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Analytical Performance of the RZ-500 HPLC-MS/MS for Serum 25(OH)D Quantification and Its Clinical Value for Thyroid Malignancy

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Article

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Abstract

Objective

To verify the analytical performance characteristics of the RZ-500 high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Reigncom company, Shanghai, China) for serum 25-hydroxyvitamin D [25(OH)D] quantification and to evaluate its clinical value for thyroid malignancy.

Methods

In accordance with CNAS-GL037:2019, the precision, accuracy, linear range, lower limit of quantification, and carryover of the RZ-500 system were verified. A total of 101 patients with thyroid malignancy and 101 age- and sex-matched healthy controls were enrolled in the clinical study from May to September 2026. The fasting serum samples were collected from all the participants and measured by LC-MS/MS. 25(OH)D_2, 25(OH)D3, and total 25(OH)D levels were compared between groups, and receiver operating characteristic (ROC) curve analysis was used to assess discriminative performance.

Results

All analytical performance indicators met the predefined acceptance criteria. Serum 25(OH)D_3 and total 25(OH)D were significantly lower in the thyroid malignancy group than those in controls (12.00 vs. 17.70 ng/mL for 25(OH)D_3, $P < 0.001$; 14.27 vs. 20.06 ng/mL for total 25(OH)D, $P < 0.001$, respectively). And vitamin D insufficiency was present in 87.1% of patients and 48.5% of healthy controls ($P < 0.001$). ROC analysis used for thyroid malignancy assessment showed that the area under the curve (AUC) was 0.853 for 25(OH)D_3 at the optimal cutoff value of 13.91 ng/mL, and 0.864 for total 25(OH)D at the optimal cutoff value of 16.42 ng/mL, respectively. 25(OH)D_2 exhibited no significant discriminative value for thyroid malignancy (AUC = 0.522).

Conclusion

The RZ-500 LC-MS/MS system exhibits excellent analytical performance. Owing to isomer-specific quantification, serum 25(OH)D measured by LC-MS/MS showed favorable discriminative performance (high specificity), and may serve as a reliable auxiliary biochemical marker for thyroid malignancy diagnosis.

1 Introduction

Vitamin D extends well beyond its canonical role in bone mineralization; it functions as a critical safeguard in immune regulation and cardiovascular protection [1]. In circulation, vitamin D exists predominantly as 25-hydroxyvitamin D [25(OH)D], whose long half-life has led to its international recognition as the "single reliable biomarker" of vitamin D status [2]. Yet the reality is sobering: vitamin D deficiency is widespread in the

Chinese population. When deficiency is defined as 25(OH)D < 50 nmol/L (20 ng/mL), reported prevalence rates exceed 60% in most regions, and over one billion people worldwide, including those in China, face the burden of vitamin D deficiency [3–5]. This burden is of particular concern in endocrine oncology. Thyroid malignancy is the most common malignancy of the endocrine system, and its incidence has risen steadily worldwide in recent decades, now ranking as the fifth most common malignancy among women [6]. The role of vitamin D in endocrine oncology has attracted increasing attention: it exerts core anti-tumor effects through multiple pathways, including inducing cell cycle arrest, promoting apoptosis, inhibiting epithelial-mesenchymal transition, and remodeling the tumor microenvironment immune response [7]. Several observational studies and meta-analyses have identified vitamin D deficiency as an independent risk factor for thyroid cancer and have linked it to tumor aggressiveness and poor prognosis [8, 9]. Furthermore, preoperative vitamin D deficiency significantly increases the risk of postoperative hypocalcemia and refractory hypothyroidism, underscoring the importance of perioperative vitamin D status assessment [10, 11]. Most clinical laboratories currently rely on conventional immunoassays, but these methods carry inherent limitations: poor cross-reactivity with 25-hydroxyvitamin D_2 [25(OH)D_2] (typically 40%–70%), inability to distinguish biologically inactive C3-epimers, and susceptibility to matrix effects arising from incomplete dissociation of vitamin D-binding protein [12–16]. In contrast, liquid chromatography-tandem mass spectrometry (LC-MS/MS), with its high-specificity mass spectrometric quantitation and chromatographic separation, can eliminate these blind spots and interferences at the source and is internationally recognized as the definitive "gold standard" for vitamin D measurement [17].By virtue of its precise quantitation capability and international standardization, LC-MS/MS-based vitamin D measurement provides reliable biochemical support for malignant risk prediction in thyroid nodules, noninvasive monitoring of thyroid cancer, prevention and management of postoperative complications, and the formulation of enhanced recovery pathways.

Despite the superior analytical performance of mass spectrometry platforms, high-end instrumentation does not absolve laboratories from rigorous verification before clinical deployment, the ultimate quality of results depends critically on meticulous method optimization and robust sample preparation workflows. Accordingly, this study, conducted in strict alignment with the guidance on the verification of quantitative measurement procedures used in the clinical chemistry (CNAS-GL037:2019) standard quality requirements, performs an in-depth performance verification of a newly introduced RZ-500 LC-MS/MS system (Reigncom company, Shanghai, China) in our laboratory, comprehensively evaluating its precision, accuracy, limit of quantitation, linear range, and carryover resistance. The objective is to provide definitive experimental evidence confirming the absolute reliability of this system in complex clinical practice, thereby delivering solid analytical support for precision medicine interventions.

