ELSEVIER

Journal of Chromatography A, 870 (2000) 189–198

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Determination of the *N*-methyl-D-aspartate receptor NR2B subunit blocker Ro 63-1908 in rat, cynomolgus monkey and human plasma by high-performance liquid chromatography with column switching and fluorescence detection

Ronald Wyss*, Franz Bucheli, Wolf Philipp

Pharmaceuticals Division, F. Hoffmann-La Roche Ltd., PRNS, 68/121A, CH-4070 Basel, Switzerland

Abstract

A HPLC method with automated column switching was developed and validated for the determination of Ro 63-1908 in rat and cynomolgus monkey plasma. Human plasma was used for calibration and was also included in the validation process. Ro 63-1908 belongs to a class of neuroprotective *N*-methyl-D-aspartate (NMDA) receptor blockers which were in development for the treatment of stroke and traumatic brain injury. The method involves deproteinisation of plasma samples with ethanol and direct injection of the supernatant (1.4 ml) into the HPLC column-switching system. To prevent a breakthrough of the analyte and the internal standard on the precolumn (Purospher RP-18, 75×4 mm) due to the high ethanol content, the injection solution was diluted, on-line, using an additional pump and a T-piece. 1% ammonium acetate–ethanol (100:2, v/v) was used as mobile phase for injection, as well as for on-line dilution, resulting in pre-concentration of the analyte and the internal standard on the precolumn. As Purospher RP-18 is a non-endcapped stationary phase with a special selectivity for amines, the analyte and the internal standard could then be selectively eluted with 30% acetonitrile (without any buffer in the mobile phase) and transferred to the analytical column [consisting of two coupled columns (125+250×4 mm) packed with Superspher 60 RP-select B], where they were separated by gradient elution and detected by fluorescence detection. Compared to the use of a 125 mm long precolumn and dilution of the supernatant with ammonium acetate prior to injection, the 75 mm precolumn and the on-line dilution procedure allowed about one third shorter run times (21 min) and, therefore, a higher sample throughput. The limit of quantification was 1 ng/ml using 0.4 ml plasma. The method was applied to more than 670 plasma samples from pharmacokinetic and toxicokinetic studies and is also suitable for other matrices and NMDA receptor blockers. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Column switching; Ro 63-1908; Methyl aspartate receptor blockers

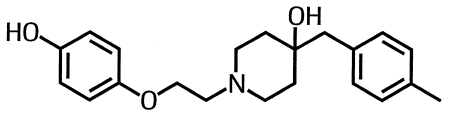
1. Introduction

Ro 63-1908 (**I**, Fig. 1) is an NMDA (*N*-methyl-D-aspartate) receptor NR2B subunit selective blocker, which was in development as a neuroprotective drug for the treatment of brain stroke and traumatic brain injury. Its pharmacological action is similar to that of Ro 25-6981 (**II**, Fig. 1) [1]. Metabolic studies in vitro, using hepatocytes of different species, produced mainly conjugates and oxidation products of the methyl group (the corresponding alcohol and carboxylic acid) [2]. In vivo intravenous (i.v.) bolus and infusion studies in rats showed that the compound was mainly excreted in bile where a glucuro-

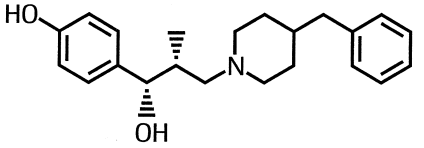
*Corresponding author. Tel.: +41-61-6884-781; fax: +41-61-6882-908.

E-mail address: ronald.wyss@roche.com (R. Wyss)

0021-9673/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(99)01016-X



I: Ro 63-1908



II: Ro 25-6981 (internal standard)

Fig. 1. Chemical structures of the compounds.

nide of **I** and conjugates of the carboxylic acid were found. In urine the alcohol, the carboxylic acid and glucuronides of the acid and of parent compound were the main metabolites. The latter was also found to be the main metabolite in rat plasma. However, the activity of all metabolites tested was significantly lower than that of the parent compound [2]. During continuous infusion of 15 and 5 mg/kg/h to rats and cynomolgus monkeys over two weeks, mean steady-state plasma concentrations of **I** were about 1300 ng/ml in male rats and about 2800 ng/ml in male cynomolgus monkeys [3].

