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Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 429–441

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Development of a sensitive bioanalytical method for determination of PNU-83757 in rat, monkey and human plasma: from LC-UV to LC-MS/MS

M.G. Williams, E.M. Shobe, B.E. Bothwell, W.Z. Zhong *

Pharmacokinetics and Bioanalytical Research, Pharmacia, 301 Henrietta Street, Kalamazoo, MI 49007-4940, USA

Received 18 May 2001; received in revised form 12 February 2002; accepted 1 April 2002

Abstract

To support pre-clinical pharmacokinetic/toxicokinetic (PK/TK) evaluation, a sensitive bioanalytical method for determination of *N*-cyano-*N*′-(*tert*-pentyl)-*N*″-(3-pyridinyl) guanidine (PNU-83757), in rat and monkey plasma was required. Although the UV response of PNU-83757 was quite decent and the extracts using solid phase extraction (SPE) were very selective and concentrated, the best limit of quantitation (LOQ) achieved was 0.4 ng ml^{-1} using 0.5 ml plasma for extraction and 2 ng ml^{-1} using 0.1 ml plasma for extraction, which was insufficient for PK/TK evaluation at lower doses. When using liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometric detection (LC-APCI-MS/MS, positive ions) and SPE, a LOQ of 0.045 ng ml^{-1} for PNU-83757 was reached. Quantitation was accomplished using the precursor → product ion combinations of m/z 232 → 162 for PNU-83757 and m/z 236 → 166 for the internal standard, [$^2H_4$]PNU-83757, in the multiple reaction monitoring mode. This method has been successfully utilized for PK/TK evaluation in pre-clinical studies and proved to have sufficient sensitivity to determine plasma concentrations for a dose level as low as 1 μg kg^{-1} day^{-1} in the rat and monkey. Further improvement of this method by using electrospray mass spectrometric detection (LC–ESI–MS/MS, positive ions) and automated membrane SPE, gave an LOQ of 0.008 ng ml^{-1}, and allowed analysis of large numbers of samples to support clinical PK studies in μg dose levels.

Keywords: Bioanalytical method; PNU-83757; Erectile dysfunction; LC-APCI-MS/MS; LC-ESI-MS/MS; Solid-phase extraction; Automation

1. Introduction

PNU-83757, *N*-cyano-*N*′-(*tert*-pentyl)-*N*″-(3-pyridinyl) guanidine (Fig. 1), is a potassium channel opener being developed as a second generation injectable for erectile dysfunction and is currently under clinical evaluation [1]. To support pre-clinical pharmacokinetic (PK) and toxicokinetic (TK)

* Corresponding author. Tel.: +1-616-833-1123; fax: +1-616-833-7788
E-mail address: wei-zhu.zhong@pharmacia.com (W.Z. Zhong).

0731-7085/02/$ - see front matter
PII: S0731-7085(02)00221-2

evaluation, a bioanalytical method was developed initially using a commonly available high performance liquid chromatography system with ultraviolet detection (HPLC-UV), feasible due to the decent UV absorbance of PNU-83757 at 260 nm. To maximize the assay sensitivity of the HPLC-UV method, a selective and concentrated extract was necessary from the sample preparation. By using solid phase extraction (SPE), and evaporation and reconstitution of the extracts for HPLC-UV analysis, a limit of quantitation (LOQ) of 0.4 ng ml^{-1} using 0.5 ml plasma for extraction and 2 ng ml^{-1} using 0.1 ml plasma for extraction could be reached depending on the plasma sample size available. This method was extensively used to support the preclinical PK/TK evaluation.

With the progress of PNU-83757 development, a bioanalytical method with a better sensitivity was required to support the toxicology studies at doses as low as 1 μg kg^{-1} day^{-1} in the rat and monkey. The expected maximum plasma concentrations of PNU-83757 in the rat and monkey following a 1 μg kg^{-1} day^{-1} intravenous administration would be in the range of 0.5–1 ng ml^{-1}. The sensitivity of the HPLC-UV method was apparently insufficient for these low dose TK studies, particularly for the rat study due to the limited available plasma size for sample preparation. The LOQ levels reported in the literature using HPLC with UV or electrochemical detection for determination of pinacidil, an analog of PNU-83757, in plasma were all in ng ml^{-1} range [2–4]. Since the extraction method for the HPLC-UV method was already optimized, the sensitivity could only be improved by using alternative detection methods. In addition, a major systemic circulating metabolite of PNU-83757 was identified to be PNU-95931 (unpublished data). Characterization of PK and TK of this metabolite in animals was also required for full understanding of the results from toxicology studies. Therefore, alternative detection methods for both the parent and metabolite were widely evaluated. Based on the chemical structure of PNU-83757, the protonation of *N*-cyano-*N*′-(3-pyridinyl)guanidine could be formed with subsequent cleavage of N–C bond in the *N*′-*tert*-pentyl moiety. Therefore, the tandem mass spectrometric detection appeared to be quite promising for lowering the LOQ. Still using the SPE method for obtaining a clean and concentrated extract, a liquid chromatographic method with atmospheric pressure chemical ionization tandem mass spectrometric detection (LC-APCI-MS/MS) for simultaneous quantitation of both PNU-83757 and PNU-95931 was developed. This method achieved an LOQ of 0.045 ng ml^{-1} for PNU-83757 and 0.09 ng ml^{-1} for PNU-95931. The LC-APCI-MS/MS method has been successfully utilized for PK/TK evaluation in pre-clinical studies and proved to have sufficient sensitivity to determine plasma concentrations for the dose level as low as 1 μg kg^{-1} day^{-1}.

Although the chromatographic run time for the LC-APCI-MS/MS method was only 5 min per sample, time efficiency was lost in the labor intensive and time consuming SPE preparation, evaporation, reconstitution and sample transferring.