2 Materials and Methods

2.1 Detection System and Reagents

Detection system: This study was conducted in the Department of Clinical Laboratory, an ISO 15189 accredited laboratory, in Beijing Tsinghua Changgung Hospital affiliated to Tsinghua University from May to

September 2026. The detection system was a high-performance liquid chromatography-tandem mass spectrometer (RZ-500) equipped with an atmospheric pressure chemical ionization (APCI) source. Ancillary equipment included a vortex mixer, an ultrapure water system, a high-speed refrigerated centrifuge, a multi-tube vortex mixer, a nitrogen evaporator, and an ultrasonic bath. Instrument maintenance and calibration were performed according to the manufacturer's operating procedures to ensure the system remained in optimal condition. Quality control measurements were performed prior to all testing to confirm that QC results were within acceptable limits before experimental data were collected.

Reagents and calibrators: The 25-hydroxyvitamin D assay kit (Reigncom company, Shanghai, China), which was compatible with the RZ-500 LC-MS/MS system, was used for the quantification of 25(OH)D_2 and 25(OH)D_3.Total 25(OH)D was calculated as the sum of the two isoform concentrations. The principal kit components included: mobile phase A (aqueous solution containing 0.1% formic acid, 120 mL) and mobile phase B (methanol solution containing 0.1% formic acid, 150 mL). Sample preparation reagents comprised: precipitant (acetonitrile-based, 30 mL), extraction solvent (tert-butyl methyl ether-based, 80 mL), and reconstitution solution (20% methanol in water, 15 mL). Internal standard solution: methanol-based solution containing 25(OH)D_2 and 25(OH)D_3 internal standards (3 mL). Calibrators and quality controls: lyophilized preparations containing 25(OH)D_2, 25(OH)D_3, and bovine serum albumin (BSA) (six calibrator concentration levels at 0.2 mL each; three QC concentration levels at 0.2 mL each).

2.2 LC-MS/MS Analytical Conditions

Chromatographic conditions: An analytical column (Reigncom, Shanghai, China) compatible with the RZ-500 LC-MS/MS system was used. The column oven temperature was maintained at 40 °C, with an injection volume of 10 μL and a flow rate of 0.6 mL/min. The gradient elution program was: 0–0.5 min (30% mobile phase B), 0.6–2.5 min (70% mobile phase B), 2.6–4.0 min (30% mobile phase B).

Mass spectrometric conditions: The scan mode was selected reaction monitoring (SRM). The ion source temperature and nebulizer temperature were both set at 350 °C. Curtain gas was set to 0 Arb, nebulizer gas to 45 Arb, auxiliary heater gas to 1.5 Arb, and collision gas was argon, with a discharge current of 6 μA. The precursor/product ion transitions used for quantitation were m/z 383.45/257.315 (collision energy 14.89 V) for 25(OH)D_3 and m/z 395.4/211.179 (collision energy 28 V) for 25(OH)D_2.

2.3 Sample Preparation Procedure

Following the manufacturer's instructions, 100 μL of test sample or QC material was transferred to a reaction tube, and 20 μL of internal standard solution was added, followed by vortex mixing for 30 s. Subsequently, 200 μL of precipitant and 100 μL of ultrapure water were added, and the mixture was vortexed for 2 min. Then, 600 μL of extraction solvent was added, and the sample was extracted by vortexing for 2 min. The mixture was centrifuged at 14,000 round per min (rpm) for 10 min in a high-speed refrigerated centrifuge at 10°C. A 550 μL aliquot of the supernatant was transferred to a 96-well receiver plate,

evaporated to near-dryness under nitrogen, reconstituted with 100 µL of reconstitution solution, mixed for 1 min, and then injected for analysis.

2.4 Method Verification Protocol

2.4.1 Precision verification: In accordance with the requirements of CNAS-GL037:2019, the low-, medium-, and high-concentration quality control (QC) materials supplied with the kit, designated as low-level QC (QCL), medium-level QC (QCM), and high-level QC (QCH), were used for method verification. The concentrations at the three QC levels were 3.45, 21.64, and 104.4 ng/mL for 25(OH)D_2, and 4.69, 28.19, and 138.79 ng/mL for 25(OH)D_3, respectively. Each concentration level was measured six times per run over three consecutive days, yielding a total of 18 measurements. Within-run repeatability precision and intermediate (within-laboratory) precision were evaluated according to CNAS-GL037:2019. The total allowable error (TEa) was 25%, which was obtained from the external quality assessment (EQA) standards issued by the National Center for Clinical Laboratories, National Health Commission of the People's Republic of China. The acceptance criteria were as follows: within-run precision coefficient of variation (CV) ≤ 1/4 of the TEa (6.25%), and intermediate precision CV ≤ 1/3 of the TEa (8.33%).

2.4.2 Accuracy verification

1. Bias assessment: Following the CNAS-GL037:2019 requirements, certified reference materials provided by the manufacturer were selected at a minimum of two concentration levels. Each level was measured at least twice daily for five consecutive days. Results were recorded, and the mean of all measurements was calculated. Absolute bias was calculated using the formula: Absolute bias = Abs (Mean result − Reference value). The verification criterion was that the absolute percentage bias for all levels should be < 1/2 TEa.

2. Spike-and-recovery: A clinical serum sample with low endogenous background was divided into three aliquots, to which 1/10 volume of blank matrix, low-concentration mixed standard, and high-concentration mixed standard were added, respectively. Recovery was calculated for each. The acceptance criterion was spike recovery within 85%–115%.

2.4.3 Linearity verification: Following the CNAS-GL037:2019 and the guideline for the verification of analytical performance of quantitative examination procedures (WS/T 408-2024, a recommended health industry standard of the People's Republic of China), six levels of calibrators provided by the manufacturer were measured, with each concentration level measured three times in a single run. First-, second-, and third-order polynomial regression coefficients, standard errors of coefficients, corresponding *t*-values, and regression standard errors were calculated using computational or spreadsheet software. Linearity was confirmed if the second- and third-order coefficients were not significantly different from zero by *t*-test. If the best-fit model was second- or third-order, the non-linearity bias was required to meet the acceptance criterion.

