Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 845 (2007) 109–113
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of the dopamine agonist rotigotine in microdialysates from the rat brain by microbore column liquid chromatography with electrochemical detection

Jan Kehr[a,b], Xiao-Jing Hu[b], Takashi Yoshitake[a], Dieter Scheller[c,*]

[a] *Department of Physiology and Pharmacology, Karolinska Institutet, SE-171 77, Stockholm, Sweden*
[b] *Pronexus Analytical AB, Karolinska Science Park, 171 77 Stockholm, Sweden*
[c] *Department Pharmacology/Toxicology, Schwarz Biosciences GmbH, Alfred-Nobel-Strasse 10, D-40789 Monheim, Germany*

Received 7 October 2005; accepted 28 July 2006
Available online 18 September 2006

Abstract

Rotigotine, an investigational dopamine agonist formulated as a patch, is being studied in Parkinson's disease. A microdialysis technique, in combination with microbore column liquid chromatography and electrochemical detection, was developed to monitor rotigotine levels in the brain. Microdialysis probes were inserted into the striata of anesthetized rats, and samples were collected during perfusion with Ringer's solution. Rotigotine was separated using a C18 reversed-phase column. The mobile phase consisted of 50 mM $Na_2HPO_4 \cdot 2H_2O$, 2.5 mM sodium octyl sulfonate, and pH 4.5; 35% volume to volume acetonitrile. The flow rate was 30 μl/min, and the potential of the glassy carbon electrode was set to +850 mV. The method allowed monitoring of the time course of brain extracellular rotigotine levels with a detection limit of 1 nM following either intravenous (0.5 mg/kg) or subcutaneous (5.0 mg/kg) rotigotine injection.

Keywords: Rotigotine; Microdialysis; Electrochemical detection; Liquid chromatography

1. Introduction

Rotigotine is a new, non-ergolinic dopamine receptor agonist currently in development for the treatment of Parkinson's disease and restless leg syndrome. From a pharmacokinetic point of view, characterizing rotigotine levels in the brain is of interest.

With in vivo *microdialysis*, the extracellular concentrations of neurotransmitters, neuromodulators, and other endogenous molecules [1], as well as exogenously administered compounds, can be determined in the extracellular space of the brain or other organs following their systemic administration [2]. With the microdialysis probe inserted into the area of interest in the brain and with suitable analytical techniques, the extracellular dynamics of small molecules in the extracellular space can be monitored without the need of complex purification procedures, thus allowing evaluation of pharmacokinetic/pharmacodynamic profiles of drugs more or less continuously [3]. So far, rotigotine and its pharmacokinetics have never been determined in animal or human tissues using this approach. Considering the potential use of rotigotine as an antiparkinsonian drug, the development of a suitable analytical methodology that would allow for its determination in the brain extracellular space was of interest.

The molecular structure of rotigotine, (−)-5,6,7,8-tetrahydro-6-[propyl-[2-(2-thienyl) ethyl] amino]-1-naphthalenol hydrochloride (Fig. 1), includes a tetrahydronaphthalenol moiety. This structural characteristic suggests that rotigotine can undergo anodic oxidation in a 3-electrode electrochemical cell [4]. This reaction would afford electrochemical detection of the drug in combination with high-performance liquid chromatography (LCEC). Earlier, several studies described LCEC methods for separating 5-hydroxytetralins from biological samples such as plasma and urine [4,5] or the use of HPLC with UV detection [6]. Halogenated or substituted phenols can be measured using electrochemical and fluorescence detection [7] or using fluorescence detection following postcolumn derivatization, such as with Ce^{3+} ions [8]. Spectrophotometric detection modes such

* Corresponding author. Tel.: +49 2173 48 1927; fax: +49 2173 48 1608.
E-mail address: Dieter.Scheller@schwarzpharma.com (D. Scheller).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.07.066

