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Development of a high throughput 96-well plate sample preparation method for the determination of trileptal (oxcarbazepine) and its metabolites in human plasma

C. Souppart*, M. Decherf, H. Humbert, G. Maurer

Drug Metabolism and Pharmacokinetics, Novartis Pharma SA, 2–4 Rue Lionel Terray, 92506 Rueil-Malmaison, France

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Abstract

A high throughput preparation method for the determination of trileptal (oxcarbazepine, OXC) and its mono (MHD) and dihydroxy (DHD) metabolites in human plasma, using 96-well plate technology, has been developed and validated according to international regulatory requirements. Preparation of plasma samples (50 μl) containing the compounds to be analysed involved solid-phase extraction (SPE) on Empore C_{18} 96-well SPE plates. Eluates from the plate were injected onto a reversed-phase column (Hypersil C_{18}, 3 μm) with UV detection at 210 nm. Detector response was linear over the ranges 0.2–10, 0.1–200 and 0.1–20 μmol/l, for OXC, MHD and DHD, respectively, with relative standard deviations from 1 to 10% and mean accuracies within 4% of the nominal values (number of standard curves=3 in duplicate). The limits of quantitation were 0.2, 0.1 and 0.1 μmol/l, respectively. The overall mean accuracies ranged from 96 to 106% and precision was in the range 4 to 11%. Cross validation indicated no significant difference between plasma concentrations obtained using the 96-well method and the previous method using a traditional SPE method with a 50 mg C_{18} cartridge. About a threefold increase in sample throughput and a twofold decrease of plasma volume required for the assays, were the main advantages obtained from the previous method. The method was applied for the determination of 3000 plasma samples from clinical studies.

Keywords: 96-Well plates; Trileptal; Oxcarbazepine

1. Introduction

Trileptal is the trademark of oxcarbazepine (10,11-dihydro-10-oxo-5H-dibenz[*b*,*f*]azepine-5-carboxamide, Fig. 1), an antiepileptic drug currently registered in over 50 countries and recently accepted for registration worldwide.

Oxcarbazepine (OXC) undergoes reductive metabolism of the keto group to form the active monohydroxy derivative, MHD (10-hydroxy-10,11-dihydro-10-oxo-5H-dibenz[*b*,*f*]azepine-5-carboxamide, Fig. 1). This metabolite predominates in plasma after oral dosing whereas OXC reaches only low levels. MHD is thus the main compound responsible for the antiepileptic activity of OXC in man. A minor amount is oxidised to an inactive dihydroxy derivative, DHD (10,11-dihydro-10,11-*trans*-dihydrocarbamazepine, Fig. 1).

The need for fast sample preparation in bioanalytical analysis is growing rapidly as it is often the

*Corresponding author. Tel.: +33-1-5547-6889; fax: +33-1-5547-8167.

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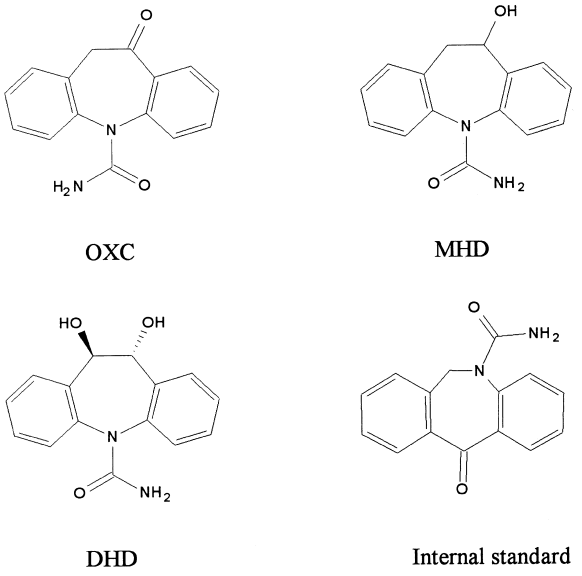

Fig. 1. Chemical structures of oxcarbazepine, its metabolites and the internal standard.

bottleneck step avoiding speeding up the overall analysis process. There is a recent trend for batch sample preparation in the 96-well format, the samples being processed simultaneously. Another advance in the area of solid-phase extraction (SPE) in the 96-well format is the availability of membrane-extraction discs instead of conventional packings: the Empore membrane [1–5]. It consists of bonded silica particles immobilised within an inert matrix of polytetrafluoroethylene (90% particles) and graded density polypropylene filters.

