Translational pharmacology of dopamine receptor agonists and antagonists : prolactin and oxytocin as biomarkers

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Online solid phase extraction with liquid chromatography-tandem mass spectrometry to analyse remoxipride in small plasma-, brain homogenate-, and brain microdialysate samples

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ABSTRACT

Remoxipride is a selective dopamine D2-receptor antagonist, and useful as a model compound in mechanism-based pharmacological investigations. To that end, studies in small animals with serial sampling over time are needed. For these small volume samples currently no suitable analytical methods are available. We propose analytical methods for the detection of low concentrations remoxipride in small sample volumes of plasma, brain homogenate, and brain microdialysate, using online solid phase extraction with liquid chromatography–tandem mass spectrometry.

Method development, optimization and validation are described in terms of calibration curves, extraction yield, lower limit of quantification (LLOQ), precision, accuracy, inter-day- and intra-day variability. The 20 µl plasma samples showed an extraction yield of 76%, with a LLOQ of 0.5 ng/ml.
For 0.6 ml brain homogenate samples the extraction yield was 45%, with a LLOQ of 1.8 ng/ml. The 20 µl brain microdialysate samples, without pre-treatment, had a LLOQ of 0.25 ng/ml. The precision and accuracy were well within the acceptable 15% range.
Considering the small sample volumes, the high sensitivity and good reproducibility, the analytical methods are suitable for analyzing small sample volumes with low remoxipride concentrations.

INTRODUCTION

Mechanism-based pharmacokinetic–pharmacodynamic (PK–PD) modeling of dopaminergic agents following different routes of administration of in preclinical studies *in vivo* is anticipated to provide key knowledge to predict efficacious doses and the time-course of dopaminergic drug effects in man. Remoxipride is a substituted benzamide and is a weak, but very selective, dopamine D2-receptor antagonist. It was originally intended to be used as an atypical antipsychotic (Roxiam®) but taken from the market in 1993. Remoxipride is, however, very useful as a model compound in mechanism-based PK–PD studies on the dopaminergic system in small animals.
With our ultimate aim being to build a mechanism-based PK–PD model of remoxipride, we first need to characterize the pharmacokinetic part of the model, i.e. determining the transport of remoxipride across the blood–brain barrier (Hammarlund-Udenaes, et al., 1997) and consecutive distribution between extracellular and intracellular space. Especially the free drug concentrations in the brain extracellular fluid surrounding the membrane receptors, as can be measured with intracerebral microdialysis, are of interest as membrane receptors are important targets for many drugs including remoxipride (Nadal, 2001). Plasma pharmacokinetics can be derived from serial plasma samples, brain extracellular fluid pharmacokinetics can be determined from collecting brain microdialysates, and total brain concentrations can be obtained from the brain, obtained from each animal at the end of the experiment.

As summarized in table 1, in 1987, De Ruiter *et al*. (de Ruiter, et al., 1987) described an analytical method for remoxipride in plasma using reversed-phase (RP) liquid chromatography (LC) with direct injection of 1ml untreated plasma diluted with 10mM phosphate buffer (1:1, v/v) into a disposable precolumn (C-18) treated with hexadecylytrimethylammonium bromide. A post-column ionpair extraction with 9,10-demethoxy-anthracene-2-sulphonate allowed detection with a fluorescence detector. In 1990 Nilsson (Nilsson, 1990) described a more simple and robust analytical method using a two-step liquid–liquid extraction, with higher sensitivity. This was followed by the method of Chiou and Lo (Chiou and Lo, 1992) who, in 1992, described a faster way of measuring remoxipride concentrations, using an easier extraction step, RP high-pressure liquid chromatography (HPLC) with ultraviolet detection (UV). In 1997, DePuy *et al*. (DePuy, et al., 1997) described an analysis using a chiral OD-R column, HPLC, and UV detection, allowing the separate measurement of the remoxipride enantiomers.
In the research, evidence was provided that *in vivo* conversion of the R- to S-

enantiomer does not occur, and confirmed stability in repetitive freeze–thaw cycles. Pharmacological investigations comprise full understanding of the distribution of drug throughout the body, including the determination of free drug concentrations in brain. Therefore, Main *et al*. (Main, et al., 1996) measured remoxipride in cerebrospinal fluid using the method described by Nilsson (Nilsson, 1990).

The earlier published analytical methods presented above (Chiou and Lo, 1992; Main, et al., 1996; de Ruiter, et al., 1987; DePuy, et al., 1997; Widman, et al., 1993; Nilsson, 1990) require rather large plasma or cerebrospinal fluid samples ranging from 0.5 to 2 ml, which can be obtained from humans in clinical studies (Table 1). In preclinical PK–PD studies, however, small laboratory animals are mostly used, thereby limiting the sample size. Based on Dutch legislation, quality of research, and animal ethics, the maximal obtainable blood volume in rats is 8 ml/kg/day (Van Zutphen, et al., 2001).

Table 1 Overview of analytical methods to determine remoxipride concentrations in plasma.

