FUZZY LOGIC-BASED MOBILE PHASE OPTIMIZATION AND DIFFERENTIAL ANALYSIS OF LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY DETECTION OF COENZYME Q10 AND 25-HYDROXYVITAMIN D

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Abstract:

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) serves as a key tool for the test of lipophilic substances in laboratory medicine and is widely employed in the analysis of coenzyme Q10 (CoQ10) and 25-hydroxyvitamin D (25OHD). In this paper, fuzzy concept was applied to improve the LC-MS/MS methods used for CoQ10 and 25OHD detection. The focus was placed on selecting the optimal mobile phase for CoQ10 analysis and examining the differences between LC-MS/MS and chemiluminescence immunoassay (CLIA) methods for 25(OH)D measurement. Through screening various organic phase combinations and employing fuzzy inference, the optimal mobile phase ratio for CoQ10 test is determined to be methanol and isopropanol at a ratio of 8:2. Additionally, fuzzy logic was employed to analyze the variations in 25OHD concentrations across different sexes and age groups. The results showed that women aged 30 – 40 exhibited greater differences in 25(OH)D levels compared to other groups. This study shows that the use of fuzzy concepts can enhance the adaptability and accuracy of LC-MS/MS detection, offering a novel approach to the analysis of lipophilic substances.

Keywords:

LC-MS/MS, fuzzy, CoQ10, 25(OH)D, reasoning

1. Introduction

With the rapid advancement of science and technology, artificial intelligence (AI) has made remarkable progress, demonstrating powerful computational capabilities and high accuracy, and has been widely applied across various fields [1]. As a critical component of the medical domain, laboratory medicine has achieved a high degree of automation with emphasis on the ability to reason at the human level. In recent years, the application of AI in laboratory medicine has garnered significant attention. The vast number and variety of testing items, coupled with the continuous iteration, updating of testing methods, and technological advancements, highlight the potential of AI in this field [1].

However, in the biomedical field, many concepts are inherently ambiguous or uncertain [2], making precise definitions challenging. As illustrated in Figure 1, some concepts are often described using imprecise terms such as “good” or “poor.” For example, the statement “poor public health conditions exacerbate the spread of infectious diseases” uses “poor” as a term that has different ways to quantify. Among various AI technologies, fuzzy logic offers a significant advantage in addressing such ambiguous concepts by providing reasoning. By transforming vague linguistic variables into quantifiable data, a fuzzy system enables quantitative reasoning, thereby facilitating the resolution of such fuzzy problems [3, 4].

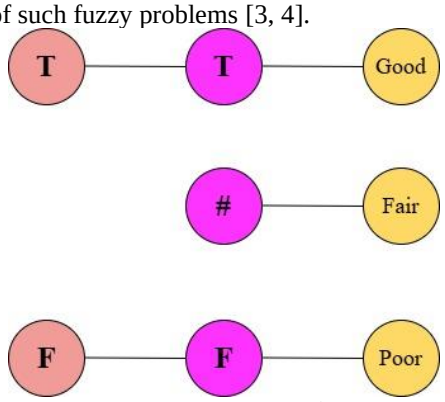

FIGURE 1. Fuzzy logic operation diagram. In fuzzy logic, there is typically no clear distinction between "good" and "poor"; instead, it often lies in between.

In recent years, the application of LC-MS/MS in laboratory medicine testing has shown a significant increase. Compared to many other testing technologies, LC-MS/MS offers high specificity and strong resistance to cross-reaction interference, enabling effective

differentiation of similar compounds [5, 6], such as vitamin D2 and D3. Its exceptional sensitivity, with detection limits reaching pg/ml, allows for the precise detection of substances at extremely low concentrations [6]. Furthermore, this technology decreases manual intervention, significantly improving reproducibility [5]. Its integration with AI technologies is also on the rise, demonstrating considerable potential in areas such as optimization of experimental conditions and ion source selection [7].

Lipid-soluble substances have consistently played a significant role in LC-MS/MS analysis, with 25-hydroxyvitamin D (25(OH)D) and coenzyme Q10 (CoQ10) being key components. The levels of these two substances are closely related to various diseases. 25(OH)D serves as a critical indicator of vitamin D status in the body, positively contributing to bone health and chronic disease prevention [8]. On the other hand, CoQ 10 is significantly linked to the occurrence and progression of cardiovascular diseases (CVD) [9].

