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Journal of Pharmaceutical and Biomedical Analysis 34 (2004) 1063–1070

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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Assay for nipecotic acid in small blood samples by gas chromatography–mass spectroscopy

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Received 8 April 2002; received in revised form 28 August 2003; accepted 23 November 2003

Abstract

An analytical method for nipecotic acid quantification in rat blood was developed utilizing a stable isotope internal standard and capillary gas chromatography–mass spectroscopy. The method involves a solid phase extraction step followed by a two-step derivatization. The analytes are separated by capillary gas chromatography and detected by selected ion monitoring of their base peaks at 180 and 185 *m/z*, respectively. The assay has a limit of detection (LOD) of 10 ng/ml and a limit of quantification of 26 ng/ml in 200 μl of rat whole blood. The linear range of the assay covers from 26 to 6500 ng/ml ($r^2 = 0.9996$, $n = 9$). The coefficient of variation was less than 10% at concentrations of 50, 1000 and 5000 ng/ml. The assay was used to characterize the pharmacokinetics of *R*-(-)-nipecotic acid in a rat. *R*-(-)-nipecotic acid clearance was 4.2 ml/min, its half-life was 1.5 h and its volume of distribution at steady state was 325 ml.

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Keywords: Nipecotic acid; Gas chromatography–mass spectrometry; Pharmacokinetics

1. Introduction

Gamma amino butyric acid (GABA) is the major inhibitory neurotransmitter in the brain, and nipecotic acid is a very selective inhibitor of the GABA re-uptake pump found on neurons and glial cells in the central nervous system (CNS). Low levels of GABA are thought to play a major role in epileptic seizures, pain and anxiety [1,2]. Thus, nipecotic acid and analogs of it have received considerable attention over the past 30 years as possible therapeutic agents [3–6]. In spite of the attention directed towards this deceptively simple molecule (Fig. 1) and the number of years since it was first reported in the literature, there is no simple, reliable and sensitive assay for nipecotic acid published in the literature. The methods that have been used, either have inadequate specificity and sensitivity [7], or do not quantify nipecotic acid at all, but measure instead GABA levels in the brain or some other biological response after nipecotic acid administration [8–10].

The quantification of nipecotic acid is more complex than it might at first appear from the structure of this molecule (Fig. 1). This zwitterion is

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doi:10.1016/j.jpba.2003.11.019

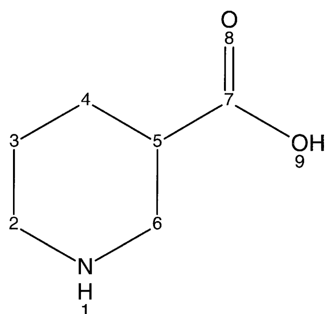

Fig. 1. Structure of nipecotic acid. The carboxylic acid functional group is attached to a chiral carbon (5) in the nipecotic acid structure. Both the nitrogen and the carboxylic acid groups are charged at physiological pH.

extremely soluble in water and cannot be extracted easily from aqueous biological samples by simple organic solvents or solvent mixtures. Solid phase extraction would seem a natural method for isolation of nipecotic acid. However, most solid phase materials do not retain this zwitterion, including a variety of cation and anion exchange columns. The similarity of nipecotic acid to amino acids further complicates efforts to quantify low levels of this analyte in biological samples where amino acids are prevalent. The lack of any chromophore in nipecotic acid precludes its quantification by simple UV or fluorescence methods without derivatization. The addition of a chromophore to the molecule is complicated by two factors. First, the secondary amine group in nipecotic acid makes it more difficult to derivatize with chromophores typically used for amino acids with primary amine groups. Second, any derivatization of nipecotic acid also produces a derivative of amino acids that are extracted with it and at concentrations higher than that of nipecotic acid. For these reasons, the high resolving power of capillary gas chromatography was considered the most appropriate place to start for developing a selective and specific assay for nipecotic acid. However, even this analytical method was complicated by the instability/decomposition of a common derivative often utilized to facilitate volatilization of polar molecules for gas chromatography.