	De Ruiter, et al., 1987	Nilsson, 1992	Chiou and Lo, 1992	DePuy, et al.,1997
sample volume plasma (ml)	>0.5	1	2 (*)	0.5
sample preparation	PCE	LLE	LLE	LLE+P
internal standard	-	3B	-	3B
injection volume (ml)	1	0.2	0.05	0.1
flow rate (ml/min)	1	1.3	1.3	0.58-0.70
chromotography	RP LC	RP LC	RF HPLC	HP LC
detection	FL	FL	UV	UV
run time (min)	8	-	6	>40.6
retention time (min)	-	-	2.8	22.6
Extraction yield (%)	88 +/- 4	85 (*)	75	88.1 +/- 10.2 (**)
LLOD (ng/ml)	1	-	-	0.01
LLOQ (ng/ml)	-	0.742	12.5	20
intraday (%)	3.5	5.2	0.6-9.7	7
interday (%)	3.5	2.3	1.1-4.4	4.5

RP, reverse phase; LC, liquid chromatography; HP, high pressure; LLOD, lower limit of detection (3* noise ratio); LLOQ, lower limit of quantification; PCE, post column extraction; LLE, liquid-liquid extraction; p, prefilter; 3B, 3-bromo-N-[(1-propyl-2-pyrrolidinyl)methyl]-2,6, -dimethoxybenzamide; -, unknown; (*), for low concentration range only; (**), extraction recovery only.

Together with the strong need for sampling over time in pharmacokinetic studies, 9–10 blood samples of 200 µl can be taken per day, resulting in approximately 100 µl plasma samples, to analyze often both PK and PD parameters.
In rats, total brains of ± 1.8 g are obtained and homogenized. Only one sample can be taken, therefore the sample size is large. There is currently no analysis available for the measurement of remoxipride in brain homogenate. Brain microdialysis studies are bound to low perfusion speed resulting in typical microdialysate sample volumes of 10–60 µl (Chaurasia, et al., 2007; De Lange and Danhof, 2002; Nadal, 2001).
Analytical procedures for measuring both plasma and brain homogenate samples are complicated by the presence of interfering compounds. For plasma samples, dilution and/or protein precipitation are standard methods to clean up the samples before analysis. To analyze brain homogenate samples liquid–liquid extraction is often used as a pre-treatment. The microdialysis samples are far less polluted with interfering compounds, therefore, it remains to be seen whether sample pre-treatment is a necessity.

For experiments in rats, we here propose analytical methods for the detection of low concentrations remoxipride in small sample volumes of plasma, brain homogenate, and microdialysate, using online solid phase extraction (SPE) coupled to liquid chromatography (LC) with tandem mass spectrometry (MS–MS). The analytical method should cover the PK concentration range in all tissue types in our future experiments. For the plasma analysis a simple precipitation step was needed, and for the brain homogenates a liquid–liquid extraction step was implemented. The measurement of remoxipride in microdialysate could be performed without pre-treatment of the samples.

MATERIALS AND METHODS

To validate our method and increase the sensitivity and throughput, we tested plasma dilution and protein precipitation by acetonitrile or perchloric acid for positive contribution to the remoxipride analysis. Several SPE cartridges were tested; hysphere silica based cyanopropyl phase (CN), resin general phase (GP), and resin strong hydrophobic phase (SH) (Spark, Emmen, The Netherlands), mixed mode cationic exchange (MCX), and weak cationic exchange (WCX) cartridges (Waters, Etten-Leur, The Netherlands). The performance of these cartridges was tested alone as well as in combination with micro-column LC. Remoxipride was injected at the SPE column under acidic- and alkaline conditions. The effect of the addition of trifluoro acetic acid was investigated as well as different mobile phase ratios, to

increase peak performance. The final method is presented hereunder, together with an illustrative application of these analyses after remoxipride administration in individual rats. Because of its structural resemblance and previous use as internal standard in remoxipride measurements in plasma (Ogren, et al., 1993), we used raclopride as internal standard.

Chemical and reagents

For all procedures Purified Millipore water (MQ, 18.2 MΩ cm) from a Milli-Q® PF Plus system was used (Millipore B.V., Amsterdam, The Netherlands). Remoxipride ((S)-3-bromo- N-[1-ethyl-2-pyrrolidinyl)methyl]-2,6-dimethoxybenzamide, 371.27 g/mol, p*K*a 8.9 (Budavari, et al., 1996), was obtained from TOCRIS, Bristol, United Kingdom. The internal standard raclopride (IS, 3,5-dichloro-N-[[(2S)-1-ethyl-2-pyrrolidinyl]methyl]-2-hydroxy-6-methoxybenzamide, 347.24 g/mol, p*K*a,basic 9.5, p*K*a,acidic 6.11 (Bouchard, et al., 2002)), and L-cysteine were obtained from Sigma, Zwijndrecht, The Netherlands. Acetonitrile, acetic acid (both ULC/MS grade), trifluoro acetic acid (TFA, ULC/MS grade), ammonium acetate and t-butyl-methyl-ether (HPLC grade) were obtained from Biosolve B.V., Valkenswaard, The Netherlands. Ammonium hydroxide (25%), ammonium acetate (99.1%), sodium carbonate (99.9%), potassium iodide (99.9%), ethylenediaminetetraacetic acid (EDTA), perchloric acid, and L-(+)-ascorbic acid were obtained from Baker, Deventer, The Netherlands. All pH measurements were performed using a Schott CG 820 pH meter (Schott-Geräte, Germany). 10 mM Sodium phosphate homogenization buffer (pH 7.4) was prepared using di-sodium hydrogen phosphate di-hydrate (99.5%, E. Merck Nederland B.V., Amsterdam, The Netherlands) and sodium di-hydrogen phosphate monohydrate (99%, Sigma–Aldrich, Zwijndrecht, The Netherlands). Anti-oxidant (0.1M acetic acid, 3.3mM l-cysteine, 0.27M EDTA, 0.0125 mM L-(+)-ascorbic acid; S. Sarre, personal communications) and microdialysis perfusion fluid (PF (Moghaddam and Bunney, 1989)) was prepared, filtered, stored in glass vials (–20 °C), and sonified before use.

