Journal of Chromatography B, 876 (2008) 76–82

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of 4-dimethylaminophenol concentrations in dog blood using LC–ESI/MS/MS combined with precolumn derivatization

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ARTICLE INFO

Article history:
Received 30 November 2007
Accepted 17 October 2008
Available online 1 November 2008

Keywords:
DMAP
LC–MS/MS
Precolumn derivatization

ABSTRACT

A sensitive and reproducible LC–ESI/MS/MS method, which was combined with the precolumn dansyl chloride derivatization to enhance the signal intensity of analytes, was developed to determine blood 4-dimethylaminophenol (DMAP) concentrations. The linearity of the method was observed within the concentration range of 2–2000 ng/mL. The precision, accuracy, stability, recovery and matrix effect of the method were also investigated and found to meet the requirements for pharmacokinetic studies of the drug. By using this method, pharmacokinetic studies were conducted in dogs after i.m. and i.v. administrations. The results showed that DMAP could not only be absorbed into blood quickly after i.m., but also can be eliminated rapidly. Both i.m. and i.v. routes are appropriate for DMAP to be used in field first-aid. It has been proved that this LC–MS/MS combined with precolumn derivatization method can be used as a routine analytical method to provide enhanced measurements for blood DMAP concentrations. It is also useful for DMAP pharmacokinetic evaluation.

1. Introduction

4-dimethylaminophenol (DMAP) forms ferrihemoglobin quickly in dogs and humans. This effect differs from ferrihemoglobin production by other substances in that it takes only 5–10 min after an i.v. injection to raise blood ferrihemoglobin contents to the maximal level corresponding to the dose applied. DMAP, therefore, has been proposed for the treatment of cyanide poisoning [1–3]. However, for treatment of cyanide poisonings under field conditions and under the circumstance of mass casualties of poisonings, an intravenous application of DMAP by physicians would keep relatively few people alive. An i.m. therapy of cyanide poisoning by a self and fellow aid with DMAP would be most welcome [4]. As a result of efforts to develop DMAP as a potential agent for i.m. administration, there is a need to evaluate its pharmacokinetic profiles in preclinical models as well as in clinical trials. To support the pre-clinical pharmacokinetic study, it is desirable to have a sensitive method for the determination of DMAP in biological samples. To our knowledge, previous available analytical methods developed for DMAP were based on radioactive isotope labeling assay and phosphorescence colorimetry [5]. They were either less sensitive or involved complicated pre-treatment procedures.

The objective of the current study was to develop a liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method to determine DMAP in dog blood following a simple derivatization reaction. The derivatization step is added to significantly enhance signal intensity and to increase the retention time for the purpose of avoiding ion suppression effects. By utilizing the developed method, the pharmacokinetic study of DMAP was conducted in dogs after i.m. and i.v. injection to investigate the potential of DMAP as a field first-aid drug in cyanide poisoning.

2. Materials and methods

2.1. Chemicals and reagents

DMAP (99.3% purity) and butyl paraben (internal standard, I.S., 97.5% purity) were synthesized by the Academy of Military Medical Sciences (AMMS). Acetonitrile was of HPLC grade and other chemicals used were of analytical grade. The derivatization agent, dansyl chloride (DNS-CL), was purchased from Sigma–Aldrich. The HPLC grade water that was used throughout this study was produced in-house by a water purification system (Millipore, Simplicity, USA).

2.2. Instrumentation

A Thermo Finnigan TSQ tandem mass spectrometer equipped with ESI source (San Jose, CA, USA) and a Surveyor LC pump were

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doi:10.1016/j.jchromb.2008.10.025

used for LC–MS/MS analysis. Data acquisition was performed with Xcalibur software (Thermo Finnigan). The data processing was carried out using the Thermo Finnigan LCQuan 2.0 data analysis program.

2.3. Chromatographic conditions

A XBP-C18 column (50 mm × 2.1 mm, 5 μm, 100 Å, Agela Technologies Inc. USA) was used for the analysis. The temperature of the column was kept at 20 °C. The mobile phase composed of acetonitrile–water–formic acid in the ratio of 85:15:0.1 (v/v/v) and the flow rate was 0.2 mL/min. The tray was kept at the temperature of 4 °C.

