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Simultaneous determination of human neutrophil elastase inhibitor (ONO-5046) and its metabolite in plasma and urine by direct injection column-switching HPLC

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Abstract: The direct injection method for the simultaneous determination of a human neutrophil elastase inhibitor (ONO-5046) and its metabolite (ONO-EI-601) in plasma and urine has been developed using a column-switching HPLC system. The system was set up with a pre-treatment column, a pre-concentration column and an analytical column which were connected in series via two automatic switching valves. The calibration lines showed a good linearity for concentrations of ONO-5046 and ONO-EI-601 in a range of 156–20000 ng ml^{-1} in plasma and 1.56–100 μg ml^{-1} in urine, all correlation coefficients being greater than 0.9999. The limit of quantitation of ONO-5046 was 156 ng ml^{-1} in plasma, that of ONO-EI-601 was 313 ng ml^{-1} in plasma, and those of ONO-5046 and ONO-EI-601 were 1.56 μg ml^{-1} in urine. The developed method is rapid and sensitive with automated operation, allowing untreated samples to be analysed every 45 min. The application of the present method to the real plasma and urine samples proved to be useful for pharmacokinetic, toxicological and clinical studies.

Keywords: *Human neutrophil elastase inhibitor (ONO-5046); HPLC; direct injection; column-switching; plasma; urine; metabolite.*

Introduction

The analysis of drugs and their metabolites in biological fluids by high-performance liquid chromatography (HPLC) often necessitates pre-treatment procedures such as deproteinization, extraction and pre-concentration in order to avoid deterioration of separation efficiency or to gain high reproducibility. However, these procedures are time-consuming, and often cause low recovery and poor reproducibility. In an attempt to overcome these problems, direct injection techniques using various new-type HPLC columns [1–5] have been explored. For instance, direct injection methods with restricted-access columns [1, 4, 5] and column-switching [6–9] techniques have been reported, which allow the plasma samples to be analysed successfully without protein precipitation and column clogging. However, there are few reports dealing with the simultaneous determination of unchanged drug and its metabolites in plasma or urine by the direct injection method using a column-switching HPLC.

ONO-5046 [10], sodium N-[2-[4-(2,2-dimethylpropionyloxy)phenylsulphonylamino]-benzoyl]aminoacetate tetrahydrate, is a newly synthesized inhibitor of human neutrophil elastase (HNE). ONO-EI-601 was identified as the main metabolite of ONO-5046 in rat, dog and humans. The present paper describes an automated direct injection HPLC system for the simultaneous determination of ONO-5046 and its metabolite (ONO-EI-601) in plasma and urine using a column-switching technique with ultraviolet detection. The system uses a semipermeable surface (SPS) column [5] as a pre-treatment column which selectively adsorbs small molecules such as a drug and its metabolites, but excludes macromolecules such as plasma proteins in a manner comparable to an internal surface reversed-phase (ISRP) column [1, 3]. The present method was also applied to the pharmacokinetic study in dogs during and after constant-rate intravenous infusion of ONO-5046 sodium salt.

As a conventional method, a solid-phase extraction and HPLC method for the simultaneous determination of ONO-5046 and

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ONO-EI-601 was developed and the present paper also considers the correlation between direct injection and solid-phase extraction methods.

Experimental

Reagents and materials

ONO-5046, its metabolite (ONO-EI-601) and internal standards (ONO-EI-547 and ONO-EI-537) were synthesized in ONO laboratories. Their chemical structures are shown in Fig. 1. The solvents of HPLC grade and the reagents of analytical grade were purchased from Wako Pure Chemical (Osaka, Japan) and Katayama Chemical (Osaka, Japan).

Direct injection method

Preparation of human plasma and urine samples. Fresh plasma was obtained by centrifugation of human blood treated with the anticoagulant heparin sodium. The known amounts of ONO-5046, ONO-EI-601 and internal standards (ONO-EI-547 and ONO-EI-537) were dissolved in the plasma to yield the planned concentrations, and the aliquots (40 μl) of plasma solution were filtered through a 0.22-μm membrane filter and applied to the chromatographic system.

The weighed amounts of ONO-5046, ONO-EI-601 and internal standards (ONO-EI-547 and ONO-EI-537) were dissolved in human urine and the aliquots (10 μl) were filtered through a 0.22-μm membrane filter and applied to the HPLC system.

