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Analysis of Ecteinascidin 743, a new potent marine-derived anticancer drug, in human plasma by high-performance liquid chromatography in combination with solid-phase extraction

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Abstract

A reversed-phase high-performance liquid chromatographic method has been developed and validated for the quantification of the novel anticancer drug Ecteinascidin 743 in human plasma. The sample pretreatment of the plasma samples involved a solid-phase extraction (SPE) on cyano columns. Propyl-*p*-hydroxybenzoate was added after the sample pretreatment to correct for variability in injection volumes. The separation was performed on a Zorbax SB-C_{18} column (75×4.6 mm I.D., particle size 3.5 μm) with acetonitrile–25 m*M* phosphate buffer, pH 5.0 (70:30, v/v) as the mobile phase. The flow-rate was 1.0 ml/min and the eluent was monitored at 210 nm. The accuracies and precisions of the assay fall within ±15% for all quality control samples and within ±20% for the lower limit of quantitation, which was 1.0 ng/ml using 500 μl of plasma. The overall recovery of the sample pretreatment procedure for Ecteinascidin 743 was 87.0±5.9%. The drug was found to be stable in human plasma at −30°C for at least 2 months. At room temperature Ecteinascidin 743 was stable in human plasma for 5 h at most.

Keywords: Ecteinascidin 743

1. Introduction

In the search for novel anticancer drugs from natural sources cytotoxic compounds originating from the marine ecosystem attract much attention. Examples of this class of compounds currently under early clinical evaluation are didemnin B from the Caribbean tunicate *Trididemnum solidum* [1–3], bryostatin 1 from the bryozoan *Bugula neritina* [4,5], and dolastatins from the mollusk *Dollabella auricularia* [6]. Didemnin B was the first marine compound to enter in clinical trials. This compound has completed the phase II evaluation with indications of significant activity in heavily pretreated patients with low grade non-Hodgkin's lymphoma. Ecteinascidin 743 (ET-743) is another example of a

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drug isolated from a Caribbean tunicate (*Ecteinascidia turbinata*). In vitro studies indicated activity in melanoma, non-small-cell lung cancer, renal and breast human tumor lines. Moreover, in vivo data in nude mice bearing human tumors demonstrated significant activity in breast, non-small-cell lung cancer, ovarian, melanoma and renal cancers. ET-743 exerts its antitumor activity by interacting with minor groove DNA guanine-rich sequences and modifying the cell microtubule network [6]. It is now tested in phase I clinical trials in Europe and the US.

The marine derived anticancer compounds including ET-743, share high potencies and are administered in $\mu g/m^2$ dosages which demands special requirements in terms of sensitivity for the analytical method supporting pharmacokinetic studies. For pharmacokinetic monitoring of ET-743 in phase I studies we have designed a sensitive and selective assay of the drug in human plasma based on solid-phase extraction (SPE) as sample pretreatment procedure followed by reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection.

2. Experimental

2.1. Chemicals

Ecteinascidin 743 (ET-743, lot SL 0010; E94/455, Fig. 1) reference was obtained from Pharma Mar (Madrid, Spain). The internal standard, propyl-*p*-hydroxybenzoate (POB) originated from Fluka (Bornem, Belgium). Acetonitrile (HPLC gradient grade) was obtained from Biosolve (Amsterdam, The Netherlands) and methanol (ChromAR®) from Promochem (Wesel, Germany). Ammonium acetate, glacial acetic acid, hydrochloric acid 37%, disodium hydrogen phosphate dihydrate and sodium dihydrogen phosphate dihydrate (all analytical grade) were from Merck (Darmstadt, Germany). Double distilled water was used throughout. Drug free human plasma originated from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands).

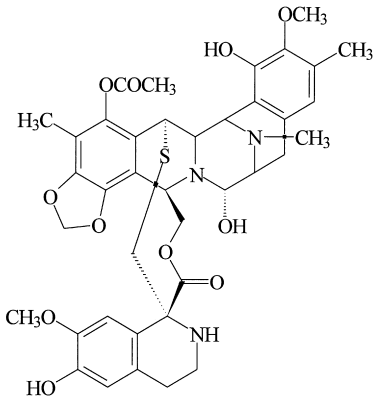

Fig. 1. Chemical structure of ET-743.

