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Simultaneous determination of nikethamide and lidocaine in human blood and cerebrospinal fluid by high performance liquid chromatography

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Abstract

Nikethamide and lidocaine are often requested to be quantified simultaneously in forensic toxicological analysis. A simple reversed-phase high performance liquid chromatography (RP-HPLC) method has been developed for their simultaneous determination in human blood and cerebrospinal fluid. The method involves simple protein precipitation sample treatment followed by quantification of analytes using HPLC at 263 nm. Analytes were separated on a 5 μm Zorbax Dikema C_{18} column (150 mm × 4.60 mm, i.d.) with a mobile phase of 22:78 (v/v) mixture of methanol and a diethylamine–acetic acid buffer, pH 4.0. The mean recoveries were between 69.8 and 94.4% for nikethamide and between 78.9 and 97.2% for lidocaine. Limits of detection (LODs) for nikethamide and lidocaine were 0.008 and 0.16 μg/ml in plasma and 0.007 and 0.14 μg/ml in cerebrospinal fluid, respectively. The mean intra-assay and inter-assay coefficients of variation (CVs) for both analytes were less than 9.2 and 10.8%, respectively. The developed method was applied to blood sample analyses in eight forensic cases, where blood concentrations of lidocaine ranged from 0.68 to 34.4 μg/ml and nikethamide ranged from 1.25 to 106.8 μg/ml. In six cases cerebrospinal fluid analysis was requested. The values ranged from 20.3 to 185.6 μg/ml of lidocaine and 8.0 to 72.4 μg/ml of nikethamide. The method is simple and sensitive enough to be used in toxicological analysis for simultaneous determination of nikethamide and lidocaine in blood and cerebrospinal fluid.

Keywords: Nikethamide; Lidocaine; Reversed-phase HPLC; Forensic toxicological analysis

1. Introduction

Nikethamide, one of the respiratory central stimulants, is used to treat respiratory failure in clinical practice. Meanwhile, nikethamide is one of the abused drugs, banned for athletes. Therefore, it is important to develop analytical methods to determine nikethamide in biological samples for both forensic and clinical medical practice.

Non-invasive topical administration of local anesthetics is preferred in clinics for the relief of local pain, owing to its convenience of application as well as reduced adverse effects. Lidocaine is the most widely used local anesthesia agent. It is also used for the regional management of major pain via either spinal and epidural or peripheral administration. Moreover, lidocaine has also been utilized as an antiarrhythmic agent in emergency treatment for ventricular arrhythmias. For the analyses of lidocaine in biological matrix, many analytical methods have been reported, including gas chromatography (GC) [1–7], GC–MS [8–13], HPLC [14–24] and high performance liquid chromatography–mass spectrometry (HPLC–MS) [25,26].

Therapeutic blood concentration range of lidocaine is usually between 2 and 5 μg/ml, which is rather narrow. Toxic symptoms such as slurred speech, confusion, jerk and vertigo will occur when blood concentration of lidocaine reaches 6 μg/ml or above. When toxic effect of lidocaine increases severely, it is difficult to maintain the vital signs of patients. In clinical practice, medicines such as nikethamide are often used to rescue the patient under the above situation. Medical disputes quite often occurred regarding the anesthetic accidents caused by lidocaine. Usually, lidocaine and nikethamide were requested to be

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quantified in plasma and cerebrospinal fluid in the forensic toxicological analyses. Due to their similarity in chemical structures, quantification of nikethamide is expected to be easily interfered with lidocaine. Therefore, it is important to develop an analytical method to determine nikethamide and lidocaine simultaneously. Although there are methods developed for simultaneous identification of hundreds of drugs including nikethamide and lidocaine in biological matrix [27–31], there is no method reported on the simultaneous quantitative determination of nikethamide and lidocaine by HPLC in biological fluid.

The purpose of the current study was to develop a sensitive, accurate and comparatively simple method for the simultaneous quantification of nikethamide and lidocaine in plasma and cerebrospinal fluid by HPLC.