Apparatus. The HPLC system constructed for direct injection analysis was equipped with a L-6200 pump and two L-6000 pumps, a L-4000 UV detector, a 655A-40 autosampler and two 655-0723 automatic switching-valves (Hitachi, Tokyo, Japan). The chromatograms were recorded on 860 data system (Waters, Milford, MA, USA). An SPS-C_{18} column (Semipermeable-Surface: 5 μm, 150 × 4.6 mm i.d.; Regis, Morton, IL, USA), a YMC-A602 column (5 μm, 150 × 4.6 mm i.d.; YMC, Kyoto, Japan) and a Capcell Pak C_{18} column (5 μm, 150 × 4.6 mm i.d.; Shiseido, Tokyo, Japan) were used as a pre-treatment column, a pre-concentration column and an analytical column, respectively. The guard column packed with SPS-C_{18} (5 μm, 10 × 4.6 mm i.d. Regis, Morton, IL, USA) was connected between injector and pre-treatment column.

HPLC conditions and column-switching procedures. The column-switching assembly used in the developed system is shown in Fig. 2. Three pumps (P1, P2 and P3) were used to deliver mobile phase 1, 0.05 M phosphate buffer (pH 7)–CH_3CN (90:10, v/v); mobile phase 2, H_2O–CH_3CN (90:10, v/v); and mobile phase 3, 0.02 M KH_2PO_4 (pH 3.8)–CH_3CN (4:1, v/v), respectively, at a flow rate of 1 ml min^{-1}. In column-switching procedures, the back flush technique [8] was employed in order to shorten analysis time, to sharpen the drug peak and to increase the recovery of drug.

Aliquots (40 μl of plasma or 10 μl of urine spiked with internal standards: ONO-EI-547 and ONO-EI-537) were injected through an autosampler onto the pre-treatment column and the column was washed with the mobile phase 1 for 7 min to remove proteins and hydrophilic substances. ONO-5046, ONO-EI-

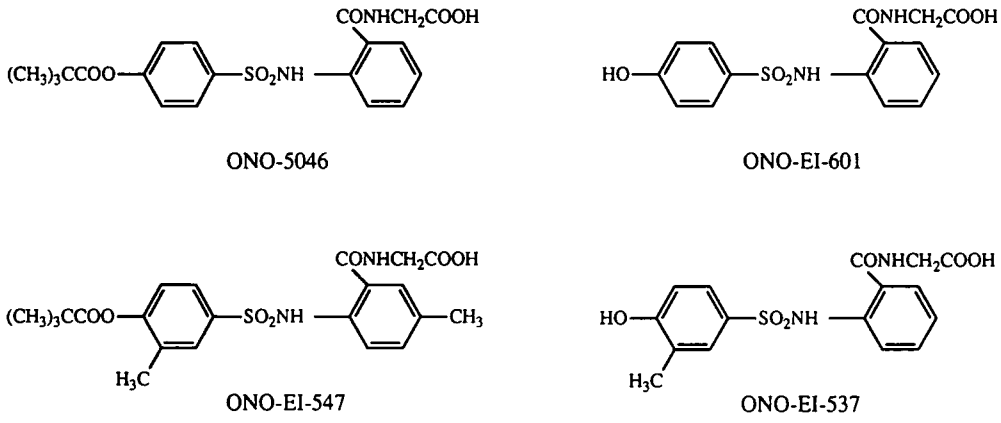
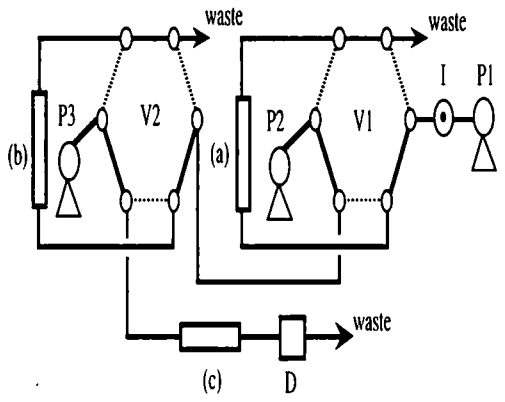


Figure 1
Chemical structures of ONO-5046, its metabolite (ONO-EI-601) and the internal standards (ONO-EI-547 and -537).