To investigate **I** for the i.v. treatment of acute ischemic damage, an analytical method was needed for sample analysis from pharmacokinetic, neuroprotection and toxicity studies in several species. Although the protective plasma levels after i.v. infusion of this drug were in the range of several hundred ng/ml, a limit of quantification of about 1 ng/ml was needed to characterise the pharmacokinetics of the compound in the elimination phase, especially for a planned single ascending dose study in human volunteers. This report describes a high-performance liquid chromatography (HPLC) column-switching method with fluorescence detection for the analysis of plasma samples from rats and cynomolgus monkeys. The validation of the method, according to good laboratory practice (GLP) requirements, was also performed for human plasma which was used for the calibration samples.

The method is based on a general procedure which was used successfully for other NMDA receptor blockers [4], as well as for a preliminary method of **I** [5]. The latter involved deproteinisation of plasma samples with three volumes of ethanol and dilution of the supernatant with 2 ml of 2% ammonium acetate–ethanol (100:2, v/v) to decrease the elution strength of the injection solution prior to injection. Moreover, the procedure relied on a selective elution of the analyte and the internal standard from the precolumn (Purospher RP-18, 125×4 mm I.D.) with 25% acetonitrile (without any buffer in the mobile phase). The limit of quantification of the preliminary method was 5 ng/ml using 0.4 ml plasma [5]. The main difference of the presented method from the preliminary method for **I** [5] is the use of a shorter precolumn (75 mm length) and on-line dilution of the injection solution using an additional pump and a T-piece. This resulted in shorter run times and, therefore, in a higher sample throughput.

2. Experimental

2.1. Materials, reagents and solvents

Glacial acetic acid (100%), ammonium acetate and absolute ethanol (all analytical-reagent grade) were obtained from E. Merck (Darmstadt, Germany), and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, UK). Water was distilled twice from an all-glass apparatus. Compounds **I** (Ro 63-1908/000) and **II** (Ro 25-6981/000, I.S.) were provided by Hoffmann-La Roche (Basel, Switzerland). Spiked plasma samples were prepared using fresh frozen plasma, either from EDTA human blood, obtained from a blood bank (Blutspendezentrum SRK, Basel, Switzerland), or from EDTA/NaF blood from our own laboratories (rat and cynomolgus monkey).

2.2. Solutions and standards

A 10% (w/v) solution of ammonium acetate was made up in bidistilled water (100 g/l).

A stock solution of the internal standard was prepared by dissolving 10.0 mg of **II** in 100 ml of

ethanol (100 μg/ml). An internal standard working solution was prepared by diluting 4 ml of the stock solution with ethanol to 1000 ml (400 ng/ml). This solution could be stored at room temperature for several weeks. The stock solution could be stored at 4°C for several months.

A stock solution of **I** was prepared by dissolving 10.0 mg in 100 ml of ethanol. Ultrasonication was used for complete dissolution. The stock solution was diluted with ethanol to give working solutions in the range 100–0.1 μg/ml. These working solutions were used as calibration standards by adding 0.1 ml to 9.9 ml of human plasma, yielding concentrations of 1000, 500, 100, 20, 5 and 1 ng/ml. The plasma calibration standards were stored at −20°C for several weeks. Spiked plasma standards for validation or quality control samples were prepared by spiking blank plasma with a small volume (normally 1%) of an adequately prepared standard working solution.

2.3. Sample preparation

To 0.4 ml of plasma, 1.2 ml of the internal standard working solution were added for protein precipitation. After vortex-mixing and storing for at least 15 min in the deep freezer at −20°C, the sample was centrifuged (6 min at ca. 18 000 *g* and 10°C), and the supernatant transferred to a 2-ml autosampler vial and 1.4 ml was injected. If less than 0.4 ml was used, blank plasma was added at 0.4 ml.

