Mass spectrometry approaches for vitamin E research

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Abstract
Vitamin E is an important nutrient with antioxidant and non-antioxidant functions, and certain evidence suggests that it has a cardiovascular protective role. It is therefore important to maintain an optimal vitamin E status. In the present paper, a number of MS applications to monitor vitamin E status and its interactions, including the use of stable-isotope-labelled vitamin E and metabonomics, are highlighted. Specifically, stable-isotope studies have been used to monitor vitamin E absorption, hepatic processing and lipoprotein transport. As oxidative stress may influence vitamin E status, a number of studies comparing vitamin E biokinetics and metabolism in cigarette smokers and non-smokers have been able to show differences in vitamin E processing in smokers. Metabonomics represents a method to identify changes to metabolite profiles, offering the potential to investigate interactions between vitamin E and metabolic pathways. These applications represent innovative approaches to investigate the role of vitamin E in health and disease.

Introduction
Vitamin E is a group of eight compounds (α-, γ-, β- and δ-tocopherols and -tocotrienols), which differ in their methyl substitution and saturation. The predominant form in the body, comprising over 90% of vitamin E, is α-tocopherol [1]. This form has been widely researched owing to its antioxidant and non-antioxidant functions and its potential as a cardioprotective agent. The most well-researched role for vitamin E is as a chain-breaking antioxidant protecting polyunsaturated fatty acids within biological membranes from lipid peroxidation. Vitamin E also appears to have a variety of roles which are independent of its antioxidant properties, including modulation of monocyte function, inhibition of platelet aggregation, inhibition of smooth muscle cell proliferation and modulation of gene expression [2].

These roles for vitamin E suggest that it is an important nutrient for the vasculature and provide a rationale for the use of vitamin E in clinical trials as a cardioprotective agent. Therefore it is important to have an adequate vitamin E status. As the measurement of plasma vitamin E levels are inadequate markers of status, being strongly associated with lipids and reflective of dietary intake, other means of monitoring vitamin E status are required. The use of stable-isotope-labelled vitamin E and MS analysis to monitor vitamin status was pioneered by the research groups of Burton, Ingold and Traber, and we have used this technique in a number of studies aimed at investigating factors which can influence vitamin E status. The more novel technique of LC (liquid chromatography)–MS-based metabonomics represents a method to understand how vitamin E influences metabolite profiles, offering insights into the perturbation of metabolic pathways.

Stable-isotope studies for monitoring vitamin E physiology
A typical study protocol involves subjects, in a fasted state, consuming a capsule containing 150 mg of hexadeuterated RRR-α-tocopheryl acetate. Blood samples are then taken at various intervals up to 48 h following dosing, and 24 h urine samples taken 2 days before and for 3 days after the supplement. From the blood samples, the plasma, erythrocytes, platelets and lymphocytes are isolated using standard methodology, and the deuterated and undeuterated tocopherols in these samples are measured by MS using trideuterated α-tocopherol as an internal standard. We have developed methods for the sensitive analysis of deuterated and undeuterated tocopherols in blood components using LC–TOF (time-of-flight)-MS [3] and urinary vitamin E metabolites [4], which we later developed further for MS.

Vitamin E transport and delivery within the body is governed by the kinetics of plasma lipoprotein metabolism, and much of what we now know about vitamin E transport has been concluded from using stable-isotope approaches. Intestinal absorption requires the presence of adequate fat; however, the amount of fat required for optimal vitamin E absorption remains in doubt. We have previously compared deuterium-labelled α-tocopherol plasma uptake with differing intakes of fat representative of typical breakfast meals [5]. We found limited plasma uptake of newly absorbed α-tocopherol following a meal containing 2.7 g of fat (cereal plus semi-skimmed milk), similar to that when vitamin E was taken with 0 g of fat (water). However, significantly higher labelled α-tocopherol concentrations were achieved with meals containing 17.5 g of fat, and, interestingly, we...
also found that not only the amount of fat, but also the food matrix, influenced the extent of vitamin E absorption [5].