2. Materials and methods

2.1. Materials and reagents

Nikethamide injection (0.375 g/1.5 ml) and lidocaine hydrochloride injection (100 mg/5 ml) were purchased from Zhaohui Pharmaceutical Factory (Shanghai, PR China). Vauqueline, used as internal standard, was obtained from Forensic Identification Center of Public Security Ministry of China (Beijing, PR China).

Methanol (HPLC grade) and acetonitrile were obtained from Dikema (Richmond Hill, USA). Ultra pure water (resistance >18 mΩ) was produced by a Millipore apparatus. Other reagents were all of analytical grade. The drug-free human blood for preparing spiked samples was supplied by Chengdu Blood Station (Chengdu, PR China) and the drug-free human cerebrospinal fluid for preparing spiked samples was obtained from forensic pathological laboratory of Huaxi Forensic Identification Center of Sichuan University (Chengdu, PR China).

2.2. Instrumentation and chromatographic conditions

Chromatography was performed using a quanternary gradient pump (G1311A, Agilent 1100) with a 100 μl fixed volume injector coupled with an autosampler (G1313A, Agilent 1100) and a photodiode array detector (G1315A, Agilent 1100). Chemeworking software was used for system control, data acquisition and process. The separation column (150 mm × 4.6 mm i.d., 5 μm Zorbax $C_{18,}$ Dikema) was maintained at 25 °C and connected with a precolumn (4 mm × 4.6 mm i.d., 5 μm, C_{18}, Dikema).

Elution was performed with a mobile phase containing methanol and buffer (22:78, v/v) at a constant flow rate of 1.0 ml/min. The buffer was prepared by diluting diethylamine (5 ml) in 500 ml ultra pure water followed by adjusting the pH to 4 with acetic acid. The final buffer mixture was filtered through a 0.45 μm filter prior to use. The analytes were monitored at a wavelength of 263 nm.

2.3. Stock solutions

Stock solutions of nikethamide and lidocaine were prepared by dissolving appropriate amounts of each analytes in methanol to reach a final concentration of 1.25 and 2.0 mg/ml, respectively. The stock and working solutions of vauqueline were prepared in methanol at concentrations of 1.25 and 0.125 mg/ml, respectively. All prepared stock and working solutions were stored at 4 °C.

2.4. Sample preparation

An aliquot of 20 μl of the internal standard working solution (0.125 mg/ml) was added to 1 ml of collected blood or cerebrospinal fluid sample followed by the addition of 5 ml of acetonitrile. The mixture was vortexed for 5 min and centrifuged at 3000 × *g* for 10 min. The supernatant was collected and evaporated to dryness with airflow. The residue was reconstituted with methanol and the mixture was centrifuged at 13,000 × *g* for 10 min. The supernatant was transferred and concentrated to 200 μl, and 20 μl was injected into HPLC for analysis.

2.5. Validation of the method

The extraction recoveries were determined at three concentration levels by comparing the analytes peak areas obtained from the quality control samples to those obtained from the corresponding reference standards prepared at same concentrations.

For linearity study, calibration curves of all analytes were obtained in the same day from both blood and cerebrospinal fluid. Quantification was performed by calculating the peak-area ratio of each analyte versus that of the internal standard. The limits of detection (LOD) were defined as the lowest concentration of the analytes that can be detected with a signal-to-noise ratio greater than 3:1. The limits of quantification (LOQs) were defined as the lowest concentration of analytes that can be quantified with an accuracy of within 10% of the true value and a coefficient of variation (CV) less than 15%.

Precision and accuracy of the method were monitored for 3 days. Two calibration curves with nine determinations of three concentrations of quality controls were analysed on each day. The obtained results were analysed with variance analysis (ANOVA), which provides the intraday-assay and interday-assay standard deviations and consequently the corresponding coefficients of variation. The intraday-assay CV took into account the variability of the three replicates each day for 3 days while the interday-assay CV refers to the analysis variability between the 3 days. The accuracy was determined by comparing the mean calculated concentration with the spiked target concentration of the quality control samples.

