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Use of microbore LC–MS/MS for the quantification of oxcarbazepine and its active metabolite in rat brain microdialysis samples

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Abstract

A microbore LC–MS/MS method is developed and validated for the quantification of the anti-epileptic drug oxcarbazepine (OXC) and its active metabolite 10,11-dihydro-10-hydroxycarbamazepine (MHD) in rat brain microdialysates, together with the internal standard for microdialysis probe calibration, 2-methyl-5H-dibenz(b,f)azepine-5-carboxamide (*m*-CBZ). The benefits of gradient versus isocratic separation are shown, next to the improved sensitivity resulting from the addition of 0.1% formic acid to the mobile phase. The coupling of microdialysis with ESI-MS requires sample desalting for which column switching was applied. Using weighed regression to calculate the calibration curves (1–1000 ng/mL), the assay was validated in terms of linearity, accuracy and precision, yielding a sensitive (limit of quantification is 1 ng/mL) and selective method for quantification of OXC, MHD and *m*-CBZ. By applying this method, we were able to determine the extracellular concentrations of OXC and MHD during at least 4 h after intraperitoneal (i.p.) administration of 10 mg/kg OXC.

Keywords: Oxcarbazepine; 10,11-Dihydro-10-hydroxycarbamazepine; Microbore LC–MS/MS; Column switching; Rat brain microdialysis

1. Introduction

The tricyclic anti-epileptic drug oxcarbazepine (OXC) (Fig. 1) is the keto tautomer of the 10-hydroxy analogue of carbamazepine (CBZ) and has proven efficacy in patients with partial-onset seizures, with or without secondary generalisation. In comparison with CBZ, OXC has a reduced occurrence of side effects that can be ascribed to differences in biotransformation. OXC is a prodrug in humans and it is metabolised to the pharmacologically active 10,11-dihydro-10-hydroxycarbamazepine (MHD) (Fig. 1). This predominant pathway of hydroxylation of OXC to MHD in humans is only a minor pathway in rats [1].

We are interested in the pharmacokinetic and pharmacodynamic properties of OXC in the rat. In vivo brain microdialysis sampling is used to simultaneously monitor extracellular drug and neurotransmitter concentrations. In a previous study, we carried out quantitative microdialysis of OXC and its active metabolite, using retrodialysis of 2-methyl-5H-dibenz(b,f)azepine-5-carboxamide (*m*-CBZ) (Fig. 1) as in vivo calibration method [2]. This method allows to correct the dialysate concentrations for in vivo probe recovery, since dialysate concentrations are only a reflection of the real extracellular concentrations of the compounds. Several LC methods have been reported for the analysis of OXC and its active metabolite in plasma or serum [3–8]. We have previously described a validated LC assay with ultraviolet (UV) detection for the analysis of OXC and its major metabolite in rat brain and blood microdialysates [9]. However, with this method, depending on the experimental conditions, MHD concentrations were not always detectable in the small volume rat brain dialysates. Furthermore, we discovered that the peak of ketoprofen, currently used as post-operative analgesic in the rats, interfered with the one of MHD, pointing to a lack of selectivity of the method. In addition, this method was not validated for the quantitative analysis of *m*-CBZ. Therefore, we developed a microbore LC method coupled to tandem MS (MS/MS) detection for the quantification of OXC, MHD and *m*-CBZ, in brain microdialysates of the rat. The combination of LC with MS/MS yields a highly sensitive and selective technique, which is cru-

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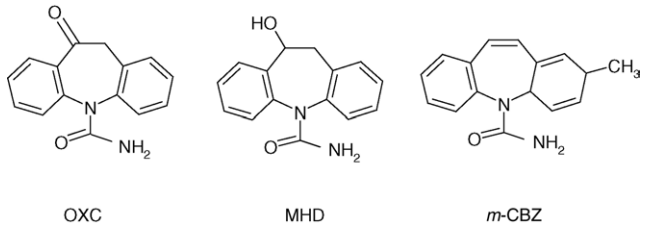

Fig. 1. Chemical structure of oxcarbazepine (OXC), 10,11-dihydro-10-hydroxycarbamazepine (MHD) and 2-methyl-5H-dibenz(b,f)azepine-5-carboxamide (*m*-CBZ).

cial for the analysis of low concentrated compounds in complex mixtures.

The coupling of microdialysis and electrospray ionisation (ESI-) MS is useful because microdialysis provides a highly filtered, low volume (20–40 μL), aqueous solution [10]. Therefore, microdialysis samples need little sample clean-up. However, desalting of the samples is necessary for analysis by ESI-MS/MS, since dialysates contain a large amount of salts. We chose to apply the column switching technique, because salts and other non-volatile hydrophilic contaminants are guided to waste before the introduction of the sample into the ion source.

The benefits of the application of the column switching technique, gradient elution and the importance of the addition of acid to the mobile phase for the analysis of these compounds in rat brain dialysis samples with LC–ESI-MS/MS were investigated. The method was applied to study the pharmacokinetic profile of OXC and MHD in rat brain after intraperitoneal (i.p.) injection of 10 mg/kg OXC.

