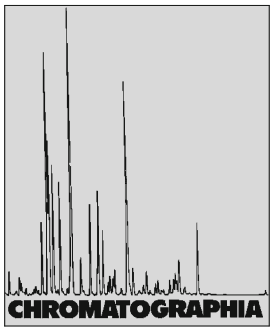
LC–MS–MS Determination of Nikethamide in Human Plasma



2009, *69*, 1067–1071

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Received: 13 November 2008 / Revised: 29 December 2008 / Accepted: 16 January 2009
Online publication: 17 March 2009

Abstract

A sensitive LC–MS–MS method with electrospray ionization has been developed for determination of nikethamide in human plasma. After addition of atropine as internal standard, liquid–liquid extraction was used to produce a protein-free extract. Chromatographic separation was achieved on a 150 mm × 2.1 mm, 5 μm particle, Agilent Zorbax SB-C_{18} column, with 45:55 (*v/v*) methanol–water containing 0.1% formic acid as mobile phase. LC–MS–MS was performed in multiple reaction monitoring mode using target fragment ions *m/z* 178.8 → 107.8 for nikethamide and *m/z* 289.9 → 123.8 for the internal standard. Calibration plots were linear over the range of 20.0–2,000 ng mL^{-1}. The lower limit of quantification was 20.0 ng mL^{-1}. Intra-day and inter-day precisions were better than 4.2 and 6.1%, respectively. Mean recovery of nikethamide from human plasma was in the range 65.3–71.1%.

Keywords

Column liquid chromatography
Tandem mass spectrometry
Nikethamidein human plasma

Introduction

Nikethamide is widely used in clinical practice to stimulate the activity of the central nervous and cardiovascular systems [1]. In sports, nikethamide is listed by the World Anti-Doping Agency as a banned substance. Therefore, it is important to develop analytical methods to determine nikethamide in biological samples for both forensic and clinical medical practice.

Several analytical methods for nikethamide determination in biological samples have been reported, including phosphorimetry [2], thin-layer chromatography (TLC) [3], gas chromatography (GC) [4], gas chromatography–mass spectrometric (GC–MS) [5] and liquid chromatography coupled with UV detection [6–8]. However, GC and GC–MS methods include analyte derivatization with a generally complicated sample preparation procedure. In the LC-UV method, UV detection has a number of limitations such as lack of specificity and poor sensitivity for non-UV absorbing species, which have driven scientists to pursue the use of MS detectors as an alternative.

Mass spectrometry (MS) is widely recognized as a powerful analytical tool which can provide both qualitative and quantitative data that may not be readily available by other techniques [9]. An advantage of MS detection over conventional UV detection is that it offers information about the chemical composition of an analyte, thus providing a second dimension of analysis. The high sensitivity of MS detection for a wide variety of analytes also allows for trace quantification. Additionally, MS can be used to detect molecules without chromophores, negating the need for derivatization of samples.

Reliable analytical methods are required and analytical chemists have to contend with a variety of compounds, including biological fluids and their

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metabolites, that have to be determined at very low concentration levels in complex matrices. Undoubtedly, optimized chromatographic methods have to be employed, and coupling with mass spectrometric techniques is compulsory in order to satisfy the sensitivity and selectivity requirements, and to confirm the identities of compounds detected in this type of analysis [10].

The large numbers of samples in clinical medicine need a rapid and reliable method. An ideal method should have simple sample preparation, fast on-column separation, and sensitive and specific detection. LC–MS–MS has become an analytical tool that meets most of the above needs. The use of LC-MS has rapidly increased in the last few years. Currently, LC-MS is one of the dominant analytical techniques in the qualitative and quantitative determination of low molecular weight compounds [11]. During recent years, LC–MS has repeatedly been proven to be a powerful technique for the rapid, quantitative determination of drugs and metabolites in biological fluids [12–16].