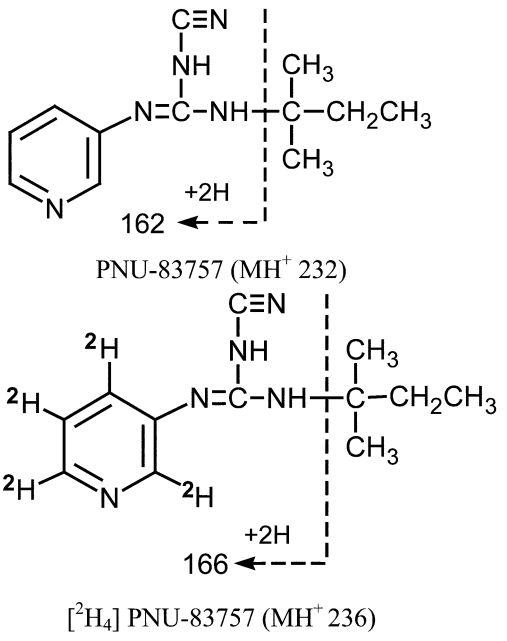

Fig. 1. Chemical structures of PNU-83757, and [$^2H_4$]PNU-83757 plus postulated structures of the product ion.

Manual preparation of 100 plasma samples using SPE required at least 6 h. Particularly, due to the sensitive nature of this assay, cross contamination during sample preparation can significantly bias results. Extreme precaution was taken during SPE and extracts evaporation. Additional clean up steps were also required to eliminate any cross contamination. Therefore, it was difficult to prepare large numbers of samples using this method for human studies. Furthermore, the dosage for clinical trials was quite low, with plasma concentrations of PNU-83757 expected to be considerably less than 0.1 ng ml^{-1}. Development of an automated SPE sample preparation method with improved assay sensitivity became most critical for supporting clinical PK evaluation. Automated SPE sample preparations for bioanalytical analyses have used robotics to process individual SPE columns [5–7] and more recently have used the 96-well plate in combination with mass spectrometric detection [8–10]. After further evaluation of LC–MS/MS methods and SPE automation, we developed a liquid chromatographic method with electrospray mass spectrometric detection (LC-ESI-MS/MS) and automated SPE for sample preparation using the Packard MultiPROBE system. This method was able to quantitate the PNU-83757 plasma concentration as low as 0.008 ng ml^{-1}, sufficient to support the clinical PK evaluation. In this report, liquid chromatographic methods with UV, APCI-MS/MS, or ESI–MS/MS detection are described. The strategy in the method development and SPE automation is also discussed in this paper.

2. Experimental

2.1. Chemical and reagents

PNU-83757 (Fig. 1) and internal standards (I.S.), PNU-80521 (for HPLC-UV) and [$^2H_4$]PNU-83757 (Fig. 1) (for LC-APCI-MS/MS and LC-ESI-MS/MS), were provided by Medicinal Chemistry of Pharmacia & Upjohn (Kalamazoo, MI). HPLC grade methanol and acetonitrile were obtained from EM Science (Gibbstown, NJ). Monobasic and dibasic potassium phosphate, ammonium acetate, and ammonium hydroxide were of analytical reagent grade and purchased from Mallinckrodt Inc (Paris, KY). Trifluoroacetic acid (TFA) was purchased from Aldrich Chemical Co, Inc (Milwaukee, WI). Purified water was produced by a Milli-Q reagent water system (Millipore Corp, Bedford, MA).

2.2. Preparation of standards and quality control samples

Stock standard solution was prepared by accurately weighing 1 mg of PNU-83757 into a 10-ml volumetric flask containing 2 ml of methanol. After dissolving the compound in the methanol, deionized water was added to volume to yield a stock standard solution containing 100 μg ml^{-1} of PNU-83757. The stock standard solution was diluted with water to give working standard solutions with appropriate concentrations of PNU-83757. The I.S. solution of PNU-80521 was prepared by weighing 0.1 mg of PNU-80521 into a 100-ml volumetric flask, dissolving the compound with 50 ml of methanol, and diluting to volume with water to yield a 1000 ng ml^{-1} I.S. working solution. The I.S. solution of [$^2H_4$]PNU-83757 for the LC-APCI-MS/MS method was prepared by weighing 0.2 mg of [$^2H_4$]PNU-83757 into a 100-ml volumetric flask and dissolving the compound with methanol to volume to yield a 2000 ng ml^{-1} I.S. working solution. The I.S. solution of [$^2H_4$]PNU-83757 for the LC–ESI–MS/MS method was prepared by weighing 0.1 mg of [$^2H_4$]PNU-83757 into a 100-ml volumetric flask and dissolving the compound with water to volume to yield a 1000 ng ml^{-1} I.S. stock solution. The stock solution was further diluted with water to yield a 0.5 ng ml^{-1} I.S. working solution. The stock and working standard solutions and the I.S. stock and working solutions were stored under refrigeration, at or below 4 °C.

Plasma standards were prepared by aliquoting 10 μl of appropriate working standard solution into 0.1 ml rat plasma, 0.5 ml monkey plasma, or 0.5 ml human plasma in a 6 ml polypropylene centrifuge tube. This provided a calibration standard series for the concentration range as indicated in Table 1 for each method and matrix.