2.4.4 Limit of quantitation (LOQ) verification: The LOQ is the lowest amount of analyte that can be measured with a specified measurement uncertainty under defined measurement conditions. For this assay,

the LOQ was 1.70 ng/mL for 25(OH)D_2 and 2.63 ng/mL for 25(OH)D_3. 10 aliquots of the lowest calibrator sample (LOQ sample) were measured 10 times each. The acceptance criteria were: deviation of the measured value from the nominal value within ±15%, and CV < 20%.

2.4.5 Carryover Verification:

Carryover was assessed using a high-low QC alternation pattern (five cycles) versus a low-low QC alternation pattern (five cycles). The acceptance criterion was that the difference between the mean of high-low transition samples and the mean of low-low transition samples should be less than 3 standard deviations (SD) of the low-low transition sample mean.

2.5 Clinical Application Study

This clinical study was conducted at Beijing Tsinghua Changgung Hospital, Tsinghua University (Beijing, China) from May to September 2026. This study received ethical approval from the Ethics Committee of Beijing Tsinghua Changgung Hospital (approval number: 24678-4-03) and obtained informed consent from all participants. All methods were performed in accordance with the relevant guidelines and regulations, and this study involving human participants was conducted in accordance with the Declaration of Helsinki.

A total of 101 thyroid malignancy patients were enrolled in this study. The thyroid malignancy diagnosis was established by postoperative histopathology or preoperative ultrasound-guided fine-needle aspiration (FNA) cytology, in accordance with the Guidelines for the Diagnosis and Treatment of Thyroid Nodules and Differentiated Thyroid Carcinoma (Second Edition). The control group comprised 101 healthy individuals matched for age and sex who underwent health examinations at the health management center during the same period. The main exclusion criteria were severe hepatic or renal insufficiency, parathyroid disease, intestinal malabsorption, other malignancies, and pregnancy or lactation. Patients who had recently supplemented vitamin D or used medications known to substantially affect vitamin D metabolism were also excluded.

Fasting venous blood specimens (4 mL) from the subjects were collected into additive-free serum tubes between 7:00 and 9:00 in the morning. Specimens with visible hemolysis, lipemia, or icterus were rejected. Clotted blood was centrifuged at 1,500 × g for 10 min at room temperature to separate serum. Serum samples were analyzed within 4 h of collection. Otherwise, samples were aliquoted and stored at −80 °C, with no more than three freeze–thaw cycles, and thawed at room temperature before analysis. Serum total 25(OH)D was calculated as the sum of 25(OH)D_2 and 25(OH)D_3. Vitamin D insufficiency was defined as total 25(OH)D < 20 ng/mL

2.6 Statistical Analysis

Statistical analyses were performed using SPSS 27.0.1 (IBM, USA), GraphPad Prism 11.0.0 (GraphPad Software, USA), and Python 3 (Python Software Foundation, USA). Normally distributed continuous variables were expressed as mean ± standard deviation and compared between groups using the independent-

samples t test. Non-normally distributed variables were expressed as median (interquartile range) and compared using the *Mann–Whitney U* test. Categorical variables were expressed as counts (percentages) and compared using the chi-square test or *Fisher's exact* test. Receiver operating characteristic (ROC) curve analysis was used to assess the discriminative performance of serum 25(OH)D for thyroid malignancy. The area under the curve (AUC) was reported, and the optimal cutoff was determined by the *Youden* index, with the corresponding sensitivity and specificity calculated. A two-sided *P* value < 0.05 was considered statistically significant.

3 Results

3.1 Method Verification Results

3.1.1 Precision Verification Results

Following the CNAS-GL037:2019 requirements, within-run precision and intermediate (within-laboratory) precision were calculated. The acceptance criteria were: within-run precision CV ≤ 1/4 TEa (6.25%); intermediate precision CV ≤ 1/3 TEa (8.33%). As shown in Table 1, within-run precision for all concentration levels was below the 1/4 TEa acceptance limit, and intermediate precision was below the 1/3 TEa acceptance limit.

Table 1. Precision of the assay

Analytes	QC Concentration Level (ng/mL)	Within-Run Precision (acceptance criteria ≤6.25%)	Intermediate Precision (acceptance criteria ≤8.33%)	Verification result
$25(OH)D_2$	QCL (3.45)	1.4%	4.6%	Pass
	QCM (21.64)	2.5%	3.7%	
	QCH (104.4)	3.5%	4.4%	
$25(OH)D_3$	QCL (4.69)	3.1%	6.1%	
	QCM (28.19)	2.7%	5.6%	
	QCH(138.79)	3.7%	6.0%	

3.1.2 Accuracy Verification Results

(1) Certified Reference Material Bias Assessment

Three concentration levels of manufacturer-supplied certified reference materials were measured across three consecutive runs. The overall mean of all results was calculated, and relative bias was computed against the reference values, with an acceptance criterion of percentage bias ≤ 12.5% (1/2 TEa). As shown

in Figure 1, the relative bias for all concentration levels of both 25(OH)D_2 and 25(OH)D_3 fell within the acceptable range, confirming satisfactory method accuracy.

(2) Spike Recovery Results

A clinical serum sample with low endogenous background was divided into three aliquots and spiked with blank matrix, low-concentration standard, and high-concentration standard, respectively. After simultaneous processing and analysis, spike recoveries were calculated, with an acceptance criterion of 85%–115%. As shown in Table 3, the low- and high-concentration spike recoveries for both 25(OH)D_2 and 25(OH)D_3 were within the acceptable range, confirming satisfactory method accuracy.