In the present work, a sensitive, specific and simple LC–MS–MS method was employed to determine nikethamide in human plasma. The analytical procedure was fully validated and successfully used to determine nikethamide in biological samples for forensic and clinical studies. To our knowledge, this is the first report dealing with the determination of nikethamide in biological samples by LC-MS-MS.

Experimental

Chemicals and Reagents

Nikethamide injection solution (0.375g /1.5 mL) was purchased from Tianjin Pharmaceuticals Group Corporation (Tianjin, China). Atropine, used as internal standard, was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

LC-grade methanol was from Merck (Darmstadt, Germany). While LC-grade formic acid was from Tedia (Cincinnati, USA). Ultra pure water (resistance > 18 mΩ) was produced by a Milli-Q purification apparatus from Millipore (Bedford, USA). Other reagents were all of analytical grade. Blank human plasma was obtained from The First Affiliated Hospital of Wenzhou Medical College (Wenzhou, China). Human Plasma samples were obtained from the Forensic Laboratory of Wenzhou Medical College (Wenzhou, China).

Preparation of Calibration Standards and Quality-Control Samples

Individual stock solutions of nikethamide (1.0 mg mL^{-1}) and atropine (internal standard, IS) (1.0 mg mL^{-1}) were prepared in methanol. Working standard solution (200, 500 ng mL^{-1}, and 1, 2, 5, 10, 15, 20 μg mL^{-1}) of nikethamide were prepared by serial dilution of the stock solution with methanol. 1.0 μg mL^{-1} working standard solution of IS was prepared by dilution of the IS stock solution with methanol.

Nikethamide calibration standards were prepared by spiking blank human plasma with appropriate amounts of the working solutions. Eight different concentrations covering the expected ranges of 20.0–2,000 ng mL^{-1} for nikethamide in human plasma (concentrations 20.0, 50.0, 100, 200, 500, 1,000, 1,500, and 2,000 ng mL^{-1}) were prepared. Quality-control (QC) samples were prepared in the same way as the calibration standards at, three different plasma concentrations (20.0, 200, and 1,500 ng mL^{-1}).

Sample Preparation

Atropine solution (1.0 μg mL^{-1}, 50 μL) was added to 0.5 mL plasma in a glass tube and the mixture was made to be alkaline by addition of aqueous ammonia (25% *v*/*v*, 100 μL). Ethyl ether (5 mL) was added, after shaking, the mixture was centrifuged at 3,500 rpm for 10 min. The supernatant was transferred to another glass tube and evaporated to dryness at 40 °C under a stream of nitrogen. The residue from each sample was reconstituted with 200 μL mobile phase and centrifuged at 15,000 rpm for 5 min. The supernatant (10 μL) was injected for LC–MS–MS analysis.

Chromatography-Tandem Mass Spectrometry

All performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment, and a Bruker Esquire HCT ion-trap mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software.

Chromatographic separation was achieved on a 150 mm × 2.1 mm, 5 μm particle, Agilent Zorbax SB-C_{18} column at 25 °C, with 45:55 (*v*/*v*) methanol–water containing 0.1% formic acid as mobile phase. The flow rate was 0.3 mL min^{-1}.

Analyses were performed with the ESI source operated in the positive ion mode at 350 °C with a spray potential of 4.0 kV. The nebulizer gas pressure was 30 psi and the dry gas was adjusted to a constant flow rate of 7 L min^{-1}. Collision-induced dissociation (CID) studies were performed with a collision energy of 40 V. The ion-spray interface and mass spectrometric conditions were optimized to achieve maximum sensitivity at unit resolution.

Nikethamide was detected as the protonated molecule $[M + H]^+$ at *m*/*z* 178.8; this ion was chosen as the precursor ion. The compound fragmented almost exclusively by collision-induced decomposition to produce an intense product ion at *m*/*z* 107.8. The protonated species $[M + H]^+$ at *m*/*z* 289.9 was the predominant ion in the full-scan spectrum of atropine (IS), and was chosen as the precursor ion. The compound fragmented almost exclusively to produce an intense ion at *m*/*z* 123.8. The multiple reaction monitoring (MRM) transitions 178.8 → 107.8 and 289.9 → 123.8 were chosen for nikethamide and atropine (IS), respectively.