V1, V2: Automatic six-way valves
P1~P3 : Pump D : UV detector 240nm
I : Autosampler (a) : Pre-column
(b) : Pre conc.-column (c) : Anal.-column
Inj. volume: 40μl (plasma) ; 10μl (urine)

Figure 2
Column-switching assembly and HPLC conditions in developed method.

601 and internal standards (ONO-EI-547 and ONO-EI-537) adsorbed on the pre-treatment column were transferred to the pre-concentration column in the back flush mode by automatically switching the six-way valve (V1) for 3 min. These compounds adsorbed on the pre-concentration column were then transferred to the analytical column, by automatically switching the six-way valve (V2) in the back flush mode. At the same time V1 was returned to the initial position, and 3 min later V2 was also returned to the initial position, so that the pre-treatment and pre-concentration columns were equilibrated with their respective mobile phases to provide for the next injection. The gradient was started after 10 min over a linear gradient pump (P3) and mobile phase composition was changed from 0.02 M KH_2PO_4 (pH 3.8)–CH_3CN (4:1, v/v) to 0.02 M KH_2PO_4 (pH 3.8)–CH_3CN (5:4, v/v) for 20 min. The separation was carried out within 45 min for each sample.

Determination of recoveries, assay linearity, precision and accuracy. Quantitations of ONO-5046 and ONO-EI-601 in plasma and urine were accomplished based on the calibration lines obtained by peak area ratio to each internal standard (ONO-EI-547 and ONO-EI-537, respectively). The percentage recoveries of ONO-5046 and ONO-EI-601 in plasma and urine from the SPS column were determined by comparing the peak areas of replicate analyses under the same conditions with the peak areas of these compounds in an aqueous mobile phase, injected into the chromatographic system.

Calibration lines were obtained on five different days by the direct injection method using spiked plasma or urine samples as described in *Preparation of human plasma and urine samples.*

Precision and accuracy of the assay by the direct injection method were determined by preparing spiked plasma or urine samples as described in *Preparation of human plasma and urine samples* in intra-assay (within-day) or in inter-assay (between-day). The plasma and urine solutions were injected onto the HPLC system five times and the precision [SD mean × 100(%)] and accuracy [(mean − theoretical)/theoretical × 100(%)] were calculated.

Pharmacokinetic study. Plasma was obtained during and after constant-rate intravenous infusion of ONO-5046 sodium salt at a dose of 10 mg kg^{-1} h^{-1} for 2 h in male beagle dogs. The plasma samples were stored frozen at −20°C until analysis and ONO-5046 and ONO-EI-601 in plasma were stable for up to 3 months in this condition.

After the internal standards (ONO-EI-547 and ONO-EI-537) were added, the concentrations of ONO-5046 and ONO-EI-601 in plasma were determined by direct injection method. The plasma concentration–time curves of ONO-5046 and ONO-EI-601 were obtained and these pharmacokinetic parameters in plasma were calculated. The elimination half-life ($T_{1/2}$) were determined by linear regression analysis of the log–linear terminal phase of the plasma concentration–time curve. Area under the plasma concentration–time curve (AUC) was estimated by the linear trapezoidal method with end correction (last concentration/the elimination half-life).

Solid-phase extraction method

Solid-phase extraction procedures. ONO-5046, ONO-EI-601 and internal standards (ONO-EI-547 and ONO-EI-537) were dissolved in human plasma or urine at known concentrations. The plasma (0.5 ml) or urine (0.1 ml) was diluted with distilled water and 1 N HCl and applied to a Sep-Pak C_{18} short column (Waters, Milford, MA, USA). ONO-5046, ONO-EI-601 and internal standards

(ONO-EI-547 and ONO-EI-537) were eluted with MeOH from the short column, and the methanolic solution was applied to a Bond Elut SAX short column (Varian, Harbor City, CA, USA). ONO-5046, ONO-EI-601 and internal standards (ONO-EI-547 and ONO-EI-537) were eluted with 0.1 N HCl–CH_3OH (40:60, v/v) solution from the short column and the compounds in this solution were extracted with ethylacetate. The organic phase was evaporated to dryness and the residue was dissolved in 150 μl of mobile phase and subjected to HPLC.

Apparatus. A liquid chromatograph system equipped with a pump, a UV detector and an autosampler (LC-module-1: Waters, Milford, MA, USA) was used. A Chemcosorb 3Dph column (3 μm, 150 × 4.6 mm i.d.; Chemco, Osaka, Japan) was used as an analytical column.

