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Microbore liquid chromatography with UV detection to study the *in vivo* passage of compound 21, a non-peptidergic AT_2 receptor agonist, to the striatum in rats

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ABSTRACT

A microbore liquid chromatography method coupled to UV detection was developed and validated in order to monitor the passage of compound **21** (C**21**), a non-peptide angiotensin II type 2 receptor agonist, to the striatum of rats. For this purpose, sampling from the striatum was performed using the *in vivo* microdialysis technique. Separations were performed on a C_{18} microbore (1 mm i.d.) column using gradient elution. The retention time for C**21** was found to be 6.3 min. The calibration curve was linear between 10 and 200 ng/ml with a correlation coefficient ≥0.999. The limit of detection and the limit of quantification were 3 and 10 ng/ml respectively. The intra-day and the inter-day precision (RSD%) ranged between 0.5 and 4.6% with an average recovery of 101.5 ± 10.0% (mean ± SD, $n = 15$). *In vivo* experiments were performed on rats to measure the concentration of C**21** in striatal dialysates after intraperitoneal (10 or 50 mg/kg) or intravenous injection (10 mg/kg or 20 mg/kg) of C**21** and suggest minimal passage of the compound to the striatum.

1. Introduction

In addition to the well-known systemic renin–angiotensin system (RAS), local RAS systems have been identified in different organs and tissues including the brain (Fyhrquist and Saijonmaa, 2008). Several studies have linked the central RAS to neurological disorders such as ischaemia, Alzheimer's and Parkinson's diseases (Phillips and de Oliveira, 2008; Horiuchi et al., 2010; Mertens et al., 2010a).

The major effector peptide of the RAS, angiotensin II (Ang II), mainly mediates its actions via two receptor subtypes namely the Ang II type 1 (AT_1) receptor and the Ang II type 2 (AT_2) receptor. Most of the pharmacological effects of central Ang II, including vasoconstriction, vasopressin release, retention of salt and water, cell growth and aldosterone release from the adrenal gland, are mediated by the AT_1 receptor (de Gasparo et al., 2000; Phillips and de Oliveira, 2008; Lemarié and Schiffrin, 2010). The AT_1 receptor is widely expressed at relatively constant levels in adults, while expression of the AT_2 receptor is high during fetal development and rapidly declines in most organs after birth (de Gasparo et al., 2000; Mertens et al., 2010b; Unger and Dalhöf, 2010). In adults, the AT_2 receptor has been localized in the heart, kidney, adrenal gland, brain areas involved in cognition and behavior, uterus, pancreas, retina, skin and endothelial and vascular smooth muscle cells (Jones et al., 2008; Mertens et al., 2010a). The activation of the AT_2 receptor is assumed to be associated with cell proliferation, cell differentiation, tissue regeneration and apoptosis (Stroth et al., 1998; de Gasparo et al., 2000; Culman et al., 2002; Gendron et al., 2003; Mertens et al., 2010a). Moreover, some researchers reported that stimulation of the AT_2 receptor has a neurotrophic effect on central nervous system neurons *in vitro* and *in vivo* (Stoll and Unger, 2001; Okada et al., 2006). An increase in the AT_2 receptor expression has been observed under several pathological conditions, including neurodegenerative diseases such as Huntington's and Alzheimer's diseases (Steckelings et al., 2005; Mertens et al., 2010a). In Parkinson's disease on the other hand, AT_2 receptor expression in the brain was diminished (Ge and Barnes, 1996) or remained unaltered (Ge and Barnes, 1996; Grammatopoulos et al., 2007).

Although the exact mechanism of Parkinson's disease remains unidentified, inflammation and oxidative stress have been suggested as key factors in the pathogenesis and progression of the disease. Accumulating evidence indicates that the brain RAS is involved in the degeneration of dopaminergic neurons and

Abbreviations: Ang II, angiotensin II; AT_1, angiotensin II type 1 receptor; AT_2, angiotensin II type 2 receptor; BBB, blood–brain barrier; C**21**, compound **21**; i.p., intraperitoneal; i.v., intravenous; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrometry; RAS, renin–angiotensin system; RL, relative loss; QC, quality control.

