Received 22 November 2014
Received in revised form 23 December 2014
Accepted 24 December 2014
Available online 28 December 2014
Peer Reviewed
2157118X.3.3.P4

Qualitative and Quantitative Analysis of Clonazepam and its Metabolite 7-aminoclonazepam in Blood by LC-tandem QTOF/MS and LC-MS/MS

DING Jing [a], ***SHI Yintao*** [a], ***WANG Junwei*** [*, a]

[a] Chongqing Engineering Researh Center for Criminal Investigation Technology, Institute of Forensic Science, Chongqing Public Security Bureau, Chongqing 400021, China.

[*] Corresponding author: Senior Coroner *WANG Junwei*. E-mail: dingjing_111@126.com

Abstract *Objective* A highly sensitive and specific analytical method was developed for the qualitative and quantitative analysis of clonazepam (CLOZ) and its metabolite 7-aminoclonazepam (7-AC) in blood. The analytes were identified by automated solid phase extraction-liquid chromatography-tandem quadrupole time-of-flight mass spectrometry (ASPE-LC-QTOF/MS) and quantitatively determined by automated solid phase extraction-liquid chromatography-tandem triple quadrupole mass spectrometry (ASPE-LC-MS/MS).

Methods After purified by automated solid-phase extraction (ASPE), the qualitative analysis of CLOZ and 7-AC in blood was performed by separation on an Agilent Eclipse Plus C_{18} HPLC column (50 mm×2.1 mm, 1.8 μm) using methanol-0.1%(v/v) formic acid aqueous solution. The detection was performed on a quadrupole time-of-flight mass spectrometer. The quantitative analysis of CLOZ and 7-AC in blood was carried out on LC-MS/MS. The chromatography separation was performed on a Waters Atlantis TM dC18 column (150mm×3.9mm,5.0μm) using methanol-water with 0.1%(v/v) formic acid as mobile phase. The detection was carried out on a triple quadrupole mass spectrometer under electrospray ion source (ESI) in positive ionization mode and multiple reaction monitoring (MRM).

Results The result screening database of antidepressants was built using Agilent Mass Hunter Personal Compound Database Library (PCDL) Manager software and then used for the analysis of spiked samples. The results showed that both CLOZ and 7-AC could be correctly identified with low deviation of retention time (<0.1 min), mass (<1 mDa) and MS matching scores, isotopic abundance matching scores, isotope spacing matching scores (all> 95 points). Furthermore, good linear responses were obtained between the concentration range, 2-1000 ng/mL with a regression coefficient (r^2) higher than 0.99 for both CLOZ and 7-AC. The LOD values for CLOZ and 7-AC were 0.5 ng/mL and 0.2 ng/mL, respectively. The LOQ values for CLOZ and 7-AC were 2 ng/mL and 1 ng/mL, respectively. The RSD values obtained from intra-day and inter-day experiments ranged from 4.2 to 10.3%. The recovery ranges for CLOZ and 7-AC were all above 70% for three spiked levels.

Conclusion The developed method was further applied for the analysis of poisoning case, both CLOZ and 7-AC in blood were confirmed and quantified. The combination of ASPE-LC-QTOF/MS and ASPE-LC-MS/MS is suitable for the identification and quantification of benzodiazepines and their metabolites in forensic toxicology.

Keywords: Forensic science, Toxicology, Clonazepam, 7-aminoclonazepam, Metabolite, Automated solid phase extraction, Liquid chromatography, Tandem quadrupole time-of-flight mass spectrometry, Triple quadrupole mass spectrometry, Blood, ASPE-LC-QTOF/MS, ASPE-LC-MS/MS.

1 Introduction

Clonazepam (CLOZ) is a benzodiazepine derivative that was approved for use as an anticonvulsant in the US in 1975[1]. At present, CLOZ has been one of the most frequently prescribed psychoactive drugs world wide due to its hypnotic, anxiolytic, anticonvulsant and muscle-relaxant properties[2-3]. However, CLOZ, because of its pharmacological effects has been identified as a compound frequently used in drug-facilitated crimes (DFC) such as robberies and sexual assaults in recent years[4-6].

CLOZ has a plasma half-life varying from 19 to 60 h, where the mean value is 40 h[7]. It is extensively metabolized in the liver, primarily by CYP3A4, to its major metabolite 7-aminoclonazepam (7-AC)[8]. 7-AC is excreted mainly in urine. Only about 0.5% of parent drug is

excreted unchanged in the urine[6]. In clinical studies oral administration of a single 2 mg dose resulted in an average plasma concentration of 17 ng/mL (range: 7-24 ng/mL) of CLOZ between 1 and 4 h after ingestion[9]. In studies of patients receiving 6 mg/day chronic therapy, the plasma concentration of CLOZ and 7-AC were reported as 29-75 ng/mL and 23-137 ng/mL, respectively[10].

Because of above metabolic characteristics, sometimes parent drug CLOZ cannot be directly detected while only its major metabolite 7-AC can be detected[7-8]. This indicated the detection of 7-AC can be regarded as evidence of CLOZ intake. It has been reported that postmortem bioconversion to 7-amino metabolites may also occur, to the extent that little or no parent drug may be present; the identification and quantification of the 7-amino metabolites are of importance toxicologically as they are often the only indication of nitrobenzodiazepine use prior to death[11]. Consequently the simultaneous determination of CLOZ and its metabolite 7-AC is very important in the fields of forensic toxicology.

On the other hand, in most cases, victims don't often report until after some time; therefore, blood and urine samples are often detected more than 24-72 h after ingestion[6]. Moreover, only immunoassay screening and sometimes liquid chromatography-diode array detector (HPLC-DAD) and gas chromatography-mass spectrometry (GC-MS) analysis are performed on blood and urine at the hospital laboratory where the victim is admitted[12]. Since most of the drugs involved in DFC are not detectable at low levels by these techniques, resulting false negative results may lead to an inaccurate conclusion and to premature destruction of samples[12]. For this reason it is important to develop highly sensitive and specific analytical methods for the simultaneous determination of CLOZ and its metabolite 7-AC in biological fluids.

