ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 209–218

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

A chiral HPLC method for the determination of low amounts of D-carnitine in L-carnitine after derivatization with (+)-FLEC

Sascha Freimüller, Hans Altorfer *

Institute of Pharmaceutical Sciences, Federal Institute of Technology (ETH), 117 M48 Winterthurerstr, 190, CH-8057 Zurich, Switzerland

Received 10 September 2001; received in revised form 2 April 2002; accepted 13 April 2002

Abstract

An indirect enantioseparation method for robust and precise determination of D-Carnitine (D-C) in L-Carnitine (L-C) in the range of 0.1–1.0% is presented. The method is based on derivatization of Carnitine with (+)-[1-(9-fluorenyl)-ethyl]-chloroformate ((+)-FLEC). The two diastereomers are subsequently separated of on an octadecyl column using detection of the eluent by fluorescence (260 excitation, 310 nm emission monitoring). This procedure can be calibrated conveniently by diluting the derivatization solution of the sample. Hence, D-C was determined indirectly through quantification of L-C thereby strongly increasing the robustness and reducing the costs. During the development of the method a study was undertaken to prove that the method is suitable to determine enantiomeric purity of L-C indeed. Moreover, the method was validated according to the ICH guidance, which required the additional performance of a collaborative study. The proposed assay can be carried out using an autoinjector because the derivatives are very stable. Hence, we believe that this method will become popular for reliable determination of enantiomeric purity of L-C. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Carnitine; High-performance liquid chromatography; Enantiomer separation; System suitability test; Validation

1. Introduction

L-Carnitine (L-C) is a vitamin-like amino acid derivative, which is an essential cofactor in fatty acid metabolism. Therein, it plays an important role in the transport of fatty acids across the mitochondrial membrane [1]. L-C is highly therapeutically effective and, therefore, used worldwide for various nutritional and pharmaceutical applications. In contrast, D-Carnitine (D-C) unfortunately displays serious side-effects, and hence the content of D-C must be precisely determined and limited in pharmaceutical and nutritional formulations [2–4]. So far, the content of D-C in the European Pharmacopoeia [5] and United States Pharmacopoeia [6] is limited to about 4%, as determined by optical rotation measurements. However, this method is neither selective nor sen-

* Corresponding author. Tel.: +41-1-635-6064; fax: +41-1-635-6885
E-mail address: altorfer@pharma.anbi.ethz.ch (H. Altorfer).

PII: S0731-7085(02)00341-2

sitive. This analytical shortcoming excludes the setting of lower limits although currently used manufacturing processes are capable to produce purer L-C. A new method with an adequate sensitivity range and robustness is highly desired.

Generally, the determination of enantiomeric purity of L-C can be performed in two different ways. The first is to directly determine the content of L-C without any previous separations by measurement of the optical rotation, by integrating signals reagents due to the two enantiomers which were separated through chiral shift reagents by NMR or by enzyme-mediated assays. None of the published methods is suitable considering the required sensitivity and precision for determination of D-C in L-C below levels of 1% [7,8]. The second strategy is based on separation by HPLC or CZE. Due to the fact that CZE is less utilized in common labs, more complex and yields less reproducible results HPLC is the preferred technique. The injected compound may either be chemically derivatized with a chiral compound and subsequently separated into the corresponding diastereomers on an achiral column. Alternatively, either chiral mobile phase additives or chiral stationary phases may be utilized to directly separate the enantiomers. Unfortunately, due to weak absorption of Carnitine in the UV/VIS region and the lack of fluorescence, the direct separation approach lacks to achieve adequate sensitivity and hence the pre-column derivatization is necessary. The synthetically most easily accessible route for derivatization involves the introduction of a UV or fluorescent active group by esterification of the chiral hydroxyl group. Thereby, the difference in physicochemical properties of the two stereoisomers increase and a higher sensitivity is achieved. Chiral derivatization reagents such as (+)-[1-(9-fluorenyl)-ethyl]-chloroformate ((+)-FLEC) and L-alanine-β-naphtylamide [9–12] and achiral reagents such as 9-anthroylnitrile or 9-anthroyldiazomenthane [13–15] have been described in the literature. In order to gain adequate sensitivity for precise enantio-purity testing at levels of less than 1% of D-C in L-C, both of the parameters, the response of the derivatives and the injected amount have to be maximized. For this, achiral derivatization in general is unsuitable as separation of the enatiomers requires utilization of chiral columns. These columns have limited capacities thereby being not sufficiently loaded with the analyte. On the other parameter, only the introduction of a fluorescent active group enables sufficient sensitive detection of the derivatives, hence (+)-FLEC is the preferred reagent for chiral derivatization, as L-alanine-β-naphtylamide does not show fluorescence activity. Furthermore, octadecyl columns, which are used in most laboratories and compendial monographs can be used to separated the (+)-FLEC derivatives [9–11].