Table 2. Spike recovery results

Items	Blank+D_2	Blank+D_3	Low Spike+D_2	Low Spike+D_3	High Spike+D_2	High Spike+D_3
Mean (ng/mL)	1.53	5.93	7.56	14.57	16.40	26.12
Theor. spike (ng/mL)	0.00	0.00	6.24	9.04	14.14	19.18
Calc. spike (ng/mL)	0.00	0.00	6.03	8.63	14.88	20.19
Recovery (%)	—	—	96.65%	95.49%	105.20%	105.27%

As shown in Table 2, the recovery rates for each parameter ranged from 95% to 115%, thereby meeting the performance verification requirements.“—”No recovery rate measurement required.

3.1.3 Linearity Verification Results

Following CNAS-GL037:2019 requirements, WS/T 408-2024, and the kit manufacturer's claimed performance specifications, six gradient concentrations of 25(OH)D_2 and 25(OH)D_3 verification samples were prepared by serial dilution, covering the range from the lower to the upper limit of the manufacturer's claimed linear interval. Each concentration level was measured three times. Outlier testing, polynomial fitting (from higher to lower order), and non-linearity bias assessment were performed sequentially. The acceptance criteria were: no statistical significance for the higher-order terms of second- and third-order polynomials, and mean non-linearity bias ≤ 1/2 TEa (12.5%). The results showed no outliers at any concentration level for either analyte, and the first-order linear equation was the best-fit model (Figure 3). By t-test, the second- and third-order coefficients were not significantly different from zero, and the higher-order terms lacked statistical significance. For 25(OH)D_2, the manufacturer's claimed linear range was 1.96–144.49 ng/mL, the actual verified range was 1.86–140.48 ng/mL, and the mean non-linearity bias was 0.60%. For 25(OH)D_3, the manufacturer's claimed linear range was 2.73–202.27 ng/mL, the actual verified range was 2.40–196.45 ng/mL, and the mean non-linearity bias was 1.31%. Both analytes showed non-

linearity bias substantially below the acceptance limit and far exceeded the manufacturer's claimed 15% specification, confirming that both linearity evaluations met the requirements.

3.1.4 Limit of Quantitation (LOQ) Verification Results

The LOQ is the lowest amount of analyte that can be measured with a specified measurement uncertainty under defined measurement conditions. The lowest calibrator sample (LOQ sample) was measured 10 times. The LOQ for 25(OH)D_2 was 1.70 ng/mL (SD = 0.13, CV = 7.59%; nominal value 1.991 ng/mL; bias = −14.60%). The LOQ for 25(OH)D_3 was 2.63 ng/mL (SD = 0.30, CV = 11.51%; nominal value 2.628 ng/mL; bias = 0.20%). Deviations of measured values from nominal values were all within ±15%, and CV values were below 12%, meeting the performance verification requirements of CV ≤ 1/2 TEa and bias within ±20%.

3.1.5 Carryover Verification Results

Carryover was evaluated using alternating high-low QC and low-low QC measurement patterns. The results showed that for 25(OH)D_2, the mean of high-low transition samples was 3.23 ng/mL, the mean of low-low transition samples was 3.30 ng/mL, the difference was 0.0616, and 3 SD was 0.14. For 25(OH)D_3, the mean of high-low transition samples was 4.47 ng/mL, the mean of low-low transition samples was 4.72 ng/mL, the difference was 0.246, and 3 SD was 0.39. All carryover differences were below their corresponding 3 SD limits, indicating no significant carryover in this system.

3.2 Clinical Application Results

3.2.1 Comparison of Clinical Characteristics Between Groups

This study included 101 patients with thyroid malignancy as the experimental group and 101 healthy examination subjects as the control group. We further analyzed serum 25(OH)D_2 and 25(OH)D_3 subfractions: there was no significant difference in serum 25(OH)D_2 levels between the two groups (2.42 [IQR 2.12, 2.72] ng/mL vs. 2.42 [IQR 2.18, 2.68] ng/mL; *P*=0.581). However, serum 25(OH)D_3 levels were significantly lower in the experimental group than in the control group (12.00 [IQR 9.83, 14.94] ng/mL vs. 17.70 [IQR 15.51, 20.14] ng/mL; *P*<0.001). Serum 25(OH)D levels were significantly lower in the experimental group than in the control group (14.27 [*IQR* 12.33, 18.01] ng/mL vs. 20.06 [*IQR* 18.17, 22.69] ng/mL; *P* < 0.001). The proportion of vitamin D insufficiency was significantly higher in the experimental group than in the control group (88/101, 87.1% vs. 49/101, 48.5%; Table 3; *P* < 0.001). No statistically significant differences in sex or age were observed between the two groups.

Table 3. Comparison of baseline characteristics between the test and control groups

Variables	Control Group (n = 101)	Patients Group (n = 101)	Statistical value ($t/Z/\chi^2$)	P value
Sex [n (%)] female/male	71 (70.3%)/30 (29.7%)	60(59.4%) 41 (40.6%)	2.628[a]	0.105
Age (years, $\bar{x} \pm s$)	43.66 ± 12.61	46.58 ± 12.58	1.651[b]	0.100
25(OH)D_2 [ng/mL, *M (Q1, Q3)*]	2.42 (2.12, 2.72)	2.42 (2.18, 2.68)	−0.553[c]	0.581
25(OH)D_3 [ng/mL, *M (Q1, Q3)*]	17.70 (15.51, 20.14)	12.00 (9.83, 14.94)	−8.678[c]	<0.001
25(OH)D [ng/mL, *M (Q1, Q3)*]	20.06 (18.17, 22.69)	14.27 (12.33, 18.01)	-8.930[c]	< 0.001
25(OH)D insufficiency proportion [n (%)]	49 (48.5%)	88 (87.1%)	34.502[a]	< 0.001

[a] The χ^2 values were derived from the Pearson chi-square test: χ^2 = 2.628 for the sex distribution comparison and χ^2 = 34.502 for the 25(OH)D insufficiency proportion comparison.