Numerous analytical methods including spectrophotometry[13-14], gas chromatography (GC)[15-19], gas chromatography-mass spectrometry (GC-MS)[20-24] and high-performance liquid chromatography (HPLC)[3, 4, 9, 25-26] have been presented for testing CLOZ in biological fluids. However, when these methods are used, some problems of low sensitivity, specificity and reproducibility are encountered[20]. Due to its ability to analyze thermally unstable or polar compounds, such as 7-AC, liquid chromatography (LC) based methods offer a distinct advantage over GC related techniques. Recently, detection of CLOZ and its metabolite 7-AC in biological fluids has become available with the development of more and more sensitive LC-MS apparatuses[27]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as the most suitable tool to test for low concentrations of CLOZ[2, 28-31] and its metabolite 7-AC[5, 7, 12, 27, 32].

At present, LC-MS/MS is widely used in the determination of benzodiazepines and their related drugs due to its strong specificity, high sensitivity, good reproducibility, wide linear dynamic range and other characteristics[30]. But LC-MS/MS can only analyze the target compound which is included in established database, while it can't screen the unknown compound which is not included in established database no matter how much concentration of the unknown compound is. These lacks of LC-MS/MS assays limit qualitative analysis and rapid screening of target compound containing multiple components at trace levels (e.g., ng/L)[29].

Recent advances in LC-MS technology have lead to the availability of time-of-flight (TOF) LC/MS systems. These provide a greater level of the analyte information as a result of high resolution and the ability to collect accurate mass information to the sub ppm levels[33]. This significantly increases the confidence in the analyte identification by limiting the possible number of candidate compounds. The coupling of a quadrupole and a collision cell to the TOF analyzer, the fragmentation of pre-selected ions and the identification of compounds based on their product ion spectra. Accurate mass determination of both the precursor and product ions is therefore possible[34-35]. Rapid screening and confirmation of the analyte can be achieved with MS matching scores, deviation of retention time, measured mass, isotopic abundance matching scores and isotope spacing match scores and MS/MS matching scores[36-37].

With the exception of some immunoassays[1], liquid liquid extraction (LLE)[3, 9, 20, 21, 25] and solid phase extraction (SPE)[28, 31, 38-40] are commonly included in sample pretreatment methods for the determination of CLOZ and 7-AC in biological fluids. Compared with LLE, SPE can improve the extraction recovery of CLOZ and 7-AC in biological fluids due to its advantages such as less use of organic solvents, stronger selectivity, shorter separation time, better reproducibility and better the clean-up effect. Furthermore, a large-diameter extraction disk is equipped with fully automatic solid phase extraction system (ASPE) for SPE, and the sample pretreatment procedure becomes simple and efficient[29].

The aim of this report is to develop a highly sensitive and specific analytical method for qualitative and quantitative analysis of CLOZ and its metabolite 7-AC in blood by using ASPE-LC-QTOF/MS combined with ASPE-LC-MS/MS. This study details the use of a ASPE coupled to LC-QTOF/MS system for the qualitative determination of CLOZ and its metabolite 7-AC in blood. The screening database of antidepressants is built using Agilent Mass Hunter

Personal Compound Database Library (PCDL) Manager software and then used to screen and confirm CLOZ and its metabolite 7-AC in blood. In addition, a ASPE coupled to LC-MS/MS system is used for the quantitative determination of CLOZ and 7-AC in blood. In order to evaluate the reliability of the developed method, it was applied for the simultaneous determination of CLOZ and 7-AC in case of poisoning.

2 Materials and Experiments

2.1 Chemicals and reagents

CLOZ and 7-AC were purchased from Sigma-Aldrich (St Louis, USA). HPLC-grade methanol and acetonitrile were obtained from TEDIA (Ohio, USA). All other chemicals were of analytical-grade in the highest purity available. Water was distilled and purified using a Millipore Milli-Q Plus system (Bedford, USA).

2.2 Preparation of stock solutions and working solutions

Stock solutions of CLOZ and 7-AC were prepared by dissolution of each drug in methanol to obtain a concentration of 1 mg/mL. Working solutions of CLOZ and 7-AC were prepared respectively each day by making appropriate dilutions of the stock solutions in methanol. All these solutions were stored at -20 °C in the absence of light.

2.3 Sample pretreatment

1 mL blood sample was added a 16 mm × 125 mm screw top test tube and diluted with 2 mL of Milli-Q water and then buffered with 2 mL of saturated sodium carbonate (Na_2CO_3) solution to give a final pH of 9. The tube was capped and submitted to vortex mixing for 5 min.

2.3.1 Liquid-liquid extraction (LLE)

The resultant mixture was shaken for 5min with 5 mL ethyl acetate. After centrifugation at 9500 r/min for 5 min, the upper organic phases were transferred to conical tubes and separated. The aqueous phase was re-extracted with 5 mL ethyl acetate. The organic phases were evaporated at 40 °C under flow of nitrogen to obtain good results. The residue was dissolved in 1 mL of the mobile phase and filtered with a Nylon filter (0.22 μm).

2.3.2 Automatable solid-phase extraction (ASPE)

A C18 cartridge (500 mg/3 mL, Waters technologies, USA) was activated with 3 mL of methanol and washed with 3 mL of Milli-Q water. After that, the resultant mixture was loaded and washed with 3 mL of Milli-Q water. The elution was performed with 3 mL of methanol. The whole SPE procedure was performed on a Gilson GX-274 fully automatable solid phase extraction apparatus (Wisconsin, USA). The collected solvent was evaporated at 40 °C under flow of nitrogen and the residue was dissolved in 1 mL of the mobile phase and filtered with a Nylon filter (0.22 μm).