This study presents a precise, fast and robust method to determine the content of D-C in L-C in the range of 0.1–1%. The complete procedure was validated according to the ICH Q2B guidance [16] and a collaborative study was performed to demonstrate the applicability of the method.

2. Experimental

2.1. Reagents and chemicals

L-C reference standard, racemic D/L-C and the samples for the collaborative study were supplied by Lonza Ltd. (Visp, Switzerland), D-C Inner Salt (98% pure) was purchased from Sigma (St. Louis, USA) and (+)-[1-(9-fluorenyl)-ethyl]-chloroformate ((+)-FLEC) was obtained as a 50 mg solution in 10 ml acetone ($c \geq 18$ mM, p.a., chira select $> 99.5\%$,) from Fluka (Buchs, Switzerland). Sodiumcarbonate (p.a.), sodiumhygrogenecarbonate (p.a.), triethylamine (p.a.), phosphoric acid (84%) and sodium hydroxide (p.a.) were from Fluka (Buchs, Switzerland), acetic acid (100%, p.a.) was from Merck (Darmstadt, Germany), acetonitrile or ACN (p.a.) was from Scharlau (Barcelona, Spain). Water was distilled with a destillator of Kleiner AG (Wohlen, Switzerland). The 0.05 M carbonate buffer was prepared by dissolving 338 mg of anhydrous sodium carbonate and 152 mg of sodium hydrogencarbonate in 100 ml of water. The 0.05 M acetate buffer was prepared by dissolving 3.0 g of glacial acetic acid in 900 ml of water, adjusting the pH with sodium hydroxide to 4.2 and diluting to 1000 ml with water.

For any spiking procedures the L-C reference standard from Lonza Ltd. (Visp, Switzerland) was used. The content of Carnitine determined by titration ($HClO_4$) was 99.8% and the content of D-C determined by the procedure as described here was about 0.01%. The spiking was always carried out with racemic Carnitine (water content below 0.1%, angle of rotation was 0 °).

2.2. Apparatus and chromatographic conditions

HPLC measurements were performed on a LaChrome apparatus (Merck, Switzerland) equipped with an autosampler L-7200, a pump L-7100 with a 30 μl sample loop, degasser L-7612, Fluorescence detector L-7480 (medium sensitivity), interface D-7000, software D-7000 HSM and hardware 590 (Compaq Deskpro). The HPLC column used was LiChrospher 100, RP-18 endcapped (5 μm), 250 × 4,6 mm I.D. (Merck, Switzerland) with a pre-column LiChrospher 100, RP-18 endcapped (5 μm), 4 × 4,6 mm I.D. (Merck, Switzerland). The flow rate was kept at 2.0 ml/min at ambient temperature. The mobile phase for the separation was prepared by mixing triethylamine phosphate buffer and acetonitrile at a ratio of about 73:27. The buffer was prepared by diluting 6.8 ml triethylamine with water to 950 ml, the pH adjusted with 84% phosphoric acid to 2.6 and finally diluted to 1000 ml with water. The system was programmed to provide variable mixtures of acetonitrile and the mobile phase (see Table 1). The wavelength for excitation was 260 nm and for emission monitoring 310 nm.