Assay Validation

Specificity was determined by analysis of blank human plasma, without addition of nikethamide and the internal standard to determine possible interference with these compounds.

Matrix effects were investigated by extracting blank plasma from five different sources, reconstituting the final extract in mobile phase containing a known amount of nikethamide. Then the peak areas obtained from the reconstituted extracts were compared to those obtained from unextracted standard working solutions in the same solvent.

Calibration standards containing nikethamide at eight concentrations were extracted and assayed. A calibration plot was constructed by plotting nikethamide to atropine (IS) area ratios against the concentration of nikethamide in plasma. The lower limit of quantification (LLOQ) of nikethamide in human plasma was selected as the lowest concentration used in the calibration curve.

The intra-day precision and accuracy of nikethamide were measured by analyzing three QC samples (20, 200, and 1,500 ng mL^{-1}) with three determinations for each. The inter-day precision and accuracy were determined by analyzing QC samples with three determinations for each over three days.

Extraction recovery experiments were performed by comparing the peak areas obtained for extracted nikethamide plasma samples to unextracted standard working solutions at three concentrations in the same solvent (low: 20.0, medium: 200, high: 1,500 ng mL^{-1}).

Sample stability was tested by analyzing QC samples (20.0, 200, and 1,500 ng mL^{-1}) after short-term (6 h) storage at room temperature, 12 h storage in an autosampler, after three freeze (−20 °C)-thaw (room temperature) cycles, and after long-term (15 days) storage at −20 °C and immediate extraction.

Results and Discussion

Method Development

Sample preparation is a key step for the determination of drugs in biological samples. At the beginning of this work, we tried using protein precipitation with acetonitrile for the sample extraction that has been reported [7]. The recovery of nikethamide was found to be about 70%, but with some interference, not clean enough for MS–MS. Then alkalization of plasma samples followed by liquid-liquid extraction with ethyl ether for the sample preparations. This simple and rapid procedure produced a clean chromatogram for a blank plasma sample and yielded satisfactory recovery for analytes from plasma.

The separation of nikethamide from matrix by a mobile phase mixture of methanol and a diethylamine–acetic acid buffer [6, 7] was reported. A simple mobile phase of mixture methanol–water containing 0.1% formic acid was used in this work. The tailing of nikethamide was significantly improved by adding formic acid. The formic acid was added to the mobile phase also to supply H^+ for ionization, which dramatically increased the ESI efficiency of the analyte [17].

