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利用液相層析串聯質譜儀搭配離子遷移光譜術分析

尿液中之苯二氮類藥物

Analysis of Benzodiazepine in Urine Using Liquid Chromatography Ion Mobility Spectrometry Tandem Mass Spectrometry

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中文摘要

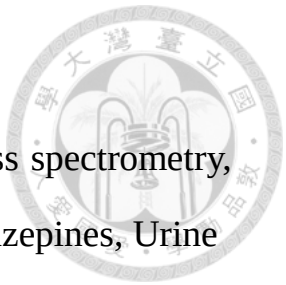
本研究利用液相層析串聯質譜儀(Ultra-performance liquid chromatography; UPLC-MS/MS)搭配離子遷移光譜術(Differential Ion Mobility Spectrometry; DMS) 開發一快速、高選擇性以及高靈敏度的方法分析人類尿液中之濫用藥物及其代謝物。DMS 最重要的功能為可以提高選擇性以及改善訊噪比(signal-to-noise ratio)，以實現定量樣品中的微量分析物。在 DMS 中，高分離電壓 (SV) 與有機溶劑之修飾劑（例如，異丙醇、乙腈及其混合物）將加入 DMS 的漂移氣體裡。苯二氮類藥物之半衰期較快，是一種常見的醫療鎮靜安眠劑，但是，在刑事案件上常發現被用來降低被害者的知覺能力，使其喪失意志，而對被害者進行身體或財物的侵害。在苯二氮類藥物裡，alprazolam 為常見的濫用藥物之一。Alprazolam 的半衰期是大約 12 到 15 小時左右。使用與分析物相關的補償電壓（Compensation voltage; CoV），以選擇性 DMS 離子通過質量分析器，找出利用液相層析串聯質譜儀與搭配離子遷移光譜術，可檢測尿液中微量之殘留代謝產物，可以測定吃藥後 6 天在尿液樣品中之 alprazolam 和它代謝物 α-hydroxyalprazolam 的含量。再現性分為 intra-day, inter-day (n=3) 皆少於 14%，線性範圍為 0.1–100 ng mL^{-1}，線性回歸係數 R^2 ≧0.998。本方法延展一般鎮靜安眠藥在尿液中的檢出時間(12–48 小時)，至藥物使用後之 6 天，有利於釐清此類藥物在醫療上或犯罪上之使用角色。

中文關鍵字: 液相層析串聯質譜儀、離子遷移光譜術、苯二氮類藥

物、有機溶劑之修飾劑、Alprazolam

English Abstract

The present work describes a rapid, selective and sensitive approach coupling ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) and modifier-assisted differential ion mobility (DMS) spectrometry mass spectrometry to investigate drugs of abuse and their metabolites in urine. The most important feature of DMS is the increase the selectivity and improving the signal-to-noise ratio to achieve lower limits of detection in the range of sample. In DMS the combination of a high separation voltage (SV) together with organic modifier (e.g., IPA, ACN) added in the drift gas. An analyte-dependent compensation voltage (CoV) was applied to selective ions through the DMS cell to the mass analyzer. Benzodiazepines are selected as the analytes, which are common sedative hypnotic agents. Recently they have been found to reduce the defensing ability of assault victims in crime. Using our investigated method, alprazolam and its metabolites, α-hydroxyl-alprazolam was identified in real urine samples after administration of alprazolam for 5-6 days. We here showed the development of a sensitive technique and looked for stable metabolites to detect in urine compounds of interest at trace level. The linear range of the method was 0.1 to 100 ng mL^{-1} for all benzodiazepines, linear plots yielded $R^2 \geqq 0.998$. And the limits of detection (LODs) ranged from 0.1 to 1 ng mL^{-1}, the residual standard deviation (RSD) ranged from 2~14%.



Keywords: Ultra-performance liquid chromatography-mass spectrometry, Differential ion mobility spectrometry, Modifier, Benzodiazepines, Urine

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Chapter 1 Introduction

1.1 Background and Motivation

1.1.1 Ultra-performance liquid chromatography (UPLC)

Ultra-performance liquid chromatography (UPLC) is a technique in analytic chemistry used to separate the components in a mixture, to identify each component, and to quantify each component [1].

It is commonly used for the analysis of organic molecules and ions because the system is well suitable for dissolving and separating samples [2].

Division of function in UPLC is mobile-phase supply and sample injection, separation, detection and data systems. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components [3].

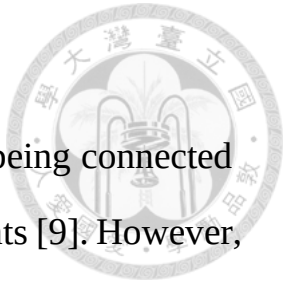
UPLC involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase. The mobile phases used in UPLC are solvents or mixtures of solvents. Separation of the

components in a solution is based on the difference in the relative distribution ratios of the solutes between the stationary phase and the mobile phase [4, 5].

1.1.2 Mass spectrometry (MS)

Over the last two decades, mass spectrometry (MS) has involved from an esoteric technology used by specialized labs into an indispensable tool used by scientists and analysts in all types of laboratories around the world [6]. A modern mass spectrometer is a device that typically will include at least an ion source, a mass analyzer, a detector and a computer with a printer. The ion source is used to produce ions in the gas phase, the mass analyzer separates the ions according to their mass-to-charge ratio (m/z) , and the detector will count the ions for every m/z ratios [7, 8].

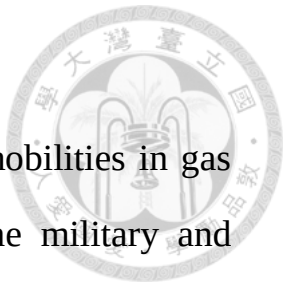
MS is to generate ions from either in-organic or organic compounds by any suitable method, to separate these ions by their m/z and to detect them qualitatively and quantitatively by their respective m/z and abundance. Electrospray is another example of atmospheric pressure ionization. The analytical solution is pumped through a capillary. The spray is produced by a high voltage of several kV between the capillary and the first lens that acts as counter-electrode [8]. After the production of ions in the gas phase, they have to be separated in a mass analyzer. Charasteristics of a mass analyzer include quadrupole and ion traps, these mass analysers are based on the stability of the trajectory of the ions. The



quadrupoles are composed of four parallel rods, the rods being connected two by two. These instruments are low resolution instruments [9]. However, with the ion traps, quite high resolution can be obtained by scanning slowly on a very limited mass range [10]. UPLC-MS-MS analysis has various advantages, for example, has high sensitivity, fast and can be directly analyzed the thermolabile compounds, contained with large molecules compounds [11]. However, UPLC-MS-MS analysis still has the issue such as chemical background interference , isomers (such as pesticides, drugs) isolation and quantitative analysis for target compounds in complex metrics [12]. So we can use recently developed the ion mobility spectrometry analysis method to cover these UPLC-MS-MS problem.

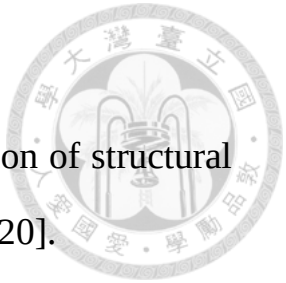
1.1.3 Differential ion mobility spectrometry (DMS)

Differential ion mobility spectrometry (DMS-MS) is a rapidly advancing technology for gas-phase ion separation. As a result, the development of fast pre-separation techniques prior to mass spectral analysis is critical. This is especially significant for analysis of small molecules contained in complex mixtures; where the probability of appearance of isobaric and isomeric interferences with m/z values similar to targeted ions can be high. This happens due to the presence of endogenous mixture components and an increasing number of channels of fragmentation of heavy mixture components with increasing sample complexity [13]. It is an electrophoretic technique that allows ionized



analyte molecules to be separated on the basis of their mobilities in gas phase. The technique has found wide application in the military and security fields, but has not until recently been similarly exploited in other areas of analytical measurement [14]. The principle of operation of DMS detector is using ions migration rate to separation of ions with high and low electric fields under high atmosphere pressure [15]. DMS composed by the separation voltage (SV) and compensation voltage (CoV), which are 2 set of electrodes [16]. The role of SV is using different electric field to remove ions with different mobility rate, finally the ions will collision with electrode plate and turn in to neutral and then detected [17]. The role of CoV which is using filters voltage to select ions, and added the correct voltage to enable selected ion that can detect in the mass spectrometer. The main difference between these measurement techniques is that in mass spectrometry ions are moving in the vacuum.

In DMS, they are moving in the gas [18]. In the classic DMS detector, ions are generated in an ionization region that may be external to the drift region, as for example in electrospray, or close coupled to the instrument like the ^{63}Ni sources currently used in explosives detectors[19]. A combination of gas flow and electrical fields are used to move the ions towards the drift region, where they encounter an ion shutter or gate which pulses the ions into the drift tube, its many attractive features such as high sensitivity, short response time, and comparatively low cost. In addition, it



also provides sensitive and enabled the gas-phase separation of structural isomers and selective detection after separation of UPLC [20].

1.1.4 Benzodiazepines

Now, drug-related crimes caused by the use of illicit drugs are a serious social problem. Some drugs will cause the body to produce which caused them dependence or inhibit nerve functions. So, it is often used for crime victims for physical or property damage. Commonly abused drugs are heroin, cocaine, amphetamine, MDMA, etc. Benzodiazepines (BZDs) is one of the commonly abused drugs[21].

BZDs have been approved for treating many illnesses. One of the most common therapeutic uses is to treat anxiety. The major advantages of BZDs as anxiolytics are their rapid onset of action and their safety [22]. The major disadvantages are the development of tolerance or dependence with long-term use, and their potential negative effects on psychomotor for long-term use. Another use of benzodiazepines is as hypnotic agents for treating insomnia [23].

Benzodiazepines and their close relatives bind to the gamma-aminobutyric acid (GABA) receptor in the central nervous system, which affecting chloride movement through ion channels [24]. In particular, the type A subtypes ($GABA_A$ receptor), have received considerable attention as the site of action of drug action as anxiolytics, sedatives, anticonvulsants, and muscle relaxants. These clinically beneficial effects are exhibited by

the benzodiazepines, which act by allosterically binding to $GABA_A$ receptors and enhancing the ability of GABA to increase chloride conductance [25]. The pharmacokinetics varies substantially between members from short acting hypnotics and midazolam to long acting anti-anxiety agents diazepam, alprazolam. BZDs are often misused in combination with illicit drugs [26]. The combination of BZDs with opiates has been reported to produce an enhanced high [27]. The most commonly encountered BZDs among illicit drug abusers are diazepam and alprazolam [28]. Additionally, according to statistics of the Department of Health Administration in Taiwan, Neurological hospitals in Taiwan informed drug abusers use drugs and drugs distribution of types. BZDs are also drug abusers used drugs one of them [http://www.fda.gov.tw/; Taiwan food and drug administration]. And statistical data showed BZDs are the most has connection with case in drug abuse occurred accidents related emergency department patient in Taipei Veterans General Hospital and China Medical University Hospital [29]. Recently, the development of LC–MS as a routine toxicological tool. A number of assays have been developed for BZDs [30]. The main purpose is use for related to psychiatric syndrome, for example, to treatment insomnia. Commonly used BZDs are about a dozen, in these types of BZDs, Alprazolam has short half-life compared with other BZDs [31]. So soon to be excluded. Because has these property, it is may be used for criminal, physical or property infringement with

higher probability.

