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ОДНОВРЕМЕННЫЙ КОЛИЧЕСТВЕННЫЙ ^{1}H ЯМР АНАЛИЗ МЕТАНОЛА, ЭТАНОЛА И ИХ МЕТАБОЛИЧЕСКИХ ПРОДУКТОВ В ПЛАЗМЕ ЧЕЛОВЕКА: РАННИЙ ДИАНОЗ И МОНИТОРИНГ ВО ВРЕМЯ ЛЕЧЕНИЯ ОСТРОГО ОТРАВЛЕНИЯ МЕТАНОЛОМ ВО ВЬЕТНАМЕ

Та Тхи Тхао, Нгуен Тхи Нган, Ву Ан Фуонг, Ха Тран Хунг, Нгуен Ван Тхык, Фам Куанг Трунг

Та Тхи Тхао, Нгуен Ван Тхык, Фам Куанг Трунг*

Химический факультет, Университет естественных наук, Ханойский государственный университет, Ле Тхан Тонг, 19, Хоан Кием, Ханой, Вьетнам, 110401
E-mail: tathithao@hus.edu.vn, nguyenvanthuc@vnu.edu.vn, trungpham781@hus.edu.vn*

Нгуен Тхи Нган, Ву Ан Фуонг

Центр контроля ядов, Больница Бах Май, Ханой, Вьетнам, 116301
E-mail: ngannt05101993@gmail.com, vuanhphuong_pcc@yahoo.com

Ха Тран Хунг

Отделение неотложной помощи, интенсивной терапии и токсикологии, Ханойский медицинский университет, тон Тхат Тунг, 1, Донг да, Вьетнам, 116001
E-mail: hatranhung@yahoo.com

В данной работе был установлен метод ядерного магнитного резонанса (^{1}H-ЯМР) для одновременного количественного определения метанола, этанола и продуктов их метаболизма (муравьиная кислота и уксусная кислота) в плазме человека. Эффект белка в образце плазмы был удален 25% трихлоруксусной кислотой (TCA) с соотношением TCA и образца плазмы при 1/5 (об./об.). Небольшое количество D_2O (приблизительно 1/10 об./об.) было выбрано из-за того, что влияние водяного сигнала было устранено с помощью предварительного подавления воды с помощью последовательности импульсов NOESY. Были проверены различные аналитические параметры, такие как линейность, точность, точность и специфичность, предел обнаружения прибора (IDL) и предел количественного определения прибора (IQL). Предел обнаружения прибора четырьмя соединениями в пустой плазме варьировался от 0,68 (для метанола) до 2,88 мг/л (для муравьиной кислоты). Среднее извлечение концентрации в динамических диапазонах составило от 96 до 105%. Хорошая линейность между концентрацией, рассчитанной по интегральному сигналу, и измеренной, полученной по калибровочным кривым, подтвердила, что концентрацию анализируемых веществ в плазме можно непосредственно определить из сигнала протона, полученого из спектроскопии ядерного магнитного резонанса (^{1}H-ЯМР). В данной работе предложенный метод был успешно применен для прямого определения пяти соединений в 9 образцах плазмы пациента с удовлетворительными результатами по сравнению с методом GC/FID. Аналитическая процедура может быть полезна для диагностики и оценки лечения отравления метанолом во вьетнамских больницах.

Ключевые слова: метанол, этанол, Вьетнам, магнитный, протон

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SIMULTANEOUS QUANTITATIVE ^{1}H NMR ANALYSIS OF METHANOL, ETHANOL AND THEIR METABOLIC PRODUCTS IN HUMAN PLASMA: EARLY DIANOSIS AND MONITORING DURING TREATMENT OF ACUTE METHANOL POISONING IN VIETNAM

Ta Thi Thao, Nguyen Thi Ngan, Vu Anh Phuong, Ha Tran Hung, Nguyen Van Thuc, Pham Quang Trung

