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Development and validation of a direct enantiomeric separation of pregabalin to support isolated perfused rat kidney studies☆

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ABSTRACT

Pregabalin (Lyrica™) is the first compound approved to treat the neural pain associated with fibromyalgia. Pregabalin is the *S*-enantiomer of a γ-amino acid analogue and chiral separation from its *R*-enantiomer must be achieved to support metabolic studies. The direct chiral separation of pregabalin from its *R*-enantiomer has been developed and HPLC/MS/MS assays have been validated to support isolated perfused rat kidney studies. The separation was developed through serial coupling of various macrocyclic glycopeptide stationary phases until partial separation of the enantiomers was achieved. Identification of the resolving stationary phase followed by optimization of the mobile phase enabled the baseline resolution of the enantiomers using mass spectrometry compatible solvents and modifiers. Assays were developed and validated for quantitation of the enantiomers from rat urine, isolated rat kidney perfusate, and isolated rat kidney perfusate ultrafiltrate to support pregabalin metabolic studies.

1. Introduction

Pregabalin (Lyrica™) is an antiepileptic drug approved for a number of indications in the US and Europe that include adjunctive therapy of partial seizures in adults, pain from diabetic neuropathy or post-herpetic neuralgia in adults, and the treatment of anxiety disorders [1]. More recently pregabalin has been approved by the FDA for the treatment of spinal cord injury and as the first drug indicated for the treatment of fibromyalgia [2,3]. Pregabalin is the 3-isobutyl substituted analogue of γ-amino butyric acid (GABA) but is inactive at GABA receptors [1]. The pharmacological activities of pregabalin result from its binding to the alpha-2-delta (α2δ) protein, an auxiliary protein associated with voltage-gated calcium channels in the central nervous system [4]. Although there is disagreement on pregabalin's effect on calcium currents in neuronal cell bodies [1], several studies agree that pregabalin subtly reduces the synaptic release of several neurotransmitters, apparently by binding to α2δ subunits, possibly accounting for its actions *in vivo* to reduce neuronal excitability and seizures [1].

3-Isobutylgaba is a γ-amino acid that exists as *S*- and *R*-enantiomers (Fig. 1), with (*S*)-3-(aminomethyl)-5-methylhexanoic acid (pregabalin) having approximately 10-times more affinity for the α2δ binding site than the *R*-isomer [5]. Achiral separation of the enantiomers is sufficient for assays that seek to measure general parameters such as exposure. Early reports of pregabalin analysis from biological matrices employ achiral HPLC-UV and HPLC-fluorescence assays that require pre-column derivatization of the molecule with a chromophore [6–8]. Other assays have avoided the derivatization step by achiral separation followed by pregabalin detection using mass spectrometry [9]. Achiral separation of the enantiomers is not sufficient for more detailed characterization of pregabalin and its properties. Efficient and reliable chiral separation and bioanalytical assays are needed to distinguish the enantiomers in stability tests and to establish the pharmacodynamics and metabolic fate of the isomers. One such metabolic study is the isolated perfused kidney (IPK) model [10] in rat. The IPK model is used for characterizing renal excretion mechanisms, screening for clinically significant drug interactions, studying renal drug metabolism, and correlating renal drug disposition with drug-induced changes in kidney function [11–13]. Support of the IPK model requires chiral separation of pregabalin and its enantiomer from rat urine, kidney perfusate and kidney perfusate ultrafiltrate to evaluate the effects of renal processes on pregabalin, and to evaluate the possible metabolic interconversion of pregabalin to the less active isomer. While the literature is replete with examples of the

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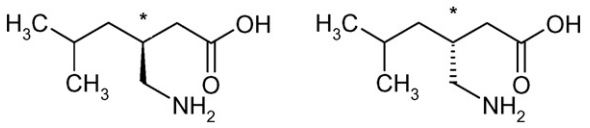

Fig. 1. Pregabalin (left) and its *R*-enantiomer (right). *Denotes the chiral center.

direct chiral separation of α- and β-amino acids, the direct chiral separation of γ-amino acids has proven to be more challenging. Here we describe a direct chiral separation of pregabalin and its *R*-isomer using a macrocyclic glycopeptide chiral stationary phase and validation of HPLC/MS/MS assays to support rat IPK studies.