Table 1
Gradient programming for separation and column cleaning

Time (min)	Mobile phase (%)	ACN (%)	Comment
0	100	0	separation
ca.30	100	0	
ca.31	2	98	cleaning
ca.45	2	98	
ca.46	100	0	equilibration
ca.51	100	0	

2.3. Derivatization procedure and determination of the purity

About 25 mg of L-C was diluted with water in a 5 ml-volumetric flask. 30 μl of this solution was pipetted into a 5 ml-volumetric flask, 30 μl of carbonate buffer and 80 μl of (+)-FLEC reagent were added and the flask was closed and gently swirled for some seconds. This solution was then heated at 45 °C for 1 h. After cooling to room temperature the mixture was diluted to volume with acetate buffer (sample solution). The reference solution for the calibration was prepared by pipetting 500 μl of the sample solution into a 100 ml-volumetric flask and diluting to volume with 0.05 M acetate buffer. The relative retention time was about 0.85 for the derivative of D-C and 1.0 for the derivative of L-C. The content of D-C inpercentage in the sample was calculated according to the formula:

$$0.5 \times \left(\frac{a_D}{a_L}\right)$$

in which a_D is the peak area of the D-C derivative peak obtained from the sample solution and a_L is the peak area of the L-C derivative peak obtained from the reference solution.

3. Results and discussion

3.1. Sensitivity

The initial efforts were directed to gain maximum sensitivity in order to precisely and reproducibly determine traces of the derivatized chiral impurity. Therefore, several key factors during derivatization and chromatographic separation were defined and optimized, such as the injection volume, the detector sensitivity, the molar ratio of (+)-FLEC to Carnitine and the injected amount of Carnitine derivatives. To avoid working at the performance limit of the HPLC system, the injected volumes and the detector sensitivity were adjusted to values in the center of the commonly applied ranges (30 μl, medium sensitivity). Up to a molar excess of (+)-FLEC to Carnitine of more than 2.5 the intensity of the detected signal

due to the derivatized alcohols was increasing. Therefore, at an even lower ratio the reaction yield for Carnitine was below 100%. However, since enantiopure (+)-FLEC is quite expensive and larger excesses do not improve sensitivity significantly, only a slight excess of Carnitine and (+)-FLEC at a molar ratio of 1.5 was chosen. As shown in the validation section by analyzing samples at different excess ratios and by analyzing samples of different D-C contents this does not have a negative impact on the accuracy and precision of the method. Since the special procedure for calibration, the potential risk of a lower precision due to the absence of a higher excess of the derivatization reagent was minimized (see Section 3.3). The pH of the buffers used was based on a previous publication of Vogt et al. [9]. Thirty microlitre of carbonate buffer was sufficient to buffer hydrochloric acid formation during the chemical reaction. The sensitivity of the method was mainly optimized through maximizing the amount of injected Carnitine derivatives (injection volume × concentration of derivatives in solution). An adequate amount of injected derivatives was determined as 1 μg (calculated as underivatized, pure L-C), but other values within the range of 0.3–3 μg work equally well (no peak broadening due to column overloading). Thus, the amount of (+)-FLEC per derivatization was set to 80 μl (18 mM) for 30 μl (31 mM) of a Carnitine solution. A dilution to 5 ml of this reaction mixture (for an injected volume of 30 μl) was found to be adequate maintaining sufficient sensitivity to reliably quantitate the chiral impurity at a content lower than 1%.

3.2. Reaction time and reaction temperature

The derivatization reaction was also optimized with respect to reaction-time and -temperature by integrating peak areas from reaction products taken during a time course of 0–60 min both at 45 and 60 ° C. The reaction scheme is shown in Fig. 1 and the results for racemic Carnitine are displayed in Fig. 2. For both of the derivatized enantiomers the integrated peak areas were equal (differences below 3%) indicating that there is no significant difference in the fluorescence properties

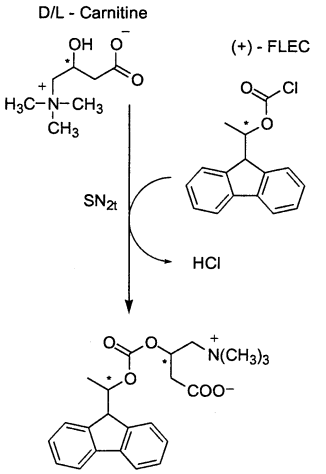

Fig. 1. Reaction of D/L-Carnitine with (+)-FLEC to a diastereomeric derivative.

and reaction rates. The equilibrium occurred after about 30 min (45 °C) and between 30 and 60 min (60 °C). The slope of the curves as well as the influence of an increased temperature of 15 °C on the areas was low. In order to maximize the robustness, the derivatization was performed at 45 ° for 60 min.

