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A liquid chromatography and tandem mass spectrometry method for the determination of potential biomarkers of cardiovascular disease

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ABSTRACT

A simple, accurate and sensitive liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed and validated for the quantitation of α-ketoglutaric acid (α-KG), L-carnitine (L-CAR) and acetyl-L-carnitine (acetyl-L-CAR) in human urine as potential biomarkers of cardiovascular disease. The separation was performed using an isocratic elution of 0.1% formic acid in water and acetonitrile (97:3, v/v) on an Acclaim 120 C8 column (150 mm × 4.6 mm, 3.0 μm). The flow rate of the mobile phase was 1.2 mL/min and the total assay run time was 3 min. Detection was performed on a triple-quadrupole mass spectrometer in selected reaction monitoring (SRM) mode via an electrospray ionization (ESI) source in positive and negative ion modes. This method covered a linearity range of 0.1–500 ng/mL for L-CAR and acetyl-L-CAR and 1–1000 ng/mL for α-KG with lower limits of quantification (LLOQ) of 0.08 ng/mL for L-CAR, 0.04 ng/mL for acetyl-L-CAR and 0.8 ng/mL for α-KG. The intra-day and inter-day precision and accuracy of the quality control samples exhibited relative standard deviations of less than 5.54% and relative error values from −5.95% to 3.11%. Analyte stability was evaluated under various sample preparation, analysis and storage conditions and varied from −9.89% to −0.47%. A two-step solid-phase extraction (SPE) procedure using silica gel and quaternary amine cartridges was used for urine sample cleanup. The average recoveries for all analyzed compounds were better than 86.64% at three concentrations. The method was successfully applied for the quantitation of α-KG, L-CAR and acetyl-L-CAR in human urine samples.

1. Introduction

Cardiac failure is an increasingly common problem in developed countries. This is mainly due to a longer average lifetime and a higher level of medical care. In the development of heart failure, the metabolism of cardiomyocytes is significantly altered. As a result, there are specific metabolites in the plasma and urine that could help in early prognosis, treatment and prevention of disease. Such biomarkers are essential tools in diagnosing disease and monitoring progression as well as response to therapy. There is thus intense interest in the identification of further biomarkers for heart failure. Distinct patterns of several such markers may eventually help in identifying specific classes of the syndrome with improved predictive power in terms of diagnosis, prognosis and treatment options. Due to the significant participation of L-carnitine (L-CAR), acetyl-L-carnitine (acetyl-L-CAR) and α-ketoglutaric acid (α-KG) in the metabolism of cardiac cells, these three compounds were investigated for correlations between their levels in urine and the clinical status of patients with heart failure.

Several experimental studies have shown that L-CAR reduces myocardial injury after ischemia and reperfusion by counteracting the toxic effect of high levels of free fatty acids, which occur in ischemia, and by improving carbohydrate metabolism. In addition to increasing the rate of fatty acid transport into mitochondria, L-CAR reduces the intramitochondrial ratio of acetyl-CoA to free CoA, thus stimulating the activity of pyruvate dehydrogenase and increasing the oxidation of pyruvate. Supplementation of the myocardium with L-CAR results in an increased tissue carnitine content, a prevention of the loss of high-energy phosphate stores, ischemic injury, and improved heart recovery on reperfusion. Clinically, L-CAR has been shown to have anti-ischemic properties. In small, short-term studies, L-CAR acts as an antianginal agent that reduces ST segment depression and left ventricular end-diastolic pressure. These short-term studies also showed that L-CAR releases lactate in coronary artery disease patients subjected to either exercise testing or atrial pacing. These cardioprotective effects have been confirmed during aortocoronary bypass grafting and acute myocardial infarction. L-CAR could improve ischemia and

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reperfusion by (1) preventing the accumulation of long-chain acyl-CoA, which facilitates the production of free radicals by damaged mitochondria; (2) improving repair mechanisms for oxidation-induced damage to membrane phospholipids; (3) inhibiting malignancy arrhythmias caused by accumulation of long-chain acyl-CoA within the myocardium; and (4) reducing the ischemia-induced apoptosis and subsequent remodeling of the left ventricle [1–4].

Studies of the content of the derivatives of L-CAR according to the severity of cardiac disease was performed only in the plasma and tissues. Regitz et al. [5] reported reduced myocardial carnitine levels in patients undergoing cardiac transplantation for heart failure. Both free and total carnitine levels were decreased compared to controls but free myocardial carnitine was decreased to a greater extent. Kobayashi et al. [6] found significant reductions in free carnitine with elevations in long-chain acylcarnitine in the myocardium of heart failure patients at necropsy. Papillary muscle biopsies taken at the time of mitral valve surgery have shown the same marked reduction in free carnitine with elevations of both short- and long-chain acylcarnitines [7].

Several methods have been developed for L-CAR and acetyl-L-CAR quantitation. They include high performance liquid chromatography with UV or FL detection (HPLC-UV or HPLC-FL) [8–15], liquid chromatography mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS/MS) [16–22]. Because L-CAR and acetyl-L-CAR have only weak and non-specific chromophoric absorption, derivatization is required for UV and fluorescence detectors coupled with liquid chromatography. In addition, capillary electrophoresis (CE), capillary electrochromatography (CEC) and enzyme and radioenzyme methods have been proposed for the separation of L-CAR or acetyl-L-CAR [23–27]. Sample preparation for these methods has been performed mainly by protein precipitation [22–24] and solid-phase extraction (SPE) [28,29].

α-KG is a major intermediate of the tricarboxylic acid cycle (also known as the citric acid or Krebs cycle) that occupies a central place in energy metabolism and is one of the 12 major precursors for the synthesis of most biochemical substances. It is also substantially decreased in heart failure patients. In recent years, it has become increasingly clear that heart failure is characterized by alterations in energy metabolism. Recent findings using metabolomics to investigate alterations during cardiac ischemia showed decreases in several constituents of the Krebs cycle. This result suggested that the metabolic state of the heart and/or peripheral tissues is at least in part reflected in serum metabolites, which can be harnessed as markers of disease. Further mechanistic studies regarding this issue are therefore warranted [30,31].

Methods using gas chromatography (GC) [32,33] and capillary electrophoresis (CE) [34] have been proposed, but separation was mainly performed by HPLC coupled with ultraviolet (UV) [35,36], fluorescence (FL) [37] or mass spectrometry (MS) detection [38].

Although markers of heart failure are currently available in the clinic, there are no markers specific for individual conditions or clinical situations. The application of novel metabolomics technologies could help to identify changes in metabolic status in these groups of patients. The aim of the present work was therefore to develop and validate an LC–ESI-MS/MS method for the simultaneous determination of L-CAR, acetyl-L-CAR and α-KG as potential biomarkers of cardiovascular disease in human urine. An additional aim was a simple sample preparation method that maintained specificity and sensitivity. To the best of our knowledge, each individual compound (L-CAR, acetyl-L-CAR and α-KG) has been analyzed separately by different methods, but not analyzed simultaneously by one LC–ESI-MS/MS method in urinal matrices. The developed method, after application to a large group of potential patients, could be useful in the detection of heart failure before symptoms of the disease appear.

