Method Validation

The developed bioanalytical method for DE-EDCP and EDCP in mouse serum was validated for its selectivity, linearity of calibration dependencies, accuracy and precision, limit of quantitation, recovery, matrix effect, and stability.

Selectivity

The method was found to be highly selective for the analytes since no interfering peaks from endogenous compounds were observed at the retention time for DE-EDCP and EDCP in any of the six independent blank serum extracts evaluated.

Linearity of calibration dependencies

The linear regressions of the peak area ratios versus concentration were fitted over the concentration range in mouse serum of 1.3–26.7 ng mL^{-1} and 0.3–6.7 μg mL^{-1} for DE-EDCP and EDCP, respectively. The typical equation of the calibration curves was as follows: DE-EDCP: $y = 0.0461x + 0.0895$, $r = 0.9978$; EDCP: $y = 0.1527x + 0.0045$, $r = 0.9987$ where y represents the peak area ratio of analyst to IS and x represents the concentration of the analyst in serum. The correlation coefficient (r) exceeded 0.99, showing a good linearity over the concentration range.

Accuracy and precision

Table I shows the results of the QC samples at four concentration levels analyzed in six replicates for DE-EDCP and EDCP, respectively. The precision (%CV) and the accuracy (%RE) ranged from 3.5% to 16.0% and from 1.8% to 14.4%, respectively.

Table I. The precision (%CV) and accuracy (%RE) for DE-EDCP and EDCP

Compound	Concentration (expected) ng mL^{-1}	Mean concentration (measured) ng mL^{-1}	CV (%)	RE (%)
DE-EDCP	1.33 – LOQ	1.3 ± 0.6	15.99	3.0
	3.33	3.8 ± 1.0	13.68	12.6
	10.00	10.6 ± 0.4	2.25	6.3
	20.00	20.4 ± 1.1	3.49	1.8
EDCP	0.33 – LOQ	0.4 ± 0.1	5.58	6.1
	0.83	0.8 ± 0.1	4.43	−2.4
	2.50	2.4 ± 0.2	5.32	−3.2
	5.00	4.3 ± 0.3	4.01	−14.4

Limit of quantitation

The lower limit of quantitation (LOQ) was 1.3 ng mL^{-1} for DE-EDCP and 0.3 μg mL^{-1} for EDCP. The intra- and inter-relative standard deviations (RSD) were both less than 20%, and the %RE was within ±9.7% at LOQ level, which were within the accepted limits.

Recovery %

The recovery for DE-EDCP and for EDCP ranged from 90% to 99% and from 76% to 100%, respectively, through four concentration levels. The recovery for the IS was 98%.

Matrix effect

The calculated matrix effects were in the 95.5–108.2% range. Therefore, ion suppression or enhancement effect deriving from serum was negligible under the current conditions.

Stability studies

All the results showed that the analyte was stable under the conditions in which the stability assessment was performed: DE-EDCP, EDCP, and IS were stable in methanol in stock solutions at all storage temperatures (at room temperature, 5 °C, and −20 °C) for 7 days; DE-EDCP, EDCP, and IS were stable in methanol in standard solutions at all storage temperatures (at room temperature, ~5 °C, and −20 °C) for 3 days.

DE-EDCP, EDCP, and IS were found to be stable in supernatants obtained after sample preparation during 24-hour period spent in autosampler at 4 °C regardless the concentration.

Conclusion

The newly developed and validated ultra high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (UHPLC–MS/MS) is rapid and specific. Therefore, the observed method is sensitive, allowing determination of very low concentrations of DE-EDCP (LOQ was 1.3 ng mL^{-1}) and EDCP (LOQ was 0.3 μg mL^{-1}) in mouse serum. The recovery was in the range of 90% to 100%, respectively. The method may be successfully applied to drug monitoring in nonclinical studies in selected animal model.

Acknowledgment

This work was partly supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, as a part of Project No. 172041.

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