2.4 Instrumentation

The qualitative detection of analyses was performed on the Agilent 1290-6550 ultra-high performance liquid chromatography-tandem quadrupole time-of-flight mass spectrometer equipped with Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI) (Agilent Technologies, Santa Clara, USA). The quantitative detection of analyses was carried out using the LC-20A liquid chromatography (Shimadzu, Japan)-API2000 tandem triple quadrupole mass spectrometer (AB Technologies, USA).

2.5 Analytical conditions

2.5.1 Chromatographic conditions

Qualitative conditions: the chromatographic separation was performed on Agilent Eclipse Plus C18 (50 mm × 2.1 mm, 1.8 μm; Agilent Technologies, Santa Clara, USA) at 35 °C with the eluents A = methanol and B = water (containing 0.1% formic acid) and the following time program of the gradient: 0.0-2.0 min 20% A, linear to 95% A at 7.0 min, const. 95% A to 9.0 min, back to 20% A at 9.1 min and equilibration for 3 min. The flow rate was 0.3 mL/min.

Quantitative conditions: gradient elution was performed on separation column (Waters Atlantis TM dC18, 50 mm × 3.9 mm, 5.0 μm; Waters technologies, USA). The mobile phase consisted of methanol (eluent A) and water containing 0.1% formic acid (eluent B). The gradient was programmed as follows: 0.0-5.0 min: gradient increases from 20% to 100% eluent A; 5.0-12.0 min: gradient 100% eluent A; 12.0-12.1 min: gradient decreases to 20% eluent A; 12.1-16.0 min: gradient 20% eluent A. The column oven was set at 35 °C and the flow rate was 1.0 mL/min.

2.5.2 MS conditions

Qualitative conditions: The QTOF-MS instrument was operated under electrospray ion source (ESI) in positive ionization mode with full scanning range of *m/z* 50-1000 Da. The source parameters were: dry gas temperature 200 °C, dry gas flow rate 16.0 L/min, nebulizer pressure 35 psi, sheath gas temperature 350°C, sheath gas flow rate 12.0 L/min, VCap voltage 4000 V and fragmentor voltage 135 V, reference ions for mass calibration: purine 121.050873 $[M+H]^+$, HP-921 = hexakis (1H,1H,3H-tetrafluoropropoxy) phosphazine 922.009798 $[M+H]^+$.

Data acquisition and qualitative management were performed by Agilent Mass Hunter Workstation Data Acquisition and Agilent Mass Hunter Qualitative Analysis (B.06.00) software, respectively. The screening database of antidepressants was established by Agilent Mass Hunter Personal Compound Database Library (PCDL) Manager software and then used for the analysis of spiked samples.

Quantitative conditions: For detection and quantification the following ESI inlet conditions were

applied: gas 1, nitrogen (35 psi); gas 2, nitrogen (40 psi); ion spray voltage, 5200 V; ion source temperature, 450 °C; curtain gas, nitrogen (20 psi). The mass spectrometer was operated in the information dependent acquisition (IDA) mode. As survey scan the multi-reaction monitoring mode (MRM) was used, followed by the dependent scan, which was an enhanced product ion scan (EPI). The settings for the MRM mode were as follows: collision gas was set at 4, the dwell time was set at 20 ms. All other settings were analyte-specific and were determined using Analyst software in the quantitative optimization mode. The MRM transitions per analyte and respective settings for both LC-MS/MS systems are summarized in Table 1.

2.6 Method validation

The method was validated for the following parameters: sensitivity (limit of detection LODs and limit of quantification LOQs), linearity, intra- and inter-day precision, recovery and matrix effect.

2.6.1 Linearity

Analytes were quantified by means of calibration curves constructed from seven known concentrations of analyte standards in pure solvent (2, 5,10, 50, 200, 500 and 1000 ng/mL). Linearity of calibration curves based on peak area as function of the nominal concentration was assessed by least square regression[25].

2.6.2 Sensitivity

The limit of detection (LOD) was considered the lowest concentration of these analytes corresponding to three times the background noise or relationship signal to noise ratio 3:1. The limit of quantification (LOQ) was defined as the lowest point of the calibration curve and fulfilled the requirement of LOQ signal-to-noise ratio of 10:1[39].

2.6.3 Precision and recovery

Intra- and inter-day precision of the analytical method were shown by following the analysis of three different spiked blood samples (50, 200 and 800 ng/mL). Precision was calculated as relative standard deviation (RSD) of the experimental concentrations[33].

The recoveries of COLZ and 7-AC were determined by comparing the response of the analyte from pure standard (n = 6) with the response of the analyte from extracted spiked blood sample at equivalent quantity[27]. Recovery was determined at low, mid, and high quality control concentrations, 50, 200 and 800 ng/mL respectively.

2.6.4 Matrix effect

The matrix effect of biological samples can affect the analyte signals, enhance background noises or suppress the analyte responses[41]. This matrix effects may result as positive or negative responses depending on the level of ion suppression and can greatly affect the method reproducibility and accuracy[42].

3 Results and Discussion

3.1 Optimization of sample pretreatment

3.1.1 Liquid-liquid extraction (LLE) and Automated solid phase extraction (ASPE)

The impact of the extraction pretreatment (LLE or ASPE) of blood sample on extraction recoveries of analyte in blood sample was investigated in LC-QTOF/MS system. Extraction recoveries were calculated from peak area ratios between extracted blood samples spiked at three concentrations (50, 200 and 800 ng/mL) before and after the extraction pretreatment[45].

It was shown in Table 2 that extraction recoveries of CLOZ and 7-AC in blood sample by LLE ranged between 56.4% and 82.5%, while extraction recoveries of CLOZ and 7-AC in blood sample by ASPE ranged between 68.4% and 91.8%. The results suggested that the extraction efficiency of CLOZ and 7-AC in blood sample by ASPE were better than that by LLE. On the other hand, the RSD values of CLOZ and 7-AC in blood sample by ASPE were obviously lower than those by LLE. The results indicated that compared with LLE, ASPE provided superior stability, better repeatability and lower susceptibility to matrix interferences for the extraction pretreatment of CLOZ and 7-AC in blood sample.