2. Experimental

2.1. Chemicals and reagents

L-Carnitine (L-CAR) (purity 98%), acetyl-L-carnitine (acetyl-L-CAR) (purity 98%), α-ketoglutaric acid (α-KG) and mildronate dihydrate (MLD) (purity ≥98%) (IS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC–MS grade), methanol (HPLC–MS grade) and water (HPLC grade) were purchased from Merck (Darmstadt, Germany). Formic acid (minimum of 95%) and ammonium hydroxide (25%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All reagents were of analytical grade or better and were used prior to their respective expiration dates.

2.2. Preparation of calibration standards (CS) and quality control (QC) samples

Stock solutions of L-CAR, acetyl-L-CAR and α-KG were prepared by dissolving an accurately weighed amount of the compound in water to obtain a final concentration of 1.0 mg/mL and stored at 4 °C. The stock working solution was appropriately diluted with mobile phase (water with 0.1% formic acid and acetonitrile (97:3, v/v)) to prepare a series of solutions at concentrations ranging from 1 to 100 μg/mL. The IS stock solution (MLD) (1 mg/mL) was prepared in water. A working standard solution of IS (10 μg/mL) was prepared by diluting the stock solution with mobile phase and stored at 4 °C.

L-CAR, acetyl-L-CAR and α-KG are endogenous compounds, making it impossible to obtain urine free of these compounds. The presence of endogenous L-CAR, acetyl-L-CAR and α-KG thus makes it difficult to use urine as a matrix for QC samples. To facilitate the determination of L-CAR, acetyl-L-CAR and α-KG in urine, calibration standards (CS) and quality control (QC) samples were prepared by spiking the proper amounts of each working solution into synthetic urine during validation [39]. In synthetic urine was contained of urea at the concentration of about 17 g/L (normal concentration of urea in urine is lower but to simplify the organic composition only urea was used as an organic component) and a saline solution at a similar concentration about 17 g/L (saline components: Na^+ – 5.4 g/L; K^+ – 0.2 g/L; Mg^{2+} – 0.65 g/L; Ca^{2+} – 0.2 g/L; Cl^- – 9.6 g/L; SO_4^{2-} – 1.35 g/L). Urine was adjusted to pH 6.0 with nitric acid.

The pH of normal urine is also generally around 6.0. Given that urine is mostly water (~95%), but also contains some other substances dissolved in the water, its density is expected to be close to, but slightly greater than, 1.0. The components of normal urine are: urea, uric acid, creatinine and other substances/molecules (e.g. carbohydrates, enzymes, fatty acids, hormones, pigments, and mucins). Another components which are present in urine are ions (sodium, potassium, chloride, magnesium, calcium; small groups formed from a few different elements: ammonium, sulphates, phosphates) [40].

CS and QC samples were prepared by spiking pretreated synthetic urine with working standard solutions and IS (10 μg/mL). The synthetic urine used for preparation of CS and QC samples was extracted in the same way as the patient urine samples (Section 2.3).

A set of eight calibration standards (CS) were freshly prepared at concentrations in the range of 0.1–500 ng/mL for L-CAR and acetyl-L-CAR, and 1–1000 ng/mL for α-KG.

According to the US-FDA Bioanalytical Method Validation Guidance, three QC concentrations representing the entire range of the calibration curve were selected [41]. The lower QC was selected within three times the lower limit of quantification (LLOQ). QC samples at different levels of high (HQC), middle (MQC) and low quality control (LQC) were selected to perform various validation

parameters. All the solutions were stored at 4 °C and were brought to room temperature before use.

2.3. Solid phase extraction (SPE)

Urine samples were obtained from 20 donors (10 healthy people and 10 patients with cardiac failure). This study was approved by the Ethics Committee (Institutional Review Board of Regional Specialist Hospital, Wroclaw, Poland). Urine samples were stored in the freezer at −20 °C.

Extraction was performed with a BAKERBOND spe-12G system (J.T. Baker Inc., Deventer, Netherlands). Solid-phase extraction (SPE) of α-KG was performed using silica gel cartridges (3 mL, 200 mg, J.T. Baker Inc.). Quaternary amine cartridges (3 mL, 200 mg, J.T. Baker Inc.) were then used for the extraction of L-CAR and acetyl-L-CAR.

For human samples, 10 μL of urine, 20 μL of 10 μg/mL working IS solution (which corresponds to a concentration of 50 ng/mL in analyzed samples) and 2 mL of acetonitrile were mixed. Then, the samples were adjusted to pH 10 with 25% ammonium hydroxide and the mixture was vortexed for 30 s. The mixtures were loaded onto silica gel cartridges pre-conditioned with 3 mL of water and 3 mL of 5% formic acid in water. The loaded cartridges were left for at least 2 min at room temperature and then α-KG was eluted with 1 mL of 5% formic acid in acetonitrile and 1 mL of 5% formic acid in water. Sample collected from loading step from one-step SPE were next loaded onto quaternary amine cartridges pre-conditioned with 3 mL of methanol and 3 mL of water. The loaded cartridges were left at room temperature for at least 2 min and then, the L-CAR and acetyl-L-CAR were eluted with 1 mL of 5% formic acid in acetonitrile and 1 mL of 5% formic acid in water. The eluates obtained from the two extraction steps (using silica gel cartridges for α-KG and using quaternary amine cartridges for L-CAR and acetyl-L-CAR) were combined, shaken for 10 s and transferred to LC inserts in injection vials for further analysis on LC–MS/MS by separate injections.

2.4. Liquid chromatographic and mass spectrometric conditions

The chromatographic separations were performed on a Dionex UPLC system (Dionex Corporation, Sunnyvale, CA, USA) equipped with an UltiMate 3000 RS (Rapid Separation) pump, an UltiMate 3000 autosampler, an UltiMate 3000 column compartment with a thermostable column area and an UltiMate 3000 variable wavelength detector, all of which were operated using Dionex Chromeleon™ 6.8 software. Chromatographic separations were performed on an Acclaim 120 C8 column (150 mm × 4.6 mm, 3.0 μm) equipped with an Acclaim 120 C8 precolumn (10 mm × 4.3 mm, 5.0 μm) (Dionex, Olten, Switzerland) using a mobile phase consisting of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) (97:3, v/v). Separations were conducted under isocratic conditions. The flow rate was set at 1.2 mL/min and resulted in a total run time of 3 min. To assure the reproducibility of the retention time, the column temperature was maintained at 35 °C. For optimal stability, the autosampler temperature was set at 5 °C. The sample volume injected was 10 μL.

Mass spectrometric analyses were performed using an AB SCIEX 4000 Q TRAP triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source. The data acquisition software used was Analyst™ version 1.4. The mass spectrometer was operated in the positive and negative ion modes using a TurboIonSpray™ ion source.