[b] The t value was derived from the independent-samples t-test for the age comparison (t = 1.651).

[c] The Z values were derived from the Mann-Whitney U test for comparisons of 25(OH)D_2, 25(OH)D_3, and total 25(OH)D, which did not follow a normal distribution (Z = −0.553, −8.678, and −8.930, respectively).

3.2.2 Serum 25(OH)D Status in the Patients Group

The median serum 25(OH)D level in female patients was 13.96 ng/mL (*IQR*: 12.21–18.21 ng/mL), which was lower than the median serum 25(OH)D level of 14.81 ng/mL (*IQR*: 13.07–17.11 ng/mL) in male patients (two-sided P = 0.4031). Similarly, the prevalence of vitamin D deficiency (total 25(OH)D < 20 ng/mL) was 84.51% (60/71) in female patients and 93.33% (28/30) in male patients (P = 0.3344). A significant difference in age was observed between female and male patients: the mean age of female patients was 48.9 years, which was higher than the mean age of 41.1 years in male patients (P = 0.0042).

3.2.3 ROC Curve Analysis of Serum 25(OH)D for Thyroid Malignancy

The ROC curve analysis is shown in Figure 4, demonstrating that both total 25(OH)D and 25(OH)D_3 possessed high auxiliary diagnostic value for thyroid malignancy, with AUCs of 0.864 for total 25(OH)D and 0.853 for 25(OH)D_3, respectively. Using the *Youden* index, the optimal cut-off values were 16.42 ng/mL for total 25(OH)D (sensitivity 69.3%, specificity 96.0%) and 13.91 ng/mL for 25(OH)D_3 (sensitivity 72.3%,

specificity 93.1%). The AUC for 25(OH)D_2 was 0.522, indicating low discriminatory performance, which is consistent with the fact that the vast majority of endogenous circulating vitamin D is in the D_3 form.

4 Discussion

Current clinical vitamin D testing predominantly relies on automated immunoassays, which, despite offering high throughput and operational convenience, carry intrinsic methodological limitations that are incompatible with the demands of precision medicine. Mainstream reagents show 25(OH)D_2 cross-reactivity of only 40%–70%, leading to potential underestimation of true vitamin D status in subjects receiving 25(OH)D_2 supplementation [12–14]. Furthermore, biologically inactive C3-epimers are structurally highly similar to active vitamin D and can account for 10%–60% of total measured 25(OH)D in infants, young children, and subjects receiving high-dose supplementation; immunoassay antibodies cannot distinguish these epimers, potentially causing falsely elevated results [15]. In circulation, over 99% of 25(OH)D is tightly bound to vitamin D-binding protein [4], and the chemical dissociation reagents used in immunoassays may fail to achieve complete dissociation in samples from pregnant women, critically ill patients, and subjects with chronic kidney disease, leading to matrix-effect-driven inaccuracies [18].

LC-MS/MS does not rely on antibody recognition; instead, it achieves absolute quantitation through specific precursor/product ion mass transitions and is internationally recognized as the gold standard for vitamin D measurement [17]. This method can independently measure 25(OH)D_2 and 25(OH)D_3 concentrations and calculate total levels, thereby eliminating the 25(OH)D_2 recognition blind spot at the source [2]. Through chromatographic separation, it can precisely remove C3-epimer interference, ensuring that results reflect only biologically active vitamin D concentrations. Combined with liquid-liquid extraction sample preparation and stable isotope internal standardization, it effectively corrects for matrix ion suppression effects, delivering accurate quantitative results even for complex pathological samples [19].

In the present study, the RZ-500 LC-MS/MS system demonstrated excellent overall performance, with precision, accuracy, linear range, LOQ, and carryover resistance all meeting industry standards and the manufacturer's specifications. Clinical analysis revealed that serum total 25(OH)D levels in patients with thyroid malignancy were significantly lower than those in healthy controls (14.27 vs 20.06 ng/mL, $P < 0.001$), with vitamin D insufficiency reaching 87.1% in the patient group, corroborating the close association between vitamin D deficiency and thyroid malignancy. ROC curve analysis showed an AUC of 0.864 for total 25(OH)D and 0.853 for 25(OH)D_3, indicating good auxiliary diagnostic value; the AUC for 25(OH)D_2 was only 0.522, showing no meaningful predictive performance, consistent with the fact that 25(OH)D_2 originates primarily from exogenous intake, circulates at low concentrations, and lacks an endogenous association with tumor biology, a finding that also underscores the clinical significance of the mass spectrometric ability to distinguish between isoforms.

Because this is a retrospective case-control study, it can only establish an association between low 25(OH)D and thyroid cancer, not a causal relationship. The observed association may instead reflect reverse causation, whereby thyroid cancer itself reduces vitamin D intake or sun exposure, or residual confounding

by factors such as age and season. It is likewise entirely plausible that individual patients with thyroid cancer have normal 25(OH)D levels, since tumour heterogeneity, disease stage, and prior vitamin D supplementation, and outdoor exposure all vary across patients; a population-level trend does not determine the outcome in any single individual. Nearly half of the subjects in the health checkup group still had low vitamin D levels. This likely reflects the fact that individuals referred for mass-spectrometry-based vitamin D testing carry other causes of vitamin D deficiency beyond thyroid cancer, such as limited sunlight exposure. Despite this, the overall trend still showed that patients with thyroid cancer had significantly lower vitamin D levels than the checkup group. Vitamin D, therefore, cannot be used alone to diagnose thyroid cancer, for which confirmation relies on ultrasonography combined with fine-needle aspiration cytology; it should be regarded only as an auxiliary reference for risk assessment, or as one variable to be incorporated into future multi-marker integrative models.