The present selective and specific assay for nipecotic acid resulted from a painstaking process of trial and error that eliminated other methods and approaches that proved too non-specific and/or to insensitive to be acceptable. After assay development and validation, the method was applied to the quantification of (*R*)-nipecotic acid in one rat after its intravenous administration to assess its pharmacokinetic profile.

2. Experimental

2.1. Materials

Rac-Nipecotic acid and nicotinic acid were obtained from Sigma (St. Louis, MO 63178, USA). *R*-(-)-nipecotic acid (>98% purity and >95% optical purity) was purchased from FCRD (Haifa Bay 26111, Israel). Deuterium, deuterium oxide (>99.9%), platinum(IV) oxide, bis(trimethylsily)trifluoroacetamide (BSTFA, derivatization grade) and trifluroacetic acid anhydride (>99%) were purchased from Aldrich (Milwaukee, WI 53233, USA). Sodium pentobarbital (50 mg/ml) was obtained from Abbott Labs. (North Chicago, IL 60064, USA). Saline (0.9%, injectable) was purchased from Baxter Healthcare Corp. (Deerfield, IL 60015, USA). Waters Oasis MCX LP extraction columns (3 cm^3, 60 mg) were purchased from Waters Corporation (Milford, MA 01757, USA). Analytical-reagent grade ethyl alcohol, methanol, acetonitrile, and all other reagents were purchased from Fisher Scientific (Fair Lawn, NJ 07410, USA). A 200 μl positive displacement pipette and pipette tips were purchased from Wheaton (Midville, NJ 08332, USA). Male rats (250 g) were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN 46229, USA). All chemicals were used without further purification.

2.2. Synthesis of internal standard

d_5-Nipocotic acid was synthesized from nicotinic acid and deuterium gas [11,12]. For this synthesis, nicotinic acid hydrochloride salt (1 g) was first dissolved in deuterated water (D_2O, 20 ml). To this mixture was added 0.1 g of catalyst (platinum oxide). The solution was sealed in a parr bomb, flushed with deuterium gas, and the bomb pressurized with 3 atm of deuterium for 2 h at room temperature with mixing. The resulting solution was first filtered to remove the

catalyst, and the filtrate dried under reduced pressure. The d_5-nipecotic acid produced was re-crystallized from hot ethanol. Total yield was 90%. The d_5/d_0 ratio of the *N*-trifluoroacetyl, *C*-trimethylsilyl derivative of d_5-nipecotic acid was determined by GC–MS and found to be greater than >99% pure. NMR was used to further characterize the final product which produced a spectrum similar to *rac*-nipecotic acid, but with extensive proton–proton de-coupling relative to d_0-nipecotic acid.

2.3. Standards and sample preparation

The calibration standards were prepared by serial dilution of a *rac*-nipecotic acid stock solution in water from which standard concentrations of 5.2, 13, 26, 52, 130, 260, 520 and 1300 ng/40 μl were prepared. The d_5-*rac*-nipecotic acid solution was fixed at 86 ng/20 μl. Blank whole rat blood (200 μl) was spiked with 40 μl of a *rac*-nipecotic acid standard and with 20 μl of the d_5-*rac*-nipecotic acid internal standard. Actual blood samples (200 μl) collected for the pharmacokinetic study were spiked only with the internal standard—d_5-nipecotic acid. Eight hundred microlitres of acidified acetonitrile (1 N HCl/acetonitrile; 1/10 (v/v)) was added to all tubes. The samples were mixed on a vortex shaker for 30 s, and then centrifuged at 10,000 rpm for 4 min in an Eppendorf 5415C micro centrifuge. The aqueous-acetonitrile supernatant was transferred to an unconditioned Waters Oasis MCX extraction column. All column loading and elution was by gravity. The column was washed with a 3 ml solution of 0.1 N HCl followed by 3 ml of 100% methanol. The analytes were eluted from the column with 1 ml of a 5% ammonium hydroxide/methanol (v/v) solution. The eluate was evaporated to dryness under nitrogen at room temperature, and a derivative made utilizing two separate derivatizing reagents. For derivatization of the nipecotic acid amine group, 400 μl of trifluroacetic acid anhydride was added to each sample and allowed to react for 30 min at room temperature. This solution was blown off with nitrogen gas at room temperature prior to the addition of the second reagent, BSTFA. For derivatization of the carboxylic acid group, 25 μl of BSTFA in pyridine was added to each sample and allowed to react for at least 30 min at room temperature before an aliquot was injected into the GC–MS system.