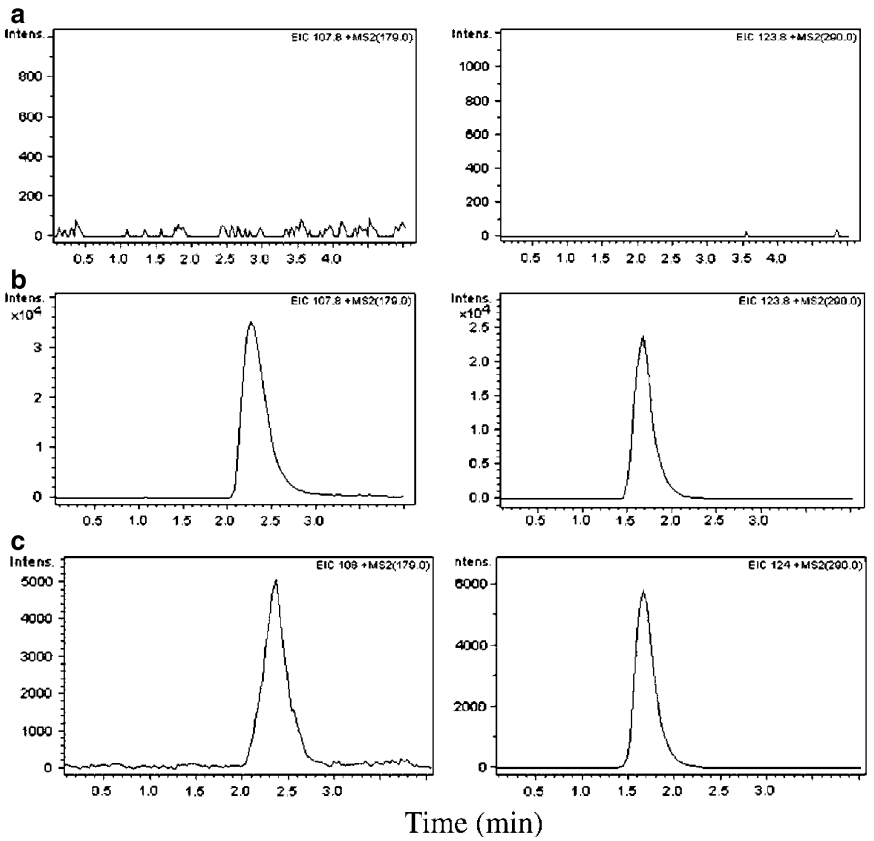

Fig. 1. Representative chromatograms of: **a** blank plasma; **b** plasma spiked with atropine and nikethamide; **c** plasma sample obtained from forensic case

ESI interface was used in this work because it gave better sensitivity and repeatability for nikethamide determination compared to an atmospheric pressure chemical ionization (APCI) interface.

Several compounds were tried as internal standards and atropine was found to be optimal for our work. Compounds such as diazepam, nitrazepam, estazolam, carbamazepine and atropine were screened during the selection of an appropriate internal standard for the current assay. After investigations, atropine was chosen as the internal standard since (i) its extraction recovery from the samples was similar to that of nikethamide at the conditions used; (ii) its ionization MS condition was similar to that of nikethamide.

Selectivity

At the chromatographic conditions described in the experimental part, no interfering compound was found in blank plasma. The selectivity for the

Table 1. Accuracy and precision for nikethamide of quality control sample in human plasma ($n = 3$)

Nominal concentration (ng mL^{-1})	Mean found concentration (ng mL^{-1})	Precision (RSD, %)	Accuracy (%)
Intra-day			
20.00	19.20	4.2	96.0
200.0	198.6	2.3	99.3
1500	1503	1.1	100.2
Inter-day			
20.00	20.44	6.1	102.2
200.0	194.0	3.4	97.0
1500	1510	1.6	100.7

Table 2. Stability of nikethamide in human plasma ($n = 3$)

Nominal concentration (ng mL^{-1})	Mean found concentration (ng mL^{-1})	Precision (RSD, %)	Accuracy (%)
Short-term stability for 6 h in plasma at room temperature			
	20.23	4.5	101.2
200.0	197.31	3.6	98.7
1500	1490.23	1.3	99.3
Short-term stability in an autosampler 12 h			
200.0	19.32	6.1	96.6
200.0	202.21	4.6	101.1
1500	1485.16	2.6	99.0
Three freeze-thaw cycles			
200.0	19.37	7.6	96.9
200.0	197.10	5.6	98.6
1500	1481.02	5.3	98.7
Storage in plasma at −20 °C for 15 days			
200.0	19.46	7.8	97.3
200.0	197.21	6.7	98.6
1500	1479.45	5.2	98.6

plasma sample is shown in Fig. 1a and b. By multiple reaction monitoring with MS–MS, the precursor ion was isolated and fragmented by CID. Subsequently, one or more product ions were isolated and scanned, allowing specific precursor product ion transition to be followed and increasing sensitivity by total elimination of background noise. MRM in the positive mode was used for quantification. The most intense ions of nikethamide and IS were m/z 178.8 and m/z 289.9, respectively. The collision energy was determined by observing the maximum response obtained for the fragment ion peak. The product ions of nikethamide for MRM acquisition were m/z 107.8 and m/z 123.8 for the IS.