Sample preparation in the 96-well format is mainly used in conjunction with liquid chromatography–tandem mass spectrometry (LC–MS–MS) because this technique allows short analysis times due to its high specificity. However, when a high sensitivity is not required, optical detection is still used because it is less expensive than MS–MS detection and requires less expertise and less maintenance. When UV detection is applied, sample preparation in the 96-well format can also enhance sample throughput.

The described method was validated with respect to accuracy, precision, selectivity and limit of quantitation (LOQ) according to Good Laboratory Practice Guidelines [6].

2. Experimental

2.1. Solvents and chemicals

OXC, MHD and DHD were synthesised by Novartis Pharma (Basle, Switzerland). An analog of OXC (5-carboxamide) was also provided by Novartis Pharma and used as internal standard (I.S.). Analytical-grade methanol and acetonitrile were obtained from Carlo-Erba (Nanterre, France). Potassium dihydrogenorthophosphate was obtained from Fisher (Elancourt, France). Water was deionised, filtered and purified on a Milli-Q Reagent Grade Water System from Millipore (St. Quentin en Yvelines, France).

Drug-free human plasma was obtained from Les Etablissements Français du Sang where blood was collected from volunteers in tubes containing citrate–phosphate–dextrose or heparin. After centrifugation, the plasma was transferred and stored at −20°C.

2.2. Standard solutions

Primary stock solutions were prepared by dissolving the compounds or internal standard in water–methanol (50:50, v/v). Appropriate dilutions of the stock solutions with the same diluent were then made in order to prepare the working solutions. Two different series of stock solutions were prepared from different weightings for calibration and quality control samples. All the solutions were prepared in glass flasks and stored at 4°C.

2.3. High-performance liquid chromatography (HPLC) apparatus and chromatography

The HPLC system consisted of a Model 305 pump from Gilson (Villiers-le-Bel, France), a Model 234 injector equipped with a Model 832 rack temperature regulator from Gilson, and a Model UV-783 Kratos detector from Perkin-Elmer (Les Ulis, France) monitoring at a wavelength of 210 nm. A chromatography workstation, Model X-Chrom from LabSystems (Issy-Les-Moulineaux, France), was used to perform data acquisition.

The chromatographic separations were performed as follows:

A Hypersil C_{18} column, 40 mm×4.6 mm ID, 3

μm particle size, supplied by A.I.T.chromato (St. Nom-la-Bretèche, France), was used for the determination of the three compounds with a KS 11/4 Nucleosil 100-5 C_{18} pre-column (Macherey–Nagel, Hoerdt, France), 11 mm×4 mm, 5 μm particle size. The chromatography was performed at 35°C. The mobile phase, methanol–acetonitrile–0.01 *M* KH_2PO_4 (11:9:80, v/v) strongly degassed with helium, was delivered at a flow-rate of 2 ml/min.

2.4. Sample preparation

A 3M Empore C_{18}-SD 96-well disk plate (supplied by Varian, Les Ulis, France) containing 14 mg sorbent per well was placed on top of a 3M vacuum manifold.

After thawing, the plasma was centrifuged and aliquots of 50 μl were transferred to a deep-well collection plate. A 50-μl volume of the suitable internal standard solution was then added. After addition of aliquots (5 μl) of calibration or quality control solutions, the plate was vortex-mixed for 10 s. The samples were transferred to the wells of the Empore C_{18} plate after the conditioning step within 15 min. The SPE procedure is detailed in Table 1. Following the elution step, the extracts were diluted and the collection tubes were placed on the autosampler rack refrigerated at 4°C.

The vacuum manually controlled allowed the different solvents to pass through the extraction membranes: the disks should be kept wet after the conditioning and loading steps and dried after the washing and elution. To protect the wells for being dried at the conditioning and loading steps, the vacuum was stopped as soon as the first wells were empty.

All liquid transfers were done manually using Biohit Proline electronic pipettors (Bonnelles, France). Twelve-channel pipettors were used in the multi-dispensing mode.

Table 1
SPE procedures for the determination of oxcarbazepine and its metabolites in plasma

Step	Fluid
Sorbent	200 μl methanol
conditioning	200 μl water
Loading	100 μl diluted plasma
Washing	50 μl water
	50 μl water–methanol (80:20)
Elution	75 μl×2 methanol
Dilution	400 μl water
Injection	300 μl

3. Results

3.1. Retention times and specificity

Observed retention times were 3.5, 4.5, 8.5 and 12.5 min for DHD, MHD, OXC and I.S., respectively. They were well separated from plasma components within 15 min (Fig. 2).