2. Experimental

2.1. Chemicals

OXC and MHD were kindly donated by Novartis (Basel, Switzerland) and *m*-CBZ was bought from Aldrich Chemical (Milwaukee, USA). Methanol (MeOH, HPLC grade) was purchased from Fisher Scientific (Leicestershire, UK); acetonitrile (ACN, HPLC grade) and formic acid (FA, analytical-reagent grade) from Acros Organics (New Jersey, USA). The water used for preparing solutions was obtained from a Seralpur Pro 90 CN purification system (Seral, Ransbach-Baumbach, Germany).

Stock solutions of OXC (100 μg/mL), MHD (100 μg/mL) and *m*-CBZ (50 μg/mL) were made monthly in MeOH and stored in the refrigerator. Standards were made daily by further dilution with MeOH/Ringer's solution (10:90, v/v). The modified Ringer's solution was prepared weekly and filtered (0.2 μm filter) and consisted of 147 mM NaCl (Merck, Darmstadt, Germany), 4 mM KCl (Merck) and 2.3 mM $CaCl_2 \cdot 6H_2O$ (Fluka, Buchs, Switzerland).

For the i.p. injection of OXC, 10 mg was suspended in 600 μL propylene glycol (Vel, Leuven, Belgium) and 400 μL absolute ethanol (Carlo Erba reagenti, Milan, Italy) and consequently placed in an ultrasonic bath type Branson 2000 (Vel, Leuven, Belgium) for 30 min.

2.2. Liquid chromatography

The LC system consisted of an Ultimate™ quaternary low pressure gradient pump (LC Packings, Dionex, Amsterdam, The Netherlands). Injections (5 μL) were made in full loop mode using a refrigerated (12 °C, precautionary measure) Famos™ micro autosampler (LC Packings). The needle wash solvent was a water/ACN (50:50, v/v) solution. For column switching operations, a Switchos™ micro switching module (LC Packings) loaded the samples onto a precolumn. The LC was connected to an Ultimate™ UV detector, equipped with a U-Z View™ capillary flow cell (45 nL) (LC Packings). UV detection was performed during method development. The solvents for the mobile phase were degassed for 15 min on a Branson 2000 ultrasonic bath after preparation and by helium (Alphagaz 2®, Air Liquide, Liege, Belgium) during the analyses.

After injection, standards and dialysates were loaded for 5 min on to a Pepmap™ C18 precolumn (5 mm × 300 μm I.D., 5 μm particles) (LC Packings) at a flow-rate of 30 μL/min, to trap the compounds of interest and to direct the hydrophilic contaminants (mainly salts) to the waste. The loading solvent, delivered by the Switchos™ pump, consisted of water/ACN/FA (98:2:0.1, v/v). After the sample clean-up, the Switchos valve changed position and switched the precolumn in-line with the analytical column. The compounds of interest were eluted in back-flush mode and directed to the C18 separation column (15 cm × 1.0 mm I.D., 5 μm particles) (Unijet, BAS, Indiana, USA) at a flow-rate of 50 μL/min. Gradient elution was performed by the Ultimate™ pump, using solvent A (water/ACN/FA 98:2:0.1, v/v) and solvent B (water/ACN/FA 20:80:0.1, v/v). The linear LC gradient for separation was 30–100% B in 10 min, 100% B for 5 min, 100–30% B in 0.5 min and 30% B for 5 min. The total sample analysis time was 20 min. All LC parts were controlled by Chromeleon® software (Dionex, Amsterdam, The Netherlands).

2.3. Mass spectrometry

The LC eluent was directed on-line to the Quattro Premier™ triple quadrupole mass spectrometer (Waters-Micromass, Manchester, UK). Ionisation was performed by the standard electrospray (Z-spray®) source in the positive ion mode, with a 125 μm I.D. spray capillary. The source parameters were optimised as follows: capillary voltage was set at 3.0 kV, extractor and RF lens, respectively, at 3.0 and 0.0 V. The source temperature was kept constant at 110 °C and the nitrogen cone gas flow rated at 55 L/h. Desolvation temperature and nitrogen gas flow were 200 °C and 500 L/h. The nitrogen gas was produced by a Peak Scientific NM30LA nitrogen generator (Alkmaar, The Netherlands).

The full product ion spectra obtained with this system are similar to the ones described by Maurer et al. [8]. The analyser was operated in SRM mode and the following transitions were monitored with a dwell time of 0.3 s: OXC m/z 253.0 > m/z 180.0; MHD m/z 255.0 > m/z 194.0 and *m*-CBZ m/z 251.1 > m/z 193.0. The sensitivity was optimised for each singly charged compound by varying the cone voltage and collision energy.

