Short communication

A Novel Derivatization Procedure and Chiral Gas Chromatographic Method for Enantiomeric Purity Screening of L-Carnitine

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Abstract

L-Carnitine is used extensively in functional foods and food supplements; consequently, the control of its enantiomeric purity is of paramount importance. A new derivatization procedure and chiral gas chromatographic method with flame ionization detection, using a cyclodextrin based stationary phase, enables prompt, simple, and inexpensive screening of the enantiomeric ratio of L- and D-carnitine in samples with different matrices. Conversion of carnitine to β-acetoxy-γ-butyrolactone was optimized for maximum conversion (98% of the desired product lactone was formed and 2% of the side product γ-crotonolactone) and minimum racemization (no changes at the chiral center were detected) and time consumption. As it is shown in this study, a fast gas chromatographic method, with total run time of 7 min, together with the new derivatization procedure enables an effective enantiomeric purity screening of L-carnitine in real samples such as food supplements and L-carnitine raw ingredient.

Keywords: L-carnitine, D-carnitine, GC-FID, derivatization, enantiomeric separation, food supplements.

1. Introduction

Carnitine (3-hydroxy-4-(trimethylazaniumyl)butanoate, **1**) (Fig. 1) and its ester analogues are chiral compounds that can play an important biochemical role and possess properties which are beneficial to human health.[1–4] In fact, only *R*-enantiomer (L-carnitine) is significant in the energy producing pathway; the *S*-enantiomer (D-carnitine) is believed to be inactive and to even cause inhibition of carnitine acetyl-transferase.[5,6]

The zwitterionic L-carnitine is non-volatile and is biosynthesized from lysine and methionine. It facilitates the transport of long-chained fatty acids from the cytosol into the mitochondria, where the breakdown of fatty acids and energy production occurs.[7] Human genetic disorders such as primary and secondary carnitine deficiencies lead to disturbances in energy production and in the intermediary metabolism of the organism.[8,9]

Due to the increasing use of L-carnitine in functional foods and food supplements, monitoring of the enantiomeric purity of L-carnitine in these products is essential. This also applies to the L-carnitine feedstock used in food production. Consequently, a fast and inexpensive analytical procedure is needed.

There are many high-performance liquid chromatographic (HPLC)[10–13] and capillary electrophoretic (CE)[12–18] methods available for the separation of the enantiomeric L- and D-carnitine; even ^{1}H NMR methods using chiral shift reagents have been utilized.[19] These methods give good results but are tedious and require expensive chiral derivatization reagents or chiral buffer selectors, which makes a routine analysis impractical. On the other hand, no simple gas chromatographic (GC) methods exist for the screening of L- and D-carnitine in real samples; this is probably due to the non-volatility barrier of carnitine. In this regard, an efficient transformation of this amino acid from non-volatile to volatile form is crucial. Di Tullio et al. reported on the first

and only gas chromatographic resolution of L- and D-carnitine, based on standards.[20] The attempt to repeat this experiment in our laboratory proved unfruitful.

The aim of the present study was to develop a new derivatization procedure and chiral GC method for the rapid screening of the enantiomeric purity of L-carnitine in various samples, irrespective of the sample matrix. The derivatization procedure should also be useful for the GC analysis of acylcarnitines.

2. Experimental

2. 1. Materials

Food supplement samples in capsules: L-carnitine (Food Supplement 1 – FS1) was purchased from Goerlich Pharma (Edling, Germany), L-karnitin 500 (FS2) from Encian d.o.o. (Donji Stupnik, Croatia), ActivLab L-Carnitine Plus Green Tea (FS3) from Regis Ltd. (Krakow, Poland), and Liver Aid (FS4) from 4Life® (Farmington, CT, USA). Raw ingredient samples: L-carnitine (Raw Ingredient 1 – RI1) and L-carnitine tartrate (RI2) were purchased from Maypro (Shanghai, China), L-carnitine tartrates (RI3 and RI4) from Lonza (Basel, Switzerland), and L-carnitine (RI5) from Brenntag Chemicals Trading Co. Ltd. (Shanghai, China).