3.2. Linearity

Calibration samples were prepared at five different concentrations in duplicate in the ranges 0.2–10, 0.1–200 and 0.1–20 μmol/l, respectively, for OXC, MHD and DHD, by spiking drug-free plasma with the corresponding working solutions.

The calibration curves, represented by the plots of the peak height ratio (parent compound/I.S.) versus parent compound concentration, were generated using weighted ($1/x^2$) linear regression (Fig. 3). Linear calibration curves were obtained with a coefficient of correlation (r) usually higher than 0.99.

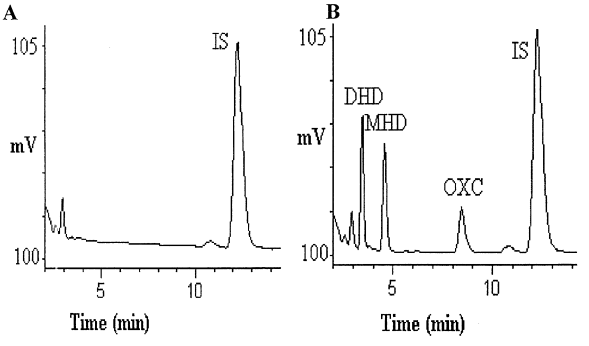

Fig. 2. Representative chromatograms obtained following extraction of oxcarbazepine, its metabolites and the internal standard. (A) Drug-free plasma spiked with I.S. (19.8 μmol/l), (B) plasma spiked with 2 μmol/l of the three compounds and I.S.

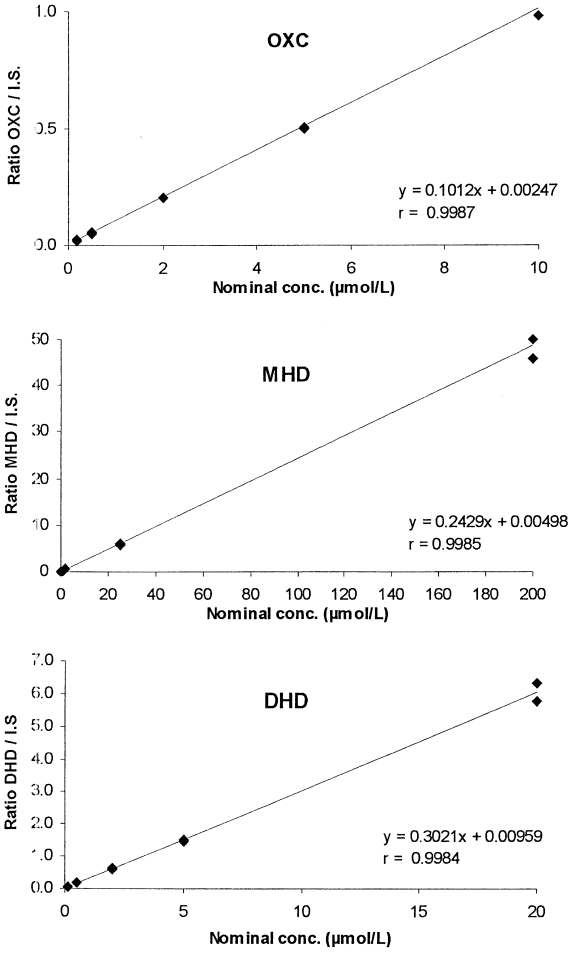

Fig. 3. Representative calibration curves for the determination of oxcarbazepine and its metabolites.

Inter-day repeatability was determined on 3 different days (Table 2).

For each calibration standard level, the concentration was back-calculated from the calibration curve equation. Inter-day variability is presented in Table 3: the precision ranged from 1 to 10% and mean accuracies were within 4% of the nominal values for the three compounds.

Table 3
Back-calculated concentrations from calibration curves

OXC nominal concentration (μmol/l)	0.2	0.5	2	5	10
Mean accuracy (%), $n=6$	99	102	100	99	100
Precision (%)	4	2	2	1	7
MHD nominal concentration (μmol/l)	0.1	0.5	2	25	200
Mean accuracy (%), $n=6$	100	104	99	98	100
Precision (%)	6	1	3	2	9
DHD nominal concentration (μmol/l)	0.1	0.5	2	5	20
Mean accuracy (%), $n=6$	100	100	99	98	103
Precision (%)	3	2	3	2	10

Accuracy: (found/nominal concentration)·100.

