

Liquid chromatographic assay using a microcolumn coupled to a U-shaped optical cell for high-sensitivity ultraviolet absorbance detection of oxcarbazepine and its major metabolite in microdialysates

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Abstract

An isocratic LC assay using a microcolumn (800 μm I.D.) coupled to a U-shaped optical flow cell (cell volume 70 nl; optical path length 8 mm) for high-sensitivity UV absorbance is described for the detection of oxcarbazepine and its major and active metabolite, 10,11-dihydro-10-hydroxycarbamazepine in microdialysates. Using the combination microcolumn–capillary UV detector, a ten-fold increase in sensitivity was obtained resulting in a limit of detection of 10 pg/10 μl . This assay is sufficiently sensitive to allow quantification of drug and metabolite in 10- μl aliquots of rat blood and hippocampus microdialysates, using carbamazepine-10,11-epoxide as external standard.

1. Introduction

Carbamazepine is a major drug in the treatment of epilepsy and exhibits excellent antiepileptic properties with less pronounced toxicity compared to other antiepileptic drugs [1,2]. However, carbamazepine treatment must be discontinued because of serious side effects in 5% of the patients [3].

Oxcarbazepine (OX) (10,11-dihydro-10-oxo-carbamazepine: GP 47680), a compound chemically closely related to carbamazepine, was developed to reduce occurrence of side effects. OX

exhibits a pharmacologic spectrum and potency similar to that of carbamazepine but with a lower frequency of side effects [4,5].

In humans, OX is a prodrug, immediately metabolized to the pharmacologically active 10,11-dihydro-10-hydroxycarbamazepine (MHC) (GP 47779), and present at low concentrations [6]. In contrast, the metabolism of OX is less pronounced in rats, resulting in low concentrations of the metabolite [7].

Microdialysis, an *in vivo* sampling technique, allows to study distribution, metabolism and elimination of drugs simultaneously in different tissues of freely moving laboratory animals [8]. Due to dialysis recovery, small fractions of unbound drug and metabolite are collected in small

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volume samples. Therefore, low dialysate concentrations need to be analyzed.

The increase in mass sensitivity, improved separation efficiency, higher speed of analysis and reduction of sample and reagent consumption are the most important advantages of microcolumn separations [9,10].

However, ordinary UV detectors can not be combined with microcolumns due to their large cell volumes (8–12 μl ; path length 5–10 mm) and most UV detectors used in microseparation techniques suffer from a loss in sensitivity due to small cell volumes (10–100 nl; path length 50–320 μm) necessary to avoid peak dispersion.

Chervet et al. [11] successfully constructed a sensitive optical U-shaped UV flow cell with a total volume of 70 nl and an optical path length of 8 mm with a special design (Fig. 1) and capillary connections to minimize dead volume.

A limitation in microseparations is the lack of reliable instrumentation for the delivery of highly reproducible microflows. The use of standard pumps with higher flow-rates (50–400 $\mu\text{l}/\text{min}$) and flow-splitting improves the reduction of baseline noise due to flow fluctuations [12].

We report an LC system using a microcolumn coupled to a U-shaped optical cell for high-sensitivity UV absorbance detection of oxcarbazepine and its major metabolite in small volumes of blood and brain dialysates of the rat using carbamazepine-10,11-epoxide (CBZ-EPO) as external standard.

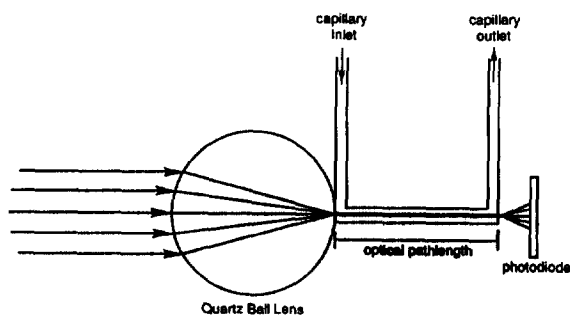


Fig. 1. High-sensitivity UV absorbance U-shaped optical flow cell (cell volume 70 nl; path length 8 mm) with 75 μm I.D. capillary.

2. Experimental

2.1. Chemicals and reagents

OX, MHC and CBZ-EPO were gifts from Ciba-Geigy (Basle, Switzerland). All other chemicals were analytical grade or better and purchased from Merck (Darmstadt, Germany).

Stock solutions of OX, MHC and CBZ-EPO (100 $\mu\text{g}/\text{ml}$) were made in methanol and kept at -20°C . Further dilutions were made in filtered (0.2 μm membrane filter) fresh water purified by a Seralpur pro 90 CN (Belgolabo, Overijse, Belgium).