3.1.2 pH values of blood sample

In the present study the effect of different pH values of blood sample extracted by ASPE on extraction recoveries of the analyte at medium

Table 1. The MRM transitions per analyte and respective settings for LC-MS/MS system (CE: collision energy [V], DP: declustering potential [V], EP: entrance potential [V], CEP: collision cell exit potential [V]).

Analyte	Ion pairs (m/z)	CE	DP	EP	CEP
CLOZ	316.1/270.0* 316.1/241.1	26,36	20	10	8
7-AC	286.2/222.2* 286.2/250.1	36,28	20	10	8

* stands for the quantitative ion pair.

Table 2. Extraction recoveries of analytes in blood sample by liquid-liquid extraction(LLE) and automated solid phase extraction (ASPE) (n = 6, LC-QTOF/MS system)

Analyte	Concentration added(ng/mL)	LLE		ASPE	
		Recovery (%)	RSD(%)	Recovery(%)	RSD(%)
CLOZ	50	56.4	8.5	68.4	6.8
	200	70.3	7.9	78.7	5.9
	800	78.9	5.4	87.3	5.4
7-AC	50	61.7	8.9	73.7	7.1
	200	75.3	9.2	86.9	5.4
	800	82.5	5.8	91.8	4.4

concentration was also investigated. The results were shown in Table 3. It was seen that increasing sample pH value provided higher extraction recoveries for CLOZ and 7-AC in blood sample. Nevertheless, in order to avoid analyte decomposition in strong alkaline solutions (pH ≥11) and blood solidification resulting in low extraction recovery, pH 9 was chosen as final pH value of blood sample extracted by ASPE.

3.2 Establishment and optimization of qualitative method in LC-QTOF/MS system (*Database building and screening)*

A large number of antidepressants, which included CLOZ and 7-AC, were selected as target compounds and the screening database of them was built using Agilent Mass Hunter PCDL Manager software. The target compound name, molecular formula, retention time and extract mass for (de)protonated compounds, and information on the elemental compositions for their main fragment ions were included in the database[43]. The resulting screening database was built using Agilent Mass Hunter PCDL Manager software and then used to screen unknown samples based on a combination of retention time, observed spectral molecular weight and isotope ratio, generating a match score for any identified compounds[35]. Further confidence in analyte identification can then be obtained through the selected fragmentation of any identified compounds, and comparing the MS/MS data with that stored as library spectra[36].

Thus, the built screening database was used for the analysis of spiked samples. MS matching scores, isotopic abundance matching scores, isotope spacing matching scores (all> 95 points) and MS/MS matching scores (> 70 points)[3] were applied to identify the analytes (CLOZ and 7-AC). The results were shown in Table 4. As it can be seen that both CLOZ and 7-AC could be correctly identified with low deviation of retention time (<0.1 min), mass (<1 mDa) and MS matching scores, isotopic abundance matching scores, isotope spacing matching scores (all> 95 points).

Moreover, MS/MS matching scores of 7-AC were 73.87 points (> 70 points) while MS/MS matching scores of CLOZ were 65.42 points (< 70 points). The main reason was that this drug CLOZ might produce the low signal response (e.g. in sensitivity) and might be highly affected by matrix effect[44]. As shown in Fig. 1, the agreement of the spectra (similarity index and specific fragments m/z of identity) as well as of the retention time were used as criteria for identification. This indicated that CLOZ was screened and identified.

3.3 Establishment and optimization of quantitative method in LC-MS/MS system

3.3.1 Optimization of chromatographic Separation

Table 3. Extraction recoveries of C18 disk under different pH values (n = 3, LC-QTOF/MS system)

Analyte	Extraction recoveries (%)				
	pH 3.0	pH 5.0	pH7.0	pH9.0	pH 11.0
CLOZ	42.5±8.4	58.2±5.9	72.5±6.3	80.3±5.6	78.6±6.4
7-AC	51.8±7.8	64.2±8.1	80.4±6.0	85.9±5.7	87.2±7.1

Table 4. Screening results of analytes from Agilent MassHunter PCDL Manager software (LC-QTOF/MS system)

Name	MS matching scores	Theoretical Mass/Da	Measured Mass/Da	Deviation of Mass/mDa	Deviation of retention time/min	Isotopic abundance matching scores	Isotope spacing matching scores	MS/MS matching scores
CLOZ	91.68	315.0411	316.0499	-1.58	-0.031	95.85	99.18	65.42
7-AC	97.68	285.0675	286.0748	-0.63	-0.064	96.76	99.38	73.87

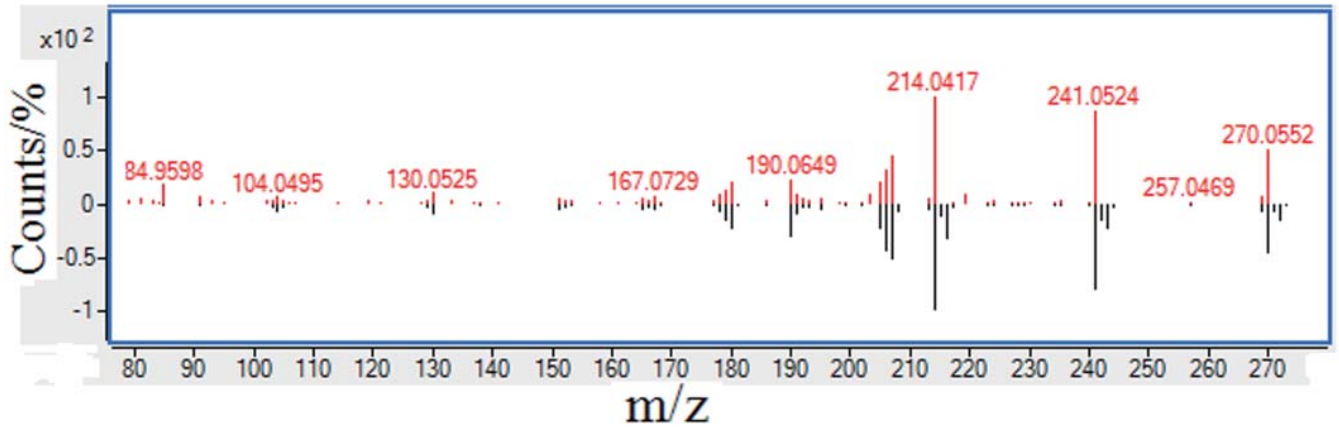

Fig 1. A chromatogram identifying CLOZ in blood sample using the custom PCDL(retention time < 0.1 min, mass < 1 mDa).