2.2. HPLC instrumentation and conditions

The HPLC system was composed of a SP 8800 pump and a SP 8880 autosampler (Thermo Separation Products (TSP), Fremont, CA, USA). Chromatographic separation was conducted on a Zorbax SB-C_{18} column (75×4.6 mm I.D., particle size 3.5 μm, Rockland Technologies, Newport, DE, USA). The mobile phase consisted of acetonitrile–25 m*M* phosphate buffer, pH 5.0 (70:30, v/v). The flow-rate of the mobile phase was 1.0 ml/min and the eluent was monitored at 210 nm with a Spectra 100 detector (TSP) using the recorder output at 10 mV, a range of 0.02 a.u.f.s. and a rise time of 1 s. Chromatographic data was processed by a PC1000 data system (TSP).

2.3. Preparation of stock solutions, working solutions and plasma standards

ET-743 stock solutions were prepared in methanol at concentrations of 2.0 mg/ml. The stock solution was diluted further in methanol to obtain working solutions with concentrations of 100 μg/ml and 10 μg/ml, respectively. All solutions were stored at approximately −30°C and were stable for at least 2 months.

The stock solution of the internal standard, POB, (100 μg/ml) was prepared in methanol and was stored at 4–8°C for, at most, 3 months. A reconstitution solvent was prepared as follows: the POB stock solution was diluted in 0.2 *M* ammonium acetate, pH 5.0–methanol (60:40, v/v) to achieve a POB concentration of 0.5 μg/ml. This solvent was used to redissolve the dry extracts after SPE. The solution

was stored at room temperature and prepared freshly every 3 months.

For the validation of the ET-743 assay, a plasma standard of 100 ng/ml was prepared by adding 50 μl of the ET-743 working solution (10 μg/ml) to 5.0 ml of control human plasma. This plasma standard (100 ng/ml) was further diluted in control human plasma to achieve analyte concentrations of 1.0, 2.5, 5.0, 10.0, 25.0 and 50 ng/ml. Plasma calibration standards were prepared freshly for every analytical run.

2.4. Sample processing

The extraction columns (non-endcapped cyano columns, 100 mg, Varian, Harbour City, CA, USA) were first activated and washed with 1 ml of 0.1 *M* hydrochloric acid in methanol, 2 ml of methanol and 2 ml of 0.01 *M* ammonium acetate, pH 5.0. A 1000-μl sample volume (plasma–0.2 *M* ammonium acetate, pH 5.0 (1:1, v/v)) was loaded onto the columns with a dispense flow of approximately 1 ml/min. After successive washings with 2 ml of 0.01 *M* ammonium acetate, pH 5.0 and 2 ml of acetonitrile, elution of ET-743 was performed with 1 ml of 0.1 *M* of hydrochloric acid in methanol. The elution solvent was evaporated to dryness under a nitrogen stream at approximately 30°C. The dry residues were then dissolved in 50 μl of reconstitution solvent by mixing the tubes for 1 min. A 25-μl volume was injected onto the HPLC column.

2.5. Validation procedures

2.5.1. Linearity

Six plasma calibration standards were prepared (see Section 2.3) and analyzed in duplicate in three separate analytical runs. The linear regression of the ratio of the areas of the analyte and internal standard peaks versus the concentration were weighted by $1/x^2$ (reciprocal of the squared concentration). The *F* test for lack of fit ($\alpha = 0.05$) was used to evaluate the linearity of the calibration curves [7].

2.5.2. Accuracy and precision

For the validation of the ET-743 assay, four quality control samples containing 1.0, 2.5, 10.0 and 40.0 ng/ml were prepared in control human plasma by dilution of a plasma standard containing 100 ng/ml (see Section 2.3). Replicates of each quality control sample were processed and analyzed in three different runs with plasma calibration standards to determine the ET-743 concentration. The accuracy was calculated at each test concentration: the measured concentration was divided by the nominal concentration and multiplied by 100%. The assay precisions were obtained by one-way analysis of variance (ANOVA) for each test concentration using the run day as the classification variable (software package statistical product and service solutions, version 6.1 for Windows, SPSS, Chicago, IL, USA). The following formulas were used to calculate the precisions:

$$\text{Within-run precision} = \frac{\sqrt{MS_{WG}}}{GM} \cdot 100\%$$

$$\text{Between-run precision} = \frac{\sqrt{\frac{(MS_{BG} - MS_{WG})}{n}}}{GM} \cdot 100\%$$

where, MS_{WG} is the mean square of the within-groups-runs, MS_{BG} the mean square of the between-groups/runs, GM the grand mean of the measured quality control concentration, and *n* the number of determinations per group/run.

