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Simultaneous determination of deuterated and non-deuterated α-tocopherol in human plasma by high-performance liquid chromatography

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Abstract

Labelled tocopherol is used to evaluate its absorption by biodiscriminating the dietary intake from the endogenous tocopherol pool of subject. A normal-phase high-performance liquid chromatographic method is described for the easy separation and quantification of deuterated (d_6) and non-deuterated α-tocopherol. The α-tocopherol isotopomers were extracted from plasma triacylglycerol-rich lipoproteins in hexane, separated by two EC Nucleosil columns in series with a mobile phase of hexane–isopropanol (659.34:0.786, w/w) running isocratically. The detection of d_6-α-tocopherol was performed by its UV absorbance at 297 nm with a limit of detection of 34 pmol/ml, a limit of quantification of 83 pmol/ml and a range of determination of 34–9905 pmol/ml. Between- and within-assay RSDs were 2.4% (n=10) and 2.7% (n=5), respectively.

Keywords: α-Tocopherol

1. Introduction

A strong correlation exists between antioxidant micronutrient intake and several degenerative diseases, in particular for vitamins E, C, β-carotene and selenium [1,2]. Epidemiological and clinical assays suggest that the fat-soluble vitamin E, vitamin A and β-carotene protect against coronary heart disease and they may be important in the prevention in some cancers [3,4]. There is still an interest and a need to study the absorption of vitamin E in humans.

High-performance liquid chromatography (HPLC) has been widely applied for the determination of fat-soluble vitamins in several biological matrices. Both normal- and reversed-phase columns were used, the elution being either isocratic or with gradient and the quantification performed with fluorimetric, ultraviolet or electrochemical detectors [5,6]. However, HPLC methods with UV detection are currently the most commonly used [7,8] as fluorimetric and colorimetric methods are hampered by interferences [9]. Several reports have docu-

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mented the recent development of analytical methods for studying the metabolism of α-tocopherol in man. The improvements in the analytical methodology consist of a reduction of sample preparation process as well as analysis time [10–13] and an increase of the sensitivity up to the fmol level [14–16].

Progress in the understanding of α-tocopherol metabolism has been acquired by the use of the stable isotope allowing the labelling of α-tocopherol. The main advantage of using α-tocopherol labelled with stable isotope is the possibility to selectively differentiate the absorbed tocopherol from the endogenous tocopherol pool of the living organism. Determination of deuterated α-tocopherol concentration in biological matrices involved the use of either mass spectrometry (MS) or tandem MS [17–20]. These methodologies have several limitations, i.e., the requirement of another labelling of tocopherol as internal standard which is not commercially available, i.e., α-tocopherol-d_3 as well as the use of sophisticated and expensive mass spectrometry equipment which is not often available in clinical situations.

The absorption of α-tocopherol in human is evaluated in a clinical trial generating a vast amount of biological samples. Therefore, the use of analytical procedure with high sensitivity, high sample throughput as well as a classical instrumentation is required. The aim of this work is to develop a rapid, sensitive and specific method for the simultaneous determination of deuterated and non-deuterated α-tocopherol in plasma lipoprotein fractions without the need of an internal standard as well as mass spectrometry instrumentation. The separation of both α-tocopherol molecules is achieved using a normal-phase HPLC column. These molecules are subsequently detected and quantified with a photodiode array detector. The utility of this method is demonstrated in a pilot study in subjects who had consumed a 30 mg dose of d_6-α-tocopherol. The application of the extraction and analysis protocols is assessed by following the appearance of d_6-α-tocopherol in human triacylglycerol-rich lipoproteins (TRLs) containing both chylomicrons and very-low-density lipoprotein (VLDL) particles. The good sensitivity of this HPLC method offer great promise for investigating the absorption of α-tocopherol in humans.

2. Materials and methods

2.1. Chemicals

(2*R*,4′*R*,8′*R*)-d_6-α-Tocopherol having a chemical purity of greater than 99% and an isotopic purity of approximately 98% was purchased from Orphachem (Clermont-Ferrand, France). α-Tocopherol was purchased from Merck (Darmstadt, Germany). Sodium bromide was from Merck, deferoxamine mesylate from Sigma, 2,6-di-*tert*.-butyl-*p*-cresol (BHT) from Fluka, ethylenediaminetetraacetic acid (EDTA) from Sigma. All organic solvents were HPLC grade with water content less than 0.02% for hexane and 0.05% for isopropanol.