1.1.5 Alprazolam

Alprazolam (Xanax® , Kalma®) is primarily used to treat anxiety and depression. It is white powder with pK_a of 2.4, soluble in methanol and ethanol but insoluble in water [32]. Following oral administration, alprazolam is well absorbed, with a bioavailability of approximately 90%. Alprazolam is metabolized to α-hydroxy-alprazolam and 4-hydroxyalprazolam by cytochrome P450 3A4 [33]. A comparison of pharmacokinetics of alprazolam 1 mg after oral and sublingual routes in healthy male volunteers showed that peak plasma levels are reached significantly later after sublingual 2.8 h than after oral administration 1.8 h [32]. Other pharmacokinetic parameters do not differ significantly between these routes of administration. The alprazolam, which mechanisms of action is easily to crosses the blood brain barrier and enters central nervous system (CNS) [34]. Although the exact mechanism of action of benzodiazepines is unknown, alprazolam binds non- selectively to the gamma-amino butyric acid-A ($GABA_A$)-benzodiazepine receptor complex. Most $GABA_A$ receptors are composed of three classes of subunits. Almost all of a single dose of alprazolam is excreted within 72 h, with 80% excreted in urine and 7% in feces; 20% is excreted as unchanged alprazolam [35]. A quick examination technique to confirm the presence of drugs of abuse may be a necessary for toxicology laboratory.

1.2. Research purpose

A major challenge for analyzing BZDs has been the quantitation and quantification after being administrated longer than 72 hr. identifying the types of BZDs and their metabolites in urine is important in certain crimes, such as sexual assault. Herein, developing a fast, simple technique and sensitive technique is inevitable. The purpose of the study is to use UPLC-MS-MS coupled with DMS mass spectrometry to measure BZDs especially alprazolam and its metabolites α-hydroxy-alprazolam in urine.

Chapter 2 Material and methods

2.1 Chemicals and Reagents

Benzodiazepines, alprazolam (ALP), α-hydroxy-alprazolam (α-OH-ALP), clonazepam (CNZ), 7-aminoclonazepam (7-CNZ), flunitrazepam (FNZ), 7-aminoflunitrazepam (7-FNZ), diazepam (DIZ), nordiazepam (NDZ), lorazepam (LRZ), midazolam (MDZ), doxepin (DXP) were obtained from Sigma-Aldrich (Cerilliant, U.S.A.). Methanol (MeOH, HPLC grade) was obtained from Macron. Isopropanol (IPA, HPLC grade) was obtained from J.T. Baker. Acetonitrile (ACN, HPLC grade) and water (HPLC grade) obtained from Merck (Darmstadt, Germany). Formic acid was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). A stock solution containing the set of 11 drugs, mixture was prepared in MeOH/H_2O (50/50, v/v) at a final concentration of 500 ng mL^{-1}. This mixture was then used for sample spiking to provide calibration curves in urine.

2.2 Instrument

2.2.1 Liquid Chromatography-Mass Spectrometry (LC-MS)

The LC separation of the set of drugs of abuse and their metabolites

(250 ng mL^{-1} each) was performed on a Eksigent 100 UPLC system coupled with an AB SCIEX 6500 Qtrap mass spectrometer with a DMS cell placed in front of the orifice plate (AB Sciex, Concord, ON, Canada). A HPLC column (Restek 5 μm, 50 × 2.1 mm, Allure) was used. The column oven was at 40 ℃. The mobile phases used for the gradient separation was aqueous 2% MeOH and 0.1% formic acid (mobile phase A), and MeOH + 0.1% formic acid (mobile phase B). The mobile phase eluted under the following linear gradient conditions: (A:B; v/v) from 100:0 to 28:72 in 8 min, fast gradient to 10:90 in 1 min and then 100:0 until 11 min for re-equilibration. The flow rate was stable 0.5mL min^{-1} for the first 5 min, increased to 0.9 mL min^{-1} from 5.01 min to 8.5 min and return to 0.5mL min^{-1} for re-equilibration. The analysis run time was 11min and the injection volume was 5 to 50μL. Acquisitions were performed in the scheduled multiple reaction monitoring (MRM)-MS mode.

2.2.2 Differential Ion Mobility and Mass Spectrometry (DMS-MS)

Experiments were performed in positive ionization mode on a QTRAP 6500 mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a prototype DMS cell (AB Sciex, Concord, ON, Canada) placed in front of the orifice plate. The organic modifiers were 2-propanol (IPA) and Acetonitrile (ACN).

2.3 Method validation

2.3.1 Calibration curve

Standard curves were prepared by adding known amounts of ten benzodiazepines and DXP was used as internal standard (IS). The internal standards selected should be a material that is not expected to appear in the specimen, has good stability, has a retention time reasonably close to the analytes, and does not interfere with other peaks that may be present. IS improves analytical precision by eliminating the effect of small variations in injection volumes and that peak should always give nearly the same response, it also lets the chromatographer monitor method performance. Calibration curve is determined by injection of the same volume of each of several standards of various concentrations. The response is plotted as ordinate, and the concentration is plotted as abscissa. After the line that fits these data has been determined, the same amount of analytes solution is injected, and the concentration of the material of interest is read from the curve or calculated from the equation for the curve. The concentrations of the calibration curve were 0.1, 0.5, 1, 5, 10, 50, 100 ng mL^{-1} dissolved in blank urine. The calibration curve a correlation coefficient ($R^2 \geqq 0.995$) was considered satisfactory.

2.3.2 Accuracy

The quality control (QC) samples were prepared by appropriate dilutions using separate stock solutions of different batch to obtain final concentration of 1 ng mL^{-1}. QC samples were processed in five replicates in order to evaluate the intra- and inter-assay precision and accuracy. Accuracy was defined as the relative difference between the calculated and theoretical concentrations of the ten benzodiazepines. The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to signal to noise ratio (S/N=3 and 10, respectively) and from the equations of the calibration curves.

2.4 Preparation of real sample

2.4.1 Collection urine sample

Authentic urine samples were donated from 3 male or female patients known to be receiving oral doses 1 mg of alprazolam or clonazepam and collected 7 days of urine after administration. The real urine samples were stored at -20 ℃.

2.4.2 Acid hydorolysis of urine samples

Upon collection, the sample was frozen at -20 ℃ before analysis. A

14 mL volume of urine was centrifuged at 15,000 rpm for 5 min. Then, the supernatant from urine was taken 2 mL and added 0.6 mL of 6M HCl in a 15 mL conical tube that were soaked in water bath at 80 ℃ for 2 hrs. After cooling to room temperature, the solution was mixed with 5 M NaOH to obtain a pH between 9.5 and 11 [23].

2.5 Software and equation for data acquisition

Analyst 1.5 software (AB Sciex) was used for mass spectrometer control and data collection. A dedicated driver provided by Advion BioSciences. was used to create and launch the batches. PeakView software (v. 1.0, AB Sciex) was used for data processing. MultiQuant software (v. 2.0, AB Sciex) was used for processing of quantitative data by LC−SRM/MS.

Chapter 3 Result and discussion

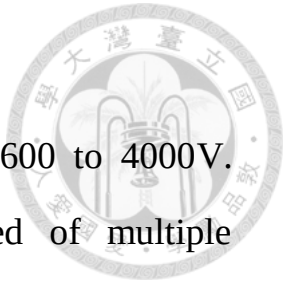
3.1 Evaluation of optimized condition

3.1.1 Scan multiple reaction monitoring (MRM) mode parameters

Before beginning the experiment, it is necessary to optimize MS parameters of for each analyte. The MRM scan mode was applied in the study. The MRM parameters are mainly used after for the use of gas phase for specific mass collision fragmentation reaction, and then specific molecular fragments (selected ion fragments) performed scan detection. The main advantage of this method is that the specific mass screening analysis through twice. Scanning analysis can reduce the error probability of the molecules (high specificity), and increase the credibility of quantitative for these molecules. Declustering potential (DP) is the voltage applied to the orifice plate. Entrance potential (EP) is the voltage between the skimmer (ground) and the entrance to Q0. Collision energy (CE) is the potential difference between the Q0 and Q2. Collision cell exit potential (CXP) is the potential difference between Q2 and Q3 **(Table. 1)**.

3.1.2 Consider of optimized modifier

Five kinds of organic modifiers have been demonstrated. The mixtures of BZDs at 250 ng mL^{-1} in 50% MeOH were used to evaluate the DMS conditions **(Fig. 1)**. The mixture was first infused into the MS



and tuned with different the separation voltage (SV) 2600 to 4000V. According to past research, using modifier composed of multiple components, where each component accomplishes a specific task on mixture of peptides and small drug molecules [36]. Use of a higher proton affinity modifier (e.g. IPA, acetone) provides increased peak capacity and separation, and use of a lower proton affinity modifier (e.g. ACN, MeOH, EA) can significantly increasing signal intensity and sensitivity for low proton affinity analytes [36]. While the DMS cell, it is reliant on ion/molecule interactions for its performance. So we can make use of the ion/molecule interactions between the analyte ion and modifier molecules to optimize the performance of such a device.

First of all, we used infusion to confirm the separation ability for each modifier **(Fig. 2)**. When no modifier, all of analytes were overlapped, and when used a higher proton affinity-modifier, such as IPA and acetone, especially the separation ability was improved by adding IPA compared with acetone, but these modifiers caused decrease intensity. While using a lower proton-affinity modifier such as ACN, EA and MeOH, the intensity was increased significantly by adding ACN or EA. However, the resolution of the chromatograph chart is not easy to choose optimize modifier. So we made up 2D plot figure by useing CoV v.s. each SV (3200 to 4000V). This 2D plot figure's characteristics visualize alteration of separation ability and intensity with the relationship of analytes and modifier.

With no modifier **(Fig. 3A)**, the CoVs of all analytes were not completely separation but increasing SV, the CoVs were only separated slightly. With ACN **(Fig. 3B)** and IPA **(Fig. 3C)**, the CoVs of compounds were separated significantly in the scale. It is important increase compared to with no modifier. Adding ACN and IPA, max 8 peaks were obtained. Moreover, when using MeOH **(Fig. 3D)** and EA **(Fig.3 E)**, the separation of BZDs was not improved, compared with ACN and IPA. Only 7 peaks were obtained. If using acetone, only 5 points were shown in the figure **(Fig. 3F)**.

Considering the effect of each modifier on the intensity of BZDs, data showed when no modifier, peak areas of all compounds were higher compared with those obtained from other modifiers. With different organic modifiers, such as acetone, methanol, ethylacetate, ACN, IPA and mixture of IPA and ACN 5% **(Fig. 4)**, results showed when adding no modifier, the highest intensity was 4.34E+05 at SV 3300 **(Fig. 4A).** If adding ACN as modifier, the highest intensity value was decreased approximately 59% at SV 3800V **(Fig. 4B)**. In addition, when adding IPA, the highest intensity dropped down 70% approximately at SV 3600 compared with no modifier **(Fig. 4C)**. However, when using the mixture of IPA/ACN (95:5, v/v) as modifier, the highest intensity was only decreased 53% approximately at SV 3500 **(Fig. 4D)**. According to above results we found, when using the mixture of IPA/ACN (95:5, v/v) as modifier, the intensity was increased

about 14 to 37 % compared with ACN and IPA, and the same data used for the standpoint of CoV separation.

Examining the above data, ACN or IPA obtained well separation ability and less intensity loss. We reasonably assumed that mixing ACN and IPA might improve the CoV separation and sensitivity of BZDs at the same time.

The hypothesis is to use different ratios of IPA and ACN might improve not only the gas phase separation ability but also the signal to noise coupled with UPLC-MS/MS. Therefore, the different mixtures of IPA and ACN (IPA: 5%, 15%, 25%, 35%, 45%, 55%, 65%, 75%, 85%, 95%, v/v) were subsequently demonstrated in the following study. The result showed when using organic modifier mixture of IPA + 85% ACN, the intensity was the highest than the other mixtures **(Fig. 5)**.

As the results mentioned above, IPA + 85% ACN was selected as modifier added in drift gas. With increasing the SV from 2600 to 4000V, the separation of CoV was also improved largely. In the next step, we examined the separation ability after added IPA + 85%ACN as organic modifier. We used 2D plot figure by CoV value v.s. retention time.

As a matter of fact the relationship, between retention times (LC) and CoV with modifier (IPA, ACN and IPA + 85%ACN) or with no modifier was observed. The result has showed that with no modifier, some CoVs of analytes (like α-OH-ALP and LRZ) were so closed **(Fig. 6A)**, additionally,

when added IPA, some analytes (like CNZ and NDZ), their CoV value were also similar **(Fig. 6B)**. However, when using ACN or IPA + 85% ACN, the separation ability increased **(Fig. 6C,D)**. Expressly compared with IPA and IPA+85%ACN, mixture of IPA+85% ACN performed better the separation ability than IPA.