2. 2. Chemicals and Standards

HPLC grade methanol (MeOH) was purchased from Baker (Derenter, Netherlands), analytical grade acetyl chloride from Merck (Darmstadt, Germany), analytical grade pyridine from Carlo Erba Reagenti (Milano, Italy). HPLC grade acetonitrile (MeCN), *N*,*N*-Diisopropylethylamine (DIPEA) (>99%), dimethylformamide (DMF) (>99.9%), A.C.S. Reagent grade dimethyl sulfoxide (DMSO) (99.9%), L- and D-carnitine (98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Pyridine was distilled prior to use. Twice distilled water was used.

2. 3. Sample Preparation Prior to Derivatization

No sample preparation was used for pure carnitine (inner salt) samples RI1, RI5 (500 mg) and standards L- and D-carnitine (80 mg).

Sample preparation for RI2 – RI4. The sample (500 mg) was dissolved in water (1 mL), filtered through a 0.45 μm Millipore Millex-HV hydrophilic polyvinylidene difluoride-PVDF membrane filter (Middlesex, MA, USA) and concentrated to the point at which the sample remained in solution.

In the case of food supplements, the contents of four identical capsules were mixed and homogenized with a pestle and mortar. Then, from this homogenized material, the amount of sample was accurately weighed for each individual analysis (~500 mg for FS1, FS2, and ~700 mg for FS3, FS4). DMSO (10 mL) was added to the sample, vortexed for 30 s, and centrifuged for 4 min at 3600 rpm. Supernatant was decanted and to the solid residue MeOH (1 mL) and then H_2O (1 mL) were added. The suspension was vortexed for 30 s, filtered through a 0.45 μm PVDF filter and concentrated to the point at which the sample remained in solution.

2. 4. Derivatization of Carnitine

To the prepared samples and standards DMF (2 mL) and DIPEA (0.4 mL – only when analyzing RI2 – RI4 and FS1 – FS4) were added and the suspension was refluxed for 1h; to simplify this step the cyclization of carnitine standard was alternatively achieved under microwave radiation at 170 °C for 5 min in MeCN (1.5 mL). Volatile components were subsequently evaporated under reduced pressure; the remaining β-hydroxy-γ-butyrolactone was redissolved in pyridine (2 mL) and filtered through a 0.45 μm PVDF filter. Acetyl chloride (0.3 mL) was added drop wise to a solution of the lactone in cold pyridine, and the solution was heated to reflux and immediately cooled to room temperature. MeOH (2 mL) was added to the solution. A volume of 0.2 mL was taken from the solution and all volatile components were removed under reduced pressure to obtain β-acetoxy-γ-butyrolactone. The solid residue was redissolved in MeOH (1 mL) without purification, filtered through a 0.45 μm PVDF filter, and subjected to chiral GC analysis. In the case of the analysis of FS4 the sample subjected to GC was concentrated 10-fold relative to the other samples.

Microwave reactions were conducted using a focused microwave unit (Discover by CEM Corporation, Matthews NC). The machine consists of a continuous, focused microwave power delivery system with an operator-selectable power output from 0 to 300 W. Reactions were performed in glass vessels (capacity 10 mL) sealed with a septum. The mixtures were stirred with a Teflon-coated magnetic stir bar in the vessel.

2. 5. GC-FID Analysis

GC-FID analyses were conducted on a VARIAN 3900 system (Varian, Palo Alto, CA, USA) using a fused-silica capillary CP-Chirasil-Dex CB column (0.25 μm; 25 m × 0.25 mm I.D., Varian). Conditions were as follows: carrier gas (H_2) at 1.5 mL/min; injection port T = 200 °C, FID T = 225 °C, 130 °C isotherm. The injection volume was 0.3 μL, and split ratio 1:25. Galaxie Workstation software version 1.7.403.22 was used for the evaluation of the data collected.

3. Results and Discussion

Carnitine and its analogues are non-volatile compounds and are soluble almost exclusively in protic sol-

vents due to their zwitterionic nature. Volatility and solubility of derivatized carnitine in organic solvents are, however, enhanced; hence the amenability of carnitine to GC analysis.

This report is focused on an innovative derivatization procedure for carnitine (also applicable to acylcarnitines) in GC analysis. Additionally, a fast chiral GC-FID method, based on a cyclodextrin capillary column, was developed, which enables the resolution of L- and D-carnitine. The new derivatization procedure combined with chiral GC method permits the analysis of a large number of samples in a limited amount of time.