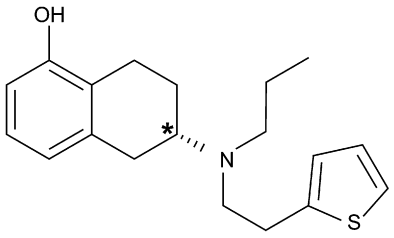

Fig. 1. Rotigotine formula.

as photodiode-array detection generally do not provide enough sensitivity for quantitative analysis of trace levels of rotigotine in biological samples [6]. Liquid chromatography and mass spectroscopy (LC/MS) technology represents a possible alternative for highly specific detection of rotigotine in biological tissues; however, the high concentrations of inorganic salts (physiological perfusion media) can interfere with its sensitivity, and therefore LC/MS may be a poor choice for use with microdialysate samples.

The objectives of the present study were to define and optimize the chromatographic conditions for determination of rotigotine by LCEC that would allow continuous measurement of the drug in the extracellular space of the rat striatum following its systemic administration at clinically relevant doses.

2. Experimental

2.1. Animals and drug administration

Male Sprague-Dawley rats (B&K Universal, Sollentuna, Sweden) weighing between 200 and 350 g were used for the experiment. The rats were fed lab chow (Ewos R36; Ewos, Sweden) and water ad libitum. Animals were housed in pairs at an ambient temperature of 20 °C with a 12 h light-dark cycle and were approximately 7–8 weeks in age at the time of experiment.

Rotigotine was obtained from Schwarz Biosciences (Monheim, Germany; Batch number WE11471), stored at −20 °C as a 10^{-2} M stock solution in deionized water, and thawed immediately prior to final dilution and administration. Two treatment groups received rotigotine, either infused intravenously ($n = 5$; 0.5 mg/kg; 150 μl/min) into the femoral vein or injected subcutaneously ($n = 3$; 5 mg/kg; 1 ml/min) into the scruff of the neck at a concentration of 0.5 mg/l ml. All animal experiments were approved by the local ethical committee (Stockholm Norra Forsoksdjurs Etiska Kommittee) following the directives of the "Principles of Laboratory Animal Care" (NIH publication No. 85-23) and the Council of the European Communities (86/809/EEC).

2.2. Surgery and brain microdialysis

Microdialysis experiments were carried out on anesthetized rats using the protocol developed by Kehr [9]. Briefly, the rats were anesthetized with enflurane (3% N_2O:O_2 [1:1] at 0.8 l/min) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) using a flat skull position with incisor bar set to −3.2 mm. The body temperature of the animal was controlled by a rectal thermometer and maintained at 37 °C, using a CMA/105 temperature controller (CMA/Microdialysis, Stockholm, Sweden). An incision was made in the middle of the scalp, approximately 2–3 cm long, and the skin flaps were kept aside using the homeostatic forceps. After exposing the skull, a hole was made using a fine trephine drill for insertion of a microdialysis probe. The CMA/12 microdialysis probe (4 mm membrane length; 20,000 Da) was implanted into the right striatum at the coordinates AP + 1.2 mm; L −3.0 mm; V −4.2 mm (from bregma and the dural surface) according to the atlas of Paxinos and Watson, 1997 [10]. The dialysis probe was perfused with Ringer's solution (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 2.3 mM) at a flow rate of 1.0 μl/min, using a CMA/100 microinjection pump. The dialysates were collected every 20 min with a CMA/170 refrigerated fraction collector. The fractions corresponding to the first 60 min after the probe insertion were discarded; thereafter, two microdialysis samples (blank) were collected prior to drug administration, and sampling continued for 240 min after the drug was administered. Thus, a total of 15 samples were collected, each containing 20 μl. After finalizing the experiment, the rats were sacrificed by carbon dioxide, followed by dislocation of the neck.

