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Ultrasensitive determination of NE-100, a novel sigma ligand, in human plasma by liquid chromatography and electrospray ionization tandem mass spectrometry combined with a column-switching technique

Jun-ichi Yamaguchi[a,*], Yumiko Watanabe[a], Mari Ohmichi[a], Shigeji Jingu[a], Naoyoshi Ogawa[a], Jun Kokatsu[a], Kiyomi Fukushima[a], Junichi Goto[b]

[a]*Drug Metabolism Laboratory, Pharmaceutical Research Laboratories, Taisho Pharmaceutical Co., Ltd., 403, Yoshino-cho 1-chome, Ohmiya-shi, Saitama 330-8530, Japan*

[b]*Faculty of Pharmaceutical Sciences, Tohoku University, Aobayama, Sendai, 980-8578, Japan*

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Abstract

For the highly sensitive and selective determination of NE-100, a novel sigma ligand, at levels of low picogram per milliliter of human plasma, a method with excellent reliability employing liquid chromatography (LC)–electrospray ionization (ESI) tandem mass spectrometry (MS–MS) combined with a column-switching technique has been developed. The method involves the use of a stable isotope labeled compound as the internal standard (I.S.), liquid–solid extraction of a plasma specimen with a C_8 cartridge, automated on-line clean-up on a short trapping column, subsequent separation on a micro-bore C_{18} column and detection with ESI-MS–MS using m/z 356 ($[M+H]^+$) as a precursor ion and m/z 105 as a product ion in a selected reaction monitoring mode. The detection and the quantification limits of NE-100 in plasma were 0.5 pg/ml with a signal-to-noise ratio (S/N) of 3 and 2.3 pg/ml, respectively, with an S/N of 21. The good linearity of the calibration graph was obtained in the range of 2.3~907.0 pg/ml with excellent reliability. The developed method was applied to the determination of NE-100 in plasma obtained from the clinical trail. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Electrospray ionization; NE-100; Sigma ligands

1. Introduction

The importance of the pharmacokinetic study for clarifying the pharmacological efficacy and toxicity of drugs in vivo has been emphasized [1]. For this purpose, a reliable method for the determination of drugs in biological fluids with sufficient sensitivity, specificity, accuracy, precision and reproducibility is essential. NE-100, *N,N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride (Fig. 1A), has been shown to be a selective sigma 1 receptor ligand, which may be a new therapeutic drug for schizophrenia [2–5], and due to the marked potency of NE-100 [2], an ultrasensitive bioanalytical method was required for metabolic and pharmacokinetic studies in clinical trails. Recently, a quantitative bioanalytical procedure involving the

*Corresponding author. Tel.: +81-48-663-1111; fax: +81-48-652-7254.

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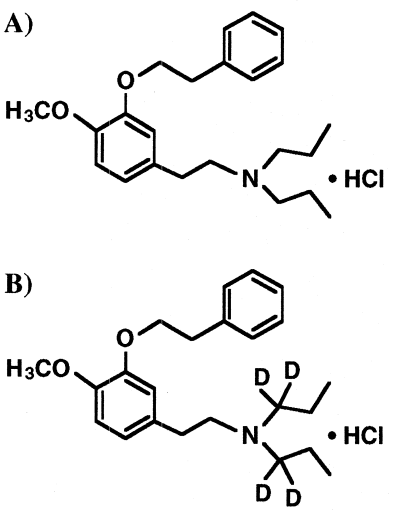


Fig. 1. Chemical structures of (A) NE-100 and (B) NE-100-D_4.

extraction of analytes in a biological matrix, and the liquid chromatographic separation with a sensitive detection system such as UV and fluorescence detectors has been widely employed. However, these methods were not suitable in sensitivity and selectivity for the determination of NE-100 in plasma.

In recent years, with the development of novel interfaces, such as thermospray ionization [6], atmospheric pressure chemical ionization [7] and electrospray ionization (ESI) [8,9], mass spectrometry (MS) hyphenated with liquid chromatography (LC) as a new approach for the separation and determination of trace compounds in biological fluids is now available. Among these interfaces, ESI has been considered to be a very useful tool for the highly sensitive determination of drugs and their metabolites having an ionic function in biological fluids. It is also known that the use of the hyphenated method of LC with tandem mass spectrometry (MS–MS) enhances selectivity to a significant extent [10]. However, even though LC–ESI-MS–MS is employed, the clean-up procedure, which becomes time consuming, is needed to avoid the ion suppression effect [11,12], which is responsible for lowering sensitivity due to coexisting materials. Moreover, for obtaining the effective formation of characteristic ions of target compounds in the ESI mode, a microbore or a narrow-bore column with a flow-rate of 20~200 μl/min has usually been used [13,14], resulting in the limited loading of a sample. Although, this defect may be overcome by using a solvent having a much lower eluting strength as a dissolving solution which is injected into a column [13,15–18], this technique would give rise to the loss of analytes due to adsorption on the surface of the devices. For these reasons, an on-line clean-up procedure, in which no sample evaporation and reconstitution steps, may probably be most preferable for the ultrasensitive assay.