The operating parameters of the ion source, including the source-dependent and the compound-dependent ones, were optimized to obtain the best performance from the mass spectrometer for the analysis of compounds. The source-dependent parameters for all analyzed compounds were the nebulizer gas, the curtain gas, the collision gas, the ion spray voltage, and the temperature of the heater gas. In the positive mode, the MS/MS setting parameters were as follows: 30 psi curtain gas (CUR); 90 psi nebulizer gas (GS1); 80 psi drying gas (GS2); 3000 V ion spray voltage; 600 °C drying gas; and 150 ms dwell time. In the negative mode, the MS/MS setting parameters were as follows: the same curtain gas, nebulizer gas and turbo gas as above; −3000 V ion spray voltage; 600 °C heater gas; and 150 ms dwell time.

To increase the sensitivity of the qualification and quantification, the compound-dependent parameters were optimized by infusion of each individual analyte (500 ng/mL) using a Harvard syringe pump at a flow rate of 10 μL/min. Continuous mass spectra were obtained by scanning from 50 to 800 *m/z*. For good sensitivity and peak shape, a product ion was selected for the optimization of the MS parameters (i.e., declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP)).

Data acquisition was performed via selected reaction monitoring (SRM) using the parent ions and the corresponding product ions. The ions representing the $[M+H]^+$ species for L-CAR, acetyl-L-CAR, and MLD (IS) as well as the ion representing the $[M-H]^-$ species for α-KG were selected in the first quadrupole (Q1) and dissociated with nitrogen gas to form specific product ions. These ions were subsequently monitored by the third quadrupole (Q1). Two pairs of transitions were chosen for each compound metabolite using one parent ion and two product ions. One of two product ions was the quantitative ion and the other was the qualitative ion for each compound.

2.5. Method validation

Analytical method validation assays were performed as per the United States Food and Drug Administration (US-FDA) Bioanalytical Method Validation Guidance [41]. The analytical method was validated for selectivity, linearity, sensitivity, precision, accuracy, recovery, matrix effect and stability.

2.5.1. Selectivity

The selectivity of the method was evaluated by analyzing synthetic urine samples and synthetic spiked urine samples at the lower limit of quantification (LLOQ) from six different sources. The SRM chromatograms of the synthetic urine as blank samples were compared with SRM chromatograms obtained when the blank samples from the same source were spiked with the analytes and IS.

2.5.2. Linearity and sensitivity

Linearity was assessed by assaying calibration curves in urine at eight concentrations (0.1–500 ng/mL for L-CAR and acetyl-L-CAR; 1–1000 ng/mL for α-KG). A calibration curve was constructed by plotting the ratio of the analyte peak area/IS peak area versus analyte concentration. The linearity of the calibration curve was evaluated by linear regression analysis. The sensitivity of the developed method was determined using LLOQ. Blank samples were analyzed to confirm the absence of interference and the LLOQ was set as the lowest amount of analyte in a sample that could be quantitatively determined with acceptable precision and accuracy.

2.5.3. Precision and accuracy

The precision and accuracy of the method were assessed by assaying QC samples at four different concentrations: 0.08 ng/mL (LLOQ), 0.5 ng/mL (LQC), 80 ng/mL (MQC) and 400 ng/mL (HQC) for L-CAR; 0.04 ng/mL (LLOQ), 0.5 ng/mL (LQC), 80 ng/mL (MQC) and 400 ng/mL (HQC) for acetyl-L-CAR; and 0.8 ng/mL (LLOQ), 15 ng/mL

Table 1
The MS/MS parameters for the analyzed compounds.

Analyte	t_R [a] (min)	Parent type	Q1[b] (*m*/*z*)	Q3[c] (*m*/*z*)	DP[d] (V)	EP[e] (V)	CE[f] (V)	CXP[g] (V)
α-KG	2.19	$[M-H]^-$	144.9	101.0	−45	−11	−12	−3
			144.9	73.0		−9	−20	−1
L-CAR	1.57	$[M+H]^+$	162.1	103.0	40	3	17	4
			162.1	84.9		5	29	6
Acetyl-L-CAR	2.32	$[M+H]^+$	204.2	85.0	30	3	15	6
			204.2	144.9		5	19	10
MLD (IS)	1.66	$[M+H]^+$	147.2	58.1	56	5	37	4
			147.2	59.1		3	27	4

[a] Retention time.
[b] Parent ion.
[c] Fragment ion.
[d] Declustering potential.
[e] Entrance potential.
[f] Collision energy.
[g] Cell exit potential.

(LQC), 200 ng/mL (MQC) and 800 ng/mL (HQC) for α-KG. To evaluate intra-day accuracy and precision, QC samples were analyzed in six replicates at each concentration level. The inter-day accuracy and precision were determined by analysis of QC samples on three consecutive days. The concentration of each sample was determined using the calibration curve. The precision of the developed method was expressed as a relative standard deviation (RSD). Accuracy, defined as the relative error (RE), was calculated using the formula RE (%) = [(measured value − theoretical value)/theoretical value] × 100.

2.5.4. Recovery and matrix effect

The extraction recoveries for the analytes were calculated by comparing the mean area response of pre-spiked synthetic urine samples (set A – samples spiked before extraction) to the extracts with post-spiked samples (set B – samples spiked after extraction) at three QC levels. Recovery was obtained by comparing set A and set B; Recovery (%) = (set A/set B) × 100. The extraction recovery of each IS was determined in a similar way using the QC samples at high concentration as a reference.

The absolute matrix effect was assessed by comparing the mean area response of post-spiked synthetic urine samples (set B – samples spiked after extraction) with mean area of neat standard solutions (set C – in mobile phase). Matrix effects were obtained by comparing set B and set C; ME (%) = (set B/set C) × 100.

2.5.5. Stability

The stability of L-CAR, acetyl-L-CAR and α-KG in human urine under different storage conditions was determined by the analysis of six replicates of QC samples (LLOQ, LQC, MQC, HQC). For the short-term stability study, urine samples were kept at room temperature for 12 h, which exceeded the routine preparation time of the samples. To determine long-term stability, QC samples were stored at −20 °C for one month, which exceeded the time between sample collection and sample analysis. The freeze–thaw stability of the analytes was determined over three freeze–thaw cycles within three days. In each freeze–thaw cycle, the spiked urine samples were frozen for 24 h at −20 °C and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12–24 h at −20 °C. The post-preparative stability was measured by analyzing QC samples kept under autosampler conditions (5 °C) for 24 h. All the stability studies were done with six replicates. Samples were then processed, extracted, analyzed and compared with the freshly prepared calibration standard samples. For all experiments, concentration variations were expressed as a percentage of the nominal concentration measured at the beginning of the stability study (T_0).

3. Results and discussion

3.1. Method development

The MS/MS conditions were optimized for each compound by direct injection of each standard solution (500 ng/mL). Identification of parent ions was performed in the full scan mode by recording from 50 to 800 (*m*/*z*) in both positive (ESI^+) and negative (ESI^-) ionization modes. The results showed that the responses of the $[M-H]^-$ ions generated from α-KG under the ESI^- mode were higher than their $[M+H]^+$ ions generated under ESI^+ mode. On average, however, positive-ion ESI showed better sensitivity than negative-ion ESI for L-CAR, acetyl-L-CAR and MLD (IS). Therefore, the positive ion mode was used for the detection of these compounds. The mass spectrometer has a switch of polarity in the middle of run. Changing the polarity in the middle of the run (from positive to negative) has been tested, but more stable conditions for the analytical signal were obtained, when the samples were injected twice, once in positive mode, and once in negative mode.