2.4. Mass spectrometric conditions

The Thermo Finnigan TSQ tandem mass spectrometer was operated in positive ionization mode for LC–MS/MS analyses. In order to optimize MS parameters, the samples of the DMAP derivative and I.S. derivative (1 μg/mL) were constantly added at the flow rate of 20 μL/mL by a syringe pump in the infusion mode. The optimized instrument conditions were as follows: transfer capillary temperature, 300 °C; spray voltage, 4800 V; sheath gas, 35 psi; auxiliary gas, 25 psi. For both analytes, selected reaction monitoring (SRM) mode were used and transitions selected for quantification were m/z 371 → m/z 137 for DMAP derivative and m/z 428 → m/z 171 for I.S. derivative. Optimized collision energy values for DMAP derivative and I.S. derivative were 37 and 51 eV, respectively. Scan time was 0.5 s per transition.

2.5. Preparation of stock solutions

DMAP and I.S. were accurately weighed and added into acetonitrile to make the primary solutions at 1 mg/mL. Working stock solutions of DMAP at concentrations of 4,10, 100, 200, 1000, 2000, 4000 ng/mL were prepared from the primary stock solution with acetonitrile containing 0.1% formic acid. A working solution of I.S. (500 ng/mL) was prepared by diluting the primary I.S. stock solution with acetonitrile. The stock and working solutions of I.S. were kept at −20 °C. The stock and working solutions of DMAP was freshly prepared daily to avoid DMAP degradation. Structures of DMAP and the I.S. are shown in Fig. 1.

2.6. Calibration standards and quality control samples

There are some differences from routine samples in the preparation of calibration standards and quality control (QC) samples. A series of 100 μL of pooled blank dog heparinized blood samples were added to 200 μL acetonitrile containing 0.1% formic acid to destroyed the Hb. The blood samples were then vortexed vigorously and were spiked with 50 μL of the above mentioned working solutions and 50 μL I.S. working solution to prepare a series of calibration solutions, in which the calibration curve points in blood were equivalent to 2, 5, 50, 100, 500, 1000 and 2000 ng/mL of DMAP. Blank blood samples (without I.S.) were also analyzed.

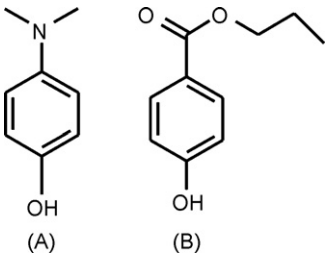

Fig. 1. Structures of DMAP (A) and butyl paraben (B).

The QC samples were prepared to the target concentrations of 4, 750, 1500 ng/mL in the same way as the blood samples for calibration, representing low, medium and high concentration levels separately. During the study, calibration standards and QC samples were prepared and analyzed along with unknown samples in each analytical batch.

2.7. Extraction and derivatization

To a 100 μL aliquot of dog blood, 50 μL I.S. working solution and 250 μL acetonitrile were added. After vortex-mixing for 1 min the mixtures were centrifuged at 10,000 × g for 5 min at 4 °C to remove the precipitate. A 200 μL aliquot of the supernatant was transferred into a 1.5 mL plastic centrifugal tube, added with 50 μL $NaHCO_3$–NaOH buffer solution (pH = 11), and was derivatized with 100 μL of 1 mg/mL DNS-CL at 60 °C for 10 min. The reaction mixture was then vortex-mixed by centrifuging at 10,000 × g for 2 min at 4 °C. The supernatant was taken and then transferred to an analytical vial. A 5 μL aliquot was injected onto the LC–MS/MS system.

2.8. Method validation

The following elements were used to validate the methods: sensitivity, linearity, intra- and inter-day precision, accuracy, stability and selectivity. The sensitivity of the method was assessed by determining low limit of detection (LLOD) and low limit of quantification (LLOQ). The LLOD and LLOQ were calculated based on the signal-to-noise ratio 3 and 10, respectively. The calibration curve $y = ax + b$ was obtained by assigning the concentration of DMAP and the peak area ratio of DMAP to I.S. as x and y, respectively. Subsequently, a $1/x^2$ weighted linear regression was performed. To evaluate linearity, blood calibration curves were prepared and assayed on three separate days. Precision and accuracy were determined by replicate analyses ($n = 6$) of QC samples at three concentration levels over 5 different days spaced over 2–3 weeks. During these 5 days also a new calibration curve had been obtained, thereby allowing the evaluation of the reproducibility of the calibration curve. The precision of the method was defined as the coefficient of variation

