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Short communication

# Simultaneous determination of dibucaine and naphazoline in human serum by monolithic silica spin column extraction and liquid chromatography–mass spectrometry

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ABSTRACT

A simple, sensitive, and specific liquid chromatography–mass spectrometry (LC–MS) method for simultaneous determination of dibucaine and naphazoline from serum was developed and validated. The extraction procedure was performed using a monolithic silica spin column. Chromatographic separation of dibucaine and naphazoline was achieved on a $C_{18}$ reverse phase column with a mobile phase gradient (mobile phase A: 10 mM ammonium formate and mobile phase B: acetonitrile) at a flow rate of 0.2 mL/min. LC–MS was operated under the selective ion monitoring mode using the electrospray ionization technique in the positive mode. The retention times for naphazoline, dibucaine, and the internal standard (IS) were 6.7, 7.8, and 8.0 min, respectively. A linear graph was obtained for dibucaine and naphazoline with correlation coefficients >0.998 for all analytes by this method. The limit of quantification of dibucaine and naphazoline was 10 and 25 ng/mL, respectively. The mean recoveries were greater than 70%. Both compounds were stable under conditions of short-term storage, long-term storage as well as after freeze–thaw cycles. Monolithic spin column extraction and LC–MS analysis enabled the separation of dibucaine and naphazoline within 20 min.



## 1. Introduction

Dibucaine, 2-butoxy-*N*-(2-diethylaminoethyl)-4-quinolinecarboxamide hydrochloride (Fig. 1), is a long-acting local anesthetic used for disorders associated with painful skin. The toxicity of seven local anesthetics that are potentially lethal to the central nervous system and muscle fibers such as lidocaine and dibucaine, has been well described [1]. Dibucaine is considered to be 10–20 times more toxic than lidocaine and procaine [2]. Further, three accidental fatal poisoning cases due to dibucaine ingestion have been reported [3].

Naphazoline, 2-(1-naphthylmethyl)-2-imidazoline hydrochloride (Fig. 1), is a relatively long-lasting vasoconstrictor, which acts on the alpha receptors of smooth vascular muscle. Clinically, patients poisoned with naphazoline may exhibit miosis, mydriasis, palpitations, hypertension or hypotension, bradycardia, pallor, cyanosis, diaphoresis, anxiety, insomnia, tremor, agitation, hallucinations, seizures, lethargy, obtundation, and coma [4]. Cases of naphazoline poisoning by accidental over dose have also been reported [5].

The patients exhibit severe cardiovascular effects as a result of dibucaine and/or naphazoline intoxication. Several products containing dibucaine hydrochloride and naphazoline hydrochloride are produced by different manufacturers in Japan. These products are sold as anti-infective compounds, which include approximately 75 mg benzalkonium chloride, 75 mg dibucaine hydrochloride, 75 mg naphazoline hydrochloride, and 150 mg chlorpheniramine maleate per 75 mL solution.

There are several analytical methods to determine the dibucaine content; the most common among these are the chromatographic methods, namely, gas chromatography–mass spectrometry (GC–MS) [6], high performance liquid chromatography (HPLC) [7,8], and LC–MS [9,10].

Some chromatographic methods have been also reported for the separation or quantitative measurement of naphazoline, e.g., GC–MS [11], sequential injection chromatography [12], and capillary electrophoresis [13] methods.

