Journal of Chromatography B, 878 (2010) 963–968

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Rapid determination of 25-hydroxy vitamin D3 in swine tissue using an isotope dilution HPLC-MS assay

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ARTICLE INFO

Article history:
Received 29 September 2009
Accepted 20 February 2010
Available online 26 February 2010

Keywords:
25-Hydroxy vitamin D3
Isotope dilution assay
Tissue contents in swine
New method validation

ABSTRACT

A rapid method for quantification of 25-hydroxy vitamin D3 in different swine tissues based on isotope dilution HPLC-MS has been developed and validated. Six times deuterated analyte is used as internal standard. The method is fast and can be performed with only 1 g sample. Sample preparation for kidney, liver, muscle and spleen requires only homogenisation and extraction with methanol. An additional enzymatic digest is required for skin, and clean-up of the extract by solid-phase extraction (SPE) is used for adipose tissue and skin. The lower limit of detection varies from 1 ng/g (muscle) to 5 ng/g (adipose and skin). The method has been successfully applied to various tissue samples of pigs fed for 119 days either 2000 IU of vitamin D3 or 50 μg of 25-hydroxy vitamin D3 per kg feed. For animals ingesting 25-OH-D3 supplements the highest tissue contents were observed in the skin (24.8 ± 3.5 ng/g), followed by kidney (14.2 ± 1.5 ng/g), liver and muscle (5.7 ± 0.6 ng/g). The 25-OH-D3 content in the skin was significantly higher in animals ingesting 2000 IU/kg of vitamin D3 (39.5 ± 13.4 ng/g). Levels in selected tissues of some animals were below the lower limit of quantification. No measurable amounts of 25-OH-D3 were found in spleen, abdominal fat and subcutaneous fat of the animals of both groups as well as in the liver, kidney and muscle of the animals ingesting 2000 IU/kg of vitamin D3.

1. Introduction

25-Hydroxy vitamin D3 (25-OH-D3) is one very important active metabolite of vitamin D3. Amongst others, and at least as a precursor of 1,25-$(OH)_2$-D3, it plays a prominent role in bone health and calcium metabolism, and its efficacy is often complementary to vitamin D3. However, optimal body levels for many animal species, including chicken and swine, can only be reached by supplementation. For scientific monitoring of feeding trials, fast and robust analytical methods are needed that can be used for high sample numbers in a short time. Plasma analysis has been established as the method of choice for monitoring of 25-OH-D3 status in clinical chemistry [1]. However, determination of tissue contents, particularly those playing a role in human consumption, is equally important. While different techniques for analysis of 25-OH-D3 in plasma have been established and are readily available for routine analysis, the varying matrix complicates matters for tissue analysis. Techniques for plasma analysis are typically immuno-assay-based methods or liquid chromatography with MS detection using isotopically labelled 25-OH-D3 as internal standard. The application of an isotope dilution assay with tandem-MS analysis of 25-OH-D3 in plasma has been recently described [2]. These previous reports were extensively covered in two excellent recent reviews [1,3]. Due to its superior selectivity, HPLC-MS is considered the reference methodology for plasma and our method of choice for tissue analysis. While plasma can be analysed directly after protein precipitation, tissue analysis requires a more complex sample preparation, specifically adapted to different matrices such as kidney, liver, muscle, spleen, adipose and skin. In addition to a more complex matrix, tissue contents are presumably much lower than plasma levels (compared on a per weight base), as a tissue storage of 25-OH-D3 has not been reported. Up to now, only a few methods for tissue analysis have been published [5–7]. These are based on HPLC-UV or radio immuno-assay (RIA) for quantification, and HPLC-MS seems not to be in use for tissue analysis thus far. HPLC-UV requires rather large sample amounts and a complex sample preparation, including saponification. It has therefore limited use for trials with smaller animals such as chicken and rat, and large animal numbers. Also, limited stability of 25-OH-D3 during saponification needs to be critically considered. The RIA methodology requires handling of radioactive educts and has to cope with specificity issues [3], requiring its validation prior to use for analysis of a new type of tissue or samples from a different species. Therefore, neither of the described methods is suited for fast routine

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doi:10.1016/j.jchromb.2010.02.026