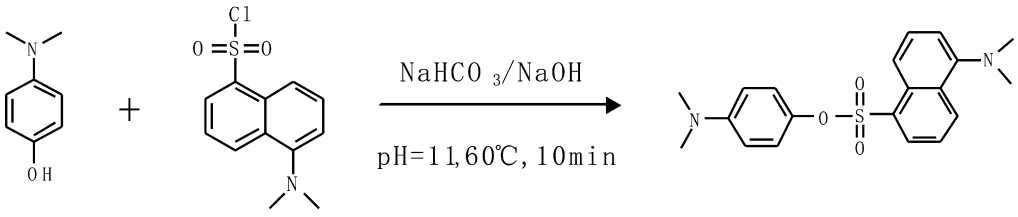

Fig. 2. Reaction scheme of DMAP with dansyl chloride.

(C.V.) calculated from replicate measurements of QCs. The accuracy of the assay was defined as the relative error (R.E.) for the mean of the replicate measurements of QCs compared to the theoretical values. Acceptance criteria were established to be <15% C.V. for accuracy and precision for low, medium and high QC samples in the inter-day and intra-day assay.

2.9. Matrix effects and recovery

The matrix effects were investigated by the following experiment. DMAP and the I.S. were spiked separately into dog blank blood as well as into water at the same three concentration levels and each level had five samples ($n = 5$), then prepared with exactly