2.3. Chromatographic conditions

Rotigotine (Fig. 1) was detected by microbore column liquid chromatography with electrochemical detection. The chromatographic apparatus consisted of a LC100 micropump (ALS, Inc., Tokyo, Japan), a CMA/260 degasser (CMA/Microdialysis, Stockholm, Sweden), and a CMA/200 refrigerated microsampler (CMA/Microdialysis). An electrochemical detector LC4B (Bioanalytical Systems, West Lafayette, IN, USA) was equipped with a radial flow cell (ALS, Inc., Tokyo, Japan). At optimal conditions, the potential of a glassy carbon electrode (6 mm in diameter) was set to +850 mV, versus the Ag/AgCl/3 M NaCl reference electrode. Data were collected using an EZChrom data acquisition system (Scientific Software, Pleasanton, CA, USA). A microbore column (Luna C18 silica; 3 μm particle size; 150 mm × 1 mm I.D.; Phenomenex, Torrance, CA, USA) was maintained at room temperature (22–25 °C). The mobile phase was pumped at a flow rate of 30 μl/min.

Liquid chromatography calibration standards were prepared daily by diluting stock solutions in Ringer's solution to concentrations ranging between 1 and 100 nM. Standard solutions of dopamine, noradrenaline, and serotonin, and their major metabolites (3,4-dihydroxyphenylacetic acid [DOPAC]; homovanillic acid [HVA]; and 5-hydroxyindoleacetic acid [5-HIAA]) were assessed for rotigotine interference LCEC studies. Molecular standards were prepared as 10 mM stock solutions in deionized water and kept frozen at −20 °C. Fifteen microliters of standard solution or microdialysis sample was injected into the chromatographic column. All chemicals and solvents were of analytical or HPLC purity grade and purchased from Sigma-RBI (St. Louis, MO, USA), Merck (Darmstadt, Germany), or Fluka Chemie (Buchs, Switzerland).

In vitro rotigotine recovery studies were performed with the CMA/12 microdialysis probe (4 mm membrane length; 20,000 Da). Micropump flow rates were evaluated to optimize rotigotine recovery from a standard solution kept at 24 °C. Twenty-microliters sample volumes were collected and used for quantitative analysis by LCEC. The recovery of rotigotine at varying microdialysis conditions was estimated as the ratio of peak area of rotigotine found in the microdialysates to rotigotine concentrations in the standard solution. Chromatography data were collected and quantitated with an EZChrom data acquisition system (Scientific Software, Pleasanton, CA, USA).

3. Results

3.1. Optimization of analytical conditions

The initial studies focused on evaluation of sensitivity and optimization of chromatographic conditions for LCEC determination of rotigotine. At given chromatographic conditions, the electric currents generated by oxidation of rotigotine (100 nM in water) at the glassy carbon working electrode were measured. The electrode operated at potentials ranging from +700 mV to +950 mV versus the Ag/AgCl reference electrode. Increasing the electrode potential caused a marked increase in rotigotine signals, reaching the maximal value at a potential of +900 mV. The increase in oxidation potential also caused a strong increase in the background noise signals. In order to evaluate the maximal sensitivity for each tested potential, modified hydrodynamic voltammograms were constructed, expressing the signal-to-noise ratios (S/N) for rotigotine as a function of applied potential at the glassy carbon working electrode. The maximal sensitivity was achieved at +850 mV (S/N ratio = 8840); thereafter, the S/N values tended to decrease as a consequence of rapid increase in background noise levels.