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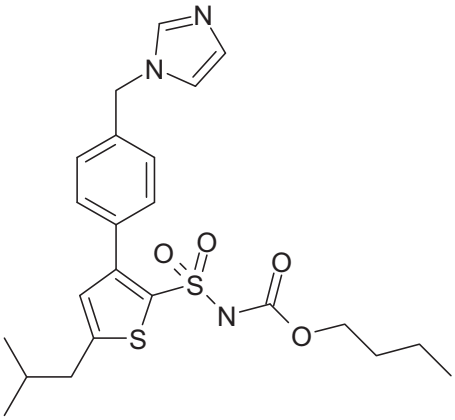

Fig. 1. Chemical structure of C**21**.

the progression of Parkinson's disease (Mertens et al., 2010a). Stimulation of the AT_2 receptor is considered a plausible approach to achieve neuroprotection in Parkinson's disease. Until recently, the study of AT_2 receptor mediated effects was hampered by the lack of a non-peptidergic and highly selective AT_2 receptor agonist (Unger and Dalhöf, 2010; Mertens et al., 2010a). The development of the selective (K_i = 0.4 nM for AT_2 receptor, K_i > 10 μM for the AT_1 receptor) and potent non-peptide AT_2 receptor agonist compound 21 (C**21**) (Fig. 1) allowed to study the role of the AT_2 receptor in dopamine release and synthesis in the striatum of the rat (Wan et al., 2004; Okada et al., 2006; Mertens et al., 2010b). Indeed, we recently showed that C**21** is involved in the modulation of dopamine synthesis since perfusion of C**21** into the striatum of rats did not alter dopamine release but reduced extracellular levels of its major metabolite 3,4-dihydroxyphenylacetic acid *in vivo* (Mertens et al., 2010b). Furthermore, intraperitoneal (i.p.) injection of C**21** had no effect on the striatal levels of dopamine and its major metabolite questioning whether C**21** crosses the blood–brain barrier (BBB) and reaches the striatum after systemic administration. Therefore, the aim of this work was to develop and validate a liquid chromatographic (LC) method coupled to UV detection to quantify C**21** in striatal samples, in order to monitor its passage to the striatum. For this purpose, sampling from the striatum was performed using the *in vivo* microdialysis technique.

2. Experimental

2.1. Chemicals

C**21** was kindly donated by Vicore Pharma (Göteborg, Sweden). Acetonitrile (ACN, HPLC-grade) and formic acid (FA, LC–MS grade) were obtained from Acros Organics (NJ, USA). Glacial acetic acid (analytical reagent grade) was purchased from Fisher Scientific (Loughborough, UK). The purified water used for preparing solutions was obtained from a Seralpur Pro 90 CN purification system (Seral, Ransbach-Baumbach, Germany). The modified Ringer's solution was prepared and filtered through a 0.2 μm pore size membrane filter, and consisted of 147 mM NaCl (Sigma–Aldrich, Bornem, Belgium), 4 mM KCl (Merck, Darmstadt, Germany) and 1.1 mM $CaCl_2 \cdot 6H_2O$ (Fluka, Buchs, Switzerland). The Ringer's solution was stored in the refrigerator at 4 °C for one week.

2.2. Stock and working solutions

The stock solution of C**21** (1 mg/ml) was made in ACN. The in-between stock solution of 10 μg/ml was obtained by diluting 100 μl of the stock solution up to 10 ml ACN. The stock solution and the in-between stock solution were made every six weeks. The working standard solutions of serial concentrations (0.2, 0.4, 0.6, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 μg/ml) were made weekly by diluting the in-between stock solution with purified water. Stock and working solutions were stored at 4 °C.

2.3. Preparation of standards and quality control (QC) samples

Calibration standards (10–200 ng/ml) were prepared daily by mixing 10 μl of the working standard solutions with 90 μl of purified water and 100 μl of mobile phase B (water:ACN:FA 20:80:0.1 V/V/V). The same procedure was applied to prepare the QC samples using 90 μl of blank dialysate instead of 90 μl water.

2.4. Preparation of solutions for intraperitoneal (i.p.) and intravenous (i.v.) injection

Two solutions were prepared for i.p. injection of C**21**, i.e. 10 mg of C**21** was dissolved in 1 ml physiological saline to administer a dose of 10 mg/kg. A solution containing 50 mg/ml physiological saline was prepared for the i.p. injection of 50 mg/kg. The same procedure was used to obtain the solutions for i.v. injection (10 and 20 mg/kg).