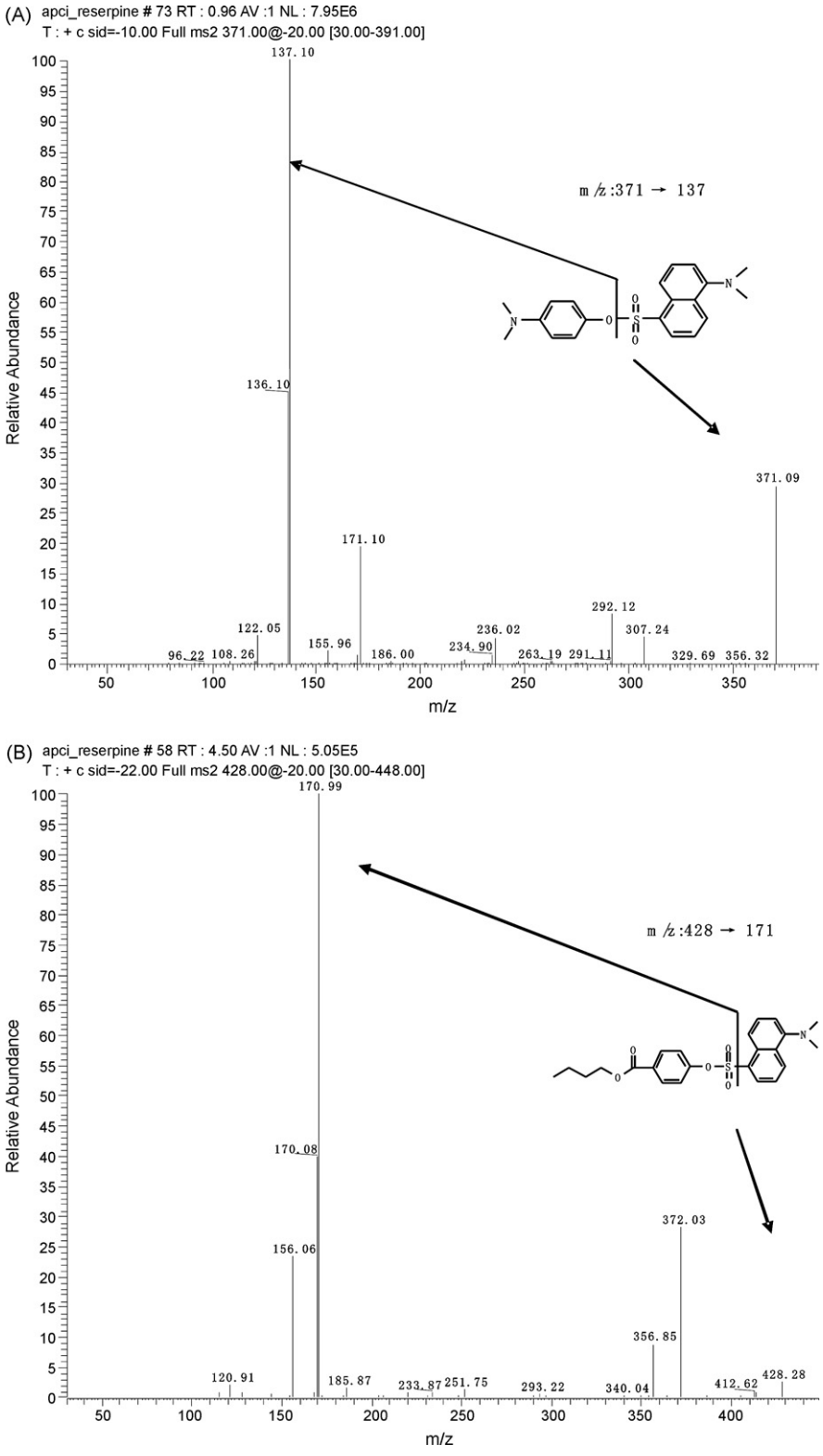

Fig. 3. Full scan of the parent ions and product ions of the DMAP derivative (A) and I.S. derivative (B)

the same procedures as described in the Section 2.7. Matrix effects were evaluated by taking the peak area of each standard spiked blood and finding its percentage of the peak area of reference neat samples.

The recovery of the method was determined by spiking analyte neat solution to the processed blank blood samples with acetonitrile to reach three concentrations. Samples were derivatized and analyzed. Recovery was calculated by comparing DMAP peak area of the samples with same three concentrations to post-processed blood blanks fortified with the known amount of analyte neat solutions.

2.10. Stability

The stability tests were conducted to cover the experimental conditions that real samples may experience, including the stability of DMAP in biological samples (dog blood) that were treated with acetonitrile to precipitate proteins and the stability of the derivative (dansylated ethinyl DMAP) in the aliquots placed on the injection tray at 4 °C ready for LC–MS/MS analysis. The stability of dansylated ethinyl DMAP in the processed samples was also assessed under the storage condition at −20 °C for 3 days. In the study these samples at three different concentration levels with three replicates were prepared and analyzed. The resulted values for these samples were then compared to the values of the respective freshly prepared blood samples. The analytes were considered stable in the different conditions when 85–115% of the initial concentrations were found.

2.11. In vivo study

The applicability of the method for DMAP pharmacokinetics study was assessed by analyzing blood samples collected from beagle dogs ($n = 5$) at 1, 2, 3, 5, 8, 12, 16, 20, 28, 36, 50, 60 and