Matrix Effect [18]

Ion suppression caused by the plasma matrix was evaluated. Comparison of peak areas obtained from reconstituted extracts (blank plasma from five different sources) and from unextracted working solutions standards in the same solvent showed there was no matrix effect and no interferences from endogenous compounds. The relative standard deviation (RSD) of the peak areas for the five reconstituted extracts was 3.1%, indicating that the extracts did not interfere with ionization of the analyte.

Linearity and Lower Limit of Quantification

The linearity for nikethamide was investigated by linear regression of peak area ratios against concentrations. The regression equation for the calibration plot ($n = 8$) was $Y = 0.41778C–0.019234$ (Y is the peak ratio of nikethamide to IS, and C is the concentration of nikethamide in plasma), correlation coefficient 0.998, for concentrations in the range 20.0–2,000 ng mL^{-1}. The detection limit, defined as the concentration giving a signal–noise ratio of 3, was 5.0 ng mL^{-1} in plasma for nikethamide. The lower limit of quantitation (LLOQ) for nikethamide in plasma, defined as the concentration giving a signal–noise ratio of 10, was 20.0 ng mL^{-1}, which is sufficient for forensic and clinical studies.

Accuracy and Precision

The intra-day and inter-day precision and accuracy of nikethamide are shown in Table 1. The precisions (RSD) are all less than 7%. The accuracy of nikethamide ranged from 96.0 to 100.2% for intra-day, and 97.0 to 102.2% for inter-day.

Recovery

Extraction recoveries of nikethamide in plasma were assessed using three concentration levels (20.0, 200, and 1,500 ng mL^{-1}) with three determinations for each. Comparison of the peak areas obtained for extracted nikethamide plasma samples with unextracted standard working solutions enabled calculation of mean extraction recovery of 65.3, 71.1, and 68.9% for concentrations of 20.0, 200, and 1500 ng mL^{-1}, respectively. And RSD ranged between 1.2% and 4.1%.

Stability

All the stability studies of nikethamide in plasma were conducted at three concentration levels (20.0, 200, and 1,500 ng mL^{-1}) with three determinations for each under different storage conditions. The RSD of the mean test responses was within 8% in all stability tests of nikethamide in plasma (Table 2). No effect on the quantitation was observed for plasma samples kept at room temperature for 6 h. Samples were also stable for at least 12 h in an autosampler. There was also no significant degradation when samples of nikethamide in plasma were taken through three freeze–thaw cycles. Nikethamide in plasma was stable at −20 °C for 15 days.

Application

In clinical practice, medicines such as nikethamide are often used to rescue the

Table 3. Representative cases involved nikethamide analyses in human plasma

Case no.	Age	Gender	Concentration (ng mL^{-1})
1	12	Male	40.62
2	19	Female	820.74
3	24	Male	1868.41
4	40	Male	700.64
5	64	Female	1474.56

patient when overdosed with anesthetic. Medical disputes quite often occurred regarding the anesthetic accidents. Usually, nikethamide was requested to be quantified in plasma in the forensic toxicological analyses.

The developed method has been successfully applied to the plasma determination of nikethamide in our laboratory since 2007. Between July 2007 and June 2008, five samples were obtained from the forensic laboratory. The results in Table 3, show plasma samples from five cases analyzed with plasma concentration of nikethamide ranging from 40.62 to 1868.41 ng mL^{-1}.

Conclusion

A sensitive, rapid and specific LC–MS–MS method has been developed for the determination of nikethamide in human plasma. A very low limit of quantitation was obtained and a simple procedure for pretreatment of plasma samples was used. The method was validated to meet the requirements for both forensic and clinical medical determination of nikethamide in human plasma, and could be suitable for pharmacokinetic studies.

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