However, several uncertainties persist in studies utilizing LC-MS/MS for the detection of lipid-soluble substances such as CoQ10 and 25(OH)D [10]. For instance, in the methodological development for CoQ10, selecting the optimal mobile phase ratio requires balancing safety and optimal peak time. Regarding 25(OH)D, differences in results between LC-MS/MS and chemiluminescence immunoassay (CLIA), beyond methodological variations, may be influenced by other factors, potentially affecting the accuracy of LC-MS/MS detection [6].

The fuzzy concept offers promising potential to address these relatively ambiguous issues to some extent. Thus, this study aims to investigate the potential of using fuzzy in optimizing LC-MS/MS for the testing of lipid-soluble substances (CoQ10 and 25(OH)D), by thoroughly analyzing and addressing methodological uncertainties. Specifically, the study will focus on optimizing the mobile phase ratio for CoQ10 detection to achieve an ideal balance between safety and peak time. Concurrently, it will explore the factors contributing to discrepancies in 25(OH)D results between LC-MS/MS and CLIA, assessing the impact of potential variables beyond methodological differences on detection accuracy. By using fuzzy logic for quantitative reasoning of these ambiguous issues, this study seeks to resolve key challenges in CoQ10 and 25(OH)D detection and also to provide valuable insights for addressing similar issues in the testing of other substances. Ultimately, this study strives to improve the precision and reliability of LC-MS/MS testing, offering innovative technical support for laboratory medicine testing.

2. Methods

LC-MS/MS is considered the gold standard for detection 25(OH)D due to its ability to differentiate between 25(OH)D2 and 25(OH)D3 [6]. However, in clinical practice, CLIA is widely used for its simplicity and speed [6]. Investigating the differences between these two methods can provide valuable insights for clinical testing and offer a robust basis for optimizing LC-MS/MS. Thus, this research collected 138 random serum samples from routine physical examinations to analyze the differences in detection results while employing fuzzy logic to explore whether these discrepancies are influenced by additional factors such as age, gender. Furthermore, to improve the testing method for CoQ10, this study focuses on screening mobile phases by employing fuzzy concept to determine the optimal mobile phase ratio.

2.1. Samples used

In this paper, we have utilized the samples collected by the Sichuan Taikang Hospital (Chengdu, Sichuan Province, China). The samples provided were collected from patients undergoing 25(OH)D chemiluminescence immunoassay (CLIA) testing during routine physical examinations at Sichuan Taikang Hospital (Chengdu, Sichuan Province, China) between August 2024 and January 2025. Samples that were positive for cardiovascular diseases or severe infectious diseases (hepatitis B, hepatitis C, syphilis, or HIV) were excluded. The data used in this study were provided by Sichuan Taikang Hospital and have gained approval by the ethics committee of the Sichuan Taikang Hospital.

2.2. Using Fuzzy to analyse the testing of 25(OH)D

The differences between the two detection methods (LC-MS/MS and CLIA), beyond methodological factors, could also be influenced by other variables such as age and gender. This study establishes a multi-input-one-output (MISO) structural model, as shown in Figure 2. To establish the fuzzy rules from the input-output data, Generating Fuzzy Inference System (GENFIS) in the Matlab toolbox was used to build the fuzzy rule inference system. This study uses the 25(OH)D concentration values from the collected samples detected by LC-MS/MS and CLIA, with age and gender as the input variables and the difference coefficient between the two methods as the output variable. The input and output variables were standardized within the interval of [0,1].

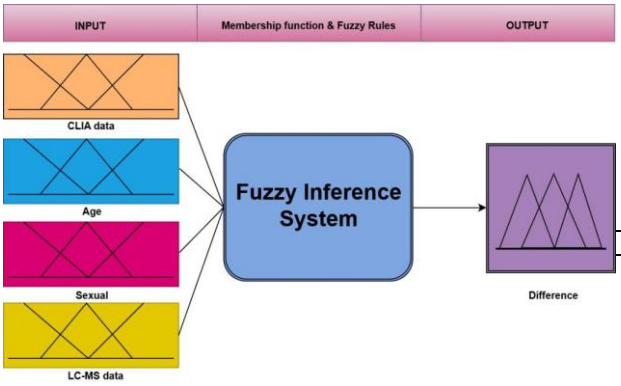

FIGURE 2. The MISO Fuzzy model. CLIA and LC-MS individually represent their testing results of 25(OH)D

GENFIS provided in Matlab Fuzzy Toolbox utilizes FCM clustering to create the fuzzy inference system. The overall equation of the GENFIS is given as follows (1):

$$J_m = \sum_{i=1}^{D} \sum_{j=1}^{N} \mu_{ij}^{m} \|x_i - c_j\|^2 \quad (1)$$

D is the total number of data points, *N* represents the number of sets, *m(>1)* shows the fuzzy overlap degree, x_i is the *i-th* data point, c_j is the *j-th* cluster centre, and μ_{ij} means the membership degree of x_i in the *j-th* sets.