Ta Thi Thao, Nguyen Van Thuc, Pham Quang Trung*

Faculty of Chemistry, VNU University of Science - Hanoi, Le Thanh Tong Str., 19, Hanoi, 110401, Vietnam
E-mail: tathithao@hus.edu.vn, nguyenvanthuc@vnu.edu.vn, trungpham781@hus.edu.vn*

Nguyen Thi Ngan, Vu Anh Phuong

Poison Control Center, Bach Mai Hospital, Hanoi, 116301, Vietnam
E-mail: ngannt05101993@gmail.com, vuanhphuong_pcc@yahoo.com

Ha Tran Hung

Department of Emergency, Intensive Care Medicine and Toxicology, Hanoi Medical University, Ton That Tung, 1, Dong Da, Hanoi, 116001, Vietnam
E-mail: hatranhung@yahoo.com

The ^{1}H-NMR method for simultaneous quantification of methanol, ethanol and their metabolic products (formic acid and acetic acid) in human plasma was established. The effect of protein in plasma sample was removed by 25% trichloroacetic acid (TCA) with the ratio of TCA and plasma sample at 1/5 (v/v). The small amount of D_2O (estimated 1/10 v/v) was chosen due to the effect of water signal was eliminated by using the advance water suppression with NOESY pulse sequence. The different analytical parameters such as linearity, precision, accuracy, and specificity, instrument detection limit (IDL) and instrument quantification limit (IQL) were validated. The instrument detection limit of four compounds in blank plasma varied from 0.68 (for methanol) to 2.88 mg/L (for formic acids). The average recoveries of concentration in dynamic ranges were found to be 96 to105%. The good linearity between concentration calculated by integrated signal and measured obtained by calibration curves confirmed that concentration of analytes in plasma can be directly determined from the ^{1}H-NMR signal. The proposed method has been successfully applied for direct determination of five compounds in 9 patient's plasma samples with the satisfactory results compared to GC/FID method. The analytical procedure can be useful for diagnosis and evaluation of treatment of methanol poisoning in Vietnamese hospitals.

Key words: methanol, ethanol, Vietnam, magnetic, proton

INTRODUCTION

In recent years, the methanol poisoning in Vietnam is increasing with a large number of poisonous patients. In 2014, there were only 16 methanol poisoning patients transferred to Poison Control Center – Bach Mai hospital, but it reached 33 and 46 patients in 2015, 2016, and it was 48 patients within the first six months of 2017. It is alarming that the group poisoning cases (40 methanol poisoning victims and 9 deaths) happened in Lai Chau or Gia Lai province in 2017. Practically, the early diagnosis [1, 2] of methanol poisoning is difficult because that clinical symptom of methanol poisoning is quite similar to other ones such as those of ethanol, ethylene glycol, and isopropanol poisoning. This led to the delay application of the specific treatments for the patients that resulted in a relatively high mortality rate of 18-54%. The toxicity of methanol for human is caused by very toxic formic acid formed from formaldehyde which is metabolite of methanol [3, 4]. In methanol poisoning treatment, ethanol is used as antidote for limiting the metabolism to formic acid, and then methanol and ethanol should be eliminated by haemodialysis. Therefore, an early monitoring of ethanol, methanol and their metabolic products simultaneously in human plasma just after patients enter to the hospital and during treatment is very important in methanol poisoning treatment.

Traditionally, the simultaneous quantification of methanol, ethanol and selected metabolic products can be done by UV-VIS[5], Raman spectroscopy [6, 7], GC-MS [8, 9], GC-FID [10-12], eletropheresis[13] or cyclic voltammetry [14]. These methods have good sensitivity, but they often require highly skilled analysts and complicated sample preparation [15].

Nuclear magnetic resonance (NMR) spectroscopy is gaining more and more application in quick, simple and non-destructive analysis of samples with complicated matrix, whereas it can allow simultaneously determine different compounds in a mixture without sample preparation and separation before quantification [16-18]. Accepting the NMR detection limits, blood is still a complex mixture that, in addition to this complication, presents additional challenges. The presence of protein and other components in blood and plasma can affect to the quantification of selected compounds [19, 20] when several signals overlap, particularly those of ethanol and glucose. This phenomenon could result in disadvantages of ^{1}H-NMR quantitative analysis in biological samples [19, 21, 22]. A simple deproteinization and solvent suppression step can not only improve the spectrum resolution, but also simplify the quantification analysis.