Next, experiments aiming to optimize the elution conditions of rotigotine from the microbore column were performed. The pH of the mobile phase was the first tested variable using the electrode potential of +900 mV and at the constant elution strength of the mobile phase (50 mM phosphate buffer; 35% acetonitrile [ACN]). Variation in pH between 3.0 and 6.0 had only a minor effect on the S/N of rotigotine (mean S/N ratio 7377×10^3, variation coefficient 23.8%). The maximal value was achieved at pH 4.5; therefore, this pH was chosen as optimal. The optimal ACN concentration for rotigotine elution was also determined, according to its effects on S/N. For the mobile phase consisting of 50 mM $Na_2HPO_4{\cdot}2H_2O$, 2.5 mM sodium octyl sulfonate, pH 5.5, the maximal S/N was obtained when using 35% ACN. The effects of varying concentrations of the phosphate buffer and the ion-pairing reagent on rotigotine detection sensitivity were also explored. When the concentrations of octyl sulfonate were varied from 1.25 to 3.75 mM, the optimal separation and sensitivity for rotigotine peak were found at a concentration of 2.5 mM octyl sulfonate in the mobile phase of 50 mM $Na_2HPO_4{\cdot}2H_2O$ at pH 5.5 and 50% ACN. Similarly, the concentration of 50 mM $Na_2HPO_4{\cdot}2H_2O$ was found to be optimal for a mobile phase composed of 2.5 mM sodium octyl sulfonate and 50% ACN at pH 5.5. Thus, at optimal chromatographic conditions, the mobile phase consisted of 50 mM $Na_2HPO_4{\cdot}2H_2O$, 2.5 mM sodium octyl sulfonate, pH 4.5, and 35% volume to volume ACN. The flow rate was 30 μl/min, and the potential of the working electrode was +850 mV. At these conditions, rotigotine was eluted from the microbore column approximately 18 min after injection.

3.2. Detection limit

After the completion of experiments performed to optimize rotigotine detection, rotigotine standards were used to assess the limit of detection. The method detection limit (MDL) and the limit of quantification (LOQ) were calculated according to the Guidance for Industry, Bioanalytical Method Validation, 2001. The MDL value of 1.05 nM was calculated using the estimated standard deviation of seven injections of rotigotine standard at concentrations of 5 nM each; the corresponding LOQ value was 3 nM.

Using optimized conditions, it was possible to detect rotigotine down to 1-nM levels. The chromatograms in Fig. 2 show the elution of rotigotine standards at concentrations of 10^{-7}, 10^{-8}, and 10^{-9} M, and a blank (Ringer's solution) in 15 μl each injected onto the column.

Subsequent studies were performed to ensure that monoamines (dopamine [DA], noradrenaline [NA], serotonin [5-HT]) and their major metabolites (DOPAC, HVA and 5-HIAA) did not interfere with rotigotine peak at given chromatographic conditions (Fig. 3). Each of these substances injected onto the microbore column eluted in the front peak of the chromatogram, without interfering with the elution and detection of

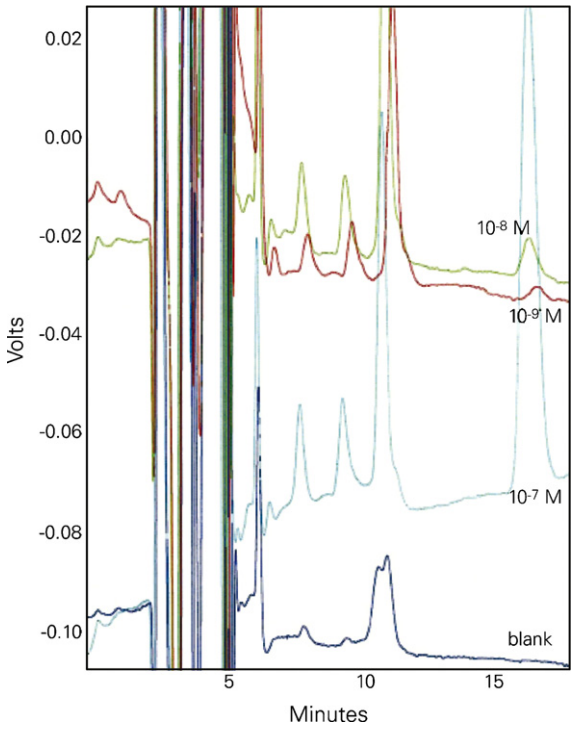

Fig. 2. Superimposed chromatograms of rotigotine standards at concentrations of 10^{-7} M, 10^{-8} M, 10^{-9} M, and a Ringer's solution blank. Fifteen microliters of each sample was injected onto the column.