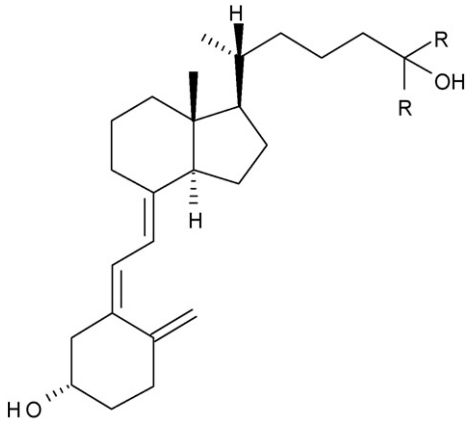

Fig. 1. 25-hydroxy vitamin D3 (25-OH-D3), R = CH_3 and the deuterated isomer used as internal standard (25-OH-D3-d6), R = CD_3.

measurements for our applications. Thus, the goal of the current work was to develop and validate a fast HPLC-MS method, suitable for routine analysis of 25-OH-D3 in different tissues of swine. The method was based on our current procedure for plasma, and adapted to the analysis of different tissues. After a complete validation, the method was applied for monitoring levels in application trials of 25-OH-D3 in swine.

2. Experimental

2.1. Materials, reagents, and solvents

All solvents and reagents used were of analytical grade or equivalent. 25-Hydroxycholecalciferol monohydrate (25-hydroxy vitamin D3 monohydrate, Dr. Ehrenstorfer, Augsburg, Germany) was used for spiking experiments and as reference material. For quantification by HPLC-MS deuterium labelled 25-OH-D3 was used as internal standard (ISTD). This compound, 26,26,26,27,27,27-hexadeutero-25-hydroxycholecalciferol (25-OH-D3-d6, Fig. 1) was supplied by Prof. Mourino, University of Santiago de Compostela, Spain. For homogenisation of tissue samples a knife mill at 7500 rpm and a ball mill with 20 mL-grinding beaker and 20 mm steel balls at a frequency of 30 s^{-1} were used (MM 301 and Grindomix GM 200, respectively, Fa. Retsch, Haan, Germany). For enzymatic digest of skin samples, collagenase with a specific activity of >1200 CDU/mg (Sigma, C7657) was used. 2.5 mg were dissolved in Tris buffer (50 mM) with calcium acetate (0.1 M) at pH 7.2. The buffer was prepared by dissolving 0.6 g tris-(hydroxymethyl)-aminomethane hydrochloride in 25 mL water, addition of 45 mL 0.1 N HCl and 1.58 g calcium acetate, and completion to 100 mL with water. For solid-phase extraction Chromabond SPE, C18ec, 500 mg columns were used (Macherey & Nagel, Düren, Germany). HPLC-MS analysis was performed on an Agilent 1100 LC/MSD-system with APCI source, two binary pumps and column switching valve (Agilent Technologies, Santa Clara, CA).

2.2. Samples used for method validation

Samples originated from different animal feeding trials with growing/fattening pigs conducted by DSM Nutritional Products, and were not identical with samples from the trial presented in the current work. Tissue samples were frozen in liquid nitrogen and then stored at −80 °C until analysis. The delay between slaughtering and freezing was about 15–20 min.

2.3. Sample extraction procedures

Samples were thawed and further processed at room temperature. Due to their different textures, extraction procedures were adapted to the different tissues.

2.3.1. Kidney, liver, muscle, and spleen

For analysis of kidney, liver, muscle and spleen, 20–100 g tissue has been homogenised for approx. 1 min using a knife mill. Then, 1 g of the resulting homogenate was accurately weighted into a 20 mL-grinding beaker containing a steel ball. 5 mL methanol and 40 ng ISTD in 100 μL methanol were added. Grinding time was 2 min. The resultant extract was transferred into a 15 mL centrifugation vial and centrifuged for 15 min at 3000 × *g* and 10 °C. 650 μL of the resulting clear supernatant were diluted with 350 μL water. An aliquot of 75 μL was used for analysis on the HPLC system.