2.2. Human study design

Three healthy males participated to the pilot clinical study, which was approved by the Nestlé Ethical Committee. Subjects signed informed consent before entering the study and could withdraw from the study at any time point if desired. After an overnight fast of 12 h, subjects consumed 30 mg of d_6-α-tocopherol incorporated into a standard meal consisting of 35 g peanut oil, 70 g semolina (cooked in 200 g water), 40 g bread and 60 g egg whites. Blood samples were collected from the forearm of the subject into EDTA-containing tubes before and at 2, 2.5, 3, 4, 5, 6, 7, 8 and 9 h post-meal. These blood samples were immediately placed in an iced water bath. Blood was centrifuged at 3000 *g* at 4 °C for 10 min. Plasma samples were stored at −20 °C until analysis.

2.3. Plasma triacylglycerol-rich lipoprotein isolation and extraction

Plasma (4 ml) was thawed and introduced in an ultracentrifuge tube. A 1.006 g/ml sodium bromide solution was deposited on the top of the plasma solution without mixing these two solutions. The tube was filled with this 1.006 g/ml solution and subjected to an ultracentrifugation at 100 000 *g*, at 15 °C for 30 min. The upper phase containing TRLs, i.e., mainly chylomicrons with low amount of very low-density lipoproteins was collected. TRLs (200–

400 μl) were adjusted up to 1 ml with distilled water. Subsequently, 1 ml ethanol, 5 μl deferoxamine mesylate (10 mg/ml water) and 2 ml hexane–BHT (350 mg/l) were added. The tube was mixed vigorously for 20 s with vortex and then centrifuged at 3000 rpm at room temperature for 10 min in a tabletop centrifuge. The organic phase was collected while the water phase was extracted again with an additional 2 ml hexane–BHT. The hexane layers were combined and evaporated to dryness under a stream of nitrogen. The sample was dissolved in 200 μl hexane–BHT and 60 μl was injected into the HPLC system. The lipid extract of the biological sample is quite stable for 1 week at 4 °C and 2 days at room temperature prior to its injection into the HPLC system.

2.4. HPLC instrumentation

A HPLC HP 1100 system (Hewlett-Packard, Geneva, Switzerland) was equipped with an autosampler working at room temperature and a photodiode array detector. Two columns EC Nucleosil (250 mm×4.6 mm I.D., 5 μm, 100 Å) (Macherey–Nagel, Oensingen, Switzerland) were coupled in series. The separation was achieved at room temperature using isocratic condition with a mobile phase consisting of hexane–isopropanol (659.34:0.786, w/w). The mobile phase flow-rate was 1 ml/min and the retention times were 40.8 and 42.5 min for α-tocopherol and d_6-α-tocopherol, respectively. Detection was performed at 297 nm. The batch-to-batch reproducibility of the columns has not been tested.

2.5. Reproducibility and precision of the method

Between- and within-assay relative standard deviations (RSDs) were determined using a pool of plasma collected from different volunteers having consumed a dose of 30 mg d_6-α-tocopherol. Plasma was subjected to ultracentrifugation as previously described and TRLs were isolated. This pool of TRLs is aliquoted and stored for up to 9 months at −20 °C. On the day of analysis, an aliquot of TRL was thawed. TRLs were extracted with organic solvents and tocopherol isotopomers were separated and quantified by HPLC. This pool of TRLs contained 4.4 and 0.37 μmol/l of α-tocopherol and d_6-α-tocopherol, respectively. For the determination of the within-assay precision, 10 aliquots of the pool TRLs were analyzed on the same day whereas for the between-assay variation, five aliquots of the pool TRLs were analyzed over 5 consecutive days.

2.6. Accuracy determination–standard dilution curve for d_6-α-tocopherol in TRLs

The accuracy was determined using a spiking procedure. TRLs were spiked rather than plasma because the d_6-α-tocopherol added to plasma will not be incorporated into the TRL fraction and therefore the isolation of TRLs by ultracentrifugation process will induce a loss of d_6-α-tocopherol which could not be attributed to a loss of recovery due to the extraction process. An increasing amount (from 37 up to 148 ng) of d_6-α-tocopherol was added to the same quantity of TRL. The sample processing was performed on all of these spiked samples and the recovery was assessed by the ratio of d_6-α-tocopherol determined in the spiked sample divided by the amount added into the TRL multiplied by 100.