The acceptance criteria for accuracy and precision are not more than 15% and at the lower limit of quantitation, 20% is acceptable [9].

2.5.3. Selectivity and specificity

Six batches control human plasma were processed and analyzed to determine whether endogenous plasma constituents coeluted with ET-743 and/or POB. To investigate the potential interference of the comedication with the analytical method, drugs were added to control human plasma in therapeutic concentrations. These samples were then processed and assayed according to the described method. The following drugs were tested: paracetamol (20 μg/ml), morphine (50 ng/ml), dexamethason (100 ng/ml), ondansetron (40 ng/ml), domperidone (500 ng/ml), metoclopramide (50 ng/ml) and omeprazole (200 ng/ml).

2.5.4. Recovery

The overall extraction recoveries were determined by comparing the slopes of the processed calibration curves to a standard curve prepared in reconstitution solvent [0.2 *M* ammonium acetate, pH 5.0–methanol (60:40, v/v)].

2.5.5. Stability

In methanol, the stability of ET-743 has been investigated at a concentration of 10 μg/ml when stored at approximately −30°C during 2 months. The stability of ET-743 in plasma has been studied at a concentration of 40 ng/ml during 2 months at approximately −30°C, and at ambient temperature at a concentration of 10 ng/ml for 24 h. The processed sample stability of ET-743 in plasma extracts after dissolution in reconstitution solvent [0.2 *M* ammonium acetate, pH 5.0–methanol (60:40, v/v)] has been studied at a concentration of 50 ng/ml during 48 h.

2.6. Pharmacokinetic case study

A patient with a solid tumor was treated with ET-743 in a phase I clinical study at a dose level of 330 $\mu g/m^2$ given as a 1-h infusion. Whole blood samples were taken from the contralateral arm receiving the infusion. Sampling times were: prior to the start of the infusion, at 30 min and 1 min before the end of the infusion. Postinfusion samples were taken at 5, 10, 15, 30 min and 1, 2, 4, 6, 8, 10, 12 and 24 h. The blood samples (8 ml) were collected in heparinized tubes and immediately centrifuged (15 min at 4000 *g*). The plasma layer was then removed and stored at approximately −30°C until analysis.

The ET-743 concentration vs. time curve was fitted using the MW\PHARM® software program (Medi\Ware, Groningen, The Netherlands) [8]. The postinfusion ET-743 kinetics were best described by the use of a two compartment model ($n=2$), with the general equation:

$$C_t = \sum_{i=1}^{N} C_i e^{(-\lambda_i t)}$$

where λ_i is the slope of the *i*th exponential term, and C_i the initial concentration of the ith component of the curve. Curve fitting yields the parameters for λ_i and C_i. The elimination halflives ($t_{1/2,\alpha}$ and $t_{1/2,\beta}$) were calculated from the equation $t_{1/2}=0.693/\lambda$. Other pharmacokinetic parameters were determined by noncompartmental analysis. The total area under the curve ($AUC_{0\rightarrow\infty}$) was calculated using the trapezoidal rule with extrapolation of the terminal phase to infinity (C_{last}/λ_2, where C_{last} is the last measured concentration). Total plasma clearance (Cl_{tot}) was calculated by dividing the total dose by the $AUC_{0\rightarrow\infty}$. The volume of distribution at steady state was calculated out of the total plasma clearance and slope of the second exponential term: $V_{ss}=Cl_{tot}/\lambda_2$.