Samples

Blank plasma-, whole brain-, and brain microdialysis samples were obtained from Male Wistar WU rats (245 ± 18 g, Charles River, The Netherlands). All animal procedures were performed in accordance with Dutch laws on animal experimentation. The study protocol was approved by the Animal Ethics Committee of Leiden University (UDEC nr. 05156). A microdialysis guide was implanted in the striatum, and 6 days later replaced by a microdialysis probe (CMA/12, 4 mm polycarbonate membrane, cut-off 20 kD,

Aurora Borealis Control, Schoonebeek, The Netherlands). The next day the probe was perfused with 2 µl/min perfusion fluid, and blank microdialysate samples were collected in a cooled fraction collector (Univentor 820 Microsampler, Antec, Netherlands) for 5 hours. After administration of an overdose of Nembutal, blank blood was obtained by open heart puncture. The whole brains were harvested after transcardial transfusion with 50 mM phosphate buffer (pH 7.4), and the blank brains were stored at –80 °C.

■ Pharmacokinetic study in rats

The described analytical methods have been applied to the pharmacokinetic study of remoxipride in rats. Intranasal delivery of remoxipride was investigated to directly target the brain. Intranasal delivery is a promising alternative for oral or parenteral administration, since it avoids the high first-pass clearance of remoxipride (American Academy of Pediatrics: Comittee on Drugs, 1997; Widman, et al., 1993; Graff and Pollack, 2005; Jansson and Bjork, 2002). All animal procedures were performed in accordance with Dutch laws on animal experimentation. The study protocol was approved by the Animal Ethics Committee of Leiden University (UDEC 6132). Rat experiment were performed as described by Stevens *et al.* (Stevens, et al., 2009) in seven male Wistar WU rats. In short, during anaesthetized surgery, two blood cannulas were implanted in the femoral artery and -vein for blood sampling and drug administration, respectively. Next to that, a microdialysis guide was implanted in the striatum, and 6 days later replaced by a microdialysis probe (CMA/12, 4 mm polycarbonate membrane, cut-off 20 kD). The next day the probe was perfused with 2 µl/min perfusion fluid, and microdialysate samples were collected in the mentioned cooled fraction collector. After a 30 min blank perfusion period, remoxipride in saline (B. Braun Melsungen AG, Melsungen, Germany) was administered (16 mg/kg, t = 0) during a 30 min intravenous infusion, using an automated pump (Harvard apparatus 22, model 55-2222, Holliston, MA, USA). A total number of 10 blood samples per animal, of 200 µl each, were collected from the arterial cannula in EDTA-coated vials at 10 time points during the experiment (t = 0, 5, 10, 20, 35, 60, 90, 120, 150, and 180 min). Blood was centrifuged for 15 min at 5000 rpm and the plasma was stored at –20 °C. Microdialysate samples were collected every 20 min for the first 2 h, and every 30 thereafter, until the end of the experiments. The microdialysate samples were stored at –80 °C. At t = 240 min, transcardial perfusion was performed with 50 mM phosphate buffer (pH 7.4), and the whole brain was removed and stored at –80 °C. The concentrations of remoxipride in the samples were measured using the described methods. Plasma- and microdialysate geometric means (± standard deviation (S.D.)) were plotted over time. The geometric mean of

the brain homogenates were used to calculate the ratio of the concentration remoxipride in the brain versus the microdialysate concentrations.

■ Sample preparation, calibration curves and quality controls

For the plasma analysis a calibration curve of 0, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/ml remoxipride in MQ was prepared from a stock solution (100 µg remoxipride per ml MQ). For the quality controls (QCs), standard solutions containing remoxipride were added to 1.9 ml blank plasma (0.05:0.95, v/v), resulting in concentrations of 5, 50, 200, and 2000 ng/ml remoxipride. To 20 µl experimental plasma samples and QCs, IS (100 ng/ml) and MQ (1:1:1, v/v/v) was added and vortexed. Standard calibration solutions were mixed with IS (100 ng/ml) and blank plasma (1:1:1, v/v/v). 6% perchloric acid ($HClO_4$) was added to these mixtures (2:3, v/v) to precipitate the proteins. After brief vortexing (Vortex-Genie CM-9, Wilton en Co B.V., Etten-Leur, The Netherlands), the samples were centrifuged in eppendorf vials at 8000 rpm during 10 min (Labofuge GL centrifuge, Micro CL 21R, Thermo Fisher Scientific, Breda, The Netherlands) and 60 µl supernatant was added to 45 µl 0.5 M Na_2CO_3 and vortexed. 10 µl of sample was injected onto the online SPE.
Brains were defrosted and homogenized with a Potter S apparatus (B. Braun, Melsungen) in phosphate buffer in a 1:5 ratio (m/v) and kept on ice.
To 0.6 ml blank brain homogenate (100 mg brain) samples 100 µl of calibration standard was added resulting in concentrations of 0, 5, 10, 20, 50, 100, 200, 400, and 500 ng remoxipride/gram brain. The quality controls (QCs) con-tained 5, 20, 100, and 400 ng/gram brain. To the spiked homogenates (calibration curve), QCs and samples, 20 µl of IS (1000 ng/ml) and/or MQ was added (60:2:10, v/v/v). After vortexing, potassium iodide and 1 M Na_2CO_3 were added (72:10:10, v/v/v) and subsequently vortexed. t-Butyl-methyl-ether was added (0.92:5, v/v), and thorough vortexing supplied transfer of remoxipride and IS from aqueous to organic phase. After centrifugation at 4000 rpm during 10 min, organic phase was transferred to clean glass tubes and evaporated in a vacuum vortex at 40 °C (Labconco vortex evaporator, Beun de Ronde, Breda, The Netherlands). 100 µl SPE solvent (Section Online SPE-LC-MS-MS system) was added, mixed and sonified for 20 s. After spinning down the liquid, sample was transferred to eppendorf vials and centrifuged at 15000 rpm. 10 µl of supernatant was injected onto the online SPE.
For the microdialysate analysis, calibration standards were prepared in PF, resulting in concentrations of 0, 0.5, 1.0, 2.0, 5.0, 10, and 20 ng/ml remoxipride, all containing 10 ng/ml IS. The QCs were prepared containing 0.5, 5 and 20 ng/ml remoxipride. To 20 µl calibration standards, QCs, or micro-

