Countercurrent Approach to the Enrichment of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 Isomers by Urea Complexation

David W.L. Ma[a,b], Catherine J. Field[a,b,c], and M.T. Clandinin[a,b,c,*]

[a]Nutrition and Metabolism Research Group, and Departments of [b]Medicine and [c]Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5

ABSTRACT: CLA refers to a group of geometrical and positional isomers of linoleic acid. CLA has been shown to have potentially beneficial effects on cancer, atherosclerosis, and body metabolism in animals. Mixtures containing equal amounts of these isomers are commonly used in many research studies because of their greater availability and lower cost relative to pure isomers. This has hindered progress in elucidating the biological properties of specific isomers and their relevance in animal and human biology. A method was developed that offers a compromise between cost and utility to make available enriched mixtures of either the Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 isomers for use in a wide range of experimental applications. A countercurrent approach was developed to separate the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers from an equal mixture of these two isomers by urea complexation. After three successive rounds of complexation using an equal amount of CLA and urea, a fraction enriched in Δ9*c*,11*t*-18:2 containing 42.5 and 17.4% of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2, respectively, was recovered. After a single round of complexation using 2.5 g urea/g CLA, a fraction enriched in Δ10*t*,12*c*-18:2 was recovered containing 29.7 and 69.1% of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2, respectively.

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CLA refers to a group of isomers of the essential FA, linoleic acid (LA) (1,2). Research devoted to CLA is a growing area of interest owing to the discovery of its anticancer properties in the mid-1980s (3,4). CLA has also been shown to have anti-atherogenic properties (5) and to modulate body composition (6), which may be therapeutic for weight reduction (7) and diabetes (8,9).

Progress in elucidating the biological activity of specific isomers in large animal trials has been hindered by the lack of availability of large quantities of specific isomers. Alkali isomerization of LA is a common and inexpensive method used to synthesize 100-g quantities of CLA containing a mixture of equal amounts of the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers (1). The large-scale production of single isomers is not as easily accomplished. Methodologies to synthesize specific CLA isomers, particularly the Δ9*c*,11*t*-18:2 and Δ10*t*,12*c*-18:2 isomers, are limited. Several methods describe the synthesis or preparation of these isomers by low-temperature crystallization (10), selective lipase esterification (11), dehydration of methyl ricinoleate (12–14), and bacterial synthesis (15). There are some limitations to these methods, including low yield, and, to some degree, difficulties in implementation.

Separation of FA can readily be accomplished by fractional crystallization procedures (16). Typically, FA mixtures are crystallized in various types of organic solvents at various low temperatures. The specific combination of organic solvent and temperature results in selective crystallization of specific FA. Separation of FA isomers is not readily accomplished by this type of conventional process. A variation of this conventional system is the countercurrent system. This system entails partitioning two compounds that are similar but have slightly different affinities between two different and immiscible phases. The two phases are then separated, and the process is repeated until the desired purity is obtained. An alternative approach to the extraction of FA by crystallization is complexation of FA in urea (17–19). Urea forms complexes and preferentially occludes straight-chain FA, thus providing a rationale for an approach to separation of CLA isomers based on the overall geometry of the FA molecule.

We endeavored to develop a simple method that takes advantage of readily available CLA produced *via* alkali isomerization of LA to produce enriched mixtures of CLA isomers. The preparation of such enriched mixtures may be a suitable compromise between cost and utility in a wide range of experimental applications from cell culture to human studies. Described herein is a countercurrent approach to prepare enriched mixtures of either the Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 isomer by urea complexation.

EXPERIMENTAL PROCEDURES

Preparation of CLA. A mixture of isomers containing predominantly Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 in equal proportions was prepared from safflower oil as described by Ma *et al.* (20).

Urea complexation of CLA. CLA (50 g) was complexed with an equal weight of urea that was fully dissolved in warmed methanol (MeOH) (2 g/mL) so as to ensure effective complexation. CLA rapidly complexes with the urea and is allowed to cool at room temperature or 5°C briefly before chilling overnight at –25°C. Next day, the urea complex (UC) fraction was separated from the mother liquor (ML) fraction by vacuum filtration. The UC were washed with chilled satu-

*To whom correspondence should be addressed at 4-10 Agriculture/Forestry Centre, University of Alberta, Edmonton, Alberta T6G 2P5 Canada. E-mail: tclandin@ualberta.ca

rated urea in MeOH and pressed with a glass stopper to ensure dryness.

Extraction of CLA from ML fraction. The ML fraction was transferred to a separatory funnel. Double-distilled water (ddH_2O) (50 mL) was added, and the mixture was acidified with hydrochloric acid (HCl) (6 N, 50 mL) to pH <2. CLA was extracted with hexane (2 × 50 mL) and transferred to a clean separatory funnel. The hexane fraction was washed with ddH_2O (25 mL) and acidified with HCl (6 N, 25 mL). The hexane fraction was further washed with MeOH in ddH_2O (30% vol/vol, 3 × 50 mL) and with ddH_2O (3 × 50 mL). The hexane fraction was dried over anhydrous sodium sulfate and removed by rotary evaporator.