3.3. Precision and accuracy

Series of five quality control samples were prepared at three different concentrations in the range 0.2–8, 0.1–160 and 0.1–16 μmol/l, respectively, for OXC, MHD and DHD, by spiking drug-free plasma with the corresponding working solutions.

The intra-day, the overall (intra-/inter-day) accuracy and precision of the method were assessed by assaying these series of quality controls on three different days. The LOQs were defined as the lowest drug concentration which can be determined with an accuracy of 80–120% and a precision ≤20% on a day-to-day basis [6].

The results (Table 4) satisfactory met the acceptance criteria: mean accuracy within 85–115% and RSD≤15% (80–120% and ≤20% at the limit of quantitation, LOQ).

LOQs were set at 0.2, 0.1 and 0.1 μmol/l for OXC, MHD and DHD, respectively.

Table 2
Assay linearity of the method

Inter-day repeatability, mean values of 3 days	Slope±SD	*y*-Intercept±SD	Coefficient of correlation±SD
OXC	0.1008±0.0014	0.00078±0.00329	0.9973±0.0021
MHD	0.2389±0.0047	0.00471±0.00174	0.9958±0.0052
DHD	0.2938±0.0080	0.00527±0.00553	0.9935±0.0088

Table 4
Assessment of the accuracy and precision of the method

	Low QC	Medium QC	High QC
OXC nominal concentration (μmol/l)	0.2	1.6	8
Intra-day mean accuracy (n=5)	94	100	100
Precision: RSD (%)	7	5	7
Intra-/inter-day accuracy (n=15)	96	100	104
Precision: RSD (%)	7	4	5
MHD nominal concentration (μmol/l)	0.1	1.6	160
Intra-day mean accuracy (n=5)	107	100	98
Precision: RSD (%)	8	4	8
Intra-/inter-day accuracy (n=15)	105	99	102
Precision: RSD (%)	11	4	6
DHD nominal concentration (μmol/l)	0.1	1.6	16
Intra-day mean accuracy (n=5)	98	101	102
Precision: RSD (%)	6	4	9
Intra-/inter-day accuracy (n=15)	96	99	106
Precision: RSD (%)	7	4	6

Accuracy: (found/nominal concentration)·100.

3.4. Extraction recovery

The extraction yields from plasma were estimated at three different concentration levels: 1, 5 and 10 μmol/l for OXC and DHD and 1, 20 and 100 μmol/l for MHD. The mean extraction efficiencies were 86, 87 and 91%, respectively, for OXC, MHD and DHD. I.S. efficiency, estimated at the concentration used for the assays (19.8 μmol/l) was 51%. The recovery was assessed by comparing the peak heights of the processed samples to the ones of drug-free samples processed identically and spiked with the corresponding concentration after their processing.

To ensure reproducible extraction yields, the same plasma volume (50 μl) needs to be used in calibration, quality control or unknown samples with same dilution factor (1:1). If drug-free samples are required, the plasma needs to be diluted with water–methanol (50:50, v/v).

3.5. Stability

At about −70°C in frozen human plasma (actual samples), OXC and DHD have been found to be stable for at least 1 year; the mean recoveries (from the first determination)±SD were 99±13% and 103±14%, n=26 for OXC and DHD, respectively. MHD has been found stable for at least 3 years with a mean recovery of 103±11%, n=4.

At about −20°C in frozen human plasma (spiked samples), OXC was not stable. MHD has been found stable for at least 4 months with a recovery of 103%, n=1. DHD stability at this temperature was not performed.

Stock solutions were found stable at least 3 months at +4°C.

Extracts were found stable on the autosampler at +4°C for at least 33 h.

3.6. Productivity and application of the method

The method was applied to a clinical trial. The plasma samples were collected from 108 patients. Concentrations of OXC and DHD were determined from about 1000 samples. With this assay, batch runs of 96 injections, from one 96-well plate, were performed which included clinical samples, 10 standards, six quality controls (QCs) and drug-free samples. 13 analytical runs over 24 h were necessary for the determination of the overall study.

The method was also applied to a bioequivalence study where two oral forms of OXC were administered to 18 volunteers. Concentrations of MHD were determined from about 1900 samples. With this assay, single batch runs up to 120 injections, from

two 96-well plates, were performed, which included clinical samples, 10 standards, six QCs and drug-free samples. 17 analytical runs over 30 h were carried out.