The optimization of LC conditions was focused on improving the shape of peaks in order to achieve good separation among major substances from complicated matrices. Thus, the effects of two different mobile phases on ionization degree were compared in the present study. Two different mobile phases were (1) acetonitrile -water with 0.1%(v/v) formic acid and (2) methanol-water with 0.1%(v/v) formic acid. The result suggested a better chromatographic resolution and sensitivity for blood samples using methanol-water with 0.1%(v/v) formic acid as mobile phase.

Furthermore, several other chromatographic conditions, such as type of column and its length, flow rate, elution gradient program and column temperature were also optimized to obtain a satisfactory chromatographic separation (good resolution and efficiency) for target analytes. These optimized chromatographic conditions were described in Section 2.5.1 and produced the results presented in Fig. 2. It can be observed that CLOZ had a retention time of 5.82 min and 7-AC of 4.03 min.

3.3.2 Optimization of MS conditions

The MS spectra were obtained in positive mode using LC-MS/MS, and the experimental conditions were optimized. Results were described in Section 2.5.2 (Table 1). As shown in Table 1, the protonated molecule $[M+H]^+$ were observed at *m/z* 316.1 and 286.2 in Q1 full scan for CLOZ and 7-AC, respectively. The major fragments observed in MS-MS spectrum of CLOZ and 7-AC were at *m/z* 270.0 and 241.1, 222.2 and 250.1, respectively. Precursor-product ion transitions were selected according to the highest sensitivity, optimal selectivity and reproducibility of the ion ratio. Confirmation of the identity of CLOZ and 7-AC was based on retention time, transitions selected and ion ratio statistics for the

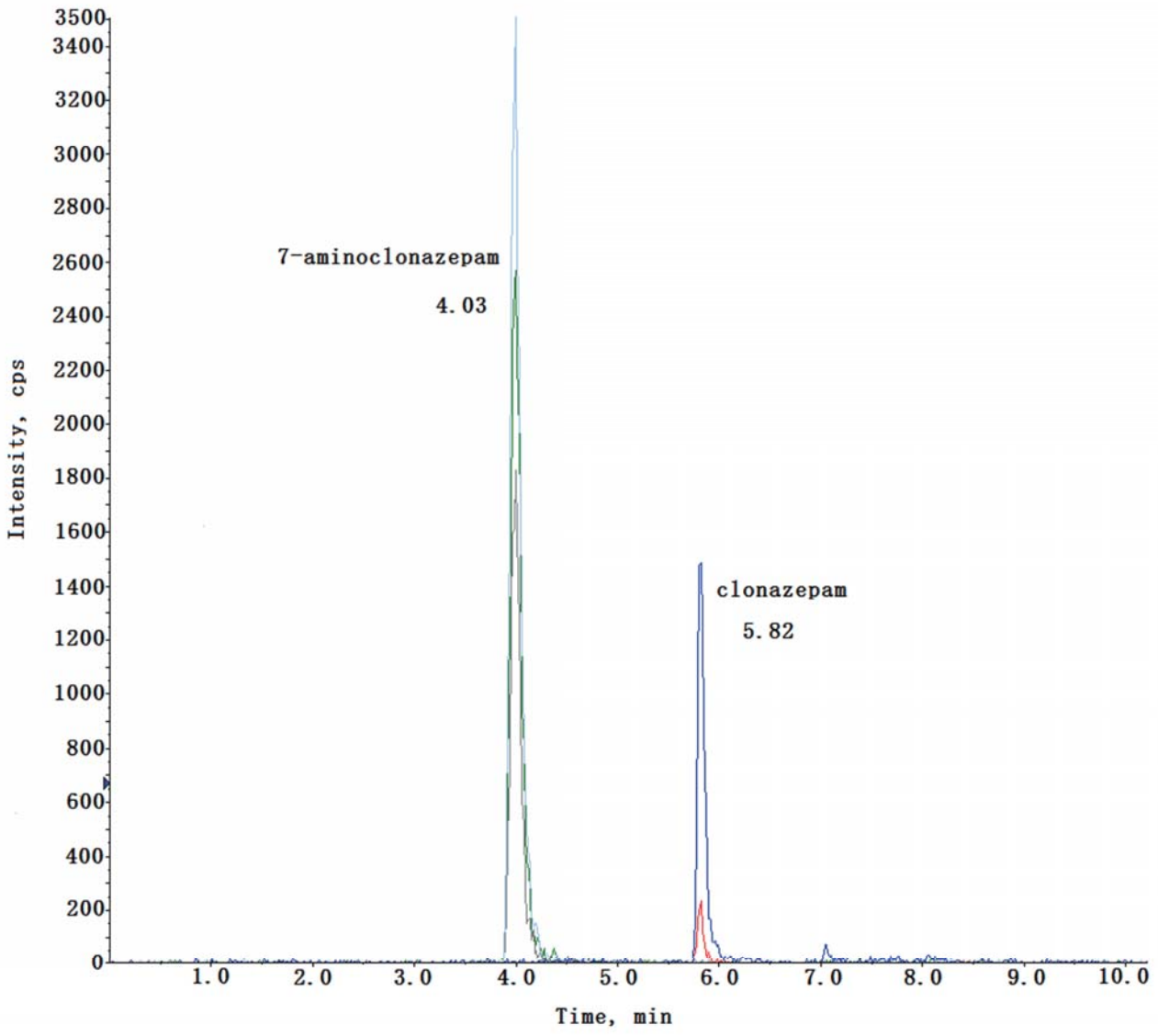

Fig 2. MRM chromatogram (LC-MS/MS system) of CLOZ(clonazepam) and 7-AC(7-aminoclonazepam) (100 ng/mL) in methanol.

transition monitored. The *m/z* ions 270.0 and 222.2 were used for the quantification of CLOZ and 7-AC, respectively, with 241.1 and 250.1 as qualifier ions. An external standard method was developed for qualifying CLOZ and 7-AC over a level of 2-1000 ng/mL. Replicate calibration standards (n=6) for each level were analyzed, and peak areas were used for quantification.