2.4. Chromatographic system and conditions

A schematic representation of the HPLC column-switching system is given in Fig. 2. A HPLC pump L-6000 (P1A; Merck), in combination with a solvent selector (SS; Labsource, Reinach BL, Switzerland), delivered mobile phase M1A (or alternatively M3). Aliquots (1.4 ml) were injected by the autosampler (AS; Model AS-4000A, Merck) onto the precolumn (PC). In order to inject large sample volumes, the autosampler was used with two 5-ml syringes as dilutors 1 and 2 [solvents: water–ethanol (100:4, v/v) and ethanol, respectively], a 3-ml sample loop, and the slow needle-down-speed. The injected sample plug was diluted, on-line, with mobile phase

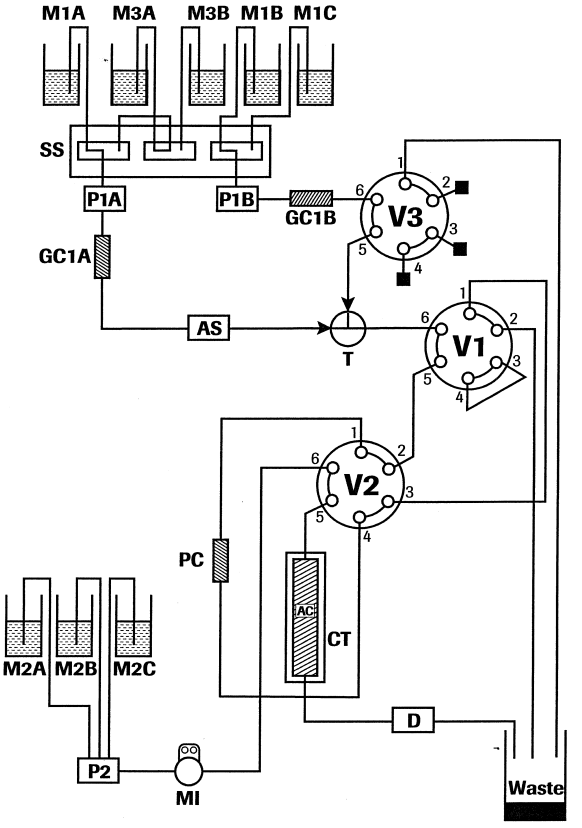


Fig. 2. Schematic representation of the HPLC column-switching system. See text for further details.

M1B by HPLC-pump P1B (L-6000, Merck) via a T-piece (Valco Instruments, Houston, TX, USA; 1/16 in., bore 0.25 mm; 1 in.=2.54 cm). The preconcentrated analyte and internal standard were eluted with mobile phase M1C using HPLC pump P1B. The gradient pump P2 (L-6200A, Merck) delivered mobile phase M2, which was degassed on-line (Solvent degaser SDU 2003, Labsource). A manual injector (MI; Model 7125 with a 200-μl loop, Rheodyne, Cotati, CA, USA) was used for direct injection onto the analytical column (e.g., for recovery experiments). Detection of the eluted compounds was carried out using a Waters 474 Scanning Fluorescence Detector (D; Waters, Milford, MA, USA; excitation 286 nm, emission 318 nm, gain 10, d.c. filter 3 s).

A HPLC ChemStation (Hewlett-Packard, Waldbronn, Germany), in connection with a laboratory-

developed laboratory information and management system (LIMS) (UNICHROM and KINLIMS [6]), was used for data acquisition and processing. The three electrically driven switching valves (V1–V3; high-speed valve 7000E, all Labsource) and the solvent selector were controlled by P2.

2.5. Columns and mobile phases

LiChroCART HPLC cartridges (all 4×4 mm I.D.) packed with LiChrospher 100 RP-18 (5 μm) were used as guard columns, GC1A and GC1B, and a LiChroCART HPLC cartridge (75×4 mm I.D.) packed with Purospher RP-18 (5 μm) was used as PC. The analytical column (AC) consisted of two LiChroCART HPLC cartridges (125+250×4 mm I.D.) packed with Superspher 60 RP-select B (4 μm) (all Merck) and was kept at 30°C by a column thermostat (CT; with Peltier controller unit PCU 1000, Labsource).