2.5. LC conditions

All experiments were performed on an Ultimate 3000 Quaternary Micro system (Dionex, The Netherlands) consisting of an autosampler, a pump, a flow manager, and a UV detector. Separations were performed on a Unijet™ BAS C_{18} (Bioanalytical Systems Inc., West Lafayette, USA) column (15 cm × 1.0 mm i.d., 5 μm particles). The flow-rate was 50 μl/min. The injection loop was 20 μl and the detection wavelength was set at 271 nm. The needle was washed with ACN:water 1:1 (V/V). Gradient elution was performed using mobile phase A (water:ACN:FA 98:2:0.1 V/V/V) and mobile phase B (water:ACN:FA 20:80:0.1 V/V/V). The mobile phases were degassed for 15 min on a Branson 5200 ultrasonic bath (Danbury, CT, USA) after preparation. The linear LC gradient was 0–100% B in 5 min, 100% B for 2 min, 100–0% B in 0.1 min and 0% B for 8 min. The total run time was 15 min. All LC parts were controlled by Chromeleon® version 6.80 (Dionex).

2.6. In vivo experiments

2.6.1. Animals

All animal experiments were carried out according to the national guidelines [Belgian guideline on the protection of laboratory animals (KB Nov 1993) and the Revised European Guideline (Appendix E to ETS123)] on animal experimentation and were approved by the Ethical Committee for Animal Experiments of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel. All efforts were made to minimize animal suffering and the minimal number of animals necessary to produce reliable scientific data was used. The experiments were performed on male albino Wistar rats (Charles River, Brussels, Belgium), weighing 250–320 g. During the actual experimental procedures, rats were placed in experimental cages and allowed to move freely. All animals had access to tap water and standard laboratory chow *ad libitum*.

2.6.2. Surgical procedures and stereotaxic implantation of the microdialysis probe

Rats were first anaesthetized with a mixture of ketamine (66.7 mg/kg i.p.; Ketamine 1000 Ceva®, Ceva Sante Animale, Brussels, Belgium) and diazepam (5 mg/kg i.p.; Valium®, Roche, Brussels, Belgium) and placed on a Kopf stereotaxic frame (ear bars positioned symmetrically). The skull was exposed and two burr holes were drilled to implant the guide cannulae (MAB 2/6/9.14.IC,

Microbiotech/se AB, Stockholm, Sweden) positioned 4 mm above the left and right dorsal striata according to the atlas of Paxinos and Watson (1998) (coordinates relative to bregma:L: ± 2.4, A:+1.2 and V:+2.3). The guide cannula was fixed to the skull with dental acrylic cement. After surgery, a microdialysis probe (MAB 6.14.4, Microbiotech/se AB) with a membrane length of 4 mm and a molecular cut-off value of 15 kDa was introduced via the cannula in both striata. For i.v. administration of C**21** an indwelling cannula was placed in the right femoral vein and was subcutaneously tunneled to the back of the neck of the rats and exteriorized. Cannula (i.d. = 0.58 mm; o.d. = 0.96 mm) were made from 20 cm pyrogen-free polyethylene tubing (Portex Limited, Hythe, Kent, UK). The correct placement of the i.v. catheter was evaluated after the experiment by killing the rats with an overdose Nembutal® (Ceva Sante Animale). In all rats the catheter was correctly placed. The animals received ketoprofen (4 mg/kg i.p.; Ketofen®, Merial, Brussels, Belgium) as analgesic. The probes were continuously perfused with modified Ringer's solution at a constant flow rate of 0.5 μl/min using a microdialysis pump (CMA 100; CMA Microdialysis, Solna, Sweden). Animals were allowed to recover from surgery overnight and dialysate collection was started the following day.

2.6.3. *In vivo microdialysis experiments*

Samples were collected every 45 min, yielding 22.5 μl dialysate, in vials containing 22.5 μl of mobile phase B. The samples from both striata were gathered and mixed. Eighty microliter was transferred to the injection vial and injected immediately on the LC system. At least two dialysate samples were collected before the administration of C**21**, either i.p. at a dose of 10 or 50 mg/kg or i.v. at a dose of 10 or 20 mg/kg. Three samples were collected, starting immediately after systemic administration of the compound.

2.6.4. *In vivo probe recovery*

In order to correctly estimate the real free extracellular C**21** concentration based on its striatal dialysate concentration, the *in vivo* probe recovery needs to be determined.