3.4 Quantitative method validation in LC-MS/MS system

3.4.1 Linearity, LOD and LOQ

Good linear responses were obtained between the concentration range, 2-1000 ng/mL with a regression coefficient (r^2) higher than 0.99 for both CLOZ and 7-AC. Each calibration equation was fitted by the linear regression equation $y=mx+c$ where y was the signal peak area of the spiked analyte and x is the corresponding concentration of the spiked analyte. Slopes, intercepts, and regression coefficients were summarized in Table 5.

LOD and LOQ for all analyzing CLOZ and 7-AC were calculated in the range of 2-1000 ng/mL and were also presented in Table 5. It can be seen that the LOD values for CLOZ and 7-AC were 0.5 ng/mL and 0.2 ng/mL, respectively. The LOQ values for CLOZ and 7-AC were 2 ng/mL and 1 ng/mL, respectively. This indicated that the proposed method was suitable for the high sensitive determination of CLOZ and 7-AC in blood samples.

3.4.2 Precision and recovery

In this work, the precision of intra- and inter-day of low, medium and high (50, 200 and 800ng/mL) spiked blood samples were determined. The RSD values obtained from intra-day (five successive injections) and inter-day (three successive days) experiments ranged from 4.2 to 10.3%. Recovery study was carried out by preparing the spiked blood samples at three concentrations of low, intermediate and high spiked levels as mentioned in precision. The recovery ranges for CLOZ and 7-AC were all above 70% for three spiked levels. Results of both precision and recovery tests were shown in Table 6.

3.4.3 Evaluation of matrix effect

Due to the complexity and high inter-subject variability of blood, ESI-MS signals may seriously impair the reliability of a method[41]. The assessment of matrix effects in different batches of blood has been included as part of the validation procedure. In the present study, the matrix effect was calculated by comparing peak areas of each analyte standard in pure solvent (S) with the those of corresponding peak areas of spiked blood extracted by ASPE, according to the following equation[42].

Ion suppression or enhancement (%) = (Y - X) / X ×100

Where, X is the peak area of an analyte standard in S and Y is the peak area of the analyte standard in blood extracted by ASPE. The results showed that the values of matrix effects for CLOZ and 7-AC in extracted blood samples spiked at three concentrations (50, 200 and 800 ng/mL) were 6-14% and -14-1%, respectively. The ion suppression or enhancement by blood was within ±15% for all for both CLOZ and 7-AC and was within acceptable experimental errors. It was confirmed that after spiked blood sample extracted by ASPE, no serious matrix interferences with the analyte's signal occurred.

3.5 Case examples

The practical application of the developed method using ASPE-LC-QTOF/MS combined with ASPE-LC-MS/MS was demonstrated at two examples.

3.5.1 Case #1

This is a sample of blood taken from a 58-year-old male. He was robbed at home after having had a cup of coffee offered by two men who spent the night at his home. Blood was collected at the hospital 24h after the crime.

3.5.2 Case #2

This is a sample of blood taken from a victim of drug-facilitated sexual assault. The 23-year-old female suspected that she had been victim of a sexual assault during her stay at a retiring room of a nightclub after having had two drinks in the nightclub. Blood was collected at the hospital 48h after the crime.

The blood samples of two case examples were analyzed by the present methodology. The CLOZ and 7-AC levels of Case #1 were 24.5 and 30.8 ng/mL, respectively. The CLOZ and 7-AC levels of Case #2 were 12.5 and 20.2 ng/mL, respectively. The results indicated that this

Table 5. Quantitative performance of the analytes in LC-MS/MS system

Analyte	Calibration range (ng/mL)	Calibration curve	Regression coefficient (r^2)	LOD (ng/mL)	LOQ (ng/mL)
CLOZ	2-1000	$y = 90.2x+11.6$	0.9964	0.5	2
7-AC	2-1000	$y = 167x+1050$	0.9918	0.2	1

Table 6. Recoveries and RSDs of the analytes in LC-MS/MS system (n = 6)

Analyte	Concentration added(ng/mL)	Recovery (%)	RSD (%)	
			Intra-day	Inter-day
CLOZ	50	72.6	5.6	10.3
	200	78.6	5.2	8.7
	800	85.6	4.8	7.6
7-AC	50	79.1	6.2	9.4
	200	84.2	5.4	8.2
	800	96.3	4.2	6.8

Recovery = peak area of the analyte spiked in blood after ASPE/peak area of the analyte spiked with the same concentration in blank matrix extract×100%

metabolite 7-AC can be regarded as the target analyte to prove ingestion of clonazepam. Furthermore, CLOZ and 7-AC were judged to be the significant contributors in the two poisoning cases.

4 Conclusion

In the present work, the combination of ASPE-LC-QTOF/MS and ASPE-LC-MS/MS proved to be a practicable and efficient way for the simultaneous identification and quantification of CLOZ and 7-AC in blood. The developed method using ASPE-LC-QTOF/MS is regarded as a sensitive and specific analytical method successfully applied to the simultaneous screening and confirmation of CLOZ and 7-AC in blood due to QTOF analyzer's higher sensitivity, its much higher separation power by retention time as well as by accurate mass. Additionally, by using ASPE-LC-MS/MS, a sensitive and robust analytical method for the simultaneous quantification of CLOZ and 7-AC in blood has also been developed with good linearity, LOD, LOQ, precision and recovery as well as its low susceptibility to matrix interferences.