3. Results

A review of the various analytical methods described in the literature for the determination of d_6-α-tocopherol points out the use of MS with previous isolation by either gas chromatography (GC) or HPLC [17–19]. These methodologies required another labelling of tocopherol (for example d_3-α-tocopherol) as internal standard which is not commercially available, as well as a sophisticated MS instrumentation which is also not available in all clinical laboratories. Their sensitivity was quite high with a limit of detection of 20 pmol injected [20] which have been recently improved to reach sub-fmol level [18].

We developed a HPLC method for performing large-scale stable isotope studies in humans. This HPLC method allows selective and sensitive de-

tection of d_6-α-tocopherol in TRLs by HPLC–UV. It does not necessitate any derivatisation as for GC analysis as well as an internal standard for MS quantification. A HPLC trace of standard mixture of α-tocopherol isotopomers is presented in panel A of Fig. 1 and shows a good separation of these two molecules. The peak at 27.5 min corresponds to BHT that does not interfere with the peaks of α-tocopherol isotopomers. The ratio of the two solvents in the mobile phase has been found by trial and error experiments and is expressed by weight in order to be more accurate since this ratio is a key parameter for an efficient separation of the two α-tocopherol isotopomers. Panel B shows the HPLC chromatogram of TRLs isolated from plasma of a healthy subject. No peaks are present around the peak of α-tocopherol demonstrating that α-tocopherol is efficiently isolated from the other lipids present in this lipoprotein fraction. The lower tracer (panel C) exhibits the HPLC chromatogram of TRLs (similar to panel B) that have been spiked with the mixture of α-tocopherol isotopomers. A separation between α-tocopherol [retention time (t_R)=40.8 min] and d_6-α-tocopherol (t_R=42.5 min) is observed and characterized by a resolution factor of 0.26 and a selectivity factor of 1.04. These chromatograms clearly show that labelled tocopherol could be distinguished by HPLC from its unlabelled form. In the insert of panel A (Fig. 1), the UV spectrum of a standard of d_6-α-tocopherol is presented, in panel B the insert presents the UV spectra of α-tocopherol and in panel C the d_6-α-tocopherol. These spectra confirm the identity and purity of α-tocopherol and d_6-α-tocopherol peaks in the chromatogram of plasma TRLs. In addition, the identity of these two isotopomers has been confirmed by GC–MS after peak isolation by HPLC and trimethyl silylation. Reduction of the run time by modifying the mobile phase composition has been evaluated and is not possible since the ratio of solvents in the mobile phase is a key parameter for an efficient separation of α-tocopherol isotopomers. Calibration curve is linear over the following ranges: from 51 up to 18948 pmol/ml and from 34 up to 9905 pmol/ml for α-tocopherol and d_6-α-tocopherol, respectively. The equation of the curves and correlation coefficients are $y=0.131x-1.279$, $r^2=0.9994$ and $y=0.1325x-0.070$, $r^2=0.9995$, for α-tocopherol and d_6-α-tocopherol, respectively. The lowest amounts detected are 21 and 34 pmol/ml whereas the lower amounts quantified are 48 and 83 pmol/ml, for α-tocopherol and d_6-α-tocopherol, respectively. The sensitivity of this HPLC–UV methodology is similar to those detected with the conventional MS-based techniques. The lipid extract of the biological sample is quite stable for 1 week at 4 °C and 2 days at room temperature prior to its injection onto the HPLC system. Due to the high stability of the HPLC system, separation is performed 24 h a day and 7 days a week allowing the determination of 180 biological samples per week. The reproducibility of the d_6-α-tocopherol in plasma TRLs is controlled over 20 series of analyses. In each series of analysis, TRLs isolated from a pool plasma of subjects having consumed 30 mg d_6-α-tocopherol is incorporated, treated similarly to biological samples and d_6-α-tocopherol concentration is determined. The results are plotted in Fig. 2 and show that over a period of 9 months no values exceed the control limits set as mean±3 times the standard deviation that corresponds the rejection limits on a Shewhart control chart [21]. Storage of this TRL pool up to 9 months at −20 °C does not affect the concentration of two isotopomers of α-tocopherol indicating a good stability of these isotopomers.