Mobile phase 1A (M1A) and 1B (M1B) were identical and were prepared by mixing 100 ml of 10% ammonium acetate with 900 ml of water and 20 ml of ethanol. Mobile phase 1C (M1C) was prepared by mixing 300 ml of acetonitrile with 700 ml of water. Mobile phase 2 (M2) consisted of three components: (M2A) 30 ml of 10% ammonium acetate, 600 ml of water, 350 ml of acetonitrile; (M2B) 50 ml of 10% ammonium acetate, 350 ml of water, 650 ml of acetonitrile; (M2C) 5 ml of 10% ammonium acetate, 20 ml of water, 980 ml of acetonitrile. Mobile phase 3A (M3A) and 3B (M3B) consisted of acetonitrile–water (9:1, v/v) and ethanol, respectively.

2.6. Procedure

The total sequence of automated sample analysis required 21 min. Details of the parameters used are shown in Table 1. The autosampler started the

Table 1
Column-switching parameters and gradient program

Time (min)	P2 gradient A (%):B (%):C (%)	P2 flow (ml/min)	P1A flow (ml/min)	P1B flow (ml/min)	P2 timer				Comment
					V1	V2	V3	SS	
0	100:0:0	0.05	0.05		10[a]	20			Injection of the sample
0.1		1.0	0.5	1.4				30,40	onto PC using M1A and
0.2							80	70	M1B for pre-concentration
4.9			0.5				81	71	of the analyte
5.0			3.4						
7.6		1.0	3.4						
7.7		0.7	0				80		PC was eluted with M1C
8.0			0			21			Transfer of the retained
8.7			2.0		11			31	components from PC to
9.0	100:0:0						81		AC. In the meantime, the
9.2		0.7				20			capillaries between AS and
9.3		1.0						70	waste were purged with
12.0				0				41	M3
14.0								40	
15.0								30	Re-equilibration of PC with
17.0	0:100:0								M1
19.0	0:100:0		2.0						
19.1	0:0:100		2.5						
20.0			2.5						
20.1			2.0						
20.9	0:0:100				10		80		
21.0	100:0:0	1.0	2.0						

[a] The first digit of the timer signal represents the address and the second one the activation.

gradient program of P2 and the HPLC ChemStation. P2 controlled the flow of P1A. In addition, the timer signals of P2 were also used for: (a) switching the valves (10/11, 20/21 and 80/81 means switching of V1, V2 and V3, respectively), (b) the solvent selector [30=M1A, 31=M3 (either M3A or M3B), 40=M3A, 41=M3B, 70=M1B, 71=M1C], and (c) to stop (62) P1B.

2.7. Calibration and calculations

Together with the unknown and quality control samples, six calibration standards, distributed over the whole set of samples, were processed as described above. The calibration curve ($y=a+bx$) was obtained by weighted linear least-squares regression (weighting factor $1/x^2$) of the measured peak-height ratios **I**/**II** (y) versus the concentration of **I** (x).

3. Results and discussion

3.1. Sample preparation

A HPLC method with automated column switching, which had been used successfully for the determination of several NMDA receptor blockers in biological samples [4], was adapted to the determination of the NMDA receptor NR2B subunit selective blocker **I**. The method is highly automated; the only off-line step is deproteinisation of the biological fluids, using three volumes of ethanol for precipitation of one volume of plasma. However, large volumes of ethanol-containing solutions would lead to an early breakthrough of the analytes on the precolumn. In the first method for **I** [5] and in all previous methods for NMDA receptor blockers [4], the elution strength of the supernatant was decreased by adding 2 ml of 2% ammonium acetate–ethanol (100:2, v/v) to the supernatant, followed by injection of 3.3 ml of the mixture. As the injection of such a large volume by the autosampler is time consuming, the supernatant in this method was diluted, on-line, using pump P1B and a T-piece. Mobile phase M1B had the same composition as M1A and contained mainly ammonium acetate. The low volume of ethanol in M1A and M1B was used for stability reasons, as it prevents bacterial growth in the vessels.

The addition of the internal standard to the plasma sample together with the solvent for protein precipitation had no influence on the recovery of the internal standard or the precision of the method and was, therefore, used to keep the manual steps as simple as possible.

The analysis of cerebrospinal fluid, urine and brain homogenates was performed with the first method for **I** [5] using off-line dilution of the supernatants. However, on-line dilution would also work for these matrices although no routine determinations were performed.