HPLC conditions. The aliquot (50 μl) was injected through an autosampler onto an analytical column and the mobile phase composition was changed from 0.02 M KH_2PO_4 (pH 3.8)–CH_3CN (4:1, v/v) to 0.02 M KH_2PO_4 (pH 3.8)–CH_3CN (5:4, v/v) in the linear gradient mode for 40 min. The separation was completed within 55 min for each sample and quantitations of ONO-5046 and ONO-EI-601 in plasma and urine were carried out based on the calibration lines obtained by peak area ratio to each internal standard (ONO-EI-547 and ONO-EI-537, respectively). Assay precision and accuracy in solid-phase extraction method were also determined as described in *Direct injection method.*

The correlation between direct injection and solid-phase extraction method

Plasma and urine were obtained during and after constant-rate intravenous infusion of ONO-5046 sodium salt for 2 h in healthy volunteers. After the internal standards [ONO-EI-547 and ONO-EI-537 (2 μg)] were added, the concentrations of ONO-5046 and ONO-EI-601 in human plasma and urine were determined by both direct injection and solid-phase extraction methods. The correlations between the values determined by these two methods were investigated.

Results and Discussion

Direct injection method

Recovery from plasma and urine samples. In general, a drug in blood is bound to plasma proteins to a greater or less extent, and there is an equilibrium between bound and unbound species. In conventional methods, the drug can be almost completely recovered by pre-treatment procedures such as deproteinization and solvent extraction. The use of SPS or ISRP columns allows direct injection of plasma samples without pre-treatment, because the bound drug is released from plasma proteins in the aqueous mobile phase and the proteins are excluded from the column. However, the extent of the release depends on the degree of dilution and the change of the higher-order structure of proteins around the binding sites. It is clear that binding of drug to plasma protein affects the recovery of a drug from SPS or ISRP columns [11, 13]. Furthermore, in direct injection method, it is advisable to use as low a concentration of organic modifier in the mobile phase (<20%, v/v) in order to prevent coalescence of proteins [3, 12]. Therefore, the mobile phase conditions such as the pH, ionic strength and concentration of organic modifier, should be optimized not only to give good separation of drug peaks but also to avoid the excessive denaturation of proteins.

Table 1
Effect of phosphate buffer concentration in mobile phase on absolute recoveries of ONO-5046 and its metabolite (ONO-EI-601) from plasma and urine samples in the presence of a constant concentration of CH_3CN in the mobile phase (10%, v/v) in the direct injection method

Sample	Phosphate buffer concentration in mobile phase (M)	*n*	Recovery (%) ONO-5046	Recovery (%) ONO-EI-601
Plasma	0.05	6	99.9 ± 6.4	101.3 ± 4.4
	0.02	3	91.7 ± 2.4	101.4 ± 7.7
Urine	0.05	6	104.6 ± 2.5	101.9 ± 2.6
	0.02	—	N.T.	N.T.

Concentrations of ONO-5046 and ONO-EI-601 in plasma samples were 0.125 μg ml^{-1}.
The data represent the mean ± SD. N.T., not tested.

Thus, the effect of mobile phase conditions such as pH, ionic strength and concentration of organic modifiers in an SPS column on the separation and recoveries of ONO-5046 and ONO-EI-601 from plasma or urine samples were investigated. When the phosphate buffer concentration in mobile phase 1 was decreased from 0.05 to 0.02 M in the presence of a constant concentration of CH_3CN in the mobile phase (10%, v/v), recoveries of ONO-5046 from plasma samples were lowered (Table 1) because ONO-5046 exhibits strong binding to plasma proteins. Over 95% of ONO-5046 and ONO-EI-601 in plasma and urine was recovered from the SPS column using mobile phase 1. Since plasma proteins and the hydrophilic small substances in plasma and urine were eluted within 5 min, their elution having been detected by their absorbance at 240 nm, so the pre-treatment column was washed with the mobile phase 1 for 7 min in column-switching procedures.

In the present method, the choice of the pre-concentration column was important for the simultaneous determination for ONO-5046 and ONO-EI-601 in plasma and urine. First, a Chemocosorb SAX (anion exchange phase, 7 μm, 100 × 4 mm i.d.) and a conventional ODS column were chosen as the pre-concentration columns, ONO-EI-601 was not absorbed on these columns, because ONO-EI-601 is the relative polar metabolite of ONO-5046. Since both ONO-5046 and ONO-EI-601 were absorbed on a YMC-NH_2 (aminopropyl phase) column, the column was chosen as the pre-concentration column and the simultaneous determination was accomplished.

