An Improved Method for Acetaldehyde Determination in Blood by High-Performance Liquid Chromatography and Solid-Phase Extraction

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Abstract

This study reports on an improved method for acetaldehyde (ACH) determination in blood by high-performance liquid chromatography (HPLC). In the case of HPLC analysis, ACH is generally converted to derivatives for ultraviolet detection (for example 2,4-dinitrophenylhydrazine [DNPH] derivative). Nevertheless, elevation of the background during protein precipitation, hydrazone synthesis, or both frequently results in a serious loss of accuracy and precision of the analysis. The method in this study is developed to minimize the increase in nonspecific ACH–DNPH with a view to optimize mainly the synthetic condition of ACH–DNPH. The background is decreased dramatically by gentle deproteination, optimization of the DNPH amount and reaction pH, and reversed-phase solid extraction for the elimination of excess DNPH reagent. The standard curves show good linearity between 0 and 100µM and minimal background is observed, indicating that the method is useful for monitoring the ACH concentration in blood.

Introduction

Acetaldehyde (ACH) is a volatile toxicoid derived in vivo from ethanol mainly by enzymatic oxidation catalyzed by alcohol dehydrogenase (ADH), microsomal ethanol oxidizing system (MEOS), and catalase. ACH also has various biological activities (which are generally expressed as toxicity) by means of its own chemical reactivity (1–4). Therefore, ACH is usually eliminated immediately from blood and converted to its oxidized metabolite (acetic acid) by ACH dehydrogenase (ALDH), largely in the hepatocytes (5,6). In addition, it is considered that the binding with scavenger-like receptors on leukocytes and red blood cells and adduct formation with plasma proteins (e.g., Schiff base) may contribute to the partial excretion of toxic ACH in blood and tissues (7,8).

It is well known that the activity of enzymatic ACH detoxication decreases with long-term intake of alcohol (9,10). Moreover, genetic poor metabolizers of ACH have an inclination to elevate the ACH level in blood compared with genetic extensive metabolizers of ACH. In particular, in the case of ALDH-type I-deficient subjects, the intake of alcohol rarely leads to significant symptoms such as the acute alcoholic poisoning seen as "Oriental flushing", which is correlated to ACH concentration in blood (11). For this reason, research of ACH levels in blood is very important to investigate the relationship between the precise ACH concentration and various symptoms. ACH determination methods are continuously improving (12–20), but its physicochemical properties prevent the determination of the precise concentration in the biological matrices. For example, the boiling point of ACH is very low (approximately 21°C), thus it is difficult to suppress any volatilization completely before ACH–2,4-dinitrophenyl hydrazine (DNPH) formation even if all the procedures are pre formed in ice-chilled conditions. Moreover, nonenzymatic oxidation of ethanol during protein precipitation in the acidic condition usually results in considerable in vitro artifactual formation of the background in ACH determination (21). The regulative techniques of this phenomena still remain to be improved.

In this study, we focused on a high-performance liquid chromatographic (HPLC) method that was easy to use for biological matrices in comparison with the gas chromatographic method. The background elevation was investigated quantitatively to assess the improved method with minimal background. Artifactual formation of the background was also analyzed mass spectrometrically (MS) with deuterium-labeled ethanol (d_6-EtOH). In order to achieve diminishment and stabilization of the background, the sample preparation was optimized with regard to the DNPH amount, reaction time and pH, solvent, and solid-phase extraction. Thus, we proposed an improved HPLC method for ACH determination in blood.

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Experimental

Chemicals and apparatus

ACH was obtained from Merck (Darmstadt, Germany). d_6-EtOH and deuterium-labeled ACH (d_4-ACH) were purchased from Aldrich (Milwaukee, WI) and Sigma (St. Louis, MO), respectively. Authentic *n*-butylaldehyde (BUH)–DNPH as an internal standard and DNPH were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Human serum albumin (HSA), essentially fatty acid free, was also obtained from Sigma. Other reagents were of analytical grade. The Sep-Pak Vac 1-cc C_{18} cartridge was purchased from Waters (Milford, MA).

HPLC analytical condition

Samples including ACH–DNPH were injected into an HPLC system using an automatic injector (Shimadzu, Kyoto, Japan). A CAPCELL PAK C18 UG120 prepacked column (250- × 4.6-mm i.d., 5 µm, 120 Å) (Shiseido, Tokyo, Japan) was used at 40°C in this analysis. The quantitative determinations were performed isocratically at a flow rate of 1.5 mL/min using BUH–DNPH as an internal standard. The mobile phase consisted of acetonitrile and water (50:50). Both the ACH–DNPH and BUH–DNPH peaks were detected at an absorbance of 365 nm with a diode-array detector.