Given that the present study is limited by its single-center, small-sample, retrospective design, the generalizability of the findings should be interpreted with caution. Leveraging the high-throughput mass spectrometry platform established in our laboratory, future multi-center cohort studies will validate this association across broader clinical populations. Moreover, the exceptional specificity of mass spectrometry-based detection positions this standardized biomarker measurement system to extend beyond single-disease assessment, providing a rigorous and methodologically robust foundation for future exploration of the complex pathophysiological links between thyroid dysfunction and other major degenerative diseases.

Conclusion

The RZ-500 LC-MS/MS system met the manufacturer's declared specifications and relevant guideline requirements for precision, accuracy, linear range, lower limit of quantification, and carryover. In clinical application, serum total 25(OH)D was significantly lower in patients with thyroid malignancy than in matched controls (14.27 vs. 20.06 ng/mL, $P < 0.001$). Vitamin D insufficiency was present in 87.1% of patients. Owing to isomer-specific chromatographic separation and stable isotope internal standards, the AUCs of total 25(OH)D and 25(OH)D_3 for discriminating thyroid malignancy were 0.864 and 0.853, respectively. At the optimal cutoff of 16.42 ng/mL, the specificity reached 96.0%. These results indicate that LC-MS/MS is the gold-standard method for vitamin D measurement and may serve as a reliable auxiliary biochemical marker for thyroid malignancy diagnosis.

Declarations

Author Contributions

Yujian Xie and Runqing Li conceptualized and wrote the manuscript. Other authors participated in the operation and data collection. All authors edited the manuscript and approved the final version.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare that they have no competing interests. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Figures

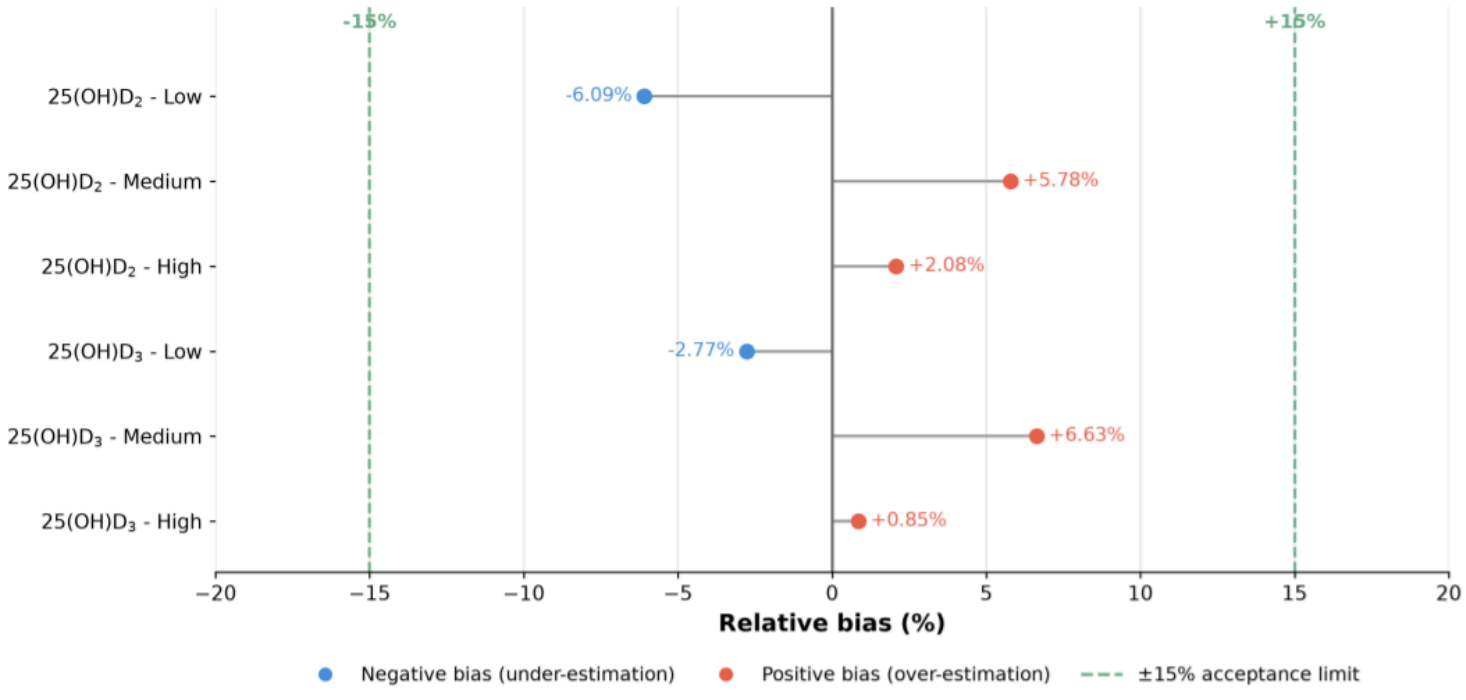


Figure 1

Bias assessment using certified reference materials

Relative bias assessment for 25(OH)D_2 and 25(OH)D_3 measured by the RZ-500 system against certified reference materials. The bias for all concentration levels was within ±1/2 TEa (12.5%), indicating excellent method accuracy.