The present study, *in vivo* probe recovery was estimated using the retrodialysis technique. In this approach, the relative loss (RL) was used to estimate the probe recovery. RL is defined as the ratio of the difference between perfusion fluid (C_{in}) and dialysate (C_{out}) concentration of the compound of interest over the perfusion fluid concentration (Scheller and Kolb, 1991). For the estimation of the RL of C**21** *in vivo*, two probes were stereotactically implanted in the left and the right striatum of rats weighing 250–320 g and continuously perfused with a 400 ng/ml solution of C**21** in modified Ringer's solution at a flow-rate of 0.5 μl/min. Following overnight equilibration, collection of the samples started the next day. Samples were collected every 90 min in a vial containing 45 μl of mobile phase B. The samples were transferred to the injection vial and injected on the LC system. Three samples from each striatum were collected. The RL of the compound was calculated using the following formula.

$$\mathrm{RL} = \frac{C_{in} - C_{out}}{C_{in}} \times 100\%$$

2.7. *Assessment of plasma protein binding*

Binding of drugs to plasma proteins is one of many factors that influences drug disposition. The effect of a drug is related to the exposure of a patient to the unbound concentration of drug in plasma rather than the total concentration (Kratochwil et al., 2002). Since only the unbound fraction of the drug is able to cross the BBB, the knowledge of the plasma protein binding of this compound is important.

Plasma was obtained by withdrawing blood from the heart of the rat after being euthanized by CO_2. The blood samples were collected in EDTA collection tubes and centrifuged at 12,000 rpm for 3 min. 190 μl of pooled plasma was spiked with 10 μl of 150, 333.3 or 666.7 μg/ml of C**21** standard solutions resulting in nominal plasma concentration of 7.5, 16.7 and 33.3 μg/ml respectively. The spiked plasma was vortexed for 1 min before being incubated at 37 °C for 30 min. The mixture was divided into two equal portions. The first portion was used to determine the total plasma drug concentration. For this, protein precipitation was performed on 100 μl by adding 300 μl of ACN. After mixing for 1 min, the sample was centrifuged at 10,000 rpm for 10 min. The supernatant was diluted and analyzed in duplicate for C**21**.

The second portion was used to determine the free drug concentration. Plasma was centrifuged at 13,000 rpm for 20 min. The supernatant was diluted and injected in duplicate to obtain the free concentration. Based on the total and unbound fractions, the plasma protein binding was estimated according to

$$\mathrm{PB\%} = \frac{C_t - C_f}{C_t} \times 100\%$$

where C_t is the total plasma drug concentration and C_f is the free drug concentration.

3. Results and discussion

3.1. *Method development and optimization*

Previously, Wan et al. (2004), to verify the synthesis of the compound, developed an analytical LC–mass spectrometry (MS) method using a Zorbax SB-C_8 column as stationary phase and water, ACN and 0.05% FA as mobile phase. No further details concerning the mobile phase composition were given. For evaluating the oral absorption of C**21** in rats, a LC–MS/MS was described. This method consisted of a Polaris column as stationary phase, and ACN and 10 mM aqueous ammonium acetate as mobile phase. Gradient elution was performed (Wan et al., 2004). Also in this case, no further information concerning the gradient program was available.

Since low concentrations of C**21** need to be detected to evaluate its passage to the striatum after systemic administration, a miniaturized microbore LC–UV system (1 mm i.d. column) was chosen in this study. As starting conditions, a C_{18} stationary phase in combination with a mobile phase consisting of water, ACN and acetic acid (65:35:1 V/V/V) was chosen.

The influence of the acid in the mobile phase was studied by comparing the separation performance of acetic acid (1%) with formic acid (0.1%). Shorter retention times and smaller peak widths were obtained when using formic acid. In addition, a more stable baseline was obtained. Therefore, formic acid was used in all further experiments. To further decrease the peak width, gradient elution was investigated. The optimized LC gradient was as follows: from 0 to 100% B in 5 min, 100% B for 2 min, 100 to 0% B in 0.1 min and 0% B for 8 min. The total run time was 15 min. Under these conditions peak width decreased from 0.44 min to 0.14 min.