Table 1
Calibration curve data for PNU-83757

Method	Assay number		Rat plasma[a]	Monkey plasma[b]	Human plasma[b]
HPLC-UV	1	Linearity	$Y = 0.01124x$	$Y = 0.04832x$	
		r^2	0.9996	0.9998	
	2	Linearity	$Y = 0.01065x$	$Y = 0.0534x$	
		r^2	0.9993	0.9979	
	3	Linearity	$Y = 0.01075x$	$Y = 0.0471x$	
		r^2	0.9991	0.9997	
	Linear range (ng ml^{-1})		2–500	0.4–100	
	LOQ (ng ml^{-1})		2	0.4	
LC-APCI-MS/MS	1	Linearity[c]	$y = 1.068x + 0.030$	$y = 1.558x + 0.020$	
		r^2	0.9998	0.9997	
	2	Linearity[c]	$y = 1.129x + 0.010$	$y = 1.175x + 0.008$	
		r^2	0.9998	0.9998	
	3	Linearity[c]	$y = 1.258x - 0.020$	$y = 1.488x + 0.025$	
		r^2	0.9994	0.9999	
	Linear range (ng ml^{-1})		0.1–1500	0.045–500	
	LOQ (ng ml^{-1})		0.1	0.045	
LC–ESI–MS/MS	1	Linearity[d]			$Y = 1.072x + 3.6$
		r^2			0.9970
	2	Linearity[d]			$Y = 0.840x + 0.7$
		r^2			0.9975
	3	Linearity[d]			$Y = 0.938x + 2.5$
		r^2			0.9967
	Linear range (ng ml^{-1})				0.0078–1
	LOQ (ng ml^{-1})				0.0078

[a] Validated using 0.1 ml plasma.
[b] Validated using 0.5 ml plasma.
[c] 1/concentration weighting was used for the linear fit.
[d] 1/concentration^2 weighting was used for the linear fit.

Quality control (QC) samples were prepared by aliquoting the standards (from separate weighing) to blank plasma to produce concentration pools as listed in Table 2 for each method and matrix. QC samples were stored frozen at -10 °C or colder.

2.3. Plasma sample preparation

2.3.1. SPE for HPLC-UV and LC-APCI-MS/MS (rat and monkey plasma)

The SPE was operated manually using a vacuum manifold (Supelco, Bellefonte, PA). The plasma standards, QC samples and unknown plasma samples (0.1 ml for rat plasma and 0.5 ml for monkey plasma) were mixed with 10 μl I.S. working solution (1000 ng ml^{-1} of PNU-80521 for the HPLC-UV method and 2000 ng ml^{-1} of [$^2H_4$]PNU-83757 for the LC-APCI-MS/MS method) and 0.5 ml water. If the plasma sample size was less than 0.5 ml, additional 0.1 M KH_2PO_4 solution was added to make a total sample volume equal to 0.5 ml. The mixture was loaded on C18 SPE columns (100 mg 1.0 ml^{-1}, Varian, Harbor City, CA), which had been preconditioned with 1 column volume of methanol followed by 1 column volume of water under vacuum aspiration ($\approx$86 kPa). The SPE columns were then washed consecutively with 1 ml each of 30% (v/v) methanol in water, 0.1 M KH_2PO_4, and water. After the SPE columns were dried with vacuum aspiration ($\approx$27 kPa) for 10 min, the compounds of interest were eluted with 0.5 ml methanol into 6 ml polypropylene tubes under

slight vacuum aspiration. The extracts were taken to dryness in a Zymark Turbo-Vap evaporator (Hopkinton, MA) at 40 °C under nitrogen. The residues were reconstituted in 0.2 ml of 0.1% TFA in acetonitrile–water (20:80, v/v) and 150 μl of extract was injected for HPLC-UV analysis. The residues were reconstituted in 0.1 ml of freshly prepared 0.6% TFA in methanol and 20 μl of the extract was injected when using LC-APCI-MS/MS analysis.

2.3.2. Automated SPE for LC-ESI-MS/MS (human plasma samples)

The Packard MultiPROBE (Packard, Downers Grove, IL) grid was set up with a rack to hold 12×75 cm^2 culture tubes and a rack for the 96-well plate manifold. Five troughs were set up with the following reagents: the working I.S. solution, methanol, 30% methanol in water, and 0.1 M KH_2PO_4 buffer solution. Approximately 1 ml of plasma standards, QC samples and unknown plasma samples were placed in 12×75 mm culture tubes in the sample rack. A C18 SD Empore (3M, ST. Paul, MN) 96-well SPE disc plate was placed in the manifold. Deionized water was used as the system liquid and was checked for sufficient volume prior to each run. The MultiPROBE was programmed to perform the following steps: the disc plate was preconditioned with 0.5 ml methanol followed by 0.5 ml water through vacuum aspiration; before the disc plate dried, a 0.5 ml plasma sample with 0.5 ml of the I.S. solution (0.5 ng ml^{-1} of [$^2H_4$]PNU-83757) was loaded into the appropriate SPE wells; after the plasma/I.S. solution was completely through the disc plate under vacuum aspiration, a 0.25 ml of the 30% methanol in water followed by 0.25 ml of the 0.1 M KH_2PO_4 buffer solution was applied to wash each SPE well; after the disc plate was dry for 5 min by aspiration, the 96-well collection plate with 0.3 ml capacity of each well was placed on the disc plate; 50 μl of methanol was loaded into each SPE well and was allowed to sit for 2 min before adding 0.2 ml of water; the methanol/water

Table 2
Intra- and inter-assay accuracy and precision for determination of PNU-83757 in rat, monkey, or human plasma