2. Experimental

2.1. Chemicals and reagents

(*S*)-3-(Aminomethyl)-5-methylhexanoic acid (Pregabalin, 100.0%), (*R*)-3-(aminomethyl)-5-methylhexanoic acid (99.4%) and PD-0299685 (96%, an analogue compound for internal standard, structure not shown) were synthesized by Pfizer Inc. HPLC grade water, ethanol, methanol, and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI, USA). Ammonium acetate and glacial acetic acid (HOAc) were purchased from J.T. Baker (Phillipsburg, NJ, USA). All other reagents used in this study were of analytical grade and purchased commercially.

2.2. HPLC conditions

Assay development and sample analysis were conducted on an HPLC system consisting of Shimadzu (Kyoto, Japan) LC-10 series components (two Shimadzu LC-10ADVP pumps and one DGU-14A degasser, controlled by an SCL-10ADVP system controller), and an HTC PAL autosampler (Carrboro, NC, USA). Separation of the pregabalin enantiomers was achieved on a Chirobiotic T (teicoplanin, 15 cm × 0.46 cm, 5 μm) column (Advanced Separation Technologies Inc., Whippany, NJ, USA), with a 0.5-μm stainless steel inline prefilter (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using an isocratic mobile phase of ethanol: 10 mM ammonium acetate in water, pH 5.5 (adjusted with acetic acid) (80:20, v/v) at a flow rate of 1.2 mL/min. The column was maintained at ambient temperature and the sample injection volume was 6 μL.

Two other macrocyclic glycopeptide stationary phases were also evaluated, Chirobiotic V (vancomycin) and Chirobiotic R (ristocetin) (15 cm × 0.46 cm, 5 μm, Advanced Separation Technologies Inc., Whippany, NJ, USA).

2.3. Mass spectrometry conditions

Detection of the enantiomers was achieved using an API 4000 triple–quadrupole tandem mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, Canada) in positive-ion mode. Analytes were ionized using the Turboionspray™ interface at 460 °C with an ionization voltage of 4000 V. The nebulizer, curtain, auxiliary, and collision gases were nitrogen and set at 20, 12, 60 and 6, respectively. The declustering potential (DP), collision energy (CE), collision exit potential (CXP) and entrance potential (EP) for both enantiomers and internal standard were set at 20, 15, 15, and 10, respectively. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode using the mass-to-charge transitions of m/z 160 → 142 for the two enantiomers and m/z 188 → 170 for the internal standard. The acquisition dwell times for the enantiomers and internal standard were 300 ms and 200 ms, respectively. Quadrupoles Q1 and Q3 were set at unit resolution (0.7 amu at half height). The HPLC/MS/MS system was controlled and the analytical data were processed using Sciex Analyst software (Version 1.4.1).

2.4. Preparation of stock solutions and internal standard working solution (ISWS)

Primary stock solutions of pregabalin, its enantiomer and the internal standard were prepared in DMSO (1000 μg/mL). Separate aliquots were weighed for the preparation of standards and quality control (QC) samples. These solutions were stored at 4 °C and found to be stable for at least 1 month (data not shown). The stock solutions were combined and diluted to give a working solution consisting of 500 μg/mL of each enantiomer. The working solution was prepared fresh daily.

The internal standard primary stock solution was diluted in ethanol to give an ISWS of 2000 ng/mL. This solution was prepared fresh daily.

2.5. Preparation of isolated kidney perfusate and perfusate ultrafiltrate

Blank isolated kidney perfusate and protein-free perfusate ultrafiltrate were prepared as described by Lepsy et al. [14] for the preparation of standards, QC samples and blanks. The perfusate and perfusate ultrafiltrate were not passed through isolated rat kidney prior to their use as blank matrices.