According to the results, the ability for improve matrix interferences in DMS (no modifier) or DMS (adding IPA+85% ACN) were tested. The analytes were spiked in urine. Data showed when adding no modifier, the interference from urine was higher, so the signals of analytes signal were undetected **(Fig. 7)**.

On the other hand, when adding IPA+85% ACN, signal-to-noise was improved importantly. This means that the DMS (adding IPA+85% ACN) has better ability of reducing interferences.

BZDs were spiked in blank urine, and IPA+85% ACN increased signal-to-noise ratio significantly compared with other modifier such as DMS no modifier, DMS off, IPA and ACN **(Fig. 8)**. It was up to 7 times than adding no DMS. The analytical platform allows enhancing intensity of target compounds by eliminating interferences significantly. So we found when use IPA+85% ACN, the separation ability was improved and matrix interference was reduced.

Therefore, we decided to choose IPA+85% ACN as modifier. Then we found the optimal SV value with using IPA+85% ACN. With

increasing the SV from 2600 to 4000V, the separation of CoV was also improved largely **(Fig. 9)**. Results showed in **Fig. 10**, the highest intensity (7-aminoflunitrazepam, 6.05E+05) at SV 3700V. However, when SV was at 3600V the highest intensity for alprazolam (3.03E+05) was obtained. In addition, the peak area of alprazolam begun to decrease from the value. So we used SV 3600V as our study optimized condition.

To achieve the sensitivity, using DMS in the gas-phase separation, each standard of BZDs was injected into the UPLC-MS/MS system with the addition of IPA, ACN and IPA+ACN85% or without applying DMS respectively **(Fig. 11)**. It indicated that the intensity of the majority BZDs had been enhanced by IPA + ACN85%, especially on midazolam, of which was 4 times higher than applying no modifier or no DMS.

3.2 Validation

3.2.1 Linearity

Linearity, regression coefficient (R^2) was investigated under optimized experimental conditions. The linearity of the method was evaluated using DI- water/urine (50:50, v/v) spiked with the selected compounds at various concentrations.

The linear calibration of the targeted benzodiazepine was examined in the range 0.5 to 100 ng mL-1. Linear in the corresponding dynamic ranges

with square correlation coefficient (R^2) $\geqq$ 0.998 **(Table. 2)**.

3.2.2 Limit of detection (LOD)

The LOD is defined as 3 times of signal to noise. The LODs of all BZDs tested were found between 0.1~1 ng mL^{-1} respectively, using the equation of the calibration curve **(Table 2)**.

3.2.3 Accuracy

The RSD (%) of intra- and interday accuracy were found to be less than 11% and less than 14%, respectively.

3.3 Application in real urine sample

In forensic toxicology, when determination whether a suspect administrates drugs of abuse or if a victim is poisoned, urine samples are the most commonly analyzed to identify drugs and their metabolites. We have collected urine samples from who had administrated alprazolam. The study was performed on LC-MS-MS using IPA + 85% ACN as the DMS modifier to detect alprazolam and its metabolites, α-hydroxy-alprazolam. Results showed in case 1 and case 2, the free form alprazolam was detected in the urine samples till 6 days **(Fig.12)**. However α-hydroxy-alprazolam is the main compound which existed in case 3. In three cases, either alprazolam or α-hydroxy-alprazolam was still able to be confirmed at

approximately 1 to 4 ng mL^{-1} in day 6.

Chapter 4 Conclusion

A rapid, selective, and sensitive approach combining LC-MS-MS and modifier-assisted differential ion mobility spectrometry mass spectrometry (DMS) analysis platform to BZDs and their metabolites. By removing interferences, DMS allows increasing the selectivity and improving the signal-to-noise ratio to achieve lower limits of detection for analytes in urine. Different modifiers have been assessed, such as IPA, ACN, EA, MeOH and acetone. The results indicate that different selectivity is provided in function of the nature of each modifier, for example, IPA and acetone enhance the separation ability. Acetonitrile, EA and MeOH improve the intensity and sensitivity. We found that the most suitable modifier for analysis BZDs and their metabolites is the mixture of IPA+85% ACN which improves the separation ability and signal to noise ratio. The application of LC-MS-MS coupled with DMS for the analysis of trace amout of such as BZDs and their metabolites from urine samples demonstrates the great potential of the gas-phase separation technique as an alternative to liquid-phase chromatography and may be very important for compounds at trace amount, which are difficult to be analyzed, after being administrated for 72 hr. Using our method, ALP can be detected till the sixth days after administration. The platform could also be used as a powerful tool for metabolites profiling within the scope of early drug

metabolism study.

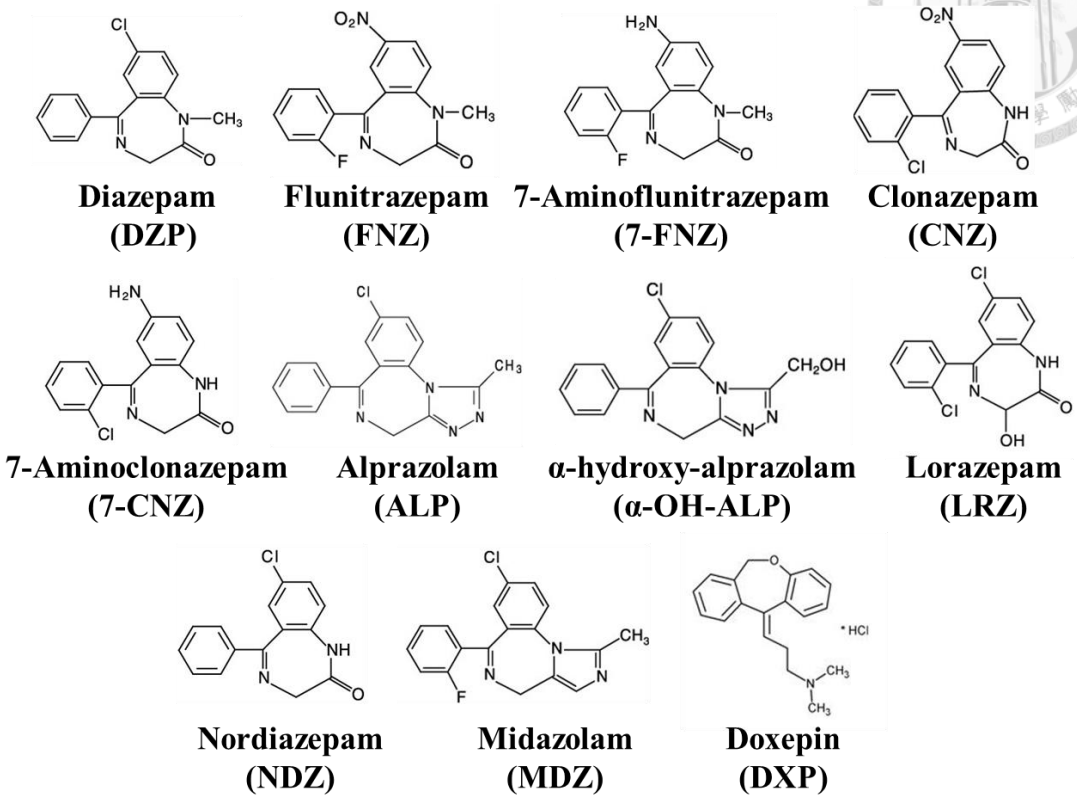


Fig. 1 The structure of investigated analysis.