Modified Ringer's solution (147.5 mmol/l Na^+ , 4 mmol/l K^+ , 2.3 mmol/l Ca^{2+} and 156.1 mmol/l Cl^-) and physiological saline were filtered (0.2 μm membrane filter) and used as perfusion solutions in the microdialysis experiment.

2.2. Chromatographic system

The LC system consisted of a Gilson 305 piston pump (Villers le Bel, France) with a manometric module 805 as pulse dampener, operating at a flow-rate of 0.25 ml/min over a narrow-bore column (Ultrasphere ODS, 2 mm \times 25 cm, 5 μm , Beckman, San Ramon, CA, USA) responsible for the high pressure. Separation was performed on a microcolumn (Hypersyl C18 BDS, 800 μm \times 15 cm, 3 μm , LC Packings, Amsterdam, Netherlands). Due to the use of a splitter, the flow-rate through the microcolumn was 25 $\mu\text{l}/\text{min}$.

The column inlet was connected with a Kontron 465 HPLC autosampler (Milano, Italy) using capillary fused-silica (50 μm I.D.).

The column outlet capillary was directly connected via a small piece of Teflon (Teflon tubing kit, LC Packings) to the U-shaped flow cell with a quartz ball lens [13] (Fig. 1) (cell volume 70 nl; optical path length 8 mm) (LC Packings), to minimize the dead volume. The flow cell was mounted in a Kontron 433 capillary detector.

The chromatograms were integrated with an integration computer program (Integration Pack, Kontron).

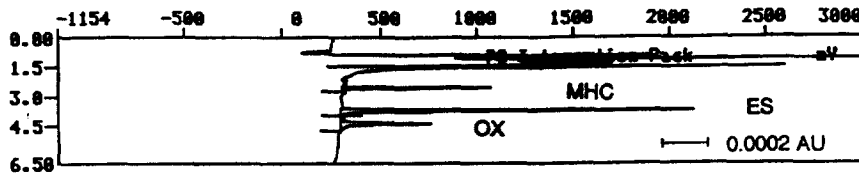


Fig. 2. Chromatogram of a 25 ng/ml standard of OX and MHC.

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile and filtered water (27:73). The sensitivity of the detector was set at 0.002 (blood dialysates) or 0.001 (brain dialysates) AUFS and the detection wavelength was 220 nm.

To 10 μ l of standard and dialysate samples 2.5 μ l of external standard solution (CBZ-EPO, 1 μ g/ml) was added. A 10- μ l aliquot of this mixture was injected onto the column.

2.4. Microdialysis experiment

Microdialysis samples were collected from the hippocampus and the jugular vein of freely moving animals to determine the transport across the blood–brain barrier of OX and its metabolite.

A male albino Wistar rat (\pm 250 g) was anaesthetized with a mixture of ketamine and diazepam (50 and 5 mg/kg). An intracranial guide cannula (CMA, Stockholm, Sweden) was placed 3 mm above the hippocampus and a flexible microdialysis probe (CMA20, 1 cm membrane length) was placed in the jugular vein. After surgery, the inner cranial guide was replaced by a rigid microdialysis probe (CMA10, 3 mm membrane length). The rats were allowed to recover from surgery for 24 h.

The probes were connected to a microinjection pump (CMA 100) and perfused with modified Ringer's solution (hippocampus) or physiological saline (blood) at a flow-rate of 2 μ l/min.

Dialysates were collected every 20 min in basal (pre-dose) conditions for 1 h. Then OX was administered (10 mg/kg; intraperitoneally (i.p.)) and dialysates were collected for another 4 h.

Dialysates are clean and free of proteins so no sample preparation is necessary.

3. Results and discussion

3.1. Assay characteristics

The mean capacity factors (k') [$= (t_r - t_0)/t_0$, where t_r and t_0 are the elution times of retarded and unretarded solutes, respectively] and standard deviations (S.D.) of MHC, CBZ-EPO and OX over 6 days were 2.50 (0.10), 4.00 (0.08) and 4.83 (0.08), respectively.

A chromatogram of a 25 ng/ml standard of OX, MHC with CBZ-EPO as the external standard is shown in Fig. 2. Fig. 3 shows a chromatogram of a blank blood dialysate and Fig. 4 a blood dialysate after i.p. administration of OX (10 mg/kg). No interference of carbamazepine, phenobarbital or phenytoin appeared.