3. Results and discussion

Chromatography of ET-743, with basic properties, was optimal using an endcapped Zorbax SB-C_{18} column in combination with a mobile phase at pH 5.0. Chromatograms typical for the analysis of ET-743 are presented in Fig. 2. Various detection modes to quantify the analyte have been investigated. ET-743 has no fluorescence properties and poor electrochemical properties. A current–potential curve showed that the analyte can undergo oxidation, however, at a nonselective, relative high potential of +900 mV. The UV spectrum of the compound in water exhibited an absorbance maximum at 285 nm with a molar absorptivity of 7010 $M^{-1}\,cm^{-1}$. To improve sensitivity chromatographic detection was performed at 210 nm (molar absorptivity of 432 280 $M^{-1}\,cm^{-1}$). A significant signal-to-noise improvement (factor 3) was obtained when the recorder output of the detector at 10 mV was used in stead of the integrator output. Detection at 210 nm requires a very selective sample pretreatment. Different SPE sorbents were tested (octadecyl, aromatic sulfonic acid as a strong cation exchanger and cyanopropyl). The cyano group gave an unique selectivity due to nonpolar–polar interactions and cation-exchange interactions with ET-743, allowing to use 100% acetonitrile as a washing solvent. The analyte can be eluted from the sorbent with methanolic hydrochloric acid, a solvent that disrupts both ionic and nonpolar interactions simultaneously. Using aromatic sulfonic acid as extraction sorbent clean chromatograms were