The above developed method was further applied for the analysis of poisoning cases, both CLOZ and 7-AC in blood were confirmed and quantified. In conclusion, the developed method using ASPE-LC-QTOF/MS combined with ASPE-LC-MS/MS can be used in future studies for the simultaneous identification and quantification of benzodiazepines and their metabolites in forensic toxicology.

Acknowledgements

The authors would like to thank Agilent Technologies Inc. for generous technical support of these investigations and in particular Dr. JIANG Chang (Chengdu, China), Mr. GUO Jingqi and Mr. WANG Huijun(Chongqing, China) for helpful cooperation and discussions.

This paper is supported by the Natural Science Foundation of Chongqing of China (cstc,2011pt-gc00004), and the Key Science and Technology Program of Chongqing Public Security Bureau (G2013-04).

Reference

[1] A A Elian. ELISA detection of clonazepam and 7-aminoclonazepam in whole blood and urine. Forensic Sci Int, 2003, 134: 54-56.
[2] E N Sauve, M Langødegård, D Ekeberg, Å M L Øiestad. Determination of benzodiazepines in ante-mortem and post-mortem whole blood by solid-supported liquid–liquid extraction and UPLC–MS/MS. J Chromatogr B: Biomed Appl, 2012, 883: 177-188.
[3] A E Mahjoub, C Staub. Simultaneous determination of benzodiazepines in whole blood or serum by HPLC/DAD with a semi-micro column. J Pharm Biomed Anal, 2000, 23: 447-458.
[4] W M Mullett, J Pawliszyn. Direct LC analysis of five benzodiazepines in human urine and plasma using an ADS restricted access extraction column. J Pharm Biomed Anal, 2001, 26: 899-908.
[5] M Chèze, M Villain, G Pépin. Determination of bromazepam, clonazepam and metabolites after a single intake in urine and hair by LC-MS/MS application to forensic cases of drug facilitated crimes. Forensic Sci Int, 2004, 145: 123-130.
[6] L W Storhaug, A Enger, K Hjelmeland, E L Øiestad, V Vindenes. Prolonged excretion of 7-aminoclonazepam in urine after repeated ingestion of clonazepam: A case report. Forensic Sci Int, 2012, 222: 33-35.
[7] A Steentoft, K Linnet. Blood concentrations of clonazepam and 7-aminoclonazepam in forensic cases in Denmark for the period 2002-2007. Forensic Sci Int, 2009, 184: 74-79.
[8] A A Salem, B N Barsoum, E L Izake. Spectrophotometric and fluorimetric determination of diazepam, bromazepam and clonazepam in pharmaceutical and urine samples. Spectrochim Acta Part A, 2004, 60: 771-780.
[9] I F Bares, F Pehourcq, C Jarry. Development of a rapid RP-HPLC method for the determination of clonazepam in human plasma. J Pharm Biomed Anal, 2004, 36: 865-869.
[10] J Hackett, A A Elian. Extraction and analysis of clonazepam and 7-aminoclonazepam in whole blood using a dual internal standard methodology. Forensic Sci Int, 2007, 166: 209-217.
[11] M D Robertson, O H Drummer. High-performance liquid chromatographic procedure for the measurement of nitrobenzodiazepines and their 7-amino metabolites in blood. J Chromatogr B: Biomed Appl, 1995, 667: 179-184.
[12] M Chèze, G Duffort, M Deveaux, G Pépin. Hair analysis by liquid chromatography-tandem mass spectrometry in toxicological investigation of drug-facilitated crimes: Report of 128 cases over the period June 2003-May 2004 in metropolitan Paris. Forensic Sci Int, 2005, 153: 3-10.
[13] F R Gil, J A Daros, A Salvador, M de la Guardia. Direct derivative spectrophotometric determination of nitrazepam and clonazepam in biological fluids. J Pharm Biomed Anal, 1991, 9: 539-545.
[14] F R Gil, A Salvador, M de la Guardia. Influence of the differentiation system on the analytical parameters for the spectrophotometric determination of clonazepam in urine. Microchem J, 1991, 44: 249-257.
[15] A G de Boer, J R Kaiser, H Bracht, D D Breimer. Assay of underivatized nitrazepam and clonazepam in plasma by capillary gas chromatography applied to pharmacokinetic and bioavailability studies in humans. J Chromatogr B: Biomed Appl, 1978, 145: 105-114.
[16] N R Badcock, A C Pollard. Micro-determination of clonazepam in plasma or serum by electron-capture gas-liquid chromatography. J Chromatogr B: Biomed Appl, 1982, 230: 353-361.
[17] P Lillsunde, T Seppälä. Simultaneous screening and quantitative analysis of benzodiazepines by dual-channel gas chromatography using electron-capture and nitrogen-phosphorus detection. J Chromatogr B: Biomed Appl, 1990, 533: 97-110.
[18] J Naestoft, N Larsen. Quantitative determination of clonazepam and its metabolites in human plasma by gas chromatography. J Chromarogr, 1974, 93: 113-122.
[19] D Song, S Zhang, K Kohlhof. Quantitative determinationof clonazepam in plasma by gas chromatography-negative ion chemical ionization mass spectrometry. J Chromatogr B: Biomed Appl, 1996, 686: 199-204.
[20] M Augsburger, L Rivier, P Mangin. Comparison of different immunoassays and GC-MS screening of benzodiazepines in urine. J Pharm Biomed Anal, 1998, 18: 681-