Based on the confirmation of parent ions, two product ions for each parent ion were selected according to the highest sensitivity and optimal selectivity for the target compounds. The selected reaction monitoring (SRM) mode was developed for quantification.

The source-dependent parameters for all analyzed compounds were optimized by observing the maximum responses of the product ions. MS/MS operating conditions were systematically evaluated with standard solutions to optimize the ionization of the analytes and IS: CUR (10–50 psi, selected 30 psi), GS1 (40–90 psi, selected 90 psi), GS2 (30–80 psi, selected 80 psi), drying gas temperature (300–750 °C, selected 600 °C), and ion spray voltage (1500–4500 V, selected 3000 V).

The compound-dependent parameters were also optimized. DP was tested between 0 and 400 V for ESI^+ and between −400 and 0 V for ESI^-; CE between 0 and 130 V for ESI^+ and between −130 and 0 V for ESI^-; EP between 0 and 15 V for ESI^+ and between −15 and 0 V for ESI^-; and CXP between 0 and 55 V for ESI^+ and −55 and 0 V for ESI^-. DP were selected according to the sensitivity of the parent ions, whereas CE were chosen to give the maximum intensity of the fragment ions. EP and CXP did not greatly affect the analyte response. In CE and DP, however, the analyte response was better at lower values. The optimal conditions for all analyzed compounds are presented in Table 1 together with the *m*/*z* ratios of the $[M+H]^+$ and $[M-H]^-$ molecular ions. Fig. 1 shows the product ion mass spectra, the structures and possible fragmentations of all compounds in the SRM mode.

Different dwell times (from 50 to 250 ms) were used to identify the best detection parameters for obtaining a sufficient number

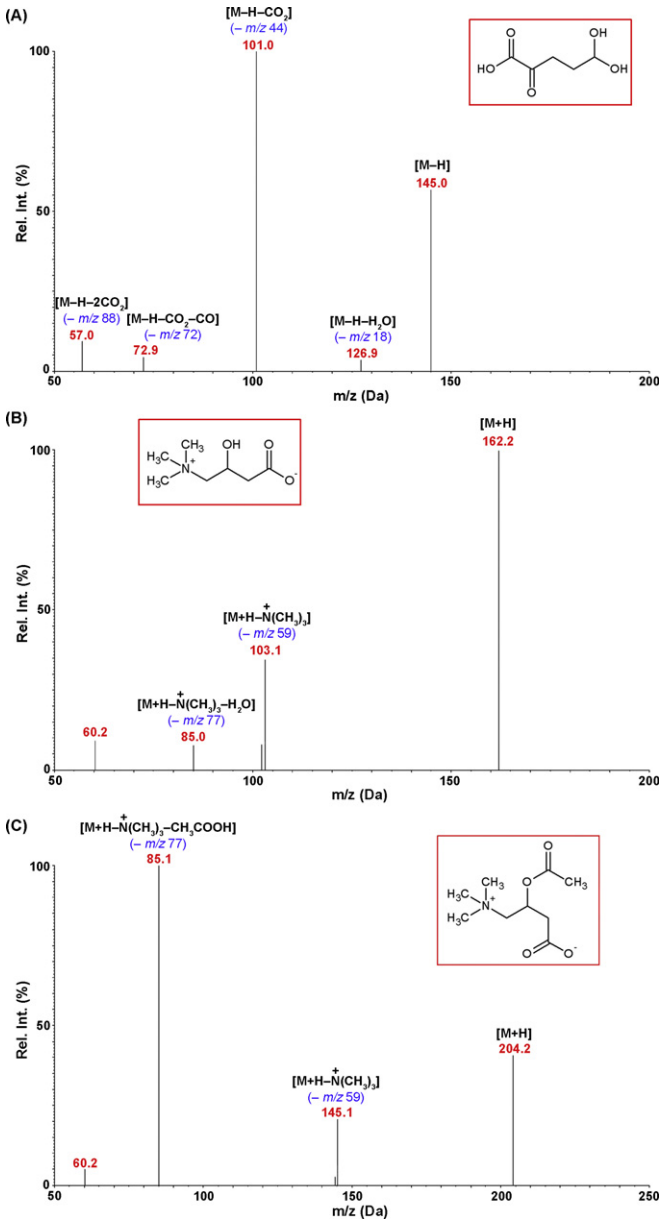

Fig. 1. Mass spectra of product ions of (A) α-KG in negative electrospray ionization mode and (B) L-CAR and (C) acetyl-L-CAR in positive electrospray ionization mode.

of data points across the peak. Negligible differences were found at low dwell times. An optimum dwell time of 150 ms was thus selected, resulting in at least 10 points per peak and using this dwell time gave reproducible results for determination and confirmation.

Analytical column selection and method development were carried out in regard to physical–chemical properties, the stability of the analytes and mass spectrometry detection, which limits the solvents that can be used. Chromatographic conditions, particularly the composition of the mobile phase, were optimized to increase analyte signals, achieve good separation and minimize running times. The composition of the mobile phase must also be concerned with the ionization efficiency, which is correlated with high sensitivity.

In the present study, chromatographic separation was optimized after investigating different aqueous and organic phase combinations in both isocratic and gradient programs. To achieve a suitable mobile phase, several solvent mixtures were tested, including acetonitrile, methanol, and water in various ratios. Better separation was achieved with water and acetonitrile than water and methanol in an isocratic elution. Specifically, significant peak tailing was observed for L-CAR, acetyl-L-CAR, α-KG and MLD (IS) when using a methanol mobile phase. Therefore, several combinations of acetonitrile and water were evaluated to sufficiently resolve each compound while minimizing both noise and peak tailing effects. The inclusion of formic acid in the mobile phase improved the chromatographic peak shape and increased the MS/MS response.

The analyzed compounds are weak acids with pK_a values of 3.80 for L-CAR, 3.60 for acetyl-L-CAR and 2.37 for α-KG. Thus, the analytes are protonated in an acidic mobile phase and hence show greater retention in reversed-phase separations. Chromatographic separation performed at low pH for the analysis of acid compounds is associated with improved chromatographic resolution due to higher retention of the compounds on the analytical support. Different concentrations of formic acid were therefore tested as an additive, and 0.1% was found to be the optimum because it gave higher peak intensity, better peak shape, a lower background and promoted ionization. The addition of 0.1% formic acid to the mobile phase also increased the accuracy of the analytes. An aqueous solution of 0.1% formic acid had sufficiently low pH and thus was used as the aqueous mobile phase, while acetonitrile was used as the organic modifier.