2.3.2. Adipose

Adipose tissue (20–100 g) was homogenised using a knife mill for approx. 1 min. Then, 0.5 g of the resulting homogenate were weighted into a 20 mL-grinding beaker containing a steel ball, and 2 mL 0.2 N sodium dodecyl sulfate was added. After a grinding time of 2.0 min, 2.0 mL methanol and 250 ng ISTD in 50 μL methanol were added. The sample was then ground for another 1.0 min. The resultant extract was transferred into a 15 mL centrifugation vial and centrifuged for 10 min at 3000 × *g*. 1000 μL of the supernatant were diluted with 4000 μL water/methanol 1/1 (v/v) and subjected to solid-phase extraction (SPE). SPE was carried out using Chromabond SPE, C18ec, 500 mg cartridges under reduced pressure. The cartridges were activated with 1.0 mL methanol, and equilibrated with 1.0 mL water. Aliquots of 1000 μL extract corresponding to 25 mg equivalents of adipose were then loaded onto the cartridges. The columns were washed with 1.0 mL water, and the analytes were eluted with 2 mL of methanol. The eluate was reduced to dryness under reduced pressure, and reconstituted with 250 μL water/methanol 1/1 (v/v). 50 μL were injected into the HPLC system.

2.3.3. Skin

From a representative piece of skin the underlying fat was discarded with a scalpel. Approx. 1 cm^2 of skin tissue, corresponding to 0.5 g, were then cut into small pieces (<1 mm^2) with scissors and weighted into a 15 mL centrifuge tube. 2.0 mL collagenase solution and 100 ng ISTD in 20 μL methanol were added. The tube was accurately closed, and kept overnight at 30 °C in a heater block. Then, 2.0 mL of methanol were added, and the mixture was briefly mixed with a vortex and ground for 1.0 min with a polytron. The resultant extract was centrifuged for 10 min at 3000 × *g*. 1000 μL of the supernatant were diluted with 4000 μL water/methanol 1/1 (v/v). SPE was carried out using Chromabond SPE, C18ec, 500 mg cartridges under reduced pressure. The cartridges were activated with 1.0 mL methanol, and equilibrated with 1.0 mL water. Aliquots of 1000 μL diluted skin extract, corresponding to 25 mg equivalents of skin, were then loaded onto the cartridges. The columns were washed with 1.0 mL water. Analytes were eluted with 2 mL of methanol. The eluate was reduced to dryness under reduced pressure. After reconstitution with 250 μL water/methanol 1/1 (v/v), 50 μL were injected into the HPLC system.

2.4. HPLC-MS analysis

The HPLC system included a column switching set-up and gradient elution (Fig. 2). Analytical column was an Aquasil C18, 3 mm × 100 mm, 3 μm, linked to pump 1, while pump 2 was linked to the injector and an Aquasil C18, 3 mm × 10 mm, 5 μm trapping column. After 1.85 min the trapping column was switched

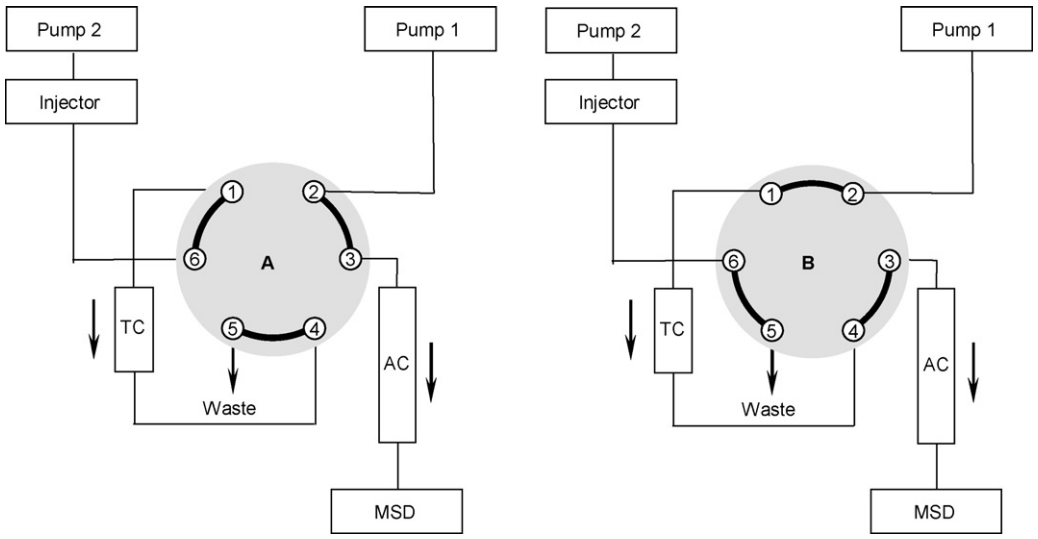

Fig. 2. Column switching system ("forward flush-mode") used for analyses of 25-OH-D3 in tissue extracts. The extract is loaded onto the trapping column (TC, position A) and then eluted and analysed on the analytical column (AC, position B).