Microdialysis offers the opportunity to investi-

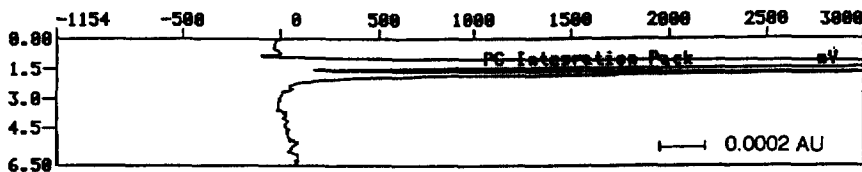


Fig. 3. Chromatogram of a blank blood dialysate.

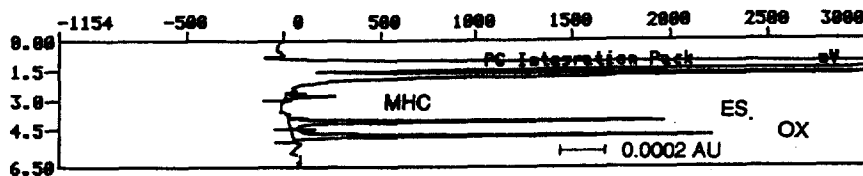


Fig. 4. Chromatogram of a blood dialysate after i.p. administration of OX (10 mg/kg).

gate simultaneously the transport of drug and metabolite across the blood–brain barrier [14] and the effect on endogenous compounds such as neurotransmitters [15]. The limitation of this sampling technique is the small sample volume (40 μ l) which needs to be split for the analysis of exogenous and endogenous compounds.

Noirfalise and Collinge [16] described a conventional LC method with determination limits of 150 ng/ml (OX) and 1000 ng/ml (MHC). This method is not sensitive enough to determine drug and metabolite in the collected dialysates. In a previous paper [10], the separation of OX and its metabolite was performed after injection of a 50- μ l aliquot on a narrow-bore column (I.D. 2.1 mm) and the limit of detection obtained was 10 ng/ml. The purpose of this work was to gain at least the same sensitivity for a 5-fold reduced injection volume. Therefore, the narrowbore column was replaced by a microcolumn (I.D. 0.8 mm). Theoretically, the gain in sensitivity by reducing the I.D. of the column, for the same injection volume, is given by the following equation:

$$\frac{\text{I.D.}_{\text{narrow}}^2}{\text{I.D.}_{\text{micro}}^2} = \frac{(2.1)^2}{(0.8)^2}$$

For a 5-fold decrease in injection volume this would result in a 1.38-fold increase in sensitivity. Practically, this sensitivity cannot be achieved using a standard capillary flow cell due to the small path length (10–100 nl; path length 50–320 μ m). Therefore we combined the separation on a microcolumn with the ultra-sensitive U-shaped flow cell (cell volume 70 nl; optical path length 8 mm) for UV absorbance detection.

The sensitivity criteria were calculated for six standards: the limit of detection was taken as the

amount corresponding to a signal-to-noise ratio of 3 and was 1 ng/ml (= 10 pg on column) for OX and its metabolite with coefficients of variation (C.V.) of 10.5% and 10.6%, respectively.

The combination of a microcolumn with the U-shaped flow cell provides a 10-fold increase in sensitivity for a 5-fold decrease of the injection volume.

The quantification limit was 5 ng/ml (= 50 pg on column) for both compounds (C.V. for OX 5.7%, C.V. for MHC 5.4%).

Good linearity ($n = 6$) was exhibited for OX and MHC over the concentration range 5–500 ng/ml. The coefficients of correlation (mean and C.V.) were respectively 0.9997 (0.03%) and 0.9996 (0.02%).

3.2. Analytical precision

Within-run precision was determined by comparing the peak-area ratios for six standard curves ranging from 5 to 500 ng/ml on the same day. The run-to-run precision of standard curves in the range from 5 to 500 ng/ml was determined on six different days. Results of within-run and run-to-run precision are summarized in Table 1.

3.3. Method precision and accuracy

Pools of blank blood and hippocampus dialysates were spiked, resulting in OX and MHC concentrations of 100 ng/ml. From each pool (hippocampus, blood) six samples were analyzed. The recoveries were calculated by dividing the peak-area ratios (compound to external standard) of the spiked dialysates by the peak-area ratio of the 100 ng/ml standard and

