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Determination of the principal metabolites of vitamin D in blood by means of on-line solid phase extraction with liquid chromatography-tandem mass spectrometry

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Summary
The determination of metabolites of vitamin D is very important in bone metabolism, in coronary disease, cancer, innate immunology, etc. Unfortunately, variation in methods for determining the metabolites of vitamin D limits the ability of clinicians to monitor the status, supplementation and toxicity of vitamin D. In this work, an automatic method of determining the most important metabolites of vitamin D is presented. 0.2 ml of serum is injected into an XLC-MS/MS (eXtraction Liquid Chromatography-tandem Mass Spectrometry) platform to be cleaned and preconcentrated through extraction in the solid phase (SPE). The analytes retained in the SPE cartridge are eluated directly by the mobile chromatographic phase containing 10% water in methanol, with 5 mM of ammonium formate as ionizing agent, at a flow of 0.3 ml/min for the separation of the analytes, and their later detection through triple quadrupole mass spectrometry (MS/MS).
The limits of detection varied between 3.5 and 8.2 pg/ml. The coefficients of variation within the trial varied between 1.5 and 2.3% during the same day, and between 2.5-3.9% over a week. The recuperation varied between 97 and 99.7% for all analytes. The total time taken for the analysis was 20 minutes.
Thus, the proposed method is robust, cheap and appropriate for use in clinical and research laboratories.

Key words: *Metabolites of vitamin D, Healthy population, Deficiency in vitamin D.*

Introduction

Lack of vitamin D constitutes one of the most prevalent deficiencies in the world. It affects more than half of the population: babies, young people, adults, postmenopausal women and older people, in whom, if they have osteoporotic fractures, the prevalence of low levels of vitamin D reaches 100%[1]. In Spain, in spite of its geographical position and climate which facilitate adequate sunshine, this situation is faithfully reproduced.

Deficiency in vitamin D, in addition to its role in the etiopathology and treatment of rickets or osteomalacia, contributes to multiple extra-skeletal pathologies[1,2]. In fact vitamin D deficiency is associated with an increased risk of suffering diabetes mellitus[4], arterial hypertension[5], cardiac insufficiency[6], cardiovascular disease[7], peripheral arterial disease, acute myocardial infarction[8], cancer[9], as well as risk of suffering infections[10], autoimmune and inflammatory diseases 11, and mortality[12,13]. In addition, the taking of the usual doses of treatment of osteoporosis is associated with a reduction in rates of mortality[14]. All of which has increased interest in the metabolism of the endocrine system of vitamin D, and the quantification of its key metabolites.

The state of vitamin D is determined by the blood concentration of 25-hydroxyvitamin D [25(OH)D][1,2], which includes the concentrations of 25(OH)D_3 and [25(OH)D_2, although it is not clear if both have the same activity, or the same weight as vitamin D_2 or D_3 in the maintenance of the state of 25-(OH)D in humans[15,16]. For its quantification, methods based on liquid chromatography (LC)[17,18], on protein competition tests by chemiluminescence[19], on high and low frequency radioimmunoassay by sampling[20,21], on automatic methods of chemiluminscence[19], and on liquid chromatography tandem mass spectrometry (LC-MS/MS)[22,23], are normally used. These new methods have generated much controversy due to the fact that the interlaboratory studies carried out have not shown concordant results between these different methods , nor among those based on immunoassay[24-26]. The clinical application of LC-MS/MS has improved the selectivity in the determination of 25(OH)D, although the coefficients of variation continue to be high (20%) among the different laboratories which use it, due to the nonexistence of standardised procedures for the analysis of vitamin D, since they are methods developed by and, generally very dependent on, the operator. The implementation of international measures of standardisation which certify the quality of the methodology, such as those conducted by DEQAS (vitamin D External Quality Assessment Scheme)[27], have shown the differences in the determination vitamin D and have developed measures for their standardisation, such as the use of the same standards for the calibration of the method[28].

The determination of 1,25$(OH)_2$ dihydroxyvitamin D_3 is necessary in cases of renal insufficiency, hypoparathyroidism, pseudohypoparathyroidism, screening for hypercalcemia, etc. This determination is more complicated than with 25(OH)D because the concentration is much lower and its stability less. Most of the methods use I^{125} as a marker in radioimmunoassay after a process of extraction. The structural similarity between the metabolites of vitamin D means that the specificity of the method is always in question, since there is no robust study of interference, due to the difficulty of finding a reference method. LC-MS/MS is used for the determination of calcitriol, using precipitation of proteins and previous extraction in solid phase[29] and, recently, applying a derivation of Diels-Alder to improve the ionisation efficiency[30]. In spite of the fact that current methods based on LC-MS/MS provide high sensibility and sensitivity, a consistent platform, totally automated, with high frequency of sampling, precision and exactitude, which makes unnecessary the presence of expert operators, is demanded. From the experience of the group it is thought that the most appropriate platform is XLC-MS/MS (eXtraction Liquid Chromatography-tandem Mass Spectrometry), since it is a closed system which avoids the loss of analytes, and is totally automatic, for which reason it has low coefficients of variation.