for 0.90 min to pump 2, inline with the analytical column for transfer of the trapped 25-OH-D3 and ISTD. Retention time of 25-OH-D3 was approx. 5.7 min. Eluent A was water containing 0.05% formic acid; eluent B was methanol containing 0.05% formic acid. The gradient program for pump 1 operated with a flow of 0.6 mL/min: 0.0–6.4 min, 90% B; 6.4–6.5 min, linear gradient to 100% B; 6.5–9.0 min, 100% B; 9.0–9.1 min linear gradient to 90% B; 9.1–12.0 min, 90%B. For pump 2 a gradient program with a flow of 0.7 mL/min was used: 0.0–1.0 min, linear gradient from 60% B to 85% B; 1.0–2.7 min, 85% B; 2.7–2.8 min, linear gradient to 100% B; 2.8–9.0 min, 100% B; 9.0–9.1 min, linear gradient to 60% B; 9.1–12.0 min, 60% B.

For quantification, an Agilent 1100 Single mass spectrometer was operated with an APCI source in positive mode at unit resolution. 25-OH-D3 and the ISTD were detected in SIM mode using the quasi-molecular ions: 25-OH-D3 mass 401.3 amu, 25-OH-D3-d6 mass 407.3 amu. The following ion source and other MS-instrument parameters were used: drying gas 9.5 L/min at 225 °C, spray gas 50 psig at 250 °C, ionisation potential 3000 V, corona current 10 μA, fragmentor 90 V.

Within each analytical sequence the relative response factor (RRF) between 25-OH-D3 and 25-OH-D3-d6 with regard to the MS detection was determined by analysing a solution containing both compounds at nominal 50 ng/mL ($n = 4$). As acceptance criteria for validity of the sequence a relative standard deviation (RSD) < 5% was used. Calculation of 25-OH-D3 content in tissue (ng/g) using internal standardisation was done as following: $c(25-OH-D3) = (A(25-OH-D3) \times m(ISTD))/(A(ISTD) \times E \times RRF)$, where A(25-OH-D3) and A(ISTD) are the peak areas of the corresponding compounds, m(ISTD) is the mass of 25-OH-D3-d6 (ng) added to sample aliquot, RRF is the Relative Response Factor and E is the sample weight (g).

2.5. Validation experiments

Method validation was performed including the following parameters: selectivity, linearity, accuracy by recovery, precision and robustness. As far as possible, experiments were performed using samples with incurred 25-OH-D3 content. If these were not available, especially for the upper content levels, spiking experiments with tissue containing no measurable 25-OH-D3 content were performed. If not stated otherwise, statistical calculations were performed using the Origin software, version 7.0, OriginLab Corporation, Northampton, USA.

Linearity was tested by spiking blank tissue with different amounts of 25-OH-D3. Experiments were carried out in triplicate at each spike level. The resulting area ratio 25-OH-D3/ISTD was plotted against the nominal spiked content, and a linear regression through zero was calculated using the least square method. Linearity was assumed for a range where the deviation of the measured values from the trend line was equal to or less than 15% [20% at the level of the lower limit of quantification (LLQ)]. The LLQ was defined as the lowest tested analyte content where the acceptance criteria of all validation parameters were met. The lower limit of detection (LOD, Table 1) was estimated based on chromatograms of blank tissue extracts taking into account selectivity and a signal to noise ratio of 3:1.

Accuracy by recovery was determined also by spiking experiments with blank tissue. Analytical results were compared with the nominal values. Experiments were carried out with three replicates (6 at the LLQ level).