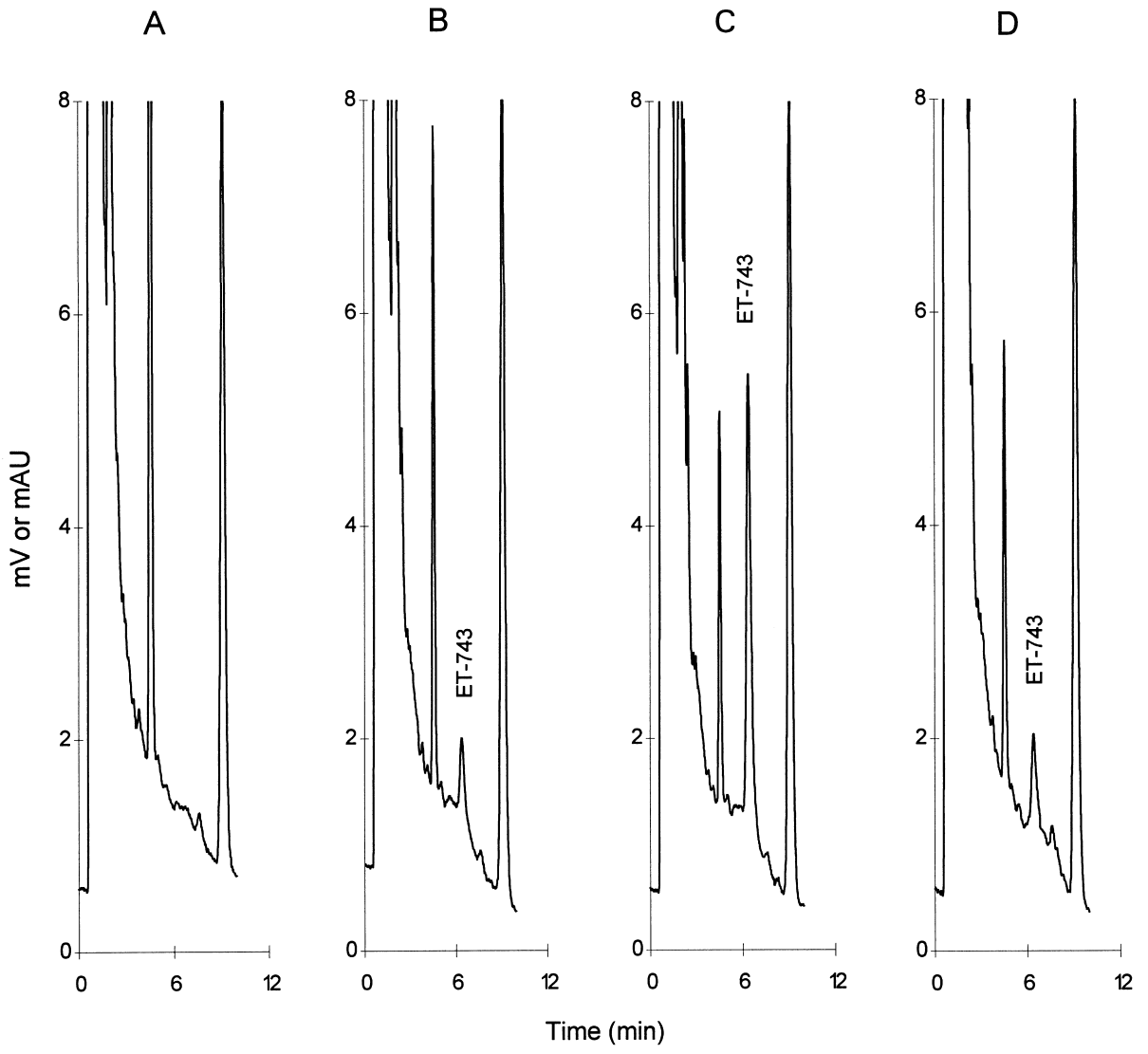

Fig. 2. Chromatograms typical for the analysis of ET-743 in human plasma: control human plasma (A), plasma calibration standard of 5.0 ng/ml (B), plasma calibration standard of 25.0 ng/ml (C) and a sample taken from a patient treated with ET-743 at a dose level of 330 $\mu g/m^2$ (30 min after the start of the infusion, D) with ET-743 concentration of 5.1 ng/ml. POB elutes after 9.1 min.

obtained, however, elution of the compound was not complete.

ET-729 (N-desmethyl ET-743) was tested as potential internal standard for the bioanalytical assay at a concentration of 50 ng/ml in plasma. Irreproducible recoveries were obtained and the chromatographic characteristics of ET-729 were not ideal in terms of symmetry. It was then decided to execute the validation programme with a semi-internal standard (POB) added after the sample pretreatment to correct for variability in injection volumes.

The assay was linear over a concentration range of 1–50 ng/ml for ET-743 in human plasma as determined by the F test for lack of fit ($\alpha = 0.05$). For every calibration curve the calibration concentrations were back-calculated from the ratio of the peak areas from ET-743 and POB (see Table 1). The deviation of the nominal concentration for all concentrations

Table 1
Calibration concentrations ($n=2$) back-calculated from the ratio of the peak areas from ET-743 and POB

Run number	Concentration (ng/ml)					
	1.0	2.5	5.0	10	25	50
1	1.03	2.36	4.81	10.71	26.05	49.62
2	1.03	2.34	4.71	10.49	25.52	51.06
3	1.01	2.42	5.20	9.95	24.06	51.35
Mean	1.02	2.37	4.91	10.38	25.21	50.68
C.V. (%)	1.1	1.8	5.3	3.8	4.1	1.8
Dev. (%)	2.0	−5.2	−1.8	3.8	0.8	1.4

C.V., coefficient of variation; Dev., deviation of the nominal concentration.

were less than 6%. The assay performance data for the quantification of ET-743 is presented in Table 2. The within-day and between-day precisions at 2.5, 10.0 and 40.0 ng/ml were less than the required 15% [9]. At the lower limit of quantitation (1.0 ng/ml) a within-day precision of 13.4% was measured, within the accepted range of 20% [9]. The accuracies were all within ±10%. Chromatograms of six batches control human plasma samples contained no endogenous peaks coeluting with ET-743 and/or POB. None of the tested concomitant drugs interfered with the assay. The mean overall extraction recovery of ET-743 from control human plasma was 87.0±5.9% ($n=3$).

In Table 3 the stability data for ET-743 is summarized. ET-743 was found to be stable in human plasma for at least 2 months, when stored at −30°C. The drug is, however, not stable in plasma at ambient temperature. After 24 h the concentration was 91±4.6% of the initial concentration. The sample preparation procedure should be performed within 5 h (Table 3). ET-743 was found to be stable in reconstitution solvent for at least 48 h at ambient temperature. This allows the use of an autosampler injection device with samples stored for 48 h.