Extraction of CLA from UC fraction. The UC fraction was dissolved in ddH_2O (50 mL) and heated to dissolve the urea. CLA should appear as a separate, distinct upper phase upon complete dissolution of urea. The heated mixture was briefly cooled, then transferred to a separatory funnel. HCl (6 N, 50 mL) was added to acidify to pH <2. CLA was extracted with hexane (2 × 50 mL) and transferred to a clean separatory funnel. The hexane fraction was washed with ddH_2O (25 mL) acidified with HCl (6 N, 25 mL). The hexane fraction was further washed with MeOH in ddH_2O (30% vol/vol, 3 × 25 mL) and with ddH_2O (3 × 25 mL). The hexane fraction was dried over anhydrous sodium sulfate and removed by rotary evaporator.

*Enrichment of Δ9*c,*11*t*-18:2 and Δ10*t,*12*c*-18:2.* The initial ML fraction, enriched in Δ10*t*,12*c*-18:2, was subsequently recomplexed in an equal weight of urea and prepared as previously described. Each successive ML fraction was again recomplexed to achieve greater enrichment. The initial UC fraction, enriched in Δ9*c*,11*t*-18:2 content, was subsequently recomplexed in an equal weight of urea and prepared as previously described. Each successive UC fraction was again recomplexed to achieve increased enrichment.

Analysis of CLA by GLC. CLA (1–2 mg) was methylated with 14% BF_3-MeOH (1 mL) and hexane (2 mL) in a screw-cap tube (9 mL) with Teflon™ cap for 30 min at room temperature with shaking. The reaction was halted with addition of ddH_2O (1 mL). Phases were separated by centrifuging at 300 × *g* for 10 min. The upper hexane phase was extracted and analyzed by GLC as described by Ma *et al.* (2).

RESULTS AND DISCUSSION

Initial complexation of CLA with urea resulted in an enrichment of Δ9*c*,11*t*-18:2 in the solid UC fraction and an enrichment of Δ10*t*,12*c*-18:2 in the solvent ML fraction. The recovered fractions were recomplexed, and the nomenclature identifying these fractions reflects the sequential order from which the fraction is derived (Table 1) (i.e., UC ML is the ML fraction recovered from the recomplexation of material from the initial UC fraction). The apparent preferential occlusion of the Δ9*c*,11*t*-18:2 isomer may be attributed to the more overall linear geometry of its methyl tail compared with the Δ10*t*,12*c*-18:2 isomer. By assuming that the *trans* double bond of Δ9*c*,11*t*-18:2 is essentially linear, one can see that the

TABLE 1
Composition of CLA Mixtures After Successive Equal Weight Urea Complexations

Fraction	Initial composition	First complexation		Second complexation				Third complexation							Fourth complexation			
		ML[a]	UC[b]	ML ML	ML UC	UC ML	UC UC	ML ML ML	ML ML UC	ML UC ML	ML[c] UC UC	UC ML ML	UC ML UC	UC UC ML	UC UC UC	ML ML ML ML	ML ML ML UC	ML ML UC UC
% CLA[d]	93.5	95.6	91.0	96.0	95.6	94.5	89.5	94.8	96.3	97.4	92.2	96.5	90.3	90.3	79.3	93.2	95.9	
% Δ9*c*,11*t*-18:2[e]	45.6	44.6	50.4	42.5	46.8	51.4	50.8	42.2	45.1	43.2	55.6	48.0	58.2	53.5	42.5	40.1	46.3	
% Δ10*t*,12*c*-18:2[e]	50.3	52.6	42.4	55.3	50.2	46.2	37.4	53.6	53.1	54.6	40.6	48.4	34.6	36.9	17.4	57.9	51.3	
% Δ11*c*,13*t*-18:2[e]	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0	0.2	0.1	0.1	0.3	0.1	0.1	0.1	0.2	
cis/*cis* CLA[e]	0.8	0.8	2.6	0.5	1.5	1.3	3.1	0.6	0.3	0.6	1.5	0.9	4.0	5.0	18.7	0.3	0.8	
trans/*trans* CLA[e]	3.1	1.9	4.4	1.6	1.4	1.1	8.6	3.4	1.5	1.4	2.1	2.5	2.9	4.4	21.3	1.6	1.4	
9,11:10,12[f]	0.9	0.8	1.2	0.8	0.9	1.1	1.4	0.8	0.8	0.8	1.4	1.0	1.7	1.5	2.4	0.7	0.9	
10,12:9,11[f]	1.1	1.2	0.8	1.3	1.1	0.9	0.7	1.3	1.2	1.3	0.7	1.0	0.6	0.7	0.4	1.4	1.1	

[a]ML, mother liquor fraction collected after filtration.
[b]UC, urea complex fraction collected after filtration.
[c]Fractions are identified by the lineage of complexations from which they are derived: i.e., ML UC UC and ML UC ML are derived from the first ML fraction, which was recomplexed; then the recovered UC fraction was recomplexed, producing the fractions ML UC UC and ML UC ML.
[d]Values are expressed as a percentage of total FA by GLC analysis.
[e]Values are expressed relative to total CLA FA.
[f]Ratio of Δ9*c*,11*t*- to Δ10*t*,12*c*-18:2 or vice versa.

methyl tail adopts a linear configuration between carbons 10 and 18. In comparison, the *cis* double bond of Δ10*t*,12*c*-18:2 kinks the FA at carbon 12; therefore, the methyl tail is linear only between carbons 13 and 18. It should be noted that although the "UC UC UC" fraction is greatly enriched in Δ9*c*,11*t*-18:2 compared with the Δ10*t*,12*c*-18:2 isomer, it also becomes enriched in *cis/cis* and *trans/trans* CLA isomers and other FA that may also be occluded by urea (Table 1).