Extraction efficacy for liver and muscle was determined by analysing samples as detailed in Section 2.3.1 with the exception that the ISTD was not added prior to extraction, but was given into the final extract before HPLC analysis. Amounts added were adjusted to the extract volume, taking into account a water content of the tissue of 73%. The remaining extract was then discarded, and the solid sample residue was extracted again with 5 mL methanol.

Precision of the method was assessed by analysing six aliquots of homogenised samples each on two different days. The relative standard deviation (RSD) for the determinations on day 1, and for all 12 replicates was calculated, respectively.

Robustness of the method was investigated for different parameters including auto sampler stability of sample extracts,

Table 1
Linear range for the determination of 25-OH-D3 in selected tissues determined by analysis of spiked tissue. The lower end of the linear range equals the LLQ. The LOD has been estimated based on selectivity and sensitivity results (Section 2.5) and is given for comparison.

Tissue	Linear range (ng/g)	R^2	Slope	LOD (ng/g), estimated
Liver	10–200	0.9906	0.022	2
Muscle	5–1000	0.9984	0.023	1
Adipose	20–500	0.9956	0.0020	5
Skin	20–500	0.9994	0.0051	5

freeze–thaw stability of samples with incurred 25-OH-D3 content, storage stability of these samples and stock solution stability for 25-OH-D3.

2.6. In vivo swine experiment

Fifty 28-day-old Large-White × Landrace weaner piglets having an initial body weight of 7.9 ± 0.74 kg were used. The animals were allocated into two equal groups (A and B) and housed in cages in sub-groups (two of 8 and one of 9 animals for each group) in an environmentally controlled room. Each cage had a plastic-coated welded wire floor and was equipped with two water nipples and two stainless-steel feeders. Room temperature was initially 27 °C and was lowered weekly by about 2 °C until 21–22 °C was reached. Environment humidity percentage throughout the experiment was 50%.

Each group of animals was fed for 32 days either a vitamin D3 free basal diet with the addition of 2000 IU/kg (50 μg/kg) of vitamin D3 (group A – actual content of vitamin D3 – 1900 IU/kg) or the basal diet with the addition of 50 μg/kg 25-OH-D3 (Rovimix Hy.D®) (group B – actual content of 25-OH-D3 – 57.1 μg/kg). The method for quantification of 25-OH-D3 in feed was published earlier [8]. Both diets were distributed *ad libitum* in a mash form. Blood was withdrawn by jugular puncture for the determination of the plasmatic concentration of 25-OH-D3 at day 32 of the observation. Analysis of 25-OH-D3 was performed at DSM following an established method [9]. At the end of the post-weaning phase the 20 heaviest animals of each group were used for the growing-fattening period. They had an initial body weight of 19.4 ± 1.36 kg for the group A and of 21.5 ± 1.46 kg for the group B. The animals were housed in floor-pen cages in sub-groups of four animals each in an environmentally controlled room. Each pen had a plastic-coated welded wire floor and was equipped with two water nipples and four stainless-steel individualised feeders. Room temperature was 21–22 °C and humidity percentage was 50%. The pigs were fed for 87 days *ad libitum* a mash form diet without vitamin D3 supplemented either with 2000 IU/kg of vitamin D3 (group A, animals ingesting during the post-weaning phase 2000 IU/kg; actual content of vitamin D3 2350 IU/kg) or with 50 μg/kg of 25-OH-D3 (group B, animals ingesting during the post-weaning phase 50 μg/kg; actual content of 25-OH-D3 54 μg/kg). Blood was withdrawn by jugular puncture for the determination of the plasmatic concentration of 25-OH-D3 at day 80 of the observation.

Both basal diets used in the two phases were formulated to meet the animals' requirements according to Henry et al. [10] and NRC [11], with the exception of the vitamin D3 supply.

Animals of each lot were fasted for 12 h and weighed just before they were slaughtered after tranquilization and stunning. Samples of the *longissimus dorsi* muscle, liver, kidney, spleen, abdominal fat, subcutaneous fat and skin were taken and frozen in liquid nitrogen within 15–20 min after slaughtering for the determination of the 25-OH-D3 content.