Method	Matrix	Added (ng ml^{-1})	Intra-assay ($n \geqslant 3$)			Inter-assay ($n \geqslant 9$)		
			Mean (ng ml^{-1})	CV (%)	Bias (%)	Mean (ng ml^{-1})	CV (%)	Bias (%)
HPLC-UV	Rat	10	10.8	13	8	10.8	10	8
	Plasma	20	18.6	9	−7	19.3	8	−3
		200	224	2.2	12.1	223	2.3	11
	Monkey	3	3.48	4	15	3.28	6	9
	Plasma	30	33.5	1.7	11.5	32.2	6	7
		90	96.1	1.3	6.8	96	6	7
LC-APCI-MS/MS	Rat	0.580	0.580	3	0	0.590	12	2
	Plasma	11.6	11.4	3	−2	11.4	8	−2
		1160	1170	7	1	1170	6	1
	Monkey	0.116	0.119	12	3	0.115	12	−1
	Plasma	2.32	2.53	6	9	2.47	7	7
		232	237	3	2	234	4	1
LC-ESI-MS/MS	Human	0.015	0.0143	7	2	0.0146	10	−3
	Plasma	0.030	0.0315	7	5	0.0292	8	3
		0.300	0.310	4	3	0.289	8	4
		0.900	0.962	1.7	6.9	0.910	5	1

was then drawn into each corresponding collection well by slight vacuum aspiration and the collection plate was covered with an adhesive plastic sheet that could be pierced by an autosampler needle. The 96-well plate was then transferred to the autosampler and a 150 μl aliquot was injected for LC-ESI-MS/MS analysis.

2.4. Instrumentation

HPLC-UV—The HPLC system employed for the HPLC-UV method consisted of a Perkin–Elmer Series 200 LC pump, a Perkin–Elmer Series 200 autosampler (Norwalk, CT) and a Waters 486 UV detector (Milford, MA). Chromatographic separation was performed on a Zorbax SB-phenyl reversed-phase column (250 × 4.6 mm ID, 5 μm particle size, Mac-Mod Analytical, Inc, Chadds Ford, PA) with a mobile phase of acetonitrile-0.1% TFA in water (20:80, v/v) delivered at 1 ml min^{-1}. The UV absorbance of the column effluent was monitored at 260 nm. An analog of PNU-83757, PNU-80521, was used as the I.S.

LC-APCI-MS/MS—A Hewlett Packard Series 1050 HPLC system was equipped with a Hewlett Packard Series 1050 autosampler (Avondale, PA) for sample injection and a Finigan MAT TSQ 700 triple quadrupole mass spectrometer (San Jose, CA) for detection. The Zorbax 300 SCX column (150 × 4.6 mm ID, 5 μm particle size, Mac-Mod Analytical, Inc, Chadds Ford, PA) was used for the chromatographic separation. The isocratic mobile phase consisted of 0.3% TFA in water (pH adjusted to 3.0 with ammonium hydroxide)–methanol–acetonitrile (40:30:30, v/v/v) and was delivered at 1.5 ml min^{-1}. The quantitation was accomplished using the positive ion APCI mode with selected reaction monitoring for the transitions m/z 232 (MH^+:PNU-83757) → m/z 162 for PNU-83757 and m/z 236 (MH^+:[$^2H_4$]PNU-83757) → m/z 166 for the I.S. The APCI interface was operated with a vaporizer temperature of 400 °C and capillary temperature of 250 °C. Resolution on Q1 and Q3 was less than unit to achieve higher sensitivity. The scan time was 0.25 s for PNU-83757 and I.S. The collision gas was Argon (99.999% pure, AGA, Maumee, OH) with a collision cell pressure of 2.0 mtorr with respect to manifold.

LC–ESI–MS/MS—The LC–MS/MS system consisted of a Perkin Elmer Series 200 pump, a Perkin Elmer Series 200 autosampler, and a Finigan MAT TSQ 700 triple quadrupole mass spectrometer. The prepared samples were chromatographed on a Waters Symmertry C-8 column (150 × 2.1 mm ID, 5 μm particle size, Norwalk, CT) with a mobile phase of 30:30:40 (v/v/v) acetonitrile–methanol – 0.3% TFA in water (pH adjusted to 3.0 with ammonium hydroxide) delivered at a flow rate of 0.25 ml min^{-1}. The quantitation was accomplished using the positive ion ESI mode utilizing selected reaction monitoring for the transitions m/z 232 (MH^+:PNU-83757) → m/z 162 for PNU-83757 and m/z 236 MH^+:[$^2H_4$]PNU-83757) → m/z 166 for the I.S. The precursor and product resolution windows was slightly open (less than unit) with a scan time of 0.3 s. for PNU-83757 and I.S. The collision gas was Argon (99.999% pure, AGA, Maumee, OH) with a collision cell pressure of 2.0 mtorr with respect to manifold. The electrometer gain was 8 and the capillary temperature was 260 °C.

2.5. Quantitation

Quantitation for the HPLC-UV method was accomplished by peak height ratio of the drug to I.S. using an UPACS software on an Harris Nighthawk Computer System. The calibration curve was computed by forced through-the-origin linear regression with no weighting. Data generated from the Finnigan MAT TSQ-700 mass spectrometry were exported to the UPACS Computer System and were analyzed by generating calibration curves of peak area ratios of PNU-83757 to the I.S. versus theoretical concentrations using linear fit with intercept and either 1/concentration weighting or 1/concentration^2 for the LC-APCI-MS/MS or LC–ESI–MS/MS methods, respectively. Unknown and QC sample concentrations are determined by inverse prediction against the calibration curve.

2.6. Validation experiments

The methods were validated in rat, monkey, and human plasma using the validation approach

as described by Shah et al. [11] and Wieling et al. [12]. The linear range, LOQ, accuracy, and precision of the assay were determined from standard curves prepared fresh on at least three occasions along with the low, medium, and high QC samples assayed in triplicate. The intra- and inter-assay precision was determined from the coefficient of variation (CV) of QC sample concentrations analyzed within-run and between-run, respectively. The assay accuracy was evaluated by comparing the known concentrations with those measured against the calibration curve (bias). The LOQ was estimated from the lowest concentration standard in the calibration curve with acceptable accuracy and precision (CV or bias $\leqslant 20\%$).