2.3. Fuzzy inference for mobile phase optimization in LC-MS/MS detection of CoQ10

The selection of the mobile phase is crucial for mass spectrometry detection, as its composition ratio directly determines the elution time and sensitivity. The experiment involved preliminary screening of mobile phase combinations using standards and clinical samples, which were subjected to LC-MS/MS testing with different mobile phases after pretreatment. Regarding the selection of the mobile phase, due to the large molecular weight (863.34) and strong hydrophobicity of CoQ10, which resulted in strong retention in the chromatographic column and difficulty in elution, a pure organic phase was used for elution. Conventional elution reagents (methanol, acetonitrile, ethanol, and isopropanol, all containing 0.1% formic acid) and their combinations were screened (as shown in Table 1). Based on the results in Table 2, groups 1, 2, 5, 7, and 8 were excluded due to low signal-to-noise ratio (S/N), poor retention, or excessively long elution time, as evaluated by retention time and S/N. For groups 3, 4, and 6, a comprehensive comparison of toxicity and cost was conducted: toxicity ranked as acetonitrile > methanol > isopropanol > ethanol, and price ranked as ethanol > acetonitrile > isopropanol > methanol. Ultimately, group 3 (a methanol-isopropanol solution containing 0.1% formic acid) was selected as the mobile phase.

TABLE 1. Mobile phase grouping information

Group	Component 1 (0.1% by volume)	Component 2 (99.9% by volume)
1	Formic acid	Methanol
2	Formic acid	Acetonitrile
3	Formic acid	Methanol:Isopropanol (7:3)
4	Formic acid	Methanol:Ethanol (5:5)
5	Formic acid	Methanol:Acetonitrile (5:5)
6	Formic acid	Ethanol:Acetonitrile (5:5)
7	Formic acid	Ethanol
8	Formic acid	Isopropanol

TABLE 2. Comparison of chromatographic parameters across different groups

Group	Peak Area (mV.s)	Peak Height (mV)	Retention Time(min)	Half Peak Width (min)	S/N
1	481500	67650	3.77	0.11	4290.6
2	351800	24360	8.76	0.22	1825.6
3	470500	133800	1.24	0.05	3948
4	522100	156600	1.12	0.05	2820.8
5	512000	47720	6.27	0.16	4323.1
6	75640	21650	1.24	0.05	2562.3
7	1222000	376900	0.59	0.05	309.7
8	896900	266500	0.39	0.05	3669.1

However, the earliest elution time is 1.16 min (with an isopropanol-to-methanol ratio of 3:7), while the optimal elution time is approximately 33.33%-66.66% of a single injection duration [11]. Therefore, fuzzy inference is employed to decrease the appropriate elution time and select the most suitable elution time. The model inputs were the proportions of methanol (Figure 3A) and isopropanol (Figure 3B), with initial concentrations set at 70% methanol and 30% isopropanol (y-axis value of 1). The output was the elution time, with the initial starting point being the earliest elution time of 1.16 min (y-axis value of 1). The input function adopted the triangular function with an equidistant distribution, which is most commonly used in fuzzy inference for biomedical applications; in the output model, each membership function followed an equilateral triangular distribution. The midpoint of the membership function for the medium level of elution time is selected at the median value of a single injection duration, 1.75 min, with the two endpoints determined as 1.455 min and 2.045 min based on the equidistant distribution principle (equal intersection areas), as shown in Figure 3. The fuzzy rules were based on the "IF-THEN" rule:

1. If methanol is high and isopropanol is low, the retention

time is high.

2. If methanol is medium and isopropanol is medium, the retention time is medium.
3. If methanol is low and isopropanol is high, the retention time is low.

The calculation formula is adopted in (2):

$$\mu A \cap B(x) = min\,[\mu A(x), \mu B(x)] \quad (2)$$

The model predicts the ratio range of methanol to isopropanol from 7:3 to 9:1.