In the present study, Acclaim 120 C8 (150 mm × 4.6 mm, 3.0 μm), Acclaim 120 C18 (150 mm × 2.1 mm, 5.0 μm), Acclaim 120 C18 PAII (150 mm × 4.6 mm, 5.0 μm), and Acclaim C18 PAII (150 mm × 2.1 mm, 3.0 μm) (Dionex, Thermo Scientific) reverse phase columns were compared based on the peak shape and retention time. The C18 columns did not provide a good separation for the compounds of interest. The Acclaim 120 C8 column (150 mm × 4.6 mm, 3.0 μm) was thus chosen for the analysis of L-CAR, acetyl-L-CAR and α-KG because of its symmetrical peak shape, high intensity and favorable selectivity. L-CAR was eluted first at 1.57 min outside the dead volume of the column. As shown in Fig. 2, analytes were eluted within 3 min, with acetyl-L-CAR eluted last at 2.32 min. The retention times were sufficiently short for high throughput sample determination in these studies.

3.2. Method validation

3.2.1. Selectivity

The selectivity of this method in relation to endogenous compounds was established by confirming the absence of significant chromatographic peaks at the retention times of α-KG, L-CAR, acetyl-L-CAR and IS in six different individual human urine samples. The method was selective toward the matrix, as no interference was observed at the retention times of the analytes and the IS in synthetic urine samples. α-KG, L-CAR, acetyl-L-CAR and IS were eluted with good peak shapes. Furthermore, no interfering peaks were observed at any of the eluting positions for analytes and IS in any of the human urine samples collected from healthy individuals and from patients with cardiac failure.

3.2.2. Linearity and sensitivity

The calibration curve ranges for α-KG and L-CAR and acetyl-L-CAR were 1–1000 ng/mL and 0.1–500 ng/mL, respectively. Mean correlation coefficients of 0.9992, 0.9995 and 0.9997 were obtained for α-KG, L-CAR and acetyl-L-CAR, respectively ($n = 6$). The LLOQ samples for all compounds were analyzed with acceptable precision and accuracy (Table 2). Based on the evaluation results for selectivity, precision and accuracy, the LLOQ of biomarkers in human urine were determined to be 0.8 ng/mL for α-KG, 0.08 ng/mL for L-CAR and 0.04 ng/mL for acetyl-L-CAR.

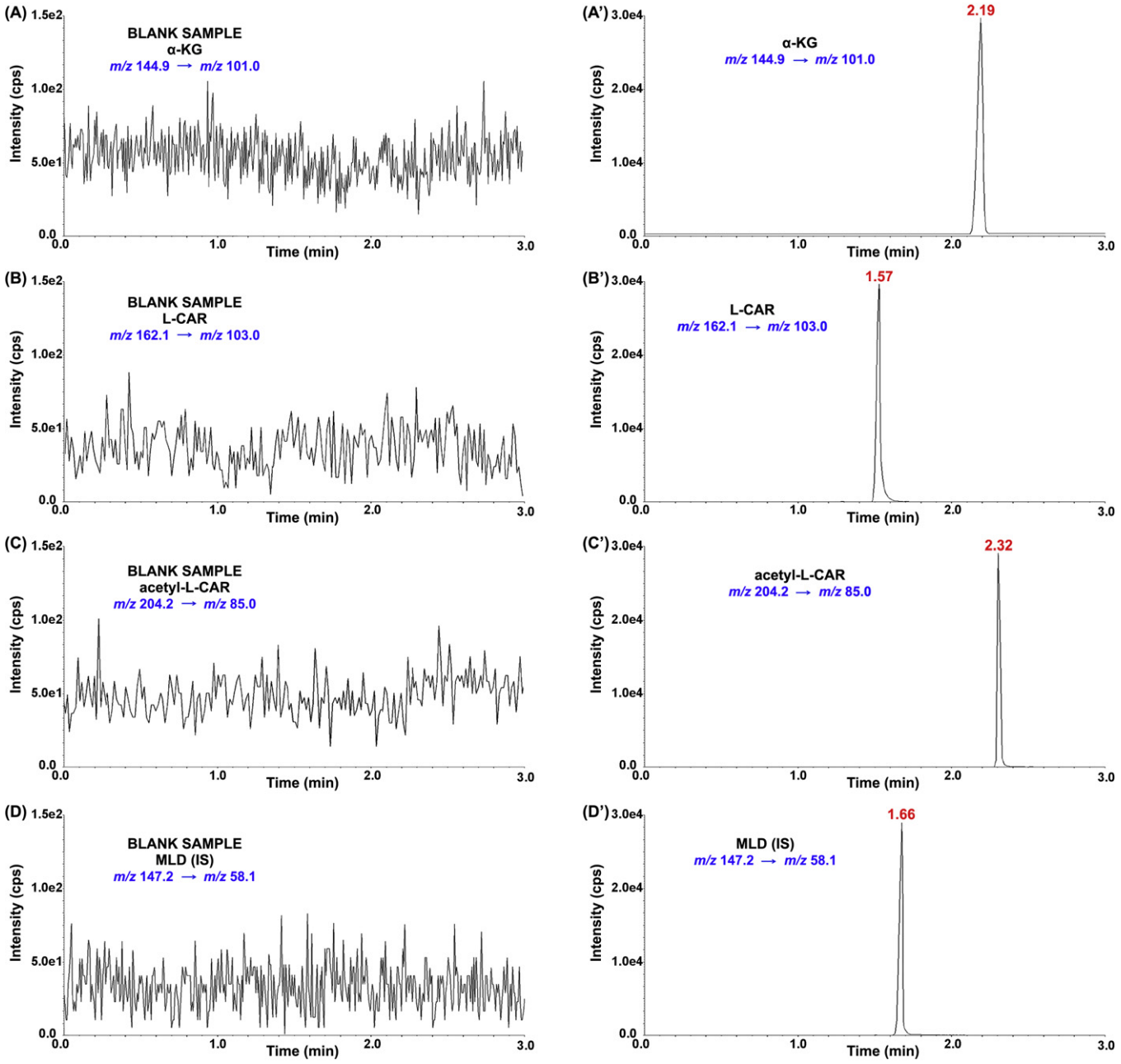

Fig. 2. Representative SRM chromatograms obtained from a blank synthetic urine sample (A–D) and from a mixed standards solution (A′–D′) containing (A) α-KG, (B) L-CAR, (C) acetyl-L-CAR and (D) MLD (IS) in synthetic urine sample.

3.2.3. Precision and accuracy

The intra-assay precision and accuracy for this method were determined by analyzing the QC samples at four concentrations (LLOQ, LQC, MQC and HQC; $n = 6$ for each level). The inter-assay precision and accuracy were also determined by analyzing the QC samples in three batches on different days. The precision and accuracy were presented as RSD (%) and RE (%), respectively and data for α-KG, L-CAR and acetyl-L-CAR in synthetic urine at four different concentration levels are presented in Table 2. The intra-day precision and accuracy ranged from 0.58% to 3.98% and from −3.98% to −1.20% for α-KG; from 1.20% to 5.01% and from −0.64% to 3.11% for L-CAR; from 2.02% to 2.87% and from −2.54% to 0.36% for acetyl-L-CAR. The inter-day precision and accuracy ranged from 0.45% to 4.23% and from −4.21% to −3.03% for α-KG; from 0.66% to 5.54% and from −5.95% to −2.32% for L-CAR; from 0.41% to 4.89% and from −2.65% to −0.37% for acetyl-L-CAR. These results suggested that the method assessed in this study had satisfactory accuracy, precision, and reproducibility.