Assay linearity. Linear regressions of spiked human plasma or urine samples with standard calibration lines are listed in Table 2. The calibration lines showed a good linearity for concentrations of ONO-5046 and ONO-EI-601 in a range of 156–20000 ng ml^{-1} in plasma and 1.56–100 μg ml^{-1} in urine, respectively, all correlation coefficients being greater than 0.9999. The slopes of these calibration lines for ONO-5046 and ONO-EI-601 were essentially equal.

Assay precision and accuracy. Tables 3 and 4 show that the precision for ONO-5046 and ONO-EI-601 was less than 9.7% in intra-assay and 6.3% in inter-assay and also indicated that all concentrations showed a good accuracy (<6.9%) in intra-assay and in inter-assay.

Table 2
Calibration lines of the direct injection method (plasma: 0.156 ~ 20 μg ml^{-1}, urine: 1.56 ~ 100 μg ml^{-1})

Sample	Compound	Day	Slope	Intercept	Correlation coefficient
		1	0.5918	−0.0033	0.99991
		2	0.6026	−0.0001	0.99997
Plasma	ONO-5046	3	0.5960	−0.0037	0.99998
		4	0.5922	−0.0018	0.99999
		5	0.5895	−0.0027	0.99997
		mean ± SD	0.5944 ± 0.0051	−0.0023 ± 0.0014	—
		1	0.5732	−0.0001	0.99998
		2	0.5748	−0.0013	0.99999
Plasma	ONO-EI-601	3	0.5789	−0.0019	0.99999
		4	0.5807	−0.0024	0.99999
		5	0.5807	−0.0028	0.99999
		mean ± SD	0.5777 ± 0.0035	−0.0017 ± 0.0011	—
		1	0.5921	0.0019	0.99995
		2	0.6086	0.0032	0.99993
Urine	ONO-5046	3	0.5917	0.0005	0.99997
		4	0.6097	−0.0023	0.99995
		5	0.5877	−0.0024	0.99999
		mean ± SD	0.5980 ± 0.0104	0.0002 ± 0.0025	—
		1	0.5780	−0.0014	0.99999
		2	0.5731	−0.0017	0.99998
Urine	ONO-EI-601	3	0.5764	0.0005	0.99997
		4	0.5767	−0.0018	0.99999
		5	0.5761	−0.0014	0.99999
		mean ± SD	0.5761 ± 0.0018	−0.0012 ± 0.0009	—

Table 3
Intra-assay precision* and accuracy† of the direct injection method

Sample	Compound	Theoretical conc. ($\mu g\ ml^{-1}$)	Measured conc. ($\mu g\ ml^{-1}$) Mean ± SD	Precision (%)	Accuracy (%)
Plasma	ONO-5046	20.00	19.88 ± 0.08	0.4	−0.6
		5.00	4.91 ± 0.14	2.9	−1.8
		1.25	1.23 ± 0.08	6.5	−1.6
		0.31	0.33 ± 0.02	6.1	5.6
Plasma	ONO-EI-601	20.00	20.05 ± 0.07	0.3	0.3
		5.00	4.90 ± 0.12	2.4	−2.0
		1.25	1.24 ± 0.02	1.6	−0.8
		0.31	0.31 ± 0.03	9.7	−0.8
Urine	ONO-5046	100.00	100.33 ± 0.47	0.5	0.3
		25.00	24.78 ± 0.29	1.2	−0.9
		6.25	6.07 ± 0.20	3.4	−2.9
		1.56	1.58 ± 0.07	4.3	1.1
Urine	ONO-EI-601	100.00	99.35 ± 0.46	0.5	−0.7
		25.00	25.02 ± 0.10	0.4	0.1
		6.25	6.43 ± 0.25	3.9	2.9
		1.56	1.65 ± 0.08	4.8	5.6

* Precision: SD mean × 100(%).
† Accuracy: (mean − theoretical)/theoretical × 100(%).
The data represent the mean ± SD of five experiments.

