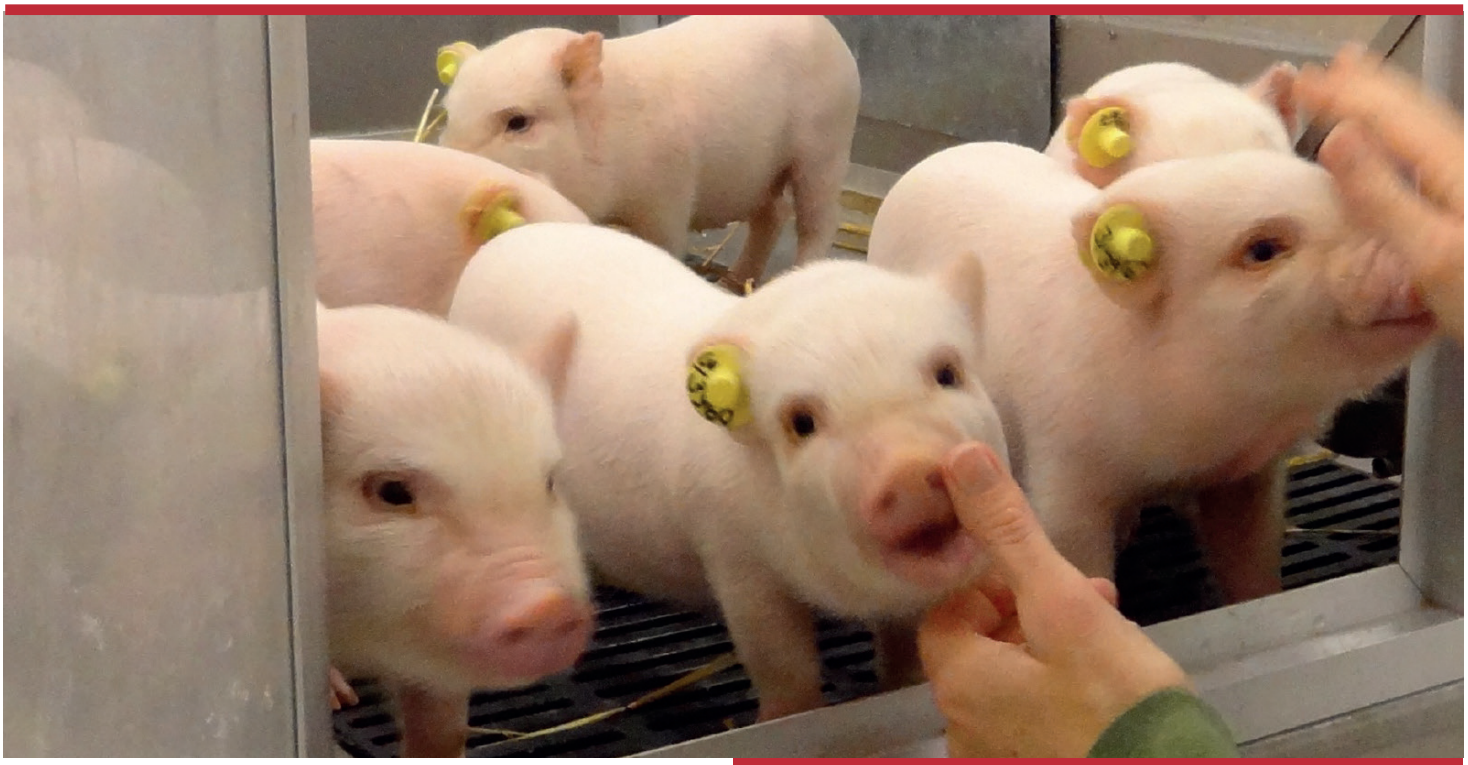
Vitamin D_3 in Pigs: Distribution, Storage and Turnover under Various Input Conditions



Anders Burild
PhD Thesis
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DTU Food
National Food Institute

Vitamin D_3 in Pigs

Distribution, Storage and Turnover

under Various Input Conditions

PhD thesis

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Division of Food Chemistry

National Food Institute

Technical University of Denmark

2014

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Preface

This PhD-thesis is submitted as a partial requirement for the attainment of my PhD-degree. The research was conducted in Dec. 2010-April 2013 and Jan. 2014-July 2014. The animal studies were carried out at the Division of Toxicology and Risk Assessment, DTU Food and the bioanalysis of vitamin D_3 was performed at the Division of Food Chemistry, DTU Food. The mathematical modelling was done at the Pharmacometric Group, the Department of Pharmaceutical Sciences, Uppsala University, Sweden from 1-30 November 2012 and from 17-31 March 2014. The PhD-project was funded by a grant from the Ministry of Higher Education and Science.

Senior Scientist Jette Jakobsen from Division of Food Chemistry was my main supervisor and Senior Scientist Henrik Frandsen from Division of Food Chemistry and Senior Scientist Morten Poulsen from the Division of Toxicology and Risk Assessment were my co-supervisors. Professor Mats Karlsson and Post Doc Elke Krekels from Uppsala University were supervisors for me during my externals stays at Uppsala University.

Several people have contributed to my completion of this work, and I would like to express my gratitude to the following:

My three supervisors, Jette Jakobsen, Henrik Frandsen and Morten Poulsen for their guidance and support throughout the PhD-project and for investing in my personal development as a researcher and communicator.

Mats Karlsson for welcoming me in Uppsala and for teaching me the art of modelling. The modelling work in this thesis would not have been possible without Elke Krekels, who has also been helping me after I returned to Denmark. Thank you so much for all the interesting discussions around modelling.

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Alicja Mortensen, Louise Isling and Charlotte Madsen from Division of Toxicology and Risk Assessment for contributing with their veterinary expertise.

All my colleagues from DTU Food who have helped me on a daily basis and made me feel very welcome at DTU Food, Mørkhøj.

Finally, I would like to thank my friends and family for their support and highly appreciated distractions during the PhD-project.

Anders Burild

DTU Food, Mørkhøj, July 2014

Summary

Vitamin D_3 is important for the mineralization of the skeleton to prevent the deficiency diseases rickets and osteoporosis, and to maintain a healthy skeleton throughout life.

Vitamin D_3 is synthesized in the skin after exposure to the sun. Due to the low angle of the sun during wintertime at high latitudes, no or only a negligible amount of vitamin D_3 is synthesized and the body needs to rely on its storages of vitamin D_3, or dietary vitamin D_3 in the form of vitamin D_3 and 25-hydroxyvitamin D_3. The information of the size of the storages of vitamin D_3 in humans is sparse, but very low levels of vitamin D_3 is found in tissues from animals fed physiologically relevant doses of vitamin D_3. The natural synthesis of vitamin D_3 might, however, influence on the storages of vitamin D_3. The different inherent properties of the two forms of vitamin D_3 might also affect the tissue distribution of vitamin D_3 and 25-hydroxyvitamin D_3 and how the distribution associates with serum 25-hydroxyvitamin D_3.

To study the association between vitamin D_3 and 25-hydroxyvitamin D_3 in serum and tissue, two analytical methods were developed and validated.

The difference in tissue distribution of vitamin D_3 and 25-hydroxyvitamin D_3 after supplementation of vitamin D_3 and 25-hydroxyvitamin D_3 was investigated in slaughter pigs. Tissue 25-hydroxyvitamin D_3 was significantly higher in pigs fed 25-hydroxyvitamin D_3 compared to vitamin D_3, but vitamin D_3 in tissue was higher in the pigs fed vitamin D_3. The content of 25-hydroxyvitamin D_3 in the different tissues correlated with the serum 25-hydroxyvitamin D_3 level, but the correlation between the tissue content of vitamin D_3 and the serum 25-hydroxyvitamin D_3 concentration was dependent on the form of the ingested vitamin D_3.

Göttingen minipigs were used to investigate the endogenous storages of vitamin D_3 after UV-exposure to stimulate synthesis of vitamin D_3 and after oral supplementation of vitamin D_3. Furthermore, the minipigs were used to study the turnover of synthesized vitamin D_3 in skin and adipose tissue during vitamin D_3 shortages.

Daily UV-exposure of minipigs stimulated the cutaneous synthesis of vitamin D_3. The results showed an increase in serum vitamin D_3 and 25-hydroxyvitamin D_3, but also tissues and organs contained vitamin D_3 and 25-hydroxyvitamin D_3. The vitamin D_3 content in adipose tissue from the UV-exposed minipigs was 150-260 ng/g while the content was 90-150 ng/g in the orally

supplemented minipigs. Vitamin D_3 and 25-hydroxyvitamin D_3 declined from the skin and the adipose tissue after the UV-exposure had ceased.

A comprehensive pharmacokinetic-model was established to describe the relation between vitamin D_3 in tissue and vitamin D_3 and 25-hydroxyvitamin D_3 in serum by taking both synthesized and orally supplemented vitamin D_3 into account.

Resumé (in Danish)

D_3-vitamin er vigtigt for knoglemineraliseringen for at forebygge mangelsygdommene engelsk syge og knogleskørhed samt for at sikre sunde knogler igennem hele livet.

D_3-vitamin dannes i huden efter soleksponering. Da solen står lavt på himmelen på de høje breddegrader om vinteren, dannes intet eller negligerbare mængder af D_3-vitamin. Kroppen må derfor klare sig med dets eget D_3-vitamin lager eller D_3-vitamin fra kosten, enten i form af D_3-vitamin eller som 25-hydroxy D_3-vitamin. Viden om D_3-vitamin lagerets størrelse er yderst sparsom, men væv fra dyr, som har fået fysiologisk relevante mængder af D_3-vitamin, indeholder kun små mængder af D_3-vitamin. Den naturlige dannelse af D_3-vitamin i huden kan dog tænkes at påvirke D_3-vitamin lageret.

De to forskellige former af D_3-vitamin har forskellige kemiske egenskaber, hvilket muligvis kan påvirke vævsfordelingen af D_3-vitamin og 25-hydroxy D_3-vitamin, samt sammenhængen mellem vævsfordelingen og koncentrationen af 25-hydroxy D_3-vitamin i serum.

To analytiske metoder blev udviklet og valideret til at belyse sammenhængen mellem D_3-vitamin og 25-hydroxy D_3-vitamin i serum og væv.

Forskellen i vævsfordelingen mellem D_3-vitamin og 25-hydroxy D_3-vitamin blev belyst i slagtegrise fodret med enten D_3-vitamin eller 25-hydroxy D_3-vitamin. Vævskoncentrationen af 25-hydroxy D_3-vitamin var signifikant højere i de grise, som blev fodret med 25-hydroxy D_3-vitamin i forhold til grisene fodret med D_3-vitamin, men disse grise havde til gengæld højere D_3-vitamin i vævene. Vævskoncentrationen af 25-hydroxy D_3-vitamin korrelerede med 25-hydroxy D_3-vitamin i serum, men korrelationen mellem koncentrationen af D_3-vitamin i væv og 25-hydroxy D_3-vitamin i serum var afhængig af, hvilken form af D_3-vitamin, som grisene var blevet fodret med.

Göttingen minigrise blev anvendt til at undersøge det endogene lager af D_3-vitamin efter UV-belysning for at stimulere den endogene syntese og efter oral administration af D_3-vitamin. Desuden blev minigrise anvendt til at undersøge hvordan D_3-vitamin i hud- og fedtvæv omsættes ved D_3-vitamin mangel.

Daglig UV-belysing af minigrisene stimulerede syntesen af D_3-vitamin i huden og resulterede i øget serum indhold af D_3-vitamin og 25-hydroxy D_3-vitamin, samt indhold af D_3-vitamin og 25-hydroxy D_3-vitamin i grisevævene. Indholdet af D_3-vitamin i fedtvævet fra de UV-belyste minigrise var

150-260 ng/g og indholdet i de oralt D_3-vitamin administrerede minigrise var 90-150 ng/g. D_3-vitamin og 25-hydroxy D_3-vitamin indholdet faldt i hud og fedtvæv efter UV-belysningen ophørte.

En omfattende farmakokinetisk model som beskriver sammenhængen mellem D_3-vitamin i væv og D_3-vitamin og 25-hydroxy D_3-vitamin i serum efter både syntese af D_3-vitamin og oral administration af D_3-vitamin blev udviklet.

List of publications

Paper I: **Anders Burild**, Henrik L. Frandsen, Jette Jakobsen, 2014. Simultaneous quantification of vitamin D_3, 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 in human serum by LC-MS/MS. Scandinavian Journal of Clinical and Laboratory Investigation 74, 418-423. Reprinted with permission from Informa Healthcare.

Paper II: **Anders Burild**, Henrik L. Frandsen, Morten Poulsen, Jette Jakobsen, 2014. Quantification of Physiological Levels of Vitamin D_3 and 25-Hydroxyvitamin D_3 in Porcine Fat and Liver by use of Subgram Sample Sizes. Journal of Separation Sciences 37, 2659-2663. Reprinted with permission from Wiley.

Paper III: **Anders Burild**, Charlotte Lauridsen, Nasrin Faqir, Helle M. Sommer, Jette Jakobsen. Tissue content of vitamin D_3 and 25-hydroxyvitamin D_3 and the relation to serum 25-hydroxyvitamin D_3 in pigs supplemented with two forms of vitamin D_3 at four different levels. Submitted to British Journal of Nutrition.

Paper IV: **Anders Burild**, Henrik L. Frandsen, Morten Poulsen, Jette Jakobsen. Tissue Content of Vitamin D_3 and 25-hydroxyvitamin D_3 in Minipigs after cutaneous synthesis, supplementation and deprivation of vitamin D_3. Submitted to Steroids.

List of abbreviations

APCI	Atmospheric pressure chemical ionization
BW	Body weight
CL	Clearance
CYP	Cytochrome P450 monooxygenase
DBP	Vitamin D-binding protein
DEXA	Dual-energy X-ray absorptiometry
ESI	Electrospray ionization
GI	Gastrointestinal
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LC-MS/MS	High performance liquid chromatography coupled with tandem mass spectrometry
LLE	Liquid-liquid extraction
LOQ	Limit of quantification
m/z	Mass to charge ratio
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NP	Normal phase
OFV	Objective function value
PDA	Photodiode array
PFP	Penta fluoro phenyl
PK	Pharmacokinetic
ppb	Parts per billion
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
PTH	Parathyroid hormone
RIA	Radioimmunoassay
RP	Reverse phase
RSE%	Relative standard error
SED	Standard erythema dose
SEM	Standard error mean
SPE	Solid phase extraction
UV	Ultraviolet
V	Volume of distribution
VDR	Vitamin D-binding receptor
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3

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1 Introduction

Vitamin D exists in the two major forms, vitamin D_2 and vitamin D_3. Vitamin D_3 is the vitamer naturally synthesized in the skin after exposure to the sun. Due to the low angle of the sun during wintertime at high latitudes, no or only a negligible amount of vitamin D_3 is synthesized (Webb et al., 1989). During wintertime, vitamin D_3 is acquired from dietary sources of vitamin D_3 and endogenous storages of vitamin D_3, although the importance of the endogenous storages of vitamin D_3 is still debated (Brannon et al., 2008).

The information of the size of the storages of vitamin D_3 in humans is sparse. Very low levels of vitamin D_3 is found in tissues from animals fed physiological relevant doses of vitamin D_3, which can only provide limited reserves of vitamin D_3 (Heaney et al., 2009). The cutaneous synthesis of vitamin D_3 might, however, influence on the storages of vitamin D_3 (Brannon et al., 2008).

Natural dietary sources of vitamin D_3 are products of animal origin where the metabolite 25-hydroxyvitamin D_3 ($25(OH)D_3$) also contribute to the vitamin D_3 content (Ovesen et al., 2003). Oral $25(OH)D_3$ is more potent than oral vitamin D_3 assessed by the biomarker of vitamin D_3 status, measured as serum $25(OH)D_3$ (Jetter et al., 2014;Cashman et al., 2012). Jakobsen *et al.* previously showed that the two forms of vitamin D_3 affect the tissue composition of vitamin D_3 and $25(OH)D_3$ (Jakobsen et al., 2007). The different properties of the two forms of vitamin D_3 might also affect how the tissue distribution of vitamin D_3 and $25(OH)D_3$ associates to serum $25(OH)D_3$.

A minipig animal model for vitamin D_3, an analytical platform for vitamin D_3 and a pharmacokinetic (PK)-model for vitamin D_3 in minipigs were established during the PhD-project to study:

1) The storage of vitamin D_3 and 25-hydroxyvitamin D_3 in minipigs after UV-exposure and oral supplementation of vitamin D_3
2) The turnover of vitamin D_3 during shortages of vitamin D_3.

Data from slaughter pigs generated by Charlotte Lauridsen and Jette Jakobsen was used to study:

3) Differences between oral supplementation of vitamin D_3 and $25(OH)D_3$ in slaughter pigs in terms of storages, and how the storages associate to the serum $25(OH)D_3$ concentration.

The thesis includes two published articles (**Paper I** and **Paper II**) and two submitted manuscripts (**Paper III** and **Paper IV**), which will be referred to throughout the thesis. Furthermore the thesis also includes a novel PK-model describing the concentrations of vitamin D_3 and 25(OH)D_3 under various input conditions in minipigs. The work on the PK-model is still in progress and the PK-model may improve for a future publication.

The structure of the thesis is as follows:

Background information of vitamin D_3 is reviewed in chapter 2. The analytical platform to quantify vitamin D_3 (**Paper I** and **Paper II**) is described in chapter 3. Chapter 4 contains information about the animal models and the results from the animal experiments (**Paper III** and **Paper IV**). The data from the minipigs was analysed by use of a PK-model described in chapter 5, which can be read independently. The conclusion of the thesis and the perspectives are found in chapter 6 and 7, respectively.

2 Background of vitamin D_3

Chapter 2 focuses on some of the aspects of vitamin D_3, which are important to understand the rationale for the PhD-project. Vitamin D exists in two major forms, vitamin D_2 and vitamin D_3. Vitamin D_3 is synthesized in the skin or provided as a natural nutrient from mainly food of animal origin, whereas vitamin D_2 is mainly provided as a nutrient from fungal sources (Ovesen et al., 2003). This thesis only focuses on vitamin D_3. Vitamin D will only be referred to as vitamin D_3 throughout the thesis unless otherwise stated. Most of the biological behaviour of vitamin D_3 will, however, also apply to vitamin D_2 although the equivalence of vitamin D_2 and vitamin D_3 is questionable (Houghton and Vieth, 2006). This will not be discussed any further, as it is regarded beyond the scope of this thesis.

2.1 Vitamin D_3 and health

Vitamin D_3 is involved in the homeostasis of calcium in vertebrates which is important for the mineralization of the skeleton to prevent rickets and osteoporosis and to maintain a healthy skeleton throughout life. The role of vitamin D_3 in maintaining the calcium level is exerted through the binding of the active metabolite 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) to the vitamin D binding receptor (VDR). In the intestines vitamin D_3 increases the uptake of calcium and phosphate by inducing the synthesis of the proteins involved in the active transport of calcium and phosphate. In the bones, vitamin D_3 is important for the activation of bone resorption to mobilize calcium from the bone to the rest of the body. Finally, vitamin D_3 also promotes the calcium reabsorption in the kidney to reduce renal losses of calcium. It is becoming increasingly clear that vitamin D_3 is not only important for bone health, but that it is also associated with the functioning of other organs, and may play a role in supporting a healthy immune system and the prevention of cancer (reviewed by DeLuca, 2004).

Vitamin D_3 intoxication leads to hypercalcemea, nephrocalcinosis and death. Intoxication occurs after an excessive intake of vitamin D_3 or in individuals failing to catabolize $1,25(OH)_2D_3$ (reviewed by Jones, 2008;reviewed by Jones et al., 2011)

2.2 Vitamin D_3 synthesis and metabolism

The metabolism of vitamin D_3 is shown in Figure 2.1. Vitamin D_3 is formed in stratum basale in the skin after UV-exposure where 7-dehydrocholesterol is converted to previtamin D_3. During exposure to sunlight the synthesis occurs at wavelengths between 290 and 315 nm. The previtamin D_3 is converted to vitamin D_3 after thermal isomerisation of previtamin D_3 at body temperature. During excessive sun exposure previtamin D_3 is photoisomerized into two inert metabolites and this photodegradation probably accounts for the most important regulation of the cutaneous synthesis of vitamin D_3 (Webb et al., 1989;MacLaughlin et al., 1982). The vitamin D_3 synthesized in the skin is translocated into the circulating system by binding itself to the vitamin D-binding protein (DBP) (Haddad et al., 1993).

The vitamin D_3 absorbed from the intestine enters the chylomicrons. The vitamin D_3 in the chylomicrons is probably taken up by the peripheral tissue including adipose and muscle tissue via action of the lipoprotein lipase, but vitamin D_3 can also be transferred to DBP. The vitamin D_3 left in the chylomicron is taken up by the liver (reviewed by Jones, 2008;Silver and Berry, 1982;Dueland et al., 1982).

In the liver, vitamin D_3 is hydroxylated by non-tight regulated cytochrome P450 monooxygenases (CYP) including CYP2R1, CYP27A1, CYP2J2/3 and CYP3A4 (reviewed by Jones, 2008;reviewed by Prosser and Jones, 2004;Aiba et al., 2006), although Gupta *et al.* did not observe any 25-hydroxylation by CYP3A4 (Gupta et al., 2004). Extrahepatic 25-hydroxylases are also evident (Wamberg et al., 2012;reviewed by Karlgren et al., 2005). In the kidney, the $25(OH)D_3$ is activated by CYP27B1 to $1,25(OH)_2D_3$ which is tightly regulated by the level of calcium, phosphate and parathyroid hormone (PTH) (reviewed by DeLuca, 2004;reviewed by Prosser and Jones, 2004). $1,25(OH)_2D_3$ also promotes its own inactivation by inducing CYP24A1, which catabolizes $1,25(OH)_2D_3$. The CYP24A1 also hydroxylates $25(OH)D_3$ to 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) mainly considered a catabolite (Wagner et al., 2011), although it might also have some unique biological functions as well (reviewed by Prosser and Jones, 2004). $25(OH)D_3$ and $1,25(OH)_2D_3$ can also be epimerized to the corresponding 3-epimers resulting in a reduced biological activity (Kamao et al., 2004;Molnár et al., 2011).

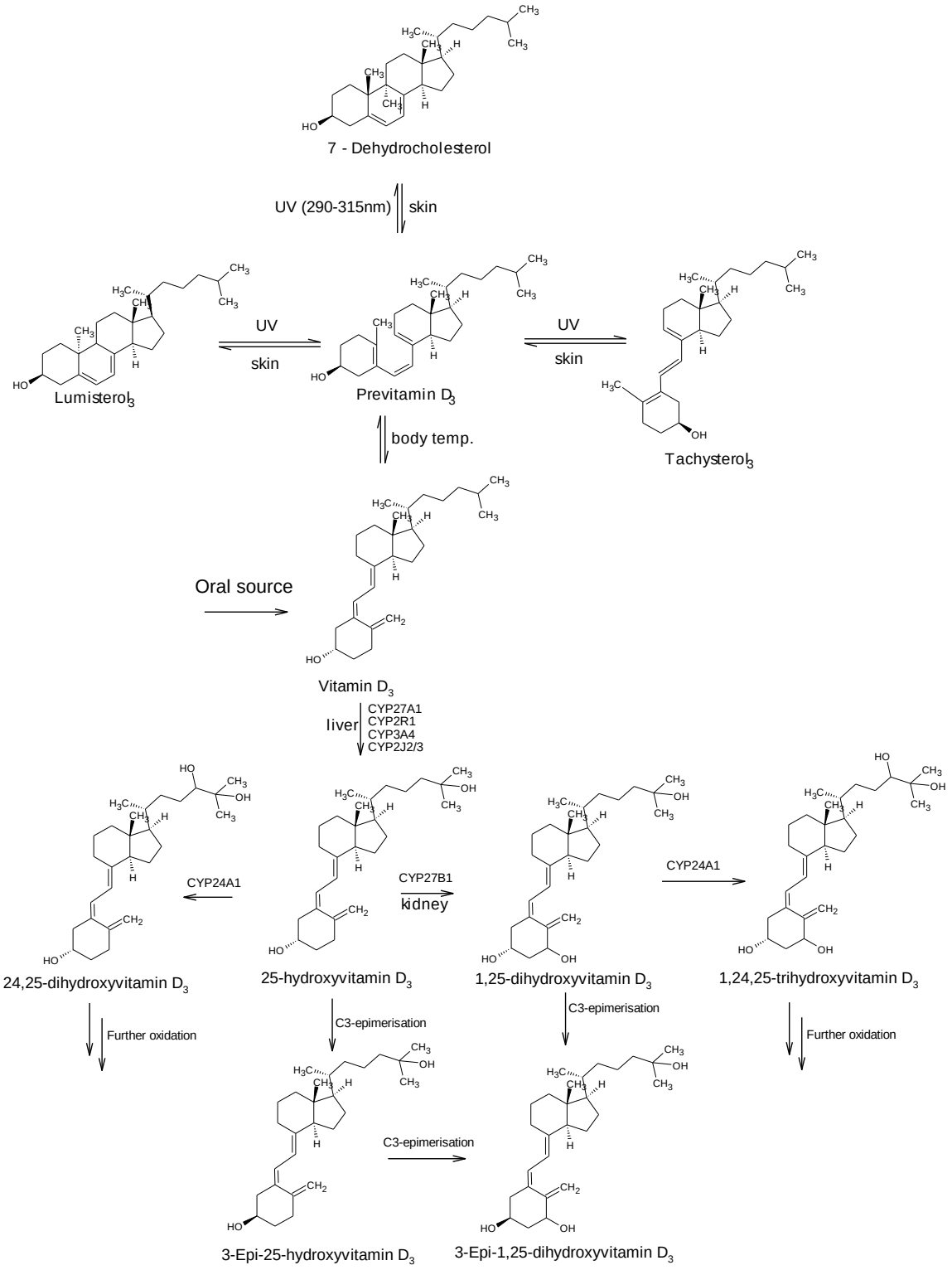

Figure 2.1 Metabolism of vitamin D_3 (after Webb et al., 1989;MacLaughlin et al., 1982;Kamao et al., 2004;reviewed by van den Ouweland et al., 2013).

2.3 Vitamin D_3 status

The circulating vitamin D_3 itself is conventionally considered a poor biomarker of the vitamin D_3 status due to its short half-life and its fluctuation after recent ingested vitamin D_3 or cutaneously synthesized vitamin D_3. $25(OH)D_3$ is considered a better biomarker of vitamin D_3 status because of its non-tight formation and its half-life of 2-3 weeks (reviewed by van den Ouweland et al., 2013). Plasma/serum $25(OH)D_3$ is routinely used in the clinic to assess the vitamin D_3 status (reviewed by Higashi et al., 2010;reviewed by Jones, 2012), and most studies only measure the serum level of $25(OH)D_3$ (Heaney et al., 2009).

Different single nucleotide polymorphisms of CYP2R1 and CYP24A1 involved in the 24-hydroxylation and 25-hydroxylation of vitamin D_3 are associated with the $25(OH)D_3$ status (Engelman et al., 2013;Zhang et al., 2013;Wang et al., 2010), and serum $24,25(OH)_2D_3$ appears to have a close correlation to serum $25(OH)D_3$ (Wagner et al., 2011). Furthermore, the relation of serum $25(OH)D_3$ to serum D_3 is reported to appear non-linear, saturable and regulated (reviewed by Jones et al., 2011;Heaney et al., 2008;Hollis et al., 2007).

2.4 Vitamin D_3 and latitude

Previtamin D_3 is only synthesized during the summer at high latitudes because only few high energy solar photons (<315 nm) reach the surface of the earth during winter. The winter sunlight still consist of less energy containing photons (315-330 nm) promoting the photodestruction of vitamin D_3, which could potentially also influence the vitamin D_3 status (Webb et al., 1989). It is estimated that no cutaneous synthesis of vitamin D_3 occurs in Denmark from October to April. A large seasonal fluctuation in serum $25(OH)D_3$ is also observed for many populations with the lowest serum $25(OH)D_3$ during wither time (Brot et al., 2001;Kuchuk et al., 2009).

2.5 Dietary sources to vitamin D_3

The natural sources of vitamin D_3 are products of animal origin e.g. meat, eggs, milk and fish. $25(OH)D_3$ may contribute to the content of vitamin D_3 in food, either due to the metabolism of vitamin D_3 in all vertebrates (Ovesen et al., 2003), or if the animals are fed high amounts of $25(OH)D_3$ (Jakobsen et al., 2007).

2.6 Storage of vitamin D_3

Vitamin D_3 is belonging to the group of fat-soluble vitamins and it is known to accumulate in human fat after oral supplementation (Heaney et al., 2010). Vitamin D_3 is also found in human fat removed after gastric bypass surgery (Pramyothin et al., 2011;Blum et al., 2008), and in fat sampled *post mortem* (Lawson et al., 1986b).

In rats supplemented with vitamin D_3 the adipose tissues contained the main part of the vitamin D_3. Half of the vitamin D_3 quantified was found as vitamin D_3 and the other half as polar metabolites and esters of vitamin D_3 (Rosenstreich et al., 1971). Based on estimations from data acquired in pigs, it is suggested that the total body of vitamin D_3 corresponds to 370 µg for a 70 kg adult woman. 65% consisted of vitamin D_3 and 35% consisted of 25(OH)D_3. 75% of vitamin D_3 was distributed in the fat, while 25(OH)D_3 was more evenly distributed throughout the body (Heaney et al., 2009).

2.6.1 Differences in storages between oral vitamin D_3 and oral 25-hydroxyvitamin D_3

The potency of dietary 25(OH)D_3 relative to dietary vitamin D_3 is debated (Jakobsen, 2007). In humans, Cashman *et al.* found a potency factor of 5 in men and women >50 years old supplemented for 10 weeks, while Jetter *et al*. recently reported the potency between 25(OH)D_3 and vitamin D_3 to be 2-3, assessed in 50-70 years old women supplemented for 15 weeks (Jetter et al., 2014;Cashman et al., 2012). For pigs, the factor is reported to vary from one up to three (Jakobsen et al., 2007;Lauridsen et al., 2010;Witschi et al., 2011;Höller et al., 2010).

Although differences in the potency is observed for oral vitamin D_3 and 25(OH)D_3 in terms of increment in serum 25(OH)D_3, it is not known how the serum 25(OH)D_3 relates to the tissue content of vitamin D_3. Since 25(OH)D_3 is more polar than vitamin D_3, and the affinity of 25(OH)D_3 for the DBP is more than 500 times stronger than that of vitamin D_3, more D_3 is assumed to be on its free form, which will allow it to diffuse directly into adjacent tissues. These inherent differences between 25(OH)D_3 and vitamin D_3 might be important for the distribution and storage of the different forms of vitamin D_3 (Schuster, 2011).

2.6.2 Differences between oral vitamin D_3 and cutaneously synthesized vitamin D_3

The information of differences of storages of vitamin D_3 and 25(OH)D_3 after cutaneously synthesized vitamin D_3 and oral vitamin D_3 is sparse. The cutaneously synthesized vitamin D_3 is chemically identical to the orally absorbed vitamin D_3. The absorption and the transport differ,

however, between cutaneous synthesized vitamin D_3 and oral vitamin D_3 as described in section 2.2, which could influence on the fate of vitamin D_3 in the body (Brannon et al., 2008).

It is hypothesized that more vitamin D_3 is shunted down the catabolic pathway when the vitamin is presented to the liver via chylomicrons compared to vitamin D_3 bound to DBP (Fraser, 1983). Indeed, Clements *et al.* found a higher biliary excretion rate of orally dosed vitamin D_3 compared to intravenously dosed vitamin D_3 (Clements et al., 1984). Fraser *et al.* also found that dietary vitamin D_3 is mainly esterificated at the site of absorption and during circulation in plasma, whereas there is no evidence of esterification in the liver (Fraser and Kodicek, 1968b).

Exposure to sunlight is the major source of vitamin D_3 for humans (Holick and Chen, 2008), which could be a rationale for finding larger storages of vitamin D_3 in individuals exposed to sun during summertime compared to individuals only relying on dietary sources of vitamin D_3.

2.7 Turnover of vitamin D_3 in tissue during shortage of vitamin D_3

Although vitamin D_3 is found in tissues of animals and humans as discussed in section 2.6, the turnover of vitamin D_3 from endogenous storages is still mainly unknown. The question whether vitamin D_3 and 25(OH)D_3 is sequestered in the tissue or if it is available during periods of shortages of vitamin D_3 is still unanswered (Brannon et al., 2008).

The half-life of vitamin D_3 in serum is 12-26 hours in humans (Smith and Goodman, 1971;Hahn et al., 1972), but a whole body half-life of ~2 months (reviewed by Jones, 2008) could indicate that stored vitamin D_3 is transferred to serum.

The turnover of vitamin D_3 in tissue is studied in rats where an exponential decline of vitamin D_3 in adipose tissue was observed in vitamin D_3-replete rats placed on a vitamin D-free diet (Rosenstreich et al., 1971;Lawson et al., 1986a), whereas a rapid decrease in vitamin D_3 and its metabolites initially was observed in blood, liver and kidney followed by a slower exponential decline (Rosenstreich et al., 1971). Vitamin D_3 in adipose tissue increased in fasting rats prior fed vitamin D_3, probably due to a preferential loss of triglycerides (Brouwer et al., 1998).

3 Quantification of vitamin D_3

To study the association between vitamin D_3 and $25(OH)D_3$ in serum and tissue two analytical methods were developed and validated. Detailed descriptions of the methods are found in **Paper I** and **Paper II**. Chapter 3 focuses on the background for the method development, but does also include some unpublished results generated during the development of the serum method.

3.1 Why LC-MS/MS?

The two major principles for quantification of vitamin D_3 metabolites are protein binding assays such as radioimmunoassay (RIA) and chromatographic methods coupled to a detector, e.g. high performance liquid chromatography (HPLC) coupled with UV-detection or mass spectrometry (MS). The immunoassays are widely used in serum/plasma matrices, but are rarely used for tissue. The immunoassays require very little sample preparation, but they do not distinguish between the vitamin D_2 and D_3 form. In addition, a cross reaction between $25(OH)D_3$ and $24,25(OH)_2D_3$ is reported (reviewed by van den Ouweland et al., 2013;reviewed by Higashi et al., 2010;reviewed by Zerwekh, 2008). MS can distinguish between the vitamin D_2 and D_3 as well as vitamin D_3, $25(OH)D_3$ and $24,25(OH)_2D_3$. Furthermore, the MS can also separate deuterated and 13C-labeled compounds from non-labelled compounds by the mass to charge ratio (*m/z*). That enables the inclusion of labelled internal standards with almost identical chemical properties to correct for losses of analytes during sample preparation and ion suppression in the ion source (reviewed by van den Ouweland et al., 2013). Furthermore, MS also enables the use of stable isotopic compounds in intervention studies. High performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was chosen because of its high specificity and sensitivity when operated in the multiple reaction monitoring (MRM) mode. Briefly, MRM selects a fragmentation reaction by focusing the first analyzer on a selected *m/z* allowing the precursor ion to pass through to the collision cell. In the collision cell the molecular ion is fragmented after colliding with an inert gas. The second mass analyzer is set up to transmit the *m/z* of a specific product ion to the detector (Figure 3.1).

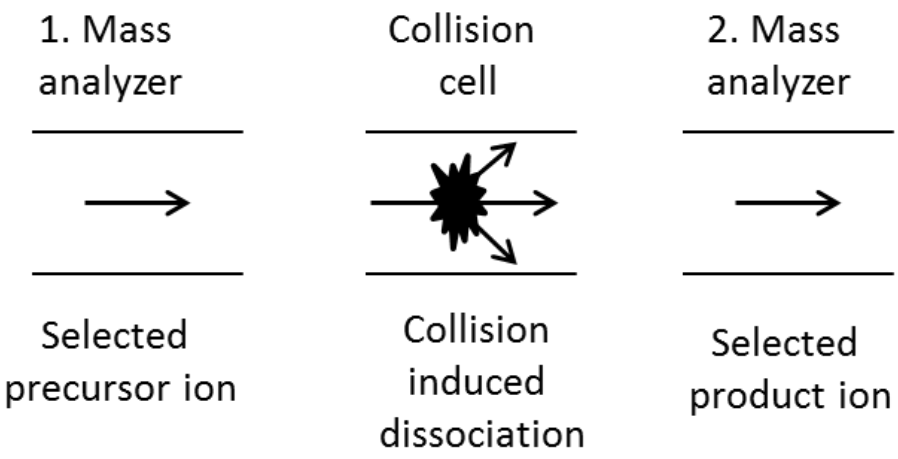

Figure 3.1 Schematic representation of a tandem mass spectrometer operated in the multiple reaction monitoring (MRM) mode.