In this research, by setting up a suitable procedure for deproteinization and water suppression by advance NOESY pulse sequence, a simple, rapid and inexpensive procedure utilizing quantitative ^{1}H-NMR (qHNMR) with solvent suppression was established to quantify simultaneously methanol, ethanol and their aldehyde- and acid-metabolic products in human plasma. The obtained results by the proposed method can be applied for rapid diagnosis of methanol poisoning as well as its treatment monitoring.

MATERIALS AND METHODES

Reagents, Reference and Standard solutions

LC grade chemicals namely methanol (99.99%), ethanol (99.99%), formaldehyde (solution, 38%), acetaldehyde, formic (solution, 90%) and acetic acid (100%) obtained from Merck (US) were used for preparing stock standards in deionized water. Working calibration standard solutions were daily prepared by dilution of stock standards in deionized water. Maleic acid (MA) (99.94% deuterated) purchased from Sigma-Aldrich (Singapore) and deionized water was used for preparing the stock internal standard. Deuterium oxide D_2O (Armar Chemical) was used as solvent for qHNMR. Tetramethylsilane (TMS, 99% deuterated) purchased from Sigma-Aldrich (Singapore) was used as NMR reference to correct resonance chemical shifts (δ in ppm). A 25% (v/v) trichloroacetic acid (TCA) solution was used for deproteinization of the plasma samples.

Sampling and sample preparation

To ensure the efficiency and accuracy of analytical method, plasma samples obtained from healthy people in Vietnam Institute of Haematology and Blood Transfusion (Hanoi, Vietnam) were use as blank samples and standard reference (SRM) samples (by spiking known amount of standards into blank samples).

Nine blood samples of patients infected by methanol poisoning were collected from methanol poisoning patients (in 2018) in Poison Control Center of Bach Mai Hospital, Hanoi and transferred into plastic tubes containing heparin. The samples were then centrifuged at 4000 rpm for 15 minutes. The upper plasma was taken into capped vial and stored at -4 °C. 0.2 mL of 25% trichloroacetic acid (TCA) solution was added to 1 mL of protein-separated plasma, then the sample was again centrifuged for 10 minutes at 4000 rpm, and the supernatant was taken for NMR analysis. The methanol concentrations in blood samples determined by GC/FID at Department of Chemical Forensic Medicine – National Institute of Forensic Medicine (Hanoi, Vietnam) were used as references data.

qHNMR measurement

Analytical samples were analyzed in the standard 5 mm NMR tubes. 0.9 mL of sample were mixed with 0.1 mL of D_2O containing tetramethylsilane (TMS). The ^{1}H-NMR spectra were acquired at 300 K on a Bruker Avance III 500 MHz (at Faculty of Chemistry, VNU University of Science, Vietnam National University, Hanoi) equipped with a 5 mm BBFO probe including automated locking, matching, tuning and calibration of the 90° hard pulse and operating at 11.7 T. The NMR protocol applied was based on the Bruker standard protocol NOESYGGPPR1D established for solvent suppression using advance NOESY sequence. The two successive ^{1}H-NMR experiments used for the acquisition of each sample were as follows:

Experiment 1 (measure the exact resonance frequency of water using standard water suppression, Bruker sequence ZGPR): First, a one-scan standard ^{1}H NMR spectrum was recorded. The peak of water at around 4.7 ppm was chosen as offset frequency o1. Then, the exact frequency of water in each sample was determined by using a standard Bruker water presaturation pulse program ZGPR. This pulse program only suppresses the signal of OH protons by applying a presaturation scheme with low-power o1 frequency irradiation. In the experiment, a 25-Hz RF field was used for the presaturation, the relaxation delay (*RD*) and acquisition time (*AQ*) were set to 4 s and 3.27 s, respectively, resulting in a total recycle time of 7.27 s. Two dummy scans (*DS*) were applied, and four free induction decays (FIDs) (number of scans, *NS* = 4) were collected into a time domain (*TD*) of 65536 (65 k) complex data points using a 19.9947 ppm spectral width and a receiver gain (*RG*) of 3.2. The FID signals were

multiplied with an exponential function, which corresponded with line broadening 0.3 Hz prior to Fourier transformation. Repeat this step until the OH signal of water was totally suppressed to a negative peak (Fig. 1). This frequency was used for optimization the shape pulse that is required for the advance water suppression in second experiment.