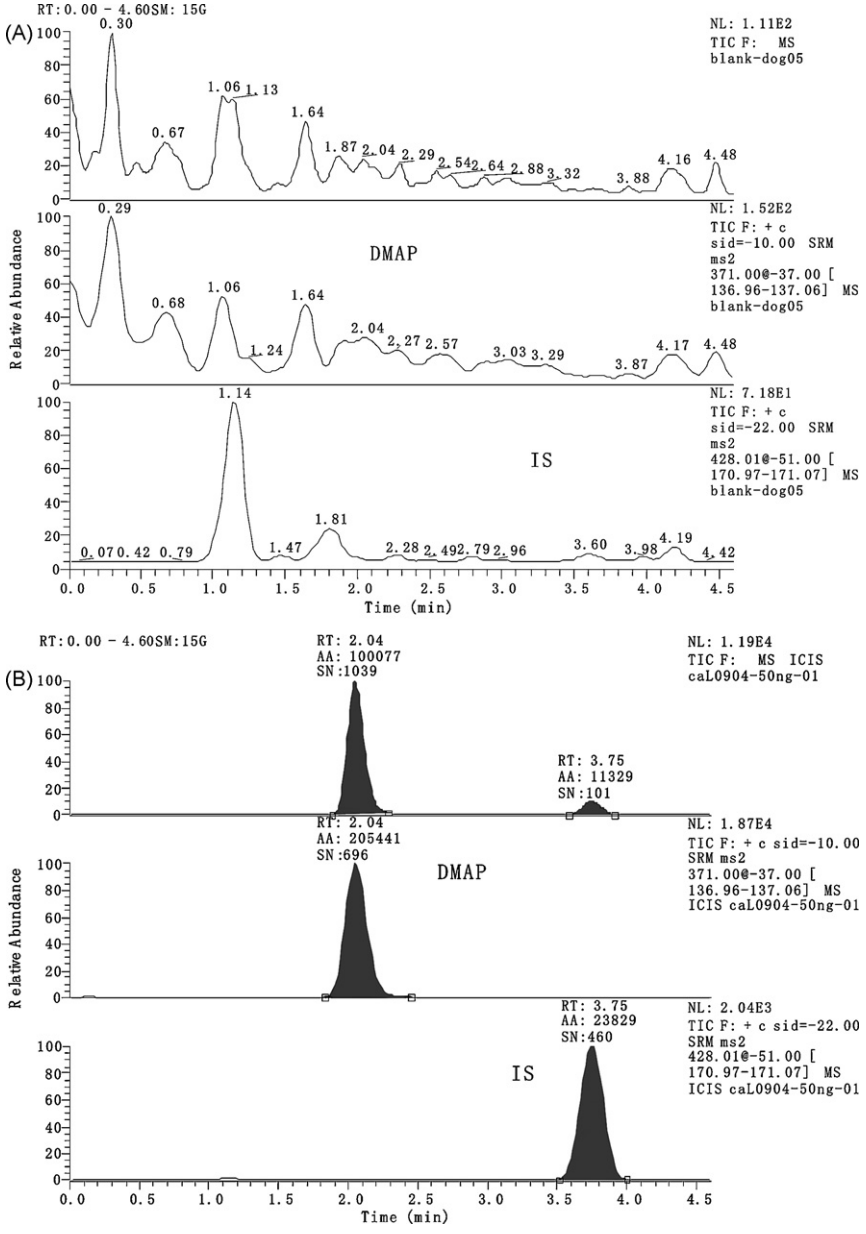

Fig. 4. Representative SRM chromatograms of derivatized DMAP and I.S. in dog blood. (A) Blank dog blood, (B) blood sample spiked with DMAP (50 ng/mL) and I.S. (50 ng/mL), (C) blood sample of 16 min after i.m. administration of 3.25 mg/kg DMAP to a dog.

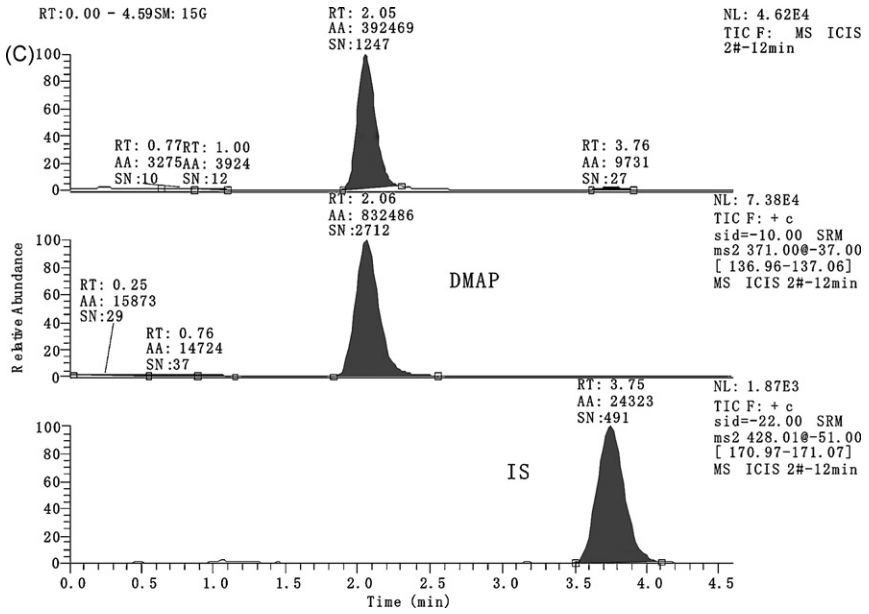

Fig. 4. (*Continued*).

75 min after intravenous and intramusclar administration of DMAP at 3.25 mg/kg. DMAP was dissolved in physiologic saline and was administrated at a dose volume of 1 mg/kg. All animal use procedures were in accordance with the regulation of experimental animal administration issued by the State Committee of Science and Technology of People's Republic of China. 0.3 mL blood samples were collected to heparinized glass tubes and a portion of 100 μL was transferred immediately to labeled vials containing 200 μL acetonitrile with 0.1% formic acid to destroy the Hb and to avoid DMAP autoxidation. The samples were then immediately processed within 1 h.