3. Results

3.1. Linearity

The linearity of the three methods was established for the concentration range as indicated in Table 1 for each matrix. Analysis of variance indicated that the linear model for the HPLC-UV method was observed for PNU-83757 up to 500 ng ml^{-1} with correlation coefficients greater than 0.999 in all cases during the validation exercise when using the forced through-the-origin linear regression model without weighting. The LC-APCI-MS/MS method had a linear range up to 1500 ng ml^{-1}. The best fit linear model with a weighting factor of 1/concentration was most appropriate for establishing the relationship between the concentration and the response with correlation coefficients greater than 0.999. The linearity of the LC–ESI–MS/MS method was observed up to 1 ng ml^{-1} with correlation coefficients greater than 0.996 when using the best fit linear model with a weighting factor of 1/concentration^2.

3.2. Sensitivity and specificity

The LOQ was reached as low as 0.4 ng ml^{-1} for the HPLC-UV method when using 0.5 ml plasma for extraction, indicating excellent sensitivity for an HPLC-UV method. A 10-fold improvement in detection limit was observed for the LC-APCI-MS/MS method with a LOQ of 0.045 ng ml^{-1} when using 0.5 ml plasma for extraction. The LC–ESI–MS/MS method was most sensitive, which had a LOQ of 0.0078 ng ml^{-1}. No interference was found either in extracted rat, monkey, or human blank plasma from several plasma pools using each method, showing the excellent selectivity of these methods combined with SPE techniques. The precision and accuracy were $< 20\%$ at the LOQ level during the validation exercises. However, precaution in sample preparation must be taken to avoid any contamination or carry over owing to the low detectable levels of these methods. Representative chromatograms for each method are shown in Figs. 2–4.

3.3. Precision and accuracy

The intra- and inter-assay precision (CV) and accuracy (Bias) expressed by the QC data from each validation exercise for each method and matrix are summarized in Table 2. Both CV and Bias were $\leqslant 15\%$, $\leqslant 12\%$, and $\leqslant 10\%$ in all cases as can be concluded from Table 2, for the HPLC-UV, LC-APCI-MS/MS, and LC-ESI-MS/MS methods, respectively. Several analytical columns were also compared for each method during the validation exercise and no apparent differences were observed in the chromatographic responses, slopes, or QC results.

3.4. Stability

Deionized water solutions of PNU-83757 and the methanol solution of [$^2H_4$]PNU-83757 were stable for a minimum of 8 weeks when stored at room temperature in the dark. There was no evidence of instability of the prepared samples sitting in an autosampler tray at room temperature for 8 h or in a temperature controlled autosampler tray (at 4 °C) for 24 h before injection. PNU-83757 in plasma samples stored frozen at or below -10 °C was stable for a minimum of 4 weeks.

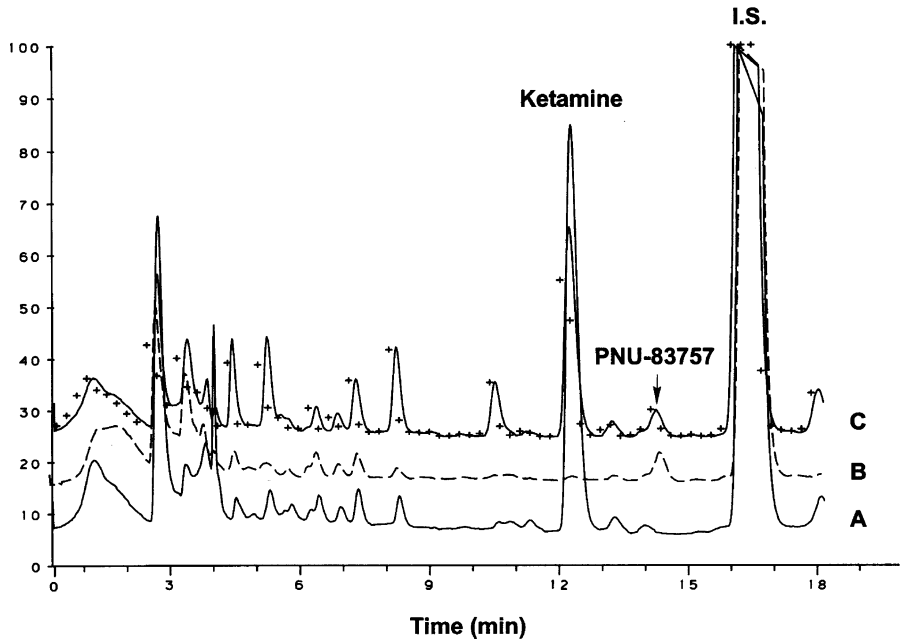

Fig. 2. Representative chromatograms using HPLC-UV: (A) pre-dose monkey plasma (ketamine was administered prior to blood sampling), (B) a fortified monkey plasma standard at 12.5 ng ml^{-1}, and (C) a 15 min plasma sample from a monkey dosed at 5.3 mg kg^{-1} intracavernously.

3.5. Application

The performance and ruggedness of these methods were tested by analyzing more than 1000 plasma samples from pre-clinical PK and toxicity studies in rats and monkeys and in clinical trials. The sensitivity of the LC-MS/MS methods was demonstrated to be sufficient for evaluation of TK of PNU-83757 in rat and monkeys administered PNU-83757 intravenously at 1 μg kg^{-1} day^{-1} and in humans administered PNU-83757 intracavernously at a low dose of 5 μg per subject. As examples, plasma concentration-time profiles of PNU-83757 in the monkey after a single 8 mg kg^{-1} intravenous and 5 mg kg^{-1} intracavernous administration determined using the HPLC-UV method are presented in Fig. 5; plasma concentration-time profiles of PNU-83757 and PNU-95931 determined using the LC-APCI-MS/MS method for the rat and monkey administered 1 μg kg^{-1} day^{-1} PNU-83757 intravenously for 28 days are shown in Fig. 6; and plasma concentration-time profiles of PNU-83757 determined using the LC–ESI–MS/MS method for patients administered 5 μg PNU-83757 intracavernously are presented in Fig. 7.