Experiment 2 (advance water suppression using NOESY pulse sequence - NOESYGPPR1D). The parameters *RD*, *P(90°)*, *AQ* and *TD* were set the same asin Experiment 1; *DS* was 4, *NS* was 32 and the mixing time was set to 100ms. The optimum *RG* was set to 20.2, which resulted in a considerable gain in signal-to-noise ratio (S/N) compared with Experiment 1 (with pulse sequence ZGPR). The shape pulse was calculated using command *pulsecal* in Topspin 3.2. Total time required for analyses was about 10 minutes per sample.

All ^{1}H-NMR were automatically baseline corrected using the Topspin 3.2 package (Bruker Biospin, Germany) by the command *absn*.

For quantification, NMR signals in the specific regions for each compound were integrated and summarized. Samples were normally quantified using linear calibration curves. Besides, the concentration of X compound could be also directly calculated from equation 1, and were compared with the concentrations determined by standard calibration curves.

$$C_x = \frac{A_x}{A_{IS}} \frac{N_{IS}}{N_x} \frac{M_x}{M_{IS}} \frac{V_{IS}}{V_x} C_{IS} \quad (1)$$

where C_x and C_{IS} were concentration of X and internal standard MA, *A* was the peak integration, M was the molar mass, *N* was the number of resonant protons, and *V* was the volume of X and internal standard.

Validation of analytical method

The linearity was studied in a concentration range of 15.00 mg/L to 10.50 mg/mL of each compound in a mixture of methanol, ethanol, acetaldehyde, formic acid and acetic acid in water. The mixture of 0.6 mL of solution of 5 substances at different concentrations, 0.3 mL of 200 mg/L MA solution and 0.1 mL D_2O was analysed by ^{1}H-NMR. The method validation for four analytes (methanol, ethanol, formic acid and acetic acid) was conducted using blank and spiked plasma samples.

Signal-to-noise calculation

To quantitatively evaluate the enhancement in intensity (the amplitude of the highest peak in the signal region) and signal-to-noise ratio (*SNR*), the Bruker *sino* routine was applied to determine *SNR* [23]. The *sino* command in the TopSpin 3.2 software package gives the SNR of a 1D spectrum evaluated by the formula SNR = *maxval/(2*noise)*, where *maxval* is the highest intensity in the spectral region and noise is the average level of noise in the noise region. The instrument detection method (*IDL*) and instrument quantification limit (*IQL*) were calculated by equations:

$$IDL = 3 \times SNR / b \quad (2)$$

$$IQL = 10 \times SNR / b \quad (3)$$

where b is the slop of calibration curve. The line broadening of all spectra was set at 0.3 Hz, and all spectra were automatically shimming by command topshim tunea to ensure the same shim quality.