Liquid chromatography–MS analytical condition

d_4-ACH, an oxidized metabolite of d_6-EtOH, was determined by liquid chromatography (LC)–MS. The HP LC–MS system HP1100 LC/MSD (Hewlett Packard, Palo Alto, CA) was used in this study. A ZORBAX eclipse C18 prepacked column (150- × 2.1-mm i.d., 3.5 mm) (Agilent Technologies, Palo Alto, CA) joined to a Develosil packed precolumn (Nomura Chemical, Seto, Japan) was used for d_4-ACH–DNPH separation at a flow rate of 0.3 mL/min. As an internal standard, nonlabeled BUH–DNPH was also used in LC–MS analysis. The peaks of ACH–DNPH, d_4-ACH–DNPH, and BUH–DNPH were detected as molecular ion peaks of *m/z* 223, 227, and 251, respectively, by an electron-spray ionization method (negative mode).

Background derived from DNPH

For an investigation of the origin of the background, DNPH reagent was analyzed. Saturated DNPH solution in an HPLC mobile phase was injected directly to the HPLC system.

Background derived from ethanol

For an investigation of the origin of the background, ACH derived from ethanol in blood was analyzed. The transformation from ethanol to ACH was studied with d_6-EtOH. Saline-diluted d_6-EtOH (5mM) was added to fresh human blood obtained from a healthy donor to a final concentration of 50µM. Aliquots of 100 µL of the blood sample were transferred to each tube and some of the samples were analyzed immediately by the method mentioned. The other blood samples were stored at –40°C, and the time course of artifactual ACH formation was examined until 3 weeks after sample preparation.

Background derived from HSA

For an investigation of the origin of the background, ACH derived from HSA was analyzed. An aqueous solution containing 0.5mM of HSA (the physiological concentration in normal subjects) was used as a matrix of DNPH derivatization, and the volume of HSA solution for the reaction was varied from 0 to 0.5 mL. DNPH derivatization and determination of the analyte were carried out by the procedure mentioned in the "Sample preparation" and "Reversed-phase solid extraction" sections.

Optimization of ACH–DNPH synthesis

For an investigation of the optimal condition for DNPH derivatization of ACH, the reaction was examined under various conditions. ACH solution was prepared with saline to a final concentration of 50µM for this investigation. Into 0.1 mL of the ACH solution, 0.3 mL of 0.1M glycine–HCl buffer (pH 3), 0.1M acetate buffer (pH 4 and 5), or phosphate buffered saline (pH 7) was added followed by 0.1 mL of 10mM DNPH in dimethyl sulfoxide (DMSO). The reaction mixtures were shaken vigorously and analyzed by the HPLC system immediately. The injections of the samples to the HPLC were carried out at 5-min intervals for the investigation of the time course of the reaction.

The correlation between the efficiency and DNPH concentration in the reaction was examined by the following procedure. ACH solution (50µM) was diluted fourfold with 0.1M acetate buffer (pH 4), followed by the addition of 0.1 mL of 0.08, 0.4, 2, or 10mM DNPH in DMSO (a final of 0.016, 0.08, 0.4, and 2mM), respectively. After vigorous shaking, the reaction mixtures were analyzed by the HPLC system immediately. The injections of the samples to the HPLC were carried out at 5-min intervals for the investigation of the time course of the reaction.

Sample preparation

Blood samples were pretreated by the following method. An aliquot of the chilled blood sample (0.1 mL) was deproteinated with 0.3 mL of 3M perchloric acid on ice, followed by the addition of 0.8 mL of 3M sodium acetate immediately. After centrifugation, the supernatant was recovered and mixed with 0.5 mL of 2mM DNPH solution (0.1M acetate buffer (pH 4)–DMSO = 16:9), and then the mixture was reacted for 10 min at room temperature. A methanol solution of 20µM BUH–DNPH was added to the reaction mixture before purification by a solid-phase cartridge (Sep-Pak).

