Supporting Information

Rapid simultaneous determination of 7 fat-soluble vitamins in human serum by ultra high performance liquid chromatography tandem mass spectrometry

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This supplemental file includes:

Method validation

Table S1, Fig. S1, Fig. S2.

Method validation

The method validation was based on the European Medicines Agency (EMA) and U.S. Food and Drug guidelines and C62-A on bioanalytical method validation. The following parameters were evaluated during method validation: linearity, carry-over, accuracy and precision, limits of detection (LOD), limits of quantification (LOQ), matrix effect, recovery and stability.

Linearity and carryover

Concentrations of the analytes were calculated from their respective linear-regression calibration curves with the analyte-to-internal standard peak area ratios measured in each sample solution.

Martrix-matched calibration curves were constructed on each validation day, by using vitamin free Human Serum. For each calibration curve, DB (not containing analyte or IS), BL

(not containing analyte but containing IS) and seven non-zero calibrators (S1-S7) were included. The standard series should be treated in the same way according to that described in section sample preparation. Calibration curves were constructed from peak area ratios of calibrators MRMs and internal standards versus concentrations and a 1/x weighting was used for the linear regression. Quantification is achieved by comparing peak area ratios of analytes MRMs and internal standards with that of calibrators.

The carry-over effect was evaluated in this experiment and operated as follows: a blank (DB) was injected after the highest calibrator. Criteria for acceptability included that the blank sample area following the highest calibrator should be less than 20.0% of the LOQ area for each vitamin and 5.0% for the internal standards.

The intra-day and inter-day precision

Precision was assessed by spiking QC working solutions (low, medium and high) to 2% bovine serum albumin (BSA) solution. For the determination of the precision, all QC levels were measured (n=6) in three different analytical batches on three consecutive days. Six replicates of each QC level were analysed on per day to estimate intra-day precision. Eighteen replicates of each QC level were combined to calculate inter-day precision.

The acceptance criterion for precision was 15% for each QC level, except for the LOQ, where it was 20%.

Limit of quantification (LOQ)

LOD was considered as the lowest analyte concentration which correspond to the analyte peak detected at a signal-to-noise ratio (S/N) of 3. LOQ was defined as the lowest concentration of analyte that gave precise and accurate values within the limits ± 20.0%, and a signal-to-noise ratio (S/N) was at least 10: 1[38]. In the present procedure, which were set at the level of the lowest calibrator.

Matrix effect

Samples such as serum and plasma are complex mixture of biologically active compounds that can bond to the target analyte, interfere with determination or have a negative impact on the stability of the observed compound. In order to avoid the matrix effect, appropriate pretreatment methods and a stable isotope-labeled internal standard were used. Matrix effect was assessed by using six different human serum specimens. The same

specimen was divided into two portions. After sample pretreatment, one portion was added into a certain amount of standard solution, the other was added into blank solvent of the standard solution. By comparing the response of the two portions to the corresponding response of standard solution in methanol , the relative matrix effects was calculated as formula (1):

$$relative\ matrix\ effect = \frac{response_{\ spiked\ analyte} - response_{\ endogenous}}{response_{\ standard\ \ in\ solution}} \quad (1)$$

$response_{\ spked\ analyte}$: area ratio of sample pretreated as section 2.3 and spiked a certain amount of standard solution ;

$response_{\ endogenous}$: area ratio of sample pretreated as section 2.3 and spiked a certain amount of blank solution ;

$response_{\ standard\ in\ methanol}$: area ratio of a certain amount of standard in solution.

Recovery

Recovery as a measure of accuracy was assessed by comparing the concentrations of analyte before and after addition of known amounts of the analyte[38]. Spiked and unspiked human serum samples were used in the current study. For validation, serum from six presumed healthy persons were spiked with mixed vitamin standard solutions with known amounts of the analytes at three different concentration levels (LQC, MQC, HQC). Recovery was calculated as the formula (2).

$$recovery = \frac{measured\ \ concentration - endogenous\ \ concentration}{added\ \ concentration} \times 100\% \quad (2)$$

Stability

All FSV, particularly vitamin A and vitamin E, are susceptible to oxidation and can degrade during the sample preparation and analysis. Therefore, it is very necessary to study the stability of samples.The stability of the samples included the stability of processed samples and the unprocessed serum specimens. They were examined under different storage time. Processed serum samples in 96-well collecting microplate were stored in refrigerator (2-8℃, protected from light). Regarding the stability studies, 12 extracted patient samples were repeated measured after 15 hours, 34 hours, 48 hours. Unprocessed serum specimens were stored in refrigerator (2-8℃, protected from light). To satisfy the demands of a short intra-

laboratory turn-around time, patient samples were detected as soon as possible, so more attention was paied to the sample stability within 48h.