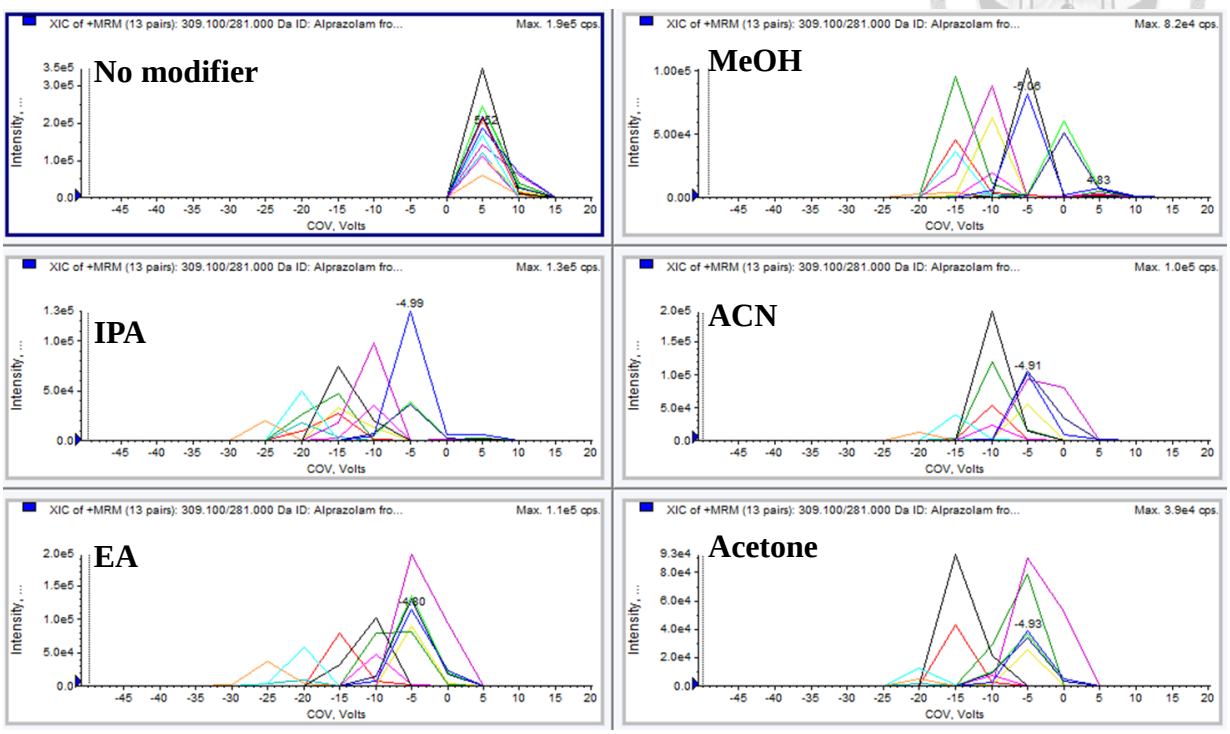
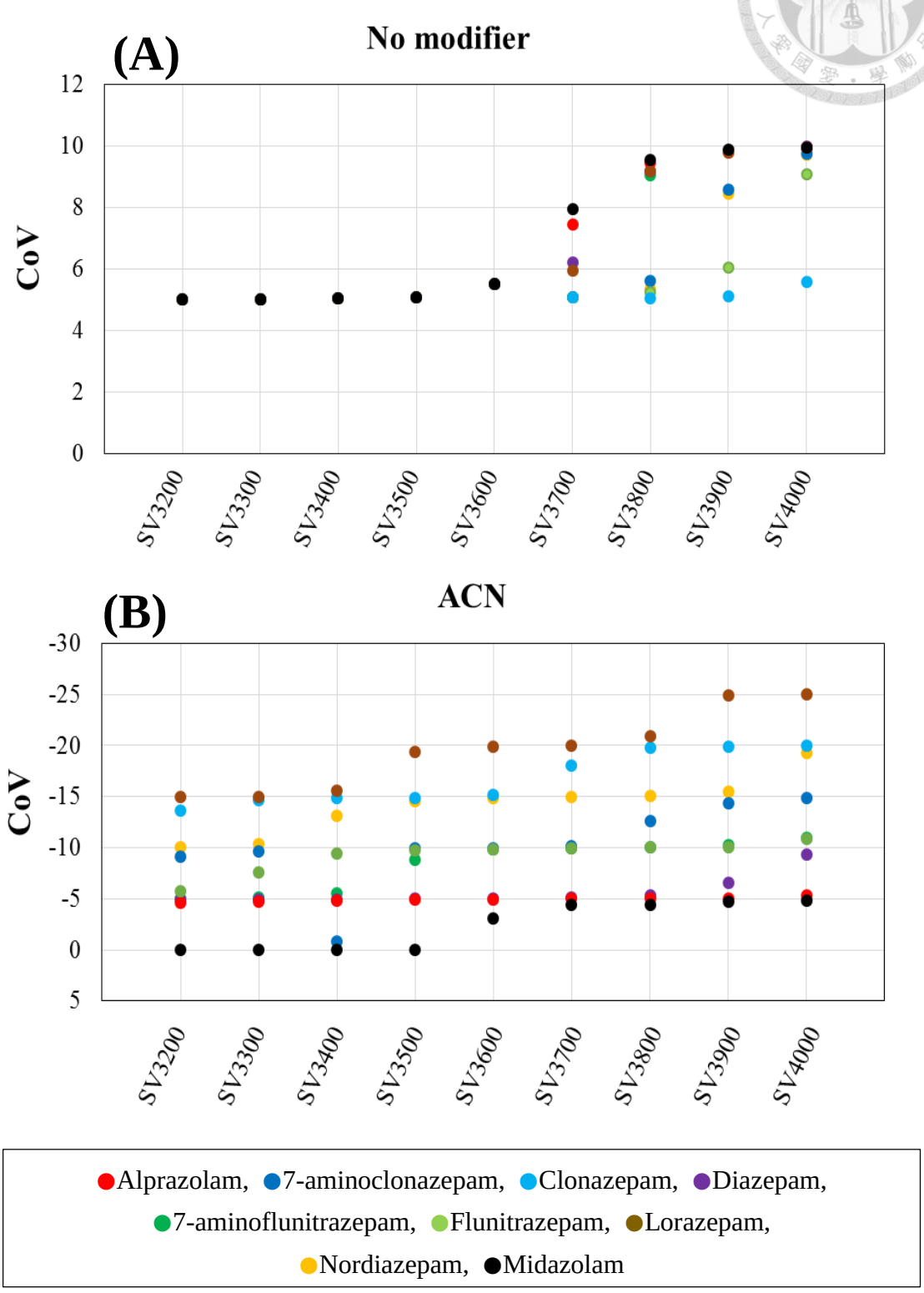
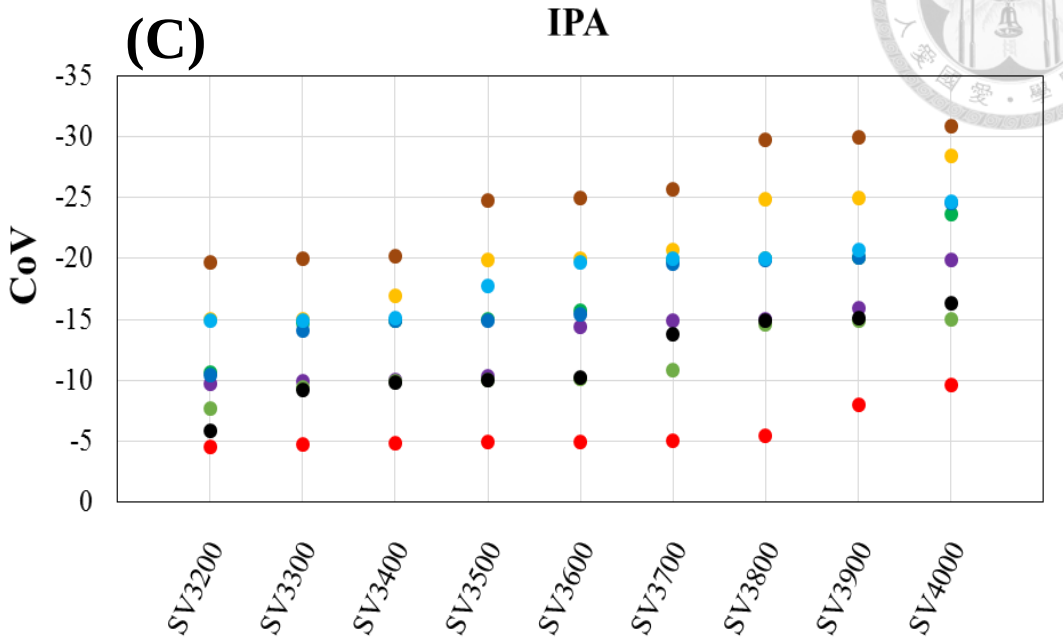
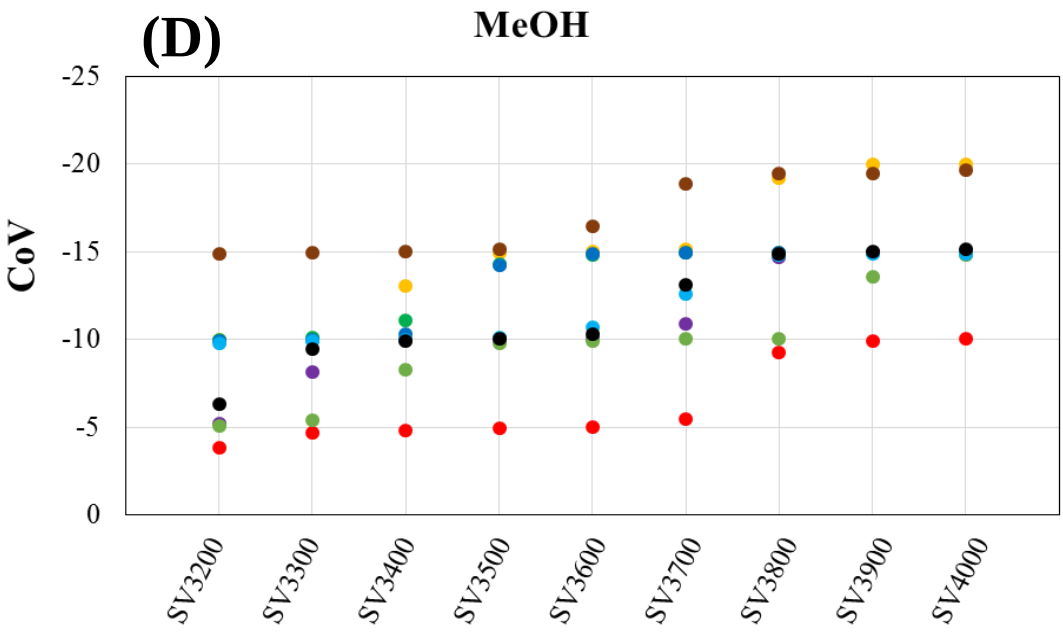


Fig. 2 Representative chromatogram of analytes with using different modifier.


(A)
No modifier
CoV
12
10
8
6
4
2
0
SV3200
SV3300
SV3400
SV3500
SV3600
SV3700
SV3800
SV3900
SV4000
(B)
ACN
CoV
-30
-25
-20
-15
-10
-5
0
5
SV3200
SV3300
SV3400
SV3500
SV3600
SV3700
SV3800
SV3900
SV4000
Alprazolam, 7-aminoclonazepam, Clonazepam, Diazepam,
7-aminoflunitrazepam, Flunitrazepam, Lorazepam,
Nordiazepam, Midazolam


(C)
IPA
CoV
-35
-30
-25
-20
-15
-10
-5
0
SV3200
SV3300
SV3400
SV3500
SV3600
SV3700
SV3800
SV3900
SV4000


(D)
MeOH
CoV
-25
-20
-15
-10
-5
0
SV3200
SV3300
SV3400
SV3500
SV3600
SV3700
SV3800
SV3900
SV4000

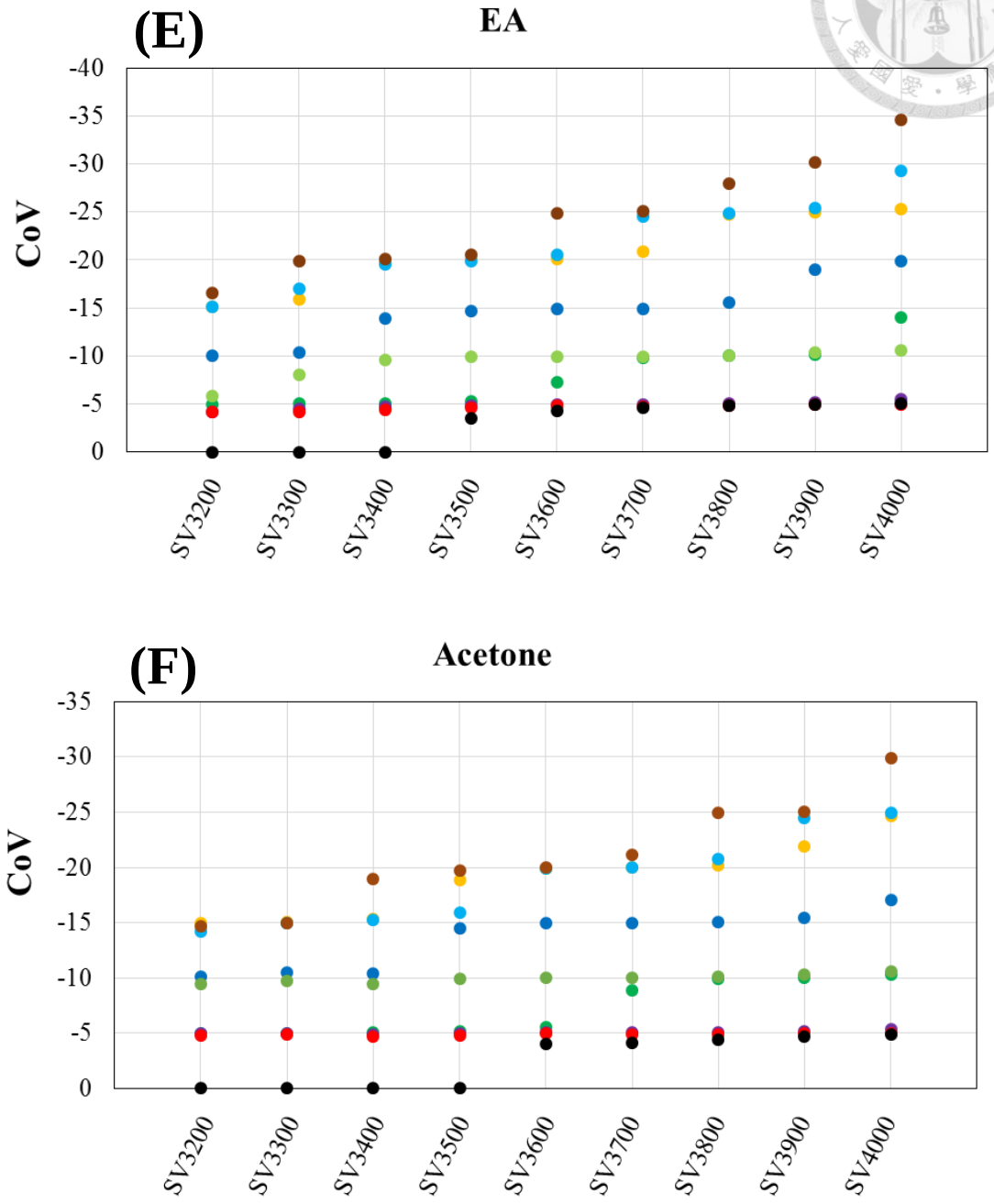
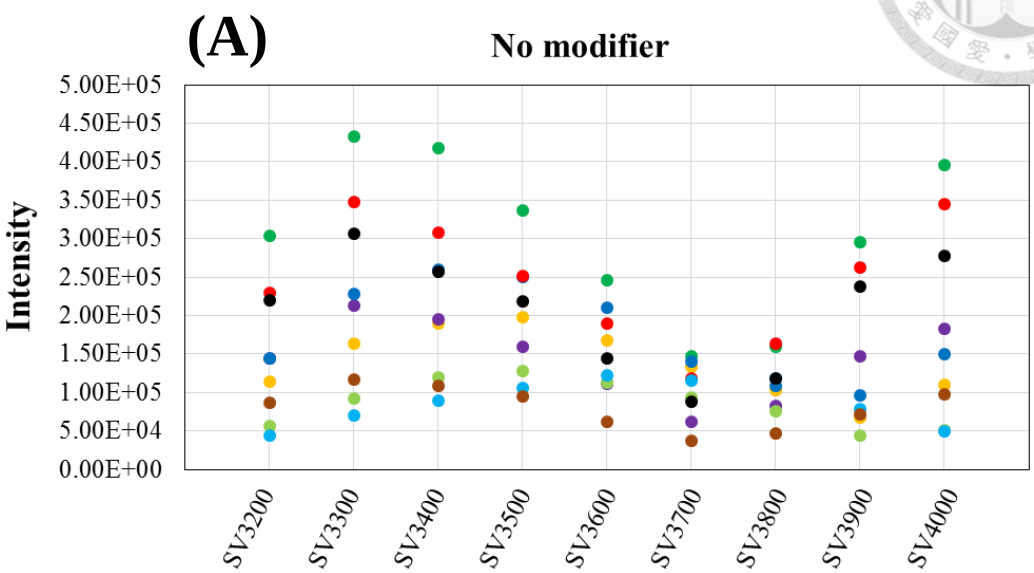
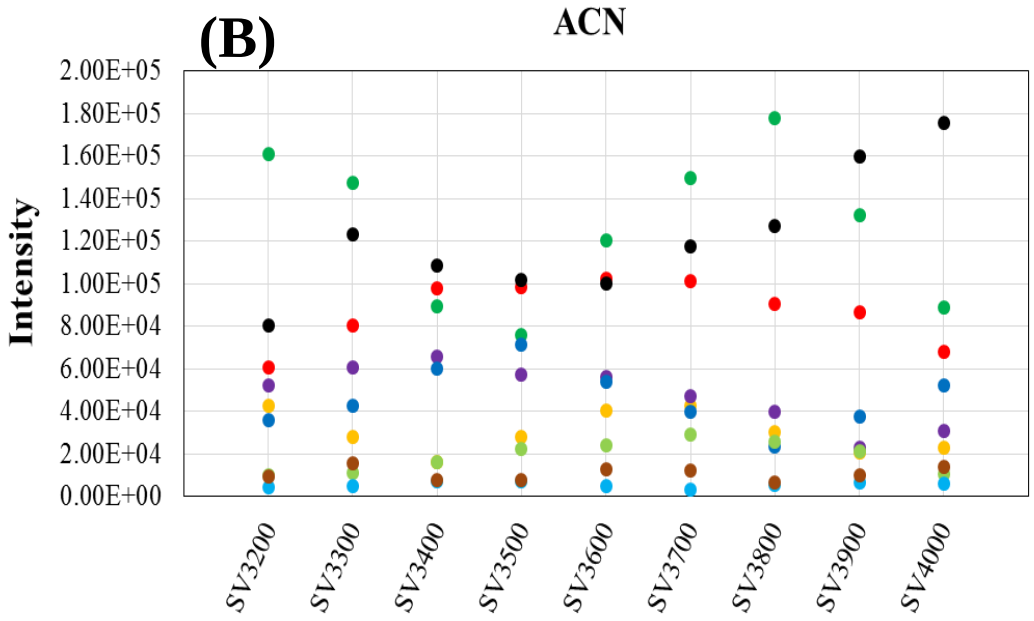