Reversed-phase solid extraction

ACH–DNPH synthesized in biological matrices was purified by the solid-phase extraction method with a reversed-phase Sep-Pak cartridge. Each Sep-Pak C18 cartridge was conditioned with 2 mL of methanol and water sequentially. The reaction mixture applied on the cartridge was washed with 1 mL of water, followed by additional washing with 1 mL of 50% methanol to remove excess DNPH. After these washings, the retained ACH–DNPH and internal standard were eluted with 2 mL of methanol. Solid-phase extraction was carried out using an Extraction Manifold (Waters, Milford, MA) without aspiration or pressure. The recovered fraction was dried under a nitrogen stream and reconstituted in 0.1 mL of an HPLC mobile phase.

Repeatability

Repeatability was determined at concentration levels of 2.5, 10, and 50µM ACH in blood. Spiked ACH in five identical samples was

measured by the method described previously, and the calculated values in the assay were expressed as within-run accuracy and precision.

Results and Discussion

Source of nonspecific background

DNPH

Many assays of ACH by HPLC involve the chemical conversion of ACH to its UV-absorbable derivative. In these cases, DNPH is often used for the labeling of ACH (22). This strategy is sensitive (0.1µM under limit of detection) but DNPH reagent usually contains slight amounts of its adducts with various ketones contaminated from the atmosphere or container because of its high reactivity to carbonyl groups. As shown in Figure 1, the peak indicated with an arrow had an identical retention time with ACH–DNPH when 10 µL of the saturated solution of DNPH was injected to an HPLC directly. Moreover, this peak also showed the typical molecular ion peak of ACH–DNPH (*m/z* 223, API ES-negative) by LC–MS analysis. Recrystallization of DNPH in ethanol was effective but incomplete for the elimination of contamination (data not shown). Nevertheless, the amount of contamination was slight (< 0.1µM per assay). An assay using recrystallized DNPH was reported (23), but it was considered that DNPH recrystallization was not essential for the improvement of accuracy and precision in the ACH determination when the most suitable solvent and concentration of DNPH were chosen.

Ethanol

It is well known that ACH is the first metabolite of ethanol in vivo. This oxidative metabolism is mainly catalyzed by hepatic enzymes such as cytosolic ADH and MEOS (5,6), but ACH is also formed from ethanol nonenzymatically in the process of deproteination of the plasma protein in an acidic condition (24). Therefore, nonenzymatic formation of ACH from ethanol was investigated with 20mM of d_6-EtOH-spiked blood, which is a model of the ethanol level in blood after alcohol ingestion. A time course study on nonenzymatic ACH formation revealed that d_4-ACH was generated from d_6-EtOH during protein precipitation even with diluted 0.6M perchloric acid (data not shown). No peak for d_4-ACH was observed in the LC–MS chromatogram of control blood. This result suggested that a part of EtOH might be transformed to ACH in acidic protein precipitation, but the amount was slight and not significant.

Anticoagulants

In our preliminary study, the addition of anticoagulants to blood had a mere inhibitory effect on background formation from EtOH in blood. Lucas et al. (15) reported that ethylenediaminetetraacetic acid potassium salt (EDTA-K) decreased nonspecific ACH formation in blood and its possibility to inhibit background was higher than heparin. In our study, this inhibitory effect of EDTA-K was compared with heparin and sodium citrate in our assay condition. Time-dependent ACH formation in control blood spiked with each anticoagulant and 50mM EtOH was monitored. In comparison with blank blood (anticoagulant free), no significant decrease or increase in the amount of nonspecific ACH formation was observed in the blood spiked with heparin, EDTA-K, or sodium citrate (data not shown). This result is not coincident with the findings in a previous study (15). ADH activity is theoretically inhibited by EDTA-K, which strips zinc ion from ADH; but in our case, rapid handling of blood may cause ADH activity to be neglected.

HSA

Instead of ACH levels in blood, there are some reports about ACH levels in plasma or serum (25,26). Therefore, background formation from serum albumin (a typical and major plasma protein) was investigated. As shown in Figure 2, the increase in a nonspecific ACH level was well correlated with the spiked HSA amount. Also, the amount of background from 0.5mM HSA was