The column-switching technique has been known as a useful technique for automated on-line clean-up with a short trapping column packed with a special support for solid-phase extraction [19,20]. Recently, a procedure for the direct injection of a large volume of an aqueous solution obtained by liquid–liquid extraction of human blood [21] or of a supernatant obtained by adding perchloric acid to human plasma [22,23] has been reported for an LC–ESI-MS–MS analysis coupled with a column-switching technique. However this procedure still has a limitation in application to polar analytes and unstable compounds under acidic or basic conditions. It seems that liquid–solid extraction employing a chemically bonding silica cartridge is more preferable for the direct injection of a sample specimen, making possible on-line clean-up. From this point of view, the present paper deals with the development of an ultrasensitive and simple method for the determination of NE-100 in human plasma by LC–ESI-MS–MS combined with on-line clean-up employing a column-switching technique.

2. Experimental

2.1. Chemicals

NE-100 and NE-100-D_4 (Fig. 1B) as an internal standard (I.S.) were synthesized in the Pharmaceutical Research Laboratories of Taisho Pharmaceutical Co., Ltd. Tritium labeled NE-100 (NE-100-$^3H_3$, *N,N*-dipropyl-2-[4-(methoxy-$^3H_3$)-3-(2-phenylethoxy)phenyl]-ethylamine monohydrochloride, 2.96 TBq/mmol) was obtained from Amersham (Buckinghamshire, UK). Methanol and acetonitrile were of HPLC grade from Wako Pure Chemicals (Osaka, Japan), and all other reagents were of analytical reagent grade.

2.2. Plasma collection

A blood specimen (10 ml) from a healthy volunteer was placed in a Venojest Π tube containing sodium heparin (Terumo, Tokyo, Japan) followed by immediate centrifugation at 2056 *g* for 10 min at 4°C, and then the plasma was stored at approximately −20°C.

2.3. LC–ESI-MS–MS

ESI-MS–MS with a positive ion detection mode was carried out with a Finnigan MAT TSQ7000 triple-stage quadrupole tandem mass spectrometer equipped with a Finnigan MAT ESI source (San Jose, CA, USA). A manifold temperature was set at 70°C and ESI was performed at a voltage of 4.5 kV with a heated capillary temperature of 200°C, a sheath gas (N_2) pressure of 50 p.s.i., and an auxiliary gas (N_2) flow of 10 units. The mass resolution as a peak width at half height on Q1 and Q3 was adjusted to 0.8 and 2.0 Da, respectively. The selected reaction monitoring (SRM) was operated at a scan time of 2.0 s for each target compound with a collision gas (Ar) pressure of 2.4 mTorr and a collision offset voltage of −35 V. The precursor ion used was *m*/*z* 356 for NE-100 and *m*/*z* 360 for the I.S., and the product ion was *m*/*z* 105 for both target compounds. The apparatus was combined with a column switching LC system.

2.4. Column-switching LC

The column-switching LC system was constructed with components of Shiseido Nanospace SI-1 series (Tokyo, Japan). Three pumps (model 2001), a six-port switching valve (model 2012), a column oven (model 2004), an auto-sampler (model 2003) and two degassing units (model 2009) were arranged as shown in Fig. 2A. The columns used in the system were a short trapping column of Shiseido Capcell Pak C_{18} UG120A (5 μm, 10 mm×2.0 mm I.D.) and a micro-bore analytical column of YMC J'sphere ODS H-80 (4 μm, 150 mm×1.0 mm I.D., Kyoto, Japan). These columns were maintained at 50°C. As the mobile phases for directing the sample specimen to the trapping column and the column refreshing, mixtures of Solvent A (5 m*M* ammonium acetate buffer (pH 4.0) delivering from Pump 1) and Solvent B (methanol delivering from Pump 2) were used. By use of a by-pass tube, which splits the mobile phase at a ratio of 1:9, a 9/10 aliquot of the mobile phase flow passed the auto-sampler, and the separated mobile phases were mixed continuously before reaching the switching valve. As the mobile phase for the analytical column, Solvent C (the mixed solution of 5 m*M* ammonium acetate buffer (pH 4.0) and acetonitrile (65: 35, v/v)), delivering from Pump 3 at a flow-rate of 50 μl/min, was used.