Quantification of vitamin D_3 and its metabolites is difficult due to the very low levels (<ppb) found in serum and tissue (Jakobsen et al., 2007;reviewed by van den Ouweland et al., 2013). Additionally, the lack of easily charged groups in the vitamin D_3 molecule makes ionization of the molecule difficult in the electrospray ion source (reviewed by van den Ouweland et al., 2013;reviewed by Higashi et al., 2010), but derivatization of vitamin D_3 with Cookson-type reagents improve the sensitivity of the molecule in the mass spectrometer equipped with an electrospray ionization interface (Higashi et al., 2001;Wilson and Wu, 1993). Recently, several LC-MS/MS methods, which quantify vitamin D_3 metabolites in serum, have been described. Aronov *et al.* described an assay for vitamin D_3 metabolite profiling where the metabolites were derivatized with the Cookson-type reagent 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) to improve the ionization ability of the vitamin D_3 metabolites. PTAD reacts selectively with the diene C-10-19 and C-5-6 in the vitamin D_3 molecule which is retained for all the requested metabolites. Both the 6R and 6S epimers are formed (Figure 3.2), but the derivatization still increases sensitivity by 100-1000 fold (Aronov et al., 2008). The sample is derivatised before the introduction to the LC-MS/MS with no need for special equipment or plumbing to the LC-MS/MS. Indeed, the derivatization reaction has proven its reliability and has been used elsewhere (Wang et al., 2011;Higashi et al., 2011;Duan et al., 2010;Ding et al., 2010). An addition of methylamine to the mobile phase further increases the sensitivity when using the methylamine adduct ion as precursor ion for the quantification (Ding et al., 2010).

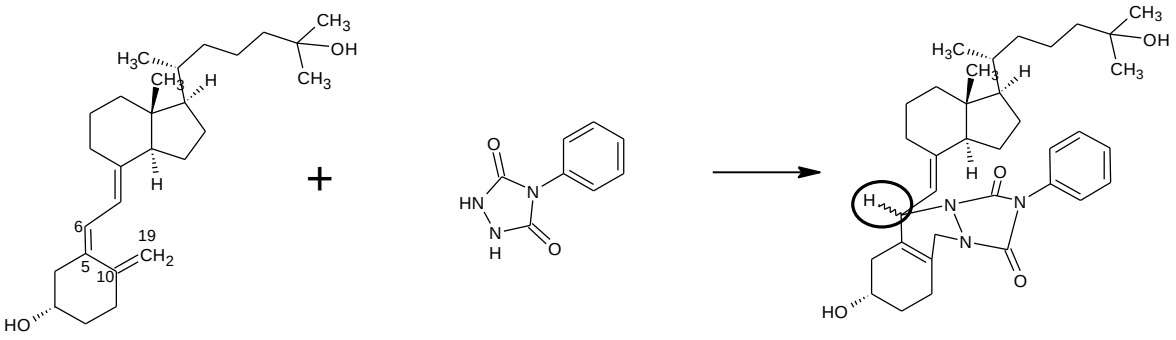

Figure 3.2 The derivatization of 25(OH)D_3 with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) forms the 6R and 6S epimers.

3.2 The chromatography of vitamin D_3, 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3

A C18 analytical column and a gradient consisting of methanol, formic acid and methylamine was developed **(Paper I)** and 24,25$(OH)_2D_3$, 1,25$(OH)_2D_3$ and 25(OH)D_3 and vitamin D_3 eluted between 4 and 14 minutes. For 24,25$(OH)_2D_3$, 1,25$(OH)_2D_3$ and 25(OH)D_3 two peaks were observed which correspond to the 6R and 6S epimers formed after the reaction with PTAD as discussed in section 3.1. The two PTAD-1,25$(OH)_2D_3$ epimers were only partly separated (Figure 3.3).

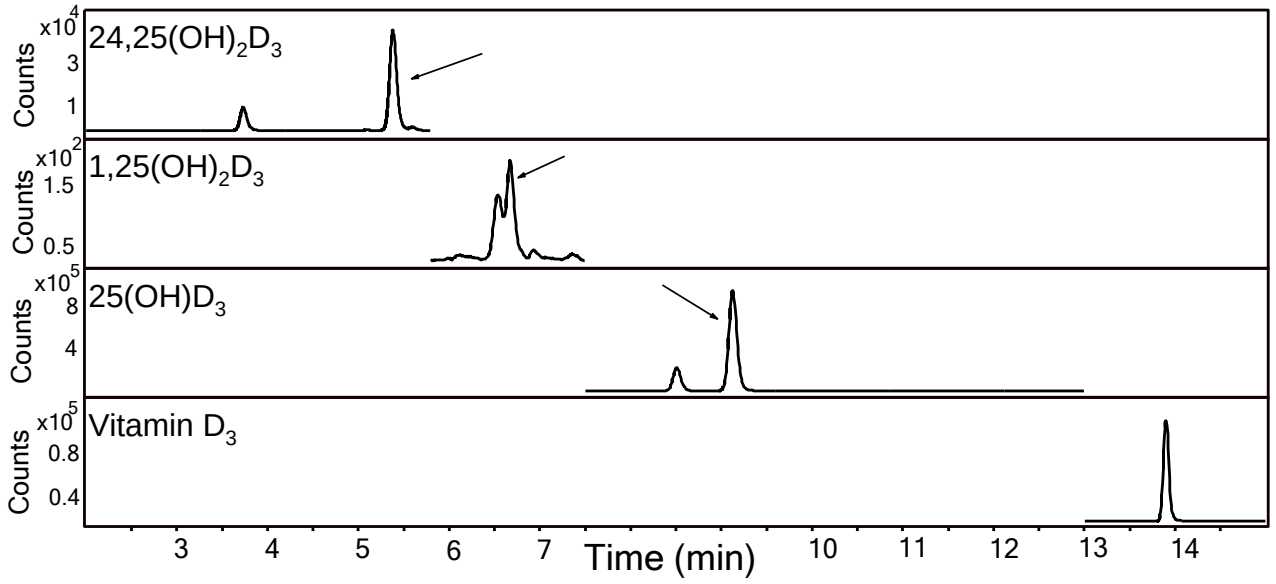

Figure 3.3 The chromatogram of standards of PTAD-24,25$(OH)_2D_3$, PTAD-1,25$(OH)_2D_3$, PTAD-25$(OH)D_3$ and PTAD-vitamin D_3 on a C18 analytical column. See **Paper I** for chromatographic details.

3.3 Quantification of serum vitamin D_3, 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 (Paper I)

Serum 25$(OH)D_3$ is the established biomarker of vitamin D_3, but the metabolism of vitamin D_3 appears complex and the serum vitamin D_3 and 24,25$(OH)_2D_3$ may add valuable information to the vitamin D_3 metabolism as described in section 2.3.

Several methods are described for the determination of either serum vitamin D_3 and 25$(OH)D_3$ (Adamec et al., 2011) or serum 25$(OH)D_3$ and dihydroxylated vitamin D_3 metabolites (Aronov et al., 2008;Wang et al., 2011;Duan et al., 2010;Ding et al., 2010), but no methods are available for the determination of both serum vitamin D_3, 25$(OH)D_3$ and dihydroxylated vitamin D_3.

The purpose was to develop a method for the simultaneous quantification of serum vitamin D_3, 25$(OH)D_3$, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$, although 1,25$(OH)_2D_3$ was not included in the final method due to coeluting interferences as described in section 3.3.2.

3.3.1 Development of the sample preparation

Many published methods for vitamin D_3 in serum/plasma use protein precipitation to release vitamin D_3 metabolites from DBP followed by reverse phase (RP) solid phase extraction (SPE) purification (Aronov et al., 2008;Duan et al., 2010;Ding et al., 2010), or liquid-liquid extraction (LLE) (Wang et al., 2011;Tai et al., 2010;Schleicher et al., 2011). Both principles of extractions were initially tested and the SPE purification described by Ding *et al.* (Ding et al., 2010) resulted in cleaner chromatograms compared to LLE (Figure 3.4). The recovery of vitamin D_3 was <5%, and a low recovery of vitamin D_3 has also previously been reported (Aronov et al., 2008).

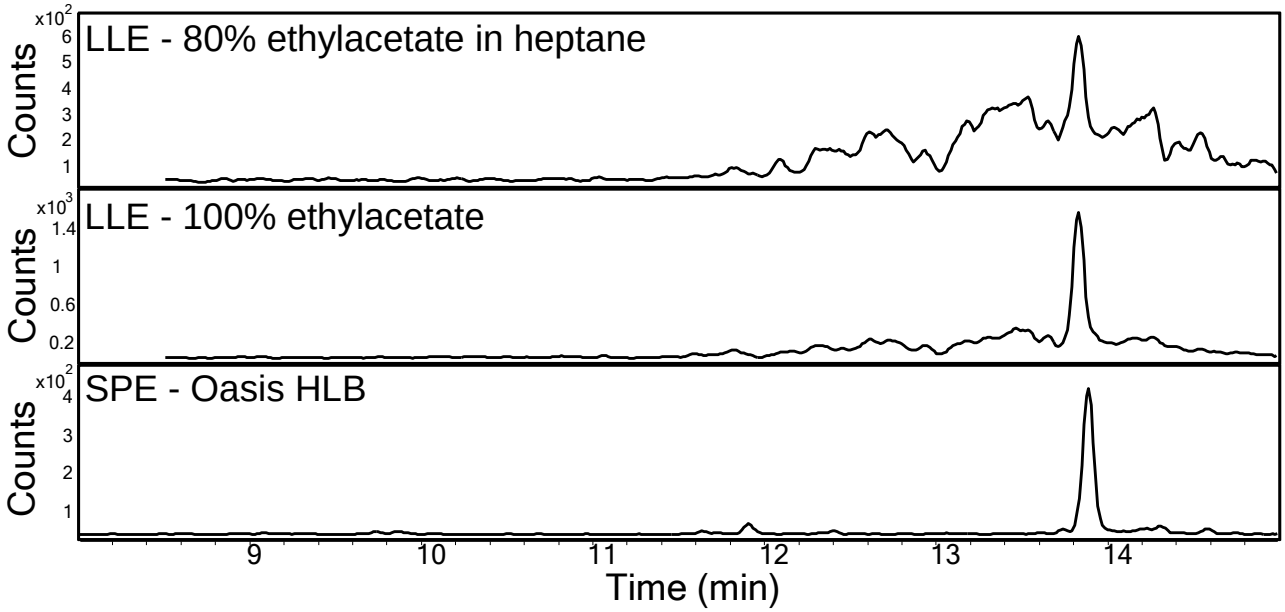

Figure 3.4 Chromatograms of vitamin D_3 in 0.2 mL human serum after either LLE with 2.5 mL of organic solvent or SPE. The cleanest chromatogram was obtained after SPE purification.

The low recovery of vitamin D_3 was hypothesized to be due to ion suppression in the ion source of the mass spectrometer caused by coeluting phospholipids. A precursor scan of *m/z* = 184 of the SPE extract showed large amounts of coeluting interferences (Figure 3.5A), most likely to be coeluting phospholipids (Brugger et al., 1997). As described in **Paper I**, the interferences were successfully removed by a HybridSPE column (Figure 3.5A) which selectively retains phospholipids and the response of vitamin D_3 was improved by 100% (Figure 3.5B).

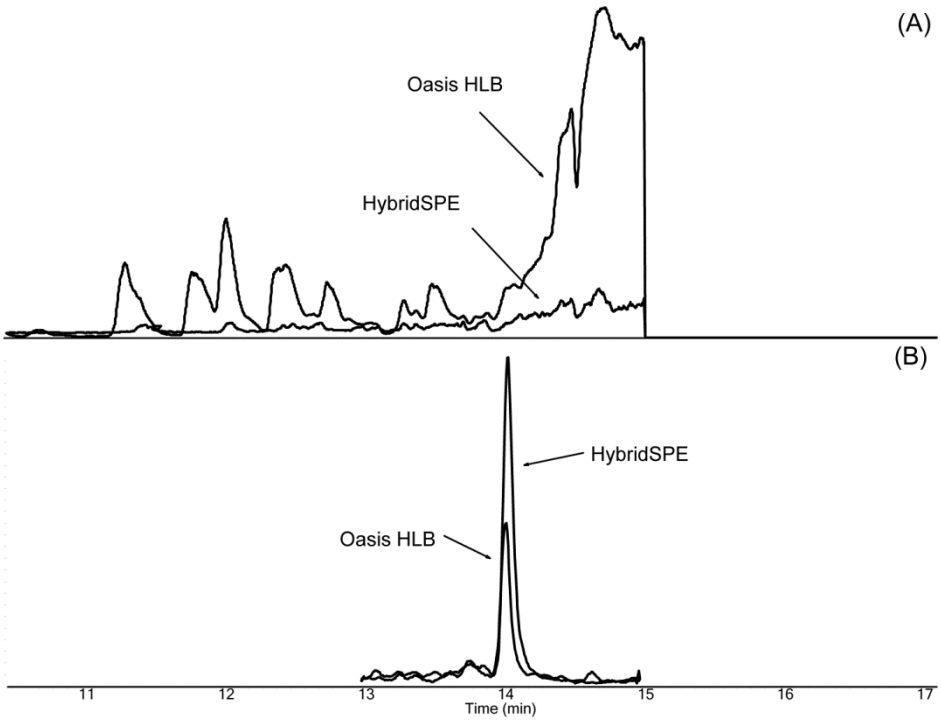

Figure 3.5 Chromatograms of 200 µL serum spiked with deuterated vitamin D_3 after sample clean up by Oasis HLB and HybridSPE. A precursor scan of *m/z* = 184 in (A) and an extracted ion chromatogram of the deuterated vitamin D_3 (B) are shown (Modified figure and legend from **Paper I**).

3.3.2 Coeluting interferences

Recently, it has been evident that the 3-epi-25(OH)D_3 is important to quantify separately, but most methods are not able to distinguish 3-epi-25(OH)D_3 from 25(OH)D_3. The epimer cannot be resolved by MS since the *m/z*-values of both the precursor and the product ions of the 3-epi-25(OH)D_3 do not differ from the 25(OH)D_3.

Penta fluoro phenyl (PFP) analytical columns are reported to have enhanced selectivity for the 3-epi-25(OH)D_3, but have only been tested on the underivatized vitamin D_3 analytes (Schleicher et al., 2011;van den Ouweland, 2011). A C18 analytical column was compared to a PFP analytical column, but neither of the columns were able to separate the PTAD-3-epi-25(OH)D_3 from the PTAD-25(OH)D_3 (Figure 3.6 and **Paper I**). Even separation of 3-epi-25(OH)D_3 from 25(OH)D_3 is desired, it is most relevant when assessing the vitamin D_3 status in infants where the fraction of 3-epi-25(OH)D_3 is substantial, but less important for the adult population (Bailey et al., 2013).

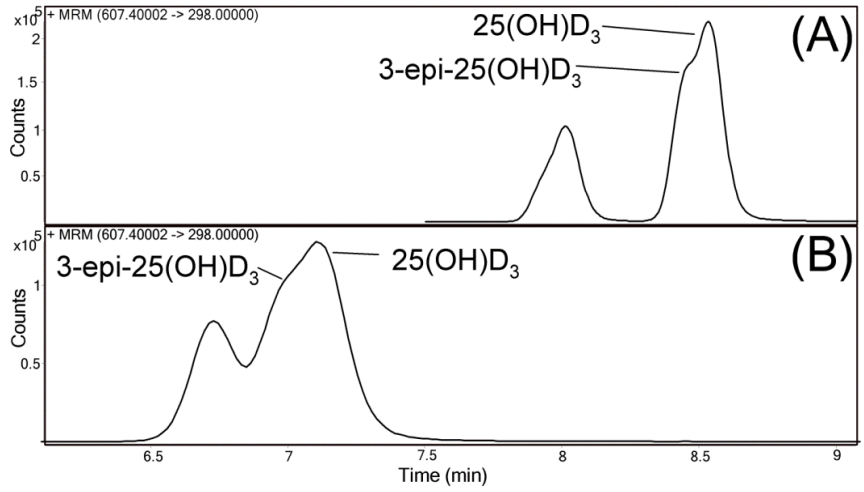

Figure 3.6 Chromatogram of PTAD-3-epi-25(OH)D_3 and PTAD-25(OH)D_3 on a C18 column (A) and on a penta fluoro phenyl (PFP) column (B). For chromatographic details, see **Paper I** (Figure and legend modified from **Paper I**, *supplementary material*).

Serum 1,25$(OH)_2D_3$ was also initially intended to include in the serum method. 1,25$(OH)_2D_3$ eluted at 6.5 minutes and a partial separation of the 6S and 6R epimers were observed for the internal standard and for the standard (Figure 3.7A+B). No separation was, however, observed for 1,25(OH)D_3 in a human serum sample (Figure 3.7C), probably caused by a coeluting interference. Since the interference also gave rise to a product ion with *m/z* = 314 similar to PTAD-1,25$(OH)_2D_3$ (Figure 3.8) it was hypothesized that the interference could be due to another vitamin D_3 metabolite with two hydroxyl groups in the A-ring. Such metabolites have previously been reported and they could be removed by periodate, which selectively cleaves C-C bonds with vicinal hydroxyl groups (Wang et al., 2011).

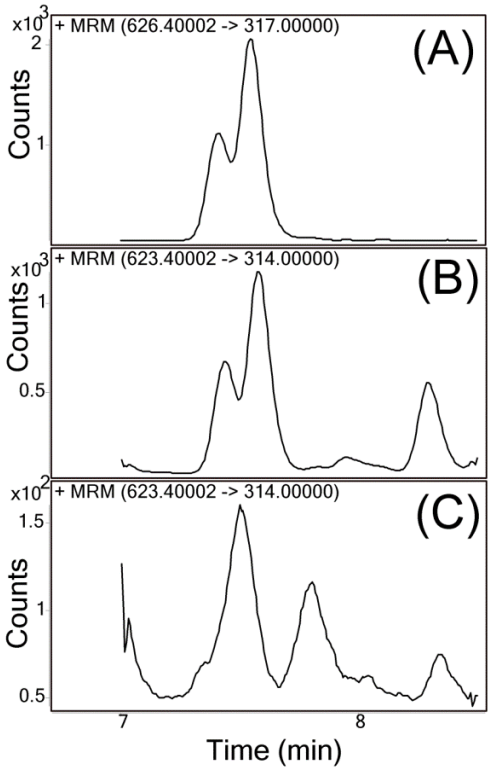

Figure 3.7 Chromatogram of PTAD-*d3*-1,25$(OH)_2D_3$ (A) in serum, PTAD-1,25$(OH)_2D_3$ in 60% methanol (B) and *m/z* 623.4→ 314 corresponding to the mass of PTAD-1,25$(OH)_2D_3$ in serum (C).

To test the hypothesis, 0.5 mL serum containing *d3*-1,25$(OH)_2D_3$ was purified by SPE according to Ding *et al* (Ding et al., 2010). After derivatization with PTAD for 2 hours the sample was dried under a gentle stream of nitrogen and reconstituted in 300 uL of methanol. The sample was treated with periodate as described by Wang *et al.* (Wang et al., 2011), except that potassium periodate was used instead of sodium periodate. The final sample was reconstituted in 200 uL 50% acetonitrile before the analysis by LC-MS/MS as described in **Paper I**. As shown in Figure 3.9A, periodate did not affect *d3*-1,25$(OH)_2D_3$. In contrast, the periodate removed the coeluting interference (Figure 3.9B) indicating an interference due to 2,25-dihydroxyvitamin D_3 or 4,25-dihydroxyvitamin D_3. Periodate as a routine treatment is not possible since the 24,25$(OH)_2D_3$ will also be removed selectively by periodate as shown in Figure 3.9C.

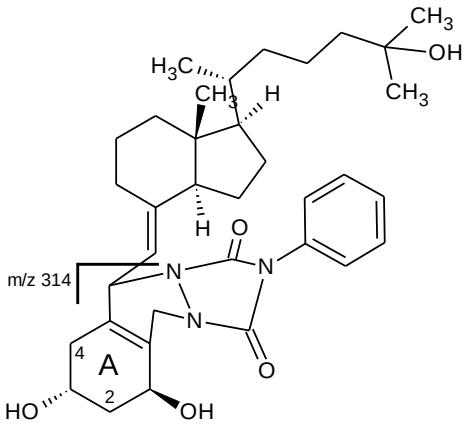

Figure 3.8 Fragmentation of PTAD-1,25$(OH)_2D_3$ gave rise to a product ion of *m/z* = 314. PTAD-2,25$(OH)_2D_3$ and PTAD-4,25$(OH)_2D_3$ would also result in product ions with *m/z* = 314.

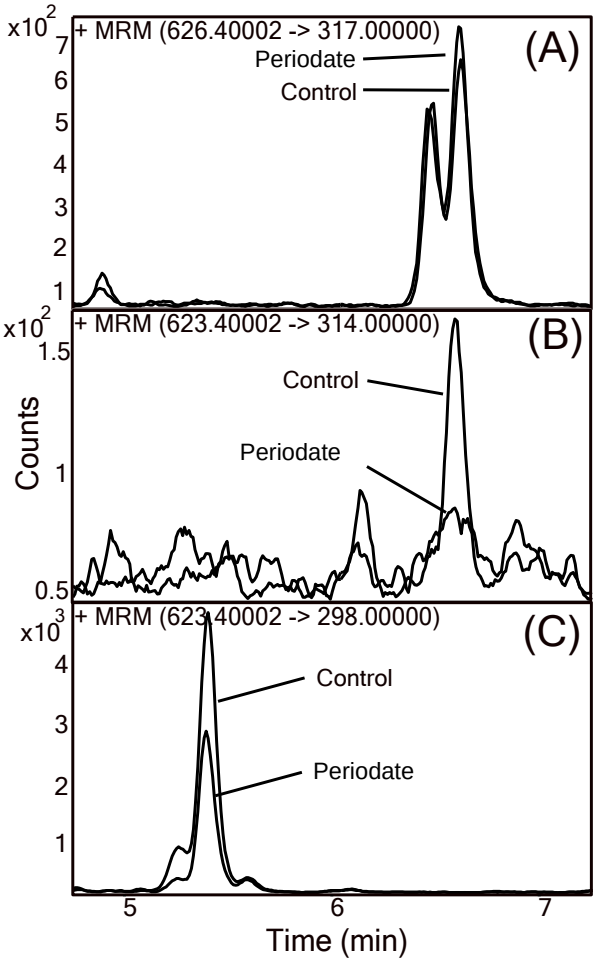

Figure 3.9 Chromatograms of *d3*-1,25$(OH)_2D_3$ (A), *m/z* 623.4→ 314 corresponding to the mass of 1,25$(OH)_2D_3$ (B) and 24,25$(OH)_2D_3$ (C) in 0.5 mL of serum treated with periodate.

3.3.3 Future applications for the serum method

A novel method for the quantification of vitamin D_3, 25(OH)D_3 and 24,25$(OH)_2D_3$ in serum was developed **(Paper I)** and used for the studies in minipigs as described in section 4.3.2 and 4.3.3.

The method was validated in human serum to make the method applicable for use in clinical research where it could be used to further explore the vitamin D_3 metabolism and to investigate new associations between vitamin D_3 and its metabolites. Single nucleotide polymorphisms in enzymes involved in the 25-hydroxylation of vitamin D_3 are associated with the serum 25(OH)D_3 level (Wang et al., 2010). Different genotypes might affect the ratio of serum vitamin D_3 and 25(OH)D_3 and could possibly be used as a future biomarker for predicting the increase of serum 25(OH)D_3 to an oral intervention with vitamin D_3. Chapter 5 demonstrated that the relation between serum vitamin D_3 and 25(OH)D_3 appears to be nonlinear for minipigs. The relation between serum vitamin D_3 and 25(OH)D_3 could be further elucidated by the method. The serum vitamin D_3 can also be used to investigate the contribution of endogenous storages of vitamin D_3 during periods of vitamin D_3 shortages, as demonstrated in **Paper IV**. The serum 24,25$(OH)_2D_3$ is interesting to study for several reasons. It is believed to exert some biological functions for the cartilage repair (van Leeuwen et al., 2001) and might be interesting to study for that reason. Single nucleotide polymorphisms is also reported for the enzymes involved in the 24-hydroxylation (Wang et al., 2010), where the ratio of serum 24,25(OH)D_2D_3 and serum 25(OH)D_3 at baseline predicts the response in serum 25(OH)D_3 of healthy individuals to an oral intervention with vitamin D_3 (Wagner et al., 2011). Furthermore, some individuals with mutations in CYP24A1, known as idiopathic infantile hypercalcemia, are more susceptible to vitamin D intoxication due to impaired catabolism of 1,25$(OH)_2D_3$ to 1,24,25-trihydroxyvitamin D_3 (Schlingmann et al., 2011). The serum 24,25$(OH)_2D_3$ is, however, also decreased in these individuals making the quantification of serum 24,25$(OH)_2D_3$ interesting from a diagnostic perspective, but also as a possible screening tool to identify susceptible individuals to avoid intoxication caused by vitamin D_3 prophylaxis.

3.4 Quantification of vitamin D_3 and 25-hydroxyvitamin D_3 in porcine tissue (Paper II)

A method for the quantification of vitamin D_3 and 25(OH)D_3 as well as the 13C-labelled analytes in different types of tissues in minipigs was essential. Some of the samples were biopsies of subgram sizes so the method needed to be a highly sensitive MS method.

Several methods are available for the analysis of vitamin D_3 and 25(OH)D_3 in tissue (Table 3.1). Many of the methods capable of quantifying physiological levels of vitamin D_3 and 25(OH)D_3 in tissues are developed for food stuff analysis. When analysing foodstuffs, an unlimited amount of sample will usually be available for the analysis and the methods need sample sizes of 7.5 g to 50 g (Strobel et al., 2013;Bilodeau et al., 2011;Jakobsen et al., 2004). Some tissue methods use cold saponification prior to the extraction of the analytes whereas other methods only homogenize the sample before the extraction (Table 3.1). Saponification was used in the method to hydrolyse potentially esterified vitamin D_3 metabolites and to remove lipids. Existing methods require a very extensive sample preparation using LLE, SPE and normal phase (NP) preparative HPLC prior to the quantification by HPLC coupled to a UV or MS detector (Bilodeau et al., 2011;Jakobsen et al., 2004). These methods are laborious and use large amounts of organic solvents.

Our existing HPLC-UV method for vitamin D_3 and 25(OH)D_3 was downscaled and the preparative HPLC purification step was made redundant by changing the principle of detection from UV to MS/MS **(Paper II)**. The sample size was reduced from 10 g to 0.2 g which enables the quantification of vitamin D_3 and 25(OH)D_3 in samples obtained from biopsies. Additionally, the throughput was increased 3-4 times and the amount of organic solvents was decreased by 90% in the tissue method described in **Paper II** compared to a HPLC-UV method (Jakobsen et al., 2004).

Table 3.1 Different methods for the quantification of vitamin D_3 and 25(OH)D_3 in tissue.

Method	Sample Size (g)	LOQ vitamin D_3 / 25(OH)D_3 (ng/g)	Sample preparation	Detection	Matrix	Reference
RIA	1	-/?	Homogenization, NP-SPE, NP-HPLC	RIA	Muscle tissue	Wertz et al., 2004
UV/PDA	50g	0.3/0.5	Saponification, LLE, NP-SPE, NP-HPLC	RP-HPLC-UV-PDA	Muscle tissue	Jakobsen et al., 2004
UV-PDA/MS	20	0.4/0.4	Saponification, LLE, NP-SPE, NP-HPLC	RP-HPLC-UV-PDA (vitamin D_3) RP-HPLC-APCI-MS/MS (25(OH)D_3)	Muscle tissue	Bilodeau et al., 2011
MS	7.5	0.3/0.5	Saponification, diatomaceous earth SPE	NP-HPLC coupled to APCI-MS/MS (iontrap) equipped with APCI	Muscle tissue	Strobel et al., 2013
MS	0.5	-/1	Homogenization, extraction with methanol, enzymatic digestion, RP-SPE	Online RP-SPE coupled to APCI-MS	Muscle and skin tissue	Höller et al., 2010
MS	0.2	?/-	Saponification, LLE, RP-SPE	RP-HPLC-APCI-MS	Adipose tissue	Blum et al., 2008
MS	0.08	0.1/0.1	Homogenization, LLE, RP-SPE, PTAD-derivatization	RP-HPLC-ESI-MS/MS	Muscle tissue	Lipkie et al., 2013

APCI, Atmospheric pressure chemical ionization; ESI, Electrospray ionization; HPLC, High performance liquid chromatography; LOQ, Limit of quantification; LLE, Liquid-liquid extraction; MS, Mass spectrometry; MS/MS, Tandem mass spectrometry; NP, Normal phase; PDA, Photodiode array; RP, Reverse phase; RIA, Radioimmunoassay; SPE, Solid phase extraction; UV, Ultraviolet

4 Animal experiments

In chapter 4, the pig and minipig as an animal model in vitamin D_3 research will be discussed and the lamp used to stimulate vitamin D_3 synthesis will be described.

The main findings from one study in slaughter pigs and two studies in minipigs are presented and discussed. The samples from the slaughter pigs **(Paper III)** were obtained from an animal study done at Research Centre Foulum, Aarhus University as described by Lauridsen *et al.* (Lauridsen et al., 2010). The two studies in minipigs **(Paper IV)** were done at National Food Institute, Technical University of Denmark. Ethical approval was given by *The Danish Animal Experiments Inspectorate.*

4.1 Choosing the animal model

4.1.1 Pig

In this thesis the slaughter pig was used as a model to study the influence of oral supplementation of vitamin D_3 and 25(OH)D_3 on the distribution and storages of vitamin D_3 and 25(OH)D_3 in tissues.

The pig has previously been used extensively in nutritional research. The gastrointestinal (GI) tract has a similar anatomy, morphology and physiology as the human GI and the digestive and absorptive abilities of the pig and the human are comparable (Cooper et al., 1997).

The pig is a commonly used animal within the field of vitamin D_3 research and has previously been used to study the differences between vitamin D_3 and 25(OH)D_3 (Jakobsen et al., 2007;Höller et al., 2010;Coffey et al., 2012), the distribution of 1,25$(OH)_2D_3$ (Rungby et al., 1993), and to investigate the influence of UV-exposure on vitamin D_3 (Cooper et al., 1997).

The metabolic system of the pig discriminates between vitamin D_2 and vitamin D_3, most likely at the 25-hydroxylase level of the metabolism (Horst et al., 1982), but it is irrelevant for the studies in this thesis which only focuses on vitamin D_3.

4.1.2 Göttingen Minipig

When choosing the animal model for the cutaneous synthesis of vitamin D_3, it was pertinent to be able to distinguish cutaneously synthesized vitamin D_3 from orally ingested vitamin D_3.

The Göttingen minipig was chosen, although most of the vitamin D studies from the past used the rat as an animal model (Lawson et al., 1986b;Rosenstreich et al., 1971;Fraser and Kodicek, 1968b;Lawson et al., 1986a;Brouwer et al., 1998;Gaylor and Sault, 1964;Okano et al., 1978;Okano et al., 1977;Holick et al., 1977;Fraser and Kodicek, 1968a). The reason for not choosing rats was, that UV-exposed rats can possibly be unintended orally exposed to vitamin D_3 in the fur after grooming itself (reviewed by Carpenter and Zhao, 1999). The vitamin D_3 in the fur probably originates from 7-dehydrocholesterol present in the sebum secreted to the fur and skin surface (Gaylor and Sault, 1964). After irradiation, the 7-dehydrocholesterol is converted to vitamin D_3 (reviewed by Carpenter and Zhao, 1999).

The Göttingen minipig is a cross between the Minnesota minipig, the Vietnamese potbelly swine and the German landrace. The boars are sexually mature at the age of 3-4 months and the sows at the age of 4-5 months. The Göttingen minipig has a slower growth curve compared to normal sized pigs (Ellegaard Göttingen Minipigs A/S;Mount and Ingram, 1971), which was important for the study design to reduce dilution on the storage of vitamin D_3 due to growth during times of vitamin D_3 deprivation. The minipig gains roughly 0.5 kg/week in body weight (BW) during the first year of its life. The BW of an adult minipig is 35-40 kg after ~2 years (Ellegaard Göttingen Minipigs A/S;Bollen and Ellegaard, 1997). Female minipigs reach a higher BW and have a thicker backfat layer than male minipigs when fed *ad libitum* (Bollen et al., 2005). The fat content of female minipigs is 10% for lean minipigs and 15% for obese minipigs determined by dual-energy X-ray absorptiometry (DEXA) scanning (Johansen et al., 2001).

Despite of the small size, the minipig is still suitable for repeated blood and tissue sampling, which was important for the study design where multiple data sampling was required. The use of multiple data sampling from each animal during the study also compensated for the relatively low number of minipigs used.

The porcine skin resembles the human in terms of architecture, composition, vascularization, lymphatic drainage and the attachment of the skin to the subcutaneous tissue (Suenderhauf and Parrott, 2013). The unpigmented skin of the minigpig (Bollen and Ellegaard, 1997) is of particular relevance for studying cutaneously synthesized vitamin D_3.

The CYP enzyme system in minipigs is at a mature level at the age of 2 months (Bollen and Ellegaard, 1997). The activity of the CYP is slightly higher in Göttingen minipigs than in conventional pigs. Both male and female minipigs were used in the studies. No sex differences are observed for the total concentration of hepatic CYP, but certain isoforms of the CYP including CYP3A4 associated with the 25-hydroxylation of vitamin D_3 is higher in female minipigs (Skaanild and Friis, 1999).

4.2 UV-lamp

The UV-lamp was developed to stimulate cutaneous synthesis in vertebrate animals. The cutaneous vitamin D_3 synthesis can be stimulated by irradiation of the skin to artificial UV-light, which has previously been shown for rats (Lawson et al., 1986a;Okano et al., 1978;Okano et al., 1977;Holick et al., 1979), pigs (Cooper et al., 1997) and humans (Bogh et al., 2012). The maximum synthesis of previtamin D_3 occurs at 295-300 nm, but monochromatic UV-light at 295 nm is reported to impact on the degradation of previtamin D_3 to lumisterol3 and tachysterol3, probably due to different UV absorption spectra (MacLaughlin et al., 1982). Therefore, a broadband UV-light source was chosen to mimic the natural synthesis and the degradation of vitamin D_3. The lamp was characterized from 250-400 nm **(Paper III)** in the distance corresponding to the distance between the lamp and the minipigs used in the experiments. The light spectrum for the lamp is shown in Figure 4.1. In order to be able to relate the UV-exposure to other experiments and to natural sunlight, the standard erythema dose (SED) was used to describe the UV dose. The UV weighed spectrum was calculated from the erythema action spectrum (CIE, 1999) (Figure 4.1) and one SED was defined as 100 J/m^2 (Diffey et al., 2011). One minute of light exposure was calculated to be 0.9 SED, which equals ~10 minutes of full body exposure of midday summer sun at 56°N (Bogh et al., 2012).

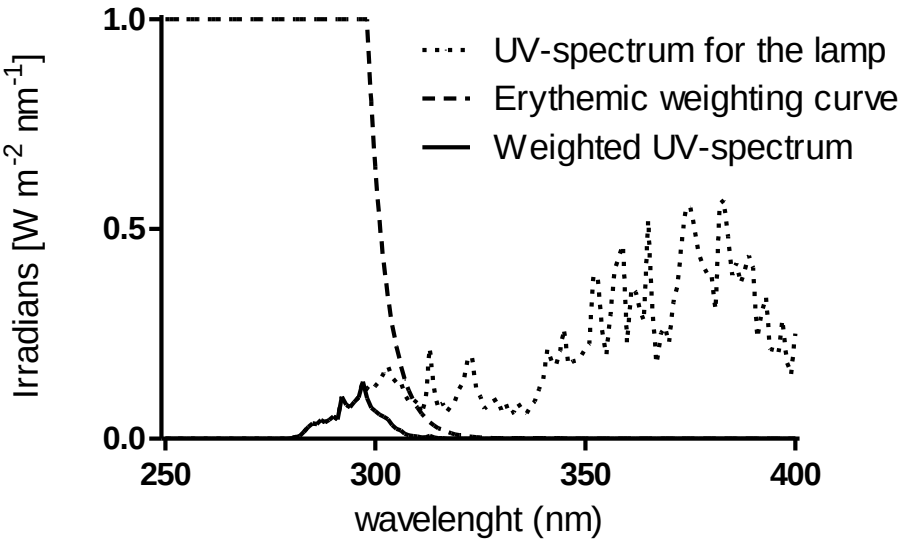

Figure 4.1 Characterization of the UV-lamp used to irradiate the minipigs to stimulate the cutaneous synthesis of vitamin D_3.

4.3 Animal studies

4.3.1 Oral 25-hydroxyvitamin D_3 versus oral vitamin D_3 in slaughter pigs (Paper III)

The objective of the study was to investigate the tissue distribution of vitamin D_3 and 25(OH)D_3 in slaughter pigs after oral supplementation of either vitamin D_3 or 25(OH)D_3. Furthermore, the objective was to investigate the association of the serum 25(OH)D_3 concentration with the tissue content of vitamin D_3 and 25(OH)D_3.