For OXC, MHC and *m*-CBZ, the cone voltage was, respectively, 25, 20 and 35 V and the collision energy was set at 27, 20 and 30 eV. The parameters of the first quadrupole were as follows: LM resolution 13.0; HM resolution 13.0 (15/15 are the arbitrary units) and ion energy 0.6. Non-compound specific collision cell parameters for optimal transmission and fragmentation were: entrance 1.0 V and exit 1.0 V. The parameters of the second quadrupole measure: LM resolution 12.5; HM resolution 12.5 and ion energy 0.7. Argon (Alphagaz 2®, Air Liquide) was used as collision gas and the pressure in the gas cell was 3.8×10^{-3} mbar. The multiplier voltage was set at 650 V. All measurements were controlled by Masslynx® version 4.0 operating software (Waters-Micromass). Integration of the MRM chromatograms after smoothing (window size = 3; number of smooths = 2) was performed by Masslynx (Quanlynx).

2.4. Microdialysis

Protocols for animal experiments described in this study were carried out on freely moving rats, according to the national guidelines on animal experimentation and were approved by the Ethical Committee for Animal Experimentation of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel. The experiments were performed on male albino Wistar rats (Iffa Credo, Brussels, Belgium), weighing 280–360 g. The rats were given free access to food and water. The implantation of the cannula with a replaceable guide (CMA Microdialysis, Solna, Sweden) in the hippocampus of the rat was previously described by Clinckers et al. [11,12]. Immediately after surgery, animals received an i.p. injection of 4 mg/kg ketoprofen for postoperative analgesia and the guide was replaced by a 3 mm CMA 12 microdialysis probe that was continuously perfused with modified Ringer's solution at a constant flow-rate of 2 μL/min, using a CMA 100 microdialysis pump. Animals were allowed to recover from surgery overnight. The dialysate collection was started the day after the surgery. In the experiments on the metabolism of OXC to MHD in the rat, the probe was continuously perfused with 1000 ng/mL *m*-CBZ in Ringer's solution, as internal standard to correct for probe recovery variations [2], at a flow-rate of 2 μL/min. After equilibration for one hour, 10 mg/kg OXC was administered via an i.p. catheter and dialysates were collected every 10 min for another 4 h from the freely moving animals.

3. Results and discussion

3.1. Method development

LC separation before ESI-MS/MS detection proved to be indispensable to prevent sensitivity loss due to competition for ionisation between the analytes of interest. Indeed, we manually injected a standard mixture of 100 ng/mL OXC, MHD and *m*-CBZ in Ringer's/MeOH (90:10, v/v) three times directly into the ESI source via the divert valve (loop 5 μL), fitted on the MS, and almost no peaks were detected. Direct injection of a single standard of 100 ng/mL OXC in the same solvent, clearly yielded responses (data not shown). This observation points to competition between the three compounds for ion evaporation, resulting in a complete loss of ionisation efficiency. After all, the compounds have similar ionisation characteristics due to their structural analogy.

The possible differences between gradient and isocratic elution (water/ACN/FA 70:30:0.1) were explored and the results are summarised in Table 1. The sensitivity is reflected by the mean signal-to-noise ratio (S/N) ± standard deviation (S.D.) of six replicate analyses of a standard of 100 ng/mL OXC, MHD and *m*-CBZ. Although an isocratic method is generally preferred because of convenience, simplicity and reproducibility, we can observe from Table 1 that it is inappropriate to elute the compounds of interest in an isocratic way, at least for the later eluting compounds. With isocratic elution, the S/N for *m*-CBZ is significantly lower (Cochran test, $p < 0.01$) compared with gradient elution and this is the result of band broadening. Next to the higher separation efficiency, gradient elution results in a shorter analysis time (20 min in stead of 60 min). With gradient elution (Fig. 2), the retention times (mean ± S.D.) of OXC, MHD and *m*-CBZ are, respectively, 15.66 ± 0.04 min; 13.78 ± 0.04 min and 18.17 ± 0.03 min (100 ng/mL, $n = 10$).

In the future, this method will be miniaturised to capillary and nano LC–MS/MS dimensions. To enhance the concentra-

Table 1
Influence of the column switching technique, the addition of formic acid to the mobile phase and gradient elution vs. isocratic elution on the sensitivity of the assay, expressed as S/N ± S.D. ($n = 6$) of a 100 ng/mL standard (in Ringer's/MeOH 90:10, v/v) containing OXC, MHD and *m*-CBZ

	S/N ± S.D. with column switching ($\times 10^3$, $n = 6$)		S/N ± S.D. without column switching ($\times 10^3$, $n = 6$)	
	Gradient	Isocratic	Gradient	Isocratic
With FA				
MHD	0.90 ± 0.10*	2.01 ± 0.09	1.05 ± 0.07*	1.12 ± 0.13*
OXC	1.57 ± 0.22*,§	1.12 ± 0.04*	1.50 ± 0.08*,§	0.79 ± 0.08*
m-CBZ	2.25 ± 0.38*,§	0.54 ± 0.03*	2.04 ± 0.20*,§	0.61 ± 0.09*
Without FA				
MHD	0.28 ± 0.06	2.45 ± 0.30	0.16 ± 0.06	0.22 ± 0.06
OXC	0.35 ± 0.06	0.67 ± 0.08	0.30 ± 0.11§	0.11 ± 0.07
m-CBZ	0.30 ± 0.04§	0.06 ± 0.01	0.87 ± 0.15§	0.08 ± 0.04

* Significant improvement of S/N due to addition of 0.1% FA to the mobile phase (Cochran test $p < 0.01$).
§ Significantly higher S/N with gradient elution compared to isocratic separation (Cochran test, $p < 0.01$).