This research seeks to fill this gap through the development of an automatic method based on the on-line coupling of solid phase extraction with liquid chromatography and tandem mass spectrometry to determine vitamins D_3 and D_2, the metabolites 25-hydroxyvitamin D_3 and D_2, 24,25$(OH)_2$ dihydroxyvitamin D_3, and 1,25$(OH)_2$ dihydroxyvitamin D_3, and their application in serum from blood donors.

Material and method

Solvents and standards

Ammonium formate, 25-hydroxyvitamin D_3 (25(OH) D_3) and 25-hydroxyvitamin D_2 (25(OH)D_2), vitamin D_2 and vitamin D_3, were obtained from Sigma (Sigma–Aldrich, St. Louis, MO, USA). 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) and 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$), were provided by Roche (Basle, Switzerland). Methanol, acetonitrile and formic acid were obtained from Scharlau (Barcelona, Spain).

The reserve solutions were prepared by dissolving a known quantity of analytes (25(OH)D_2, 25(OH)D_3, vitamin D_3, vitamin D_2, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$) in methanol. From the reserve solutions the working solutions were prepared by dilution of an appropriate volume in methanol, calculating the exact concentration by photometry.

Instrumentation

The separation chromatography was carried out in reverse phase mode with an Agilent 1200 Series (Palo Alto, CA, USA) chromatograph, followed by electrospray ionisation (ESI) in positive mode, and detection by means of mass spectrometry in tandem (Agilent 6410 Triple Quadrupole). The analyses were processed through the MassHunter Workstation Software (Agilent) programme for qualitative and quantitative analysis. The automa-

Table 1. Method of extraction in solid phase

	Flow rate	Volume	Solvent	Commentary
New cartridge				
Autosampler				Load sample
Solvation	5 mL/min	2 mL	Methanol	
Solvation	5 mL/min	4 mL	30% ACN-0.2% FA	
Equilibration	0.4 mL/min	0.4 mL	30% ACN-0.2% FA	
Load of sample	0.4 mL/min	2 mL	30% ACN-0.2% FA	
Washed	2 mL/min	4 mL	30% ACN	
Elution			Mobile phase	7 min
Tube cleaning	5 ml/min	2 mL	Methanol	
Tube cleaning	5 mL/min	2 mL	Water	

tic solid phase extractor used was a Prospekt2 (Spark Holland, Emmen, Netherlands) system, and (Midas) autosampler with a sample loop of 0.2ml. The solid phase extraction cartridge was a Hysphere C18 (Spark Holland) of 10 x 2 mm. The analytic column used was Synergi Hydro-RP (Phenomenex, Torrance, CA, USA) of 2.5 µm particle size, 100 x 2,0 mm.

The extraction of blood was carried out using the standard process. Once obtained the blood was centrifuged at 4° for 10 minutes, aliquoted and frozen at -80°C until used.

Procedure

The autosampler fills the sample loop (0.2ml) and initiates the sequence of operations described in Table 1. Basically, the process of extraction starts with the preparation of the cartridge by means of an activation of the stationary phase with methanol, conditioner and equilibration with an aqueous solution of 0.2% formic acid in 30% acetonitrile. With this same solution, the sample is pulled towards the cartridge. In these conditions the analytes are retained in the absorbent contained in the cartridge, and then 30% acetonitrile is used as a washing solution for interferences. Subsequently, the stages of chromatographic elution and separation commence, putting the mobile phase in contact with the extraction cartridge by the twisting of a valve. The elution time is 7 minutes. The initial mobile phase was 5 mM of ammonium formate contained in 90% methanol at a flow rate of 0.3 ml/min. In minute 2 a lineal gradient of 5 minutes was programmed to obtain 5 mM of ammonium formate in 100% methanol. The temperature of the column was 15° C. The total time for the analysis was 20 minutes.