Serum 25(OH)D_3 and vitamin D_3 and 25(OH)D_3 in muscle and adipose tissues were analysed in slaughter pigs fed either vitamin D_3 or 25(OH)D_3 at four different doses (Figure 4.2 and **Paper III**). Adipose tissue and muscle tissue were chosen for the analysis to represent the major tissues in the pig.

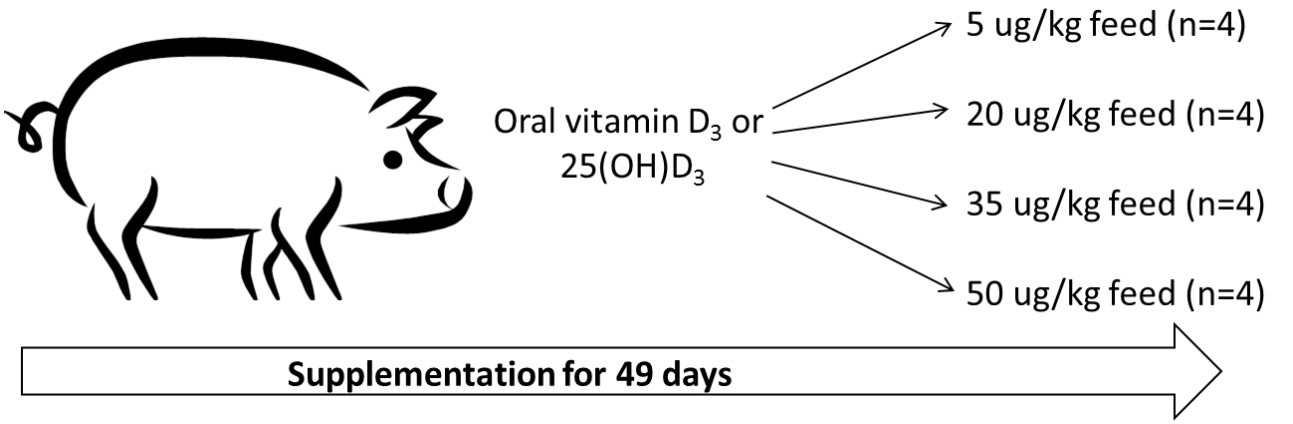

Figure 4.2 Slaughter pigs were fed 4 different doses of vitamin D_3 or 25(OH)D_3 for 49 days where the pigs were euthanized and serum and tissue were collected.

The tissue content of 25(OH)D_3 was significantly higher in the pigs supplemented with 25(OH)D_3 compared to the pigs supplemented with vitamin D_3 at all dietary concentrations. On the other hand, a significantly higher content of vitamin D_3 was found in all tissues originating from pigs fed vitamin D_3 **(Paper III)**. This is in consistency with a previous study in slaughter pigs fed a single level of either vitamin D_3 or 25(OH)D_3 (Jakobsen et al., 2007).

Serum 25(OH)D_3 was linearly correlated to both the tissue content of vitamin D_3 and 25(OH)D_3, but the correlation was also depended on the ingested form of vitamin D_3 (Figure 4.3 and **Paper III**). It has previously been hypothesized that vitamin D_3 accumulates in the body above a certain threshold of serum vitamin D_3 (Heaney et al., 2008). Serum vitamin D_3 was not measured in this

study, but the data does, however, not suggest any threshold for accumulation of tissue vitamin D_3 at serum 25(OH)D_3 concentrations within the observed range (< 80 ng/mL).

The study demonstrated that the adipose and white muscle tissue content of 25(OH)D_3 could be predicted from serum 25(OH)D_3 independently of the ingested form of vitamin D_3. The content of vitamin D_3 in these tissues was also related to the serum 25(OH)D_3, but the correlation was depending on the dietary source of vitamin D_3. Therefore, serum 25(OH)D_3 will be a poor biomarker for tissue vitamin D_3 if the dietary vitamin D_3 source contains both vitamin D_3 and 25(OH)D_3, which is the case for many food items contributing with vitamin D_3 in a natural diet (Ovesen et al., 2003).

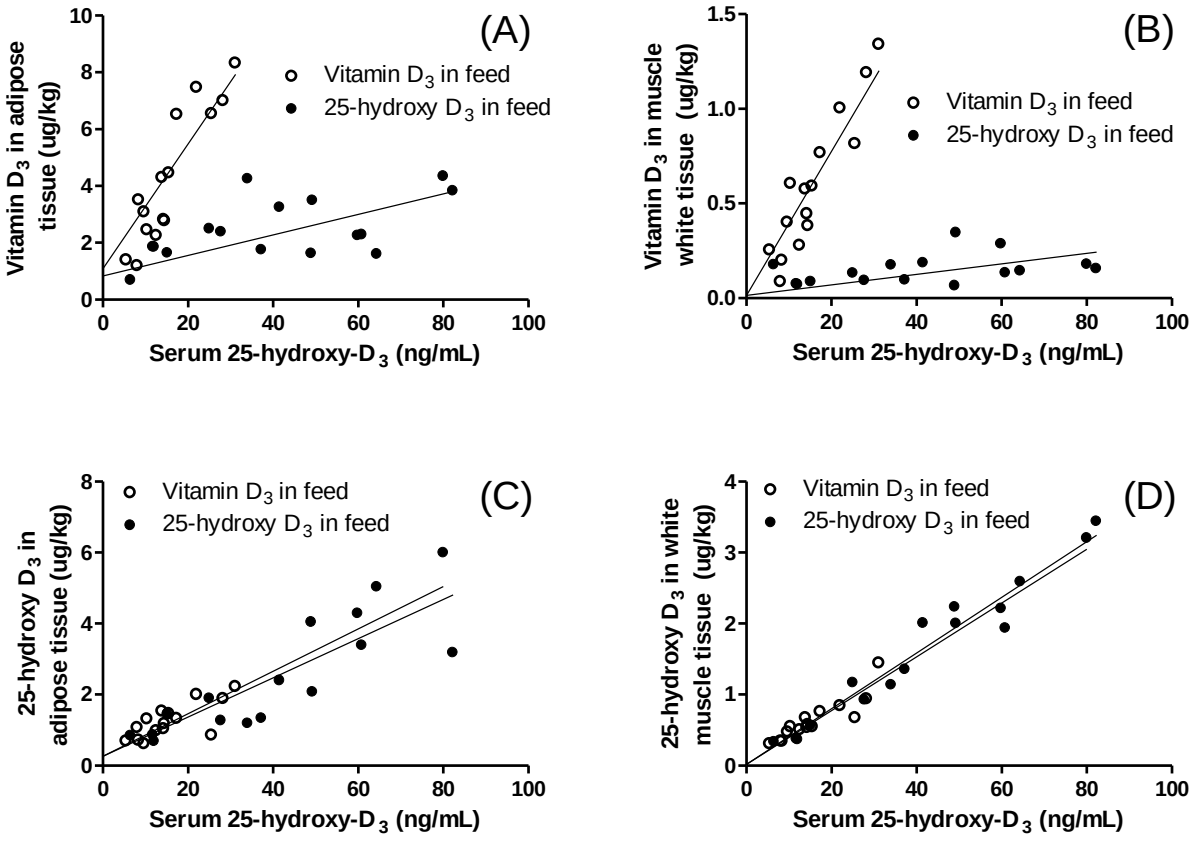

Figure 4.3 Serum 25(OH)D_3 plotted against the content of vitamin D_3 in adipose and muscle tissues (A and B) and against the content of 25(OH)D_3 in adipose and muscle tissues (C and D) for pigs fed either vitamin D_3 or 25(OH)D_3 (Figure and legend adopted from **Paper III**).

4.3.2 Storage of vitamin D_3 and 25-hydroxyvitamin D_3 in minipigs after UV-exposure and oral vitamin D_3 (Paper IV)

The purpose of the study was to investigate the tissue content of vitamin D_3 and 25(OH)D_3 in minipigs exposed to UV-light or in minipigs orally dosed with vitamin D_3.

The study design is shown in Figure 4.4 and housing conditions are described in **Paper IV**. One group of minipigs was initially irradiated with UV-light corresponding to ~10 minutes of daily sun exposure, and towards the end of the study the exposure time was doubled equalling ~20 minutes of daily sun exposure. For the orally dosed minipigs the oral dose was increased twice from 15 µg/day to 27.5 µg/day reflecting the increase in BW and the last dose of 60 µg/day was three-fold higher than the vitamin D_3 obtained from a standard minipig diet containing 60 µg/kg feed (Ellegaard Göttingen Minipigs A/S;SDS Speciel Diet Service).

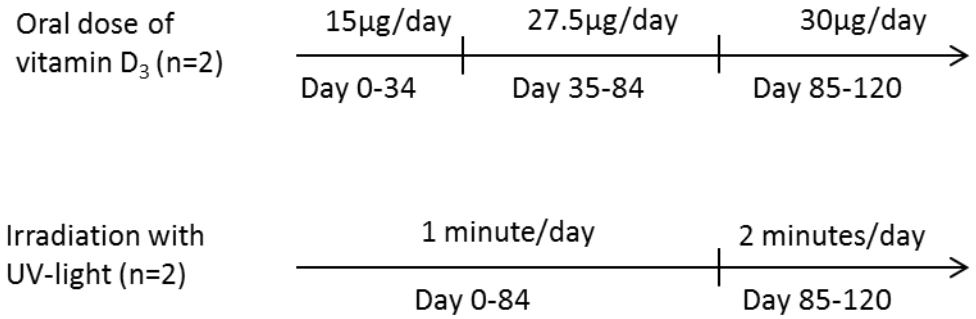

Figure 4.4 The experimental design of the study in male minipigs to investigate the tissue concentrations of vitamin D_3 and 25(OH)D_3 after oral vitamin D_3 and UV-exposure (Figure and legend adopted from **Paper IV**).

Figure 4.5 shows that irradiation with UV-light stimulated the endogenous synthesis of vitamin D_3 and resulted in increasing serum vitamin D_3 and 25(OH)D_3. Doubling the UV-exposure time only resulted in minor increases of serum vitamin D_3 and 25(OH)D_3 which could indicate that the cutaneous synthesis of vitamin D_3 has reached its maximum. The stepwise increment of oral vitamin D_3 resulted in corresponding increases in serum vitamin D_3 and 25(OH)D_3.

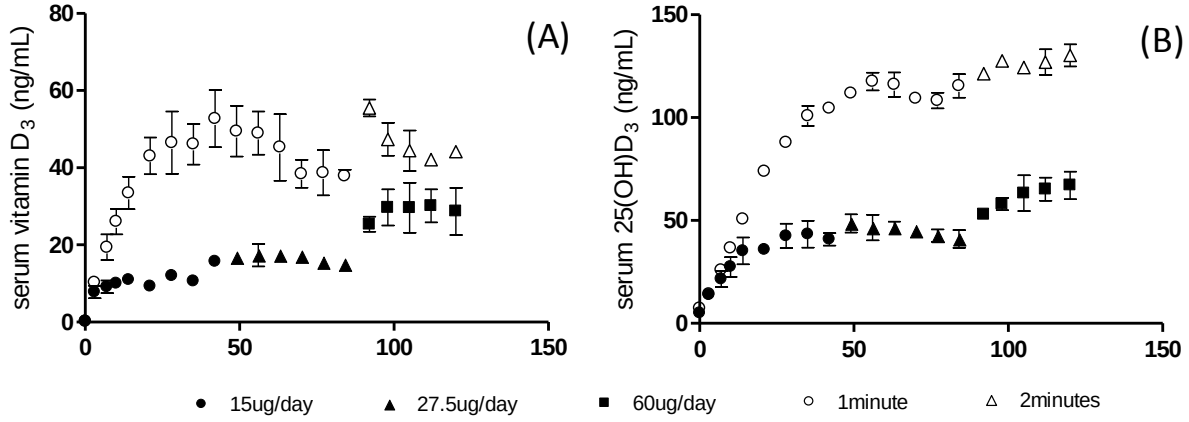

Figure 4.5 Serum levels (mean ± SEM) of vitamin D_3 (A) and 25(OH)D_3 (B) in minipigs supplemented daily with vitamin D_3 (filled symbols) and pigs exposed daily to UV-light (285-400 nm) (open symbols) (Figure and legend adopted from **Paper IV**).

The tissue content of vitamin D_3 and 25(OH)D_3 in the different analysed tissues are found in Figure 4.6. The vitamin D_3 content in adipose tissue from the minipigs UV-exposed for 120 days was 150-260 ng/g. In comparison, Lawson *et al.* found ~90 ng/g of vitamin D_3 in rats exposed daily to UV-light for 25 days (Lawson et al., 1986b). For the UV-exposed minipigs large differences of vitamin D_3 and 25(OH)D_3 were observed between skin sampled from the abdomen compared to skin sampled from the neck of the animals where the pigs were the most prone to UV-exposure. The supplemented minipigs received 60 µg/day of vitamin D_3 during the last period of the study, which is three times more vitamin D_3 than obtained from a standard minipig diet containing 60 µg/kg feed (Ellegaard Göttingen Minipigs A/S;SDS Speciel Diet Service). The resulting findings of 90-150 ng/g vitamin D_3 in the minipigs are expectedly higher compared to slaughter pigs fed 20-30 µg/kg feed of vitamin D_3 where the content of vitamin D_3 in adipose tissues was 3.2-7.9 ng/g (Clausen et al., 2003) and the mean of vitamin D_3 of 7.5 ng/g in subcutaneous adipose tissue quantified in slaughter pigs fed 55 µg/day of vitamin D_3 (Jakobsen et al., 2007).

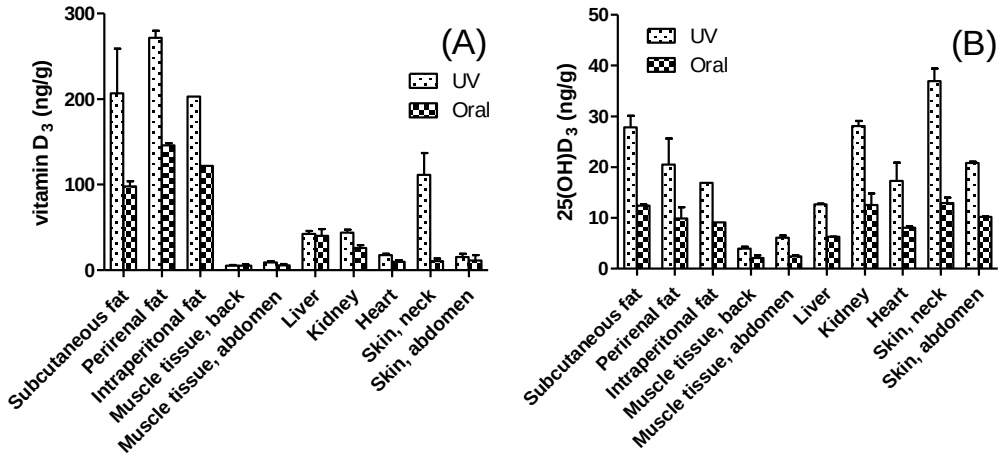

Figure 4.6 The distribution (mean + SEM) of vitamin D_3 (A) and 25(OH)D_3 (B) in minipigs after daily exposure to UV-light (n=2) or a daily oral dose of vitamin D_3 (n=2). For intraperitonal adipose tissue (n=1) (Figure and legend adopted from **Paper IV**).

Differences in storages of vitamin D_3 after sun exposure or oral vitamin D_3 is unknown for humans, but a seasonal variation of serum 25(OH)D_3 is observed for populations in many countries. The highest serum 25(OH)D_3 concentrations are reached during summertime (Brot et al., 2001;Kuchuk et al., 2009), indicating that the contribution from the cutaneous synthesis of vitamin D_3 in humans is substantial. It is reasonable to believe that vitamin D_3 will also accumulate in human tissue during periods of excessive sun exposure.

An estimation of the size of the storage of vitamin D_3 in a 70 kg woman is previously done from slaughter pigs feed 50-55 µg/day vitamin D_3 (Heaney et al., 2009). By using the same referent values for a 70 kg woman and the vitamin D_3 content found in the minipigs, the size of the storage of vitamin D_3 was reestimated (Table 4.1). The total content of cutaneously synthesized vitamin D_3 in a human body was estimated to ~7460 µg, where ~15% was in the form of 25(OH)D_3. The total content of vitamin D_3 originating from oral vitamin D_3 is ~3870 µg, where ~14% was in the form of 25(OH)D_3. Heaney *et al.* estimated the total body content to 367µg and 35% of the content was in the form of 25(OH)D_3 (Heaney et al., 2009).

In conclusion, the study showed that the adipose tissue concentration of vitamin D_3 was 10-30 folds higher in the minipigs compared to other studies of vitamin D_3 in pigs (Jakobsen et al., 2007;Clausen et al., 2003). The orally supplemented minipigs received three times more vitamin D_3

than recommended, whereas the irradiated minipigs were exposed to UV-light corresponding to 10-20 minutes of daily summer sun.

Table 4.1 Estimation of the size of the human storages of vitamin D_3 and 25(OH)D_3. The tissue concentrations are mean values from the UV-exposed minipigs and minipigs orally supplemented with vitamin D_3. A 70 kg woman was used as a referent.

	Tissue concentrations in minipigs (µg/kg)		Tissue distribution of a referent woman (kg)*	Estimated storages in a referent woman (µg)	
	UV-exposed	Orally dosed		UV-exposed	Orally dosed
	Vitamin D_3			Vitamin D_3	
Adipose tissue	231.9	121.9	25	5682	2987
Muscle tissue	7.2	5.3	31	223	162
Liver	42.3	40.2	1	59	56
Serum	44.2	28.7	2	102	66
Remainder¤	35.4	8.1	7	248	56
Sum (vitamin D_3)				6313	3328
	25(OH)D_3			25(OH)D_3	
Fat	22.7	10.7	25	556	263
Muscle	5.0	2.3	31	154	70
Liver	12.7	6.3	1	18	9
Serum	130.2	67.1	2	299	154
Remainder¤	16.9	6.9	7	119	48
Sum (25(OH)D_3)				1146	545
Total vitamin D_3 + 25(OH)D_3				~7460	~3870

*From (Heaney et al., 2009)

¤Average of skin and muscle tissues

4.3.3 Changes of tissue vitamin D_3 and 25-hydroxyvitamin D_3 in minipigs during shortages of vitamin D_3 (Paper IV)

In section 4.3.1 and 4.3.2 it was shown that vitamin D_3 and 25(OH)D_3 accumulates in the tissue and that the tissue content was correlated with the dose of vitamin D_3 and the serum 25(OH)D_3 concentration. Based on the findings in section 4.3.2 it was decided to investigate the changes of vitamin D_3 and 25(OH)D_3 in skin and subcutaneous adipose tissue in UV-exposed minipigs during deprivation and supplementation of 13C-labelled vitamin D_3 as described in detail in **Paper IV**. The study design is shown in Figure 4.7.

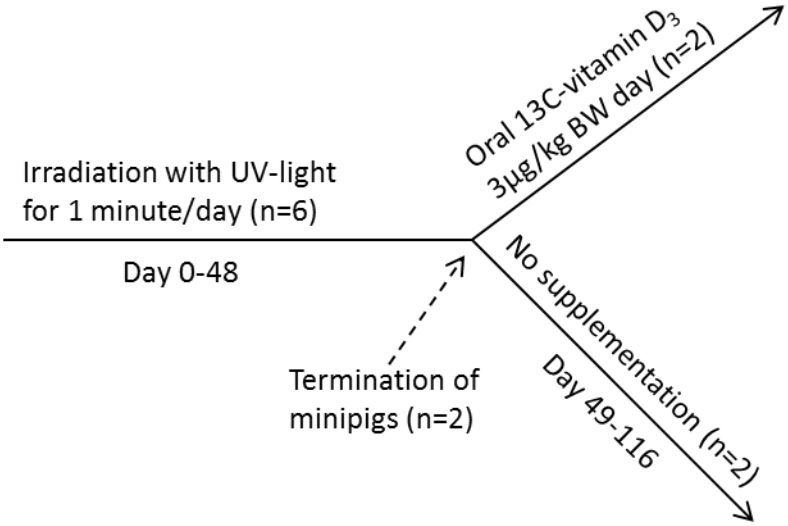

Figure 4.7 The experimental design of the study in female minipigs to investigate the change in vitamin D_3 and 25(OH)D_3 after UV-exposure had ceased. Four biospies were sampled during the end of the study starting at day 49 (Figure and legend adopted from **Paper IV**).

After the UV-exposure had ceased the serum vitamin D_3 initially declined quickly during the first two weeks followed by a slower decline suggested to be of 1st-order. The decline of serum vitamin D_3 did not seem to be influenced by the concomitant oral 13C-labelled vitamin D_3. The decline of 25(OH)D_3 appeared nonlinear with a declining elimination rate (Figure 4.8).

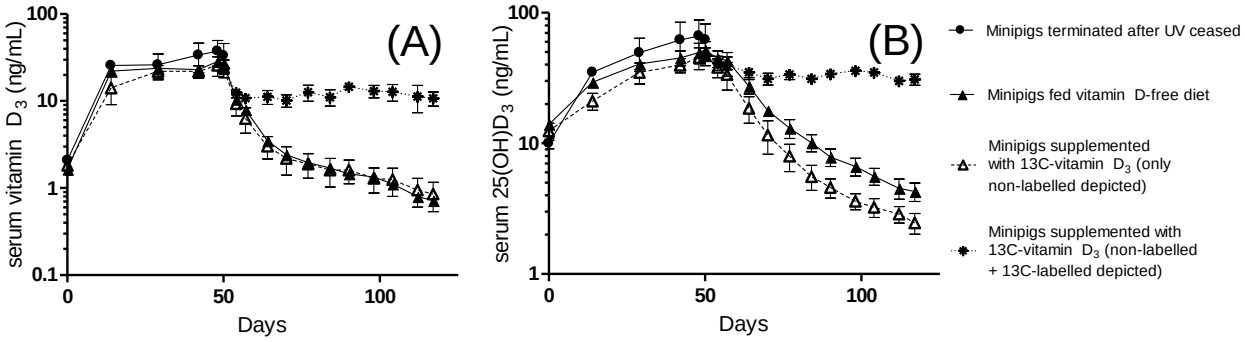

Figure 4.8 Serum (mean + SEM) vitamin D_3 and 13C-vitamin D_3 (A) and serum 25(OH)D_3 and 13C-vitamin D_3 (B) in minipigs after daily UV-exposure for 49 days. After the UV-exposure, two pigs were terminated, two pigs were kept on a vitamin D-free diet and two pigs were supplemented daily with 13C-vitamin D_3 (Figure and legend adopted from **Paper IV**).

1st-order decline of vitamin D_3 in adipose tissue and 25(OH)D_3 in adipose tissue and skin was suggested after UV-exposure had ceased. For rats dosed with vitamin D_3 a 1st-order decline of vitamin D_3 in adipose tissue was also observed in vitamin D_3-repleted rats placed on a vitamin D-free diet (Rosenstreich et al., 1971;Lawson et al., 1986a). For vitamin D_3 in the skin a quick decline was initially observed followed by a 1st-order decline of vitamin D_3 (Figure 4.9). Similar kinetic behaviour was also observed for vitamin D_3 and its metabolites in liver and kidney in rats fed a vitamin D-free diet (Rosenstreich et al., 1971).

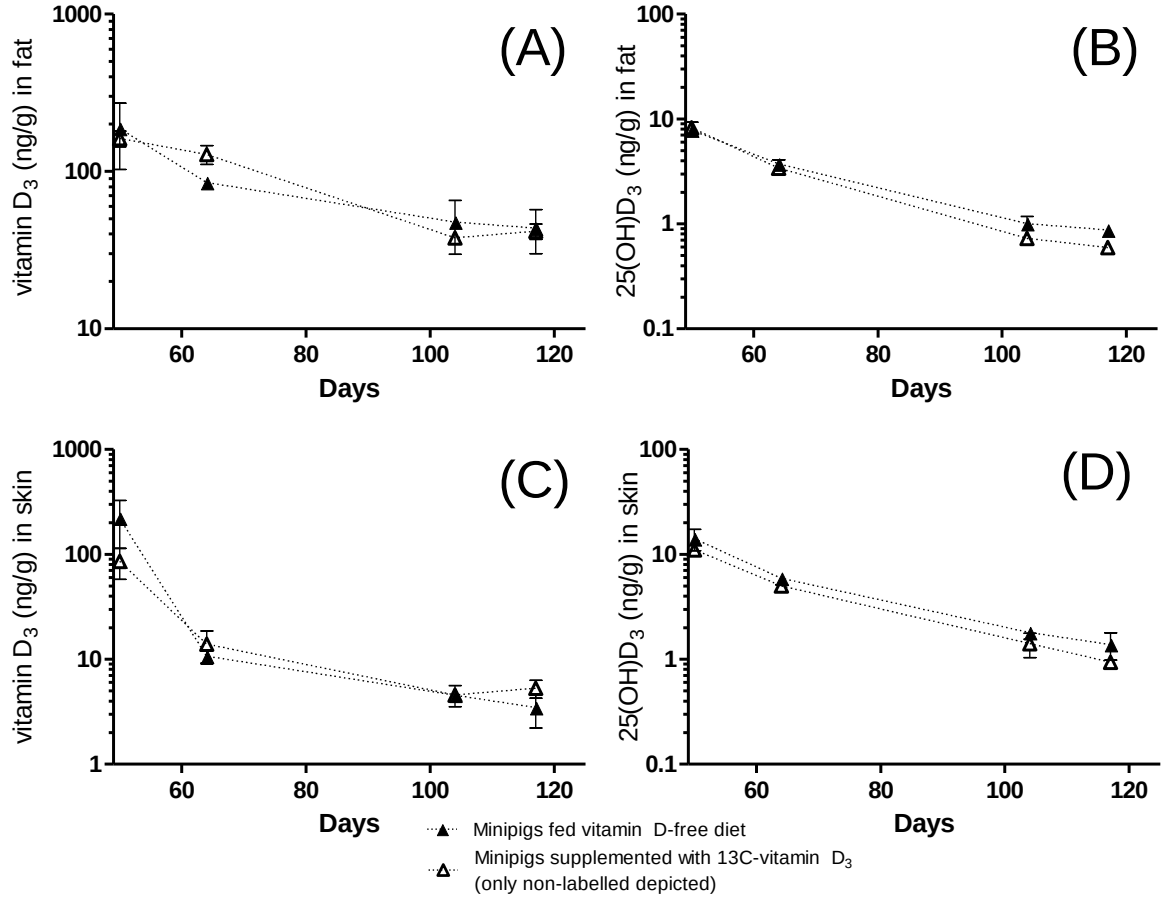

Figure 4.9 Content (mean ± SEM) of vitamin D_3 (A) and 25(OH)D_3 (B) in biopsied subcutaneous adipose tissue and vitamin D_3 (C) and 25(OH)D_3 (D) in biopsied skin in minipigs after UV-exposure. After the UV-exposure had ceased the minipigs were fed a vitamin D-free diet (n=2) or supplemented with 13C-vitamin D_3 (n=2) (Figure and legend adopted from **Paper IV**).

The storages of vitamin D_3 and 25(OH)D_3 generated during periods of UV-exposure declined when UV-exposure had ceased and the decline was independent of a concomitant supplementation of 13C-labelled vitamin D_3. This suggests that the transfer of vitamin D_3 from the adipose tissue is determined by the partitioning of vitamin D_3 between the adipose tissue and blood rather than biologically regulated by the vitamin D_3 status. This is supported by the fact that tissue concentrations of 13C-vitamin D_3 was build up simultaneously with the depletion of endogenously synthesized vitamin D_3 in the supplemented minipigs **(Paper IV)**.

Finally, it was also shown that minipigs become vitamin D_3 deficient within 20 days without UV-exposure when serum 25(OH)D_3 < 20 ng/mL was used as the cut-off value.

5 Pharmacokinetic-modelling of vitamin D_3 and 25-hydroxyvitamin D_3 in minipigs

Chapter 5 focuses on the analysis of the data from the two studies in minipigs reported in section 4.3.2 and 4.3.3, but the chapter can be read independently of the rest of the thesis. The work was carried out under supervision of Post Doc Elke H.J. Krekels and Professor Mats O. Karlsson from the Pharmacometric group at Uppsala University, Sweden. The work on the PK-model is still in progress and the final work is intended for submission to an international peer-reviewed journal in the future.

5.1 The Pharmacokinetic-model

Vitamin D_3 is stored in tissue after cutaneous synthesis of vitamin D_3 and after oral supplementation of vitamin D_3 in minipigs. The amount of vitamin D_3 in tissue declines during times of deprivation of vitamin D_3 and the decline of serum vitamin D_3 during deprivation appears to be biphasic **(Paper IV)**. That underpins the need to understand the kinetics of serum vitamin D_3 and the contribution of endogenous storages of vitamin D_3 to understand and predict the serum concentration of 25(OH)D_3 under various input conditions.

One way to describe the complexity of the vitamin D_3 synthesis, oral absorption and metabolism is by use of PK-modelling, also known as non-linear mixed effects modelling, referring to the mixture of fixed and random effects. Fixed effects are included in a structural model describing the time-course of drug concentrations or concentrations of endogenous compounds. Random effects quantify the variability in the system and comprise both intra-individual and inter-individual variability and residual variability. Covariates such as BW can be incorporated to partly explain some of the intra-individual and inter-individual variability.

The aim of this study was to establish a comprehensive PK-model for vitamin D_3 and 25(OH)D_3, describing the cutaneous synthesis, oral absorption, distribution and metabolism of vitamin D_3 in the minipig.

5.2 Data

Data for vitamin D_3 and 25(OH)D_3 in serum, subcutaneous adipose tissue and skin used for the analysis are described in **Paper IV**, briefly:

Study 1

Two 7-week-old male minipigs were daily orally dosed with vitamin D_3. From day 0 to day 34 the minipigs were dosed with 15 µg vitamin D_3/day corresponding to 1.5-2.5 µg/kg BW and from day 35 to day 84 with 27.5 µg vitamin D_3/day, to maintain the dose within the range of 1.5-2.5 µg/kg BW with the increasing BW. From day 85 to day 120 the dose was increased to 60µg vitamin D_3/day corresponding to 3.7-4.4 µg/kg BW.

Two 7-week-old male minipigs were UV-exposed daily. From day 0 to day 84 the minipigs were UV-exposed daily for one minute corresponding to 0.9 SED/day and from day 85 to day 120 the daily exposure time was increased to two minutes corresponding to 1.8 SED/day.

From day 0 to day 14, the blood was sampled twice a week, followed by once a week until the termination of the experiment at day 120 where the pigs were euthanized.

Study 2

Six 5-week-old female Göttingen minipigs were UV-exposed daily for one minute corresponding to 0.9 SED/day until day 48 when the UV-exposure had ceased. At day 49 two animals were terminated. The remaining four animals were divided into two groups. One group (n=2) was daily orally dosed with 3 µg vitamin 13C-labelled vitamin D_3/ kg BW. The other group of animals (n=2) did not receive any vitamin D throughout the remainder of the study period. The study was terminated at day 116.

From day 0 to 42, the blood was sampled every second week, and from day 48 to 56 the blood was sampled twice a week followed by once a week throughout the rest of the study.

The minipigs were punch biopsied at day 49, 63, 103 and prior to exsanguination of the animals at day 116.

The two animal experiments were carried out at the animal facilities at the National Food Institute, the Technical University of Denmark (Mørkhøj, Denmark) and the studies were financed by a grant from the Ministry of Higher Education and Science. Ethical approval was given by *The Danish Animal Experiments Inspectorate*.

Bioanalysis of serum and tissue concentrations

Serum and tissues were analyzed for vitamin D_3 and 25(OH)D_3 by LC-MS/MS as described in **Paper I** and **Paper II**. The precision of 25(OH)D_3 in serum was assessed in a non-spiked house reference of human serum (n=27) and found to be 4.4%. For the tissue analysis, the precision was determined by a house reference of porcine fat (n=14) and found to be 5.9% and 5.0% for vitamin D_3 and 25(OH)D_3, respectively **(Paper IV)**.

5.3 The Pharmacokinetic-model building

The analysis in this study was performed using NONMEM 7.2 (Icon Development Solutions, Ellicott City, Maryland) with a 1st-order conditional estimation method (FOCE), facilitated by Pirana 2.6.1 (Keizer et al., 2011) and PsN 5.16.1 (Lindbom et al., 2005). Xpose (Jonsson and Karlsson, 1998) run in R 3.0.2 (R Core Team, 2012) was used for the visualization of the results. The ADVAN6 subroutine in NONMEM was used in all models and all data were fit simultaneously.

For the animals supplemented with 13C-labelled vitamin D_3 the sum of endogenous vitamin D_3 analytes and 13C-labelled analytes were used. To maintain the mass balance the concentrations of vitamin D_3 and 25(OH)D_3 were expressed as nmol/L and nmol/kg in serum and tissue, respectively.

Model building was performed in 2 steps:

1) Choice of a structural model

2) Choice of an error model

Nonmem reports an objective function value (OFV) for each analysis, which the model selection was based on. A lowering of the OFV corresponds to a model improvement and a decrease in OFV of more than 3.84 points between different (sub)-models was considered to be statistically significant. This correlates with a value of $p<0.05$ assuming a χ^2-distribution. Model evaluation was performed by visually assessing basic goodness-of-fit plots (population predicted concentration *versus* observed concentration, conditional weighted residual *versus* time, and conditional weighted residual *versus* population predicted concentration). The uncertainty in the parameter estimates calculated as the relative standard error (RSE%), was considered acceptable when lower than 50%.

Structural model

The distribution volume was estimated for serum vitamin D_3 (V1) and for vitamin D_3 in skin (V2). The distribution volume for serum 25(OH)D_3 (V3) was fixed to V1 as this could not be separately estimated. The distribution volume of vitamin D_3 in adipose tissue (V5) could not be estimated and was fixed to 10% of the BW (Johansen et al., 2001). BW was included as a covariate during the

initial development of the structural model, due to the relatively large change in BW of the minipigs during the studies. BW was normalized to the average BW of all minipigs at all occasions. BW was tested as a linear function on V and as an exponential function on clearance with both a fixed allometric exponent of 0.75 and an estimated exponent (a).

1st-order absorption was used for the oral absorption, but a dose-dependent sigmoidal relationship was also tested. The skin was assumed to represent the compartment where vitamin D_3 synthesis takes place. 100% of the oral dose was assumed to be absorbed in the system and the amount of vitamin D_3 synthesized in the skin was estimated relative to this oral dose (F). Since the data suggested no linear correlation between cutaneous vitamin D_3 synthesis and the time of UV-exposure, the bioavailability of synthesized vitamin D_3 at two minutes/day of UV-exposure was estimated as a multiple of the UV-exposure of one minute/day.

Differences in serum vitamin D_3 after UV-exposure were observed between study 1 and study 2. Therefore, a separate clearance for serum vitamin D_3 (Cl1) and a separate fractional amount of cutaneous vitamin D_3 synthesis for study 2 estimated as fractions of the parameter for study 1, were both estimated, to see what explained the differences in vitamin D_3 levels between the minipigs in the two studies best.

The transfer of vitamin D_3 between V1 and V2 was estimated by an inter-compartmental clearance constant Q. The transfer of vitamin D_3 between V1 and V5 was estimated by the rate constants, k15 and k51. All serum vitamin D_3 were assumed to be metabolized into serum 25(OH)D_3 by Cl1 and all serum 25(OH)D_3 were cleared from the system by Cl3.

Cl1 was initially included as a 1st-order clearance, but later a sigmoidal function was tested, where the 25-hydroxylation is decreased by increasing vitamin D_3 or 25(OH)D_3 serum concentrations:

$$Cl1\left(1-\frac{[serum_{conc}]^n}{EC_{50}{}^n+[serum_{conc}]^n}\right)$$

where *n* is the hill-factor, $serum_{conc}$ is the serum vitamin D_3 or serum 25(OH)D_3 concentration and EC_{50} is the serum vitamin D_3 or 25(OH)D_3 concentration where the 25-hydroxylation is 50% of its maximum (Cl1).

To account for endogenous vitamin D_3 and 25(OH)D_3 at the start of the experiments, the system was initialized assuming no vitamin D_3 in the oral compartment and estimating a serum vitamin D_3

concentration that was in equilibrium with all other compartments. The estimated serum vitamin D_3 concentration in study 2 was estimated as a fraction of the value in study 1 due to observed differences in baseline serum vitamin D_3 and 25(OH)D_3 concentrations.

Error model

Each parameter has a typical value, which describes the median value for a log-normal distribution in the population. The typical individual has typical parameter values for each of its parameters. Inclusion of inter-individual variability was tested on Cl1, Cl3, Q, k15, k51 and F using a log-normal distribution:

$$\theta_i = \theta_p \cdot e^{\eta i} \qquad \text{Equation 1}$$

in which θ_i represents the individual parameter value, θ_p represents the parameter value for a typical individual and η_i represents a random variable from a normal distribution with a mean of 0 and estimated variance ω^2.