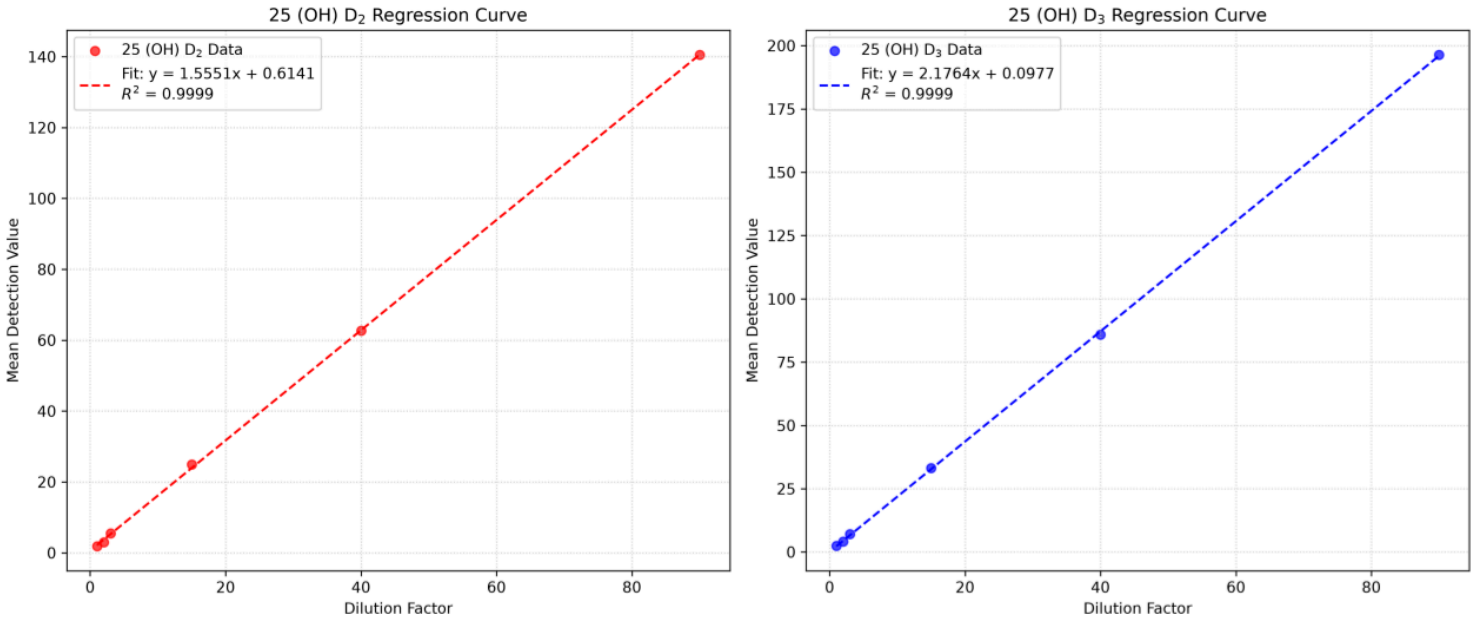


Figure 2

Linearity verification results

Evaluation of the linear response range for 25(OH)D_2 and 25(OH)D_3. Both analytes exhibited excellent linearity, with non-linearity bias well below the 1/2 TEa acceptance limit, fully meeting clinical requirements.