In order to further increase the sensitivity of the method, several detection wavelengths were investigated. The highest peak areas were achieved at the maximal detection wavelength of C**21**, i.e. 271 nm. In addition, to obtain a linear relationship between the concentration and the peak area of C**21**, it was necessary to dilute all standards and samples with mobile phase B, i.e. water:ACN:FA 20:80:0.1 (V/V/V), in a 1:1 ratio.

Fig. 2D shows the chromatogram of a 100 ng/ml standard solution of C**21** using the optimized conditions. The retention time of this AT_2 receptor agonist is 6.33 ± 0.01 min (mean ± SD, n = 12).

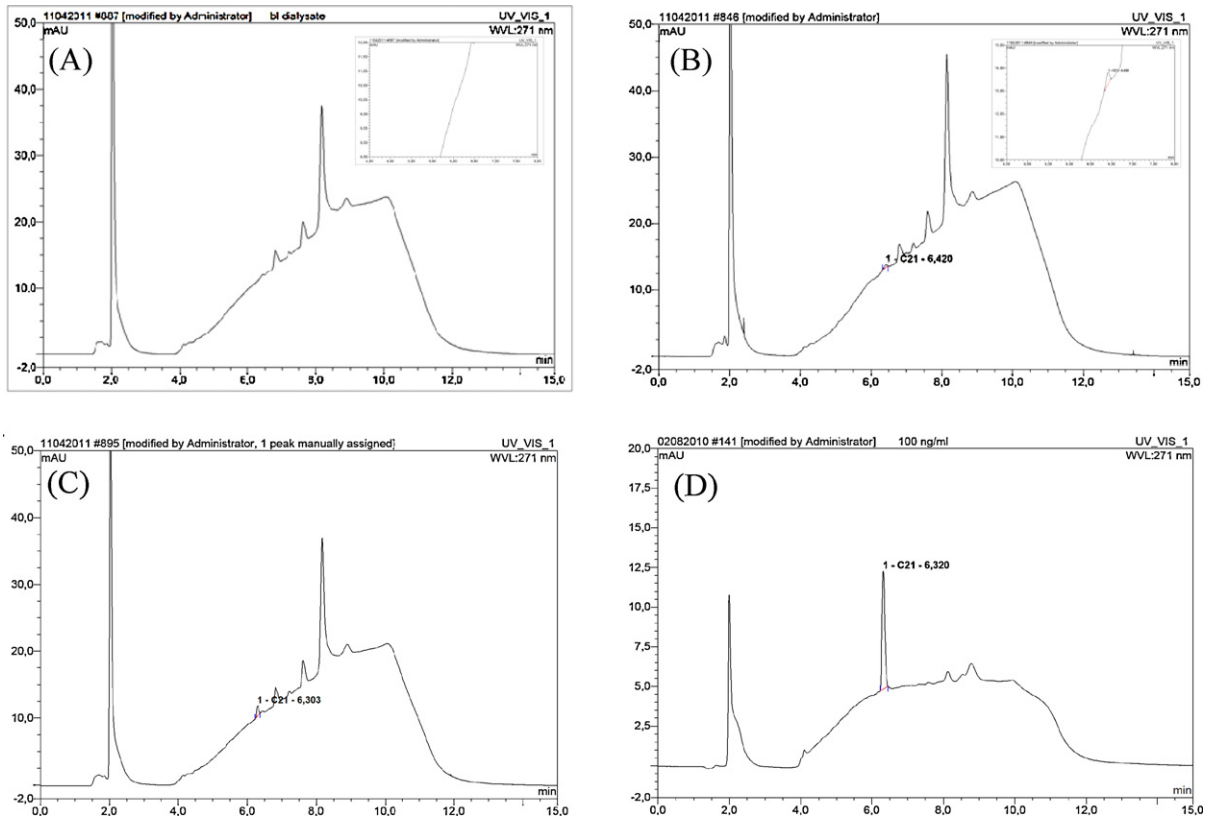

Fig. 2. Typical chromatograms of (A) blank dialysate, (B) blank dialysate spiked at 3 ng/ml (LOD), (C) blank dialysate spiked at 10 ng/ml (LOQ) and (D) 100 ng/ml of C**21** standard solution. Peak 1 indicates C**21**.

3.2. Method validation

The optimized method was validated according to the FDA guidelines for bioanalytical method validation, in terms of selectivity, linearity, accuracy, precision and stability (Guidance for Industry, 2001).