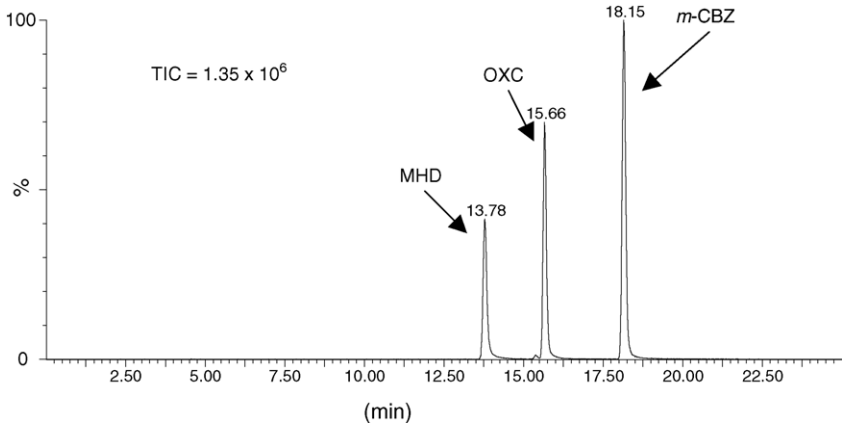

Fig. 2. MRM chromatogram of a 100 ng/mL standard mixture containing MHD, OXC and *m*-CBZ after gradient elution. The peaks in the MRM chromatograms show the relative responses towards the maximal abundances (TIC = total ion counts). Experimental conditions see Sections 2.2 and 2.3.

tion sensitivity of miniaturised methods, preconcentration of the sample is required. Therefore, we tested the suitability of the column switching technique within this microbore application. Table 1 shows that applying the column switching technique does not result in a clear gain in sensitivity. This is to be expected, since for this microbore LC–MS/MS application the device was not intended to be used for preconcentration of the sample but rather for salt removal. Without this sample clean up, large amounts of non-volatile contaminants, such as the salts in the dialysis samples, accumulate around the orifice of the ESI interface during continuous analysis, resulting in a decrease in sensitivity after a certain time [13]. The benefit of the application of the column switching technique is the improvement in robustness of the microbore LC–MS/MS method, thus making the assay suited for routine analysis. Otherwise, non-volatile contaminants can interfere with the operation of the ESI ion