3. 1. Derivatization Procedure and Chiral GC-FID Method

Using our approach, little or no sample preparation is required prior to derivatization of carnitine.

The derivatization process involves two steps (Fig. 1).

First, carnitine (**1**) cyclizes to give β-hydroxy-γ-butyrolactone (**2**); in cases where carnitine does not form an inner salt, DIPEA was added. Secondly, the β-hydroxy-γ-butyrolactone was acetylated with acetyl chloride to give β-acetoxy-γ-butyrolactone (**3**). The second step should be omitted when acylcarnitines are subjected to gas chromatography, consequently even further simplifying the derivatization.

With the introduction of microwave technology the intramolecular cyclization of carnitine occurs significantly more rapidly than similar known transformations.[21] The second step is at least comparable in speed and efficiency to other state-of-the-art acylations.[22] The acetylation step was done merely to drastically improve peak shape, and consequently resolution, and to substantially shorten the retention times of L- and D-carnitine to ~6 minutes when applied to chiral GC analysis.

The transformation of carnitine to β-acetoxy-γ-butyrolactone was carefully conducted to maximize the overall conversion, minimize by-product formation and to make it as rapid as possible. In the first step, 98% β-hydroxy-γ-butyrolactone was formed but also 2% γ-crotonolactone, which did not interfere in the subsequent transformations and analyses. In the second step, β-hydroxy-γ-butyrolactone (**2**) was fully transformed into β-acetoxy-γ-butyrolactone (**3**). Furthermore, the chiral centre in the carnitine molecule was not affected and consequently no sign of racemization was observed after either of the two derivatization steps. The excellent overall yield underlies the good sensitivity of the chiral GC method.

In the first (and only) report of the gas chromatographic resolution of L- and D-carnitine standards, Di Tullio et al. converted D- or L-carnitine, respectively, to the corresponding β-hydroxy-γ-butyrolactones which were separated in 20 min using a β-cyclodextrin stationary phase column that differed from the one we used.[20] It should be stressed that we made every endeavour to repeat these experiments but were unable to achieve reported results. Firstly, a problem with solubility of carnitine in nitromethane, the injection solvent for GC, was encountered. And secondly, a considerable amount of side products formed in the injector

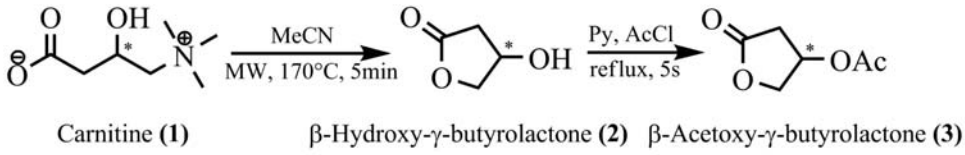

Fig. 1. Two-step derivatization procedure for L- and D-carnitine.

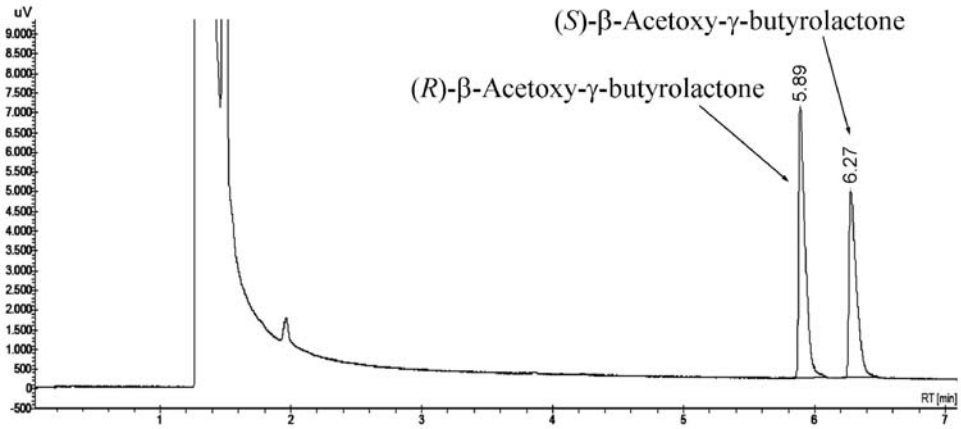

Fig. 2. GC-FID separation of (*R*) and (*S*)-β-acetoxy-γ-butyrolactone. Chromatographic conditions are given in the experimental section.

port at 200 °C where the cyclization of carnitine takes place, and this greatly reduces the sensitivity of the method. The array of side products may result from different GC injector types, which detrimentally affects the wide applicability of this method. The aforementioned work was done on standards; however, real sample matrices often include additional compounds which may even further complicate the analysis. We overcame these problems with our two step pre-chromatographic derivatization described above. In addition, the new GC method enables baseline resolution of carnitine enantiomers in under 7 min (Fig. 2).