2.4. Gas chromatography–mass spectrometer conditions

The GC–MS analysis was performed with a Varian 3400 capillary GC connected to a Finnigan INCOS 50 ion-trap mass selective detector (Thermo Finnigan, San Jose, CA 95134, USA). Chromatographic separation was achieved with a DB5-MS capillary column (15 m × 0.25 mm, JNW, Folsom, CA 95630, USA) at a helium flow rate of 1.2 ml/min. The injector temperature was set at 280 °C and the oven temperature was programmed at 60 °C for 1 min, followed by a temperature increase of 10 °C/min to a final temperature of 280 °C followed by a rapid return to 60 °C. The injector was operated in the splitless mode for the first minute to facilitate detection of the low analyte levels. The temperature of the transfer line from the GC to the MS was set at 280 °C, and the MS ion source was fixed at 150 °C. The MS was operated in the electron impact ionization mode (electron energy 70 eV), and ions from the ion source were selectively monitored at *m*/*z* 180 (d_0-nipecotic acid) and 185 (d_5-nipecotic acid).

2.5. Animal study

Investigational animal care and use committee (IACUC) approval was obtained for this research prior to the animal work. A 250 g Sprague–Dawley rat was anesthetized with sodium pentobarbital (25 mg per animal) and after attainment of surgical anesthesia, two cannulae were implanted in the rat. The first cannula was inserted into the jugular vein for drug dosing, and the second cannula was inserted into the femoral vein and threaded into the inferior vena cava for purposes of blood sampling. Immediately following surgery, and while the animal remained anesthetized, a small dose (0.6 mg/0.05 ml saline) of *R*-(-)-nipecotic acid was administered via the jugular vein cannula. Blood samples (200 μl) were collected from the femoral vein prior to drug administration and at 1, 3, 5, 10, 20, 30, 40, 60, 120, 240 and 360 min after drug administration. Samples were stored at −20 °C, until processed as described earlier.

2.6. Quality control samples

A large quantity of rat blood (16–18 ml) was collected from two rats and placed in tubes with heparin

as the anticoagulant. The blood was divided into three portions of 5 ml each. A nipecotic acid stock solution was prepared and added in appropriate proportions to each blank 5 ml of blood to produce a final concentration of 50, 1000 and 5000 ng/ml. Two hundred microliter aliquots were removed, placed in pre-marked 1.5 ml eppendorf tubes, and stored at −20 °C until needed. These quality controls were removed and used with each analytical run.

2.7. Data analysis

Concentration-time profiles of *R*-(-)-nipecotic acid were fit to a two compartment model by with the WinNonlin® computer program purchased from Pharsight (Mountain View, CA 94040, USA). The terminal half-life was determined by dividing the 0.693 by the rate constant calculated from the best-fit line through the terminal portion of the concentration-time profile (e.g., 45–300 min). The area under the concentration time (AUC) profile was determined by the log-trapazoidal method with extrapolation to infinite time. The area under the first moment curve (AUMC) was determined by the trapezoidal method with extrapolation to infinite time. The systemic clearance (Cl) of *R*-(-)-nipecotic acid was determined from the equation:

$$\mathrm{Cl} = \frac{\mathrm{dose}}{\mathrm{AUC}}$$

The volume of distribution at steady state (V_{ss}) for *R*-(-)-nipecotic acid was determined from the equation:

$$V_{ss} = \frac{\mathrm{dose} \times \mathrm{AUMC}}{(\mathrm{AUC})^2}$$

3. Results

3.1. Methods development

A number of problems presented themselves throughout the development of this assay. The first was the difficulty in concentrating nipecotic acid from a biological matrix. From the start, it was known pharmacologically active concentrations of nipecotic acid would fall in the low μg/ml to low ng/ml range. Thus, it would be necessary to accurately quantify nipecotic acid in that concentration range. However, there was no good way to get nipecotic acid out of a biological matrix. Nipecotic acid is simply too polar. Organic solvent mixtures did not work well and nearly every solid phase material tried, except for the waters oasis columns, did not retain nipecotic acid. However, the oasis column created another problem, what internal standard to use. Initially, isonipecotic acid (an isomer of nipecotic acid) was used, but on the oasis column its recovery was markedly different from that of nipecotic acid (<20% versus 50–60%). This resulted in the need to use a better matched internal standard in the assay and led to the incorporation of the stable isotope (d_5-nipecotic acid) into this analytical method. However, the use of a stable isotope required MS analysis of the samples. Originally, LC/MS was tried, but the sensitivity of that method and the resolving power of the LC column were not adequate for nipecotic acid quantification in the low nano grams per milliliter range. This was probably more a reflection of the LC/MS equipment available than anything else. Thus, capillary GC–MS analysis was selected as the preferred route. The selection of capillary GC–MS, however, led to yet another problem, volatilization of the analyte. Most polar and charged drugs are derivatized with BSTFA and initially that seemed to work, but re-injection of samples indicated the peak heights decreased with time. That led to the discovery that a simple BSTFA derivatization of nipecotic acid resulted in a re-arrangement of the BSTFA-nipecotic acid derivative. That problem could only be circumvented if the nitrogen group was derivatized first with trifluroacetic acid anhydride, followed by a derivatization of the carboxylic acid group by BSTFA. Trifluroacetic acid anhydride is not stable enough to block carboxylic acid groups. It was only after each of these problems was solved that it was possible to obtain reliable, reproducible and consistent nipecotic acid concentrations in spiked biological samples.

3.2. Analytical validation

The nipecotic acid assay was validated by performing a standard series of assessments. The linearity of the assay was determined by visual and fitting programs that determined how well a straight line defined

the relationship between the area of d_0/d_5-nipecotic acid against the concentration of d_0-nipecotic acid. The reproducibility in estimated concentrations of nipecotic acid at three separate concentrations were determined 10 times. The accuracy of the estimated nipecotic acid concentration was determined 10 times on quality control samples analyzed on the same day and on different days to define intra- and inter-day variations in the nipecotic acid assay. The limit below which it would be difficult to detect the presence of nipecotic acid was defined as the concentration at which the d_0-nipecotic acid produced a signal that was three times that of the background noise of the GC–MS system after injection of a blank blood sample. The limit at which accuracy of the assay fell below acceptable limits was defined as the concentration at which the variance in the assay was >20%. Finally, the analytical method was applied to actual biological samples to assess its ability to generate useful pharmacokinetic information. The following sections highlight each of these aspects of the analytical methods validation.

3.3. Analytical performance

The standard calibration curve displayed excellent linearity over a wide range of concentrations (26–6500 ng/ml). No effort was made to push the assay limits further to determine the point at which the assay would become non-linear. A simple linear regression analysis was performed, and the data were fit to the best-fit line without weighting of the points. The regression equation associated with nine separate analyses was: $y = 0.608(\pm 0.029\,\text{S.D.})x + 0.0125(\pm 0.0101\,\text{S.D.})$, where y is the observed area ratio for the ions formed from nipecotic acid (*m/z* 180/185) and x is the amount (μg/ml) of nipecotic acid in the biological sample. The correlation coefficient (r) was 0.9996. Visual inspection indicated a strong linear relationship between concentration and