2.6. Preparation of calibration standards and quality control samples

Calibration curves were prepared by spiking the enantiomer working solution into the appropriate matrix (rat urine, rat kidney perfusate, or perfusate ultrafiltrate), to give concentrations of 50.0 ng/mL, 100 ng/mL, 240 ng/mL, 480 ng/mL, 2400 ng/mL, 6000 ng/mL, 18,000 ng/mL and 20,000 ng/mL. Similarly, quality control samples were prepared to give concentrations of 150 ng/mL, 1200 ng/mL, and 16,000 ng/mL and a lower level of quantitation (LLOQ) pool was prepared at 50.0 ng/mL.

2.7. Sample preparation

Kidney perfusate and perfusate ultrafiltrate blanks, standards, QC samples, and study samples were prepared by combining 50 μL of the samples with 200 μL of ISWS into the wells of a polypropylene 96-well plate. The plate of samples was then capped, mixed for 10 s (multi-tube vortexer, VWR, West Chester, PA, USA), and centrifuged (Eppendorf Model 5810R, Westbury, NY, USA) at 4000 rpm, 6 °C for 20 min. The resulting supernatants were transferred to a clean polypropylene 96-well plate and sealed for HPLC/MS/MS analysis. Rat urine blanks, standards, QC samples, and study samples were prepared by combining 50 μL of the sample with 200 μL of the ISWS, capping, mixing, and then injecting without centrifugation or transfer.

2.8. Method validation

2.8.1. Specificity

Specificity was defined as no signal greater than or equal to 20% of the signal achieved at the lower limit of quantitation at the retention times of the enantiomers. Assay specificity for the analytes from endogenous matrix components in the processed blank matrix and specificity from cross-interference between the enantiomers or internal standard were established. Specificity for the internal standard was established as no signal greater than or equal

to 5% of the signal of the internal standard. Six lots of urine and one lot each of perfusate buffer and perfusate ultrafiltrate buffer were evaluated to establish the assay specificity.

2.8.2. Linearity of calibration curve

Calibration curves were generated by plotting the peak area ratios (analyte/internal standard) versus the theoretical concentration. The calibration curves were run in singlet and the linearity of the calibration curve was evaluated by linear regression analysis using a $1/(\text{concentration})^2$ weighting.

2.8.3. Precision and accuracy

The intra-run assay accuracy and precision were established through the performance of six replicates of the QC samples at the three concentrations described. The inter-run assay accuracy and precision were established through the performance of three consecutive intra-day runs. The assay accuracies were evaluated by the deviation of the mean concentration measurement of the replicates versus the theoretical concentration value expressed as a percentage (%Error). The assay precisions were evaluated from the relative standard deviation (R.S.D.) of the concentration measurements and expressed as the percent coefficient of variation (%CV) from the mean concentration of the replicates. Intra- and inter-run accuracies and precisions at all QC concentrations of less than or equal to ±15% were deemed to be acceptable.

2.8.4. Lower limit of quantitation

The lower limit of quantitation of the assay was established for each matrix from the performance of six replicates of the LLOQ pool. Intra- and inter-run accuracies and precisions of the measured concentrations of the pool less than or equal to ±20% were deemed to demonstrate acceptable performance at the LLOQ.

2.8.5. Recovery

The extraction recovery of the analytes in this method was not assessed.

2.8.6. Interconversion of the enantiomers and stability

Interconversion of the enantiomers during stock solution storage and during the processing of the samples from all three matrices was qualitatively evaluated. Enantiomers were separately spiked into DMSO or the blank matrix at a level of 150 ng/mL and incubated at 50 °C for 24 h. Chiral separation of the resulting samples revealed no evidence of enantiomer interconversion under these conditions.

The freeze–thaw and ambient condition storage stability of the enantiomers in matrix were evaluated from stability pools prepared by spiking each enantiomer to a concentration of 150 ng/mL and 16,000 ng/mL into each matrix. Triplicate aliquots of the stability pools were subjected to either three −20 °C freeze/ambient thaw cycles or storage under ambient conditions for 4 h. The enantiomer stability was assessed by comparing the mean measured concentration to the theoretical concentration with a difference of ≦ ±15% deemed to establish stability.