In general, three types of fractions evolve. (i) Complexation, recovery, and subsequent recomplexation of only the UC or ML fractions produce highly enriched mixtures of Δ9*c*,11*t*- and 10*t*,12*c*-18:2, respectively. (ii) Intermediary fractions can be further recomplexed and purified, but only those fractions with the greatest number of successive complexations of the same fraction will continue to be useful (i.e., "ML UC UC" and "UC ML ML"). (iii) Fractions derived from nonsuccessive recomplexation of UC and ML fractions are of poorest enrichment and can be pooled to begin the process anew.

The ratio of urea to CLA is also a factor affecting the level of isomer enrichment and weight yield between the UC and ML fractions. Starting with 52 g of CLA complexed with an equal weight of urea, three successive rounds of complexation resulted in the recovery of 0.3 g of material in the "UC UC UC" fraction enriched in Δ9*c*,11*t*-18:2 containing 42.5 and 17.4% of Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2, respectively. In comparison, 18 g of material was recovered in the "ML ML ML" fraction with modest enrichment of the Δ10*t*,12*c*-18:2 isomer relative to the Δ9*c*,11*t*-18:2 isomer. The "ML ML ML" fraction was recomplexed, and only a small enrichment of Δ10*t*,12*c*-18:2 was gained in the "ML ML ML ML" fraction. In a separate trial, starting with 50 g of a CLA mixture complexed with a 2.5-fold amount of urea, a highly enriched Δ10*t*,12*c*-18:2 ML fraction was recovered containing 29.7 and 69.1% of the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers, respectively (Table 2). Relative to the initial 50 g of CLA, 12 and 34 g of material were recovered in the ML and UC fractions, respectively. Based on these results, an equal ratio of urea to CLA or less is favorable for the enrichment of the Δ9*c*,11*t*-18:2 isomer in the UC fractions; conversely, a 2.5-fold or higher ratio of urea to CLA is favorable for the enrichment of the Δ10*t*,12*c*-18:2 isomer in the ML fractions.

TABLE 2
Composition of CLA Mixtures After Complexation with 2.5-fold Greater Urea

		First complexation	
Fraction[a]	Initial composition	2.5 ML	2.5 UC
% CLA	93.5	94.4	87.4
% Δ9*c*,11*t*-18:2	45.6	29.7	50.4
% Δ10*t*,12*c*-18:2	50.3	69.1	44.1
% Δ11*c*,13*t*-18:2	0.2	0.1	0.5
cis/*cis* CLA	0.8	0.3	2.3
trans/*trans* CLA	3.1	0.8	2.7
9,11:10,12	0.9	0.4	1.1
10,12:9,11	1.1	2.3	0.9

[a]These fractions were recovered after a mixture of CLA isomers was complexed with 2.5-fold greater urea. See Table 1 for abbreviations.

The primary limitation of this method is diminished recovery on a weight basis of the desired CLA isomer, because the FA mixture is partitioned into increasing numbers of fractions after each successive round of complexation. There is also some loss of material due to experimental handling in the recovery process. Additionally, the ratio of urea to CLA apparently determines the distribution of material between the UC and ML fractions (19). In general, when CLA was complexed on an equal weight basis with urea, approximately two-thirds of the CLA was recovered in the ML fraction and one-third was recovered in the UC fraction. This was reversed when CLA was complexed with 2.5-fold greater urea. This is not surprising, because as the amount of urea increases, the number of urea complexes also increases relative to the fixed amount of FA; thus, more FA are occluded, in particular the Δ9*c*,11*t*-18:2 isomer. It is likely that with further modifications, the ratio of urea to CLA can be optimized to increase enrichment and yield for a desired CLA isomer. Although the method is fairly simple in its approach, some proficiency is required to ensure a satisfactory level of recovery of material, and there must be a realization that gains in overall enrichment are always offset by a loss in yield.

The two CLA isomers that are of primary research interest are the Δ9*c*,11*t*- and Δ10*t*,12*c*-18:2 isomers. Overall, the method described is a simple approach to selectively enrich the yield of Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 isomers. Owing to its simple procedures and use of common reagents, the countercurrent method described can be readily scaled up to produce large quantities of enriched isomers. With these enriched mixtures, it is feasible to produce sufficient amounts of specific enriched isomer CLA to conduct human or large animal studies to examine the effect of Δ9*c*,11*t*- or Δ10*t*,12*c*-18:2 on health.

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