To show the applicability of the method, the plasma concentration vs. time profile of ET-743 of a patient treated with a dose level of 330 μg/m^2 in a 1-h infusion is presented in Fig. 3. The maximum concentration (C_{max}) was found at the end of the ET-743 administration. Postinfusion, the drug concentration declines rapidly. The plasma concentration vs. time profile was biphasic. The relevant pharmacokinetics of ET-743 are given in Table 4. The short halflife of the drug rationalizes the selection of prolonged schedules for administration, i.e. 24-h infusions in other clinical studies. No indications for metabolism were found in the chromatograms.

Table 2
Assay performance data for the determination of ET-743 in human plasma

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	Within-day precision (%)	Between-day precision	Number of replicates
1.0	1.06	106	13.4	5.4	15
2.5	2.33	93	7.1	2.7	15
10.0	10.08	101	6.0	5.3	15
40.0	41.06	103	3.9	1.0	15

Table 3
Stability data of ET-743

Storage condition	Initial conc. (ng/ml)	Recovery (%)	C.V. (%)	Number of replicates
Methanol at −30°C for 2 months	10 000	99	2.3	2
Plasma at −30°C for 2 months	40.0	96	4.5	2
Plasma at ambient temperature for 5 h	10.0	99	1.0	2
Plasma at ambient temperature for 24 h		91	4.6	2
Reconstitution solvent after plasma extraction at ambient temperature during 48 h	50.0	99	0.9	2

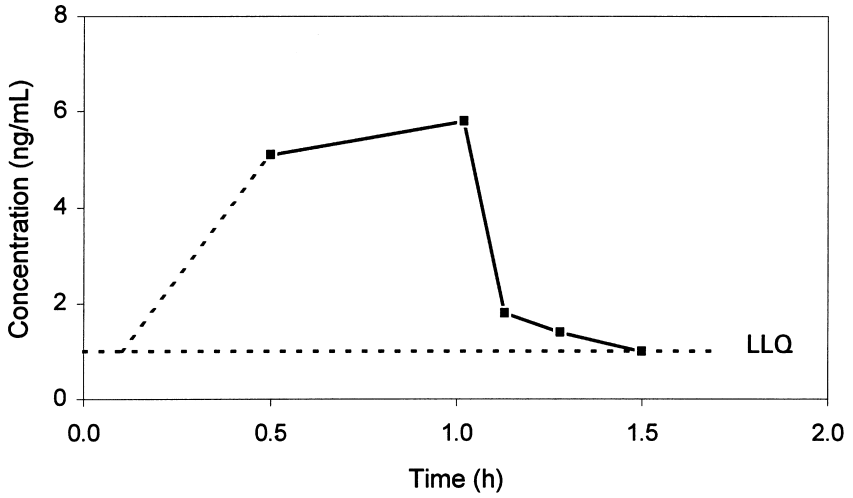

Fig. 3. Plasma concentration vs. time profile of ET-743 in a patient treated with 330 μg/m^2 ET-743 in a 1-h infusion (LLQ=lower limit of quantitation).

4. Conclusions

An accurate, reproducible and selective HPLC assay, utilizing solid-phase extraction on cyano columns as a sample pretreatment procedure has been developed for the quantification of ET-743 in human plasma. The assay quantitates ET-743 concentrations of 1.0–50.0 ng/ml using a 500-μl sample volume. This assay is used now to support pharmacokinetic research in a phase I study. At the starting dose of this study (50 μg/m^2) only C_{max} concentrations of ET-743 at the end of infusion could be detected and quantified in plasma of patients. An analytical method with more sensitivity should be developed to monitor complete pharmacokinetic profiles at these low dosages. The use of liquid chromatography combined with mass spectrometry may be appropriate to overcome these sensitivity problems and is currently under investigation.

Table 4
Pharmacokinetics of ET-743

Pharmacokinetic parameter	
Dose	330 μg/m^2
Total dose	700 μg
Infusion duration	1.0 h
C_{max}	5.8 ng/ml
$AUC_{0\rightarrow\infty}$	6.0 h.ng/ml
Halflife $t_{1/2,\alpha}$	3.2 min
Halflife $t_{1/2,ß}$	53 min
Clearance, Cl	117 l/h
Distribution volume atsteady state, V_{ss}	149 l

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