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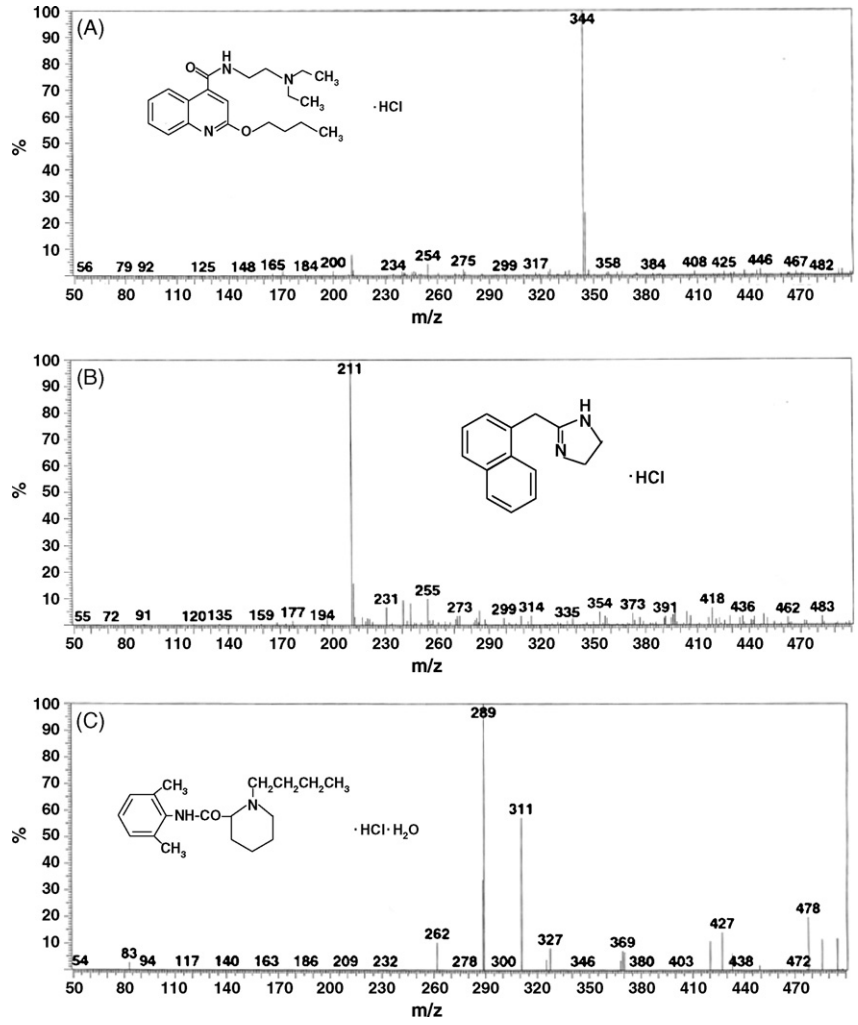


**Fig. 1.** Electrospray ionization mass spectrum and chemical structures of dibucaine (A), naphazoline (B), and bupivacaine (IS) (C).

We have recently reported a specific and sensitive LC–MS method to characterize amitraz and its metabolite in serum by monolithic silica spin column extraction [14]. This extraction method can be used to simultaneously analyze many samples; further, sample preparation for this method involves only centrifugation and no evaporation is required. The purpose of this study was to develop a sensitive and specific LC–MS method to determine the level of dibucaine and naphazoline in human serum. This sensitive method was employed for toxicological analysis of dibucaine and naphazoline in poisoned patients. Unlike dibucaine and naphazoline, chlorpheniramine does not cause heart toxicity. Furthermore, the LC–MS analysis of chlorpheniramine in human plasma has been published [15,16]. Therefore, we did not examine chlorpheniramine.

## 2. Experimental

### 2.1. Chemicals

Dibucaine hydrochloride and naphazoline hydrochloride were purchased from Sigma (St. Louis, MO, USA) and bupivacaine hydrochloride from ICN Biomedicals Inc. (Ohio, USA) (Fig. 1). HPLC-grade methanol and acetonitrile were purchased from Wako Pure Chemicals (Osaka, Japan). The monolithic spin extraction column was purchased from GL Science (Tokyo, Japan). All other reagents were commercially available and of reagent grade.

### 2.2. Preparation of stock solutions, standard and quality control (QC) samples, and internal standard (IS)

Stock solutions of dibucaine, naphazoline, and IS (bupivacaine hydrochloride) were prepared in methanol (1 mg/mL). A 10 μg/mL solution of IS was prepared by diluting the stock IS solution with methanol. A series of standard working solutions (1.0, 10, 100 μg/mL) for each constituent were prepared by further dilution of the dibucaine and naphazoline stock solutions with methanol.