The time program for column-switching is also summarized in Fig. 2B. After injection of a 200 μl aliquot of a sample solution using the auto-sampler, the sample was directed to the trapping column with the mobile phase 1 (Solvent A/B=9:1 v/v) at a flow-rate of 1.0 ml/min, followed by flushing by further elution with the same mobile phase for 3 min. For delivering the analyte from the trapping column into the analytical column by eluting Solvent C for 5 min, the valve was switched following cessation of the delivery of the mobile phase 1. The switching valve was then turned back to the initial position, and the trapping column was subjected to flushing by eluting the mobile phase 2 (Solvent A/B=5:95 v/v) for 4 min to wash out any retained endogenous compounds, and re-equilibrated with mobile phase 1 for 6 min. The effluent corresponding to a retention time of 12~22 min from the analytical column was lead to an ESI source through an inactivated fused-silica capillary (0.05 mm I.D.) by using an SPCLS-LCA01 divert valve (Valco Instruments, Houston, TX, USA).

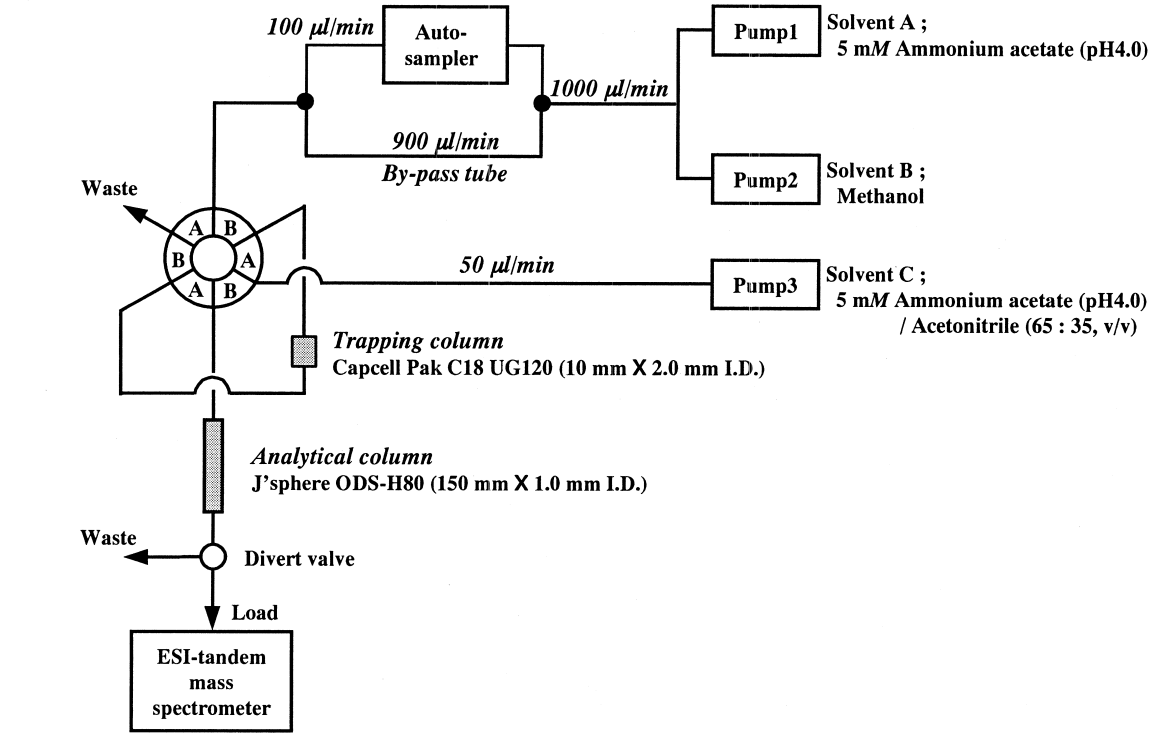
2.5. Standard solutions

The NE-100 was dissolved in methanol to prepare a primary stock solution (9.07 mg as its free amine/10 ml) and a standard solution of NE-100 (0.1~907.0 pg/50 μl in methanol) was prepared from the primary stock solution. A working I.S. solution (90.8 pg/50 μl) was also prepared by dilution of the primary stock solution (9.08 mg as its free amine/10 ml) with methanol. All standard solutions were stored at 4°C.

2.6. Pretreatment of plasma sample

A 50 μl aliquot of the working I.S. solution was added to plasma (1 ml) and the entire mixture was thoroughly mixed. After standing for at least 1 h, the plasma sample was re-mixed and passed through an ISOLUTE C_8 cartridge (100 mg) (International

A)



B)

Position of the switching valve	B	A	B		
Mobile phase for the trapping column	Mobile phase 1	Solvent C	-	Mobile phase 2	Mobile phase 1
Flow rate(μl/min) at the trapping column	1000	50	0	1000	1000
Situation of the trapping column	Enrichment	Transfer	-	Refreshing	Equilibration
Situation of the analytical column	Idling	Transfer	Separation		
Flow rate(μl/min) at the analytical column	50				
Mobile phase for the analytical column	Solvent C				
Position of the divert valve	Waste		Load		
Situation of the tandem mass spectrometer			Data acquisition		

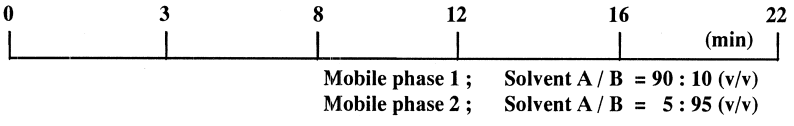


Fig. 2. (A) Schematic diagram and (B) time schedule of the column-switching system.