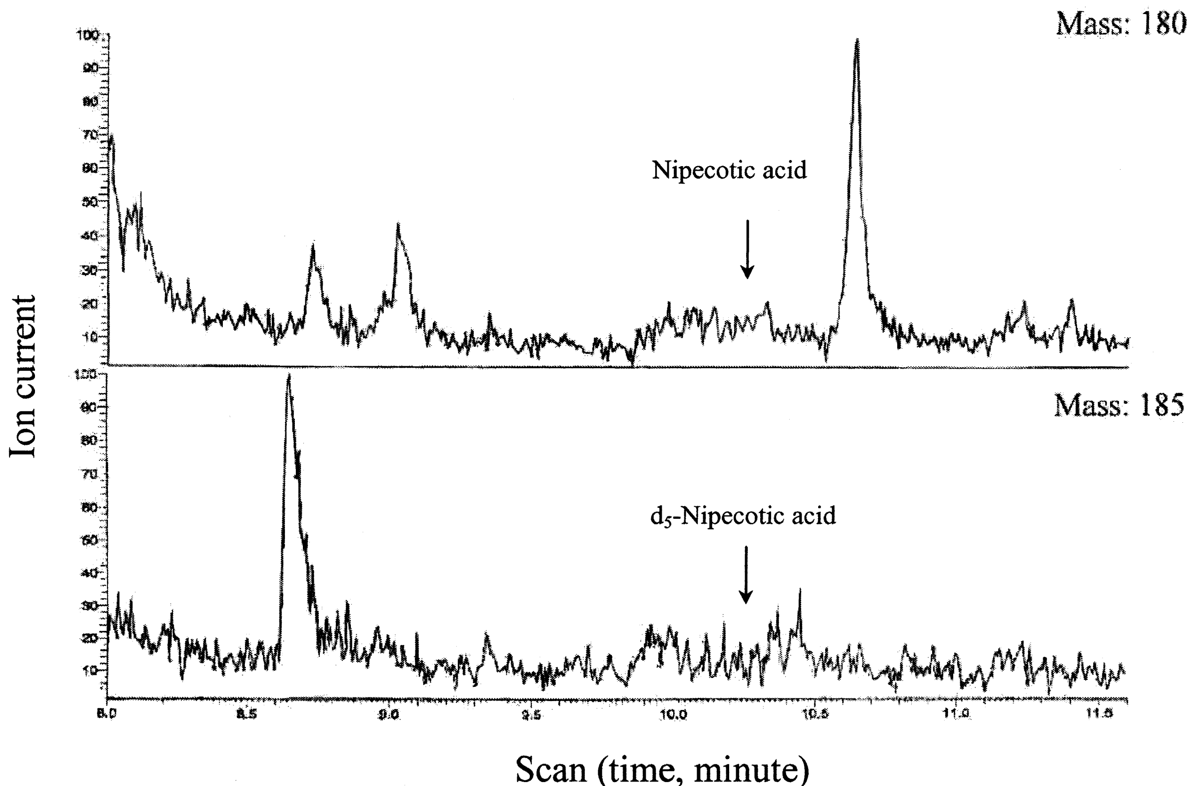

Fig. 2. Ion tracings from gas chromatograph at *m/z* 180 (upper trace) and *m/z* 185 (lower trace) for blank rat blood sample.