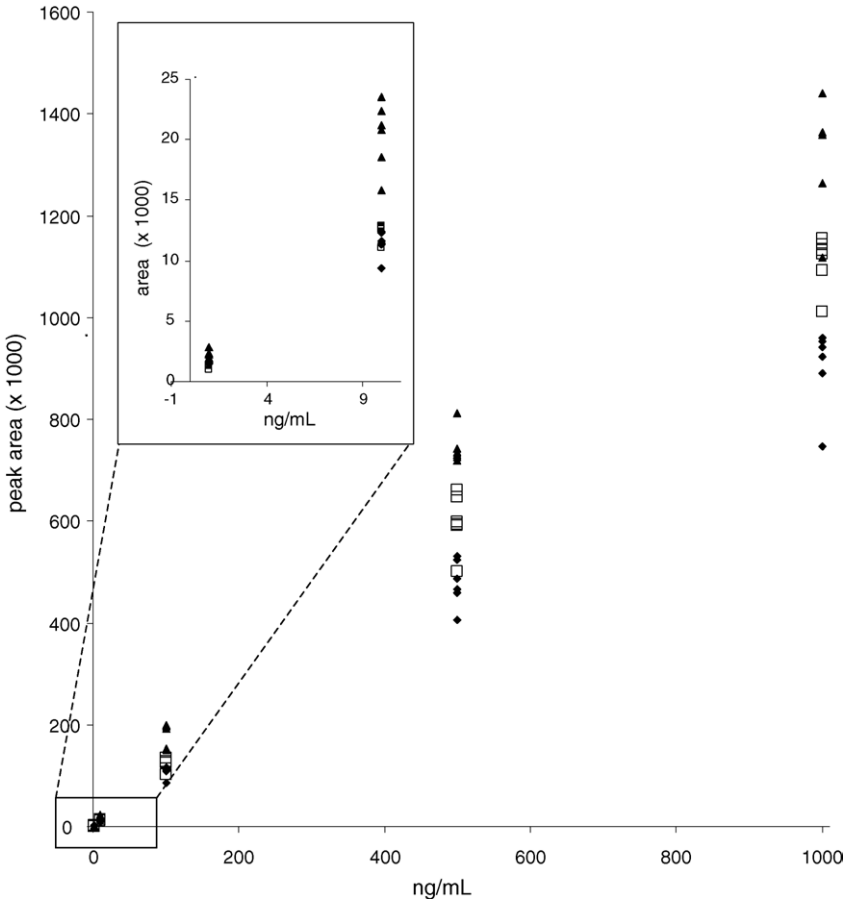

Fig. 3. Peak area–concentration relationship for MHD (♦), OXC (□) and *m*-CBZ (▲) at five concentration levels (1, 10, 100, 500 and 1000 ng/mL), with six replicates at each concentration.

source by clogging the skimmer and they can diminish the column lifetime.

Finally, Table 1 shows that the addition of formic acid to the mobile phase significantly (Cochran test, $p < 0.01$) improves the S/N ratio of a 100 ng/mL standard ($n = 6$) in most cases. The results confirm that the use of acid mobile phases is beneficial for analysis in ESI positive mode of the compounds of interest. Indeed, the acid pH promotes the protonation of the analyte molecules [14]. The concentration of acid however should be kept low (<0.2%, v/v), since it can reduce the MS signal [15].

3.2. Validation

3.2.1. Linearity of the calibration line

Standard solutions for the calibration curves, containing OXC, MHD and *m*-CBZ, were made daily in five different concentrations (1, 10, 100, 500 and 1000 ng/mL) by diluting the stock solutions in Ringer's/MeOH (90:10, v/v). The calibration curves were investigated in this range because of its relevance for pharmacokinetic studies of these anti-epileptic drugs. Linearity of the calibration lines was evaluated on six consecutive days. As can be seen from Fig. 3 the peak area variances for the three compounds increase with increasing concentration. Heteroscedasticity was confirmed by a significant *F*-test, for which the variances of the area of the six replicates at the levels of 1 and 1000 ng/mL were compared ($F_{0.05;6,6}$). Since the data showed non-uniform variance, a weighed least-squares procedure with weighing factor $1/x$ was used to calculate the calibration curve. For each compound, six calibration curves were analysed on consecutive days and the calibration curve for OXC was $y = (1196.7 \pm 47.9)x + (361.1 \pm 244.5)$; the equation for MHD was $y = (960.3 \pm 22.9)x + (335.1 \pm 163.7)$ and for *m*-CBZ $y = (1398.1 \pm 100.2)x + (2086.9 \pm 1308.2)$; with $y = (\text{mean} \pm \text{S.D.})x + (\text{mean} \pm \text{S.D.})$ and $n = 6$ for the three compounds. The regression curves were linear and the squares of the correlation coefficients exceeded 0.995.

3.2.2. Analytical precision

Repeated injections of standard solutions in Ringer's/MeOH (90:10, v/v) were carried out to test the instrument's performance (analytical precision). Intra-day precision was expressed by the relative standard deviations (%R.S.D.) of 10 repeated injections of the five standard concentrations (1, 10, 100, 500 and 1000 ng/mL) of the calibration curves for OXC and MHD. The inter-day precision for OXC and MHD was evaluated over 6 days in the same range of 1–1000 ng/mL. The intra- and inter-day precision for *m*-CBZ was evaluated at concentration levels of 500 and 1000 ng/mL. Indeed, for this compound, only the concentration range between 500 and 1000 ng/mL is relevant for the pharmacokinetic microdialysis experiments, since *m*-CBZ is added to the perfusate in a concentration of 1000 ng/mL (see Section 2.4). Table 2 summarises the results of the intra-day (a) and inter-day (b) precision. The %R.S.D. remains fairly constant across the concentration range of OXC and MHD, confirming the observed heteroscedastic data of the calibration curve. The %R.S.D. values for the intra-day precision are smaller than 3.0, 3.5 and 4.0% for OXC, MHD and *m*-CBZ, respectively, while for the inter-day precision the maximal percentages are, respectively, 13.0, 10.5 and 8.6%.

Table 2
Analytical precision was tested for OXC and MHD by injecting the standards of the calibration curve (1, 10, 100, 500 and 1000 ng/mL in Ringer's/MeOH 90:10, v/v) 10 times into the LC–MS/MS system in 1 day (intra-day precision) (a) and 1 time on 6 different days (inter-day precision) (b)

	1 ng/mL	10 ng/mL	100 ng/mL	500 ng/mL	1000 ng/mL
(a) Intra-day precision ($n = 10$)					
OXC	2.9	1.1	1.9	1.2	0.8
MHD	3.5	2.4	2.2	1.2	1.4
m-CBZ				4.0	1.6
(b) Inter-day precision ($n = 6$)					
OXC	13.0	6.0	9.4	9.4	4.7
MHD	3.9	9.4	10.5	9.7	9.7
m-CBZ				4.6	8.6

For *m*-CBZ the same protocol was followed at the concentration levels 500 and 1000 ng/mL. The precisions are expressed as relative standard deviations (%R.S.D.).