Fig. 3 Comparison between in-solution (LC) and DMS separations using different organic modifiers. (A) No modifier (B) ACN (C) IPA (D) MeOH (E) EA (F) Acetone. Plotted against the retention time obtained for analytes after their separation (CoV).





Alprazolam, 7-aminoclonazepam, Clonazepam, Diazepam, 7-aminoflunitrazepam, Flunitrazepam, Lorazepam, Nordiazepam, Midazolam

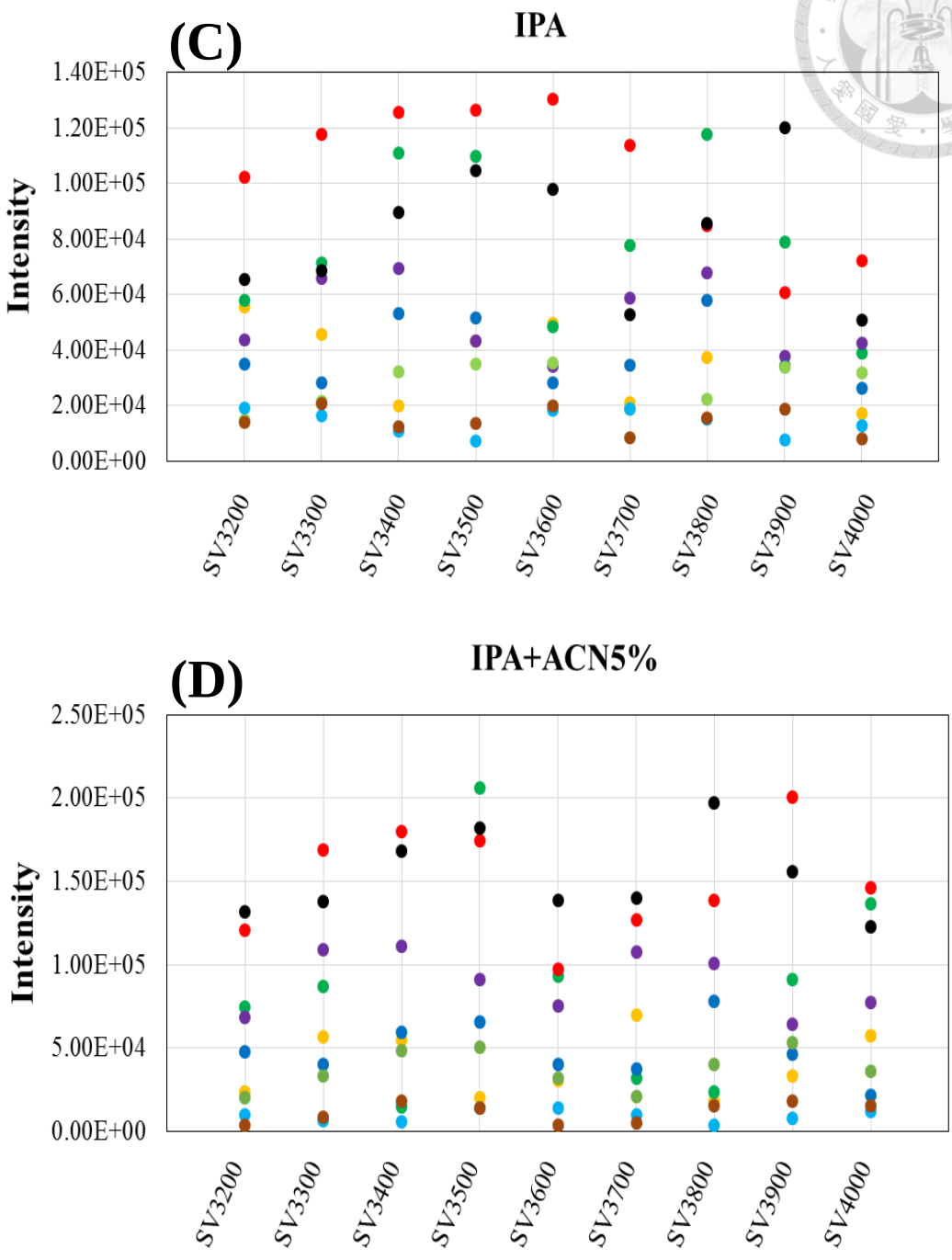


Fig. 4 Comparison between in-solution (LC) and DMS separations using different organic modifiers. (A) no modifier (B) ACN (C) IPA and (D) IPA+15%ACN, plotted against the retention time obtained for these analytes after their intensity.

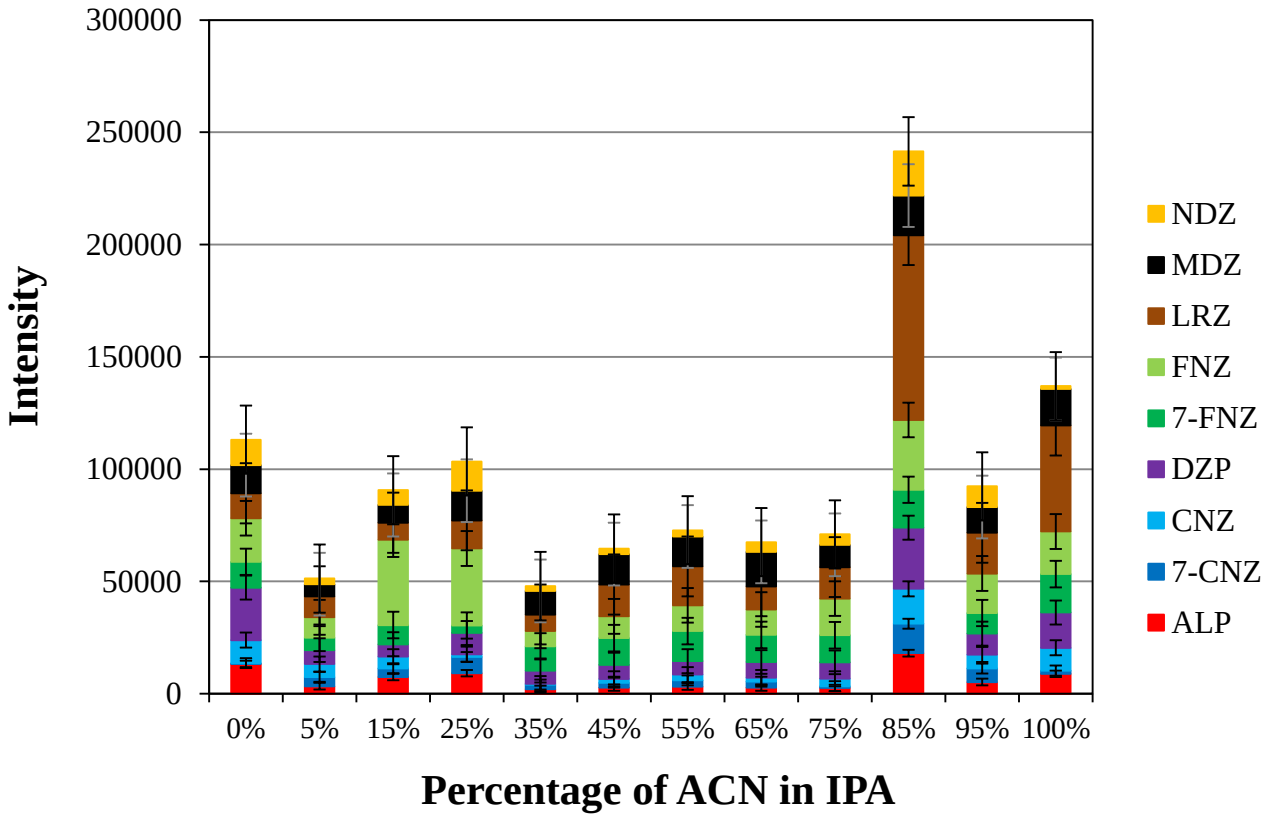
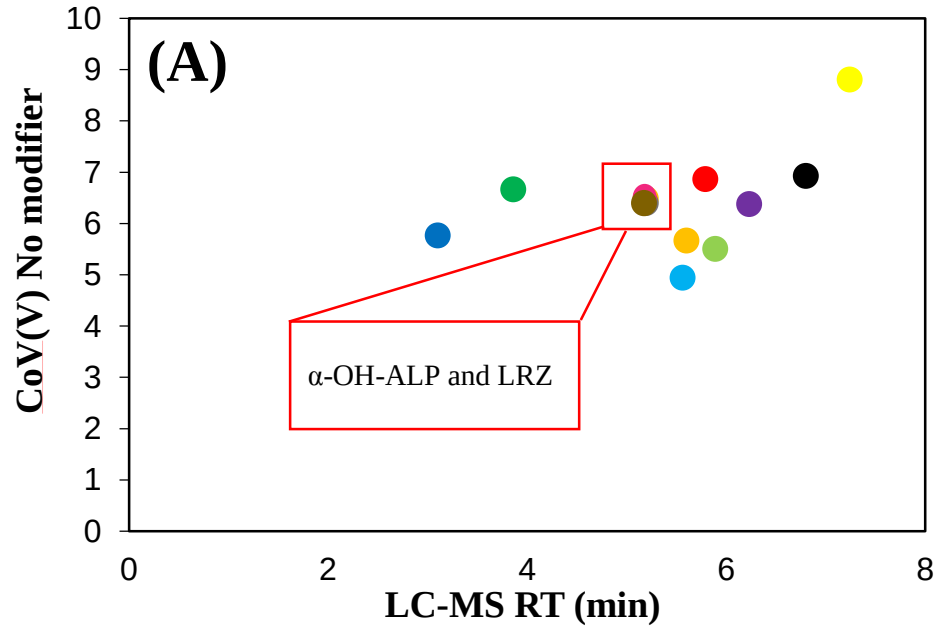
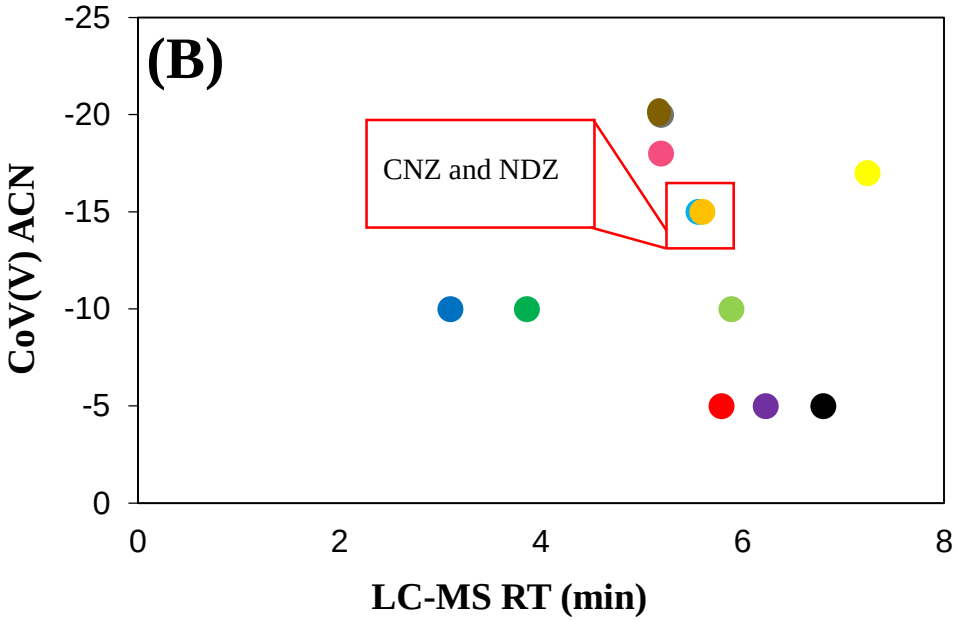