3.3. Calibration

In the proposed procedure the calibration of the fluorescence-detected signal is a critical factor. Several options such as percentage area or calibration using a D-C or L-C standard were com-

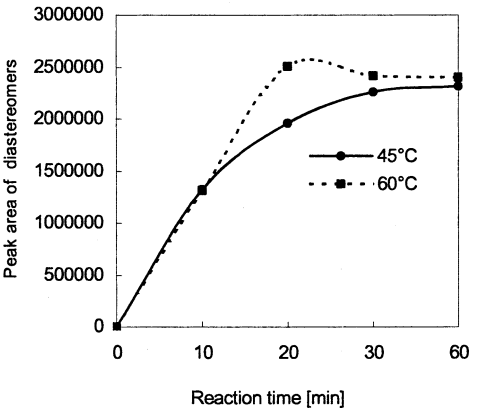

Fig. 2. Effect of reaction time and reaction temperature.

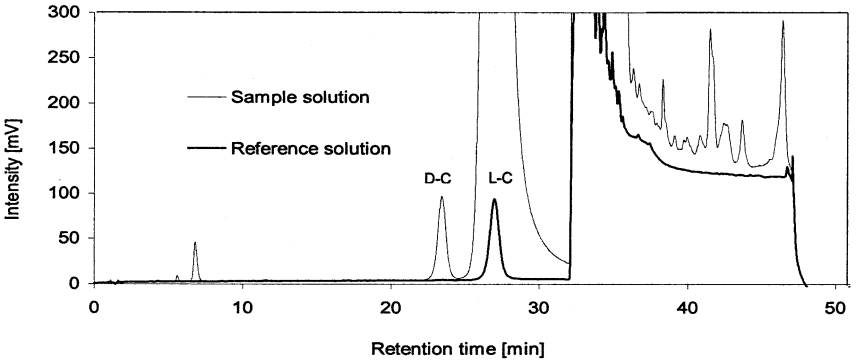

Fig. 3. Chromatograms for quantification of a sample containing 0.5% of D-C.

pared. Determination of low amounts of D-C requires overloading of the system with L-C in order to yield precise integrals of D-C and hence prevents the use of this percentage area method. Pure D-C is highly expensive, not commonly available and, therefore, not used as a standard. However, the fluorescence signal due to the two enantiomers for a given quantity is expected to be equally strong and hence the signal of the derivatized D-C component may conveniently be calibrated from the signal of derivatized L-C. The simple procedure of diluting a derivatized solution of the L-C sample (sample solution) to a reference solution of known L-C content was found to be more economically and robust. Any variations caused by sample preparation and derivatization occur for both the internal standard as well as for the sample. Moreover, this procedure avoids the error from absorption of water during the weighting of this hygroscopic compound. We could show that a single determination is sufficient for calibration. In contrast, when an external standard of L-C was used, at least three independent determinations were required to gain comparable results. Due to the high linearity of the fluorescence curve and because the intercept was close to zero (see section validation), a single calibration point was set at a content of 0.5%, in the middle of the desired range (0.1–1%). When the sample solution is diluted accurately by 1:200 (500 μl/100.0 ml) into the reference solution signal integration can be performed with proper precision. Typical chromatograms of a sample and a reference solution are shown in Fig. 3. We injected 1 μg of L-C derivatives through a sample solution and only 5 ng of L-C derivatives through a reference solution. Therefore, to avoid systematic errors we measured and limited residuals of L-C derivatives after injecting the sample solution by a system suitability test.