3.2.3. Accuracy and method precision

The accuracy of the LC–MS/MS method was assessed by analysing six replicates of blank hippocampal microdialysate matrix, obtained with Ringer's solution as perfusion fluid, spiked with OXC, MHD and *m*-CBZ. Table 3 summarises the recovery percentages, representing the ratios of the theoretical concentrations after spiking, to the experimentally obtained concentrations (i.e. concentrations of the spiked microdialysis samples as estimated using the calibration curve made with standards in Ringer's/MeOH). For OXC and MHD, the blank dialysates were spiked at a level of 1, 10, 100, 500 and 1000 ng/mL. For the same reason as described in Section 3.2.2, the accuracy for *m*-CBZ was assessed at the levels of 500 and 1000 ng/mL, with six replicates at each level. Overall, the recoveries ranged from 92 to 111%. Since these values are within the acceptance criteria (<15%) proposed at the Washington conference [16], the method can be considered accurate for this application. Consequently, we can conclude that there is no significant matrix effect.

For the evaluation of the method precision, the data of the accuracy testing were used (Table 3). Blank hippocampal dialysate matrix, obtained with Ringer's solution as a perfusion fluid, was spiked with different concentrations (1, 10, 100, 500 and 1000 ng/mL) of OXC and MHD. Again the investigated

Table 3
Accuracy was calculated at each test concentration of the calibration curve for OXC and MHD

	1 ng/mL	10 ng/mL	100 ng/mL	500 ng/mL	1000 ng/mL
OXC	104 ± 11	100 ± 1	107 ± 1	106 ± 1	98 ± 6
MHD	107 ± 6	111 ± 1	111 ± 3	108 ± 3	98 ± 5
m-CBZ				102 ± 1	92 ± 5

The accuracy was tested at two levels for *m*-CBZ. Recoveries are calculated as the ratios of the theoretical concentration after spiking blank dialysate matrix, to the experimentally obtained concentration (concentration of spiked dialysis samples as estimated using the calibration curve, made with standards in Ringer's/MeOH 90:10, v/v) and are expressed as percentages. The data are shown as mean % ± S.D. ($n = 6$).

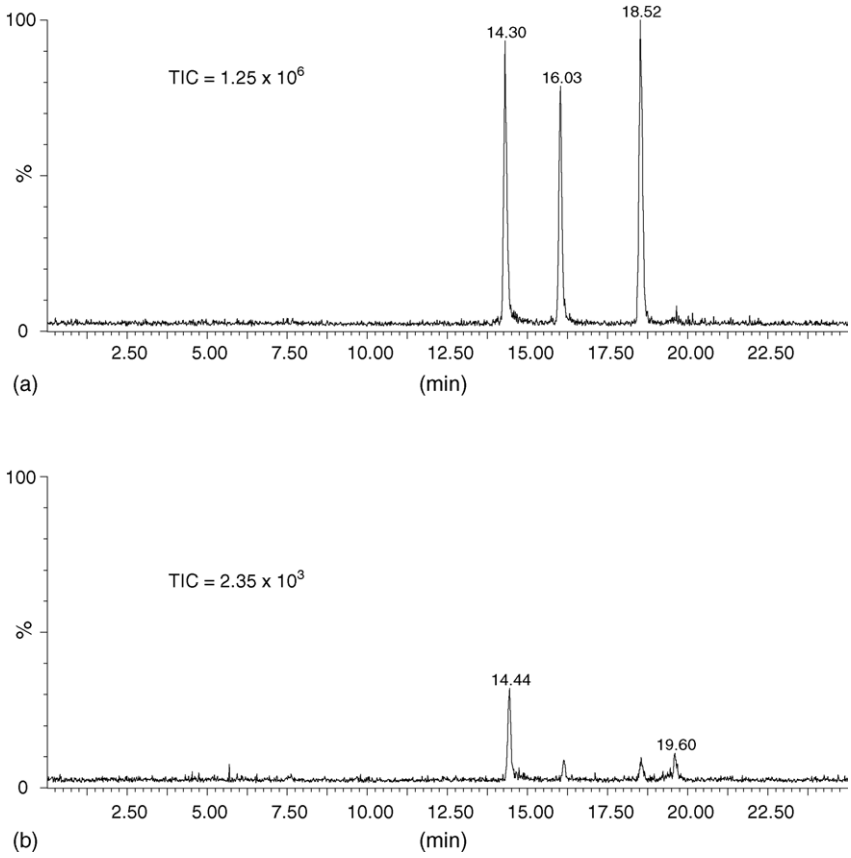

Fig. 4. MRM chromatogram of a blank hippocampal dialysate matrix (b) injected after a dialysate matrix spiked with MHD, OXC and *m*-CBZ at 100 ng/mL (a). The peaks in the MRM chromatograms show the relative responses towards the maximal abundances of both (a) and (b) (TIC = total ion counts). Experimental conditions see Sections 2.2 and 2.3.