Stability was assessed by relative deviation, which was calculated as formula (3), within the pre-set limit of 10% deviation from original value was accepted. Study found that for the processed FSV samples in the auto-sampler, 25-OHD was the most stable one, which showed no significant degradation for at least 48 h, the relative deviation of 25-OHD was within 7.0%. For vitamin A, there was no clear degradation when stored at 2-8℃ for 34 h, while the relative deviation increased near to 10% when stored for 48 hours. Significant differences in concentrations were noticed for vitamin E when stored at the 2-8℃ for 34 h, and the relative deviation rised up to 25%, which continue increased near to 30% over a 48 h period. A summary of the stability data can be found in Fig. S1.

$$relative\ deviation = \left| \frac{measured\ value - original\ value}{original\ value} \right| \times 100\% \quad (3)$$

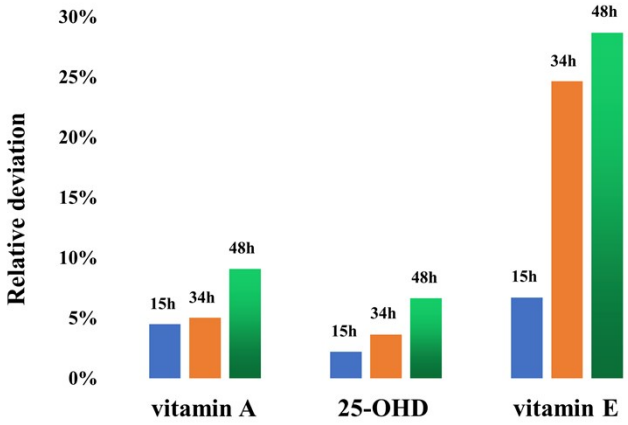

Fig. S1. Stability studies of fat soluble vitamins in microplate under various storage time (n = 12)

For the unprocessed serum samples, when stored in 2-8℃ for 48h, results showed the average bias of vitamin A, 25-OHD and E were all within 10%. Since the concentration of vitamin K in the blood was very low compared to other FSV, even if the detection result had a small change, the relative deviation was too large, so which was not calculated here for both processed and unprocessed serum samples.

Therefore, for vitamin A, 25-OHD and E, processed samples were stable for 15 h stored in 96-well collecting microplate at 2-8℃; nonprocessed samples were stable for 48 h at 2-8℃.