The eluite of the chromatographic column was ionised using ESI in positive mode and monitored by MS/MS in multiple reaction monitoring (MRM) mode. The flow and temperature of the drying gas (nitrogen) in the ESI was 13 l/min and 350° C, respectively. While the pressure of the nebulizer was 35 psi and the capillary voltage 4,000 V. The scanning time for each transition of MS/MS was 50 minutes.

Results

The limit of detection (LOD) according to the definition of the IUPAC (International Union of Pure and Applied Chemistry) is the minimum quantity of analyte detectable, and is calculated experimentally as the concentration which corresponds to 3 times the standard deviation of the noise to signal ratio calculated in 10 samples. The limit of quantification (LOQ) according to IUPAC is the minimum quantifiable quantity of analyte, generally corresponding to the smallest concentration of the calibration line and is calculated as the concentration which corresponds to a signal 10 times the standard deviation of noise to signal ratio in 10 samples. The individual values of these limits and the regression coefficients are found in Table 2.

Evaluation of the precision of the method

The coefficients of variation, intra- (repeatability) and inter-trial (reproducibility) were calculated over seven days, carrying out daily measurements with replicas in a serum of known concentration[31]. The results are shown in Table 2.

Evaluation of the exactitude of the method

The exactitude of the method and the matrix effect was studied using samples with or without being fortified with standard solutions. The recuperation was calculated with two configurations of the Prospekt2, a double cartridge for non-fortified samples and a single cartridge for fortified samples[32]. This was done because the recuperation may not be adequate for two reasons: first, due to

Table 2. Figures of merit. *Expressed as a percentage of the relative standard deviation

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Coefficient of correlation	Repeatability* (%)	Reproducibility* (%)
$24,25(OH)_2D_3$	0.055	0.184	0.9978	1.6	2.5
$1,25(OH)_2D_3$	0.0035	0.012	0.9977	1.8	2.9
$25(OH)D_3$	0.082	0.272	0.9987	1.5	3.1
$25(OH)D_2$	0.080	0.267	0.9973	1.7	2.8
Vitamin D_2	0.084	0.284	0.9943	2.3	3.9
Vitamin D_3	0.085	0.281	0.9915	2.1	3.5

LOD: limit of detection; LOQ: limit of quantification

a poor retention in the cartridge of the compound under study, which can be seen in the double cartridge, because what is not retained in the first one is retained in the second; the second reason is a low elution, which may be probed by means of a fortified sample whose concentration is known.

The recuperation in the double cartridge system is calculated as the quantity in the first cartridge/[quantity in cartridge 1+quantity in cartridge 2]. The configuration of a single cartridge is calculated as [final concentration - initial concentration/concentration added evaluated in a single sample in five repetitions on the same day under identical conditions. It is understood that the initial concentration is the concentration of analyte present in the sample before adding a known quantity of it, that is to say, the quantity of a blank sample. The results are found in Table 3.

Application of the method

Samples were analysed from 92 blood donors. A representative chromatogram of a sample fortified with standard solutions appears in Figure 1. Levels of vitamin D_3 were detected: 10.4 ± 4.8 ng/ml; but levels of vitamin D_2 were not. Blood levels of 25(OH)D (21.3 ± 5.7 ng/ml) correspond to the sum of $25(OH)D_2$ and $25(OH)D_3$. The share of $25(OH)D_2$ out of a total 25(OH)D is 1.9 %. 5% of the population studied had blood levels of 25(OH)D < 10 ng/mL, 42% < 20 ng/ml, 40% between 20 and 30 ng/ml, and only 18% were > 30 ng/ml. Blood levels of $24,25(OH)_2D_3$ were 4.1 ± 1.6 ng/ml and of $1,25(OH)_2D_3$, 48.2 ± 11.4 pg/ml.

Discussion

The analysis of vitamin D and its metabolites represent a great challenge, due to the high lipophilic nature of these compounds, which means that they are found strongly bonded to their transporter proteins; bonds which need to be broken for their analysis by liquid chromatography. The cleaning of the extracts is essential, since other endogenous lipids will be co-extracted along with the vitamin D metabolites, which results in unclean extracts which may distort the shape of the chromographic peaks and curtail the life of the column. This makes necessary the use of selective and highly sensitive systems such as mass spectrometry for an exact quantification. It also means that the use of a closed system is critical, which avoids the breakdown of the metabolites of vitamin D by light. The advance in methodologies to ensure the determination of vitamin D has not improved the variations previously shown in the measurement of 25(OH)D[24,25].