RESULTS AND DISCUSSION

Optimization of sample preparation and HNMR measurement conditions

The optimization of the sample preparation and measurement protocol was the key factor for the development of the quantitative NMR analyses, which should be applicable for the complicated blood matrix. In order to simplify the sample preparation, water itself in blood acted as solvent and only 10% of D_2O were added. Due to the very strong signal of water that hindered all the small signals (Fig. 1-(1)), a solvent suppression protocol was needed. The simplest solvent suppression (ZGPR in Bruker's standard library) uses a long continuous but weak intensity pulse that have same frequency with resonance frequency of water just before the main pulse (water presaturation). The method could suppress not only part of water signal, but also the proton exchange signal of OH, NH groups [23, 24]. Therefore, the signal of formic acid was partially eliminated (Fig. 1-(2)). Another NOESYGPPR1D solvent suppression method applied the water presaturation scheme in combination with NOESY pulse (NOE: Nuclear Overhausser Effect) was conducted for observing NH and OH group signals. Pulse program allows observing all signals that have strong proton exchange in the water medium, hereby the CH group of formic acid (Figure 1-(3)). Practically, it is possible to directly quantify five compounds around the suppressed water signal. Each sample underwent an automatic tuning of the probe head, followed by an optimization of the power for water saturation and pulse determination using *pulsecal* command in Topspin. As none signal selected for quantification of the target compounds was very close to water suppressed signal, no correction was necessary. The performance of solvent suppression NOESYGPPR1D allowed the used of water as solvent, so only 10% of D_2O needed for frequency calibration, that reduced the cost of experiment.

By using NOESYGPPR1D pulse, the measurement of the standard solutions was relatively simple with no peak overlapping observed. The chemical shifts (Table 1) of methanol, ethanol and their respective aldehyde and acid metabolites except formaldehyde showed a clearly separation and could be used for

their simultaneous quantification in a mixture. Under the experimental condition, the ^{1}H - NMR spectra of a plasma sample without protein precipitation also clearly showed the methanol and formic acid signals, but the CH_3 signals of ethanol and acetic acid were overlapped (Fig. 2-(3)), and quantification of formaldehyde in a mixture was impossible. But due to very short life-time of formaldehyde and acetaldehyde in blood [2] (change to formic acid and acetic acid after that) so the determination of aldehydes in this study was ignored.

Table 1

Chemical shift of five compounds in standard solution (in water) and in plasma samples [22, 25]

***Таблица 1.* Химический сдвиг пяти соединений в стандартном растворе (в воде) и в образцах плазмы**

Compound	Signal	Chemical shift in standard solutions (ppm)	Chemical shift in plasma samples (ppm)
Methanol	CH_3 (s)	3.37	3.20
Ethanol	CH_3 (t)	1.19*	1.06*
	CH_2 (q)	3.54	3.47
Acetaldehyde	CH_3 (m)	1.22	-
Acetic acid	CH_3 (s)	2.09	1.93
Formaldehyde	CH (s)	9.60	-
Formic acid	CH (s)	8.27	8.08
Maleic acid	CH (s)	6.35	6.31

Notes: s): singlet, (t): triplet, (q): quadruplet, (m): multiplet
(*: The chemical shift was used for quantification of ethanol)
Примечания: синглет, (t): триплет, (q): квадруплет, (m): мультиплет
(*: Химический сдвиг использовался для количественного определения этанола)

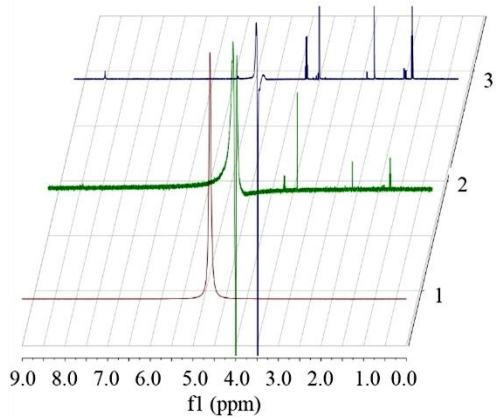

Fig. 1. The ^{1}H- NMR spectra of a standard solution containing 5 analytes (1) without solvent suppression, (2) solvent suppression by ZGPR pulse program and (3) solvent suppression by NOESYGPPR1D pulse program
Рис.1. Спектры ^{1}H-ЯМР стандартного раствора, содержащего 5 аналитов: (1) без подавления растворителя, (2) подавление растворителя с помощью импульсной программы ZGPR и (3) подавление растворителя с помощью импульсной программы NOESYGPPR1D

The spectra of plasma samples (with deproteinization) showed good baseline and no peak overlapping of all studied signals (Fig. 2-(1)). The CH_3 triplet signal of ethanol at 1.06 ppm was chosen for ethanol quantification study (Fig. 3). This procedure was used for subsequent experiments.