To assess for the liquid–liquid extraction procedure on the determination of d_6-α-tocopherol, a pool of TRLs containing only unlabelled α-tocopherol is aliquoted, spiked with increasing amount of d_6-α-tocopherol mimicking the calibration curve. Recoveries are above 86% and indicate that the use of an internal standard is not mandatory. The intra-assay variabilities are 4.9 and 2.4% based on 10 determinations while the inter-assay variabilities are 4 and 2.7% based on five determinations for α-tocopherol and d_6-α-tocopherol, respectively.

To evaluate the relative absorption of α-tocopherol, the pharmacokinetic of d_6-α-tocopherol in plasma TRL is assessed in three healthy subjects having consumed an oral dose of 30 mg d_6-α-tocopherol. As expected, no d_6-α-tocopherol is detected in the TRL drawn at 0 h (Fig. 3). The TRL d_6-α-tocopherol concentration increases rapidly and subsequently decreases. The level of TRL d_6-α-tocopherol is still quite high 9 h post-absorption. This pharmacokinetic of tocopherol is similar to those previously described in the literature [17] and

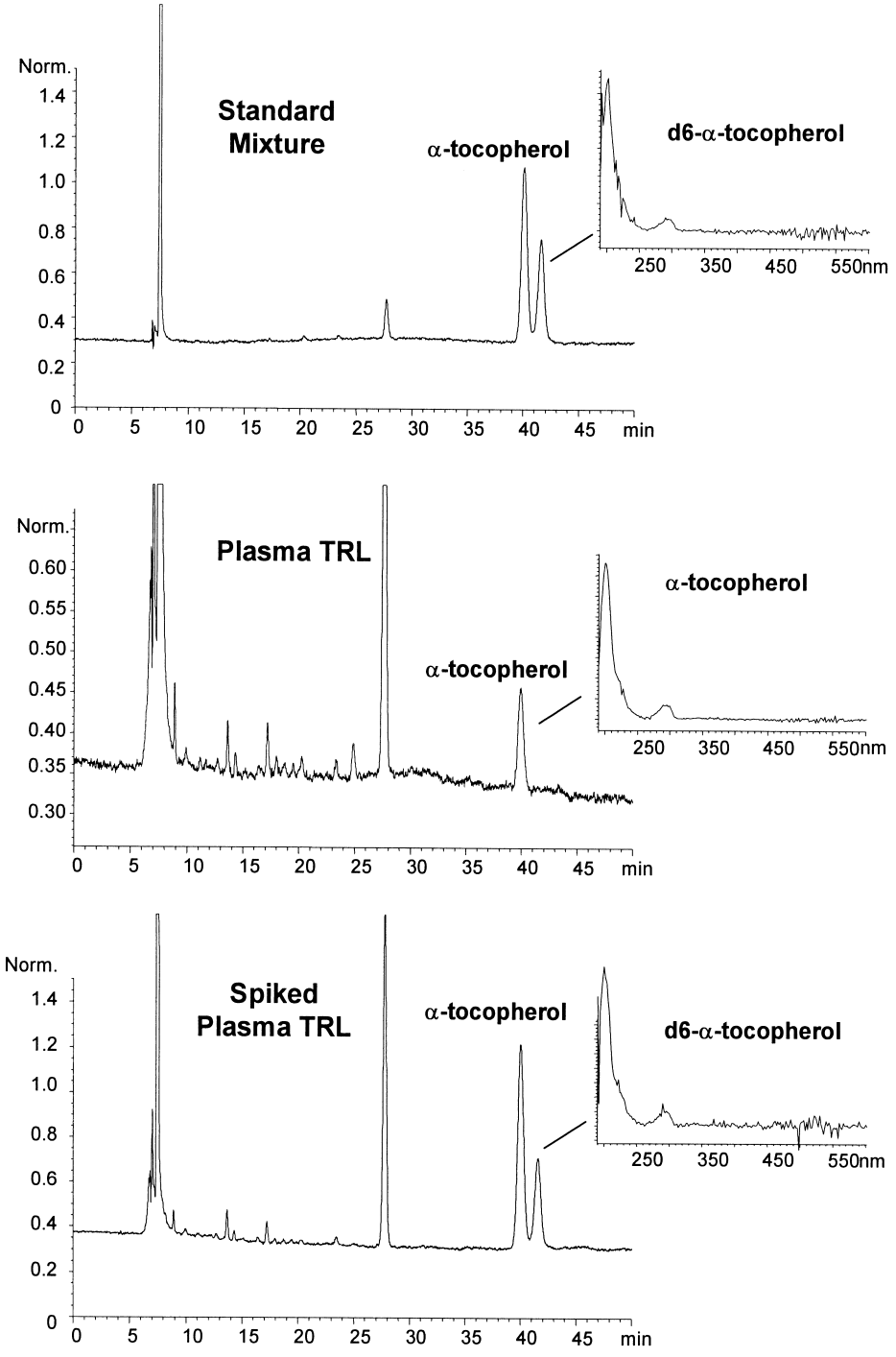

Fig. 1. LC–UV chromatograms of a mixture α-tocopherol (176 pmol) and d_6-α-tocopherol (102 pmol) standard (panel A), of TRLs isolated from a healthy male subject (panel B) and TRLs spiked with the above amount of the mixture of tocopherol isotopomers (panel C). In the insert, spectra of a standard of d_6-α-tocopherol is presented in panel A, of α-tocopherol in plasma TRLs in panel B and of d_6-α-tocopherol in the spiked TRLs in panel C.