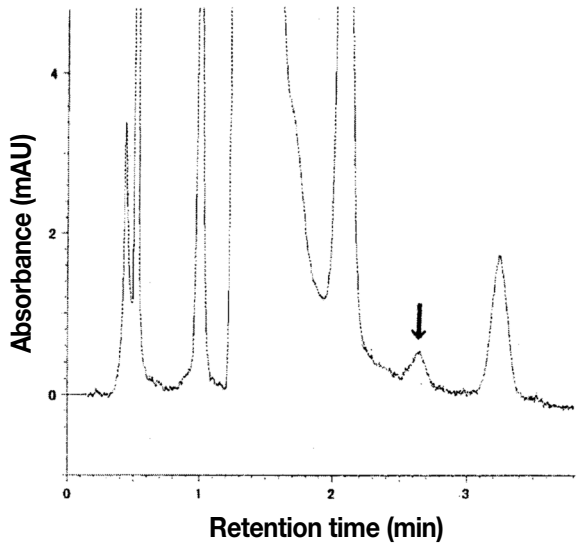

Figure 1. Contamination of ACH–DNPH in analytical-grade DNPH reagent. The impure peak at approximately 2.65 min (arrow) indicated the same retention time with ACH–DNPH.

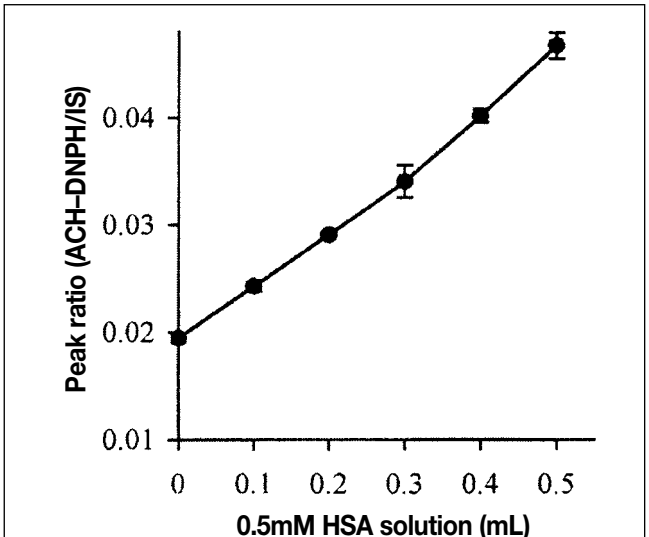

Figure 2. Albumin-dependent increase in ACH–DNPH. The error bar represents the standard deviation of triplication.

nearly equal to that from blank human blood, thus there was a good possibility that most background would be generated from HSA. It is suggested that ACH forms adducts with HSA and hemoglobin (7,8,27,28), but the detailed mechanisms of ACH–DNPH formation from plasma components are still not clear and are worthy of further study. This result suggests that HSA may be one of the key molecules in inhibiting background formation.

As mentioned previously, almost all of the background in ACH determination may be formed from plasma proteins such as HSA. In this case, the determination with plasma or serum rather than blood may be ineffectual from the standpoint of diminishment of the background. Needless to say, the plasma or serum levels are necessary for the calculation of the blood-to-plasma distribution ratio (R_B), which is one of the critical parameters for the evaluation of toxicokinetics and pharmacokinetics of ACH in vivo. However, the R_B value is generally changeable in accordance with the concentration of compounds, and thus it is often difficult to estimate the total concentration in blood from the plasma concentration. Thus, blood concentration is more reliable for the analysis of kinetics in vivo. It goes without saying that the free ACH concentration in blood is very important in these kinetic analyses. In these situations, headspace gas chromatography is usually recommended for the determination of the free ACH level (17,26). The total blood level of ACH by this study's method results in the precise bound-form ACH level in blood by subtraction of the free level from the total level.

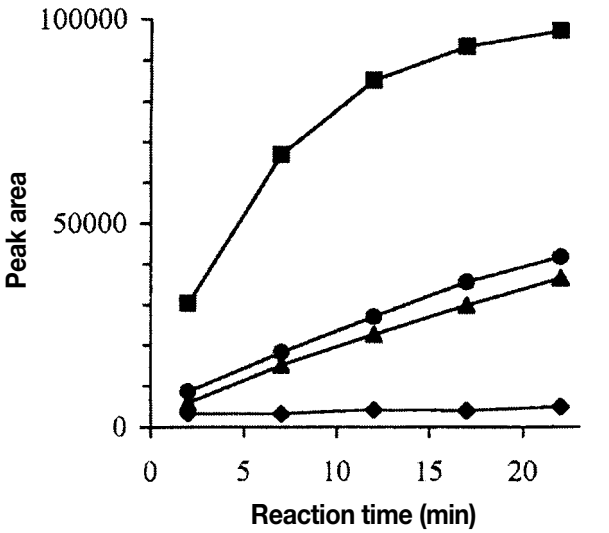

Figure 3. DNPH derivatization at various pH levels: (circle) pH 3, (square) pH 4, (triangle) pH 5, and (rhombus) pH 7, respectively.