Method validation was performed by evaluating intra-assay accuracy and precision of low, medium, and high QC concentrations. The QC samples of 30 (low QC), 90 (medium QC), and 400 (high QC) ng/mL were prepared separately. All the solutions were stored at −30 °C and brought to room temperature before use.

### 2.3. Sample preparation

Serum (0.2 mL), 20 ng IS (2 μL of 10 μg/mL solution), and 0.2 mL water were vortexed together for 15 s.

The spin columns were conditioned by addition of 0.2 mL acetonitrile followed by centrifugation at 2500 rpm for 1 min. Distilled

water (0.2 mL) was then added, followed by further centrifugation at 2500 rpm for 1 min. The samples were then applied to the conditioned spin columns and centrifuged at 2500 rpm for 2 min. Distilled water (0.2 mL) was then added to the columns and centrifuged at 2500 rpm for 1 min. Following this spin, 0.2 mL 5% methanol was added and centrifugation was conducted at 2500 rpm for 1 min. Finally, 50 μL acetonitrile was added to the columns and the residual compounds were eluted by centrifugation at 2500 rpm for 1 min. Samples (10 μL) were then injected into the LC system.

### 2.4. Chromatography

Chromatography was performed using a Shimadzu LC10 system (Shimadzu Corp., Kyoto, Japan). HPLC separation was conducted on a XTerra® MS C18 column (2.1 mm × 150 mm, 3.5 μm) (Waters Corp., Milford, MA, USA) at 50 °C, with a flow rate of 0.2 mL/min using a gradient mobile phase comprising 10 mM ammonium formate (A) and acetonitrile (B). The mobile phase contained 0% of component B at the initiation of each chromatographic run and this concentration was increased to 100% in a linear gradient within 5 min. This concentration was maintained for 4.5 min and then reduced to 0% B for 10 min. The total run time was 10 min. Prior to next injection, the mobile phase B was maintained at 0% for 5 min.

### 2.5. Mass spectrometry

LC–MS analyses were performed on a QP8000α quadruple single mass spectrometer (Shimadzu Corp., Kyoto, Japan) using an electrospray ionization (ESI) source in the positive ion mode under conditions defined by the mass characterization. High-purity nitrogen gas was used as a nebulizer gas and was set at 4.5 L/min. The instrumental parameters of ESI were as follows: curve desolvation line (CDL) temperature, 250 °C; CDL voltage, −45 V; deflector voltage, 25 V; interface voltage, 4.5 kV. For full-scan MS analysis, the spectra were recorded in the range $m/z$ 50–500. Instrument control, data acquisition, and analysis were performed using the Class 8000 version 1.2 data system software.

### 2.6. Calibration curve

Calibration curves were acquired by plotting the peak-area against the nominal concentration of calibration standards. For both analytes, standard solutions were prepared with human serum to yield final concentrations of 10, 25, 50, 100, 250, and 500 ng/mL. The results were fitted to linear regression analysis using $1/\chi^2$ as the weighting factor. The minimum acceptable correlation coefficient ($r^2$) for the calibration curve was 0.99 or greater. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at the lowest limit of quantitation, which was set at ±20%.

### 2.7. Precision and accuracy

The intra-day precision and accuracy were estimated by analyzing six serum replicates containing dibucaine and naphazoline at the three QC concentrations. The inter-assay precision was determined by analyzing three QC samples in three different runs. Accuracy was calculated on the basis of the quotient of the averaged measurements and the nominal value and expressed in percent. The criteria for acceptability of data included accuracy within ±15% deviation from the nominal values and precision within ±15% relative standard deviation (RSD), except for the lowest limit of quantitation, where it should not exceed 20% of RSD.

### 2.8. Recovery

The relative recoveries of dibucaine and naphazoline from serum were determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 3$) and IS with the response of analytes prepared from non-extracted standard solutions at equivalent concentrations. The recovery amount was determined by comparing the peak-area ratios of dibucaine or naphazoline with the IS for the extracted samples, which were calculated from the respective calibration curves.