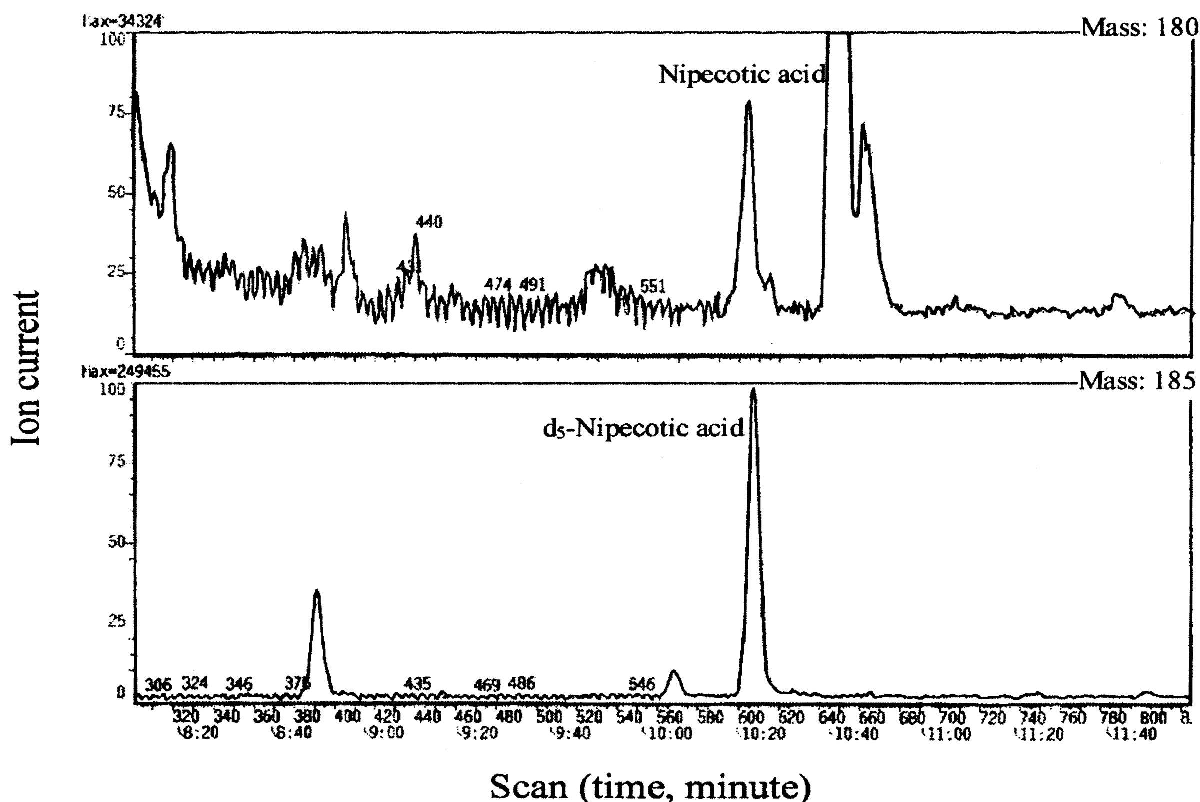

Fig. 3. Ion tracings from gas chromatograph at *m/z* 180 (upper trace) and *m/z* 185 (lower trace) for a rat blood sample containing 65 ng/ml of nipecotic acid and 430 ng/ml of d_5-nipecotic acid internal standard (retention time = 10.2 min).

the *m/z* 180/185 area ratio. A sample chromatogram indicates the nipecotic acid was easily detectable above the noise in a 200 μl whole blood sample containing 65 ng/ml of nipecotic acid (Fig. 2, blank blood, Fig. 3, blood containing nipecotic acid).

3.4. Limit of detection and quantification

The limit of detection (LOD) (S/N = 3) was estimated by verifying the observed noise at *m/z* 180 in whole blood sample and set at three times the background noise. The LOD for this assay was estimated to be 10 ng/ml.

The limit of quantitation (LOQ) was defined by the lowest concentration in the standard curve (26 ng/ml) that could be quantified with a coefficient of variation less than 20%. The lowest concentration used (26 ng/ml) had a coefficient of variation associated with it of 14%, and consequently this level was defined as the limit of quantification for the assay.

3.5. Intra- and inter-day assay variation

The intra- and inter-day variability in the assay of nipecotic acid from rat whole blood is provided in Table 1. Intra- and inter-day assay variation was measured at the three concentrations selected by the quality control samples (50, 1000 and 5000 ng/ml). Quality control samples were evaluated 10 times in 1 day (intra-day variation) and once at each concentration on 10 different days (inter-day variation). The inter-day shows a slightly greater variation than the intra-day variability, but the coefficient of variation at each concentration was less than 10%.

3.6. Application

The pharmacokinetics of a racemate is considered meaningless because it is a composite of two enantiomers. To avoid generating pharmacokinetic parameters of limited utility, the pure *R*-(-)-nipecotic

Table 1
Intra- and inter-assay variability of nipecotic acid GC–MS method ($n = 10$)