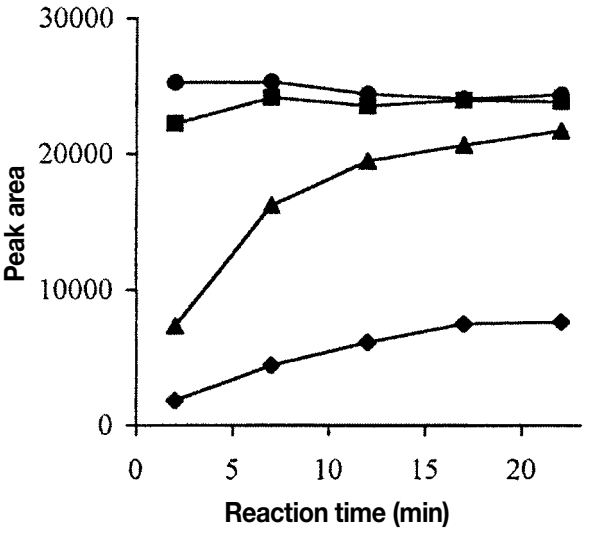

Figure 4. DNPH derivatization at various DNPH concentrations: final concentrations of (circle) 2mM, (square) 0.4mM, (triangle) 0.08mM, and (rhombus) 0.016mM DNPH.

Assay optimization

Reaction buffer and pH

Aldehydes including ACH have a high reactivity to DNPH with their own carbonyl group. Because of the strong nucleophilicity of hydrazine components, there have been many reports that various hydrazines form adducts with various components in blood (7,8,27,28). It is considered that this high reactivity is very important for the quantitative recovery of analytes, but the optimization of the reaction is essential for the prevention of background formation from blood components.

The synthesis rate of ACH–DNPH was examined in acidic to neutral conditions (pH 3, 4, 5, and 7). As shown in Figure 3, the synthesis rate was maximum at pH 4 in the tested conditions. This result suggested that pH 4 is the most suitable condition for ACH–DNPH formation; however, the precise relationship between reaction matrices and recovery still remains unclear and is worthy of further study.

DNPH solution

DNPH is usually used as a 10–15mM solution in 6N HCl for ACH–DNPH derivatization (15,22,25), but in our preliminary study analytical-grade DNPH was poorly soluble in 6N HCl and compulsory dissolution of DNPH in 6N HCl generated an increase of nonspecific ACH–DNPH background. Therefore, a DNPH solution for the synthesis was optimized on its solvent and concentration. As a result of solvent optimization, DMSO was chosen instead of 6N HCl on account of its dissolvable capacity and no

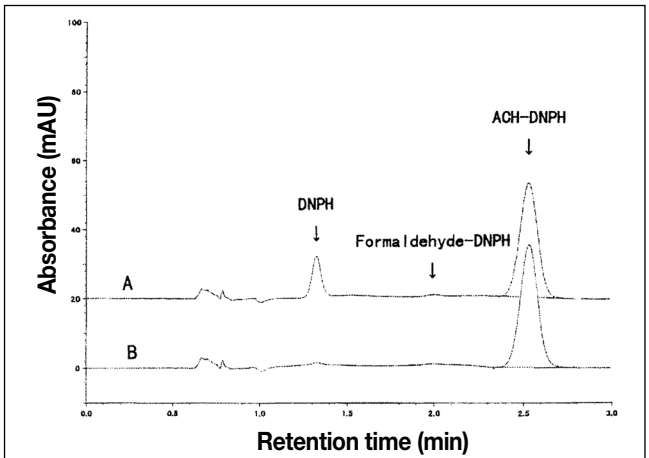

Figure 5. Purification of ACH-DNPH by solid-phase extraction: HPLC chromatograms of purified fraction by (A) deionized water and (B) 50% methanol washing.

increase in background (data not shown). The final ratio of DMSO to the reaction buffer (0.1M acetate, pH 4) was 36%. Next, the relationship between DNPH concentration and the synthesis rate was examined. As shown in Figure 4, ACH–DNPH was synthesized immediately at final concentration levels of 2mM and 0.4mM DNPH. These results indicate that the final concentration level of 0.4mM DNPH is sufficient for the ACH–DNPH synthesis in this study's method. Furthermore, it was considered that the complete reaction required only a few minutes. The shorter time for ACH–DNPH synthesis may contribute to the decrease in background.