Sorbent Technology, Mid-Glamorgan, UK), which was previously conditioned with 1 ml of methanol and then 1 ml of 0.1 *M* aqueous ammonium acetate. After flushing with 1 ml of 25% methanol in 0.1 *M* ammonium acetate buffer (pH 4.0), the cartridge was set in a glass centrifuge tube and subjected to elution of the analyte with 300 μl of methanol by centrifugation at 21 *g* for 1 min followed by additional centrifugation at 2056 *g* for 5 min using a GS-6KR centrifuge (Beckman, Fullerton, CA, USA). A 200

μl aliquot of the eluate was directly injected into the column switching system.

A known amount of NE-100-$^{3}H_{3}$ (93.1 pg (195.6 Bq), 136.1 pg (198.5 Bq) and 997.8 pg (181.6 Bq)/ml) was spiked into human plasma, which in turn, was subjected to the extraction procedure on a ISOLUTE C_{8} cartridge according to the procedure described above. The resulting effluent was dissolved in 10 ml of the Pico-Fluor™ 40 liquid scintillation cocktail (Packard, Meriden, CT, USA), and the radioactivity was measured with a Beckman LS6000TA liquid scintillation counter to estimate the recovery rates.

2.7. Calibration graph

The calibration graph was constructed by plotting the ratio of the peak area of NE-100 to that of the I.S. versus the concentration of NE-100 (2.3, 4.5, 9.1, 45.3, 226.7 and 907.0 pg/ml) to that of the I.S. (90.8 pg/ml) using a weighted ($1/y^{2}$) linear regression.

2.8. Validation study

The novel method was validated according to the procedure recommended by V.P. Shah et al. [24]. The accuracy, precision, and stability in plasma were evaluated by analyzing the plasma spiked NE-100 in concentrations of 2.3, 4.5, 45.3, 453.3 or 907.0 pg/ml. The concentration was determined using the calibration equation.

3. Results and discussion

For the clinical trails with an oral dose of NE-100, which has a marked potency as a selective sigma 1 receptor ligand, an ultrasensitive method, which is able to determine the low picogram per milliliter of the drug in human plasma, is required. In acidic and neutral solutions, NE-100 is ionized due to its characteristic basic moiety, that is the N-dipropyl group, and the liquid chromatographic separation