The long-term storage stability of the enantiomers in matrix was evaluated from the same pools of stability samples. All stability pools were analyzed in triplicate on their day of preparation and the mean measured concentrations defined as the 0-h concentration. Triplicate measurements of the pools were repeated after 30 days of storage at −20 °C and the stabilities of the enantiomers assessed by comparing the mean measured concentration of the stored aliquots to the 0-h concentration. A difference between the means of ≦ ±15% was deemed to establish stability.

The stability of the enantiomers in sample extracts was evaluated for each matrix from validation inter-run data. Final sample extracts were stored under ambient conditions for 28 h and re-analyzed. The meeting of the inter-run accuracy and precision acceptance criteria was deemed to establish stability.

3. Results and discussion

The direct chiral separation of amino acid enantiomers requires three simultaneous points of interaction between the functionalities of the amino acid and the stationary phase [15]. The separation is more effective when the analyte functionalities are adjacent to the chiral center as they are for α- and β-amino acids. The direct separation of pregabalin from its enantiomer has proven difficult because pregabalin is a γ-amino acid with both the amine and carboxylic functionalities separated from the chiral center by a methylene group. Attempts at the direct chiral separation of the enantiomers have been reported by Chen et al. who were unable to achieve baseline resolution using Chiralpak AD and AD-H, Chiralcel OD, Ceramospher chiral RU-1, and chiral RU-2, and CROWNPAK CR (+) columns [16]. Indirect HPLC-enantiomeric separation of the pregabalin enantiomers for chiral purity evaluation has been reported by pre-column chiral derivatization of the enantiomers to their corresponding diastereomers using Marfey's reagent followed by reverse phase separation [16–19].

Macrocyclic glycopeptide-based stationary phases were introduced in 1994 [20] and have been used to separate an array of enantiomeric compounds that includes amino acids [21–29]. The macrocyclic glycopeptide provides a number of chiral centers, binding pockets, sites of aromaticity, and hydrogen donors/acceptors enabling a variety of interactions such as hydrophobic, hydrogen bonding, dipole, π–π, steric repulsion, and ionic. The large diversity and combinations of binding site, and interaction geometry and mechanism between the γ-amino acid the stationary phase greatly increases the likelihood that three points of simultaneous interaction can be achieved. A broad number of specificities can be interrogated on a single macrocyclic phase through systematic investigation of different mobile phase types, such as polar ionic, polar organic, normal phase, and reversed phase modes. Of equal utility is that these specificities can be achieved with mass spectrometer-compatible solvents and additives enabling the development of specific and sensitive assays.

3.1. Optimization of the HPLC chiral separation

3.1.1. Column coupling

The large number of column–analyte interactions that can be evaluated using macrocyclic glycopeptide stationary phases lends itself to mobile phase screening strategies. Wang et al. [30] have employed a column coupling strategy to speed method development and optimize the chiral separation. In their strategy macrocyclic glycopeptide stationary phases are connected serially using low dead volume connectors. The racemic pair of analytes is injected onto the tandem columns under a variety of mobile phase types and compositions until some level of resolution is achieved. The resolving glycopeptide is identified and the chiral separation is optimized.

This screening strategy was employed to evaluate 15 cm × 0.46 cm columns of Chirobiotic R, V, and T (connected in this order), for the separation of pregabalin and its *R*-isomer. The three columns were joined using low dead volume connectors and plumbed to the HPLC system using stainless steel tubing (0.0005′ i.d.) to withstand the high backpressure. The mobile phase flow rate was set at 0.4 mL/min so that the column back pressure was maintained within the vender suggested range [31]. This low flow rate offered the additional benefit of increased column-analyte partitioning

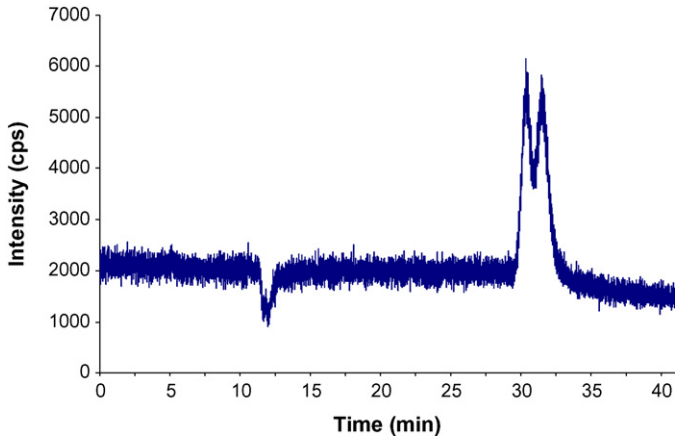

Fig. 2. Chromatography from the initial screening by serial coupling of Chirobiotic R, V, and T columns; mobile phase, MeOH:water; 80:20 (v:v); flow rate, 0.4 mL/min.