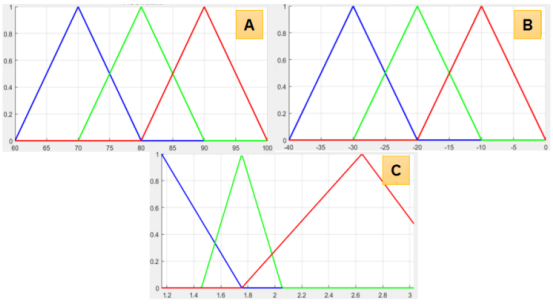

FIGURE 3. The membership function of the mobile phase optimization in testing CoQ10; The blue, green, and red lines represent low, medium, and high levels, respectively.

3. Results

3.1 Fuzzy analysis of testing 25(OH)D

GENFIS generates membership functions by extracting the relationship between input and output variables from 138 samples (as shown in Figure 4). The results indicated that the variability across age groups in males exhibits no significant change. The variability in females aged 30-40 is higher than that in other age groups and all male age groups (see Table 3).

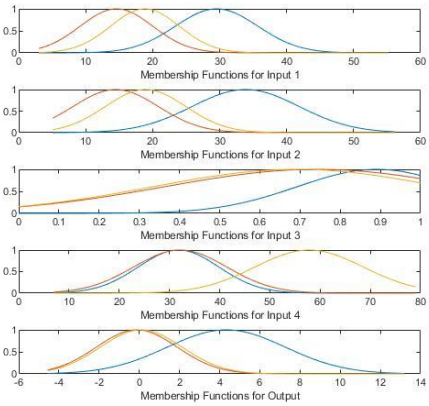

FIGURE 4. The membership function of the fuzzy analysis of 25(OH)D; Input1, input2, input3, and input4 are the CLIA testing results, LC-MS/MS testing results, gender, and age, respectively; Bule, red and yellow lines separately represent cluster1, 2 and 3.

TABLE 3. The prediction of differences in the women's between different ages by the fuzzy system

Age	The prediction of difference
20	0.299
30	0.348
35	0.349
40	0.342
50	0.285
>60	< 0.27

3.2 Fuzzy inference for mobile phase optimization

The model predicts the elution times for methanol-to-isopropanol concentration ratios of 8:2, 8.5:1.5, and 9:1, acquiring results of 1.76 min, 2.29 min, and 3.2 min, respectively. The model of the 8:2 and 8.5:1.5 are shown in Figure 5. The 3.2 min elution time is close to the end of a single injection duration and is therefore discarded. The optimal elution time range is between 1/3 and 2/3 of a single injection duration (33.33%-66.66%) [11]. Although 2.29 min falls within this range, it is too close to the lower limit. Considering that experimental errors may cause the elution time to vary and not remain consistent each time, the 8:2 ratio is selected as the mobile phase concentration ratio for LC-MS/MS testing of CoQ10.

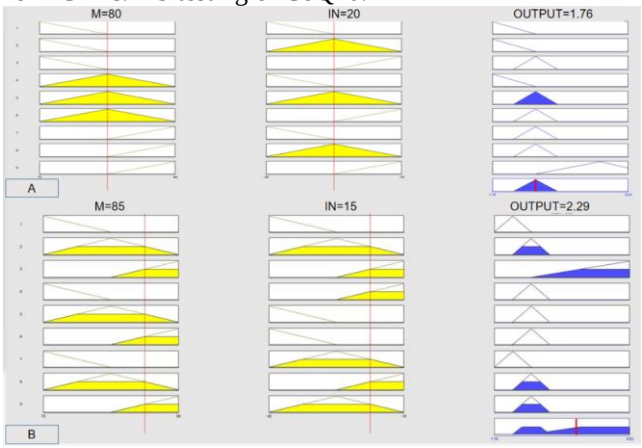

FIGURE 5. The prediction elution times of the fuzzy inference system; A for a methanol-to-isopropanol ratio of 8:2, and B for 8.5:1.5.

3.3 Fuzzy inference validation

To analyze the difference inference between LC-MS/MS and CLIA in testing 25(OH)D by the fuzzy system, an additional 24 samples that were not used when establishing the model were used as testing. The additional

data were from a test group (10 women aged 30-40 years) and a control group (14 individuals from other demographics). The threshold is set as the mean difference value of these additional samples between the two methods, determined to be 2.95 ng/mL. The relative risk (RR) between the two groups is evaluated as 2.1 (95% CI: 0.7953-5.5442) (as shown in Table 4). This verification result provides early support for the fuzzy inference. However, as the confidence interval includes 1, further validation with an expanded sample size may be necessary to confirm the results in future.