3.2.1. Selectivity

The selectivity of the method was assessed by comparing the chromatograms of six blank dialysates obtained from different rats with dialysate samples spiked at 10 ng/ml. As shown in Fig. 2(A and C), no significant interference peaks appeared at the retention time of C**21**, indicating good selectivity of this method.

3.2.2. Limits of detection and quantification

The limit of detection (LOD) was estimated as the concentration yielding a signal to noise of 3. The limit of quantification (LOQ) was defined as the lowest concentration that can be determined with acceptable accuracy and precision (<20%). The LOD and LOQ were determined as 3 and 10 ng/ml respectively (Fig. 2 B and C).

3.2.3. Linearity

Linearity of the calibration curve was evaluated using calibration standards of 10, 20, 30, 50, 75, 100, 125, 150 and 200 ng/ml. All standard solutions were analyzed in duplicate and on five different days. The calibration curves were constructed using linear regression of the peak area versus the nominal concentration. A linear relationship within the studied concentration range was obtained. These calibration curves were compared to calibration curves made with standard solutions of C**21** dissolved in Ringer's solution. No significant difference was observed between the calibration curves made in water and Ringer's solution ($F_{1;12}$; $p = 1$). Therefore, all calibration curves were constructed using standard solutions prepared with water. The linear regression equations are given in Table 1.

3.2.4. Analytical precision

Analytical precision was determined by repeatability (intra-day) and intermediate precision (inter-day), determined by repeated injection of standard solution of 10, 100 and 200 ng/ml on the same day and on five different days. The intra-day and interday precision ranged from 1.6 to 7.7% and 2.5 to 10.8% RSD respectively.

3.2.5. Accuracy and method precision

Accuracy was assessed by analyzing five replicates of blank striatal dialysate spiked with 10, 100 and 200 ng/ml of C**21**. The percentage of recovery was determined comparing the experimental values, calculated using the calibration line constructed with standards in water, to the nominal values. An average recovery of $101.5 \pm 10.0\%$ (mean $\pm$ SD, $n = 15$) was obtained. Method precision was evaluated using the data of the accuracy testing. Method repeatability was assessed by analyzing five replicates of each sample within one day and it was found to range from 2.8 to 3.8% RSD. Intermediate precision was obtained by analyzing each concentration level on five days. The RSD values for intermediate precision ranged from 0.5 to 4.6%. The obtained values are within the acceptance criteria (not more than 20% at LOQ and not more than 15% for the others). Therefore, the method can be considered accurate and precise for this application. Table 2 summarizes the obtained results.

3.2.6. Stability

The stability experiments were performed in triplicate and at three concentration levels in striatal dialysate (30, 100 and

Table 1
Linear regression equations of the calibration curves made with standard solutions prepared in water or Ringer's solution. The equations are expressed as mean ± SD ($n = 5$).

	Slope	Intercept	Correlation coefficient (r)
Water	0.00666 ± 0.00009	−0.0205 ± 0.0108	0.9996 ± 0.0002
Ringer's	0.00610 ± 0.00025	−0.0031 ± 0.0141	0.9994 ± 0.0003

Table 2
Method precision and accuracy for C**21** in dialysates. The observed concentration is expressed as mean ± SD ($n = 5$).

Nominal concentration (ng/ml)	Observed concentration (ng/ml)	Method precision RSD %	Accuracy %
Intra-day			
10	11.4 ± 0.3	3.1	114
100	94.9 ± 3.5	3.8	95
200	195.6 ± 5.4	2.8	98
Inter-day			
10	11.5 ± 0.4	4.6	115
100	92.7 ± 1.0	1.1	93
200	190.4 ± 0.9	0.5	95

Table 3
Stability (%) = mean measured concentration ($n = 3$) at the indicated time divided by mean measured concentration ($n = 3$) at zero time × 100.

Nominal concentration (ng/ml)	Stability %					
	Room temperature		Freeze–thaw			Long term freezer storage at −20 °C
	5 h	24 h	Cycle 1	Cycle 2	Cycle 3	8 weeks
30	97.8	91.2	98.6	95.9	95.0	92.2
100	98.7	96.4	99.6	99.0	99.0	97.6
200	99.4	98.2	99.8	99.5	99.5	98.4

200 ng/ml). Three different stability experiments were performed. During the first experiment, three aliquots of each sample were allowed to stand on the bench-top for 5 h and 24 h at room temperature. Secondly, the freeze and thaw stability was assessed by storing three aliquots of each concentration at −20 °C for 24 h, whereafter they were thawed unassisted at room temperature. The samples were injected on the LC system and afterwards restored in the freezer. This procedure was repeated two times to obtain three freeze-thaw cycles. Thirdly, samples were stored at −20 °C for 8 weeks before being analyzed to assess long term freezer storage stability. C**21** was stable under all conditions tested (Table 3).