time thereby increasing the opportunity to achieve analyte resolution.

Partial resolution of the enantiomers was observed using the tandem columns and a reversed phase mobile phase of methanol:water (80:20, v:v) (Fig. 2). Decoupling and investigation of the independent columns revealed that all of the separation was achieved on the Chirobiotic T phase with no contribution to the enantiomeric resolution by the Chirobiotic R and V phases. This observation is consistent with other reports that the Chirobiotic T has enabled the enantiomeric recognition of a variety of native α- and β-amino acids [26–29].

3.1.2. Optimization of the mobile phase composition

Optimization of the separation on the Chirobiotic T stationary phase took place at an increased mobile phase flow of 0.8 mL/min. Methanol:water mobile phase compositions over the range of 50–80% methanol were evaluated. It was observed that retention of the isomers decreased with increasing percentage of methanol with no increase in the separation factor (α) or isomer resolution (R_s).

Ethanol:water mobile phase compositions over the range of 30–95% ethanol demonstrated much different behaviors (Fig. 3). As the percentage of ethanol increases there is a nonlinear increase of the retention factor (k) and a linear increase in α and R_s. Nearly baseline separation ($R_s = 1.26$) was achievrd with a mobile phase composition of 95:5 ethanol:water (v/v) but because the retention time of the isomers was on the order of 45 min ($k = 19.1$), further optimization focused on a solvent composition of 80:20 ethanol:water ($k = 6.58$, $\alpha = 1.07$, $R_s = 0.85$).

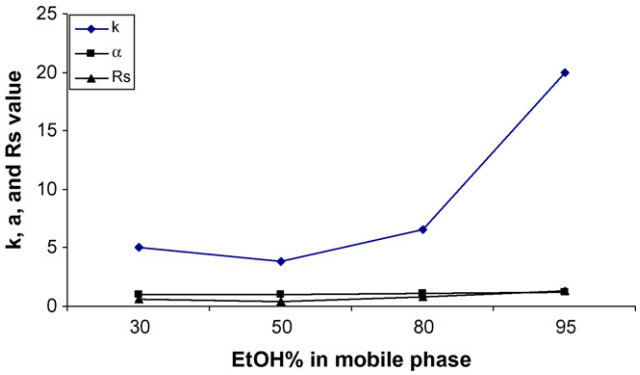

Fig. 3. Pregabalin chromatographic parameters k, α, and R_s on a Chirobiotic T column as a function of ethanol:water mobile phase composition (0.8 mL/min).

3.1.3. Optimization of ionic strength and pH

The pK_a's of pregabalin are 4.2 and 10.6 [2] and those of teicoplanin are of 2.5 and 9.2 [31] so that at in organic/water mobile phase compositions both pregabalin and teicoplanin exist as zwitterions. This indicates that a charge–charge electrostatic interaction between the analyte and the stationary phase likely plays a role in the chiral recognition of pregabalin. Ammonium acetate concentrations of 2 mM, 5 mM, and 10 mM adjusted to pH 5.5 with glacial acetic acid were evaluated to maintain the reproducibility of the separation and the charge states of the molecules. A significant increase in resolution was observed at buffer concentrations of 5 mM and 10 mM ($R_s = 1.84$ and 1.98, respectively).