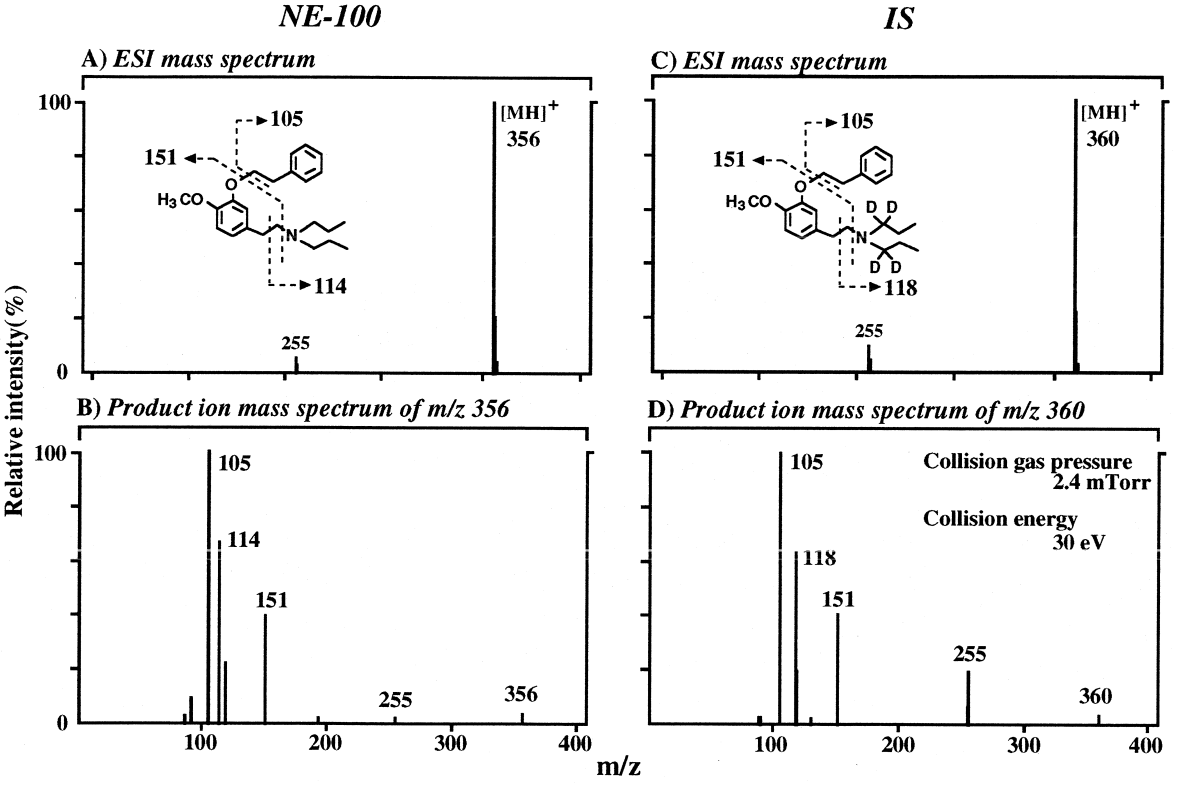


Fig. 3. ESI mass spectra of (A) NE-100 and (C) I.S., and product ion mass spectra of (B) NE-100 and (D) I.S.

hyphenated with ESI-MS–MS is therefore considered to be suitable for the determination of the drug in plasma.

The ESI full scan mass spectrum of NE-100 showed an abundant ion at m/z 356 as a $[M+H]^+$ (Fig. 3A), which underwent extensive fragmentation by collision-induced dissociation (CID) to give an abundant product ion at m/z 105 corresponding to the phenetyl group of the molecule (Fig. 3B). The intensity of the fragment ion at m/z 105 was maximized at a collision energy of 35 eV and a collision gas pressure of 2.4 mTorr. From these observations, the precursor/product combination of m/z 356–105 under the described CID conditions was selected.

In the mass spectrometric determination of trace compounds in biological specimens, the use of a stable isotope-labeled compound as an I.S. is recommended for obtaining reliable results. In the SRM mode, the I.S. which gives the same product ion with the parent compound is much preferred. Therefore, NE-100 having four deuterium atoms at the *N*-dipropyl group of the molecule was designed and used as the I.S. The ESI mass spectrum and the product ion mass spectrum are depicted in Figs. 3C and D, respectively.

For the pharmacokinetic study, the successive analysis with many samples is required and a simple sample pretreatment procedure is needed. Accordingly, the liquid–solid extraction on a C_8 cartridge was employed. When a plasma specimen spiked $^3H_3$-NE-100 was subjected to the liquid–solid extraction, the recovery rate was more than 80% in all the concentration ranges.

For on-line clean-up using a column-switching technique, it is very important to retain the analyte on the trapping column, which removes a large excess of the coexisting compound, even when injecting a large volume of a methanolic effluent. For this purpose, a by-pass tube, which is for dilution of the methanolic effluent, allowed its eluting strength to be decreased. When the drug in 200 μl of methanol was delivered to the trapping column without any dilution, NE-100 was not retained on the

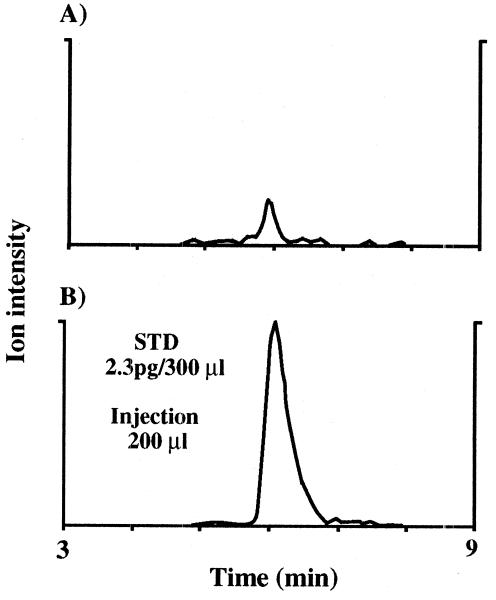


Fig. 4. Enrichment of NE-100 onto the trapping column (A) without and (B) with a by-pass tube. A 200 μl aliquot of NE-100 in methanol (2.3 pg/300 μl) was delivered to the trapping column with 5 m*M* ammonium acetate buffer (pH 4.0) at a flow-rate of 1.0 ml/min. After 3 min, the switching-valve was switched to transfer the analyte from the trapping column to the tandem mass spectrometer.