4. Discussion

4.1. Chromatography

The HPLC-UV method utilized a reversed-phase analytical column (Zorbax SB-phenyl, 250 × 4.6 mm I.D., 5 μm particle size) with a mobile phase of acetonitrile—0.1% TFA in water (20:80, v/v). Under this condition, PNU-83757 and PNU-95931 were eluted at approximately 14 and 4 min, respectively. Owing to the interference of plasma endogenous components at the retention time of PNU-95931, quantitation of this metabolite was impossible under this condition. It was desirable to shorten the retention times and retain good separation and peak shape for both PNU-83757 and PNU-95931. Attempts resulted in the plasma components completely masking the

PNU-83757 and the metabolite signals when UV detection was used with faster chromatographic elution. Since PNU-83757 is a basic compound with two pK_a's; 3.5 for the pyridinyl nitrogen and 11.5 for the guanidino nitrogen, a cation ion-exchange HPLC column, Zorbax 300 SCX (4.6 × 150 mm, 5 μm particle size), was the first choice of alternative column for testing. With a mobile phase of 0.3% TFA in water (pH adjusted to 3.0)-methanol-acetonitrile (40:30:30, v/v/v), the cation ion-exchange chromatography provided sharp peaks of PNU-83757 and PNU-95931 with a retention time of 4.4 and 1.2 min, respectively. By using the ion-exchange column, each chromatographic run time was reduced to 5 min and the detection responses were also correspondingly increased due to the sharp peaks of the analytes. With UV detection, there were too many interfering peaks from the plasma to use this column. With the selectivity of the LC-APCI-MS/MS method, however, this column and mobile phase were used successfully.

Although the LC–ESI–MS/MS detection response was higher than the LC-APCI-MS/MS, the required assay sensitivity for supporting clinical PK studies also needed injection of a greater percentage of the prepared sample to the LC-MS/MS system. Broader peak shape for the analytes and I.S. was observed when injecting more than 20 μl of the prepared sample to the ion-exchange column. After evaluating a variety of columns, the Waters Symmetry C8 column (2.1 × 150 mm, 5 μm particle size) was the best choice to provide sharp PNU-83757 and I.S. peaks within 4 min with an injection volume as large as 150 μl. Combining this chromatographic system with the ESI–MS/MS detection, the assay sensitivity was increased at least 5-fold further as compared to the LC-APCI-MS/MS.

4.2. Sample preparation

Sample preparation using protein precipitation was not appropriate for the HPLC-UV method due to the interference of plasma endogenous components. It was also not suitable for LC–MS/MS due to severe matrix suppression. Therefore, the SPE method used for the LC-APCI-MS/MS

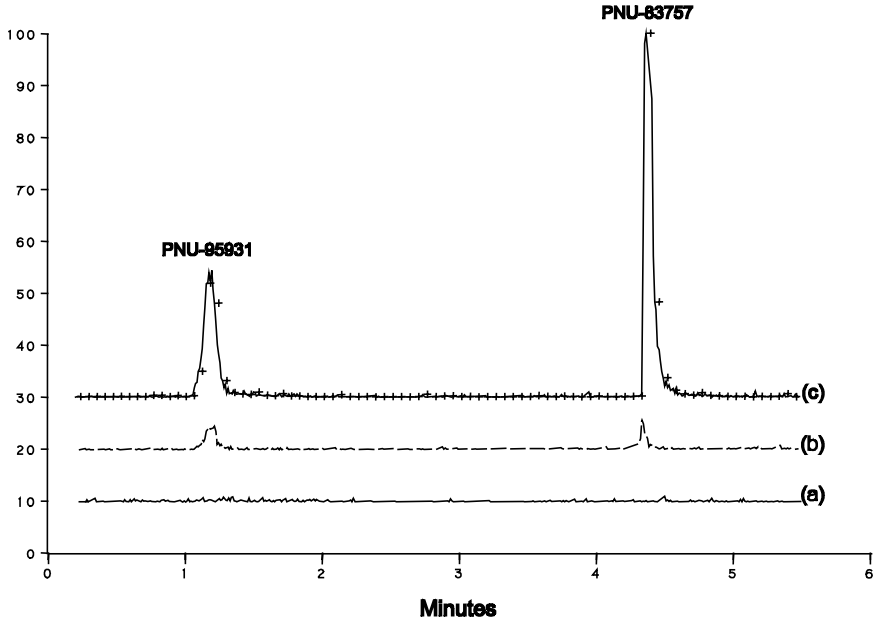

Fig. 3. Representative chromatograms using LC-APCI-MS/MS: (a) a predose monkey plasma sample, (b) a fortified plasma standard containing 0.088 ng ml^{-1} PNU-83757 and 0.197 ng ml^{-1} of PNU-95931, and (c) a 0.25 h post-dose monkey plasma sample for PNU-83757 and PNU-95931.