3.4. Separation and column cleaning

Based on the conditions published by C. Vogt et al. [9] several parameters were analyzed concerning optimum, reproducibility and robustness. Therefore, the influence of the pH, the composition of the mobile phase, the flow rate, the column efficiency and the column type on the chromatographic resolution of D-C- and L-C derivatives was investigated. These investigations were carried out by multiple injections of a solution containing racemic D/L-C. The solution was prepared as described under section Experimental, but additionally diluted by a factor of 1:100. Increased buffer content led to better resolution, as shown in Table 2. The retention time strongly varied (up to 5 min) on the buffer content (1%). Additionally, within a buffer concentration series, the column efficiency significantly changed accompanied by differences in resolution of the derivatives. Thus, it was decided to adjust the composition of the mobile phase in a system suitability test to yield the desired robustness. In our case the optimum content of acetonitrile was

Table 2
Influence of different conditions on the separation of D/L-C derivatives

Buffer/ACN	pH	Flow (ml/min)	N_{th}[a]	R_s[b]	t_r[c] (min)
74/26	2.6	2.00	high	3.16[d]	22.2
74/26	2.6	2.00	low	2.38[d]	28.0
73/27	2.6	2.00	low	2.26	22.9
68/32	2.6	2.00	low	1.72	9.33
73/27	2.4	2.00	high	3.03	22.9
73/27	2.6	2.00	high	3.05[d]	22.9
73/27	2.8	2.00	high	2.93	21.4
73/27	2.6	1.90	high	3.1	23.5
73/27	2.6	2.00	high	3.0	22.8
73/27	2.6	2.10	high	3.0	21.2

[a] N_{th} (Separation efficiency) = Number of theoretical plates; high corresponds to ca. 9000 and low to ca. 4500
[b] R_s (Resolution) = $2\,(t_2-t_1)/(W_1+W_2)$ whereas t_1 and t_2 are the retention times of the peaks, W_1 and W_2 are the bandwidths at the baseline of the two peaks.
[c] Corresponds to the retention time of the peak due to the derivative of D-C.
[d] Sample series in-between the column was regenerated.

found to vary between 25 and 30%, depending on the column efficiency. The complete removal of excessive (+)-FLEC and hydrolysis products after each injection from the chromatographic system was an important aspect to secure stable separation efficiency and guarantee maximum column lifetime. This was achieved by using a highly lipophilic gradient for column washing. The time required for cleaning at 98% acetonitrile varied between 6 and 14 min. Additionally, after more than 100 injections the column had to be regenerated. As shown in Table 2, this helped to completely regain the column efficiency (number of plates from low to high). Even long-term effects due to repetitive treatment of the column with excesses of reactive (+)-FLEC reagent were undetectable. Moreover, neither the pH nor the flow rate had a relevant influence on the resolution. The pH is limited by the pK_a-value of the derivatives (estimated at 4–5) and the flow was maximized to shorten the run time. Finally, the separation was repeated on three different columns from different manufacturer (LiChrospher, Nucleosil and Spherisorb; 250 × 4.6 mm × 5 μm) and the data indicate very similar reproducibilities. End-capped columns were preferred because they gave sharper peaks and higher peak symmetry even if triethylamine (a deactivator) was added.

3.5. Stability of derivatives and (+)-FLEC reagent

The stability of the buffered sample solution was evaluated by measuring peak areas and peak symmetries of derivatives over 60 h at ambient temperature and under protection from light, typical conditions of the autoinjector. The results for a sample containing 0.5% of D-C are shown in Fig. 4. The peak areas as well as the peak symmetries did not show any significant variations (tailing factors between 0.95 and 1.05). Thus, acetate buffer was effectively stopping the reaction without hydrolyzing the derivatives. Finally, other degradation pathways or adhesion effects of the polar derivatives could not be detected. To

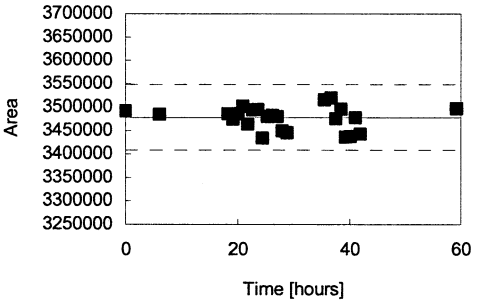

Fig. 4. Stability testing of derivative showing bandwidths at 2%.