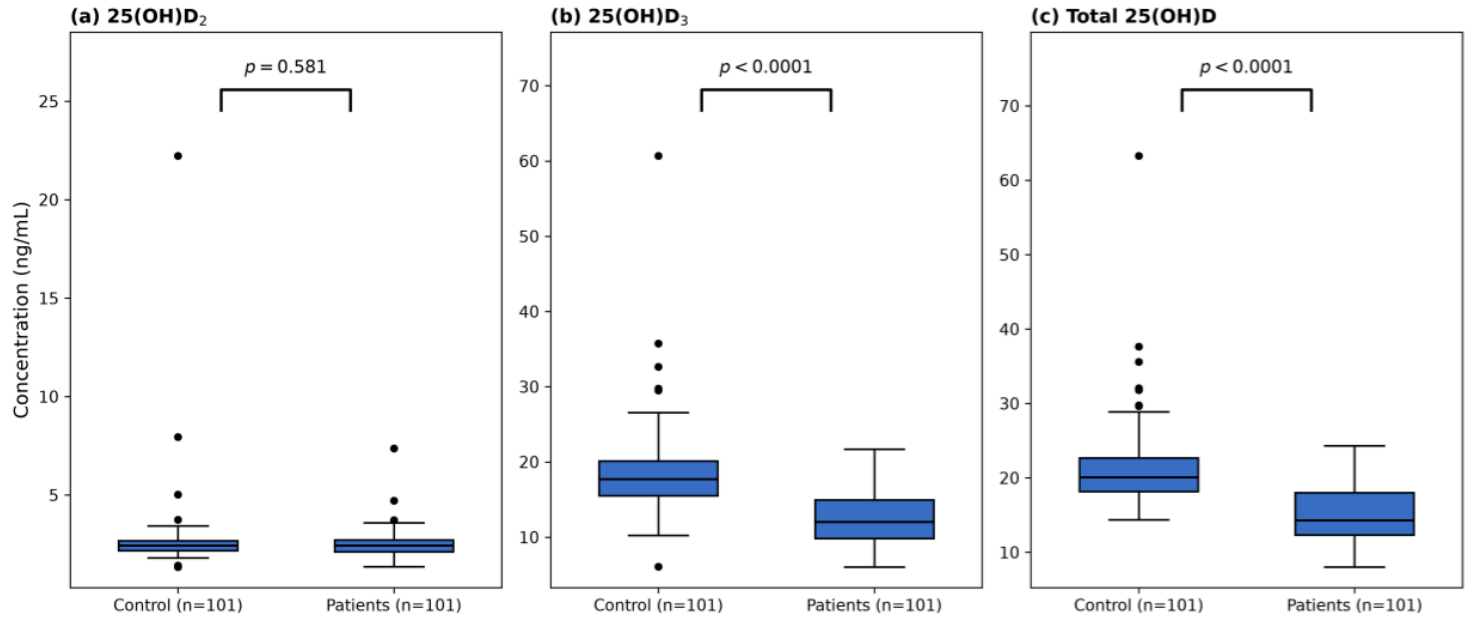


Figure 3

Comparison of Serum 25(OH)D_2, 25(OH)D_3, and Total 25(OH)D Levels between the Thyroid Malignancy and Control Groups

Each box represents the interquartile range (IQR), with the median indicated by the central line and whiskers extending to 1.5 times the IQR. The plots compare 25(OH)D_2, 25(OH)D_3, and total 25(OH)D levels between the control and patient groups, with 25(OH)D_3 and total 25(OH)D significantly lower in patients ($p < 0.0001$).

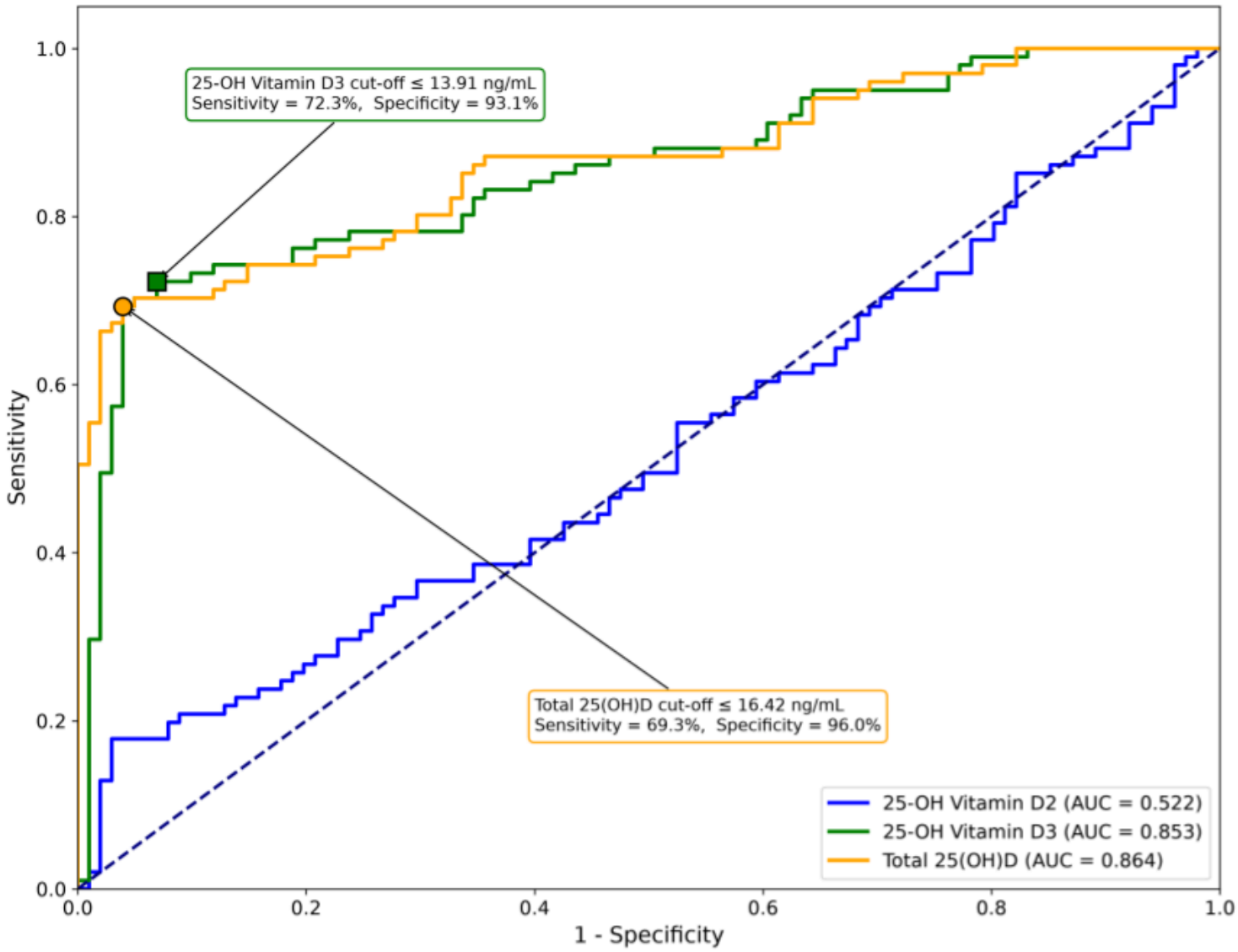


Figure 4

ROC curve depicting the predictive value of serum 25(OH)D levels for thyroid malignancies

ROC curves of serum 25-hydroxyvitamin D markers for discriminating thyroid malignant tumor from healthy controls. The AUC was 0.864 for total 25(OH)D, 0.853 for 25(OH)D_3, and 0.522 for 25(OH)D_2. By the Youden index, the optimal cut-offs were ≤ 13.91 ng/mL (sensitivity 72.3%, specificity 93.1%) for 25(OH)D3 and ≤ 16.42 ng/mL (sensitivity 69.3%, specificity 96.0%) for total 25(OH)D.