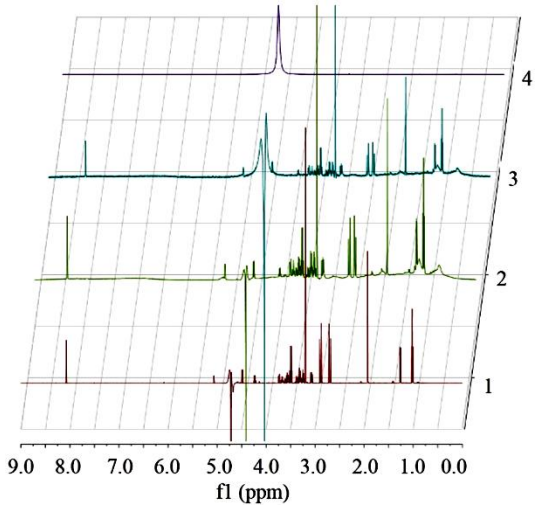

Fig. 2. ^{1}H-NMR spectra of spiked plasma sample (4) without solvent suppression but no deproteination, (3) solvent suppression by ZGPR pulse programbut no deproteination, (2) solvent suppression by NOESYGPPR1D pulse program but no deproteination, (1) solvent suppression by NOESYGPPR1D pulse program, with protein precipitation
Рис. 2. Спектры ^{1}H-ЯМР пробы плазмы с добавкой: (4) без подавления растворителем, но без депротеинации, (3) подавление растворителя с помощью импульсной программы ZGPR, но без депротеинизации, (2) подавление растворителем с помощью импульсной программы NOESYGPPR1D, но без депротеинации, (1) подавление растворителем импульсной программой NOESYGPPR1D с осаждением белка

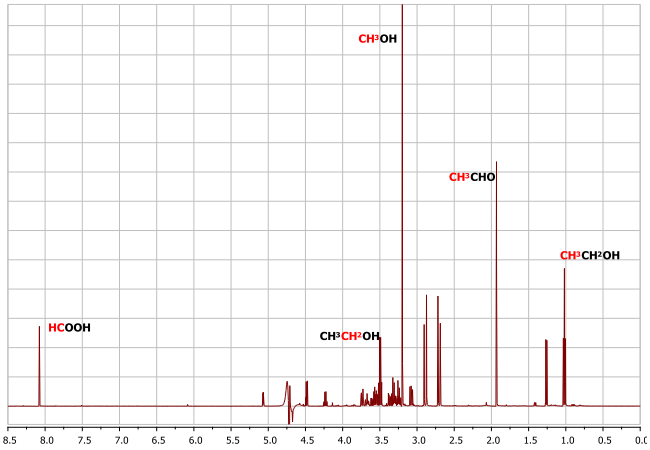

Fig. 3. ^{1}H NMR spectra of a spiked plasma sample (after protein precipitation) using NOESYGPPR1D pulse program
Рис. 3. Спектры ^{1}H-ЯМР пробы плазмы с добавкой (после осаждения белка) с использованием импульсной программы NOESYGPPR1D

Validation of analytical method

Linearity

The calibration curves were done based on signals of five standard solutions and spiked plasma sample containing of five or four compounds. The relationship between integrated peak areas and concentration showed good linearity with high correlation coefficient (R^2value > 0.999) (Fig. 4a). The instrument detection method (*IDL*) and instrument quantification limit (*IQL*) of five compounds were calculated according to equations 2 and 3 base on signal-to-noise [23, 25]. Results were given in Table 2. Methanol determination exhibited the most sensitivity with the lowest *IDL* and *IQL* of 0.02 mg/L and 0.07 mg/L respectively. The instrument detection limit (*IDL*) and instrument quantification limit (*IQL*) of four compounds in blank plasma was from 0.68 (for methanol) to 2.88 mg/L (for formic acid) and 2.25 (for methanol) to 9.60 mg/L(for formic acid), respectively.