Pharmacokinetic parameters of DMAP were subsequently obtained by processing the data with the non-compartmental model of pharmacokinetic software DAS 2.0. The area under the curve from time 0–∞ min ($AUC_{0-\infty}$) was calculated by the linear trapezoidal rule. The other pharmacokinetic parameters obtained were maximum plasma concentration (C_{max}), time of maximum plasma concentration (T_{max}), half-life ($T_{1/2}$), mean residence time (MRT).

The experimental data and the pharmacokinetic parameters were expressed as mean ± S.D.

3. Results and discussion

3.1. Method development

MS has certain distinct advantages over conventional methods, such as radioactive labeling assays. The most attractive advantage of MS is its generic nature of detection based solely on the mass-to-charge ratio of an ion, which is measured with exquisite accuracy and high sensitivity in modern times [6].

DMAP is a compound with low molecular weight and strong polarity. These properties cause the difficulty for DMAP to be retained on common C18 columns. Initially, we had attempted to optimize mobile phase conditions for direct detection of DMAP with HPLC. However, changing organic phase could not get the appropriate retention time for DMAP to reduce the ion suppression effects of endogenous, polar and non-retained matrix components (solvent front, salts, etc.), as they eluted at the same time [7]. Therefore, we investigated the feasibility of chemical modification on DMAP molecule in an effort to improve the separation of DMAP from endogenous substances.

Dansyl chloride was chosen as the derivatization reagent, as DMAP contains a phenolic hydroxyl that is known to be oxidized easily in solutions and to be reactive with dansyl chloride. Furthermore, recent reports indicated that dansyl chloride could be used to improve ESI signal intensity [8]. By introducing a dansyl functional group, the retention time of the DMAP derivative on the column could be significantly prolonged. At the same time, dansylated ethinyl DMAP is more stable than DMAP (the reaction scheme is shown in Fig. 2).

First, we optimized the derivatization conditions for dansylated ethinyl DMAP, such as reaction time, temperature and pH of the reaction system. The results showed that 10 min of reaction time was enough for completion of derivatization at 60 °C and the optimum pH value for the reaction mixture was pH 11 [9].

Quantitative extraction of DMAP from blood samples before derivatization was a challenge, as the compound was unstable and strongly bound to the red blood cell. Eyer and Gaber reported that 1 min after the end of the i.v. injection of DMAP only 25% unchanged DMAP was detected. After 10 min no DMAP could be detected [10]. After testing different solvents, we selected acetonitrile to immediately precipitate blood proteins and Hb. This pre-treatment could avoid the autoxidation of DMAP during the RBC and plasma separation and reduce the blood sample volume to 0.5 mL.This method was successfully used to determine DMAP concentrations in dog blood samples. DMAP could be determined quantitatively from 1 min till 60 min after i.v. doses.

Full scan and product ion mass spectra for derivatized DMAP and I.S. were obtained in positive ion mode. The full scan spectra showed intense signals for their protonated molecular ions ([*M*+H]+) at *m*/*z* 371 and 428, respectively. Both product ion spectra of DMAP and I.S. have a fragment ion at *m*/*z* 171. To get the best sensitivity the mass transition in the selected reaction monitoring mode was set at 371 → 137 and 428 → 171 for DMAP and I.S., respectively (Fig. 3).

3.2. Selectivity and sensitivity of the method

From the chromatograms of the blank blood sample and the spiked blood samples (see Fig. 4), it was noted that the peaks of

Table 1
Accuracy and precision of determination of DMAP in dog blood from the method validation (n = 3 days, six replicates per day).

	QC low (4 ng/mL)	QC medium (750 ng/mL)	QC high (1500 ng/mL)
Intra-assay precision and accuracy			
Mean ± SD	4.04 ± 0.41	765 ± 58.5	1490 ± 130
C.V. (%)	10.1	7.65	8.72
R.E. (%)	1.03	2.00	−0.48
n	6	6	6
Inter-assay precision and accuracy			
Mean ± SD	4.02 ± 0.31	759 ± 40.5	1560 ± 115
C.V.(%)	7.79	5.33	7.33
R.E.(%)	0.44	0.25	4.25
n	18	18	18

the target compound and the I.S. were not interfered by matrices. The retention times for the derivatives of DMAP and the I.S. were at about 2.04 and 3.72 min, respectively. The derivatization method showed that there were no significant differences in peak areas of the derivatives of the analytes prepared from five different drug-free blood samples (data not shown). The current assay has a LLOD of 0.5 ng/mL and LLOQ of 2 ng/mL, respectively, based on a 100 μL blood volume.