dialysate samples, 5 µl anti-oxidant was added and vortexed prior to injection of 10 µl into the LC system. Anti-oxidant was used in microdialysate to prevent oxidation of molecules, other than remoxipride, that we might like to measure in the future.

Online SPE-LC–MS–MS system

After each injection (8 °C, Surveyor auto sampler, Thermo Fischer Scientific, Breda, The Netherlands), the injection needle was washed with 100 µl acetonitrile in MQ (1:9, v/v) and acetonitrile in MQ with acetic acid (1:3, v/v) to reduce carry-over. A Gynkotek P580 Solvent Delivery pump (Dionex Benelux B.V., Amsterdam, The Netherlands) flushed sample onto the SPE column (Hysphere Resin GP SPE in SPE cartridge holder, Spark, Emmen, The Netherlands: ambient temperature). SPE solvent A (SPE-A) consisted of acetonitrile in 10mM ammonium acetate/ammonium hydroxide (pH 8.3, 1:4, v/v). SPE solvent B (SPE-B) consisted of 50mM ammonium acetate/ammonium hydroxide (pH 8.3), and SPE solvent C (SPE-C) consisted of acetonitrile mixed with 16 mM acetic acid (7:3, v/v, 0.1% TFA, pH 2). The flow rate was 1 ml/min. At basic conditions, including t = 0 min, the SPE was flushed with SPE-A solvent. After 1 min of washing, a six-port switching valve diverted the SPE to the LC system. At 5 min the SPE was switched back to the SPE pump, and flushed with SPE-C solvent C, SPE-A, and SPE-B for 3, 3, and 2 minutes, respectively, to precondition the column for a next injection. The SPE was used only during one LC-sequence with a maximum of about 90 injec-tions, finalizing with QCs to verify quality. After switching to the LC system in back flush mode, the MS pump (Surveyor MS pump, Thermo Fischer Scientific, 100 µl/min) was used to separate the analytes on an Alltima HP C18 HPLC column (ambient temperature, 150 mm × 1.0 mm, 5 µm, Alltech Applied Science, Breda, The Netherlands). The mobile phases (MP) consisted of acetonitrile, 10 mM acetic acid/ammonium acetate at pH 3.7, and 0.05 % TFA. The latter was added to enhance peak shape and resolution. For the plasma- and brain analysis the ratio for MP-1 was 1:3 (v/v) and for MP-2 the ratio was 2:3 (v/v). A linear gradient (0–6 min, MP-1–MP-2) was applied to elute remoxipride and IS. At 10 min the LC system was reset to MP-1. The microdialysate samples were directly injected into the LC system using a mixture of MP-A and MP-B (6:4, v/v). Since no SPE was used, the flow was diverted to waste at the onset of the elution, and after 2 minutes the switching valve directed the flow to the MS–MS.

The Finnigan TSQ Quantum Ultra Mass Spectrometer System (Thermo Fischer Scientific) was tuned by infusing standard solutions of remoxipride or IS with the aid of a T-piece in the LC-eluent. Since remoxipride and the

IS are charged, electro spray-ionization was preferred above atmospheric pressure chemical ionization, and nitrogen was used as the desolvation gas. After collision-induced dissociation by argon gas (0.8 psi), the total ion current (TIC) was measured using the fragmented ions of remoxipride (MH + = 371) and raclopride (MH + = 347). Data acquisition was performed using the software package LC-Quan provided by Thermo Scientific. For all three analysis a separate tuning of the mass spectrometer was performed.

After analysis, linear regression was used to determine the slope, intercept and correlation coefficient (R^2) of the relation between the peak-area ratio and the drug concentration in the calibration standards data, and accepted when $R^2 > 0.98$. For the calibration curves weighting factors of 1, 1/Y and $1/Y^2$ were compared.

■ Extraction yield, LLOD, LLOQ, accuracy, precision, inter- and intra-day variability

Peak-area ratios of remoxipride over IS were calculated, and remoxipride concentrations were corrected for the dilution with IS and anti-oxidant. The extraction yield was calculated by dividing the peak area's of remoxipride either before and after LLE or LC with and without SPE. The lower limit of detection (LLOD) was defined as the lowest concentration measurable by a signal–noise ratio (S/N) of 3, lower limit of quantification (LLOQ) as the lowest concentration measurable by a S/N of 10. Intra-day, inter-day variability, and accuracy for the analysis were determined by measuring replicates of the QCs. Precision was calculated as the relative standard deviation (RSD) for both intra-day and inter-day variability, and accuracy as the degree off closeness of the determined value to the true value. Acceptable precision was defined by a RSD <15% for the standards, and <20% at LLOQ. Accuracy was allowed to deviate <15% for the standards, and <20% at LLOQ compared to the nominal values. Extraction yield, LLOD, LLOQ, accuracy, precision, inter- and intra-day variability methods and acceptation ranges are based on guidelines distilled from the Third Bioanalytical Workshop of the American Association of Pharmaceutical Scientists and Food and Drug Administration in 2006 (Viswanathan, et al., 2007).