2.6. Clinical application

The developed method has been applied to analyse lidocaine and/or nikethamide in 10 forensic cases received in our institute from 2004 to 2006 (Table 1). All of the reported patients were admitted to hospitals and received operations owing to various diseases. Majority of them obtained continuous epidural anesthesia with lidocaine and were rescued with nikethamide. Eventually, these patients passed away either during or after operations. Their families subsequently requested

Table 1
Representative forensic cases involved lidocaine and nikethamide analyses in human blood and cerebrospinal fluid

No.	Disease	Age	Gender	Concentration (μg/ml)			
				Lidocaine		Nikethamide	
				Blood	Cerebrospinal fluid	Blood	Cerebrospinal fluid
1	Pregnancy hypertension	23	Female	34.4	48.0	68.8	46.5
2	Intestinal obstruction	35	Male	18.8	168.7	99.8	64.8
3	Ectopic pregnancy	31	Female	32.0	185.6	106.8	72.4
4	Gallbladder polipi	42	Female	NA[a]	ND[a]	NA	8.0
5	Appendicitis	8	Male	ND	ND	24.6	8.6
6	Bone fracture	47	Male	NA	20.3	NA	ND
7	Abdominal pain	52	Female	ND	NA	8.5	NA
8	Cardiopalmus	71	Male	0.68	NA	5.6	NA
9	Acute gastroenteritis	45	Male	2.20	NA	17.0	NA
10	Abdominal pain	47	Male	ND	NA	1.25	NA

[a] NA, not applicable; ND, not detectable.

the determination of lidocaine and/or nikethamide in blood and/or cerebrospinal fluid. One milliliter of the collected blood or cerebrospinal fluid was treated and analysed as described in Sections 2.4 and 2.2, respectively. Three representative cases are described as follows.

2.6.1. Case 1

A 35-year-old male patient (No. 2 case in Table 1) was diagnosed with intestinal obstruction and toxic shock. He received continuous epidural anesthesia by lidocaine prior to the implementation of laparotomy. Ten minutes after 2% lidocaine (12 ml of volume dose) was punctured from lumbar 1, 2, the patient suffered from headache, chest distress, nasal obstruction, short of breath, hypopiesis and cyanotic lips. Medicines including nikethamide and adrenaline were used immediately for rescue. However, the patient died eventually. Quantification of lidocaine and nikethamide were subsequently requested in his cerebrospinal fluid (4.5 ml) and blood (2 ml) samples.

2.6.2. Case 2

A 45-year-old male patient (No. 9 case in Table 1) was admitted to hospital due to acute abdominal pain and subsequently diagnosed with acute appendicitis. During his appendectomy operation, a continuous epidural anesthesia by lidocaine was utilized. Four hours after operation, the patient unexpectedly experienced dyspnea and heartthrob. Medicines including nikethamide and adrenaline were used immediately for rescue. The patient died 5 days later. Blood concentrations of nikethamide and lidocaine were requested for forensic analysis purpose.

2.6.3. Case 3

A 71-year-old male patient (No. 8 case in Table 1) was admitted to hospital due to common cold. During transfusion, the patient encountered heartthrob and received lidocaine and nikethamide afterwards. The patient died 1 day later. Around 2 ml of his blood was collected for the analyses of lidocaine and nikethamide concentrations.

3. Results and discussion

3.1. Sample preparation procedure

Alkalization of blood and cerebrospinal fluid samples followed by liquid–liquid extraction was tried for the sample preparations. However, the extraction recoveries of nikethamide at low concentrations were found to be less than 30%, which is due to its relatively good aqueous solubility. When protein precipitation with acetonitrile was used for the sample extraction, the recoveries of nikethamide and lidocaine increased dramatically to be greater than 70% with no interference. Therefore, the protein precipitation method was chosen for the sample treatment in the current study.