3.3. Calibration curve and reproducibility

Three consecutive calibration analyses were performed on different days with freshly aliquoted standards. Calibration curves for DMAP in blood were linear within the concentration range of 2–2000 ng/mL, with correlation coefficients equal to or greater than 0.995.

Precision and accuracy of the assay were determined by analyzing replicates (n = 6) of QC samples at three concentration levels on three validation days. The data from these QC samples were examined by a one-way analysis of variance (ANOVA). For all QC levels the intra-day CV was less than10.1%, and the inter-day CV was less than 7.79%. The accuracy ranged from −0.48 to 4.25% for each QC level. The results are summarized in Table 1. All intra- and inter-day precision and accuracy values were acceptable, and spanned the entire concentration range.

3.4. Recovery and matrix effects

The results of recovery and matrix effect are summarized in Table 2. For all the test groups the recovery rates were high and consistent, which indicated that protein precipitation with acetonitrile containing 0.1% formic acid was optimal for the polar analyte.

To develop a reliable and reproducible LC–MS/MS method, it is very important to investigate the matrix effects. It was reported that

Table 2
Recovery rate and matrix enhance effect of DMAP in dog blood (mean ± SD, n = 5).

	5 ng/mL	100 ng/mL	1000 ng/mL
Recovery rate (%)	82.2 ± 10.6	84.6 ± 8.21	82.4 ± 6.82
Matrix effect (%)	–	1500 ± 100	301 ± 30.1

the suppression or enhancement effects of matrix might be caused by polar, non-retained matrix components (solvent front, salts, etc.) [11]. The selectivity results did not show significant influences of co-eluting 'unseen' compounds on the ionization of the derivatives of DMAP and I.S. However, it was observed in this study that the biomatrix (dog whole blood) could significantly enhance the signal intensity. The signal at low concentrations of the neat samples was too weak to assay, so the matrix effect of low concentration could not be calculated. The possible reason may be the improvement of the derivatization efficiency by the matrix component in dog blood, such as endogenous phospholipids. Furthermore, the linear slope of neat samples was different from the whole blood samples, this indicated that the matrix effects varied with concentrations of analytes.

3.5. Stability

Due to the fact that DMAP contains a phenolic hydroxyl, which is known to be oxidized easily in aqueous solutions, whole blood or plasma, the compound was found very unstable in dog blood at room temperature. It was noted in this study that 5 min after addition of DMAP into blood only 10% of DMAP was not oxidized or changed. The lack of stability of test solutions during the measurement period may result in a significant drawback to the experimental method [12]. In order to keep DMAP stable in test solutions as well as in samples, it was a challenge for us to find appropriate experimental conditions. In the study, working solutions of DMAP were freshly prepared daily with acetonitrile containing 0.1% formic acid. Calibration and QC samples were prepared by spiking 100 μL of blank blood samples each with 200 μL acetonitrile firstly to destroy the Hb, then 50 μL I.S. and DMAP working solutions were added. Blood samples were processed by drawing 100 μL whole blood at each time points and added immediately with 200 μL acetonitrile containing 0.1% formic acid to destroy the Hb, as well as to avoid the auto oxidation of DMAP. The stability of DMAP in the mixture of dog blood with acetonitrile was evaluated during different time frames. The results showed that DMAP was stable in the mixture for 1 h. Consequently, all the samples were derivatized within 1 h after mixing with acetonitrile.

Fortunately, dansylated ethinyl DMAP is more stable than DMAP. The stability of dansylated ethinyl DMAP in extract was assessed after 3 days at −20 °C. For all the concentration levels tested, the

Table 3
Summary of the stability of DMAP and dansylated ethinyl DMAP in samples (n = 3).