RESULTS AND DISCUSSION

■ Method development and optimization SPE-LC–MS–MS

Online SPE was used for rat plasma and brain homogenate samples in order to provide an adequate sample clean up. As cleaning and preconditioning

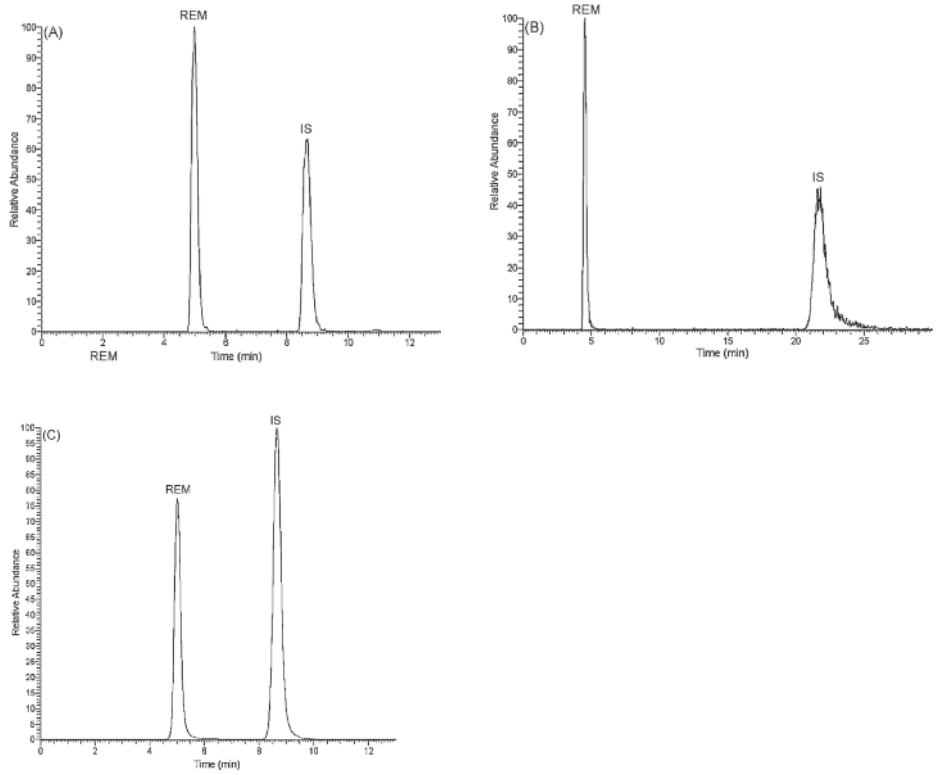
the SPE could be performed during elution of remoxipride and IS at the LC column, time loss due to the cleansing step was reduced.
Several SPE cartridges were tested for the optimal adsorption and elution properties. Hysphere CN was suitable for remoxipride adsorption and elution, but the internal standard raclopride already eluted during the washing phase. On the WCX phase, remoxipride behaved as a neutral and already eluted at 100% methanol, so no proper washing could take place. The Waters MCX showed bad resolution for remoxipride, so this method was abandoned. When using the Hysphere Resin SH material, the IS behaved differently from remoxipride and showed a poor peak shape. The Hysphere Resin GP SPE gave adequate separation, retention and peak shape for both remoxipride and IS. As we wanted to use material with another separation mechanism for the SPE as for the LC, this phase was chosen. Stability of this type of material over a wide range of pH (0–14) was favorable for our application. Both remoxipride and IS are trapped on the SPE at pH 8.3 and released when the SPE is switched to the LC system that is flushing MP at pH 3.7. To optimize the peak shape, several mobile phases (MPs) were tested by varying acetonitrile content, buffer composition and pH. This resulted in the MPs as described in the section Online SPE-LC-MS-MS system. In the plasma- and brain analysis the addition of TFA (0.05%) enhanced the peak shape of both remoxipride and IS. After tuning, using a syringe at 5 µl/min and LC flow of 100 µl/min, the main fragments of remoxipride and IS were identified (Table 2). For the plasma analysis the retention times of remoxipride and IS were 5.0 and 8.7 min, respectively, for the brain analysis 5.1 and 8.7 min, respectively, and for the microdialysate analysis the retention times were 4.8 and 20.7 min, respectively (Figure 1).

Table 2 Tandem mass spectrometer settings.

	20 µl plasma or 0.6 ml brainhomogenate		20 µl microdialysate	
	REM	IS	REM	IS
measure window (min)	0-7	7-13	0-12	12-25
desolvation gas (psi)	27	27	25	10
ionization voltage (kV)	3.5	3.5	3	3.5
fragments (m/z)	112	112	112	112
	228	129	228	129
	242	219	242	204

REM, remoxipride; IS, internal standard.

Figure 1 Relative abundances of remoxipride (REM) and raclopride (IS) in different matrices. A; Chromatogram of plasma spiked with 20 ng/ml remoxipride. B; 2 ng/ml remoxipride in microdialysate. C; 20 ng/ml remoxipride in brain homogenate.