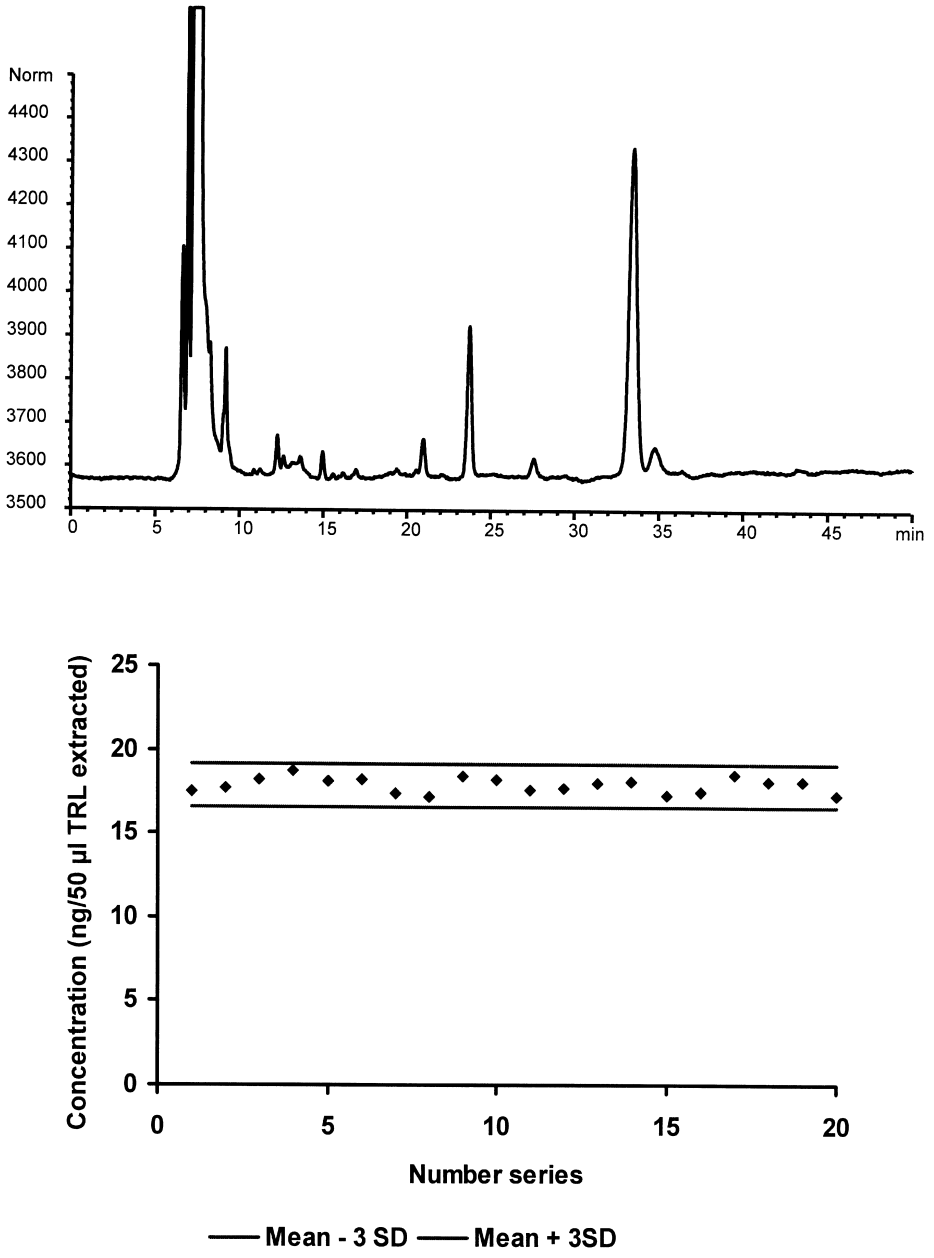

Fig. 2. Reproducibility of the d_6-α-tocopherol in plasma TRLs. In each series of analysis, TRLs isolated from a pool plasma of subjects having consumed 30 mg d_6-α-tocopherol is incorporated, treated similarly to biological samples and d_6-α-tocopherol concentration is determined. The upper panel presents the chromatogram of the pool TRLs and the lower panel the evolution of the concentration of d_6-α-tocopherol over a 9-month period.

confirms that this proposed method is sensitive enough to allow the determination of pharmacokinetic d_6-α-tocopherol in TRL of subjects having consumed a relatively low dose of deuterated tocopherol, i.e., 30 mg. The calculation of the area under the curve of this concentration–time curve

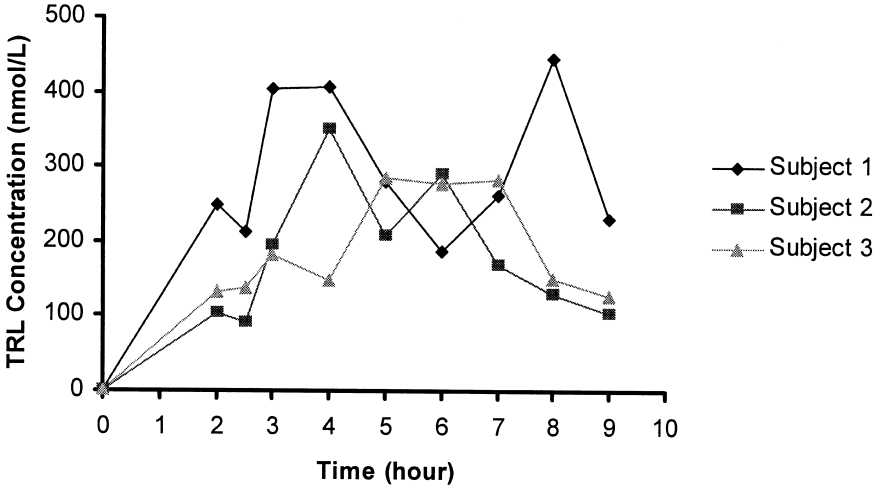

Fig. 3. d_6-α-Tocopherol concentration in plasma TRLs of three healthy male subjects after ingestion of an oral dose of 30 mg of d_6-α-tocopherol.

would provide information on the relative absorption of α-tocopherol by humans.

4. Conclusion

The development of a simple and sensitive method to study the absorption of α-tocopherol in humans is of considerable interest. The use of tocopherol labelled with stable isotope allows the biodiscrimination of tocopherol consumed from the endogenous tocopherol pool of the studied subject. Up to now, the determination of both isotopomers of tocopherol requires the use of MS techniques which are time-consuming, require multiple sample manipulation steps, the need of another labelling of tocopherol as internal standard which is not commercially available as well as sophisticated and expensive MS instrumentation. The HPLC–UV method presented here is a simple and straightforward technique that could be used for the assessment of tocopherol absorption in human clinical trials.

The quantification of isotopomers of α-tocopherol is in the range of pmol similar to most of the described MS methods. In addition, the stability of the HPLC system allows continuous processing of biological samples 24 h a day and 7 days a week leading to analysis of 180 per week. The utility of this method has been demonstrated in a pilot study involving human subjects who consumed an oral dose of 30 mg of d_6-α-tocopherol. The advantages of this new technique are now being exploited in a clinical study examining the effect of various dietary compounds on the absorption of α-tocopherol in man.

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