3.2. Selection of HPLC chromatography conditions

The aim of the current study was to develop a HPLC method using UV detection for the simultaneous determination of nikethamide and lidocaine in blood and cerebrospinal fluid. Nikethamide has UV maxima at 263 and 255 nm, while lidocaine at 263 and 278 nm. The 263 nm was selected for the current assay since at this wavelength both nikethamide and lidocaine can be detected with adequate sensitivities and no interference from the endogenous impurities. Although the wavelengths of 200–230 nm were employed in the previous studies by others [14,15,17–20,22–24] to provide higher sensitivity of lidocaine detection, such short wavelength may lead to interference of endogenous materials, necessitating more complicated sample preparation methods such as extraction and back extraction [14,15,19,20] or solid phase extraction (SPE) [18,23].

Peaks of nikethamide and lidocaine are often tailing due to their basic properties. Therefore, diethylamine was added to the buffer of the mobile phase to reduce the tailing. The effect of diethylamine concentrations on the peak shapes of lidocaine and nikethamide was investigated and the results are shown in Fig. 1. It is seen that the symmetry factors of nikethamide do not alter significantly with the increase of diethylamine concentration (0.9–1.0). However, tailing of

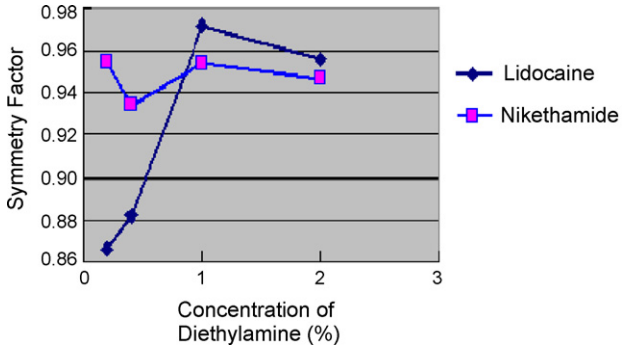

Fig. 1. Effect of diethylamine concentrations on the symmetry factor of HPLC peaks of nikethamide and lidocaine.

lidocaine was significantly improved with the increase of diethylamine concentration. Symmetry factor of lidocaine was over 0.95 when diethylamine concentration was above 1%. Therefore, 1% diethylamine was used in the mobile phase.

In the course of optimizing the mobile phase composition full coincidence of the peaks of the two analytes was found when ratio of methanol to buffer was at 35:65 (v/v). With reducing the proportion of methanol the analytes were gradually separating from each other. Baseline separation was achieved when the ratio was 22:78 (v/v), which was eventually selected to be the final composition.

The HPLC chromatograms of a blank blood sample and a blank cerebrospinal fluid sample are shown in Fig. 2A and B. The chromatographic separations of the analytes obtained from the spiked drug-free blood and cerebrospinal fluid are shown in Fig. 2C and D, respectively. All peaks are completely resolved without any interference from endogenous substances. Retention times for lidocaine, nikethamide, vauqueline were 12.5, 15.1, 19.1 min, respectively. The complete elution of the three analytes was obtained in less than 22.5 min.

3.3. Selection of internal standard

Compounds with similar structures of nikethamide such as isoniazid, pyrazinamide and protionamide were screened during the selection of an appropriate internal standard for the current assay. However, all of the above analytes were eluted too rapidly to be fully separated from the endogenous impurities in either blood or cerebrospinal fluid samples. After pilot investigations, vauqueline was chosen as the internal standard since (1) it has never been used in clinical practice; (2) it could be well separated from both analytes with no endogenous interfering peaks appeared at its retention time; (3) its extraction recovery from samples is similar to that of lidocaine under the conditions used; (4) and its absorption maximum is at 265 nm, which is close to the wavelength (263 nm) used in the current assay.

3.4. Method validation

3.4.1. Recovery

As indicated in Table 2, the mean recoveries of nikethamide in blood samples were between 69.8 and 80.7% and that of lidocaine were between 78.9 and 92.5%. In cerebrospinal fluid samples, the mean recoveries of nikethamide were in the range of 72.3–94.4% and those of lidocaine were between 91.0 and 97.2%.