3.1.4. Final HPLC conditions

The mobile phase flow rate was increased from 0.8 mL/min to 1.2 mL/min without a significant sacrifice in resolution so that the final chromatographic mobile phase of ethanol: 10 mM ammonium acetate in water, pH 5.5 produced chromatographic parameters of $\alpha = 1.14$, $R_s = 1.7$ and $k = 5.2$ for pregabalin. The high percentage of organic in the final mobile phase composition enabled sensitive mass spectrometric detection using ionspray ionization. As a result, there was no need to concentrate the analyte during the sample preparation to achieve the desired 50 ng/mL lower limit of quantitation. A representative chromatogram of an extracted LLOQ sample (50 ng/mL pregabalin and the *R*-isomer in perfusate) is shown in Fig. 4.

3.2. Validation

3.2.1. Specificity

The specificity of the extraction and chromatographic method tested the ability of the method to differentiate and quantitate the analyte in the presence of other endogenous constituents in the sample and to detect potential interferences. No interfering peaks were observed and no significant peaks were found at the reten-

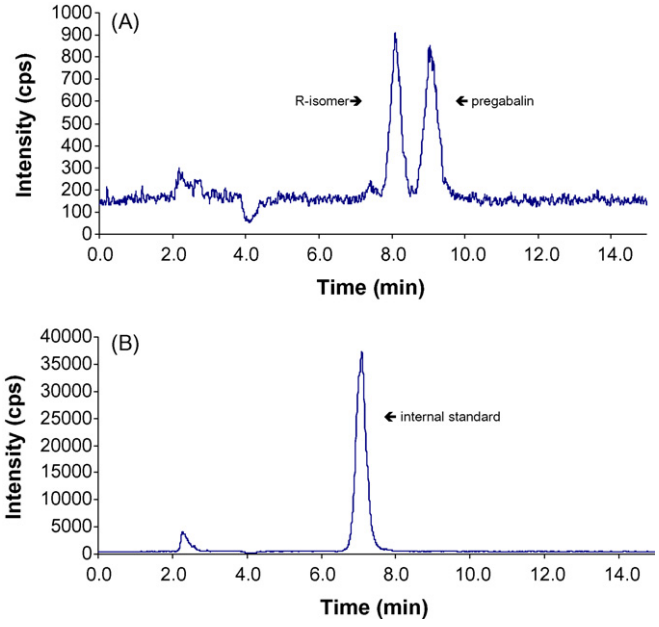

Fig. 4. A representative chromatogram of an extracted LLOQ sample. MRM monitoring of 50 ng/mL pregabalin and 50 ng/mL *R*-isomer (Trace A) and the internal standard (Trace B) in isolated rat kidney perfusate.

tion times of the analytes for both enantiomers using the sample preparation procedure described above (Fig. 5).

3.2.2. Linearity of calibration curve

The calibration curves for pregabalin and *R*-enantiomer were linear over the concentration range of 50–20,000 ng/mL. Calibration curve parameters are provided in Table 1.

3.2.3. Accuracy and precision

A three-batch validation was performed for perfusate whereas single-batch partial validations were performed for urine and perfusate ultrafiltrate. Intra- and inter-run (for perfusate only) accuracies for both pregabalin and *R*-enantiomer were within ±20% for the LLOQ and ±15% over the validation range of 50–20,000 ng/mL for the other QC samples. Intra- and inter-run (for perfusate only) assay precisions (CV%) for both pregabalin and *R*-enantiomer were within the acceptable range of 20% at LLOQ and 15% over the validation range for other QC samples. Accuracy and precision data are summarized in Table 2.

3.2.4. Ruggedness

It was observed that over 1000 injections of the prepared rat urine, rat IKP and rat IKP ultrafiltrate could be made onto the same chromatography column without degradation of the chromatographic parameters α, k, and R_S.