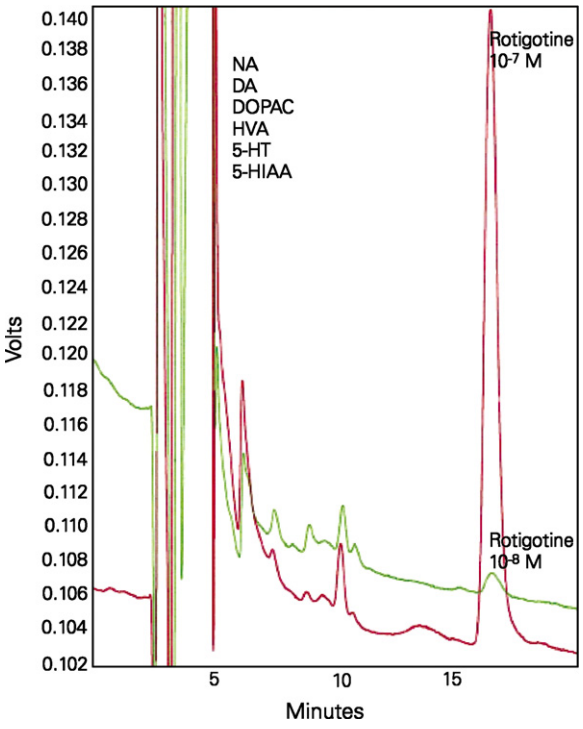

Fig. 3. Superimposed chromatograms illustrating separation of rotigotine (10^{-8} M) from endogenous monoamines (NA, DA, 5-HT; 10^{-7} M each) and their metabolites (DOPAC, HVA, 5-HIAA; 10^{-7} M each). Fifteen microliters of each sample was injected onto the column.

rotigotine. Fig. 3 shows superimposed chromatographs of the rotigotine standard at a concentration of 10^{-8} M, and molecular standards for NA, DA, 5-HT, DOPAC, HVA, and 5-HIAA, each at a concentration of 10^{-7} M.

3.3. Variability

The intra-day repeatability of the assay calculated as CV from eight injections of 200 nM rotigotine standard during a 10-h period was 10.1%, whereas the inter-day repeatability calculated for the same number of 200 nM rotigotine standard injections during a 3-day period was 28%. The low inter-day reproducibility led us to find a way to improve the assay conditions; to do so, the solution of the rotigotine standard was prepared fresh every day, and the working electrode was cleaned daily with methanol. Thereafter, systematic inter-day comparisons were not performed as the subsequent linear response of the electrochemical detector for the rotigotine standard was within the range of at least 3 orders of magnitude (1 nM to 1 μM).

3.4. In vitro recovery

Another series of experiments was performed to optimize the in vitro recovery of rotigotine via dialysis through the CMA/12 microdialysis probe. Using the micropump to perfuse Ringer's solution at various flow rates, a volume of 100 nM rotigotine standard solution was dialyzed from a 1.5 ml Eppendorf tube kept at room temperature. The perfusates were collected in 20 μl volumes and analyzed for rotigotine content; experiments were

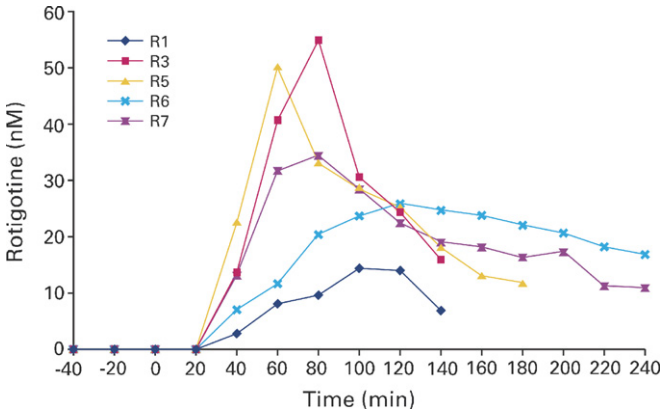

Fig. 4. Determination of rotigotine in microdialysate samples from five rats following rotigotine administration (0.5 mg/kg iv) at 0 min; 15 μl of each sample was injected onto the column.

run in triplicate. The highest recovery of about 31% was achieved at the lowest flow rate (0.5 μl/min) tested, whereas at the flow rate of 1 μl/min, the mean recovery was 18% (data not shown).