Table 4
Inter-assay precision* and accuracy† of the direct injection method

Sample	Compound	Theoretical conc. ($\mu g\ ml^{-1}$)	Measured conc. ($\mu g\ ml^{-1}$) Mean ± SD	Precision (%)	Accuracy (%)
Plasma	ONO-5046	20.00	20.03 ± 0.09	0.4	0.2
		5.00	5.00 ± 0.10	2.0	0.0
		1.25	1.26 ± 0.05	4.0	0.8
		0.31	0.32 ± 0.02	6.3	2.4
Plasma	ONO-EI-601	20.00	19.98 ± 0.02	0.1	−0.4
		5.00	4.99 ± 0.06	1.2	−0.2
		1.25	1.24 ± 0.01	0.8	−0.8
		0.31	0.32 ± 0.01	3.1	2.4
Urine	ONO-5046	100.00	99.70 ± 0.42	0.4	−0.3
		25.00	25.03 ± 0.40	1.6	0.1
		6.25	6.09 ± 0.17	2.8	−2.6
		1.56	1.63 ± 0.08	4.7	4.3
Urine	ONO-EI-601	100.00	99.83 ± 0.31	0.3	−0.2
		25.00	24.96 ± 0.19	0.8	−0.2
		6.25	5.91 ± 0.28	4.7	−5.4
		1.56	1.67 ± 0.10	6.2	6.9

* Precision: SD mean × 100(%).
† Accuracy: (mean − theoretical)/theoretical × 100(%).
The data represent the mean ± SD of five experiments.

Within acceptable intra- and inter-assay precision and accuracy (<10%), the limit of quantitation of ONO-5046 was 156 ng ml^{-1} and the limit of quantitation of ONO-EI-601 was 313 ng ml^{-1} in plasma, and the limits for both ONO-5046 and ONO-EI-601 in urine were 1.56 $\mu g\ ml^{-1}$.

Pharmacokinetic study. Figure 3 shows the plasma concentration–time curves of ONO-5046 and ONO-EI-601 during and after constant-rate intravenous infusion of ONO-5046 sodium salt at a dose of 10 mg $kg^{-1}\ h^{-1}$ for 2 h in male beagle dogs. Steady state plasma concentrations of ONO-5046 and

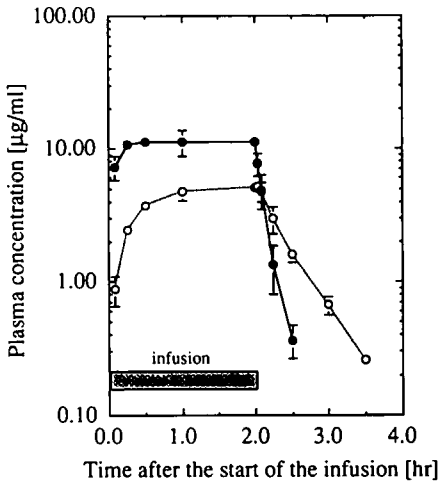


Figure 3
Plasma concentrations of ONO-5046 and its metabolite (ONO-EI-601) during and after constant-rate intravenous infusion of ONO-5046 sodium salt at a dose of 10 mg kg^{-1} h^{-1} for 2 h in male beagle dogs determined by direct injection column-switching HPLC. The data represent the mean ±SD of three animals. —●— ONO-5046; —○— ONO-EI-601.

ONO-EI-601 (11.2 and 5.1 μg ml^{-1}, respectively) were reached 2 h after the start of the infusion. The areas under the plasma concentration–time curve (AUC) of ONO-5046 and ONO-EI-601 were 22.87 ± 3.32 and 10.60 ± 0.81 μg h ml^{-1} (n = 3, mean ± SD), and plasma elimination half-lives ($T_{1/2}$) were 0.14 ± 0.01 and 0.36 ± 0.04 h (n = 3, mean ± SD), respectively.

Solid-phase extraction method

Recovery, assay precision and accuracy. Recoveries of ONO-5046 and ONO-EI-601 from plasma samples (0.2 μg ml^{-1}) in the solid-phase extraction method were 80.6 ± 8.3% and 83.1 ± 4.6% (n = 3, mean ± SD), respectively. Recoveries of these compounds from plasma samples by the solid-phase extraction method were significantly lower compared to those by the direct injection method, because more pre-treatment procedures were required prior to chromatographic injections in the solid-phase extraction method than the direct injection method.