Method validation

Table 2 provides an overview of the MS settings regarding measure window, desolvation gas pressure, ionization voltage, and measured fragment sizes derived from the total ion currents, for the plasma, brain microdialysate, and brain homogenate analysis. Specific information for the different types of samples is described below. For the plasma analysis in which 20 µl plasma sample was used, the online SPE allowed us to only use a simple precipitation step, in contrast with the liquid–liquid extraction or post-column extraction described in literature (DePuy, et al., 1997; de Ruiter, et al., 1987; Nilsson, 1990; Chiou and Lo, 1992). Evaluation of the pretreatment methods showed that using acetonitrile or precipitation with perchloric acid gave similar results. The results were better when compared to diluting

plasma, which caused clogging of the system. Because of the extra evaporation step needed in the acetonitrile method, we proceeded with the more simple perchloric acid precipitation method. Rigid flushing of the SPE after injection, prevented non-volatile ions from entering the LC-MS-MS system. The thus developed sample preparation method caused an extraction yield of remoxipride of 76% (Table 3).
This extraction yield was the product of the recovery of the pre-treatment, the SPE and the matrix effect. Three measurements of the SPE recovery revealed a mean recovery ± S.D. of 92 ± 2.6%. The combined mean recovery of the pre-treatment and matrix effect was 80 ± 4.6% (n = 17). LLOD and LLOQ were 0.15 and 0.5 ng/ml, respectively. This indicates that while using a smaller sample volume a similar LLOD and LLOQ were obtained when compared to literature (Table 1). Using a weighting factor of $1/Y^2$, calibration was performed within two calibration ranges. Both calibration ranges were measured during the same run. The high standards caused a considerable memory effect. R^2 for the concentration range 100 – 2000 ng/ml was acceptable: 0.990 ± 0.017 (± S.D.). The lower concentration range gave an average R^2 of 0.966 ± 0.017, which was most likely caused by the memory effect that increased the error on the lowest concentrations of the calibration curve. The memory effect could later on be circumvented by injection of blanks after samples in the high range of the calibration curve. The memory effect also influenced the intra-day RSD for QCs in the low (5 ng/ml) concentration samples. The inter-day RSD for the other QCs ranged from 4.9 to 14.7%, with an average of 9.0%. The intraday variability is thereby higher than described with other methods (Table 1), which should decrease by reducing the memory effect. For the intra-day RSD this range was 1.8–3.6%, with an average of 2.6 ng/ml which is low compared to literature, and the average accuracy ± S.D. was 103.7 ± 5.3%. Despite the memory effect, the precision, as indicated by the RSDs and accuracy, are within ranges (Table 3) which were suitable for the samples derived from our *in vivo* remoxipride experiments in rats.

For the analysis of the brain homogenate we needed a liquid–liquid extraction step (LLE) for the clean up of our samples. Adding potassium iodide resulted in a low, but reproducible extraction yield of 45 ± 2.3% (mean ± S.D.). The extraction yield consisted of the recovery of remoxipride during the LLE, the SPE and the matrix effect of brain homogenate extracts. The mean recovery ± S.D. of the SPE was 96 ± 5.2% (n = 12), and of the matrix effect 68 ± 11% (n = 8). The recovery of the LLE could therefore be calculated to be approximately 69%. LLOD and LLOQ were 0.53 and 1.8 ng/ml, respectively. The calibration curves (1/Y) showed an average R^2 ± S.D. of 0.998 ± 0.001. The inter-day RSD ranged from 5.6 to 11.3%, with an average of 8.7%.

For the intra-day RSD this range was 2.2 – 4.0%, with an average of 2.7% and average accuracy ± S.D. of 94.8 ± 4.7%. The RSD and accuracy were within the acceptable ranges. Of the 100 µl supernatant we needed 10 µl for injection, so scaling down the amount of brain tissue needed for sample preparation would be feasible. Although from analytical perspective the extraction yield could be improved, for our experiments it is sufficient to draw conclusions about the amount of remoxipride in the intracellular brain fluid (Section pharmacokinetic study in rats).

Table 3 Validation results of remoxipride analysis.

Plasma (n = 5 over 5 consecutive days)

LLOD (ng/ml)	0.15
LLOQ (ng/ml)	0.5
REM Extraction yield (%)	76

added (ng/ml)	mean (ng/ml)	RSD (%) interday	RSD (%) intraday	Accuracy (%)
5	5.1	18.1	11.8	100.6
50	48.3	4.9	3.6	108.6
200	192.5	14.7	2.1	109.8
200	195.9	11.4	1.8	97.2
2000	2091.0	5.0	2.9	102.1

Brain homogenate (n = 5 over 7 consecutive days)

LLOD (ng/ml)	0.53
LLOQ (ng/ml)	1.8
REM Extraction yield (% ± SD)	45 ± 2.3

added (ng/ml)	mean (ng/ml)	RSD (%) interday	RSD (%) intraday	Accuracy (%)
5	5.0	11.0	4.0	100.3
20	18.7	6.8	2.3	93.3
100	96.4	11.3	2.2	96.4
400	357.3	5.6	2.4	89.3

Microdialysate (n = 6 over 4 consecutive days)

LLOD (ng/ml)	0.08
LLOQ (ng/ml)	0.25

added (ng/ml)	mean (ng/ml)	RSD (%) interday	RSD (%) intraday	Accuracy (%)
0.5	0.6	9.2	4.1	117.8
5	4.7	10.1	7.5	93.5
20	20.7	11.3	7.0	103.7

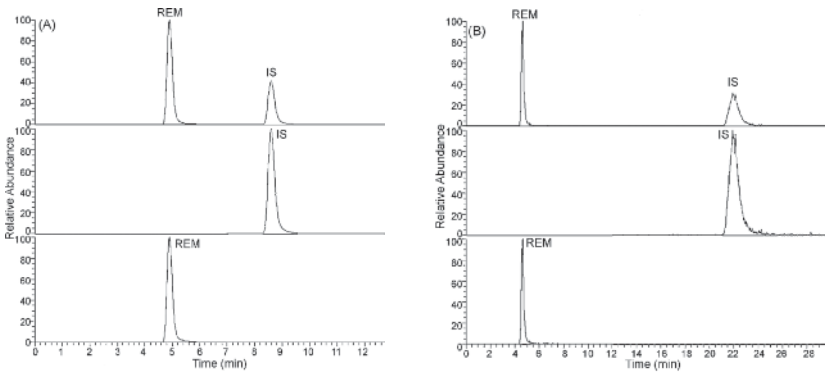
LLOD, lower limit of detection; LLOQ, lower limit of quantification; REM, remoxipride; S.D., standard deviation; RSD, relative standard deviation.