	Intra-day deviation			Inter-day deviation		
Theortical concentration (μg/ml)	0.05	1.0	5.0	0.05	1.0	5.0
Calculated concentration(μg/ml)	0.047	0.99	4.95	0.047	1.00	5.17
S.D.	0.003	0.01	0.11	0.004	0.06	0.26
Deviation (%)	6.3	1.01	2.2	8.7	6.0	5.0
Bias	−0.003	−0.01	−0.05	−0.003	0	+0.17

acid (the most potent enantiomer) was investigated in the rat since the assay is achiral and unable to distinguish *R*- from *S*-nipecotic acid. The blood levels of *R*-(-)-nipecotic acid were measured in one male Sprague–Dawley rat (Fig. 4). The levels of nipecotic acid were monitored for full 6 h after administration and used to generate the first detailed pharmacokinetic profile for this enantiomer in a rat. The area under the curve (AUC) was calculated to be 2.39 mg h/l and the volume of distribution at steady state (V_{ss}) was 325 ml. The terminal half-life of nipecotic acid was 1.5 h. The clearance of nipecotic acid was 4.25 ml/min.

4. Discussion

This assay is the most sensitive and specific assay for nipecotic acid reported to date. The use of a stable isotope of nipecotic acid as the internal standard proved essential to improve the precision and reproducibility of the assay. Isonipecotic acid (an isomer of nipecotic acid) was tried as an internal standard, but proved to be unreliable due to significant differences in its recovery from extracted blood samples (≤20%) relative to nipecotic acid (≈50–60%). A two-step process for making a derivative of nipecotic acid following its extraction from whole blood samples was also critical. Initial efforts indicated a simple BSTFA derivative of nipecotic acid was not sufficiently stable to produce good quantitative measurements of nipecotic acid. Unless the nitrogen group was masked with a more stable functional group before the carboxylic acid was reacted with BSTFA, the final product underwent a re-arrangement at room temperature that produced two different products over time, with different chromatographic characteristics.

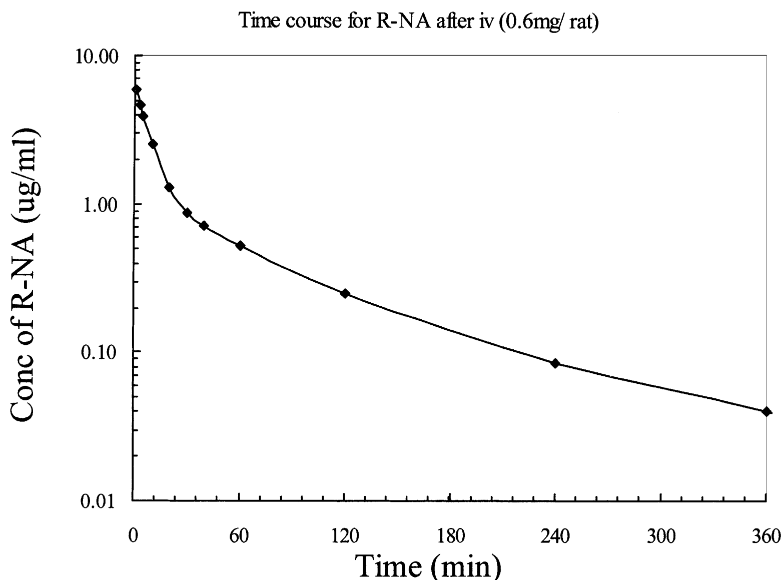

Fig. 4. *R*-(-)-nipecotic acid blood levels after intravenous administration of 0.6 mg of *R*-(-)-nipecotic acid to a 250 g male Sprague–Dawley rat.

The assay has been applied to other samples (brain tissue, not shown) and found to provide an accurate quantification of nipecotic acid in this tissue with no problems from background noise. This represents the first time *R*-(-)-nipecotic acid has been successfully monitored in small biological samples and its pharmacokinetics determined in rat blood. This assay will facilitate efforts to assess the advantages and disadvantages of various nipectoic acid prodrugs as vehicles for delivery of nipectoic acid to the brain.

Acknowledgements

This manuscript is dedicated to Dr. Jan St. Pyrek, who contributed greatly to our efforts to develop this assay for nipecotic acid. The untimely death of Dr. Pyrek has left a void that will not be easily filled. As a token to the unwavering demand Dr. Pyrek expressed for excellence in MS analytical methods, and his expert advice and help in this work the authors hereby dedicate this manuscript in his remembrance.

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