3.2. Chromatographic system

Purospher RP-18 is a non-endcapped stationary phase which has an interesting selectivity for amines [4,7]. A Purospher RP-18 (125×4 mm) column used as precolumn allowed pre-concentration of different NMDA receptor blockers with 1% ammonium acetate–acetic acid–acetonitrile (100:1:5, v/v/v) [4,5], or 1% ammonium acetate–ethanol (100:2, v/v) as in this method, followed by a selective elution of the analyte and the internal standard with about 25% acetonitrile and subsequent separation on a second reversed-phase column [4,5]. This resulted in a separation from most plasma interferences when a precolumn longer than 4 mm was used. Therefore, 25 and 75 mm long precolumns were also tested, allowing a reduction of the analysis time compared to the 125 mm precolumn. Although the 25 and 75 mm columns are not in the supplier's catalogue, they are available on request. However, the quality of the packing is not always as good as for 125 mm columns. Finally, 75 mm precolumns were used for this method, allowing sufficient stability for routine determinations. Thirty percent acetonitrile in M1C was used for the transfer of the analyte and the internal standard from the 75 mm precolumn to the analytical column. This percentage of acetonitrile gave more robust conditions for replicate analyses than 25%. Compared to the use of a 125 mm long precolumn and dilution of the supernatant prior to injection, the 75 mm long precolumn and the on-line dilution procedure allowed one third shorter run times (21 min) and, therefore, a higher sample throughput. About 200 injections of deproteinised

plasma samples could be made onto one 75 mm precolumn before it was replaced.

3.3. Selectivity

The Purospher RP-18 precolumn allowed a selective separation of the analyte and the internal standard from plasma interferences. The procedure may be considered as a two-dimensional chromatographic separation. It worked not only for **I**, **II** and compounds with similar structures [4], but also for Ro 24-6173, an NMDA receptor blocker with a morphinan structure [4]. All investigated compounds were tertiary amines, which could explain the similar selectivity. As with other modern stationary phases from other producers, Purospher RP-18 was especially developed for the elution of amines with minimal interaction with metal ions and silanol groups [7]. However, carboxylic acids appear to respond quite differently to the hydrophilic endcapping of this stationary phase [8]. This may be a disadvantage for its general use as an analytical column, but it is certainly a great advantage for its use as first column in two-dimensional chromatography of amines. All other tested stationary phases from other producers did not show this effect.

UV detection was also possible for the method for **I**. However, fluorescence detection was preferred due to its better selectivity. Typical chromatograms for plasma samples are shown in Fig. 3–6.

3.4. Recovery

Recovery from plasma was determined during replicate analysis, by comparison of peak heights of spiked plasma samples, processed as described above, with blank plasma samples to which the same amount of analyte was added after protein precipitation.

The results are presented in Table 2. The mean recoveries of **I** from rat, cynomolgus monkey and human plasma were 83.0%, 83.3% and 88.9%, respectively.

3.5. Linearity

The plasma method was linear in the range 1–1000 ng/ml, at least. Standard curves for **I** were calculated by means of weighted least-squares regression, using $1/x^2$ as weighting factor.

3.6. Limit of quantification

The limit of quantification was 1 ng/ml using 0.4

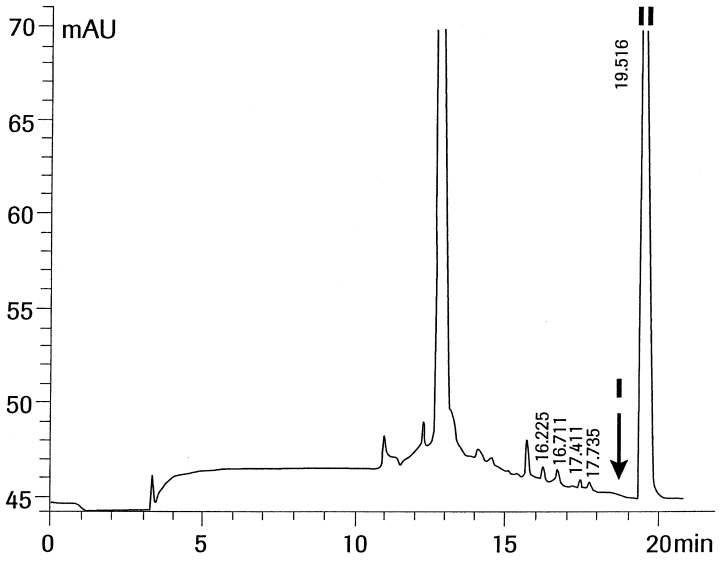


Fig. 3. Chromatogram of a rat blank plasma sample containing the internal standard **II**.