For the microdialysate analysis no extraction step was needed, allowing direct injection of the 20 µl brain microdialysate samples in the LC system, although this caused the LLOD to be slightly higher than in the plasma analysis. Diverting the first 2 min to waste reduced the noise, thereby reducing the LLOD and LLOQ to 0.08 and 0.25 ng/ml, respectively. The calibration curves ($1/Y^2$) showed an R^2 of 0.986 ± 0.015. The average intra-day and inter-day RSD were 6.2 and 10.2%, respectively. The average accuracy ± S.D. was 105 ± 12.2%. The RSD and accuracy were within the acceptable ranges. When previous reported analysis in cerebrospinal fluid (Main, et al., 1996) would be used, sample sizes ranging from 500 to 1500 µl would be necessary. Our method allows 20 µl sampling over time in an individual animal using microdialysis techniques.

■ Pharmacokinetic study in rats

The concentration of remoxipride was successfully measured in plasma-, brain homogenate-, and brain microdialysate samples after intravenous administration of 16 mg/kg remoxipride. Figure 2 depicts examples of real sample chromatograms in all three matrices. Figure 3 shows the concentration-time profiles in plasma and brain microdialysate.

Figure 2 Relative abundances of remoxipride (REM) and raclopride (IS) in different matrices in an individual rat after administration of 16 mg/kg remoxipride at t = 0. The upper panel shows the chromatogram of the sample. The lower panels show the peaks of the IS (middle panel) and remoxipride (lower panel) within that sample. (A) Chromatograms of plasma sample at t = 40 min, (B) chromatograms of microdialysate sample at t = 140-160 min and (C) chromatograms of a brain homogenate sample at t = 240 min.



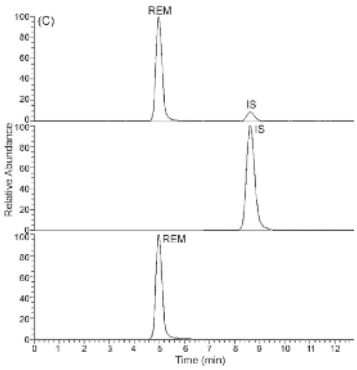
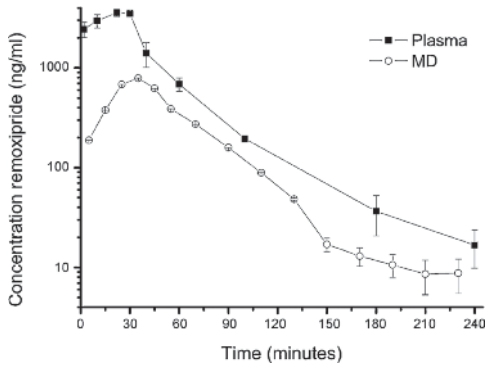


Figure 3 Remoxipride concentrations (geometric mean ± standard deviation) in plasma (closed symbols) and brain microdialysate (MD, open symbols) after a 30 min intravenous infusion of 16 mg/kg remoxipride at t = 0 in 7 Wistar rats.



The lowest measured concentrations are above the LLOQ. As expected, the maximum concentration (C_{max}) in plasma is reached at the end of the infusion; 30 min. The C_{max} of the microdialysate concentrations lies between 30 and 40 min. The delay in the microdialysate time to C_{max} implies activity at the site of the blood–brain barrier (BBB). Remoxipride acts on dopaminergic neurons that are located on the cell membranes of dopaminergic neurons. When investigating the PD in terms of efficacy and safety of compounds, understanding of the time to onset of action is vital. Interaction with the BBB has, in the case of remoxipride, evidently an effect on the time to onset of action. Although from a preclinical perspective, these results endorse the assumption that full understanding of the distribution of compound to the site of action is essential for dose-finding studies.

Moreover, the geometric mean (± S.D.) of the concentration in brain homogenate at t = 240 min was found to be 1096 ± 153.3 ng/ml, with corresponding microdialysate concentration of 8.77 ± 1.46 ng/ml in the microdialysate. The ratio that is present in the brain normalized by the microdialysate concentrations is therefore 125. Since this is larger than one, it is proven that a significant portion of bound remoxipride is still present in the brain, thereby increasing the complexity of the PK of remoxipride in a rat.

A single dose study does not provide sufficient information on the distribution over the BBB. To fully understand the mechanistic interpretation of the brain distribution, these studies must first be expanded with several other dosages. Secondly, after expansion, population approach non-linear mixed effect modeling should be performed to fully comprehend the PK of remoxipride in a mechanistic way. Full understanding of the PK then allows building of a mechanism-based PK–PD model of remoxipride in rat. The small sample volumes used in the analytical methods described in this article allow, for the first time, in-depth analysis of the PK of remoxipride over time in small laboratory animals.