The precision of the assay in the solid-phase extraction method was determined by preparing spiked plasma or urine samples as described in *Solid-phase extraction procedures* in intra-assay (within-day) or inter-assay (between-day). ONO-5046, ONO-EI-601 and the internal standards (ONO-EI-547 and ONO-EI-537) were purified and extracted from the plasma and urine solutions by the pre-treatment procedures as previously described and injected onto HPLC five times. Tables 5 and 6 show that the precision for ONO-5046 and ONO-EI-601 assays in plasma and urine were less than 9.8% in intra-assay and 7.7% in inter-assay, respectively. Tables 5 and 6 also show that the accuracy determinations for ONO-5046 and ONO-EI-601 in plasma and urine were less than 9.4% in intra-assay and 7.5% in inter-assay, respectively. Based upon

Table 5
Intra-assay precision* and accuracy† of the solid-phase extraction method

Sample	Compound	Theoretical conc. (μg ml^{-1})	Measured conc. (μg ml^{-1}) Mean ± SD	Precision (%)	Accuracy (%)
Plasma	ONO-5046	4.000	4.021 ± 0.101	2.5	0.5
		0.500	0.501 ± 0.011	2.2	0.2
		0.125	0.134 ± 0.003	2.5	6.9
Plasma	ONO-EI-601	4.000	4.125 ± 0.045	1.1	3.1
		0.500	0.504 ± 0.006	1.2	0.8
		0.125	0.137 ± 0.001	0.8	9.4
Urine	ONO-5046	20.00	21.092 ± 2.074	9.8	5.5
		5.000	5.018 ± 0.266	5.3	0.4
		0.625	0.680 ± 0.028	4.1	8.8
Urine	ONO-EI-601	20.00	19.622 ± 0.493	2.5	−1.9
		5.000	5.034 ± 0.218	4.3	0.7
		1.250	1.270 ± 0.020	1.6	1.6

*Precision: SD mean × 100(%).
†Accuracy: (mean − theoretical)/theoretical × 100(%).
The data represent the mean ± SD of five experiments.

Table 6
Inter-assay precision* and accuracy† of the solid-phase extraction method

Sample	Compound	Theoretical conc. (μg ml^{-1})	Measured conc. (μg ml^{-1}) Mean ± SD	Precision (%)	Accuracy (%)
Plasma	ONO-5046	4.000	4.029 ± 0.222	5.5	0.7
		0.500	0.514 ± 0.026	5.0	2.9
		0.125	0.130 ± 0.004	3.1	4.0
Plasma	ONO-EI-601	4.000	4.117 ± 0.123	3.0	2.9
		0.500	0.518 ± 0.017	3.3	3.6
		0.125	0.134 ± 0.006	4.4	7.5
Urine	ONO-5046	20.000	19.690 ± 1.525	7.7	−1.6
		5.000	5.064 ± 0.144	2.8	1.3
		0.625	0.668 ± 0.045	6.8	6.9
Urine	ONO-EI-601	20.000	19.990 ± 0.579	2.9	−0.1
		5.000	5.189 ± 0.517	3.0	3.8
		1.250	1.248 ± 0.043	3.5	−0.1

*Precision: SD mean × 100(%).
†Accuracy: (mean − theoretical)/theoretical × 100(%).
The data represent the mean ± SD of five experiments.

acceptable intra- and inter-assay precision and accuracy (<10%), the limits of quantitations of both ONO-5046 and ONO-EI-601 were 125 ng ml^{-1} in plasma and 1.25 μg ml^{-1} in urine.

Correlation between direct injection and solid-phase extraction methods

Typical HPLC chromatograms obtained by the direct plasma and urine injection methods are shown in Figs 4 and 5, respectively. There were no interfering peaks at 240 nm from components in human plasma or urine before dosing of ONO-5046 sodium salt to healthy volunteers. It can be seen that, though both ONO-5046 and its metabolite (ONO-EI-601) were detected in plasma, only the metabolite was excreted in urine during and after constant-rate intravenous infusion of ONO-5046 sodium salt to healthy volunteers.