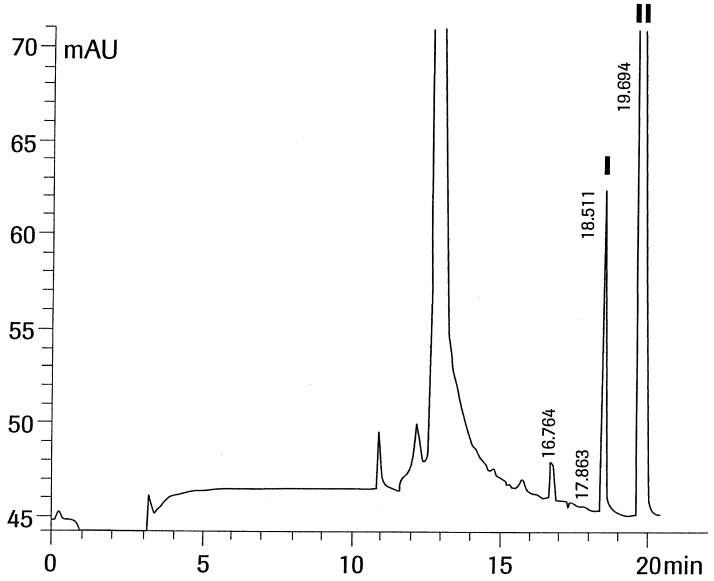


Fig. 4. Chromatogram of an unknown rat plasma sample taken 168 h after the start of a 14-day continuous intravenous infusion study (7.5 mg/kg/h). Measured concentration of **I**: 481 ng/ml. **II** is the I.S.

ml of plasma. The inter-assay ($n=6$) relative standard deviation (RSD) at this concentration was 2.9–7.2% and the accuracy was 99–109% (see Table 3). A chromatogram showing the limit of quantification of the method is presented in Fig. 7.

3.7. Precision and accuracy

The inter-assay precision (defined as the RSD of replicate analyses) and the accuracy (defined as the deviation between found and added concentration) of

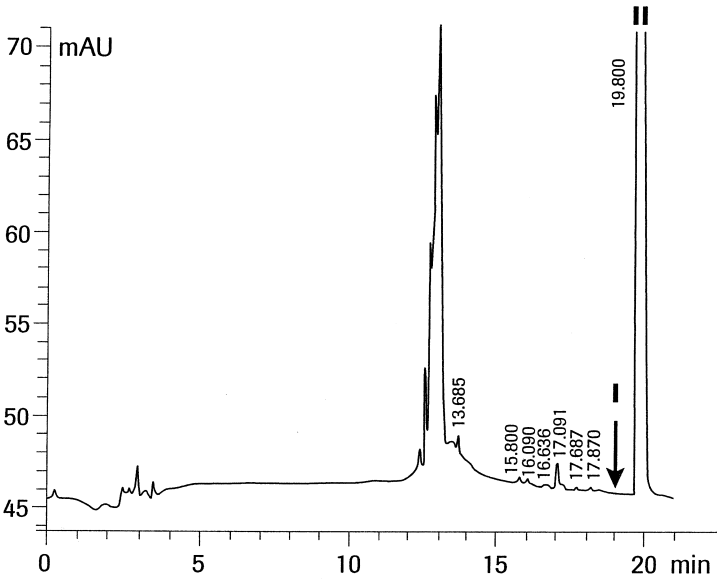


Fig. 5. Chromatogram of a cynomolgus monkey blank plasma sample containing the internal standard **II**.