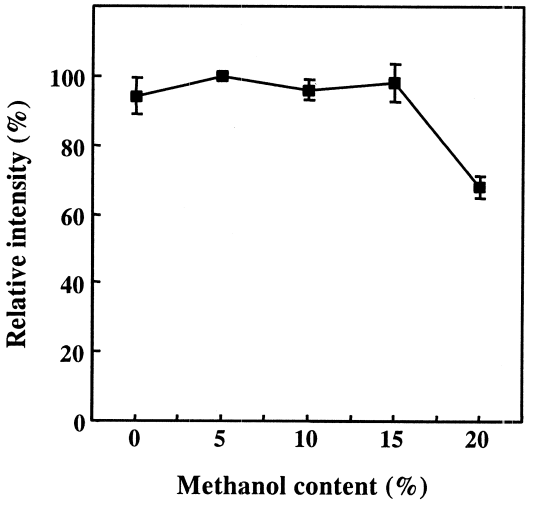


Fig. 5. Effect of a methanol content in the mobile phase on the enrichment of NE-100 onto the trapping column. A 200 μl aliquot of NE-100 in methanol (2.3 pg/300 μl) was delivered to the trapping column with 5 m*M* ammonium acetate buffer (pH 4.0) containing methanol ranging 0 to 20% at a flow-rate of 1 ml/min. After 3 min, the switching-valve was switched to transfer the analyte from the trapping column to the tandem mass spectrometer. Each point represents mean±SD (N=3).

column (Fig. 4A). On the other hand, with a 10-times dilution of a methanolic solution with 5 m*M* ammonium acetate buffer (pH 4.0), improved retention of NE-100 on the column was observed (Fig. 4B). The increasing methanol content up to 15% in the buffer did not affect the retention of the drug (Fig. 5). On the basis of these findings, 10% methanol in 5 m*M* ammonium acetate buffer (pH 4.0) was chosen as a suitable solvent for directing the analyte to the trapping column.

The next effort was used to select a mobile phase for eluting the analyte from the trapping column and directing it to the analytical micro-bore column, on which the drug was effectively separated. It is well known that the use of a mobile phase having a low surface tension to form a shorter radius droplet in the ESI process shows good ionization efficiency [25]. Since the surface tension decreases with the higher content of an organic solvent in an aqueous solution, it is generally accepted that a high organic content in a mobile phase improves the response towards the analyte [26]. As NE-100 is more strongly retained on an ODS column with a neutral solvent compared with an acidic mobile phase due to its dissociation, a higher organic modifier content is required for elution of the drug. As shown in Fig. 6A, with use of a neutral mobile phase (5 m*M* ammonium acetate (pH 6.9)–acetonitrile (48:52, v/v), no peaks on the chromatogram obtained with a spiked plasma specimen (2.3 pg/ml) were observed, because of the co-elution of interfering endogenous compounds from the trapping column by the high acetonitrile content. On the other hand, with use of an acidic mobile phase (5 m*M* ammonium acetate (pH 4.0)–acetonitrile (65:35, v/v)), the background level was dramatically reduced, and NE-100 in plasma corresponding to 2.3 pg/ml was successfully detected (Fig. 6B), indicating the excellent on-line clean-up with the trapping column.

It is possible that NE-100, having a tertiary amino group in the molecule, is metabolized into its N-oxide derivative [27]. In the ESI mass spectrum, the