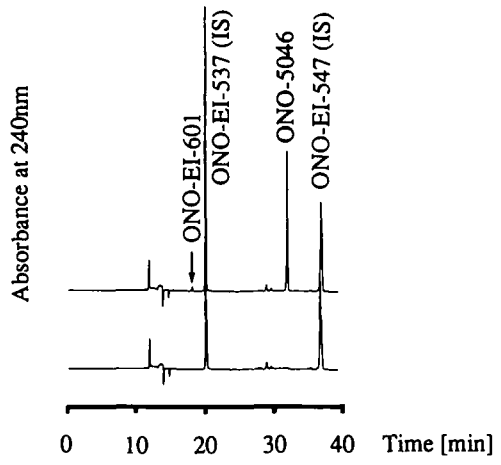


Figure 4
Typical HPLC chromatograms with direct plasma injection before and 2 h after constant-rate i.v. infusion of ONO-5046 sodium salt (0.5 mg kg^{-1} h^{-1}) to a healthy volunteer (lower: before, upper: 2 h after).

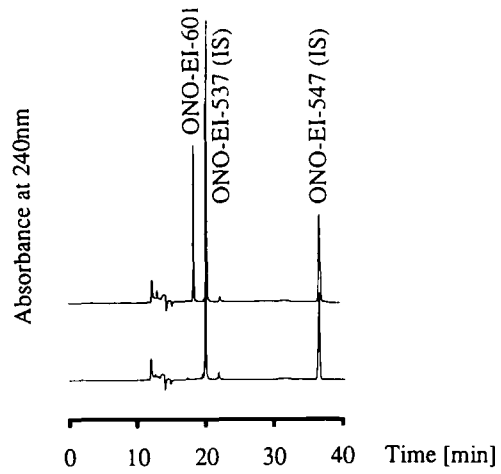


Figure 5
Typical HPLC chromatograms with direct urine injection before and until 24 h constant-rate i.v. infusion of ONO-5046 sodium salt (0.5 mg kg^{-1} h^{-1}) to a healthy volunteer (lower: before, upper: until 24 h).

Correlation between the values determined by both the direct injection and the solid-phase extraction methods are shown in Fig. 6. The slopes of the regression lines were 0.995, 1.005 and 1.032 for ONO-5046 in plasma, and ONO-EI-601 in plasma and in urine, respectively. All correlation coefficients are greater than 0.995. Thus the results determined by these two methods correlated well.

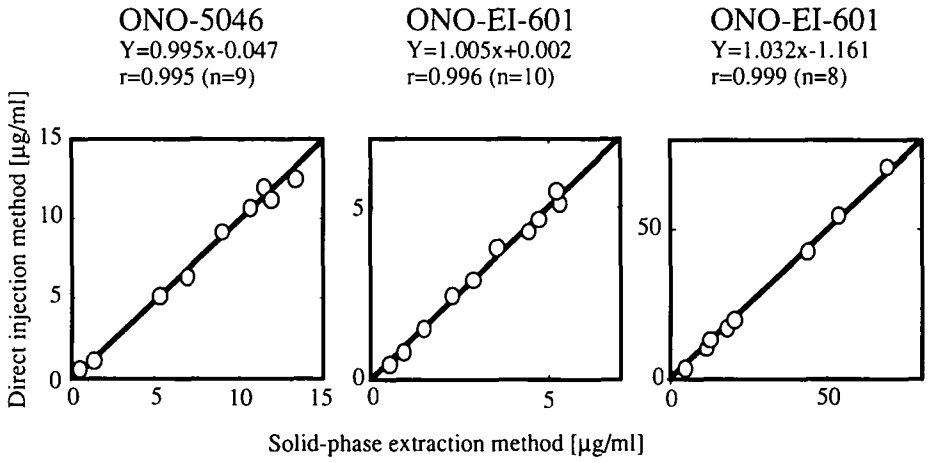


Figure 6
Correlation between the values determined by direct injection and solid-phase extraction methods (plasma: left and centre, urine: right).

The solid-phase extraction method was not only time-consuming but allowed only a low recovery (about 80%), whereas the direct injection method had a high recovery (>95%). And though the limits of detection of ONO-5046 and its metabolite in both the direct injection method and the solid-phase extraction method were similar to one another, the direct injection method demands less plasma volume (0.1 ml) or urine volume (0.02 ml) to analyse than the solid-phase extraction method (plasma 0.5 ml or urine 0.1 ml). In addition, the direct injection method showed a higher reproducibility than the solid-phase extraction method. Thus the direct injection method is rapid and sensitive with automated operation, allowing untreated samples to be analysed every 45 min. Application of the direct injection method to the real plasma and urine samples proved to be useful for pharmacokinetic, toxicological and clinical studies.

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