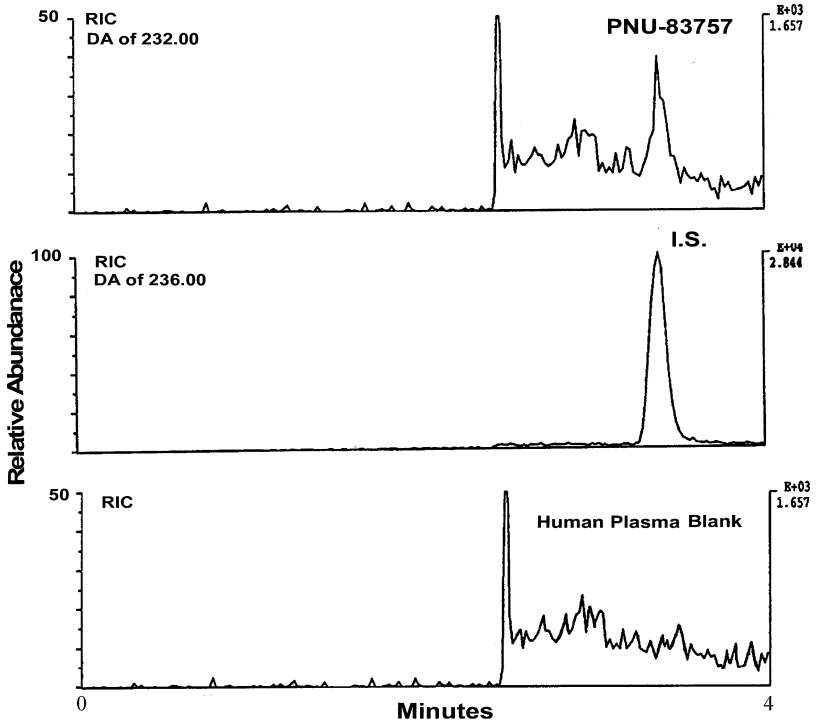

Fig. 4. Representative chromatograms in human plasma using LC–ESI–MS/MS: (A) 8 pg ml^{-1} PNU-83757 plasma standard, (B) a 5 ng ml^{-1} I.S. ([$^2H_4$]PNU-83757) and (C) a human plasma blank.

assays was adapted from the HPLC-UV method with minor modification. Based on the validation results of the HPLC-UV method, the absolute extraction recovery for PNU-83757 averaged 90% by comparison of the peak area of the analyte after extraction with those of unextracted corresponding standards. When using a plasma sample size less than 0.5 ml for extraction, the extraction recovery was greatly decreased, particularly for PNU-95931. It is likely due to reduced retention of the analytes on the SPE column and increased efficiency of column washing step using 30% methanol. With mixing additional 0.1 M KH_2PO_4 to the plasma samples before loading to the SPE column, the recovery increased to what had been achieved when using 0.5 ml plasma for extraction. This is probably attributable to the facilitation of the secondary interaction between the C18 sorbent and the analytes by the acidic buffer. The SPE column washing with 1 ml of each 30% (v/v) methanol in water, 0.1 M KH_2PO_4, and water after sample loading, facilitated the removal of most plasma endogenous components retained on the SPE column without elution of the analytes. A

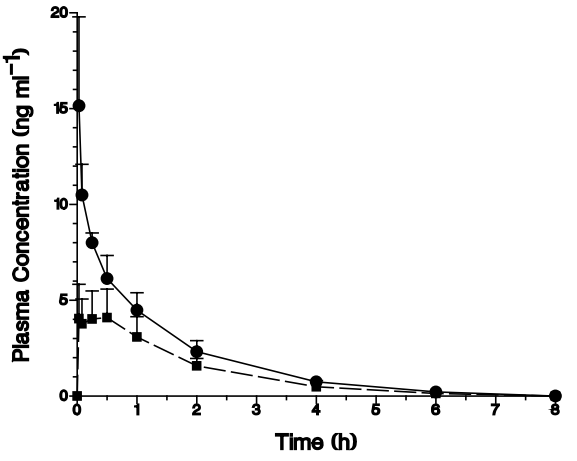

Fig. 5. Mean ($n = 4$) plasma concentration-time profiles for PNU-83757 in the monkey after 8 mg kg^{-1} intravenous (—●—) and 5 mg kg^{-1} intracavernous (---■---) administration.

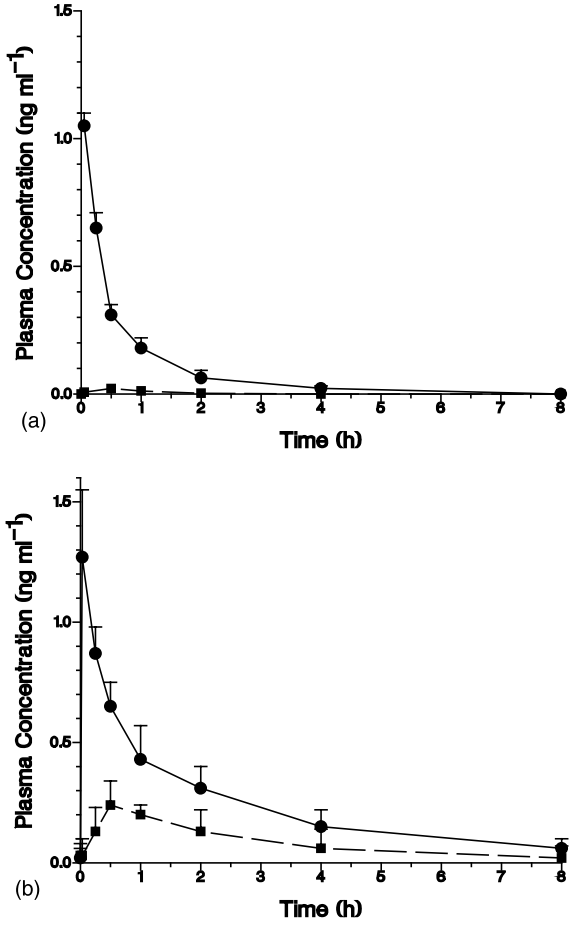

Fig. 6. Mean plasma concentration-time profiles of PNU-83757 (—●—) and PNU-95931 (---■---) on treatment day 28 for male rats (upper) and monkeys (lower) administered 1 μg kg^{-1} day^{-1} PNU-83757 intravenously for 28 days.