For the residual variability a proportional model was used for all compartments:

$$Y_{ij} = c_{pred,ij} + c_{pred,ij} \cdot \varepsilon_{ij} \qquad \text{Equation 2}$$

in which Y_{ij} represents the observed concentration at time-point *j* in the *i*th individual, c_{pred} is the predicted concentration at time-point *j* in the *i*th individual, and ε_{ij} is a random variable with a mean of zero and an estimated variance σ^2. A correlation in the residual error between serum vitamin D_3 and serum 25(OH)D_3 was included using an L2 data item in NONMEM, since values of serum vitamin D_3 and serum 25(OH)D_3 originated from the same sample and were determined simultaneously (**Paper I** and **Paper II**).

5.4 Results from the Pharmacokinetic-model

Figure 5.1 presents a schematic representation of the *final model* and Table 5.1 presents all the parameter estimates obtained in the final model fit. The RSE% is also presented for each parameter. The code for the *final model* is found in the Appendix.

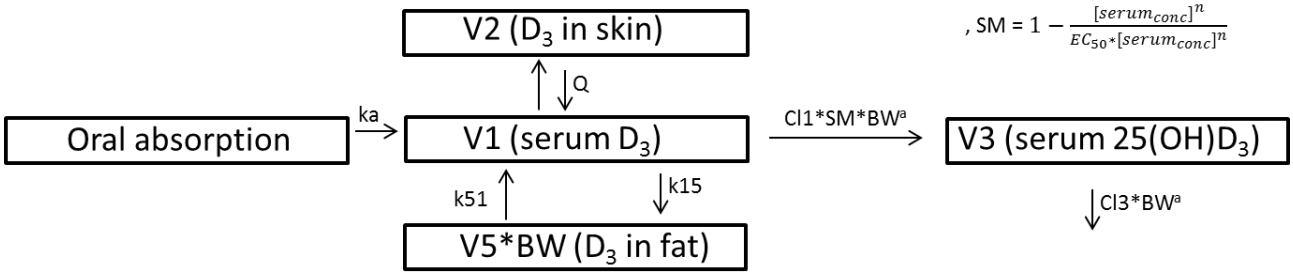

Figure 5.1 Schematic representation of the structural model for the PK-model of vitamin D_3 and 25(OH)D_3 in minipigs. V1 = volume of distribution for vitamin D_3 in serum, V2= volume of distribution for vitamin D_3 in skin, V3= volume of distribution for 25(OH)D_3 in serum, V5= volume of distribution for vitamin D_3 in adipose tissue, ka = oral absorption rate, Q = inter-compartmental clearance of vitamin D_3, k15 = rate constant for vitamin D_3 from the central to the adipose compartment, k51= rate constant for vitamin D_3 from adipose to central compartment, Cl1= formation clearance of serum vitamin D_3 to 25(OH)D_3, Cl3= elimination clearance of serum 25(OH)D_3, a= exponential scaling-factor, n = hill factor, EC_{50}= the serum vitamin D_3 concentration where the 25-hydroxylation is 50% of Cl1.

Table 5.1 Parameter estimates of the *final model*.

Parameter	Value	RSE(%)
Fixed Effects		
V1	1.22	38
V2	5.36	31
ka	0.21	42
Cl1	6.18 ¤	56
Cl3	0.53	28
Q	0.21	32
k15	0.123	35
k51	0.016	15
Initial serum vitamin D_3 (Study 1)	2.47	51
Initial serum vitamin D_3 (Study 2)	3.29*	24
F (Study 1, one minute of UV-light)	0.16	30
F (Study 1, two minutes of UV-light)	1.67*	14
F (Study 2, two minutes of UV-light)	0.436*	18
n	0.55	29
EC50	4.98	195
a	1.42	20
Inter-individual variability		
ω^2 (Cl1)	0.06	29
ω^2 (Cl3)	0.30	22
Residual error		
σ^2 (serum vitamin D_3)	0.075	7
$\sigma^{2,\,corr.}$ (serum 25(OH)D_3)	0.033	8
σ^2 (skin vitamin D_3)	0.80	6
σ^2 (adipose vitamin D_3)	0.065	13

*proportional

¤ covariate (weight)

V = volume of distribution, Cl = clearance, ka = oral absorption rate, Q = inter-compartmental clearance, k = rate constants, F = fractional bioavailability of cutaneous vitamin D_3, n = hill factor, a = allometric scaling factor, ω^2 = variance, σ^2 = intra-individual variance, $\sigma^{2,\,corr.}$ = correlated proportional intra-individual variance, RSE = relative standard error

The distribution volumes V1 and V2 were estimated with good precision (RSE%<50). The distribution volume for serum 25(OH)D_3 (V3) was fixed to V1, as this could not be independently estimated from the data. The distribution volume of vitamin D_3 (V5) could not be estimated either and was fixed to 10% of the BW, which is the fat content in minipigs according to Johansen *et al.* (Johansen et al., 2001). A linear BW dependency was included on V5 and an exponential BW dependency with an estimated exponential exponent was included on Cl1 and Cl3.

1st-order oral absorption described the data adequately. Inclusion of dose-dependent absorption improved the OFV of the model, but the serum vitamin D_3 concentration predictions became biased as observed in the goodness-of-fit plots (not shown).

Doubling the UV-exposure time in study 1, yielded an increase in vitamin D_3 synthesis of 67%. A separate estimation of the synthesis of vitamin D_3 for study 2 better explained the difference between study 1 and 2 than a separate estimation of Cl1 for study 2. The synthesis of vitamin D_3 in study 2 was 44% of the synthesis observed for study 1. The serum vitamin D_3 concentration at the initialization of the experiment was estimated to be 33% higher in study 2 compared to study 1

The amount of cutaneously synthesized vitamin D_3 was estimated relative to the oral dose. Assuming 100% oral bioavailability, the cutaneous synthesis of vitamin D_3 in study 1 was 61 µg/day for one minute of UV-exposure and 103 µg/day after two minutes of UV-exposure. For study 2 the cutaneous vitamin D_3 synthesis was estimated to be 28 µg/day.

The individual profiles for serum vitamin D_3 for a model without and with a sigmoidal function for the 25-hydroxylation are shown in Figure 5.2. The peak concentrations and the fast decline of serum vitamin D_3 concentrations after UV-exposure had ceased, was poorly described without the sigmoidal function (Figure 5.2A). The model fit was improved by inclusion of a sigmoidal relationship based on serum vitamin D_3 concentrations in the final model, as assessed by the individual profiles (Figure 5.2B).

Inter-individual variability could be identified on Cl1 and Cl3 with good precision (RSE%<50), but was not identifiable on k15, k51 and F. Inter-individual variability was identifiable on Q, but skewed the population prediction of both serum vitamin D_3 and serum 25(OH)D_3 (not shown).

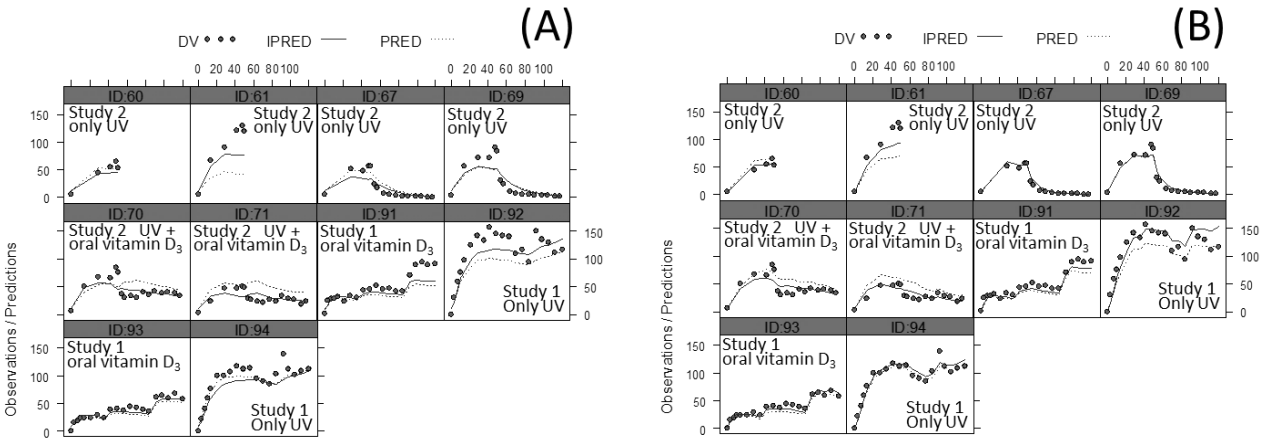

Figure 5.2 The individual plots of the serum vitamin D_3 concentrations for a model *without* sigmoidal kinetics (A) and for the *final model* including sigmoidal kinetics (B).

No biases were observed for serum vitamin D_3 or 25(OH)D_3 concentrations in the goodness-of-fit plots. Only a limited number of observations were available for the vitamin D_3 concentration in adipose tissue and skin, probably causing the skewed smoother in the goodness-of-fit plots for vitamin D_3 in adipose tissue which appeared unbiased, whereas some biases were observed for vitamin D_3 in skin (Figure 5.3).

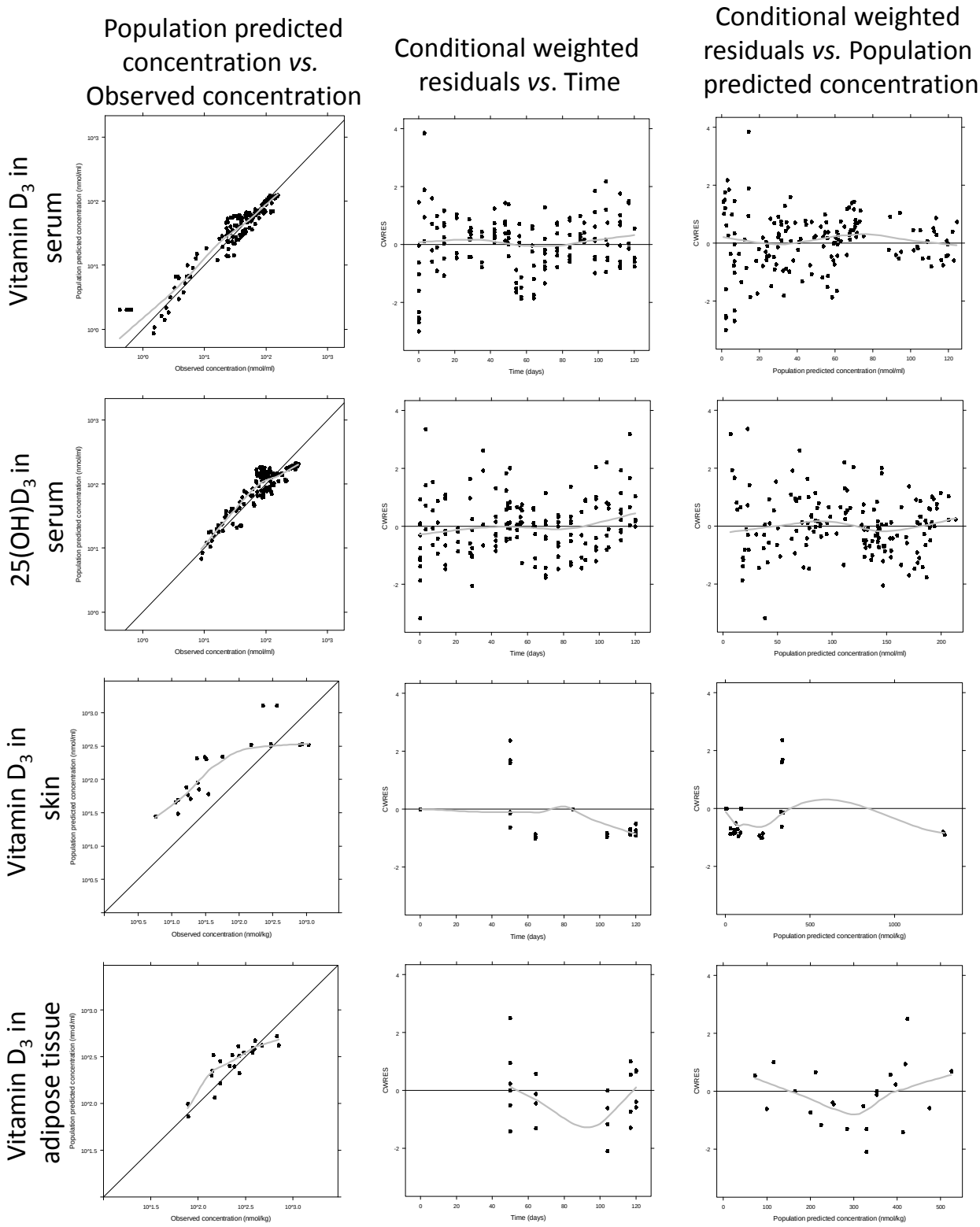

Figure 5.3 Goodness-of-fit plots for vitamin D_3 in serum, 25(OH)D_3 in serum, vitamin D_3 in skin and vitamin D_3 in adipose tissue for the *final model*.

5.5 Discussion of the Pharmacokinetic-model

In this study a comprehensive and novel PK-model for vitamin D_3 was developed, and it accurately describes tissue and serum vitamin D_3 concentrations and serum 25(OH)D_3 concentrations after oral vitamin D_3 supplementation and cutaneous synthesis of vitamin D_3 after UV-exposure in growing minipigs.

The PK-model was developed using data from 10 minipigs. Although the number of individuals in this study was limited, dense data and observations in multiple compartments were available from each individual, allowing an identification of a complex model structure. Most of the RSE% was below 50%, indicating that the data support the model.

The serum vitamin D_3 and serum 25(OH)D_3 levels were higher for study 1 compared to study 2 in which female minipigs were used instead of male minipigs used in study 1. Initially, it was hypothesized, that the observed differences could be gender specific. Although the expression of the major CYP responsible for the 25-hydroxylation in minipigs are unknown, gender differences for the common CYP isoforms are reported for minipigs including CYP3A4 (Skaanild and Friis, 1999), also associated with the 25-hydroxylation of vitamin D_3 (reviewed by Prosser and Jones, 2004). Therefore, it was tested if differences in 25-hydroxlyation could account for the observed difference in serum vitamin D_3 and serum 25(OH)D_3 levels between the two studies, but this difference was better described by separate estimations of the cutaneous vitamin D_3 synthesis for the two studies.

Vitamin D_3 belongs to the fat-soluble vitamins and is distributed peripherally (**Paper III** and **Paper IV**). That is also reflected by the different peripheral compartments (V2 and V5) in the PK-model. The transfer of vitamin D_3 between blood and skin and blood and adipose tissue was described by 1^{st}-order kinetics. The observed quick drop of serum vitamin D_3 after UV-exposure had ceased could not be accurately described by the distribution of vitamin D_3 to these tissues. Therefore, a serum vitamin D_3 or 25(OH)D_3-dependent 25-hydroxylation clearance was tested. This suggested the cutaneous synthesis of serum 25(OH)D_3 to be up-regulated at low serum vitamin D_3 concentrations, which could result from a feedback mechanism regulating serum vitamin D_3 and 25(OH)D_3 levels. Heaney *et al*. reports a saturation of the 25-hydroxylase at high serum vitamin D_3 concentrations based on ratios of serum vitamin D_3 and serum 25(OH)D_3 in human subjects (Heaney et al., 2008).

The PK-model shows that the concentrations of vitamin D_3 and 25(OH)D_3 in minipigs are described by a system of multiple non-linear processes and that the kinetics of serum 25(OH)D_3 in minipigs cannot be described by a simple half-life. The serum 25(OH)D_3 concentration is not only determined by the oral input of vitamin D_3 and the cutaneous synthesis of vitamin D_3, but also by the concentrations of vitamin D_3 in skin, adipose tissue and serum, making predictions of the time-course of serum 25(OH)D_3 concentration complex.

In conclusion, this is the first PK-model describing serum and tissue vitamin D_3 concentrations and serum 25(OH)D_3 concentrations. The model allows input from both dietary vitamin D_3 and cutaneously synthesized vitamin D_3. A similar approach could be applied to human data to investigate and get a better understanding of the kinetics of vitamin D_3 and 25(OH)D_3, as well as the contribution of the endogenously synthesized vitamin D_3 to the serum 25(OH)D_3 concentration. Such information would be helpful when planning future public health strategies to improve the nutritional status of vitamin D_3 and to avoid intoxications.

6 Conclusion

The aim of the thesis was to study the storages of vitamin D_3 and 25(OH)D_3 in pigs under various input conditions. The investigation required sensitive and reliable methods for the quantification of vitamin D_3 and its metabolites in serum and tissue. Therefore, two methods for the quantification of vitamin D_3 and its metabolites were developed. A novel method for the quantification of vitamin D_3, 25(OH)D_3 and 24,25$(OH)_2D_3$ in serum was developed, and it has the potential to investigate new associations between vitamin D_3 and its metabolites.

To study the changes in vitamin D_3 and 25(OH)D_3 in biopsies of subgram sizes, an existing HPLC-method (Jakobsen et al., 2004) for the quantification of vitamin D_3 and 25(OH)D_3 in tissue was downscaled. The sensitivity of the method was retained by replacing the detection by UV with MS/MS, and this also enabled the separation of 13C-labelled vitamin D_3 and 25(OH)D_3 from the endogenous forms. Compared to the existing HPLC-method (Jakobsen et al., 2004), the required sample size was reduced 50 times, the sample throughput was increased 3-4 times and the required amount of organic solvent was reduced by 90%.

Oral supplementation of vitamin D_3 and 25(OH)D_3 were investigated by use of slaughter pigs. The tissue concentration and distribution of vitamin D_3 and 25(OH)D_3 in slaughter pigs proved to be linearly dependent on the dose of vitamin D_3 and 25(OH)D_3, but the tissue content also depended on the ingested form of vitamin D_3. The serum 25(OH)D_3 concentration was linearly associated with the tissue concentration of 25(OH)D_3. The serum 25(OH)D_3 concentration was also linearly associated with the tissue concentration of vitamin D_3, but the relation was dependent on the ingested form of vitamin D_3 in slaughter pigs.

The Göttingen minipig was successfully used to study vitamin D_3 and it was demonstrated that minipigs synthesize vitamin D_3 when exposed to UV-light. The adipose tissue concentration of vitamin D_3 was 10-30 folds higher in the minipigs compared to other studies of vitamin D_3 in pigs (Jakobsen et al., 2007;Clausen et al., 2003). The orally supplemented minipigs received three times more vitamin D_3 than recommended, whereas the irradiated minipigs were only exposed to UV-light corresponding to 10-20 minutes of the daily summer sun.

The turnover of vitamin D_3 and 25(OH)D_3 in skin and adipose tissue can be studied in biopsies in minipigs and a decline of both vitamin D_3 and 25(OH)D_3 during deprivation of vitamin D_3 was observed.

Finally, a comprehensive and novel PK-model for vitamin D_3 that accurately describes tissue and serum vitamin D_3 concentrations and serum 25(OH)D_3 concentrations after oral vitamin D_3 supplementation and cutaneous synthesis of vitamin D_3 after UV-exposure in growing minipigs was developed.

7 Perspectives

This PhD-thesis demonstrated that the storages and turnover of vitamin D_3 and 25(OH)D_3 in biopsies from minipigs could be investigated by use of sensitive analytical methods. This thesis also showed, that such types of data describing the time-course of vitamin D_3 and 25(OH)D_3 concentrations in different tissues could be analysed by means of PK-modelling. The same approach could also be applied to investigate the storages of vitamin D_3 and 25(OH)D_3 in humans and to elucidate the contribution of the storages under various input conditions of vitamin D_3. The PK-model for humans could also be expanded to test the relevance of other covariates, e.g. demographic factors or different genotypes. Depending on the number of individuals, the design of the human studies as well as the quality of the dataset, it might also be possible to establish a predictive PK-model. The authorities in several countries are concerned about the vitamin D status in the general population and are discussing how to improve the vitamin D status. A predictive PK-model could be used to simulate the efficacy of different intervention regimens, e.g. mandatory fortification on the vitamin D_3 status in a population before a certain strategy is implemented.

8 References

Adamec, J., Jannasch, A., Huang, J., Hohman, E., Fleet, J.C., Peacock, M., Ferruzzi, M.G., Martin, B. and Weaver, C.M., 2011. Development and optimization of an LC-MS/MS-based method for simultaneous quantification of vitamin D2, vitamin D3, 25-hydroxyvitamin D- and 25-hydroxyvitamin D3. J. Sep. Sci. 34, 11-20.

Aiba, I., Yamasaki, T., Shinki, T., Izumi, S., Yamamoto, K., Yamada, S., Terato, H., Ide, H. and Ohyama, Y., 2006. Characterization of rat and human CYP2J enzymes as Vitamin D 25-hydroxylases. Steroids 71, 849-856.

Aronov, P.A., Hall, L.M., Dettmer, K., Stephensen, C.B. and Hammock, B.D., 2008. Metabolic profiling of major vitamin D metabolites using Diels–Alder derivatization and ultra-performance liquid chromatography–tandem mass spectrometry. Anal. Bioanal. Chem. 391, 1917-1930.

Bailey, D., Veljkovic, K., Yazdanpanah, M. and Adeli, K., 2013. Analytical measurement and clinical relevance of vitamin D3 C3-epimer. Clin. Biochem. 46, 190-196.

Bilodeau, L., Dufresne, G., Deeks, J., Clément, G., Bertrand, J., Turcotte, S., Robichaud, A., Beraldin, F. and Fouquet, A., 2011. Determination of vitamin D3 and 25-hydroxyvitamin D3 in foodstuffs by HPLC UV-DAD and LC–MS/MS. J. Food. Compost. Anal. 24, 441-448.

Blum, M., Dolnikowski, G., Seyoum, E., Harris, S.S., Booth, S.L., Peterson, J., Saltzman, E. and Dawson-Hughes, B., 2008. Vitamin D 3 in fat tissue. Endocrine 33, 90-94.

Bogh, M., Schmedes, A., Philipsen, P., Thieden, E. and Wulf, H., 2012. A small suberythemal ultraviolet B dose every second week is sufficient to maintain summer vitamin D levels: a randomized controlled trial. Br. J. Dermatol. 166, 430-433.

Bollen, P. and Ellegaard, L., 1997. The Göttingen minipig in pharmacology and toxicology. Pharmacol. Toxicol. 80, 3-4.

Bollen, P.J., Madsen, L.W., Meyer, O. and Ritskes-Hoitinga, J., 2005. Growth differences of male and female Gottingen minipigs during ad libitum feeding: a pilot study. Lab. Anim. 39, 80-93.

Brannon, P.M., Yetley, E.A., Bailey, R.L. and Picciano, M.F., 2008. Overview of the conference" Vitamin D and Health in the 21st Century: an Update". Am. J. Clin. Nutr. 88, 483S.

Brot, C., Vestergaard, P., Kolthoff, N., Gram, J., Hermann, A.P. and Sørensen, O.H., 2001. Vitamin D status and its adequacy in healthy Danish perimenopausal women: relationships to dietary intake, sun exposure and serum parathyroid hormone. Br. J. Nutr. 86, S97-S103.

Brouwer, D.A.J., Beek, J.V., Ferwerda, H., Brugman, A.M., Klis, F.R.M., Heiden, H.J. and Muskiet, F.A.J., 1998. Rat adipose tissue rapidly accumulates and slowly releases an orally-administered high vitamin D dose. Br. J. Nutr. 79, 527-532.

Brugger, B., Erben, G., Sandhoff, R., Wieland, F.T. and Lehmann, W.D., 1997. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. Proc. Natl. Acad. Sci. U. S. A. 94, 2339-2344.

Carpenter, K.J. and Zhao, L., 1999. Forgotten mysteries in the early history of vitamin D. J. Nutr. 129, 923.

Cashman, K.D., Seamans, K.M., Lucey, A.J., Stöcklin, E., Weber, P., Kiely, M. and Hill, T.R., 2012. Relative effectiveness of oral 25-hydroxyvitamin D3 and vitamin D3 in raising wintertime serum 25-hydroxyvitamin D in older adults. Am. J. Clin. Nutr. 95, 1350-1356.

CIE - International Commission on Illumination; Erythema Reference Action Spectrum and Standard Erythema Dose. ISO/CIE Standard ISO 17166; 1999.

Clausen, I., Jakobsen, J., Leth, T. and Ovesen, L., 2003. Vitamin D3 and 25-hydroxyvitamin D3 in raw and cooked pork cuts. J. Food. Compost. Anal. 16, 575-585.

Clements, MR, Chalmers, TM, Fraser, DR, 1984. Enterohepatic circulation of vitamin D: a reappraisal of the hypothesis. The Lancet 323, 1376-1379.

Coffey, J., Hines, E., Starkey, J., Starkey, C. and Chung, T., 2012. Feeding 25-hydroxycholecalciferol improves gilt reproductive performance and fetal vitamin D status. J. Anim. Sci. 90, 3783-3788.

Cooper, D.A., Berry, D.A., Spendel, V.A., Kiorpes, A.L. and Peters, J.C., 1997. The domestic pig as a model for evaluating olestra's nutritional effects. J. Nutr. 127, 1555S.

DeLuca, H.F., 2004. Overview of general physiologic features and functions of vitamin D. Am. J. Clin. Nutr. 80, 1689S.

Diffey, B., Jansen, C., Urbach, F. and Wulf, H., 2011. The standard erythema dose: a new photobiological concept. Photodermatol. Photoimmunol. Photomed. 13, 64-66.

Ding, S., Schoenmakers, I., Jones, K., Koulman, A., Prentice, A. and Volmer, D.A., 2010. Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS. Anal. Bioanal. Chem. 398, 779-789.

Duan, X., Weinstock-Guttman, B., Wang, H., Bang, E., Li, J., Ramanathan, M. and Qu, J., 2010. Ultrasensitive Quantification of Serum Vitamin D Metabolites Using Selective Solid-Phase Extraction Coupled to Microflow Liquid Chromatography and Isotope-Dilution Mass Spectrometry. Anal. Chem. 82, 2488-2497.

Dueland, S., Pedersen, J., Helgerud, P. and Drevon, C., 1982. Transport of vitamin D3 from rat intestine. Evidence for transfer of vitamin D3 from chylomicrons to alpha-globulins. J. Biol. Chem. 257, 146.

Ellegaard Göttingen Minipigs A/S. Taking good care of Ellegaard Göttingen Minipigs. [Rev 2010 Feb 10; cited 2014 Jun 6]. Available from:

http://minipigs.dk/fileadmin/filer/pdf/Taking_good_care_of_Ellegaard_Goettingen_Minipigs_04.02.10.pdf

Engelman, C.D., Meyers, K.J., Iyengar, S.K., Liu, Z., Karki, C.K., Igo, R.P., Truitt, B., Robinson, J., Sarto, G.E. and Wallace, R., 2013. Vitamin D Intake and Season Modify the Effects of the GC and CYP2R1 Genes on 25-Hydroxyvitamin D Concentrations. J. Nutr. 143, 17-26.

Fraser, D.R., 1983. The physiological economy of vitamin D. Lancet 1, 969-972.

Fraser, D. and Kodicek, E., 1968a. Investigations on vitamin D esters synthesized in rats. Detection and identification. Biochem. J. 106, 485-490.

Fraser, D. and Kodicek, E., 1968b. Enzyme studies on the esterification of vitamin D in rat tissues. Biochem. J. 109, 457.

Gaylor, J. and Sault, F.M., 1964. Localization and biosynthesis of 7-dehydrocholesterol in rat skin. J. Lipid Res. 5, 422.

Gupta, R.P., Hollis, B.W., Patel, S.B., Patrick, K.S. and Bell, N.H., 2004. CYP3A4 is a Human Microsomal Vitamin D 25-Hydroxylase. J. Bone Miner. Res. 19, 680-688.

Haddad, J.G., Matsuoka, L.Y., Hollis, B., Hu, Y.Z. and Wortsman, J., 1993. Human plasma transport of vitamin D after its endogenous synthesis. J. Clin. Invest. 91, 2552.

Hahn, T., Birge, S., Scharp, C. and Avioli, L., 1972. Phenobarbital-induced alterations in vitamin D metabolism. J. Clin. Invest. 51, 741.

Heaney, R.P., Horst, R.L., Cullen, D.M. and Armas, L.A.G., 2009. Vitamin D3 distribution and status in the body. J. Am. Coll. Nutr. 28, 252.

Heaney, R.P., Armas, L.A.G., Shary, J.R., Bell, N.H., Binkley, N. and Hollis, B.W., 2008. 25-Hydroxylation of vitamin D3: relation to circulating vitamin D3 under various input conditions. Am. J. Clin. Nutr. 87, 1738.

Heaney, R.P., Recker, R.R., Grote, J., Horst, R.L. and Armas, L.A., 2010. Vitamin D3 is more potent than vitamin D2 in humans. J. Clin. Endocrinol. Metab. 96, E447-E452.

Higashi, T., Awada, D. and Shimada, K., 2001. Determination of 24, 25-dihydroxyvitamin D3 in human plasma using liquid chromatography-mass spectrometry after derivatization with a Cookson-type reagent. Biomedical Chromatography 15, 133-140.

Higashi, T., Suzuki, M., Hanai, J., Inagaki, S., Min, J.Z., Shimada, K. and Toyo'oka, T., 2011. A specific LC/ESI-MS/MS method for determination of 25-hydroxyvitamin D(3) in neonatal dried blood spots containing a potential interfering metabolite, 3-epi-25-hydroxyvitamin D(3). J. Sep. Sci. 34, 725-732.

Higashi, T., Shimada, K. and Toyo'oka, T., 2010. Advances in determination of vitamin D related compounds in biological samples using liquid chromatography-mass spectrometry: A review. J. Chromatogr. B 878, 1654-1661.

Holick, M.F. and Chen, T.C., 2008. Vitamin D deficiency: a worldwide problem with health consequences. Am. J. Clin. Nutr. 87, 1080S.

Holick, M.F., 2002. vitamin D. Clin. Rev. Bone Miner. Metab. 1, 181-207.

Holick, M.F., Richtand, N.M., McNeill, S.C., Holick, S.A., Frommer, J.E., Henley, J.W. and Potts Jr, J.T., 1979. Isolation and identification of previtamin D3 from the skin of rats exposed to ultraviolet irradiation. Biochemistry (N. Y.) 18, 1003-1008.

Holick, M., Frommer, J., McNeill, S., Richtand, N., Henley, J. and Potts, J., 1977. Photometabolism of 7-dehydrocholesterol to previtamin D3 in skin. Biochem. Biophys. Res. Commun. 76, 107-114.

Höller, U., Quintana, A.P., Gössl, R., Olszewski, K., Riss, G., Schattner, A. and Nunes, C.S., 2010. Rapid determination of 25-hydroxy vitamin D3 in swine tissue using an isotope dilution HPLC-MS assay. J. Chromatogr. B 878, 963-968.

Hollis, B.W., Wagner, C.L., Drezner, M.K. and Binkley, N.C., 2007. Circulating vitamin D3 and 25-hydroxyvitamin D in humans: An important tool to define adequate nutritional vitamin D status. J. Steroid Biochem. Mol. Biol. 103, 631-634.

Horst, R.L., Napoli, J. and Littledike, E.T., 1982. Discrimination in the metabolism of orally dosed ergocalciferol and cholecalciferol by the pig, rat and chick. Biochem. J. 204, 185.

Houghton, L.A. and Vieth, R., 2006. The case against ergocalciferol (vitamin D2) as a vitamin supplement. Am. J. Clin. Nutr. 84, 694-697 doi: 84/4/694 [pii].

Jakobsen, J., 2007. Bioavailability and bioactivity of vitamin D3 active compounds–Which potency should be used for 25-hydroxyvitamin D3? International Congress Series 1297, 133-142.

Jakobsen, J., Maribo, H., Bysted, A., Sommer, H.M. and Hels, O., 2007. 25-Hydroxyvitamin D3 affects vitamin D status similar to vitamin D3 in pigs–but the meat produced has a lower content of vitamin D. Br. J. Nutr. 98, 908-913.

Jakobsen, J., Clausen, I., Leth, T. and Ovesen, L., 2004. A new method for the determination of vitamin D3 and 25-hydroxyvitamin D3 in meat. J. Food Compost. Anal. 17, 777-787.

Jetter, A., Egli, A., Dawson-Hughes, B., Staehelin, H.B., Stoecklin, E., Goessl, R., Henschkowski, J. and Bischoff-Ferrari, H.A., 2014. Pharmacokinetics of oral vitamin D3 and calcifediol. Bone 59, 14-19.

Johansen, T., Hansen, H.S., Richelsen, B. and Malmlof, K., 2001. The obese Gottingen minipig as a model of the metabolic syndrome: dietary effects on obesity, insulin sensitivity, and growth hormone profile. Comp. Med. 51, 150-155.

Jones, G., 2012. Metabolism and biomarkers of Vitamin D. Scand. J. Clin. Lab. Invest. 72, 7-13.

Jones, G., Prosser, D. and Kaufmann, M., 2011. 25-hydroxyvitamin d-24-hydroxylase (cyp24a1): its important role in the degradation of vitamin d. Arch. Biochem. Biophys. .

Jones, G., 2008. Pharmacokinetics of vitamin D toxicity. Am. J. Clin. Nutr. 88, 582S.

Jonsson, E.N. and Karlsson, M.O., 1998. Xpose—an S-PLUS based population pharmacokinetic/pharmacodynamic model building aid for NONMEM. Comput. Methods Programs Biomed. 58, 51-64.

Kamao, M., Tatematsu, S., Hatakeyama, S., Sakaki, T., Sawada, N., Inouye, K., Ozono, K., Kubodera, N., Reddy, G.S. and Okano, T., 2004. C-3 epimerization of vitamin D3 metabolites and further metabolism of C-3 epimers: 25-hydroxyvitamin D3 is metabolized to 3-epi-25-hydroxyvitamin D3 and subsequently metabolized through C-1alpha or C-24 hydroxylation. J. Biol. Chem. 279, 15897-15907.

Karlgren, M., Miura, S. and Ingelman-Sundberg, M., 2005. Novel extrahepatic cytochrome P450s. Toxicol. Appl. Pharmacol. 207, 57-61.

Keizer, R.J., Van Benten, M., Beijnen, J.H., Schellens, J.H. and Huitema, A.D., 2011. Pirana and PCluster: a modeling environment and cluster infrastructure for NONMEM. Comput. Methods Programs Biomed. 101, 72-79.

Kuchuk, N.O., van Schoor, N.M., Pluijm, S.M., Chines, A. and Lips, P., 2009. Vitamin D status, parathyroid function, bone turnover, and BMD in postmenopausal women with osteoporosis: global perspective. J. Bone Miner. Res. 24, 693-701.

Lauridsen, C., Halekoh, U., Larsen, T. and Jensen, S.K., 2010. Reproductive performance and bone status markers of gilts and lactating sows supplemented with two different forms of vitamin D. J. Anim. Sci. 88, 202-213.

Lawson, D., Sedrani, S. and Douglas, J., 1986a. Interrelationships in rats of tissue pools of cholecalciferol and 25-hydroxycholecalciferol formed in uv light. Biochem. J. 233, 535.

Lawson, D., Douglas, J., Lean, M. and Sedrani, S., 1986b. Estimation of vitamin D3 and 25-hydroxyvitamin D3 in muscle and adipose tissue of rats and man. Clin. Chim. Acta 157, 175-181.

Lindbom, L., Pihlgren, P. and Jonsson, N., 2005. PsN-Toolkit—a collection of computer intensive statistical methods for non-linear mixed effect modeling using NONMEM. Comput. Methods Programs Biomed. 79, 241-257.

Lipkie, T.E., Janasch, A., Cooper, B.R., Hohman, E.E., Weaver, C.M. and Ferruzzi, M.G., 2013. Quantification of vitamin D and 25-hydroxyvitamin D in soft tissues by liquid chromatography–tandem mass spectrometry. J. Chromatogr. B 932, 6-11.

MacLaughlin, J.A., Anderson, R. and Holick, M.F., 1982. Spectral character of sunlight modulates photosynthesis of previtamin D3 and its photoisomers in human skin. Science 216, 1001.

Molnár, F., Sigüeiro, R., Sato, Y., Araujo, C., Schuster, I., Antony, P., Peluso, J., Muller, C., Mouriño, A. and Moras, D., 2011. 1α, 25 (OH) 2-3-epi-vitamin D3, a natural physiological metabolite of vitamin D3: its synthesis, biological activity and crystal structure with its receptor. PloS one 6, e18124.

Mount. L.E and Ingram, D.L., 1971. THE PIG as a Laboratory Animal. Academic Press, London.

Okano, T., Mizuno, K. and Kobayashi, T., 1978. Identification and determination of 25-hydroxyvitamin D3 in the blood and liver of vitamin D-deficient rats irradiated with ultraviolet light. J. Nutr. Sci. Vitaminol. (Tokyo) 24, 511-518.