Table 3
System suitability test

Controlled parameter	Suitability test
Composition of mobile phase	The content of ACN in the mobile phase has to be adjusted that the resolution (R_s) of the derivatives of L-C and D-C obtained with the system suitability solution is not less than 2.0
Reproducibility of integral system	The coefficient of variation calculated for the peak areas of the derivatives of D-C and L-C for five replicate injections of the system suitability solution is not greater than 2.0%
Purity of (+)-FLEC	Any peak area in the blank solution corresponding to the retention time of the derivative of D-C is not greater than 5% of the area of the peak corresponding to the derivative of D-C obtained with the sample solution
Remained L-C	Any peak area in the blank solution corresponding to the retention time of the derivative of L-C is not greater than 5% of the area of the peak corresponding to the derivative of L-C obtained with the reference solution

conclude, under the chosen conditions the derivatives were stable for at least 24 h, making autoinjection possible.

Special attention was paid to the age of the reagent. A one-year old reagent and a freshly bought one were used for preparing separate blanks. In these experiments the old reactant was found to be unsuitable, because co-eluting by-products up to 25% of the D-C content of a 0.1% D-C solution were detected. These impurities were absent in the freshly bought (+)-FLEC solution. Since the presence of these impurities was identified as critical and variable, this parameter was involved into the system suitability test to avoid unacceptable systematic errors.

3.6. System suitability test

The system suitability test ensures that the validity of the analytical procedure is maintained whenever used [16]. The critical parameters for this method were mentioned in previous sections and tests proposed to ensure validity are summarized in Table 3. To determine the minimal acceptable resolution of the derivatives in the sample solution, and thus to adjust the acetonitrile or buffer content in the mobile phase a system suitability solution containing racemic Carnitine was used. This substance is well defined with respect to relative content of enantiomers, cheap and commonly available. The solution was prepared as described above (see Section 2), but additionally diluted by 1:100. Thereby, the concentration of both D-C and L-C in the solution was equal to a sample containing 0.5% of D-C. Due to the high differences of the enantiomeric ratios in the system suitability solution (50:50) and the sample solution ($\approx$99.5:0.5) the differences in chromatographic behavior of the derivatives had to be taken into account. Therefore, the relation between the resolution of the derivatives in the system suitability solution and the corresponding integration error for the peak of D-C from a sample solution was investigated and is

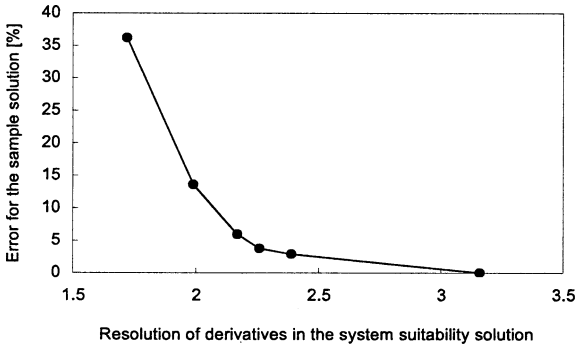

Fig. 5. Relation between the resolution of the derivatives in the system suitability solution (enantiomeric ratio of 50:50) and the corresponding integration error for the peak of D-C from a sample solution (enantiomeric ratio of 99.5:0.5). The concentration of D-C and L-C in the system suitability solution was equal to the D-C content in the sample solution (0.5%). Separations were carried out with a mobile phase of different contents of ACN (26–32%) at a pH of 2.6 and a flow rate of 2.00 ml/min.

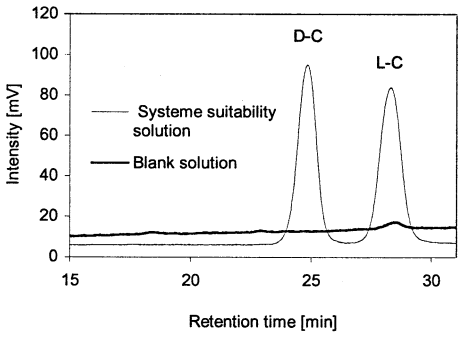

Fig. 7. Chromatogram of a system suitability solution and a blank solution. The small peak in the blank solution is stemming from remained L-C in the system caused by previous high injected amounts of L-C (sample solutions).