### 2.9. Stability

Serum QC samples of dibucaine and naphazoline at three QC concentrations were used for stability experiments. The short-term stability of the drugs in serum samples was examined by analyzing replicates of the three QC samples at room temperature for 24 h and at 4 °C for 1 week. Each freeze–thaw cycle consisted of a minimum of 24 h freezing at −30 °C followed by a complete thaw at room temperature. Samples were analyzed after the third freeze–thaw cycle. For long-time stability of the drugs in human serum was tested after storage at −30 °C for 4 weeks. The concentration of each compound after each storage period was compared with the initial concentration of the compound as determined in samples that were freshly prepared and immediately processed.

### 2.10. Application

An 84-year-old man was found ingesting an anti-infective compound, containing dibucaine and naphazoline. The estimated amount ingested was unclear on his arrival at our emergency department. However, 2.3 h after ingestion, the amount ingested was apparent. He gradually presented with bradycardia with a heart rate of under 30. Therefore, he was provided temporarily with a pacemaker, and 13 days later, he was transferred to another hospital. Toxicological analyses were performed on his serum samples 3 and 9 h after ingestion.

## 3. Results and discussion

The accuracy, precision, recovery, and stability of the analytical method were validated in accordance with the US Food and Drug Administration guidelines [17].

The MS was operated in the positive ESI mode with selected ion monitoring (SIM) acquisition. Fig. 1 shows the full-scan mass spectra ($m/z$ 50–500) of dibucaine, naphazoline, and IS. The major ions observed were $m/z$ 344 for dibucaine, $m/z$ 211 for naphazoline, and $m/z$ 289 for IS. These base peak ions were used for quantitative detection.

The typical chromatograms obtained for blank serum, low QC, and IS are shown in Fig. 2. Fig. 2 shows that this analytical method allows for a complete separation of dibucaine, naphazoline, and IS. The retention time for naphazoline (a), dibucaine (b), and IS (c) was 6.7, 7.8, and 8.0 min, respectively.

### 3.1. Calibration curves

The calibration curve was obtained using two calibration samples in the range of 10–500 ng/mL for dibucaine and 25–500 ng/mL for naphazoline from serum. The standard curve had a reliable reproducibility with standard concentrations of all the analytes across the calibration range. Calibration curves were prepared by