concentration levels for *m*-CBZ were 500 and 1000 ng/mL. Each concentration was analysed on six consecutive days (intermediate precision) and six replicates of each sample were analysed within 1 day (method repeatability). The data were evaluated by one-way ANOVA, yielding estimates for both method repeatability and intermediate precision. The method repeatability for OXC, MHD and *m*-CBZ, expressed as %R.S.D., is respectively 5.4, 3.6 and 3.6%. The intermediate precision for OXC, MHD and *m*-CBZ was calculated as, respectively, 9.3, 12.4 and 17.7%. Since the %R.S.D. values of the inter-day analytical precision and the intermediate method precision are rather large compared to the ones of the intra-day analytical precision and the method repeatability, we suggest that a calibration curve is made daily and that samples of a microdialysis experiment are analysed within 1 day.

3.2.4. Limit of detection and quantification

For the determination of the limits of detection (LOD), blank hippocampal dialysates were collected for 20 min, during the perfusion of the microdialysis probe with Ringer's solution at a flow-rate of 2 μL/min. The LOD is expressed as $3.3\sigma/S$, where σ is the S.D. of the responses of the blanks ($n = 6$) and S is the slope of the calibration curve [17]. For OXC, MHD and *m*-CBZ the LOD is, respectively, 0.04, 0.06 and 0.03 ng/mL. The limit of quantification (LOQ) is the lowest concentration that can be determined with acceptable accuracy and precision. The validated LOQ was 1 ng/mL for the three compounds. A further gain in sensitivity can be obtained by miniaturisation of the LC–(ESI-)MS method and this is currently being investigated in our laboratory. Comparison with the LOD values formerly obtained by Maurer et al. [8] and by Van Belle et al. [9], reveals that our LC–MS/MS method is, respectively, about 200 and 20 times more sensitive. This allows the detection of OXC, MHD and *m*-CBZ in the dialysis samples for a longer time period after i.p. administration of OXC and/or with a higher microdialysis sampling frequency.

Due to the low LOD of this method, carry-over problems are more visible than with the less sensitive assay using UV detection. When a blank hippocampal dialysate is injected after a series of standard mixtures (100 ng/mL), carry-over peaks are present for the three compounds (Fig. 4a and b). Changing the composition of the needle wash solvent had no apparent effect on these carry-over peaks. Since the degree of carry-over remains rather constant, the responses for the blanks were subtracted from the responses for the three compounds in the standards and the dialysates. The degree of carry-over was used as a system suitability test, and should not exceed 0.5% when injecting a blank dialysate after a series of six standard mixtures of 100 ng/mL. When this limit is exceeded, several blank injections have to be performed to diminish the percentage carry-over.

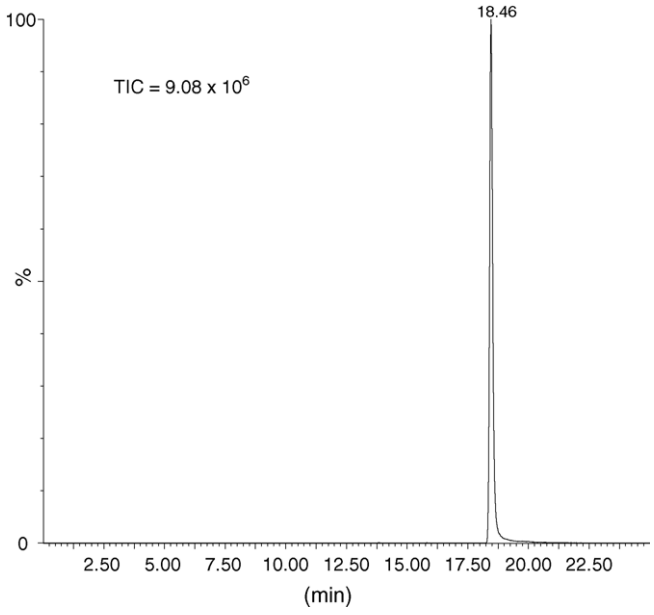

Fig. 5. MRM chromatogram of a hippocampal dialysis sample, collected during perfusion of the microdialysis probe with 1000 ng/mL *m*-CBZ in Ringer's solution. Experimental conditions see Sections 2.2, 2.3 and 2.4.

3.2.5. Selectivity

In Fig. 5, a chromatogram is shown of a hippocampal dialysate, collected during perfusion of the microdialysis probe with 1000 ng/mL *m*-CBZ in Ringer's solution (see Section 2.4) and before i.p. injection of 10 mg/kg OXC. Besides the peak for *m*-CBZ, no other peaks (from ketoprofen or endogenous compounds) are present which may interfere with the analytes. Additionally, when we performed the same LC method coupled to UV detection, ketoprofen was well separated from the other compounds of interest (data not shown).