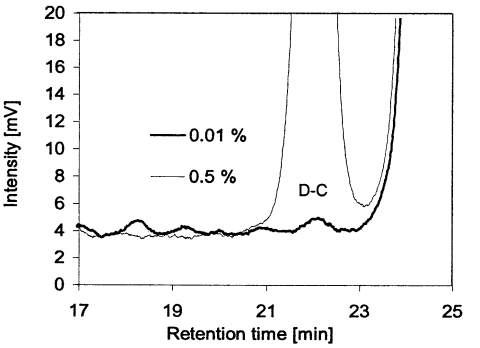

Fig. 6. Chromatograms of sample solutions obtained with two samples containing 0.5 and 0.01% of D-C.

shown in Fig. 5 (D-C content was 0.5%). The error from peak integration strongly increased at resolutions lower than 2.0. Hence, the minimal adequate resolution was defined as 2.0 taking into account that the error caused by insufficient resolution of the derivatives in a sample solution may be around 5%. In general, a resolution of at least 2.0 was achieved even with less efficient columns. The limits for reproducibility were set at a value which is still sufficient [5,6] but easy to achieve. The use of a system suitability solution ensures the control of these parameters for both, the D-C and L-C derivatives (see Fig. 7). The detection of impurities in the blank solution at positions close to the genuine peaks in the sample and reference solution serves to limit systematic errors caused to both, to integrals of D-C as well as of L-C peaks, at 5% over the complete range (see Fig. 7). The blank solution was similarly to the sample solution, however without sample weighting. Since the sample solution was diluted by 1:200 to the reference solution, a test for impurities coeluting due to L-C derivatives was found to be not necessary.

3.7. Validation and range

The complete procedure was validated according to the ICH guidelines [16] for specificity, precision, accuracy, linearity and limit of quantification. The specificity was investigated using two different samples with D-C contents of 0.01 and 0.5%. The results in Fig. 6 prove that the method is very specific for the chiral purity of L-C even with as little as 0.01% of D-C. Importantly, no racemization occurred in the derivatization procedure. The absence of a negative impact of the slight excess of (+)-FLEC to Carnitine was proofed by analyzing a sample which was derivatized with different reagent excess ratios (1.5, 2.0, 2.5 and 2.7). For all the different derivatization conditions the D-C content was found to be within a range of $0.5 \pm 0.02\%$. The accuracy, precision (repeatability) and linearity was investigated by analyzing spiked samples at five different contents of D-C ranging between 0.1 and 1.0%. The numbers of independent determinations were varied and all sample and reference solutions were injected twice. The results are shown in Table 4. For complex procedures, with hygroscopic analytes and when investigations are carried out at these low levels, recovery rates are usually below 100%. Pipetting inaccuracy, absorption of humidity during weighting procedure and impurities in

Table 4
Results of the validation study

Theoretical content (%)	Recovery (%)	Variation coefficient (%)	n[a]
0.10	82.9	3.11	3
0.25	77.8	1.82	3
0.50	83.9	1.95	6
0.75	83.2	0.24	1
1.00	90.8	1.09	2

[a] Number of independent determinations

Table 5
Equipment- and condition differences for the collaborative study

Equipment	Laboratory A	Laboratory B
RP-Column	LiChrospher 100	Nucleosil-100
Dimensions	250 × 4 mm, 5 μm	250 × 4 mm, 5 μm
Conditions	25 °, 280 bar	40 °C, 250 bar
Detector	L-7480 (Merck Hitachi)	RF-551 (Shimadzu)
Sensitivity	Medium	Low

the substance used to spike (content set as 100%) have a direct influence on the determined accuracy. The tendentious increase of the recovery and precision over the investigated range and the results of the collaborative study additionally indicate that it is not the analytical method itself that causes recovery values below 100% but rather operational errors during determination of recovery (spiking). The reproducibility of the average recovery rates was adequate incorporating variance homogeneity, which was confirmed by the F-test ($P = 0.95$). In order to verify the influence of operational and environmental variables, the ruggedness was tested by a collaborative study (reproducibility). Four different samples of homogenous lots were analyzed separately in the two different Laboratories A and B. Differences in equipment employed by the participating laboratories are described in Table 5. However, significant differences could not be detected for any of the determined contents. Thus, the procedure seems to be robust (see Table 6.). The deviations of measured versus theoretical values of D-C was determined to linearly depend on the concentrations in the range of 0.1–1%. The regression line passed through the origin (no significant difference from zero) with a high correlation of 0.9952 (r^2) and a slope of 0.9079 (average recovery). Hence, no systematic error was found and good linearity was confirmed. The response equivalence of D-C and L-C derivatives in the fluorescence-detected signal was investigated through separate determinations of the response linearity of reference samples of D-C and L-C over the concentration range between 0.04 and 2%. In each case, three independent measurements consisting of seven different concentrations per series were performed. We found no significant differences between the corresponding curves (slopes) and hence the response factor of the two substances is equal. The quantification limit (signal to noise ratio of 10) was determined to be lower than 0.05%. Thus, the limit is at least 50% below the lowest D-C content in the investigated range and the sensitivity, therefore, sufficient. Finally, samples from three different manufactures were analyzed (data not shown), demonstrating the robustness for impurity profiles of different routes of synthesis.