3. Results and discussion

3.1. HPLC-MS analysis

Compared with UV-detection [5] and RIA [4] that have previously been described for tissue analysis of 25-OH-D3, MS detection has two major advantages. It is more selective than UV-detection, and its application to tissues and species other than those it was developed for usually requires less additional validation work as it would be needed for a RIA. Pre-cleaning of the sample extracts by column switching technique together with the selectivity of the MS detection led to a good chromatographic resolution of the 25-OH-D3 and ISTD peaks. Example chromatograms of a muscle extract, and an extract of the same sample spiked with both 25-OH-D3 and ISTD are given in Fig. 3. If necessary, fragment ions resulting from the loss of one and two molecules of water from the target molecules in the ion source could be used as qualifiers. This fragmentation is dependent both on ion source temperature and voltage, and can be optimised accordingly. As the fragmentation occurs in the ion source, it can add additional selectivity to the detection without requiring the use of triple-MS instruments [2]. Sensitivity of the detection was high enough to allow quantification of 25-OH-D3 tissue contents below 5 ng/g. Ion suppression was compensated by the ISTD and did not strongly influence the sensitivity. However, no acceptable accuracy and precision could be reached for these low contents (Section 3.3).

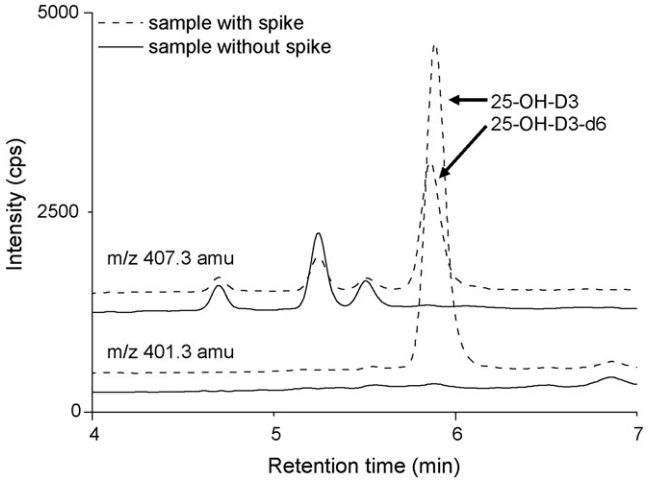

Fig. 3. Mass chromatograms of an extract of a blank pig muscle (solid lines), and the same sample spiked with 25-OH-D3 and 25-OH-D3-d6 (dashed lines). The retention time for both compounds is approx. 5.9 min. The two lower mass chromatograms have been recorded at *m/z* 401.3 amu, the upper two at 407.3 amu. For better visibility, the upper three chromatograms are shown with an offset of 250, 1000 and 1250 cps, respectively.

3.2. Sample extraction

Due to the low contents, and the instability of 25-OH-D3 when exposed to elevated temperatures and light, sample extraction is a difficult step during the analysis. The availability of deuterated 25-OH-D3 as internal standard allowed for compensation of losses occurring during most work up procedures. The final procedure had to fulfil two requirements: it should be applicable also for animals other than swine, where only limited amounts of tissue are available, e.g., mice, and it should be easy to perform to allow processing of larger sample numbers. In case of kidney, liver, muscle and spleen a homogenisation in a ball mill using methanol as extraction solvent proved to be sufficient. The tissue was very finely homogenised, and the methanol freed the 25-OH-D3 from possible protein complexes by denaturation. Extraction efficacy in liver and muscle was determined by repeated extractions of the same samples ($n = 3$). For liver 84% of 25-OH-D3 were shown to be extracted within the first step (absolute content of the samples 69.6–86.4 ng/g), and for muscle 89% (absolute content of the samples 23.7–43.0 ng/g). The 25-OH-D3 content in the third extract was already below the LLQ. Due to the high fat content, adipose tissue required a more complex procedure. The addition of sodium dodecyl sulphate as surfactant yielded a homogenous extract, whereas the clean-up of the extract by SPE was necessary to remove lipids that would otherwise interfere with the reversed-phase HPLC. Due to their strong texture, skin samples could not be sufficiently homogenised in the ball mill and

Table 2
Accuracy by recovery for the determination of 25-OH-D3 in selected tissues by analysis of spiked tissue. Data is based on analysis of six sample aliquots for the lowest level, and three for all other levels; RSD (%): relative standard deviation.