Table S1 the detection results of 30 serum samples using the current procedure

Number	Vitamin A (ng/ml)	25-OH-D2 (ng/ml)	25-OH-D3 (ng/ml)	Vitamin E (ng/ml)	Vitamin K1 (ng/ml)	MK-4 (ng/ml)	MK-7 (ng/ml)
1	541.8	0.4	29.5	6436.0	3.12	0.12	0.38
2	346.1	0.9	20.7	7101.0	0.21	0.09	0.2
3	301.3	0.4	19.4	10555.0	0.3	0.1	0.48
4	297.9	0.3	19.8	7780.0	0.11	0.08	0.38
5	302.7	1.7	34.6	10594.0	0.2	0.14	0.22
6	317.9	0.5	26.9	9599.0	0.4	0.27	0.43
7	277.8	0.4	28.8	7640.0	0.22	0.21	0.19
8	431.4	0.3	42.7	11064.0	0.54	0.22	0.63
9	582.4	0.8	26.5	6557.0	0.43	0.21	0.22
10	513.2	4.5	36.3	9319.0	0.16	0.14	0.25
11	395.3	0.7	40.2	14762.0	0.69	0.12	0.19
12	295.3	8.5	18.2	14737.0	0.2	0.19	0.3
13	485.6	0.1	18.6	8745.0	0.7	0.14	0.28
14	330.5	0.5	25.2	11099.0	0.15	0.12	0.23
15	368.4	8.2	28.1	9506.0	0.65	0.23	0.24
16	334.4	0.3	21.7	8746.0	0.66	0.26	0.17
17	397.8	0.4	29.3	8550.0	1.35	0.11	0.16
18	278.0	0.2	11.5	7273.0	1010	0.1	0.22
19	364.0	2.7	26.8	10984.0	0.31	0.2	0.42
20	192.5	0.9	17.7	8860.0	0.73	0.24	0.16
21	282.4	1.7	28.0	9440.0	0.46	0.09	0.15
22	360.1	1.7	44.8	10990.0	0.42	0.14	0.18
23	399.6	0.8	27.3	9952.0	0.23	0.14	0.34
24	326.3	3.2	38.4	10360.0	0.25	0.11	0.44
25	305.4	1.2	30.0	8556.0	0.26	0.12	0.18
26	303.3	1.2	37.5	7573.0	0.21	0.12	0.22
27	276.2	1.1	32.7	9277.0	1.61	0.39	0.29
28	287.1	1.0	30.9	10734.0	1.62	0.69	0.44
29	409.7	1.8	15.7	10036.0	0.44	0.16	0.17
30	330.6	1.2	19.3	9272.0	0.25	0.13	0.21

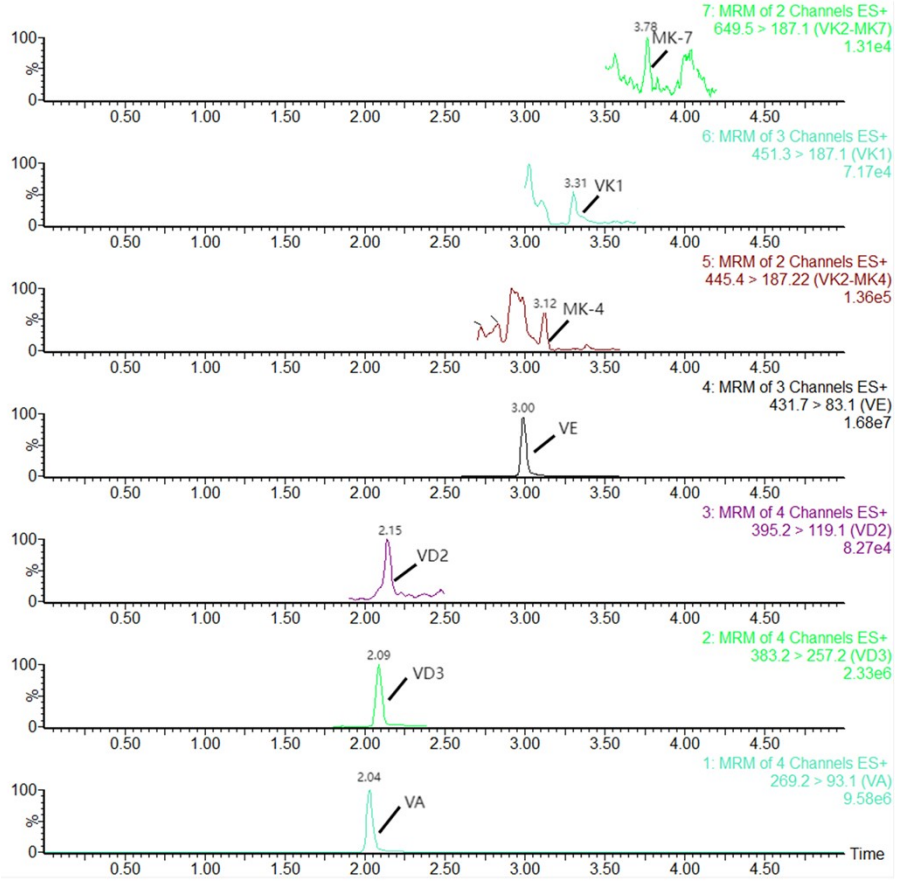

Fig. S2. A chromatogram obtained from human serum