Table 6
Results of the collaborative study

Sample[a]	Laboratory A (%)	Laboratory B (%)
1	0.01	<0.03
2	0.10	0.09
3	0.13	0.10
4	0.47	0.42

[a] One independent determination

Due to the results described above, the working range for this method could be set from 0.1 to 1.0% of D-C in L-C. Widening the range to 3% required no changes to the method. After several cycles of adaptation, the method could be used for determinations of contents of D-C significantly lower than 0.1% as well.

4. Conclusions

The new method proposed in this paper integrates chiral derivatization during 1 h and convenient separation of the Carnitine diastereomers on octadecyl columns. The separation was completed within 50 min using a mobile phase consisting of a mixture of triethylamine phosphate buffer and acetonitrile and including gradient programming for column cleaning. The calibration was carried out conveniently by injecting a diluted derivatization solution of a sample, and thereby D-C was quantified indirectly through L-C. This method increased the robustness and decreased the costs for the complete procedure. A system suitability test was established and the complete procedure was validated according to ICH-criteria. Thereby,

reliability and validity for this new method to quantificate D-C in L-C samples in the range of 0.1–1% was ensured.

Acknowledgements

All measurements performed by C. Bernet and support provided through D. Andris and Dr O. Zerbe from ETH Zurich as well as from Lonza Ltd. are gratefully acknowledged.

References

[1] N. Siliprandi, L. Sartorelli, M. Di Lisa, F. Di Lisa, Clin. Chim. Acta 183 (1989) 3.
[2] H. Jung, K. Jung, H.P. Kleber, in: A. Fiechter (Ed.), Advances in Biotechenical Engineering Biotechnology, Springer, Berlin, 1993.
[3] B. Stieger, B. O'Neill, S. Krähenbühl, Biochem. J. 309 (1995) 643.
[4] D.B. Shennan, A. Grant, R.R. Ramsay, C. Burns, V.A. Zammit, Biochim. Biophys. Acta 1393 (1998) 49.
[5] The European Pharmacopoeia Convention Inc., European Pharmacopoeia, 3rd edition, (1997).
[6] The United States Pharmacopoeia Convention Inc., The United States Pharmacopoeia, 24, (2000).
[7] A. Marzo, G. Cardace, E. Martelli, E. Arrigoni, Chirality 4 (1992) 247.
[8] J. Bounoure, J. Souppe, Analyst 113 (1988) 1143.
[9] C. Vogt, A. Greorgi, G. Werner, Chromatographia 40 (1995) 287.
[10] P. De Witt, R. Deias, S. Muck, B. Galetti, D. Meloni, P. Celletti, A. Marzo, J. Chromatogr. B 657 (1994) 67.
[11] W. Engewald, H. Engelhardt, P. Goetzinger, P. Klossek, H.P. Kleber, Pharmazie 45 (8) (1990) 629.
[12] M. Kagawa, Y. Machida, H. Nishi, J. Chromatogr. A 857 (1999) 127.
[13] M. Takahashi, K. Terashima, M. Nishijima, K. Kamata, J. Pharm. Biomed. Anal. 14 (1996) 1579.
[14] T. Hirota, K. Minato, K. Ishii, N. Nishimura, T. Sato, J. Chromatogr. A 673 (1994) 37.
[15] C. Mardones, A. Rios, M. Valcarcel, R. Cicciarelli, J. Chromatogr. A 849 (1999) 609.
[16] International Conference of Harmonization, Q2B document; Validation of Analytical Procedures, (1996).