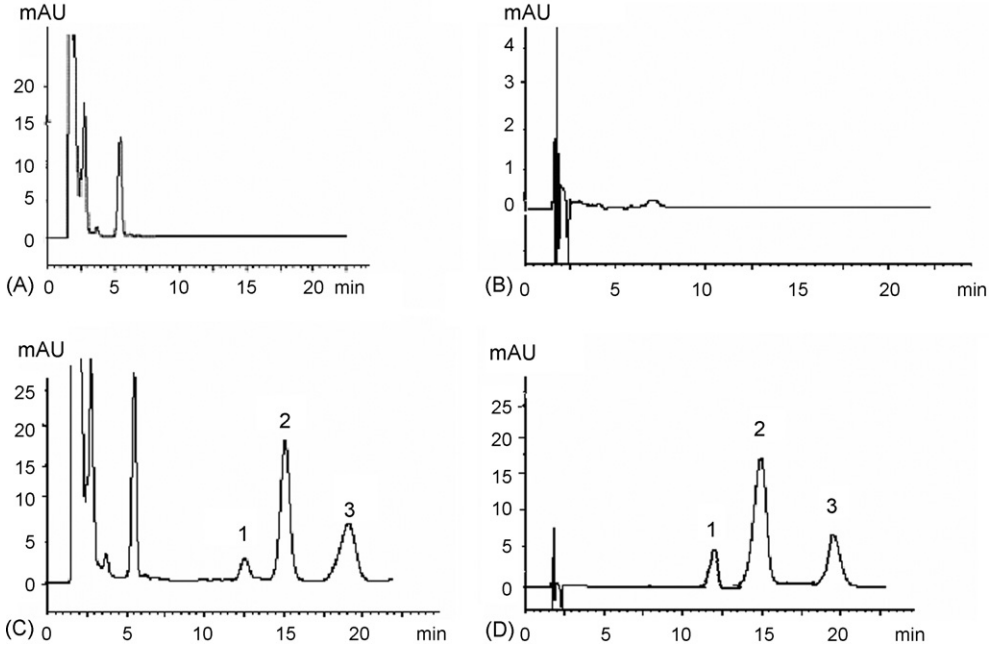

Fig. 2. Representative HPLC chromatograms of: (A) blank plasma; (B) blank cerebrospinal fluid; (C) plasma spiked with lidocaine (5.00 μg/ml), nikethamide (6.25 μg/ml) and their internal standard (2.50 μg/ml); (D) cerebrospinal fluid spiked with lidocaine (5.00 μg/ml), nikethamide (6.25 μg/ml) and their internal standard (2.50 μg/ml). Chromatographic peaks: (1) lidocaine; (2) nikethamide and (3) vauqueline (internal standard).

Table 2
Preparation recoveries of lidocain and nikethamide in blood and cerebrospinal fluid samples ($n = 3$)

Samples	Concentration (μg/ml)	Recovery (mean ± S.D., $n = 3$) (%)
Nikethamide		
Blood	1.25	80.7 ± 4.2
	12.5	75.9 ± 2.2
	125	69.8 ± 2.4
Cerebrospinal fluid	1.25	94.4 ± 4.3
	12.5	85.1 ± 3.8
	125	72.3 ± 4.4
Lidocaine		
Blood	1.00	92.5 ± 3.1
	10.0	78.9 ± 2.7
	100	81.6 ± 4.4
Cerebrospinal fluid	1.00	95.6 ± 1.4
	50.0	91.0 ± 2.7
	200	97.2 ± 3.8

3.4.2. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

As indicated in Table 3, the calibration curves for nikethamide and lidocaine in the blood and cerebrospinal fluid were linear with correlation coefficients (r^2 values) greater than 0.9987. LOD were 0.008 μg/ml for nikethamide and 0.16 μg/ml for lidocaine in blood (Table 3). LOD were 0.007 μg/ml for nikethamide and 0.14 μg/ml for lidocaine in cerebrospinal fluid (Table 3).

In some earlier studies, the LOQs of lidocaine in biological matrix by HPLC ranged from 20 [24,25] to 300 ng/ml [22], which were higher than that of ours. The effect of the selection of the wavelength on the sensitivity and selectivity was discussed in Section 3.2. As low an LOD as 1 ng/ml was described for lidocaine when fluorescence detector was used after treatment of the samples with 9-fluorenylmethylchloroformate [16], however, such derivatization is rather tedious.