Liver			Muscle			Adipose			Skin		
Level (ng/g)	RSD (%)	Recovery (%)	Level (ng/g)	RSD (%)	Recovery (%)	Level (ng/g)	RSD (%)	Recovery (%)	Level (ng/g)	RSD (%)	Recovery (%)
10	6.5	92.5	5.0	5.3	81.6	20	3.4	104.5	20	4.0	100.8
50	12.0	88.7	50	1.0	90.5	50	6.0	101.2	50	6.2	97.6
200	4.4	80.3	200	4.8	89.1	200	1.4	105.1	200	1.5	102.6
1000	26.1	81.0	1000	3.8	86.6	500	5.8	101.6	500	1.5	101.5

Table 3
Intra-day and inter-day precision for the determination of 25-OH-D3 in tissues with incurred content. Data is based on analysis of 12 sample aliquots, six on each of the 2 days. Intra-day precision was calculated with the results of the analyses performed on day 1, inter-day precision included all 12 results; RSD (%): relative standard deviation.

	Kidney		Liver		Muscle		Spleen		Adipose		Skin	
	Mean (ng/g)	RSD (%)	Mean (ng/g)	RSD (%)	Mean (ng/g)	RSD (%)	Mean (ng/g)	RSD (%)	Mean (ng/g)	RSD (%)	Mean (ng/g)	RSD (%)
Intra-day $n=6$	11.9	3.4	60.8	4.4	27.3	8.6	79.1	7.8	50.1	2.8	116	5.4
Inter-day $n=12$	12.3	6.1	62.0	5.5	25.2	11.2	n.d.	n.d.	47.8	7.9	111	7.2

n.d., not determined.

required an enzymatic digest. Adhering lipids, and peptides and proteins from the digest were removed also by SPE.

3.3. Validation results

The method was validated for the use in the different tissues following international standards [12]. Linearity was assessed by spiking into blank matrix. The results are given in Table 1, together with the estimated LOD. The different slopes result from the different amounts of ISTD added during extraction of the different tissues. Unfortunately, the LLQ estimated from the selectivity and sensitivity of the HPLC-MS could not be realised as the deviation of the measured values from the trend line in that region was >20%.

Accuracy by recovery could be shown to be reproducible and consistent over the content range investigated. Mean recovery was 81.0–92.5% for liver and muscle, and close to 100% for adipose and skin. Results are given in Table 2.

Precision was investigated using samples with incurred 25-OH-D3 content. Inter-day precision is characterised by an RSD of 3.4–8.6%, while inter-day precision is characterised by an RSD of 5.5–11.2%. Typical results are shown in Table 3.

Robustness of the method was investigated for different parameters including auto sampler stability of sample extracts, freeze–thaw stability of samples with incurred 25-OH-D3 content, storage stability of these samples and stock solution stability for 25-OH-D3. Auto sampler stability was given for at least 24 h. Tissue samples were stable for 2–3 freeze–thaw cycles, depending on tissue, and stable for at least 6 months at −80 °C. Stock solution was stable for more than 5 months.

3.4. In vivo swine experiment

The observed vitamin D3 and 25-OH-D3 content in the supplemented feed used was in good agreement with the programmed inclusion levels (Section 2.6).

The animals did not present symptoms of illness during both phases of the experiment. The plasma concentration of 25-OH-D3 was very significantly higher ($P<0.001$) in the animals ingesting 25-OH-D3 supplements in both phases of sampling. In the post-weaning phase the absolute values were 16.8 ± 1.3 ng/ml and 47.9 ± 4.9 ng/ml in the groups A and B, respectively. For the measurements performed during the growing-fattening phase the corresponding values were 38.0 ± 5.1 ng/ml and 92.3 ± 9.7 ng/ml, respectively. The higher plasma concentration in the growing-fattening phase can be explained by the quantities of 25-OH-D3 ingested. In the growing-fattening phase the individual animals were ingesting about 3 kg of feed per day (corresponding to 150 μg of 25-OH-D3) whilst in the post-weaning phase the individual animals were ingesting only about 500 g of feed per day (corresponding to 25 μg of 25-OH-D3). Furthermore, a higher storage of 25-OH-D3 in plasma over the longer growing-fattening phase cannot be excluded.