Fig. 5 The effect of the organic modifier which each concentration mixture IPA and ACN ratio on peak capacity (n=3).


(A)
CoV(V) No modifier
LC-MS RT (min)
α-OH-ALP and LRZ
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8


(B)
CoV(V) ACN
LC-MS RT (min)
CNZ and NDZ
-25
-20
-15
-10
-5
0
0
2
4
6
8

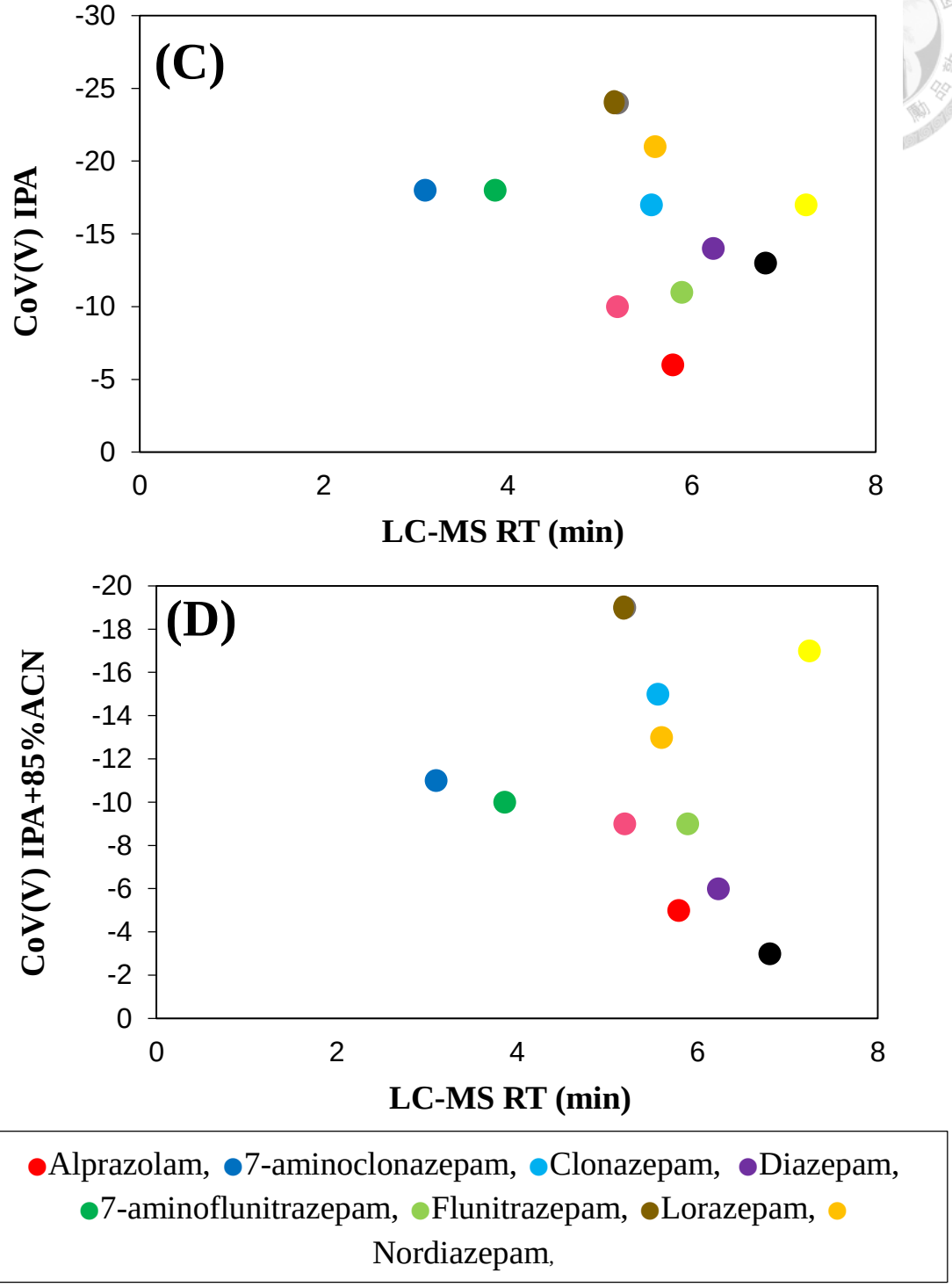


Fig. 6 Comparison of different modifier for the DMS separation when SV 3600V. CoV values of each analyte plotted at a retention time. (A) no modifier (B) ACN (C) IPA and (D) IPA+85% ACN.

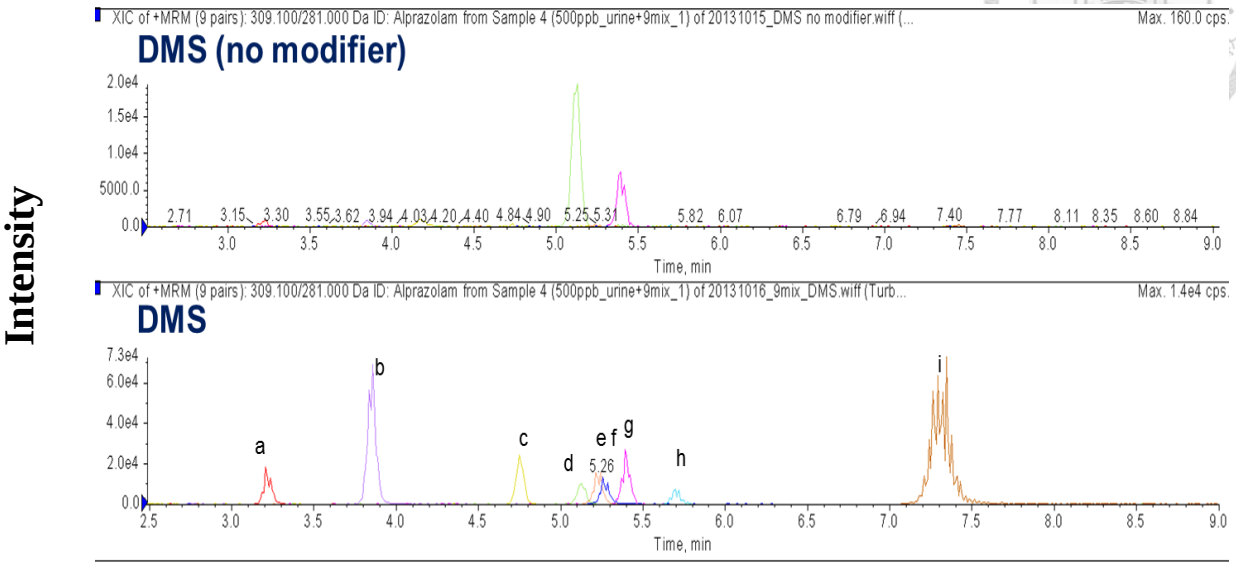


Fig. 7 Comparison with DMS (no modifier) and DMS (IPA+85% ACN) Results showed DMS (IPA+85% ACN) improved the background interference in urine. (a)7-CNZ (b) 7-FNZ (c) LRZ (d) CNZ (e) NDZ (f) ALP (g) FNZ (h) DZP (i) MDZ.

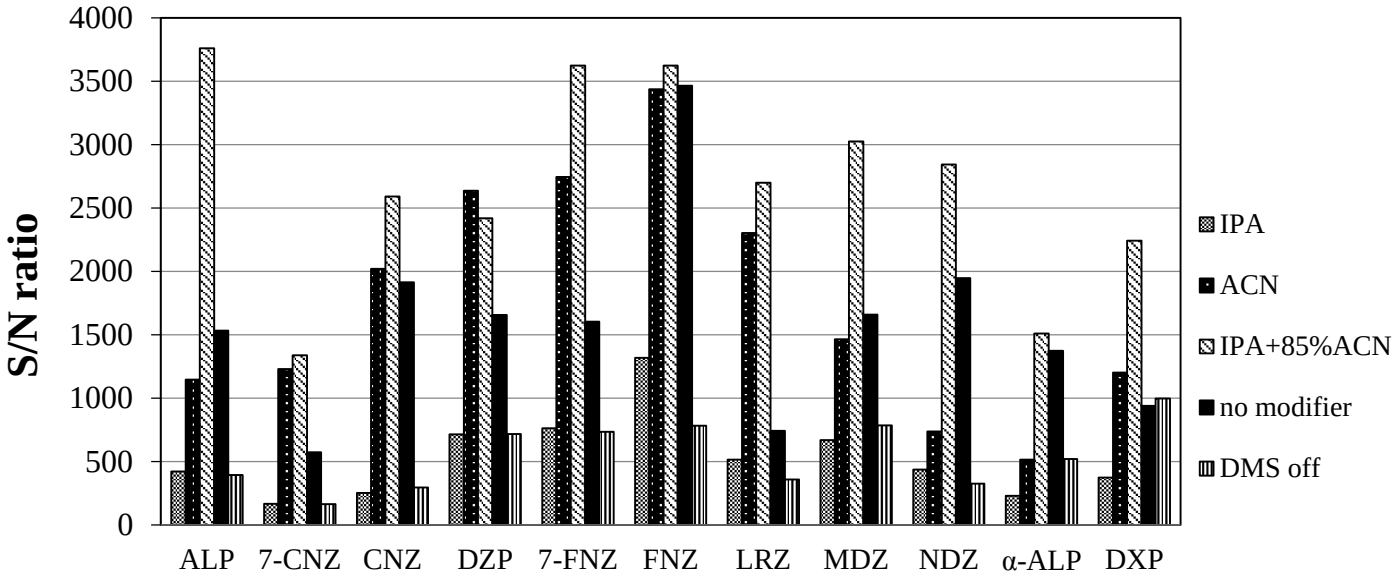


Fig. 8 Results in comparison with analytes spiked in urine using each different organic modifier on signal to noise (S/N) ratio (n=3).