The concentration of selected compounds in spiked samples evaluated by standard calibration curve is in good linear correlation with that by direct calculation using equation (1) (Table 2). It is clearly that methanol, ethanol, acetic acid and formic acid could be directly determined in plasma sample by using the integrated signal with the calibration factors.

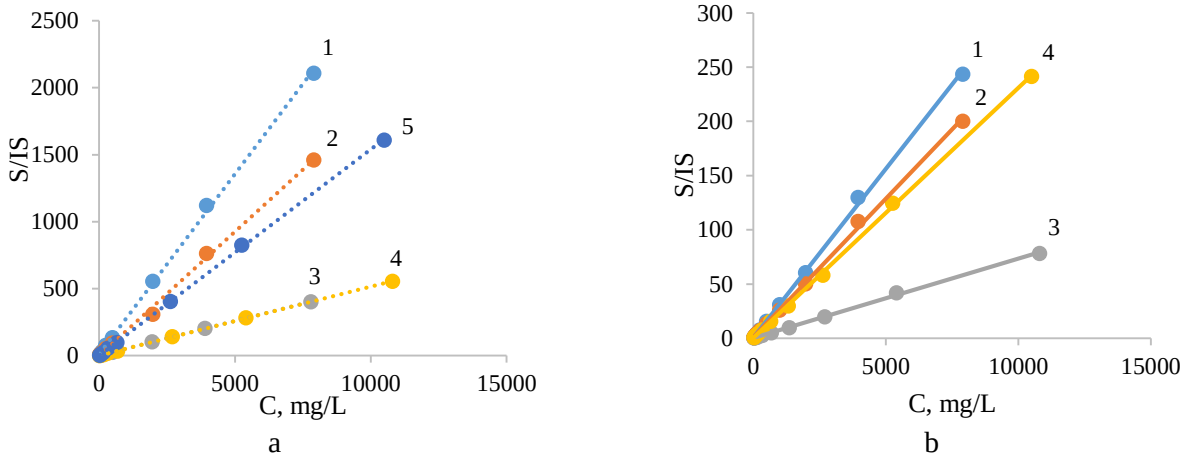

Fig. 4. a: Calibration curves of 1-methanol, 2-ethanol, 3-formic acid, 4-acetaldehyde and 5-acetic acid in standard solutions. b: Calibration curves of 1-methanol, 2-ethanol, 3-formic acid and 4-acetic acid in spiked plasma samples

Рис.4. a: Градуировочные кривые для 1-метанола, 2-этанола, 3-муравьиной кислоты, 4-ацетальдегида и 5-уксусной кислоты в стандартных растворах. b: Калибровочные кривые 1-метанола, 2-этанола, 3-муравьиной кислоты и 4-уксусной кислоты в пробах плазмы с добавками

Table 2

IDL and IQL of selected compounds in standard solutions (water) and in plasma samples

***Таблица 2*. IDL и IQL выбранных соединений в стандартных растворах (вода) и в образцах плазмы**

Analytes	Standard solutions			Plasma samples		
	Correlation Coefficient between measured* and calculated **concentrations	*IDL* (mg/L)	*IQL* (mg/L)	Correlation Coefficient between measured* and calculated** concentrations	*IDL* (mg/L)	*IQL* (mg/L)
Methanol	1.01	0.02	0.074	0.69	0.68	2.25
Ethanol	1.02	0.03	0.09	0.81	0.82	2.74
Acetaldehyde	0.95	0.10	0.23	-	-	-
Formic acid	1.22	0.10	0.324	0.67	2.88	9.60
Acetic acid	0.94	0.03	0.11	0.96	0.91	3.04

Note: (*concentration of analytes determined based on calibration curves; ** concentration of analytes directly calculated by using equation (1); «-» did not measure)

Примечания: (* концентрация аналитов, определенная на основании калибровочных кривых; ** концентрация аналитов, рассчитанная непосредственно с использованием уравнения (1); «-» не измерено)

Accuracy (precision and trueness) of the analytical method

The relative standard deviations (%RSD) were evaluated based on analyzing of replicate spiked samples and were below than 5% for all concentrations in the dynamic range. The percentage recoveries varied in the range of 96-107% (Table 3) confirmed satisfactory reliability of the analysis.