The 25-OH-D3 content determined in several tissues (*longissimus dorsi* muscle, liver, kidney, spleen, abdominal fat, subcutaneous fat and skin) showed that in the animals ingesting 25-OH-D3 supplements the highest content was observed in the skin (24.8 ± 3.5 ng/g, measurable in 14 animals in a total of 20), followed by the kidney (14.2 ± 1.5 ng/g, measurable in 17 animals in a total of 20), the liver (11.4 ± 0.1 ng/g, but only measurable in 2 animals in a total of 20) and the muscle (5.7 ± 0.6 ng/g, measurable in 8 animals in a total of 20), Table 4. No measurable amounts of 25-OH-D3 were found in the spleen, abdominal fat and subcutaneous fat of the animals of both groups as well as in the liver, kidney and muscle of the animals ingesting 2000 IU/kg of vitamin D3. The 25-OH-D3

Table 4
Effects of the addition of 25-OH-D3 (Hy.D®) to the diet on the muscle, liver, kidney, spleen, abdominal fat, subcutaneous fat and skin content of 25-OH-D3 (ng/g) in the growing-fattening pig. Diet A contained only vitamin D3 (2000 IU/kg), whereas diet B contained only 25-OH-D3 (50 μg/kg).

Animal group A	Animal group B
Muscle	
LLQ[a]	5.7 ± 0.6[b]; 8[c]
Liver	
LLQ	11.4 ± 0.1; 2[c]
Kidney	
LLQ	14.2 ± 1.5; 17[c]
Spleen	
LLQ	LLQ
Abdominal fat	
LLQ	LLQ
Subcutaneous fat	
LLQ	LLQ
Skin	
39.5 ± 13.4[d]; 9[c]	24.8 ± 3.5[d]; 14[c]

[a] LLQ, content below the lower limit of quantification, cf. Table 1.
[b] Mean ± standard deviation of the mean of samples with content above the LLQ.
[c] Number of samples in a total of 20 having a content above the LLQ.
[d] Mean values were significantly different, $P<0.01$.

content in the skin was significantly higher in animals ingesting 2000 IU/kg of vitamin D3 than that observed in the animals ingesting 50 μg/kg of 25-OH-D3 (39.5 ± 13.4 ng/g detectable in 9 animals in a total of 20 versus 24.8 ± 3.5 ng/g, $P < 0.001$), Table 4.

Today, few reports of 25-OH-D3 tissue levels are available, which is to some extend due to the fact that no simple assay is available. Most of these studies concentrate on meet and fat analysis, whereas values for liver and other organs are scarce. Mammal species investigated include swine [5,13–15], lamb [4] and cattle [4,7,15]. Reported levels in pig tissues range from about 1 ng/g in loin [14] to 20 ng/g in lard [13]. However, as study designs and thus also the daily intake of vitamin D3 or 25-OH-D3 by the animals varied greatly, these results are not directly comparable with our study. Some reported contents are below the LOD of our method, suggesting its application preliminary to studies were 25-OH-D3 was administered, as the one presented in this work.

If a calculation of the potential daily ingestion of 25-OH-D3 coming from pigs ingesting 50 μg of Hy.D® per kg of feed is performed, it appears that the ingested quantities are very low. For example, the ingestion of 100 g of pig skin, tissue with the highest content in the supplemented group, represents as a mean less than 2.5 μg of 25-OH-D3. Such quantity represents less than about half of the daily dose recommended by the NIH for an adult human with an age between 19 and 50 years and one fourth of that recommended for an adult human with an age between 51 and 70 years [16]. Furthermore, the mean content in that tissue was significantly lower than that measured in pigs ingesting vitamin D3.

4. Conclusions

This is the first report of an isotope dilution HPLC-MS method for determination of 25-OH-D3 in five different animal tissues. The method is fast and easy to perform and thus suited for analysis of larger sample numbers from application trials. It needs only a small sample amount, and is adapted to the different textures and compositions of muscle, liver, kidney, adipose and skin. After validation, the method was applied to an application trial of vitamin D3 and 25-OH-D3 in swine. The results show that levels of 25-OH-D3 in the tissues after supplementation are low with the highest content found in the skin. This is in agreement with earlier findings that 25-OH-D3 is not stored in body tissues but rather present in the blood stream.

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