3.3. *In vivo experiments*

3.3.1. *Quantification of C**21** in rat striatal dialysates after systemic administration*

The validated microbore LC–UV method was applied to investigate the passage of C**21** to the striatum of the rat. For this purpose, a microdialysis probe was implanted in both striata of the rat. C**21** was given either i.p. or i.v. to the rats. After i.p. administration of 10 mg/kg C**21** to the rats ($n = 3$), C**21** was only detected in the striatal dialysate of one out of three rats. In this rat a low concentration of 35 ng/ml was detected and only during one collection period. Since C**21** was not, or almost not, detected after i.p. injection of 10 mg/kg, a higher dose of 50 mg/kg was also investigated ($n = 3$). Only in one rat, C**21** was detected in the striatal dialysate collected immediately after i.p. administration of 50 mg/kg. In this case, only 10 ng/ml was detected which is even lower than the concentration measured after i.p. injection of 10 mg/kg.

To be sure that all C**21** administered to the rat reaches the blood stream, i.v. injection of this AT_2 receptor agonist was investigated. Both i.v. administration of 10 mg/kg and 20 mg/kg were performed. After the 10 mg/kg i.v. dose, 15 ng/ml was measured in one rat. Approximately the same concentration was obtained when administering 20 mg/kg i.v. to the rat.

3.3.2. *In vivo probe recovery*

The average *in vivo* probe recovery was estimated to be 43.0 ± 2.3% (mean ± SD, $n = 6$). Accordingly, extracellular C**21** concentration was estimated to be maximal 82 ng/ml.

3.4. *In vitro plasma protein binding*

The average plasma protein binding was found to be 8.25 ± 2.75% (mean ± SD, $n = 24$). These values are consistent with a previous report suggesting that C**21** may be widely distributed into the tissues (Wan et al., 2004). Table 4 summarizes the obtained results.

Despite the favorable small protein binding of C**21** and sufficient microdialysis probe recovery, our *in vivo* experiments suggest that C**21** may not be able to reach the striatum after systemic application, at least not in a consistent, reproducible way. These results are further supported by the fact that the striatal levels of dopamine and its major metabolite remained unchanged after systemic administration of C**21** (50 mg/kg i.p.), despite its effect on dopamine synthesis after local perfusion of the compound (0.1 μM) (Mertens et al., 2010b). In adult animals, the AT_2 receptor expression is limited, also in brain and restricted to a few organs. AT_2 receptor is only re-expressed under pathological conditions and traumatic injuries (Unger and Dalhöf, 2010). Even so, we cannot exclude the impact of C**21** binding to the receptor on free extracellular concentration in striatum.

Table 4
C**21** plasma protein binding. The % plasma protein binding is expressed as mean ± SD ($n = 8$).

Nominal spiked plasma concentration (μg/ml)	C**21** plasma protein binding %
7.5	9.04 ± 2.95
16.67	8.63 ± 2.75
33.33	7.07 ± 2.48

Beneficial effects of systemic administration of C**21** were observed in spontaneously hypertensive stroke-prone rats (Gelosa et al., 2009). Disruption of the BBB has been detected in many neurodegenerative diseases, including stroke (Van Hemelrijck et al., 2005; Sironi et al., 2004; Guerrini et al., 2002). In our study, BBB intactness was evaluated by i.v. injection of Evans blue (2 ml of 5% solution). A dialysate sample was collected during 2 h after injection and afterwards measured at 655 nm using a Microplate reader (Bio Rad Laboratories Ltd., UK). The BBB was shown to be intact.

4. Conclusions

To our knowledge, this is the first paper describing a validated microbore (1 mm i.d. column) LC–UV method for the determination of the selective non-peptidergic AT_2 receptor agonist compound 21 in striatal dialysate samples of the rat. This method was used to investigate the *in vivo* passage of this compound to the striatum of rats. The results showed minimal passage of this non-peptidergic AT_2 receptor agonist to the striatum.

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