In summary, sensitivity of the determination of nikethamide and lidocaine obtained with our method is higher than most of those reported in literatures. Although the sensitivity for lidocaine is not as high as for some of the reported methods, our sample preparation method is easier. Our LOD and LOQ satisfy the requirement from forensic lidocaine analysis since the LOQ is at least 10 times lower than the minimum toxic blood concentration.

3.4.3. Accuracy and precision

The accuracy and precision of the assay are summarized in Table 4. The intra-assay coefficients of variation for both medicines were ≤9.2% and all inter-assays CVs were below 10.8% in blood and cerebrospinal fluid. The intra-assay and inter-assay accuracies for both compounds were found to be from 91.6 to 109.3%.

Table 3
Linearity, LOD and LOQ of the current assay for simultaneous determination of nikethamide and lidocaine in blood and cerebrospinal fluid ($n = 3$)

	Range (μg/ml)	Slope (mean)	Intercept (mean)	Mean coefficient of correlation	Limit of detection (μg/ml)	Limit of quantification (μg/ml)
Nikethamide						
Blood	0.25–125	0.1864	0.0015	0.9997	0.008	0.25
Cerebrospinal fluid	0.25–125	0.2151	−0.0736	0.9987	0.007	0.25
Lidocaine						
Blood	0.50–100	0.0194	−0.0012	0.9992	0.16	0.50
Cerebrospinal fluid	0.50–200	0.0199	−0.0058	0.9992	0.14	0.50

Table 4
Intra-assay and inter-assay precision and accuracy

Compound	Concentration (μg/ml)	Intra-assay CV (%)	Intra-assay accuracy (mean ± S.D.) (%)	Inter-assay CV (%)	Inter-assay accuracy (mean ± S.D.) (%)
Nikethamide					
Blood	1.25	6.5	98.5 ± 6.4	8.9	106.4 ± 9.5
	12.5	8.5	107.6 ± 9.1	10.8	106.4 ± 11.4
	125	2.3	91.6 ± 2.1	5.9	94.1 ± 5.5
Cerebrospinal fluid	1.25	9.2	100.0 ± 9.2	2.3	97.6 ± 2.2
	12.5	6.7	106.8 ± 6.9	2.3	109.3 ± 2.4
	125	7.6	100.9 ± 3.8	2.3	102.0 ± 2.3
Lidocaine					
Blood	1.00	8.3	101.8 ± 7.9	3.9	101.5 ± 3.7
	10.0	2.5	102.6 ± 2.5	2.9	102.9 ± 2.9
	100	0.8	98.6 ± 0.7	3.5	97.2 ± 3.4
Cerebrospinal fluid	1.00	7.6	100.0 ± 7.5	6.3	101.6 ± 6.8
	50.0	1.9	103.1 ± 2.0	3.5	107.6 ± 3.8
	200	4.1	100.7 ± 3.9	0.9	102.9 ± 0.9

3.5. Results of the forensic cases application

The developed method has been successfully applied to the blood and cerebrospinal fluid analyses of lidocaine and/or nikethamide in 10 forensic cases and the results are shown in Table 1. Blood samples from eight forensic cases were analysed with blood concentrations of lidocaine ranging from 0.68 to 34.4 μg/ml and nikethamide ranging from 1.25 to 106.8 μg/ml. From the six cases requested the cerebrospinal fluid analyses, there were 20.3 to 185.6 μg/ml of lidocaine and 8.0 to 72.4 μg/ml of nikethamide found in the collected samples.

4. Conclusion

A simple and selective RP-HPLC method for the simultaneous determination of nikethamide and lidocaine in plasma and cerebrospinal fluid was developed. This method has been fully validated with satisfactory accuracy and adequate reproducibility. The successful application of the developed method to the 10 forensic cases demonstrated that the current assay method could be readily used in toxicological screening tests for the simultaneous determination of nikethamide and lidocaine in biological materials such as blood and cerebrospinal fluid.

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