CONCLUSIONS

We have successfully developed online SPE-LC–MS–MS methods for the rapid analysis of remoxipride in plasma, brain microdialysates and brain homogenate.
A simple protein precipitation proved to be sufficient as plasma sample (20 µl) clean up, before online SPE. One SPE cartridge could be used for about ninety injections, with a high extraction yield. For the measurement of remoxipride in rat brain homogenate (0.6 ml), an additional LLE was performed before online SPE. This is the first report for analyzing remoxipride in whole brain samples and although the extraction yield is relatively low, it is feasible for our *in vivo* experiments. For brain microdialysate samples (20 µl), no pre-treatment was needed and the samples were directly injected in the LC system.

For all three sample types a good precision and accuracy of the remoxipride analysis was obtained. The LLODs are appropriate for the use in preclinical experiments. When compared to previous reports, we accomplished the analysis of remoxipride in small sample volumes, to allow collection of concentration data suitable for mechanistic pharmacological studies on remoxipride in small laboratory animals.

REFERENCES

- American Academy of Pediatrics: Comittee on Drugs (1997) Alternative Routes of Drug Administration---Advantages and Disadvantages (Subject Review). *Pediatrics* **100**:143-152.
- Bouchard G, Pagliara A, Carrupt PA, Testa B, Gobry V and Girault HH (2002) Theoretical and experimental exploration of the lipophilicity of zwitterionic drugs in the 1,2-dichlor oethane/water system. *Pharm Res* **19**:1150-1159.
- Budavari S, O'Neil MJ, Smith A, Heckelman PA and Kinneary JF (1996) *The Merck Index, an encyclopedia of chemicals, drugs, and biologicals.* Merck & Co., Inc., Whitehouse Station, N.J., U.S.A.
- Chaurasia CS, Muller M, Bashaw ED, Benfeldt E, Bolinder J, Bullock R, Bungay PM, De Lange ECM, Derendorf H, Elmquist WF, Hammarlund-Udenaes M, Joukhadar C, Kellogg DL, Jr., Lunte CE, Nordstrom CH, Rollema H, Sawchuk RJ, Cheung BWY, Shah VP, Stahle L, Ungerstedt U, Welty DF and Yeo H (2007) AAPS-FDA Workshop White Paper: Microdialysis Principles, Application, and Regulatory Perspectives. *J Clin Pharmacol* **47**:589-603.
- Chiou RH and Lo MW (1992) Determination of remoxipride in human plasma and urine by reversed-phase ion-pair high-performance liquid chromatography. *J Chromatogr* **581**:300-305.
- De Lange EC and Danhof M (2002) Considerations in the use of cerebrospinal fluid pharmacokinetics to predict brain target concentrations in the clinical setting: implications of the barriers between blood and brain. *Clin Pharmacokinet* **41**:691-703.
- De Ruiter C, Brinkman UAT and Frei RW (1987) Liquid Chromatographic Determination of a Substituted Benzamide in Biological Fluids Using Preconcentration and Post-Column Extraction. *Journal of Liquid Chromatography & Related Technologies* **10**:1903-1916.
- DePuy ME, Demetriades JL, Musson DG and Rogers JD (1997) Stereoselective determination of R-(+)- and S-(-)-remoxipride, a dopamine D2-receptor antagonist, in human plasma by chiral high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* **700**:165-173.
- Graff CL and Pollack GM (2005) Nasal drug administration: potential for targeted central nervous system delivery. *J Pharm Sci* **94**:1187-1195.
- Hammarlund-Udenaes M, Paalzow LK and de Lange EC (1997) Drug equilibration across the blood-brain barrier--pharmacokinetic considerations based on the microdialysis method. *Pharm Res* **14**:128-134.
- Jansson B and Bjork E (2002) Visualization of in vivo olfactory uptake and transfer using fluorescein dextran. *J Drug Target* **10**:379-386.
- Main DC, Waterman AE and Kilpatrick IC (1996) Disposition of remoxipride in plasma and cerebrospinal fluid in sheep. *J Vet Pharmacol Ther* **19**:402-404.
- Moghaddam B and Bunney BS (1989) Ionic composition of microdialysis perfusing solution alters the pharmacological responsiveness and basal outflow of striatal dopamine. *J Neurochem* **53**:652-654.
- Nadal R (2001) Pharmacology of the atypical antipsychotic remoxipride, a dopamine D2 receptor antagonist. *CNS Drug Rev* **7**:265-282.
- Nilsson LB (1990) Determination of remoxipride in plasma and urine by reversed-phase column liquid chromatography. *J Chromatogr* **526**:139-150.
- Ogren SO, Lundstrom J and Nilsson LB (1993) Concentrations of remoxipride and its phenolic metabolites in rat brain and plasma. Relationship to extrapyramidal side effects and atypical antipsychotic profile. *J Neural Transm Gen Sect* **94**:199-216.
- Stevens J, Suidgeest E, Van der Graaf PH, Danhof M and De Lange EC (2009) A New Minimal-Stress Freely-Moving Rat Model for Preclinical Studies on Intranasal Administration of CNS Drugs. *Pharm Res* **26**:1911-1917.
- Van Zutphen LFM, Baumans V and Beynen AC (2003) *Handboek Proefdierkunde; Proefdieren, dierproeven, alternatieven en ethiek*. Elsevier Gezondheidszorg.

- Viswanathan C, Bansal S, Booth B, DeStefano A, Rose M, Sailstad J, Shah V, Skelly J, Swann P and Weiner R (2007) Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays. *Pharmaceutical Research* **24**:1962-1973.
- Widman M, Nilsson LB, Bryske B and Lundstrom J (1993) Disposition of remoxipride in different species. Species differences in metabolism. *Arzneimittelforschung* **43**:287-297.