687.
[21] I I Papoutsis, S A Athanaselis, P D Nikolaou, C M Pistos, C A Spiliopoulou, C P Maravelias. Development and validation of an EI-GC-MS method for the determination of benzodiazepine drugs and their metabolites in blood: Applications in clinical and forensic toxicology. J Pharm Biomed Anal, 2010, 52: 609-614.
[22] H Maurer, K Pfleger. Identification and differentiation of benzodiazepines and their metabolites in urine by computerized gas chromatography-mass spectrometry. J Chromatogr B: Biomed Appl, 1987, 422: 85-101.
[23] M Yegles, F Mersch, R Wennig. Detection of benzodiazepines and other psychotropic drugs in human hair by GC/MS. Forensic Sci Int, 1997, 84: 211-218.
[24] A Negrusz, C M Moore, J L Kern, P G Janicak, M J Strong, N A Levy. Quantitation of clonazepam and its major metabolite 7-aminoclonazepam in hair. J Anal Toxicol, 2006, 24: 614-620.
[25] C L Guellec, M L Gaudet, M Breteau. Improved selectivity for high-performance liquid chromatographic determination of clonazepam in plasma of epileptic patients. J Chromatogr B: Biomed Appl, 1998, 719: 227-233.
[26] J C Spell, J T Stewart. Analysis of clonazepam in a tablet dosage form using smallbore HPLC. J Pharm Biomed Anal, 1998, 18: 453-460.
[27] K Y Rust, M R Baumgartner, N Meggiolaro, T Kraemer. Detection and validated quantification of 21 benzodiazepines and 3 “z-drugs” in human hair by LC-MS/MS. Forensic Sci Int, 2012, 215: 64-72.
[28] C A A de Almeida, C G B Brenner, L Minetto, C A Mallmann, A F Martins. Determination of anti-anxiety and anti-epileptic drugs in hospital effluent and a preliminary risk assessment. Chemosphere, 2013, 93: 2349-2355.
[29] S L Yuan, X F Li, X M Jiang, H X Zhang, S K Zheng. Simultaneous determination of 13 psychiatric pharmaceuticals in sewage by automated solid phase extraction and liquid chromatography-mass spectrometry. Chin J Anal Chem, 2013, 41: 49-56.
[30] M Jang, H Chang, W Yang, H Choi, E Kim, B H Yu, Y Oh, H Chung. Development of an LC-MS/MS method for the simultaneous determination of 25 benzodiazepines and zolpidem in oral fluid and its application to authentic samples from regular drug users. J Pharm Biomed Anal, 2013, 74: 213-222.
[31] R Verplaetse, E Cuypers, J Tytgat. The evaluation of the applicability of a high pH mobile phase in ultrahigh performance liquid chromatography tandem mass spectrometry analysis of benzodiazepines and benzodiazepine-like hypnotics in urine and blood. J Chromatogr A, 2012, 1249: 147-154.
[32] R C Irving, S J Dickson. The detection of sedatives in hair and nail samples using tandem LC-MS-MS. Forensic Sci Int, 2007, 166: 58-67.
[33] S Broecker, S Herre, F Pragst. General unknown screening in hair by liquid chromatography-hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). Forensic Sci Int, 2012, 218: 68-81.
[34] D DeTata, P Collins, A M Kinley. A fast liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QToF-MS) method for the identification of organic explosives and propellants. Forensic Sci Int, 2013, 233: 63-74.
[35] C Boix, M Ibáñez, J V Sancho, N León, V Yusá, F Hernández. Qualitative screening of 116 veterinary drugs in feed by liquid chromatography-high resolution mass spectrometry: Potential application to quantitative analysis. Food Chem, 2014, 160: 313-320.
[36] S Broecker, F Pragst, A Bakdash, S Herre, M Tsokos. Combined use of liquid chromatography–hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) and high performance liquid chromatography with photodiode array detector (HPLC-DAD) in systematic toxicological analysis. Forensic Sci Int, 2011, 212: 215-226.
[37] A E Mahjoub, C Staub. Simultaneous determination of benzodiazepines in whole blood or serum by HPLC/DAD with a semi-micro column. J Pharm Biomed Anal, 2000, 23: 447-458.
[38] P M Kabra, E U Nzekwe. Liquid chromatographic analysis of clonazepam in human serum with solid-phase (Bond-Elut®) extraction. J Chromatogr B: Biomed Appl, 1985, 341: 383-390.
[39] K B Borges, E F Freire, I Martins, M E P B (de) Siqueira. Simultaneous determination of multibenzodiazepines by HPLC/UV : Investigation of liquid-liquid and solid-phase extractions in human plasma. Talanda, 2009, 78: 233-241.
[40] N Badawi, K W Simonsen, A Steentoft, I M Bernhoft, K Linnet. Simultaneous screening and quantification of 29 drugs of abuse in oral fuild by solid-phase extraction and ultraperformance LC-MS/MS. Clin Chem, 2009, 55: 2004-2018.
[41] The fitness for purpose of analytical methods, a laboratory guide to method validation and related topics. EURACHEM guide, Teddington, UK, 1998.
[42] H Jin, A P Kumar, D H Paik, K C Ha, Y J Yoo, Y I Lee. Trace analysis of tetracycline antibiotics in human urine using UPLC-QToF mass spectrometry. Microchem J, 2010, 94: 139-147.
[43] M C Y Wong, W T K Lee, J S Y Wong, G Frost, J Lodge. An approach towards method development for untargeted urinary metabolite profiling in metabonomic research using UPLC-QToF/MS. J Chromatogr B: Biomed Appl, 2008, 871: 341-348.
[44] F Hernández, L Bijlsma, J V Sancho, R Díaz, M Ibáñez. Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry. Anal Chim Acta, 2011, 684: 96-106.
[45] F Badoud, E Grata, L Perrenoud, M Saugy, S Rudaz, J L Veuthey. Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography–quadrupole time-of-flight mass spectrometry. II: Confirmatory analysis. J Chromatogr A, 2010, 1217: 4109-4119. ■

ISSN 2157-118X

Printed in the United States of America
10 9 8 7 6 5 4 3 2 1 0