Following intestinal absorption, vitamin E is packaged into chylomicrons. At this stage, studies have shown that there is no discrimination between vitamin E forms [6,7]. Hydrolysis of circulating chylomicrons results in the transfer of lipids and vitamin E to peripheral tissues [8]. Chylomicron remnants then enter the liver, and it is here that vitamin E regulation occurs. Natural (RRR)-α-tocopherol is preferentially incorporated into VLDL (very-low-density lipoprotein) via the hepatic α-1TP (α-tocopherol transport protein) for systemic distribution. A number of biokinetic studies have demonstrated the preferential secretion of α-tocopherol in VLDL over other vitamin E forms [6,7], so that there is a retention of α-tocopherol in the body. During VLDL hydrolysis and LDL formation, vitamin E can again be transferred to tissues [9], and there is also a constant flux of vitamin E between circulating lipoproteins [10]. The monitoring of α-tocopherol in lipoproteins demonstrates that α-tocopherol transport is dependent on lipoprotein kinetics [11,12], with peaks in α-tocopherol concentration coinciding with lipoprotein dynamics.

Overall, following plasma uptake of newly absorbed α-tocopherol, there is a simultaneous decrease in pre-existing or endogenous α-tocopherol concentration. This rapid turnover of α-tocopherol in the plasma, such that the ‘new’ constantly replaces the ‘old’, is an established phenomenon identified by stable-isotope techniques [11,13,14] and also occurs within individual lipoproteins [11]. We have also investigated labelled α-tocopherol uptake into blood components [11]. Whereas erythrocyte uptake follows a similar profile to that of plasma, uptake into platelets and lymphocytes follows a different pattern, with a slower uptake reaching a plateau between 24 and 48 h, and limited turnover of endogenous α-tocopherol [11], and we believe that, since these components predominantly obtain their vitamin E through a more controlled receptor-mediated process, this is representative of α-tocopherol tissue uptake.

**Other factors influencing vitamin E bioavailability**

We have investigated other physiological factors that could influence vitamin E bio kinetics [15]. In a study investigating the influence of the plasma lipid status, we found differences in α-tocopherol bio kin etics between normolipida emics and hyperlipidaemics [16]. A decreased uptake of labelled α-tocopherol into plasma, lipoproteins, erythrocytes, platelets and lymphocytes was found in the hyperlipidaemics, showing that differences in steady-state lipid status influence the uptake of newly absorbed α-tocopherol into plasma [16]. Vitamin E status itself also influences uptake of newly absorbed vitamin E. We performed single-dose biokinetic studies before and after an extensive supplementation period (which saturates total plasma α-tocopherol concentration), and found decreased bioavailability of newly absorbed (labelled) α-tocopherol post-supplementation, indicating that, following plasma saturation, the ability for the plasma to take up newly absorbed α-tocopherol is diminished [17,18].

We have suggested that genetic heterogeneity is an important determinant of vitamin E status and greatly contributes to interindividual variation in response to vitamin E [19] and proceeded to show that individuals with apoE (apolipoprotein E) 3/3 and apoE3/4 have different biokinetic profiles of newly absorbed α-tocopherol following a single dose [15].

These studies highlight that vitamin E homoeostasis is ultimately dependent upon a combination of factors that control its absorption, transport, distribution and metabolism, and that approaches using stable isotopes are essential to monitor vitamin E status in vivo.

**Stable isotope studies for monitoring vitamin E status in smokers as in vivo models of oxidative stress**

Cigarette smokers are under sustained oxidative stress because of the large number of reactive oxygen and nitrogen species contained in both the gas and tar phases of the cigarettes. Although studies have consistently demonstrated lower levels of certain plasma antioxidants in smokers [20] and increased levels of oxidative products such as F₂-isoprostanes [21], an effect of smoking on vitamin E status is less clear, with some studies having observed comparable circulating levels [22], whereas others report decreased concentrations in smokers relative to non-smoking groups [23]. As plasma vitamin E concentrations are regulated, this may not be the best approach to monitor status. Indeed we have shown that smokers have significantly lower α-tocopherol levels in lymphocytes and platelets even though plasma levels were similar in non-smokers [24], whereas others have found significantly lower concentrations of vitamin E in arterial tissue of smokers compared with non-smokers even though plasma levels were similar [25]. These studies highlight the necessity of alternative approaches to investigate vitamin E status and disease interactions.