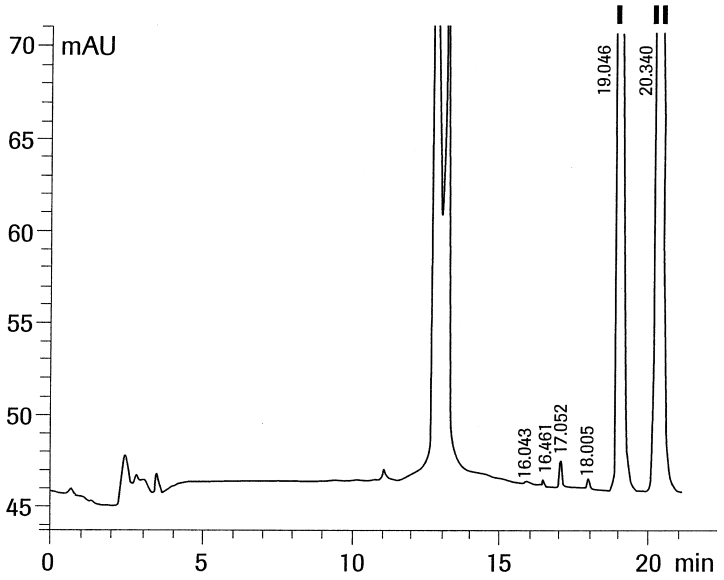


Fig. 6. Chromatogram of an unknown cynomolgus plasma sample taken on day 4 (at 0 h) after a continuous infusion of 1.5 mg/kg/h. Measured concentration of **I**: 856 ng/ml. **II** is the I.S.

the method were evaluated in rat, cynomolgus monkey and human plasma, by analysing spiked samples on several days, using a separate calibration set on each day. The results are compiled in Table 3. The mean inter-assay precision in the range 1–1000 ng/ml was 3.0%, 3.6% and 1.6% and the mean accuracy was 104.8%, 105.3% and 99.2% for rat, cynomolgus monkey and human plasma, respectively.

3.8. Stability

Stability tests were conducted according to our internal guidelines, which are based on a published procedure [9]. However, a decrease of 15% is now considered relevant, in contrast to 10% in the published procedure [9]. Storage of plasma samples at room temperature for 24 h or at −20°C for three months did not show a relevant decrease (see Tables 4 and 5).

Table 2
Recovery of **I** from plasma (n=5)

Species	Concentration (ng/ml)	Recovery (%)	RSD (%)
Rat	2	82.3	5.3
	1000	83.6	2.3
Cynomolgus monkey	2	85.0	1.5
	1000	81.6	1.4
Man	2	90.2	2.8
	1000	87.5	0.6

3.9. Application of the method to biological samples

The method described was successfully applied to the analysis of more than 670 plasma samples from pharmacokinetic and toxicokinetic studies in rats and cynomolgus monkeys. Figs. 3–6 show typical chromatograms from these studies, demonstrating the validity of the assay.

4. Conclusions

A HPLC method with automated column switching was developed and validated (for GLP requirements) for the determination of the NMDA receptor NR2B subunit selective blocker I in rat and

Table 3
Inter-assay precision and accuracy ($n=6$)

Species	Concentration added (ng/ml)	Concentration found (ng/ml)	RSD (%)	Accuracy (%)
Rat	1	1.06	4.7	106.0
	20	20.7	2.7	103.5
	1000	1050	1.5	105.0
Cynomolgus monkey	1	1.09	7.2	109.0
	20	20.6	2.3	103.0
	1000	1040	1.2	104.0
Man	1	0.99	2.9	99.0
	20	19.5	1.0	97.5
	1000	1010	0.8	101.0