3.3. Quantification of OXC and MHD in hippocampal rat brain dialysates

The validated LC–MS/MS method was applied to quantify OXC and MHD in rat brain microdialysates after i.p. administration of 10 mg/kg OXC. To estimate extracellular concentrations from the dialysate concentrations, in vivo probe recovery needs to be determined. The extracellular concentrations of OXC and MHD were calculated as described by Van Belle et al. [2]. Briefly, retrodialysis of an internal standard was used as in vivo calibration method for microdialysis. The in vivo microdialysis probe recoveries of OXC and MHD were estimated by the loss of *m*-CBZ, the internal standard, from the perfusion fluid. The ratios of the in vivo relative recovery (RR) for OXC and MHD to the relative loss (RL) for *m*-CBZ are constant and these f-values were found to be, respectively, 0.42 ± 0.10 and 0.19 ± 0.06 ($n = 6$). In Fig. 6, the extracellular (i.e. dialysate concentrations corrected for in vivo probe recovery) hippocampal concentration-time curves for OXC and MHD after i.p. administration of OXC (10 mg/kg) are shown. The concentrations are presented as means with standard error on the mean ($n = 5$). The maximal mean extracellular concentrations

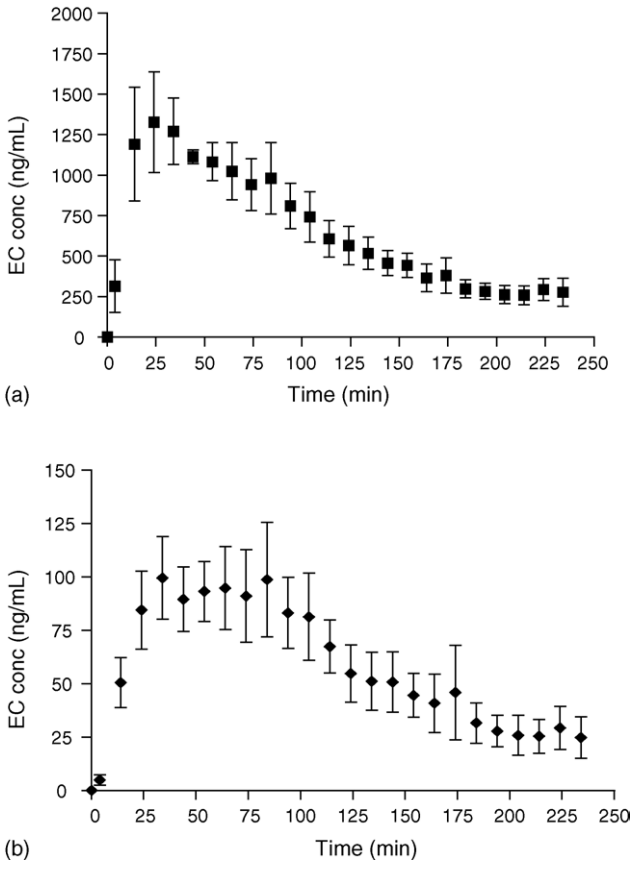

Fig. 6. Hippocampal extracellular concentrations (i.e. dialysate concentrations corrected for in vivo probe recovery, EC) of OXC (a) and MHD (b) as a function of time after i.p. injection of 10 mg/kg OXC. The data are presented as means with standard error on the mean ($n = 5$) and the dialysate sampling times are corrected for lag time and time-averaged. The dialysate concentrations of OXC and MHD are less than 10% of the real extracellular concentrations (see Section 3.3). Experimental conditions see Sections 2.2, 2.3 and 2.4.

(C_{max}) of OXC and MHD are, respectively, 1329 ± 24 ng/mL and 100 ± 2 ng/mL ($n = 5$). By comparing the area under the curves (AUC) of OXC and MHD, we estimated that the AUC of MHD is only 8.8% of the AUC of the parent compound. Based on the RL for *m*-CBZ and the previously determined f-values, the mean in vivo probe recoveries for OXC and MHD were determined as $4.3 \pm 1.5\%$ and $9.6 \pm 3.4\%$ (mean% ± S.D., $n = 5$). Thus, the dialysate concentrations obtained with this assay are less than 10% of the extracellular concentrations (Fig. 6). The feasibility of the quantification of the low concentrations of MHD in the rat brain during this 4 h collection period after i.p. admininistration of OXC, is due to the high sensitivity of the assay.

4. Conclusions

This is the first paper describing a column switching microbore LC–MS/MS method with gradient elution for the quantitative analysis of OXC, its active metabolite (MHD) and *m*-CBZ in rat brain microdialysis samples. The addition of formic acid to the mobile phase was proven to enhance sensitivity. This highly sensitive analysis technique was shown to be able to reliably

determine OXC and MHD in rat brain microdialysates uptil 4 h after i.p. administration of 10 mg/kg OXC.

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