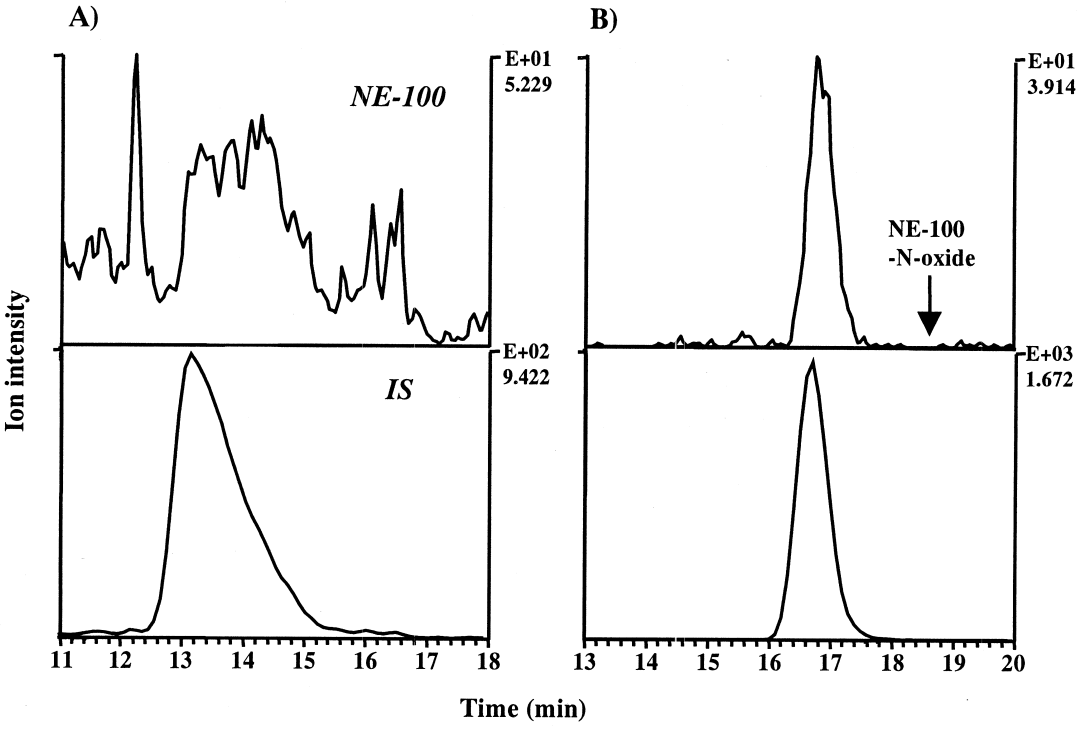


Fig. 6. Typical SRM chromatograms of NE-100 (2.3 pg/ml) and I.S. (90.8 pg/ml) in spiked human plasma. The effluent from the trapping column with (A) neutral and (B) acidic mobile phases at a flow-rate of 50 μl/min was subjected to the separation of the micro-bore analytical column and then detection with ESI-MS–MS. The arrow indicates the retention time of NE-100-N-oxide.

N-oxide showed a weak fragment ion at m/z 356 (0.6% of the base peak) with the most abundant $[M+H]^+$ ion at m/z 372, indicating elimination of an oxygen atom to generate NE-100 under the ESI process (data not shown). Since NE-100 and its N-oxide are completely resolved on the analytical column (Fig. 6B), the N-oxide does not interfere with the peak corresponding to the parent drug, even if a large amount of NE-100-N-oxide exists in human plasma.

The validation study for the newly developed method was then undertaken. Initially, to elucidate the specificity of the method, independent blank plasma from fasted- and non-fasted subjects (each six) was subjected to the analysis. A typical SRM chromatogram of a blank human plasma is shown in Fig. 7A, in which no interference on the chromatogram was observed. Also, as shown in Fig. 7B–E, the detection limit of the method was 0.5 pg/ml with a signal to noise ratio (S/N)=3. It is generally accepted that the S/N value of the lower limit for quantification should be better than ten. In this study, the lower limit of quantification was set at 2.3 pg/ml (S/N=21), by considering the robustness in the pharmacokinetic study.

The linearity of the calibration graph was observed in the range of 2.3~ 907.0 pg/ml; this range allowed for the determination of NE-100 in plasma obtained in clinical trails. Moreover, when the calibration graph was determined on different six days, the mean back calculated values for the calibration standards did not exceed ±3.3% for all the concentrations, thus indicating excellent linearity (Table 1). The typical parameters for the calibration graph were as follows; slope: 0.0103, intercept: −0.0049, correlation coefficient: 0.9992. The intercept value of the calibration graph was nearly equal to zero, showing excellent specificity of the method as well as no interference by non-labeled NE-100 contained in the I.S.

The precision and accuracy were further examined by analyzing spiked plasma samples with concentrations of 2.3, 45.3 and 907.0 pg/ml (Tables 2 and 3). The intra-assay and the inter-assay precisions (C.V.%) were within 4.5 and 8.0%, respectively, and intra-assay and inter-assay accuracies (% Bias) at 2.3