Typical time for sample preparation of a 96-well plate was about 1 h.

Suitable sensitivity, robustess and reproducibility were obtained for both studies.

4. Discussion

The described method has been adapted from a previously published one [7] which involved classic SPE on cartridge automated by the ASPEC system (Gilson).

The Empore extraction membrane, used in the present method, is less than 0.8 mm thick and replaces conventional SPE devices, used in the previous method, containing several times more sorbent mass. It leads to significantly reduce the solvent volumes used in the previous method: the volume of plasma required for the assays could have been divided by two without modifying the LOQs of the three compounds; the volumes of solvent for conditioning were divided by five and the ones for washing divided by about seven; the diluent and injection volume were divided by about two.

Above the extraction disk of the Empore plate is a patented prefilter (graded density polypropylene) which retained 98% of all particles larger than 10 μm and 50% of particles as small as 2 μm. This results in an eluate free from particles which could be responsible for column plugging as this often occurs in conventional SPE. Consequently in the present method no pressure at the top of the pre-column was observed and the life time of the analytical column lasted for more than 1000 injections (more than twofold increase).

In the previously described method, when using the ASPEC system for on-line determination, one sample was prepared at a time, during the chromatography of the previous one; so the diluted plasma samples were left on the ASPEC rack for several hours, waiting to be extracted. As the thermal instability of OXC in plasma prevented from leaving the samples for more than 6 h at room temperature (or more than 10 h at 4°C), only eight clinical samples (or 24 when using a thermostated rack) could be determined during one analytical run where 10 calibration samples and six QC samples were required.

With this present method the extraction step of a block of 96 samples could have been reduced down to 1 h and so prevents from OXC instability concerns. The eluates (in methanol–water) were found stable at 4°C for at least 33 h. So the number of clinical samples determined in one analytical run was threefold increased. As a consequence of the analytical run number decrease, the required number of calibration and quality controls for an overall clinical study was threefold decreased.

The agreement of the two methods was confirmed by comparison of OXC and DHD measurement of 26 actual samples. Fig. 4 shows the good relationship between both methods.

No interference from anti-epileptic drugs such as phenobarbital, phenytoin, valproic acid and carbazepine was found previously in patients treated

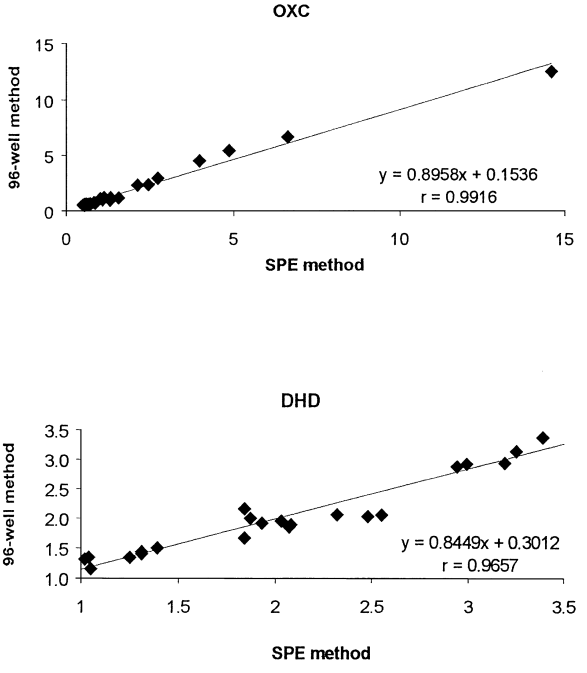

Fig. 4. Comparison of oxcarbazepine and its dihydroxy metabolite measurements of 26 patient plasma samples by the classic SPE method and the 96-well plate method.

concomitantly with OXC and one of these drugs as described in Ref. [7].

5. Conclusion

A simple and robust SPE procedure from plasma has been developed and validated using the Empore microtiter plate. The membrane extraction disc technology, as a result of low bed volume and dense packing, allows for reduced solvent and plasma volume requirement compared with traditional SPE technique without loss of sensitivity. A reduction of plasma used has ethical benefits and a reduction of solvent used is beneficial from a cost point of view but also helps in minimize the impact of solvent waste treated in our ecological system.

The use of the 96-well format and the quick preparation of the plate have allowed a significant increase in sample throughput. The method was applied successfully to the analysis of over 3000 samples generated during human trials. The system could be easily automated.

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