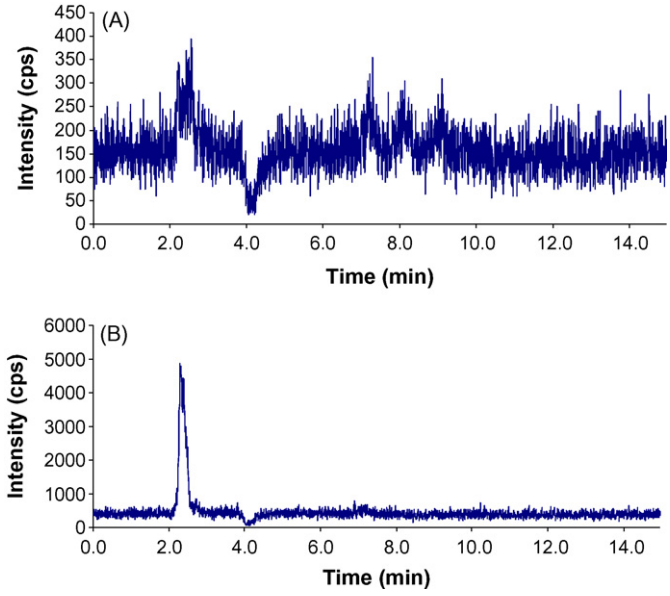

Fig. 5. A representative chromatogram of an extracted blank rat kidney perfusate sample without internal standard. MRM monitoring of pregabalin and its *R*-isomer (Trace A) and the internal standard (Trace B) in isolated rat kidney perfusate.

Table 1
Summary of standard calibration curve parameters

Curve Number	Perfusate[a]			Urine[b]			Perfusate ultrafiltrate[b]		
	a	*b*	*r*	*a*	*b*	*r*	*a*	*b*	*r*
Pregabalin									
1	0.000345	0.00740	0.9957	0.000335	0.00450	0.9977	0.000256	0.00457	0.9983
2	0.000303	0.00631	0.9939						
3	0.000279	0.00850	0.9948						
Mean	0.000309	0.00740	0.9948						
S.D.	0.0000334	0.00109	0.000900						
%CV	10.8	14.8	0.0900						
n	3	3	3	1	1	1	1	1	1
R-Enantiomer									
1	0.000264	0.00331	0.9939	0.000317	0.00662	0.9971	0.000209	0.00437	0.9929
2	0.000282	0.00229	0.9964						
3	0.000259	0.00241	0.9948						
Mean	0.000268	0.00267	0.9950						
S.D.	0.0000121	0.000557	0.001270						
%CV	4.51	20.8	0.130						
n	3	3	3	1	1	1	1	1	1

Linear regression $y = ax + b$ ($1/x^2$ weighing).
[a] Three batch run validation.
[b] Single batch run validation.

Table 2
Summary of QC sample validation results

Target concentration (ng/mL)	Perfusate[a]				Urine[b]				Perfusate ultrafiltrate[b]			
	50	150	1200	18000	50	150	1200	18000	50	150	1200	18000
Pregabalin												
Intra-assay precision (CV%)	7.2	3.0	7.4	3.8	8.3	4.3	6.4	6.5	4.9	6.1	2.7	4.7
% Error ($n = 6$)	−5.3	−2.9	−8.1	−6.8	−9.5	−4.1	13	10.9	1.9	6.0	3.2	3.5
Inter-assay precision	7.9	9.2	3.2	8.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
% Error ($n = 18$)	−4.0	2.1	6.2	7.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
R-Enantiomer												
Intra-assay precision (CV%)	6.5	3.2	4.6	4.5	8.6	7.3	12.8	7.7	7.9	9.2	3.2	8.6
% Error ($n = 6$)	−9.6	−4.5	−5.0	−5.4	−4.9	−8.7	9.1	8.5	−3.3	1.1	10	2.7
Inter-assay precision	14	8.3	8.8	8.7	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
% Error ($n = 18$)	−2.7	4.0	7.6	7.3	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

[a] Three batch run validation.
[b] Single batch run validation.

4. Conclusions

A direct chiral separation of the γ-amino acid analogue pregabalin and its *R*-isomer has been achieved using the macrocyclic glycopeptide stationary phase Chirobiotic T. The chromatographic separation was coupled to triple–quadrupole mass spectrometry and the resulting assays were validated to support isolated perfused rat kidney metabolic studies. The assay successfully met validation criteria for rat kidney perfusate, perfusate ultrafiltrate and urine. The assay demonstrated acceptable ruggedness as the assay was successfully used to support IPK studies and there was not a degradation in the chiral separation after at least 1000 injections of the sample extracts.

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