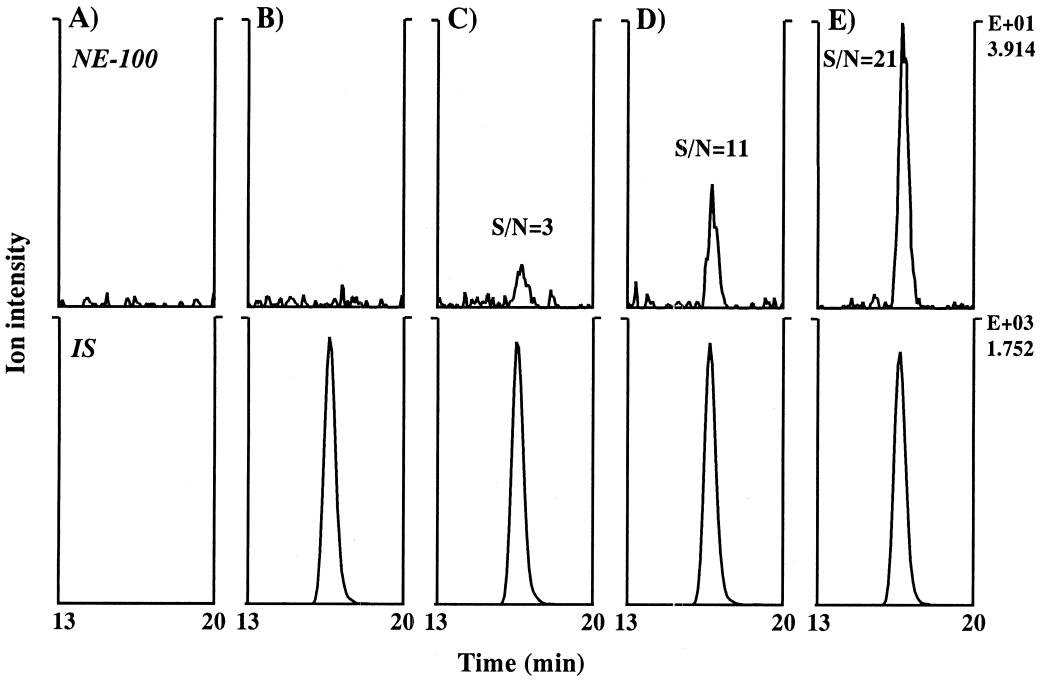


Fig. 7. Typical SRM chromatograms of NE-100 and I.S. in (A)blank human plasma, and spiked human plasma in NE-100 concentrations of (B) 0, (C) 0.5, (D) 0.9 and (E) 2.3 pg/ml with 90.8 pg/ml of the I.S.

Table 1
Back-calculated concentrations from the calibration graphs over six days

Nominal concentration (pg/ml)	Back-calculated concentration[a] (pg/ml)	C.V. (%)	Bias (%)
2.3	2.3	2.3	0.0
4.5	4.6	5.8	2.2
9.1	9.4	4.5	3.3
45.3	45.1	3.0	−0.4
226.7	225.5	2.5	−0.5
907.0	893.8	2.9	−1.5

[a] Each value represents mean concentration from six experiments

pg/ml were 4.3 and 0.0%, respectively, thus exhibiting a very high reliability of the method. The stability of NE-100 in plasma at concentrations of 4.5 and 453.3 pg/ml was also evaluated. As a result, no changes were found in the concentration at least during two freeze/thaw cycles and a one-year storage period at approximately −20°C.

Finally, the developed method was applied to a pharmacokinetic analysis of NE-100 in a clinical trail. The results obtained with one subject after oral administration of NE-100 at a starting dose of 1 mg are presented (Fig. 8). As can be expected, the method enabled us to completely follow the concentration-time course up to 24 h.

The utility of LC–ESI-MS–MS combined with a column-switching technique for the ultrasensitive determination of NE-100 in human plasma with excellent selectivity and reliability was demonstrated. The developed method involves the use of a stable isotope labeled compound as the I.S., direct injection of a large volume of a methanol effluent into the trapping column, subsequent automated on-line clean-up and separation on a C_{18} column followed by detection with ESI–SRM. The assay performance was unchanged at least after the successive analysis of 300 samples. This novel method was applied to the pharmacokinetic profiling of NE-100 from the clinical study. It is hoped that the availabili-

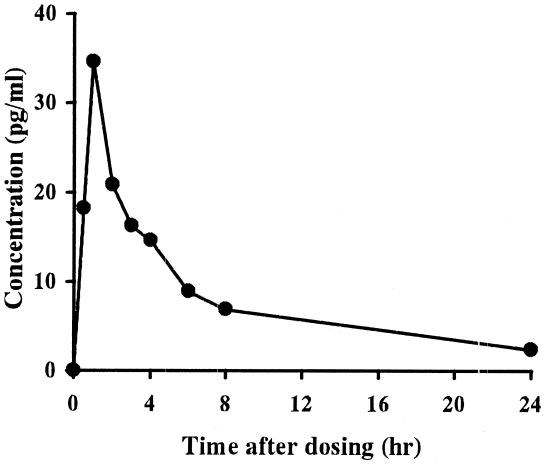


Fig. 8. Typical plasma concentration-time profile of NE-100 in a subject after an oral dose of 1 mg.

Table 2
Intra-assay precision and accuracy for the determination of NE-100 in human plasma

Nominal concentration	Found (pg/ml)					Mean	C.V.	Bias
(pg/ml)	1	2	3	4	5	(pg/ml)	(%)	(%)
2.3	2.4	2.4	2.3	2.4	2.6	2.4	4.5	4.3
45.3	44.1	44.3	42.9	46.2	44.5	44.4	2.7	−2.0
907.0	925.5	895.3	943.4	958.7	933.5	931.3	2.5	2.7

Table 3
Inter-assay precision and accuracy for the determination of NE-100 in human plasma

Nominal concentration	Found (pg/ml)						Mean	C.V.	Bias
(pg/ml)	Day1	Day2	Day3	Day4	Day5	Day6	(pg/ml)	(%)	(%)
2.3	2.4	2.2	2.1	2.2	2.6	2.2	2.3	8.0	0.0
45.3	44.1	47.0	46.4	45.3	45.4	46.8	45.8	2.4	1.1
907.0	925.5	880.9	862.3	936.8	897.4	931.7	905.8	3.3	−0.1

ty of the new method for the determination of a trace amount of NE-100 in plasma may provide much more precise knowledge about the pharmacological efficacy of the drug.

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