Determination of methanol, ethanol and their metabolic products in plasma samples of patients

Nine plasma samples collected from 9 methanol poisoning patients (in 2018) in Poison Control

Center of Bach Mai hospital were analysed using the above proposed analytical procedure. It was confirmed that the patients were overexposure with methanol. They accidentally or deliberately ingested methanol as a substitute for ethanol.

The amount of methanol, ethanol, formic and acetic acid found in the samples by qHNMR compared with methanol and ethanol concentrations determined by GC/FID were given in the Table 4. Despite of the very complicated matrix of plasma, the successful quantification of all four substances in human plasma samples with acceptable deviation of 2-23%makes qHNMR possible method for a quick and precise determination of methanol in blood and treatment monitoring. The different ratios of formic acid to methanol concentration or of acetic acid to ethanol concentrations in patient's plasma samples suggested that the changes of methanol and ethanol to acids dependent on the duration since patients drunk alcohol. Therefore, the determination of formic acids and acetic acid together with methanol and ethanol is necessary to helpful doctors to decide suitable treatments.

Table 3

Extraction and %RSD of four analytes in plasma samples
***Таблица 3.* Извлечение и % RSD четырех аналитов в образцах плазмы**

	Concentration (mg/L) in spiked plasma samples	RSD %	Recovery percentage (%)
Methanol	79	3.95	101±2
	790	4.67	106±2
	3950	1.90	100±1
Ethanol	79	4.79	99±2
	790	4.75	105±2
	3950	1.83	100±1
Formic acid	108	3.55	97±2
	1080	4.62	104±2
	5400	3.44	102±2
Acetic acid	105	2.60	103±1
	1025	4.13	108±2
	5250	0.92	99±2

Table 4

Concentrations of methanol, ethanol and their respective acids in 9 plasma samples
***Таблица 4.* Концентрации метанола, этанола и их соответствующих кислот в 9 образцах плазмы**

N	Patient	Methanol (mg/L)			Ethanol (mg/L)			Formic acid, mg/L	Acetic acid, mg/L
		NMR	GC/FID	*Bias (%)*	NMR	GC/FID	*Bias (%)*	NMR	NMR
1	Case 1	969	867	*+12*	427	514	*-17*	354	154
2	Case 2	2301	2348	*-2*	N.D.	N.D.	-	779	-
3	Case 3	1045	1312	*-20*	539	571	*-5*	N.D.	-
4	Case 4	2274	1973	*+15*	N.D	N.D.	-	1001	-
5	Case 5	N.D	N.D	-	4525	3680	*+23*	N.D.	102
6	Case 6	N.D.	N.D.	-	1954	1900	*+3*	N.D.	116
7	Case 7	473	407	*+16*	600	580	*+3*	518	63.7
8	Case 8	2250	2061	*+9*	787	794	*-1*	641	62.8
9	Case 9	408	366	*+12*	423	450	*-6*	104	182

Note: (N.D. not detected; "-" could not be determined or calculated)
Примечания: (N.D. не обнаружен; "-" не могло быть определено или рассчитано)

CONCLUSION

A rapid qHNMR method for simultaneous quantification of methanol, ethanol and their acid metabolic products in plasma samples was developed, validated and applied for analysis of 9 patient's plasma samples. Compared to a traditional GC/FID method that needs derivation reactions of acetic acid and formic acid before chromatographic analysis, the quantitative ^{1}H-NMR method with good linearity and accuracy was found to be more suitable for simultaneous determination of selected compounds. The simple plasma preparation using TCA deproteinization and absolute quantification could make the proposed qHNMR method turn to be more comprehensive method rather than GC/FID that must use standard solutions for making calibration curves. Thus, qHNMR was shown as a good alternative method in the diagnosis and evaluation of methanol treatment poisoning in Vietnamese hospitals.

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