In a single-dose study in smokers and non-smokers, we found that, following administration of deuterium-labelled RRR-α-tocopheryl acetate, the elimination rate of unlabelled (endogenous) α-tocopherol in the first 12 h following administration was significantly higher in smokers compared with non-smokers and this resulted in a significantly shorter half-life of unlabelled α-tocopherol [26]. In smokers, unlabelled α-tocopherol concentration decreased almost 2-fold more than that in non-smokers. However, even though smokers had a faster and more extensive disappearance of unlabelled α-tocopherol, smokers took up less of the newly absorbed labelled α-tocopherol than non-smokers, as demonstrated by a lower $C_{max}$ (maximum plasma concentration) and AUC (area under the curve) from labelled α-tocopherol concentration against time profiles [26]. These results suggest that smokers and non-smokers differ in their processing of α-tocopherol. In other studies with multiple dosing regimes, a faster disappearance of newly absorbed labelled α-tocopherol was demonstrated in smokers [27]. The disappearance rate was found to correlate with vitamin C status [27], and the same group went on to show that the
higher disappearance rate in smokers could be normalized by vitamin C supplementation [28]. This important observation is a good indication of vitamin E–vitamin C interactions in vivo induced by oxidative stress. There have been no direct oxidation products of α-tocopherol found in vivo; however, nitrated oxidation products of γ-tocopherol have been shown to be significantly higher in smokers compared with non-smokers [29], and nitrated γ-tocopherol does increase in plasma exposed to cigarette smoke in vitro [29]. These various lines of evidence suggest that smokers and non-smokers differ in their hepatic processing of vitamin E, and oxidative stress may be a cause of these differences.

**Vitamin E metabolism and smoking**

If there are differences in the hepatic processing of α-tocopherol in smokers, this may also be observed by monitoring vitamin E metabolite production. Vitamin E is metabolized in the liver by CYP (cytochrome P450)-induced ω-oxidation followed by consecutive β-oxidation, yielding CEHCs (carboxyethylhydroxychromans) which are present in both plasma and urine. Tocopherols appear to be preferentially metabolized by different CYPs and to different extents. α-Tocopherol is metabolized primarily by CYP3A4, but only a small fraction of the dose (<1%) is found as α-CEHC [30], whereas γ-tocopherol is primarily metabolized by CYP4F2 and is extensively converted into γ-CEHC [31]. In a study using labelled synthetic (all rac) and natural (RRR) α-tocopherol (when given simultaneously), the synthetic form of α-tocopherol was metabolized to a greater extent than the natural form [32]. These studies highlight the selective metabolism of vitamin E forms other than α-tocopherol. However, α-tocopherol metabolism is more extensive during periods of supplementation when intakes of α-tocopherol saturate α-TTP [33]. In a recent study, we found decreased urinary excretion of the specific α-tocopherol metabolite α-CEHC in smokers compared with non-smokers [26]. Similarly, separate biokinetic studies have also demonstrated decreased plasma α-CEHC and γ-CEHC in smokers compared with non-smokers [27,34]. We also found that, following extensive vitamin E supplementation (400 mg/day for 4 weeks) which should normalize the metabolic response between smokers and non-smokers (as α-TTP would be saturated leading to extensive α-CEHC production [33]), smokers still excreted approx. 50% of α-CEHC as compared with non-smokers [26]. Although it has been suggested that the difference in vitamin E metabolites is due to oxidative stress [34], it must be taken into account that smokers have decreased activity of CYP3A [35] which catalyses the primary step in α-tocopherol metabolism and which provides an alternative explanation for the differences, at least in the metabolism of vitamin E, between smokers and non-smokers.