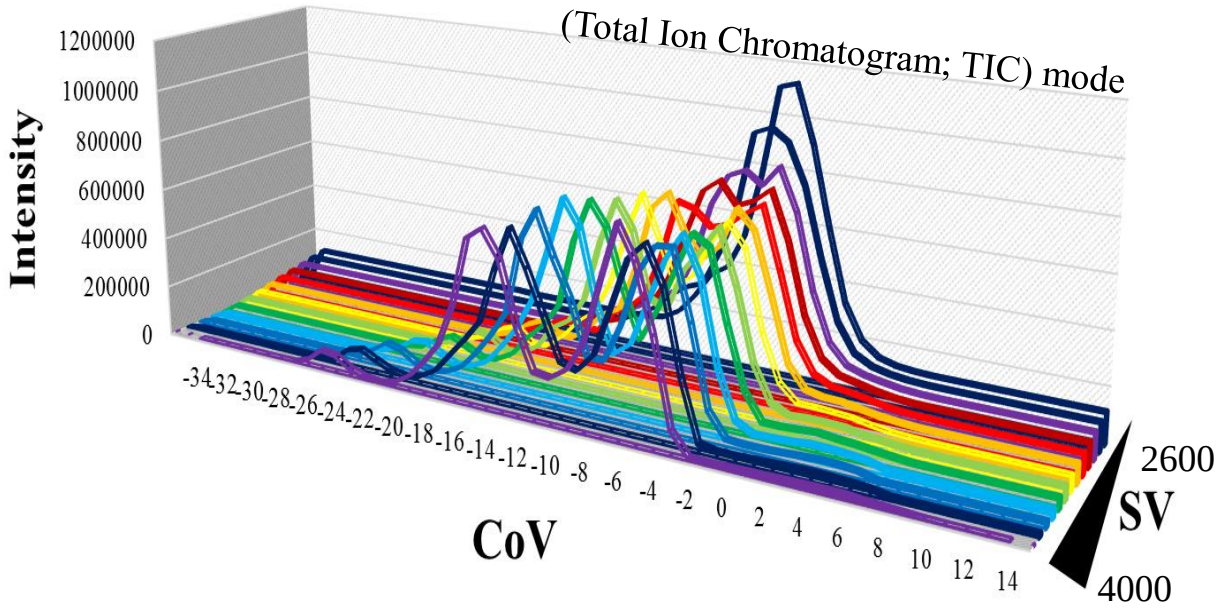


Fig. 9 The effect of the separation voltage and of the addition of IPA+85% ACN in the drift gas on the DMS separation.

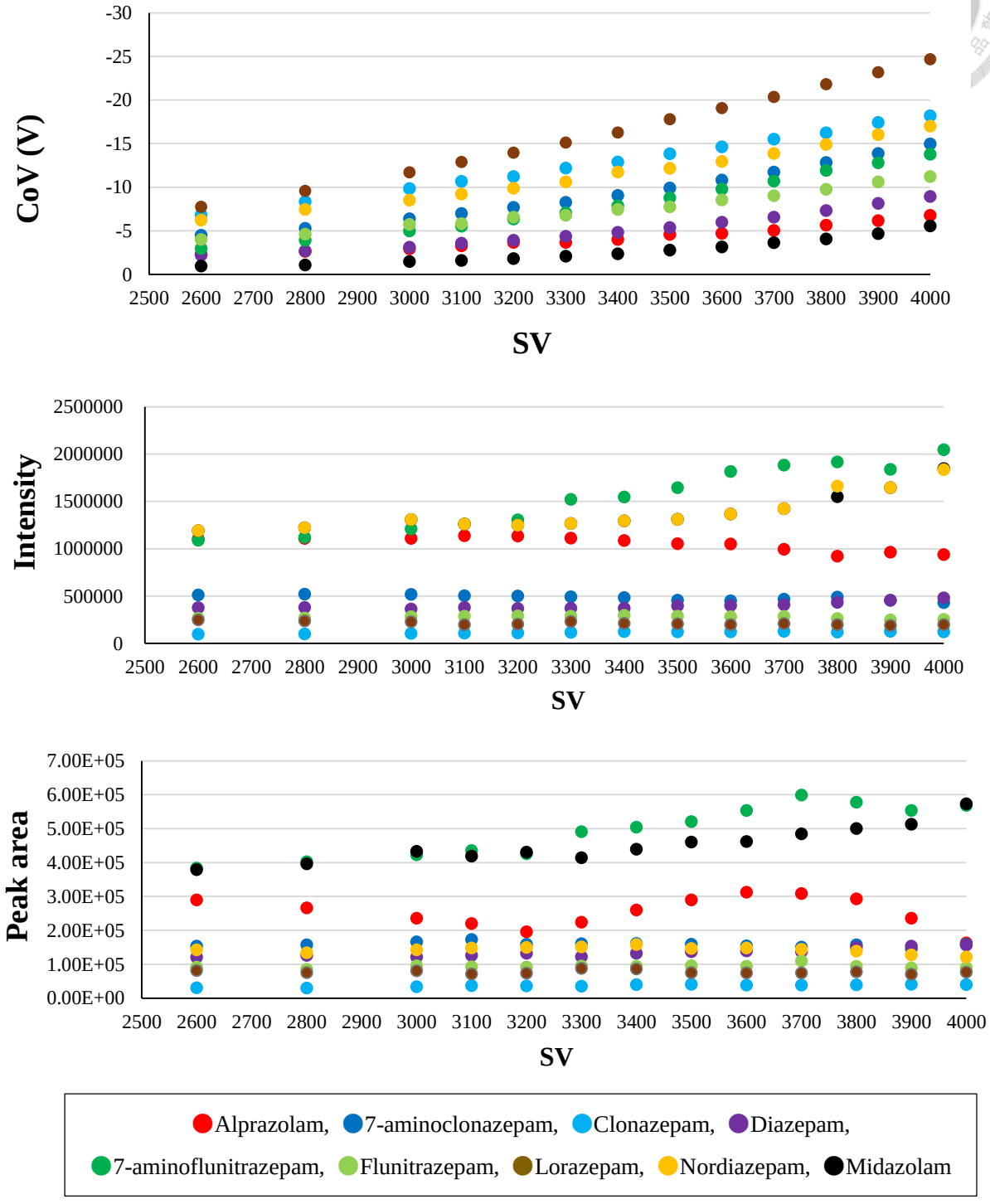


Fig. 10 The effect of IPA+85% ACN on the DMS separation, intensity and peak area in the drift gas with different SV.

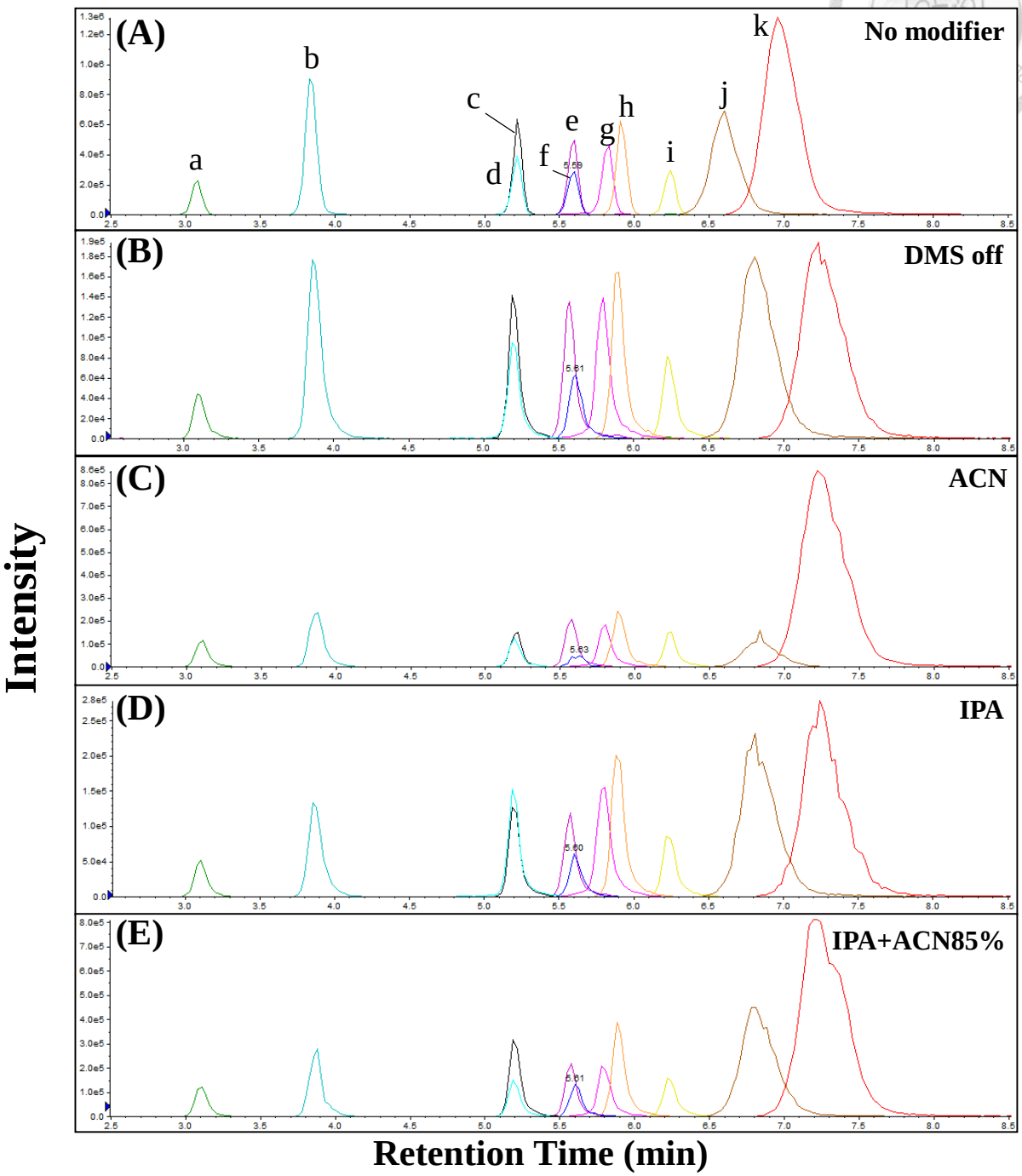


Fig. 11 Effect of different organic modifiers on the separation of a set of 11 drugs BZDs in blank urine with MRM mode. (A) no modifier (B) DMS off (C) ACN (D) IPA (E)IPA+85% ACN, and the analytes were (a) 7-CNZ (b) 7-FNZ (c) LRZ (d) *α*-OH- ALP (e) CNZ (f) NDZ (g) ALP (h) FNZ (i) DZP (j) MDZ (k) DZP at 250 ng mL^{-1}.