Okano, T., Yasumura, M., Mizuno, K. and Kobayashi, T., 1977. Photochemical conversion of 7-dehydrocholesterol into vitamin D3 in rat skins. J. Nutr. Sci. Vitaminol. (Tokyo) 23, 165-168.

Ovesen, L., Brot, C. and Jakobsen, J., 2003. Food contents and biological activity of 25-hydroxyvitamin D: a vitamin D metabolite to be reckoned with? Ann. Nutr. Metab. 47, 107-113.

Pramyothin, P., Biancuzzo, R.M., Lu, Z., Hess, D.T., Apovian, C.M. and Holick, M.F., 2011. Vitamin D in Adipose Tissue and Serum 25-Hydroxyvitamin D After Roux-en-Y Gastric Bypass. Obesity 19, 2228-2234.

Prosser, D.E. and Jones, G., 2004. Enzymes involved in the activation and inactivation of vitamin D. Trends Biochem. Sci. 29, 664-673.

R Core Team, 2012. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing .

Rosenstreich, S.J., Rich, C. and Volwiler, W., 1971. Deposition in and release of vitamin D3 from body fat: evidence for a storage site in the rat. J. Clin. Invest. 50, 679.

Rungby, J., Mortensen, L., Jakobsen, K., Brock, A. and Mosekilde, L., 1993. Distribution of hydroxylated vitamin D metabolites [250HD3 and 1, 25 (OH) 2D3] in domestic pigs: Evidence that 1, 25 (OH) 2D3] is stored outside the blood circulation? Comp. Biochem. Physiol. A Mol. Integr. Physiol. 104, 483-484.

Schleicher, R.L., Encisco, S.E., Chaudhary-Webb, M., Paliakov, E., McCoy, L.F. and Pfeiffer, C.M., 2011. Isotope dilution ultra performance liquid chromatography-tandem mass spectrometry method for simultaneous measurement of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 in human serum. Clin. Chim. Acta 412, 1594-1599.

Schlingmann, K.P., Kaufmann, M., Weber, S., Irwin, A., Goos, C., John, U., Misselwitz, J., Klaus, G., Kuwertz-Bröking, E. and Fehrenbach, H., 2011. Mutations in CYP24A1 and idiopathic infantile hypercalcemia. N. Engl. J. Med. 365, 410-421.

Schuster, I., 2011. Cytochromes P450 are essential players in the vitamin D signaling system. Biochim. Biophys. Acta 1814, 186-199.

SDS Speciel Diet Service. Minipig-Expanded [Catalogue Rev 4; cited 2014 Jun 6]. Available from: http://www.sdsdiets.com/pdfs/mini-Pig.pdf[23] Clausen I, Jakobsen J, Leth T, Ovesen L. Vitamin D3 and 25-hydroxyvitamin D3 in raw and cooked pork cuts. J Food Compost Anal 2003;16:575-85.

Silver, J. and Berry, E., 1982. Vitamin D uptake by the perfused rat liver is determined by its transport protein. Miner. Electrolyte Metab. 7, 298-304.

Skaanild, M.T. and Friis, C., 1999. Cytochrome P450 sex differences in minipigs and conventional pigs. Pharmacol. Toxicol. 85, 174-180.

Smith, J.E. and Goodman, D.S., 1971. The turnover and transport of vitamin D and of a polar metabolite with the properties of 25-hydroxycholecalciferol in human plasma. J. Clin. Invest. 50, 2159-2167.

Strobel, N., Buddhadasa, S., Adorno, P., Stockham, K. and Greenfield, H., 2013. Vitamin D and 25-hydroxyvitamin D determination in meats by LC–IT-MS. Food Chem. 138, 1042-1047.

Suenderhauf, C. and Parrott, N., 2013. A physiologically based pharmacokinetic model of the minipig: data compilation and model implementation. Pharm. Res. 30, 1-15.

Tai, S.S., Bedner, M. and Phinney, K.W., 2010. Development of a candidate reference measurement procedure for the determination of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human serum using isotope-dilution liquid chromatography− tandem mass spectrometry. Anal. Chem. 82, 1942-1948.

van den Ouweland, Johannes MW, Vogeser, M. and Bächer, S., 2013. Vitamin D and metabolites measurement by tandem mass spectrometry. Rev. Endocr. Metab. Disord. 14, 159–184.

van den Ouweland, Johannes MW, Beijers, A.M. and van Daal, H., 2011. Fast separation of 25-hydroxyvitamin D3 from 3-Epi-25-hydroxyvitamin D3 in human serum by liquid chromatography–tandem mass spectrometry: variable prevalence of 3-Epi-25-hydroxyvitamin D3 in infants, children, and adults. Clin. Chem. 57, 1618-1619.

van Leeuwen, J.P.T.M., van den Bemd, G.J., van Driel, M., Buurman, C.J. and Pols, H.A.P., 2001. 24, 25-Dihydroxyvitamin D3 and bone metabolism. Steroids 66, 375-380.

Wagner, D., Hanwell, H.E., Schnabl, K., Yazdanpanah, M., Kimball, S., LEI, F., Sidhom, G., Rousseau, D., Cole, D.E. and Vieth, R., 2011. The ratio of serum 24, 25-dihydroxyvitamin D3 to 25-hydroxyvitamin D3 is predictive of 25-hydroxyvitamin D3 response to vitamin D3 supplementation. J. Steroid Biochem. Mol. Biol. 126, 72-77.

Wamberg, L., Christiansen, T., Paulsen, S., Fisker, S., Rask, P., Rejnmark, L., Richelsen, B. and Pedersen, S.B., 2012. Expression of vitamin D-metabolizing enzymes in human adipose tissue—the effect of obesity and diet-induced weight loss. Int. J. Obes. 37, 651-657.

Wang, T.J., Zhang, F., Richards, J.B., Kestenbaum, B., Van Meurs, J.B., Berry, D., Kiel, D.P., Streeten, E.A., Ohlsson, C. and Koller, D.L., 2010. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. The Lancet 376, 180-188.

Wang, Z., Senn, T., Kalhorn, T., Zheng, X.E., Zheng, S., Davis, C.L., Hebert, M.F., Lin, Y.S. and Thummel, K.E., 2011. Simultaneous measurement of plasma vitamin D3 metabolites including 4 [beta], 25-dihydroxyvitamin D3 using liquid chromatography-tandem mass spectrometry. Anal. Biochem. 8,126-33.

Webb, A., DeCosta, B. and Holick, M., 1989. Sunlight regulates the cutaneous production of vitamin D3 by causing its photodegradation. J. Clin. Endocrinol. Metab. 68, 882.

Wertz, A., Knight, T., Trenkle, A., Sonon, R., Horst, R., Huff-Lonergan, E. and Beitz, D., 2004. Feeding 25-hydroxyvitamin D3 to improve beef tenderness. J. Anim. Sci. 82, 1410-1418.

Wilson, S.R. and Wu, Y., 1993. Applications of electrospray ionization mass spectrometry to neutral organic molecules including fullerenes. J. Am. Soc. Mass Spectrom. 4, 596-603

Witschi, A.K.M., Liesegang, A., Gebert, S., Weber, G. and Wenk, C., 2011. Effect of source and quantity of dietary vitamin D in maternal and creep diets on bone metabolism and growth in piglets. J. Anim. Sci. 89, 1844-1852.

Zerwekh, J.E., 2008. Blood biomarkers of vitamin D status. Am. J. Clin. Nutr. 87, 1087S-1091S.

Zhang, Z., He, J., Fu, W., Zhang, C. and Zhang, Z., 2013. An analysis of the association between the vitamin D pathway and serum 25-hydroxyvitamin D levels in a healthy Chinese population. J. Bone Miner. Res. 28,1784-92.

9 Appendix

Code for the *final PK-model* used in Nonmem

```
$INPUT ID AMT TIME DV EVID CMT MDV ADDL II WT FLAG DOSE SEX FLAGII L2 DOSEII

$SUBROUTINES ADVAN6 TOL=5

$MODEL
COMP= (CENTRAL)      ;vitamin D3 in serum
COMP= (LIGHT)        ;vitamin D3 in skin
COMP= (METAB)        ;25(OH)D3 in serum
COMP= (ORAL)         ;oral absorption
COMP= (PERIF)        ;vitamin D3 in adipose tissue

$PK
TVCL1 = THETA(1)     ;formation clearance of serum vitamin D3 to 25(OH)D3
TVV1  = THETA(2)     ;volume of distribution for vitamin D3 in serum
TVQ1 = THETA(3)      ;inter-compartmental clearance of vitamin D3
TVKA2 = THETA(4)     ;oral absorption rate
TVV3  = TVV1         ;volume of distribution for 25(OH)D3 in serum
TVCL3 = THETA(5)     ;elimination clearance of serum 25(OH)D3
TVK15  = THETA(6)    ;rate constant for vitamin D3 from the central to the adipose compartment
TVK51  = THETA(7)    ;rate constant for vitamin D3 from adipose to central compartment
TVV2  = THETA(13)    ;volume of distribution for vitamin D3 in skin
V5=0.1*WT            ;volume of distribution for vitamin D3 in adipose tissue

CL1   = TVCL1*((WT/10.0)**THETA(16))*EXP(ETA(1))*(1-
((1*(DOSEII**THETA(14))/(THETA(15)**THETA(14)+DOSEII**THETA(14)))))
V1    = TVV1
V2    = TVV2
KA2   = TVKA2
Q1    = TVQ1
V3    = V1
CL3   = TVCL3*((WT/10.0)**THETA(16))*EXP(ETA(2))
K15    = TVK15
K51    = TVK51

; micro constants
K30=CL3/V3
K13=CL1/V1
K12=Q1/V1
K21=Q1/V2
K41=KA2

S1=V1
S2=V2
S3=V3
S5=V5

; Initialization of the system
BASE= THETA(8)
IF (SEX.EQ.2) BASE= THETA(8)*THETA(9)

A_0(1)= BASE
A_0(2)= (K12/K21)*BASE
A_0(3)= (K13/K30)*BASE
A_0(5)= (K15/K51)*BASE

;Amount of cutaneously synthesized vitamin D3
F2= THETA(10)
IF (ID.EQ.92..AND.TIME.GT.85.OR.ID.EQ.94.AND.TIME.GT.85) F2= THETA(10)*THETA(11)
IF (ID.EQ.60.OR.ID.EQ.61.OR.ID.EQ.67.OR.ID.EQ.69.OR.ID.EQ.70.OR.ID.EQ.71) F2= THETA(10)*THETA(12)
```

```
$DES
DADT(1) = - K13*A(1)+ K21*A(2)+ K41*A(4)+K51*A(5) -K15*A(1)-K12*A(1)
DADT(2) = - K21*A(2)+K12*A(1)
DADT(3) = K13*A(1) - K30*A(3)
DADT(4) = - K41*A(4)
DADT(5) = -K51*A(5) + K15*A(1)
;DADT(6) = -K61*A(6) + K16*A(1)

$ERROR
COM1=0
IF(CMT.EQ.1) COM1=1
COM3=0
IF(CMT.EQ.3) COM3=1
COM5=0
IF(CMT.EQ.5) COM5=1
COM2=0
IF(CMT.EQ.2) COM2=1

IPRED = F
IRES = DV - IPRED
W = IPRED
IF(W.EQ.0) W = 1
IWRES =IRES/W
Y1= IPRED + W*EPS(1)                    ;vitamin D3 in serum
Y2= IPRED + W*EPS(2)                    ;25(OH)D3 in serum
Y3= IPRED + W*EPS(3)                    ;vitamin D3 in adipose tissue
Y4= IPRED + W*EPS(4)                    ;vitamin D3 in skin
Y= Y1*COM1+Y2*COM3+Y3*COM5+Y4*COM2

$THETA
(0, 6.01215) ; 1.CL1
(0, 1.20908) ; 2.V1
(0, 0.20788) ; 3.Q1
(0, 0.20678) ; 4.KA2
(0, 0.5287) ; 5.CL3
(0, 0.12347) ; 6.K15
(0, 0.01617) ; 9.K51
(0, 2.47093) ; 10.INIT_STUDY1
(0, 3.27446) ; 11.INIT_STUDY2
(0, 0.15814) ; 12.F_LIGHT1min
(0, 1.65255) ; 13.F_LIGHT2min
(0, 0.44309) ; 14.F_LIGHT_study 2
(0, 5.38631) ; V2
(0, 0.557) ; Hill factor
(0, 5.38436) ;EC50
(0, 1.42662) ; Allometric scaling factor

$OMEGA
 0.057129
 0.159854

$SIGMA BLOCK(2)
 0.074354 ;EPS1
 0.01 0.032819 ;correlation EPS2

$SIGMA
 0.065164 ;EPS3
 0.803355 ;EPS4

$EST MAX=9999 METHOD=1 INTERACTION NOABORT POSTHOC PRINT=5
```

10 Publications

Paper I: **Anders Burild**, Henrik L. Frandsen, Jette Jakobsen, 2014. Simultaneous quantification of vitamin D_3, 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 in human serum by LC-MS/MS. Scandinavian Journal of Clinical and Laboratory Investigation 74, 418-423. Reprinted with permission from Informa Healthcare.

Paper II: **Anders Burild**, Henrik L. Frandsen, Morten Poulsen, Jette Jakobsen, 2014. Quantification of Physiological Levels of Vitamin D_3 and 25-Hydroxyvitamin D_3 in Porcine Fat and Liver by use of Subgram Sample Sizes. Journal of Separation Sciences 37, 2659-2663. Reprinted with permission from Wiley.

Paper III: **Anders Burild**, Charlotte Lauridsen, Nasrin Faqir, Helle M. Sommer, Jette Jakobsen. Tissue content of vitamin D_3 and 25-hydroxyvitamin D_3 and the relation to serum 25-hydroxyvitamin D_3 in pigs supplemented with two forms of vitamin D_3 at four different levels. Submitted to British Journal of Nutrition.

Paper IV: **Anders Burild**, Henrik L. Frandsen, Morten Poulsen, Jette Jakobsen. Tissue Content of Vitamin D_3 and 25-hydroxyvitamin D_3 in Minipigs after cutaneous synthesis, supplementation and deprivation of vitamin D_3. Submitted to Steroids.

Paper I

Supplementary Material

(Published in Scandinavian Journal of Clinical and Laboratory)

Scandinavian Journal of Clinical & Laboratory Investigation, 2014; 74: 418–423

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ORIGINAL ARTICLE

Simultaneous quantification of vitamin D_3, 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 in human serum by LC-MS/MS

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Abstract

Introduction. Serum 25-hydroxy-vitamin D is the established biomarker of vitamin D status although serum concentrations of vitamin D and 24,25-dihydroxyvitamin D may also be of interest to understand the *in vivo* kinetics of serum 25-hydroxyvitamin D. ***Method.*** An LC-MS/MS method was developed and validated to quantify vitamin D_3, 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 in serum. After protein precipitation of the serum it was loaded on a HybridSPE column to separate vitamin D metabolites from phospholipids. Vitamin D_3, 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 in the eluate were derivatized by 4-phenyl-1,2,4-triazoline-3,5-dione to improve sensitivity in the following LC-MS/MS analysis. ***Results.*** Using only 100 µL serum the limit of quantification was <0.2 ng/mL for vitamin D_3, 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3. The method was validated up to 100 ng/mL (260 nmol/L) for vitamin D_3, up to 100 ng/mL (240 nmol/L) for 24,25-dihydroxyvitamin D_3 and up to 200 ng/mL (499 nmol/L) for 25-hydroxyvitamin D_3. Precision was $<6.5\%$ for vitamin D_3 and 25-hydroxyvitamin D_3 and $<10.2\%$ for 24,25-dihydroxyvitamin D_3. ***Conclusion.*** We demonstrate that a method including not only serum 25-hydroxyvitamin D_3 but also vitamin D_3 and 24,25-dihydroxyvitamin D_3 could easily be implemented in most modern biochemical laboratories. The method could be used to study the metabolism of endogenous synthesized vitamin D_3 as well as vitamin D_3 in intervention studies.

Key Words: *Liquid chromatography, vitamin D, mass spectroscopy*

Introduction

Vitamin D is essential for the body to prevent rickets and osteomalacia and to maintain a healthy skeleton throughout life and it is becoming increasingly clear that vitamin D is not only important for bone health but also for the functioning of other organs, e.g. the immune system and prevention of cancer [1].

Serum or plasma 25-hydroxyvitamin D (25(OH) D) is the established biomarker of vitamin D status routinely used in the clinic [2,3] as well as in intervention studies and epidemiological studies [4]. However, a large inter-individual variation in serum 25(OH)D response to oral supplementation is observed in populations given the same dose [5].

Therefore a better understanding of the *in vivo* kinetics of 25(OH)D seems pertinent for planning public health strategies to improve the vitamin D nutritional status and to avoid intoxications. It has recently become evident that serum concentrations of vitamin D and 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) may be of interest to understand the kinetics of serum 25(OH)D [4,6,7]. For serum 25(OH)D the relation to serum vitamin D appears to be non-linear, saturable and controlled [4,6].

Although $24,25(OH)_2D$ might play a biological role itself [8] it is mainly considered a catabolite with a close correlation to serum 25(OH)D [9]. CYP24A1 is the enzyme responsible for the 24-hydroxylation of vitamin D metabolites and several mutation/polymorphisms are reported for this enzyme [7] most likely affecting the turnover rate of 25(OH)D and the level of $24,25(OH)_2D$.

To our knowledge, no single methods are currently available for quantification of serum vitamin D, 25(OH)D and $24,25(OH)_2D$ probably due to the large inherent differences in polarity

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between vitamin D and 24,25(OH)$_2$D. The method described by Adamec et al. focused on the quantification of serum vitamin D and 25(OH)D [10] whereas other methods focused on the quantification of dihydroxylated metabolites of vitamin D including 24,25(OH)$_2$D along with the 25(OH)D in serum [11–15]. Indeed Aronov et al. attempted to include vitamin D to a multi method for 25(OH)D, 24,25(OH)$_2$D and 1,25-dihydroxy D (1,25(OH)$_2$D), but was unsuccessful [11].

Since vitamin D$_3$ is the dominant form of vitamin D in humans [16,17] we developed and validated an LC-MS/MS method for simultaneous quantification of serum vitamin D$_3$, 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$. The method could be used to study the metabolism of endogenous synthesized vitamin D$_3$ as well as vitamin D$_3$ in intervention studies.

Materials and methods

Chemicals and reagents

Methanol and acetonitrile were HPLC grade (Rathburn Chemicals Ltd, Walkerburn, Scotland). Formic acid for mass spectrometry, anhydrous acetonitrile, methylamine and 4-Phenyl-1,2,4-Triazoline-3,5-Dione (PTAD) as well as standards for vitamin D$_3$, 25(OH)D$_3$, (24R)-24,25(OH)$_2$D$_3$ and 3-epi-25(OH)D$_3$ were obtained from Sigma-Aldrich (Steinheim, Germany). The deuterated internal standards 26, 26, 26, 27, 27, 27-*d6*-vitamin D$_3$ (*d6*-vitamin D$_3$) and 26, 26, 26, 27, 27, 27-*d6*-25(OH) D$_3$ (*d6*-25(OH)D$_3$) were from Chemaphor Inc., Ottawa, Canada, CHE012 and CHE011, respectively.

Serum for method development was processed from blood drawn from an employee at the National Food Institute in Denmark not taking vitamin D$_3$ supplements.

Milli-Q water was made in house (18.2 MΩ, Millipore, Billerica, MA).

Stock solutions and calibration curves

Stock solutions were prepared in ethanol and the concentrations were determined spectrophotometrically at 265 nm with $\varepsilon = 18{,}466$ m^2/mol for vitamin D$_3$ [18], $\varepsilon = 18{,}584$ m^2/mol for 25(OH)D$_3$ [19] and $\varepsilon = 18{,}300$ m^2/mol for the 24,25(OH)$_2$D$_3$ [20].

Calibration standards were prepared from a mixed solution of PTAD-derivatized vitamin D$_3$, 25(OH)D$_3$ and (24R)-24,25(OH)$_2$D$_3$ in acetonitrile. Using serial dilution calibration standards containing 0.005, 0.100, 1.00, 10.0, 50.0 and 100 ng/mL of vitamin D$_3$ and (24R)-24,25(OH)$_2$D$_3$ and 0.010, 0.200, 2.00, 20.0, 100, 200 ng/mL for 25(OH)D$_3$ as well as PTAD-derivatized vitamin *d6*-vitamin D$_3$ (25 ng/mL) and *d6*-25(OH) D$_3$ (50 ng/mL) were prepared. The calibration standards were evaporated under a gentle stream of nitrogen and reconstituted in 60% methanol. Calibration standards were included in all series of analysis.

For vitamin D$_3$ and 25(OH)D$_3$ deuterated internal standards were used for quantification while external standard was used for 24,25(OH)$_2$D$_3$. All standard curves were weighted over the concentrations (1/x).

Test of solid phase extraction (SPE) columns

Initially, Oasis HLB, 1 cc 30 mg (Waters, Milford, MA) was tested by a published method using 200 µL serum protein precipitated by 200 µL ice cold acetonitrile [11]. The Oasis column was later substituted by the HybridSPE 30 mg/1 mL column (Supelco Analytical, Bellefote, PA). A total of 200 µL serum was protein precipitated by 600 µL of ice cold 1% formic acid. After vortex mixing the sample was centrifuged at 10,000 *g* for 10 min and the supernatant was loaded to the HybridSPE column which was initially conditioned with 1 mL of 1% formic acid in acetonitrile. Then, 0.5 mL of 1% formic acid in acetonitrile was subsequently added. The elution fractions were evaporated to dryness and derivatized with PTAD as described below and analyzed by LC-MS/MS.

Test of analytical columns to separate 3-epi-25(OH)D$_3$ from 25(OH)D$_3$

3-epi-25(OH)D$_3$ and 25(OH)D$_3$ were derivatized with PTAD prior to injection on C18 (Ascentis Express C18, 2.1 mm × 10 cm, 2.7 µm particles) and pentafluorophenyl-propyl (PFP) (Ascentis Express F5, 2.1 mm × 10 cm, 2.7 µm particles) analytical columns from Supelco Analytical (Bellefonte, PA). Gradient, mobile phases and column temperature as described below were used.

Method for analysis of vitamin D metabolites in serum

To 100 µL of serum 20 µL internal standard solution with a content of 125 ng/mL vitamin *d6*-vitamin D$_3$ and 250 ng/mL *d6*-25(OH)D$_3$ in ethanol was added. After vortex mixing the sample was allowed to equilibrate for 20 min. The proteins were precipitated by adding 300 µL ice cold 1% formic acid in acetonitrile to the sample. After vortex mixing the sample was centrifuged at 10,000 *g* for 10 min. A HybridSPE 30 mg/1 mL cartridge placed in a vacuum manifold was conditioned with 1 mL of 1% formic acid in acetonitrile. The supernatant was loaded followed by addition of 0.5 mL of 1% formic acid in acetonitrile. The total eluates from the HybridSPE columns were evaporated to dryness

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under a gentle stream of nitrogen (25°C) taking approximately 30 min. Then 50 µL of 0.75 mg/mL PTAD in anhydrous acetonitrile was added and the sample was allowed to derivatize for 2 h at ambient temperature. The sample was dried under nitrogen (25°C), reconstituted in 100 µL 60% methanol and transferred to a vial for LC-MS/MS analysis.

LC-MS/MS

Separation was performed on an Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA) equipped with an Ascentis Express C18 (2.1 mm × 10 cm, 2.7 µm particles) column and an Ascentis Express C18 (2.1 mm × 5 mm, 2.7 µm particles) guard column from Supelco Analytical (Bellefonte, PA) thermostated at 50°C. Mobile phase A consisted of Milli-Q water, methylamine (5 mM) and formic acid (0.1%) and mobile phase B was made of methanol, methylamine (5 mM) and formic acid (0.1%). The flow was 0.5 mL/min and the following gradient was used: 0–1 min, 60%B; 1–9 min, linear gradient to 72%B; 9–10 min, linear gradient to 75%B; 10–14 min, linear gradient to 100%B; 14–18 min, 100%B; 18–20 min, linear gradient to 60%B; 20–25 min, 60%B. The injection volume was 10 µL.

For quantification an Agilent 6460 series Triple Quad MS (Agilent Technologies, Santa Clara, CA) operated in positive multi reaction monitoring (MRM) mode and equipped with a Jetstream ion source was used. The following instrument parameters were used: Drying gas temperature (310°C), drying gas flow (8 L/min), nebulizer (24 psi), capillary voltage (4000 V), sheath gas temperature (375°C), sheath gas flow (11 L/min), nozzle voltage (0 V), fragmentor (120), collision energy (17) and dwell time (200 ms). Minor changes were used for the precursor scan: Fragmentor (135), collision energy (20). Nitrogen was used as the collision gas.

The optimal fragmentor and collision energy settings were determined for each derivatized analyte after multiple injections. The methylamine adducts were used as parent ions and the following transitions were used: *m/z* 591.4→298.0 and *m/z* 597.4→298.0 for vitamin D_3 and *d6*-vitamin D_3, respectively, *m/z* 607.4→298.0 for 25(OH)D_3 and for 3-epi-25(OH)D_3, *m/z* 613.4→298.0 for *d6*-25(OH)D_3 and *m/z* 623.4→298.0 for 24,25$(OH)_2D_3$.

Validation of the method for vitamin D metabolites in serum

For validation, four different levels of vitamin D_3, 25(OH)D_3 and 24,25$(OH)_2D_3$ were spiked to human serum containing endogenous levels of vitamin D metabolites. Non-spiked human serum was included in the validation to determine the endogenous level of vitamin D metabolites. The validation was repeated over three different days and all samples were each time prepared in triplicates. Accuracy was calculated from the results from the spiked samples corrected for the endogenous levels of vitamin D_3 metabolites. For external validation of serum 25(OH)D_3 level the standard reference material '1950 Metabolites in Human Plasma' (National Institute of Standards and Technology, Gaithersburg, MD) was analyzed four times and the results for 25(OH)D_3 were compared with the certified concentration.

Limit of quantification was calculated from the lowest calibration point with S/N > 10 corrected for loss of analyte during extraction and ion suppression.

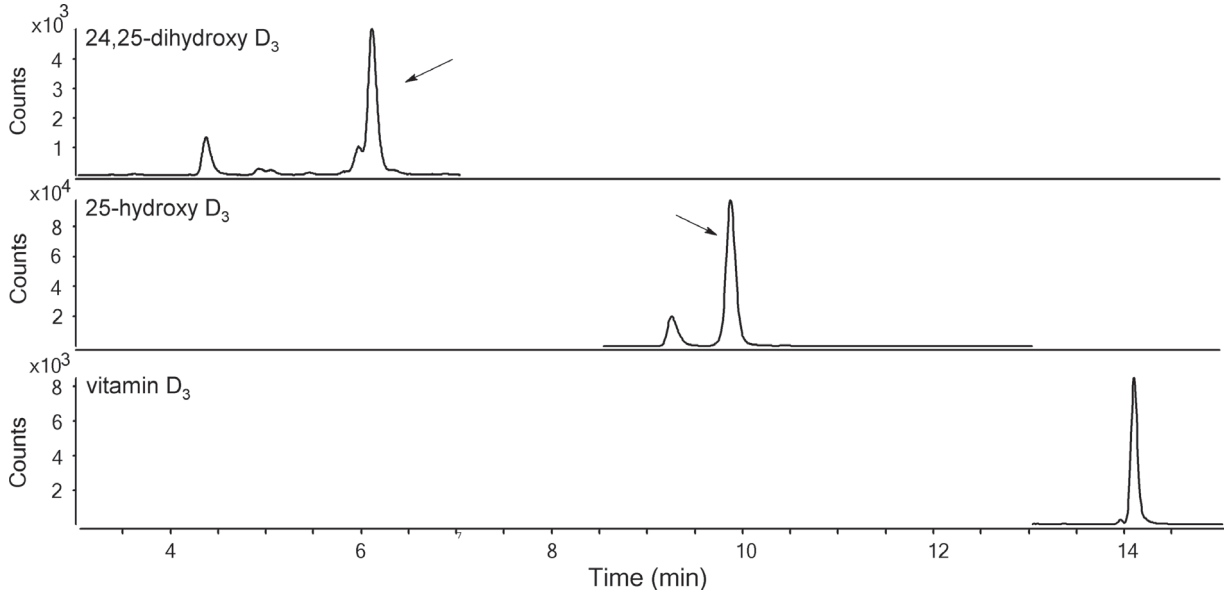

Figure 1. Chromatograms of 24,25$(OH)_2D_3$ (2.1 ng/mL), 25(OH)D_3 (25 ng/mL) and vitamin D_3 (3.5 ng/mL) in the '1950 Metabolites in Human Plasma' reference material. The peaks indicated with an arrow were used for quantification. For chromatographic details see the Materials and Methods section.

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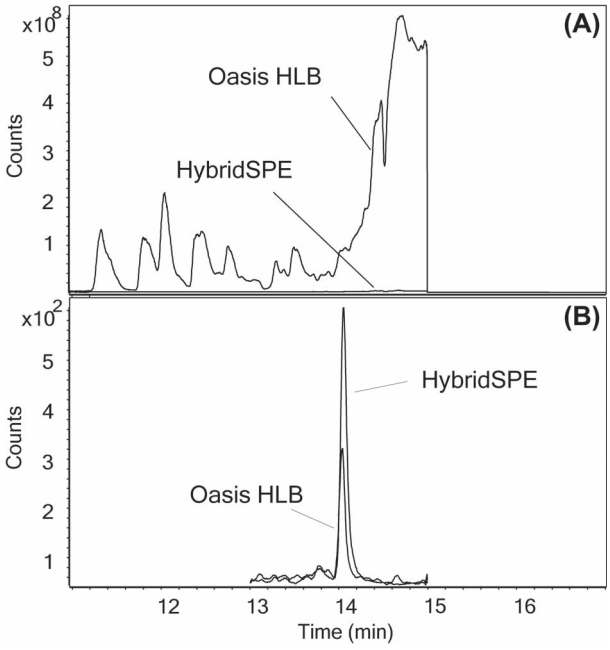

Figure 2. Chromatograms of 200 µL serum spiked with deuterated vitamin D_3 after sample clean up by Oasis HLB and HybridSPE. A precursor scan of m/z 184 in (A) and an extracted ion chromatogram of the deuterated vitamin D_3 (B) are shown.

For vitamin D_3 and 25(OH)D_3 the correction was made by comparing intensities of the internal standards in the cleaned up sample with intensities of the internal standards in solvent. For 24, 25$(OH)_2D_3$ the correction was made from the accuracy which reflects the recovery of analyte during sample preparation and the subsequent analysis by LC-MS/MS.

The highest spiking level of each of the vitamin D analytes defined the upper range for the method.

Statistical analysis

Precision expressed in terms of %CV was calculated according to the model of variance in [21] by use of Excel (version 2007, Microsoft, Redmond, WA).

Results

Analytical method

A chromatogram of the vitamin D metabolites is shown in Figure 1. The analytes eluted between 4 min and 14 min. Two peaks were observed for 24,25$(OH)_2D_3$ and 25(OH)D_3 corresponding to the two epimers 6S and 6R formed after reaction of PTAD with the diene in the vitamin D structure [11]. The major peak was used for quantification. 3-epi-25(OH)D_3 was not separated by the C18 or the PFP analytical columns under the tested chromatographic conditions (data not shown).

Solid phase extraction using Oasis HLB for quantification of hydroxylated and dihydroxylated vitamin D metabolites in serum has previously been used [11], but we found low recovery of vitamin D_3 in preliminary experiments. The signal for vitamin D_3 was reduced to ~50% when derivatized vitamin D_3 was spiked to serum purified by Oasis HLB compared to solvent (data not shown). A precursor scan in positive mode for *m/z* 184 (Figure 2) showed interferences coeluting with vitamin D_3 on the analytical column for the samples purified on the Oasis HLB. The interferences were most likely phospholipids [22] causing severe ion suppression. The interferences were successfully removed by replacing the Oasis HLB by the HybridSPE columns which selectively retains phospholipids and the signal for vitamin D_3 was improved by ~100% (Figure 2).

Deuterated 1,25$(OH)_2D_3$ has previously been used as an internal standard for 24,25$(OH)_2D_3$ [11,12,14], but preliminary experiments revealed an accuracy of 24,25(OH)2D_3 at 156–159% when using *d3*-1,25$(OH)_2D_3$ for quantification. Presumably due to coeluting interferences causing ion suppression for *d3*-1,25$(OH)_2D_3$ (vide infra). More precise and accurate results for 24,25$(OH)_2D_3$ were obtained using an external standard curve.

An attempt was made to include 1,25$(OH)_2D_3$ in the method. 1,25$(OH)_2D_3$ eluted at 6.5 min and a partial separation of the 6S and 6R epimers were observed for the internal standard and the standards.

Table I. Precision and accuracy of vitamin D_3 metabolite measurement in human serum. The analyses were done on three different days *in triplo* ($n = 9$).

Compound	Concentration, ng/mL (nmol/L)	Intra-assay precision (%)	Inter-assay precision (%)	Accuracy (%)
Vitamin D_3	2.5 (6.5)	3.7	3.6	83.8
	25 (65)	2.3	3.8	98.8
	100 (260)	4.0	3.5	104.9
25(OH)D_3	5 (12.5)	5.4	6.5	92.9
	50 (125)	2.5	2.6	98.6
	200 (499)	4.4	4.3	101.6
24,25$(OH)_2D_3$	2.5 (6)	7.4	10.2	76.2
	25 (60)	2.1	4.2	86.5
	100 (240)	4.0	4.2	89.4

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No separation was, however, observed for the analyte in the serum samples, probably due to coeluting interferences (data not shown).

The precision for vitamin D_3 and 25(OH)D_3 was $<10\%$ and the accuracy 90–110% except at the lowest spiking level of vitamin D_3 (~84%). The lack of internal standard can account for the slightly poorer precision and underestimation of 24,25$(OH)_2D_3$ (Table I).

For all standard curves the regression coefficient was >0.99. Limit of quantification was 0.2 ng/mL (0.52 nmol/L) for vitamin D_3, 0.02 ng/mL, (0.05 nmol/L) for 25(OH)D_3 and 0.2 ng/mL (0.48 nmol/L) for 24,25$(OH)_2D_3$. Inter-assay precision at limit of quantification was 11.8%, 13.0% and 4.1% for vitamin D_3, 25(OH)D_3 and 24,25$(OH)_2D_3$, respectively.

The 25(OH)D_3 in '1950 Metabolites in Human Plasma' was determined ($n=4$) to 25.53 ng/mL (± 0.40 ng/mL), which was in accordance with the certified value at 24.78 ng/mL $\pm$ 0.77 ng/mL.

Discussion

Serum 25(OH)D is the established biomarker for vitamin D status [2,3]. Serum 25(OH)D_3 is related to serum vitamin D_3 [4,6] but serum 24,25$(OH)_2D_3$ also seems to be of interest in understanding the kinetics of serum 25(OH)D_3 [7] in intervention studies with vitamin D_3.

Recently, several LC-MS/MS methods measuring different hydroxylated vitamin D metabolites have been published [11–14]. These methods all use Oasis HLB SPE columns and derivatization of vitamin D metabolites with PTAD to improve the sensitivity [2]. PTAD reagent is usually used in concentrations between 0.5 mg/mL and 1 mg/mL and derivatization time is reported to be from 1 h to overnight derivatization [11,13,14]. We chose initially 0.75 mg/mL and 2 h which resulted in satisfying sensitivity as well as robustness, hence no optimization was conducted. We aimed to develop an LC-MS/MS method for quantification of serum vitamin D_3, 25(OH)D_3 and 24,25$(OH)_2D_3$. We managed to include vitamin D_3 in our method by switching to HybridSPE columns. Contrary to Oasis HLB SPE the HybridSPE columns selectively retained the phospholipids improving the ionization of the derivatized vitamin D_3.

We were not able to separate PTAD derivatized 3-epi-25(OH)D_3 from 25(OH)D_3 by analytical columns with stationary phases consisting of either C18 or PFP. The PFP phase is reported to have enhanced selectivity for the 3-epi-25(OH)D_3, but has only been tested on the underivatized vitamin D analytes [23,24]. The separation of 3-epi-25(OH)D_3 from 25(OH)D_3 is important for vitamin D assays used for the clinical assessment of vitamin D status in the infant and pediatric population although less relevant for the adult population [25].

The validation confirmed that our method was capable of simultaneously quantifying vitamin D_3, 25(OH)D_3 and 24,25$(OH)_2D_3$ by use of 100 µL serum with high robustness. For external validation we used the NIST reference and our results were in accordance with the certified value. The limit of quantification for vitamin D_3 and 24,25$(OH)_2D_3$ was <0.2 ng/mL which is well below normal physiological baseline levels [4,9]. We expect that an inclusion of deuterated 24,25$(OH)_2D_3$ as an internal standard would further improve precision and accuracy of serum 24,25$(OH)_2$ D_3 which was slightly lower than for vitamin D_3 and 25(OH)D_3.