**LC–MS-based metabonomic approaches for the study of vitamin E interactions**

Metabolomics and metabonomics are emerging techniques for the characterization of biological samples that are gaining increasing interest in disease diagnostics and treatment. Whereas metabolomics aims to identify and quantify the metabolome, metabonomics has been defined as the “quantitative measurement of time-related multiparametric responses of multicellular systems to pathophysiological stimuli or genetic modifications” [36]. Owing to the complexity of biological fluids and the large number of compounds present, data from biological mixtures are highly complex and are therefore processed using multivariate statistical analysis and pattern recognition. These chemometric measurements generate metabolic profiles or patterns of metabolites from which it is then possible to detect changes to the normal phenotype induced by, for example, drugs, toxins and disease (pathophysiological stimuli) or distinguish between gender or strain (genetics). In essence, metabonomics is a non-invasive method for the investigation of global metabolite profiles in biological fluids, and, as these profiles reflect the metabolic status, this is potentially a powerful technique for defining diet- and disease-induced metabolic changes. Metabonomics has been used recently for several types of investigation in humans, including diagnosis of disease [37,38], toxicity [39] and nutritional studies [40–42]. There have also been many reviews suggesting the potential value for this technology in human studies (e.g. [43–45]).

Metabonomic work using a combination of the newly developed UPLC (ultra-performance liquid chromatography) combined with TOF-MS has been shown to have improved peak resolution, increased sensitivity and speed (10-fold) over conventional LC [46]. The superior separation and improved spectral quality greatly enhances MS analysis, improving MS sensitivity and reducing ion suppression [46], and is now a powerful analytical tool for such research.

Although the potential of this technology is without question, the actual methodological optimization for the analysis of human biological fluids with MS is not yet firm. Because the technique requires optimization of sample extraction as well as LC and MS conditions, there can be considerable analytical variation [47,48]. This is aside from the considerable biological variation in metabolite profiles that exists owing to sex, age or body mass index [49]. Following preliminary investigations (M. Wong and J.K. Lodge, unpublished work) in which we have attempted to optimize LC–MS conditions based on the total number of features observed in both positive- and negative-ionization modes, intra- and inter-individual variation in ion intensity and the discriminatory power of multivariate analysis in a model group of male and female subjects, we have applied this technique to study vitamin E–metabolome interactions. Principal component analysis of samples obtained before and after vitamin E supplementation (400 mg/day for 4 weeks) showed weak discrimination between groups; however, it was still possible to highlight a number of features that were either significantly increased or decreased following supplementation, and we are currently in the process of identifying those metabolites. Although preliminary, such approaches demonstrate that
vitamin E can influence the plasma metabolome, opening up new areas of vitamin E research.

Conclusions

Vitamin E has a variety of antioxidant and non-antioxidant functions relating to a cardioprotective role, and it is therefore important to maintain an optimal vitamin E status. To monitor vitamin E status and the factors influencing status in vivo, it is clear that certain approaches are required and it is not enough to simply measure plasma vitamin E concentrations.

We, and others, have successfully used stable isotopes and subsequent MS analysis to study vitamin E status. Such techniques have been vital for the understanding of vitamin E physiology, hepatic vitamin E processing and vitamin E metabolism. They have also been used to show that, smoking, as an in vivo model of oxidative stress, influences vitamin E kinetics and metabolism indicating that oxidative stress has an impact on vitamin E status. With the development of newer technologies such as LC–MS-based metabolomics, it is now possible to gain insights into the influence of vitamin E on metabolic pathways and changes to the metabolome, potentially opening novel areas of vitamin E research and its role in health and disease.

Yvonne Jeanes, Wendy Hall and Arina Proteggente performed the vitamin E studies, and Max Wong performed the metabolomic analysis. Thank you to Christine Gartner and Dr James Clark of Cognis Health and Nutrition for the gifts of the deuterated tocopherol acetates. The British Heart Foundation, BASF Aktiengesellschaft and the University of Surrey provided financial support.

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Received 28 April 2008
doi:10.1042/BST0361066