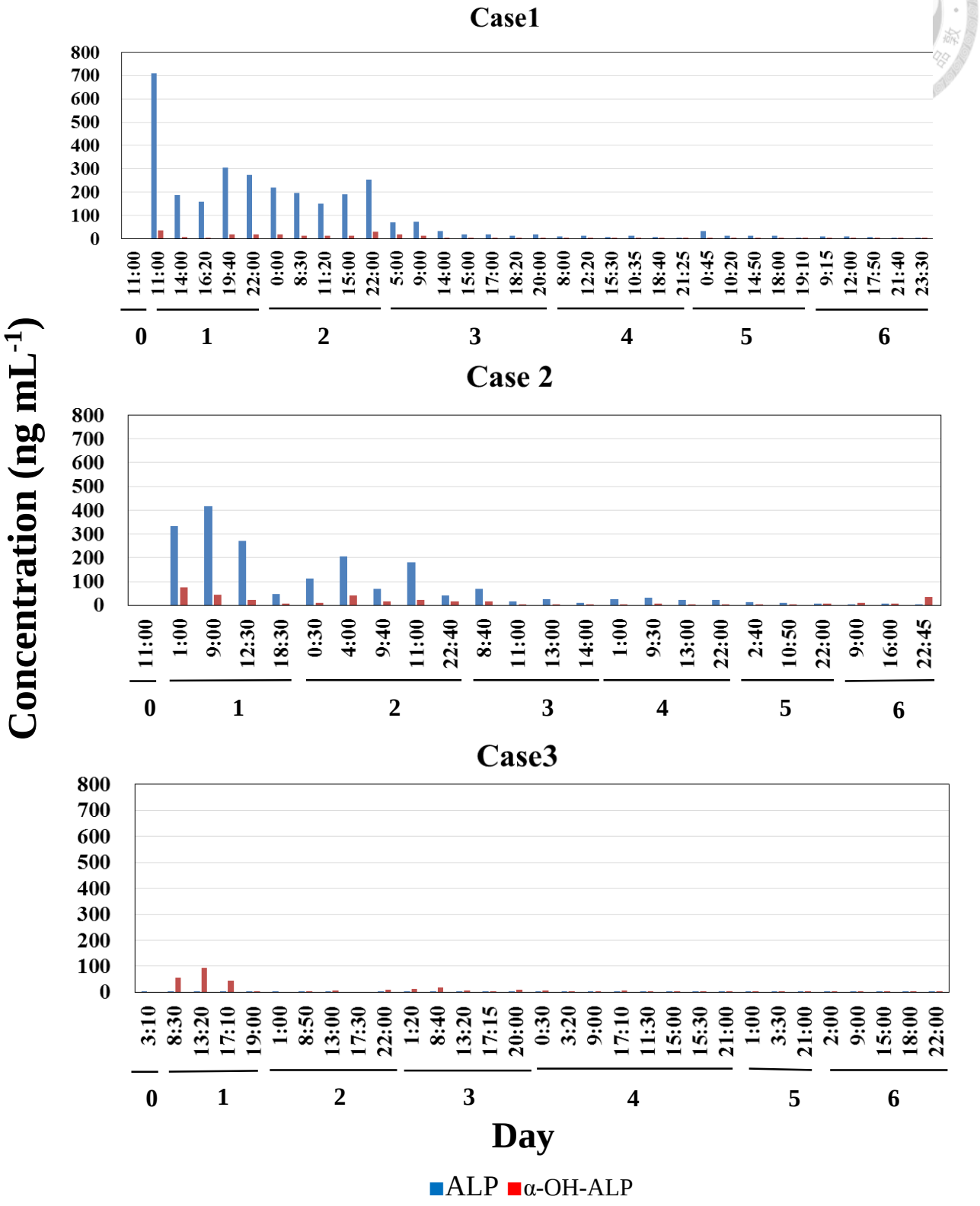


Fig.12 Quantification of ALP, α-OH-ALP in real urine sample from case 1 to 3.

Table. 1 MRM transitions monitored.

Analytes	Q1	Q3	DP	EP	CE	CXP
Nordiazepam	271	140	56	10	37	8
7-Aminoflunitrazepam	284	135	60	10	35	4
Diazepam	285	154	100	12	36	10
7-Aminoclonazepam	286	222	120	6	33	14
Alprazolam	309	281	74	7	31	15
Flunitrazepam	314	268	76	10	35	17
Clonazepam	316	270	81	9	36	16
Lorazepam	321	275	85	8	28	18
Midazolam	326	291	68	9	35	19

DP: Declustering potential

EP: Entrance potential

CE: Collision energy

CXP: Collision cell exit potential

Table. 2 Linearity results and LOD of the proposed method for BZDs in human

Analytes	**Calibration curve**	**R^2**	**LOD (ng mL^{-1})**
Nordiazepam	y=9840x-1860	0.9996	0.5
7-Aminoflunitrazepam	y=8540x-1040	0.9991	0.5
Diazepam	y=7260x-918	0.9993	0.1
7-Aminoclonazepam	y=9370x-2510	0.9992	0.5
Alprazolam	y=15900x-1220	0.9983	0.5
Flunitrazepam	y=14400x-4850	0.9982	0.5
Clonazepam	y=13500x-5920	0.9988	0.5
Lorazepam	y=70600x-29100	0.9994	0.1
Midazolam	y=63400x-2340	0.998	1.0
α-hydroxyl-alplazolam	y=5270x-1260	0.9994	0.5

Reference

1. Swartz, M.E., *UPLC™: An Introduction and Review.* Journal of Liquid Chromatography & Related Technologies, 2005. **28**(7-8): p. 1253-1263.

2. Wren, S.A.C. and P. Tchelitcheff, *Use of ultra-performance liquid chromatography in pharmaceutical development.* Journal of Chromatography A, 2006. **1119**(1–2): p. 140-146.

3. Snyder, L.R., J.J. Kirkland, and J.W. Dolan, *Introduction to modern liquid chromatography*. 2011: John Wiley & Sons.

4. Weston, A. and P.R. Brown, *High Performance Liquid Chromatography & Capillary Electrophoresis: Principles and Practices*. 1997: Academic Press.

5. Meyer, V.R., *Practical high-performance liquid chromatography*. 2013: John Wiley & Sons.

6. Takats, Z., et al., *Mass spectrometry sampling under ambient conditions with desorption electrospray ionization.* Science, 2004.

306(5695): p. 471-473.

7. Berkenkamp, S., et al., *Measurements of mean initial velocities of analyte and matrix ions in infrared matrix-assisted laser desorption ionization mass spectrometry*. Journal of the American Society for Mass Spectrometry, 2002. **13**(3): p. 209-220.

8. Gross, J.H., *Mass spectrometry: a textbook*. 2004: Springer.

9. Hoffmann, E., *Mass spectrometry*. 1996: Wiley Online Library.

10. Fernández-Maestre, R., C. Wu, and H.H. Hill Jr, *Using a buffer gas modifier to change separation selectivity in ion mobility spectrometry.* International Journal of Mass Spectrometry, 2010. **298**(1–3): p. 2-9.

11. Nováková, L., L. Matysová, and P. Solich, *Advantages of application of UPLC in pharmaceutical analysis.* Talanta, 2006. **68**(3): p. 908-918.

12. Galhena, A.S., et al., *Enhanced Direct Ambient Analysis by Differential Mobility-Filtered Desorption Electrospray Ionization-*

Mass Spectrometry. Analytical Chemistry, 2010. **82**(22): p. 9159-9163.

13. Lapthorn, C., F. Pullen, and B.Z. Chowdhry, *Ion mobility spectrometry-mass spectrometry (IMS-MS) of small molecules: Separating and assigning structures to ions.* Mass Spectrometry Reviews, 2013. **32**(1): p. 43-71.

14. Levin, D.S., et al., *Characterization of Gas-Phase Molecular Interactions on Differential Mobility Ion Behavior Utilizing an Electrospray Ionization-Differential Mobility-Mass Spectrometer System.* Analytical Chemistry, 2005. **78**(1): p. 96-106.

15. Blagojevic, V., et al., *Differential Mobility Spectrometry of Isomeric Protonated Dipeptides: Modifier and Field Effects on Ion Mobility and Stability.* Analytical Chemistry, 2011. **83**(9): p. 3470-3476.

16. Schneider, B.B., et al., *Chemical Effects in the Separation Process of a Differential Mobility/Mass Spectrometer System.* Analytical Chemistry, 2010. **82**(5): p. 1867-1880.

17. Kanu, A.B., et al., *Ion mobility–mass spectrometry.* Journal of Mass Spectrometry, 2008. **43**(1): p. 1-22.

18. Schneider, B., T. Covey, and E. Nazarov, *DMS-MS separations with different transport gas modifiers.* International Journal for Ion Mobility Spectrometry, 2013. **16**(3): p. 207-216.

19. Creaser, C.S., et al., *Ion mobility spectrometry: a review. Part 1. Structural analysis by mobility measurement.* Analyst, 2004. **129**(11): p. 984-994.

20. Porta, T., E. Varesio, and G. Hopfgartner, *Gas-Phase Separation of Drugs and Metabolites Using Modifier-Assisted Differential Ion Mobility Spectrometry Hyphenated to Liquid Extraction Surface Analysis and Mass Spectrometry.* Analytical Chemistry, 2013. **85**(24): p. 11771-11779.

21. Nutt, D.J., L.A. King, and L.D. Phillips, *Drug harms in the UK: a multicriteria decision analysis.* The Lancet. **376**(9752): p. 1558-1565.

22. Schweizer, E. and K. Rickels, *Benzodiazepine dependence and withdrawal: a review of the syndrome and its clinical management.*

Acta Psychiatrica Scandinavica, 1998. **98**: p. 95-101.

23. Poyares, D., et al., *Chronic benzodiazepine usage and withdrawal in insomnia patients.* Journal of Psychiatric Research, 2004. **38**(3): p. 327-334.

24. Koob, G.F., *Drugs of abuse: anatomy, pharmacology and function of reward pathways.* Trends in Pharmacological Sciences, 1992. **13**(0): p. 177-184.

25. Rowlett, J.K., et al., *Different GABAA receptor subtypes mediate the anxiolytic, abuse-related, and motor effects of benzodiazepine-like drugs in primates.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(3): p. 915-920.

26. Chan, A.W.K., *Effects of combined alcohol and benzodiazepine: A review.* Drug and Alcohol Dependence, 1984. **13**(4): p. 315-341.

27. Martin, L., et al., *Enhanced recognition of facial expressions of disgust in opiate users receiving maintenance treatment.* Addiction, 2006. **101**(11): p. 1598-1605.

28. O'Brien, C.P., *Benzodiazepine use, abuse, and dependence.* J Clin Psychiatry, 2005. **66**(Suppl 2): p. 28-33.

29. Chun-Jen Chen, I., et al., *Drug abuse-related accidents leading to emergency department visits at two medical centers.* Journal of the Chinese Medical Association, 2012. **75**(5): p. 234-239.

30. Marin, S.J., et al., *Quantitation of Benzodiazepines in Urine, Serum, Plasma, and Meconium by LC-MS-MS.* Journal of Analytical Toxicology, 2008. **32**(7): p. 491-498.

31. Schuckit, M.A., *Drug and alcohol abuse: A clinical guide to diagnosis and treatment*. 2006: Springer.

32. de Armas, H.N., et al., *Polymorphism of alprazolam (Xanax®): A review of its crystalline phases and identification, crystallographic characterization, and crystal structure of a new polymorph (form III).* Journal of Pharmaceutical Sciences, 2007. **96**(5): p. 1114-1130.

33. Wennerholm, A., et al., *Alprazolam as a probe for CYP3A using a single blood sample: pharmacokinetics of parent drug, and of α- and 4-hydroxy metabolites in healthy subjects.* European Journal of

Clinical Pharmacology, 2005. **61**(2): p. 113-118.

34. Verster, J.C. and E.R. Volkerts, *Clinical Pharmacology, Clinical Efficacy, and Behavioral Toxicity of Alprazolam: A Review of the Literature.* CNS Drug Reviews, 2004. **10**(1): p. 45-76.

35. Levine, B., *Forensic toxicology.* Analytical chemistry, 1993. **65**(5): p. 272A-276A.

36. Blagojevic, V., G. Koyanagi, and D. Bohme, *Multi-Component Ion Modifiers and Arcing Suppressants to Enhance Differential Mobility Spectrometry for Separation of Peptides and Drug Molecules.* Journal of The American Society for Mass Spectrometry, 2014. **25**(3): p. 490-497.