TABLE 4. The RR value of the two groups' differences

	High difference	Low difference
Test group	0.6 (6/10)	0.4 (4/10)
Control group	0.2857 (4/14)	0.71 (10/14)
RR (95% CI)	2.10 (0.7953-5.5442)	

To verify the fuzzy reasoning method for mobile phase selection, 10 mobile phases were prepared with methanol and isopropanol using the 8:2 ratio. These were tested on five samples from the same batch, with peak times recorded. The mean peak time across the 10 mobile phases is 1.749 ± 0.119 min, with a coefficient of variation (CV) of 6.8%. The deviation between the average peak time and the model-predicted time is 0.63%, indicating a minor difference from the predicted time by the fuzzy system, thus confirming the effectiveness of some content.

4. Discussions

The primary aim of this study is to leverage the capability of fuzzy in handling uncertainty or incomplete data by applying it to specific processes in the LC-MS/MS measuring of 25(OH)D) and CoQ10 [12]. This method aims to optimize detection conditions and enhance the precision and reliability of the testing method, thereby providing valuable insights and evidence. The results show that the differences in 25(OH)D detection between LC-MS/MS and CLIA may be more pronounced in women aged 30-40 years. Analysis of input data features using the fuzzy system reveals that the age membership function (Input4) shows significant overlap between cluster 3 and clusters 1 and 2, indicating the notable contribution of the 30-40 age group to the observed differences. For the gender membership function (Input3), all clusters trend toward 1 (male = 0, female = 1), highlighting a more substantial influence of female gender on the differences. However, this study has certain limitations. The sample used is predominantly female (104 females, 38 males), primarily because the dataset obtained is a retrospective study based on health checkup participants. In China, women may prioritize health checkups more than men [13], which could be a significant factor contributing to sample bias. However, the male sample size is still more than the minimum statistical threshold (20 cases) for biomedical studies. Furthermore, an important strength of fuzzy logic lies in its ability to perform analysis despite limitations in data. The validation results partially show the accuracy of the predictions. Another advantage is the use of human understandable rules which provide strong reasoning and justifications for the outcomes,

In the selection of mobile phases, the fuzzy system demonstrated its accuracy, as evidenced by the verification results showing a mere 0.63% deviation between the average peak time and the predicted time. The effective application of fuzzy reasoning in the LC-MS/MS detection of CoQ10 significantly reduces experimental time and resource consumption, thereby improving research efficiency.

This study provides some initial outcomes to demonstrate the potential of using fuzzy logic in the application of LC-MS/MS detection. By using fuzzy logic in the testing of two representative lipid-soluble substances (25(OH)D and CoQ10), research efficiency has been enhanced, largely avoiding unnecessary experiments and massive data collection. The accuracy and capability to handle incomplete experimental data underscore the promise of fuzzy logic in biomedical applications [14]. Indeed, numerous studies have shown that fuzzy logic holds significant potential in areas such as laboratory medicine-related epidemiology, disease prediction, and optimization of experimental techniques [14-16]. Given that collecting comprehensive and high-quality datasets is often challenging for most research institutions, fuzzy can help to minimise this issue [17]. However, fuzzy logic is not without limitations [3，18]. Nonetheless, it can streamline or eliminate numerous steps in biomedical research [19], substantially enhancing research efficiency.

This study, while enhancing the liquid LC-MS/MS testing of CoQ10 and25(OH)D, also provided valuable insights for applying fuzzy logic to the LC-MS/MS detection of other lipid-soluble substances and beyond. It provides some support for improving the precision and reliability of LC-MS/MS techniques.

5. Conclusions

This study focuses on two lipid-soluble substances, CoQ10 and 25(OH)D, by applying fuzzy logic to their detection via LC-MS/MS. The validation results provided initial confirmation of the accuracy of fuzzy reasoning predictions. By using fuzzy logic in the LC-MS/MS detection of these two lipid-soluble substances, the study

can improve the test effectiveness and provide valuable reference for the application of LC-MS/MS to other substances. Further research in optimizing the use of fuzzy in this area could improve the accuracy and workflow of such detection.

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