3.2.4. Recovery and matrix effect

The extraction recoveries of α-KG, L-CAR and acetyl-L-CAR from synthetic urine were determined at three concentrations (LLOQ, LQC, MQC and HQC; $n = 6$ for each level) by comparing the peak area ratios (analyte/IS) of the QC samples (spiked before extraction) with those of the corresponding samples spiked post-extraction. In a similar manner, the recovery of IS was also evaluated based on the peak area ratios (IS/analyte). The results for the recovery evaluation are shown in Table 2. The mean extraction recoveries ranged from 96.89% to 106.49% for α-KG; from 92.27% to 98.96% for L-CAR and from 86.64% to 94.86% for acetyl-L-CAR. The precision of

Table 2
Intra-day and inter-day precision, accuracy, recovery and matrix effect of the method for determination of α-KG, L-CAR and acetyl-L-CAR in urine ($n = 6$).

Analyte	Nominal concentration (ng/mL)	Intra-day			Inter-day			Recovery (mean ± SD[c]) (%)	Matrix effect (mean ± SD[c]) (%)
		Calculated concentration (ng/mL)	RSD[a] (%)	RE[b] (%)	Calculated concentration (ng/mL)	RSD[a] (%)	RE[b] (%)		
α-KG	0.80	0.768	3.98	−3.98	0.766	4.23	−4.21	106.49 ± 4.91	108.65 ± 4.43
	15.00	14.490	3.77	−3.40	14.496	3.84	−3.36	102.03 ± 4.62	105.76 ± 4.32
	200.00	194.736	0.58	−2.63	193.934	0.45	−3.03	96.89 ± 6.15	99.65 ± 2.78
	800.00	790.392	0.76	−1.20	766.944	1.06	−4.13	99.83 ± 6.41	98.91 ± 3.21
L-CAR	0.08	0.082	5.01	3.11	0.075	5.54	−5.87	94.60 ± 1.83	96.21 ± 1.23
	0.50	0.512	4.34	2.34	0.470	3.54	−5.95	92.27 ± 5.67	95.89 ± 3.21
	80.00	81.145	1.59	1.43	77.512	2.27	−3.11	94.29 ± 4.46	94.89 ± 3.87
	400.00	397.432	1.20	−0.64	390.716	0.66	−2.32	98.96 ± 1.23	101.21 ± 1.54
Acetyl-L-CAR	0.04	0.039	2.87	−2.54	0.039	4.89	−2.65	94.24 ± 1.28	98.76 ± 5.21
	0.50	0.494	2.73	−1.15	0.493	4.72	−1.45	91.06 ± 4.05	96.43 ± 4.01
	80.00	79.798	2.06	−0.25	79.704	4.30	−0.37	86.64 ± 4.35	94.78 ± 3.72
	400.00	401.452	2.02	0.36	381.636	0.41	−4.59	94.86 ± 4.50	97.63 ± 2.37

[a] Relative standard deviation.
[b] Relative error.
[c] Standard deviation.

the extraction procedures for the studied analytes was lower than 6.41%. The IS recovery from synthetic urine was 97.9% with a RSD of 3.65%.

Matrix effect is an important issue when a new LC–MS/MS method is established, especially when using a Turbo Ionspray ionization interface. ME occurs when molecules coeluting with the compounds of interest alter the ionization efficiency of the electrospray interface. The importance of ME on the reliability of LC–ESI-MS/MS has been shown in terms of accuracy and precision. In the present study, the evaluation of the ME was conducted in synthetic urine samples following the procedures described above. The matrix effects were observed after the analysis of urine samples without pre-treatment. Therefore, it was necessary to use solid phase extraction to remove impurity. Table 2 contains the matrix effects for the analytes at different concentration levels. Values less than 100% indicate ion suppression, while values higher than 100% indicate ion enhancement by matrix components. Matrix effects on the analytes and IS were between 94.78% and 108.65%, and the RSD values from six lots of urine were less than 5.21%, indicating that no co-eluting substances influenced the ionization of either the analytes or the IS.

3.2.5. Stability

Stability assessments were carried out to demonstrate that α-KG, L-CAR, and acetyl-L-CAR were stable under typical sample storage and processing conditions. The stability experiments for urine were performed using QC samples at the LLOQ and low, medium and high QC levels. The mean values of the stability of QC samples at each level were compared to nominal concentrations. Several stability tests, including urine short-term, long-term and freeze–thaw storage stability, were performed, and the results are summarized in Table 3. α-KG, L-CAR, and acetyl-L-CAR in urine QC samples were stable at room temperature for 12 h, at least 30 days at both −20 °C and through three freeze–thaw cycles. The compounds of interest were also found to be stable in the autosampler (5 °C) for at least 24 h. These results suggested that human urine samples containing α-KG, L-CAR, and acetyl-L-CAR could be handled under normal laboratory conditions without any significant compound decomposition.

3.3. Application to patient samples

The main goal of this study was to develop a method for quantifying the compounds of interest in urine and to validate these markers using a controlled pool of patients and healthy subjects. This optimized and validated method was successfully utilized to analyze human urine samples collected from 20 donors (10 healthy people and 10 patients with cardiac failure).

Silica gel cartridge was used to adsorb α-KG from urine matrices with subsequent elution of the compounds in an organic solvent. Retention of an analyte under normal phase conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. These include hydrogen bonding, π–π interactions, dipole–dipole interactions, and dipole-induced dipole interactions, among others. A compound adsorbed by these mechanisms is eluted by passing a solvent that disrupts the binding mechanism – usually a solvent that is more polar than the sample's original matrix.

Quaternary amine cartridge was used to extract L-CAR and acetyl-L-CAR from urine samples. A quaternary amine is a strong base and exists as a positively charged cation that exchanges or attracts anionic species in the contacting solution – thus the term strong anion exchanger (SAX). The primary retention mechanism of the compound is based mainly on the electrostatic attraction of the charged functional group in the compound to the charged group that is bonded to the silica surface. The pK_a of a quaternary amine is very high (greater than 14), which makes the bonded functional group charged at all pHs when in an aqueous solution. As a result, in most cases, the compounds of interest are strong or weak acids [42].

Urine has become a popular medium for biomarker discovery due to its noninvasive nature, and patients often prefer to give urine samples rather than blood samples. In this study, human urine was analyzed for L-CAR, acetyl-L-CAR and α-KG as potential cardiovascular markers, and these compounds were quantified using internal standards and multiple-point standard curves. The samples were analyzed six times, and the resulting analyte concentrations in each urine sample are shown in Table 4. The variability in these determinations demonstrating the reproducibility and precision of this approach. As an example, the repeatability test of six replicates (each injected six times) of one urine sample in a single run (Table 4, Sample 1) gave a RSD in range 1.72–6.54% for α-KG, 0.65–5.20% for L-CAR and 0.98–5.49% for acetyl-L-CAR. In separate runs, 1 week, 2

Table 3
Stability of α-KG, L-CAR and acetyl-L-CAR in urine ($n = 6$).