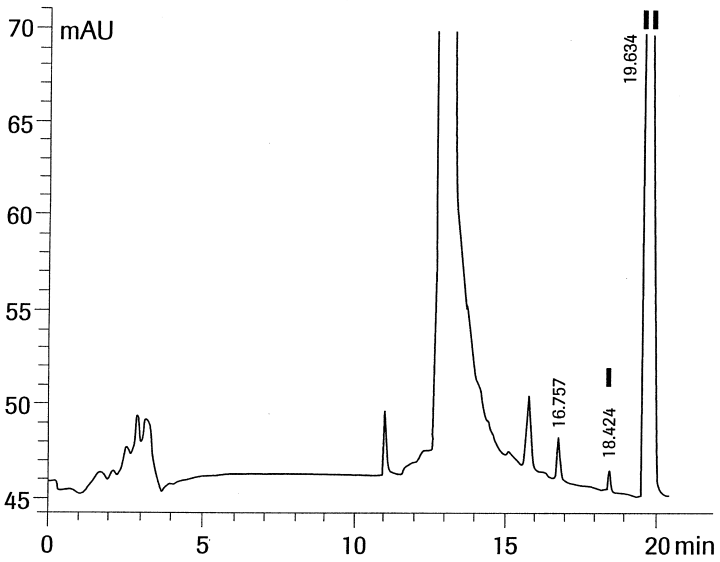


Fig. 7. Chromatogram showing the quantification limit of the method. Human plasma sample spiked with 1 ng/ml of **I**. **II** is the I.S.

Table 4
Stability of **I** in plasma after storage for 24 h at 22°C ($n=5$)

Species	Concentration (ng/ml)	Deviation (%)	90% confidence interval (%)
Rat	5	−1.9	−5.2/+1.4
	1000	+1.8	−0.5/+4.2
Cynomolgus monkey	5	−2.3	−4.0/−0.5
	1000	−1.0	−3.1/+1.1
Man	5	+1.2	−1.0/+3.5
	1000	+0.5	−1.1/+2.2

Table 5
Stability of **I** in plasma after storage for three months at −20°C (n=5)

Species	Concentration (ng/ml)	Deviation (%)	90% confidence interval (%)
Rat	5	−10.9	−12.4/−9.3
	1000	−6.6	−7.3/−5.8
Cynomolgus monkey	5	−8.6	−10.1/−7.1
	1000	+2.7	+1.1/+4.5
Man	5	−8.8	−10.1/−7.4
	1000	−7.7	−8.6/−6.8

cynomolgus monkey plasma. Human plasma was used for calibration and was also included in the validation process. Plasma samples were deproteinised with ethanol and the supernatant directly injected. To prevent a breakthrough of the analyte and the internal standard on the precolumn due to the high ethanol content, the supernatant was diluted, on-line, using an additional pump and a T-piece. Highly selective pre-concentration and elution on a Purospher RP-18 precolumn (75×4 mm I.D.) resulted in high sensitivity and selectivity. Compared to the use of a 125 mm long precolumn and dilution of the supernatant prior to injection, the shorter precolumn and the on-line dilution procedure allowed one third shorter run times (21 min) and, therefore, a higher sample throughput. The limit of quantification was 1 ng/ml using 0.4 ml plasma which should also be sufficient for a single ascending dose study in human volunteers. The method was applied to more than 670 plasma samples from pharmacokinetic and toxicokinetic studies in rats and cynomolgus monkeys and is also suitable for the determination of other NMDA receptor blockers.

Acknowledgements

The authors thank Dr. D. Lubda for fruitful discussions on the Purospher RP-18 column, Drs. D. Dell and G. Hopfgartner for reading the manuscript and Mr. Hp. Suter for the drawings.

References

[1] G. Fischer, V. Mutel, G. Trube, P. Malherbe, J.N.C. Kew, E. Mohacsi, M.P. Heitz, J.A. Kemp, J. Pharmacol. Exp. Ther. 283 (1997) 1285.
[2] A. Viger-Chougnet et al., personal communication.
[3] M. Schmitt et al., personal communication.
[4] R. Wyss, F. Bucheli, W. Philipp, J. Chromatogr. A 797 (1998) 211.
[5] R. Wyss, F. Bucheli, W. Philipp, unpublished data.
[6] U. Timm, B. Hirth, in: E.J. Karjalainen (Ed.), Scientific Computing and Automation (Europe) 1990, Elsevier, Amsterdam, 1990, p. 329.
[7] D.V. McCalley, J. Chromatogr. A 769 (1997) 169.
[8] D. Lubda, personal communication.
[9] U. Timm, M. Wall, D. Dell, J. Pharm. Sci. 74 (1985) 972.