The disparity in the results affects all laboratories using the same, or different, methodologies. The use of methods with a low level of automisation makes the measurement of vitamin D highly dependent on the user, and requires rigorous quality control to assure the results. Methods based on RIA are not all equal, given different specificity for $25(OH)D_3$ and $25(OH)D_2$; therefore, there are some which overestimate levels of 25(OH)D and others which give lower values for some of the metabolites[26,33].

HPLC is commonly recognised as the gold standard for the determination of vitamin D metabolites[3,17,18,34], but it has high equipment costs and possesses a low frequency of samples, given the obligatory precipitation of proteins and/or liquid-liquid extraction using the methods described above, which has made its establishment in laboratories as a routine technique difficult. The methods which exist for the quantification of $1,25(OH)_2D_3$ are laborious and take much time; therefore, more rapid, cheaper and simpler methods are required, which also reduce the risk to health associated with the use of radioisotopes. In recent studies an EIA kit had a poor correlation with a typical analysis by RIA35. Kissmeyer et al.[29], published in 2001 an method using LC-MS/MS for determining $1,25(OH)_2D_3$, but this required 1-mL of serum, in addition to an earlier precipitation of proteins and drying stage in a flow of nitrogen, and later reconstitution, which leads to low fre-

quency of samples and relatively high coefficients of variation, because all the stages of treatment of the sample before the chromatographic analysis were manual.

In conclusion, the proposed method is an improvement on existing methods since it allows the rapid and automated determination of the concentrations of vitamins D_3 and D_2, and the metabolites $24,25(OH)_2D_3$, $1,25(OH)_2D_3$, $25(OH)D_3$ and $25(OH)D_2$ using a small quantity of serum, permitting both research into the physiology and physiopathology of the endocrine system, and clinical association studies, or their use in normal practice.

The use of an on-line system for the extraction means that there is no loss of analytes by degradation, and the total automation of the process of analysis means that the precision and accuracy is improved and the need for an expert user is avoided.

Therefore, the proposed method is rapid, with a high sensitivity, exactitude and precision. The main inconvenience it presents is the high cost of the equipment used, although the application of the method is cheap.

Figure 1. Chromatogram of a fortified blood sample
(1) $24,25(OH)_2D_3$; (2) $1,25(OH)_2D_3$; (3) $25(OH)D_3$; (4) $25(OH)D_2$; (5) vitamin D_2, (6) vitamin D_3

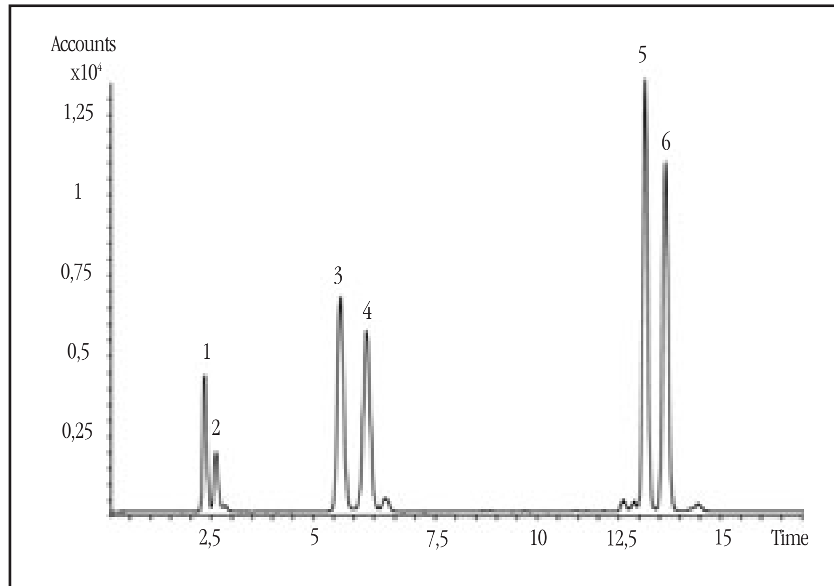

Table 3. Recuperation of each analyte: (1) two-cartridge configuration, (2) single cartridge configuration

Analyte	Recuperation (1)	Recuperation (2)
$24,25(OH)_2D_3$	97.0	96.5
$1,25(OH)_2D_3$	100.2	99.5
$25(OH)D_3$	99.8	99.3
$25(OH)D_2$	98.9	99.0
Vitamin D_2	99.1	99.4
Vitamin D_3	98.3	98.4

Acknowledgements

The authors thank Sanyres for funding the research and the regional centre for blood donation for their collaboration in the collection of samples. And to the Roche laboratories for kindly and disinterestedly providing the metabolites 1,25 dihydroxyvitamin D_3 and 24,25 dihydroxyvitamin D_3.

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