3. 2. Enantiomeric Purity Screening of L-carnitine in Real Samples

The new derivatization procedure/chiral GC method for screening of enantiomeric purity of L-carnitine was tested on five raw ingredient samples and four different food supplement samples with different matrices, giving the results shown in Table 1. The quantitation limit for L- and D-carnitine was determined at 2mg/g (S/N ≥ 10).

Table 1. Enantiomeric purity of L-carnitine in samples.

Sample	D-carnitine (relative to L-carnitine) found in sample (%)
FS1	0.4 ± 0.2
FS2	< 0.2
FS3	< 0.2
FS4	< 0.2
RI1	< 0.2
RI2	< 0.2
RI3	< 0.2
RI4	< 0.2
RI5	0.2 ± 0.2

All samples showed good enantiomeric purity of L-carnitine and no matrix effects were observed during the derivatization procedure or GC analysis. Some of the samples analyzed possessed dense matrices; different amino acids, choline, α-ketoglutaric acid, α-lipoic acid, citric acid, silymarin and green tea extract, gelatin, microcrystalline cellulose, magnesium stearate and lactose and other substances were present.

4. Conclusions

Although the analytics of carnitine and acylcarnitines is already extensively covered in the literature, the enantioseparations in this field, especially GC, are still not well covered, thus, new rapid, efficient, and reliable chromatographic methods for the resolution of L- and D-carnitine are needful.[23,24]

In this study we developed a fast chiral GC method for baseline enantiomeric resolution of L- and D-carnitine using a cyclodextrin-based capillary column, with prior derivatization for enhanced volatility and solubility of carnitine in organic solvents. No racemization was observed during the derivatization process. The enantiomeric composition of L- and D-carnitine was determined as the enantiomeric composition of the β-acetoxy-γ-butyrolactones.

This new derivatization procedure, together with the newly developed GC method excels in simplicity, applicability, and minimal sample loss (maximum conversion). The total analysis time is greatly shortened in comparison to other enantiomeric determinations of L- and D-carnitine and the overall analysis is relatively inexpensive. The general applicability of the method was demonstrated by screening the enantiomeric purity of L-carnitine in four food supplements and five raw ingredients.

The future work will be directed towards the quantification of L-carnitine. The chemical properties of D-carnitine are identical to those of L-carnitine and this should make the use of the former, as an internal standard, ideal for the determination of L-carnitine in various samples, where side reactions and other interferences are possible during the derivatization process and GC analysis.

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6. References

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Povzetek

L-karnitin se intenzivno uporablja v obliki funkcionalnih živil in prehranskih dopolnil, zato je nadzor njegove enantiomerne čistote izjemnega pomena. V ta namen smo razvili nov derivatizacijski postopek in kiralno plinsko kromatografsko metodo s plamensko ionizacijsko detekcijo, pri čemer smo uporabili ciklodekstrinsko stacionarno fazo. Ta kombinacija omogoča hitro, enostavno in poceni kontrolo enantiomernega razmerja L- in D-karnitina v vzorcih z različnimi matriksi. Pretvorbo karnitina v β-acetoksi-γ-butirolakton smo optimizirali z namenom maksimalne konverzije (nastalo je 98 % želenega laktonskega produkta in 2% stranskega produkta γ-krotonolaktona) ter minimalne racemizacije (na kiralnem centru ni bilo sprememb) in porabe časa. Pokazali smo, da nova plinska kromatografska metoda, s časom analize 7 minut, v povezavi z novim derivatizacijskim postopkom omogoča učinkovito kontrolo enantiomerne čistote L-karnitina v realnih vzorcih kot so prehranska dopolnila in vhodne surovine.