Table 1
Analytical precision for OX and MHC

OX			MHC		
Concentration (ng/ml)	Peak-area ratio (mean \pm S.D.)	C.V. (%)	Concentration (ng/ml)	Peak-area ratio (mean \pm S.D.)	C.V. (%)
<i>Within-run precision (n = 6)</i>					
0	–	–	0	–	–
5	0.0123 \pm 0.0007	5.7	5	0.0204 \pm 0.0011	5.4
25	0.0774 \pm 0.0041	5.3	25	0.1155 \pm 0.0047	4.1
50	0.1301 \pm 0.0049	3.8	50	0.1695 \pm 0.0061	3.6
100	0.2537 \pm 0.0066	2.6	100	0.3267 \pm 0.0046	1.4
250	0.7322 \pm 0.0167	2.3	250	0.8711 \pm 0.0177	2.0
500	1.4478 \pm 0.0290	2.0	500	1.7100 \pm 0.0156	0.9
<i>Run-to-run precision (n = 6)</i>					
0	–	–	0	–	–
5	0.0119 \pm 0.0009	7.6	5	0.0183 \pm 0.0012	6.6
25	0.0824 \pm 0.0054	6.6	25	0.1102 \pm 0.0061	5.5
50	0.1284 \pm 0.0073	5.7	50	0.1647 \pm 0.0094	5.7
100	0.2503 \pm 0.0099	3.9	100	0.3398 \pm 0.0113	3.3
250	0.6823 \pm 0.0240	3.5	250	0.8798 \pm 0.0255	2.9
500	1.4120 \pm 0.0434	3.1	500	1.7901 \pm 0.0177	1.0

Table 2
Method precision and accuracy for OX and MHC

Compounds	Sample	Recovery (n = 6) (mean) (%)	C.V. (%)
OX	Blood	100.2	2.8
	Hippocampus	99.8	2.6
MHC	Blood	99.9	1.6
	Hippocampus	100.1	1.4

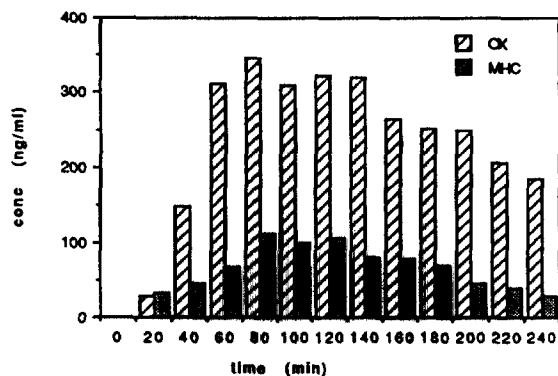


Fig. 5. Exact OX and MHC extracellular concentration–time curves for hippocampus after i.p. administration of OX (10 mg/kg).

expressed in percentage. These recoveries are given in Table 2.

3.4. Measurement of OX and MHC in rat blood and hippocampus dialysates

The described LC method was used to measure the OX and MHC concentrations in rat blood and hippocampus dialysates after i.p. administration of OX (10 mg/kg).

The dialysate concentrations are smaller than the real extracellular tissue and unbound blood concentrations due to the recovery of the dialysis membrane which is smaller than 100%. Therefore, exact tissue and unbound blood concentrations of OX and MHC were calculated using the internal standard technique [17] as in vivo calibration method for the microdialysis.

Fig. 5 shows the exact OX and MHC concentration–time curves for hippocampus, and Fig. 6 the unbound blood concentration–time curves for OX and metabolite after i.p. administration of OX (10 mg/kg).

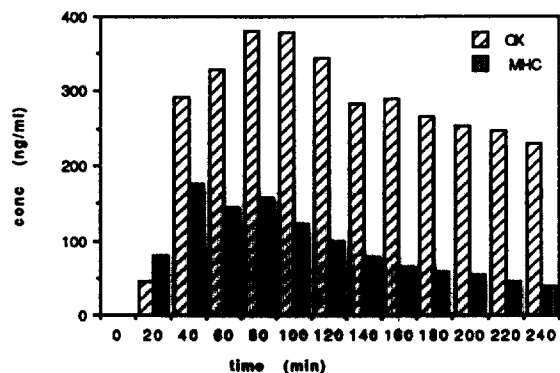


Fig. 6. Unbound blood concentration–time curves for OX and MHC after i.p. administration of OX (10 mg/kg).

4. Conclusion

A capillary LC assay is described giving adequate separation and sensitivity to determine OX and its active metabolite in rat hippocampus and blood dialysates.

The advantage of increased mass sensitivity and efficiency in microseparations coupled to the ball-lens-modified U-shaped cell provides a breakthrough for UV absorbance detection. A 10-fold sensitivity gain in 10 μ l of sample was obtained. This allows to determine endogenous compounds in the remaining 30 μ l of the microdialysis sample.

Sensitive LC systems combined with microdialysis makes it possible to study simultaneously the pharmacokinetics and pharmacology of drugs.

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