Storage conditions	Fresh sample, mean ± SD (ng/mL)	Stored sample, mean ± SD (ng/mL)	Stability (% of initial)	C.V. (%)
DMAP in the mixture of dog blood and acetonitrile at room temperature for 1 h				
	4.58 ± 0.39	4.64 ± 0.31	101	6.68
	94.3 ± 8.94	104 ± 8.39	110	8.08
	958 ± 78.9	919 ± 55.4	95.9	6.03
Dansylated ethinyl DMAP in extract on tray at 4 °C for 15 h				
	4.76 ± 0.29	4.84 ± 0.15	102	3.10
	103 ± 9.25	106 ± 0.36	103	0.34
	927 ± 53.3	907 ± 9.70	97.8	1.07
Dansylated ethinyl DMAP in extract at −20 °C for 3 days				
	4.65 ± 0.43	4.77 ± 0.16	103	3.35
	98.8 ± 8.76	103 ± 1.55	104	1.51
	1020 ± 34.5	1010 ± 32.9	99.2	3.25

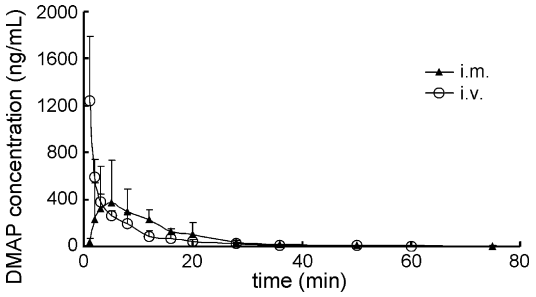

Fig. 5. Mean blood concentration–time curve of DMAP in dogs after i.v. and i.m. at 3.25 mg/kg (mean ± 1 SD, $n = 5$).

Table 4
Pharmacokinetics parameters for DMAP in dogs after i.v. and i.m. administration (3.25 mg/kg, $n = 5$, mean ± SD).

Parameters	Unit	i.m.	i.v.
$AUC_{(0-t)}$	ng/mL × min	5494 ± 2256	6061 ± 1161
$AUC_{(0-\infty)}$	ng/mL × min	5561 ± 2199	6094 ± 1154
$MRT_{(0-t)}$	min	14.5 ± 4.2	5.9 ± 2.9
$MRT_{(0-\infty)}$	min	16.1 ± 6.2	6.1 ± 2.9
$t_{1/2z}$	min	13.9 ± 6.4	5.9 ± 0.8
T_{max}	min	8.4 ± 3.5	–
CLz/F	L/min/kg	0.7 ± 0.3	0.5 ± 0.1
Vz/F	L/kg	15.0 ± 13.0	4.7 ± 1.1
C_{max}	ng/mL	449 ± 321	–

results did not show a significant decline of dansylated ethinyl DMAP. The derivative was stable at least for 3 days, which allows re-analysis of samples, should a chromatographic or instrument malfunction occur during the initial analysis. The derivative in extracts placed on injection tray at 4 °C was also stable within 15 h. The results were summarized in Table 3.

3.6. Application of the method to a pharmacokinetic study

Mean blood concentrations of DMAP (ng/mL) following i.v. and i.m. doses at 3.25 mg/kg in dogs ($n = 5$) are depicted in Fig. 5.

Mean pharmacokinetic parameters of DMAP in dogs are presented in Table 4.

After dogs received a single i.m. dose of DMAP at 3.25 mg/kg, the maximum DMAP concentration (C_{max}) of 449.4 ± 321.3 ng/mL appeared between 5 and 12 min. The half-life was 13.9 ± 6.4 min. DMAP was not detectable after 75 min post-dosing. The data showed that DMAP could not only be absorbed into blood quickly after i.m., but also be eliminated rapidly. The data obtained from the study suggested that i.m. injection would also be a convenient and practical administrative route for DMAP to be used in field aid as an emergent treatment drug.

4. Conclusions

A novel high-performance liquid chromatographic–mass spectrometric method to quantitative analysis of DMAP in blood was established using a simple precolumn dansyl chloride derivatization. Derivatization with dansyl chloride could significantly improve detection limit and sample stability were in positive electrospray ionization. The method was characterized by a high degree of sensitivity, precision, accuracy and stable under some certain conditions, and it was capable to accurately determine DMAP concentration as low as 2.0 ng/mL.

The method has been applied successfully, for the first time, to quantitatively analyze DMAP concentrations in dog blood for determination of DMAP pharmacokinetics.

Acknowledgement

The authors acknowledge the invaluable comments provide by Professor H. Li in the preparation of this manuscript.

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