The method enables a more holistic approach to vitamin D_3 status and metabolism by including assessment of vitamin D_3 and 24,25$(OH)_2D_3$. The method has the potential to add new insight to the kinetics of vitamin D_3 as well as the large inter-individual variation in serum 25(OH)D_3 observed in the clinic.

The quantification of vitamin D_3, 25(OH)D_3 and 24,25$(OH)_2D_3$ in serum from human intervention studies are now in progress in our laboratory and the data will be reported in the near future.

Acknowledgements

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Declaration of interest: The authors have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] DeLuca HF. Overview of general physiologic features and functions of vitamin D. Am J Clin Nutr 2004;80: 1689–96.

[2] Higashi T, Shimada K, Toyo'oka T. Advances in determination of vitamin D related compounds in biological samples using liquid chromatography-mass spectrometry: a review. J Chromatogr B 2010;878:1654–61.

[3] Jones G. Metabolism and biomarkers of vitamin D. Scand J Clin Lab Invest 2012;72:7–13.

[4] Heaney RP, Armas LAG, Shary JR, Bell NH, Binkley N, Hollis BW. 25-Hydroxylation of vitamin D3: relation to circulating vitamin D3 under various input conditions. Am J Clin Nutr 2008;87:1738–42.

[5] Vieth R. Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety. Am J Clin Nutr 1999;69: 842–56.

[6] Hollis BW, Wagner CL, Drezner MK, Binkley NC. Circulating vitamin D3 and 25-hydroxyvitamin D in humans: an important tool to define adequate nutritional vitamin D status. J Steroid Biochem Mol Biol 2007;103:631–4.

[7] Jones G, Prosser D, Kaufmann M. 25-hydroxyvitamin d-24-hydroxylase (cyp24a1): its important role in the degradation of vitamin D. Arch Biochem Biophys 2012;523:9–18.

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[8] van Leeuwen JPTM, van den Bemd GJ, van Driel M, Buurman CJ, Pols HAP. 24, 25-Dihydroxyvitamin D3 and bone metabolism. Steroids 2001; 66:375–80.

[9] Wagner D, Hanwell HE, Schnabl K, Yazdanpanah M, Kimball S, Lei F, Sidhom G, Rousseau D, Cole DEC, Reinhold V. The ratio of serum 24, 25-dihydroxyvitamin D3 to 25-hydroxyvitamin D3 is predictive of 25-hydroxyvitamin D3 response to vitamin D3 supplementation. J Steroid Biochem Mol Biol 2011;126:72–7.

[10] Adamec J, Jannasch A, Huang J, Hohman E, Fleet JC, Peacock M, Ferruzzi MG, Berdine M, Weaver CM. Development and optimization of an LC-MS/MS-based method for simultaneous quantification of vitamin D2, vitamin D3, 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3. J Sep Sci 2011;34:11–20.

[11] Aronov PA, Hall LM, Dettmer K, Stephensen CB, Hammock BD. Metabolic profiling of major vitamin D metabolites using Diels–Alder derivatization and ultra-performance liquid chromatography–tandem mass spectrometry. Anal Bioanal Chem 2008;391:1917–30.

[12] Wang Z, Senn T, Kalhorn T, Zheng XE, Zheng S, Davis CL, Hebert MF, Lin YS, Thummes KE. Simultaneous measurement of plasma vitamin D3 metabolites including 4 [beta], 25-dihydroxyvitamin D3 using liquid chromatography-tandem mass spectrometry. Anal Biochem 2011; 418:126–32.

[13] Duan X, Weinstock-Guttman B, Wang H, Bang E, Li J, Ramanathan M, Qu J. Ultrasensitive quantification of serum vitamin D metabolites using selective solid-phase extraction coupled to microflow liquid chromatography and isotope-dilution mass spectrometry. Anal Chem 2010;82:2488–97.

[14] Ding S, Schoenmakers I, Jones K, Koulman A, Prentice A, Volmer DA. Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS. Anal Bioanal Chem 2010;398:779–89.

[15] Baecher S, Leinenbach A, Wright JA, Pongratz S, Kobold U, Thiele R. Simultaneous quantification of four vitamin D metabolites in human serum using high performance liquid chromatography tandem mass spectrometry for vitamin D profiling. Clin Biochem 2012;45:1491–6.

[16] Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. Am J Clin Nutr 2008; 87:1080–6.

[17] Ovesen L, Brot C, Jakobsen J. Food contents and biological activity of 25-hydroxyvitamin D – a vitamin D metabolite to be reckoned with? Ann Nutr Metab 2003;47:107–113.

[18] The Pharmaceutical Codex. 11th edn. London: The Pharmaceutical Press; 1979. pp. 190–91.

[19] Mattila PH, Piironen VI, Uusi-Rauva EJ, Koivistoinen PE. Contents of cholecalciferol, ergocalciferol, and their 25-hydroxylated metabolites in milk products and raw meat and liver as determined by HPLC. J Agric Food Chem 1995; 43:2394–9.

[20] Shabahang M, Buras RR, Davoodi F, Schumaker LM, Nauta RJ, Evans SRT. 1, 25-Dihydroxyvitamin D3 receptor as a marker of human colon carcinoma cell line differentiation and growth inhibition. Cancer Res 1993;53:3712.

[21] NMKL-Procedure No 4. Validation of chemical analytical methods. Oslo: NMKL; 2009.

[22] Brügger B, Erben G, Sandhoff R, Wieland FT, Lehmann WD. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. Proc Natl Acad Sci USA 1997;94:2339–44.

[23] Schleicher RL, Encisco SE, Chaudhary-Webb M, Paliakov E, McCoy LF, Pfeiffer CM. Isotope dilution ultra performance liquid chromatography-tandem mass spectrometry method for simultaneous measurement of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 in human serum. Clin Chim Acta 2011;412:1594–9.

[24] van den Ouweland J, van Daal H. Fast separation of 25-hydroxyvitamin D3 from 3-Epi-25-hydroxyvitamin D3 in human serum by liquid chromatography–tandem mass spectrometry: variable prevalence of 3-Epi-25-hydroxyvitamin D3 in infants, children, and adults. Clin Chem 2011;57:1618–9.

[25] Bailey D, Veljkovic K, Yazdanpanah M, Adeli K. Analytical measurement and clinical relevance of vitamin D3 C3-epimer. Clin Biochem 2013;46:190–96.

Supplementary material available online

Supplementary Figures 1 and 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/00365513.2014.900694.

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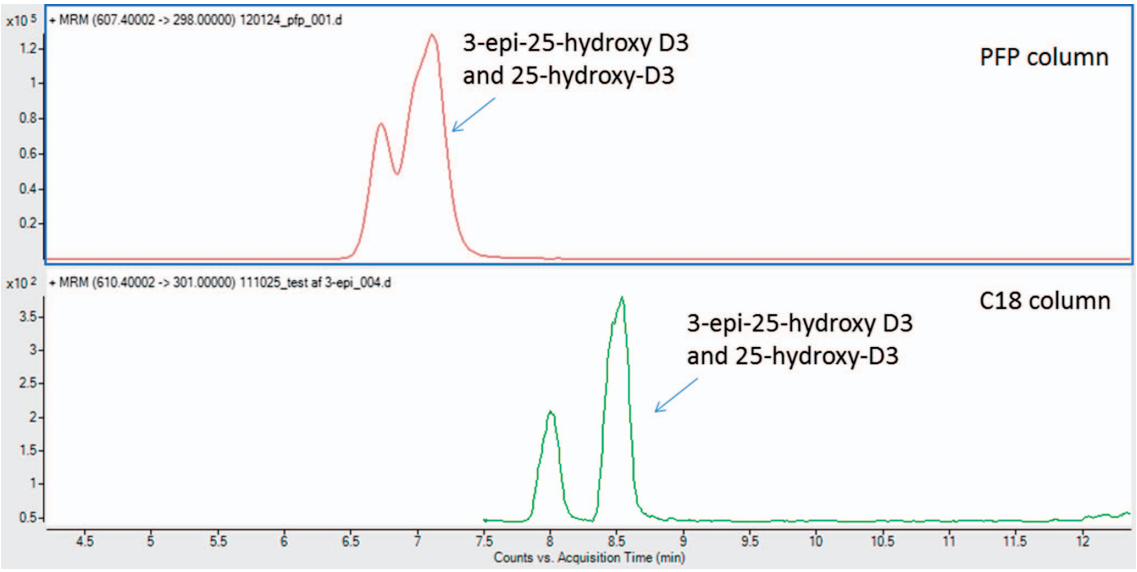
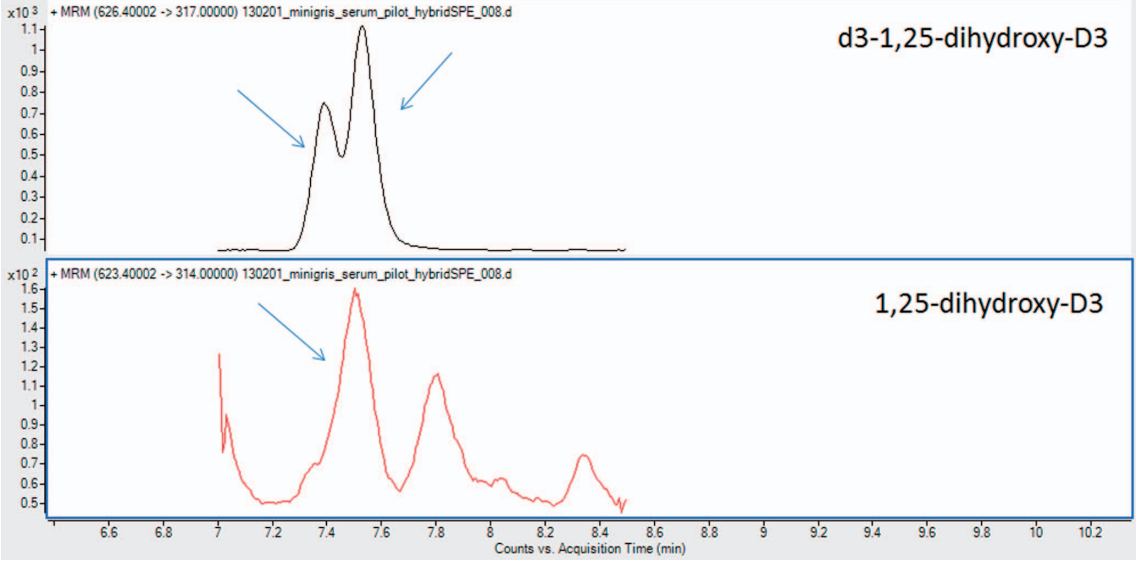
Supplementary material for Burild A, et al. Simultaneous quantification of vitamin D_3, 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 in human serum by LC-MS/MS. Scand J Clin Lab Invest 2014;74:418–23.



Supplementary Figure 1. Test of PFP and C18 analytical columns to separate 3-epi-25-hydroxy-D3 from 25-hydroxy-D3.



Supplementary Figure 2. Partial separation of 1,25-dihydroxy-D3, but no separation of the peak in human serum indicating coeluting interferences on top of the peak of 1,25-dihydroxy-D3.

Paper II

(Published in Journal of Separation Sciences)

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Research Article

Quantification of physiological levels of vitamin D_3 and 25-hydroxyvitamin D_3 in porcine fat and liver in subgram sample sizes†

Most methods for the quantification of physiological levels of vitamin D_3 and 25-hydroxyvitamin D_3 are developed for food analysis where the sample size is not usually a critical parameter. In contrast, in life science studies sample sizes are often limited. A very sensitive liquid chromatography with tandem mass spectrometry method was developed to quantify vitamin D_3 and 25-hydroxyvitamin D_3 simultaneously in porcine tissues. A sample of 0.2–1 g was saponified followed by liquid–liquid extraction and normal-phase solid-phase extraction. The analytes were derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione to improve the ionization efficiency by electrospray ionization. The method was validated in porcine liver and adipose tissue, and the accuracy was determined to be 72–97% for vitamin D_3 and 91–124% for 25-hydroxyvitamin D_3. The limit of quantification was <0.1 ng/g, and the precision varied between 1.4 and 16% depending on the level of spiking. The small sample size required for the described method enables quantification of vitamin D_3 and 25-hydroxyvitamin D_3 in tissues from studies where sample sizes are limited.

Keywords: Derivatization / Method validation / Tissues / Vitamin D_3
DOI 10.1002/jssc.201400548

1 Introduction

Vitamin D belongs to the group of fat-soluble vitamins and vitamin D is important for the calcium homeostasis in the body to prevent rickets and osteomalacia and to maintain a healthy skeleton throughout life. In addition, vitamin D is also associated with the functioning of other organs, and may play a role in supporting a healthy immune system and the prevention of cancer [1].

Vitamin D exists in two major forms, vitamin D_2 and vitamin D_3. Vitamin D_3 is formed in the skin after being exposed to sunlight [2] and vitamin D_3 is the predominant form found in animals [3]. Due to the low angle of the sun on high latitudes during winter time, vitamin D_3 is mainly synthesized during the summer [4]. Therefore, populations living on high latitudes have to rely on endogenous reserves of vitamin D_3 as well as dietary sources, including the supplementation of vitamin D during the winter.

It is known that vitamin D_3 accumulates in the body, after excessive doses of vitamin D_3 has been fed to rats [5]. The differences between the cutaneous vitamin D_3 synthesis and orally administered vitamin D_3 might impact on storage on vitamin D_3 in animals and humans. To our knowledge, this information is sparse, probably due to lack of sufficient sensitive quantitative methods.

Recently, several LC–MS/MS methods for the quantification of vitamin D_3 metabolites in serum and plasma have been published [6]. Derivatization of the vitamin D_3 with Cookson-type reagents improve the sensitivity of the molecule in the mass spectrometer equipped with an electrospray ionization interface [7, 8]. The use of the commercially available 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) for derivatization, is now widely used in LC–MS/MS methods to reduce the size of the sample required for the analysis and to include several analytes beyond the clinical biomarker 25-hydroxyvitamin D_3 (25(OH)D_3) [9–13].

Many existing methods capable of quantifying the total vitamin D_3 and 25(OH)D_3 in tissues at physiological levels have been developed for analyzing foodstuff, where an unlimited amount of samples usually will be available for the analysis. These methods are precise and accurate but require sample sizes between 7.5 and 50 g [14–16]. Recently, Lipkie et al. quantified vitamin D_3 and 25(OH)D_3 at physiological levels in rat tissue by the use of subgram sample sizes. The sample was homogenized and purified by LLE and SPE followed by PTAD derivatization prior to the analysis by LC–MS/MS [17].

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Abbreviations: **PTAD**, 4-phenyl-1,2,4-triazoline-3,5-dione; **25(OH)D_3**, 25-hydroxyvitamin D_3

†This paper is included in the virtual special issue sample preparation in mass spectrometry available at the Journal of Separation Science website.

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Vitamin D_3 is, to some extent, stored as esters [18, 19]. Potential esterified vitamin D_3 in the sample will, however, be liberated during the alkaline hydrolysis used in methods for the quantification of vitamin D_3 in animals and humans [14–16, 20].

The aim of this method was to quantify physiological levels of vitamin D_3 and 25(OH)D_3 by using LC–MS/MS in small tissue samples after alkaline saponification. For the validation, a liver matrix was used because it requires an extra cleanup step when using UV detection [21]. Adipose tissue was chosen because it in itself is the tissue matrix with the highest content of fat and therefore, the matrix most critical for the alkaline hydrolysis.

2 Materials and methods

2.1 Chemicals and reagents

Methanol, acetonitrile, 2-propanol, and heptane were of HPLC-grade (Rathburn Chemicals, Walkerburn, UK). Formic acid for MS, anhydrous acetonitrile, methylamine and PTAD as well as vitamin D_3 and 25(OH)D_3 were obtained from Sigma–Aldrich (Steinheim, Germany). Deuterated standards 26, 26, 26, 27, 27, 27-[D_6]vitamin D_3 ([D_6]vitamin D_3) and 26, 26, 26, 27, 27, 27-[D_6]25(OH)D_3 ([D_6]25(OH)D_3) were from Chemaphor (Ottawa, Canada).

For method development, adipose tissue was rendered porcine fat purchased from the local grocery store. The liver tissue was homogenized liver from one single pig supplemented with 25(OH)D_3 from a previous feeding trial. Water was of Milli-Q grade made in house (18.2 MΩ, Millipore, Billerica, MA).

2.2 Stock solutions and calibration curves

Stock solutions were prepared in ethanol and the concentrations were determined spectrophotometrically at 265 nm with $\varepsilon = 18{,}466\ m^2/mol$ for vitamin D_3 [22] and $\varepsilon = 18{,}584\ m^2/mol$ for 25(OH)D_3 [23].

Calibration standards were prepared from a mixed solution of PTAD-derivatized vitamin D_3 and 25(OH)D_3 in acetonitrile. Using serial dilution calibration standards containing 0.01, 0.025, 0.1, 1, 10, 25, 50, and 100 ng/mL of vitamin D_3 and 25(OH)D_3, as well as PTAD-derivatized vitamin [D_6]vitamin D_3 (8 ng/mL) and [D_6]25(OH)D_3 (8 ng/mL) were prepared. The calibration standards were evaporated under a gentle stream of nitrogen and reconstituted in 60% methanol. Calibration standards were included in all series of analysis. All standard curves were weighted over the concentrations ($1/x$).

2.3 Tissue

For the analyses of vitamin D_3 and 25(OH)D_3 in tissue, an extraction procedure was developed and validated. To 0.2–1 g of tissue, 25 μL of an internal standard solution (400 ng/mL [D_6]25(OH)D_3 and 400 ng/mL [D_6]vitamin D_3 in ethanol), 0.2 g sodium ascorbate, 3 mL KOH 60%, and 9 mL of ethanol were added. Atmospheric air was replaced by nitrogen and the sample was left for saponification overnight (16–18 h) at ambient temperature. Thirteen milliliters of water was added followed by extraction with 10 mL 20% ethyl acetate in *n*-heptane for 1 min. The organic phase was transferred to a clean tube and the water phase was reextracted twice. The pooled organic phases were washed with 20 mL of water. The organic phase was evaporated under reduced pressure at 35°C using a rotary evaporator and the sample was reconstituted in 5 mL of 1% 2-propanol in *n*-heptane. The sample was loaded on a 6 mL silica 500 mg SPE cartridge (Isolute, IST, Hengoed, UK) conditioned with 5 mL *n*-heptane. The cartridge was washed twice with 4 mL 0.5% 2-propanol in *n*-heptane and the analytes were eluted twice with 4 mL 6% 2-propanol in *n*-heptane followed by 4 mL 10% 2-propanol in *n*-heptane. The combined eluates were evaporated under a gentle stream of nitrogen and reconstituted in 1 mL 1% 2-propanol in *n*-heptane. Two hundred microliters of the solution was evaporated to dryness under a gentle stream of nitrogen. Two hundred and fifty microliters of 0.75 mg/mL PTAD in anhydrous acetonitrile was added and the sample was derivatized for 2 h at ambient temperature. The sample was dried under nitrogen and redissolved in 250 μL 60% methanol. After centrifugation at 10 000 × *g* for 10 min, the supernatant was transferred to an LC vial for analysis.

2.4 LC–MS/MS

The LC–MS/MS system used for quantification of vitamin D_3 and 25(OH)D_3 has been described in detail elsewhere [13].

Briefly, an Agilent 1200 series HPLC was coupled to an Agilent 6460 series triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) via a jet stream interface. Separations were performed on an Ascentis Express C_{18} (2.1 mm × 10 cm, 2.7 μm particles) column and an Ascentis Express C18 (2.1 × 5 mm, 2.7 μm particles) guard column from Supelco Analytical (Bellefonte, PA) thermostatted at 50°C. Mobile phase A consisted of Milli-Q water, methylamine (5 mM) and formic acid (0.1%) and mobile phase B was made of methanol, methylamine (5 mM) and formic acid (0.1%). The flow was 0.5 mL/min and the following gradient was used: 0–1 min, 60% B; 1–9 min, linear gradient to 72% B; 9–10 min, linear gradient to 75% B; 10–14 min, linear gradient to 100% B; 14–18 min, 100% B; 18–20 min, linear gradient to 60% B; 20–25 min, 60% B. The injection volume was 10 μL.

The mass spectrometer was operated in a positive multiple reaction monitoring (MRM) mode and the following transitions were used: m/z 591.4 → 298.0 and m/z 597.4 → 298.0 for vitamin D_3 and [D_6]vitamin D_3, respectively, m/z 607.4 → 298.0 for 25(OH)D_3 and m/z 613.4 → 298.0 for [D_6]25(OH)D_3.

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Table 1. Precision and accuracy of vitamin D_3 and 25(OH)D_3 in liver. The analyses were done on three different days in triplicate

	Matrix	Endogenous level (ng/g)	Spiked level (ng/g)	Within day precision (%)	Between day precision (%)	Accuracy
Vitamin D_3	Liver	0.12	0	16	14	n.r.
		0.12	2.5	9.2	8.8	82.2
		0.12	25	2.9	2.9	89.8
25(OH)D_3	Liver	3.38	0	1.4	8.9	n.r.
		3.38	10	6.9	13	115
		3.38	100	8.3	11	124

n.r., not relevant.

Table 2. Precision and accuracy of vitamin D_3 and 25(OH)D_3 in porcine adipose tissue. The analyses were done on three different days in triplicate

	Matrix	Endogenous level (ng/g)	Spiked level (ng/g)	Within day precision (%)	Between day precision (%)	Accuracy
Vitamin D_3	Adipose tissue	5.87	0	8.4	4.8	n.r.
		5.87	10	6.2	6.4	83.6
		5.87	100	2.8	2.7	96.6
25(OH)D_3	Adipose tissue	2.15	0	5.2	4.3	n.r.
		2.15	2.5	17.1	15.1	114
		2.15	25	5.1	8.6	113

n.r., not relevant.

2.4.1 Quality assurance for the quantification of vitamin D_3 and 25(OH)D_3 in tissues

Precision and accuracy of the method was determined in both porcine liver and adipose tissue. One gram of porcine liver and 1 g of adipose tissue containing endogenous levels of vitamin D_3 and 25(OH)D_3 were each spiked at two different levels of vitamin D_3 and 25(OH)D_3 (Tables 1 and 2). Non-spiked liver and adipose tissue samples were included in the validation to determine the endogenous level of vitamin D_3 metabolites. The experiment was repeated on three different days in triplicate.

To test if sample size influences the accuracy, 0.2, 0.5, and 1 g of porcine adipose tissues were spiked at 20 and 100 ng/g with vitamin D_3 and at 5 ng/g and 25 ng/g for 25(OH)D_3. The experiment was done in triplicate.

The LOQ was calculated from the lowest calibration point with S/N > 10 corrected for sample dilution, loss of analytes during extraction, and ion suppression. Loss of analytes during extraction and ion suppression of the analyte were determined from areas of the peaks from the internal standards spiked to samples. These areas were compared to the areas of the peaks from internal standards spiked to solvent. The accuracy was calculated by using the results from the spiked samples corrected for the endogenous levels of vitamin D_3 analytes.

2.5 Statistical analysis

Precision was calculated according to the model of variance [24]. A two-tailed *t*-test assuming unequal variances was used to compare the performance of the LC–MS/MS method to our existing LC–UV method [16]. Excel (version 2007, Microsoft, Redmond, WA) was used for the statistical analysis.

3 Results

25(OH)D_3 and vitamin D_3 eluted after 10 and 14 min, respectively. Two peaks were observed for 25(OH)D_3, which are the epimers 6*S* and 6*R* of 25(OH)D_3 formed after reacting with the PTAD [9]. The largest peak was used for quantification (Fig. 1). The regression coefficients for 25(OH)D_3 and vitamin D_3 were >0.999. The accuracy was determined on four spiked levels in porcine liver and adipose tissue and was calculated to be 82–97% for vitamin D_3 and 113–124% for 25(OH)D_3 (Tables 1 and 2). The precision varied between 1.4 and 16% depending on the level of spiking. The precision for vitamin D_3 was <10% except for the native level in the liver. This is probably due to the low concentration (0.12 ng/g) close to the LOQ, which was <0.1 ng/g. For 25(OH)D_3, the precision was ≤ 13% except at the 2.5 ng/g level in adipose tissues where the precision was 15 and 17%, respectively (Tables 1 and 2). The accuracy ranged from 72 to 84% for vitamin D_3 and 91 to 114% for 25(OH)D_3 using 0.2 to 1 g of sample size (Table 3). The vitamin D_3 and 25(OH)D_3 levels in a house reference of porcine fat were determined ($n = 21$) to be 5.62 ng/g (±0.47) and 2.26 ng/g (±0.22), respectively. The levels were similar ($p > 0.05$) to the levels assessed by our existing LC–DAD/UV method [16] accredited according to ISO17025 [25] where the

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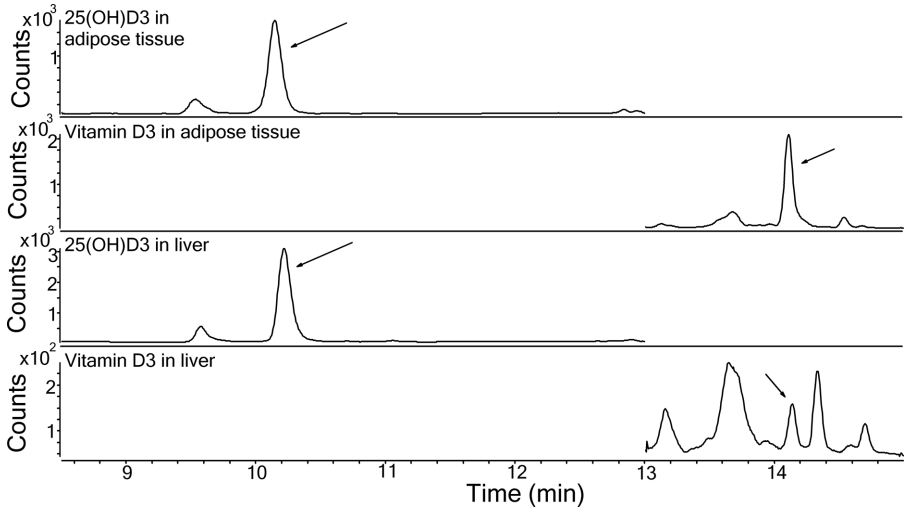

Figure 1. Chromatograms of vitamin D_3 and 25(OH)D_3 in porcine adipose and liver tissue at physiological levels. Peaks used for the quantification are indicated by arrows.

Table 3. The impact of sample size on accuracy and precision. Adipose tissue was spiked with vitamin D3 and 25(OH)D3 to 0.2–1 g of sample. The analysis was done in triplicate

	Matrix	Endogenous level (ng/g)	Sample size (g)	Spiked level (ng/g)	Accuracy	Precision (%)
Vitamin D_3	Adipose tissue	5.32	1	100	82.6	9.7
		5.32	0.5	100	84.8	3.4
		5.32	0.2	100	82.6	7.1
		5.32	1	20	83.7	1.9
		5.32	0.5	20	81.3	4.7
		5.32	0.2	20	72.4	2.3
25(OH)D_3	Adipose tissue	2.34	1	25	99.8	2.0
		2.34	0.5	25	98.9	4.1
		2.34	0.2	25	92.4	5.1
		2.34	1	5	114	8.8
		2.34	0.5	5	100	5.6
		2.34	0.2	5	91.7	6.3

levels were determined to be 5.70 ng/g (±0.39) for vitamin D_3 and 2.28 ng/g (±0.15) for 25(OH)D_3 ($n = 9$).

4 Discussion

To determine the concentration of vitamin D_3 and 25(OH)D_3 in different tissues, we developed and validated an LC–MS/MS method for the quantification of vitamin D_3 and 25(OH)D_3 in porcine tissues.

Vitamin D is, to some extent, stored as esters [18, 19]. To measure the total amount of vitamin D_3, the sample was subjected to alkaline hydrolysis to liberate the potential esterified vitamin D metabolites. Additionally, the saponification also enables the exclusion of fat content by the subsequent LLE. The conditions for the saponification, the LLE and the normal phase SPE were adopted and downscaled from our existing method [16], which reduced the amount of organic solvent from >600 to 70 mL during sample preparation The analytes were derivatized with PTAD to improve ionization in the mass spectrometer. The method is intended for use on many different matrices. Therefore, only one fifth of the sample is derivatized after the SPE purification, which leaves enough sample to perform a preparative HPLC for further sample purification if needed.

The validation confirmed that the method was capable of quantifying vitamin D_3 and 25(OH)D_3 in porcine liver and adipose tissue with high robustness. The method was compared to our existing method [16] and the results for vitamin D_3 and 25(OH)D_3 were not significantly different. The method meets the AOAC method validation criteria for precision and recovery, except for the recovery for 25(OH)D_3 in liver at 100 ng/g [26]. The LOQ for vitamin D_3 and 25(OH)D_3 of <0.1 ng/g is below the content of 0.5 ng/g of vitamin D_3 found naturally in lean meat from pigs, [16] and below human values in fat of 45.3 ng/g (±22.2) [27] and 39.49 ng/g (±16.13) [20].

The method requires a sample size of 0.2–1 g of tissue for the quantification of physiological levels of vitamin D_3 and 25(OH)D_3. In fact, the amount of required sample can easily be reduced by 4/5 without compromising the LOQ if the

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entire sample is used for the derivatization. However, using small amounts of sample make representative sampling and homogeneity of the sample very critical.

The small required sample size for the described method enables a quantification of vitamin D_3 and 25(OH)D_3 in studies where sample sizes are limited. We are currently monitoring the changes in vitamin D_3 and 25(OH)D_3 over time in skin and subcutaneous fat repeatedly sampled by punch biopsies from pigs exposed to UV light.

5 Concluding remarks

We developed an LC–MS/MS method for the quantification of physiologically relevant levels of vitamin D_3 and 25(OH)D_3 using only 0.2–1 g of porcine tissues with a LOQ <0.1 ng/g. The amount of organic solvent required is reduced nearly tenfold compared to conventional LC–UV methods. The sensitivity and the small sample size needed enables the study of vitamin D_3 and 25(OH)D_3 in samples obtained from living animals.

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The authors have declared no conflict of interest.

6 References

[1] DeLuca, H. F., *Am. J. Clin. Nutr.* 2004, *80*, 1689–1996.

[2] Holick, M. F., Chen, T. C., *Am. J. Clin. Nutr.* 2008, *87*, 1080–1086.

[3] Ovesen, L., Brot, C., Jakobsen, J., *Ann. Nutr. Metab.* 2003, *47*, 107–113.

[4] Webb, A., DeCosta, B., Holick, M., *J. Clin. Endocrinol. Metab.* 1989, *68*, 882–887.

[5] Brouwer, D. A. J., Beek, J. V., Ferwerda, H., Brugman, A. M., Klis, F. R. M., Heiden, H. J., Muskiet, F. A. J., *Br. J. Nutr.* 1998, *79*, 527–532.

[6] van den Ouweland, J. M. W., Vogeser, M., Bächer, S., *Rev. Endocrinol. Metab. Disord.* 2013, *14*, 1–26.

[7] Wilson, S. R., Wu, Y., *J. Am. Soc. Mass Spectrom.* 1993, *4*, 596–603.

[8] Higashi, T., Awada, D., Shimada, K., *Biomed. Chromatogr.* 2001, *15*, 133–140.

[9] Aronov, P. A., Hall, L. M., Dettmer, K., Stephensen, C. B., Hammock, B. D., *Anal. Bioanal Chem.* 2008, *391*, 1917–1930.

[10] Wang, Z., Senn, T., Kalhorn, T., Zheng, X. E., Zheng, S., Davis, C. L., Hebert, M. F., Lin, Y. S., Thummel, K. E., *Anal. Biochem.* 2011, *418*, 126–132.

[11] Ding, S., Schoenmakers, I., Jones, K.; Koulman, A., Prentice, A., Volmer, D. A., *Anal. Bioanal Chem.* 2010, *398*, 779–789.

[12] Eyles, D., Anderson, C., Ko, P., Jones, A., Thomas, A., Burne, T., Mortensen, P. B., Nørgaard-Pedersen, B., Hougaard, D. M., McGrath, J., *Clin. Chim. Acta.* 2009, *403*, 145–151.

[13] Burild, A., Frandsen, H. L., Jakobsen, J., *Scand., J. Clin. Lab. Invest.* 2014, 1–6 (early online).

[14] Strobel, N., Buddhadasa, S., Adorno, P., Stockham, K., Greenfield, H., *Food Chem.* 2013, *138*, 1042–1047.

[15] Bilodeau, L., Dufresne, G., Deeks, J., Clément, G., Bertrand, J., Turcotte, S., Robichaud, A., Beraldin, F., Fouquet, A., *J. Food Compost. Anal.* 2011, *24*, 441–448.

[16] Jakobsen, J., Clausen, I., Leth, T., Ovesen, L., *J. Food Compost. Anal.* 2004, *17*, 777–787.

[17] Lipkie, T. E., Janasch, A., Cooper, B. R., Hohman, E. E., Weaver, C. M., Ferruzzi, M. G., *J. Chromatogr. B* 2013, *932*, 6–11.

[18] Fraser, D., Kodicek, E., *Biochem. J.* 1968, *106*, 485–490.

[19] Rosenstreich, S. J., Rich, C., Volwiler, W., *J. Clin. Invest.* 1971, *50*, 679.

[20] Blum, M., Dolnikowski, G., Seyoum, E., Harris, S. S., Booth, S. L., Peterson, J., Saltzman, E., Dawson-Hughes, B., *Endocrine* 2008, *33*, 90–94.

[21] Jakobsen, J., Maribo, H., Bysted, A., Sommer, H. M., Hels, O., *Br. J. Nutr.* 2007, *98*, 908–913.

[22] *The Pharmacetical Codex*, 11th ed., The Pharmaceutical Press, London 1979, p. 190.

[23] Mattila, P. H., Piironen, V. I., Uusi-Rauva, E. J., Koivistoinen, P. E., *J. Agric. Food Chem.* 1995, *43*, 2394–2399.

[24] NMKL, Validation of chemical analytical methods. In NMKL-Procedure No. 4 NMKL, Oslo 2009.

[25] ISO, ISO Standard 17025, ISO 2013.

[26] AOAC Official Methods of Analysis, Appendix F: Guidelines for Standard Method Performance Requirements, AOAC International Standard 2012.

[27] Lawson, D., Douglas, J., Lean, M., Sedrani, S., *Clin. Chim Acta.* 1986, *157*, 175–181.

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Paper III

(Submitted to British Journal of Nutrition)

Tissue content of vitamin D_3 and 25-hydroxyvitamin D_3 and the relation to serum 25-hydroxyvitamin D_3 in pigs supplemented with two forms of vitamin D_3 at four different levels

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Running title: Distribution of vitamin $D_3$and 25(OH)D_3

Abbreviations: 25-hydroxyvitamin D_3 (25(OH)D_3)

Key words: vitamin D3, 25-hydroxy vitamin D_3, bioavailability

Abstract

Both vitamin D_3 and 25-hydroxyvitamin D_3 are sources to dietary vitamin D_3 but the bioavailability assessed by serum 25-hydroxyvitamin D_3 concentration is reported to be different. The relationship of serum 25-hydroxyvitamin D_3 to tissue content of vitamin D_3 and 25-hydroxyvitamin D_3 is, however, unknown. The objective of the study was to investigate the tissue distribution after oral vitamin D_3 and 25-hydroxyvitamin D_3 intake in a pig model, and to investigate the association of the tissue content to serum 25-hydroxyvitamin D_3. Adipose tissue, white and red muscle, liver and serum was sampled from pigs of a larger animal study in which dietary treatments containing four concentrations of either vitamin D_3 or 25-hydroxyvitamin D_3 at 5, 20, 35, and 50 µg/kg feed provided for seven weeks. Samples were analyzed for the concentration of vitamin D_3 and 25-hydroxyvitamin D_3. Tissue 25-hydroxyvitamin D_3 was significantly higher in pigs fed 25-hydroxyvitamin D_3 compared to vitamin D_3, but vitamin D_3 in tissue was higher in the pigs fed vitamin D_3. The content of 25-hydroxyvitamin D_3 in the different tissues fully correlated with the serum 25-hydroxyvitamin D_3 level, whereas the correlation between tissue content of vitamin D_3 and serum 25-hydroxyvitamin D_3 was dependent on the source of the ingested vitamin D_3.

Introduction

Vitamin D is belonging to the group of lipophilic vitamins and vitamin D accumulates in the rat after supraphysiological doses of vitamin D [1] whereas only low concentrations of vitamin D are found in pigs fed physiological relevant doses of vitamin D [2]. Serum/plasma 25-hydroxyvitamin D (25(OH)D) is considered the best biomarker of vitamin D status [3], but its correlation to the tissue concentration of vitamin D is unknown [4].