Analyte	Nominal concentration (ng/ml)	Short-term stability			Long-term stability			Freeze–thaw stability			Post-preparative stability		
		Calculated concentration (ng/mL)	RSD[a] (%)	RE[b] (%)	Calculated concentration (ng/mL)	RSD[a] (%)	RE[b] (%)	Calculated concentration (ng/mL)	RSD[a] (%)	RE[b] (%)	Calculated concentration (ng/mL)	RSD[a] (%)	RE[b] (%)
α-KG	0.80	0.779	3.04	−2.65	0.721	4.19	−9.89	0.754	4.54	−5.76	0.768	3.24	−3.98
	15.00	14.546	3.11	−3.00	13.721	4.77	−8.53	14.079	3.53	−6.14	14.549	3.82	−3.00
	200.00	198.898	1.40	−0.55	180.580	3.15	−9.71	190.114	1.49	−4.94	196.234	2.49	−1.88
	800.00	782.064	1.87	−2.24	726.608	0.92	−9.17	747.024	1.61	−6.62	791.832	1.48	−1.02
L-CAR	0.08	0.077	4.38	−3.43	0.074	3.76	−7.32	0.077	4.96	−4.31	0.077	2.12	−4.12
	0.50	0.485	4.86	−3.07	0.463	3.11	−7.39	0.467	5.30	−6.53	0.482	1.76	−3.64
	80.00	79.263	1.03	−0.92	74.096	2.52	−7.38	78.152	2.02	−2.31	77.696	1.48	−2.88
	400.00	386.632	0.77	−3.34	371.516	1.06	−7.12	380.712	0.92	−4.82	383.032	1.16	−4.24
Acetyl-L-CAR	0.04	0.039	4.56	−3.12	0.036	2.32	−9.01	0.038	4.51	−4.91	0.039	2.38	−2.91
	0.50	0.486	4.22	−2.73	0.457	1.69	−8.65	0.482	3.43	−3.55	0.491	1.69	−1.90
	80.00	77.336	1.57	−3.33	73.624	1.63	−7.97	76.102	0.46	−4.87	79.624	3.62	−0.47
	400.00	387.636	0.68	−3.09	362.472	2.03	−9.38	393.428	1.02	−1.64	396.596	0.65	−0.85

[a] Relative standard deviation.
[b] Relative error.

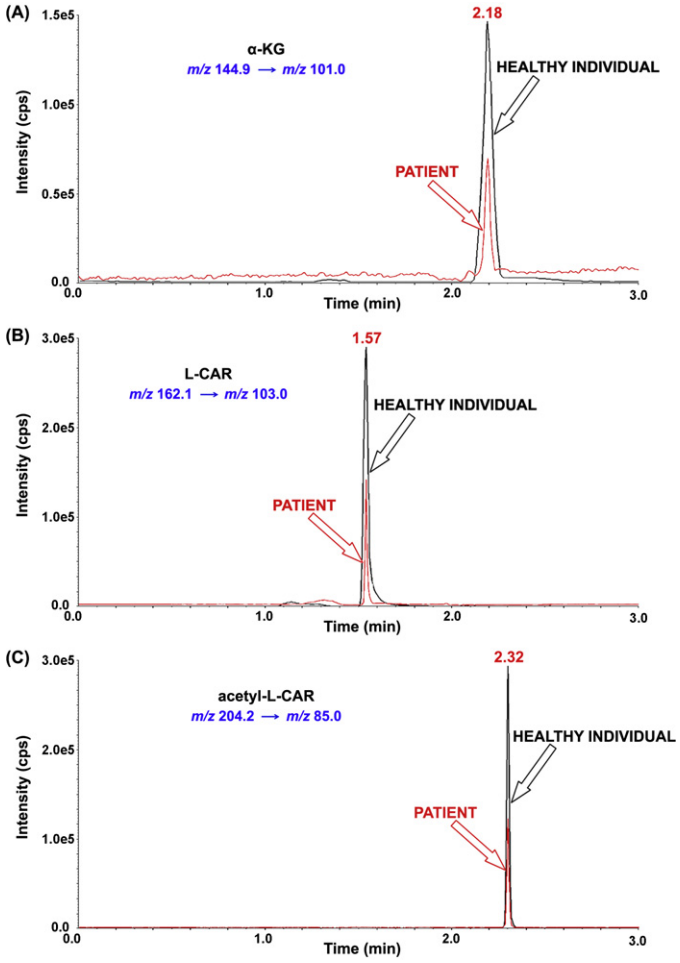

Fig. 3. SRM chromatograms of extracts of urine samples obtained from healthy individuals (black line) and patients (red line) (after dilution of urine samples 400 times) for (A) α-KG (healthy individuals: 131 ng/mL; patients: 34 ng/mL), (B) L-CAR (healthy individuals: 61 ng/mL; patients: 13 ng/mL) and (C) acetyl-L-CAR (healthy individuals: 58 ng/mL; patients: 11 ng/mL). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

weeks and 3 weeks later the RSD values were 1.20–7.80% for α-KG, 1.55–5.98% for L-CAR and 1.76–7.03% for acetyl-L-CAR. Application of this strategy to biological samples is illustrated in Fig. 3. Representative SRM chromatograms show the extract of a urine samples obtained from healthy individuals (black line) and patients (red line) for (A) α-KG, (B) L-CAR, and (C) acetyl-L-CAR. In this figure, the differences in the α-KG, L-CAR and acetyl-L-CAR and profiles are clearly demonstrated.

Application to patient urine samples proved that the developed HPLC–ESI-MS/MS method was able to identify α-KG, L-CAR and acetyl-L-CAR as potential biomarkers and can therefore be used for non-invasive diagnosis. The developed HPLC–ESI-MS/MS method can also be used to monitor concentrations of these biomarkers to find correlations between their levels in urine and the clinical status of patients with cardiovascular disease.

In this study the concentrations of α-KG, L-CAR and acetyl-L-CAR in human urine were examined with the use of two analytical techniques (HPLC-DAD for L-CAR, acetyl-L-CAR and HPLC-FL for α-KG). Thus, the procedures previously described in the literature for L-CAR and acetyl-L-CAR [11] and for α-KG [35] were applied. The results obtained with different methods for the same samples were compared. Based on the obtained results it can be concluded that both the methods provided similar results (healthy individuals: L-CAR: 20–160 μg/mL; acetyl-L-CAR: 15–130 μg/mL; α-KG:

Table 4
Analyte concentrations in urine samples obtained from healthy individuals and heart disease patients ($n = 6$).