The relative recovery of rotigotine as determined here relates to the recovery of membrane, depending critically on the flow rate and the length of the membrane. For the CMA/12 microdialysis probes with 4-mm membrane length and the flow rate of 1 μl/min, the in vitro recovery of rotigotine was $18.5 \pm 1.5\%$ ($n = 3$ fractions). This estimation of in vitro recovery of the microdialysis probes is necessary to validate the uniform performance of the probes and assure minimal variation in recovery before using the probes in vivo.

3.5. Rotigotine determination in striatal microdialysates

The final in vivo studies aimed to provide a proof of concept for the devised LCEC methodology to detect extracellular rotigotine levels in rat striatal microdialysates. Fig. 4 shows the time courses of rotigotine levels in the extracellular space of the striatum of five anesthetized rats that received 0.5 mg/kg intravenously at time 0 min. The peak concentration of extracellular rotigotine (30.3 ± 7.6 nM) was found approximately

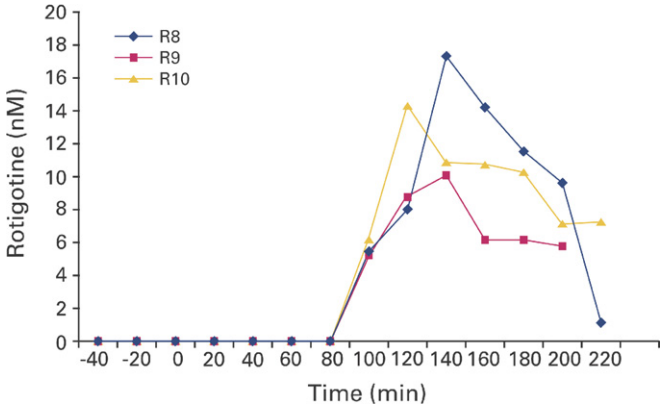

Fig. 5. Determination of rotigotine in the microdialysate samples from three rats given rotigotine (5.0 mg/kg s) at 0 min; 15 μl of each sample was injected onto the column.

80 min after rotigotine infusion (data not corrected for dead volume/time of the system or for relative recovery). Striatal rotigotine concentrations were also monitored in anesthetized rats after they received a subcutaneous injection of rotigotine (5.0 mg/kg), as shown in Fig. 5. The highest dialysate concentrations of rotigotine from three rats were observed in fractions collected between 120 and 140 min after drug administration (data not corrected for dead volume/time of the system or for relative recovery), and the concentration of rotigotine in the peak fractions was found to lie between 14 and 17 nM.

4. Discussion

Microdialysis is a powerful method of continuously detecting and measuring drug concentrations in the target tissues of both anesthetized and awake animals. This procedure eliminates having to sacrifice many animals, which otherwise would be necessary for analysis of drug levels at multiple time points. The microdialysate technique also allows for the sampling of small molecules and the use of liquid chromatographic methods without requiring additional purification steps, which greatly simplifies drug analysis in the tissue. The data shown here demonstrate that LCEC can be used in conjunction with in vivo microdialysis for measuring rotigotine levels in the extracellular space of rat brains after systemic rotigotine administration. The LCEC method developed and optimized using rotigotine standard solutions was sufficient to measure rotigotine in rat striatal tissues at nM levels. The present method offers an advantage over other highly sensitive liquid chromatography methods that require chemical derivatization reactions and removal of excess fluorescent reagents [11] or other technical complications [12]. The poor inter-day reproducibility, as observed during a 3-day continuous operation of the electrochemical detector, might be attributed to a gradual loss of the detector's sensitivity, probably caused by accumulation of reaction products on the surface of the working electrode operating at a relatively high potential (+850 mV) and a mobile phase containing a high level of acetonitrile. Daily cleaning of the working electrode and a weakly refill of the reference electrode with fresh aqueous 3 M NaCl maintained the highest possible sensitivity, and the within-a-day reproducibility was significantly improved for 12–16 h, which allowed for the determination of rotigotine levels at the low nM range.