Solid-phase extraction

A moderate excess of DNPH is essential for an immediate reaction for ACH–DNPH synthesis, but residual DNPH in the samples produces various adducts with components of the matrices. These adducts often interfere in the quantitative analysis of ACH. We used a solid-phase extraction method for the purification of the analytes, ACH–DNPH, and the internal standard. The reaction mixture including analytes and excess DNPH was applied onto a Sep-Pak reversed-phase C18 cartridge conditioned with methanol and distilled water, and the eluent was recovered following washing with 50% methanol. As shown in Figure 5, more than 99% of excess DNPH was eliminated from the recovered fraction by this procedure. Furthermore, the ACH–DNPH was efficiently recovered (92%) with low variation (relative standard deviation [RSD] = 4.9%). These results indicate that ACH–DNPH purification by solid-phase extraction may be useful as an ACH determination method with low background.

Binding et al. (29) reported ACH–DNPH synthesis with a solid-phase binding-DNPH reagent. This idea is suitable for diminishment of the DNPH amount, but in our examination the reaction efficiency on the solid phase was relatively lower than that on conventional liquid conditions. The combination method of solid-phase extraction and an HPLC analysis was also examined by Ma et al. (30); however, excess DNPH still remained in the recovered fraction.

Standard curve and intraday variation

The strategy of this study's method is shown by the procedure for sample preparation: (*a*) mix 0.3 mL of 3M perchloric acid and 0.1 mL of the blood sample, (*b*) quench the acidified sample immediately with 0.8 mL of 3M sodium acetate, (*c*) centrifuge for 1500 × g for 10 min, (*d*) transfer the supernatant to a new tube, (*e*) add 0.5 mL of 2mM DNPH solution (0.1M acetate buffer (pH 4)–DMSO = 16:9), (*f*) react for 10 min at room temperature, (*g*) add 0.1 mL of 20mM BUH–DNPH solution (internal standard), (*h*) apply to the Sep-Pak cartridge pretreated with methanol (1 mL × 2) and distilled water (1 mL × 2), (*i*) wash with 1 mL of water and 50% methanol sequentially, (*j*) elute ACH–DNPH with 2 mL of methanol, (*k*) dry up the eluate with gentle heating and a nitrogen gas stream, and (*l*) reconstitute in an appropriate volume of the HPLC mobile phase. By this procedure, the standard curve for ACH determination was prepared and resulted in good linearity in the range of 0 to 100µM (Figure 6). Table I lists the result of the intraday variation of this method. Intraday variation was also good at 2.5 to 100µM ACH (accuracy < ±12%, RSD < ±17.2%). These results suggested that this method might be useful for the quantitative determination of ACH in blood.

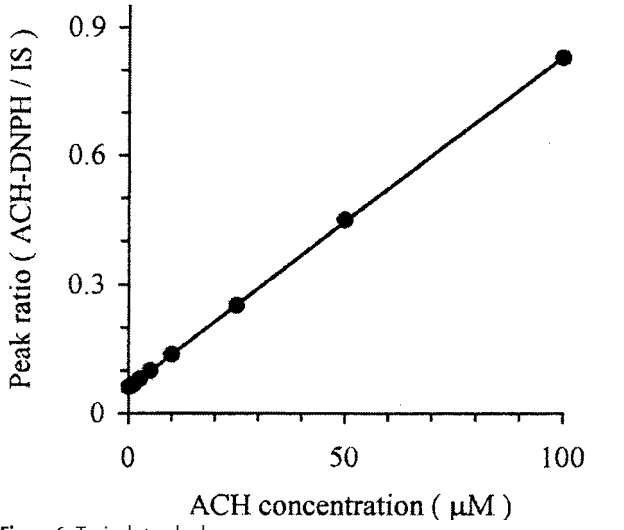

Figure 6. Typical standard curve.

Table I. Intraday Variation (*n* = 5)

Spiked ACH concentration (µM)	Measured ACH concentration (µM)	Standard deviation	%Accuracy	%RSD
2.5	2.58	0.18	103	7.0
10	11.2	1.92	112	17.2
50	49.4	1.82	99	3.7

Conclusion

The increase in the background is always discussed in ACH measurement methods. But as far as we know, this is the first quantitative report on the background formation in ACH determination. This method with minimal background is recommended in the ACH analysis in whole blood. Thus, efficacious compounds that inhibit the toxicity of ACH are now under investigation.

Acknowledgments

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