	L-CAR			Acetyl-L-CAR			α-KG		
	Concentration (ng/mL)[a]	Concentration (μg/mL)[b]	SD[c] (μg/mL)	Concentration (ng/mL)[a]	Concentration (μg/mL)[b]	SD[c] (μg/mL)	Concentration (ng/mL)[a]	Concentration (μg/mL)[b]	SD[c] (μg/mL)
Healthy individuals									
Sample 1	85.432	34.173	1.561	53.918	21.567	1.092	154.402	61.761	2.561
Sample 2	77.822	31.129	1.389	50.563	20.225	1.348	107.903	43.161	1.987
Sample 3	79.205	31.682	1.461	57.712	23.085	1.154	107.020	42.808	2.141
Sample 4	79.820	31.928	0.987	81.402	32.561	1.871	214.716	85.886	5.431
Sample 5	85.817	34.327	1.832	51.208	20.483	0.926	129.184	51.674	2.655
Sample 6	60.755	24.302	1.209	217.679	87.072	4.210	131.865	52.746	2.104
Sample 7	387.707	155.083	7.541	326.825	130.730	6.365	205.382	82.153	4.076
Sample 8	115.106	46.042	2.121	40.806	16.322	0.161	60.012	24.005	1.102
Sample 9	167.766	67.106	3.053	204.348	81.739	4.002	118.221	47.288	2.644
Sample 10	223.116	89.247	3.423	90.075	36.030	1.015	45.757	18.303	0.151
Patients									
Sample 11	3.914	1.565	0.073	8.652	3.461	0.107	31.433	12.573	0.627
Sample 12	12.777	5.111	0.155	11.383	4.553	0.247	26.444	10.577	0.529
Sample 13	1.607	0.643	0.031	3.526	1.410	0.035	10.699	4.279	0.021
Sample 14	0.623	0.249	0.015	3.163	1.265	0.063	12.182	4.873	0.246
Sample 15	1.461	0.584	0.022	7.510	3.004	0.152	24.747	9.899	0.594
Sample 16	0.130	0.052	0.002	1.581	0.632	0.038	34.488	13.795	0.689
Sample 17	0.546	0.218	0.010	6.546	2.618	0.121	22.710	9.084	0.252
Sample 18	4.190	1.676	0.088	10.867	4.347	0.113	67.581	27.032	1.016
Sample 19	0.523	0.209	0.015	6.995	2.798	0.109	51.662	20.665	1.332
Sample 20	2.189	0.876	0.048	5.205	2.082	0.101	20.448	8.179	0.309

[a] Concentration of analytes in 1 mL of eluate, after SPE procedure, calculated from calibration curve (after dilution of urine samples 400 times).
[b] Concentration of analytes in 1 mL of urine samples.
[c] Standard deviation.

20–90 μg/mL in urine sample and patients: L-CAR: 0.05–6 μg/mL; acetyl-L-CAR: 0.5–5 μg/mL; α-KG: 4–30 μg/mL in urine sample). Fig. 4 presents (A) the HPLC-DAD (for α-KG) and (B) HPLC-FL (for L-CAR and acetyl-L-CAR) chromatograms of the extract of a human urine sample. Concentrations of α-KG, L-CAR, and acetyl-L-CAR in human urine obtained by the methods are generally comparable, but differ in accuracy, sensitivity and precision. HPLC–ESI-MS/MS method for the detection of selected compounds in urine is more sensitive than HPLC-DAD and HPLC-FL. Furthermore, the method developed in this study is much simpler to be executed and allows for simultaneous determination of α-KG, L-CAR, acetyl-L-CAR as potential cardiac biomarkers.

This research represents the development of a method that allows the simultaneous determination of three potential biomarkers for heart disease. However, it is still unclear how L-CAR, acetyl-L-CAR, and α-KG can be used as reliable diagnostic biomarkers. Therefore, more detailed clinical studies are needed to validate these markers. In any case, this LC–MS/MS method provides a valuable tool for urinary analysis of α-KG, L-CAR and acetyl-L-CAR in heart disease.

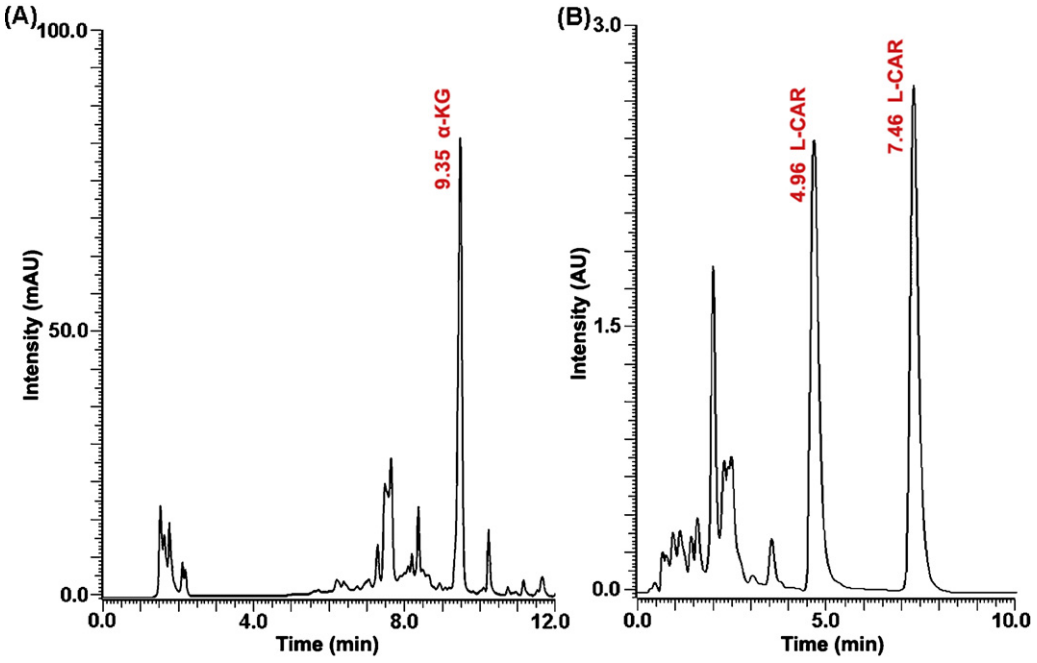

Fig. 4. (A) HPLC-DAD and (B) HPLC-FL chromatograms obtained from an extract of a human urine sample.

4. Conclusions

A reliable, fast, and simple HPLC–ESI-MS/MS method was developed to simultaneously separate and detect three potential biomarkers of heart disease (L-CAR, acetyl-L-CAR, and α-KG) in urine samples. Parameters affecting LC separation and MS/MS detection were investigated and optimized to allow complete separation of the above compounds within 3 min. The proposed HPLC–ESI-MS/MS method was fully validated and showed appropriate specificity, linearity, sensitivity and precision for all the analytes studied. The developed method is the first direct method for the simultaneous analysis of the studied compounds. It is expected that this method can be applied in clinical research to study correlations between the levels of L-CAR, acetyl-L-CAR and α-KG in urine samples and the clinical status of patients with cardiovascular disease. However, a large representative group of patients and a large group of healthy subjects must be studied to confirm these compounds as reliable biomarkers.

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