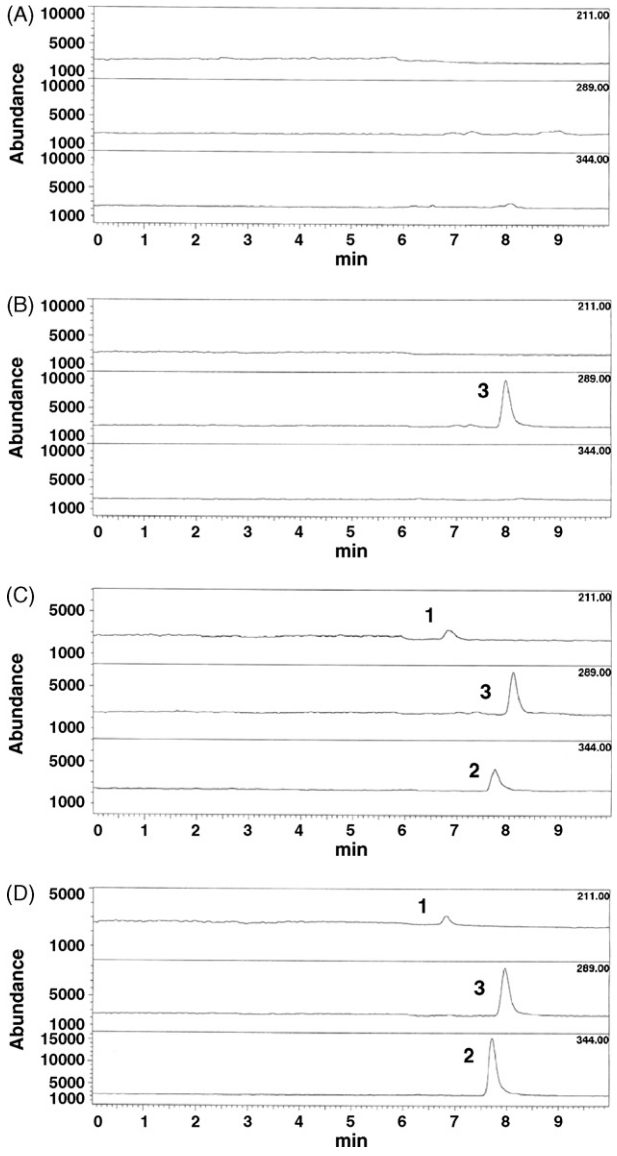


**Fig. 2.** LC–MS–SIM chromatograms obtained by monolithic silica spin column extraction of (A) blank human serum, (B) blank human serum with internal standard (IS), (C) blank human serum with medium QC (naphazoline, 90 ng/mL and dibucaine, 90 ng/mL) and IS. (D) Serum from naphazoline and dibucaine poisoned patient with IS. 1: naphazoline, 2: dibucaine, and 3: bupivacaine (IS).

determining the best fit of the peak-area ratios versus concentration, and they were then fitted to linear regression using the weighing factor ($1/\chi^2$). The regression equations for dibucaine and naphazoline were $y = 0.0031x - 0.0039$ and $y = 0.0006x + 0.0093$, respectively. The coefficient of determination ($r^2$) in both cases was >0.998. The average regression was always greater than 0.998. The experimental peak-area ratios were interpolated on the calibration curve, and the concentrations were back-calculated. The mean back-calculated concentrations approached the spiked concentrations by an RSD of <15%.

### 3.2. Limits of detection and quantification

The limit of detection (LOD) is defined as the signal equivalent to three times the noise; the LOD of dibucaine and naphazoline were 5 and 10 ng/mL, respectively. The lower limit of quantitation (LLOQ) of the assay, defined as the lowest concentration on the standard curve that can be quantitated with the %RSD, was <15% and the accuracy was within ±20% of the nominal value. The LLOQ was determined as 10 and 25 ng/mL for dibucaine and naphazoline, respectively.

**Table 1**
Validation data of dibucaine and naphazoline from human serum

| | 30 ng/mL ($n = 6$) | 90 ng/mL ($n = 6$) | 400 ng/mL ($n = 6$) |
|---|---|---|---|
| Dibucaine | | | |
| Accuracy (%) | 92.3 | 102.4 | 94.2 |
| Precision RSD (%) | 5.68 | 4.75 | 3.26 |
| Intra-day RSD (%)[a] | 7.3 | 4.3 | 3.6 |
| Inter-day RSD (%)[b] | 4.2 | 3.6 | 2.4 |
| Extraction recovery (%)[c] | 75.2 ± 4.3 | 78.6 ± 5.1 | 73.7 ± 6.4 |
| Naphazoline | | | |
| Accuracy (%) | 90.7 | 91.7 | 94.7 |
| Precision RSD (%) | 6.84 | 5.23 | 4.33 |
| Intra-day RSD (%)[a] | 5.8 | 3.7 | 2.2 |
| Inter-day RSD (%)[b] | 4.0 | 2.1 | 2.8 |
| Extraction recovery (%)[c] | 70.2 ± 3.8 | 72.8 ± 2.3 | 75.7 ± 4.8 |

[a] Intra-day accuracy and precision results were obtained from six duplicate samples ($n = 6$) for each concentration of the analyte analyzed on a single day.
[b] Inter-day accuracy and precision results were obtained by analyzing six duplicate samples for each concentration of the analyte on three separate days.
[c] Data are expressed as mean ± SD.

### 3.3. Precision

The intra-day precision and accuracy of the assay were evaluated by running three validation batches on three separate days. Each batch consisted of one set of calibration standards and six duplicates of three QC samples. The inter-day precision and accuracy were also assessed in a similar manner. Table 1 summarizes the means, standard deviation, precision, and accuracy for dibucaine and naphazoline at each QC concentration. Precision was expressed as %RSD. As shown in Table 1, the intra- and inter-day precision (%RSD) over the three QC concentrations was 2.4–7.3% and 2.1–5.8% with an accuracy range of 92.3–102.4% and 90.7–94.2% for dibucaine and naphazoline, respectively. These data confirm the good precision of the method.