Vitamin D exists in two major forms, vitamin D_2 and vitamin D_3. Vitamin D_3 is synthesized in the skin after UV exposure [5] and is naturally found as vitamin D_3 and 25-hydroxyvitamin D_3 ($25(OH)D_3$) in products of animal origin e.g. meat and eggs [6, 7]. Within the European Union vitamin D_3 is the main source for animal feeding, but recently, $25(OH)D_3$ has been approved for supplementary use in poultry and pig nutrition [8].

The potency between the two vitamin D_3 sources assessed by serum/plasma $25(OH)D_3$ is generally found to be higher for oral $25(OH)D_3$ compared to oral vitamin D_3, although data are inconsistent. In humans the factor has been found from two up to five [9, 10] and in pigs the factor is assessed from one up to three [2, 11-13].

To our knowledge the difference in the distribution of oral $25(OH)D_3$ and oral vitamin D_3 is sparse. Since $25(OH)D_3$ is more polar than vitamin D_3, and the affinity of $25(OH)D_3$ for the vitamin D binding protein is more than 500 times stronger than that of D_3, more D_3 is assumed to be on its free form, which will allow it to diffuse directly into adjacent tissues. These inherent differences between $25(OH)D_3$ and vitamin D_3 might be important for the distribution and storage of the different forms of vitamin D_3 [14].

The purpose of this study was to investigate the influence of increasing dietary levels of vitamin D_3 in the form of vitamin D_3 and $25(OH)D_3$ in the feed on the distribution and the tissue content of vitamin D_3 and $25(OH)D_3$ and to assess the association of serum $25(OH)D_3$ to tissue concentration of vitamin D_3 and $25(OH)D_3$.

Experimental methods

Animal experiment

A subpopulation of 32 pigs was selected from experiment 1 of a large animal trial as described in detail by Lauridsen *et al.* [11]. Briefly, female pigs were randomly assigned from the first estrus until d 28 of gestation to dietary treatments containing four different concentrations of either vitamin D_3 or 25(OH)D_3 at 5, 20, 35, and 50 µg/kg feed. In total, the pigs were provided the dietary treatments (4 pigs per diet, unless otherwise stated) for 7 weeks where after the pigs were terminated by exsanguination.

Sampling

Blood samples obtained from vena jugularis were collected in vacutainer tubes containing no additives and processed to serum, which was immediately stored at -80°C until analysis. After the carcasses were eviscerated, samples of the liver, longisimus dorsi (loin), and of the psoas major (red muscle tissue) were obtained. Adipose tissue and muscle tissue (white muscle tissue) were carefully dissected from the loin. All samples were stored in plastic bags at -20ºC until analysis. Before analysis each sample were slowly thawed and homogenized for 2 min (1094 Homogenizer, Tecator, Paris).

Analysis of 25(OH)D_3 and vitamin D_3 in tissue and 25(OH)D_3 in serum and tissues

The tissue samples were analyzed by a previous published method using HPLC [2]. In short, the internal standard of vitamin D_2 and 25(OH)D_2 were added to the test sample. The samples were saponified, liquid/liquid extracted, cleaned-up in a solid-phase step, followed by a preparative normal phase HPLC-steps. For the final separation, detection and quantification reversed phase chromatography coupled to UV- and DAD-detector was used. The analysis were performed for samples with content of vitamin D_3 and 25(OH)D_3 above 1 µg/kg with a precision at 5·6% and 5·1%, respectively. For samples with contents below 1 µg/kg the precision was 0·06µg/kg for both compounds.

Serum was analyzed for 25(OH)D_3 by HPLC equipped with DAD and UV detector for detection and quantification as described in details elsewhere [15].

Analysis of fat content in muscle tissue

The content of fat in the muscle tissue was determined by the gravimetric method by a modified Schmid-Bondzynski-Ratslaff (SBR) [16]. For the red muscle tissues fat content was only determined on samples from pigs receiving 20 and 35 µg/kg feed. In short, the sample was boiled with

hydrochloric acid followed by the addition of ethanol and extraction of the lipids with diethyl ether:petroleum ether (1:1). After evaporation of the solvent, the fat was weighed.

Data analysis

The effect of vitamin D_3 form (vitamin D_3, 25(OH)D_3) and level in feed (5, 20, 35, 50 µg/kg) on content of 25(OH)D_3 and vitamin D_3 in tissues were analyzed by the regression model:

$$y_{ijk} = \beta_{0,i} + \beta_1 form_{ij} + \beta_2 feeding\ level_{ik} + \beta_3 (feeding\ level * form)_{ijk} \quad \textbf{Eq.1}$$

Where "*y*" is the response variable for eight measurements (*i*=1,2,…,8) of vitamin D i.e. vitamin D_3 or 25(OH)D_3 in adipose tissue, white muscle tissue, red muscle tissue, and liver, . β_0 is referred to as the intercept, β_1 is a categorical parameter, and β_2 and β_3 are regressor parameters.

For each response variable (*i*) β_0 and β_1 represent the cut offs and β_2 and β_3 represent the slopes of the regression lines for feeding level of vitamin D_3 and 25(OH)D_3, respectively. The two regression lines for each response variable were analyzed simultaneously. This way not only the power of the test was increased by increased degree of freedom but the simultaneously estimation also served the purpose of being able to determine whether the two cut offs were significantly different from each other (β_1 significant), and to determine whether the slopes were significant different from each other (β_3 significant).

The association between serum 25(OH)D_3, vitamin D_3 form and vitamin D_3 and 25(OH)D_3 in tissues were investigated by a similar type of model as shown above (Eq. 1) except that the explanatory variable "*feeding level*" was replaced by "*serum*".

In the data set one outlier was detected using the methods described by [17]. Results in the tables and figures are given as mean ± SEM. All data was analyzed by using proc glm, SAS version 9.3 (SAS Institute, Cary, NC,) and a significant level of α=5% was used as cut off value for the p-values. For plotting the program Prism 5 for Windows (GraphPad Software, San Diego, CA) was used.

Results

All results (mean± SD) of the content of vitamin D_3 and 25(OH)D_3 in meat cuts i.e. adipose tissue, white muscle tissue, red muscle tissue, liver, and serum 25(OH)D_3 are shown in Table 1 and 2, Overall, the content of 25(OH)D_3 in serum was between 8·7 and 67·1 ng/mL. In the tissues the content of 25(OH)D_3 was between 0·37 and 5·96 µg/kg, while the content of vitamin D_3 were between 0·10 and 8·41 µg/kg.

Effect of dose of vitamin D_3 and 25(OH)D_3 *on tissue content of vitamin D_3 and 25(OH)D_3*

Increasing doses of either vitamin D_3 or 25(OH)D_3 in feed increased the content of 25(OH)D_3 and vitamin D_3 in all tissues ($\beta_{2:}$ $p< 0·001$). The vitamin D_3 content in all analyzed tissues was significantly ($\beta_{3:}$ $p< 0·001$) higher for pigs fed vitamin D_3, whereas the tissue content of 25(OH)D_3 was significantly higher ($\beta_{3:}$ $p<0·002$) in all tissues when 25(OH)D_3 was provided the feed. The parameter β_1 was not significant for any of the analyses meaning that for dose 0 the content of vitamin D_3 and 25(OH)D_3 in all tissues was the same for the two regression lines (Figure 1). The baseline values for the two groups are thus the same. The highest content of vitamin D_3 was found in liver followed by the adipose tissue, red muscle tissue and white muscle tissue. The content of fat in red muscle tissue and in white muscle tissues was 3·7±1·2% (n=32) and 2·1±0·7% (n=16), respectively.

Correlation of tissue vitamin D_3 and 25(OH)D_3 with serum 25(OH)D_3

As shown in Figure 2A and 2C, the content of vitamin D_3 in adipose and white muscle tissues was linearly correlated with serum 25(OH)D_3 (β_2: $p<0·001$). Furthermore, the concentration was dependent on the dietary vitamin D_3 form, as the interaction term was significant ($\beta_{3:}$ $p<0·001$).

The content of 25(OH)D_3 in adipose and white muscle tissues (Figure 2B and 2D) was also linearly correlated with serum 25(OH)D_3 ($\beta_{2:}$ $p<0·001$). The correlation coefficient was, however, independent of the dietary vitamin D_3 form ($\beta_{3:}$ $p>0·72$).

Discussion

The pigs were fed between 5 and 50µg/kg feed where 50µg/kg is the maximum allowed content of vitamin D in feed for slaughter pigs [18].

The content of vitamin D_3 and 25(OH)D_3 as well as the serum 25(OH)D_3 in slaughter pigs fed different doses of vitamin D_3 and 25(OH)D_3 was analyzed. Adipose tissue, liver and different muscle tissues were chosen for the analysis to represent the major tissues in the pig. The tissue content of 25(OH)D_3 was significantly higher in the pigs supplemented with 25(OH)D_3 compared to the provision of vitamin D_3 at all dietary concentrations. On the other hand, significantly higher content of vitamin D_3 was found in all tissues originating from pigs fed vitamin D_3. This result is in consistency with our previous study in slaughter pigs [(2)] fed a single level of each vitamin D feeding source.

We expect the higher content of vitamin D_3 and 25(OH)D_3 in red muscle tissue compared to white muscle tissue to be due to the higher fat content in red muscle tissue, though in this study we were not able to verify this because of the limited amount of samples of red muscle tissue. However, vitamin D_3 is a lipophilic vitamin and it was previously shown that the tissue content of vitamin D_3 depends on the fat content of the given tissue [(2, 19)].

The study demonstrated that the adipose and white muscle tissue content of 25(OH)D_3 could be predicted from serum 25(OH)D_3 independently of the ingested form of vitamin D_3. The content of vitamin D_3 in these tissues was also related to the serum 25(OH)D_3, but the correlation was depending on the dietary source of vitamin D_3.

Data on vitamin D in human tissues is available mainly from *post mortem* sampling or from fat removal [(20-22)]. Extrapolation from pig data has previously been done to estimate the size of the storage of vitamin D in humans [(23)]. Due to the resemblance in body composition and digestion abilities between pigs and humans we believe that our findings would be transferable to humans. When neglecting the contribution of endogenous synthesis of vitamin D_3 after UV exposure in humans our results indicate that the tissue content of 25(OH)D_3 is reflected by the serum 25(OH)D_3. The serum 25(OH)D_3 will, however, be a poor biomarker for tissue vitamin D_3 if the dietary vitamin D source contains both vitamin D_3 and 25(OH)D_3, which is the case for many food items contribution with vitamin D in a human diet [(24)]. We also demonstrated that the body concentration and distribution of vitamin D_3 metabolites are depending on the ingested form of vitamin D_3. The importance for the accessibility and eventually the biological effect of vitamin D_3 in target organs is unknown.

An issue which seems relevant to elucidate in future research since our study also showed that introduction of 25(OH)D_3 as a vitamin D source in the production of pigs will provide the consumer pork with higher content of 25(OH)D_3 at the expense of lower content of vitamin D_3.

Conflict of interest

None

Acknowledgements

Charlotte Lauridsen was responsible for the design of the pig trial and performed all sample collection. Jette Jakobsen was responsible for the design of this nutritional related subproject and performed the chemical analyses. Anders Burild, Jette Jakobsen, Nasrin Faqir and Helle M. Sommer designed the statistical test, and Nasrin Faqir performed the statistical analysis. Anders Burild wrote the draft paper. All authors approved the final version to be published.

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Legends

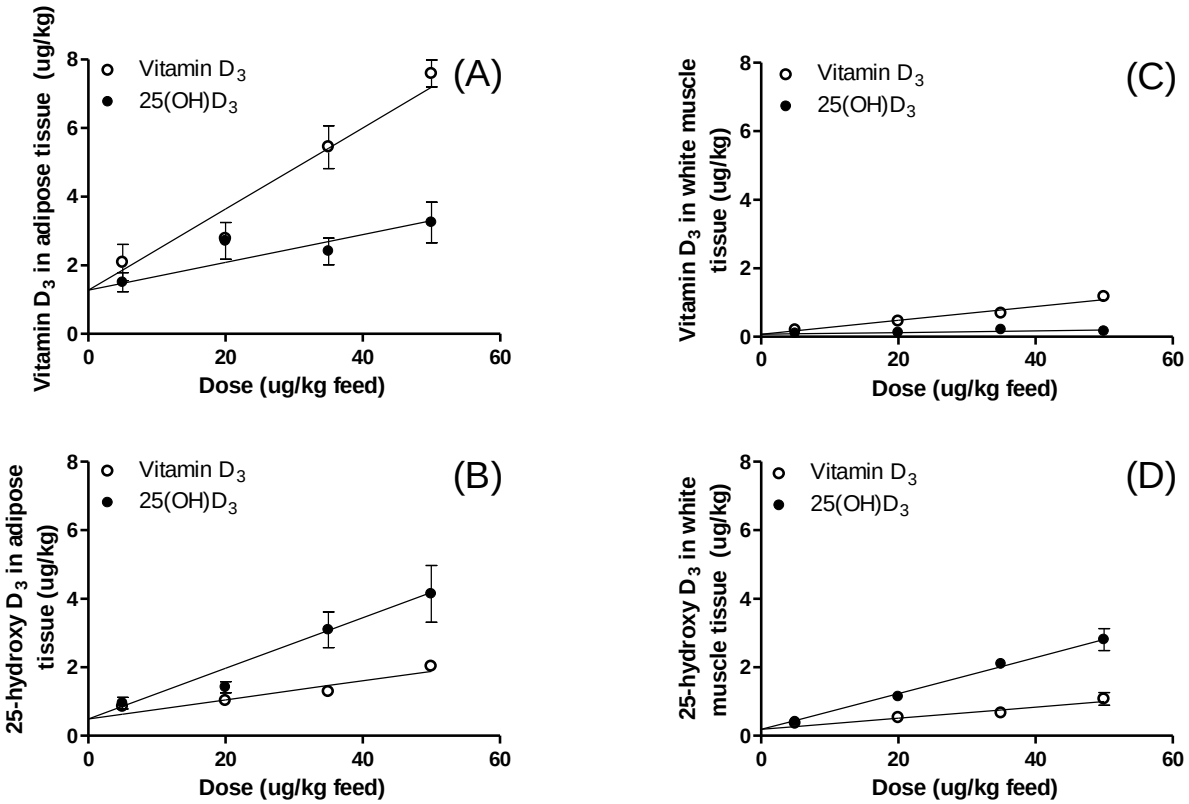
Figure 1 Vitamin D_3 (A and B) and 25(OH)D_3 (C and D) in adipose tissue and white muscle tissue plotted against content of vitamin D_3 or 25(OH)D_3 in feed.

Figure 2 Serum 25(OH)D_3 plotted against content of vitamin D_3 in adipose and muscle tissues (A and B) and against content of 25(OH)D_3 in adipose and muscle tissues (C and D) for pigs fed either vitamin D_3 or 25(OH)D_3.

Table 1 Content of vitamin D_3 and 25(OH)D_3 in adipose tissue, (subcutaneous fat from loin), white muscle tissue (lean meat from loin), red muscle tissue (chain muscle), and in the liver following feeding for 49 days with 5, 20, 35 and 50 µg pr. kg feed of vitamin D_3. Four animal in each group except five animals were fed 50 µg/kg.

Table 2 Content of vitamin D_3 and 25(OH)D_3 in adipose tissue, (subcutaneous fat from loin), white muscle tissue (lean meat from loin), red muscle tissue (chain muscle), and in the liver following feeding for 49 days with 5, 20, 35 and 50 µg pr. kg feed of 25(OH)D_3. Four animal in each group except three animals were fed 35 µg/kg.

204 **Figure 1**



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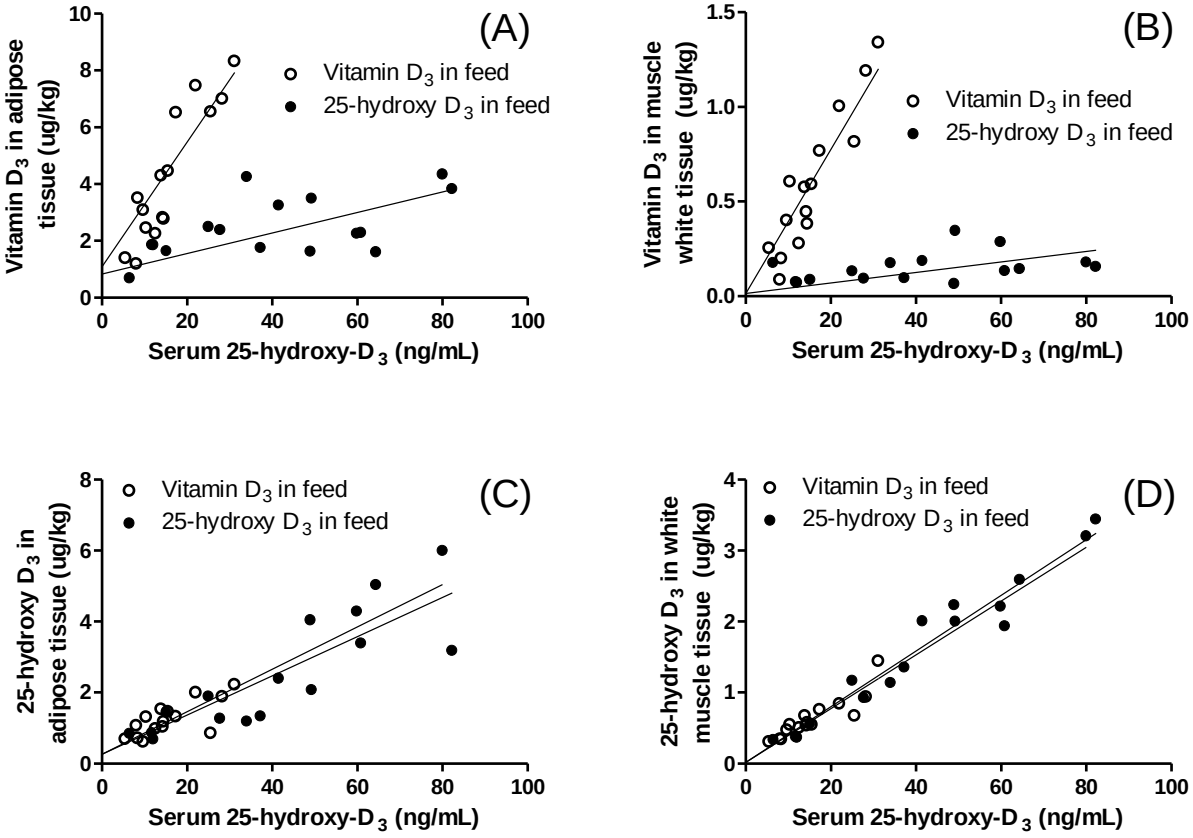
206 **Figure 2**



207

208 **Table 1**

Vitamin D form in feed	Vitamin D_3							
µg/kg feed	5		20		35		50	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Serum								
- 25(OH)D_3 (ng/mL)	8·7	0·75	12·2	0·75	21·7	0·65	27·2	0·98
Adipose tissue								
- vitamin D_3 (µg/kg)	2·08	0·27	2·78	0·07	5·45	0·31	7·59	0·25
- 25(OH)D_3 (µg/kg)	0·86	0·05	1·03	0·08	1·29	0·075	2·03	0·20
White muscle tissue								
- vitamin D_3 (µg/kg)	0·20	0·02	0·46	0·03	0·69	0·03	1·18	0·06
- 25(OH)D_3 (µg/kg)	0·37	0·02	0·53	0·01	0·66	0·02	1·07	0·18
Red muscle tissue								
- vitamin D_3 (µg/kg)	0·49	0·08	n.a.		n.a.		2·50	0·15
- 25(OH)D_3 (µg/kg)	0·54	0·09	n.a.		n.a.		1·81	0·14
Liver								
- vitamin D_3 (µg/kg)	0·68	0·04	n.a.		n.a.		8·41	0·79
- 25(OH)D_3 (µg/kg)	1·33	0·03	n.a.		n.a.		4·52	0·21

209 **Table 2**

Vitamin D form in feed	$25(OH)D_3$							
µg/kg feed	5		20		35		50	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Serum								
- $25(OH)D_3$ (ng/mL)	11·4	0·90	31·1	1·40	49·2	2·73	67·1	4·70
Adipose tissue								
- vitamin D_3 (µg/kg)	1·51	0·14	2·71	0·27	3·37	0·45	3·25	0·30
- $25(OH)D_3$ (µg/kg)	0·95	0·09	1·42	0·08	3·09	0·23	4·15	0·42
White muscle tissue								
- vitamin D_3 (µg/kg)	0·10	0·02	0·12	0·05	0·46	0·11	0·17	0·01
- $25(OH)D_3$ (µg/kg)	0·41	0·03	1·14	0·05	1·85	0·11	2·81	0·16
Red muscle tissue								
- vitamin D_3 (µg/kg)	0·34	0·02	n.a.		n.a.		0·45	0·04
- $25(OH)D_3$ (µg/kg)	0·67	0·04	n.a.		n.a.		4·02	0·34
Liver								
- vitamin D_3 (µg/kg)	0·64	0·22	n.a.		n.a.		0·20	0·04
- $25(OH)D_3$ (µg/kg)	2·74	0·16	n.a.		n.a.		5·96	0·98

References

1. Brouwer DAJ, Beek JV, Ferwerda H *et al.* (1998) Rat adipose tissue rapidly accumulates and slowly releases an orally-administered high vitamin D dose. *Br J Nutr* **79**(06), 527-532.

2. Jakobsen J, Maribo H, Bysted A *et al.* (2007) 25-Hydroxyvitamin D3 affects vitamin D status similar to vitamin D3 in pigs–but the meat produced has a lower content of vitamin D. *Br J Nutr* **98**(05), 908-913.

3. Jones G (2012) Metabolism and biomarkers of Vitamin D. *Scand J Clin Lab Invest* **72**(S243), 7-13.

4. Heaney RP, Armas LAG, Shary JR *et al.* (2008) 25-Hydroxylation of vitamin D3: relation to circulating vitamin D3 under various input conditions. *Am J Clin Nutr* **87**(6), 1738.

5. Holick MF & Chen TC (2008) Vitamin D deficiency: a worldwide problem with health consequences. *Am J Clin Nutr* **87**(4), 1080S.

6. Mattila PH, Piironen VI, Uusi-Rauva EJ *et al.* (1995) Contents of cholecalciferol, ergocalciferol, and their 25-hydroxylated metabolites in milk products and raw meat and liver as determined by HPLC. *J Agric Food Chem* **43**(9), 2394-2399.

7. Mattila P, Lehikoinen K, Kiiskinen T *et al.* (1999) Cholecalciferol and 25-hydroxycholecalciferol content of chicken egg yolk as affected by the cholecalciferol content of feed. *J Agric Food Chem* **47**(10), 4089-4092.

8. EUR-Lex (2009) *Commission Regulation (EC) No 887/2009*; Available at http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:254:0068:0070:EN:PDF

9. Cashman KD, Seamans KM, Lucey AJ *et al.* (2012) Relative effectiveness of oral 25-hydroxyvitamin D3 and vitamin D3 in raising wintertime serum 25-hydroxyvitamin D in older adults. *Am J Clin Nutr* **95**(6), 1350-1356.

10. Jetter A, Egli A, Dawson-Hughes B *et al.* (2014) Pharmacokinetics of oral vitamin D_3 and calcifediol. *Bone* **59**, 14-19.

11. Lauridsen C, Halekoh U, Larsen T *et al.* (2010) Reproductive performance and bone status markers of gilts and lactating sows supplemented with two different forms of vitamin D. *J Anim Sci* **88**(1), 202-213.

12. Witschi AKM, Liesegang A, Gebert S *et al.* (2011) Effect of source and quantity of dietary vitamin D in maternal and creep diets on bone metabolism and growth in piglets. *J Anim Sci* **89**(6), 1844-1852.

13. Höller U, Quintana AP, Gössl R *et al.* (2010) Rapid determination of 25-hydroxy vitamin D3 in swine tissue using an isotope dilution HPLC-MS assay. *J Chromatogr B* **878**(13), 963-968.

14. Schuster I (2011) Cytochromes P450 are essential players in the vitamin D signaling system. *Biochim Biophys Acta* **1814**(1), 186-199.

15. Jakobsen J & Saxholt E (2009) Vitamin D metabolites in bovine milk and butter. *J Food Compost Anal* **22**(5), 472-478.

16. Nordic Committee on Food Analysis (1989) *Fat. Determination According to SBR (Schmid-Bondynzki-Ratslaff) in Meat and Meat Products. Method no. 131.* Oslo: Nordisk Metodik Komitee for Næringsmidler.

17. Cook RD & Weisberg S (1982) *Residuals and Influence in Regression.* New York: Champman and Hall.

18. EFSA (2009) Scientific Opinion: Safety and efficacy of 25-hydroxycholecalciferol as a feed additive for poultry and pigs. *The EFSA Journal* **969,** 1-32.

19. Clausen I, Jakobsen J, Leth T *et al.* (2003) Vitamin D_3 and 25-hydroxyvitamin D_3 in raw and cooked pork cuts. *J Food Compost Anal* **16**(5), 575-585.

20. Lawson D, Douglas J, Lean M *et al.* (1986) Estimation of vitamin D_3 and 25-hydroxyvitamin D_3 in muscle and adipose tissue of rats and man. *Clin Chim Acta* **157**(2), 175-181.

21. Blum M, Dolnikowski G, Seyoum E *et al.* (2008) Vitamin D 3 in fat tissue. *Endocrine* **33**(1), 90-94.

22. Pramyothin P, Biancuzzo RM, Lu Z *et al.* (2011) Vitamin D in Adipose Tissue and Serum 25-Hydroxyvitamin D After Roux-en-Y Gastric Bypass. *Obesity* **19**(11), 2228-2234.

23. Heaney RP, Horst RL, Cullen DM *et al.* (2009) Vitamin D3 distribution and status in the body. *J Am Coll Nutr* **28**(3), 252.

24. Ovesen L, Brot C, Jakobsen J (2003) Food contents and biological activity of 25-hydroxyvitamin D: a vitamin D metabolite to be reckoned with? *Ann Nutr Metab* **47**(3-4), 107-113.

Paper IV

(Submitted to Steroids)

Tissue Content of Vitamin D_3 and 25-hydroxy vitamin D_3 in Minipigs after cutaneous synthesis, supplementation and deprivation of vitamin D_3

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Key words: vitamin D_3; 25-hydroxy vitamin D_3; vitamin D_3-kinetics; minipigs

Abbreviations

25-hydroxy vitamin D_3, 25(OH)D_3; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; standard erythema dose, SED

Abstract:

Information regarding the endogenous storages of vitamin D_3 after cutaneous vitamin D synthesis compared to oral vitamin D_3 supplementation is sparse. Furthermore it is not known whether vitamin D_3 can be stored for later use during periods of shortages of vitamin D_3. To investigate the endogenous storages of vitamin D_3 two studies were carried out in Göttingen minipigs. In study 1 one group of minipigs (n=2) was daily exposed to UV light corresponding to 10-20 minutes of midday sun and another group (n=2) pigs were fed up to 60µg vitamin D_3/day corresponding to 3.7-4.4µg/kg body weight.

Study 1 demonstrated that daily UV-exposure of minipigs stimulated the cutaneous synthesis of vitamin D_3 and resulted in increasing serum vitamin D_3 and 25-hydroxy vitamin D_3, but also carcasses containing vitamin D_3 and 25-hydroxy vitamin D_3. The vitamin D_3 content in adipose tissue from the UV-exposed minipigs was 150-260ng/g and the content was 90-150ng/g in the orally supplemented minipigs.

In study 2, minipigs were UV-exposed daily for 49 days. Subsequently, one group (n=2) was fed a vitamin D-free diet and another group (n=2) was dosed daily with 13C-labelled vitamin D_3. The concentrations of vitamin D_3 and 25-hydroxy vitamin D_3 in serum and skin- and subcutaneous adipose tissue biopsies were repeatedly monitored. Vitamin D_3 and 25-hydroxy vitamin D_3 were eliminated from the skin and the adipose tissue after UV-exposure was ceased. Supplementation of 13C-vitamin D_3 did not seem to affect the decline in the endogenous vitamin D_3 in the adipose tissue formed during UV-exposure.

Introduction

Vitamin D_3 is belonging to the group of fat-soluble vitamins. Exposure to sunlight stimulates the cutaneous synthesis of vitamin D_3 [1], but due to the low angle of the sun on high latitudes during winter time vitamin D_3 is mostly synthesized during summer time [2]. Populations such as the Scandinavians and many Americans living on high latitudes have to rely on endogenous reserves of vitamin D_3 as well as dietary sources including supplementation of vitamin D_3 during winter time.

It is known that vitamin D_3 accumulates in the body after feeding excessive doses of vitamin D_3 to rats [3] whereas only low concentrations of vitamin D_3 are found in animal fed physiological relevant doses of vitamin D_3 [4]. Most of the data available on endogenous pools of vitamin D_3 are derived from animals orally dosed with vitamin D_3.

Sparse information is available about the differences in endogenous storages of vitamin D_3 after cutaneous vitamin D_3 synthesis or oral administration of vitamin D_3. In fact, the importance of stored vitamin D_3 for later use during periods of shortages of vitamin D_3 is still debated [5].

Information about the contribution and the magnitude of the endogenous reserves of vitamin D_3 would be of interest for authorities when formulating vitamin D_3 recommendation to optimize the vitamin D_3 status and to avoid intoxications.

The aim of the study was to investigate the endogenous storages of vitamin D_3 in minipigs exposed to UV-light to stimulate cutaneous synthesis of vitamin D_3, and in minipigs orally supplemented with vitamin D_3. Secondly, to investigate if the storages of vitamin D_3 are available during shortages of vitamin D_3 and if oral supplementation of vitamin D_3 interacts with the endogenous reserves of vitamin D_3.

We monitored the serum and tissue concentrations of vitamin D_3 metabolites in the minipigs to elucidate the changes of vitamin D_3 during various input conditions, e.g. UV-exposure, vitamin D_3 supplementation and shortages of vitamin D_3.

Experimental

Housing conditions

Göttingen minipigs were purchased from Ellegaard Göttingen Minipigs (Dalmose, Denmark). The animals were five weeks old at the arrival and they were allowed to exercise daily on the floor. The room temperature was 22°C, the humidity was 50-60% and the air change was 8-10 times/h. Laboratory light (TLD58W/3, Phillips, Eindhoven, Holland) was switched on from 8.00 to 20.00. At the location of the breeder the pigs had been fed a standard diet including vitamin D_3. After arrival the pigs were fed a vitamin D-free diet (Altromin Special diet C 9000, Altromin GmbH, Lage, Germany) according to the feeding scheme recommended by the breeder, and supplemented with apples for titbits. The animals had access to water *ad libitum*.

Study design

Two different studies in minipigs (Figure 1) were carried out at the animal facilities at National Food Institute, Technical University of Denmark (Mørkhøj, Denmark). Ethical approval was given by *The Danish Animal Experiments Inspectorate*. The authorization number given: 2012-15-2934-00089 C12. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use. In both studies the minipigs were anesthetized prior to euthanization by an intramuscular injection of a mixture of Narcoxyl®vet., Ketaminol®vet. from Intervet International BV (Boxmeer, The Netherlands), Torbugesic®vet. Pfizer Inc (Memphis, TN) and Zoletil®50 from Virbac (Carros, France).

After exsanguination, organs and soft tissues were examined visually for gross pathology by a pathologist.

Study 1 – Storages of vitamin D_3 and 25(OH)D_3 after UV-exposure and vitamin D_3 supplementation in minipigs

Four male Göttingen minipigs from the same litter were divided into two groups and kept in 4.6m^2 cages. After 6 weeks the pigs were placed in individual cages (2.3m^2) where visual contact was possible within each group.

After two weeks of acclimatization the intervention was initiated (day 0). One group of animals (designated oral-group) was dosed orally every day with vitamin D_3 (Sigma Aldrich, Steinheim, Germany) dissolved in 0.9mL corn oil. From day 0 to day 34 the oral-group was dosed with 15µg vitamin D_3/day corresponding to 1.5-2.5µg/kg body weight (BW) and from day 35 to day 84 with 27.5µg vitamin D_3/day to maintain the dose within the range of 1.5-2.5 µg/kg BW with the increasing BW. From day 85 to day 120 the dose was increased to 60µg vitamin D_3/day corresponding to 3.7-4.4µg/kg BW. The strength of the doses was verified by analysis using an HPLC-based method described elsewhere [4].

The other group of animals (designated light-group) was UV-exposed daily and concurrent with the oral-group this group received daily 0.9mL/day of corn oil vehicle. From day 0 to day 84 the light-group was UV-exposed daily for one minute and from day 85 to day 120 the daily UV-exposure time was increased to two minutes to further stimulate the cutaneous synthesis of vitamin D_3.

From day 0 to day 14, the blood was sampled twice a week, followed by once a week until the termination of the experiment at day 120 where the pigs were euthanized.

Study 2 – Changes of stored vitamin D_3 under various input conditions in minipigs

Six female Göttingen minipigs from two different litters were kept together in a 9.2m^2 cage. At day 49 two minipigs were terminated and the four remaining pigs were separated pairwise into two cages (4.6m^2).

At day 0 the intervention was initiated. The minipigs were UV-exposed daily for one minute until day 48 where the UV-exposure was ceased. At day 49 two animals were terminated. The remaining four animals were divided into two groups. One group was dosed orally every day from day 49-115 with 3µg vitamin [$^{13}C_2$]-vitamin D_3/ kg BW hereafter referred to as 13C-vitamin D_3. 13C-vitamin D_3 (Cambridge Isotope Laboratories Inc. Andover, MA, USA) was dissolved in corn oil (30µg vitamin D_3/mL) and the dose was adjusted on a weekly basis from the average of the animals weight. The other group of animals was dosed daily with corn oil vehicle but did not receive any vitamin D throughout the rest of the study period. The four pigs were terminated at day 116.

The remaining four pigs were punch biopsied at day 49, 63, 103 and prior to exsanguination of the animals at day 116. From day 0 to 42, the blood was sampled every second week. From day 48 to 56 the blood was sampled twice a week followed by once a week until the termination of the experiment.

Blood sampling

Blood samples were taken from vene saphena on the back leg. For study 1 the leg was locally anesthetized with Xylocain 5% cream (AstraZeneca A/S, Copenhagen, Denmark). From day 49 in study 1 and for study 2 the animals were tranquilized with 8mg Stresnil/kg BW (Janssen-Cilag, Neuss, Germany) before blood sampling. The blood sample was allowed to clot for 15-30minutes at room temperature before centrifugation at 3000*g* for 15minutes. Serum was removed and kept at -80°C until analysis.

Biopsy sampling

The animals were fasted overnight prior to the sampling of the biopsies. The animals were anaesthetized with an intramuscular injection of a mixture of Narcoxyl®vet., Ketaminol®vet., Torbugesic®vet. Pfizer Inc (Memphis, TN) and Zoletil®50. The area surrounding the point of sampling was shaved, scrubbed with soap and disinfected with 70% ethanol. Two adjacent punch biopsies of skin and subcutaneous adipose tissue were removed by a disposable 6mm biopsy punch (kai Europe GmbH Solingen, Germany). The skin was sutured with two stitches of synthetic suture (Monocryl 2/0 FS-1, Ethicon, Somerville, NJ). The first biopsy was sampled from the left side of the neck and succeeding biopsies alternated from side to side. The following biopsies were sampled laterally from the previous point of sampling. The biopsies were packed in air-tight containers, air was replaced by nitrogen and the samples were stored at -80°C until analysis.

Tissue sampling

After exsanguination of the pigs the following tissues were sampled: subcutaneous, perirenal and intraperitoneal adipose tissues, liver, kidney, heart, skin from the neck, abdominal skin and muscle tissues (logissimus dorsi and rectus abdominis). Visible adipose tissue surrounding the organs and muscle tissue were removed. All the tissues were washed in saline to remove blood. Heart, liver, kidneys and muscle tissue were homogenized. Subcutaneous adipose tissue was removed from the skin. All the tissues were packed in air-tight containers, air was replaced by nitrogen and the samples were stored at -80°C until analysis.

Light-source

The lamp used to stimulate the cutaneous vitamin D_3 was developed to mimic the UVA and UVB light spectrum emitted by the sun as described elsewhere [6]. The light source was placed 2.3m above the floor of the cages. The lamp was characterized on day 80 by an OL 756 spectroradiometer (Optronics Laboratories, Orlando, FL) scanning at 1nm increment from 250nm to

400nm. Furthermore, the stability of the lamp was monitored at 312nm throughout the study by an ILT 1400-BL photometer equipped with a SEL005/TLS312/TD detector (International Light Technologies, Peabody, MA). No changes in the intensity of the lamp were observed throughout the study.

The UV erythemally weighted dose for one minute exposure was calculated to correspond to ~0.9 standard erythema dose by use of the erythema reference action spectrum [7] and one standard erythema dose (SED) was considered to be $100J/m^2$ [8].