0.5 ml of methanol as the elution solvent was sufficient to recover 90% of the analytes from the SPE column. Due to the sensitive nature of this assay, cross contamination during sample preparation can significantly bias results. Precaution was taken during the sample preparation to avoid any cross contamination. By carefully washing each female luer receptacle and solvent guide needle on the SPE manifold cover with methanol before each extraction run, the contamination during SPE was found to be minimal. The evaporation using the Turbo-Vap was also found to be a source of contamination. This problem can be solved by lowering the pressure of the nitrogen gas to eliminate splattering and also by washing the gas nozzles with methanol between sample sets. With precautions in the sample preparation, the assay was demonstrated to be rugged as well as accurate, precise, and specific as shown in Table 2 and Fig. 3. However, this SPE procedure was quite labor intensive and time consuming. Preparation of 100 plasma samples typically required at least 6 h. Other extraction methods, although possible, either did not provide the needed selectivity that SPE afforded or were also labor intensive and time consuming. Confronted with large numbers of clinical samples, automation of the sample preparation procedure was considered to reduce the sample preparation time. Therefore, automated SPE method using 96-well plates for sample preparation was consequently developed for the clinical studies. The membrane SPE system using C18 SD Empore 96-well extraction disc plate was focused on due to their small bed volumes. This allowed the use of small solvent volumes for compound elution [13,14], thereby eliminating the evaporation and reconstitution steps to make the sample preparation completely automated. A 50 μl volume of methanol was able to elute PNU-83757 and I.S. from the solid phase. Adding an additional of 200 μl water enforced the complete elution of analytes from the SPE well to the collection plate, as well as limiting

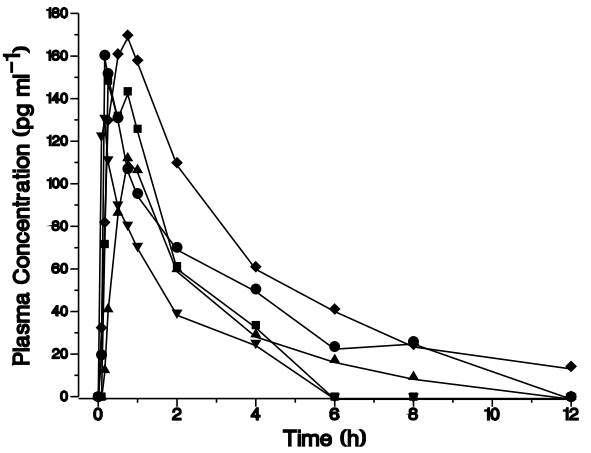

Fig. 7. Plasma concentration-time profiles of PNU-83757 for 5 patients administered 5 μg PNU-83757 intracavernously.

the strong solvent effect on the reversed phase chromatography. The 96-well format allowed automation using the Packard MultiPROBE work station and direct injection from the 96-well collection plates to the LC–MS/MS system, eliminating the need to transfer samples to autosampler vials. The automated SPE provided the necessary selectivity and concentrated extracts to make the quantitation of PNU-83757 possible at the plasma level as low as 7.8 pg ml^{-1}. However, the retention of PNU-95931 on the C-18 SD disc failed due to the polar nature of this metabolite. As a result, the quantitation of the LC–ESI–MS/MS method with automated sample preparation was restricted to the parent compound only.

4.3. LC-MS/MS

The positive ion APCI mass spectrum of PNU-83757 (molecular mass 231) revealed that the protonate molecule at m/z 232 was formed to the largest extent. Under MS/MS conditions, the m/z 232 ion was observed to fragment almost exclusively to a m/z 162 product ion, which is likely the protonated form of *N*-cyano-*N'*-(3-pyridinyl) guanidine resulting from the cleavage of N–C bond in the *N'-tert*-pentyl moiety as presented in Fig. 1. Similarly, for the stable isotope of PNU-83757 (MH^+ 236), the product ion at m/z 166 was found to be exclusive (Fig. 1). By monitoring the precursor → product ion combination at m/z 232 → 162 for PNU-83757 and m/z 236 → 166 for the I.S. in the multiple reaction monitoring mode using the conditions described previously, the detection for PNU-83757 was found to be quite sensitive and selective. As shown in Fig. 3, the extracted plasma blank had no interference peak at either the PNU-95931 or PNU-83757 position, although the elution of PNU-95931 was at 1 min, which might co-elute with early eluting plasma components. The fragmentation of PNU-83757 to a dominant product ion at m/z 162 proved favorable with the plasma matrix. In addition, the favorable ionization characteristics of PNU-83757 may also provide more complete protonation of PNU-83757 in the column effluent (pH 3), resulting in the excellent sensitivity of this method. The ESI mode showed similar mass spectrum for positive ion but had approximately 2-fold higher sensitivity for PNU-83757 over the APCI mode. Therefore, the ESI mode was selected for use in the assay to support clinical PK studies.

5. Conclusion

Method development from using HPLC-UV to LC-MS/MS resulted in a straightforward and highly sensitive LC-ESI-MS/MS method. This method combines the universality of liquid chromatographic separation with the sensitivity and selectivity of mass spectrometric detection as well as the high efficiency of the automated SPE technique for plasma sample preparation. This method was demonstrated to be most suitable for the assay of samples from clinical trials with PNU-83757 because of the sufficient sensitivity, robustness and minimal labor requirement. The described LC-APCI-MS/MS method was also demonstrated to be sensitive, selective, and reproducible. The method was able to quantitate both the parent compound and the metabolite and has quite large linear detection range, which appeared to be most suitable for pre-clinical PK/TK evaluation. The HPLC-UV method was simple and rugged and uses commonly available instrumentation. It has adequate sensitivity to provide initial PK and TK information for the early stages of drug development. All three methods were validated and can be used to support the preclinical and clinical studies depend on the assay sensitivity and PK information required.

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