One obvious advantage of the technique is illustrated by the observed differences in rotigotine levels and pharmacokinetics in the rat brain microdialysates after iv and sc rotigotine administration. For those rats receiving a dose of 0.5 mg/kg iv, the peak levels of striatal rotigotine (30.3 ± 7.6 nM) occurred approximately 80 min after infusion, whereas those receiving sc injections of 5.0 mg/kg showed the peak of striatal rotigotine levels (14–17 nM) at 120–140 min. These results reflect the different time intervals necessary for absorption after sc administration in comparison to iv injection. It is noteworthy that the results are not corrected for the dead volume of the system. In addition, the long sampling periods necessary for detection of rotigotine at low concentrations did not allow highly precise time resolution of extracellular rotigotine levels. These initial in vivo data demonstrate that while there is some interanimal variability in the peak levels of rotigotine, there is good reproducibility in the time courses of extracellular rotigotine levels in the rat brain. These results suggest that microdialysis in combination with optimized LCEC technique can be conveniently used for studies of rotigotine pharmacokinetics and pharmacodynamics (monitoring dopamine agonist levels) in animal models of Parkinson's disease.

5. Conclusion

The present study was designed to develop a liquid chromatographic method for sensitive detection of rotigotine in microdialysis samples from rat brains. An optimized chromatographic protocol was applied to monitor the time courses of extracellular rotigotine in the rat striatum following systemic rotigotine administration. Present data illustrate the suitability of microdialysis sampling combined with the LCEC technique in monitoring extracellular rotigotine pharmacokinetics at nM levels in the brains of anesthetized rats.

Acknowledgment

The study was sponsored by Schwarz Biosciences, Monheim, Germany.

References

[1] T.E.D. Robinson, J.B.J. Justice, Microdialysis in the neurosciences, techniques in the behavioral and neural sciences, Elsevier, Amsterdam, 1991.
[2] W.F. Elmquist, R.J. Sawchuk, Adv. Drug Deliv. Rev. 45 (2000) 123.
[3] X. Wang, P.N. Patsalos, Seizure 12 (2003) 330.
[4] P.J. Swart, B.F. Drenth, R.A. de Zeeuw, J. Chromatogr. 528 (1990) 464.
[5] S.C. Ruckmick, B.D. Hench, J. Chromatogr. 565 (1991) 277.
[6] D.L. Walters, C.R. Strong, S.V. Green, M.A. Curtis, J. Chromatogr. B Biomed. Appl. 670 (1995) 299.
[7] J. Meyer, A. Liesener, S. Gotz, H. Hayen, U. Karst, Anal. Chem. 75 (2003) 922.
[8] G. Lamprecht, J.F.K. Huber, J. Chromatogr. A 667 (1994) 47.
[9] J. Kehr, in: U. Windhorst, H. Johansson (Eds.), Modern Techniques in Neuroscience Research, Springer-Verlag, GmbH, Heidelberg, Germany, 1999, p. 1149, Ch. 41.
[10] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, New York, 1998.
[11] A. Kaddoumi, M.N. Nakashima, T. Maki, Y. Matsumura, J. Nakamura, K. Nakashima, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 791 (2003) 291.
[12] J.P. Qiao, Z. Abliz, F.M. Chu, P.L. Hou, L.Y. Zhao, M. Xia, Y. Chang, Z.R. Guo, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 805 (2004) 93.