Analysis of serum and tissue

Serum and tissues were analyzed for vitamin D_3 and 25(OH)D_3 by LC-MS/MS as described in details elsewhere [9,10]. Where appropriate, measurement of 13C-vitamin D_3 and 13C-25(OH)D_3 were included using the following transitions: *m/z* 593.4→298.0 for 13C-vitamin D_3 and *m/z* 609.4 →298.0 for 13C-25(OH)D_3. The internal standards and standard curves for D_3 and 25(OH)D_3 were used for the quantification of 13C-vitamin D_3 and 13C-25(OH)D_3, respectively. The concentration of 13C-vitamin D_3 and 13C-25(OH)D_3 were corrected for the contribution from the natural abundant 13C-vitamin D_3 and 13C-25(OH)D_3 in the non-labeled analytes.

The precision of 25(OH)D_3 in serum was assessed in a non-spiked house reference of human serum (n=27) to be 4.4% at 13.0ng/mL. For the tissue analysis the precision was determined by a house reference of porcine adipose tissue (n=14) and found to be 5.9% at 5.1ng/g and 5.0% at 2.3ng/g for vitamin D_3 and 25(OH)D_3, respectively.

A modified Schmid-Bondzynski-Ratslaff (SBR) gravimetric method was used to determine the content of fat in the adipose and muscle tissues [11]. Briefly, the sample was boiled with

hydrochloric acid followed by the addition of ethanol and extraction of the lipids with diethyl ether:petroleum ether (1:1). After evaporating of the solvent, the fat was weighed.

Data plotting

Prism 5 for Windows (version 5.00; GraphPad Software, San Diego, CA) was used to plot the data. Data is reported as mean ± SEM.

Results

The daily visual clinical inspection of the minipigs revealed no signs of treatment-related effects and the food and water intake was comparable between the different groups within each study. The pigs were following known growth curves and reached a BW of 16.1-16.8kg for study 1. In study 2 the pigs terminated at day 49 weighed 6.9-8.6kg and the remaining pigs weighed 13.8-17.1kg on the day the study ended. For study 2 one of the two minipigs terminated at day 49 was suffering from a few irregular epileptic-like attacks. The attacks were observed in stressful situations especially during feeding time. The phenomenon is known to the breeder and not related to the intervention. All animals appeared lean with only little abdomal and intraperitoneal adipose deposits. No signs of intoxication were apparent after a gross pathologic examination of soft tissues and organs of the minipigs.

Study 1

Serum vitamin D_3 was 1-2 ng/and serum 25(OH)D_3 was 5-8ng/mL for 25(OH)D_3 before the intervention had begun.

Both groups of animals responded to the treatment with either UV-exposure or oral vitamin D_3 supplementation and an increase in both serum vitamin D_3 and 25(OH)D_3 was observed for all animals. The light-group was exposed daily to 0.9-1.8 SED/day which corresponds to 10-20 minutes of midday sun exposure at 56ºN during summertime [12]. The serum vitamin D_3 increased rapidly after the intervention was initiated. Increasing the UV-exposure time to two minutes only resulted in a little increase of serum vitamin D_3. In contrast, the stepwise increment of oral vitamin D_3 resulted in an increase in serum vitamin D_3 (Figure 2A). The oral-group of minipigs was fed up to 60µg vitamin D_3/day but it only resulted in ~50% of the serum 25(OH)D_3 level observed in the light-group as shown in Figure 2B.

Tissue content of vitamin D_3 and 25(OH)D_3

The tissue content of vitamin D_3 and 25(OH)D_3 in the carcasses from the minipigs is found in Figure 3. Briefly, the content of vitamin D_3 in the muscle tissues was 5-11ng/g for the light-group and 3-7ng/g for the oral-group. For the three different adipose tissues the vitamin D_3 content was 150-260ng/g and 90-150ng/g for the light-group and oral-group, respectively. For the light-group vitamin D_3 in the skin was 82-137ng/g in skin sampled from the neck and 7-13ng/g in the skin sampled from the abdomen (Figure 3A). The tissue concentration of 25(OH)D_3 was lowest in muscle tissues and highest in kidney, subcutaneous adipose tissue and skin (Figure 3B).

The fat content ranged from 1-2% in muscle and 66-79% in the adipose tissue (Figure 4).

Study 2

The initial serum value for vitamin D_3 were 1-2ng/mL and 9-15ng/mL for 25(OH)D_3.

All the minipigs responded to the UV-exposure and reached serum values of 18.7-45.9ng/mL for vitamin D_3 and 39.5-82.2ng/mL for 25(OH)D_3 after 49 days of daily UV-exposure. After the following 67 days without any UV-exposure the non-supplemented pigs declined to 0.6-0.9ng/mL for vitamin D_3 and 3.5-5.0ng/mL for 25(OH)D_3 (Figure 5). For the supplemented animals the serum 13C-vitamin D_3 and 13C-25(OH)D_3 reached a plateau at 9-14ng/mL and 25-33ng/mL (Figure 6A+B), respectively, after 27 days of intervention.

The elimination of serum vitamin D_3 appears to be biphasic on a logarithmic scale (Figure 5A) with a very fast decline of vitamin D_3 in the first two weeks after UV-exposure ceased. The elimination of vitamin D_3 did not seem to be different for the group orally supplemented with 13C-vitamin D_3.The half-life of vitamin D_3 was determined graphically to be < 4 days on the first part of the

curve whereas the half-life of vitamin D_3 of the second part of the curve was >20 days. The elimination of 25(OH)D_3 appeared nonlinear with a declining elimination rate (Figure 5B).

Four biopsies were sampled from each of the animals after the UV-exposure ceased. For the two animals supplemented with 13C-vitamin D_3, the content of 13C-vitamin D_3 and 13C-25(OH)D_3 in adipose tissue and skin increased until day 104 (Figure 6). After UV-exposure ceased, 60-300ng/g of vitamin D_3 and 7-17ng/g of 25(OH)D_3 was found in skin and adipose tissue (Figure 7). The elimination of vitamin D_3 in adipose tissue, and 25(OH)D_3 in adipose tissue and skin appeared to be linear on a log-scale (Figure 7). For vitamin D_3 in the skin a quick drop was initially observed followed by a constant elimination rate of vitamin D_3 (Figure 7C). The half-life of vitamin D_3 was graphically determined to be 30-40 days. Based on the graphs, no differences in the elimination of vitamin D_3 or 25(OH)D_3 between the pigs on vitamin D-free diet and pigs supplemented with 13C-vitamin D_3 were observed in neither skin nor adipose tissue biopsies. The BW of the minipigs was increasing two-fold after UV-exposure ceased until the study ended, but the concentration of vitamin D_3 in the adipose tissue biopsies was decreased by four-fold during the same period of time.

The two minipigs terminated immediately after the period of UV-exposure had concentrations of vitamin D_3 in the adipose tissue between 72ng/g and 143ng/g (Figure 8A). The vitamin D_3 content in the skin was 306-414ng/g in the skin sampled from the neck compared to 49-56ng/g for the skin sampled from the abdomen.

The four animals which were kept for an additional 67 days after the end of the UV-exposure, had vitamin D_3 tissue levels between 0.2 and 1.7ng/g except from the skin and the adipose containing vitamin D_3 up to 52ng/g. For 25(OH)D_3 the content was 0.1-1.1ng/g in all tissues. In the two animals supplemented with 13C-vitamin D_3 all the analyzed tissues had a content of 13C-vitamin

244 D_3 and 13C-25(OH)D_3 (Figure 8) . The highest levels of 13C-vitamin D_3 were found in the different

245 adipose tissues (Figure 8A).

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Discussion

During times of deprivation of vitamin D the body needs to rely on endogenous pools of vitamin D. Although vitamin D_3 is fat soluble and known to accumulate in adipose tissue after supraphysiological doses of oral vitamin D_3 [3] only low levels of vitamin D_3 is found in animals fed the recommended doses of vitamin D_3 [4]. An attempt to estimate the size of the endogenous pool of vitamin D_3 based on the knowledge of vitamin D_3 in pigs indicated that the pool of vitamin D_3 would only provide 7 days of vitamin D_3 reserve in humans [13].

To our knowledge the differences in storages of vitamin D_3 after either cutaneous synthesis of vitamin D_3 or oral has not been investigated before and the information about the turnover of vitamin D_3 is also sparse. The need for such data seems highly relevant since the information on the storage of vitamin D_3 and its importance for the vitamin D status is still debated [5]. To address the question we established an animal model to investigate the changes of tissue content of vitamin D_3 under various input conditions. We chose to use a pig model for several reasons. Like the humans the pig is a true omnivore with similar digestive abilities [14]. The pig is extensively used in nutritional research [15], and has previously been used to assess the vitamin D_3 status in relation to UV-exposure [16] and as a model to estimate the endogenous pool of vitamin D_3 in humans [13].

To reduce the influence of growth on the dilution on the storage of vitamin D_3 during times of vitamin D_3 deprivation we chose a minipig, which has a slower growth curve compared to normal sized pigs [17,18]. Despite of the small size, the minipig is still suitable for repetitive blood and tissue sampling, which was important as repeated data sampling was required. The use of multiple data sampling from each animal during the study also compensated for the relatively low number of minipigs used. The Göttingen minipig model is novel in the vitamin D_3 research, but its

unpigmented skin [19] makes it particularly relevant when studying cutaneously synthesized vitamin D_3.

Repeated tissue samples were obtained by punch biopsies. Except for one study on differences between vitamin D_2 and vitamin D_3 [20] such types of experiments have to our knowledge not been done before, probably due to the lack of methods for vitamin D_3 tissues analysis applicable for sample sizes obtained by biopsies. Recently, we published such a quantitative method for vitamin D_3 and 25(OH)D_3 in porcine tissue using only 0.2g [10] which was applied in the present study.

In study 1, we tested the response of oral vitamin D_3 and UV-exposure in minipigs and the resulting tissue content of vitamin D_3 and 25(OH)D_3. The serum 25(OH)D_3 level was very low (<8ng/mL) for all animals before the intervention was initiated and the contribution from endogenous storages of vitamin D_3 and 25(OH)D_3 in the minipigs at the beginning of the study is assumed to be negligible.

Study 1 demonstrated that daily UV-exposure of minipigs stimulated the cutaneous synthesis of vitamin D_3 and resulted in increasing serum concentrations of vitamin D_3 and 25(OH)D_3, but also tissues containing vitamin D_3 and 25(OH)D_3. In study 1, the vitamin D_3 content in adipose tissue from the minipigs UV-exposed for 120 days was 150-260ng/g. In comparison, Lawson *et al.* found ~90ng/g of vitamin D_3 in rats daily exposed to UV-light for 25 days [21]. The supplemented minipigs in study 1 received 60µg/day of vitamin D_3 during the last period of the study, which is three times more vitamin D_3 than obtained from a standard minipig diet [17,22]. The reported content of 90-150ng/g vitamin D_3 in the minipigs is, as expected, higher compared to slaughter pigs fed 20-30µg/kg feed of vitamin D_3 where the content of vitamin D_3 in adipose tissues was 3.2-7.9ng/g [23] and the mean of vitamin D_3 of 7.5ng/g in subcutaneous fat quantified in slaughter pigs fed 55µg/day of vitamin D_3 [4].

In skin, the content of vitamin D_3 and 25(OH)D_3 sampled from the UV-exposed pigs were higher at the back where the pigs were more prone to UV-exposure compared to the abdomen. In general, the vitamin D_3 content was higher in the adipose tissues compared to muscle tissue, most likely due to the differences in fat content, previously shown to correlate to the vitamin D_3 content [4,23].

In study 2, we investigated how the storages of vitamin D_3 and 25(OH)D_3 are changing in biopsies sampled from skin and subcutaneous adipose tissue in UV-exposed minipigs during deprivation and oral supplementation of vitamin D_3.

To imitate the dietary input of vitamin D_3 from either supplements or natural food sources we chose to use 13C-vitamin D_3, which we expect to exhibit a similar biological profile as non-labelled vitamin D_3. The advantage being, that the supplemented 13C-vitamin D_3 can be distinguished from endogenous synthesized vitamin D_3 by mass spectrometry detection used in the analytical methods.

A fast decline was initially observed for skin and serum vitamin D_3 after the UV-exposure ceased followed by a slower elimination rate of vitamin D_3 suggested to be of 1^{st}-order. Similar kinetic behaviour was also observed for vitamin D_3 and its metabolites in the liver and kidney in rats fed a vitamin D-free diet [24]. A 1^{st}-order elimination rate of vitamin D_3 from the adipose tissue was suggested. This is in accordance with previous findings in rats dosed with vitamin D_3 where a 1^{st}-order decline of vitamin D_3 in adipose tissue was also observed in vitamin D_3-repleted rats placed on a vitamin D-free diet [24,25]. In contrast, a non-linear elimination rate of serum 25(OH)D_3 was observed in the minipigs.

Supplementation of 13C-vitamin D_3 did not seem to affect the decline in the endogenous vitamin D_3 in the adipose tissue formed during UV-exposure, which could suggest that the transfer of vitamin D_3 from the adipose tissue is predominantly determined by the partitioning of vitamin D_3 between the adipose tissue and blood rather than biologically regulated by the vitamin D_3 status. This is

supported by the fact that tissue concentrations of 13C-vitamin D_3 was build up simultaneously with the depletion of endogenously formed vitamin D_3 in the supplemented minipigs.

In summary, we used the Göttingen minipig to study the distribution of vitamin D_3 and 25(OH)D_3 during and after vitamin D_3 was provided orally or cutaneously synthesized after UV-exposure. We were able to generate a dense data set showing how UV-exposure and oral supplementation of vitamin D_3 affect the serum and tissue content of vitamin D_3 and 25(OH)D_3 in minipigs. Study 1 showed that the adipose tissue concentration of vitamin D_3 was 10-30 fold higher in the minipigs compared to other studies of vitamin D_3 in pigs [4,23]. The orally supplemented minipigs did also receive three times more vitamin D_3 than recommended, whereas the irradiated minipigs were only exposed to UV-light corresponding to 10-20 minutes of the daily summer sun.

We successfully biopsied skin and subcutanoues adipose tissue to demonstrate that the storages of vitamin D_3 generated during periods of UV-exposure were eliminated when UV-exposure was ended, and that the elimination of vitamin D_3 seemed to be independent of concomitant supplementation of 13C-vitamin D_3. Finally, we also showed that minipigs become vitamin D_3 deficient within 20 days without UV-exposure when serum 25(OH)D_3 < 20ng/mL was used as the cut-off value.

Our study demonstrated that it is possible to monitor the change of vitamin D_3 and 25(OH)D_3 in tissue obtained by simple punch biopsies. A similar study design could be applied for future studies in humans to investigate the turnover of endogenous storages of vitamin D_3 in humans.

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References

[1] Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. Am J Clin Nutr 2008;87:1080S.

[2] Webb A, DeCosta B, Holick M. Sunlight regulates the cutaneous production of vitamin D3 by causing its photodegradation. J Clin Endocrin Metab 1989;68:882.

[3] Brouwer DAJ, Beek JV, Ferwerda H, Brugman AM, Klis FRM, Heiden HJ et al. Rat adipose tissue rapidly accumulates and slowly releases an orally-administered high vitamin D dose. Br J Nutr 1998;79:527-32.

[4] Jakobsen J, Maribo H, Bysted A, Sommer HM, Hels O. 25-Hydroxyvitamin D3 affects vitamin D status similar to vitamin D3 in pigs–but the meat produced has a lower content of vitamin D. Br J Nutr 2007;98:908-13.

[5] Brannon PM, Yetley EA, Bailey RL, Picciano MF. Overview of the conference" Vitamin D and Health in the 21st Century: an Update". Am J Clin Nutr 2008;88:483S.

[6] Jäpelt RB, Jensen SK, Hymøller L, Andersen EW, Kaas P, Burild A et al. Increase of vitamin D_3 in milk from dairy cows housed indoor by exposure to a special designed UVB lamp. Forthcoming

[7] International Commission on Illumination; Erythema Reference Action Spectrum and Standard Erythema Dose. ISO/CIE Standard ISO 17166; 1999.

[8] Diffey B, Jansen C, Urbach F, Wulf H. The standard erythema dose: a new photobiological concept. Photodermatol Photoimmunol Photomed 2011;13:64-6.

[9] Burild A, Frandsen HL, Jakobsen J. Simultaneous quantification of vitamin D3, 25-hydroxyvitamin D3 and 24, 25-dihydroxyvitamin D3 in human serum by LC-MS/MS. Scand J Clin Lab Invest 2014; Early Online: 1-6.

[10] Burild A, Frandsen HL, Poulsen M, Jakobsen J. Quantification of Physiological Levels of Vitamin D_3 and 25-Hydroxyvitamin D_3 in Porcine Fat and Liver by use of Subgram Sample Sizes. J Sep Sci. Forthconing 2014.

[11] Nordic Committee on Food Analysis; Fat. Determination According to SBR (Schmid-Bodynzki-Ratslaff) in Meat and Meat Products. Metgod no. 131; 1989.

[12] Bogh M, Schmedes A, Philipsen P, Thieden E, Wulf H. A small suberythemal ultraviolet B dose every second week is sufficient to maintain summer vitamin D levels: a randomized controlled trial. Br J Dermatol 2012;166:430-3.

[13] Heaney RP, Horst RL, Cullen DM, Armas LAG. Vitamin D3 distribution and status in the body. J Am Coll Nutr 2009;28:252.

[14] Swindle MM, Smith A. Comparative anatomy and physiology of the pig. Scand J Lab Anim Sci 1998;25:1-10.

[15] Miller E, Ullrey D. The pig as a model for human nutrition. Annu Rev Nutr 1987;7:361-82.

[16] Cooper DA, Berry DA, Spendel VA, Kiorpes AL, Peters JC. The domestic pig as a model for evaluating olestra's nutritional effects. J Nutr 1997;127:1555S.

[17] Ellegaard Göttingen Minipigs A/S. Taking good care of Ellegaard Göttingen Minipigs. [Rev 2010 Feb 10; cited 2014 Jun 6]. Available from: http://minipigs.dk/fileadmin/filer/pdf/Taking_good_care_of_Ellegaard_Goettingen_Minipigs_04.02.10.pdf

[18] Mount. L.E, Ingram DL. THE PIG as a Laboratory Animal. London: Academic Press; 1971.

[19] Bollen P, Ellegaard L. The Göttingen minipig in pharmacology and toxicology. Pharmacol Toxicol 1997;80:3-4.

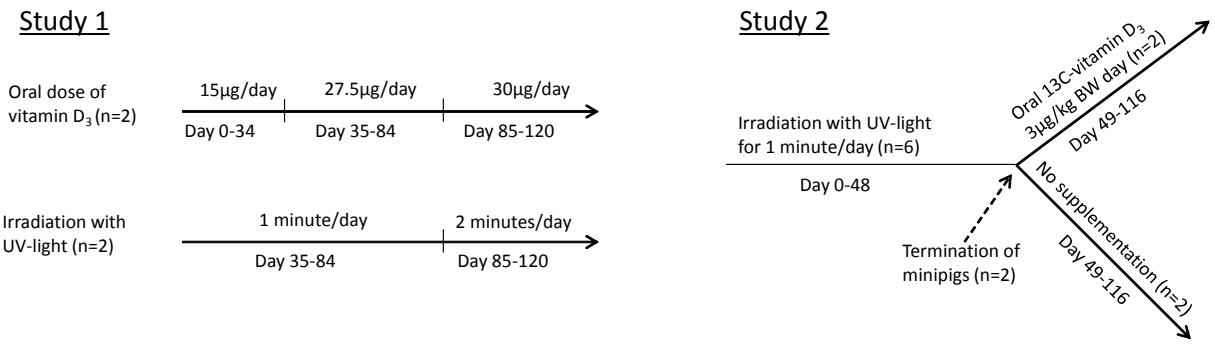
[20] Heaney RP, Recker RR, Grote J, Horst RL, Armas LA. Vitamin D3 is more potent than vitamin D2 in humans. J Clin Endocrinol Metab 2010;96:E447-52.

[21] Lawson D, Douglas J, Lean M, Sedrani S. Estimation of vitamin D3 and 25-hydroxyvitamin D3 in muscle and adipose tissue of rats and man. Clin Chim Acta 1986;157:175-81.

[22] SDS Speciel Diet Service. Minipig-Expanded [Catalogue Rev 4; cited 2014 Jun 6]. Available from: http://www.sdsdiets.com/pdfs/mini-Pig.pdf[23] Clausen I, Jakobsen J, Leth T, Ovesen L. Vitamin D3 and 25-hydroxyvitamin D3 in raw and cooked pork cuts. J Food Compost Anal 2003;16:575-85.

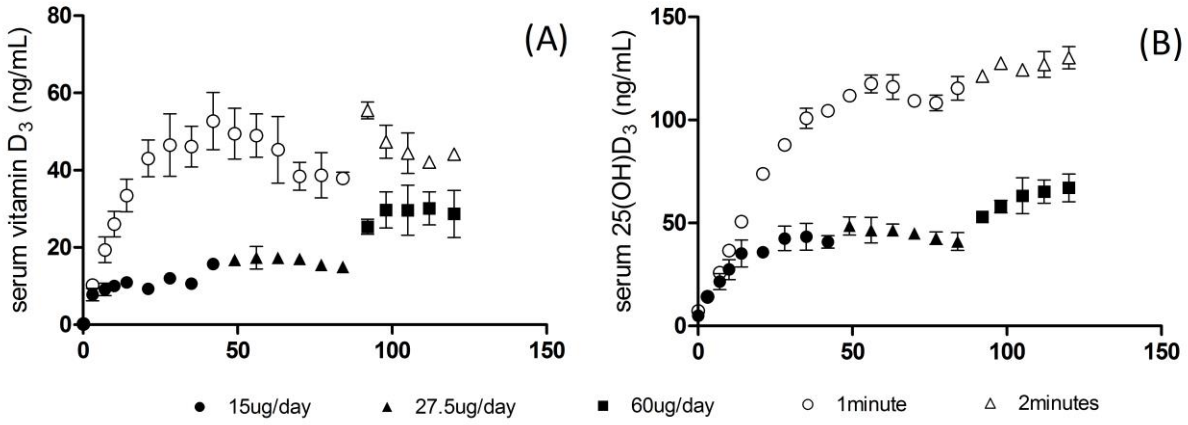
[24] Rosenstreich SJ, Rich C, Volwiler W. Deposition in and release of vitamin D3 from body fat: evidence for a storage site in the rat. J Clin Invest 1971;50:679.

[25] Lawson D, Sedrani S, Douglas J. Interrelationships in rats of tissue pools of cholecalciferol and 25-hydroxycholecalciferol formed in uv light. Biochem J 1986;233:535.



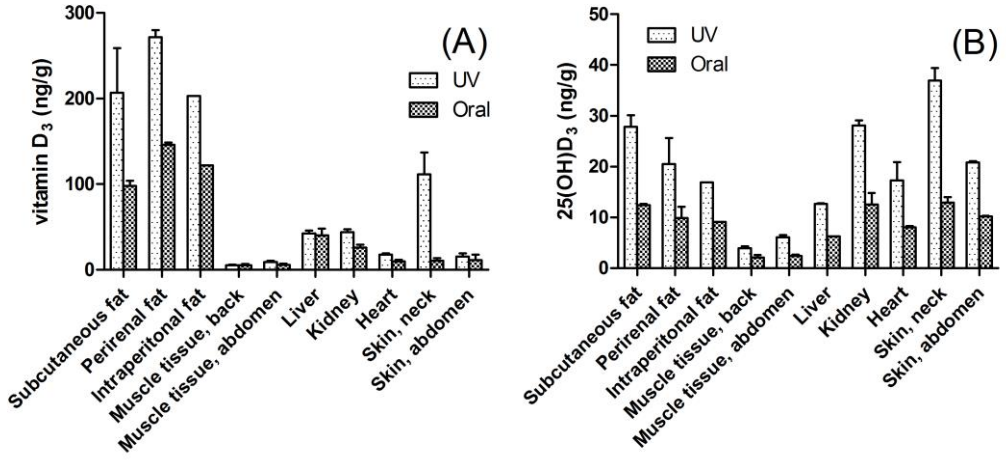
403

404 **Figure 1** Study 1 & 2: The experimental design. Blood was sampled throughout the two studies.
405 For study 2 skin and adipose tissue biopsies were sampled repeatedly after UV-exposure ceased on
406 day 48.



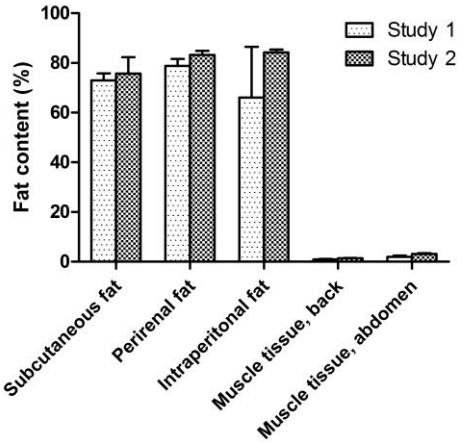
407

408 **Figure 2** Study 1: Serum levels of vitamin D_3 (A) and 25(OH)D_3 (B) in minipigs supplemented
409 daily with vitamin D_3 (filled symbols) and pigs exposed daily to UV-light (285-400nm) (open
410 symbols).



411

412 **Figure 3** Study 1: The distribution of vitamin D_3 (A) and 25(OH)D_3 (B) in minipigs after daily
413 exposure to UV-light (n=2) or a daily oral dose of vitamin D_3 (n=2) for 120 days. For intraperitonal
414 adipose tissue (n=1).



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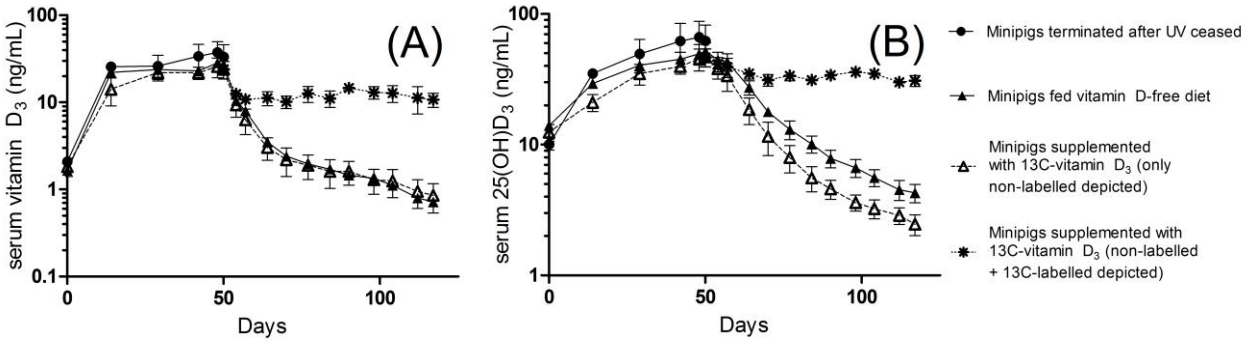
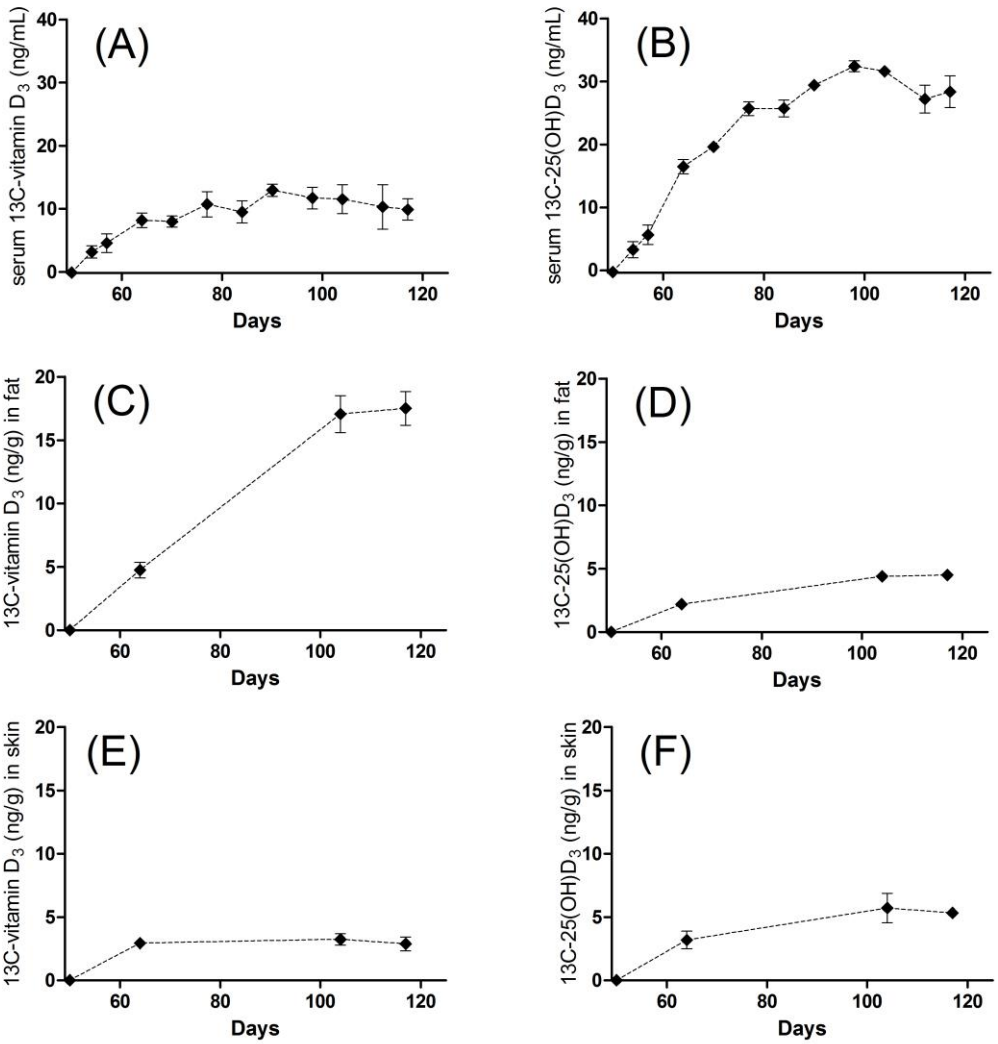
416

417 **Figure 4** Study 1 & 2: Fat content in adipose tissue and muscle tissue in minipigs from study 1 and
418 2.



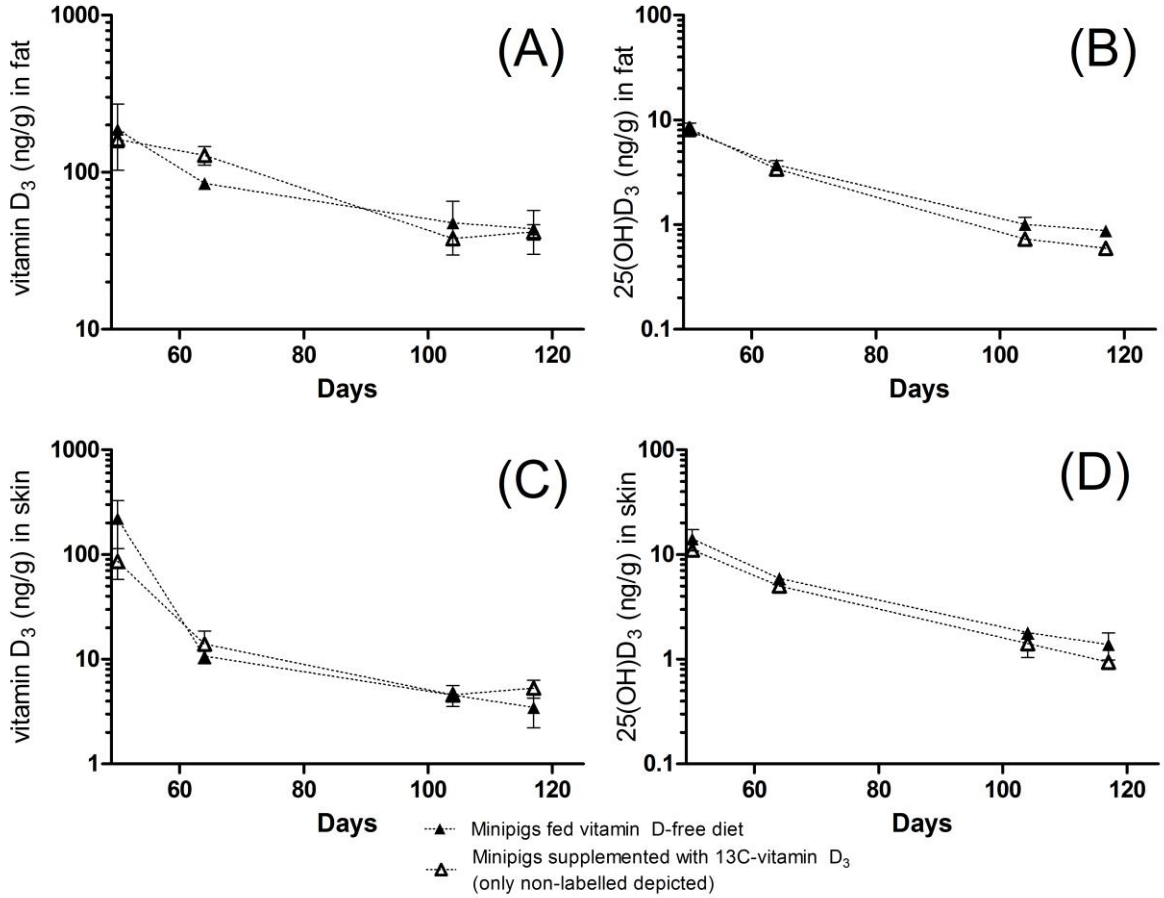
419

420 **Figure 5** Study 2: Serum vitamin D_3 and 13C-vitamin D_3 (A) and serum 25(OH)D_3 and 13C-
421 25(OH)D_3 (B) in minipigs after daily UV-exposure for 49 days. After the UV-exposure, two pigs
422 were terminated, two pigs were kept on a vitamin D-free diet and two pigs were supplemented daily
423 with 13C-vitamin D_3.



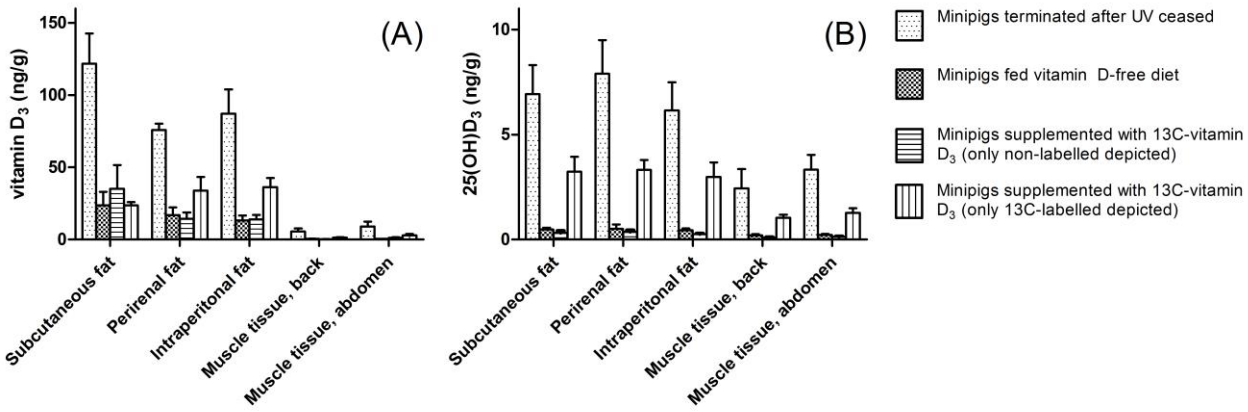
424

425 **Figure 6** Study 2: Serum and biopsies from the neck of the minipigs after oral supplementation of
426 13C-vitamin D_3 (n=2). 13C-vitamin D_3 and of 13C-25(OH)D_3 in serum (A+B) and in biopsies of
427 the subcutaneous adipose tissue (C+D) and skin (E+F).



428

429 **Figure 7** Study 2: Content of vitamin D_3 (A) and 25(OH)D_3 (B) in biopsied subcutaneous adipose
430 tissue and vitamin D_3 (C) and 25(OH)D_3 (D) in biopsied skin in minipigs after the UV-exposure
431 ceased. Following the UV-exposure the minipigs were fed a vitamin D-free diet (n=2) or
432 supplemented daily with 13C-vitamin D_3 (n=2).



433

434 **Figure 8** Study 2: The distribution of vitamin D_3 (A) and 25(OH)D_3 (B) in minipigs terminated
435 after daily UV-exposure (n=2) for 49 days, in minipigs subsequently fed a vitamin D-free diet (n=2)
436 and in minipigs subsequently supplemented with 13C-vitamin D_3 (n=2) for 67 days.

437

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