### 3.4. Recovery

The relative recovery was evaluated by comparison of the mass ion peak-area ratios of the extracted samples at three QC levels with the standard solutions of equivalent concentrations. The mean extraction recoveries of dibucaine and naphazoline from serum were 73.7–78.6% and 70.2–75.7%, respectively.

### 3.5. Matrix effect

The matrix effect was evaluated by calculating the matrix factor (MF). MF is defined as follows: peak response in the presence of matrix ions/peak response in the absence of matrix ions. Serum QC samples of dibucaine and naphazoline at three QC concentrations were tested. The matrix effect was considered to be acceptable. Indeed, the MFs were between 0.89 and 1.12, and the variability among the MFs, which was measured using the %RSD method, was less than 15%.

### 3.6. Stability

Table 2 presents the results of the short-term stability study (24 h at room temperature and 4 °C for 1 week), 4-week stability at −30 °C, and freeze–thaw stability (3 cycles). Samples were con-

**Table 2**
Stability of dibucaine and naphazoline in human serum

| | Nominal concentration (ng/mL) | Room temperature (24 h) | 4 °C (1 week) | Freeze–thaw (3 cycles) | −30 °C (4 weeks) |
|---|---|---|---|---|---|
| Dibucaine | 30 ng/mL | 29.6 ± 2.7 | 30.0 ± 2.2 | 30.4 ± 2.3 | 30.3 ± 1.4 |
| | %RSD | 9.0 | 7.2 | 7.4 | 4.7 |
| | 90 ng/mL | 86.9 ± 6.7 | 87.0 ± 7.1 | 89.5 ± 5.4 | 90.4 ± 4.8 |
| | %RSD | 7.7 | 8.2 | 6.0 | 5.3 |
| | 400 ng/mL | 395.4 ± 10.2 | 391.3 ± 16.6 | 395.7 ± 9.9 | 404 ± 14.7 |
| | %RSD | 2.6 | 4.2 | 2.5 | 3.6 |
| Naphazoline | 30 ng/mL | 29.5 ± 2.6 | 28.8 ± 3.4 | 29.0 ± 1.0 | 30.6 ± 2.1 |
| | %RSD | 8.9 | 11.9 | 3.5 | 6.9 |
| | 90 ng/mL | 88.1 ± 7.4 | 85.2 ± 8.5 | 90.8 ± 3.5 | 86.9 ± 7.8 |
| | %RSD | 8.4 | 10.0 | 3.8 | 8.9 |
| | 400 ng/mL | 405.1 ± 10.5 | 385.0 ± 5.9 | 391.6 ± 17.8 | 394.9 ± 27 |
| | %RSD | 2.6 | 1.5 | 4.5 | 6.8 |

$n = 3$ each.

sidered stable if the deviation of the freshly prepared standard was less than 10%. However, dibucaine and naphazoline were stable in human serum under these conditions.

### 3.7. Applications

This method has been applied successfully for the quantitative analysis of dibucaine and naphazoline in a poisoned patient's serum. Dibucaine and naphazoline concentrations in the serum were determined to be 675.7 and 37.8 ng/mL, respectively, 3 h after ingestion and 218.6 and 28.0 ng/mL, respectively, 9 h after ingestion. Fig. 2(D) shows the chromatograph of the serum extract after 3 h ingestion in an actual poisoned patient.

## 4. Conclusion

The proposed LC–MS system and monolithic spin column extraction were proven to be a rapid and efficient tool for the extraction, separation, and determination of dibucaine and naphazoline in human serum. Dibucaine and naphazoline were separated within 20 min with the monolithic spin column extraction in conjunction with LC–MS analysis. The simultaneous analysis of dibucaine and naphazoline has not been previously described.

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